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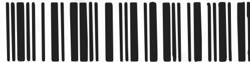
METABOLISM OF LACTIC ACID BACTERIA

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University, New Zealand.

Terence David THOMAS

1968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ABSTRACT

Streptococcus lactis organisms were grown in lactose-limited batch culture and the survival characteristics of washed organisms were examined at the growth temperature. Washed suspensions had high initial viabilities (>99%) which were maintained for varying periods depending on the presence of certain added materials in the buffer and the conditions of incubation. Added Mg^{2+} markedly prolonged survival, while high bacterial concentrations also extended survival times, probably because Mg^{2+} was excreted by the bacteria. Surviving organisms in some conditions showed prolonged division lags, especially in the absence of Mg^{2+}. Addition of trace amounts of EDTA decreased the death rate by removing toxic cation impurities, while the buffer salt concentration had little effect on survival within wide limits. The optimum pH value for survival was near 7.0 and survival times increased considerably at lower temperatures. Agitation and aeration tended to decrease survival and the death rate was not influenced by the phase of growth at which the organisms were harvested from a lactose-limiting medium.

Addition of casamino acids increased survival times markedly in the presence of Mg^{2+}; arginine was almost as effective as the complete mixture of amino acids while other individual amino acids tested gave only slight increases in survival times. Fermentable carbohydrates accelerated death of starved organisms irrespective of the growth phase from which they were harvested and of the limiting nutrient; the accelerated death rate was reduced by addition of Mg^{2+}. Glucose metabolism proceeded at a much faster rate than arginine metabolism, theoretically producing about 7.5 times as much ATP. This rapid generation of ATP may be responsible for the more rapid

death rates with added carbohydrates. Arginine substantially reduced the lethal effect of adverse pH values and suppressed the leakage of free intracellular amino acids into the external medium.

Survival studies were followed by an investigation of the changes which took place in starved organisms and their relation to survival. No polyglucose or poly-β-hydroxybutyrat was detected and starved organisms had a negligible respiration rate. Soluble protein was released from viable organisms into the suspending buffer and the intracellular free amino acid pool declined steadily with the components appearing in the suspending buffer; a net increase in the total amount of free amino acid indicated some protein hydrolysis. Chloramphenicol reduced the death rates in some environments, possibly by suppressing protein degradation. RNA was hydrolysed with the release of u.v.-absorbing bases and ribose from the organisms. Conditions which promoted rapid RNA breakdown also produced rapid death rates and long cell division lags in surviving organisms. There was no appreciable degradation of carbohydrate or DNA. After 28 hr. starvation in buffer containing Mg^{2+}, the bacterial dry wt. decreased by 26%; loss of RNA, protein and free amino acids accounted for 10.3%, 7.3% and 2.7% of the total bacterial mass loss. The products of polymer hydrolysis appeared to be released in an undegraded form into the external buffer and there was no appreciable formation of lactate, ammonia or volatile fatty acids possibly indicating the absence of any important endogenous energy sources.

Protein synthesis, determined by the incorporation of valine-^{14}C into TCA-insoluble material, was barely detectable when organisms were starved in buffer containing Mg^{2+}. Addition of an energy source allowed limited protein synthesis while glucose produced a much higher rate of valine-^{14}C uptake and incorporation than arginine.

Although arginine prolonged survival this was not due to the limited protein synthesis which took place. The survival capacity of starved organisms could be correlated with the ability to synthesize protein which in turn may be correlated with RNA stability.

A new method was developed for the assay of glycolytic activity in microorganisms. Organisms were incubated with glucose-U-^{14}C and samples removed at intervals. Samples were chromatographed on DEAE-cellulose paper strips in deionized water which separated the radioactive anionic products of glycolysis (lactate, acetate and formate) from the unfermented glucose. The activity of the two fractions was then determined by liquid scintillation counting. The glycolytic activity of starved organisms declined steadily and was not correlated with survival.

Phospholipid was broken down on prolonged starvation and the permeability properties of the organism were gradually lost. Addition of spermine gave enhanced survival and suppressed the release of u.v.-absorbing material. Lactic dehydrogenase and DNA were released as the death rate increased in buffer containing Mg^{2+} and eventually, well after death, cell lysis occurred. Electron micrographs indicated that addition of amino acids maintained cell structures for a much longer period and in this system cell lysis occurred as the death rate increased. It was concluded that the death rate of starved *S. lactis* organisms in phosphate buffer was partly dependent on the presence of Mg^{2+}, which probably acted by promoting polymer stability, particularly that of RNA. In this environment, a suitable exogenous energy source further enhanced survival which may ultimately be a function of cell wall and membrane stability.

ACKNOWLEDGEMENTS

The author is indebted to the New Zealand Dairy Research Institute for providing the opportunity to undertake this investigation.

Particular thanks are extended to:

Professor R.D. Batt and Dr. W.A. McGillivray for their advice and encouragement throughout the course of this work;

Dr. R.C. Lawrence and Mr. J.G. Robertson for helpful comments on the manuscript;

Mrs. P. Lyttleton and Mr. K.I. Williamson for preparing the electron micrographs;

Dr. C.R. Boswell for assisting with atomic absorption spectrometry measurements;

Mr. J.G. Dingle for assisting with amino acid analyses;

Mr. R.V. Toms for the reproduction of figures and plates;

Mrs. J. Crompton and Mrs. R.V. Haggett for typing the manuscript.

PREFACE

A major function of the Microbiology Department of the New Zealand Dairy Research Institute is the maintenance of active cultures of various lactic acid bacteria for use in the cheese industry. This work is vital for the efficient functioning of one of the country's major industries. The most important cheese 'starter' organisms are the lactic streptococci. This group consists of *Streptococcus lactis*, *Streptococcus cremoris* and variants. No detailed measurements have been reported on factors affecting the survival and activity of lactic streptococci, or closely related bacteria, at growth temperatures. Accordingly, it was decided to undertake such an investigation which could possibly produce results of considerable practical significance.

The Introduction is divided into two parts. Part I contains a literature review on the metabolism of lactic acid bacteria which provides a background for subsequent discussions. Part II contains a summary of the reports most relevant to studies on the survival of *S. lactis*.

Publications to date from results presented in this thesis are entitled:

'Survival of *Streptococcus lactis* in starvation conditions', *J. gen. Microbiol.*, *50*, 367, (1968),

'A new method for the assay of glycolytic activity with special reference to microorganisms', *Anal. Biochem.*, in Press.

CONTENTS

INTRODUCTION — Page

PART I. THE METABOLISM OF LACTIC ACID BACTERIA WITH SPECIAL REFERENCE TO STREPTOCOCCUS LACTIS — 1
- (i) General characteristics — 1
- (ii) Carbohydrate fermentation — 3
- (iii) Metabolism of non-carbohydrate compounds — 9
- (iv) Growth requirements — 11
- (v) Summary — 12

PART II. SURVIVAL OF VEGETATIVE MICROORGANISMS — 13
- (i) Definition of terms — 13
- (ii) Methodology — 13
- (iii) Results of viability measurements on microorganisms starved at the growth temperature under 'minimum-stress' conditions — 17
  - (a) Effect of ionic environment — 17
  - (b) Effect of exogenous substrates — 19
  - (c) Effect of bacterial density — 21
- (iv) Catabolism and turnover of cell constituents in viable, starved bacteria — 23
- (v) The role of endogenous metabolism in the survival of starved bacteria — 28

AIMS OF THE PRESENT INVESTIGATION — 35

METHODS — 37
- Microbiological procedures — 37
- Analytical methods — 45
- Glycolytic activity assay — 54
- Electron microscopy — 62

		Page
EXPERIMENTAL		64
PART I.	SURVIVAL OF <u>STREPTOCOCCUS LACTIS</u>	64
	Growth of the organism	64
	Bacterial numbers in resuspended systems	66
	Survival in resuspended systems	70
	Effect of: EDTA	70
	divalent metal ions	70
	bacterial concentration	71
	growth phase and media composition	72
	salt concentration	72
	pH value	73
	temperature	74
	atmosphere and agitation	75
	metabolic inhibitors	75
	added carbohydrates	77
	added amino acids and other growth medium components	77
	Growth characteristics of survivors	79
PART II.	CHANGES IN VIABLE ORGANISMS IN STARVATION CONDITIONS	79
	(i) Chemical studies	79
	'Reserve' polymers	79
	Oxygen uptake studies	80
	Changes in bacterial protein and total nitrogen	80
	Release of amino acids and ammonia	83
	Changes in nucleic acids	87
	Changes in carbohydrate	90
	Changes in lipids	90
	(ii) Protein synthesis in starved *S. lactis*	95
	(iii) Metabolism of arginine and glucose	100
	(iv) Changes in permeability and ultra-structure	102
	Measurement of bacterial lysis	102

Page

Effect of spermine 105
General ultrastructural features of the cell 105
Ultrastructural changes in starved cells 106

DISCUSSION 108
Survival of starved bacteria 108
Survival measurement 108
Effect of: Mg^{2+} 109
bacterial density 109
added substrates 111
temperature and pH 111
Changes in starved organisms 112
Polymer degradation 116
Metabolism of arginine and glucose 117
Changes in permeability and ultrastructure 121
Conclusions 122

REFERENCES 124

INTRODUCTION

PART I. THE METABOLISM OF LACTIC ACID BACTERIA WITH SPECIAL REFERENCE TO STREPTOCOCCUS LACTIS

i) GENERAL CHARACTERISTICS

The lactic acid bacteria are members of a single family, the *Lactobacillaceae*, the species of which ferment glucose with the predominant formation of lactic acid. The genus *Streptococcus* (Table 1) contains the largest and most varied group of lactic acid bacteria. The best known species in this genus are rather specialized ecologically as a result of their exacting nutritional requirements and strong fermentative metabolism. The natural habitat of *Streptococcus lactis* was thought to be on the surface of plants (Stark & Sherman, 1935), where the organism grows on secreted plant materials. Following the domestication of lactating animals, lactic streptococci have adapted to a milk environment and these organisms generally predominate in raw milk which has turned sour (Orla-Jensen, 1942).

Members of the family *Lactobacillaceae* are non-spore forming and with a few exceptions, they are non-motile. They are strongly Gram-positive in young cultures but often appear Gram-negative in old cultures. The organisms are generally catalase-negative (see Whittenbury, 1964) and microaerophilic or facultative aerobes. The lactic group of streptococci (Table 1) consist of closely related organisms. Strains of *S. lactis* grow mainly as diplococci or in short chains while those of *Streptococcus cremoris* tend to form long chains, the morphology being determined largely by growth conditions. Both organisms occur as ovoid cells elongated in the direction of the chain with individual cells 0.5 - 1.0μ in diameter. Most strains of *S. lactis* produce the antibiotic nisin which is considered

Table 1. Classification of lactic acid bacteria (Bergey, 1957).

Order Eubacteriales

Family Lactobacillaceae

Tribe I Streptococceae II Lactobacilleae

Peptostreptococcus

Leuconostoc

Lactobacillus etc

Pediococcus

Diplococcus

Streptococcus

pyogenic gp

viridans gp

enterococcus gp

lactic gp

lactis, cremoris
(diacetilactis, thermophilus)

by Hurst (1966, 1967) to be involved in cell regulation, particularly in the processes connected with the initiation and cessation of growth.

Many strains of lactic acid bacteria are of considerable industrial and agricultural importance, primarily because of their fermentative activities. Applications in which they are involved include (a) the preparation of a wide range of fermented milk products, (b) curing of meats, (c) manufacture of lactic acid and (d) the fermentation of vegetable and other plant products. The lactic streptococci are of particular importance in the manufacture of cheese because of their capacity for consistent, rapid, lactic acid production and their ability to impart desirable flavour and texture to the final product. Lactic acid bacteria are also involved in the spoilage of food products.

Few biochemical investigations have been undertaken with S. lactis (see reviews by Marth, 1962; Reiter & Moller-Madsen, 1963). This may be a result of the restricted nature of their metabolism and the difficulty in maintaining experimental cultures with reproducible activities. The bulk of the published work on lactic streptococci concerns microbiological aspects of their use as cheese 'starter' organisms. As a consequence of this emphasis, there are considerable gaps in our basic knowledge of their metabolism. The following discussion deals with aspects of the metabolism of some closely related species of microorganisms.

ii) CARBOHYDRATE FERMENTATION

The ability of lactic acid bacteria to ferment carbohydrates other than glucose varies widely and these differences are often used in microbial classification. Lactic streptococci can utilize glucose, galactose, lactose, mannose and fructose

as energy sources for growth (Sandine, Elliker & Hays, 1962). Many strains, especially of S. cremoris, are unable to ferment maltose, presumably because of their inability to induce the appropriate specific transport or degradative enzymes (Citti, Sandine & Elliker, 1967). The β-galactosidase of S. lactis has been shown to have similar properties to that of Escherichia coli (Citti, Sandine & Elliker, 1965). Fermentation of pentoses and other carbohydrates is restricted to only a few strains of lactic streptococci (Sandine et al., 1962). There are no reports of ribose fermentation by S. lactis.

Two metabolic groups were recognized among lactic acid bacteria by Orla-Jensen (1919). The homofermentative or homolactic group was characterized by the formation of mainly lactic acid from glucose fermentation. This group included all members of the genera Streptococcus, Diplococcus, Pediococcus and most members of the genus Lactobacillus. The second group, the heterofermentative lactic acid bacteria, produced considerable amounts of CO_2, ethanol or acetic acid, in addition to lactic acid (for reviews see Wiken, 1959; Wood, 1961; Reiter & Moller-Madsen, 1963). This group included all members of the genera Leuconostoc and Peptostreptococcus (Bergey, 1957).

Early investigations involving kinetic, inhibitor and enzyme studies, provided evidence that the pathway for homolactic glucose fermentation was essentially the same as that found in muscle glycolysis or in yeast fermentation. Isotopic studies with specifically labelled glucose-^{14}C (Gibbs, Dumrose, Bennett & Bubeck, 1950) confirmed the presence of the Embden-Meyerhof pathway of glycolysis in homofermentative lactic acid bacteria. These studies

demonstrated that the glucose molecule was split symetrically into two 3-carbon units, leading to a defined distribution of the glucose carbon among the carbon atoms of the fermentation products (Fig. 1). Gibbs *et al.*, (1950) demonstrated that under both anaerobic and aerobic conditions, *Lactobacillus casei* fermented glucose-1-^{14}C almost exclusively to methyl labelled lactate with a 50% dilution of the specific activity in the methyl group compared with that of carbon atom 1 of glucose. When glucose-3,4-^{14}C was fermented, carboxy-labelled lactate was formed without dilution of the specific activity. No fixation of labelled CO_2 occurred during fermentation. The distribution of carbon atoms in the final products is consistent with the operation of the Embden-Meyerhof glycolytic pathway (Fig. 1).

This pathway is assumed to operate in all homolactic bacteria, although it has been conclusively demonstrated in only a few species. Direct evidence for the involvement of this pathway in *S. lactis* is limited to brief reports of the presence of some of the glycolytic enzymes in cell-free extracts (Buyze, Hamer & Haan, 1957; Shahani, 1960; Shahani & Vakil, 1962). The latter authors have not reported any experimental details.

It is well recognized that products other than lactic acid may result from glucose fermentation by homolactic organisms, although these products are formed in small amounts only. Small quantities of formate, acetate, propionate, butyrate, ethanol, CO_2, 2,3-butanediol, acetoin, diacetyl and acetaldehyde have been reported as glucose fermentation products of such organisms (Hammer, 1920; Foster, 1921; Langwill, 1924; Platt & Foster, 1958; Harvey, 1960). Although none of these authors demonstrated that the end products actually arose from glucose, the oxidation-reduction indices and the carbon

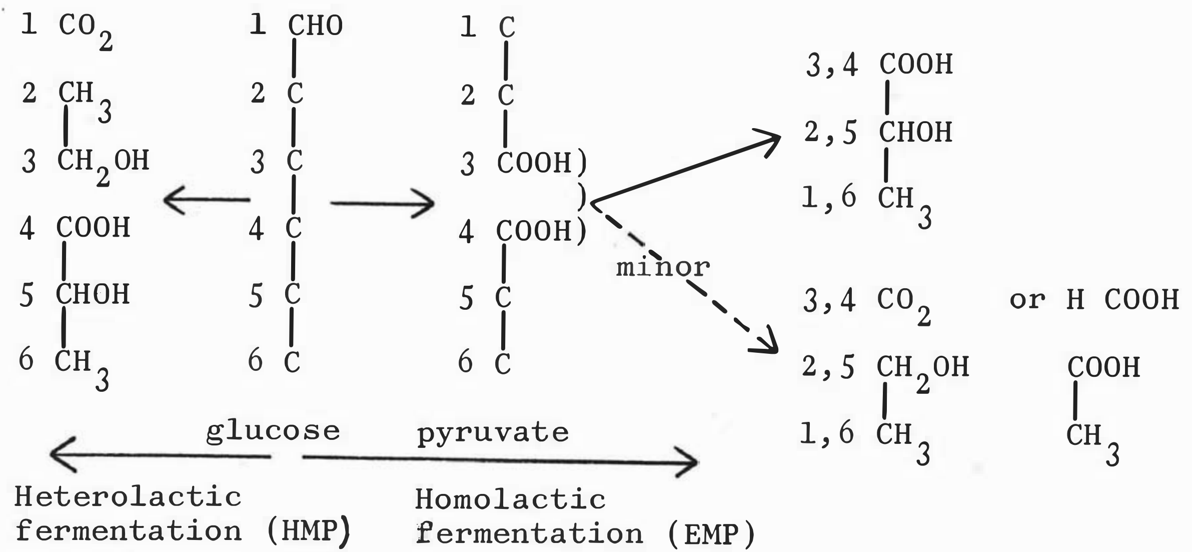


Fig. 1. Distribution of glucose carbon in the major fermentation products of lactic acid bacteria (Busse, 1966).

recoveries reported by Platt & Foster (1958) were so close to those expected theoretically, that it seems likely that the wide variety of end products were formed from glucose rather than from other carbon sources in the medium. Platt & Foster (1958) measured anaerobic glucose fermentation balances for seven typical homofermentative streptococci. The combined fermentation products other than lactate (acetate, formate, CO_2, ethanol, glycerol, diacetyl, acetoin and 2,3-butanediol) accounted for less than 18% of the recovered carbon in all cultures. For S. lactis and S. cremoris these products amounted to about 5% of the recovered carbon at pH 7.0. Platt & Foster (1958) suggested that these compounds were produced by glycolytic reactions but the equimolar relationship of one- and two-carbon products to be expected from pyruvate was not observed experimentally.

White & Sherman (1943) examined the effect of aeration on glucose fermentation by a range of streptococci. While little difference was observed between anaerobic and 'normal' aerobic conditions, vigorous aeration markedly inhibited growth and in all cases lactic acid was produced in reduced amounts. Gunsulas & Niven (1942) found that the production of formate, acetate and ethanol by Streptococcus liquefaciens was markedly increased at alkaline pH at the expense of lactate. By contrast, White, Steele & Pierce (1955) reported no marked change in the relative amounts of glucose fermentation products of Streptococcus pyogenes over the pH range 6.0-7.8. With this organism, galactose fermentation resulted in only 25-57% conversion to lactate, while glucose fermentation resulted in 90-95% conversion to lactate. According to Shahani & Vakil (1962), a transition of S. lactis from a homofermentative to a heterofermentative type as a result of changing glucose for another carbohydrate is a

characteristic finding. However, *S. lactis* is known to ferment galactose via the Embden-Meyerhof pathway (Kandler, 1961). Subsequently, Fukuyama & O'Kane (1962) demonstrated that *Streptococcus faecalis* also fermented galactose via the Embden-Meyerhof pathway, so presumably the alteration of end products occurs at the pyruvate level.

Various enzymes of the hexose monophosphate (HMP) pathway have been demonstrated in cell-free extracts of *S. lactis* by Buyze *et al*. (1957). Busse (1963) found that four strains of *S. lactis* produced much more $^{14}CO_2$ from glucose-1-^{14}C than from glucose-6-^{14}C, suggesting that as well as homolactic fermentation, some of the glucose was oxidized to gluconate and then decarboxylated to pentose (see review by Busse, 1966). It has also been suggested that *S. lactis* may be capable of fermenting glucose via the Entner-Doudoroff pathway (Reiter & Moller-Madsen, 1963). There is however, no experimental evidence for this type of metabolism and the Embden-Meyerhof glycolytic pathway is undoubtedly the quantitatively significant pathway in lactic streptococci.

Until 1950, the Embden-Meyerhof pathway was believed to be the only route for the dissimilation of carbohydrates. However DeMoss, Bard & Gunsalus (1951) found that *Leuconostoc mesenteroides* produced CO_2, ethanol and lactic acid in a constant ratio of 1:1:1 which is not consistent with the glycolytic pathway. Furthermore, it was found that the enzymes aldolase and triose phosphate isomerase were absent in *L. mesenteroides* and the ratio of products could not be varied significantly by changing the pH of the culture. Isotopic studies by Gunsalus & Gibbs (1952) confirmed the existence of an alternative pathway. Fermentation of glucose-1-^{14}C by *L. mesenteroides* resulted in all the isotope being found in the CO_2, whereas with

glucose-3,4-^{14}C, the isotope was contained in carbon 2 of the ethanol and the carboxyl carbon of the lactate (Fig. 1). These findings were later confirmed and extended to elucidate the complete HMP pathway for this organism (see review by Wood, 1961).

The release of glucose carbon 1 as CO_2 is characteristic of glucose fermentation by the HMP pathway (Gibbs, Sokatch & Gunsalus, 1955). By the assymetric breakdown of the glucose molecule, the various products of heterolactic fermentation are not formed by side reactions as was the case in the glycolytic pathway. Homolactic fermentation results in isotope dilution, while in heterolactic fermentation no dilution occurs (Fig. 1).

Presence of the Entner-Doudoroff pathway (Entner & Doudoroff, 1952), which includes the formation of 2-keto-3-deoxy-6-phosphogluconate from glucose, has been demonstrated in S. faecalis (Sokatch & Gunsalus, 1957) but this route appears to be of limited significance in lactic acid bacteria. Similarly, the fructose-6-phosphate pathway is not common in these organisms, although Lactobacillus bifidus ferments glucose to lactate and acetate in a ratio of about 1:2 (Scardovi, 1964; Scardovi & Trovatelli, 1965), as theoretically required by this route.

The ability of an organism to produce enzymes of the Embden-Meyerhof and HMP pathways has been suggested as a basis for the differentiation of the genus Streptococcus from Leuconostoc and for the separation of lactic acid bacteria into homo- and heterofermentative groups (Buyze et al., 1957). As a result of the enzymic assays of a large number of lactic acid bacteria, Buyze et al. (1957) concluded that three fermentative types of lactic acid

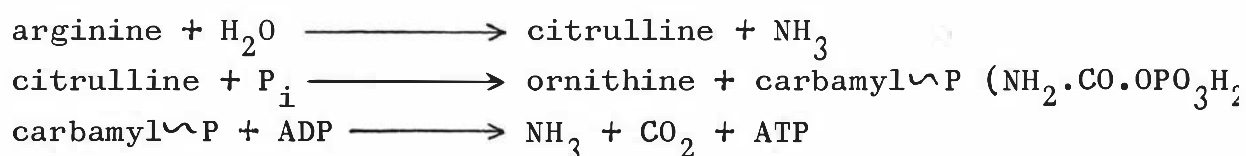
bacteria exist: (a) obligate homofermenters possessing aldolase but lacking dehydrogenases for glucose-6-phosphate and 6-phosphogluconate; (b) obligate heterofermenters possessing both these dehydrogenases but lacking aldolase; (c) facultative homofermenters possessing aldolase, as well as the C_6-dehydrogenases but generally metabolizing carbohydrate by means of the Embden-Meyerhof glycolytic pathway. *S. lactis* appears to belong to the last group, the total and relative amounts of by-products being determined by the structure of the carbohydrate, the strain and growth conditions of the organism, a number of environmental factors such as pH, the presence or absence of oxygen and CO_2, and inherent factors such as changes in the metabolic state of the organism during continued cultivation. From the information now available, it seems that the terms homo- and heterofermentative may have outlived their usefulness in describing the carbohydrate metabolism of lactic acid bacteria. A fuller description of enzyme activities and fermentation products would be required to provide a satisfactory grouping of the main types of lactic acid bacteria.

iii) METABOLISM OF NON-CARBOHYDRATE COMPOUNDS

Certain species of enterococci in the genus *Streptococcus*, are able to metabolize pyruvate, citrate, malate, glycerol or gluconate alone as sources of energy for anaerobic growth (see review by Deibel, 1964). Fermentation of citrate by lactic acid bacteria involves the splitting to acetate and oxalacetate, decarboxylation of oxalacetate to pyruvate and conversion of pyruvate to lactate or to acetoin and CO_2 (Kandler, 1961). *Streptococcus diacetilactis* was unable to use citrate as a source of energy for growth but the addition of citrate to a lactose containing medium increased the specific growth rate by 35% (Harvey & Collins, 1963). These authors suggested that

citrate fermentation provided a carbon source for essential cell constituents which were synthesized at a slower rate in the absence of citrate, all excess pyruvate being converted to acetoin. Citrate uptake by S. diacetilactis is enzymically mediated by an energy dependent, inducible transport system (Harvey & Collins, 1962), which is absent from the other lactic streptococci.

Apart from carbohydrate, arginine is the only substrate reported from which S. lactis can obtain energy (see Barker, 1961). The catabolism of arginine by S. lactis was first demonstrated by Niven, Smiley & Sherman (1942) and the enzymes catalysing these reactions were subsequently characterized by Korzenovsky & Werkman (1953, 1954). Arginine is converted to ornithine, ammonia and CO_2, probably by the following reactions (Barker, 1961):



arginine + H_2O ⟶ citrulline + NH_3
citrulline + P_i ⟶ ornithine + carbamyl∼P ($NH_2.CO.OPO_3H_2$
carbamyl∼P + ADP ⟶ NH_3 + CO_2 + ATP

Arginine is catabolized by the same pathway in S. faecalis but the energy produced did not permit growth in a defined medium (Bauchop & Elsden, 1960).

The production of carbonyls and other volatile compounds by lactic streptococci has received much attention in recent years because of the possible role of these compounds in flavour enhancement or flavour defects in dairy products (see reviews by Mabbitt, 1961; Marth, 1963; Vedamuthu, Sandine & Elliker, 1966a,b). The formation of some of these products from secondary reactions of pyruvate during carbohydrate or citrate fermentation has already been discussed. Certain strains of lactic streptococci can also produce volatile carbonyl compounds and fatty acids from some amino acids, although normally in trace amounts only (MacLeod & Morgan, 1955, 1956, 1958; Nakae

& Elliott, 1965a, b). For example, it has been reported that Streptococcus lactis var. maltigenes produces 3-methylbutanal, 2-methylbutanal, 2-methylpropanal, 3-methylthiopropanal and phenylacetaldehyde by transamination followed by decarboxylation of the α-keto acids formed from leucine, isoleucine, valine, methionine and phenylalanine respectively (MacLeod & Morgan, 1958).

There is no evidence for the operation of a tricarboxylic acid cycle in lactic acid bacteria or for the presence of cytochromes but several species can actively respire through a flavoprotein mediated hydrogen transport system (see reviews by Gunsalus, 1958; Dolin, 1961).

iv) GROWTH REQUIREMENTS

Some species of enterococci can grow with ammonium ion as the sole nitrogen source and a non-carbohydrate energy substrate (Diebel, 1964). However, all strains of lactic streptococci require for growth, a carbohydrate, amino acids, vitamins and inorganic salts (see review by Reiter & Moller-Madsen, 1963). Reiter & Oram (1962) used a synthetic medium in their studies and determined the essential growth requirements of S. lactis and S. cremoris by the omission of individual substances from the growth medium. The following amino acids were found to be (a) essential: valine, glutamic acid, methionine, leucine, isoleucine, histidine and arginine; (b) non-essential: aspartic acid and cystine; (c) occasionally required by some strains: alanine, phenylalanine, glycine, threonine and tryptophan. The requirement for proline, lysine, serine and tryosine by most strains of S. cremoris, helps to distinguish this organism from S. lactis. Essential vitamins for the growth of lactic streptococci were nicotinic acid, pantothenic acid and biotin; pyridoxal stimulated growth while some strains required riboflavin and thiamin. Vitamins or amino acids may be essential,

stimulatory or not required, depending on the extent of deficiencies or imbalances in the basal medium (see Reiter & Moller-Madsen, 1963). This nutritional data is consistent with earlier findings of Niven (1944) and Anderson & Elliker (1953).

Other growth factors have been shown to be essential or stimulatory for the growth of S. lactis under certain conditions. These include peptides (Garvie & Mabbitt, 1956), nucleic acid bases (Snell & Mitchell, 1941; Koburger, Speck & Aurand, 1963), acetate and oleate (Collins, Nelson & Parmelee, 1950) and α-lipoic acid (Reiter & Oram, 1962).

v) SUMMARY

S. lactis was shown to contain the enzymes aldolase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Buyze et al. 1957), indicating the presence of both the Embden-Meyerhof and the HMP pathways for carbohydrate fermentation. The end products of glucose fermentation by S. lactis were consistent with fermentation by the Embden-Meyerhof glycolytic pathway (Platt & Foster, 1958) and this is normally the main fermentation route. However, variation in carbohydrate structure and environmental conditions may vary fermentation patterns. All strains of S. lactis are capable of fermenting glucose, galactose, lactose, mannose, fructose and maltose (Sandine et al., 1962). There are no reports of ribose fermentation by S. lactis. Non-carbohydrate compounds do not appear to be fermented to any appreciable extent, with the exception of arginine. Arginine catabolism by S. lactis produces adenosine triphosphate (ATP) and the enzymes involved have been characterized (Korzenovsky & Werkman, 1953, 1954).

The literature contains no detailed reports on the chemical composition or structure of S. lactis organisms

and there are no indications of the presence of any 'storage' polymers, such as polysaccharide.

The industrial importance of S. lactis has led to several survival studies in milk cultures at low temperatures (Gibson, Landerkin & Morse 1965, 1966; Lamprech & Foster, 1963; Cowell, Köburger & Weese, 1966). However, there are no reports of survival studies at growth temperatures.

PART II. SURVIVAL OF VEGETATIVE MICROORGANISMS

i) DEFINITION OF TERMS

Viability of a microbial population refers to the proportion of the organisms capable of multiplication when provided with optimal growth conditions (Postgate, 1967). Therefore, viability is normally assessed by growth measurements and generally the survival potential of a population must be defined experimentally (Dawes & Ribbons, 1964). A dead organism may not necessarily be metabolically inert. For example, it has been claimed that some bacteria retain functional osmotic barriers after death (Postgate & Hunter, 1962). Some authors have used the terms ageing or senescence in connection with studies on the activities of starving microbial populations. However, the extrapolation of these concepts to unicellular microorganisms, in the sense normally applied to higher organisms, has been questioned by some workers (see Clifton, 1966; Postgate, 1967).

ii) METHODOLOGY

Many investigations of microbial survival under conditions of stress have been reported. These stresses include desiccation, freezing, heating, exposure to adverse pHs, osmotic pressure, radiation, light and

toxic materials. It may be considered that starving populations are subjected to the 'primary' stress of nutrient deficiency when they are incubated at normal growth temperatures. Starvation environments without other 'secondary' stresses maybe difficult, if not impossible, to achieve in practice (Postgate, 1967). Ideally, organisms should be removed from a growth medium without centrifugal damage, washed and re-suspended in non-nutrient buffer solution adjusted to the optimal survival pH and ionic concentration. The buffer should contain stabilizing metal ions plus a chelating agent to remove any toxic metal ions present. There should also be minimal change in pH, buffer composition, aeration or temperature during this process, since there is considerable evidence that starving organisms become hypersensitive to secondary stresses (Postgate, 1967) and many bacterial species appear to develop increased nutritional requirements under stress. Postgate & Hunter (1962) demonstrated that buffer solutions prepared from the purest available materials often contained impurities at a toxic level. Contaminant copper was identified and its effect neutralized with ethylenediaminetetra-acetate (EDTA). Garvie (1955) found that E. coli multiplied on contaminant nutrients in buffer solution prepared from specially purified salts dissolved in distilled water. Similarly, Sobek, Charba & Foust (1966) reported growth of 'starved' Azotobacter agilis in tris buffer suspensions. Many workers have routinely subjected organisms to low temperatures or distilled water washes during harvesting procedures (Burleigh & Dawes, 1967; Dawes & Ribbons, 1965; Clifton & Sobek, 1961; McGrew & Mallette, 1962). These procedures are probably best avoided in view of the considerable sensitivity of many organisms to cold shock and osmotic shock (Strange & Dark, 1962; Postgate & Hunter, 1962). However, under certain conditions

water-washed suspensions of Aerobacter aerogenes were more resistant to starvation than bacteria washed with saline-phosphate buffer (Tempest & Strange, 1966).

Apart from the findings of Postgate, Strange and collaborators (see review by Postgate, 1967), the literature contains few precise measurements of bacterial survival under essentially 'stress-free' conditions. Earlier studies on microbial survival at growth temperatures in buffer solutions may have been complicated to an appreciable extent by the secondary stresses discussed previously, difficulties in counting, problems of cell-aggregation and 'cryptic growth'.

Available methods for the assessment of viability have been critically reviewed recently by Postgate (1967). The only method which is both convenient and unambiguous appears to be the micro-slide culture method of Postgate, Crumpton & Hunter (1961). This procedure gives accurate viabilities (% viable/total organisms) in the range 5-100% viability by short term incubation of sample populations on agar films followed by differential counting of viable and dead organisms using a phase contrast microscope. This method is most suitable for populations of unicellular, aerobic or facultative organisms with uniform division lag times. Gross inaccuracies arise with organisms which tend to aggregate (Burleigh & Dawes, 1967) or with filamentous or chain-forming organisms which tend to fragment. As clumping or fragmentation of starving organisms seems quite prevalent, their effect on viability data must be assessed in the interpretation of results. With populations having varying division lag times, care must be taken to avoid overgrowth of colonies. However, sufficient incubation time should be allowed to permit division of all survivors. As well as accurate viable counts, accurate total counts are also necessary to adequately describe survival characteristics of a starving population since lysis of dead organisms or

'cryptic growth' may obscure studies on the physiology of starvation.

'Cryptic growth' (Ryan, 1959; Harrison, 1960) is caused by nutrients leaking from dead or lysed organisms allowing growth of survivors without a net increase in bacterial mass; an increase in total bacterial numbers may result. Thus a new population may be formed so that results cannot be interpreted in terms of the original population. Cryptic growth is likely to be of particular importance with organisms which can utilize a wide range of substrates for energy, especially when these organisms are starved at high population densities or in the presence of an added energy source. The probable existence of cryptic growth in many published experiments involving starved suspensions undergoing 'substrate-accelerated death', or in 'maintenance energy' experiments, may invalidate many of the conclusions drawn by some workers (see review by Postgate, 1967).

The method of routine culture of an organism, the growth rate, medium composition and the growth phase at which organisms are harvested from batch culture, may have pronounced effects on the survival of bacteria (Strange, Dark & Ness, 1961; Postgate & Hunter, 1962). Little reliance can be placed on the survival measurements of workers who failed to take all these factors into account. Herbert (1961) and Neidhardt (1963) reviewed reports which illustrate how greatly the chemical composition of micro-organisms may vary in response to changes in the composition of the growth medium.

Such complications in making survival measurements stress the necessity for care in designing experiments in studies on the survival properties of microorganisms.

(iii) RESULTS OF VIABILITY MEASUREMENTS ON MICROORGANISMS STARVED AT THE GROWTH TEMPERATURE UNDER 'MINIMUM-STRESS' CONDITIONS

(a) Effect of ionic environment on survival

Early work on the survival of starved bacteria in aqueous suspensions has been discussed briefly by Postgate & Hunter (1962). Although some of this work is of doubtful significance because most of the complications mentioned in the previous section were not appreciated at the time, it was established that the presence of the cations Mg^{2+} and Ca^{2+}, and buffer solutions at certain pH values favoured survival of coliform bacteria. The work of Postgate & Hunter (1962) confirmed these findings and established the necessity for adding traces of EDTA to the buffer.

The absolute requirement for Mg^{2+} during bacterial growth (Webb, 1948, 1949, 1951) is understandable from its known functions as a co-factor in many enzymic reactions and its role in the stabilization of membranes and ribosomes. As well as being essential for growth, Mg^{2+} decreased the death rate of starved organisms in buffered suspensions; the mechanism of this protective action has not been clearly defined. Tempest & Strange (1966) suggested that most of the intracellular magnesium in bacteria was associated with ribosomes and up to 25% of the total magnesium may be loosely attached to the cell surface. Surface adsorbed Mg^{2+} was unaffected by washing with distilled water but was desorbed by washing with saline-phosphate solutions (Strange & Shon, 1964). Water-washed *A. aerogenes* organisms were more resistant to starvation and other stresses than saline-phosphate washed bacteria (Tempest & Strange, 1966) and this has been attributed to the retention of adsorbed Mg^{2+}. Thus the important functional role that surface adsorbed Mg^{2+} may have in bacteria should be considered when preparing

washed bacterial suspensions for studies of metabolic activity or survival.

Strange & Hunter (1967) considered that the decreased death rates of starved A. aerogenes and E. coli in the presence of exogenous Mg^{2+} may have been due to: (1) A stabilizing action on bacterial ribosomes as shown by the lower rates of death and RNA degradation during starvation of strains of these organisms at growth and higher temperatures. (Mg^{2+} is believed to be involved in at least three aspects of ribosome structure and function: (i) conformation of the RNA strucutre, (ii) association of RNA with protein and (iii) ribosomal aggregation (see Rodgers, 1966)). (2) An effect on bacterial metabolism indicated by the abolition of the lethal effect of carbon energy sources on starved bacteria. (3) A stabilizing effect on the permeability control mechanisms of bacteria which, in the absence of Mg^{2+}, are susceptible to cold shock. Although it has been shown that exogenous Mg^{2+} decreases the death rate of Gram-negative bacteria starved at the growth temperature, Burleigh & Dawes (1967) have found that added Mg^{2+} did not affect the death rate of the Gram-positive organism Sarcina lutea, despite the suppression of RNA degradation.

Further evidence for the importance of the ionic environment in bacterial survival is found in the considerable body of evidence showing that EDTA disorganizes the outer layer of bacterial cell walls, presumably by binding or extracting certain cations responsible for structural integrity; the process leading to the rapid death of sensitive organisms (see Gray & Wilkinson, 1965; Asbell & Eagon, 1966). Other ionic factors influencing the survival of microorganisms were discussed by Postgate (1962, 1967).

(b) Effect of exogenous substrates on survival

Postgate & Hunter (1962) reported that cells of *A. aerogenes* which had ceased growing because of glycerol exhaustion in the medium, died more rapidly when washed and starved in phosphate buffer containing glycerol than in phosphate buffer alone. This phenomenon was termed 'substrate-accelerated death' and has since been investigated intensively for *A. aerogenes* (see review by Postgate, 1967). The phenomenon was demonstrated with organisms from carbon-, nitrogen- and phosphate- limited media but not with organisms grown under limitations of sulphate or magnesium ions (Postgate & Hunter, 1964). A high degree of substrate specificity was suggested and it was shown that the addition of either Mg^{2+} or the 'uncoupling' agents 2,4-dinitrophenol and azide, abolished glycerol-accelerated death. The increased death rate was not accompanied by accelerated breakdown of the osmotic barrier or cell polymers and surviving organisms showed long division lags which were considered by Postgate & Hunter (1964) to indicate a slow reclamation of lost cell material or the recovery from repression of enzyme synthesis.

Strange & Dark (1965) repeated these experiments with the same strain of *A. aerogenes* and found that substrate-accelerated death was less general than claimed by Postgate & Hunter (1964) and was in fact restricted to a non-specific lethal effect of carbon energy sources metabolized by *A. aerogenes* in the absence of added Mg^{2+}. However, Strange & Hunter (1966) later withdrew this conclusion having succeeded in observing nitrogen-accelerated death. The discrepancies in these results from adjacent laboratories arose from the appearance of a variant organism which replaced the parent strain of *A. aerogenes* after prolonged continuous culture (Strange & Hunter, 1966). These experiments emphasize the possible dangers arising from mutation in the growth culture. Strange & Dark (1965) reported that the death rate increased with the rate of

substrate metabolism while exogenous Mg^{2+} decreased the rate of degradation of intracellular RNA without significantly affecting substrate metabolism.

