THE CITRATE CYCLE AND RELATED METABOLISM OF *LISTERIA MONOCYTOGENES*

by

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LIST OF ABBREVIATIONS AND DEFINITIONS

acetyl-CoA:	S-acetyl coenzyme A.
acetyl-P:	acetyl phosphate.
ADP:	adenosine diphosphate.
AMP:	adenosine monophosphate.
ATP:	adenosine triphosphate.
BHI:	brain-heart infusion.
CE:	crude extract.
CFM:	cofactor mixture.
CoA:	coenzyme A.
DCPIP:	2,6-dichlorophenol indophenol.
DPT:	diphosphothiamine.
EDTA:	ethylenediamine tetraacetic acid.
FAD:	flavin adenine dinucleotide.
$FADH_2$:	reduced flavin adenine dinucleotide.
FMN:	flavin mononucleotide.
$FMNH_2$:	reduced flavin mononucleotide.
GDP:	guanosine diphosphate.
IDP:	inosine diphosphate.
INT:	p-iodonitrotetrazolium violet.
M^{++}:	divalent cation.
mgN, μgN:	milligrams nitrogen, micrograms nitrogen.
mgP:	milligrams protein.
NAD:	nicotinamide adenine dinucleotide.
NADH:	reduced nicotinamide adenine dinucleotide.

LIST OF ABBREVIATIONS AND DEFINITIONS

NADP:	nicotinamide adenine dinucleotide phosphate.
NADPH:	reduced nicotinamide adenine dinucleotide phosphate.
NDP:	nucleoside diphosphate.
NTP:	nucleoside triphosphate.
Oxidation ratio:	µmoles O_2 consumed/µmole of substrate added.
P_i:	inorganic phosphate.
PSS:	protamine sulfate supernatant extract.
$Q_{O_2}(N)$:	µl O_2 consumed/hr/mg bacterial nitrogen.
Resting cells:	non-proliferating bacterial cells which have been washed free of the culture medium.
Tris:	tris (hydroxy methyl) aminomethane.

STATEMENT OF THE PROBLEM

The purpose of this thesis was to determine whether *Listeria monocytogenes* carries out the citrate cycle pathway and certain closely related metabolism, i.e. pyruvate oxidation and the glyoxylate bypass. In general, *Listeria* metabolism has been studied very little. Present knowledge of metabolic pathways and component enzymes of *Listeria* is fragmentary and limited, the Embden-Meyerhof glycolytic pathway being the only major pathway known to be used by this bacterium. Several lines of evidence suggest citrate cycle activity and pyruvate oxidation in *Listeria*. In this thesis, therefore, experiments were designed and executed to answer these questions:

(1) Can citrate cycle intermediates or pyruvate serve as the sole source of carbon and energy for *Listeria* growth in a defined medium?

(2) Can citrate cycle intermediates or pyruvate increase *Listeria* growth in defined medium when glucose, the usual carbon and energy source, is limiting?

(3) Can intact *Listeria* oxidize pyruvate or citrate cycle intermediates?

(4) Can the enzymes of pyruvate oxidation, the citrate cycle, and the glyoxylate bypass be detected in cell-free extracts of *Listeria*?

INTRODUCTION

I. GENERAL

Listeria monocytogenes holds a comparatively obscure status in bacteriology. Many aspects of its biology have not been studied experimentally and are poorly understood. For these reasons, some characteristics of this bacterium and the diseases it causes will be briefly reviewed. More detailed information is presented in two reviews (1, 6). The nutritional requirements and metabolism of *Listeria* will be discussed in depth because these topics relate directly to the thesis problem. Aspects of the citrate (Krebs) cycle, glyoxylate bypass, and pyruvate oxidation will then be discussed briefly because this metabolism, as it concerns *Listeria*, was the experimental subject of this research. Finally, the approaches to the problem will be detailed.

II. *LISTERIA MONOCYTOGENES* AND LISTERIOSIS

A. Discovery and nomenclature.

Listeria monocytogenes is a recently recognized bacterial pathogen of man as well as of domestic and wild animals (1). It was first cultured and described in 1926 by Murray et al. (2) who isolated the bacterium from diseased rabbits which showed a pronounced monocytosis, ergo, the specific epithet "monocytogenes". The original genus name, *Bacterium*, proposed by Murray et al. (2) was later deemed unacceptable because the bacterium lacks the characteristics of this genus. Pirie (12) chose to call this organism *Listerella*, but this name was

unsatisfactory because it had been applied earlier to a group of slime molds (1). The accepted name is now *Listeria monocytogenes*, Pirie, and it is the only species in the genus (13). Hereafter it will be referred to simply as *Listeria*.

B. Listeriosis: a disease of man and other animals.

Listeriosis is the infection of man or other animals by *Listeria*. For the sake of clarity, the adjective "listeric" is often placed before the specific disease process, e.g. listeric septicemia of man (1). The characteristic clinical pictures in listeric infections are as follows: man, septicemia with associated meningitis or meningoencephalitis; fowl, septicemia with myocardial degeneration; ruminants, localized encephalitis or meningoencephalitis; monogastric animals, e.g. mice, or young ruminants before the rumen becomes functional, septicemia with a tendency toward conspicuous focal hepatic necrosis, but usually without brain involvement (1). Many mammals including man can suffer from listeric abortions and other perinatal infections (1).

The host range of *Listeria* is very broad: man, 37 mammalian species, 17 fowl species, and assorted ticks, fish, and crustacea (1). *Listeria* is quite ubiquitous, having been isolated from stream water, silage, mud, sewage, and slaughterhouse waste in many parts of the world (1). Recently, Welshimer (34) cultured vegetation for *Listeria* in Hanover County, Virginia, an area reportedly having a low incidence of listeriosis; in 7 of the 12 fields tested, a total of 8 *Listeria* strains were isolated. The wide distribution of *Listeria* raises important questions about the natural habitat of the bacterium and the

epidemiology of listeriosis.

C. Characteristics of *Listeria monocytogenes*.

1. Morphology, ultrastructure, protoplasts, and L-forms.

The *Listeria* cell is a small gram-positive rod with rounded ends, measuring about 0.5 μ x 1.0 to 2.0 μ; coccobacillary or filamentous forms may be seen, however, depending on the method of growth and age of culture (1). It is non-sporeforming, not acid-fast, and motile by means of flagella (1). Although usually regarded as non-encapsulated (6), capsules have been observed around *Listeria* grown in an unusually rich medium of trypticase soy agar supplemented with 10% rabbit serum and 5% glucose (3). Stained smears of *Listeria* colonies less than 24 hr old show typical "diphtheroid" arrangements, i.e. palisade, V, and Y forms (1); this finding is in harmony with the fact that *Listeria* is a member of the family *Corynebacteriaceae* (13).

Electron micrographs of *Listeria* show a typical bacterial cell structure. An outer cell wall surrounds a complex plasma membrane which in turn encloses the cytoplasm filled with dense granules and a nuclear apparatus (4). A system of internal membranes is continuous with the plasma membrane. This membrane appears more complex than that seen in most gram-positive bacteria except for *B. subtilis*; it shows five distinct layers, three dark lines with two alternating light lines sandwiched between (4). Thin sections of *Listeria* protoplasts, however, show a plasma membrane having the more conventional triple layer structure (39).

Listeria protoplasts may be formed by treatment of *Listeria* cells

with lysozyme and lipase (39). L-forms of *Listeria* have been induced by growth on penicillin gradient plates (40).

2. Culture and colony characteristics.

Listeria grow readily on common media such as tryptose agar or blood agar (1). Twenty-four hr smooth type colonies appear raised, smooth, and circular with an entire margin, from translucent to opaque in density, and from white to grey in color (1, 6). Size of the colonies will vary from 0.3 to 1.5 mm in diameter. A zone of β-hemolysis usually surrounds the colony grown on blood agar due to the production of a soluble, filterable hemolysin which is capable of lysing most mammalian erythrocytes (10, 11). Broth media containing a peptone and fermentable carbohydrate such as glucose will support excellent growth in less than 24 hr (6, 8).

Listeria colonies are of two main types, smooth (S) and rough (R), but an intermediate (I) type may occur (6). In 24 hr stationary broth cultures, growth of the S type produces uniform turbidity while the R type yields sediment and often a pellicle. Growth characteristics of the I type fall between those of the S and R types (6).

Listeria prefer a pH around neutrality, although they tolerate pH extremes of 5.6 to 9.6 (6). Growth in unbuffered complex media often results in the pH dropping below 5.0 (15), but viability suffers when the pH falls below 5.6 (6). Optimal growth occurs between temperatures of 30 C to 37 C, and the growth reaches a plateau between 16 and 18 hr (6). Growth is slow at the temperature extremes of 3 C and 45 C (1). Cells grown between 4 C and 30 C are highly motile and possess from 1 to 4 flagella, but cells grown at 37 C are either sluggishly

motile or non-motile (1). Flagella are subterminal or peritrichous (5, 9). True polar flagellation has not been observed (6).

3. Serotypes.

Listeria strains from around the world are presently divided into eleven serotypes (1), based upon differences in somatic (O) and flagellar (H) antigens (5, 6, 7); these are designated 1a, 1b, 2, 3a, 3b, 4a, 4ab, 4b, 4c, 4d, and 4e. Type 4b predominates in the United States, while other serotypes are characteristically found elsewhere, e.g. type 1 in Europe (1).

4. Chemical composition.

Some attempts have been made to define the chemical composition of *Listeria*. Keeler and Gray (26) isolated a crude cell wall preparation from sonically disrupted *Listeria*. The cell wall fraction contained about 20% hexose, 5% hexosamine, and 50% protein (Kjeldahl). The hexose portion consisted of equal amounts of glucose and galactose. Paper chromatograms of acid-hydrolyzed cell walls showed the following five amino acids: alanine, glutamic acid, aspartic acid, leucine, and α,ε-diaminopimelic acid. Similar analysis of protoplasmic material demonstrated 17 to 20 amino acids. The protoplasm was rich in nucleic acids. Visible spectra of both protoplasmic and cell wall fractions failed to reveal a peak in the Soret region (400-450 mμ); it was concluded that the *Listeria* strain studied contained little if any cytochrome (26).

Ghosh and Carroll reported the composition and structure of *Listeria* plasma membrane (41). Membranes were prepared from protoplasts and shown to be essentially free of cytoplasm or cell wall

contamination. Plasma membrane constituted 7 to 10% of the dry weight of the whole cell. The membrane preparation was composed of protein (55-60%), lipid (30-35%), nitrogen (10.5-12.0%), phosphorus (3.5-4.0%), ribonucleic acid (1.5%), deoxyribonucleic acid (0.1%), carbohydrate (1.3-2.3%), amino sugar (0.17-0.38%), and rhamnose (0.2-0.4%). Membrane lipid consisted of 80 to 85 percent phospholipid (phosphatidyl glycerol type) and 15 to 20 percent neutral lipid. Membrane carbohydrate contained glucose, galactose, ribose, and arabinose. Whole *Listeria* cells contained 18 amino acids while membrane preparations contained 17; this difference was due to the membrane's lack of methionine, which was present in considerable amounts in whole cells (41). Ornithine, however, was present in considerably greater amounts in the membrane than in whole cells. Sulfur-containing amino acids were not detected in membrane preparations. Both whole cells and membranes contained large amounts of lysine, alanine, glutamic acid, and aspartic acid; this abundance of glutamic acid and aspartic acid is linked to the rationale of this thesis and will be mentioned later.

Monocytosis has been associated with listeriosis since the first description of *Listeria* by Murray et al. (2). A monocyte-producing agent (MPA) has since been isolated from *Listeria* in crude form as a chloroform-soluble lipid (33). MPA is non-toxic, non-antigenic, and is apparently associated with the cell wall-membrane fraction of the bacterium (26, 33). Since about 90 percent of the lipid of *Listeria* is membrane component (41), the lipid nature of MPA suggests that it may be of membranous origin.

Certain fractions of disrupted *Listeria* cells enhance the

pathogenicity (lower the LD_{50}) of *Listeria* when injected with the bacteria into test animals (1). The chemical nature of these substances is as yet undetermined.

5. Carbohydrate fermentation and other biochemical properties.

Listeria consistently ferment glucose, fructose, trehalose, and salicin, in all cases producing acid but no gas within 24 hr. The fermentation of arabinose, galactose, lactose, maltose, rhamnose, sucrose, dextrin, sorbitol, glycerol, esculin, melezitose, and xylose is slow (3 to 10 days) and irregular, depending on the strain tested (1).

Listeria behaves in other classical biochemical tests as follows: nitrate, not reduced; indole, not formed; gelatin or coagulated serum, not liquefied; starch, not hydrolyzed; urea, not hydrolyzed; H_2S, produced under appropriate conditions; litmus milk, slowly acidified, but not coagulated; arginine, hydrolyzed to yield NH_3; Voges-Proskauer test, positive; methyl red test, positive.

6. Nutritional requirements.

a. General.

The nutritional requirements of certain strains of *Listeria*, particularly A4413, are reasonably well understood, although a minimal medium has not yet been described. Several definitive studies have shown which carbon and energy sources, amino acids, vitamins, and inorganic ions are needed for growth. These are discussed below.

b. Requirement for carbohydrate as a carbon and energy source.

Carbohydrates are essential for good *Listeria* growth,

and glucose is most commonly used for this purpose (6, 8, 23); it probably serves as the source of both carbon and energy (23). Either fructose or cellobiose can replace glucose as an efficient carbon and energy source, but maltose or trehalose is only about half as efficient as glucose (25). Conceivably, salicin could also serve as an adequate supplement since it is among the few carbohydrates that *Listeria* ferments within 24 hr; this has not been tested. Glucose cannot be replaced by gluconate, xylose, arabinose, or ribose as a carbon and energy source (23). Little information exists concerning the suitability of other compounds, e.g. organic acids such as substrates of the citrate cycle, as carbon and energy sources. *Listeria* A4413 is unable to utilize pyruvate (24) or citrate (25) for this purpose.

c. Nitrogen sources: specific amino acid requirements.

Peptones such as those present in enzymatic digests of casein, i.e. trypticase (8), tryptose (20, 21), as well as acid hydrolyzed "vitamin-free" casein (14, 15, 18, 21) serve as adequate nitrogen sources for *Listeria* growth when present in a medium including inorganic salts, glucose, and vitamins.

Listeria was cultivated in defined media as early as 1944 by Hutner (16). The nitrogen requirement was satisfied by an amino acid mixture, the number and identity of which, with the exception of cystine, were not listed. Cury et al. (17, 18) reported *Listeria* growth on a mixture of 19 amino acids which were not specified; individual amino acid requirements were not determined. Friedman and Roessler (20), however, cultivated *Listeria* in defined medium containing inorganic salts, glucose, vitamins, and 21 amino acids which

were specified. Amino acid exclusion tests indicated that strain A4413 required cysteine, leucine, isoleucine, and valine, and was stimulated by histidine, arginine, methionine, and glutamine. Similarly, strain 9037-7 (an R-type) required cysteine, leucine, isoleucine, and valine as did strain A4413, but also required histidine and arginine, while glutamine was stimulatory. Methionine was not mentioned in connection with strain 9037-7. Neither cysteine nor sulfhydryl groups were specifically required, but only some source of organic sulfur, since either thioglycollate or cystine could replace cysteine. As a growth stimulant, glutamate could not replace glutamine, although glutamine could be replaced by NH_4^+, urea, or asparagine. Ammonium salts were unsuitable as a sole nitrogen source.

The *Listeria* grew just as well when the 21 amino acids were replaced by only 9 amino acids (20). Thus, the simplest defined *Listeria* medium contained the following components.

Friedman and Roessler's defined medium (20)*

Salts A:	NaCl, 0.25; $MgSO_4 \cdot 7H_2O$, 0.08; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.004; Na citrate, 0.04.
Salts B:	K_2HPO_4, 0.6.
Glucose:	0.6; sterilized by filtration.
Vitamins:	Riboflavin, 0.0005; thiamine, 0.0005; biotin, 0.0005; α-lipoate, 0.0005. Sterilized by filtration.
Amino acids:	L-cysteine, 0.01; L-leucine, 0.01; DL-isoleucine, 0.02; DL-valine, 0.02;

L-glutamine, 0.06; DL-methionine, 0.02; L-histidine, 0.01; L-arginine, 0.02; and DL-tryptophan, 0.02.

*Ingredients, listed in per cent, were dissolved in distilled water. Before addition to the other medium components, salts A and B were mixed and heated for 5 min, after which the resultant precipitate was discarded.

These studies (20) indicated, therefore, that a mixture of only 9 amino acids supported *Listeria* growth. Only 3 amino acids were required for strain A4413, while strain 9037-7 required 5 amino acids. Since NH_4^+ could replace glutamine and since tryptophan, although not stimulatory, was included in the mixture of 9 amino acids, it seemed that for the purposes of this thesis research, a simpler mixture of 7 amino acids might serve as a suitable nitrogen source for *Listeria*. This will be discussed further in the Methods section, in relation to the design of medium D10.

d. Vitamin requirements.

Porter and Pelczar first attempted to find specific vitamin requirements of *Listeria* (14). All 12 strains which they tested grew consistently in serial subculture in a medium consisting of acid-hydrolyzed "vitamin-free" casein, inorganic salts, and glucose, but only if riboflavin, biotin, and hemin were included. Thiamine was stimulatory, but not essential. Hutner (15) found that *Listeria* was so dependent upon riboflavin that he suggested the use of *Listeria* in

a microbiological assay for this vitamin. Cury et al. (18) reported that *Listeria* grew in a defined medium which included riboflavin, thiamine, biotin, and α-lipoate; omission of any of these four vitamins prevented growth. Growth was practically unaffected by addition of the following substances to the defined medium: pantothenic acid, pyridoxine, pyridoxal, pyridoxamine, para-aminobenzoic acid, vitamin B_{12}, folic acid, citrovorum factor, diphosphothiamine, diphosphopyridine nucleotide, adenosine triphosphate, xanthopterin, hydrolysates of ribonucleic acid or deoxyribonucleic acid, adenine, guanine, xanthine, uracil, or thymine. The requirement for α-lipoate was abolished by adding high concentrations of acetate (0.1 to 0.5%) or pyruvate (0.3 to 1.0%); lower concentrations of acetate (0.003 to 0.03%) exerted a "sparing" effect toward α-lipoate. Their *Listeria* required α-lipoate to such an extent that they suggested the use of this species in the microbiological assay for α-lipoate (18).

Listeria strains A4413 and 9037-7 were grown by Friedman and Roessler (20) in a defined medium (see previous section for composition) including riboflavin, thiamine, biotin, and α-lipoate. They demonstrated a requirement for riboflavin, thiamine, and biotin, but not for α-lipoate.

Welshimer (21) recently conducted the most definitive study of *Listeria*'s vitamin requirements to date. He tested 8 strains, including A4413 and 9037-7, and clearly demonstrated that all strains required riboflavin, thiamine, biotin, and α-lipoate. Welshimer used more precautions than earlier workers; his *Listeria* inoculum was "starved" for vitamins by passage through vitamin-free media, thus

lessening the danger of transfer of contaminating vitamins from the first growth medium. The vitamin-starved inoculum was then transferred to media containing various combinations of vitamins and the growth response was determined.

Earlier workers agreed that *Listeria* require riboflavin and biotin; they disagreed, however, on this organism's thiamine and α-lipoate requirements. Welshimer's careful study (21) suggests that thiamine and α-lipoate may have been carried over in the inoculum used in earlier experiments, whereupon the growth response led to erroneous conclusions about the requirement for these vitamins.

e. Inorganic ion requirements.

Growth of *Listeria* strains A4413 and 9037-7 in defined media requires the presence of Mg^{++}, $PO_4^{\equiv}$, and K^{+} (20). Sword (22) discovered that these strains were greatly stimulated by the addition of iron; in the defined medium of Welshimer (21), growth was roughly proportional to the amount of iron added (0.1 µg to 100 µg/ml Fe^{+++} as ferric ammonium citrate or 0.1 µg to 10 µg/ml Fe^{++} as ferrous sulfate). In the same study, *Listeria* growth was markedly inhibited, however, by Mn^{++} in amounts from 0.1 µg to 10 µg/ml.

f. Effect of oxygen tension on growth of *Listeria*.

Listeria is usually considered to be a microaerophile or facultative anaerobe (6). *Listeria* growth in semisolid medium is heaviest slightly beneath the surface, suggesting that it prefers a somewhat reduced oxygen tension (6). Growth is reportedly satisfactory if oxygen has been replaced by carbon dioxide, but very poor or absent under strict anaerobiosis (6). If *Listeria* prefers a slightly

reduced oxygen tension, agitation of broth cultures should not be stimulatory. Cury et al. (18) observed that agitation had no effect upon the growth of *Listeria* in defined medium consisting of inorganic salts, glucose, amino acids, and vitamins. Shaking has, however, reportedly increased the growth of *Listeria* as much as two-fold, provided the medium contained a peptone as the nitrogen source (20, 25); this suggests that the composition of the medium plays a role in the agitation effect.

Friedman and Roessler (20) found that the composition of their media influenced the growth response in stationary or agitated cultures. Initial *Listeria* growth in the defined medium was improved by shaking, but shaken subcultures failed to grow; stationary subcultures were always possible in this defined medium (20). Shaken subcultures could be made only when enzymatic digests of casein (dialyzed or undialyzed) were the source of nitrogen; neither acid-hydrolyzed casein, amino acid mixtures, nor synthetic peptides permitted this type of subculture. They concluded that natural peptides were apparently required for shaken subculture (20).

Listeria has been commonly grown in complex media with agitation, because this aeration increased the yield (24-26). This is difficult to reconcile with the observation that heaviest *Listeria* growth in semisolid media appears just beneath the surface (6). Possibly factors other than peptides influence growth under agitation. The recent observation (22) that iron is very stimulatory for *Listeria* may be a clue to this apparent discrepancy. It will be shown in the Results section of this thesis, as incidental to the main objectives, that

Listeria A4413 growth in defined medium was improved by agitation only when an adequate amount of iron was included.

7. Metabolism.

a. General.

A survey of the literature on *Listeria* from the first publication (2) in 1926 until the present leads to at least one conclusion: *Listeria* is poorly characterized metabolically (1). The information on the bacterium's nutritional requirements, while not complete, still dwarfs that concerning its metabolism. Most publications relate to listeriosis or general characteristics of the bacterium; very few concern metabolism. Relevant information about *Listeria* metabolism is presented below.

b. Glucose catabolism.

As stated previously, carbohydrate is essential for *Listeria* growth, and glucose is most commonly used to meet this demand. Miller and Silverman (23) investigated glucose catabolism of "resting" *Listeria*. Under fermentative conditions, they found that 95% of the glucose actually degraded could be recovered as L(+) lactic acid. Aerobically, 91 percent of the glucose oxidized was accounted for as lactate, pyruvate, and acetoin. Thus, essentially independent of aeration, two 3-carbon compounds were formed from each molecule of glucose. Growth studies (23) revealed that *Listeria* did not utilize pentoses or gluconate as sources of carbon and energy. Since the anaerobic dissimilation of glucose was homofermentative, and the pentoses were not used for growth, the hexose monophosphate shunt pathway was thought not to be important as a means of glucose catabolism.

This would not, however, suggest that the hexose monophosphate shunt is totally unimportant to the metabolism of *Listeria*. Enzymatic analyses of cell-free extracts of *Listeria* supported the hypothesis that the Embden-Meyerhof pathway was the important means of glucose degradation. All enzymes of the Embden-Meyerhof pathway were found, but only glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes of the shunt pathway were detected. The key enzyme of the Entner-Doudoroff pathway, the 2-keto-3-deoxy-6-phosphogluconate aldolase, was not found. These results led these workers to suggest that *Listeria* might be related to the homofermentative type of *Lactobacteriaceae*, since both are gram-positive, non-sporeforming rods yielding mainly lactic acid from glucose breakdown. This relationship was postulated earlier by Cury et al. (17). Luppi et al. (27) also noted that the Embden-Meyerhof pathway could explain the degradation of glucose and related carbohydrates by *Listeria*.

c. *Listeria* metabolism and virulence in relation to the glucose concentration of the growth medium.

The amount of glucose available to growing *Listeria* influences their metabolism and virulence (24, 25). *Listeria* grown in a complex medium containing 0.2% to 0.6% glucose were 4 to 9-fold more virulent for mice than those grown with 1.0% glucose (25). Similarly, the "glucose dehydrogenase system" (as reduction of methylene blue) was some six times more active in cells grown on 0.2% to 0.6% glucose than on 1.0% glucose (25). Inclusion of 0.1% cysteine in the culture medium containing 1.0% glucose, however, yielded *Listeria* with virulence and glucose dehydrogenase levels similar to those grown in 0.2%

to 0.6% glucose (25). In another study (24), *Listeria* grown in 0.2% glucose had glucose oxidation rates about 4 times greater, and total oxygen consumption (μmoles O_2/μmoles glucose) and catalase activity two times greater than cells grown on 1.0% glucose. These studies indicated that virulence, glucose dehydrogenase, glucose oxidation rate, and catalase activity of *Listeria* increased as the glucose content of the growth medium decreased (24, 25).

The catalase activity of the many *Listeria* strains studied by Stricker et al. (28) was roughly proportional to their virulence for mice; Friedman and Alm (24) confirmed this relationship. It appears, therefore, as Stricker et al. (28) suggested, that catalase activity is a useful index of *Listeria* virulence. *Listeria* catalase is a classical heme-iron type, sensitive to cyanide or azide (24).

d. Oxidation of sugars, pyruvate, and certain intermediates of the citrate cycle.

Listeria respiratory capacity has been studied by two research groups (24, 31); sugars, pyruvate, and certain intermediates of the citrate cycle were oxidized.

The work of Friedman and Alm (24) concerned the oxidation of glucose by *Listeria* strain A4413 with emphasis on the role of the glucose level during growth. Their experimental data are summarized in Table 1, page 18. The data in Table 1 indicate the following (24):

(1) High growth levels of glucose yielded *Listeria* which were only capable of slow (low $Q_{O_2}[N]$) and inefficient (low oxidation ratio = μmoles O_2/μmoles glucose) oxidation of glucose. In contrast, low growth levels of glucose produced *Listeria* capable of rapid and

TABLE 1

THE METABOLISM OF GLUCOSE BY RESTING CELLS OF *LISTERIA* A4413 (24)[a]

Growth medium[b]	$Q_{O_2}(N)$[c]	µmoles O_2 / µmole glucose	End products (µmoles/µmole glucose) Pyruvate	Lactate	Acetoin	CO_2	Carbon recovery (%)
BHI[d]	444-773	1.4	0.15	0.17	0.03	0.96	34
NS-3[e], 0.2% glucose	594	1.2	0.23	0.43	0.07	0.97	54
NS-3[e], 0.6% glucose	375-475	0.9	0.61	0.33	0.21	0.75	74
NS-4CA[e], 1.0% glucose	193-315	0.6	1.2	0.55	0.13	0.44	104

a. Warburg vessel contents/3 ml: Cell nitrogen, 1 mg; glucose 10 µmoles; potassium phosphate buffer, pH 7.0, 0.022 M; 0.1 ml 2N KOH in the center well, except for the CO_2 determination. Vessels shaken for 60 to 80 min at 36 C.

b. All cultures were agitated.

c. $Q_{O_2}(N)$ = µliters O_2 consumed/mg bacterial nitrogen/hr. Range of values represents 3-5 replicate experiments except for medium NS-3 (0.2% glucose) which was run once. There was no measurable endogenous respiration.

d. BHI = brain heart infusion which includes 0.2% glucose.

e. NS-3 and NS-4CA media contained yeast extract and pancreatic digest of casein plus glucose as indicated.

efficient glucose oxidation. The Q_{O_2} (N) or oxidation ratio values from low-glucose cells were at least twice those of high-glucose cells.

(2) Carbon recovery (as pyruvate, lactate, acetoin, and CO_2) was roughly proportional to the growth level of glucose; recovery was 104% from 1.0% glucose, but only 34 to 54% from 0.2% glucose media (BHI and NS-3, respectively).

(3) Pyruvate and lactate recovery were approximately proportional to the growth level of glucose. Cells from 1.0% glucose almost quantitatively oxidized glucose to pyruvate and lactate, 1.2 µmoles and 0.55 µmoles, respectively, for a total of 1.75 µmoles as against a theoretical yield of 3-carbon moieties = 2.0 µmoles. In contrast, pyruvate and lactate recovery from cells grown in 0.2% glucose (BHI) was 0.15 and 0.17 µmoles, respectively, for a total of 0.32 µmoles which accounts for only one-sixth of the glucose carbons consumed.

(4) Evolution of CO_2 was inversely related to the glucose level. Cells grown in either medium containing 0.2% glucose yielded about one µmole CO_2/µmole glucose while cells grown in 1.0% glucose produced less than 0.5 µmoles CO_2/µmole glucose.

(5) There was little relation between glucose level and acetoin production; more acetoin was produced by cells grown in 0.6% glucose than in other glucose levels.

The oxidation of glucose by *Listeria* grown in BHI showed an oxidation ratio of 1.4, a carbon recovery of only 34%, an evolution of 1 µmole CO_2/µmole glucose, and a recovery of only one-sixth of the glucose carbons as pyruvate and lactate combined. These data indicate that glucose oxidation proceeded beyond the formation of two pyruvate

moieties by aerobic glycolysis, because this would yield an oxidation ratio of 1.0 (μmole O_2/μmole glucose). Since Friedman and Alm (24) reported that resting *Listeria* oxidized pyruvate (Q_{O_2}[N] = 17.7), the oxidation of some of the pyruvate formed during the glucose oxidation could account for the oxidation ratio being greater than 1.0 and the failure to recover significant pyruvate. Had Friedman and Alm (24) tested for the presence of acetate and/or acetyl derivatives, the usual products of pyruvate oxidation, the importance of this oxidation could have been estimated. In summary, this work (24) demonstrates that the oxidation of glucose by *Listeria* varies significantly in cells grown on different glucose levels.

These data (24) concerning glucose oxidation by *Listeria* grown on 1.0% glucose agree with those of Miller and Silverman (23) who found that $\simeq$ 91% of the glucose consumed could be accounted for as pyruvate, lactate, and acetoin; they did not state the glucose level used to grow their *Listeria*. As the data indicate, however, *Listeria* from BHI oxidized glucose the end-products of which only 34 to 54% were accounted for as pyruvate, lactate, acetoin, and CO_2 (24). Therefore, the earlier conclusion (23) that *Listeria* yields mainly lactic acid from glucose dissimilation appears to be only partially true; the end-products of glucose breakdown are a function of the glucose level during growth. Under low glucose growth (0.2%), lactic acid accounts for only a small proportion of the end-products, the greater portion of which are as yet undetermined (24).

Kolb and Seidel (31) also studied the respiration of *Listeria*. They tested the capacity of several carbohydrates and citrate cycle

intermediates to stimulate the endogenous oxidation rate. A type 1 *Listeria* strain was employed for these studies; it is a common type in Europe, but rare in the United States (1). Cultures were grown in nutrient broth for 3 to 4 days at 37 C, harvested, washed, and resuspended in phosphate buffer, pH 7.2. Oxidation rates were measured by Warburg manometry at 37 C and pH 7.2. Resting *Listeria* exhibited an endogenous oxygen consumption of about 75 µl O_2/0.05 mg N/5 hr. This was stimulated by the following carbohydrates with the stimulation (µl O_2/0.05 mg N/5 hr) listed after each substance: glucose, 232; fructose, 134; lactose, 107; maltose, 100; sucrose, 41; (+) arabinose, 18; D-mannitol, 5; D-sorbitol, 2. Of these carbohydrates oxidized, glucose, fructose, and maltose may serve as carbon and energy sources for *Listeria* (6, 23). The very slight oxidation of arabinose is not in disagreement with its inability to act as a carbon source (23). Mannitol and sorbitol did not stimulate the oxygen consumption significantly.

Kolb and Seidel (31) also noted that oxygen consumption by *Listeria* was stimulated by three intermediates of the citrate cycle. These data are summarized in Table 2, page 22. The results in Table 2 show that without pre-incubation or with a 1 hr pre-incubation period, malate and succinate, but not α-ketoglutarate, stimulated the oxygen consumption slightly (about two-fold) over the endogenous value. All three intermediates, i.e. malate, succinate, and α-ketoglutarate, were more stimulatory after a 20 hr pre-incubation period; only α-ketoglutarate, however, required this pre-incubation for a stimulatory effect. The pre-incubation effect is interpretable as being the time

TABLE 2

OXYGEN CONSUMPTION BY TYPE 1 *LISTERIA* (31)*

Endo-genous	Substrates	Pre-incubation of cells and substrate		
		None	1 hr.	20 hr.
68	Sodium malate	140	140	275
65	Sodium succinate	150	150	231
78	Sodium α-ketoglutarate	18	18	167

*All figures indicate the μl O_2/0.05 mg N/5 hr.

required for the synthesis of a "permease" which would allow α-ketoglutarate to penetrate the *Listeria* cells (42). Friedman and Alm (24) reported that *Listeria* A4413 oxidized succinate slightly (Q_{O_2}[N] = 8.1). The report of Kolb and Seidel (31) is the only one, however, indicating that *Listeria* oxidize malate and α-ketoglutarate. The oxygen consumed under stimulation by malate, succinate, or α-ketoglutarate suggests that significant oxidation of these substrates occurred; in relation to the endogenous oxygen consumption, however, these intermediates only increased the oxygen uptakes by 2 to 4-fold over the 5 hr observation period. The endogenous oxygen consumption reported here for type 1 *Listeria* was very high compared to that reported for strain A4413, which showed no measurable endogenous oxygen consumption (8, 24). The Kolb and Seidel report suggests that type 1 *Listeria* contains certain enzymes of the citrate cycle; these data served as one reason for selecting this thesis problem.

e. Dehydrogenases which reduce tellurite and tetrazolium.

Listeria contain various dehydrogenases which have been demonstrated by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) and potassium tellurite. Stricker et al. (28), using *Listeria* suspensions, found that TTC was reduced to its formazan in the presence of glucose or lactic acid. Dias and da Silva (29) observed that TTC was reduced within 2 hr by *Listeria* from either liquid or solid media. Electron microscopy of fixed cells revealed colored formazan granules located either at one or both poles of the cell, or centrally, indicating an intracellular reduction of the TTC. This rapid formation of granules in the cells led them to suggest that *Listeria* have regions

of intense metabolic activity.

Tellurite and TTC were employed by Kawata and Inoue (30) to determine the reduction sites in *Listeria*. They mixed actively growing *Listeria* with tellurite or TTC, then fixed, sectioned, and examined the cells by electron microscopy. Observation of thin sections indicated that tellurite had been reduced to metallic tellurium crystals on or near the inner layer of the cytoplasmic membrane, but not on the intracytoplasmic membrane system. In contrast, most of the TTC was reduced to its formazan on or near the intracytoplasmic membrane system. They suggested, therefore, that the tellurite reduction system and the TTC reduction system are primarily associated with the cytoplasmic membrane and intracytoplasmic membrane, respectively.

f. Transaminases.

Glutamic-oxalacetic transaminase activity was found in many strains of *Listeria* (28). There was poor correlation between virulence for mice and the activity of this transaminase; transaminase activity was a function only of the viable cell concentration (28).

III. RATIONALE FOR THIS RESEARCH

A. General comments.

Listeria metabolism has not been studied extensively. The information about the metabolic pathways and component enzymes of this organism is limited and fragmentary. The Embden-Meyerhof glycolytic pathway is the only complete pathway known to be present in *Listeria*. Significant among the other data concerning *Listeria* metabolism is information which implies citrate cycle activity. Discoveries

relevant to this topic were mentioned in the previous section, but they are emphasized here because they form the rationale for this thesis problem.

