BRANCHED LONG CHAIN FATTY ACIDS IN BACTERIA FROM THE BOVINE RUMEN

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by

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TABLE OF CONTENTS

Chapter Page

3.3. Gas-liquid Chromatography.

3.4. Radiochemical Methods.

4 RESULTS

LIST OF TABLES

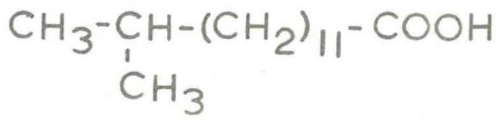
LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1. The Nomenclature of Branched Chain Fatty Acids

Fatty acids containing a branched carbon skeleton have been isolated from different sources, and in most cases the branch chain consists of a single methyl group. The branched chain fatty acids have been named according to two different conventions. According to one system the branched chain fatty acid is regarded as having a straight chain with a methyl side group on one of the carbon atoms and the name of the acid is derived from that of the straight chain acid to which the methyl group is attached. For example, a branched chain fatty acid containing C_{15} carbon atoms, with a methyl group on carbon atom 13, is known as 13-methyltetradecanoic acid (Figure 1).

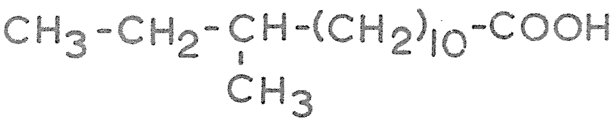


$$CH_3-\underset{\displaystyle CH_3}{\underset{|}{C}}H-(CH_2)_{11}-COOH$$

13-methyltetradecanoic acid
(iso-pentadecanoic acid)

Figure 1

The second system of naming branched chain fatty acids is applicable only in the case of fatty acids having the methyl side chain on the penultimate, or ante-penultimate carbon atom. Branched chain fatty acids having a methyl group on the penultimate carbon atom belong to the iso fatty acid series. For example, 13-methyltetradecanoic acid is also termed *iso*pentadecanoic acid (Figure 1). If the methyl group is on the ante-penultimate carbon atom the fatty acid belongs to the (+)-*anteiso* fatty acid series. An example of this series of acids is (+)-12-methyltetradecanoic acid, or (+)-*anteiso*pentadecanoic acid (Figure 2).



$CH_3-CH_2-\underset{CH_3}{\underset{|}{C}}H-(CH_2)_{10}-COOH$

12-(+)-methyltetradecanoic acid
(ante-isopentadecanoic acid)

Figure 2

Branched chain fatty acids of the (+)-*anteiso* series will exhibit optical rotation due to the stereochemical configuration of the methyl side chain about the ante-penultimate carbon atom. The direction of rotation is indicated by the insertion of the appropriate sign (+ or -) before the name of the fatty acid.

In this work the second system of nomenclature has been adhered to wherever possible, in order to show the relationship between fatty acids of the same series.

1.2. The Occurrence of Branched Chain Fatty Acids in Naturally Occurring Lipids

The earliest reported discovery of a branched chain fatty acid in a naturally occurring lipid was made by Chevreul who, in 1823, reported the isolation of a volatile fatty acid with a branched chain structure from the head and body oil of dolphins. The fatty acid was identified as *iso*valeric

TABLE 1

(Shorland and Hansen, 1957)

Branched chain fatty acids in cows' milk fat

Carbon Number of Fatty Acid	Name of Fatty Acid	Estimated per cent of Total Fatty Acids
C_{13} iso	11-methyldodecanoic acid	0.05
C_{13} anteiso	(+)-10-methyldodecanoic acid	0.01
C_{14} iso	12-methyltridecanoic acid	0.05
C_{15} iso	13-methyltetradecanoic acid	0.37
C_{15} anteiso	(+)-12-methyltetradecanoic acid	0.43
C_{17} iso	15-methylhexadecanoic acid	trace
C_{17} anteiso	(+)-14-methylhexadecanoic acid	0.41
C_{18} branched	-	trace
C_{20} multi-branched	-	trace

acid by Gill and Tucker (1930). This has since been proved to be an exceptional case, remaining the only known instance in which a branched chain fatty acid is a major constituent of a triglyceride from depot fat.

Branched chain fatty acids have been isolated from extracts of the tubercule and leprosy bacteria (Anderson and Chargaff, 1929; Anderson, 1932). The fatty acids were isolated and shown to possess more than one terminal methyl group, which indicated their branched chain structure. Later studies of this fatty acid fraction showed that the acids concerned were 10-methyloctadecanoic acid (10-methylstearic acid) and the multi-branched chain fatty acid, 3,13,19-trimethyltricosanoic acid (Ginger and Anderson, 1944). These acids were not present as components of triglycerides but were found in the unsaponifiable material, presumably esterified to alcohols to form waxes.

The waxes of wool grease have been found to contain appreciable amounts of branched chain fatty acids of both the iso and anteiso series (Weitkamp, 1945). Branched chain fatty acids have been isolated from the coccygeal glands of ducks and geese (Weitzel and Lennert, 1951; Weitzel et al., 1952a, b; Murray, 1962). Murray (1962) has determined the structure of branched chain fatty acids from this source and found that the two main fatty acids of the waxes were 2,4,6,8-tetramethyldecanoic acid, and 2,4,6,8-tetramethylundecanoic acid.

With the exception of isovaleric acid from dolphin triglycerides, all of the preceding reports concerned with the occurrence of branched chain fatty acids indicate their presence in the non-glyceride fraction of lipid extracts.

In a review of studies made on the fatty acids from butterfat, Shorland and Hansen (1957) reported that branched chain fatty acids were present in small proportions. Table 1 summarises their results.

Shorland and Hansen (1957) maintained that, in butterfat, the branched chain fatty acids occurred in the neutral glyceride fraction rather than as wax esters similar to those found in wool wax. The total branched chain fatty acids of butterfat comprised about 2 per cent of the total fatty acids, while the unsaponifiable material (mainly cholesterol) amounted to only 0.65 per cent of the total weight (Morice, 1951). Consequently the amount of branched chain fatty acids is greater than that which could be held in combination by the high molecular weight unsaponifiable material present, and thus the fatty acids must be present as glycerol esters. Branched chain fatty acids were first isolated from butterfat by procedures which included hydrogenation (Hansen and Shorland, 1951) but later investigations using extraction procedures which did not involve hydrogenation showed that branched chain fatty acids were only present in a saturated form (Shorland *et al*., 1955).

Hansen *et al*. (1952a) have reported the presence of branched chain fatty acids in ox perinephric fat. Both *iso* and *anteiso* C_{17} carbon chain fatty acids were present in trace amounts, as was the *iso* C_{16} carbon fatty acid. These workers also examined the fatty acids in mutton tallow and found evidence for the presence of *iso*pentadecanoic acid, *iso*heptadecanoic acid, and *anteiso*pentadecanoic acid in trace amounts (Hansen *et al*., 1952a, b). An examination of the milk fat of ewes by Gerson *et al*. (unpublished data quoted by Shorland, 1957) showed the presence of trace amounts of fatty acids of the *anteiso* series containing an odd number of carbon atoms. These fatty acids ranged from C_{13} to C_{17} carbon atoms in chain length.

Branched chain fatty acids have been isolated from shark liver oil by Morice (1952). She demonstrated the presence of traces of a multi-branched C_{18} carbon chain fatty acid in oil from this source. Unpublished work by Morice (quoted by Shorland, 1956) indicated that small amounts - less than 0.05 per cent - of each of the following branched chain fatty acids were found in the same sample of shark liver oil:

(a) iso branched chain series.

13-methyltetradecanoic acid.

15-methylhexadecanoic acid.

(b) anteiso branched chain series.

(+)-14-methylhexadecanoic acid.

The presence of branched chain fatty acids in the milk fat of goats has been reported by James and Martin (1956). Using Gas-liquid Chromatography (G.L.C.), these workers tentatively identified branched chain fatty acids containing C_8 to C_{18} carbon atoms from this source. These fatty acids were not positively identified, and iso and anteiso fatty acids containing the same number of carbon atoms were not resolved on the column used. The proportions of the branched chain fatty acids present in the milk fat were not reported, but observations on the chromatograms published by these authors suggests that they were only present in trace amounts. Human milk fat has been found to contain traces of branched chain fatty acids with a chain length of from C_{13} to C_{17} carbon atoms (Insull and Ahrens, 1959).

The sebum from several species of mammals (human, rat, mouse, rabbit and guinea pig) has been shown to contain branched chain fatty acids of both the iso, anteiso and multi-branched type (James and Wheatley, 1957; Wheatley and James, 1957).

The occurrence of branched chain fatty acids, and fatty acids containing an odd number of carbon atoms, in the body fat of rats fed a diet containing bovine milk fat has been reported (Bhalerao et al., 1961). However, body fats from rats fed a diet containing no milk or milk products contained none of these components. This work was interpreted as suggesting that branched chain fatty acids and fatty acids with an odd number of carbon atoms were not synthesized by the rat, but arose from a dietary source. Livingston et al. (1957) found that 10 per cent of 14-methylhexadecanoic acid fed to rats on an otherwise fat-free diet was deposited in the depot fat.

The phospholipids of a species of bacteria of the genus *Sarcina* have been found to contain a C_{15} carbon atom branched chain fatty acid (Akashi and Saito, 1960). This fatty acid has been tentatively identified as (+)-12-methyltetradecanoic acid and constitutes the sole fatty acid component of the phosphatidic acid isolated from this organism. The acetone soluble lipids of *Sarcina* were also found to contain a large proportion of this fatty acid. These workers have also reported the presence of branched chain fatty acids of the *iso* series in *Bacillus subtilis* sub sp. *natto* (Saito, 1960) A study of the fatty acid composition of *B. subtilis* (strain ATCC 7059) has been made by Kaneeda (1963a), who found that the predominant fatty acids isolated from this organism were the (+)-*anteiso* fatty acids, (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid.

Macfarlane (1961) has found that the neutral lipid from the membranes and whole cells of *Micrococcus lysodeikticus* was a diglyceride which contained branched chain fatty acids. Of the total fatty acids, 77 per cent was a branched chain fatty acid containing C_{15} carbon atoms. The fatty acids from the membrane phospholipids were also predominantly (80-90 per cent) fatty acids having a branched chain structure, of which (+)-12-methyltetradecanoic acid was the most abundant. Further work has shown that a branched chain fatty acid containing C_{15} carbon atoms comprised 42 per cent of the total fatty acids of *Micrococcus lysodeikticus* (Lennarz, 1961), while a branched chain fatty acid containing C_{17} carbon atoms comprised a further 5 per cent of the total fatty acids.

Lipid extracts from the bacterial fraction of the rumen microbial population of a lactating cow have been found to contain a large proportion of branched chain fatty acids (Keeney *et al.*, 1962). Branched chain fatty acids were also found, but to a far lesser extent, in the lipid extracts

TABLE 2

The relative percentage composition of the fatty acids of the lipid fractions from mixed rumen bacteria, mixed rumen protozoa and of the rumen organism *Ruminococcus flavefaciens*. (Keeney *et al.*, 1962)

Fatty Acid*	RUMEN BACTERIA Neutral Lipid	Free Fatty Acids	Polar Lipid	RUMEN PROTOZOA Neutral Lipid	Free Fatty Acids	Polar Lipid	*R. FLAVEFACIENS* Total Lipid
12:0	0.6	tr	1.5	-	-	-	0.7
br.13:0	tr	tr	1.2	-	-	-	1.5
13:0	tr	tr	0.7	-	-	0.7	tr
br.14:0	0.7	tr	1.3	-	-	-	2.0
14:0	2.0	0.9	3.8	1.0	tr	1.6	2.5
br.15:0	6.7	1.9	20.3	0.5	-	3.7	43.7
15:0	4.5	1.2	8.2	1.1	0.6	2.0	4.3
br.16:0	2.9	-	1.6	2.2	tr	1.1	9.3
16:0	26.1	16.9	30.6	26.5	14.2	37.5	19.0
16:1	1.5	0.8	1.3	0.7	-	1.2	3.4
br.17:0	1.5	0.5	1.2	1.2	0.6	2.8	
17:0	0.4	1.9	0.8	tr	1.3	0.8	1.8
18:0	10.7	58.9	6.5	12.3	68.3	10.3	4.1
18:1	16.2	12.5	10.2	17.1	10.0	20.3	6.9
18:2	18.1	4.3	10.7	27.5	4.9	14.6	0.8
18:3	-	-	-	-	-	-	-
18:4 or 20:1	7.9	-	-	9.7	-	3.2	-

* The shorthand notation used for the fatty acids is that used by James (1960). The figure preceding the colon denotes the number of carbon atoms in the fatty acid, while the figure following denotes the number of double bonds. br. or cy. indicate branched chain or cyclopropane fatty acids.

from a fraction comprising mainly rumen protozoa. The authors suggested that the latter fraction was unlikely to be purely protozoa, and was probably contaminated with bacteria which had adhered to the small food particles which were present in the fraction. The lipid from these bacteria could contribute to the branched chain fatty acids found in the protozoal fraction. The results of these authors are summarised in Table 2.

Keeney *et al*. (1962) also found that branched chain fatty acids were present in the milk fat and blood serum lipids of the cow from which the rumen samples were obtained. The proportions of the branched chain fatty acids found in the milk fat corresponded to those published by Shorland (1957).

These authors (Keeney *et al*., 1962) also analysed the total lipid of the rumen cellulolytic anaerobe *Ruminococcus flavefaciens* and found that the branched chain fatty acids, particularly those containing C_{15} carbon atoms, comprised a large proportion of the total fatty acids (see Table 2). Further work on *R. flavefaciens* and on the related rumen organism, *Ruminococcus albus*, (Allison *et al*., 1962b) has revealed the presence of large proportions of branched chain fatty acids in both the total lipid and phospholipid fractions from these bacteria.

The presence of trace amounts of branched chain fatty acids of both the *iso* and *anteiso* series in human faecal lipid has been reported (James *et al*., 1961; Sammons, 1961). Sammons (1961) also reported that small quantities of branched chain fatty acids found in the faeces were not present in the ileal discharge of the same human subject, indicating that these fatty acids had arisen in the large intestine. This author suggested that branched chain fatty acids may have arisen from the bacteria in the large intestine, but he presented no direct evidence for this suggestion.

The presence of branched chain fatty acids in plant lipids has been reported (Shorland, 1961; Hansen and Cook, 1957). Hansen and Cook (1957) found that tall oil, produced in the manufacture of wood pulp from *Pinus radiata* contained trace amounts of (+)-14-methylhexadecanoic acid. Shorland (1961) analysed the fatty acids of the acetone soluble lipids of ryegrass and found traces of branched chain fatty acids containing C_{14}, C_{15} and C_{17} carbon atoms, but the presence of these branched chain fatty acids in the acetone insoluble lipids was not reported. This author's work has since been criticized (Allison *et al*., 1962b) on the grounds that the fatty acids were obtained after dialysis of the lipid extract through a rubber membrane. Quoting unpublished data of Katz and Keeney, Allison *et al*. (1962b) stated that branched chain fatty acids containing C_{15} and C_{17} carbon atoms were present in hexane extracts of rubber membranes.

1.3. The Volatile Fatty Acids as Growth Factors for some Rumen Bacteria

Bryant and Doetsch (1954) showed that the rumen bacteria *Bacteroides succinogenes* would not grow in a chemically defined medium unless rumen fluid was added. Further work (Bryant and Doetsch, 1955) revealed that the growth factor necessary for culture of this organism was in the volatile fatty acid fraction of the rumen fluid. Two groups of volatile fatty acids were isolated, one of which consisted of the straight chain fatty acids containing C_5 to C_8 carbon atoms, while the other group consisted of the branched chain fatty acids *iso*butyric acid, *iso*valeric acid, and 2-methylbutyric acid. One fatty acid from each group was required in the culture medium for optimum growth of *B. succinogenes*.

Ruminococcus flavefaciens and *Ruminococcus albus* have been found to have an absolute growth requirement for *iso*valeric acid, but they do not require a straight chain volatile fatty acid other than acetic acid (Allison, Bryant and Doetsch, 1958). The degradation of cellulose by rumen bacteria *in vitro* has been found to be increased by the presence of volatile fatty acids of both branched and straight chain structure (Bentley *et al.*, 1955). Bryant and Robinson (1962) have studied the growth of a large number of strains of rumen bacteria in chemically defined media and they found that many strains, including some bacteria which were not cellulose digesting, had a requirement for growth of one or more of the branched chain volatile fatty acids.

The metabolic fate of the branched chain volatile fatty acids taken up by *R. flavefaciens* has been studied by Allison *et al.* (1962a, b; 1963). They found that *iso*valerate was incorporated into branched chain fatty acids containing C_{15} and C_{17} carbon atoms, and also into the amino acid leucine. These authors are of the opinion that because of the unique rumen environment, which has a low level of free amino acids and a high level of ammonia, there is a tendency towards selection of those organisms which are capable of synthesizing many or all of their amino acids from ammonia. It has been shown that many rumen bacteria require ammonia, even when grown in a medium containing a high level of amino acids (Bryant and Robinson, 1962)

Allison and Bryant (1963) suggested that *R. flavefaciens* required branched chain fatty acids because the mechanism for the biosynthesis of the *iso*propyl moiety was lacking or inadequate.

Dehority *et al.* (1958) showed that *in vitro* digestion of cellulose by mixed rumen cultures was stimulated by the branched chain amino acids leucine, valine, and *iso*leucine, as well as by the branched chain volatile fatty acids *iso*butyric acid, *iso*valeric acid and 2-methylbutyric acid.

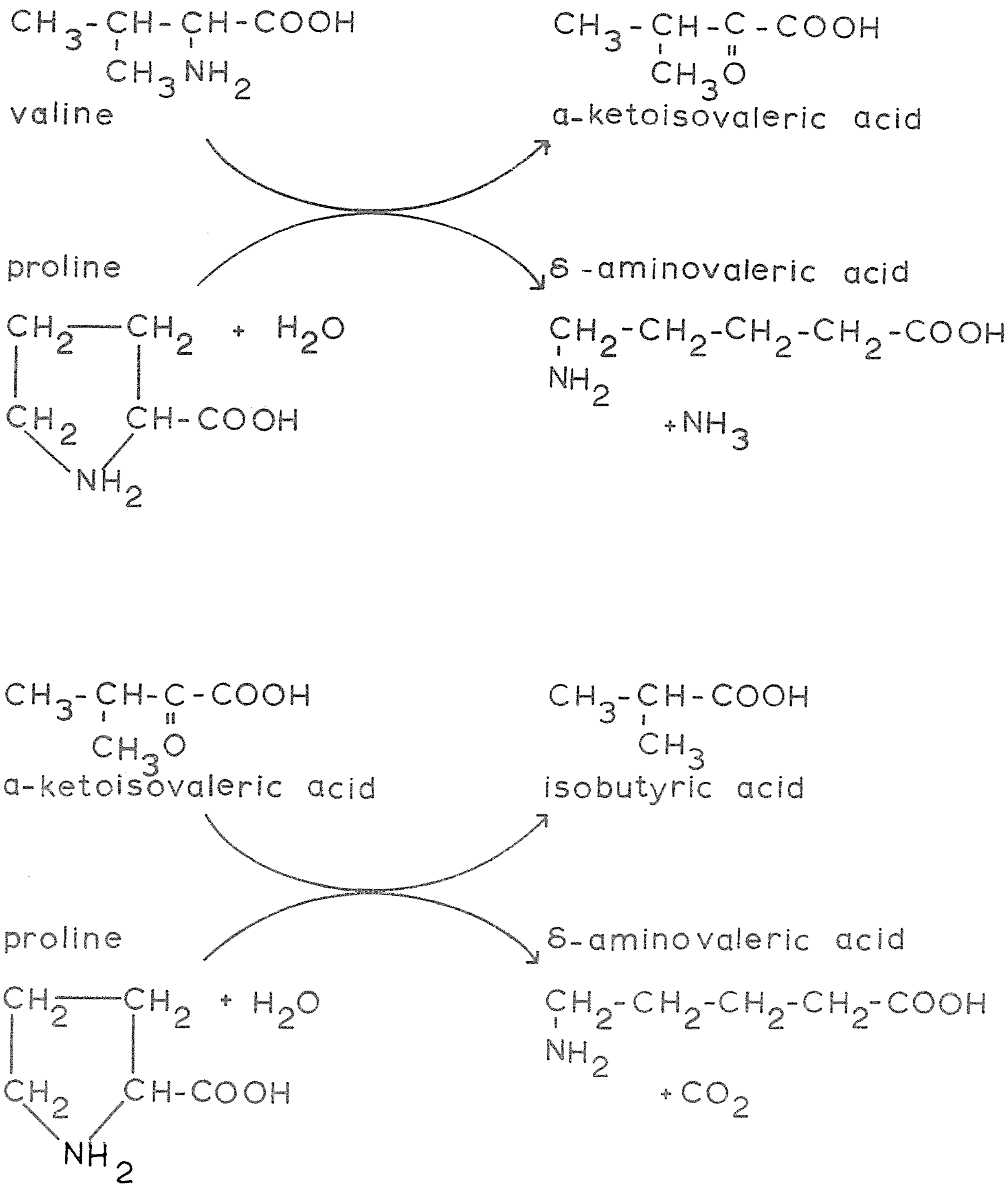
1.4. The Biosynthesis by Bacteria of Branched Chain Volatile Fatty Acids from Branched Chain Amino Acid Precursors

The presence of branched chain volatile fatty acids in the rumen has been reported by a number of workers (Grey *et al.*, 1952; el Shazly, 1952a; Annison, 1954) and their formation by the microbial degradation of amino acids has been demonstrated (el Shazly, 1952b). El Shazly (1952b) has postulated that the reactions involved could be the mutual oxidation-reduction reactions first demonstrated by Stickland (1934, 1935). Stickland found that anaerobic micro-organisms of the *Clostridium* family of bacteria contained special pathways for the deamination of amino acids. Suspensions of these bacteria did not produce ammonia when any single amino acid was added to the culture medium, but some amino acids were deaminated if an organic reducing dye was also present in the medium. Other amino acids in the culture medium were deaminated in the presence of organic oxidizing dyes.

The protein amino acids have been classified into two groups on the basis of their behaviour in this "Stickland reaction", i.e. those that act as reducing agents form one group while those which act as oxidizing agents fall into a second group. Stickland (1934) found that alanine, leucine, *iso*leucine and valine belong to the first group, while glycine, proline and hydroxyproline are members of the second group. When one amino acid from each group was added to the suspension of *Clostridium* cells there was an intermolecular oxidation-reduction reaction with the concomitant production of ammonia.

El Shazly (1952b) has suggested that the volatile fatty acids *iso*butyric acid, *iso*valeric acid and 2-methylbutyric acid could be derived from the amino acids valine, leucine and *iso*leucine by way of reactions of the type postulated by Stickland. He found that if hydrolysed casein was added to a mixed suspension of rumen bacteria, there was an increase in the proportions of the branched chain volatile fatty acids present. The amino acid proline disappeared from the culture medium during the period of incubation and a new amino acid, δaminovaleric acid, appeared. This evidence suggested that the branched chain amino acids acted as hydrogen donors and the amino acid proline was the hydrogen acceptor in these reactions (see Figure 3). Presumably amino acids other than proline could function as hydrogen acceptors in the rumen, e.g. glycine.