The mechanism of substrate-accelerated death is not fully understood. However, Strange & Dark (1965) attributed glycerol-accelerated death, at least in part, to the formation of an unidentified toxic product of glycerol metabolism. It was suggested that added Mg^{2+} prevented the accumulation or formation of this product. During glucose-accelerated death the bacterial ATP pool increased significantly whereas in the presence of glucose plus Mg^{2+} there was little change (see discussion of paper by Strange & Hunter, 1967). It was suggested in this discussion that if all the bacterial adenosine diphosphate (ADP) was converted to ATP, then oxidative phosphorylation would be impeded and toxic hydrogen peroxide might accumulate. In this situation, Mg^{2+} could exert its protective effect by increasing ATP hydrolysis. Such an explanation would be consistent with the report that 2,4-dinitrophenol abolished glycerol-accelerated death (Postgate & Hunter, 1964). However, any proposed mechanism should be consistent with the fact that while exogenous glucose accelerates death, the death rate decreases in parallel with increasing intracellular polyglucose levels (Strange et al., 1961).

Present data does not establish whether accelerated death by inorganic substrates, such as phosphate and ammonium-ion, occurs by a mechanism similar to carbon substrate-accelerated death but Postgate & Hunter (1964) have pointed out that it is likely to be intimately involved in the control mechanisms governing the synthesis of constitutive material in the cell. Although a considerable amount of data has been published on substrate-accelerated death, it is not clear whether this phenomenon occurs with organisms harvested from the logarithmic growth phase in batch culture where there are no limiting nutrients. No bacterial lysis

or change in total cell numbers occurred during substrate-accelerated death and the good agreement between viability results by plate count and slide culture (Postgate & Hunter, 1964) was considered by these authors to preclude the possibility of biosynthesis taking place and giving rise to unbalanced growth (see discussion of paper by Strange & Hunter, 1967). Substrate-accelerated death may have some features in common with the 'suicidal' behaviour of certain microbial mutants (e.g. thymine-less E. coli, Barner & Cohen, 1956) which show accelerated death when deprived of an essential metabolite in an otherwise complete growth medium.

In contrast to the effect of carbon substrates on starving organisms discussed above, there are several recent reports of decreased death rates resulting from the repeated addition of small amounts of glucose to starving populations of E. coli (Clifton, 1966; McGrew & Mallette, 1962, 1965; Mallette, 1963). However, the evaluation of these results is difficult since the experimental design did not preclude regrowth (see previous discussion).

Postgate & Hunter (1962) found that the addition of amino acids and vitamins to starving populations had no effect on survival and the protective effect of the basal medium, without a carbon source, was completely attributable to its trace element content. There appear to have been no unequivocal reports of decreased bacterial death rates in the presence of an energy source without concomitant growth, although the experiments of Tempest, Herbert & Phipps (1967) involving growth in chemostats at low dilution rates would indicate that such a situation is possible.

(c) Effect of bacterial density on survival

A bacterial density effect on survival was first investigated by Harrison (1960). Dense populations were

found to have lower death rates than sparse populations although an optimum density was claimed for survival above which the death rate increased. These findings were later confirmed by Postgate & Hunter (1963) who demonstrated that the population density effect was not an experimental artifact. However, the 'reversed' death rate at high bacterial concentrations reported by Harrison (1960), was not observed. Although limited cryptic growth occurred in Harrison's high density populations it was considered that this could not have contributed significantly to the decrease in death rate.

The intrinsic factors involved in the population density effect have not been resolved. Harrison (1960) suggested that starving organisms release substances which, if they reach a critical concentration, may be taken up by surviving organisms and permit them to maintain viability for a longer time. It was concluded that the secreted material responsible provided an energy source for maintenance.

Starving Gram-negative organisms are known to degrade intracellular RNA at rapid rates, especially in the absence of exogenous Mg^{2+}, so that the magnesium associated with ribosomes is released. This fact, together with the observations that population density effects occur in freezing damage (Harrison, 1955), cold shock (Strange & Dark, 1962), thermal death (Strange & Shon, 1964) and substrate-accelerated death (Postgate & Hunter, 1964), where exogenous Mg^{2+} is known to be protective, suggests that the secreted material responsible for the population density effect may be Mg^{2+}. However, Postgate & Hunter (1963) still observed a population density effect in the presence of optimal exogenous Mg^{2+} and could not detect Mg^{2+} in the filtrates from dying populations.

(iv) CATABOLISM AND TURNOVER OF CELL CONSTITUENTS IN VIABLE, STARVED BACTERIA

The progressive loss of cell constituents which generally occurs when bacteria are starved in buffer at the growth temperature, is normally a result of an imbalance in the total anabolic and catabolic reactions. It may also result from other processes such as the leakage or secretion of cell components and intracellular pools. Limited resynthesis or turnover of cell polymers may occur but the net metabolism is catabolic and must eventually result in death of the organism (Strange, 1967). The overall metabolic activities of starved bacteria are normally referred to as their 'endogenous' metabolism which, according to Powell (1967), is effectively a measure of the rate at which bacteria breakdown their own mass. This field has been extensively investigated with aerobic organisms and published work has been reviewed in a symposium (Lamanna, 1963) and by Dawes & Ribbons (1962, 1964). The functions of endogenous metabolism, as envisaged by Dawes & Ribbons (1962), include the provision of energy for turnover of protein and nucleic acids, osmotic regulation, pH control and the supply of suitable substrates for the resynthesis of essential bacterial constituents. Components reported to be degraded in starved organisms include carbohydrate, RNA, protein, free amino acids, peptides, lipids and certain specialized 'reserve' materials. Cellular constituents which have not been implicated as substrates for endogenous metabolism include DNA, certain cell wall and membrane polymers (Dawes & Ribbons, 1964). The rates and orders of substrate degradation and the general pattern of endogenous metabolism vary (1) in different organisms, (2) with the growth conditions and hence the chemical and physiological state of the organism and (3) with the physico-chemical conditions of the starvation environment (Strange, 1967).

In certain nutrient conditions, many bacteria accumulate relatively large amounts of polymers which have been considered as specialized carbon and/or energy-stores, analogous to the lipid and glycogen reserves in animals (see reviews by Wilkinson, 1959; Wilkinson & Duguid, 1960; Duguid & Wilkinson, 1961; Neidhardt, 1963). The principal compounds which have been implicated as carbon and energy reserves in bacteria are intracellular polysaccharides, in particular glycogen, and intracellular lipids, especially poly-β-hydroxybutyrate (PHB). These two polymers have no other known function in bacteria (Wilkinson, 1959). Capsular and cell wall polysaccharides do not normally act as energy stores according to Wilkinson (1958) and Duguid & Wilkinson (1961). Polyphosphate has been considered to act as a phosphorus and/or energy reserve (see Duguid & Wilkinson, 1961), although its energy reserve function in a strain of A. aerogenes has been questioned (Harold & Sylvan, 1963).

Before a particular substance can be considered to have a specialized reserve function, certain criteria must be met (Wilkinson, 1959; Wilkinson & Munro, 1967). These authors considered that the reserve must accumulate in conditions where the supply of the necessary components for its synthesis is in excess of growth demands. They also suggested that the reserve must be metabolized when exogenous substrates can no longer support growth, yielding energy and intermediates that can be utilized by the cell for 'maintenance'. Wilkinson (1959) considered it probable that the glycogen accumulated by E. coli (Holme & Palmstierna, 1956b) and the PHB in Bacillus megaterium (Macrae & Wilkinson, 1958) fulfilled these criteria. Accumulation and utilization of the reserve in the natural environment of the organism was also considered essential. However, this criterion cannot normally be established because of the difficulty in reproducing the natural environment of most bacteria.

The growth conditions necessary for the accumulation of glycogen in E. coli (Holme & Palmstierna, 1956a,b; Ribbons

& Dawes, 1963) and of PHB in B. megaterium (Macrae & Wilkinson, 1958; Wilkinson & Munro, 1967), have been extensively investigated. In either batch or continuous culture systems, nitrogen-limiting growth conditions or carbon source excess, were either essential or stimulatory for the accumulation of these reserves. Synthesis of these storage compounds can therefore take place independent of growth. As well as degrading their reserves on starvation, bacteria may also degrade other cell constituents, sometimes after the reserve is exhausted (Dawes & Ribbons, 1965). However, preferential utilization of reserves has only been reported for some species. Some bacterial species cannot accumulate reserves but this does not imply that these organisms are incapable of endogenous respiration (see Campbell, Gronlund & Duncan, 1963). When such bacteria are starved, the degradation of 'basal' materials such as RNA, protein and free amino acids may occur (see Dawes, 1967).

At rapid growth rates the nucleic acids of E. coli are stable, whereas in starving organisms only DNA is stable and RNA undergoes considerable degradation (Mandelstam, 1960). The degradation of RNA appears to be widespread in starved bacteria and has been demonstrated in L. casei (Holden, 1958), A. aerogenes (Strange et al., 1961), E. coli (Dawes & Ribbons, 1965), Sarcina lutea (Burleigh & Dawes, 1967) and Pseudomonas aeruginosa (Gronlund & Campbell, 1963). The ribose portion of the RNA was generally oxidized and the nitrogen normally appeared in the suspending buffer as ammonia and free bases. Wade (1961) demonstrated two possible routes of enzymic breakdown of RNA depending on the presence or absence of magnesium.

The protein fraction of E. coli is also stable at rapid growth rates according to Mandelstam (1960) but net degradation may occur in starvation conditions (Strange et al. 1961; Postgate & Hunter, 1962; Dawes & Ribbons, 1965). Loss of protein from all the ultra-centrifugal fractions of starved

A. aerogenes occurred (Strange, Wade & Ness, 1963) while Strange (1966) presented evidence that adaptively formed β-galactosidase in E. coli was degraded at a higher rate on starvation than the overall rate for cell protein; this indicates that some proteins are less resistant to degradation than others. The products of protein catabolism which appeared in the suspending buffer, were normally CO_2 and ammonia with only trace amounts of amino acids. Peptone-grown S. lutea degraded RNA and intracellular free amino acids when starved, while protein, polysaccharide and DNA were stable in this situation (Burleigh & Dawes, 1967).

The free amino acid and peptide pool in peptone-grown S. lutea was considered by Dawes & Holms (1958) to constitute the main endogenous substrate. The pool was reduced to a low level on starvation and was not replenished from cell protein. Glutamate accounted for 20% of the free amino acid pool and significant depletion of the pool could not be attributed to the secretion or leakage of amino acids. Although polyglucose is accumulated by glucose-peptone grown S. lutea organisms, this compound does not exert the same sparing action on nitrogenous substrates (Ribbons & Dawes, 1963) as it does in starved E. coli (Dawes & Ribbons, 1965).

Early reports by Stephenson & Whetham (1922) indicated that growing Mycobacterium plei accumulated large quantities of lipid, especially in the presence of acetate and it was suggested that 'neutral' lipid was degraded in starved organisms. However, the relatively crude extraction, identification and assay techniques make this data difficult to evaluate. No reports of endogenous glyceride or fatty acid catabolism by starving bacteria have since been published and an energy storage function for triglyceride in bacteria has not been established (see Robertson, 1968).

When bacteria are starved of a nitrogen source, net

protein and RNA synthesis stops but protein and RNA turnover may continue for several hours. Degradation of pre-existing protein and RNA provides amino acids and bases for resynthesis (see reviews by Mandelstam, 1960, 1963). During starvation of E. coli at the growth temperature, degradation of protein occurred at a rate of about 5%/hr. for a least 4hr. This was balanced by an equal rate of resynthesis resulting in no net protein loss in 4hr. (Mandelstam, 1957, 1958). Bacterial lysis, as measured by the release of β-galactosidase into the suspending buffer, was not appreciable in these experiments suggesting that only intracellular protein turnover occurred. Subsequently, Levine (1965) demonstrated that intercellular protein turnover occurred at a lower rate of 0.16-0.18%/hr. in starved E. coli. Not all the proteins of nitrogen-starved E. coli are subject to degradation at equal rates (Willetts, 1967). Ribosomal protein and RNA degradation occurred at similar rates (Mandelstam & Halvorson, 1960) and Mandelstam (1963) considered it probable that the starvation conditions which caused protein breakdown also caused RNA breakdown. Protein turnover was not measured over extended starvation periods by Mandelstam, but Schlessinger & Ben-Hamida (1966) reported declining, although significant protein turnover in nitrogen-starved E. coli for at least 20hr. Ben-Hamida & Schlessinger (1966) recorded a much lower rate for RNA turnover in nitrogen-starved E. coli. These authors concluded that ribosomes are not resynthesized and that the net effect of the turnover process was to transfer amino acids and nucleotides from a metabolically useless excess of ribosomes to the soluble proteins, energy supply and reserves required for subsequent adaptation.

The protein turnover rates (5%/hr.) reported by Mandelstam (1957, 1958) were considerably greater than those measured by Dawes & Ribbons (1965) in starved E. coli (0.6%/hr.). Dawes & Ribbons (1965) suggested that the difference may reflect a greater capacity for turnover in Mandelstam's

medium which was complete except for a nitrogen source, as opposed to starvation in their simple salt medium. Protein turnover occurred in starved glucose-grown E. coli containing glycogen but when all of the cellular glycogen had been used, ammonia was released which was considered by Dawes & Ribbons (1965) to indicate net protein degradation. Tryptone-grown E. coli was devoid of glycogen and net protein degradation occurred from the beginning of the starvation period (Ribbons & Dawes, 1963). These findings may be compared with the observations of Duncan & Campbell (1962), who found that starved P. aeruginosa released ammonia which was reincorporated on addition of exogenous glucose. Similarly, Clifton (1966) reported reincorporation of ammonia by starved E. coli when glucose was added and the higher viabilities with added glucose may indicate that resynthesis favours survival.

It is now clear that while protein degradation occurs at similar rates in both nitrogen- and carbon-limited media (Willetts, 1967), the rate of protein resynthesis is very dependent on the presence of a carbon source (Schlessinger & Ben-Hamida, 1966).

(v) THE ROLE OF ENDOGENOUS METABOLISM IN THE SURVIVAL OF STARVED BACTERIA

Many of the published papers on the endogenous metabolism of bacteria do not include viability measurements so that correlations with survival are not possible.

Bacteria starved in buffer at their optimum growth temperature may survive for hours or days but prolonged starvation ultimately leads to death (Strange, 1967). The reasons for bacterial death in starvation environments are probably impossible to define but some authors have claimed that certain interpretations can be excluded. For instance, starved A. aerogenes was considered to maintain functional membranes after death (Postgate & Hunter, 1962).

McGrew & Mallette (1962) claimed that the continuous addition of small amounts of glucose to starved *E. coli* sustained viability for several days without concurrent increase in cell mass or numbers. They reported that extrapolation of the curve of turbidity increment versus concentration of exogenous energy source to zero growth, gave a reproducible intercept at a definite positive level of energy source. This finding provided evidence for an 'energy of maintenance' for the survival of starving bacteria. These and other attempts to demonstrate and measure the bacterial maintenance requirement (Mallette, 1963; Marr, Nilson & Clark 1963; see review by Dawes & Ribbons, 1964) have since been widely criticized on the grounds that these results could be interpreted in terms of cryptic growth (see Dawes & Ribbons, 1964; Clifton, 1966; Postgate, 1967). However, if cell constituents are turning over and the membranes remain functional, then energy is required (see Dawes & Ribbons, 1964). While the presence of a suitable exogenous energy source may sustain viability for a limited period without growth (Clifton, 1966), there are no reports of starving, vegetative cells which survive indefinitely.

Postgate & Hunter (1962) grew *A. aerogenes* in a chemostat at decreasing dilution rates until dead organisms made a contribution to the steady state population. Over this range there was a progressive increase in doubling time and decrease in bacterial yield as measured by mass increase/glycerol oxidized (see Tempest *et al.*, 1967). This result may be interpreted in terms of a maintenance requirement. With carbon-, sulphate- or magnesium-limited chemostat cultures, Postgate & Hunter (1962) were unable to provide *A. aerogenes* organisms with just sufficient nutrient to maintain themselves indefinitely without dividing and they concluded that these bacteria were obliged to multiply or die. Essentially similar results were obtained by Tempest *et al.*, (1967) with nitrogen-limited *A. aerogenes*.

It has been suggested that the metabolism of endogenous substrates by starving bacteria is necessary for their survival, so that bacteria which contain large amounts of 'storage polymers' may have an advantage for survival (see Lamanna & Mallette, 1956; Duguid & Wilkinson, 1961). Although energy yielding reactions may occur in starved bacteria and are essential for survival, the amount of energy required for survival has not been established. For many microorganisms, indications are that if an energy source is available, then it is metabolized at a rate in excess of that which might be expected for maintenance requirements. This initial rapid utilization of reserves by some bacteria suggests that they may not have a maintenance role (see Dawes & Ribbons, 1964; Strange, 1967). The glycogen in glucose-grown E. coli, which amounted to as much as 23% of the cellular dry weight, was completely oxidized in 1-3hr. yet viability remained unchanged for the first 12hr. of starvation (Dawes & Ribbons, 1963, 1965).

A correlation between the presence of a reserve material and enhanced capacity for survival has only been found with some of the organisms studied. Glycogen-rich A. aerogenes survive better than the corresponding glycogen deficient organisms (Strange et al. 1961), whereas glycogen-rich S. lutea die more rapidly (Burleigh & Dawes, 1967). Glycogen metabolism suppressed the release of ammonia from starved E. coli and A. aerogenes but not from starved S. lutea. Dawes & Ribbons (1963) suggested that possession of glycogen was not an aid in the survival of E. coli (see review by Dawes & Ribbons, 1964). Survival of Micrococcus halodenitrificans and A. agilis appears to be related to the initial PHB content of the cells (Sierra & Gibbons, 1962; Sobek et al., 1966). In starved organisms with high PHB levels, the endogenous respiration rate and viability remained unaltered for 96hr. (Sierra & Gibbons, 1962); the constant rate of degradation perhaps indicating some degree of efficiency. When the PHB

level fell to a critical value, the respiration rate and viability fell rapidly. Similar organisms with low initial PHB content showed a rapid decline in respiration rate and viability from the onset of starvation. The presence of PHB was considered to exert a sparing effect on cell protein (Sobek et al., 1966). It would appear that organisms with low respiration rates may be better equipped for survival. However, there is no evidence to suggest that vegetative bacteria are capable of adapting their metabolism to starvation conditions.

Profound changes in the composition of starved bacteria without concurrent viability loss are well documented. Up to 25% of the total cell protein was catabolized before significant death of A. aerogenes occurred (Strange et al., 1961). It appears that RNA is to some extent expendable in that up to 50% of the ribosomal RNA of some organisms can be metabolized without death taking place. Within a single species, fast growing organisms contain much more RNA (Schaetchter, Maaloe & Kjeldgaard, 1958; Herbert, 1958; Tempest et al., 1967) and die more slowly than slow growing organisms (Postgate & Hunter, 1962). Although many conditions which delay RNA degradation, such as the presence of Mg^{2+} (Strange & Hunter, 1967) or D_2O (Lovett, 1964), also delay death, no absolute correlation between RNA degradation and death rate has been established (see Postgate & Hunter, 1963; Burleigh & Dawes, 1967).

When the DNA of E. coli was specifically labelled with tritium, the organisms died more rapidly after a period of cold storage than organisms similarly labelled in their RNA and protein (Rachmeler & Pardee, 1963). The loss of viability was attributed to radiation damage to the DNA, indicating that intact DNA is required for bacterial viability and enzyme synthesis.

Endogenous metabolism results in ATP generation but a correlation between the ATP content of starved A. aerogenes organisms and their survival properties could not be shown (Strange, Wade & Dark, 1963). It was suggested that survival may be influenced by the total amount of AMP, ADP and ATP present in the organisms, and also by their ability to form ATP when required. Strange (1961) concluded that the loss in ability of starved A. aerogenes to synthesize β-galactosidase was due to shortage of intermediates such as amino acids, and not ATP. It is possible to define energy requiring processes in starving bacteria (protein and RNA turnover, maintenance of concentration gradients across membranes, pH control, etc.,) but the control and economy of these mechanisms are ill-defined (see Dawes & Ribbons, 1962, 1964; Strange, 1967).

In the past (see Lamanna & Mallette, 1956), it was thought that respiration was necessary to sustain viability of aerobes. However, it is now well known that while many starving organisms exhibit high initial O_2- uptakes, the O_2- uptake may fall to a negligible level where significant decline in viability may not occur for a considerable period (see Burleigh & Dawes, 1967). This suggests that the amount of energy required for survival may be very small.

Glycerol dehydrogenase activity and the respiration rate with glycerol as substrate, declined in parallel with the viability of starved A. aerogenes which were grown in a glycerol-limited chemostat (Postgate & Hunter, 1962). Burleigh & Dawes (1967) concluded that the survival of peptone-grown S. lutea in aerobic starvation conditions could be correlated with the ability to oxidize exogenous glucose and L-glutamate. These results suggest that the activity of the enzymes catabolizing the energy source may be critical for survival. Burleigh & Dawes (1967) suggested that death of S. lutea was probably caused by relatively non-specific inactivation of enzymes, although it was not possible to attribute death to the degradation or inactivation of any single cell constituent.

Turnover may represent a mechanism whereby cell constituents, whose loss is particularly likely to result in death, may be selectively reformed from dispensible material. Schaechter (1961) removed organisms at intervals from a synchronized growth culture of Salmonella typhimurium and transferred them after washing, to a non-nutrient phosphate buffer. Each group of organisms proceeded to divide in the buffer at the same time as they would have divided in the growth medium. No bacterial mass increase occurred, so presumably extensive turnover was involved (see also Dean, 1967).

In experiments on the slow growth of A. aerogenes in a chemostat, attempts to demonstrate what Postgate & Hunter (1962) termed 'inheritance of longevity' were unsuccessful. However, Harrison & Lawrence (1963) demonstrated that 'starvation-resistant' mutants could be obtained from batch culture populations of A. aerogenes. It would appear that the outstanding difference between these mutants and the wild type was a slower exponential growth rate.

From a consideration of the growth rates of glycerol-limited A. aerogenes at decreasing dilution rates, Tempest et al. (1967) concluded that there is a finite, temperature dependent minimum growth rate for bacteria. As the dilution rate was decreased, the doubling time tended to a maximum and the 'steady state' culture viability progressively diminished. In natural environments such as seas, lakes, rivers and soil, the concentration of substrates is generally low and possibly nitrogen-limiting. The viable bacteria in such environments must be able to grow at very low generation rates and it seems possible that the ability to grow very slowly facilitates survival (see Elsden, 1967). Other factors favouring survival in natural environments probably include the ability to form spores, metabolic versatility and the ability to withstand such stresses as temperature shock and desiccation. In these habitats, organisms may be subjected to greater stress

than in a constant experimental environment but despite these stresses, many organisms must survive for very long periods. Whereas experimental environments probably permit maximum rates of metabolic activity (i.e. organisms at the optimum growth temperature in aqueous buffer) and hence maximum rates of breakdown and death, such factors as lower temperatures or partial desiccation may restrict metabolic activity and possibly death rates, in natural habitats.

The faster A. aerogenes organisms grow, the slower they die (Postgate & Hunter, 1962). Maximum growth rates produce organisms of maximum size and RNA content and the greater ability of such organisms to maintain viability may be a result of an excess of some components in the organism. Then the levels of components essential for viability may have further to fall before death. Organisms growing at the minimum rate for complete viability are comparatively small, they have greatly reduced RNA and DNA levels and they die very rapidly on complete starvation (Postgate & Hunter, 1962; Tempest et al., 1967). These organisms appear to be in a state of 'minimum subsistence' near death, so that any breakdown of essential cell components is lethal. When placed in a rich growth medium, a considerable proportion of these organisms die in the division lag phase (Postgate & Hunter, 1962).

It has been shown that A. aerogenes organisms cannot be maintained indefinitely in a non-proliferating, viable, steady state (Postgate & Hunter, 1962; Tempest et al., 1967). Starving bacteria can be considered to have a negative mass growth rate while endogenous metabolism continues (Powell, 1967). For a particular organism, this rate and the death rate depend on such factors as the growth medium composition (hence cell composition), the growth rate which produced the organism and the properties of the starvation environment.

AIMS OF THE PRESENT INVESTIGATION

Maintenance of bacterial cultures with high acid producing activities is an important function of research laboratories serving the dairy industry. An understanding of the factors affecting the survival and activity of cheese 'starter' organisms is therefore necessary and could also be important in evaluating the role of 'starter' organisms in cheese flavour.

Only a small number of reports on the metabolism of starved lactic acid bacteria have been published, possibly because oxygen consumption is not normally appreciable with these organisms. No correlations between viability and endogenous metabolism have been reported. Most of the reported studies on the metabolism of viable, starved bacteria have involved organisms which can accumulate 'reserves' such as glycogen or PHB. Lactic streptococci have no reported 'reserves' and very limited catabolic activities, it was considered that extension of endogenous metabolism studies to *Streptococcus lactis* could be informative. In addition, the exacting growth requirements of *S. lactis* should allow for uncomplicated survival studies in environments which would lead to cryptic growth of other organisms.

Most reports on the endogenous metabolism and survival of bacteria have been concerned with Gram-negative organisms. The report that exogenous Mg^{2+} suppressed RNA degradation but had no effect on the survival of starved *Sarcina lutea* (Burleigh & Dawes, 1967), has suggested that some of the important survival characteristics of this Gram-positive organism may be markedly different from Gram-negative organisms. Similar findings were made by Dawes with two other Gram-positive organisms (see discussion, Strange & Hunter, 1967). Further investigations with Gram-positive organisms have been indicated to define more clearly the general parameters for bacterial survival.

Although milk is the normal culture medium for *S. lactis*, studies on the physiology of bacterial survival require defined environments and highly reproducible bacterial cultures. While continuous culture propagation could be the best growth procedure, the appropriate equipment and techniques were not available for the present investigation. Accordingly, preliminary experiments were undertaken to achieve controlled growth of *S. lactis* in batch culture. Studies on the metabolism of viable, starved organisms are likely to be significant only if factors affecting survival are investigated concurrently. 'Minimum-stress' conditions must be defined for the organism under study since it is not possible to accurately extrapolate from data obtained with other organisms. Within the framework of these defined conditions, further investigations were undertaken to study the chemical changes which occurred in starved *S. lactis* organisms with special reference to changes in viability.

METHODS

MICROBIOLOGICAL PROCEDURES

Organism. *Streptococcus lactis* ML_3, used in the present investigation, was obtained from the collection of cheese 'starter' organisms of the New Zealand Dairy Research Institute. Strain ML_3 was selected because its short chain morphology facilitated the measurement of more accurate viability data for reasons already discussed.

Identification. The data used to check the identity of *S. lactis* ML_3 periodically was obtained using the methods described by Robertson (1961) and is summarized in Table 2. The reactions observed were similar to those reported for *S. lactis*. All temperatures are given in ^{o}C.

Culture maintenance. Since the method of propagation of lactic streptococci has a profound effect on the properties of the organisms, a detailed procedure for the maintenance of *S. lactis* ML_3 is outlined in Table 3. This procedure gave more reproducible cultures than were obtained by maintaining the organism on agar slopes at $0-4^{o}$ with subculture at intervals of up to one month.

Daily subculture subjects the organisms to intermittent starvation when growth ceases. However, in this environment complete viability was maintained until the next transfer and the appearance of 'starvation-resistant' mutants on prolonged subculture could not be demonstrated.

Innocula were normally 1% except when initially transferring from skim milk to the routine medium when a 5% innoculum was required for consistent growth. The reasons for the necessity of a large innoculum are not clear. Reiter & Oram (1962) found that some *S. lactis* strains would not grow when transferred

Table 2. Identification of *Streptococcus lactis* ML_3.

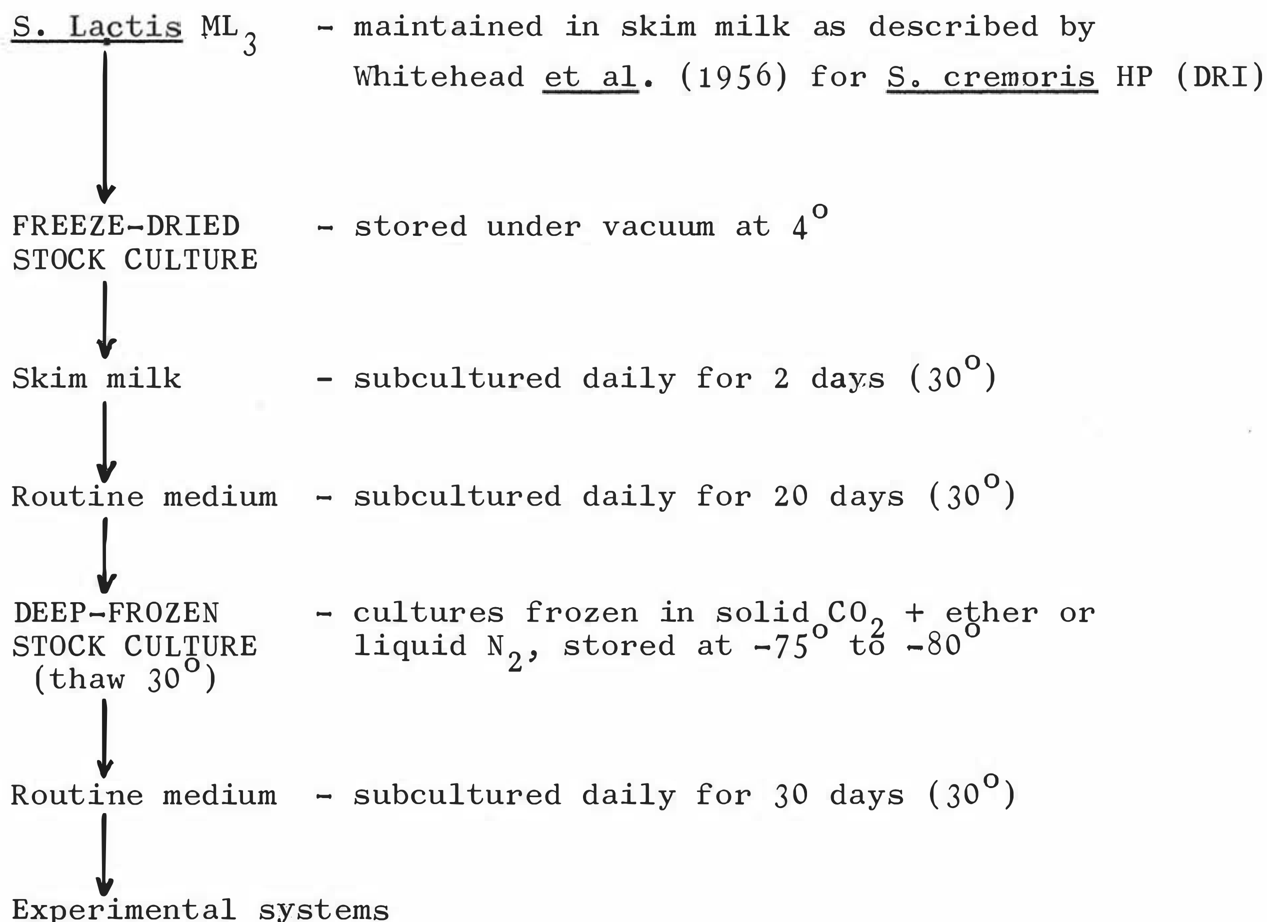
Test	Present study	Robertson (1961)	Breed *et al.* (1957)
Cell shape	ovoid cocci	cocci	ovoid cocci
Cell arrangement	diplococci		
Gram staining	+	+	+
Catalase	−	−	−
Growth at 10°	+	+ (15°)	+
" " 45°	−	−	−
Growth in 4.0% NaCl	+	+	+
" " 6.5% NaCl	−	?	−
NH_3 from arginine	+	+	+
CO_2 from tyrosine	−	−	−
CO_2 from citrate	−	±	±
Growth at pH 9.0	+	+	+
Acid from: Glucose	+	+	+
lactose	+	+	+
maltose	+	+	+
dextrin	+	+	nm
Serological group	nm	N	N

+ = positive reaction   ± = variable reaction

− = negative reaction   ? = reaction ill-defined

nm = not measured

Table 3. Procedure for maintenance of *Streptococcus lactis* ML_3.



Note: the deep-frozen stock cultures provided the regular source of organisms, this stock was renewed from the freeze-dried stock culture every six months.

directly from skim milk to a synthetic medium and required an intermediate subculture in a broth medium. It is well established that repeated subculture of lactic streptococci in skim milk over long periods may result in the development of a 'less active' culture (Whitehead, Briggs, Garvie & Newland, 1956). Since it is not understood why this occurs it was decided to limit the period of subcultivation according to Table 3. Cultures deep-frozen for six months were 50-80% viable on thawing.

Culture media. Synthetic media for the growth of *S. lactis* were reported by Niven (1944), Anderson & Elliker (1953) and Reiter & Oram (1962). Attempts to grow *S. lactis* ML_3 in each of these media proved unsuccessful in the present study. Reiter & Moller-Madsen (1963) concluded that vitamins or amino acids may be essential, stimulatory or not required, depending on the extent of deficiencies or imbalances in the basal medium. Growth antagonisms varied for different strains of the same organism and antagonisms between structurally related amino acids could be overcome by the use of peptides. Rather than reinvestigate the nutritional requirements of *S. lactis* ML_3, casamino acids were used to replace the synthetic amino acid mixture. Even so, peptone was required for maximum growth rates. The culture medium finally adopted contained (g./l.): lactose monohydrate, 5.0; sodium acetate, 1.0; glycerol, 1.0; casamino acids (Difco), 5.0; peptone (Difco), 0.5; L-asparagine, 0.1; DL-tryptophan, 0.05; L-arginine, 0.1; adenine, 0.005; guanine, 0.001; uracil, 0.005; xanthine, 0.005; pyridoxine hydrochloride, 0.001; pyridoxal hydrochloride, 0.0002; nicotinic acid, 0.001; calcium pantothenate, 0.001; biotin, 0.0001; riboflavin, 0.0001; thiamine hydrochloride, 0.0001; folic acid, 0.0001; *p*-aminobenzoic acid, 0.0002; $NaHCO_3$, 4.2; Na_2HPO_4, 4.25; KH_2PO_4, 2.7; $MgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.001; $MnSO_4.4H_2O$, 0.001; disodium ethylenediaminetetra-acetate (EDTA), 0.004. The

lactose, $NaHCO_3$ and vitamin components were sterilized separately using a Seitz filter, which had been pre-washed with 200ml. de-ionized water, and added aseptically to the other components which had been autoclaved at 115° for 20 min. The medium had a final pH of 7.3. Such a medium is not completely defined because of the presence of casamino acids and peptone, in subsequent descriptions it will be referred to as the routine medium.

The broth medium which was used occasionally, contained: lactose, 0.5%, Casamino acids (Difco), 0.5%; peptone (Difco), 0.1%; yeast extract (Difco), 0.25%; $MgSO_4$, 0.01%; 20μ M-EDTA; Na_2HPO_4-KH_2PO_4 buffer, 0.075M, pH 7.3.

Growth conditions and resuspension procedures. All growth cultures and washed suspensions were incubated in a water bath at 30° in static conditions unless otherwise stated. Organisms were prepared for survival studies as follows. After 10-20 daily transfers (70-140 generations) in the routine medium, growth phase organisms were inoculated into 25ml. medium and growth was followed turbidimetrically. At the end of the growth phase, a sample (usually 6ml.) was removed and after centrifugation (30,000g/1 min.), the pellet of organisms was rinsed thoroughly and then dispersed in sterile phosphate buffer (see below). Following further centrifugation (30,000g/1 min.), the organisms were rinsed and finally suspended in 5ml. sterile phosphate buffer (pH 7.0). Small volumes (0.2ml.) of the washed suspension were used to inoculate 10ml. phosphate buffer (plus substrates etc., were specified) in metal-capped test tubes which were incubated in a water bath at 30°. Suspensions prepared in this manner contained the equivalent of 20 $\pm$ 2 μg. dry wt. organisms/ml. (equivalent to about 6 x 10^7 chains/ml. or 1.6 x 10^8 cocci/ml.) and had an initial viability of more than 99%. Usually samples were removed at intervals and

placed directly on agar slides for viability measurements. In experiments with dense populations some dilution was necessary before doing viability determinations. Where the effect of a toxic metal or growth inhibitor was being examined the organisms were centrifuged and washed in phosphate buffer before the viability determination.

The phosphate buffer used contained Na_2HPO_4 + KH_2PO_4 (0.075M-PO_4, pH 7.0) plus 10 μM-EDTA, unless otherwise stated. No measurable change in pH occurred with suspensions equivalent to 10mg. dry wt. organisms/ml. All washing and resuspension of cells in buffers was carried out with aseptic precautions at 30°. Changes in temperature and chemical environment were thus minimized and manipulations were completed in about 15 min. These procedures left insignificant traces of growth medium (equivalent to a 10^{-7} dilution) and repeated centrifugation had no measurable effect on the survival curves of the organisms. A vortex-mixer was used for all mixing and resuspension operations.

Similar procedures were adopted for experiments on the metabolism of starved _S. lactis_ organisms (see experimental results for details). Some settling of organisms occurred after prolonged starvation; in these experiments gentle agitation was provided by a magnetic stirrer and correction was made for evaporation loss by adding the appropriate amount of sterile water. Samples of supernatant buffer solutions were obtained for chemical analyses by centrifugation and filtration through a membrane filter (0.45 μ; Millipore Filter Corp., Bedford, U.S.A.). When analyses on the bacteria were required, the packed organisms were washed in phosphate buffer and normally resuspended in water. Bacterial mass determinations were carried out at the sampling time, while suspension samples and cell-free supernatant solutions were stored at -20° when not analysed immediately.

Growth measurement and cell counting procedures. Growth

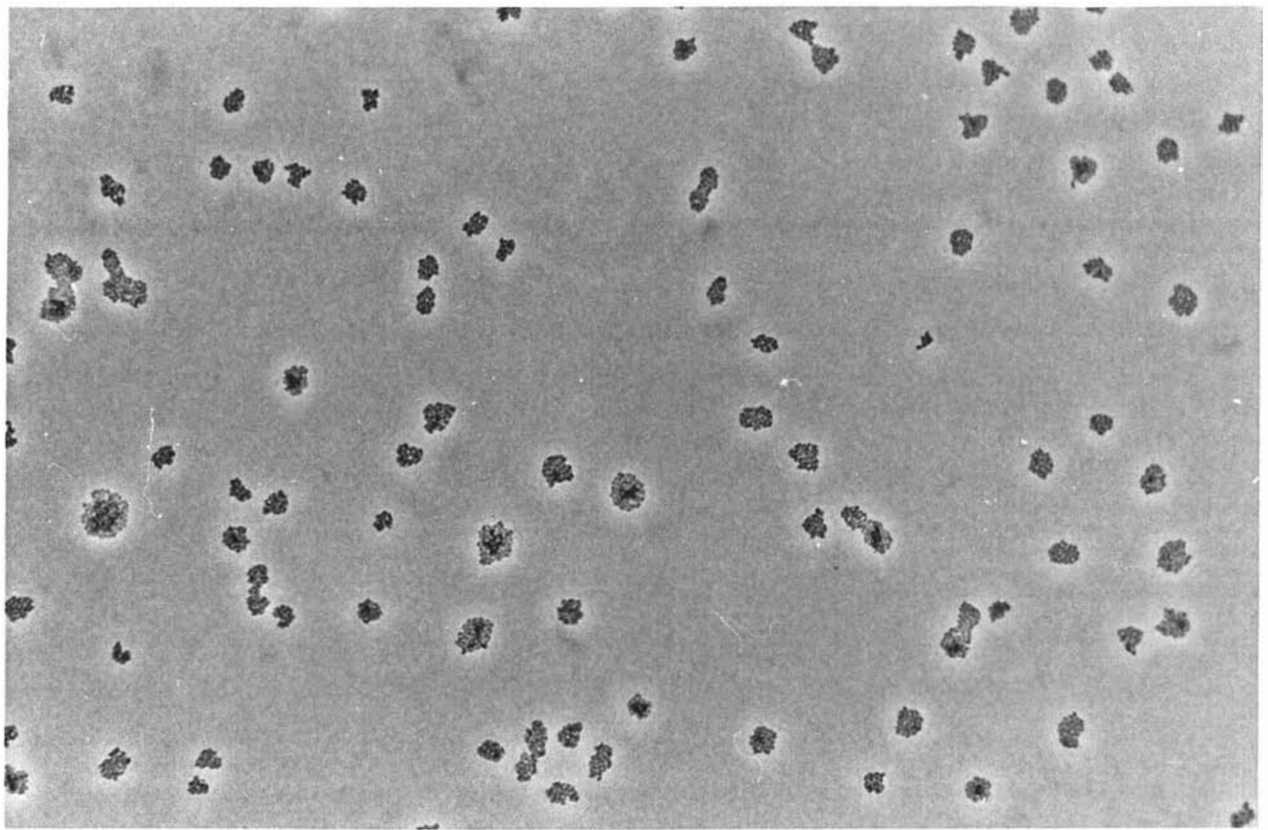
was followed by taking samples at intervals from the culture and measuring the turbidity at 600 mμ with either a Beckman DB or a Zeiss PMQII spectrophotometer. Dilutions were made in distilled water where necessary, equivalent dilutions in phosphate buffer gave the same turbidities. A correlation between bacterial dry weights and the turbidities of suspensions was obtained by calibration with suspensions of known dry wt./ml. Fixed volumes of suspensions in distilled water or phosphate buffer were dried to constant weight at 100°, appropriate correction being made for the amount of solids present in the buffer. The proportionality between bacterial mass and turbidity was examined with a range of starvation environments after varying periods of incubation and was found to be constant in all cases. All cell masses are recorded as mass units dry wt. bacteria/ml.

