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STUDIES ON THE GROWTH,
VIABILITY AND LIPIDS OF
STREPTOCOCCUS BOVIS

A thesis presented in partial fulfilment of the requirements
for the degree of Master of Agricultural Science
in Animal Science

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CHAPTER 1

INTRODUCTION

In their comprehensive review of rumen metabolism, Annison and Lewis (1959) emphasised the symbiotic relationship between the metabolic activities of the mixed population of anaerobic bacteria and ciliated protozoa, and the digestion of fodder consumed by the host. Prominent features of the microbial activity characterising the ruminant mode of digestion have been listed by Moir (1965) and may be summarised as follows:

(1) Cellulose is hydrolysed to monosaccharides by microbial cellulases and carbohydrates fermented to volatile fatty acids. While problems in the quantitative assessment of this volatile fatty acid production were reviewed by Warner (1964), it was also suggested that the amount produced in the rumen and absorbed directly into the blood stream, was sufficient to meet about 70 % of the host's energy requirements.

(2) Microbial protein is synthesised from both plant protein and inorganic nitrogen with the energy released during carbohydrate fermentation. Although the extent of this conversion has also proved difficult to quantitate, Phillipson (1964) has stated that the microbial synthesis of essential amino acids, not always present in the diet, makes the ruminant almost independent of the quality of dietary protein.

(3) As B vitamin deficiency has never been demonstrated in animals on a balanced intake of trace elements (Annison and Lewis 1959 p. 20) it would appear that the microbial population can synthesise the B vitamins.

A more extensive review of carbohydrate metabolism in the rumen can be found in the monograph by Oxford (1964), while nitrogen metabolism was covered thoroughly by Phillipson (1964) and Blackburn (1965).

To orientate investigations on the lipids of a rumen microorganism, Streptococcus bovis, current knowledge of the metabolism of lipids in the rumen is examined with special reference to the microbial activity responsible for modifying dietary lipid. Most attention will be directed towards features not discussed by Garton (1961, 1965) in his reviews of lipid metabolism in the rumen. The converse effect of dietary lipid in microbial activity is not considered, having been recently investigated and discussed by Robertson and Hawke (1964 a, b).

For further aspects of lipid digestion in the ruminant the reviews of Garton (1961, 1963, 1965) should be consulted.

LIPID METABOLISM IN THE RUMEN

After a brief examination of the constituents of dietary lipid, the breakdown and modification of lipid is reviewed in relation to microbial activity. The section concludes with a consideration of the lipids synthesised by rumen microbes.

(1) Lipids in the Diet of Ruminants

Based on an intake of 100 lb dry matter (D.M.) per day, Garton (1961) estimated the maximum lipid consumption of a grazing cow to be of the order of 500 g per day. However, the validity of this estimate is questionable, as Hutton, Hughes, Newth and Watanabe (1964) found the maximum intake of New Zealand Jersey cows was about 30 lb D.M. per day. In pasture plants the lipid content varies from 4 - 8 % (Shorland 1961, Garton 1961), giving a maximum lipid intake of about 1,000 g/day. This figure may be increased still further, by 100 g per day, if the pasture has been sprayed with emulsified oil to prevent bloat, as recommended by Reid and Johns (1957).

Data on the lipid composition of any plant species, let alone pasture plants, is still incomplete (Allen and Good 1965) but from the work of Shorland (1961) and Weenink (1959, 1961, 1962) it was found that over half of the lipid from a variety of pasture species was acetone-soluble; of which the galactolipids, mono- and di- galactosyl glycerol esters of linolenic were major components. For example in red clover, Weenink (1962) found that galactosyl glycerides amounted to 50 % of the total lipid while triglycerides, diglycerides, sterols, sterol esters and hydrocarbons together comprised less than 4 %. Virtually all of the galactosyl glycerides are thought to be present in plant chloroplasts (Benson 1964).

Known constituents of polar lipids in plants have been listed by Allen and Good (1965). These included the choline, ethanolamine, serine, glycerol and inositol esters of phosphatidic acid, the sulphonated sugar lipid (sulpholipid), the sphingolipids (including cerebrosides), and proteolipids. No estimate of the extent to which these constituents are present in the lipids of pasture plants appears to have been published.

Before reaching the small intestine, where absorption of long chain fatty acids occurs, (Johnston 1963), the complex array of dietary lipids is subjected to the hydrolytic action of rumen microorganisms.

(2) Hydrolysis of Lipid

The discovery of lipid hydrolysis in the rumen was made by Garton, Hobson and Lough (1958) who found that free fatty acids accounted for over half of the total lipid extracted from rumen contents. In vitro incubations of linseed oil with rumen contents released 75 % of the triglyceride as free fatty acids. Subsequent experiments by Garton, Lough and Vioque (1961) showed extreme variation in the extent of hydrolysis (in vitro) which may have been due to the extent of emulsification of the linseed oil. Thus cocoa butter, a very saturated fat and hence difficult to emulsify was less extensively hydrolysed than olive and linseed oils which are more readily emulsified. Further evidence for the hydrolysis of triglycerides was obtained by Hawke and Robertson (1964), who found mono- and di- glycerides in the rumen liquor of a dairy cow fed pasture and 500 g/day of linseed oil.

Besides triglycerides a wide variety of other lipid compounds are also hydrolysed. Thus lecithin and lysolecithin (Dawson 1959), ethyl esters of fatty acids (Hill, Saylor, Allen and Jacobson, 1960), monostearin, tributyrin, and Tween 80 (Wright 1961) and galactosyl glycerides, sterol esters and methyl esters (Garton 1965) have all been shown to undergo hydrolysis. However, attempts to isolate mono- and di- glycerides during the in vitro hydrolysis of galactosyl glycerides by rumen contents were unsuccessful (Hawke and Weenink unpublished. Cited by Hawke and Robertson 1964).

Consequent upon the hydrolysis of lipid ester linkages is the release of water soluble moieties such as glycerol, galactose and phosphatide bases. While galactose and glycerol are known to be fermented to volatile fatty acids (see Garton 1965) microorganisms can probably metabolise phosphatide bases such as choline and ethanolamine.

Hydrolytic Enzymes and Microbial Activity

Although enzyme preparations, capable of releasing the water soluble moieties from lipids, have been extracted from rumen bacteria and protozoa, attempts to demonstrate lipase activity in cell-free extracts have not been entirely successful. Thus Bailey (1962) obtained α - and β - galactosidase activity in cell-free extracts of rumen bacteria, but the preparations were inactive on intact galactosyl glycerides, depending upon their prior deacylation. Deacylation was demonstrated, however, with cell suspensions of the same bacteria, showing that bacteria could be responsible for total hydrolysis of the galactolipids.

Similarly Bailey and Howard (1963) and Howard (1963) reported the extraction of α - and β - galactosidase activity from four species of protozoa. While these enzymes were capable of hydrolysing the intact galactosyl glycerides no release of free fatty acids was observed.

The partial success in preparing cell-free lipase activity is typified by the reports of Dawson (1959) and Wright (1961). Butanol extracts of rumen bacteria prepared by Dawson (1959) gave complete hydrolysis of lysolecithin but were inactive against lecithin. Likewise Wright (1961) obtained extensive hydrolysis of tributyrin and trihexanoïn with extracts of rumen bacteria or protozoa, but little activity was observed on esters of long chain fatty acids.

On the other hand Hobson and Mann (1961) repeatedly isolated an unidentified lipolytic bacterium from $1/10^9$ dilutions of rumen fluid from a sheep fed hay and concentrates with and without linseed oil. Clear zones were observed around colonies on agar containing an emulsion of linseed oil, indicating that the organism may have secreted an extracellular lipase.

Further evidence to implicate bacteria as agents in the hydrolysis of lipids comes from studies with antibiotics. It is well known that some antibiotics are able to prevent the onset of bloat (Mangan, Johns and Bailey 1959) and some evidence for their mode of action accrues from the studies of Hill (1960 Cited by Garton 1965) and Wright (1961). Both of these workers showed that the lipase activity of rumen contents decreased when some antibiotics were fed and it was argued that reduced lipolysis would favour the continued antifoaming action of the intact lipids. However, Shellenberger (1964 Also cited by Garton 1965) was unable to confirm the earlier results.

Although Oxford (1958 a) observed the ingestion of chloroplasts by a protozoal species it is not clear to what extent the hydrolysis of lipids may occur as an intra- or extra- cellular process. Thus studies to define the location and extent of lipid hydrolysis merit high priority, especially if it should be shown that the presence of intact lipid in the rumen liquor is necessary for the prevention of bloat in cattle.

(3) Hydrogenation of Unsaturated Fatty Acids

Although the composition of the body fat of monogastrics reflects the nature of the dietary fatty acids, ruminant fats retain a highly saturated fatty acid composition in spite of the ingestion of predominantly linoleic and linolenic acids from plant lipids (Garton 1963). The studies of Reiser (1951) pointed to the occurrence of hydrogenation in the rumen to account for this phenomena, as a reduction in linolenic acid concentration was observed in incubations with rumen contents. Further evidence for the hydrogenation of plant fatty acids was obtained by Shorland, Weenink and Johns (1955) who showed that the high proportions of linolenic and linoleic acids present in herbage were not reflected in the fatty acid composition of rumen contents.

Hydrogenation and Microbial Activity

The hydrogenating activity of protozoa was indicated by Wright (1959) who incubated linseed oil, sodium linoleate and chloroplast lipids with washed suspensions of protozoa in the presence of antibiotics to minimise the activity of any bacteria also present.

Analysis of the fatty acids after incubation showed a decrease in iodine numbers or changes in fatty acid composition consistent with the occurrence of hydrogenation. More recently Gutierrez, Williams, Davis and Warwick (1962) have demonstrated the uptake of 1-C¹⁴ oleic acid by washed suspensions of two ciliate protozoal species and its conversion to stearic acid.

Although initial attempts to demonstrate that rumen bacteria could also hydrogenate fatty acids were unsuccessful, it was later found by Wright (1960 a) that glucose and rumen fluid were necessary for hydrogenation to occur and were possibly needed for the fermentative activity of the bacterial suspensions. Recently, Polan, McNeill and Tove (1964) have developed an assay system for measuring hydrogenation activity of washed suspensions of bacteria in the presence of boiled rumen fluid and 0.25 - 4.0 mg/ml of 1-C¹⁴ linoleic acid as substrate. In view of the high concentration of substrate, compared with the levels of fatty acids found in rumen contents by Hawke and Robertson (1964), the claim by Polan et.al. (1964) that carbon dioxide inhibited hydrogenation is not necessarily valid; especially as Ward, Scott, and Dawson (1964) obtained extensive hydrogenation with a gaseous phase of carbon dioxide/nitrogen in an artificial rumen, where microorganisms were supplied with a diet of hay and oats, and only 0.001 - 0.28 mg/ml of a C¹⁴ - labelled unsaturated acid employed as substrate. These levels approximated to the concentration in rumen contents reported by Hawke and Robertson (1964). Polan et.al. (1964) also found it necessary to gas their system with hydrogen in order to effect measurable rates of hydrogenation.

A survey of rumen bacterial species by Polan et. al. (1964) for hydrogenation activity indicated that only Butyrivibrio fibrisolvens was active, but further studies revealed a distinct loss of activity with aged cultures of this species. In the light of this finding it is disturbing that the survey of bacterial species was not repeated using suspensions known to be viable or capable of fermentative activity. These authors also found that suspensions of mixed species of bacteria were capable of hydrogenation whereas pure species, presumably of a similar age were inactive. Attempts to define the nature of this symbiosis were inconclusive but made no allowance for the possibility that the death or lysis of one species was providing fermentable substrate for another.

It was also reported by Polan et. al. (1964) that glucose, formate or amino acids were unable to replace rumen fluid and thereby act as hydrogen donors. However, as Wright (1960 a) found that both rumen fluid and glucose were needed for the hydrogenating activity of washed suspensions of bacteria, the experiments of Polan et. al. (1964) probably failed to provide conditions necessary to test their hypothesis viz.; that glucose, formate or amino acids served as hydrogen donors.

Evidence for the location of hydrogenation on or within the cell wall was sought by Polan et. al. (1964) by measuring the extent of hydrogenation in the precipitate and supernatant fractions of their incubation system. As identical activity was noted in each fraction their claim that hydrogenation occurred on or within the cell wall presupposed that acids were released into the supernatant as rapidly as they were hydrogenated. No evidence for

the validity of this assumption was considered and consequently the results fail to support their claim.

Although the nature of the hydrogenation reaction remains obscure, Shorland, Weenink, Johns and McDonald (1957) and Ward, Scott and Dawson (1964) have both made an intensive examination of the reaction products. Shorland *et. al.* (1957) incubated relatively large amounts of oleic, linoleic and linolenic acids with rumen contents and using classical fractionation procedures demonstrated the formation of trans - unsaturated acids and positional isomers of mono- and di- enoic acids among the products of hydrogenation, indicating migration of the double bond during the reaction. On the other hand Ward *et. al.* (1964) employed radioactive substrates in an artificial rumen and drew conclusions from the radioactivity recovered in fractions obtained from thin-layer and gas-liquid chromatographic separations. Unsaturated acids were oxidised and the products examined for radioactivity. As well as substantiating the results obtained by Shorland *et. al.* (1957) further evidence was obtained for the formation of a conjugated dienoic acid from linolenic acid and the migration of the C-15 double bond toward the methyl group in non-conjugated acids. Monoenoic acids formed were predominantly of the trans configuration with double bond mainly at C-13 and C-14.

Both Shorland *et. al.* (1957) and Ward *et. al.* (1964) have noted the resemblance between the products of hydrogenation in the rumen and the products of catalytic hydrogenation of natural oils. As the reducing potential of the rumen is only -0.35 V (Hungate 1963) at a temperature of 39°C, little if any, catalytic hydrogenation would be expected and the presence of enzymes is implied. Polan *et. al.* (1964) obtained some evidence for the presence of iron and sulphhydryl groups in a

hypothetical enzyme as both cyanide and arsenite at 0.1 M concentration inhibited hydrogenation whereas 0.1 M azide had no effect. It was also found that B. fibrisolvens, while capable of converting linoleic to a monoenoic acid, was unable to form the saturated acid, indicating the possible existence of specific enzymes for each stage of the reaction.

The relationship between hydrolysis and hydrogenation has yet to be fully clarified, but both Garton, Lough and Vioque (1961) and Hawke and Robertson (1964) have obtained evidence for the preferential hydrogenation of free fatty acids over triglycerides, as higher proportions of saturated acids were found in the free fatty acids released during hydrolysis, than in the unhydrolysed triglycerides. However the unhydrolysed triglycerides also contained a higher proportion of saturated acids than the linseed oil employed as substrate, suggesting that either some hydrogenation of intact triglycerides occurred or that preferential hydrolysis of more unsaturated triglyceride species was taking place. Transesterification reactions could also be invoked to explain these results but no consideration to their occurrence in the rumen has been given.

(4) Degradation of Fatty Acids

An evaluation of the extent of degradation of long chain fatty acids is hampered by a paucity of experimental data and only the studies of Wood, Bell, Grainger and Teekell (1963) give positive indication of the occurrence of breakdown. These workers added 1-C¹⁴ linoleic acid to the rumen contents of sheep, then recovered steam volatile and long chain fatty acids after 48 hours. With the reticulo-omasal orifice ligated, loss of digesta from the rumen was prevented and 90 % of the radioactivity added was recovered as long chain acids. Less than 1 %

of the original radioactivity was present in steam volatile fatty acids from the rumen, while further small amounts of labelled volatile and long chain fatty acids were found in the jugular blood, having been absorbed from the rumen. Thus only minor proportions of long chain fatty acids are thought to be broken down in the rumen and no evidence is available to implicate microorganisms in this capacity.

The ω -oxidation of hydrocarbons to fatty acids observed by McCarthy (1964) in ruminants does not appear to be undertaken by rumen microorganisms as following the administration of C^{14} - hexadecane or C^{14} - octadecane no labelled fatty acids were found in rumen contents.

Thus through the hydrolytic and hydrogenating reactions occurring in the rumen, substantial changes to dietary lipids are made prior to their absorption from the small intestine. However, as microbial growth proceeds with fermentation (Hungate 1963) lipids are synthesised by microorganisms for cell membranes, or cell walls (Kates 1964). With the outflow of digesta and microbes from the rumen, a wide variety of lipids of both dietary and microbial origin are subjected to further hydrolysis by pancreatic lipases and absorbed from the small intestine (Borgstrom 1960).

(5) Lipids of Rumen Microorganisms

Apart from studies on the formation of branched chain fatty acids, lipid metabolism by rumen microorganisms has received scant attention. However Kates (1964) in a general review of bacterial lipids, their distribution in the cell, and metabolism emphasised the similarities of lipid composition shown by closely related species. Although Bryant (1959) has indicated that many of the bacterial species isolated from the rumen are unique to this environment, it is expected that most aspects of their lipid metabolism will resemble that discovered in other

anaerobic bacteria. In this review attention is confined to three aspects of microbial fatty acid synthesis -

- (a) The pathway of fatty acid synthesis in anaerobic bacteria.
- (b) The incorporation of free fatty acids.
- (c) The synthesis of branched chain fatty acids and aldehydes by rumen bacteria.

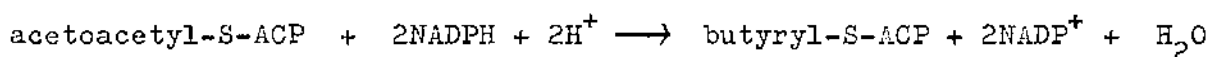
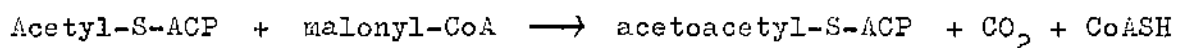
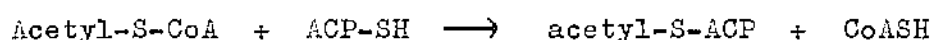
(a) Fatty Acid Synthesis by Bacteria

Early evidence for the bacterial synthesis of fatty acids from acetate was obtained by Stephenson and Whetham (1922) who showed that the lipid content of Mycobacterium phlei was increased by the addition of acetate to the growth medium. A similar effect of acetate on Escherichia coli lipid was also observed by Dagley and Johnson (1953). More recently, Goldfine and Bloch (1961) working with Clostridium butyricum, and Thorne and Kodicek (1962 b) working with Lactobacillus casei, have shown that labelled acetate was incorporated into long chain fatty acids.

For many years fatty acid synthesis from acetyl - CoA was thought to occur by reversal of the pathway of β -oxidation (O'Leary 1962). However Wakil (1958) showed that fatty acid synthesis in pigeon liver proceeded via carboxylation of acetyl-CoA to form malonyl-CoA. Subsequently Wakil and Ganguly (1959) found the enzyme which catalysed the synthesis of fatty acids by coupling the decarboxylation of malonyl-CoA with the elongation of the acyl chain. A similar enzyme was found in yeast by Lynen (1961) who also carried out an extensive investigation of this, the condensation reaction.

Recently Vagelos and co-workers have extracted soluble enzymes for the condensation reaction from Clostridium kluyveri and E. coli.

Fractionation of these enzymes yielded a heat stable protein which was purified and found to contain one sulphhydryl group. As this enzyme was shown to bind the acyl group during condensation with successive two-carbon fragments, it has been called the acyl carrier protein (ACP) (Vagelos 1964). The following reactions were catalysed by the complete enzyme system;



and shown to be intermediates in the formation of fatty acids from malonyl-CoA (Alberts, Goldman and Vagelos 1963, Goldman, Alberts and Vagelos 1963 a, 1963 b, Goldman 1964). These results constitute strong evidence for the bacterial synthesis of fatty acids by the malonyl-CoA pathway similar to that found in yeast (Lynen 1961) and pigeon liver (Wakil 1961).

On the other hand biosynthesis of unsaturated fatty acids in anaerobic bacteria proceeds by a different pathway from the aerobic desaturation of fatty acids common to the actinomycetes, yeasts and higher organisms (Bloch 1962). Current knowledge of the anaerobic pathway, which involves elongation of β , γ - decenoic acid to cis-vaccenic acid, has been reviewed by Vagelos (1964) who also presents evidence for the occurrence of this pathway in facultative as well as obligate anaerobes.

(b) Incorporation of Free Fatty Acids

In addition to the synthesis of fatty acids, Goldfine and Bloch (1961) and Thorne and Kodicek (1962 c) have shown that bacteria can incorporate preformed fatty acids from the media. In view of the existence of long chain fatty acids in rumen fluid (Garton, Lough and Vioque 1961, Hawke and Robertson 1964), it is obvious that the fatty acids found in bacteria

harvested from the rumen, are not necessarily those synthesised from short chain precursors.

For example, using gas liquid chromatography, Keeney, Katz and Allison (1962), Erwin, Sterner and Marco (1963) and Tweedie (1965) have all found polyenoic acids in bacteria harvested from the rumen, yet despite intensive investigation no polyenoic acids have been found to be synthesised by bacteria (Bloch, Baronowski, Goldfine, Lennarz, Light, Norris and Scheuerbrandt 1961, Kates 1964, Erwin, Hulanicka and Bloch 1964).

To overcome this problem fatty acid metabolism in rumen microorganisms can only be studied with radioactive precursors or in media known to be free of long chain fatty acids.

Similar arguments also apply to the long chain fatty acid metabolism of rumen protozoa, but, to date no studies of the fatty acids synthesised by comparable species of anaerobic protozoa, let alone rumen species, appear to have been reported. However, Gutierrez, Williams, Davies and Warwick (1962) showed that palmitic, stearic, oleic and linoleic acids were taken up from the media by washed suspensions of two species of rumen protozoa.

(c) Branched Chain Fatty Acids and Aldehydes

Evidence for the occurrence of iso- and anteiso- long chain acids in butterfat was reviewed by Shorland and Hansen (1957). With the discovery of branched chain fatty acids in the lipids of bacteria harvested from the rumen by Keeney, Katz and Allison (1962), it was realised that these bacteria were the probable origin of branched chain acids in butterfat. Similarly Katz and Keeney (1964) have suggested that the branched chain fatty aldehydes of complex plasmalogen lipids in ruminant tissues are synthesised by rumen bacteria. These authors isolated fatty aldehydes from rumen bacterial lipids and by gas-liquid chromatography of the

reduced aldehydes showed that 45 % of the total aldehyde fraction had a branched chain structure.

Tweedie (1965) has reviewed the occurrence of branched chain fatty acids in bacterial lipids and the metabolism of branched chain volatile fatty acids which are considered to be precursors of the higher branched chain acids. Several studies claiming to demonstrate the incorporation of branched volatile fatty acids into their higher homologues have been reported. Thus Allison, Bryant, Katz and Keeney (1962) found that Ruminococcus flavefaciens incorporated 1-C^{14} isovalerate into long chain fatty acids and aldehydes while R. albus, another cellulolytic bacterium, incorporated 1-C^{14} isobutyrate. Similarly Wegner and Foster (1963) found that both 1-C^{14} valerate and 1-C^{14} isobutyrate were incorporated into the long chain fatty acid and aldehyde moieties of an ethanolamine plasmalogen, in the cellulolytic Bacteroides fibrisolvens. Tweedie (1965) using mixed cultures of rumen bacteria demonstrated the incorporation of 1-C^{14} isobutyrate into bacterial lipids. These workers then used preparative gas-liquid chromatography to separate the methyl esters of the fatty acids. In each case the highest activity was associated with fractions tentatively identified as the homologous branched chain fatty acids. However, no evidence for the purity of the fractions collected was quoted in the literature, nor was evidence other than retention volumes on gas-liquid chromatography obtained for the chemical structure. Despite these deficiencies the results conform to the theory of branched chain fatty acid biosynthesis. This visualises the elongation of a branched volatile fatty acid with two-carbon units derived from malonyl-CoA (Kates 1964).

The fatty acid composition of a non-cellulolytic rumen microorganism, Streptococcus bovis was studied by Tweedie (1965) using gas-liquid

chromatography (Table 1). Unlike the cellulolytic bacteria mentioned above, S. bovis did not incorporate isobutyrate into long chain fatty acids, nor could the formation of any iso- acid be induced by the presence of isobutyrate.

As Tweedie (1965) had found lipids to constitute 7 % of the dry weight of S. bovis, the present investigations were commenced with the long-term objective of assessing the importance of lipids as endogenous reserves. To keep this aim in perspective, the importance of S. bovis as a member of the rumen population is deduced from current knowledge of rumen microbiology and metabolism. Subsequent sections of this review cover the general biochemistry of S. bovis, lipid metabolism in the lactic acid bacteria and evidence for the function of lipids in bacteria.

S. BOVIS IN THE RUMEN

Bryant (1959) and Hungate (1963) have both made comprehensive reviews of the variety of bacterial species found in sufficient numbers to account for some aspect of rumen metabolism. One of these species, S. bovis, has been isolated from rumen contents by MacPherson (1953), Mann, Masson and Oxford (1954), Perry, Wilson, Newland and Briggs (1955), Hungate (1957), Bailey and Oxford (1958 a), Krogh (1963) and Clarke (1964). All of these authors obtained positive evidence for the identification of their isolates and many were made from dilutions of greater than $1/10^6$, while Gall and Huhtanen (1951), Hungate, Dougherty, Bryant and Cello (1952), Higginbottom and Wheeler (1954) and Krogh (1959, 1960, 1961) have also found more than a million streptococci per ml of rumen contents. Problems encountered in making repeatable counts of rumen bacteria were discussed by Bryant (1959), but the results obtained indicate that sufficient streptococci are present to have some role in the overall metabolism in the rumen.

Isolates have been predominantly facultative anaerobes, but Hungate (1957) claimed that most of his isolates showed an obligate requirement for anaerobiosis.

The characteristic fermentation pattern for S. bovis is the formation of lactate from hexose (Annison and Lewis 1959), but only when diets rich in soluble carbohydrate are fed does the concentration of lactic acid in rumen fluid reach a measurable concentration (Balch and Rowland 1957). Furthermore, studies on the turnover of lactic acid by Jayasuriya and Hungate (1959) demonstrated that lactic acid formation accounted for less than 1 % of the total fermentation of a hay diet, but could reach 8 % of the total on grain feeding. Thus the metabolic activity of S. bovis is normally of minor importance to fermentation in the rumen.