B. Evidence suggesting citrate cycle activity in *Listeria*.

1. Oxidation of certain citrate cycle intermediates by whole *Listeria* type 1.

Kolb and Seidel (31) found that resting *Listeria* oxidized malate, succinate, and α-ketoglutarate. They tested the effect of these substrates on the oxidative rate only at pH 7.2. These three citrate cycle intermediates stimulated oxygen consumption from 2 to 4-fold over the endogenous rate. Other substrates of the citrate cycle were not tested for a stimulatory effect. Other workers have also reported a very slight oxidation of succinate (24), but the rate was so slow ($Q_{O_2}[N] = 8.1$) as to be of doubtful significance. Although these data (31) are incomplete in that more citrate cycle intermediates were not used, the cellular dehydrogenases found for malate, succinate, and α-ketoglutarate imply citrate cycle activity in *Listeria*.

2. Lack of a growth requirement for glutamate and aspartate, and the presence of transaminase.

Growth of *Listeria* in defined media does not require glutamate or aspartate (20). A mixture of 9 amino acids satisfied the nitrogen requirement, and glutamate and aspartate did not stimulate growth. Since these two amino acids are present in considerable quantities in *Listeria* (41), these bacteria are obviously capable of efficiently synthesizing them. The common pathway of glutamate or

aspartate biosynthesis involves transamination of the α-keto acids, α-ketoglutarate and oxalacetate, respectively (35). A glutamic-oxalacetic transaminase has been demonstrated in *Listeria* (28). In other organisms glutamate may also be synthesized by a reductive amination of α-ketoglutarate by NH_3 plus NADH or NADPH, catalyzed by glutamic dehydrogenase (35). Aspartate may also be formed by the aspartase-catalyzed addition of NH_3 to fumarate (35). Thus, biosynthesis of glutamate and aspartate most commonly depends upon carbon skeletons which are intermediates of the citrate cycle: α-ketoglutarate, oxalacetate, and fumarate. Assuming that *Listeria* employ such pathways to glutamate and aspartate, the biosynthetic demand for these intermediates suggests that *Listeria* possess the citrate cycle enzymes requisite for their syntheses.

3. Pyruvate oxidation in *Listeria*.

Listeria grown in BHI (0.2% glucose) oxidized glucose giving an oxidation ratio of 1.4 and a carbon recovery of only 34% as pyruvate, lactate, acetoin, and CO_2 (see Table 1) (24). In this same experiment, *Listeria* oxidized pyruvate, but at a rate about 1/34th that of glucose. The data (see Table 1) suggest that under these conditions, some pyruvate, produced by glucose catabolism, was in turn oxidized. An oxidative decarboxylation of pyruvate (pyruvate dehydrogenase system) could at least partially explain the oxidation ratio (1.4) because a single-step oxidation of half of the pyruvate produced by aerobic glycolysis to the level of acetate would give an oxidation ratio of 1.5, just slightly greater than observed (24). Moreover, such pyruvate breakdown would help explain the poor carbon recovery,

because no attempt was made to detect acetate or acetyl derivatives after glucose oxidation had stopped. Friedman and Alm (24) did not comment about pyruvate oxidation.

Citrate synthase, the first enzyme of the citrate cycle, demands acetyl-CoA as one substrate; therefore, the operation of the entire citrate cycle depends on a ready supply of acetyl-CoA. The pyruvate dehydrogenase system represents perhaps the commonest pathway to provide acetyl-CoA from pyruvate. Because of the intimate relation between pyruvate oxidation and the citrate cycle, this oxidation was studied as a first step in this research.

C. Working hypothesis.

Since *Listeria* contain glutamic-oxalacetic transaminase, can synthesize glutamate and aspartate at rates sufficient for growth, and can apparently oxidize pyruvate, malate, succinate, and α-ketoglutarate, a working hypothesis was constructed asserting that this bacterium contains (a) the enzymes of the citrate cycle, and (b) a mechanism for the oxidation of pyruvate to provide acetate for entrance into this cycle. It was the purpose of this thesis to test this hypothesis. The approaches used in this study are contained in section V.

IV. ASPECTS OF THE METABOLISM OF OTHER BACTERIA WHICH CLOSELY RELATE TO THE OBJECTIVES OF THIS RESEARCH

A. General.

The purpose here is to briefly review those aspects of general bacterial metabolism which relate directly to this thesis problem, i.e.

oxidation of pyruvate, the citrate cycle, and the glyoxylate bypass. The volume of literature on these subjects forbids an exhaustive treatment in this thesis; however, excellent reviews are available which discuss in depth the oxidation of pyruvate (47-49) as well as the citrate cycle and glyoxylate bypass (36, 37, 45). This presentation will attempt to outline the features and functions of this metabolism, and the approaches used to delineate it in some bacteria. It is intended that this will furnish a frame of reference for the experimental methods and discussion which follow.

B. Oxidation of pyruvate.

There appear to be several pathways for the oxidation of pyruvate in bacteria as studied in cell-free extract systems. Most of these pathways liberate CO_2 and all form acetate or an acetyl derivative, i.e. acetyl-P, acetyl-CoA (47). Considerable variety becomes apparent, however, when one compares the reaction mechanisms, coenzymes, and hydrogen transport involved.

1. Phosphoroclastic systems.

Some bacteria, e.g. *Clostridium butyricum* (52) and *Escherichia coli* (47) grown anaerobically, oxidize pyruvate by a mechanism often termed "phosphoroclastic" because of the role of phosphate in the breakdown process. The products are acetyl-P + formate (*E. coli*) or acetyl-P + CO_2 + 2H (*C. butyricum*). The reaction requires DPT, CoA, and Fe^{++}, but not α-lipoate or NAD (52). Acetyl-CoA may be formed before acetyl-P, but is usually converted rapidly to acetyl-P by phosphotransacetylase. Whereas *Clostridium* may either liberate the

hydrogen atoms as H_2 or reduce flavins (riboflavin, FMN, FAD) (52), *E. coli* couples the CO_2 and hydrogens to yield formate which may then be cleaved enzymatically to H_2 and CO_2 (47, 49).

A simpler version of the phosphoroclastic reaction is present in *Lactobacillus delbrueckii* (47). This pyruvate oxidase is a flavoprotein which requires DPT and apparently uses phosphate as the only acetyl acceptor. The products are acetyl-P, CO_2, and reduced flavoprotein enzyme; the latter can react with oxygen. Anaerobically, a second pyruvate molecule can be reduced to lactate by a flavoprotein lactate dehydrogenase.

2. The *Proteus* type of pyruvate oxidase.

Proteus vulgaris and other bacteria have yet another type of pyruvate oxidase (53). This enzyme system involves flavoprotein (47) and produces free acetate and CO_2 without the intermediate formation of acetyl-CoA or acetyl-P. The reaction requires DPT, and also involves cytochrome components b_1 as well as a_1 and a_2 which are autooxidizable in air. These cytochrome components greatly increase the rate of reduction of artificial electron acceptors such as ferricyanide (53). NAD, CoA, and α-lipoate are not required in this system (53).

3. The pyruvate dehydrogenase complex (PDC).

This is the essential mechanism for pyruvate oxidation in animal cells and is also a prominent one in many aerobic and facultative bacteria. *E. coli*, with a cytochrome-type terminal oxidase (49), and *Streptococcus faecalis*, which has a flavoprotein terminal oxidase (54), both use this reaction. PDC catalyzes the following overall

reaction:

$$CH_3COCOOH + NAD + CoASH \xrightarrow[Mg^{++}]{DPT,\alpha\text{-lipoate}} CH_3CO\cdot SCoA + NADH + H^+ + CO_2$$

This enzyme complex requires DPT, α-lipoate, CoA, FAD, and NAD for activity (49, 50). This multi-enzyme reaction is similar to that of the α-ketoglutarate dehydrogenase complex (KDC) to be discussed (see section 3-b-4), except that the first enzyme (E_a) in the PDC is pyruvate decarboxylase and not α-ketoglutarate decarboxylase as illustrated. In the reaction sequence (see section 3-b-4), R = $-CH_3$ for PDC and $-CH_2CH_2COOH$ for KDC. The three enzymes involved in the PDC are the following:

E_a: Pyruvate decarboxylase (coenzyme: DPT) decarboxylates the pyruvate to form acetyl-DPT + CO_2.

E_b: Lipoate reductase-transacetylase (coenzymes: α-lipoate, CoASH) first reductively acetylates the lipoyl residue, then transfers the acetyl group from the lipoyl to CoASH giving acetyl-CoA and dihydrolipoate.

E_c: Dihydrolipoate dehydrogenase (coenzymes: FAD, NAD) dehydrogenates the dihydrolipoate to give $FADH_2$ which then reduces NAD to yield NADH + H^+.

The PDC of *E. coli* has been isolated and characterized enzymically (50) and structurally by electron microscopy (51). In a beautiful correlation of enzymatic, electron microscopic, and other physical data, Koike and Reed (51) found PDC to be a highly organized multi-enzyme

system, a structured mosaic of three enzymes in a particle some 300 by 200 Å large. The component enzyme subunits appear to be efficiently arranged in the particle so as to implement consecutive reactions.

The pyruvate dehydrogenase complex reaction is an important one; the product acetyl-CoA is a common intermediate in many phases of metabolism, one of the most important being the citrate cycle which requires acetyl-CoA in the synthesis of citrate. The NADH formed in this reaction may be oxidized by cellular oxidases, which, if of the cytochrome type, are able to couple the electron transport to phosphorylation, giving ATP. Of the oxidases for pyruvate, only the PDC system is known to require α-lipoate as a coenzyme (47).

C. The citrate cycle and glyoxylate bypass.

1. Discovery.

The aerobic breakdown of carbohydrates, fatty acids, and some amino acids leads to the formation of a common C_2 intermediate, acetyl-CoA. The acetate moiety (as acetyl-CoA) is then oxidized to $CO_2 + H_2O$ through the citrate cycle pathway[1], yielding, by mediation of the electron transport sequence most of the energy necessary for aerobic life. Extensive work with animal extracts and tissues led to the discovery of this pathway, and it has also been found in plants and many microorganisms, with and without some deviations (37).

Krebs and Johnson first proposed the citric acid cycle in 1937 to account for the intermediary oxidation of carbohydrate in pigeon breast

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1. The term citrate cycle is used here for brevity; equivalent terms are the citric acid cycle, Krebs cycle, or tricarboxylic acid cycle.

muscle (38). Their original scheme was essentially similar to that presented in Figure 1, although information since 1937 has clarified (a) the complicated nature of the α-ketoglutarate dehydrogenase complex, and (b) that citrate arises from the condensation of oxalacetate with acetyl-CoA, and not from pyruvate as originally proposed. The crucial finding of Krebs and Johnson (38) was that pigeon breast tissue could synthesize citrate from oxalacetate and pyruvate, thereby linking the oxidation of the C_4 dicarboxylic acids to that of pyruvate; the cyclic nature of the pathway was then clear. It is now understood that not pyruvate, but acetyl-CoA, produced from carbohydrates through pyruvate or from the oxidation of fatty acids and other substances, is the co-substrate required with oxalacetate in the synthesis of citrate (55).

2. Evidence for the citrate cycle in animal tissues.

The following observations in muscle tissue support the concept of a citrate cycle (37):

(1) All of the component oxidations of the cycle have been demonstrated, and they proceed at rates sufficient to explain the maximum respiratory rate.

(2) The reactions of the cycle are the only known series of rapid respiratory reactions in muscle which give CO_2 and water as the main products.

(3) The effect of the intermediates on minced muscle tissue respiration is catalytic, not stoichiometric. This catalytic effect is satisfactorily explained by the citrate cycle concept; for each molecule of oxalacetate condensed into citrate, another is generated by

one turn of the cycle.

(4) Malonate specifically inhibits succinate dehydrogenase, a component enzyme of the citrate cycle. Muscle homogenates respiring in the presence of malonate accumulate succinate. This suggests that succinate dehydrogenase is indeed a component of the citrate cycle.

Citrate cycle activity has been found in the respiring tissues of all animals from mammals to protozoa, in plants, and in many bacteria (36, 37).

3. Features of the citrate cycle and glyoxylate bypass.

a. Scheme of these pathways.

A scheme of the citrate cycle and glyoxylate bypass as presently understood (37) is shown in Figure 1. Acetate is completely oxidized to CO_2 and water by the citrate cycle and the electron transport system (not illustrated). Details of the enzymes and their coenzymes are presented in the following section.

b. Enzymatic reactions of these pathways.

(1) Citrate synthase (condensing enzyme).

The first enzyme of the citrate cycle is citrate synthase which catalyzes the reversible condensation of acetyl-CoA and oxalacetate to give citrate; the reaction equilibrium greatly favors citrate (37).

$$\text{acetyl-CoA} + \text{oxalacetate} + H_2O \rightleftharpoons \text{citrate} + \text{CoASH} + H^+$$

(2) Aconitase (aconitate hydratase).

Aconitase catalyzes the reversible dehydration of either citrate or isocitrate to cis-aconitate.

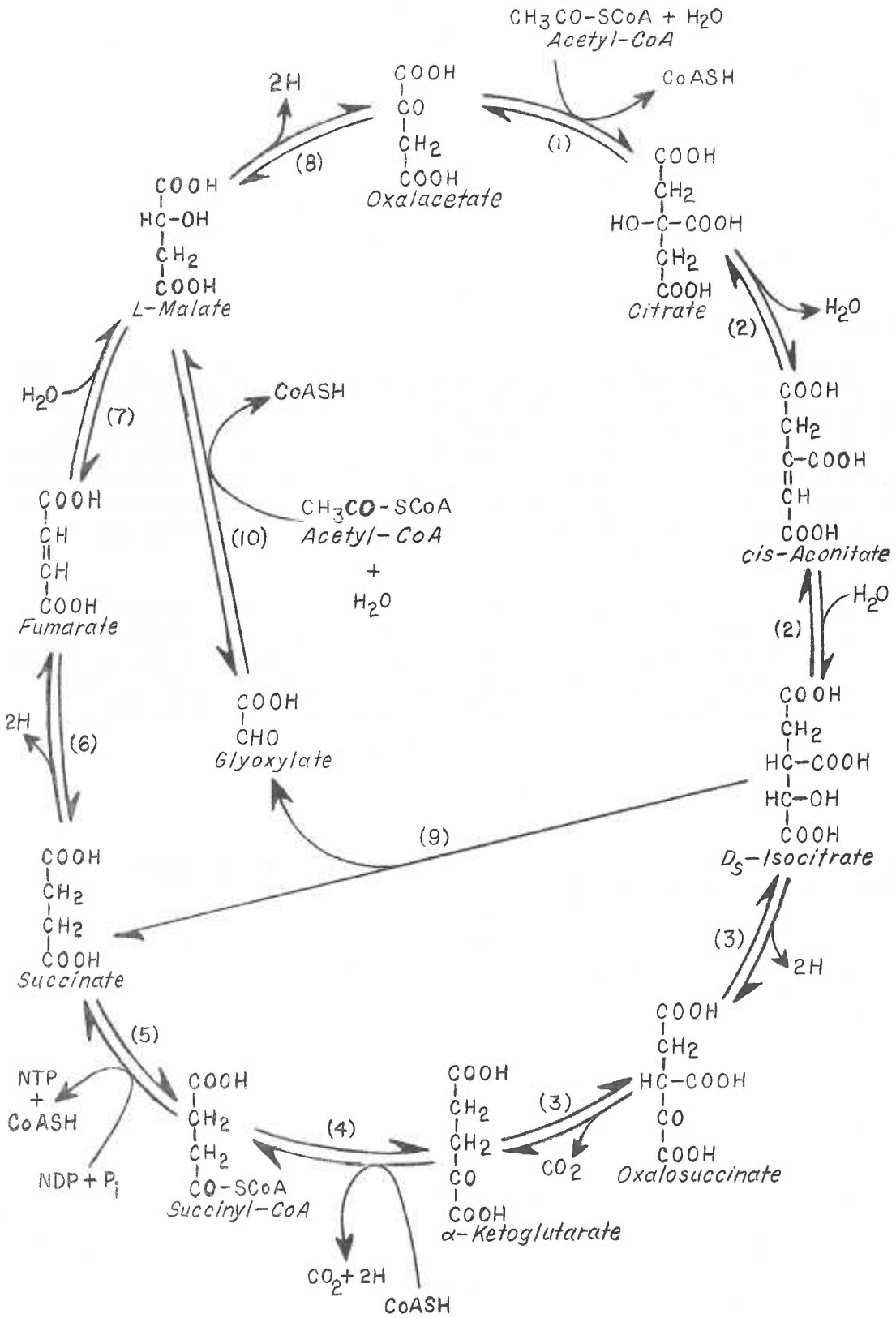
Figure 1.

Scheme of the citrate cycle and glyoxylate bypass (37). The enzymes which catalyze the component reactions of the citrate cycle are: (1) citrate synthase (condensing enzyme); (2) aconitase (aconitate hydratase); (3) isocitrate dehydrogenase; (4) α-ketoglutarate dehydrogenase complex; (5) succinate thiokinase; (6) succinate dehydrogenase; (7) fumarase (fumarate hydratase); and (8) malate dehydrogenase. The glyoxylate bypass enzymes are: (9) isocitratase (isocitrate lyase) and (10) malate synthase.

The scheme of the citrate cycle shows the complete oxidation of acetate (as acetyl-CoA) yielding CO_2, H_2O, and hydrogen atoms. Four pairs of hydrogen atoms are accepted by various coenzymes (detailed in the text) which may react with the electron transport chain to form water. The net balance for the oxidation of acetic acid is then:

$$CH_3COOH + 2O_2 \longrightarrow 2CO_2 + 2H_2O$$

The formulas shown are those of the free acids, but are named as the anions (salts) for convenience.


CH3CO-SCoA + H2O
Acetyl-CoA
CoASH
(1)
COOH
CO
CH2
COOH
Oxalacetate
2H
(8)
COOH
HC-OH
CH2
COOH
L-Malate
COOH
CH2
HO-C-COOH
CH2
COOH
Citrate
(2)
H2O
H2O
(7)
CoASH
(10)
CH3CO-SCoA
Acetyl-CoA
+
H2O
COOH
CH2
C-COOH
CH
COOH
cis-Aconitate
COOH
CH
CH
COOH
Fumarate
(2)
H2O
2H
(6)
COOH
CHO
Glyoxylate
COOH
CH2
HC-COOH
HC-OH
COOH
Ds-Isocitrate
(9)
COOH
CH2
CH2
COOH
Succinate
(3)
2H
COOH
CH2
HC-COOH
CO
COOH
Oxalosuccinate
(5)
NTP
+
CoASH
NDP + Pi
COOH
CH2
CH2
CO-SCoA
Succinyl-CoA
(4)
(3)
CO2
COOH
CH2
CH2
CO
COOH
α-Ketoglutarate
CO2 + 2H
CoASH

$$\text{citrate} \rightleftharpoons \text{cis-aconitate} + H_2O \rightleftharpoons \text{isocitrate}$$

It has the unusual property of catalyzing two reactions; evidence obtained with purified pig-heart aconitase indicates that both reactions are catalyzed by one enzyme (37, 55). The equilibrium favors citrate; at 25 C and pH 7.4, the equilibrium mixture contains 90.9% citrate, 6.2% isocitrate, and 2.9% cis-aconitate. Aconitase is stabilized and activated by Fe^{++} and reducing agents (37, 55).

(3) Isocitrate dehydrogenase.

Isocitrate dehydrogenases catalyze the general reaction,

$$\text{isocitrate} + \text{NAD(P)} \xrightleftharpoons{M^{++}} \alpha\text{-ketoglutarate} + CO_2 + \text{NAD(P)H} + H^+$$

Several types of enzyme occur. All isocitrate dehydrogenases require a divalent cation (Mg^{++} or Mn^{++}), but some enzymes require NAD, others NADP; the NAD-dependent enzyme may also require AMP or ADP (37). Animal tissues generally contain both NAD- and NADP-specific enzymes, while bacteria usually contain only the NADP-specific enzyme; of 28 diverse bacterial species tested for isocitrate dehydrogenase activity, only one, *Xanthomonas pruni*, contained the NAD-specific enzyme (56). Yeast possess two isocitrate dehydrogenases; one is NADP-specific and the other, NAD-specific, also requires AMP (37). Mammalian NAD-specific enzyme characteristically requires ADP. The usual isocitrate dehydrogenase of bacteria requires NADP and either Mg^{++} or Mn^{++}. A two-step reaction is catalyzed as follows (37);

$$\text{(a) isocitrate} + \text{NADP} \rightleftharpoons \text{oxalosuccinate} + \text{NADPH} + H^+$$

(b) oxalosuccinate + H+ $\xrightleftharpoons{M^{++}}$ α-ketoglutarate + CO_2.

The first step requires NADP but no metal ion, whereas the second step requires only the metal ion.

(4) α-ketoglutarate dehydrogenase complex (KDC).

The overall reaction below is brought about by the multi-enzyme α-ketoglutarate dehydrogenase complex (KDC).

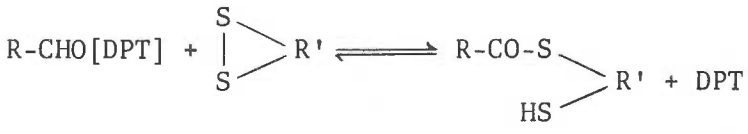
α-ketoglutarate + CoASH + NAD $\rightleftharpoons$ succinyl-CoA + CO_2 + NADH + H^+

This complex resembles the pyruvate dehydrogenase complex (PDC) already described; it consists of three enzyme components which require 5 co-enzymes. The overall reaction entails a number of steps as illustrated below (37, 55).

(a) α-ketoglutarate decarboxylase (coenzyme: DPT).

$HOOCCH_2CH_2COCOOH + DPT \rightleftharpoons HOOCCH_2CH_2CHO[DPT] + CO_2$

(b) lipoyl reductase-transsuccinylase (coenzymes: α-lipoate, CoASH) catalyzes two reactions,



and,

where R = $-CH_2CH_2COOH$ in the case of KDC or $-CH_3$ in the case of PDC, and R' = $-CH_2CH_2\underset{|}{C}H(CH_2)_4COOH$.

(c) Dihydrolipoate dehydrogenase (coenzymes: FAD, NAD) also catalyzes a two-step reaction,

and

$$FADH_2 + NAD \rightleftharpoons FAD + NADH + H^+.$$

The KDC of *E. coli* has been isolated as a soluble complex of three enzyme components (a, b, and c above); the three enzymes were separated, purified, and then recombined to form a KDC which contained about half the original activity (57).

(5) Succinate thiokinase (succinyl-CoA synthetase).

The succinyl-CoA formed by KDC may be converted to succinate by succinate thiokinase (55). The general reaction is as illustrated below.

$$\text{succinyl-CoA} + \text{NDP} + \text{Pi} \rightleftharpoons \text{succinate} + \text{NTP} + \text{CoASH}$$

Mammalian enzymes require IDP or GDP for activity while those from *E. coli* or spinach require ADP (37).

(6) Succinate dehydrogenase.

Succinate dehydrogenase catalyzes the reversible dehydrogenation of succinate to fumarate as expressed below.

$$\text{succinate} + \text{FAD} \rightleftharpoons \text{fumarate} + FADH_2$$

The enzyme contains non-heme iron and FAD in a ratio of about 4:1 (37). In the purified soluble state, succinate dehydrogenase will reduce only a limited number of artificial electron acceptors, e.g. phenazine

methosulfate or ferricyanide, but in the native particulate state it reacts readily with natural electron acceptors, e.g. cytochrome of the b-type, to form a succinoxidase system; coenzyme Q may be the physiological acceptor of reducing equivalent (37, 55). Purified succinate dehydrogenases from animals, yeast, or *Micrococcus lactilyticus* also catalyze the reduction of fumarate to succinate, but these preparations evidently do not contain a fumarate reductase distinct from succinate dehydrogenase (37). In contrast, *E. coli* shows two enzymes, distinct and separable by mutation, each of which can catalyze either succinate dehydrogenation or fumarate reduction (58). Physiologically, one enzyme apparently acts as a succinate dehydrogenase and the other as a fumarate reductase, the latter when anaerobic growth conditions dictate a need for fumarate to serve as a hydrogen acceptor (58).

(7) Fumarase (fumarate hydratase).

Fumarase catalyzes the reversible hydration of fumarate to L-malate as illustrated.

$$\text{fumarate} + H_2O \rightleftharpoons \text{L-malate}$$

Water is added to fumarate in the trans position. The equilibrium favors L-malate (37).

(8) Malate dehydrogenase.

Malate dehydrogenase catalyzes the reversible dehydrogenation of L-malate to give oxalacetate as shown.

$$\text{L-malate} + \text{NAD} \rightleftharpoons \text{oxalacetate} + \text{NADH} + H^+$$

NAD is required as a coenzyme (37). Although the reaction equilibrium

strongly favors L-malate, physiological concentrations of oxalacetate are so low as to permit the reaction to proceed from left to right (37).

The oxalacetate produced from L-malate by malate dehydrogenase may be condensed with acetyl-CoA by citrate synthase to give citrate, and the citrate cycle is complete, having turned once.

(9) Isocitratase (isocitrate lyase).

Isocitrate is cleaved into succinate and glyoxylate by action of isocitratase as shown.

$$\text{isocitrate} \rightleftharpoons \text{succinate} + \text{glyoxylate}$$

The reaction is reversible and favors the formation of succinate and glyoxylate (75).

(10) Malate synthase.

Malate synthase condenses acetyl-CoA and glyoxylate to form malate as represented below.

$$\text{acetyl-CoA} + \text{glyoxylate} + H_2O \rightleftharpoons \text{malate} + \text{CoASH}$$

All the enzymes of the citrate cycle ([1]-[8] above) are to be found in animal cell mitochondria, although many of them are also present in the extramitochondrial portions of the cell (37). Bacteria lack mitochondria. In bacteria, the enzymes mentioned above ([1]-[10]), in general, appear to be either soluble or solubilized easily; however, succinate dehydrogenase and fumarate reductase are membrane-bound in facultative *E. coli*, while obligate aerobes contain succinate dehydrogenase and malate dehydrogenase which are membrane bound (32,

46, 71).

c. Functions of the citrate cycle.

The citrate cycle makes two recognized contributions to the overall biochemistry of the cell: the production of energy (catabolic) and the biosynthesis of other cell constituents (anabolic) (36, 55). It thus has a dual, or in the terminology of Davis (59), an "amphibolic" nature.

(1) Catabolism and the production of energy.

Oxidation of the reduced coenzymes of the citrate cycle dehydrogenases by the electron transport sequence is ordinarily coupled to a phosphorylation of ADP to yield ATP, i.e. oxidative phosphorylation. Thus the cell produces energy by oxidizing acetate to CO_2 and water; each turn of the cycle generates some 12 ATP molecules under proper conditions (55). In fact, this oxidation of reduced coenzymes is necessary for the continued turning of the citrate cycle because oxidized coenzymes are required by the several dehydrogenases; an accumulation of reduced coenzymes would bring the cycle to a standstill (55).

One enzyme of the citrate cycle, i.e. succinate thiokinase, is coupled to the phosphorylation of a nucleoside diphosphate (substrate level phosphorylation) to give a nucleoside triphosphate. This reaction is energetically useful, but quite limited in comparison to the quantity of energy derived from oxidative phosphorylation (55).

(2) Biosynthetic role of the citrate cycle.

The citrate cycle forms a cellular metabolic hub. Its intermediates are substrates for a host of important ancillary reactions: (a) transamination or reductive amination of α-ketoglutarate to yield

glutamate, (b) transamination of oxalacetate or reductive amination of fumarate to give aspartate, (c) oxalacetate may give rise to glucose and other sugars through phosphoenolpyruvate and a reversed glycolytic pathway, and (d) succinyl-CoA leads to porphyrin synthesis (55). It will be recognized, however, that the drain of intermediates from the citrate cycle for biosynthetic purposes effectively stops the cycle unless such intermediates are replenished by other reactions. Cells growing on glucose or other substrate degradable to pyruvate or phosphoenolpyruvate may replace these intermediates by fixation of CO_2 to yield the C_4 compounds oxalacetate or malate (72). On the contrary, microorganisms grown on C_2 substrates, i.e. acetate, do not have important CO_2 acceptors such as pyruvate or phosphoenolpyruvate available for C_4 synthesis and are forced to use other mechanisms to supply these C_4 compounds; such needs are met by means of the glyoxylate bypass of the citrate cycle, often termed the glyoxylate cycle, which is a modified citrate cycle (73).

d. Features and function of the glyoxylate bypass.

This pathway deviates from the conventional citrate cycle so as to bypass the two oxidative decarboxylations (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) which liberate CO_2 (Figure 1). The key enzymes of the glyoxylate bypass are isocitratase which splits isocitrate to succinate and glyoxylate, and malate synthase which condenses acetyl-CoA with glyoxylate to give malate. The reaction sequence catalyzed by citrate synthase, aconitase, isocitratase, malate synthase, and malate dehydrogenase create a glyoxylate cycle, which, by avoiding the loss of carbon (CO_2) as seen in the

citrate cycle, leads to a net increase in the organic acid content of the cell (37, 74). Two reducing equivalents are transferred to NAD by malate dehydrogenase; this reaction and malate synthase provide the driving force for the glyoxylate cycle (74).

The glyoxylate bypass plays a necessary role in the utilization of acetate (or other C_2 compounds such as ethanol) for growth because it permits the net synthesis of C_4 intermediates from C_2 compounds. For example, *E. coli* mutants devoid of isocitratase are unable to grow on acetate as do the wild type, but grow well on glucose or a usable citrate cycle intermediate. Revertants of these mutants simultaneously regain the ability to grow on acetate and to synthesize isocitratase (60). High levels of isocitratase and malate synthase are normally found in cells grown on acetate or other C_2 compounds, but lower enzyme levels are usually present in cells grown on glucose or intermediates of the citrate cycle (37). The isotope distribution in amino acids of *Pseudomonas* grown on labelled acetate indicates that both the citrate cycle and glyoxylate cycle are metabolically important under these conditions (37).

4. Approaches used to demonstrate the citrate cycle in bacteria.

The rapid oxidation of citrate cycle intermediates by animal cells is important evidence that this cycle is their main pathway for the oxidation of acetate (45). Bacteria, however, possess a cytoplasmic membrane which is often impermeable to such compounds as citrate cycle intermediates; early workers erroneously concluded that the failure of bacteria such as *E. coli* to oxidize citrate indicated the absence of citrate cycle activity (45). Addition of such intermediates

to impermeable bacteria would not be expected to stimulate oxygen consumption. *E. coli*, as well as certain other species, is unable either to grow on citrate as a sole source of carbon or to oxidize citrate. Extracts of such bacteria, however, usually oxidize citrate or related compounds, indicating that cellular impermeability prevents the oxidation by intact cells. Many bacteria are also impermeable to malonate; certain other bacteria decarboxylate this inhibitor. Failure of malonate to inhibit bacterial respiration therefore does not imply the absence of succinate dehydrogenase and citrate cycle activity (45).

Once the permeability problem involved with bacteria was realized, steps were taken to solve it and to demonstrate the reactions and usefulness of the citrate cycle. Lara and Stokes (61), for instance, discovered that resting *E. coli* suspensions could not oxidize citrate. However, dried cells readily oxidized citrate; in one experiment citrate was completely oxidized to CO_2 and H_2O. Similarly, cell-free extracts from *E. coli* oxidized citrate (62). Subsequently, the citrate cycle enzymes in *E. coli* have all been detected and separated; their relative abundance has been estimated and the coenzymes required by each enzyme determined (63).

The oxidation of radioactive acetate by *E. coli* suspensions indicates the importance of the citrate cycle pathway to this bacterium (64). Resting *E. coli* were allowed to oxidize acetate-2-C^{14}, after which the cells were broken; intracellular citrate cycle intermediates were first separated, then their concentration and specific activities quantitated. The results showed that citrate, α-ketoglutarate, succinate, fumarate, malate, acetate, and CO_2 all contained considerable

radioactivity, sufficient radioactivity in fact to account for the original acetate-2-C^{14}. The citrate cycle was thereby indicated as an important mechanism for the oxidation of acetate (45, 64).

Citrate cycle metabolism has also been studied a great deal in *Pseudomonas*. Barrett and Kallio studied the capacity of *P. fluorescens* extracts to carry out certain reactions of the citrate cycle (65). By identification of substrate and product, they found these conversions: citrate $\longrightarrow$ α-ketoglutarate, α-ketoglutarate $\longrightarrow$ succinate, citrate $\longrightarrow$ succinate, acetate + oxalacetate $\longrightarrow$ citrate, cis-aconitate $\longrightarrow$ citrate, and isocitrate + NADP $\longrightarrow$ α-ketoglutarate + NADPH + H^+. In addition, intact cells oxidized each citrate cycle intermediate tested, including citrate. This study (65) not only demonstrated many citrate cycle reactions, but also showed that this *Pseudomonas* differed from *E. coli* in being permeable to citrate.

In *E. coli*, it has been shown that the citrate cycle is important as a means to supply carbon skeletons for amino acid biosynthesis (45, 76); more than 50 percent of the carbon skeletons required for protein synthesis were provided by the citrate cycle (76). Aspartate and glutamate, as well as the amino acids derived from them, contained considerable radioactivity (C^{14}) incorporated from either glucose or acetate (76).

The various reports just mentioned indicate that the citrate cycle serves essentially similar dual functions in both animal and bacterial cells.

5. Factors affecting the syntheses of citrate cycle enzymes in bacteria.

a. Carbon source.

The syntheses of citrate cycle enzymes is often a function of the carbon source during bacterial growth. It has been observed repeatedly in a variety of bacteria that growth on glucose yields lower levels of citrate cycle enzymes than growth on substrates such as intermediates of the citrate cycle. For example, Halpern (66) found that cell-free extracts from *E. coli* grown on succinate oxidized succinate, malate, α-ketoglutarate, pyruvate, or lactate at rates two to three times higher than similar glucose-grown cells. Similarly, Gray et al. (43) found that the specific activity of the citrate cycle enzymes of *E. coli* grown on either malate, pyruvate, or glycerol was from 2 to 10-fold higher than from glucose-grown cells. This glucose repression (effect) on the formation of citrate cycle enzymes has also been noted in *P. natriegens* (67).

Considerable variation is also found in the enzymic or oxidative patterns between bacteria which have been grown on different citrate cycle substrates. A given bacterium will ordinarily show certain patterns of whole cell oxidations of various intermediates of the citrate cycle in relation to the carbon source. For example, Barrett and Kallio (65) found that citrate-grown *P. fluorescens* oxidized citrate with no lag period while acetate was oxidized only after a lag. Conversely, acetate-grown cells oxidized acetate with no lag, but a lag period was seen in the oxidation of citrate. However, essentially equal activities of the component citrate cycle enzymes were found in

cell-free extracts of citrate- or acetate-grown cells; this suggested that permeability was involved in the lag phenomenon (65). Clarke and Meadow (42) also found a distinctive adaptive pattern in *P. aeruginosa* which differed somewhat from that of *P. fluorescens*. On the basis of this work, and that of others, these authors (42) proposed that most if not all citrate cycle intermediates enter the bacterial cell by means of specific permeases which may be either constitutive or induced. They interpreted the lag period in the cellular oxidative response as being the time required for permease synthesis. According to their interpretation the absence of lag indicates the presence of adequate permease, a short lag (5-15 min) indicates only a little permease, and if no oxidation occurs although the appropriate intracellular enzymes can be demonstrated, then no permease is present. The relief of the lag period was inhibited by chloramphenicol or 8-azaguanine, but not penicillin; this suggests that protein synthesis was involved in the penetration of these intermediates into the bacteria, and that permeases were involved (42).

b. Biosynthetic demand during growth.

