OME ASPECTS OF THE MICROBIOLOGY OF SILAGE

by

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CONTENTS

Note. Abbreviations: μM was used as an abbreviation for micromoles, mM was used as an abbreviation for millimoles, and M as an abbreviation for molar.

SUMMARY

This thesis records the results of investigations into certain aspects of silage microbiology and discusses their implications.

The aim of the investigations was to gain more knowledge of the chemical changes brought about by microorganisms during a silage fermentation.

The investigations were divided into four sections.

1. Sugar metabolism of lactic acid bacteria

The heterofermentative, mannitol producing, fructose fermentation of Lactobacillus brevis and Leuconostoc mesenteroides was investigated. The lack of acetaldehyde dehydrogenase (acetyl phosphate NADH oxi-reductase) in L. brevis accounted for its inability to ferment glucose, whereas fructose was fermented, the reduction of acetyl phosphate to ethanol being replaced by the reduction of fructose to mannitol.

Aerobic sugar metabolism of several lactic acid bacteria was studied. Growth yields showed no ability to obtain energy from oxidative phosphorylation except for Streptococcus faecalis when in a medium supplemented with haematin.

Anaerobic rates of glucose and fructose metabolism were measured and on the basis of the results Streptococcus faecalis and Lactobacillus plantarum were judged to be the most suitable organisms for use in a silage inoculum.

2. Haematin enzymes and electron transport in lactic acid bacteria

Lactic acid bacteria were shown to form haematin enzymes when their growth medium was supplemented with haematin.

Streptococcus faecalis formed cytochrome pigments, cytochrome b_2 being identified and shown to be functional in electron transport. Cell free extracts were obtained in which ATP production coupled to oxidation of NADH was demonstrated.

Several lactic acid bacteria formed catalase when grown on media supplemented with haematin. Formation of the apoenzyme of catalase in the absence of haematin was demonstrated. When haematin was added to a resting cell suspension catalase was formed immediately. If chloramphenicol was present the catalase was still formed whilst the adaptive enzymes concerned in malate oxidation were formed to a reduced extent in the presence of the antibiotic.

3. Organic acid metabolism of lactic acid bacteria

The rates of citrate and malate breakdown by various lactic acid bacteria were measured. The effect of pH on the rate of citrate and malate breakdown was studied and the products of citrate breakdown by Streptococcus faecalis and of malate breakdown by Lactobacillus plantarum were determined.

These experiments indicated the likely fate of citrate and malate during a silage fermentation.

4. Silage inoculation experiments

A laboratory scale experiment showed an advantage could be obtained from an inoculation of herbage prior to ensiling with a S. faecalis/L. plantarum mixture. This was followed up with a farm scale experiment using 1 ton silos. This showed less effect due to the inoculation as all the silages were well preserved. There were, however, smaller dry matter losses with the inoculated silages than in the controls.

INTRODUCTION

The bacteriological changes occurring when herbage is ensiled have been well documented (Watson and Nash, 1960; Gibson and Stirling, 1959). Lactic acid bacteria increase from low numbers on growing plant material to relatively large numbers when the material reaches the silo (Stirling and Whittenbury, 1963), and then grow rapidly after ensilage of the material, reaching maximum numbers in the first few days. As the proliferation of the lactic acid bacteria occurs, there is a decrease in the numbers of obligately aerobic bacteria, which are the dominant flora of the growing plant.

If sufficient acid is produced by the lactic acid fermentation to lower the pH to around pH 4, the growth of undesirable anaerobic bacteria of the genus Clostridium is inhibited and the silage is well preserved. Clostridia are inhibited by a combination of low pH and osmotic pressure (Wieringa, 1958), so that the drier the silage, the smaller the pH fall needed to inhibit clostridial growth.

The effects of the bacteria on the chemical composition of silage is less well understood. That a lactic acid fermentation, either homolactic or heterolactic fermentation, takes place is accepted, but the rates at which different lactic acid bacteria ferment the different sugars is not known.

It is also apparent that lactic acid bacteria possess very active aerobic pathways (Dolin, 1955; Whittenbury, 1963),

many growing better under aerobic conditions than under anaerobic conditions. This fact could be of interest in explaining the rapid increase in lactobacilli in a cut crop before ensilage (Stirling and Whittenbury, 1963) and the rapid growth of lactobacilli in the initial, aerobic, stages of ensilage.

Organic acids, especially citric acid and malic acid, present in fresh herbage, disappear in the first day or two of storage in a silo. Various workers (Gunsalus and Campbell, 1944; Campbell and Gunsalus, 1944; Whittenbury, 1961) have shown that many lactic acid bacteria ferment these acids, either in energy yielding pathways or in the presence of an alternative energy source. The rates of breakdown, losses as carbon dioxide, and products of breakdown are not well documented, and an investigation has been made into organic acid breakdown by silage lactic acid bacteria.

Many attempts have been made to improve the quality of silage by the addition of various substances. The A.I.V. process, in which mineral acids are used to lower the silage pH, and the use of molasses have become accepted practices but other additives are rarely used.

The possible additives can be divided into three types:

a) those which directly inhibit bacterial growth, especially of clostridia; this type includes acids, antibiotics, sulphur dioxide and sodium metabisulphite,

b) substances which act as energy sources for the growth

of lactic acid bacteria and therefore as sources of lactic acid,

c) the addition of an inoculum of lactic acid bacteria.

The addition of mineral acids is established in the A.I.V. process and the addition of formic acid is widely used in Scandinavia and is currently receiving a great deal of attention. Both these additives are theoretically added at a concentration which lowers the pH of the herbage to a level which inhibits the growth of clostridia and therefore preserves the silage. The disadvantage of acid as an additive is the difficulty of handling it, although this problem is somewhat reduced if formic acid is used.

Antibiotics appear to be a much simpler additive to apply to herbage. Some success has been claimed for bacitracin (Dexter, 1957; Russoff, Breidenstein and Milstead, 1959; Owen, 1962) and as 'silotracin' it has been used commercially in the U.S.A. A recent suggestion has been that nisin might prove effective in aiding the preservation of silage. Nisin is used in processed cheese to inhibit clostridial growth and it is claimed that it inhibits spore germination. Flam (1967) found nisin did not prevent carbon dioxide production from carbohydrates by Clostridium butyricum and adding it to herbage did not improve silage produced from this herbage. In Flam's experiments all the silages were of good quality and it is possible that in silage made from low sugar herbage nisin may usefully inhibit the growth of clostridia.

Generally inconsistent and unconvincing results have come from experiments designed to show that antibiotics can be useful as an aid in silage making.

Sulphur dioxide, metabisulphite, common salt and other additives have been tried but have not been persisted with in this country.

Of the second group of additives molasses is by far the most useful. If a crop is low in water soluble carbohydrate, e.g. cocksfoot often has less than 6-7% W.S.C., then acid production may be insufficient to preserve the crop (Smith, 1962), and addition of a cheap carbohydrate will overcome this deficiency and enable good silage to be made.

Lastly, various workers (Allen, Watson and Ferguson, 1937; McDonald, Stirling, Henderson and Whittenbury, 1964) have inoculated herbage with lactic acid bacteria to aid the rapid establishment of a lactic acid fermentation. This method would only appear to be of use when the grass is low in water soluble carbohydrates or is of high buffering capacity, in other words when the crop is below the borderline between potentially good and bad silage.

The addition of sugar and an inoculum of lactic acid bacteria seems the most logical application of the second and third types of additives (Whittenbury, McDonald and Bryan-Jones, 1967) especially for crops with low sugar content or high buffering capacity.

The work presented in this thesis is considered in four

sections.

The first section compares the sugar metabolism of six strains of lactobacilli selected as representative of the lactic acid forming silage microflora.

The second section reports the results of an investigation into the electron transport systems of lactic acid bacteria, with a detailed consideration of haematin enzyme systems.

The third section is an investigation into the organic acid breakdown by the same lactic acid bacteria as used in the sugar metabolism studies.

The last section considers the inoculation of herbage as an aid in silage making and gives the results of experiments on both laboratory silo scale and small farm silo scale to investigate the effect of inoculating herbage prior to ensiling it.

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SECTION ONE

Sugar Metabolism of Lactic Acid Bacteria

Introduction

The fermentation of sugars by anaerobic bacteria to produce lactic acid has been studied by many workers.

Recognition that there were two distinct types of lactic acid producing fermentations led to a division of the bacteria concerned into two groups: heterofermentative lactic acid bacteria producing carbon dioxide and other products when fermenting hexoses, and homofermentative lactic acid bacteria producing only lactic acid.

Detailed studies of the reactions involved have led to the elucidation of two completely different anaerobic pathways of glucose fermentation.

Gibbs, Dumrose, Bennett and Bubeck (1950) have shown by the labelling patterns of the products of glucose-1-C^{14} and glucose-3-C^{14} fermentation by Lactobacillus casei, Lactobacillus pentosus (plantarum) and Streptococcus faecalis that the Embden-Meyerhof glycolytic pathway is the mechanism involved in homolactic fermentation.

Heterolactic fermentation of glucose normally results in the production of lactic acid, ethanol and carbon dioxide in a 1:1:1 ratio (DeMoss, Bard and Gunsalus, 1951). Gunsalus and Gibbs (1952), using labelled glucose, showed that carbon atom one of the glucose became carbon dioxide, C^2 and C^3 became ethanol and C^4, C^5 and C^6 became lactate. This demonstrated a marked divergence from the Embden-Meyerhof glycolytic

pathway. Heath, Hurwitz, Horecker and Ginsburg (1958) have purified an enzyme, phosphoketolase, from Lactobacillus plantarum which splits pentose phosphate into a two-carbon and a three-carbon fragment. In the homofermentative bacteria this enzyme is concerned with pentose fermentation but in heterofermentative organisms glucose is converted to pentose phosphate by a hexose monophosphate pathway. The pentose phosphate is then split by phosphoketolase to form acetate and pyruvate, which, under anaerobic conditions, are reduced to ethanol and lactic acid respectively.

Several deviations from this normal heterofermentative pathway have been noted.

Nelson and Werkman (1935) recorded that in Lactobacillus brevis a pathway with glycerol as an end product (see appendix 2) accounts for a proportion of the sugar fermented.

Fructose fermentation by heterofermentative organisms yields mannitol, lactic acid, acetic acid, ethanol and carbon dioxide (Eltz and Vandemark, 1959). The ethanol is completely absent from the products of fructose fermentation by Lactobacillus brevis, and present in reduced quantities, compared to normal heterofermentative stoichiometry, in other organisms. The reduction of two molecules of fructose to mannitol balances the oxidation of one molecule of fructose to lactic acid, acetic acid and carbon dioxide.

These pathways operate under anaerobic conditions but many lactic acid bacteria can use oxygen as a terminal hydrogen

acceptor (Whittenbury, 1963).

As cytochromes are usually lacking from lactic acid bacteria the terminal step of the electron transport system to oxygen involves flavoprotein enzymes (Strittmatter, 1959).

This ability to use oxygen results in several differences in the products of sugar metabolism. Pyruvate is spared to some extent from further reduction and may be fermented or oxidised to produce lactic acid, acetic acid and carbon dioxide. In heterofermentative organisms acetate may also be spared from further reduction, resulting in less ethanol amongst the products of sugar breakdown.

In order to further elucidate the pathways of silage lactic acid bacteria, and therefore to understand the chemical changes occurring during the ensilage process, several aspects of sugar metabolism by lactic acid bacteria have been investigated.

The heterofermentative, mannitol producing, pathway has been investigated in detail with strains of Lactobacillus brevis and Leuconostoc mesenteroides.

The rates of oxygen uptake and the molar quantities of oxygen used by resting cell suspensions of lactic acid bacteria metabolising various sugars have been measured manometrically. These oxidative reactions could be of great importance during the early stages of a silage fermentation before anaerobic conditions are established.

Under anaerobic conditions the rates of sugar breakdown

and carbon dioxide evolution have been studied, comparing six silage bacteria that were potentially useful as organisms for the inoculation of herbage in order to control the subsequent silage fermentation. A choice could then be made of the most suitable organisms for use as an inoculum.

Growth yield experiments have been conducted to determine the energy yield to the bacteria from various substrates under different conditions.

The evidence from all these sources has been combined to determine the pathways each organism uses for sugar metabolism, and therefore to determine the possible pathways operating in a silage fermentation.

EXPERIMENTAL

Methods

Organisms. The organisms investigated were as outlined below:

Organism	Strain No.	Source
Streptococcus faecalis	581	N.C.D.O.
Streptococcus faecium	HGH 511	
Pediococcus	507	
Lactobacillus plantarum	5914	N.C.I.B.
Lactobacillus brevis	18	
Leuconostoc mesenterioides	60	
	(All supplied by Dr R. Whittenbury)	
Lactobacillus viridescens	1655	N.C.D.O.

Media

Basal medium. A basal medium consisting of 0.5% peptone, 0.5% yeast extract and 0.5% lemco (all Oxoid) at pH 6.5 was used. For a solid medium 1.5% of agar was added. Energy sources were added as filter sterilised solutions as required.

Soft agar medium. Soft agar was as the basal medium with 0.15% agar added and bromocresol purple added as an indicator (Whittenbury, 1963). Energy sources were added as filter sterilised solutions as required.

All purpose tween medium. Lactobacillus viridescens was grown on the all purpose tween medium as described by

Evans and Niven (1951).

Heated blood, o-dianisidine agar. Heated blood o-dianisidine agar was used to detect hydrogen peroxide production and was prepared as described by Whittenbury (1964).

Roux bottles containing agar slopes were used to grow cells aerobically. Anaerobically, cells were grown in deep broth cultures in one litre, wide bottomed conical flasks filled to the neck with medium.

All cultures were incubated at 30°C.

Washed cell suspensions. Cell suspensions were prepared by centrifuging (3000 r.p.m. for 10 min.) either broth cultures or growth washed from agar slopes. The harvested cells were washed twice with buffer and resuspended in buffer. The quantity of cell material was estimated by optical density and expressed as cell dry weight per ml.

Cell free extracts. Heavy cell suspensions (about 30 mg. cell dry wt. per ml.) were disrupted either by ultrasonic oscillations or by shaking with Ballotini beads with a Mickle shaker. Unbroken cells and cell debris were removed by centrifugation at 23,000 x g. for 30 min.

The concentration of protein in cell free extracts was measured by the Folin-Ciocalteau phenol reagent method, as described by Lowry, Rosebrough, Farr and Randall (1951).

Oxygen requirements. Oxidative, microaerophilic and fermentative growth on various substrates was investigated using soft agar media.

Manometry. Conventional manometric techniques as described in Umbreit, Burris and Stauffer (1951) were used to follow the uptake of oxygen and the evolution of carbon dioxide by resting cell suspensions. When a quantitative estimate of total carbon dioxide evolution was required any carbon dioxide dissolved in the buffer was released by the addition of 0.5 ml. of 0.1 N HCl, from a second side arm, at the end of the experiment.

Anaerobic conditions in manometer flasks were achieved by sparging the flasks with nitrogen for 15 minutes. Checks for any residual oxygen, by measuring oxygen uptake with a suitable substrate, always resulted in negligible amounts of residual oxygen being detected.

Enzyme assays. The oxidation of reduced pyridine nucleotides with oxygen as the terminal hydrogen acceptor was assayed spectrophotometrically in cuvettes containing 1-4 mg. protein of cell free extract; 150 μM potassium phosphate buffer, pH 6.8; 0.03 μM NADH or NADPH (as their sodium salts); and distilled water to a total volume of 2.5 ml. Oxidation of the reduced pyridine nucleotide was followed as the decrease in optical density at a wavelength of 340 mμ., on a Unicam SP500

spectrophotometer.

Oxidation of reduced pyridine nucleotides with hydrogen acceptors other than oxygen was measured in cuvettes sparged with nitrogen for 5 minutes and then maintained under a nitrogen atmosphere. The reactions were initiated by the addition of the hydrogen acceptor.

Mannitol dehydrogenase (fructose-NAD/NADP oxidoreductase) was assayed by following the oxidation of reduced pyridine nucleotide in the system as outlined above plus 10 μM fructose and 25 μM $MgCl_2$ in each cuvette.

Acetaldehyde dehydrogenase (acetyl phosphate-NAD/NADP oxidoreductase) was assayed in a similar system with 10 μM acetyl phosphate replacing the fructose.

Ethanol dehydrogenase (acetaldehyde-NAD/NADP oxidoreductase) was assayed in a similar system with 10 μM acetaldehyde replacing the fructose.

Glucose and fructose estimation. Glucose and fructose were measured by the method described by Fuller, Lampitt and Coton (1955) in which the reduction of potassium ferricyanide by the sugar was measured by subsequent titration against ceric sulphate with xylene cyanol FF as an indicator.

Rates of glucose and fructose fermentation. Resting cell suspensions were incubated with glucose or fructose under an atmosphere of nitrogen in the apparatus shown in Figure 1.

Fermentation balance experiments.

In the apparatus shown in Figure 1 a U-tube was incorporated between the fermentation flask and the first Drechsel bottle. This U-tube was surrounded by an ice-bath and trapped any volatile products of the fermentation reactions. The contents of the U-tube were added to the bulk of the supernatent from the fermentation flask at the end of the experiment.

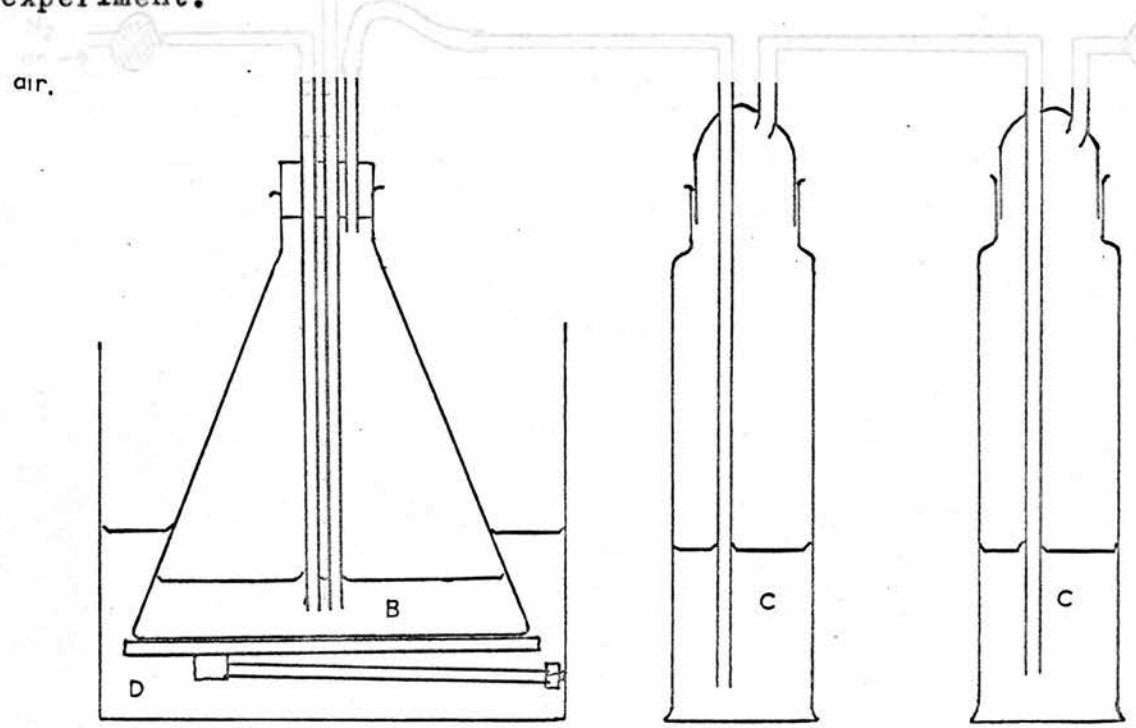

FIGURE 1. Apparatus used in fermentation and oxidation balance experiments.

KEY:
- A Carbon dioxide trap.
- B Reaction flask.
- C Dreschel bottles containing 50ml. 0·1N $Ba(OH)_2$.
- D Shaking water bath.
- E Inlet for additions to reaction flask and removal of samples.

Each flask contained 20 mg. cell dry wt.; 2.5 mM potassium phosphate buffer pH 6.0; 40 mg. of glucose or fructose and distilled water to a total volume of 100 ml. The experiment was started by the addition of the glucose or fructose solution and 1 ml. samples were removed at 15 minute intervals and assayed for either glucose or fructose.

Growth yield experiments. Growth yields were measured in the basal, broth medium, except for Streptococcus faecalis, when the partially defined medium of Bauchop and Elsden (1960) was used, and for Lactobacillus viridescens, when the APT medium of Evans and Niven (1951) was used.

Substrates were added to the media as filter sterilised solutions. Aseptically harvested and washed cells were used as inocula to avoid carrying any energy source from the growth medium to the test medium. In all cases very small inocula were used.

Aerobic growth yields were measured in 10 ml. broth cultures growing in 50 ml. Erlenmeyer flasks incubated at 30°C on a shaker.

Anaerobic growth yields were measured in 10 ml. broth cultures in $\frac{5}{8}$" test tubes. The broth was briefly boiled, cooled, the substrate added and inoculated. Anaerobic conditions were maintained by covering the medium with a 1 cm. thick seal of 2% water agar. Incubation was at 30°C.

Samples were examined on a Unicam SP1300 colorimeter and

TABLE 1. Growth (as acid production) of some heterofermentalactobacilli in soft agar media.

Substrate:	No Sugar	Glucose		Fructose		Arabinose		Glucose (autoclaved in medium)	
Incubated for:	7 days	2 days	7 days	2 days	7 days	2 days	7 days	2 days	7 days
Organism									
Lactobacillus brevis 6	-	-	+	-	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
L. brevis 18	-	+	+	+	+	-	+	+	+
	-	-	-	+	+	+	+	+	+
	-	-	-	+	+	+	+	-	+
L. brevis 27	-	-	-	-	+	-	+	+	+
	-	-	-	+	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
L. brevis 38	-	-	-	-	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
L. brevis 42	-	-	-	-	-	-	+	+	+
	-	-	-	-	+	+	+	+	+
	-	-	-	+	+	+	+	+	+
L. brevis 84	-	-	+	-	-	+	+	+	+
	-	-	-	-	-	+	+	-	+
	-	-	-	-	(+)	-	+	-	+
L. viridescens	-	+	+	+	+	-	-	+	+
	-	-	-	-	-	-	-	+	+
	-	-	+	-	(+)	-	-	+	+
Leuconostoc mesenteroides	-	+	+	+	+	-	-	+	+
	-	+	+	+	+	+	+	+	+
	-	-	+	+	+	+	+	+	+

KEY:
+ growth aerobically
+ growth microaerophilically
+ growth anaerobically

- no growth aerobically
- no growth microaerophilically
- no growth anaerobically

(+) mutant growth (one or two colonies only)
n.t. not tested

TABLE 2. Carbon dioxide evolution by washed cell suspensions of Lactobacillus brevis and Leuconostoc mesenteroides with glucose and fructose as substrates (anaerobically).

Substrate: Organism	$Q_{CO_2}^{N_2}$ @ 30°C Endogenous	Glucose	Fructose
Lactobacillus brevis 18	0.0	0.0	8.0
Leuconostoc mesenteroides	0.1	6.7	33.0

N.B. Cells grown anaerobically on fructose. Each flask contained 1-5 mg. cell dry weight; 10 μM fructose or glucose; 150 μM potassium phosphate buffer pH 6.8; total volume 3 ml. Flasks sparged with nitrogen for 15 minutes. Incubated at 30°C.

Growth yield methods.

Aerobic growth yields were measured with four flasks at each substrate concentration. Three of the flasks were used to follow growth and when growth had ceased the contents of the fourth flask were made up to 10ml. with distilled water and the optical density measured.

All growth yields were measured at several substrate concentrations and the results plotted on a graph. Final growth yield results were then calculated from the gradient of the linear portion of the graph.

the optical density recorded when growth had ceased. The dry weight of cells present was obtained from a standard optical density against dry weight curve, prepared for each organism investigated.

Results

The heterofermentative mannitol producing fermentation

Soft agar studies (Table 1) showed an inability of Lactobacillus brevis to ferment glucose, whilst fructose was fermented. This is in agreement with the findings of Whittenbury (1963).

Carbon dioxide evolution by Lactobacillus brevis and Leuconostoc mesenteroides anaerobically with glucose and fructose as substrates was measured manometrically. The results are summarised in Table 2. The rates of carbon dioxide evolution bear out the results obtained in the soft agar studies. Lactobacillus brevis only fermented fructose whilst Leuconostoc mesenteroides fermented both glucose and fructose, the fermentation of fructose proceeding at a faster (5x) rate than the fermentation of glucose.

The total quantities of carbon dioxide evolved by L. brevis and Leuconostoc mesenteroides were measured. A figure of 0.34 M. of carbon dioxide per Mole of fructose was obtained with L. brevis and a figure of 0.32 M. of carbon

TABLE 3. Use of various hydrogen acceptors by cell free extracts of anaerobically grown Lactobacillus brevis and Leuconostoc mesenteroides.