However, a sudden excessive intake of soluble carbohydrate causes an acute indigestion accompanied by the accumulation of lactic acid in the rumen (Hungate et. al. 1952). This was also associated with a sharp increase in the numbers of viable streptococci as the pH of the rumen fell to 5.0. Below pH 5.0, Krogh (1959, 1960, 1961) found that lactobacilli predominated, suggesting that these species were more tolerant of acidity than the streptococci and had taken over the role of lactic acid production. Death of the animal was commonly observed as the result of indigestion.

If however, a gradual change was made to a diet rich in soluble carbohydrate, no symptoms of indigestion occurred and it is currently thought (Annison and Lewis 1959 p. 166) that the increased lactic acid production is matched by an increase in the numbers of lactic acid fermenting bacteria. Thus Jayasuriya and Hungate (1959) have demonstrated the conversion of 2-C¹⁴ lactate to a mixture of acetic, propionic and butyric acids in rumen contents, while a number of lactate fermenting bacteria have been isolated from the rumen. (see Annison and Lewis 1959 p. 48). On the other hand, evidence for the slow absorption of lactate

from the rumen has been presented by Williams and Mackenzie (1965).

From the data at present available, it seems as though S. bovis is of minor importance during normal metabolism in the rumen, however, its capacity for extremely rapid growth (Hungate 1963) places it at an obvious advantage when given access to the soluble carbohydrates it is capable of fermenting.

BIOCHEMISTRY OF S. BOVIS

Strain I of S. bovis, which was cultured throughout the present investigation, was isolated by Bailey and Oxford (1958 a). According to "Bergey's Manual of Determinative Bacteriology" (Breed, Murray and Smith 1957), S. bovis is a member of the "viridans" group within the Group D streptococci. All Streptococcus species are members of the Lactobacteriaceae family within the order Eubacteriales.

To introduce a study on the lipids of S. bovis, current knowledge of the biochemistry of the species, the lipids of other lactic acid bacteria and the function of lipids in bacteria will be reviewed.

In previous studies of S. bovis most attention was directed toward the synthesis and degradation of polysaccharides or the ability of some strains to utilise ammonia as the sole source of nitrogen. Although other aspects of S. bovis metabolism have received scant attention, the biochemistry of S. faecalis has been extensively investigated and was recently reviewed by Deibel (1964) with particular emphasis on the similarities and differences between the various members of the Group D streptococci. Therefore in a review of the biochemistry of S. bovis under the headings indicated below, it is relevant to discuss literature dealing with related streptococci and lactobacilli as many aspects of their metabolism appear to be similar.

- (1) Carbohydrate Metabolism of S. bovis.
- (2) Nitrogen, Sulphur, Vitamin and Growth Factor Requirements.
- (1) Carbohydrate Metabolism
 - (a) Carbohydrases
 - (b) Intracellular polysaccharides
 - (c) Capsular polysaccharides
 - (d) Extracellular polysaccharides
 - (e) Fermentation of carbohydrate for energy

(a) Carbohydrases of S. bovis

Identification of lactic acid bacteria according to "Bergey's Manual of Determinative Bacteriology" (Breed, Murray and Smith 1957), relies upon the ability of a species to hydrolyse and ferment a characteristic range of poly- and oligo-saccharides. The rationale of this criterion of classification would appear to be that the enzymes responsible for hydrolysis (the carbohydrases) are relatively constant characteristics of the species. Thus amylolytic activity was employed by MacPherson (1953) for the selective isolation of streptococci from the rumen. The isolates were similar to cultures identified as S. bovis. Similarly Merdek and Barnes (1962) examined the characteristics of 170 isolates of S. bovis. Hydrolysis and fermentation of starch, inulin, lactose and raffinose was characteristic of all but one of the isolates.

Enzyme activities responsible for the hydrolysis of these carbohydrates, together with melibiose and sucrose, were observed in cell-free extracts by Bailey and Bourne (1961) and Bailey (1963). From the range of substrates hydrolysed these authors suggested that S. bovis contains amylase, α -(1 \rightarrow 6) glucosidase, α -galactosidase, invertase, sucrose phosphorylase and isomaltase. In addition Walker (1965 a) has

isolated cell-bound and extracellular α -amylases from S. bovis. The properties of both enzymes were similar and evidence was presented for the extracellular secretion of the enzyme into the medium, rather than its release from lysed cells.

(b) Intracellular polysaccharides

Hobson and Mann (1955) observed the formation of an intracellular iodophilic polysaccharide, comprised almost entirely of glucose in α -(1 \rightarrow 4) linkages, when S. bovis was cultured on maltose or various amylopectins, but no formation was observed on glucose, sucrose, trehalose, cellobiose or amylose. Accumulation of the polysaccharide was only observed during resting phase and in a media buffered between pH 5 and 6.

Recently Walker (1965 b) has studied the properties of an intracellular transglucosylase of S. bovis which was capable of forming maltodextrins with α -(1 \rightarrow 4) glucosidic linkages. However, this enzyme does not appear to be responsible alone for the synthesis of the intracellular iodophilic polysaccharide studied by Hobson and Mann (1955) as the enzyme had pH optimum between 6 and 8 and utilised amylose or glucose as substrates, while the accumulation of iodophilic polysaccharide by whole cells required a pH of 5 - 6 and was not observed in amylose or glucose media.

(c) Capsular polysaccharides

Capsular polysaccharides from four strains of S. bovis were extracted by Hobson and MacPherson (1954). Galactose, rhamnose, and uronic acids were found in the acid hydrolysate of the dialysed polysaccharide. In addition to these sugars Bailey and Oxford (1959) found glucose in the capsular polysaccharide of S. bovis (Strain I).

(d) Extracellular polysaccharides

Following the reports by Niven, Smiley and Sherman (1941) Hehre and Neill (1946) and Dain, Neal and Seeley (1956) that extracellular polysaccharides were formed when "viridans" streptococci were grown in sucrose media, Bailey and Oxford (1958 a, b), Oxford (1958 b), and Bailey (1959 a) showed that S. bovis (Strain I) was able to synthesise an unbranched α -(1 \rightarrow 6) linked anhydroglucose polymer (dextran) from sucrose, in the presence of carbon dioxide. The enzyme responsible for dextran formation was found by Bailey (1959 b) to be present in the cell free filtrate and was called dextransucrase after similar enzymes produced by Leuconostoc species. Carbon dioxide gave a four fold increase in enzyme activity and while the enzyme was produced by cultures grown on either glucose or sucrose, only the glucose moiety from sucrose was utilised for the formation of dextran (Bailey 1959 b), leaving free fructose in the culture medium.

In addition to the formation of dextran, Bailey and Bourne (1959) and Bourne, Hutson and Weigel (1961) found small amounts of the oligosaccharides leucrose, isomaltulose, isomaltotriose and 5-O- α -isomaltosyl-D-fructose, with traces of isomaltose and isomaltotriulose, all of which were thought to be by-products of the dextransucrase reaction.

(e) Fermentation of Carbohydrate for Energy

As lactic acid was the principal product of the fermentation of glucose, S. bovis was classified with the homofermentive lactic acid bacteria (Bailey and Oxford 1958 a). Consequently energy requirements are met by the production of two moles of ATP per mole of hexose fermented via the Embden-Meyerhof pathway (Gunsalus and Shuster 1961).

Although Smith and Sherman (1942) reported that 91.7 % of the glucose fermented by a strain of S. bovis was recovered as lactic acid, other products may be formed under some circumstances. Clarke (1964) recovered as lactic acid, only 60-80 % of the glucose utilised by four strains of S. bovis and Forrest, Walker and Hopgood (1961) noted the formation of volatile fatty acids by the classically homofermentive S. faecalis on fermentation of glucose.

The range of monosaccharides fermented by lactic acid bacteria is another characteristic commonly employed to identify species (Wilson and Miles 1964, Breed, Murray and Smith 1957). In the case of S. bovis the hexoses fermented are glucose, fructose and mannose (Bailey and Oxford 1958 a) but not sorbitol (Robertson 1961). On the other hand arabinose fermentation varied between different isolates of S. bovis (Seeley and Dain 1960, Merdrek and Barnes 1962, Clarke 1964). Dain, Neal and Seeley (1956) and Merdrek and Barnes (1962) have also encountered a few strains which unlike the others, fermented mannitol, were non-capsulated and did not produce dextran from sucrose.

Little information is available on the enzymes or pathways of carbohydrate fermentation in S. bovis. However Wiken (1959), in a review of the carbohydrate metabolism of lactic acid bacteria, cited work by Buyze, van den Hamer and de Haan indicating that homofermentive activity was dependent on the presence of aldolase. Obligate heterofermentive species contained no aldolase activity in cell-free extracts but the presence of hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase indicated that glucose possibly was fermented through a pentose phosphate pathway. On the other hand Buyze et. al. (loc. cit.) observed that the facultative homofermenters possessed aldolase as well as dehydrogenases for glucose-6-phosphate and 6-phosphogluconate. As Burchall, Niederman and Wolin (1964) found no glucose-6-phosphate dehydrogenase activity

in cell-free extracts of their strain of S. bovis it would be classified among the obligate homofermenters. However, the strain variability which exists for the fermentation of arabinose (Seeley and Dain 1960, Merdrek and Barnes 1962, Clarke 1964) suggests that some strains contain enzymes for the metabolism of pentose sugars, which in the lactic acid bacteria are usually cleaved and the products metabolised to yield equimolar amounts of lactate and acetate (Wood 1961).

From the data presented by Wiken (1959) and Wood (1961) it is obvious that homofermentation and heterofermentation have outlived their usefulness as terms to describe carbohydrate metabolism of lactic acid bacteria, and should be replaced by a description of the products of fermentation from a range of carbohydrates, and the enzyme activities present in cell-free extracts. So far for S. bovis, Wolin (1964) has demonstrated that the lactic dehydrogenase of one strain required fructose-1,6-diphosphate and phosphate for maximal activity, but the same enzyme from other strains was less dependent upon fructose-1,6-diphosphate.

Evidence for other pathways of energy metabolism in Group D streptococci was recently reviewed by Deibel (1964), who differentiated the enterococcal species S. faecalis and S. faecium from one another on their ability to utilise glycerol, pyruvate, arginine, citrate, serine, agmatine, gluconate and malate for the production of energy. Apart from a negative response on glycerol and an inability to release ammonia from arginine (Deibel 1964, Robertson 1961), S. bovis has not been rigorously tested for the metabolism of compounds listed above.

(2) Nitrogen, Sulphur, Vitamin and Growth

Factor Requirements of S. bovis

Members of the Lactobacteriaceae generally require a number of essential amino acids and vitamins for satisfactory growth (Guirard and Snell 1962).

However some strains of S. bovis have extremely simple requirements and do not conform to the pattern for the family. Nutritional requirements are reviewed under the following headings.

- (a) Nitrogen nutrition
- (b) Sulphur nutrition
- (c) Vitamin requirements for growth and dextran production
- (d) Effect of carbon dioxide
- (e) Acetate incorporation
- (f) Ammonia fixation
- (g) Long chain fatty acids as growth factors

(a) Nitrogen Nutrition

Working in the U.S.A. Niven, Washburn and White (1948) found that seven bovine strains of S. bovis grew on a media containing only arginine or glutamic acid as nitrogen source. No single amino acid was essential but for arginine to supply all the nitrogen, Prestcott and Stutts (1955) showed that carbon dioxide was required. Experiments were also carried out by Prestcott, Williams and Ragland (1959) to find nitrogen sources, which would substitute for arginine and support growth of two strains of S. bovis in the presence of carbon dioxide. Glutamine, citrulline, serine, glucosamine and histidine were almost as good as arginine, but a third strain failed to grow on any single amino acid and required a casein hydrolysate. It was thus completely unexpected that all three strains would grow in a medium containing ammonium salts as the sole source of nitrogen. Under these conditions carbon dioxide was required while acetate was found to stimulate growth and be incorporated by the cells.

Wolin, Manning and Nelson (1959) also reported the growth of bovine strains of S. bovis in media containing either L-glutamine or ammonium chloride as the sole source of nitrogen provided that carbon dioxide was present. Subsequently Wolin and Weinberg (1960) found that growth on

ammonium chloride would not occur at pH greater than 7.0 unless glucose was autoclaved with the media to yield products of the non-enzymic browning reaction. Similar requirements have been shown by other members of the Lactobacteriaceae (Guirard 1958).

A number of amino acid antagonisms have also been observed in the growth of some strains of S. bovis on chemically defined media. Washburn and Niven (1948) reported that the addition of isoleucine, leucine, nor-leucine or threonine inhibited growth on a simple source of nitrogen, but if valine was added with any of the inhibitory amino acids, growth was normal. This suggested that synthesis of valine had been blocked by the presence of similar amino acids. Another antagonism was noted by Washburn and Niven (1948) between tryptophane synthesis and the presence of DL-phenylalanine and L-tryosine in the medium. Later Prestcott, Ragland and Stutts (1957) showed that these antagonisms were overcome by the addition of carbon dioxide to the growth medium.

Exhaustive studies of the minimal amino acid requirements of strains of S. bovis isolated in other parts of the world do not appear to have been undertaken, but Oxford (1958 b) reported that none of five strains of S. bovis isolated in United Kingdom and New Zealand would grow in the simple medium of Niven, Washburn and White (1948). Similarly in Australia, Paul (1961) found that six rumen strains of S. bovis required a peptide-like substance for growth while three faecal strains would grow in simple media containing only six amino acids as nitrogen source.

From these reports it is obvious that strains of S. bovis vary enormously in their nitrogen requirements, but it is not clear whether the strains studied in the U.S.A. received attention because of their unusually simple nitrogen requirements or because they were typical of the majority of strains isolated. Wolin, Manning and Nelson (1959) suggested that strains with simple nitrogen requirements were commonly encountered but no

report of their occurrence in other countries appears to have been published.

(b) Sulphur Nutrition

The sulphur requirements of S. bovis were examined by Prestcott (1961) who showed that cultures would grow in media containing a variety of sulphur compounds including thioglycollate, thiomalate, thiourea, thiouracil, sulphide, and thiosulphate as the sole source of sulphur but best growth was obtained in media containing either cysteine or cystine. Methionine was unable to meet the requirements of S. bovis for growth when used as the sole source of sulphur. However, as the presence of methionine reduced the requirement for other sulphur compounds, Prestcott (1961) concluded that methionine supplied sulphur to the cells, but that enzymes were lacking for the transfer of sulphur from methionine to some of the essential cellular constituents of S. bovis.

(c) Vitamin Requirements for Growth
and Dextran Production

The vitamin requirements of S. bovis, like the nitrogen requirements show wide variation between different strains. Niven, Washburn and White (1948) found that only biotin was needed to initiate growth in a chemically defined media and while other B vitamins gave variable growth responses, only thiamine, nicotinic acid and panthothenic acid were essential for prolonged serial cultivation. Part of the variability in response was no doubt due to the lack of attention to anaerobiosis as Ford, Perry and Briggs (1958) found that none of the twenty-six strains of S. bovis studied had a B vitamin requirement for growth under anaerobic conditions. However, the presence of 0.1 % Tween 80 would have satisfied the biotin requirement (Barnes, Seeley and VanDemark 1961). Under aerobic conditions

Ford, Perry and Briggs (1958) noticed that some strains required nicotinic acid, biotin and thiamine.

Differences between another five strains of S. bovis were found by Oxford (1958 b) and it was concluded that growth and dextran production had separate vitamin requirements. On the other hand, Barnes, Seeley and VanDemark (1961) claimed that growth of seven other strains of S. bovis was always accompanied by dextran production, as measured by a very sensitive antiserum precipitation technique. In this study the magnitude of dextran production was not considered but growth requirements for biotin, pantothenate and thiamine were observed.

The growth of S. bovis on simple sources of nitrogen by Wolin, Manning and Nelson (1959) and Prestcott, Williams and Ragland (1959) also required the presence of vitamin solutions. One of these strains was examined for specific vitamin requirements by Barnes, Seeley and VanDemark (1961) who found that only biotin and thiamine were needed.

(d) Effect of Carbon Dioxide

Carbon dioxide has been found to have a variety of effects on the metabolism of S. bovis.

(i) Higher weights of cells were harvested from complex media containing a source of carbon dioxide. (Bailey and Oxford 1958 a, Wright 1960 b).

(ii) Dextranucrase activity in the cell free supernatant was greater when cultures were grown in the presence of carbon dioxide. (Bailey 1959 b).

(iii) Growth from simple nitrogen sources was dependent upon the presence of carbon dioxide in the media. (Prestcott and Stutts 1955, Wolin, Manning and Nelson 1959).

(iv) Antagonism between amino acids was relieved if the media contained carbon dioxide. (Prestcott, Ragland and Stutts 1957).

All of these effects have a common basis in amino acid metabolism or protein synthesis and evidence for a mechanism of the carbon dioxide stimulation was obtained by Wright (1960 b) working with S. bovis, Strain I, (Bailey and Oxford 1958 a). In this strain, which required casein hydrolysate as a nitrogen source, C^{14} - carbon dioxide was incorporated mainly into aspartate but also into glutamate and threonine. Degradation of aspartate and glutamate showed that the label was located in the carboxyl groups of aspartate and the α -carboxyl group of glutamate. A similar fixation of carbon dioxide into aspartate was observed by Lardy, Potter and Burris (1949) and MacDonald (1958) in the homofermentive Lactobacillus arabinosus and by Martin and Niven (1960) in the "minute streptococci" (S. anginosus). Labelling patterns showed the localisation of C^{14} in the β -carboxyl of aspartate which was consistent with carboxylation of a C-3 acid formed from glucose by the Embden-Meyerhof pathway. Lardy, Potter and Burris (1949) noted that carbon dioxide fixation was dependent upon biotin. Conversely Broquist and Snell (1951) showed that biotin spared the need for exogenous aspartate in L. arabinosus. As exogenous aspartate was not readily incorporated into protein, Wright (1960 b) suggested that aspartate synthesis could limit cell growth or dextransucrase production.

Wright (1960 b) also found that C^{14} - carbon dioxide was incorporated into the pyrimidine and purine nucleotides, illustrating the wide variety of cellular compounds synthesised by a strain of S. bovis that is allegedly quite fastidious in its growth requirements. From the effects of carbon dioxide in other strains of S. bovis it might be expected that C^{14} - carbon dioxide would be incorporated into a much wider variety of cellular compounds.

As an alternative argument Barnes, Seeley and VanDemark (1961) suggested that S. bovis was capable of producing carbon dioxide from an organic constituent of the medium. Although experimental details are only vaguely described, it would seem that these authors have ignored the action of lactic acid on potassium carbonate as the probable source of carbon dioxide in their manometric growth experiments.

(e) Acetate Incorporation

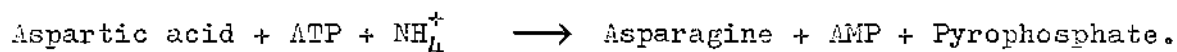
The incorporation of C^{14} - acetate was demonstrated by Prestcott, Williams and Ragland (1959) and Burchall, Niderman and Wolin (1964) and found to be more extensive when S. bovis was grown in a medium with ammonia as the sole source of nitrogen. The fate of the C^{14} - acetate was not followed but it was postulated by Burchall, Niderman and Wolin (1964) that acetate would be incorporated into α -ketoglutarate by the reactions of the tricarboxylic acid cycle. However, of the enzymes responsible for this pathway, only isocitric dehydrogenase activity was detected in cell-free extracts of S. bovis.

(f) Ammonia Fixation

Following the isolation of S. bovis strains which would grow on ammonium salts as a sole source of nitrogen, Burchall, Niderman and Wolin (1964) carried out an extensive survey of the enzymes likely to be involved in ammonia fixation. Of the amino acid dehydrogenases, only L-Glutamic acid dehydrogenase activity was present in cell-free extracts. When this strain of S. bovis was grown on a complex media or when nitrogen requirements were met by acid hydrolysed casein, glutamic acid dehydrogenase activity was repressed, suggesting that the activity of this enzyme controlled ammonia uptake. However, this hypothesis was not supported by the finding that a complete amino acid mixture in the medium decreased ammonia uptake without affecting the activity of glutamic acid

dehydrogenase (Burchall, Niederman and Wolin 1964).

Ammonia was also found to be incorporated into the amide group of asparagine by asparagine synthetase. Burchall, Reichelt and Wolin (1964) extracted this enzyme from cell-free extracts and showed that the reaction comprised:-



Asparagine was found to inhibit the enzyme activity.

Should these two sites of ammonia fixation be the only means of incorporating nitrogen from a simple medium, it would be expected that these strains of S. bovis would contain a rich array of transaminases and enzymes for the synthesis of carbon skeletons of amino acids.

(g) Long Chain Fatty Acids as Growth Factors

In many of the lactic acid bacteria unsaturated long chain fatty acids have been found to have a growth stimulating effect. O'Leary (1962) has postulated that this is due either to their physicochemical action or to the requirement of the cell for essential fatty acids.

Evidence for the physicochemical action was presented by Williams, Broquist and Snell (1947). These authors found that concentrations of oleic acid toxic to growth could be made stimulatory by adding to the media Tween 20 or Tween 40 (polyoxyethylene sorbitan mono-esters of laurate and palmitate respectively). These results were attributed to the surface active properties of the Tweens, as lecithin or saponins exerted a similar action. It was also found by Wright (1960 b), that in the presence of 1 % Tween 80 (polyoxyethylene sorbitan mono-oleate) uptake of C^{14} - aspartic acid from the medium by S. bovis, during growth, was enhanced three-fold. It was suggested that the Tween - oleic acid combination exerted its stimulatory effect by increasing cell permeability to constituents of the media.

On the other hand O'Leary (1962) favoured the concept of fatty acids as essential metabolites for the cell and evidence to support this theory was obtained by Guirard, Snell and Williams (1946). These authors showed that for a number of lactic acid bacteria, including Lactobacillus casei and Lactobacillus plantarum, myristic, oleic, linoleic and linolenic acids, lecithin, cholesterol, ergosterol or calciferol could replace the requirement for acetate. In addition Williams and Feiger (1946) and Broquist and Snell (1951) found that oleic acid and aspartate would together replace the biotin requirement of L. casei, L. plantarum, S. faecalis and Leuconostoc mesenteroides.

Hofmann, O'Leary, Yoho and Liu (1959) also found that biotin was spared by L. casei, L. delbrueckii and L. plantarum in the presence of the shorter chain fatty acids, cis-5-dodecenoic, cis-7-tetradecenoic and palmitoleic; all of which are thought to be intermediates in the biosynthesis of cis-vaccenic acid (Kates 1964). Synthesis of fatty acids from labelled acetate was also increased by the addition of biotin to the culture medium (Thorne and Kodicek 1962 b).

Camien and Dunn (1957) argued in favour of fatty acids having a metabolic effect, as the growth of several Lactobacillus sp. in media free of fatty acids was inhibited by the addition of saturated C-14 to C-20 fatty acids. The inhibition was overcome by the addition of Tween 80, or oleic, cis-vaccenic, behenic or lactobacillic acids. Camien and Dunn (1957) suggested that the saturated fatty acids inhibited the synthesis of an unsaturated fatty acid while in the presence of an unsaturated fatty acid the pathway was by-passed. Furthermore O'Leary (1959) demonstrated the uptake of cis-vaccenic and oleic acids by L. plantarum and their metabolism to cyclopropane derivatives.

Although the exact mechanism by which fatty acids act as growth factors for streptococci remains to be elucidated clearly, it appears as though some of the effects arise through the replacement of acids which would otherwise have to be synthesised.

LIPIDS OF LACTIC ACID BACTERIA

In an extensive review of bacterial lipids, Kates (1964) discussed factors which were found to affect the lipid composition reported by various authors. It was shown that age of culture, temperature and the composition of the growth medium all had considerable effect on the lipid composition reported. These factors may explain the discrepancies which occur between different reports on the lipids of lactic acid bacteria (Table 1). However, it is also pertinent to examine the method of extraction employed and procedures used in the fractionation of lipid classes.

