LACTATE METABOLISM BY VEILLONELLA PARVULA

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TABLE OF CONTENTS

LIST OF TABLES

Table

Page

PART I

PART II

PART III

PART IV

LIST OF FIGURES

ABSTRACT

Lactate Metabolism by *Veillonella parvula*

by

Stephen Kam-Chi Ng

Culture of *Veillonella* species were isolated from human saliva on lactate agar plates incubated anaerobically at 37 C. The isolated organisms were subjected to various taxonomic tests and a strain classified as *V. parvula* was used for the study of anaerobic lactate metabolism.

Pure cultures of *V. parvula* grew readily in 1% lactate tryptone broth in atmospheres of nitrogen or N_2 + 5% CO_2 with a typical growth rate of 0.68 generations per hour. Unlike *V. alcalescens*, cells of *V. parvula* did not require carbon dioxide for growth on lactate, but clumping was advoided by the use of N_2 + 5% CO_2 as the gas phase. Lactate metabolism by growing and resting cells of *V. parvula* resulted in the formation of propionate, acetate, carbon dioxide and hydrogen.

Although carbon dioxide was not required for lactate metabolism, the presence of carbon dioxide in the gas phase influenced the quantities of propionate produced. Increasing amounts of carbon dioxide increased the absolute amount of propionate produced, the $C^{14}O_2$ evolution from lactate-U-C^{14} and cellular incorporation of lactate carbon, although the rates of lactate utilization were not affected.

When resting cells of *V. parvula* were incubated with lactate-U-C^{14}

as the substrate, both carbon dioxide fixation and CO_2-pyruvate exchange reactions were observed. These reactions were directly proportional to the partial pressure of carbon dioxide in the atmosphere with increased levels of carbon dioxide always resulting in the increased production of propionate (from carbon dioxide fixation) and $C^{14}O_2$ evolution (from the carbon dioxide-pyruvate exchange reaction) from the radioactive lactate substrate. At 50% carbon dioxide in the atmosphere, 80% of the decrease in radioactivity of propionate was due to the fixation of unlabelled carbon dioxide, while 20% of it was due to the CO_2-pyruvate exchange reaction.

Whole cells of V. parvula also metabolized, in addition to lactate, other substrates such as oxaloacetate and pyruvate. However, although the acidic end-products produced were quanlitatively the same, the rates of utilization for these substrates were slower than that observed for lactate. The ability of V. parvula to utilize pyruvate and oxaloacetate suggested that these substrates may be the intermediates in the propionate production pathway.

The inability of cell-free extracts of V. parvula to utilized lactate confirmed previous results that a conventional lactate dehydrogenase was not present in this organism. The utilization of lactate in the presence of oxaloacetate suggest that, like V. alcalescens, a malic-lactic transhydrogenase functions in this organism coupling the oxidation of lactate to pyruvate with the reduction of oxaloacetate to malate.

The direct detection of oxaloacetate formation from carbon dioxide and pyruvate or phosphoenolpyruvate by enzyme preparations of V. parvula gave further evidence that CO_2-fixation occurs in cells of this organism. The ability of V. parvula to form oxaloacetate confirms that propionate formation in this organism occurs by the conversion of lactate to pyruvate, pyruvate to oxaloacetate, oxaloacetate to malate, fumarate, and succinate with the decarboxylation of succinate to form propionate. Pyruvate degradation to acetate, CO_2 and H_2 also occurs and produces reducing power for propionate formation. The formation and accumulation of lactate when cell-free extracts were incubated with pyruvate as the substrate suggests that the acrylate pathway for propionate formation does not operate in V. parvula.

This culture study with V. parvula confirms that both species of the veillonellae, V. parvula and V. alcalescens, appear to form propionate according to the oxaloacetate scheme. V. parvula, however, differs from V. alcalescens, in that carbon dioxide was not required for growth in lactate broth or for the metabolism of lactate by resting cells.

Mixed culture studies with V. parvula and S. salivarius have shown that lactic acid produced by S. salivarius from substrate glucose was readily utilized by V. parvula for energy. Changes in the pH of the reaction medium depended on the ratio of the two bacteria and the number of cells in the system.

GENERAL INTRODUCTION

The presence of man in a microbe-laden environment has led to the establishment of micro-organisms indigenous to various regions of the body. During the process of birth and thereafter throughout life, man is exposed to environmental contamination. As man ingests a wide variety of substances by way of the mouth, it is not therefore surprising that the oral cavity supports one of the most dense and varied microbial populations indigenous to man.

Leeuwenhoek was the first to describe this in 1683 when he observed the presence of 'animalcules' in human saliva and teeth scrapings, but several centuries elapsed before people began to realize the importance of his findings. Only relatively recently have microbial surveys of the human mouth (Morris, 1954 a, b; Morris et al, 1954; Richardson and Jones, 1958; Bisset and Davis, 1960; Socransky et al, 1963; Gibbons et al, 1963; Gibbons, Kapsimalis and Socransky, 1964; Gordon and Gibbons, 1966; Ritz, 1967) led to the conclusion that the oral microflora, like many other indigenous microbial populations of man, is not composed of causal contaminants derived from outside sources, but rather is a collection of specialized parasites (Bisset and Davis, 1960).

Furthermore, these specialized parasites exist in a variety of ecological microcosms within the oral cavity itself, which are not homogenous in composition, but vary according to the particular

environment. Studies on the microbiota of the gingival crevice area (Gibbons et al, 1963), the surface of the tongue, (Gordon and Gibbons, 1966) and dental plaque (Gibbons et al, 1964) have shown that the microbial composition of these different ecological areas is different (Table I). Investigations by Gibbons and coworkers (1964) confirmed the findings of Krasse (1953, 1954 a, b) that the major source of the microorganisms in saliva was not dental plaque or gingival debris, but the dorsum of the tongue. For this reason, the tongue and saliva have essentially the same microbiota.

Inspection of Table I illustrates the variation between the types of microorganisms found in saliva, dental plaque, and the gingival crevice area. Saliva has a high proportion of Streptococcus and Veillonella species compared to the other areas, while dental plaque, on the other hand, has a high proportion of gram-positive, facultative and anaerobic diptheroids. The inability to detect the presence of obligately aerobic bacteria in these studies indicates that the microflora of the human oral cavity is anaerobic, or at least, microaerophilic.

The oral cavity is an ideal environment for the growth of micro-organisms. The mouth is warm and continually supplied with fresh nutrients in saliva (Jenkins, 1966) and ingested food materials with the result that the microbes, which make up the various ecological

TABLE I

Distribution of the microorganisms in the human oral cavity

Organism		Gram characteristics	Regions of the Oral Cavity				Type genus
			Saliva (a)	Saliva and tongue (b, c)	Gingival crevices (d)	Plaque (e)	
Rod	Facultative	positive	-	13.0	15.3	24	Corynebacterium
		negative	0.1*	3.6	1.2	4	Fusobacterium
	Anaerobic	positive	-	7.4	20.2	18	Actinomyces
		negative	-	5.3	16.1	10	Bacteroides
Coccus	Facultative	positive	27.7	38.3	28.8	27	Streptococcus
		negative	3.1	2.3	0.4	0.4	Neisseria
	Anaerobic	positive	-	7.4	7.4	13	Diptheroids
		negative	26.2	14.5	10.7	6	Veillonella

Reference: (a) Richardson and Jones, 1958.
(b) Gibbons, Kapsimalis and Socransky, 1964.
(c) Gordon and Gibbons, 1966.
(d) Gibbons et al, 1963.
(e) Gibbons et al, 1964.

* percentages

populations in this area, exhibit considerable metabolic activity. This has been shown in investigations with dental plaque (Kleinberg, 1961) and with "salivary sediment" preparations (Sandham, 1963, 1967; Kleinberg, 1967), which have demonstrated the rapid utilization of glucose and the formation of intracellular polysaccharide.

In saliva, organisms such as Streptococcus and Veillonella species are the major microbial components and appear to have readily adapted to this environment, while other less numerous types, such as species of Neisseria and Vibrio, apparently have been less successful in this regard. The reasons for the variability in adaptation to such matural ecological environments is unknown, but probably reflects the pressures of evolution. One can imagine that continual exposure to the metabolites in saliva and foodstuffs has produced a group of bacteria, which, through competition and selection, possess varying biochemical capabilities. Those organisms found in the highest numbers appear to dominate the metabolism of the entire population probably because their metabolic pathways are more suited to the environment. Despite the dominance of certain microbial species, however, each ecological system undoubtedly exists in dynamic equilibrium. The probability of such biological equilibrium is enhanced by the evidence of Kraus and Gaston (1956) who showed that the relative numbers of the various

microbial groups in saliva was constant within each individual.

In view of the small amount of information in the literature concerning the role of predominant bacteria in natural microbial populations, a long range study was undertaken in our laboratory using saliva as the source of material. Essential to this study was the selection of the predominant bacteria and the determination of their biochemical properties. One of the species selected for study was the gram-negative coccus, Veillonella parvula.

Veillonella species represent 11.0% of the total bacteria in saliva (Richardson and Jones, 1958) and 14.5% of all bacteria on the tongue (Gordon and Gibbons, 1966), although the former authors have reported a value as high as 36% in some samples. Examination of Table I shows that species of Veillonella are also important microbial components of dental plaque and the gingival crevice area.

As species of Veillonella are incapable of utilizing carbohydrates, it has been suggested (Douglas, 1950) that in saliva these organisms are dependent upon saccharolytic bacteria, such as the streptococci, for the production of metabolic acids which they in turn can metabolize for energy. A portion of the research reported here (Part IV) was designed to provide a definitive answer to this suggestion. Resting-cell experiments with mixtures of Veillonella parvula and Streptococcus

salivarius, incubated in a model ecological system with glucose, have shown that the lactic acid produced by S. salivarius from the catabolism of this glucose was further metabolized by V. parvula. The ability of V. parvula to metabolize lactic acid under these conditions, therefore, appears to be an important factor in the metabolism of the salivary microflora.

As little has been reported in the literature concerning the metabolic properties of V. parvula, a detailed study on the characteristics and properties of the lactate-metabolizing system in cells (Part II) and cell-free extracts (Part III) of this organism was undertaken.

PART I

BACTERIOLOGY OF VEILLONELLA PARVULA

INTRODUCTION

Early studies in oral microbiology were mainly concerned with the etiology of oral disease. As a consequence of this, the microbes receiving the most attention were pathogens, while many of the non-pathogenic species were neglected. Furthermore, most of these studies were carried out by investigators who were primarily concerned with dental caries, a disease thought to be caused by the dissolution of tooth enamel by acids accumulating in the oral cavity as the result of microbial metabolism. As acid production by bacteria was associated with the degradation of carbohydrates, early studies on the biochemistry of oral microbes was limited largely to the metabolism of carbohydrate fermenting species. Although they represent a significant quantity of the microflora in the oral cavity, few investigations were concerned with the non-carbohydrate utilizing microbes, such as the veillonellae. In fact, past treatises on the normal flora in the oral cavity either failed to mention the presence of Veillonella species or gave them scant attention (Wilson and Miles, 1946; Appleton, 1944).

Despite this general lack of attention in the non-carbohydrate fermenting microbes, however, the presence of the veillonellae in the oral cavity has been known for some 70 years. Species of this genus were first isolated from the mouth by Veillon and Zuber (1898), while

investigating the pathological role of certain obligate-anaerobes. As this species was difficult to classify at that time it was given two names, Staphylococcus parvula and Micrococcus parvula. In 1901, while working as a student under Veillon, Lewkowicz, isolated a similar anaerobe from a healthy infant's mouth, which he named Micrococcus gazogenes alcalescens anaerobius, to differentiate it from the anaerobe isolated by Veillon and Zuber. Despite the later conclusion of Jugano and Distaso (1910) that the strains of Veillon-Zuber and Lewkowicz were identical, considerable uncertainty as to the classification of these species prevailed in the literature for a number of years (Ozaki, 1912). Holman and Krock (1923 a, b.) isolated a strain of S. parvula (M. parvula) from the oral cavities of the human, rabbit and guinea pig, which they thought resembled Bacterium pneumosintes because it passed through Berkefeld-Mandler filters (Olitsky and Gates, 1922).

During the isolation of sporulating anaerobes from saliva, Hall (1925) frequently observed large numbers of small gram-negative non-sporulating anaerobes. These observations prompted a study of these minute bacteria, which included a re-examination of Holman and Krock's strain (Hall and Howitt, 1925). This investigation showed that both the species isolated by Holman and Krock and their own strains were non-filterable and were, in fact, biochemically similar to the species

studied by Lewkowicz. Furthermore, the S. parvula strain of Veillon and Zuber (1898) was shown to be different from the Lewkowicz strain because of its ability to produce hydrogen sulphide. As Lewkowicz's quadrinomial designation (Micrococcus gazogenes alcalescens anaerobius) was contrary to the rules of scientific nomenclature, the name was changed to M. gazogenes.

Although Hall and Howitt considered S. parvula and M. gazogenes to be different bacterial species, they were generally considered to be the same organism. For example, Branham (1927), during the investigation of these microorganisms in nasopharyngeal washing, used both names synonymously. Further work by Branham (1928), in fact, demonstrated that M. gazogenes and S. parvula were indeed similar serologically and that the differences in hemolytic action, gas, hydrogen sulphide and indole production previously reported were quantitative rather than qualitative.

Prevot (1933) confirmed the observations of Branham that the Micrococcus and Staphylococcus species were similar and proposed that they be designated as Veillonella. By Prevot's classification, species of Veillonella were divided into three main species; V. parvula, V. variabilis and V. alcalescens. The organisms isolated by Lewkowicz and by Hall and Howitt were grouped into V. alcalescens,

while those studied by Veillon and Zuber, and by Branham were placed in the V. parvula group.

Prevot's classification of the veillonellae remained unchanged until Forbert and Douglas (1948 a) observed that lactate-fermenting strains of Veillonella growing in lactate media were gram-positive in the early logarithmic phase of growth, but rapidly became gram-negative as growth approached the stationary phase. This observation prompted the transfer of these organisms from the genus Veillonella to the genus Micrococcus and the species designated as 'lactilyticus' to reflect their lactate fermenting ability. Later, however, Langford, Faber and Pelczar (1950) found that Prevot's Veillonella species and the species of M. lactilyticus studied by Forbert and Douglas were consistently gram-negative and, therefore, identical. As a result, the genus name Veillonella was reaffirmed.

The 7th edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith, 1957) recognized six species of Veillonella divided into two distinct groups on the basis of gas production. One group contained the gas producers, V. parvula, V. alcalescens and V. discoides, while the other was composed of the non-gas producers, V. remiformis, V. orbiculus and V. vulvovaginitidis.

After an extensive taxonomic investigation of the genus, Veillonella

(Rogosa, 1964; Rogosa and Bishop, 1964, a, b), Rogosa (1965) concluded that both Bergey's Manual and Prevot's classification were incorrect in some pertinent differentiating characteristics, such as, sugar fermentation, indole and hydrogen sulphile production, and nitrate reduction. In addition to a complete re-examination of their biochemical properties, these bacteria were also subjected to serological techniques as another means of differentiation.

As a result of this work, the genus Veillonella was reclassified into two main species: V. parvula and V. alcalescens. In this new classification, the main differences between these two species are as follows: (1) each species has distinctive non-crossing agglutinogens; (2) V. alcalescens is catalase-positive, while V. parvula is catalase-negative, and (3) V. alcalescens has an absolute growth requirement for putrescine or cadaverine in a nutritionally-defined media.

Despite the classification of this genus in the 7th edition of the Bergey's Manual (1957) and more recently by Rogosa (1965), publications continue to use the genus names, Veillonella and Micrococcus, for the same organism (McCormick, Ordal and Whiteley, 1962, a, b; Bahn and Tiecke, 1967).

The purpose of the present investigation was to isolate species of the veillonellae and to determine the taxonomic and biochemical

properties of a representative strain. All of the organisms isolated were given species designations according to the classification of Rogosa (1965).

METHODS

Bacteriological

Fresh human saliva was the source of the Veillonella strains isolated in this study. Initially, several loopfuls of saliva were inoculated into lactate broth and incubated overnight in an atmosphere of N_2 + 5% CO_2 at 37 C. Samples from these overnight cultures were then streaked on lactate agar plates and incubated anaerobically for several days. Veillonella-like colonies were selected from these plates, sub-cultured in broth and those possessing the general staining and morphological characteristics of the veillonella retained for purification and identification.

The lactate medium used for the isolation and growth of these Veillonella strains was similar to that proposed by Rogosa (1964) and contained tryptone, 10 g; yeast extract, 5 g; Tween 80, 1 ml; sodium lactate (42.5%), 24 ml; KH_2PO_4, 6.9 g; and sodium thioglycollate, 0.75 g per litre of deionized water. The pH of this medium was adjusted to 6.5 with Na_2CO_3. Where lactate agar was required, 20 g agar was added to the above medium. Sterilization was always carried out for 15 minutes at 121 C.

The isolation of Veillonella species free of small gram-positive cocci was difficult, but was facilitated by screening the veillonellae in

lactate medium containing streptomycin or vancomycin essentially as proposed by Rogosa et al (1956, 1958).

Taxonomic tests were carried out according to precedures of the Society of American Bacteriologist Manual of Microbiological Methods (1957) and according to the methods of Rogosa (1964) and Rogosa and Bishop (1965). Classification of the species isolated was carried out according to that proposed by Rogosa (1965).

Stock cultures were maintained in an atmosphere of N_2 + 5% CO_2 at -10 C, either in lactate broth or on lactate agar plates. The viability and purity of these cultures was checked at monthly intervals by staining procedures and routine taxonomic tests carried out for the production of hydrogen sulphide, catalase, the conversion of nitrate to nitrite and the production of acid from a variety of carbohydrates. When contamination was observed, cultures were grown in broth containing streptomycin or vancomycin followed by triple-streaking on lactate agar plates. The fresh, pure cultures obtained after this procedure were employed to maintain the stock collection and also used to replace the cultures transferred daily. These cultures, transferred every one to two days, were grown anaerobically at 37 C in 5 ml of lactate broth sterilized in screw-capped tubes.

Growth

The growth characteristics of the strains of Veillonella isolated in this study were determined in lactate broth in an atmosphere of N_2 + 5% CO_2 by monitoring cell mass with time. Increases in cell mass were determined bymeasuring the turbidity of cell suspensions in a Klett-Summerson Colorimeter containing a red filter (640-700 mμ). A standard curve for Klett units vs dry weight of cell material was prepared with the test organism prior to this growth study. All cell concentrations are expressed on the basis of mg dry weight of cell material.

Turbidity measurements of these anaerobic cultures were facilitated by fitting the growth flasks with a two-hole stopper: one hole containing the gassing tube, while the other was fitted with an inverted Klett tube. Inverting the growth flask in the colorimeter permitted the determination of turbidity without disturbing the gas phase in the culture flask.

RESULTS

The isolation of pure cultures of Veillonella species from human saliva was difficult because of persistent contamination by small gram-positive cocci even after the use of a variety of streaking and

subculturing techniques. These tiny contaminants adhered tenaciously to the soft, liquescent veillonella colonies on lactate agar streak-plates such that any attempt to transfer single colonies of veillonella always resulted in the carry-over of the contaminants. Rogosa et al (1958) had previously solved this problem by selectively inhibiting the growth of these contaminants by the use of streptomycin or vancomycin. The species of Veillonella isolated by Rogosa were found to be resistant to 5 µg, streptomycin or 20 µg, vancomycin, while other oral bacteria were inhibited significantly at these concentrations. Of these two antibiotics, Veillonella species were recovered more readily in media containing vancomycin than streptomycin. Two to three subsequent transfers of the impure cultures in the antibiotic-containing media were usually sufficient to remove the undesirable contaminants.

The Veillonella strains isolated in this work were subjected to increasing concentrations of streptomycin and vancomycin lactate broth to determine their tolerance to these antibiotics. Their effect on cell growth was determined by comparing the relative turbidity of the innoculated broth after a 24 hour incubation period and the presence or absence of contamination determined microscopically. The results obtained for a representative strain are illustrated in Table 2. The antibiotics were found to have no effect on the morphology of the

Table 2

Antibiotic tolerance of V. parvula and contaminant(s).

Antibiotic	Concentration (µg/ml)	Growth (turbidity)	Presence of contaminant
Streptomycin	0	++++	+++
	5	++++	++
	10	+++	+
	15	+++	-
	20	++	-
	25	+	-
Vancomycin	0	++++	+++
	5	++++	++
	10	+++	+
	15	+++	+
	20	+++	-
	25	+++	-
	50	+++	-

Lactate broth tubes containing either streptomycin or vancomycin were incubated with a 1% inoculum of V. parvula isolated from saliva, the tubes incubated in an atmosphere of N_2 + 5% CO_2 for 24 hours.

veillonellae cells, although streptomycin had a greater inhibitory effect on growth of both Veillonella species and the contaminant compared to vancomycin at equivalent concentrations. The contaminant was completely inhibited in broth containing streptomycin or vancomycin at concentrations at or above 15 and 20 μg /ml, respectively. With this in mind, Veillonella cultures were successfully isolated by serial transfer in broth containing either streptomycin at 20 μg/ml or vancomycin at 50 μg/ml. The cultures so isolated, however, were maintained as stock cultures in lactate broth free of antibiotic. Stock cultures subsequently found to be contaminated were purified by serial transfer in streptomycin or vancomycin-containing broth for short periods of time to remove the contaminants.

Of the several strains of Veillonella isolated in this study, one representative culture was selected for future study. This gram-negative, obligate-anaerobic coccus was subjected to a series of taxonomic tests to facilitate classification, the results of which are outlined in Table 3. The principle characteristics of this organism were as follows: (1) inability to utilize carbohydrates as a source of energy; (2) H_2S gas was detected only when a heavy cell inoculum was used, and (3) indole and catalase formation were negative. From this information, the organism was classified as Veillonella parvula

Table 3

Taxonomic characteristics of Veillonella parvula

Morphology	Coccoid, very small (0.4 microns in diameter) occuring in masses pairs or short chains.
Environment	Strictly anaerobic.
Gram reaction	Negative.
Growth in lactate broth	Young growth turbid to sediment with age. CO_2 enhances growth.
Optimal temperature	30 - 37 C.
Optimal pH	pH 6.0 - 8.0
Fermentation tests	
glucose, sucrose, lactose, arabinose, raffinose, inulin, fructose, monnose, mannitol, glycerol, galactose	Not utilized.
lactic acid	Products formed: CO_2, H_2 acetic acid and propionic acid.
Hydrogen sulphide medium (Rogosa and Bishop, 1964)	H_2S produced.
Indole production	Negative.
Nitrate reduction to nitrite	Positive.
Catalase	Negative.
Gelatine liquification	Negative.
Starch utilization	Negative.
Blood agar	β-hemolysis.
Litmus milk	No change.
Antibiotic limits	Vancomycin 50 µg/ml. Streptomycin 20 µg/ml.
Habitat	Oral cavity.

according to the recent classification of Rogosa (1965).

The conditions for optimal growth of V. parvula were found to be very specific. Growth of this strict anaerobe was inhibited in the presence of small amounts of oxygen and for this reason cultures were always incubated in an atmosphere of either high purity nitrogen or N_2 + 5% CO_2. Freshly prepared broth, containing a reducing agent such as sodium thioglycollate was also essential if rapid growth was required as liquid media absorbed enough oxygen to inhibit growth when allowed to stand in contact with air for short periods of time. The use of a large inoculum (greater than 2%), consisting of actively growing cells, also reduced the lag period of growth and better growth was always obtained in broth medium compared to growth on the surface of lactate agar medium.

A typical growth curve for V. parvula in lactate broth at 37 C in an atmosphere of N_2 + 5% CO_2 is shown in Figure 1(A). This curve was obtained by inoculating 500ml of freshly prepared 1% lactate broth with a 4 ml of a 12 hour culture. The growth rate of V. parvula in this medium was 0.682 cell divisions/hour, which was calculated from an average generation time of 88 minutes. The growth rate was constant between 30 to 37 C with marked inhibition of growth at temperatures of 40 C or higher. Although the growth rate was constant between 30 to 37 C, the cells tended to produce more extracellular slime as the incubation temperature approached 37 C. As this extracellular mucoid material hindered the harvesting of these cells by centrifugation in preparation for resting cells experiments, temperature for

Figure 1

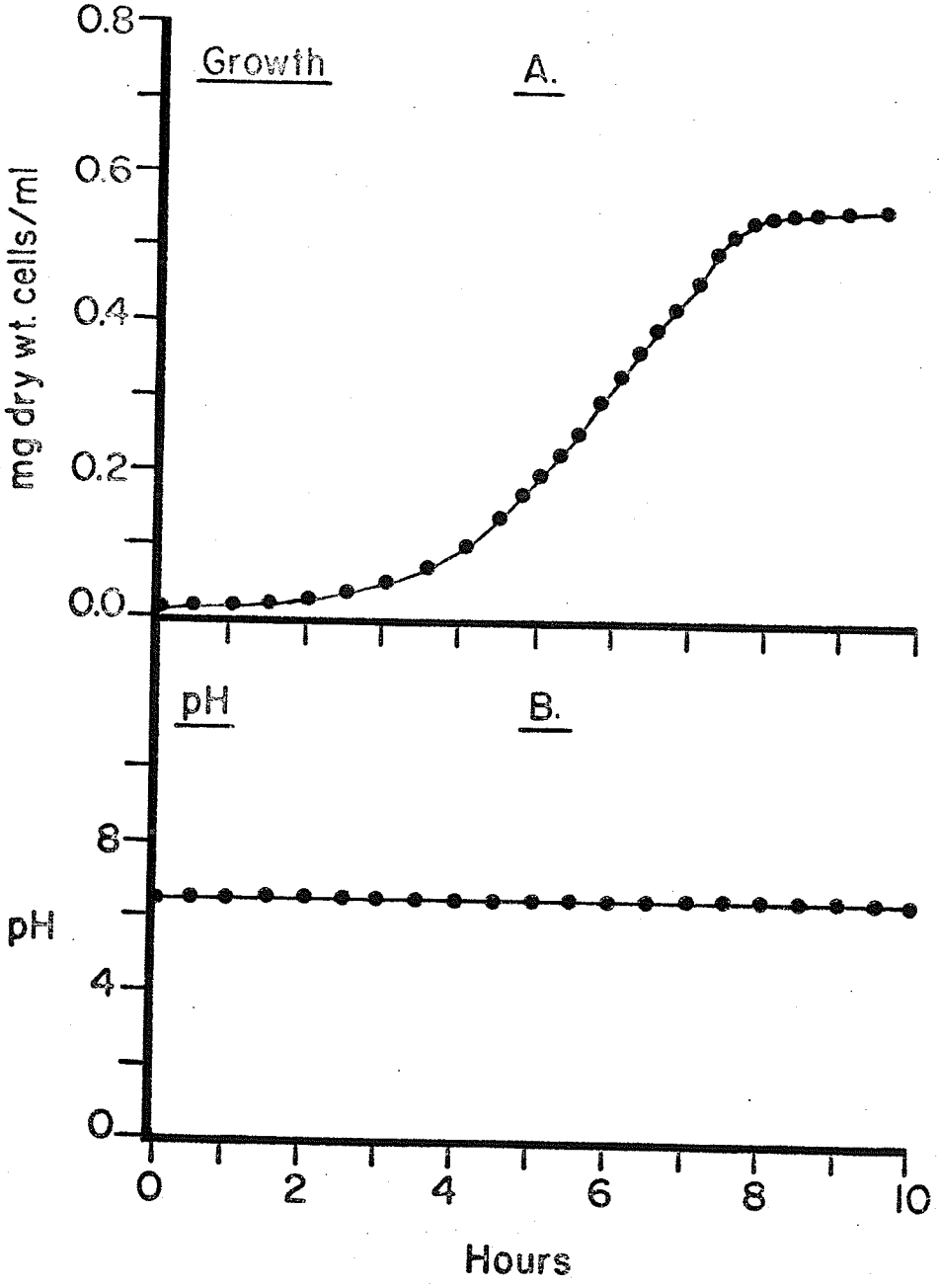
Growth of V. parvula in a 1% lactate broth medium in an atmosphere of N_2 + 5% CO_2 at 37 C.

A. Growth as measured by the increase in cell mass with time.

Four mls of an 18 hour culture of V. parvula were inoculated into 200 ml of freshly prepared 1% sodium lactate broth in a 500 ml Erlenmeyer flask fitted with a two-hole stopper. Growth was followed turbidometrically in a Klett-Summerson colorimeter containing a red filter.

B. pH of the culture during the growth period.

One ml of broth culture was aseptically withdrawn from growth flask by syringe and the pH was measured.


Growth
A.
mg dry wt. cells/ml
0.8
0.6
0.4
0.2
0.0
pH
B.
pH
8
4
0
0
2
4
6
8
10
Hours

growth was subsequently maintained at 31 C.

As shown in Figure 1(b), lactate metabolism by V. parvula did not result in a decrease in the pH of the medium. The initial pH (6.5) of the growth medium was maintained during the 10 hour growth period, although the substrate sodium lactate was completely utilized in this time.

DISCUSSION

Contrary to the observations of Foubert and Douglas (1948), the cultures isolated in this study were consistently gram-negative throughout the growth cycle and hence could not be a member of the gram-positive family Micrococcaceae as proposed by these workers, but rather belong to the family Neisseriaceae. In this family, all the species are parasitic spherical, occuring in pairs or in masses, non-motile gram negative, aerobic, facultative or anaerobic, (Bergey's Manual, 1957). The family Neisseriaceae was further divided into two genera, Neisseria and Veillonella, on the basis of the aerobic, facultative or anaerobic characteristics. Neisseria species are strict anaerobes. The taxonomic results obtained in this work (Table 2), placed the present strains in the genus Veillonella within the family Neisseriaceae.

In Bergey's Manual (1957), species of Veillonella were subdivided into six groups, however, as previously stated, the re-investigation of this genus by Rogosa (1965) resulted in a new classification con-

sisting of only two main species. Furthermore, Rogosa (1964, 1965) observed that the classification for V. alcalescens and V. parvula in Bergey's Manual was erroneous because species of V. gazogenes (V. alcalescens, Rogosa) were differentiated from V. parvula species because they did not ferment carbohydrates, reduce nitrates to nitrites, or produce indole H_2S. Rogosa reported that V. parvula should not ferment carbohydrates and did not produce indole, while V. alcalescens (V. gazogenes, Bergey) could produce H_2S and reduced nitrates to nitrites. Comparing the results in Table 2 with those of Rogosa (1964, 1965) indicates that the species isolated in this study showed a close resemblance to V. parvula as it was catalase-negative and did not require putrescine or cadaverine for growth. The only characteristic of this species which differed from Rogosa's classification was the β-hemolytic reaction following growth of this organism on blood agar plates. However, this difference did not affect the final classification of the present strain because the hemolytic properties of the veillonella are not considered to be a major differentiating characteristic.

As V. parvula is a strict anaerobe, its growth and metabolism was readily inhibited by the presence of trace amounts of oxygen. This inhibitory effect of oxygen was so pronounced that special techniques had to be developed for the growth of this organism and for the study of its metabolism. These special techniques will be discussed in the sections concerned with the metabolism of resting cells (Part II)

and cell-free extracts of this organism (Part III).

As stated previously, the isolation of pure species of V. parvula was accompanied by the use of the antibiotics, vancomycin or streptomycin at higher concentrations than those employed by Rogosa (1956). In this study, vancomycin at 50 µg/ml was used whenever contamination was observed rather than streptomycin at 20 µg/ml for the following reasons. Firstly, streptomycin, a product from Streptomyces griseus, was shown not only to have an inhibitory effect on the gram-positive contaminant (Table 2), but also had an adverse influence on the growth of V. parvula as the concentration was increased. These results can be explained by the fact that streptomycin is active against many gram-negative and gram-positive bacteria and is used medically to cure infections caused by organisms of both types (Merck Index, 1960).

On the other hand, vancomycin, an antibiotic from Streptomyces orientalis, was reported to be particularly active against gram-positive bacteria and spirochetes with high concentrations required for the inhibition of gram-negative organisms, (McCormick et al, 1955-56). The present results agree with the observations of McCormick and co-workers as V. parvula was unaffected by concentrations of vancomycin up to 50 µg/ml, although contamination was eliminated at vancomycin concentrations of 20 µg/ml or higher (Table 2). The mode of action of vancomycin on the gram-positive streptococci and diptheriod contaminants is unknown, but Best and Durham (1964, 1965)

reported that vancomycin inhibited the growth and cell-wall synthesis of gram-positive Bacillus subtilus.

An additional reason for selecting vancomycin in this work is the well-known fact that both gram-positive and gram-negative microorganisms readily develop resistance to streptomycin (Alexander and Leidy, 1949; Gazon and Carpenter, 1953-54), while resistance to vancomycin by gram-positive micrococci occurs slowly and then only to a slight extent (Ziegler, Wolfe and McGurie, 1955-56; Grace et al, 1956-57). However, despite the apparent resistance to vancomycin, prolonged exposure of V. parvula cells to this antibiotic produced an adverse effect on the growth as long lag periods were always observed with cultures continually transferred in media containing this compound. For this reason, pure stock and daily cultures were always maintained in lactate broth without vancomycin.

PART II

WHOLE CELL METABOLISM OF VEILLONELLA PARVULA.

INTRODUCTION

The microorganism most predominant in the oral cavity are members of the genus, Streptococcus (Gibbons et al, 1964; Socransky et al, 1963; Gibbons, Kapsimalis, Socransky, 1963), which catabolize carbohydrates with the concomitant production of large quantities of lactic acid. Sandham (1963), however, has shown that little free lactic acid can be found as the result of glucose metabolism by salivary sediment material, which contained primarily salivary bacteria. Thus, in vivo lactic acid does not accumulate, but is rapidly removed from saliva. Because of their abundant numbers, species of the veillonellae are thought to be primarily responsible for the conversion of this lactic acid to other weaker acids such as acetate and propionate (Foubert and Douglas, 1948 b). Thus, from the stand point of the microbial ecology of saliva, species of Veillonella are important because their ability to utilize lactate for energy probably constitutes an intermediate stage in the metabolic chain of events.