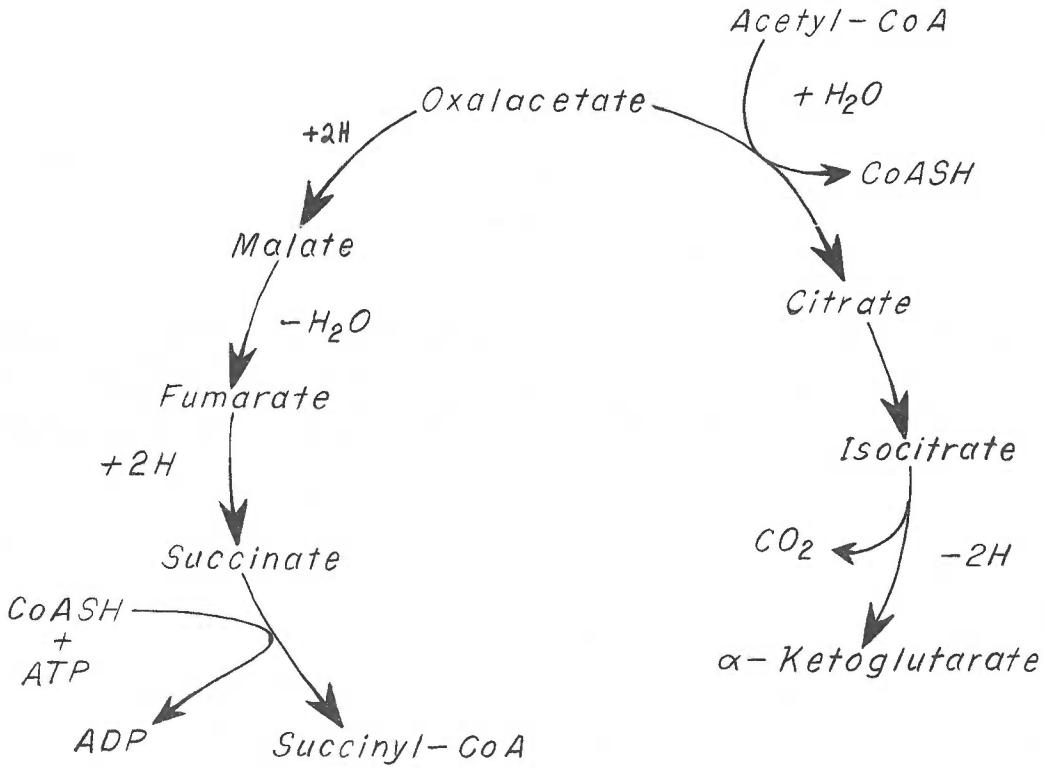
The citrate cycle would be expected to function if bacterial growth in synthetic media forced the synthesis of key amino acids such as glutamate or aspartate, whether or not glucose was the carbon source. Gray et al. (43) compared the activity of citrate cycle enzymes in *E. coli* grown in synthetic medium with that of cells grown in complex (acid hydrolyzed casein) medium, both of them including glucose. The citrate cycle enzymes were an average of 3.6 times more active from cells grown in synthetic medium than in complex medium.

This indicates that glucose repression of citrate cycle enzyme synthesis is partially relieved when the cycle is needed for biosynthetic purposes (43).

c. Effect of oxygen.

As early as 1937, Krebs clearly showed that *E. coli* could use fumarate effectively as a hydrogen acceptor in the anaerobic catabolism of such compounds as malate, lactate, and acetate (69). One might predict that the citrate cycle could operate in the absence of oxygen provided the cell possessed mechanisms for the dehydrogenation of those reduced coenzymes coupled to the citrate cycle enzymes. This has been confirmed experimentally, since most citrate cycle enzymes are found in anaerobically grown facultative bacteria, e.g. *E. coli* and *Pasteurella pestis*, but in lower concentration than in aerobically grown cells. Englesberg and Levy (68) found that the level of most citrate cycle enzymes in aerobically grown *P. pestis* was from 3 (malate dehydrogenase) to 61 (isocitrate dehydrogenase) times higher than in anaerobically-grown cells; however, succinate dehydrogenase concentration was similar in both cases. Comparable differences have been found in *E. coli* grown either on complex or synthetic medium. Gray et al. (43) discovered that the concentration of citrate cycle enzymes was from 2.5 (fumarase) to 20.4 (malate dehydrogenase) fold greater in extracts of aerobically-grown than in extracts of anaerobically-grown *E. coli*, harvested from synthetic medium plus glucose. However, whereas extracts of *E. coli* grown anaerobically on complex medium plus glucose contained slight α-ketoglutarate dehydrogenase activity, those from synthetic medium plus glucose contained none (43).

A special case of the citrate cycle may exist in bacteria growing anaerobically in synthetic medium. Certain facultative bacteria, i.e. *E. coli*, when grown anaerobically in synthetic medium seem not to carry out a citrate cycle as such, but use a modified, split, non-cyclic pathway needed primarily for biosynthesis (Figure 2). Amarsingham and Davis (70) found that α-ketoglutarate dehydrogenase was not synthesized by *E. coli* grown anaerobically in synthetic medium plus glucose, but was formed at high levels aerobically. Very similar results were obtained by Gray et al. (43) with *E. coli* also grown anaerobically in synthetic medium plus glucose. Their work (43) showed that while anaerobiosis diminished the concentration of most citrate cycle enzymes, α-ketoglutarate dehydrogenase was absent under these conditions, yet present in aerobic extracts. These data suggest that *E. coli* grown under these conditions uses a non-cyclic pathway as shown in Figure 2. This pathway consists of a "left" reductive and "right" oxidative portion of the citrate cycle, but lacks α-ketoglutarate dehydrogenase which is apparently absent under these anaerobic conditions. Such a pathway provides certain intermediates needed for biosynthesis. This pathway would require an active fumarate reductase and such has been demonstrated. *E. coli*, grown anaerobically on synthetic medium, contains an active fumarate reductase which is distinct from succinate dehydrogenase (58). Because the fumarate reductase showed a much lower K_m for fumarate than succinate (1.7×10^{-5}M vs. 1.0×10^{-3}M) it is probably important physiologically; this is also suggested by the fact that the succinate dehydrogenase of these anaerobically grown cells is much less active than the fumarate


Acetyl – CoA
+ H_2O
Oxalacetate
+2H
CoASH
Malate
Citrate
$-H_2O$
Fumarate
Isocitrate
+2H
CO_2
$-2H$
Succinate
CoASH
+
ATP
α – Ketoglutarate
ADP
Succinyl – CoA

reductase, a situation just opposite of that observed in aerobically grown cells (58). An active fumarate reductase is undoubtedly important to a non-cyclic pathway (Figure 2) to accept reducing equivalents and to assure the production of succinate.

In summary, then, at least three factors influence the biosyntheses of citrate cycle enzymes in bacteria: oxygen, the carbon source during growth, and the balance between catabolic and anabolic requirements imposed by the nutritional environment.

V. OBJECTIVES OF THIS THESIS AND APPROACHES TO THE PROBLEM

The overall objectives of this research were to determine whether *Listeria* carries out the citrate cycle pathway and certain closely related metabolism, i.e. the oxidation of pyruvate and the glyoxylate bypass.

As a first research step, the effect of glucose, iron, and aeration on the growth response of *Listeria* was determined. A defined medium was then designed which contained only those nutrients necessary for rapid growth; it was used to grow the *Listeria* in subsequent studies.

Listeria was tested for its capacity to utilize either pyruvate or citrate cycle intermediates as sources of carbon and energy; the growth response was determined using these compounds alone or in conjunction with a low concentration of glucose. A study was then made of the oxidative response of resting *Listeria* suspensions toward pyruvate or the several intermediates of the citrate cycle.

The major objective of this research was to determine whether cell-free extracts of *Listeria* contain the enzymes of pyruvate oxidation, the citrate cycle, and the glyoxylate bypass. It was also intended that the growth characteristics, oxidative capacity, and enzymatic activities would help clarify whether *Listeria*, as a facultative bacterium, prefers an aerobic environment.

MATERIALS

Chemicals - source, purity, and storage conditions.

The chemicals used in this research are listed by supplier. Chemical purity is that specified by the manufacturer. Unless stated otherwise, these chemicals were stored in the original containers at room temperature; special storage conditions are as recommended by the manufacturer.

1. Nutritional Biochemicals Corporation, Cleveland, Ohio. Unless designated otherwise, quality was the "highest purity commercially available".

   a. L-cysteine·HCl; L-leucine (99.5%); DL-isoleucine; DL-valine; L-arginine·HCl; L-histidine·HCl (monohydrate, C.P.); DL-methionine; DL-tryptophan; L-glutamine (97%); riboflavin; thiamine·HCl; D-biotin; succinate, sodium; DL-isocitrate, trisodium; L-malic acid; fumarate, monopotassium; glycine; caproic acid; and oleic acid.

   b. DL-thiotic acid (α-lipoic acid). This was refrigerated.

2. Sigma Chemical Company, St. Louis, Mo.

   a. Nicotinamide adenine dinucleotide (NAD), grade III, 98%; nicotinamide adenine dinucleotide phosphate (NADP), sodium, Sigma grade (98-100%); reduced nicotinamide adenine dinucleotide phosphate (NADPH), tetrasodium, type I, 95%; flavin adenine dinucleotide (FAD), disodium, grade III:; flavin mononucleotide (FMN), sodium, commercial grade; coenzyme A (CoA), trilithium, grade 1-L, 85%; S-acetyl coenzyme A (acetyl-CoA), sodium, grade II, 70%; diphosphothiamine (DPT), 98%; cis-oxalacetic acid, grade I; and glyoxylic acid, sodium. The above

chemicals were stored frozen and desiccated. Care was taken to protect DPT and NADPH from light.

b. Pyruvic acid, sodium, type II, 99+%; α-ketoglutaric acid; and bovine serum albumin (BSA), lyophilized crystals. These chemicals were stored under desiccation in the refrigerator.

c. Reduced nicotinamide adenine dinucleotide (NADH), disodium, 98%; this was stored desiccated at room temperature.

d. Phenazine methosulfate; 2,6-dichlorophenol indophenol (DCPIP), sodium, grade I; tris (hydroxymethyl) aminomethane (Tris), Sigma 7-9 grade, 99.0-99.5%; imidazole, grade I; p-iodonitrotetrazolium violet (INT), grade III.

3. Calbiochem, Los Angeles, Calif.

a. Reduced glutathione (GSH), A grade, 99.86%; this was refrigerated.

b. Nitrilotriacetic acid, C grade, 99.5%.

4. Fisher Scientific Company, Fairlawn, N. J.

Ethylenediaminetetraacetic acid (EDTA), disodium, reagent grade.

5. Mallinckrodt Chemical Works, St. Louis, Mo.

a. These chemicals were reagent grade unless noted otherwise: magnesium sulfate heptahydrate; potassium cyanide; manganese chloride; metaphorphoric acid; potassium acid phthalate; sodium hydroxide; potassium iodide; cupric sulfate pentahydrate; ferric chloride hexahydrate; and ferric ammonium citrate, U.S.P. grade.

b. Hydrogen peroxide, 3%, and 30%; this was refrigerated.

6. Merck and Company, Rahway, N. J.

These chemicals were of reagent grade: citric acid, sodium; D-glucose; ammonium chloride; ammonium sulfate; magnesium chloride; sulfuric acid; potassium bromide; potassium dihydrogen phosphate; sodium dihydrogen phosphate monohydrate; and potassium sodium tartrate.

7. Matheson Coleman and Bell, Cincinnati, Ohio.

These chemicals were of reagent grade: phenylhydrazine·HCl; n-heptane; potassium permanganate; dipotassium hydrogen phosphate; trichloroacetic acid; and potassium hydroxide.

8. J. T. Baker Chemical Company, Phillipsburg, N. J.

Sodium iodide, and hydrochloric acid; both were of reagent grade.

9. Industrial Air Products, Portland, Oregon.

Nitrogen gas, pre-purified.

10. Baltimore Biological Laboratories (BBL), Baltimore, Md.

Trypticase; gelatin.

11. Difco Laboratories, Detroit, Michigan.

Tryptose agar; blood agar base; brain-heart infusion (BHI); tryptose.

12. Krishell Laboratories, Portland, Oregon.

Protamine sulfate.

METHODS

I. BACTERIA USED IN THIS STUDY

a. Strains of *Listeria* used in this study.

(1) Strain A4413, serotype 4b.

This *Listeria* strain has been studied by many workers and found to be virulent (20, 22, 24, 25) and of the smooth (S) type. Its nutritional requirements are better understood than most strains (20-22, 24, 25), and it can probably be regarded as a "typical" virulent *Listeria*; it was studied most extensively in this research.

(2) Strain 9037-7, serotype unknown.

This *Listeria* has been found to be avirulent (20, 22, 25) and is nutritionally almost as well characterized as strain A4413 (20, 22). In contrast to strain A4413, which manifests smooth colony characteristics, strain 9037-7 is a rough (R) type (20). In this research, strain 9037-7 was used primarily for comparison with A4413 in the determination of acceptable carbon sources.

b. Other bacteria used in this study.

Escherichia coli K-12 (λ) was used in certain experiments as a positive control. This strain was graciously furnished by Dr. E. L. Oginsky.

II. ORIGIN, GROWTH, AND PRESERVATION OF STOCK *LISTERIA* CULTURES

a. Origin.

Listeria strains A4413 and 9037-7 were kindly furnished by Dr. M. E. Friedman, U. S. Army Biological Laboratories, Fort Detrick,

Frederick, Md. Strain A4413 arrived in the lyophilized state and was reconstituted with sterile distilled water. Strain 9037-7 was received as a trypticase soy agar slant culture.

b. Growth.

Upon receipt, the *Listeria* cultures and *E. coli* K-12(λ) were transferred to tryptose agar slants (Difco) in screw cap tubes (16 x 150 mm). The entire surface of the slants was streaked to give confluent growth. With their caps slightly loosened, the slants were incubated at 37 C for 16 hr.

c. Preservation.

Immediately following the 16 hr growth period, the slant cultures of *Listeria* were quick frozen by immersion in an ethanol-dry ice bath and then stored in the freezer at -20 C. Routinely, batches of 50 or more slants were thus treated. No difficulty was ever encountered in recovering viable *Listeria* from frozen cultures for periods of at least 6 months. Before freezing, each culture was visually inspected for gross contamination and several cultures from each batch were selected randomly and subcultured to tryptose agar plates. After incubation at 37 C for 24 hr, colony characteristics, gram-reaction, morphology, and motility (after 25 C incubation) were determined to check the culture's purity. Frozen slant cultures of *Listeria* were thawed in a water bath (37 C) for 5 min before being subcultured.

III. DETERMINATION OF THE COLONY CHARACTERISTICS, MORPHOLOGY, AND MOTILITY OF *LISTERIA*

The colony characteristics of *Listeria* were observed after growth

on tryptose agar or blood agar plates (Difco Blood Agar Base + 6% human blood) at 37 C for 24 to 48 hr. Bacterial morphology was studied microscopically at 1000X in gram-stained smears. Motility was observed at 450X by a hanging drop technique (77); a tryptose broth (Difco) culture, grown at 25 C for 18 hr, was diluted 1/10 in tryptose broth for this purpose.

IV. DETERMINATION OF THE GROWTH RESPONSE OF *LISTERIA* A4413

A. The ability of the glucose concentration to affect *Listeria* growth.

The object of this experiment was to determine the effect of glucose concentration on the rate and extent of *Listeria* A4413 growth in a complex medium.

1. The medium employed was a phosphate-buffered solution of trypticase, made up as follows: trypticase (BBL), 20.0 gm; K_2HPO_4, 9.0 gm; and $NaH_2PO_4 \cdot H_2O$, 1.0 gm, were dissolved in 850 ml of distilled water and autoclaved at 15 lb for 15 min. The pH was 7.4. The medium was thus concentrated 20/17 to allow for the addition of glucose and inoculum.

2. Pyrex test tubes (18 x 150 mm) were selected for optimal uniformity and used as cuvettes for the turbidimetric growth assay. Buffered trypticase medium, 5.1 ml, was then combined in each tube with 0.6 ml of glucose (10X concentrated, sterilized by Millipore filtration, Swinny type, 0.45 μ [Millipore Filter Corp., Bedford, Mass.]), to give final glucose concentrations of 0.05, 0.1, 0.2, 0.5, and 1.0%. Glucose-free control tubes received distilled water instead of glucose. Duplicate growth tubes were used to test the effect of each glucose

concentration.

3. Inoculum for the growth tubes was prepared as follows:

a. Seventy ml of buffered trypticase supplemented with 0.1% glucose was inoculated with stock *Listeria* A4413 and incubated stationarily at 37 C for 10 hr.

b. Twenty ml of this culture was transferred to a 50 ml polycarbonate centrifuge tube (International Equipment Co., Boston, Mass.) and centrifuged cold (≃ 4 C) in a refrigerated centrifuge, model RC-2 (Ivan Sorvall, Norwalk, Conn.) at 3,500 X g for 15 min to pack the *Listeria* into a pellet.

c. Supernatant fluid was removed from the *Listeria* cell pellet by a vacuum apparatus: an electric vacuum pump (Scientific Supplies Co., Portland, Ore.) applied suction to a Pasteur pipette by means of latex tubing with an Erlenmeyer flask trap between to catch the fluid.

d. The cell pellet was washed once in an equal volume of sterile cold 0.1M potassium phosphate buffer, pH 7.2. The cell pellet was first thoroughly dispersed in 5 ml of buffer, then sufficient buffer was added to equal the volume of culture centrifuged. The tube was centrifuged (step b) and the supernatant wash fluid suctioned off (step c) to give a washed cell pellet.

e. The washed cell pellet was resuspended in buffer to an optical density (620 mμ) of 0.50 as read against a buffer blank in a 13 mm cuvette with the Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N. Y.). This *Listeria* suspension was used as inoculum; it was equivalent to ≃ 2.6 X 10^9 cells/ml.

4. All growth tubes (step 2) except a blank received 0.3 ml of inoculum (step e) which was equivalent to ≃ 9 X 10^8 cells/tube. After mixing with a Vortex (Scientific Industries, Inc., Queens Village, N. Y.) the growth tubes were incubated stationarily at 37 C.

5. At time zero and at hourly intervals up to 6 hr and at 8, 10, 11, and 24 hr, the turbidity (optical density at 620 mμ) of each tube was read against a medium blank in a Spectronic 20 spectrophotometer. The tubes were Vortex-mixed immediately before each reading.

B. The ability of iron and agitation to affect growth.

The two objectives of this experiment were to determine the effect of iron and of agitation on the growth of *Listeria* in the defined medium of Welshimer (21).

1. The defined medium of Welshimer (21) was used; it contained the following components: KH_2PO_4, 3.28 gm; Na_2HPO_4, 8.20 gm; $MgSO_4 \cdot 7H_2O$, 0.40 gm; glucose, 10.0 gm; L-cysteine·HCl, 100 mg; L-leucine, 100 mg; DL-isoleucine, 200 mg; DL-valine, 200 mg; DL-methionine, 200 mg; L-arginine·HCl, 200 mg; L-histidine·HCl, 200 mg; DL-tryptophan, 200 mg; L-glutamine, 600 mg; riboflavin, 1.0 mg; thiamine·HCl, 1.0 mg; D-biotin, 100 μg; DL-thioctic (α-lipoate) acid, 1 μg; and distilled water, 850 ml. The salts, glucose, and amino acids were weighed and added to 75 ml distilled water, but the vitamins (the last 4 components above) were added as 100 ml of 10X concentrated solution, prepared in a manner similar to that described for the 100X vitamin solution used in medium D10 (Appendix 2-e). The medium was sterilized by Millipore filtration (0.45 μ); its pH was 7.0, and as prepared above, was

concentrated 20/17 to allow for the addition of iron and inoculum. Note that the medium included 1.0% glucose.

2. The iron supplement used was ferric ammonium citrate (22) which contains about 15% Fe^{+++} (79). Solutions of ferric ammonium citrate (10, 100, and 1000 µg Fe^{+++}/ml) were prepared and sterilized as in step A-2.

3. Growth tubes were prepared essentially as detailed in step A-2, i.e. 5.1 ml of Welshimer's medium (step B-1) was combined in each tube with 0.6 ml of ferric ammonium citrate (10X) to give final Fe^{+++} concentrations of 1.0, 10, and 100 µg Fe^{+++}/ml.

4. Inoculum for the growth tubes was prepared as in step A-3 (a-e) except that the medium used was that of Welshimer and the incubation was for 12 hr at 37 C. Buffer washed *Listeria* were resuspended to a cell density of ≃ 4.4 x 10^8/ml.

5. Growth tubes were inoculated as in step A-4, except that the inoculum furnished an initial cell concentration of ≃ 1.3 x 10^8/tube. Incubation was at 37 C, either stationary (waterbath) or with agitation on a shaker (Eberbach and Sons, Ann Arbor, Mich.). The agitated tubes were held at an angle of ≃ 60° to the horizontal; the throw was ≃ 3 cm and the speed ≃ 180 strokes/min which provided "moderate" agitation.

6. Growth was measured turbidimetrically as in step A-5, except that readings were made at zero time and at 10, 12, 14, 16, 18, 23, 34, and 40 hr. Duplicate growth tubes were used to test each iron concentration.

7. It can be seen in the Results that ferric ammonium citrate was an effective growth stimulant; it was not used as a source of iron

for medium D10 (section V), however, because when included in significant amounts (1-100 μg Fe^{+++}/ml), the increase in *Listeria* growth with time was accompanied by the formation of a fine dark blue precipitate in rough proportion to the amount of iron present; no precipitate formed in the absence of ferric ammonium citrate. Because the precipitate interfered somewhat with the turbidimetric readings, ferric ammonium citrate was not used as a source of iron in medium D10. The nature of this blue precipitate was not determined.

V. DESIGN OF THE DEFINED MEDIUM D10

A. Need for a defined medium in this research.

The growth medium can greatly influence the metabolism of bacteria, as discussed in the Introduction, i.e. certain amino acids such as glutamate and aspartate can repress the synthesis of biosynthetic enzymes of the citrate cycle. For instance, the addition of glutamate to a minimal glucose medium caused complete repression of aconitase in *Bacillus subtilis* and *B. licheniformis* (80). If the objectives of this thesis were to be satisfied, a defined medium was needed--one able to support rapid *Listeria* growth yet simple enough to force its biosynthetic pathways into action.

B. Inadequacies of available defined media for *Listeria* growth in this research.

Two defined media have been described for *Listeria* culture (20, 21). The ingredients of the medium of Friedman and Roessler (20) are given in the Introduction while Welshimer's medium (21) is described in

step IV-B-1. Both media contain the same 9 amino acids and 4 vitamins; however, the inorganic salts and glucose contents are different. Although both are satisfactory for general purposes, neither medium was used in this research for the following reasons:

1. Glutamine and tryptophan are included in the amino acid mixtures (20, 21) although neither amino acid is essential (20); glutamine could be replaced by NH_4^+, and tryptophan is not stimulatory, according to their amino acid exclusion data (20). For the purpose of this thesis, glutamine was an undesirable component in the medium because of its possible repressive effect on the citrate cycle enzymes, if these are present. Since tryptophan was not stimulatory for *Listeria* growth, it need not be included in a defined medium.

2. Iron has been shown to be important to *Listeria* growth (22). Iron is not included in Welshimer's medium (21), and in the medium of Friedman and Roessler (20) the quantity of iron available in the medium is in doubt because "salts A and B were mixed, heated for 5 min, and the resultant precipitate discarded" (20).

3. Manganese salts inhibit the growth of *Listeria* A4413 in concentrations as low as 0.1 μg Mn^{++}/ml; this component of Friedman and Roessler's medium (20) is therefore undesirable in a medium for this research.

4. Citrate, as included in Friedman and Roessler's medium (20), was similarly undesirable in the medium for this thesis because citrate was one substrate to be tested as a carbon and energy source for *Listeria*.

Taking the above into consideration, a defined medium, designated

D10, was designed which met the needs of this research.

C. Medium D10: design features.

This medium was designed to support rapid growth of *Listeria* and yet contain no excess nutrients. For reasons just detailed, the defined media described by Friedman and Roessler (20) and Welshimer (21) were unsuitable for this research. Grateful adknowledgment is due these workers, however, because their work formed the basis for the design of medium D10.

The composition of medium D10 and the details of its assembly are presented in the Appendix. The vitamins used in this medium are as described by Welshimer (21). The amino acid mixture is similar to that of Welshimer (21) except that the L-glutamine and DL-tryptophan are excluded, and NH_4^+ replaces glutamine (20). Mg^{++} and K^+ salts are incorporated in medium D10 because these ions are required for *Listeria* growth (20). Iron (Fe^{+++}) is very stimulatory to *Listeria* (22) and is included along with nitrilotriacetic acid which chelates the Fe^{+++}.

The medium is called D10 because it is defined and contains Fe^{+++} in a concentration of 10 µg/ml. In the Results it can be seen that medium D10, when supplemented with glucose, supports excellent *Listeria* growth. This medium is simpler than any yet described for the cultivation of *Listeria*.

VI. ABILITY OF MEDIUM D10, WHEN SUPPLEMENTED WITH GLUCOSE, TO SUPPORT THE GROWTH OF *LISTERIA* A4413 AND 9037-7

The purpose of this experiment was to determine the growth of

Listeria A4413 and 9037-7 in medium D10 supplemented with 0.5% glucose. The turbidimetric growth response in this medium was compared with that in similar media, one of which lacked iron (medium D), and the other contained twice the iron content of medium D10 (medium D20).

1. Medium D10 + 0.5% glucose was prepared as described in the Appendix except that a glucose supplement was included. Glucose was autoclaved and added in a 100X concentration to give a final concentration of 0.5%. Medium D + 0.5% glucose was prepared similarly, but lacked iron. Medium D20 + 0.5% glucose contained twice as much iron (20 μg Fe^{+++}/ml) as medium D10. These media were prepared in 20/19 concentration to allow for the inoculum.

2. Optically uniform test tubes (18 x 150 mm) were used for growth assay. Each tube received 5.7 ml of one of the media mentioned in step 1, and duplicate tubes were used to test the growth response in each medium.

3. All growth tubes were inoculated with 0.3 ml of *Listeria* culture which had been grown in Welshimer's defined medium (21) for 12 hr at 37 C. The initial cell density in the growth tubes after inoculation was $\simeq 1.5 \times 10^8$/tube (A4413) or $\simeq 3 \times 10^7$/tube (9037-7). Incubation was at 37 C, either stationary (waterbath) or with agitation (step IV-B-5).

4. *Listeria* growth was measured turbidimetrically as in step IV-B-6 except that readings were taken at time zero, and at 15, 19, and 23 hr.

It can be seen in the Results that medium D10 + 0.5% glucose supported excellent *Listeria* growth; this growth was much greater than

that seen in medium D + 0.5% glucose and just as great as that seen in medium D20 + 0.5% glucose. It was concluded that medium D10 + glucose was suitable for the growth of *Listeria* in this research.

VII. DETERMINATION OF THE SURVIVAL OF *LISTERIA* IN VARIOUS MENSTRUA

Resting bacteria are usually non-proliferative because their exogenous substrates have been removed by washing procedures. Obviously, solutions used to wash and suspend bacteria should not be inimical to them or significant error might result during physiological studies such as respirometry. The purpose of this experiment was to determine and compare the ability of four menstrua to maintain the viability of *Listeria* A4413 as estimated by plate counts.

1. The following solutions were tested: distilled water, 0.9% NaCl, 0.1M potassium phosphate buffer, pH 7.2, and 2% trypticase. These solutions were dispensed (18 ml) into 1 x 6 in. dilution tubes, autoclaved at 15 lb for 10 min, then cooled to refrigerator temperature before use.

2. Fifty ml of Brain-Heart Infusion (BHI) was inoculated with 0.5 ml of a *Listeria* A4413 culture (grown in BHI at 37 C for 16 hr), and incubated at 37 C for 3 hr at which time the optical density (620 mμ) was ≈ 0.10 (determined as in step IV-A-3e).

3. Serial ten-fold dilutions were prepared by transfer of 2.0 ml of the above culture to 18 ml of each menstruum to give final dilutions of 10^{-1} to 10^{-5}. Each dilution was vigorously mixed with the Vortex mixer before the next 2.0 ml was transferred.

4. The final (10^{-5}) dilution was plated immediately by spreading

0.1 ml of the suspension on each of three tryptose agar plates. The cell suspension was then refrigerated and replated after 4 hr and 21 hr. All plates were incubated at 37 C for 24 hr after which the colonies were counted.

VIII. CORRELATION OF THE TURBIDITY, NITROGEN CONTENT, AND VIABLE COUNT OF SUSPENSIONS OF *LISTERIA* A4413 AND 9037-7

The objective of this experiment was to correlate the turbidity of a *Listeria* suspension with its nitrogen content and number of viable cells. These relationships were then used to estimate the nitrogen content or viable count of a suspension by simply measuring its turbidity (optical density at 620 mμ). Potassium phosphate buffer (0.1M) was used to wash and suspend the *Listeria* cells because it best maintained their viability (see Results). The procedure up to and including viable count determinations was carried out aseptically to avoid contamination.

1. *Listeria* A4413 and 9037-7 were each inoculated into 100 ml of medium D10 + 0.5% glucose and incubated stationarily at 37 C for 24 hr. Eighty ml of each culture was harvested and washed twice as previously detailed (IV-A-3b to d). The twice-washed *Listeria* pellets were each resuspended in buffer to give a series of suspensions having turbidity values from 0.01 to 0.60; the turbidity was determined as in step IV-A-3e. The nitrogen content and viable count of each suspension was then estimated as described below.

2. Viable counts were estimated by making six serial 10-fold dilutions of each *Listeria* suspension in cold buffer and plating in

triplicate the dilutions 10^{-5} and 10^{-6} as detailed in procedure VII-3 to 4.

3. Bacterial nitrogen was determined by a Nesslerization procedure (82) on duplicate 1.0 ml samples of each *Listeria* suspension. Nessler's solution was obtained from Unitech Chemical Manufacturing Co., Panorama City, California. The Nesslerized solutions were placed in 13 x 100 mm Spectronic 20 cuvettes and read at 440 mμ in the Spectronic 20 spectrophotometer. The blank consisted of Nesslerized distilled water which had been treated as a sample. A series of $(NH_4)_2SO_4$ standards (10 to 400 μgN/ml) were determined simultaneously with the bacterial suspensions. Bacterial nitrogen was read from the linear relationship obtained between the nitrogen content and optical density at 440 mμ.

IX. ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE TO SERVE AS A SOURCE OF CARBON AND ENERGY FOR *LISTERIA* A4413 AND 9037-7

The experimental objective was to determine turbidimetrically the growth response of *Listeria* A4413 and 9037-7 in medium D10 with pyruvate or citrate cycle intermediates as the sole source of carbon and energy. One of the overall objectives of this thesis was to determine whether the enzymes of pyruvate oxidation and the citrate and glyoxylate cycles were present. The aim of this particular experiment was more far-reaching, therefore, than the mere observation of growth or no growth on these substrates, because pyruvate or citrate cycle intermediates, if suitable for *Listeria* growth, could be used to produce

cells containing higher levels of the relevant enzymes than glucose-grown cells.

1. The medium employed was D10 (see Appendix).

2. Duplicate growth tubes were used to test the suitability of the sodium salts of pyruvate, acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, and malate as carbon and energy sources. The growth tubes were prepared essentially as detailed in step IV-A-2 except that 5.1 ml of D10 was used as the medium, and instead of glucose, 0.6 ml of a 2% solution of each substrate above was added to appropriate tubes to give a final concentration of 0.2%. One set of controls lacked any supplement, while another set was supplemented with glucose. Pyruvate and α-ketoglutarate solutions were prepared immediately before use.

3. Inocula of *Listeria* A4413 and 9037-7 were prepared as in procedure IV-A-3 except that medium D10 + 0.5% glucose was used for growth, and the washed *Listeria* were resuspended to give cell concentrations of $\simeq 1 \times 10^9$/ml (A4413) or 1×10^8/ml (9037-7).

4. All growth tubes received 0.3 ml of one inoculum (A4413) or 9037-7) equivalent to $\simeq 3 \times 10^8$ cells/tube (A4413) or $\simeq 3 \times 10^7$ cells/tube (9037-7). Incubation was either stationary or with agitation at 37 C as in step IV-B-5.

5. Growth was estimated as in step IV-A-5, except that readings were made at time zero, and at 24 and 48 hr.

X. ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE, WHEN SUPPLEMENTED WITH GLUCOSE, TO SERVE AS A SOURCE OF CARBON AND ENERGY FOR *LISTERIA* A4413 AND 9037-7

Definite yet restricted *Listeria* growth occurs in a medium containing only 0.05% glucose (see Results). Since a second carbon source may be required for compounds such as citrate to be utilized by some bacteria (44), inclusion of 0.05% glucose with each intermediate might permit its utilization as denoted by increased growth relative to glucose alone.

The protocol for this experiment was identical to that of section IX except that medium D10 + 0.05% glucose was used instead of medium D10.

XI. GROWTH OF *LISTERIA* A4413 IN MEDIUM D10 + 0.2% GLUCOSE IN RELATION TO THE INOCULUM SIZE

This experiment delineated the growth curve of *Listeria* A4413 in relation to the inoculum size. Information of this sort was needed in order that cultures to be studied physiologically might be harvested in the late log phase of growth.

1. Nephelo flasks (Bellco Glass Co., Vineland, N. J.) of 300 ml capacity, each having a 13 mm sidearm for the convenient measurement of turbidity, were used for growth. Each flask contained 100 ml of medium D10 + 0.2% glucose.

2. Stock *Listeria* A4413 cultures had been grown and frozen as in step II-bc. Three of these cultures were thawed at 37 C. To the small

pool of culture fluid in each thawed slant was added 0.6 ml sterile distilled water to give a total volume of ≃ 1.1 ml. The *Listeria* growth was gently removed from the surface and mixed thoroughly with the fluid by means of a 1 ml pipette.

3. From each of the three thawed slants, 0.25 ml of inoculum was transferred to a corresponding Nephelo growth flask. A fourth flask received only 0.125 ml of inoculum.

4. The flask cultures where shaken at 37 C in a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at medium speed.

5. At time zero and at 9, 11.25, 13, 14, 15, and 16 hr, the turbidity of each flask was measured as in step IV-A-5.

The resultant growth response is shown in the Results, but is summarized here to provide a basis for the conditions used to grow *Listeria* A4413 cultures destined for metabolic studies: the 0.25 ml inoculum yielded maximum growth in ≃ 14.0 hr while the 0.125 ml inoculum required ≃ 16.0 hr to give similar growth. Thus to obtain late log phase *Listeria*, a 100 ml culture from a 0.25 ml inoculum whould be harvested between 13.0 and 13.5 hr, while that from a 0.125 ml inoculum should be harvested between 15.0 and 15.5 hr.

XII. THE GROWTH OF *LISTERIA* A4413 DESTINED FOR MANOMETRIC OR ENZYMATIC STUDIES; THE PREPARATION OF RESTING CELLS AND CELL-FREE EXTRACTS

A. Growth conditions.

Listeria cultures, whether intended for use as resting cells or for disruption to give cell-free extracts, were grown similarly; any

deviations from the procedure below are noted in the appropriate assay procedure or in the Results. Medium D10 + 0.2% glucose was used for *Listeria* culture. Glucose was included as a carbon and energy source, as no intermediate of the citrate cycle or pyruvate supported growth (see Results). The 0.2% level of glucose was a slight compromise; higher concentrations (0.5-1.0%) give more *Listeria* growth than 0.2%, but yield cells showing lower catalase and glucose oxidase activity (24) and presumably other enzymes as well.

1. *Listeria* were grown in 500 ml Erlenmeyer flasks containing 200 ml of medium D10 + 0.2% glucose. Growth was initiated by the addition of either 0.25 ml or 0.50 ml of inoculum (see XI-2); these volumes are equivalent, respectively, to the 0.125 ml and 0.25 ml inocula used to initiate growth in 100 ml of D10 + 0.2% glucose in section XI. From 400 to 800 ml of culture was used for most experiments, although as much as 2400 ml was occasionally needed.