(1) Method of Extraction

Different methods of extraction and analysis could also account for some of the variations in the reported lipid composition of these organisms. In their early work on lipids of lactic acid bacteria, Hofmann and co-workers used relatively mild extraction procedures to obtain free lipids. Cold acetone or diethyl ether were commonly used (Hofmann, Lucas and Sax 1952). Bound lipids were defined as those extracted after acid hydrolysis (2N sulphuric acid) at 118°C for 1¼ hours. For L. casei, L. plantarum and the Group C Streptococcus bound lipid comprised 60-80 % of the total (Table 1). More recently chloroform - methanol mixtures have gained wide popularity and with these it has been possible to extract much larger amounts of lipids from cells than was possible with acetone or

TABLE 1

LIPIDS OF THE LACTOBACTERIACEAE (LACTIC ACID BACTERIA)

ORGANISM AND STRAIN	LIPID % OF DRY WEIGHT	FRACTIONS AND % OF TOTAL LIPID	FATTY ACIDS (% OF TOTAL) (1)							OTHER ACIDS FOUND, METHOD OF ANALYSIS AND REMARKS	AUTHOR
			C _{12:0}	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{19:0}		
LACTOBACILLI											
<u>L. acidophilus</u>	7	Free fatty acids } Neutral Lipids } Polar lipids }	3	6	28		25	37		Dihydroxy stearic Fractional Distillation	Crowder and Anderson (1932, 1934 a, b)
<u>L. acidophilus</u> (ATCC 4963)		Saponifiable lipids	0.3	5.0	10.9	10.5	1.0	39.2	29.0	12:1, 14 br, 15:0, 15 br, 17:1 G.L.C.	Thorne and Kodicek (1962 d)
<u>L. casei</u> (ATCC 7469)	2.9	Free lipid 21 % } Bound lipid 79 % }			23		4	38	16	Fractional distillation	Hofmann and Sax (1953)
<u>L. casei</u> (ATCC 7469)		Saponifiable lipids	0.2	0.9	9.8	10.0	1.0	26.0	47.3	12:1, 14:1, 15:0, 15 br, 17:1 G.L.C.	Thorne and Kodicek (1962 d)
<u>L. casei</u> (ATCC 7469)	4.7	Acetone soluble 23 % Polar lipid 54 % Bound lipid 23 %								Polar lipid contained lysine but little choline	Ikawa (1963)
<u>L. casei</u> (ATCC 7469)		Phospholipids: % P								C _{20:0} G.L.C.	Thorne (1964)
		Fraction 1 12.5			8.6	11.7	9.3	24.4	44.7	1.3	
		Fraction 2 23.8			8.0	14.5	trace	33.3	42.5	2.7	
		P-choline 36.3			7.3	11.3	3.0	33.4	41.0	4.0	
		P-ethanolamine 1.0			9.0	13.6	1.9	37.2	37.5	1.6	
		Cardiolipin 6.4			7.2	14.0	2.6	41.8	33.1	1.3	
<u>L. delbrueckii</u> (ATCC 9649)	1.8	Saponifiable lipids	1.1	2.5	27.5		10.8	45.5	6.4	10:0 Rubber Column Chromatography	Hofmann, Henis and Panos (1955)
<u>L. plantarum</u> (ATCC 8041)	2.5	Free lipid 20 % } Bound lipid 80 % }			37		2	20	31	Fractional Distillation	Hofmann, Lucas and Sax (1952)
<u>L. plantarum</u> (ATCC 8041)	1.9	Saponifiable lipids	2.3	1.2	18.7			35.6	30.1	10:0 Rubber Column Chromatography	Hofmann, Hsiao, Henis and Panos (1955)
<u>L. plantarum</u> (ATCC 8014)		Saponifiable lipids	0.2	0.8	7.4	13.4	1.0	26.5	47.7	12:1, 14:1, 15:0, 15 br, 17:1 G.L.C.	Thorne and Kodicek (1962 d)
<u>L. plantarum</u> (ATCC 8014)	2.6	Total lipid		0.9	38.5	7.6	1.8	40.1	10.8	14:1 High biotin	Croom, McNeill and Tove (1964)
	2.3	" "		0.8	43.7	8.2	1.9	38.6	6.5	" Low "	
<u>L. plantarum</u> (ATCC 8014)	2.4	Saponifiable lipids	3.9	2.6	29.8			22.4	39.6	10:0 <u>cis</u> -Vaccenic acid in media	O'Leary (1959)
	2.3	" "	2.8	2.9	32.2			26.6	33.2	" Oleic acid in media	
<u>L. plantarum</u> (ATCC 8014)	2.9	Acetone soluble 17 % Polar lipid 24 % Bound lipid 59 %								Acetone soluble fraction contained glucose and galactose	Ikawa (1963)
<u>L. plantarum</u> (B-264)										Two glycolipid fractions	Vorbeck and Marinetti (1965 a)

diethyl ether alone. Vorbeck and Marinetti (1965 a) claimed that lipids were released from lipoprotein complexes by refluxing with methanol, and after extraction with cold chloroform - methanol they also claimed that little degradation of the lipid molecules had occurred. However, even after extraction with chloroform - methanol, Ikawa (1963) found that prolonged acid hydrolysis enabled the extraction of a further 1-2 % of the cellular dry weight (or 23-59 % of total lipid) as "bound lipid" from a variety of lactic acid bacteria (Table 1).

It was claimed by Thorne and Kodicek (1962 d), that refluxing cells for 3 hours with 17 % (v/v) conc. hydrochloric acid in methanol to extract total fatty acids, also caused degradation of the cyclopropane lactobacillic acid (cis-methylene octadecenoic). This report requires confirmation by examining the direct effect of hot acid conditions on lactobacillic acid itself. Until then the percentage composition of lactobacillic acid in the lipids of lactic acid bacteria (Table 1) must be accepted with reserve, but it would also be prudent to avoid methylation of fatty acids for gas-liquid chromatography under strongly acidic conditions.

(2) Fractionation of Lipids

A complete separation of lipids into fractions, which can be characterised by the structure of the water soluble moiety, has yet to be accomplished for any single organism of the family Lactobacteriaceae. Most workers have been content to separate as broad classes, neutral lipids from polar lipids using silicic acid chromatography (MacLeod, Jensen, Gander and Sampugna 1962), thin-layer chromatography MacLeod and Brown (1963), or acetone solubility (Ikawa 1963), (see Table 1). However, more recently Thorne (1964) has obtained chromatographic evidence for the identity of three phospholipids of L. casei, Ibbott and Abrams (1964) have

isolated two phospholipids from membrane ghosts of S. faecalis, and Vorbeck and Marinetti (1965 b) have separated and identified five polar lipid components in the membrane fraction of S. faecalis (Table 1). Partial identification of six phospholipids from S. lactis was published by MacLeod and Brown (1964).

No fractionation of neutral lipids into mono-, di- and tri- glyceride fractions appear to have been reported, nor has positive evidence for the identification of these glycerides been obtained.

(3) Fatty Acid Composition

Apart from the occurrence of minor amounts of fatty acids, which might have arisen from the casein hydrolysate used in the medium (Demain, Hendlin and Newkirk 1959), the fatty acid spectrum of the lactic acid bacteria seems to consist of only dodecanoic, tetradecanoic, hexadecanoic, hexadecenoic, octadecanoic, octadecenoic and cis-methylene octadecenoic acids (Table 1). The biosynthesis and metabolism of these acids was recently reviewed by Kates (1964) and evidence summarised for the precursor-product relationship between cis-vaccenic and lactobacillic acids. Apart from the occurrence of these two acids in varying proportions which seemed to depend upon the age of the culture, only subtle differences in the fatty acid composition existed between the various species (Table 1). However, differences existing between the methods of analysis make it difficult to compare fatty acid compositions reported by different workers (Horning, Ahrens, Lipsky, Mattson, Mead, Turner and Goldwater 1964).

Reports on the fatty acid composition of different lipid fractions, and of cells cultured under varying conditions have been common in literature pertaining to bacterial lipids, but little consideration has been given to variations in the fatty acid spectrum between replicate cultures. Data obtained by MacLeod and Brown (1963) showed considerable variation between

nine replicate cultures of S. lactis var. multigenes and should emphasise the danger of drawing conclusions from a restricted number of cultures, without undertaking a formal statistical analysis of the variance. For this reason the changes in fatty acid composition with different treatments imposed upon cultures, reviewed by (Kates 1964), and change in fatty acid composition of L. plantarum under high and low levels of biotin (Croom, McNeill and Tove (1964), Table 1) must be interpreted with caution.

FUNCTION OF LIPIDS IN MICROORGANISMS

Evidence for the role of lipids in the cell membranes was reviewed by Burgen (1962). Studies carried out by Thorne and Kodicek (1962 d), Shockman, Kolb, Bakay, Conover and Toennies (1963), Ibbott and Abrams (1964) and Vorbeck and Marinetti (1965 b) have shown that the protoplast membranes of the various lactic acid bacteria studied contained lipids, which are thought to be complexed as lipoproteins (McQuillen 1960) and indispensable to the physiological function of the membrane (Brown 1964).

For cells to remain alive after they have depleted their exogenous energy source, Dawes and Ribbons (1964) have asserted that the energy of maintenance must be derived from reserves accumulated within the cell; the endogenous reserves. These authors (Dawes and Ribbons 1962) have also suggested that lipids may act as endogenous reserves, but only in the case of a Mycobacteria sp. and Bacillus cereus was there any evidence available to implicate lipids other than poly- β -hydroxybutyrate. However, for an aerobic actinomycete, Nocardia corallina, Midwinter (1962) indicated that lipids may act as endogenous reserves, while Robertson (1964) observed the oxidation of palmitic, stearic and oleic acids to carbon dioxide during resting phase.

On the other hand, in anaerobic bacteria, it is doubtful whether fatty acids could liberate any more than a fraction of their potential energy, for even if there was a pathway of β -oxidation for the production of C_2 units, there is no tricarboxylic acid cycle to effect their complete oxidation. In studies of the β -oxidation reaction in Clostridium kluyveri, a strict anaerobic bacterium, Stadtman and Barker (1949) found that dried cells incubated aerobically had a higher oxygen uptake in the presence of fatty acids, up to octanoic in chain length. Stadtman (1953) also presented evidence for the β -oxidation of butyrate by enzyme preparations from the same organism.

Studies on the anaerobic endogenous metabolism of S. faecalis by Walker and Forrest (1964) showed that cells, grown in the presence of $2-C^{14}$ acetate to label the lipid fraction, released no activity on resuspension in a phosphate buffer. On the basis of this data alone, Walker and Forrest (1964) concluded that lipids did not constitute the endogenous reserves of S. faecalis.

From the equivocal results obtained in these studies on the metabolism of lipids for energy by anaerobic bacteria, it appeared that a study of lipid metabolism in S. bovis offered scope for investigation.

CHAPTER 2

AIMS OF THE PRESENT INVESTIGATION

Tweedie (1965), in an investigation of the fatty acid composition of S. bovis reported that the lipid content was of the order of 7.2 %, and the investigations reported in this thesis were commenced with the objective of obtaining information on the lipids present, their metabolism and function in the cells. Studying the lipid metabolism of Mycobacterium corallina, Robertson (1964) had found that the uptake of radioactive long chain fatty acids could be used to study, not only fatty acid metabolism, but also the metabolic nature of the lipid pools. It was therefore decided to test whether long chain fatty acids were incorporated into S. bovis lipids.

However, before any experiments on lipid metabolism of S. bovis could be commenced, it was essential to grow cultures under carefully defined conditions (Kates 1964). A search of the literature showed that most of the previous studies with S. bovis had been carried out in media of low buffering capacity (Hobson and Mann 1955, Bailey and Oxford 1958 a, Bailey 1963, Burchall, Neiderman and Wolin 1964) where a low pH (4.0 - 4.4) resulted from production of lactic acid during growth. As growth was halted at this pH, the medium could hardly be expected to favour viability during resting phase with the utilisation of endogenous reserves. On the other hand a medium of high buffering capacity was employed by Wright (1960 b) and growth ceased at pH 4.8. However as acetate (0.36 M) was the main buffer salt, it was feared that this constituent, at four times the concentration found in the rumen, might provide an unnaturally large carbon source for lipids (Stephenson and Whetham 1922).

To permit the full growth of S. bovis and achieve cell stability during resting phase, the effect of varying concentrations of phosphate and bicarbonate buffers on growth were studied. In the medium that was finally chosen for studies of lipid metabolism, an estimate of the change in cell viability with time was made to indicate the general extent of endogenous reserves and to enable investigations of resting phase metabolism to be integrated with cell viability.

Investigations on the lipids of S. bovis began by attempting to verify the lipid content reported by Tweedie (1965). Possible reasons for the discrepancy discovered were investigated and lead on to a study of the origin of some of the lipid components. As a basis for further work on the lipid metabolism of S. bovis, methods for the separation of lipid components were investigated and preliminary identification sought for some of the components. The incorporation of a long chain fatty acid was followed by radioautography.

CHAPTER 3

MATERIALS AND METHODS

MICROBIOLOGICAL

Culture

Streptococcus bovis Strain I (NCDO 1253) was obtained as a freeze-dried culture from the National Collection of Dairy Bacteria, National Institute for Research in Dairying, Reading, England.

Subculture

S. bovis was subcultured daily in the Glucose - Tryptose - Yeast Extract - Phosphate (GTYP) medium (see Appendix). Stab cultures were made into the GTYP medium with 1.25 % Davis Agar added, and were stored at 0-4°C for up to three weeks without loss of viability.

Microscopic Appearance

Smears of S. bovis were made periodically and showed that the organism was a Gram-positive coccus. Cells were mainly in pairs but chaining and clumping were also prevalent.

Fermentation Reactions

As a further check on the identity of the organism, some fermentation reactions, which distinguished S. bovis from other Streptococcus species, were kindly undertaken by Dr P.S. Robertson, of the Dairy Research Institute, using standardised methods (Robertson 1961). The results of these tests are presented in Table 2, along with the reactions published by Bailey and Oxford (1958 a) who isolated Strain I, the tests carried out by Dr E.I. Garvie (pers. comm.) on the same strain, and the usual

T A B L E 2

TESTS EMPLOYED TO IDENTIFY S. BOVIS FROM OTHER GROUP D STREPTOCOCCI.

TEST	PRESENT STUDY	BAILEY AND OXFORD (1958 a)	GARVIE (pers. comm.)	ROBERTSON (1961)
Growth at 10°C			-	
" " 45°C			+	+
Resists 60°C for 30 min.			+	-
Growth in: 2 % Na.Cl			+	+
4 % Na.Cl			+	±
6.5 % Na.Cl			-	
Arginine Hydrolysis	-		-	-
Starch Hydrolysis			+	
β Haemolysis			-	-
Acid from : Arabinose	-	-	-	?
Dextrin	+			±
Fructose		+		
Glucose	+	+	+	+
Glycerol			-	?
Inulin		+		
Maltose	+			±
Mannitol		-	-	+
Raffinose	+	+	+	+
Sorbitol	-		-	-
Sucrose	+	+	+	+
Trehalose			-	+
Xylose			-	?

+ = Positive Reaction

- = Negative Reaction

± = Usually Positive

± = Usually Negative

? = Reaction Ill-defined

reactions of S. bovis strains, as collated by Robertson (1961). The results indicated that the culture had characteristics similar to Streptococcus bovis.

Growth Experiments

Culture Media

S. bovis was grown in a basal medium of glucose (B.D.H. reagent grade) Bacto-Tryptose (Difco) and Bacto-Yeast Extract (Difco) as used by Bailey (1963) while sodium salts of acetate, bicarbonate, mono- and di-hydrogen phosphate, and potassium di-hydrogen phosphate (all B.D.H. reagent grade) were employed as buffers in the various experiments. Tryptose was used at a concentration of 1.4 % (w/v) and Yeast Extract at 0.5 % (w/v) throughout, while details of the varying concentrations of glucose and buffer salts are given with other experimental data in Chapter 4. Experimental cultures were grown in 250 ml of media, previously sterilised by autoclaving at 121°C for 15 min. in 500 ml Erlenmeyer flasks plugged with cotton wool. Initially glucose was included with other constituents of the media but severe browning, especially at high buffer concentrations, was observed on autoclaving. Glucose was therefore autoclaved separately and aliquots of the 12.5 % (w/v) solution added to the sterile media with aseptic precautions just prior to inoculation. Sodium bicarbonate, when used, was also autoclaved as a separate solution to avoid carbon dioxide loss which occurred when a neutral solution was heated.

Although the concentration of sodium bicarbonate quoted throughout is in terms of the concentration present in the media after the addition of the concentrated sterile solution, some loss of HCO_3^- and H^+ ions occurred at neutral pH, as no control over carbon dioxide evolution was possible.

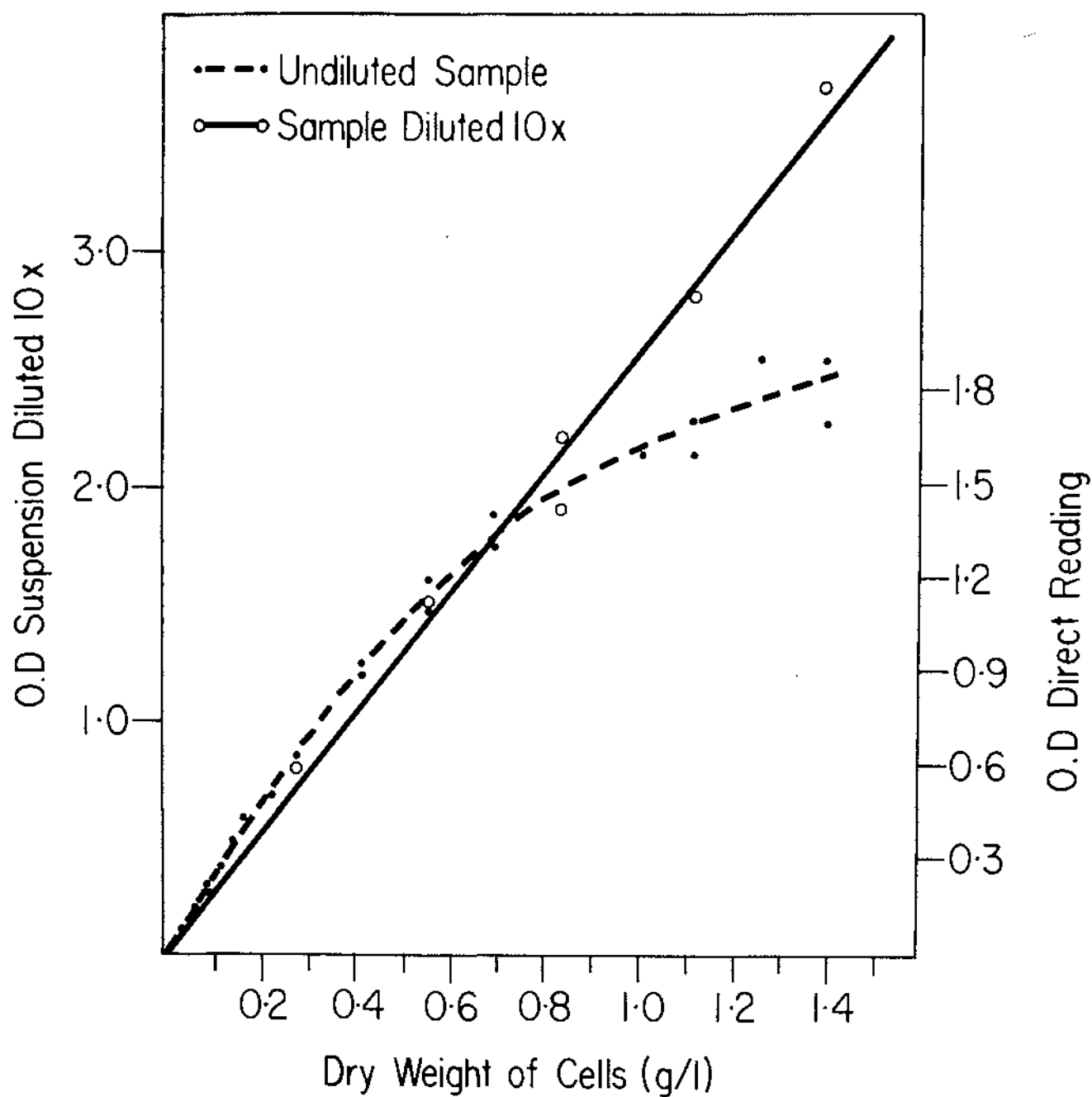


Figure 1. Relationship between optical density (O.D.) and dry weight of bacteria, showing linearity of response obtained after dilution of cell suspension.

On addition of 0.10 M Bicarbonate to 0.10 M Phosphate there was a gradual rise in pH from 6.9 to 7.5 before logarithmic growth of the culture commenced. From this rise in pH it can be calculated, using the Henderson-Hasselbach equation (Dawson, Elliot, Elliot and Jones 1959), that no more than one third of the bicarbonate added was evolved as carbon dioxide.

Cell Growth and pH Measurements

All cultures were grown at 37°C in an air incubator and before inoculation the media was warmed to this temperature. Inoculation was from a 12-16 hour culture grown in the GTYP medium (see Appendix) and 1 ml of inoculum was used for each 250 ml of media. Bacterial growth was followed turbidimetrically using a Hilger colorimeter with a green filter (Filter 58). In initial growth experiments, where the media had browned extensively, a direct reading of the culture was made against an uninoculated blank. However, bacterial dry weight was not linear with optical density above a reading of 0.4 (see Figure 1 and DeMoss and Bard 1957) and tenfold dilutions of the culture were required to remain below this (Figure 1).

pH of the culture medium was measured on a 5-10 ml aliquot using a Radiometer pH meter (Model 23).

Viable Counts

Duplicate 1 ml samples, withdrawn aseptically from well agitated cultures were diluted through eight tubes, each containing 9 mls of diluent (0.02 M Sodium acetate + 0.02 M Sodium/potassium di-hydrogen phosphate pH 5.8). The mixing and pipetting techniques described by Whitehead and Sargent (1950) were followed closely. Glucose - Tryptose - Yeast Extract - Phosphate - Agar (see Appendix), kept molten at 45°C, was poured into sterile petriplates containing 1 ml of the diluted cell suspension and gently swirled. Plates were incubated at 37°C for

approximately 36 hours, by which time colonies were approximately 0.5 mm in size and clearly countable against a dark background. A further count was made one day later. Usually two plates having between 20 and 600 colonies per plate were obtained from aliquots ranging between 10^{-4} and 10^{-8} ml of the original cell suspension.

Large Scale Cultures

Cells were grown in 6-8 litre batches in 10 litre boiling flasks. Media preparation, sterilisation, inoculation and growth assessment were as already described for growth experiments (see page 43). At the end of logarithmic growth cells were harvested at 15,000 x g in a continuous flow "Servall" refrigerated centrifuge, at a flow rate of 4-6 litres/hour. Thus all cells were chilled to 0-4°C within three hours from the end of logarithmic growth. The cells were washed twice by suspending in distilled water and recentrifuging at 0-4°C, freeze-dried and weighed.

CHEMICAL

Solvents

All solvents used for lipid manipulations were either of "Analar" grade or reagent grade purified by redistillation. Chloroform was washed twice with water before distilling and the early distillate which contained water was discarded. Methanol was distilled over calcium oxide.

Extraction of Lipids from *S. bovis*

Up to 10 g of cells were refluxed twice in 300 ml of chloroform - methanol (2:1) for 4 hours (Robertson 1964) and the cold extract filtered through ether-washed No.1 Filter paper. Water soluble impurities were removed by the procedure of Folch, Lees and Sloane-Stanley (1957) and the washed lipid solution taken to dryness in a tared flask on a rotary evaporator under reduced pressure. The lipid was dried to constant weight

in a vacuum desiccator over potassium hydroxide pellets and stored for analysis at -10°C . The lipid was freely soluble in chloroform.

Separation of Lipids

Column Chromatography

For column chromatography of total lipids the method of Vorbeck and Marinetti (1965 a) was followed with two modifications.

- (1) Mallinkrodt's silicic acid (100-200 mesh) was used in place of Clarkson's "Unisil" silicic acid.
- (2) In this laboratory Rumsby (in press) had found chloroform - acetone (3:1) eluted a monoglycolipid, present in brain tissue, from Mallinkrodt's silicic acid, while the separation of phospholipids, obtained by Vorbeck and Marinetti (1965 a) with chloroform - methanol mixtures, was not required in this study. Therefore the elution sequence comprised:

60 ml	Chloroform
60 ml	Chloroform - Acetone (3:1)
75 ml	Chloroform - Acetone (1:1)
75 ml	Acetone
100 ml	Methanol

Thin-Layer Chromatography

TLC (thin-layer chromatography) was used extensively throughout the study to monitor separations from column chromatography, for tentative identification of some lipid components and to effect separation into broad classes on a preparative scale. Following the procedures outlined by Mangold (1961), glass plates 20 x 20 cm and 20 x 10 cm were coated with silica Gel G. (E. Merck and Co.) to a thickness of 250 μ for

analytical studies and 500 μ for preparative work. The plates were activated at 110°C for at least 1 hour before use. Lipids dissolved in chloroform were applied to the plates which were developed in one of the following solvent systems;

Hexane - Diethyl Ether - Acetic Acid (30:70:1)

(Malins and Mangold 1960)

Chloroform - Methanol (185:15)

(Gray 1965)

Chloroform - Methanol - Water (65:25:4)

(Thorne 1964)

in tanks lined with filter paper to facilitate equilibration of the solvent with the atmosphere. After the solvent front had migrated 10-15 cm from the origin, the plates were removed from the tank and allowed to dry before spraying with 20% sulphuric acid and charring at 110°C. To test for the presence of phosphate, plates were sprayed with the molybdenum spray of Dittmer and Lester (1964). On standing for a few minutes phospholipids gave a distinct blue colour, while subsequent charring with sulphuric acid revealed the presence of other lipid components.

For preparative TLC the plates were spotted with total lipid and developed in a solvent of chloroform - methanol (185:15). Spots were visualised under ultra-violet light after spraying with 0.2 % dichlorofluorescein in 95 % ethanol. Fractions were scraped from the plate into funnels plugged with glass wool and lipids eluted with chloroform - methanol (2:1).

Detection of Glycolipids

The presence of glycolipids was determined by refluxing approx. 10 mg of lipid with 3 ml 2N sulphuric acid for 1 hour, extracting lipid residues three times with petroleum ether and neutralising acid in the

aqueous phase with barium carbonate. After centrifuging to remove the precipitate, the supernatant was evaporated to dryness at reduced temperature and pressure. Sugars were redissolved in 0.2 ml water and 10 μ l applied to No. 1 Whatman paper for chromatography by the procedure of Jermyn and Isherwood (1949) using their ethyl acetate - pyridine - water (2:1:2) solvent. Papers were sprayed with an alkaline silver nitrate spray (Trevelyan, Procter and Harrison 1950).

Sterol Analysis

Total sterol was determined as cholesterol using the method of Sackett as described by Varley (1958). Absorption at 620 $m\mu$ was measured with a Unicam S.P. 500 spectrophotometer.

RADIOCHEMICAL

Materials

$1-C^{14}$ Sodium acetate, 29 $mC/mmole$, and $U-C^{14}$ palmitic acid, 93 mC/m mole, were purchased from the Radiochemical Centre, Amersham, England.

Determination of Activity

Non-Aqueous Samples

Lipid samples in chloroform were evaporated to dryness in a scintillation vial and 15 ml of toluene scintillation fluid added. This solution contained 0.5 % (w/v) 2,5-diphenyloxazole (PPO) and 0.005 % (w/v) 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

Aqueous Samples

15 ml of a 60:40 mixture of Toluene and Ethanol containing 0.5 % (w/v) PPO and 0.002 % (w/v) POPOP was added to 0.5 ml of sample in the scintillation vial (Ziegler, Chleck and Brinkerhoff 1957).

Combustion of Cells

C^{14} remaining in the cells after extraction of lipids was determined by Folch - Van Slyke wet combustion as described by Sakami (1955). Carbon dioxide liberated was trapped in 3.5 ml of ethanolamine - ethylene glycol monomethyl ether (1:2 v/v) as described by Jeffay and Alvarez (1961). For counting, 1 ml of this solution was added to the Toluene - PPO - POPOP solution described above.

