Terminal anaerobic interactions in a microbial association isolated from landfill

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ABSTRACT

A multi-stage (5-vessel) chemostat was used to enrich and isolate from landfill a microbial association capable of dissimilating hexanoic acid (5 mM) under anoxic conditions. Two possible catabolic mechanisms existed : (i) that hexanoate was mineralised completely to carbon dioxide, although this has only been reported in sulphate-reducing bacteria (Widdel, Kohring & Mayer, 1983); and (ii) that a syntrophic association was involved in which the hydrogen produced, during hexanoate catabolism to acetate, was removed by a hydrogen-oxidising species such as a methanogen or sulphate-reducing bacterium.

The multi-stage chemostat was primarily used to facilitate spatial separation of the component species of the microbial association. Examination of the formation and subsequent utilisation of metabolites, however, indicated that no such separations were attained. Sulphate concentration increases from 1.4 to 5 mM and from 5 mM to 10 mM did, eventually, result in a partial shift in the metabolic activity of the methanogens from the first vessel to the second at the final concentration.

The results indicated that at least four groups of bacteria were present in the association : a hydrogen-producing acetogen, a hydrogen-utilising sulphate reducer, a hydrogen-oxidising homo-acetogen and an aceticlastic methanogen. It was thus apparent that a syntrophic association was present in which in the presence of sulphate a sulphate-reducing bacterium was the dominant hydrogen

utiliser whereas in the absence of this electron acceptor an association between a homo-acetogen and a methanogen dominated. Acetate was metabolised exclusively by the methanogen both in the presence and absence of sulphate.

To assess the effect of a non constant dilution rate regime on the microbial association a 3-stage chemostat was constructed in which the volume was increased from an initial 310 ml in the first vessel to 700 ml in the second and finally to 1600 ml in the third. The imposed dilution rate of 0.05 h^{-1} in the first vessel, together with an influent sulphate concentration of 1.4 mM, resulted in the displacement of the methanogenic population. Although maximum sulphate reduction remained in the first vessel complete dissimilation of hexanoic acid was only effected in the presence of the whole association.

To investigate the metabolic processes of the microbial association closed culture studies were made in which it was found that maximum rates of hexanoate degradation, by β-oxidation, required the intervention of sulphate reduction. Since the overall pattern of metabolism remained unchanged it was apparent that the sulphate-reducing bacteria outcompeted both the methanogens and acetogens for hydrogen.

The methanogenic component of the association was found to catabolise acetate to methane via an aceticlastic reaction although this mechanism was inhibited in the presence of hydrogen supplementation. Thus it was apparent that hydrogen removal to

facilitate not only catabolism of hexanoate but also the subsequent dissimilation of the metabolic intermediate, acetate, was an essential requirement.

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CONTENTS

1.1 Introduction

The problems caused by the increases in the total volumes of wastes generated globally are compounded by the presence of an expanding array of pollutants. As a consequence all waste streams are being viewed as potential threats to the environment thus necessitating the development of innovative waste disposal strategies. Although novel recycling processes are developed the disposal of solid waste to landfill is still the most common method and is likely to remain so in many countries for the forseeable future since it is relatively cheap, the technology and environmental control measures are reasonably well understood, and the re-establishment of contours and low-lying land are permitted.

Latterly, however, with increasing concern over conventional energy supplies and their constant price increases, novel biological sources including landfill gas have come under consideration and site practices have changed accordingly. It is particular unfortunate therefore that fundamental studies of the microbiology and biochemistry of landfill catabolisms have been noticeably lacking and as a consequence have limited progress.

1.2 The Landfill Ecosystem

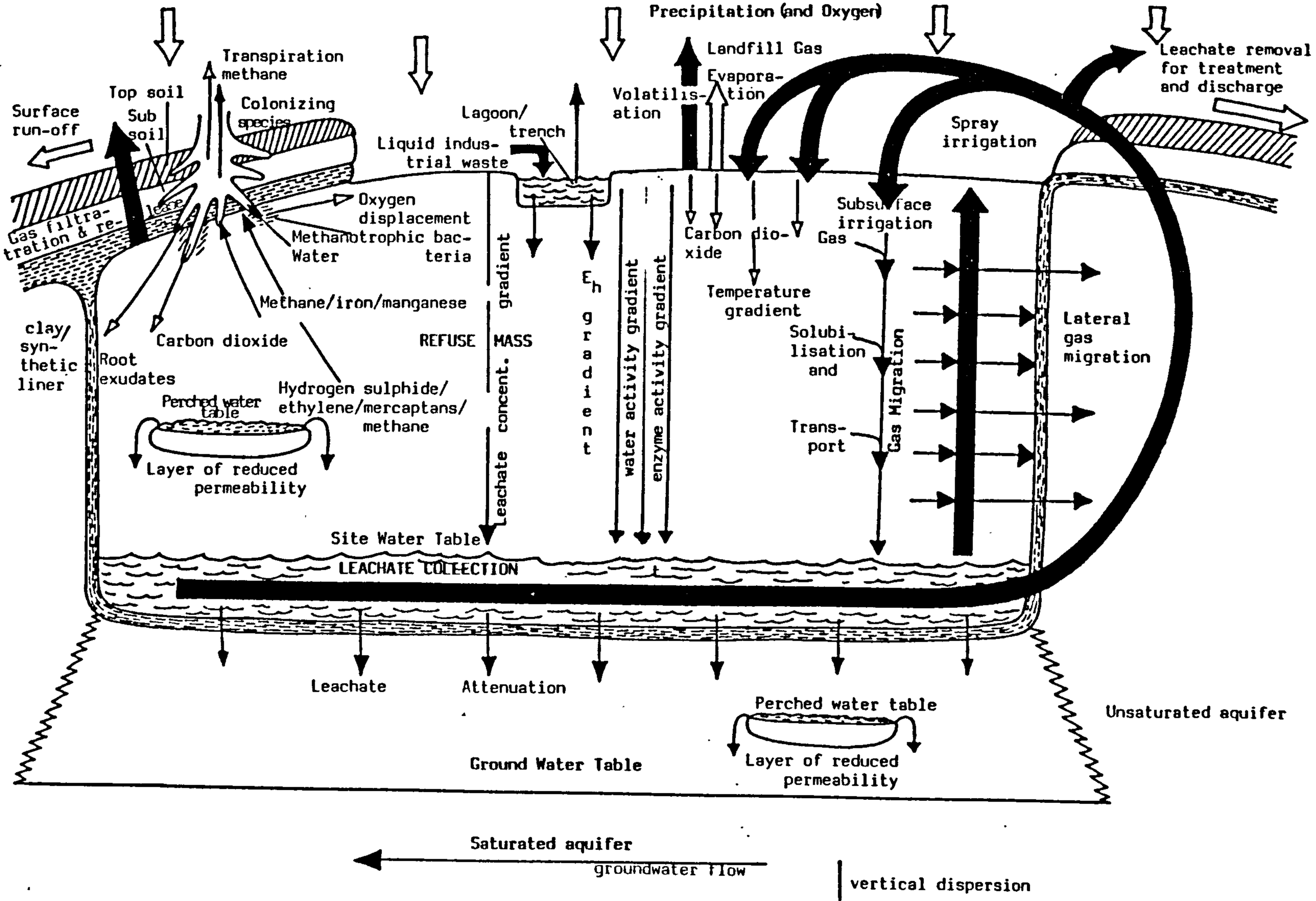
The landfill ecosystem differs from many natural environments in one major aspect, namely, that fixed carbon is presented to the species as domestic refuse and microorganisms are confronted by single, dual or most likely by multiple heterogeneous electron donors and electron acceptors which are often initially present in

non-limiting concentrations. The resulting microbial interactions depend primarily on the outcome of competition for substrates in a mixotrophic situation which is usually determined by the ratio between organic and inorganic substrates (Kuenen & Robertson, 1984). This, however, is unlikely to occur in recently emplaced refuse since substrates are rarely, if ever, growth limiting. Thus, although the aqueous phase of most natural anoxic ecosystems is usually oligotrophic (Marshall, 1980), this is unlikely to be the case in landfill.

As a consequence of the methods of refuse emplacement, stratification in landfill is initially most likely to be temporal rather than vertical. However, vertical stratification tends to develop with time as the concentrations of exogeneous electron acceptors become limited, although the picture (Figure 1) is usually complicated by the presence of horizontal and temporal gradients (Senior & Balba, 1985) of temperature, gas, liquid, Eh, pH and enzyme activity, and vectoral solute flow.

Rees (1980) described landfill sites as anaerobic, solid-state, partially-open bioreactors without temperature control. This statement, however, oversimplifies the situation which has been comprehensively reviewed by Senior & Balba (1985). Although very little work has been done to investigate catabolic pathways within landfill ecosystems analogies can be made with processes elucidated in other anoxic ecosystems.

Figure 1. Diagrammatic representation of the landfill ecosystem. From Senior and Balba (1985).


Precipitation (and Oxygen)
Landfill Gas
Transpiration
methane
Top soil
Sub
soil
Surface
run-off
Colonizing
species
Lagoon/
trench
Liquid indus-
trial waste
Volatilis-
ation
Evapora-
tion
Leachate removal
for treatment
and discharge
Spray
irrigation
Oxygen
displacement
Methanotrophic bac-
teria
Water
Gas filtra-
tration & re-
Carbon dio-
xide
Subsurface
irrigation
Gas
Temperature
gradient
Methane/iron/manganese
gradient
E_h
gradient
water activity gradient
enzyme activity gradient
clay/
syn-
thetic
liner
Carbon dioxide
REFUSE
MASS
Solubi-
lisation
and
Lateral
gas
migration
Root
exudates
Hydrogen sulphide/
ethylene/mercaptans/
methane
Perched water
table
Trans-
port
Gas Migration
Leachate concent.
Layer of reduced
permeability
Site Water Table
LEACHATE COLLECTION
Leachate
Attenuation
Perched water table
Unsaturated aquifer
Layer of reduced
permeability
Ground Water Table
Saturated aquifer
groundwater flow
vertical dispersion

1.2.1 Comparison of the landfill ecosystem with other anoxic environments

As already discussed, very little work has been done on the microbiology of landfill and hence hypothetical schemes have previously sufficed (Senior & Balba, 1985). Organic polymer transformations within landfill, however, most likely follow the patterns established for other habitats such as fresh and saltwater sediments, anaerobic digesters and the rumen.

In relation to landfill, sediments are often electron donor poor environments, but, especially in marine sediments, electron acceptor rich. Landfill, however, could be considered electron donor rich and electron acceptor diverse with transient high concentrations of sulphate recorded. Thus the microbial interactions elucidated in sediments cannot simply be extrapolated to the landfill ecosystem.

Anaerobic digesters are more like landfill than are sediments in that they are electron donor rich, although they are relatively homogeneous compared to landfill which can be considered to be heterogeneous and variable.

Initially there are considerable similarities between the rumen and the landfill ecosystem as the two have comparable temperatures and both operate under fed-batch conditions. The major V.F.As in the rumen produced from the fermentation of plant material, unlike other anaerobic habitats, are absorbed through the rumen cell wall and are then removed from the ecosystem. It is therefore apparent

that the rumen ecosystem although resembling landfill in terms of concentrations and variety of electron donors could not be used as a model for the landfill methanogenic ecosystem as the major sink for volatile fatty acids is the animal.

1.3 Landfill Catabolic Pathways

It is clear that landfill is considerably more heterogeneous than the methanogenic habitats described above. Although little work has been done on landfill catabolic processes, analogies can be drawn with other anoxic ecosystems.

1.3.1 Aerobic (Composting)

The processes of microbial carbon decomposition in the presence and absence of oxygen differ dramatically, since under aerobic conditions fixed carbon is characteristically completely degraded by the intervention of single species. For example, carbohydrate and lignin polymers are completely degraded by white-rot fungi (Kirk, 1971). Landfill is usually characterised by periods of intense aerobic metabolic activity when the refuse has been recently emplaced or after the ingression of water. Although these periods are relatively short, due to depletion of oxygen, they usually lead to increases in temperature which are analogous to composting. As a consequence of the rapid depletion of oxygen together with the downward flush of CO_2, microaerophilic and then anoxic conditions develop.

1.3.2 Microbial metabolism under anaerobic conditions

Complete mineralisation of organic polymers under anaerobic conditions requires the involvement of a number of metabolically diverse groups (Zeikus, 1983b) (Figure 2). The problem facing these bacteria is that they require some means, other than an oxygen-linked terminal system, of reoxidising any NAD(P)H produced by catabolism in excess of that required for anabolism. To effect this two general mechanisms may be employed;

1) fermentation; and

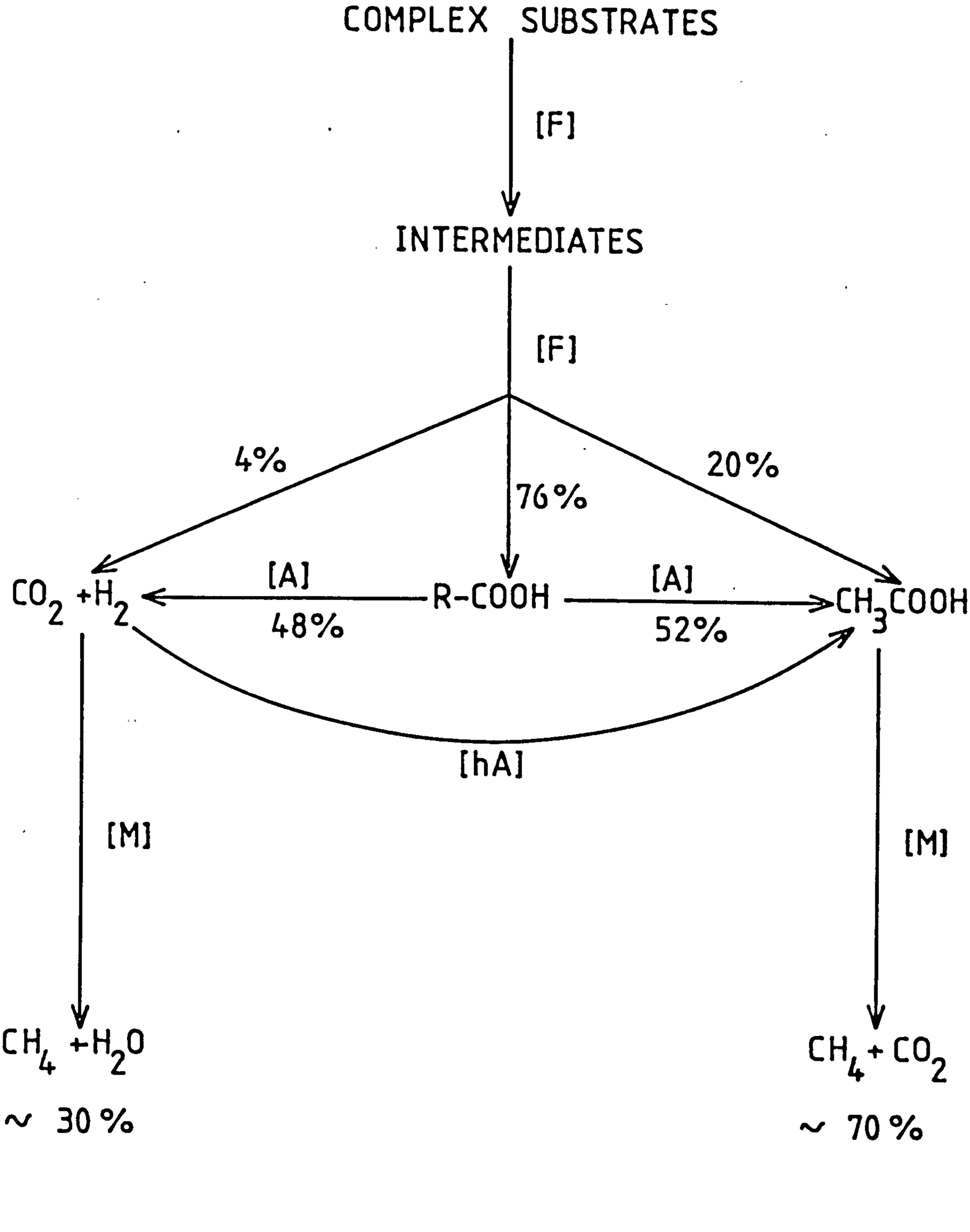
2) anaerobic respiration.

Fermentation

Fermentation is the process where reducing equivalents are directly transferred through the coupling of the oxidation of one substrate to the reduction of another substrate. The second substrate is usually the end product of a catabolic pathway where the fermentation pathway is internally balanced.

Fermentation mechanisms are found in most microorganisms including yeasts and species of Enterobacteriaceae, Staphylococcus, Bacillus and Clostridium. However, apart from the rumen environment (Bryant, 1959; Hungate, 1968), little is known about the fermentative bacteria active in other anaerobic environments. These bacteria are generally the primary organisms involved in the degradation of organic polymers which results in the formation of compounds such as hydrogen, carbon dioxide, ethanol, formate,

Figure 2: Degradation of complex organic substrates such as polysaccharides, proteins and lipids to methane by anaerobic fermentative processes. Bracketed letters denote mixed populations of : F, fermentative; A, aceto-genic; hA, homo-acetogenic; and M, methanogenic bacteria.


COMPLEX SUBSTRATES
[F]
INTERMEDIATES
[F]
4%
76%
20%
$CO_2 + H_2$
[A]
48%
R-COOH
[A]
52%
CH_3COOH
[hA]
[M]
[M]
$CH_4 + H_2O$
~ 30 %
$CH_4 + CO_2$
~ 70 %

acetate, propionate, butyrate, iso-butyrate, succinate, lactate, valerate, iso-valerate, hexanoate, heptanoate and methanol.

When substrates are fermented by chemoheterotrophs with other metabolic groups of bacteria, significant variations are noted which are of great ecological significance (Laanbroek & Veldkamp, 1982).

Anaerobic respiration

The ability to couple an electron transport chain with terminal acceptors other than O_2 is essentially confined to bacteria. The major electron acceptors are nitrate, nitrite, fumarate, sulphate and bicarbonate. The actual utilisation sequence of these can be predicted on thermodynamic grounds (Thauer et al., 1977) according to the amount of free energy liberated from a common electron donor as it is oxidised. The actual significance of each of the electron acceptors varies and ultimatley depends on the availability. Thermodynamic criteria suggest a sequence of nitrate, fumarate, sulphate and bicarbonate as the aceptors become less oxidised and less energy is available.

In this study only the latter two (sulphate and bicarbonate) will be considered, the mechanisms of which will be reviewed in the sections 1.3.4 (sulphate-reduction) and 1.3.5 (methanogenesis) respectively.

1.3.3 Acetogenesis

Acetogenesis under anoxic conditions is generally carried out by fermentative bacteria; with acetate alone or with other

compounds (for example, butyrate or propionate) as the end products.

The methanogenic fermentation of low molecular mass compounds other than direct methanogenic precursors can be achieved by the collaboration of proton-reducing acetate-forming chemoheterotrophs and H_2-oxidising methanogens.

Early work with 'mono' cultures of methanogenic bacteria indicated that they were greatly restricted in substrate specificity (Barker, 1956). At that time the assumption was made that only the normal fatty acids (C_1-C_6), the normal and iso-alcohols, which contained 1 to 5 carbon atoms, and the gases H_2, CO and CO_2 were used as substrates. Much of this information, however, was based on studies of Methanobacillus omelianskii (Barker, 1940; Wolin, Wolin & Wolfe, 1963) and Methanobacterium suboxydans (Stadtman & Barker, 1951).

Subsequently, however, Methanobacillas omelianskii was found to be a monoxenic (two-membered) rather than an axenic culture (Bryant et al., 1967). The culture was composed of a chemoheterotrophic non-methanogen, the 'S' organism, and a methanogen, Methanobacterium bryantii. Ethanol was oxidised by the 'S' organism to acetate and H_2 with the latter subsequently catabolised to CH_4 provided that CO_2 was present. H_2 removal by the methanogen was essential as the 'S' organism was sensitive to the H_2 end product and as a consequence further metabolism of ethanol was inhibited.

In addition to the above, ethanol has been shown to be metabolised by Desulfovibrio desulfuricans and Desulfovibrio vulgaris provided that either sufficient sulphate was added or that a co-culture with Methanobacterium bryantii (MoH) was established (Bryant et al., 1977). When a limiting concentration of sulphate was added to the co-culture methane was reduced by an amount equal to the reducing equivalents required for the reduction of sulphate. Similarly, if excess sulphate was added CH_4 was inhibited. In this case it was clear that sulphate was the preferred electron acceptor compared with CO_2 for interspecies H_2 transfer.

Similar results have also been found for other low molecular weight compounds such as pyruvate, lactate and volatile fatty acids (Table 1).

Reddy, Bryant & Wolin (1972) showed that the 'S' organism was able to metabolise pyruvate to ethanol, acetate, CO_2 and H_2 although, when co-cultured with Methanobrevibacter smithii, acetate, CH_4 and CO_2 were the resulting end products. The increase in acetate was equivalent to the decreased ethanol with the concentration of CH_4 formed stoichiometrically related to the quantity of reducing equivalents diverted from ethanol formation. Thus, in the presence of the methanogen CH_4 formation was the final step for electron disposal rather than to ethanol, and consequently acetate, a more oxidised end product, accumulated.

McInerney & Bryant (1981) reported that lactate was completely mineralised to CH_4 and CO_2 by a co-culture of D. desulfuricans and

Methanosarcina barkeri. The molecule was initially converted to acetate, CH_4 and CO_2 after which the acetate was further dissimilated to CH_4 and CO_2 by the methanogen (McInerney & Bryant, 1981). Acetate was not metabolised until H_2/CO_2 the preferred methanogenic substrate, was utilised.

Volatile fatty acid metabolism was studied extensively by Smith (1980) who demonstrated in enrichment cultures that H_2 was formed during the fermentation of both propionate and butyrate. Large numbers of H_2-oxidising methanogens were present in these and the fact that H_2 was shown to inhibit V.F.A. metabolism was good evidence that H_2 removal by the methanogens was essential for the degradation of the two acids.

Butyrate oxidation to acetate and CH_4 by a co-culture was first reported by McInerney, Bryant & Pfennig (1981) who showed that a methanogen or a sulphate reducer was responsible for the removal of H_2. The primary butyrate-oxidising, acetogenic organism was subsequently named Syntrophomonas wolfei (McInerney et al., 1981) and it was shown that this species was able to obtain energy from the β-oxidation of straight-chain volatile fatty acids (C_4 to C_8) and iso-heptanoate when the species was co-cultured with a H_2-utilising partner.

The results obtained with this co-culture confirmed the earlier reports that proton-reducing acetogenic bacteria were in fact responsible for the utilisation of low molecular weight compounds, rather than methanogens, as previously accepted.

The presence of the H_2 remover in the co-culture makes the overall change in free energy negative and, hence, thermodynamically favourable:

$$CH_3CH_2CH_2COO^- + 2H_2O \longrightarrow 2CH_3COO^- + H^+ + 2H_2$$

$$\Delta G_o^1 = +48.1 \text{ kJmol}^{-1} \qquad (1)$$

$$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O$$

$$\Delta G_o^1 = -135.6 \text{ kJmol}^{-1} \qquad (2)$$

$$2CH_3CH_2CH_2COO^- + HCO_3^- + H_2O \longrightarrow 4CH_3COO^- + H^+ + CH_4$$

$$\Delta G_o^1 = -39.4 \text{ kJmol}^{-1} \qquad (3)$$

Other similar types of co-cultures have been reported by Boone & Bryant (1980) who described an association which oxidised propionate to CH_4 and acetate in which the acetogenic organism Syntrophobacter wolinii metabolised propionate in the presence of a H_2-utilising methanogen. Clostridium bryantii, which oxidised fatty acids with up to 10 carbon atoms in syntrophic association with H_2-utilising organisms, was isolated by Stieb & Schink (1985).

Sulphate-reducing bacteria which are capable of utilising similar substrates as S. wolfei and S. wolinii have recently been isolated by Widdel & Pfennig (1982). These bacteria have been shown to oxidise volatile fatty acids to CO_2 and H_2O with concomitant reduction of sulphate to sulphide. It would, thus, appear that these bacteria are dominant in sulphate-rich environments such as marine sediments where they act as terminal oxidisers of both organic end products and H_2. However, syntrophic associations have also been isolated which degrade carbohydrates to CH_4 and other end

products (Table 1).

In all these methanogenic couplets, chemoheterotrophic bacteria can be involved in interspecies H_2 transfer although in this instance there is less dependence.

Cellulose, glucose and fructose have been reported as substrates for several fermentative chemoheterotrophic and methanogenic couplets (Table 1). In axenic culture Clostridium cellobioparum was shown to ferment glucose to ethanol, formate acetate and H_2. In the presence of M. ruminantium, however, the end products were shifted quantitatively to acetate at the expense of ethanol and formate (Chung, 1976). H_2 was oxidised by the methanogen although the species could be substituted chemically by palladium black.

In most reported cases the fermentation pattern was shifted to more oxidised end products in the presence of H_2-removing bacteria. This situation, however, is in direct contrast to obligate proton reducers which degrade volatile fatty acids and ethanol where no shift is observed. Thus, co-existence, in this case with a H_2-utilising partner, is an essential requirement for the disposal of excess electrons (Mah, 1982).

It is clear that both hydrogen and acetate play important roles as intermediates in the anaerobic degradation of organic polymers and will be further considered in the following two sections.

Table 1: Characteristics of acetogenic H_2-producing bacteria and their syntrophic partnerships. (Adapted from Mah, 1982).

CHEMOHETEROTROPHIC H_2 PRODUCER	SUBSTRATE	MONO-CULTURE PRODUCTS	H_2 USING METHAOGEN	MIXED CULTURE PRODUCTS
ANAEROPLASMA SP.	GLUCOSE	BUTYRATE PROPIONATE H_2	METHANOPLASMA ELIZABETHII	ACETATE (↑) BUTYRATE (↓) PROPIONATE (↓). CH_2
ACETOBACTERIUM WOODII	FRUCTOSE	ACETATE	METHANOBREVIBACT-ER ARBORIPHILUS	ACETATE (↓) CH_4
CLOSTRIDIUM BUTYRICUM	PECTIN	METHANOL ETHANOL ACETATE BURYRATE H_2	M. BARKERI	ACETATE (↓) METHANOL (↓) CH_4
RUMINOCOCCUS FLAVEFACIENS	CELLULOSE	ACETATE SUCCINATE FORMATE H_2	M. SMITHII	ACETATE (↓) SUCCINATE (↓) CH_4
CLOSTRIDIUM THERMOCELLUM	CELLULOSE	ACETATE BUTYRATE ETHANOL LACTATE. H_2	M. THERMOAUTO-TROPHICUM	ACETATE (↓) BUTYRATE (↓) LACTATE. ETHANOL (↓ CH_4
ACETIVIBRIO CELLULYTICUS	CELLULOSE	ACETATE ETHANOL H_2	M. BARKERI ± DESULFOVIBRIO	ACETATE (↓) ETHANOL (↓) CH_4
CLOSTRIDIUM THERMOCELLUM	CELLULOSE	ACETATE ETHANOL LACTATE H_2	ACETOGENIUM KIVUI	ACETATE
ESCHERICHIA COLI	SUCROSE	ACETATE FORMATE ETHANOL LACTATE H_2	ACETOBACTERIUM WOODII DESULFOVIBRIO VULGARIS M. BARKERI M. FORMICICUM	ETHANOL (↓) CH_4
UNNAMED	BENZOATE	NONE	M. HUNGATEI	ACETATE. CH_4
DESULFOVIBRIO VULGARIS	LACTATE	NONE	M. BARKERI	ACETATE CH_4
DESULFOVIBRIO SP.	CHOLINE	TRIMETHYLAMINE ACETATE ETHANOL	M. BARKERI	CH_4. NH_4 H_2S
UNNAMED	ACETATE	NONE	METHANO-BACTERIUM SP.	CH_4
ACIDAMINOBACTER HYDROGENOFORMANS	GLUTAMATE	ACETATE NH_4 H_2	M. HUNGATEI OR M. ARBORIPHILICUS OR DESULFOVIBRIO	ACETATE (↓) PROPIONATE (↓) CH_4
RUMINOCOCCUS PASTEURII	TARTRATE	ACETATE FORMATE	M. HUNGATEI	ACETATE CH_4
ILYOBACTER TARTARICUS	AS EXAMPLE ABOVE.			

CHEMOHETEROTROPHIC H_2 PRODUCER	SUBSTRATE	MONO-CULTURE PRODUCTS	H_2 USING METHANOGEN	MIXED CULTURE PRODUCTS
THERMOANAEROBIUM BROCKII	ETHANOL	ACETATE (TR) H_2 (TR)	METHANOBACTERIUM THERMOAUTOTROPHICUM	ACETATE (↓) CH_4
S. ORGANISM	ETHANOL	ACETATE (TR) H_2 (TR)	M. BRYANTII	ACETATE (↓) CH_4
"	OTHER ALCOHOLS	NONE	METHANOBREVIBACTER SMITHII	CORRESPONDING ACID CH_4
"	PYRUVATE	ETHANOL ACETATE H_2	M. SMITHII	ACETATE (↓) CH_4
DESULFOVIBRIO DESULFURICANS OR D. VULGARIS	ETHANOL	ACETATE (TR) H_2 (TR)	M. BRYANTII	ACETATE (↓) CH_4
"	LACTATE	TRACE PRODUCTS	M. BRYANTII	ACETATE (↓) CH_4
"	LACTATE	TRACE PRODUCTS	METHANOSARCINA BARKERI	CH_4, CO_2
SELENOMONAS RUMINANTIUM	LACTATE	ACETATE PROPIONATE	M. SMITHII	ACETATE (↓) PROPIONATE (↓) CH_4
ANAEROVIBRIO LIPOLYTICA	LACTATE	ACETATE PROPIONATE H_2 (TR)	M. RUMINANTIUM	ACETATE (↓) PROPIONATE (↓) CH_4
SYNTROPHOMONAS WOLFEI	BUTYRATE	NONE	METHANOSPIRILLUM HUNGATEI	ACETATE, CH_4
"	VALERATE	NONE	M. HUNGATEI	ACETATE, PROPIONATE CH_4
SYNTROPHOBACTER WOLINII	PROPIONATE	NONE	DESULFOVIBRIO SP + SO_4	ACETATE H_2S
"	PROPIONATE	NONE	DESULFOVIBRIO +M. HUNGATEI	ACETATE, CH_4
CLOSTRIDIUM BRYANTII	HEXANOATE	NONE	M. HUNGATEI	ACETATE, CH_4
EUBACTERIUM CELLULOSOLVENS	CELLOBIOSE	BUTYRATE, LACTATE H_2	M. RUMINANTIUM	ACETATE (↑) BUTYRATE (↓) LACTATE (↓) CH_4
SELENOMONAS RUMINANTIUM	GLUCOSE	LACTATE ACETATE PROPIONATE	M. SMITHII	ACETATE (↓) LACTATE (↓) PROPIONATE, CH_4
CLOSTRIDIUM CELLOBIOPARUM	GLUCOSE	ACETATE FORMATE ETHANOL, H_2	M. SMITHII	ACETATE (↓) ETHANOL (↓) FORMATE (↓), CH_4
THERMOANAEROBIUM BROCKII	GLUCOSE	ACETATE ETHANOL	M. THERMOAUTOTROPHICUM	ACETATE (↓) ETHANOL (↓) LACTATE (↑), CH_4

Interpsecies hydrogen transfer

Interactions between non-methanogenic chemoheterotrophs and methanogens were predicted by Hungate (1966) for the rumen fermentation when he postulated that the electrons which were produced from the fermentation of sugars were used to reduce CO_2 to CH_4.

This phenomenon, interspecies H_2 transfer, was first studied by using a co-culture of Ruminococcus albus and Vibrio succinogenes fermenting glucose (Ianotti et al., 1973). R. albus monocultures were found to produce acetate, ethanol, H_2 and CO_2. However, in co-culture with V. succinogenes and added fumarate (which R. albus cannot metabolise), fumarate served as the terminal electron acceptor for V. succinogenes and as a consequence was reduced to succinate. The end products of the fermentation also shifted completely to acetate and CO_2. V. succinogenes derived energy by H_2 oxidation (and fumarate reduction), which by-passed ethanol formation in R. albus. The end results of this led to higher cell yields, increased substrate metabolism, elevated oxidised end product formation and a greater energy production (Ianotti et al., 1973; Wolin, 1976; Thauer, Jungerman & Decker, 1977).

Fermentation of organic substrates by chemoheterotrophs alone yields products which are determined by the terminal electron acceptors which serve as electron sinks. Usually mixtures of organic end products such as acetate, butyrate, ethanol and butanol are produced (1.3.2). H_2 and CO_2 are also common end products

although in the presence of, for example, H_2-oxidising methanogens proton reduction becomes the major electron sink for the chemoheterotrophs. Since acetate is the major end product other reduced products are formed either in low concentrations or not at all (Mah, 1982). If present, these compounds may be further dissimilated to acetate by a specialised group of bacteria in syntrophic association with methanogens (Zeikus, 1983b).

In the presence of H_2-utilising species the ultimate fate of acetate varies depending on the microbial ecosystem. In ruminants, acetate, together with other volatile fatty acids, is absorbed through the cell wall and most, if not all, the methane is formed from either H_2/CO_2 or formate (Hungate et al., 1970). In marine environments and other sulphate-rich sediments, however, sulphate-reducing bacteria, which act as the major H_2 oxidisers, can compete for acetate and oxidise it to H_2S and CO_2.

However, in sulphate-limited environments such as fresh water sediments, aged landfill and anaerobic digesters, acetate is the major methanogenic precursor (Jeris & McCarty, 1965; Smith &Mah, 1966; Zinder et al., 1984).

Acetate; a key intermediate in anerobic metabolism

During the anaerobic decomposition of organic material the production and subsequent utilisation of acetate are quantitatively the most important metabolic process such that this molecule has been estimated to be the major intermediate in 67% of all anaerobic

fermentations (Pfennig & Widdel, 1982). Under anoxic conditions acetate can be produced from a variety of sources including carbohydrates (Schink & Zeikus, 1982), aromatic monomers (Balba, Clarke & Evans, 1979), volatile fatty acids (McInerney et al., 1979), alcohols (Eichler & Schink, 1984) and hydrogen/carbon dioxide (Braun & Gottschalk, 1982) by the intervention of complex populations of interacting bacteria.

Anoxic acetate utilisation has been reported in several groups of bacteria including methanogens (Weimer & Zeikus, 1978), sulphate- (Widdel & Pfennig, 1981) and sulphur- (Pfennig & Biebl, 1976) reducing bacteria, and also in a component species of a syntrophic association (Zinder & Koch, 1984).

In the absence of sulphate it has been estimated that approximately 70% of the methane in most anaerobic environments is derived from acetate (Jeris & McCarty, 1965; Mah et al., 1977) although in the presence of this electron acceptor it is recognised that methylated amines and methanol are major methanogenic precursors (Oremland & Polcin, 1982; King, 1984).

In view of the above it is somewhat surprising therefore that of all the methanogens so far described only Methanosarcina sp. and Methanothrix sp. have been found to catabolise acetate to methane (Smith et al., 1980; Zehnder et al., 1980; Huser et al., 1982; Fathepure, 1983; Patel, 1984).

With respect to these two species the more metabolically diverse is Methanosarcina since in addition to acetate, H_2/CO_2,

methanol, carbon monoxide and methylamines can be utilised, whereas Methanothrix can only utilise acetate.

From the above discussion, three key species; sulphate-reducing bacteria,methanogens and homo-acetogens are the major H_2-utilising bacteria in terminal metabolic processes.

1.3.4 Dissimilatory-sulphate reduction

The phrase 'sulphate-reducing bacteria' is usually reserved for the group of bacteria that conduct dissimilatory sulphate-reduction. This process, which is analogous to respiration, uses sulphate as the terminal electron acceptor rather than O_2. In this way most of the sulphate is released from the bacteria as sulphide which, in turn, is often hydrolysed to H_2S.

Sulphate is used as an electron acceptor by a heterogeneous group of bacteria which utilise organic acids, fatty acids and alcohols as electron donors. Many of these organisms also possess the enzyme hydrogenase and can hence oxidise hydrogen. Although as a group they are morphologically diverse they can be considered as physiologically unified.

Eight genera of sulphate-reducing bacteria are currently recognised (Table 2) which are divided into two broad groups. The genera in Group I, Desulfovibrio, Desulfomonas and Desulfotomaculum, utilise lactate, pyruvate, ethanol or certain fatty acids as carbon and energy sources whilst reducing sulphate to hydrogen sulphide whlst the bacteria in Group II, Desulfobulbus, Desulfobacter, Desulfococcus, Desulfosarcina and Desulfonema utilise fatty acids,

Table 2: Characteristics of sulphate-reducing bacteria. Modified from Laanbroek and Veldkamp (1982) and Brock, Smith and Madigan (1984).

NR - Not recorded.

GENUS	CHARACTERISTICS	SPECIES EXAMPLE	formate	acetate	propionate	n-butyrate	n-valerate	n-stearate	lactate	pyruvate	malate	benzoate	glucose	ethanol	proan-1-ol	butan-1-ol	H_2	Complete oxidation of organic substrates, electron donors
Group I sulphate reducers																		
Desulfovibrio	Polarly flagellated, curved rods, no spores; Gram negative; contain desulfoviridin; seven species recognised, one thermophilic	desulfuricans	+	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-
		baarsii	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+
Desulfomonas	Long, fat rods; nonmotile; no spores Gram negative; contain desulfoviridin; habitat, intestinal tract; one species.	pigra	-	-	-	-	-	-	+	+	NR	NR	-	NR	NR	NR	NR	-
Desulfotomaculum	Straight or curved rods; motile by peritrichous or polar flagellation; Gram negative; desulfoviridin absent; produce endospores; four species, one thermophilic; one species capable of utilising acetate as energy source.	acetoxidans	+	+	-	+	+	-	-	-	-	-	-	+	-	+	-	+
		nigrificans	-	-	-	-	-	-	+	+	NR	NR	+	NR	NR	NR	NR	-

Genus	Characteristic	Species Example	formate	acetate	propionate	n-butyrate	n-valerate	n-stearate	lactate	pyruvate	malate	benzoate	glucose	ethanol	propan-1-ol	butan-1-ol	H_2	complete oxidation of organic substrates electron donors
Group II Sulphate reducers																		
Desulfobulbus	Ovoid or lemon-shaped cells; no spores; Gram negative; desulfoviridin absent; if motile, by single polar flagellum. One species.	propionicus	-	-	+	-	-	-	+	+	-	-	-	+	+	+	+	-
Desulfobacter	Rods; no spores; Gram negative; desulfoviridin absent; if motile, by single polar flagellum; utilises only acetate as electron donor and oxidises it to CO_2; one species	postgatei	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Desulfococcus	Spherical cells; nonmotile; Gram negative; desulfoviridin present, no spores; utilises C_1-C_{14} fatty acids as electron donor with complete oxidation to CO_2; one species	multivorans	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	+
Desulfonema	Large filamentous gliding bacteria; gram positive; no spores; desulfoviridin present or absent; utilises C_1 - C_{12} fatty acids as electron donor, with complete oxidation to CO_2.	limicola	+	+	+	+	+	-	+	+	-	-	-	-	-	-	+	+
		magnum	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	+
Desulfosarcina	Cells in packets (sarcina arrangement); Gram negative; no spores; desulfoviridin absent; utilises C_1-C_{14} fatty acids as electron donor with complete oxidation to CO_2; one species	variabilis	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+

particularly acetate. Although all the sulphate-reducing bacteria are strict anaerobes and anaerobic techniques must be used in their isolation and cultivation they are not as fastidious with respect to oxygen as methanogens.

Sulphate-reducing bacteria are found in most terrestrial and aquatic environments but are predominant in sulphate-rich environments such as marine sediments where sulphate concentrations in excess of 32 mM are common.

Physiology: The range of electron donors used by sulphate-reducing bacteria has already been outlined in Table 2. The most universally used are lactate and pyruvate although many Group I bacteria also utilise malate, formate and certain alcohols. Some strains of Desulfotomaculum grow on glucose but this is a rather unique property. Group I sulphate-reducers tend to incompletely oxidise their substrates to acetate whilst Group II organisms completely oxidise fatty acids such as lactate and succinate and even benzoate to CO_2. Also, Desulfosarcina and Desulfonema can grow lithotrophically with H_2 as the electron donor, sulphate as the electron acceptor and CO_2 as the sole carbon source (Table 2).

In addition to sulphate reduction many sulphate-reducing bacteria grow fermentatively in the absence of sulphate. The most common fermentative substrates are pyruvate and lactate, which are converted to acetate, CO_2 and H_2. As with other fermentative bacteria metabolism can be affected (usually favourably) by the

presence of hydrogen-removing organisms such as methanogens thus resulting, in this case, in the production of methane (1.3.3). However, in the presence of sulphate the free energy change is more favourable (Thauer et al., 1977).

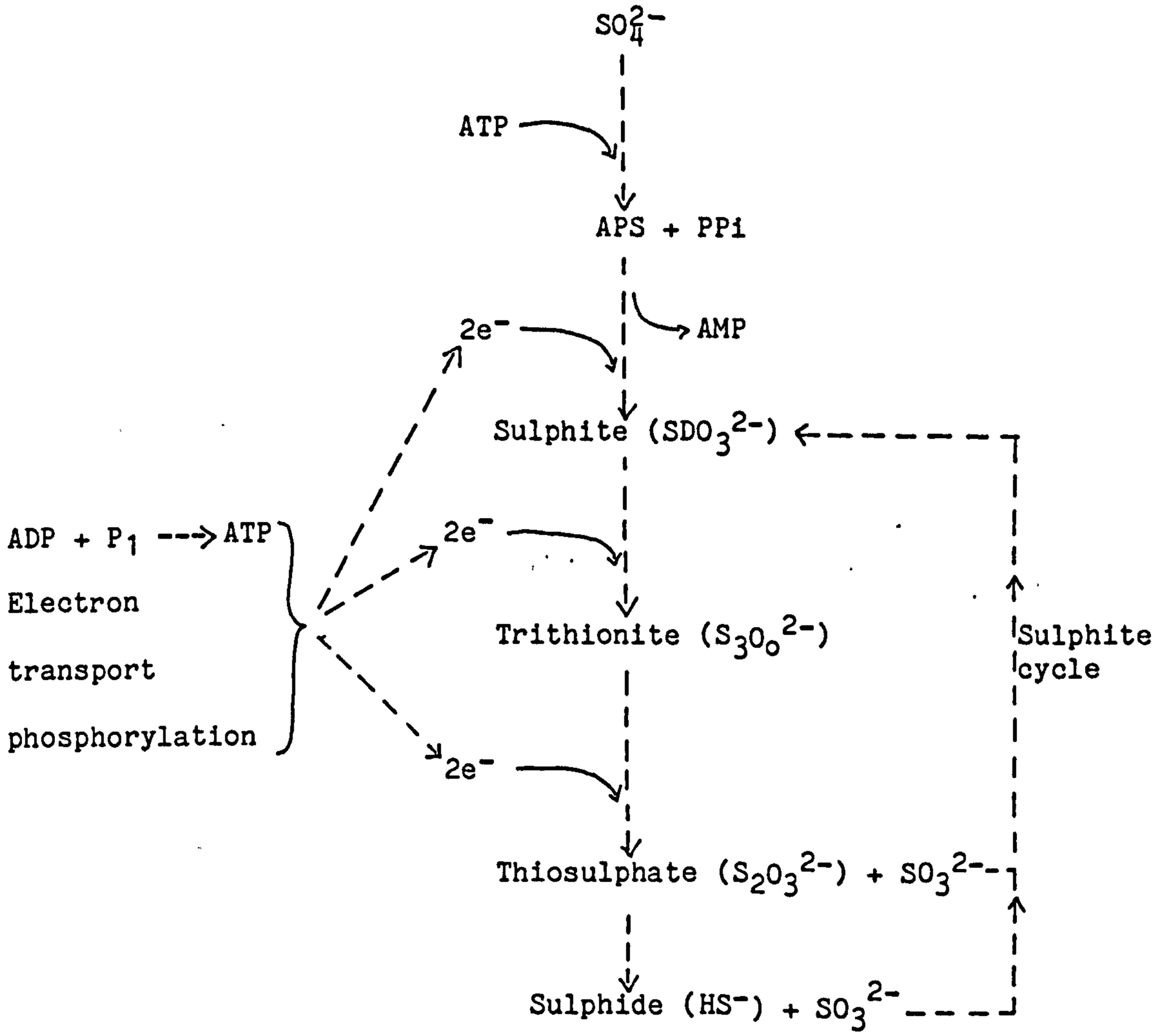
Biochemistry: The enzymology of sulphate reduction to sulphide has been extensively studied (Peck & Le Gall, 1982). The first step is the formation of adenosine phosphosulphate (APS) from ATP and sulphate. Then, APS reductase catalyses the reduction of the sulphate moiety to sulphite. The sulphite in turn is most likely reduced via trithionite and thiosulphate which then probably permits ATP synthesis. Most sulphate-reducing bacteria contain desulfoviridin (Lee, LeGall and Peck, 1973a) or desulforubidin (Lee, Yi, LeGall & Peck, 1973b) which are bisulphite reductases. ATP is most likely generated by electron transport, involving the cytoplasmic membrane as reviewed by Peck & LeGall (1982) (Figure 3).

1.3.5 Methanogenesis

Bergey's Manual (Bryant, 1974) describes the methanogens as "Methanobacteriaceae, strict anaerobes, in muds and sediments, giving marsh gas which ignites as 'will-o-the-wisp', in the guts of animals, notably ruminants, in anaerobic sewage treatment plants where methane can be used as a fuel; most commonly utilise hydrogen and carbon dioxide produced by other anaerobic organisms as substrate for methane production".

If methanogens are considered in a general organisational sense they are clearly prokaryotes with representatives of a variety of

Figure 3: Scheme for eight electron reduction of sulphate to sulphide via tetrathionate and thiosulphate. (Brock, Smith & Madigan, 1984).


SO_4^{2-}
ATP
APS + PPi
AMP
$2e^-$
Sulphite (SDO_3^{2-})
ADP + P_1 ---> ATP
Electron
transport
phosphorylation
$2e^-$
Trithionite ($S_3O_o^{2-}$)
Sulphite
cycle
$2e^-$
Thiosulphate ($S_2O_3^{2-}$) + SO_3^{2-}
Sulphide (HS^-) + SO_3^{2-}

sizes and shapes (and Gram reaction) typical of bacteria. They do not have a nuclear membrane or organelles and their ribosomes are more like bacterial examples than eukaryotic ones. However, in relation to their 16 S rRNA homologies they are no nearer to general bacteria than they are to eukaryotes. They represent a coherent phylogenetic group quite distinct from other typical bacteria. However, the methanogens as a group based on their 16 S rRNA homologies are themselves very diverse; the remotest branches are as different from one another as are the enteric bacteria and Bacillus. These taxonomically significant differences are indicated by the separation of the methanogens, together with halobacteria and two thermoacidophiles, into a new primary kingdom - the Archaebacteria (Woese, 1982).

The Archaebacteria do not have murein cell walls, the lipid components of their membranes are unusual ether-linked polyisoprenoid (branched chain) lipids and they possess characteristic transfer RNAs and rRNA.

As mentioned previously methanogens are extreme, strict anaerobes which grow on a limited range of substrates such as H_2/CO_2, CO, HCOOH, CH_3OH, CH_3COOH and methylamines; they produce methane (and in some cases CO_2) and can be best described as obligate anaerobic unicarbonotrophs (Zeikus, 1983a). Analysis of mono cultures of methanogenic bacteria has revealed the following unique properties (Table 3):

Table 3 : Unique Properties of Methanogens

A. Requirement of low redox potential.

B. Presence of novel coenzymes such as Co M, factors F_{420} and F_{430}, 7-methylpterin, methanopterin, methanyl-tetrahydromethanopterin, methanofuran.

C. 16 S rRNA distantly related to other prokaryotes.

D. Differences in the common arm of the tRNA.

E. Absence of D-amino acids and muramic acids in their cell walls.

F. Lipid composition of phytanyl ether glycerols (acyclic, cyclic) and squalenes.

G. Low genome size (approximately 30% of E. coli).

H. Absence of quinones, presence of cytochromes in a limited number of cases.

I. Presence of methanophosphagen (cyclic 2,3-diphosphoglycerate) as a possible phosphorus reserve material.

J. Metabolic products, CH_4, CO_2.

K. Presence of a novel CO_2 fixation pathway.

---

some of which will be discussed later in detail.

Taxonomically, classification of methanogens is not easy as there is wide variation both morphologically and physiologically as shown in Table 4. However, classification ultimately depends on whether emphasis is placed on morphology or physiology. Bergey's Manual of Determinative Bacteriology has favoured the morphology and hence the methane bacteria have been placed in families and genera

Table 4: Characteristics of methanogens. Adapted from Dubach & Bachofen (1985).

	DSM NO.	MORPHOLOGY	GRAM	TEMP.	GEN (H)	MOL % G + C	PH	SPECIAL CHARACTERSTICS	H_2/CO_2	FORMATE	ACETATE	METHANOL	METHYLAMINES
M. BACTERIUM FORMICICUM MF	1535	RODS	+	43	8.7	40.7	7.0	NONMOTILE	+	+	-	-	-
M. BACTERIUM BRYANTII M.O.H.	863	RODS	+	37-39	24	32.7	7.1	REQUIRES B VITAMINS					
								NONMOTILE	+	-	-	-	-
M. BACTERIUM THERMOAUTOTROPHICUM	1053	RODS	+	65-70	2.2	47.9	7.2-7.6	THERMOPHILIC, NONMOTILE	+	-	+	-	-
M. BREVIBACTER RUMINANTIUM M.I.	1093	RODS	+	37-42	4	30.6	6.5-7.7	REQUIRES ACETATE, SEVERAL AMINO ACIDS, 2-METHYL-BUTYRATE, AND COENZYME M	+	-	+	-	-
M. BREVIBACTER ARBORIPHILUS D.H.I.	1125	RODS	+	30-37	10	27.5	7.5-8.0	FOUND IN WET WOOD OF LIVING TREES, NON-MOTILE	+	-	-	-	-
M. BREVIBACTER SMITHII P.S.	861	COCCI	+	37-39	ND	31.0	6.9-7.4	NONMOTILE; REQUIRES ACETATE	+	+	-	-	-
M. THERMUS FERVIDUS V24S	2088	RODS	+	83	2.43	33.0	6.5	REQUIRES YEAST EXTRACT	+	-	-	-	-
M. COCCUS VANNIELII S.B.	1224	COCCI	-	36-40	8	31.1	7.0-9.0	MOTILE, REQUIRES SELE-NIUM OR TUNGSTEN	+	+	-	-	-
M. COCCUS VOLTAE P.S.	1537	COCCI	-	35-45	1.2	30.7	6.0-7.0	MOTILE	+	+	-	-	-
M. COCCUS MAZEI S-6	-	COCCOID	-/+	30-40	7.7	ND	6.1-8.0	UNDERGOES LIFE CYCLE; REQUIRES NaCl	-	-	+	+	+
M. COCCUS DELTAE R.C.	-	COCCI	ND	3.7	2	40.5	ND	NONMOTILE, REQUIRES 3% NaCl	+	+	-	-	-
M. MICROBIUM MOBILE B.P.	1539	RODS	-	35-42	ND	48.5	6.7-7.4	HIGHLY MOTILE; REQUIRES RUMEN FLUID	+	+	-	-	-
M. MICROBIUM PAYNTERI	1545	COCCI	-	40	4.8	44.9	7.0	MARINE. REQUIRES NaCl	+	-	-	-	-
M. GENIUM CARIACI J.R.I.	1497	COCCI	-	20-25	11	52	6.8-7.3	MARINE, REQUIRES NaCl ACETATE AND YEAST EXTRACT	+	+	-	-	-
M. GENIUM MARISNIGRI J.R.I.	1498	COCCI	-	20-25	10	6.1	6.2-6.8	MARINE, REQUIRES NaCl AND TRYPTICASE	+	+	-	-	-
M. GENIUM OLENTANGYI RC/ER	-	COCCI	-	37	10.9	54.4	ND	REQUIRES ACETATE	+	-	-	-	-
M. GENIUM TATII	2702	COCCI	-	37-40	12	54	7		+	+	-	-	-
M. GENIUM THERMOPHILICUM C.R.I.	2373	COCCI	-	55	.25	59	7	MARINE, REQUIRES NaCl TRYPTICASE, AND VITAMINS; THERMOPHILE	+	+	-	-	-
M. SPIRILLUM HUNGATEI J.F.I.	864	SPIRILLUM	-	35-45	17	45	6.6-7.4	MOTILE	+	+	-	-	-

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	DSM NO.	MORPHOLOGY	GRAM	TEMP.	GEN (H)	MOL % G+C	PH	SPECIAL CHARACTERSTICS	H_2/CO_2	FORMATE	ACETATE	METHANOL	METHYLAMINES
M. THRIX SOEHNGENII	2139	RODS	-	37	3.5D	51.9	7	UNABLE TO USE $H_2 + CO_2$	-	-	+	-	-
M. COCCOIDES METHYLUTENS	-	COCCI	-	30-35	5.2	42.0	7.0-7.5	MARINE, UNABLE TO USE $H_2 + CO_2$ ACETATE, OR FORMATE FOR METHANOGENESIS; NON MOTILE; COLONIES YELLOW PIGMENTED; YEAST EXTRACT STIMULATES GROWTH	-	-	-	+	+
M. SARCINA BARKERI	800	SARCINA	+	40	12	38.8	7	MOST METABOLIC VERSATILE OF ALL METHANOGENS	+	-	+	+	+
M. PLANUS LIMICOLA	2279	PLATES	-	40	7	47.5	7	ACETATE REQUIRED FOR GROWTH BUT NOT FOR METHANOGENESIS	+	+	-	-	-
M. LOBUS LINDARIUS	2278	COCCI	-	25	6	45.9	6.5	MARINE, MOTILE; YEAST EXTRACT DOES NOT STIMULATE GROWTH	-	-	-	+	+
M. PLASMA ELIZABETHII	-	PLASMA	ND	43	ND	7.2			+	+	-	-	-

M. = METHANO

N.D. = NOT DETERMINED

depending on the better-known bacterial forms such as Methanobacterium, Methanobacillus, Methanococcus and Methanosarcina.

However, the most widely used mode of classification tends to group the organisms based on physiology.

In 1956 Barker clustered the methanogens into a single family, the Methanobacteriaceae, which contained three genera and seventy species. However, according to Balch et al.(1979), the methanogens may be divided into three orders, the Methanobacteriales, Methanococcales and Methanomicrobiales which were further expanded by Dubach and Bachofen (1985).

Characteristics of Methanogens: Methanogens as a group are represented by a variety of morphological types including sarcina, rods and cocci. One good example of this morphological diversity is Methanococcus vanielii, a highly motile coccus with fragile walls and large cell forms (Balch et al., 1979). The large variety in morphology suggests either considerable evolutionary divergence of individual species or convergence from different origins. One would not, therefore, suspect a common physiology among the methanogens, although, as already demonstrated (Table 4), the methanogens as a group do share a number of similar attributes :

Oxygen: All the known species are strictly anaerobic and can only grow under low redox conditions (< -300 mV, Hungate, 1969). This considerable sensitivity to oxygen is one of the reasons why these organisms have proved difficult to isolate in the past.