Foubert and Douglas (1948 b) were the first to report in detail on metabolism by species of the veillonellae when they observed that cells of M. lactilyticus (now V. alcalescens) fermented lactic acid with the production of propionate, acetate, carbon dioxide and hydrogen. Pyruvate and malate were also catabolized by the same cells with the

production of abundant gas. In the same year, Johns (1948) isolated a 'new micrococcus' unable to ferment carbohydrate, but capable of degrading lactate, pyruvate, oxaloacetate, malate and fumarate with the formation of propionate, acetate, hydrogen and carbon dioxide. The fermentation of succinate, on the other hand, resulted only in the formation of propionate and carbon dioxide.

Later, Johns (1951 a, b) identified the 'new micrococcus' as V. gazogenes (now V. alcalescens) and showed that the products of lactate fermentation by this organism and a strain obtained from the Institut Pasteur (Prevot's organism) were identical. In this study, Johns observed that the utilization of lactate by his strain had a mandatory requirement for bicarbonate or carbon dioxide. Although the V. gazogenes strain isolated by Johns (1951 a) and the strain of M. lactilyticus studied by Foubert and Douglas (1948 a, b) are now considered to be the same organism (V. alcalescens), a comparison of the fermentation balances carried out by these workers shows significant differences in the quantities of the end-products formed by these two strains when metabolizing sodium lactate (Table 7).

As pyruvate, oxaloacetate, malate, fumarate and succinate were fermented by cells of V. gazogenes in the presence of carbon dioxide, Johns (1951 b) proposed a pathway for the production of propionate from

lactate which was similar to that proposed by Delwiche (1948) for propionate formation by Propionibacterium pentosaceum:

lactate ⟶	pyruvate	(1)
pyruvate ⟶	CO_2 + H_2 + acetate	(2)
pyruvate + CO_2 ⟶	oxaloacetate	(3)
oxaloacetate + NADH ⟶	malate + NAD	(4)
malate ⟶	fumarate	(5)
fumarate + NADH ⟶	succinate + NAD	(6)
succinate ⟶	propionate + CO_2	(7)

Although the veillonellae do not appear to contain a complete set of Kreb's cycle enzymes, reactions (4) to (6) are typical of this cycle, functioning, of course, in the reverse direction. In this hypothetical scheme, lactate is converted to pyruvate via a lactic dehydrogenase-type reaction and succinate is decarboxylated directly to propionate and carbon dioxide.

The oxidative decarboxylation of pyruvate (reaction 2) was included to account for the production of acetate, carbon dioxide and hydrogen by these bacteria. The production of molecular hydrogen was known, at that time, to occur by one of two reactions: (1) the formation of molecular hydrogen by the direct activation of a proton by the enzyme, hydro-

genase, or (2) the splitting of formate by the enzyme, formic hydrogenlyase, producing carbon dioxide and H_2. Johns concluded, however, that because sodium formate was not utilized by washed cells at pH 6.2, formate was not an intermediate in the production of H_2 and CO_2 by V. gazogenes.

During these studies, Johns (1951 b) observed that resting cells of V. gazogenes, grown on D-tartrate, quantitatively decarboxylated succinate, and rapidly degraded tartrate, pyruvate, oxaloacetate, malate, and fumarate, but not lactate. Washed lactate-grown cells, on the other hand, did not attack D-tartrate. These results prompted Johns to conclude that the enzymes responsible for tartrate fermentation were adaptive (inducible), while those for the dissimilation of oxaloacetate, malate, fumarate and succinate were constitutive. The inability of tartrate-grown cells to utilize lactic acid suggests that the enzymes responsible for lactate fermentation in V. gazogenes were also inducible, although there is no additional evidence to support this.

While species of the Veillonella readily ferment short chain carboxylic acids, they do possess the ability of degrading more complex organic compounds for energy. For example, cells of M. lactilyticus (V. alcalescens) were observed to grow very poorly in a peptone yeast extract medium unless certain high molecular weight nitrogenous acids

were added to the medium (Whiteley and Douglas, 1951). It was subsequently shown that the organic acids required in the growth medium were the purines—hypoxanthine, xanthine, uric acid, adenine and guanine. Furthermore, these purines were fermented by washed, resting cells of M. lactilyticus, incubated under anaerobic conditions although various strains of this organism had different activities towards the same substrate. The type of gas phase was also found to have a significant effect on metabolism.

This was shown by a comparison of strains 221 and 416, which varied not only in the rate of hypoxanthine and xanthine fermentation, but also differed in the quantities of products formed from the degradation of these compounds. In an atmosphere of nitrogen, strain 221 readily fermented hypoxanthine to large and equivalent amounts of xanthine and hydrogen gas, as well as lesser quantities of carbon dioxide, propionate and ammonia. However, in an atmosphere of hydrogen, although equivalent quantities of xanthine and H_2 were observed, only small quantities of CO_2 were produced and the rate of fermentation was inhibited. Under these conditions, propionate and ammonia were not formed.

In contrast, strain 416 in an atmosphere of nitrogen, fermented hypoxanthine to small amounts of hydrogen, uric acid, acetate,

propionate, and large quantities of carbon dioxide and ammonia. When hydrogen was the gas phase, xanthine appeared as the major product of metabolism with reduced quantities of CO_2 and H_2. Again the rate of metabolism was reduced as it was for strain 221.

Xanthine fermentation by M. lactilyticus, strain 221, in an atmosphere of nitrogen, produced large and equivalent quantities of uric acid, carbon dioxide and hydrogen and smaller amounts of acetate, propionate and ammonia, while strain 416, under the same conditions, produced CO_2 and ammonia as the major products of metabolism. Furthermore, xanthine was more readily degraded by strain 416 than strain 221. In the presence of H_2 gas, both strains produced hypoxanthine and carbon dioxide from the fermentation of xanthine with the concomitant uptake of molecular hydrogen. The hydrogen assimilated by these resting cells was equivalent to the quantity of hypoxanthine formed, indicating that hydrogen provided the required reducing power for the conversion of xanthine to hypoxanthine.

The anaerobic fermentation of purines by bacteria has been studied in only a few instances, but a comparison between Clostridium acidi-urici (Barker and Beck, 1941; Karlson and Barker, 1949) and V. alcalescens (Whiteley and Douglas, 1951) indicates that their purine degradative pathways are different. The major differences appear to be: (1) the decomposition of hypoxanthine, but not xanthine by C. acidi-urici was an adaptive process (Barker and Beck, 1941), while strain 416 of V. alcalesces decomposed both substrates readily indicating that

the enzymes responsible in this organism were constitutive; (2) glycine was an intermediate in the decomposition of hypoxanthine by C. acidi-urici, but it was not detected during hypoxanthine fermentation by V. alcalescens; and (3) the anaerobic fermentation of hypoxanthine by C. acidi-urici produced carbon dioxide, acetate, and ammonia, whereas strain 416 of M. lactilyticus (V. alcalescens) produced in addition to the above compounds, hydrogen, propionate and urea.

Although species of Veillonella are incapable of metabolizing carbohydrates because the glucose phosphorylating enzyme, hexokinase, is absent (Rogosa, 1965), gluconeogenesis appears to occur in these bacteria. Mergenhagen (1965) has reported the production of extracellular polysaccharide-lipid complexes by cells of V. parvula, grown in a trypticase-yeast extract medium with sodium lactate as the sole carbon source. These extracellular slime-layers were shown to contain glucose, galactose, glucosamine, galactosamine and a methylpentose suggesting that lactate carbon is readily converted to hexoses and pentoses by reverse-glycolysis. This would appear possible as Rogosa, Krichevsky and Bishop (1965) have shown that although hexokinase is absent in species of Veillonella, all of the remaining enzymes in the glycolytic pathway were present in these bacteria.

Michaud and Delwiche (1967) have recently reported the incorpora-

tion of small amount of glucose-C^{14} radioactivity into nucleic acid, protein and lipid components of V. alcalescens cells. However, as the high specific activity glucose-C^{14} employed in these experiments was autoclaved with the growth medium, and as a relatively small amount of the C^{14} actually appeared in these cellular components, the direct utilization of glucose for synthesis, rather than a hydrolysis product from glucose, is open to question.

METHODS

Growth

For the washed, whole cell experiments reported here, large quantities of active cells were required. To obtain such cells, batch cultures of V. parvula were grown anaerobically in 500 ml of the reduced lactate broth described in Part I, to which was added a further 100 ml of an overnight cell culture as inoculum. These cultures were then incubated for 3 hours at 31 C in one litre flasks fitted with a one-hole rubber stopper at which time the cells were in the exponential phase of growth. Rubber tubing in the stoppers permitted the evacuation and filling of the flasks at least four times with pure N_2 + 5% CO_2. As growth on lactate broth always resulted in the production of abundant gas, a de-pressurizing device was always connected to the stopper to prevent the shattering of the growth flask or loss of the stopper due to high internal positive pressure. However, growth was always established before the clamps on the connecting rubber tubing were removed. At this time, the cultures were generating gas within the growth flasks and the majority of this was permitted to escape. Better growth was always observed in flasks maintained with a slight positive pressure than flasks incubated under negative pressure.

Resting Cell Experiments.

Exponential phase cells were collected at 4 C by anaerobic centrifugation at 13,000 x g for 10-15 minutes in an LRA Lourdes regrigerated centrifuge. These cells were then washed once in de-gassed phosphate buffer (0.1 M, pH 6.5), containing 2.03×10^{-2} M $MgSO_4$ and 2.0×10^{-2} M 2- mercaptoethanol and finally suspended in the same buffer. The cell concentration was obtained by turbidity measurements in a Klett-Summerson colorimeter containing a red filter (640-700 mμ). A standard curve had been previously established for Klett units vs dry weight of cells with 2 mg dry weight of cells per ml routinely used in the reaction mixtures. All manipulations involving cells were carried out in a stream of nitrogen in order to maintain anaerobic conditions.

Experiments measuring the utilization of lactate were carried out in 25-50 ml Erlenmeyer flasks fitted with tight-fitting serum stoppers. The flasks were evacuated and gassed with nitrogen via a syringe needle 3-4 times after the cells and buffer had been added. After a 10-15 minute equilibration period, the substrate sodium lactate was added by syringe to start the reaction. One ml samples were withdrawn at various times by syringe and the reactions stopped by adding the sample either to 2 ml, 0.5 N NaOH or to 0.1 ml, 25% $ZnSO_4$ in plastic centrifuge tubes. Following neutralization to pH 7.0, the samples were

diluted to a known volume and the cells removed by centrifugation. The supernatant fractions were recovered and frozen until analysed for lactic acid.

A variety of other experiments were carried out to measure the evolution of $C^{14}O_2$ from radioactive lactate, the evolution of CO_2 and H_2 from unlabelled lactate, as well as the non-gaseous products of lactate fermentation. The details for these experiments will be dealt with under Analyses, although the method of handling the samples in these experiments was essentially the same as that described above.

Analyses.

Gas Analysis. When the gaseous products of lactate utilization were measured, washed cells or cell-free preparations were incubated anaerobically in one of two ways: (1) Tube Method - The measurement of radioactive carbon dioxide was carried out in 5 x 5/8 inch test tubes, with separate test tubes representing different intervals of time. Cells (4 mg dry weight) were incubated in pH 6.5 buffer with lactate-1-C^{14} (9.38×10^{-4} dpm/mmole) in a final volume of 2 ml. The tubes were fitted with a tight-fitting serum cap containing a small plastic rod to which was attached a 0.5 ml plastic cup inside the tube. The tubes were gassed with nitrogen, equilibrated at 37 C and the reactions started by the addition of the cells or extracts by syringe. The $C^{14}O_2$ evolved was

either absorbed during the reaction or was permitted to accumulate. In the former case, 0.2 ml, 1 M hyamine hydroxide was added to the plastic cup at the start of the experiment to absorb the CO_2 continually throughout the reaction period. In the latter situation, however, the hyamine-OH was not added until after the reaction had been stopped by addition of 0.2 ml of 4 N H_2SO_4 directly into the reaction mixture by syringe. In either case, a two hour equilibration period at 0 C always followed the acid addition to permit the complete absorption of all of the evolved carbon dioxide. Following this, the entire contents of the plastic cup were washed into a counting vial with 3 ml of methanol and 10 ml of scintillation solution composed of 1, 4-di(2, 5-phenyloxazolyl)-benzene (POPOP), 50 mg and 2, 5 diphenyloxazole (PPO), 4 mg in one litre of toluene. The radioactivity was measured in a two-channel Nuclear Chicago liquid scintillation counter and the counts corrected to 100% efficiency either by the channels-ratio method or by internal standardization. The addition of 3.0 ml methanol to the toluene-fluor solution also permitted the counting of aqueous radioactive samples, provided the sample volume was never more than 0.15 ml.

(2) Manometric Methods - The evolution of carbon dioxide and hydrogen by washed cells of V. parvula incubated in an atmosphere of N_2 was assayed manometrically by a slight modification of the

triple-side-arm technique of Hamilton, Burris and Wilson (1965), which permitted the determination of both gases in a single flask. In time-course experiments with this method, each flask represented a separate time interval. The main chamber of the flasks contained the substrate in phosphate buffer in a volume of 2.6 ml. These flasks had two side-arms (A) one was equipped with a double sac containing 0.2 ml, 4 N H_2SO_4 in one sac and 0.2 ml, freshly prepared 20% KOH in the other, (B) the second was a normal side-arm fitted with a tight-fitting serum stopper. The serum cap replaced the normal ground-glass stopper to permit the addition of cell or extract material to the flasks after the gassing procedure had been completed. This modification was necessary because V. parvula, being an obligate anaerobe and sensitive to oxygen, was found to be rapidly inactivated at room temperature during the gassing procedure.

The gassing procedure consisted of flushing the flasks with high purity nitrogen with the Brodie's solution raised in each manometer and with both side-arms of the flask open to the atmosphere. After flushing had proceeded in this fashion for one minute, the serum cap was fitted into the normal side-arm and the gas flow continued for 4 additional minutes, during which time the venting glass stoppers were inserted in the open position into the side-arms containing the double

sacs. At the end of this 5 minute flushing period, the venting stoppers were closed, the Brodie's solution lowered and the flasks equilibrated for 10-15 minutes in a shaking water-bath at 37 C.

The cells employed in these experiments were collected, washed and resuspended in cold buffer in an atmosphere of nitrogen at a high cell concentration (30 mg dry wt/ml) in a test tube fitted with a serum stopper. The tube was then stored in an ice bath until the flasks and manometers had been prepared, gassed and partially equilibrated. During the equilibration period, 0.2 ml of the cell suspension was added to the normal side-arm by syringe and the flasks further equilibrated until the manometer readings were stable.

To start the experiments, the cells were carefully added to the main chamber of the flasks after the initial reading on the manometers had been recorded (R_1). At zero time and at various time intervals thereafter, the reaction in the appropriate flask was stopped by the addition of the sulphuric acid to the main chamber from the twin side-arm (A), with care being taken not to disturb the KOH in the other sac. The concentration of acid was sufficient to reduce the pH in the main chamber to 1.0 to 2.0 thereby releasing all of the dissolved carbon dioxide. To permit the absorption of this carbon dioxide by the KOH, the flasks were equilibrated until no change in the manometers was

observed, at which time, the readings were recorded (R_2). Following this, the KOH in the double side-arm was added to the acidified contents of the main chamber and the flasks again equilibrated in the shaking water bath. As the concentration of H_2SO_4 was still high enough to maintain the pH below 3.0, the CO_2 previously absorbed by the KOH solution was released into the gas phase. The manometer readings (R_3) made after equilibrium had been reached represented both the hydrogen and carbon dioxide evolved by the cells during the reaction, in addition to any CO_2 dissolved in the buffer at the start of the experiment. Corrections for this latter CO_2 and any endogenous evolution of gas (E) were made at various times in each experiment from control flasks containing no sodium lactate. The amount of gas evolved at each sampling time was calculated as follows: (1) <u>hydrogen evolution</u> -

$$(R_2 - R_1) \times FC_{H_2(X)} = \text{Total } \mu l\ H_2 \qquad (8)$$

$$(E_2 - E_1) \times FC_{H_2(Y)} = \text{End. } \mu l\ H_2 \qquad (9)$$

$$(\text{Total} - \text{End.})\ \text{ul } H_2 = \Delta \mu l\ H_2\ (H_2) \qquad (10)$$

(2) <u>Carbon dioxide evolution</u> -

$$(R_3 - R_2) \times FC_{CO_2(X)} = \text{Total } \mu l\ CO_2 \qquad (11)$$

$$(E_3 - E_2) \times FC_{CO_2}(Y) = \text{End. } \mu l\ CO_2 \quad (12)$$

$$(\text{Total} - \text{End.})\ \mu l\ CO_2 = \Delta\ \mu l CO_2\ (CO_2) \quad (13)$$

where R_1 was the reading at zero time; R_2, the reading after the acid addition; R_3, the reading after the addition of the KOH (in flask (X), containing the substrate). E_1, E_2, E_3 were the corresponding readings in endogenous flask (Y) containing no substrate. $FC_{H_2}(X)$ and $FC_{CO_2}(X)$ are the flask constant for hydrogen and carbon dioxide in flask (X), while $FC_{H_2}(Y)$ and $FC_{CO_2}(Y)$ are the corresponding constants for the endogenous flask (Y).

The net production of gas at time t was obtained by subtracting the zero time values according to the following equations:

$$(H_2)_t - (H_2)_o = \text{net hydrogen evolution} \quad (14)$$

$$(CO_2)_t - (CO_2)_o = \text{net } CO_2 \text{ evolution} \quad (15)$$

Substrate Analysis. (1) Lactic acid was routinely assayed by a modification of the lactic dehydrogenase method of Cohen and Noell (1960). Each assay tube contained the following: 0.6 ml, 0.25 M glycine-NaOH buffer (pH 10.5); 0.1 ml, (10 mg/ml) nicotinamide adenine dinucleotide (NAD); 4 µl lactic dehydrogenase (360 units/mg - Boehringer Mannheim Corp.); 0.2 ml of sample containing 0 - 10 µg lactic acid. Zn lactate standards were included in each assay. All tubes

were covered with parafilm and incubated at room temperature for 2 hours before being read at 366 mμ in a Unicam Sp 500 spectrophotometer. The colorimeter method of Barker and Summerson (1941) was unsuitable for this work because propionic acid, which interferes with this assay, was usually present in these samples.

(2) Pyruvic acid was assayed colorimetrically by the modified 2, 4-dinitrophenylhydrazine method of Hamilton, Burris and Wilson (1965) and enzymatically with lactic dehydrogenase. In the latter method, the oxidation of NADH to NAD by lactic dehydrogenase in the presence of pyruvate was carried out in 0.05 M phosphate buffer (pH 7.5). Each assay reaction contained 0.6 ml buffer, 70 μg NADH, 10 μg lactic dehydrogenase and 0.2 ml sample containing 0 - 10 μg pyruvate. The reaction was over in 30 minutes at room temperature, at which time, the remaining NADH was measured at 340 mμ. Standard potassium pyruvate was measured in each assay.

(3) Oxaloacetic acid was measured either by the 2, 4-dinitrophenylhydrazine method or enzymatically with malic dehydrogenase (Hohorst and Reim, 1965). In this latter method, the conversion of oxaloacetate to malate is accompanied by the oxidation of NADH to NAD in the presence of malic dehydrogenase. Each assay tube contained 0.2 ml sample (0 - 10 μg oxaloacetate) and 0.8 ml of buffer-NADH-enzyme solution

consisting of NADH, 60 μg and 0.05 μg malic dehydrogenase (720 units/mg - Boehringer Mannheim Corp.) in 0.05 M phosphate or Tris buffer (pH 7.5). The reaction mixtures were incubated for 30 minutes at room temperature and the remaining NADH measured at 340 mμ. Freshly prepared sodium oxaloacetate standards were included in each assay.

Oxaloacetate was also assayed by the direct and rapid spectrophotometric method of Allen (1966), which utilizes the absorbance of oxaloacetate at 258 mμ.

Product Analysis.

The non-gaseous end-products resulting from the fermentation of sodium lactate and other substrates by cells and extract preparation of V. parvula were identified by partition chromatography on silicic acid columns by a modification of the method of Bulen, Varner and Burell (1952). All of the acidic end-products from these fermentations could be separated by employing a 0-15% butanol-in-chloroform gradient (all solvents were saturated with 0.5 N H_2SO_4). The acidified 15% butanol-in-chloro-form was added to a 250 ml mixing-flask containing 100 ml of acid-saturated chloroform agitated by a magnetic stirring bar. Solvent addition was made through acidflex tubing (1/16 inch inside diameter - Technicon, Inc.) by a Buchler peristaltic pump at a rate of 0.5 ml per minute. The contents of the mixing flask were then added to a glass cylinder containing a 1.3 x 20 column of 100 mesh silicic acid via 1/16 inch diameter polyethylene

tubing. The preparation of this column was similar to that described by Hamilton, Burris and Wilson (1965). Only samples obtained after $ZnSO_4$ precipitation were used for partition chromatographic analysis. Standard acids were run under the same conditions and their position in the eluent used as reference for the identification of the end-products.

Three ml fractions were collected in an automatic fraction collector and the acid content of each fraction determined as follows: one ml of each fraction was either added to 5 ml of degassed, deionized water and titrated to the phenolphthalein end-point with standard NaOH, or counted by liquid scintillation counting, or both, depending on whether a radioactive substrate was used in the experiment. For liquid scintillation counting, one ml samples were added to 10 ml of the fluor-toluene solution and counted as previously described. As the chloroform-butanol mixtures caused considerable quenching in this system, the counting efficiency was always low and varied from sample to sample. For this reason, all end-product-containing samples were recounted with a toluene-C^{14} internal standard in order to determine the absolute radioactivity.

Preparation of Lactate-U-C^{14}.

Lactate-U-C^{14} was prepared by two methods: (1) the homofermentative oral microbe, Streptococcus salivarius, was used to convert

glucose-U-C^{14} to sodium lactate-U-C^{14}. Cells, grown on 0.1% glucose, were harvested as previously described and incubated in 0.05 M (pH 7.0) phosphate buffer (Hamilton, 1968) with 40 mM high specific activity glucose-U-C^{14} at a cell concentration of 4.0 mg dry weight per ml of reaction mixture; the total volume of the reaction mixture was 15 ml. The degradation of the glucose-U-C^{14} was carried out for 30 minutes at 37 C and the reaction stopped by the addition of 1.5 ml, 25% $ZnSO_4$ to precipitate the cells. After neutralization to pH 7.0 with NaOH, the contents of the reaction flask were rinsed into a centrifuge tube and the supernatant retained after centrifugation at 13,000 x g at 4 C for 15 minutes. Preliminary analysis showed that the labelled glucose had been almost completely metabolized, primarily to lactic acid. The lactate-U-C^{14} in the supernatant solution was partially purified and concentrated by passage through a 8 cm Dowex-1 (Cl^- form) column with an internal diameter of 1 cm. After sample addition, the column was first washed with deionized water to remove any non-utilized glucose-U-C^{14}, and then the lactate-U-C^{14} and other minor labelled metabolic acids were eluted from the column by the addition of 3 ml, 6 N HCl. This acid fraction was neutralized, streaked on washed 10 x 12 inch Whatman No. 1 sheets and the chromatograms run in a solvent system of ethyl acetate-acetic acid-H_2O) (3-1-1) in the

ascending direction with commercial lactate-1-C^{14} used as a standard. The position of the lactate-U-C^{14} and the standard were located by scanning in a Packard Radiochromatogram Scanner and the lactate-U-C^{14} band cut from the paper and eluted with water at 4 C. After the removal of paper fibers by centrifugation, the resulting solution was neutralized and concentrated by freeze-drying. The dried material obtained from this procedure was dissolved in phosphate buffer (0.1 M, pH 6.5), the resulting lactate-U-C^{14} calibrated to determine its specific activity and the solution stored at -10 C until required.

(2) Lactate-U-C^{14} was also prepared enzymatically from sodium pyruvate-U-C^{14} by the method of Krebs et al (1966). In this method, sodium pyruvate-U-C^{14} was reduced to lactate-U-C^{14} in the presence of lactate dehydrogenase and NADH. The reaction was carried out in 0.02 M phosphate buffer at pH 8.0.

RESULTS

Lactate metabolism was first studied with washed, whole cells of V. parvula incubated in nitrogen-free phosphate buffer. These resting cell conditions permitted only catabolic reactions to proceed as synthesis was eliminated in the absence of nitrogenous precursors.

Optimum Conditions. The optimum conditions for the metabolism of

lactate by V. parvula were established as a basis for future experiments on the characteristics and properties of this system. The optimum pH for lactate utilization by washed, whole cells of V. parvula was 6.5, with the rate of degradation markedly decreased on either side of this optimum (Figure 2 (A)). As with the growth of V. parvula in lactate broth, the metabolism of lactate by resting cells did not result in a change in the pH of the incubation medium during degradation (Figure 2(B)).

Not only was the hydrogen ion concentration important to metabolic activity and cell stability, but also the concentration of the phosphate salts used in the V. parvula was optimum at a concentration of 0.1 M (Table 4), with marked inhibition at the 0.2 M level. Furthermore, the addition of salts and a reducing agent to this buffer was also shown to influence the rate of lactate degradation. Table 5 illustrates the influence on the rate of lactate utilization by washed cells of various combinations of $MgSO_4$, $MnCl_2$, $NaHCO_3$, $FeSO_4$, and 2-mercaptoethanol when added to 0.1 M phosphate buffer. These compounds were selected from those employed by McCormick, Ordal and Whiteley (1962) during the study of pyruvate degradation by M. lactilyticus (V. alcalescens). From these combinations, a phosphate buffer (0.1 M, pH 6.5), containing 2×10^{-3} M, $MnCl_2$ or 2×10^{-2} M, $MgSO_4$ and 2-mercaptoethanol at 2×10^{-2} M,

Figure 2

Effect of pH on lactate metabolism by resting cells of *V. parvula*

A. Lactate utilization.

Washed resting cells (2 mg/ml) obtained from a 1% lactate broth culture were incubated anaerobically at different pH values in tubes containing 20 mM sodium lactate. Potassium acid phthalate buffer was used between pH 4.0 and 5.5, phosphate buffer from pH 6.0 to 7.5 and Tris buffer at pH 8.0. The phthalate and Tris buffers received an amount of phosphate equivalent to that in the phosphate buffer. The reaction was stopped after 40 and 80 minutes at 37 C in an atmosphere of N_2 + 5% CO_2 by the addition of 2 ml, 0.5 N NaOH to the tubes. Only the 80 min values appear in this Figure.

B. pH during metabolism.

The hydrogen ion concentration in each tube was measured just prior to the addition of the NaOH to the 40 and 80 min tubes. Only the pH 6.5 values are given as they were typical of the reactions at other pH values.

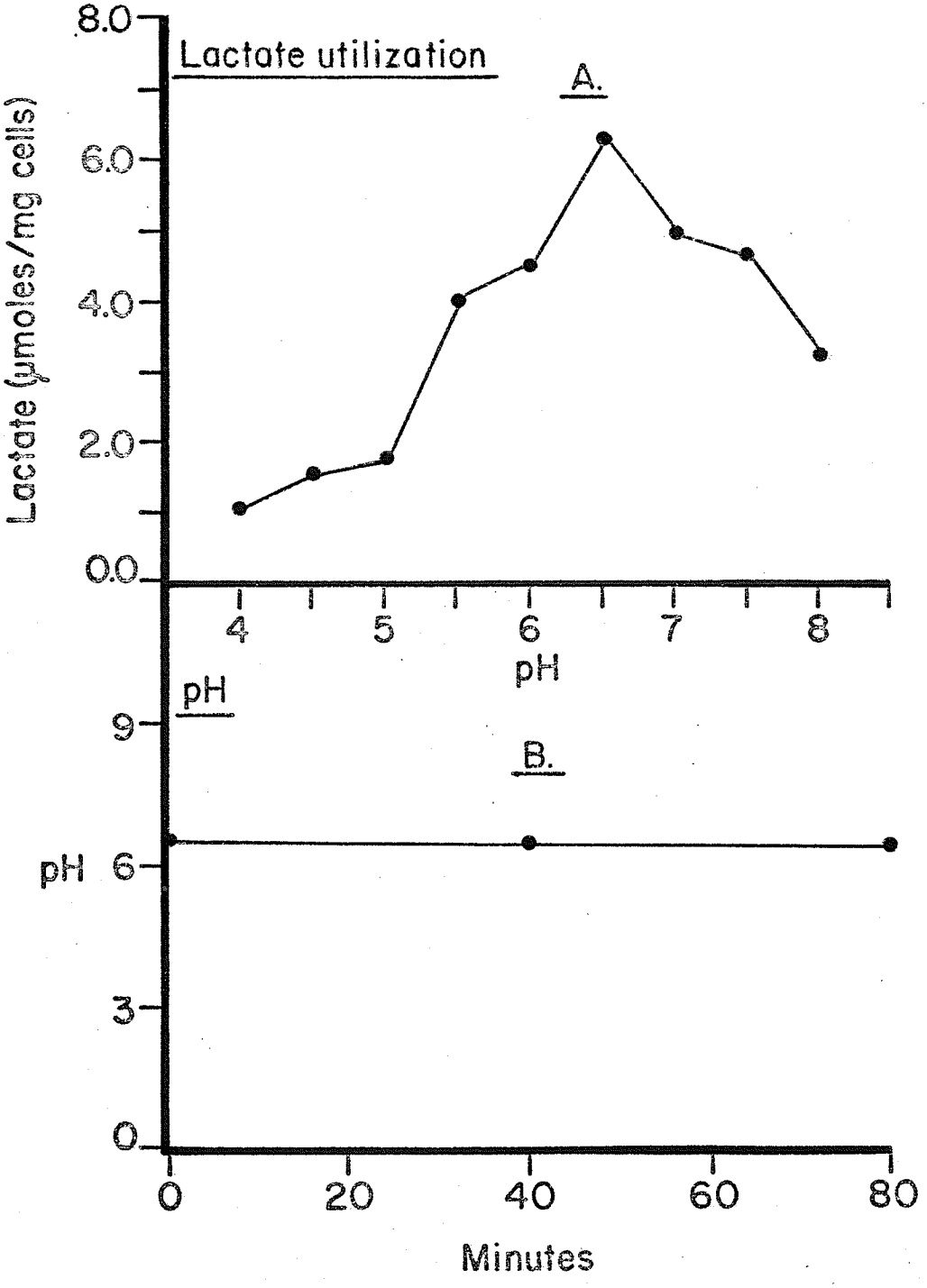

Lactate utilization
A.
Lactate (μmoles/mg cells)
8.0
6.0
4.0
2.0
0.0
4
5
6
7
8
pH
pH
9
B.
pH 6
3
0
0
20
40
60
80
Minutes

Table 4

The influence of phosphate buffer concentration on lactate utilization by washed cells of V. parvula

Phosphate concentration (x 10^{-3})	Lactate utilization
1	66.00*
5	66.30
10	69.00
50	75.00
100	78.00
200	61.50

* µmoles sodium lactate utilized /mg dry wt cells/hr.

Cells (2 mg dry wt/ml) were incubated with 30 mM sodium lacate in phosphate buffer (pH 7.0) at various concentrations in a final volume of one ml. Duplicate tubes at each concentration were incubated in an atmosphere of nitrogen for 0, 10, 20 and 40 minutes. The reactions were stopped by the addition of 0.1 ml, 12 N H_2SO_4 to each tube, the samples neutralized and the remaining lactate analyzed as described in Methods.

Table 5

The rate of anaerobic lactate utilization by washed cells of V. parvula in 0.1 M phosphate buffer with the addition of various salts and a reducing agent.

Components (x 10^{-3} M)					Lactate utilization
$MgSO_4$	$MnCl_2$	$NaHCO_3$	$FeSO_4$	MSH*	
8.1	--	--	--	--	98.40[t]
20.2	--	---	--	--	99.90
30.4	--	--	--	--	91.80
38.5	--	--	--	--	90.60
20.2	2.0	--	--	--	93.60
20.2	2.0	1.5	--	--	74.70
20.2	2.0	1.5	0.2	20.0	99.90
20.2	--	1.5	0.2	--	83.70
20.2	--	--	0.2	20.0	90.30
20.2	--	--	--	20.0	102.00
--	1.0	--	--	--	83.10
--	2.0	--	--	--	87.60
--	3.0	--	--	--	100.20
--	4.0	--	--	--	101.10
--	2.0	--	--	20.0	101.10
--	2.0	1.5	--	20.0	102.00
--	2.0	--	0.2	20.0	91.80
--	--	--	0.2	--	99.90

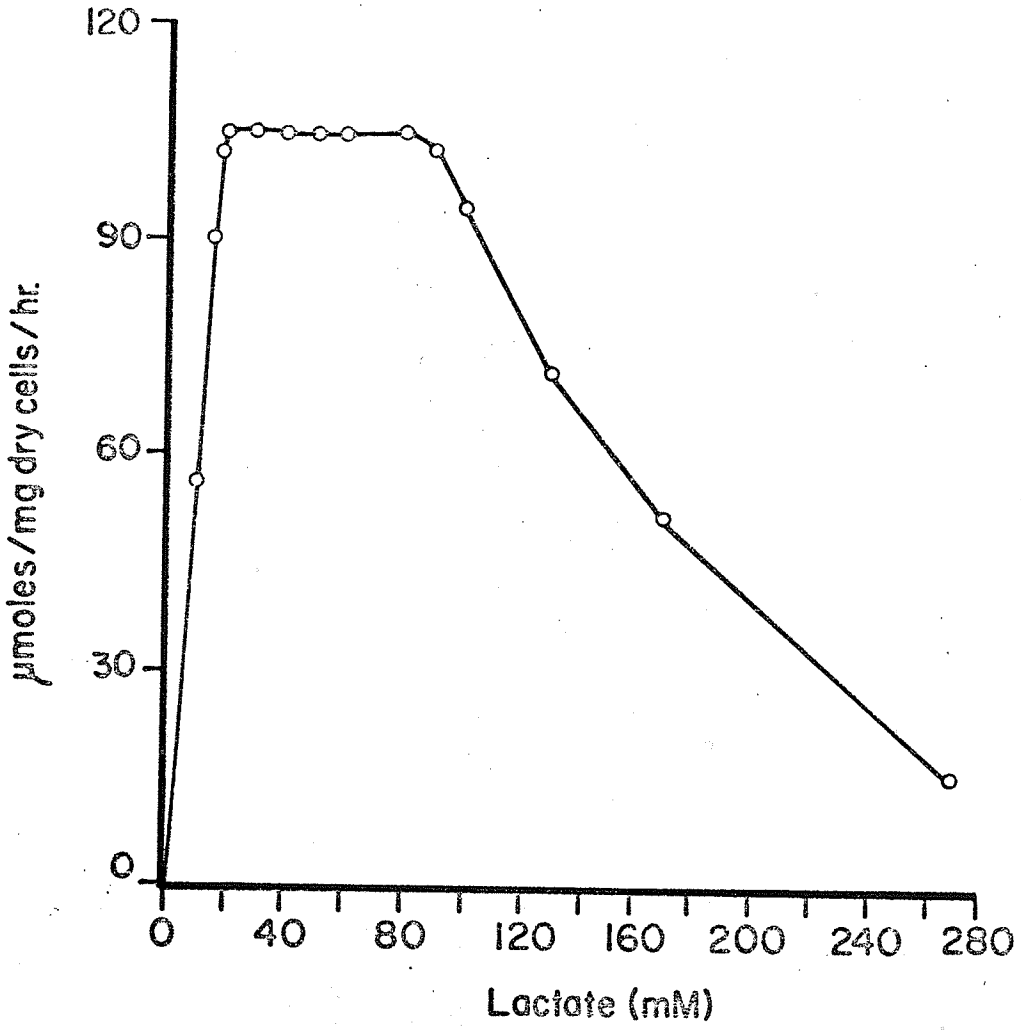
* MSH = 2-mercaptoethanol
t - μmoles sodium lactate utilized /mg dry wt cells /hour.