Counting Samples

All samples were counted in a Packard Instrument Company Series 4000 Scintillation Spectrometer with window settings of 50-800 and gain 7.5 %. Background counts were determined for each of the scintillation solutions containing the sample solvent. Efficiency of counting was assessed by the addition of 50 μ l of C^{14} -toluene having a total activity of 21,050 d.p.m. (Packard Instrument Company) and enabled all activities to be calculated as d.p.m.

Radioautography of TLC Plates

Thin-layer plates were radioautographed by placing X-Ray film (Kodak) in close contact with the absorbent layer in a specially constructed box and exposing for two weeks. The positions of spots on the developed autograph were compared with the positions of lipids on the plate after charring with 20 % sulphuric acid.

STATISTICAL ANALYSES OF DATA

Growth Experiments

Optical density measurements were statistically analysed and the significance of differences assessed by the "F test" (Snedecor 1956 p.244).

Times and Treatments mean squares were tested against the Treatments x Times mean square, which in turn was tested against the Flasks mean square (Flasks and Days where the replication was by days instead of by duplicate flasks within the day).

To restrict the number of comparisons made between treatments, sets of orthogonal comparisons (Snedecor 1956 p. 330) were devised to partition Treatments and Treatments x Times mean squares and to give tests for comparisons of interest.

As orthogonal comparisons were devised on the basis of scientific interest rather than on the basis of differences obtained, the risk of finding significant differences purely on the one-in-twenty probability was substantially reduced. However, in one analysis (Table 7) separate sets of orthogonal comparisons, within a subclass of the analysis of variance table, were required to make the pertinent comparisons.

Only comparisons of interest have been presented in the analysis of variance tables, and in several instances comparisons lacking significance within an orthogonal set have been grouped. If overall treatment effects were highly significant (when the Treatment x Times interaction formed the denominator of the "F test") they were not analysed further by the use of orthogonal components, provided that the growth curves in question remained apart from those for all other treatments. However, to correctly interpret the effect of most treatments it was necessary to make orthogonal comparisons within the Treatment and Times interaction with the time scale arbitrarily subdivided into three periods.

(a) Differences in the rate of growth were examined as deviations from the common linear regression of the treatments in question if sufficient samples had been taken during logarithmic growth.

(b) Differences in the yield of cells between treatments were examined by comparing mean optical densities over two samples near the end of logarithmic growth and if the curves in question had similar regression coefficient during resting phase an indirect assessment of yield was possible by taking the mean optical density over the stationary phase.

(c) Differences in the rate of cell lysis were tested by examining deviations from their common linear regression during resting phase.

Flasks variance appeared to be higher during growth than during the resting phase, and was in part a reflection of the variable lag phase between flasks. Comparisons within the Treatments x Times interaction were therefore made using appropriate subdivisions of the Flasks variance (Cochran and Cox 1957), while the reduced "error degrees of freedom" in these comparisons made the tests of significance more conservative.

Colony Counts

Colony counts of bacteria normally confirm to a Poisson distribution in which the variance is equal to the mean of all possible samples (Stearman 1955). Computation of the variance for counts at each sampling time showed that variance was proportional to the mean, but became independent of it if counts were transformed to their square roots, as recommended by Snedecor (1956 p.315).

An analysis of variance on the transformed data was made using an hierarchical classification (Kempthorne 1956). The mean square of each level of the hierarchy was tested for significance by "F tests", using as error, the mean square from the underlying level. Differences between counts made at intervals during resting phase were examined for significance by the combination of analysis of variance and "t tests" (Snedecor 1956 p.251). The fall in viable numbers with time was sufficiently obvious to make the use of more delicate techniques (Studentized Ranges or Range Tests) appear unwarranted.

Cell Yields and Lipid Content

Analyses of variance of the data obtained from cell yields of large scale cultures and the lipid content of the cells, were carried out as described by Snedecor (1956 p. 268) for samples of unequal sizes. The significance of differences between media were assessed by the use of a studentised difference, also described by Snedecor (1956 p. 251).

CHAPTER 4

MICROBIOLOGICAL STUDIES

GROWTH OF S. BOVIS AND CONTROL OF MEDIA pH BY BUFFERS

Before studying the lipids of a microorganism the conditions under which cultures were to be grown had to be carefully defined (Kates 1964). In the case of a homofermentive organism such as Streptotoccus bovis producing large quantities of lactic acid during growth, control over a changing media pH was of primary concern. Furthermore, where the relationship between lipids and cell viability was of interest any effect of pH on viability had to be eliminated.

Annison and Lewis (1959 p. 124), referred to "a pH range of 5.5 - 6.5, normally associated with rumen contents" but Bryant (1964) found a pH range of 6.8 - 7.6 in the rumen liquor of a pasture fed cow. In view of the suggestion by Krogh (1959) that rumen streptococci lost their viability below pH 5.0 in animals fed large quantities of soluble carbohydrate, control within the limits of pH 5.0 - 8.0 was imperative while control within the range of pH 6.0 - 7.0 would simulate the rumen environment.

One method of achieving this objective was to grow the organism in buffers having dissociation constants in the range, pKa 6 - 7. Mono-hydrogen phosphate and bicarbonate were two salts selected for this purpose as both are constituents of parotid saliva, the natural source of rumen fluid buffers, (McDougall 1948), and both are important for the effective buffering of rumen pH (Turner and Hodgetts 1955).

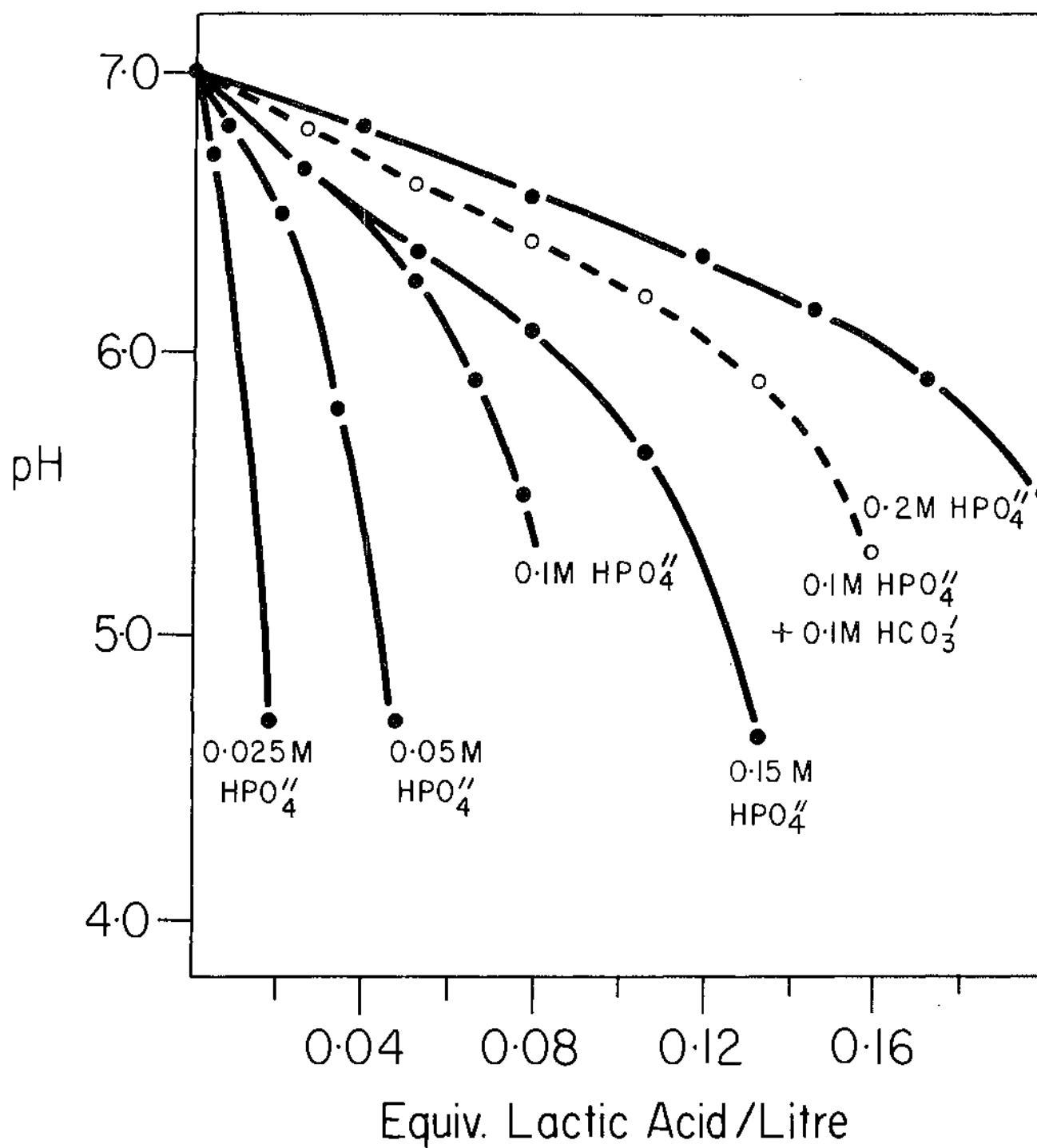


Figure 2. Buffering capacity of phosphate solutions as determined by change of pH on titration with lactic acid ($\frac{4}{3}$ N).

A study of the buffering capacity of monohydrogen phosphate - dihydrogen phosphate (pH 7.0) at varying concentrations was made by observing the pH change of a 50 ml solution on titration with $\frac{4}{3}$ N lactic acid. The results are presented in Figure 2.

Assuming the production of two moles of lactic acid for each mole of glucose fermented it is evident that 0.15 M phosphate is the minimum molarity of phosphate needed to maintain media pH within the limits 5.0 - 7.0 when 0.055 M (1.0 %) glucose is fermented. However, if S. bovis could withstand higher concentrations of phosphate, a higher cell yield should theoretically be obtained from the fermentation of greater quantities of glucose. The tolerance of S. bovis to phosphate was therefore studied.

Tolerance of S. bovis to Phosphate

A preliminary experiment to test the tolerance of S. bovis to phosphate showed that growth could occur on 0.05 - 0.15 M Phosphate solutions. Confirmation of the effect of variations in phosphate concentration was obtained in a subsequent experiment using 0.05 M, 0.15 M, and 0.30 M Phosphate buffers with two glucose concentrations (0.055 M and 0.11 M) at each level of phosphate. Growth curves and pH changes are presented in Figure 3 and the analysis of variance appears in Table 3.

No significant difference was observed either in final optical density or rate of cell growth during logarithmic phase between cultures grown in 0.05 M and 0.15 M Phosphate. Growth in 0.30 M Phosphate was lower ($P < 0.01$) than the mean for the lower phosphate levels. As expected (see Figure 2), 0.05 M Phosphate was unable to neutralise the lactic acid produced and at the final pH of this buffer (4.2) the cells

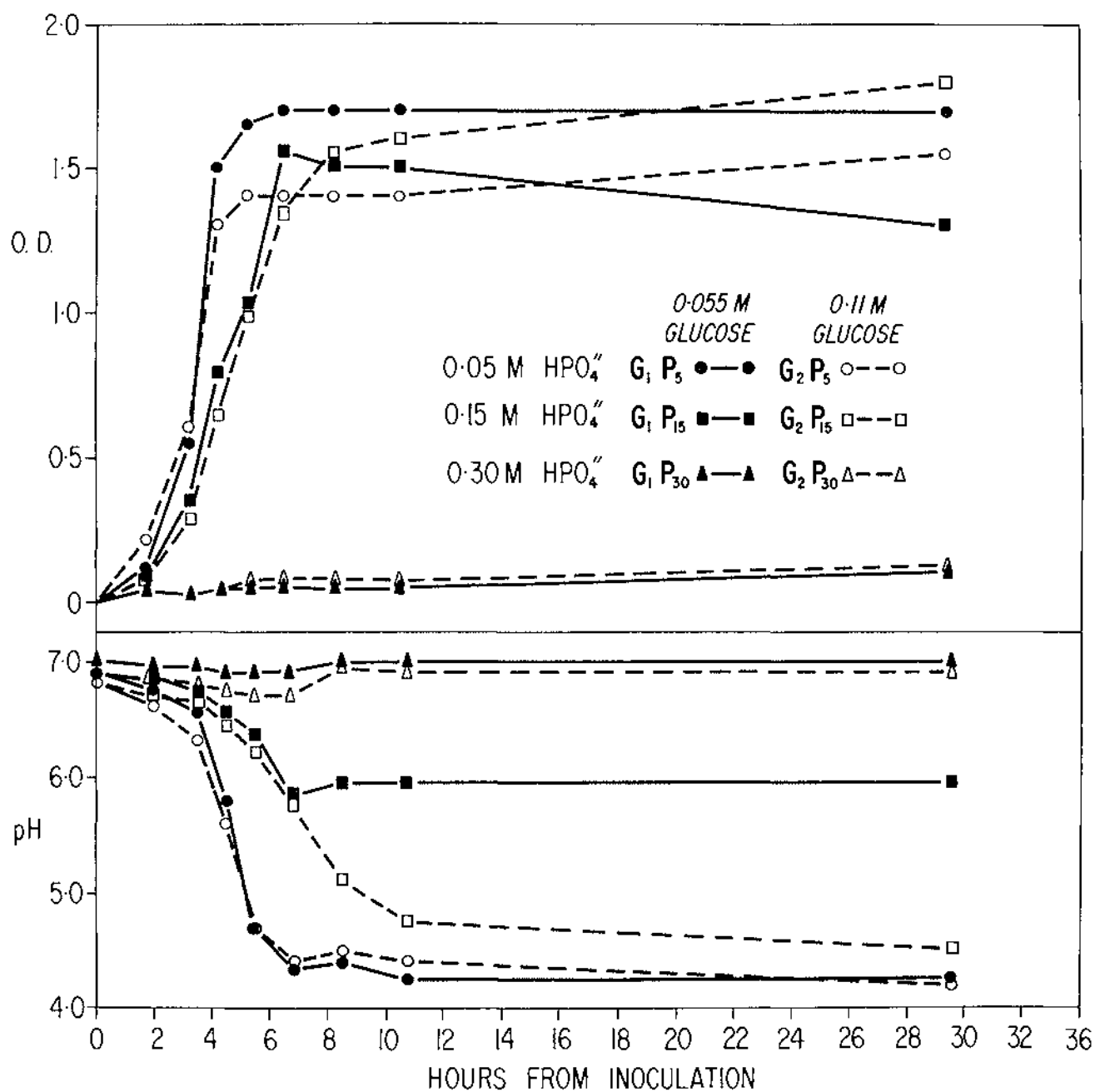


Figure 3. Growth of *S. bovis* (in O.D. units) and pH change of the media with fermentation of 0.055 M and 0.11 M Glucose in 0.05 M, 0.15 M and 0.30 M Phosphate buffers.

TABLE 3

ANALYSES OF VARIANCE FOR GROWTH OF S. BOVIS AND pH OF MEDIUM ON 0.055 M AND 0.11 M GLUCOSE IN 0.05 M, 0.15 M, AND 0.30 M PHOSPHATE BUFFERS.

SOURCE OF VARIANCE	GROWTH, (O.D. UNITS)		pH OF MEDIA	
	D.F.	MEAN SQUARE	D.F.	RESULTS OF F TESTS
Total	95	- -	107	- -
Times	7	1.7235 P < 0.001	8	3.885 P < 0.001
Treatments	5	5.0998 P < 0.001	5	9.486 P < 0.001
Glucose levels within phosphate $G_1P_5 - G_2P_5, G_1P_{15} - G_2P_{15}, G_1P_{30} - G_2P_{30}$ (1)	3	0.0893 N.S.	3	0.7142 N.S.
High v low + medium phosphate $2(G_1P_{30} + G_2P_{30}) - (G_1P_5 + G_2P_5 + G_1P_{15} + G_2P_{15})$	1	24.5174 P < 0.001	1	33.528 P < 0.001
Low v medium phosphate $(G_1P_5 + G_2P_5) - (G_1P_{15} + G_2P_{15})$	1	0.7504 P < 0.06	1	11.761 P < 0.001
Treatments x Times (Residual for testing Times and Treatments)	35	0.1946 P < 0.001	40	0.5580 P < 0.001
Deviations from Common Linear Regression 1:45 - 5:15 hrs				
Low v medium phosphate $(G_1P_5 + G_2P_5) - (G_1P_{15} + G_2P_{15})$	1	0.0334 P < 0.09	-	- -
Mean Values of O.D. 8:15 - 11:30 hrs.				
Glucose levels at medium phosphate $G_1P_{15} - G_2P_{15}$	1	0.0113 P < 0.10	1	2.10 P < 0.001
Flasks 1:45 - 29:30 hrs (Residual for testing Treatment x Times)	48	0.0041 -	54	0.0337 -
$G_1P_5 + G_2P_5 + G_1P_{15} + G_2P_{15}$ 1:45 - 6:30 hrs	16	0.0098 -	-	- -
$G_1P_5 + G_2P_5 + G_1P_{15} + G_2P_{15}$ 8:15 - 10:30 hrs	8	0.0031 -	8	0.005 -

(1) G_1P_5, G_2P_5 etc. refer to treatments - see Figure 3. legend.

G subscripts = grams glucose/100 ml.

P, B, A subscripts = m.moles buffer/100 ml.

N.S. Probability of effect being due to chance is greater than 0.10; .°. not significant.

P < 0.001 Probability of effect being due to chance is less than 0.001.
etc.

were presumably killed by the acid conditions. In 0.15 M Phosphate, the final pH of the media was 6.0 from the fermentation of 0.055 M Glucose, compared with pH 5.6 on 0.11 M Glucose, indicating a greater yield of lactic acid from the higher level of glucose. However, the higher glucose concentration gave no increase in cell growth as determined by optical density measurements.

Experiments were also carried out to test the tolerance of S. bovis to 0.20 M Phosphate. Logarithmic growth occurred in only four out of six flasks inoculated and moreover all four flasks showed an extended lag phase of 10-14 hours as opposed to 2-4 hours for 0.15 M Phosphate. It seemed therefore that 0.20 M Phosphate approached the limit of phosphate tolerance for S. bovis but the variability in growth precluded its further use as a buffer. On the other hand, as the combination of 0.15 M Phosphate buffer with a substrate of 0.055 M Glucose gave good yield of cells (0.80 g/litre) with effective control over pH, it was adopted as a control media against which other buffer mixtures involving bicarbonate and phosphate could be compared.

Growth of S. bovis in Phosphate and Bicarbonate Buffers

Wright (1960) reported that carbon dioxide increased the growth of S. bovis (Strain I) on a complex medium, and as bicarbonate is an important buffer in rumen fluid (Turner and Hodgetts 1955) it was decided to explore its usefulness as a buffer and/or growth stimulant. Early evidence of its value as a buffer was obtained when, on titration of a solution of 0.10 M Phosphate + 0.10 M Bicarbonate with lactic acid, little change of pH occurred (see Figure 2). To examine the effect of bicarbonate on the growth of S. bovis, an experiment was conducted comparing 0.15 M Phosphate with 0.15 M Phosphate + 0.10 M Bicarbonate at 0.055 M Glucose. The effect

T A B L E 4

GROWTH OF S. BOVIS IN MEDIA CONTAINING BICARBONATE (RESULTS FOR INDIVIDUAL FLASKS)

TREATMENT	O.D. AT END OF LOG. GROWTH	TIME TO END OF LOG. GROWTH	MEDIA pH AT END OF LOG. GROWTH	LYSIS OBSERVED (1)
0.055 M Glucose 0.15 M Phosphate	1.65	11:00 hrs	6.2	+
" " " "	1.65	12:30 hrs	5.3	-
0.055 M Glucose 0.10 M Phosphate + 0.10 M Bicarbonate	1.8	9:10 hrs	6.7	+
" " " " " "	1.6	9:10 hrs	6.7	+
0.055 M Glucose 0.15 M Phosphate + 0.10 M Bicarbonate	1.2	12:30 hrs	6.7	+
" " " " " "	1.2	15:40 hrs	6.7	+
0.0825 M Glucose 0.15 M Phosphate + 0.10 ⁵ M Bicarbonate	1.5	15:40 hrs	6.4	+
" " " " " "	1.3 (2)	24:00 hrs (2)	6.4	+

(1) + = 10-20 % fall in O.D. during 10 - 12 hours of resting phase.

- = No fall in O.D. observed.

(2) Log. Growth completed overnight and 24:00 hours value presented.

of an increased level of glucose (0.0825 M) in 0.15 M Phosphate + 0.10 M Bicarbonate was also examined. Reducing the level of phosphate to 0.10 M with the addition of bicarbonate (0.10 M) gave a further comparison with the best treatment (0.15 M Phosphate) from the experiments on phosphate tolerances.

No statistical analyses were made on the data from this experiment as full interpretation of the results was hampered by inaccurate assessment of growth (see page 44), and the possibility of extensive glucose degradation through browning during autoclaving. Observations made for each treatment are summarized in Table 4 from which the following provisional conclusions were drawn to form the basis of hypotheses tested in later experiments.

(1) At 0.15 M Phosphate and 0.055 M Glucose, the addition of 0.10 M Bicarbonate reduced the yield of cells as measured by optical density. In the same buffer solution (0.15 M Phosphate + 0.10 M Bicarbonate) the higher level of glucose (0.0825 M) increased cell yield and lactic acid production as estimated by change in pH. However, in both cases the cell yield was lower than that obtained with 0.055 M Glucose on either 0.15 M Phosphate alone or 0.10 M Phosphate + 0.10 M Bicarbonate, suggesting that the overall osmotic strength of the media, rather than glucose concentration was limiting cell growth.

(2) No evidence was obtained for the existence of any bicarbonate stimulation of growth, as any difference between 0.15 M Phosphate and 0.10 M Phosphate + 0.10 M Bicarbonate in cell yield or rate of growth could be due to the reduced phosphate concentration, rather than the presence of bicarbonate.

(3) A 10-20 % fall in optical density (Table 4) occurred during the 10 - 12 hours of resting phase in those flasks containing bicarbonate or where the pH of the media remained above 6.0. Although no cell counts were made, the fall in optical density was fully indicative of cell lysis (see page 70).

Using better techniques for the conduct of growth experiments (see page 43), a more detailed study of the combined effects of phosphate and bicarbonate on growth and lysis of S. bovis was carried out by comparing four different buffer mixtures;

1. 0.15 M Phosphate
2. 0.15 M Phosphate + 0.05 M Bicarbonate
3. 0.15 M Phosphate + 0.10 M Bicarbonate
4. 0.10 M Phosphate + 0.10 M Bicarbonate

each with 0.055 M Glucose as substrate. An analysis of variance for optical densities throughout the experiment is presented in Table 5. Growth curves and the pH of the media at the end of logarithmic growth are shown in Figure 4.

As in the previous experiment poor growth was observed on 0.15 M Phosphate + 0.10 M Bicarbonate but with optical density measured in a manner that gave a linear relationship with cell yield (see Figure 1) the effect was greater than indicated by Table 4. Mean optical density (over all times) on this treatment (0.15 M Phosphate + 0.10 M Bicarbonate) was significantly less ($P < 0.01$) than the average optical density of all other treatments (0.15 M Phosphate, 0.15 M Phosphate + 0.05 M Bicarbonate, 0.10 M Phosphate + 0.10 M Bicarbonate), hence no further examination of this comparison was made within the Treatments x Times interaction.

To examine difference between 0.15 M Phosphate, 0.15 M Phosphate + 0.05 M Bicarbonate and 0.10 M Phosphate + 0.10 M Bicarbonate, the Treatment x Times interaction was subdivided into three periods - 3:00 - 8:15 hours, 9:30 - 10:30 hours and 12:30 - 32:30 hours.

From 3:00 - 8:15 hours similar rates of growth occurred with 0.15 M Phosphate and 0.15 M Phosphate + 0.05 M Bicarbonate, whereas with 0.10 M Phosphate + 0.10 M Bicarbonate a higher rate of growth was obtained

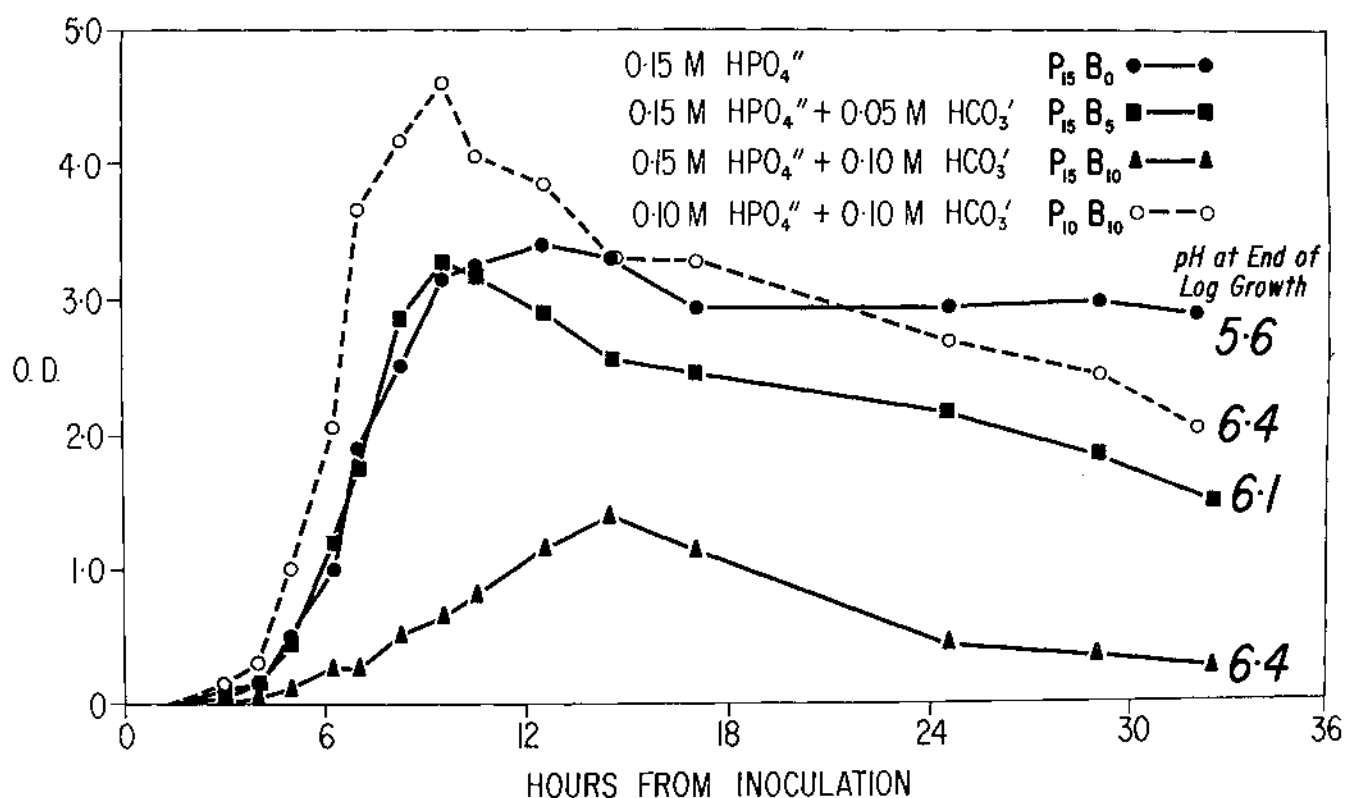


Figure 4. Growth of *S. bovis* (in O.D. units) with fermentation of 0.055 M Glucose in media containing phosphate and bicarbonate buffers.