Units: mμM NADH/NADPH oxidised/min./mg. protein

Organism:	Lactobacillus brevis				Leuconostoc mesenteroides			
Pyridine nucleotide:	NADH		NADPH		NADH		NADPH	
H Acceptor		+ $MgCl_2$		+ $MgCl_2$		+ $MgCl_2$		+ $MgCl_2$
Fructose	1.30	1.58	0.38	0.24	37.6	96.0	0.29	n.t.
Glucose	0.0	n.t.	n.t.	n.t.	0.05	0.05	0.05	n.t.
Acetyl phosphate	0.0	0.06	0.10	0.05	4.56	0.98	0.19	0.05
Acetaldehyde	9.6	9.6	42.8	n.t.	4.61	9.36	69.8	101.5

KEY: n.t. - not tested

dioxide per Mole of fructose was obtained with Leuconostoc mesenteroides. Both these figures are as would be expected if two Moles of fructose were reduced to mannitol for each Mole of fructose oxidised. This suggests that both these organisms use the mannitol producing pathway for fructose fermentation.

Eltz and Vandemark (1959) studied the enzymes present in extracts of L. brevis which had been grown on fructose. All the enzymes of the normal heterofermentative pathway were detected except acetaldehyde dehydrogenase. Also mannitol dehydrogenase activity, which is not considered part of the normal pathway, was detected.

The dehydrogenase enzymes of L. brevis and Leuconostoc mesenteroides were examined in cell free extracts. Table 3 shows the results obtained in these experiments.

A NADH-linked mannitol dehydrogenase which reduced fructose to mannitol in the presence of NADH but not NADPH was detected in both L. brevis and Leuconostoc mesenteroides. This enzyme was similar to the one described by Eltz and Vandemark (1959) but differed from the one described by Wolff and Kaplan (1956) in that it did not require phosphorylation of the fructose prior to reducing it to mannitol.

Neither the L. brevis nor the Leuconostoc mesenteroides extracts could use glucose as a hydrogen acceptor.

The reduction of fructose could theoretically produce either sorbitol or mannitol but identification of the products

of fructose fermentation (MacGregor and Bryan-Jones, 1966) showed mannitol to be the product of this reaction.

The mannitol dehydrogenase enzyme in both L. brevis and Leuconostoc mesenteroides extracts was stimulated when magnesium chloride (10 μM/ml.) was included in the reaction mixture.

A NADH-linked acetaldehyde dehydrogenase was detected in the Leuconostoc mesenteroides extracts but not in the L. brevis extracts. It was assumed to be absent from the latter organism as extracts of both organisms had been prepared in an identical manner, from cells grown anaerobically on fructose. To check that cell disintegration by ultrasonic oscillations was not destroying the enzyme in L. brevis, extracts were prepared by the use of a Mickle shaker. No differences were observed with the extracts prepared by this second method compared to extracts prepared by ultrasonic disintegration of the cells.

Seegmillar (1953) found a 'TPN-linked aldehyde dehydrogenase' in yeast which was activated by Cu, Mg, Ba and Mn. Some acetaldehyde dehydrogenase assays were therefore carried out with added $MgCl_2$ (10 μM/ml.) but with the Leuconostoc mesenteroides extracts this resulted in a decrease in enzyme activity.

King and Cheldelin (1956) found a 'DPN/TPN-linked acetaldehyde dehydrogenase' in Acetobacter suboxydans not stimulated by KCl or $MgCl_2$ (up to 40 μM/ml.), which did not require phosphate or Co-enzyme A, had an optimum pH of 8.7 and

TABLE 4. Effect of L-cysteine and Co-enzyme A on acetaldehyde dehydrogenase activity in cell free extracts of Lactobacillus brevis and of Leuconostoc mesenteroides.

mμM NADH/NADPH oxidised/min./mg. protein

Pyridine nucleotide:	NADH			NADPH	
Co-factors:	None	L-cyst.	Co-A	None	L-cyst.
Organism					
Lactobacillus brevis	0.0	0.29	0.24	0.10	0.05
Leuconostoc mesenteroides	4.56	3.24	0.72	0.19	0.05

TABLE 5. Ethanol dehydrogenase activity in cell free extracts of Lactobacillus brevis and of Leuconostoc mesenteroides.

mμM NADH/NADPH oxidised/min./mg. protein

Pyridine nucleotide: / Organism	Cells grown aerobically		Cells grown anaerobically	
	NADH	NADPH	NADH	NADPH
Lactobacillus brevis	0.33	10.3	9.6	42.8
Leuconostoc mesenteroides	2.34	73.9	9.4	101.5

was stimulated by cysteine, glutathione and E.D.T.A. The *L. brevis* extract was therefore assayed for acetaldehyde dehydrogenase activity in the presence of L-cysteine, and the *Leuconostoc mesenteroides* extract was assayed for acetaldehyde dehydrogenase in the presence of L-cysteine and Co-enzyme A. The results of these experiments with added L-cysteine and Co-enzyme A are shown in Table 4. L-cysteine showed a slight inhibitory effect on the *Leuconostoc mesenteroides* acetaldehyde dehydrogenase and Co-enzyme A reduced the activity even more. *L. brevis* still showed no acetaldehyde dehydrogenase activity with L-cysteine added. The values of less than 0.3mμM NADH or NADPH oxidised per minute per mg. protein were of the same order of magnitude as the endogenous rates of NADH or NADPH oxidation.

Extracts of both *Lactobacillus brevis* and *Leuconostoc mesenteroides* showed very active ethanol dehydrogenase enzymes, reducing acetaldehyde to ethanol whilst oxidising reduced pyridine nucleotides. Results of ethanol dehydrogenase assays are shown in Table 5. The enzyme in *L. brevis* extracts was not stimulated by $MgCl_2$ (10 μM/ml.) but the *Leuconostoc mesenteroides* ethanol dehydrogenase was, therefore when assaying ethanol dehydrogenase activity in extracts of *Leuconostoc mesenteroides* 10 μM/ml. $MgCl_2$ was added to the system.

The very high levels of activity of this enzyme are of interest as if it is specific for acetaldehyde this enzyme would have no substrate in *L. brevis*, and a very low

TABLE 6. Oxygen uptake during glucose metabolism by *Lactobacillus brevis* and *Leuconostoc mesenteroides*.

Substrate: / Organism	$Q_{O_2}^{air}$ 30°C Endogenous control	$Q_{O_2}^{air}$ 30°C Glucose	M O_2/M Hexose Glucose
Streptococcus faecalis	0.2	44.7	1.11
Streptococcus faecium	0.2	21.5	1.34
Pediococcus	0.0	2.1	-
Lactobacillus brevis	0.2	22.5	1.0
Leuconostoc mesenteroides	0.3	21.6	0.74
Lactobacillus plantarum	0.2	3.8	0.30
Lactobacillus viridescens	0.2	91.0	1.23

concentration of substrate in Leuconostoc mesenteroides as the ethanol dehydrogenase activity was 25x greater than the acetaldehyde dehydrogenase activity. There was a lower level of ethanol dehydrogenase activity in extracts prepared from aerobically grown cells than in extracts prepared from anaerobically grown cells. As this enzyme is unnecessary for aerobic growth there is therefore an adaptive mechanism controlling the amount of enzyme present. However, with the lack of substrate in L. brevis it would seem unlikely that the substrate acts as the inducer. The ethanol dehydrogenase was more active with NADPH than with NADH.

Oxidative reactions

Seven strains of lactic acid bacteria were examined for an ability to use oxygen as a hydrogen acceptor during glucose metabolism. Rates of oxygen uptake with glucose as a substrate were measured and the molar quantities of oxygen used per Mole of glucose metabolised were measured.

Table 6 shows the results obtained in these experiments. Streptococcus faecalis cells used oxygen at a rapid rate and 1.1 Moles of oxygen were used per Mole of glucose metabolised. S. faecalis showed an increased rate of oxygen uptake if haematin had been included in the growth medium, and this phenomenon was further investigated, the results being recorded and discussed in Section Two.

S. faecium also showed a rapid rate of oxygen uptake and

1.34 Moles of oxygen were used per Mole of glucose metabolised. S. faecium but not S. faecalis produces free H_2O_2 (Whittenbury, 1964) and this probably accounts for the increased oxygen uptake compared to S. faecalis.

The Pediococcus strain took up oxygen at a very slow rate and a measure of the molar quantity of oxygen used for each Mole of glucose metabolised was not obtained.

The Lactobacillus plantarum strain also had a slow rate of oxygen uptake and only used 0.3 Moles of oxygen for each Mole of glucose utilised.

It would seem likely, therefore, that only a small proportion of the glucose was metabolised oxidatively by L. plantarum and the Pediococcus and the balance followed the normal anaerobic pathway.

The three heterofermentative organisms studied all used oxygen as an acceptor of hydrogen during glucose metabolism. The Lactobacillus brevis would only metabolise glucose in the presence of oxygen as a hydrogen acceptor and would not use glucose anaerobically. The use of 1 Mole of oxygen per Mole of glucose metabolised by L. brevis suggests 2 Moles of NADH are oxidised with oxygen as the hydrogen acceptor. This is the number of Moles of NADH normally oxidised when acetate is reduced to ethanol, which is the pathway which is lacking in this organism. When metabolising fructose L. brevis used 1.34 Moles of oxygen per Mole of fructose utilised which is a larger amount than expected. The extra oxygen was probably

TABLE 7. Oxidation of reduced pyridine nucleotides by cell free extracts of lactic acid bacteria.

mμM NADH/NADPH oxidised/min./mg. protein

Cells grown aerobically

Pyridine nucleotide:	NADH				
Co-factor:	None	$MgCl_2$	None	None	None
Inhibitor:	None	None	NaN_3 $4x\ 10^{-3}M$	NaN_3 $2x\ 10^{-2}M$	KCN $1x\ 10^{-2}M$
Organism					
Lactobacillus brevis	37.1	44.5	37.3	34.9	-
Leuconostoc mesenteroides	-	-	-	-	-
Streptococcus faecalis	5.2	-	10.0	-	4.0

Cells grown anaerobically

Pyridine nucleotide:	NADH		NADPH	
Co-factor:	None	$MgCl_2$	None	$MgCl_2$
Inhibitor:	None	None	None	None
Organism				
Lactobacillus brevis	6.1	11.0	0.6	0.0
Leuconostoc mesenteroides	25.5	23.3	0.0	-
Streptococcus faecalis	-	-	-	-

KEY: - not tested

due to the oxidation of NADH otherwise used to reduce pyruvate to lactate, and the possible oxidation of some of the pyruvate.

Lactobacillus viridescens used more than 1 Mole of oxygen per Mole of glucose but if catalase was added to the Warburg flask at the end of the experiment 0.15 Moles of oxygen were evolved, reducing the net oxygen uptake to 1.08 Moles of oxygen per Mole of glucose. Therefore the extra oxygen uptake by L. viridescens was due to peroxide formation. This result suggested that L. viridescens was using the same oxidative pathway as L. brevis, probably producing acetate in place of ethanol.

Leuconostoc mesenteroides used less than 1 Mole of oxygen per Mole of glucose metabolised and this was presumed to be due to the simultaneous use of the fermentative and oxidative pathways for glucose metabolism so resulting in a reduced oxygen uptake compared to that of L. brevis.

The oxidative pathways of Lactobacillus brevis, Leuconostoc mesenteroides and Streptococcus faecalis were further investigated with cell free extracts oxidising reduced pyridine nucleotides in the presence of oxygen. The rates of NADH oxidation are shown in Table 7. Cell free extracts of all three organisms oxidised NADH in the presence of oxygen but showed little or no oxidation of NADPH, showing the oxidase enzymes to be NADH-linked. With Lactobacillus brevis aerobically grown cells gave extracts with greater NADH-oxidase activity than did anaerobically grown cells. Mg^{++} (as

10 μM/ml. $MgCl_2$) showed a stimulatory effect on the rate of NADH oxidation by L. brevis extracts but did not stimulate Leuconostoc mesenteroides extracts.

Sodium azide (4 μM/ml.) showed no inhibitory effect on NADH oxidation by L. brevis and only 6% inhibition was noted when the concentration of azide was increased to 20 μM/ml. The S. faecalis extracts showed slight inhibition with 10 μM/ml. KCN (10^{-2} M), but a faster rate of NADH oxidation in the presence of 10 μM/ml. sodium azide than in controls lacking azide.

Extracts of L. brevis and Leuconostoc mesenteroides were examined spectrophotometrically, an oxidised extract being compared with a reduced extract (sodium dithionite added to reduce the contents of the cuvette) and the spectrum plotted over a range of wavelength. The oxidised extracts showed marked peaks at 450 mμ (Fig. 2) showing the presence of flavoproteins which are probably functional in the NADH-oxidase system.

Growth yield experiments (see below) showed no extra energy from oxidation and therefore no evidence for any oxidative phosphorylation by these organisms. The exception to this was Streptococcus faecalis grown in the presence of haematin, and this is discussed in the section on haematin enzymes in lactic acid bacteria.

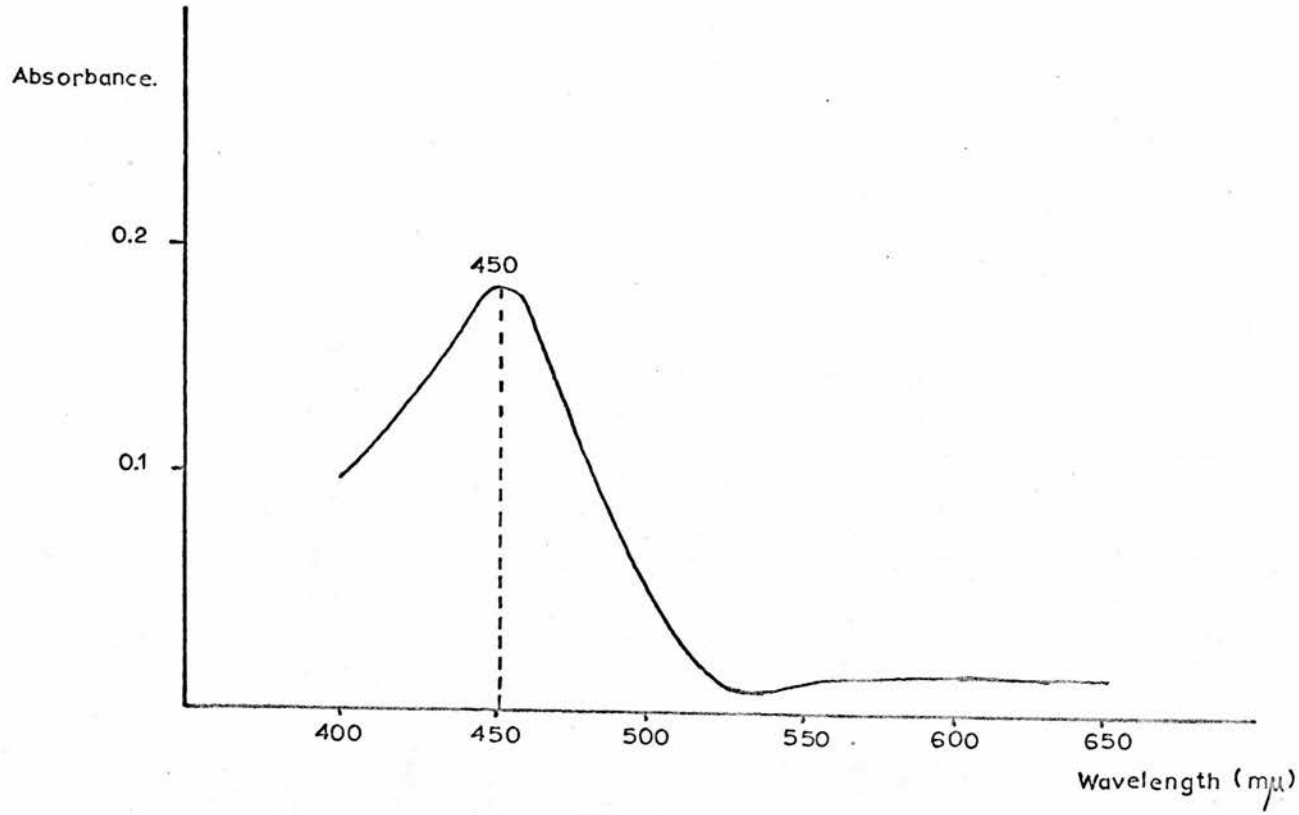

FIGURE 2. L. brevis. Difference spectrum. Oxidised cell free extract plotted against a reduced (with sodium dithionite) cell free extract.

TABLE 8. Comparison of rates of sugar breakdown by six silage lactic acid bacteria.

Organism	μM Glucose	μM Fructose	Maximum μM of acids
Streptococcus faecalis	6.4	-	12.8 lactate
Streptococcus faecium	4.4	-	8.8 lactate
Pediococcus	1.2	-	2.4 lactate
Lactobacillus plantarum	3.2	-	6.4 lactate
Leuconostoc mesenteroides	1.9	3.3	on fructose: 2.4 lactate 2.4 acetate
Lactobacillus brevis	0.0	1.8	on fructose: 0.6 lactate 0.6 acetate

All figures are as measured per hour per mg. of cell dry weight.

Anaerobic sugar metabolism

When considering the usefulness of various organisms as inocula to improve silage fermentations the rate of acid production from sugars was thought to be important. The rate of glucose utilisation gives an indication of the rate of acid production by lactic acid bacteria. The rates of glucose utilisation by Streptococcus faecalis, S. faecium, Pediococcus, Lactobacillus plantarum, L. brevis and Leuconostoc mesenteroides were measured, and, in view of their unusual fructose fermentation, the rates of fructose fermentation by Leuconostoc mesenteroides and Lactobacillus brevis were also measured.

Table 8 summarises the rates of sugar utilisation and therefore acid production (assuming the pathways outlined in the appendices) by the six organisms investigated.

Streptococcus faecalis metabolised glucose most rapidly of the organisms tested, followed by S. faecium. S. faecalis is not very acid tolerant but its rapid acid production can make a useful contribution in the early stages of a silage fermentation.

Of the other organisms Lactobacillus plantarum on glucose and Leuconostoc mesenteroides on fructose were the fastest acid producing organisms. However, Leuconostoc mesenteroides produces mannitol and carbon dioxide whereas Lactobacillus plantarum produces only lactic acid and is acid tolerant and is therefore more efficient in an ideal silage fermentation.

Carbon dioxide evolution by lactic acid bacteria

TABLE 9. Carbon dioxide evolution by lactic acid bacteria fermenting glucose and fructose.

$Q \begin{matrix} N_2 \\ CO_2 \end{matrix}$ 30°C

Organism	Growth substrate for the cells (anaerobically)	Endogenous control	Glucose	Fructose
Streptococcus faecalis	Glucose	0.0	1.6	1.3
Streptococcus faecium	Glucose	0.0	0.4	0.4
Pediococcus	Glucose	0.0	0.0	0.4
Lactobacillus plantarum	Glucose	0.2	0.3	0.3
Leuconostoc mesenteroides	Fructose	0.1	6.7	33.0
Lactobacillus brevis	Fructose	0.0	0.0	8.0

fermenting glucose and fructose was measured manometrically. Table 9 outlines the results of these experiments.

There was some carbon dioxide evolution by the homofermentative organisms but it was at a negligible level compared to the rates of carbon dioxide evolution by the heterofermentative organisms. One possible exception to this was Streptococcus faecalis which gave a steady but slow rate of carbon dioxide evolution from both glucose and fructose. Mention has already been made of the differences between glucose and fructose fermentation by Lactobacillus brevis and Leuconostoc mesenteroides.

One interesting phenomenon was the anomalous fermentation of glucose which had been autoclaved in the medium. Whittenbury (1961) noted that if glucose was autoclaved in a medium, such as the basal medium used in this work, then Lactobacillus brevis would grow anaerobically in the medium, whereas if separately sterilised glucose was added to the medium no growth of Lactobacillus brevis occurred. This was presumably due to either the formation of some reducible compound which acted as a hydrogen acceptor or to the oxidation of the glucose to give a compound such as gluconic acid which L. brevis can ferment.

To investigate this phenomenon, glucose was autoclaved with the separate constituents of the basal medium. Carbon dioxide evolution by Lactobacillus brevis (washed cell suspension of cells grown anaerobically on fructose) was measured on this autoclaved glucose. Results of this experiment are

TABLE 10. Fermentation of glucose, autoclaved with the constituents of the basal medium, by a washed cell suspension of *Lactobacillus brevis*.

Substrate (autoclaved at 15 lb./sq.in. for 15 minutes)	$Q \frac{N_2}{CO_2}$ 30°C
0.5% glucose (in distilled water)	0.0
0.5% glucose with 0.5% yeast extract	5.0
0.5% glucose with 0.5% peptone	0.9
0.5% glucose with 0.5% lemco	0.0
0.5% glucose with the complete basal medium	6.9

shown in Table 10. Autoclaving glucose with 0.5% yeast extract therefore causes changes in the glucose which permits the L. brevis cells to ferment this substrate.

A sample of the spent growth medium, obtained by centrifuging down cells after growth in a medium with autoclaved glucose, was examined chromatographically (a solvent of n-butanol:acetic acid:water 4:1:5 v/v and developed with silver nitrate/ammonia) and glycerol was detected amongst the products. Glycerol was not detected when supernatant from growth medium with separately sterilised fructose added was similarly examined. However, when glucose plus gluconate (filter sterilised) was added to the basal medium glycerol was detected amongst the fermentation products.

No gluconate was detected in the medium with autoclaved glucose when it was examined chromatographically (a solvent of n-butanol:ethanol:water 10:1:2 v/v on a descending chromatogram and detecting spots with bromocresol purple and dilute ammonia).

Another possibility is the breakdown of glucose to glyceraldehyde during autoclaving, and the use of the glyceraldehyde as a fermentation substrate, some being oxidised by normal Embden-Meyerhof pathway enzymes, and some reduced to glycerol.

These experiments were of an essentially exploratory nature and therefore not quantitative.

TABLE 11. Results of growth yield experiments.

a) Aerobic

Substrate:	Glucose		Fructose		Arabinose	
	G/M'	Y_{ATP}''	G/M	Y_{ATP}	G/M	Y_{ATP}
Organism						
Streptococcus faecalis	26.8	3.0	26.2	2.9	0.0	0.0
Leuconostoc mesenteroides	5.8	0.7	2.9	0.4	0.0	0.0
Lactobacillus brevis	21.2	2.3	20.0	2.2	19.3	2.1
Lactobacillus viridescens	19.2	2.1	-	-	0.0	0.0

b) Anaerobic

Substrate:	Glucose		Fructose		Arabinose	
	G/M	Y_{ATP}	G/M	Y_{ATP}	G/M	Y_{ATP}
Organism						
Streptococcus faecalis	16.0	1.8	18.0	2.0	0.0	0.0
Streptococcus faecium	14.0	1.5	13.0	1.4	19.3	2.1
Pediococcus	14.4	1.5	15.3	1.6	15.9	1.7
Lactobacillus plantarum	12.2	1.3	11.6	1.2	19.0	2.0
Leuconostoc mesenteroides	11.1	1.2	8.4	0.9	16.0	1.8
Lactobacillus brevis	0.0	0.0	12.0	1.3	18.6	2.0
Lactobacillus viridescens	18.0	2.0	18.7	2.0	0.0	0.0

KEY: ' G. cell dry weight per Mole of substrate

'' Moles of ATP per Mole of substrate

Growth yield experiments

The yield of cell dry weight from the complete utilisation of several substrates was measured as a method of estimating energy yield from the pathways involved.

Bauchop and Elsden (1960) have shown that the dry weight of cells produced in a culture, when the energy source is the growth limiting factor, is proportional to the number of Moles of ATP produced. If a known amount of energy source is provided a figure of Moles of ATP produced per Mole of substrate metabolised can be calculated.

The fermentation of arabinose by L. brevis was assumed to produce 2 Moles of ATP per Mole of pentose fermented, and from the dry weight of cells produced a figure of 9.0 ± 0.5 g. cell dry weight per Mole of ATP was calculated. This figure is of the same order of magnitude as the cell yields per Mole of ATP obtained by Bauchop and Elsden (1960). The figure of 9.0 g./Mole ATP was used to calculate ATP yields from the oxidative and fermentative breakdown of glucose, fructose and arabinose. Table 11 gives the results of the growth yield experiments, expressed as g. cell dry weight per Mole of substrate and the figure for Moles of ATP per Mole of substrate which was calculated from the growth yield.

Anaerobically the homofermentative pathway theoretically gives 2 Moles of ATP per Mole of sugar fermented and the figures for Streptococcus faecalis on glucose and fructose and for Lactobacillus plantarum and Streptococcus faecium on

arabinose agreed very closely with this figure. There was no growth by S. faecalis on arabinose. S. faecium and L. plantarum on glucose and fructose and Pediococcus on all three substrates gave lower growth yields than expected. These lower figures may be due to incomplete use of the energy source, formation of reserve materials or some other deviation from the standard homofermentative energy producing reactions.