2. The growth flasks were incubated at 37 C in a gyrotory shaker at medium speed. Cultures were removed from the shaker at 15.0 to 15.5 hr (0.25 ml inoculum) or 13.0 to 13.5 hr (0.50 ml inoculum).

B. The harvest and washing of the cells, and the preparation of resting cell suspensions.

1. Cell harvest. Each 200 ml of culture was transferred to a 250 ml flat-bottom centrifuge bottle (Nalge Company, Rochester, N. Y.) and centrifuged cold at 6,000 X g for 15 min in a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant culture fluid was suctioned from the *Listeria* cell pellet as

described in step IV-A-3c.

2. Cell washing. The white *Listeria* pellet was washed by resuspension in an equal volume (200 ml) of cold 0.05M potassium phosphate buffer, pH 7.0, and centrifuged as in step 1. The supernatant wash fluid was removed as in step IV-A-3c.

3. Preparation of resting cells. The washed *Listeria* pellet was resuspended in a small volume of buffer to give a dense resting cell suspension. When destined for disruptive procedures, the washed cell pellets (usually two) were resuspended in 10 ml 0.005M potassium phosphate buffer, pH 7.0 (or as indicated in the specific enzyme assay method). Various buffers were used to resuspend the cell pellets intended for manometric studies; these will be specified in section XIII.

C. Cell disruption and the preparation of cell-free extracts.

1. Cell disruption.

Ten ml of *Listeria* suspension and 10 ml of 0.11 mm glass beads (Bronwill Scientific Co., Inc., Rochester, N. Y.) were placed in a precooled (≃ 4 C) 50 ml stainless steel flask (Bronwill) with a threaded cap. Vaseline was lightly smeared on the threads and the cap was tightened as much as possible to prevent the leakage of *Listeria*. The flask was shaken in a Braun Cell Homogenizer, Model MSK (Bronwill) for 45 sec. A continuous stream of CO_2 maintained the flask temperature between 1 and 10 C; CO_2 was delivered to the homogenizer by a metal capillary (Bronwill) and the flow was regulated with a micrometer valve (Bronwill) at a setting of 1.

2. Preparation of crude extract.

a. The disruption flask was removed from the homogenizer and left undisturbed for ≃ 1 min to allow the glass beads to settle. The cell extract + debris (≃ 3 ml) was transferred to a 50 ml centrifuge tube kept on ice. The flask was flushed with 10 ml of cold 0.005M potassium phosphate buffer, pH 7.0; this flush fluid was added to the cell extract + debris. This mixture was centrifuged cold at 5,000 X g for 5 min in the RC-2; the glass beads and most of the unbroken *Listeria* were thus sedimented.

b. The supernatant extract from step a was recentrifuged at 5,000 X g for 10 min. A small pellet from this centrifugation revealed, upon staining with crystal violet and microscopic observation, typical *Listeria* rod forms and some large cell fragments. The supernatant fluid was translucent and colored a faint brownish-yellow; although bacterial forms were not recognized, numerous small subcellular particles were observed. This supernatant extract was termed crude extract (CE); its pH was 7.0.

About 30 mg of total crude extract protein (estimated by a biuret method [83] with bovine serum albumin as standard) was recovered from each 200 ml of *Listeria* culture. Approximately 90% of the *Listeria* were broken by the procedure in step 1, as estimated by the relative size of the centrifuged cell pellets before and after cell disruption. When the cell disruption (step 1) was carried out for 90 sec or 180 sec, there was an insignificant increase in the total extract protein and no increase in the specific activity of aconitase, the test enzyme. Cooling the disruption flask was difficult to regulate when cell

disruption continued for 90 sec or 180 sec, but was easy in the 45 sec exposure. The 45 sec cell disruption seemed most satisfactory, and was subsequently used exclusively.

3. Treatment of crude extracts with protamine sulfate.

Protamine sulfate solution was used to precipitate nucleic acids from the crude extract in order to minimize its ultraviolet absorbance. Crude extract was treated in the cold with protamine sulfate (1.0%, pH 6), added dropwise with agitation, until no further "stringiness" developed; 1 mg of protamine sulfate for every 3 to 5 mg of crude extract protein was usually required. The treated extract was centrifuged cold at 10,000 X g for 10 min in the RC-2. The supernatant extract was decanted and designated as protamine sulfate supernate (PSS). Such treatment co-precipitated 30-40% of the crude extract protein. The pH of PSS was 7.0.

XIII. THE ABILITY OF *LISTERIA* A4413 TO OXIDIZE GLUCOSE, PYRUVATE, INTERMEDIATES OF THE CITRATE CYCLE, AND CERTAIN FATTY ACIDS

A. Manometric methods.

The same general technique (84) was used to determine the oxidative capacity of *Listeria* A4413 toward a variety of substrates. Oxygen consumption was measured in a conventional Warburg apparatus (Precision Scientific Co., Chicago, Ill.), i.e. a double-sidearm flask + manometer + constant temperature waterbath system (84).

The total volume of the reaction mixtures was 3.0 ml which included 0.1 ml of 10% KOH in the center well of each flask for the absorption of CO_2. Small pieces of filter paper, slightly taller than

the center well, were inserted into the KOH solution to increase the surface area for CO_2 absorption.

After the appropriate components had been added, the flasks were fitted to the proper manometers with heavy vaseline as the lubricant for the ground glass joints. The flask + manometer unit was equilibrated in the waterbath at 37 C for 15 min with the stopcocks open. The stopcocks were closed and equilibration continued for 15 min after which the substrates were tipped from the sidearm into the main vessel. Oxygen consumption was then measured at 37 C in an atmosphere of air. A thermobarometric control (3.0 ml water) was run with each experiment. Appropriate flask constants were employed to convert the changes in pressure (mm Brodie's fluid [84]) to µl of oxygen consumed.

B. Reaction mixtures.

1. Components.

a. Resting *Listeria* A4413 suspensions.

Listeria A4413 were grown, harvested, and washed as detailed in section XII. The washed cell pellets were resuspended in sufficient 0.01M potassium phosphate buffer to give a cell suspension equivalent to 1.8 mgN/ml; the pH of the 0.01M buffer used to suspend the cells was similar to that of the 0.2M buffer used to control the pH of the reaction mixture as indicated in the Results.

b. Buffers.

Potassium phosphate buffer, pH 5.0 to 8.0, or phthalate-NaOH buffer, pH 4.0 to 5.0, was used as required; all buffers were 0.2M.

c. Substrates.

The following substrates were dissolved in distilled water (or sodium hydroxide as required for neutralization of the acids) to give a concentration of 50 μmoles/ml: glucose, and the sodium salts of acetate, α-ketoglutarate, citrate, fumarate, DL-isocitrate, L-malate, succinate, pyruvate, oleate, and caproate.

d. Coenzymes.

(1) A cofactor mixture (CFM) was prepared which contained (in μmoles/0.2 ml): NAD, 1; NADP, 1; DPT, 0.5; CoA, 0.1; α-lipoate, 0.1; L-cysteine, 1; and $MgCl_2$, 1. This CFM was made by dissolving twenty times the above quantities of cofactors in 80 μmoles of NaOH solution for neutralization, then diluted to a total volume of 4.0 ml with distilled water. The α-lipoate was first dissolved in 0.1 ml absolute ethanol. This CFM was included in certain reaction mixtures which tested for the oxidation of glucose or the intermediates of the citrate cycle.

(2) In the oxidation of pyruvate, the following coenzymes (in μmoles/0.1 ml) were added singly and in concert: NAD, 0.5; DPT, 1; CoA, 0.5; α-lipoate, 0.5; FAD, 0.5; $MgCl_2$, 1; and $MnCl_2$, 1.

2. Procedure.

Each Warburg flask received the following general reaction mixture:

Main vessel: buffer, 1.0 ml, 200 μmoles, the type and pH as identified in the Results; *Listeria* cells, 0.5 ml, 0.9 mgN; coenzymes as specified in the Results, in the concentration and volume indicated above; and distilled water to give a total volume of 3.0 ml after

allowing for the fluids in the sidearm and center well.

Sidearm: substrate (identified in the Results), 0.2 ml, 10 μmoles.

Center well: KOH, 0.1 ml, 10%. Endogenous flasks contained complete reaction mixtures except substrate.

XIV. METHODS OF ASSAY FOR CERTAIN ENZYMES IN CELL-FREE EXTRACTS OF *LISTERIA* A4413

A. General comments.

An important phase of this research was to determine whether cell-free extracts of *Listeria* contain the enzymes of the citrate cycle and the glyoxylate bypass, as well as pyruvate oxidase. Oxidases for reduced pyridine nucleotides were also sought and estimated.

The oxidases for pyruvate and α-ketoglutarate were determined manometrically. Citrate synthase was estimated by using a chemical assay for citrate, whereas the activities of all other enzymes were measured spectrophotometrically. When a certain enzyme was apparently absent in *Listeria* extracts, cell-free extracts were prepared from *E. coli* K-12(λ) and assayed by the same method as a positive control; this precaution strengthened the evidence that the enzyme was absent in *Listeria* A4413 and not merely inactive due to methodology. The growth, harvest, washing, and preparation of cell-free extracts of *E. coli* were as described for *Listeria* in section XII unless noted otherwise in the specific assay method.

The reaction mixture given for each method provided approximately optimal conditions for enzyme activity, as determined from experimental

changes in pH and in the concentration of buffer, substrate, and co-factor; the effects of alterations in this mixture are presented in the Results. Distilled water was added to each reaction mixture as required to give the total volume indicated.

Absorbancy changes of all spectrophotometric assay reaction mixtures were measured in Beckman "standard silica" cuvettes (Beckman Instruments, Inc., Fullerton, Calif.) with a 1 cm light path by means of a Zeiss spectrophotometer, model PMQ II (Carl Zeiss Oberkochen/ Württ, West Germany) at room temperature (≃ 25 C). Absorbancy changes were recorded at intervals of 15 to 60 sec for up to 5 min, depending upon the reaction in question. After addition of the last reaction component and before the absorbancy determination, the reaction mixture was mixed briefly with a stream of air.

The concentration of substrate required to give half maximal velocity was estimated by the method of Lineweaver and Burk (101). Activity was ascribed to a given enzyme only when the appropriate assay changes (spectrophotometric, manometric) were dependent upon the presence of both substrate and cell-free extract. The specific activity for each enzyme was calculated from the initial (linear) reaction rate under optimal conditions, and such reactions are illustrated in the various Figures in the Results.

B. Assay methods.

1. Pyruvate oxidase system.

The activity of the pyruvate oxidase system was estimated by oxygen consumption using the Warburg manometry techniques of section

XIII-A. The reaction mixture contained the following components in a total volume of 3.0 ml: *Listeria* A4413 CE, 6.6 mgP; sodium pyruvate, 20 µmoles; potassium phosphate buffer, pH 7.0, 200 µmoles; NAD, 1 µmole; DPT, 0.5 µmole; CoA, 0.1 µmole; α-lipoate, 0.1 µmole; L-cysteine, 1 µmole; $MgCl_2$, 1 µmole; and 0.1 ml of 10% KOH in the center well. Specific activity is expressed as the number of µl O_2 consumed/hr/mgP at 37 C.

2. α-ketoglutarate oxidase system.

The activity of the α-ketoglutarate oxidase system was determined manometrically as described for the pyruvate oxidase system, except that α-ketoglutarate replaced pyruvate as the substrate. Since α-ketoglutarate oxidase was apparently absent in *Listeria* extracts, the sensitivity of the assay was verified by using a crude extract of *E. coli* grown on the minimal medium of Davis et al. (85) at pH 7.0 and 37 C for 32 hr with shaking (XII-A-2); acetate (0.4%) was the carbon and energy source. Specific activity is expressed as the number of µl O_2 consumed/hr/mgP at 37 C.

3. Citrate synthase.

Citrate synthase was estimated by the chemical determination of citrate in the protein-free filtrate derived from a reaction mixture of *Listeria* A4413 extract, acetyl-CoA, and oxalacetate. This method is similar in principle to that of Englesberg and Levy (68) which tested the ability of *P. pestis* extracts to synthesize citrate.

Procedure: the complete reaction mixture contained potassium phosphate buffer, pH 7.5, 25 µmoles; $MgCl_2$, 1 µmole; L-cysteine, 0.5 µmole; acetyl-CoA, 1.5 µmole; sodium oxalacetate, 20 µmole; and

Listeria A4413 extract (PSS) 2.16 mgP, in a total volume of 2.5 ml contained in a 4 x 5/8 in. test tube stoppered with a soft rubber stopper. Each tube was flushed with nitrogen for 30 sec and incubated at 37 C. The reaction was stopped at 15 min and 30 min by the addition of 2.5 ml 1.5% trichloroacetic acid, and protein-free filtrates were prepared by filtration through Whatman #42 filter paper. The filtrates were boiled for 15 min to decompose residual oxalacetate which interferes with citrate determination (86). One ml of each filtrate was assayed for citrate by the procedure of Lane and Chen (87). The citrate content of each filtrate was calculated from a standard curve based on the determination of known quantities (2.5 to 20 µg) of citrate.

Of three methods for the determination of citrate which were tried in this research, the method of Lane and Chen (87) was the only one which combined relative simplicity and sensitivity (≃ 1 µg citrate); whereas the method of Ettinger et al. (88) was sensitive to ≃ 1 µg citrate, it was laborious and smelly due to the use of pyridine, and the method of Kraus (86) lacked sensitivity although it was quite simple.

Specific activity is expressed as the number of µmoles of citrate formed/hr/mgP at 37 C.

4. Aconitase.

This enzyme was determined spectrophotometrically by the method of Racker (89). This was the complete reaction mixture in 3.0 ml: potassium phosphate buffer, pH 7.5, 300 µmoles; sodium DL-isocitrate, 20 µmoles, or sodium citrate, 90 µmoles; *Listeria* A4413 extract (PSS), from 0.25 to 0.50 mgP. The increase in absorbance at

240 mμ was observed for 3 min after the addition of substrate against a reference cell which contained the complete reaction mixture except substrate. Specific activity is expressed as the number of μmoles of cis-aconitate formed/hr/mgP at room temperature. A millimolar extinction coefficient of 3.5 (89) for cis-aconitate was used to calculate the specific activity.

5. Isocitrate dehydrogenase.

Isocitrate dehydrogenase activity was measured by a modification of Kornberg's method (90). The complete reaction mixture included these compounds in a volume of 3.0 ml: potassium phosphate buffer, pH 7.5, 200 μmoles; NADP, 0.5 μmole; sodium DL-isocitrate, 30 μmoles; $MnCl_2$, 1 μmole; and *Listeria* A4413 extract (PSS), 1.0 mgP. The increase in absorbance at 340 mμ was observed for 4 min after the addition of isocitrate against a reaction mixture which lacked only isocitrate. Specific activity is expressed as the number of μmoles of NADPH formed/hr/mgP at room temperature. A millimolar extinction coefficient of 6.2 (91) for NADPH was used to calculate the specific activity.

6. Succinate dehydrogenase.

Succinate dehydrogenase activity was estimated by two methods. The method of Ells (92), modified essentially according to Arrigoni and Singer (93) was used most extensively; it is based on the succinate-dependent reduction of 2,6-dichlorophenol indophenol (DCPIP) mediated by phenazine methosulfate. The complete reaction mixture, in 3 ml, contained the following components added in the order indicated: potassium phosphate buffer, pH 6.5, 100 μmoles; sodium succinate, 60

μmoles; *Listeria* A4413 extract (CE), 1.1 mgP; KCN (adjusted to pH 8), 3 μmoles, was mixed with the other components by a brief nitrogen bubbling and the mixture was allowed to stand for ≃ 5 min; DCPIP, 0.083 μmoles, and phenazine methosulfate, 1.0 mg, were then added in quick succession and mixed briefly by bubbling nitrogen through the mixture. The decrease in absorbance at 600 mμ was observed for 2.5 min against a water reference. Specific activity is expressed as the number of μmoles of DCPIP reduced/hr/mgP at room temperature, based on a millimolar extinction coefficient of 19.1 for DCPIP (94). Phenazine methosulfate was protected from light at all times.

The method of Nachlas et al. (95), as modified by Hirsch et al. (58), was also used to assay succinate dehydrogenase. This method employs phenazine methosulfate to mediate the succinate-dependent reduction of p-iodonitrotetrazolium violet (INT) under anaerobic conditions. An anaerobic cuvette was made by Mr. Gunther Weiss, glassblower, University of Oregon Medical School, by fusing a Beckman "standard silica" cell (1 cm light path) to a double-sidearm by means of a ground glass joint; this cuvette was used in this assay. The reaction mixture contained the following substances:

Cuvette: INT, 0.6 ml of a 0.1% solution in 0.05% gelatin; phenazine methosulfate, 1.0 mg; potassium phosphate buffer, pH 7.5, 120 μmoles.

Sidearm: sodium succinate, 20 μmoles, and *Listeria* A4413 extract (CE), 0.86 mgP. The cuvette was closed, evacuated (Cenco Hypervac 4, Central Scientific Co., Chicago, Ill.) for 1 min, then flushed with nitrogen; the evacuation and flushing were repeated twice after which

the cuvette was evacuated and closed. After mixing the succinate and extract with the other reaction components, the increase in absorbance at 490 mμ was observed for 3 min against a water reference. The height of the anaerobic cuvette required that a dark cloth, rather than the normal cover, be used as a light shield for the Zeiss spectrophotometer. The reaction mixture was at all times shielded from light to protect the phenazine methosulfate. The specific activity is expressed as the number of μmoles of INT reduced/hr/mgP at room temperature, using the millimolar extinction coefficient of 14.2 for reduced INT (58) as a basis for the calculation.

7. Fumarate reductase.

The fumarate reductase assay was essentially that of Hirsch et al. (58). The method measures spectrophotometrically the fumarate-dependent dehydrogenation of either $FMNH_2$ or $FADH_2$ by an increase in absorbance at 450 mμ under anaerobiosis. The anaerobic cuvette previously described was used as the reaction vessel. The reaction mixture contained the following components in a volume of 2.0 ml:

Cuvette: FMN, 0.5 μmole; potassium phosphate buffer, pH 7.0, 100 μmoles.

Sidearms: (1) *Listeria* A4413 extract (CE), 0.86 mgP; (2) sodium fumarate, 20 μmoles.

The cuvette was evacuated and flushed with nitrogen as described in the previous section. After two evacuations, approximately 0.3 mg of sodium hydrosulfite was added to the cuvette and mixed briefly with bubbling nitrogen to reduce (bleach) the flavin component; the cuvette was quickly evacuated and flushed with nitrogen (two cycles) and

finally evacuated. The increase in absorbance at 450 mμ was observed for 2 min against a water reference after the fumarate and extract had been mixed with the other components. Specific activity is expressed as the number of μmoles of $FMNH_2$ (or $FADH_2$) dehydrogenated/hr/mgP at room temperature; this was calculated using a millimolar extinction coefficient of 12.4 for FMN (58). The level of oxygen exclusion achieved by the above evacuation procedure was sufficient to prevent the oxidation of a solution of $FMNH_2$ for at least 2 hr; nevertheless, the absorbance at 450 mμ was observed for 2 min after tipping only the extract to check for fumarate-independent dehydrogenation of $FMNH_2$; none was encountered.

8. Fumarase.

Fumarase was estimated spectrophotometrically by the method of Racker (89). The complete reaction mixture contained in 3.0 ml: Tris·HCl buffer, pH 8.0, 200 μmoles; sodium L-malate, 30 μmoles; and *Listeria* A4413 extract (PSS), 0.5 mgP. The increase in absorbance at 240 mμ was measured for 5 min after the addition of malate against a reference containing all components except malate. Specific activity is expressed as the number of μmoles of fumarate formed/hr/mgP at room temperature. A millimolar extinction coefficient of 2.1 for fumarate (89) was used in the calculation.

9. Malate dehydrogenase.

Malate dehydrogenase was determined spectrophotometrically by a modification of the method of Mehler (96). The complete reaction mixture included the following in a volume of 3.0 ml: glycine·NaOH buffer, pH 9.0, 100 μmoles; sodium L-malate, 60 μmoles; NAD, 0.5 μmole;

$MnCl_2$, 1 μmole; and *Listeria* A4413 extract (PSS), 1.0 mgP. The increase in absorbance at 340 mμ was measured for 3 min after the addition of malate against a reference containing all components except malate. The specific activity is expressed as the number of μmoles of NADH formed/hr/mgP at room temperature. The millimolar extinction coefficient of 6.2 for NADH (91) was used in the calculation.

10. Isocitratase.

Isocitratase was estimated by the method of Kornberg (97) which measures the glyoxylate formed by trapping it with phenylhydrazine to give glyoxylate phenylhydrazone. Before the actual assay, certain reaction components were pre-mixed to give what Kornberg termed "R_{im}"; this was a mixture of 1.0 ml 0.5M imidazole buffer of pH 6.4, 6.8, or 7.2, 1.0 ml of 0.1M $MgCl_2$, 0.2 ml 0.1M EDTA, 0.8 ml 0.1M phenylhydrazine·HCl (pre-neutralized), and 1.0 ml distilled water. This mixture was used fresh or up to 2 hr if kept on ice. The complete reaction mixture contained in a volume of 2.0 ml: R_{im}, 0.4 ml; reduced glutathione (pre-neutralized), 5 μmoles; *Listeria* A4413 extract (CE), 1.29 mgP, and sodium DL-isocitrate, 2 μmoles. The increase in absorbance at 324 mμ was observed for 3 min after the addition of isocitrate against a reference which contained all components except isocitrate. Specific activity is expressed as the number of μmoles of glyoxylate phenylhydrazone formed/hr/mgP at room temperature; this was calculated using a millimolar extinction coefficient of 16.8 for glyoxylate phenylhydrazone (97).

11. Malate synthase.

Malate synthase was assayed by the method of Dixon and

Kornberg (98) as modified by Dixon et al. (99) so as to avoid the use of phosphate in the procedure. The preparation of bacterial extracts was as indicated in section XII except that (1) harvested cells were washed twice with equal volumes of 0.02M Tris·HCl buffer + mM $MgCl_2$, pH 8.0, and (2) the washed cell pellets were resuspended in 0.005M Tris·HCl buffer + mM $MgCl_2$, pH 7.0, prior to cell disruption, and this buffer was also used to flush the disruption flask after the breakage procedure. The complete reaction mixture contained the following components in a volume of 2.0 ml: Tris·HCl buffer, pH 7.5, 100 µmoles; $MgCl_2$, 2 µmoles; acetyl-CoA, 0.1 µmole; sodium glyoxylate, 1 µmole; and *Listeria* A4413 extract (PSS), 0.52 mgP. As soon the glyoxylate had been added, the decrease in the absorbance at 232 mµ was observed for 3 min against a reference which contained all components except glyoxylate, and which was set to read 0.500 against water so that the decrease in absorbancy of the complete system could be conveniently measured. Specific activity is expressed as the number of µmoles of acetyl-CoA cleaved/hr/mgP at room temperature. A millimolar extinction coefficient of 4.5 for acetyl-CoA (98) was used in the calculation.

12. NAD(P)H oxidase.

The oxidases for NADH or NADPH were estimated spectrophotometrically. The complete reaction mixture contained these components in a volume of 3.0 ml: potassium phosphate buffer, pH 7.0 (NADH) or 6.0 (NADPH), 200 µmoles; NADH, 0.5 µmole, or NADPH, 0.375 µmole; and *Listeria* A4413 extract (PSS), 0.25 mgP. Following the addition of NADH or NADPH the decrease in absorbance at 340 mµ was observed for 3 min against a water reference. Specific activity is expressed as the

number of μmoles of NADH or NADPH oxidized/hr/mgP at room temperature, using the millimolar extinction coefficient of 6.2 for NADH or NADPH (91) in the calculation.

XV. DETECTION OF CYTOCHROME PIGMENTS IN *LISTERIA* A4413

A dense *Listeria* suspension (≃ 0.5 mgN/ml) or crude extract (4.2 mgP/ml) was examined spectrophotometrically between 400 and 650 mμ by the difference spectra technique (100), using a Cary model 14 recording spectrophotometer (Cary Instruments, Monrovia, California). A few crystals of $Na_2S_2O_4$ were added to the cell suspension or extract before the reduced spectrum was determined. The spectra were graciously performed by Dr. Jack Fellman, Department of Biochemistry, University of Oregon Medical School.

RESULTS

I. *LISTERIA* A4413 AND 9037-7: CULTURAL AND MORPHOLOGICAL CHARACTERISTICS

Listeria A4413 and 9037-7 grew readily on either tryptose agar or blood agar. After incubation at 37 C for 24 hr the *Listeria* colonies were small, convex, smooth, circular, moist, entirely marginated, slightly opaque, and greyish in color. Whereas the average diameter of the A4413 colony was about 0.5 mm, the 9037-7 colony varied from about 0.5 mm to 1 mm. In size and general colonial appearance, each of these two *Listeria* strains was similar whether grown on tryptose agar or blood agar. On blood agar, the colonies from each strain produced a narrow (< 1 mm) zone of β-hemolysis in 24 hr. Between 24 and 48 hr at 37 C the size of the *Listeria* colonies increased somewhat and the hemolytic zone increased about two-fold.

Gram-stained smears of the two *Listeria* strains revealed that A4413 cells were small, uniformly stained gram-positive rods with rounded ends and about 0.5 x 1.0 μ in size. Strain 9037-7 cells stained like those of A4413, but tended to be longer and form some filaments.

Both *Listeria* strains grew well in tryptose broth in 24 hr at 37 C; strain A4413 presented a homogeneous turbidity while 9037-7 produced a sediment of growth, which is in harmony with its R type and tendency to form filaments.

The above characteristics of *Listeria* A4413 and 9037-7 agree in general with those described previously by other workers (1).

II. THE GROWTH RESPONSE OF *LISTERIA* A4413

A. Effect of the glucose concentration on *Listeria* growth.

Carbohydrate is required for the growth of *Listeria* and glucose is most commonly employed to meet the demand, as mentioned in the Introduction. The effect of various concentrations of glucose on the growth response of *Listeria* A4413 in buffered trypticase broth at 37 C is shown in Figure 3. The results indicate that this organism grew only when glucose was present, because no increase in turbidity occurred without glucose. Definite yet restricted growth occurred in the presence of only 0.05% glucose, and higher concentrations increased the growth. The 0.5% level supported maximum growth, and 1.0% glucose increased it no further. The extent of growth, based on the maximum turbidity attained, was roughly proportional to the glucose concentration from 0.05 to 0.5%. The slopes of the growth curves indicate that the logarithmic growth rates were similar on 0.1 to 0.5% glucose, but slightly slower on 0.05%.

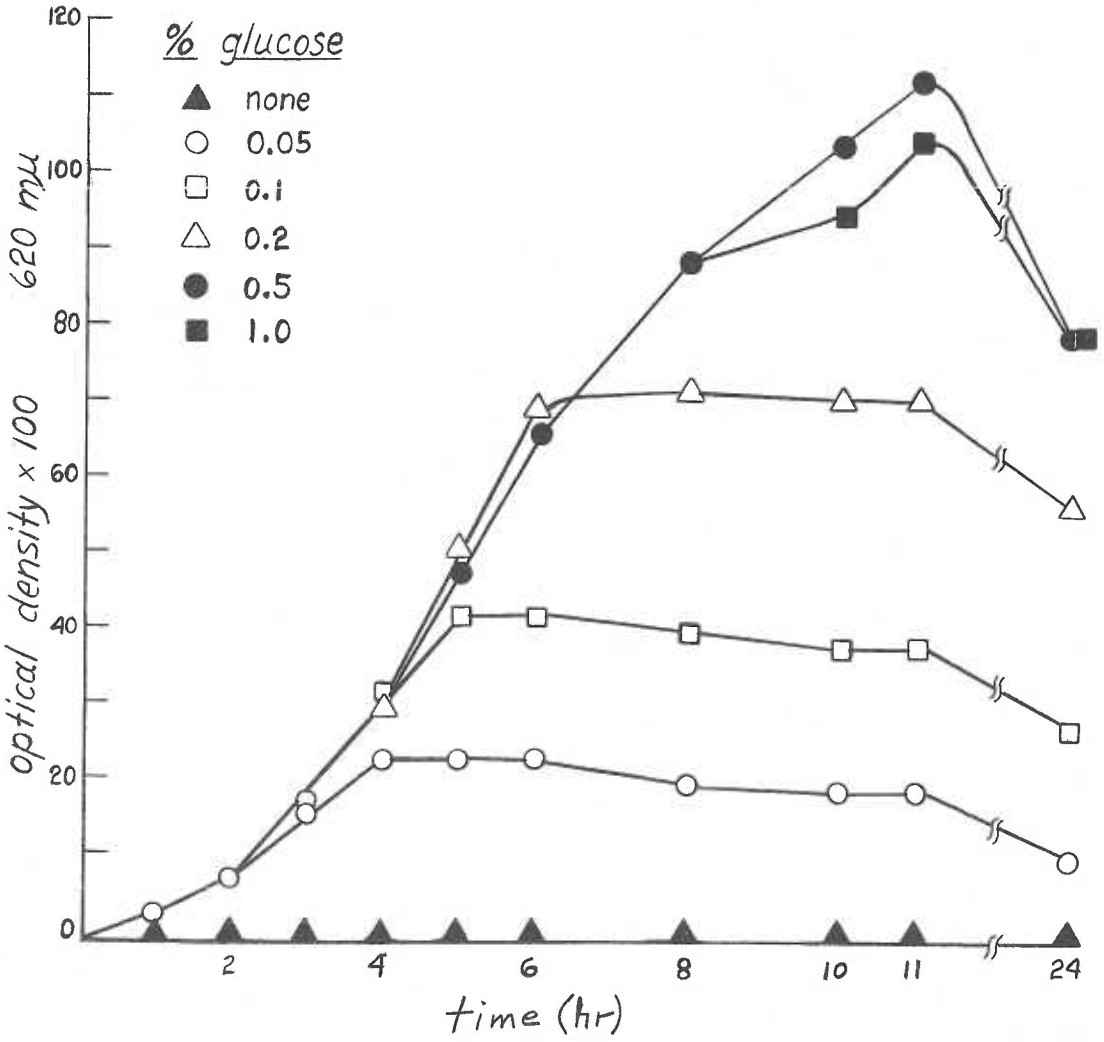
It was concluded from this experiment that the glucose concentration affects the growth of *Listeria*, as might be expected. Maximum growth occurred in the presence of 0.5% glucose, but 0.05% supported limited growth. The extent of growth on 0.2% glucose, the level which gave the most oxidative and virulent cultures in the work of Friedman and Alm (24), was about two-thirds of that seen on 0.5% glucose.

B. Effect of iron (as ferric ammonium citrate) and agitation on *Listeria* growth.

The growth response of *Listeria* A4413 was now determined in the

Figure 3.

Effect of the glucose concentration on the growth of *Listeria* A4413. Cultures were incubated without agitation in buffered trypticase broth at 37 C. The optical density of the growth tubes was measured at 620 mμ.

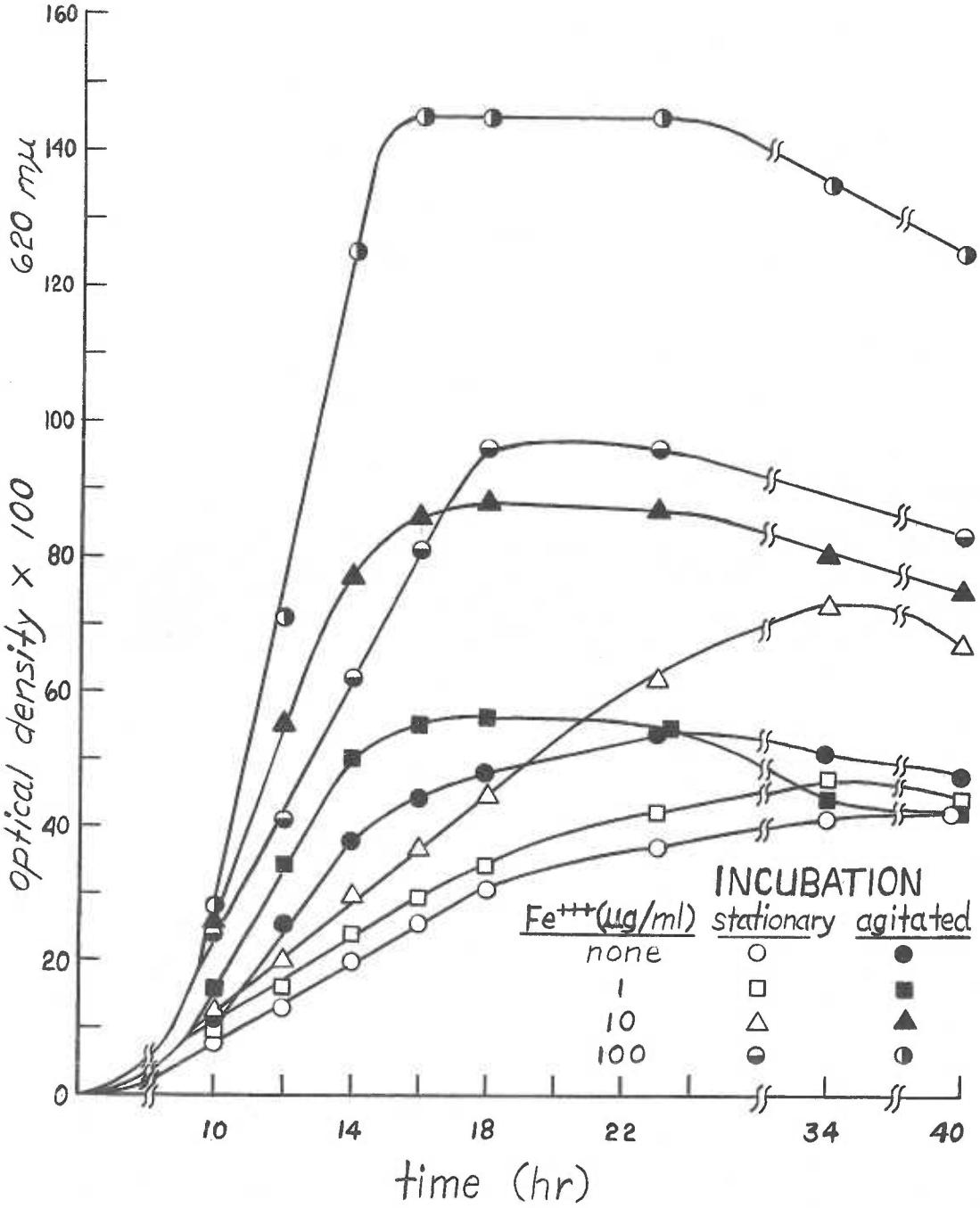

% glucose
none
0.05
0.1
0.2
0.5
1.0
optical density x 100
620 mμ
120
100
80
60
40
20
0
2
4
6
8
10
11
24
time (hr)

defined medium described by Welshimer (21). The composition of this medium is given in the Methods, step IV-B-1. Since iron is stimulatory for *Listeria* (22) and since the effect of agitation on *Listeria* growth in defined medium is controversial (20), it was decided to test the effects of iron and agitation on the growth of *Listeria* A4413 in Welshimer's defined medium. The results of this experiment are presented in Figure 4.

Listeria A4413 grew in this medium with or without iron and whether agitated or not. Iron and agitation each increased the growth; however, the greatest growth resulted in agitated tubes containing large amounts of iron. As little as 1 µg Fe^{+++}/ml stimulated *Listeria* A4413 growth, and 10 or 100 µg Fe^{+++}/ml was very stimulatory, especially under agitation. With or without agitation, growth was roughly proportional to the amount of iron included in the medium, although at each level of iron the growth was increased significantly by agitation. The combined effect of iron and agitation was most pronounced with 100 µg Fe^{+++}/ml at the 14 hr turbidity determination in which the growth was about 6 times greater than in iron-deficient stationary controls. The growth of *Listeria* A4413 in relation to the iron concentration, as observed here, is similar to that reported by Sword (22), who used shallow layers of medium without agitation and obtained approximately the same growth as found in this experiment with agitated tubes. Sword (22) also found that $FeSO_4$, within the limits of its solubility (10 µg/ml), was as effective as ferric ammonium citrate as an iron source; therefore the Fe^{+++} and not the citrate and ammonium content of ferric ammonium citrate was

Figure 4.