TABLE 5

ANALYSIS OF VARIANCE FOR GROWTH OF S. BOVIS ON 0.055 M GLUCOSE IN PHOSPHATE AND BICARBONATE BUFFERS.

SOURCE OF VARIANCE	GROWTH (O.D. UNITS)		
	D.F.	MEAN SQUARE	RESULTS OF F TESTS
Total	102	-	-
Times	12	4.24	P < 0.01
Treatments	3	25.58	P < 0.001
High phosphate high bicarbonate v remainder $3P_{15}B_{10} - (P_{15}B_0 + P_{15}B_5 + P_{10}B_{10})$	1	66.83	P < 0.001
High bicarbonate at low phosphate v high phosphate $2P_{10}B_{10} - (P_{15}B_0 + P_{15}B_5)$	1	8.12	P < 0.025
Low bicarbonate at high phosphate $P_{15}B_0 - P_{15}B_5$	1	1.77	N.S.
Treatments x Times (Residual for tests of Times and Treatments)	36	1.30	P < 0.001
Deviations from Common Linear Regressions 3:00 - 8:15 hrs			
High bicarbonate at low phosphate v high phosphate $2P_{10}B_{10} - (P_{15}B_0 + P_{15}B_5)$	1	4.312	P < 0.001
Low bicarbonate at high phosphate $P_{15}B_0 - P_{15}B_5$	1	0.0298	N.S.
Mean Values at End of Log. Growth 9:30 - 10:30 hrs			
High bicarbonate at low phosphate v high phosphate $2P_{10}B_{10} - (P_{15}B_0 + P_{15}B_5)$	1	3.375	P < 0.001
Low bicarbonate at high phosphate $P_{15}B_0 - P_{15}B_5$	1	0	N.S.
Deviations from Common Linear Regressions 12:30 - 32:30 hrs			
Bicarbonate at low and high phosphate $2P_{15}B_0 - (P_{10}B_{10} - P_{15}B_5)$	1	1.679	P < 0.001
High bicarbonate low phosphate v low bicarbonate high phosphate $P_{10}B_{10} - P_{15}B_5$	1	0.099	P < 0.05
Flasks 4:00 - 32:30 hours (Residual for testing Treatments x Times)	51	0.119	-
$P_{15}B_0 + P_{15}B_5 + P_{10}B_{10}$ 3:30 - 10:30 hrs	23	0.242	-
$P_{15}B_0 + P_{15}B_5 + P_{10}B_{10}$ 12:30 - 32:30 hrs	18	0.0211	-

 $P_{15}B_{10}$ etc. refer to treatments - see Fig. 4 legend.

For other footnotes see Table 3 - page 58.

($P < 0.01$ for deviations from common linear regression).

Yield of cells at the end of logarithmic growth (9:30 - 10:30 hrs) showed similar differences. Hence not only did cultures in 0.10 M Phosphate + 0.10 M Bicarbonate grow more rapidly than in 0.15 M Phosphate or 0.15 M Phosphate + 0.10 M Bicarbonate but the extent of growth was also greater.

Thereafter (12:30 - 32:30 hrs) a drop in optical density, associated with a pH of greater than 6.0 or bicarbonate in the media, occurred in both 0.10 M Phosphate + 0.10 M Bicarbonate and 0.15 M Phosphate + 0.05 M Bicarbonate. On the other hand in 0.15 M Phosphate alone, where the pH fell to 5.6, the culture remained at almost constant optical density. ($P < 0.01$ for deviations from common linear regression).

Despite the occurrence of lysis in media containing bicarbonate, the higher yield of cells and similarity to the rumen environment warranted the selection of 0.10 M Phosphate + 0.10 M Bicarbonate along with 0.15 M Phosphate for studies on the lipids of S. bovis (see page 81).

Growth of S. bovis in Phosphate, Bicarbonate and Acetate Buffers and Comparison of Two Cation mixtures.

Tweedie (1965) found the lipid content of S. bovis to be 7.2 % of the cell dry weight, but chloroform - methanol extractions of the cells grown in the present study yielded only 0.7 - 1.7 % (Table 11). One of the reasons for this discrepancy was thought to be the high concentration of acetate (0.36 M) employed by Tweedie (1965) to buffer the medium. By comparison, the concentration of acetate in the rumen fluid seldom exceeds 0.11 M (Annison and Lewis 1959 p. 61).

It was therefore decided to determine whether acetate had any effect on the lipid content of S. bovis, but to interpret these results it was also necessary to know the effect of acetate on cell growth. For a study of the effect of acetate on the growth of S. bovis, a concentration of 0.10 M was chosen, to be added to the buffers found most useful in previous experiments (0.15 M Phosphate and 0.10 M Phosphate + 0.10 M Bicarbonate). Substitution of 0.10 M acetate for 0.10 M Bicarbonate in a solution of 0.10 Phosphate was also examined.

Hence the buffer treatments were,

1. 0.15 M Phosphate
2. 0.15 M Phosphate + 0.10 M Acetate
3. 0.10 M Phosphate + 0.10 M Bicarbonate
4. 0.10 M Phosphate + 0.10 M Bicarbonate + 0.10 M Acetate
5. 0.10 M Phosphate + 0.10 M Acetate

each with 0.055 M Glucose.

Superimposed upon each of these treatments was a comparison of two cation mixtures. Although previously all buffers had been made up from only sodium salts, it was realised at this stage that a sodium-potassium mixture would bear more relation to rumen fluid. To provide a potassium concentration within the range of 0.01 - 0.07 mole/litre, as found in rumen fluid by Reid (1965), potassium dihydrogen phosphate (0.045 M) replaced the sodium salt in half of the flasks assigned to each buffer treatment. Growth curves and pH of the media at the end of logarithmic growth are shown for all treatments in Figure 5. An analysis of variance is presented in Table 6.

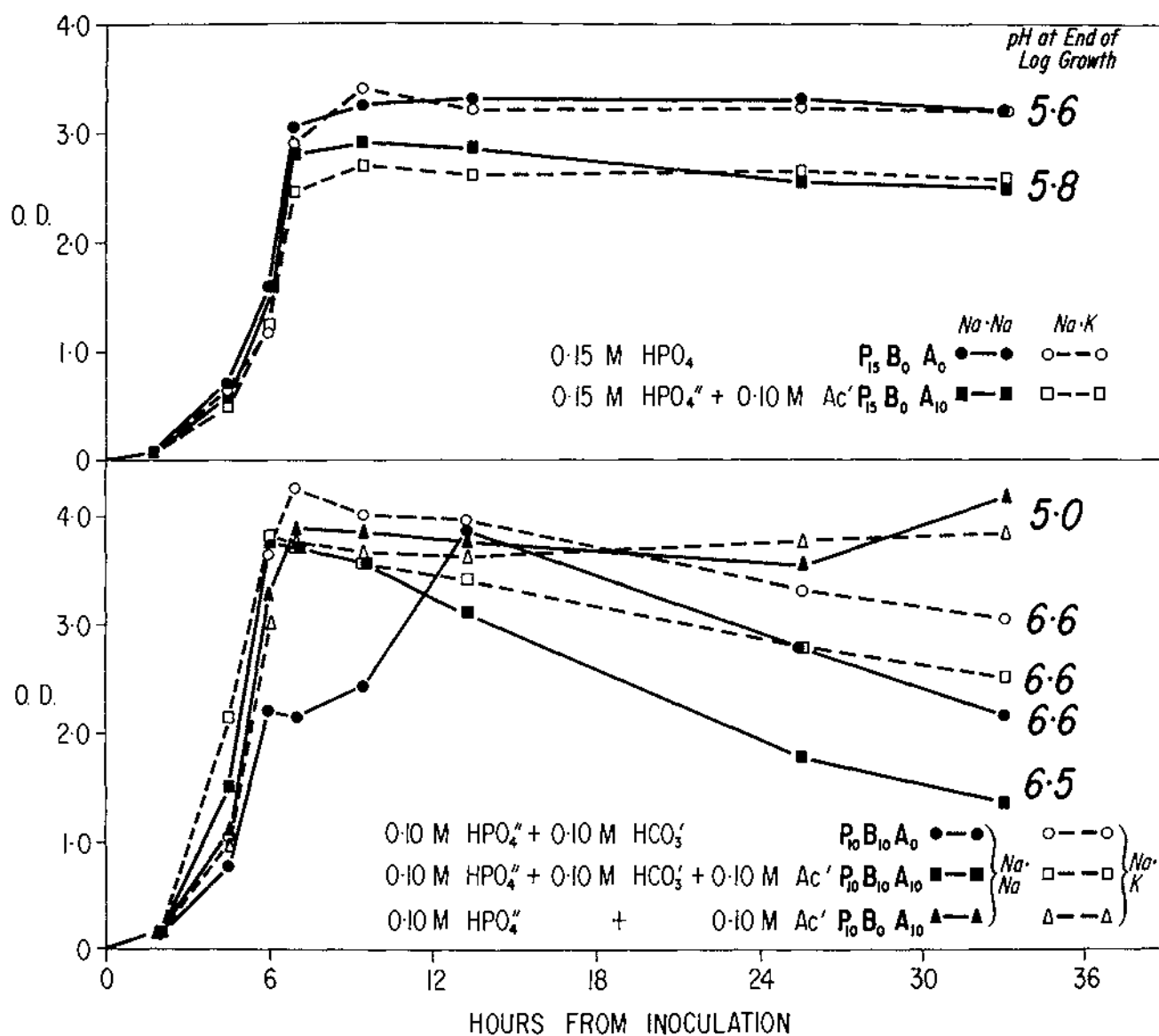


Figure 5. Growth of *S. bovis* (in O.D. units) with fermentation of 0.055 M Glucose in phosphate, bicarbonate and acetate buffers.

TABLE 6

ANALYSIS OF VARIANCE FOR GROWTH OF *S. BOVIS* ON 0.055 M GLUCOSE IN PHOSPHATE, BICARBONATE AND ACETATE BUFFERS.

SOURCE OF VARIANCE	GROWTH (O.D. UNITS)		
	D.F.	MEAN SQUARE	RESULTS OF F TESTS
Total	179	-	-
Times	8	10.35	P < 0.001
Treatments	9	4.826	P < 0.001
Acetate at high phosphate $P_{15}B_0A_0 - P_{15}B_0A_{10}$	1	9.200	P < 0.01
Bicarbonate at low phosphate $2 P_{10}B_{10}A_{10} - (P_{10}B_{10}A_0 + P_{10}B_{10}A_{10})$	1	41.317	P < 0.01
Acetate at low phosphate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	0.0178	N.S.
Cations without bicarbonate Na.Na - Na.K in $P_{15}B_0A_0, P_{10}B_0A_{10}, P_{15}B_0A_{10}$	3	0.1087	N.S.
Cations with bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0$	1	14.761	P < 0.001
Cations with bicarbonate + acetate Na.Na - Na.K in $P_{10}B_{10}A_{10}$	1	7.347	P < 0.01
Treatments x Times (Residual for tests of Treatments and Times)	72	0.8866	P < 0.001
Mean Values at End of Log. Growth 6:45 - 10:45 hrs			
Cations at low phosphate + bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0$	1	6.66	P < 0.01
Cations Remainder Na.Na - Na.K in $P_{15}B_0A_0, P_{15}B_0A_{10}, P_{10}B_{10}A_0, P_{10}B_{10}A_{10}$	4	0.5307	N.S.
Mean Values during Resting Phase 13:15 - 33:00 hrs			
Acetate at high phosphate $P_{15}B_0A_0 - P_{15}B_0A_{10}$	1	2.734	P < 0.001
Acetate at low phosphate + bicarbonate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	2.470	P < 0.001
Cations at low phosphate + bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0 + P_{10}B_{10}A_{10}$	1	2.734	P < 0.001
Deviations from Common Linear Regressions 13:15 - 33:00 hrs			
Bicarbonate $2 (P_{15}B_0A_0 + P_{15}B_0A_{10} + P_{10}B_0A_{10}) - 3 (P_{10}B_{10}A_0 + P_{10}B_{10}A_{10})$	1	3.9647	P < 0.001
Differences between Treatments with Bicarbonate			
Cations Na.Na - Na.K in $P_{10}B_{10}A_0 + P_{10}B_{10}A_{10}$	1	0.8382	P < 0.01
Acetate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	0.0002	N.S.
Remainder	1	0.0068	N.S.
Differences between Treatments without Bicarbonate			
Cations Na.Na - Na.K in $P_{15}B_0A_0 + P_{15}B_0A_{10} + P_{10}B_0A_{10}$	1	0.0031	N.S.
Phosphate levels $2 P_{10}B_0A_{10} - (P_{15}B_0A_0 + P_{15}B_0A_{10})$	1	0.2895	P < 0.07
Acetate at high phosphate $P_{15}B_0A_0 - P_{15}B_0A_{10}$	1	0.0115	N.S.
Remainder	2	0.0401	N.S.
Flasks + days 4:15 - 73:00 hrs (Residual for testing Treatments x Times)	90	0.3422	-
6:45 - 10:15 hrs	30	0.5183	-
13:15 - 73:00 hrs	50	0.0786	-

TABLE 6

ANALYSIS OF VARIANCE FOR GROWTH OF S. BOVIS ON 0.055 M GLUCOSE IN PHOSPHATE, BICARBONATE AND ACETATE BUFFERS.

SOURCE OF VARIANCE	GROWTH (O.D. UNITS)		
	D.F.	MEAN SQUARE	RESULTS OF F TESTS
Total	179	-	-
Times	8	10.35	P < 0.001
Treatments	9	4.826	P < 0.001
Acetate at high phosphate $P_{15}B_{10}A_0 - P_{15}B_{10}A_{10}$	1	9.200	P < 0.01
Bicarbonate at low phosphate $2 P_{10}B_{10}A_{10} - (P_{10}B_{10}A_0 + P_{10}B_{10}A_{10})$	1	41.317	P < 0.01
Acetate at low phosphate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	0.0178	N.S.
Cations without bicarbonate Na.Na - Na.K in $P_{15}B_{10}A_0, P_{10}B_{10}A_{10}, P_{15}B_{10}A_{10}$	3	0.1087	N.S.
Cations with bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0$	1	14.761	P < 0.001
Cations with bicarbonate + acetate Na.Na - Na.K in $P_{10}B_{10}A_{10}$	1	7.347	P < 0.01
Treatments x Times (Residual for tests of Treatments and Times)	72	0.8866	P < 0.001
Mean Values at End of Log. Growth 6:45 - 10:45 hrs			
Cations at low phosphate + bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0$	1	6.66	P < 0.01
Cations Remainder Na.Na - Na.K in $P_{15}B_{10}A_0, P_{15}B_{10}A_{10}, P_{10}B_{10}A_0, P_{10}B_{10}A_{10}$	4	0.5307	N.S.
Mean Values during Resting Phase 13:15 - 33:00 hrs			
Acetate at high phosphate $P_{15}B_{10}A_0 - P_{15}B_{10}A_{10}$	1	2.734	P < 0.001
Acetate at low phosphate + bicarbonate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	2.470	P < 0.001
Cations at low phosphate + bicarbonate Na.Na - Na.K in $P_{10}B_{10}A_0 + P_{10}B_{10}A_{10}$	1	2.734	P < 0.001
Deviations from Common Linear Regressions 13:15 - 33:00 hrs			
Bicarbonate $2 (P_{15}B_{10}A_0 + P_{15}B_{10}A_{10} + P_{10}B_{10}A_{10}) - 3 (P_{10}B_{10}A_0 + P_{10}B_{10}A_{10})$	1	3.9647	P < 0.001
Differences between Treatments with Bicarbonate			
Cations Na.Na - Na.K in $P_{10}B_{10}A_0 + P_{10}B_{10}A_{10}$	1	0.8382	P < 0.01
Acetate $P_{10}B_{10}A_0 - P_{10}B_{10}A_{10}$	1	0.0002	N.S.
Remainder	1	0.0068	N.S.
Differences between Treatments without Bicarbonate			
Cations Na.Na - Na.K in $P_{15}B_{10}A_0 + P_{15}B_{10}A_{10} + P_{10}B_{10}A_{10}$	1	0.0031	N.S.
Phosphate levels $2 P_{10}B_{10}A_{10} - (P_{15}B_{10}A_0 + P_{15}B_{10}A_{10})$	1	0.2895	P < 0.07
Acetate at high phosphate $P_{15}B_{10}A_0 - P_{15}B_{10}A_{10}$	1	0.0115	N.S.
Remainder	2	0.0401	N.S.
Flasks + days 4:15 - 73:00 hrs (Residual for testing Treatments x Times)	90	0.3422	-
6:45 - 10:15 hrs	30	0.5183	-
13:15 - 73:00 hrs	50	0.0786	-

 $P_{15} B_{10}$ etc. refer to treatments - see Fig.5 legend.

For other footnotes see Table 3 page 58.

(a) Cation Effects

In solutions containing bicarbonate where cell lysis was always observed, a highly significant difference in rate of lysis was found between the cation mixtures. Over the 13:15 - 33:00 hour period cells grown in the sodium-potassium mixture lost optical density at only half of the rate observed for cultures in the sodium-sodium mixture. As no other differences due to the changed cation complement were found, the sodium-potassium mixture was adopted for subsequent experiments.

(b) Acetate Effects

A prolonged lag phase in one flask containing 0.10 M Phosphate + 0.10 M Bicarbonate (sodium-sodium) was responsible for the atypical mean growth curve of this treatment presented in Figure 5. Individually, each culture exhibited a normal growth curve but a variable lag phase was also found to be associated with this treatment in other experiments. Because of the increased variability of this treatment, together with the restricted number of samples taken during logarithmic growth, no significant effect of acetate addition to either 0.15 M Phosphate or 0.10 M Phosphate + 0.10 M Bicarbonate was observed in the period 6:45 - 10:15 hours. However during resting phase the addition of acetate to both buffers resulted in a 20 % reduction in the average optical density reading ($P < 0.01$). As deviations from the common linear regressions due to acetate did not contribute to the variance ($F < 1$) for either treatment, it was unlikely that acetate made any difference to the slope of the regression during resting phase. Hence differences in the mean values during resting phase may be extrapolated back to infer a decreased cell yield from the addition of 0.10 M Acetate to both 0.15 M Phosphate and 0.15 M Phosphate + 0.10 M Bicarbonate. Similar decreases in growth were observed from the addition of 0.10 M Bicarbonate to 0.15 M Phosphate (see page 62).

While substitution of 0.10 M Acetate for 0.10 M Bicarbonate in the presence of 0.10 M Phosphate permitted adequate growth, it also allowed the pH of the media to fall to pH 5.0. As Krogh (1959) suggested that viability of S. bovis fell as pH decreased below 5.0, no further use of this buffer was made.

Despite the possibility of diminished growth of S. bovis, 0.10 M Acetate was retained in subsequent media to provide an enhanced buffering capacity below pH 6.0 and to simulate the composition of rumen liquor.

Control of Cell Lysis

In previous experiments, the inclusion of bicarbonate as a constituent of the buffer mixture led to a distinct fall in optical density during resting phase, and was attributed to cell lysis. Also associated with the use of bicarbonate was a higher buffering capacity, so that on no occasion did pH fall below 6.0.

Shockman, Conover, Kolb, Phillips, Riley and Toennies (1961 a) in a study of Streptococcus faecalis, observed that lysis occurred above pH 5.9 when cells were depleted of a media constituent (e.g. glucose or lysine) essential for cell wall synthesis, whereas at a lower pH the cells remained intact. In a preliminary experiment with S. bovis, the addition of hydrochloric acid to one flask of a fully grown culture lowered the pH from 6.4 to 5.2 and prevented the fall of optical density. Cells in a duplicate flask left at pH 6.4 lysed extensively.

Thus to control lysis in media employing bicarbonate as buffer it was necessary to forfeit the original aim of a media buffered at about pH 6.0 for studies of resting phase metabolism, and instead adopt a media buffered in the range pH 5.2 - 5.7. Three alternative methods of achieving this were possible.

(1) Commencing growth at a lower pH was undesirable, as in acid conditions, there could be extensive conversion of bicarbonate to carbon dioxide which, in the open incubation system adopted, would be evolved before logarithmic growth commenced.

(2) By increasing glucose concentration, a greater production of lactic acid could be expected, with possibly some increase in cell yield.

(3) Reducing the concentration of a buffer component (such as bicarbonate) would allow pH to fall to the desired level.

To test the two latter hypotheses and confirm that lysis could be controlled by adjusting media pH, the following combinations of glucose and buffer were compared;

- (1) 0.055 M Glucose, 0.10 M Phosphate + 0.10 M Acetate + 0.10 M Bicarbonate
- (2) " " " " "

+ lactic acid at end of logarithmic growth.

- (3) 0.0825 M Glucose, 0.10 M Phosphate + 0.10 M Acetate + 0.10 M Bicarbonate
- (4) 0.11 M Glucose, " " "
- (5) 0.055 M Glucose, " " + 0.07 M "
- (6) " " " + 0.05 M "

Growth curves for each treatment are presented in Figure 6 and the analysis of variance in Table 7.

(a) Addition of Lactic Acid

Decreasing the media pH to 5.7 at the conclusion of logarithmic growth, by the addition of lactic acid, counteracted cell lysis ($P < 0.05$ for deviations from common linear regression).

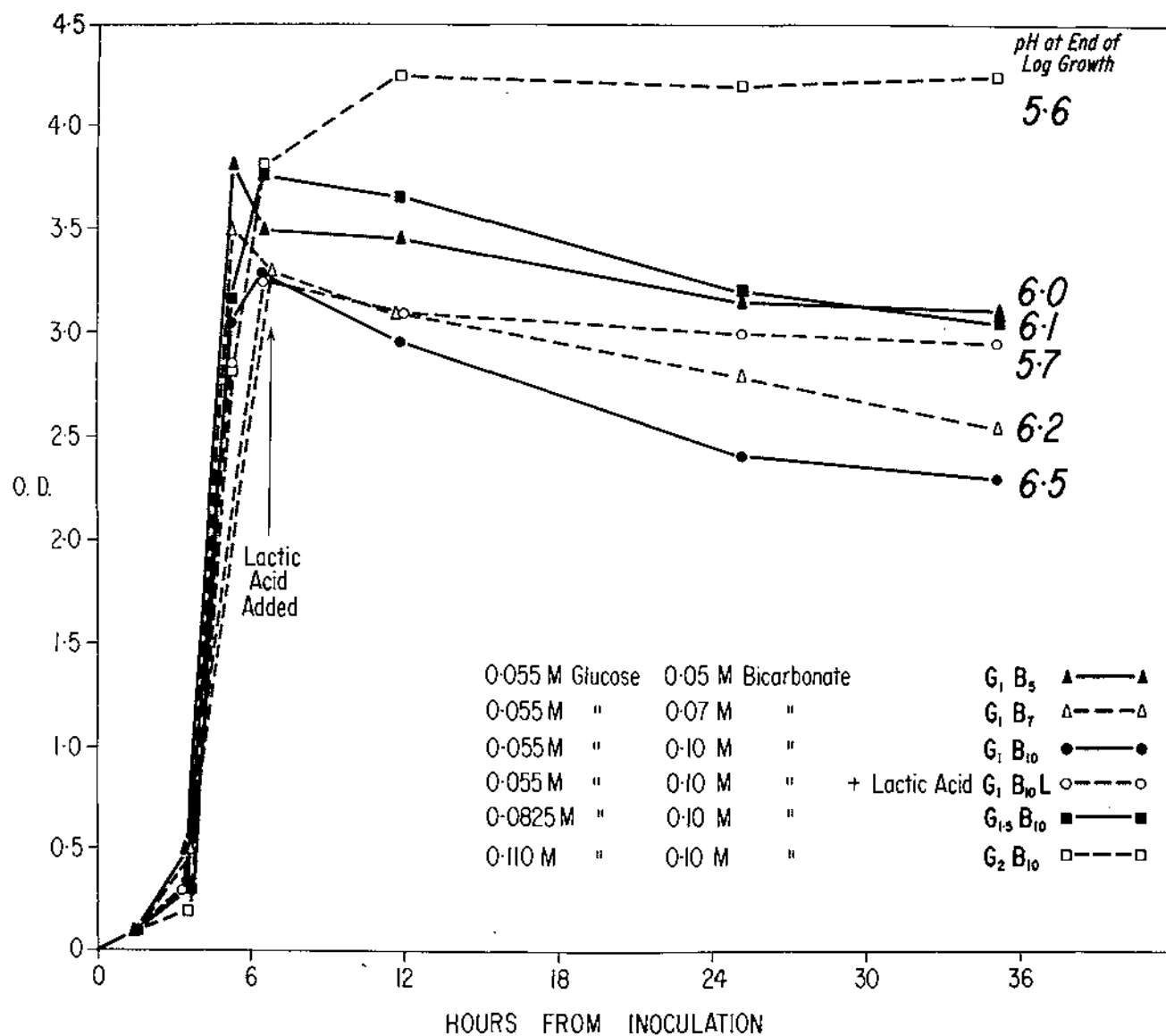


Figure 6. Growth of *S. bovis* (in O.D. units) with fermentation of varying glucose concentrations and in media of varying bicarbonate concentrations. Addition of lactic acid to media containing 0.055 M Glucose and 0.10 M Bicarbonate gave stable O.D. during resting phase.