FIGURE 3



The oxidative deamination and decarboxylation of valine to isobutyric acid by the "Stickland" reaction.

The results of Dehority *et al*. (1958) supported those of el Shazly and showed that when mixed suspensions of rumen bacteria were incubated in a medium containing one of the branched chain amino acids, together with proline, there was an increase in the proportion of the corresponding volatile fatty acid in the medium. The amino acid δaminovaleric acid, initially absent from the culture, was present at the end of incubation. The addition of valine-1-C^{14} to the culture medium resulted in a large proportion of the radioactivity being recovered as $C^{14}O_2$, as suggested by the pathways shown (Figure 3). By slowing the rate of reaction, Dehority *et al*. (1958) were able to show the presence of α-ketoisovalerate-1-C^{14} as an intermediate in the formation of *iso*butyric acid from valine-1-C^{14}.

An amino acid catabolising strain of *Bacteroides ruminicola* has been isolated and shown to produce *iso*valerate-1-C^{14} from leucine-2-C^{14} (Bladen, Bryant and Doetsch, 1961). To date this is the only rumen organism isolated in a pure culture which has been shown to be capable of forming branched chain volatile fatty acids in this way.

1.5. The Biosynthesis of Branched Chain Fatty Acids in Animal Tissues

The discovery of small proportions of branched chain fatty acids and fatty acids with an odd number of carbon atoms in the milk and body fat of certain animals, especially ruminants, has raised the question of their biological origin. As the configuration of the carbon skeleton of these fatty acids resembles that of the branched chain volatile fatty acids found in the rumen, el Shazly (1952b) has suggested that branched long chain fatty acids arise by condensation of several molecules of acetate with one molecule of the appropriate branched chain volatile fatty acid precursor. A number of workers have attempted to demonstrate, in animal tissues, the synthesis of these long chain fatty acids from branched chain volatile fatty acids, and also from fatty acids with an odd number of carbon atoms (Gerson *et al*., 1960; Verbeke *et al*., 1959; Horning *et al*., 1961).

Gerson et al. (1960) injected carboxyl C^{14} labelled n-valeric acid into the jugular vein of a lactating cow and examined the radioactivity which occurred in the fatty acids of the milk. They found that the distribution of the C^{14} label in the fatty acid molecules was consistent with the breakdown of n-valeric acid to propionate and acetate and the subsequent incorporation of these breakdown products into the long chain fatty acids of the milk fat. The incorporation of propionate from the blood into the fatty acids of milk fat has been demonstrated by James et al. (1960), but Gerson et al. (1960) could find no evidence to indicate the direct incorporation of unchanged n-valeric acid into long chain fatty acids containing an odd number of carbon atoms.

The incorporation of carboxyl C^{14} labelled isovaleric acid into the long chain fatty acids of milk fat has been studied by Verbeke et al. (1959). In his experiments one half of an isolated cow's udder was perfused with blood containing isovalerate-1-C^{14} while the other half udder was perfused with blood containing leucine-U-C^{14}. In the half udder perfused with leucine-1-C^{14} the radioactivity was recovered mainly from the protein of the milk while very little radioactive carbon dioxide was recovered, suggesting that leucine was not metabolised to any great extent in the udder tissue.

When isotopic isovaleric acid was perfused through the other half of the udder a measurable proportion of the added radioactivity (6 per cent) was recovered as $C^{14}O_2$, which suggested that isovaleric acid was metabolised to some extent by the udder tissue. The pattern of radioactivity in the molecules of the fatty acids of the milk and of the fat of the udder tissues indicated that isovaleric acid was not incorporated specifically into the branched chain fatty acids, but was broken down into a radioactive two-carbon unit which was presumed to be acetic acid. This two-carbon unit was then incorporated universally into the fatty acids of the milk fat. The breakdown of *iso*valerate to acetate and aceto-acetate has been demonstrated in rat liver slices and in intact rats (Coon, 1950).

The *in vitro* synthesis of branched long chain fatty acids, and of long chain fatty acids with an odd number of carbon atoms, has been demonstrated (Horning *et al*., 1961). The precursors were shown to be the Coenzyme A derivatives of volatile fatty acids containing either an odd number of carbon atoms or a branched chain structure.

These authors used a partially purified enzyme system from rat epididymal adipose tissue and the mechanism postulated involved the addition of malonyl Coenzyme A units to the appropriate Coenzyme A derivative of the precursor involved. Horning *et al*. (1961) accounted for the very low proportions of branched chain fatty acids in mammalian tissue by suggesting that there was probably a correspondingly low concentration of the appropriate branched chain acyl Coenzyme A precursors available to the enzyme system. Keeney *et al*. (1962) are of the opinion that the biosynthesis of branched chain fatty acids in animal tissues is not of great importance and state that "It remains to be demonstrated *in vivo* that the appropriate short chain odd and branched chain Coenzyme A esters become available to the Horning enzyme system."

It is probable that propionate could be incorporated by animal tissues into straight chained fatty acids containing an odd number of carbon atoms. James *et al*. (1960) found that, on injection of radioactive propionate into the jugular vein of a lactating cow the activity was located specifically in the fatty acids of milk fat which had an odd number of carbon atoms.

However, the results of Gerson, Verbeke and Horning cast doubts upon the biosynthesis in animal tissues of fatty acids containing a branched carbon skeleton. Thus, a site other than in animal tissues must be found for the biosynthesis of branched long chain fatty acids.

1.6. The Biosynthesis of Branched Chain Fatty Acids by Bacteria

Branched chain fatty acids of the *iso* and *anteiso* series have been isolated from milk and depot fats of cattle and sheep, and from the wool wax of sheep, and it has been suggested that these fatty acids might have arisen from the micro-organisms inhabiting the digestive tracts of these animals (Keeney *et al.*, 1962).

Traces of branched chain fatty acids in the milk and depot fats of humans, and other non-ruminant animals, could have originated from branched chain fatty acids present in the diet when it included fat of ruminant origin. Bhalerao *et al.* (1961) and Livingston (1959) have demonstrated that when rats are fed a diet containing branched chain fatty acids, then these acids are found in the depot fat.

The theory of the bacterial biosynthesis of branched chain fatty acids has been supported by the discovery that a large proportion of the fatty acids from several species of bacteria have a branched chain structure (Saito, 1961; Akachi and Saito, 1961; Macfarlane, 1961; Lennarz, 1961; Kaneeda, 1963a; Allison *et al.*, 1962b), and that branched chain fatty acids also constitute a large proportion of the fatty acids in the lipid extract from mixed rumen bacteria (Keeney *et al.*, 1962). These findings have led to a considerable volume of work on individual bacterial species to determine the role of branched chain volatile fatty acids in the biosynthesis of branched long chain fatty acids of the bacteria.

Wegner and Foster (1961) reported that *iso*butyrate-1-C^{14} and *iso*valerate-1-C^{14} were incorporated into an uncharacterized fatty acid from the rumen cellulolytic organism *Bacteroides succinogenes*. The incorporation of 2-methylbutyrate-U-C^{14} and *iso*leucine-U-C^{14} into the branched chain fatty acids of *Micrococcus lysodeikticus* has been followed by Lennarz (1960) and the radioactivity from both of these metabolites was incorporated specifically into the branched chain fatty acids containing

C_{15} and C_{17} carbon atoms.

Ruminococcus flavefaciens, a rumen cellulolytic bacterium with an obligate growth requirement for either *iso*butyric or *iso*valeric acid, has been found to incorporate radioactivity from *iso*valerate-1-C^{14} approximately equally into the cellular protein (as the amino acid leucine) and into the cellular lipid fraction (Allison *et al.*, 1962a, b). In the latter fraction, 74 per cent of the radioactivity was present in a branched chain fatty acid containing C_{15} carbon atoms, and 13 per cent in a similar fatty acid containing C_{17} carbon atoms. The phospholipids were obtained from the total lipid extract of *R. flavefaciens* by precipitation with acetone and about 63 per cent of the total radioactivity was found in this fraction. The total lipid and phospholipid fractions contained similar proportions of branched chain fatty acids.

Allison *et al.* (1961) also noted the presence of long chain fatty aldehydes in the phospholipids of *R. flavefaciens* and suggested that they were probably present in plasmalogens. A proportion of the aldehydes present had a branched chain structure similar to that of the branched chain fatty acids and those aldehydes with a branched chain containing C_{15} and C_{17} carbon atoms contained more isotopic carbon than those of a straight chain configuration. This suggested that *iso*valerate was a precursor of branched chain fatty aldehydes as well as of branched chain fatty acids in this organism.

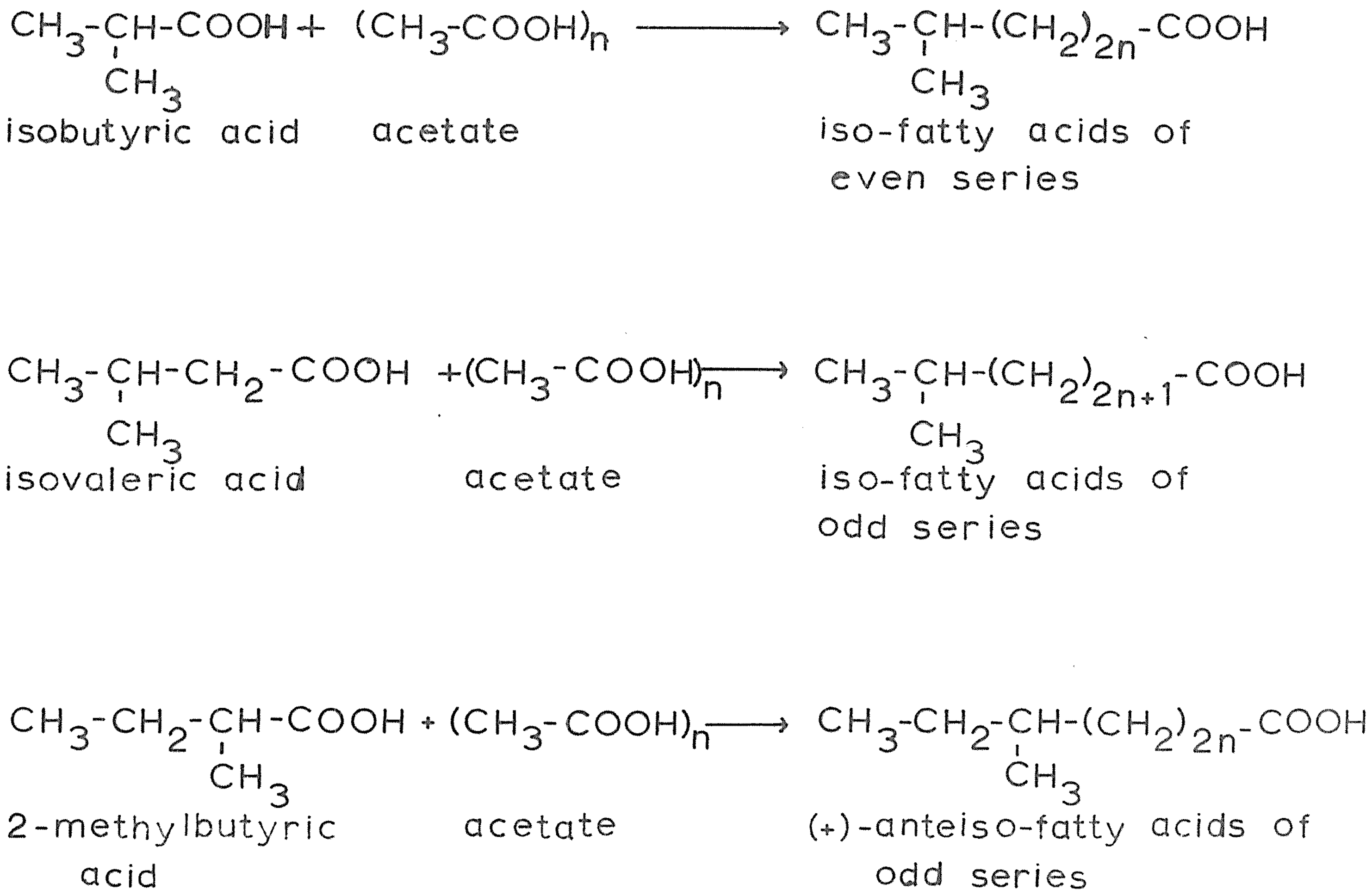
Allison *et al.* (1962b) has studied the incorporation of *iso*butyrate-1-C^{14} into the cellular lipid of *Ruminococcus albus*. These workers found that this branched chain volatile fatty acid was incorporated specifically into the branched long chain fatty acids containing C_{14} and C_{16} carbon atoms. In this experiment very little of the *iso*butyrate-1-C^{14} was incorporated into the protein fraction of the bacteria, while 96 per cent of the incorporated radioactivity was recovered in the lipid fraction.

The incorporation of branched chain volatile fatty acids into the long chain fatty acids of Bacillus subtilis has been studied by Kaneeda (1963b). Addition of the volatile fatty acids isobutyrate, isovalerate and 2-methylbutyrate individually to the culture medium resulted in an increase in the corresponding branched long chain fatty acids (Figure 4). When C^{14} labelled branched chain volatile fatty acids were added to the culture medium, the isotopic labelling pattern found in the newly synthesized fatty acids was also in agreement with this scheme. Growth of B. subtilis in the presence of valine-U-C^{14} resulted in the incorporation of some of the label into the iso branched chain fatty acids containing C_{14} and C_{16} carbon atoms. However, when valine-1-C^{14} was substituted for valine-U-C^{14} in a similar experiment, no radioactivity was found in any of the fatty acids. This is further evidence that the carboxyl carbon atom of valine is lost during the conversion of valine to isobutyrate.

A more detailed study has been made (Kaneeda, 1963c) of the pattern of radioactivity in the fatty acids of B. subtilis grown in the presence of valine-U-C^{14}, and the results have indicated that, while most of the radioactivity was found in the iso fatty acids containing C_{14} and C_{16} carbon atoms (as would be expected according to Figure 4), there was also a significant incorporation of the isotopic label into the iso C_{15} and iso C_{17} carbon chain fatty acids.

The work quoted in this section supports the theory that the branched chain fatty acids in bacteria are formed by condensation of several two-carbon units with a branched chain precursor such as isobutyrate or isovalerate. In this scheme, isobutyrate is incorporated into the branched chain fatty acids of the iso configuration containing an even number of carbon atoms. Similarly, isovalerate is incorporated into iso fatty acids with an odd number of carbon atoms, while the anteiso volatile fatty acid, 2-methylbutyrate, would be the precursor of the anteiso fatty acids with

FIGURE 4



$$CH_3\text{-}\underset{CH_3}{CH}\text{-}COOH + (CH_3\text{-}COOH)_n \longrightarrow CH_3\text{-}\underset{CH_3}{CH}\text{-}(CH_2)_{2n}\text{-}COOH$$

$$CH_3\text{-}\underset{CH_3}{CH}\text{-}CH_2\text{-}COOH + (CH_3\text{-}COOH)_n \longrightarrow CH_3\text{-}\underset{CH_3}{CH}\text{-}(CH_2)_{2n+1}\text{-}COOH$$

$$CH_3\text{-}CH_2\text{-}\underset{CH_3}{CH}\text{-}COOH + (CH_3\text{-}COOH)_n \longrightarrow CH_3\text{-}CH_2\text{-}\underset{CH_3}{CH}\text{-}(CH_2)_{2n}\text{-}COOH$$

The pathways of formation of the branched chain fatty acids of the iso and anteiso series (Shorland, 1957).

an odd number of carbon atoms (Shorland, 1956) (see Figure 4).

It is interesting to note that there are no naturally occurring *anteiso* branched chain fatty acids with an even number of carbon atoms. The probable reason for this is that there is no naturally occurring amino acid which can undergo a Stickland reaction to form a volatile fatty acid with an *anteiso* carbon skeleton and an even number of carbon atoms.

The bacteria which are able to synthesize branched chain fatty acids appear to fall into two groups. The first group consists of bacteria, such as *Bacillus subtilis* and *Micrococcus lysodeikticus*, which can produce branched long chain fatty acids from both the branched chain volatile fatty acids and from the branched amino acid precursors. The second group consists of bacteria, so far found only in the rumen, which have an obligate growth requirement for the branched chain volatile fatty acids and cannot synthesize branched long chain fatty acids from branched chain amino acid precursors.

1.7. The Transfer of Branched Long Chain Fatty Acids of Rumen Bacteria to the Milk and Depot Fat of Ruminants

Many bacteria in the rumen perish and are broken down by the bacterial and protozoal enzymes, which include lipolytic enzymes, capable of hydrolysing triglycerides (Garton *et al.*, 1958) and phospholipids (Dawson, 1959). Thus, it is probable that some of the branched long chain fatty acids would be released into the rumen content as free fatty acids. The presence of branched chain fatty acids in the extracellular rumen fluid has been reported (Hawke and Robertson, 1964).

McCarthy (1962) has demonstrated the absorption of long chain fatty acids directly from the rumen into the blood of the ruminal vein, but the physiological importance of this is not clear. Probably the great majority of bacteria in the rumen pass unchanged into the abomasum and then into the small intestine. Here the bacterial cells are broken down by the intestinal and pancreatic enzymes, which include lipolytic enzymes (Keller *et al*., 1958).

Following digestion, the long chain fatty acids are absorbed into the blood of the ruminant, where Keeney *et al*. (1962) have demonstrated the presence of branched long chain fatty acids. Presumably these acids are present in the blood in a form which is available for uptake into the depot and milk fats of the animal. The direct incorporation of long chain fatty acids from the blood into the fatty acids of milk has been demonstrated by a number of workers (see Garton, 1963). This includes those long chain fatty acids present in the blood plasma as free fatty acids, as glycerides and as cholesteral esters.

1.8. The Classification of *Streptococcus bovis* and its Importance in the Rumen

Streptococcus bovis belongs to the genus *Streptococcus* of the tribe *Streptococcaceae* within the family *Lactobacteriaceae* which belong to the sub-order Eubacteriineae. This sub-order is the major sub-order of the order Eubacterials (Bergey's Manual of Determinative Bacteriology, 1948).

Although most rumen bacteria are strict anaerobes, certain groups of facultatively anaerobic bacteria are always present in the rumen. Streptococci, especially *S. bovis*, are generally the most numerous facultatively anaerobic bacteria cultivated from the rumen (Hungate, Bryant and Mah, 1964) but they are usually outnumbered by the anaerobic non spore-forming rumen bacteria, regardless of the diet of the ruminant (Bryant, 1959; Bryant *et al*., 1961).

S. bovis is an amylolytic rumen bacteria and is involved in the fermentation of starch and soluble carbohydrates. However, Bryant (1959) stated that, on the basis of numbers of this organism found in the rumen, it is probable that under most conditions other groups of amylolytic bacteria, particularly various non spore-forming anaerobes, are of greater importance. Only in cases of acute indigestion, caused by heavy feeding of carbohydrates, is the proportion of S. bovis increased at the expense of the anaerobic bacteria (Hungate, 1952).

Hungate, Bryant and Mah (1964) are of the opinion that a consistent prevalence of colonies of facultative anaerobes in cultures inoculated from the normal rumen indicates that the culture medium used does not provide the necessary conditions for growth of the obligate anaerobes.

Studies made on the nutritional requirements of S. bovis have shown that these requirements differ somewhat from those of other non-ruminal Streptococci. Wright (1960) has reported that S. bovis utilises exogenous carbon dioxide for the synthesis of aspartic acid in preference to incorporating this amino acid from the culture medium. The results of Bryant and Robinson (1961) indicate that the endogenous biosynthesis of amino acids in several strains of rumen bacteria is not greatly inhibited by an exogenous supply of amino acids. These results, together with the finding that the nitrogen requirements of S. bovis are less complex than those of other Streptococci (Wolin et al., 1959; Niven et al., 1948; Prescott and Stutts, 1955), suggest that the metabolism of this organism has been modified by the rumen environment. This is indicative of the position of S. bovis as a true rumen bacteria, even if it is not of great significance in the overall metabolism of the rumen.

1.9. The Fatty Acids of the Family Lactobacteriaceae

While the fatty acids of Streptococcus bovis have not been examined, several workers have reported the fatty acid spectra of a number of related Streptococci and Lactobacilli including L. arabinosis, L. casei and L. delbrukii (Hofman et al., 1952, 1953, 1957). All of these bacteria were found to contain a C_{19} cyclopropane fatty acid, lactobacillic acid (cis-11,12-methylene octadecanoic acid). The octadecenoic acid in these bacteria was found to be *cis*-vaccenic acid (*cis*-11-octadecenoic acid) and not oleic acid (*cis*-9-octadecenoic acid). In a study of the fatty acids of *L. casei*, *L. arabinosis* and *L. acidophilus*, Thorne and Kodicek (1962) found that the major fatty acid of these bacteria was a C_{19} cyclopropane acid, with smaller amounts of octadecenoic, hexadecenoic and palmitic acid.

The lipids of *Streptococcus cremoris* and *S. lactis* have been investigated by MacLeod and Brown (1963) and MacLeod *et al*. (1962). The fatty acid composition of these bacteria is very similar to that of the lactobacilli, with lactobacillic, octadecenoic, hexadecenoic, palmitic and myristic being the major fatty acids. An exception to this family similarity in fatty acid composition is *Streptococcus haemolyticus* (Hofman and Tausig, 1955) which has not been found to contain any lactobacillic acid.

The synthesis of *cis*-vaccenic acid has been suggested to occur by a process of chain elongation by the addition of two-carbon units to an unknown, unsaturated, short chain precursor (O'Leary and Hofman, 1957; Hofman *et al*., 1959). Evidence for this is that the position of the double bond in the unsaturated fatty acids of the lactobacilli is the same with respect to the methyl end of the fatty acid. Lengthening of an unsaturated short chain precursor would produce palmitoleic and *cis*-vaccenic acids, both of which are routinely found in lactobacilli.

Lactobacillic acid has been shown to be synthesized by the addition of a one-carbon fragment from methionine across the double bond of *cis*-vaccenic acid (Lui and Hofman, 1962; O'Leary, 1959; Hofman and Lui, 1960).

Chapter 2

THE OBJECT OF THE PRESENT WORK

The discovery of branched chain fatty acids in the milk and depot fat of ruminants (Shorland, 1957), together with the inability of the animal to synthesize these fatty acids (Verbeke *et al*., 1959; Gerson *et al*., 1960), has prompted the investigation of the fatty acids of several species of rumen bacteria (Allison *et al*., 1962b; Wegner and Foster, 1960). These studies have revealed that the fatty acids of certain rumen bacteria contain a large proportion of acids which possess a branched chain structure. However these investigations have all involved cellulolytic bacteria which have a growth requirement for branched chain volatile fatty acids (Bryant and Robinson, 1962).

Since a number of non-ruminal bacteria, which do not require volatile fatty acid growth factors, have also been shown to contain branched chain fatty acids (Lennarz, 1960; Saito, 1960; Akashi and Saito, 1960; Kaneeda, 1963a), it was decided that an investigation of the fatty acids of a non-cellulolytic rumen bacteria was warranted. *Streptococcus bovis* was chosen for this investigation because the conditions required for its culture are less stringent than those of other more anaerobic rumen bacteria, and because pure cultures of the organism were readily available.