The effect of iron (as ferric ammonium citrate) and agitation on the growth of *Listeria* A4413 in Welshimer's defined medium (21). Cultures were incubated at 37 C either with or without agitation. The optical density of the growth tubes was measured at 620 mμ.


160
140
120
100
80
60
40
20
0
620 mμ
optical density × 100
10
14
18
22
34
40
time (hr)
INCUBATION
Fe+++(μg/ml)
stationary
agitated
none
1
10
100

responsible for the stimulation of *Listeria* growth (22).

III. THE ABILITY OF MEDIUM D10, WHEN SUPPLEMENTED WITH GLUCOSE, TO SUPPORT GROWTH OF *LISTERIA* A4413 AND 9037-7, AND THE EFFECT OF AGITATION AND IRON (AS $FeCl_3$) ON THE GROWTH RESPONSE

The need for a defined medium in this research and the design features of medium D10 are mentioned in the Methods, section V, and its composition and assembly are detailed in the Appendix. The objectives of this experiment were to determine whether *Listeria* A4413 and 9037-7 would grow in the new defined medium (D10); and to compare the growth response in this medium with that in medium D (no iron) and medium D20 (20 µg Fe^{+++}/ml) in relation to agitation. A supplement of 0.5% glucose in each medium provided the necessary carbon and energy source.

The growth of *Listeria* A4413 and 9037-7 is shown in Figures 5 and 6, respectively. While both *Listeria* strains grew in all three media, the quantity of growth was 2 to 3-fold greater in medium D10 and D20 than in medium D. The response of both strains in the three media was similar. In medium D, which contained no iron supplement, growth was only moderate and was not increased significantly by agitation. In medium D10 or D20 the growth was much improved over that in medium D, whether agitation was employed or not, although agitation improved the growth over that of stationary cultures. Growth was not greater in medium D20 than in D10, which indicates that 10 µg Fe^{+++}/ml is sufficient for *Listeria* growth under these conditions.

The finding that agitation increased the *Listeria* growth in these

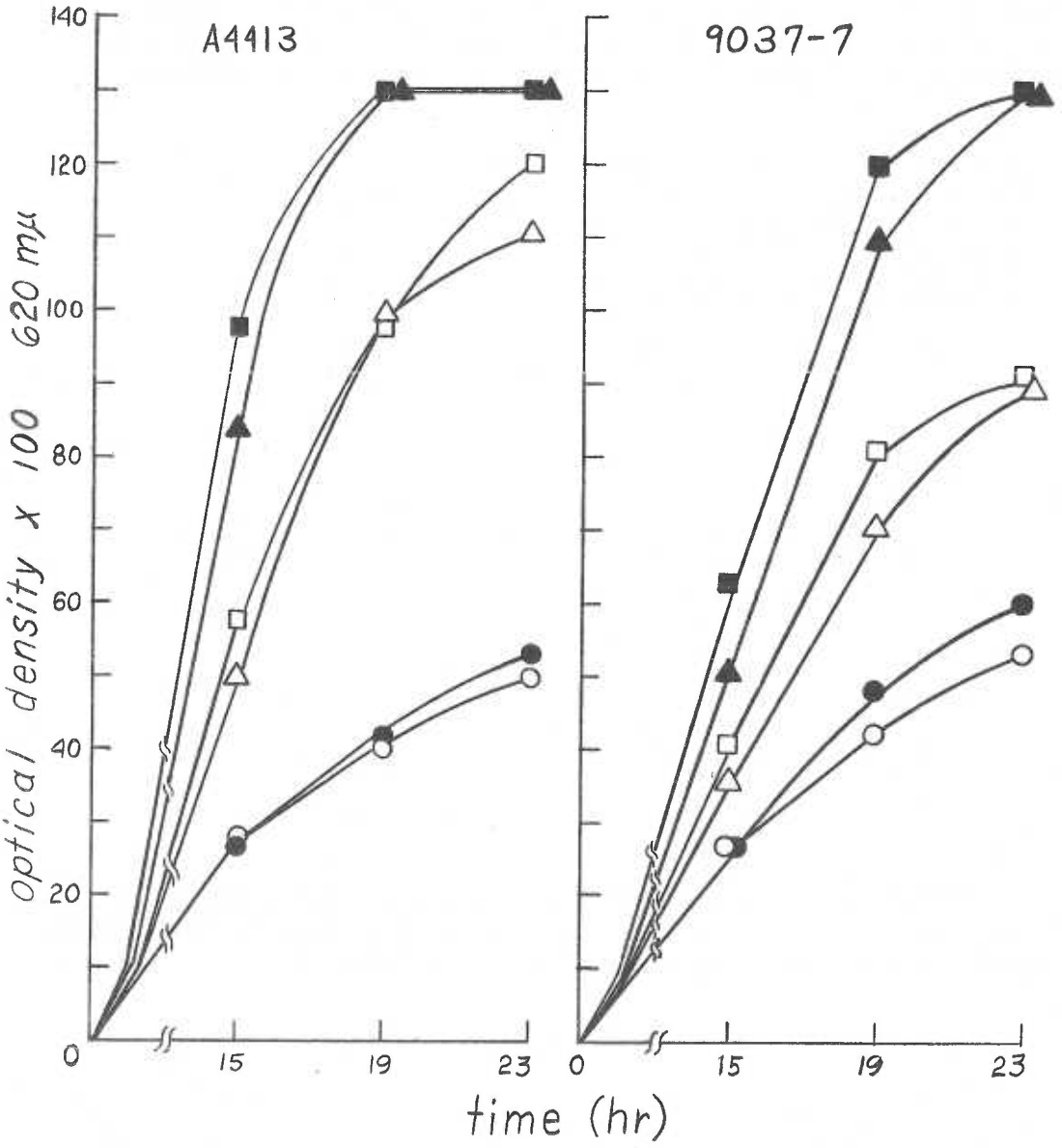
Figure 5.

The effect of agitation and iron (as $FeCl_3$) on the growth of *Listeria* A4413 in medium D, medium D10, and medium D20. Growth tubes were incubated at 37 C with or without agitation and the optical density was measured at 620 mμ. All media contained 0.5% glucose.

Circles: medium D (no iron).
Squares: medium D10 (10 μg Fe^{+++}/ml).
Triangles: medium D20 (20 μg Fe^{+++}/ml).
Open symbols: stationary.
Closed symbols: agitated.

Figure 6.

The effect of agitation and iron (as $FeCl_3$) on the growth of *Listeria* 9037-7 in medium D, medium D10, and medium D20. The growth conditions and symbols used are indicated in Figure 5.


A4413
9037-7
optical density x 100 620 mμ
140
120
100
80
60
40
20
0
15
19
23
time (hr)

defined media only when iron was included reinforces the similar results obtained in Welshimer's defined medium, as mentioned previously. Figures 4 to 6 indicate that iron (as Fe^{+++}) is important in *Listeria* growth, thus confirming a previous report (22); however, the finding that *Listeria* apparently require iron for improved growth in defined medium under agitation has not been reported previously, as far as is known. This relationship of iron and agitation may be relevant to the previous report that agitated subculture of *Listeria* A4413 required peptides (20), because the defined medium of Friedman and Roessler (20) was probably iron-deficient (see Methods, V-2).

Medium D10 and 0.5% glucose is thus excellent for the cultivation of *Listeria* A4413 or 9037-7. It is simpler, in terms of the amino acid complement (seven), than any medium yet described for *Listeria*, and it met the needs of this research for a defined medium. Medium D10 + 0.2% glucose was used for the growth of all *Listeria* intended for manometric or enzyme studies (see Methods, XII).

IV. THE SURVIVAL OF *LISTERIA* A4413 IN VARIOUS MENSTRUA

The purpose of this experiment was to compare the survival of *Listeria* in four different menstrua. The menstruum which best maintained the viability of these cells would then be used in subsequent cell washing and resuspending procedures.

Distilled water, saline (0.9% NaCl), 0.1M K phosphate buffer, pH 7.2, and 2% trypticase were the solutions tested. *Listeria* A4413 culture was diluted to 10^{-5} in the appropriate menstruum and plated in triplicate, as detailed in the Methods, section VII. After the

plating at zero time, the suspensions were replated after refrigeration for 4 and 21 hr. The results of this experiment are summarized in Table 3.

Trypticase solution not only best maintained the viability of *Listeria* A4413, but also supported a slight increase in growth. Since trypticase is a nutrient medium, however, it was not considered as a possible menstruum for washing and resuspension procedures; it was included in this experiment as a control. Of the non-nutrient menstrua, phosphate buffer most effectively preserved viability. After 21 hr, 93% of the original cells were viable in phosphate buffer while corresponding viabilities in 0.9% NaCl and distilled water were 67% and 0. Phosphate buffer, of the menstrua tested, therefore, was most suitable for washing and suspending *Listeria*; it was subsequently used for these purposes in this research.

V. THE CORRELATION OF THE TURBIDITY, NITROGEN CONTENT, AND VIABLE COUNT OF SUSPENSIONS OF *LISTERIA* A4413 AND 9037-7

The objective of this experiment was to correlate the turbidity of a washed *Listeria* suspension with its nitrogen content and number of viable cells, so that the nitrogen content or viable count could be estimated by a simple measurement of turbidity.

The procedures used in this experiment are given in the Methods, section VIII. The experimental data are summarized in Table 4 and presented graphically in Figures 7 and 8. In Figure 7 it is shown that the nitrogen content of either *Listeria* A4413 or 9037-7 is linearly related to the turbidity of the bacterial suspension. The

TABLE 3

THE SURVIVAL OF *LISTERIA* A4413 IN VARIOUS MENSTRUA

Menstruum	Plate Counts[a] 0 hr[b]	4 hr	21 hr	% Viability[c]
Distilled water	340 ± 11	174 ± 45	0[d]	0
0.9% NaCl	345 ± 14	368 ± 22	230 ± 28	67
0.1M K phosphate	328 ± 50	322 ± 18	306 ± 28	93
2% trypticase	393 ± 5	408 ± 41	458 ± 35	117

a. The mean number of *Listeria* colonies on three plates is indicated; the ± number denotes the range of individual counts.

b. The time indicates how long the diluted *Listeria* suspensions were refrigerated before plating. Before plating, each suspension was vigorously mixed with the Vortex. All plates were counted after incubation at 37 C for 24 hr, except the 4 and 21 hr platings from distilled water which were incubated for 48 hr.

c. This % represents the plate count at 21 hr divided by the original (0 hr) count.

d. Two of three plates were contaminated; the third showed no colonies after 48 hr.

TABLE 4

THE CORRELATION OF THE TURBIDITY, NITROGEN CONTENT, AND VIABLE COUNT OF SUSPENSIONS OF *LISTERIA* A4413 AND 9037-7[a]

Listeria strain	Turbidity[b]	Nitrogen[c] (mg/ml)	Turbidity[b]	Dilution plated	Plate counts[d]	*Listeria*/ml
A4413	14	0.018	01	10^{-5}	55 ± 14	5.5×10^{7}
	28	0.026	08	10^{-5}	380 ± 37	3.8×10^{8}
	58	0.045	20	10^{-6}	94 ± 20	9.4×10^{8}
			42	10^{-6}	216 ± 26	2.16×10^{9}
			58	10^{-6}	288 ± 39	2.9×10^{9}
9037-7	17	0.020	07	10^{-5}	36 ± 34	3.6×10^{7}
	33	0.029	14.5	10^{-5}	81 ± 10	8.1×10^{7}
	60	0.048	29	10^{-5}	160 ± 30	1.6×10^{8}
			56	10^{-6}	34 ± 22	3.4×10^{8}

a. *Listeria* suspensions were washed twice in 0.1M K phosphate buffer.

b. Turbidity = optical density x 100, as determined in a 13 mm cuvette at 620 mμ.

c. Determined by Nesslerization (see Methods, step VIII-3).

d. Value given is the mean colony count of at least 6 plates. The ± indicates the range of individual plate counts.

Figure 7.

The nitrogen content of *Listeria* A4413 and 9037-7 as determined by Nesslerization. The values plotted here are taken from Table 4.

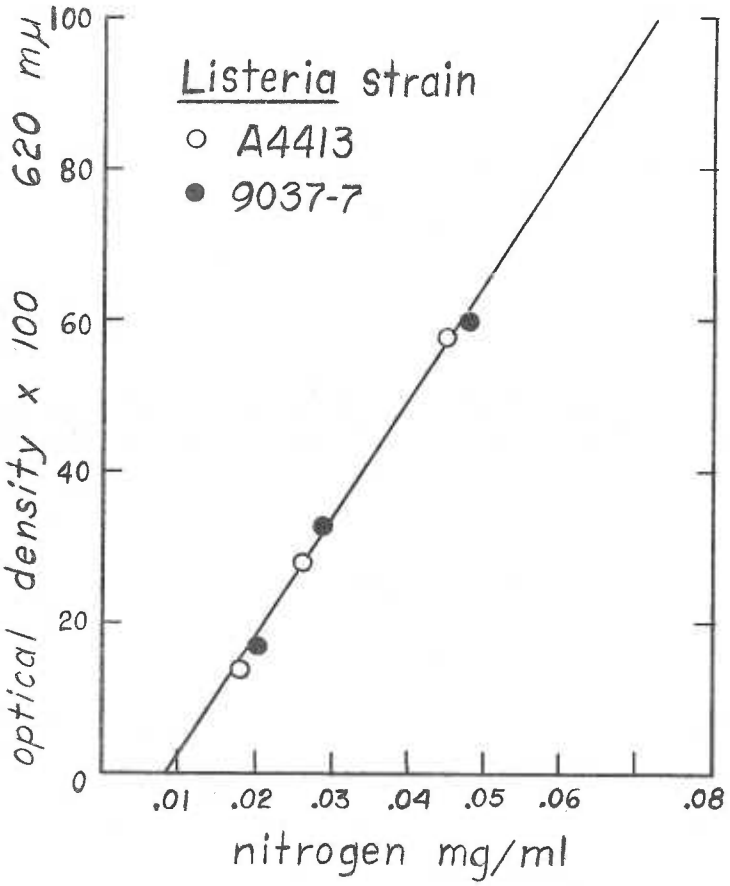
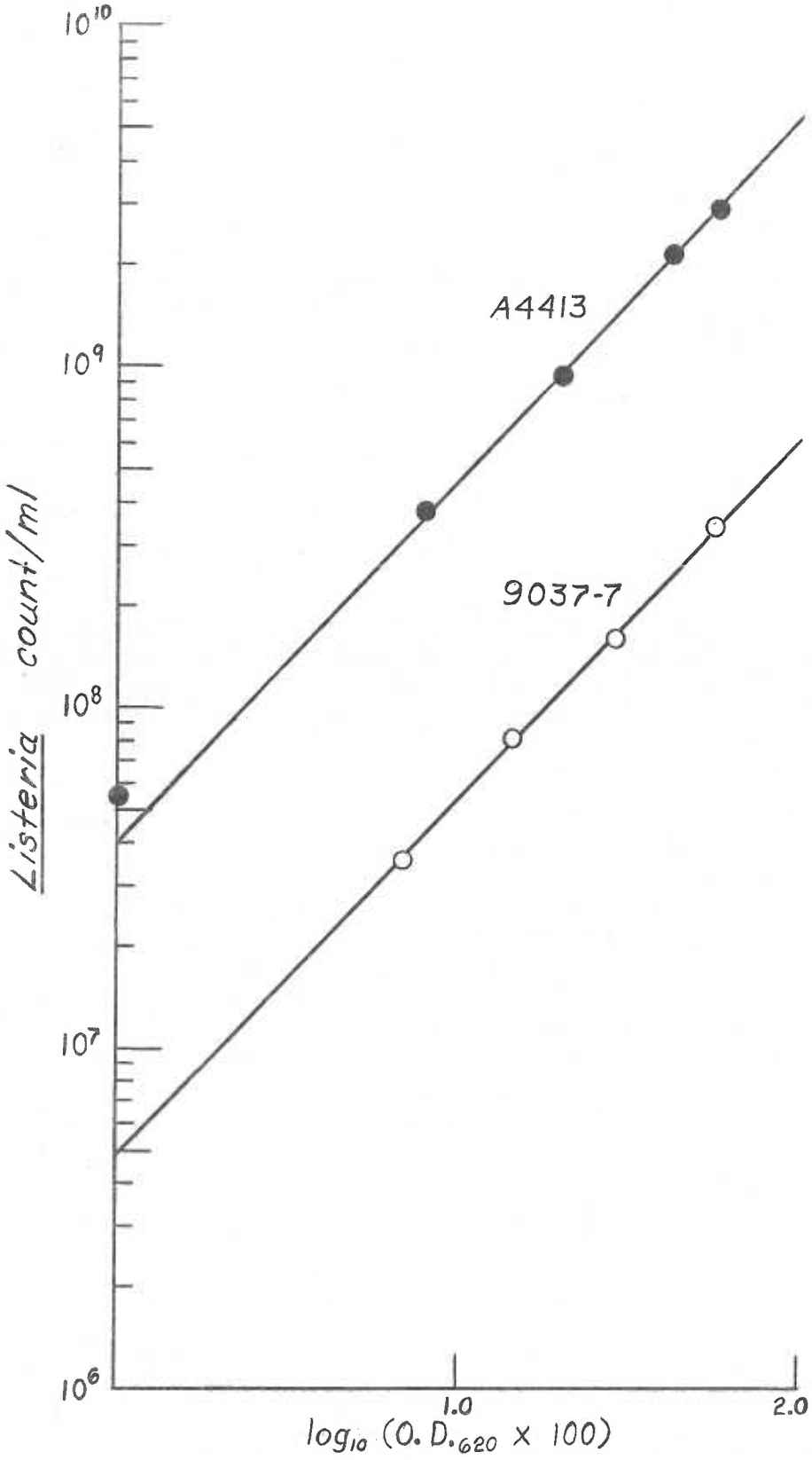

Listeria strain
A4413
9037-7
optical density x 100 620 mμ
0
20
40
60
80
100
.01
.02
.03
.04
.05
.06
.08
nitrogen mg/ml

Figure 8.

The viable count of *Listeria* A4413 and 9037-7 in relation to turbidity. The number of *Listeria* are plotted against the $\log_{10}$ of the optical density x 100 determined at 620 mμ in a 13 mm cuvette. The anti-logarithm of the value on the abscissa is the optical density x 100.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
Listeria count/ml
A4413
9037-7
1.0
2.0
$\log_{10}$ (O. D.$_{620}$ × 100)

nitrogen contents of strains A4413 and 9037-7 were equivalent; the line on the graph (Figure 7) connects the points which represent the nitrogen content of each *Listeria* strain.

Figure 8 indicates that a linear relationship exists between the $\log_{10}$ of the turbidity x 100 and the number of viable *Listeria*. At a given turbidity, the number of *Listeria* 9037-7 was about a tenth of the number of strain A4413. This difference may be explained by the tendency of strain 9037-7 to form filaments which would be expected to give lower colony counts.

VI. THE ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE TO SERVE AS A SOURCE OF CARBON AND ENERGY FOR *LISTERIA* A4413 AND 9037-7

The experimental results presented thus far are important to this thesis, but do not directly concern the central problem of the citrate cycle and related metabolism of *Listeria*. This problem was now confronted directly.

The first question for which answers were sought concerned whether pyruvate or any individual intermediate of the citrate cycle could serve as a source of carbon and energy for *Listeria* A4413 and 9037-7. The experimental aims here were broader than to simply note whether growth occurred on a given intermediate, because citrate cycle intermediates, if acceptable to *Listeria*, could be used in later experiments to produce cells containing higher levels of citrate cycle enzymes than glucose-grown cells (see Introduction, IV-C-5).

Medium D10 was supplemented with either 0.2% citrate, isocitrate,

α-ketoglutarate, succinate, fumarate, malate, pyruvate, acetate, or glucose (control) and used as a test medium. Growth of *Listeria* A4413 and 9037-7 was measured turbidimetrically at 37 C either with or without agitation. The results of this experiment are summarized in Table 5.

The results were clear-cut in that neither *Listeria* A4413 nor 9037-7 grew on pyruvate or any intermediate of the citrate cycle. Growth of both *Listeria* strains occurred on glucose, the control source of carbon and energy. In the presence of pyruvate or any of the citrate cycle intermediates tested, the turbidity level either remained constant at the initial level following inoculation or declined slightly in 48 hr similar to the control tubes which received no supplement. This was the case with either *Listeria* A4413 or 9037-7 whether incubated with or without agitation. Clearly, none of the compounds tested served as a carbon and energy source for *Listeria* because no growth occurred. These experimental results confirm two previous reports that neither citrate (25) nor pyruvate (24) are suitable carbon sources for *Listeria* growth.

VII. THE ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE, WHEN SUPPLEMENTED WITH GLUCOSE, TO SERVE AS A SOURCE OF CARBON AND ENERGY FOR *LISTERIA* A4413 AND 9037-7

Because *Listeria* did not utilize pyruvate or the citrate cycle intermediates as a sole source of carbon and energy (section VI), further experiments were conducted similar in every detail to those of section VI except that 0.05% glucose was included in medium D10. This

TABLE 5

THE ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE TO SUPPORT THE GROWTH OF *LISTERIA* A4413 AND 9037-7 IN MEDIUM D10*

	Listeria A4413 Type and time of incubation						*Listeria* 9037-7 Type and time of incubation					
	Stationary			Agitated			Stationary			Agitated		
Compound (0.2%)	0	24 hr	48 hr	0	24 hr	48 hr	0	24 hr	48 hr	0	24 hr	48 hr
None	02	02	01	01	01	01	05	04	04	06	05	05
Glucose	01	61	47	04	70	17	03	67	32	07	72	66
Citrate	01	01	01	02	02	02	03	02	02	04	03	03
Isocitrate	02	01	01	02	02	02	02	02	01	05	04	04
α-ketoglutarate	03	03	02	03	02	02	02	02	02	06	05	05
Succinate	02	02	01	01	00	00	03	02	02	05	04	04
Fumarate	02	02	02	02	01	01	02	02	02	05	05	05
Malate	02	02	02	03	02	02	02	02	02	04	04	03
Pyruvate	02	02	01	03	02	02	05	04	04	04	03	02
Acetate	02	02	01	01	00	00	05	04	04	05	05	05

* Growth is expressed as the optical density x 100 at 620 mμ determined at 0, 24, and 48 hr. Incubation was at 37 C. The results are representative of two experiments, each of which used duplicate growth tubes.

low glucose concentration allows limited yet definite *Listeria* growth (see Figure 3) such that increased growth in response to a supplement of pyruvate or other intermediate could be easily detected turbidimetrically. The experiment was so designed because certain bacteria such as *E. coli* may require a second carbon source in order to utilize compounds such as citrate (44). The experimental results are presented in Table 6.

With reference to the level of growth in the controls, there was no significant difference in the growth of either *Listeria* strain in the presence of any supplement tested. The maximum turbidity (O.D. at 620 mμ x 100) in the presence of any supplement varied no more than 2 from that with glucose alone, with the exception of strain 9037-7 under stationary incubation with fumarate which showed a turbidity increase of 3 over that with glucose alone. Under these growth conditions, it was concluded that neither pyruvate nor any intermediate of the citrate cycle was apparently suitable as a carbon and energy source for these *Listeria*.

VIII. THE GROWTH CURVE OF *LISTERIA* A4413 IN MEDIUM D10 + 0.2% GLUCOSE IN RELATION TO THE INOCULUM SIZE

The purpose of this experiment was to determine when a *Listeria* A4413 broth culture, destined for physiological studies, should be harvested in order to obtain late log phase cells. Since *Listeria* is apparently unable to utilize pyruvate or intermediates of the citrate cycle for growth purposes, glucose was used as a source of carbon and energy in this and in all subsequent experiments reported

TABLE 6

THE ABILITY OF PYRUVATE OR SINGLE INTERMEDIATES OF THE CITRATE CYCLE TO INCREASE THE GROWTH OF *LISTERIA* A4413 AND 9037-7 IN MEDIUM D10 + 0.05% GLUCOSE*

	Type of Incubation			
	Stationary		Agitation	
	Listeria strain			
Supplement (0.2%)	A4413	9037-7	A4413	9037-7
None	17	16	18	18
Citrate	16	16	18	18
Isocitrate	15	16	16	18
α-ketoglutarate	16	16	17	19
Succinate	16	16	17	18
Fumarate	16	19	19	20
Malate	16	16	17	19
Pyruvate	16	16	18	19
Acetate	16	15	20	18

*The data are expressed as the optical density x 100 at 620 mμ. Each value represents the highest optical density observed (after correction for the initial reading at inoculation) throughout the incubation period at 37 C; readings were made at 0, 8, 16, and 24 hr. Every value is the mean from two experiments which used duplicate growth tubes.

in this thesis. According to Friedman and Alm (24), growth of *Listeria* A4413 on 0.2% glucose yields cells with more oxidative activity than higher glucose levels (see Table 1). This (0.2%) concentration of glucose was employed in this and in all subsequent experiments.

Listeria A4413 were grown in medium D10 + 0.2% glucose contained in Nephelo sidearm flasks; the growth response in relation to the inoculum size is shown in Figure 9. The cultures grew well under these conditions and yielded an optical density x 100 of about 90 regardless of the inoculum size. Maximum growth was attained in approximately 14 hr (0.25 ml inoculum) or 16 hr (0.125 ml inoculum). Based on these growth curves, it was decided to harvest future cultures grown in D10 + 0.2% glucose either at 13.0 to 13.5 hr or 15.0 to 15.5 hr, depending on which inoculum had been used to initiate growth. The pH of the cultures at maximum growth was 6.8, indicating that there was a drop in pH of about 0.5 from the time of inoculation (the pH of medium D10 is 7.3 to 7.4).

IX. THE OXIDATIVE ABILITY OF RESTING *LISTERIA* A4413

A. The oxidation of glucose.

Glucose was rapidly oxidized by resting *Listeria* A4413 as shown in Figure 10. Oxidation was most rapid at pH 6.0, but it also occurred at pH 7.0 and 8.0. The rate and extent of glucose oxidation was slightly stimulated by the cofactor mixture (CFM). At pH 6.0, with the CFM included, the $Q_{O_2}(N)$ was 767 based on the oxygen consumed between 10 and 20 min, after correction for the endogenous oxygen consumption ($Q_{O_2}[N] = 32$). Under these same conditions, the oxidation

Figure 9.

The growth curve of *Listeria* A4413 in medium D10 + 0.2% glucose in relation to the inoculum size. Growth was initiated by either 0.25 ml or 0.125 ml of inoculum as detailed in the Methods step XI-2. Cultures were shaken at 37 C and the optical density was determined at 620 mμ by means of the 13 mm sidearm on each Nephelo flask. Three flasks received the larger inoculum (0.25 ml) and their growth range is indicated by the vertical bars.

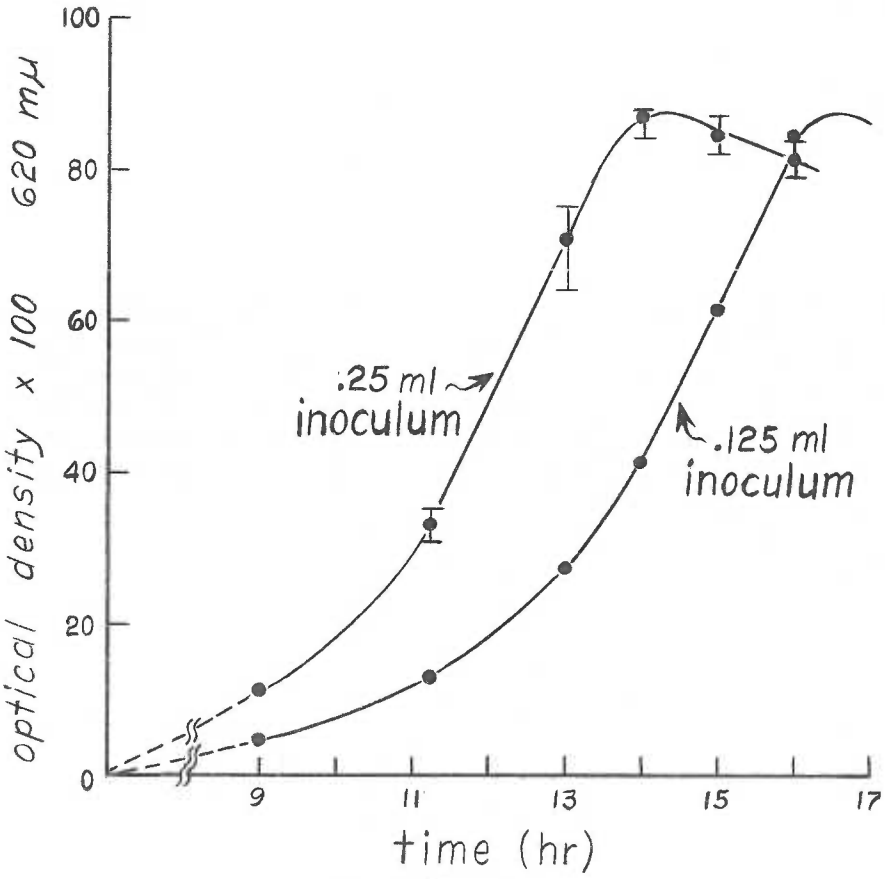
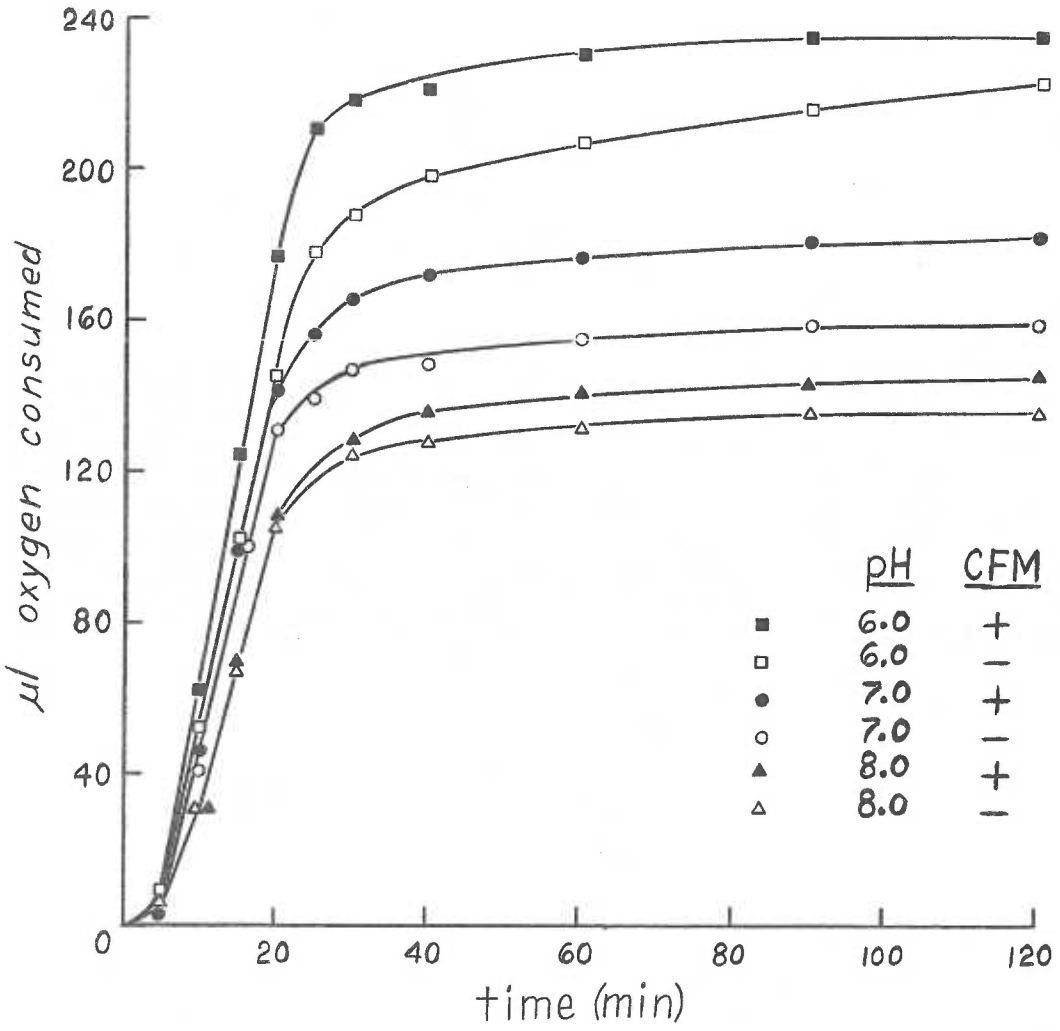

optical density x 100 620 mμ
100
80
60
40
20
0
9
11
13
15
17
time (hr)
.25 ml inoculum
.125 ml inoculum

Figure 10.

The oxidation of glucose by resting *Listeria* A4413 at various pH values. The Warburg vessels contained bacterial nitrogen, 0.9 mg, K phosphate buffer, 200 µmoles (pH indicated), and glucose, 10 µmoles, in a total volume of 3.0 ml. The center well contained 0.1 ml of 10% KOH. A cofactor mixture (CFM) (contained in µmoles): NAD, 1; NADP, 1; DPT, 0.5; CoA, 0.1; α-lipoate, 0.1; L-cysteine, 1; and $MgCl_2$, 1) was included (+) or excluded (-) in each reaction mixture as indicated. The temperature was 37 C. The oxidation rates have been corrected for endogenous oxidation, with or without CFM.


240
200
160
120
80
40
0
µl oxygen consumed
20
40
60
80
100
120
time (min)
pH
CFM
6.0 +
6.0 −
7.0 +
7.0 −
8.0 +
8.0 −

ratio (μmoles O_2/μmole glucose) at 120 min was 1.05, which is compatible with a pathway of aerobic glycolysis giving rise to two pyruvates per glucose molecule.

B. The oxidation of pyruvate.

Pyruvate was oxidized by resting *Listeria* A4413, but only in a moderately acid medium. The effect of pH on the rate of pyruvate oxidation in the absence of coenzyme supplements is summarized in Table 7. No oxidation was observed at pH 8.0, 7.0, or 4.0. Pyruvate was oxidized, however, between pH 4.5 and 6.5, and the rate was most rapid in phosphate buffer at pH 5.0, which yielded a Q_{O_2}(N) of 133. The rate of oxidation in phthalate·NaOH buffer at pH 5.0 was slightly less than in phosphate buffer at the same pH.

Certain coenzymes involved in the pyruvate dehydrogenase system of other organisms affect the rate and extent of pyruvate oxidation by resting *Listeria* A4413, as presented in Figure 11.

The addition of DPT, FAD, or $MgCl_2$ did not stimulate the rate or extent of pyruvate oxidation and the oxidation was only slightly increased by either CoA, NAD, or $MnCl_2$. Oxidation was increased considerably, however, by α-lipoate and slightly more by the complete coenzyme mixture. Since α-lipoate was nearly as stimulatory as the complete coenzyme mixture, this coenzyme was apparently the most active supplement. Inclusion of α-lipoate in the reaction mixture increased the Q_{O_2}(N) from 120 (unsupplemented) to 220, and increased the oxidation ratio (at 2 hr) from 0.42 to 0.55. This oxidation ratio suggests that *Listeria* A4413 oxidized pyruvate to the level of acetate.