Temperature: Most methanogens have been found to be active in either the mesophilic range of about 32° to 37°C or in the thermophilic range of 50 to 55°C (Ward, 1978; Zinder, Cardwell, Anguish & Koch, 1984). Of the two, it has been shown that the rate of methanogenesis is favoured at the higher temperatures (McCarty, 1964).

pH: Methanogens isolated so far are most active between pH 6.4 and 7.4 (Barker, 1956; McCarty, 1964; van den Berg et al., 1976) with values below 6 and above 8 inhibitory to the bacteria. However, one exception to this is the formate-metabolising species Methanococcus vannielii which grows best in alkaline conditions of pH 8 to 9 (Barker, 1956; Jones & Stadtman, 1976; Balch et al., 1979). There have been few reports on acidophilic methanogens although methane production has been noted in peatlands at pH values of 3.9 to 4.3 (Williams & Crawford, 1984).

Methane formation: Most methanogenic bacteria obtain their energy for growth from the reduction of carbon dioxide by hydrogen. Early in the 1900s two conflicting theories of methanogenesis were postulated. Barker (1956), suggested that methane was formed directly from carbon dioxide and hydrogen:

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \qquad (4)$$

(Barker, 1956)

which was further expanded when carbon dioxide was also shown to be

an electron acceptor for the oxidation of reduced organic compounds such as volatile fatty acids:

$$4H_2A \longrightarrow 4A + 8H \qquad (5)$$

$$CO_2 + 8H \longrightarrow CH_4 + 2H_2O \qquad (6)$$

with the net reaction:

$$4H_2A + CO_2 \longrightarrow 4A + CH_4 + 2H_2O \qquad (7)$$

The second theory was based on an experiment by Buswell & Sollo (1948) who found that 2- ^{14}C acetate was fermented to ^{14}C $CH_4 + CO_2$ and concluded that the methyl group of acetate was directly converted to methane whilst carbon dioxide arose from the carboxyl group :

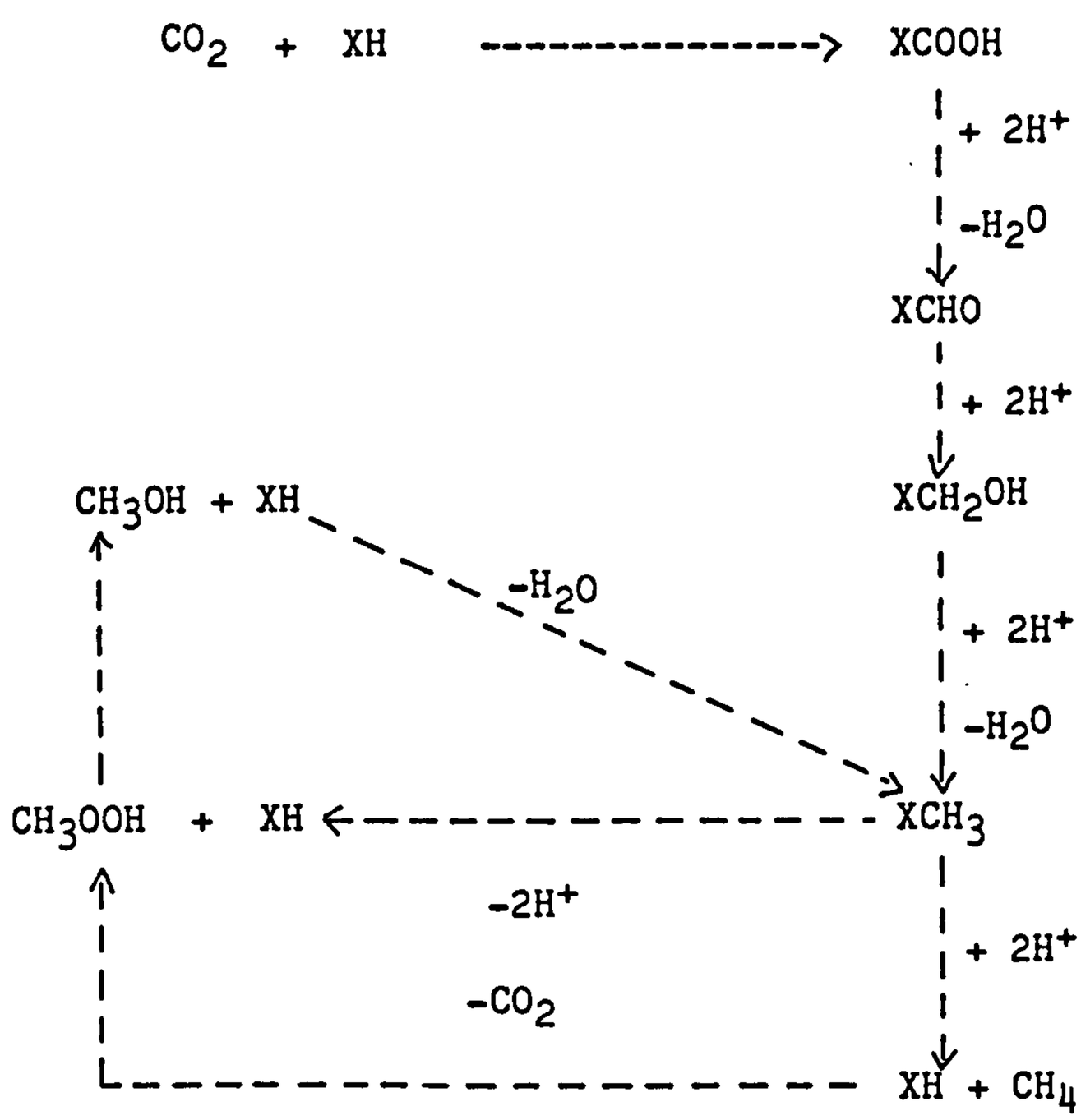
$$C^{14}H_3\,COOH \longrightarrow C^{14}H_4 + CO_2 \qquad (8)$$

(Kirsch & Sykes, 1971)

Recognising the apparent anomaly, Barker (1956) proposed a schematic unification of the two mechanisms which incorporated both the carbon dioxide reduction and the direct substrate reduction theories (Figure 4). Carbon dioxide was postulated to combine with an unidentified carrier XH which formed the carboxylate derivative of X. This was then reduced in stages to produce methane and a regenerated XH. Acetate and methanol also reacted with XH to give the intermediate XCH_3 with eventual methane production.

The modern concept of methane formation has altered little since, with the reduction of CO_2, usually by H_2, thought to occur via several intermediates which are most likely different oxidation states at the levels of formate, formaldehyde and methanol, as

Figure 4: Unified concept for carbon dioxide reduction and direct substrate reduction. (Barker, 1956).


CO_2 + XH
XCOOH
+ $2H^+$
$-H_2O$
XCHO
+ $2H^+$
CH_3OH + XH
XCH_2OH
$-H_2O$
+ $2H^+$
$-H_2O$
CH_3OOH + XH
XCH_3
$-2H^+$
+ $2H^+$
$-CO_2$
XH + CH_4

follows:

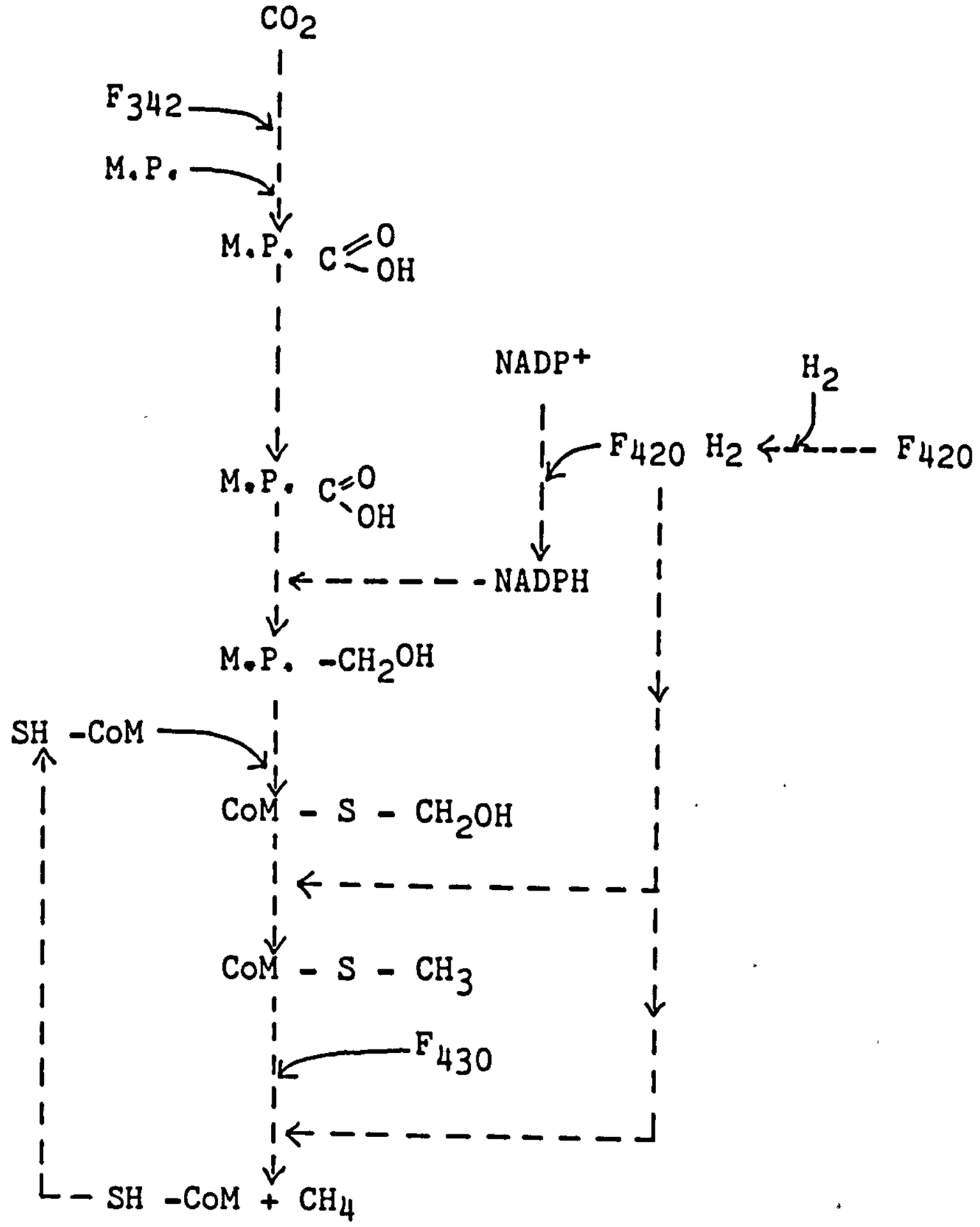
1. H_2 is activated by hydrogenase and used to reduce F_{420};
2. Reduced F_{420} reduces $NADP^+$ to NADPH;
3. CO_2 is bound by F_{342} (methanopterin, M.P.) to give MP-C$\overset{O}{\underset{OH}{<}}$;
4. MP - C$\overset{O}{\underset{OH}{<}}$ is reduced by an unknown electron donor to MP-C$\overset{O}{\underset{OH}{<}}$ in the presence of a $^1CO_2$ reduction factor;
5. The C$\overset{O}{\underset{OH}{<}}$ group bound to MP or an unidentified carrier is reduced to the level of $-CH_2OH$. The source of electrons is probably reduced F_{420} or NADPH;
6. The $-CH_2OH$ group is transferred to SH-CoM to give $HOCH_2$-S-CoM;
7. $HOCH_2$ - S-CoM is reduced to H_3C-S-CoM probably by reduced F_{420}; and
8. H_3C-S-CoM is reduced to HS-CoM and CH_4 by the methylreductase - F_{430} complex with F_{420} as the probable source of electrons.

This reaction sequence is summarised in Figure5.

Since there is no possibility of substrate level phosphorylation during growth on any of the methanogenic substrates, ATP synthesis most likely occurs via electron transport phosphorylation. Under standard conditions the overall reaction yields a ΔG_o^1 of -131 $kJmol^{-1}$. However, concentrations of H_2 are usually very low in natural environments (< 1 μM) and since these would influence the above rection it is unlikely that more than 1 ATP would be formed per mole of CH_4.

Formation of methane from acetate is still the subject of intense investigation although one certain fact is that tri-

Figure 5: Reaction sequence for the reduction of CO_2 to methane.

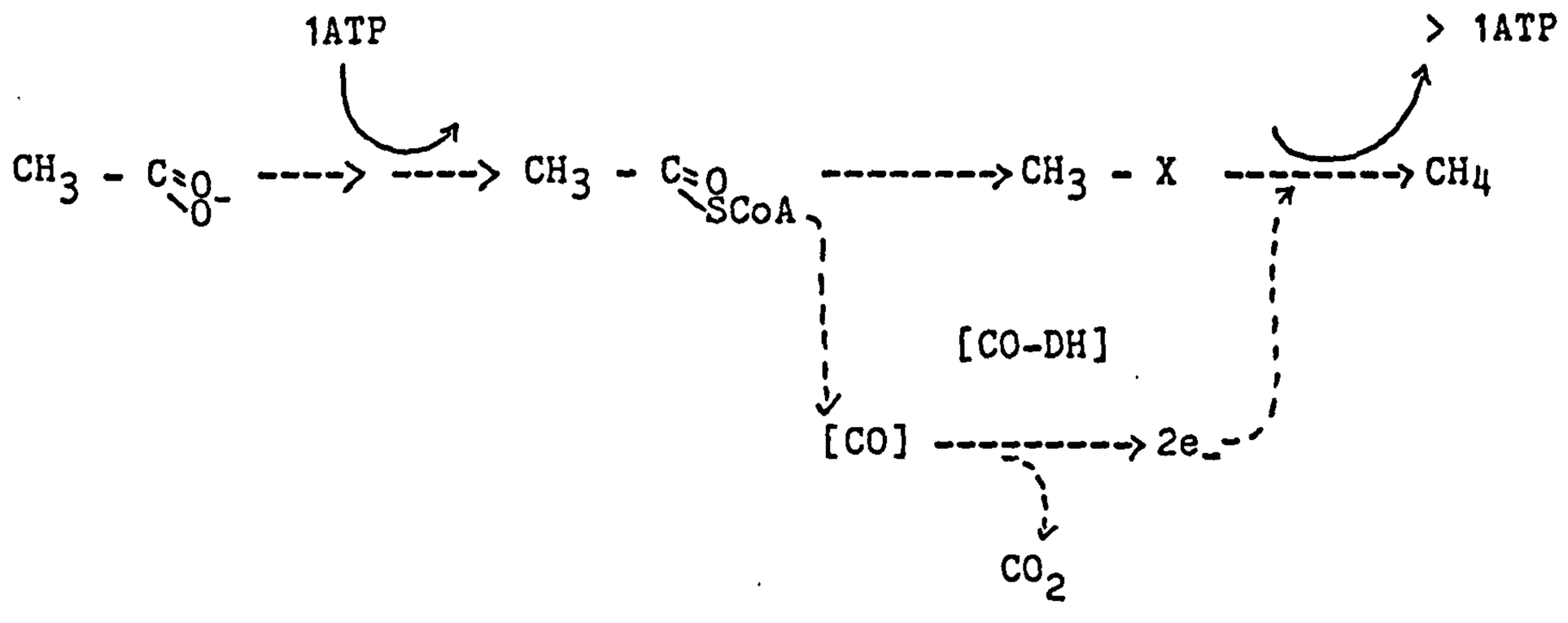

CO2
F342
M.P.
M.P. C=O OH
NADP+
H2
F420 H2
F420
M.P. C=O OH
NADPH
M.P. -CH2OH
SH -CoM
CoM - S - CH2OH
CoM - S - CH3
F430
SH -CoM + CH4

deuterate acetate gives rise to tri-deuterate methane, which thus excludes the possibility that acetate is first oxidised to two carbon dioxides, one of which is then reduced to CH_4 (Blaut & Gottschalk, 1982). It has also been found that the formation of methane from acetate, but not carbon dioxide or methanol, was specifically inhibited by cyanide, an inhibitor of carbon monoxide dehydrogenase (Eikmanns & Thauer, 1983). Figure 6 shows a proposed mechanism of acetate fermentation to methane and carbon dioxide with bound carbon monoxide as an intermediate (Thauer & Morris, 1984).

Assimilation of carbon dioxide to cell material: Methanogens carry out two distinct reductive processes with CO_2, one of which, as discussed above, leads to the formation of methane whilst the other to cell carbon assimilation. Actively growing methanogens convert approximately 5% of the available CO_2 to cell carbon with the remainder used for energy generation. As yet, there is no evidence for a Calvin cycle (Daniels & Zeikus, 1976; Zeikus et al., 1977) a hexulose phosphate cycle (Fuchs & Stupperich, 1978) or a serine pathway, or for the presence of key enzymes of the reductive tricarboxylic acid cycle (Daniels & Zeikus, 1978) which is thought to operate in the green photosynthetic bacterium, Chlorobium. Hence it would seem that methanogens have a unique mechanism for autotrophic carbon dioxide fixation.

Examination of $^{14}CO_2$-fixation products in Methanobacterium thermoautotrophicum by Fuchs & Stupperich (1980) revealed that alanine and aspartate appeared as the earliest labelled products

Figure 6 Acetate metabolism to CH_4 and CO_2 with carbon monoxide as an intermediate. CH_3 denotes activated methanol; and CO-DH, carbon monoxide dehydrogenase (Adapted from Thauer & Morris, 1984).


1ATP
> 1ATP
CH3 - C
O
O-
CH3 - C
SCoA
CH3 - X
CH4
[CO-DH]
[CO]
2e
CO2

with significant concentrations of radioactivity also apparent in glutamate and coenzyme M derivatives. These studies led to the proposal of an "activated acetic acid pathway" (Figure 7). The first identified product of this pathway was activated acetic acid which appeared to be synthesised from two CO_2 molecules via bound one carbon units in a process related to the clostridial homoacetate fermentation (Ljungdahl & Wood, 1982).

1.3.6 Homoacetogenesis

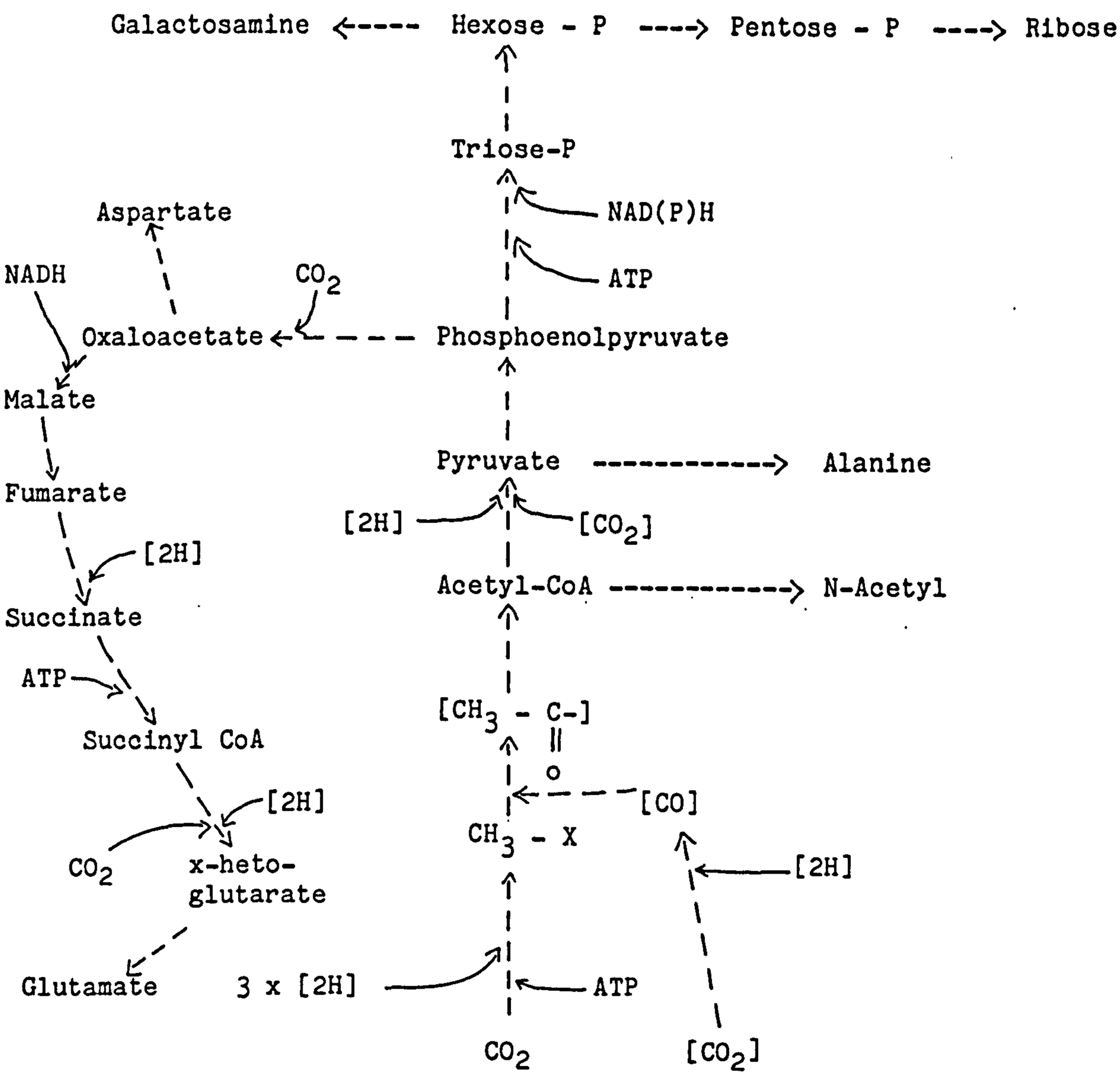
In addition to methanogens there is a second group of bacteria which are capable of growing lithotrophically on H_2 and CO_2 although the product of CO_2 reduction in this case is acetate instead of methane. Organisms such as Acetobacterium woodii (Balch et al., 1977) carry out homoacetate metabolism as follows :

$$2CO_2 + 4H_2 \longrightarrow CH_3COOH + 2H_2O \qquad (9)$$

$$\Delta G_o^1 = -104.6 \text{ kJmol}^{-1}$$

Acetate production from H_2 + CO_2 by monocultures was first demonstrated by Wieringa (1940). The isolated bacterium, Clostridium aceticum, however, was reported to have been lost for many years but recently was resuscitated from an old endospore preparation (Braun, Mayer & Gottschalk, 1981). Similar isolates have also been described by Adamse (1980) and Ohwaki & Hungate (1977). Other species with the capability of reducing CO_2 to acetate include Acetobacterium (Balch et al., 1977; Braun & Gottschalk, 1982), Acetogenium (Leigh, Mayer & Wolfe, 1981),

Figure 7: Proposed pathway of autotrophic cell carbon fixation from CO_2 in Methanobacterium thermoautotrophicum (Fuchs & Stupperich, 1982).


Galactosamine
Hexose - P
Pentose - P
Ribose
Triose-P
NAD(P)H
ATP
Aspartate
NADH
CO_2
Oxaloacetate
Phosphoenolpyruvate
Malate
Pyruvate
Alanine
Fumarate
[2H]
[CO_2]
[2H]
Acetyl-CoA
N-Acetyl
Succinate
ATP
[CH_3 - C-]
Succinyl CoA
[2H]
[CO]
CO_2
CH_3 - X
[2H]
x-heto-
glutarate
Glutamate
3 x [2H]
ATP
CO_2
[CO_2]

Eubacterium (Sharak-Genther, Davis & Bryant, 1981), Clostridium (Wiegl, Braun & Gottschalk, 1981), and Acetoanaerobium (Sleat, Mah & Robinson, 1985), including a thermophilic species (Leigh et al., 1981; Wiegel et al., 1981).

Table 5 compares the physiological characteristics of several homoacetogens.

Although the contribution of homoacetogens to the anoxic cycling of carbon has yet to be elucidated it was found by Jones & Simon, 1985 that homoacetogens accounted for a considerable amount of the available H_2 even though the free energy available from this reaction is less than the reduction of CO_2 to CH_4 by methanogens (Thauer et al., 1977).

Unlike methanogens, homoacetogens have the capacity to grow heterotrophically on sugars (Braun & Gottschalk, 1981) with acetate characteristically the sole end product. Biochemical studies have revealed that homo-acetogens do not possess the unique set of coenzymes found in methanogens and it would appear that they resemble typical fermentative anaerobes much more than they do methanogens. Formation of acetate from CO_2 has been extensively studied and has resulted in the proposed model shown in Figure 8 (Kerby, Niemczura & Zeikus, 1983).

1.4 Interspecies Interactions

Microbial associations are known to be ubiquitous in nature although very few associations have been fully characterised. Microbial associations were comprehensively reviewed by Meers (1973)

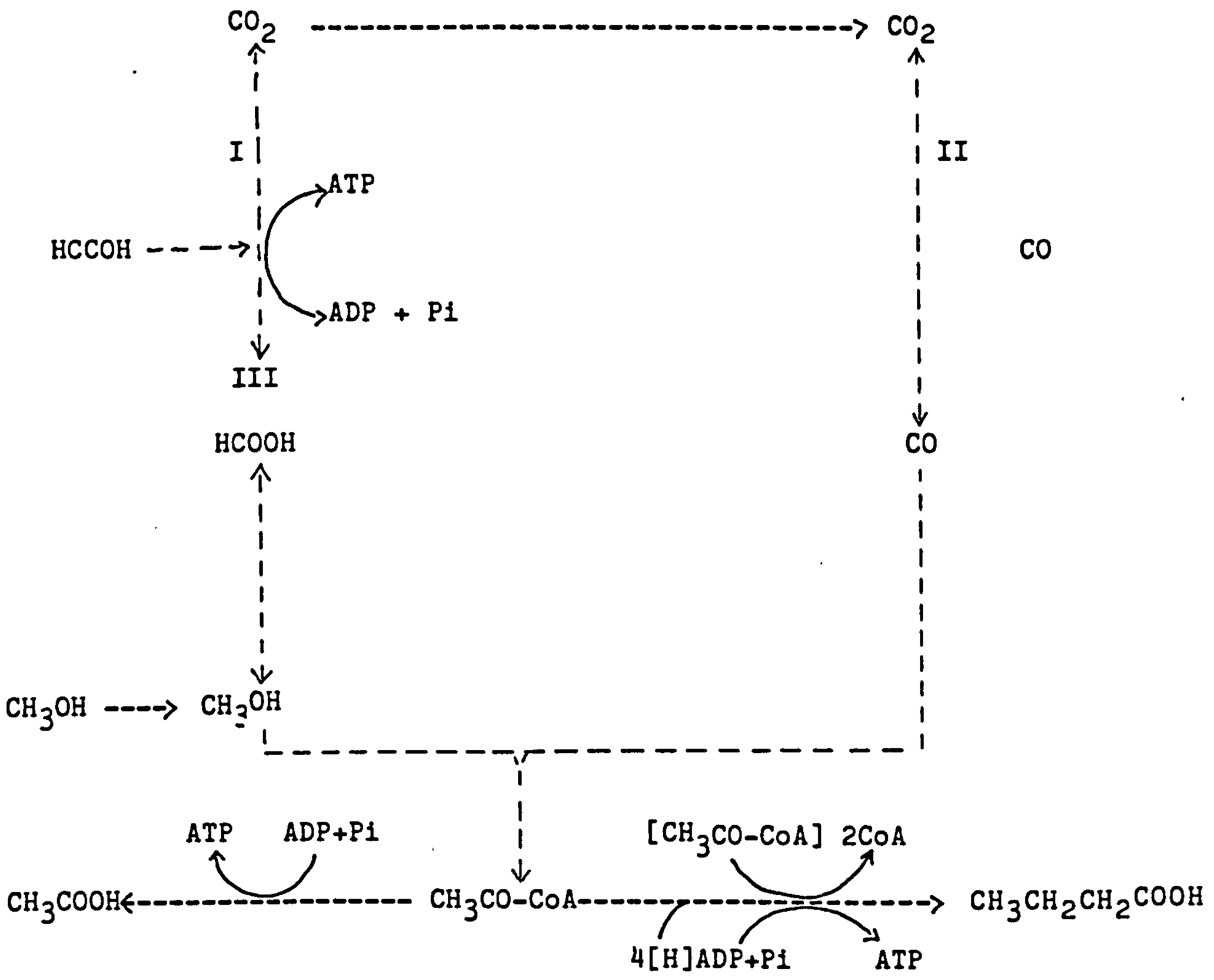
Table 5: Characteristics of homoacetogenic bacteria. From Sleat, Robinson & Mah (1985).

CHARACTERISTIC	C. ACETICUM DSM 1496	ACETOBACTERIUM WOODII ATCC 29683	ACETOBACTERIUM WEIRINGAE DSM 1911	ACETOGENIUM KIVUI ATCC 33488	ACETOANAEROBIUM NOTERAE ATCC 35199
CELL MORPHOLOGY	RODS	OVAL RODS	OVAL RODS	RODS	RODS
CELL WIDTH (μM)	0.8 - 1.0	1	1	0.7 - 0.8	0.8
CELL LENGTH (μM)	5	2	1 - 2	2 - 7.5	1 - 5
GRAM REACTION	-	+	+	-[A]	-[A]
MOTILITY	+	+	+	-	+
FLAGELLA	PETRICHOUS	SUBTERMINAL	SUBTERMINAL	NONE	PERITRICHOUS
SPORE FORMATION	+	-	-	-	-
PH OPTIMUM	8.3	NR[B]	7.2 - 7.8	6.4	7.6
TEMP OPTIMUM (°C)	30	30	30	66	37
SUBSTRATES FERMENTED					
FRUCTOSE	+	+	+	+	-
GLUCOSE	-	+	-	+	+
MALTOSE	-	-	-	-	+
PYRUVATE	+	-	-	+	-
YEAST EXTRACT REQUIRED FOR H_2 OXIDATION	+	-	-	-	+
GUANINE-PLUS-CYTOSINE CONTENT (MOL %)	33	39	43	38	37

A TRANSMISSION ELECTRON MICROGRAPHS SHOW GRAM-POSITIVE CELL WALL STRUCTURE OF ACETOGENIUM KIVUI BUT AN ATYPICAL DOUBLE-LAYERED WALL OF STRAIN NOT-3

B NR, NOT REPORTED.

Figure 8: Single-carbon catabolism flow model proposed for acetogenic bacteria which synthesise either acetate or butyrate from single carbon compounds (Kerby, Niemczura & Zeikus, 1983).


CO2
CO2
I
II
ATP
HCCOH
CO
ADP + Pi
III
HCOOH
CO
CH3OH
CH3OH
ATP
ADP+Pi
[CH3CO-CoA] 2CoA
CH3COOH
CH3CO-CoA
CH3CH2CH2COOH
4[H]ADP+Pi
ATP

who described the growth of bacteria in mixed cultures as one of six types of interactions: competition, amensalism, predation, parasitism, commensalism and mutualism. However, an approach which is probably more relevant to the present study would be to consider microbial interactions on a biochemical basis : nutritional interactions, bioenergetic requirements or differences or changes in the metabolic capabilities of the interacting microbial population (Bull & Slater, 1982) an approach which was further refined to consider seven classes of microbial association :

1. Structure due to the provision of specific nutrients between different members of the community;
2. Structure due to the removal of metabolic products which may be inhibitory to the producing member of the community, including H_2-transfer associations;
3. Structure and stability due to interactions which may result in the modification of individual population growth parameters resulting in a more competitive and/or efficient community;
4. Structure due to the effect of a concerted combined metabolic capability, not expressed by the individual populations acting alone;
5. Structure due to a co-metabolic stage;
6. Structure due to the transfer of hydrogen ions; and
7. Structure due to the presence of more than one primary substrate utiliser.

In this study two major interactions were envisaged:

1. syntrophic interaction between H_2-producing acetogens and H_2-utilising organisms; and
2. competition for common substrates between sulphate-reducing bacteria and methanogens.

The former was reviewed in the sections on acetogenesis and interspecies hydrogen transfer (1.3.3).

1.4.1 Interaction between sulphate-reducing bacteria and methanogens

Terminal mineralisation of organic polymers may proceed in two directions depending on the environmental conditions which are competitive but not exclusive. In environments with sufficient sulphate it is the sulphate-reducing bacteria that function predominantly as terminal oxidisers (Laanbroek & Veldkamp, 1982) whereas in sulphate-limited environments it is the methanogens, together with hydrogen producing acetogenic bacteria, which carry out terminal oxidation. In other words sulphate-reduction is more common in marine ecosystems and methanogenesis in freshwater and terrestrial environments. The reason why sulphate-reducing bacteria outcompete methanogens for common substrates in sulphate sufficient environments is that the former have higher affinities for acetate and hydrogen. When H_2 concentrations drop below between 5 and 10 uM, as they often do in sulphate-rich environments, methanogens are no longer able to grow since their H_2-uptake systems are unable to function at such low concentrations. Sulphate-reducing bacteria, on

the other hand, can grow at these low partial pressures of H_2, effectively 'inhibiting' H_2-mediated methanogenesis. A possible explanation for this inhibition is that the affinity of sulphate-reducing bacteria for H_2 (and acetate) is more than 10 times that of methanogens (Pfennig, 1984).

Thus, although very little work has been done on interacting microbial associations isolated from landfill, postulated interactions can be derived (Senior & Balba, 1985) (Figure 9).

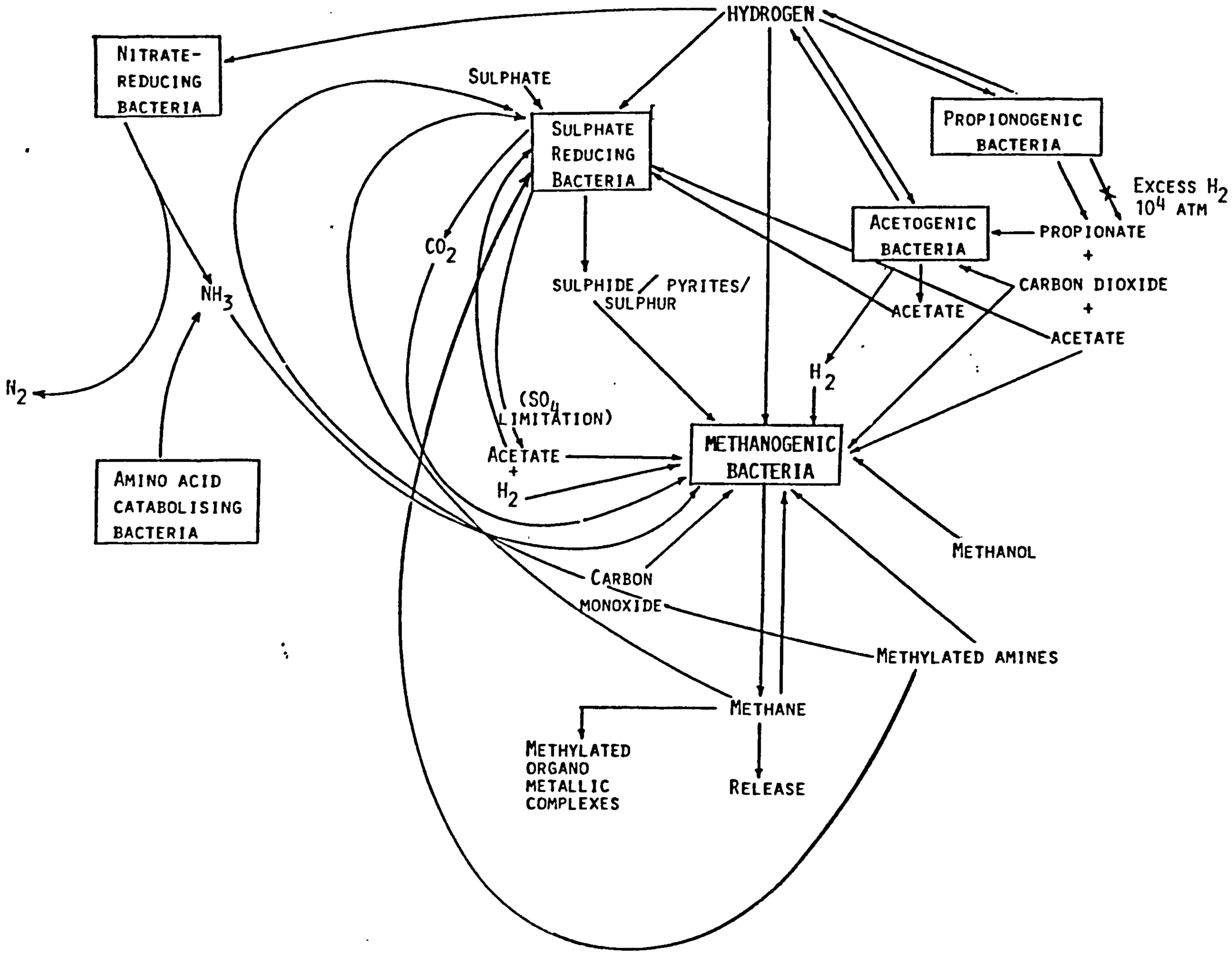
1.5 Enrichment and Isolation of Interacting Microbial Associations

Most natural ecosystems contain a considerable amount, both in terms of numbers and metabolic types, of microorganisms and, hence, if one is to study a particular process, it is often necessary to change the environment such that the specific microbial association, or individual species, is increased at the expense of others. This process, enrichment, is highly selective and ideally all but the selected for association will be competitively displaced under the conditions imposed by the enrichment conditions.

A wide number of enrichment methods have been reviewed by, amongst others: Hutner (1962), who considered nutrition; Stanier (1953),methods; Veldkamp (1977), history and chemostat; Pochon and Tardieux (1967), methods for soil bacteria; Parkes (1982), laboratory systems; and Parkes & Senior (in press), multi-stage chemostats.

As one would expect the majority of examples from the above reviews have dealt largely with enrichment of aerobic species

Figure 9: Interactions in anoxic environments.


HYDROGEN
NITRATE-REDUCING BACTERIA
SULPHATE
SULPHATE REDUCING BACTERIA
PROPIONOGENIC BACTERIA
EXCESS H_2 10^4 ATM
ACETOGENIC BACTERIA
PROPIONATE
+
CARBON DIOXIDE
+
ACETATE
CO_2
NH_3
SULPHIDE / PYRITES/ SULPHUR
ACETATE
H_2
H_2
(SO_4 LIMITATION)
METHANOGENIC BACTERIA
ACETATE
+
H_2
AMINO ACID CATABOLISING BACTERIA
METHANOL
CARBON MONOXIDE
METHYLATED AMINES
METHANE
METHYLATED ORGANO METALLIC COMPLEXES
RELEASE

whilst, until latterly (Parkes & Senior, in press), anaerobes have been essentially neglected. For the purpose of this discussion only examples of the latter will be considered.

Enrichment cultures can be initiated in two ways : closed and open. In closed culture enrichments a fixed amount of enriching material is added at the initiation of the experiment whereas in open culture enrichments fresh quantities are added continuously. All enrichments, however, have the following points in common (Veldkamp, 1970):

1. The water content of the medium should be high enough to allow proliferation of the selected organism(s);
2. Media should contain the elements and energy source required by the organism(s) in order to grow and these should be present in a form available to the organism(s); and
3. The organism(s) to be enriched should be present in the inoculum.

The type of closed enrichment used most commonly is the batch flask liquid culture in which the inoculum is taken from an anoxic ecosystem such as an anaerobic digester, sediment or landfill. In this type of system a number of environmental conditions can be specified such as medium composition, pH, Eh, temperature and light. Closed culture enrichments have been used successfully to enrich, for example, populations and single species of nitrate and sulphate reducers and methanogens. For example, Otto et al. (1980) used glass-stoppered bottles, which were completely filled with medium

which contained lactate and nitrate, to enrich for the nitrate-reducing bacterium Pseudomonas stutzeri from the sludge of a potato starch mill. Widdel & Pfennig (1977) similarly used closed cultures to enrich acetate-catabolising sulphate reducers such as Desulfotomaculum acetoxidans from both fresh water and marine sediments. Samples from these two types of environment together with sewage sludge were used to enrich for sulphate-reducing bacteria that could metabolise vanillate, syringate and 3,4,5-trimethyleinamate (Bache & Pennig, 1981). Finally, the anoxic serum bottle was used by Shlomi,Lankhorst & Prins (1978) to enrich from anoxic muds a methanogenic association which degraded benzoate.

Two variations of the closed culture enrichment are the Winogradsky soil column and the batch culture with gas collection of Balba (1978). The Winogradsky (1949) soil column mimics the habitat formed by anaerobic muds covered by water. The soil is amended at the beginning of the experiment by the addition of a fixed quantity of the enriching substrate and the column left to stabilise over a period of time. The system developed by Balba (1978) was first used to enrich for benzoate-degrading species under anoxic conditions. This system has the advantage of maintaining a high gas head space : liquid ratio thus negating any possible feedback inhibition by gaseous products (Hanson & Molin, 1981). This method has been further used successfully to enrich from anoxic freshwater sediments microbial associations which degraded veratric and syringic acids to methane (K.K. Abdul-Halim, unpublished observations). A

characteristic of closed culture enrichments is that nutrients become exhausted and metabolic products accumulate. Thus, competitive selection of microorganisms is based solely on their maximum specific growth rate (u_{max}) which could be considered analogous to the initial stages of anoxic landfill catabolism.

Open culture systems operate under a totally different set of conditions as fresh nutrients are continuously supplied and metabolic products continuously removed. Two types of open culture enrichment systems have been recognised by Schlegel & Jannasch (1967), the homo- and hetero-continuous culture. The two types of homo-continuous culture are the turbidostat and chemostat of which the latter has been the more commonly used in enrichment studies. According to Parkes (1982) chemostat enrichment cultures have the following advantages :

1. The conditions throughout can be constant and as a consequence the enrichment is reproducible;
2. The dilution rate of the system determines the specific growth rate of the enrichment organism(s);
3. The displacement of metabolites and cells prevents the accumulation of potentially inhibiting products, which, together with the ability to use low concentrations of inhibitory substrates make continuous culture ideal in the enrichment of organisms capable of degrading toxic compounds;
4. Because chemostats can provide stable conditions these systems are ideal for enrichment of 'natural' microbial associations

which are ubiquitous in the environment;

5. The long term effects of potentially toxic compounds can be investigated in continuous culture, for example, the degradation of pesticides, recalcitrant molecules, oils and metals; and

6. Steady states develop when the microorganism's growth rate equals the dilution rate and the biomass and environmental parameters remain constant.

Chemostats have only been used to a limited extent in anaerobic environment studies (1.6) although the mechanisms of enrichment together with the possible effects of enrichment conditions have been fully discussed by Parkes (1982).

Following enrichment and isolation of a microbial association, isolation of the component monocultures may be accomplished by the use of non-selective and selective media, and on the basis of morphology and growth by use of methods such as the roll tube, Hungate technique (1969), gas jars and anaerobic chambers. There are many examples in the literature of the isolation of monocultures by the above techniques, for example, sulphate-reducing bacteria (Widdel & Pfennig, 1977, 1981; Badziong, Thauer & Zeikus, 1978; Mountfort & Bryant, 1982), methanogens (Edwards & McBride, 1975; Huser, Wuhrmann & Zehnder, 1982; Sowers & Ferry, 1983) and homoacetogens (Wiegel, Braun & Gottschalk, 1981; Sleat, Robinson & Mah, 1985).

1.6 Methods of Study

Complex microbial interactions of the type typified by terminal anerobic processes present many problems to the investigator. However, it is generally accepted that the most satisfactory method of investigating 'natural' microbial processes is to use a chemostat (Pfennig, 1984). The major advantage of this approach is the accurate simulation of substrate limitation and slow growth rate (Jannasch, 1967), although in, for example, landfill, substrate is usually present in excess (Senior & Balba, 1983). Although chemostats are ideal for studying submaximal growth rates, the major limitation is that continuous culture enforces growth under constant environmental conditions in a steady state. The physiological characteristics of the microbial population in a single stage chemostat are, therefore, not dependent on preceding states but on the dilution rate and as a consequence the system becomes homogeneous. Single-state chemostats have been used by several investigators to study microbial interactions in anaerobic processes (Hanson & Molin, 1981; Keith, Herbert & Harfoot, 1982; Traore et al., 1983a; Boone, 1984).

Heterogeneity, however, is essential for realistic modelling of anoxic microbial interactions as most anoxic ecosystems are spatially organised (Figure 2) with the environment and substrate availability determining the stratification. Thus, an ideal cultivation system should, even under continuous conditions, pass through stages of development which will influence its final state.

The very nature of anaerobic degradation, of sequential metabolism by different bacterial groups (Zeikus, 1983b), thus excludes the use of single-stage chemostats, which are homogeneous and rely on steady-state kinetics. In addition chemostats have only limited value when studying mutually exclusive niches, such as nitrate reduction and methanogenesis, or niches with limited overlap such as sulphate-reduction and methanogenesis.

Temporal heterogeneity can be modelled with a batch culture, where growth is dependent on the result of sequential physiological states which change with time, with each physiological state dependent on the previous one. Unfortunately, since closed cultures are characterised by conditions of excess substrate then their applicability to studies of anoxic microbial interactions are somewhat limited. Despite these considerations, batch culture still appears to be the most widely used technique to study anaerobic degradation (McInerney & Bryant, 1981; Fiebig & Gottschalk, 1983; Eichler & Schink, 1984). However, as already noted, most landfills are characterised by excess substrate and hence this method could be used to monitor microbial processes.

In addition to batch cultures, temporal distribution may be simulated by spatial distribution in an ideal plug flow reactor (Ricica, 1968) where the culture is variable in space but constant in time. Settlement of cells, however, together with longitudinal and radial irregular movement caused by gas bubbles and flow instabilities cause a plug flow reactor to become an imperfectly-

mixed reactor, the contents of which are variable both in time and space.

An example of this type of reactor is the multi-stage chemostat which was used by Thompson et al. (1983) to examine carbon flow in marine sediments. Five vessels, each of 800 ml capacity, were connected in series and influent medium was introduced into the first vessel of the array to give a dilution rate of 0.009 h^{-1} which was equivalent to an overal dilution rate of 0.0018 h^{-1}. Glucose (10 mM) and benzoate (10 mM) were individually used as limiting carbon and energy sources. With both substrates, spatial separation of individual groups resulted with sulphate reduction preceding methanogenesis.

The same approach was used by Parkes & Taylor (1983a) to isolate bacterial associations of different respiratory types (aerobic, facultative aerobic and facultative anaerobic) from marine sediments. Basic mineral salts medium supplemented with glucose (1 gl^{-1}), cellulose (0.1 gl^{-1}) and sulphate (6mM) was introduced into the first vessel of a multi-stage array which consisted of four linked stirred chemostats. The dilution rate was set at 0.008 h^{-1} in the first vessel and was subsequently decreased to 0.006 h^{-1} in the remaining three vessels. Comparison of the analysis of the fatty acid distribution of the system with that of the sediment distribution showed good correlation and thus it would appear that this method facilitated 'realistic' modelling of marine sediments.

There is now increasing use of multi-stage chemostats to study

anoxic metabolism and the characteristics and applicability of these systems have been fully revivewed by Parkes & Senior (in press).

2. MATERIALS AND METHODS

2.1 Source of inoculum

Partially-decomposed domestic refuse was used in a closed culture enrichment to isolate an interacting microbial association capable of catabolising hexanoate. The culture was then used to inoculate a 5-stage chemostat (Senior & Balba, 1984) the first vessel of which was subjected to a dilution rate of 0.025 h^{-1} for 1 year prior to initiation of this study.

2.2 Growth medium and culture conditions

Two basic mineral salts media were used:

2.2.1 Basic mineral salts medium (A)

Contained the following (g l^{-1} glass-distilled H_2O): K_2HPO_4, 1.5; Na $H_2PO_4.2H_2O$, 0.85; NH_4Cl, 0.9; $MgCl_2.6\ H_2O$, 0.2; $NaHCO_3$, 0.5; Na_2CO_3, 0.2; trace elements, 1.0 ml; trace minerals, 1.0 ml; nickel solution (1 mmol l^{-1}), 1.0 ml; vitamins, 1.0 ml; resazurin (0.01% w/v), 1.0 ml.

The trace elements contained (mg l^{-1}): $FeCl_2.4H_2O$, 1500; NaCl, 9000; $MnCl_2.4H_2O$, 197 ; $CaCl_2$, 900; $CoCl_2.6H_2O$, 238; $CuCl_2.2H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; H_3BO_3, 62; $NiCl_2.6H_2O$, 24.

The trace minerals contained (mg l^{-1}): $NaMoO_4.2H_2O$, 48.4; $NaSeO_3.xH_2O$ (31% Se), 2.55; $Na_2WO_4.2H_2O$, 3.3.

The vitamins contained (mg l^{-1}): biotin, 10; p-amino benzoic acid, 19; x-lipoic acid, 20; folic acid, 10; pyridoxine HCl, 20; thiamine HCl, 20; riboflavin, 30; nicotinic acid, 50; D (+) (Ca-

pantothenate, 30; cyanocobalamine, 20.

For use in batch cultures the medium was prepared in three parts. Firstly, a solution which contained the basic mineral salts minus $NaHCO_3$, Na_2CO_3, vitamins and trace elements was diluted to 900 mls with distilled H_2O. This was distributed into bottles or flasks and was sterilised by autoclaving at 15 lb psi (121°C) for 20 minutes. After cooling, appropriate volumes of a filter sterilised (2.2.4) 100 ml solution, which contained the remainder of the medium with the pH poised at 7.0, were added aseptically to the bottles to give a final ratio of 1:9.

Finally, filter sterilised (2.2.4) sulphide solution ($Na_2S.9H_2O$, 12 g 100 ml^{-1}) was added to give a final concentration of 18 mg l^{-1}. The bottles were then overgassed with oxygen-free nitrogen (O.F.N., British Oxygen Company) after removal of residual O_2 with an oxygen trap (Phase Separations Limited) to give an Eh of less than -30 to -40 mV.

The bottles were maintained at 30°C overnight prior to addition of the carbon source and inoculum.

10 litre volumes of medium were prepared for chemostats in two stages. Firstly, the correct weight of reagents was diluted to 900 ml with glass-distilled H_2O. Sodium sulphate (various concentrations) and hexanoic acid (6.3 ml) were then added with the latter solubilised in the medium by conversion to sodium hexanoate. The resulting solution was diluted to 10 litres with glass-distilled H_2O and filter sterilised into a sterile 10-litre aspirator by

passage through a Sartorius Membrane Filter (0.2 μm, SM 11307) in this case under an atmosphere of O.F.N.

2.2.2 Basic mineral salts medium (B)

This medium was adapted from Kaiser and Hanselmann (1982) and contained the following (g l^{-1} glass-distilled H_2O) : KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1.0; $MgCl_2.6H_2O$, 0.4; KCl, 0.5; $CaCl_2.2H_2O$, 0.15; HCl (1.0N), 5.0 ml. The following additions were made to the autoclaved (15 lb psi, 121°C, 20 minutes), cooled medium from sterile stock solutions (litre^{-1}): acidic trace elements, 1.0 ml; $NiCl_2.6H_2O$ (1 mmol l^{-1}), 1.0 ml; $NaHCO_3$ (1 mol l^{-1}), 20 ml; basic trace elements, 1.0 ml; vitamins, 1.0 ml; resazurin (5 mmol l^{-1}), 1.0 ml. The acidic trace elements were dissolved in 50 mM HCl and contained the following (mg l^{-1}): $FeCl_2.4H_2O$, 1491; H_3BO_3, 61.8; $ZnCl_2$, 68.1; $AlCl_3$, 50; $MnCl_2.4H_2O$, 90; $CuCl_2.2H_2O$, 17; $CoCl_2.6H_2O$, 238; $NiCl_2.6H_2O$, 23.8. The basic trace elements were of the same composition as those described above (2.2.1) but in this case were dissolved in 100 mM NaOH. The vitamin solution was the same as before (2.2.1). Finally the $Na_2S.9H_2O$ solution (100 mM), 2.5 ml l^{-1}, C-source, sodium dithionite (52.5 mg l^{-1}) and the inoculum were aseptically mixed under a constant stream of O.F.N.

The procedure used to prepare agar-solidified basic mineral salts medium A and B was as follows. The media were prepared in double-strength aliquots and filter sterilised (2.2.4). Similarly, the agar, 1.2% w/v (Agar Technical, Agar No. 3, Oxoid) was also constituted double strength and sterilised by autoclaving (15 lb

psi, 121°C, 20 minutes). After the agar had cooled to approximately 50°C the two solutions were aseptically mixed and the plates immediately poured.

The plates were then transferred to the interchange lock of an Anaerobic Chamber (Forma Scientific, 1024) and O_2 eliminated. After setting, the plates were placed inside the Anaerobic Chamber and maintained under an atmosphere of CO_2, H_2, N_2 (5%: 10%: 85%) for between 24 and 48 hours prior to use.

2.2.3 Preparation of carbon sources

Solutions: 1 M stock solutions were prepared of the oxidisable carbon sources from either the sodium salts or their acids. When the sodium salts were used these were dissolved directly in glass-distilled water. Preparation of stock solutions from the acids, however, involved the following : to 50 ml of glass-distilled H_2O, 3g NaOH pellets were added. Appropriate volumes of acid were then added followed by 3 N NaOH to give a final pH of 9.0 ± 0.1. After dilution to 100 ml the solutions were autoclaved at 15 lb psi, 121°C, for 20 minutes.

Gases: Where CO_2 was used as a Carbon source (and H_2 as a source of electrons) this was supplied as H_2/CO_2 (80:20) (B.O.C.).

2.2.4 Filter sterilisation

Growth media and solutions such as hydrogen sulphide (2.2.1) were sterilised by passage through a Whatman 0.2 um cellulose acetate membrane filter, under vacuum.

2.2.5 Culture conditions

Batch cultures were made in flasks or bottles with 10 to 50 ml culture, depending on the volume of the container (Appendix 1), under an atmosphere of O.F.N. with the flasks sealed with Suba Seals and bottles with serum caps (MacFarlane Robson Ltd., UK). Except where otherwise stated the cultures were incubated, inverted at 30°C in the dark, stationary.

Agar plates were incubated in either the Anaerobic Chamber (Forma Scientific, 1024) inverted, under a gas atmosphere of CO_2, H_2, N_2 (5:10:85) or in anaerobic jars with a specified atmosphere (O.F.N. or H_2/CO_2, 80:20). All culture manipulations were made within the chamber.