Total counts were made in a Petroff-Hausser chamber with a phase contrast microscope (magnification x 310). The precautions recommended by Norris & Powell (1961) were taken except that there was no instrument available to standardize chamber depth. The four large squares at each corner of the grid were counted for each sample (400-500 chains each). Counts are of individual chains separated by at least two coccal diameters unless otherwise specified. The number of cocci/chain was determined by counting the individual cocci in a total of 250 chains for each sample (magnification x 1100). These determinations were made on buffer suspensions using a phase contrast microscope with oil immersion and a glass coverslip. The criterion used for establishing when cocci had divided was, of necessity, subjective. Cell division was considered to be completed when the outline of the individual cocci in the chain resembled touching spheres. The estimate of the cocci/chain given by this method is probably subject to a systematic error giving slightly lower ratios (see Schaechter, Williamson, Hood & Koch, 1962).

Viability measurement. The percentage of viable organisms in resuspended systems was determined by the slide-culture method of Postgate *et al*. (1961). The only modification to the method found necessary was the use of stainless steel annuli instead of brass annuli, to avoid copper toxicity. The agar medium used had the following composition (g./100ml.); lactose monohydrate, 2.0; Casamino acids (Difco), 1.0; peptone (Difco), 0.8; yeast extract (Difco), 0.1; agar (Davis), 1.0; Na_2HPO_4, 0.25; $MgSO_4 . 7H_2O$, 0.05; final pH 7.0. The agar was filtered (Whatman no. 15) before autoclaving and centrifuged hot before slide preparation to remove debris. Slide-cultures were incubated at 30° for times sufficient to allow 4-5 generations. This provided adequate differentiation between live and dead organisms. Representative slide-cultures from populations with high and low viabilities are illustrated in Fig.2. *S. lactis* ML_3 occurred mainly as diplococci but chains with up to six cocci were observed. Slide-cultures were counted in green light using a phase contrast microscope with an eyepiece grid (magnification x 310). As a routine, eight typical fields with 40-60 objects/field were counted on duplicate slide-cultures, significant overcrowding of microcolonies did not result. For slide-cultures of organisms with long division lags (see Fig.15a) the incubation time was increased appropriately and ten fields were counted with 20-35 objects/field to minimize errors from overgrowth of dead organisms. Conventional colony counts were made by the standard pour-plate method (Cruickshank, 1965) using 0.1/100 or 1/100 serial dilutions in phosphate buffer and the agar medium described above. Plates were incubated until a constant count was obtained, normally for 24-48 hours.

Cell division lag times were measured by a method similar to that described by Postgate & Hunter (1963b, 1964). Populations were starved in various systems and at intervals samples

Fig. 2. Representative slide-cultures from starved populations of _Streptococcus lactis_ ML_3. Organisms were starved at 30° in 0.075M-phosphate buffer, containing 10 μM-EDTA and 1mM-$MgSO_4$. Slide-cultures for viability determination were prepared after 2 hr. (a) and 24 hr. (b) starvation and incubated at 30° for 3 hr. and 5 hr. respectively. The viability was determined and typical fields photographed with a phase contrast microscope. Viabilities were 99% (a) and 20% (b).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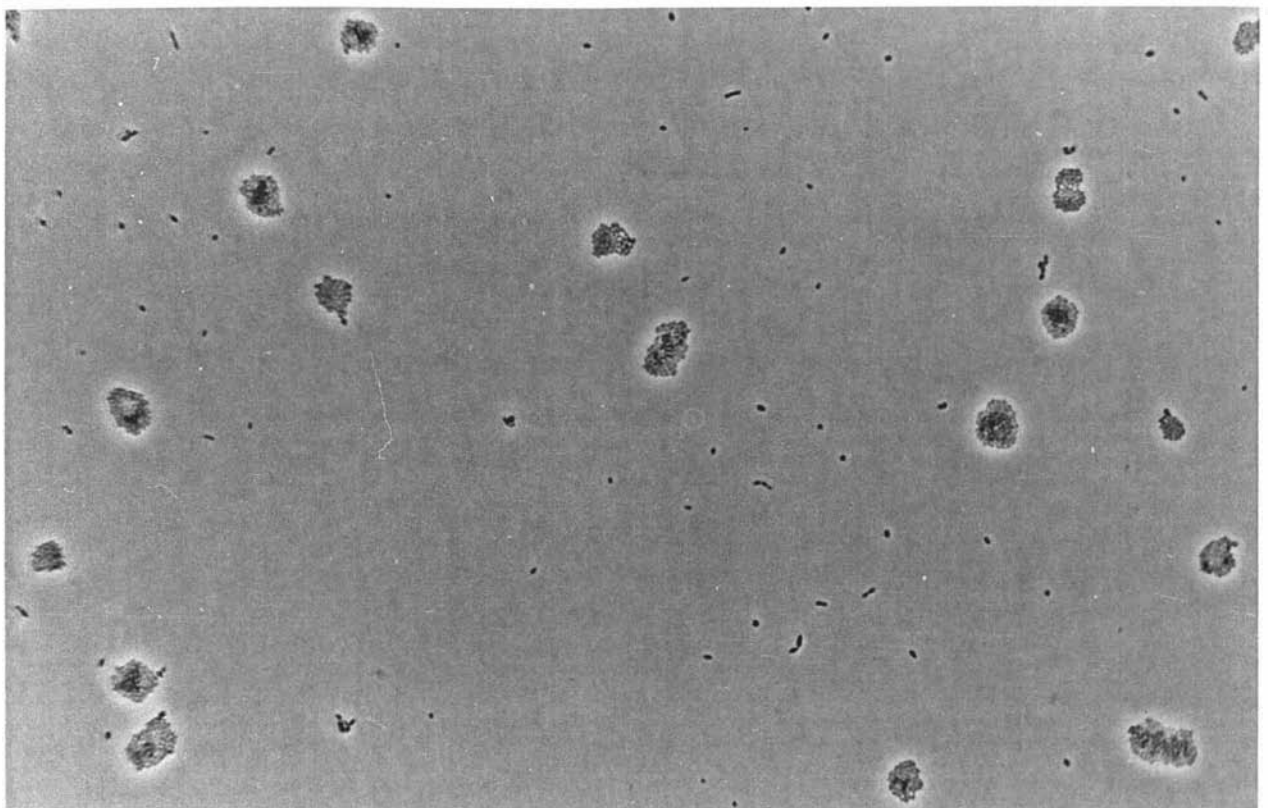
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were removed, centrifuged and the packed organisms resuspended in broth medium at 30°. The viability of the population was immediately determined by slide-culture and its turbidity measured at intervals. From the extrapolation of a semi-logarithmic plot of turbidity increase to the turbidity of the viable proportion of the original innoculum, an estimate of the division lag time was obtained (see Fig. 15b).

ANALYTICAL METHODS

All colorimetric and spectrophotometric measurements in the visible and ultraviolet (u.v.) region were made with either a Beckman DB or a Zeiss PMQII spectrophotometer using 1cm. glass or silica cells.

Magnesium and copper. The magnesium and copper contents of cell-free supernatant fluids and buffer solutions were measured using a Techtron AA3 atomic absorption spectrophotometer (Techtron Pty. Ltd., Australia) under standard operating conditions. Standard magnesium solutions and samples contained in phosphate buffer were diluted 1/10 with deionized water to appropriate concentrations. Phosphorus interference was suppressed by using lanthanum chloride. Cellular magnesium content was determined by the method of Webb (1966). Perchloric acid was added to copper standards and samples to a final concentration of 10% (v/v). Ammonium pyrrolidine dithiocarbamate solution and methyl isobutyl-ketone were added (Allan, 1961), and the copper was determined in the organic phase after shaking.

'Reserve' polymers. Polyglucose and poly-β-hydroxybutyrate were assayed by the methods of Strange *et al*. (1961) and Williamson & Wilkinson (1958) respectively.

Manometry. Conventional manometric methods were used

(Umbreit, Burris & Stauffer, 1957). Oxygen uptake was determined at 30° with a shaking rate of 50 oscillations/min.

Protein. Protein was determined by the biuret method (Stickland, 1951). Suspensions of *S. lactis* required heating at 100° for 20 min. in 0.75N-NaOH for maximum colour development. Supernatant buffer and standard samples were not heated. It was found that heating produced higher blank readings so the appropriate corrections were used. Dried bovine serum albumin (Sigma, Agrade), containing 13.6%N, was used as the standard. The alternative method of Lowry, Rosebrough, Farr & Randall (1951) gave similar results to the biuret method with samples of both supernatant and alkali-hydrolysed cell suspension.

The reproducibility of the biuret method was tested by performing twelve analyses on a suspension of 1.0mg. dry wt. bacteria/ml. The mean protein content and standard deviation was 0.48 ± 0.028mg./ml.

Total nitrogen. Total cell N and supernatant N were determined by the micro-Kjeldahl method described by Humphries (1956) using a Se/K_2SO_4 catalyst and methyl red-methylene blue indicator.

Enzyme assays. Supernatant protein was assayed for tributyrinase activity (see Lawrence, Fryer & Reiter, 1967) and for proteolytic activity by the Folin-Ciocalteu method (see Cowman & Speck 1967).

Lactic dehydrogenase was assayed using the method described by Kornberg (1955). Supernatant samples (2.8ml.) were adjusted to pH 7.4, 10mM-Na pyruvate (0.1ml.) and 4mM-nicotinamide-adenine-dinucleotide (NADH, 0.1ml.) were added and the rate of oxidation of NADH at 30° was determined using a recording spectrophotometer at 340 mμ. The unit of activity was taken as the amount of enzyme which caused an initial rate of oxidation of 1 μmole NADH per minute.

β-Galactosidase activity of supernatant samples was tested for by the o-nitrophenyl-β-D-galactopyranoside hydrolysis method as described by Citti et al. (1965).

The assay for glycolytic activity developed in the present investigation is described later in the Methods.

Amino acids and ammonia. Amino acids in bacterial extracts and supernatant samples were measured colorimetrically by the ninhydrin method of Yemm & Cocking (1955). Glycine standards were used and $(NH_4)_2SO_4$ standards were included to correct for NH_3, which was determined separately by the micro-diffusion method of Conway (1947). Buffer samples were adjusted to pH 5.0 before amino acid analysis by addition of HCl or dilution in citrate buffer (pH 5.0).

Amino acids were separated by thin-layer chromatography (TLC) on mixed cellulose-silica gel layers and detected with ninhydrin (Turner & Redgwell, 1966). Plates were developed with phenol-water (80:20, w/v) in the first direction (5 hr.) and dried overnight at 40 - 50°. Butanol:acetic acid:water (5:1:4, v/v/v, upper phase) was used in the second direction (4 hr.).

Two-dimensional, descending paper chromatography of the amino acids in bacterial extract and supernatant samples was performed at 22° using Whatman no.1 paper and the procedures of Roberts, Abelson, Cowie, Bolton & Britten (1957). Supernatant samples were desalted by the ion-exchange methods of Dreze, Moore & Bigwood (1954). Paper chromatography solvents were (i) first direction (16 hr.); sec-butanol : formic acid:water (7:1:2, v/v/v); (ii) second direction (18 hr.); phenol:aq. NH_3 (sp.gr. 0.88) : water (80:0.3:20, w/v/v). Papers were dried, dipped in acetone containing ninhydrin (0.25%) and heated at 100° for 5 min. (Toennies & Kolb, 1951). Amino acids on thin-layer and paper chromatograms were tentatively identified by

comparison with R_F values of standards.

More positive identification and quantitative measurement of amino acids was obtained using a Beckman model 120C automatic amino acid analyser with Beckman custom spherical ion exchange resin. The short column for basic amino acids contained resin type PA 35 to a height of 8cm. The elution buffer pH was 5.28 and the flow rate 68ml./hr. The long column for acidic and neutral amino acids contained resin type PA 28 to a height of 58cm. Buffer, pH 3.28, was used as eluant being replaced after 90 min. by a second buffer at pH 4.25. The ninhydrin flow rate was 34ml./hr. and analyses took place over a 4 hr. period. Peaks were identified from standard elution times and integrated by the height-width method. Using standard calibration constants the amino acids were estimated with an accuracy of $\pm$ 1% for the major peaks.

The total cellular amino acid composition was determined on organisms harvested at the end of the growth phase and washed twice in distilled water. Washed organisms were freeze-dried, hydrolysed (6N-HCl, 24 hr., 110° in vacuum sealed tube) and the hydrolysate analysed.

Extraction of soluble intracellular compounds. The complete extraction of water soluble intracellular compounds from bacteria is probably best achieved by heating the organisms in boiling water (see Holden, 1962). The minimum heating time at 100° for complete release of the amino acid and nucleotide pools of *S. lactis* was found to be 12 $\pm$ 3 min. For routine extracts, bacteria were washed and resuspended in deionized water and the suspensions heated for 20 min. at 100° in stoppered tubes. Cell debris was removed by centrifugation and the clear extract pipetted off. A second extraction yielded only a further 1 - 3% of ninhydrin-positive material and was therefore not carried out routinely.

Ribonucleic acid. Bacterial RNA was determined by the method described by Munro & Fleck (1966). Perchloric acid-washed bacteria (10 min. at 0° in 0.5N-$HClO_4$) were subjected to alkaline hydrolysis in 0.3N-KOH at 37° for 60 min. This was shown to give a complete extraction of ribonucleotides. (For convenience, samples and standards were often stored at -20° in alkaline suspension before hydrolysis. It was shown that storage for up to 24 hr. at -20° did not affect analyses). The hydrolysate was chilled to 0° and ice-cold $HClO_4$ was added to a final concentration of 0.5N. After 10 min. at 0° the insoluble fraction was sedimented and washed twice in ice-cold $HClO_4$ (0.5N). Centrifugation was carried out in a Sorvall refrigerated centrifuge (30,900g/1 min.) The alkali extract and washings were combined and made up to 25ml. Samples were filtered through a sintered glass filter (porosity 5/3) before extinction measurement at 260 mμ. Soluble yeast RNA (Sigma, type III) was used as a standard and was treated in the same way as samples. The standard RNA contained 7.85% phosphorus and was assumed to be 83% RNA (see Strange et al., 1961). The method was highly reproducible; twelve analyses on a suspension with 1.0mg. dry wt. bacteria/ml. gave a mean RNA content with standard deviation of 0.195 $\pm$ .002mg. RNA/ml.

Deoxyribonucleic acid. Bacterial DNA was extracted and estimated by the methods of Burton (1956). Deoxyribose was used as the standard.

Extracellular u.v.-absorbing compounds. Before measuring the extinction of supernatant samples at 257 mμ, the solutions were deproteinized with 5% TCA; the appropriate blanks were included.

Tentative identification of the u.v.-absorbing compounds, which were released from starved S. lactis organisms, was established by the following procedures. Supernatant samples were applied in bands to preparative TLC plates

(MN-Cellulose, 300G) and chromatographed in deionized water following the method of Randerath (1964). The bands, which were distinctly separated, were marked under u.v. light, removed and the u.v.-absorbing material eluted with three portions of 0.1N-$HClO_4$. After filtration through sintered glass (porosity 5/3), the u.v. spectra of the eluates were recorded on a Beckman DK-2A ratio recording spectrophotometer along with spectra of standards in 0.1N-$HClO_4$. The R_F values and spectra of the unknown compounds were compared with those of standards and literature data (Dawson, Elliott, Elliott & Jones, 1959).

Lactose. Lactose was estimated in cell-free samples of growth medium by the anthrone method of Richards (1959).

Ribose. Free and purine-bound ribose was estimated in cell-free supernatant or alkali-soluble nucleotide extracts using the orcinol method (Mejbaum, 1939).

Hexose. Total cellular hexose was estimated as glucose by the anthrone procedure of Trevelyan & Harrison (1952) or by the reducing sugar method of Nelson (1944). For comparison of the glycolytic activity assay (described later) with chemical analyses, glucose was determined by the more precise neocuproine method of Dygert, Li, Florida & Thoma (1965) (see Table 4).

Lactic acid. Lactic acid was measured in deproteinized supernatant samples by the method of Barker & Summerson (1941).

Steam-volatile acids. These compounds were separated by exhaustive steam distillation in a Markham still, after adjusting the supernatant and standard samples to pH 1 with H_2SO_4. Distillates were titrated with 0.01N-NaOH in a stream of 'dry' N_2, using phenolphthalein as the indicator.

Phosphorus. Phosphorus was estimated by the method of Burton & Petersen (1960).

Lipids. Extraction of a 50g. sample of casamino acids with diethyl ether for 30 hr. in a soxhlet apparatus, yielded 15mg. of semi-solid material. However, all of this material was removed by washing with aqueous $CaCl_2$ (Folch, Lees & Stanley, 1957) confirming that there was no appreciable lipoid material introduced into the routine growth medium from the casein hydrolysate. Similar observations were reported by Ikawa (1963) and Vorbeck & Marinetti (1965).

Lipids were extracted directly from washed organisms by the methanol-chloroform procedure of Vorbeck & Marinetti (1965). A second extraction recovered a further 6 - 9% of the total lipid, and acid hydrolysis (2.5N-HCl, 16 hr. at 100°) of the residue yielded a further 3-4% of the total lipid. Two methanol-chloroform extractions were normally performed. Extracts were washed with aqueous $CaCl_2$ (Folch *et al*., 1957) to remove non-lipid material and the solvents were removed using a rotary evaporator at 40°. The tared flasks were finally dried to constant weight in a vacuum desiccator over P_2O_5 and the lipids were estimated gravimetrically.

Silicic acid column chromatography was used to separate lipids into neutral and polar fractions (Wren, 1960; Hirsch & Ahrens, 1958). Lipid (200mg.) was applied to the top of the column (250 x 17mm.) in the minimum amount of chloroform-methanol (4:1) and the neutral lipid was completely eluted with ethanol-free chloroform containing 1% methanol (500ml.). Polar lipid was then eluted completely using chloroform-methanol (2:1, v/v, 300ml.). Column separations were monitored routinely by both TLC and phosphorus analyses. Fractions obtained were dried and weighed as described previously.

Thin-layer chromatography was carried out on glass plates with a layer of silicic acid (silica gel G, Merck) following the procedures of Mangold (1961). Chromatograms, which had been developed in either hexane:diethyl ether:acetic acid (70:30:1, v/v/v) or diisobutyl ketone:acetic acid:water (80:50:8, v/v/v), were dried before spraying with ninhydrin and the spots giving a positive reaction were marked. Phosphorus-containing components were detected by using the molybdenum spray reagent of Dittmer & Lester (1964). Finally the plate was sprayed with 10% H_2SO_4 and charred at 110° to show all components. The presence of a glycol-ipid was demonstrated by refluxing a sample eluted from a preparative TLC plate in 3ml. 2N-H_2SO_4 for 1 hr., extracting the lipid residues with petroleum ether and neutralizing the aqueous phase with barium carbonate. After centriguation, the supernatant was removed and evaporated to dryness at reduced temperature and pressure. Residual sugars were redissolved in water and samples applied to Whatman no. 1 paper. Papers were developed in ethyl acetate:pyridine: water (2:1:2:, v/v/v) by the procedure of Jermyn & Isherwood (1949) and sprayed with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950).

Glycerides were hydrolysed by refluxing with aqueous 5N-KOH:CH_3OH (1:1, v/v) for 5 hr.; non-saponifiable material was removed by ether extraction. After acid-ification, free fatty acids were extracted into diethyl ether, dried over anhydrous Na_2SO_4 and esterified with diazomethane (Schlenk & Gellerman (1960)).

Analysis of the methyl esters was carried out on an Aerograph 600 gas chromatograph (Wilkins Instrument & Research, U.S.A.) using a hydrogen flame ionization detector. Apiezon L on 80-100 mesh celite (10%, w/w) at 196° and polyethylene glycol adipate at 180° were used as stationary phases and the columns were prepared as described by James (1960). The detector and columns

were checked periodically with the fatty acid standards and procedures recommended by Horning, Ahrens, Lipsky, Mattson, Mead, Turner & Goldwater (1964). Peaks were identified by comparison with retention data of standards or with published values and the percentage composition of the fatty acids was estimated by the height-width method (Horning et al., 1964).

Materials. Solid reagents for analytical procedures were recrystallized and solvents redistilled if analytical reagent grade was not available. All water was distilled and then deionized by passage through a mixed bed ion-exchange resin (Permutit 'Biodeminrolit').

Measurement of Protein Synthesis. Protein synthesis in starved organisms was estimated by following the incorporation of valine-^{14}C into cell protein using the procedures outlined by Willetts (1967). Culture samples (2ml.) were added to an equal volume of trichloroacetic acid (TCA, 10%, w/v) and, after heating for 30 min. at 90° (see Marchesi & Kennell, 1967), the precipitates were filtered off on membrane filters (25mm., pore size 0.45μ; Millipore Filter Corp., U.S.A.). Each filter was washed successively with three 1ml. volumes of TCA (5%, w/v) containing DL-valine (150μg./ml.), TCA (5%, w/v) alone and acetic acid (1%). Material isolated by this procedure may include cell wall substance as well as 'true' protein. However, lactic acid bacteria contain little valine in the cell walls (Ikawa & Snell, 1960) and the procedure seemed to be adequate for the purpose of the present investigation.

For the measurement of valine-^{14}C uptake by whole cells, culture samples (2ml.) were filtered on membrane filters, washed with ten 2ml. volumes of 0.075M-phosphate buffer containing L-valine (200μg./ml.) and dried. The washing procedures used for incorporation and uptake measurements

removed all radioactivity from control systems without cells and the reproducibility of the measurements is shown in Table 4.

The radioactivity on the dried membrane filter discs was measured with a Packard Tri-Carb 2000 series liquid scintillation spectrometer (gain settings 10%, window settings 50-1000) with an efficiency of 70.5% as determined by channels ratio measurements. The scintillation solution consisted of 2,5-diphenyloxazole (5.0g.) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.3g.) per litre of toluene.

GLYCOLYTIC ACTIVITY ASSAY

In view of the correlation between bacterial survival and the activity of the enzymes catabolizing the energy source (see Introduction), it seemed appropriate to examine the glycolytic activity of starved *S. lactis* organisms since glycolysis represents the only major catabolic pathway in this organism. Existing methods for determining glycolytic activity are based on the measurement of lactate formation rates either by direct titration, manometric techniques, colorimetric or enzymic analyses. However, these methods were either too insensitive, inaccurate or laborious for the purposes of the present investigation. The assay developed involves the measurement of the radioactive anionic products (lactate, acetate and formate) and the remaining unfermented labelled substrate (glucose) by liquid scintillation counting following separation on DEAE-cellulose paper strips.

Materials. DEAE-cellulose paper (DE81, free base form) was obtained from Whatman, England. D-Glucose-U-^{14}C, sodium-DL-lactate-2-^{14}C, sodium acetate-2-^{14}C, sodium formate-^{14}C and glycerol-1-^{14}C, were obtained from The

Table 4. Reproducibility of valine-^{14}C incorporation and uptake measurements. Washed organisms were resuspended at 0.91mg. dry wt./ml. in four 20ml. volumes of 0.075M-phosphate buffer (pH 7.0; containing 10μM-EDTA, 1mM-$MgSO_4$, DL-valine-1-^{14}C (.25μc./ml., 6μg./ml.), L-valine (200μg./ml.) and 10mM-D-glucose). After incubation for 1 hr. at 30°, two 2ml. samples were removed from each culture and the valine-^{14}C uptake by cells determined (see text). The remainder of the cultures was cooled rapidly to -10°, four 2ml. samples were removed from each culture and the valine-^{14}C incorporation into cell protein estimated. Results are given as the mean $\pm$ standard deviation, with the number of measurements in parentheses.

	Valine-^{14}C incorporation and uptake (c.p.m./mg. dry wt. bacteria/hr.)
Protein	844 $\pm$ 37 (16)
Cells	3096 $\pm$ 189 (8)

Radiochemical Centre, Amersham, England. Under the influence of its own radiation, glucose-U-^{14}C decomposes in the freeze-dried state at a rate of about 1% per year at -20°. The decomposition products remain firmly bound to DEAE-cellulose paper and cannot be eluted with deionized water. To obtain a sample of glucose-U-^{14}C essentially free from decomposition products, freeze-dried glucose-U-^{14}C was dissolved in a small volume of deionized water and applied to a DEAE-cellulose paper strip 2cm. wide. After running 10cm. in deionized water by ascending chromatography, the paper was dried, cut into bands and eluted with deionized water; 98% of the applied activity was eluted from the upper 4 x 2cm. of the paper. This glucose-U-^{14}C, free from anionic impurities, was used in subsequent experiments. Since glucose fermentation by S. lactis may produce a variety of end products and may proceed to a limited extent via the HMP pathway (see Introduction and Fig.1), it is desirable to use a uniformly labelled substrate for this assay.

The liquid scintillation solution consisted of 2,5-diphenyl-oxazole (5.0g.) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.3g.) per litre of toluene.

Routine counting procedure. Standard radioactive compounds were used in preliminary experiments. Dried DEAE-cellulose strips (2 x 2.5cm., folded where necessary) with adsorbed labelled compounds were placed vertically in standard counting vials. Scintillation solution (10ml.) was added and vials were counted in a Packard Tri-Carb 2000 series liquid scintillation spectrometer with gain settings of 10% and window settings of 50-1000 in both channels. The absolute counting efficiency for glucose-U-^{14}C and lactate-2-^{14}C on DEAE-cellulose paper with the

above settings was 55.3%, determined from channels ratio measurements on aqueous solutions of the two compounds in toluene-ethanol scintillation solvent. For most labelled samples at least 10^4 counts were recorded and samples near background activity were counted for 20 min.

For the scintillation counter used, the orientation of the DEAE-cellulose papers had little effect on count rate; for discs lying flat on the bottom of the vial the activities were 3% lower than the values obtained with vertical strips. Vertical placement of strips was therefore adopted routinely. Removal of papers after counting did not significantly increase the background count showing that all the C^{14}- labelled compounds used remained adsorbed to the DEAE-cellulose paper in the scintillation solvent. There was a linear relationship between the applied activity and the count rate and the count rate was independent of the distribution of the C^{14}-activity on the DEAE-cellulose paper. When several 2 x 2.5cm. blank DEAE-cellulose strips were placed on either side of a paper containing C^{14}- labelled compounds, the count rate was unchanged showing that the paper was transparent to photons.

Chromatographic separation of compounds. Using a number of different solvents, adsorbed glucose-U-^{14}C could not be washed off DEAE-cellulose papers using techniques described by Sherman (1963) without removing much of the adsorbed lactate-^{14}C. It was therefore necessary to separate glucose-^{14}C from the anionic fermentation products by chromatography and deionized water was found to be the most efficient solvent. Samples of solutions containing C^{14}- labelled compounds were applied to 2 x 15cm. DEAE-cellulose strips in a narrow band. These samples were not dried following the observation

that irradiation from infra-red lamps resulted in a substantial breakdown of the glucose-U-^{14}C to products which remained firmly bound to the paper and subsequently interfered with assays. The paper strips were eluted with deionized water by ascending chromatography, the solvent front being allowed to ascend 10cm. from the sample band. After drying under an infra-red lamp the strip was cut along pre-ruled lines, normally into 2 x 5cm. strips, folded when necessary and placed vertically in counting vials. Glucose-U-^{14}C travelled at the solvent front while lactate-^{14}C remained at the origin (Fig.3).

The presence of salts in the sample applied to the DEAE-cellulose strip had a pronounced effect on the chromatographic separation of glucose-^{14}C from lactate-^{14}C in deionized water. When 20-50 μl. samples in 0.075M-phosphate buffer were applied to 2 x 15cm. DEAE-cellulose strips, the lactate-^{14}C tended to move away from the origin and separation from glucose-^{14}C was not precise. With 10 μl. samples in 0.075M-phosphate buffer there was no salt effect.

Effect of metabolic products other than lactate. Sodium acetate-2-^{14}C and sodium formate-^{14}C, dissolved in phosphate buffer (pH 7.0), were spotted on separate DEAE-cellulose strips which were chromatographed, dried, cut up and counted as usual. All the applied activity remained at the origin so that any acetate or formate produced during glucose fermentation in routine assays was counted together with lactate. Glycerol-1-^{14}C was found to travel at the solvent front on DEAE-cellulose paper strips.

Routine assay procedure. For routine glycolytic activity assays of starved *S. lactis* organisms, fermentation systems

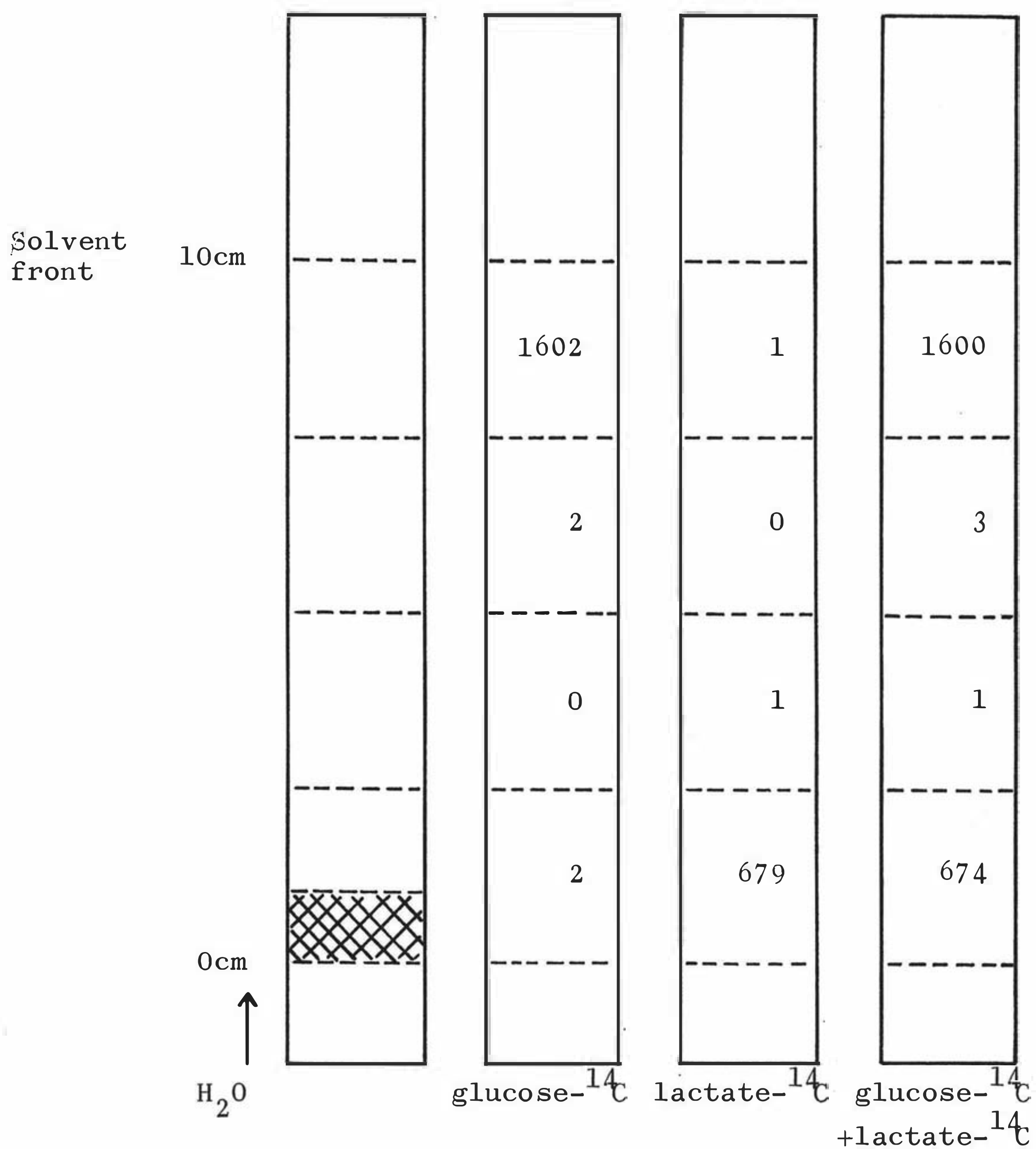


Fig. 3. Separation of standard D-glucose-U-^{14}C from standard Na-DL-lactate-2-^{14}C on DEAE-cellulose paper. Samples (20 μl.) of aqueous solutions of glucose-^{14}C (0.065 μc./ml.) and lactate-^{14}C (0.027 μc./ml.) were spotted on 2 x 15 cm. DEAE-cellulose strips as shown by the crossed area. After elution with deionized water by ascending chromatography, the strips were dried, cut into four 2 x 2.5 cm. strips along pre-ruled lines, and counted. Counts are given in c.p.m. minus background, figure to scale.

contained; D-glucose-U-^{14}C (1.66×10^3 c.p.m./10μl.), 10mM; $MgSO_4$, 2mM; 0.075M-phosphate buffer, pH 7.0; and organisms, approximately 0.5mg. dry wt./ml. Two samples were removed at times designed to give measurements in the range of 20-60% glucose utilization. Each sample was placed on a 2 x 15cm. DEAE-cellulose paper strip and chromatographed in deionized water. When the solvent front had ascended 10cm. the paper was dried, two 2 x 5cm. portions containing the labelled compounds were cut out and counted. The mean glycolytic activity of the two samples was expressed as μmoles lactate/mg. dry wt. bacteria/min. The change in buffer pH after 20-60% glucose utilization was less than 0.14 units.

Time course of glycolysis. The conversion of glucose-U-^{14}C to lactate-^{14}C by whole cells of *S.lactis* took place at a linear rate until almost all of the glucose was fermented (Fig.4). This rate produced 0.295μmoles lactate/mg. dry wt. bacteria/min. With a bacterial mass equivalent to 0.69mg. dry wt./ml., 1.5-2.0% of the total activity remained cell-bound during glycolysis. Most of this activity was released when all of the glucose had been fermented. In routine assays of glycolytic activity with bacterial masses similar to that given above, bacteria were not removed from samples prior to the assay of glycolytic activity so that cell-bound activity was measured as lactate.

Relationship between bacterial mass and glycolytic activity. The glycolytic activity of *S. lactis* was directly proportional to bacterial mass when measured in suspensions ranging from 0.11-1.94mg. dry wt. bacteria/ml. (Fig.5). Low bacterial masses may be assayed more accurately by increasing the specific activity of the glucose-U-^{14}C.

Effect of glucose concentration. With bacterial masses of

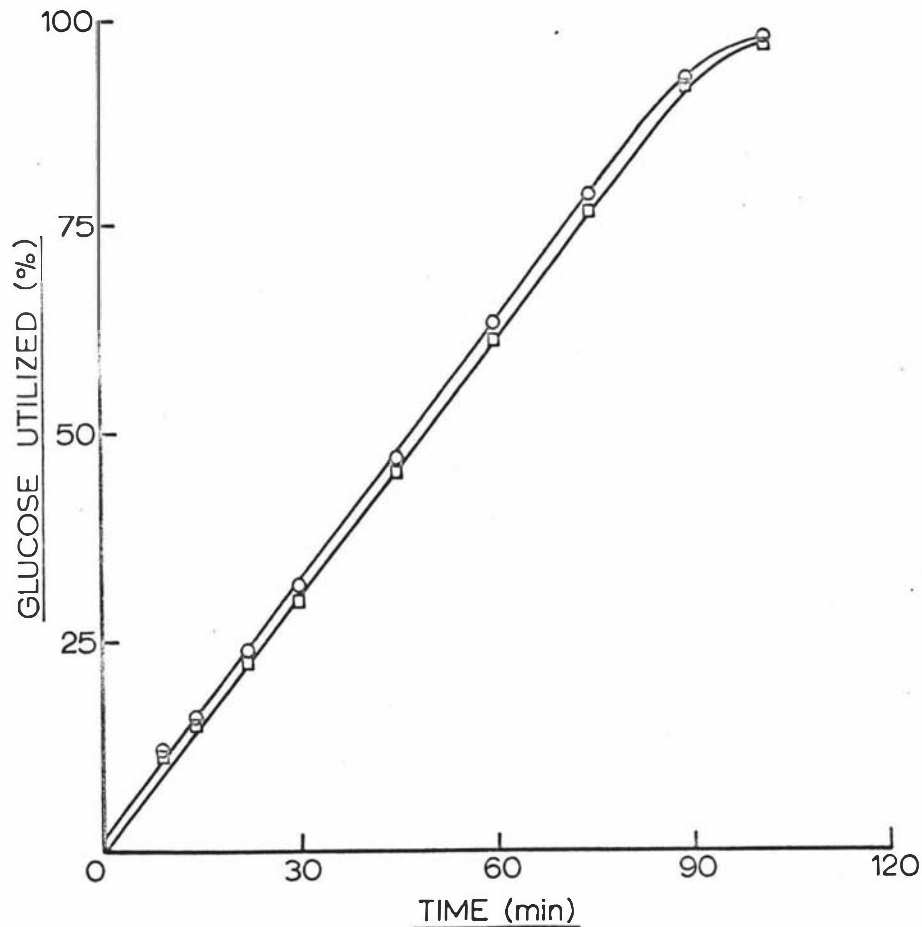


Fig. 4. Time course of glycolysis. Organisms from the end of the growth phase were washed and resuspended in 10ml. phosphate buffer at 30° ($0.075M-Na_2HPO_4-KH_2PO_4$, pH 7.0, containing 10 μM-EDTA + 1mM-$MgSO_4$). An aliquot of the washed suspension (5.7ml.) was added to 300 μl. of 0.2M-glucose-^{14}C + 0.02M-$MgSO_4$ solution to give final concentrations; 10mM-glucose-^{14}C (1600 c.p.m./10 μl.), 2mM-$MgSO_4$, and 0.69 mg. dry wt. bacteria/ml.

The suspension was incubated at 30° without agitation. At intervals, samples (0.5ml.) were removed and immediately chilled to 0°. Aliquots (10 μl.) of the suspension were removed with a syringe and placed on DEAE-cellulose strips, the remaining chilled suspension was centrifuged and 10 μl. samples of the cell-free supernatant were placed on DEAE-cellulose strips. Strips were chromatographed, dried, cut up and counted as described in the text; ○ , cell suspension; □ , cell-free supernatant.