The conditions in this experiment are essentially similar to those described in Table 3.

Figure 3

Effect of substrate concentration on the rate of lactate utilization by washed cells of V. parvula.

Resting cells of V. parvula were harvested as previously described and incubated at 2 mg dry wt/ml in flasks containing 0.1 M, pH 6.5 phosphate buffer with various concentrations of sodium lactate at 37 C in an atmosphere of 100% N_2. The final volume of the incubation mixture was 10 ml in each flask. One ml samples were taken at 0, 5, 10, 15, 20, 30, 40 & 60 minutes and the remaining lactate analyzed. Initial rates were calculated from the initial linear portion of the lactate utilization vs time curve.


120
90
60
30
0
μmoles/mg dry cells/hr.
0
40
80
120
160
200
240
280
Lactate (mM)

concentration was selected as the incubation medium. However, as concentrated solutions of $MgCl_2$ tended to crystalize readily upon storage at room temperature, and also caused the formation of a precipitate in phosphate buffer at low temperature, $MgSO_4$ was employed in all experiments. Sodium thioglycolate was used as the reducing agent in lactate broth or agar medium because 2-mercaptoethanol was unsuitable for autoclaving.

Utilizing these optimum conditions (0.1 M, pH 6.5 phosphate buffer), the effect of lactate concentration on the rate of utilization at constant cell concentrations was determined. This was carried out under zero order conditions by incubating washed cells of V. parvula with sodium lactate in an atmosphere of nitrogen. As shown in Figure 3, increased concentrations of lactate produced three effects on the rate of utilization. In the region extending from 0 to 15 mM sodium lactate, the rates were proportional to substrate concentration (first order kinetics) while in the region extending from 15 to 80 mM, the rate of utilization was maximum at 102.0 μmoles/mg dry wt of cells/hour (zero order kinetics). Above 80 mM, severe and progressive substrate inhibition was observed up to the highest concentration tested, 270 mM sodium lactate. As concentrations above 80 mM showed substrate inibition, sodium lactate was employed at concentrations between 15 and 80 mM in all succeeding experiments.

Products of lactate metabolism.

V. alcalescens has been shown to produce propionic and acetic acids, carbon dioxide and hydrogen as a result of lactate fermentation (Foubert and Douglas, 1948 b; and Johns, a, b). As the literature contains little or no information on lactate metabolism by V. parvula, studies were undertaken to examine the products of lactate fermentation with the strain isolated in this work.

Gaseous Products: The measurement of the gaseous products evolved during the metabolism of lactate by V. parvula encountered difficulties when conventional manometric methods were used. Three methods of analysis were attempted: (1) the indirect method (Umbreit, Burris and Stauffer, 1964), in which carbon dioxide and hydrogen were measured in separate flasks, was unsuitable. Measurement of gas production by this method was inconsistent and the carbon dioxide evolved did not compare with the evolution of $C^{14}O_2$ by washed cells of V. parvula incubated under the same conditions with lactate-C^{14}. (2) The triple side-arm Warburg method described by Hamilton, Burris, and Wilson, (1965) was also unsatisfactory, although by this technique, the gaseous end-products were produced in a single flask under the same conditions. In this method, exposure of the cells to oxygen during the gassing procedures, rapidly reduced cell activity thereby producing variable results. (3) By modifying the triple side-arm Warburg technique to permit the addition of the cells by syringe to the flasks after the gassing procedure, the previous problems were overcome. The measurement of carbon dioxide

evolution by this method gave consistent results which were in good agreement with the evolution of radioactive CO_2 from lactate-1-C^{14} determined by the Tube Method (Table 6).

The stoichiometry of carbon dioxide and hydrogen evolution in relationship to lactate utilization is shown in Figure 4 (A). The evolution of gas ceased when the substrate lactate was completely utilized and the amount of CO_2 produced was much greater than the amount of hydrogen formed.

Acidic Products. The non-gaseous end products formed from the metabolism of sodium lactate by resting cells of V. parvula (Figure 4 (B)) were acetic and propionic acids, with the latter acid predominating. All products of the lactate fermentation increased until lactate became exhausted except for the production of acetate which reached a plateau approximately five minutes before the substrate in the medium was completely exhausted. Negligible quantities of lactate were incorporated into cellular materials under the conditions of this experiment.

The complete fermentation balance for the utilization of sodium lactate by washed cells of V. parvula is given in Table 7 along with the results obtained by Foubert and Douglas (1948) and Johns (1951) with V. alcalescens for comparison.

Effect of CO_2 on lactate metabolism. Johns (1951) reported that V. gazogenes would not ferment lactate in the absence of carbon dioxide. In the presence of carbon dioxide or bicarbonate, however, lactate was utilized with the production of acetate and propionate.

Table 6

Carbon dioxide evolution from sodium lactate or sodium lactate-U-C^{14} by resting cells of V. parvula as measured by different methods.

Methods	Type	μmoles CO_2 evolved per 100 μmoles lactate
MANOMETRY	Indirect Method (Umbreit, Burris, Stauffer 1964)	0
	Triple-side-arm Technique (Hamilton, Burris, Wilson, 1965)	10.2 - 15.8
	Modified triple-side-arm technique	38.3
$C^{14}O_2$ counting	Tube method	41.5

Cells were incubated in pH 6.5 phosphate buffer in an atmosphere of N_2 at 37 C as previously described.

Figure 4

Products of lactate metabolism by washed, resting cells of *V. parvula* incubated in a nitrogen atmosphere.

Cells, at concentration of 2 mg/ml, were incubated in 0.1 M phosphate buffer, pH 6.5 with 30 mM sodium lactate-U-C^{14} in an atmosphere of N_2 at 37 C according to the modified triple-side-arm technique as described in Methods.

A. Sodium lactate utilization and gas production.

B. Acid end-products of lactate metabolism analysed by silicic acid column chromatography.

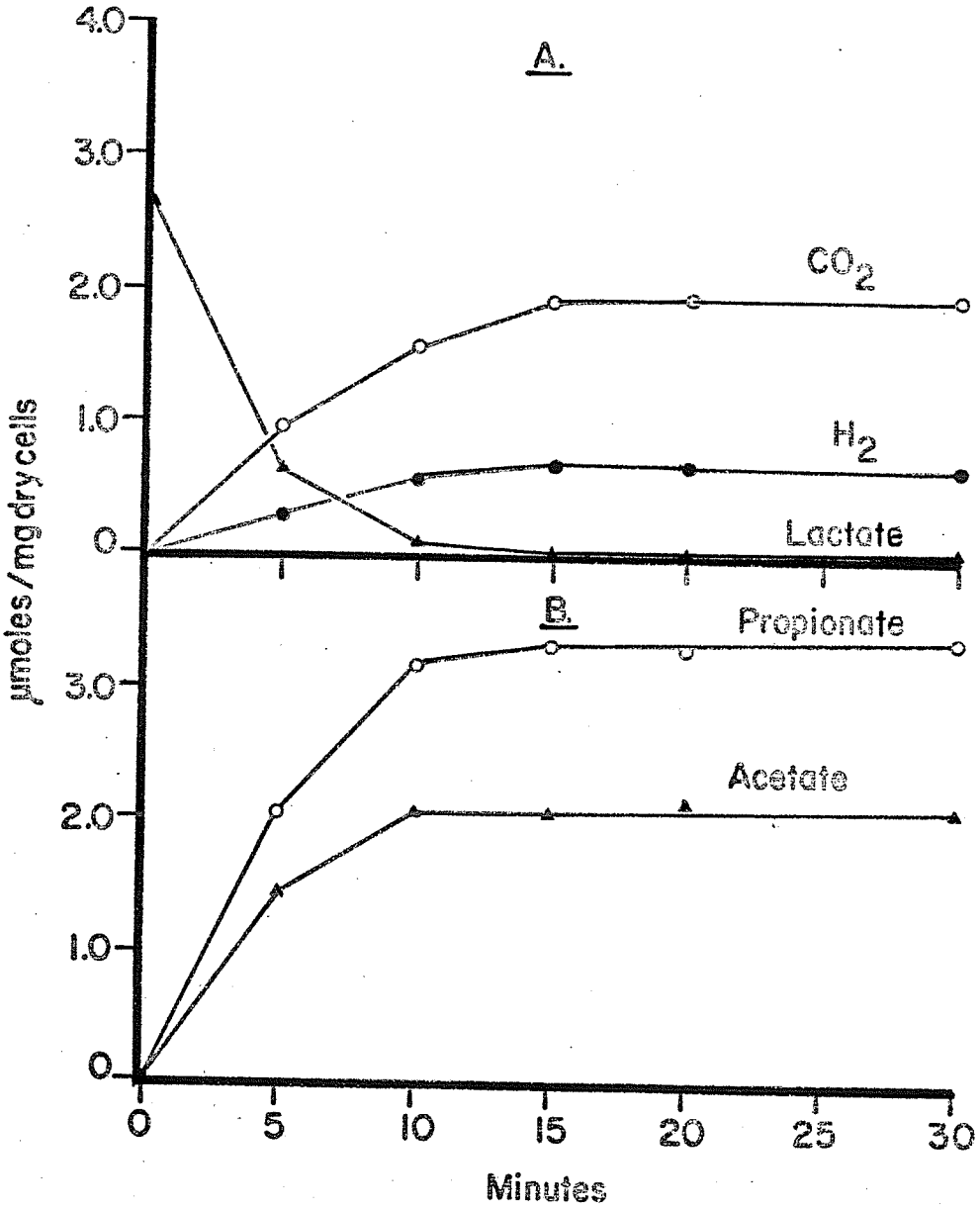

A.
B.
CO_2
H_2
Lactate
Propionate
Acetate
μmoles / mg dry cells
Minutes
4.0
3.0
2.0
1.0
0
0
5
10
15
20
25
30

Table 7

The products of lactate fermentation obtained by Foubert and Douglas (1948) and Johns (1951) with V. alcalescens compared to those obtained with V. parvula in this study.

Product	mmoles/100 mmoles sodium lactate fermented		
	Veillonella alcalescens		Veillonella parvula
	Foubert-Douglas (a)	Johns (b)	
Propionate	63.7	58.5	66.0
Acetate	39.5	48.7	39.6
Carbon Dioxide	39.1	35.2	38.3
Hydrogen	14.2	14.8	13.6
Carbon Recovery	103.6	102.0	105.0
$\frac{O}{R}$ * Ratio	0.9	1.04	0.96

(a) Foubert, E.L. Jr. and H. C. Douglas; J. Bacteriol. 56, 35-36 (1948).

(b) Johns, A. T.; J. Gen. Microbiol. 5, 317-325 (1951).

* $\frac{\text{Oxidation}}{\text{Reduction}}$ Ratio.

As the production of propionate required the presence of CO_2 in the medium, Johns concluded that carbon dioxide was required for the synthesis of this acid.

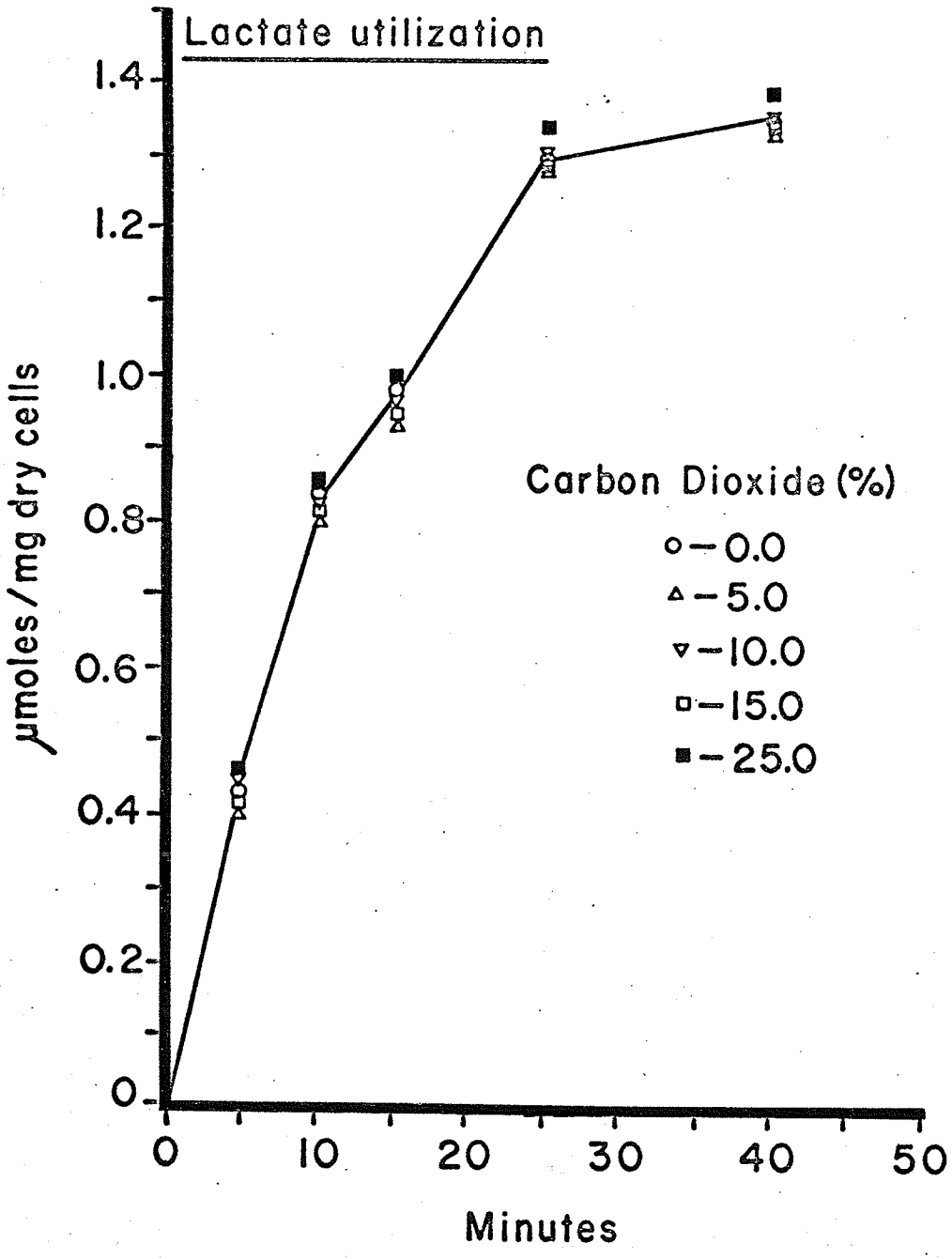
Carbon dioxide does not appear to play a similar role in lactate metabolism by the V. parvula strain isolated in this study. This organism grew and metabolized lactate readily in a nitrogen atmosphere free of carbon dioxide, suggesting that some aspects of its metabolism differ from that of V. alcalescens. To further examine this difference, lactate metabolism by cells of V. parvula was carried out at varying partial pressures of CO_2 in an atmosphere of nitrogen. Washed, resting cells were incubated with 30 mM sodium lactate in flasks which contained 0, 5, 10 15, and 25% of carbon dioxide in an atmosphere of nitrogen. The appropriate concentration of sodium bicarbonate was added to the buffer in each flask to ensure that the pH of the reaction mixture was near pH 6.5 during the incubation period. As shown in Figure 5, increasing the partial pressure of carbon dioxide up to a level of 25% did not significantly alter the rate of lactate utilization indicating that lactate metabolism by this organism was not CO_2-dependent.

This was confirmed in an experiment where cells were incubated with lactate-U-C^{14} at high concentrations of atmospheric carbon dioxide. In this experiment, the distribution of radioactivity into cellular components and into evolved carbon dioxide was followed in addition to the utilization of the substrate, lactate-U-C^{14}.

Figure 5

Influence of increased concentrations of carbon dioxide on the rate of lactate utilization by resting cells of *V. parvula.*

Cells, at a concentration of 2 mg/ml, were incubated in 25 ml Erlenmeyer flasks sealed with serum stoppers with 30 mM sodium lactate in pH 6.5, 0.1 M phosphate buffer in a final volume of 10 ml. The flasks were gassed by a special gassing device such that the final composition of gas phase varied from 0 to 25% CO_2 in nitrogen as indicated.


Lactate utilization
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
µmoles / mg dry cells
0
10
20
30
40
50
Minutes
Carbon Dioxide (%)
o—0.0
Δ—5.0
▽—10.0
□—15.0
■—25.0

As shown in Figure 6 (A), an atmosphere of 25 or 50% carbon dioxide did not affect the rate of lactate utilization by resting cells of V. parvula when compared to control cells incubated in an atmosphere of N_2. However, the presence of this gas did influence the products of metabolism as increased levels of atmospheric carbon dioxide increased the evolution of radioactive CO_2 from lactate-U-C^{14} under conditions where the $C^{14}O_2$ was allowed to accumulate for varying periods of time. (Figure 6 (B)). In the tubes containing 50% carbon dioxide the evolution of $C^{14}O_2$ reached a plateau before lactate was exhausted, while at 25% atmospheric CO_2 and in the control, $C^{14}O_2$ continued to be evolved.

In addition to carbon dioxide evolution, a small but significant amount of C^{14} was incorporated into cellular material with the quantity assimilated increasing as the partial pressure of carbon dioxide in the atmosphere was increased (Figure 6 (C)). Although substrate sodium lactate -U-C^{14} was still present, incorporation of radioactivity in both the 25 and 50% tubes reached a plateau after 12 to 15 minutes while that in nitrogen showed a steady increase with time.

Carbon dioxide was also shown to influence the concentration of non-gaseous end-products formed as a result of lactate metabolism. A comparison of the acidic end-products formed in flasks in which the CO_2 evolved during lactate metabolism was allowed to accumulate (-KOH), and in flasks where the CO_2 was absorbed by KOH demonstrates this point (Table 8). In the flasks without KOH,

Figure 6

Effect of atmospheric carbon dioxide on the metabolism of lactate-U-C^{14} by resting cells of V. parvula.

Employing gas phases of N_2, 25% CO_2 + 75% N_2 and 50% CO_2 + 50% N_2, cells (2 mg dry wt/ml) were incubated in test tubes with 30 mM sodium lactate-U-C^{14} (17.91×10^5 dpm/mmole). The total volume was 1.0 ml with each tube representing a separate time inteval. The tybes were closed with serum caps and the tubes evacuated and filled with high purity carbon dioxide and nitrogen five times via a syringe needle. $C^{14}O_2$ analysis was according to the Tube Method. Cellular radioactivity was analyzed by washing the cells at each sample time free of exogenous radioactivity three times with buffer and finally with deionized water. These washed cells were then plated on cupped planchets and counted in a low background Nuclear Chicago Geiger counter.

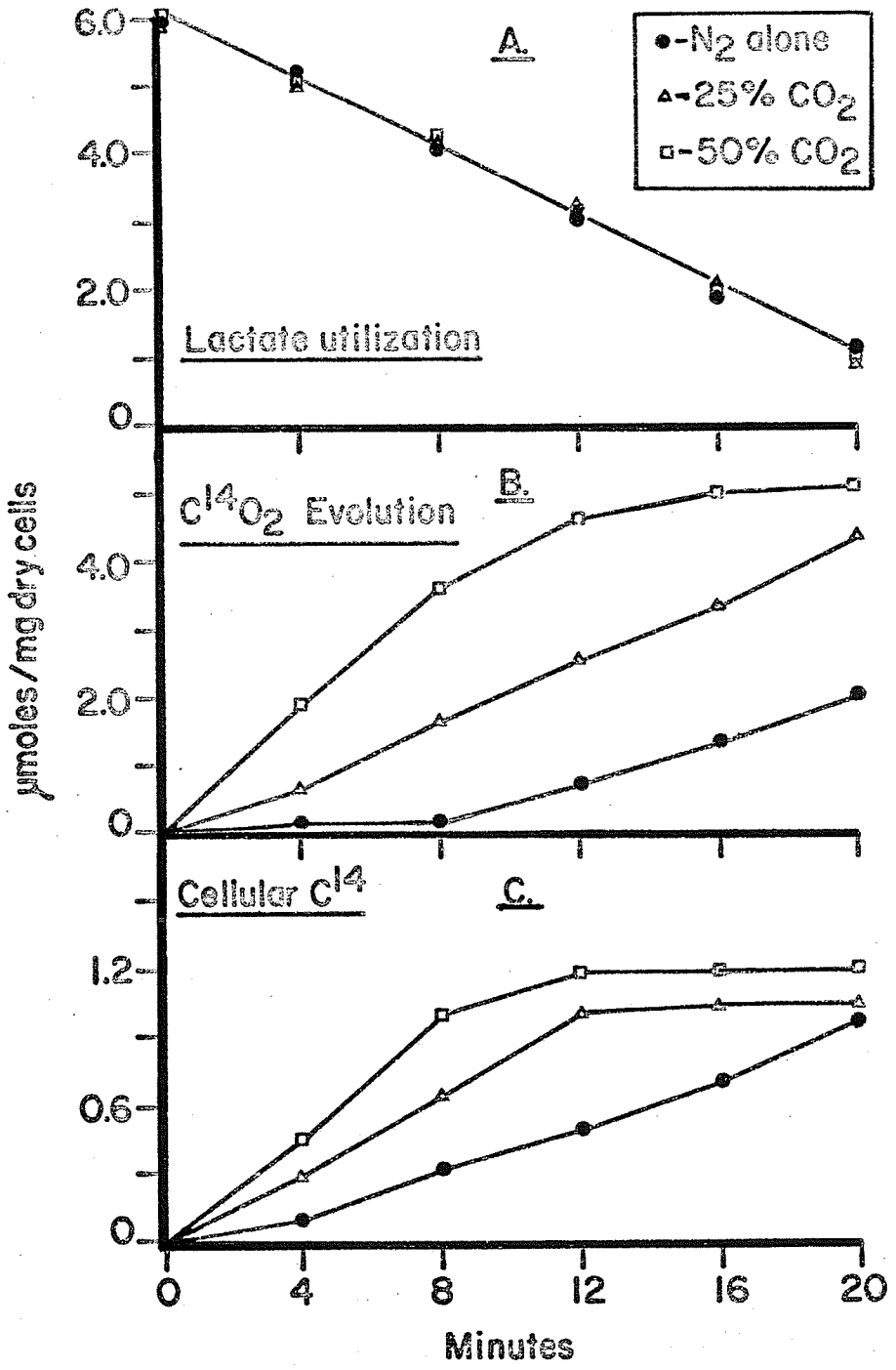

A.
●-N_2 alone
△-25% CO_2
□-50% CO_2
Lactate utilization
B.
$C^{14}O_2$ Evolution
C.
Cellular C^{14}
μmoles/mg dry cells
6.0
4.0
2.0
0
4.0
2.0
0
1.2
0.6
0
0
4
8
12
16
20
Minutes

Table 8

Production of non-gaseous end-products of lactate metabolism by resting cells of V. parvula in the presence and absence of carbon dioxide.

	mmoles/100 mmoles lactate fermented	
Product	-KOH	+KOH
Propionate (P)	64.4	52.6
Acetate (A)	37.4	45.8
$\frac{P}{A}$ Ratio	1.72	1.15

Cells, 2 mg dry wt/ml, were incubated in an atmosphere of N_2 at 31 C. with 30 mM sodium lactate-U-C^{14} in Warburg Flasks with and without KOH. The contents of the flasks were collected as described in Methods and the acid end-products analyzed by column chromatography.

propionate formation was much higher than that in the flasks containing KOH, while the acetate production, on the other hand, was slightly higher in the flasks with KOH. The propionate/acetate (P/A) ratio increased from 1.15 to 1.72 under conditions which permitted the accumulation of carbon dioxide. Propionate formation, therefore, increased at the expense of acetate production.

These results were confirmed by analyzing the quantity and radioactive content of the propionate and acetate formed in the experiment described in Figure 6, where washed cells of V. parvula were incubated with lactate-U-C^{14} at high levels of atmospheric carbon dioxide. As shown in Table 9, the total propionate concentration increased as the partial pressure of atmospheric CO_2 increased from 0 to 50%. At the same time, however, the specific activity of propionate at each carbon dioxide level decreased markedly. On the other hand, the concentration and specific activity of acetate at each level of CO_2 remained almost constant. As a result, the P/A ratio increased significantly as the atmospheric CO_2 concentration increased, such that at the 50% level the P/A ratio (1.66) was close to that obtained under conditions where CO_2 was permitted to accumulate during the incubation period (Table 8, (-KOH)). As both the absolute quantity and the specific activity of propionate were markedly affected by the amount

Table 9

Influence of increasing partial pressures of unlabelled atmospheric carbon dioxide on the metabolism of lactate-U-C^{14} by resting cells of V. parvula.

Percent Carbon Dioxide	Propionate (P)		Acetate (A)		P/A
	mmoles*	Specific Activity (dpm/mmole)	mmoles	Specific Activity (dpm/mmole)	
		(x 10^5)		(x 10^5)	
0	64.5	3.29	45.3	1.78	1.42
25	67.5	2.83	46.2	1.74	1.42
50	76.5	2.35	46.2	1.72	1.66

*mmoles/100 mmoles lactate fermented

Experimental conditions were described in Figure 6. Reaction mixtures were recovered, neutralized and analyzed as described in Methods.

of unlabelled carbon dioxide in the atmosphere, carbon fixation appears to be involved in the production of propionic acid during lactate metabolism by V. parvula.

Non-lactate Metabolism.

As reported by Johns(1951 b), M. lactilyticus (V. alcalescens), in addition to metabolizing lactate, also fermented pyruvate, oxaloacetate, malate, and succinate with the production of abundant gas. As a comparison with V. alcalescens, lactate-grown, resting cells of V. parvula were incubated with these substrates in time course experiments and their rates of degradation determined. As shown in Figure 7, lactate (A), pyruvate (B) and oxaloacetate (C) were readily fermented by these cells in a nitrogen atmoshpere at rates of 108.0, 69.6 and 48.0 μmoles/mg cells/hr, respectively. However, when such cells were incubated with both lactate and oxaloacetate together (D), the rate of utilization for each substrate was markedly decreased. The rates, 66.0 μmoles/mg cells/hr for lactate and 26.4 μmoles/mg cells/hr for oxaloacetate, were almost exactly half the rate obtained when the cells were incubated with each substrate alone.

Partition chromatographic analyses of the samples from the above experiments have shown that propionic and acetic acids were the principle end-products from the fermentation of all the substrates

Figure 7

Metabolism of lactate, oxaloacetate and pyruvate by resting cells of V. parvula in an atmosphere of nitrogen.

Resting cells of V. parvula (2.0 mg dry weight/ml) were injected by syringe into equilibrated 50 ml Erlenmeyer flasks, fitted with serum stoppers and gassed with high purity nitrogen. The flask contained the following substrates such that the final concentration for each was 10 mM in a final volume of 10 ml.

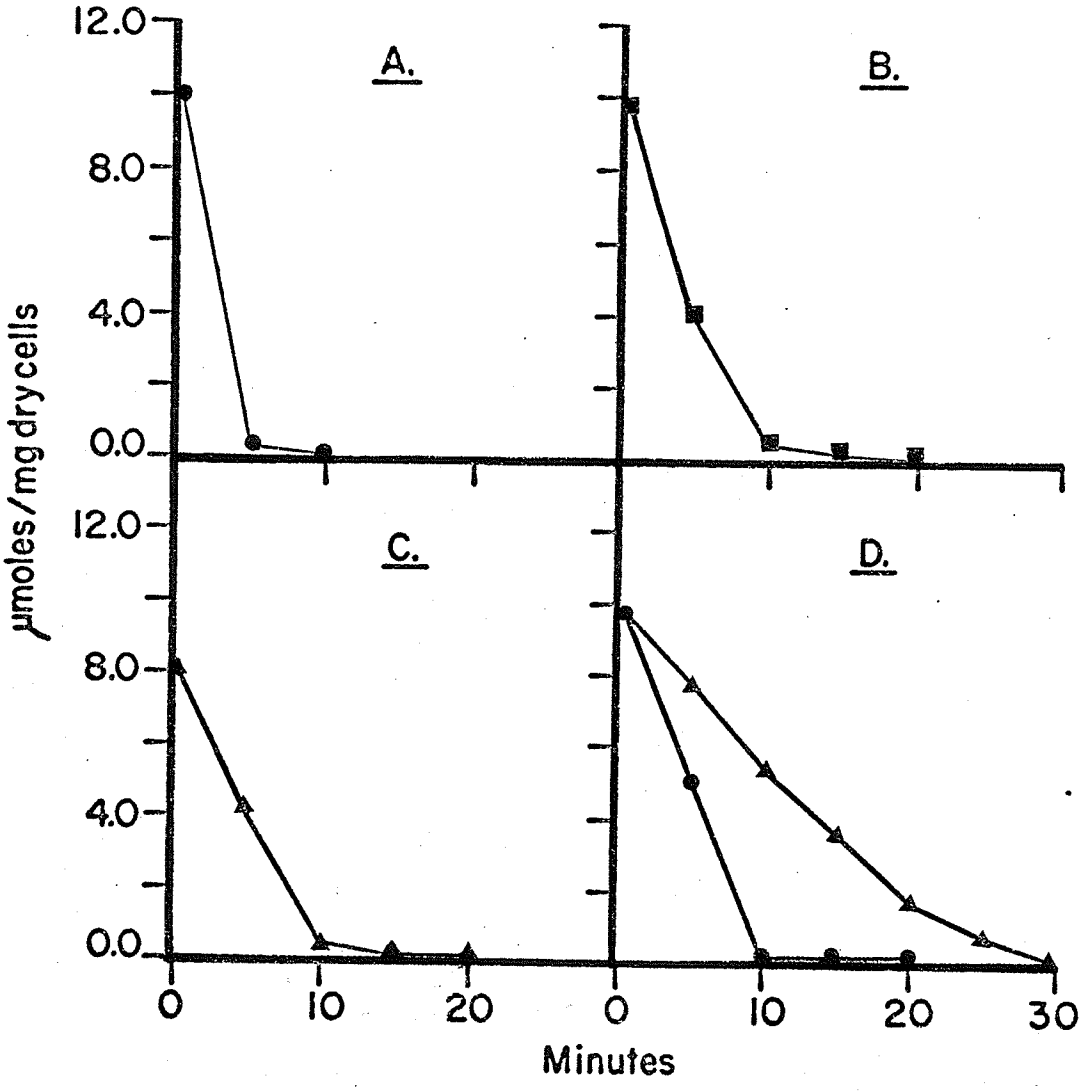
Flask A Sodium lactate-U-C^{14} (specific activity 17.91×10^5 dpm/mmole)

Flask B Potassium pyruvate

Flask C Sodium oxaloacetate

Flask D Sodium lactate-U-C^{14} and sodium oxaloacetate

At the time the cells were added (zero time) and at regular intervals thereafter, 1.0 ml samples were removed by syringe and the reaction stopped with $ZnSO_4$. The samples were then neutralized, made to volume, centrifuged and the supernatant solution analyzed for the substrate remaining by procedures described in Methods.


12.0
8.0
4.0
0.0
A.
B.
C.
D.
μmoles/mg dry cells
0
10
20
30
Minutes

(Table 10). The fermentation of lactate produced the greatest amount of propionic acid (68.7 mmoles/100 mmoles of lactate) with much less from either pyruvate (34.8 mmoles) or oxaloacetate (29.0 mmoles). Only a slight decrease in the production of propionate was observed in the flask (D) containing both lactate-U-C^{14} and oxaloacetate as the substrates (62.6 mmoles). However, while the absolute amount of propionic acid was almost the same, the specific activity of the propionic acid formed in the latter case decreased from 14.28×10^5 to 12.26×10^5 dpm/mmole when unlabelled oxaloacetate was added as an additional substrate.

On the other hand, the greatest quantity of acetate (104 mmoles/100 mmoles lactate fermented), was produced in the flask containing both lactate-U-C^{14} and oxaloacetate, while the flask containing lactate alone formed only 49.8 mmoles. The acetate specific activity, however, decreased from 9.40×10^5 dpm/mmole with lactate alone to 5.46×10^5 dpm/mmole with both lactate and oxaloacetate as substrates indicating that unlabelled oxaloacetate carbon had been converted to acetate in considerable quantities. This was further shown by the marked decrease in the propionic acid to acetic acid ratio (P/A) in this flask compared to that with lactate alone. For example, the P/A ratio for the lactate flask was 1.38, while that for lactate + oxaloacetate was

Table 10

Production of propionate and acetate by resting cells of V. parvula incubated in an atmosphere of nitrogen, with lactate, oxaloacetate and pyruvate (Figure 7).

Flask	Substrate	Propionate (P)		Acetate (A)		P/A
		mmoles[a]	sp. act. (x 10^5)	mmoles	sp. act. (x 10^5)	
A	Lactate-U-C^{14}[b]	68.7	14.28	49.8	9.4	1.38
B	Pyruvate	34.8	-	80.0	-	0.46
C	Oxaloacetate (O.A.A.)	29.0	-	45.4	-	0.63
D	Lactate-U-C^{14} + O.A.A.	62.6	12.26	104.0	5.46	0.60

(a) - mmoles/100 mmoles substrate

(b)-Lactate-U-C^{14} specific activity = 17.91 x 10^5 dpm/mmole

Experimental conditions as described in Figure 7. Reaction mixtures were recovered, neutralized and analyzed as described in Methods. Data given were obtained from the 30 minute samples.

only 0.60. This latter ratio was similar to that obtained when cells were incubated with oxaloacetate alone (0.63). When pyruvate was the substrate for fermentation the P/A ratio decreased even further to 0.46.