The first section of the work was an investigation of the fatty acids of *S. bovis* with particular emphasis being placed on the possibility of branched chain fatty acids being present. Following this work an investigatio into the formation of branched long chain fatty acids by mixed rumen bacteria was undertaken. Branched chain fatty acids have been reported in mixed rumen bacteria (Keeney *et al*., 1960) and several workers have demonstrated their synthesis by rumen bacteria from branched chain volatile fatty acids

(Allison *et al*., 1962; Wegner and Foster, 1960). However there have not been any reports on this pathway of synthesis either *in vivo* or in mixed cultures of rumen bacteria. The second section of this investigation was undertaken to establish further evidence for the pathways of synthesis of branched long chain fatty acids in mixed cultures of rumen bacteria.

Chapter 3

MATERIALS AND METHODS

3.1. Microbiological Methods

3.1.1. Experiments with *Streptococcus bovis*

(a) Culture medium

Preliminary experiments were carried out to determine a suitable medium for the culture of *Streptococcus bovis* and the results are shown in Appendix I. The medium finally chosen was a modification of that used by Wright (1960) and had the composition shown below.

Composition of the medium used for the culture of *S. bovis*

Component	Concentration in gm./litre
Sucrose	20
Bactocasamino acids (Difco)	10
Bactoyeast extract (Difco)	5
Sodium acetate (hydrated)	49
Di-sodium hydrogen phosphate	2.2
Sodium thioglycollate	0.3
Magnesium sulphate (hydrated)	0.2
L-cysteine-HCl	0.15
L-tryptophan	0.15

Before sterilisation, the pH of the medium was adjusted to pH 7 with concentrated ammonia solution. Sterilisation was carried out by steaming in an a~~o~~utoclave for 20 minutes on each of three consecutive days so as to reduce the darkening of the medium which occurred on autoclaving.

(b) Source of *Streptococcus bovis*

Streptococcus bovis strain I (Bailey and Oxford, 1958) was obtained as a freeze-dried culture from Plant Chemistry Division, D.S.I.R., Palmerston North.

(c) Maintenance of stock cultures of *Streptococcus bovis*

The organism was maintained in agar stab cultures, in 20 ml. screw capped bottles and the medium used was that described above with the addition of 2 per cent of agar (N.Z. Davis) prior to sterilisation. After subculturing, which was carried out at weekly intervals, the cultures were incubated at 37°C. for 12 hours to initiate growth. They were then stored in the refrigerator at 4°C.

(d) Growth of *Streptococcus bovis* in large scale experiments

Several workers have shown that *Streptococcus bovis* requires a high concentration of carbon dioxide in the medium before maximum growth will occur (Barnes, Seeley and Van Demark, 1961; Bailey and Oxford, 1958; Prescott and Stutts, 1955; Wright, 1960). A comparison of the yield of bacterial cells was made between cultures grown in air, in an atmosphere of carbon dioxide, and with the addition of calcium carbonate to the medium. The results of these experiments (shown in Appendix I) indicated that the addition of calcium carbonate to the medium produced the highest yield of bacterial cells. In all subsequent experiments 5 per cent of sterile (360°C. for four hours) calcium carbonate was added to the medium prior to inoculation.

Growth of *S. bovis* on a large scale was carried out in 2 litre conical flasks which contained 1 litre of medium and which were loosely plugged with cotton wool. After the addition of calcium carbonate, and inoculation with 10 mls. of a 12 hour sub-culture of *S. bovis* in liquid medium, the flasks were incubated at 37°C. for 36 hours. As the bacteria grew, the pH of the medium fell, due to the production of lactic acid and, after 6 to 10 hours, had fallen to pH 5.6. Sterile 0.75N sodium hydroxide was then added to bring the pH back to neutrality. The amount of 0.75N sodium hydroxide needed was calculated from information gained in an early growth trial which is shown in Appendix II.

(e) Harvesting of bacterial cells

The medium was first centrifuged at low speed (200 x G) for 5 minutes in an International No. 1 centrifuge to remove any suspended calcium carbonate from the medium. The supernatant, which contained the bacterial cells, was decanted and centrifuged for 15 minutes at 13,000 x G in a "Spinco" Model L ultracentrifuge. The pellets of bacterial cells were washed into a weighed centrifuge tube, washed with distilled water, and recentrifuged. Washing and recentrifuging was repeated twice. The bacterial cells were then freeze-dried in the centrifuge tubes and the last traces of water were removed, by storage overnight in a vacuum dessicator containing phosphorous pentoxide. The centrifuge tube containing the bacteria was then weighed and the yield of bacterial cells obtained.

3.1.2. Experiments with washed suspensions of mixed rumen bacteria

(a) Culture medium

The composition of the medium used for the culture of washed suspensions of rumen bacteria is shown below.

Composition of the medium used for the culture of washed suspensions of mixed rumen bacteria

Component	Concentration in ml./litre or gm./litre
Sterile buffer	500 ml.
Clarified rumen fluid	500 ml.
Cellulose (Whatman's)	10 gm.
Cellobiose	2 gm.

The composition of the buffer solution used in the culture medium above, and for the preparation of washed suspensions of rumen bacteria, is shown below.

Composition of the buffer solution used in the culture of washed suspensions of rumen bacteria

Component	Concentration in gm./litre
Di-potassium hydrogen phosphate	0.5
Potassium di-hydrogen phosphate	1.0
Sodium chloride	3.0
Magnesium sulphate (hydrated)	0.1
Sodium acetate (hydrated)	1.8
L-cysteine-HCl	0.1
Calcium chloride	0.008
Sodium bicarbonate	1.0

The buffer was made up without sodium bicarbonate, sterilised by autoclaving at 15 psi for 20 minutes, and stored at 4°C. until required. Immediately before the buffer was used, sodium bicarbonate was added as a freshly prepared sterile 6 per cent (w/v) solution.

Clarified rumen fluid was prepared from rumen contents taken from a fistulated non-lactating Jersey cow. The rumen content was first strained through two thicknesses of cheese cloth to remove coarse food particles and the filtrate was centrifuged at 200 x G in an International No. 1 centrifuge to remove protozoa and small food particles. The supernatant was decanted and recentrifuged at 30,000 x G for 15 minutes in the "Spinco" Model L ultracentrifuge. The clarified rumen fluid was then decanted as a clear straw coloured fluid, practically free of bacteria, which was stored in the refrigerator at 4°C. for no longer than 48 hours before use.

(b) Preparation of washed suspensions of mixed rumen bacteria

Two fistulated non-lactating Jersey cows were confined to a small yard and fed a diet of lucerne hay *ad lib* and concentrates (Tui dairy meal, 5 lb. per day). This feeding pattern was commenced at least 10 days before rumen content was collected and bacterial suspensions were always prepared from rumen content from the same animal, to eliminate any differences that may have occurred in the bacterial population between the cows.

About 1 litre of rumen content was removed from the animal through the fistula and strained through two thicknesses of cheese cloth to remove coarse food particles. Protozoa and fine food particles were immediately removed by centrifugation at 200 x G for 5 minutes in an International No. 1 centrifuge. The supernatant, containing the bacteria, was decanted and 450 mls. were centrifuged at 11,000 x G for 15 minutes in the "Spinco" ultracentrifuge. This operation and all subsequent steps were carried out at 4°C.

The supernatant rumen fluid was discarded and the pellet of sedimented bacteria immediately resuspended in sterile buffer, which had been previously cooled to 4°C. The resuspended bacteria were then centrifuged and the process of washing with buffer and centrifuging was repeated twice. After the last centrifugation the bacteria were again suspended in sterile buffer and made up to the original volume of 450 mls. One hundred mls. of this washed suspension of mixed rumen bacteria were then immediately added to each litre of culture medium in the growth flasks.

(c) Culture flasks for washed suspensions of mixed rumen bacteria

Mixed rumen bacteria were grown in conical flasks which were closed with rubber stoppers through which passed three glass tubes. One tube was connected to a bunsen valve which permitted gas to escape from the flask while, through a second tube, ammonia, radioisotope solutions and carbon dioxide were passed into the culture medium. Through the third tube aliquots of the culture medium were removed during the incubation. Both the second and third tubes were closed with screw clips when not in use.

(d) Conditions for the culture of mixed rumen bacteria

After the addition of the washed suspension of mixed rumen bacteria the flask containing the culture medium was placed in a water bath maintained at 39°C. and carbon dioxide was bubbled through the medium for one hour to ensure that the oxygen tension was as low as possible. The pH changes in the culture medium during growth were followed by measuring the pH of a 5 ml. aliquot taken at regular intervals. Each time the pH of the medium fell below pH 5.8, concentrated ammonia solution was added until the medium reached its initial value of pH 6.8 - 7.0. Carbon dioxide was bubbled through the medium for 15 minutes after each sample was withdrawn.

Non-radioactive *iso*butyric acid was added to several of the cultures of mixed rumen bacteria before inoculation. The amount added was 0.44 gm./litre which gave a concentration of 5 μ moles of *iso*butyric acid per litre. In experiments where radioactive sodium *iso*butyrate-1-C^{14} and dl-valine-4-C^{14} were added to the medium, the addition of the labelled substrate was not made until there was a visible increase in the turbidity of the medium in the flasks, indicating that bacterial growth had started.

All cultures of mixed rumen bacteria were incubated for 36 hours and at the end of this time the bacterial cells were harvested and freeze-dried in the same way as described for *Streptococcus bovis* on page 25.

3.2. Chemical Methods

3.2.1. Solvents and reagents

All solvents used were purified according to Vogel (1956). All reagents were "Analar" or "technical" grade unless otherwise specified.

3.2.2. The extraction of the total lipid from bacterial cells

In some early experiments the lipid was extracted from the freeze-dried bacterial cells using 2:1 diethyl ether:ethanol as the extracting solvent. The bacterial cells were heated under reflux for 3 hours with three 50 ml. portions of the solvent. After each extraction the solvent was decanted and the 3 extracts were combined and the solvent removed on a rotary evaporat below 40°C. However further extraction of the bacterial cell residues with chloroform:methanol (2:1) showed that further lipid material could be extracted with this solvent. Consequently, in all subsequent experiments chloroform:methanol (2:1) replaced diethyl ether:ethanol in the procedure outlined above.

3.2.3. The saponification of the total lipid and extraction of the fatty acids

The total lipid fraction was dissolved in 20 mls. of a 2 per cent solution of potassium hydroxide in 95 per cent ethanol and heated under reflux for 3 hours. The ethanolic solution was then reduced to a small volume by distillation and transferred to a separating funnel, where the non-saponifiable material was removed by washing the mixture with diethyl ether. The aqueous fraction, containing the potassium salts of the fatty acids, was acidified with 6N hydrochloric acid and the fatty acids extracted into diethyl ether, which was then removed on a rotary evaporator below 40°C.

3.2.4. Methylation of the fatty acids

The fatty acids were dissolved in 10 mls. of dry methanol containing 1 per cent of concentrated sulphuric acid, and heated under reflux for 2 hours. The contents of the flask were reduced to half volume on a rotary evaporator and transferred to a separating funnel containing 3 volumes of diethyl ether and 0.5 volumes of 10 per cent potassium carbonate solution. After shaking to neutralise the sulphuric acid the aqueous phase was separated and the ether layer containing the fatty acid methyl esters was washed with distilled water until the pH of the wash water was neutral.

3.2.5. Bromination of the fatty acid methyl esters

Bromination was carried out according to the method of Farquhar *et al.* (1959) as follows: The methyl esters (up to 100 mg.) were dissolved in 10 mls. of dry diethyl ether and the solution was cooled to below -10°C. in a dry ice:ethanol mixture. A 22 per cent solution of dry bromine in diethyl ether was also cooled to -10°C. and added drop by drop to the solution of methyl esters until the yellow colour of the bromine persisted. Nitrogen was then bubbled through the solution at room temperature to remove the diethyl ether and any excess bromine present. The resulting mixture, in which the unsaturated fatty acid methyl esters had been brominated, was stored at -20°C. until analysed by gas-liquid chromatography.

3.2.6. The purification of the methyl esters of fatty acids after bromination

Fatty acid methyl esters which contained radioactive fatty acids, and which were to be separated by preparative gas chromatography, were purified in the following manner.

The mixture of brominated and unbrominated methyl esters was dissolved in hexane and any insoluble material discarded. The hexane soluble fraction (which contained all of the radioactivity) was chromatographed on a preparativ thin layer of silicic acid (Silica gel G, Merck and Co.) in a solvent of hexane:diethyl ether (60:40) to remove those bromo fatty acids which were not insoluble in hexane. Under these conditions the fatty acid methyl esters had an Rf of between 0.9 and 0.95 while the Rf of the bromo derivatives was between 0.1 to 0.5. The position of the fatty acid methyl esters was revealed by spraying the plate with 2,7-dichlorofluorescein and the esters were recovered by scraping off the appropriate area of the thin layer and eluting this with diethyl ether until no further radioactive material could be detected in the eluate.

The purified methyl esters were then treated with diazomethane to methylate any free fatty acids formed during the procedures above. The purified fatty acid methyl esters were then analysed by preparative gas-liquid chromatography.

3.2.7. Steam distillation of volatile fatty acids from the culture medium

Total volatile fatty acids were obtained from the culture medium by steam distillation in a Markham (1942) apparatus. Five mls. of the culture medium and 2 mls. of 10N sulphuric acid (saturated with magnesium sulphate) were added to the apparatus and steam distilled. Sixty mls. of distillate were collected, followed by a further 60 mls., on which a blank determination was carried out to correct for organic acids which were slightly steam volatile. The distillates were titrated against 0.05N sodium hydroxide under carbon dioxide free conditions using phenolphthalein as an indicator. A small excess of alkali was then added and the sodium salts of the fatty acid were reduced to dryness in a drying oven and stored at -20°C. until the individual fatty acids could be analysed by gas-liquid chromatography.

3.3. Gas-liquid Chromatography

3.3.1. Separation of the methyl esters of long chain fatty acids

(a) Apparatus

A Pye-Argon Chromatograph, having an argon ionisation detector with a 20 millicurie strontium 90 radiation source (Lovelock, 1959), was used for the separation of the fatty acid methyl esters. The columns used were of glass, 1 metre in length and 4 mm. in internal diameter.

(b) Liquid and gas phases

The liquid phases used were 20 per cent Apiezon M or L grease (Edwards High Vacuum Ltd.) or 20 per cent polyethylene glycol adipate (P.G.A.) supported by acid washed celite 545 (85-100 mesh). After the stationary phase had been packed into the glass column it was preconditioned by heating to

225°C. (Apiezon M or L) or 200°C. (P.G.A.) and flushing with a stream of dry nitrogen for 24 hours.

Argon (99.95 per cent pure) was obtained from N.Z. Industrial Gases Ltd.

(c) Operating conditions

The operating conditions used for gas-liquid chromatography were as follows:

	Apiezon M or L	P.G.A.
Column temperature	180-205°C.	150-180°C.
Argon flow rate	33-35 ml./minute	35-50 ml./minute
Detector voltage	1250 volts	1250 volts

The sample of fatty acid methyl esters was introduced onto the column with a 0.05 or 0.1 μ litre micro-pipette (Pye Ltd.). The flow of argon was stopped and the inlet opened to the air during this procedure. On restarting the gas flow a negative air peak was recorded and this was used as the starti point for the measurement of the relative retention volumes of the fatty acid esters.

(d) Preparative gas-liquid chromatography

Fatty acid methyl esters from the experiments with C^{14} labelled compound were analysed by preparative gas-liquid chromatography and the individual fatty acids were collected for the determination of their radioactivity.

The separation of the fatty acid methyl esters was carried out in a Wilkens "Autoprep" instrument with a thermal conductivity detector. Columns 5 feet long were used and packed with 12 per cent diethylene glycol succinate on celite 545. The column conditions were as follows:

Column temperature	148-158°C.
Injector "	266°C.
Detector "	290°C.
Collector "	248°C.
Helium flow rate	60 ml./minute

Collection of the individual fatty acids was carried out manually using drying tubes packed with siliconised glass fibre, wetted with methanol. The fatty acid methyl esters were washed with ethanol from the drying tubes into counting vials and the solvents were evaporated. Five mls. of toluene (containing 0.6 per cent P.P.O. and 0.05 per cent P.O.P.O.P.) were added to the counting vials and the sample was counted in a Packard Tri Carb liquid scintillation counting system (Model 314 Ex) which gave an efficiency of 55-60 per cent.

(e) The identification of the fatty acid methyl esters

Individual fatty acid methyl esters were identified by comparison of their relative retention volumes on two liquid phases, with those reported by Hawke *et al*. (1959) and James (1960). Pure samples of many of the fatty acid methyl esters were available and these were chromatographed under the same conditions as the unknown samples and the relative retention volumes compared. Co-chromatography with a pure sample of a known fatty acid methyl ester was carried out in order to identify some of those fatty acids with similar relative retention volumes. The identification of the fatty acid methyl esters was checked by plotting the logarithm (base 10) of the relative retention volume against the number of carbon atoms in the fatty acid to verify that the relationship demonstrated by Hawke *et al*. (1959) was satisfied

The proportions of the individual fatty acids in a sample were obtained by measurement of the peak areas with a planimeter. Each peak was measured three times and the mean value calculated. The relative proportion of each fatty acid was expressed as a percentage of the sum of the total peak areas on the chromatogram.

3.3.2. Separation of the individual volatile fatty acids by gas-liquid chromatography

Separation of the individual volatile fatty acids from acetic acid to butyric acid was carried out on the gas-liquid chromatograph described by

James and Martin (1952). Behenic acid replaced stearic acid in the column packing (Hawke, 1957) and ethyl cellosolve (B.P. 137°C.) was used in the vapour jacket. Samples of the concentrated steam distillate of fatty acids from the culture medium were applied to the column as aqueous solutions of their sodium salts. One drop (approximately 0.05 ml.) was added to a 50:50 (w/w) mixture of celite 545 and anhydrous sodium bisulphate in a metal boat. This was quickly slid into the heated region of the column and the nitrogen gas flow started.

A number of separations were carried out on each sample, the first of which indicated the time of emergence of each fatty acid from the column. During subsequent separations the operating conditions were not changed, so that the time of emergence of each fatty acid would be the same. After the first separation the indicator in the titration cell was replaced by 10 mls. of a dilute (0.05N) solution of sodium hydroxide and the automatic titration apparatus disconnected. The second and subsequent samples were applied to the column as outlined above, and when sufficient time had elapsed for acetic acid to emerge, the sodium hydroxide was removed and the titration cell quickly washed out with distilled water. A further 10 mls. aliquot of sodium hydroxide was added and propionic acid collected. The procedure was repeated for isobutyric acid and butyric acid as they emerged from the column. The aliquots of dilute sodium hydroxide containing the same volatile fatty acid from several separations of each sample were combined. These were evaporated to dryness as their sodium salts and made up to a known volume for the determination of their radioactivity.

The separation of the volatile fatty acids was checked in the following way. Ten separations of the fatty acids in one sample were made and the fractions corresponding to each fatty acid were combined and rechromatographed On rechromatography the first fraction was found to contain only acetic acid, the second only propionic acid, the third fraction contained isobutyric acid and some butyric acid while the fourth fraction contained butyric and higher fatty acids.

As the amount of fatty acid applied to the column for each separation wa unknown, it was not possible to determine the specific radioactivity for each fatty acid in terms of counts per minute per mg. of fatty acid. Comparison of the total radioactivities of each fatty acid in a sample enabled the presence of radioactivity in any fatty acid to be detected.

3.4. Radio-chemical Methods

3.4.1. Source of radio-isotopes

Sodium *iso*butyrate-1-C^{14} and dl-valine-4-C^{14} were obtained from the Radiochemical Center, Amersham, England.

3.4.2. Radio-isotope counting

(a) Apparatus

Radioactivity was measured by liquid scintillation counting in a Nuclear Enterprises Ltd. scintillation head, which was connected to a Phillips pulse height analysing system.

(b) Scintillation fluid

The liquid scintillator was a solution of 0.5 per cent of 2,5-biphenyloxazole (P.P.O.) and 0.03 per cent 1,4-bis-2-(5-phenyloxazolyl)-benzene (P.O.P.O.P.) in scintillation grade toluene. P.P.O., P.O.P.O.P. and scintillation grade toluene were obtained from Nuclear Enterprises Ltd., England.

(c) Counting of radioactive samples

With water soluble compounds, such as sodium *iso*butyrate-1-C^{14}, a measured aliquot (usually 10 μ litres) of the culture medium, or of an aqueous solution containing the isotope, was applied to a half inch square of filter paper (Whatman's No. 1). This was then dried under a heat lamp and placed in a quartz scintillation vial containing 2 mls. of liquid scintillator and counted. Background determinations were carried out under the same conditions using a clean piece of filter paper.

To test the efficiency of counting using this method a standard solution of sodium isobutyrate-1-C^{14} was prepared which contained an activity of 0.02 μcuries/ml. Ten μlitres of this solution were applied, using a micro-syringe, to a square of filter paper which was then dried. The amount of radioactivity on the filter paper was 0.0002 μcuries which has a theoretical yield of 456 disintegrations per minute. However the actual number of counts per minute recorded was 250, which showed that the efficiency of counting under these conditions was 55 per cent.

When lipid fractions were to be counted they were first dissolved in a small known volume (2-4 mls.) of diethyl ether. A small aliquot of this solution (20-50 μlitres) was measured into a counting vial containing 2 mls. of the liquid scintillator and counted.

Chapter 4

RESULTS

4.1. The Identification of the Methyl Esters of the Fatty Acids of *Streptococcus bovis* by Gas-liquid Chromatography

Gas-liquid chromatography of the methyl esters of the fatty acids from the total lipid extract of *S. bovis* was carried out on two liquid phases, Apiezon M at 200°C., and polyethylene glycol adipate (P.G.A.) at 180°C. The retention volumes, relative to methyl palmitate (P.G.A. columns), and methyl myristate (Apiezon M columns) were calculated and compared with those reported in the literature (Hawke, Hansen and Shorland, 1959; James, 1960). Pure samples of several fatty acid methyl esters were also chromatographed on these columns and their relative retention volumes calculated. All relative retention volumes are shown in Table 3.

The relative retention volumes of fatty acid methyl esters of a homologous series can be shown to exhibit a logarithmic relationship with the number of carbon atoms in the fatty acid (Hawke *et al.*, 1959). When the $\log^{10}$ of the relative retention volume is plotted against the carbon number of the fatty acid, those acids belonging to a homologous series, such as the straight chain saturated acids, fall along a straight line. Fatty acids of a second homologous series (e.g. mono-unsaturated fatty acids) fall along a second line, parallel to, but slightly displaced from the first. This relationship for the fatty acids of *S. bovis* on Apiezon M and P.G.A. liquid phases is shown in Figures 5 and 6.