TABLE 7

THE EFFECT OF pH ON THE RATE OF PYRUVATE OXIDATION BY RESTING *LISTERIA* A4413[a]

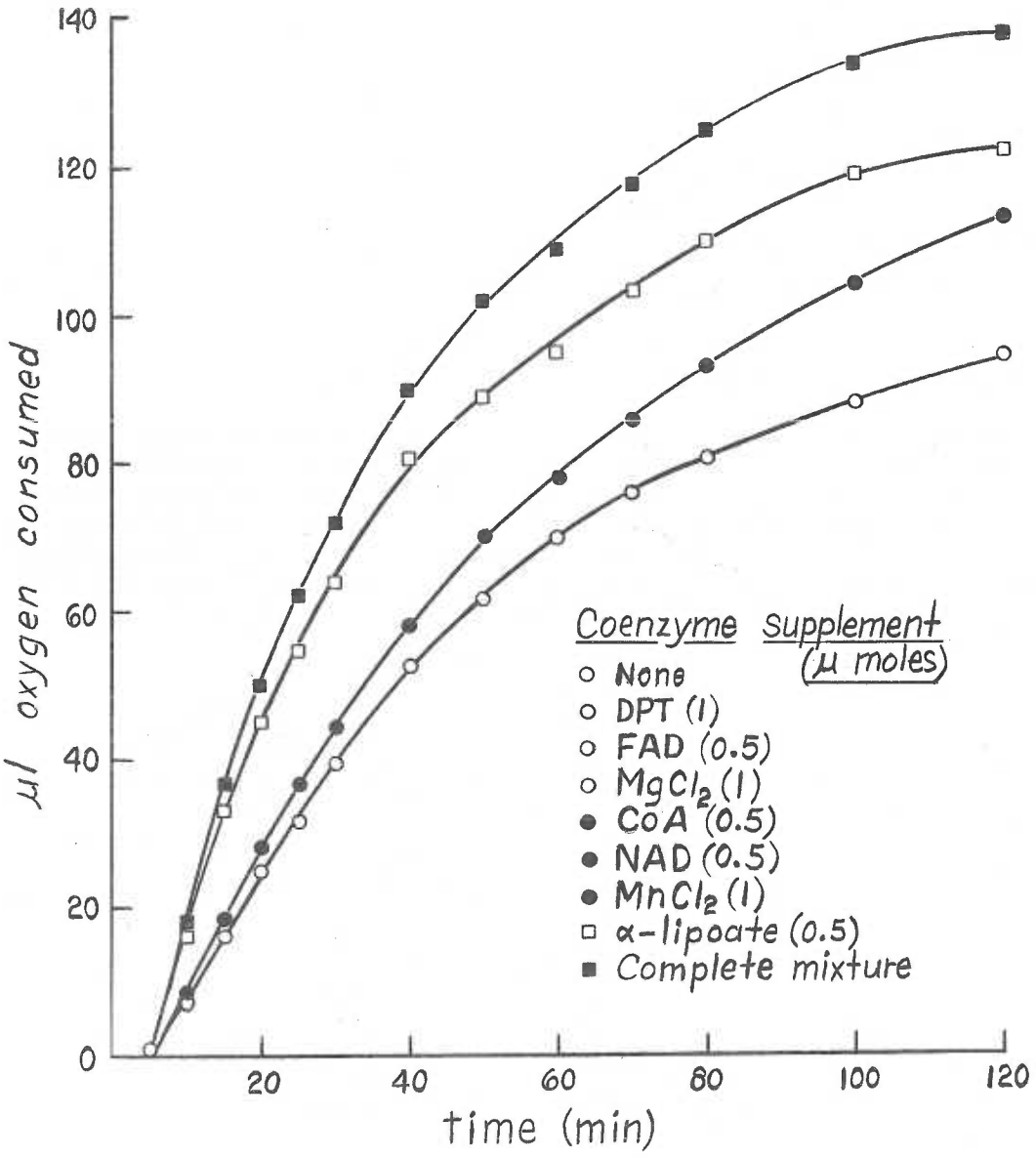
Buffer, pH	Q_{O_2} (N)[b]
K phosphate, 8.0	0
" 7.0	0
" 6.5	10
" 6.0	26
" 5.5	40
" 5.0	133
K phthalate·NaOH, 5.0	93
" 4.5	16
" 4.0	0

a. The Warburg vessels contained bacterial nitrogen, 0.9 mg, buffer (type and pH indicated above), 200 µmoles, and sodium pyruvate, 10 µmoles. The center well contained 0.1 ml 10% KOH. The total volume was 3.0 ml, and the temperature was 37 C.

b. Corrected for endogenous oxidation.

Figure 11.

The effect of coenzyme supplements on the oxidation of pyruvate by resting *Listeria* A4413. Each Warburg vessel contained the following in 3.0 ml: K phosphate buffer, pH 5.0, 200 μmoles; bacterial nitrogen, 0.9 mg; sodium pyruvate, 10 μmoles; coenzyme supplements as indicated; and 0.1 ml 10% KOH in the center well. The temperature was 37 C. The *Listeria* suspension had been refrigerated for 24 hr when used. There was no significant endogenous oxidation.


140
120
100
80
60
40
20
0
µl oxygen consumed
20
40
60
80
100
120
time (min)
Coenzyme supplement (µ moles)
None
DPT (1)
FAD (0.5)
MgCl2 (1)
CoA (0.5)
NAD (0.5)
MnCl2 (1)
α-lipoate (0.5)
Complete mixture

Since the pyruvate dehydrogenase complex is the only known pathway for the oxidation of pyruvate which requires α-lipoate (see Introduction, IV-B-3), the stimulation of pyruvate oxidation in *Listeria* A4413 by α-lipoate suggests that this organism oxidizes pyruvate by an enzyme system identical with or similar to pyruvate dehydrogenase complex. Doubling the concentration of α-lipoate produced no further stimulation of pyruvate oxidation.

C. The oxidation of citrate cycle intermediates and caproic and oleic acids.

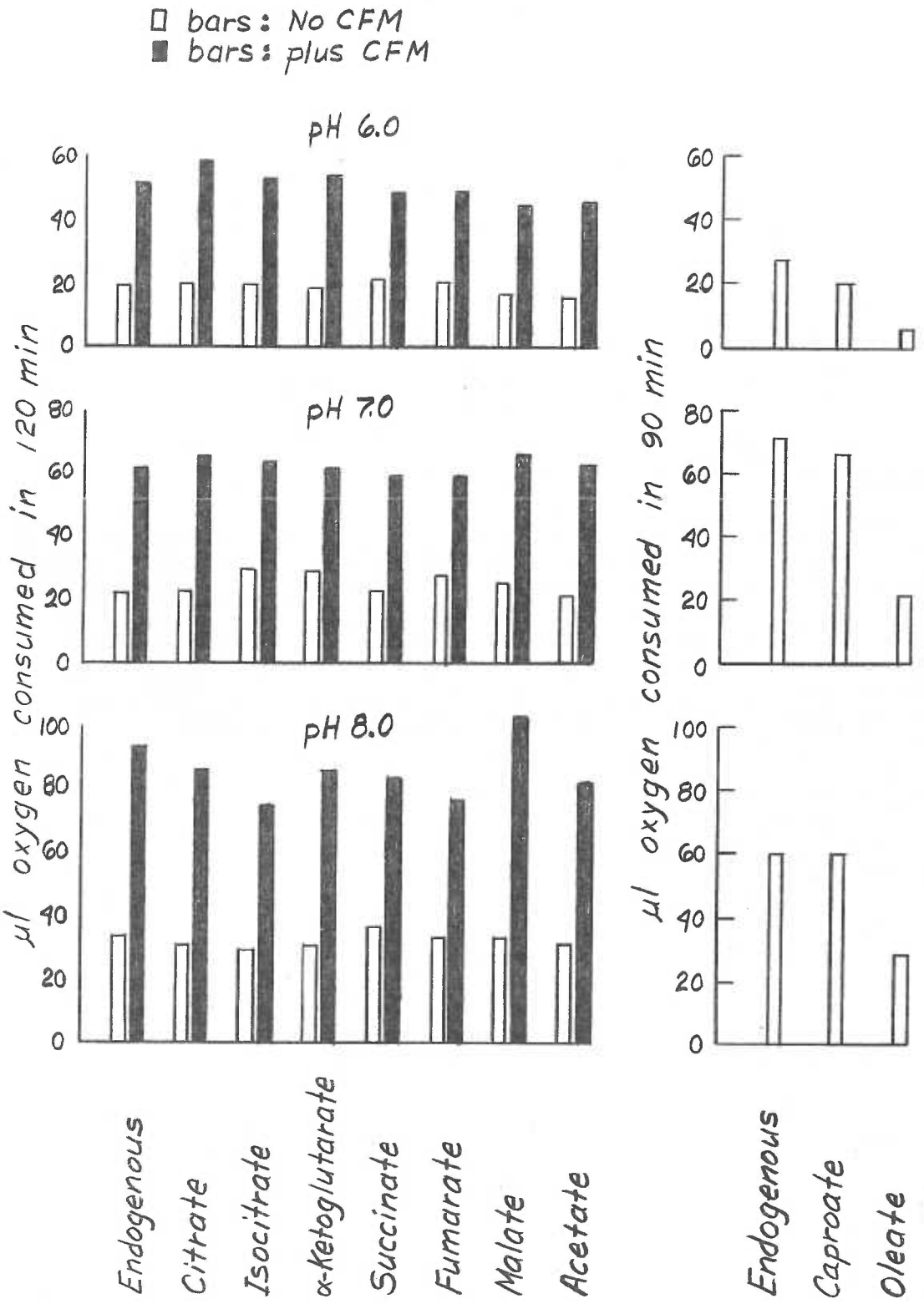
The effect of citrate cycle intermediates or caproate or oleate on the oxygen consumption by resting *Listeria* A4413 is shown in Figure 12.

With the endogenous oxygen consumption as a reference, there was apparently no significant stimulation of oxygen consumption by any citrate cycle intermediate at any pH, in the presence or absence of CFM. Other similar experiments, one of which was at pH 5.0, yielded results little different from those in Figure 12. The evidence obtained in these several experiments indicates that resting cells of *Listeria* A4413, grown and tested as described, lack the capacity to oxidize exogenous citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, or acetate.

Figure 12 also indicates that *Listeria* A4413 failed to oxidize sodium caproate or sodium oleate. Caproate had no apparent effect on endogenous oxidation by resting *Listeria*, but oleate seemed definitely inhibitory. At pH 6.0, 7.0, or 8.0, the oxygen consumption

Figure 12.

The oxidation of citrate cycle intermediates or caproate or oleate by resting *Listeria* A4413 at pH 6.0, 7.0, and 8.0. The Warburg vessels contained the following in a volume of 3.0 ml: K phosphate buffer, 200 μmoles, pH indicated; bacterial nitrogen, 0.9 mg; substrate, 10 μmoles; a cofactor mixture (CFM) as detailed in Figure 10, included as indicated; and 0.1 ml 10% KOH was added to the center well. The temperature was 37 C. In the oxidation of caproate and oleate, *Listeria* equivalent to 1.8 mg of bacterial nitrogen were used.


□ bars: No CFM
■ bars: plus CFM
pH 6.0
pH 7.0
pH 8.0
µl oxygen consumed in 120 min
µl oxygen consumed in 90 min
0
20
40
60
80
100
Endogenous
Citrate
Isocitrate
α-Ketoglutarate
Succinate
Fumarate
Malate
Acetate
Endogenous
Caproate
Oleate

in the presence of oleate decreased by at least 50%.

In this research so far, no evidence has been obtained to indicate the existence of a citrate cycle in *Listeria* A4413. None of the citrate cycle intermediates supported *Listeria* growth, nor were they oxidized. Experiments were now directed toward determining whether cell-free extracts of *Listeria* A4413 contain the enzymes of the citrate cycle, a pyruvate oxidation system, and oxidases for the reduced pyridine nucleotides.

X. THE RESULTS OF ASSAYS FOR THE OXIDATION OF PYRUVATE, THE ENZYMES OF THE CITRATE CYCLE AND GLYOXYLATE BYPASS, AND REDUCED PYRIDINE NUCLEOTIDE OXIDASES IN CELL-FREE EXTRACTS OF *LISTERIA* A4413

In the results which follow, the reaction mixture given for each assay provided essentially optimal enzyme activity as was determined by experimental changes in the pH, the type and concentration of certain buffers, substrates, and coenzymes. The effects of experimental variations in the reaction mixture on the optimal reaction rate (illustrated in each figure) are mentioned in the text. As detailed in the Methods, all control cell-free extracts of *E. coli* K-12(λ) were from *E. coli* grown under conditions identical to those of *Listeria* growth, unless specified otherwise.

A. Pyruvate oxidation system.

Since resting *Listeria* A4413 oxidize pyruvate, as shown previously, it was expected that pyruvate oxidation would be demonstrable in cell-free extracts from this organism. This was the case. The enzyme

was estimated manometrically.

In Figure 13 is shown the oxidation of pyruvate by *Listeria* A4413 crude extract (CE) at pH 7.0. The effect of deleting single coenzymes or cysteine from the complete mixture is illustrated.

Maximal oxidation of pyruvate was observed at pH 7.0; the specific activity (µl O_2 consumed/hr/mg protein) was 47.3 at pH 7.0 as compared to 25.5 at pH 7.5 and 15.5 at pH 6.5. In the absence of all coenzymes and cysteine there was no oxidation of pyruvate. All coenzymes (except Mg^{++}) and cysteine were required for maximal oxidation. The deletion of Mg^{++} caused no significant change in the oxidative response. The deletion of DPT, CoA, NAD, α-lipoate, or cysteine, however, in increasing order of importance, significantly decreased the oxidation rate. Without α-lipoate, for example, the oxidation rate fell about 71%. The requirement for DPT, CoA, NAD, and especially α-lipoate suggests that this oxidation of pyruvate by *Listeria* extract involves the pyruvate dehydrogenase complex (see Introduction, IV-B-3). In the presence of all coenzymes and cysteine, the oxidation ratio was 0.47, which suggests, as did the oxidation of pyruvate by resting *Listeria*, that pyruvate was oxidized to the level of acetate.

B. α-ketoglutarate oxidation system.

The oxidation of α-ketoglutarate, like that of pyruvate, was estimated manometrically. The reaction mixture was similar to that of the pyruvate oxidation assay except that α-ketoglutarate replaced pyruvate as the substrate.

The experimental results are presented in Figure 14. A pH range

Figure 13.

The oxidation of pyruvate by *Listeria* A4413 CE, and the effect of individual coenzyme deletions. The complete reaction mixture in Warburg vessels included the following components in a volume of 3.0 ml: K phosphate buffer, pH 7.0, 200 μ moles; sodium pyruvate, 20 μmoles; *Listeria* A4413 CE, 6.6 mg protein; and the following (in μmoles): $MgCl_2$ (1), DPT (0.5), CoA (0.1), NAD (1), α-lipoate (0.1), and L-cysteine (1); the center well contained 0.1 ml 10% KOH. The temperature was 37 C. No oxidation occurred in the absence of pyruvate.

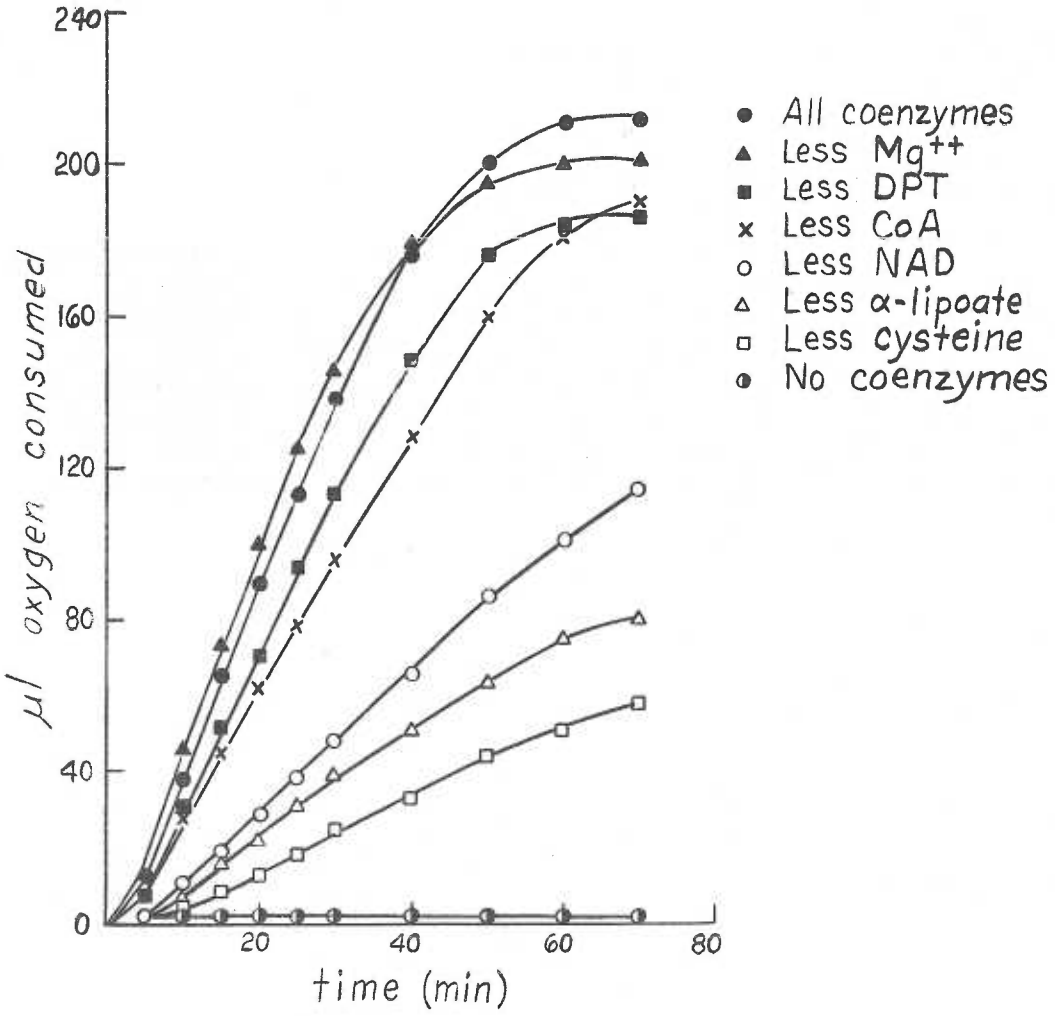
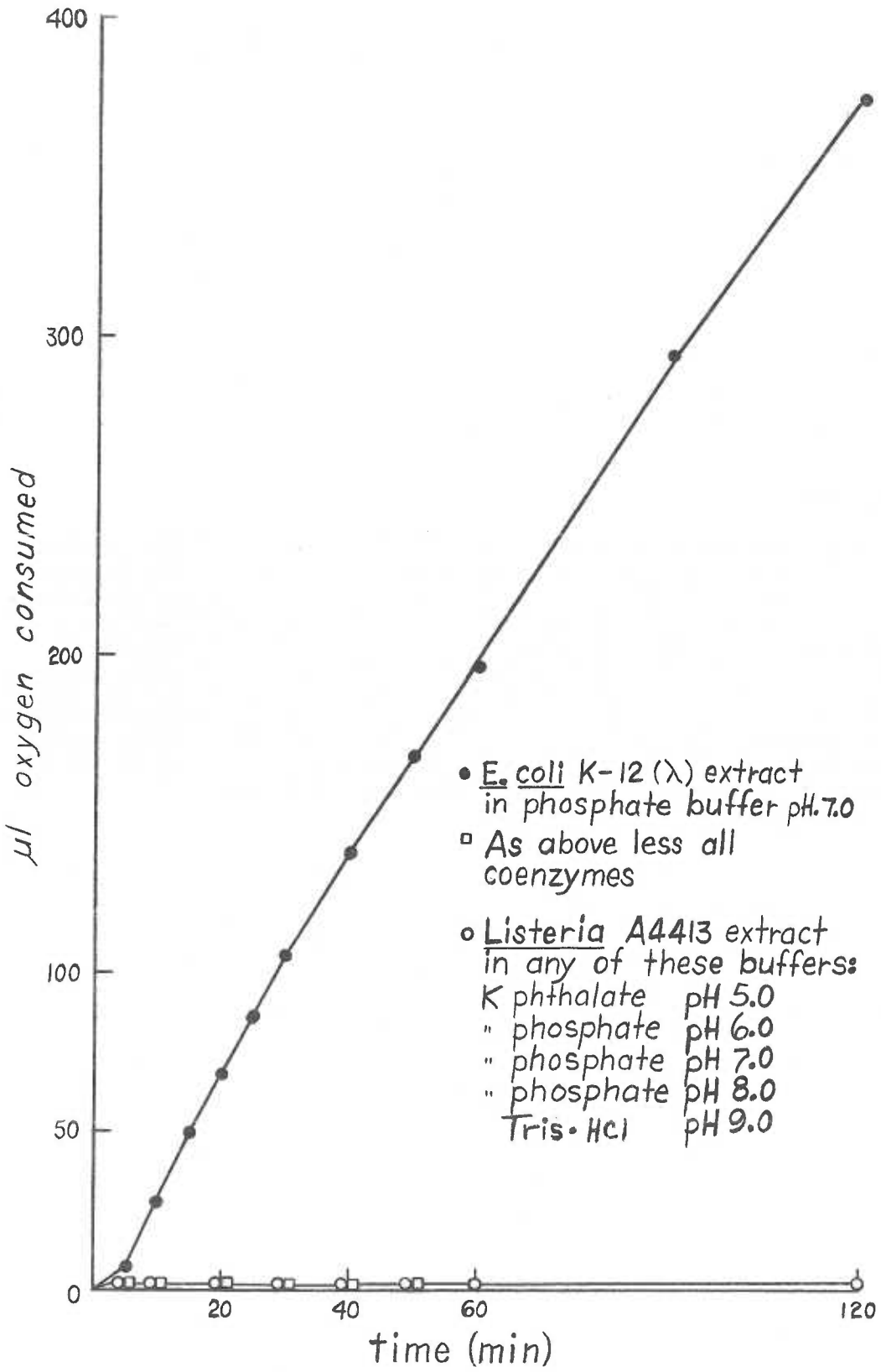

240
200
160
120
80
40
0
μl oxygen consumed
20
40
60
80
time (min)
All coenzymes
Less Mg++
Less DPT
Less CoA
Less NAD
Less α-lipoate
Less cysteine
No coenzymes

Figure 14.

The oxidation of α-ketoglutarate by *Listeria* A4413 CE. A CE of *E. coli* K-12(λ), acetate-grown, was used as a control. The complete reaction mixture in Warburg vessels included the following components in 3.0 ml: K phosphate buffer, pH 7.0, 200 μmoles (or buffers as indicated); sodium α-ketoglutarate, 20 μmoles; *Listeria* A4413 CE, 6.25 mg protein, or *E. coli* K-12(λ) CE, 6.9 mg protein; the following in μmoles: $MgCl_2$ (1), DPT (0.5), CoA (0.1), NAD (1), α-lipoate (0.1), and L-cysteine (1); and 0.1 ml of 10% KOH in the center well. The temperature was 37 C. The oxidation of α-ketoglutarate by *E. coli* extract was corrected for slight endogenous oxidation.


400
300
200
100
50
0
µl oxygen consumed
20
40
60
120
time (min)
• E. coli K-12 (λ) extract in phosphate buffer pH. 7.0
▫ As above less all coenzymes
o Listeria A4413 extract in any of these buffers:
K phthalate pH 5.0
" phosphate pH 6.0
" phosphate pH 7.0
" phosphate pH 8.0
Tris·HCl pH 9.0

of 5.0 to 9.0 was employed in the search for α-ketoglutarate oxidation in *Listeria* A4413 CE, but no oxidation was detected. A control extract of *E. coli* K-12(λ), however, was tested and found oxidative under identical conditions.

α-ketoglutarate oxidation by *E. coli* K-12(λ) CE was most rapid about pH 7.0, showing a specific activity (μl O_2 consumed/hr/mg protein) of 34.7 as compared to 18.5 and 28.8 at pH 6.5 and 7.5, respectively. Oxidative activity was dependent upon the mixture of coenzymes and cysteine.

Listeria A4413 CE failed to oxidize α-ketoglutarate in any buffer system from phthalate·NaOH, pH 5.0, to Tris·HCl, pH 9.0. The oxidation of α-ketoglutarate by extracts from acetate-grown *E. coli* K-12(λ) suggests that the assay method was valid, and that this enzyme system is probably absent in *Listeria* A4413 under the assay conditions. α-ketoglutarate oxidation by extracts from acetate-grown *E. coli* K-12(λ) was about 4-fold greater than by extracts from glucose-grown cells.

C. Citrate synthase.

Citrate synthase is a key enzyme in the citrate cycle and experiments were conducted to determine whether *Listeria* A4413 extract (PSS) could synthesize citrate from acetyl-CoA and oxalacetate. The procedure used is detailed in the Methods, section XIV-B-3, and the results of the experiments are summarized in Table 8. The results show that citrate was synthesized by the *Listeria* extract. The amount of citrate present after 30 min was twice that at 15 min, so the synthesis was apparently linear from zero time to 30 min. Both acetyl-CoA

TABLE 8

THE SYNTHESIS OF CITRATE BY CELL-FREE EXTRACTS OF *LISTERIA* A4413

Reaction Components	Incubation at 37 C (min)	Citrate (μmoles/reaction)
Complete mixture[a]	15	0.37
Complete mixture	30	0.73
- less acetyl-CoA	30	0.039
- less oxalacetate	30	0.013
Complete mixture, using boiled extract[b]	30	0.086

a. The complete reaction mixture contained the following components in a volume of 2.5 ml (in μmoles): K phosphate buffer, pH 7.5, 25; $MgCl_2$, 1; L-cysteine, 0.5; acetyl-CoA, 1.5; sodium oxalacetate, 20; and *Listeria* A4413 extract (PSS), 2.16 mg protein.

b. The extract (PSS) was boiled for 40 min.

and oxalacetate were required substrates, and enzyme activity was destroyed by boiling. The specific activity of citrate synthase (μmoles of citrate synthesized/hr/mg protein) was about 0.68.

D. Aconitase.

Aconitase was present in *Listeria* A4413 extracts. Citrate and isocitrate were used as substrates for this enzyme. The difference in activity with these respective substrates is shown in Figures 15 and 16. A comparison of the activities illustrated in these figures, although they appear equivalent, reveals that aconitase was about two-fold more active in the presence of isocitrate than with citrate; this follows from the observation that only 0.25 mg of protein in the presence of isocitrate brought about the same absorbancy increase at 240 mμ as did 0.50 mg of protein with citrate. Activity with either substrate was maximal at either pH 7.5 or 8.0, but activity at pH 7.0 or 8.7 was only about 10% less than maximal. At pH 8.0, the activity in phosphate buffer was about twice that in Tris·HCl buffer. Lowering the final concentration of phosphate buffer from 0.1M (300 μmoles/3 ml) to 0.067M (200 μmoles/3 ml) decreased the activity 20%, with either citrate or isocitrate as substrate. The activity exhibited saturation kinetics, as illustrated by the Lineweaver-Burk (101) plot in Figure 17a. The concentration of sodium citrate or sodium DL-isocitrate required for half maximal velocity (the K_m) was about 8×10^{-4}M or 4.4×10^{-4}M, respectively. The K_m for DL-isocitrate was, therefore, about half that for citrate. Aconitase activity remained essentially constant on storage for 4 hr at 1-2 C, making it one of

Figure 15.

Aconitase activity of *Listeria* A4413 extract with sodium citrate as the substrate. A reaction mixture of 3.0 ml contained K phosphate buffer pH 7.5, 300 μmoles, sodium citrate, 90 μmoles, and *Listeria* A4413 extract (PSS), 0.50 mg protein. The increase in absorbance at 240 mμ was observed at room temperature against a reference which lacked only sodium citrate.

Figure 16.

Aconitase activity of *Listeria* A4413 extract with sodium DL-isocitrate as the substrate. The reaction mixture of 3.0 ml contained K phosphate buffer, pH 7.5, 300 μmoles, sodium DL-isocitrate, 20 μmoles, and *Listeria* A4413 extract (PSS), 0.25 mg protein. The increase in absorbance at 240 mμ was observed at room temperature against a reference which lacked only sodium DL-isocitrate.

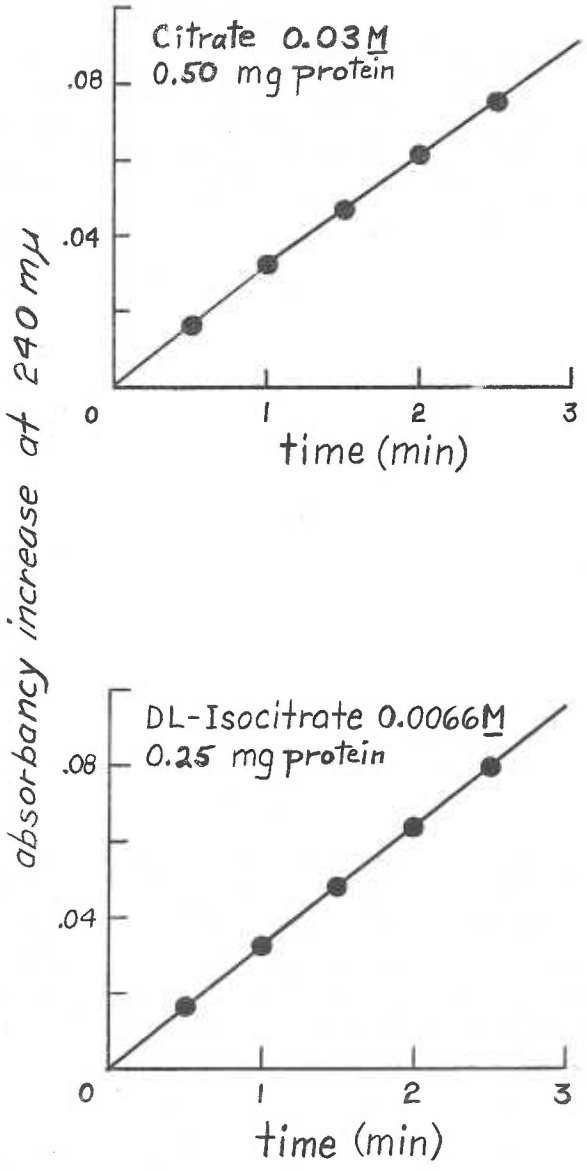
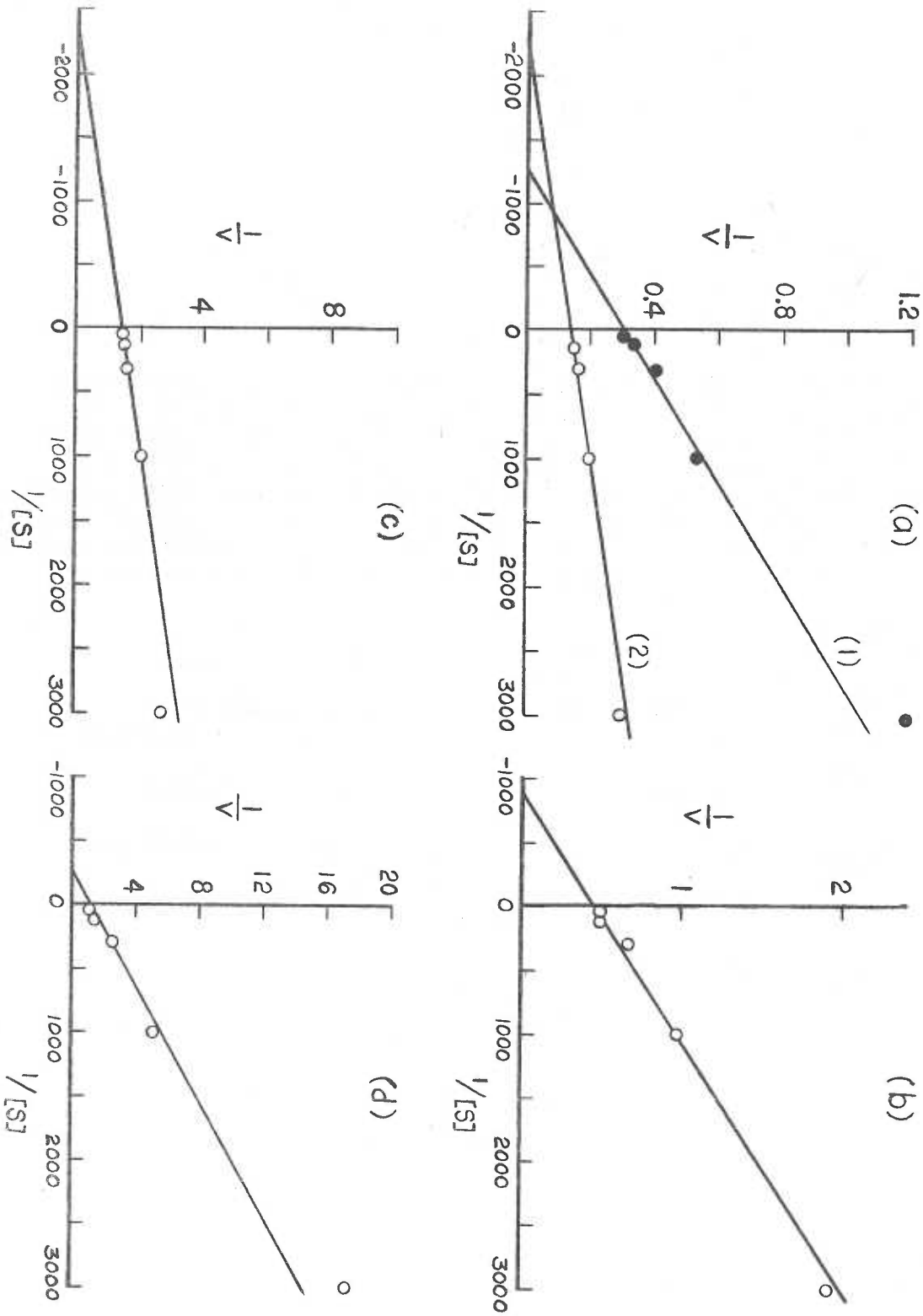

Citrate 0.03M
0.50 mg protein
.08
.04
0
1
2
3
time (min)
absorbancy increase at 240 mμ
DL-Isocitrate 0.0066M
0.25 mg protein
.08
.04
0
1
2
3
time (min)

Figure 17.

Lineweaver-Burk plots (101) for aconitase, fumarase, isocitrate dehydrogenase, and malate dehydrogenase activity of *Listeria* A4413 extract (PSS). Experimental assay conditions were as described for aconitase (Figures 15 and 16), fumarase (Figure 22), isocitrate dehydrogenase (Figure 18), and malate dehydrogenase (Figure 23), except for the variation in the substrate concentrations. The reciprocal of the initial velocity (v) in μmoles substrate transformed/hr/mg protein was plotted against the reciprocal of the substrate concentrations (M).

Diagram	Enzyme	Substrate
(a)	Aconitase	(1) sodium citrate (2) sodium DL-isocitrate
(b)	Fumarase	sodium L-malate
(c)	Isocitrate dehydrogenase	sodium DL-isocitrate
(d)	Malate dehydrogenase	sodium L-malate


(a)
(b)
(c)
(d)
(1)
(2)
1/V
1/[S]
-2000
-1000
0
1000
2000
3000
0.4
0.8
1.2
1
2
4
8
12
16
20

the more stable enzymes in the *Listeria* extracts. Boiling the extract for 15 min abolished the activity. The specific activity (μmoles cis-aconitate formed/hr/mg protein), was about 3.33 (citrate) or 6.66 (isocitrate).

E. Isocitrate dehydrogenase.

Isocitrate dehydrogenase was detected in *Listeria* A4413 extracts. NADP was reduced in the presence of extract and isocitrate as shown in Figure 18. NAD was not reduced by this reaction mixture, suggesting that this enzyme is NADP-specific.