A general comparison between the absolute quantities of the end-products formed in these experiments shows that the presence of lactate as a substrate favored the formation of propionic acid while oxaloacetate and pyruvate favored acetic acid production.

Using the date obtained in Table 10, it was possible to calculate the distribution of C^{14} label from lactate-U-C^{14} to propionate and acetate, when lactate was the sole exogenous carbon source. The contribution of endogenous sources to both end-products was also possible by calculating the reduction in the specific activity of both propionate and acetate compared to the original lactate-U-C^{14}. Furthermore, assuming that the endogenous contribution was the same when both oxaloacetate + lactate-U-C^{14} were present as it was with lactate alone, the distribution of the carbon from these substrates into the products was also determined.

When resting cells were incubated with lactate-U-C^{14} alone, 80% of the carbon in the propionate formed originated with the substrate, lactate-C^{14}, with the remaining 20% arising from endogenous material (Table 11). In experiments with both lactate-U-C^{14} and

Table 11

Percent contribution of lactate-U-C^{14} and oxaloacetate carbons to the productions of propionate and acetate by resting cells of V. parvula incubated in an atmosphere of nitrogen.

Flask	Substrate	Propionate			Acetate		
		Lactate -C^{14}	Oxaloacetate	Endogenous	Lactate -C^{14}	Oxaloacetate	Endogenous
A	Lactate -U-C^{14}	80*	0	20	79	0	22
B	Lactate-U-C^{14} + Oxaloacetate	69	11	20	46	32	22

*percentage

Experimental conditions as described in Figure 7.

oxaloacetate, only 69% of the propionate carbon originated with lactate, 11% was contributed by the unlabelled oxaloacetate thereby explaining the decrease in the propionate specific activity observed in Table 10.

The majority of the acetate carbon (79%) originated with lactate-C^{14} when it was the sole exogenous substrate, but the lactate contribution decreased to only 46% when oxaloacetate was added as an additional substrate. In this case, oxaloacetate provided 32% of the carbon in acetate.

Endogenous material contributed significantly to the formation of both end-products. With lactate as sole exogenous carbon source, 20% of the carbon in propionate arose from endogenous sources, while 22% of the acetate carbon originated with this source.

DISCUSSION

In the discussion of the previous section (Part I), the inhibitory effect of oxygen on the growth of V. parvula cells was mentioned. This inhibitory effect was also observed in resting cell studies with the same organism, particularly during the measurement of gas production in the Warburg experiments.

In the earlier stages of this work, the indirect technique of Umbreit, Burris and Stauffer (1964) was employed to measure carbon dioxide and

hydrogen evolution by cells incubated with lactate. However, this technique was found an unsatisfactory method of monitoring lactate metabolism as gas production was not often observed despite the handling of cells in a stream of nitrogen, the employment of a reducing agent in the buffer, and the removal of oxygen from the reaction chamber by flushing the flasks with nitrogen for five minutes. This lack of metabolic activity by resting cells of V. parvula was due either to one of two possibilities or both: (i) cells of V. parvula, like V. alcalescens (Johns, 1951 b), required the presence of carbon dioxide for lactate metabolism and since the reactions were incubated in an atmosphere of nitrogen, metabolic activity was not observed; and/or (ii) the cells were inactivated during the process of gassing the Warburg flasks even before the reaction was started.

The first explanation is not applicable to the V. parvula system, since this organism has been shown to grow readily in lactate broth in atmospheres of nitrogen free of carbon dioxide. Furthermore, resting cells have also been shown to metabolize lactate repeatedly in an atmosphere of 100% nitrogen (e.g. Tables 4, 5 and Figure 3). As cells of V. parvula do not have a CO_2 requirement, inactivation of these cells during gassing procedures by oxygen would appear to be the explanation for the observed lack of activity.

Furthermore, the indirect method of Umbreit, Burris and Stauffer (1964) was shown to be unsatisfactory for reasons other than cell inactivation. On those occasions when gas production was observed during indirect Warburg analysis, significant differences were noted between flasks with and without KOH, in the quantity of the non-gaseous end-products formed (Table 8). The variability in the results obtained accompanied by a high frequency of cell inactivation prompted a change in the method of gas analysis.

The second procedure employed for the measurement of gas production was the triple side-arm technique of Hamilton, Burris and Wilson (1965). However, the adoption of the triple side-arm technique did not decrease the frequency with which V. parvula cells were inactivated by oxygen because the gassing procedure for this method was similar to that of the indirect method, i.e. the strict anaerobe V. parvula was exposed to oxygen during the initial gassing period. This short initial period of exposure to oxygen apparently was sufficient to inactivate the cells.

To solve the problem of inactivation, a modification of the triple side-arm Warburg technique was developed to permit the addition of the cells to the flasks by syringe after the gassing procedure had been completed. Modification of the triple side-arm technique, in this

manner, permitted the cells to be stored in reduced phosphate buffer at 0 C ice in an atmosphere of nitrogen until the Warburg flasks were prepared and gassed. The loss of cell activity, due to prolonged exposure to oxygen at room temperature while suspended in a phosphate buffer without a substrate, was therefore avoided.

It was noted during early resting cell experiments that the activity and stability of V. parvula cells was dependent not only on phosphate salts in the incubation medium, but also to other components of the buffer. In the process of determining the optimum phosphate concentration for this medium (Table 4), cells of V. parvula were observed to lose their activity and lyse after a 10 minute incubation period in a buffer composed of phosphates salts alone. However, when magnesium or maganese salts were added to this phosphate buffer, the cells remained stable and active throughout incubation periods as long as two hours. As the experiments in this study often required active and stable cells for at least one hour and frequently longer, the optimal concentration of these additional inorganic salts was of some importance.

Winslow and Haywood (1931) observed that when bacteria were suspended in distilled water, the addition of simples salts either enhanced or decreased the activity of the test microorganisms. Most

salts were toxic at high concentrations, but promoted cell survival and growth at low concentrations, with different effects being observed for different ions of the same valency. A similar observation can be made from the effect of various inorganic salts on the metabolism of lactate by cells of V. parvula (Table 5). Resting cells of this organism incubated in a phosphate buffer containing 20.2×10^{-3} M $MgSO_4$ exhibited a lactate utilization rate of 99.9 μmoles/mg dry cell wt./hr, while concentrations higher or lower than this showed adverse effects. On the other hand, increasing concentrations of $MnCl_2$ increased the rate of metabolism, but beyond 4.0×10^{-3} M concentration, the problem of solubility became the governing factor as $MnCl_2$ at this concentration crystallized out of solution on prolonged storage. Thus, although 4.0×10^{-3} M $MnCl_2$ gave a slightly better rate than 20.2×10^{-3} M $MgSO_4$, the latter salt was used in subsequent investigations.

The use of various other salts, such as $NaHCO_3$, $FeSO_4$ and $MnCl_2$ in combination with $MgSO_4$ did not increase the rate of lactate utilization by washed cells and as the results obtained with these combinations showed no obvious trend, they were not included in the phosphate buffer selected for use. However, the reducing agent, 2-mercaptoethanol, was incorporated into this buffer to protect the cells from oxygen inactivation. The buffer combination finally selected

for resting cells of V. parvula (0.1 M phosphate buffer, pH 6.5, containing 20.0×10^{-3} M $MgSO_4$ and 20.0×10^{-3} M 2-mercaptoethanol) gave an optimal lactate utilization rate of 102.0 μmoles/mg dry cell wt./hr.

The mechanism by which these salts influenced lactate utilization by cells of V parvula is unknown, but magnesium is required by many gram-negative bacteria, both for survival (Winslow and Haywood, 1931) and for growth (Lodge and Hinshelwood, 1939). In addition, many enzymes are now known to require magnesium and these enzymes may, in turn, exert a regulating influence on lactate metabolism.

The effect of substrate concentration on the rate of lactate utilization by V. parvula is an interesting aspect of its metabolism. The plot of utilization rate vs concentration (Figure 3) can be divided into 3 distinct sections: (i) from 0 to 15 mM of lactate, (ii) 15 to 80 mM, and (iii) 80 mM and above. In the first section, the rate of lactate utilization was directly proportional to the concentration of lactate in the medium, which can be represented by the equation:

$$\frac{dc}{dt} = kc_t \qquad (16)$$

(where c_t is the concentration of lactate at time t).

This equation is similar to a first order rate equation of a enzyme

catalyzed reaction. The rate of utilization between 15 and 80 mM sodium lactate was optimal, being independent of substrate concentration. The rate in this region can be represented mathematically by the equation:

$$\frac{dc}{dt} = kc_o \qquad (17)$$

It can be seen that equation 17 is similar to a zero order rate equation for enzymic reactions.

The similarity between the first two sections of the lactate utilization rate curve for whole cells of V. parvula and typical enzyme kinetics leads to the conclusion that cellular lactate utilization is limited by a single enzyme, possibly a substrate-transporting enzyme located in the cell-wall or cell-membrane. This postulation, however, is undoubtedly greatly over-simplified as many other possible explanations exist, e.g., the substrate binding capacity of the cells, the availability of free substrate binding sites on the cell surface, or the rate of substrate utilization within the cells.

The third section of the lactate rate curve (Figure 3), which includes the rates obtained at concentrations at 80 mM or above, has a negative slope typical of progressive substrate inhibition. The reason for this severe inhibition at high substrate concentrations is unknown,

but may again be involved with the transport of the lactate into the cell.

By employing modified triple side-arm technique, resting cells of V. parvula were shown to metabolize 100 mmoles of lactate to the following concentrations of propionate, acetate, carbon dioxide and hydrogen: 66.0, 39.6, 38.3 and 13.6 mmoles, respectively (Table 7). These results are reasonably close to those obtained by Foubert and Douglas (1948 b) with M. lactilyticas, while those of Johns (1951 b) with V. gazogenes showed significant differences, particularly in the quantity of the acids formed. As previously noted, the organisms investigated by Foubert and Douglas, and by Johns were classified by Rogosa (1965) as the same organism under the name V. Alcalescens. Although the fermentation balance obtained for V. parvula and for M. lactilytus were similar, the procedures employed to obtain these results were different. Foubert and Douglas (1948 b) utilized growing batch cultures of M. lactilyticus, while washed, resting cells of V. parvula were used in the present study. A possible reason for the different results obtained by Johns with V. gazogenes may be the use of a method similar to the indirect method of Umbreit, Burris and Stauffer (1964) for product analysis. Although cell inactivation apparently was not an obstacle in Johns' work, differences in the acidic end-products formed in Warburg flasks with and without KOH (i.e. with and without carbon dioxide) may have influenced his results.

Some changes would appear to be necessary before applying the

propionate-synthesizing scheme outlined by Johns (1951 b) for V. gazogenes to the results obtained with V. parvula. One of the major differences between these two organisms concerns the influence of carbon dioxide on the lactate metabolism by resting cells. Johns (1951 a, b) postulated that V. gazogenes (V. alcalescens) had a requirement for carbon dioxide in the degradation of lactate and concluded that carbon dioxide fixation was involved in the process of propionate formation. In the present work, however, cells of V. parvula were capable of growing and metabolizing lactate in the atmosphere of nitrogen free of carbon dioxide. Furthermore, the presence or absence of carbon dioxide in the reaction gas phase did not affect the rate of lactate utilization (Figures 5 and 6), but did influence the production of propionic acid.

While carbon dioxide did increase the absolute quantity of propionate formed from cellular metabolism, increased amounts of unlabelled CO_2 in the gas phase did decrease the specific activity of the propionate produced to a greater extent than could be accounted for by CO_2-fixation alone. These results suggested that the carbon dioxide-pyruvate-exchange reaction (reaction 18), which Whiteley and McCormick (1963) had demonstrated for V. alcalescens was also occurring in cells of V. parvula.

$$CH_3COCOOH + C^*O_2 \rightleftharpoons CH_3COC^*OOH + CO_2 \quad (18)$$

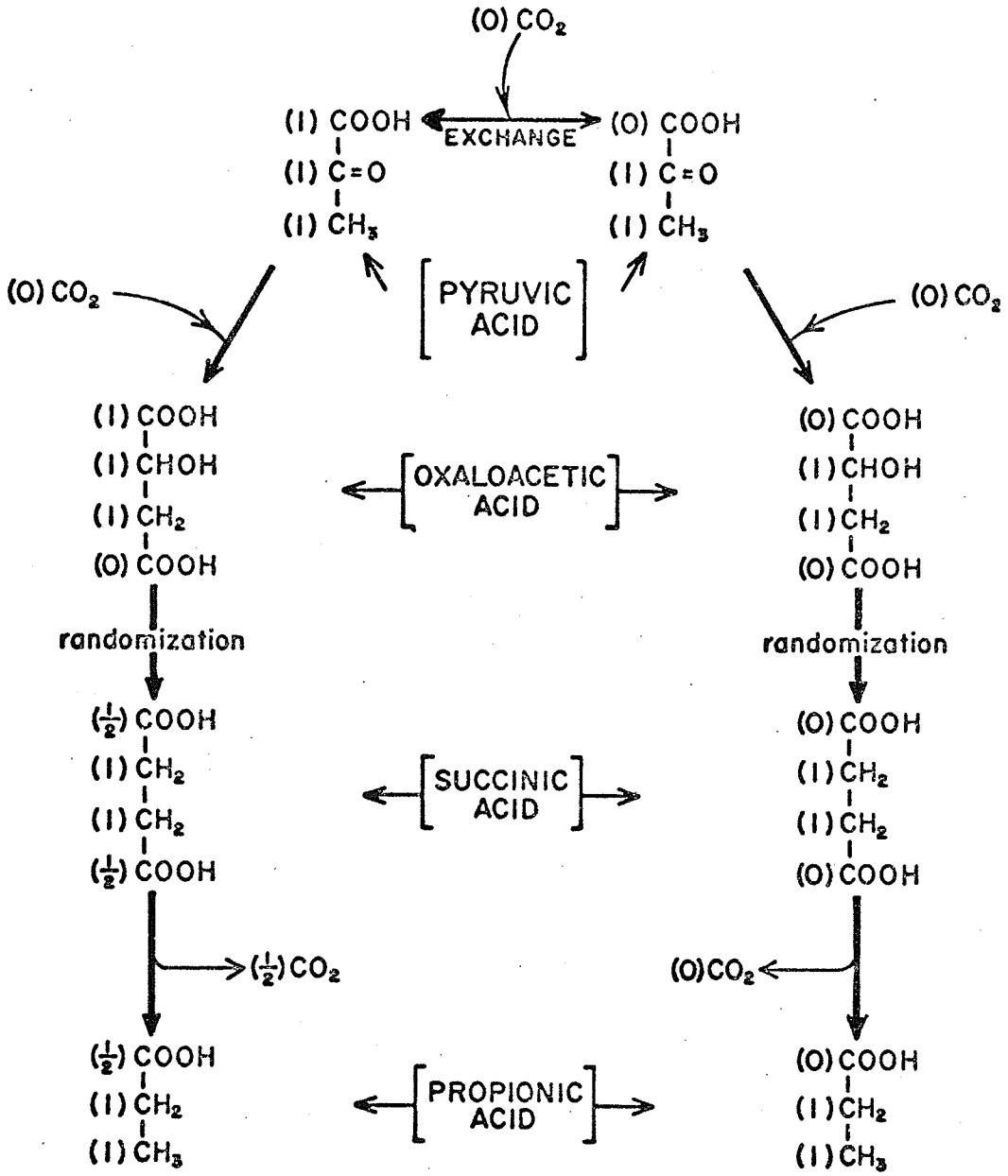
The existence of an exchange reaction in this organism was further substantiated by the observation that increased levels of $C^{12}O_2$ in the atmosphere resulted in the increased evolution of $C^{14}O_2$ (Fig. 6).

The influence of both CO_2-exchange and CO_2-fixation on the metabolism of lactate by V. parvula can best be illustrated by reference to the hypothetical pathway for propionate production (Fig. 8). The pathway on the left considers the labelling pattern in propionate if the fixation of unlabelled carbon dioxide occurred with uniformly labelled pyruvate. The pathway on the right, on the other hand, would be the labelling pattern if both fixation and the carbon dioxide-pyruvate exchange reaction operated in the presence of excess quantities of unlabelled CO_2. If only the fixation of unlabelled carbon dioxide occurred without exchange, (left pathway), the oxaloacetate formed would be labelled only in the carbons derived from pyruvate, while the C-4 carboxyl carbon, obtained from CO_2, would be unlabelled. As randomization of the label occurs at the succinate step, the radioactivity originally in C-1 would be distributed randomly between both carboxyl groups of this acid. The propionate formed as a result of this would have a specific activity of 2.5 compared to a specific activity of 3.0 in the original pyruvate.

If, however, the exchange reaction is superimposed on this

Figure 8

The postulated influence of carbon dioxide fixation and the CO_2-pyruvate exchange reaction on propionate synthesis via the oxaloacetate pathway.


(0) CO2
(1) COOH
(1) C=O
(1) CH3
EXCHANGE
(0) COOH
(1) C=O
(1) CH3
(0) CO2
PYRUVIC ACID
(0) CO2
(1) COOH
(1) CHOH
(1) CH2
(0) COOH
OXALOACETIC ACID
(0) COOH
(1) CHOH
(1) CH2
(0) COOH
randomization
randomization
(1/2) COOH
(1) CH2
(1) CH2
(1/2) COOH
SUCCINIC ACID
(0) COOH
(1) CH2
(1) CH2
(0) COOH
(1/2) CO2
(0) CO2
(1/2) COOH
(1) CH2
(1) CH3
PROPIONIC ACID
(0) COOH
(1) CH2
(1) CH3

distribution of C^{14}, and if the unlabelled CO_2 in the atmosphere is at a high concentration such that very little of the C^{14} evolved from the C-1 of pyruvate as $C^{14}O_2$ has an opportunity of reappearing in pyruvate, the carboxyl group of the pyruvate would be unlabelled. This is shown on the right side of Figure 8. Again the conversion of pyruvate to oxaloacetate would occur by $C^{12}O_2$-fixation such that both the carboxyl groups in oxaloacetate would be unlabelled. In this case, the resulting propionate would have specific activity of 2.0 compared to a value of 3.0 for the original pyruvate.

By comparing the propionate specific activities of the 0 and 25% carbon dioxide flasks in Table (8), a ratio of 2.6 was obtained. However, calculation of the amount of $C^{12}O_2$ fixed or exchanged is only valid for the 20 minutes samples in the 50% carbon dioxide flasks because the production of $C^{14}O_2$ and the incorporation of C^{14} into the cells had reached a steady state (Figure (B) and (C)). The propionate specific activity thus obtained from these flasks was 2.1. This value can only be obtained if the exchange reaction had occurred simultaneously with carbon dioxide fixation. By further comparing the specific activity for propionate at CO_2 levels of 0, 25 and 50% (Table 8), it can be calculated that 80% of the C^{12} incorporated into propionate arose from $C^{12}O_2$ fixation, while the exchange reaction accounted for 20% of

the propionate carbon. It can be concluded, therefore, that increased partial pressures of carbon dioxide, not only increased the absolute amount of propionate formed, but also enhanced the CO_2-pyruvate exchange reaction.

Foubert and Douglas (1948) postulated that lactate fermentation by M. lactilyticus occurred by the following reactions:

$$CH_3CHOHCOOH \longrightarrow CH_3COOH + CO_2 + 4\,H \quad (19)$$

$$2H^+ \longrightarrow H_2 \quad (20)$$

$$CH_3CHOHCOOH + 2\,H \longrightarrow CH_3CH_2COOH \quad (21)$$

$$2\,CH_3CHOHCOOH \longrightarrow CH_3CH_2COOH + CH_3COOH + CO_2 + H_2 \quad (22)$$

According to these reactions, two moles of lactate were fermented to give one mole of propionate, acetate, carbon dioxide and hydrogen giving ratios of 2:1:1:1:1 for lactate, propionate, acetate, carbon dioxide and hydrogen, respectively. These equations, however, do not fit the fermentation balances obtained by these workers because the actual substrate to the end-product ratios were 10:6:4:4:1.

Similar substrate to end-product ratios were obtained with V. parvula in resting cell experiments. Employing the values obtained in the present fermentation study, the following equations can be written:

$$2\ CH_3CHOHCOOH + H_2O \longrightarrow 2\ CH_3COOH + 2\ CO_2 + 8\ H^+ + 8e^- \quad (23)$$

$$3\ CH_3CHOHCOOH + 6\ H^+ + 6e^- \longrightarrow 3\ CH_3CH_2COOH + 3\ H_2O \quad (24)$$

$$2\ H + 2e^- \longrightarrow H_2 \quad (25)$$

$$5\ CH_3CHOHCOOH \longrightarrow 3\ CH_3CH_2COOH + 2\ CH_3COOH + 2\ CO_2 + H_2 + 2\ H_2O \quad (26)$$

It is obvious that equations such as these, based on fermentation studies with whole cells, can only express the net result of lactate metabolism. Enzymatic studies are required for more definite answers as to the intermediate steps involved. Because these enzymatic reactions were of interest, the fermentation of lactate and other substrates was carried out with cell-free enzyme preparations obtained from washed exponential phase cells of V. parvula. These experiments are the subject of Part III.

PART III

METABOLISM BY CELL-FREE PREPARATIONS FROM VEILLONELLA PARVULA

INTRODUCTION

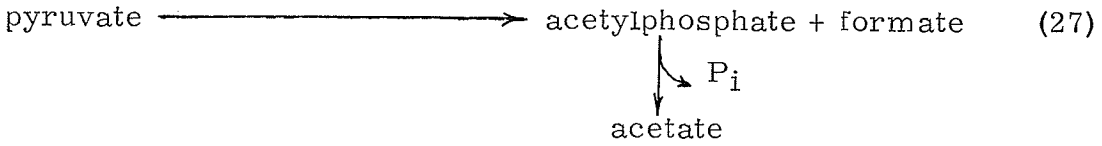
Although the oxaloacetate scheme proposed by Johns (1951 b) for the fermentation of lactate to propionic acid was established from studies with whole cells alone, it did form the basis for further research on the metabolism of short-chain carboxylic acids by enzyme preparations from species of the veillonellae. In 1953, Witter isolated the enzyme, hydrogenase, from cell-free extracts of M. lactilyticus (V. alcalescens), which catalyzed the formation of molecular hydrogen from pyruvate. This evidence confirmed Johns' conclusion that formate was not an intermediate in the formation of H_2 by this organism. The activity of the isolated enzyme, measured between pH 5.0 and 8.0, was found to be optional at pH 6.2 - 6.3 decreasing rapidly and progressively as the pH was increased to 8.0.

Further studies with extracts of M. lactilyticus by Whiteley and Ordal (1957) demonstrated the oxidative decarboxylation of pyruvate at pH6.5 according to the following equation:

$$\text{pyruvate} \longrightarrow \text{acetate} + CO_2 + H_2 \qquad (2)$$

Later, however, McCormick, Ordal and Whiteley (1962 a, b) showed that pyruvate degradation at pH 8.0 resulted not in the formation of acetate, carbon dioxide and hydrogen, but acetate and formate, according

to the 'phosphoroclastic' reaction (27). In this process, acetylphosphate was postulated to an intermediate in the production of acetate.



In addition to confirming the findings of Johns and Witter, these observations also revealed that cell-free extracts of M. lactilyticus were capable of performing two pH-dependent reactions for the degradation of pyruvate.

Although formate was not utilized by these extracts, an exchange reaction, activated by a diphosphothiamine-requiring enzyme, was observed between formate and the carboxyl group of pyruvate (McCormick, Ordal, Whiteley, 1962 a, b). Whiteley and McCormick (1963) further observed that carbon dioxide, either in the gaseous state or the bicarbonate form, was also exchanged with the carboxyl-carbon of pyruvate under reduced conditions by a reaction requiring coenzyme A and diphosphothiamine as cofactors.

One of the fundamental reactions for the conversion of lactate carbon to propionate proposed by Johns (1951 b) for species of Veillonella was carbon dioxide fixation with pyruvate to form oxaloacetate. In Johns' study, cells of V. gazogenes were grown in lactate broth in the

presence of C^{13}-carbonate for 24 hours followed by the isolation and degradation of the propionate formed as the result of metabolism. The results of this experiment showed that the propionate carboxyl carbon was substantially enriched with C^{13}, which led Johns to conclude that CO_2-fixation had occurred. However, in view of the later data of Whiteley and McCormick (1963) demonstrating the CO_2-pyruvate exchange reaction, the incorporation of C^{13}-carbonate into the C-1 of propionate may have resulted from the exchange reaction followed by randomization of the label at the succinate step rather than by CO_2-fixation. Little enzymatic evidence can be found for the fixation of carbon dioxide by species of Veillonella, although the CO_2-requirement for growth with some species would imply that such fixation does occur. Furthermore, oxaloacetate has never been isolated in any of the studies with cells or extracts of these organisms.

The conversion of malate to fumarate (reaction 5) fumarate to succinate (reaction 6) and succinate to propionate (reaction 7) were postulated by Johns (1951 b) on the basis of fermentation studies in which cells of V. gazogenes (V. alcalescens) were shown to degrade malate, fumarate and succinate with the concomitant formation of propionate. The production of propionate from these substrates, as well as oxaloacetate and pyruvate was confirmed by Whiteley and Ordal

(1957) with cell suspensions and Mickle extracts (Type I) of M. lactilyticus (V. alcalescens). Enzymatic preparations (Type II) obtained by Mickle disintegration or alumina grinding, however, were unable to produce propionate from pyruvate. Despite these differences in enzyme activity, both types were shown to activate and decarboxylate succinate at comparable rates. In addition, malic and succinic dehydrogenase activities in these preparations, as measured by the rate of dye reduction, were also shown to be comparable.

Contrary to many of the other reactions involved in the Johns' scheme, the decarboxylation of succinate to form propionate (reaction 7) by Veillonella species has received considerable attention. Whiteley (1953 a) showed that small quantities of coenzyme A (CoA) and adenosine triphosphate (ATP) were required for the decarboxylation of succinate by cell-free extracts of M. lactilyticus (V. alcalescens) depleted of cofactors by Norit treatment. Further work showed that succinate was initially 'activated' by an apparent ATP-dependent accumulation of an intermediate capable of forming succinhydroxamic acid (Whiteley, 1953 b). Both succinyl-phosphate and succinyl-CoA were postulated to be involved in the decarboxylation step as the 'active' intermediate.

However, as only traces and not stoichiometric amounts of ATP were required for decarboxylation, and since succinyl-CoA could be

readily decarboxylated to form propionate and CO_2, ATP was postulated to function prior to the actual decarboxylation reaction (Whiteley, 1953 c). Furthermore, the formation of propionyl-CoA, as well as succinyl-CoA, could be demonstrated from the incubation of acetyl-phosphate, CoA and succinate with cofactor-depleted Norit extracts. Since propionyl-CoA could stimulate the decarboxylation of succinate with extracts capable of decarboxylating succinyl-CoA, but not succinate, the generation of succinyl-CoA by CoA-transfer from propionyl-CoA was postulated.

From these observations, Whiteley (1953 a, b, c) proposed the following pathway for succinate decarboxylation for extracts of M. lactilyticus (V. alcalescens):

$$\text{succinate} + \text{ATP} \rightleftharpoons \text{succinyl-P} + \text{ADP} \qquad (28)$$

$$\text{succinyl-P} + \text{CoA} \rightleftharpoons \text{succinyl-CoA} + \text{P} \qquad (29)$$

Succinyl-CoA was thought then to be converted to propionyl-CoA, followed by the transfer of the CoA moiety to a new molecule of succinate by an acyl transferase, thereby regenerating succinyl-CoA and producing propionate.

$$\text{succinyl-CoA} \rightleftharpoons \text{propionyl-CoA} + CO_2 \qquad (30)$$

$$\text{propionyl-CoA} + \text{succinate} \rightleftharpoons \text{succinyl-CoA} + \text{propionate} \qquad (31)$$

Delwiche, Phares and Carson (1954, 1956) confirmed, in part, this scheme with extracts of P. pentosaceum and V. gazogenes, however, they were unable to demonstrate the presence of succinyl-P as an intermediate in the reaction sequence.

Further work with these extracts (Phares, Delwiche, and Carson, 1956) indicated that during the decarboxylation of succinate to propionate, an active C_1 fragment was produced from the free carboxyl of succinyl-CoA and that CO_2 was subsequently produced from this C_1 fragment by the action of an additional enzyme.

Further elucidation of the succinate decarboxylation step in extracts of M. lactilyticus (V. alcalescens), was shown by Allen and Galivan (1965), who demonstrated the presence of the enzyme methylmalonyl-CoA carboxylase which catalyzed the following reaction:

$$\text{methyl-malonyl-CoA} \rightleftharpoons \text{propionyl-CoA} + CO_2 \qquad (32)$$

More recently, Galivan and Allen (1968) isolated and characterized such an enzyme from extracts of M. lactilyticus and found it to be tightly bount to the 39 and 48 S ribosomes. Furthermore, these workers demonstrated that this enzyme was part of a complicated reaction sequence, for the conversion of succinate to propionate by this organism:

succinyl-CoA ⇌ R-methylmalonyl-CoA (33)

R-methylmalonyl-CoA ⇌ S-methylmalonyl-CoA (34)

S-methylmalonyl-CoA ⇌ propionyl-CoA + CO_2 (35)

propionyl-CoA + succinate ⇌ propionate + succinyl-CoA (36)

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succinate ⟶ propionate + CO_2 (37)

Reaction (32) is catalyzed by methylmalonyl CoA mutase, reaction (33) by a racemase, reaction (34) by methylmalonyl-CoA carboxylase, and reaction (35) by an acyl-CoA transferase.

It is apparent from this evidence that species of Veillonella produce propionate from succinate in a manner different from that demonstrated in Propionibacterium shermanii by Stadtman et al (1960) and Swick and Wood (1960). These workers have demonstrated the formation of propionyl-CoA from methylmalonyl-CoA coupled to the carboxylation of pyruvate to oxaloacetate, a reaction catalyzed by methylmalonyl-CoA-oxaloacetate transcarboxylase.

methylmalonyl-CoA + pyruvate ⟶ propionyl-CoA + oxaloacetate (38)

Oxaloacetate would then be decarboxylated by oxaloacetate decarboxylase to regenerate pyruvate (reaction 39).

oxaloacetate $\longrightarrow$ pyruvate + CO_2 (39)

The net result of the reactions catalyzed by the propionibacteria would be the production of propionyl-CoA and CO_2 from succinyl CoA with a requirement for catalytic quantities of pyruvate.

Implicit in the propionate synthesizing pathway proposed by Johns was the initial conversion of lactate to pyruvate (reaction 1) by a lactic dehydrogenase-type enzyme. However, as first shown by Phares and Long (1956), species of Veillonella do not contain lactic dehydrogenase, but couple the oxidation lactate to pyruvate with the reduction of oxaloacetate to malate by a transhydrogenase-type enzyme:

lactate + oxaloacetate $\rightleftharpoons$ pyruvate + malate (15)

This malate-lactate transhydrogenase was shown to be a single enzyme, devoid of lactic and malic dehydrogenase activity, containing bound NADH and NAD in the molar ratio of three to one (Dolin, Phares and Long, 1965). The NADH bound to this enzyme was not accessible to artificial electron acceptors, but the addition of pyruvate completely quenched NADH fluorescence. Furthermore, the enzyme was shown not to function as a transcarboxylase as C^{14}-label was found only in lactate and not in oxaloacetate after the reaction had been allowed to proceed in the presence of pyruvate-1-C^{14} and unlabelled malate (Allen

and Galivan, 1965; Allen, 1966). The enzyme, on the other hand, did catalyze the transfer of tritium from lactate-2-H^3 to the pyridine prosthetic group of the protein and from this moeity to oxaloacetate forming malate-H^3 (Allen, 1966). The purified enzyme had a molecular weight near 99,000 with the prosthetic group so firmly bound that it could not be removed without denaturing the protein. The reaction catalyzed by this enzyme was shown to be readily reversible with a K_{eq} of 1.8 in favour of malate-pyruvate (Table 16).

Allen (1966) postulated that as malic dehydrogenase was present in these extracts, only catalytic amounts of oxaloacetate would be required for the conversion of lactate to pyruvate. The malate formed as a result of the reaction would be reoxidized to oxaloacetate by malic dehydrogenase assuring the continual regeneration of oxaloacetate, such that the reaction would produce a net formation of pyruvate and NADH from lactate. A separate 'carboxylase'-type enzyme must, therefore, be present in cells of Veillonella species to account for the net formation of propionate from pyruvate and lactate.

Whiteley and Ordal (1957) observed that, besides the fermentation of pyruvate, cell-free extracts of M. lactilyticus (V. alcalescens) also metabolized other α-keto acids such as α-ketobutyrate and α-ketoglutarate to propionate, carbon dioxide and hydrogen. When α-ketobutyrate was

used as the substrate, the type of medium used for growth and the method of extract preparation had little influence on this metabolism, although maximum activity was only obtained with fresh preparations as aging brought on a rapid loss of metabolic activity.

The oxidative decarboxylation of α-ketoglutarate, on the other hand, was shown to be dependent on the medium in which the cells were grown, on the type of preparation employed, as well as the age of the preparation. Freshly prepared extracts of cells grown in lactate medium (Whiteley 1953 c) were required for optimum activity with the production of propionate from α-ketoglutarate postulated to proceed by the decarboxylation of this substrate to CO_2, hydrogen and succinyl-CoA in a manner analogous to that shown in mammalian tissue (Sanadi and Littlefield, 1951). The subsequent decarboxylation of succinyl-CoA to propionyl-CoA and carbon dioxide was similar to that postulated for M. lactilyticus in earlier investigations (Whiteley, 1953 a, b).

Aged extracts or extracts from cells grown in suboptimal medium produced less propionate from α-ketoglutarate and decarboxylated succinate at a lower rate, in addition to forming small amounts of acetate. The production of acetate was explained by assuming that part of the succinate formed was oxidized to fumarate, and thence via malate and oxaloacetate to pyruvate with the eventual production of acetate.

Experiments with malonate supported this postulation as essentially no acetate was formed from α-ketoglutarate or succinate in the presence of this inhibitor.