To obtain further evidence for the identity of the fatty acids a sample of their methyl esters was brominated to remove unsaturated fatty acid esters and then chromatographed on the Apiezon M and P.G.A. columns. The chromatograph charts of the fatty acid methyl esters on Apiezon M before and after bromination are shown in Figures 7 and 8. Figure 9 shows

TABLE 3

Relative retention volumes in two stationary phases of the long chain fatty acids of *S. bovis*

		RELATIVE RETENTION VOLUMES					
		Apiezon M at 200°C. (Relative to methyl myristate)			Polyethylene glycol adipate at 180°C. (Relative to methyl palmitate)		
		Observed		Hawke et al. (1959)	Observed		James (1960)
Chemical Name of Fatty Acid	Shorthand Notation*	*S. bovis*	Known standard		*S. bovis*	Known standard	
n-decanoic	10:0	0.17	0.17	0.17	0.13	0.13	0.134
—	11:1 ?	0.25	—	—	0.21	—	—
n-undecanoic	11:0	0.28	0.26	0.27	0.18	0.18	0.183
—	12:1 ?	0.37	—	—	0.28	—	—
n-dodecanoic	12:0	0.42	0.42	0.42	0.25	0.25	0.26
n-tridecenoic	13:1	0.58	—	—	0.43	—	—
n-tridecanoic	13:0	0.63	0.63	0.64	0.37	0.37	0.362
n-tetradecenoic	14:1	0.87	—	—	0.61	—	0.615
n-tetradecanoic	14:0	1.00	1.00	1.00	0.51	0.52	0.51
n-pentadecenoic	15:1	1.47	—	—	0.79	—	—
n-pentadecanoic	15:0	1.52	1.52	1.53	0.71	0.71	0.705
n-hexadecenoic	16:1	2.14	—	2.15	1.14	—	1.15
n-hexadecanoic	16:0	2.37	2.37	2.38	1.00	1.00	1.00
n-heptadecenoic	17:1	3.16	—	—	1.59	—	—
n-heptadecanoic	17:0	3.59	3.53	3.47	1.37	1.35	—
n-octadecenoic	18:1	4.80	4.80	4.82	2.22	2.21	1.93
n-octadecanoic	18:0	5.50	5.48	5.49	1.97	1.96	1.66
Lactobacillic acid	cy.19:0	6.71	—	—	2.14	—	—
n-nonodecanoic	19:0	7.17	7.15	—	2.74	2.72	—
n-eicosenoic	20:1	9.61	—	—	3.97	—	—
n-eicosanoic	20:0	10.90	10.70	—	3.52	3.50	—

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* See Table 2.

FIGURE 5

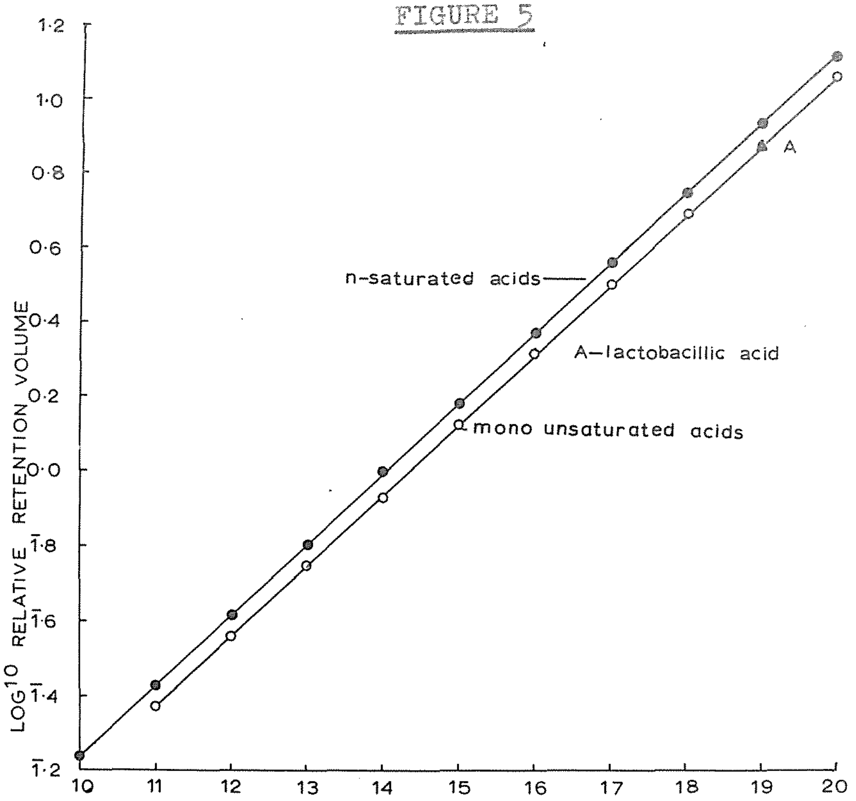
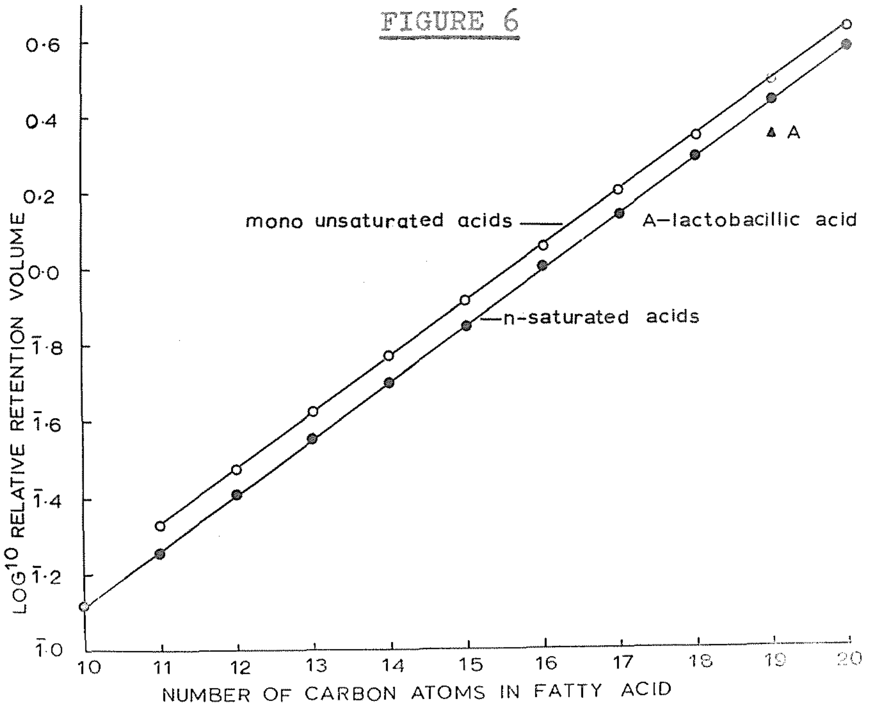


FIGURE 6



The relationship between the carbon number of the fatty acid and the log^{10} of the relative retention volume for the fatty acids of *S. bovis* on Apiezon M (Figure 5) and P.G.A. (Figure 6) liquid phases.

FIGURE 7

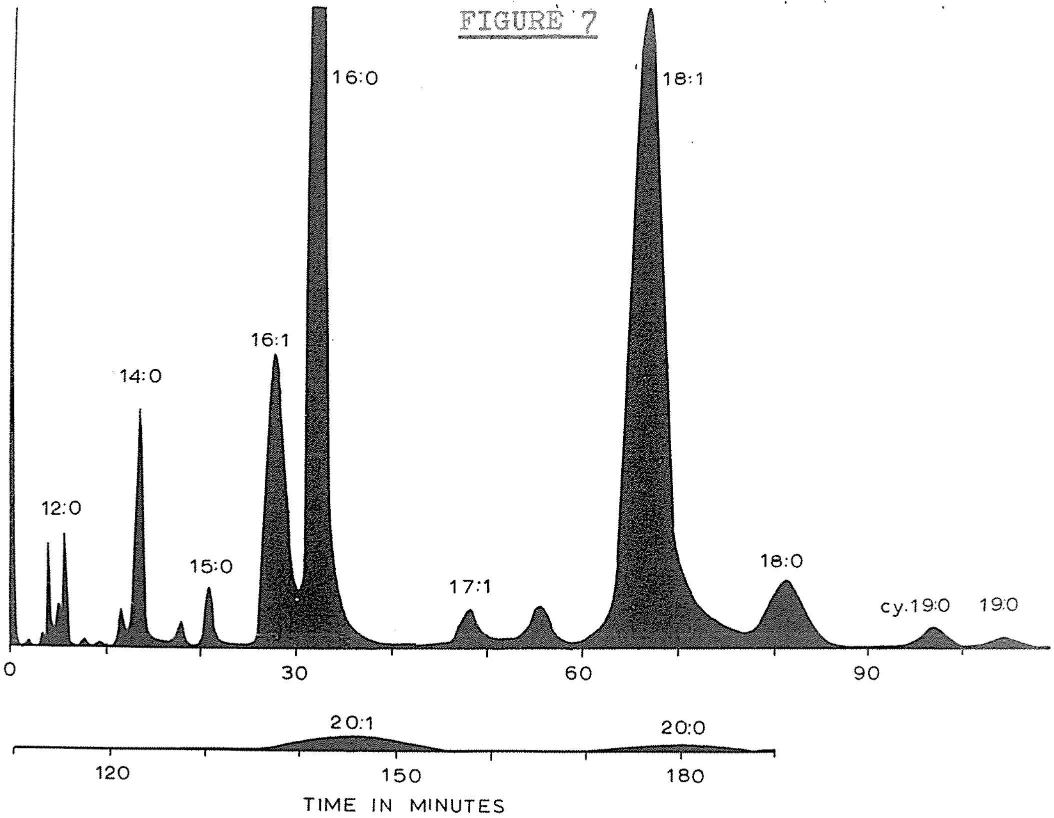
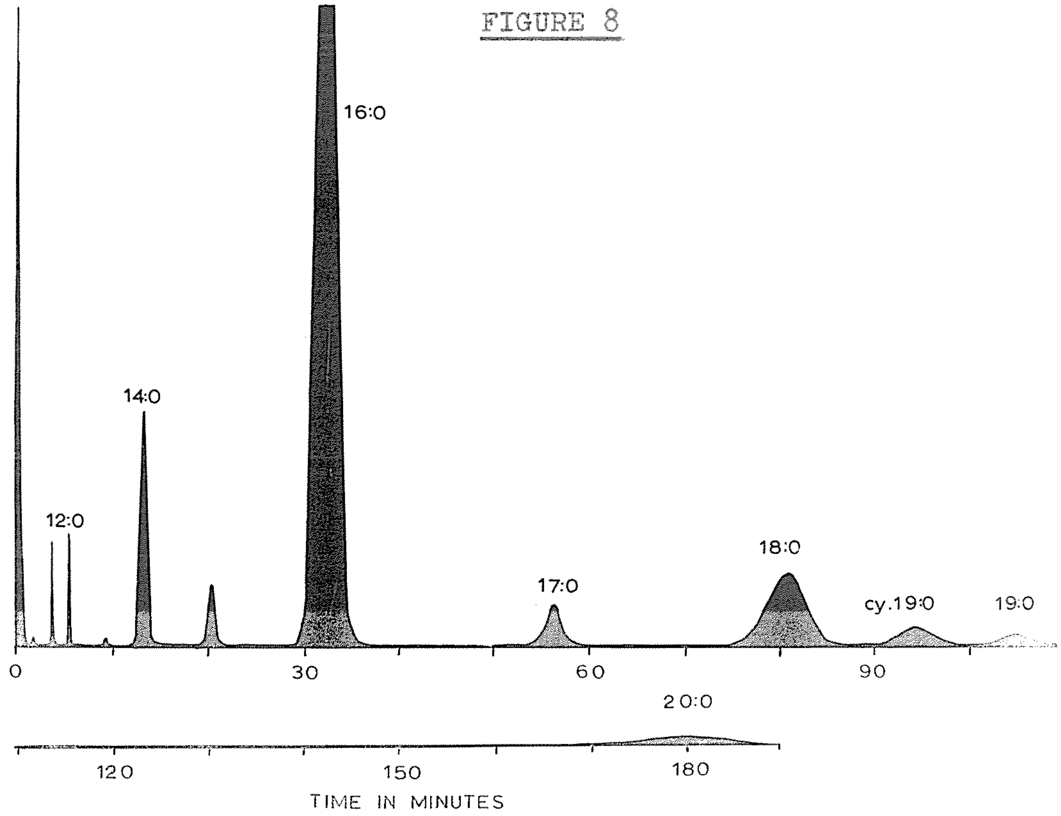


FIGURE 8



Gas-liquid chromatograms of the methyl esters of the fatty acids of *S. bovis* on Apiezon M liquid phase at 200°C.

Figure 7: Total fatty acids.

Figure 8: Saturated fatty acids.

FIGURE 9

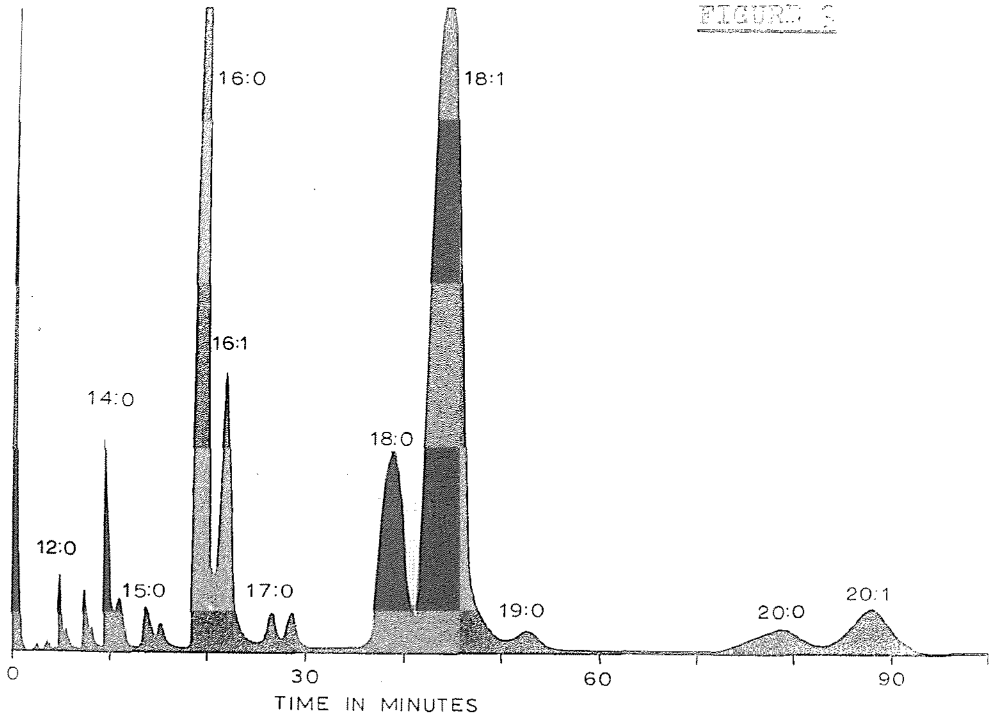
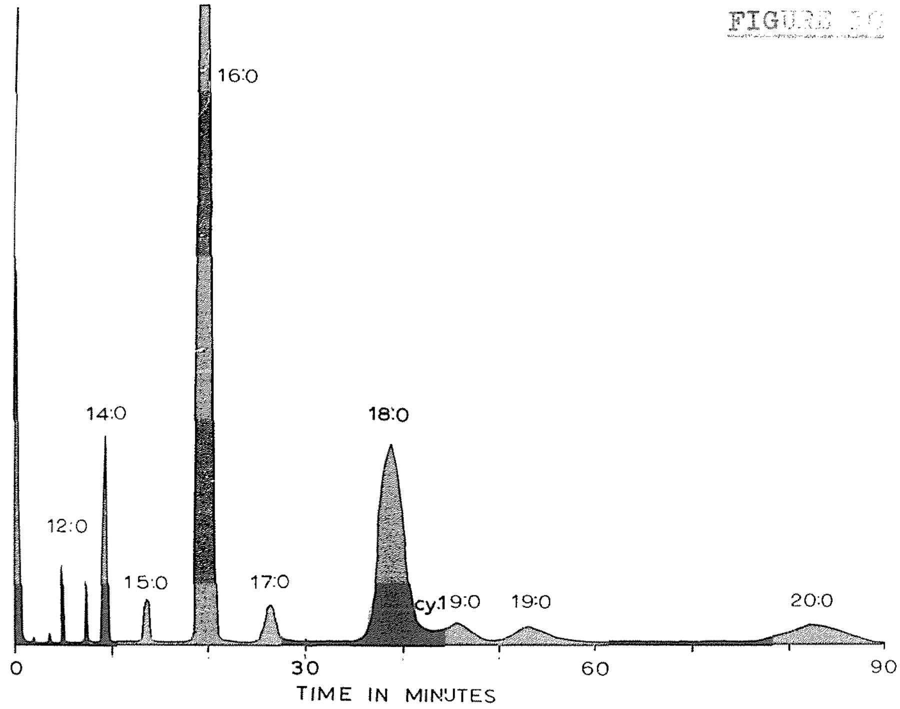


FIGURE 10



Gas-liquid chromatograms of the methyl esters of the fatty acids of *S. bovis* on P.G.A. liquid phase at 180°C.

Figure 9: Total fatty acids.

Figure 10: Saturated fatty acids.

the total fatty acid methyl esters from S. bovis chromatographed on the P.G.A column while Figure 10 is a chromatogram of the same sample after bromination Small quantities of pure methyl esters of myristic, palmitic, stearic, oleic, nonodecanoic and eicosanoic acids were each co-chromatographed separately with the methyl esters of the fatty acids from S. bovis on the Apiezon M column and in each case the peak corresponding to the additional methyl ester increased in size and no new peaks were observed.

The position of the double bond in the unsaturated fatty acids of S. bovis was not established in the present investigation. When pure methyl oleate was added to the S. bovis fatty acid methyl esters a single symetrical peak appeared on both the Apiezon M and P.G.A. columns. However it is possible that the columns used would not resolve the various positional isomers of octadecenoic acid but, as no pure samples of isomers other than oleic acid were available, the lack of separation could not be demonstrated. For the same reasons the position of the double bond was not determined for any of the other unsaturated fatty acids.

The identification of lactobacillic acid (cis-11,12-methylene-octadecanoic acid) should be considered tentative as the evidence is based solely on gas chromatography results. The proportion of this fatty acid in the lipids of S. bovis was too small to enable pure samples to be obtained by preparative gas chromatography and consequently no other tests to determine the structure of the acid could be carried out. Retention volumes for the methyl ester of this fatty acid could not be found in the literature and no pure sample of the acid was available for comparison. However it is difficult to envisage a fatty acid, other than a cyclopropane or branched chain fatty acid, which would have the relative retention volumes shown (Table 3). Lactobacillic acid was only discovered in S. bovis fatty acids when it was found that the fatty acid peak on Apiezon M, thought previously to belong to a $C_{19:1}$ fatty acid, was not reduced in size after bromination (Figures 7 and 8). Subsequent examination of the methyl esters of the saturated fatty acids on the P.G.A. column revealed the presence of a peak emerging from the column immediately before nonodecanoic acid (Figure 10). On the basis of the evidence above, this fatty acid has been tentatively identified as lactobacillic acid.

4.2. The Fatty Acid Composition of the Total Lipid of *Streptococcus bovis*

The fatty acid composition of the total lipid from several batches of *S. bovis* is shown in Table 4. This includes the fatty acids from bacteria grown on the basic medium (Table 4, columns A, B and D) and on the basic medium plus 2.0 μmoles/ml. of *iso*butyric acid (Table 4, columns C, E and F). In all these experiments the bacteria were grown in one litre of the culture medium. The cultural conditions have been described in Chapter 3.

After incubation for 36 hours the bacterial cells were harvested, freeze-dried, and the total lipid extracted. Fatty acids were obtained after saponification of the total lipid extract and the fatty acid composition was determined by gas-liquid chromatography of the methyl esters on an Apiezon M liquid phase. Peak areas were measured by planimetry and expressed as a percentage of the total area of all the peaks. Gaps in Table 4 indicate that if a fatty acid was present it did not constitute more than 0.1 per cent of the total fatty acids. In one experiment (Table 4, columns D and E) the fatty acids were extracted by alkaline hydrolysis of the wet bacterial cells according to the method of Kaneeda (1963). The fatty acid composition of *S. bovis* in this experiment was considerably different to the fatty acid composition in the experiments where the total lipid was extracted with chloroform:methanol before saponification.

The gas chromatograph charts of the methyl esters of the total and saturated fatty acids from *S. bovis* grown on the medium containing *iso*butyric acid are shown in Figures 11 and 12. These fatty acids were obtained from

TABLE 4

The relative percentage composition of the fatty acids of *S. bovis*.

Chemical Name of Fatty Acid	Shorthand Notation*	A	B	C	D	E	F
n-decanoic	10:0	—	—	—	0.1	0.1	0.1
—	11:1 ?	—	—	0.1	—	—	—
n-undecanoic	11:0	—	—	0.7	0.3	0.1	0.1
—	12:1 ?	0.5	0.5	0.4	—	—	—
n-dodecanoic	12:0	0.8	0.3	0.5	0.2	0.2	0.1
n-tridecenoic	13:1	—	—	—	—	—	—
n-tridecanoic	13:0	—	—	0.2	—	0.2	0.1
n-tetradecenoic	14:1	0.5	0.4	0.7	0.5	0.4	0.6
n-tetradecanoic	14:0	2.1	2.3	3.9	0.7	2.9	2.8
n-pentadecenoic	15:1	—	—	0.2	—	—	—
n-pentadecanoic	15:0	—	0.2	0.6	—	0.9	0.8
n-hexadecenoic	16:1	6.3	5.1	5.0	10.0	6.4	5.3
n-hexadecanoic	16:0	29.4	29.6	30.5	19.4	26.7	29.6
n-heptadecenoic	17:1	—	0.5	2.3	—	—	—
n-heptadecanoic	17:0	—	—	0.3	—	0.9	0.2
n-octadecenoic	18:1	38.6	39.0	32.0	60.8	45.2	39.5
n-octadecanoic	18:0	14.5	15.7	16.4	3.8	9.2	15.5
Lactobacillic acid	cy.19:0	1.5	1.6	3.6	1.0	0.7	1.7
n-nonodecanoic	19:0	—	0.2	—	—	—	0.2
n-eicosenoic	20:1	3.8	3.4	2.8	2.7	3.9	2.8
n-eicosanoic	20:0	1.3	1.2	1.3	0.2	—	1.2

Column A - Fatty acids from preliminary growth experiment. Bacteria grown in basic medium.
" B - Fatty acids from bacteria grown on the basic medium.
" C - " " " " " " " medium containing 2 μ moles/ml. of <u>iso</u>butyric acid.
" D - " " " " " " " basic medium.
" E - " " " " " " " medium containing 2 μmoles/ml. of <u>iso</u>butyric acid.
" F - " " " " " " " " " " " " " "

The fatty acids shown in columns D and E were extracted by alkaline hydrolysis of the wet bacterial cells according to the method of Kaneeda (1963).