Activity was greatest at pH 7.5; at pH 7.0 or pH 8.0 it was some 20% lower than at pH 7.5. Assays in Tris·HCl buffer yielded about half the activity seen in phosphate buffer at the same pH. In 0.067M phosphate buffer, activity was about 10% greater than in 0.033M. Mn^{++} was important to maximal activity and the rate fell 50% in its absence. Mg^{++} or Co^{++} (as the chloride) was only about 60% as effective as Mn^{++}, and Ca^{++} or Ni^{++} did not stimulate the activity.

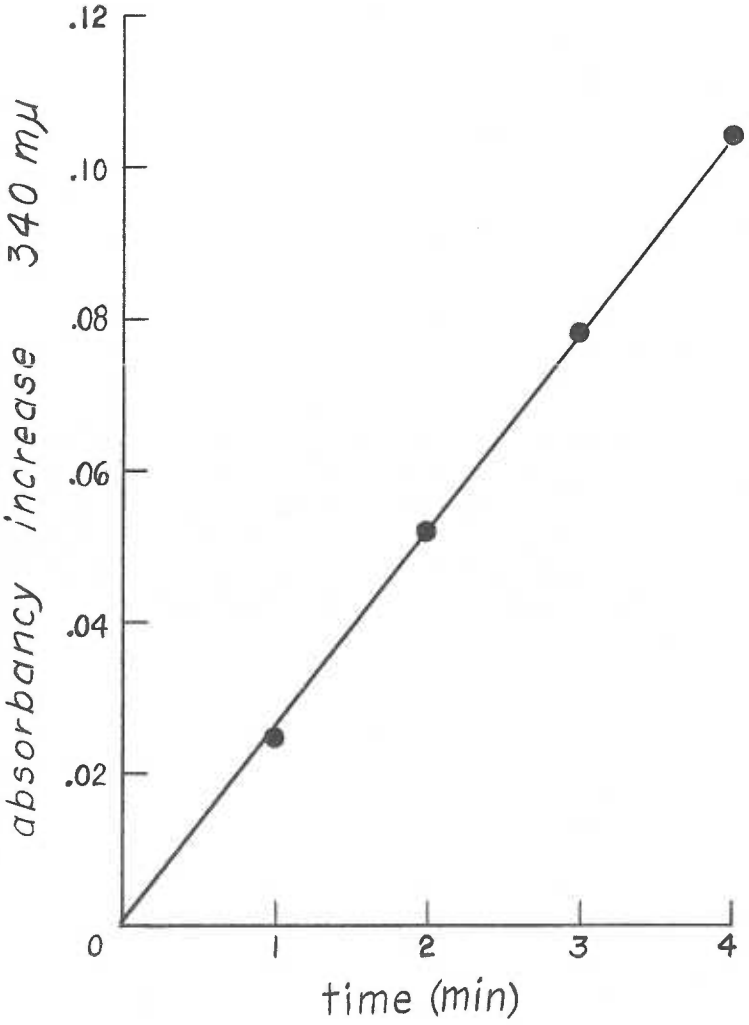
As shown in Figure 17(c), saturation kinetics were also observed with this enzyme, and about 4.4×10^{-4}M sodium DL-isocitrate gave half maximal velocity. When the extract was stored at 1-2 C, the activity declined to an almost non-measurable level by 8 hr. Activity was destroyed by boiling the extract for 15 min. The specific activity (μmoles of NADPH formed/hr/mg protein) was about 0.75 in fresh preparations of PSS.

F. Succinate dehydrogenase.

Many attempts to detect succinate dehydrogenase in *Listeria* A4413

Figure 18.

Isocitrate dehydrogenase activity of *Listeria* A4413 extract. The 3.0 ml reaction mixture contained the following (in μmoles): K phosphate buffer, pH 7.5, 200; NADP, 0.5; $MnCl_2$, 1; sodium DL-isocitrate, 30; and *Listeria* A4413 extract (PSS), 1.0 mg protein. The increase in absorbance at 340 mμ was observed at room temperature against a reference which lacked only isocitrate.


.12
.10
.08
.06
.04
.02
0
1
2
3
4
absorbancy increase 340 mμ
time (min)

extracts under a variety of conditions were not successful. *E. coli* K-12(λ) CE was used as a positive control. A modified method of Ells (92) was used most extensively to assay this enzyme. The data on the succinate dehydrogenase activities of crude extracts from *Listeria* and *E. coli* are presented in Figure 19.

The extract of *E. coli* K-12(λ) contained succinate dehydrogenase. The activity was greatest at pH 7.5, and was about 40% lower at either pH 7.0 or 8.0 in phosphate buffer. At pH 7.5 the specific activity (μmoles of 2,6-dichlorophenol indophenol [DCPIP] reduced/hr/mg protein) was about 0.97. The endogenous reduction of DCPIP at this pH was some 20% as great as the succinate-dependent reduction. In the absence of either KCN or phenazine methosulfate, succinate dehydrogenase activity fell about 50%.

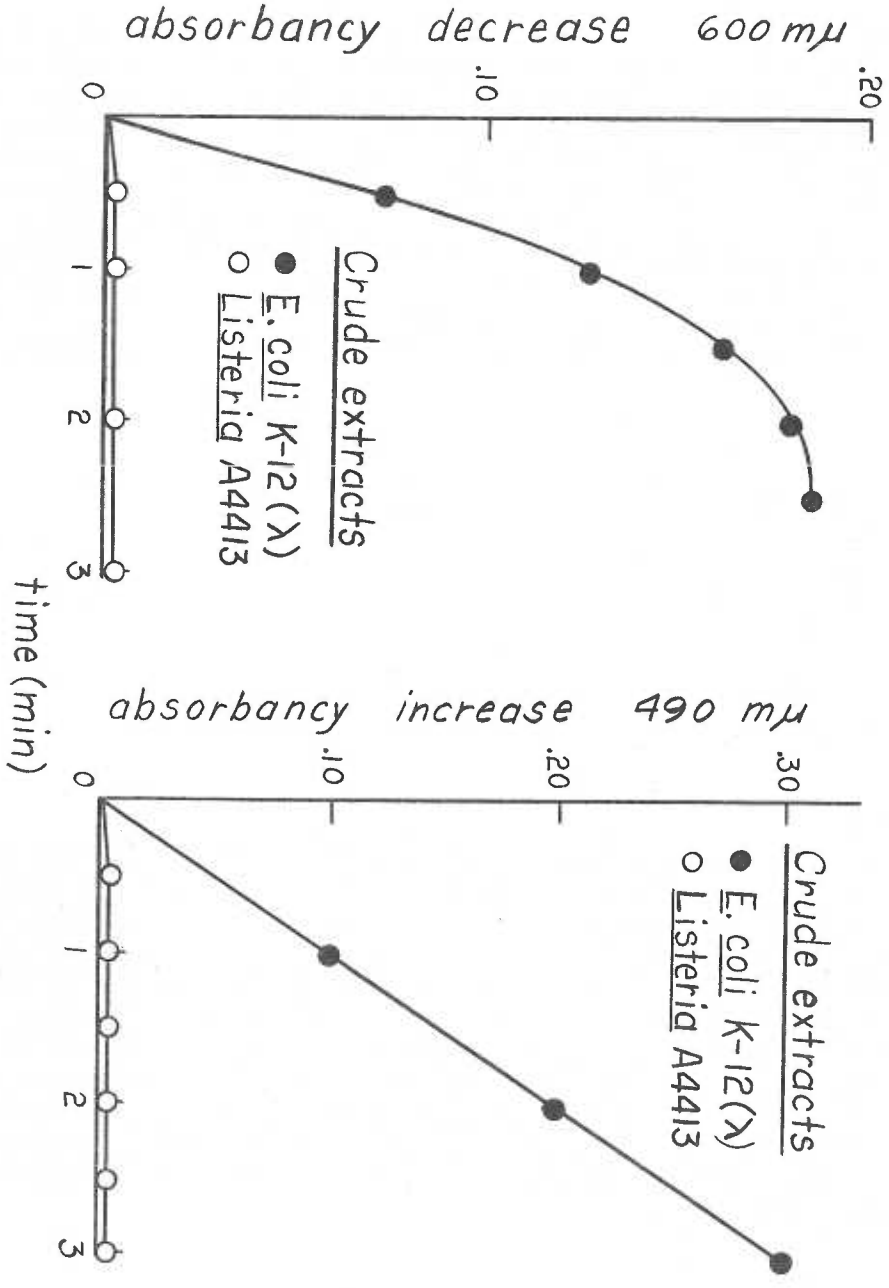
Using the same assay method, extracts of *Listeria* A4413 showed no succinate dehydrogenase activity. Attempts to detect activity were unsuccessful in any of the following buffer systems: K phosphate at pH 6.0, 6.5, 7.0, 7.5, and 8.0; Tris·HCl at pH 7.0, 7.5, 8.0, or 8.5; or glycine·NaOH at pH 9.0. *Listeria* A4413 CE showed moderate endogenous reduction of DCPIP (the decrease in absorbance at 600 mμ was 0.100 in 3 min at pH 6.5), but such activity was not increased in the presence of succinate. This endogenous activity was essentially removed from *Listeria* A4413 CE by dialysis against 0.01M K phosphate buffer, pH 7.0, in the cold for 24 hr; no succinate dehydrogenase was detected after this dialysis. Similar dialysis of crude extract in phosphate buffer supplemented with 10^{-4}M cysteine did not result in the appearance of any succinate dehydrogenase activity. The lack of

Figure 19.

Assay for succinate dehydrogenase activity in *Listeria* A4413 CE by the use of 2,6-dichlorophenol indophenol. A CE of *E. coli* K-12(λ) was used as a control. The complete reaction mixture contained the following components in a volume of 3.0 ml: K phosphate buffer, pH 6.5 (pH 7.5 for *E. coli*), 100 μmoles; sodium succinate, 60 μmoles; *Listeria* A4413 CE (1.10 mg protein) or *E. coli* K-12(λ) CE (1.40 mg protein); KCN, 3 μmoles; 2,6-dichlorophenol indophenol (DCPIP), 0.083 μmoles; phenazine methosulfate, 1.0 mg. Further details are given in the Methods, XIV-6. The decrease in absorbance at 600 mμ was observed against a water reference at room temperature. The activity illustrated has been corrected for endogenous reduction of DCPIP.

Figure 20.

Assay for succinate dehydrogenase activity in *Listeria* A4413 CE by the use of p-iodonitrotetrazolium violet. A CE of *E. coli* K-12(λ) was used as a control. The complete reaction mixture contained the following components in 3.0 ml which was made anaerobic: K phosphate buffer, pH 7.5, 120 μmoles; sodium succinate, 20 μmoles; *Listeria* A4413 CE (0.86 mg protein) or *E. coli* K-12(λ) CE (1.40 mg protein); p-iodonitrotetrazolium violet (INT), 0.6 ml of a 0.1% solution in 0.05% gelatin; and phenazine methosulfate, 1.0 mg. Further details are given in the Methods XIV-6. The increase in absorbance at 490 mμ was observed at room temperature against a reference of water. The activity illustrated has been corrected for endogenous reduction of INT.


absorbancy decrease 600 mμ
0
.10
.20
Crude extracts
● E. coli K-12 (λ)
○ Listeria A4413
1
2
3
time (min)
absorbancy increase 490 mμ
0
.10
.20
.30
Crude extracts
● E. coli K-12(λ)
○ Listeria A4413
1
2
3

detectable succinate dehydrogenase activity in *Listeria* A4413 CE illustrated in Figure 19 represents the results of assays under all the conditions tested.

The method of Nachlas et al. (95), as modified by Hirsch et al. (58) to provide anaerobiosis, was also used to assay *Listeria* extracts for succinate dehydrogenase. The results of this experiment, including a control with *E. coli* K-12(λ) extract, is summarized in Figure 20. While *E. coli* K-12(λ) CE was active (specific activity [μmoles INT reduced/hr/mg protein] was 0.90 at pH 7.5), *Listeria* A4413 CE was inactive in either phosphate buffer at pH 6.5 or 7.5, or in Tris·HCl buffer, pH 8.5. As in the previous method, moderate endogenous reduction of INT was carried out by *Listeria* A4413 CE (the increase in absorbance at 490 mμ in 3 min was 0.027, 0.040, and 0.074 at pH 6.5, 7.5, and 8.5, respectively); addition of succinate to the reaction, however, did not increase the reduction of INT.

Two sensitive succinate dehydrogenase assay methods were used, therefore, in an attempt to detect activity in *Listeria* extracts. Either method consistently revealed succinate dehydrogenase in *E. coli* extracts, but extracts of *Listeria* A4413 were always inactive. Apparently, succinate dehydrogenase is essentially absent in *Listeria* A4413 under the conditions tested.

G. Fumarate reductase.

Although succinate dehydrogenase could not be determined in *Listeria* A4413 extracts, it seemed possible that this bacterium might contain fumarate reductase which is essentially the reverse of succi-

nate dehydrogenase. Such was the case.

Fumarate reductase activity was found in *Listeria* A4413 CE as shown in Figure 21. This extract catalyzed a rapid fumarate-dependent dehydrogenation of $FMNH_2$. Activity was essentially the same in phosphate buffer at pH 6.0, 6.5, 7.0, or 7.5, but the rate in phosphate buffer at pH 8.0 was only 50% of the higher level. $FADH_2$ was substituted for $FMNH_2$ with no resultant change in activity. When the concentration of $FMNH_2$ (2.5×10^{-4}M) was doubled, a slight increase in activity occurred. Fumarate reductase activity fell 50% on 24 hr storage at 1-2 C, and boiling the extract for 20 min abolished its activity. The specific activity (μmoles $FMNH_2$ dehydrogenated/hr/mg protein) was at least 2.82.

H. Fumarase.

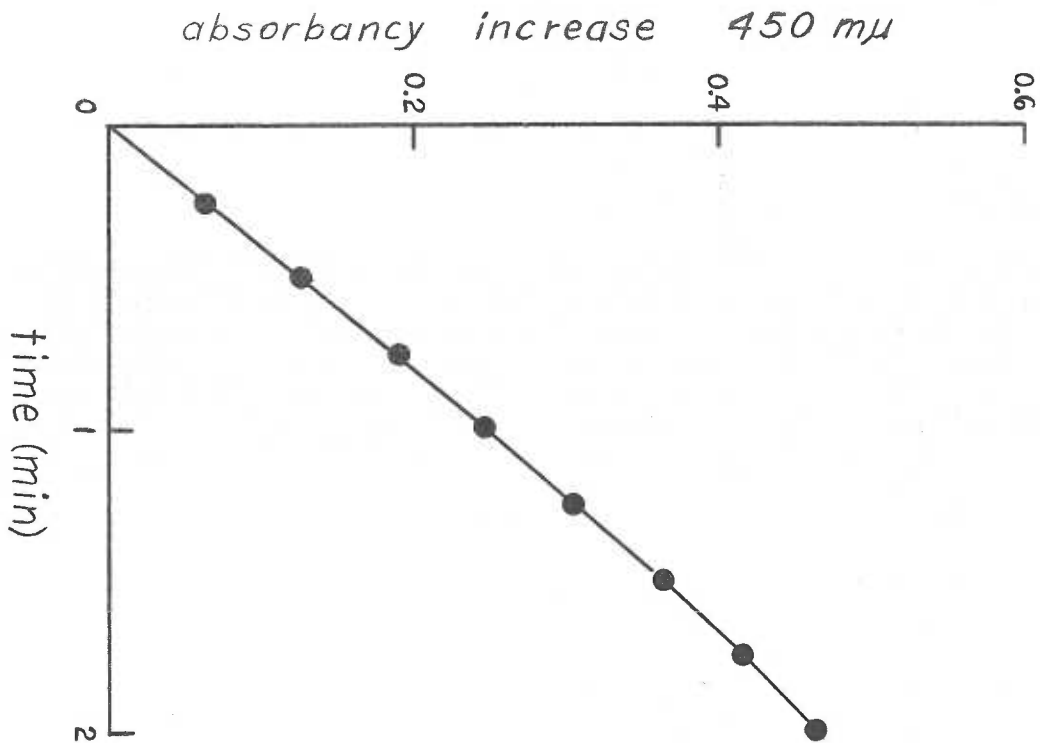
Extracts of *Listeria* A4413 contained fumarase activity as shown in Figure 22. The increase in the absorbance at 240 mμ was linear for at least 4 min. Activity was optimal about pH 8.0; as there were insignificant differences in the reaction rate in either phosphate buffer pH 7.5 or 8.0, or in Tris·HCl buffer pH 8.0 or 8.5, fumarase exhibited a rather broad pH optimum. In phosphate buffer pH 7.0 or Tris·HCl buffer pH 9.0, the activity was about 40% lower than at pH 7.5 to 8.5. The rate in 0.033M Tris·HCl (pH 8.0) was about 10% lower than in 0.067M Tris·HCl. Saturation kinetics were observed as shown in Figure 17(b) and about 1.1×10^{-3}M L-malate gave half maximal velocity. A decline in the activity of about 50% in 3 hr was noted when the extract (PSS) was held at 1-2 C, so the enzyme seemed quite

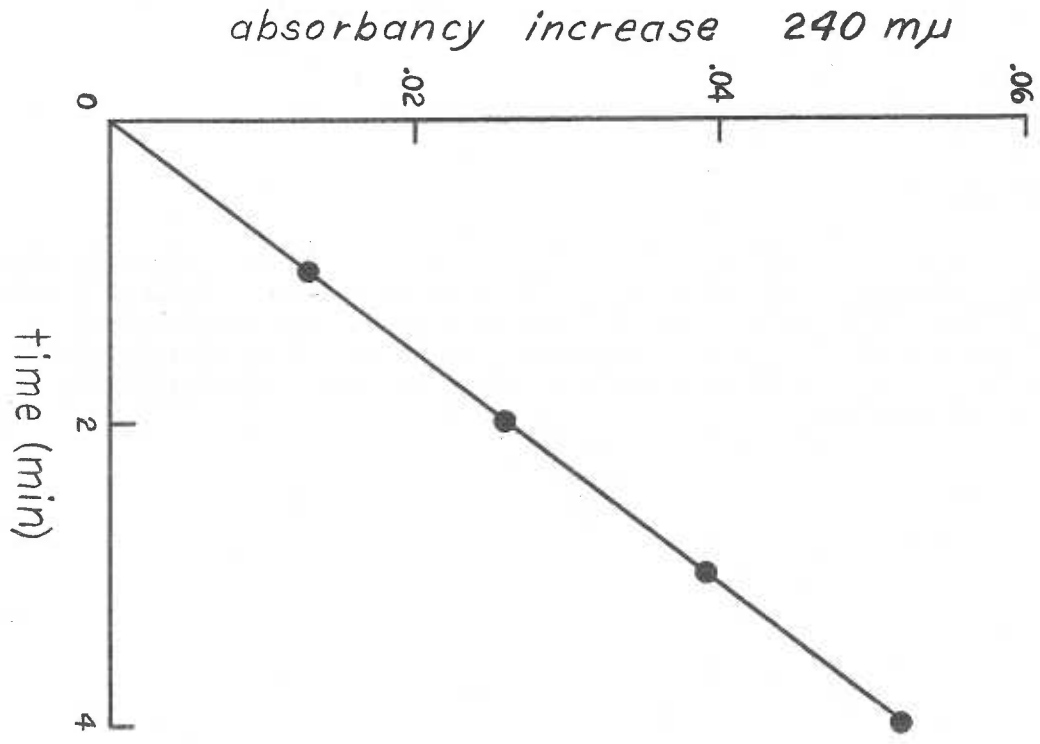
Figure 21.

Fumarate reductase activity of *Listeria* A4413 CE. The reaction mixture contained the following in 2.0 ml: K phosphate buffer, pH 7.0, 100 µmoles; sodium fumarate, 20 µmoles, $FMNH_2$, 0.5 µmole; and *Listeria* A4413 CE, 1.0 mg protein. Details of protocol are given in the Methods XIV-B-7. The increase in absorbance at 450 mµ was observed at room temperature against a water reference.

Figure 22.

Fumarase activity of *Listeria* A4413 extract (PSS). The reaction mixture contained the following in a volume of 3.0 ml: Tris·HCl buffer, pH 8.0, 200 µmoles; sodium L-malate, 30 µmoles; and *Listeria* A4413 extract (PSS), 0.50 mg protein. The increase in absorbance at 240 mµ was observed at room temperature against a reference which lacked only malate.


absorbancy increase 450 mμ
0
0.2
0.4
0.6
time (min)
1
2


absorbancy increase 240 mμ
0
.02
.04
.06
time (min)
2
4

unstable in these preparations; boiling for 15 min abolished the extract's fumarase activity. The specific activity (μmoles of fumarate formed/hr/mg protein) was about 2.24.

I. Malate dehydrogenase.

The activity illustrated in Figure 23 indicates that malate dehydrogenase was detected in extracts of *Listeria* A4413. The absorbance at 340 mμ increased linearly for at least 3 min after the addition of L-malate. Deletion of Mn^{++} lowered the reaction rate by 75%. Mg^{++}, Ni^{++}, or Co^{++} were only about half as effective as Mn^{++} in the stimulation of activity, and Ca^{++} gave no increase in the rate over that observed in the absence of added divalent cation.

The optimal pH was roughly 9.0 in glycine·NaOH buffer, and the rate was about 33% lower at pH 9.6 and about 67% lower at pH 8.6. Malate dehydrogenase showed a more narrow pH optimum than most enzymes in the *Listeria* cell-free extracts. The activity in glycine·NaOH buffer was about 10% less in 0.067M than in 0.033M. Activity was subject to substrate saturation, and about 4.0×10^{-3}M L-malate gave half maximal velocity, as indicated in Figure 17(d). The enzyme is apparently NAD-specific, as only traces of activity were observed upon substitution of NADP for NAD in the reaction mixture. Activity was essentially unchanged after storage of the extract for 4 hr at 1-2 C, but was destroyed by boiling for 15 min. The specific activity (μmoles NADH formed/hr/mg protein) was about 0.87.

J. Isocitratase.

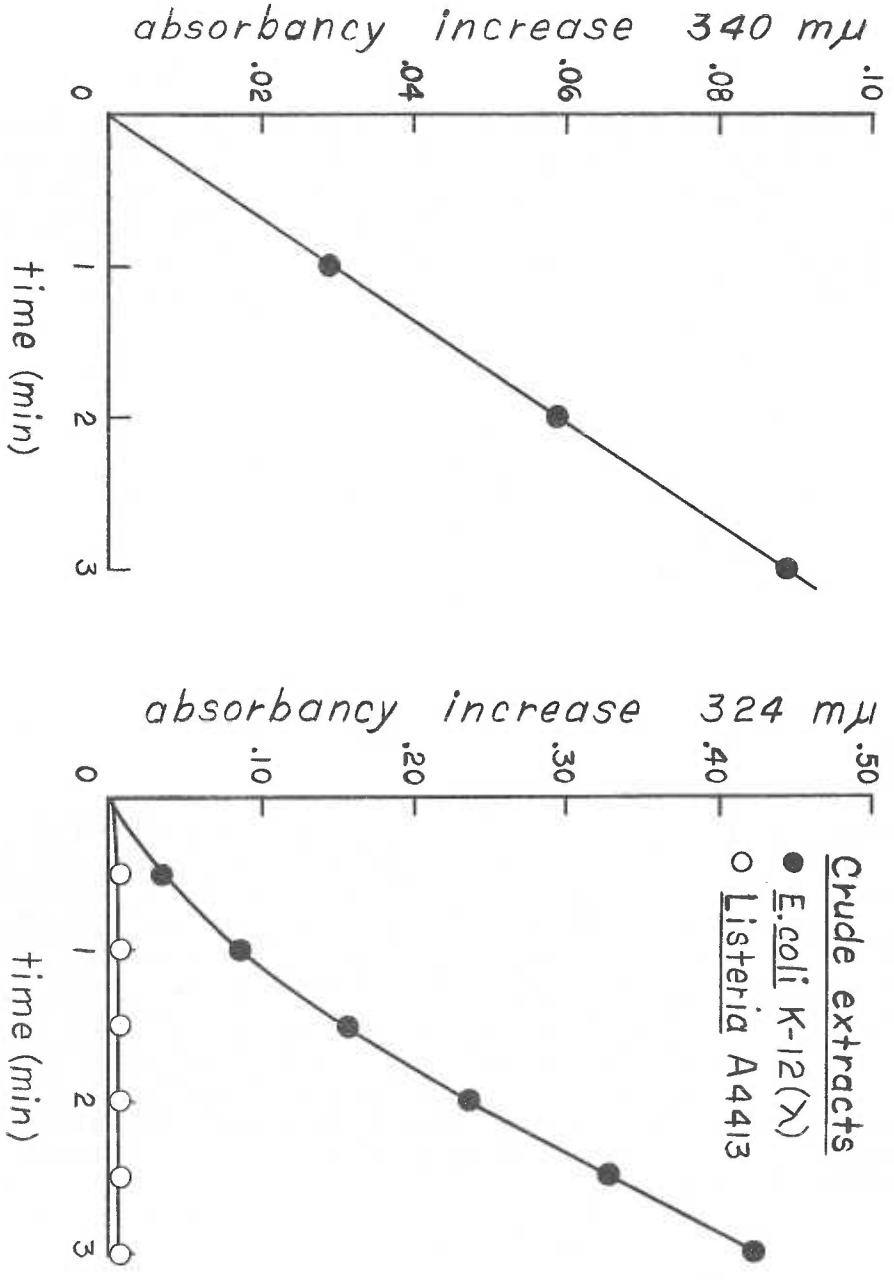
Isocitratase was not detected in *Listeria* A4413 extracts. The

Figure 23.

Malate dehydrogenase activity of *Listeria* A4413 extract (PSS). The reaction mixture contained the following in 3.0 ml: glycine·NaOH buffer, pH 9.0, 100 µmoles; sodium L-malate, 60 µmoles; NAD, 0.5 µmole; $MnCl_2$, 1 µmole; and *Listeria* A4413 extract (PSS), 1.0 mg protein. The absorbancy increase at 340 mµ was observed at room temperature against a reference which lacked only malate.

Figure 24.

Isocitratase activity of *Listeria* A4413 CE. *E. coli* K-12(λ) CE was used as a control. The protocol is detailed in the Methods, XIV-10. Either *Listeria* A4413 CE (1.29 mg protein) or *E. coli* K-12(λ) CE (0.75 mg protein) was employed as the enzyme source. The increase in absorbance at 324 mµ was observed against a reference which lacked only isocitrate. Because a slight substrate-independent absorbancy increase occurred in the presence of either bacterial extract in the reference cuvettes, a slight correction was made before each reading. The activity illustrated for *E. coli* extract was observed in imidazole buffer at pH 6.4; that for *Listeria* extract represents the inactivity observed either in imidazole buffer at pH 6.4, 6.8, or 7.2, or in Tris·HCl buffer at pH 8.0.


absorbancy increase 340 mμ
.02
.04
.06
.08
.10
0
1
2
3
time (min)
absorbancy increase 324 mμ
.10
.20
.30
.40
.50
0
1
2
3
time (min)
Crude extracts
● E.coli K-12 (λ)
○ Listeria A4413

results of the Kornberg assay (97) for this enzyme in crude extracts of *Listeria* A4413, and of *E. coli* K-12(λ) as a control, are presented in Figure 24. It is apparent that *E. coli* extract showed isocitratase activity. The increase in absorbance at 324 mμ was linear only after about 1.5 min; Kornberg (97) also observed a similar lag in the reaction rate and ascribed it to the time elapsing between glyoxylate formation and its subsequent reaction with phenylhydrazine to give the glyoxylate phenylhydrazone which absorbs at 324 mμ. Based on the linear part of the curve (between 2 and 3 min) in Figure 24, the specific activity (μmoles glyoxylate phenylhydrazone formed/hr/mg protein) in imidazole buffer, pH 6.4, was about 1.8, and the rate was insignificantly lower in imidazole, pH 6.8, or in phosphate buffer, pH 6.0.

In contrast to the isocitratase activity of *E. coli* extract, *Listeria* A4413 CE was not active when assayed under identical conditions in either imidazole buffer at pH 6.4, 6.8, 7.2, or in Tris·HCl buffer, pH 8.0.

K. Malate synthase.

Listeria A4413 extracts apparently lack malate synthase activity. The results of the assays using extracts (PSS) of *Listeria* A4413 and *E. coli* K-12(λ) are shown in Figure 25.

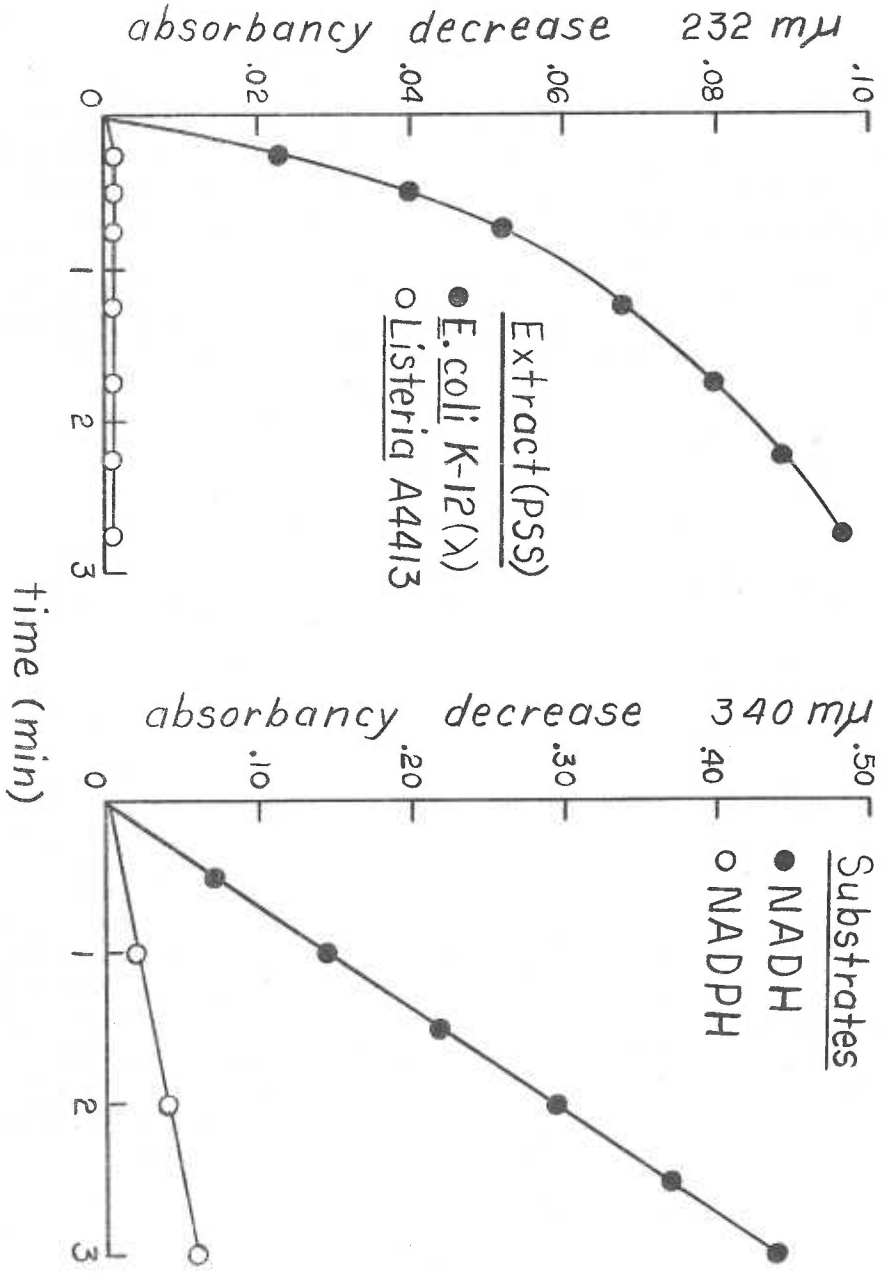
Malate synthase was present in extract (PSS) from *E. coli* K-12(λ); it was active in Tris·HCl buffer from pH 7.0 to 9.0 and was most active at pH 7.5 (illustrated). The reaction rate at pH 7.0 or 8.0 was, however, only some 5% lower than at pH 7.5. The specific activity (μmoles of acetyl-CoA cleaved/hr/mg protein) at pH 7.5 was 4.02.

Figure 25.

Malate synthase activity of *Listeria* A4413 extract (PSS). *E. coli* K-12(λ) extract (PSS) was used as a control. The reaction mixture contained the following in 2.0 ml: Tris.HCl buffer, pH 7.5, 100 μmoles; $MgCl_2$, 2 μmoles; acetyl-CoA, 0.1 μmole; sodium glyoxylate, 1 μmole; and *Listeria* A4413 extract (PSS), 0.52 mg protein, or *E. coli* K-12(λ) extract (PSS), 0.58 mg protein. The decrease in absorbance at 232 mμ was observed at room temperature against a reference which lacked only glyoxylate. Deacylase activity in the reference mixture was slight (≃ 0.01 per 3 min) and was corrected for before each reading.

Figure 26.

The oxidation of NADH or NADPH by *Listeria* A4413 extract (PSS). The reaction mixture contained the following in 3.0 ml: K phosphate buffer, pH 7.0 (pH 6.0 for NADPH), 200 μmoles; NADH, 0.50 μmole, or NADPH, 0.37 μmole; $MnCl_2$, 1 μmole (for NADPH only); and *Listeria* A4413 extract (PSS), 0.25 mg protein. The decrease in absorbance at 340 mμ was observed at room temperature against a water reference.


absorbancy decrease 232 mμ
.10
.08
.06
.04
.02
0
Extract (PSS)
● E. coli K-12 (λ)
○ Listeria A4413
absorbancy decrease 340 mμ
.50
.40
.30
.20
.10
0
Substrates
● NADH
○ NADPH
1
2
3
time (min)

In contrast, extract (PSS) from *Listeria* A4413 did not show malate synthase activity in any of the following buffers: Tris·HCl at pH 7.0, 7.5, 8.0, 8.5, or 9.0, or in glycine·NaOH at pH 9.6. The inactivity of *Listeria* A4413 extracts, under all assay conditions, is represented by the curve in Figure 25.

L. NAD(P)H oxidases.

Figure 26 illustrates that crude extracts of *Listeria* A4413 contain oxidases for NADH and NADPH. In the presence of either reduced pyridine nucleotide substrate there was a linear decrease in the absorbance at 340 mμ for at least 3 min.

NADH oxidase was about 7-fold more active than NADPH oxidase. NADH oxidase was most active about pH 7.0 (illustrated) and was about 30% less active at pH 6.0. At pH 8.0, the activity fell about 50% from the rate at pH 7.0, and at pH 9.0 (Tris·HCl) the rate was about 10% of that seen at pH 7.0. Greater activity was observed in phosphate than in Tris·HCl buffer at the same pH. Neither Mn^{++} nor Mg^{++} increased the rate of NADH oxidation.

NADPH oxidase was most active about pH 6.0, and although the rate at pH 7.0 was about half that at pH 6.0, activity was hardly detectable at pH 8.0 or above. In the absence of Mn^{++} the activity of NADPH oxidase decreased more than 50%.

The specific activity (μmoles of NAD[P]H oxidized/hr/mg protein) was about 16.2 (NADH) or 2.3 (NADPH) at pH 7.0 and 6.0, respectively.

XI. THE ASSAY FOR CYTOCHROME PIGMENTS IN *LISTERIA* A4413

Whole *Listeria* A4413 cells and crude extracts made from these cells failed to show any recognized cytochrome peaks by a difference spectrum analysis from 400 mμ to 650 mμ. The absence of cytochromes apparent in this *Listeria* strain is in harmony with the report on other *Listeria* by Keeler and Gray (26), who also found no cytochromes.