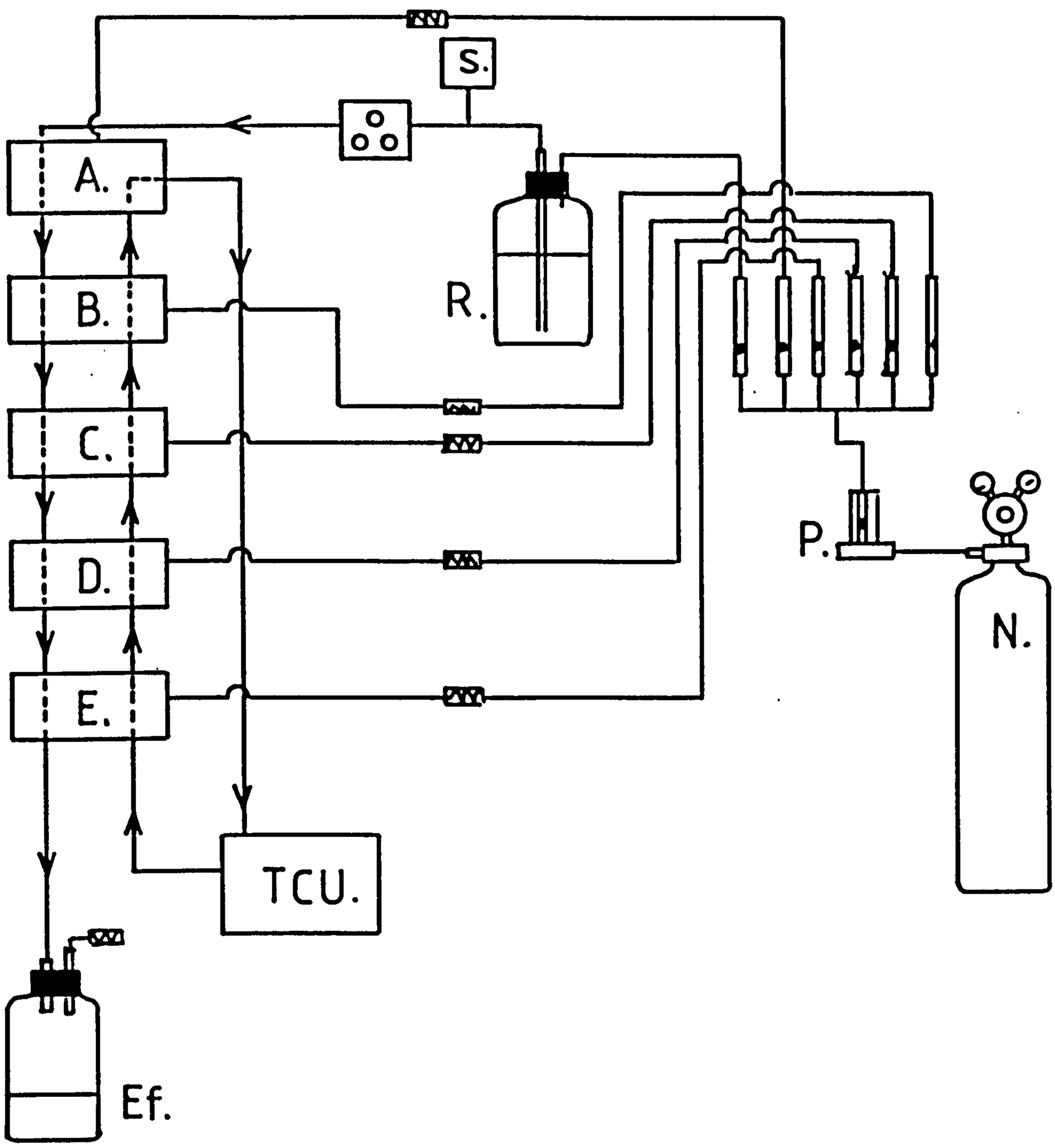
2.3 Cultivation Systems

2.3.1 Multi-stage chemostat : five vessels

A 5-stage chemostat was constructed as shown in Figures 10 and 11. Influent medium (2.2.1) was introduced into the first vessel by means of a Watson Marlow (MHRE/2) Peristaltic Pump at a constant flow rate to give a dilution rate of 0.005 h^{-1} for the whole system and 0.025 for each individual vessel. The culture vessels, each of 700 ml capacity, were maintained at a constant temperature of 25°C by water jackets (10) in conjunction with a Churchill Thermocirculator (05-CTCV, Churchill Instrument Co., Uxbridge). Each vessel was fitted with an angled effluent overflow tube (4), the height of which was calculated to give the required volume. The

Figure 10: Anoxic 5-vessel chemostat in which a medium (2.2) pumped from the medium reservoir (R) into the first vessel, A. Culture subsequently eluted down the array by weir overflows and collected in the effluent reservoir (Ef). Culture vessel headspaces purged with a flow stream of O.F.N. (N) and the vessels maintained at 25°C by a thermocirculator unit (T.C.U.)

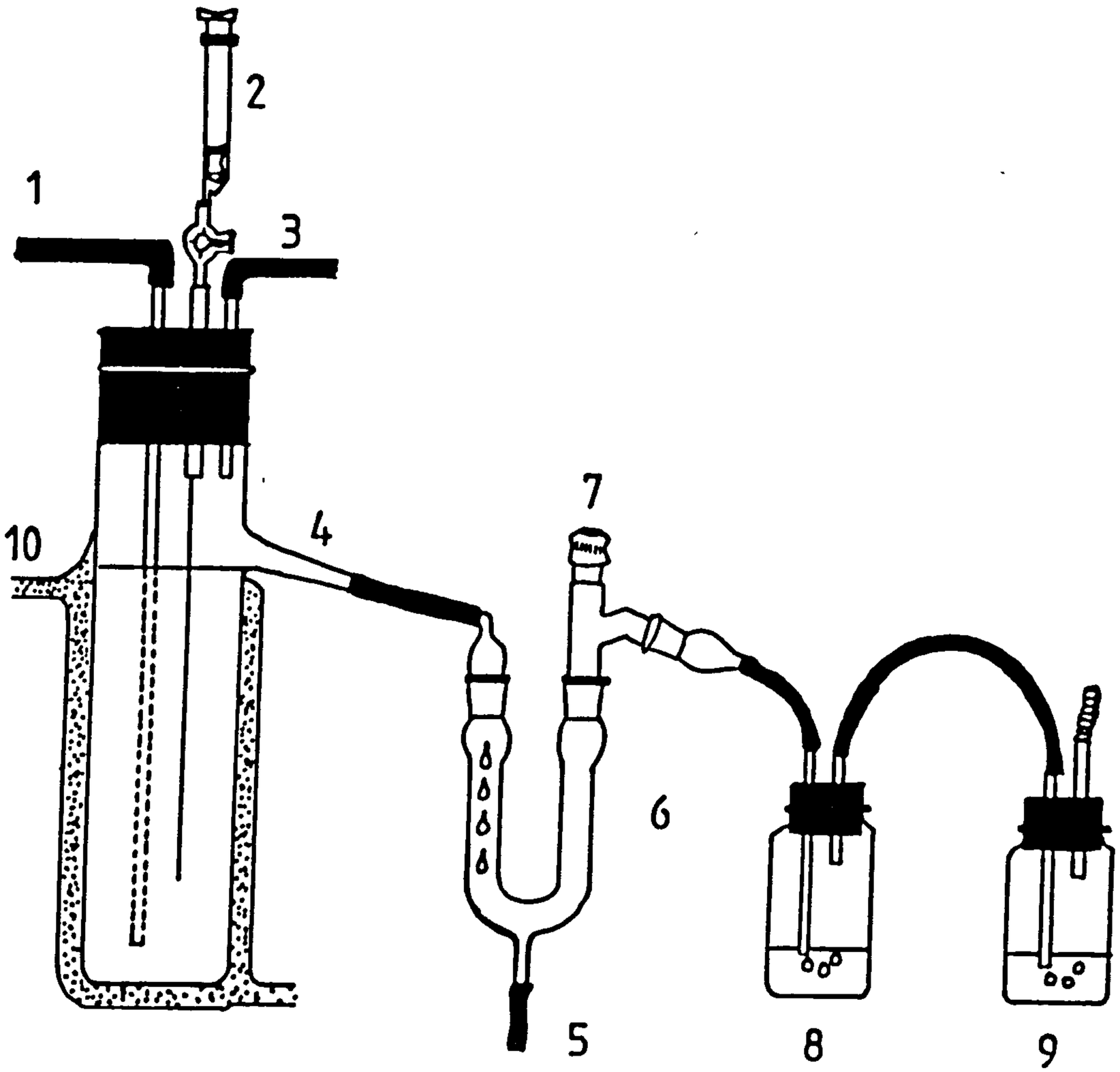
Each vessel sampled for metabolites and compared with the medium, which was sampled in the sampling bottle (S).



A - E	Culture vessels
R	Medium reservoir
Ef	Effluent vessel
TCU	Thermocirculator unit
N	OFN
P	Pressure reducing valve
	Medium pump
	Flow meter

Figure 11 : Single-stage from the multi-stage chemostat with, as detailed in the text :

1. culture inflow;
2. culture sampling syringe via a 3-way connector;
3. O.F.N. influent;
4. outflow of culture from vessel;
5. influent to subsequent vessel or effluent reservoir;
6. route of gas from vessel;
7. gas sample port - Suba Seal;
8. 1% (w/v) zinc acetate (H_2S trap);
9. 0.1N $Ba(OH)_2$ (CO_2 trap).


1
2
3
4
5
6
7
8
9
10

vessels were arranged so that the entire liquid effluent of the first vessel became the influent of the second and so on. Culture liquid from each vessel was introduced via a centrally-located medium input tube (1) which allowed influent to be emitted into the vessels at their base only. The design ensured that the bulk flow characteristics of the system approximated to a continuous but segmented plug flow. Anaerobiosis was maintained by an overpressure of O.F.N. (3) in conjunction with pressure heads of zinc acetate (1%, w/v)(8) and barium hydroxide (18 g l^{-1})(9) which were also used for trapping gaseous H_2S and CO_2 respectively. Oxygen-impermeable butyl-rubber tubing was used throughout (Esco, Rubber Ltd., Teddington). Degasification of the effluent from each stage was effected by the U-tube arrangement(6) so that no gas entered the succeeding vessel. Samples for gas analysis were withdrawn into gas syringes through the rubber Suba Seal(7) and for culture analysis samples were withdrawn directly into sterile syringes(2) from the vessels. Light was excluded from each of the vessels by covering them with aluminium foil. Effluent from the final vessel was collected in a 10 litre reservoir.

2.3.2 Multi-stage chemostat : three vessels

This system was the same as that described above with the exception that in this array the working volumes of the three individual vessels were increased from 310 ml in vessel A to 700 ml in vessel B and finally 1600 ml in vessel C (Plate 1) to

Plate 1: Three-vessel multi-stage chemostat, as detailed in the text (2.3.2).

facilitate a regime of non-constant dilution rate. The medium (2.2.1) was pumped into the first vessel by means of a Watson-Marlow (202V/AA 10) Peristaltic Pump.

2.4 Analytical Methods

2.4.1 Substrate concentration

Influent (S_R) and residual (S) substrate concentrations of hexanoic acid were determined as for volatile fatty acids (2.4.2).

2.4.2 Volatile fatty acids

0.9 ml samples of culture supernatant were taken and acidified with 0.1 ml formic acid (Aristar, B.D.H.). 1 ul of this preparation was then injected into a G.L.C. (Perkin Elmer Sigma 115) which was equipped with a flame ionisation detector (F.I.D.). The carrier gas used was O.F.N. at a flow rate of 40 ml min^{-1}. The glass column (length 2 m, internal diameter 2mm) was filled with 5% Neopentyl glycol sebacate + 1% H_3PO_4 on Anakrom Polyester (80-100 mesh) (Phase Separations Ltd). The injector and detector temperatures were maintained at 180°C. The column was maintained initially at 100°C for 5 minutes after which the temperature was increased to 130°C at a ramp rate of 40°C min^{-1}.

10 mM fatty acid standards were used and were prepared as above.

Development of this method is described in Appendix 2.

2.4.3 Methane

The methane concentration of the culture gas phase was determined by use of a Perkin-Elmer Sigma 115 G.L.C. which was equipped with a F.I.D. The carrier gas was O.F.N., with a flow rate of 40 ml min^{-1}, and the temperatures of the column, injector and detector were 70°, 150° and 150°C respectively. Gas samples, 50 µl, were injected directly onto the column (length 2 m, internal diameter 2mm) which was packed with Poropak Q (Phase Separations Ltd).

The atmospheric temperature and pressure were recorded and the methane concentrations converted to molarity at standard temperature and pressure. 100% research grade methane (B.O.C.) was used as the standard.

2.4.4 Carbon dioxide

Gaseous and soluble fractions of CO_2 were both determined.

Gaseous CO_2, evolved by the chemostat cultures, was first trapped in barium hydroxide solution (18 gl^{-1}) prior to titration with standard 0.1N HCl in the presence of phenolphthalein as an indicator.

Soluble CO_2 was first distilled (from a liquid sample) and trapped in barium hydroxide, as above.

The amount of CO_2 in the sample was found by difference and then calculated from, equation 10.

$$1 \text{ ml } 0.1N \text{ } Ba(OH)_2 = 1.119 \text{ ml } CO_2 \text{ at S.T.P.} \qquad (10)$$

2.4.5 Sulphate

Sulphate ions were estimated by the method described in Standard Methods in Water and Wastewater Analysis.

10 ml samples of culture supernatant were taken and O.F.N. bubbled through for 1 minute to remove soluble sulphide. 0.5 ml of conditioning reagent, which contained : 50 ml glycerol, 30 ml concentrated HCl, 300 ml distilled H_2O, 100 ml 95% ethyl alcohol and 75 g NaCl, were added followed by a standard 'spoonfull' of barium chloride (20-30 mesh). After mixing in a vortex mixer for 1 minute, the absorbance at λ = 410 nm was determined in a Pye Unicam PU 8800 spectrophotometer with a 1 cm light path, after a time interval of 4 minutes. The sulphate concentration was then estimated by comparison with a standard curve of sulphate (1 to 10 mg 100 ml^{-1}).

2.4.6 Sulphide

For analysis, 3 forms of sulphide : total, dissolved and unionised H_2S were recognised.

Total sulphide : this included dissolved H_2S and HS together with acid-soluble metallic sulphides. 120 ml of sample were introduced into a 2-necked vessel, the outlet of which was connected to 2 absorption tubes which contained 10 ml each of 1% (w/v) zinc acetate. The sample was then acidified with 1 ml of concentrated HCl and the gas O.F.N. bubbled through the sample for 1 hour to displace the sulphide as H_2S.

10 ml of 0.1N iodine solution (Convol, B.D.H.) was then added

to the pooled zinc acetate solutions, followed by 1 ml concentrated HCl. The solution was then back titrated with 0.1N sodium thiosulphate (Convol, B.D.H.) with starch (1g 100 ml^{-1} of boiled H_2O) as the indicator.

Since 1.0 ml of 0.1N iodine is equivalent to 1.6 mg sulphide the total sulphide was calculated as follows:

$$[HS^-] = \frac{(\text{ml iodine} - \text{ml } Na_2S_2O_3)}{\text{ml sample}} \times 1600 \text{ mg } l^{-1}$$

Dissolved sulphide: this was the sulphide which remained after the suspended solids had been removed by filtration through a Whatman 0.2 um membrane filter. The procedure then followed was the same as above.

Unionised hydrogen sulphide: the concentration of the unionised hydrogen sulphide was determined by two methods. Firstly, H_2S was estimated by multiplying the concentration of dissolved HS by a factor derived from the table in Standard Methods in Water and Waste Water Analysis after first determining the pH of the sample (2.4.7).

Secondly, gaseous H_2S from chemostat cultures was trapped as zinc sulphide in 1% (w/v) zinc acetate, and the concentration determined by the method described above.

2.4.7 pH

pH values of culture supernatants were routinely determined by use of a Pye Unicam PW 9418 pH meter fitted with a Pye Unicam electrode (No. 8401.67).

2.4.8 Biomass determinations

Biomass determinations were made by two methods : optical density and dry weight.

Optical density: Biomass was measured with a Pye Unicam PU 8800 spectrophotometer with a 1 cm light path at a wavelength of 600 nm. The concentration of iron in the medium (2.2.1) was such that it prevented precipitation of ferrous sulphides. In addition, dithionite was added to ensure that the resazurin remained colourless during the determination.

Dry weight: Culture dry weights were determined on 50 ml aliquots of culture supernatants. The samples were filtered through pre-weighed dried cellulose acetate filters, washed twice with 10 ml of sterile distilled water and dried overnight at 100°C. The filters were then reweighed and the biomass determined by difference.

2.4.9. Electron microscopy

10 ml sample of cultures were filtered through a Shandon Nuclepore membrane filter (N020 CPR 025000 0.2 µm. The filter was then placed in a bottle which contained glutarldehyde/cacodylate buffer for 4 h. The buffer contained 8 ml glutaraldehyde plus 42 ml cacodylate buffer,made up to 100 mls. The cacodylate buffer contained 50 ml sodium cacodylate plus x mls HCl to give a final pH of 7 and was diluted to 100 ml with distilled H_2O.

The filter was washed several times in the above buffer and

then fixed in osmium tetraoxide (1% osmium tetraoxide made up in a fume cupboard; 0.1 g dissolved in 10 ml distilled H_2O).

The sample was then dehydrated in increasing concentrations (30%, 50%, 70%, 95% and 100%) of alcohol.

The specimen as then placed on an electron microscope stub, air dried and then dried in a critical point drier. Finally the specimen was coated and examined under a scanning electron microscope.

2.4.10 Radiotracer analysis

^{14}C-labelled methane and carbon dioxide were measured with a gas proportional counter (E.S.I. Nuclear, UK, Model 504) interfaced with a Perkin Elmer Sigma 115 Gas Chromatograph. The gas exhaust from the G.L.C. column was passed through a 2-way splitter, which directed part of the flow to the F.I.D. detector of the G.L.C. and the rest to the detector of the radio-gas counter.

With ^{14}C compounds a carrier gas of Argon + 5% CO_2 was used. The gas stream from the splitter passed through a furnace tube which contained copper oxide maintained at 700°C with the result that ^{14}C compounds were oxidised to $^{14}CO_2$ and H_2O and were subsequently dried before passing to the detector.

CH_4 and CO_2 were separated by means of a G.L.C. glass column (length 2 m, internal diameter 2 mm) packed with Poropak N (Perkin Elmer Ltd.) maintained at 60°C with the injector and detector both at 100°C. CH_4 was quantified by G.L.C. analysis (2.4.3).

Radioactivity was expressed as total counts in each peak and was quantified from a standard curve prepared from ^{14}C-HCO_3^-.

3. Use of multi-stage chemostats for investigating the microbial interactions of a microbial association catabolising hexanoate to methane

The major objective of the present study was to investigate the microbial interactions involved in terminal anaerobic catablism of hexanoate within a microbial association enriched and isolated from a landfill site. Hexanoic acid was specifically chosen as the oxidisable carbon source as it is a representative low molecular weight molecule found in leachate of recently emplaced refuse (Harmsen, 1983).

The method of study in this case was to construct two multi-stage chemostats which operated under segmented plug flow conditions. This approach ensured that the microbial population developed under heterogeneous conditions. To enable the component species to be studied whilst still functioning within a microbial asscociation it was essential that spatial separation of the various component species occurred and to this end two approaches were used to effect this : (1) varying the concentration of electron acceptor (sulphate); and (2) varying the dilution rate of the system.

Experimental: The isolated hexanoate-catabolising microbial association was first grown in a 5-stage open culture system at a constant dilution rate of 0.005 h^{-1} and was subjected to step-wise increases in sulphate concentration of 1.4, 5 and 10mM. Each vessel was quantitatively analysed for residual substrate, intermediates and end products at regular intervals until 'stable states' were

reached, and the results are presented as the means of triplicate analyses.

Secondly, the same association was cultured in the presence of 1.4 mM sulphate in a 3-stage chemostat which was constructed to facilitate discrete dilution rates in each vessel, and was analysed as before.

3.1 Five-vessel multi-stage model : 1.4 mM sulphate

From Figure 12 and 12A (which shows the dynamics of carbon flow in the system) it can be seen that at a dilution rate of 0.005 h^{-1} residual hexanoic acid was only detected in Vessels A (0.162 mM) and B (0.093 mM). T-test statistical analysis with the probability at 5%, however, showed that these concentrations were not significantly different from zero. The multi-stage chemostat therefore appeared to be hexanoate limited, although this was not confirmed by experimentation.

Catabolism of hexanoate in the absence of oxygen has been reported by McInerney et al. (1979) and Stieb and Schink (1985). In both cases an obligate syntrophic association between the hexanoate-oxidiser and H_2-utiliser was involved. McInerney et al. (1979) isolated a Gram -ve bacterium Syntrophomonas wolfei in co-culture with either Methanospirillum hungatei or Desulfovibrio sp. whilst Stieb and Schink (1985) similarly isolated a Gram -ve spore-forming bacterium Clostridium byrantii in co-culture with M. hungatei or a Desulfovibrio sp. The major advantage of each association was that excess electrons generated by the hexanoate oxidiser were removed by

Figure 12: Changes in concentrations of metabolic intermediates and methane during the anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of sulphate (1.4 mM).

a - hexanoic acid

b - butyric acid

c - propionic acid

d - acetic acid

e - methane

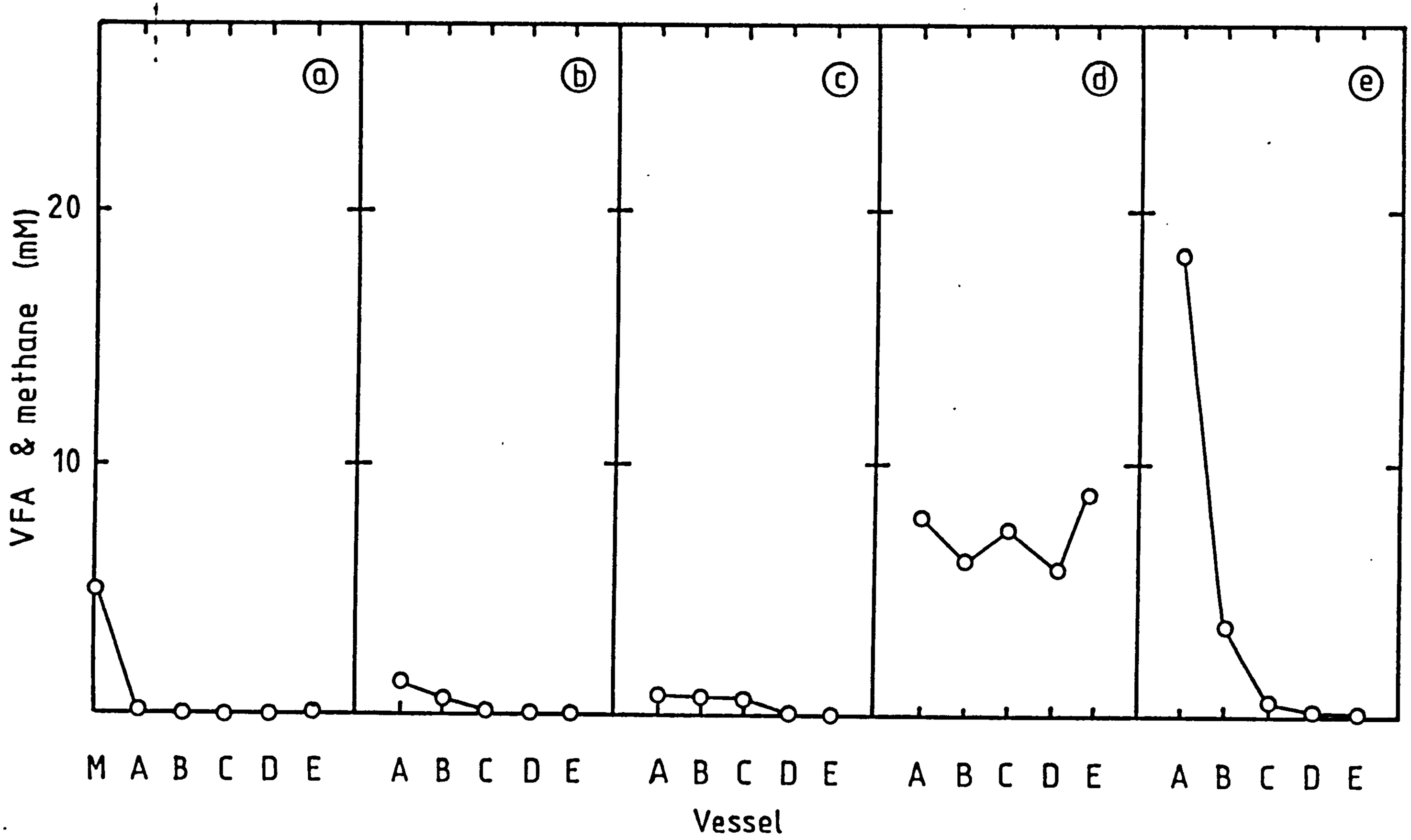
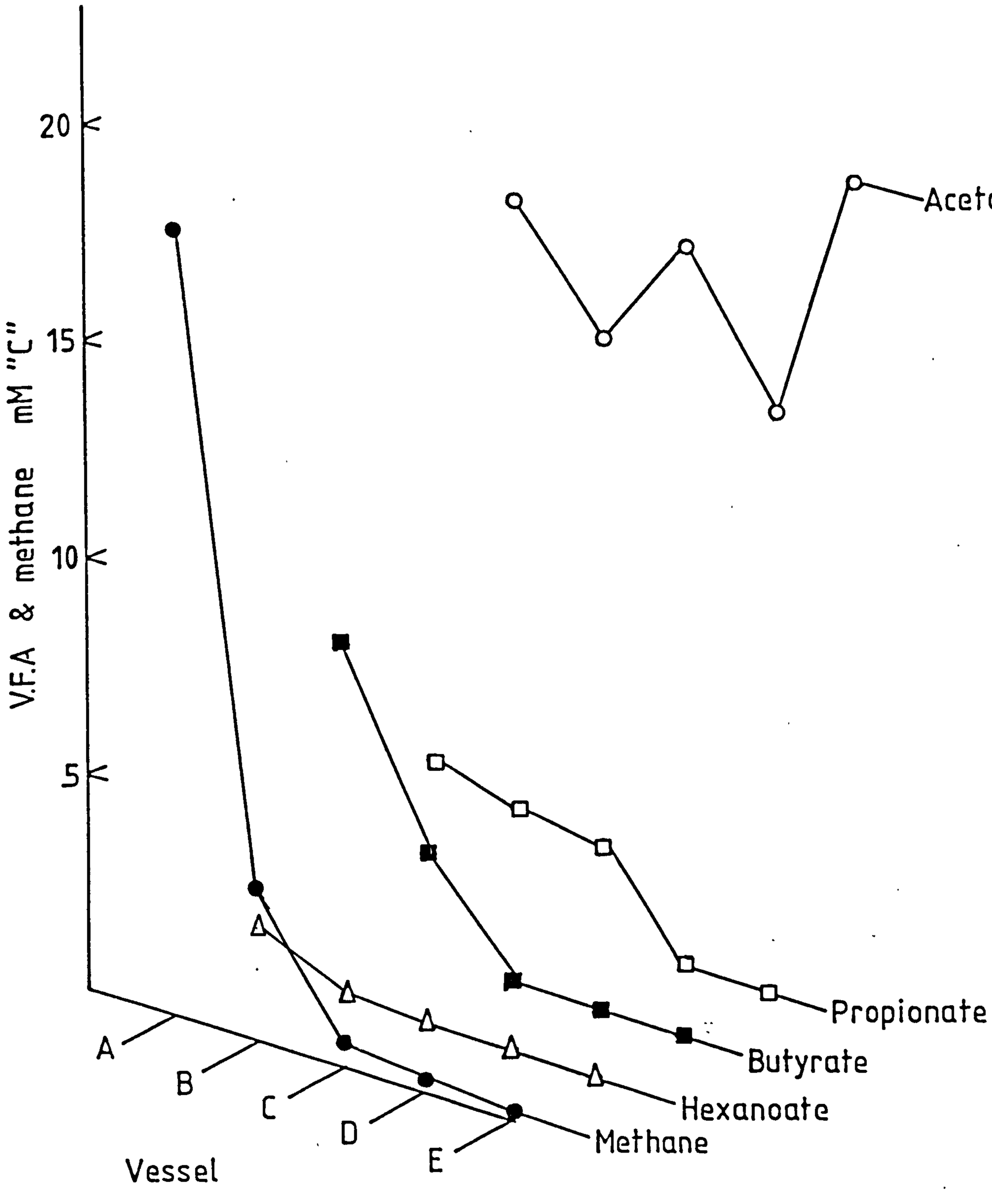

a
b
c
d
e
20
10
VFA & methane (mM)
M A B C D E
A B C D E
A B C D E
A B C D E
A B C D E
Vessel

Figure 12a: Carbon flow during anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of sulphate (1.4 mM)


20
15
10
5
V.F.A & methane mM "C"
Acetate
Propionate
Butyrate
Hexanoate
Methane
A
B
C
D
E
Vessel

the H_2-oxidising partner thus negating the requirement for electron acceptor reduction by the hexanoate-utiliser.

In addition to the two associations described above, sulphate-reducing bacteria have also been isolated which can oxidise volatile fatty acids to CO_2 in the presence of sulphate (Widdel and Pfennig, 1981). However, if this latter mechanism was operative then complete catabolism of hexanoate in the multi-stage chemostat would have required 20 mM of sulphate. Thus it is unlikely that complete oxidation of hexanoate by sulphate-reducing bacteria was occurring, since the influent concentration of sulphate was 1.4 mM.

Although complete oxidation of hexanoate to CO_2 was possible, from the results (Figure 12) it can be seen that acetate, propionate and butyrate were present in Vessel A at concentrations of 7.93, 0.96 and 1.2 mM respectively. Consideration of the carbon balance for hexanoate catabolism in this vessel showed that 30 mM hexanoate 'C' was dissimilated to 4.8 mM butyrate 'C' plus 2.88 mM propionate 'C' plus 15.86 mM acetate 'C'. Thus, 78% of the hexanoate carbon was accounted for by volatile fatty acid intermediates alone, with the remaining, 22%, probably accounted for by methane, carbon dioxide and cell assimilation.

Figure 12 shows that the concentration of butyrate and propionate both subsequently decreased down the array. The concentration of butyrate decreased from 1.2 mM in Vessel A to 0.616

mM in Vessel B and was not detected in Vessel C. Similarly, the concentration of propionate decreased from 0.96 mM in Vessel A to 0.77 mM in Vessel B and 0.7 mM in Vessel C and was not detected in Vessel D.

Oxidation of volatile fatty acids under anoxic conditions has been demonstrated by Stieb and Schink (1985) to yield acetate and hydrogen, with the latter removed by a syntrophic partner to leave acetate as the end product. From Figure 12 it can be seen that the concentrations of acetate fluctuated little between the individual vessels with 7.93, 6.05, 7.46, 5.76 and 8.85 mM respectively recorded for Vessels A to E. Thus acetate was most likely produced from volatile fatty acid dissimilation in Vessels A to D, although, the increased concentration of acetate in Vessel E, in the absence of intermediates, showed that a second acetogenic pathway was operative.

The most common catabolic pathway for volatile-fatty acids, of chain length greater than acetate, is β-oxidation which has been shown to yield acetate from even-numbered acids, or acetate and CO_2 from odd-numbered volatile-fatty acids (Jeris and McCarty, 1965). The appearance of butyrate and acetate would, therefore, suggest that β-oxidation was the operative pathway. The detection of propionate as an intermediate, on the other hand, was somewhat surprising since it is unlikely that propionate was formed directly from hexanoate. If this was the case then an atypical reaction such as w (omega) oxidation of butyrate could have resulted in

propionate production. Alternatively, propionate could have been produced from the reduction of acetate and CO_2 with H_2, which has been reported to occur in sulphate-reducing bacteria such as Desulfobulbus propionicus (Laanbroek, Abee and Voogd, 1982). Similarly, propionate formation has been linked to hydrogen utilisation in Propionispira arboris (Thompson, Conrad and Zeikus, 1984) when the addition of H_2 to glucose- or lactate-grown cells induced homo-propionogenesis.

From the results it may be postulated that butyrate and acetate were products of β-oxidation of hexanoate, with subsequent butyrate dissimilation to acetate also mediated by the same mechanism. Boone and Bryant (1980) isolated a propionate-degrading bacterium (Syntrophobacter wolinii) in co-culture with a H_2-utilising methanogen. This reaction, however, was shown to be less thermodynamically favourable than the syntrophic oxidation of even-chain volatile fatty acids and as a consequence was more sensitive to H_2 accumulation (Zinder, Cardwell, Anguish, Lee and Koch, 1984). Since the component vessels of the multi-stage chemostat were overgassed with OFN then this could have possibly enhanced propionate utilisation by maintaining a low partial pressure of hydrogen. Zinder et al. (1984) also found that propionate inhibitied methanogenesis by between 20 and 30%, although no similar inhibition was apparent in the multi-stage chemostat culture.

Concomitant with hexanoate degradation, the residual sulphate decreased from 1.4 mM in the influent medium to 0.19 mM in Vessel A, after which no further significant reductions were observed; 0.16, 0.17, 0.2 and 0.17 mM for Vessels B to E respectively (Figure 13). Thus it would appear that sulphate reduction was restricted to the first vessel. Supporting evidence for this can be seen from the concentration of gaseous H_2S, which was maximum in Vessel A, 0.68 mM, and then decreased to 0.04 mM in Vessel B and 0.02 mM in Vessel C (Figure 13). Only 56.2% of the added sulphate was accounted for in Vessel A as gaseous H_2S. However, a total sulphur balance is shown in Table 6A from which it can be seen that 65% of the sulphur was accounted for by analysis.

The initial reduction of sulphate was not accounted for by gaseous H_2S production alone (Table 6B) and thus the possibility existed that either much of the sulphide remained in solution under the pH conditions of 7.3 or the sulphur was assimilated to cell material. Methanogens also have an obligate requirement for sulphide for both biomass sulphate and maintenance of adequate redox conditions (Hungate, 1969).

Production of H_2S from sulphate is indicative of an active population of sulphate-reducing bacteria. This reaction, however, tends to make the medium alkaline since reduction of Na_2SO_4 leads to the formation of the intermediate NaS, which is subsequently hydrolysed to H_2S and NaOH. Thus any formation of NaOH in the culture would tend to make the medium basic, which was in fact

Figure 13: Changes in the concentration of residual hexanoic acid (Δ—Δ) and sulphate (O—O) gaseous hydrogen sulphide (●—●), methane (□—□) and pH (■—■) during the five-stage open culture cultivation of the isolated microbial association in the presence of hexanoic acid (5 mM) and sulphate (1,4 mM).

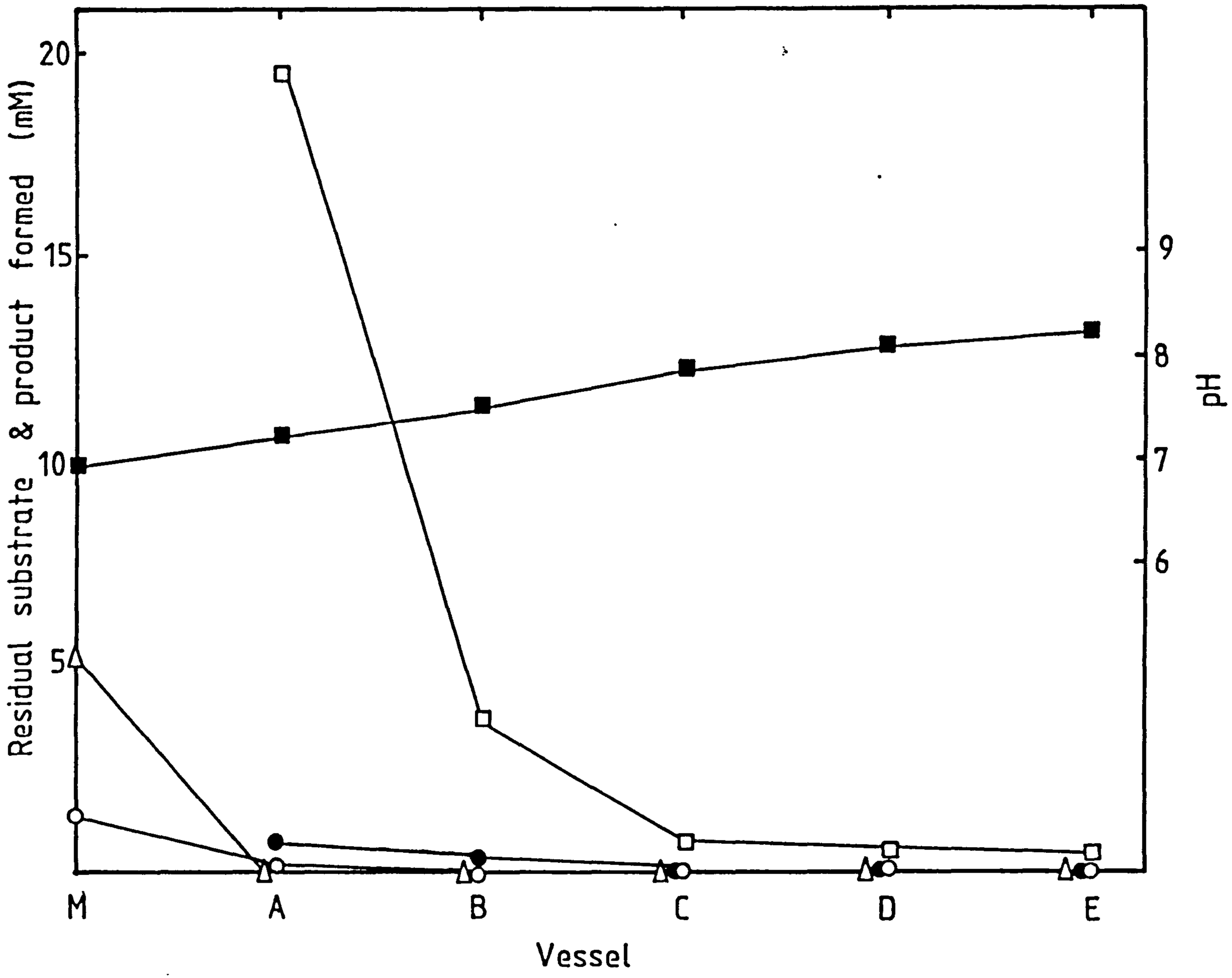

Residual substrate & product formed (mM)
20
15
10
5
9
8
7
6
pH
M
A
B
C
D
E
Vessel

Table 6A: Total sulphur balance for the dissimilation of hexanoic acid (5mM) by the interacting microbial association cultivated in a 5-vessel chemostat model in the presence of 1.4 mM SO_4^{2-}

Table 6B: Balance of sulphate removal and formation of sulphide (mM 'S') after the mode of Thompson et al. (1984)

Input sulphate - (Residual sulphate + Total gaseous H_2S)

1.4 - 0.17 + 0.74

= 0.91

Sulphur accounted for = $\frac{0.91}{1.4} \times 100 = 65\%$

Vessel	SO_4	H_2S	Total
A	-1.21	+0.68	-0.53
B	-0.03	+0.04	+0.01
C	+0.01	+0.02	+0.03
D	+0.03	0	+0.03
E	-0.03	0	-0.03
Total	-1.23	0.74	-0.49

observed. Although sulphate reduction was only noted in Vessel A, the pH progressively increased from 7.0 in the influent medium to 8.3 in Vessel E (Figure 13). This change in pH could have had sever-al effects. Firstly, it could have exerted a differential toxic effect, but since there was activity (catabolism of metabolites) down the whole array this was unlikely. Secondly, an increase in pH could have resulted in increased sulphide solubility which would possibly have accounted for the discrepancy in the sulphur balance.

From Table 6B "unaccounted" sulphur could possibly have been due to precipitated metallic sulphide which was present as a crystal-line precipitate on the glass surfaces of all the vessels. Although Fe^{2+} was continuously supplied in the influent medium, since sulphide will precipitate any iron as insoluble FeS, this mechanism could eventually have restricted growth of the sulphate-reducing bacteria (Postgate, 1979) and methanogens (Patel, Khan and Roth, 1978) since both groups of bacteria have an obligate requirement for this metal.

Another possibility exists that the observed precipitate could also have been other metal sulphides, although a more likely explanation is that the precipitate was magnesium ammonium phosphate, which forms from any Mg^{2+}, NH_4^+ and PO_4^{3-} ions in alkaline conditions (Postgate, 1979).

Sulphate-reducing bacteria use only a narrow range of substrates. Typically these are C_3- and C_4-substituted fatty acids,

such as lactate, pyruvate and fumarate, and certain alcohols (ethanol, propanol) and C_2 to C_6 volatile fatty acids (Laanbroek and Veldkamp, 1982) although other compounds are also utilised (Pfennig, 1984). The actual substrate used by the sulphate-reducing bacteria in the isolated microbial association was hard to ascertain, especially as the sulphate-concentration (1.4 mM) was so low. However, if sulphate was used as an electron acceptor for the oxidation of any of the possible substrates (hexanoate, butyrate, propionate, acetate and H_2/CO_2), then increasing its concentration should have had a marked effect on the metabolic characteristics of the system.

In addition to the above, two other forms of metabolism were possible:

(1) that the sulphate-reducing bacteria acted as H_2-producing acetogens (Bryant, Campbell, Reddy and Crabill, 1977; McInerney and Bryant, 1981). In this case sulphate could not have been utilised as an electron acceptor thus addition of excess sulphate would have dramatically affected the pathway of degradation of hexanoate; and

(2) that the sulphate-reducing bacteria were growing mixotrophically (Postgate, 1979). In this instance the substrate could have supported sulphate-reduction, but not growth, by acting as source of inorganic H_2.

Of the various possibilities it is most likely that the sulphate-reducing bacteria acted as the major electron sinks for H_2 produced

by hexanoate oxidation:

$$C_8H_{11}O_2^- + 4\ H_2O \longrightarrow 3\ C_2H_3O_2^- + 4\ H_2 + 2\ H^+$$

$$\Delta\ G_o{}^1 = +\ 96.2\ kJ\ mol^{-1} \quad (11)$$

$$4\ H_2 + SO_4^{--} + H^+ \longrightarrow HS^- + 4\ H_2O$$

$$\Delta\ G_o{}^1 = -151.9\ kJ\ mol^{-1} \quad (12)$$

$$C_6H_{11}O_2^- + SO_4^{--} \longrightarrow 3\ C_2H_3O_2^- + HS^- + H^+$$

$$\Delta\ G_o{}^1 = -58.3\ kJ\ mol^{-1} \quad (13)$$

If this assumption was correct then 5 mM hexanoate would have required 5 mM sulphate to oxidise all the H_2. However, since the medium was amended with 1.4 mM sulphate only, then a proportion of the H_2 must also have had another fate.

Since CO_2 (HCO_3^-, CO_3^{2-} and biotic CO_2) was non-limiting in the culture, then H_2 could have been removed by reduction of CO_2 to CH_4:

$$4\ H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3\ H_2O$$

$$\Delta\ G_o{}^1 = -135.6\ kJ\ mol^{-1} \quad (14)$$

$$C_6H_{11}O_2^- + H_2O + HCO_3^- \longrightarrow 3\ C_2H_3O_2^- + CH_4 + H^+$$

$$\Delta\ G_o{}^1 = -40.83\ kJ\ mol^{-1} \quad (15)$$

However, since reaction (15) is less thermodynamically favourable than the equivalent reaction with sulphate-reduction (13) then in the presence of sulphate in non-limiting concentrations the predicted end products would be sulphide and acetate due to the higher affinity for H_2 of the sulphate-reducing bacteria than the methanogens (Schonheit, Kristjansson and Thauer, 1982).

As discussed above, β-oxidation of hexanoate primarily yielded acetate and H_2, which are both potential methanogenic substrates.

Therefore, it is perhaps not surprising that there appeared to be a correlation between maximum hexanoate utilisation and maximum methane production of 18.35 mM in Vessel A. The evolved methane concentration then subsequently decreased down the array to 3.6, 0.633, 0.37 and 0.25 mM in Vessels B to E respectively.

Since total hexanoate dissimilation by sulphate-reduction was limited by the influent sulphate concentration it is reasonable to assume that both H_2 and acetate were methanogenic substrates. However, the presence of acetate in concentrations of between 7 and 8 mM in all the vessels was hard to explain as methanogenesis was only significant in Vessels A and B.

Acetate production by H_2-dependent CO_2 reduction has been demonstrated by various workers (Weiringa, 1940; Balch, Schoberth, Tanner and Wolfe, 1977; Adamse, 1980; Sleat, Mah and Robinson, 1985).

If the assumption was made that hexanoate was completely catabolised to acetate, then from Equation 16, 5 mM hexanoate could have produced 20 mM acetate:

$$5\ C_6H_{11}O_2^- + 10\ HCO_3^- \longrightarrow 20\ C_2H_3O_2^- + 5\ H^+$$

$$\Delta G_o{}^1 = -8.4\ kJ\ mol^{-1} \quad (16)$$

However, since 18 mM methane was generated in Vessel A, then if this had all originated from acetate a balance of 2 mM acetate should have remained:

$$5\ C_6H_{11}O_2^- + 18\ H_2O \longrightarrow 8\ HCO_3^- + 18\ CH_4 + 2\ C_2H_3O_2^- + 5\ H^+ \quad (17)$$

However, from the Figure 12 it can be seen that the "stable

state" concentration of acetate was 7.93 mM in Vessel A. Thus homoacetogenesis alone could not have accounted for this total.

Hexanoate can be completely dissimilated to methane and carbon dioxide:

$$5\ C_6H_{11}O_2^- + 20H_2O \longrightarrow 20\ CH_4 + 10\ HCO_3^- + 5\ H^+$$
$$\Delta\ G_o{}' = -70.4\ kJ\ mol^{-1} \quad (18)$$

Whether acetate removal by methanogens (or other bacteria in the microbial association) could have been important in driving the catabolism of hexanoate has still to be determined. Ferry and Wolfe (1976) found that the methanogenic conversion of benzoate via intermediate formation of H_2, acetate and formate, required the removal of all these intermediates. Conversely, added acetate has been shown to have little effect on the conversion of intermediates such as ethanol, propionate and butyrate to methane by enrichment cultures although this is in sharp contrast to the effect of added H_2 which completely inhibited these conversions (Bryant et al., 1967; Smith, 1980).

The metabolism of acetate by the interacting microbial association will be examined in detail later (4.2.7).

Carbon balance: A carbon balance was developed for the complete system (Table 7A) in which 111.4% was accounted for. Of this, methane was responsible for 56% with the rest as organic carbon, particularly acetate (44%). The carbon balance, however, did not take into account any cellular carbon although this is known to be limited in anaerobic systems (Zeikus, 1983a).

The distributions of organic carbon and gaseous carbon down the array are shown in Table 7B and Figure 14. Total soluble carbon at an initial concentration of 36.8 mM carbon equivalents decreased by 13.26 mM to 23.54 mM 'C' in Vessel A, and was accompanied by an evolution of 18.35 mM CH_4. Thus, 138.38% of the carbon dissimilated in Vessel A was recovered as CH_4, although the explanation for this over-estimate is not readily apparent.

Subsequently, the concentration of V.F.A. intermediates decreased slightly to 17.05 in Vessel B from which point no further significant changes were detected. Similarly, small changes in the total methane concentration were observed.

Maximum activity, in terms of dissolved carbon dissimilation and gaseous carbon production, therefore, occurred in Vessels A and B.

Table 7a: Carbon balance (mM'C') for the dissimilation of hexanoic acid by the interacting microbial association, cultured in a 5-vessel chemostat model in the presence of 1.4 mM SO_4^{2-}

Table 7B: Balance of removal of carbon and formation of CH_4 (mM 'C') after the mode of Thompson *et al.* (1984). ml^{-1} C.

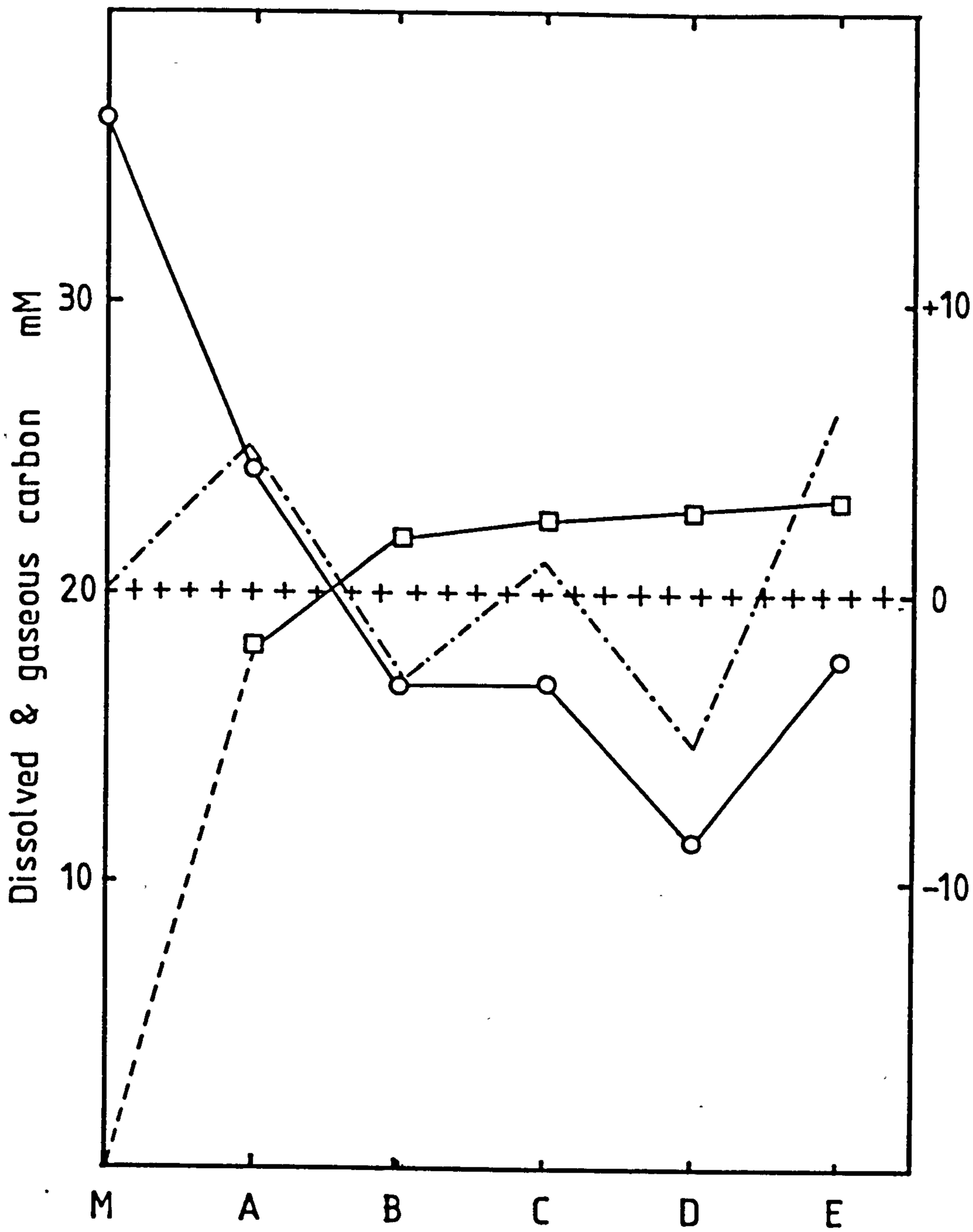
Input to Vessel A		Output from Vessel E + Total gaseous products	
Organic carbon	30	Methane	23.2
Inorganic carbon	6.8	Carbon dioxide	0
		Organic	17.7
Total	36.8		40.9

Carbon accounted for 40.9 / 36.8 x 100 = 111.4%

	Inorganic 'C'	Organic 'C'	CH_4	Difference
A	-6.8	-6.46	+18.35	5.09
B	0	-6.49	+ 3.6	-3.34
C	0	+0.63	+0.633	1.263
D	0	-5.71	+0.37	-5.34
E	-6.8	-6.8	+0.28	6.46
Total	-6.8	-12.3	23.23	4.13

Figure 14: Changes in concentrations of dissolved (O—O) and gaseous (□—□) carbon during the catabolism of hexanoate (5 mM) by the isolated microbial association in a five-stage chemostat model with an influent sulphate concentration of 1.4 mM

.-.-. denotes carbon accounted for


Dissolved & gaseous carbon mM
30
20
10
+10
0
-10
Carbon balance
M
A
B
C
D
E
Vessel

3.2 Five-vessel multi-stage model : 5 mM sulphate

As a result of increasing the influent sulphate concentration, significant changes in the fermentation balance were readily apparent (Figure 15 and 15A).

Once again hexanoate appeared to be limiting although in this instance no residual substrate was detected in Vessel A, thus it would appear that the sulphate concentration increase stimulated catabolism of hexanoate.

As discussed earlier, a sulphate concentration of 5 mM should theoretically have satisfied the complete oxidation of H_2 produced by hexanoate oxidation (19).

$$5\ C_6H_{11}O_2^- + 5\ SO_4^{2-} \longrightarrow 15\ C_2H_3O_2^- + 5\ HS^- + 5\ H^+$$

$$\Delta G_o^1 = -58.3\ kJ\ mol^{-1} \quad (19)$$

In contrast to the hexanoate results, the distribution of volatile fatty acid intermediates in the system was significantly different to that obtained with 1.4 mM sulphate. Acetate, propionate and butyrate were again found as intermediates in Vessel A, although in this case, the concentrations of 1.21, 0.12 and 0.53 mM respectively were significantly lower. 30 mM hexanoate 'C' was thus catabolised to 2.42 mM acetate 'C' plus 0.36 mM propionate 'C' and 2.12 mM butyrate 'C', which in total accounted for 16% of the initial hexanoate carbon dissimilated. Thus, it would appear that 84% of the 'C' was either metabolised to CH_4 and CO_2 or incorporated into cellular material.