FIGURE 11

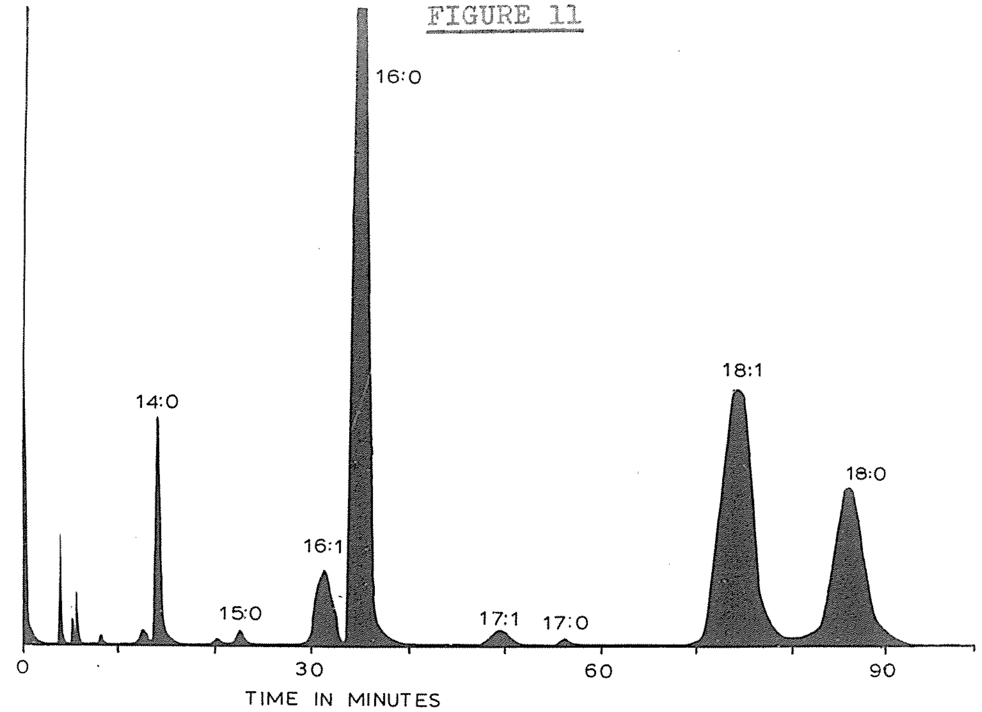
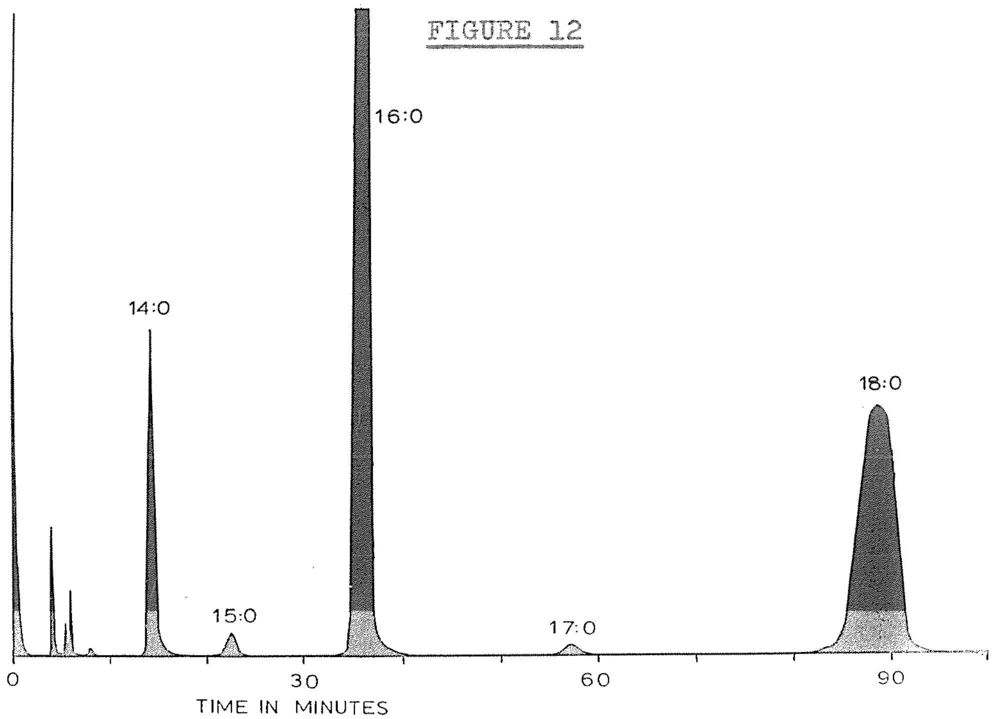


FIGURE 12



Gas-liquid chromatograms of the methyl esters of the fatty acids of S. *bovis* grown in a medium containing *isobutyric* acid. Apiezon M liquid phase at 190°C. (Fatty acids higher than $C_{18:0}$ not shown.)

Figure 11: Total fatty acids.

Figure 12: Saturated fatty acids.

the bacteria which had been freeze-dried before extraction of the lipid and not from the experiment where the fatty acids were obtained by saponification of the wet cells. These charts, together with the results in Table 4, show that no branched chain fatty acids are found in the lipids from *S. bovis* when this organism is grown under the conditions reported in these experiments. When massive sample loads were applied to the gas chromatograph column there were no peaks observed which had relative retention volumes corresponding to those expected for branched chain fatty acids of the *iso* and *anteiso* series.

As can be seen from Table 4, the addition of 2 μ moles/ml. of *iso*butyric acid to the culture medium had no pronounced effect on the fatty acid composition of *S. bovis*. Any differences in the fatty acid composition which were observed could be attributed equally well to the considerable differences which occurred in the proportions of the fatty acids when different batches of bacteria were grown on the basic medium alone. However in the fatty acids of the bacteria grown in the presence of *iso*butyric acid there was a very slight increase in the proportions of the fatty acids containing an odd number of carbon atoms (11:0, 13:0, 15:0, 17:0). These fatty acids were present in very small quantities and the small size of the chromatograph peaks made accurate measurement difficult. Despite this difficulty a slight increase in the proportion of the odd numbered fatty acids was apparent in all of the bacteria grown on the medium containing *iso*butyric acid.

4.3. The Incorporation of the Radioactivity from *iso*butyrate-1-C^{14} into the Fatty Acids of *Streptococcus bovis*

Experiments to determine the effect of unlabelled *iso*butyric acid on the fatty acids of *S. bovis* were supplemented by further work to determine whether the radioactivity from *iso*butyric acid-1-C^{14} was incorporated into the long chain fatty acids of *S. bovis*.

Thirty μ-curies of sodium *iso*butyrate-1-C^{14} were added to 1 litre of the culture medium, together with sufficient unlabelled *iso*butyric acid to give a final concentration of 2 μ moles of *iso*butyrate per ml. The medium was then inoculated with *S. bovis* and grown under the conditions described in Chapter 3.

The radioactivity of the culture medium was determined before and after harvesting of the bacterial cells (Table 5) and there was no detectable reduction in the radioactivity of the medium after removal of the bacteria. This suggested that the proportion of the *iso*butyric acid taken up by the bacteria was very small (less than 1 per cent) (Table 5). A 10 ml. aliquot of the culture medium supernatant, obtained after the bacteria were harvested was steam distilled and all of the radioactivity was recovered in the steam volatile fraction, none being detected in the non steam volatile residues (Table 5). Separation of a sample of the individual steam volatile fatty acids by gas-liquid chromatography showed that virtually all of the radioactivity in these fatty acids was located in *iso*butyric acid (Table 6).

The radioactivity of the whole bacterial cells was not determined in this experiment but the total radioactivity present in the fatty acid fraction was only 2,100 disintegrations per minute, which was only a very small proportion of the total amount added to the culture medium.

The total fatty acids from the lipid extract of *S. bovis* were then steam distilled and all of the radioactivity was recovered in the steam volatile fraction. This suggested that the radioactivity present in the total fatty acid fraction was due to the presence of *iso*butyrate-1-C^{14} from the culture medium which had not been washed from the cells after harvesting. This would then have been extracted with the total lipids and been included in the fatty acid fraction. No radioactivity was found in the long chain (non steam volatile) fatty acids from *S. bovis* grown in the presence of *iso*butyrate-1-C^{14}.

TABLE 5

The distribution of radioactivity between the steam volatile and non steam volatile fractions of the supernatant from the culture medium of *S. bovis* supplemented with 30 μ-curies of *iso*butyric acid-1-C^{14}

Fraction	Counts/Minute per ml. of Culture Medium (Corrected for Background)	Percentage of Counts in Medium Plus Bacteria
Culture medium plus bacteria	35,000	100
Culture medium less bacteria (supernatant)	34,700	99
Steam volatile fraction of supernatant	34,200	98
Residue from steam distillation of supernatant	10	-

TABLE 6

The distribution of radioactivity in the individual steam volatile fatty acids from the culture supernatant when *S. bovis* was grown in a medium containing 30 μ-curies of *iso*butyrate-1-C^{14}

Fatty Acid	Counts per 10 minutes (Corrected for Background)
Acetic	3
Propionic	4
*Iso*butyric	540
Butyric	13

An attempt was made to analyse the steam volatile fatty acids from the *S. bovis* lipid extract by gas-liquid chromatography on the apparatus described in Chapter 3 on page 33. However no results were obtained since the amount of volatile fatty acids in the sample was below the limit of detection of the apparatus.

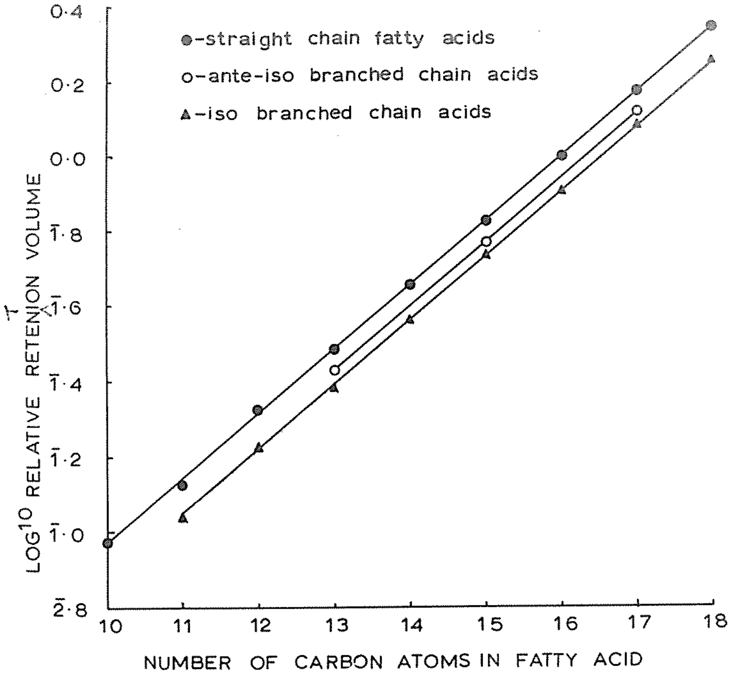
Once it had been established that there were no branched chain fatty acids in *Streptococcus bovis* it was decided to extend the investigation to the study of mixed suspensions of rumen bacteria, which are known to contain branched chain fatty acids of the *iso* and *anteiso* series (Keeney *et al*., 1962 After preliminary experiments to establish conditions for the growth of these bacteria a study was made of the uptake of the C^{14} label from *iso*butyrate-1-C and valine-4-C^{14} into the long chain fatty acids of these organisms.

4.4. The Identification of Methyl Esters of the Fatty Acids of Mixed Rumen Bacteria and Rumen Protozoa by Gas-liquid Chromatography

A preliminary experiment was undertaken to determine the fatty acid composition of the bacteria and protozoa in the rumen of a fistulated, lactating, Jersey cow which had been grazing on mixed ryegrass:white clover pasture.

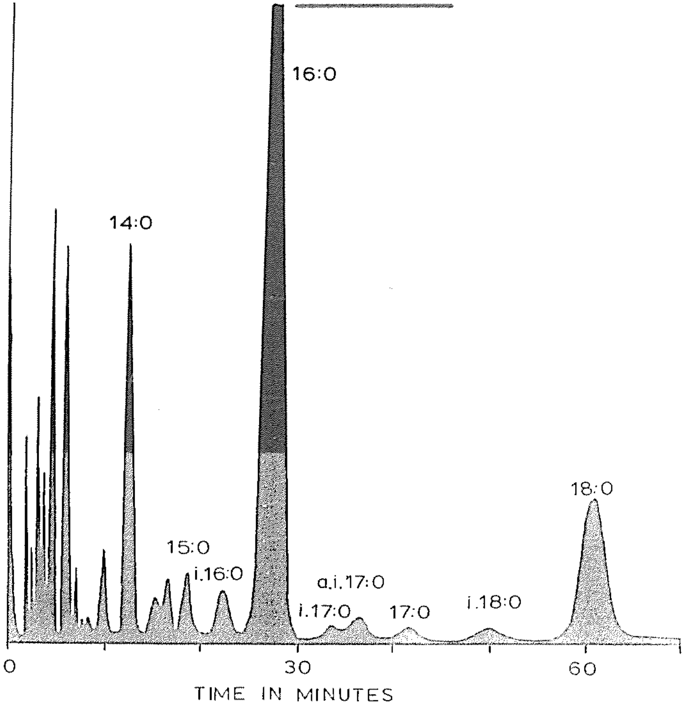
Freshly collected rumen fluid was strained twice through two layers of cheese cloth to remove food particles and then centrifuged (150 x G) to precipitate the protozoa, which were then washed twice with 0.9 per cent saline solution and recentrifuged. (This fraction was observed to contain a certain amount of finely divided food material which had passed through the cheese cloth.) The supernatant obtained after centrifuging at 150 x G was then recentrifuged at 10,000 x G for 15 minutes and the pellet of bacteria obtained was washed twice with distilled water. Both fractions (bacteria and protozoa) were then freeze-dried and the methyl esters of the fatty acids were prepared from the total lipid extract as described in

FIGURE 13



The relationship between the carbon number of the fatty acid and the $\log^{10}$ of the relative retention volume of the saturated fatty acids from mixed rumen bacteria. Apiezon L liquid phase at 200°C.

FIGURE 14



Gas-liquid chromatogram of the methyl esters of the saturated fatty acids of mixed rumen bacteria on Apiezon L liquid phase at 210°C.

Chapter 3. These were then analysed by gas-liquid chromatography on a stationary phase of Apiezon L before and after bromination to remove methyl esters of the unsaturated fatty acids. The relative retention volumes of the fatty acid methyl esters from rumen bacteria and protozoa are recorded in Table 7 together with those reported by James (1960) for these fatty acid methyl esters.

Figure 13 shows the relationship between the logarithm (base^{10}) of the relative retention volume, on an Apiezon L liquid phase, and the number of carbon atoms in the fatty acids of the normal, iso and anteiso series of fatty acids from the mixed rumen bacteria. Figure 14 is a chromatogram of the methyl esters of the saturated fatty acids from a sample of mixed rumen bacteria, showing the degree of separation achieved between iso and anteiso fatty acids with the same number of carbon atoms.

4.5. The Fatty Acid Composition of the Lipid Extract of Rumen Bacteria and Protozoa

Table 8 lists the relative percentage composition of the fatty acids from the rumen bacterial and protozoal fractions. The branched chain fatty acids containing an odd number of carbon atoms were measured as one fraction because the iso and anteiso acids were not completely separated on the columns used (see Figure 14).

The main features shown in Table 8 are that the proportion of branched chain fatty acids is far higher in the rumen bacterial fraction than it is in the protozoal fraction and that there are unsaturated fatty acids with more than one double bond in both the protozoal and bacterial fatty acids.

TABLE 7

The retention volumes (relative to methyl palmitate) of the methyl esters of the fatty acids found in mixed rumen bacteria and in rumen protozoa.

Liquid phase: 20 per cent Apiezon L on Celite 545.

Column temperature: 200°C.

Fatty Acid	Shorthand Notation*	Rf (obs)	Rf (James, 1960)
n-decanoic	10:0	0.076	0.073
n-undecanoic	11:0	0.118	0.117
10-methylundecanoic	i.br.12:0	0.142	0.15
n-dodecanoic	12:0	0.177	0.18
11-methyldodecanoic	i.br.13:0	0.220	—
10-methyldodecanoic	a.i.br.13:0	0.238	0.242
n-tridecanoic	13:0	0.250	0.250
11-methyltridecanoic	i.br.14:0	0.365	—
n-tetradecanoic	14:0	0.44	0.42
13-methyltetradecanoic	i.br.15:0	0.54	0.55
12-methyltetradecanoic	a.i.br.15:0	0.59	0.58
n-pentadecanoic	15:0	0.67	0.66
14-methylpentadecanoic	i.br.16:0	0.82	—
n-hexadecenoic	16:1	0.91	0.90
n-hexadecanoic	16:0	1.00	1.00
15-methylhexadecanoic	i.br.17:0	1.22	—
14-methylhexadecanoic	a.i.br.17:0	1.35	1.38
n-heptadecanoic	17:0	1.50	1.51
n-octadeca-di and tri-enoic	18:2 & 18:3	1.90	1.90
n-octadecenoic	18:1	2.98	2.03
16-methyloctadecanoic	i.br.18:0	1.93	—
n-octadecanoic	18:0	2.26	2.36

* See Table 2.

TABLE 8

The relative percentage fatty acid composition of the bacterial and protozoal fractions from the rumen of a lactating fistulated dairy cow.

Fatty Acid	Shorthand Notation*	Bacterial Fraction	Protozoal Fraction
n-decanoic	10:0		
n-undecanoic	11:0		
n-dodecanoic	12:0	0.7	0.3
branched tridecanoic	br.13:0	0.7	0.1
n-tridecanoic	13:0	0.3	
branched tetradecanoic	br.14:0	1.5	0.1
n-tetradecanoic	14:0	3.5	0.7
branched pentadecanoic	br.15:0	11.0	1.0
n-pentadecanoic	15:0	5.0	1.0
n-hexadecenoic	16:1	1.0	0.1
branched hexadecanoic	br.16:0	1.1	0.2
n-hexadecanoic	16:0	29.0	20.0
branched heptadecanoic	br.17:0	1.1	0.3
n-heptadecanoic	17:0	1.1	1.2
n-octadeca-di and tri-enoic	18:2 & 18:3	6.0	8.5
n-octadecenoic	18:1	4.3	6.8
branched octadecanoic	br.18:0	1.1	0.5
n-octadecanoic	18:0	32.6	59.2

* See Table 2.

Gaps in Table 8 indicate that, if a fatty acid was present, it did not constitute more than 0.1 per cent of the total acids.

4.6. Preliminary Growth Experiments carried out with Cultures of Mixed Rumen Bacteria

Before the experiments using radioactive substrates were begun, preliminary experiments (experiments 1 and 2) were carried out to establish that rumen bacterial suspensions would grow under the conditions used and also to determine the effect, if any, of the addition of unlabelled *iso*butyric acid to the medium on the fatty acids of these bacteria.

In experiment 1 a suspension of mixed rumen bacteria, prepared as described in Chapter 3, was grown in two flasks, each containing 1 litre of the culture medium described in Chapter 3 (page 25). Experiment 2 was identical with experiment 1 except that 0.44 gm. (5 μ moles/ml.) of *iso*butyric acid were added to one of the flasks (column B, experiment 2, Table 10).

The total volatile fatty acids in the cultures were estimated at the beginning and end of incubation to give a measure of the amount of fermentation which had taken place during the experiment. These results, for all of the experiments with suspensions of mixed rumen bacteria, are shown in Table 9.

At the end of the incubation period the bacteria were harvested and the methyl esters of the fatty acids prepared from the total lipid extract. The fatty acid composition of the bacteria grown in experiments 1 and 2 is shown in Table 10. The most notable features about the fatty acid compositions shown in Table 10 are the occurrence of fatty acids with more than one double bond in the sample of bacteria used to inoculate the culture, and the decrease in the proportion of branched chain fatty acids in the lipids of the mixed bacteria after incubation of the mixed suspension.

TABLE 9

The concentration (μmoles/ml.) of steam volatile fatty acids (V.F.A^{S}.) in the cultures of mixed rumen bacteria at the beginning (0 hrs.) and end of incubation. (See text for experimental details.)

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		
Flask	A	B	A	B	A	B	A	B	C
0 hrs.	47.0	46.3	53.4	58.2	51.3	56.5	49.7	55.1	49.0
End of incubation	86.5	80.9	90.5	92.4	97.6	104.9	78.2	73.8	73.7
V.F.A^{S}. produced	39.5	34.6	37.1	34.2	46.3	48.4	28.5	18.7	24.7

Experiments 1 and 2 were preliminary growth experiments.

Flask A in all experiments and flask B in experiment 1 contained the basic culture medium only.

In experiments 3 and 4 flask B contained the basic culture medium plus isobutyrate-1-C^{14} while flask C in experiment 4 contained the basic culture medium plus dl-valine-4-C^{14}.

TABLE 10

The relative percentage fatty acid composition of the mixed rumen bacteria grown in experiments 1 and 2. (Preliminary growth experiments.)

Fatty Acid	Shorthand Notation*	Experiment 1			Experiment 2		
		X**	A	B	X**	A	B
n-decanoic	10:0		0.36	0.25			
n-undecanoic	11:0	0.18	1.23	1.07	0.13	0.28	0.33
branched dodecanoic	br.12:0	0.25	0.38	0.35	0.22	0.23	0.22
n-dodecanoic	12:0	0.29	1.45	1.23	0.33	1.45	1.10
branched tridecanoic	br.13:0	0.51	0.36	0.29	0.70	0.51	0.22
n-tridecanoic	13:0	0.23	0.51	0.72	0.18	0.70	0.38
branched tetradecanoic	br.14:0	2.92	2.53	2.82	1.32	0.98	1.53
n-tetradecanoic	14:0	3.45	6.36	6.13	2.33	7.94	6.14
branched pentadecanoic	br.15:0	11.92	10.43	10.98	10.12	7.85	7.02
n-pentadecanoic	15:0	7.35	6.09	6.35	4.39	3.69	3.56
n-hexadecenoic	16:1		2.39	2.93		2.07	2.19
branched hexadecanoic	br.16:0	1.43	1.52	1.72	1.01	1.73	1.32
n-hexadecanoic	16:0	27.83	33.80	32.92	35.18	38.63	38.15
branched heptadecanoic	br.17:0	1.32	2.46	3.25	1.65	2.95	3.13
n-heptadecanoic	17:0	2.59	2.67	2.89	1.78	1.92	3.29
n-octadeca-di and tri-enoic	18:2 18:3	4.87			3.47		
n-octadecenoic	18:1	3.45	6.12	6.09	3.91	2.12	5.65
branched octadecanoic	br.18:0	1.25	1.35	1.08			
n-octadecanoic	18:0	30.17	20.00	18.93	32.00	25.70	25.45

* See Table 2.

** Column X in each experiment shows the fatty acid composition of a sample of the bacteria used to prepare the mixed suspensions for each experiment.

Columns A and B in experiment 1 and column A in experiment 2 show the fatty acid composition of mixed rumen bacteria grown in the basic medium.

Column B in experiment 2 shows the fatty acid composition of mixed rumen bacteria grown in the basic medium supplemented with 5 μ moles/ml. of isobutyric acid.

4.7. The Incorporation of the Radioactivity from isobutyrate-1-C^{14} and valine-4-C^{14} into the Long Chain Fatty Acids of Mixed Rumen Bacteria grown as Washed Suspensions.

Two experiments were carried out to determine the fate of the label from isobutyrate-1-C^{14} when this short chain fatty acid was incubated with a mixed culture of rumen bacteria. The first of these experiments (experiment 3) was similar to experiment 2 described previously (page 49), but in addition to the 5 μ moles/ml. of unlabelled isobutyric acid which was added to one flask (flask A), 10 μ -curies of sodium isobutyrate-1-C^{14} were also added to the medium in this flask. The other flask contained only the mixed suspension of rumen bacteria in the basic medium and acted as a control (flask B).