DISCUSSION

The experiments in this thesis attempted to determine whether *Listeria* carries out the citrate cycle and related metabolism. Experiments were concerned with the growth response of *Listeria* to pyruvate or intermediates of the citrate cycle, the oxidative response of resting *Listeria* cells to the same compounds, and assays for the enzymes of pyruvate oxidation, the citrate cycle, the glyoxylate bypass, and oxidases for NADH and NADPH in cell-free extracts of this organism. The following discussion will consider the results of these experiments in relation to each other and to the results obtained by other workers.

Several experiments were basic to the research although they did not relate directly to the central problems of the thesis. One such experiment determined the survival of *Listeria* A4413 in various menstrua which are often used in the washing and resuspension of bacteria. Of the non-nutrient solutions used, K phosphate buffer (0.1M, pH 7.2) best preserved the viability of *Listeria*. After refrigeration for 21 hr in the appropriate menstruum, the % viability in relation to the count at zero time was as follows: K phosphate buffer, 93; 0.9% NaCl, 67; and distilled water, 0. The superiority of K phosphate may be related to the growth requirement of *Listeria* for K^+ and $PO_4^{\equiv}$ (20, 105). In contrast, the slight inferiority of saline (0.9% NaCl) is in harmony with the report that physiological saline may kill *Listeria* and that this solution should be avoided in the preparation of tissue suspensions for culture (1). Distilled water seems to be unacceptable

for the long term maintenance of *Listeria* viability. Trypticase, the only nutrient menstruum used, permitted slight growth of the *Listeria*.

The relationship between the turbidity (optical density at 620 mμ) of a *Listeria* suspension, its nitrogen content, and number of viable cells revealed similarities and differences between strain A4413 and 9037-7. Whereas the nitrogen content of strain A4413 was essentially identical to that of strain 9037-7 at a given turbidity, the number of viable A4413 cells was almost 10-fold greater than the number of 9037-7 cells, as estimated by plate count procedures. The simplest explanation for this finding is that 9037-7 is an R-type *Listeria* which grows in broth medium in the form of a sediment, consists of longer cells than strain A4413, and often forms filaments; a bacterial suspension containing some filamentous forms would be expected to yield fewer colonies than a suspension consisting of rather uniformly sized cells.

Before determining the growth response of *Listeria* to various sources of carbon and energy, some basic observations were made concerning the effects of the glucose concentration, iron, and agitation on the growth of this bacterium. That glucose or some other carbohydrate is essential for *Listeria* growth has been reported before (6, 8, 23), and the experimental results obtained in this research confirm the earlier finding. The extent of *Listeria* A4413 growth increased in approximate proportion to the glucose content from 0.05% to 0.5%, above which the growth did not increase. A glucose concentration of > 0.5% is an excess, therefore, and according to Friedman and Alm (24), *Listeria* A4413 grown in such high amounts of

glucose are less oxidative than when grown on a lower (i.e., 0.2%) concentration.

Listeria A4413 grew in Welshimer's defined medium (21) with or without shaking. It was interesting, moreover, to find that either iron or agitation moderately increased the growth and that iron and agitation produced the greatest stimulation. Growth increased in rough proportion to the amount of iron added and at each concentration of iron, agitation further stimulated the growth. In the presence of 100 µg Fe^{+++}/ml and agitation growth was six times as great (at 14-16 hr) as the iron-deficient stationary controls. Sword (22) noted a similar stimulation of *Listeria* growth by iron (as ferric ammonium citrate) in Welshimer's medium (21), but this author did not mention the use of agitation; he did, however, employ shallow layers of medium in flasks such that considerable exchange of gases probably occurred during growth.

For reasons detailed in the Methods section V, a defined medium, designated D10, was designed and used thereafter for the growth of *Listeria* in this research. As far as is known, this is the simplest medium yet described for the cultivation of *Listeria*. The growth response of *Listeria* A4413 and 9037-7 in medium D10 was determined in relation to the content of iron and agitation. Similar to the results with the ferric ammonium citrate supplements in Welshimer's medium, iron (as $FeCl_3$) again stimulated *Listeria* A4413 growth whether agitation was used or not, but agitation increased the growth only in the presence of iron supplements. The importance of this experiment is greater than the observation that medium D10 + 0.5% glucose is an

excellent broth for the cultivation of *Listeria*; it also suggests that iron is important to *Listeria* growth in defined medium especially when agitation is employed. That the stimulatory effect of agitation on the growth of *Listeria* in defined medium is apparently iron-dependent has not been reported previously, as far as is known. This observation may be relevant to the report by Friedman and Roessler (20) that the subculture of *Listeria* A4413 grown with agitation apparently requires peptides, because the defined medium used by these authors (20) was probably somewhat iron-deficient (see Methods, V-2).

The stimulatory effect of iron on the growth of *Listeria* is interesting, furthermore, because *Listeria* are apparently cytochrome-deficient, as reported by Keeler and Gray (26) and as noted in this research. *Listeria*, however, contain catalase, of the heme-iron type (24), and the iron probably is essential in the synthesis of catalase and perhaps other enzymes. Winder et al. (102) observed that the catalase activity of iron-deficient *Mycobacterium tuberculosis* was about one-third that of normal cells. Possibly, therefore, iron-deficiency in *Listeria* leads to catalase deficiency which would be expected to adversely affect growth.

Citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, acetate, and pyruvate were not acceptable sources of carbon and energy for the growth of *Listeria* A4413 and 9037-7. It seems significant that none of the intermediates tested, under any conditions (with or without 0.05% glucose as an auxiliary carbon source, or with or without agitation) supported the growth of either *Listeria* strain. The experiment reported in Table 6 included 0.05% glucose in

the growth medium in case a secondary carbon source might enable the *Listeria* to utilize another compound, based on the finding that *E. coli* can utilize citrate only when provided a supplementary carbon source such as glucose (44); this approach was to no avail. These results may be interpreted to mean that the cells either lack a means for bringing the given compound into the cell (a permease) as in the case of citrate and *E. coli* (61), or that the necessary enzyme systems for its utilization are absent or at low levels, or both. Of the compounds tested, citrate (25) and pyruvate (24) have been reported previously as being unsatisfactory carbon and energy sources for *Listeria* growth. Many species of bacteria, including *E. coli* (43) and *P. fluorescens* (65), are able to use one and usually many of the citrate cycle intermediates or pyruvate for growth. Such is not the case with *Listeria* A4413 and 9037-7, however, at least under the conditions tested.

The oxidative ability of resting *Listeria* A4413 cells toward glucose, pyruvate, and intermediates of the citrate cycle was determined. Glucose was more rapidly oxidized at pH 6.0 than at 7.0 or 8.0, and the addition of the cofactor mixture stimulated the oxidation slightly. The maximum Q_{O_2}(N) of 767 observed here at pH 6.0 (+ CFM) for the oxidation of 10 µmoles of glucose is in agreement with the highest Q_{O_2}(N) reported by Friedman and Alm (24) of 773 for *Listeria* A4413 grown on BHI. The oxidation ratio (at 120 min) of 1.05 is, however, less than the 1.4 reported by these authors (24). The observed oxidation ratio (1.05) suggests that the oxidation of glucose continued little beyond the formation of two pyruvates from each glucose molecule by aerobic

glycolysis. In Figure 11 and Figure 13 it can be seen that pyruvate was oxidized by either intact *Listeria* or cell-free extracts, respectively, and an oxidation ratio of ≃ 0.5 was found in either case. It seems a bit strange that the *Listeria* cells did not oxidize glucose to the level of acetate which would have given an oxidation ratio of ≃ 2.0.

Pyruvate was oxidized by *Listeria* A4413 only at acid pH (see Table 7), showing the most rapid rate ($Q_{O_2}[N] = 133$) in phosphate buffer, pH 5.0. This acid-dependent oxidation by intact cells, when cell-free extracts oxidized pyruvate maximally at pH 7.0, suggests that the pyruvate molecule must be in the undissociated state (i.e. pyruvic acid) to penetrate the *Listeria* cell. Friedman and Alm (24) noted that *Listeria* A4413 oxidized pyruvate at pH 6.5 ($Q_{O_2}[N] = 17.7$) which is in harmony with the results of the experiment shown in Table 7 in which a $Q_{O_2}(N)$ of 10 was found at this pH. The rate of pyruvate oxidation at pH 5.0 was 13 times greater than at pH 6.5. Certain coenzymes stimulated the oxidation of pyruvate by resting *Listeria* cells. Whereas the oxidation rate was stimulated only slightly by CoA, NAD, or Mn^{++}, the rate was almost doubled in the presence of α-lipoate. The addition of α-lipoate increased the $Q_{O_2}(N)$ from 120 to 220, and increased the oxidation ratio at 120 min from 0.42 to 0.55. The oxidation ratio (0.55) suggests that pyruvate was oxidized to the level of acetate. The stimulation by α-lipoate, and to a lesser extent by CoA, and NAD, suggests that *Listeria* oxidize pyruvate by means of a system which involves the pyruvate dehydrogenase complex.

In contrast to their ability to oxidize pyruvate, resting

Listeria cells demonstrated no significant oxidation of any intermediate of the citrate cycle or of the fatty acids caproate or oleate. This inability to oxidize these intermediates was shown at pH 5.0, 6.0, 7.0, and 8.0, in the presence or absence of the cofactor mixture. The cofactor mixture stimulated the endogenous oxidation about two-fold at all pH levels used. Apparently *Listeria* A4413 lack the capacity to oxidize exogenously added citrate cycle intermediates or caproate or oleate.

The results concerning the oxidative capacity of *Listeria* A4413, a type 4b, are in disagreement with the report of Kolb and Seidel (31) that a type 1 *Listeria* oxidized malate, succinate, and α-ketoglutarate (see Table 2, Introduction). The possibility exists that type 1 *Listeria* differ from *Listeria* A4413 in having this oxidative capacity.

Six enzymes of the citrate cycle, i.e. citrate synthase, aconitase, isocitrate dehydrogenase, malate dehydrogenase, fumarase, and fumarate reductase, as well as the oxidases for NADH and NADPH were found in cell-free extracts of *Listeria* A4413. Of the remaining three enzymes of the citrate cycle, the α-ketoglutarate oxidation system and succinate dehydrogenase could not be demonstrated, while succinate thiokinase was not assayed. Thus, this organism contains the intracellular enzymes necessary for the oxidation of several intermediates of the citrate cycle, but apparently lacks the required permeases which would transport the appropriate intermediates across the cell membrane. According to Clarke and Meadow (42), if the intracellular enzymes for the oxidation of a given citrate cycle

intermediate are shown to be present, and no cellular oxidation of the intermediate occurs after about 2 hr, then the required permease may be assumed to be absent.

The results of assays for the enzymes of pyruvate oxidation, the citrate cycle and glyoxylate bypass, and of oxidases for NADH and NADPH have been presented in Figures 13 to 26 and in Table 8. The presence or absence of the *Listeria* enzymes, their specific activity and other aspects, are summarized in Table 9 along with the specific activities reported for these enzymes in *E. coli* or *P. aeruginosa* to provide a convenient point of reference and comparison for the following discussion. The specific activities reported by Gray et al. (43) are utilized for comparison in Table 9, when appropriate, because the *E. coli* in their study were grown either aerobically or anaerobically in a salts-glucose medium. The enzyme activity from aerobically and anaerobically grown *E. coli* furnish a useful comparison with the activities found in cell-free extracts of *Listeria* A4413.

A pyruvate oxidation system was demonstrated in cell-free *Listeria* A4413 extracts. Although this enzyme system is not part of the citrate cycle, it is intimately related to this pathway because it provides acetyl-CoA, one co-substrate for citrate synthase. The optimal pH observed for the cell-free extract oxidation of pyruvate was about 7.0, which is in contrast to the pH (5.0) required for maximal oxidation of pyruvate by resting *Listeria* A4413 cells. DPT, CoA, NAD, and α-lipoate are each required for maximal oxidation, and no oxidation of pyruvate occurred in the absence of all coenzymes; this oxidative dependence upon these coenzymes suggests that the mechanism

TABLE 9

SUMMARY OF ENZYME ASSAYS OF CELL-FREE EXTRACTS OF *LISTERIA* A4413 AND A COMPARISON OF THE SPECIFIC ACTIVITIES WITH THOSE REPORTED IN OTHER BACTERIA

Enzyme	*Listeria* A4413				Other bacteria		
	Activity detected?	Specific activity[a]	Coenzyme requirement	K_m (M)	Specific activity[a]	Bacterium	Ref.
Pyruvate oxidation system	yes	47.3[b]	DPT, CoA, NAD, α-lipoate	ND[c]	10.0[b,i]	*E. coli*	66
α-ketoglutarate oxidation system	no[h]	--	--	--	--		
Citrate synthase	yes	0.68	ND	ND	0.63[f], 3.08[g]	*E. coli*	43
Aconitase	yes	3.33[d], 6.66[e]	ND	8.0×10^{-4}[d] 4.4×10^{-4}[e]	0.97[f], 19.1[g]	*E. coli*	43
Isocitrate dehydrogenase	yes	0.75	NADP, Mn^{++}	4.4×10^{-4}	8.32[f], 85.3[g]	*E. coli*	43
Succinate dehydrogenase	no[h]	--	--	--	--		
Fumarate reductase	yes	2.82	$FMNH_2$ or $FADH_2$	ND	4.56[j]	*E. coli*	58
Fumarase	yes	2.24	ND	1.1×10^{-3}	20.6[f], 50.8[g]	*E. coli*	43
Malate dehydrogenase	yes	0.87	NAD, Mn^{++}	4.0×10^{-3}	11.0[f], 225[g]	*E. coli*	43
Isocitratase	no[h]	--	--	--	--		
Malate synthase	no[h]	--	--	--	--		
NADH oxidase	yes	16.2	See note (k)	ND	5.33[f], 38.2[g]	*E. coli*	43
NADPH oxidase	yes	2.3	Mn^{++}	ND	0.18[g]	*P. aeruginosa*	71

Explanatory notes are listed on the following page.

EXPLANATORY NOTES FOR TABLE 9 (SEE PREVIOUS PAGE)

a. Specific activity = μmoles of substrate transformed or product formed/hr/mg protein, unless noted otherwise.

b. Specific activity = μl O_2 consumed/hr/mg protein.

c. ND = not determined.

d. With citrate.

e. With DL-isocitrate.

f. Anaerobically grown in synthetic medium + glucose.

g. Aerobically grown in synthetic medium + glucose.

h. Control enzyme preparations of *E. coli* showed activity.

i. Assay mixture lacked coenzymes.

j. Fumarate-grown.

k. Mg^{++} or Mn^{++} not stimulatory.

of pyruvate oxidation in *Listeria* A4413 is similar to or identical with the pyruvate dehydrogenase complex of *E. coli* and other organisms, because this is the only mechanism of pyruvate oxidation which involves α-lipoate (see Introduction IV-B-3).

The oxidation of pyruvate by cell-free *Listeria* A4413 extracts showed, at the termination of the experiment, an oxidation ratio of 0.47, suggesting that pyruvate is oxidized to the level of acetate (and probably in the form of acetyl-CoA because the pyruvate dehydrogenase complex seems to be involved). The specific activity (47.3) was about 5 times greater than that for *E. coli* in one report (66), although the *E. coli* extract was not supplemented with coenzymes, upon which the oxidation of pyruvate by *Listeria* extract depends. It is apparent from this experiment that *Listeria* is capable of providing acetyl-CoA from pyruvate, thus connecting the degradation of glucose to the first reaction of the citrate cycle.

The first and key enzyme of the citrate cycle, citrate synthase, was detected in *Listeria* A4413 extracts. Citrate synthase showed a specific activity of 0.68, slightly greater than that reported for anaerobically grown *E. coli* but about one-fourth that of aerobically grown *E. coli* (43).

Aconitase showed the highest specific activity of any of the citrate cycle enzymes found in extracts of *Listeria* A4413, showing values of 3.33 (citrate) or 6.66 (DL-isocitrate). The value of 3.33 should be used in comparison with the specific activity in *E. coli* because citrate was the substrate used in the *E. coli* aconitase determination (43). The specific activity of *Listeria* aconitase falls

between the activities found in anaerobically- and aerobically-grown *E. coli* (43). The finding that the *Listeria* aconitase activity in the presence of DL-isocitrate was twice that with citrate as the substrate, and that twice as much citrate as DL-isocitrate is required to drive the reaction at one-half its maximal velocity, are in harmony with the observation that the equilibrium of aconitase from other sources favors citrate (37). The K_m values for *Listeria* aconitase (8.0×10^{-4}M, citrate, and 4.4×10^{-4}M, DL-isocitrate) are close to those reported by Racker (89) for a mammalian-heart aconitase preparation (1.1×10^{-3}M, citrate, and 4.0×10^{-4}M, D-isocitrate). Racker (89) also observed that aconitase activity was from 2 to 7-fold greater with isocitrate than with citrate as the substrate.

Isocitrate dehydrogenase activity was found in extracts of *Listeria* A4413, but the specific activity (0.75) was low compared to that in *E. coli* (43), being about 9% as great as in anaerobically-grown and less than 1% as great as in aerobically-grown *E. coli* (43). The K_m for isocitrate dehydrogenase was about 4.4×10^{-4}M (DL-isocitrate) which is identical to that observed with aconitase in the presence of the same substrate. The isocitrate dehydrogenase is apparently NADP-specific, because no activity was observed when NAD replaced NADP in the reaction mixture. In contrast to the NADPH oxidase, the NADH oxidase of *Listeria* is very active at pH 7.5 (the pH of the isocitrate dehydrogenase assay) and this oxidase would be expected to interfere with any attempt at measuring isocitrate-dependent reduction of NAD; it cannot be stated with certainty, therefore, that *Listeria* A4413 extracts do not contain a NAD-dependent isocitrate

dehydrogenase. However, NADP-specific isocitrate dehydrogenases are the rule in bacteria, while NAD-specific enzymes occur only rarely (56). Mn^{++}, of all the divalent cations tested, was the most stimulatory to isocitrate dehydrogenase activity.

The α-ketoglutarate oxidation system could not be demonstrated in *Listeria* A4413 extracts. In reaction mixtures which included many coenzymes (see Figure 14), and in which the control extract of acetate-grown *E. coli* showed great oxidation of α-ketoglutarate, the *Listeria* extract did not oxidize α-ketoglutarate over a pH range of 5.0 to 9.0. It appears, therefore, that *Listeria* A4413 grown under these conditions, contain no significant oxidation system for α-ketoglutarate. With respect to this apparent enzyme deficiency, *Listeria* seems to resemble *E. coli* grown anaerobically on a synthetic medium plus glucose (43, 70).

Neither was succinate dehydrogenase found in extracts of *Listeria* A4413. Two methods (92, 95) were used in the attempt to detect this enzyme, both of which proved capable of demonstrating succinate dehydrogenase activity in control extracts of *E. coli*. No activity was ever observed with the *Listeria* extracts, however, and it appears that significant levels of succinate dehydrogenase are not present in *Listeria* A4413 as assayed.

Fumarate reductase, an enzyme which catalyzes the reverse of the succinate dehydrogenase reaction, i.e. the reduction of fumarate to succinate (58), was, however, found in extracts of *Listeria* A4413. The specific activity demonstrated by fumarate reductase from *Listeria* (2.82) is of the same order of magnitude as that reported from

fumarate-grown *E. coli* (4.56) by Hirsch et al. (58). Either $FMNH_2$ or $FADH_2$ was dehydrogenated by the enzyme in the presence of fumarate, and similar reaction rates were observed when either of these reduced flavins was the substrate. Since an α-ketoglutarate oxidation system and succinate dehydrogenase are apparently absent in *Listeria* A4413, the organism lacks a conventional means to provide succinate and succinyl-CoA. By means of fumarate reductase, however, fumarate may serve as a hydrogen acceptor and the production of succinate is possible. It would seem, therefore, that fumarate reductase is of considerable importance to *Listeria* A4413 metabolism. It is not known, however, whether *Listeria* can form succinyl-CoA from succinate because succinate thiokinase was not assayed.

Listeria A4413 extracts contained fumarase activity. The specific activity of this enzyme was, however, lower even than anaerobically grown *E. coli* (43), being about 11% as active as the enzyme from anaerobic *E. coli* and some 5% as active as the enzyme from aerobic *E. coli* (43). A K_m of about 1.1×10^{-3} M was found using L-malate as the substrate. Because the equilibrium of fumarase favors malate, a lower K_m would undoubtedly result with fumarate as the substrate, but the high absorbance of fumarate at 240 mμ discouraged attempts to measure the reaction from fumarate to malate by the spectrophotometric method (89).

Malate dehydrogenase was also detected in extracts of *Listeria* A4413. Again, however, the specific activity (0.87) was rather low, being about 8% of that found in anaerobic *E. coli* and a mere 0.4% of that observed in aerobic *E. coli* (43). The enzyme was demonstrated

only at the moderately high pH of 9.0, which seems to be a common finding with malate dehydrogenases. A pH of 9.0 was used by Robinson and Katznelson to assay the malate dehydrogenase of *Pseudomonas nigrifaciens* (103). The *Listeria* malate dehydrogenase, in contrast to the isocitrate dehydrogenase in this organism, required NAD as its pyridine nucleotide coenzyme, and only traces of activity were observed when NADP replaced NAD in the assay mixture. NAD is the usual pyridine nucleotide coenzyme required by malate dehydrogenases (37), although at least one strain of *P. aeruginosa* contains an NADP-specific malate dehydrogenase (not decarboxylating) (71, 104). Mn^{++} was required by the *Listeria* malate dehydrogenase for full activity, and the rate was 75% lower without Mn^{++}. The observed K_m for this enzyme was 4.0×10^{-3} M for L-malate; because the equilibrium of the reaction favors malate (37), a lower K_m would be expected if oxalacetate were the substrate.

Neither enzyme of the glyoxylate bypass (isocitratase and malate synthase) was detected in *Listeria* A4413 extracts. Control extracts from *E. coli* K-12(λ) contained activity of both enzymes. Under the assay conditions, therefore, the glyoxylate bypass is apparently absent in *Listeria* A4413. Since this pathway is required for bacteria to grow on C_2 compounds such as acetate, it is not surprising that *Listeria* neither grew on acetate nor contained the enzymes of the glyoxylate bypass.

Extracts of *Listeria* A4413 catalyzed the oxidation of NADH and NADPH. The specific activity of NADH oxidase (16.2) was higher than that of any citrate cycle enzyme or the pyruvate oxidation system

(note that the specific activity of the pyruvate oxidation system is defined in µl O_2 and not in µmoles O_2). *Listeria* NADH oxidase specific activity was about half that reported for aerobic *E. coli* (43) and over 3-fold greater than that in anaerobic *E. coli* (43). NADPH oxidase specific activity (2.3) was about one-seventh as great as the NADH oxidase in *Listeria*, but was about 12-fold greater than that reported for *P. aeruginosa* (71). Whereas the pH optimum for NADH oxidation was near 7.0, that for NADPH oxidation was near 6.0. Mn^{++} stimulated the rate of NADPH oxidation but did not affect that of NADH oxidation. Any interference by the reduced pyridine nucleotide oxidases with the isocitrate dehydrogenase or malate dehydrogenase assays was probably minimal because NADH oxidation was barely detectable at pH 9.0 (the assay pH for malate dehydrogenase) and NADPH oxidation was minimal at pH 7.5 (the assay pH for isocitrate dehydrogenase).

In review, the following enzymes were found in *Listeria* A4413: the pyruvate oxidation system (which probably involves the pyruvate dehydrogenase complex), citrate synthase, aconitase, isocitrate dehydrogenase, malate dehydrogenase, fumarase, fumarate reductase, and the oxidases for NADH and NADPH. The α-ketoglutarate oxidation system and succinate dehydrogenase of the citrate cycle, and both enzymes of the glyoxylate bypass (isocitratase, malate synthase) could not be detected in *Listeria* A4413. Succinate thiokinase was not determined.

The enzymes of the citrate cycle which have been found in *Listeria* A4413 form a pattern which resembles that apparently present in *E. coli* grown anaerobically in a synthetic medium with glucose as the source of carbon and energy. Because α-ketoglutarate dehydrogenase

is absent in *E. coli* grown under these conditions (43, 70), a crucial connecting link is missing in the cyclic chain of enzymatic reactions and there is no functional citrate cycle as such. Instead, a split, non-cyclic pathway exists which consists of a "right" oxidative portion from citrate to α-ketoglutarate, and a "left" reductive portion from oxalacetate to succinate and succinyl-CoA (43, 70), as diagramed in Figure 2 in the Introduction. Such a pathway appears to be of little consequence for the supply of energy, and enough energy (ATP) could be derived from glycolysis to support the cell (43). The pathway, however, would be important in biosynthesis, to supply carbon skeletons demanded in the formation of amino acids and porphyrins. In fact, the biosynthetic demand for α-ketoglutarate is thought to keep the level of this intermediate so low that the α-ketoglutarate dehydrogenase in *E. coli* grown anaerobically in synthetic medium plus glucose is completely repressed (43), while *E. coli* grown anaerobically on complex medium plus glucose show low levels of α-ketoglutarate dehydrogenase; this is explained on the basis that the glutamate supplied in the complex medium relieves the drain of α-ketoglutarate to an extent that the dehydrogenase for this substrate is partially derepressed.

The results of the enzyme assays of *Listeria* A4413 suggest that this organism, although grown aerobically, shows a pattern similar to that shown by anaerobically grown *E. coli* (43, 70). The demonstration of fumarate reductase, and the absence of succinate dehydrogenase, supports the concept of a non-cyclic citrate pathway (Figure 2) in *Listeria* A4413 having essentially a biosynthetic role. In harmony with this concept is the observation that the levels of citrate cycle

enzymes found in *Listeria* A4413 resemble more closely the level of similar enzymes in anaerobic than in aerobic *E. coli* (43).

If the pathway (Figure 2), suggested as being present in *E. coli* grown anaerobically on synthetic medium plus glucose (70), and apparently representative also of *Listeria* A4413, is to operate, oxalacetate would have to be synthesized in order that both the oxidative and reductive branches may function. Oxalacetate or malate synthesis would be expected by means of a carbon dioxide fixation reaction with either pyruvate or phosphoenolpyruvate. One or more of the following CO_2 fixation reactions might occur (49): malic enzyme, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, or phosphoenolpyruvate carboxylase. No attempts were made to demonstrate any of these enzymes in *Listeria* A4413.

With the results of the enzyme assays in mind, more conclusions can now be drawn concerning the growth and oxidative response of *Listeria* A4413 toward the intermediates of the citrate cycle. Since citrate synthase, aconitase, isocitrate dehydrogenase, malate dehydrogenase, and fumarase were demonstrated in *Listeria* extracts, the required enzymes for the oxidation of citrate, isocitrate, malate, and fumarate appear to be present. Therefore, the failure of *Listeria* A4413 to oxidize these intermediates is probably due to the lack of appropriate permeases (42). The requisite intracellular enzymes for the oxidation of succinate or α-ketoglutarate could not be shown, and this would account for the inability of the *Listeria* A4413 to oxidize these substrates. No enzyme assay attempted to use acetate, so few conclusions can be drawn regarding whether permease or intracellular

enzymes are lacking for the oxidation of this compound. The failure of *Listeria* to grow on any of the citrate cycle intermediates is also explainable either in terms of these cells being impermeable to citrate, isocitrate, malate, and fumarate, or lacking the needed intracellular enzymes for the utilization of α-ketoglutarate and succinate. Even if the *Listeria* had permeases for these compounds, the low level of enzymes found would not be expected to permit significant growth on any of the substrates tested.

The absence of detectable cytochromes in *Listeria* A4413 confirms the previous report (26) that *Listeria* are cytochrome-deficient. This apparent cytochrome deficiency is in agreement with the suggested biosynthetic, not energetic, role of the non-cyclic citrate pathway in *Listeria* A4413, because an efficient electron transport sequence would be primarily needed in a cell which contained high concentrations of each citrate cycle enzyme in order to maintain a sizeable pool of oxidized pyridine nucleotide coenzymes and to couple the transport of electrons to the production of ATP. In a cell such as *Listeria*, which appears to contain significant but low levels of most but not all citrate cycle enzymes, and which is unable to oxidize any intermediate of the citrate cycle or use it for growth, cytochromes would be less necessary.

Are the results of this research with *Listeria* A4413 representative of other *Listeria* strains as well? A certain answer cannot be given at this time. Seeliger (6), an investigator having long experience with *Listeria*, feels that aside from minor differences in fermentations, et cetera, the different *Listeria* serotypes and strains

may be regarded as a cultural and biochemical entity. *Listeria* A4413, serotype 4b, has been the main experimental subject of this research because it is thought to be a typical virulent *Listeria* strain, the nutritional requirements and metabolism of which have received more study than other *Listeria*.

SUMMARY AND CONCLUSIONS

This thesis describes experiments which determined whether the bacterium *Listeria monocytogenes* carries out the oxidation of pyruvate, the citrate cycle, and the glyoxylate bypass.

Experiments revealed that pyruvate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, and acetate were not acceptable sources of carbon and energy for the growth of *Listeria* A4413, a virulent smooth strain, or *Listeria* 9037-7, an avirulent rough strain. This conclusion followed the observation that no *Listeria* growth occurred when each individual compound mentioned above was the sole source of carbon and energy; furthermore, a supplement of any one of these compounds, when added to a medium containing glucose, failed to increase the level of *Listeria* growth over that resulting with glucose alone. For the purposes of these experiments and for the growth of *Listeria* used in all manometric and enzyme studies, a defined (synthetic) medium was developed and used; this medium, termed D10, is thought to be simpler than any yet described for the growth of *Listeria*.

Resting *Listeria* A4413 cells failed to oxidize citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, or acetate between pH 5.0 and 8.0. Pyruvate, however, was oxidized at pH 5.0 by a process which was stimulated significantly by α-lipoate, and which apparently oxidized the pyruvate to the level of acetate.

Enzyme assays with cell-free extracts of *Listeria* A4413 indicated that pyruvate was oxidized by an enzyme system which is

apparently either identical with or similar to the pyruvate dehydrogenase complex. Citrate synthase, aconitase, isocitrate dehydrogenase, malate dehydrogenase, fumarase, and fumarate reductase enzymes of the citrate cycle were detected. Succinate dehydrogenase and the α-ketoglutarate oxidation system of the citrate cycle, and the two enzymes of the glyoxylate bypass (isocitratase and malate synthase), however, could not be found. Oxidases for NADH and NADPH were present, but no evidence of cytochromes was found in *Listeria* A4413 cells or extracts. When an enzyme could not be demonstrated in *Listeria* A4413 extract, a cell-free extract of *E. coli* was used as an activity control to verify the assay method.

The presence of citrate synthase, aconitase, isocitrate dehydrogenase, malate dehydrogenase, and fumarase in cell-free extracts of *Listeria* A4413 suggests that the non-oxidation of citrate, isocitrate, malate, and fumarate by intact *Listeria* A4413 cells is due to cellular impermeability, i.e. lack of the appropriate permease. The apparent absence of an α-ketoglutarate oxidation system and succinate dehydrogenase in cell-free extracts explains the inability of intact *Listeria* A4413 to oxidize α-ketoglutarate or succinate. The absence of either the appropriate permease or intracellular enzyme may also explain why no intermediate of the citrate cycle supported the growth of *Listeria* A4413.

The pattern of citrate cycle enzymes in *Listeria* A4413, although the cells were grown aerobically on a synthetic medium plus glucose, resembles that shown by *E. coli* grown anaerobically in synthetic medium plus glucose, i.e. α-ketoglutarate dehydrogenase is absent.

An α-ketoglutarate oxidation system and succinate dehydrogenase were not found in *Listeria* A4413.

The enzyme pattern found in *Listeria* A4413 is interpreted as forming not a functional citrate cycle, but a split, non-cyclic citrate pathway having an oxidative portion (citrate synthase, aconitase, and isocitrate dehydrogenase) and a reductive portion (malate dehydrogenase, fumarase, fumarate reductase, and succinate thiokinase [not assayed for]), the function of which is predominantly biosynthetic for the synthesis of α-ketoglutarate and succinyl-CoA.

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APPENDIX

MEDIUM D10

1. Composition.

	Component	Quantity/liter
SALTS	K_2HPO_4	8.5 gm
	$NaH_2PO_4 \cdot H_2O$	1.5 gm
	NH_4Cl	0.5 gm
	$MgSO_4 \cdot 7H_2O$	0.41 gm
	$FeCl_3 \cdot 6H_2O$	0.048 gm
	NaOH	0.24 gm
	Nitrilotriacetic acid	0.48 gm
AMINO ACIDS	L-cysteine·HCl	100 mg
	L-leucine	100 mg
	DL-isoleucine	200 mg
	DL-valine	200 mg
	DL-methionine	200 mg
	L-arginine·HCl	200 mg
	L-histidine·HCl	200 mg
VITAMINS	Riboflavin	1 mg
	Thiamine·HCl	1 mg
	D-biotin	0.1 mg
	α-lipoic acid	1 μg

2. Details of assembly.

NOTE: It is important to assemble the medium in the order described to avoid the formation of precipitates.

To make 1000 ml of medium D10, the above quantities of each component are assembled as described below.

a. Dissolve the K_2HPO_4, $NaH_2PO_4 \cdot H_2O$, and NH_4Cl in 500 ml of distilled water.

b. (1) Make up solution A by dissolving the NaOH then the nitrilotriacetic acid in 40 ml distilled water; solution A need not be fresh.

(2) Make up solution B by dissolving the $FeCl_3$ in 40 ml distilled water; this solution must be fresh!

(3) Mix solutions A and B to give mixture AB (80 ml).

c. To the 500 ml solution from step a, add:

(1) Mixture AB (80 ml).

(2) The $MgSO_4 \cdot 7H_2O$ dissolved in 30 ml of distilled water.

(3) The amino acids.

(4) 390 ml distilled water.

d. Mix the solution and autoclave at 15 lb for 15 min. Allow to cool. Autoclaving causes the loss of ≃ 2% of the fluid volume of the medium; this is utilized for the addition of vitamins (100X), and, when desired, glucose (100X), detailed as follows:

e. Add 10 ml vitamin solution (100X), made as follows: An α-lipoic acid solution is first made by dissolving 5 mg α-lipoic acid in 200 ml 70% ethanol. Two ml of this solution is then combined with 5 mg biotin, 50 mg thiamine, and 50 mg riboflavin in 125 ml 95% ethanol, after which the final volume is brought to 500 ml with distilled water. This solution is sterilized by Millipore filtration (0.45 μ).

After the addition of vitamin solution, the medium acquires a

pale yellow color but remains clear. It may be stored in the refrigerator for up to 2 months with no change in appearance and with no effect on the growth of *Listeria*. The final pH is 7.3-7.4. Supplements of glucose or other carbon and energy source (in 100X concentration) may be added.

3. Obstacles encountered in the design of medium D10.

Except for the vitamin components (the vitamins were not autoclaved because at least the thiamine component is heat-labile [20]), medium D10 was sterilized by autoclaving. The heat from autoclaving causes the precipitation of iron and magnesium phosphates unless a chelating agent is included in the medium. Iron (either Fe^{+++} or Fe^{++}) is stimulatory for *Listeria* (22) and was included in medium D10. Ferric ammonium citrate (22) was not used as a source of iron for the reasons mentioned (step IV-B-7 of Methods); instead, $FeCl_3$ was used.

Nitrilotriacetic acid was chosen as the chelating agent because it tends to specifically bind the Fe^{+++} (81). Nitrilotriacetic acid is insoluble in water but dissolves in NaOH solution. If the nitrilotriacetate solution was too alkaline, massive precipitates formed upon addition of the $FeCl_3$ component of medium D10. When nitrilotriacetic acid and NaOH were combined in solution in a ratio of 2:1 (w/w) as in solution A (step 2-b-1), however, no precipitate formed during any phase of the assembly process. After autoclaving, the medium appeared water-clear.