Only Streptococcus faecalis of the homofermentative organisms was tested aerobically. On glucose and fructose the cell dry weight yield approximated to the production of 3 Moles of ATP per Mole of glucose or fructose metabolised. This extra ATP compared to the anaerobic ATP yield was probably obtained by further oxidation of pyruvate to form acetyl phosphate and thence ATP. The aerobic metabolism of S. faecalis is discussed in more detail in Section Two.

The growth yields of the heterofermentative organisms, Lactobacillus brevis, L. viridescens and Leuconostoc mesenteroides, require more detailed explanation. The heterofermentative pathway theoretically gives 1 Mole of ATP per Mole of hexose fermented. If oxygen is available all or some of the acetyl phosphate may be spared from further reduction and the high energy phosphate trapped as ATP, so giving a possible yield of 2 Moles of ATP per Mole of glucose or fructose metabolised oxidatively. Likewise when arabinose is fermented there is no reduction of acetyl phosphate and the high energy phosphate is incorporated into ATP, so that 2 Moles of ATP are

produced per Mole of arabinose fermented.

The aerobic growth yields of Lactobacillus brevis on glucose and fructose were approximately 2 Moles of ATP per Mole of hexose as was expected. The slightly higher values could be due to some oxidation of pyruvate yielding acetyl phosphate, replacing the normal reduction of pyruvate to lactate. L. brevis did not grow anaerobically on glucose, and on fructose the anaerobic growth yield was equivalent to the production of 1.3 Moles of ATP per Mole of fructose fermented. If the pathway where 2 Moles of fructose are reduced to mannitol for each Mole of fructose oxidised was operating the net energy yield would be 0.66 Moles of ATP per Mole of fructose fermented. The extra ATP detected in the experiments could be due to the presence of some other hydrogen acceptor so sparing some fructose from reduction to mannitol.

DeMoss, Bard and Gunsalus (1951) have detected a growth yield of 1.4 Moles of ATP per Mole of glucose fermented by Leuconostoc mesenteroides and as yet this figure has not been explained experimentally. A sparing of acetyl phosphate if there was an electron accepting substance in the medium would allow more ATP production.

Lactobacillus viridescens gave the same growth yield on glucose and fructose under anaerobic conditions, equivalent to 2 Moles of ATP per Mole of substrate fermented. This figure is double the figure of 1 Mole of ATP per Mole of glucose for the normal heterofermentative pathway. The anaerobic growth

of L. viridescens on glucose and fructose was very slow to appear and this, and the results of soft agar studies (Table 1), suggested that the anaerobic metabolism detected was the result of the growth of mutants. The L. viridescens strain did not grow on arabinose and when utilising glucose oxidatively it produced 2 Moles of ATP per Mole of substrate used.

Leuconostoc mesenteroides gave very little growth aerobically due to peroxide production inhibiting growth. On a solid medium with added haematin (50 µg./ml.) there was better colony growth aerobically than on the same medium without added haematin. As no oxidative phosphorylation occurred in this organism this enhanced growth was due to the destruction of H_2O_2 by haematin overcoming the peroxide inhibition. Anaerobically there was a growth yield equivalent to just over 1 Mole of ATP per Mole of glucose and just under 1 Mole of ATP per Mole of fructose. The lower figure of the fructose growth could be due to some of the fructose being fermented by the mannitol producing pathway and therefore giving less energy than if fermented by the normal heterofermentative pathway. It is still an unexpected, and so far unexplained, result that Lactobacillus brevis gave a higher growth yield on fructose anaerobically than did Leuconostoc mesenteroides.

Lactobacillus viridescens also seems to have an atypical hexose metabolism, more growth than expected occurring anaerobically. Also the metabolism of fructose was not constitutive

TABLE 12. Adaptive oxidation of fructose by Lactobacillus viridescens.

$Q \begin{matrix} N_2 \\ CO_2 \end{matrix}$ 30°C

Substrate:	Fructose	Glucose
Cells grown on		
Glucose	0.0	91.0
Fructose	34.0	62.0

in this organism as was shown by the figures for oxygen uptake on glucose and fructose by cells grown either on glucose or fructose (Table 12).

Discussion

Following these experiments several conclusions may be drawn concerning the probable pathways of sugar utilisation in silage fermentations. Table 13 outlines these pathways.

In the initial, aerobic stages of a silage fermentation lactic acid bacteria proliferate and are able to use oxygen as a terminal hydrogen acceptor.

The homofermentative lactic acid bacteria use oxygen as a hydrogen acceptor to regenerate NAD and therefore spare some pyruvate from reduction to lactate (see reaction 1; Table 13). The pyruvate will be either oxidised or used fermentatively and products include CO_2, formate, lactate and acetoin. The heterofermentative lactic acid bacteria use oxygen as a hydrogen acceptor in place of the reduction of acetyl phosphate to ethanol, so obtaining an extra Mole of ATP per Mole of hexose oxidised (see reaction 3; Table 13) and producing acetate. Lactobacillus brevis, unable to ferment glucose, can grow on glucose in the presence of oxygen, producing CO_2, acetate and lactate as end products.

In the anaerobic stages of the silage fermentation the

TABLE 13. Summary of the pathways of sugar metabolism discussed in the first section of this thesis.

Homofermentative type

Aerobic: Streptococcus faecalis

(1) Glucose (or fructose) $\xrightarrow{O_2}$ lactate + pyruvate + H_2O

(The pyruvate is further oxidised to acetoin, acetate, formate and CO_2)

Anaerobic: Streptococcus faecalis, S. faecium, Lactobacillus plantarum and Pediococcus

(2) Glucose (or fructose) $\longrightarrow^{2}$ lactate

Heterofermentative type

Aerobic: Lactobacillus brevis and Leuconostoc mesenteroides

(3) Glucose (or fructose) $\xrightarrow{O_2}$ acetate + lactate + CO_2 + $2H_2O$

Anaerobic: Lactobacillus brevis

(4) 3 Fructose $\longrightarrow$ 2 Mannitol + acetate + lactate + CO_2

2 Fructose + glucose $\longrightarrow$ 2 Mannitol + acetate + lactate + CO_2

Leuconostoc mesenteroides

(5) 3 Fructose $\longrightarrow$ 2 Mannitol + acetate + lactate + CO_2

2 Fructose + glucose $\longrightarrow$ 2 Mannitol + acetate + lactate + CO_2

(6) Glucose (or fructose) $\longrightarrow$ Ethanol + lactate + CO_2

homofermentative lactic acid bacteria produce predominantly lactic acid from glucose or fructose (see reaction 2; Table 13). The heterofermentative lactic acid bacteria produce several products. Lactobacillus brevis, unable to use acetyl phosphate as a hydrogen acceptor, only ferments glucose if there is an exogenous hydrogen acceptor present. Fructose can function as a hydrogen acceptor, being reduced to mannitol. The reduction of 2 Moles of fructose to mannitol is coupled to the oxidation of 1 Mole of fructose or glucose to CO_2, acetate and lactate (see reaction 4; Table 13). Leuconostoc mesenteroides carries out the same fermentations (see reaction 5; Table 13) as Lactobacillus brevis, but in addition has the ability to reduce acetyl phosphate to ethanol and so can ferment glucose or fructose to CO_2, ethanol and lactate (see reaction 6; Table 13).

Therefore anaerobically homofermentative lactic acid bacteria produce lactic acid, and heterofermentative lactic acid bacteria produce CO_2, acetate, ethanol, lactate and mannitol. The proportions of these products occurring in a given silage will depend on the ratios of the different lactic acid bacteria present in the silage, and on the chemical composition of the plant material.

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SECTION TWO

Haematin Enzymes in Lactic Acid Bacteria

Introduction

Lactic acid bacteria normally lack haematin enzymes in their electron transport systems (Dolin, 1953), and until recently were thought to lack catalase. However, various investigators (Whittenbury, 1964; Johnston and Delwiche, 1965a, 1965b) have revealed an ability of these organisms to form catalase if grown on a medium containing haematin compounds.

Whittenbury (1964) has also noted the presence of cytochrome pigments in lactic acid bacteria grown in media containing haematin, and has suggested that apparent increased growth of Streptococcus faecalis in the presence of haematin could be due to oxidative phosphorylation. In contrast to this suggestion Davidson and Hartree (1968) noted the presence of cytochromes in lactic acid bacteria grown on haematin but state that there was no evidence that these cytochromes were functional in the normal sense.

Gallin and Vandemark (1964) have demonstrated oxidative phosphorylation by extracts of Streptococcus faecalis 101C, lacking haematin enzymes. Gallin and Vandemark claimed that the site of phosphorylation could be at the 'DPNH/flavin' level or alternatively during the oxidation of naphthoquinone, shown to be present in S. faecalis 101C by Baum and Dolin (1963).

An investigation has been made of the haematin enzymes

developed in S. faecalis when grown aerobically in media containing haematin. Evidence is presented for a cytochrome electron transport system and for oxidative phosphorylation coupled to the oxidation of reduced pyridine nucleotide. Results of experiments designed to indicate the properties of haematin catalase in several strains of lactic acid bacteria are also reported.

EXPERIMENTAL

Methods

Organisms. The organisms used in this investigation were as follows:

Organism	Strain No.	Source
Streptococcus faecalis	581	N.C.D.O.
Lactobacillus plantarum	5914	N.C.I.B.
Lactobacillus brevis	18	
Pediococcus	507	

(All supplied by Dr R. Whittenbury)

Media

Basal medium. A basal medium identical to that described in Section One was used.

Haematin medium. Haematin medium was the basal medium plus haematin (50 µg./ml.) added as a sterile solution. The haematin stock solution was prepared by dissolving 0.1 g. of haematin (BDH) in a minimum volume of triethanolamine and then diluting to 100 ml. with distilled water. This stock solution was sterilised by heating to 100°C for 15 min. on two consecutive days, and was stored at 4°C and freshly prepared every two months.

Partially defined medium. The partially defined medium as described by Bauchop and Elsden (1960) was used for growth yield studies on S. faecalis.

Substrates were added to media as filter sterilised solutions.

Aerobic growth was either on agar slopes in Roux bottles or in shallow broth cultures incubated in a shaking incubator. Anaerobic growth was in deep broth cultures. All cultures were incubated at 30°C.

Washed cell suspensions. Washed cell suspensions were prepared as described in Section One.

Cell free extracts. Cell free extracts were prepared as described in Section One. Broken cell suspensions were suspensions of disrupted cells, centrifuged at 3000 x g for 15 min. to remove any remaining whole cells.

The concentration of protein in cell free extracts was measured by the Folin-Ciocalteau phenol reagent method described by Lowry, Rosebrough, Farr and Randall (1951), using crystalline bovine serum albumin as a standard.

Preparation of cell membranes. Cell membranes were prepared by the method of Shockman, Kolb, Bakay, Conover and Toennies (1963), using lysozyme, osmotic shock and centrifugation. Any remaining whole cells were removed at the end of the preparation by centrifugation at 3000 x g for 15 min.

Manometry. Oxygen uptake was measured by conventional Warburg techniques with air as the gas phase (Umbreit, Burris and Stauffer, 1951). Each flask contained 1-5 mg. cell dry weight and 150 μM potassium phosphate buffer pH 6.5 in the main compartment; 0.2 ml. of 10% KOH (w/v) plus a filter paper wick in the centre well and 10 μM of substrate in the side arm; total volume to 2.5 ml. with distilled water.

Enzyme assays. The oxidation of NADH and NADPH was followed spectrophotometrically with a S.P. 800 recording spectrophotometer at a wavelength of 340 mμ. Cuvettes contained 13 μM glyglycine buffer, pH 7.2; 3 μM potassium phosphate buffer, pH 7.2; 3 μM $MgCl_2$; 3 μM potassium fluoride; 0.15 μM NADH (or NADPH) and 0.1 to 0.5 mg. protein of cell extract, the total volume being made to 2 ml. with distilled water.

The extinction coefficient of Horecker and Kornberg (1948) was used to convert optical density at 340 mμ to molar quantities of reduced pyridine nucleotide.

In experiments to detect oxidative phosphorylation adenosine triphosphate (ATP) was measured by the method of Pinchot (1957). Each cuvette contained 13 μM glyglycine buffer pH 7.2; 3 μM potassium phosphate buffer, pH 7.2; 3 μM $MgCl_2$; 3 μM potassium fluoride; 0.03 mg. bovine serum albumin; 10 μM glucose; 0.3 μM ADP or AMP; 0.15 μM NADH; with or without 0.15 μM NADP; 5 units hexokinase (BDH

ex-yeast); 100 units glucose-6-phosphate dehydrogenase and 0.1 to 0.5 mg. of protein. Total volume was made to 2 ml. with distilled water.

In most experiments a preparation of glucose-6-phosphate dehydrogenase extracted from Leuconostoc mesenteroides cells grown anaerobically on glucose was used. Ragland, Kawaski and Lowenstein (1966) have shown that L. mesenteroides has a very high level of glucose-6-phosphate dehydrogenase activity. A cell free extract containing 5-10 mg. of protein per ml. was treated with 0.1 of its volume of 0.25 M manganese chloride ($MnCl_2$), agitated and allowed to stand at room temperature for 10 min. The precipitate was centrifuged off at 23,000 x g for 10 min. and discarded. The supernatant was treated with saturated ammonium sulphate and the fraction precipitated between 45% and 60% saturation was retained for its glucose-6-phosphate dehydrogenase activity. The precipitate was dissolved in 0.02 M tris-HCl buffer, pH 7.2, and an aliquot containing 0.2 mg. of protein was used in each cuvette for the assay of ATP.

This method of preparing a fraction containing partially purified glucose-6-phosphate dehydrogenase was based on the method of Scott and Cohen (1953).

Lactic dehydrogenase was assayed by observing the reduction of triphenyltetrazolium chloride in the presence of lactate in Thunberg tubes. Each tube contained 1mM potassium phosphate buffer, pH 7.5; 6 μM NAD; 6 μM triphenyltetrazolium

chloride; 1.3 μM phenazonium methosulphate; 100 μM DL-sodium lactate and 5-10 mg. of protein, the total volume being made up to 10 ml. with distilled water. Each tube was evacuated and refilled with nitrogen twice and then the reaction was initiated by tipping cell extracts from the side-arm.

Catalase activity was measured by following the splitting of hydrogen peroxide, using the method of Herbert (1955) as described by Whittenbury (1964).

Analysis of the products of glucose and lactate oxidation. Residual glucose was measured by the method of Fuller, Lampitt and Coton (1955) as described in Section One.

Formic, acetic and lactic acids were separated chromatographically on a silica gel column with a benzene/butanol gradient solvent (Lessard and McDonald, 1966). The effluent from the column was titrated against 0.01 N sodium hydroxide using a Radiometer (Denmark) automatic titration assembly.

Carbon dioxide was absorbed in 0.1 N barium hydroxide and estimated by titration against 0.1 N hydrochloric acid.

Pyruvate was determined by the method of Friedemann and Haugen (1943) using benzene to extract the phenyl hydrazone.

Acetoin was determined by the method of Langlykke and Peterson (1937) in which acetoin was distilled off, and then assayed by an iodoform reaction.

Growth yield experiments. Aerobic growth yield measure-

ments were made in shallow broth cultures in flasks which were incubated on a shaker. The partially defined medium of Bauchop and Elsden (1960) was used and haematin and substrates were added as separately sterilised solutions. Optical density measurements were made of cultures with a Unicam S.P.600 spectrophotometer at 540 mμ and the cell dry weight calculated from a dry weight against optical density curve.

Results

The first indication that haematin enzymes were of importance in the energy metabolism of Streptococcus faecalis came from the increased size (Fig. 3) of colonies on agar media containing 50 μg/ml. of haematin and incubated aerobically.

This enhanced growth could either be due to the catalase formed removing the inhibitory hydrogen peroxide or due to a haematin enzyme system which could yield energy to the cell by oxidative phosphorylation.

Growth yield experiments

Bauchop and Elsden (1960) have shown that with anaerobically growing cells the dry weight of cells produced is proportional to the Moles of ATP formed, per Mole of substrate fermented by that culture. If the growth limiting factor is

a)

b)

FIGURE 3. Streptococcus faecalis, 72 hour old colonies on basal medium plus glucose. Incubated aerobically at 30 °C.
a) Without added haematin.
b) Haematin (50 µg/ml.) added to medium.

TABLE 14. Streptococcus faecalis. Growth yields on glucose, fructose and arabinose.

Incubation conditions	Additions to basal medium	Glucose G/M	Glucose Y_{ATP}	Fructose G/M	Fructose Y_{ATP}	Arabinose G/M	Arabinose Y_{ATP}
Anaerobic	None	16.0	1.8	18.0	2.0	0.0	0.0
	None	26.8	3.0	26.2	2.9	0.0	0.0
Aerobic	50 µg./ml. haematin	55.2	6.1	55.2	6.1	-	-
	50 µg./ml. Proto-porphyrin IX	31.9	3.5	35.9	4.0	-	-

KEY: G/M G. cell dry weight per Mole of substrate

Y_{ATP} Moles ATP per Mole of substrate

\- Not tested

the energy source a figure can therefore be calculated for the energy made available to the bacteria when metabolising that energy source.

It has been argued (Hernandez and Johnston, 1967) that in cultures growing aerobically incorporation of the energy source into cell material is such that the proportion used for energy production is too small for accurate measurements of ATP yields to be made. However, if the ATP yield of an aerobic pathway is in the range of 3-6 Moles of ATP per Mole of substrate, as appears to be the case with S. faecalis, it could be expected that the incorporation of the energy source into the cells would be a small experimental error and therefore tolerable, in contrast to an aerobic pathway yielding upwards of 30 Moles of ATP per Mole of substrate which would give an intolerable error due to substrate incorporation.

Therefore aerobic and anaerobic growth yields of S. faecalis were measured in the presence and the absence of haematin.

Table 14 outlines the results obtained with glucose, fructose and arabinose as sources of energy.

Growth yields of 16 g. cell dry weight per Mole of glucose, and 18 g. cell dry weight per Mole of fructose used anaerobically were obtained, which, assuming a theoretical ATP yield of 2 Moles of ATP per Mole of hexose fermented, approximates to 9 g. cell dry weight per Mole of ATP, a figure of the same order of magnitude as the results of Bauchop and Elsden (1960).

TABLE 15. Streptococcus faecalis. Growth yields on acetate, pyruvate and lactate.

Incubation conditions	Additions to basal medium	Acetate G/M	Acetate Y_{ATP}	Pyruvate G/M	Pyruvate Y_{ATP}	Lactate G/M	Lactate Y_{ATP}
Anaerobic	None	0.0	0.0	0.0	0.0	0.0	0.0
	50 μg./ml. haematin	0.0	0.0	0.0	0.0	0.0	0.0
Aerobic	None	0.0	0.0	5.0	0.6	2.0	0.2
	50 μg./ml. haematin	2.0	0.2	5.0	0.6	15.6	1.8

KEY: As for Table 14

Aerobic growth yields on glucose and fructose approximated to 3 Moles of ATP per Mole of substrate oxidised. If all the pyruvate produced was oxidised to acetate and no lactate was produced, a theoretical ATP yield of 4 Moles of ATP per Mole of hexose oxidised would be possible. Chemical analysis of the products of glucose oxidation (Table 30) showed lactate to be a product of aerobic breakdown, hence accounting for the less than maximum ATP yield.

In the presence of haematin a yield of 6 Moles of ATP per Mole of hexose was measured. This yield is in excess of the theoretical maximum of 4 Moles of ATP by substrate level phosphorylations and the suggestion is that the extra energy is obtained from oxidative phosphorylation coupled to a haematin-enzyme electron transport system.

In view of the marked effect of haematin, it was of interest to determine whether the normal precursor of haematin, protoporphyrin IX had a similar effect. The results obtained (Table 14) suggested that any ability to synthesise haematin enzymes from protoporphyrin IX was very limited as there was only a marginal increase in growth yield.

Growth yields were measured with pyruvate, lactate and acetate as substrates, aerobically in the presence and absence of haematin, and also anaerobically. The results are outlined in Table 15.

If energy was obtained from oxidative phosphorylation theoretically there would be a greater cell dry weight yield

TABLE 16. Streptococcus faecalis. Oxygen uptake on glucose, lactate, acetate and pyruvate.

Cells grown aerobically on basal medium plus	Endogenous control	Glucose	Lactate	Pyruvate	Acetate
Glucose	0.16	44.7	0.35	0.42	0.15
Glucose plus 50 µg./ml. haematin	0.35	56.6	2.86	0.92	0.43

All figures expressed as Q_{O_2} at 30°C

with lactate than with pyruvate in the presence of haematin. This was in fact found to be the case. No growth was obtained anaerobically on acetate, pyruvate or lactate, although slight growth might have been expected on pyruvate.

London (1968) obtained growth of S. faecium on lactate. The growth yield on lactate aerobically quoted in Table 15 is slightly greater than that obtained by London. This aerobic growth on lactate in the absence of haematin is presumably due to energy obtained from pyruvate breakdown following oxidation of lactate to pyruvate, rather than due to oxidative phosphorylation.

From the results obtained (Table 15) there was obviously energy obtained from lactate in the presence of haematin, and this was sufficient to support good growth. This energy was presumed to be from oxidative phosphorylation and so different from the energy used in the growth of S. faecium on lactate as observed by London.

Manometric measurement of oxygen uptake

Comparisons were made of oxygen uptake by resting cells of S. faecalis grown aerobically with or without haematin added to the growth medium.

Table 16 shows the results of this comparison. There was an increased rate of oxygen uptake by cells grown in the presence of haematin. With glucose as a substrate control cells used 1.1 Moles of oxygen per Mole of glucose oxidised,

TABLE 17. Streptococcus faecalis. Oxygen uptake on glucose and lactate by cells grown aerobically with haematin in the medium.

Substrate:	Glucose	Lactate
Energy source in the growth medium		
Glucose	56.6	2.9
Lactate	58.2	12.4

All figures expressed as Q_{O_2} at 30°C

whilst cells grown on media supplemented with haematin used 1.4 Moles of oxygen per Mole of glucose oxidised. Cells grown on glucose anaerobically took up oxygen at a rate of 18.9 ml. per mg. cell dry weight per 60 minute and 0.7 Moles of oxygen were used per Mole of glucose oxidised.

With pyruvate as a substrate, control cells used 0.03 Moles of oxygen per Mole of substrate whereas haematin grown cells used 0.18 Moles of oxygen per Mole of pyruvate oxidised. On lactate, control cells used 0.04 Moles of oxygen per Mole of lactate whilst haematin grown cells used 0.6 Moles of oxygen per Mole of lactate.

The oxygen uptake by haematin grown cells on pyruvate and lactate is considerable whilst that of control cells is almost negligible, which accounts for the greater oxygen uptake by the former during glucose oxidation.

As S. faecalis grew readily aerobically with lactate as an energy source in haematin media the oxygen uptake of cells grown on lactate was compared to that of cells grown aerobically on glucose in haematin containing media. Table 17 gives the rates of oxygen uptake by these cells on glucose and lactate.

Cells grown on lactate plus haematin aerobically used oxygen on glucose at the same rate as cells grown aerobically on glucose plus haematin, but the lactate grown cells showed a marked adaptation to lactate. The lactate grown cells used 1.4 Moles of oxygen per Mole of glucose oxidised and 0.34 Moles

TABLE 18. Streptococcus faecalis. Comparison of NADH and NADPH oxidation by extracts of cells grown aerobically with or without haematin.

System: Cell extract	Growth medium additions	NADPH	NADH	NADH plus 10^{-2} M KCN	NADH plus 10^{-2} M NaN_3
Cell free extracts	None	1.15	5.21	4.00	10.0
	50 µg./ml. haematin	0.76	17.6	3.01	14.6
Membrane preparation	None	-	-	-	-
	50 µg./ml. haematin	-	323.0	18.5	340.0

All figures as mµM NADH/NADPH oxidised/min./mg. of protein

- : Not tested

of oxygen per Mole of lactate.

Growth on lactate must be supported either by energy from pyruvate breakdown via acetyl phosphate or alternatively by energy from oxidative phosphorylation via haematin enzymes.

It is likely that a proportion of substrates are used fermentatively, giving a lower figure for oxygen uptake per Mole of substrate oxidised than would be the case if all the substrate was used oxidatively.

Oxidation of reduced pyridine nucleotides

Most of the NADH oxidising enzymes appeared to be associated with cell membranes in haematin-grown cells (Table 18). Haematin-grown cells yielded extracts with a higher level of NADH oxidising activity, which, in comparison to the NADH oxidising activity of cells grown without haematin, was sensitive to cyanide.

The cyanide sensitive NADH oxidising activity was concentrated in a cell membrane fraction prepared as outlined in the methods section. It was found that a broken cell suspension, containing membranes, cell debris and soluble proteins, but with whole cells removed by centrifugation, was the most convenient fraction for the study of the oxidation of reduced pyridine nucleotides.

Oxidative phosphorylation coupled to NADH oxidation

Before attempting to detect ATP production during NADH

TABLE 19. Control experiments prior to ATP assay during NADH oxidation by extracts of *Streptococcus faecalis*.