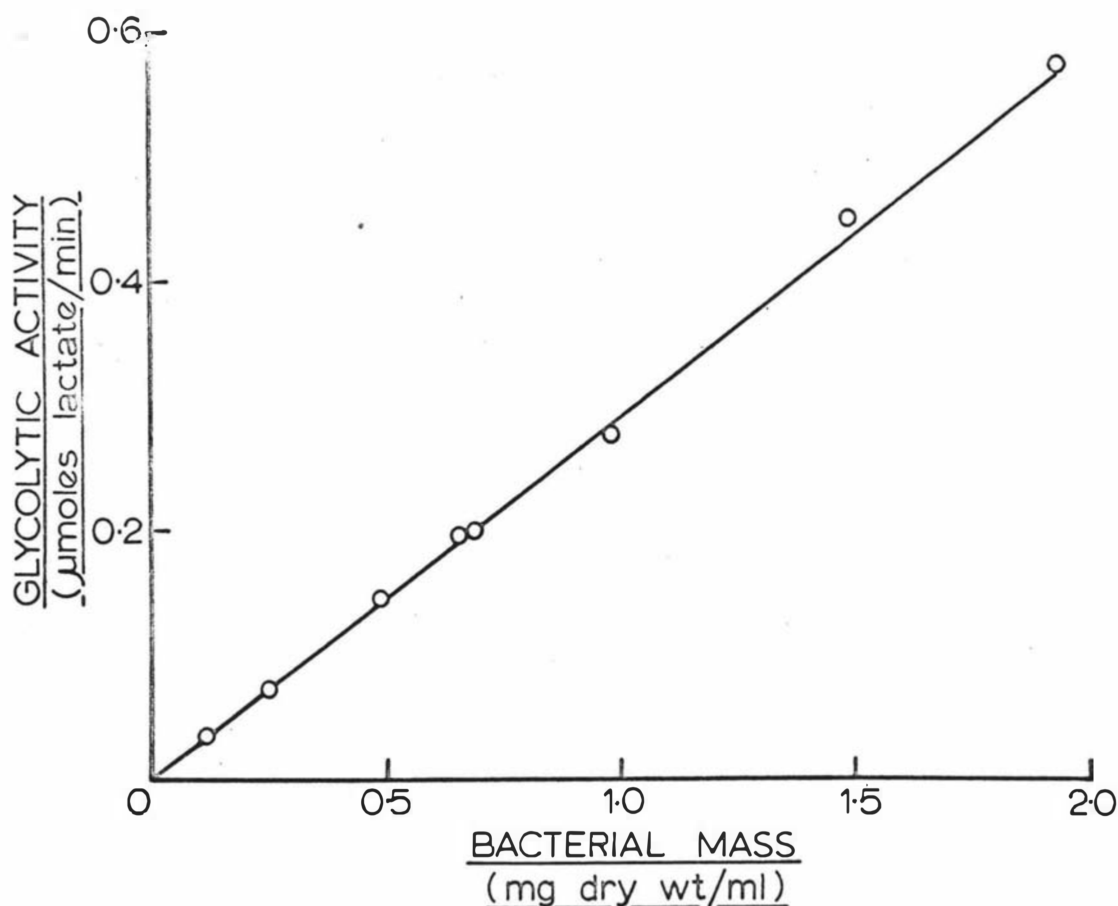


Fig. 5. Relationship between glycolytic activity and bacterial mass. Washed suspensions of Streptococcus lactis were prepared in a similar manner to those described in Fig. 4. A range of bacterial concentrations were prepared by dilution in 0.075M-phosphate buffer. Diluted samples (1.9ml.) were added to 100 μl. of 0.2M-glucose-^{14}C + 0.02M-$MgSO_4$ and the tubes were incubated at 30°. Two samples (0.5ml.) were removed at times designed to give measurements between 20-60% glucose-^{14}C utilization. After rapid chilling and centrifugation at 0°, 10 μl. samples of the cell-free supernatants were placed on DEAE-cellulose strips which were assayed as described in the text.

0.66 and 0.13mg. dry wt./ml., a constant glycolytic activity (0.29 μmoles lactate/mg. dry wt. bacteria/min.) was observed with glucose concentrations in the range 0.67mM to 100mM. Each system contained the same amount of radioactivity. This result would be consistent with the presence of an active transport system for glucose which was not rate-limiting.

Comparison of isotopic assay with chemical analyses. The glycolytic activity determined separately by chemical analyses on the rates of glucose utilization and lactate formation was in agreement with results of the isotopic assay (Table 5).

The major advantages of this assay over existing methods are that as well as being rapid, sensitive and highly reproducible, it measures simultaneously the rates of glucose utilization and lactate formation in a single operation. All anionic products (lactate, acetate, formate etc.,) are measured together, while any non-anionic products (e.g. glycerol) are measured as glucose, resulting in lower assay. Calculation of any loss of radioactivity from the system (total activity minus glucose activity minus lactate) would provide a measurement of volatile product formation (ethanol, CO_2 etc.). Hence in a single assay of glycolytic activity, considerable information can also be provided about the glucose fermentation products of a microorganism. Incorporation of label into cell components may result in loss of counts from some systems. However, only a small percentage of the total activity was cell-bound at normal cell densities of starved *S. lactis* and most of this was released when all the glucose had been fermented.

This method would appear to be much more rapid, efficient and precise than the Sephadex column method recently described by Riley (1968) for the separation and measurement of C^{14}-labelled glucose and lactate.

Table 5. Comparison of isotopic assay with chemical analyses. Washed suspension (50ml.) was prepared from 50ml. growth culture as for Fig.4. Final concentrations were 10mM-glucose-^{14}C, 2mM-$MgSO_4$, bacterial mass 0.54mg. dry wt./ml. Samples (5ml.) were removed at intervals, rapidly chilled to 0°, centrifuged and the cell-free supernatants analysed as below. Values are given as the mean ± standard deviation, with the number of independent determinations given in parentheses.

Time	Isotopic assay		Glucose assay		Lactate assay	
(min.)	% conversion glucose ⟶ lactate		% glucose utilization		% conversion glucose ⟶ lactate	
21	18.06±.17	(5)	20.6±.5	(6)	17.8±.6	(6)
49	40.72±.26	(5)	42.2±.7	(6)	39.5±.8	(6)
75	61.04±.28	(5)	63.5±.4	(6)	61.7±.9	(6)
100	81.60±.24	(5)	83.0±.3	(6)	78.9±.6	(6)
160	97.46±.15	(5)	97.9±.4	(6)	93.5±.6	(6)

ELECTRON MICROSCOPY

To 20 ml. culture was added 2 ml. of fixative (0.095M-phosphate buffer, pH 6.1, containing 6mM-$MgCl_2$ and 1% glutaraldehyde). After mixing, the culture was centrifuged (1000g/5 min.) and the organisms resuspended in fixative (2 ml.) and left for 3 hr. at 4°. Following further centrifugation, the organisms were washed twice in 0.1M-phosphate buffer (pH 6.1) containing 0.2M-sucrose. The pellet was then resuspended in 0.1M-phosphate buffer containing 1% osmium tetroxide and left for 2 hr. at 4°. After two further washes in phosphate buffer the organisms were set in 2% agar at 45°. The solidified block was cut into small pieces and immersed in uranyl acetate (0.5%, aq.) for 2 hr. at room temperature. The agar blocks were then dehydrated in graded aqueous ethanol solutions according to the following schedule: 25%-15 min., 75%-16 hr.(4°), 95%-30 min., 100%-30 min. (twice). The blocks were then washed with propylene oxide and embedded in araldite resin using the method of Luft (1961).

Sections were cut with either a glass or diamond knife using a L.K.B. ultramicrotome and picked up on copper grids having either a collodion-carbon supporting film or no supporting film. Sections were stained by floating the grids on lead salt solutions (Millonig, 1961; Reynolds, 1963) for 1 min. followed by washing with distilled water. Some sections were floated on 7.5% H_2O_2 for 5 min. prior to lead staining to reduce the density of the background cytoplasm and thus increase the contrast of ribosomes (Silva, 1967). Some organisms were fixed with osmium tetroxide and postfixed with uranyl acetate according to the method of Kellenberger, Ryter & Sechand (1958). These organisms were then dehydrated and embedded as described above and sections were treated with Reynold's (1963) lead stain.

Sections were examined with a Philips EM200 electron

microscope equipped with a single condenser system and a 40μ objective operating at 60Kv.

EXPERIMENTAL

PART I. SURVIVAL OF STREPTOCOCCUS LACTIS

Growth of the organism. The physical and chemical properties of the growth environment not only determine the growth rate and chemical composition of bacteria but also profoundly affect their subsequent metabolism and survival when starved (Strange *et al.*, 1961). In the present study it was therefore necessary to define the growth conditions for *Streptococcus lactis* as completely as possible. Use of a chemically defined medium was impracticable for reasons already discussed. Preliminary experiments indicated that an amino acid-limiting medium produced lower growth rates and in view of the reports of unbalanced cell wall synthesis and subsequent lysis of *Streptococcus faecalis* in certain amino acid-limiting media (Shockman, Conover, Kolb, Phillips, Riley & Toennies, 1961), it was decided to use a lactose-limiting medium. The choice of lactose appears to have been particularly fortunate in view of the recent finding by Moustafa & Collins (1968) that growth of lactic streptococci on glucose resulted in lysis. It was established that glucosamine, which was essential for complete cell wall synthesis, was formed from galactose but not from glucose.

With an organism such as *S. lactis*, which produced large quantities of lactate during growth, some control over changing medium pH was necessary. Experiments were undertaken to determine the lactose and buffer concentrations required to obtain an adequate cell yield at the maximum growth rate with a minimum change in pH. Some results from typical experiments are listed in Table 6. From the limited number of experiments undertaken it would appear that bacterial mass yield was proportional to the amount of energy substrate used, as postulated by Bauchop & Elsden (1960) for anaerobic glycolysis (see Gunsalus & Shuster, 1961). The term 'molar growth yield coefficient (Y)', has been defined as the grams dry weight of

Table 6. Growth of *Streptococcus lactis* ML_3. The routine medium was used except for variation of the lactose and buffer components. Experimental media (200ml.) were inoculated with growth phase organisms from a similar medium and incubated at 30° in a water bath.

Lactose (%)	Buffer[a]	pH		Cell yield (mg./ml.)	Generation time (min.)[b]
		Initial	Final		
1.0	0.1M-PO_4	7.0	4.6[c]	1.09	52
1.0	0.2M-PO_4	7.0	5.3	1.21	79
1.0	0.1M-PO_4 +0.08M-$NaHCO_3$	7.3	6.4	1.32	61
0.5[d]	0.05M-PO_4 +0.05M-$NaHCO_3$	7.3	6.3	0.65	46

a — PO_4 buffer was Na_2HPO_4 -KH_2PO_4.

b — Mean time for doubling of turbidity in logarithmic growth phase.

c — Growth limited by pH, other cultures were lactose-limited.

d — Routine medium.

organisms produced per mole of substrate metabolized (Monod, 1949). The molar growth yield coefficient $\{Y_{glucose}\}$ and the ATP yield coefficient $\{Y_{ATP}\}$ for anaerobic glycolysis by S. faecalis were 21.0 ± 4.0 and 10.5 ± 2.0 respectively (Bauchop & Elsden, 1960). Growth of S. lactis in the routine medium gave $Y_{lactose} = 47$ which is similar to the value reported for Streptococcus diacetilactis (Harvey & Collins, 1962). These authors considered that this value was consistent with the utilization of more than 95% of the fermented lactose as an energy source, indicating that only a small amount of lactose carbon is used for cell synthesis. This $Y_{lactose}$ value for S. lactis also suggests that the Embden-Meyerhof pathway is the only major pathway for ATP production in this organism (see Oxenburgh & Snoswell, 1965).

Growth characteristics with the routine medium finally adopted are shown in Fig. 6. Growth was highly reproducible in this medium. The mean generation time (doubling of turbidity) of S. lactis ML_3 during the logarithmic growth phase in batch culture was 46 ± 4 min. Analyses showed that lactose was the growth-limiting substrate. The pH changed from 7.3 to 6.3 ± 0.1 at the end of growth and the cell yield was 0.65 ± 0.03 mg. dry wt. organism/ml. corresponding to about 5×10^9 cocci/ml.

Bacterial numbers in resuspended systems. It was important in the present investigation to determine whether the total cell numbers in a given suspension remained constant on prolonged incubation. Accordingly, total counts/ml. were made as well as viability counts by the method of Postgate et al. (1961), which determines only the ratio of viable/total organisms. Four washed suspensions (approx. 50 μg. dry wt. organisms/ml.) in phosphate buffer were sampled over a 28 hr. period. For total chain counts, quadruplicate samples from one suspension and single samples from the other suspensions were counted. The viable chain counts given in Table 7 are the means of plate counts from samples of all flasks diluted in duplicate and

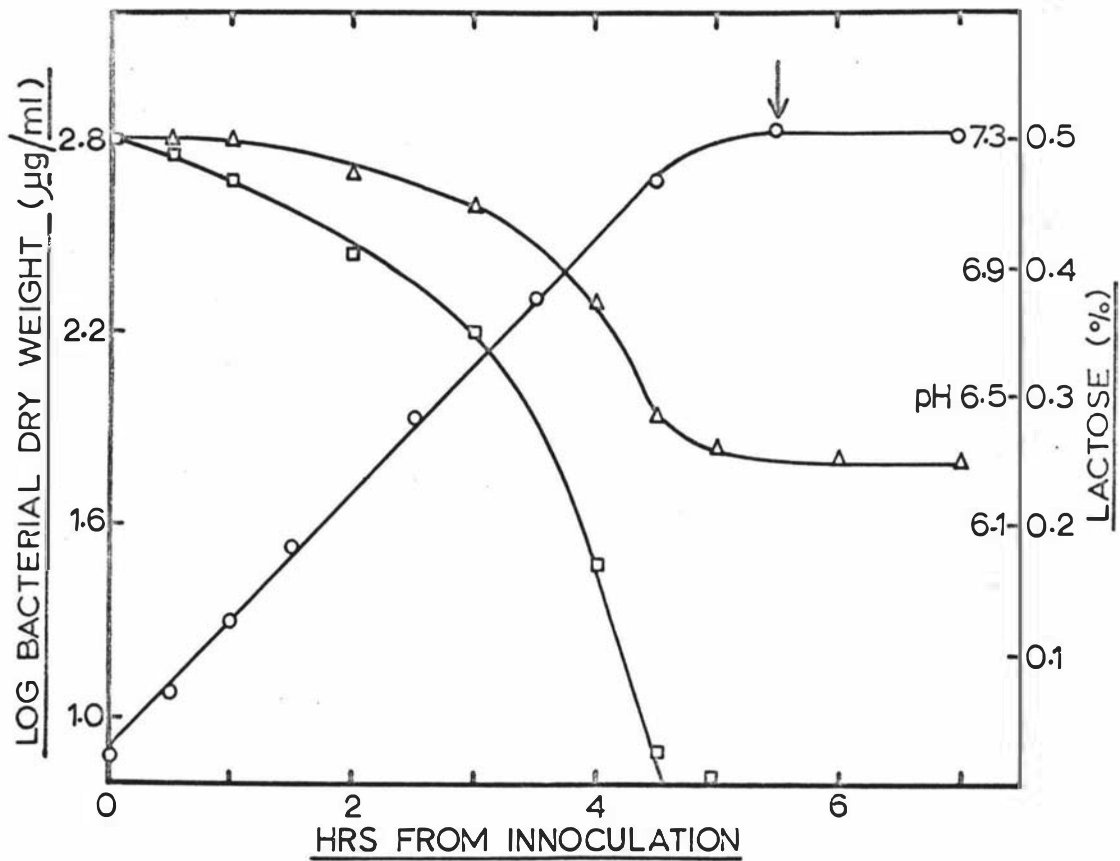


Fig. 6. Growth of Streptococcus lactis ML_3 in the routine medium. The medium was inoculated with log-phase culture: ○ , bacterial mass; □ , lactose concentration; △ , pH are shown. For subsequent experiments, organisms were harvested at the point on the growth curve indicated by the arrow, unless otherwise specified.

plated in duplicate (i.e., 16 plates). The number of cocci/chain and the % viability figures from slide-cultures are means of duplicate counts from each suspension (see Methods).

The results in Table 7 show that S. lactis ML_3 organisms had a tendency to clump in phosphate buffer on prolonged storage. This was the reason for the decrease in total chain counts but there was no significant decrease in total numbers of cocci for at least 28 hr. The increase in cell numbers in starved suspensions of Salmonella typhimurium (Schaechter, 1961) and Aerobacter aerogenes (Dean, 1967) was not observed with starved S. lactis. The 20% decrease in turbidity was probably a result of endogenous metabolism and leakage of cellular material into the suspending buffer. There appeared to be a constant systematic error in the % viability results obtained by normal methods, similar to that noted by Norris & Powell (1961), Postgate et al. (1961) and Strange et al. (1961). These low figures were probably the result of errors in the total count determination, since the survival curves were very similar when plate count viabilities were expressed as percentages of the initial plate count (taken as 99%).

The significance of viability measurements cannot be accurately assessed unless they are accompanied by statistical data. The slide-culture technique of Postgate et al. (1961) was evaluated for S. lactis ML_3 in the following way. Six washed suspensions were prepared in phosphate buffer (see Methods). Duplicate samples were removed from each suspension at times designed to give measurements at high, intermediate and low viabilities. Slide preparation and counting were performed as described in Methods. An analysis of variance was carried out on the % viability figures for each sampling time. The standard deviation from the mean was computed together with the 95% confidence interval for two slides prepared from one suspension (Fig. 7). At high viabilities (94-99%), variance between tubes was negligible and the accuracy of the method was high but variance increased with

Table 7. Constancy of total cell numbers and comparison of survival measurements on Streptococcus lactis ML_3 in washed suspensions at 30°. Organisms were harvested at the end of the growth phase, washed twice and resuspended in 0.075M-phosphate buffer (pH 7.0, containing 10 μM-EDTA) at approx. 50 μg. dry wt. organism/ml. with gentle agitation provided by a magnetic stirrer.

Suspension incubation time (hr.)	1	3	5	7	10	24	28	S.D.[a]
(1)Total chain count ($x10^{-8}$/ml.)	1.52	1.47	1.36	1.45	1.42	1.12	1.10	±0.07 (7)
(2)Viable chain count ($x10^{-8}$/ml.)	1.19	1.16	1.10	0.52	0.22	0.03		±0.08 (16)
(3)% Viability ((2)/(1) x 100)	78	79	81	36	15	3		
(4)% Viability (slide-culture)	99.5	98	96.5	65	28	6		b
(5)Cocci/chain or clump		2.64[c]		2.71		3.56	3.45	±0.05 (8)
(6)Total coccal count ((1).(5)$x10^{-8}$/ml.)		3.88		3.93		3.99	3.79	±0.22
(7)Turbidity	0.173	0.165	0.157	0.149	0.143	0.140	0.139	

a — Standard deviation (no. of measurements/sample).

b — See Fig. 7.

c — Calculated from chain length distribution, cocci/chain (% total chains); 1(2%), 2 (60%), 3 (17%), 4 (15%), 5 (5%), 6 (1%).

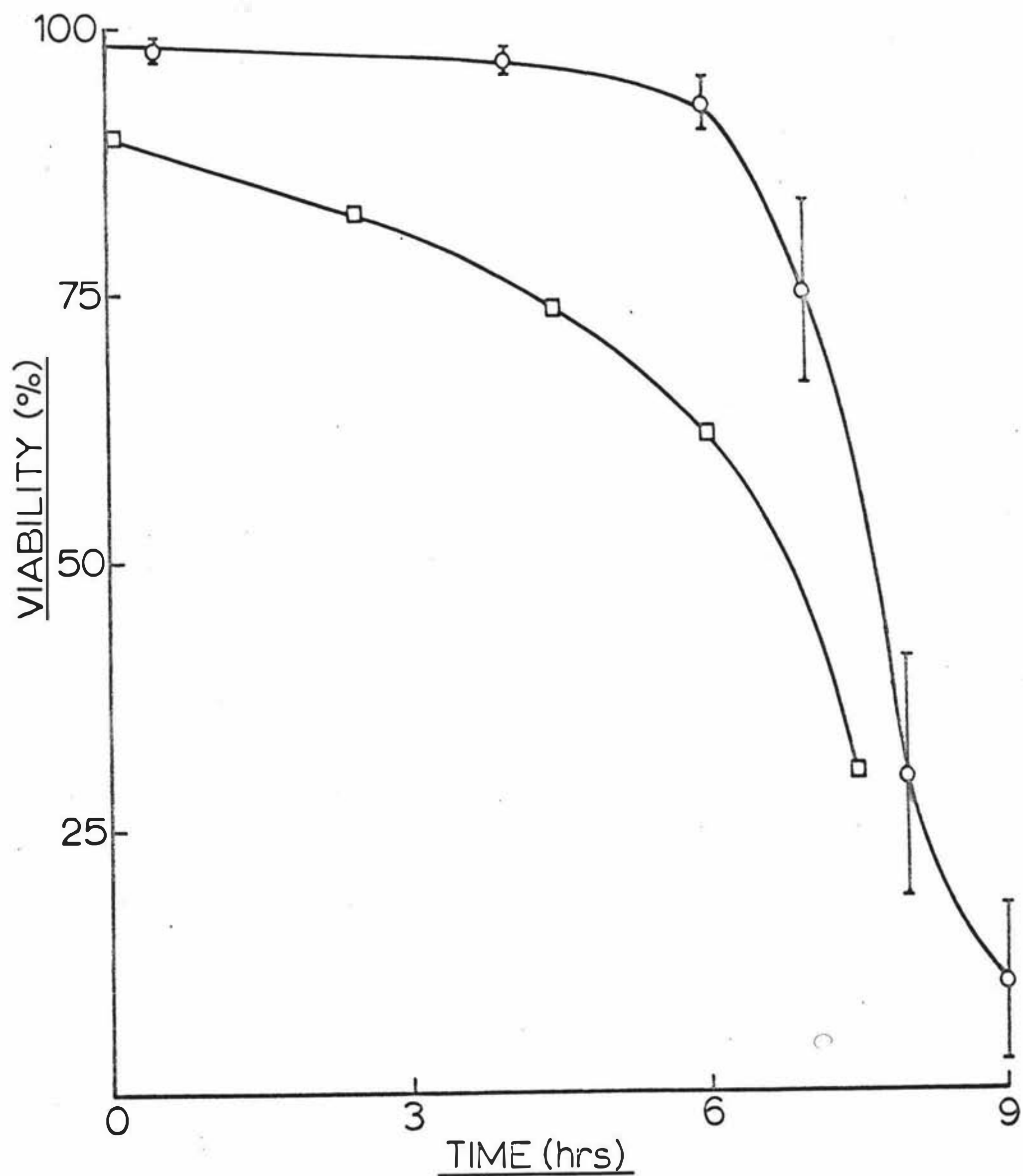


Fig. 7. Range of possible viabilities for starved Streptococcus lactis ML_3 using the slide-culture method. Mean viabilities, ○ , and the variance for 95% confidence limits with the routine counting procedure are indicated by vertical bars. For details see text and Methods. For a random distribution of viable cocci among chains, the estimated individual coccal viabilities are shown, □ (see text).

dying populations which had variable cell division times. Variance was largely a function of division lag time.

The viability measurements of Burleigh & Dawes (1967) on Sarcina lutea, and probably those of many other workers (for example Tempest et al., 1967), were complicated by clumping or aggregation of individual cells. Assuming the individual cells in a clump are independent and have varying capacities for survival then an erroneously high estimate of viability is obtained with any direct growth method. In this situation correlations between endogenous metabolism and survival are made more difficult. Similar problems occur with chain-forming bacteria and it is obviously preferable to determine the numbers of individual viable cells rather than viable clumps or chains.

Using the distribution of chain lengths after 3 hr. starvation (Table 7), estimates of the individual coccal viability were obtained using the probability equations described by Robertson (1968). A random distribution of viable cocci among chains was assumed. These estimates thus represent the minimum possible viability values of starved cultures (Fig. 7) and the slide-culture viability represents a maximum value (see Burleigh & Dawes, 1967). The difference between these extreme estimates can be considerable, even with short chain organisms such as S. lactis ML_3 (Fig. 7) but the death curve was very steep in most subsequent experiments. Starved S. lactis organisms showed a slight tendency to clump (Table 7) but the resulting change in chain (clump) size distribution had a negligible effect on the calculated individual coccal viabilities for at least 7 hr. If the individual cocci in a chain have varying capacities for survival, then as a population begins to die the dead organisms should have a higher proportion of short chains than the total population. However, from observations of dying populations on slide-cultures it appeared that the distribution of chain lengths of dead organisms was very similar to that of the total population. Attempts to obtain a statistical correlation

to substantiate this observation were unsuccessful due to the difficulty in differentiating between the individual cocci of dead chains on slide-cultures.

Survival in resuspended systems

Survival measurements on all resuspended systems were repeated at least three times. Survival curves showed small fluctuations with different batches of organisms but trends between systems were always the same.

Effect of ethylenediaminetetra-acetate (EDTA). Early experiments on survival in phosphate buffer gave variable results and rapid death rates which were decreased on addition of EDTA (Fig. 8). The minimum effective EDTA concentration was approximately 5 μM. High concentrations of EDTA (10 mM) accelerated death, possibly as a result of its capacity to destabilize cell walls (Gray & Wilkinson, 1965; Asbell & Eagon, 1966) or ribosomes (Wade, 1961). It seemed likely that a toxic metal impurity was present in the buffer (Postgate & Hunter, 1962). Extraction of phosphate buffers with organic metal complexing reagents (see Methods) and analysis by atomic absorption spectroscopy showed a copper concentration of about 0.06 p.p.m. (1 μM). When trace amounts of Cu^{2+} (10 μM) were added to suspensions containing 5 μM-EDTA, the survival curve was similar to that for buffer without EDTA (Fig. 9a). MacLeod, Kuo & Gelinas (1967) reported that contaminant Cu^{2+} in the plating diluent caused metabolic damage to A. aerogenes resulting in increased nutritional requirements (see also Burke & McVeigh, 1967).

Effect of divalent metal ions. The effect of divalent metal ions was studied with suspensions containing sufficient EDTA to remove the toxic effect of contaminant copper. Mercuric ions (1 μM) caused complete death within 5 min. while the toxicity of other ions tested decreased in the order $Cu^{2+} > Ni^{2+} > Fe^{2+} > Co^{2+} > Zn^{2+} > Pb^{2+}$ (Fig. 9a). This appears to be the approximate order of decreasing stability of the metal-EDTA complexes (Perrin, 1964) so that it is unlikely

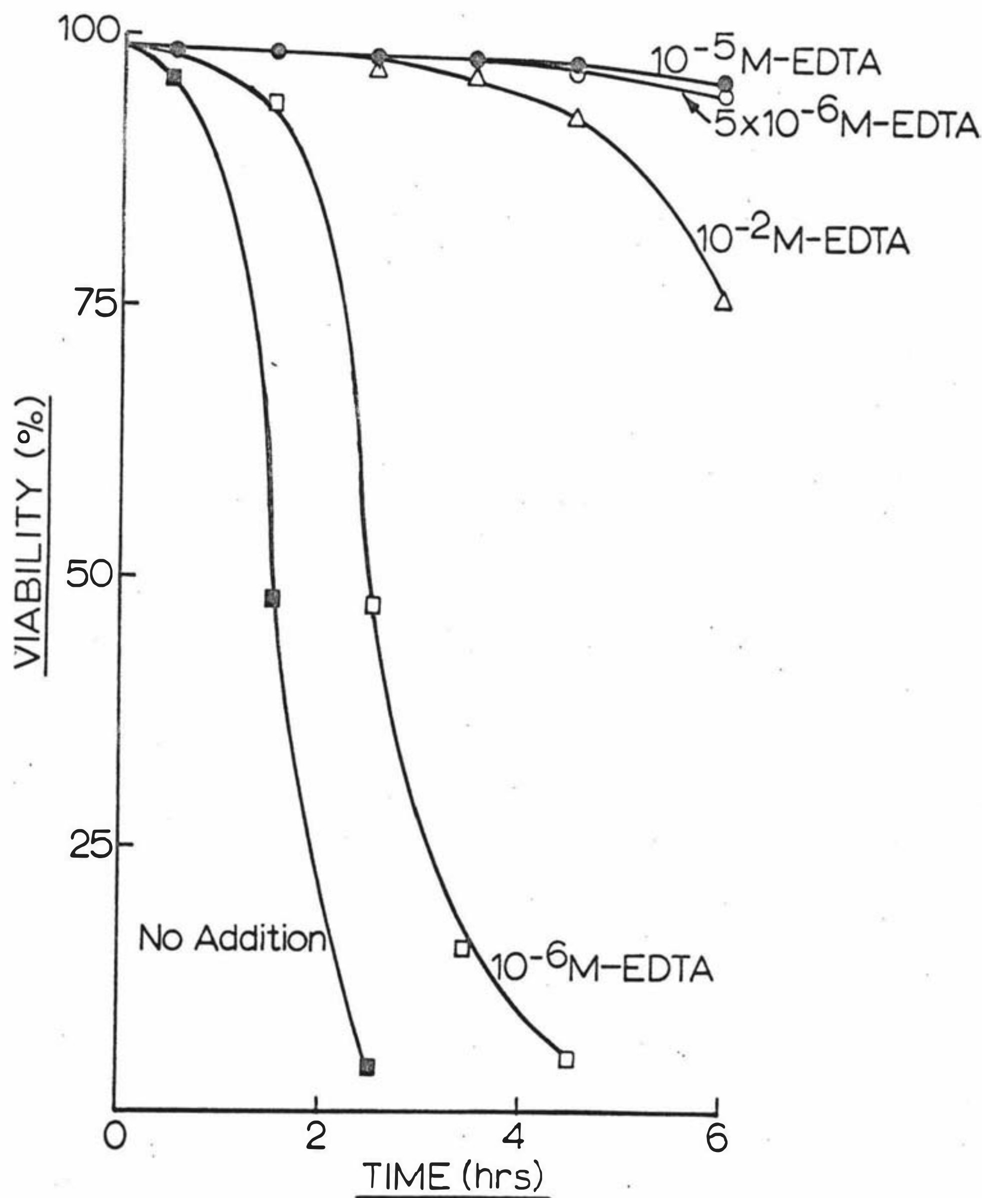


Fig. 8. Effect of EDTA on survival of Streptococcus lactis ML_3. Organisms were harvested at the end of the growth phase, washed and resuspended in 0.075M-phosphate buffer at approx. 20 μg. dry wt./ml. Organisms were incubated at 30°, pH 7.00 $\pm$ 0.05, without agitation (for details see Methods). Buffer contained the following concentrations of EDTA: △ , 10^{-2}M; ● , 10^{-5}M; ○ , $5x10^{-6}$M; □ , 10^{-6}M; ■ , no EDTA. Viabilities were determined by the slide-culture method.

that any of these ions displaced the contaminant copper from its complex. Calcium ions, Mn^{2+}, and Sn^{2+} (10 μM and 100 μM) were without effect.

Addition of Mg^{2+} produced greatly increased survival times (Fig. 9b), maximum survival being afforded with about 100 μM-Mg^{2+}. Magnesium ions also prolonged survival of organisms in phosphate buffer without EDTA. When organisms were incubated with Mg^{2+}+ Cu^{2+}, the toxic effect of Cu^{2+} was decreased (Fig. 9b). These observations are similar to those made by MacLeod & Snell (1950) for *Lactobacillus arabinosus* and by Abelson & Aldous (1950) for *Escherichia coli*. The experiments described show that added Mg^{2+} may produce two effects: (i) a decrease in Cu^{2+} toxicity and (ii) a separate effect decreasing the death rate.

Effect of bacterial concentration. Very dense suspensions (equiv. 7.8 mg. dry wt. organism/ml. or about 6×10^{10} cocci/ml.) survived best, with decreasing survival times at lower bacterial concentrations (Fig. 10a). Measurements of Mg^{2+} excretion by the organisms (see Methods), showed that at high bacterial concentrations Mg^{2+} leakage from the organisms was sufficient to produce protective concentrations in the suspending buffer (Fig. 10a). Analyses of whole bacteria immediately after washing in buffer indicated 0.41% (w/w) magnesium. The concentration of excreted Mg^{2+}(700 μM) in the suspending buffer containing an equivalent bacterial concentration of 7.8 mg. dry wt./ml., represented a loss of 0.22% (w/w) magnesium from the organisms (i.e. 54% of the cellular magnesium). From these results it seemed likely that Mg^{2+} excretion by the organisms was the cause of extended survival at high bacterial concentrations. Additional support for this conclusion was obtained in a similar experiment where the addition of 100 μM-Mg^{2+} gave almost identical survival curves at each bacterial concentration (Fig. 10b). High bacterial concentrations also prolonged survival in phosphate buffer without EDTA, presumably because the copper toxicity was decreased by the excreted Mg^{2+} in a similar

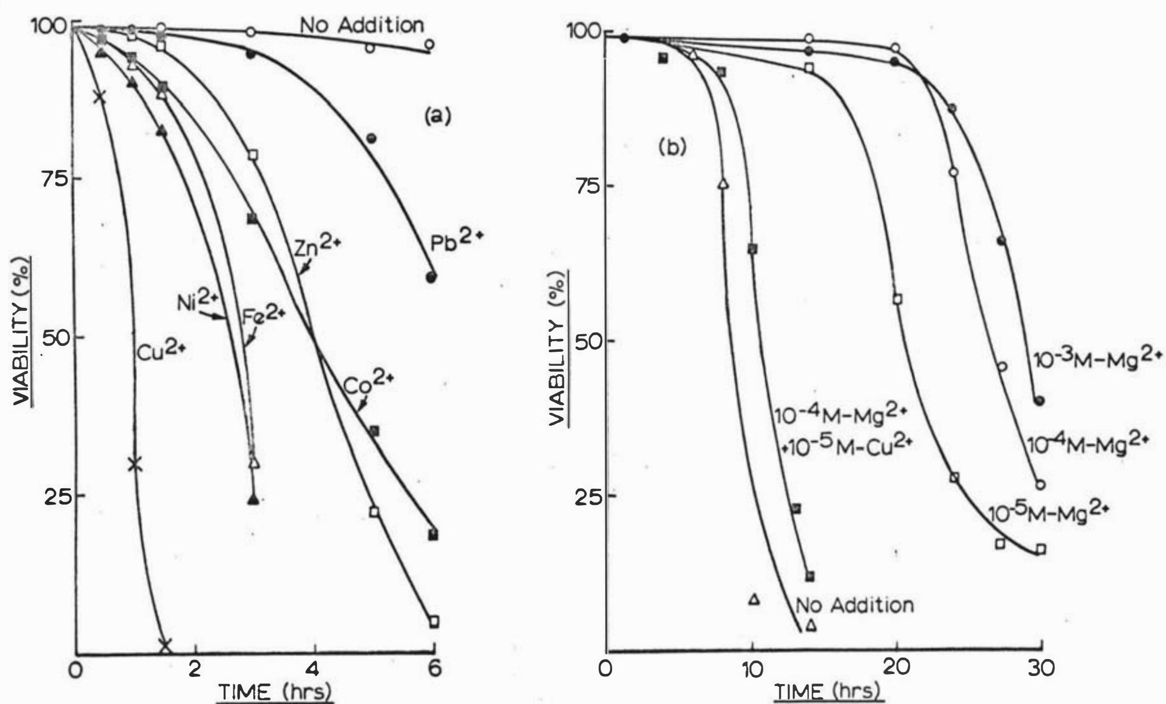


Fig. 9. Effect of divalent metal ions on survival of Streptococcus lactis ML_3. Cell suspensions were prepared as for Fig. 8. Tubes contained 5×10^{-5}M-EDTA in 0.075M-phosphate buffer. Part (a): ○ , no addition; ● , $+Pb(NO_3)_2$; □ , $+ZnSO_4$; ■ , $+CoCl_2$; △ , $+FeSO_4$; ▲ , $+NiCl_2$; × , $+CuSO_4$; all salts 10^{-5}M. Part (b): △ , no addition; remaining tubes contained $MgSO_4$ at concentrations; ● , 10^{-3}M; ○ , 10^{-4}M; □ , 10^{-5}M; ■ , 10^{-4}M + $CuSO_4$ (10^{-5}M).

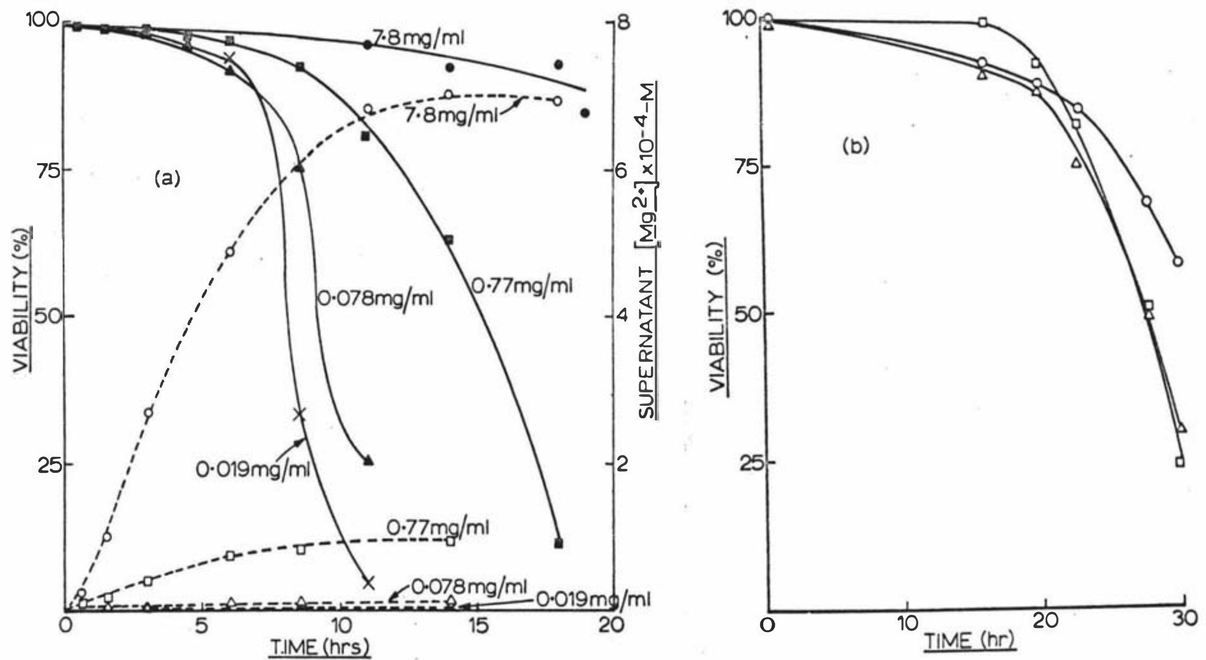


Fig. 10. Effect of cell concentration on survival of Streptococcus lactis ML_3. Cells were harvested from 500ml. of culture at the end of the growth phase, washed and resuspended in 60ml. 0.075M-phosphate buffer with 10 μM-EDTA. Part (a). Viabilities of washed suspensions (50ml.) containing 7.8mg. dry wt. bacteria/ml., 0.77mg./ml., 0.078mg./ml., and 0.019mg./ml., are shown ●, ■, ▲, and X respectively. Supernatant Mg^{2+} concentrations at the same cell densities were ○, □, △, and X. At intervals viability determinations were made, after sample dilution where necessary, and 5 ml. samples withdrawn from each suspension. The samples were centrifuged and the cell-free supernatants carefully removed and filtered through sintered glass (porosity 5) into tubes. These samples were frozen and later analysed for magnesium (see Methods). Part (b). Cell suspensions were prepared as in Part (a) containing; ○, 8.1 mg./ml., □, 0.80mg./ml., △, 0.079mg./ml. Each suspension contained 100 μM-Mg^{2+}.

manner to that described in the preceding section.

Effect of growth phase and media composition. The survival of organisms taken from different growth phases in the lactose-limiting medium were compared. Organisms from the mid-logarithmic phase, the end of the growth phase and from 1 and 2 hr. after growth had ceased, showed no measurable differences in the survival curves. This is in agreement with the observations of Postgate & Hunter (1962) who stressed the importance of the nutritional status of the population and suggested that for a genetically uniform population the growth phase has only a small effect on the survival time during starvation. Attempts to find a satisfactory non-carbohydrate-limiting medium were unsuccessful. When the lactose concentration was increased to 1%, growth ceased because of the inhibitory acid conditions produced (about pH 4.7); analyses indicated the presence of 0.08% lactose at the end of growth. These organisms, when washed and resuspended, showed a slightly increased death rate compared with organisms from the routine medium. When the amino acid concentration was decreased from 0.5% to 0.05% the growth rate was substantially decreased (doubling time 2 - 2.5 hr. compared with about 46 min. for the routine medium), and resuspended organisms from this medium also showed higher death rates than normal (50% decrease in viability in 4.5 - 5 hr.). Postgate & Hunter (1962) quoted death rates in %/hr. since their survival curves for starved *A. aerogenes* tended to be linear. Survival curves for *S. lactis* were generally sigmoid in shape, so that it is probably more reasonable to quote the death rate as the time taken to reach 50% viability.

Effect of salt concentration. In de-ionized water containing 10 μM-EDTA the viability of resuspended organisms decreased to 50% in 3.6 hr. In Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) containing 10 μM-EDTA, survival times decreased slightly with increasing buffer concentration. Organisms in phosphate buffer at 0.075M, 0.150M and 0.300M decreased to 50% viability in 7.2 hr.,

6.1 hr., and 6.0 hr., respectively. Ringer solution, with and without 10 μM-EDTA, gave 50% viabilities after 6.7 hr., and 3.8 hr. All times were $\pm$ 0.3 hr. for replicate samples.