TABLE 7

ANALYSIS OF VARIANCE FOR GROWTH OF S. BOVIS IN MEDIA OF VARYING GLUCOSE AND BICARBONATE CONCENTRATIONS.

SOURCE OF VARIANCE	GROWTH (O.D. UNITS)		
	D.F.	MEAN SQUARE	RESULTS OF F TESTS
Total	107	-	-
Times	8	20.343	P < 0.001
Treatments	5	2.60	P < 0.001
High Glucose v remainder 5 $G_{2B_{10}} - (G_{1.5B_{10}} + G_{1B_{10}} + G_{1B_{10}L} + G_{1B_7} + G_{1B_5})$	1	9.2302	P < 0.001
Low Glucose high bicarbonate v remainder 4 $G_{1B_{10}} - (G_{1.5B_{10}} + G_{1B_{10}L} + G_{1B_7} + G_{1B_5})$	1	2.7386	P < 0.01
Remaining orthogonal comparisons	3	0.3437	N.S.
Treatments x Times (Residual for testing Treatments and Times)	40	0.3435	P < 0.001
Mean Values at end of Log. Growth 5:15 - 6:30 hrs			
Low v high bicarbonate at low glucose 2 $G_{1B_5} - (G_{1B_{10}} + G_{1B_{10}L})$	1	1.1516	P < 0.01
Medium v mean of low and high bicarbonate at low glucose 4 $G_{1B_7} - (2 G_{1B_5} + G_{1B_{10}} + G_{1B_{10}L})$	1	0.0010	N.S.
Glucose levels ($G_{2B_{10}} + G_{1.5B_{10}}$) - ($G_{1B_{10}} + G_{1B_{10}L}$), $G_{2B_{10}} - G_{1.5B_{10}}$	2	0.1603	N.S.
Mean Values during Resting Phase 11:45 - 35:00 hours			
High glucose v remainder 5 $G_{2B_{10}} - (G_{1.5B_{10}} + G_{1B_{10}} + G_{1B_{10}L} + G_{1B_7} + G_{1B_5})$	1	7.8130	P < 0.001
Deviations from Common Linear Regressions 11:45 - 35:00 hrs			
Addition of lactic acid $G_{1B_{10}} - G_{1B_{10}L}$	1	0.1238	P < 0.04
High v medium glucose $G_{2B_{10}} - G_{1.5B_{10}}$	1	0.1865	P < 0.02
Low v high bicarbonate at low glucose $G_{1B_5} - G_{1B_{10}}$	1	0.1515	P < 0.025
Medium v mean of low and high bicarbonate at low glucose 2 $G_{1B_7} - (G_{1B_5} + G_{1B_{10}})$	1	0.0233	N.S.
Flasks + Days 1:30 - 73:00 hrs (Residual for testing Treatment x Times)	54	0.0209	-
5:15 - 6:30 hrs	18	0.1100	-
11:45 - 35:00 hrs	18	0.0233	-

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Glucose levels $(G_2B_{10} + G_{1.5}B_{10}) - (G_1B_{10} + G_1B_{10}L), G_2B_{10} - G_{1.5}B_{10}$	2	0.1603	N.S.
Mean Values during Resting Phase 11:45 - 35:00 hours			
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High v medium glucose $G_2B_{10} - G_{1.5}B_{10}$	1	0.1865	P < 0.02
Low v high bicarbonate at low glucose $G_1B_5 - G_1B_{10}$	1	0.1515	P < 0.025
Medium v mean of low and high bicarbonate at low glucose $2 G_1B_7 - (G_1B_5 + G_1B_{10})$	1	0.0233	N.S.
Flasks + Days 1:30 - 73:00 hrs (Residual for testing Treatment x Times)	54	0.0209	-
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Remaining orthogonal comparisons	3	0.3437	N.S.
Treatments x Times (Residual for testing Treatments and Times)	40	0.3435	P < 0.001
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Glucose levels ($G_2B_{10} + G_{1.5}B_{10}$) - ($G_1B_{10} + G_1B_{10}L$), $G_2B_{10} - G_{1.5}B_{10}$	2	0.1603	N.S.
Mean Values during Resting Phase 11:45 - 35:00 hours			
High glucose v remainder 5 $G_2B_{10} - (G_{1.5}B_{10} + G_1B_{10} + G_1B_{10}L + G_1B_7 + G_1B_5)$	1	7.8130	P < 0.001
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Low v high bicarbonate at low glucose $G_1B_5 - G_1B_{10}$	1	0.1515	P < 0.025
Medium v mean of low and high bicarbonate at low glucose 2 $G_1B_7 - (G_1B_5 + G_1B_{10})$	1	0.0233	N.S.
Flasks + Days 1:30 - 73:00 hrs (Residual for testing Treatment x Times)	54	0.0209	-
5:15 - 6:30 hrs	18	0.1100	-
11:45 - 35:00 hrs	18	0.0233	-

 G_1B_{10} , $G_{1.5}B_{10}$ etc. refer to treatments - see Fig. 6 legend

For other footnotes see Table 3 - page 58.

(b) Increased Glucose Concentrations

The use of 0.11 M Glucose gave a significant improvement in total growth over all other treatments. However, within the Treatments x Times interaction a similar comparison (High Glucose v Remainder, Table 6) was only significant during resting phase. Again the restricted sampling schedule masked the probable effect of increased glucose; viz. an extended period of growth.

Despite a 100 % increase in glucose concentration, optical density only increased by 30 % while media pH at the conclusion of logarithmic growth (5.6) was not as low as expected from the complete fermentation of 0.11 M Glucose to lactic acid. Thus although correcting cell lysis, 0.11 M Glucose was unsatisfactory in a media where cells were required to fully utilise their exogenous supply of carbohydrate for growth.

(c) Decreased Bicarbonate Concentration

When compared against 0.10 M Bicarbonate (with 0.055 M Glucose) the reduction of Bicarbonate to 0.05 M resulted in a small but highly significant improvement in logarithmic growth and a lower rate of lysis ($P < 0.05$ for deviations from common linear regression). At a concentration of 0.07 M Bicarbonate, results were intermediate between 0.05 M and 0.10 M Bicarbonate. Even at the lowest level of bicarbonate tested (0.05 M), pH did not fall below 6.0 but a further experiment showed that reduction of Bicarbonate to 0.033 M had no deleterious effect on growth but permitted pH to fall to 5.6, thereby preventing cell lysis.

VIABILITY OF S. BOVIS

A study of the viability of S. bovis during resting phase was carried out on cells left suspended in the medium after the fermentation of 0.055 M Glucose. The buffer system comprised 0.10 M Phosphate + 0.10 M

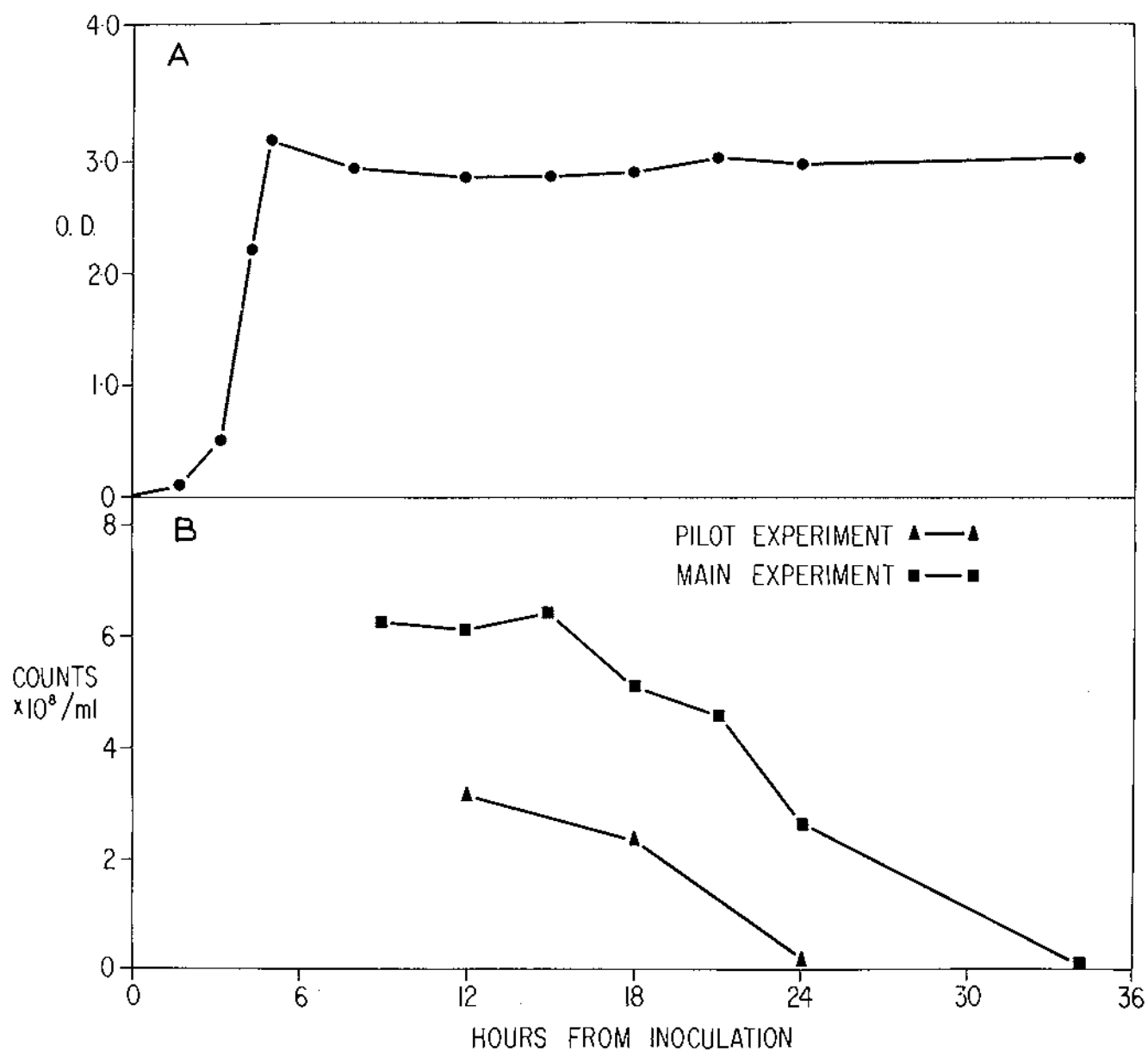


Figure 7. Viability of *S. bovis* cells suspended in spent growth medium.

A. Optical Density (O.D.) of cultures remaining constant during resting phase.

B. Mean numbers of viable cells, showing loss of viability with time.

TABLE 8

HIERARCHICAL ANALYSES OF VARIANCE FOR VIABLE COUNTS OF S. BOVIS AND OPTICAL DENSITY OF CULTURE DURING RESTING PHASE.

SOURCE OF VARIANCE	PILOT EXPERIMENT			MAIN EXPERIMENT								
	VIABLE COUNTS (1)			VIABLE COUNTS (1)			VIABLE COUNTS (1) (2)			OPTICAL DENSITY (3)		
	D.F.	M.S.	F	D.F.	M.S.	F	D.F.	M.S.	F	D.F.	M.S.	F
Total	23	-	-	110	-	-	106	-	-	26	-	-
Times	2	4337.2	17.40 *	6	10482.8	68.97 **	6	12847.0	114.0 **	6	0.0267	< 1 NS
Flasks within Times	-			7	152.00	1.15 NS	7	112.65	2.03 NS	7	0.0471	4.36 *
Samples within Flasks	3	249.24	2.62 NS	14	131.94	3.13 **	14	55.39	1.46 NS	13	0.0108	-
Plates within Samples	6	95.09	8.27 **	28	42.170	35.41 **	28	37.96	30.8 **	-		
Counts within Plates	12	11.50	-	55	1.907	-	51	1.23	-	-		

(1) Data analysed = $\sqrt{\text{Count}}$

(2) Values for sample plated with semi-solidified agar replaced by missing plot analysis (Snedecor 1956 p. 310)

(3) No transformation of data

NS Not Significant ($0.05 < P$)** Highly Significant ($P < 0.01$)* Significant ($0.01 < P < 0.05$)

TABLE 9

SIGNIFICANCE OF DIFFERENCES (4) IN VIABLE COUNTS WITH TIME.

HOURS FROM INOCULATION	HOURS FROM END OF LOGARITHMIC GROWTH	MEAN OF TRANSFORMED COUNTS	
		PILOT EXPERIMENT	MAIN EXPERIMENT (2)
9	4	-	78.17
12	7	55.55	77.89
15	10	-	79.94
18	13	47.81	71.24
21	16	-	67.63
24	19	11.91	50.64
34	29	-	7.35
		L.S.D. 5 % = 18.57	L.S.D. 5 % = 6.02
		L.S.D. 1 % = 35.82	L.S.D. 1 % = 8.74

(4) Means not linked by a single line are significantly different ($P < 0.05$)" " " " double " " highly significantly different ($P < 0.01$)

Acetate + 0.033 M Bicarbonate and had a pH throughout resting phase of 5.6.

Counts at 12, 18 and 24 hours after inoculation were made in a pilot experiment using only one flask of cells. Mean counts have been plotted against time in Figure 7 and the analysis of variance is presented in Table 8. A significant fall in number of viable cells was observed in the 24 hour sample (Table 9).

To furnish a more detailed estimate of the loss in viability, and to assess the importance of separate sources of variance, a further experiment was also carried out with duplicate flasks of cells and an increased frequency of sampling. In this, the main experiment, the cultures were grown overnight and optical densities recorded throughout resting phase are shown in Figure 7. Data for the logarithmic phase were recorded from separate flasks grown under identical conditions. Viable counts made at 9, 12, 15, 18, 21, 24 and 34 hours after inoculation are depicted in Figure 7 and the analyses of variance are included in Table 8. The first sampling time to show a significant fall in viable cell numbers occurred 18 hours after inoculation (Table 9), or after approximately 13 hours of resting phase. Six hours later only half of the cells remained viable. No change in optical density occurred throughout the entire resting phase.

There was a large difference between the counts obtained in the two experiments. This may have been due to a failure of the agar medium, used in the pilot experiment, to fulfil optimum growth conditions. S. bovis colonies, which on this medium had previously reached a countable size within 36 hours, did not on this occasion reach a countable size until 72 hours. If this indicates that unfavourable conditions for growth prevailed, the lower number of colonies could result from a high mortality rate in the establishment of growth on the agar.

From the results obtained under the conditions of these experiments, it is obvious that any experiments to study the metabolism of living cells

of S. bovis can only be conducted within 13 hours from the end of logarithmic growth.

Information obtained from the analyses of variance (Table 8), pertinent to the procedures employed in undertaking a study of viability, was as follows:

(1) The value of duplicate flasks and an increased frequency of sampling is illustrated by the higher level of significance for "Times" in the main experiment.

(2) In the first analysis of the data from the main experiment, "Samples within Flasks" was found to be a significant source of variance. However, in the course of one sampling, plates were poured with semi-solidified agar and gave a much lower number of colonies than other samples taken at the same time. Replacement of the data from the defective sample, by missing plot analyses (Snedecor 1956 p. 310) eliminated the significance of this level of the hierarchy when variances were recalculated, and illustrates the need for uniformity in preparing plates for each sampling.

(3) In the measurement of O.D., a difference of 0.2 O.D. units between duplicate flasks attained significance only because of its consistency throughout resting phase and the small variance of duplicate samples.

(4) The efficiency of the experimental design can be judged by calculating the components of variance for each level of the hierarchy, then making changes in the layout and noting the effect on the "Within Times" variance. Estimates of the components of variance were calculated from the formulae given by Cochran (1956) and are as follows;

<u>Source</u>	<u>Mean Square</u>	<u>% of Total</u>
Flasks (σ_f^2)	7.15	23
Samples (σ_s^2)	4.36	14
Dilutions (σ_d^2)	18.36	59
Plates (σ_p^2)		
Counts (σ_c^2)	1.23	4

Repeated counts made a negligible contribution to the total "Within Times" variance, showing that this procedure could be omitted. The highest individual component was plates, however this component could theoretically be subdivided into two parts; that due to the dilution tube from which the plate was prepared, and that due to sampling from the dilution tube. As only one plate per tube was poured in this experiment, it was not possible to separate the two components.

The variance of a mean count at any point in time is given by the formula:

$$\sigma_{\bar{T}}^2 = \frac{\sigma_f^2}{f} + \frac{\sigma_s^2}{fs} + \frac{\sigma_d^2}{fed} + \frac{\sigma_p^2}{fsdp} + \frac{\sigma_c^2}{fsdpc}$$

(Cochran 1956),

where $\sigma_{\bar{T}}^2$ = variance of mean count,

f = number of flasks sampled,

s = number of samples per flask,

d = number of dilutions per sample giving countable plates,

p = number of plates per dilution tube,

c = number of times each plate counted.

Hence the variance of a mean count in the main experiment was;

$$\frac{7.15}{2} + \frac{4.36}{4} + \frac{18.36}{8} + \frac{1.23}{16} = 7.05$$

The effect of changes in the layout is predicted by substituting the appropriate values for f, s, d, p and c in these equations, and it can be shown that by sampling four flasks, but taking only one sample per

flask would decrease the variance of the mean count. Alternatively if three flasks were sampled only once, there would be fewer dilutions to make without any great change from the variance calculated above.

Further experimentation is needed to show the most profitable method of reducing variance from the combined source of dilutions plus plates. However it would seem that the introduction of fivefold dilutions over the range expected to give countable plates, would be the most practicable method for increasing the number of dilution replicates, while pouring duplicate plates from each tube would enable the contribution of each source to the total variance to be estimated.

CHAPTER 5

STUDIES ON THE LIPIDS OF *S. BOVIS*

Lipid Content

Studies on the lipids of *S. bovis* were commenced with the extraction of lipids from freeze-dried cells grown in media containing 0.15 M Phosphate and 0.10 M Phosphate + 0.10 M Bicarbonate as buffers. Gravimetric estimation of the Folch-washed lipid gave lipid contents of 0.8 % (of cell dry weight) for cells grown in 0.15 M Phosphate and 1.7 % for cells from the medium buffered with 0.10 M Phosphate + 0.10 M Bicarbonate (Table 11). Both estimates were considerably lower than the figure of 7.2 % reported by Tweedie (1965) and may have been due to any of the following factors.

1. The absence of acetate from the medium may have decreased the lipid content of *S. bovis*.
2. The nitrogen source employed may have affected the formation of lipid.
3. Extraction and washing procedures may have reduced the material weighed as lipid.

Acetate Effect. As Stephenson and Chetham (1922), Dagley and Johnson (1963) and Brown and MacLeod (1964) had all shown that acetate in the media increased the lipid content of the bacteria studied, it was thought that the high level of acetate (0.36 M) employed by Tweedie (1965) might have been responsible for the higher lipid content of his cells. Adopting an acetate concentration of 0.10 M, which was more likely to be encountered in rumen fluid than the level used by Tweedie (1965), the effect of acetate on the growth of *S. bovis* was studied (see page 69) and found to cause only a slight reduction in total yield of cells. Therefore comparable

T A B L E 10

ANALYSES OF VARIANCE FOR CELL YIELD AND LIPID CONTENT OF S. BOVIS
GROWN IN DIFFERENT MEDIA.

SOURCE	CELL YIELD g/litre			LIPID CONTENT mg/g		
	D.F.	MEAN SQUARE	RESULT OF F TEST	D.F.	MEAN SQUARE	RESULT OF F TEST
Total	9	-	-	8	-	-
Treatments	4	0.35	$P < 0.001$	4	27	$P < 0.10$
Residual	5	0.01	-	4	6	-

For footnotes see Table 3 - page 58.

T A B L E 11

CELL YIELD AND LIPID CONTENT OF S. BOVIS GROWN IN DIFFERENT MEDIA

MEDIA AND BUFFER	CELL YIELD g/litre		LIPID CONTENT % OF DRY WT.	
	(1)		(1)	
Glucose-tryptose-yeast extract + 0.15 M $\text{HPO}_4^{''}$	0.8	a	0.8	ab
Glucose-tryptose-yeast extract + 0.10 M $\text{HPO}_4^{''}$ + 0.10 M $\text{HCO}_3^{'}$	1.3	b	1.7	b
Glucose-tryptose-yeast extract + 0.10 M $\text{HPO}_4^{''}$ + 0.033 M $\text{HCO}_3^{'}$	1.2	b	0.7	a
Glucose-tryptose-yeast extract + 0.10 M $\text{HPO}_4^{''}$ + 0.033 M $\text{HCO}_3^{'}$ + 0.10 M Acetate ¹	1.5	b	0.9	ab
Sucrose-casamino acids-yeast extract - thioglycollate etc + 0.36 M Acetate ¹ (Medium of Tweedie 1965)	0.5	a	1.3	ab
Studentised Differences 5 %	0.37		0.91	

(1) Means having common letters are not significantly different from one another ($P < 0.05$) using Studentised Differences (Snedecor 1956).

batches of cells were grown in media containing 0.10 M Phosphate + 0.033 M Bicarbonate as buffer with and without 0.10 M Acetate, but no increase in lipid content was found in the cells grown on acetate (Table 11). This result indicated that under the conditions of growth, S. bovis did not utilise exogenous acetate to markedly increase its lipid reserves and that acetate was unlikely to account for the discrepancy of the results.

Nitrogen Source in the Media. With acetate unable to account for the difference in lipid content, the effect was attributed to some other constituent of the media. In place of the tryptose used in this study, Tweedie (1965) had employed casamino acids, thioglycollate, cysteine, and tryptophane which could have resulted in the depletion of different amino acids at the end of growth. Furthermore Toennies, Shockman and Kolb (1963) found that valine depleted cells of S. faecalis, harvested at the completion of growth, contained 10.96 % lipid whereas cells harvested during logarithmic phase had only 4.93 % lipid. To test whether this effect was causing the variation in lipid content of S. bovis, cells were grown in the medium used by Tweedie (1965). Growth on this medium was slow and yielded only 0.45 g of cells/litre compared with the 0.8 - 1.5 g/litre obtained from the tryptose media (see Table 11). Lipid content remained low at 1.3 % (Table 11), indicating that differences in the growth media were not responsible for the different results.

Washing Procedure. Tweedie (1965) in his experiments did not wash the lipids extracted from S. bovis prior to weighing. In the course of extractions in the present study, it was found that up to 60 % of the "lipid" extracted with chloroform - methanol (2:1) was removed by washing.

However this factor alone could not account for the differences observed. It was then discovered that Tweedie (pers. comm.) had not allowed the chloroform - methanol extract to cool prior to filtering; consequently the "lipids" of Tweedie (1965) would have contained a considerable amount of material insoluble in cold chloroform - methanol. This probably accounts for the discrepancy of the results obtained in the two studies.

Statistical analysis of the data obtained from lipid extractions (Table 10 and 11) indicated that the cells grown on 0.10 M Phosphate + 0.10 M Bicarbonate had a significantly higher lipid content (1.7 %) than cells grown on 0.10 M Phosphate + 0.033 M Bicarbonate (0.7 %). However the cells grown in the former medium lysed extensively (page 65), and in view of the requirements for cell stability during resting phase, reasons for the variation in lipid content were not examined further.

Fractionation of Lipids and Evidence for the Presence of Glycolipids and a Sterol

Before the metabolism and importance of various lipid components from S. bovis could be studied, it was necessary to separate lipids into the broad categories of neutral and polar lipids and obtain some indication of the nature of the compounds present in each. In view of the elegant separation of S. faecalis glycolipids from phospholipids obtained by Vorbeck and Marinetti (1965 a, b) it was decided;

- (a) to determine whether glycolipids were present in S. bovis and if so
- (b) to employ column chromatography for their separation from neutral and phospholipids.

Presence of Glycolipid. The aqueous extract from an acid hydrolysate of total lipid (See Methods page 48) was examined for reducing sugars by paper chromatography. Using silver nitrate spray (Trevelyan, Proctor and

Harrison 1950) only one spot, which co-chromatographed with glucose in the ethyl acetate - pyridine - water solvent system, was visible. No spots corresponding to galactose, mannose or inositol were detected, and as no spot equivalent to glucose was obtained from the unhydrolysed lipid, the experimental results indicated that glucose was bound to lipids through an acid-labile bond.

Column Chromatography. Using the procedure described in the Methods (page 47) column chromatography of S. bovis total lipid on silicic acid was expected to yield fractions corresponding to, neutral lipids in the chloroform eluate, glycolipids in the chloroform - acetone eluates and phospholipids in the methanol eluate.

Fractions obtained from the column were monitored by TLC (see Methods page 47) using chloroform - methanol (185:15) and chloroform - methanol - water (65:25:4) as solvent systems. On developing spots with 20 % sulphuric acid charring, it was found that neutral lipids, which ran near the solvent front in chloroform-methanol (185:15), were not completely eluted from the column with 60 ml of chloroform, but tailed into subsequent fractions. Tailing of the glycolipids was also observed, as paper-chromatography of the acid hydrolysed lipid from each fraction showed a strong glucose spot in the methanol eluate.

Vorbeck and Marinetti (1965 a, b) claimed that neutral lipids were eluted as a single fraction with 60 ml chloroform, but their use of carbohydrate and phosphate analyses to monitor the fractions would not have detected tailing of neutral lipids. In view of the results obtained in this study it appeared that better separations of total lipid could be achieved by the more graphic method of preparative TLC.