System being tested	Additions made to the basic buffer system (see methods for details)	mμM NAD/NADP reduced/min./mg. mg. protein
Reduction of NADP by *S. faecalis* extract (1.0 mg. prot./ cuvette)	NADP, extract and glucose	0.0
	NADP, extract, glucose and ADP	6.4
	NADP, extract, glucose, ADP and hexokinase	16.1
Reduction of NAD by *S. faecalis* extract (1.0 mg. prot./ cuvette)	NAD, extract and glucose	0.0
	NAD, extract, glucose and ADP	0.0
	NAD, extract, glucose and ATP	0.0
	NAD, extract, glucose, ATP, G-6-P dehydrogenase and hexokinase	0.0
	NAD, extract, glucose, ATP, G-6-P dehydrogenase, hexokinase and NADP (+ve control)	41.6
Enzymic ATP assay system controls (with NADP)	NADP, glucose and ADP	0.0
	NADP, glucose, ADP and *S. faecalis* extract	6.0
Enzymic ATP assay system controls (with NAD)	NAD, ADP and *S. faecalis* extract	0.0
	NAD, ATP and *S. faecalis* extract	0.0
	NAD, ATP, *S. faecalis* extract and NADP (+ve control)	40.0

KEY: G-6-P : Glucose-6-phosphate

TABLE 20. Streptococcus faecalis. Comparison of oxidative phosphorylation in extracts of cells grown under various conditions.

Reaction system	Growth		mμM/min./mg. protein NADH oxid.	NADP redn.	P:O
Complete	Aerobic	Gluc. + haematin	215.0	73.0	0.34 +
"	"	"	189.2	64.4	0.34 +
"	"	"	202.0	64.4	0.32 +
"	"	Lact. + haematin	53.2	15.0	0.28
AMP in place of ADP	"	"	48.5	9.7	0.20 +
"	"	"	48.8	7.2	0.15 +
Complete	"	Glucose	90.0	0.0	0.0 +
"	"	"	95.0	2.7	0.03 +
"	Anaerobic	Gluc. + haematin	54.5	0.0	0.0
"	"	Glucose	57.8	0.0	0.0

KEY: Gluc. : Glucose

Lact. : Lactate

\+ : Results of several separate investigations are displayed

oxidation by extracts of S. faecalis control experiments were conducted on all components of the reaction mixture. Results of these control experiments are outlined in Table 19.

Reduction of NADH by the S. faecalis extract in the presence of ADP indicates slight myokinase, hexokinase and glucose-6-phosphate dehydrogenase activities in the extract. If excess ADP was present in the ATP assay system there was a steady rate of NADH reduction due to these reactions which was corrected for when ATP production was assayed.

The ATP assay system did not reduce NAD in the presence of ATP, with or without the extract of S. faecalis in the system.

The glucose-6-phosphate dehydrogenase preparation from Leuconostoc mesenteroides gave similar results to the commercial (BDH) glucose-6-phosphate dehydrogenase except for a slight myokinase activity which was corrected for along with the S. faecalis myokinase activity.

Cells grown aerobically in media containing haematin yielded extracts which produced ATP during oxidation of NADH, whilst cells grown without haematin did not (Table 20). Broken cell suspensions were the extracts used in this comparison. A P/O ratio of about 0.3 was measured for the haematin grown extracts, which fell to a P/O ratio of 0.15 to 0.2 when AMP was used in place of ADP in the reaction mixture.

The lack of oxidative phosphorylation by extracts of cells grown in the absence of haematin contrasts with the

TABLE 21. Effect of various inhibitors of oxidative phosphorylation on extracts of *S. faecalis* grown aerobically on glucose plus haematin.

Extract	Inhibitor	Conc. (Molar)	mμM/min./mg. protein NADH oxid.	NADP redn.	P:O	
S. faecalis (broken cell preparation)	None	-	215.0	73.0	0.34	
	KCN	1×10^{-2}	88.2	7.9	0.09	
		3×10^{-2}	12.8	0.0	0.0	
	NaN_3	1×10^{-2}	197.0	58.2	0.30	
		1.5×10^{-2}	176.0	74.8	0.42	
S. faecalis (membrane preparation)	None	-	505.0	221.0	0.44	
	Antimycin A	1.6×10^{-4}	480.0	0.0	0.0	+
		1.6×10^{-4}	456.0	0.0	0.0	+

KEY: + : Results of two separate investigations are displayed.

results obtained by Gallin and Vandemark (1964) who demonstrated oxidative phosphorylation by extracts of S. faecalis grown in media which contained no haematin.

Extracts prepared from cells grown anaerobically on glucose, whether in the presence or the absence of haematin gave no detectable phosphorylation linked to NADH oxidation.

Effects of inhibitors on oxidative phosphorylation by S. faecalis extracts

The effects of various inhibitors on oxidative phosphorylation by S. faecalis were investigated.

Sodium azide (10 μM/ml. and 15 μM/ml.); potassium cyanide (10 μM/ml. and 30 μM/ml.) and Antimycin A (0.16 μM/ml.) were used and the results are shown in Table 21.

With 1×10^{-2} M cyanide there was a 59% inhibition of the rate of NADH oxidation and a 74% fall in the P/O ratio, indicating a cyanide sensitive enzyme system. With 3×10^{-2} M cyanide phosphorylation was completely inhibited and NADH oxidation was reduced to only 6% of the original rate.

With 1×10^{-2} M azide there was only a marginal drop (8%) in the rate of NADH oxidation, and no inhibition of coupled phosphorylation. When the azide concentration was increased to 1.5×10^{-2} M the rate of NADH oxidation fell to 82% of the control value but the P/O ratio was slightly increased.

These results with cyanide and azide contrast with those obtained by Gallin and Vandemark (1964) who detected no

inhibition of oxidative phosphorylation in their S. faecalis extracts in the presence of either cyanide or of azide. They used lower concentrations (4×10^{-4} KCN and 1×10^{-4} NaN_3) and this may explain the difference. Alternatively Gallin and Vandemark were measuring oxidative phosphorylation in a different, non-haematin, enzyme system.

With Antimycin A, at a concentration of 1.6×10^{-3} M, there was complete inhibition of oxidative phosphorylation, although the rate of NADH oxidation was only 10% inhibited. Gallin and Vandemark had obtained a reduced P/O ratio in the presence of 8×10^{-4} M Antimycin A.

Some observers (Smith, 1954; Breummer, Wilson, Glenn and Crane, 1957; Lightbown and Jackson, 1956) have noted a lack of sensitivity to Antimycin A amongst bacterial oxidative phosphorylation systems. However, the S. faecalis system was sensitive to this antibiotic.

The results obtained with these three inhibitors of oxidative phosphorylation add further to the evidence for a haematin enzyme system being involved in the oxidative phosphorylation by S. faecalis extracts.

The effect of adding haematin to extracts of S. faecalis grown in the absence of haematin

An experiment was conducted to test whether there was a preformed enzyme in cells grown without haematin, which on addition of haematin to a cell extract would form the haematin-

TABLE 22. Effect of adding haematin to a broken cell suspension of S. faecalis grown aerobically on glucose.

Additions to the cell suspension	mμM/min./mg. protein		P:O
	NADH oxid.	NADP redn.	
None	90.0	0.0	0.0
15 μg./ml. haematin	93.0	0.0	0.0
15 μg./ml. haematin and 3×10^{-2} M KCN	72.0	0.0	0.0

enzyme NADH oxidase system. Several workers have noted a preformed apoenzyme for catalase in cells unable to synthesise haematin, the enzyme being activated when haematin was added to the cells (Whittenbury, 1965) or even to a cell free extract (Beljanski and Beljanski, 1957).

A broken cell preparation of cells grown on glucose aerobically was incubated with haematin for 30 min. at 30°C and then tested for the rate of NADH oxidation and for oxidative phosphorylation. The results are outlined in Table 22.

There was no evidence for the formation of haematin enzymes oxidising NADH. This experiment, incidentally, acts as a control in the sense that it shows that all the activities noted as haematin enzymes are not due to haematin itself but are due to an enzyme synthesised by S. faecalis in the presence of haematin.

Division of oxidative phosphorylation between different fractions of S. faecalis extracts

Investigations were carried out to determine the site of the haematin enzymes in the cell. Soluble and particulate fractions of S. faecalis extracts, prepared from cells grown aerobically on glucose or lactate in the presence of haematin, were examined and compared to determine whether the haematin enzymes were cytoplasmic or membrane bound.

S. faecalis cells grown aerobically on lactate plus haematin were treated ultrasonically and the whole cells and

TABLE 23. Division of NADH oxidising enzymes between cell debris and a membrane plus soluble enzymes fraction of S. faecalis.

Cell extract fraction	mμM/min./mg. protein	
	NADH oxid.	NADH oxid. with 3×10^{-2} M KCN
Cell debris	53.0	49.0
Membranes plus soluble enzymes	372.0	37.0

Cells grown aerobically on glucose plus haematin.

TABLE 24. S. faecalis. Division of ADP myokinase activity between soluble and membrane fractions of cell extracts.

Cell fraction	mμM NADP redn./min./mg. protein
Soluble fraction	67.8
Membrane fraction	29.0

Assay system - as for ATP assay with no NADH and 1 μM ADP added and 0.4 - 0.6 mg. protein per cuvette

TABLE 25. S. faecalis. Division of NADH oxidising enzymes between soluble and membrane fractions of cell extracts.

Cells grown on	Cell fraction(s)	mμM/min./mg. protein NADH oxid.	NADP redn.	P:O
Glucose and Haematin	Soluble fraction	294.0	77.0	0.26
	Membrane fraction	650.0	195.0	0.30
	Soluble fraction (1.0)+ and Membrane fraction (0.8)+	467.0	149.6	0.32
		397.0	127.0	0.32
	Soluble fraction (spun 40,000 r.p.m./30 min.)	89.5	14.3	0.16
Lactate and Haematin	Soluble fraction	191.0	52.1	0.27
	Membrane fraction	109.0	46.5	0.43
	Soluble fraction (1.0)+ and Membrane fraction (0.7)+	201.0	90.5	0.45

+ Figures in parenthesis denote ratios of weight of protein of each fraction.

debris removed by centrifuging at 3000x g for 15 minutes. Table 23 shows that most of the NADH oxidising enzymes were in the supernatant, which contained soluble enzymes and membranes. This agrees with the experimental results shown in Table 18, using cell membranes prepared by lysozyme treatment.

The cell free extract/membrane fraction was centrifuged at 23,000 x g for 35 minutes to precipitate the membranes, so separating a soluble fraction and a membrane fraction, the latter being resuspended in buffer (pH 6.5, 0.05 M phosphate).

As Table 24 shows, the ADP myokinase activity was greater in the soluble fraction than in the membrane fraction. This reduced one possible error in oxidative phosphorylation determinations when membrane fractions were being studied.

Results of experiments on cell fractions to determine the site of oxidative phosphorylation are shown in Table 25. The membrane fraction of cells grown aerobically on glucose plus haematin oxidised NADH faster than did the soluble fraction of the same cells. The P/O ratio, however, was only slightly greater in the membrane fraction than in the soluble fraction. Ultracentrifugation (40,000 r.p.m. for 30 min.) of the soluble fraction reduced the rate of NADH oxidation by this fraction and the P/O ratio was reduced to half that measured with the membrane fraction. This suggested that the enzymes concerned with oxidative phosphorylation were associated with the membranes, the residual activities in the soluble fraction being due to small particles of membranes left in suspension. The

reduced P/O ratio suggested that there was a soluble enzyme in the extracts, oxidising NADH, which was not coupled to phosphorylation.

With the cells grown on lactate plus haematin the P/O ratio was higher in the membrane fraction than the soluble fraction, although the rate of NADH oxidation was faster in the soluble fraction. The lactate grown extract was not ultracentrifuged.

With extracts of both glucose plus haematin and lactate plus haematin grown cells there was no increase in the P/O ratio when soluble and membrane fractions were combined, suggesting that, unless the preparation of cell free extracts destroyed a site of oxidative phosphorylation, there was only one site of oxidative phosphorylation in the extracts.

Similar results were obtained with the cell membrane preparations prepared by the use of lysozyme and osmotic shock as were obtained with the cell membrane fractions prepared by differential centrifugation of ultrasonically treated cells.

Reduction of cytochrome pigments in the extracts

In the presence of cyanide (10^{-2} M) and an excess of NADH reduced cytochrome peaks were detected in membrane fractions of cells grown aerobically on lactate plus haematin and on glucose plus haematin.

Figure 4 shows a typical spectrum. This reduced spectrum is typical of a 'b type' cytochrome, and to support this the

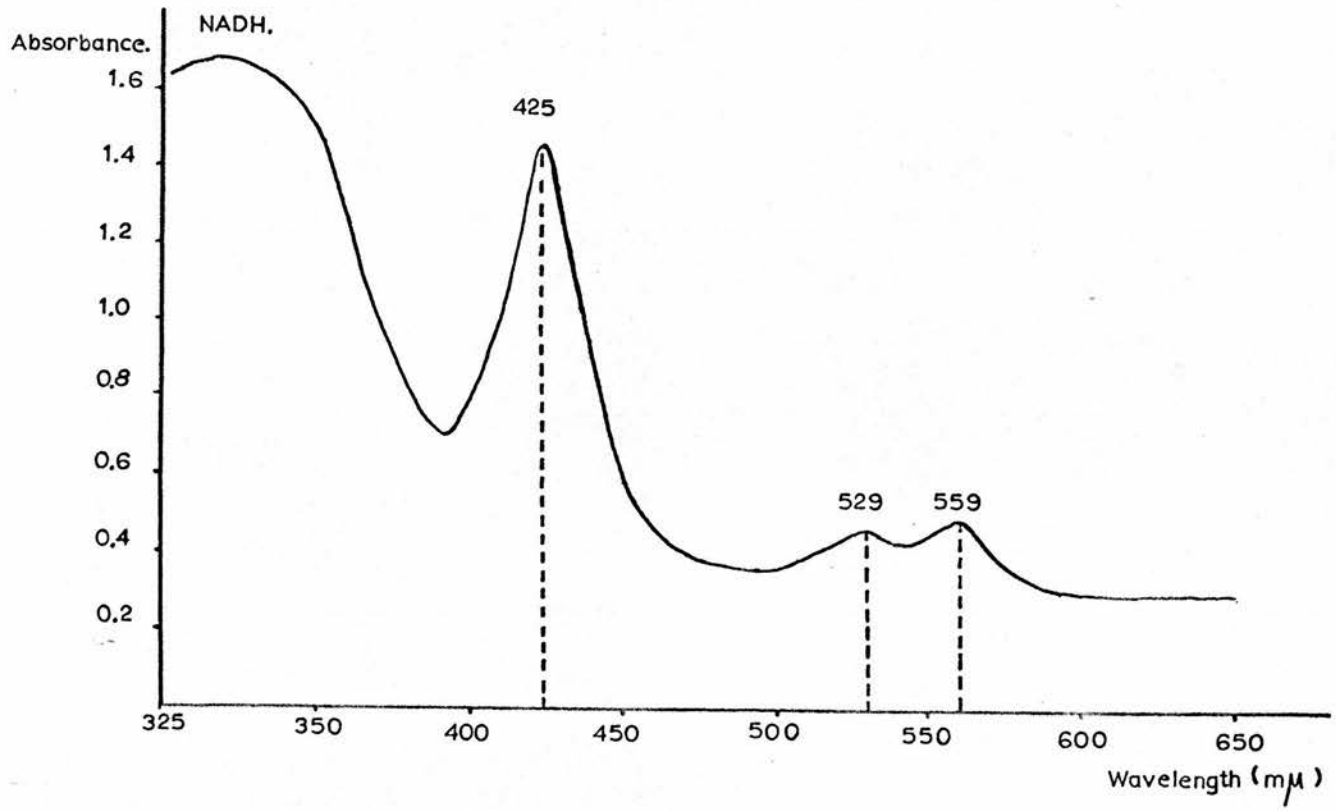

FIGURE 4. S.faecalis ; absorbance spectrum.
Broken cell suspensions of cells grown aerobically on glucose plus haematin. Cuvette contained KCN (10^{-2}M), ADP (1μM/ml.) and excess of NADH.
Blank was the broken cell suspension alone.

TABLE 26. Increases in absorption at the cytochrome maxima in the presence of KCN (10 μM/ml.) and NADH.

Optical density (wavelength): System and additions	412 mμ	425 mμ	529 mμ	559 mμ
Haem grown cells - broken cell suspension	0.25	0.21	0.01	0.00
As above plus 10 μM KCN/ml.	0.27	0.26	0.02	0.01
As above plus 10 μM KCN/ml. plus 0.3 μM NADH (after 2 min.)	0.28	0.36	0.05	0.04

(All compared to a blank of a broken cell suspension of *S. faecalis* grown aerobically on glucose)

extracts have an oxidised peak at 412 mμ.

No water soluble cytochrome was detected and attempts to isolate a cytochrome oxidase, using sodium cholate extraction techniques as described by Yonetani (1960) were unsuccessful.

When haematin (15 μg/ml.) was added to a broken cell preparation of cells grown aerobically on glucose, and then cyanide (10^{-2} M) and excess NADH were added as above, there were no detectable cytochrome peaks.

Haematin itself gave a peak of 425 mμ, but this was not altered by the presence of NADH or S. faecalis enzymes. This shows that the cytochrome peaks were not artefacts due to residual haematin carried over from the growth medium.

A broken cell suspension prepared from S. faecalis cells grown anaerobically on glucose plus haematin showed no cytochrome peaks when tested as above. Therefore oxygen is necessary to induce cytochrome formation.

The reduction of cytochrome was followed at 425, 529 and 559 mμ, in a system containing an extract of cells grown on glucose plus haematin aerobically. Cyanide (10^{-2} M) and excess NADH were added to the cuvette and the optical density at the three peaks was compared to a blank containing a similar extract (broken cell suspension) to which no additions of cyanide or NADH were made. The results of this experiment are shown in Table 26.

The reduction at 425 mμ was found to have a steady rate of 0.02 O.D. units per minute per mg. of protein, up to a maximum

TABLE 27. Reduction of 2,3,5-triphenyltetrazolium chloride by extracts of S. faecalis in the presence of lactate.

Cells grown on	Time in min. to reduce TTC (by broken cell suspensions) + lactate	- lactate	+ lactate + haematin
Glucose	>120	>120	>120
Glucose + haematin	40	>120	-
Lactate + haematin	40	>120	-

KEY: >120 No reduction after 2 hours

\- Not tested

TABLE 28. The apparent slower rate of NADH oxidation by an extract of S. faecalis (grown aerobically on lactate plus haematin) in the presence of lactate

System	mμM NADH oxidised/min./mg. protein
Control	37.2
+ 5 μM/ml. lactate	29.1

value of 0.21 O.D. units per mg. of protein.

Oxidation of lactate coupled to ATP production by oxidative phosphorylation

Several observations were made which showed an ability of extracts of S. faecalis cells grown on lactate plus haematin to reduce NAD in the presence of lactate and then obtain energy from oxidative phosphorylation coupled to the oxidation of the NADH.

In an experiment in Thunberg tubes 2-3-5-triphenyltetrazolium chloride was reduced in the presence of lactate (Table 27). Only the extracts of cells grown in the presence of haematin reduced the tetrazolium salt, and there was no tetrazolium reduction in the absence of lactate.

Difficulty was encountered in demonstrating the reduction of NAD in the presence of lactate due to the very active NADH oxidases present in extracts. However, differences between rates of NADH oxidation in systems with and without lactate, and a greater ATP assay figure in the presence of lactate indicated that the oxidation of lactate is coupled to the production of energy from oxidative phosphorylation (Table 28).

These experiments were carried out using a combined membrane/soluble fraction of a cell free extract of S. faecalis cells grown aerobically on lactate plus haematin.

A system containing AMP (1 μM/ml.); 5 μM/ml. lactate and 0.03 μM NAD/ml. was compared to the same less lactate. When

TABLE 29. Reduction of NADP in the presence of ATP, ATP assay system, S. faecalis extract, AMP, NAD and with or without lactate.

System	mμM NADP redn./min./mg. protein	Total mμM NADP redn./system
Control (no lactate)	6.5	202.0
+ lactate	7.7	238.0

TABLE 30. S. faecalis. Products of glucose and lactate oxidation.

Growth conditions of cells:	Glucose (aerobically)		Glucose and Haematin (aerobically)	Lactate and Haematin (aerobically)	
Incubation conditions in experiment:	Anaerobic	Aerobic	Aerobic	Aerobic	
Substrates					
Glucose	100	100	100	100	-
Lactate	-	-	-	-	100
Products					
Lactate	187	77	29	25	-
Acetate	0	64	61	108	38
Formate	0	0	8	4	0
Pyruvate	3	1	3	4	9
Acetoin	0	26	43	10	23
Carbon dioxide	6	130	171	153	91
% C Recovery	96	99	95	101	95

Figures expressed as mM/mM of substrate used

the ATP assay system was added no NADP reduction (indicative of ATP) was noted; however, when 1 μM ATP was added a faster rate of NADP reduction and a greater total NADP reduction was recorded in the system containing lactate than in the system lacking lactate (Table 29).

It was considered that this difference could be attributed to the reduction of NAD by lactate dehydrogenase and the subsequent oxidation of the NADH coupled to production of ATP.

The system appeared to need ATP present to initiate the reaction, although resulting in a net gain of ATP.

It is suggested that this is the mechanism whereby growth energy is obtained to support the growth observed aerobically on lactate in the presence of haematin.

Products of glucose and lactate breakdown

The products detected from glucose and lactate breakdown by washed cell suspensions of S. faecalis are shown in Table 30.

Cells grown aerobically without haematin converted 94% of the glucose to lactate under anaerobic conditions, whereas aerobically lactate only accounted for 38% of the glucose oxidised. The remainder of the pyruvate, which would otherwise have been reduced to lactate, was either oxidised to acetate and carbon dioxide or fermented to acetoin and carbon dioxide.

Cells grown aerobically on glucose plus haematin converted only 14% of the glucose into lactate. Cells grown aerobically on lactate plus haematin converted 13% of the glucose to

TABLE 31. Effect of haematin in the growth medium on the catalase activity of lactic acid bacteria (cells grown aerobically).

Organism	μM H_2O_2 decomposed/min./mg. cell dry wt.	
	Control	+ Haematin
Streptococcus faecalis	0.5	31.5
Pediococcus	0.5	43.5
Lactobacillus plantarum	1.3	42.5
Lactobacillus brevis	0.4	7.3

lactate. Cells grown on lactate plus haematin oxidised lactate to produce carbon dioxide, acetate and acetoin.

Catalase of lactic acid bacteria

Streptococcus faecalis, Pediococcus strain 507, Lactobacillus plantarum and Lactobacillus brevis all formed catalase when grown aerobically in the presence of haematin (Table 31). In the absence of haematin there was no catalase production.

This catalase activity was of the same order of magnitude as that noted by Whittenbury (1964). Sodium azide (10^{-3} M) completely inhibited the catalase activity of all four strains investigated. The S. faecalis catalase, in a cell free preparation, was 78% inhibited by 2×10^{-5} M sodium azide, which compares with the 50% inhibition of pure catalase by the same concentration of sodium azide quoted in 'The Enzymes' (Dixon and Webb, 1964).

Whittenbury (1964) noted an ability of resting cell suspensions to form catalase when provided with haematin, suggesting that the apo-enzyme of catalase was formed constitutively. Johnston and Delwiche (1965) working with 30 different cultures, however, found no evidence for any apo-enzyme.

Experiments conducted with the bacterial strains noted above confirmed the results of Whittenbury (1964). Resting cell suspensions of L. plantarum, L. brevis and Pediococcus

TABLE 32. Formation of catalase by resting cell suspensions, of organisms grown in the absence of haematin, when incubated with haematin (1 µg./ml.).

Organism	µM H_2O_2 decomposed/min./mg. cell dry wt.	
	5 min. incubation with haematin	10 min. incubation with haematin
Streptococcus faecalis	0.0	0.0
Pediococcus	59.0	-
Lactobacillus plantarum	15.0	15.7
Lactobacillus brevis	4.7	4.7

KEY: - Not tested

TABLE 33. Formation of catalase in cell free extracts of S. faecalis and Pediococcus (cells aerobically grown).

Organism	μM H_2O_2 decomposed/min./mg. protein		
	Haematin in the growth medium	No haematin in the growth medium	
		Control	Incubated 5 min. with 1 μg. haematin/ml.
Streptococcus faecalis	156.0	0.5	3.5
Pediococcus	107.0	0.5	10.0

all formed catalase when incubated with haematin (1 μg/ml.) and the catalase was fully active after 5 minutes incubation (Table 32).

The L. plantarum cell suspension formed catalase in the presence of 20 μg/ml. chloramphenicol, an inhibitor of protein synthesis (Dagley and Sykes, 1959), showing no protein synthesis was concerned in this formation of catalase by resting cell suspensions and therefore that the apo-enzyme was preformed (Fig. 5).