Effect of pH value. In phosphate-citrate buffer at 30°, *S. lactis* ML_3 had a sharp pH optimum for survival near 7.0 (Fig. 11a). Survival times decreased sharply on decreasing the pH value and a 50% decrease in viability occurred in 0.5 hr. at pH 4.0. It was therefore of interest to re-examine the observations of Harvey (1965) who concluded that *S. lactis* ML_3 organisms held in a broth growth medium adjusted to pH 4.2 were damaged but maintained complete viability for 5 hr. Organisms were grown and placed in a broth medium adjusted to pH 4.2 as described by Harvey (1965). It was found that 97-99% of the organisms survived for at least 6 hr. This indicated a very marked protective effect of nutrients at low pH values. Organisms harvested from Harvey's growth medium (pH 6.5) and starved in phosphate buffer gave similar survival times to washed organisms from the routine medium starved in phosphate buffer.

Since Dawes & Ribbons (1962, 1964) have suggested that an energy source is required for intracellular pH control in starved bacteria it seemed appropriate to re-examine the pH effect on *S. lactis* in the presence of an added energy source. As addition of either carbohydrate or arginine alone produced accelerated death of starved *S. lactis* organisms (see Fig. 14), it was decided to re-examine the pH effect with added Mg^{2+}, which abolished arginine-accelerated death (see Fig. 14b), in addition to incubations with arginine + Mg^{2+}. Addition of Mg^{2+} alone decreased the death rate at all pH values (Fig. 11b), while arginine + Mg^{2+} produced a further remarkable decrease in the death rates (Fig. 11c). Note the different time scales in Fig. 11a, b and c. Results are summarized in Fig. 11d. The initial pH values in Fig. 11a, b, did not change significantly over the starvation period. After 48 hr. starvation with 10mM-arginine (Fig. 11c), the

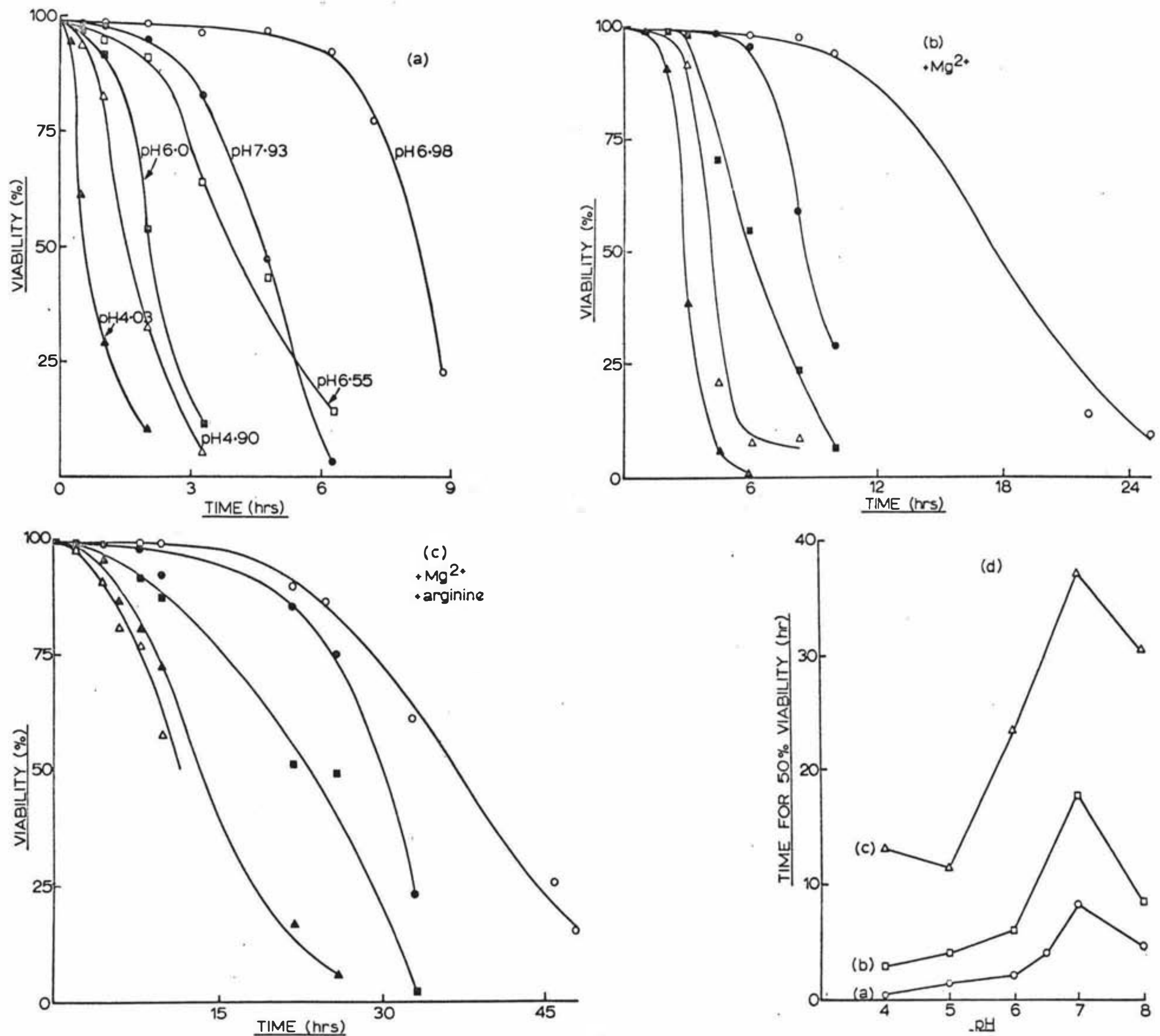


Fig. 11. Effect of pH on survival of <u>Streptococcus lactis</u> ML_3. Buffers were prepared from 0.1M-Na_2HPO_4 and 0.05M-citric acid containing 10 μM-EDTA. Part (a). Bacteria were harvested from 20ml. culture at the end of the growth phase, washed and resuspended in 5ml. phosphate-citrate buffer (pH 7.0), 0.5ml. of this suspension was added to 10ml. buffer at 30° to give 190 μg./ml. and final pH values ($\pm$0.05) of: ● , 7.93; ○ , 6.98; □ , 6.55; ■ , 6.00; △ , 4.90; ▲ , 4.03. Part (b). Suspensions prepared as in Part (a) at similar pH values. Each suspension contained 1 mM-Mg^{2+}. Part (c). Suspensions prepared as in Part (a) but at 80 μg. dry wt. bacteria/ml. The buffer contained 10mM-arginine and pH values were adjusted to those above by addition of HCl. Part (d). Summary of results from Parts a, b, and c. Before viability determination samples were diluted in phosphate-citrate buffer containing 1 mM-Mg^{2+} (pH 7.0).

initial pH values of 7.98, 6.98, 6.05, 4.92 and 4.05 had changed to 8.16, 7.08, 6.14, 5.11 and 4.19 respectively. This was presumably due to ammonia production.

Harvey (1965) reported that when growing S. lactis ML_3 organisms were subjected to rapid pH changes from 4.2 - 4.7 to 5.2 - 6.3, a lag resulted before the normal growth rate for the new pH was assumed. This lag did not occur when the initial pH was 5.0 or above. It was suggested that growth below pH 5.0 resulted in some unspecified damage to the cells. Growth was measured by turbidity increase. Similar experiments have now been repeated with additional measurements of total chain counts, plate counts and slide-culture viability. While the turbidity increased by about 50% in 6 hr. at pH 4.2, there was no significant increase in total chain counts or plate counts (Fig. 12). No aggregation of cells was evident so presumably cell growth and division were no longer in a steady state. Synthesis of important cell components at very different rates is known to occur when certain nutritional or physiological imbalances exist in a growth medium (see Duguid & Wilkinson, 1961; Shockman, 1965; Bazill, 1967; Elsden, 1967). Assuming that a mass increase occurred without cell division at low pH, then transfer to a medium with a pH allowing cell division would be expected to produce a lag in the growth curve until the normal cell size distribution was resumed. If the growth curve is extrapolated back to the 'normal turbidity' corresponding to the total cell numbers at zero time, then the lag is partially eliminated. Further, the plots of total chain counts and plate counts do not show corresponding lags. Results of a typical experiment are shown in Fig. 12. This may partially explain some of the results reported by Harvey (1965).

Effect of temperature. The death rate decreased markedly on lowering the incubation temperature of washed suspensions from 45° to 3° (Fig. 13a,b). Note the time scale for Fig. 13b is ten times that for Fig. 13a.

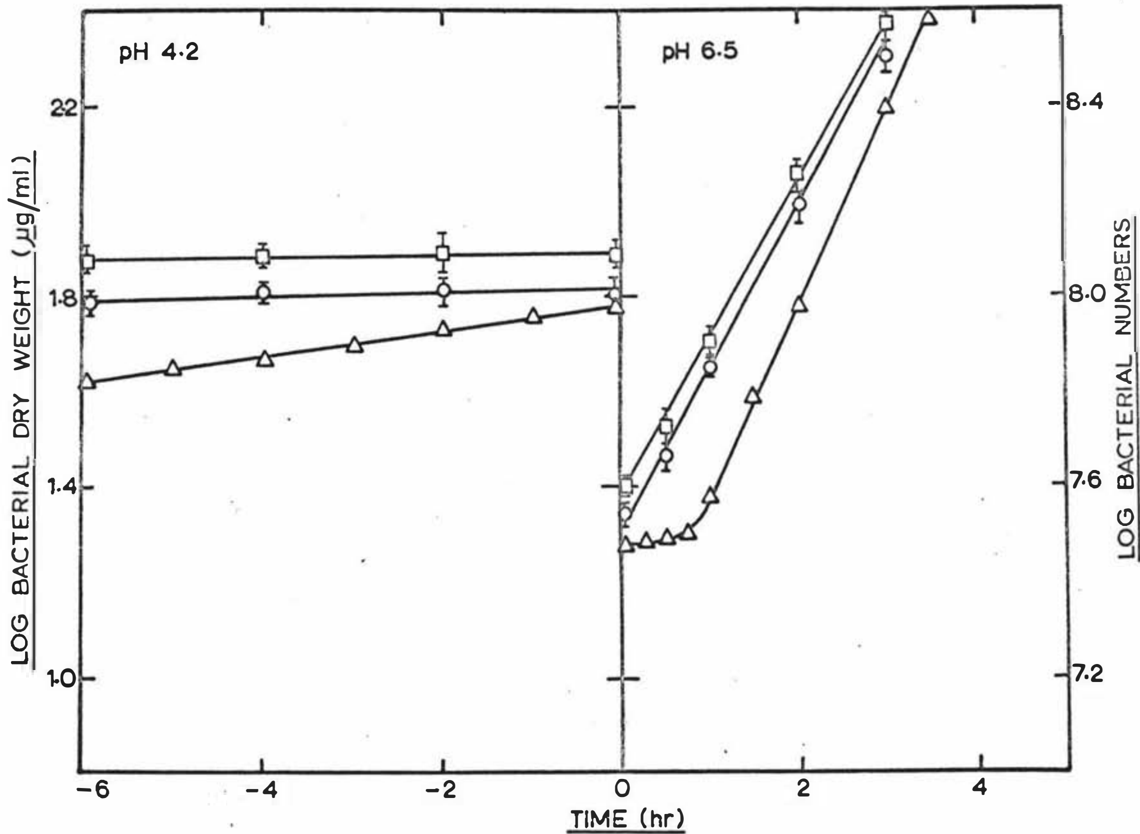


Fig.12. Effect of growth pH on Streptococcus lactis ML_3. Organisms were grown, harvested and subjected to pH changes by addition of fresh medium as described by Harvey (1965). Measurements were made of bacterial mass, △ ; total chain count, □ ; and plate count, ○ ; as described in Methods. Some of the samples were cooled and stored at 4° for short intervals before total count determinations. Vertical bars represent standard deviations from the mean, total counts were determined in quadruplicate and ten plates were counted for each determination. Slide-cultures indicated 97-99% viability throughout the experiment.

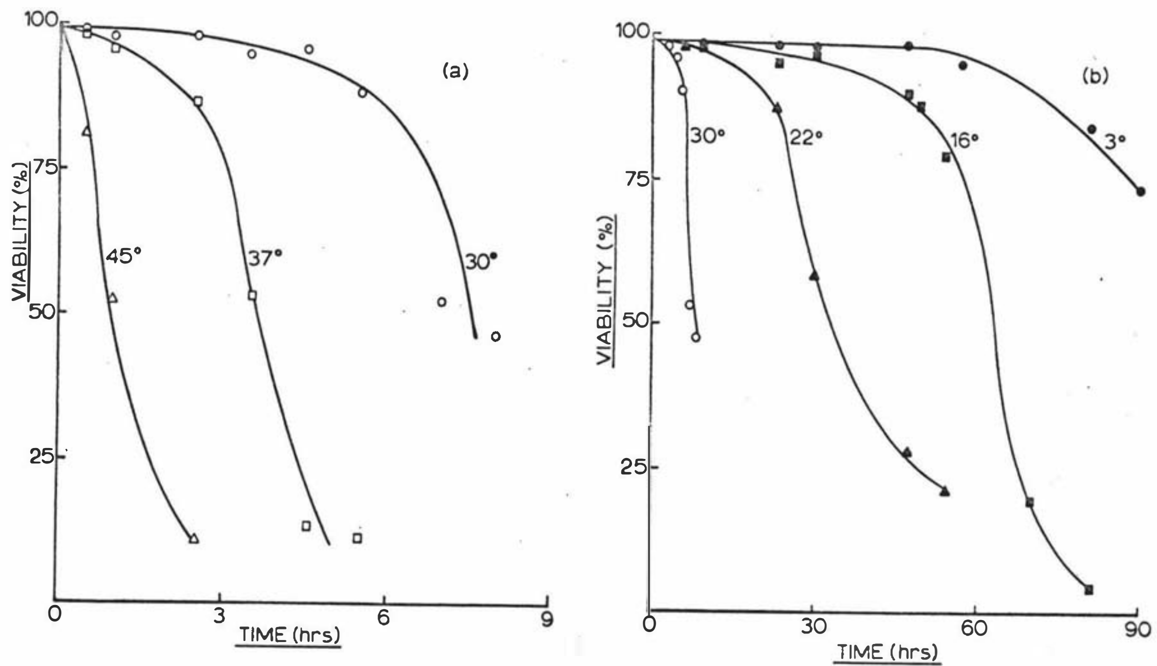


Fig. 13. Effect of temperature on survival of Streptococcus lactis ML_3. Cell suspensions were prepared as for Fig. 8, the washed cells being inoculated into phosphate buffer equilibrated to the storage incubation temperature. Part (a): △ , 45°; □ , 37°; ○ , 30°. Part (b): ○ , 30°; ▲ , 22°; ■ , 16°; ● , 3°. Temperatures are $\pm$ 1°, slide-cultures were incubated at 30°.

Effect of atmosphere and agitation. Both air and commercial 'O_2-free' N_2 increased the death rate when gently bubbled through washed suspensions (50% decrease in viability in 4.5 - 5 hr. for air and in 3 - 3.5 hr. for N_2). Addition of sodium thioglycollate (20 mM) to static suspensions did not affect the death rate. Toxic H_2O_2 accumulated when growth cultures of S. lactis organisms were aerated (Dr. G. R. Jago, pers. comm.). However, it seems unlikely that sufficient H_2O_2 would accumulate in starved suspensions, at low bacterial densities, to increase the death rate. The results imply that special E_h values were required for survival, as would befit a micro-aerophilic organism.

Gentle agitation produced by a magnetic stirrer had no measurable effect on survival curves compared with those of static suspensions, but vigorous agitation (magnetic stirrer) generally gave more rapid death (50% decrease in viability in 2-3 hr.). Vigorous agitation did not appear to produce chain breakage but may have increased the rate of leakage of soluble intracellular components. The agitation effect appears to be distinct from the aeration effect.

Effect of metabolic inhibitors. The addition of 0.01mM-iodoacetate decreased survival times sharply (Table 8). Methylene blue at concentrations above 0.02mM showed an immediate bactericidal effect, while at 0.001mM the death rate increased slightly. The addition of 2.7mM-sodium arsenate and 2.4mM-sodium fluoride also produced a slight increase in death rate. Sodium azide, 2.4-dinitrophenol, potassium cyanide and sodiummalonate did not significantly influence the survival curves, although all experiments were made under conditions of low O_2-tension. Before slide-cultures were prepared the inhibitors were removed by washing (see Methods), this procedure had no noticeable effect on the viability of control populations.

Table 8. Effect of metabolic inhibitors on survival of *Streptococcus lactis* ML_3 in buffered suspensions at 30°. Organisms were harvested at the end of the growth phase, washed twice and resuspended in 0.075M-phosphate buffer at equiv. 20 μg. dry wt. organism/ml. Buffer (pH 7.0, except where indicated) contained 10 μM-EDTA and inhibitor as below.

Inhibitor	Concentration (mM)	Death rate[a]
None (pH 7.0)		7.2
None (pH 6.5)		6.0
2,4-Dinitrophenol	1.10	6.8
	0.11	6.6
Iodoacetic acid[b]	0.11	1.1
	0.01	1.9
Methylene blue	0.010	2.9
	0.005	5.0
	0.001	6.7
Potassium cyanide	1.54	7.0
	0.77	6.8
Sodium arsenate	2.70	6.1
	0.54	6.8
Sodium azide	1.54	6.8
	0.15	7.5
Sodium fluoride	2.38	6.9
	0.24	7.3
Sodium malonate[b]	6.75	6.3
	0.67	6.0

a — Time (hr.) for 50% decrease in viability ($\pm$ 0.3 hr.).

b — Buffer adjusted to pH 6.5.

Effect of added carbohydrates. Lactose, glucose, galactose and fructose (10 mM; Fig. 14a) produced markedly increased death rates in washed suspensions (50% decrease in viability in 1 - 1.5 hr.) irrespective of the growth phase of the organisms and the limiting nutrient (50% decrease in viability in 0.5 - 0.7 hr. for organisms from amino acid-limited medium). The suspension remained at pH 7.0 and analyses for lactic acid indicated that fermentation of all carbohydrates took place. Sodium lactate (10 mM) had no effect on survival times. Accelerated death was markedly reduced in all cases on addition of 1 mM-Mg^{2+}, giv ing survival times similar to control systems without carbohydrate or Mg^{2+} (Fig. 14a). Addition of 10 mM-ribose had no effect. Analysis for lactic acid showed that *S. lactis* ML_3 did not ferment exogenous ribose to lactate in washed suspensions. A population density effect, similar to that shown in Fig. 10a, was observed in the presence of added glucose (10 mM).

Effect of added amino acids and other growth medium components. Organisms resuspended in routine medium without lactose, vitamins and $NaHCO_3$, survived for a longer period than organisms resuspended with Mg^{2+} only (Fig. 14b). In the former system no significant change in the total number of cocci occurred up to 23 hr. in two suspensions containing 100 μg. dry wt. organism/ml., one suspension contained penicillin G to prevent cell growth and provide a control (Table 9). These results indicate that cell division or cryptic growth is unlikely to occur in starved suspensions of *S. lactis* ML_3.

Casamino acids + Mg^{2+} gave equivalent survival as in routine medium without lactose, vitamins and $NaHCO_3$, while arginine + Mg^{2+} was almost as effective (Fig. 14b). Arginine produced accelerated death in the absence of added Mg^{2+}(Fig. 14b). Alanine, aspartic acid and glutamic acid, which were subsequently found to comprise most of the free amino acid pool in these organisms, produced a marginal increase in survival time when incubated together with washed organisms +Mg^{2+}. Glycerol + Mg^{2+} also produced a marginal increase in survival time

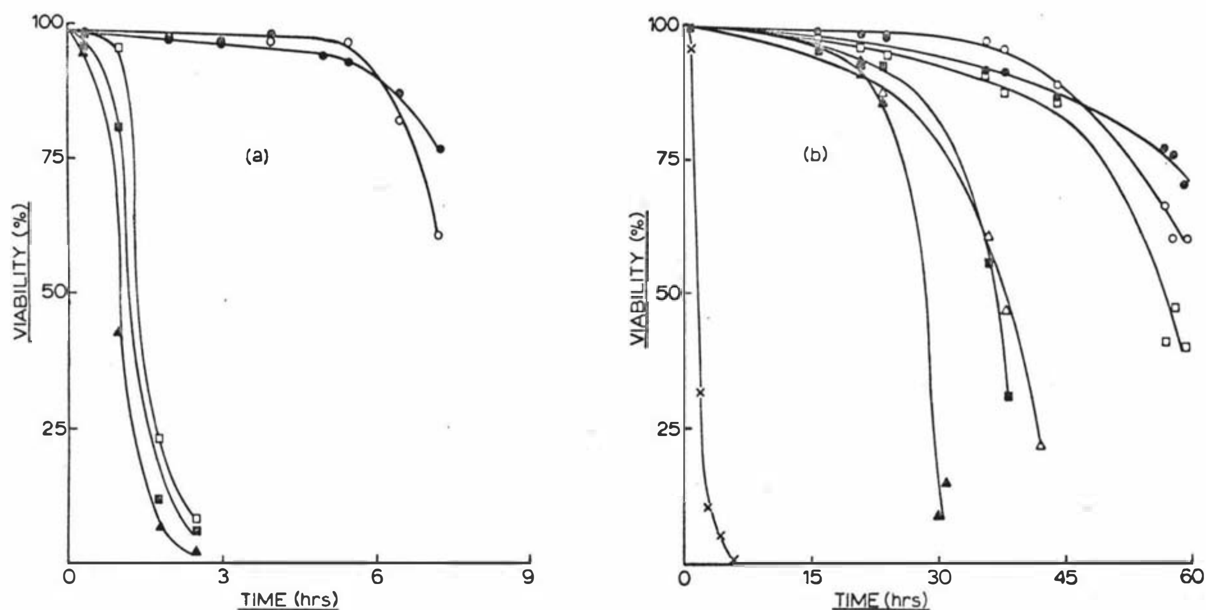


Fig. 14. Effect of nutrients on survival of <u>Streptococcus lactis</u> ML_3. Cell suspensions were prepared as for Fig. 8 in phosphate buffer containing 10 μM-EDTA. Part (a): ○ , no addition; ▲ , + lactose; ■ , + glucose; □ , + galactose; ● + lactose + Mg^{2+} (1 mM). All carbohydrate supplements were 10mM. Part (b): ▲ , + Mg^{2+}; ○ , + Casamino acids (Difco, 0.5%) + Mg^{2+}; □ , + arginine (10mM) + Mg^{2+}; × , + arginine (10mM); ■ , + alanine, aspartic acid, glutamic acid (each 10mM) + Mg^{2+}; △ , + glycerol (10mM) + Mg^{2+}; (all Mg^{2+} supplements 1mM); ● , growth medium minus lactose, vitamins and $NaHCO_3$.

Table 9. Constancy of total cell numbers of <u>Streptococcus lactis</u> ML_3 starved in the presence of amino acids. Suspensions were prepared as described in Table 7 at approx. 100 μg. dry wt. bacteria/ml. Resuspension buffers contained 0.075M-phosphate (pH 7.0), 10 μM-EDTA, 1mM-$MgSO_4$, plus the following additions; **A**, no addition; B, casamino acids (Difco, 0.5%) + arginine (10mM); C, as for B + penicillin G (100 units/ml.). Turbidity and total cocci were determined as described in Methods, standard deviations are for four determinations.

	Additions to above buffer				
	None (A)	Amino acids (B)		Amino acids + penicillin (C)	
Incubation time (hr.)	Turbidity	Turbidity	Total cocci ($\times 10^{-8}$)	Turbidity	Total cocci ($\times 10^{-8}$)
0.25	.359	.355		.358	
1.5	.346	.349	8.27 ± .23	.351	8.01 ± .25
4.5	.320	.331		.334	
10	.285	.317		.311	
23	.268	.293	8.04 ± .31	.282	8.13 ± .27
29	.263	.291		.278	
47	.141	.272	7.47 ± .44	.253	7.39 ± .52
53	.130	.260		.222	

Note: Microscopic examination revealed some cell lysis in all suspensions at 47 hr.

while 10 mM-sodium acetate + 1 mM-Mg^{2+} was ineffective (Fig.14b). Washed organisms incubated with the vitamins, purines and pyrimidines supplied at the routine medium concentrations were without effect in the presence of 1 mM-Mg^{2+}.

Growth characteristics of survivors. Surviving organisms from some resuspended systems had considerably longer division times than exponentially growing organisms. These times were measured (as shown in Fig. 15b) in four resuspended systems giving the maximum scatter in survival times. Division lags tended to increase just before the onset of bacterial death, the increase being particularly sharp in systems without Mg^{2+} (Fig. 15a). This method gives only an approximate division lag time and errors are likely to be considerable at low viabilities (see Fig. 7).

In most experiments where organisms were incubated in the absence of Mg^{2+}, surviving organisms on slide-cultures showed a large proportion of morphologically aberrant colony forms once the starved organisms started to die; instead of the typical circular colonies many organisms produced elongated colonies and these organisms generally showed longer division lags.

PART II. CHANGES IN VIABLE ORGANISMS IN STARVATION CONDITIONS

i) Chemical Studies

'Reserve' polymers. Organisms were harvested from the end of the growth phase in routine medium and extracted for polyglucose and poly-β-hydroxybutyrate (PHB) as described in the Methods. Subsequent analysis of the extracts revealed no trace of either polymer. However, the routine medium was lactose-limiting so that optimum conditions may not have existed for polymer accumulation. When organisms were harvested at the end of the growth phase and resuspended in the routine medium minus the casamino acids and peptone components, no detectable synthesis of either polyglucose or PHB occurred. Conditions similar

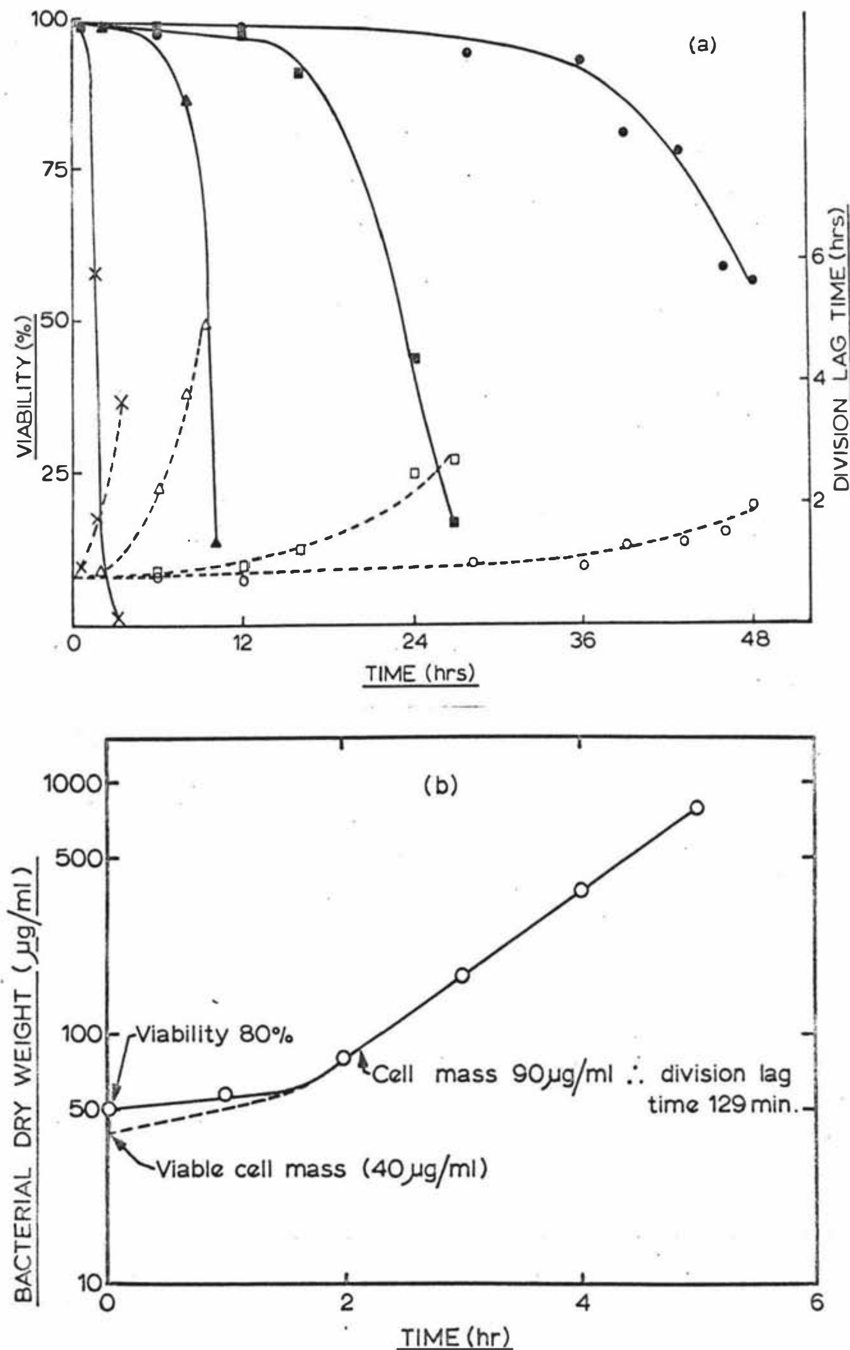


Fig. 15. Division lag times of surviving Streptococcus lactis ML_3 organisms in various resuspended systems. Cell suspensions were prepared as for Fig. 8 in phosphate buffer containing 10 μM-EDTA. Part (a). Viabilities for the suspensions: + 0.5% Casamino acids (Difco) + 1mM-Mg^{2+}; + 1mM-Mg^{2+}; no addition; + 10mM-lactose; are indicated ●, ■, ▲, ×, respectively. Division lags of surviving cells in the same systems are indicated ○, □, △, ×. Part (b). Method of calculation of division lag time.

to these were found to be optimal for intracellular polyglucose synthesis in *Streptococcus salivarius* (Hamilton, 1968). Therefore it seemed unlikely that *S. lactis* was capable of synthesizing these polymers which are found in many bacterial species. Electron micrographs of thin sections showed no evidence of polyphosphate granules.

Oxygen uptake studies. Spendlove, Weiser & Harper (1957) claimed that a strain of *S. lactis* was capable of 'active aerobic respiration' and that starved organisms began to 'oxidize some endogenous substrate after a lag'. It was suggested that this substrate was either lactate or succinate. In view of this unconfirmed report it was decided to measure the oxygen uptake of *S. lactis* ML_3 in the presence of various substrates. The oxygen uptake of organisms starved in phosphate buffer was insignificant, and of the substrates added only gluconate and pyruvate produced significant oxygen uptakes (Table 10) although other substrates may not have been taken up by the cells. During the fermentation of added carbohydrates, variable but small amounts of oxygen were taken up by the organisms (Table 11). The oxygen uptakes measured in the presence and absence of glucose were similar to those reported for *S. diacetilactis* (Oberman, 1962).

Changes in bacterial protein and total N. Only slight, if any, net protein breakdown occurred in starved suspensions (Fig. 16). Protein was estimated by the biuret method, which measures peptide bonds and by the Folin-Ciocalteu procedure which measures tyrosine or indolyl residues (see Methods). Only traces of free tyrosine were released from starved organisms (Table 13). Protein accounted for 48% of the initial bacterial dry wt. and starvation for the 28 hr. period resulted in a 26% bacterial mass loss (Fig. 16).

Soluble protein (0.22 mg./ml.), amounting to 10% of the total bacterial protein, was released from starved organisms into the external medium (Fig. 16). The total N in this culture was constant at 0.49 ± 0.018 mg./ml. and the amount

Table 10. Oxygen uptake by <u>Streptococcus lactis</u> ML_3 starved in presence of potential substrates. Each Warburg flask contained 15 mg. dry wt. washed bacteria, 30 μmoles substrate and 1mM-$MgSO_4$ in 3.0 ml. 0.075M-phosphate buffer (pH 7.0)

Substrate	Total O_2-uptake (μl.) 1 hr.	2 hr.	3 hr.	6 hr.
-	8	11	15	18
Acetate	7	9	15	21
Butyrate	5	8	14	22
Citrate	8	11	14	25
Glycerol	5	6	8	15
Gluconate	21	53	82	99
Lactate	7	9	12	20
Malate	8	12	14	16
Propionate	6	8	11	19
Pyruvate	33	48	65	83
Succinate	9	11	14	23

Table 11. Oxygen uptake by Streptococcus lactis ML_3 starved in the presence of carbohydrates. Each Warburg flask contained 5 mg. dry wt. washed organisms, 10 μmoles substrate (except where specified) and 1mM-$MgSO_4$ in 3.0 ml. 0.075M-phosphate buffer (pH 7.0).

Substrate	Total O_2-uptake (μl.)				
	10 min.	30 min.	60 min.	120 min.	240 min.
-	2	2	2	3	5
Glucose	19	28	32	36	39
Gluclose (30 μmole)	24	53	86	98	105
Galactose	33	87	135	174	191
Lactose	27	66	87	95	97
Fructose	23	54	98	149	165
Maltose	17	24	27	31	32
Arabinose[a]	1	3	3	4	5
Ribose[a]	0	2	3	3	6

a — Substrate not fermented.

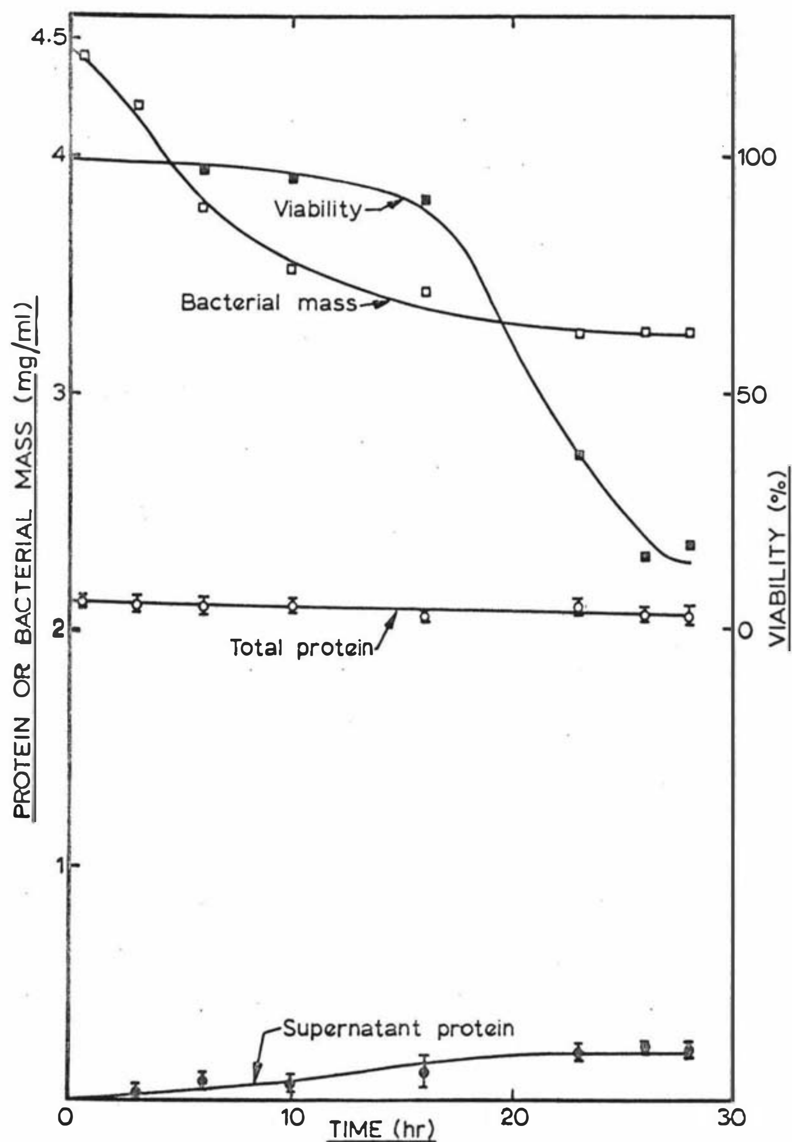


Fig. 16. Changes in protein in starved Streptococcus lactis. Bacteria were harvested from the end of the growth phase, washed and resuspended at 30° in phosphate buffer (0.075M, pH 7.0, containing 1mM-$MgSO_4$ + 10 μM-EDTA). At the times indicated, samples of the suspension were removed and a portion immediately deep-frozen together with supernatant samples which were obtained after centrifugation and filtration. Protein analyses were performed on whole suspension (○) and supernatant samples (●). Vertical bars represent S.D. from mean for 4 determinations. At each sampling time, bacterial density (□) was determined by both turbidity and dry wt. measurement, and viability (■) by slide-culture.

of N released into the suspending buffer after 28 hr. starvation (0.091 ± 0.016 mg./ml.) suggested that a considerable amount of non-protein N was involved (protein release accounted for approx. 0.02 mg.N/ml.). The turbidity of supernatant samples was always less than 0.005 (initial turbidity of the whole suspension was 23.1). Protein release appeared to be reduced at about the same time as the death rate increased. However, the rate and amount of protein released was not influenced by the presence of exogenous amino acids which reduced the death rate (Table 12). The protein released from S. lactis organisms after 18.5, 23 and 42 hr. starvation in the experimental systems (1) and (2) (Table 12) was assayed for proteinase and tributyrinase activity (see Methods). However, no detectable activity was found.

Release of amino acids and ammonia. The intracellular amino acid pool of S. lactis accounted for more than 3% of the dry wt. of freshly suspended S. lactis organisms and this pool was rapidly depleted on starvation (Fig. 17). Ninhydrin-reactive material appeared concurrently in the external medium and there was a net increase in the total free amino acids on starvation. A chromatographic investigation of the ninhydrin-reactive material was undertaken. Organisms were always thoroughly washed to avoid carry over of amino acids from the growth medium.

By two-dimensional thin-layer and paper chromatography the major components of the intracellular pool were tentatively identified from standard R_F values as alanine, aspartic acid, glutamic acid, glycine and threonine. The glutamic acid spot was the most intense. These observations on the composition of the amino acid pool are similar to those reported by Bottazzi (1959) for S. lactis and by Holden (1962a) for various species of lactic acid bacteria. However, it is well established that the amino acid pool composition of an organism may be markedly influenced by the growth medium composition. There appeared to be a progressive loss of

Table 12. Release of protein from starved *Streptococcus lactis*. Suspensions were prepared as for Fig. 16 at an initial density of 4.5 mg. dry wt./ml. but with organisms resuspended in (1) 0.075M-phosphate buffer + 1mM-$MgSO_4$ + 10 μM-EDTA and (2) as for (1) + casamino acids (0.5%, Difco) + arginine (0.1%). Supernatant protein was precipitated with 5% TCA, washed and estimated by the method of Lowry *et al*. (1951).

Starvation period (hr.)	Suspension (1)		Suspension (2)	
	Viability (%)	Super-natant protein[a]	Viability (%)	Super-natant protein[a]
2.5	99	.03	-	.02
6	98	.06	99	.06
18.5	93	.17	99	.17
23	71	.20	97	.21
42	0	.29	48	.28

a — Expressed as mean mg./ml. of 4 determinations.