In 1965, Rogosa employed cell-free extracts of V. parvula and V. alcalescens to show that these organisms were unable to utilize carbohydrates because the glucose-phosphorylating enzyme, hexokinase, was absent. However, if extracts from these organisms were supplemented with yeast-hexokinase, glucose was readily metabolized to acetate, pyruvate, propionate, lactate and CO_2 indicating that the remaining enzymes in glycolysis were present.

The data obtained in metabolic experiments with resting cells of the strain of V. parvula isolated in this study have indicated that enzymatic differences exist between this organism and V. alcalescens (Part II). However, except for the above study by Rogosa (1965), very little information is available in the literature on the enzymatic properties of V. parvula which would permit a useful comparison on a sub-cellular basis.

Futhermore, one must keep in mind that the results from such whole cell experiments are only a reflection of the net changes involved in cell metabolism, i.e. the uptake of substrate into the cells and the exit of end-products from these cells into the medium. The mechanism

involved in the transport of substrates, the enzymes involved in the pathway(s) of catabolism or the extent of endogenous metabolism are unknown. With this in mind, metabolic studies with cell-free enzyme preparations of V. parvula were undertaken.

METHODS

Cell-free Experiments

Large batch cultures of V. parvula were grown anaerobically in 2 to 4 litre volumes, the cells harvested and washed as previously described. Cell-free extracts from these exponential-phase, washed cells were obtained by two methods:

(1) Sonic disruption. Cells (20 - 30 ml at a concentration of 30 mg dry wt/ml) were disrupted in a Raytheon Sonic Oscillator for 15 minutes at approximately 4 C in a stream of nitrogen. Following sonication, the suspensions were centrifuged anaerobically at 13,000 x g for 15 minutes at 4 C and the dark, reddish-brown supernatant solution collected by syringe and injected into 25 ml Erlenmeyer flasks containing tight-fitting serum caps previously gassed with N_2. These flasks were then kept in ice until used.

(2) French pressure cell. Cell-free extracts were also obtained by passing washed, whole cells (30 mg dry wt/ml) through the French Pressure Cell (American Instrument Co.) 3 - 4 times at a pressure of 6-9000 lb/sq in. The supernatant was obtained by centrifugation and stored as described above. In all cases, the concentration of protein was determined by the method of Layne (1957).

To start each reaction, the crude enzyme preparation (4 - 5 mg

protein/ml and equilibrated at 37 C) was injected into the appropriate flask previously gassed with either nitrogen or 5% CO_2 + N_2 and containing the substrate(s) (10 μmoles/ml) in 0.1 M phosphate buffer (pH 6.5), such that the final total volume was 10 ml. Samples were taken at various intervals by syringe and added to centrifuge tubes containing either 0.1 ml, cold 25% $ZnSO_4$ or 10% trichloroacetic acid (TCA) to stop the reaction. The supernatant solution obtained after centrifugation at 13,000 x g was subjected to analysis.

The $ZnSO_4$ method was used in those experiments where the metabolic end-products, propionate and acetate, were determined by silicic acid column chromatography. TCA was not used in this method because it elutes from silicic acid columns in the acetate region. When α-keto acids were to be analyzed in the reaction mixtures, one volume of sample was added to one volume of 0.1% 2,4 dinitrophenolhydrazine in test tubes and these tubes allowed to stand overnight at 4 C. Some of the hydrazones formed would precipitate during this procedure, but could be separated from the sedimented protein after centrifugation by extraction with ethyl acetate.

Measurement of Oxaloacetate Formation.

The fixation by cell-free extracts of carbon dioxide with pyruvate to form oxaloacetate (reaction 13) was measured by coupling this

reaction to the conversion of oxaloacetate and NADH to malate and oxidized pyridine nucleotide (reaction 4). By following the formation of NAD spectrophotometrically, the kinetics of oxaloacetate formation from various substrates and CO_2 was determined. Reactions were carried out in 0.1 M, pH 6.5, phosphate buffer containing cell-free extracts (5 mg protein/ml); NADH at 30 mμmoles; 10 μmoles of the substrates, lactate, pyruvate or phosphoenolpyruvate, with or without malate dehydrogenase (Boehringer Mannheim Corp.) at 12.5 μg protein/ml (activity 720 units/mg) in a total volume of 1.0 ml. The formation of NAD was recorded at 340 mμ with a DB-spectrophotometer and recorder.

Analyses:

The analyses for lactate, pyruvate, oxaloacetate, propionate and acetate were carried out as described in Part II. Intermediates of lactate and pyruvate metabolism were analyzed by thin layer chromatography.

Thin Layer Chromatography: Thin layer chromatography was used in product analyses by employing silica gel G as the inert phase. The gel was prepared by adding 80 ml of deionized water to 40 gm silica gel G in a stoppered Erlenmeyer flask and the mixture rapidly shakened until a homogenous solution was obtained. This colloidal mixture was then spread on glass plates (2 x 8 x 10 x 8 inch) previously cleaned with

petroleum ether, using a spreader adjusted to give a gel thickness of 0.5 mm. These plates were then dried at room temperature for at least four hours and either stored in a dust-free container or activated for use in a preheated oven at 120 C for 30 - 40 minutes. These activated plates were then allowed to cool in a closed chamber containing a dessicant (silica gel crystals) to prevent the reabsorption of moisture by the plates during cooling.

This method was used primarily to determine the products formed from the degradation of pyruvate-3-C^{14} and lactate-U-C^{14} by extracts of V. parvula. Concentrated samples were applied in small volumes (20 ul) to the plates in a stream of air from a hair-dryer. Small 2 x 8 inch plates were used for the radioactive analysis of single samples as labelled areas could be located by passing the developed plates through a radioactive chromatogram scanner (Packard Instruments). Larger 10 x 8 inch plates were divided into six to seven sections and were used to analyse multiple samples in the same solvent system. The separated acids spots on these plates from unknown and standard acid mixtures were located by spraying with the dye mixture of Ting and Dugger (1965), which contained 0.3% bromophenol blue and 0.1% methyl red in 95% ethanol.

The solvents used to develop the plates differed according to the

type of product being investigated. For the separation of fumarate, succinate, malate, pyruvate, oxaloacetate and acetate, a 7:1 water-saturated diethyl ether and formic acid (88%) solvent was used (Ting and Dugger, 1965). On the other hand, α-keto acid hydrazones, such as pyruvate and oxaloacetate were separated in a solvent system composed of n-butanol-ethanol-ammonia (0.5 N) in the ratio of 70-10-20 (Hawari and Thompson, 1953).

RESULTS

As a preliminary step, the ability of cell-free extracts of V. parvula to produce gas from the fermentation of short chain carboxylic acids, such as lactate, pyruvate and oxaloacetate, was studied by the modified triple side-arm Warburg technique previously described. Extracts were prepared by sonication and incubated in an atmosphere of nitrogen with either lactate, pyruvate, oxaloacetate or a combination of both lactate and oxaloacetate. Reports in the literature have indicated that lactate cannot be metabolized by cell-free extracts of V. alcalescens in the absence of oxaloacetate (Phares and Long, 1956; Dolin, Phares and Long, 1964, 1965; Allen and Galivan, 1965; Allen, 1966).

As shown in Table 12, very little total gas was produced from the flask containing lactate alone (A), while a somewhat greater quantity was

Table 12

Gas production by cell-free extracts of V. parvula incubated with a variety of short chain carboxylic acids in an atmosphere of nitrogen.

Flask	Substrates*	Total gas production+ (µl/hr.)
A	Lactate	20
B	Pyruvate	75
C	Oxaloacetate	> 300
D	Lactate + Oxaloacetate	> 300

* Cell-free extracts at a concentration of 5.0 mg protein/ml were incubated with 10 mM substrate(s) in pH 6.5 phosphate buffer at 37 C as previously described (Manometric Methods - Part II).

+ Values from endogenous controls (without substrate) were subtracted to give the net total gas production.

observed in the flask containing 10 mM sodium pyruvate (B). However, abundant and rapid gas production occurred in the flask containing oxaloacetate alone (C) and in the flask containing oxaloacetate + lactate (D). The production of gas in these later flasks was so rapid that an accurate reading was not obtained before the Brodie's solution was displaced from the open side of the manometers. A comparison between flasks (A) and (D) readily shows that more gas was produced when oxaloacetate was incubated with lactate (D) than when extracts were incubated with lactate alone (A). This observation would seem to indicate that a malate-lactate transhydrogenase, already reported in cell-free preparations of V. alcalescens (Phares and Long, 1956), was also present in cells of V. parvula.

The ability of cell-free extracts of V. parvula to ferment lactate, pyruvate and oxaloacetate was further examined in a time course experiment. Extracts were obtained by sonication from washed exponential-phase cells and incubated in flasks containing the same substrates as the preliminary experiment (Table 12) and samples removed by syringe at various time intervals. As shown in Fig. 9, lactate (A) was not utilized by the cell-free extracts throughout the 60 minutes incubation period, while pyruvate (B) was metabolized at an initial rate of 13.3 μmoles/mg protein/hr, but was not completely exhausted during

Figure 9

Metabolism of lactate, oxaloacetate, pyruvate, and oxaloacetate + lactate by cell-free extracts of V. parvula in an atmosphere of nitrogen.

Cell-free extracts of V. parvula (5.0 mg protein/ml) were injected by syringe into equilibrated 25 ml Erlenmeyer flasks fitted with serum stoppers and gassed with high purity nitrogen. The flasks contained the following substrates such that the final concentration for each was 10 mM in a final volume of 10 ml.

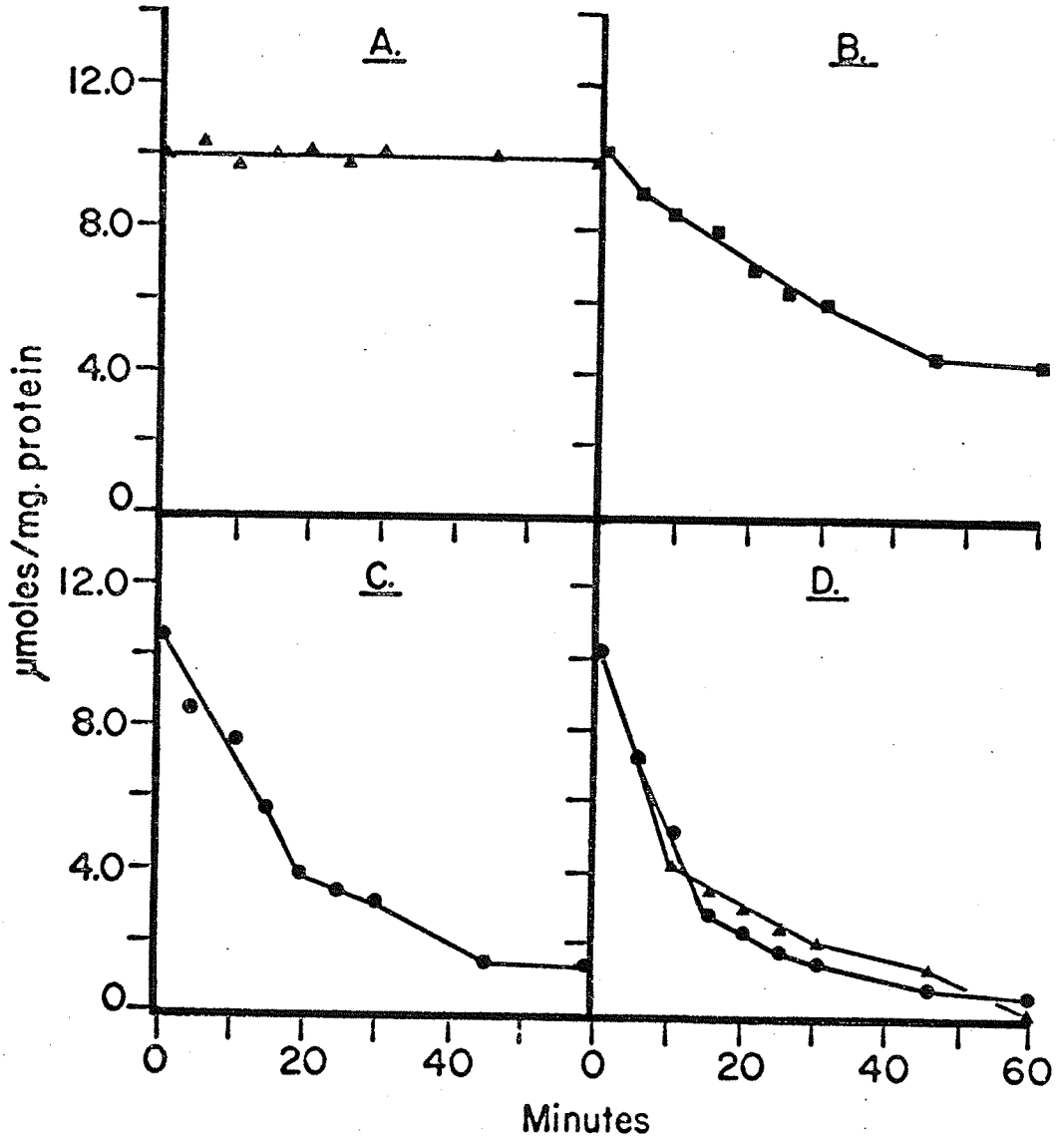
Flask A Sodium lactate-U-C^{14} (specific activity 17.91 x 10^5 dpm/mmole).

Flask B Potassium pyruvate

Flask C Sodium oxaloacetate

Flask D Sodium lactate-U-C^{14} and sodium oxaloacetate.

At the time the extracts were added (zero time) and at regular intervals thereafter, 1.0 ml samples were removed by syringe and the reaction stoped with $ZnSO_4$. The samples were then neutralized, made to volume, centrifuged and the supernatant solution analyzed for the substrate remaining by procedures described in Methods.


A.
B.
C.
D.
12.0
8.0
4.0
0
μmoles/mg. protein
0
20
40
60
Minutes

this period. The metabolism of oxaloacetate (C) was similar to that for pyruvate in that it was not completely utilized in 60 minutes, but, it was metabolized at a faster initial rate (19.5 μmoles/mg protein/hr).

Interestingly, when oxaloacetate was incubated with lactate-U-C^{14} (D) in the same flask, not only was lactate metabolized, but both of these substrates were utilized at the same initial rate of 36.0 μmoles/mg protein/hr, which was almost twice as fast as that for oxaloacetate alone. The rate of utilization of these different substrates was, however, slower than that observed with washed whole cells.

These results confirm the preliminary evidence that malate-lactate transhydrogenase and not lactate dehydrogenase is involved in the metabolism of lactate by V. parvula.

End-product analysis of the 60 minute sample from flask (D) demonstrated that the combined metabolism of lactate-U-C^{14} and unlabelled oxaloacetate resulted in the formation of radioactive acetate and propionate (Table 13). As this data indicates, 34.1 mmoles of propionate and 47.8 mmoles of acetate were produced per 100 mmoles of the substrates, lactate and oxaloacetate, when calculated on a per carbon basis. The P/A ratio in this case was 0.71, which was just slightly higher than the P/A ratio (0.60) obtained when some substrates were incubated with resting cells (Table 10).

Table 13

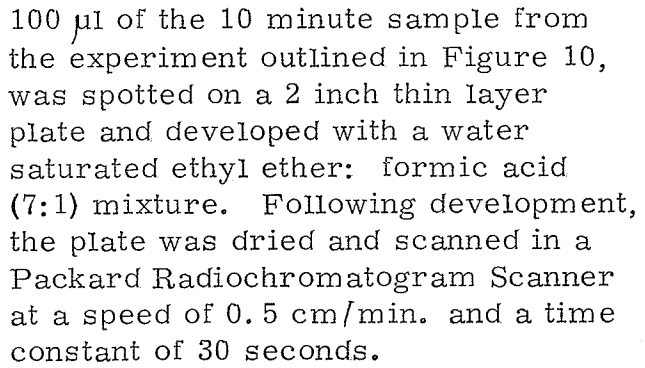
Distribution of radioactive carbon in propionate and acetate resulting from the utilization of lactate-U-C^{14} and oxaloacetate by cell-free extracts of *V. parvula*.

Products	mmoles carbon/100 mmoles of total substrate carbon	Specific Activity (dpm/mmole of carbon)	Percent Contribution	
			Lactate	Oxaloacetate + Endogenous
Propionate (P)	34.1	538	30.0	70.0
Acetate (A)	47.8	776	43.4	56.6
P/A ratio	0.71			

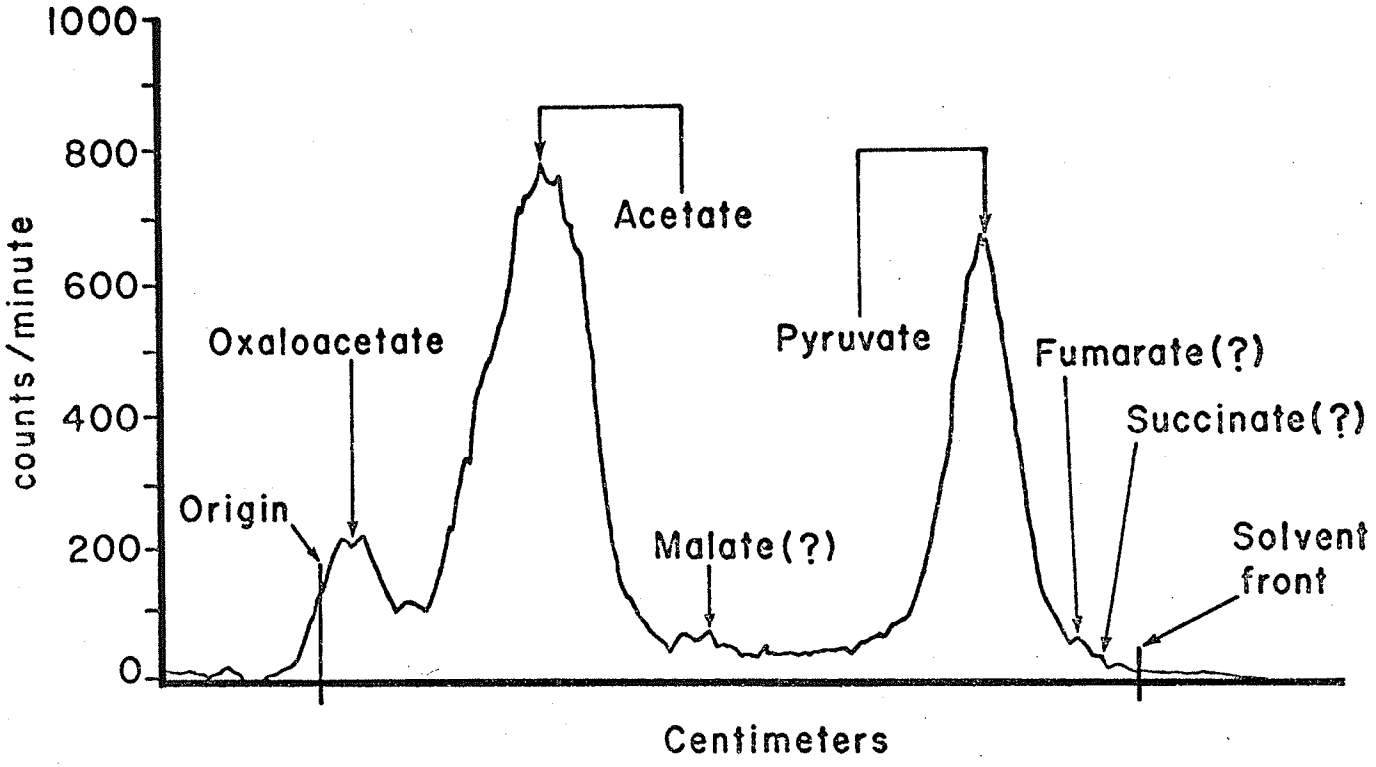
Conditions: 10 μmoles of uniformly labelled lactate-U-C^{14} (specific activity - 17.91×10^5 dpm/mmole) and 10 μmoles oxaloacetate incubated with 5.0 mg protein/ml in an atmosphere of nitrogen as in Figure 6.

Figure 10

Radioactive components obtained from the metabolism of pyruvate-3-C^{14} by extracts of V. parvula and separated by thin layer chromatography.



100 µl of the 10 minute sample from the experiment outlined in Figure 10, was spotted on a 2 inch thin layer plate and developed with a water saturated ethyl ether: formic acid (7:1) mixture. Following development, the plate was dried and scanned in a Packard Radiochromatogram Scanner at a speed of 0.5 cm/min. and a time constant of 30 seconds.


1000
800
600
400
200
0
counts/minute
Origin
Oxaloacetate
Acetate
Malate(?)
Pyruvate
Fumarate(?)
Succinate(?)
Solvent front
Centimeters

As the specific activity of the original lactate-C^{14} was known (e.g. 17.91×10^5 dpm/mmole), it was possible to calculate from the propionate and acetate specific activities, the contribution of lactate and oxaloacetate carbons in these two end-products. By this type of analysis, it was shown that 30.0% of the propionate carbon was contributed by the lactate-C^{14}, while the remaining 70% originated with oxaloacetate and endogenous material. The distribution in acetate was somewhat different with 43.4 and 56.6 percent of the molecules originating with lactate-C^{14} and oxaloacetate (plus endogenous sources), respectively.

The incorporation of lactate carbon into propionate by cell-free extracts of V. parvula again raises the question as to the pathway of propionate formation. Johns (1951 b) originally postulated that propionate formation in cells of V. alcalescens (V. gazogenes) as determined from fermentation studies, occurred by the formation of oxaloacetate from pyruvate and CO_2 (reaction 3), with the oxaloacetate subsequently converted to malate, fumarate, succinate and finally to propionate by the decarboxylation of this latter acid. Although, the conversion of succinyl-CoA to propionyl-CoA (Whiteley 1953 a, b, c; Galivan and Allen, 1968) and the malate-lactate transhydrogenation (Allen, 1966) have been studied with cell-free preparation from V.

alcalescens, the remaining enzymes of the Johns' pathway have not been elucidated.

Of primary concern in this study was an examination of the CO_2-fixation step (reaction 3), a clear demonstration of which would be strong confirmation of the oxaloacetate pathway for propionate formation in V. parvula. Because of the CO_2-pyruvate exchange (reaction 18), pyruvate-3-C^{14} was used as the substrate and the distribution of C^{14} in the various end-products examined. The reaction mixture included cell-free extract prepared by sonic disruption at a concentration of 5.0 mg protein/ml and 10 umoles of potassium pyruvate-3-C^{14} (specific activity, 102.02×10^6 dpm/mmole) incubated in an atmosphere of 5% CO_2 + 95% N_2. Samples were removed at intervals over a 40 min. period and the concentration of the end-products and remaining substrate determined.

As a preliminary step, a small portion of the 10 minute sample from the reaction mixture was run on a small 2 inch silica gel G thin layer plate to determine the general distribution of radioactivity. Following development of the plate with an ethyl ether-formic acid (7:1) solvent, the plate was scanned in a chromatrogram scanner to locate the radioactivity. Such a scan showed that large quantities of C^{14} were present in the pyruvate and acetate regions of the chromatrogram

with smaller amounts in oxaloacetate near the origin (Figure 10). To confirm the presence of oxaloacetate, 1% acidic 2, 4, dinitrophenol hydrazine was added to another portion of the same 10 minute sample to precipitate all the keto acids. The precipitate thus obtained was extracted by ethyl acetate and a sample of the ethyl acetate-extract applied to thin layer plates, developed and analyzed as previously described. The results from these plates confirmed that pyruvate was readily converted to oxaloacetate by cell-free extracts of V. parvula incubated in an atmosphere of 5% CO_2 and nitrogen.

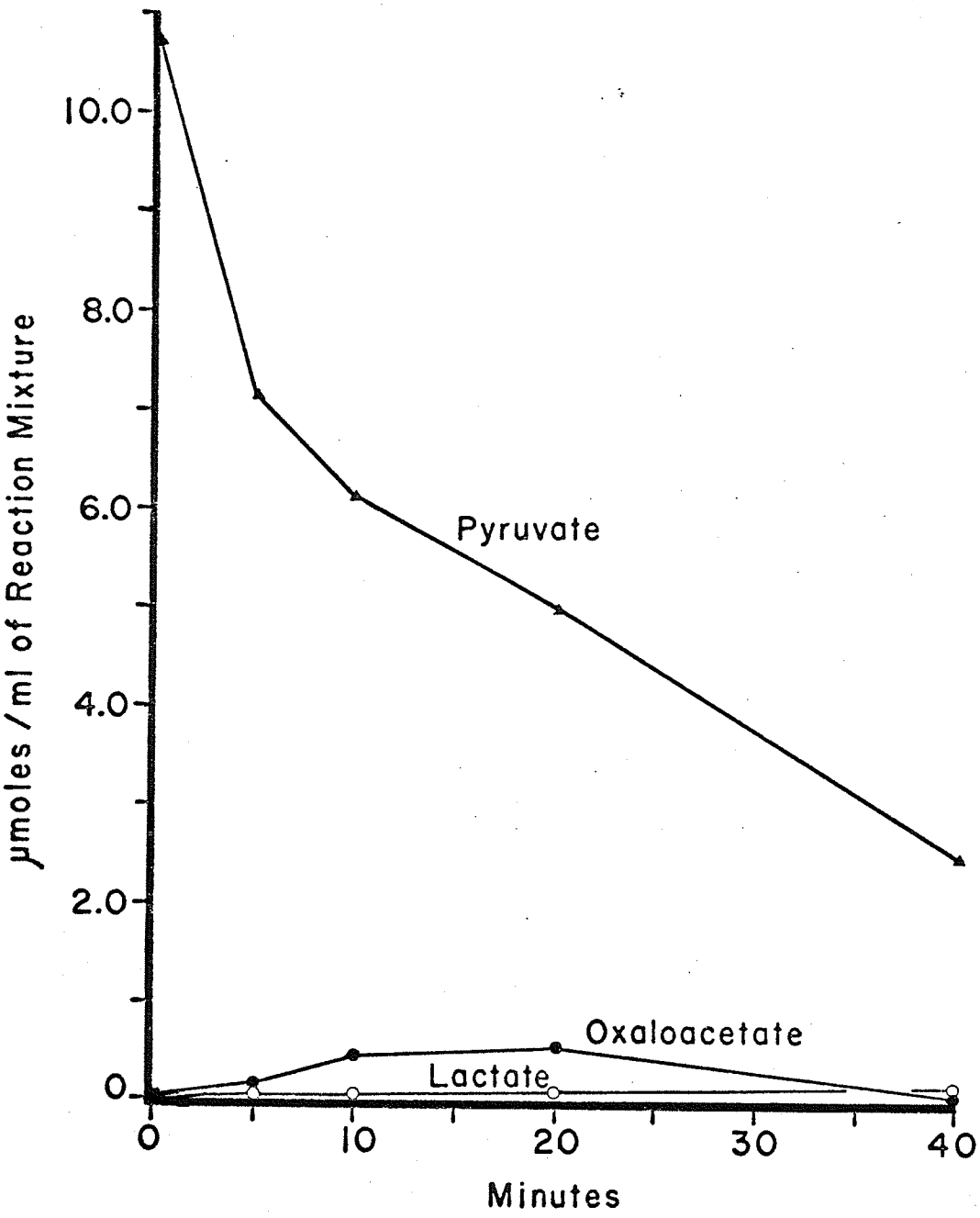
Figure 10 indicates that small quantities of malate, fumarate and succinate were also formed during pyruvate metabolism, as small peaks of radioactivity were observed at the Rf's for each of these acids. However, as the radioactivity in these peaks was relatively small compared to that for pyruvate and acetate, no definite conclusion could be made as to the presence of these dicarboxylic acids as intermediates in propionate synthesizing pathway.

As shown in Figure 11, although pyruvate was not completely utilized in the 40 min incubation period, degradation was characterized by a rapid initial rate of utilization (8. 64 μmoles/mg protein/hr) during the first 5 min period followed by a slower second phase (1. 44 μmoles/mg/hr) during the rest of the experiment. Very small amounts of

Figure 11

Degradation of pyruvate-3-C^{14} by cell-free extracts of V. parvula in an atmosphere of 5% CO_2 + 95% N_2.

Cell-free extracts of V. parvula (5.0 mg protein/ml) was incubated in pH 6.5, 0.1 M phosphate buffer with 10 mM pyruvate-3-C^{14} (sp. act. 102.02 $\bar{x}$ 10^6 dpm/mmole) to a total volume of 12 ml. Samples of 2 ml were taken at various time intervals and the reactions stopped by the addition of 1 ml to 2,4 dinitrophenol hydrazine and the remaining 1 ml to 25% $ZnSO_4$. Sample analyses were carried out as described in the Methods section.


10.0
8.0
6.0
4.0
2.0
0
μmoles / ml of Reaction Mixture
Pyruvate
Oxaloacetate
Lactate
0
10
20
30
40
Minutes

lactate were formed during this metabolism (final concentration of 0.14 mM) while oxaloacetate initially increased for the first 20 minutes and then decreased during the remainder of the incubation period.

Partition chromatographic analysis of the 5 and 40 minute samples from the pyruvate-3-C^{14} experiment demonstrated that in addition to minor quantities of oxaloacetate and lactate, most of the pyruvate carbon was converted to acetate and propionate (Table 14). The amount of propionate produced was very small (3.0 and 3.6 mmoles/100 mmoles pyruvate), while much larger quantities of acetate were formed (95.0 and 86.6 mmoles/100 mmoles pyruvate). Only 3.6 - 4.0% of the pyruvate carbon was found in propionate, while 94.6 - 95% of this substrate was present in acetate. Calculation of the P/A ratio for the two samples showed a slight increase from 0.032 at 5 minutes to 0.042 at 40 minutes as more propionate was formed in the later stages of the experiment.

Comparison of the specific activities for propionate and acetate indicate that while the value for acetate remained essentially the same as that for the substrate pyruvate, the specific activity for propionate was half the original value. This decrease in the specific activity of propionate would have occurred had small quantities of endogenous intermediates been present in the cells prior to extract preparation.

Table 14

Anaerobic metabolism of pyruvate-3-C^{14} by cell-free extracts of V. parvula in an atmosphere of 5% CO_2 in nitrogen.

	Time (minutes)	mmoles/100 mmoles pyruvate metabolized	Specific Activity (dpm/mmole x 10^6)	Specific Acticity Ratio*	Percent contribution of pyruvate carbon
PROPIONATE	5	3.0	54.0	0.53	3.6
	40	3.6	51.0	0.50	4.0
ACETATE	5	05.0	100.6	0.99	95.0
	40	86.6	100.9	0.99	94.6
P/A RATIO	5	0.032			
	40	0.042			

* Specific Activity Ratio = $\frac{\text{Specific activity of product}}{\text{Specific activity of pyruvate-3-}C^{14}}$

Conditions as described for Figure 11. (Specific activity of pyruvate-3-C^{14} = 102.02 x 10^6 dpm/mmole).

Although it was readily shown that oxaloacetate was formed from pyruvate-3-C^{14} by carbon dioxide fixation with cell-free extracts of V. parvula, the exact nature and rate of this reaction was unknown. In fact, it was not known whether pyruvate was indeed the immediate substrate for this process. To obtain more information on the nature of the carbon dioxide fixation reaction in V. parvula, the kinetics of oxaloacetate formation from lactate, pyruvate and phosphoenolpyruvate in the presence of bicarbonate was determined by converting the oxaloacetate to malate in the presence of NADH and recording the rate of NAD formation spectrophotometrically at 340 mμ.

As shown in Table 15, when lactate was supplied as the substrate, no net oxidation of NADH was observed suggesting that when lactate was the sole substrate, production of oxaloacetate did not occur. This is not surprising in view of the previous results that lactate was not utilized by cell-free extracts (Figure 8(A)). However, when pyruvate was added to the system, oxaloacetate was produced at the rate of 10 mμmoles per hour. This result supports previous data that cell-free extracts of V. parvula could form oxaloacetate from pyruvate and carbon dioxide.

When phosphoenolpyruvate was added as the substrate, oxaloacetate was formed at almost six times the rate (58 mμmoles/hr) observed with

Table 15

Oxaloacetate formation from lactate, pyruvate and phosphoenolpyruvate by cell-free extracts of V. parvula in the presence of sodium bicarbonate.

Addition to Basic System (a)		Rate Oxaloacetate Formation (b) (mμmoles/hr)
Substrate	Cofactors	
Lactate	-	0
Pyruvate	-	10
Pyruvate	ATP	10
Phosphoenol-pyruvate	-	58
Phosphoenol-pyruvate	ADP	58

(a) Basic System: V. parvula cell-free extract at a final concentration of 5 mg protein/ml incubated with 30 mumoles NADH; malic dehydrogenase, 12.5 ug (activity = 720 units/mg); substrates (10 μmoles final concentration) in pH 6.5 phosphate buffer containing 10^{-2} M sodium bicarbonate.

(b) Oxaloacetate formation as mμmoles NADH oxidized to NAD per hour.

(c) Cofactor concentration = 1.5 μmoles per assay.

pyruvate. No change was observed in the rate of oxaloacetate formation upon the addition of 1.5 μmoles ADP, indicating a cofactor-dependent carboxylase was not involved in this carbon fixation. The addition of 1.5 μmoles to the pyruvate reaction mixture had no effect on the CO_2 fixation reaction suggesting that pyruvate was not converted to phosphoenol-pyruvate in significant quantities.