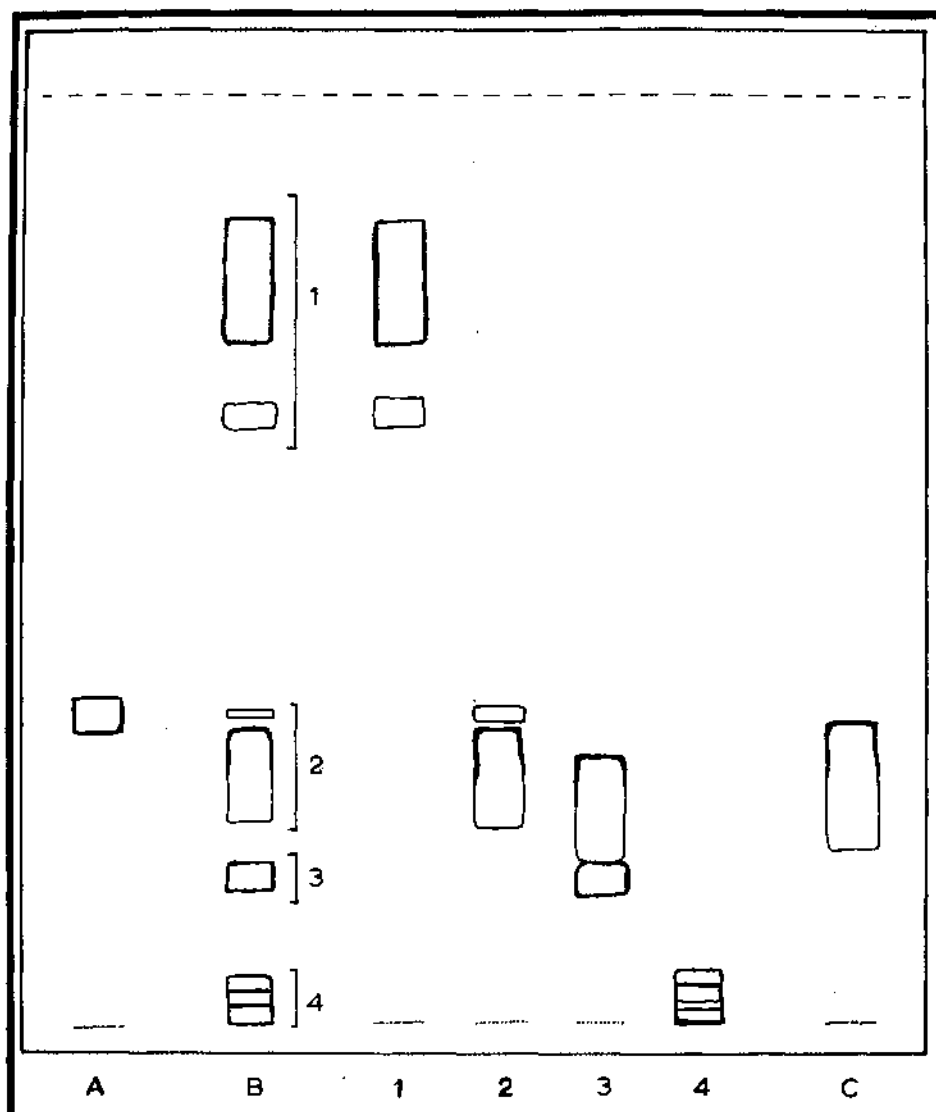


Figure 8. Thin-layer chromatoplate of total lipid from *S. bovis* in solvent system, chloroform - methanol (185:15). Spots detected by charring after spraying plate with 20% sulphuric acid.

A = Monopalmitin.

B = Total lipid.

1, 2, 3 and 4 = Fractions obtained by preparative TLC.

C = Oleic acid.

Preparative TLC. Following the procedures outlined in the Methods (page 48), four fractions corresponding to those noted in Figure 8 were scraped from the plate. Fractions were rechromatographed in the same solvent system and Figure 8 shows the separations achieved. Only fractions 2 and 3 contained a common component which must have been due to the streaking of the main component in fraction 2 in the neutral solvent system.

Fractions were stored in a solution of chloroform and methanol and with time increasing amounts of a component running near the solvent front were observed. The component was probably the methyl esters of fatty acids, formed by transesterification in the methanolic solution.

Lipid Composition of *S. bovis*. Evidence for the lipid composition of *S. bovis* was obtained from the chromatographic behaviour of different components, from their reaction to TLC spray reagents, and from the products of acid hydrolysis of the fractions prepared by TLC.

Fraction 1 contained only non-polar lipids which travelled near the solvent front, while fraction 2 chromatographed with free fatty acids (oleic and palmitic acid) in chloroform - methanol (185:15) and hexane - diethyl ether - acetic acid (30:70:1). The spot corresponding to monoglyceride was only present in minor quantities as judged by charring with 20 % sulphuric acid.

The contamination of fraction 3 with free fatty acids, due to their streaking in the neutral solvent (Mangold 1961), illustrates the need for a small quantity of acetic acid if complete separation of these components is required. Alternatively the separation could have been improved by extracting free fatty acids from the lipids prior to thin-layer chromatography.

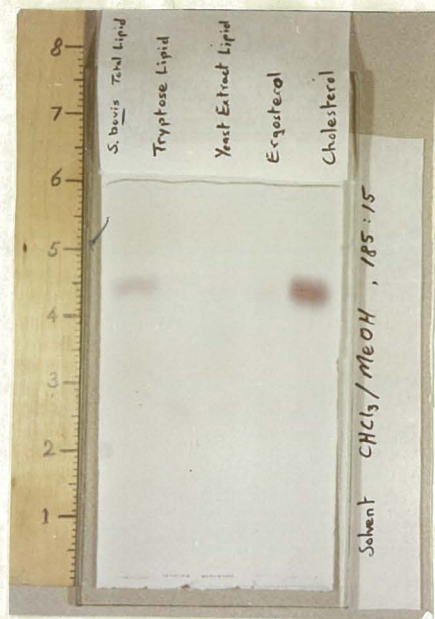


Figure 9. Thin-layer chromatoplate of total lipid from S. bovis, Bacto- Tryptose, and Bacto- Yeast Extract, with ergosterol and cholesterol standards. Solvent system; chloroform - methanol (185:15). Cholesterol and S. bovis sterol detected by charring plate for 5 - 10 min. at 110°C after spraying with 20% sulphuric acid. (Film; Agfa CN 17 Exposed; $\frac{1}{2}$ sec. f.11. Lighting; 2 x 150W. Tungsten lamps.)



Figure 10. Thin-layer chromatoplate similar to Figure 9, but charring allowed to continue for 1 hour.

Acid hydrolysis (see Methods page 48) of the lipid fractions and paper chromatography of the aqueous extracts showed that both fractions 3 and 4 contained lipid bound glucose, but none was detected in fractions 1 and 2. Hence it seemed probable that the second component in fraction 3 was a glycolipid, and as its mobility on TLC using chloroform - methanol (185:15) as solvent system was similar to that observed by Gray (1965) for monogalactosyl diglycerides from plant lipids, it is suggested that the structure may be monoglucosyl diglyceride.

On spraying thin-layer chromatoplates developed in chloroform - methanol (185:15) with the phosphate spray of Dittmer and Lester (1964), only compounds remaining at the origin (i.e. Fraction 4) were positive for phosphate. Rechromatography of Fraction 4 in the more polar solvent system chloroform - methanol - water (65:25:4) indicated that the major component of Fraction 4 was not phosphate positive. However, as acid hydrolysis of this fraction yielded glucose, the major component may correspond to the diglycosyl diglyceride reported in S. faecalis by Vorbeck and Marinetti (1965 a, b).

Presence of Sterol. During thin-layer chromatography of S. bevis lipid in the solvent system chloroform - methanol (185:15), a component was observed to run just behind other neutral lipids of Fraction 1 (Figure 8) and to produce a deep red-purple colour during charring with 20 % sulphuric acid. Figure 9 shows the charring colour which is considered to arise by the reaction of sulphuric acid with Δ^{-5} or Δ^{-7} sterols (M.G. Rumsby pers. comm.). A purple colour was also produced by glycolipids during charring but confusion with the sterol was avoided by a slower rate of colour development and the greater polarity of the glycolipids.

However it has been claimed by Fiertel and Klein (1959) and Asselineau and Lederer (1960) that bacteria, with a few notable exceptions, are unable to synthesise sterols. Therefore analyses were undertaken to

TABLE 12

STEROL ANALYSIS OF S. BOVIS LIPID, BACTO- TRYPTOSE
AND BACTO - YEAST EXTRACT.

SOURCE OF LIPID (1)	LIPID mg/g CELL DRY WT. (2)	STEROL CONTENT	
		mg/litre OF GROWTH MEDIUM	mg IN CELLS HARVESTED FROM 1 LITRE OF CULTURE
<u>S. bovis</u> cells grown in;			
0.10 M $\text{HPO}_4^{''}$ + 0.033 M $\text{HCO}_3^{'}$	7.0		0.19
0.10 M $\text{HPO}_4^{''}$ + 0.10 M $\text{HCO}_3^{'}$	17.0		0.31
Tryptose (14 g/litre of media)	134.0	1.6	
Yeast Extract (5 g/litre of media)	8.0	0.06	

(1) Tryptose in medium = 14 g/litre

Yeast Extract in medium = 5 g/litre

(2) Lipid content of Tryptose and Yeast Extract
expressed on a mean cell yield of 1.25 g/litre
(Table 11).

determine whether sufficient sterol was present in the organic constituents of the medium to account for the amount of sterol found in lipids of S. bovis. The results of the analyses are presented in Table 12, and show that only 12 - 19 % of the sterol added to the medium with Tryptose, was present in the cells harvested at the end of logarithmic growth. TLC of lipids from Yeast Extract and Tryptose in chloroform - methanol (185:15) are shown in figures 9 and 10. Although sterol was not detected in Tryptose on these plates, a positive sulphuric acid charring reaction was observed when the loading of tryptose lipid was increased. It was therefore suggested that S. bovis incorporated sterol from the media into the cellular lipids.

Lipids Synthesised by S. bovis

In the course of estimating the sterol content of tryptose and yeast extract it was found that these two constituents of the media would provide as much as 180 mg of lipid per litre of medium (see Table 12). A thin-layer chromatoplate of the lipids extracted from Yeast Extract and Tryptose is shown in Figure 10 and illustrates the variety of lipids present. However, S. bovis cells harvested from 1 litre of media yielded only 10-20 mg of lipid and therefore it seemed possible that S. bovis might fulfil its lipid requirements by the uptake of fatty acids from the medium (Camien and Dunn 1957), as appears to be the case with sterols.

To determine which lipids were synthesised by S. bovis, a culture was grown in one litre of medium containing 3 μ C of 1-C¹⁴ sodium acetate (carrier free) and buffered with 0.10 M Phosphate + 0.033 M Bicarbonate. Cells were harvested at the end of logarithmic growth, while lipids were extracted in the usual manner (Methods page 46) and chromatographed on thin layer plates with chloroform - methanol (185:15).

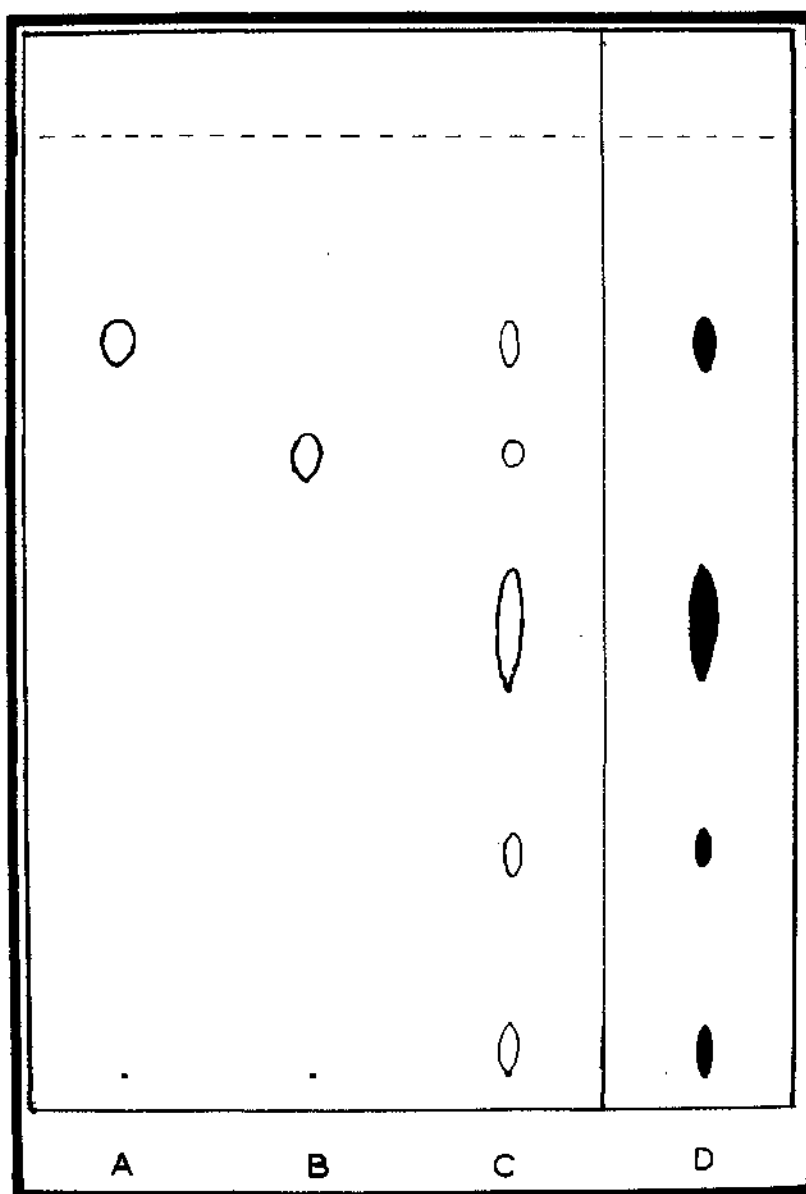


Figure 11. Thin-layer chromatoplate and radioautograph of S. bovis total lipid after 1-C¹⁴ acetate added to growth medium. Solvent system, chloroform - methanol (185:15). Spots detected by charring after spraying plate with 20 % sulphuric acid.

A = Tripalmitin B = Cholesterol
 C = S. bovis total lipid (radioactive)
 D = Radioautograph of C.

After radioautography the plate was charred with 20 % sulphuric acid and the results are illustrated in Figure 11. No trace of activity was observed in the sterol spot, indicating that S. bovis did not utilise acetate for the synthesis of sterols. Previous experiments on the analysis of lipids using TLC (page 47) suggested that the spots containing activity corresponded to (in descending order);

- (a) neutral lipid fraction (which could consist of triglycerides, diglycerides or methyl esters of fatty acids).
- (b) free fatty acids
- (c) monoglycolipid
- (d) polar lipids (comprising phospholipids and a second glycolipid compound).

Thus, in spite of the relatively large amounts of lipid present in the media, it appeared that S. bovis was capable of synthesising lipids from acetate.

Incorporation of a Long Chain Fatty Acid

Despite the demonstration of lipid synthesis from acetate by S. bovis, the incorporation of preformed fatty acids from the media remained an alternative source of fatty acids for cellular lipids. In addition it was possible that bacteria in the rumen incorporated long chain fatty acids, as Garton, Lough and Vioque (1961) had found the majority of lipid in the rumen to be free fatty acid while Hawke and Robertson (1964) had found levels of free fatty acids varying from 20 - 300 mg/litre in the rumen of a pasture fed cow. It was also possible that the incorporation of labelled fatty acid could be employed to specifically label certain lipid pools as an introduction to the study of their turnover and the metabolism of the fatty acid.

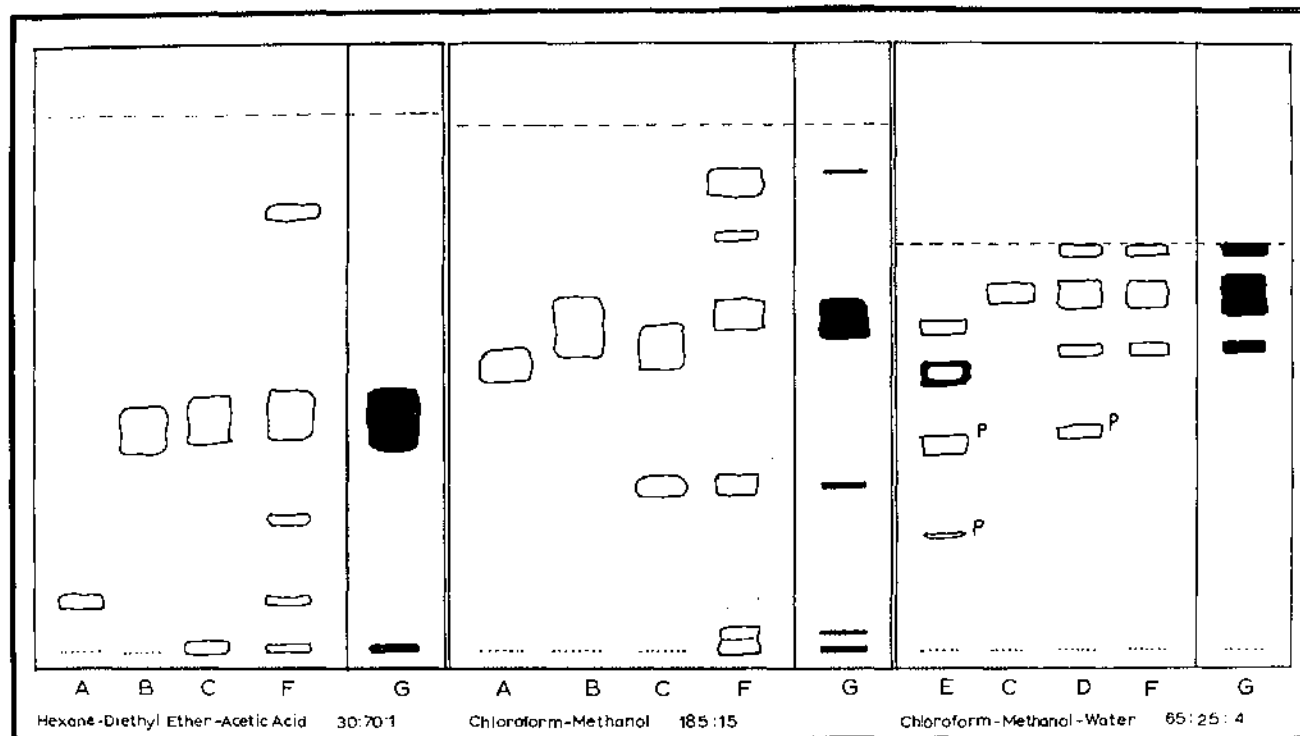


Figure 12. Thin-layer chromatography and radioautography of *S. bovis* total lipid after U- ^{14}C palmitate added to growth medium. Plate on right sprayed with phosphate spray (P = phosphate + ve) and all plates charred after spraying with sulphuric acid.

- A = Monopalmitin.
- B = Oleic acid.
- C = Fraction 3 (Figure 8).
- D = Fraction 4 (Figure 8).
- E = Phosphatidylethanolamine + digalactolipid (Gray 1965).
- F = *S. bovis* total lipid (radioactive).
- G = Radioautograph of F.

Therefore it was decided to obtain direct evidence of incorporation, by the addition of a radioactive long chain fatty acid to the medium and determining its uptake into the cellular lipids. In this experiment S. bovis was grown in one litre of medium containing 0.10 M Phosphate + 0.033 M Bicarbonate and 0.10 M Acetate as buffers, and 5 μ C of U- C^{14} palmitate (carrier free). To permit maximum metabolism of the palmitate, cells were harvested after 12 hours of resting phase or just prior to the loss of viability (see page 74). In an attempt to remove fatty acids adhering to the cell surface, the harvested cells were washed twice in an alkaline solution of phosphate buffer (0.10 M, pH 7.5). The cells were freeze dried and the lipids extracted and separated by TLC in three different solvent systems; hexane - diethyl ether - acetic acid (30:70:1), chloroform - methanol (185:15) and chloroform - methanol - water (65:25:4). After radioautography the plates were sprayed with 20 % sulphuric acid for plates from the first two solvents and the phosphate spray (Dittmer and Lester 1964) followed by 20 % sulphuric acid for the plate from the third solvent.

The results are illustrated in Figure 12, and show that the majority of the activity occurred in the free fatty acid spot, as judged by the intensity of the radioautograph, while some was incorporated into other lipid constituents. These constituents appear to be the monoglycolipid and a compound that would appear to be a diglycolipid (see page 89) since this component :

- (a) remained negative to the phosphate spray (Dittmer and Lester 1964),
- (b) remained near the origin in chloroform - methanol (185:15) and
- (c) ran only slightly ahead of an authentic sample of plant digalactolipid in chloroform - methanol - water (65:25:4).

This sample of digalactolipid was prepared in this laboratory by Mr I.K. Gray and was known to contain minor amounts of phosphatidylethanolamine.

A study of the C^{14} remaining in the cells after extraction of lipid was carried out by Folch-Van Slyke combustion of the cells (Methods page 50) and determining the radioactivity of the carbon dioxide collected. The results indicated that only 63 % of the total activity present in the harvested cells was extracted as lipid. The remainder may have been present as bound lipid as Ikawa (1963) had found bound lipids to account for up to 3.1 % of the bacterial dry weight after extraction with chloroform - methanol (2:1) (Table 1).

CHAPTER 6

DISCUSSION

GROWTH EXPERIMENTS

Growth experiments with S. bovis (Chapter 4) were carried out to devise a suitably buffered medium which would provide high cell yields with a minimum change in pH over the entire growth curve. To meet these conditions various concentrations and combinations of phosphate, bicarbonate and acetate were examined in turn, for their effect on the growth of S. bovis and their ability to neutralise lactic acid. The conclusions from these experiments were based on the results of analyses of variance. In undertaking these analyses, observations from duplicate flasks at the same point in time were summed and the results presented as the mean value in the graphs (Figures 3, 4, 5 and 6). As comparable sampling times were needed for each flask, it was necessary to refer all growth curves within an experiment to a common time scale and in view of the irregular sampling schedule, the only one suitable was "hours after inoculation". However, a consideration of the physiology of bacterial growth (Clifton 1958) shows that this time scale has one serious disadvantage; small variations in the size of the inoculum or the length of the lag phase could be responsible for most of the variation between duplicate flasks through lateral displacement of the whole growth curve. In the case of Figure 5 this effect was most noticeable and resulted in the presentation of an atypical growth curve for one treatment (0.10 M Phosphate + 0.10 M Bicarbonate Na.Na), where for some obscure reason a prolonged lag phase occurred in one of the two flasks.

To correct lateral displacements of the growth curve and still maintain a valid basis for the statistical analysis of data would require the readjustment of the time scale for all growth curves to an arbitrary point during logarithmic growth (say O.D. 1.0). As S. bovis grows rapidly (cell mass doubles in approximately 30 minutes), an almost continuous record of the growth curve would be needed with samples taken at no more than 15 minute intervals. Growth data in this form would also be useful for the description of growth as some exponential function with time, instead of the separate linear regressions used in this study (e.g. Table 5). However, means for obtaining data of this calibre were not available during the present investigation and it is therefore concluded that the statistical analyses of existing data provided a fair assessment of the significance level of the results.

The Effects of Buffer Constituents on
Growth of S. bovis

1. Phosphate

The first buffer to be examined for its effect on the growth of S. bovis and on buffering capacity of the medium was monohydrogen/dihydrogen phosphate. This buffer was chosen because it is one of the buffers in rumen fluid and was effective within the desired pH range of 6 - 7 (Turner and Hodgetts 1955).

In these experiments (page 56) satisfactory growth of S. bovis was observed in a medium of 0.15 M Phosphate (Figure 3) while the pH of the medium fell from 6.9 to 5.3 - 6.0 (Figure 3 and Table 4) on fermentation of 0.055 M Glucose. Variations in final pH may have been due to the destruction of glucose during autoclaving by non-enzymic browning as in

later experiments (Figures 4 and 5) where browning was avoided (Methods page 43), the final pH was always 5.6 from the fermentation of 0.055 M Glucose in the presence of 0.15 M Phosphate.

At higher concentrations of phosphate, growth of S. bovis was poor in comparison with that in 0.15 M Phosphate. In 0.20 M Phosphate logarithmic growth (when it occurred) was preceded by a prolonged lag phase (page 59) and at 0.30 M Phosphate no logarithmic growth occurred (Figure 3). These results are in agreement with the observation of Bailey and Oxford (1958 b); that S. bovis (Strain I) was unable to grow in 0.25 M Phosphate.

However, the phosphate tolerance of other Streptococcus spp. appears to be higher than that of S. bovis. Shockman (1965) reported a doubling time of 31 - 33 minutes for cells of S. faecalis in a medium containing 0.30 M Phosphate, while S. lactis has been grown in a medium containing 0.20 M Phosphate (T.D. Thomas pers. comm.).

The phosphate concentrations employed throughout this study were higher than the levels of 0.02 - 0.03 M reported to occur in the rumen (Garton 1951, Parathasary, Garton and Phillipson 1952, Turner and Hodgetts 1955). Although these higher concentrations may have had subtle effects on the metabolism of S. bovis, the control over pH was considered to be of primary importance to the studies of resting phase metabolism which were to follow. (see Aims page 39.)

2. Bicarbonate

The effects of bicarbonate on the growth of S. bovis and buffer capacity of the medium were examined (page 59) because Turner and Hodgetts (1955) had shown that bicarbonate was an effective buffer in the rumen fluid and because Bailey and Oxford (1958 a) and Wright (1960 b) had reported increased cell yields in the presence of carbon dioxide.

The experiments showed that, with the fermentation of 0.055 M Glucose, in a medium containing 0.10 M Phosphate + 0.10 M Bicarbonate, the final pH was 6.4 - 6.6 (Figures 4 and 5) and cell yield was 40 % greater than that obtained in a medium buffered with 0.15 M Phosphate (Figure 4). Use of bicarbonate, to give a medium of high buffer capacity, achieved greater control over pH change and an improved cell yield, two of the objectives of the growth experiments. However, it also introduced the problem of cell lysis (page 103).

The increase in cell yield could have been due, either to the reduction in phosphate concentration, or to the presence of a source of carbon dioxide but using the present system of inoculation no separation of these two effects was possible. In a medium containing only 0.10 M Phosphate, the fermentation of 0.055 M Glucose resulted in a final pH of 4.4 which could have limited cell growth, while the addition of 0.10 M Bicarbonate to 0.15 M Phosphate gave depressed growth of S. bovis (Figure 4), presumably due to the excessive concentration of cations or to the interaction of the two anions. Therefore to assess the effect of carbon dioxide on cell growth under comparable conditions it would be necessary to use a closed incubation system with and without a gaseous phase containing carbon dioxide, as employed by Wright (1960 b).