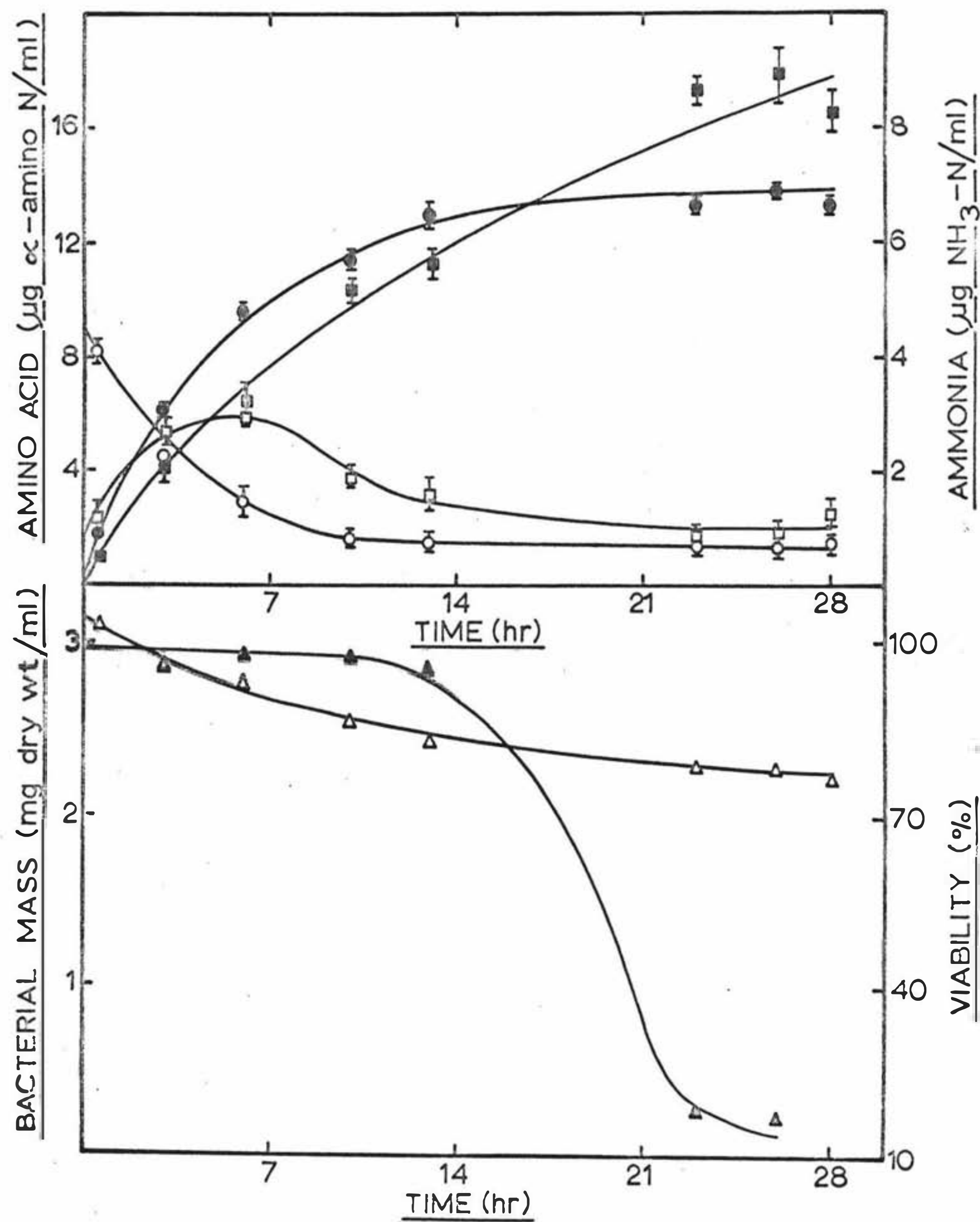


Fig. 17. Release of ninhydrin-reactive material and ammonia from starved Streptococcus lactis organisms. Bacteria were harvested, resuspended and sampled at intervals, as described for Fig. 16. Bacterial extracts were prepared and all samples were deep-frozen and later analysed (see Methods). Intracellular free amino acid and ammonia are shown as ○ , □ ; supernatant amino acid and ammonia are shown as ● , ■ . Vertical bars represent S.D. from mean for 4 determinations. Bacterial mass (△) and viability (▲) were determined as described in Methods.

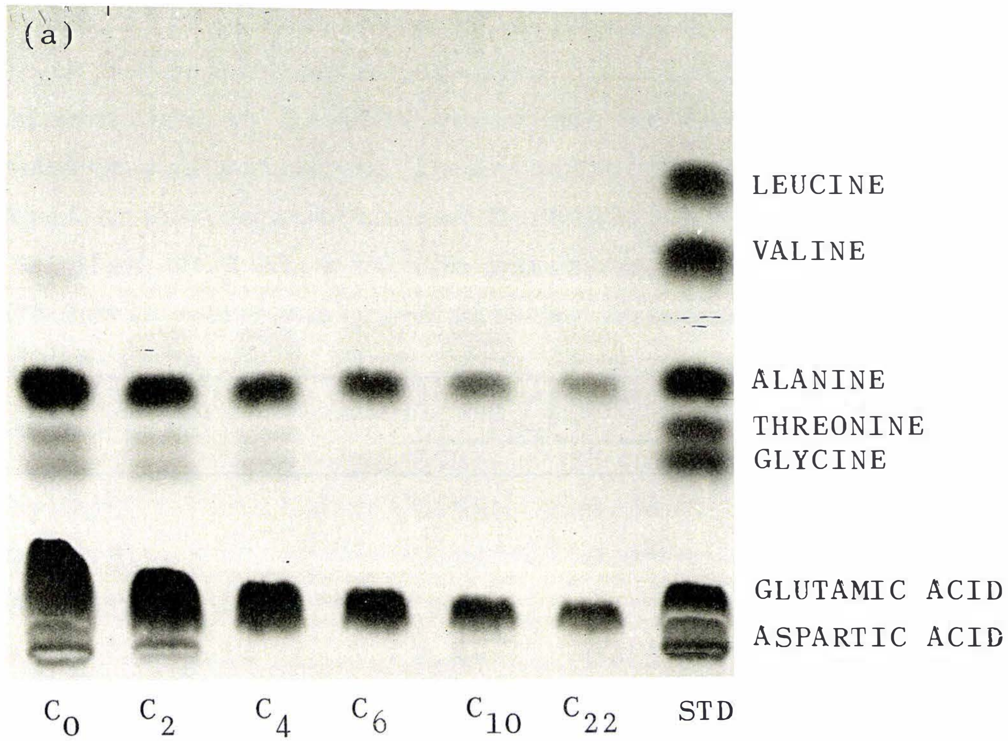
intracellular amino acids with a corresponding increase in the supernatant amino acids (Fig. 18), suggesting that leakage of the intracellular pool occurred on starvation. Later in the investigation, an amino acid analyser was available. This confirmed that the major components present and the total amounts of amino acids in the pool (Table 13) were similar to those found previously from colorimetric and chromatographic analyses.

The 73% net increase in the total free amino acids after 28 hr. starvation (Table 13) suggested that, in addition to leakage of intracellular amino acids, some protein degradation occurred. This increase in amino acid concentrations would correspond to a 5% breakdown of the total bacterial protein. Previous colorimetric methods of protein estimation were too insensitive to estimate these small changes accurately (see Fig. 16). No low molecular weight peptides were observed on the amino acid traces but it is possible that if they were present they may have been removed when the samples were deproteinized. The total amount of amino acids obtained from hydrolysed organisms (Table 13) was consistent with earlier analyses for bacterial protein.

Glutamic acid, alanine and aspartic acid made up 49.2%, 22.0% and 12.8% of the initial amino acid pool. The total amount of free aspartic acid was substantially reduced on starvation while the glutamic acid level showed a slight decrease (Table 13). There are no reports of the catabolism of these amino acids by S. lactis. The total amount of free lysine increased considerably on starvation while other amino acids increased by varying amounts. The levels of amino acids in the intracellular pool reflected the total amino acid composition of the organisms (Table 13). Neither free arginine nor ornithine were detected in any samples. These results would indicate that it is unlikely that starved S. lactis could obtain substantial energy from the amino acid pool.

Walker & Forrest (1964) claimed that 'energy from the

Fig. 18. Thin-layer chromatograms of the intracellular amino acid pool and supernatant samples of starved *Streptococcus lactis*. Bacteria were washed twice and resuspended (17 mg. dry wt./ml.) in buffer at 30° (see Fig. 16). Samples were removed at intervals and centrifuged. The packed cells were washed once, resuspended at the original density in deionized water and extracted, the supernatant buffer samples were desalted (see Methods). Equal volumes of cellular extracts (C) and supernatant samples (S) were placed on TLC plates (a) and (b) respectively. Plates were developed in phenol-water, dried and sprayed with ninhydrin (see Methods). Subscripts denote sampling times in hr. The standard sample contained 5 μg. of each amino acid indicated.


(a)
LEUCINE
VALINE
ALANINE
THREONINE
GLYCINE
GLUTAMIC ACID
ASPARTIC ACID
C_0 C_2 C_4 C_6 C_{10} C_{22} STD

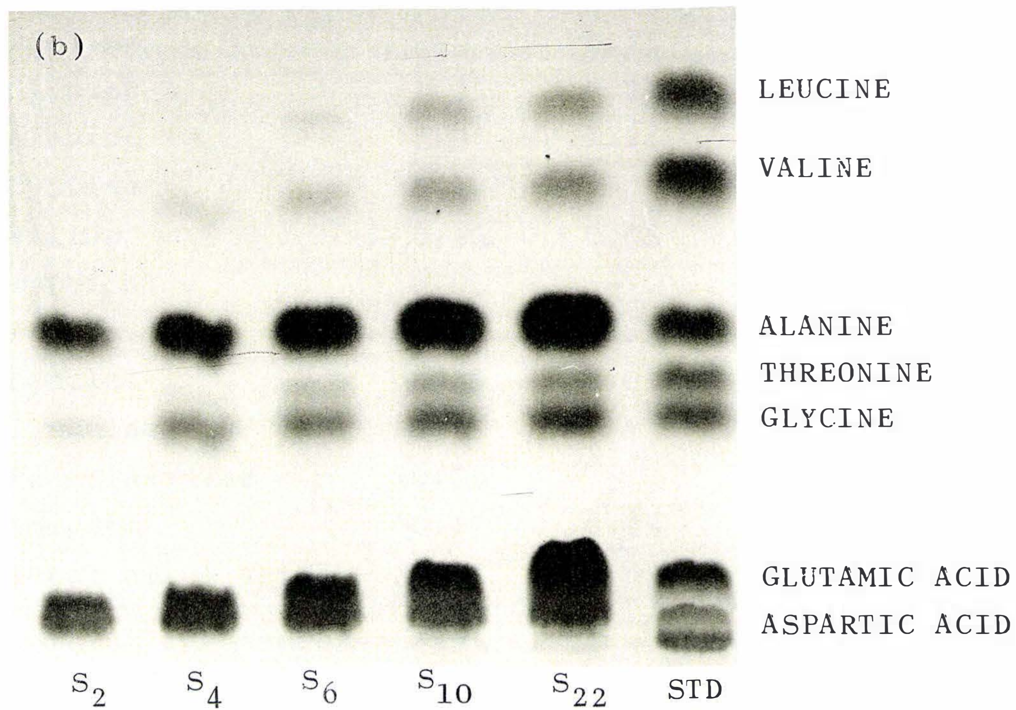

(b)
LEUCINE
VALINE
ALANINE
THREONINE
GLYCINE
GLUTAMIC ACID
ASPARTIC ACID
S_2 S_4 S_6 S_{10} S_{22} STD

Table 13. Release of amino acids from starved Streptococcus lactis organisms. Bacteria were harvested, washed twice and resuspended at a cell density of 15 mg. dry wt./ml. in buffer (Fig. 16). The intracellular amino acid pool was extracted with deionized water at zero time (see Methods). After 28 hr. starvation at 30° the organisms in the buffer suspension were washed and extracted in deionized water. The supernatant buffer was deproteinized by addition of TCA (final conc. 5%). Samples (0.5 ml.) of the extracts, supernatant and hydrolysed bacteria were analysed with an amino acid analyser (see Methods).

Amino acid	Intracellular Pool		Super-natant	Total Pool	Hydrolysed bacteria
	0.hr.	28 hr.	28 hr.	(28 hr.)	(0 hr.)
Lysine	0.95	0.38	8.9	9.3	48.5
Histidine	0.24	0.03	0.71	0.74	8.0
Arginine	-	-	-	-	18.6
Aspartic acid	4.25	0.15	0.70	0.85	67.1
Threonine	1.32	0.10	2.24	2.34	24.9
Serine	0.20	0.03	0.32	0.35	22.5
Glutamic acid	16.3	3.44	11.6	15.0	88.2
Proline	-	0.11	1.27	1.38	19.0
Glycine	1.05	0.22	5.2	5.5	28.8
Alanine	7.3	1.24	15.1	16.4	56.6
Cystine	0.19	-	0.21	0.21	-
Valine	0.33	0.05	1.37	1.42	23.2
Methionine	0.05	-	0.25	0.25	9.7
Isoleucine	0.09	0.03	0.81	0.84	18.1
Leucine	0.35	0.05	1.40	1.45	32.3
Tyrosine	0.17	-	0.57	0.57	13.3
Phenylalanine	0.35	-	0.71	0.71	16.4
Ammonia	0.17	0.25	2.18	2.43	11.5
Total amino acid	33.14[a]	5.83	51.36[b]	57.31	495.2[c]

Results are expressed as μg. amino acid or NH_3/mg. dry wt. bacteria at 0 hr.

a — plus 6 unidentified peaks which comprised 3.5% total pool.

b — plus 4 unidentified peaks which comprised $<$ 1% total pool.

c — plus 1 peak which comprised 3% total pool (probably hydroxylysine).

degradation of amino acid-containing material was responsible for maintenance and organization' of starved S. faecalis organisms. The process was not defined but did not involve catabolism of amino acids. These authors also reported that the total carbon liberated by S. faecalis organisms, when starved under anaerobic conditions, could be completely accounted for by amino acid release. The dichromate oxidation method used by them for total carbon determination (Halliwell, 1960) is not quantitative for all amino acids. Most of the major components of the amino acid pools of lactic acid bacteria——alanine, aspartic acid, glutamic acid and glycine (Holden, 1962a)——are only partially oxidized by this method (Table 14). Therefore it seems likely that other carbon compounds were released. No indication was given by Forrest & Walker (1963) or Walker & Forrest (1964) concerning the standard used for total carbon determination.

Changes in nucleic acids. Preliminary experiments indicated that a substantial amount of material, with an absorption maximum of 257 mμ, was released from starved S. lactis organisms. This suggested nucleic acid breakdown with the release of ultraviolet (u.v.)-absorbing purine and pyrimidine fragments. Results from more detailed measurements are shown in Fig. 19.

In the absence of added Mg^{2+}, bacterial RNA was broken down at a rapid rate from the onset of starvation, the degradation products being released into the suspending buffer. Organisms initially contained 20.8% RNA which was reduced after 28 hr. starvation to 5.5% RNA (calculated on the initial bacterial dry wt.). With added Mg^{2+}, RNA was broken down only after a considerable lag and the death rate was also reduced; the initial RNA level of 20.7% dry wt. was reduced to 10.4% after 28 hr. (Fig. 19). Without added Mg^{2+}, most organisms were still viable when 50% of the cellular RNA had been lost, although with added Mg^{2+} most organisms were non-viable at this point (Fig. 19).

In both suspensions loss of cellular orcinol-reactive

Table 14. Dichromate-oxidation of organic materials. The following compounds (500 μg.) were subjected to the H_2SO_4-$K_2Cr_2O_7$ oxidation method for the determination of 100-700 μg. organic material (Halliwell, 1960). Results were compared with dichromate blanks and sulphite-reduced dichromate blanks.

Compound	Dichromate reduction (%)
L-alanine	0.0
L-aspartic acid	5.5
L-glutamic acid	6.7
glycine	0.0
L-valine	71.4
L-threonine	45.1
L-leucine	88.1
L-serine	46.5
L-lysine. HCl	46.2
D-glucose	68.2
Na_2SO_3 (0.1ml., 20%, w/v)	100.0

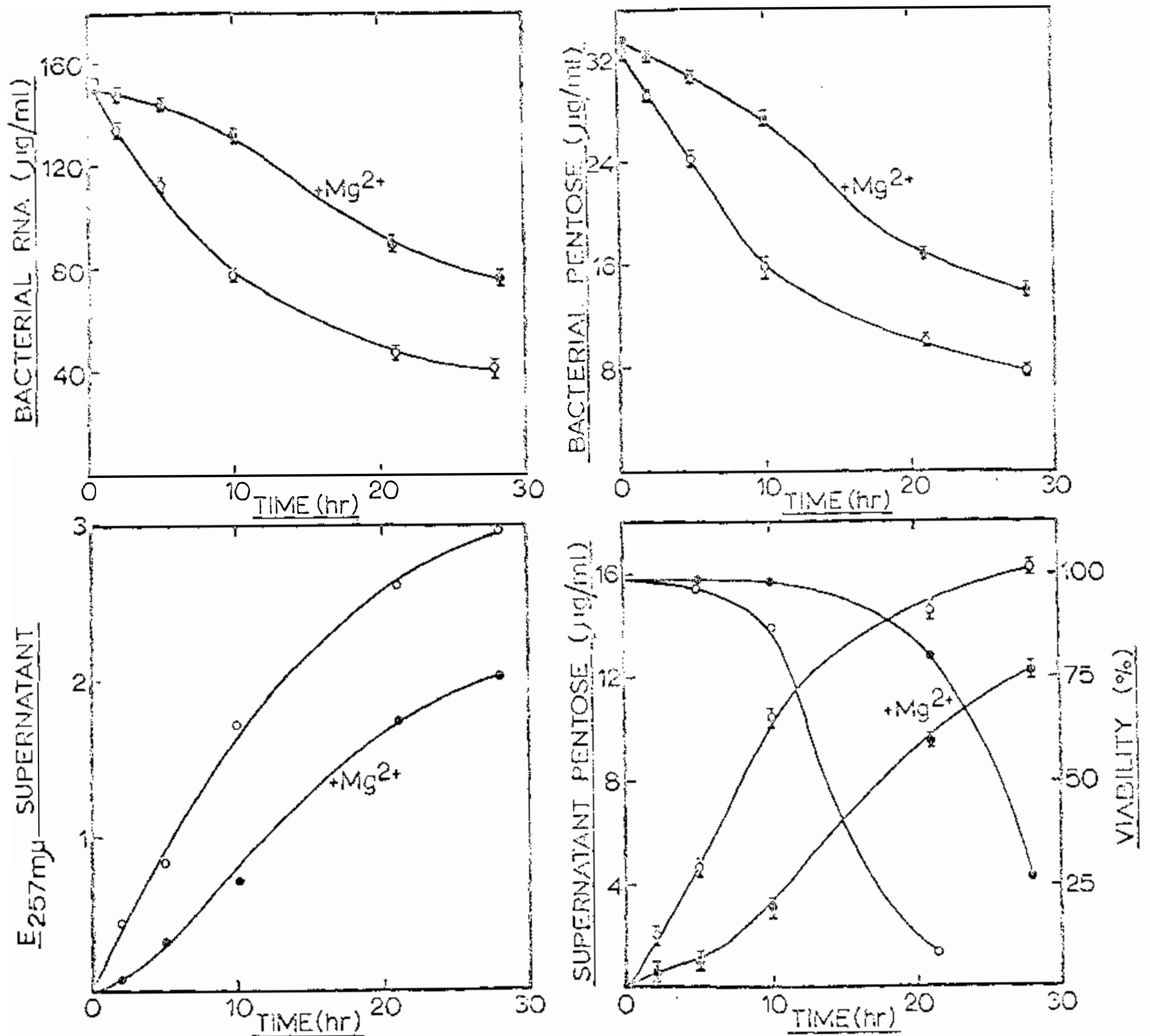


Fig. 19. Breakdown of RNA in starved Strept●coccus lactis organisms. Bacteria were harvested from routine growth medium at the end of the log-phase, washed once and resuspended at 30° in 0.075M-phosphate buffer (pH 7.0, + 10µM-EDTA) containing: O ; no addition; ● , 1mM-$MgSO_4$. Bacterial masses were initially 0.74 and 0.73mg. dry wt./ml. respectively. Samples were removed at the times indicated and immediately centrifuged. Supernatant and cellular extracts were prepared as described in Methods, frozen, and later analysed. Vertical bars represent S.D. from the mean for 4 determinations.

material was similar to RNA loss but was not balanced by a corresponding increase in supernatant orcinol-reactive material (Fig. 19), suggesting that some of the ribose may have been catabolized. However, preliminary experiments showed that lactic acid was not produced from exogenous ribose and since the orcinol reaction does not estimate pyrimidine bound ribose, it is not possible to balance ribose concentrations. The $E_{257m\mu}$ supernatant values, when converted to equivalent yeast RNA, corresponded closely with the bacterial RNA loss, suggesting that no metabolism of nucleic acid bases took place. In later experiments, therefore, measurements of the release of u.v.-absorbing material into the suspending buffer were used as a measure of RNA breakdown after samples had been treated with TCA to remove u.v.-absorbing protein (see Methods).

The identity of the u.v.-absorbing compounds released from starved organisms was studied by TLC and u.v. spectroscopy (see Methods). A suspension with 5.3 mg. dry wt. organisms/ml. was prepared in phosphate buffer without Mg^{2+}. After 24 hr. starvation at 30° the supernatant showed an extinction coefficient at 257 mμ equal to 21.0. Supernatant samples gave four distinct u.v.-absorbing bands on cellulose thin-layer chromatograms. There were no detectable bands with R_F values corresponding to nucleotides. When the bands were eluted and their u.v. spectra recorded, the extinction ratios were not in good agreement with those published for bases and nucleosides (Dawson et al., 1959) suggesting that each band contained more than one compound. However, from R_F values and absorption maxima, the major components may have been uridine, cytidine, hypoxanthine and adenosine.

The rates of RNA breakdown (release of $E_{257\ m\mu}$ material) and loss of viability were measured in the presence of substrates which produced the maximum range of death rate and cell division lag time (Fig. 20). Glucose-accelerated death was accompanied by extremely rapid RNA breakdown. Organisms in phosphate buffer also showed rapid rates of death and RNA breakdown. Additions

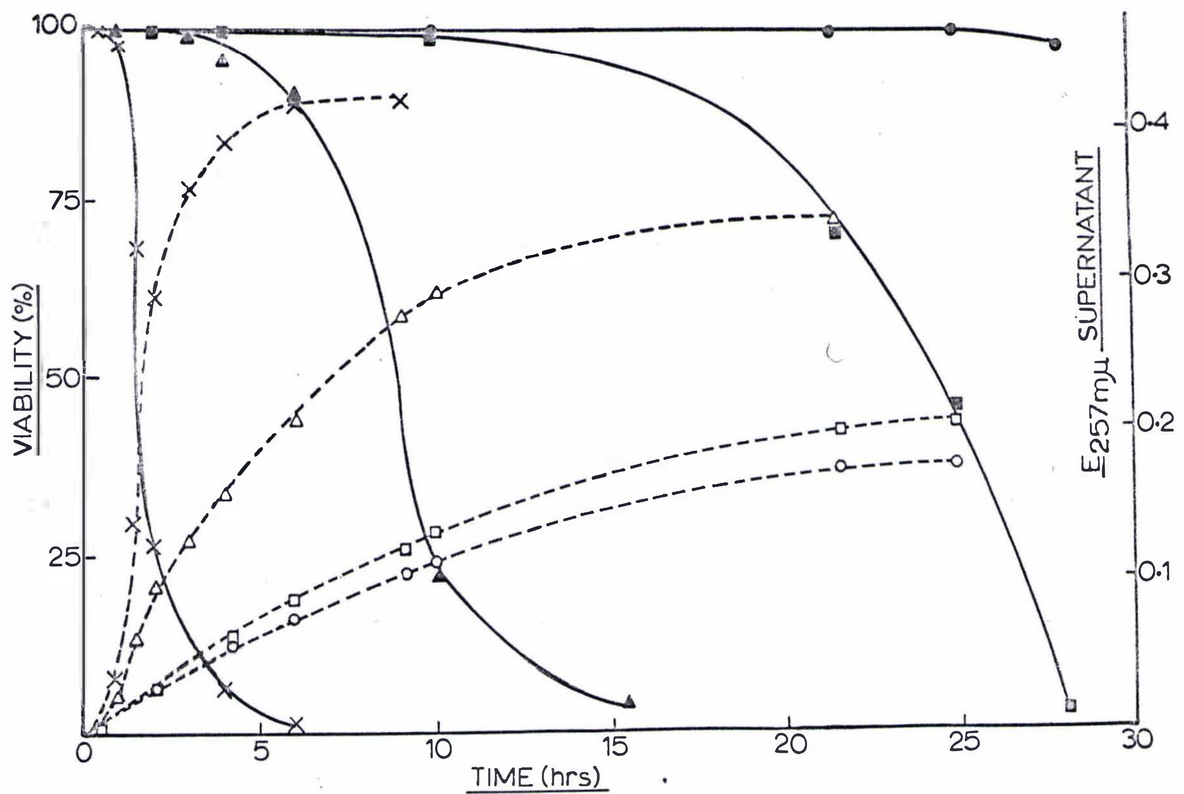


Fig. 20. RNA breakdown and death rate of starved Streptococcus lactis organisms. Suspensions were prepared as for Fig. 15 at 0.09mg. dry wt. bacteria/ml. in phosphate buffer containing 10 μM-EDTA. Supernatant $E_{257m\mu}$ values for the suspensions: + 0.5% casamino acids (Difco) + 0.1% arginine + 1 mM-Mg^{2+}; + 1 mM-Mg^{2+}; no addition; + 10mM-glucose; are indicated by dashed lines and the symbols ○ , □ , △ , X , respectively. Viability curves of organisms in the same systems have solid lines.

of Mg^{2+} suppressed RNA degradation and the death rate but the presence of exogenous amino acids had only a slight effect on RNA breakdown although they markedly reduced the death rate. Hence there was no general correlation between loss of viability and RNA breakdown.

No detectable change in bacterial DNA occurred in two experiments where organisms were starved for 16 hr. at 0.24 mg. dry wt./ml. in phosphate buffer. The DNA content of the suspensions remained at 8.84 ± 0.17 µg./ml. (i.e. DNA comprised 3.7% of the bacterial dry wt.).

Changes in carbohydrate. Previously, S. lactis organisms were shown to contain no carbohydrate which could be extracted by the methods appropriate for polyglucose. Analyses for cellular anthrone-positive material and reducing sugar indicated that there was little, if any, carbohydrate breakdown in starved S. lactis (Table 15). Carbohydrate fermentation would be expected to produce predominantly lactic acid. The small amount of lactate produced (Table 15) suggested that about 2% of the total cellular carbohydrate may have been fermented.

Most of the hexose in S. lactis is likely to have a structural role. 0 ther investigators working with other organisms (Strange et al., 1961; Ribbons & Dawes, 1963; Dawes & Ribbons, 1965; Burleigh & Dawes, 1967) have shown that structural carbohydrate is only slightly degraded, if at all, in starved bacteria.

Changes in lipids. S. lactis ML_3, grown in the routine medium, contained 3.8% lipid on a dry wt. basis (Table 16). A net loss of 10% of this lipid occurred during a 55 hr. starvation period. During this time the polar lipid fraction decreased from 85% to 70% of the total lipid and the neutral lipid fraction increased from 12% to 30% of the total lipid (Table 16). Spot intensities on H_2SO_4-charred thin-layer chromatograms indicated a marked increase in the free fatty acid component of the neutral lipid on starvation. Hydrolysis of polar lipid may

Table 15. Cellular carbohydrate in starved *Streptococcus lactis* organisms.
Bacteria from the end of the growth phase were washed and resuspended in 0.075M-phosphate buffer (pH 7.0, + 10 μM-EDTA) at 30° and a density of 1.0 mg. dry wt. bacteria/ml. At intervals, supernatant samples were prepared and frozen. Anthrone-reactive carbohydrate was determined directly on washed organisms. Reducing sugar was measured in washed organisms after storage at -20° in 2.5N-H_2SO_4 before hydrolysis.

Starvation period (hr.)	Viability (%)	Cellular carbohydrate		Supernatant	
		Anthrone +ve material[ab]	Reducing sugar[ac]	Anthrone +ve material[a]	Lactate[d] (μg./ml.)
0.2	98	77	107	0.2	0.6
1.2	97	77	102	0.6	0.7
2	95	74	105	0.5	0.9
3	95	75	109	0.7	1.2
4	96	74	106	1.0	1.0
5	93	72	106	0.9	1.6
10	68	73	103	0.9	1.8
22	12	74	102	0.8	1.9

a — Expressed as mean μg. glucose equiv./ml. of four determinations.
b — S.D. $< \pm$ 2.9.
c — S.D. $< \pm$ 2.2.
d — S.D. $< \pm$ 0.08.

explain these changes. The polar lipid phosphorus and nitrogen values observed (Table 16) were similar to those recorded by Ikawa (1963) for a range of lactic acid bacteria.

Separation of the polar lipid fraction by TLC revealed several components all containing phosphorus except for the one nearest the solvent front. A sample of this component was separated by preparative TLC and eluted. Subsequent hydrolysis and paper chromatography (see Methods) revealed that this component was a glycolipid containing glucose. Several spots near the origin were ninhydrin-positive.

Analysis of the fatty acids in the neutral and polar lipid fractions from S. lactis revealed a composition very similar to that previously reported by MacLeod, Jensen, Gander & Sampugna (1962). Starvation brought about changes in the fatty acid composition of the two fractions (Table 17). The relative amount of hexadecanoic acid decreased in the neutral lipid fraction while tetradecanoic acid increased. The cyclopropane acid, lactobacillic acid, increased in both neutral and polar lipid fractions with a corresponding decrease of its immediate precursor, cis-vaccenic acid. Formation of cyclopropane acids involves the transfer of a methylene group from S-adenosyl-methionine across the double bond of the corresponding monoenoic acid (Liu & Hofmann, 1962). Knivett & Cullen (1967) reported that E. coli, in the post-exponential growth phase, showed increases in cyclopropane fatty acids with decreases in the corresponding monoenoic acids and similar changes have been reported with many bacterial species after growth has stopped (see Kates, 1964).

In a recent report by Steinhauer, Flentge & Lechowick (1967), it was suggested that the lipid patterns of micro-organisms, as determined by gas-liquid chromatography of the fatty acid esters, could be used in identification and differentiation of bacteria. These authors examined the fatty acids obtained from the lipids of S. lactis and reported the major components as $C_{18:3}$ or C_{21}(50%) and $C_{18:2}$(20%). Di- and trienoic acids

Table 16. Changes in lipid components of starved *Streptococcus lactis*. Bacteria were grown at 30° in 3 x 10l. flasks containing 10l. routine medium, agitation was provided by a magnetic stirrer. Growth was followed turbidimetrically and at the times indicated from the end of growth, bacteria were harvested using a Sorvall continuous flow centrifuge. With a flow rate of 6l./hr. at 31,000*g*, turbidity measurements on the supernatant indicated recovery of >99% bacterial dry wt. The packed bacteria were washed twice in distilled water and the lipids extracted, washed and analysed as described in Methods.

Time from end of growth (hr.)	0	24	55
Bacterial yield[a] (gm. dry wt.)	6.72	5.73	4.97
Total lipid (gm.)[a]	0.256	0.244	0.228
(% bacterial dry wt.)	(3.81)	(4.25)	(4.59)
Neutral lipid (gm.)[a]	0.032	0.036	0.070
(% total lipid)	(12.5)	(14.6)	(30.6)
Polar lipid (gm.)[a]	0.218	0.201	0.159
(% total lipid)	(85.2)	(82.5)	(69.7)
Lipid recovery (%)	97.7	97.1	100.3
Polar lipid P (%)	2.37	2.28	1.86
Polar lipid N (%)	0.72	0.67	0.95
Viability (%)	99	96	32

a — Mean of two or more batches.

Table 17. Changes in fatty acids in starved *Streptococcus lactis*. The neutral and polar lipid fractions of the lipid extracts in Table 16 were hydrolysed, the fatty acids isolated, esterified and finally identified and quantitatively measured by gas-liquid chromatography (see Methods). Results are the means of four analyses and are expressed as % of the total fatty acid ester in each sample.

Fatty acid (probable identity)	Neutral lipid			Polar lipid		
	0 hr.	24 hr.	55 hr.	0 hr.	24 hr.	55 hr.
$C_{12:0}$	1.1	1.3	0.9	0.1	0.0	0.2
$C_{14:1}$	0.5	0.6	0.8	0.4	1.1	1.3
$C_{14.0}$	8.7	10.3	13.9	13.7	17.4	14.9
$C_{16:1}$	3.9	4.1	4.2	4.1	3.7	4.2
$C_{16:0}$	34.7	26.8	25.9	20.8	20.7	21.4
$C_{18:1}$[a]	24.6	26.8	21.0	30.2	20.3	18.1
$C_{18:0}$	2.7	2.4	1.4	0.1	1.1	0.8
$C_{19:0}^{\nabla}$	24.8	27.6	31.7	30.8	35.7	39.2

a — *Cis*-vaccenic acid.

The ∇ sign is used to indicate the presence of a cyclopropane ring in lactobacillic acid.

had not previously been reported in bacterial lipids (Kates, 1964) and it has been claimed that anaerobic or facultative organisms cannot synthesize such acids (Bloch, Baronowsky, Goldfine, Lennarz, Light, Norris & Scheuerbrandt, 1961). Furthermore, lactobacillic acid, which has been found in the lipids of all lactic acid bacteria previously investigated (Kates, 1964) was not found by Steinhauer et al. (1967). The disagreement between the results of Steinhauer et al. (1967) and those of the present investigation, which confirmed the earlier findings of MacLeod et al. (1962), suggests that some erroneous fatty acid identifications have been made by Steinhauer et al. (1967).

A small amount of volatile fatty acid (0.6 μeq./mg. dry wt. bacteria/24 hr.) was released into the suspending buffer (0.075M-phosphate + 1 mM-Mg SO_4) by starved S. lactis organisms. The source of this material has not been defined.

In starved anaerobic or facultative bacteria it seems unlikely that fatty acids could provide appreciable amounts of energy for, even if there were a β-oxidation pathway for the production of acetyl-CoA, there is no tricarboxylic acid cycle to effect its complete oxidation.

(ii) Protein synthesis in starved Streptococcus lactis

Protein synthesis, as determined by the measurement of valine -^{14}C incorporation into cell protein, was dependent on the presence of an exogenous energy source (Table 20); there appeared to be no endogenous energy source capable of supporting protein synthesis. Exogenous glucose produced approximately four times as much protein synthesis as arginine. It was subsequently shown that glucose fermentation by S. lactis generated ATP at about 7.5 times the rate for arginine metabolism and this may account for the different rates of protein synthesis.

Uptake of valine -^{14}C by starved organisms occurred without an exogenous energy source but to a much lesser extent (Table 19). Retention of the accumulated TCA soluble valine -^{14}C by starved

Table 18. Summary of changes in components of _Streptococcus lactis_ organisms starved at 30° in 0.075M-phosphate buffer (pH 7.0, 10 μM-EDTA, 1mM-$MgSO_4$).

(1) Depletion of polymers and amino acid pool (results are expressed as % initial bacterial dry wt.).

Starvation period (hr)	Protein[a]	Amino acid pool[c]	RNA[d]	DNA[e]	Carbo-hydrate[f]	Lipid[g]	Total	Bacterial dry wt.[a]
0	48	3.3	20.7	3.7	7.7	3.8	87.2	100
28	40.7[b]	0.6	10.4	3.7	7.2	3.6	66.2	74
Loss	7.3	2.7	10.3	0.0	0.5	0.2	21.0	26

(2) Formation of products (results of analyses on the suspending buffer expressed as % initial bacterial dry wt.).

Starvation period (hr.)	Lactate[f]	NH_3[c]	Volatile fatty acid[h] (as acetic acid)	Amino acid[c]
28	0.21	0.22	0.36	5.73

a — Fig. 16.

b — Estimated from protein released[a] and increase in total free amino acids[c].

c — Table 13.

d — Fig. 19.

e — Page 90.

f — Table 15, (carbohydrate = anthrone + ve).

g — Table 16 (24 hr.).

h — Page 95 (24 hr.).

Table 19. Valine-^{14}C uptake by starved <u>Streptococcus lactis</u>. Washed organisms, 0.64 mg. dry wt./ml., were starved at 30° in 0.075M-phosphate buffer (pH 7.0) containing 10μM-EDTA, 1mM-$MgSO_4$, DL-valine-1-^{14}C (.25μc./ml., 6μg./ml.) and L-valine (200μg./ml.). Additions or omissions from this buffer are given in the first column. Samples (2ml.) were removed at intervals and treated as described in Methods.

Additions to above buffer	Time (min.)							
	7	17	30	45	60	90	120	190
	Valine-^{14}C uptake (c.p.m./mg. dry wt. bacteria[a])							
None	619	659	446		318		185	154
+ Glucose (10mM)	1513	1981	2227		3144		3090	3180[b]
+ L-arginine (10mM)	705	898	934		1364		1381	1378
+ L-arginine (10mM) + casamino acids (2.78mg./ml.) -L-valine	499	703	845		904		912	856
+ Glucose (10mM) + chloramphenicol (200μg./ml.)				1934		2224		
+ L-arginine (10mM) + chloramphenicol (200μg./ml.)				981		1209		

a - Based on initial bacterial mass.

b - Equivalent to approx. 0.5% total valine-^{14}C.

Table 20. Valine-^{14}C incorporation into cell protein of starved Streptococcus lactis. This experiment was performed at the same time as the valine-^{14}C uptake experiment (Table 19) using the same conditions. Samples (2 ml.) were removed at intervals and valine-^{14}C incorporation into cell protein determined (see Methods).

Additions to buffer	Time (min.)							
	7	17	30	45	60	90	120	190
	Valine-^{14}C incorporation (c.p.m./mg. dry wt. cells)[a]							
None	9	11	11		14		20	11
+ Glucose (10mM)	247	471	626		801		1080	1137
+ L-arginine (10mM)	44	91	167		243		242	265
+ L-arginine (10mM) + casamino acids (2.78 mg./ml.)-L-valine	61	115	228		319		331	340
+ Glucose (10mM) + chloramphenicol (200 μg./ml.)				152		214		
+ L-arginine (10mM) + chloramphenicol (200 μg./ml.)				28		34		

a — Based on initial bacterial mass.

organisms was dependent on the presence of an exogenous energy source. At the bacterial density used in Tables 19 and 20, 10mM-glucose would be completely fermented in approx. 2 hr. (see Fig. 4), while 10mM-arginine would require about 7 hr. for complete metabolism (see Fig. 22).

Net protein synthesis appeared to stop when the glucose was exhausted. By contrast, net synthesis stopped in the presence of excess arginine, even with casamino acids present (Table 20). S. lactis ML_3 organisms contained 23 μg. valine/mg. dry wt. (Table 13). Assuming that all the valine is present in cell protein and that the protein synthesized in starved organisms contained the above proportion of valine, then the amount of protein synthesis in starved organisms can be calculated from the rates of valine $-^{14}C$ incorporation. With exogenous glucose and arginine, protein synthesis amounted to about 2% and 0.5% of the total cell protein in the first hour of starvation. The apparent cessation of protein synthesis after a period of starvation in the presence of arginine may have resulted from a balance being reached between protein synthesis and degradation. However, no relevant data was available to estimate the rate of protein degradation in the presence of exogenous arginine. Any TCA-insoluble protein released from starved organisms into the external medium during these experiments would be measured as cell protein.

Chloramphenicol caused 78% and 86% inhibitions of protein synthesis in the presence of glucose and arginine respectively (Table 20). Chloramphenicol produced little or no inhibition of valine $-^{14}C$ uptake into the TCA soluble pool (Table 19). (For these calculations, results from Tables 19 and 20 were graphed to obtain the 45 and 90 min. values). This is consistent with the reports that chloramphenicol does not inhibit the transport or accumulation of glutamate by micro-organisms (Gale and Paine 1951; Holden, 1965).

The ability of starved organisms to take up valine $-^{14}C$ and to synthesize protein appeared to be correlated with survival

(Fig. 21). Protein synthesis was presumably influenced by RNA degradation (see Fig. 19). With the bacterial concentration used in Fig.21, 10mM-arginine would be metabolized in approx. 4 hr. (see Fig. 22) and consequently survival was not enhanced to the same extent as at lower bacterial densities.

It has been suggested that vegetative bacteria may be able to undergo 'adaptation' in starvation conditions, presumably through turnover mechanisms, and that this may favour survival (Willetts, 1967; see also Clifton 1966). This could explain the decreased death rate of S. lactis starved in the presence of arginine or casamino acids where some protein synthesis took place. In fact, however, chloramphenicol appeared to reduce the death rate in some systems (Table 21). As well as inhibiting protein synthesis, chloramphenicol may also inhibit protein degradation (Willetts, 1967) and hence may exert some sparing action on proteins essential for survival.

(iii) Metabolism of arginine and glucose

Arginine was metabolized at a linear rate of 2.35 μmoles/mg. dry wt. bacteria/hr. (Fig.22). This rate theoretically corresponds to 2.35 μmoles ATP/mg. dry wt. bacteria/hr. (based on the stoichiometric reaction:

Arginine + H_2O + P_i + ADP $\longrightarrow$ ornithine + $2NH_3$ + CO_2 + ATP).

The glycolytic activity of S. lactis ML_3 was 0.295 μmoles lactate/mg. dry wt. bacteria/min. (Fig.4), and since anaerobic fermentation of glucose produces 1 mole of ATP/mole lactate, this glycolytic activity represents the formation of 17.7 μmoles ATP/mg. dry wt. bacteria/hr. Therefore the theoretical rate of ATP generation by S. lactis is about 7.5 times greater in the presence of 10mM-glucose than in the presence of 10mM-arginine.

Arginine metabolism yielded the theoretical amount of ornithine and slightly less than the theoretical amount of ammonia (Fig.22) confirming the findings of Korzenovsky & Werkman (1954). In earlier survival experiments, washed suspensions with 20 μg. dry wt. bacteria/ml. were incubated with 10mM-arginine. Assuming the above linear rate, these organisms would require more than 200 hr. for complete arginine metabolism.