An experiment with the same preparation of L. plantarum on the adaptive oxidation of malate and of sucrose showed that chloramphenicol inhibited enzyme synthesis (Fig. 6).

Cell free extracts of S. faecalis and Pediococcus also formed catalase when incubated with haematin although cell free extracts of L. plantarum and L. brevis did not (Table 33).

One anomalous finding, previously noted by Whittenbury (1964), was the inability of S. faecalis resting cells to form catalase when incubated with haematin, possibly due to permeability problems, although this seems unlikely as growing cells readily assimilate haematin. As mentioned above cell free extracts of S. faecalis would form catalase when incubated with haematin.

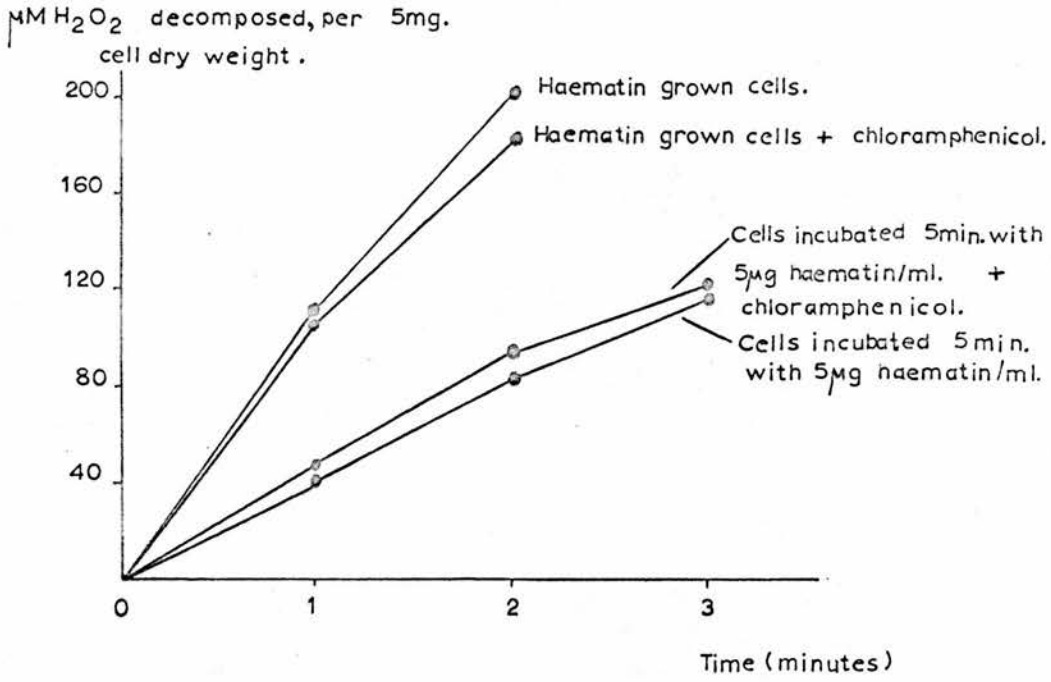

FIGURE 5. Lactobacillus plantarum. Effect of chloramphenicol (20μg./ml.) on catalase formation.

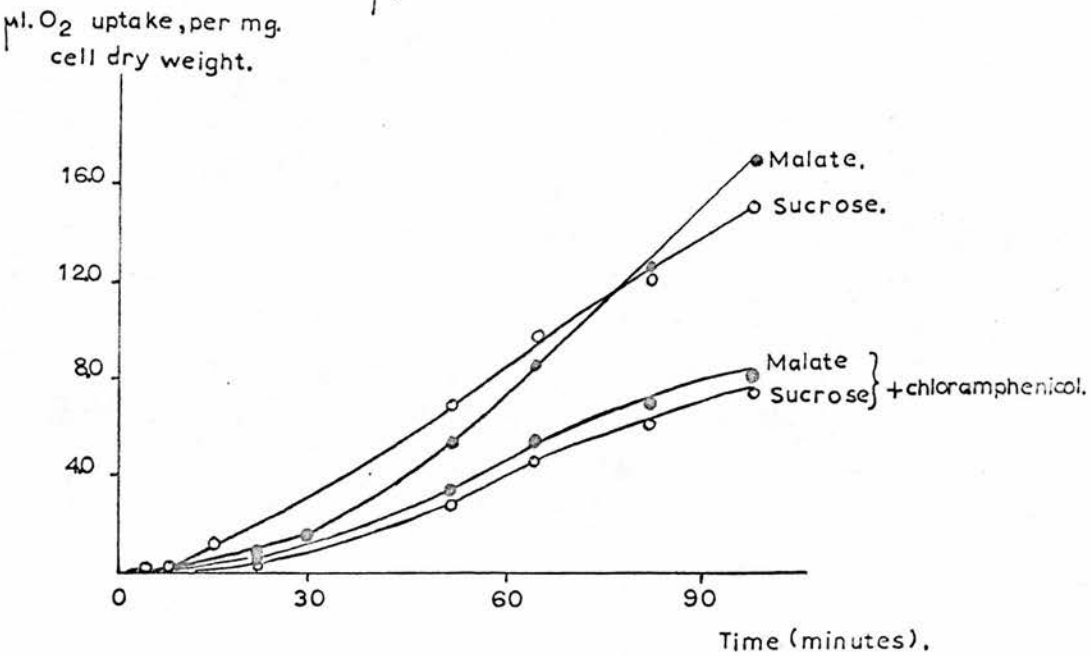

FIGURE 6. Lactobacillus plantarum. Effect of chloramphenicol (20μg./ml.) on adaptive malate and sucrose oxidation.

Discussion

The evidence presented points conclusively to the development of a pathway in S. faecalis grown aerobically in the presence of haematin in which the oxidation of NADH is catalysed by haematin enzymes and is coupled to oxidative phosphorylation.

Gallin and Vandemark (1964) demonstrated oxidative phosphorylation in S. faecalis 10C1 and suggested that potential sites for phosphorylation were at the NADH/flavin level and during the oxidation of reduced naphthoquinone. Their experiments studied a phosphorylation which was insensitive to cyanide and azide. However, the present study revealed no oxidative phosphorylation of this type in cells grown in the absence of haematin. The different results may be due to the use of different strains or else due to cultural and extract preparation differences.

The P/O ratios observed here were relatively low, the highest value obtained being 0.44, in comparison with the work of Pinchot (1957) who reported a P/O ratio of 0.78 in Alcaligenes faecalis and of Hartmann, Brodie and Gray (1957) who reported a P/O ratio approaching 1 in Azotobacter extracts.

The value for the P/O ratio for S. faecalis, and the results of experiments where the soluble and membrane fractions were examined separately and in combination, suggested that there was only one site of phosphorylation coupled to NADH

oxidation in S. faecalis extracts. It is known that the preparation of cell extracts affects the activity of the sites of oxidative phosphorylation. Racker (1965) noted that the preparation of submitochondrial particles by sonic oscillation and mechanical fragmentation reduced the efficiency of phosphorylation sites, and especially of the site coupled to the oxidation of cytochrome c and to a lesser extent of the site coupled to the oxidation of NADH by flavoproteins. The third site, coupled to the oxidation of NADH by cytochrome b, was relatively unaffected.

Antimycin A inhibits at the level of this last mentioned site (Racker, 1965) and this could well be the site in operation in S. faecalis extracts.

Growth yield experiments on glucose where oxygen uptake was measured manometrically (with Hg. as manometer fluid replacing Brodie's solution) yielded a figure of 0.16 g. cell dry weight per g. of oxygen used, a low figure in comparison to the 0.853 g. cell dry weight per g. of oxygen used obtained by Hernandez and Johnsonn (1967) with Pseudomonas fluorescens. The lower growth yield with S. faecalis again points to only one phosphorylation site, as Hernandez and Johnsonn suggested, the pseudomonad probably has up to three oxidative phosphorylation sites.

Assuming only one oxidative phosphorylation site, it is possible to account for the production of 1 Mole of ATP per Mole of NADH oxidised. If 4 Moles of ATP are obtained from

glycolysis and pyruvate oxidation, and if the 2 Moles of NADH produced per Mole of glucose oxidised are not used to reduce pyruvate to lactate, and if the 2 Moles of NADH produced per Mole of pyruvate oxidised, are oxidised via the haematin enzymes a potential yield of 8 Moles of ATP per Mole of glucose oxidised can be calculated.

Experimentally a yield of 6.5 Moles of ATP per Mole of glucose oxidised was obtained. This less than maximum yield was partly accounted for by the presence of lactate and acetoin amongst the products of glucose oxidation, showing some competition from fermentative pathways.

Also some NADH is probably oxidised by normal S. faecalis flavoprotein NADH oxidases (Dolin, 1955) as shown by the residual rate of NADH oxidation in the presence of cyanide. This oxidation would also partly account for the low P/O ratio.

The spectrum of the reduced cytochrome pigment of the S. faecalis extract was very similar to cytochrome b_2 of yeast as reported in the yeast lactate dehydrogenase system by Appleby and Morton (1959). A strain of S. faecalis (H.69.D5) has been shown to form cytochromes b_1, a_1 and a_2 (Whittenbury, 1964). However, no a type cytochromes were detected in the present study but the methods used may not have been adequately sensitive.

Jacobs, Maclosky and Conti (1967) examined the effect of haematin on Staphylococcus epidermis. Normal anaerobic growth gave cells lacking cytochromes but in the presence of

haematin anaerobic growth resulted in cells containing pigments with spectra similar to cytochromes b_2 and o. No cytochromes were formed when haematin was added to a washed cell suspension.

This is similar to the situation in S. faecalis except that in S. faecalis haematin enzymes were only formed under aerobic conditions. Haematin added to a suspension of resting cells grown in the absence of haematin resulted in no cytochrome formation.

These results with cytochromes in S. faecalis, and with haematin catalase in the four strains of lactic acid bacteria studied raise interesting evolutionary and taxonomical questions. The appearance of residual, either degenerate or undeveloped, aerobic pathways suggests that the dividing line between, say, streptococci and staphylococci may be less than at first appears to be the case. However, Krebs cycle enzymes appear to be absent from lactic acid bacteria so their aerobic pathways are still incomplete compared to true aerobic bacteria.

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SECTION THREE

Organic acid metabolism of lactic acid bacteria

Introduction

Non-volatile organic acids, especially malic, citric and glyceric acids, have a widespread occurrence in plants. In grass the total organic acid level is 3.5 - 5% of the dry matter and in legumes 6 - 8% of the dry matter (Fouconneau and Jarrige, 1954).

Playne and McDonald (1966) have shown that the buffering capacity of plant material, especially in the range from pH 4 to pH 6, is largely due to the organic acids. Protein of plants has relatively little effect and the increase in buffering capacity following ensilage (Playne, 1963) is due to the production of lactic and volatile acids and not due to proteins (Playne and McDonald, 1966; Greenhill, 1964).

In Italian rye-grass the main buffers are malate and citrate and in red clover, malate and glycerate (Playne and McDonald, 1966).

Citric and malic acids are rapidly metabolised in ensiled herbage (Playne, Stirling and McDonald, 1967; Hirst and Ramsted, 1957), and this breakdown has shown to be due to bacterial action (Playne et al., 1967). Succinic acid is increased by plant and bacterial action (Playne et al., 1967) and is present in the ensiled material and persists throughout storage.

McDonald and Whittenbury (1967) have calculated the percentage losses possible when malate and citrate are

TABLE 34. Fermentation pathways from pyruvate in *Streptococcus faecalis* (Gunsalus and Campbell, 1944).

$$2\ \text{Pyruvate} \longrightarrow \text{Lactate} + \text{Acetate} + CO_2$$

$$\text{Pyruvate} \longrightarrow \text{Acetate} + \text{Formate}$$

$$2\ \text{Pyruvate} \longrightarrow \text{Acetoin} + 2\ CO_2$$

decarboxylated. They calculated that decarboxylation of malate can result in a maximum loss of 1% of the dry matter of silage and that decarboxylation of citrate in a maximum loss of 0.5% of the dry matter of silage. These figures are the maximum normally possible and not necessarily the probable losses. The actual losses will vary with the proportion of citrate and malate dissimilated by each of the various pathways from malate and citrate outlined by McDonald and Whittenbury (1967) and by Whittenbury, McDonald and Bryan-Jones (1967).

Campbell and Gunsalus (1944) and Gunsalus and Campbell (1944) have indicated that Streptococcus faecalis breaks down citrate via oxaloacetate to pyruvate, acetate and CO_2 and then ferments the pyruvate by one of three pathways (Table 34). Lactate was a major product at pH values below 7.0 but at pH 8.5 no lactate was produced and formate was a major product.

Playne et al. (1967) found formate in silages prepared from aseptically grown grass inoculated with S. faecalis, suggesting that the formate was accounting for some of the citrate present in the original grass. Wilson and Tilly (1964) noted the presence of formic acid in high pH silages (the exact pH was not given but was higher than pH 4.76) which agrees with the pH effect noted by Gunsalus and Campbell, whereby a higher percentage of formate was produced at higher pH values.

Very few silages have been examined for the presence of acetoin but in one recent examination no acetoin was detected

(McDonald, Henderson and MacGregor, 1968), although traces of 2,3-butanediol were found in some silages, which could have been formed by a similar pathway to the acetoin formation and subsequent reduction of the acetoin.

Korkes and Ochoa (1948) showed a pathway of malate breakdown in Lactobacillus arabinosus in which the malate was decarboxylated to pyruvate and the pyruvate reduced to lactate. Radler (1966) noted an identical pathway in lactic acid bacteria occurring in wine. If the malate is broken down in the presence of a hydrogen acceptor other than the pyruvate produced in the reaction, there is a possibility of sparing pyruvate from further reduction which can then be fermented to a variety of products, namely acetoin (Whittenbury, 1956), formate, acetate, lactate and CO_2.

Whittenbury (1961) has carried out an extensive survey of the ability of lactic acid bacteria to decarboxylate citrate and malate in the presence and absence of fermentable carbohydrate. The ability was widespread amongst lactic acid bacteria, and although many need another energy source, some could use citrate or malate for growth.

The overall similarity of metabolic processes within the lactic acid bacteria would suggest that the oxaloacetate to pyruvate pathway would operate throughout the group for citrate breakdown. Likewise the pathway of malate breakdown to lactate and CO_2 would be present in all malate fermenting lactic acid bacteria.

Experiments have been carried out to obtain more information on the organic acid metabolism of six silage lactic acid bacteria with special efforts being made to determine the most probable pathways under conditions prevailing in a normal silage fermentation.

Methods

Organisms

Organism	Strain Number	Source
Streptococcus faecalis	581	N.C.D.O.
Streptococcus faecium	HGH 511	
Pediococcus	507	
Leuconostoc mesenteroides	60	
Lactobacillus plantarum	5914	N.C.I.B.
Lactobacillus brevis	18	

Media

Basal medium. A basal medium identical to that described in Section I was used.

Organic acids were added as a filter sterilised solution of their sodium salts to a final concentration of 0.5% w/v. Sugars were also added as filter sterilised solutions to a final concentration of 0.5% w/v.

All cultures were deep broth cultures, and incubation was at 30°C.

Washed cell suspensions. Washed cell suspensions were prepared as described in Section I.

Warburg manometry. Conventional manometric techniques were used as described by Umbreit, Burris and Stauffer (1951).

Each flask contained 50 μM potassium (or sodium) phosphate buffer, 1-3 mg. cell dry weight; 10 μM of sodium citrate or DL-sodium malate were added from a side arm at zero time. In experiments to measure citrate breakdown 10 μM $MgSO_4$ was present in each flask and in experiments to measure malate breakdown 25 μM $MnCl_2$ was present in each flask.

When required 2 μM of glucose or 2 μM of arabinose was also added from a side arm at zero time. The total liquid volume in each flask was 2 ml. All manometric experiments were conducted at 30°C.

Anaerobic conditions in manometric flasks were achieved by sparging the flasks with nitrogen for 15 minutes. Checks for any residual oxygen, by measuring oxygen uptake with a suitable substrate, always resulted in less than 2 μl. of residual oxygen being detected.

In experiments where carbon dioxide evolution was measured at pHs above pH 6.0 several double side-armed flasks were set up identically and at suitable time intervals 0.2 ml. of 1 N HCl was added from the second side arm, so releasing any dissolved CO_2 and enabling a measure of CO_2 evolution to be made.

The pH of the buffer at the end of experiments was checked and found to remain within 0.1 pH units of the initial figure, except where HCl had been added.

Fermentation balance experiments. Fermentation balances

were measured in a system as shown in Figure 1. Each flask contained 5 mM potassium phosphate buffer of the required pH, 25-30 mg. cell dry weight, 100 μM sodium citrate or 100 μM Dl-sodium malate and/or 100 μM glucose, total volume 100 ml. Nitrogen was bubbled through the system for 10 minutes to remove oxygen. 50 ml. 0.1 N $Ba(OH)_2$ was placed in each of the two Drechsel bottles. The experiment was started by the addition of the cell suspension and concluded by the addition of 1 ml. 10 N H_2SO_4, after which the nitrogen flow was continued for 10 minutes to flush all CO_2 from the system. Cells were removed by centrifugation, the supernatant neutralised using 10 N KOH, and phenolphthalein as an indicator, the volume reduced to 20 ml. on a rotary evaporator under reduced pressure and then the sample was stored at -20°C until analysis of the fermentation products.

Analysis of fermentation products. Carbon dioxide was measured by titration of the barium hydroxide from the Dreschel bottles against 0.1 N HCl and the carbon dioxide calculated by the difference between the titre before and after the experiment.

Formic, acetic and lactic acids were separated chromatographically on a silica gel column with a benzene/butanol gradient solvent (Lessard and McDonald, 1966). The effluent from the column was titrated against 0.01 N NaOH using a Radiometer (Denmark) automatic titration and recording assembly.

Pyruvate was determined by the method of Friedemann and

TABLE 35. A comparison of the rate of CO_2 production from citrate by various silage lactic acid bacteria (@ pH 6.0).

	Cells grown on citrate		Cells grown on citrate and glucose		
Substrate: / Organism	Endogenous	Citrate	Endogenous	Citrate	Citrate and glucose
Streptococcus faecalis	0.5	559.0	-	-	-
Streptococcus faecium	-	-	0.1	23.2	23.4
Pediococcus	-	-	0.2	0.3	2.8
Leuconostoc mesenteroides	-	-	0.5	1.1	1.2 *
Lactobacillus plantarum	-	-	0.2	0.3	3.2
Lactobacillus brevis	-	-	0.1	3.1	15.8 *

All results are Q_{CO_2} (μl CO_2 evolved/hour/mg. cell dry wt.)

* Arabinose replaced glucose in manometer flasks with heterofermentative organisms

Haugen (1943) using benzene to extract the phenyl hydrazone.

Citrate was estimated by the method of Ettinger, Goldbaum and Smith (1952).

Acetoin was measured by the method of Langlykke and Peterson (1937). A duplicate fermentation flask, treated identically to the one used for all the other determinations, was used to estimate acetoin, the whole supernatant being used for each determination.

Glucose was determined by the reduction of ferricyanide and subsequent titration against 0.01 N ceric sulphate with xylene cyanol FF as an indicator (Fuller, Lampitt and Coton, 1955).

Results

Citrate dissimilation

Comparison of various silage lactic acid bacteria

Manometric experiments showed that Streptococcus faecalis produced carbon dioxide from citrate at a very fast rate (Table 35). S. faecium also fermented citrate but the rate of carbon dioxide production was 25 times slower than that of S. faecalis. Lactobacillus brevis dissimilated citrate in the presence of a fermentable carbohydrate as an energy source and Leuconostoc mesenteroides, Pediococcus and Lactobacillus plantarum did not produce carbon dioxide from citrate in the

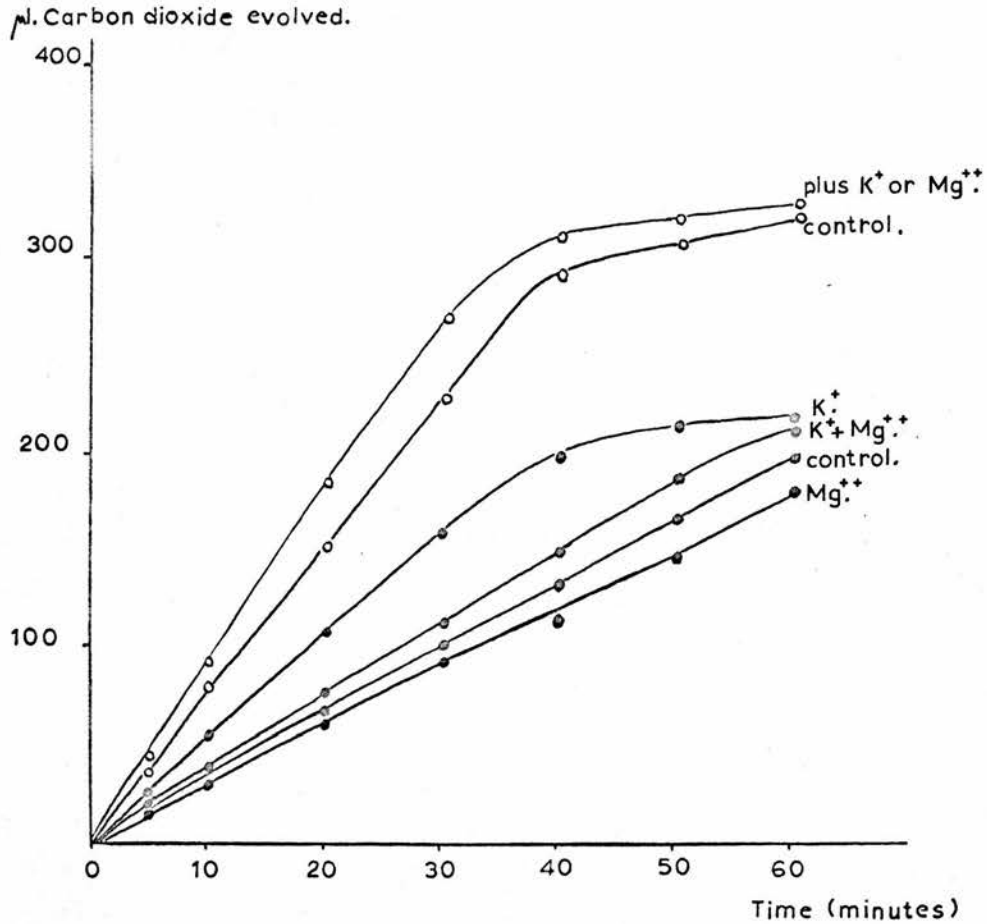

FIGURE 7. Effect of K^+ and Mg^{++} on citrate breakdown by Streptococcus faecalis and S.faecium. CO_2 evolution, measured manometrically.

KEY: o—o S.faecalis, 1mg. cell dry weight per flask.
●—● S. faecium, 5mg. cell dry weight per flask.

presence or absence of a fermentable carbohydrate.

The very fast rate of citrate dissimilation by S. faecalis suggests that this organism could be mainly responsible for the disappearance of citrate in the first few days of the silage fermentation.

Requirement for potassium and magnesium ions

The requirement for potassium and magnesium or manganese ions of the citrate 'lyase' system was noted by Keddie (1959). An experiment was conducted to test the effect of potassium and magnesium ions on the rate of carbon dioxide production from citrate by S. faecalis and S. faecium. The results of this experiment are illustrated in Figure 7.

S. faecalis showed a faster rate of carbon dioxide production from citrate in the presence of K^+ or Mg^{++} compared with a system lacking both K^+ and Mg^{++}. There was no increased rate with both K^+ and Mg^{++} present compared to when only one of them was present.

S. faecium produced carbon dioxide from citrate faster in the presence of K^+ but showed a reduced rate in the presence of Mg^{++}, both instances being compared to when both K^+ and Mg^{++} were absent.

S. faecalis therefore requires the presence of either potassium ions or magnesium ions for the maximum rate of citrate dissimilation and S. faecium requires potassium ions for the maximum rate of citrate dissimilation.