Figure 15: Changes in concentrations of metabolic intermediates and methane during the anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of sulphate (5 mM)

a - hexanoate acid

b - butyric acid

c - propanoic acid

d - acetic acid

e - methane

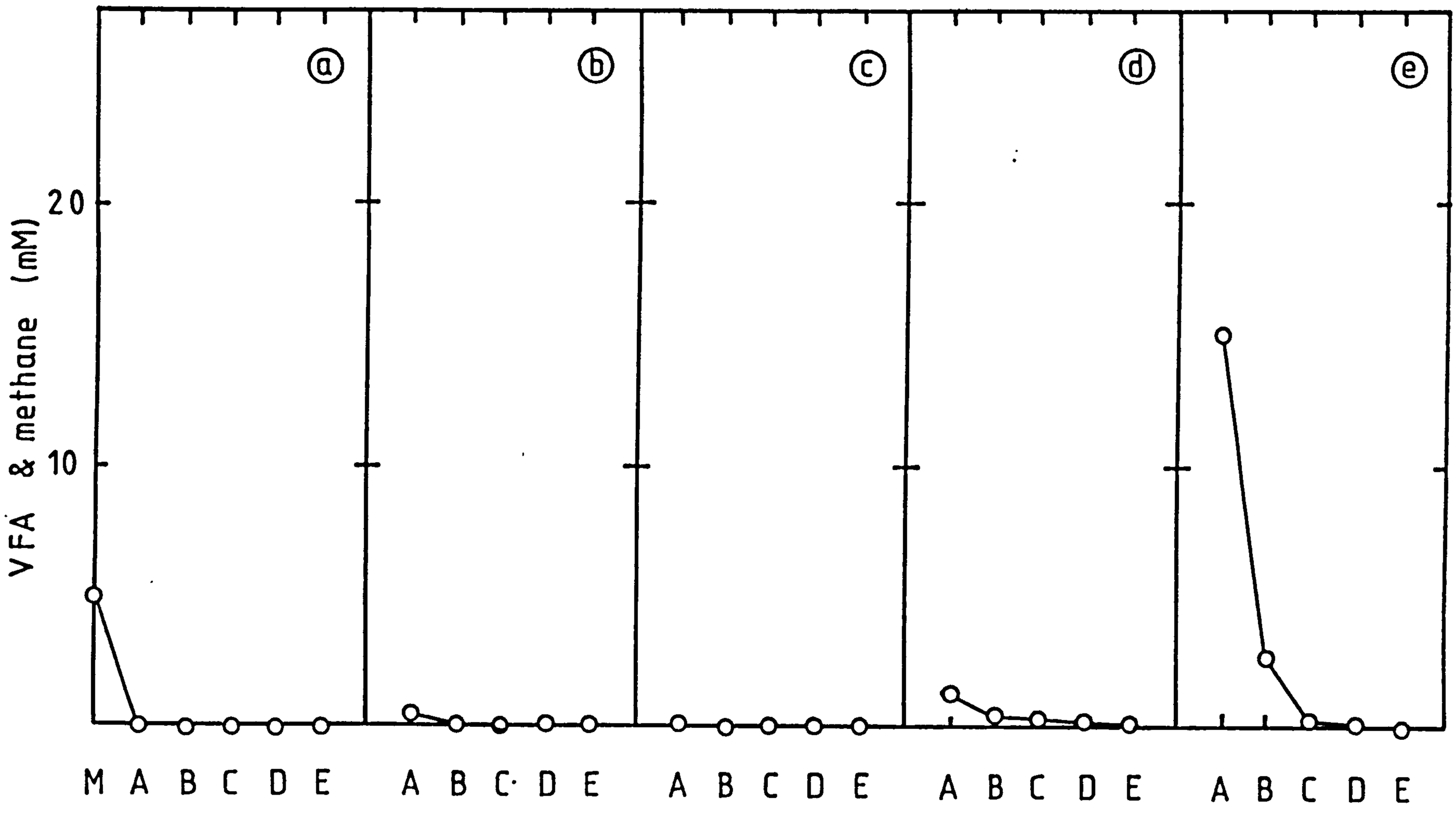
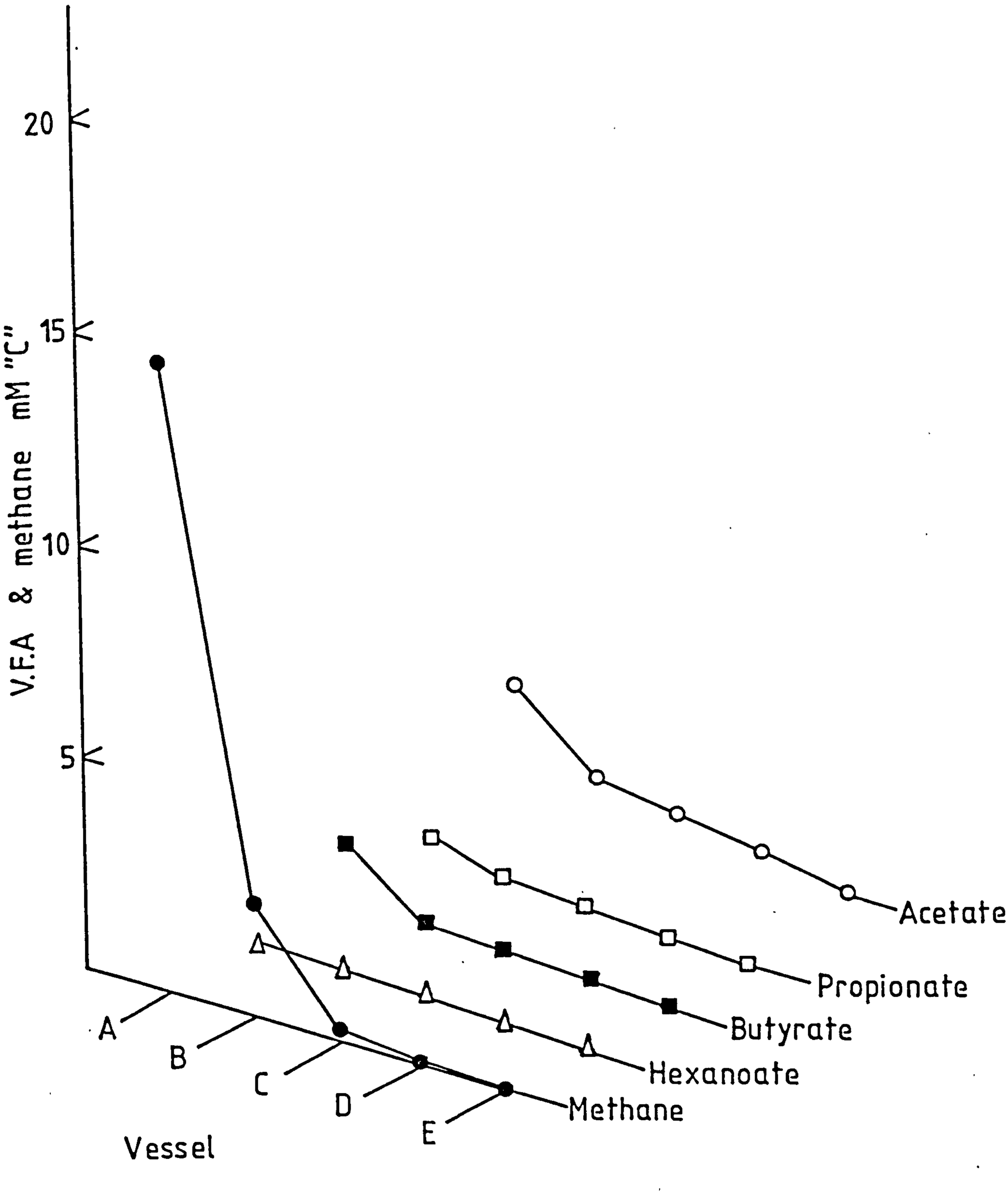

a
b
c
d
e
20
10
VFA & methane (mM)
M A B C D E
A B C D E
A B C D E
A B C D E
A B C D E
Vessel

Figure 15a: Carbon flow during anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of sulphate (5 mM).


20
15
10
5
V.F.A & methane mM "C"
A
B
C
D
E
Vessel
Acetate
Propionate
Butyrate
Hexanoate
Methane

Unlike the lower concentration of sulphate, however, the two intermediates, propionate and butyrate, were not detected in the proceeding vessels. Thus it would appear that the provision of a higher sulphate concentration resulted in accelerated dissimilation of these.

Once again, acetate was detected in each of the five vessels, although in this case, in decreasing concentrations of 1.21, 0.579, 0.49, 0.32 and 0.23mM in Vessels A to E respectively. The most probable explanation for these dramatic decreases in acetate concentrations was that sulphate-reducing bacteria were outcompeting the homo-acetogenic bacteria for H_2 (Eq. 20 and 21):

$$4\ H_2 + SO_4^{2-} + H^+ \longrightarrow HS^- + 4\ H_2O$$

$$\Delta G_o{}^1 = -151.9\ kJ\ mol^{-1} \quad (20)$$

$$2\ HCO_3^- + 4\ H_2 + H^+ \longrightarrow CH_3COOO^- + 4\ H_2O$$

$$\Delta G_o{}^1 = -104.6\ kJ\ mol^{-1} \quad (21)$$

and as a consequence oxidation of H_2 should have resulted in H_2S production rather than acetate, which, in fact, the results substantiated.

A further explanation for the reduction in acetate concentration could be that the sulphate-reducing bacteria were utilising the acetate. However, since the initial concentration of sulphate was 5 mM this could not have facilitated both the complete mineralisation of all the acetate as well as the oxidation of H_2 (Eq. 22 to 25).

$$5\ C_6H_{11}O_2^- + 20\ H_2O \longrightarrow 15\ C_2H_3O_2^- + 20\ H_2 + 10\ H^+ \quad (22)$$

$$20\ H_2 + 5\ SO_4^{2-} + 5\ H^+ \longrightarrow 5\ HS + 20\ H_2O \qquad (23)$$

$$15\ CH_3COOO^- + 15\ SO_4^{2-} + 15\ H^+ \longrightarrow 10\ CO_2 + 15\ H_2S + 10\ H_2O \qquad (24)$$

with the overall reaction :

$$5\ C_6H_{11}O_2^- + 20\ SO_4^{2-} \longrightarrow 10\ CO_2 + 20\ HS^- + 30\ H_2O \qquad (25)$$

The concentration of residual sulphate decreased from 5 mM in the influent reservoir to 1.94 mM in Vessel A, 1.51, 1.96, 1.53 and 2.11 mM in Vessels B to E respectively (Figure 16). Hence from Equation 25 it would appear that only 3 mM hexanoate could have been catabolised in association with sulphate-reducing bacteria, therefore the remaining hexanoate dissimilation must have been linked with a carbon dioxide-utilising organisms such as a methanogen as the mode of electron disposal.

Concomitant with sulphate-reduction was gaseous H_2S production in each of the vessels, with concentrations of 0.84, 0.46, 0.22, 0.06 and 0.058 mM detected in Vessels A to E respectively. The total concentration of 1.638mM H_2S was 54.8% higher than that monitored at the lower sulphate concentration. The concentrations of total and/or soluble sulphide were also measured in the liquid phase of each vessel and were 6.89, 5.41, 2.92, 2.45 and 7.38 mM in Vessels A to E respectively.

Although the total sulphur balance was greatly in excess of theoretical maximum (Table 8), in the absence of acid soluble sulphide the percentage sulphur recovered was 74.9%, which was almost 10% higher than the figure obtained in the first experiment. The possibility also existed that the total sulphide concentration

Figure 16: Changes in the concentrations of residual hexanoic acid (Δ—Δ) and sulphate (O—O), gaseous sulphide (●—●), methane (□—□), carbon dioxide (▲—▲), pH (■—■), and total sulphide (▽—▽) during the 5-stage open culture cultivation of the isolated microbial association in the presence of hexanoic acid (5 mM) and sulphate (5 mM).

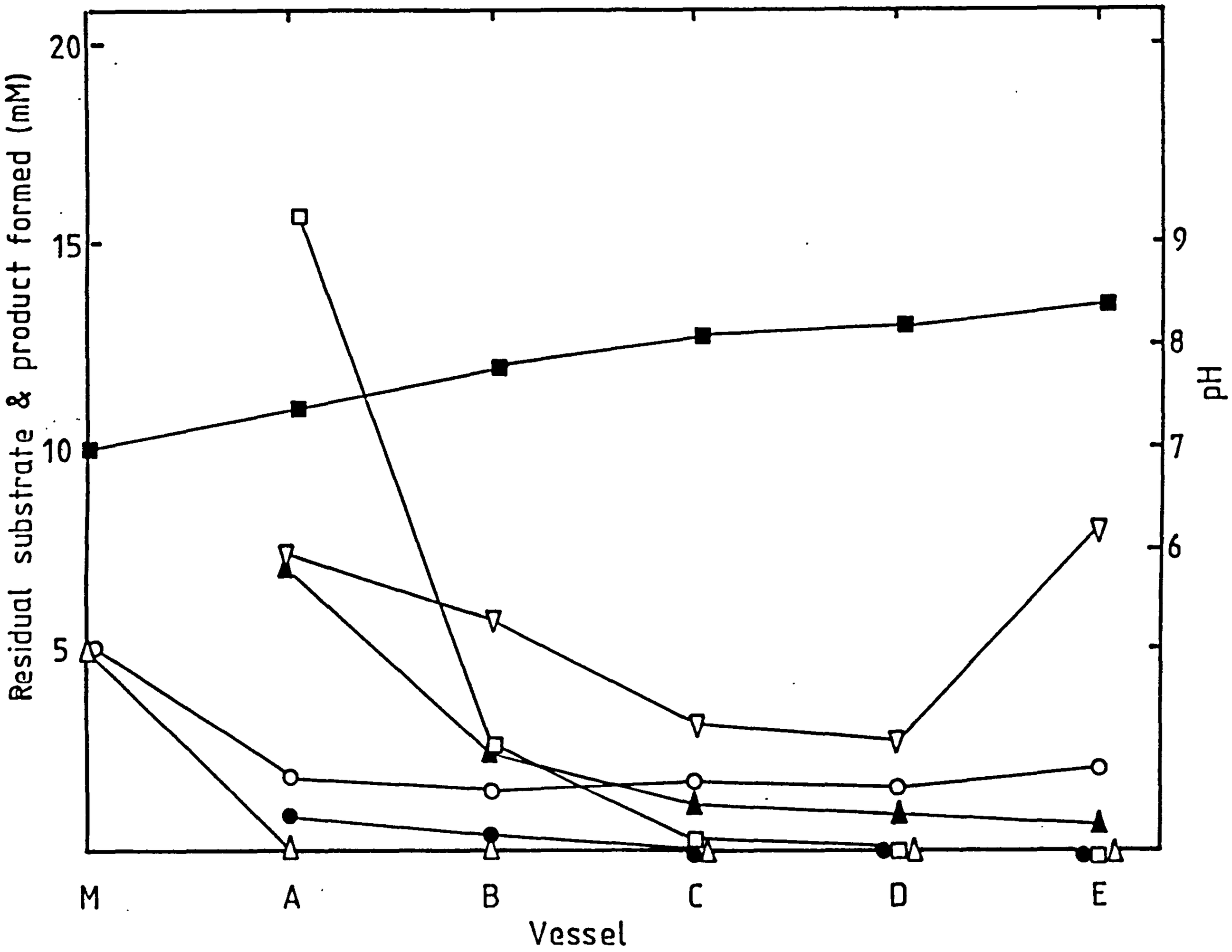

Residual substrate & product formed (mM)
20
15
10
5
9
8
7
6
pH
M
A
B
C
D
E
Vessel

Table 8A: Total sulphur balance (mM 'S') for the 5-stage system.

Table 8B: Balance of sulphate removal and formation of sulphide (mM 'S') after the mode of Thompson *et al.* (1984)

Input sulphate = Residual sulphate + Total $H_2S + HS^-$

5 = 2.11 + 1.638 + 7.38

= 11.97

% accounted for = $\frac{11.97}{5} \times 100 = 239.4\%$

	SO_4	H_2S	HS^-	Total
A	-3.06	+0.84	+6.89	+4.67
B	-0.43	+0.46	-1.48	-1.45
C	+0.45	+0.22	-2.49	-1.82
D	-0.43	+0.06	-0.47	-0.84
E	+0.58	+0.058	+4.93	5.568
Total	-2.89	1.638	+7.38	6.12

was increasing with time, possibly due to, for example, the accumulation of FeS.

As for the lower sulphate concentration, the pH of the system gradually increased from 7.0 in the influent medium to 8.4 in Vessel E, which was again possibly due to sulphate-reduction.

Methane concentrations followed the same pattern as that obtained with 1.4 mM sulphate, with a maximum CH_4 concentration (15.0 mM) in Vessel A, which subsequently decreased down the array to 2.6, 0.28, 0.04 and 0.1 mM in Vessels B to E respectively. Thus it would appear that increasing the sulphate concentration resulted in partial displacement of methanogenesis.

The interactions between methanogens and sulphate-reducing bacteria have been studied extensively (Cappenberg, 1974; Winfrey & Zeikus, 1977; Abram & Nedwell, 1978; Oremland & Taylor, 1978). Although the initial findings of Cappenberg (1974) suggested that sulphide inhibited methanogenesis it would now appear that the two groups of bacteria were in fact competing for either H_2 and/or acetate. This has been subsequently confirmed by Schonheit, Kristjansson & Thauer (1982) who reported that the apparent k_S for acetate for Desulfobacter postgatei was lower, 0.2 mM, than that of Methanosacrina barkeri, 3 mM. Similarly the apparent k_S for H_2 of Desulfovibrio sp. was 1.0 μM and that of Methanobrevibacter sp. 6.0 μM (Kristjansson et al., 1982).

Competition for both acetate and H_2 should, therefore, theoretically have resulted in the competitive displacement of the

methanogens.

The results presented here, however, showed clearly that maximum sulphate-reduction and methanogenesis occurred within the same vessel, A, which suggested that the interaction was not competitive. As discussed above a possible explanation for this was that the sulphate-reducing bacteria were utilising H_2 and the methanogens acetate.

Stoichiometrically, the theoretical and experimental data fit. Equation 26 shows the theoretical stoichiometry of the reaction:

$$5\ C_6H_{11}O_2^- + 3\ SO_4^{2-} + 17\ H_2O \longrightarrow 3\ HS + 17\ CH_4 + 13\ HCO_3^- + 5\ H^+ \quad (26)$$

assuming that approximately 3 mM sulphate was utilised by the microbial association, thus leaving a residual sulphate concentration of approximately 2 mM.

Theoretically, carbon dioxide was both utilised and produced during the methanogenic fermentation of hexanoic acid. Firstly, it was utilised as an electron acceptor by component species of the syntrophic association once sulphate was depleted and secondly, it was produced from the methanogenic fermentation of acetate. Gaseous carbon dioxide was monitored in all the vessels in decreasing concentrations of 6.4, 3.5, 1.4, 1.19 and 0.83 mM in Vessels A to E respectively. From these results an experimental Equation can be derived as follows :

$$5\ C_6H_{10}O_2^- + 3.SO_4 + 17\ H_2O \longrightarrow 1.6\ HS^- + 18.02\ CH_4 + 13.32\ HCO_3^- \quad (27)$$

Although the expected concentration of hydrogen sulphide (gaseous and soluble) was 3 mM, only 1.6 mM H_2S was in fact

detected. As discussed earlier, this discrepancy was possibly due to the masking of soluble HS^- by the accumulation of sulphide precipitate within the system. Methane (18.02 mM) and CO_2 (13.32 mM) on the other hand approximated to the theoretical values even though soluble CH_4 and CO_2 were not assayed.

Carbon balance: The carbon balance for the system is shown in Table 9A from which it can be seen that 86.14% of the carbon was accounted for. The major difference between these results and those presented in Table 7 is that soluble carbon was almost completely dissimilated with as little as 1.63 mM 'C' detected in Vessel E.

This dissimilation was, however, accompanied by gas production of 31.34mM 'C' by Vessel E.

Table 9B shows the distribution of carbon down the array with major imbalances monitored in Vessels A and B. In vessel A more soluble carbon was dissimilated than was accounted for, by gaseous products, although this was possibly assimilated into cellular material. Conversely, in Vessel B the concentrations of gaseous end-products produced were higher than the total concentration of soluble organic materials dissimilated, although, in this case, the "unaccounted" carbon from the previous vessel was possibly serving as a carbon source. Figure 17 shows the relationship between soluble and gaseous carbon in the system, with the majority of the soluble carbon (hexanoate and bicarbonate) dissimilated into gaseous products (CH_4 and CO_2). Thus, it was apparent that by increasing

Table 9: Carbon balance (mM 'C') for the dissimilation of hexanoic acid by the interacting microbial association, cultured in a 5-vessel chemostat model in the presence of 5 mM SO_4^{2-}.

Table 9: Balance of removal of carbon and formation of methane and CO_2 (mM 'C'), after the mode of Thompson et al. (1984).

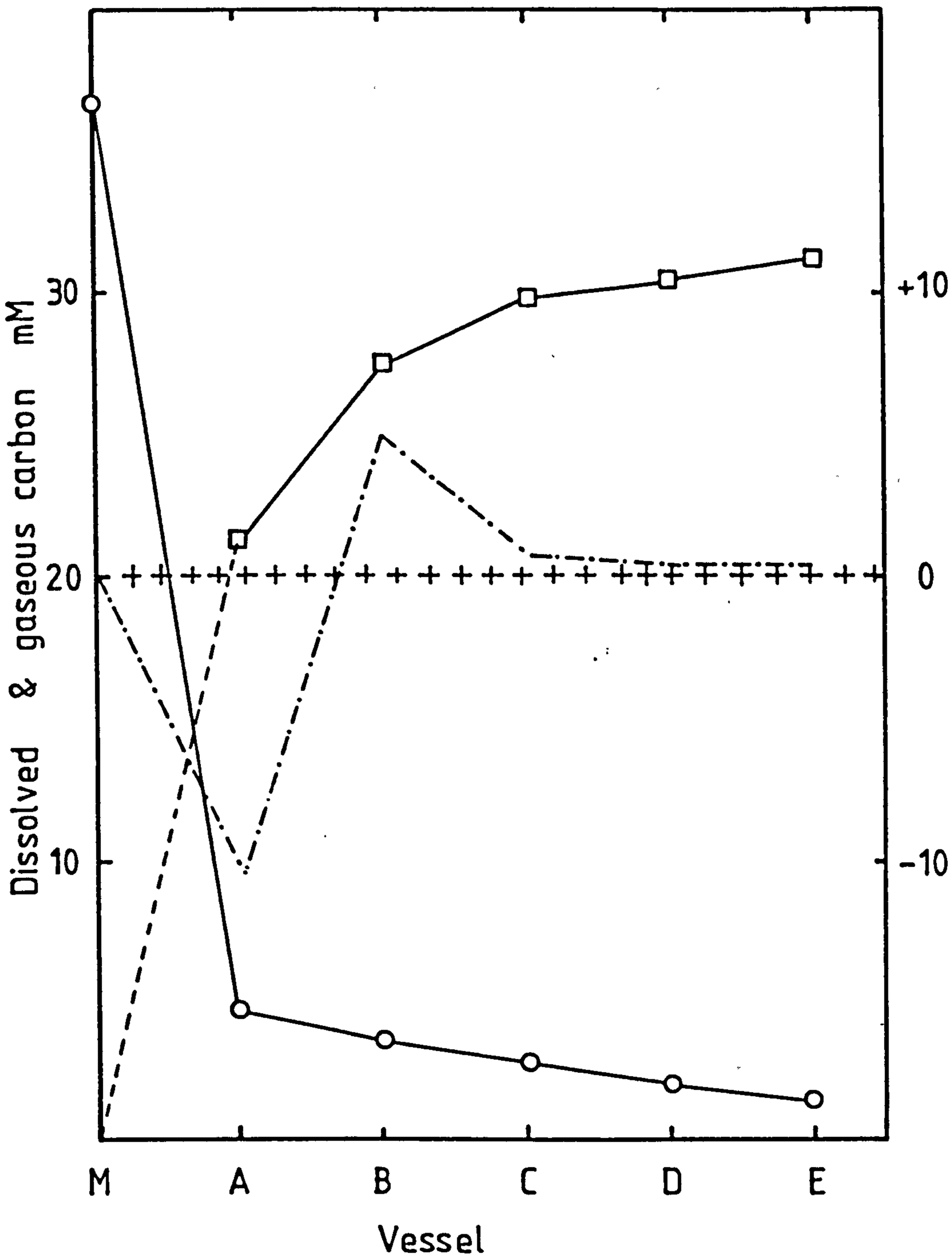
Input to Vessel A		Output from Vessel E + Total gaseous products	
Organic carbon	30	Methane	18.02
Inorganic carbon	6.8	Carbon dioxide	13.32
		Organic	0.46
Total	36.8		31.57

Carbon accounted for 31.57 / 36.8 x 100 = 86.14%

	Inorganic 'C'	Organic 'C'	Gas	Difference
A	6.8	-25.1	+21.4	-10.5
B	0	- 1.158	+ 6.1	4.9
C	0	- 0.98	+ 1.68	0.7
D	0	- 0.68	+ 1.23	0.55
E	0	- 0.46	+ 0.93	0.47
Total	-6.8	-28.37	31.34	-3.88

Figure 17: Changes in concentrations of dissolved (o—o) and gaseous (□—□) carbon during the catabolism of hexanoate (5 mM) by the isolated microbial association in a five-stage chemostat model in the presence of SO_4^{2-} (5 mM).

-·-·- denotes carbon accounted for.


Dissolved & gaseous carbon mM
30
20
10
+10
0
-10
Carbon balance
M
A
B
C
D
E
Vessel

the concentration of sulphate from 1.4 to 5 mM conversion of soluble carbon to gaseous products was significantly enhanced.

3.3 5-vessel multi-stage model : 10 mM sulphate

In the presence of 10 mM sulphate as with the previous concentration, hexanoic acid was completely dissimilated in Vessel A (Figure 18 and 18A). As already discussed, degradation of volatile fatty acids such as hexanoate and butyrate by obligate syntrophic co-cultures has been shown to proceed faster when the component H_2 sink organism was a sulphate-reducing bacterium (Boone & Bryant, 1980; McInerney et al., 1979; Mountfort & Bryant, 1982). This was explained on the basis that, in comparison with, for example, methanogens, the sulphate-reducing bacteria have a higher V_{max} and lower k_S for H_2 and hence act as energetically superior electron sink organisms (Kristjansson et al., 1982; Robinson & Tiedje, 1984).

Theoretically, the previous concentration of sulphate of 5 mM was sufficient to enable the sulphate-reducing bacteria to catabolise all the H_2 which resulted from hexanoate degradation, although the results clearly showed that only 61.2% of the influent sulphate was, in fact, catabolised. With the sulphate now increased to 10 mM the sulphate should have been well in excess of the theoretical maximum, if the sulphate-reducing activity was confined solely to the removal of H_2.

Figure 18: Changes in concentrations of metabolic intermediates and CH_4 during the anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of SO_4^{3-} (10 mM)

a - hexanoic acid

b - butyric acid

c - propanoic acid

d - acetic acid

e - methane

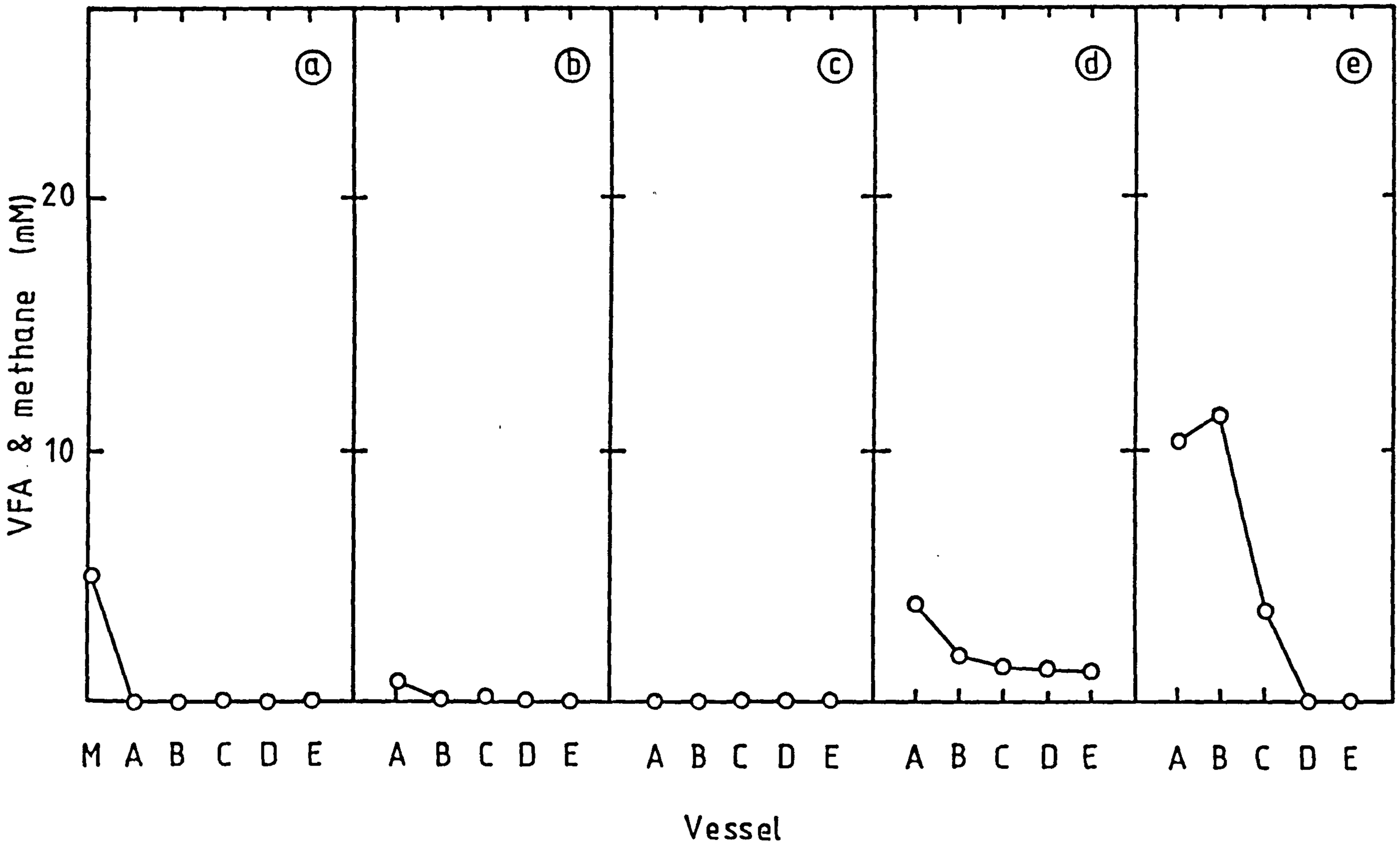
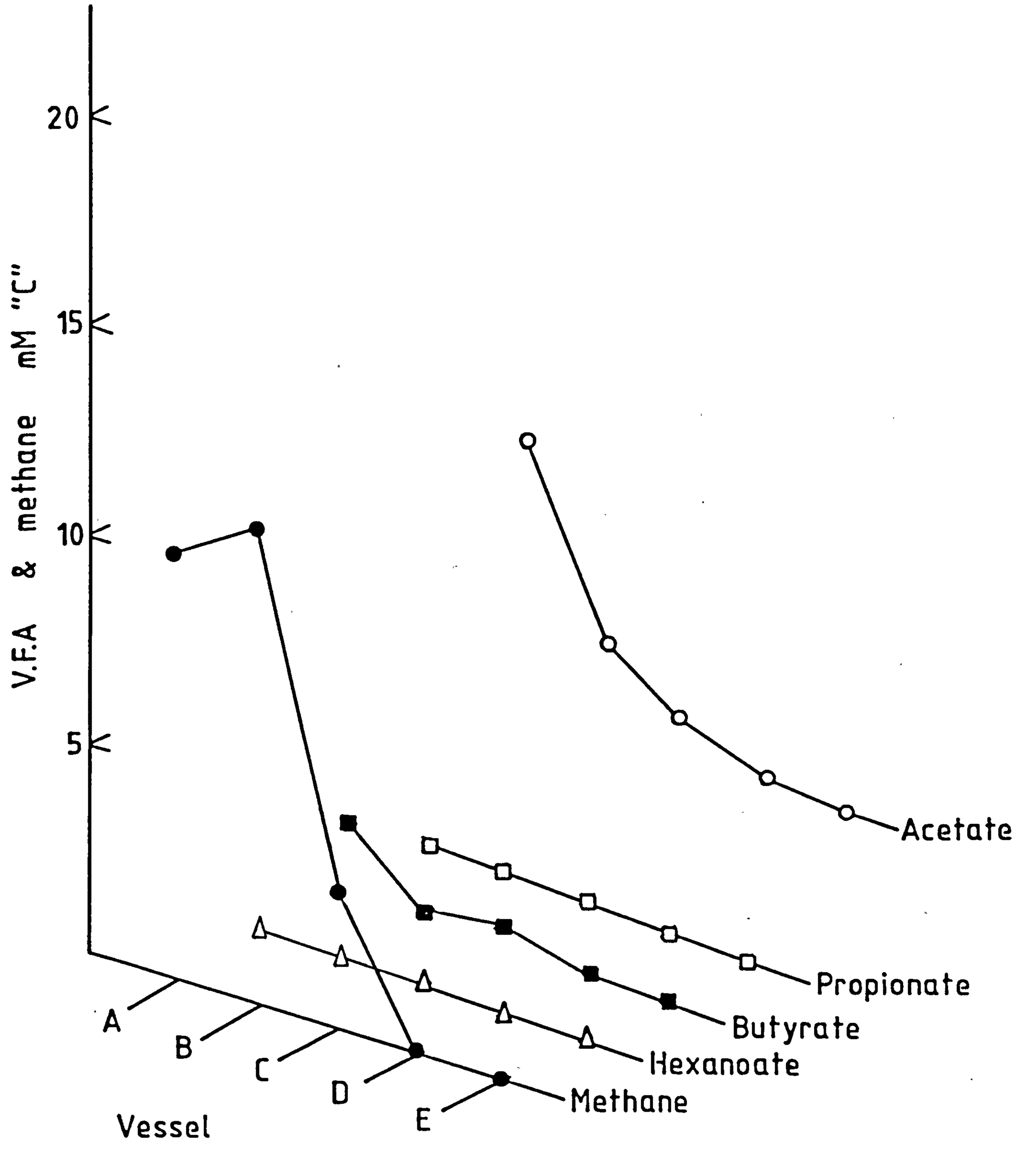

a
b
c
d
e
VFA & methane (mM)
20
10
M A B C D E A B C D E A B C D E A B C D E A B C D E
Vessel

Figure 18a: Carbon flow during anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a 5-vessel chemostat model in the presence of sulphate (10 mM).


V.F.A & methane mM "C"
20
15
10
5
A
B
C
D
E
Vessel
Acetate
Propionate
Butyrate
Hexanoate
Methane

Concomitant with hexanoic acid dissimilation butyrate and acetate were detected as intermediates. Butyrate was present at concentrations of 0.68 mM in Vessel A, zero in B and 0.229 mM in Vessel C. This reappearance of butyrate in Vessel C was hard to explain although it could possibly have been due to cell lysis and/or formation from H_2/CO_2. The appearance of butyrate was similar, in respect to vessels and concentrations, to that found in the previous experiment which suggested that this intermediate was catabolised at a slower rate than hexanoate. One possible explanation for this is that since butyrate was detected in the culture supernatant either as an excreted metabolite or a cell lysis product then further β-oxidation would have necessitated cell uptake although the mechanism of transport of metabolites into the bacteria responsible for the oxidation of volatile acids is still unknown.

A major difference between this experiment and the previous one was the absence of propionate as a metabolic intermediate, which was perhaps not surprising since the first sulphate concentration increase also resulted in reduced concentrations of volatile fatty acid intermediates. As previously discussed, the pathway of propionate formation was not immediately apparent although acetate plus HCO_3^- in the presence of H_2 was suggested as the precursors. The absence of propionate, however, was hard to explain.

However, as with the previous experiment, acetate was detected in all the vessels at concentrations of 3.9, 1.97, 1.37, 1.23, and

1.2 mM in Vessels A to E respectively. Although the concentrations of acetate were somewhat higher than the results obtained with an influent SO_4^{2-} concentration of 5 mM, they were considerably lower than the concentrations found in the initial experiment (1.4 mM sulphate).

The effects of excess sulphate on metabolic interactions in anaerobic environments has been discussed fully earlier (1.4.1). However, even in the presence of excess sulphate here, it would appear that the activities of the sulphate-reducing bacteria were most likely restricted to that of oxidation of H_2.

The concentration of residual sulphate decreased from 10 mM in the influent medium to 3.46 mM in Vessel A (Figure 19) after which it remained approximately the same with concentrations of 3.41, 3.35 and 3.68 mM recorded for Vessels B to E respectively. Concomitant with sulphate utilisation was the formation of sulphide as:

(1) HS^-;5.33 mM (A), 3.88 mM (B), 4.85 mM (C), and 3.88 mM (D); and

(2) H_2S; 1.22 mM (A), 0.659 mM (B), 1.15 mM (C), and 0.659 mM (D).

The sulphur balance (Table 10 A) shows that 112.4% of the influent sulphur was accounted for, with the balance of removal of sulphate and generation of sulphide (Table 10B) for each Vessel (A to D) showing that in Vessels B and D sulphur was removed whereas in Vessel C sulphur was generated.

Compared with the previous two experiments, the results

Figure 19: Changes in the concentrations of residual hexanoic acid (Δ—Δ) and sulphate (○—○), gaseous hydrogen sulphide (●—●), soluble sulphide (◒—◒), methane (□—□), pH (■—■), and optical density (▼—▼) during the 5-stage open culture cultivation of the isolated microbial association in the presence of hexanoic acid (5 mM) and sulphate (10 mM)

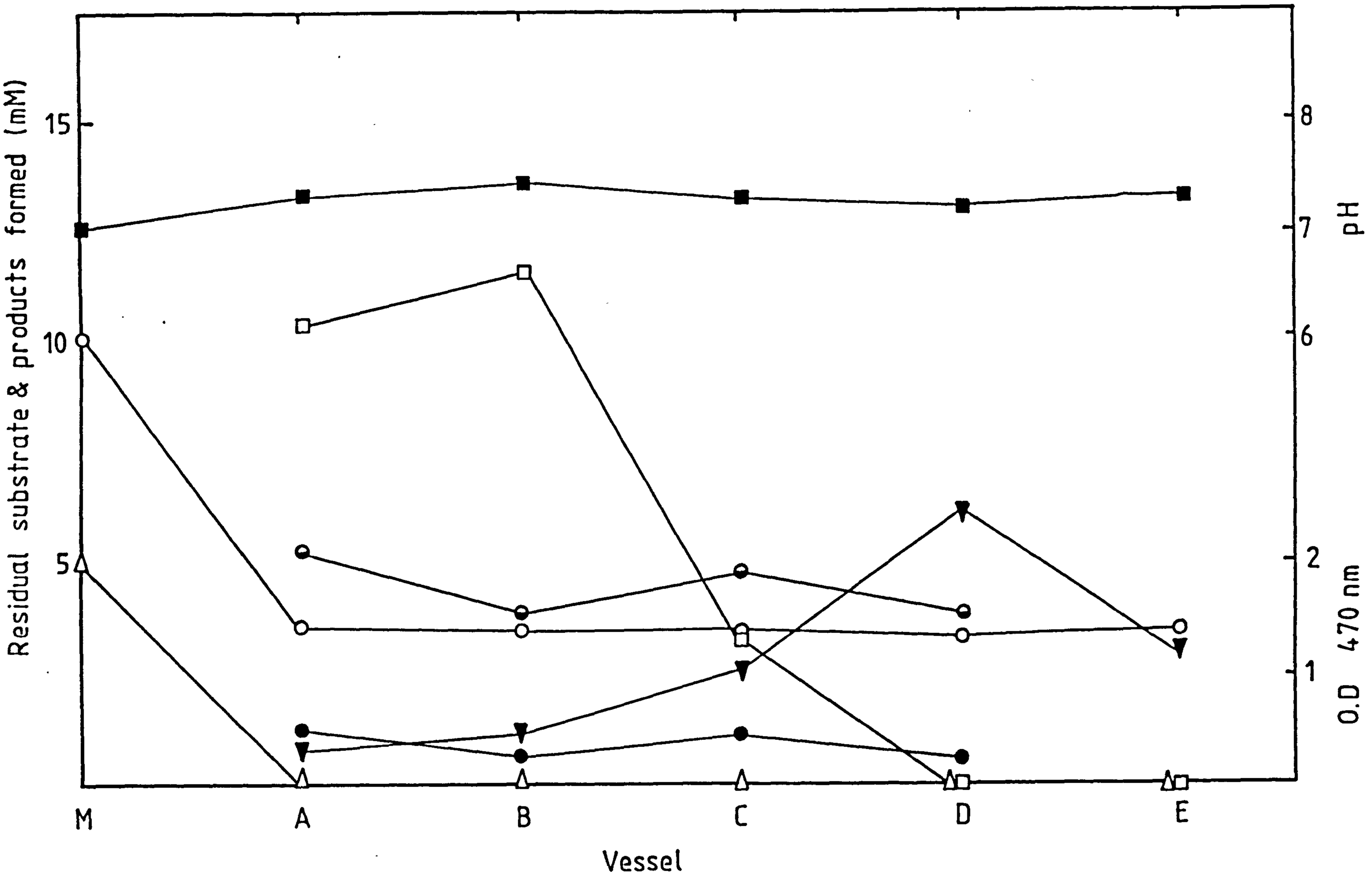

Residual substrate & products formed (mM)
15
10
5
pH
8
7
6
O.D 470 nm
2
1
M
A
B
C
D
E
Vessel

Table 10A: Total sulphur balance (mM 'S') for the 5-stage system with initial sulphate at 10 mM.

Table 10B: Balance of sulphate removal and formation of sulphide (mM 'S') after the mode of Thompson et al. (1984)

$$\text{Input } SO_4^{2-} = HS^- + H_2S + SO_4$$

$$10 = 3.88 + 3.688 + 3.68$$

$$\text{Sulphur accounted for} = \frac{11.24}{10} \times 100 = 112.4\%$$

	SO_4	HS^-	H_2S	Total
A	-6.54	+5.33	+1.22	0.01
B	-0.049	-1.45	+0.659	-0.84
C	+0.129	+0.97	+1.15	2.249
D	-0.19	-0.97	+0.659	-0.501
Total	-6.65	+3.88	3.688	0.918

suggested that sulphate was playing a more significant role as an electron acceptor as higher concentrations were catabolised than were required for the oxidation of H_2 (5 mM). This suggested that either the sulphate-reducing bacteria were also utilising a substrate other than H_2, for example acetate, or that sulphate was utilised by another group of bacteria. However, since sulphate removal was almost balanced by sulphide production, this was unlikely.

Compared with the results of the previous experiments the pH of the system did not increase significantly, with values of 7.3, 7.4, 7.3, 7.2 and 7.3 recorded for Vessels A to E respectively. These results are rather hard to explain as the previous rise in pH was attributed to sulphate-reduction and since there was considerably more sulphate-reduction in this experiment, one would have expected a greater or steeper increase in pH.

As a result of this second sulphate concentration increase it was anticipated that methane production would have been affected. However, instead of a reduction in the concentration a shift in methanogenic activity was noted. A concentration of 10.25 mM was recorded in Vessel A with 11.48 mM in Vessel B and 3.46 mM in Vessel C. Increasing the sulphate concentration in incremental steps 1.4 to 5 and 5 to 10 mM resulted in total methane concentrations of 23.2, 18.0 and 25.2 mM respectively, hence it would appear that the sulphate did not significantly alter the overall methane concentrations.

The carbon balance for the system is shown in Table 11A, from which it can be seen that 91.9% of the carbon was accounted for. The distribution of carbon is shown clearly in Table 11B and Figure 20 where there was a deficit of carbon in Vessels A, D and E, which was almost balanced by excess carbon in Vessels B and C. The deficit in Vessel A could possibly be explained as assimilated carbon which subsequently became available as lysis products in Vessel B and C and as a consequence accounted for the excess carbon in these two. Unfortunately, the biomass determinations did not support the above possibility as the O.D. increased from 0.36 in Vessel A to 0.48 (B) to 1.076 (C) and then peaked in Vessel D at 2.48 before falling to 1.21 in Vessel E. However, interpretation of these results was somewhat difficult since the recorded O.Ds could have been due to a combination of biomass and insoluble compounds such as metal sulphides.

Displacement of organisms down the array was also apparent, although it was clear that metabolic activity and optical density were not linked, as Vessels A and B contained the most active population of bacteria but had the two lowest O.D. values, unless either interspecies interactions were occurring or the responsible organisms were attached to the surfaces of the chemostats.

3.4 Kinetic interpretation

The multi-stage chemostat consisted of a series of 5 individual vessels which, in total, approximated to a segmented but continuous

Table 11A: Carbon balance for the dissimilation of hexanoic acid by the interacting microbial association cultured in a 5-vessel chemostat model in the presence of 10 mM SO_4^{2-}.

Table 11B: Balance of removal of carbon and formation of methane and CO_2 (mM 'C'), after the mode of Thompson *et al.* (1984).

C input = 30.00 output + CH_4

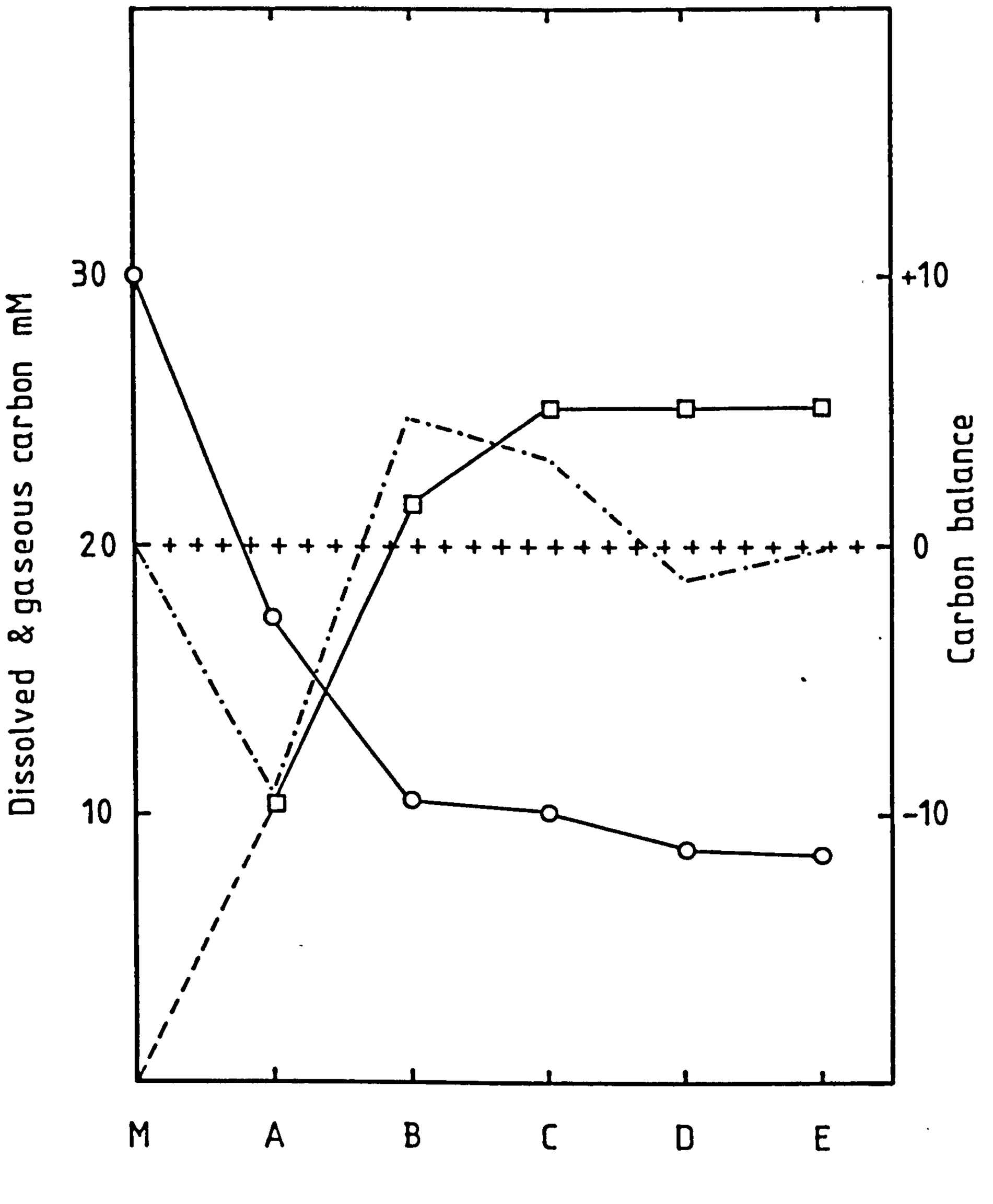
= 27.59

∴ % accounted for = ' 91.9%

	Organic	CH_4	Total
A	-19.48	+10.25	-9.23
B	- 6.58	+11.48	+4.9
C	- 0.284	+ 3.46	+3.19
D	- 1.19	-	-1.19
E	- 0.06	-	-0.06
Total	-27.59	25.19	-2.4

Figure 20: Changes in concentrations of dissolved (O—O) and gaseous (□—□) carbon during the catabolism of hexanoate (5 mM) by the isolated microbial association in a five-stage chemostat model with an influent sulphate concentration of 10 mM.

- • - • denotes the carbon balance


30
20
10
+10
0
-10
Dissolved & gaseous carbon mM
Carbon balance
M
A
B
C
D
E
Vessel

plug-flow reactor. The results, therefore, may be interpreted by considering either the individual vessels or, alternatively, by considering combinations of vessels and thus cumulative volumes, which may be equated with decreasing dilution rates.

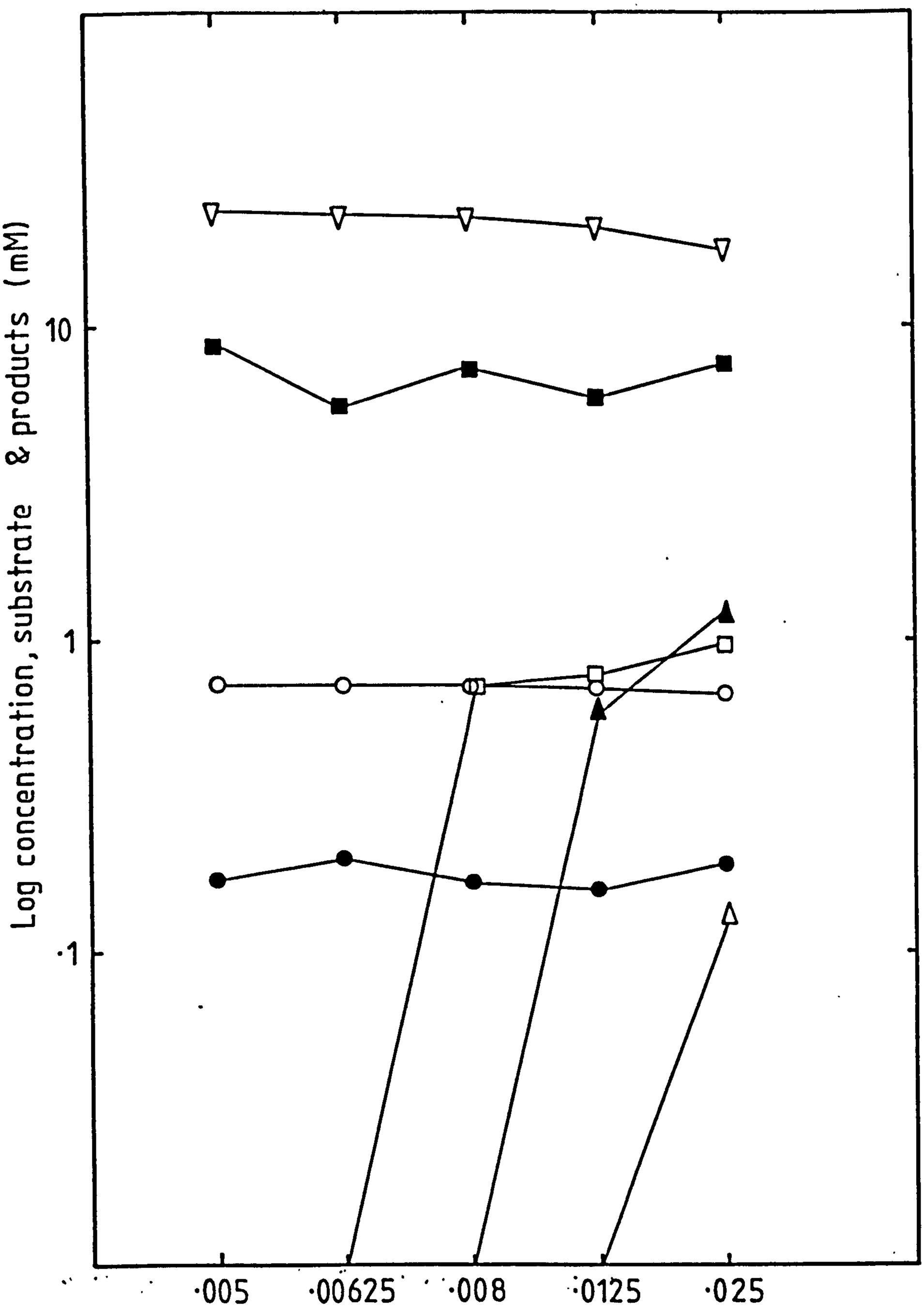
Data from the first sulphate concentration are shown in Figure 21 where the concentrations of metabolites are plotted against accumulating and, thus, decreasing dilution rates.

Cumulative concentrations of methane and hydrogen sulphide, together with residual sulphate and acetate all reached their approximate terminal values at a dilution rate of 0.025 h^{-1} (Vessel A), and then remained relatively constant as the dilution rate decreased. This, however, was not found with hexanoate, butyrate and propionate which were all detected at an initial dilution rate of 0.025 h^{-1} before their concentrations decreased to zero at dilution rates of 0.025, 0.008 and 0.00625 h^{-1} respectively.

The results indicated that the hexanoate degrader had a D_{crit} < 0.025 h^{-1} > 0.0125, whilst the butyrate-degrading organisms would have been eventually displaced at a dilution rate of 0.008 h^{-1} and the propionate degrading organisms at 0.00625 h^{-1} if the results were equated with a single-stage homogeneous chemostat.

This situation could have been analogous to that described by Pirt (1975), who found that when a mixture of carbon sources were supplied to a chemostat they were utilised simultaneously over a wide range of dilution rates, if growth was carbon limited. If, however, the carbon sources were not growth limiting then

Figure 21: Concentrations of hexanoate (Δ—Δ), butyrate (▲—▲), propionate (□—□),acetate (■—■), methane (▽—▽), sulphate(○—○), and hydrogen sulphide (●—●), plotted against cumulative dilution rates during the open culture cultivation in the 5-vessel chemostat model, of the isolated microbial association in the presence of hexanoate (5 mM) and sulphate (1.4 mM). The key at the bottom of the figure denotes the individual vessel or combination of vessels which corresponded with each discrete dilution rate.


Log concentration, substrate & products (mM)
10
1
·1
·005
·00625
·008
·0125
·025
D (h⁻¹)
E
D
C
B
A

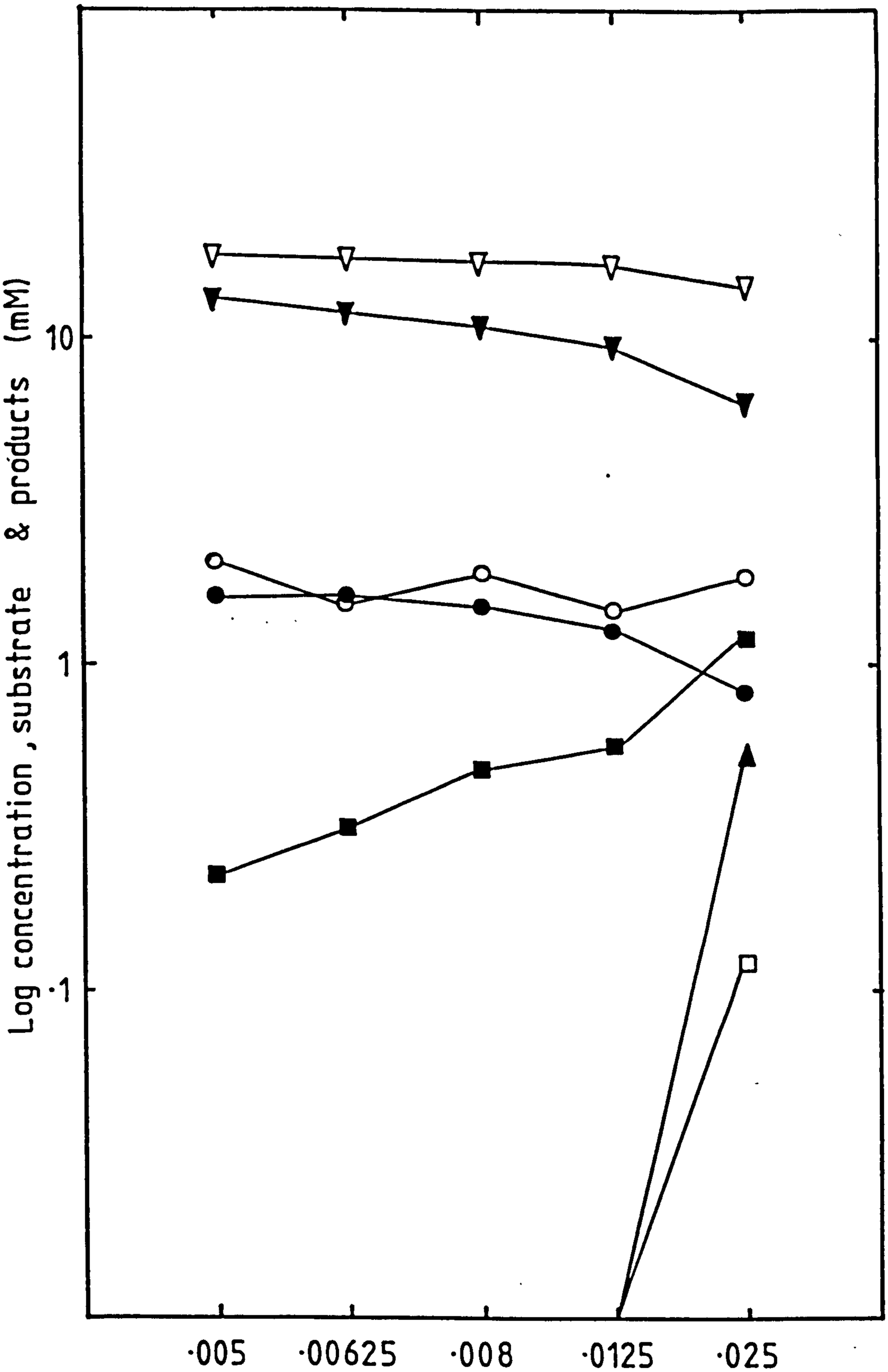
preferential utilisation was apparent. For example, acetate was used before glucose in a nitrogen-limited culture of Pseudomonas sp. (Ng and Dawes, 1973).