The volatile fatty acid production by the mixed bacteria was determined by measurement of the concentration of these acids at the beginning and end of incubation. These results are shown in Table 9.

Samples of the whole culture, supernatant from the culture and steam distillate of the culture supernatant were obtained at the end of incubation from the suspension which contained isobutyrate-1-C^{14} (flask B) and the radioactivity of these fractions was determined and is shown in Table 11. The difference in radioactivity between the whole culture and the culture supernatant represents approximately the amount of isobutyrate-1-C^{14} which was taken up from the culture medium by the bacteria. However, as the activity of the whole cells was not determined, it was not possible to obtain an accurate estimate of the total radioactivity taken up by the bacteria.

A sample of the steam volatile fatty acids obtained from the culture supernatant from flask B by steam distillation was separated by gas-liquid chromatography and all of the C^{14} was located in the isobutyric acid fraction. The radioactivity of the other steam volatile fatty acids was not significantly above background.

TABLE 11

The radioactivity (counts per minute) of fractions of the mixed suspension of rumen bacteria at the end of incubation in the basic medium plus 5 μ moles/ml. of isobutyric acid-1-C^{14} (10 μ -curies).
(Experiment 3, flask B.)

Fraction	Counts/Minute per ml. of Culture Medium (Corrected for Background)	Percentage of Radioactivity in Whole Suspension
Whole suspension	4,900	100
Supernatant of suspension	3,800	77
Steam distillate of supernatant	3,700	75
Residue from steam distillation	-	-

The lipids were extracted from the bacterial cells from flasks A and B after freeze-drying and the methyl esters of the fatty acids were prepared. The relative percentage fatty acid composition of the bacterial lipid was determined by gas-liquid chromatography and is shown in Table 12 (columns A and B), together with the fatty acid composition of the mixed rumen bacteria used to prepare the suspension (column X).

The radioactivity of the total lipid and total fatty acid fractions from the bacteria grown in flask B was determined, but the results obtained were inconclusive due to the presence of isobutyrate-1-C^{14} from the medium which had remained bound to the surface of the cells and was extracted into the total lipid fraction and from here into the fatty acids (cf. experiments with S. bovis fatty acids, page 43). Since isobutyric acid is soluble in water, as well as in lipid solvents, the various washing procedures involved in the saponification of the lipids resulted in a considerable loss of radioactivity between the total lipid and total fatty acid fractions. Investigation of the radioactivity present in the washings from the fatty acid fraction after saponification showed that this activity was located in a steam volatile component, which was assumed to be isobutyrate-1-C^{14}.

The total fatty acids from the lipid extract were then steam distilled and, of the total activity of the fatty acids from the bacteria grown in flask B (7,500 counts per minute), one third (2,800 counts per minute) was found in the steam volatile fraction, while the remainder (4,500 counts per minute) was in the long chain fatty acids. An unsuccessful attempt was made to separate the steam volatile fatty acids by gas-liquid chromatography, as described in Chapter 3, but the total amount of fatty acids present was below the limits of detection of the apparatus used. It was probable that most of the radioactivity in the steam volatile fatty acids of the bacteria in this experiment was due to isobutyrate-1-C^{14} from the culture medium which had been extracted into the fatty acid fraction.

TABLE 12

The relative percentage fatty acid composition of the mixed rumen bacteria grown in experiment 3.

Fatty Acid	Shorthand Notation*	X	A	B
n-decanoic	10:0	0.79	0.58	0.61
branched undecanoic	br.11:0	0.07		
n-undecanoic	11:0	0.70	0.32	0.37
branched dodecanoic	br.12:0	0.25	0.27	0.24
n-dodecanoic	12:0	0.41	1.89	1.34
branched tridecanoic	br.13:0	0.67	0.74	0.61
n-tridecanoic	13:0	0.22	0.87	0.55
branched tetradecanoic	br.14:0	1.31	1.43	1.95
n-tetradecanoic	14:0	2.16	8.23	7.95
branched pentadecanoic	br.15:0	10.50	6.58	7.32
n-pentadecanoic	15:0	4.87	4.99	5.12
n-hexadecenoic	16:1	-	3.98	3.35
branched hexadecanoic	br.16:0	1.72	1.23	1.52
n-hexadecanoic	16:0	31.60	37.93	36.85
branched heptadecanoic	br.17:0	2.42	2.87	3.17
n-heptadecanoic	17:0	2.96	1.32	1.40
n-octadeca-di & tri-enoic	18:2 + 18:3	4.38		
n-octadecenoic	18:1	1.54	5.43	4.51
branched octadecanoic	br.18:0	2.34	0.42	0.67
n-octadecanoic	18:0	31.80	20.85	22.85

* See Table 2.

Column X - Fatty acids from the suspension of mixed rumen bacteria used to inoculate the cultures.

Column A - Fatty acids from mixed rumen bacteria grown in the basic medium (flask A).

Column B - Fatty acids from mixed rumen bacteria grown in the basic medium plus isobutyrate-1-C^{14} (flask B).

After methylation and bromination the methyl esters of the long chain fatty acids from the bacteria grown in flask B were purified as described in Chapter 3 and the resulting methyl esters gave 2,350 counts per minute. The methyl esters were then separated by preparative gas chromatography and the radioactivity of the individual fractions was determined and is shown in Table 13. It was not practicable to collect individual fatty acid methyl esters with a chain length of less than C_{10} carbon atoms because of the closeness with which these acids emerged from the column. In this experiment (experiment 3) no radioactivity was found in the fatty acids with a chain length shorter than tridecanoic acid and it was presumed that these had been distilled off with the steam volatile fraction, although any fatty acids with a chain length of more than C_{10} carbon atoms would be expected to be in the non steam volatile fraction.

The branched and straight chain fatty acids of shorter chain length than $C_{15:0}$ proved very difficult to separate on a preparative scale on the gas chromatograph used. For this reason the radioactivity found in n-tetradecanoic and n-pentadecanoic acids was probably due to some of the labelled branched chain fatty acid being carried over from the preceding fraction. Table 13 shows that as the chain length of the fatty acid increased, the degree of separation of the branched and straight chain fatty acids improved. The separation of the straight and branched chain isomers of hexadecanoic, heptadecanoic and octadecanoic acids was complete and none of the radioactivity occurred in the straight chain fatty acids after hexadecanoic. It was probable that, in fact, all of the radioactivity present in the fatty acids was located in the branched chain fatty acids and none occurred in the straight chain acids of the bacteria grown in the presence of <u>iso</u>butyrate-1-C^{14}.

The details of experiment 4 were the same as for experiment 3 except that each flask contained 2 litres of medium, and 100 μ-curies of

TABLE 13

The relative specific activities (counts per 30 minutes per unit area of chromatogram peak) of the fatty acids from mixed rumen bacteria grown in a medium containing isobutyrate-1-C^{14}.
(Flask B, experiment 3.)

Fatty Acid Fraction	Total Radioactivity in Fraction (Counts per 30 minutes) (Corrected for Background)	Percentage of Total Radioactivity	Relative Specific Activity
n-dodecanoic and lower fatty acids	—	—	—
branched and n-tridecanoic	5,575	21.2	4,820
branched tetradecanoic	8,230	31.4	4,830
n-tetradecanoic	2,140	8.2	269
branched pentadecanoic	5,900	22.5	806
n-pentadecanoic	1,250	4.8	244
branched hexadecanoic	2,633	10.0	1,730
n-hexadecanoic	—	—	—
branched heptadecanoic	160	0.6	50
n-heptadecanoic	—	—	—
branched octadecanoic	340	1.3	507
n-octadecanoic	—	—	—

isobutyrate-1-C^{14} were added to the flask containing added isobutyric acid (flask B). In addition a third flask (flask C) was set up, to which was added 100 μ-curies of dl-valine-4-C^{14}, in an attempt to demonstrate the production of isobutyrate-3-C^{14} from valine by deamination and decarboxylation (see Figure 3, Chapter 1).

The volatile fatty acids produced by the bacteria during incubation were determined by steam distillation and are shown in Table 9. In this experiment the production of volatile fatty acids in all 3 flasks was only approximately one half of that which occurred in the previous experiments. It was also observed that the "lag" phase of growth which occurred at the start of incubation was increased to 7 hours, compared with the "lag" phase in the previous experiments of from 3-4 hours. (The "lag" phase was taken to be the period of time before gas production occurred in the cultures.)

The radioactivity of the whole culture, culture supernatant and steam distillate of the supernatant from flasks B and C was determined at the end of incubation and the results are reported in Table 14. A sample of the culture medium from flask C taken at the time of addition of valine-4-C^{14} was also steam distilled and the radioactivity of the steam distillate determined. No radioactivity was detected in the steam volatile fraction at this time, indicating that the activity of this fraction at the end of incubation was due to the presence of a radioactive steam volatile component and not to mechanical carry over of valine-4-C^{14} during distillation.

As was the case in experiment 3, it was difficult to obtain an accurate estimate of the extent of uptake of the labelled compounds into the bacterial cells. However from observation of Table 14, a much larger proportion of the valine-4-C^{14} (approximately 40 per cent) was taken up by the bacteria than was the case with isobutyrate-1-C^{14}, where only approximately 13 per cent of the radioactivity in the total culture was lost when the bacteria were removed.

TABLE 14

The radioactivity (counts per minute per ml. of culture) of fractions of the mixed rumen bacterial suspensions at the end of incubation (experiment 4).

	Medium plus *iso*butyrate-1-C^{14} (Flask B)		Medium plus dl-valine-4-C^{14} (Flask C)	
Fraction	Radioactivity in Counts/Minute per ml. of Culture (Corrected for Background)	Percentage of Counts in Total Suspension	Radioactivity in Counts/Minute per ml. of Culture (Corrected for Background)	Percentage of Counts in Total Suspension
Whole suspension	57,500	100	51,900	100
Supernatant of suspension	50,100	87	31,600	60
Steam distillate of supernatant	49,800	87	2,000	4
Residue from steam distillate	—	—	29,300	56

TABLE 15

The radioactivity present in a sample of the steam volatile fatty acids from the culture at the end of incubation of a mixed suspension of rumen bacteria in the basic medium together with isobutyrate-1-C^{14} and dl-valine-4-C^{14}. (Experiment 4)

	Radioactivity (counts per 10 minutes) (corrected for background)	
Fatty Acid	Medium plus isobutyrate-1-C^{14}	Medium plus dl-valine-4-C^{14}
Acetic	3	3
Propionic	4	29
Isobutyric	350	125
Butyric	9	6

TABLE 16

The relative percentage fatty acid composition of the mixed rumen bacteria grown in experiment 4.

Fatty Acid	Shorthand Notation*	Percentage of total fatty acids			
		X	A	B	C
n-decanoic	10:0	0.45	1.07	0.50	1.14
branched undecanoic	br.11:0		1.26	1.47	
n-undecanoic	11:0	2.67	0.93	0.76	1.73
branched dodecanoic	br.12:0	0.34	2.98	3.02	2.63
n-dodecanoic	12:0	0.51	3.49	3.19	3.27
branched tridecanoic	br.13:0	0.67	1.42	1.56	2.58
n-tridecanoic	13:0	0.17	0.86	0.29	0.53
branched tetradecanoic	br.14:0	1.24	2.16	2.27	2.37
n-tetradecanoic	14:0	3.14	9.12	8.08	8.23
branched pentadecanoic	br.15:0	12.81	3.69	3.19	3.27
n-pentadecanoic	15:0	4.83	2.12	2.18	2.49
n-hexadecenoic	16:1		4.98	4.23	3.64
branched hexadecanoic	br.16:0	1.74	1.42	1.57	1.93
n-hexadecanoic	16:0	32.52	37.03	39.90	38.34
branched heptadecanoic	br.17:0	1.01	2.16	2.77	2.98
n-heptadecanoic	17:0	0.67	1.12	1.39	1.36
n-octadeca-di & tri-enoic	18:2 18:3	6.85			
n-octadecenoic	18:1	3.51	11.95	10.57	10.62
branched octadecanoic	br.18:0	1.10	1.07	1.39	1.52
n-octadecanoic	18:0	25.38	11.52	11.20	11.53

* See Table 2.

Column X - Fatty acids from the suspension of mixed rumen bacteria used to inoculate the cultures.

Column A - Fatty acids from mixed rumen bacteria grown in the basic medium (flask A).

Column B - Fatty acids from mixed rumen bacteria grown in the basic medium plus isobutyrate-1-C^{14} (flask B).

Column C - Fatty acids from mixed rumen bacteria grown in the basic medium plus dl-valine-4-C^{14} (flask C).

TABLE 17

The relative specific activities (counts per 10 minutes per unit area of chromatogram peak) of the fatty acids from mixed rumen bacteria grown in a medium containing *iso*butyrate-1-C^{14} (Flask B, experiment 4.)

Fatty Acid Fraction	Total Radioactivity in Fraction (Counts per 10 Minutes) (Corrected for Background)	Percentage of Total Radioactivity	Relative Specific Activity
Less than undecanoic	4,200	27.0	—
n-undecanoic and branched undecanoic	1,960	12.5	850
n-dodecanoic and branched dodecanoic	1,830	11.8	570
n-tridecanoic and branched tridecanoic	1,880	12.1	990
branched tetradecanoic	1,500	9.7	660
n-tetradecanoic	1,420	9.1	170
branched pentadecanoic	1,070	6.9	330
n-pentadecanoic	390	2.5	179
branched hexadecanoic	500	3.2	320
n-hexadecanoic	—	—	—
branched heptadecanoic	650	4.2	235
n-heptadecanoic	—	—	—
branched octadecanoic	150	1.0	100
n-octadecanoic	—	—	—

TABLE 18

The relative specific activities (counts per 10 minutes per unit area of chromatogram peak) of the fatty acids from mixed rumen bacteria grown in a medium containing dl-valine-4-C^{14} (Flask C, experiment 4.)

Fatty Acid Fraction	Total Radioactivity in Fraction (Counts per 30 minutes) (Corrected for Background)	Percentage of Total Radioactivity	Relative Specific Activity
Less than decanoic	11,750	44.5	—
branched decanoic and n-decanoic	2,100	8.1	1,760
branched undecanoic and n-undecanoic	2,450	9.4	1,630
branched dodecanoic and n-dodecanoic	1,930	7.4	327
branched tridecanoic and n-tridecanoic	1,730	6.6	423
branched tetradecanoic	2,310	9.0	1,005
n-tetradecanoic	780	3.0	85
branched pentadecanoic	300	1.3	70
n-pentadecanoic	90	0.3	36
branched hexadecanoic	220	0.8	105
n-hexadecanoic	—	—	—
branched heptadecanoic	1,590	6.1	548
n-heptadecanoic	450	1.7	521
branched octadecanoic	30	0.1	20
n-octadecanoic	—	—	—
branched nonadecanoic	320	1.2	413

A sample of the steam volatile fatty acids obtained from the medium containing *iso*butyrate-1-C^{14} and valine-4-C^{14} (flasks B and C) at the end of incubation, was separated by gas-liquid chromatography and the results are shown in Table 15. All of the C^{14} in the steam volatile fraction was shown to be present in *iso*butyrate-1-C^{14} in the culture from flask B, to which this labelled metabolite had been added prior to incubation.

Most of the radioactivity in the steam volatile fatty acids from the culture containing valine-4-C^{14} (flask C) was also located in *iso*butyric acid although a small but significant fraction was found in the propionic acid (Table 15).

The lipids were extracted from the bacterial cells grown in flasks A, B, and C, and fatty acid methyl esters were prepared. The fatty acid compositio was determined by gas-liquid chromatography and is shown in Table 16. After bromination and purification, as described in Chapter 3, the methyl esters from bacteria grown in the presence of *iso*butyrate-1-C^{14} (flask B) contained 5,000 counts per minute and those bacteria grown in a medium containing valine-4-C^{14} contained 1,200 counts per minute. These fatty acid methyl esters were then separated by preparative gas chromatography and the radioactivity of the individual fatty acids was determined and is shown in Tables 17 and 18.

Difficulty was again experienced in separating the branched chain fatty acid esters from their straight chain isomers, especially those of fatty acids with a chain length of less than C_{16} carbon atoms. It was probable that most of the C^{14} in the fatty acids shown in Tables 17 and 18 was located in the branched chain fatty acids. However the labelling of n-heptadecanoic acid in the experiment in which valine-4-C^{14} was added to the culture medium (Table 18 did not appear to be due to contamination with the branched chain isomer since the branched and normal isomers of hexadecanoic acid, which emerge before heptadecanoic acid, were separated cleanly. It was probable that pentadecanoic and tridecanoic acids were also labelled in this experiment but this could not be determined because of the carry over of radioactivity from the preceding branched chain fatty acid.

A large proportion of the radioactivity in the fatty acid methyl esters from the bacteria in flasks B and C was found in the fatty acids with a chain length of less than C_{11} carbon atoms. It was likely that a large proportion of this radioactivity was due to the presence in the fatty acid fraction of isobutyrate-1-C^{14}, which had adhered to the bacterial cells after washing, and had been extracted into the fatty acid fraction. The total fatty acids from the lipid extract of the bacteria grown in this experiment (experiment 4) were not separated by steam distillation, as was done in experiment 3 (page 54). Consequently the radioactivity from the labelled isobutyric acid from the culture medium was separated by preparative gas chromatography and could account for the high activity of the fraction containing all the methyl esters of the fatty acids with a chain length of less than C_{11} carbon atoms (Tables 17 and 18).

Chapter 5

DISCUSSION

5.1. The Fatty Acids of *Streptococcus bovis*

The total lipid extracted from *S. bovis* with chloroform:methanol by the methods described in Chapter 3 comprised 7.2 per cent of the freeze-dried bacterial cells, which is somewhat more than the 5.5 and 5.3 per cent of total lipid reported for *S. lactis* and *S. cremoris* respectively (MacLeod and Brown, 1963). However these authors washed the chloroform:methanol extracts according to the method of Folch *et al.* (1957). This procedure, which removes water soluble non lipid material from the chloroform:methanol extract, was not used in the present investigation. Hofman and Tausig (1955) found that the total lipid content of *S. haemolyticus* was 4.5 per cent of the dry cell weight of this organism. The extraction methods used by these authors involved acetone extraction of "free" lipids followed by acid hydrolysis and extraction of the "bound" lipids with ether. *S. faecalis* has been found to contain only 2.9 per cent of chloroform:methanol extractable lipids (Vorbeck and Marinetti, 1965).

The medium used in the present work for the culture of *S. bovis* was based on an acetate buffer (Chapter 3, page 23). Since acetate is known to be a precursor of long chain fatty acids (Wakil, 1961; Lynen, 1962), it is possible that this metabolite promoted lipogenesis in *S. bovis* in the experiments reported in this work and resulted in the high proportion of lipid reported. Differences in lipid extraction methods could also account for some of the differences in total lipid content in the work discussed above The stage of growth which the culture has reached at the time of harvesting is a further possible cause of variation in the lipid content of the bacteria.

The identity of the isomer of octadecenoic acid found in *S. bovis* was not conclusively established in this investigation. Oleic acid (*cis*-9-octadecenoic) and *cis*-vaccenic acid (*cis*-11-octadecenoic) are not likely to be separated on the gas chromatograph liquid phases used (Apiezon M and P.G.A.) although, according to James (1960), it is theoretically possible to separate them on a liquid phase of Apiezon M. Because of this difficulty in separation the fact that a single octadecenoic acid peak was obtained when methyl oleate was co-chromatographed on Apiezon M with the fatty acid methyl esters of *S. bovis* does not prove that the fatty acid is oleic acid. Reports of several authors on the fatty acids of other *Lactobacteriaceae* indicate that, in all of the members of this family studied to date, *cis*-vaccenic acid is the major octadecenoic acid present (Hofman *et al*., 1952; Hofman and Sax, 1953; Hofman and Tausig, 1955). MacLeod and Brown (1963) have commented on the difficulty of resolving oleic and *cis*-vaccenic acids by gas-liquid chromatography and have suggested, on the basis of the work of Hofman reported above, that the octadecenoic acid present in these organisms was *cis*-vaccenic acid. Consequently the octadecenoic acid found in *S. bovis* in this investigation was considered to be *cis*-vaccenic acid.

The identification of lactobacillic acid in the fatty acids of *S. bovis* is open to question as it is only based on gas chromatography data (see Chapter 4, page 39). However in all other *Lactobacteriaceae* previously studied, with the possible exception of *Streptococcus haemolyticus*, lactobacillic acid has been shown to be a major component of the fatty acid spectrum, comprising up to 50 per cent of the total fatty acids (Hofman *et al*.. 1952; Hofman and Sax, 1953; Hofman and Tausig, 1955; MacLeod *et al*., 1962; MacLeod and Brown, 1963). Hofman and Tausig (1955) could find no lactobacillic acid in *S. haemolyticus* after separation of the fatty acid methyl esters of this organism by fractional distillation and subsequent examination of the fractions by infra-red spectroscopy. The possibility that lactobacillic acid is present in *S. haemolyticus* but occurs in proportions too low to be detected by these methods should not be discounted.

The fatty acid spectrum of S. bovis shown in Table 4 is similar to that reported by MacLeod and Brown (1963) for the fatty acids of S. lactis and S. cremoris. The major differences between the fatty acids of these three bacteria is in the proportion of cis-vaccenic and lactobacillic acids. In S. bovis, under the conditions of the present work, the octadecenoic acid considered to be cis-vaccenic is the most abundant fatty acid, comprising 40 per cent of the total acids, while lactobacillic acid constitutes only 4 per cent of the total fatty acids. S. cremoris and S. lactis both have higher proportions of lactobacillic acid (44 per cent and 20 per cent) but less cis-vaccenic acid (4.8 per cent and 17 per cent) (MacLeod and Brown, 1963) than S. bovis.

Lui and Hofman (1962) have shown that lactobacillic acid is formed by the addition of the methyl group of S-adenosyl methionine across the double bond of cis-vaccenic acid. Because of this metabolic relationship between these two fatty acids it is conceivable that bacteria harvested at a late stage in their growth cycle would have a greater proportion of lactobacillic acid and a lesser proportion of cis-vaccenic acid than bacteria harvested at an earlier stage. An inverse relationship of this type has been demonstrated by Robertson (1964) between oleic acid and 10-methylstearic acid in the lipids of Nocardia corallina. The metabolic relationship between these two fatty acids (oleic and 10-methylstearic) is analogous to that which occurs between lactobacillic acid and cis-vaccenic acid since Lennarz et al. (1962) have demonstrated the formation of 10-methylstearic acid by addition of a methyl group across the double bond of oleic acid. Robertson (1964) showed that during the exogenous, or logarithmic, phase of growth of N. corallina, oleic acid was present in greater proportions than 10-methylstearic, and during the endogenous phase the oleic acid was converted to 10-methylstearic acid and the proportion of the two fatty acids changed.