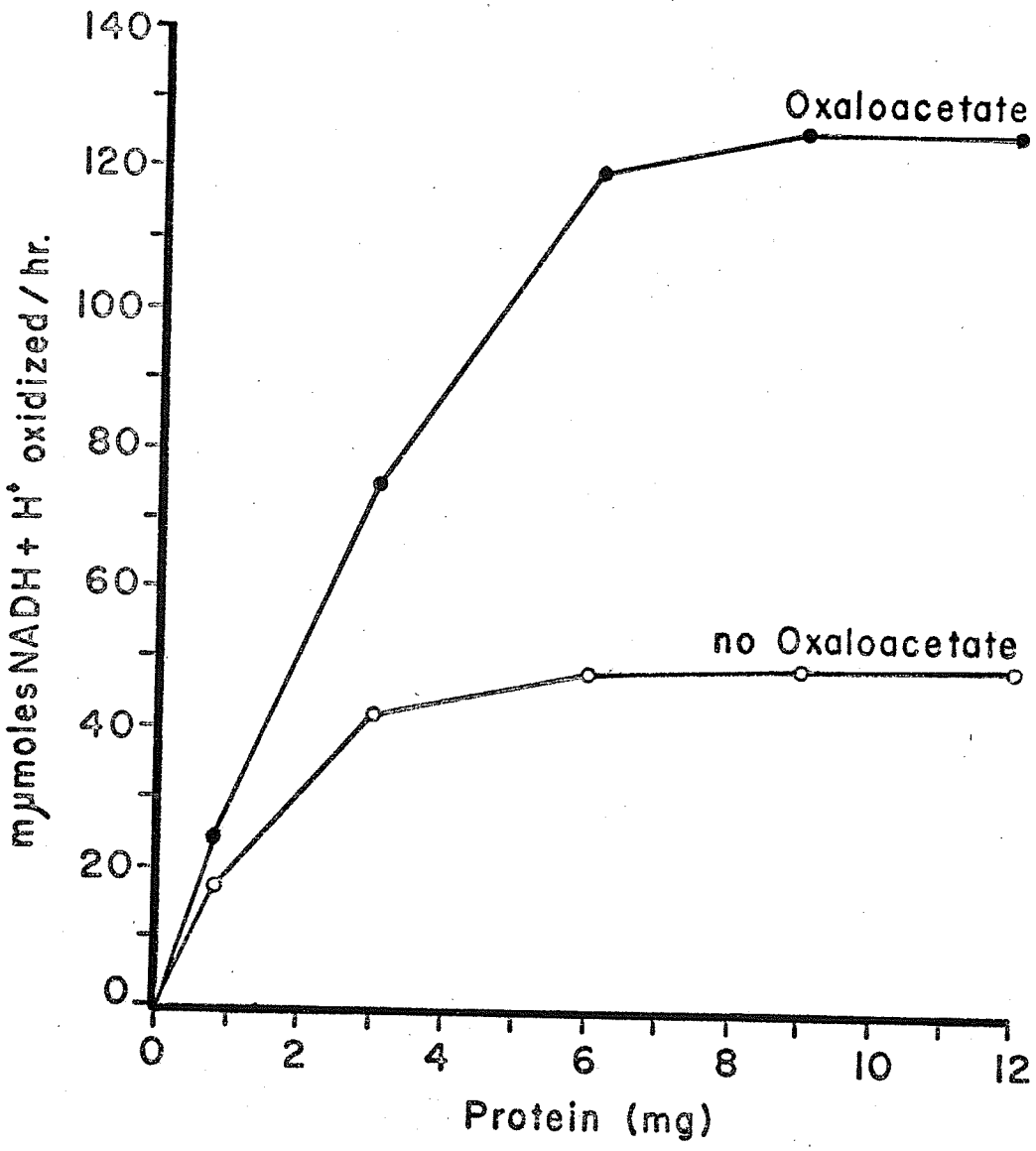
The values given in Table 15 were obtained after substracting the endogenous rate of NADH oxidation, which in many preparations was substantial. This can be seen in Figure 12, where increasing concentrations of a V. parvula extract preparation was incubated with NADH (30 mμmoles) without commercial malic dehydrogenase and the endogenous oxidation recorded (lower curve). At concentration above 9.0 mg protein, a constant rate of 49 mumoles NAD was formed per hour from endogenous substrates. The rate of oxidation was increased to 126 mμmoles/hr upon the addition of 10 μmoles oxaloacetate (upper curve) indicating that the V. parvula extract preparations contained malic dehydrogenase.

The zero order rate of endogenous NADH oxidation in the presence of both V. parvula and commercial malic dehydrogenase carried out as a control for the experiment in Table 15 was 82 mμmoles NAD formed per hr indicating that significant quantities of endogenous oxaloacetate was

Figure 12

The utilization of oxaloacetate and the endogenous oxidation of NADH by cell-free extracts of V. parvula.

Conditions: Increasing amounts (0-12 mg) of a cell-free extract from V. parvula was added to a cuvetter containing 0.1 M, pH 6.5, phosphate buffer containing 10^{-2} M sodium bicarbonate, NADH (30 mμmoles) with or without 10 μmoles oxaloacetate. The zero order rate of NADH oxidation was read and recorded at 340 mμ in a DB-spectrophotometer.


140
120
100
80
60
40
20
0
mμmoles NADH + H⁺ oxidized / hr.
Oxaloacetate
no Oxaloacetate
0
2
4
6
8
10
12
Protein (mg)

present in the extract preparation.

The cell-free preparations used in this study varied in enzymatic activity with respect to carbon dioxide fixation such that consistent results were often difficult to obtain. This variability undoubtedly reflects inadequate technical procedures involved in the spectrophotometric assay.

DISCUSSION

As shown in Part II, oxygen had an inhibiting influence on lactate metabolism by washed cells of V. parvula, which necessitated a variety of modifications in procedure. However, cell-free extracts prepared from these cells often gave unconsistent results when studied under identical conditions to those employed for successful cell experiments. Early experiments demonstrated that extracts were more sensitive to oxygen inhibition than cells and brief exposure of the extract preparation to air reduced activity considerably. The method of cell breakage was found to be a critical procedure in this regard. Although, both the Raytheon sonication and the French pressure cell methods were equally efficient in breaking cells, the sonic disruption was used because by this method the cells were never exposed to atmospheric oxygen. High purity nitrogen was flushed over the cells in the Raytheon

chamber to prevent oxidation during the disintegration period, a procedure which could not be readily applied to the French pressure cell.

In addition to exposure to oxygen, the activity of the cell-free extracts of V. parvula was also affected by the temperature of cell suspension during breakage. When the cell compartment of the Raytheon sonic oscillator was cooled to 10 C by circulating ice-water, the activity of the cell-free extracts obtained after breakage exhibited low activity. However, when the cell compartment was maintained at 0 - 3 C by means of a refrigerated circulating pump, the preparations had consistently higher enzymatic activities.

To date, most of the discussion on lactate metabolism by Veillonella species has been centered on the oxaloacetate scheme proposed by Johns (1951 b). In this pathway, one of the most important steps is the conversion of pyruvate to oxaloacetate by carbon dioxide fixation. However, little enzymatic evidence has been obtained in past investigations, demonstrating the formation of oxaloacetate from lactate or pyruvate by species of the veillonella.

Evidence obtained in this study with V. parvula (Part II), has demonstrated both carbon dioxide fixation and the pyruvate-CO_2 exchange reaction in resting cells of this organism. However, like

previous investigations, these results only served to demonstrate that carbon dioxide was incorporated into the final end-product, propionic acid. The data did not indicate whether oxaloacetate was an intermediate in the propionate pathway. Experiments with cell-free preparations were required to definitely establish this information.

When pyruvate-3-C^{14} was metabolized by cell-free extracts in an atmosphere of 5% CO_2 + 95% N_2, three main products were formed: propionate, acetate and carbon dioxide. However, further analysis of the samples obtained in this time course study showed that small quantities of oxaloacetate and lactate were also formed within the 40 minute incubation period (Fig. 10). Oxaloacetate increased steadily in the first 20 minutes and then decreased again in the latter periods of incubation suggesting that oxaloacetate, while being formed from pyruvate, was also being metabolized to form an end-product. Oxaloacetate thus would appear to act as an intermediate in the propionate pathway. The initial accumulation of the oxaloacetate would indicate that at rapid rates of pyruvate degradation, malic dehydrogenase was the limiting factor in the removal of this compound.

Lactate accumulation was very small compared to oxaloacetate. Although the amount of lactate produced was very small, the net amount of this acid increased during the incubation period suggesting

that lactate was not produced as an intermediate in the metabolism of pyruvate by cell-free extracts of V. parvula, but was an end-product. This is presumptive evidence that lactate is not converted to propionate via the acrylate pathway (Cardon and Barker, 1947).

In bacteria, oxaloacetate formation by carbon dioxide fixation is known to occur by three reactions. Krampitz, Wood and Werkman (1943) demonstrated carbon dioxide fixation in M. lysodeikticus, catalyzed by oxaloacetate decarboxylase (oxaloacetate carboxy-lyase (EC 4. 1. 1. 3)), which utilized pyruvate as the substrate, but was independent of NAD, NADP (TPN) and ATP.

$$\text{pyruvate} + CO_2 \rightleftharpoons \text{oxaloacetate} \qquad (3)$$

Later, Suzuki and Werkman (1958) demonstrated another reaction for carbon dioxide fixation in Thiobacillus thiooxidans, which involved phosphoenolpyruvate instead of pyruvate. This reaction was mediated by the enzyme orthophosphate: oxaloacetate carboxy-lyase (phosphorylating) (EC 4. 1. 1. 31), which like oxaloacetate decarboxylase, did not require pyridine nucleotides or high energy compounds:

$$\text{phosphoenolpyruvate} + CO_2 \xrightarrow{Mg^{++}} \text{oxaloacetate} + P_i \qquad (40)$$

Recently, Siu and Wood (1962) demonstrated in Propionibacterium

shermanii, yet another reaction which also converted phosphoenolpyruvate and carbon dioxide to oxaloacetate, but required inorganic phosphate (P_i). The fixation observed in this organism was catalyzed by the enzyme pyrophosphate: oxaloacetate carboxy-lyase (phosphorylating) (EC 4. 1. 1. 38):

$$\text{phosphoenolpyruvate} + CO_2 + P_i \xrightleftharpoons{\text{Mg++ or Mn++}} \text{oxaloacetate} + PP_i \qquad (41)$$

In general, the reactions catalyzed by oxaloacetate decarboxylase and pyrophosphate: oxaloacetate carboxy-lyase (phosphorylating) are reversible, while the reaction catalyzed by orthophosphate: oxaloacetate carboxy-lyase (phosphorylating) is not.

Although the primary objective of the present investigation was not concerned with the mechanism of carbon dioxide fixation, the results in Table 15, permit some conclusions as to the possible mechanism of fixation in V. parvula. The marked increase in NADH oxidation when phosphoenolpyruvate was substituted for pyruvate suggests that oxaloacetate was more readily formed from phosphoenolpyruvate than pyruvate even with the addition of ATP. This data coupled with the fact that acetate was formed from oxaloacetate (Table 13) suggests that the CO_2-fixing reaction in V. parvula is reversible.

Therefore, it is possible that the enzyme responsible for carbon dioxide fixation in extracts of V. parvula is pyrophosphate: oxaloacetate carboxy-lyase (reaction 41) and not the irreversible orthophosphate: oxaloacetate carboxy-lyase (reaction 40).

The formation of acetate from oxaloacetate in this organism, therefore, might occur by the following reactions:

$$\text{oxaloacetate} + \text{PPi} \rightleftharpoons \text{phosphoenolpyruvate} + CO_2 + \text{Pi} \quad (41)$$

$$\text{phosphoenolpyruvate} \rightleftharpoons \text{pyruvate} + \text{Pi} \quad (42)$$

$$\text{pyruvate} \longrightarrow \text{acetate} + CO_2 + H_2 \quad (2)$$

$$\text{oxaloacetate} \longrightarrow \text{acetate} + CO_2 + H_2 \quad (43)$$

When the specific activities of both propionate and acetate are compared with that of pyruvate-3-C^{14} after its metabolism by cell-free extracts of V. parvula (Table 14), the specific activity ratios indicate that endogenous metabolism contributed significantly to the formation of propionic acid. The propionate specific activity ratios (Table 14) of 0.53 and 0.50 for the 5 and 40 minutes samples, respectively, indicate that slightly more than half of the propionate formed originated with endogenous substrates present in the cell-free extracts. The most probable endogenous source involved in this metabolism is either oxaloacetate or α-ketoglutarate or both. The conversion of oxaloacetate

to pyruvate

to propionate by the veillonella is well-known, while Whiteley and Ordal (1957) have reported that cell-free extracts from M. lactilyticus (V. alcalescens) were capable of metabolizing α-ketoglutarate. V. parvula probably utilizes α-ketoglutarate in a manner similar to that for V. alcalescens. In addition, oxaloacetate and α-ketoglutarate are readily obtained from the two amino acids, asparate and glutamate, important components of proteins. The glucogenic effect of amino salts is well-known and has been studied in species of the veillonellae by Rogosa (personal communication), who observed that most species of this genus were capable of utilizing asparate and glutamate for energy.

Although Johns (1951 b) postulated that lactate dehydrogenase was involved in the initial conversion of lactate to pyruvate, the results obtained with resting-cells of V. parvula (Part II) and with cell-free extracts confirmed the presence of malate-lactate transhydrogenase (Allen, 1966) in this organism (Fig. 8). However, in order for lactate to be metabolized by this enzyme, catalytic amounts of oxaloacetate are required (reaction 15):

$$\text{lactate} + \text{oxaloacetate} \rightleftharpoons \text{pyruvate} + \text{malate} \qquad (15).$$

As oxaloacetate does not normally accumulate as an intermediate in metabolic pathways in living organisms, some method of generating this

compound is required. Allen (1966) postulated that because malate dehydrogenase was also present in the M. lactilyticus system, this enzyme would couple with the transhydrogenase to give a net production of pyruvate from lactate:

lactate + oxaloacetate ⇌ pyruvate + malate (15)

malate ⇌ oxaloacetate (4)

lactate ⇌ pyruvate (1)

As there is now evidence for both malic-lactic transhydrogenase and malate dehydrogenase in extracts of V. parvula, it is logical to conclude that the lactate metabolizing system of V. parvula is the same as that for V. alcalescens. However, imspection of the kinetic data by Allen (1966) for the substrates and products of the malic-lactic transhydrogenase reaction in M. lactilyticus (V. alcalescens) shows that the generation of oxaloacetate from malate would not readily occur in an actively metabolizing systems (Table 16). The K_m for oxaloacetate was approximately 40 times lower than that for the other substrates, such as pyruvate, lactate and malate, and the K_{eq} for the reaction is in favour of malate and pyruvate formation. Therefore, the coupling reaction between the transhydrogenase and malic dehydrogenase does not appear a likely method of oxaloacetate regeneration. A more likely

Table 16

Kinetic constants for the reaction catalyzed by malic-lactic transhydrogenase enzyme from M. lactilyticus (V. alcalescens).

Reactant	K_m (x 10^{-5} M)
Lactate	190
Oxaloacetate	5
Pyruvate	240
Malate	140

$$K_{eq} = \frac{\text{(malate) (pyruvate}}{\text{(oxaloacetate) (lactate)}} = 1.8.$$

Reference: Allen, 1966.

possibility is that the pyruvate formed from the transhydrogenase reaction is converted to oxaloacetate by carbon dioxide fixation (reaction 3), which has been shown to readily occur in cells and to a lesser extent in extracts of V. parvula.

Examination of acetate production by extracts of V. parvula, incubated with pyruvate-3-C^{14}, demonstrated that the specific activities for acetate and the substrate pyruvate-3-C^{14} were almost identical suggesting that acetate was formed from the direct decarboxylation of pyruvate. The mechanism of this reaction could be similar to that postulated by Whiteley and McCormick (1963) for the decarboxylation of pyruvate in M. lactilyticus.

$$\text{pyruvate} + \text{Pi} \rightleftharpoons \text{acetyl-phosphate} + CO_2 + H_2 \quad (27)$$

The acetyl-phosphate thus formed would then be hydrolyzed to inorganic phosphate and acetate. Using the information obtained with M. lactilyticus and other organisms, these workers postulated the following sequence of reactions for pyruvate degradation:

$$\text{pyruvate} + \text{DPT} \rightleftharpoons \text{lactyl-DPT} \rightleftharpoons \text{hydroxyethyl-DPT} + {}'C'_1 \quad (44)$$

$${}'C'_1 \rightleftharpoons CO_2 \quad (45)$$

hydroxyethyl-DTP + electron carrier $\rightleftharpoons$ acetyl-DPT + reduced carrier (46)

reduced electron carrier $\rightleftharpoons$ H_2 + electron carrier (47)

acetyl-DPT + CoA $\rightleftharpoons$ acetyl-CoA + DPT (48)

acetyl-CoA + Pi $\rightleftharpoons$ acetyl-phosphate + CoA (49)

pyruvate + Pi $\rightleftharpoons$ acetyl-phosphate + CO_2 + H_2 (27)

In this scheme, pyruvate is initially cleaved to a C_2 and C_1 compound (equation 44) by a reaction which may involve the formation of a lactyl-DPT (lactyl-diphosphothiamine) complex (Holzer and Beaucamp, 1961). The one carbon or active 'C' moiety in the sequence is thought to be enzyme-bound, which could be released directly as CO_2 (reaction 46) as in reactions mediated by pyruvic carboxylase. Such a reaction would be similar to the CoA-dependent activation of CO_2 proposed for C. butyricum (Mortlock et al, 1959) and the release of carbon dioxide from the CO_2-biotin-enzyme complex by C. kluyveri (Shuster and Lynen, 1960) and C. saccharobutyricum (Delavier-Klutchko, 1961).

Reactions 46 to 49 represent the oxidation of the C_2 fragment and its transfer to phosphate, by a process which involves the oxidation of an electron carrier. Either FAD or ferrodoxin (Mortenson et al, 1962) postulated as the electron carrier. However, Whitely and McCormick (1963) demonstrated that ferredoxin increased the rate of pyruvate

decarboxylation when substituted for pyridine nucleotides and FAD suggesting it may be the natural electron acceptor involved.

This observation has suggested that the system operating in M. lactilyticus is similar to that of C. pasteriuanum (Mortenson et al, 1962) where ferridoxin is involved in hydrogen evolution. In the clostridia, reduced ferridoxin can transfer its electrons either to other electron acceptors, such as NAD or FAD, or to the enzyme, hydrogenase. Whether such a system was also operating in the veillonellae is not known, but Witter (1953) isolated a hydrogenase enzyme from M. lactilyticus which was coupled to an iron-containing electron acceptor, which activated protons to form molecular hydrogen.

Reducing power is required for the production of propionate from lactate or pyruvate as this end-product is more reduced than either of these substrates. As less reducing power would be required to convert lactate to propionate compared to pyruvate, one would expect more propionate from lactate than from pyruvate. Such a conclusion was confirmed by experimental evidence. When lactate was the substrate, 68.7 mmoles of propionate were produced per 100 mmoles of lactate compared to 34.8 mmoles from the same concentration of pyruvate. The presence of a more reduced substrate, therefore, had a substantial effect on propionate production causing a decrease in the P/A ratio 1.38

to 0.46, when the substrate was changed from lactate to pyruvate (Table 10).

The data obtained in fermentation studies with resting cells and extracts of V. parvula permit certain conclusions concerning the source and transfer of electrons required for propionate formation in this organism. In Table 6, 66 mmoles of propionate were produced from 100 mmoles of lactate by cells of V. parvula. To produce this propionate according to the oxaloacetate scheme, 132 mmoles of reduced pyridine nucleotide equivalents or 132 electron pair ($2\ e^-$) equivalents would be required.

Of the reaction in the oxaloacetate scheme, two reactions can provide this reducing power. The first of these is the conversion of lactate to pyruvate via the malic-lactate transhydrogenase enzyme (reaction 15). As shown in Table 6, as 100 mmoles of lactate were utilized in the malic-lactic transhydrogenase reaction 100 mmoles of pyruvate must have been formed as a metabolizable intermediate. However, the other portion of this coupled reaction, the oxaloacetate malate conversion only produced 66 mmoles of propionate.

As the conversion of each molecule of lactate results in the formation of NADH-enzyme, which in turn reduces oxaloacetate to malate, a net production of NADH-enzyme or reduced enzyme must

have occurred during this fermentation. Logically, although this reduced pyridine nucleotide is enzyme-bound (Allen, 1966), its reducing power should be readily available for the reduction of other compounds, such as the conversion of fumarate to succinate:

$$\text{fumarate} + \text{NADH} \rightleftharpoons \text{succinate} + \text{NAD} \qquad (6)$$

However, in order for this to happen, one must postulate the involvement of an electron acceptor, which has a redox potential near that for the NADH bound to the transhydrogenase. This postulated electron acceptor might also be linked to another or several other electron acceptors to form an electron transport system with fumarate being the ultimate acceptor. Such a simple hypothetical scheme is outline in Figure 13.

The net number of electron pairs available from the reduced form of the malic-lactic transhydrogenase would be 24 mmoles which would be utilized to form 34 mmoles of propionate at the fumarate step. Of the remaining 32 nucleotide equivalents of reducing power required for propionate formation, 26 would be available from the phosphoroclastic degradation of pyruvate (reaction 2). With cells of V. parvula (Table 7), 100 mmoles of lactate produced approximately 40 mmoles of acetate, and carbon dioxide, but only 14 mmoles of hydrogen. Stoichiometrically, one mole of pyruvate should produce one mole each of acetate, hydrogen

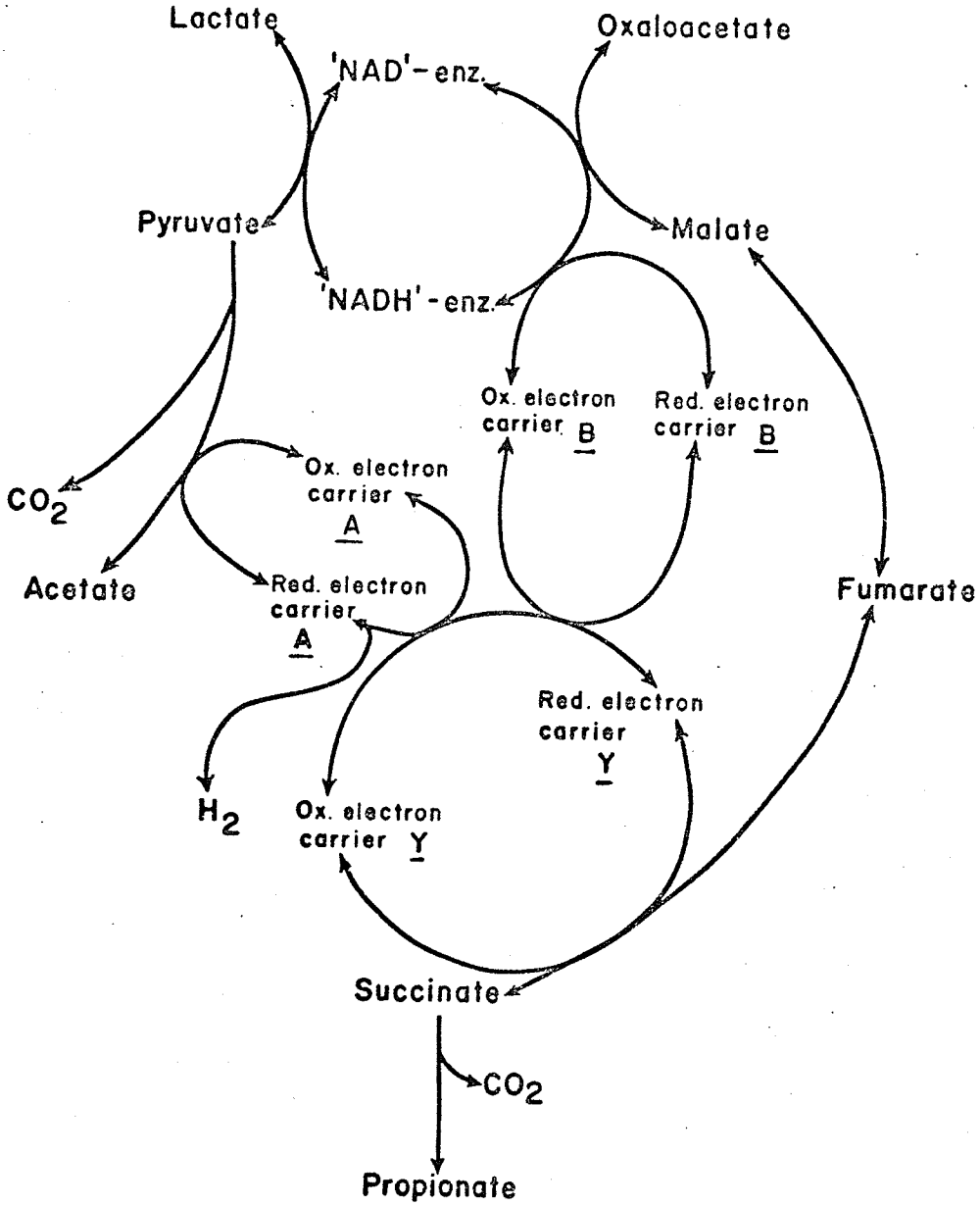
Figure 13

Hypothetical scheme for the production and transport of reducing power in V. parvula.

Legend 'NAD'- enzyme: the pyridine nucleotide-like moiety which is bound to the malic-lactic transhydrogenase enzyme.

electron carriers A: the ferredoxin-like electron carrier which is involved in the phosphoryclastic reaction of pyruvate. It can either transfer its' electrons to other electron carriers or to the enzyme, hydrogenase.

electron carrier B and Y: hypothetical electron carriers.


Lactate
'NAD'-enz.
Oxaloacetate
Pyruvate
Malate
'NADH'-enz.
Ox. electron carrier B
Red. electron carrier B
CO_2
Ox. electron carrier A
Acetate
Red. electron carrier A
Fumarate
Red. electron carrier Y
H_2
Ox. electron carrier Y
Succinate
CO_2
Propionate

and carbon dioxide, thus 40 mmoles of hydrogen should have been formed. The deficit in H_2 production undoubtedly meant that 26 electrons pair equivalents (40 - 14 = 26) were utilized for the reduction of fumarate by transporting the electrons initially via an electron carrier such as ferredoxin, which is present in the veillonellae and is coupled to hydrogenase in other bacteria (Mortenson et al 1962). However, the addition of the 26 electron pair equivalents from the pyruvate coupled with the 34 from the transhydrogenase reaction (34 + 26 = 60) leaves a requirement of 6 reduced equivalents. As cells of V. parvula were grown in lactate broth and readily assimilate this substrate, it is postulated that the 6 reduced equivalents probably arose from the utilization of 6 mmoles of endogenous lactate.

Table 17 outlines the hypothetical sources of reducing power involved in the formation of propionate from lactate metabolism. It can be seen that a small amount of endogenous lactate metabolism (line 2) would explain the fermentation results in Table 7.

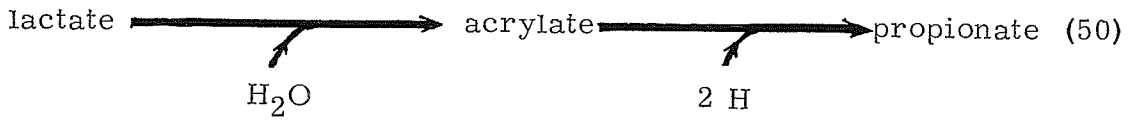
In general, the results obtained in Parts II and III of this investigation support in part the oxaloacetate scheme proposed by Johns (1951 b). However, the evidence provided by previous investigations (Whiteley, 1953 a, b, a; 1957; Delwiche, Phares and Carson, 1954, 1956; Allen and Galivan, 1965) did not exclude the possibility that another alternative

Table 17

Source of reducing power for the production of propionate from lactate by cells of V. parvula

No.			Reducing power
1.	100 lactate + 100 NAD-enz. ⟶ 100 pyruvate + 100 NADH-enz.	106 pyruvate	+ 100 NADH-enz.
2.	endogenous: 6 lactate + 6 NAD-enz. ⟶ 6 pyruvate + 6 NADH-enz.		+ 6 NADH-enz.
3.	66 pyruvate + 66 CO_2 ⟶ 66 oxaloacetate		+ 106 NADH-enz.
4.	66 oxaloacetate + 66 NADH-enz. ⟶ 66 malate + 66 NAD-enz.		- 66 NAD-enz.
5.	66 malate ⟶ 66 fumarate		+ 40 NADH-enz. or 40 electron pairs
6.		40 pyruvate ⟶ 40 acetate + 40 CO_2 + 14 H_2	+ 26 electron pairs (40 - 14 = 26)
		66 electron carriers	+ 66 electron pairs
7.	66 fumarate + 66 (electron carriers) ⟶ 66 succinate + 66 oxid. elec. carriers		- 66 electron pairs
8.	66 succinate ⟶ 66 CO_2 + 66 propionate		
Net	100 lactate + (6 endo lactate) ⟶	66 propionate + 40 acetate + 40 carbon dioxide + 14 hydrogen	0 electron pairs

propionate producing pathway similar to the acrylate scheme (reaction 50) proposed for C. propionicum (Cardon and Barker, 1947) also existed in Veillonella species:



(50)

In experiments with cell-free extracts of V. parvula direct evidence eliminating the acrylate scheme as a pathway for propionate formation was not obtained. However, the time course accumulation of small quantities of lactate, when extracts were exposed to pyruvate-3-C^{14} (Figure 11), indicates that lactate was not metabolized for the formation of a compound, such as acrylate. From this evidence one can conclude that propionate formation in cells of V. parvula occurs solely by the oxaloacetate scheme proposed in reactions (1) to (7).

PART IV

MIXED CULTURE STUDIES WITH VEILLONELLA PARVULA AND STREPTOCOCCUS SALIVARIUS

INTRODUCTION

Studies by Krasse (1953), Morris (1953 a, b; 1954 a, b), Morris et al (1954) and Bisset & Davis (1960) have demonstrated that the oral microflora of the human is a collection of specialized parasites existing in a variety of ecological microcosms, which are not homogenous in composition but vary according to the particular environment. This variability in composition was again demonstrated in studies on the gingival crevice area (Gibbons et al, 1963) the surface of the tongue (Gordon & Gibbons, 1966) and dental plaque (Gibbons et al, 1964).

Although the bacterial composition varied in the different areas of the mouth, Bibby (1939) observed that in bacterial smears from a given area of the mouth, groups of organisms tended to occur with the same relative frequency suggesting that the microbial population was in a state of equilibrium. Appleton (1940), after elaborate study on the ecological problems of oral microbiology, concluded that this state of equilibrium was a dynamic one. In this study, changes in diet, the eruption of the deciduous teeth and their replacement by the "permanent" set, changes in the state of health or disease, artificial hygienic measures or their neglect, the nature of the reparative procedures of operative and prosthetic dentistry and the loss of teeth, were all observed to influence microflora of the mouth qualitatively and

quantitatively.

Appleton concluded that, in reality, there was throughout life a succession of biotas, whose respective end-products and beginnings were not sharply marked. This gradual change in microflora was demonstrated recently by Ritz (1967) while studying the shifts in both the composition and the numbers of bacteria in the microbial populations in developing dental plaque. During the early stages of the plaque formation on cleaned tooth surfaces, members of the streptococci, neisseriae and nocardiae appeared to predominate. However, nine days after the start of the experiment, the flora shifted to the streptococci, actinomyces, veillonellae and corynebacteria, Ritz noted that the growth of anaerobic microorganisms, such as the veillonellae and fusobacteria, was dependent upon the prior growth of facultative organisms which caused an increase in plaque thickness yielding reduced conditions suitable for anaerobic growth.

During his investigation of plaque formation, Ritz observed that species of Neisseria, when incubated aerobically on plates, were occasionally contaminated with slender rods having pointed ends characteristic of species of fusobacteria, which were incapable of growing by themselves under aerobic conditions. This saprophytic interrelationship between different organisms may be characteristic of

many oral microorganisms, as Appleton recognized the existence of an interacting phenomena between bacteria in the oral cavity in his earlier investigation in 1940. However, although he was aware of such bacterial interrelationships he concluded that the nature of this interaction in a succession of biotas and the factors which influenced changes in these populations were unknown. This lack of knowledge apparently persisted throughout the next two decades prompting Rosebury, Gale and Taylor (1954), while reviewing the subject of interacting phenomena in the oral cavity, to conclude that the subject was poorly understood.

The slow development in the understanding of microbial interaction can be attributed to the interest of many investigators in the role of oral microorganisms in human disease. This orientation led to the collection of data for individual organisms in pure culture and naturally prevented the study of the biochemical relationships between the various bacterial groups in the mouth. However, as microbial activity is dependent upon the availability of nutrients for growth and energy, the understanding of the biochemical interactions between various members of a natural microbial population is undoubtedly an important one.

To date, most of the investigations with oral microorganisms from saliva have been taxonomic in character with only a limited number of

etc.

studies concerned with the biochemistry of these species. Except for the experiments by Stephan and Hemmens (1947) on the acid-producing ability of some oral bacteria in pure culture and in mixed cultures, investigations on the biochemical aspects have been confined to the overall metabolism of the natural mixed populations in dental plaque in vivo (Kleinberg & Jenkins, 1959; 1964; Kleinberg, 1961; 1962), in vitro (Miller, Muntz and Bradel, 1940; Muntz, 1943; Moore et al, 1956; Clements, Plothin and Fosdick, 1956; Englander, Carter and Fosdick, 1956) and in salivary-sediment preparations (Kleinberg, 1961, 1962, 1967; Sandham, 1963). Throughout all these investigations, carbohydrates were employed as the principal substrate with metabolism generally resulting in the formation of lactic acid as the major end-product. As the accumulation of this acid caused the pH of the reaction system to drop, it was proposed therefore, that the production of lactic acid from carbohydrates was the cause of tooth decay (Moore et al, 1956).

However, prior to this, Stephan & Hemmen (1947) had observed that many of the oral organisms investigated in their study possessed the ability to consume lactic acid and as the lactate was consumed, the pH of the reaction system increased. Sandham (1963) demonstrated that salivary-sediment preparations composed mainly of bacteria, metabolized lactic acid with the formation of the weaker acids, acetate and propionate.

This conversion from the stronger acid, lactic acid, to other weaker acids would then account, in part, for the increase in pH observed by Stephan and Hemmens with various oral bacteria.

In vivo increases in pH could lead to two effects in the oral cavity: (i) as the pH of the environment slowly returned to the physiological range (pH 6.8 - 7.2), many bacterial cells within the system which would normally lyse after prolonged exposure to conditions of low pH, would be spared, and (ii) a less acidic environment would limit the mobilization of calcium in tooth enamel, preserving the tooth surface and reducing the possibility of carious lesion formation. Thus, those organisms capable of lactic acid utilization could play a significant role in the overall metabolism of the microflora in human oral cavity.

One of these lactate-utilizing organisms was identified as a Veillonella species (Foubert and Douglas, 1948, a, b) and after further investigation, Douglas (1950) suggested that because organisms of this genus were not capable of fermenting carbohydrates, "it seems probable that the occurrence of M. lactilyticus (V. alcalescens) in this habitat (oral cavity) is dependent upon the presence of other bacteria that convert sugars to lactic acid".