Although it is recognised that a single species was used in the above study with the carbon sources supplied in the medium (and did not arise as metabolic products), the results presented in Figure 21, show that sequential utilisation of the volatile fatty acids, hexanoate, butyrate and propionate resulted in Vessels A, B and C respectively. This could have been, as suggested above, indicative of control of V.F.A. utilisation by a factor other than carbon-limitation. Since it is unlikely that the culture was limited by nutrient factors in the medium, the most likely explanation is that the overall reaction limitation was H_2 removal by interspecies hydrogen transfer.

Energetically, H_2 is removed more effectively by sulphate reduction than methanogenesis (Thauer et al., 1977) and in fact confirmation of this was obtained when the sulphate concentration was increased from 1.4 to 5 mM. Figure 22 shows the results of the 5 mM sulphate concentration experiment plotted against cumulative dilution rate.

Hexanoate was not detected in any of the vessels which implied that the hexanoate degrader had a $D_{crit} > 0.025\ h^{-1}$. Butyrate and propionate were detected at a discrete dilution rate of $0.025\ h^{-1}$ but not at $0.0125\ h^{-1}$. Thus, by increasing the sulphate concentration from 1.4 to 5 mM the critical dilution rates for hexanoate,

Figure 22: Concentrations of butyrate (▲—▲), propionate (□—□), acetate (■—■), methane (▽—▽), CO_2 (▼—▼), sulphate (○—○), and hydrogen sulphide (●—●), plotted against cumulative dilution rates during the open culture cultivation in the 5-vessel chemostat model, of the isolated microbial association in the presence of hexanoate (5 mM) and sulphate (5 mM). The key at the bottom of the figure denotes the individual vessel or combination of vessels which corresponded with each discrete dilution rate.


Log concentration, substrate & products (mM)
10
1
·1
·005
·00625
·008
·0125
·025
D (h⁻¹)
E
D
C
B
A

butyrate and propionate-metabolism shifted from 0.025 to > 0.025 h^{-1}, 0.008 to 0.0125 h^{-1} and 0.00625 to 0.00125 h^{-1} respectively.

The concentrations of methane, gaseous sulphide and sulphate as before did not change significantly over the range of dilution rates. Acetate, however, was seen to decrease gradually from 1.21 mM at a dilution rate of 0.025 h^{-1} to 0.23 mM at 0.005 h^{-1}. This was much in contrast to the results of the previous experiment where the acetate concentrations remained between 7 and 8 mM over the whole range of dilution rates.

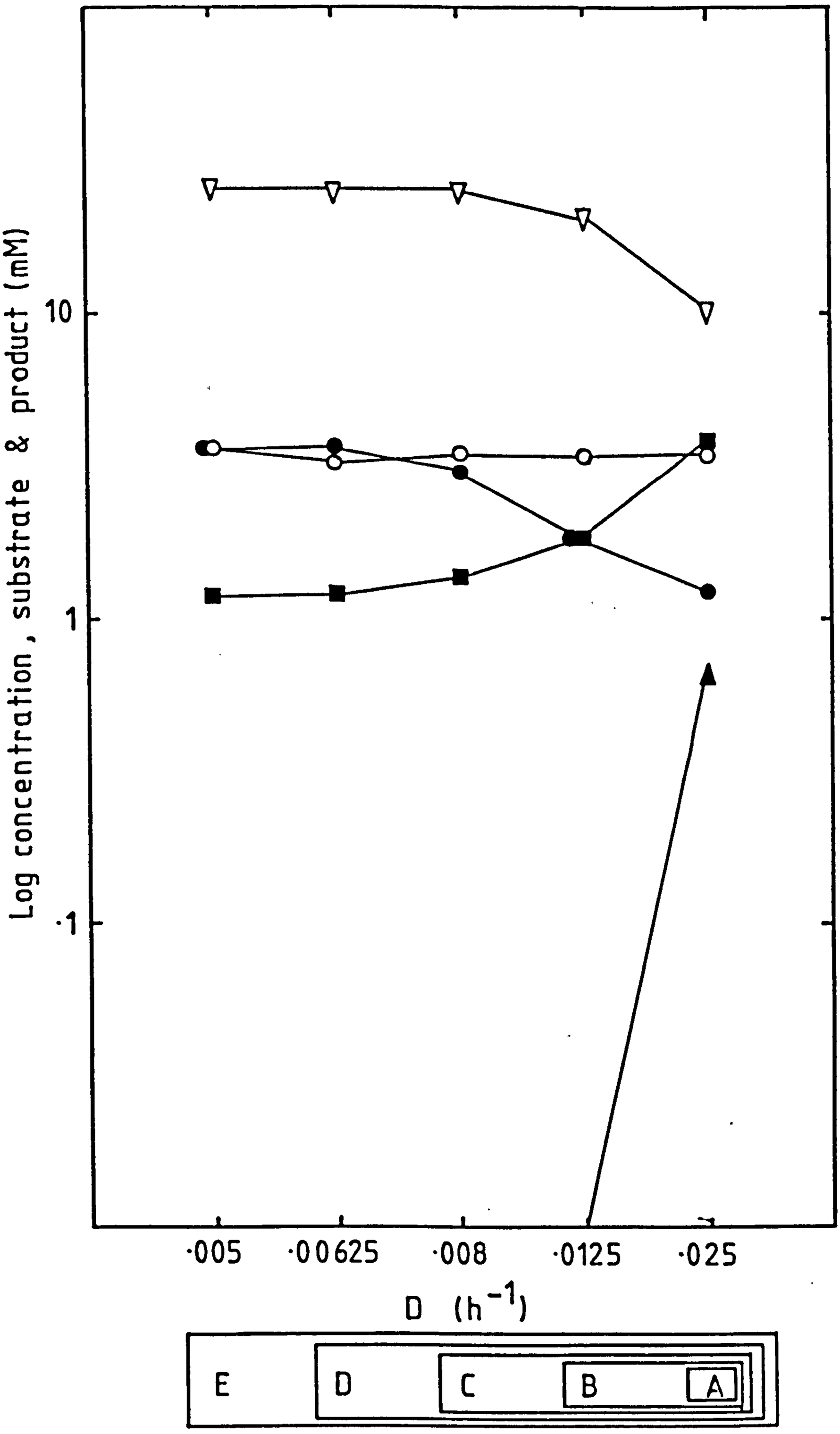
A possible explanation for these apparent increases in the specific growth rate of the V.F.A.-utilising bacteria could have been, as already discussed, due to the increase in influent sulphate concentration which facilitated an increase in the H_2-removing capacity of the microbial association.

As a result of the final increase in influent sulphate concentration to 10 mM, hexanoate once again was not detected in the array and hence, as for the results at 5 mM sulphate, the hexanoate degrader had a D_{crit} > 0.025 h^{-1} (Figure 23). These results did not, however, indicate if the critical dilution rate of the hexanoate degrader had increased as a result of the elevated sulphate concentration.

As with the previous sulphate concentration butyrate was detected in Vessel A only and thus the D_{crit} for subsequent catabolism was 0.0125 h^{-1}. Unlike the two previous experiments, propionate was not detected in the array in this experiment. Two

Figure 23: Concentrations of butyrate (▲—▲), acetate (■—■), methane (▽—▽), sulphate (○—○), and hydrogen sulphide (●—●) plotted against cumultative dilution rates during the open culture cultivation in the 5-vessel chemostat model, of the isolated microbial association in the presence of hexanoate (5 mM) and sulphate (10 mM).

The key at the bottom of the figure denotes the individual vessel or combination of vessels which corresponded with each discrete dilution rate.


Log concentration, substrate & product (mM)
10
1
·1
·005
·00625
·008
·0125
·025
D (h⁻¹)
E
D
C
B
A

possibilities exist to explain this situation; firstly, metabolism was altered to such a degree as to shift the metabolic products away from propionate, or secondly, propionate turnover was more rapid when the sulphate concentration was increased to 10 mM.

The results did show clearly that increased concentrations of sulphate had considerable effects on volatile fatty acid metabolism, with the sequential utilisation recorded with the first concentration (1.4 mM) absent in the presence of 5 and 10 mM sulphate. This result supported the hypothesis tendered earlier that volatile fatty acid metabolism was limited by H_2 removal, which was made energetically more favourable as sulphate replaced CO_2 as the terminal electron acceptor. Another possibility, however, could be that volatile fatty acid metabolism was limited by sulphate, nutritionally, although this was unlikely as complete mineralisation of hexanoate was noted in the first experiment.

Acetate, again followed the same trend as observed in the presence of 5 mM sulphate when gradual decreases were apparent. Although increased sulphate concentrations did not appear to affect acetate metabolism, methane production increased significantly from 10.25 mM at a dilution rate of 0.025 h^{-1} to 25.19 mM at 0.008 h^{-1}. Since this increase in methanogenesis was not seen in the previous 2 experiments the most probable explanation was that the non-sulphate-limited conditions resulted in an uncoupling of methanogenesis from hexanoate catabolism and as a consequence the methanogens were competitively displaced down the array.

3.5 Microbial interactions and spatial separation

The multi-stage chemostat was primarily developed to facilitate spatial separation between the various metabolic groups involved in the degradation of hexanoic acid. The results of the experiments with the two lower concentrations of sulphate, however, were indicative of the fact that no such separations were realised since hexanoic acid dissimilation, sulphate-reduction and methanogenesis all occurred in the first vessel (A). A possible explanation for this was that sulphate was limiting and depleted thus the methanogens or homo-acetogens constituted the H_2-sink metabolic group(s) and as a consequence were not displaced from the activity domain of hexanoate cataboliser (Figure 24).

This tightly interacting microbial association, however, did show signs of 'separating' in the final experiment when the sulphate concentration was increased to 10 mM. In this experiment sulphate-reduction in Vessel A was such that all the H_2 could have been accounted for by this process and thus result in the displacement of methanogens as was observed.

Figure 25 shows a diagram of this postulated configuration in which the sulphate-reducing bacteria formed an obligate syntrophic partnership with the hexanoate cataboliser.

Spatial separation of bacterial species within an interacting microbial association requires distinct habitat domains to develop in the presence of overlapping activity domains (Wimpenny, 1981). The experimental results showed clearly that the microbial

Figure 24: Hypothetical scheme for anoxic hexanoate catabolism by the interacting microbial association in which the hexanoate-catabolising species and the homo-acetogens constitute an obligate first tier association (in the absence of sulphate) and these two species together with the methanogenic species constitute a second tier association.

The solid lines represent habitat domains and the broken lines, activity domains, where the activity domains for H_2 are closely associated with the habitat domains of the first tier co-culture and the acetate activity domain is more distinct for the second tier culture.

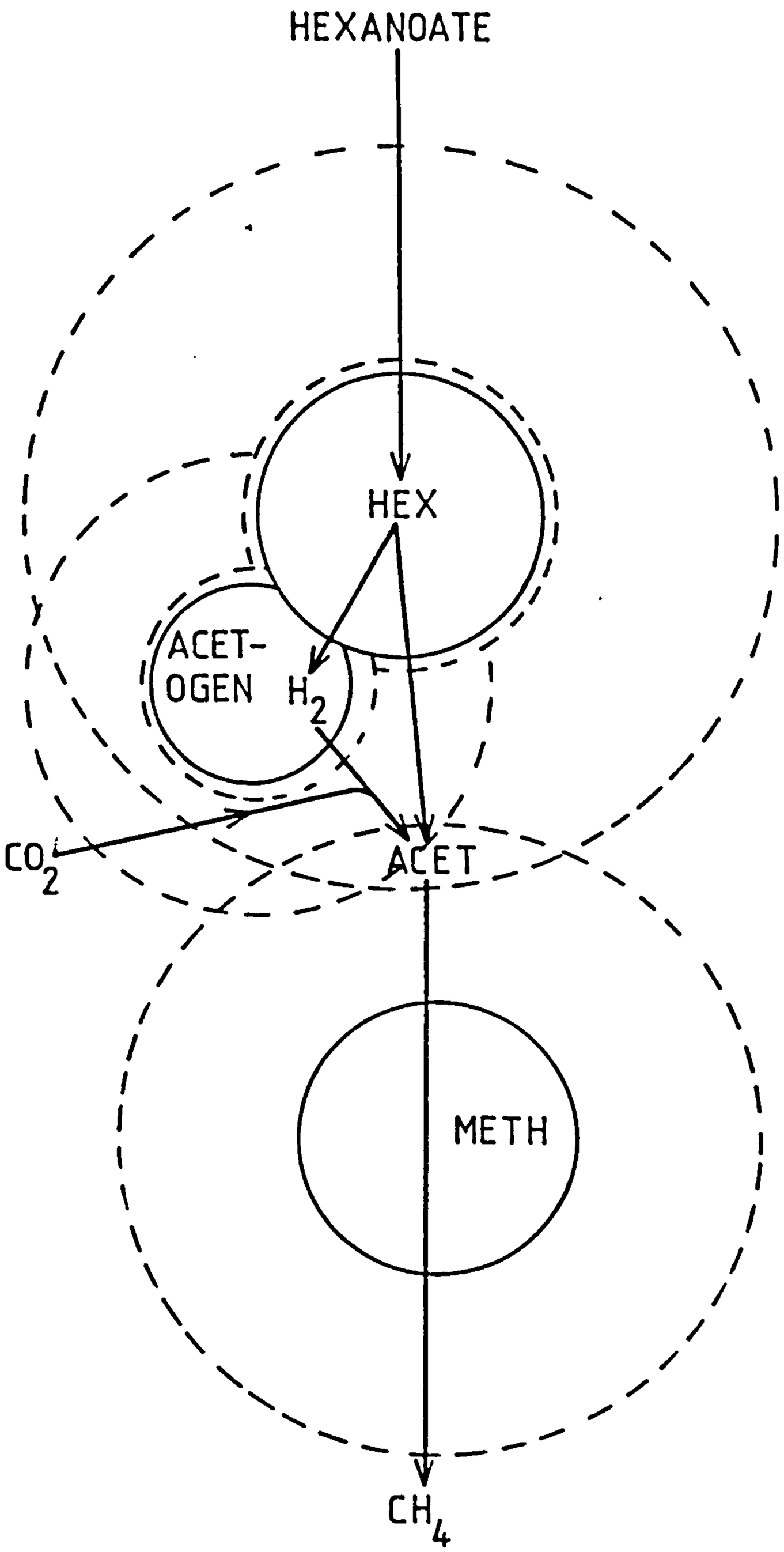
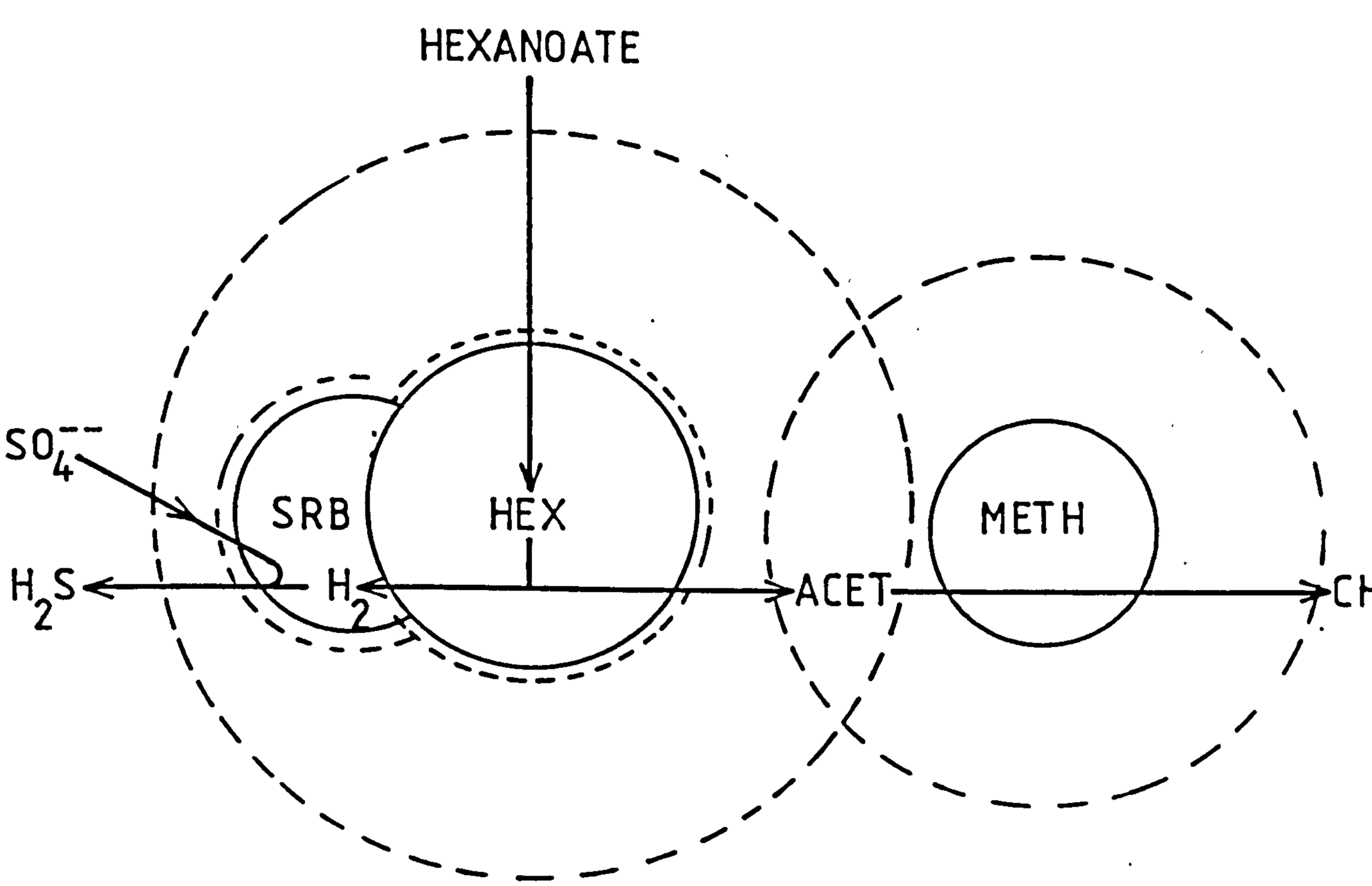

HEXANOATE
HEX
ACET-
OGEN
H_2
CO_2
ACET
METH
CH_4

Figure 25: Hypothetical scheme for anoxic hexanoate catabolism by the interacting microbial association in which the hexanoate catabolising species and the sulphate reducer constitute an obligate first tier association (in the presence of sulphate) and the two species together with the methanogenic species constitute a second tier association.

The solid lines represent habitat domains and the broken lines, activity domains, where the activity domains for H_2 are closely associated with the habitat domains of the first tier co-culture and the acetate activity domain is more distinct for the second tier culture.


HEXANOATE
SO_4^{--}
SRB
HEX
METH
H_2S
H_2
ACET
CH

association was very tightly associated since the hexanoate-degrading organism required the presence of other microorganisms to effect complete degradation. Since spatial separation of habitat domains was not effected it would appear that either considerable overlap of activity domains existed or that the activity domains were very localised around the habitat domains.

The major limitation of metabolic interactions between different organisms is diffusion of solutes. Diffusion in liquids is a slow process and in natural environments could be the rate-limiting step in the activity of particular organisms. If diffusion is the only transfer process then rates would be governed by the diffusion coefficient for the solute, the temperature and the proximity of the sources and sinks. In the absence of a sink for a solute the logarithm of the solute concentration is a linear function of the square of the distance. Hence it is clear that to interact effectively an organism which is metabolising a solute must be as close to the source as possible.

The component species of the microbial association isolated in the first vessel of the multi-stage chemostat were obviously in close physical contact which, as discussed above, is a priori for β-oxidation. Therefore, it is apparent that physical separation of the component species of the above association was extremely unlikely without a major physico-chemical perturbation. Such a perturbation, if found, could have had the effect of inhibiting the degradation of hexanoate.

Close coupling of non-syntrophically-interacting members of the associaiton is also logical since reliance on solute diffusion necessitates this, as the greater the separating distance the more solute could be 'lost' to the sink organisms.

Coupling of organisms may be made effective in three ways: first, sink organisms may completely surround source organisms, for example, Pelechromatium roseo viridae (Wimpenny, 1981); secondly, organisms may form strata, for example, the organisms of the sulphur cycle; and thirdly, microbial associations may be cultured in well-stirred, homogeneous fermenters, which would ensure good interactions as the solutes were mixed effectively amongst the population.

Anaerobic ecosystems tend to form strata, with different metabolic groups of bacteria occupying each tier. The major factor responsible for these is the availability of electron acceptors which are often utilised sequentially, reflecting the free energy released on reduction. This usually results in specialised groups of bacteria utilising each electron acceptor with each species occupying a discrete niche, for example, sulphate-reducing bacteria utilise sulphate whilst methanogens carbon dioxide. Since sulphate-reduction is thermodynamically more favourable than methanogenesis (Thauer et al., 1977), sulphate-reducing bacteria usually occupy the redox zone preceding methanogens as they are metabolically more versatile. Stratification, typical of some marine sediments was found by Thompson et al. (1984) when they used a five-vessel

chemostat to examine anaerobic catabolism of glucose and benzoate. Development of a methanogenic population was ensured as the initial sulphate concentration (10 mM) was insufficient, and hence relatively atypical (Stewart, 1983) to enable complete oxidation of the organic substrate by sulphate-reduction alone. The dilution rate was slow (D = 0.0018 h^{-1} for the whole system) which, together with the above factors, resulted in spatial separation of sulphate-reduction, and methanogenesis and acetogenesis similar to that found in other anoxic ecosystems.

The three experiments described here show that spatial separation between the sulphate-reducing bacteria and the methanogens was not achieved until the sulphate concentration was raised to 10 mM. With the exception of recently emplaced refuse this concentration, however, can be considered to be artificially high for most landfill ecosystems.

From the discussion presented here it would appear that an elevated concentration of sulphate was the critical factor for the spatial separation of sulphate-reducers and methanogens in the microbial association isolated from landfill. This conclusion, however, was in direct contrast to marine sediments (Thompson et al., 1984) where a reduced sulphate concentration was the prerequisite for separation.

3.7 Three-vessel multi-stage model : 1.4 mM sulphate

The cumulative dilution rate figures (Figures 21 to 23)

demonstrated that changes in dilution rate resulted in fermentation balance changes. Thus, this strategy was considered to further probe the isolated association.

A multi-stage chemostat with three vessels was therefore constructed to investigate the effect of a non-constant dilution rate regime on the interactions of the microbial association. The dilution rate of the first vessel was set at 0.05 h^{-1}, with the second at 0.022 h^{-1} and the third at 0.00625 h^{-1}. Thus, the second vessel of this system was equivalent to the first vessel of the original five-vessel array with the third vessel equivalent to the fourth.

An inoculum from Vessel A of the five-stage model was used in this experiment in which the influent medium contained 5 mM hexanoate and 1.4 mM sulphate. The component vessels were monitored at regular intervals over a period of 3 months at which point terminal samples were taken.

Figure 26 and 26A (which shows carbon flow in the system) shows that the concentration of residual hexanoate decreased gradually down the array from 2.2 mM (A) to 0.4 mM (B) and finally 0.09 mM (C). These results differed considerably from the results obtained with the corresponding 5-stage system)1.4 mM sulphate) where 96.7% of the influent hexanoic acid was dissimilated in the first vessel. The end result, however, was comparable as the concentration of hexanoate in the first vessel of the original multi-stage was 0.162 mM, which was approximately equivalent to that found in Vessel C of

Figure 26: Changes in concentrations of residual substrate metabolic intermediates and methane during the anoxic catabolism of hexanoic acid (5 mM) by the isolated microbial association in a three-vessel chemostat model in the presence of sulphate (1.4 mM).

a - hexanoic acid

b - butyric acid

c - propanoic acid

d - acetic acid

e - methane

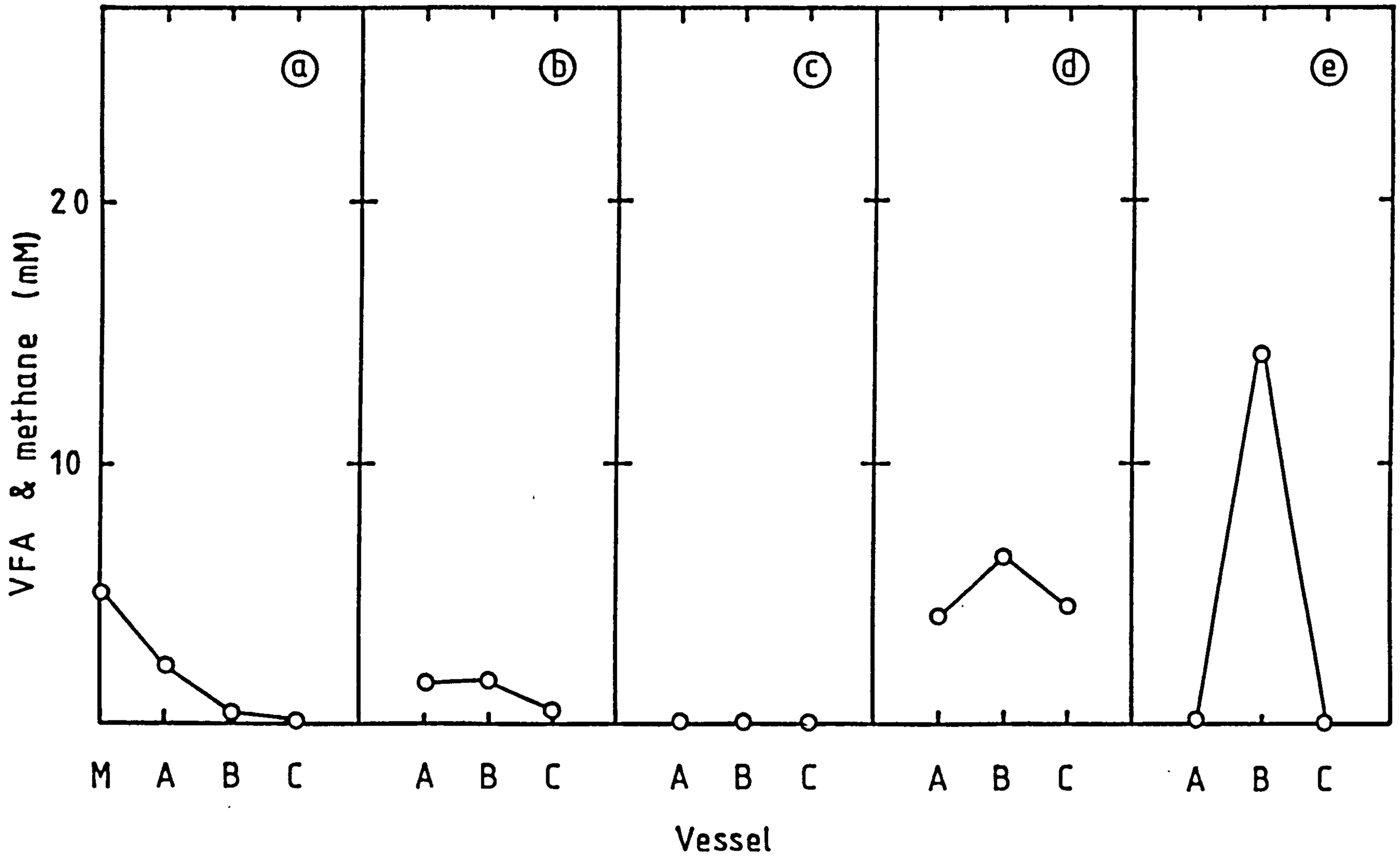
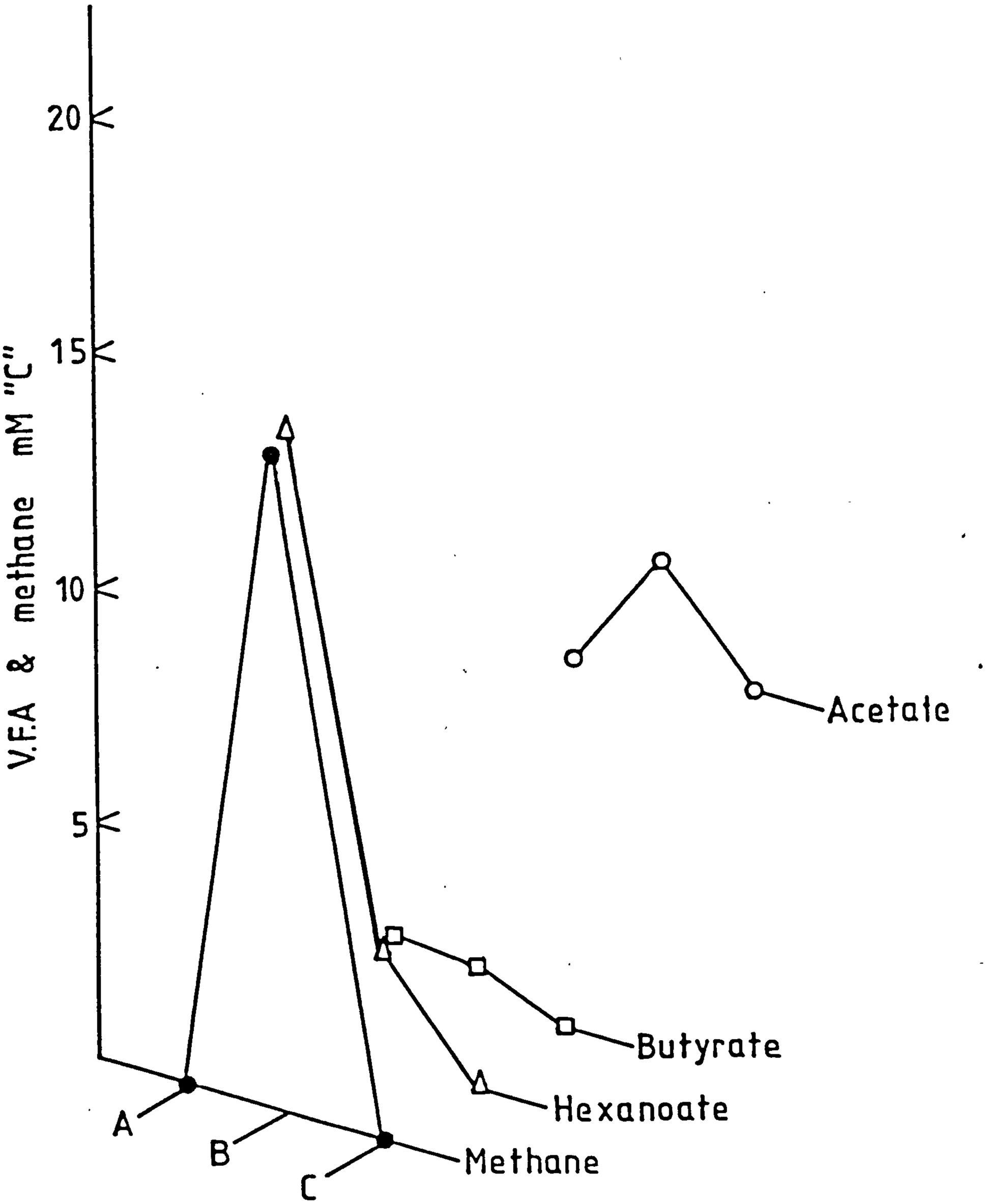

a
b
c
d
e
20
10
VFA & methane (mM)
M A B C
A B C
A B C
A B C
A B C
Vessel

Figure 26a: Carbon flow during anoxic catabolism of hexanoate (5 mM) by the isolated microbial association in a 3-vessel chemostat model in the presence of sulphate (1.4 mM).


20
15
10
5
V.F.A & methane mM "C"
Acetate
Butyrate
Hexanoate
Methane
A
B
C
Vessel

this system. As already discussed for the previous system, the results obtained here suggested that once again the overall system was hexanoate limited.

Concomitant with hexanoate dissimilation, butyrate and acetate were produced. The concentration of butyrate increased from 1.45 mM in Vessel A to 1.6 mM in Vessel B before decreasing to 0.4 mM in Vessel C. Butyrate was also found as an intermediate in the original system when the concentration decreased steadily from 1.2 mM in the first vessel to 0.616 mM in the second and finally zero in the third. However, as Vessel B of this system was comparable to the first vessel of the original five-stage array the results were comparable.

In Vessel A, at a dilution rate of 0.05 h^{-1}, 56% of the influent hexanoate was catabolised. This process, as already discussed, was probably facilitated by a syntrophic association, in which the electron sink organism used either sulphate as the terminal electron acceptor, if sulphate was present, or, if depleted, CO_2. Thus it may be speculated that in the presence of sulphate the responsible organism was probably a sulphate-reducing bacterium and in its absence a methanogen. Equation 28 shows the stoichiometry of hexanoic acid catabolism to butyrate and acetate in the presence of sulphate :

$$2\ C_6H_{11}O_2^- + SO_4^{2-} \longrightarrow 2\ C_4H_7O_2^- + 2\ C_2H_3O_2^- + HS^- + H^+ \qquad (28)$$

This demonstrates that in the presence of 1 mM sulphate 2 mM hexanoate would be dissimilated to 2 mM each of butyrate and

acetate. Thus, in the presence of 1.4 mM sulphate 2.8 mM of the respective substrate and products would potentially be dissimilated and formed. This, however, was not seen in the chemostat as the butyrate and acetate concentrations were 1.45 and 4.0 mM respectively. The concentration of sulphate used in this experiment was, therefore, not sufficient to facilitate oxidation of the butyrate with sulphate-reducing bacteria as the electron sink component species. The situation, however, was analogous to that found in the 5-vessel system, when the same concentration of sulphate was used, when more than 90% of the influent hexanoate was dissimilated in the first vessel. As fully discussed in Section 3.1.1 it would appear that when sulphate was depleted then CO_2 was reduced to CH_4, as a route for electron dispersion for the syntrophic association.

The imposed dilution rate of 0.05 h^{-1} in the first vessel of the 3-stage system resulted in the displacement of the methanogenic population since no methane was detected and as a consequence hexanoate was dissimilated to butyrate and acetate only (Equation 28).

Residual hexanoate and butyrate were subsequently catabolised in Vessel B in association with methanogens as the syntrophic partner.

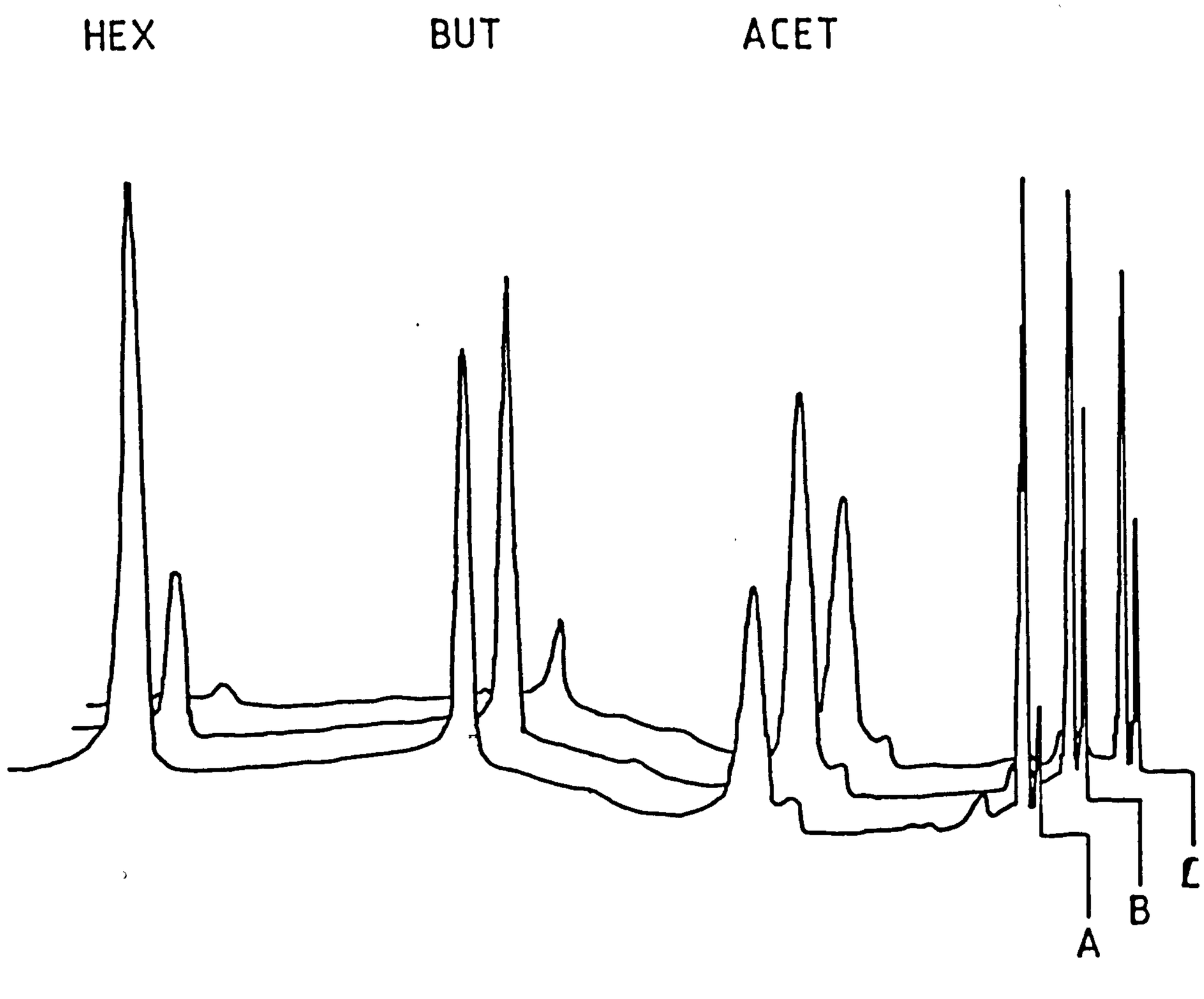
In the 5-vessel array propionate was found as a metabolic intermediate in Vessels A to C, although, in the 3-stage system, propionate did not appear as an intermediate. This result is hard to explain as the appearance of propionate previously was considered

atypical. These results then suggest that β-oxidation of hexanoate to acetate was the only metabolic pathway for volatile fatty acid degradation occuring in this system.

Although acetate was shown earlier to be the major carbon and energy surce of the component methanogens this intermediate accumulated in the vessels of the 5-stage array which suggested that acetogenic activity was proceeding at a rate in excess of methanogenesis. In this experiment acetate again accumulated in concentrations of 4.0 mM in Vessel A, 6.39 mM in Vessel B and finally 4.5 mM in Vessel C. From these results it would appear that like butyrate, acetate dissimilation was restricted and two possible factors may be implicated : the concentration of the influent sulphate; and the dilution rate. In Vessel B the increase in acetate concentration was most likely due to the further dissimilation of hexanoate which was recorded in this vessel. In the absence of acetate catabolism, the dissimilation of 5 mM hexanoate would have resulted in the production of 15 mM acetate. However, since only 6.39 mM acetate was detected it would appear that the acetate was catabolised to the most likely product, methane. The decrease in acetate concentration from Vessels B to C was indicative of acetate utilisation although the fate of this intermediate was not immediately obvious from the results.

The dynamics of V.F.A. metabolism are clearly seen in Figure 27 which was taken directly from the G.L.C. trace. As hexanoate was dissimilated, acetate and butyrate were formed and initially both

Figure 27: Volatile fatty acid dynamics of the microbial association cultivated in a three-stage chemostat model in the presence of hexanoic acid (5 mM) and sulphate (1.4 mM).


HEX
BUT
ACET
C
B
A
Vessel

increased in concentration down the array. After complete dissimilation of the substrate in Vessel C, however, the concentration of both butyrate and acetate decreased, thus it would appear that hexanoate dissimilation proceeded faster than subsequent metabolisms.

As discussed above, hexanoate catabolism was most likely linked to sulphate-reduction as a means of disposing of excess electrons in Vessel A since more than 88% of the influent sulphate was used in this stage (Figure 28) compared with 86.4% in the five-stage array. The sulphate concentration thereafter remained approximately the same and concentrations of 0.166 and 0.29 mM were recorded for Vessels B and C respectively, which were again comparable to the trend found in the 5-stage system.

Concomitant with sulphate dissimilation, gaseous hydrogen sulphide concentrations of 0.146, 0.076, and 0.037 mM and total HS^- concentrations of 2.11, 1.44 and 0.96 mM were recorded in Vessels A to C respectively which suggested that sulphate reduction was maximum in Vessel A.

The sulphur balance (Table 12A) accounted for 107.7% of the initial sulphur as residual sulphate and gaseous and total hydrogen sulphide. Table 12B shows the fluctuations in the sulphur balances in each of the vessels. In Vessel A sulphur was present in excess, mainly as total sulphide, although in subsequent vessels the concentrations of sulphide decreased although these were not accompanied by reciprocal increases in gaseous H_2S. The

Figure 28: Changes in residual hexanoic acid (Δ—Δ), and sulphate (○—○), hydrogen sulphide (●—●), total sulphide (◐—◐), methane (□—□), pH (■—■) and optical density (600 nm) (▼—▼) during the three-vessel open culture of the interacting microbial association in the presence of hexanoic acid (5 mM) and sulphate (1.4 mM).

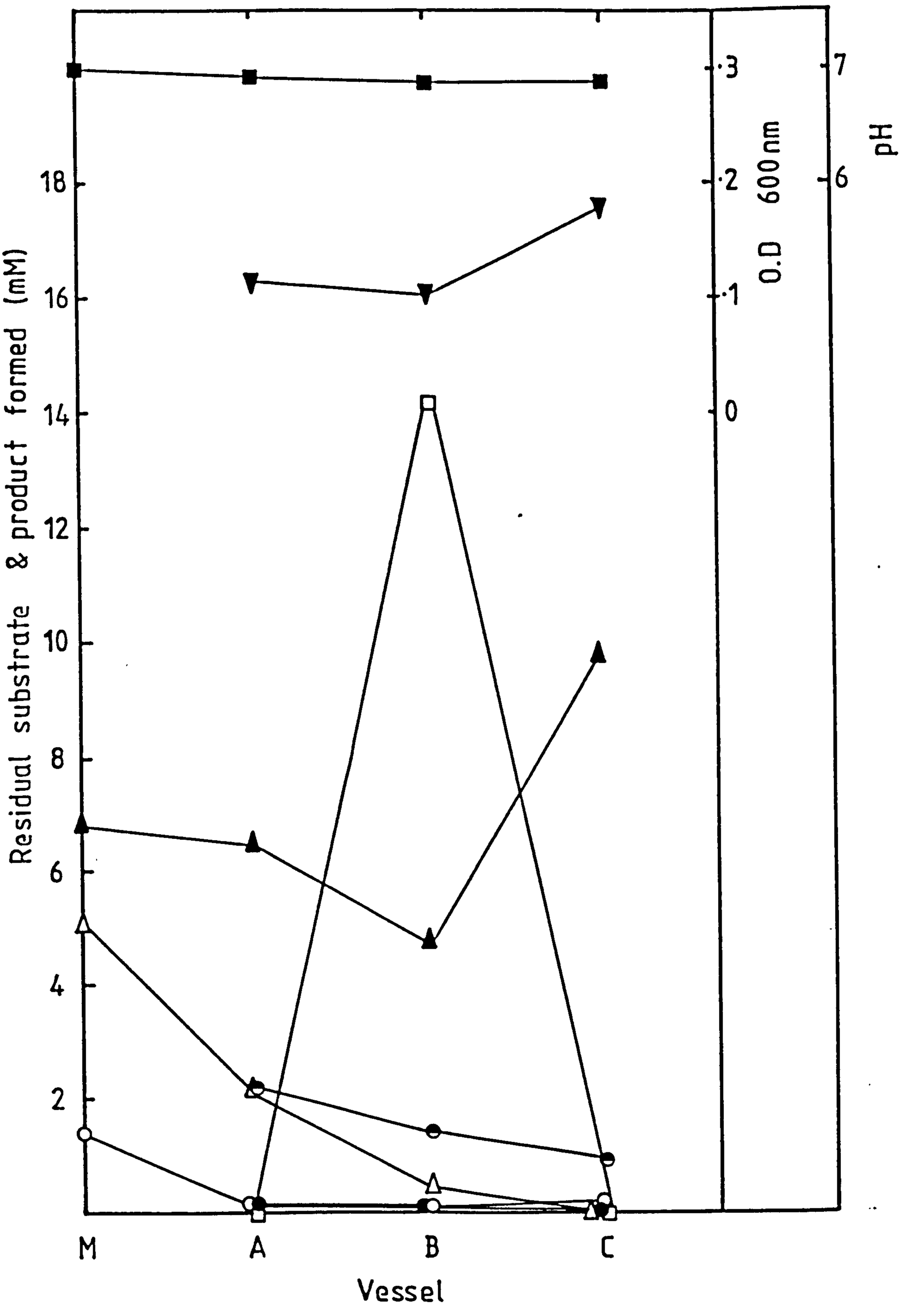

Residual substrate & product formed (mM)
18
16
14
12
10
8
6
4
2
O.D 600nm
·3
·2
·1
0
pH
7
6
M
A
B
C
Vessel

Table 12A: Total sulphur balance (mM 'S) for the 3-stage system with initial sulphate at 1.4 mM.

Table 12B: Balance of sulphate removal and formation of sulphide (mM 'S') after the mode of Thompson *et al.* (1984).

$$\text{Input sulphate} - (\text{Residual sulphate} + \text{total } H_2S + HS^-)$$

$$1.4 - (0.29 + 0.259 + 0.96)$$

$$= 1.509$$

$$\text{Sulphur accounted for} = \frac{1.509}{1.4} \times 100 = 107.7$$

Vessel	SO_4	H_2S	HS^-	Total
A	-1.21	+0.146	+2.11	1.046
B	-0.024	+0.076	-0.67	-0.618
C	+0.124	+0.037	-0.48	-0.319
Total	-1.11	+0.259	+0.96	+0.109

possibility, therefore, existed that the sulpide could have been assimilated to cellular material, as, for example, methanogens have an obligate requirement for sulphide as a source of sulphur (Ahring & Westermann, 1985).

The pH of the system remained relatively constant throughout with a drop of 0.2 units from the influent medium to Vessel C. This was in marked contrast to the earlier experiment where the pH rose to a final value of 8.3 and was attributed to active sulphate-reduction (Postgate, 1979). In this case since comparable sulphate-reduction was recorded then a possible explanation is difficult to tender although the expected rise in pH could have been negated by the presence of residual hexanoate in Vessel A.

In contrast with sulphate-reduction methanogenesis was not significant in Vessel A since the methane concentration detected was 0.036 mM which then substantially increased to 14.2 mM in Vessel B before decreasing to 0.013 mM in Vessel C. The results gave clear evidence that sulphate-reduction and methanogenesis were essentially, but not toally, spatially separated between Vessels A and B.

Carbon dioxide was measured in eachof the vessels at concentrations of 6.52, 4.78 and 9.5 mM for Vessels A to C respectively.

A carbon balance was developed (Table 13A) which showed that 94.6% of the influent carbon was accounted for, with 40.7% of final carbon as methane, 27.2% as inorganic carbon (CO_2) and the remainder

Table 13A: Carbon balance for dissimilation of hexanoic acid by the interacting microbial association cultured in a 3-vessel chemostat model in the presence of 1.4 mM sulphate.

Table 13B: Balance of removal of carbon and formation of CH_4 and CO_2 (mM 'C'), after the mode of Thompson *et al.* (1984).

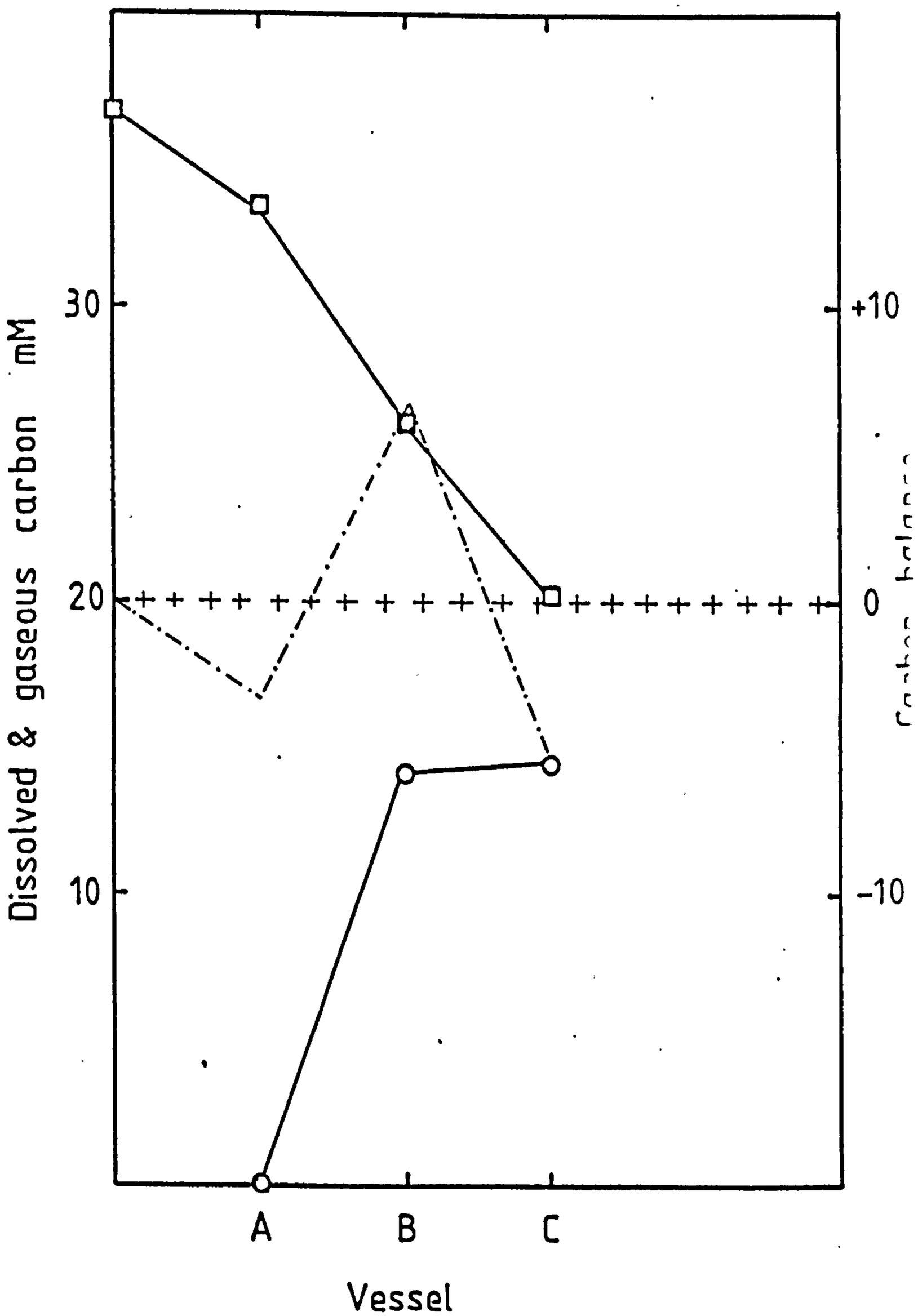
Input to Vessel A		Output from Vessel C + Total gaseous products	
Organic carbon	30	Methane	14.2
Inorganic carbon	6.8	CO_2	b.d.
		Inorganic carbon	9.5
		Organic carbon	11.14
Total	36.8	**Total**	34.84

Carbon accounted for 34.84 / 36.8 x 100 = 94.6%

	Inorganic 'C'	Organic 'C'	CH_4	Difference
A	-0.28	-3.0	+0.036	-3.244
B	-1.74	05.52	+14.2	+6.94
C	+4.72	-10.34	+0.013	-5.607
Total	+2.709	-18.86	+14.249	-1.902

Figure 29: Changes in concentrations of dissolved (□—□) and gaseous (○—○) carbon during the catabolism of hexanoate (5 mM) by the isolated microbial association in a three-stage chemostat model with an influent sulphate concentration of 1.4 mM. Dashed line represents the carbon accounted for.

Text cut off in original


30
20
10
+10
0
−10
Dissolved & gaseous carbon mM
A
B
C
Vessel

as organic carbon (volatile fatty acid intermediates). Table 13B shows the balance of removal and production of carbon in each of the vessels and Figure 29 shows the balance of carbon for the whole system. In Vessel A, more carbon was dissimilated than was accounted for by methane production. Thus it is most likely that the excess carbon was used for cell biomass. This, conversely, probably added to the influent carbon of Vessel B and as a consequence excess methane was generated. In Vessel C there was again a deficit in the carbon balance, although in this instance the reason was not readily apparent.