A glycolytic activity of 0.295 μmoles lactate/mg. dry wt. bacteria/min. corresponds to fermentation of 1.59 mg. glucose/mg.

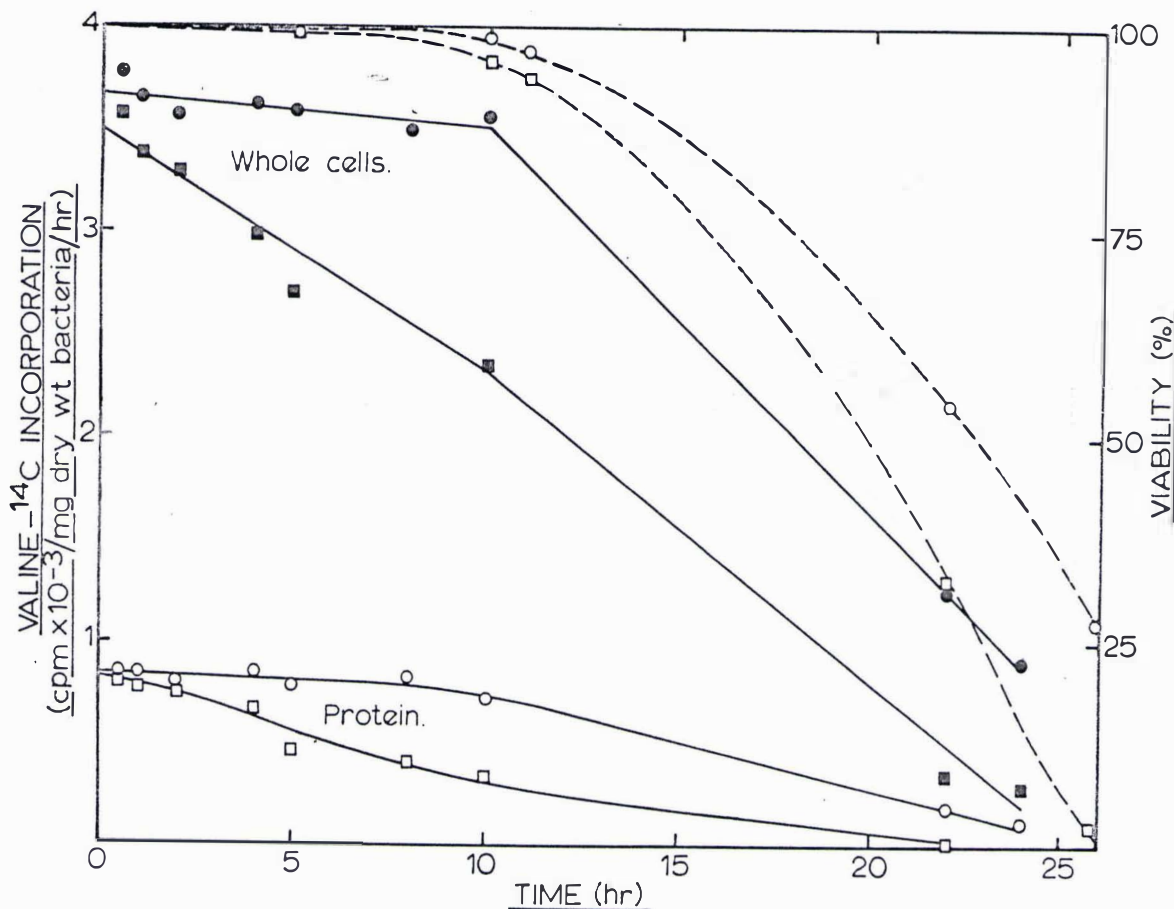


Fig..21. Ability of starved Streptococcus lactis to assimilate and incorporate valine -^{14}C. Washed organisms were resuspended at a density of 0.93 mg. dry wt./ml. in 0.075M-phosphate buffer (pH 7.0, + 10 μM-EDTA + 1mM-$MgSO_4$) containing: L-arginine (10mM), (○, ●); no addition (□, ■). The cultures were starved at 30°, samples (2 ml.) were withdrawn at intervals and added to 2 ml. 0.075 M-phosphate buffer containing $MgSO_4$ (1mM), D-glucose (20mM), DL-valine-1-^{14}C (.5 μc./ml., 12 μg./ml.) and L-valine (400 μg./ml.). After incubation for 1 hr. at 30°, the incorporation of valine-^{14}C into cells (closed symbols) and protein (open symbols) was determined (see Methods). Results are based on the initial cell mass. The dashed lines indicate slide-culture viabilities.

Table 21. Effect of chloramphenicol on the survival of Streptococcus lactis. Washed organisms, 30 μg. dry wt./ml. were starved at 30° in phosphate buffer (0.075 M, pH 7.0, + 10 μM-EDTA + 1mM-$MgSO_4$) containing the additions given in the first column. Samples were removed at intervals and the organisms washed (except where indicated). Viability was determined by the slide-culture method.

Additions to phosphate buffer	Time (hr.)							
	0	0[a]	16	20	24	28	40	45
	Viability (%)							
None	99	99	96	81	79	37	1	0
+ Chloramphenicol (200μg./ml.)	99	0	98	91	92	80	6	1
+ Glucose (10mM)	99	99	69	43	26	14	15	3
+ Glucose (10mM) + chloramphenicol (200μg./ml.)	99	0	97	87	78	82	26	1
+ Arginine (10mM)	99	99	99	96	98	94	85	52
+ Arginine (10mM) + chloramphenicol (200μg./ml.)	99	0	98	99	99	87	76	31

a — Slide-cultures prepared with unwashed organisms.

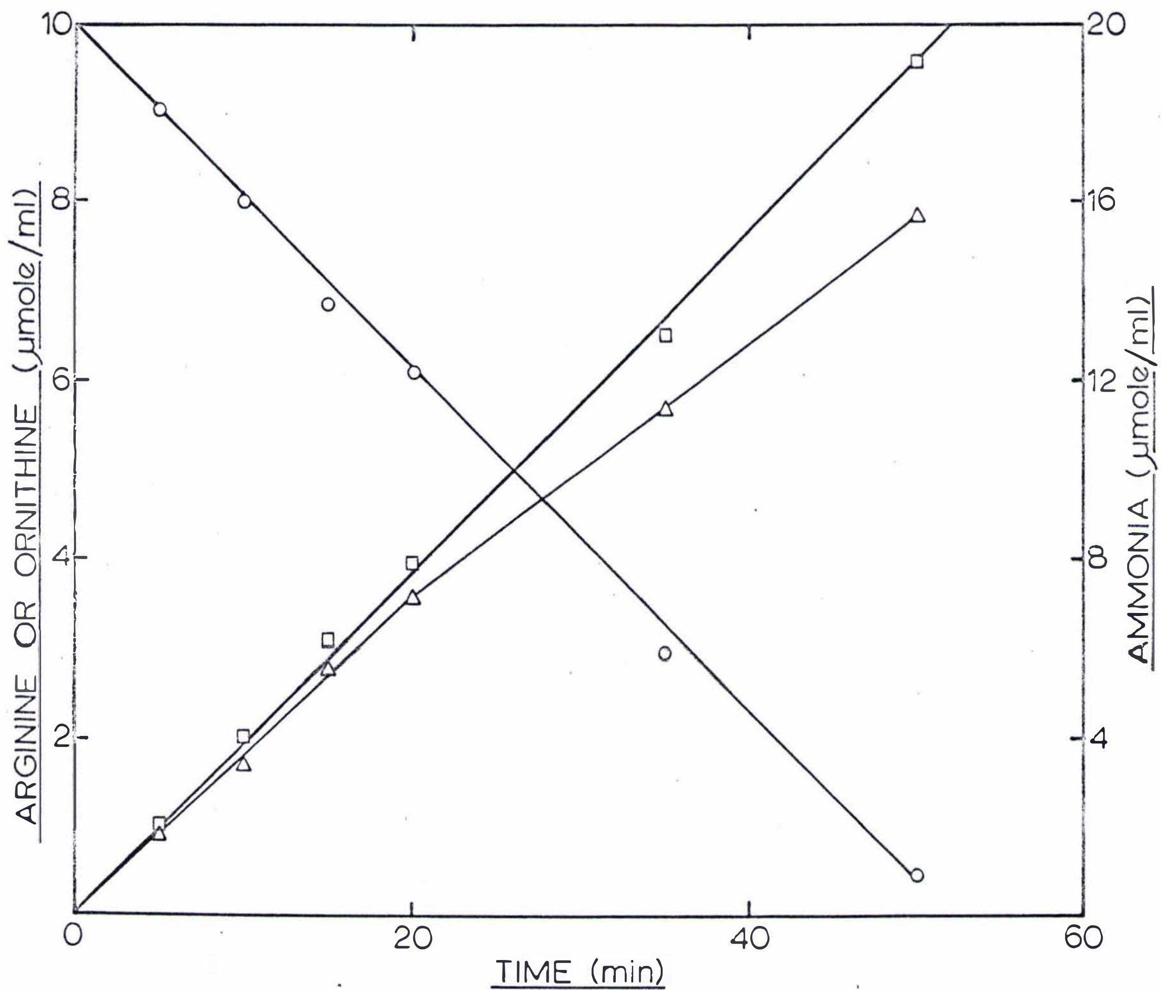


Fig. 22. Arginine metabolism by Streptococcus lactis. Bacteria were harvested from the routine medium at the end of the growth phase, washed twice and resuspended in 0.075M-phosphate buffer (pH 7.0; containing 10 μM-EDTA, 1mM-$MgSO_4$ and 10mM-L-arginine). The organisms were incubated at 30° at a density of 4.9 mg. dry wt./ml. At intervals, samples were withdrawn, immediately cooled to -10°, centrifuged and the supernatants filtered. Supernatant samples were frozen (-20°) and later analysed for arginine(○), ornithine (□) and ammonia (△), using the basic column of an amino acid analyser (see Methods).

dry wt. bacteria/hr. Even allowing for energy yield differences from glucose metabolism, this rate was much greater than the maintenance energy rate of 0.028 mg. glucose/mg. dry wt. bacteria/ hr. reported for E. coli by Marr et al. (1963). Assuming a similar maintenance energy rate for S. lactis, then clearly there is no economy of metabolic energy for maintenance of starved S. lactis and the linear rate of glucose fermentation (Fig.4) indicates that the metabolism of starved organisms continues at rapid rates.

Organisms were starved in conditions giving the maximum range of death rate and their glycolytic activity determined. No general correlation between glycolytic activity and survival was found (Table 22). It was shown that the incubation of starved organisms with glucose caused a more rapid loss of glycolytic activity (Table 22). With the bacterial concentration used, 10mM-glucose is metabolized in 5.2 hr. and 10mM-arginine in about 20 hr.

(iv) Changes in permeability and ultrastructure

Measurement of bacterial lysis. Leakage of metabolic intermediates from viable S. lactis organisms was shown to occur from the onset of starvation. The release of an intracellular enzyme, such as β-galactosidase, into the external medium has been used as an index of cell lysis (Pollock, 1961; Willetts, 1967). In the present study no detectable β-galactosidase activity was found in cell-free samples of S. lactis suspensions starved at a bacterial density of 4.8 mg. dry wt./ml. Citti et al. (1965) reported that the β-galactosidase released from five out of six strains of S.lactis by shock treatment was very unstable, so that if this enzyme had been released in the present study its activity may have been rapidly lost.

Assays for lactic dehydrogenase (LDH) indicated that this enzyme was released from viable organisms (Table 23). Release of LDH increased at the onset of cell death. Control experiments showed that the LDH in cell-free samples was unstable under the incubation conditions used. Assays of 24 hr. samples (Table 23), after 5 hr. and 22 hr. incubation, indicated a loss of 11% and 53% of the original activity. Therefore, the results for LDH release in Table 23 are only approximate.

Release of diphenylamine reactive material (expressed as DNA - Table 23) from starved organisms became pronounced as the death rate accelerated.

Table 22. Glycolytic activity of starved *Streptococcus lactis*. Organisms were washed and resuspended at 30° in 100 ml. 0.075M-phosphate buffer (pH 7.0, 10µM-EDTA) containing the additions given in the first column. The cell mass was 0.215 mg. dry wt./ml. At intervals, samples (10 ml.) were removed and centrifuged, the organisms were washed and resuspended in phosphate buffer (2 ml.) and the glycolytic activity determined as described in Methods.

Additions to above buffer	Time (hr.)							
	0.5	1.5	3	5	8	10	22	27
	Glycolytic activity[a]							
None	.274[b] (99)	.247	.230	.194 (96)	.175 (57)	.174 (15)	.155 (1)	.133
+ $MgSO_4$ (1mM)	.279 (99)	.264	.234	.206 (99)	.185 (96)	.164 (98)	.158 (72)	.155 (36)
+ $MgSO_4$ (1mM) + L-arginine (10mM)	.285 (99)	.269	.262	.218	.189	.193 (97)	.148 (98)	.144 (91)
+ $MgSO_4$ (1mM) + L-arginine (10mM) + casamino acids (.5%)	.286 (99)	.272	.241	.231	.178	.170 (98)	.157 (95)	.140 (87)
+ Glucose (10mM)	.269 (99)	.243 (72)	.181 (19)	.162 (11)	.137 (1)	.118	.052	.045
+ $MgSO_4$ (1mM) + glucose (10mM)	.277 (99)	.260	.205	.171 (99)	.121 (84)	.091 (61)	.016 (23)	.011 (4)

a — µmoles lactate/mg. dry wt. bacteria/min.

b — % viabilities in brackets.

Table 23. Release of lactic dehydrogenase (LDH) and deoxyribonucleic acid (DNA) from starved Streptococcus lactis. Washed organisms were resuspended at 4.6 mg. dry wt./ml. in phosphate buffer containing 10 μM-EDTA + 1mM-$MgSO_4$. Samples were removed at the times indicated, centrifuged and the supernatant buffers assayed as described in Methods.

Starvation Period (hr.)	Supernatant LDH (Units/ml.)	Supernatant DNA (μg./ml.)	Viability (%)
1	.000	0.0	-
3	.002	0.0	99
5	.006	0.2	-
8	.011	0.2	99
10	.069	0.7	-
12	.138	1.3	94
21	.319	4.0	32
24	.505	6.8	17
29	.547	11.2[a]	3

a — Equivalent 6.6% total cell DNA.

Effect of spermine. Certain aliphatic diamines, especially spermine, have a marked stabilizing effect on osmotically sensitive bacteria and protoplasts (Mager, 1959; Tabor, 1962), suppressing the leakage of u.v.-absorbing material and cell lysis. Incubation of starved *S. lactis* organisms with spermine reduced the death rate and the release of u.v.-absorbing material (Fig.23). This effect was less marked in the presence of Mg^{2+}.

General ultra-structural features of the cell. Typical examples of dividing cocci are shown in Plate 1. This sequence illustrates separation of the nuclear material followed by septum formation. Prominent mesosomes (Fitz-James, 1960) are associated with the ingrowing cell wall and membrane and also with the nuclear material. The structure and possible functions of bacterial mesosomes have been reviewed by Salton (1967) and Ryter (1968). It has been suggested that mesosomes may (1) function as sites for electron transport systems similar to mitochondria, (2) have a role in chromosome replication, (3) provide a mechanism for the secretion of extracellular enzymes and (4) have a role in the formation of new cell wall and membrane material thereby playing an active role in cell division. Some of these functions have been questioned since mesosomes are rarely present in Gram-negative bacteria.

The cytoplasmic membrane is 70-80A thick and consists of two prominent electron-dense layers separated by a light layer (Plate 1). The cell wall occurs as a diffuse band approximately 200-250A thick. Cole (1965) reviewed investigations on cell wall replication using an immunofluorescence technique and reported that cell wall growth in *Streptococcus pyogenes* was initiated along an equatorial ring. Cross wall grew centripetally with the peripheral wall replicating simultaneously so that the new hemispheres of the daughter cocci were initiated back-to-back. New equatorial sites of wall and cross wall formation were initiated before completion of the previous cross wall. In contrast, Chung, Hawirko & Isaac (1964) found that *S. faecalis* had only one site of cell wall synthesis per coccus. This mode of cell wall replication appears to operate in *S. lactis* since only one site of cross wall formation per coccus was seen (see Plate 2, Fig. 1). Individual cocci in negatively stained

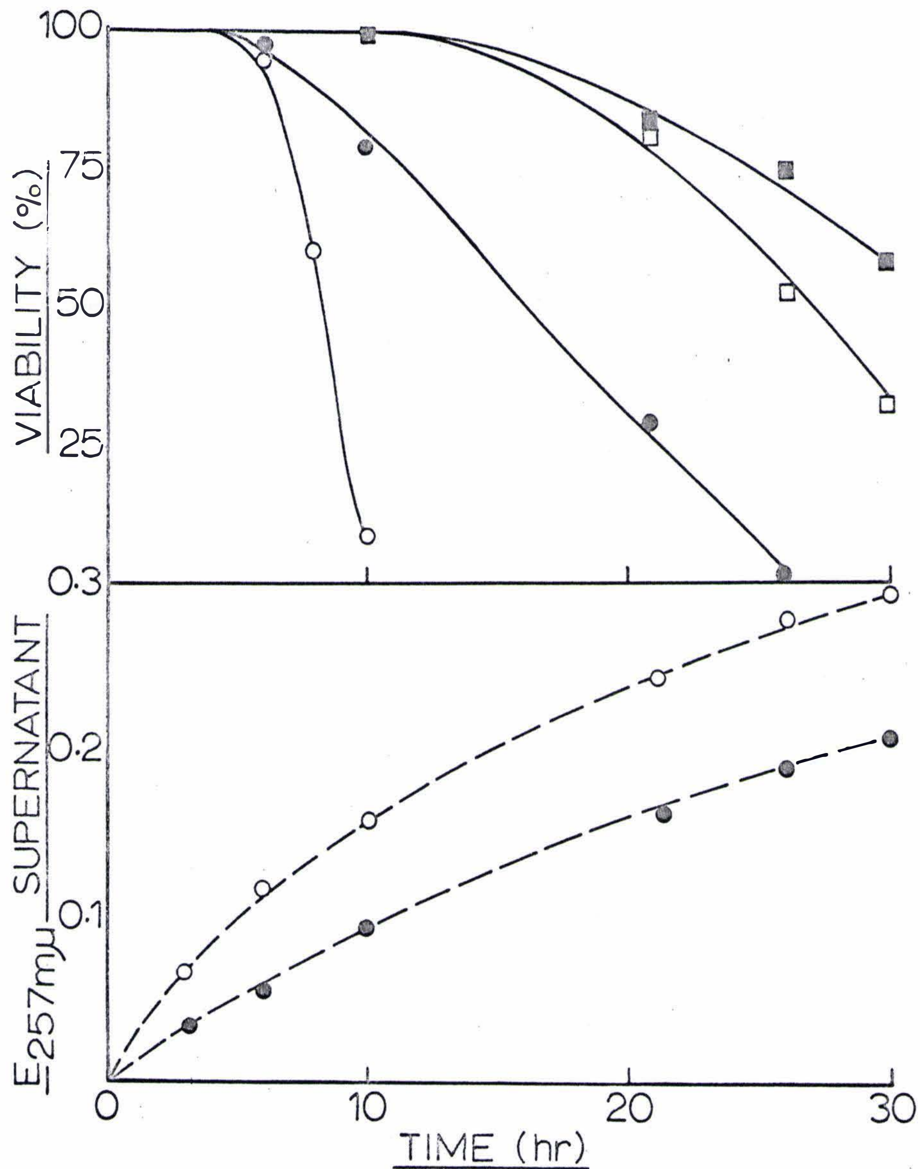


Fig. 23. Effect of spermine on starved <u>Streptococcus lactis</u>. Organisms were washed and resuspended at 20 μg. dry wt./ml. in 0.075M-phosphate buffer (pH 7.0, 10 μM-EDTA). Viabilities of suspensions containing: no addition, ○ ; spermine (1mM), ● ; $MgSO_4$ (1mM), □ ; $MgSO_4$ (1mM) + spermine (1mM), ■ , are shown. Release of u.v.-absorbing material is indicated by dashed lines, symbols as above.

Note: spermine HCl was sterilized separately in distilled water, spermine concentrations of 5mM and above produced crystaline deposits in phosphate buffer.

preparations of whole cells were 0.5-0.8 μ wide and 0.6-1.2 μ long, depending on the stage of division.

The fixation method of Kellenberger et al. (1958) preserved the nuclear material with a typical fibrillar structure (Plate 1). Bacterial nuclei do not appear to be separated from the cytoplasm by a membrane and may be regarded as a very long filament of a single, tightly coiled DNA molecule (Ryter, 1968). Other fixation procedures clearly demonstrated granular particles, 100-200A in diameter, which were sometimes concentrated near the cytoplasmic membrane (Plate 5). Most workers consider that these particles are ribosomes (see Kellenberger & Ryter, 1964; Silva, 1967).

Ultrastructural changes in starved cells. Although the appearance of ribosomes in thin sections was neither uniform nor constant, examination of a large number of sections prepared by different techniques seemed to indicate a rapid depletion of ribosomes when organisms were starved in buffer without Mg^{2+} (Plates 2,3). Magnesium starvation of *E. coli* (Morgan, Rosenkranz, Chan & Rose, 1966) and *A. aerogenes* (Kennell & Kotoulas, 1967) resulted in similar ribosome depletion. Death of *S. lactis* in buffer without Mg^{2+} occurred while cell structures remained intact. However, after 17 hr. starvation, intrusions of the membrane and cell wall were observed which appeared to be in direct contact with the nuclear material (Plate 3, Figs. 2-4). These micrographs are typical of organisms which have extruded their mesosomes (see Ryter & Landman, 1964; Beaton, 1968; Ryter, 1968). Ryter (1968) reported that mesosome extrusion may occur without loss of viability.

After 30 hr. starvation in buffer containing Mg^{2+}, the viability had fallen to 42% and examination of micrographs indicated that approximately 12% of the population had lysed. By 40 hr. most of the organisms had lysed and the viability was $<$ 1% (Plate 4). Lysis appeared to result from rupture of both the membrane and cell wall. After lysis, membranes tended to remain relatively intact while cell walls fragmented (Fig. 1, Plate 4). It is not clear whether the sites involved

in the extrusion of cytoplasmic material were randomly distributed over the cell surface, although in some cases they appeared where mesosomes would be expected. The micrographs of lysing cocci (Plate 4) appear similar to those of lipase-treated bacteria (Ghosh & Murray, 1967) where breakdown of the cytoplasmic membrane and cell wall eventually resulted in the liberation of cytoplasmic material.

Autolytic enzymes are present in most bacteria and appear to be activated whenever normal growth of the organism is disrupted (see reviews by Stolp & Starr, 1965; Shockman, 1965). The bacterial cell wall is believed to have a predominantly mechanical role, conferring cell shape, rigidity, and resistance to the high osmotic pressure created by the selectivity of the cytoplasmic membrane. As cocci lysed, the cytoplasmic membrane pulled away from the rigid wall and eventually collapsed (Plate 4) due to the decreasing osmotic pressure. The membranes of Gram-positive bacteria are not bound to the cell walls (Salton, 1967). It is interesting to note that while the cell walls of most Gram-positive bacteria are comprised mainly of mucopeptides (up to 50% dry wt.) and teichoic acids (30-40% dry wt.), Oram & Reiter (1965) reported that teichoic acids were absent from the cell walls of group N streptococci.

Exogenous amino acids maintained cell structures intact for a longer period (Plate 5). A dense ribosome pattern was still visible after prolonged starvation and lysed organisms appeared as the death rate increased.

EXPLANATION OF PLATES

Organisms were fixed as described in Methods, except where indicated. The bar represents 0.25μ on all micrographs and the magnification is x 64,400 except for Plate 1 (x 116,000) and Fig. 4, Plate 5 (x 21,000).

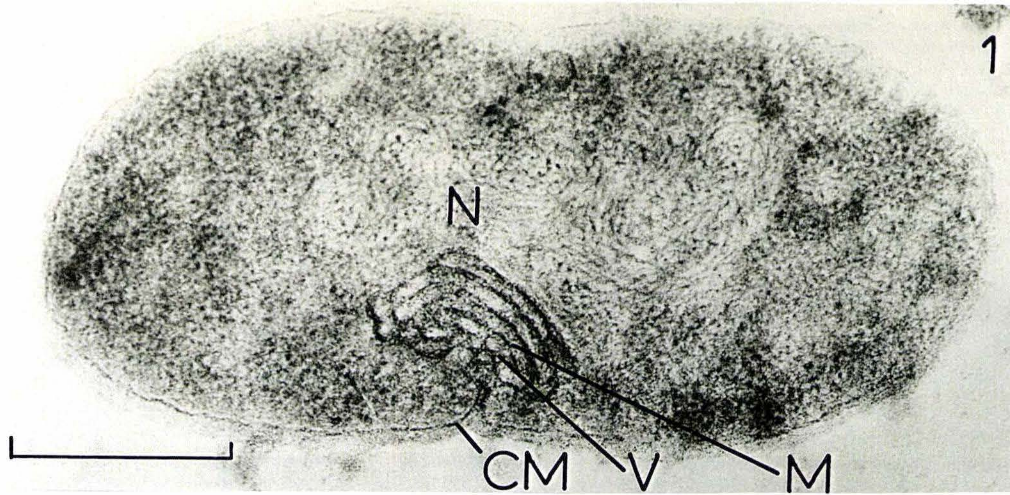
PLATE 1. Division sequence of *Streptococcus lactis* ML_3 from the logarithmic growth phase. Organisms were fixed by the method of Kellenberger *et al*. (1958) which gives best definition of the nuclear material and membranes.

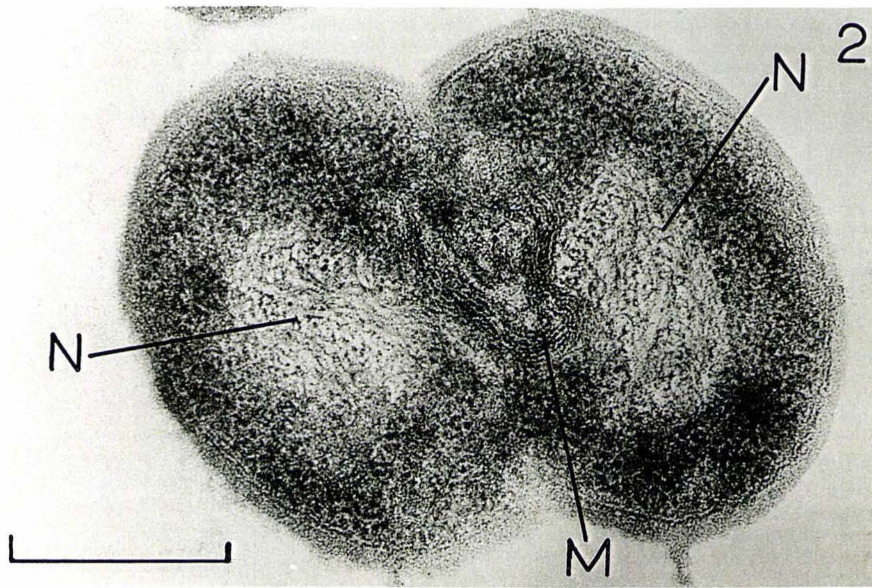
Fig. 1. Initial stage of cell division showing the undivided nuclear material (N) and small invaginations where the mesosome (M) is continuous with the cytoplasmic membrane (CM). The mesosome appears to contain vesicles (V).

Fig. 2. An intermediate stage showing the divided nuclear material (N) in close contact with the mesosome (M).

Fig. 3. Cross wall formation is complete with a clearly defined membrane. The new mesosome (M), formed at the next division site, is continuous with the cytoplasmic membrane. The mesosomes in Figs. 2, 3 are of the lamellar type consisting of concentric whorls of membrane, the appearance of mesosomes is partly dependent on the orientation of the section.

PLATE 1.





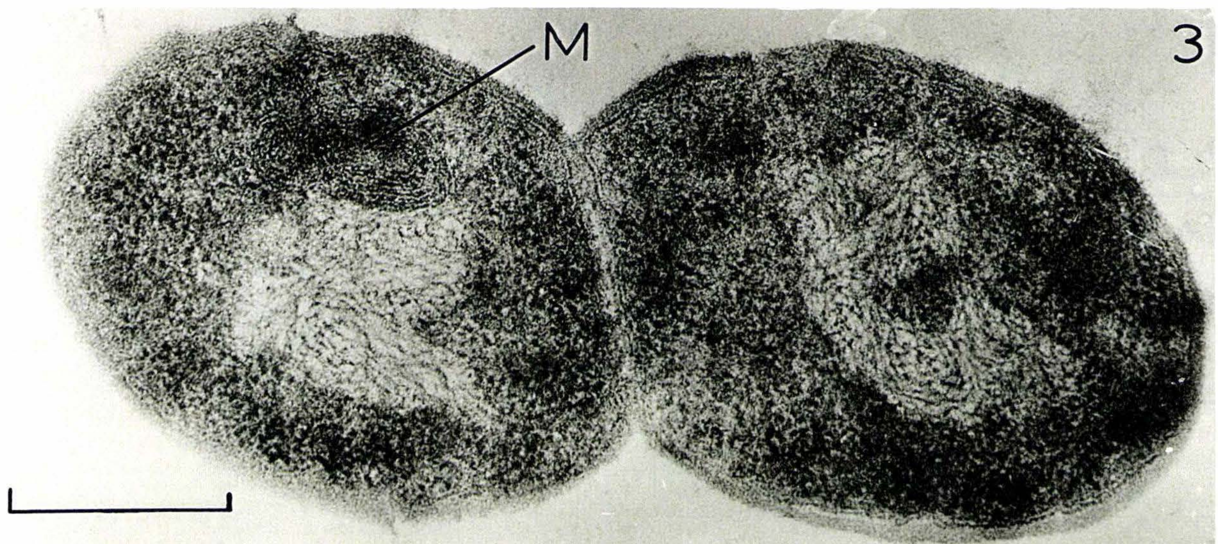


PLATE 2. Figs. 1-3. Organisms from the logarithmic growth phase. Figs. 4,5. Organisms after 5 hr. starvation in phosphate buffer, 50 μg.dry wt. bacteria/ml., viability 94%.

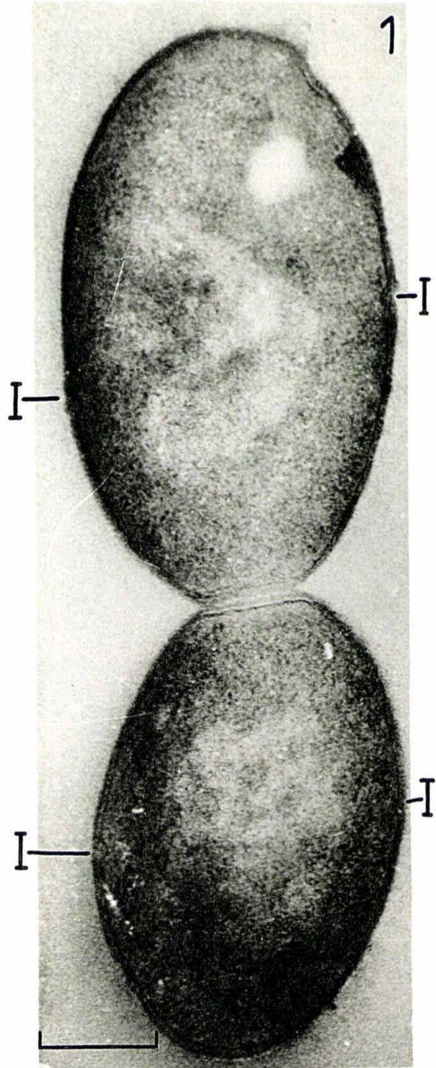
Fig. 1. Cocci are almost separated before invaginations (I) appear (Millonig's stain).

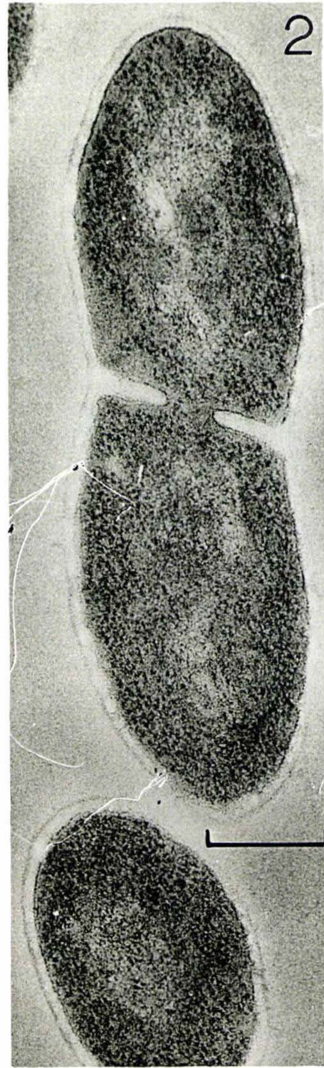
Figs. 2,3. Electron dense ribosome particles appear as granular dots (sections were floated on H_2O_2 followed by treatment with Reynold's stain. Note: H_2O_2 treatment reduces cell wall density and increases the contrast of ribosomes).

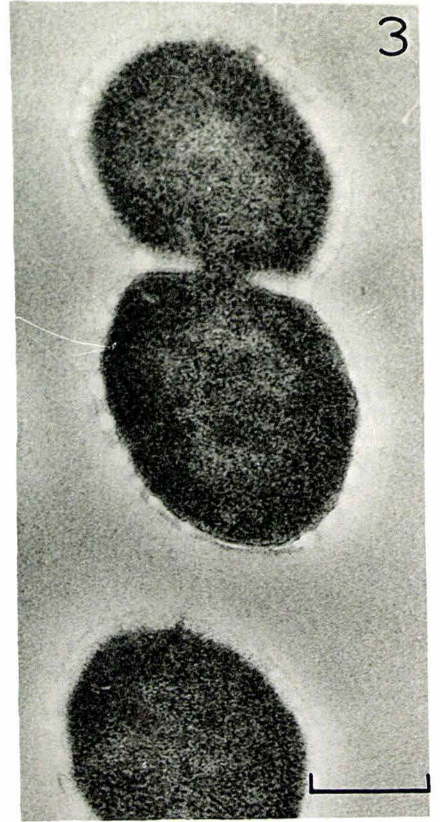
Fig. 4. Structural characteristics of starved organisms appear similar to those in Fig. 1 (Millonig's stain).

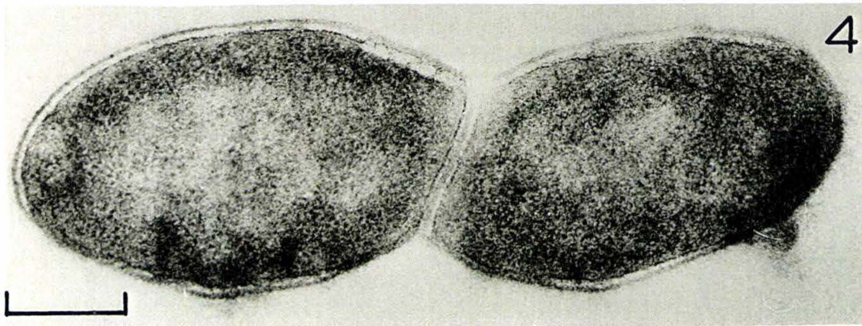
Fig. 5. Ribosomes are not as well defined as in growth phase organisms (H_2O_2 treatment, Millonig's stain).

PLATE 2.









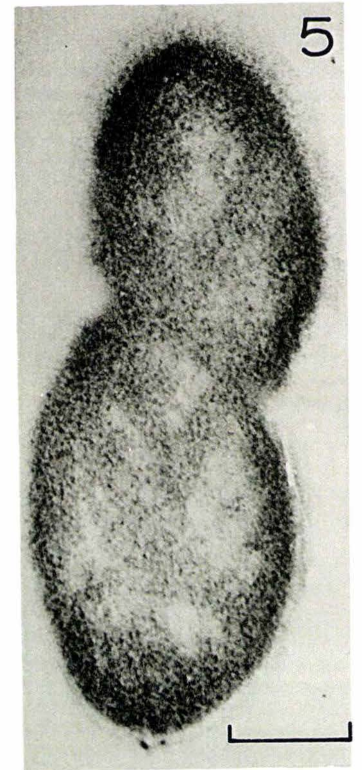
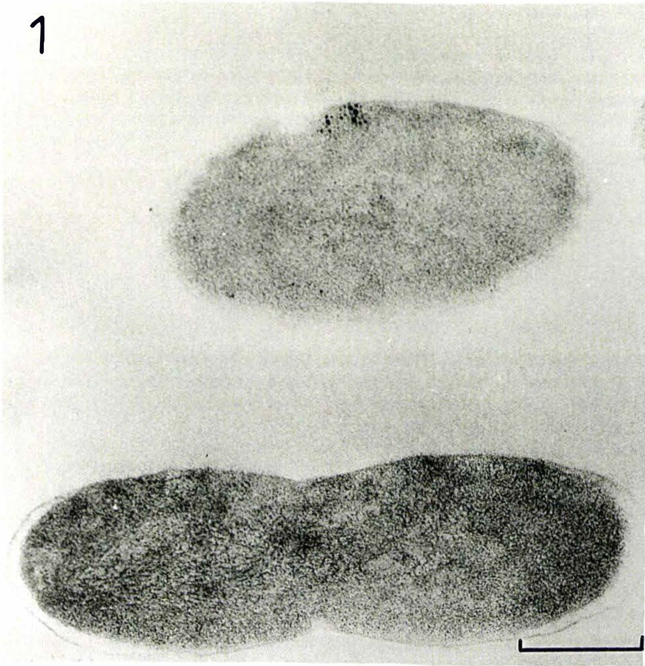


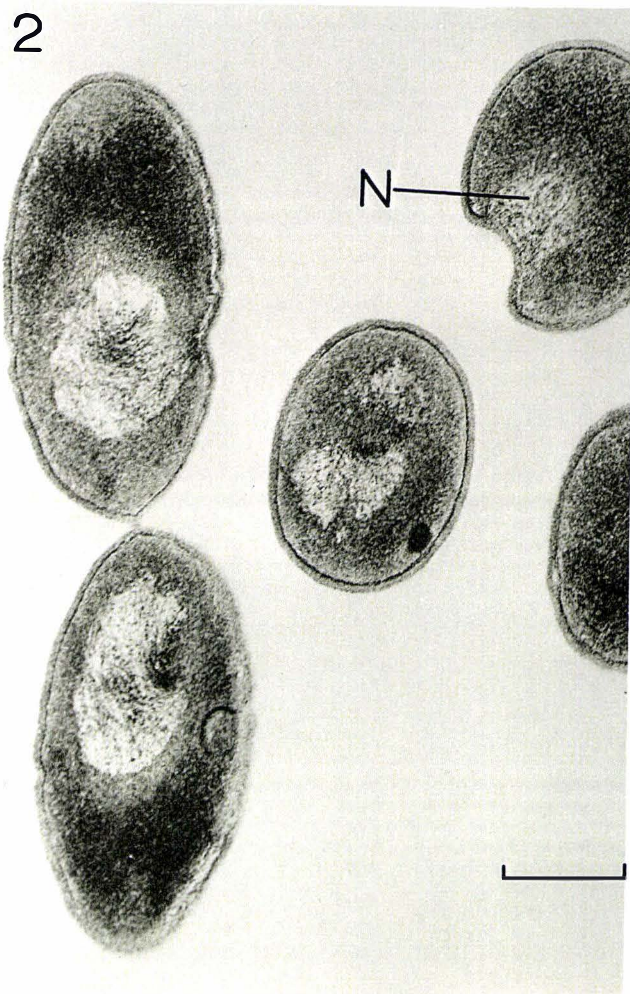
PLATE 3. Organisms after 17 hr. starvation in phosphate buffer, 50 μg. dry wt. bacteria/ml., viability 2%.