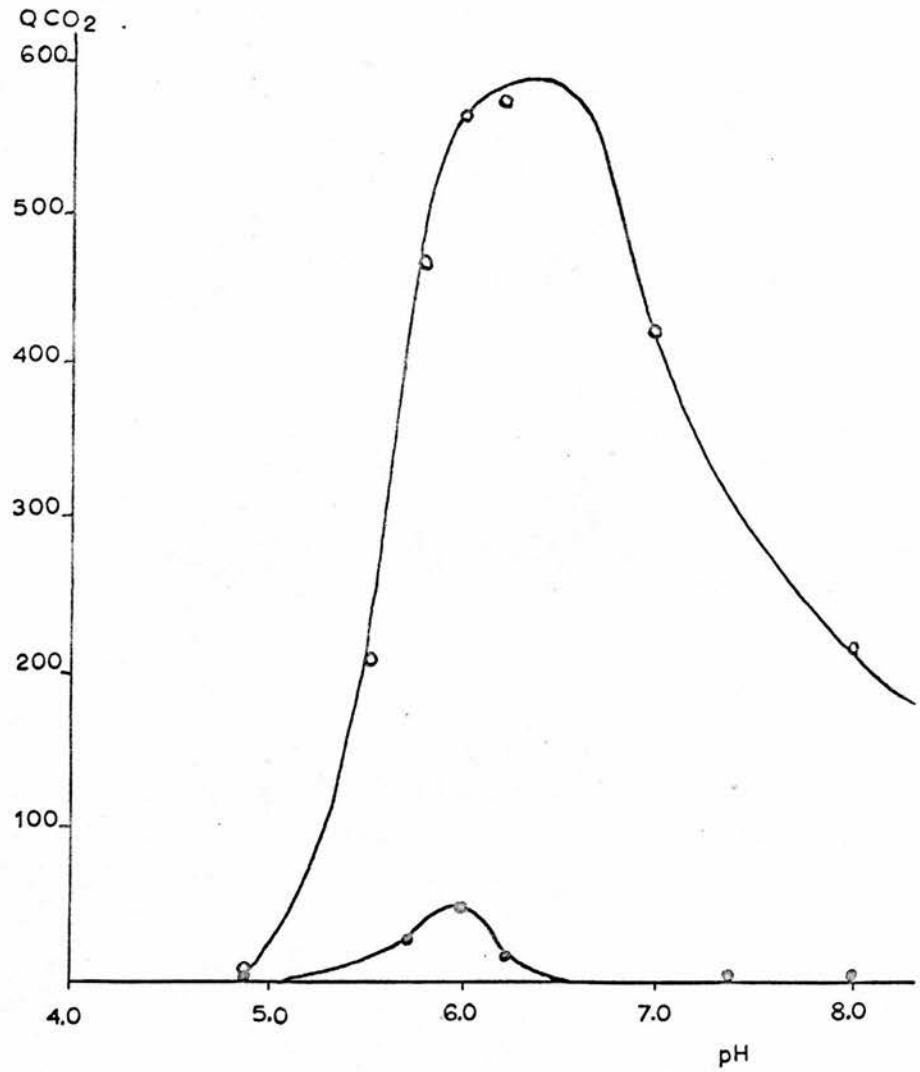

FIGURE 8. Effect of pH on the rate of citrate breakdown; CO_2 evolution by S.faecalis and S.faecium.

KEY: o——o S. faecalis.
●——● S. faecium.

TABLE 36. Molar quantities of carbon dioxide produced during citrate dissimilation by S. faecalis, S. faecium and L. brevis.

	Citrate grown cells	Citrate and glucose *(arabinose) grown cells	
Substrate:	Citrate	Citrate	Citrate and glucose
Organism			
Streptococcus faecalis	pH 6.2 1.47 pH 7.5 1.43	-	-
Streptococcus faecium	-	1.40	1.44
Lactobacillus brevis	-	-	1.43 *

* Arabinose replaced glucose in studies with the heterofermentative organism, Lactobacillus brevis

All results as Moles CO_2 produced per Mole of citrate fermented

Effect of pH on the rate of citrate dissimilation

The effect of varying the pH on the rate of citrate dissimilation by S. faecalis and S. faecium was investigated. Figure 8 shows the relationship between pH and the rate of carbon dioxide evolution obtained from these experiments.

The inability of S. faecium to dissimilate citrate at pH 7 and above, whereas S. faecalis still actively dissimilates citrate at pH 8, agrees with the observations of Whittenbury (1965) who used this character to differentiate the two species.

The rate of citrate dissimilation also falls off at low pH values. This is of interest in silage fermentations as it means that if the pH value fell rapidly, or was artificially reduced, e.g. by formic acid, citrate fermentation could be prevented and some gaseous dry matter loss avoided.

Quantitative estimation of products of citrate fermentation

Table 36 shows the molar quantities of carbon dioxide produced by S. faecalis and S. faecium fermenting citrate, and by Lactobacillus brevis dissimilating citrate in the presence of arabinose.

All produced about 1.5 Moles of carbon dioxide per Mole of citrate fermented and would therefore each produce the same gaseous loss from citrate in a silage fermentation. If grass has 1% of its dry matter as citrate (McDonald and Whittenbury, 1967) this would result in a gaseous loss of 0.38% of the dry

TABLE 37. S. faecalis. Fermentation of citrate and pyruvate at various pHs by resting cells. Analysis of products - expressed as mM/mM substrate used.

pH:	5.5		6.5		7.4	
Substrates						
Citrate	1.00	-	1.00	-	1.00	-
Pyruvate	-	1.00	-	1.00	-	1.00
Products						
CO_2	1.42	0.49	1.48	0.45	1.37	0.38
Formate	0.29	0.24	0.30	0.25	0.44	0.39
Acetate	1.63	0.51	1.58	0.56	1.65	0.59
Pyruvate	0.01	0.0	0.0	0.0	0.01	0.0
Lactate	0.31	0.25	0.25	0.29	0.19	0.20
Acetoin	0.04	0.10	0.10	0.07	0.08	0.09
% Carbon recovery	101.2	96.8	101.2	96.0	100.5	97.2
O/R ratio	1.06	1.08	1.04	1.04	1.04	1.00

System as in methods

Each set of results is the mean of two separate experiments

TABLE 38. S. faecalis. Fermentation of citrate and pyruvate in the presence of glucose. Results expressed as mM/mM of substrate used.

Substrate:	Glucose (a)	Glucose + Citrate (b)		Glucose + Pyruvate (c)	
		Products from glucose	Products from citrate*	Products from glucose	Products from pyruvate*
Substrates					
Citrate	-	-	1.00	-	-
Pyruvate	-	-	-	-	1.00
Glucose	1.00	1.00	-	1.00	-
Products					
CO_2	0.06	0.06	1.50	0.06	0.64
Formate	0.0	0.0	0.12	0.0	0.09
Acetate	0.0	0.0	1.40	0.0	0.33
Pyruvate	0.04	0.04	0.0	0.04	0.0
Lactate	1.84	1.84	0.36	1.84	0.28
Acetoin	0.0	0.0	0.12	0.0	0.20
% Carbon recovery	95.0	97.5		98.0	

System as in the methods.

* Total products less the normal products from glucose fermentation.

(a) Mean of two experiments. (b) Mean of three experiments.

(c) Results of a single experiment.

matter of the grass (37.5% of the citrate). That these organisms produce the same amounts of carbon dioxide from citrate does not prove they all have the same pathways from citrate, but in view of the similarity of metabolism within the lactic acid bacteria as a group, it is a strong possibility that they have the same pathways for citrate dissimilation.

As S. faecalis is the most active citrate dissimilating organism it would seem likely that this organism would account for the majority of the citrate dissimilated during a silage fermentation. Therefore a more detailed study was made of the products of citrate breakdown by S. faecalis. Fermentation balances were measured at three pH values, pH 5.5, pH 6.5 and pH 7.4, as Gunsalus and Campbell (1944) had noted a change in the proportions of products as the pH changed, due to different pathways from pyruvate at different pH values. Also the effect of the presence of glucose on the products of citrate fermentation was investigated.

The fermentation balances on citrate are shown in Table 37 and those on citrate plus glucose in Table 38.

Assuming the pathway of citrate dissimilation proceeds via oxaloacetate (Gunsalus and Campbell, 1944) one Mole of citrate results in one Mole each of acetate, pyruvate and carbon dioxide. The pyruvate would then be fermented by the pathways outlined in the introduction. The proportion of pyruvate fermented by each of these pathways was calculated from the proportions of formate, acetoin, lactate and acetate in the

TABLE 39. S. faecalis. Fermentation of citrate and pyruvate. Effect of pH on the proportion of pyruvate fermented by different pathways.

pH:	5.5		6.5		7.4	
Substrate:	Cit.	Pyr.	Cit.	Pyr.	Cit.	Pyr.
1. Pyr.→form. + acet.	29	24	30	25	44	39
2. Pyr.→CO_2 + acet.	34	29	28	31	21	20
3. Pyr.→ Lact.	31	25	25	29	19	20
4. 2 Pyr.→acetoin +2CO_2	8	20	20	14	16	18
Total pyruvate (%)	102	98	103	99	100	97

All figures as percentages of total pyruvate

KEY: Pyr. - Pyruvate
Form. - Formate
Acet. - Acetate

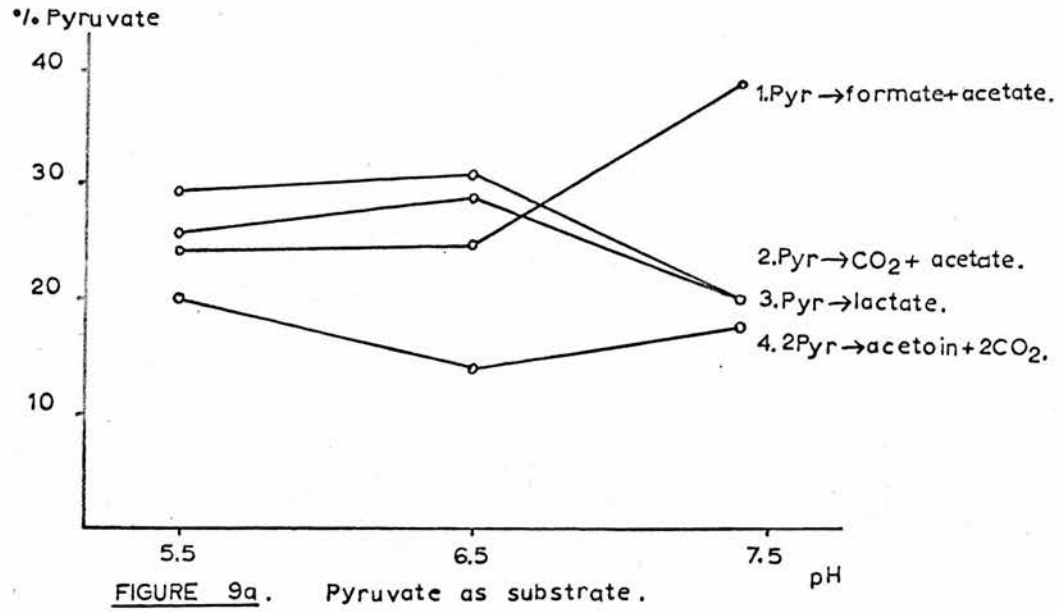

FIGURE 9a. Pyruvate as substrate.

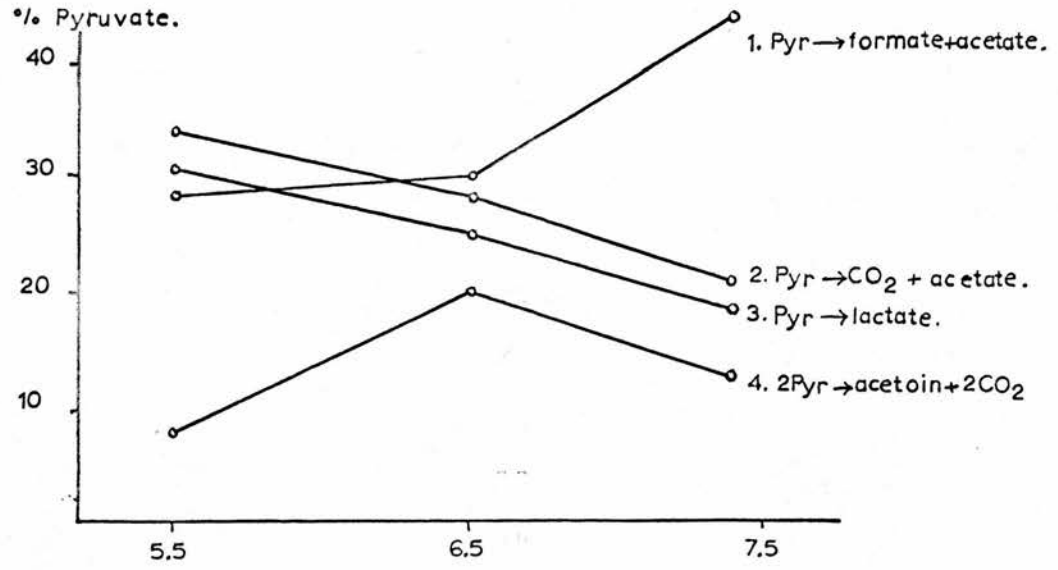

FIGURE 9b. Citrate as substrate.

FIGURE 9. Effect of pH on pyruvate metabolism of *Streptococcus faecalis*. As % pyruvate metabolised by each of 4 pathways.

TABLE 40. S. faecalis. Fermentation of citrate and pyruvate in the presence of glucose. The proportion of pyruvate fermented by different pathways.

Substrate:	Glucose and Citrate	Glucose and Pyruvate
1. Pyr.→ Form. + Acet.	12	9
2. Pyr.→ CO_2 + Acet.	28	24
3. Pyr.→Lact.	36	28
4. 2 Pyr.→Acetoin + 2 CO_2	24	40
Total pyruvate (%)	100	101

All figures as percentage of total pyruvate

KEY: Pyr. - Pyruvate
Form. - Formate
Acet. - Acetate

fermentation products and the results are shown in Table 39 and in Figure 9.

As Gunsalus and Campbell (1944) found, the proportion of formate increases at higher pH values and concurrently the proportion of lactate decreases. The proportion of pyruvate oxidised to acetate plus carbon dioxide was slightly greater than the proportion reduced to lactate, the difference probably being explained by some oxygen reaching the system when the cell suspension and the substrate were added. This also explains the O/R ratios being slightly greater than unity. All the pyruvate balance carbon recoveries were less than 100% and there may have been another product, such as 2,3-butanediol or diacetyl which were not assayed.

In the presence of glucose there was an increase in the proportion of acetoin in the products of citrate dissimilation and a decrease in the proportion of formate (Table 40).

In silage the products of citrate fermentation will vary with pH, changing during the first few days of storage as the pH falls from the initial pH of the grass (about pH 6.5). The products at pH 6.5 in the presence of glucose are as shown in Table 40, and as the pH falls the proportion of formate and acetoin will decrease and the proportion of lactate and acetate increase.

TABLE 41. A comparison of the rate of CO_2 production from malate by various silage lactic acid bacteria at pH 6.0.

	Cells grown on malate			Cells grown on malate and glucose		
Substrate: / Organism	End.	Malate	Malate + Glucose	End.	Malate	Malate + Glucose
Streptococcus faecalis	0.5	8.3	78.2	-	-	-
S. faecium	0.5	1.2	190.0	0.8	69.2	187.0
Pediococcus	0.1	6.6	15.5	0.2	7.1	8.7
Leuconostoc mesenteroides	-	-	-	0.3	13.7	42.6
Lactobacillus plantarum	0.2	3.2	32.9	0.2	38.0	90.0
L. brevis	-	-	-	0.2	0.2	2.1

All results expressed as μl. CO_2 evolved/hour/mg. cell dry weight.

Arabinose replaced glucose in experiments with the hetero-fermentative organisms.

- Not tested.

TABLE 42. Effect of K^+ and Mn^{++} on malate breakdown by *Streptococcus faecalis*.

System: Organism	Endog.	Complete	Less Mn^{++}	Less K^+	Less Mn^{++} and K^+
Streptococcus faecalis	0.5	8.3	4.1	7.7	4.1

All results expressed as μl. CO_2 evolved/hour/mg. cell dry wt.

Malate dissimilation

Comparison of various silage lactic acid bacteria

The ability to ferment malate was more widespread amongst the organisms studied than was the ability to ferment citrate (Table 41). S. faecalis, S. faecium, Pediococcus, Leuconostoc mesenteroides and Lactobacillus plantarum all fermented malate in the presence and absence of a carbohydrate energy source, although all except Pediococcus showed a much faster rate with a carbohydrate energy source than without one. The fastest rates of malate dissimilation were shown by Lactobacillus plantarum and Streptococcus faecium cell suspensions grown on glucose plus malate.

Lactobacillus brevis did not ferment malate, and only dissimilated malate very slowly in the presence of arabinose as an energy source.

The effect of potassium and manganese ions on malate dissimilation

S. faecalis showed an increased rate of carbon dioxide production from malate in the presence of Mn^{++} compared to controls without added Mn^{++} (Table 42). K^{+} had only a very slight beneficial effect on the rate of carbon dioxide production from malate by S. faecalis.

TABLE 43. Effect of acetoin and 2,3,5-triphenyltetrazolium chloride on malate breakdown by S. faecalis.

System: Organism	Endog.	Malate	Malate + Acetoin	Malate + TTC
Streptococcus faecalis	0.5	8.2	8.9	34.0

All results expressed as μl. CO_2 evolved/hour/mg. cell dry weight

Effect of exogenous hydrogen acceptors on malate dissimilation

As the pyruvate produced in malate breakdown is reduced to lactate to regenerate the NAD reduced in the initial step of the reaction, it seemed likely that this would be the rate limiting step, as the pyruvate would never be available at a high concentration. Therefore the effect of acetoin and 2,3,5-triphenyltetrazolium chloride, as hydrogen acceptors, on the rate of carbon dioxide evolution from malate by S. faecalis was tested. There was a slight increase in the rate of CO_2 evolution with acetoin present and a marked increase with the tetrazolium salt (Table 43). The provision of a hydrogen acceptor, and therefore the speed of NAD regeneration, would appear to be the rate limiting step in the dissimilation of malate by S. faecalis cells.

Effect of pH on malate breakdown by Streptococcus faecalis

Figure 10 shows the results of measurements of the rate of malate breakdown by S. faecalis over a range of pH values. As with citrate the rate slowed as the pH fell. The optimum pH for production of CO_2 from malate was at pH 7.0 which compares to an optimum of pH 6.0 found by Korkes, del Campillo and Ochoa (1950) for the 'malic' enzyme of Lactobacillus arabinosus.

Quantitative estimation of the products of malate fermentation

Manometric studies on Streptococcus faecalis, S. faecium,

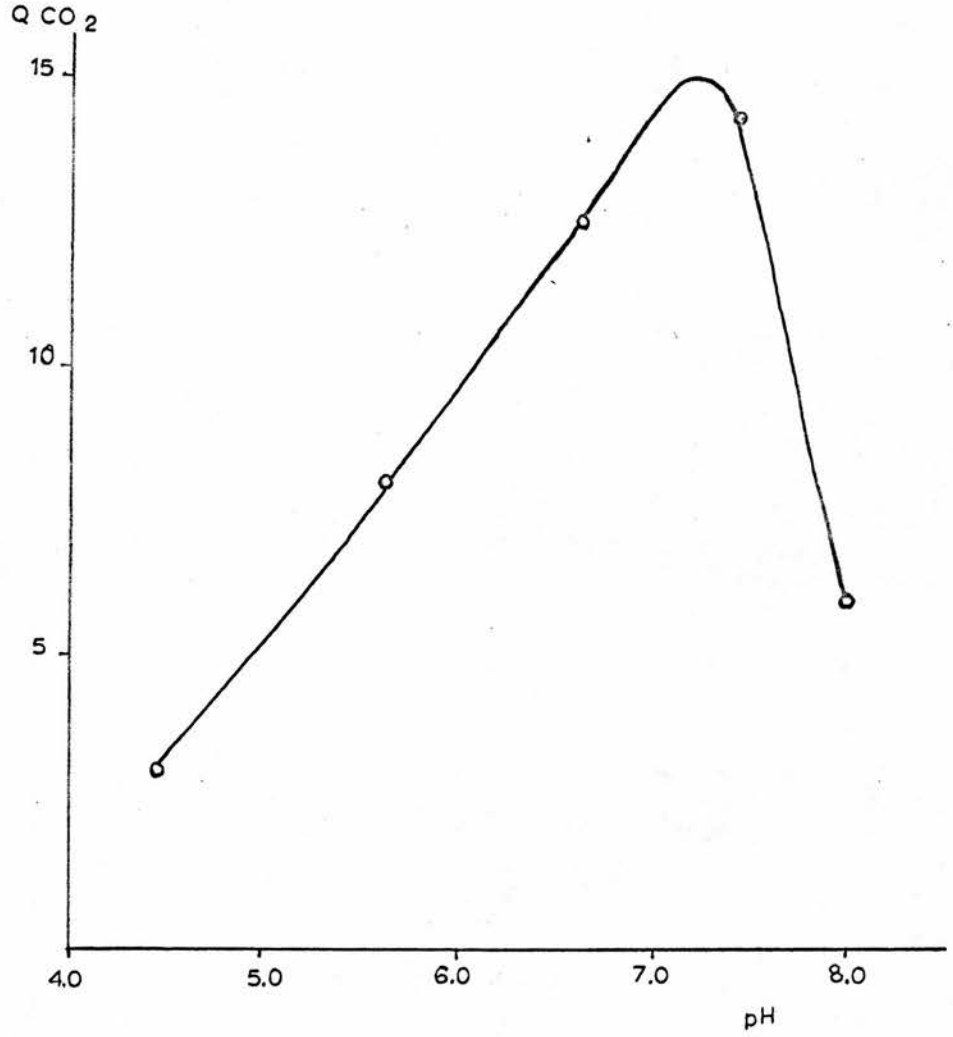

FIGURE 10. Effect of pH on the rate of malate breakdown. CO_2 evolution by Streptococcus faecalis.

TABLE 44. Molar quantities of carbon dioxide produced during malate dissimilation by various silage lactic acid bacteria.

Organism	Cells grown on malate		Cells grown on malate and glucose	
Substrate:	Malate	Malate + Glucose	Malate	Malate + Glucose
Streptococcus faecalis	1.0	-	-	-
S. faecium	-	-	0.98	1.20
Pediococcus	0.92	1.10	-	-
Leuconostoc mesenteroides*	-	-	1.04	1.18
Lactobacillus plantarum	0.88	0.93	0.90	1.14

All results expressed as Moles of CO_2 produced per Mole of malate dissimilated

\- Not tested

* Arabinose replaced glucose in studies with the hetero-fermentative organism, Leuconostoc mesenteroides

TABLE 45. Lactobacillus plantarum. Fermentation of malate and of malate in the presence of glucose. Results expressed as mM/mM of substrate used.

Substrate:	Malate	Glucose + Malate		Glucose
		Products from glucose	Products from malate *	
Substrates				
Malate	1.00	-	1.00	-
Glucose	-	1.00	-	1.00
Products				
CO_2	0.98	0.04	1.14	0.04
Formate	0.0	0.0	0.0	0.0
Acetate	0.0	0.0	0.0	0.0
Pyruvate	0.0	0.02	0.0	0.02
Lactate	0.96	1.93	0.88	1.93
Acetoin	0.0	0.0	0.08	0.0
% Carbon recovery	99.0	100.5		98.0

* Calculated from figures for total products less the products from glucose fermentation

System as in methods

Pedioccus, Leuconostoc mesenteroides and Lactobacillus plantarum (Table 44) showed a production of 1 Mole of CO_2 per Mole of malate dissimilated. In the presence of a carbohydrate source S. faecium produced 1.2 Moles of CO_2 per Mole of malate dissimilated, Leuconostoc produced 1.18 Moles of CO_2 per Mole of malate, Lactobacillus plantarum 1.14 Moles and Pediococcus 1.10 Moles of CO_2 per Mole of malate dissimilated. The full results are outlined in Table 41. The oxidative decarboxylation of malate to pyruvate plus CO_2 and the reduction of the pyruvate to lactate coupled to NAD regeneration results in the production of 1 Mole of CO_2 per Mole of malate fermented. These results therefore suggest that this is the pathway operating in the bacteria studied.

The figure of more than 1 Mole of CO_2 produced per Mole of malate fermented in the presence of fermentable carbohydrate appeared to be coupled to the production of acetoin. This was confirmed in a fermentation balance experiment with Lactobacillus plantarum (Table 45), in which lactate and CO_2 were the only products from malate but in the presence of glucose acetoin and extra CO_2 were detected in the products. This production of acetoin agrees with the results obtained by Whittenbury (1956) who detected acetoin production from L-malate fermentation in the presence of a fermentable carbohydrate.

In silage, therefore, some acetoin production could be expected from malate dissimilation although lactate and CO_2 are the main products.

Discussion

Keddie (1959) has stated that a relatively high potassium ion concentration is necessary for citrate and malate dissimilation by lactic acid bacteria. The experiments reported here showed that the presence of potassium ions increased the rate of citrate dissimilation by both Streptococcus faecalis and S. faecium. With S. faecalis magnesium ions had the same effect as K^+. There was, however, no summation of the effects of K^+ and Mg^{++} when both were present. With S. faecium Mg^{++} ions had an inhibitory effect on the rate of carbon dioxide evolution from citrate.

With S. faecalis K^+ ions had very little effect on malate breakdown but Mn^{++} had a marked stimulatory effect. This effect of Mn^{++} ions agrees with the findings of Korkes, del Campillo and Ochoa (1950).

From these brief experiments it appears that K^+ ions are necessary for citrate dissimilation and Mn^{++} ions for malate dissimilation.

The products of citrate dissimilation vary considerably with pH. The results obtained at different pH values agree with the results of Gunsalus and Campbell (1944), the proportion of formic acid increasing and the proportion of lactic acid decreasing as the pH became more alkaline.