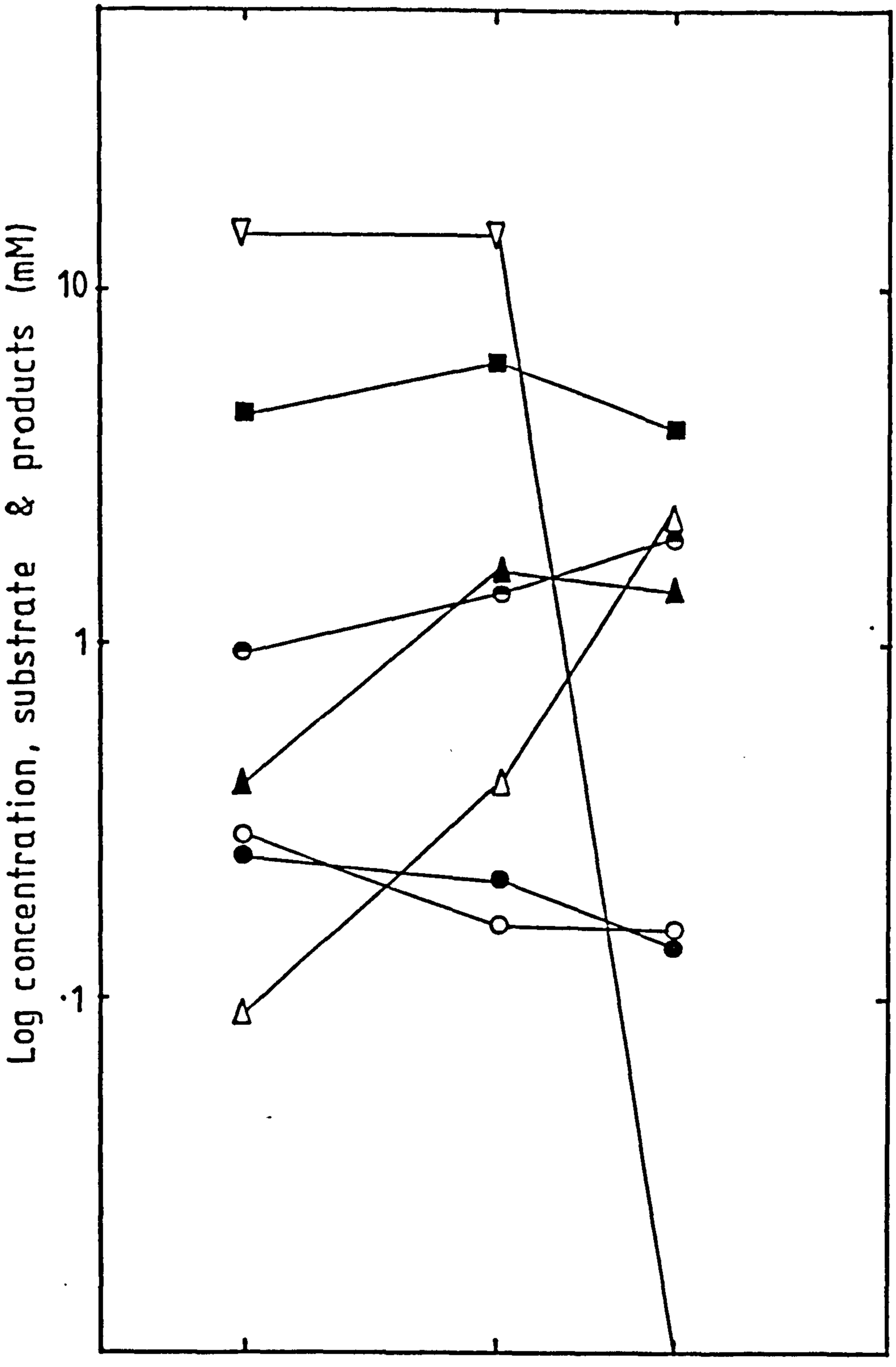
3.7 Kinetic interpretation and spatial separation

Spatial separation of the sulphate-reducing bacteria and methanogens was not apparent in the 5-vessel model at a concentration of 1.4 mM sulphate, thus it would appear that separation was effected purely due to growth kinetics. Since the methanogens could not compete successfully in the first vessel, at the imposed dilution rate of 0.05 h^{-1}, they were consequently displaced down the array. The second vessel of the three-stage system, however, was maintained at a dilution rate of 0.015 h^{-1}, which was between Vessel A and Vessel B of the 5-stage model in which maximum methanogenesis was also detected. From these results it would appear that the D_{crit} for the methanogens was < 0.05 and > 0.015 h^{-1}.

Sulphate-reduction was most active in the first vessel in the presence of active hexanoate catabolism. Thus it would appear that

Figure 30: Concentrations of hexanoic acid (Δ—Δ), butyrate (▲—▲), acetate (■—■), methane (▽—▽), sulphate (○—○), sulphide (◐—◐) and hydrogen sulphide (●—●) plotted against cumulative dilution rates during the 3-stage open culture cultivation of the isolated microbial association in the presence of hexanoic acid (5 mM) and sulphate (1.4 mM).

The key at the bottom of the figure denotes the individual vessel or combination of vessels which corresponded with each discrete dilution rate.


Log concentration, substrate & products (mM)
10
1
·1
·00625
·015
·05
D (h⁻¹)
C
B
A

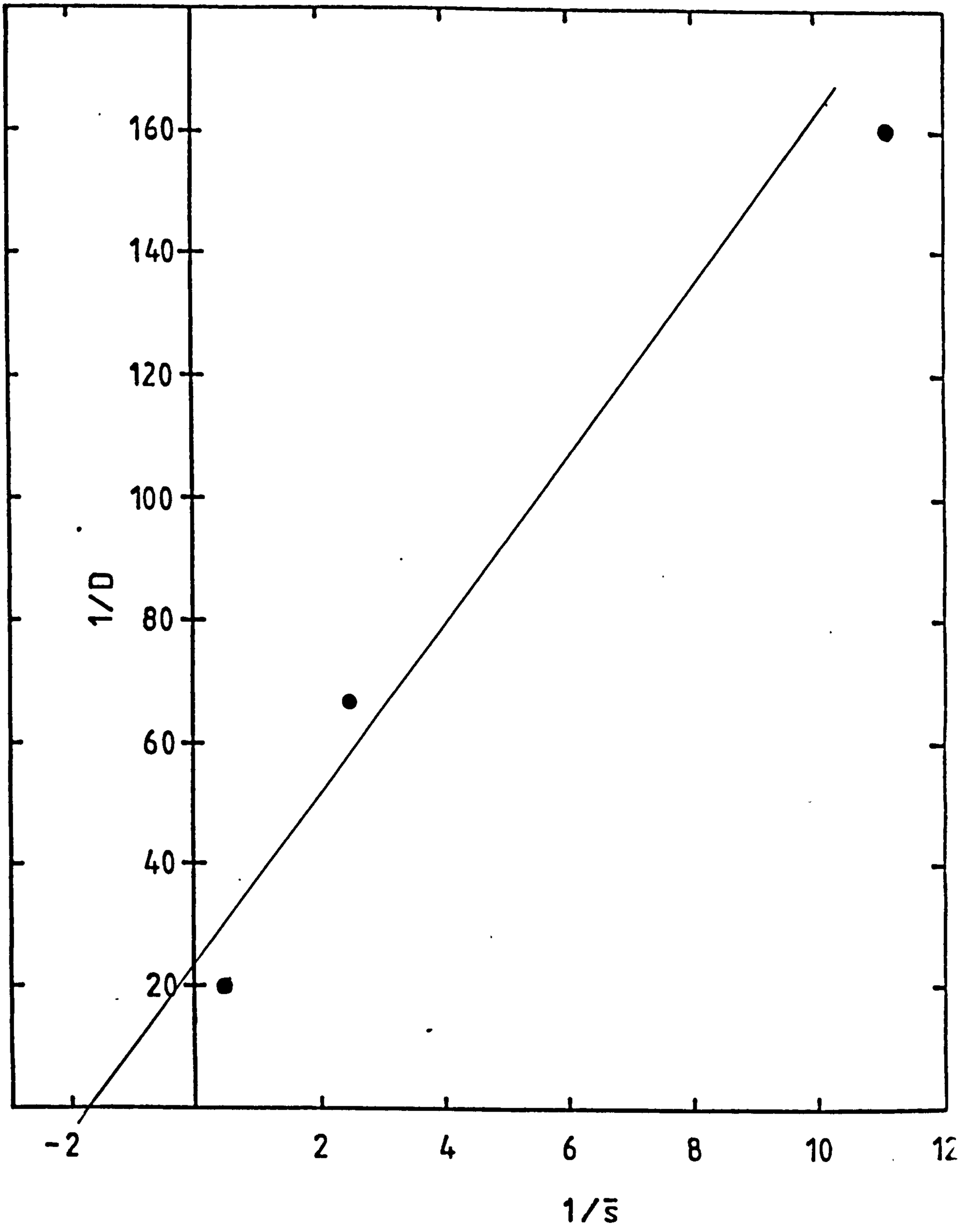
these species both had a $\mu_{max} > 0.05\ h^{-1}$. This is perhaps not surprising since sulphate-reducing bacteria are known to have a greater affinity for the common terminal products than methanogens (Pfennig, 1984) and hence should have been able to grow in open culture at higher dilution rates.

The differences in the maximum specific growth rates of the sulphate-reducing bacteria and the methanogens are exemplified in Figure 30 where the concentrations of residual substrates, intermediates and products are plotted against cumulative dilution rates.

Comparison of these results with those of Figure 21 shows that hexanoate was gradually catabolised in the three-vessel system whereas previously hexanoate was completely dissimilated in Vessel B at a dilution rate of $0.0125\ h^{-1}$. For methane, on the other hand, although initial discrepancies were apparent, the overall pattern was considered to be approximately equivalent since as in the three-stage system, the gas was first detected at a dilution rate of 0.015 h^{-1} which corresponded to the first vessel of the 5-stage system maintained at a dilution rate of $0.025\ h^{-1}$.

The saturation constant of the hexanoate catabolising co-culture was determined by plotting experimental values of 1/D against 1/S in the mode of Lineweaver & Burk (1934) when D was the dilution rate and S was the concentration of residual substrate (hexanoate). The lines were fitted by regression analysis and the abscissa intercept gave the -1/Ks. Figure 31 shows the plot derived

Figure 31: 1/D plotted against 1/S in the mode of Lineweaver and Burk (1954) where D is the dilution rate and S the concentration of residual substrate (hexanoate) for the 3-stage system as detailed in the text.


160
140
120
100
80
60
40
20
1/D
-2
2
4
6
8
10
12
1/s

from the hexanoate concentration data when the analysis yielded a line with a correlation coefficient of 0.989 and an abscissa intercept of -1.01 mM, which was equivalent to a Ks value of 0.557 mM.

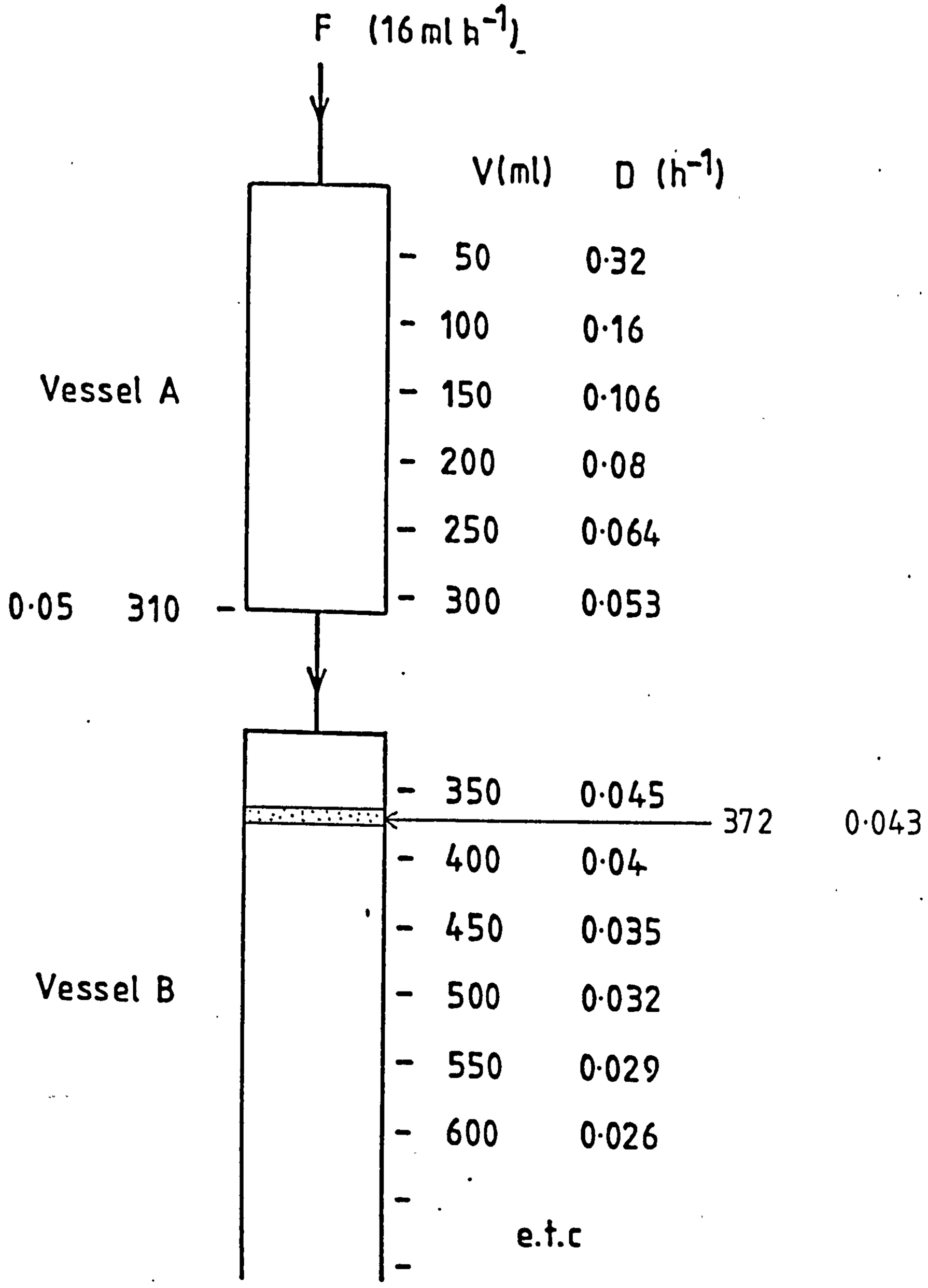
The saturation constant is a measure of the affinity the organism has for the growth-limiting substrate (hexanoate) (although in this instance, it was not a single species but a co-culture), that is the lower the K_s then the higher the affinity and the greater the capacity to grow rapidly in an environment with low growth-limiting substrate concentrations. The saturation constant is defined as that growth-limiting substrate concentration which allows the organism to grow at half the maximum specific growth rate. The maximum specific growth rate can also be estimated from Figure 31, as the intercept with the y-axis (22.9 h) is $1/\mu_{max}$ with the μ_{max} therefore 0.043 h^{-1}.

The three-stage array was not designed to be a homogeneous system, and the results show that this indeed was not the case, as the u_{max} of cultures in homogeneously-stirred chemostats is equivalent to the critical dilution rate.

The three-stage system approximated to a plug flow reactor of total volume 2610 ml, hence the lower the position in the system, the lower the dilution rate.

Figure 32 shows the first two stages of the three-stage system as it would have operated as a single plug-flow reactor. From the Lineweaver Burk plot the μ_{max} of the hexanoate-catabolising species

Figure 32: Diagram of the 3 stage system operating under plug flow conditions with cumulative volumes and their respective dilution rates shown. ▭ represents the μ_{max} (0.043 h^{-1}) of the hexanoate-catabolising species.


F (16 ml h⁻¹)
V(ml)
D (h⁻¹)
Vessel A
- 50 0·32
- 100 0·16
- 150 0·106
- 200 0·08
- 250 0·064
0·05 310 - 300 0·053
- 350 0·045
372 0·043
- 400 0·04
- 450 0·035
Vessel B
- 500 0·032
- 550 0·029
- 600 0·026
-
e.t.c
-

was 0.043 h^{-1} which was equivalent to a volume of 372.0 ml in Vessel A. Hence, one could envisage that in an ideal system the hexanoate-catabolising species would have occupied a discrete layer (stippled) as shown in Figure 32.

The results, however, (Figure 27) clearly demonstrated that hexanoate dissimilation proceeded in all three vessels and was not confined just to the second vessel, hence it would appear that some of the population of hexanoate catabolisers were growing above their maximum specific growth rate.

It was apparent from the results that the culture in Vessel A was sulphate limited, although depletion of sulphate did not cause hexanoate metabolism to cease since reduction of carbon dioxide was then used as a sink for the excess electrons produced from the oxidation of hexanoate. Therefore a shift in the kinetic properties of the microbial association would probably have been expected.

3.8 Summary

1. Multi-stage chemostats proved useful in investigating interspecies interactions within the microbial association (isolated from landfill) which catabolised hexanoic acid to methane.

2. The microbial association isolated in the multi-stage chemostat consisted of a 'tight' association of microorganisms, which could only be spatially separated when either the sulphate concentration was raised to 10 mM or the dilution rate of the

first vessel was increased to that obtained in the equivalent vessel of the three vessel system.

3. With respect to these two variables, 10 mM sulphate could be considered atypical of landfill (although transient high concentrations have been recorded). On the other hand non-constant dilution rate, and low sulphate, are more typical and could realistically be extrapolated to the landfill ecosystem.

4. The results suggested that the interacting microbial association was composed of 4 groups of organisms, hexanoate/butyrate catbolisers (H_2-producing acetogens), sulphate-reducers (H_2-oxidising, sulphate reducing), methanogens (H_2-oxidising, carbon dioxide reducing, and acetate catabolising) and homo-acetogens (acetate producing, H_2 reducing), although the exact composition was not fully resolved.

4. The anaerobic catabolism of hexanoate and its putative breakdown products by the interacting microbial association isolated in the multi-stage chemostat

The anaerobic catabolism of hexanoate can be mediated by two groups of bacteria : (1) obligate syntrophic co-cultures; and (2) sulphate-reducing bacteria (Banat & Nedwell, 1983).

In the multi-stage chemostat experiments described in Chapter 3, the former mechanism of catabolism of hexanoate was thought to be operative and as a consequence a series of batch culture experiments was devised to substantiate this finding.

Batch culture conditions were specifically chosen to enable initial growth parameters to be controlled.

Two key areas were studied : (1) Metabolism of hexanoate and its putative breakdown products by the isolated microbial association; and more specifically, (2) Methanogenesis from acetate.

The following experiments were undertaken :

In group (1):

i) Hexanoate catabolism by the isolated microbial association;

ii) Effect of sulphate on butyrate, acetate and H_2/CO_2 catabolism by the isolated microbial association;

iii) Catabolism of propionate;

iv) Confirmation of β-oxidation of volatile fatty acids;

v) Effect of metabolite inhibitors on methanogenesis by the microbial association; and

vi) Methanogenesis from labelled substrates.

In group (2) :

i) Methanogenesis from labelled acetate;

ii) Kinetics of acetate catabolism to methane; and

iii) Effect of H_2 on methanogenesis from acetate.

4.1 Hexanoate catabolism by the isolated microbial association

700 ml aliquots of basic mineral salts medium (A) supplemented with hexanoic acid (5 mM) were aseptically dispensed into sterile bottles to give a headspace : culture volume ratio of 2.5:1. The bottles were closed with Suba Seals and overgassed with O.F.N. prior to inoculation with 100 ml of culture from the first vessel of the 5-vessel multi-stage chemostat. The bottles were incubated stationary in the dark at 30°C. Headspace, gas samples were removed at regular intervals for methane analysis and the culture supernatant was assayed for volatile fatty acids, optical density (470 nm) and pH.

From Figure 33 it can be seen that the residual hexanoic acid concentration decreased steadily from time 0 to day 6 without a noticeable lag phase at a rate of 0.615 μ mol $ml^{-1}d^{-1}$ after which it was no longer detected in the culture supernatant. Hexanoic acid dissimilation was accompanied by production of butyrate and acetate. The butyrate concentration increased to a maximum of 1.475 μ mol ml^{-1} at day 5 and then sharply decreased and was not detected after 9 days. As butyrate did not reach the theoretical maximum concentration of 5 μ mol ml^{-1} it would appear that the intermediate produced by β-oxidation of hexanoate was utilised simultaneously and

Figure 33: Changes in concentrations of residual substrate (□ - □), butyrate (O - O) and acetate (Δ - Δ) and pH (● - ●), O.D. (▼ - ▼) and methane (■ - ■) during the closed culture cultivation at 30°C of the isolated microbial association in the presence of hexanoate.

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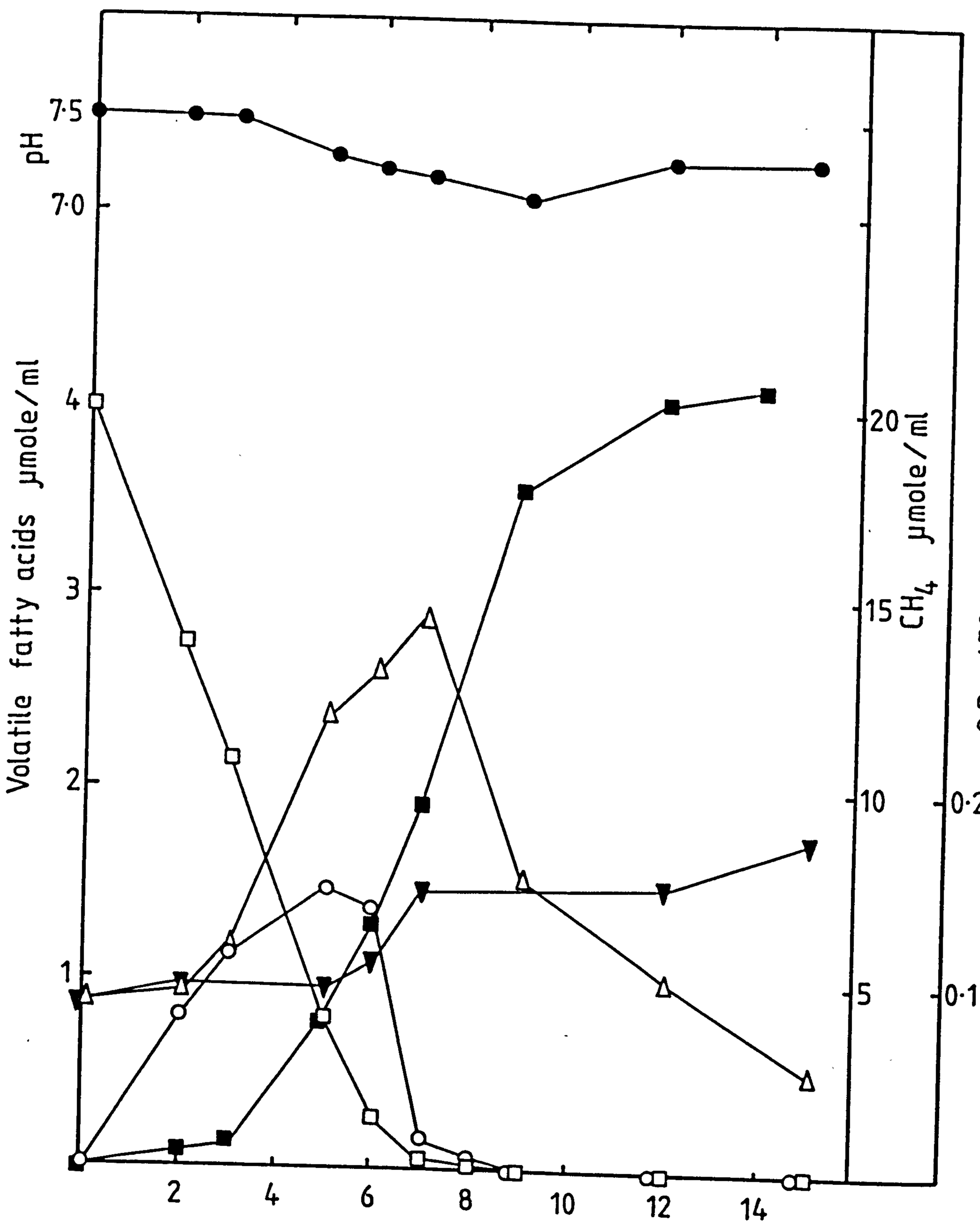

pH
7·5
7·0
Volatile fatty acids µmole/ml
4
3
2
1
CH_4 µmole/ml
20
15
10
5
0·2
0·1
2
4
6
8
10
12
14
Time (d)

not sequentially. The acetate concentration increased to a maximum of 2.9 μ mol ml^{-1} by day 7 after which it decreased, apparently at two rates. Between days 7 and 9 the rate was 0.68 μ mol ml^{-1} d^{-1} which then slowed to 0.16 μ mol $ml^{-1}d^{-1}$ between days 9 and 15, which corresponded to the log and stationary phases respectively of methane generation.

From the results it would appear that acetogenesis was relatively insignificant after day 7 since butyrate was almost completely dissimilated.

Methane production, however, by day 7 had only reached 46.5% of its final value.

Methane production increased steadily after a lag phase of 4 days at a rate of 3.5 μ mol $ml^{-1}d^{-1}$ to reach a maximum concentration of 20.7 μ mol ml^{-1} at the end of the experiment (15 days). From the results it would appear that methane production was most likely linked to acetate utilisation as the decrease in acetate catabolism recorded at day 9 corresponded with a decrease in methane production. The terminal concentration of 20.7 μ mol ml^{-1} correlated well with the theoretical concentration of 20 μ mol ml^{-1}, for the dissimilation of 5mM hexanoate, of which 5 mM originated from the H_2 and 15 mM from the acetate produced from β-oxidation of the molecule.

Concomitant with methane production was an increase in the optical density of the culture from 0.086 to 0.177. Hence it would seem that as expected there was very little increase in biomass.

These results did not give a typical batch growth curve as the lag phase was lengthy (5 days), particularly when one considers the size of the inoculum, and the logarithmic phase short (3 days) although the latter did correspond to the logarithmic phase of methane production.

Accompanying methane production was a rise in pH from 7.1 at day 9 to 7.3 by the end of the experiment. This, rather than an artefact of CH_4 production was probably due to the utilisation of acetate, the presence of which, as already discussed, would probably have caused the pH to decrease.

Hexanoate degradation has been reported previously by a number of workers incuding McInerney et al. (1981) and Stieb & Schink (1985). In both of these studies, however, a co-culture was used which contained a primary proton-reducing acetogenic organism together with a H_2-utilising species (either a methanogen or a sulphate-reducing bacterium). In both of these associations a characteristic feature, which was not repeated here, was the accumulation of acetate.

The actual metabolism of acetate by the interacting microbial association is further discussed in detail in Section 4.7 .

In conclusion it was apparent that hexanoate was completely degraded (probably by β-oxidation) to CH_4 by members of the microbial association and that at least 2 metabolically distinct groups were present : the hexanoate / butyrate degrader; and a methanogen.

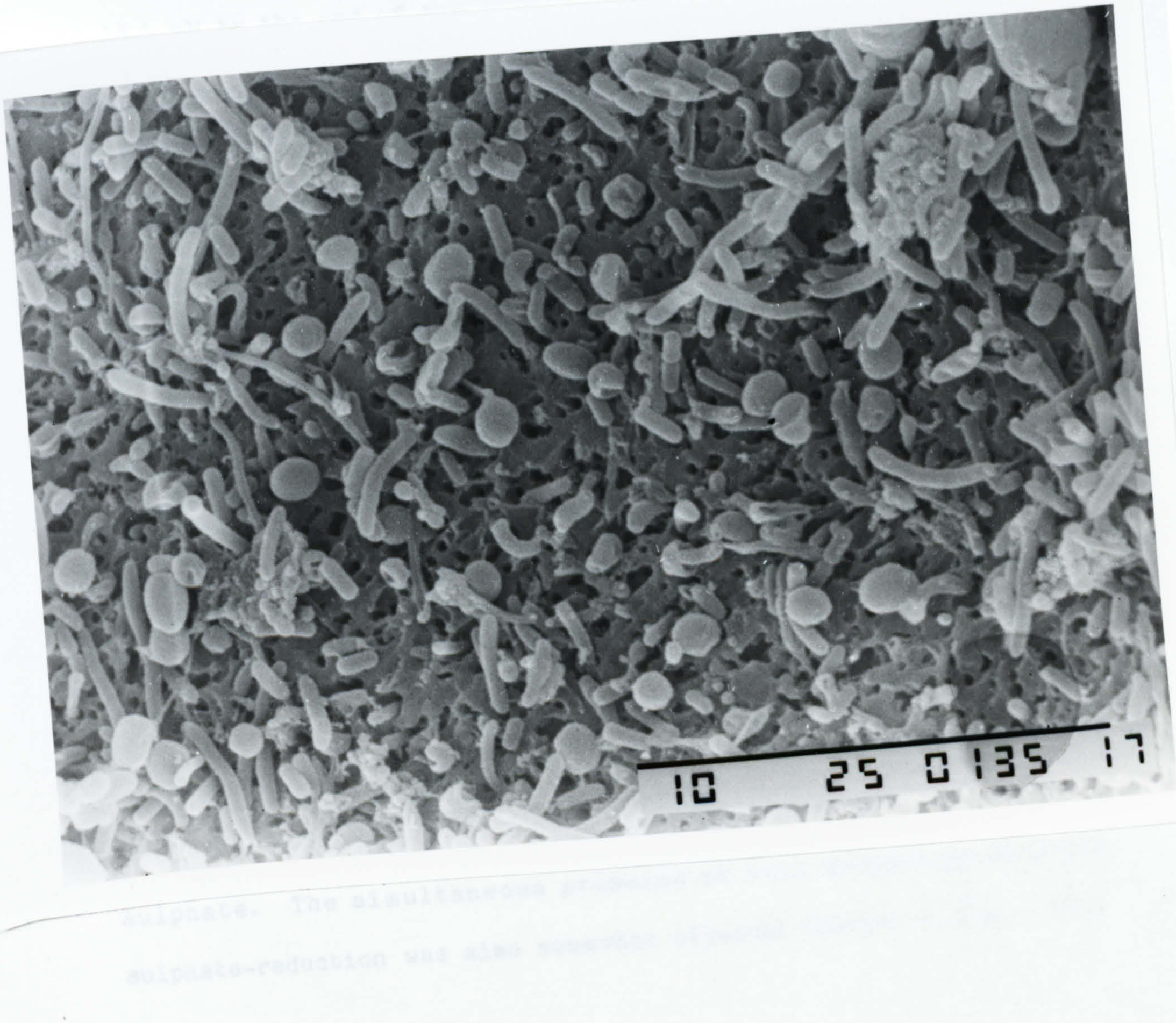
Microscopic examination of the culture: Examination of the culture under the light microscope was unable to resolve the exact composition of the microbial association. Most of the bacteria were Gram -ve rods in chains with a few Gram +ve cocci also present. Samples of the culture were then examined by scanning electron microscopy. Plate 2 shows one area of the filtered sample magnified 5000 times. The culture was seen to consist of a heterogeneous assemblage of bacteria of various morphological types : (1) large coccoid; (2) large curved rod; (3) small rod; (4) vibriod rod; and (5) thin filaments.

4.2 Effect of sulphate on butyrate, acetate and H_2/CO_2 catabolism by the isolated microbial association

Flasks were prepared in which 50 ml aliquots of basic mineral salts medium (B) were supplemented individually with butyrate (10 mM), acetate (16 mM), or H_2 CO_2 as the electron donors. With H_2/CO_2 as the carbon source, the flasks were overgassed with H_2 CO_2 (80:20) (British Oxygen Company).

In closed culture the headspace : culture volume ratio was set at 1.6:1 and the inoculum volume was 3 ml (6% v/v). The flasks were incubated stationary in the dark at 30°C and the gas and liquid phases were analysed at regular intervals for methane and V.F.A. and sulphate respectively.

Plate 2: Electron micrograph of the isolated microbial association metabolising hexanoate (5mM).

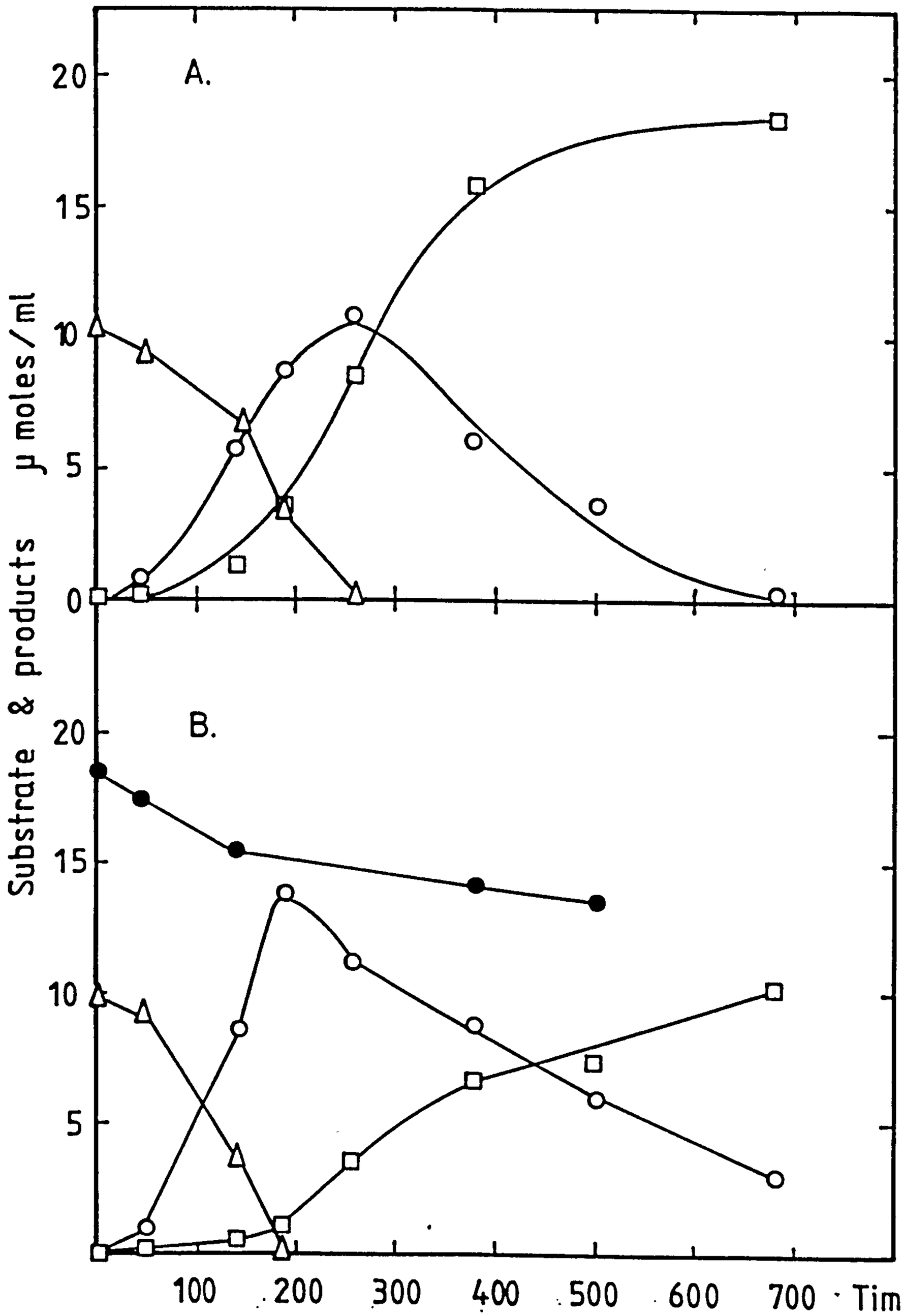

10 25 0135 17

From Figure 34 A it can be seen that in the absence of sulphate butyrate was completely dissimilated after 268 h cultivation. Concomitantly, acetate was produced and reached a peak at 268 h, after which it was utilised and not detected at the end of the experiment. Significant CH_4 production was detected only after 45 h of cultivation, and this then progressively increased to a maximum of 18.6 mM by the end of the experiment.

In the presence of excess sulphate, 18.5 mM (Figure 34 B), butyrate was once again completely dissimilated and acetate formed as an intermediate prior to CH_4 production. Significant changes were, however, apparent in the stoichiometry of the reaction since increased rates of butyrate dissimilation and acetate formation were accompanied by a decrease of 8.3 mM in the terminal CH_4 concentration. This reduction was coincident with a residual sulphate concentration decrease of 5 mM.

In the presence of excess sulphate, such as is found in marine sediments, sulphate-reducing bacteria have been shown by Balba & Nedwell (1982) to metabolise butyrate. However, it would appear that the component sulphate-reducing species in the microbial association, originally isolated from landfill, were unable to catabolise the complete reaction, even in the presence of excess sulphate. The simultaneous presence of both methanogenesis and sulphate-reduction was also somewhat atypical (Lovley & Klug, 1983).

Figure 34: Changes in residual butyrate (Δ - Δ), sulphate (● - ●), acetate (O - O), and methane concentrations (□ - □) during the closed culture cultivation of the isolated microbial association in the absence (A) and presence (B) of sulphate.


A.
B.
Substrate & products μ moles/ml
0
5
10
15
20
100
200
300
400
500
600
700
Time (h)

Dissimilation of butyrate in the absence of O_2 is thermodynamically unfavourable :

$$CH_3\ CH_2\ CH_2\ COO^- + 2\ H_2O \text{ ---- } 2\ CH_3\ COO^- + H^+ + H_2$$

$$\Delta\ G_o^1 = +4.81\ kJ\ mol^{-1} \quad (29)$$

For the reaction to proceed the partial pressure of H_2 must be maintained at a concentration of 0.8 atm or less (McInerney et al., 1981b) which may be facilitated by the presence of a H_2-utilising species. For example, degradation of volatile fatty acids (including butyrate) by an obligate syntrophic association of a H_2-producing acetogen and either a H_2-utilising methanogen or a sulphate-reducing bacterium was first reported by McInerney et al. (1979) who described the reactions as follows :

$$2\ CH_3\ CH_2\ CH_2\ COO^- + HCO_3^- + H_2O \text{ ---- } 4\ CH_3\ COO^- + H^+ + CH_4$$

$$\Delta\ G_o^1 = -39.4\ kJ\ mol^{-1} \quad (30)$$

$$2\ CH_3\ CH_2\ CH_2\ COO^- + SO_4^{2-} \text{ ---- } 4\ CH_3COO^- + H^+ + H\ S^-$$

$$\Delta\ G_o^1 = -55.7\ kJ\ mol^{-1} \quad (31)$$

From the results of this experiment it would appear that the mechanisms operative within the isolated microbial association were similar to those of the obligate syntrophic association described above. However, the results presented here differ in two major aspects to those of McInerney et al. (1979). Firstly, a microbial association was used and not a defined co-culture, and secondly, the acetate intermediate was subsequently catabolised and did not accumulate.

Although the removal of H_2 to facilitate the butyrate reaction

thermodynamically feasible is now well documented (Wolin, 1976) the possible role of acetate in butyrate dissimilation is not fully resolved.

Stoichiometrically, the majority of CH_4 produced from butyrate originates via the acetate intermediate, and in the experiment described this would appear to have been the case. Removal of acetate by methanogens could possibly enhance butyrate degradation, similar to the removal of H_2. In addition, prevention of acetate accumulation would also facilitate pH maintenance near neutrality.

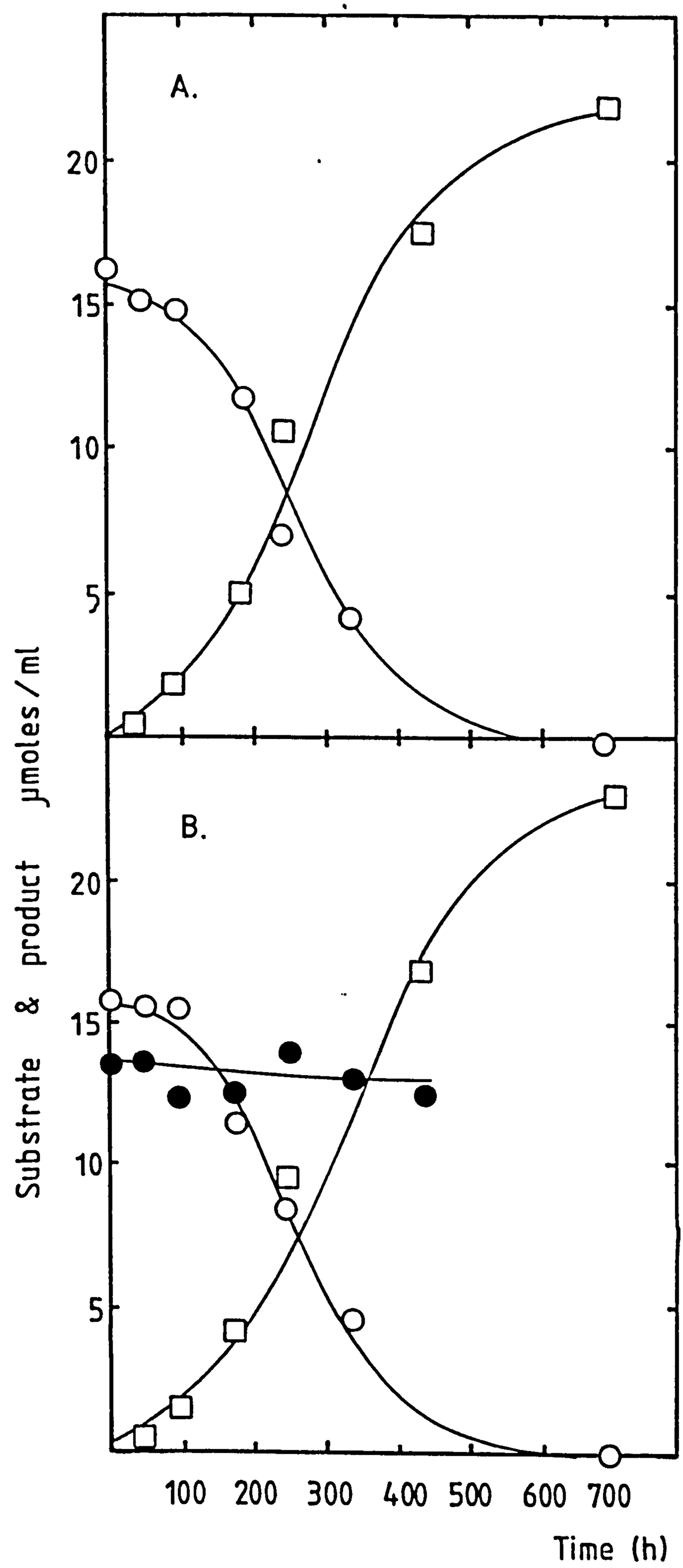
Confirmation of methane production from acetate by the microbial association was obtained, when it was found that methane was present in the headspace after 48 h cultivation in the absence of sulphate (Figure35 A). The methane concentration then progressively increased and reached a peak of 22.4 mM after 694 h cultivation, which was 7.4 mM greater than that predicted from theoretical grounds (15 mM).

Surprisingly, addition of sulphate (13.4 mM) did not appear to affect the reaction (Figure 35B) although the concentration of residual sulphate decreased slightly possibly due to assimilation by the methanogens as a source of sulphur.

Consideration of reported substrate affinities (k_s) for acetate would imply that in the presence of sulphate, acetate-utilising sulphate-reducers would outcompete methanogens for acetate.

In the experiment described, although sulphate was present in excess, only limited utilisation was recorded and methanogenesis was

Figure 35: Changes in residual acetate (O - O) and sulphate (● - ●) and methane concentrations (□ - □) during closed culture of the microbial association in the absence (A) and presence (B) of sulphate.

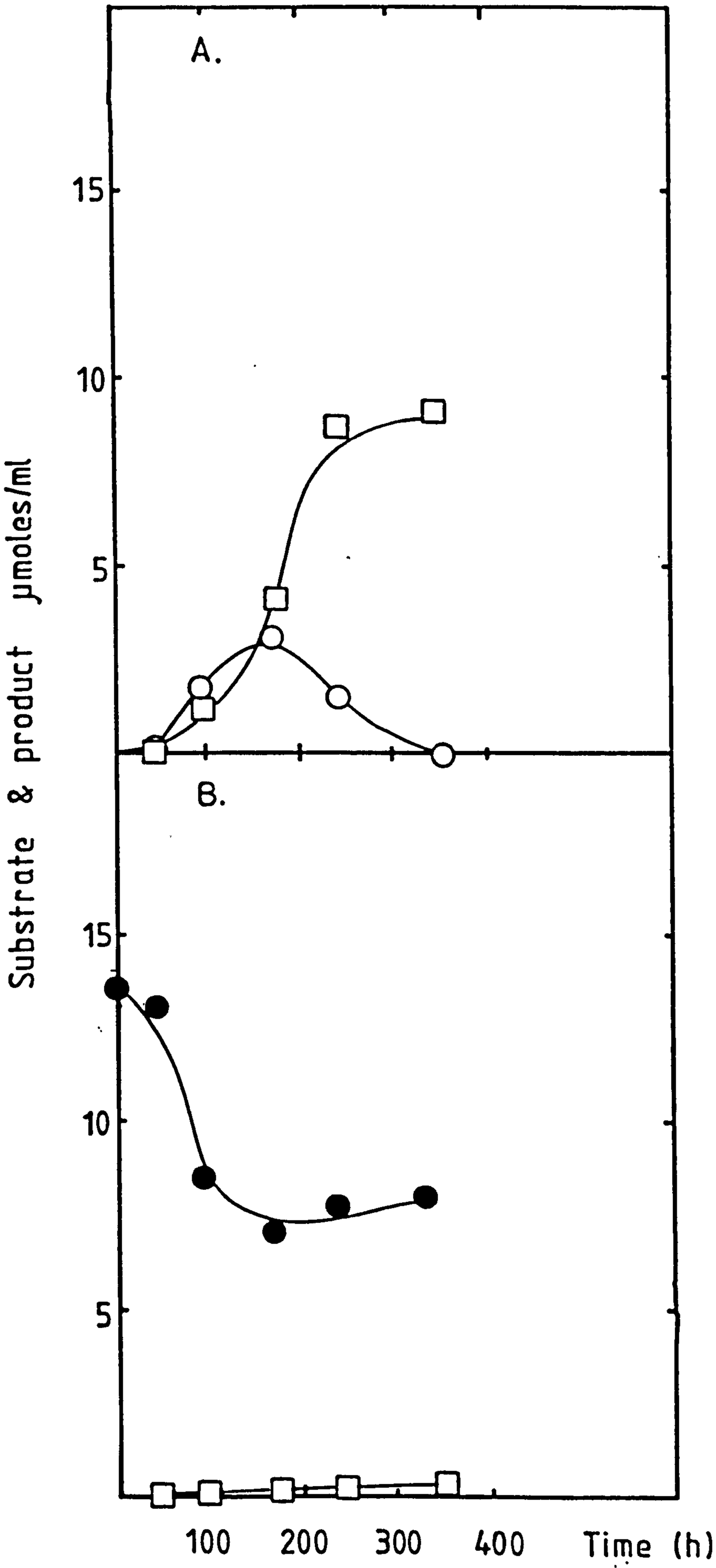

A.
B.
20
15
10
5
Substrate & product µmoles / ml
100
200
300
400
500
600
700
Time (h)

unaltered. Therefore, it would seem that in this microbial association, either the sulphate reducers did not utilise acetate or no sulphate-reducers were present. However, since sulphate-reduction was observed in the culture it would appear that the sulphate-reducers utilised a different substrate to the acetate-utilising methanogens.

Examination of CH_4 generation from H_2/CO_2 (Figure 36A) showed that in the absence of sulphate both methane and acetate were produced. The acetate reached a peak of 3.1 mM after 178 h cultivation and was then utilised and no longer detected after 337 h. Methane production, however, continued to rise to a maximum of 8.9 mM by the end of the experiment. These results were indicative of both acetogenic and methanogenic activity. The interactions between homo-acetogens and methanogens are still unclear (Adamse, Velzeboer and Janssen, 1984) since methanogens have been reported to outcompete homo-acetogenic bacteria for H_2 in the gastro-intestinal tract (Prins & Lankhorst, 1977).

The results of Figure 36A were somewhat surprising and did not resolve the question of whether the methanogens and the homo-acetogens both utilised H_2/CO_2 equally or whether there was some form of competition. However, one possibility could be that homo-acetogens produced acetate from H_2/CO_2 exclusively and then the methanogenic population utilised the acetate to form methane. Thus, a symbiotic relationship could have been operative. If this was the case then this could represent a strategy for energy conservation in

Figure 36: Changes in concentration of acetate (O - O), sulphate (● - ●) and CH_4 (□ - □) during the closed culture cultivation at 30°C of the isolated microbial association in the presence of H_2/CO_2 in the gas phase in the absence (A) and presence (B) of sulphate.


A.
15
10
5
& product µmoles/ml
Substrate
B.
15
10
5
100
200
300
400
Time (h)

which excess electrons are converted into a further energy source, acetate, prior to subsequent utilisation.

Figure 36B shows the results of H_2/CO_2 utilisation in the presence of sulphate (14 mM) where methanogenesis (and acetogenesis) were largely inhibited as sulphate was reduced. Therefore it was apparent that when sulphate was present the sulphate reducers were the major H_2 utilisers.

From the results of the experiments it would appear that when butyrate was dissimilated the H_2 produced was utilised preferentially by sulphate-reducing bacteria, provided that exogenous sulphate was present, although in the absence of this, an interacting association of homo-acetogenic and methanogenic bacteria was responsible (Figure 37). Acetate, however, was metabolised exclusively by the methanogenic bacteria.

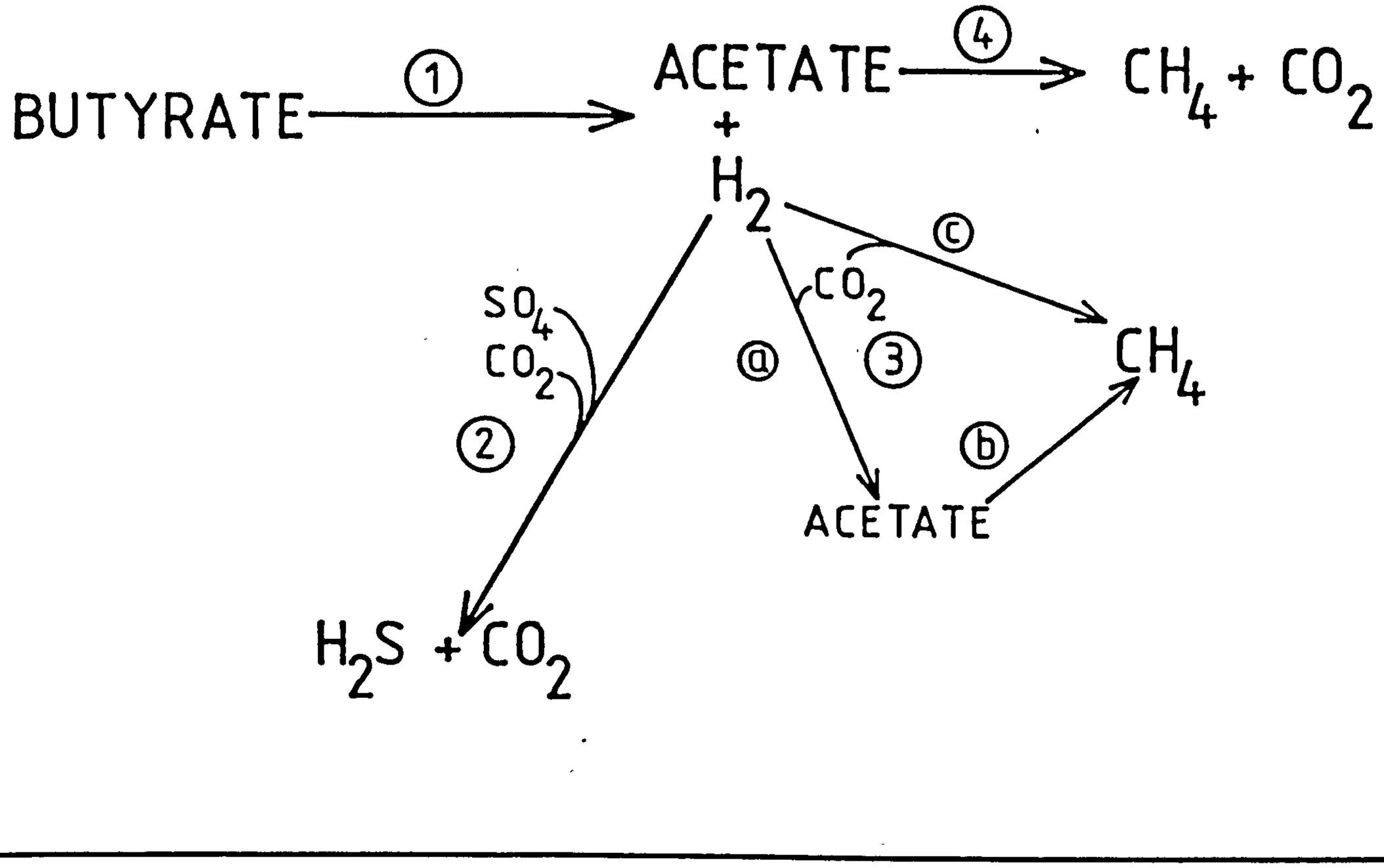
4.3 Catabolism of propionate

When propionate (10 mM) was used as the substrate for batch culture experiments in the absence and presence of sulphate it was found that in both cases neither propionate nor sulphate were dissimilated and methane was not formed. It would, thus, appear that under the conditions of this experiment propionate was recalcitrant to degradation by the microbial association.

These results were somewhat surprising as propionate was found as an intermediate of hexanoate degradation by the microbial

Figure 37: Proposed anaerobic butyrate dissimilation pathway(s) in the absence and presence of sulphate. Responsible species:

1. H_2-producing acetogen;
2. H_2-oxidising sulphate-reducer;
3. a/ acetogenic species
   b/ acetate-oxidising methanogen.
   c/ H_2-oxidising methanogen; and
4. acetate-oxidising methanogen.


BUTYRATE
①
ACETATE
④
$CH_4 + CO_2$
+
H_2
ⓒ
SO_4
CO_2
②
@
CO_2
③
CH_4
ⓑ
ACETATE
$H_2S + CO_2$

association in the 5-stage chemostat and in the presence of sulphate, at concentrations of 1.4 and 5 mM, was subsequently degraded. As already discussed (3.2) this could have possibly been facilitated by the lower H_2 partial pressures due to over-gassing the individual chemostat vessels with OFN. Propionate has, however, been found to be degraded by microbial associations to acetate and methane in batch cultures (Boone & Bryant, 1980). In this report, however, the co-culture could not degrade even number volatile fatty acids. Conversely, it has been found that co-cultures capable of degrading even-number volatile fatty acids could not degrade propionate.

The fact that propionate was not degraded by the isolated microbial association was used as a marker for the next experiment in which methyl-substituted butyrate (methyl-butyrate and iso-valerate) were assessed as potential substrates. The object of this experiment was to investigate whether β-oxidation was the operative pathway of volatile fatty acid oxidation.

4.4 Confirmation of β-oxidation of volatile fatty acids

50 ml batch cultures were initiated with basic mineral salts medium (B) supplemented with 2-methyl butyrate (11 mM) or iso-valerate (11 mM) and the gas and liquid phases were sampled as before (4.3). It should be noted, however, that the G.L.C. method of volatile fatty acid analysis could not differentiate between the two substrates/molecules.

Figure 38 shows that the concentration of 2-methyl butyrate decreased slowly from 11.48 mM to 7.3 mM during the first 330 h of cultivation, and then further decreased to 0.91 mM by 546 h. Concomitant with this degradation, formation of propionate, acetate and methane was noted.

The concentration of propionate increased slowly to 1.12 mM by 330 h, after which it increased dramatically to reach a terminal concentration of 10.5 mM (546 h). Acetate formation followed a similar pattern with a concentration of 0.69 mM recorded after 330 h, after which a significant, although less dramatic, increase was apparent until a final concentration of 2.45 mM was recorded.

Production of methane by the culture, after a lag period of approximately 330 h, reached a concentration of 13.7 mM after 643 h cultivation at a rate of 0.96 μ mol ml^{-1} h^{-1} which was comparable to that found previously (4.3).