It is quite possible that a similar relationship could occur in *S. bovis* between *cis*-vaccenic and lactobacillic acids and that this relationship could account for some, if not all, of the differences reported in the proportion of these fatty acids in various members of the family *Lactobacteriaceae*. In the experiments with *S. bovis* reported in this work it was likely that growth of the organism stopped at a relatively early stage, due to the production of lactic acid, and the consequent fall in pH to levels below optimum for the growth of the organism. Hence it is unlikely that the bacteria ever reached a true endogenous phase of growth caused by the depletion of the substrates in the culture medium, although insufficient data was obtained to confirm this hypothesis. This is one possible reason for the low proportion of lactobacillic acid and high proportion of *cis*-vaccenic acid present in the lipids of *S. bovis* reported in this work.

This type of inverse relationship could also account for the difference in the proportions of these two fatty acids in *S. lactis* and *S. cremoris* reported by MacLeod and Brown (1963). These authors found that the fatty acids of *S. lactis*, which was harvested after 16-20 hours of growth, contained only 19 per cent of lactobacillic acid, while the fatty acids of *S. cremoris*, which was not harvested until 48-60 hours after inoculation, contained 44 per cent of lactobacillic acid. These authors did not say what stage of growth the bacteria had reached at the time of harvesting.

It is apparent from the above that, in any attempt to compare the fatty compositions of a group of bacteria, it is necessary to ascertain the stage of growth reached by the culture at the time of harvesting, in addition to the conditions of culture, and the composition of the medium used.

The absence of any branched long chain fatty acids of the *iso* or *anteiso* series in *S. bovis* in the experiments reported here is consistent with the fatty acid spectra reported for other members of the same family of bacteria (Hofman and Sax, 1953; Hofman and Tausig, 1955; MacLeod *et al.*, 1962;

MacLeod and Brown, 1963). It is also consistent with reports which indicate that branched chain fatty acids of this type have been found only in those rumen bacteria which are cellulolytic and which require branched chain volatile fatty acids as growth factors (Allison *et al.*, 1958, 1962). *S. bovis* does not exhibit either of these characteristics.

A fatty acid synthesizing enzyme system isolated from rat epididymal adipose tissue has been shown to synthesize straight and branched chain fatty acids from the appropriate short chain fatty acid acyl Coenzyme A precursors (Horning *et al.*, 1961). The proportions of the various long chain fatty acids produced was shown by these authors to be dependant, to a large extent, on the relative amounts of the various short chain fatty acid precursors present in the system. If a similar enzyme system was present in *S. bovis*, the high proportion of acetate in the culture medium used in this investigation could have encouraged the production of the straight chain fatty acids and suppresse any possible production of branched chain fatty acids. However there has been no evidence reported in the literature to suggest the presence of an enzyme system similar to that reported by Horning *et al.* (1961) in *S. bovis*.

The addition of *iso*butyrate-1-C^{14} to the *S. bovis* culture medium revealed that very little (less than 1 per cent) (Table 5) of this metabolite was taken up from the medium by the bacteria. None of the C^{14} label was incorporated into the long chain fatty acids of the organism, which indicates that there was no direct incorporation of *iso*butyrate into these fatty acids. It is possible that some *iso*butyrate was metabolised by *S. bovis* to produce propionic acid by the pathway postulated by Rendina and Coon (1957) and shown in Figure 15.

Propionate produced from *iso*butyrate-1-C^{14} by this pathway would not contain any of the C^{14} label as carbon ~~C~~ 1 would have been lost as carbon dioxide If this propionic acid was subsequently incorporated into the long chain fatty acids they also would not contain any of the C^{14} label and could not be detected by radiochemical means. However the slight, but consistent, increas in the proportions of the long chain fatty acids having an odd number of carbon atoms in S. bovis grown on a medium supplemented with isobutyrate suggests that isobutyric acid was metabolised by the organism and the propionic acid produced incorporated into the long chain fatty acids with an odd number of carbon atoms. Further work using isobutyrate-3 or 4-C^{14} is necessary before any conclusions can be reached regarding the metabolism of isobutyrate by S. bovis.

The work discussed in this section shows that, under the experimental conditions imposed, S. bovis does not synthesize branched chain fatty acids of the iso or anteiso series even when isobutyric acid, a known precursor of the iso series of branched chain fatty acids, is added to the medium. This evidence, together with the fact that S. bovis is non-cellulolytic and does not require branched chain volatile fatty acid growth factors, makes it unlikely that this bacteria contributes to the high proportion of branched chain fatty acids produced in vivo in the rumen.

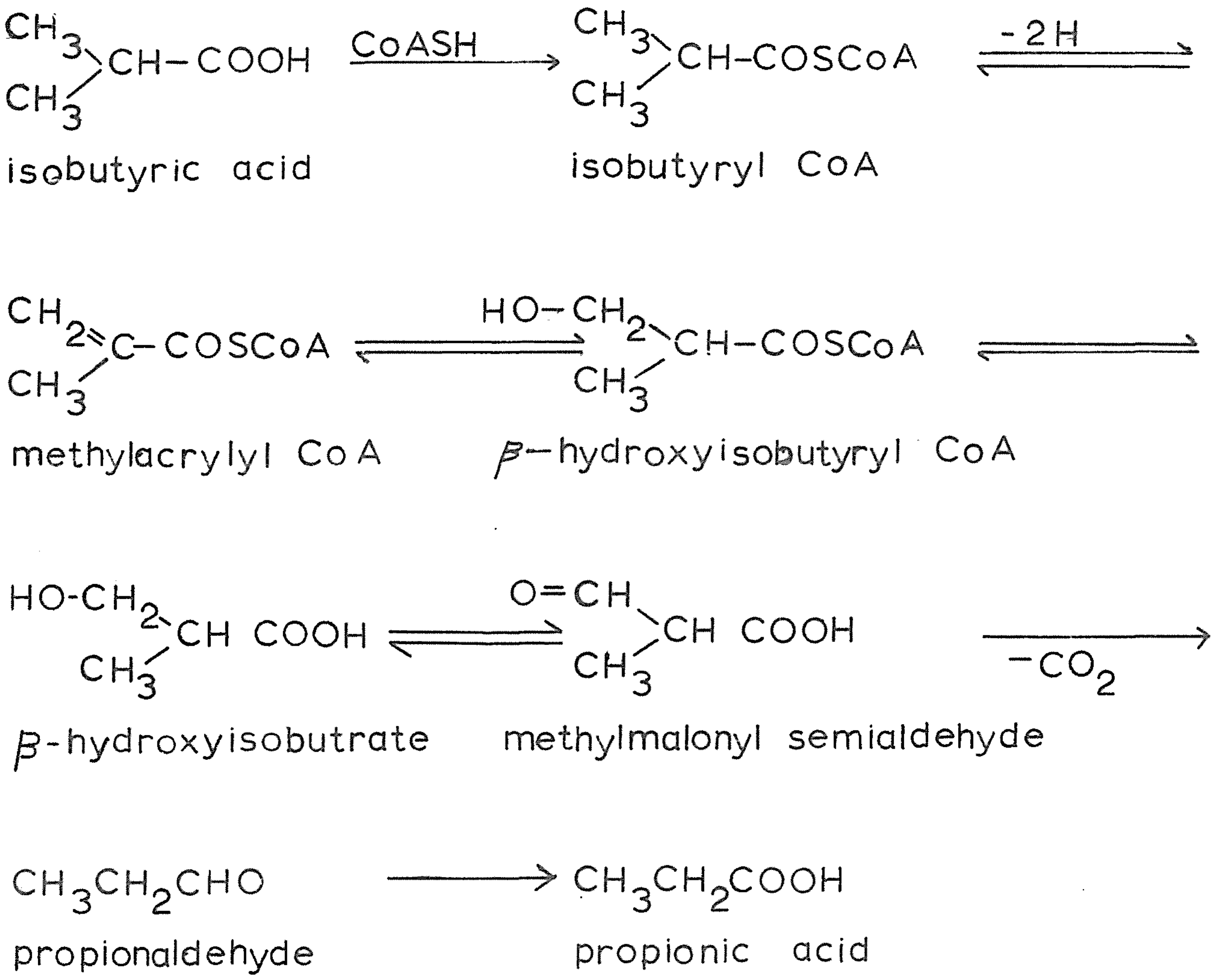
5.2. The Branched Chain Fatty Acids of Rumen Bacteria and Protozoa

The fatty acid composition of the rumen micro-organisms examined in the present work (Table 8) showed that the bacteria contained a far greater proportion of branched chain fatty acids than the protozoa. This is in agreement with the results of Keeney et al. (1962) and in fact the complete spectrum of rumen bacterial fatty acids shown in Table 8 agrees with the fatty acid composition of the rumen bacteria reported by these authors. The protozoal fraction examined in the present work was, like that of Keeney et al (1962), observed to contain some finely divided food particles which had passed through the cheese cloth filter used to separate the solid rumen ingesta from the liquid rumen fluid. Since these food particles were likely to have colonies of bacteria adhering to them, they were a probable source of bacterial contamination in the protozoal fraction. The fatty acids from these bacteria could quite reasonably have accounted for all of the branched chain acids found in the fatty acids from the protozoal fraction, both in the present work and in the work of Keeney *et al*. (1962). It would have been of interest to determine whether rumen protozoa, free of contaminating bacteria, do contain any branched chain fatty acids.

Erwin and Bloch (1963) have reported quite large proportions (up to 20 per cent) of branched chain fatty acids of the *iso* series in the fatty acids of several species of the protozoon family *Tetrahymenidae*. However the presence in the rumen of protozoa of this family has not been reported.

The presence of di- and tri-enoic fatty acids in the mixed rumen bacteria examined in this work was almost certainly due to the presence of chloroplasts and other finely divided plant material in this fraction. A green colouration which indicated the presence of chlorophyll containing material, occurred in all suspensions of rumen bacteria even after repeated washings. Weenink (1961 has shown that the fatty acids from ryegrass and clover chloroplasts contain a large proportion (up to 85 per cent) of linolenic acid. Since Erwin *et al*. (1964) has stated that no bacteria have been found to contain unsaturated fatty acids with more than one double bond it is unlikely that these fatty acids occurred in the rumen bacteria. The evidence for the occurrence of di- and tri-enoic fatty acids in the extraneous plant material, present in the bacterial fraction and not in the bacteria, was strengthened by the observation that these fatty acids disappeared from the mixed bacterial lipids after incubation and growth of the bacteria (Tables 10, 12, 16).

FIGURE 15



The pathway of catabolism of isobutyric acid.

5.3. The Production of isobutyrate-3-C^{14} from valine-4-C^{14} by Mixed Rumen Bacteria

The production of C^{14} labelled *iso*butyric acid from valine-4-C^{14} by a mixed population of rumen bacteria has been demonstrated in the present work (experiment 4, Tables 14 and 15). The mechanism involved was probably a mutual oxidation-reduction reaction between two amino acids, one of which was valine-4-C^{14}. In this type of reaction, which was first elucidated by Stickland (1935) and subsequently shown by el Shazly (1952b) and Dehority *et* (1958) to occur in mixed cultures of rumen bacteria, valine is oxidatively deaminated to form α-ketoisovaleric acid, while a second amino acid is reduced and ammonia is produced. α-ketoisovaleric acid is then oxidatively decarboxylated at the expense of another molecule of the second amino acid to form *iso*butyric acid. An outline of the reactions involved is shown in Figure 3 (Chapter 1). The formation of C^{14} labelled *iso*butyrate from valine-4-C^{14} in the present work is consistent with the occurrence of this type of reaction in mixed cultures of rumen bacteria.

The formation of C^{14} labelled propionic acid from valine-4-C^{14}, which has been demonstrated in the present work (Table 15), is in agreement with the known pathways of metabolism of this amino acid. The mechanism of formation of *iso*butyrate from valine has been outlined above and the subsequent pathway of catabolism of *iso*butyrate to propionate has been elucidated by a number of authors (Atchly, 1948; Kinnory *et al.*, 1955; Robinson and Coon, 1957; Rendina and Coon, 1957) and the reactions involved are shown in Figure 15.

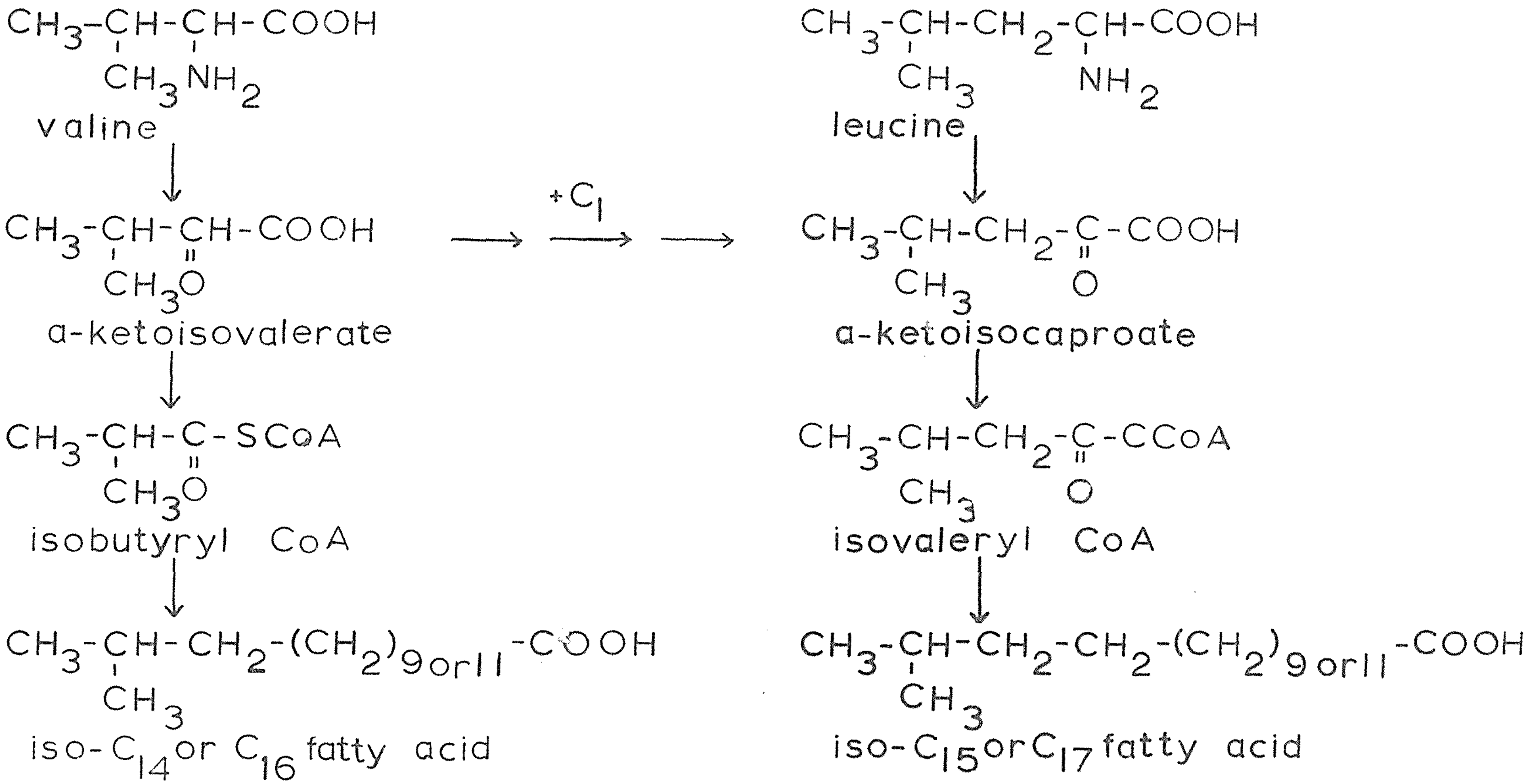
Reference to Figure 3 shows that after deamination and decarboxylation, carbon C_4 of the valine molecule becomes carbon C_3 or C_4 of *iso*butyric acid. In the subsequent metabolism of *iso*butyric acid (Figure 15) the carboxyl carbon (C_1) is lost by decarboxylation and carbon C_2, C_3 and C_4 become carbon C_1, C_2 and C_3 of propionic acid. Consequently in the experiments where *iso*butyrate-1-C^{14} was incubated with mixed rumen bacteria, the catabolism of this metabolite could not be followed because of the loss of carbon 1, as shown in the reaction scheme in Figure 15.

5.4. The Incorporation of the C^{14} Label from *iso*butyrate-1-C^{14} and valine-4-C^{14} into the Long Chain Fatty Acids of Mixed Rumen Bacteria

When mixed rumen bacteria were incubated in a medium containing *iso*butyrate-1-C^{14} the label was found to be located in the branched long chain fatty acids of the bacteria containing both odd and even numbers of carbon atoms (Tables 13 and 17). In experiment 3 (Table 13) a comparison of the relative specific activities of the fatty acids shows that the label is incorporated to a greater extent into those branched chain fatty acids with an even number of carbon atoms. However in experiment 4 (Table 17) the C^{14} label is incorporated approximately equally into both odd and even numbered branched chain fatty acids. Although the *iso* and *anteiso* isomers of the branched chain fatty acids could not be separated, it is probable that only the *iso* branched chain fatty acids were labelled in these experiments.

The incorporation of the C^{14} from *iso*butyrate-1-C^{14} into the *iso* series of branched long chain fatty acids with an even number of carbon atoms is in agreement with the pathways reported for the synthesis of branched chain fatty acids in pure cultures of several species of rumen bacteria (Wegner and Foster, 1963; Allison *et al*., 1962b). Those pathways have also been found to occur in several species of bacteria not found in the rumen (Lennarz, 1961; Kaneeda, 1963b). The mechanism postulated for their formation involves the addition of a variable number of two-carbon units (presumably acetate) to the carboxyl group of *iso*butyrate or a similar branched chain volatile fatty acid (el Shazly, 1952b) (see Figure 4).

The incorporation of the C^{14} label from *iso*butyrate-1-C^{14} or valine-4-C^{14} into the branched chain fatty acids with an odd number of carbon atoms has been demonstrated in the present work and this pathway has not previously been



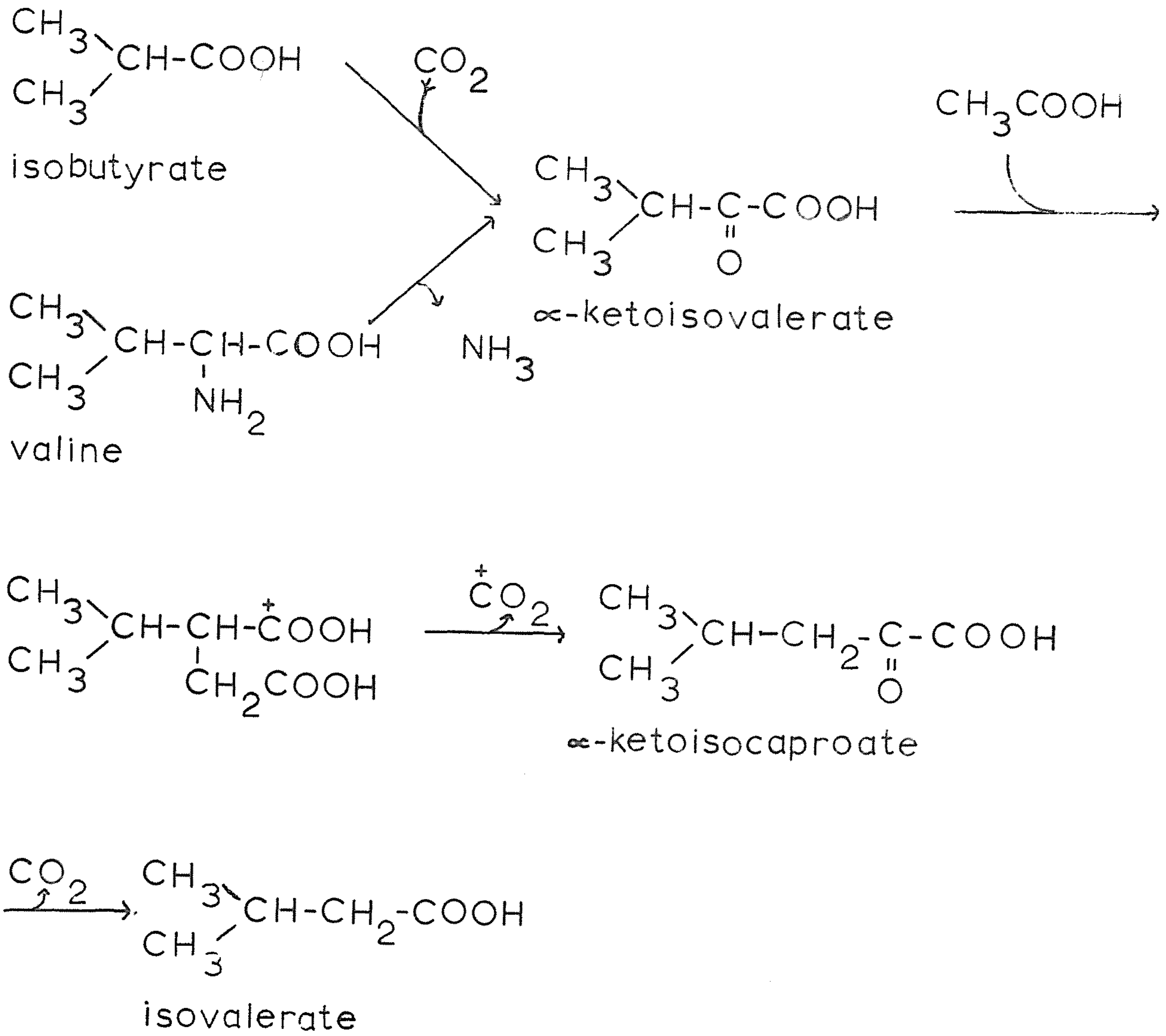
A possible sequence of reactions in the formation of <u>iso</u> branched chain fatty acids with an odd number of carbon atoms from <u>iso</u>butyric acid (Kaneeda, 1963c).

reported in any species of rumen bacteria. However Kaneeda (1963c) has reported that when *Bacillus subtilis* was grown in a medium containing valine-U-C^{14} the C^{14} label was incorporated into the *iso* series of branched chain fatty acids containing both odd and even numbers of carbon atoms. These findings are an exception to the generally accepted belief (Keeney *et a* 1962) that branched chain fatty acids of the *iso* and *anteiso* series are forme by the condensation of a number of acetate molecules with the appropriate branched chain volatile fatty acid precursor. On the basis of this theory one would expect only the *iso* series of branched chain fatty acids to be produced from *iso*butyrate.

Kaneeda (1963c) postulates a mechanism for the synthesis of the odd numbered branched chain fatty acids from valine which involves the formation of α-ketoisocaproate by condensation of α-ketoisovalerate and acetate, followed by the elimination of one carbon atom. A mechanism of this type has been shown to occur in several micro-organisms in connection with the synthesi of the amino acid leucine (Calvo *et al*., 1962; Gross *et al*., 1962; Jungwirth *et al*., 1961; Strassman and Ceci, 1962). The carboxyl carbon of α-ketoisocaproate is then lost and the resulting *iso*valerate is incorporated into the *iso* branched chain fatty acids having an odd number of carbon atoms. This reaction sequence, an outline of which is shown in Figure 16, could account for the production of C^{14} labelled odd numbered branched chain fatty acids from valine-4-C^{14} in mixed rumen bacteria since, in these organisms, α-ketoisovalerate has been shown to be an intermediate in the formation of *iso*butyrate from valine (Dehority *et al*., 1958).