Fermentation balances with S. faecalis on pyruvate agreed largely with the results obtained with citrate as the substrate,

when pyruvate would be an intermediate. However, with pyruvate fermentation balances carbon recovery was always less than 100%, possibly due to the production of 2,3-butanediol or of diacetyl which were not assayed.

The results of the pyruvate fermentation balances were similar to the results obtained by Deibel and Niven (1964) and confirmed that it is the diversity of reactions from pyruvate which accounts for the varying products when citrate is fermented at different pH values.

In the fermentation balances in the presence of glucose the assumption that the products from glucose when citrate or malate are present are the same as the products when glucose alone is fermented is possibly invalid. As pyruvate is produced from both glycolysis and citrate or malate breakdown there is presumably no means whereby the pyruvate from glucose is reduced exclusively to lactate. However, the molar quantity of lactate produced to regenerate NAD will be the same as when glucose is fermented alone. Therefore the molar quantity of pyruvate going to products other than lactate will be the amount of pyruvate produced from the citrate or malate, although not necessarily the actual molecules derived from the organic acids.

Studies with labelled glucose would be necessary to show whether the pyruvate from glucose and the pyruvate from organic acids all enters the same pool or whether the two metabolic pathways are separated, for example by substrate-

enzyme complexes (Kaufman, Korkes and del Campillo, 1951) keeping intermediates separated.

As far as organic acid breakdown in silage is concerned S. faecalis would appear to be the organism mainly responsible for citrate dissimilation. S. faecium and Lactobacillus brevis will also play a minor part in citrate dissimilation.

At the pH of silage in the first day or two of storage (pH 6.5 falling to pH 5.5 or lower), the main products of citrate dissimilation will be acetate, lactate and CO_2 with some formate and acetoin being produced. In the presence of glucose, and presumably of other carbohydrate energy sources, there will be a lower proportion of formate and a higher proportion of acetoin.

As mentioned in the introduction, acetoin and formate have not been found frequently in silage. Formate, however, was detected by Playne, Stirling and McDonald (1967) in silage prepared from aseptically grown grass inoculated with S. faecalis. 2,3-Butanediol has been detected in silage by McDonald, Henderson and MacGregor (1968) and it could have been formed by the reduction of acetoin.

There could be a loss of formate due to its further metabolism or by evaporation (B. pt. 100°C), and likewise a loss of acetoin by similar means (B. pt. 148°C), accounting for their absence when silage samples are analysed.

Lactobacillus plantarum, Streptococcus faecium and S. faecalis, judging by the rates of carbon dioxide evolution from

malate, appear to be the organisms mainly responsible for malate breakdown in silage, with Pediococcus and Leuconostoc mesenteroides playing a minor role. Carbon dioxide and lactate are the main products, although in the presence of glucose some acetoin was produced by L. plantarum. Whittenbury (1956) noted acetoin production from malate by S. faecalis and S. faecium in the presence of glucose. However, the acetoin only accounts for about 8% of the malate fermented.

This study was by no means exhaustive and there could well be other strains of lactic acid bacteria which dissimilate malate or citrate more rapidly than the strains examined. However, it seems likely that within the lactobacilli the pathways of malate and citrate dissimilation will not show much variation.

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SECTION FOUR

Effect of herbage inoculation on the silage fermentation

Introduction

As has already been mentioned in the general introduction, some advantage may be gained from adding an inoculum of lactic acid bacteria to herbage prior to ensiling it, especially if the herbage is low in water soluble carbohydrates or is of high buffering capacity.

Whittenbury (1961) has outlined the ideal characteristics of an organism for use as an inoculum and suggested either a strain of Pediococcus acidilactici or a strain of the Lactobacillus plantarum-casei group comes nearest to the ideal organism. Strains of the genus Streptococcus were not considered sufficiently acid tolerant but their extremely rapid growth and sugar metabolism, especially under aerobic conditions, suggest that streptococci could be of great advantage in controlling the early stages of the silage fermentation.

Recently attention has been drawn to the production of amines in silage (Macpherson, 1962; Macpherson and Violante, 1966a) and their possible effect on silage intake by the animal (McDonald, Macpherson and Watt, 1963). It is probable that these amines are the result of clostridial activity, which occurs even in a well preserved silage during the first few days of storage (Gibson, 1965) until the pH falls low enough to inhibit clostridial growth.

The faster the rate of pH fall in a silage the shorter

the period in which clostridia can multiply and therefore the fewer the amines which will be present in the final product (Macpherson and Violante, 1966b).

If an inoculum, which is fast-growing and biochemically very active, could significantly speed the lactic acid fermentation in the silage, then an earlier inhibition of clostridial growth could be achieved than if the same herbage was ensiled without an inoculum.

Experiments were conducted, initially on a laboratory scale and then on a small (1000 kg. capacity) tower silo scale, using an inoculum of Lactobacillus plantarum or Streptococcus or a mixture of the two. All inoculated silages were compared with uninoculated control silages.

As these lactic acid bacteria grow aerobically and anaerobically it was expected that they would increase before anaerobic conditions developed. S. faecalis is faster growing aerobically and was therefore expected to dominate in the early stages of storage but as it is not acid tolerant, the L. plantarum was expected to take over the lactic acid fermentation as the pH decreased and produce a low enough pH to preserve the silage.

Two experiments were conducted, the first in September 1966 and the second in May 1967.

Methods

Grass. In the first, small scale experiment Italian rye-grass (Lolium multiflorum) and cocksfoot (Dactylis glomerata) were ensiled in 70 g. quantities in pyrex test-tubes (20 x 3 cm.).

In the second experiment a Timothy (Phleum pratense)/ meadow fescue (Festuca pratensis) mixture was used and ensiled in 1000 kg. tower silos (McDonald et al., 1960) and also in test-tubes as described above.

Treatments. The rye-grass (dry matter 15.7; water soluble carbohydrates (WSC.) 13.8%) was chopped and one set of tubes set up as a control. Sets of tubes were then filled with grass inoculated with 10^5 organisms/g. dry wt. of grass of Streptococcus faecalis; Lactobacillus plantarum and a 1:1 mixture of S. faecalis and L. plantarum. The inoculum was applied by aerosol spray as a cell suspension in distilled water, 1 ml. of suspension being sprayed on per 70 g. of grass. One ml. of distilled water per 70 g. of grass was sprayed on the grass used in the control tubes.

The cocksfoot (dry matter 18.2%; WSC. 12.3% of D.M.) was divided into portions. One portion was used as a control and the other portion was inoculated with 10^5 org./g. dry wt. of grass of a 1:1 mixture of S. faecalis and L. plantarum. Half of each portion was ensiled in tubes and the other half was

wilted at room temperature to 27.2% dry matter and then ensiled in tubes.

In the second experiment two 1000 kg. silos were filled with uninoculated herbage and then two with inoculated herbage. Prior to filling the silos were washed with an iodine based dairy disinfectant and well rinsed. The inoculum was applied by a spray at a rate of 1.8 litres of cell suspension per 1000 kg. of grass, equivalent to an inoculum of 3×10^5 organisms of a mixture of S. faecalis and L. plantarum per g. dry wt. of grass. 1.8 litres of distilled water/1000 kg. was sprayed onto the control silages. The same herbage was used to fill test-tube silos in the manner previously described.

All test-tube silos were sealed with a sintered glass and a mercury valve to allow gases to escape but prevent the entry of air.

Bacteriological analysis. Bacteriological changes were followed in test-tube silos using methods outlined by McDonald et al. (1960). Bacteriological examinations were made of effluent from the large silos, core samples taken on the twelfth day of storage and of the final silage.

Biochemical analysis. The pH changes were followed by measuring the pH of macerates of test-tube silos and the pH of the effluent from the large silos. Chemical analysis of the samples in the second experiment was carried out in a study

concurrent with the bacteriological experiments, so that bacteriological and biochemical results could be correlated if possible. The chemical analysis used standard methods as used in the Advisory Nutrition laboratories of the Edinburgh School of Agriculture.

Results

Experiment 1

The rye-grass silages were intended to compare silages made with inocula of S. faecalis; L. plantarum and a mixture of S. faecalis and L. plantarum with silage made with no inoculum.

The silage made with uninoculated grass showed a rise in pH in the first 24 hrs. followed by a slow pH fall (Fig. 12) whilst all the inoculated silages showed little pH change in the first 24 hrs. but then the pH fell rapidly. The fastest pH drop was in the silage inoculated with L. plantarum but the lowest pH value was in the silage inoculated with the S. faecalis and L. plantarum mixture.

The fastest rise in numbers of lactic acid bacteria coincided with the fastest rate of pH fall (Fig. 11). There appeared to be an earlier increase in lactic acid bacteria in silage with S. faecalis in the inoculum than in the silages without it, although all three inoculated silages had very

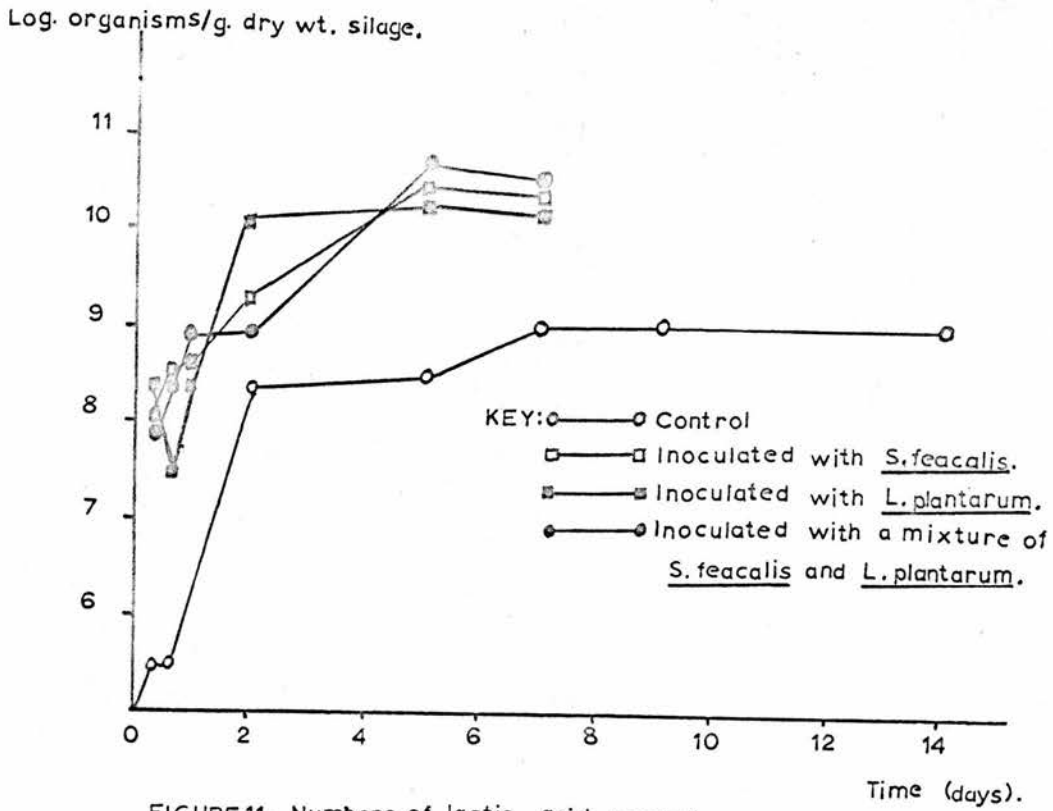

FIGURE 11. Numbers of lactic acid bacteria.
Rye grass silages; effect of inoculation of the grass.

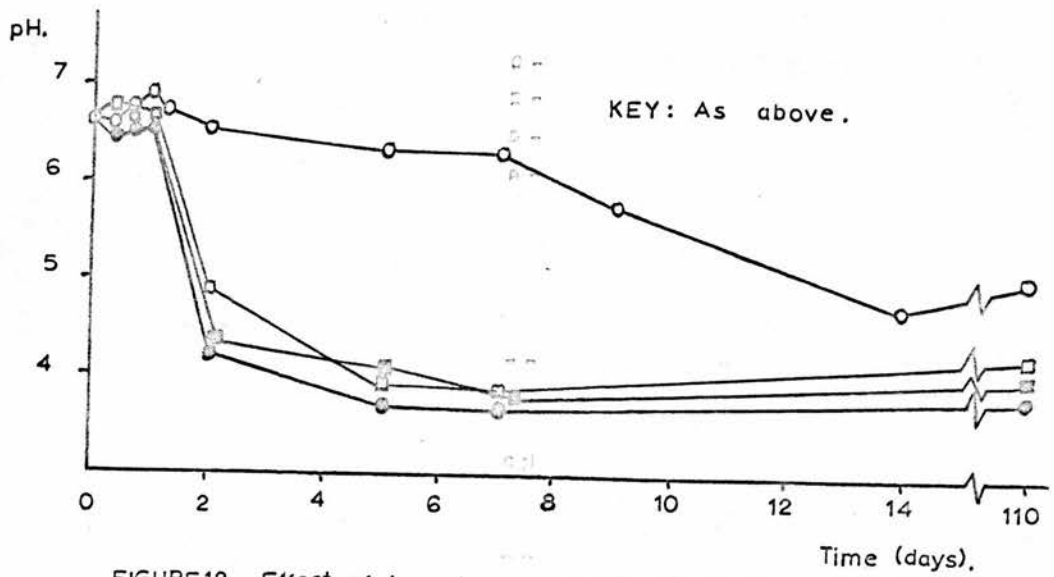

FIGURE 12. Effect of inoculation on the pH of rye grass silage.

similar lactic acid bacteria counts after 5 days of storage.

In the silage inoculated with S. faecalis alone Gram-positive cocci were dominant for the first 24 hrs., at which time the silage was at pH 5.87 but after 47 hrs. storage (pH 4.71) Gram-positive rods had replaced the cocci as the dominant flora.

The silage inoculated with L. plantarum was dominated by Gram-positive rods.

In the silage inoculated with a mixture of S. faecalis and L. plantarum the Gram-positive cocci were more numerous in the first 24 hrs. (up to 78% of the lactic acid bacteria) but by 47 hrs. Gram-positive rods were 98% of the lactic acid bacteria detected.

All the inoculated silages were well preserved and of less than pH 4.1 when opened after 110 days of storage. In contrast the control silage had a final pH of 5.1 and the lowest pH recorded in the control silages was pH 4.8 after 14 days storage. The highest lactic acid bacteria count in the control silages was 10^9 org./g. dry wt. compared to an average maximum of 3×10^{10} org./g. dry wt. in the inoculated silages. The control silage had a butyric acid smell and was not well preserved.

The silage inoculated with the S. faecalis and L. plantarum mixture was the first to reach a pH of less than pH 4 and also gave the lowest pH recorded in the experiment (pH 3.8) suggesting that this inoculum resulted in the most efficient

fermentation. This inoculum was therefore used in subsequent experiments.

The cocksfoot silages compared the effect of inoculation of herbage which was subsequently wilted with unwilted and wilted control silages. One interesting feature was a rise in the lactic acid bacteria count (org./g. dry wt.) during wilting of both inoculated and uninoculated chopped grass. This shows the aerobic tolerance of many lactic acid bacteria.

As with the rye-grass silages the pH fall in the inoculated silages was much faster than in the uninoculated controls. Also, as expected, the wilted silage had a slower lactic acid fermentation and a higher final pH than the unwilted material (Fig. 14). Both wilted silages were well preserved, although the uninoculated silage had some surface moulds.

In the unwilted silages the lactic acid bacteria count in the control rose to 5×10^8 org./g. dry wt. after 7 days storage whereas the lactic acid bacteria count in the inoculated, unwilted silage was 9×10^9 org./g. dry wt. after 7 days storage. In the wilted silage the lactic acid bacteria count rose to 1×10^7 org./g. dry wt. in the control after 11 days storage and in the inoculated silage the lactic acid bacteria count was 6×10^9 org./g. dry wt. after the same storage period. Lactic acid bacteria counts of the cocksfoot silages are shown in Figure 13.

The unwilted, uninoculated silage was poorly preserved and had a very strong butyric acid smell. All the other

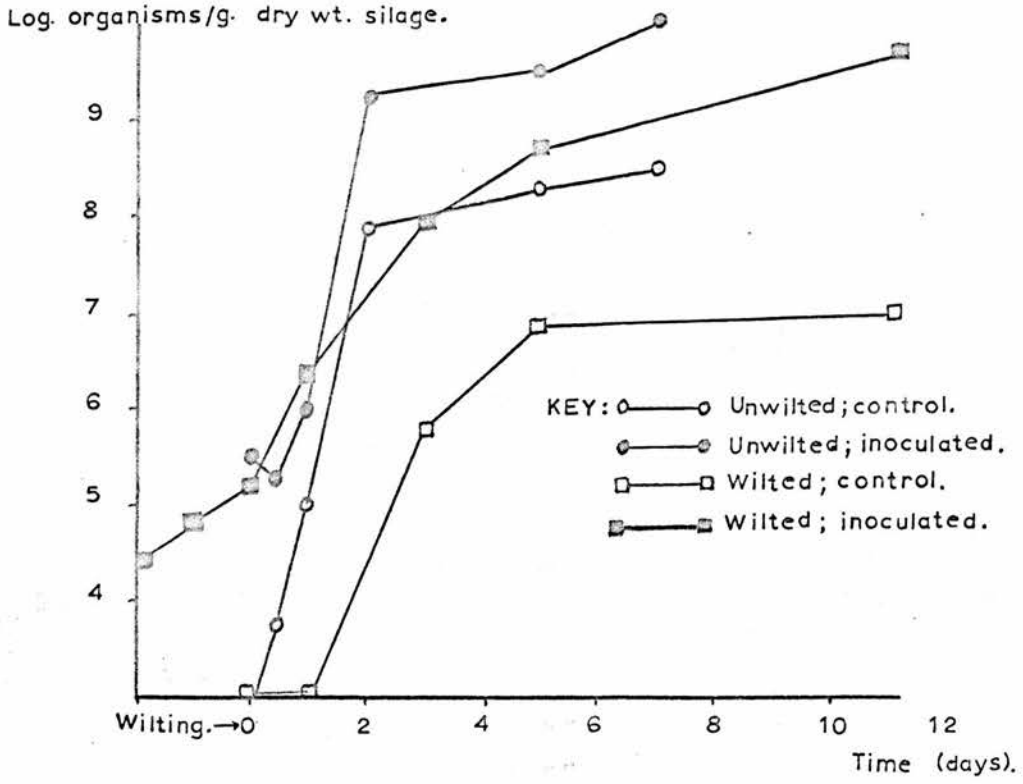

FIGURE 13. Numbers of lactic acid bacteria.
Cocksfoot silages; effect of wilting and inoculating the grass.

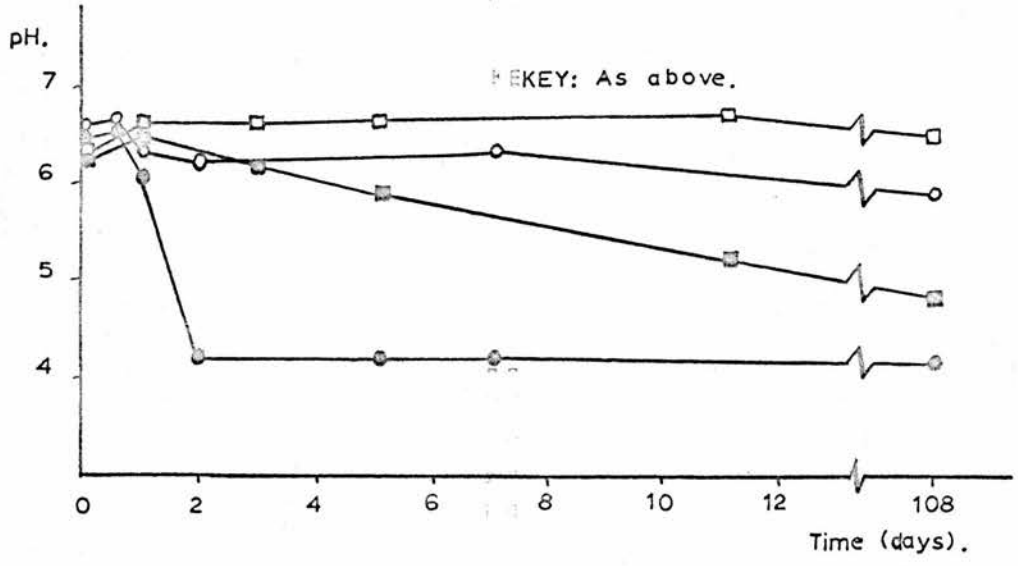

FIGURE 14. Effect of wilting and inoculation on the pH of cocksfoot silages.

cocksfoot silages were well preserved.

This first experiment indicated a definite advantage in the addition of an inoculum, especially in the rate of pH fall. A larger scale experiment was therefore conducted to follow up these results.

Experiment 2

All silages made in this experiment were well preserved. The water soluble carbohydrate level in the grass was above the minimum level considered sufficient for good silage preservation (Smith, 1962) and also the lactic acid bacteria count on the herbage immediately prior to ensiling was quite high (3×10^3 org./g. dry wt. of herbage). These two factors probably ensured a satisfactory fermentation resulting in good silage.

The laboratory, tube silos all showed a fast fall in pH and after 3 days storage the pH was pH 4 or less in both silages and after 16 days storage the pH of the inoculated silage was 3.80 and of the uninoculated silage pH 3.98 (Fig. 16).

Core samples were taken from the 1000 kg. silos after 12 days storage and the pHs of the inoculated and control silages were the same, varying between pH 4.12 and pH 4.19. All had a higher pH than laboratory silos.

The lactic acid bacteria count in both inoculated and

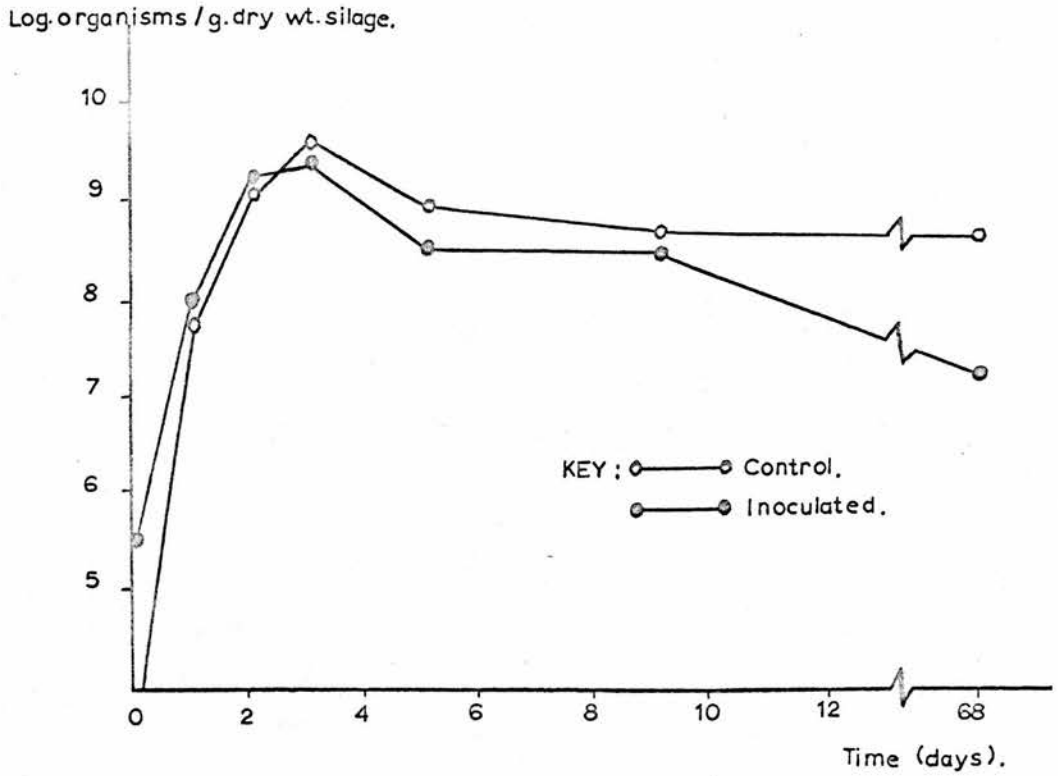

FIGURE 15. Numbers of lactic acid bacteria. Timothy / meadow fescue silages; effect of inoculation of the grass.