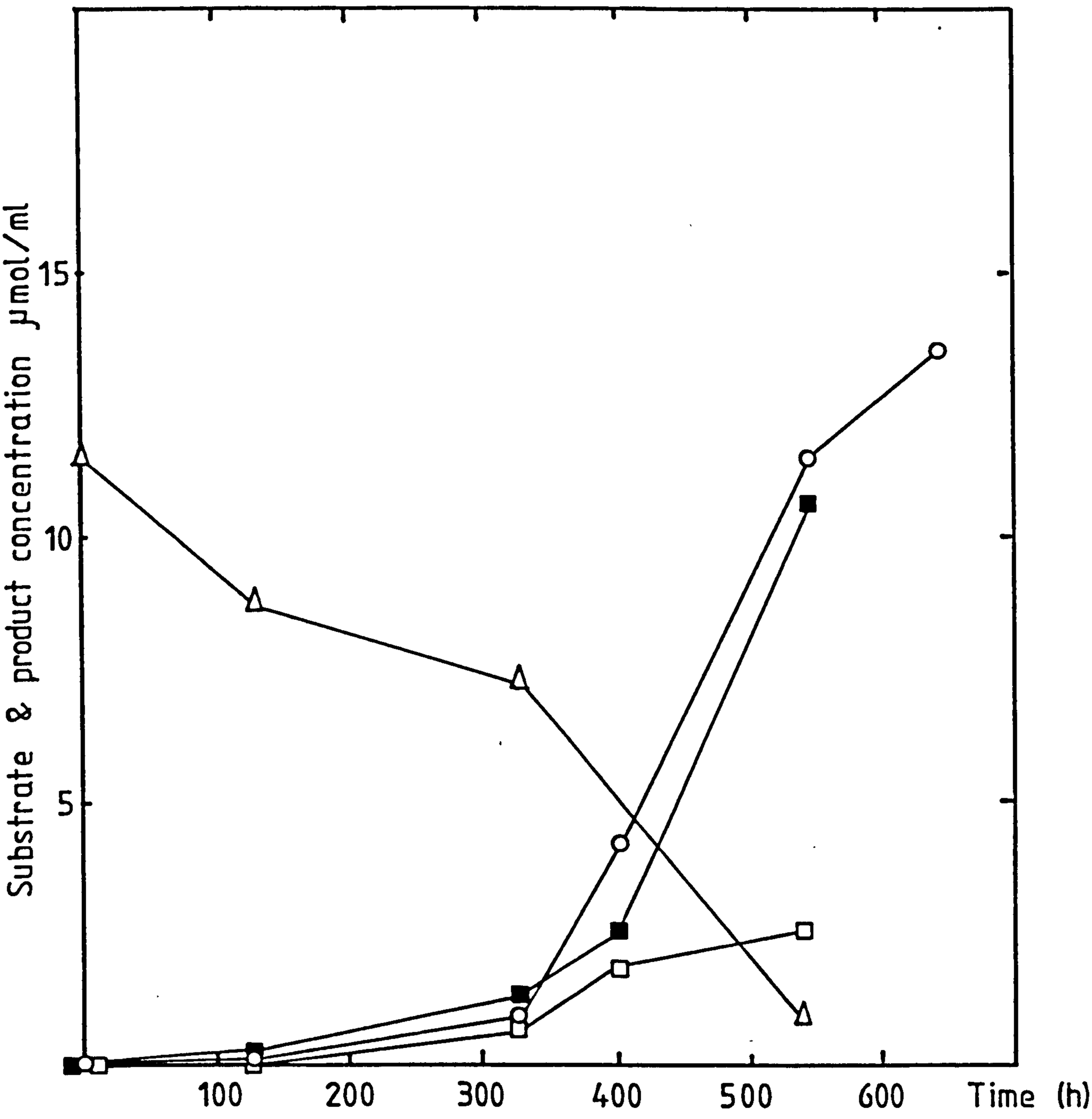
These results suggested that 2-methyl butyrate was catabolised initially to acetate and propionate, with the former then further catabolised to methane whilst the propionate remained undegraded.

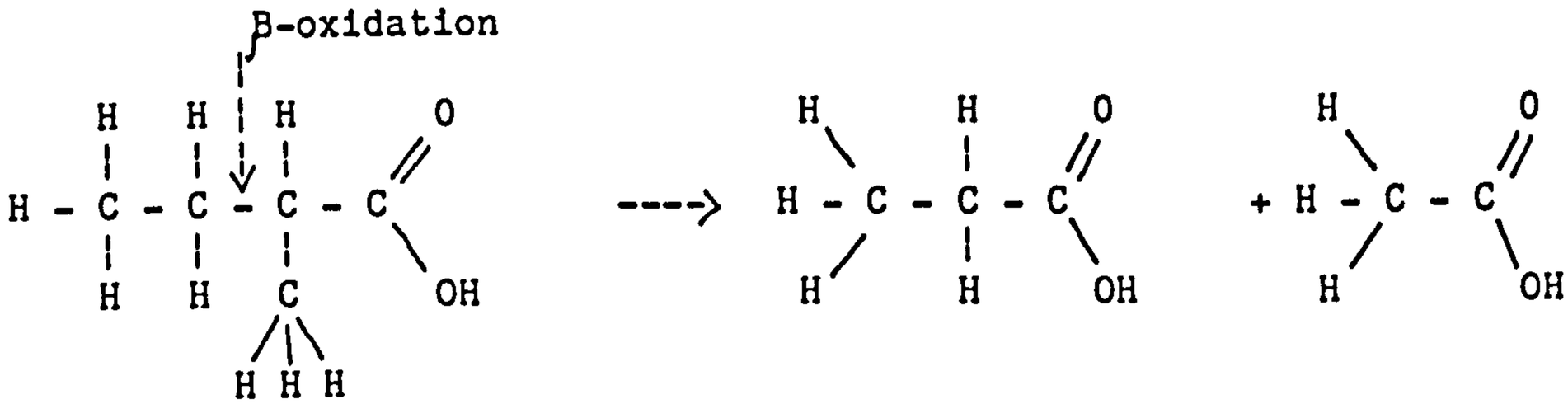
Stoichiometrically 1 mole of 2-methyl butyrate should yield 1 mole of propionate plus 2 moles of CH_4 (Equation 32):

$$C_5 H_{10} O_2 + 2 H_2O \longrightarrow C_3 H_6 O_2 + 2 CH_4 \quad (32)$$

thus, from the results obtained it would appear that the mechanism of metabolism of 2-methyl butyrate involved β-oxidation which would lead to the formation of acetate and propionate :

Figure 38: Changes in residual 2-methyl butyrate (Δ-Δ), propionate (■-■), acetate (□-□) and methane (○-○) concentrations during the closed culture cultivation at 30°C of the isolated microbial association in the presence of 2-methyl butyrate at an initial concentration of 12 mM.


Substrate & product concentration µmol/ml
15
10
5
100
200
300
400
500
600
Time (h)



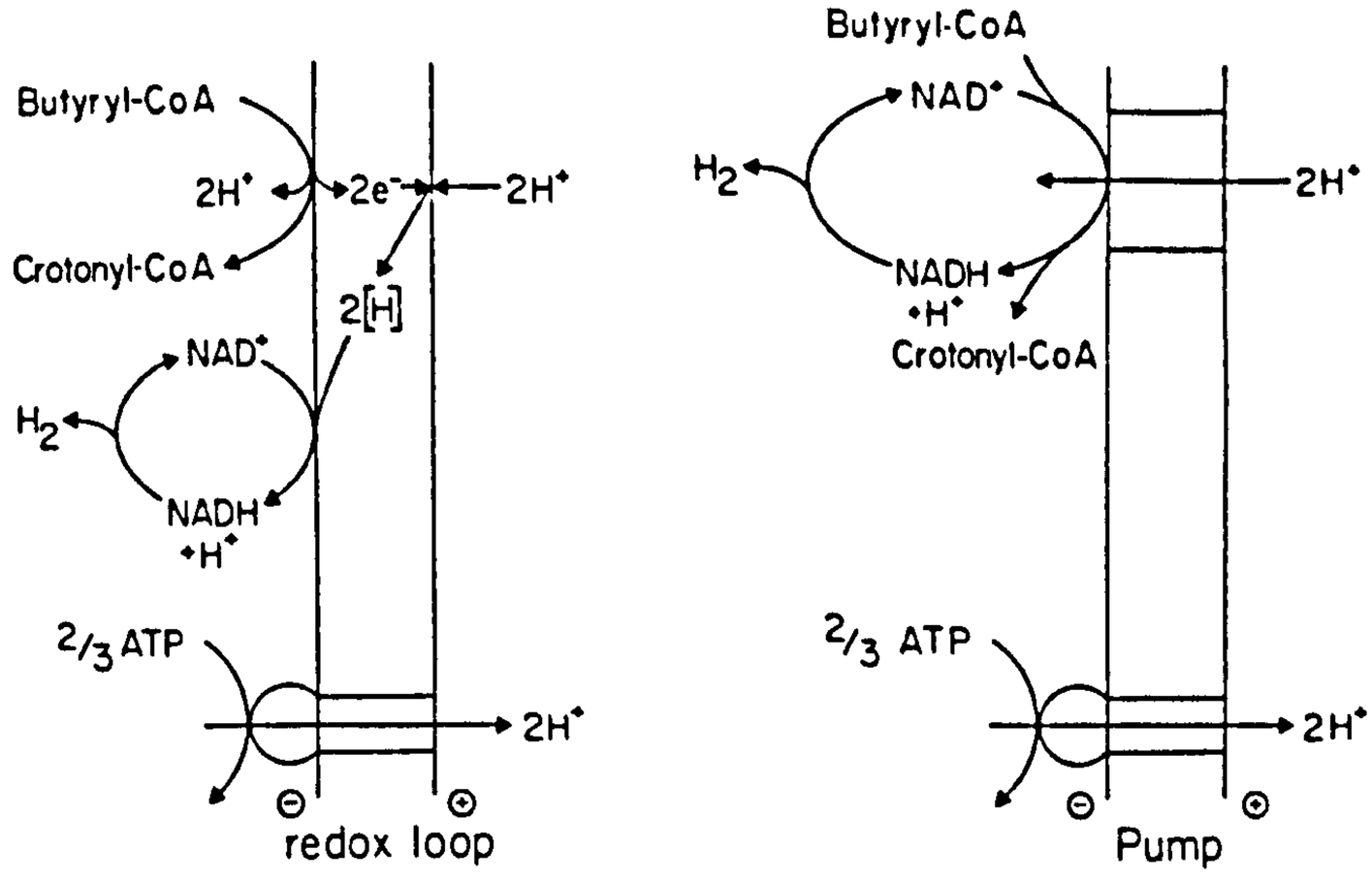
2-methyl butyrate --------> propionate + acetate (33)

This assumption was confirmed by use of iso-valerate, which blocks β-oxidation by the presence of a methyl group at the β-position, when after 643 h cultivation no substrate dissimilation and no intermediates or methane were detected.

β-oxidation by syntrophic co-cultures was examined by McInerney et al. (1979) who found that Syntrophomonas wolfei, in co-culture with Methanospirillum hungatei, degraded butyrate but surprisingly was unable to catabolise 2-methyl butyrate. Conversely, Clostridium bryantii has been shown to dissimilate, along with volatile fatty acids such as hexanoate and butyrate, 2-methyl butyrate to acetate and propionate and methane in the presence of M. hungatei (Stieb & Schink, 1985).

Theoretically, the mechanism of butyrate oxidation could have involved reverse electron transport (Thauer & Morris, 1984). This involves a fraction of the A.T.P. generated by substrate phosphorylation being used to drive a thermodynamically unfavourable rection. Figure 39 shows two hypothetical mechanisms for this process where the unfavourable reaction (butyryl-CoA ---> crotonyl CoA + H_2) could be facilitated by the hydrolysis of 2/3 mol ATP.

Figure 39: Two hypothetical mechanisms of reversed electron transport by which the endergonic oxidation of butyryl-CoA to crotonyl-CoA and H_2 could be driven by the hydrolysis of 1/3 mol ATP. This reaction only becomes favourable if the H_2 is subsequently removed. (Adapted from Thauer and Morris, 1984).


Butyryl-CoA
2H+
2e-
2H+
Crotonyl-CoA
2[H]
NAD+
H2
NADH
+H+
2/3 ATP
2H+
redox loop
Butyryl-CoA
NAD+
H2
2H+
NADH
+H+
Crotonyl-CoA
2/3 ATP
2H+
Pump

However, removal of H_2 is an essential requirement for this type of reaction.

4.5 Effect of metabolic inhibitors on methanogenesis by the microbial association

To further investigate the metabolic roles of the individual groups of the microbial association isolated in the multi-stage chemostat a series of experiments was initiated in which key metabolic inhibitors were used. In all 6 inhibitors were used, the actual mechanisms of which will be briefly considered below.

Bromo ethane sulphonic acid: B.E.S.A. has been reported to competitively inhibit the methyl transfer reaction at the terminal reductive step during methane formation from H_2/CO_2 (Gunsalus et al., 1976), with 50% inhibition observed with 10^{-6} M B.E.S.A. B.E.S.A. has also been shown to inhibit methanogenesis from acetate (Zehnder & Brock, 1979; Zehnder et al., 1980) when at a concentration of 5×10^{-7} M a 50% reduction was observed. B.E.S.A. (10^{-5} M) was used by Healey et al. (1980) to inhibit methanogenesis and thus facilitate the accumulation of intermediates from the degradation of ferulic acid. Oremland (1981) found that B.E.S.A. at a concentration of 10^{-3} M completely inhibited CH_4 production from anaerobic sediments, whereas Bouwer & McCarty (1983) found that the presence of B.E.S.A. at a concentration of 6×10^{-4} M resulted in only 41% inhibition of acetate utilisation in a continuous methanogenic fixed film column.

Chloroform: Chlorine-containing analogues of methane have been shown to be effective inhibitors of methanogenesis (MacPherson & Miller, 1964; Bauchop, 1967; Cappenberg, 1974; Zinder & Brock, 1978; Lovley & Klug, 1982). These compounds react with the reduced vitamin B_{12} to inhibit cobamide-dependent methyl transfer reactions involved in methane formation. Various con-centrations such as 6.2 mM, 120 μM and 100 μM have been used by Zinder & Brock (1978), Jones & Simon (1985) and Lovley & Klug (1982) with complete inhibition noted.

Fluoroacetate : Fluoroacetate, which specifically inhibits acetate metabolism, by the same mechanism as B.E.S.A., by inhibiting methyl transfer during acetate catabolism, was used by Cappenberg (1974) to differentiate between methanogenesis from acetate and H_2/CO_2. Banat *et al.* (1983) also used fluoroacetate (5 mM) to inhibit acetate metabolism in marine sediments.

Sulphate: Methanogens can also be inhibited by sulphate-reducing bacteria competing for substrates (Winfrey & Zeikus, 1977; Abram & Nedwell, 1978; Pfennig, 1984) and as little as 0.2 mM sulphate has been shown to inhibit methanogenesis in freshwater sediments (Winfrey & Zeikus, 1977).

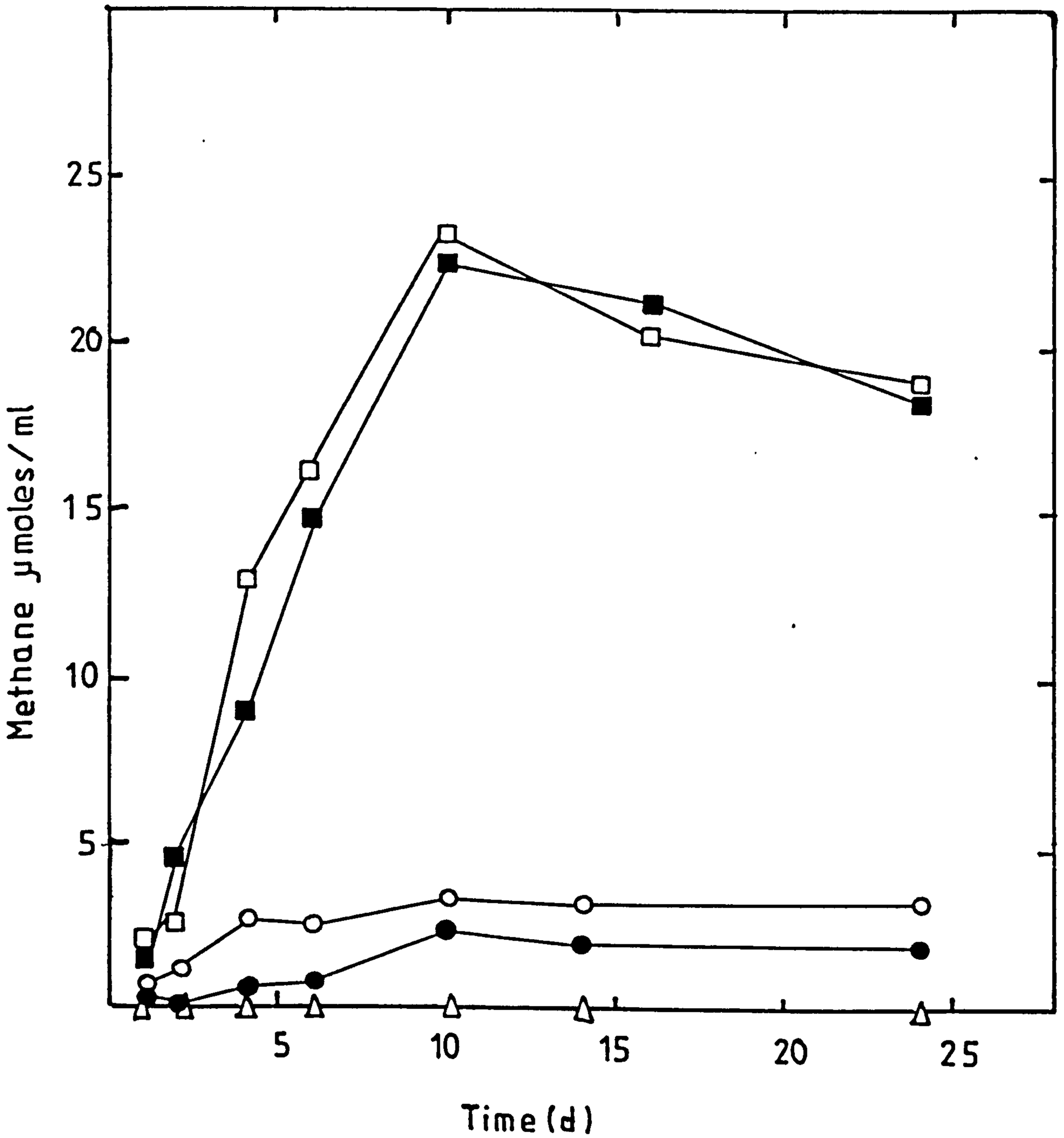
Inhibition of sulphate-reducing bacteria: Sulphate reducing bacteria can be specifically inhibited by molybdate, which is a sulphate structure analogue (Oremland & Taylor, 1978; Balba & Nedwell, 1982; Banat *et al.*, 1983). Other inhibitors of these

species include selenate cupric nitrate (Norqvist & Roffey, 1983), isothiazone (Ruseska et al., 1982), glutaraldehyde and quaternary ammonium compounds (Hardy & Syrett, 1983).

Inhibition of methanogens and sulphate-reducing bacteria in the presence of butyrate as substrate : In this experiment methanogenic species were inhibited with either chloroform (20 μM) or sulphate (10 mM). Acetate metabolism was inhibitied with fluoroacetate (10 mM) and sulphate reducing bacteria were inhibited by omission of sulphate. 30 ml serum bottles which contained 9 ml aliquots of basic mineral salts medium (A) were inoculated with 1 ml from Vessel A of the 5-stage chemostat after which the cultures were supplemented with butyrate (5mM) and electron acceptors and inhibitors as detailed above.

Figure 40 shows that methanogenesis from butyrate (5 mM) in the absence of an exogenous electron acceptor increased with no noticeable lag phase at a rate of 2.35 μ mol ml^{-1} d^{-1} to reach a concentration of 23.5 μ mol ml^{-1} after 10 days incubation. The terminal concentration then subsequently decreased to 19.0 μ mol ml^{-1} by day 24. Surprisingly, addition of sulphate (20 mM) had little effect on this production. Conversely, addition of fluoroacetate (10mM) resulted in a reduction in the terminal methane concentration of 83.1% to 3.2 μ mol ml^{-1} by day 24. The initial rate of methane production was also considerably reduced to 0.7 μ mol ml^{-1} d^{-1}.

Figure 40: Changes in methane concentration during the closed culture cultivation of the isolated microbial association on butyrate (5mM) (□ - □) and in the presence of sulphate (■ - ■), fluoroacetate (O - O) and chloroform (Δ - Δ). (● - ●) denotes the zero control.


Methane µmoles/ml
25
20
15
10
5
5
10
15
20
25
Time (d)

No detectable methane was produced when chloroform (20 μM) was used as a methanogenic inhibitor.

The results presented here support the findings detailed earlier that :

1. sulphate as an exogenous electron acceptor plays a reduced role; and
2. methane is produced mainly from acetate which is a product of β-oxidation of butyrate (and other V.F.A.s).

Although the landfill ecosystem may be considered to be analogous to freshwater sediments, in relation to sulphate concentrations, the sulphate-reducers in these latter environments have been found to out-compete methanogens for common substrates (Lovley & Klug, 1983). The results presented here, however, are much in contrast, as the presence of sulphate (10 mM) did not significantly effect methane production from butyrate even though sulphate-reducing bacteria were present in the microbial association.

The methanogenic population was completely inhibited by chloroform (20 μM) whereas only partial inhibition was recorded with fluoroacetate (10 mM). Two explanations were possible for this partial inhibition. Firstly, the effect of the inhibitor at the concentration used was not total and, secondly, the methane evolved could have originated from the H_2 ($+CO_2$) produced from the β-oxidation of butyrate. The possible effect of inhibitor

concentration will be discussed later.

The effect of different concentrations of the inhibitors fluoroacetate and B.E.S.A. on methanogenesis from acetate and H_2/CO_2

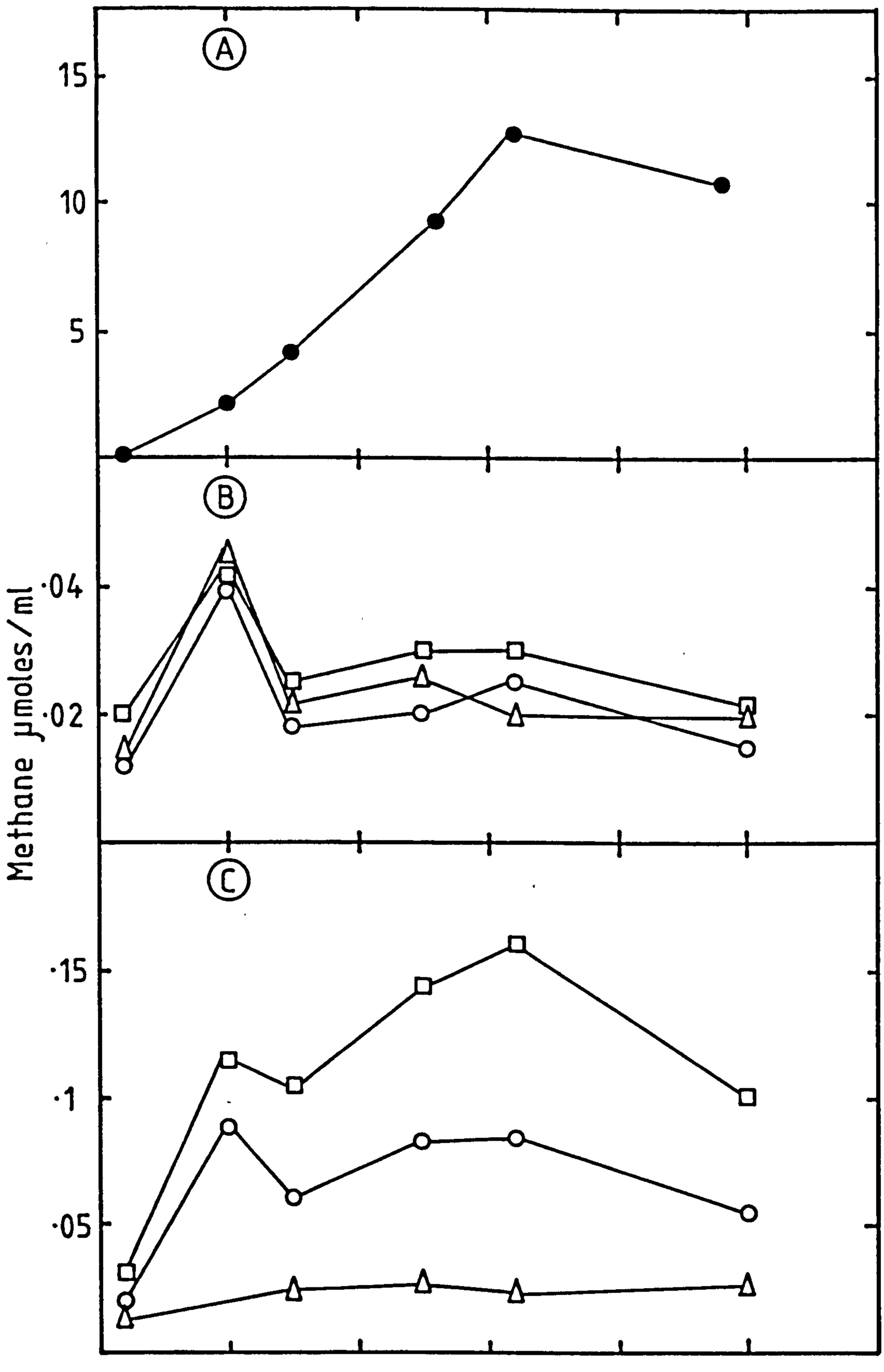
The experimental procedure used was as described above with fluoroacetate added to give concentrations of 2, 6 and 20 mM whilst the final concentrations of B.E.S.A. were 2, 4 and 12 mM.

In the presence of acetate (12 mM) (Figure 41A) the methane concentration increased at a rate of 0.95 μ mol ml^{-1} d^{-1} after an initial lag period of approximately 5 days. The methane concentration reached a maximum of 12.68 μ mol ml^{-1} d^{-1} after 16 days incubation before subsequently dropping to 10.88 μ mol ml^{-1} by the end of the experiment (24 days).

Fluoroacetate, an inhibitor of acetate metabolism, significantly decreased methanogenesis from acetate by the microbial association (Figure 41B). All three concentrations of inhibitor used (2, 6 and 20 mM) gave similar results with final concentrations of between 0.02 and 0.03 μ mol ml^{-1} which approximated to a 99.8% decrease.

B.E.S.A. also significantly inhibited methanogenesis from acetate at all three concentrations used (Figure 41C), although in this case there was a noticeable increase in inhibition as the concentration was increased. With 2 mM B.E.S.A. the maximum methane concentration recorded was 0.16 μ mol ml^{-1} which corresponded to a 98.7% reduction, whilst an increase in the concentration of the inhibitor to 4 mM resulted in a maximum methane concentration of

Figure 41: Changes in methane concentration during the closed culture cultivation of the microbial association on acetate (A) (● - ●) in the presence of fluoroacetate (B); 2 mM (□ - □), 6 mM (○ - ○) and 20 mM (△ - △) and BESA (C); 2 mM (□ - □), 4 mM (○ - ○) and 12 mM (△ - △).


A
B
C
15
10
5
·04
·02
·15
·1
·05
Methane µmoles/ml
5
10
15
20
25
Time (d)

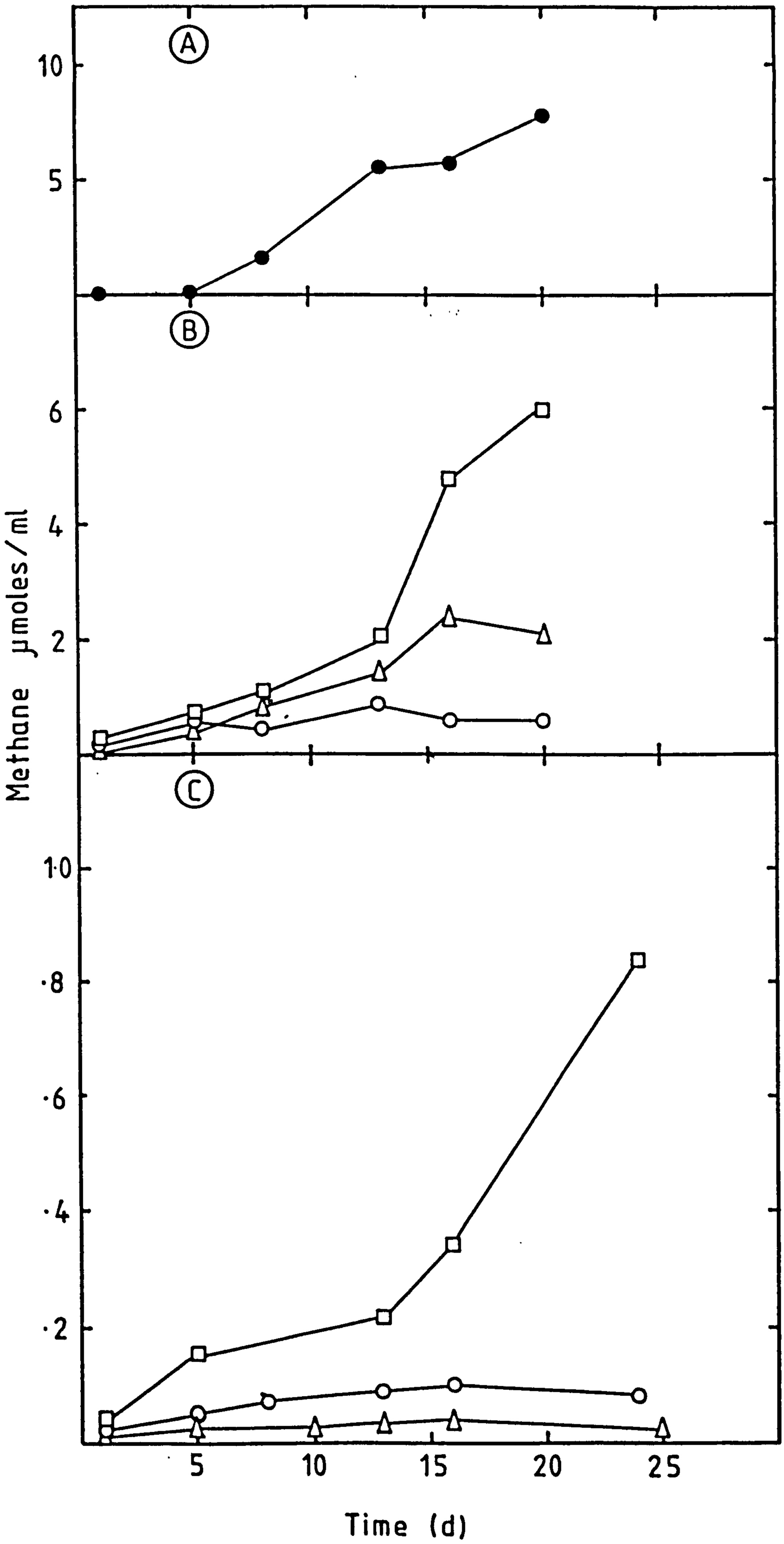
0.086 μ mol ml^{-1}, an overall decrease of 99.3%. Finally, 0.026 μ mol ml^{-1} methane, an overall drop of 99.7%, was recorded when the concentration of B.E.S.A. was increased to 12 mM.

The effects of fluoroacetate and B.E.S.A. on methane production from H_2/CO_2 are shown in Figure 42. Methane production from H_2/CO_2 (Figure 42A) after a lag period of approximately 5 days, increased to a maximum of 7.8 μ mol ml^{-1} by day 20 at a rate of 0.77 μ mol $ml^{-1}d^{-1}$.

In the presence of fluoroacetate (Figure 42B) significant effects were noted. At a concentration of 2 mM the final concentration of methane was reduced to 6.0 μ mol ml^{-1} by the end of the experiment (20 days). In addition there appeared to be a protracted lag phase of approximately 13 days, after which the methane concentration increased at a rate of 0.92 μ mol ml^{-1} d^{-1}. Although this increase in lag phase could have been due to the presence of fluoroacetate a possible explanation for this is not readily obvious.

With fluoroacetate at a concentration of 6 mM, methanogenesis was significantly inhibited and only 0.9 μ mol ml^{-1} were recorded after 13 days incubation which represented a decrease of 85%. By increasing the concentration of inhibitor to 20mM, however, further decreases were not apparent and in fact a slight increase to 2.46 μ mol ml^{-1} was recorded after 16 days incubation although this was still equivalent to an overall reduction of 59%. The explanation for this lessening of inhibitory effect at the higher concentration

Figure 42: Changes in methane concentration during the closed culture cultivation of the isolated microbial association on H_2/CO_2 (A) (● - ●) in the presence of fluoroacetate (B); 2 mM (□ - □), 6 mM (O - O) and 20 mM (Δ - Δ) and BESA (C); 2 mM (□ - □), 4 mM (O - O) and 12 mM (Δ - Δ).


A
B
C
10
5
6
4
2
1·0
·8
·6
·4
·2
Methane µmoles/ml
5
10
15
20
25
Time (d)

is hard to tender.

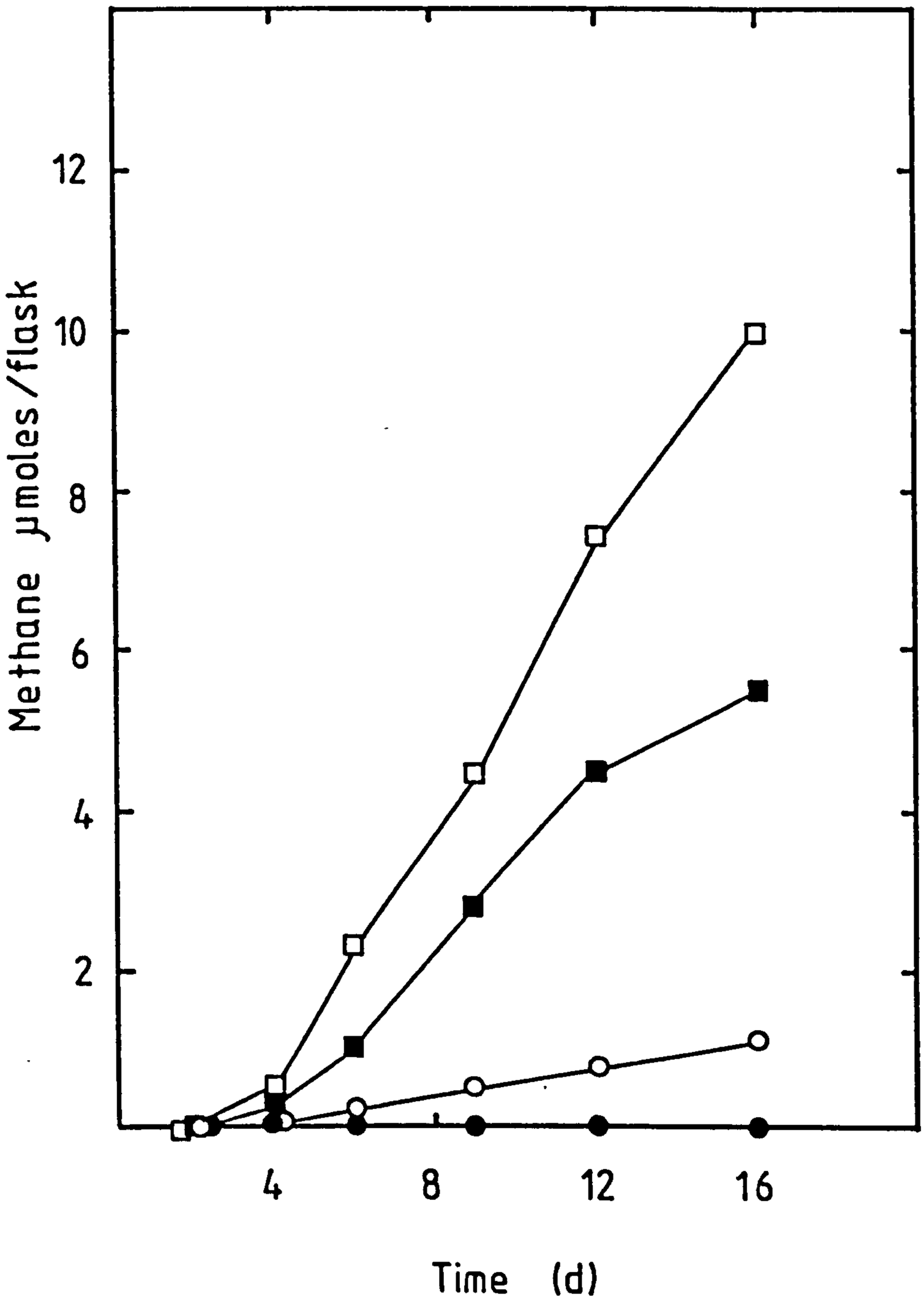
When the microbial association was incubated with H_2/CO_2, in the presence of B.E.S.A. (2 mM) (Figure 42C), although methane production increased slowly to 0.236 μ mol ml^{-1} by day 13 and then subsequently increased to 0.85 μ mol ml^{-1} by day 24 this final concentration was equivalent to a reduction of 89.1%. Further increases in the concentration of B.E.S.A. to first 4 and then 12 mM also resulted in increased inhibition of 99.0 and 99.7% respectively.

Interactions between homoacetogens and methanogens : This experiment was essentially the same as that described above with the exception that 100 ml flasks were used which contained 30 ml basic mineral salts medium and were inoculated with 3 ml from Vessel A of the 5-stage chemostat. The first 3 sets of flasks were overgassed with H_2/CO_2. Set 1 received no further supplementation, whereas sets 2 and 3 were individually amended with B.E.S.A. (2 mM) and fluoroacetate (5 mM) respectively. Set 4 was used as a control. Gas samples were taken at regular intervals and assayed for CH_4 as before.

Figure 43 shows that after a lag period of 4 days CH_4 production from H_2/CO_2 commenced and reached a mean concentration of 10.0 μ mol $flask^{-1}$ by day 16. In the presence of B.E.S.A. the lag period was extended by approximately 6 days although the subsequent rate of CH_4 production was similar to that for H_2/CO_2 alone. The

Figure 43: Changes in methane concentration during the closed culture cultivation of the isolated microbial association on H_2/CO_2 (□ - □) in the presence of BESA (■ - ■) and fluoroacetate (O - O).

(● - ●) denotes the zero control.


Methane µmoles/flask
12
10
8
6
4
2
4
8
12
16
Time (d)

terminal concentration (5.3 μ mol flask^{-1}) however was less than that of H_2/CO_2 and corresponded to a reduction of 47%.

Partial inhibition of methanogenesis was again apparent in the presence of fluoroacetate (5 mM) although in this instance the effect was more significant since a 90% reduction was recorded in the methane concentration. The lag phase again appeared to be approximately 6 days although this was hard to determine accurately as the rate of methane production was low (approximately 0.07 μ mol flask^{-1} d^{-1}).

Although 1 μ mol flask^{-1} of methane was formed in the presence of fluoroacetate this could have been due to the concentration of inhibitor being too low for total inhibition or perhaps more likely that either the methanogen had two operative pathways or that two different species of methanogen were present.

The metabolic interactions and the involvement of homo-acetogens such as Acetobacterium woodii (Balch et al., 1977) and Clostridium aceticum (Braun et al., 1981) in the terminal metabolism of organic material is poorly understood (Jones & Simon, 1985). Theoretically, it is possible for homo-acetogens to produce acetate which could be subsequently utilised by other organisms, such as methanogens. Energetically, in a freely competitive situation between homo-acetogens, methanogens and sulphate-reducing bacteria for H_2 (Eqs. 34 to 36) then the order of utilisation would be sulphate-reducing bacteria before methanogens before acetogens (Thauer et al., 1977).

$$4\ H_2 + SO_4^{2-} \longrightarrow HS^- + 4\ H_2O$$

$$\Delta G_o^1 = -151.9\ kJ\ mol^{-1} \quad (35)$$

$$4\ H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3\ H_2O$$

$$\Delta G_o^1 = -135.6\ kJ\ mol^{-1} \quad (36)$$

$$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O$$

$$\Delta G_o^1 = -104.6\ kJmol^{-1} \quad (37)$$

Jones & Simon (1985) however found that hydrogen consumption by acetogens could be as high as 50% of that utilised in methanogenesis.

In the present study it was clearly demonstrated that fluoroacetate inhibited methanogenesis from H_2/CO_2, which suggested that the pathway of H_2/CO_2 to CH_4 was via a fluoroacetate-affected intermediate. In a previous experiment (4.3) it was shown that in the absence of sulphate both acetate and methane were produced from H_2/CO_2. The acetate concentration then subsequently decreased which suggested that both homo-acetogens and acetogenic methanogens were present in the microbial association.

Although the results of the above experiments supported the hypothesis that metabolism of H_2/CO_2 to CH_4 required homo-acetogenic intervention it could be that the metabolism of H_2/CO_2 to methane proceeded through an as yet unidentified fluoroacetate-inhibited intermediate.

Summary: The collective results of the three experiments showed that :

1. Methanogenesis from butyrate was significantly inhibited by fluoroacetate and chloroform but not sulphate; and
2. Both methanogenesis from acetate and H_2/CO_2 was inhibited by fluoroacetate and B.E.S.A.

The results supported the postulated structure of the microbial association presented earlier (Figure 37) where it would appear that methane production from H_2 involves homoacetogenesis and subsequent catabolism of the acetate to methane.

4.6 Methanogenesis from Labelled Substrates

10 ml samples of the first vessel of the 5-stage chemostat, operating at 1.4 mM sulphate, were incubated under a gas atmosphere of O.F.N. in 30 ml bottles closed with Suba-Seals. The cultures were allowed to equilibrate for 1 hour at 25°C prior to the addition of radioactive substrates. Sterile, anoxic, pre-warmed radio-labels were injected into duplicate bottles to give final concentrations of 1 μCi 10 ml^{-1} [U-^{14}C] acetate (58.6 mCi m mol^{-1}; 2.1 G B_q m mol^{-1}), 2 μCi 10 ml^{-1} [^{14}C] bicarbonate (55.5 mCi $mmol^{-1}$; 2.06 G B_q $mmol^{-1}$), 1 μCi 10 ml^{-1} [^{14}C] methanol (56.9 mCi $mmol^{-1}$; 2.1 G B_q $mmol^{-1}$) and 2 μCi 10 ml^{-1} [^{14}C] formate (59.0 mCi $mmol^{-1}$; 2.18 G B_q $mmol^{-1}$). The bottles were incubated at 25°C stationary in the dark and gas samples were taken at regular intervals and assayed for ^{14}C-methane and ^{14}C carbon dioxide.

Acetate

Addition of acetate led to the immediate formation of both methane and carbon dioxide (Figure 44) with most of the methane evolved during the first 24 h at which point a count of 9.7 was recorded. This count did not thereafter increase significantly even after a protracted incubation period of 120 h when the terminal count was 10.7. The same pattern was also found for the evolved carbon dioxide with most of the label again evolved in the first 24 h.

If the aceticlastic reaction described by Eikmans and Thauer (1984) was the mechanism of methane formation from acetate, then equal concentrations of methane and carbon dioxide should have been formed :

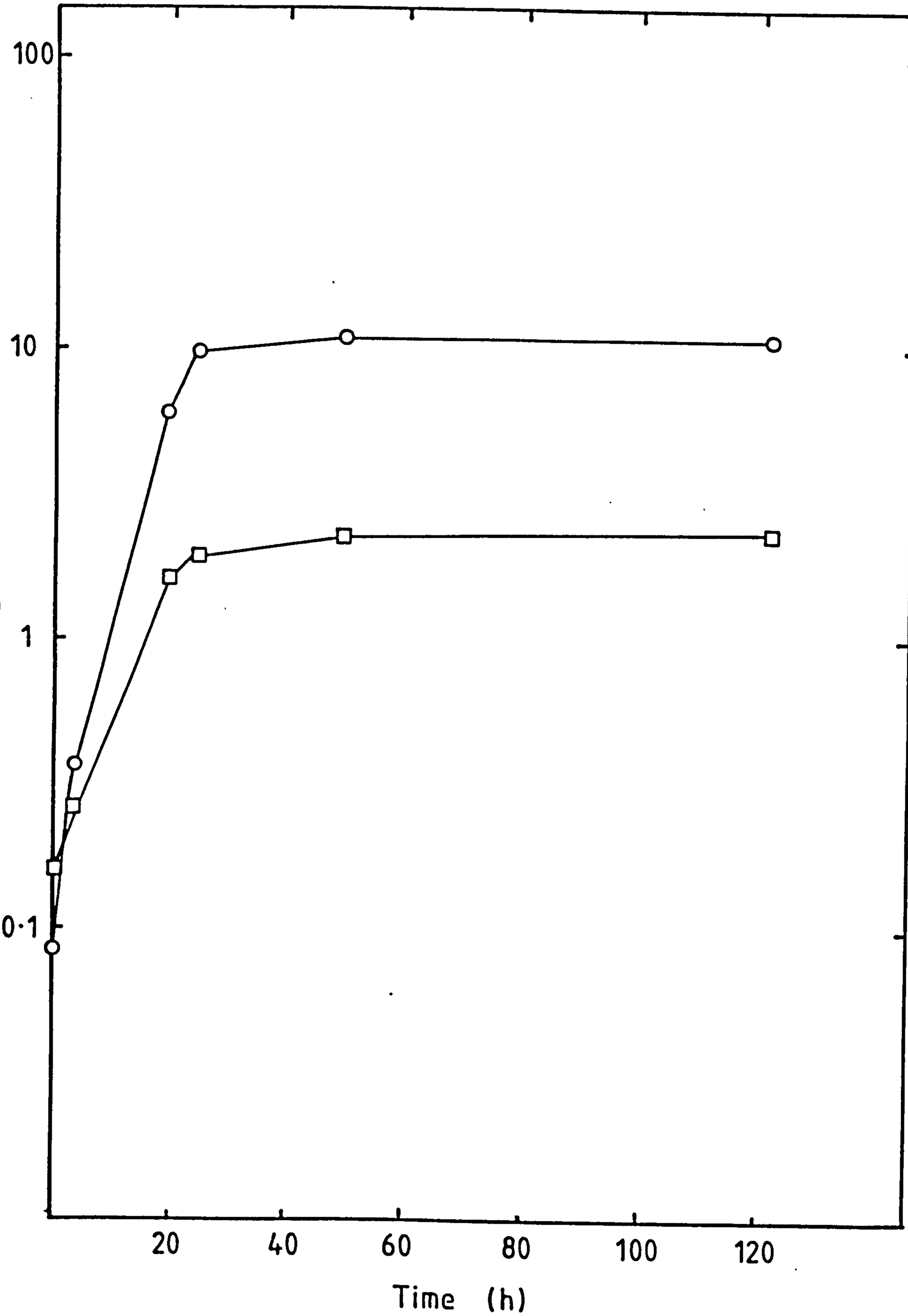
$$CH_3\,COO^- + H^+ \;\text{----}\; CH_4 + CO_2$$

$$\Delta G_o^1 = -36\ kJ\ mol^{-1} \qquad (38)$$

However, after 120 h incubation the percentage distribution of the dissimilated labelled substrate was methane (82.4%) and CO_2 (17.6%). In this case, however, the estimate for CO_2 was probably low as soluble $^{14}C\ CO_2$ (HCO_3^-) was not determined. Also, since most methanogens have a biosynthetic requirement for CO_2 it is possible that some of the CO_2 produced was assimilated into cell material (Patel, 1984).

Catabolism of hexanoate by β-oxidation presents both acetate and H_2 as potential substrates for methanogenesis. It has been found that metabolically diverse methanogens growing on hydrogen

Figure 44: Production of [^{14}C]-CH_4 (O - O) and [^{14}C]-CO_2 (□ - □) during the closed culture cultivation of the isolated microbial association in the presence of [U-^{14}C] acetate. Counts are expressed in arbitrary units.


100
10
1
0·1
Log counts
20
40
60
80
100
120
Time (h)

plus carbon dioxide required protracted time intervals (for example, 4 weeks (Zeikus, 1983a)) to adapt to growing on acetate.

The results of this experiment showed that acetate was utilised immediately, which was expected as the methanogenic population was continuously exposed to acetate as a product of hexanoate catabolism.

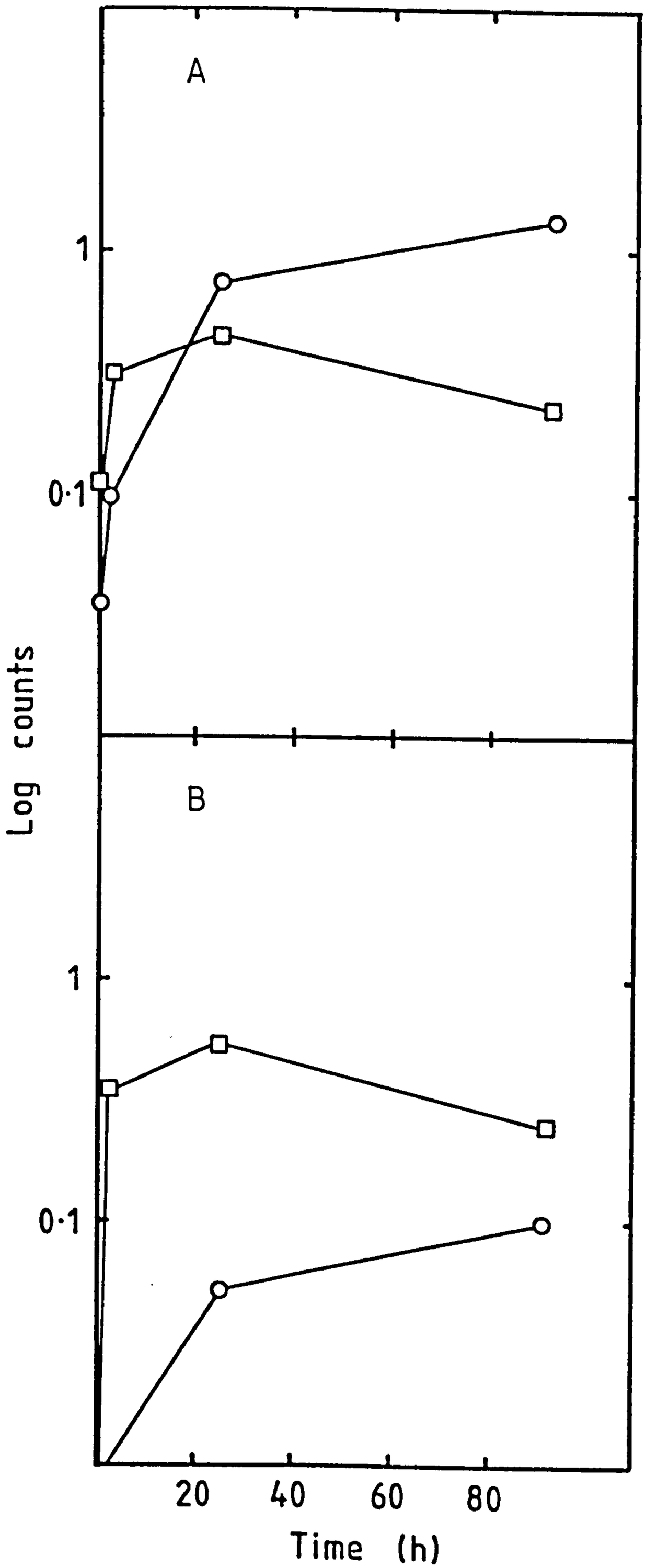
Bicarbonate

Labelled bicarbonate was added to the culture in the presence (Figure 45A) and absence (Figure 45B) of H_2. In the case of the former there was an immediate formation of both labelled CH_4 and CO_2. Methane reached a count of 1.48 after 94 h cultivation and labelled CO_2 reached a maximum count of 0.45 after 25 h incubation after which the latter decreased to 0.256 by the end of the experiment.

As expected, the addition of [^{14}C] HCO_3^- alone did not result in significant [^{14}C] methane production. Appearance of [^{14}C] CO_2 however, followed the same pattern as that found under H_2 supplementation, with maximum CO_2 (0.54 counts) detected after 25 h with a subsequent decrease to 0.27 counts by 94 h. In this instance [^{14}C] methane reached 0.105 counts by the end of the experiment and could possibly have been attributed to the availability of H_2 from residual V.F.A. catabolism.

Hydrogen, which is a key intermediate in the degradation of organic material (Robinson & Tiedje, 1982), can be terminally

Figure 45: Production of labelled CH_4 (O-O) and CO_2 (□-□) during closed culture cultivation of the isolated microbial association on [^{14}C]-HCO_3 in the presence (A) and absence (B) of H_2. Counts are expressed in arbitrary units.


A
1
0·1
Log counts
B
1
0·1
20
40
60
80
Time (h)

oxidised to H_2S (Brandis & Thauer, 1981), methane, (Zeikus & Wolfe, 1972) or acetate (Braun et al., 1981) depending on the availability of exogenous electron acceptors (sulphate or carbon dioxide). Thus, in this experiment, the availability of H_2 could also have been of considerable importance. Since H_2 has a low solubility in water the reaction rate could have been limited by the rate of absorption of the gas into the liquid phase.

Another factor could have been the availability of CO_2, as it has been found that most reactions involving reduction of CO_2 by anaerobes utilise CO_2 rather than HCO_3^- as the active species of CO_2 (Thauer et al., 1976). From the above discussion it can be seen that results obtained from ^{14}C - HCO_3^- studies can only be interpreted with a considerable degree of caution. However, the experiment did show that H_2 had a significant effect on the conversion of CO_2 to CH_4 although it is interesting to note that labelled CO_2 in the headspace followed approximately the same pattern whether H_2 was present or not. From this observation one could speculate that the immediate precursor for methane formation from HCO_3^- was dissolved in the medium and not released into the gas phase.

From the results it would appear that two possible pathways: (1) acetate; and (2) H_2 + CO_2 were operative with the former the more dominant. However, at this stage it was not possible to determine if two separate methanogenic species were present in the microbial association or whether there was one only with two

operative pathways.

Formate

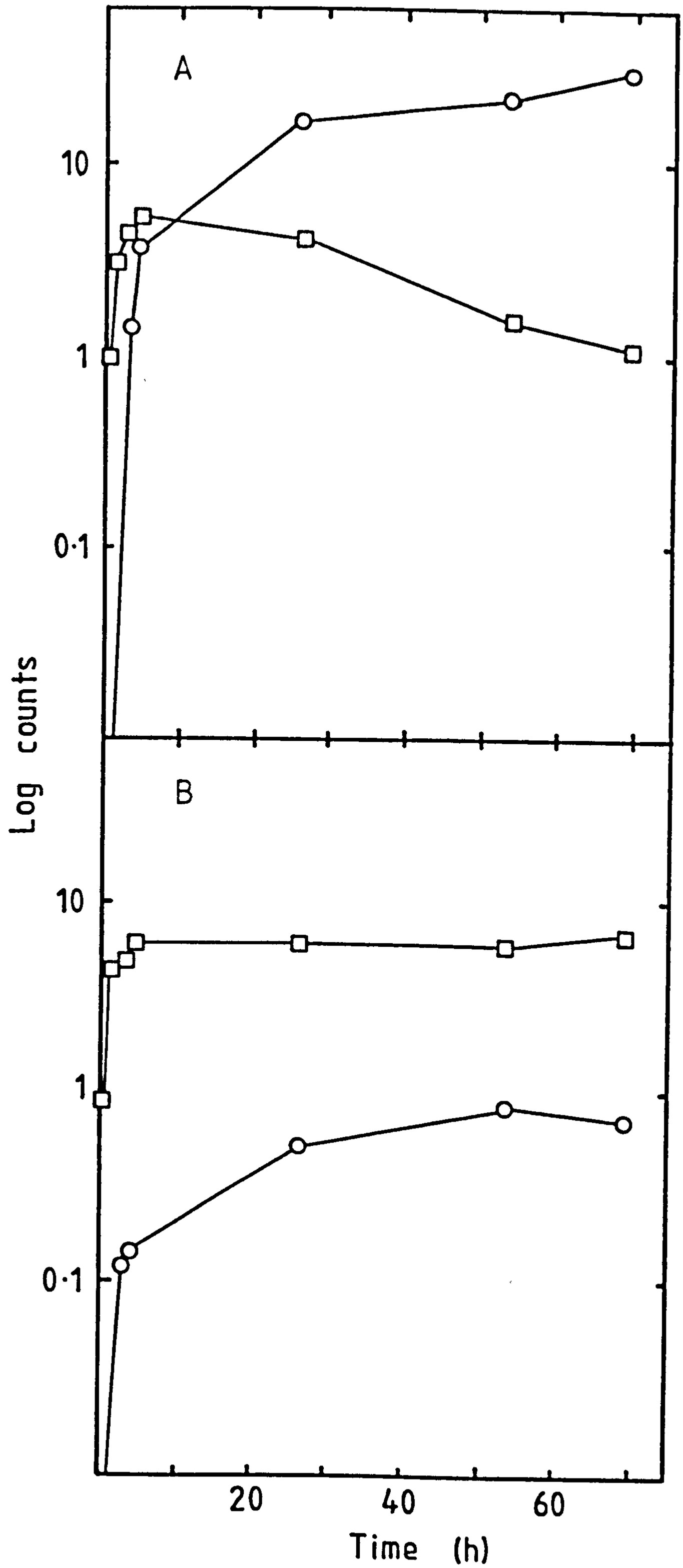
As a consequence of the above results an experiment was initiated with formate (Figure 46A and 46B) since this molecule is considered to be, oxidised to carbon dioxide and reducing equivalents with the former subsequently reduced to methane.