As shown in Table 1, recent studies have shown that two main bacterial genera are present on the human tongue: species of the genus,

Streptococcus, dominant lactic acid-producing cocci and Veillonella species, which together constitute 53% of the total viable microbial counts (Gordon & Gibbons, 1966). As Gibbons, Kapsimalis and Socransky (1964) had previously shown that the source of salivary bacteria was the tongue, these bacterial species undoubtedly predominate in saliva also. The earlier work by Richardson and Jones (1958) would support the conclusion that Streptococcus and Veillonella species are the two main genera in saliva.

With these observations in mind, resting cell experiments were initiated with S. salivarius, the most predominant of the salivary streptococci, and V. parvula in an attempt to make some simple and preliminary observations on bacterial interaction under a variety of conditions. Such experiments were not undertaken in an attempt to duplicate the actual conditions in the oral cavity, but to provide a basis for the study bacterial interaction in an artificial environment.

METHODS

S. salivarius was isolated aerobically from human saliva on 5% sucrose-tryptone agar (Niven, Smiley and Sherman, 1941) by the selection of mucoid colonies. The isolated organisms were grown in a medium containing tryptone, 10.0 g; yeast extract, 5.0 g; glucose, 10.0 g; and K_2HPO_4, 3.0 g in 1000 ml of deionized water. The growth and harvesting of the S. salivarius cells was carried by procedures similar to that previously described for V. parvula except that a different buffer system (B-1) was employed. This (0.05 M, pH 7.0) buffer contained K_2HPO_4, 5.2 g; KH_2PO_4, 2.7 g; NaCl, 2.0 g; $MgSO_4 \cdot 7\ H_2O$, 80 mg; $FeSO_4 \cdot 7\ H_2O$, 4 mg; $MgCl_2 \cdot 4\ H_2O$, 1.2 mg in 1 litre of deionized water and adjusted to pH 7.0.

The growth and harvesting of the V. parvula was as previously described with the washing and suspending procedures carried out with the B-1 buffer.

Experiments were carried out in 50 ml Erlenmeyer flasks fitted with a two-hole rubber stopper such that during the incubation period, N_2 + 5% CO_2 gas could be passed into the flask via one hole and out through the other. Each reaction system consisted of 0.05 M phosphate buffer, glucose, and a cell mixture, which had been previously prepared in a separate test tube before being injected via syringe into the reaction

flask to initiate the reaction. The final volume in each flask was 20 ml and the temperature of incubation was 37 C.

At various time intervals, one ml samples were taken and added to 0.1 ml 25% $ZnSO_4$ to stop the reaction. The samples were neutralized, the cells removed by centrifugation, and the supernatant employed to measure glucose and lactate.

The pH of the reaction mixture was determined by inserting a standardized pH microelectrode into the reaction mixture immediately prior to sample removal.

Glucose was determined by the glucose oxidase method of Kingsley and Getchell (1960). In this method, aliquots of samples were pipetted into 10 x 75 mm tubes and the volume was adjusted to 200 µl with deionized water. To these samples was added 800 µl of buffer reagent solution which contained: Na_2HPO_4, 0.8409 g; KH_2PO_4, 1.119 g; peroxidase, 5.652 mg and O-dianisidine, 56.25 mg in 500 ml of deionized water. Following this procedure, 100 µl of glucose oxidase enzyme (10 mg/ml) was added to each tube to initiate the reaction. The contents of the tubes were then mixed thoroughly and incubated at 37 C for 60 minutes. After the incubation, 400 ul of 37.4% sulfuric acid was added to stop reaction. The samples were then read at 530 mµ in a Unicam SP 500 spectrophotometer. Lactate was analyzed as previously

described. The relative concentration of the other acids formed during metabolism (presumably propionate and acetate) was determined by calculation, using the following equation:

Other acids = 2 (glucose concentration) - lactate concentration (51)

RESULTS

As early as 1915, Colebrook reported inhibition of growth of Neisseria catarrhalis and certain other grain negative cocci by a pneumococcus and by streptococci. More recently, various workers had also demonstrated the inhibitory effect of salivary streptococci on various bacteria principally C. diphtheriae and staphylococci (Muhlenbach, 1939; Thompson and Johnson, 1951; Annear, 1951). Although both S. salivarius and V. parvula were both isolated from human saliva, no information is available as to the influence of S. salivarius on the metabolism of V. parvula. Furthermore, though S. salivarius and V. parvula had been individually demonstrated to be able utilize glucose and lactate, respectively, it was not known whether V. parvula would metabolize lactate produced by S. salivarius from glucose.

In an effort to examine some of these questions, a mixed culture system was established with resting cells, in which the ratio of

S. salivarius to V. parvula varied under conditions of constant cell mass (Table 18). As the cell concentration of S. salivarius was decreased from 2.0 to 0 mg dry wt./ml, the cell concentration of V. parvula was increased from 0 to 2.0 mg dry wt./ml, with the final total cell concentration 2.0 mg/ml. This experiment was to provide information as to whether the streptococcus had an inhibitory effect on V. parvula and also whether cells of V. parvula were active at high and low concentration of S. salivarius.

As shown in Figure 14 (flask A), glucose was rapidly degraded by S. salivarius alone with the concomitant production of lactic acid, while a pure culture of V. parvula (flask F) did not utilize this substrate. As the concentration of V. parvula was increased, the amount of "free" lactate in the reaction mixture decreased until in flask(E), where 90% of the cells were V. parvula, lactate was hardly detectable. The actual "free" lactate concentration decreased with increasing numbers of V. parvula, while the rate of glucose utilization also decreased. The relative concentration of the other acids formed from the degradation of lactate, on the other hand, increased in a reciprocal manner.

Figure 15 illustrates the effect of the above cell ratios on the pH of the reaction mixture during the course of the experiment. As the concentration of S. salivarius decreased, there was a proportional

Table 18

Concentration of S. salivarius and V. parvula employed in Experiment 1.

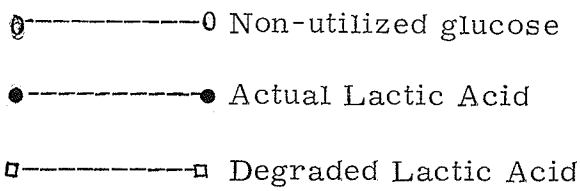
Flask	Streptococcus salivarius		Veillonella parvula	
	mg dry wt/ml	%	mg dry wt/ml	%
A	2.0	100	0	0
B	1.5	75	0.5	25
C	1.0	50	1.0	50
D	0.5	25	1.5	75
E	0.2	10	1.8	90
F	0	0	2.0	100

Total volume was 20 mls.

Figure 14

Influence of varying concentrations of washed cells of V. parvula on the glucose metabolism and subsequent lactic acid formation by resting cells of S. salivarius under conditions of constant mass.

Cells (2 mg. total dry weight per ml.) in the ratios outlined in Table 15, were incubated with 15.6 mM glucose in phosphate buffer (0.05 M, pH 7.0) at 37 C in an atmosphere of N_2 + 5% CO_2. The final volume was 20 ml. in a 50 ml. flask fitted with a rubber stopper with all procedures as described in Method.



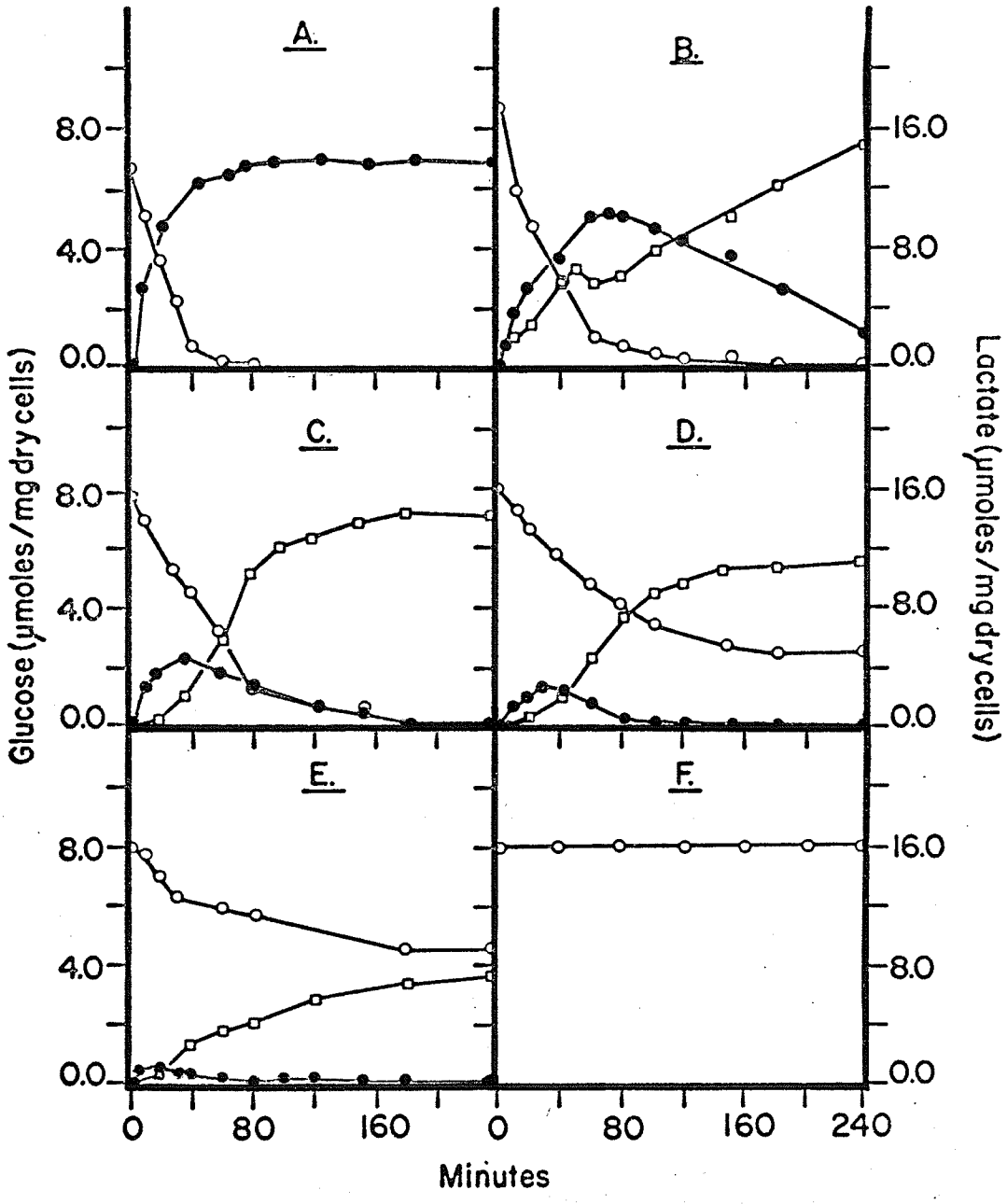
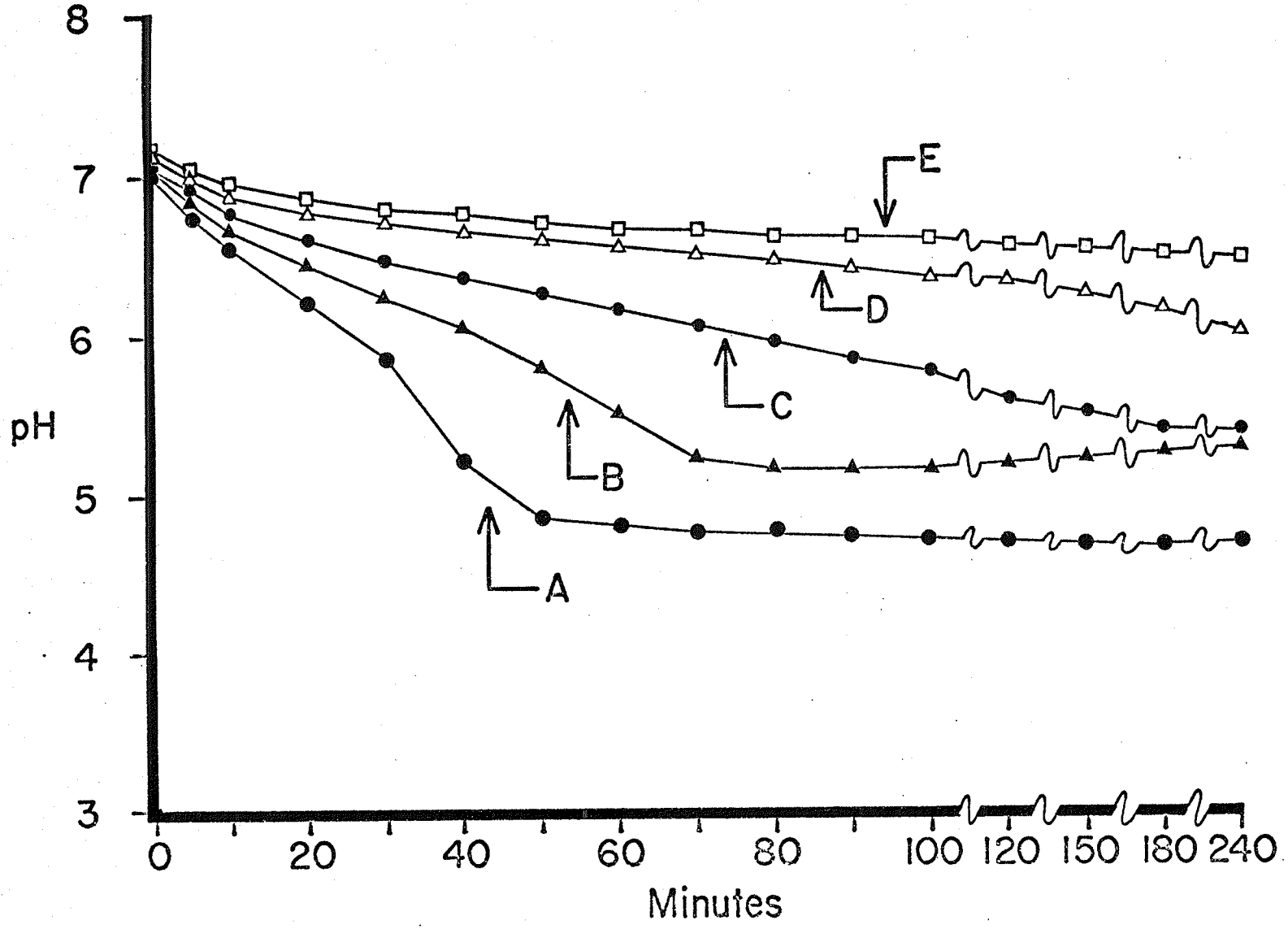

A.
B.
C.
D.
E.
F.
8.0
4.0
0.0
16.0
8.0
0.0
0
80
160
240
Minutes
Glucose (μmoles/mg dry cells)
Lactate (μmoles/mg dry cells)

Figure 15

pH curves of the mixtures incubated in Experiment 1.


8
7
6
5
4
3
pH
0
20
40
60
80
100
120
150
180
240
Minutes
A
B
C
D
E

decrease in the hydrogen ion concentration. In flask(A)(S. salivarius alone) the pH continued to drop as glucose was being metabolized until the glucose in the reaction mixture was completely degraded at 80 minutes, after which the pH tended to level off during the rest of the incubation period. An initial drop in pH was also observed in flask(B), but the pH increased slightly after 80 minutes of incubation. At this time the glucose had not been completely metabolized, but the production of "free" lactate was at its maximum. As the percentage of V. parvula cells in the incubation medium increased, the rate of pH drop decreased until in flask(E), there was only a very slight decrease in pH during the reaction period.

The above information demonstrates that resting cells of V. parvula can actively metabolize lactic acid produced during the degradation of glucose by S. salivarius.

As shown by Kraus and Gaston (1956), the number of S. salivarius cells in an individual did not vary significantly when compared to the total oral streptococci. Therefore, the conditions described in the first experiment (Figure 14) were not similar to the actual situation found in the oral cavity. In view of this, another set of conditions was established in which the V. parvula cell concentration was increased, while that for S. salivarius remained constant (Table 19). The cell

TABLE 19

Concentration of S. salivarius and V. parvula employed in Experiment 2.

Flask	Streptococcus salivarius		Veillonella parvula	
	mg dry wt/ml	%	mg dry wt/ml	%
A	0.4	100	0	0
B	0.4	62	0.25	38
C	0.4	44	0.50	56
D	0.4	29	1.00	71
E	0.4	17	2.00	83
F	0	0	2.00	100

Total volume 10 ml.

concentrations for S. salivarius was kept constant at 0.4 mg dry wt/ml in all flasks, while the V. parvula cell concentration increased from 0 to 2.0 mg dry wt/ml with flask F composed entirely of V. parvula. Thus, ratio of S. salivarius to V. parvula varied as before, but, in this case the total cell mass increased from 0.4 to 2.4 mg dry wt/ml.

In this second experiment (Figure 16) the rate of glucose degradation increased with increasing concentrations of V. parvula. The difference in the rate between flask (A) with S. salivarius alone was 6.75 μmoles glucose utilized/mg dry wt/hr while flask (E) containing 83% V. parvula was 19.2 μmoles glucose utilized/mg/hr. As previously shown in Figure 13, V. parvula alone (flask F) did not utilize glucose. This experiment again illustrates the rapid loss of lactate from the medium with increased concentrations of veillonellae cells. At a ratio of 1:1, which is similar to that for the streptococci and veillonellae in vivo, very little "free" lactate appeared on the medium (flask C).

Figure 17 illustrates that with a constant concentration of S. salivarius cells, there was a short drop in the pH for about 30 minutes in all the flasks followed by a levelling-off before the glucose was completely metabolized. This observation could be account for by the low S. salivarius cell to substrate ratio employed in this system (0.025), as compared to a higher value (0.13) in the first experiment.

FIGURE 16

Influence of increasing concentrations of V. parvula cells on the glucose metabolism and subsequent lactic acid formation by resting cells of S. salivarius under conditions of increasing cell mass.

Cells, as outline in Table 16, were incubated with 16.7 mM glucose in phosphate buffer as previously described. Total volume was 10 ml in a 25 ml. flask fitted with a rubber stopper and all procedures as previously described.

o----o Non-utilized Glucose

●----● Actual Lactic Acid

▫----▫ Degraded Lactic Acid

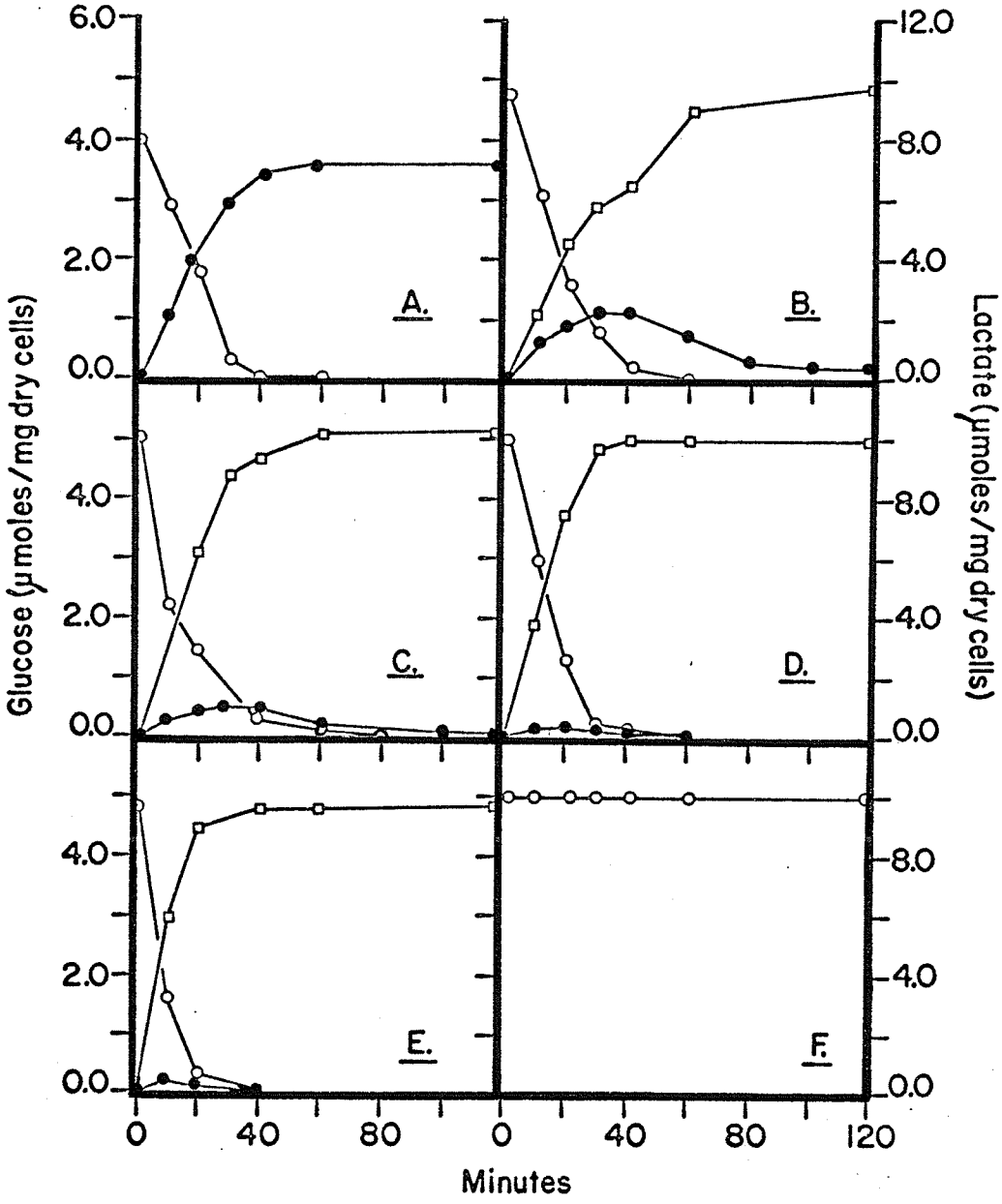
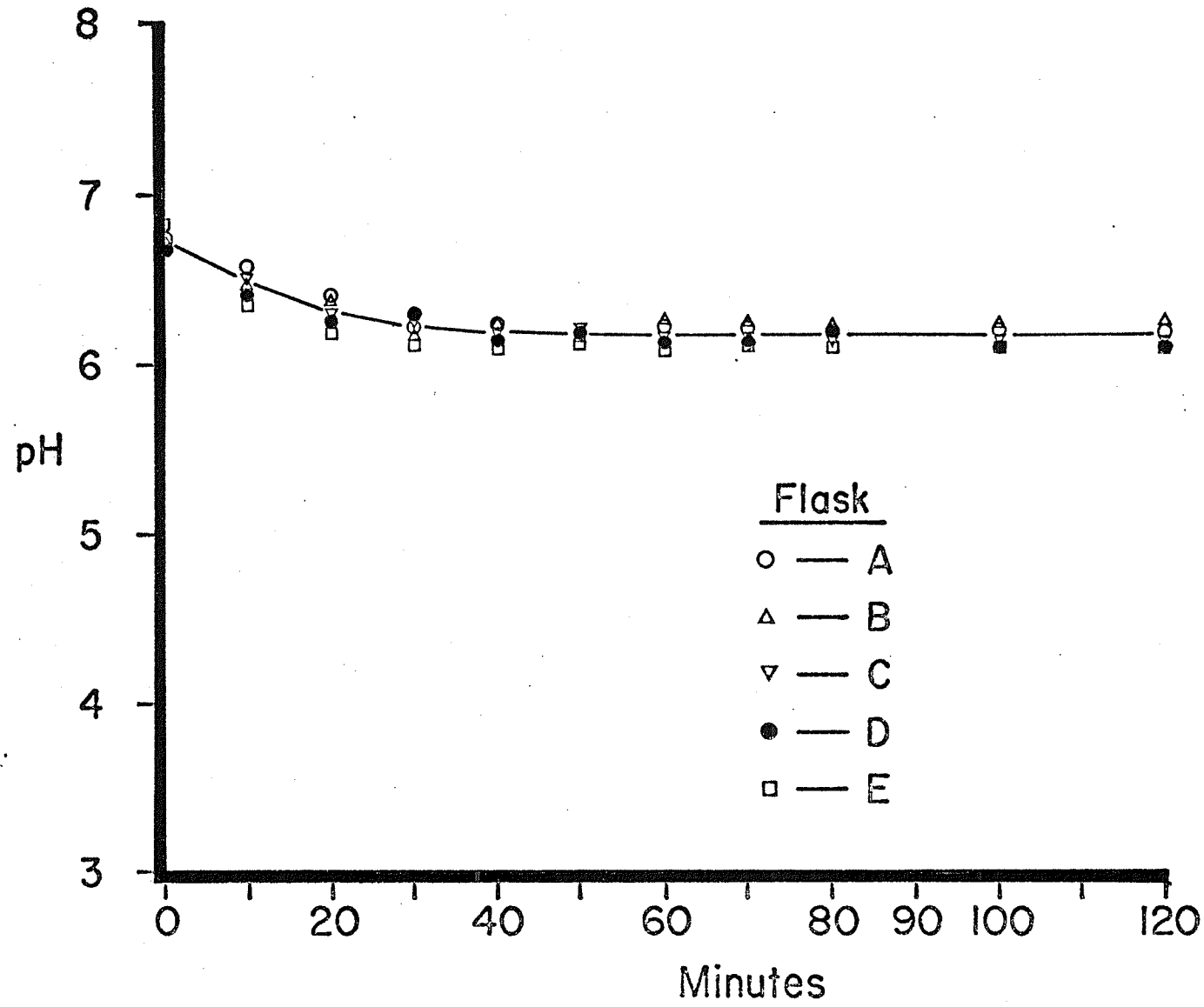

6.0
4.0
2.0
0.0
12.0
8.0
4.0
0.0
A.
B.
C.
D.
E.
F.
Glucose (μmoles/mg dry cells)
Lactate (μmoles/mg dry cells)
0
40
80
120
Minutes

FIGURE 17

pH curvesof the mixtures incubated in Experiment 2.

Conditions: Similar to those described in Figure 16.


8
7
6
5
4
3
pH
0
20
40
60
80
90
100
120
Minutes
Flask
A
B
C
D
E

DISCUSSION

The preliminary studies with mixed cultures of S. salivarius and V. parvula have demonstrated interactions very similar to a commensal system composed of Proteus vulgaris and Saccharomyces cerevisiae (Shindala, et al, 1965). In the latter investigation, growth was carried out in a continuous culture system employing a chemically defined medium, which would support the growth of S. cerevisiae, but not P. vulgaris. When the two cultures were mixed together in the chemically defined medium, growth of P. vulgaris was observed. Furthermore, this growth had no effect on the growth of S. cerevisiae. The growth of P. vulgaris (with S. cerevisiae) in a medium unable to support this organism alone was attributed to the production of a niacin-like compound by S. cerevisiae. As P. vulgaris required this compound for growth, this organism was, therefore, dependent on the growth and metabolism of the yeast.

In the S. salivarius and V. parvula system, the former organism holds a position similar to S. cerevisiae, while V. parvula functions in a manner analogous to P. vulgaris. Glucose will support the metabolism (and growth) of S. salivarius, but not V. parvula, the latter organism depending on the lactic acid produced by the streptococcus in the same way P. vulgaris required the niacin-like compound for growth.

Flask (A) of Figure 14 represents a typical glucose utilization curve for resting cells of S. salivarius (Hamilton, 1968). As most of the glucose was completely utilized in 80 minutes, the production of lactate tended to plateau and the amount of lactate produced was approximately twice that of the glucose supplied as would be expected for such a homofermentative organism. With S. salivarius alone in the incubation medium, there was no degradation of lactate, however, as shown in flasks (B) to (E), this accumulated carboxylic acid became the substrate for V. parvula as the net "free" lactate decreased as the concentration of the latter organism increased.

As the S. salivarius cell concentration decreased from flask (A) to (E), S. salivarius cell to substrate ratio decreased with the result that the rate of glucose utilization also decreased. Whether the presence of increasing concentration of V. parvula cells was a factor in the decrease in the rate of glucose utilization is unknown as the controls with decreasing concentrations of S. salivarius cell were not included in the experiment. However, previous work in our laboratory has shown that decreasing concentrations of S. salivarius cells result in decreasing rate of glucose degradation.

The pH profiles for the different flasks shown in Figure 15 can be grouped into 3 types. Flask (A) is a typical curve for the metabolism of

glucose by S. salivarius, where the pH dropped initially due to the production of lactic acid (Ka = 1.38 x 10^{-4}; pKa = 3.87). When the pH of the medium reached approximately 5.0, the rate decreased considerably as the exogenous glucose became exhausted, although the production of lactate continued at a slow rate for the remainder of the incubation period because of the utilization of endogenous carbohydrate.

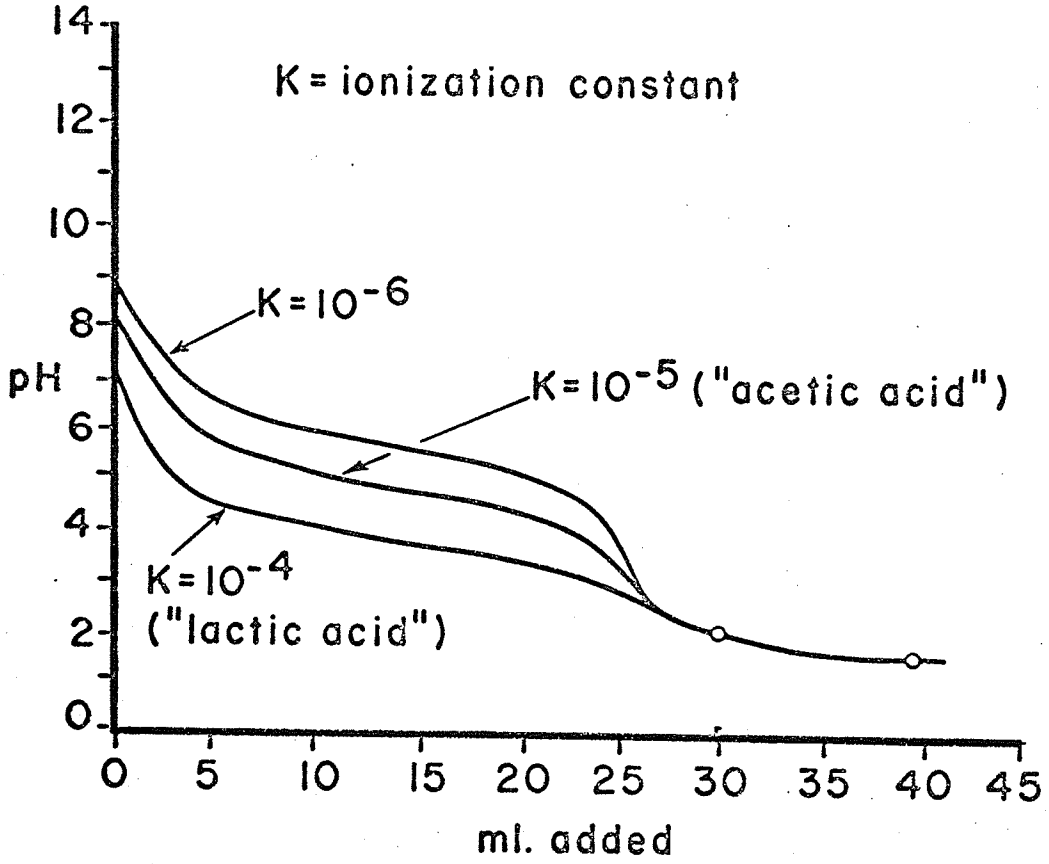
A second type of pH profile was obtained from flask (B) demonstrating an initial pH drop followed by a region of constant pH and then a final rise in the pH of the medium. In this flask, there was a continuous production of lactic acid by S. salivarius from glucose with the concomitant utilization of this acid by V. parvula to produce the end-products, propionic and acetic acids, which have pK values of 4.85 and 4.73, respectively. This type of pH curve is similar to that obtained by Stephan and Hemmen (1947) and Sandham (1963) with salivary sediment preparations.

These results are best explained by referring to a curve for the titration by a strong acid of the salts of weak acids. Figure 18 illustrates such titration curves for the salts of three weak acids having ionization constants of 10^{-4}, 10^{-5} and 10^{-6}. Lactic acid has an ionization constant in the region of 10^{-4}, while acetic and propionic acids have constants in the 1 x 10^{-5} range.

Figure 18

Curves for the titration of the salts of weak acids with a strong acid.

25 ml of 0.1 N solution of weak acid of indicated ionization constant titrated by 0.1 N hydrochloric acid. (Fischer, 1962).


K = ionization constant
14
12
10
8
6
4
2
0
pH
K = 10-6
K = 10-5 ("acetic acid")
K = 10-4
("lactic acid")
0 5 10 15 20 25 30 35 40 45
ml. added

In flask (B), the conversion of glucose to lactic acid by S. salivarius results initially in a decrease in pH, but as V. parvula utilizes this acid for energy the net result of the total metabolism is the formation of two acids weaker than lactic acid. Reference to Figure 15 shows that in the region of pH 5.0, the conversion of a stronger acid ($Ka = 1 \times 10^{-4}$) to a weaker acid ($Ka = 10^{-5}$) will result in a pH shift (arrow). This would occur despite the fact that the initial pH of the salt solutions (at 0 ml strong acid) were not the same. The shift towards the formation of weaker acids prevented the pH in flask (B) from falling as low as flask (A) containing S. salivarius alone.

When all the glucose in flask (B) had been utilized at 80 minutes, the major production of lactic acid from exogenous glucose by S. salivarius ended, but metabolism by V. parvula continued to convert lactic acid to acetic acid and propionic acid. For this reason, the shift in pH became more pronounced after 80 minutes as observed by the slight rise in pH.

Curves (C), (D) and (E) (Figure 15) demonstrate a third type of pH profile in which the percentage of S. salivarius cells in the reaction were 50% or less. In this situation, the utilization of the lactic acid by V. parvula occurs almost as rapidly as it can be produced from glucose by S. salivarius. This rapid conversion of the stronger lactic acid to the weaker acetic and propionic acids, prevented the pH from dropping lower

than curve (B). However, no rise in pH occurred in these reactions because lactic acid was continually produced throughout the incubation period.

As shown in Figure 16 as the concentation of V. parvula cells was increased with a constant number of S. salivarius cells the rate of lactate degradation increased significantly. As the presence of lactic acid is deleterious to S. salivarius this result is not surprising. The presence of V. parvula had an effect similar to the removal of a product in a simple enzyme catalyzed reaction. The rapid removal of the inhibiting product (i.e. lactic acid) caused the reaction to proceed at a faster rate. It is obvious from this result that the accumulation of acetic and propionic acids in this system did not significantly inhibit either organism in the system.