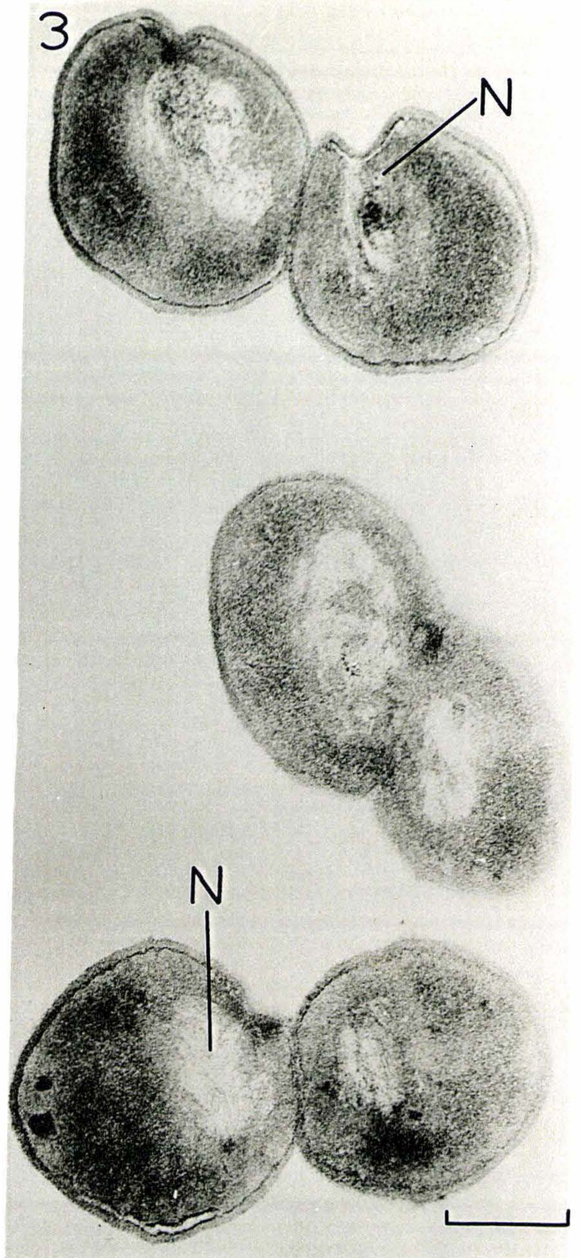
Fig. 1. No ribosome particles are visible (H_2O_2 treatment, Millonig's stain).

Figs. 2-4. Organisms showing intrusions of the membrane in direct contact with the low density nuclear material (N). (Reynold's stain. Note: the electron-dense spots are lead salt deposits.)

PLATE 3.







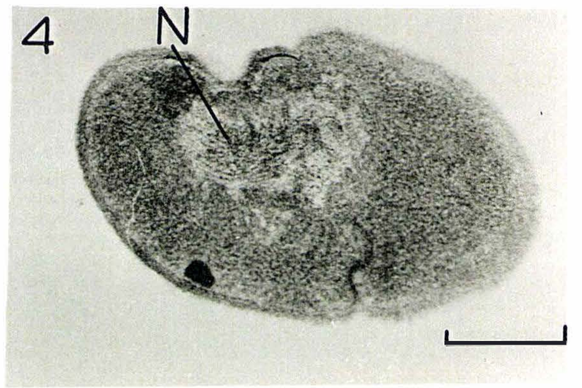
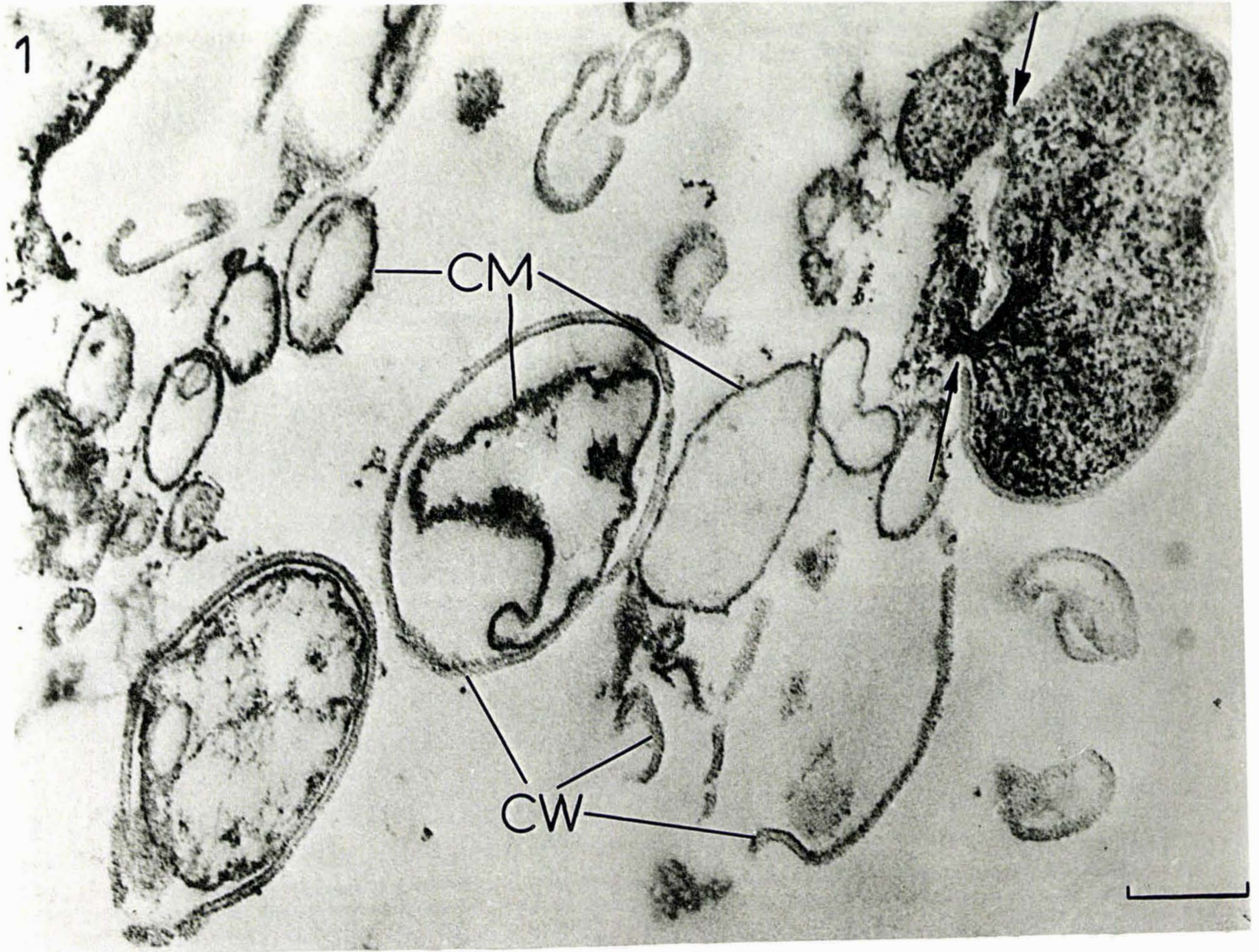


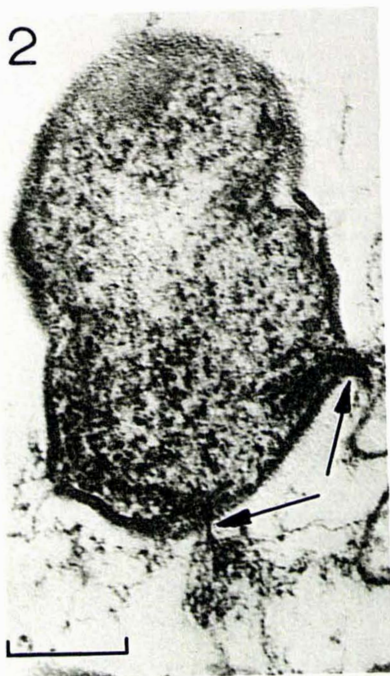
PLATE 4. Organisms after 40 hr. starvation in phosphate buffer containing 1mM-Mg^{2+}, 2mg. dry wt. bacteria/ml., viability $<1\%$ (Millonig's stain).

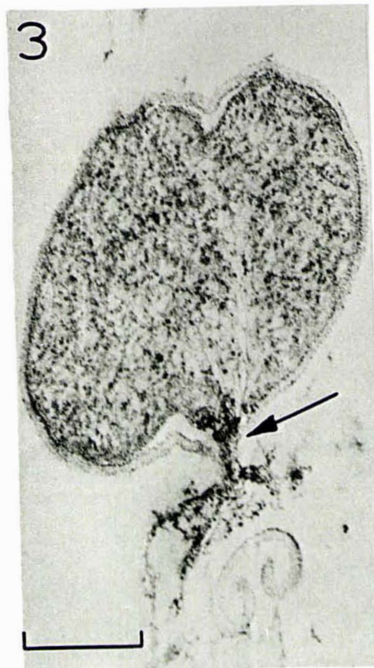
Fig. 1. Bacterial lysis has occurred leaving the remains of cytoplasmic membranes (CM) and cell walls (CW). Membranes tend to remain intact while cell walls fragment.

Figs. 1-4. Lysed organisms showing the extrusion of cytoplasmic material into the external medium through ruptures (arrowed) in the membrane and cell wall.

PLATE 4.







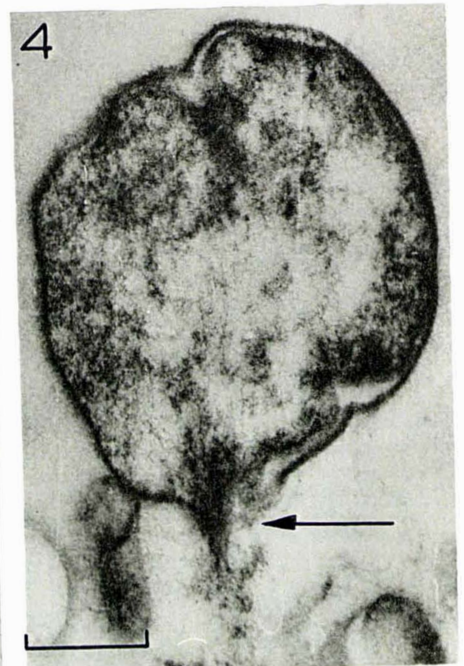
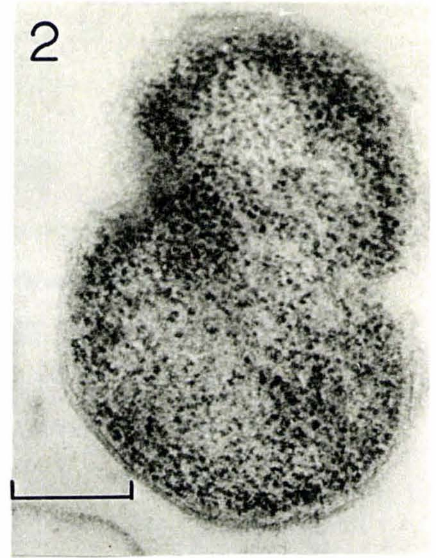
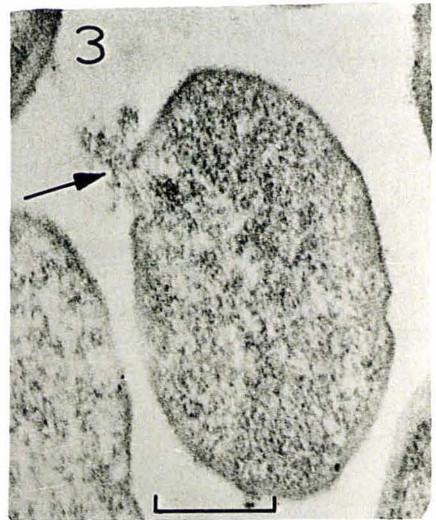


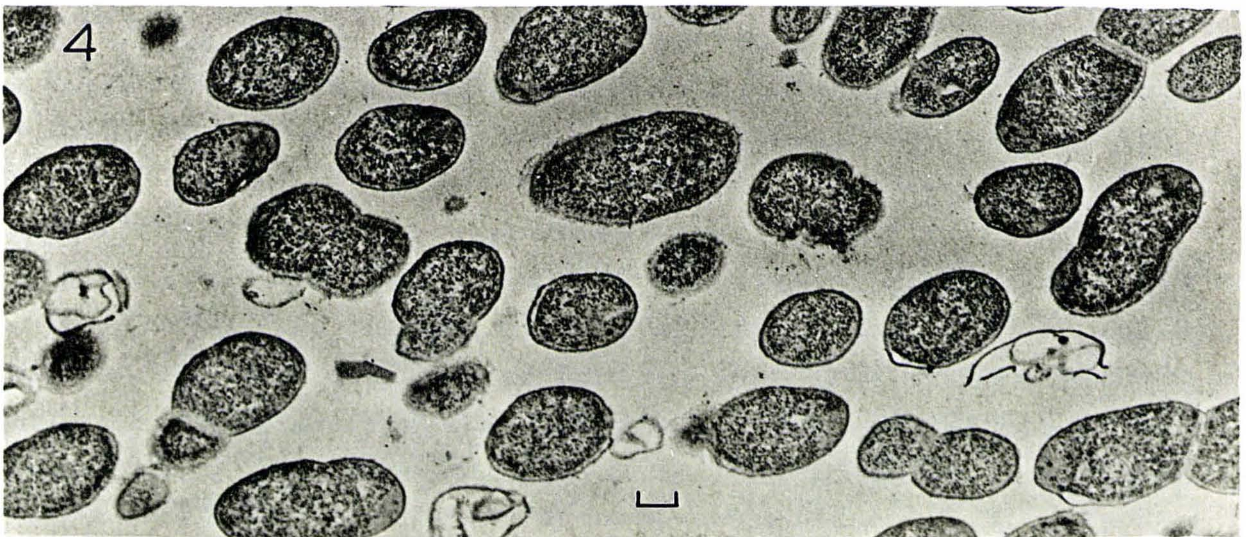
PLATE 5. Organisms after 48 hr. starvation in phosphate buffer containing 1mM-Mg^{2+}, 10mM-arginine, casamino acids (1%) and 100 μg. dry wt. bacteria/ml., viability 92%.

Figs. 1, 2, 4. Ribosome particles are still present and appear to be concentrated near the cytoplasmic membrane. Lysed cells are visible in Fig. 4 and the lytic process illustrated in Fig. 3 resembles that shown in Plate 4. (Sections shown in all figures were treated with Millonig's stain but for Fig. 2 the section was first floated on H_2O_2).

PLATE 5.







DISCUSSION

Survival of starved bacteria

Survival measurements. The slide-culture method of Postgate et al. (1961) is well suited for survival measurements with short-chain lactic streptococci such as *Streptococcus lactis* ML_3, although viability estimates have been complicated to some extent by the chain-forming nature of the organism and its tendency to clump on starvation. The maximum variance attributable to these factors has been assessed and the effect on the trends of survival measurements could only be small. The total numbers of cocci starved in phosphate buffer remained constant even when suspensions showed viabilities near zero. However, after prolonged starvation in the presence of amino acids, cell lysis occurred in viable populations as shown by a fall in the total count and the appearance of lysed organisms in electron micrographs. This could have led to some high viability estimates since lysed organisms may not always be counted on slide-cultures.

No increase in cell mass or numbers was observed in any starvation conditions. The report that *S. faecalis* did not grow when 'starved' in the presence of glucose and casamino acids (Walker & Forrest, 1964) is surprising in view of the fact that *S. lactis*, which has a more restricted biosynthetic capacity, gives limited growth under these conditions. Although metabolites were released from *S. lactis* organisms starved in phosphate buffer, it seemed unlikely that these compounds would support cryptic growth, even at high cell densities partly because of the organism's exacting nutritional requirements and partly through the absence of an appropriate amount of an energy substrate. The very limited protein synthesis, demonstrated from amino acid incorporation studies, and the constancy of total numbers of cocci in suspensions containing exogenous amino acids,

support the impression that cryptic growth did not occur to any extent under the experimental conditions adopted in this study. Since the present investigation has been primarily concerned with the survival of the first 90% of the population, these possible complications are unlikely to seriously affect the interpretation of results.

Stresses which develop during the preparation of washed suspensions have been minimized by washing and resuspending cells in a defined chemical environment at a constant temperature, and by incubating suspensions near the optimum survival pH in phosphate buffer containing sufficient EDTA to remove toxic metal ions.

Effect of Mg^{2+} on survival. Addition of Mg^{2+} (0.1mM or more) produced a marked decrease in the death rate of starved *S. lactis* organisms, a finding which is in agreement with the observations of Postgate & Hunter (1962) for *Aerobacter aerogenes*. Apart from its potential for reducing the toxic effect of certain metal ions (MacLeod & Snell, 1950; Abelson & Aldous, 1950; MacLeod *et al*., 1967), Mg^{2+}, together with Na^{+} and K^{+}, has been established as an important stabilizer of ribosomes in bacterial cells (Bowen, Dagley & Sykes, 1959; Tempest, Dicks & Hunter, 1966). Other possible functions of Mg^{2+} in starved bacteria, including membrane stabilization, have been discussed by Strange & Hunter (1967). Possible relationships between the presence of exogenous Mg^{2+}, ribosome stability and survival will be discussed later.

Effect of bacterial density. Harrison (1960) first observed the effect of bacterial concentration on survival and reported an optimum bacterial density above which the death rate increased. If the death of *A. aerogenes* at high cell concentrations is due to anoxia as Harrison has suggested, then the finding that *S. lactis*, a facultative anaerobe, did not show an optimum cell density for survival would be consistent. In general, Postgate & Hunter (1962, 1963a) confirmed the findings of Harrison but they did not observe the 'reversed' population density effect. The discrepancy may be explained if anoxia was the cause of the increased death rate at high concentrations of organisms since

Harrison (1960) did not aerate the washed suspensions, in contrast to the experimental procedure of Postgate & Hunter (1962).

In the present investigation, organisms in dense suspensions (equivalent to 7.8 mg. dry wt. bacteria/ml.) lost over 50% of their cell-bound Mg during a 12 hr. starvation period in phosphate buffer, producing a protective Mg^{2+} concentration (0.7mM) in the external medium. With the equivalent of 0.77 mg. dry wt. bacteria/ml., a protective concentration of Mg^{2+} (0.1mM) was established in the external medium but death was more rapid than at the higher cell density. It is possible that irreparable damage was done to the organisms before this effective concentration was reached. Results suggest that Mg^{2+} excretion by starved organisms was a major factor in prolonging survival in dense populations and the observation that the addition of 0.1mM-$MgSO_4$ produced almost identical survival times at all population densities is consistent with this view.

In experiments on glycerol-accelerated death, Postgate & Hunter (1964) reported that increased bacterial densities reduced the death rate. They tested the possibility that excreted Mg^{2+} may have been responsible for this effect but they were unable to detect Mg by chemical methods in concentrates of cell-free buffer solutions in which A. aerogenes had died. However, added Mg^{2+}, at concentrations below the limit detectable with their analytical method, protected organisms against glycerol-accelerated death (Postgate & Hunter, 1964). Therefore Mg excretion, although not detected, may have been responsible for at least part of the observed bacterial density effect.

Although excreted Mg^{2+} concentrations can be related to the high bacterial density effect with S. lactis, Postgate & Hunter (1963a) have demonstrated a bacterial density effect in the presence of added Mg^{2+} with A. aerogenes. Webb (1966) observed Mg^{2+} liberation by Bacillus megaterium and E. Coli when these organisms were starved in a Mg^{2+}-free salt solution. Burleigh & Dawes (1967) failed to observe a bacterial density effect with starved Sarcina lutea but their lowest density (1 mg. dry wt./ml.)

may have been too high to observe this effect.

Effect of added substrates. All of the fermentable carbohydrates tested produced accelerated death of washed organisms irrespective of the growth phase or growth-limiting nutrient. Carbohydrate-accelerated death was not caused by the lactate produced and was markedly reduced on the addition of Mg^{2+}. These results contrast with the observations of McGrew & Mallette (1962, 1965) and Clifton (1966), who reported extended survival of Escherichia coli, 'without growth', in the presence of glucose and Mg^{2+}. Postgate & Hunter (1963c, 1964) observed that the addition of certain growth-limiting nutrients to starving suspensions of certain Gram-negative bacteria increased the death-rate. This applied to nitrogen-, phosphorus- and carbon-limited populations and it was suggested that this 'substrate-accelerated death' was a fairly general phenomenon for Gram-negative bacteria.

Although carbohydrate energy sources produced accelerated death, arginine, which S. lactis converts to ornithine with the production of ATP (Korzenovsky & Werkman, 1953, 1954), produced an increase in survival time in the presence of Mg^{2+}. This indicated that the nature of the energy source may be critical for survival. It is noteworthy that in the absence of added Mg^{2+}, arginine accelerated death. Other aspects of glucose and arginine metabolism will be discussed later. The extended survival of S. lactis ML_3 without growth, in the routine growth medium minus lactose, vitamins and bicarbonate, can be attributed to its Mg^{2+} and amino acid content.

Effect of temperature and pH. Death rates were reduced when the incubation temperature was lowered, presumably due to reductions in the rates of degradative processes. This contrasts with the observed increased death rates of A. aerogenes at low temperatures (Postgate & Hunter, 1962) but death of this organism may have been due to a sensitivity to 'cold shock' which involves damage to the permeability regulating systems (see Strange & Dark, 1962; Strange & Postgate, 1964).

Although it is believed that energy is required for

intracellular pH control in starved bacteria (Dawes & Ribbons, 1962, 1964), this has not been experimentally demonstrated. S. lactis had a sharp pH optimum for survival which is perhaps consistent with the absence of an endogenous energy source. In this connection, the addition of arginine was shown to produce a marked decrease in death rates at all pH values. At acid pH values this effect could have been due to a neutralizing action of the NH_3 produced from arginine metabolism. However, arginine also prolonged survival at alkaline pH and it seemed likely that the energy produced from arginine metabolism was directly responsible for reducing the lethal effect of adverse pH values.

The growth of lactic acid bacteria in milk normally ceases when the lactic acid formed produces inhibitory acid conditions. Although growth ceases, the organisms remain viable and continue to ferment the large excess of lactose present but at a much slower rate. It is possible that the energy produced by the continuing fermentation could be an important factor for the survival of these organisms in sour milk.

The very low death rate observed in the initial incubation period followed by the rapid decline in viability of S. lactis ML_3 in most resuspended systems, suggests that intrinsic differences in survival potential between individual bacteria may be slight. The duration of this period of almost complete viability varied greatly, depending on the resuspension system and the biological history of the population. The death rate then began to increase and generally continued at a rapid rate.

Changes in starved organisms

Polymer degradation. S. lactis did not accumulate detectable amounts of polyglucose or poly-β-hydroxybutyrate in conditions which would be considered as favourable for the synthesis of these reserve materials. No reports have appeared in the literature indicating the presence of these or other storage polymers in lactic streptococci. Forrest & Walker (1965a) reported that S. faecalis synthesized reserve material under

certain growth conditions. However, the nature and metabolism of these reserves were not defined. Only one report has been published on the metabolism of S. lactis starved at growth temperatures and this claimed that (a) 'the organism had a substantial endogenous respiration' and (b) 'endogenous lactate or succinate was oxidized after a lag' (Spendlove et al., 1957). These results have not been confirmed in the present investigation. It seems unlikely that lactate or succinate could function as endogenous substrates for an organism which is a homolactic fermenter, possessing no terminal respiratory system.

Whether bacteria possess reserves or not, the constitutive material of starved organisms is ultimately degraded and death ensues (see reviews by Dawes & Ribbons, 1964; Postgate, 1967). Most of the decrease in cell mass of starved S. lactis organisms could be accounted for by RNA and protein degradation. The rates of RNA breakdown and death were reduced by the addition of Mg^{2+}; these observations are in contrast to the findings of Burleigh & Dawes (1967) with S. lutea. However, these authors examined the effect of added Mg^{2+} with suspensions of high bacterial density (8.8 mg. dry wt./ml.) and it is possible that if Mg^{2+} was liberated by the organisms as their RNA was degraded, the effect of added Mg^{2+} on survival may have been masked since RNA appeared to be to some extent expendable. The protective effect of Mg^{2+} on Gram-negative organisms under conditions of stress has been demonstrated with bacterial concentrations of 20 μg. dry wt./ml. or less (see Postgate & Hunter, 1962, 1964; Strange & Postgate, 1964; Strange & Dark, 1965). These stresses showed population density effects so that the response of S. lutea to added Mg^{2+} may not be basically different from that defined for Gram-negative organisms. S. lactis has both a high RNA content, typical of an organism grown at a rapid rate, and a high Mg content, which is consistent with the probable interdependence of these constituents (Tempest & Strange, 1966). The molar ratio of RNA/Mg was approximately 50, which is of the

same order as that observed for A. aerogenes (Tempest & Strange 1966).

Conditions which accelerated RNA breakdown - such as buffer systems which either contained glucose alone or did not contain Mg^{2+} - also produced increased death rates. However, in the presence of Mg^{2+}, arginine only slightly suppressed RNA degradation although it extended survival. Hence concommittant RNA degradation and death does not occur in all systems. Although considerable RNA may be degraded without affecting viability, in agreement with results for many other starved bacteria (see Burleigh & Dawes, 1967), it seems likely that a degree of ribosome stability is important for survival of S. lactis. Conditions which accelerated RNA breakdown in other bacteria were generally more lethal (e.g. see Strange & Shon, 1964; Strange & Dark, 1965) and Postgate (1967) has concluded that RNA degradation is a critical process in the survival of A. aerogenes. However, no absolute correlation between the rates of death and RNA breakdown existed. In contrast to these results, the 'starvation-resistant' mutants isolated by Harrison & Lawrence (1963) degraded RNA more rapidly than the wild type.

It has been suggested that RNA breakdown may continue in viable bacteria as long as mechanisms for polymer resynthesis from precursors remain intact (Burleigh & Dawes, 1967). The present results show that conditions producing maximum rates of RNA degradation and death also produce increased cell division lag times of surviving organisms. These lags are probably directly influenced by the amount of polymer degradation which has taken place, particularly that of RNA, since the RNA content of bacteria increases with the growth rate (Tempest & Strange, 1966) while the rate of protein synthesis per ribosome particle is constant in growing bacteria (Tempest et al., 1967). Mandelstam (1963) has suggested that the restoration of degraded protein and RNA takes place gradually on the resumption of growth.

Most of the RNA in bacteria is found in the ribosome fraction and Strange et al. (1963) reported that 72% of the total RNA loss

from starved A. aerogenes was from ribosomes. Electron micrographs of S. lactis from a log-phase growth culture showed a dense pattern of ribosomes which appeared to be depleted on starvation in phosphate buffer. No evidence was obtained for the catabolism of ribose or bases from degraded RNA, in contrast to findings with most other starved organisms (see Dawes & Ribbons, 1964).

The mechanism of ribosome disaggregation and RNA breakdown in starved E. coli was studied by Wade (1961; see also Mandelstam 1963). Most of the depolymerase activity was associated with the ribosomes and was present in an inactive state in the presence of Mg^{2+}. Two degradative routes were proposed depending on the presence of Mg^{2+}. The M route (dependent on low Mg^{2+} concentrations) appeared to involve a phosphodiesterase and a polynucleotide phosphorylase. The V route involved a ribonuclease which was stimulated by removal of Mg^{2+}. It appears that removal of Mg^{2+} initiates ribosome disaggregation which causes RNAase activation with rapid breakdown of RNA to acid-soluble products. Gronlund & Campbell (1965) identified polynucleotide phosphorylase in the ribosome fraction of P. aeruginosa. This enzyme was only active at low Mg^{2+} concentrations which allowed the 70s ribosomes to dissociate. More recently, Ben-Hamida & Schlessinger (1966) have concluded that the breakdown of RNA was initiated by the destabilization of polyribosomes.

Most of the protein lost from starved organisms appeared in the external medium as biuret-positive material although the net increase in total free amino acids indicated that some protein had been hydrolysed. Protein hydrolysis occurs in many starved bacteria with subsequent catabolism of the released amino acids (see Dawes & Ribbons, 1964). Although the overall level of free aspartate (and to a lesser extent glutamate) was reduced, no evidence was obtained for appreciable catabolism of components of the free amino acid pool in S. lactis. In contrast, the free amino acid pool, and in particular glutamate, provided the main

endogenous substrate for starved S. lutea (Dawes & Holms, 1958). It has been suggested that pre-existing proteases are activated by starvation conditions (Schlessinger & Ben-Hamida, 1966; Willetts, 1967) and that protease activity is determined by the levels of protein precursors, perhaps amino-acyl sRNA. Willetts (1967) had found no correlation between the level of the free amino acid pool and the rate of protein degradation.

The cellular anthrone-reacting material and reducing sugar of S. lactis (which probably occurs mainly in structural polymers) together with cellular DNA, were not appreciably degraded in starvation conditions. These results are in agreement with literature reports for other starved organisms. However, it has been pointed out by Postgate (1967) and Burleigh & Dawes (1967) that chemical analyses would not detect structural changes in DNA that could cause loss of viability.

The loss of RNA and protein from starved S. lactis appeared to involve only hydrolytic reactions with the release of products into the external medium. Viability was not immediately affected and there was no evidence for appreciable energy-yielding catabolism of endogenous substrates. This was consistent with the formation of only trace amounts of the normal end products of fermentation, namely lactate, NH_3 and volatile fatty acids. As expected, the uncoupling agents 2,4-dinitrophenol and azide had no effect on survival. In contrast, the addition of these compounds to starved A. aerogenes, which has endogenous energy sources, substantially increased the death rate (Postgate & Hunter, 1964). Forrest & Walker (1965a) found that when S. faecalis was grown with limiting energy source and then starved, the ATP pool which was initially small, declined rapidly. They concluded that 'no detectable endogenous metabolism existed.' On the other hand, 'organisms grown with excess energy source exhibited endogenous metabolism' and this could be correlated with a much higher pool concentration of ATP.

Polymer synthesis. Since macromolecule synthesis is an energy

consuming process, protein synthesis in S. lactis organisms starved in phosphate buffer was not expected as no endogenous energy source was defined. In fact, the incorporation of valine-^{14}C into cell protein could only be demonstrated if an exogenous energy source was present. The extent of the incorporation appeared to be directly related to the amount of the available ATP. The acceleration of valine-^{14}C uptake by the presence of an added energy source, was consistent with mechanisms for the accumulation of amino acids in other bacteria (see Holden, 1962b).

The ability of starved organisms to accumulate and incorporate valine-^{14}C appeared to be correlated with viability and the rate of protein synthesis is probably directly influenced by the extent of RNA degradation. It has been suggested that vegetative bacteria may be able to undergo 'adaptation' in starvation conditions, presumably by turnover of constituents, and that this process may be important for survival (see Duguid & Wilkinson, 1961; Willetts, 1967). This could explain the decreased death rate of S. lactis in the presence of amino acids where some protein synthesis takes place. However, the results from the present study show that protein synthesis does not influence survival noticeably. In fact, chloramphenicol has been shown to reduce the death rate in some cases. As well as inhibiting protein synthesis, chloramphenicol may also inhibit protein degradation (Willetts, 1967) and hence may exert some sparing action on cellular proteins which are essential for survival. Chloramphenicol, on the other hand, had little or no effect on the survival of A. aerogenes (Postgate & Hunter, 1962). All reported studies involving starved bacteria indicate that while protein degradation may be balanced by synthesis during the initial starvation period, diminishing synthesis and a net increase in catabolism eventually results.

Metabolism of arginine and glucose. Both arginine and glucose were metabolized at constant rates by starved organisms and the theoretical rate of ATP generation was about 7.5 times greater with 10mM-glucose than with 10mM-arginine, in agreement with the

results of Forrest (1965) for starved S. faecalis. This rate of glucose metabolism is more than 50 times the rate calculated for the supply of maintenance energy in starved E. coli (Marr et al., 1963). From the data given for the growth of S. lactis it can be calculated that growing organisms should produce approximately 1.2 µmoles lactate/mg. dry wt. bacteria/min. while from glycolytic activity measurements it was found that non-growing organisms produced 0.29 µmoles lactate/mg. dry wt. bacteria/min. The simplest explanation for the observed linear rates of glycolysis and arginine fermentation in starved S. lactis is that these processes are not subject to feedback control so that ATP was generated in amounts which were far in excess of requirements. This explanation is in agreement with the results of Forrest & Walker (1965b). Forrest (1965) found that the constant input of ATP to the pool of starved S. faecalis during glycolysis was eventually balanced by an exponential decay process so that the pool level rose to an upper limit. When the exogenous glucose was completely fermented the decay process alone operated and the pool level fell back to the much lower endogenous base level.

In a number of starvation environments, the glycolytic activity of S. lactis organisms fell steadily from the onset of starvation and was not correlated with viability. Other workers have, however, found a correlation between the activity of the catabolic enzymes and survival in support of an early hypothesis associating bacterial death with enzyme inactivation (Rahn & Schroeder, 1941). For example, Postgate & Hunter (1962) found that the glycerol dehydrogenase and glycerol oxidase activities of starved A. aerogenes declined in parallel with viability, while the endogenous respiration rate of the glycerol-limited organisms was not directly related and, in fact, declined more rapidly. Similarly, Burleigh & Dawes (1967) reported a correlation between the survival of aerobically starved S. lutea organisms and their ability to oxidize exogenous glutamate and glucose. In the present study the

glycolytic activity of S. lactis was not maintained with exogenous energy sources or amino acids including arginine, in contrast to the findings of Walker & Forrest (1964) and Forrest & Walker (1965a). Indeed, added glucose produced a more rapid decline in glycolytic activity.

It seemed possible that the extended survival of starved S. lactis organisms with added arginine, compared with glucose, was due to the lower rate of ATP production. This explanation may be consistent with the results of McGrew & Mallette (1962) who claimed to have extended the survival of starved E. coli by the regular addition of very small amounts of glucose, although their experimental conditions did not preclude regrowth. In contrast, most other workers have used relatively high carbohydrate/cell mass ratios and have not observed extended survival. The metabolism of exogenous glucose by S. lactis produced long division lags and more rapid RNA breakdown in agreement with the results of Postgate & Hunter (1964) and Strange & Dark (1965) with A. aerogenes. The latter authors suggested that Mg^{2+} abolished substrate-accelerated death by preventing the accumulation of toxic products but such products have not been defined. The end products of carbohydrate and arginine metabolism are not toxic to S. lactis but unfavourable imbalances may have been created in the associated metabolic pools (see Introduction). However, the factors responsible for substrate-accelerated death have not been resolved. Strange's results (cited in discussion, Strange & Hunter, 1967) indicated that ATP accumulation during glucose metabolism was suppressed with added Mg^{2+}. In this connection, Postgate & Hunter (1964) have shown that glycerol-accelerated death could be largely abolished by uncoupling agents.

The death rate of S. lactis when harvested from the lactose-limited growth medium and starved in phosphate buffer, was accelerated to similar degrees by the addition of either glucose, galactose, fructose or lactose. All these sugars were probably metabolized via the glycolytic pathway. In contrast, the death

rate of glycerol-limited A. aerogenes was not accelerated by added glucose or ribose (Postgate & Hunter, 1964). Although these substrates were shown to be metabolized, there was no indication that the rate of their metabolism was similar to that in organisms grown with glucose or ribose limitations where these substrates accelerated the death rate of starved organisms. Since the activity of constitutive enzymes may vary according to growth conditions (Pardee, 1961), it seems possible that substrates not included in the growth medium may be metabolized at slower rates by starved organisms. However, the strain of A. aerogenes used by Strange & Dark (1965) showed carbon-accelerated death with carbon sources besides the one which had been used as the growth-limiting substrate. Glycerol- and succinate-accelerated death decreased on reducing the substrate concentration and was undetectable at low concentrations (Postgate & Hunter, 1964). It could be informative to re-examine the effect of low glycerol concentrations on survival in the presence of added Mg^{2+}. Strange & Dark (1965) have observed that increased rates of glycerol oxidation produced increased death rates and hence there is now a considerable amount of evidence which suggests that the rate of substrate metabolism, and the rate of ATP production, may be critical for survival. Slow substrate metabolism may supply the maintenance energy requirement without deleterious effects.

The metabolism of exogenous substrates does not always appear to be coupled to the energy requirements of starved organisms and it seems possible that the rapid depletion of reserve polymers in some bacteria may present a similar situation with respect to endogenous substrates. Burleigh & Dawes (1967) have suggested that the rapid metabolism of polyglucose may cause substrate-accelerated death. The fact that glucose was not limiting in their growth cultures may not be inconsistent with this interpretation, since substrates apart from those limiting growth may accelerate death as discussed previously. Reserve polymers may exert a sparing action on

essential cell constituents but they seem to enhance survival only if they are broken down at comparatively slow rates (e.g. see Strange *et al*., 1961; Sierra & Gibbons, 1962; Sobek *et al*., 1966; Zevenhuizen, 1966). However, Strange & Hunter (1967) also pointed out that the enhanced survival of nitrogen-limited *A. aerogenes* may be due to the relatively high Mg^{2+} content rather than the glycogen reserves.

Changes in permeability and ultrastructure. Polar lipid and in particular phospholipid, constitutes the main lipid fraction in most lactic acid bacteria (see review by Kates, 1964). In *S. faecalis*, 94% of the total lipid was found in the membrane fraction (Vorbeck & Marinetti, 1965a). A substantial amount of phospholipid breakdown occurred on prolonged starvation of *S. lactis* ML_3. Since these compounds are known to have important structural and physiological roles in bacterial membranes (see reviews by Brown, 1964; Salton, 1967), any breakdown of phospholipids may be expected to impair permeability barriers.

There was a steady leakage of the intracellular amino acid pool from starved *S. lactis* into the external medium. Release of accumulated valine-^{14}C from the TCA-soluble pool of *S. lactis* was suppressed by the presence of an exogenous energy source. This result is similar to the observation made by Gale (1953) with *S. aureus*. In contrast to the present results Holden (1962b) has suggested that leakage of the free amino acid pool is a common property of starved Gram-negative bacteria in contrast to the retention of the pool by Gram-positive organisms. However, the important factor may be the presence of an energy supply since organisms with endogenous energy sources appear to retain their free amino acid pools when starved (Dawes & Holms, 1958; Postgate & Hunter, 1962; Dawes & Ribbons, 1965). Conditions promoting release of the intermediate metabolic pools may initiate polymer breakdown and increase the death rate and this would be consistent with the observation that spermine enhanced survival and suppressed the

release of u.v.-absorbing material from starved S. lactis.

As well as promoting ribosome stability, added Mg^{2+} may have an important function in maintaining permeability barriers. The finding that high EDTA concentrations were lethal may support this view since EDTA appears to remove cell wall and membrane Mg^{2+} by chelation. After approximately 10 hr. starvation of S. lactis in phosphate buffer containing Mg^{2+}, the death rate began to increase and was accompanied by the release of lactic dehydrogenase and DNA. No cell lysis was evident from electron micrographs prepared after 17 hr. starvation but most of the cells were lysed after 40 hr. starvation. The release of intracellular macromolecules, before lysis was detectable by electron microscopy, appeared to result from a partial rupture of the cell wall and membrane. These structures were maintained intact for a longer period in the presence of exogenous amino acids and lysis occurred as the viability fell. In this environment, death may be a direct result of cell wall or membrane degradation.

Conclusions. The existence of minimum growth rates for bacteria (Tempest et al., 1967) implies a state of minimum subsistence which must be maintained for survival. In this state, essential polymers are present at the lowest level capable of initiating regrowth and any breakdown of these components results in loss of viability. It is now well established that bacteria produced at maximum growth rates may contain material in excess of these minimum levels so that considerable degradation may take place before death. Organisms capable of only low rates of growth and catabolism may have an advantage for survival in starvation environments. The products of polymer hydrolysis in most bacteria are metabolized producing energy but with S. lactis these products generally appear to be released into the external medium in an undegraded form.

Although it has been suggested that maintenance of bacteria in a viable state requires energy, the amount of energy is ill-defined. S. lactis maintained complete viability for 15-20 hr.

when starved in buffer containing Mg^{2+}, yet no absolute requirement for an energy source could be determined. At the onset of starvation an ATP pool was probably present and small amounts of energy may have been produced by endogenous metabolism especially if S. lactis contains constitutive kinases for nucleotide metabolism (see Gronlund & Campbell, 1965). However, leakage of the intracellular free amino acid pool occurred as soon as the organisms were starved, there was no appreciable protein synthesis and, in addition, starved organisms were extremely pH sensitive. This suggested that an appreciable endogenous energy source was not involved in maintaining the viability of organisms starved in phosphate buffer. It would appear that these organisms remained viable as long as the degradation of polymers, particularly RNA, had not proceeded beyond some irreversible point and the present evidence suggests that the death rate was dependent on the presence of compounds promoting polymer stability. When Mg^{2+} was added to buffered suspensions, a suitable exogenous energy source further enhanced survival. This energy source appeared to suppress the release of the free amino acid pool and allow limited protein synthesis and pH control. In this environment survival may then be a function of cell wall and membrane stability.

S. lactis does not appear to have any survival mechanism as a result of an endogenous metabolism. The ability to withstand low pH values may be the most important factor for the survival of this organism in milk. The present results provide information which may be relevant to applied studies involving 'cheese starter' propagation and storage where it is important to produce organisms with minimum cell division lag times and maximum viabilities and rates of lactate production.

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