However both Wright (1960 b) and Bailey and Oxford (1958 a) grew S. bovis in media containing excess sucrose to favour dextran formation, and no estimate of cell yield in relation to the amount of carbohydrate fermented was possible. Under the conditions of their experiments, growth was probably limited by the low pH of the medium or the depletion of essential amino acids. On the other hand, in the present study glucose was employed to keep dextran formation to a minimum (Bailey 1959 b)

and growth was certainly not limited by the acidity of the medium. Since Hobson (1965) found that glucose utilisation by S. bovis, was complete up to a concentration of 0.055 M, in a medium similar to that used in the present study, it is assumed that growth was halted by the depletion of glucose.

As Bauchop and Elsdon (1960) postulated that the growth of bacteria under anaerobic conditions was proportional to the carbohydrate utilised for fermentation, it appears that either the reduction in phosphate concentration or the presence of carbon dioxide, in the media used in the present study has spared the energy derived from fermentation of glucose. In view of the studies by MacDonald (1958) and Wright (1960 b) indicating that the carboxylation of a C-3 acid to form aspartate was the main site of fixation of carbon dioxide, and the demonstration of an energy requirement for the uptake of aspartate from the medium (Wright 1960 b), it is possible that the increased cell yield in the presence of carbon dioxide resulted from the energy saved by the intracellular synthesis of aspartate. However further study under conditions that would permit unequivocal assessment of the effect of carbon dioxide on the energy economy of the cell, is required to determine whether synthesis of aspartate by carboxylation of a C-3 acid requires less energy than the incorporation of extracellular aspartate.

3. Acetate

As cells of S. bovis grown in the media used in the present study had a low lipid content (Table 11) compared with the results of Tweedie (1965), it was decided to examine the effect of acetate in the medium on the lipid content of S. bovis (page 65). However it was first necessary to determine whether acetate would have any effect on the growth of S. bovis. The experimental data presented in Figure 5

and Table 6 indicated that, in media already containing a high buffer concentration (i.e. 0.15 M Phosphate or 0.10 M Phosphate + 0.10 M Bicarbonate), 0.10 M Acetate caused a depression in cell yield. The effect was not as great as the depression caused by the addition of 0.10 M Bicarbonate to 0.15 M Phosphate (Figure 4) and as the cation levels in the media were identical, it is possible, that in the presence of high phosphate concentrations, the acetate ion is less inhibitory to growth than the bicarbonate ion.

As acetate is only effective as a buffer salt between pH 3.6 and 5.8 (Dawson, Elliot, Elliot and Jones 1959), the addition of acetate to the culture medium only contributed to the buffer capacity at the end of logarithmic growth when the buffering capacity of phosphate had been exhausted (i.e. at approximately pH 5.8. Dawson et. al. 1959). However in the studies of S. bovis metabolism during resting phase in a spent growth medium of pH 5.2 - 5.7, the acetate salt served as a useful buffer against further decreases in pH resulting from small variations in lactic acid production.

Growth Medium Developed for Studies on S. bovis Lipids

The medium finally chosen for the growth of S. bovis employed 0.055 M Glucose as energy source, as the increased yield of cells obtained from higher concentrations of glucose was less than proportional to the increase in glucose concentration (Figure 6). Furthermore Hobson (1965) indicated that, in a similar growth medium, glucose utilisation was virtually complete at 0.055 M.

Buffer constituents comprised 0.10 M Phosphate + 0.033 M Bicarbonate as at this concentration the fall in pH to 5.6 favoured cell stability (page 70). Acetate at a concentration of 0.10 M was also included to

simulate the rumen environment and to act as a buffer against further decreases in pH. With 0.10 M Phosphate, and Bicarbonate at the concentration of 0.033 M instead of 0.10 M, 0.10 M Acetate had no depressing effect on the cell yield harvested at the end of logarithmic growth (Table 11).

Cell Lysis

During the search for optimum growth conditions for S. bovis, it was found that if the pH of the medium was maintained above 6.0 over the entire growth period, then a distinct fall in the optical density of the bacterial suspension during resting phase was observed. Buffers capable of maintaining the pH above 6.0 during the entire growth phase were 0.10 M Phosphate + 0.10 M Bicarbonate, 0.15 M Phosphate + 0.05 M Bicarbonate and 0.15 M Phosphate + 0.10 M Bicarbonate (Figure 4) and 0.10 M Phosphate + 0.10 M Bicarbonate + 0.10 M Acetate (Figure 5). The fall in optical density was critically dependent upon pH and could be prevented by lowering the pH below 5.9 (page 70). This fall in optical density could have been due to plasmolysis, a bacteriophage or cell autolysis.

Mitchell and Moyle (1956) found that plasmolysis of Staphylococcus aureus cells in solutions of 0.10 M sodium chloride resulted in a 6 - 9 % decrease in cell volume, but in the present study the optical density of S. bovis fell to a much greater extent than this, without there being any reason to suspect that the osmolarity of the medium had changed. Therefore it seems unlikely that S. bovis cells were plasmolysed.

Cell lysis could have been due to the presence of a bacteriophage in the culture but the fall in optical density is generally precipitous (Kellenberger 1959, Anderson 1960) and is unlikely to be so critically dependent upon pH.

Autolytic enzymes were suggested by Shockman (1965) to account for the cell lysis of S. faecalis. Shockman, Toennies and co-workers have made an extensive investigation of the growth chemistry of S. faecalis following the depletion of essential amino acids from the medium (see Toennies and Shockman 1959 and Shockman 1965 for reviews). On depletion of an amino acid such as threonine or valine, which were only essential for the formation of cytoplasmic protein, cell wall synthesis was observed to continue, giving rise to the phenomenon of "post-exponential cell wall synthesis". Growth of cell wall was shown to require only a relatively simple medium of buffer, glucose, a mixture of inorganic salts, acetate, and five amino acids (Shockman, Conover, Kolb, Riley and Toennies 1961 b). When exponential growth of S. faecalis was halted by the depletion of either glucose, or one of the amino acids required for cell wall synthesis, and the medium pH was greater than 5.9, cell lysis resulted (Shockman et. al. 1961 a). In the experiments of this study where logarithmic growth of S. bovis was probably limited by the depletion of glucose, the fall in optical density was shown to be similarly dependent upon pH (Figure 6).

In view of the autolytic enzyme systems observed in a number of Gram-positive and Gram-negative bacteria, Shockman (1965) supports the hypothesis of Mitchell and Moyle (1957); that the autolytic enzymes, responsible for the cell lysis described above, have a role in the growth of cell walls by breaking polymers to allow the insertion of new material. However, this hypothesis in itself fails to explain the pH effect entirely, as both cell wall growth and cell division continued in a medium of pH lower than 5.9.

The slower rate of lysis observed when potassium replaced part of the sodium ion concentration in the medium is difficult to account for. However, Toennies and Shockman (1959) observed the formation of osmotically fragile protoplasts during the lysis of S. faecalis and Mitchell and Moyle

(1956) have shown that the stability of these forms is dependent upon the composition of the medium used for their suspension. Abrams (1959, 1960) observed that the osmotic stability of S. faecalis protoplasts was enhanced by the metabolically dependent entry of oligosaccharides requiring the presence of potassium ions. On the other hand Shockman et. al. (1961 a) noted no difference in the rate of lysis of S. faecalis in either sodium or potassium phosphate buffers, and it is possible that other cations are involved; McQuillen (1960) suggested that magnesium ions could have a stabilising effect on protoplasts.

In view of the results obtained with S. bovis, it is possible that lysis also occurs in the rumen as the pH of rumen liquor is commonly on the alkaline side of 5.9 (Annison and Lewis 1959, Bryant 1964). However, it is difficult to know whether the other factors required for lysis also exist and further studies on the stability of S. bovis cells suspended in rumen fluid are required to ascertain the practical importance of this observation.

As cell lysis of S. bovis depended on the pH of the medium, a buffer concentration of 0.10 M Phosphate + 0.033 M Bicarbonate + 0.10 M Acetate, which stabilised optical density during resting phase, was chosen for the study of S. bovis viability.

CELL VIABILITY

To form an estimate of the period of survival for S. bovis cells during resting phase, numbers of viable cells left suspended in the spent growth medium were determined by the plate count method. Further studies on the lipid metabolism of S. bovis during resting phase could then be integrated with the period of cell survival. Constant numbers of viable cells were found to last for no more than approximately 13 hours of resting

phase before a substantial drop in viability occurred (Table 9).

As Dawes and Ribbons (1964) have postulated that the presence of a utilisable endogenous reserve is required for the survival of cells in suspension, the present results would tend to indicate that S. bovis does not contain appreciable endogenous reserves. This may be due to the adaptation of S. bovis to the conditions of continuous culture prevailing in the rumen.

However the result obtained is only preliminary to further studies on the factors affecting cell viability in S. bovis. Dawes and Ribbons (1964) have listed as factors affecting cell survival; the toxicity of the suspending medium, illumination, temperature, pH, potassium and magnesium ion concentrations and degree of anaerobiosis. Although it is believed that the pH of the spent growth medium was favourable to the survival of S. bovis (Krogh 1959), further experiments are required to determine whether the death of S. bovis was caused by expiry of endogenous reserves, or the conditions under which the cells were suspended.

STUDIES ON THE LIPIDS OF S. BOVIS

Lipid Content of S. bovis

A comparison of the lipid content of other lactic acid bacteria (Table 1) with the figure of 7.2 % reported by Tweedie (1965) for S. bovis, indicated that S. bovis contained substantially larger lipid reserves than other members of the Lactobacteriaceae. The question therefore arose as to the origin of this lipid and its metabolism during the endogenous phase. However, the studies on the lipid content of cells grown in the present experiments (Table 11) showed that the lipid extractable from S. bovis with chloroform - methanol (2:1) (Methods page 46) constituted no more than 1 - 2 % of the cell dry weight. Changes in the composition of the growth

medium, including the presence of acetate at a concentration of 0.10 M were unable to fully account for the differences observed between Tweedie's (1965) result and the data of the present study. The high level of lipid in S. bovis reported by Tweedie (1965) was possibly due to the large amount of water soluble material which can be extracted if cells, refluxed with hot chloroform - methanol, are filtered while the solvent is still hot.

As the presence of 0.10 M Acetate in the medium had no detectable effect on the lipid content of S. bovis it would appear that this species is different from S. lactis (Brown and MacLeod 1964), E. coli (Dagley and Johnson 1953) and M. phlei (Stephenson and Whetham 1922) where exogenous acetate was found to increase the lipid content. Apart from the increase in lipid content of S. faecalis cells on depletion of certain amino acids (Toennies, Shockman and Kolb 1963), other factors governing the lipid content of bacteria do not appear to have been investigated. O'Leary (1962) and Kates (1964) in their reviews of bacterial lipids emphasise the effect of growth conditions on lipid composition but make no reference to the lipid content of cells.

The results of lipid extractions (Table 11) also showed that cells harvested from a medium buffered at pH 6.7 with 0.10 M Phosphate + 0.10 M Bicarbonate had a higher lipid content (1.7 %) than cells from a media buffered at pH 5.2 with 0.10 M Phosphate + 0.033 M Bicarbonate (0.7 %). It appears that either the level of bicarbonate or the final pH of the medium has resulted in a subtle change in the cell lipids. Houtsmuller and van Deenan (1964) working with Staphylococcus aureus found that the ratio of phosphatidyl glycerol to phosphatidyl glyceryl lysine depended upon the pH of the medium. Further work involving the complete extraction of all lipids from the cells is needed to show whether the changes noted

by Houtsmuller and van Deenan (1964) also resulted in a change in the lipid content of cells, or whether the present results were due to the release of bound lipids at the higher pH.

The Lipids of *S. bovis*

Despite the relatively low yield of lipid from *S. bovis* the study of lipids was continued to gain knowledge on their structure, as a prerequisite to further studies on lipid metabolism. Evidence obtained from TLC of the lipids and paper chromatography of the acid hydrolysates (pages 84-91) indicated that the lipids consisted of;

- (a) a non-polar fraction
- (b) minor amounts of monoglyceride
- (c) free fatty acids
- (d) phospholipids
- (e) two glycolipid components
- (f) a sterol component

In view of the hot chloroform - methanol extraction procedure (page 46) which has been found to transesterify fatty acyl groups to methyl esters (J.G. Robertson pers. comm.), it is impossible to propose structures for the non-polar fraction. Methyl esters of fatty acids, triglycerides and diglycerides all chromatographed together in the chloroform - methanol (185:15) solvent system. Trace amounts of a compound chromatographing with monoglyceride were observed in this solvent system, but further evidence is required for its positive identification.

In previous studies on the lipids of lactic acid bacteria (Table 1) little attention has been paid to the structure of the neutral lipids. Only MacLeod and Brown (1963) claim to have identified a triglyceride fraction in the lipids of *S. lactis* var *maltigenes* and *S. cremoris* but the

infrared spectrum, upon which they based their identification, was not published. Since these authors also used hot chloroform - methanol to extract the lipids, more adequate evidence for the identity of triglycerides is required. Ibbott and Abrams (1964) also suggested a glyceride structure for one of the lipid fractions from the membrane of S. faecalis, but only on the basis of its elution from a silicic acid column with hexane - diethyl ether (1:1).

In the lipids of S. bovis, large amounts of free fatty acids appeared to be present and in the solvent system used for preparative TLC (chloroform - methanol, 185:15) this fraction tailed and was not separated cleanly from the glycolipid component. This could be remedied either by the use of 1 % acetic acid in the solvent system or by the prior extraction of free fatty acids from the total lipid.

Asselineau and Lederer (1960) and O'Leary (1962) mentioned that large amounts of free fatty acids have been found in several species of bacteria but this may have resulted from the action of lipases during extraction.

By comparison with the lack of knowledge on the structure and occurrence of neutral lipids, the phospholipids from lactic acid bacteria have been extensively investigated (Table 1). In the present study evidence for their presence was obtained using the phosphate spray of Dittmer and Lester (1964) (page 89).

Occurrence of Glycolipids

From the results of acid hydrolysis of lipid fractions (page 89) it is apparent that two glycolipid fractions are present in the lipids of S. bovis. This enables S. bovis to be included in the growing lists of Gram-positive bacteria found to contain glycolipids (Brundish, Shaw and Baddiley 1965 a).

Brundish, Shaw and Baddiley (1965 a, b) and Vorbeck and Marinetti (1965 b) have also investigated the structures of the glycolipids by saponification and chromatography of the water soluble products. The release of glycosides containing disaccharides of galactose and glucose linked to the 1 - position of glycerol and the release of glucosylglycerol indicated that the original structures were di- and mono-hexosyl diglycerides respectively. Glucose, galactose and mannose were the hexoses released by acid hydrolysis under conditions similar to those employed in the present study (page 48).

Further experiments on the glycolipids obtained from S. bovis are required to fully determine their structure, and effect means for their complete separation from other lipid constituents. From the studies conducted in this investigation it appears that the technique of preparative TLC can be adapted to this end by the prior extraction of free fatty acids from the lipid to be chromatographed.

As yet little information has been obtained on the role or metabolism of glycolipids in bacteria. However, Distler and Roseman (1964) have observed the transfer of labelled hexose from uridinediphosphate -glucose and -galactose to glycolipids by an enzyme system from Diplococcus pneumoniae while Kaufman, Kundig, Distler and Roseman (1965) have shown that the galactosylglucosyl diglyceride was synthesised from the glucosyl diglyceride.

Presence of Sterol

The observation of a positive sterol reaction on charring thin-layer chromatoplates with sulphuric acid (page 89) appeared to be contrary to the assertion of Asselineau and Lederer (1960); that bacteria, with few exceptions, do not contain sterols. The notable exceptions included E. coli (Dauchy, Kayser and Villontreix 1956), Azotobacter chroococcum (Sifferd and Anderson 1936), and Micronospora sp. (Fiertel and Klein 1959).

In each case the bacteria were grown in media free of sterol and strong chemical evidence was obtained for the identity of the sterol. Other investigations by Lemoinge, Milhaud and Croson (1949 Cited by Fiertel and Klein 1959) in Bacillus megatherium and by Williams, Bloor and Sandholzer (1937) in E. coli, suggesting that bacteria do not contain sterols can only be regarded as inconclusive; any sterol present was below the sensitivity of the method used for their detection.

On the other hand Guirard, Snell and Williams (1946) obtained evidence for the incorporation of sterols by finding that cholesterol, ergosterol or calciferol could replace the acetate requirement of several Lactobacillus spp. Thorne and Kodicek (1962 a) also found that mevalonic acid (an intermediate in the synthesis of sterols by animals; Goodwin 1960) was able to replace the acetate requirement of L. acidophilus and L. plantarum. Other studies by Thorne and Kodicek (1962 b, c, 1963) indicated that labelled mevalonic acid was incorporated mainly into a lipid component with an isoprenoid structure. However as none of the radioactivity was precipitated with digitonin it was concluded that the compound was not a sterol. Thus the synthetic pathway for sterols in bacteria is not clearly defined, as even when A. chroococcum was grown in the presence of labelled acetate or mevalonate, radioactivity was not found in the sterol (Bloch and Amdur unpublished data. Cited by Asselineau and Lederer 1960).

In the present investigations, no indication of incorporation of acetate into the sterol fraction was observed (Figure 11), while the analyses for sterol (Table 12) shows that Bacto-Tryptose contained sufficient sterol to account for its presence in the extractable lipids of S. bovis. Proof of the ability of L. acidophilus to incorporate sterols was demonstrated by Thorne and Kodicek (1962 c) when labelled

cholesterol was added to the medium and radioactivity recovered in the harvested cells.

Origin of Lipids of *S. bovis*

In view of the low lipid content of *S. bovis* (Table 11) and the comparatively large amount of lipid in the organic constituents of the media (Table 12), attention was directed toward the origin of the lipids in *S. bovis*, and the ability of *S. bovis* to synthesize fatty acids from acetate was compared with the incorporation of palmitic acid from the medium. That both processes occurred can be seen from the distribution of radioactivity in the radioautographs of total lipids of *S. bovis* (Figures 11 and 12).

The studies of Goldfine and Bloch (1961) and Thorne and Kodicek (1962 b) showed that bacterial fatty acids were synthesised from acetate, and in the present study it seemed probable that the activity from acetate was located mainly in the fatty acid moieties. This contention is supported by the occurrence of radioactivity in the free fatty acid spot after the growth of *S. bovis* in medium containing labelled acetate (Figure 11). Thus although the presence of acetate in the medium at a concentration of 0.10 M failed to increase the lipid content of *S. bovis*, exogenous acetate obviously supplied carbon for lipid synthesis. Tweedie (1965), on the other hand, found that *iso*-butyrate in the medium was unable to effect the synthesis of branched chain fatty acids in *S. bovis* nor was radioactivity from 1-C¹⁴ *iso*-butyrate incorporated into *S. bovis* lipids. In view of these results it would seem doubtful that the branched chain fatty acids, reported by Thorne and Kodicek (1962 d) to be present in the lipids of *Lactobacillus* spp. (Table 1), were actually synthesised by the bacteria. Like the sterol discovered in this study, these acids probably arose from

the trace amounts of fatty acids present in the amino acid source (Demain, Hendlin and Newkirk 1959). As Casamino acids and Tryptose are both prepared from casein it is likely that the contaminating fatty acids and lipids would reflect the lipid composition of butterfat which has been shown to contain branched chain fatty acids (Shorland and Hansen 1957).

Where it is desired to know which fatty acids are in fact synthesised by the organism it appears that the answer can only be obtained either by growing the cells in a medium completely free of fatty acids, or by tracing the fatty acid spectrum from the amount of radioactivity present in the fatty acids extracted from cells grown on labelled acetate or glucose. Thorne and Kodicek (1962 b) found that even in the presence of unlabelled acetate, 50 % of the fatty acid carbon was derived from glucose. Some of the previous studies on the fatty acids of lactic acid bacteria (e.g. Hofmann, Lucas and Sax 1952, Table 1) have taken the precaution of ensuring that the lipid content of the medium was negligible compared with the lipids extracted. However in these studies by Hofmann and co-workers the methods of analysis would not have detected the minor amounts of the less commonly encountered acids. With the present-day techniques of gas-liquid chromatography the fatty acid spectrum must be re-examined under conditions which free the spectrum from contamination, if the acids synthesised by the organism are to be determined.

Metabolism of a Long Chain Fatty Acid

In the experiment in which palmitic acid was added to the medium it was observed that most of the label remained in the free fatty acid pool, with some incorporation into what appeared to be glycolipid fractions (Figure 12). By washing the cells twice in an alkaline phosphate solution (page 95),

it was hoped that fatty acids adhering to the cell wall could be removed, but in spite of this precaution it was impossible to be certain that the free fatty acids constituted a true intra-cellular pool of lipid. From the intensity of the free fatty acid spot on the charred thin-layer chromatoplate (Figures 8 and 10), it appeared that regardless of whether the pool was intra-cellular or adhered to the cell wall, it constituted a major fraction of the lipid extracted. Consequently the pool probably had a relatively slow turnover and this contention is supported by the observation (Figure 12) that most of the label from C^{14} - palmitate remained as free fatty acids. Since it is unlikely that a radioactive fatty acid can be incorporated into cellular lipids without going through this pool, it would seem logical to investigate methods for increasing the turnover rate, if a more effective incorporation of a long chain fatty acid is desired.

In the present study the large size of this pool may have been due to the presence of relatively large amounts of lipid in the organic constituents of the medium. Thus the use of ether-extracted casein hydrolysate and yeast extract would not only avoid many of the problems encountered in this study but might also increase the turnover rate of the free fatty acid pool.

Further studies on the lipid metabolism of S. bovis could then investigate the incorporation of fatty acids into lipid fractions during growth and resting phase separately, and subsequently follow the turnover of the lipid pools. Toennies, Shockman and Kolb (1963), Ibbott and Abrams (1964) and Vorbeck and Marinetti (1965 b) found the majority of the lipid in S. faecalis was present in the cell membrane but details of the metabolism of these lipids and their function in membrane phenomena of the cell remain to be investigated. While it is perhaps unlikely that S. bovis will degrade fatty acids to release energy during resting phase it is almost certain that the integrity of the cell membrane is essential to the viability of S. bovis.

SUMMARY

1. Growth of S. bovis occurred in media buffered with 0.15 M Phosphate. At 0.20 M Phosphate however, the lag phase of growth was prolonged while at 0.30 M Phosphate no growth occurred.
2. An enhanced cell yield of S. bovis occurred on the substitution of 0.10 M Phosphate + 0.10 M Bicarbonate for 0.15 M Phosphate.
3. The optical density of S. bovis cultures fell during resting phase in media where the pH was maintained above 6.0 by high buffer concentration. Adjustment of the buffer concentration, or the addition of acid to give a pH of less than 5.9 during resting phase, prevented the fall in optical density. Substitution of potassium ions for part of the sodium ion complement also favoured cell stability.
4. In media already containing a high concentration of buffer, the addition of 0.10 M Acetate appeared to cause a slight decrease in yield of S. bovis.
5. Cells of S. bovis, left suspended in the spent growth medium began to loose viability after approximately 13 hours of resting phase.
6. Lipids of S. bovis extracted with chloroform - methanol (2:1), varied between 0.8 % and 1.7 % of cell dry weight in media of different buffer composition. Although lipid synthesis from exogenous acetate was demonstrated, the addition of 0.10 M Acetate to the growth medium did not increase the yield of extractable lipid.
7. Two glycolipid fractions appeared to be present in the lipids of S. bovis.

8. The presence of a sterol in the lipids of S. bovis was attributed to its uptake from the Bacto-Tryptose used as nitrogen source in the growth medium.
9. C^{14} - palmitic acid was incorporated from the media into cellular lipids of S. bovis but most remained in the free fatty acid pool which seemed to constitute a large proportion of the total lipid.

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APPENDIX

MICROBIOLOGICAL MEDIA

- A. Glucose - Tryptose - Yeast Extract - Phosphate (GTYP) medium used for the daily subculture of S. bovis (Buffer; 0.15 M Phosphate).

	<u>Concentration</u>
Glucose	10.0 g/litre
Bacto - Tryptose (Difco)	14.0 " "
Bacto - Yeast Extract (Difco)	5.0 " "
Na_2HPO_4	13.0 " "
K_2HPO_4	8.0 " "

pH 6.9

Dispensed in 10 ml aliquots in subculture tubes and sterilised by autoclaving at 121°C for 15 minutes.

- B. Growth Media used for the study of lipids of S. bovis (Buffer; 0.10 M Phosphate + 0.10 M Acetate + 0.033 M Bicarbonate).

	<u>Concentration</u>
Glucose	10.0 g/litre
Bacto - Tryptose	14.0 " "
Bacto - Yeast Extract	5.0 " "
Na_2HPO_4	8.7 " "
KH_2PO_4	5.3 " "
Sodium acetate (anhyd)	8.2 " "
NaHCO_3	2.7 " "

Glucose and bicarbonate were each dissolved separately in 100 ml water and remaining constituents in 800 ml water. All solutions were sterilised by autoclaving at 121°C for 15 minutes. The glucose and bicarbonate solutions were combined with the bulk solution immediately prior to inoculation. pH of entire solution was approximately 7.1 at inoculation but thereafter rose slowly to pH 7.5 as carbon dioxide was evolved before growth of the culture reached logarithmic phase.

Flasks were incubated at 37°C.

- C. Glucose - Tryptose - Yeast Extract - Phosphate - Agar medium for growth of S. bovis on plates for viable counts (after MacPherson 1953).

	<u>Concentration</u>	
Bacto-Tryptose (Difco)	10	g/litre
Bacto-Yeast Extract (Difco)	10	" "
KH ₂ PO ₄	1	" "
Agar (Davis)	20	" "

Glucose was made up as a 5.0 % (w/v) solution and sterilised by autoclaving at 121°C for 15 minutes. Remaining constituents were made up in a separate solution, steamed to dissolve the agar, dispensed into 70 ml aliquots in Erlenmeyer flasks and sterilised by autoclaving as above.

Before pouring the plates, 10 ml of the 5.0 % glucose solution was added each flask of molten agar with aseptic precautions and gently swirled to mix the solutions. Five plates were poured, one after another, from each flask of agar.