Applying the above two experiments to mixed populations found within the oral cavity, the first set of conditions somewhat resembled the variations in oral glucose and lactate metabolism which one might expect between individual subjects. Cell concentrations are known to vary from individual to individual and a shift in the numbers of any of the predominant species, particularly the streptococci, would have a dramatic effect on the metabolism of the entire system.

The second experiment can be applied to a typical plaque system

within a single individual through daily cycles of cleaning and plaque accumulation. As Ritz (1967) has shown S. salivarius remains relatively constant for a number of days after the tooth surface had cleaned while Veillonella species began at a low concentration and increased with time.

In natural ecological populations, the number of cells and the substrate-end-product interactions, all of which affect the overall metabolism of the system, probably fluctuate within a narrow range. It is obvious that in studies with resting cells and with batch-grown bacterial cultures, many factors change simultaneously. Therefore, such closed systems are not the ideal experimental model for the study of bacterial interrelationships. A more feasible approach would be the technique of continuous cultures which permits the steady state growth of bacteria under strictly controlled conditions.

SUMMARY

SUMMARY

Cultures of Veillonella species were isolated from human saliva on lactate agar plates incubated anaerobically at 37 C. The elimination of contamination by small gram-positive cocci frequently found in these cultures was brought about by serial transfer of the organisms in growth media containing the antibiotics, vancomycin or streptomycin. The isolated organisms were subjected to various taxonomic tests and a strain classifed as V. parvula was used for the study of anaerobic lactate metabolism.

Pure cultures of V. parvula grew readily in 1% lactate tryptone broth in atmospheres of nitrogen or N_2 + 5% CO_2 with a typical growth rate of 0.68 generations per hour. Unlike V. alcalescens, cells of V. parvula did not require carbon dioxide for growth on lactate, but clumping was avoided by the use of N_2 + 5% CO_2 as the gas phase. Lactate metabolism by growing and resting cells of V. parvula resulted in the formation of propionate, acetate, carbon dioxide and hydrogen. The following stoichiometry was observed.

$$5 \text{ lactate} \longrightarrow 3 \text{ propionate} + 2 \text{ acetate} + 2 \text{ carbon dioxide} + \text{hydrogen} \qquad (26)$$

Although carbon dioxide was not required for lactate metabolism, the

presence of carbon dioxide in the gas phase influenced the quantities of propionate produced. Increasing amounts of carbon dioxide increased the absolute amount of propionate produced, the $C^{14}O_2$ evolution from lactate-U-C^{14} and cellular incorporation of lactate carbon, although the rates of lactate utilization were not affected.

When resting cells of V. parvula were incubated with lactate-U-C^{14} as the substrate, both carbon dioxide fixation and CO_2-pyruvate exchange reactions were observed. These two reactions were directly proportional to the partial pressure of carbon dioxide in the atmosphere with increased levels of carbon dioxide always resulting in the increased production of propionate (from carbon dioxide fixation) and $C^{14}O_2$ evolution (from the carbon dioxide-pyruvate exchange reaction) from the radioactive lactate substrate. At 50% carbon dioxide in the atmosphere, 80% of the decrease in radioactivity of propionate was due to the fixation of unlabelled carbon dioxide, while 20% of it was due to the CO_2-pyruvate-exchange reaction.

Whole cells of V. parvula also metabolized, in addition to lactate, other substrates such as oxaloacetate and pyruvate. However, although the acidic end-products produced were qualitatively the same, the rates of utilization for these substrates were slower than that observed for lactate. Quantitatively, lactate produced more propionate and less

acetate than pyruvate. This difference between the amounts of the end-products formed may be due to the availability of reducing power in the system. The ability of V. parvula to utilize pyruvate and oxaloacetate suggested that these substrates may be intermediate in the propionate production pathway.

The inability of cell-free extracts of V. parvula to utilize lactate confirmed previous results that a conventional lactate dehydrogenase was not present in this organism. The utilization of lactate in the presence of oxaloacetate suggest that, like V. alcalescens, a malic-lactic transhydrogenase functions in this organism coupling the oxidation of lactate to pyruvate with the reduction of oxaloacetate to malate.

The direct detection of oxaloacetate formation from carbon dioxide and pyruvate or phosphoenolpyruvate by enzyme preparations of V. parvula gave further evidence that CO_2-fixation occurs in cells of this organism. The ability of V. parvula to form oxaloacetate confirms that propionate formation in this organism occurs by the conversion of lactate to pyruvate, pyruvate to oxaloacetate, oxaloacetate to malate, fumarate and succinate with the decarboxylation of succinate to form propionate. Pyruvate degradation to acetate, CO_2 and H_2 also occurs and produces reducing power for propionate formation. The formation and accumulation of lactate when cell-free extracts were incubated with pyruvate as the

ostrate suggests that the acrylate pathway for propionate formation does not operate in V. parvula.

This pure culture study with V. parvula confirms that both species of the veillonellae, V. parvula and V. alcalescens, appear to form propionate according to the oxaloacetate scheme. V. parvula, however, differs from V. alcalescens, in that carbon dioxide was not required for growth in lactate broth or for the metabolism of lactate by resting cells. Figure 19 summarizes the reactions thought to operate during lactate metabolism by resting cells of V. parvula.

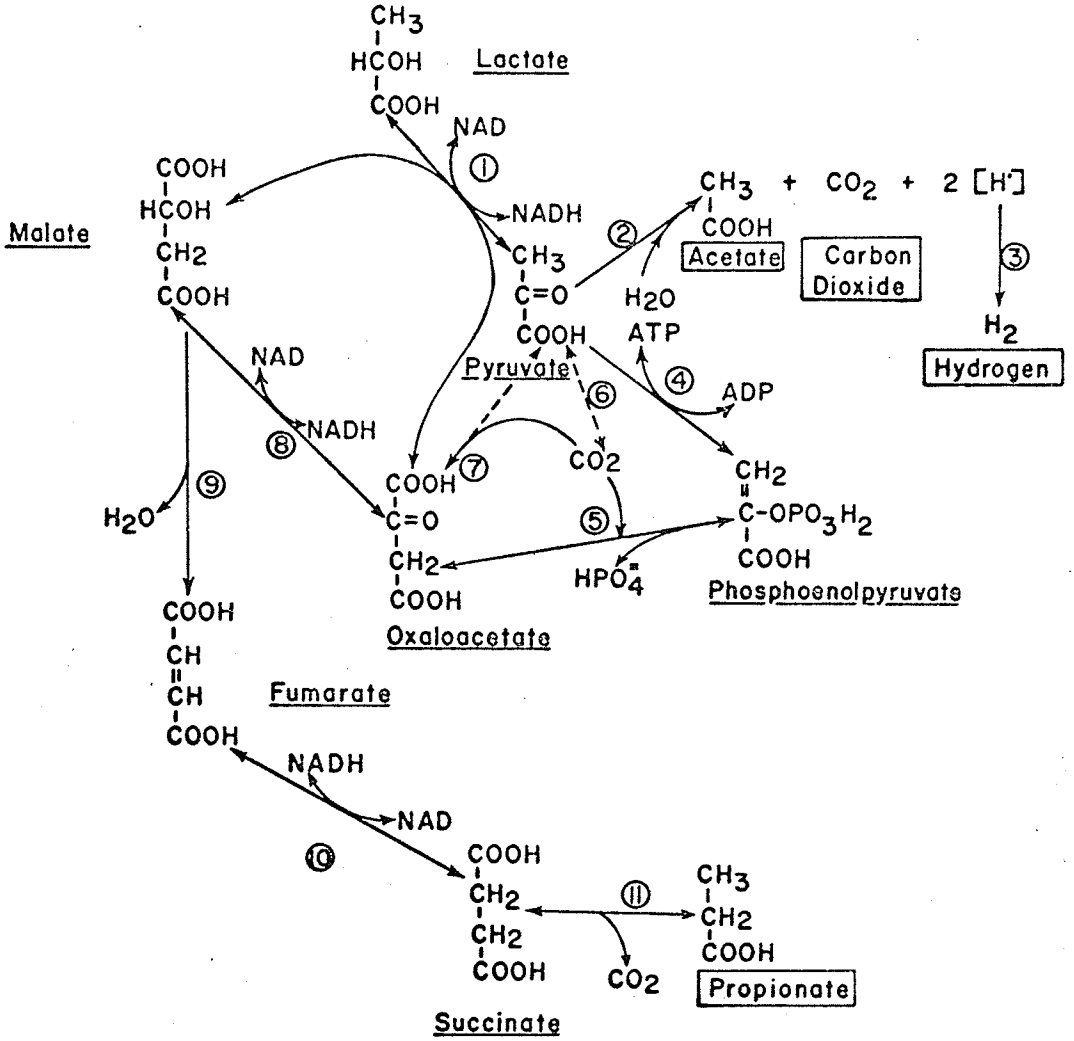
Mixed culture studies with V. parvula and S. salivarius have shown that lactic acid produced by S. salivarius from substrate glucose was readily utilized by V. parvula for energy. Changes in the pH of the reaction medium depended on the ratio of the two bacteria and the number of cells in the system.

Figure 19

Pathway of lactate metabolism in V. parvula

Legend: Enzymes involved in the pathway.

(1) Malic-lactic transhydrogenase.
(2) Enzyme complex for the pyruvate phosphoroclastic reaction.
(3) Hydrogen: ferredoxin oxidoreductase (EC 1.12.1.1).
(4) ATP: pyruvate phosphotransferase (EC 2.7.1.40).
(5) Pyrophosphate: oxaloacetate carboxyl-lyase (EC 4.1.1.38).
(6) Enzyme(s) involved in the carbon dioxide exchange reaction.
(7) Oxaloacetate carboxy-lyase (EC 4.1.1.3).
(8) L-Malate: NAD oxidoreductase (EC 1.1.1.37).
(9) L-Malate hydro-lyase (EC 4.2.1.2).
(10) Succinate: (acceptor) oxidoreductase (EC 1.3.99.1)
(11) Enzyme complex for succinate decarboxylation.


Lactate
NAD
NADH
Malate
Acetate
Carbon Dioxide
Hydrogen
Pyruvate
ATP
ADP
Phosphoenolpyruvate
Oxaloacetate
Fumarate
Propionate
Succinate

BIBLIOGRAPHY

Allen, S. H. G. 1966. The isolation and characterization of malate-lactate transhydrogenase from Micrococcus lactilyticus. J. biol. Chem., 241: 5266-5275.

Allen, S. H. G. and Galivan, J. H. 1965. Malic-lactic transhydrogenase and methylmalonyl CoA decarboxylase. Two new enzymes and their role in lactate fermentation. Federation. Proc., 24: 531.

Alexander, H. and Leidy, G. 1949. Mechanism of emergence of resistance to streptomycin in five species of gram-negative bacilli. Pediatrics, Springfield, 4: 214-221.

Annear, D. I. 1951. Mixed bacterial growth. III. Studies on the mixed growth of a viridans streptococcus, a diphtheroid and Staphylococcus albus. Aust. J. exp. Biol. med. Sci., 29: 93-99.

Appleton, J. L. T. 1940. The problems of oral Microbiology as problems in ecology, in Proc. Dent. Centen. Celeb.: 282-293. Waverly Press Inc., Baltimore.

Appleton, J. L. T. 1944. Bacterial Infection. Lea and Febiger. Philadelphia.

Bahn, A. N. and Tiecke, R. W. 1967. Microorganisms of dental significance. Editorial Office. Am. dent. Ass., Chicago.

Barker, H. A. and Beck, J. V. 1941. The fermentative decomposition of purines by Clostridium acidi-urici and Clostridium cylindro-sporum. J. biol. Chem., 141: 3-27.

Barker, S. B. and Summerson, W. H. 1941. The colorimetric determination of lactic acid in biological material. J. biol. Chem., 138: 535-554.

Best, G. K. and Durham, N. N. 1964. Effect of vancomycin on Bacillus subtilis cell walls. Archs. Biochem. Biophys., 111: 685-692.

Bibby, B. G. 1939. Oral Bacteriology: basic considerations bearing on disease and therapy. J. Am. dent. Ass., 26: 629-636.

Bisset, K. A. and Davis, G. H. G. 1959. The Oral Actinomycetes. Arch. Oral Biol., 1: 80-86.

Bisset, R.A. and Davis, G.H.G. 1960. The Microbial flora of the Mouth. Heywood and Co. Ltd., London.

Branham, S.E. 1927. Anaerobic microorganisms in nasopharygeal washings. J. infect. Dis., 41: 203-207.

Branham, S.E. 1928. An apparently undescribed hemolytic anaerobic diplococcus. J. infect. Dis., 42: 230-237.

Breed, R.S., Murray, E.G.D., Smith, N.R. 1957. Bergey's Manual of Determinative Bacteriology, 7th edition. Williams and Wilkins Co., Baltimore.

Bulen, W.A., Varner, J.E. and Burrell, R.C. 1952. Separation of Organic acids from plant tissues. Chromatographic Analyt. Chem., 24: 187-190.

Cardon, B.P. and Barker, H.A. 1947. Amino acid fermentation by Clostridium propionicum and Diplococcus glycinophilus. Arch. Biochem., 12: 165-180.

Clement, A.J., Plotkin, R., and Fosdick, L.S. 1956. The Formation of lactic acid in Dental plaques. II Oral Conditions of Primitive Bushment of the Western Kalahari Desert. J. dent. Res., 35: 786-791.

Cohen, L.H. and Noell, W.K. 1960. Glucose catabolism of Rabbit Retina before and after development of visual function. J. Neurochem., 5: 253-276.

Colebrook, L. 1915. Bacterial antagonism, with particular reference to the menigococcus. Lancet, 2: 1136-1138.

Delavier-Klutchko, C. 1961. Intervention de la biotine dans la reaction clastique du pyruvate par Clostridium saccharobutyrium. Compt. Rend., 252: 1681-1683.

Delwiche, E.A. 1948. Mechanisms of propionic acid formation by Propionibacterium pentosaceum and Veillonella gazogenes. I. Activation, decarboxylation and related reactions. J. Bactiol., 71: 598-603.

Dolin, M.I., Phares, E.F. and Long, M.V., 1964. Bound pyridine nucleotide of malic-lactic transhydrogenase. Federation Proc., 23: 427.

Dolin, M.I., Phares, E.F., and Long, M.V. 1965. Bound pyridine nucleotide of malic-lactic transhydrogenase. Biochem. biophys. Res. Commun., 21: 303-310.

Douglas, H.C. 1950. On the occurrence of the lactate fermenting anaerobe, Micrococcus lactilyticus, in human saliva. J. dent. Res., 29: 304-306.

Englander, H.R., Carter, W.J. and Fosdick, L.S. 1956. The formation of Lactic acid in Dental plaques. III Caries immune individuals. J. dent. Res., 35: 792-799.

Fischer, R.B. 1962. Quantitative Chemical analysis. W.B. Saunders Co., Philadelphia.

Foubert, E.L. Jr. and Douglas, H.C. 1948 a. Studies on the anaerobic Micrococci. I. Taxonomic Considerations. J. Bacteriol., 56: 25-34.

Foubert, E.L. Jr. and Douglas, H.C. 1948 b. Studies on the anaerobic Micrococci. II. The Fermentation of Lactate by Micrococcus lactilycus. J. Bacteriol., 56, p. 35-36.

Galivan, J.H. and Allen, S.H.G. 1968. Methylmalonyl Coenzyme A Decarboxylase. Its role in succinate decarboxylation by Micrococcus lactilyticus. J. biol. Chem., 243: 1253-1260.

Gozon, H.N. and Carpenter Jr., R.R. 1953-54. Streptomycin Resistance Acquired by Shigella through continuous exposure to the Antibiotic. Antibiotics Ann.: 576-584.

Grace, J.E. Heilman, F.R., Nichols, D.R., Wellman, W.E., and Ross, G.T. 1956-57. Some laboratory and Clinical experience with a New antibiotics, Vancomycin. Antibiotics Ann.: 90-106.

Gibbons, R.J., Kapsimalis, B. and Socransky, S.S. 1964. The source of Salivary Bacteria. Arch. Oral Biol., 9: 101-103.

Gibbons, R.J., Socransky, S.S., de Araujo, W.C., Van Honte, J. 1964. Studies of the predominant cultivable microbiota of Dental plaque. Arch. Oral Biol., 9: 365-370.

Gibbons, R. J., Socransky, S. S., Sawyer, S., Kapsimalis, B., MacDonald, J. B. 1963. The Microbiota of the Gingival crevice area of man. II, The predominant cultivable organisms. Arch. Biol., 8: 281-289.

Gordon, D. F. and Gibbons, R. J. 1966. Studies of the predominant cultivable microorganisms from the Human Tongue. Arch. Oral Biol., 11: 627-632.

Hall, I. C., and Howitt, B. 1925. Bacterial factors in pyorrhea alveolaris. IV Micrococcus gazogenes. J. infect. Dis., 37: 112-125.

Hamilton, I. R. 1968. Synthesis and degradation of intercellular polyglucose in Streptococcus salivarius. Can. J. Microbiol., 14: 65-77.

Hamilton, I. R., Burris, R. H. and Wilson, P. W. 1965. Pyruvate Metabolism by Nitrogen-Fixing Bacterium. Biochem. J., 96: 383-389.

Hawary, M. F. S. and Thompson, R. H. S. 1953. Separation and estimation of blood keto-acids by paper chrometography. Biochem. J., 53: 340-307.

Hohorst, J. H. and Reim, M. 1965. Oxaloacetate in Methods of Enzymatic Analysis edit. H.U. Bugmeyer, Academic Press, New York.

Holman, W. L. and Krock, F. H. 1923 a. An anerobe from the mouth cavity of man and rabbits morphologically suggesting B. pneumosintes. Proc. Soc. exper. Biol. Med., 20: 280-282.

Holman, W. L. and Krock, F. H. 1923 b. Microorganisms suggesting B. pneumosintes from the mouths of man and Laboratory animal. Am. J. Hyg., 3: 487-490.

Holzer, H. and Beaucampt, K. 1961. Nachweis und Charakterisierung von Zwischenprodukten der Decarboxylierung und Oxydation von Pyrvat. "aktiviertes Pyrvat" und "aktivierter Acetaldehyd". Angew. Chem., 71: 776.

Jenkins, G. N. 1966. The physiology of the Mouth. 3rd edition. Blackwell Scientific Publications, Oxford.

Johns, A. T. 1948. The production of propionic acid by decarboxylation of succinate in bacterial fermentation. Biochem. J., 42: Proc. Biochem. Soc., ii-iii.

Johns, A.T. 1951. A Isolation of a Bacterium, producing propionic Acid, from the Rumen of sheep. J. Gen. Microbiol., 5: 317-325.

Johns, A.T. 1951. The Mechanism of Propionic Acid Formation by Veillonella gazogenes. J. Gen. Microbiol., 5: 326-336.

Jungano, M. and Distuso, A. 1910. Les anaerobies, Paris, Masson.

Karlson, J.L. and Barker, H.A. 1949. Tracer experiments on the mechanisms of uric acid decomposition and acetic acid synthesis by Clostridium acidi-urici. J. biol. Chem. 178: 891-902.

Kingsley, G.R. and Getchell, G. 1960. Direct ultramicro glucose oxidase method for determination of glucose in biological fluids. Clin. Chem., 6: 466-475.

Kleinberg, I. 1961. Studies on Dental Plaque. I. The Effect of different concentration of glucose on the pH of Dental plaque in vivo. J. dental Res., 40: 1087-1111.

Kleinberg, I. 1962. Further studies on the production of in vivo plaque pH curves, in vitro. J. Can. dent. Ass., 28: 242- .

Kleinberg, I., and Jenkins, G.N. 1959. Further studies on the effect of Carbohydrates substrates on Plaque pH in vivo. J. dent. Res., 38: 704- .

Kleinberg, I. 1967. Effect of varying sediment and glucose concentrations on the pH and acid production in Human Salivary Sediment mixtures. Arch. Oral Biol., 12: 1457-1473.

Kleinberg, I. and Jenkins, G.N. 1964. The pH of dental plaques in the different areas of the mouth before and after meals and their relationship to the pH and rate of flow of resting saliva. Arch. Oral Biol., 9: 493-516.

Krampitz, L.O., Wood, H.G., and Werkman, C.H. 1943. Enzymatic fixation of carbon dioxide in oxaloacetate. J. biol. Chem., 147: 243-253.

Krasse, B. 1953. The proportional distribution of different types of streptococci in saliva and plaque material. Odont. Revy., 4: 304-312.

Krasse, B. 1954 a. The relationship between lactobacilli, Candida, and streptococci and dental caries. Odont. Revy., 5: 241-261.

Krasse, B. 1954 b. The proportional distribution of Streptococcus salivarius and other streptococci in various parts of the mouth. Odont. Revy., 5: 203-211.

Kraus, F.W. and Gaston, C. 1956. Individual constancy of numbers among the oral flora. J. Bacterio., 71: 703-707.

Krebs, H.A., Hems, R., Weidemann, M.J. and Speake, R.N. 1966. The Fate of isotopic carbon in kidney cortex synthesing glucose from lactate. Biochem. J., 101: 242-249.

Langford, G.C. Jr., Faber, J.E. Jr., and Pelczar, M.J. Jr., 1950. The occurrence of anaerobic gram-negative deplococci in the normal human mouth. J. Bacterio., 59: 349-356.

Layne, E. 1957. Spectrophotometric and Turbidimetric methods for measuring proteins. Methods in Enzymology. edit. by Colowick S.P. and Kaplan N.O., 3: 451-454.

Lewkowics, X. 1901. II Recherches sur la flore microbienne de la bouche des nourrissons. Arch. Med. Expl., 13: 633-660.

Lodge, R.M., and Hinshelwood, C.N. 1939. Physioochemical aspects of bacterial growth. V. Influence of magnesium on the lag phase in the growth of Bacterium lactis aerogenes in synthetic media containing phosphate. J. Chem. Soc.: 1692-1697.

McCormick, N.G., Ordal, E.J. and Whiteley, H.R. 1962 a. Degradation of pyruvate by Micrococcus lactilyticus. I. General Properties of the Formate-Exchange Reaction. J. Bacteriol., 83: 887-898.

McCormick, N.G., Ordal, E.J. and Whiteley, H.K. 1962 b. Degradation of pyruvate by Micrococcus lactilyticus. II. Studies of co-factors in the Formate-Exchange Reaction. J. Bacteriol., 83: 899-906.

McCormick, M.H., Stark, W.M., Pittenger, G.E., Pittenger, R.C. and McGurie, J.M. 1955-56. Vancomycin, A New Antibiotic. I. Chemical and Biologic Properties, Antibiotics Annual: 606-611.

Mergenhagen, S.E. 1965. Polysaccharide-lipid complexes from Veillonella parvula. J. Bacterio. 90: 1730-1734.

Michaud, R.N. and Delwiche, E.A. 1967. Incorporation of glucose carbon into Nucleic acids by Veillonella alcalescens. Abst. Am. Soc. Microbiol.: 108.

Miller, B.F., Muntz, J.A. and Bradel, S. 1940. Decomposition of carbohydrate substrates by Dental plaque material. J. dent. Res., 19: 473-478.

Moore, B.W., Carter, W.J., Dunn, J.K., and Fosdick, L.S., 1956. The Formation of lactic Acid in Dental Plaques. I. Caries-active individuals. J. dent. Res., 35: 778-785.

Morris, E.O., 1953 a. The Bacteriology of the Oral Cavity. I. The distribution of bacteria in and on the dental enamel. Brit. dent. J., 95: 77-82,

Morris, E.O., 1953 b. The Bacteriology of the Oral Cavity. IIA.- Methods used in the study of the Oral Flora. IIB. -Lactobacillus. Brit. dent. J., 95: 259-270.

Morris, E.O., 1954 a. The Bacteriology of the Oral Cavity, III- Streptococcus. Brit. dent. J., 96: 95-108.

Morris, E.O., 1954 b. The Bacteriology of the Oral Cavity, IV- (a) Micrococcus, (b) Neisseria, (c) Veillonella. Brit. dent. J., 96: 259-265.

Morris, E.O., 1954. The Nature of Bacteria Comprising the Oral Flora. Brit. dent. J., 97: 313-315.

Mortenson, L.E., Valentine, R.C., and Carnahan, J.E. 1962. An electron transport factor from Clostridium pasteurianum. Biochem. Biophys. Res. Commun., 7: 448-452.

Mortlock, R.P., Valentine, R.C., and Wolfe, R.S. 1959. Carbon dioxide activation in the pyruvate clastic system of Clostridium butyricum. J. biol. Chem., 234: 1653-1659.

Muhlenbach, V. 1939. Untersuchungen uber die Rolle der Speichelkeine im Dold-Weigmamischen Hemmungs phanomen. Z. Hyg. Infelkrankh., 121: 569-580.

Muntz, J.A. 1943. Production of Acids from Glucose by dental plaque material. J. biol. Chem., 148: 225-236.

Niven, C.F., Smiley, K.L., and Sherman, J.M. 1941. The production of large amounts of polysaccharides by streptococcus salivarius. J. Bacteriol., 41: 479-484.

Olitsky, P.K. and Gates, F.L. 1922. Cultural and morphologic character of Bacterium pneumosites. J. Exper. Med., 35: 813.

Ozaki, Y. 1912. Zur Keuntuis der anaeroben Bakterien der Mundhole. Zentr. Bakt. Parasitenk, I. Orig., 62: 86-88.

Phares, E.F. and Long, M.V. 1956. A couple Malic-lactic dehydrogenase from Veillonella gazogenes. Abst. Am. Chem. Soc.: 62 c.

Phares, E.F., Delwiche, E.A. and Carson, S.F. 1956. Succinic acid decarboxylation system in Propionibacterium pentosaceum and Veillonella gazogenes. II. Evidence for an active "C", Complex. J. Bacteriol., 71: 604-610.

Prevot, A.R. 1933. Etudes de systematique bacterienne. I. Lois Generales. II. Cocci anaerobics. Am. Sci. Nat. Ser. Botan., 15: 23-258.

Richardson, R.H. and Jones, M. 1958. A Bacteriologic census of Human Saliva. J. dent. Res. 37: 697-809.

Ritz, H.L. 1967. Microbial population shifts in developing Human dental plaque. Arch. Oral Biol., 12: 1561-1568.

Rogosa, M. 1956. A selective medium for the isolation and enumeration of the Veillonella from the oral cavity. J. Bacteriol., 72: 533-536.

Rogosa, M. 1964. The Genus Veillonella. I. General Cultural, Ecological, and Biochemical considerations. J. Bacteriol., 87: 162-170.

Rogosa, M. 1965. The Genus Veillonella. IV. Serological groupings, and Species Emendations. J. Bacteriol., 87: 574-580.

Rogosa, M. and Bishop, F.S. 1964. a. The Genus Veillonella. II. Nutritional Studies. J. Bacteriol., 87: 574-580.

Rogosa, M. and Bishop, F.S. 1964 b. The Genus Veillonella. III. Hydrogen sulfide production by growing cultures. J. Bacteriol. 88: 37-41.

Rogosa, M. Fritzgerald, R.J., MacKintosh, M.E. and Beaman, A.J. 1958. Improved medium for selective Isolation of Veillonella. J. Bacteriol., 76: 455-456.

Rogosa, M., Krichevsky, M.I. and Bishop, F.S. 1965. Truncated glycolytic system in Veillonella. J. Bacteriol., 90: 164-171.

Rosebury, T. 1944. The Aerobic non-hemolytic streptococci. A critical review of their characteristics and pathogenicity with special reference to the Human Mouth and to subacute Bacterial endocarditis. Medicine, 23: 249-280.

Rosebury, T., Clark, A.R., Engel, S.G., Tergis, F. 1950. Studies of Fuso-spirochetal Infection. I. Pathogenicity for Giunea Pigs of Individual and Combined Cultures of Spirochetes and other anaerobic bacteria derived from the Human Mouth. J. infect. Dis., 87: 217-225.

Rosebury, T., Clark, A.R., Tergis, F., Engel, S.G. 1950. Studies of Fuso-spirochetal Infection. II. Analysis and attempted Quantitative recombination of the flora of fuso-spirochetal Infection after repeated Guinea pig passage. J. infect. Dis., 87: 226-233.

Rosebury, T., Clark, A.R., MacDonald, J.B., S. O'Connell, D.C. 1950. Studies of Fuso-spirochetal Infection: III - Further studies of a Guinea pig passage strain of fusospirochetal infection, including the infectivity of sterile exudate filtrates, of mixed cultures through ten transfers, and of recombined pure culture. J. infect. Dis., 87: 234-248.

Rosebury, T., Gale, D., Taylor, D.F. 1954. An approach to the study of Interactive phenomena among microorganism indigenous to man. J. Bacteriol., 67: 135.

Sanadi, D.R., and Littlefield, J.W. 1953. Studies on ketoglutaric oxidase. I. Formation of "Active" succinate. J. biol. Chem., 193: 683-689.

Sandham, H.J. 1967. Studies on the carbohydrate metabolism of salivary sediment. Ph.D. Thesis, University of Manitoba, Winnipeg.

Sandham, H.J. 1963. Carbohydrates storage by the Oral Microflora in vitro. M. Sc. Thesis, University of Manitoba, Winnipeg.

Shindala, A., Bungay, H.R., III. Krieg, N.R., and Culbert, K. 1965. Mixed culture interactions. I. Commensalism of P. vulgaris and S. cerevisiae. J. Bacteriol., 89: 693-696.

Shuster, C.W. and Lynen, F. 1960. The role of cofactors and enzyme-bound biotin in the CO_2-pyruvate exchange reactions. Biochem. Biophys. Res. Commun., 3: 350-355.

Sims, W. 1960. The Characteristics of some Oral strains of Veillonella. Brit. dent. J., 108: 73-74.

Siu, P.M.L. and Wood, H.G. 1962. Phosphoenolpyruvic carboxytransphosphonylase, a CO_2-fixation enzyme from propionic bacteria. J. biol. Chem., 237: 3044-3051.

Society of American Biologists Manual of Microbiological Methods 1957 edit. Bard, R.C., Burnett, G.W., Conn, H.J. (editor), DeMoss, R.D., Evans, E.E., Jennison, M.W., McKee, A.P., Riken, A.J., Warren, J., Weeks, O.B., and Weiss, F.A. McGraw-Hill Book Co. Inc., New York.

Stadtman, E.R., Overath, P., Eggerer, H., and Lynen, F. 1960. The role of biotin and Vitamin B_{12} coenzyme in propionate metabolism. Biochem. Biophys. Res. Commun., 2: 1-7.

Stephan, R.M. and Hemmens, E.S. 1947. Studies of changes in pH produced by pure cultures of oral microorganisms. I. Effects of varying the microbial cell concentration. J. dent. Res., 26: 15-24.

Socransky, S.S., Gibbons, R.S., Dale, A.C., Bortnick, L., Rosenthal, E., MacDonald, J.B. 1963. The Microbiota of the gingival crevice area of man. I, Total microscopic and viable counts and counts of specific organisms. Arch. Oral Biol., 8: 275-280.

Suzuki, I., and Werkman, C.H. 1958. Chemoautrophic carbon dioxide fixation by extracts of Thiobacillus thiooxidans. I. Formation of oxaloacetic acid. Archs. Biochem. Biophys., 76: 103-111.

Swick, R.W. and Wood, H.G. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. U.S., 46: 28-41.

The Merck Index of Chemical and Drugs, 7th edit. 1960. P.G. Stecher (editor), M.J. Finkel, O.H. Siegmund and B.M. Szafranski, Merck and Co. Inc., Rahway, N.J.

Thompson, R., and Johnson, A. 1951. The inhibitory action of saliva on the diphtheria bacillus: hydrogen peroxide, the inhibitory agent produced by salivary streptococci. J. infect. Dis., 88: 81-85.

Ting, I.P. and Dugger, W.H. Jr. 1965. Separation and Detection of Organic Acids on Silica Gel, Analyt. Biochem., 12: 571-578.

Umbreit, W.W., R.H. Burris and J.F. Stauffer. 1964. Manometric techniques, Burgess Publishing Co., Minneapolis.

Veillon, A. and Zuber, A. 1898. II. Recherches sur quelques microbes strictment anaerobies et leur role en pathologie. Arch. Med. Exptl., 10: 517-545.

Whiteley, H.R. 1953 a. Cofactor requirements for the decarboxylation of succinate. J. Am. Chem. Soc., 75: 1518-1519.

Whiteley, H.R. 1953 b. The mechanism of propionic acid formation by succinate decarboxylation. I. The activation of succinate. Proc. Natl. Acad. Sci. U.S., 39: 772-779.

Whiteley, H.R. 1953 c. The mechanism of propionic acid formation by succinate, decarboxylation. II. The formation and decarboxylation of succinyl-CoA. Proc. Natl. Acad. Sci. U.S., 39: 779-785.

Whiteley, H.R. 1957. The fermentation of amino acids by Micrococcus aerogenes. J. Bacteriol., 74: 324-330.

Whiteley, H.R. and Douglas, H.C. 1951. The fermentation of purines by Micrococcus lactilyticus. J. Bacteriol., 61: 605-616.

Whiteley, H. R., and McCormick, N. G. 1963. Degradation of pyruvate by Micrococcus lactilyticus. III. Properties and cofactor requirements of the carbon dioxide - Exchange reaction. J. Bacterio., 85: 382-393.

Whiteley and Ordal 1957. The fermentation of alpha keto-acids by Micrococcus aerogenes and Micrococcus lactilyticus. J. Bacteriol., 74: 331-336.

Wilson, G. S. and Miles, A. A. 1946. Topley and Wilson's Principles of Bacteriology and Immunity, (3rd edit.), Williams and Wilkins Co., Baltimore.

Winslow, C. E. A. and Haywood, 1931. The specific potency of certain cations with reference to their effect on Bacterial viability. J. Bacteriol., 22: 49-69.

Witter, L. D. 1953. Studies on the hydrogenase activity and on the metabolism of pyruvic acid in Micrococcus lactilyticus. Ph. D. Thesis, University of Washington, Seattle.

Ziegler, D. W., Wolfe, R. N. and McGurie, J. M. 1955-56. Vancomycin, a New Antibiotics, II. In vitro antibacterial studies. Antibiotics Ann.: 612-618.