For the formation of *iso* branched chain fatty acids with an odd number of carbon atoms from *iso*butyrate by reactions of this type, it is necessary that the *iso*butyrate first be carboxylated to α-ketoisovaleric acid. This reaction has not been reported in rumen bacteria, but Allison *et al*. (1962b), reporting unpublished work of Wegner and Foster, have stated that

FIGURE 17



The reaction sequence postulated for the production of isovaleric acid from isobutyric acid.

*iso*butyrate-1-C^{14} is a direct precursor of valine-2-C^{14} in the rumen bacteria *Ruminococcus flavefaciens*. If the reactions involved in this transformation are the reverse of those involved in valine catabolism, and outlined in Figure 3, then it is probable that α-ketoisovalerate is an intermediate in the formation of valine from *iso*butyrate. Consequently this metabolite would be available to enter the reaction pathway leading to the formation of *iso*valeric acid suggested by Kaneeda (1963c). The reactions postulated for the formation of *iso*valerate from *iso*butyrate and valine are shown in Figure 17.

Subsequent condensation of *iso*valeric acid-2-C^{14} (formed from *iso*butyric acid-1-C^{14} by this reaction) with a number of molecules of acetate, would produce C^{14} labelled *iso* branched chain fatty acids with an odd number of carbon atoms, as has been shown to occur in the present experiments (Tables 13, 17 and 18).

An alternative explanation for the production of odd numbered branched chain fatty acids from *iso*butyrate-1-C^{14} in the experiments reported in this work could be their formation by a process of α-oxidation from the even numbered branched chain fatty acids formed from this metabolite by condensation with acetate units. This process would involve the loss of one carbon unit from the carboxyl end of the even numbered branched long chain fatty acids. Saturated and unsaturated long chain fatty acids have been shown to be degraded by α-oxidation in plant systems (Martin and Stumpf, 1959 Castelfranco *et al.*, 1955; Stumpf, 1956; Hitchcock and James, 1964) and a similar mechanism has been demonstrated for the degradation of the long chain fatty acids found in brain tissue (Mead and Levis, 1963; Levis and Mead, 1964 However the plant enzyme systems responsible for α-oxidation have an absolute requirement for molecular oxygen (Hitchcock and James, 1964) and in some cases require an exogenous source of hydrogen peroxide (Martin and Stumpf, 1959). A similar requirement for oxygen for α-oxidation in brain tissue has not been demonstrated but no work has as yet been carried out on enzyme systems isolated from this tissue. These requirements make it unlikely that a similar enzyme system could operate in the rumen where the oxygen tension is very low. It is possible that in this work the oxygen tension in the culture flasks was sufficient to allow α-oxidation to take place but this would be dependent on the assumption that rumen bacteria possess the enzyme systems required for this pathway, and that branched chain fatty acids are subject to α-oxidation.

An α-oxidation system in rumen bacteria which is not dependent on molecular oxygen is also a possibility which should not be discounted, but further work is necessary before the mechanism of formation of the odd carbon number branched chain fatty acids from *iso*butyric acid can be finally elucidated.

The incorporation of C^{14} from valine-4-C^{14} into the branched long chain fatty acids of mixed rumen bacteria has been demonstrated in the present work (Table 18). The occurrence of C^{14} labelled *iso*butyrate in the medium supernatant at the end of this experiment (Table 15) suggests that this volatile fatty acid is an intermediate in the formation of branched long chain fatty acids from valine as suggested by el Shazly (1952b). It would have been of interest to elucidate whether the complete conversion of valine-4-C^{14} to the branched long chain fatty acids was carried out by any single rumen bacterial species or whether the deamination and decarboxylation of valine to *iso*butyrate was carried out by one group of bacteria in the rumen while the synthesis of the branched chain fatty acids from *iso*butyrate was undertaken by a second group. The occurrence of C^{14} labelled *iso*butyrate in the cell free supernatant from the culture in which valine-4-C^{14} was added to the mixed suspension of rumen bacteria suggests that two bacterial species are necessary for the complete reaction pathway. This conclusion is supported by the fact that those rumen bacteria which contain branched long chain fatty acids require a branched chain volatile fatty acid as a growth factor (Bryant and Robinson, 1961) and the addition of valine, or a similar branched chain amino acid to the culture medium for these organisms did not satisfy this requirement.

The labelling of n-heptadecanoic acid in the experiment with valine-4-C^{14} (Table 18) was almost certainly due to the incorporation into this fatty acid of labelled propionate which had resulted from the catabolism of the <u>iso</u>butyrate produced from valine. It was likely that the lower straight chain acids with an odd number of carbon atoms ($C_{15:0}$, $C_{13:0}$) were also labelled in this experiment but, as there was not a clean separation of these fatty acids on the gas chromatogram, the activity of these acids could not be accurately determined. The radioactivity which was found in these fatty acid fractions ($C_{15:0}$, $C_{13:0}$) was also due to carry-over of labelled branched chain fatty acids from the previous fraction during gas chromatography. The formation of long chain fatty acids with an odd number of carbon atoms from propionate has been demonstrated in a number of bacteria and in a cell-free enzyme system from rat epididymal adipose tissue (Kaneeda, 1963b; Horning, 1961).

5.5. The Validity of Results obtained with Experiments using Mixed Cultures of Rumen Bacteria and their Application to *in vivo* Conditions in the Rumen

The results of *in vitro* experiments using mixed rumen bacteria, such as those reported in this work, must be treated with caution when drawing any conclusions about reactions taking place *in vivo* in the rumen. The conditions imposed in these experiments were markedly different to conditions *in vivo* and the effect of this must be considered in any work of this type.

The method of preparation of the mixed bacteria used for washed suspensions in the experiments reported in the present work, together with the long period of incubation of the suspensions (36 hours) makes it very likely that there were considerable changes in the bacterial population during the growth of the culture. This observation regarding mixed cultures of rumen bacteria has been made by other workers (Hungate, Bryant and Mah, 1964).

The change in the bacterial population of the mixed suspensions in the present work was reflected in the changes that occurred in the proportions of the fatty acids of the mixed bacteria before and after incubation. These changes are shown in Tables 10, 12 and 16. The change in fatty acid composition was most marked in experiment 4 (Table 16) where the proportion of the branched chain fatty acids in the bacteria dropped considerably, while the proportion of the mono-unsaturated $C_{18:1}$ fatty acid present increased fro 3 to 10 per cent. Experiment 4 differed from the previous experiments with mixed suspensions in several respects. Apart from the change in fatty acid composition mentioned above, the production of volatile fatty acids by the bacteria was only half that which occurred in the other experiments (Table 9). The time between inoculation of the culture and the commencement of gas production was also much longer in all three flasks in experiment 4. Consequently it is likely that the change in the bacterial population was much more extensive in this experiment than in experiments 1, 2 and 3.

It is reasonable to suggest, in view of the evidence above, that the conditions during preparation of the mixed suspensions of rumen bacteria in these experiments were such that the proportion of obligate anaerobes in the suspension was reduced, while the less oxygen sensitive species were not so severely affected. This would account for the reduction in the proportion of the branched chain fatty acids in the mixed rumen bacteria since the obligatory anaerobic group of rumen bacteria includes the cellulolytic organisms known to produce branched chain fatty acids (Allison _et al._, 1962b; Wegner and Foster, 1963).

It is possible that, had greater care been taken in ensuring that anaerobic conditions were maintained during the preparation of the mixed suspensions, the changes which occurred in the bacterial population, and in the fatty acid composition, would not have been so marked. A greater incorporation of the C^{14} label from both *iso*butyrate-1-C^{14} and dl-valine-4-C^{14} into the long chain fatty acids would probably have occurred if the proportio of cellulolytic bacteria in the suspensions had not been reduced.

The degree of incorporation of the C^{14} label from *iso*butyrate into the fatty acids of mixed rumen bacteria was very small in these experiments. Although the protein fraction of the bacteria was not examined, it was likely that a considerable proportion of the radioactivity found its way into this fraction. The incorporation of *iso*butyrate and *iso*valerate into the amino acids of *Ruminococcus flavefaciens* has been reported by Allison *et al.* (1962a) An unknown proportion of the total radioactivity was also lost as carbon dioxide following the metabolism of *iso*butyrate-1-C^{14} by the rumen bacteria.

A large proportion (40 per cent) of the valine-4-C^{14} added to the mixed suspension in experiment 4 was taken up by the bacterial cells (Table 14) and only a very small proportion of this was found in the fatty acids. Presumably valine-4-C^{14} was incorporated directly into the protein of the mixed bacteria. This is difficult to interpret in view of the findings of Wright (1960) and Bryant and Robinson (1961) that some species of rumen bacteria prefer to synthesize amino acids from endogenous sources.

Despite the changes that occurred in the fatty acid composition of the bacteria during the period of incubation, it is evident from the data obtained in the present work that valine and its metabolite *iso*butyric acid were the precursors of some of the branched chain fatty acids found in mixed rumen bacteria. The production of *iso* branched chain fatty acids with an odd number of carbon atoms from *iso*butyrate is a phenomenon not previously observed in rumen bacteria either *in vivo* or *in vitro*. Further *in vivo* work on this pathway will be necessary before its significance in the overall metabolism of the rumen is established. Investigations with pure cultures of rumen bacteria capable of carrying out this transformation are necessary before the reaction pathways involved can be completely elucidated.

The evidence discussed in this section on mixed rumen bacteria supports the theory postulated by Keeney _et_ _al_. (1962) that the branched long chain fatty acids found in the milk and depot fats of ruminants are synthesized in the rumen by the bacterial population of this part of the digestive system.

Chapter 6

SUMMARY

1. A study has been made of the long chain fatty acids from the rumen bacteria *Streptococcus bovis* and particular attention was paid to the possibility of branched long chain fatty acids occurring in this organism. However no branched chain fatty acids could be identified in the lipids of *S. bovis* even when the organism was grown in a medium containing *iso*butyric acid. The significance of this finding in relation to the position of *S. bovis* as a rumen organism is commented upon.

2. The C^{14} label from *iso*butyrate-1-C^{14} which was added to the medium in which *S. bovis* was grown has not been found to be incorporated into any of the long chain fatty acids of the bacteria. This substrate (*iso*butyrate-1-C^{14}) was not metabolised by *S. bovis* to any appreciable extent in these experiments.

3. Lactobacillic (*cis*-11,12-methylene-octadecanoic) and *cis*-vaccenic (*cis*-11-octadecenoic) acids were both tentatively identified in the fatty acids of *S. bovis* and the proportions of these acids in this organism are compared with the proportions found in other bacteria of the family *Lactobacteriaceae*.

4. The possible effect of the presence of acetate in the culture medium for *S. bovis* on the percentage of total lipid, and on the fatty acid composition of the bacteria, is discussed.

5. A study was made of the fatty acid composition of the lipids from the mixed bacteria, and mixed protozoa, from a pasture fed dairy cow. Branched chain fatty acids were found to be present to a greater extent in the bacterial than the protozoal fatty acids.

6. The production of C^{14} labelled *iso*butyrate from valine-4-C^{14} by mixed suspensions of rumen bacteria has been demonstrated and the mechanisms involved in this transformation are discussed.

7. The incorporation of *iso*butyrate-1-C^{14} and of C^{14} labelled *iso*butyrate produced from valine-4-C^{14} into the branched chain fatty acids of mixed rumen bacteria has been shown. The C^{14} label was found in the branched chain fatty acids which contained both odd and even numbers of fatty acids and this result is discussed in the light of current knowledge of the formation of branched chain fatty acids.

8. The catabolism of C^{14} labelled *iso*butyrate (produced from valine-4-C^{1} to C^{14} labelled propionate has been demonstrated and the uptake of C^{14} labelled propionate into long, straight chain fatty acids with an odd number of carbon atoms was followed.

9. The significance of results obtained from these *in vitro* cultures of mixed rumen bacteria and their relation to the overall metabolism in the rumen is discussed.

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Appendix I

A. Experiments carried out to Determine a Suitable Medium and Cultural Conditions for *S. bovis*

Experiments were performed to determine whether the 5 per cent sucrose medium of Wright (1960) would produce a greater yield of *S. bovis* cells than the chemically defined medium used by Oxford (1958). In addition, the effect of growth in air, in an atmosphere of carbon dioxide, and in air with the addition of 5 per cent of solid calcium carbonate to the medium, as described by Bailey and Oxford (1958), was investigated.

Culture Medium

The medium of Oxford (1958) was prepared as follows: Bactocasamino acids (Difco), 25 gm.; sodium thioglycollate, 0.3 gm.; $MgSO_4{:}7H_2O$, 0.2 gm.; $FeSO_4{:}7H_2O$, 0.005 gm.; uracil, 0.02 gm.; adenine sulphate, 0.05 gm.; guanine, 0.02 gm.; l-cysteine-HCl, 0.15 gm.; l-tryptophan, 0.15 gm.; sucrose, 50 gm.; and sodium acetate, 20 gm., were made up to one litre with distilled water and the pH adjusted to 7.0 with concentrated ammonia solution. This was sterilised by intermittent steaming and 100 mls. were added to sterile, cotton wool plugged, 150 ml. conical flasks to which had previously been added 1.0 ml. of a sterile solution of B vitamins. The solution of B vitamins contained the following ingredients: pyridoxine-HCl, nicotinic acid, calcium panthothenate, thiamine-HCl, p-aminobenzoic acid (each 200 mgm./litre); riboflavin and folic acid (each 50 mgm./litre) and biotin (0.25 mgm./litre).

The medium of Wright (1960) was identical with the medium described in Chapter 3 on page 23 but the concentration of sucrose was 5 per cent and not 2 per cent.

Cultural Conditions

One hundred mls. of each of the culture media in 150 ml. conical flasks was inoculated with a single loop from a 12 hour culture of *S. bovis*, and incubated at 37°C. for 18 hours under one of three cultural conditions described below.

(a) Growth in air.

(b) Growth in an anaerobic jar flushed with carbon dioxide for 1 hour after inoculation.

(c) Growth in air, with the addition of 5 per cent of solid, sterile, calcium carbonate to the medium.

At the end of incubation the bacterial cells were harvested by centrifugation, washed, freeze-dried and their dry weight determined by the methods described in Chapter 3.

Results

The weight of bacterial cells which was produced in each culture flask is shown in the table below.

TABLE

The dry weight (gm./100 ml. of medium) of bacterial cells produced by *S. bovis* when grown in two different media, and under three conditions of culture. Details of the media and cultural conditions are given in the text above.

Medium of Wright (1960)			Medium of Oxford (1958)		
$CaCO_3$	CO_2	Air	$CaCO_3$	CO_2	Air
0.3135	0.0772	0.0357	0.0135	0.0277	0.0107
0.2975	0.1048	0.0584	0.0411	0.0128	0.0091
0.3024	0.1072	0.0436	0.0226	0.0284	0.0098
0.3036	0.0984	-	0.0389	0.0197	0.0113

Conclusions

The results of this experiment showed that S. bovis produced a greater weight of cells when grown in Wright's (1960) medium. This was the case for all cultural conditions tested. The best cultural conditions was in air with the addition of solid calcium carbonate to the medium to provide a source of dissolved carbon dioxide and bicarbonate ions. The greater weight of cells in the flasks containing solid calcium carbonate than in those grown in an atmosphere of carbon dioxide was probably due to the neutralising effect of the calcium carbonate on the lactic acid produced by S. bovis.

Larger yields of bacterial cells were associated in this experiment with heavy production of dextran by the bacteria. Since large amounts of dextran in the medium made centrifugation of the bacterial cells difficult an experime was carried out to determine the effect on dextran production, and cell yield, of reducing the sucrose concentration of Wright's (1960) medium.

B. The effect of reducing the Sucrose Concentration in the Medium on the Yield of S. bovis cells, and on the Production of Dextran by the Bacteria

The medium used in this experiment was basically that of Wright (1960) but the concentration of sucrose was varied from 0 to 5 per cent. Five per cent of solid calcium carbonate was added to 100 mls. of the medium immediatel prior to inoculation of the flasks with a loop of a 12 hour culture of S. bovi The cultures were incubated at 37°C. for 18 hours and the bacteria were then harvested. The results are shown in the table overleaf. Dextran production was assessed by precipitation from the culture supernatant with 4 volumes of 95 per cent ethanol and centrifugation. The amount of dextran produced was then assessed visually.

Results

TABLE

The dry weight (gm./100 ml. of medium) of bacterial cells and degree of dextran production of S. bovis grown on a medium with varying concentrations of sucrose.

Sucrose in Medium (%)	Weight of freeze-dried Bacteria (gm./100 ml. of medium) (Average of two cultures)	Relative Dextran Production *
5	0.147	+++
3	0.143	++
2	0.139	+
1	0.088	+
0	-	-

* - = no dextran production, +++ showing dextran production.

Conclusions

In the flask containing 2 per cent sucrose the dextran production was reduced to such an extent that centrifuging the bacteria was greatly facilitated. As the yield of bacterial cells was not affected at this level of sucrose, this medium was chosen for all subsequent experiments with S. bovis. The composition of the medium finally chosen is shown in Chapter 3 on page 23.

Appendix II

Experiments carried out to Determine the pH Changes that occur in the Medium during Growth of S. bovis and the amount of Alkali needed to Neutralise these Changes

Measurement of the pH changes in the cultures of S. bovis in the 2 per cent sucrose medium suggested that bacterial growth was being stopped because of the fall in pH caused by the production of lactic acid, rather than because any substrates in the medium had been exhausted. Hence it was decided to try and increase the yield of S. bovis cells by restoring the pH of the medium to 7 after it had fallen to 5.6 during growth. An experiment was first undertaken to determine the amount of 0.75N sodium hydroxide needed to restore the medium to pH 7 from various pH values.

Experimental

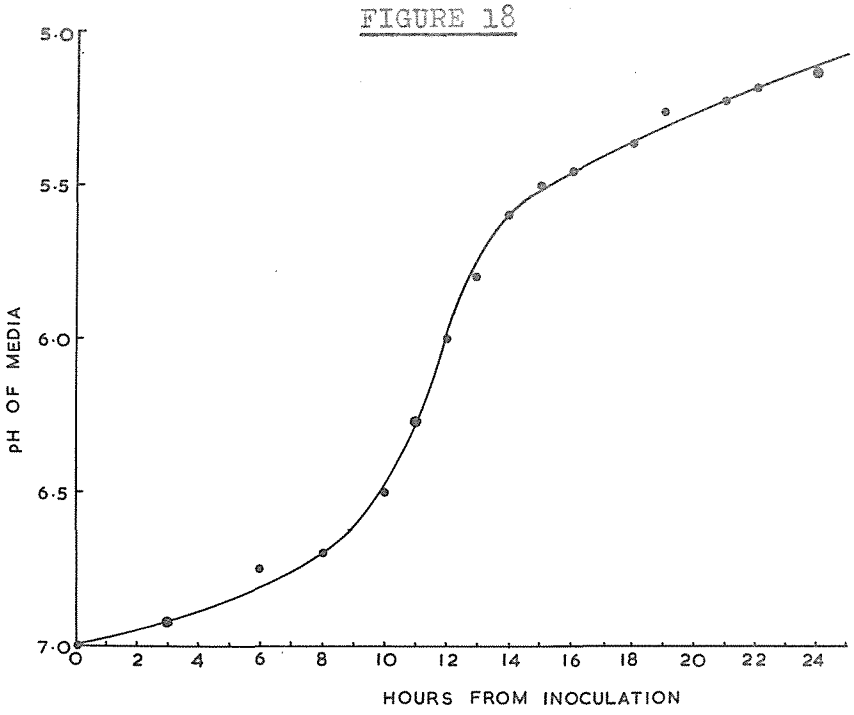
One litre of the 2 per cent sucrose medium was inoculated with 10 mls. of a 12 hour culture of S. bovis in liquid medium. Five per cent of solid calcium carbonate was added immediately before incubation. At suitable intervals 25 ml. aliquots of the culture medium were withdrawn aseptically from the flask and the pH of these was measured. The amount of 0.75N sodium hydroxide required to bring the pH of each aliquot back to pH 7 was also determined.

Results

The results of this experiment are shown graphically in Figures 17 and 18. Figure 17 shows the changes which occurred in the pH of the medium as the culture of S. bovis grew, while Figure 18 shows the amount of 0.75N sodium hydroxide required to bring the pH of the 25 ml. aliquot back to pH 7.

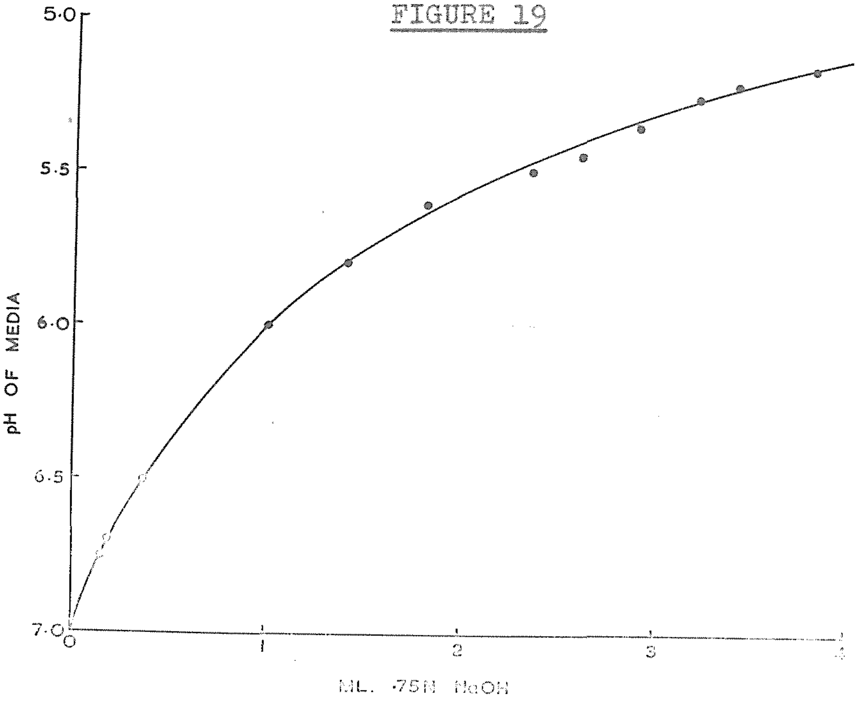
Following this trial an experiment was carried out to determine the effect of controlling the pH during growth on the yield of S. bovis cells. Two flasks, each containing 1 litre of the 2 per cent sucrose culture medium, together with 5 per cent of solid calcium carbonate, were each inoculated

FIGURE 18



The change in pH with growth of the *S. bovis* culture medium.

FIGURE 19



The amount of 0.75N NaOH required to bring the pH of 25 mls. of the *S. bovis* culture medium to pH 7 from various pH levels.

with 10 mls. of a 12 hour culture of S. bovis and incubated at 37°C. The pH changes in the medium were followed and when the pH had fallen to 5.6, sufficient 0.75N sodium hydroxide to return the pH to 7.0 (calculated from Figure 18) was added to one of the flasks. Both flasks were replaced in the incubator and were harvested after a total of 18 hours' incubation. The bacterial cells were washed, freeze-dried and the dry weight determined. The flask in which the pH had been corrected produced 1.579 gms. of dried bacterial cells while the control flask produced 1.122 gms. of cells. Since the procedure of pH correction resulted in a 30 per cent increase in the yield of cells it was used in all subsequent experiments with S. bovis.