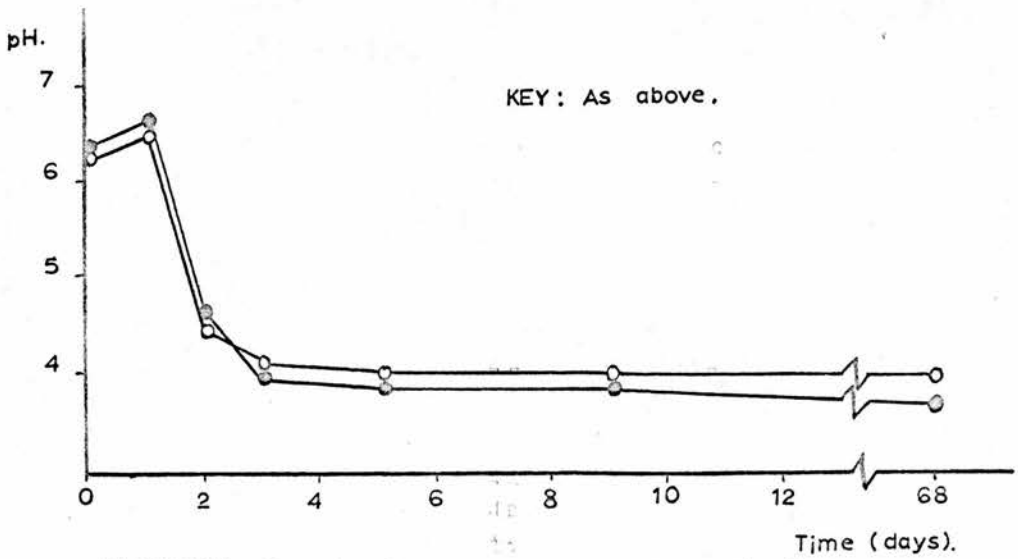

FIGURE 16. Effect of inoculation on pH of timothy / meadow fescue silages.

uninoculated tube silos was more than 10^9 org./g. dry wt. after 2 days of storage, with a slightly higher count in the inoculated silage (Fig. 15). The lactic acid bacteria numbers declined after the 3rd day of storage in the inoculated silage, possibly indicating a quicker completion of fermentation in the inoculated silage.

Anaerobic proteolytic bacteria rose in numbers simultaneously in both inoculated and control silages, rising from 10^4 org./g. dry wt. on the fresh herbage to more than 6×10^5 org./g. dry wt. after 24 hrs. storage in the test-tube silos. These levels were maintained up to the 5th day; after 9 days storage the lactic acid bacteria counts fell to 6×10^4 org./g. dry wt. and a further fall to 3×10^3 org./g. dry wt. after 16 days storage.

Anaerobic lactate fermenters were only detected in very small numbers (2×10^2 or less org./g. dry wt.) and were present equally in inoculated and control silages.

As the pH fell rapidly in all test-tube silos there was no comparison of the effect of a slow pH fall as opposed to the effect of a fast pH fall on clostridial activity in the silage. However, even with the rapid pH fall there was considerable clostridial growth, occurring in what became apparently excellently preserved silages.

Clostridia counts on the effluents from the 1000 kg. silos showed an absence of lactate fermenting anaerobes. Proteolytic anaerobes were at a level of 10^5 org./ml. of

TABLE 46. Bacteriological analysis of core samples (samples taken from 1000 kg. silos 12 days after ensiling the herbage).

	Inoculated silage		Uninoculated silage	
Silo:	A	B	C	D
pH of sample:	4.12	4.19	4.19	4.18
Bacteria				
General count	3.0×10^8	5.2×10^8	6.2×10^7	7.5×10^8
Lactic acid bacteria	7.0×10^8	1.8×10^9	2.0×10^8	1.4×10^9
Proteolytic clostridia	6.2×10^4	4.4×10^4	1.3×10^5	1.9×10^5
Lactate fermenting clostridia	6.2×10	6.2×10	3.1×10^2	1.9×10^2

Bacterial counts as organisms/g. dry wt. of silage.

TABLE 47. Losses from the 1000 kg. silos (% of dry matter ensiled).

	Inoculated silage		Uninoculated silage	
Silo:	A	B	C	D
Losses				
Effluent	1.95	1.96	2.00	1.99
Surface waste	1.78	2.94	3.24	3.32
Gaseous	2.65	1.64	3.26	3.59
Total losses	6.38	6.54	8.50	8.90

TABLE 48. Bacteriological analysis of waste material when silos (1000 kg.) opened.

	Inoculated silage		Uninoculated silage	
Silo:	A	B	C	D
pH of the waste:	8.14	8.21	8.06	8.15
Bacteria				
General count	2.5×10^9	1.3×10^9	2.5×10^9	1.9×10^9
Lactic acid bacteria	3.1×10^9	2.3×10^9	1.9×10^9	1.6×10^9
Proteolytic clostridia	6.2×10^7	6.2×10^7	6.2×10^7	6.2×10^7
Lactate fermenting clostridia	6.2×10^3	6.2×10^4	6.2×10^4	6.2×10^3
Mould count (counted on the general bacterial count plates)	6.2×10^6	1.2×10^6	1.9×10^8	6.2×10^7

Counts as organisms/g. dry wt. of silage.

effluent after 3 days of storage and started decreasing in number after the 6th day. After 16 days of storage there were only $10-10^2$ anaerobic proteolytic bacteria per ml. of effluent.

These effluent counts suggested there was considerable development of proteolytic clostridia in the 1000 kg. silos, the numbers decreasing as the pH of the effluent, and therefore presumably the pH of the silage, decreased.

Counts of core samples taken on the 12th day of storage showed slightly higher clostridial counts in the uninoculated silages compared to the inoculated silages (Table 46). Lactate fermenting anaerobes were detected in small numbers in control silages but were not detected in core samples from inoculated silages.

Lactic acid bacteria were at a similar level in all core samples of the silages.

All the silages from the 1000 kg. silos were well preserved although there were slightly greater losses from the uninoculated silages than the inoculated silages (Table 47).

Bacteriological analysis of the surface waste material (Table 48) showed 10 to 100 times more moulds in the uninoculated silages than in the inoculated ones. However, the large counts of anaerobic proteolytic bacteria suggest that excess moisture due to condensation on the polythene cover to the silo may have been the main cause of wastage, and not oxygen access.

TABLE 49. Bacteriological analysis of well preserved material when silos (1000 kg.) opened.

	Inoculated silage		Uninoculated silage	
Silo:	A	B	C	D
pH of the silage:	3.94	3.93	3.99	3.96
Bacteria				
General count	1.3×10^7	2.2×10^7	4.6×10^7	5.6×10^7
Lactic acid bacteria	3.9×10^8	5.0×10^8	2.8×10^9	2.0×10^9
Proteolytic clostridia	6.2×10^4	6.2×10^4	6.2×10^4	1.2×10^5
Lactate fermenting clostridia	6.2×10	1.2×10^3	1.2×10^3	6.2×10

Counts as organisms/g. dry wt. of silage.

There was very little difference between the bacterial counts of the well preserved material prepared from uninoculated and inoculated herbage (Table 49).

There was a higher final lactic acid bacteria count in the uninoculated silages than in the inoculated ones, a result which agreed with the results of the test-tube silos. This could be due to a slower fermentation and a slower fall off of lactic acid bacteria numbers in the uninoculated silages as suggested earlier, or alternatively due to a greater diversity of species being present in the uninoculated silage with the result that a larger proportion of aciduric organisms would be in the silage.

Discussion and Conclusions

In the first experiment the rapid pH fall in test-tubes filled with inoculated herbage showed that a definite advantage was gained from the use of an inoculum.

Both the rye-grass and the cocksfoot were fairly high in water soluble carbohydrates and might have been expected to be preserved with no inoculum. That the uninoculated silages were not well preserved indicates an inefficient fermentation, possibly due to the aerobic phase lasting too long for an adequate sugar supply to be left for the anaerobic, acid producing, fermentation phase. The use of an inoculum overcame any tendency for the inefficient sugar utilisation and adequate acid was produced to preserve the silages.

The experiment with wilted grass confirmed the beneficial effect of high dry matter content on the preservation of silage, and showed that an inoculum was not necessary for preservation, although possibly of use in making a better quality silage.

The second, larger scale experiment was inconclusive with no great advantage being detected from the use of an inoculum. Losses were slightly lower in the inoculated silages but the difference was only marginal. A slightly higher clostridia count in core samples from uninoculated silages indicated that clostridia had been inhibited earlier in inoculated silages. Also, effluent pH measurements indicated a faster pH fall in

the inoculated silages.

Gaseous losses in the inoculated silage were lower than in the uninoculated silage. Also the ratio of lactic:acetic acid was higher and there was less mannitol in the inoculated silage compared to the uninoculated silage. These facts indicated that the inoculum controlled the fermentation, producing a homolactic fermentation, whilst the uninoculated silage had a partially heterolactic fermentation.

There is a need for further investigations, if possible using low WSC., low dry matter herbage, to determine whether there is any advantage in the use of an inoculum under farm conditions.

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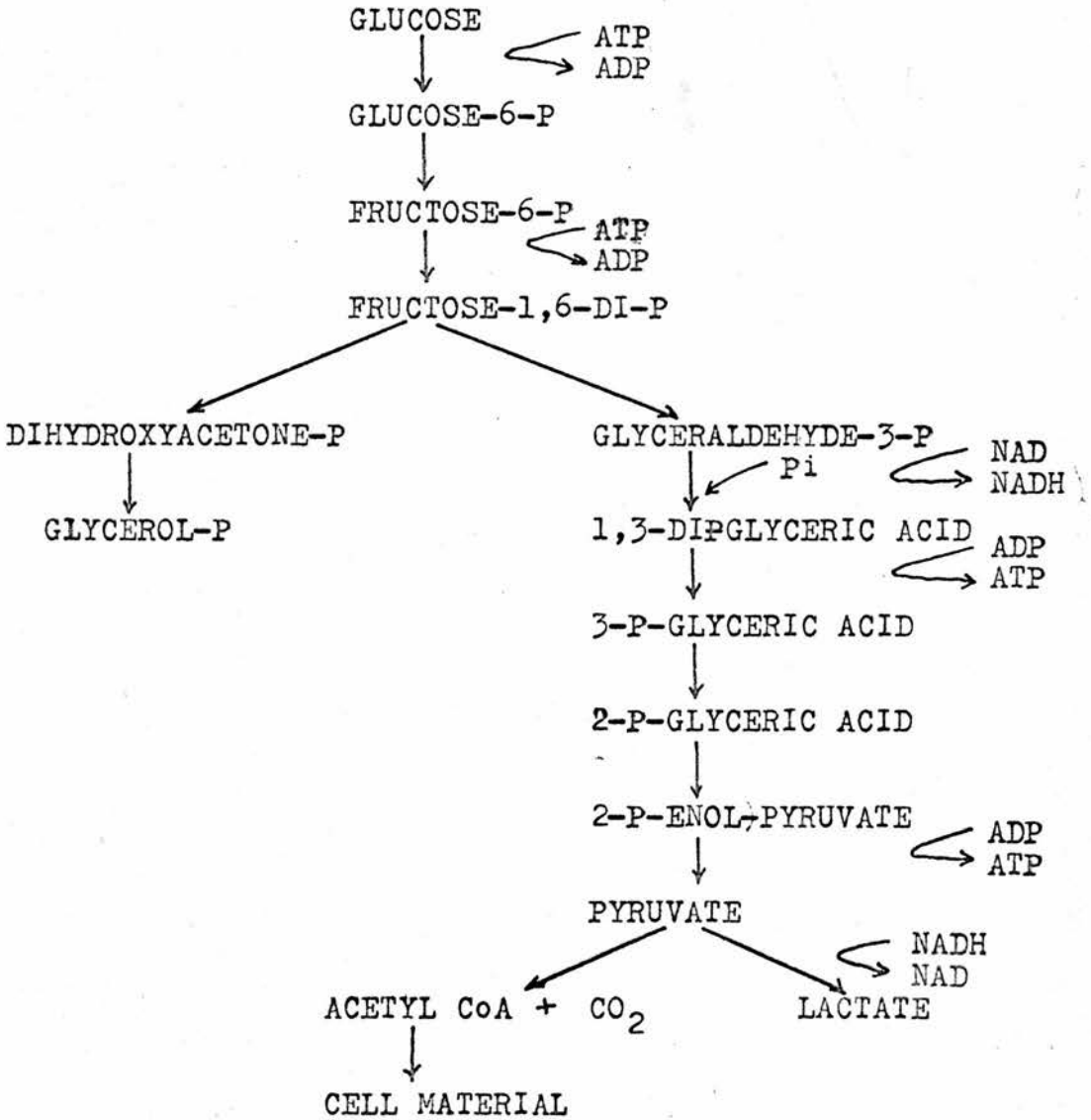
The patience of the author's wife during the preparation of the script is gratefully acknowledged.

APPENDICES

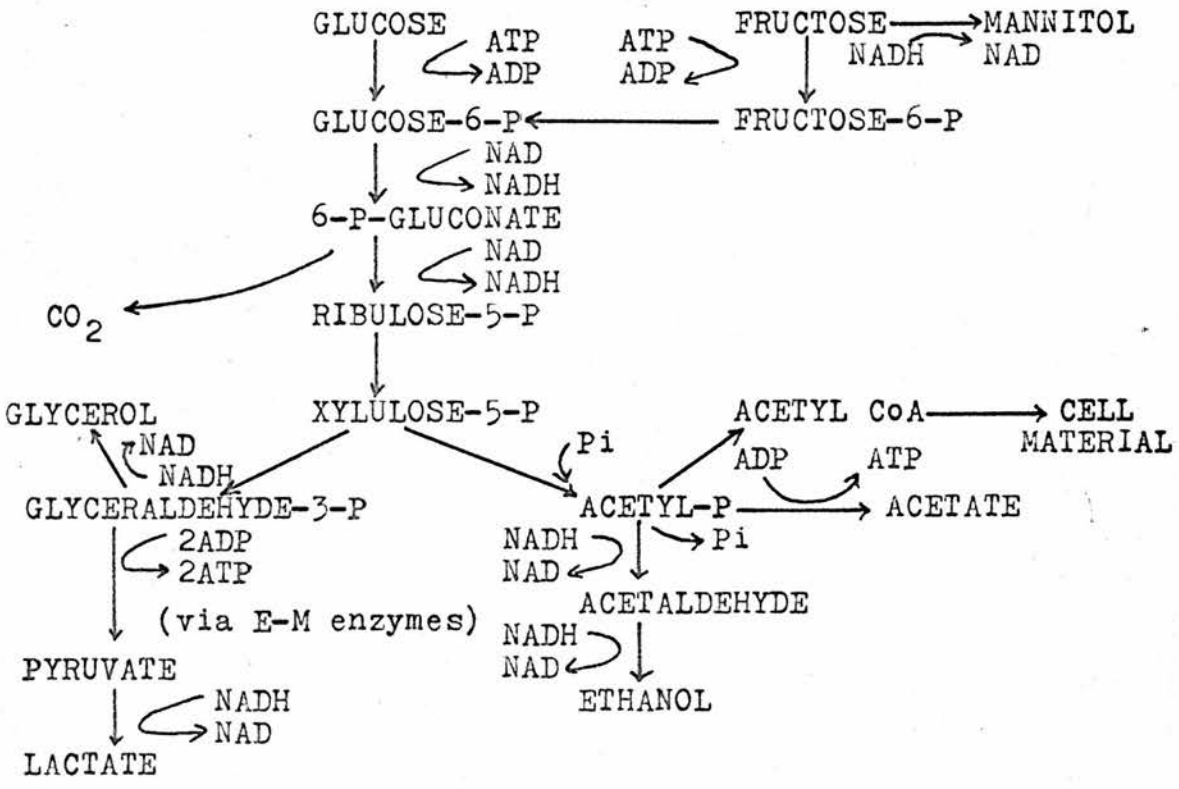
Biochemical reactions of lactic acid bacteria in silage

HOMOLACTIC FERMENTATION



GLUCOSE ⟶ 2 LACTATE (Gibbs, Dunrose, Bennett and Bubeck. J. biol. Chem. 184 545, (1950)).

HETEROLACTIC FERMENTATION.



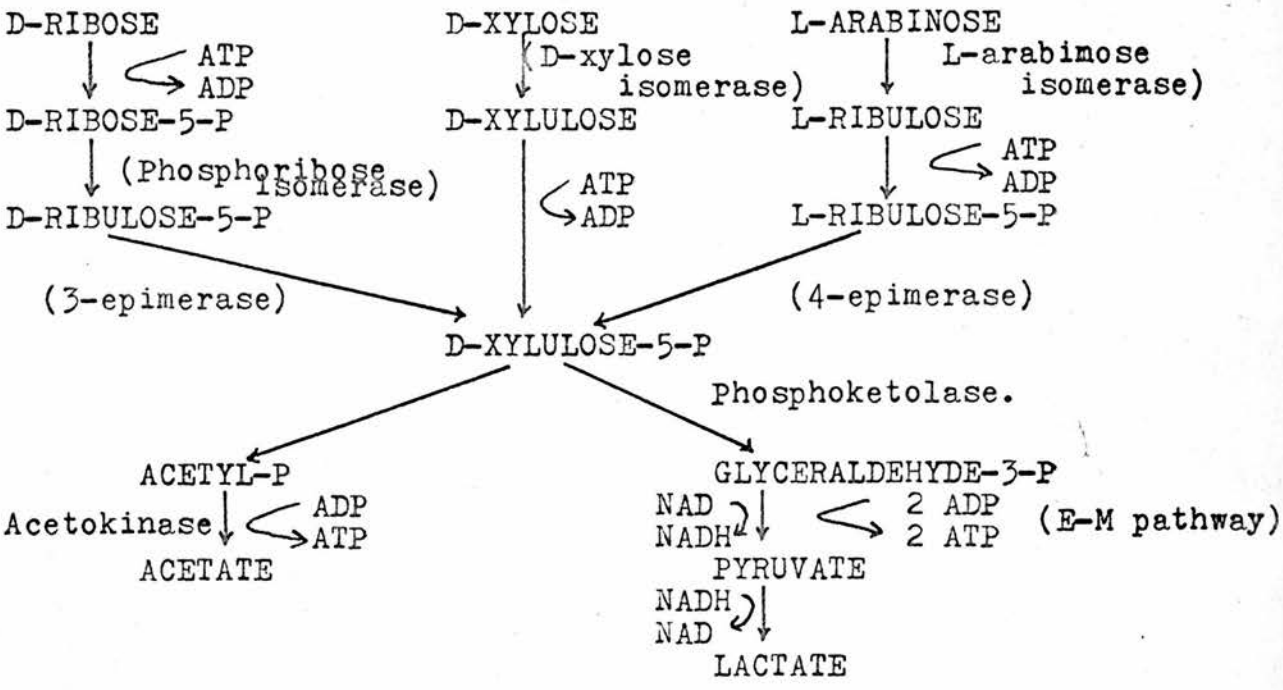
$GLUCOSE \longrightarrow ETHANOL + CO_2 + LACTATE$ — Heath et al. (J.biol. Chem. 231,1009(1958)).

$3\ GLUCOSE \rightarrow 4\ GLYCEROL + 2\ ACETATE + 2\ CO_2$ — Nelson and Werkman, (Iowa State Coll. J. Sci., 14, 359 (1940).

$2\ FRUCTOSE \longrightarrow 2\ MANNITOL$ — Eltz and Vandemark, (J.Bact. 79,763,(1959)).

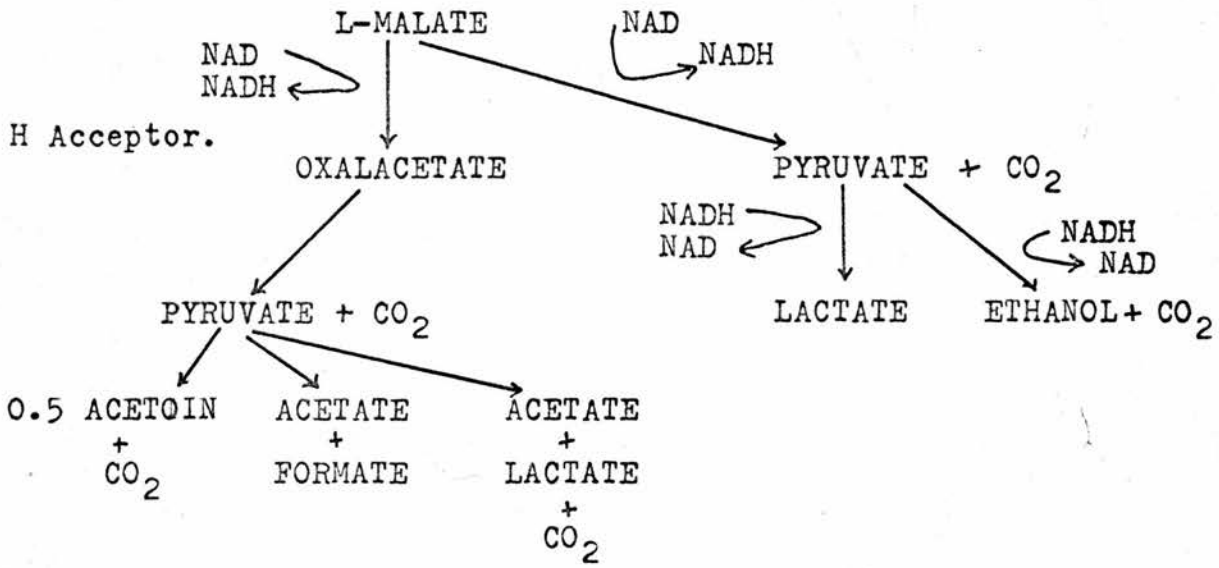
$FRUCTOSE \longrightarrow ACETATE + LACTATE + CO_2$ — and this Thesis.

PENTOSE FERMENTATION.



Heath et al.(J. biol. Chem., 231, 1009, (1958)).

MALATE FERMENTATION.

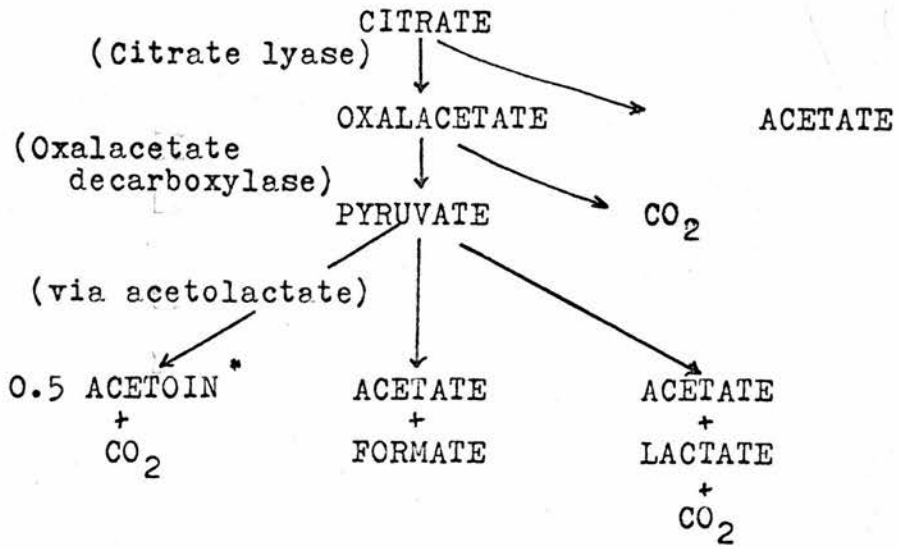


Lactate is the main product, with some ethanol and CO_2. Other products are only present when a hydrogen acceptor other than pyruvate from malate breakdown is available.

In Lactobacillus arabinosus (adaptively) Korkes and Ochoa J. biol. Chem. 176, 463. (1948).

Radler, Zbl. Bakt. II 120, 237. (1966) Malo-lactic fermentation in wines.

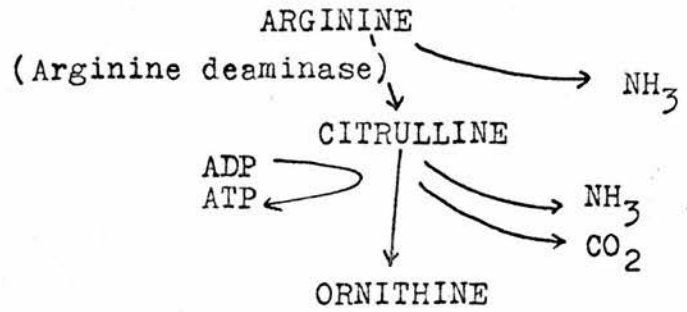
CITRATE FERMENTATION.



In Streptococcus faecalis. Campbell and Gunsalus (J.Bact 48, 71, (1944)) and Gunsalus and Campbell (J.Bact.48, 455, (1944)

* Acetoin production. Dolin and Gunsalus (J.Bact.62,199,(1951)). and Deibel (Bact. Rev. 28,330,(1964)).

ARGININE FERMENTATION.



In S. faecalis. Deibel (J.Bact. 87,988 (1964)).

Phosphorylation steps:-

$$\text{L-citrulline} + HPO_4^{=} \longrightarrow \text{L-Ornithine} + NH_2COOPO_3^{=}$$

$$NH_2COOPO_3^{=} + ADP \longrightarrow ATP + NH_3 + CO_2$$

SERINE FERMENTATION.

$$\text{SERINE} \xrightarrow{\text{(Serine dehydrase (or deaminase))}} \text{PYRUVATE} + NH_3$$

In S.faecalis. Deibel and Niven. (Bacteriol. Proc p164 (1960)).