With O.F.N. in the gas phase (Figure 46B) [^{14}C] methane was detected with most of the label evolved during 24 h (0.76 counts) from which point a count of 0.85 was reached by the end of experiment (69h).

[^{14}C] CO_2 was also detected immediately with most of the label evolved during the first 4 h (6.00 counts) with little further change then observed before a final count of 7.21 after 69 h incubation was recorded.

Addition of hydrogen to the headspace of formate-amended cultures (A) greatly stimulated [^{14}C] CH_4 production, and a count of 33.7 was obtained by the end of the experiment. CO_2 was again released initially and by 4 h had reached 5.33 counts. In contrast to the O.F.N. samples, however, the count decreased to 1.25 after 69 h incubation. The significant decrease in CO_2 with concomitant methane evolution thus supported the view that CO_2 was the preferred methanogenic substrate compared with HCO_3^- or formate itself. In fact comparison of the results from the previous experiment (HCO_3^-) (Figure 45A and B) with these results showed that they were very similar with the exception that with formate there appeared to have

Figure 46: Production of labelled CH_4 (O - O) and CO_2 (□ - □) during the closed culture cultivation of the isolated microbial association on [^{14}C]-formate in the presence of (A) and absence (B) of H_2. Counts are expressed in arbitrary units.


A
10
1
0·1
Log counts
B
10
1
0·1
20
40
60
Time (h)

been higher activity. The possibility, therefore, existed that the reductive pathway of CO_2 to CH_4 passed through a formate intermediate.

The experiment presented here indicated that formate, in the absence of H_2 supplementation, initially stimulated methanogenesis at a lesser rate than acetate. Methane production in this case, however, was most probably limited due to depletion of H_2 (Winfrey & Zeikus, 1977) since the addition of H_2 to the headspace resulted in a prolonged period of methanogenesis. With H_2 in the headspace the rate of formate conversion to methane and the terminal concentration of CH_4 were both comparable with the results obtained with acetate.

Energetically, direct metabolism of formate to methane is comparable to the metabolism of H_2 to CH_4 :

$$HCOO^- + H_2O \longrightarrow H_2 + HCO_3^- \qquad \Delta G_o^1 = +1.3 \text{ kJ mol}^{-1} \quad (39)$$

$$4\ H_2 + H^+ + HCO_3^- \longrightarrow CH_4 + 3\ H_2O \qquad \Delta G_o^1 = -135.6 \text{ kJ mol}^{-1} \quad (40)$$

$$4\ HCOO^- + H^+ \longrightarrow CH_4 + 3\ HCO_3^- \qquad \Delta G_o^1 = -130.4 \text{ kJ mol}^{-1} \quad (41)$$

Also, formate and hydrogen have been found to be equivalent sources of electrons for Methanobrevibacter symthii (Tzeng et al., 1975), Methanobacterium formicicum (Schauer & Ferry, 1984) although formate was not utilised by the metabolically diverse Methanosarcina barkeri

(Zeikus, 1983a). The above examples, however, were taken from studies of the bovine rumen where H_2/CO_2 and formate are known to be the major sources of methane. Similarly, methanogenesis in Lake Vechten sediments (Cappenberg, 1974) was also shown to be stimulated by formate, although it was thought to be a minor substrate in this instance.

The three precursors examined so far could all potentially have been intermediates of hexanoate catabolism, thus a fourth substrate, methanol, was also examined to determine if the methanogenic population retained the metabolic potential to utilise this molecule even after prolonged cultivation in its absence.

Methanol

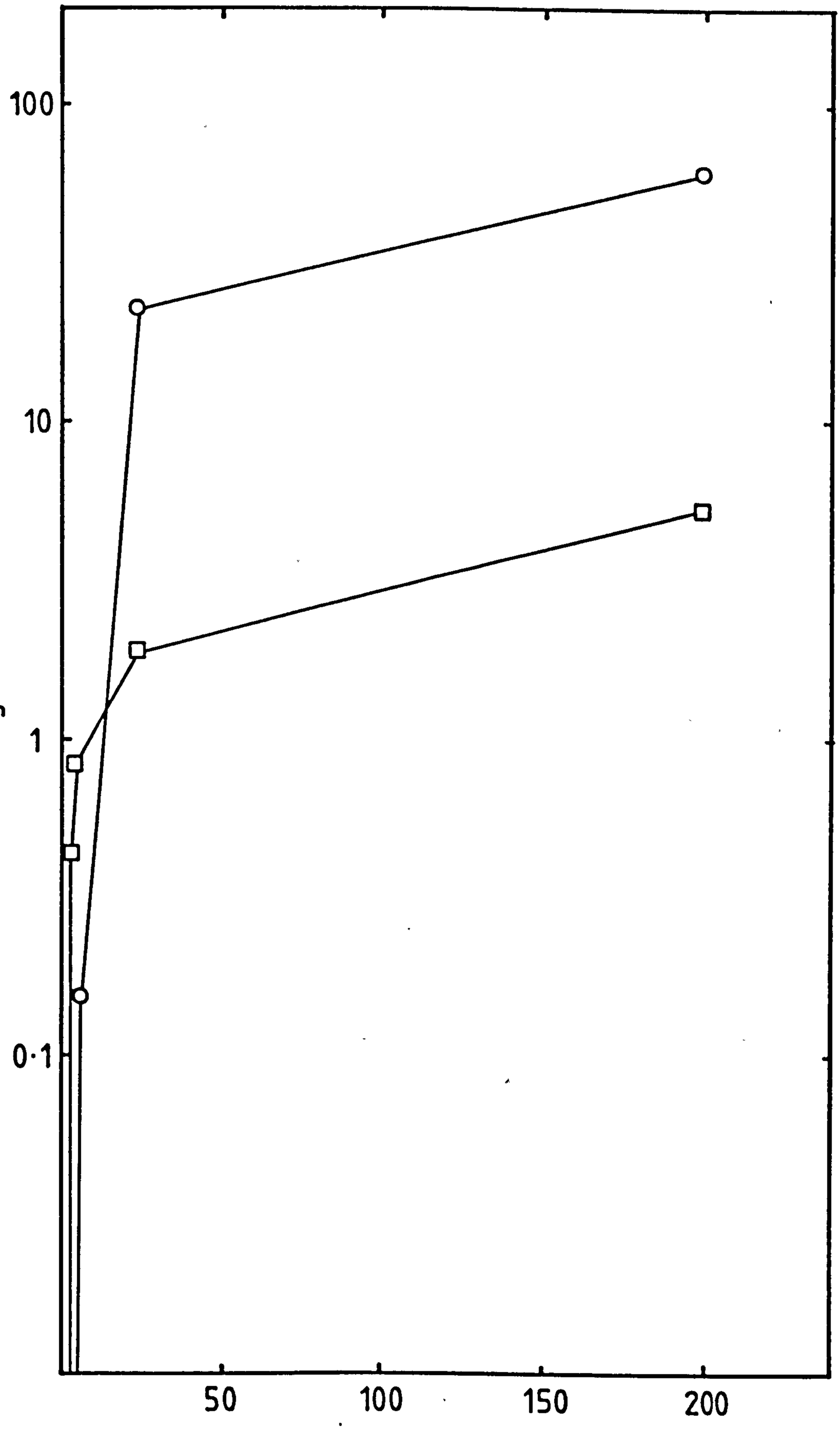
Addition of [^{14}C] methanol to the culture once again resulted in the production of both [^{14}C] methane and carbon dioxide (Figure 47). The methane concentration increased to reach 23.0 counts by 23 hours and then further increased to a count of 61.1 by the end of the experiment (193 hours). Carbon dioxide increased steadily and by 23 hours was 1.95 counts with further increases to 5.42 counts apparent after 193 hours cultivation. The terminal methane count suggested that methanol was the preferred substrate of all the substrates examined. Also, the active production of methane from methanol :

$$4\ CH_3OH \text{ ---- } 3\ CH_4 + HCO_3^- + H^+ + H_2O$$

$$\Delta G_o^1 = -105.9\ kJ\ mol^{-1} \qquad (42)$$

suggested that methanogens of comparable metabolic potential to <u>M.</u>

Figure 47: Production of labelled CH_4 (O - O) and CO_2 (□ - □) during closed culture cultivation of the isolated microbial association in the presence of [^{14}C] methanol. Counts are expressed in arbitrary units.


100
10
1
0·1
Log counts
50
100
150
200
Time (h)

barkeri were present in the microbial association.

Although methanol is not an intermediate of hexanoate catabolism, it can be a potential intermediate from demethoxylation of aromatic compounds such as 3-o methyl gallate (Tack et al., 1972) and from the degradation of pectin (Schink & Zeikus, 1982). Since pectin is a major component of plant materials, particularly in fruits, then one would expect the methanol pathway to be operative in landfill and this has recently been confirmed (G.B. Kasali, unpublished observations).

The retention of the methanol methanogenic pathway was particularly interesting since it appeared that acetate and H_2/CO_2 were the only possible substrates from hexanoate dissimilation. However, genetic stability and preservation of metabolic potential are regarded to be the norm in bacterial communities (Reanney et al., 1983). Also, consideration of methanogenic metabolism (Figure 4) shows that unification, as discussed by Barker (1956), would allow retention of methanol catabolic enzymes since these are also required for other substrates.

From the collective results of the radiotracer studies it can be seen that acetate, H_2/CO_2, formate and methanol all served as precursors for methane production by the microbial association. These results imply that either the methanogenic component of the microbial association was metabolically diverse, similar to Methanosarcina sp. for example, or was composed of more than one species.

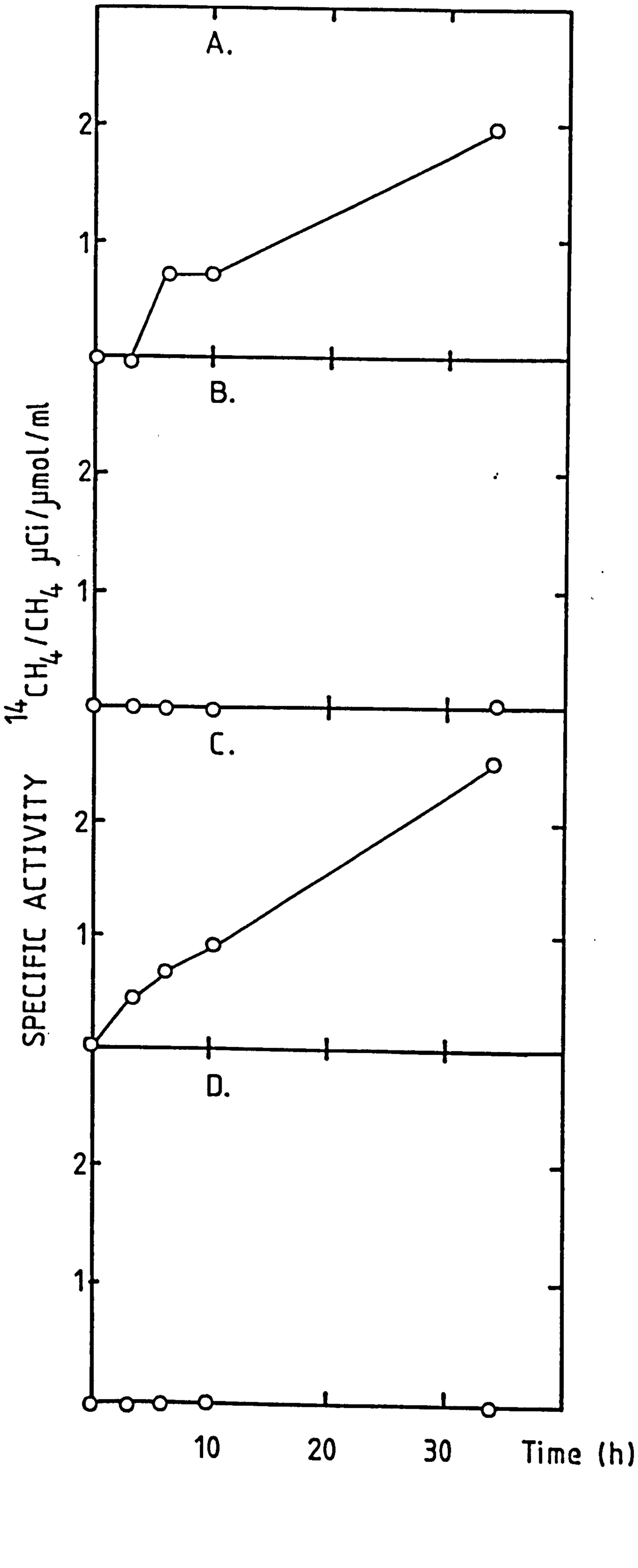
4.7 Methanogenesis from Labelled Acetate

Methanogenesis from acetate was confirmed in the previous experiment. However, the exact mechanism of acetate catabolism to methane was not considered. An experiment was initiated to investigate the mechanism of methane formation from acetate using carboxyl- and methyl- labelled acetate.

Radioactive isotopic labelling studies were made on the interacting microbial association isolated in the 5-vessel multi-stage chemostat model. 2 ml aliquots of culture from Vessel A were removed 2 days prior to initiation of the experiment to ensure that no residual acetate remained. Sterile, anoxic, labelled solutions of [U-^{14}C] acetic acid, sodium salt (58 mCi mmol^{-1}; 2.14 G B$_q$ mmol^{-1}), [1-^{14}C] acetic acid, sodium salt (57 mCi mmol^{-1}; 2.1 G B$_q$ mmol^{-1}), [2-^{14}C] acetic acid, sodium salt (54 mCi mmol^{-1}; 1.99 G B$_q$ mmol^{-1}), and sodium [^{14}C] bicarbonate (55.5 mCi mmol^{-1}; 2.06 G B$_q$ mmol^{-1}), were prepared and were individually added to the cultures to give an initial label concentration of 1 μCiml^{-1}. The cultures were incubated at 30^{o}C and headspace gas samples were removed at regular intervals and assayed for labelled and un-labelled methane.

After a lag period of more than 3 hours ^{14}C-CH_4 from [U-^{14}C] acetate was detected, the specific activity of which increased steadily to reach 1.99 μCiμmol^{-1} ml^{-1} after 34 hours cultivation (Figure 48 A). Although residual ^{14}C-acetate was not determined all the label detected was accounted for as CH_4 (42.9%) and CO_2 (57.1%). This result, however, was very different to the results of the

Figure 48: Changes in culture headspace ^{14}C-CH_4 specific activities during the anoxic closed culture cultivation of the isolated microbial association in the presence of U-^{14}C acetate (A), 1-^{14}C acetate (B), 2-^{14}C acetate (C) and ^{14}C HCO_3^- (D) under an atmosphere of OFN.


A.
B.
C.
D.
$^{14}CH_4/CH_4$ µCi/µmol/ml
SPECIFIC ACTIVITY
0
1
2
10
20
30
Time (h)

previous experiment (4.6) of 82.4% and 17.6% for CH_4 and CO_2 respectively. The explanation for this is not readily apparent. Although this result suggested that the product distribution approximated to the theoretical pattern of 50:50 it did not confirm whether an aceticlastic reaction was operative. Thus, the alternative mechanism demonstrated by Zinder and Koch (1984) for a two-membered microbial association in which the primary species oxidised acetate to $CO_2 + H_2$ and the second species, a Methanosarcina sp., reduced the CO_2 to CH_4 still also remains a possibility. If this was the case then both methyl- and carboxyl-labelled acetate should yield $^{14}C\text{-}CH_4$ with the net reaction, however, the same as that described in Equation 38. Further labelling studies were then initiated to investigate this possibility.

Figure 48 B shows that negligible specific activity of $^{14}C\text{-}CH_4$ (0.023 μCi mol^{-1} ml^{-1}) were evolved when [1-^{14}C] acetate was used as the substrate, which suggested that the latter mechanism was not operative. Conversely, addition of [2-^{14}C] acetate (Figure 48C) resulted in immediate generation of [^{14}C] CH_4 which increased to a specific activity of 2.58 μCi $umol^{-1}$ ml^{-1} after 34 h of cultivation and although slightly higher than the previous experiment (Figure 48 A)thus confirmed that the aceticlastic reaction was the sole acetate catabolic pathway. This was subsequently confirmed when no $^{14}C\text{-}CH_4$ was detected when the culture was amended with $^{14}C\text{-}HOO_3^-$ (Figure 48D).

Similar results have also been obtained with refuse subsamples (G.B. Kasali, unpublished observations).

From these results it appeared that the metabolism of acetate by the isolated microbial association followed the pathway described by Thauer & Morris (1984).

4.8 Kinetics of acetate catabolism to methane

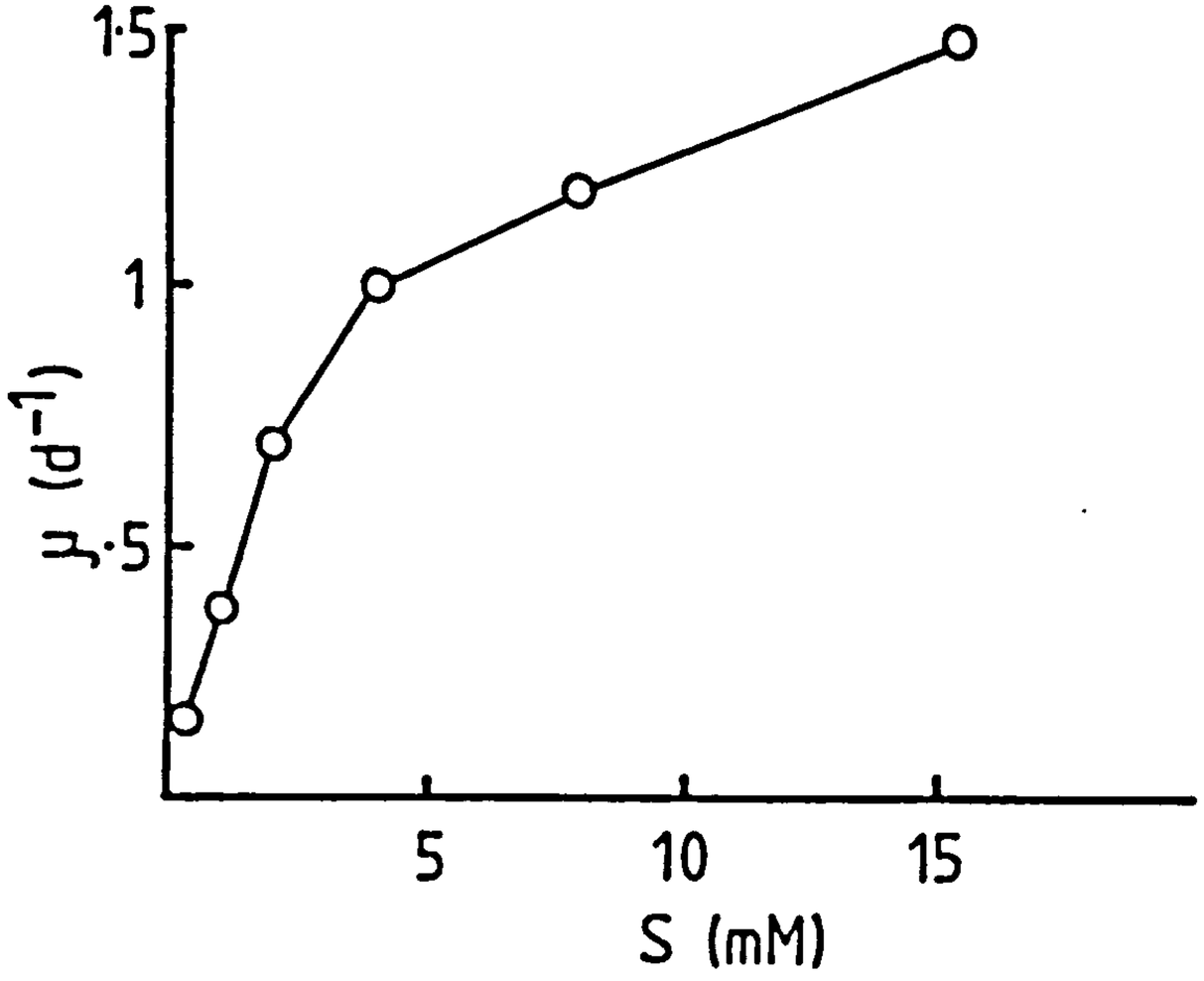
Estimation of the saturation constant (k_S) of the microbial association for acetate was undertaken in 30 ml serum bottles, which contained 9 ml basic mineral salts medium (B) under an atmosphere of O.F.N. and were closed with screw caps. The bottles were inoculated with culture (1 ml) from the first vessel of the five-stage chemostat prior to individual supplementation with acetate (0.25, 1, 2, 4, 8 and 16 mM). CH_4 evolution was monitored at regular intervals over a period of 25 days and the means of the triplicate samples were plotted against time.

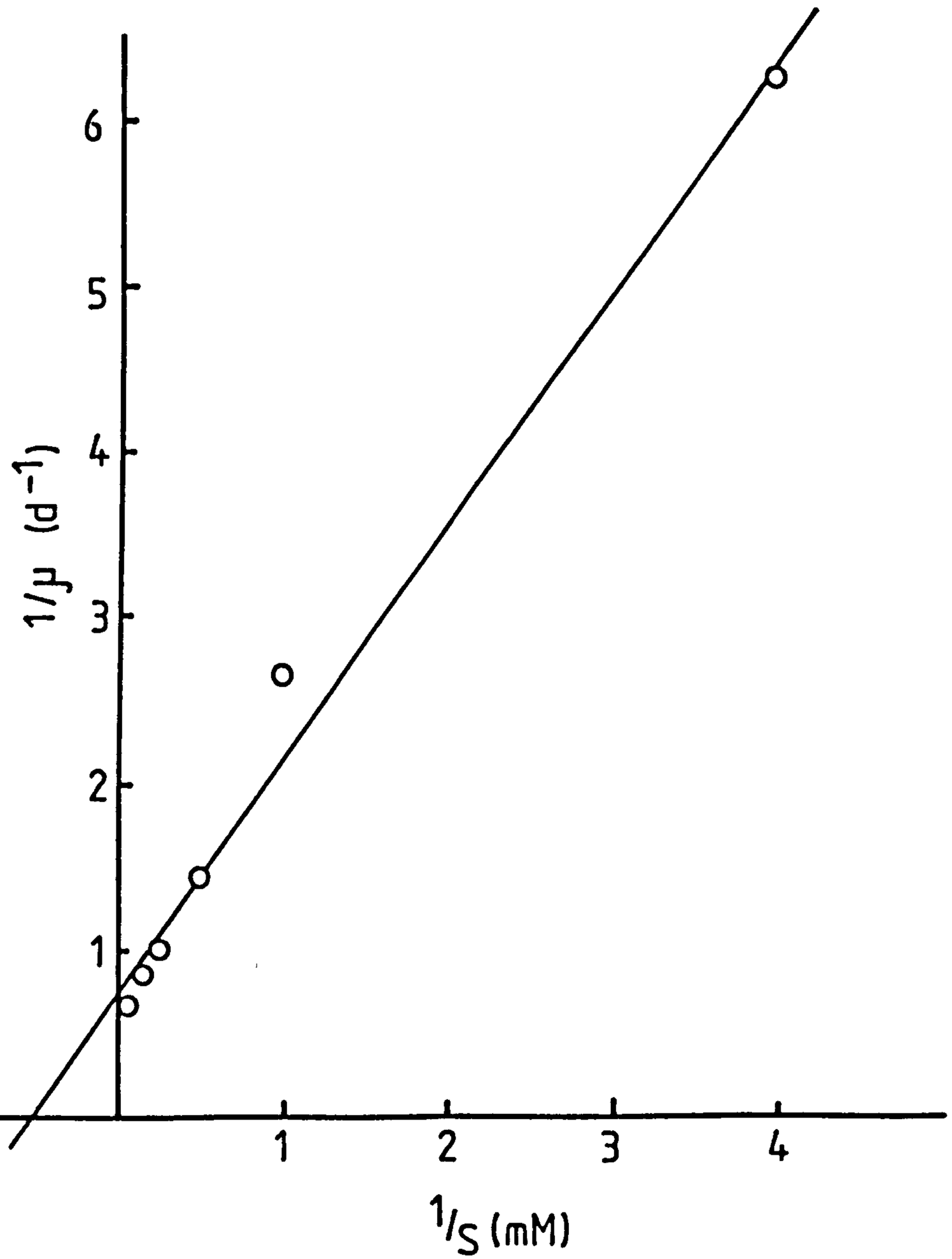
The specific growth rate at each substrate concentration was calculated from the formula $\mu = (\log_e x_t - \log_e x_o)/(t_t - t_o)\ h^{-1}$ with the assumption made that methanogenesis was growth linked.

The results were then plotted in the Monod relationship and in the Lineweaver-Burke mode (Figure 49) from which the k_S was calculated to be 1.86 mM with the μ_{max} 1.33 d^{-1}.

The method of determining the kinetic parameters were somewhat questionable since it was assumed that CH_4 was a growth-linked product. Zeikus (1983a) stated that growth and acetate utilisation

Figure 49 Kinetics of methane production from acetate and estimation of μ_{max} and k_S from Monod (A) and Lineweaver Burke (B) plots.


1·5
1
μ ·5
μ (d⁻¹)
5
10
15
S (mM)


6
5
4
3
2
1
1/μ (d⁻¹)
-1
1
2
3
4
1/S (mM)

were linked although CH_4 production occurred at the end of the growth phase. Conversely, Powell (1983) argued that CH_4 was a suitable measure of growth in batch culture when he developed an equation which showed that growth-linked gaseous products and kinetics of growth were related after several generations. As a consequence this approach was used by Kirsop (1983) and Powell (1983) to examine methanogenesis from acetate. A similar approach was used by Patel (1984) to 'accurately' determine the specific growth rate of Methanothrix concillii, an aceticlastic methanogen, when methane production was found to be proportional to the biomass formed.

The calculated k_S value of 1.86 mM of the isolated microbial association was comparable to the results obtained for Methanosarcina barkeri, 3 mM, (Schonheit, Kristjansson and Thauer, 1982) and Methanothrix soehngenii, 0.5 mM (Zehnder, Ingvorsen and Marti, 1982).

The validity of comparing k_S values of monocultures and microbial associations is however somewhat questionable. Many factors such as competition for substrate and electron acceptors, supply of growth factors, provision and/or maintenance of adequate pH and redox conditions can influence growth rate within microbial associations. In addition, the methanogens within the microbial association depend on the activities of other bacteria for their supply of substrate(s). However in this case the k_S for acetate for the association was probably a direct reflection of the k_S for

acetate of the component methanogen(s) as it has been determined previously that acetate was catabolised exclusively by the methanogenic component.

In view of the fact that at this stage it had still not been ascertained whether the production of methane from acetate was growth linked, and thus whether the k_S determination was valid, an experiment was initiated to resolve this question.

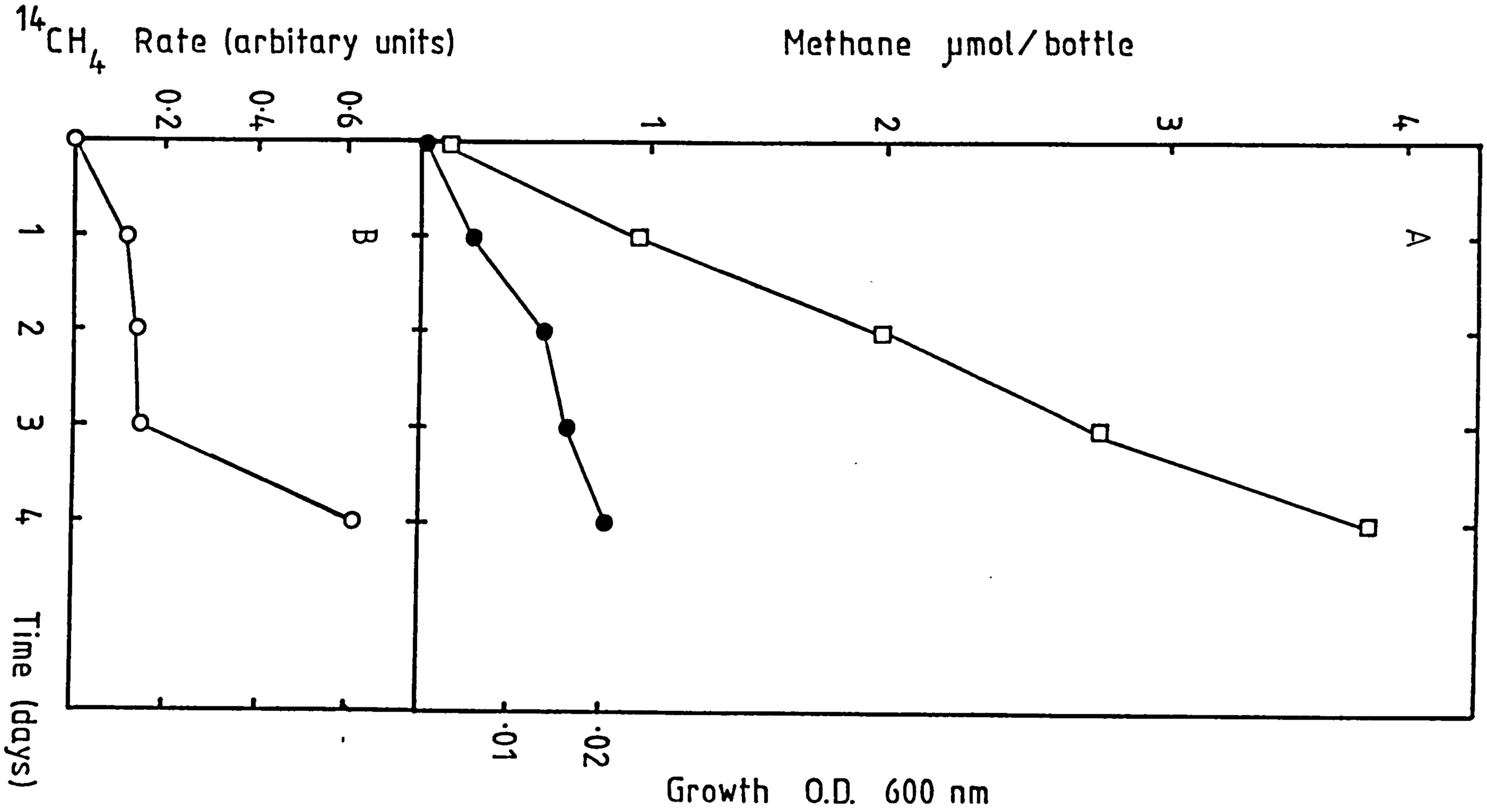
Duplicate 50 ml aliquots of basic mineral salts medium (A), maintained at 30°C under an atmosphere of OFN in 300 ml bottles were inoculated with 50 ml of culture from Vessel A of the 5-stage chemostat. Sterile pre-reduced acetate solution was added to each to give a final concentration of 15 mM. At 24 h intervals for 4 days the methane concentration and optical density (600 nm) were measured (Figure 50A).

Methane generation rate for a fixed population size was determined each day by aseptically removing aliquots from each bottle, after first correcting these for increase in O.D., by the addition of sterile anoxic mineral salts prior to inoculation with 2 μ Ci [U-^{14}C] acetate (58 m Ci mmol^{-1}; 2.14 G Bq. mmol^{-1}). The rate of [^{14}C]-CH_4 production was followed for 1 hour with gas samples taken every 15 minutes (Figure 50B).

From Figure 50A it can be seen that methane production from acetate had no measurable lag phase and the concentration increased to 4.2 μ mol.bottle^{-1} by the end of the experiment (4 days). The optical density of the culture also increased at the same time to

Figure 50A Changes in methane concentration (□ - □) and $O.D_{600}$. (● - ●) during the closed culture cultivation of the isolated microbial association in the presence of acetate.

Figure 50B: Rate of [^{14}C] methane production with time from subsamples of the above batch culture taken over a period of 4 days.


$^{14}CH_4$ Rate (arbitary units)
Methane µmol/bottle
0·2
0·4
0·6
1
2
3
4
A
B
1
2
3
4
Time (days)
·01
·02
Growth O.D. 600 nm

give a value of 0.2 units by the end of the experiment. From the results it would appear that there was a correlation between methane concentration and optical density and this in fact was confirmed when the correlation coefficient was calculated to be 0.96 (Figure 51).

The rate of [^{14}C]-CH_4 production, however (Figure 50 B), after an initial increase to 0.12 units after 1 day, remained relatively constant until day 3. Between days 3 and 4, however, the rate of methane production increased sharply to 0.6 units although this accelerated rate of production was not preceded by an accelerated increase in the methane concentration in the headspace of the initial experiment.

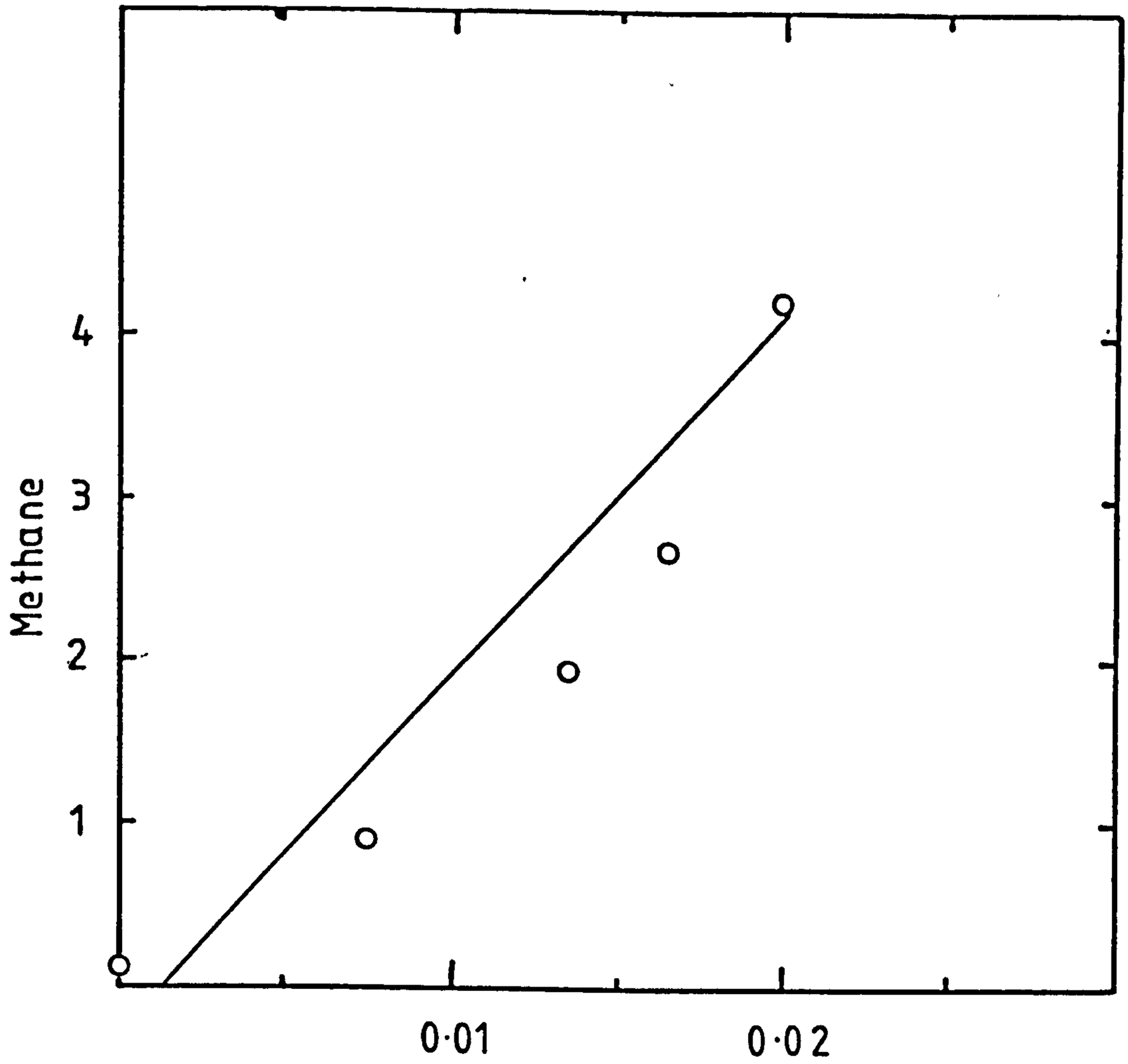
The explanation for this increase is not immediately apparent, although the possibility exists that methane production was not growth linked.

In view of the results of this experiment it was apparent that a considerable degree of caution must be exercised when using methane as a 'biomass' marker in kinetic experiments.

4.9 Effect of Hydrogen on methanogenesis from acetate

Forty five 30 ml serum bottles each containing 9 ml basic mineral salts medium (A) were inoculated with 1 ml from the first vessel of the 5-stage chemostat. 36 of these bottles were amended with sterile anoxic acetate to give a final concentration of 20 mM. The nine remaining bottles constituted the zero time controls, 3 of which were amended with acetate (alone), 3 with H_2 (alone), with the

Figure 51: Correlation between methane concentration and optical density of the isolated microbial association during closed culture cultivation in the presence of acetate.


Methane
4
3
2
1
0·01
0·02
O.D

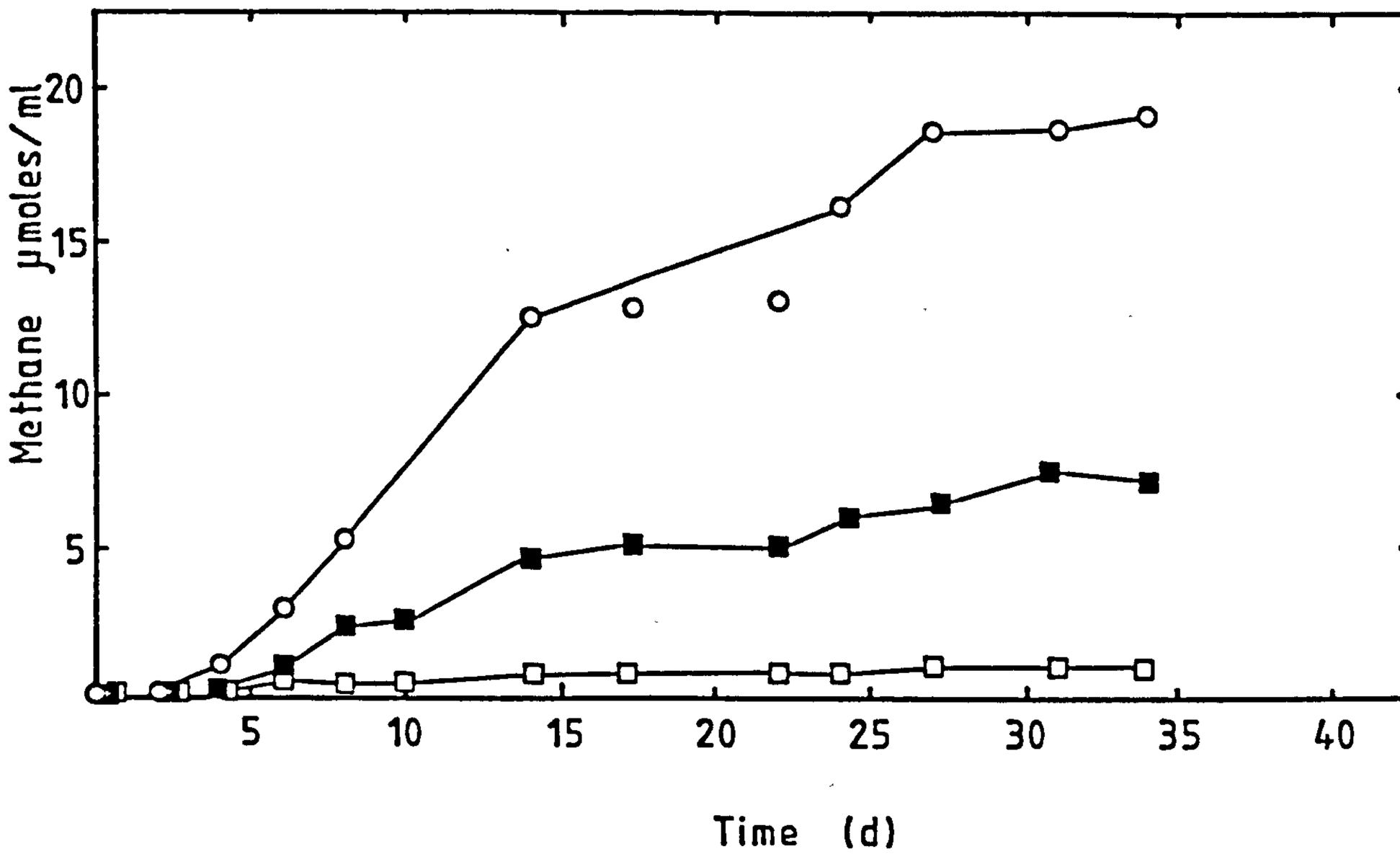
remainder untreated. Of the 36 experimental bottles, 6 were removed after 2 days incubation at 30°C and were overgassed, 3 with H_2 and 3 with O.F.N., prior to reincubation. 6 further bottles were similarly treated after 4, 6, 8, 10 and 27 days incubation. All bottles were sampled at regular intervals and were analysed for methane and in each of the cultures, CO_2 was available in the medium as HCO_3^- and CO_3^{2-}. In the presence of acetate (20 mM) alone, methane was produced after a lag period of approximately 2 days (Figure 52) and then steadily increased to a maximum concentration of 18.5 mM by day 27 at a rate of 0.96 μ mol ml^{-1} d^{-1}. The terminal concentration was equivalent to a theoretical conversion of 92.5% of the added acetate.

H_2 amendment alone, however, resulted in an increased lag period of approximately 4 days and a reduced terminal concentration of 7.5 mM. The rate of methane production (0.3 μ mol $ml^{=1}$ d^{-1}) was also considerably lower than that from acetate. The observation that acetate was a more favourable substrate than H_2/CO_2 was as expected and has been discussed earlier.

In the unamended zero control methane production was negligible thus indicating that residual substrate was absent from the cultures.

The effects of the addition of H_2 to the cultures in the presence of acetate (20 mM) are shown in Figure 53A to F for days 2 to 27 respectively.

Figure 52: Changes in concentration of methane during the closed culture cultivation at 30°C of the isolated microbial association in the presence of acetate (O - O) and H_2 (■ - ■). (□ - □) denotes untreated control.

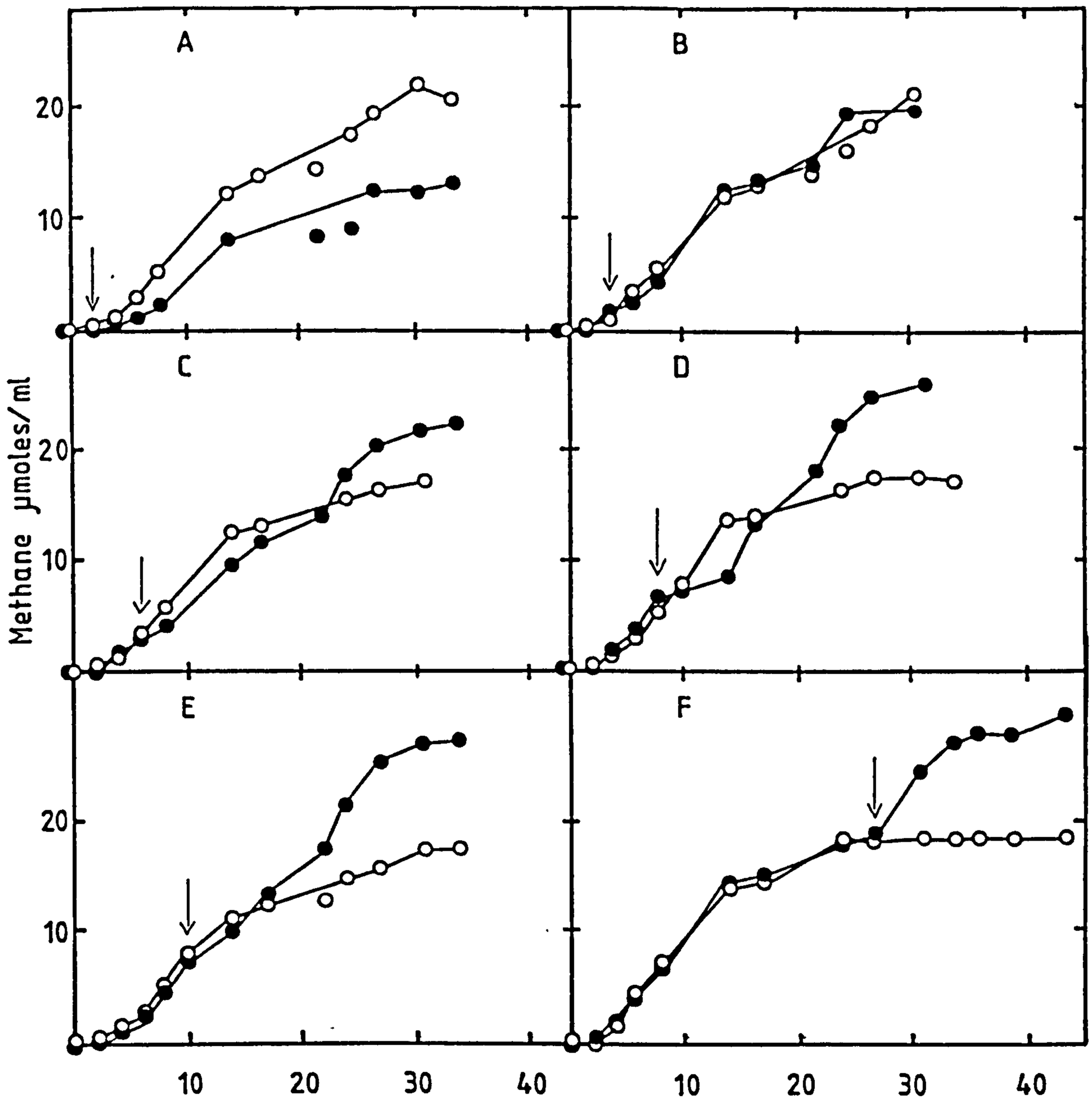

Methane µmoles/ml
20
15
10
5
5
10
15
20
25
30
35
40
Time (d)

Day 2: When the 2-day old culture was overgassed with H_2, methane was produced after a lag period of approximately 5 days and reached a concentration of 8 μ mol ml^{-1} by day 14 (Figure 53A). This then continued to increase slowly to reach 13 μ mol ml^{-1} by the end of the experiment (34 days). Overgassing the culture with O.F.N. resulted in a terminal methane concentration of 20.5 μ mol ml^{-1} which was not significantly different (at 5% probability) to the acetate alone control (Figure 52). Hence physical overgassing of the culture did not appear to affect methane production from acetate. It would, therefore, appear that the presence of H_2 resulted in partial (36.6%) inhibition of methanogenesis from acetate. The actual origin of the methane in this case, however, was hard to tender, although it is probable that the H_2 (CO_2) pathway was a contributory factor. In comparison with the H_2-supplementation alone control (Figure 52) an increase in the terminal concentration of methane of 42.3% was recorded. Thus it would appear that either 2 pathways were operative; the $H_2 + CO_2$ and a considerably inhibited acetate pathway or that the presence of acetate enhanced H_2/CO_2 conversion to methane. These possibilities will be discussed in more detail later.

Day 4: Prior to overgassing with H_2 the methane and, thus, gas displacement concentration had reached 1.5 μ mol ml^{-1}. Subsequent overgassing with H_2 then resulted in methanogenesis at a rate of 1.1 μ mol ml^{-1} d^{-1} until a concentration of 19.5 μ mol ml^{-1} d^{-1} was reached by the end of the experiment. Similarly, the bottles

Figure 53: Changes in the concentration of methane during the closed culture cultivation at 30°C of the isolated microbial association in the presence of acetate (20mM). The cultures were initiated under an atmosphere of OFN and subsequently overgassed with OFN (O - O) and H_2 (● - ●) after 2, (A); 4 (B); 6, (C); 8, (D); 10, (E), and 27 (F) days incubation.

The arrows indicate the time of overgassing.


A
B
C
D
E
F
20
10
20
10
20
10
Methane µmoles/ml
10
20
30
40
10
20
30
40
Time (d)

overgassed with O.F.N. also increased at a rate of 1.1 μmol mol^{-1} d^{-1} to reach a terminal concentration of 19.0 μmol ml^{-1}. Thus, in this case, there was very little difference between the concentrations of CH_4 produced in the H_2 and O.F.N. overgassed bottles, although compared with the previous experiment (day 2), an increase of 6.5 μ mol ml^{-1} was recorded for the H_2-overgassed experiment. Overgassing the culture with O.F.N. again resulted in no significant change compared with the acetate alone control. The increase in methane noted in the previous experiment was not due to formation from acetate prior to H_2-amendment and the exact origin of this additional methane was hard to tender although it could have been due to a greater contribution by the acetate pathway.

Days 6, 8 and 10: Overgassing the acetate-amended cultures after 6, 8 and 10 days (Figure 53C, D & E) with H_2 resulted in terminal methane concentrations of 22.0, 22.5 and 27.0 μ mol ml^{-1} respectively. As observed for the Day 2 and 4 overgassing experiments there was no significant difference between the O.F.N. controls and the acetate alone control. It would thus appear that the later the acetate cultures were overgassed with H_2, the more methane was formed. Since in each case more than 20.0 μ mole ml^{-1} methane was formed (the theoretical maximum from 20 μ mol ml^{-1} acetate) it was apparent that the H_2/CO_2 pathway was also operative. This was shown clearly in the final experiment at day 27.

Day 27 : After 27 days incubation with acetate alone (Figure 53F) the methane concentrations had reached 18.0 μ mol ml^{-1} and 17.5 μ mol ml^{-1} for the prospective H_2 and O.F.N. overgassed bottles respectively. Addition of H_2 immediately stimulated further methane production until a cncentration of 29.0 μ mol ml^{-1} was reached by the end of the experiment which was equivalent to a 37.9% increase compared with the O.F.N. control. From the results it was apparent that this increase was caused by the culture metabolising H_2 + CO_2 to methane since there was no similar increase in the O.F.N. control. The actual extent of this increase was hard to explain as in the previous H_2 alone control (Figure 52) only 7.5 μ mol ml^{-1} CH_4 were produced.

The results clearly demonstrated that the time of H_2 supplementation was critical as it appeared that the gas first inhibited and then promoted methanogenesis. In fact the results suggested that acetate catabolism to methane by the microbial association could have been regulated either by H_2 or by a product of H_2 metabolism.

The results for the control experiment (Figure 52) showed that acetate alone and H_2 alone produced 18.5 μ mol ml^{-1} and 7.5 μ mol ml^{-1} methane respectively. Hence if the two pathways were operating independently, then combination of these two substrates could collectively result in the production of 26 μ mol ml^{-1} methane. The results of the H_2-supplementation experiment showed that terminal concentrations of 13.0, 19.5, 22.0, 22.5, 27.0 and 29.0 μ mol ml^{-1}

methane were recorded for days 2, 4, 6, 8, 10 and 27 respectively. Thus supplementation on days 10 and 27 yielded results which indicated that methane was produced from both acetate and H_2 ($+CO_2$). The exact source of the methane in the other cases was difficult to ascertain, although various workers have found that the presence of H_2 inhibited acetate catabolism to methane. If this inhibition was also operative within the microbial association, it may be postulated that the earlier the H_2 was added to the culture then the less methane would be formed. Consideration of the energetics of the two pathways offers a possible explanation for this since methanogenesis from H_2/CO_2 yields considerably more free energy than methanogenesis from acetate (Thauer et al., 1977) although the actual mechanism of H_2 inhibition has not yet been resolved.

If H_2 did in fact inhibit methanogenesis from acetate then it is probable that only one species of methanogen was present which metabolised both acetate and H_2. Alternatively, if two species were present, one utilising acetate and the other H_2, then it would be unlikely that H_2 would affect methanogenesis from acetate to the extent recorded. At this stage, the former explanation seems the more likely.

In order to resolve the quesiton of whether H_2 inhibited acetate metabolism to methane, an experiment was initiated to assess the effect of H_2 on acetate catabolism without conversion of H_2 to CH_4. This was carried out by amending basic mineral salts medium in the absence of 'CO_2' (as HCO_3^- and CO_3^{2-}) with acetate under an

atmosphere of H_2.

The experimental protocol was as follows :

Acetate and hydrogen were separately and in combination added to basic mineral salts medium (A) with and without CO_2. This gave the following combinations : (1) BMS - CO_2 + OFN; (2) BMS - CO_2 + OFN + acetate; (3) BMS - CO_2 + H_2; (4) BMS -CO_2 + H_2 + acetate; (5) BMS + CO_2 + OFN; (6) BMS + CO_2 + OFN + acetate; (7) BMS + CO_2 + H_2; and (8) BMS + CO_2 + H_2 + acetate. The concentration of acetate in 2, 4, 6 and 8 was 10 mM and culture from the first vessel of multi-stage chemostat was used as the inoculum. The cultures were incubated and sampled as detailed previously.

The results are detailed in Figures 54 and 55 and Table 14.

With O.F.N. in the headspace methane was produced from both the acetate-CO_2 and acetate + CO_2 cultures (Figure 54). The presence of CO_2 in the medium in fact had little effect on the rate of methanogenesis (1.15 μ mol ml^{-1} d^{-1} for both), although there was a small difference in the terminal concentrations of 1.5 μ mol ml^{-1} in favour of the CO_2 amended cultures. These results therefore suggested that CO_2 in the medium enhanced methane production although a possible explanation for this was not readily obvious.

When O.F.N. was replaced by H_2 in the gas phase (Figure 55) significant methane production was noted in the H_2 + CO_2 and acetate + H_2 + CO_2 experiments. In the presence of H_2 +CO_2 the methane concentrations increased at a rate of 0.96 μ mol ml^{-1} d^{-1} to reach 15 μ mol ml^{-1} by day 32. With the further addition of acetate the

Figure 54 Changes in concentration of methane during the closed culture cultivation of the isolated microbial association under an atmosphere of OFN at 30°C in the presence of acetate (10 mM) in the absence (● -●) and presence (o- o) of CO_2.(■ -■) and (□ -□) denote unamended controls in the absence and presence of CO_2.

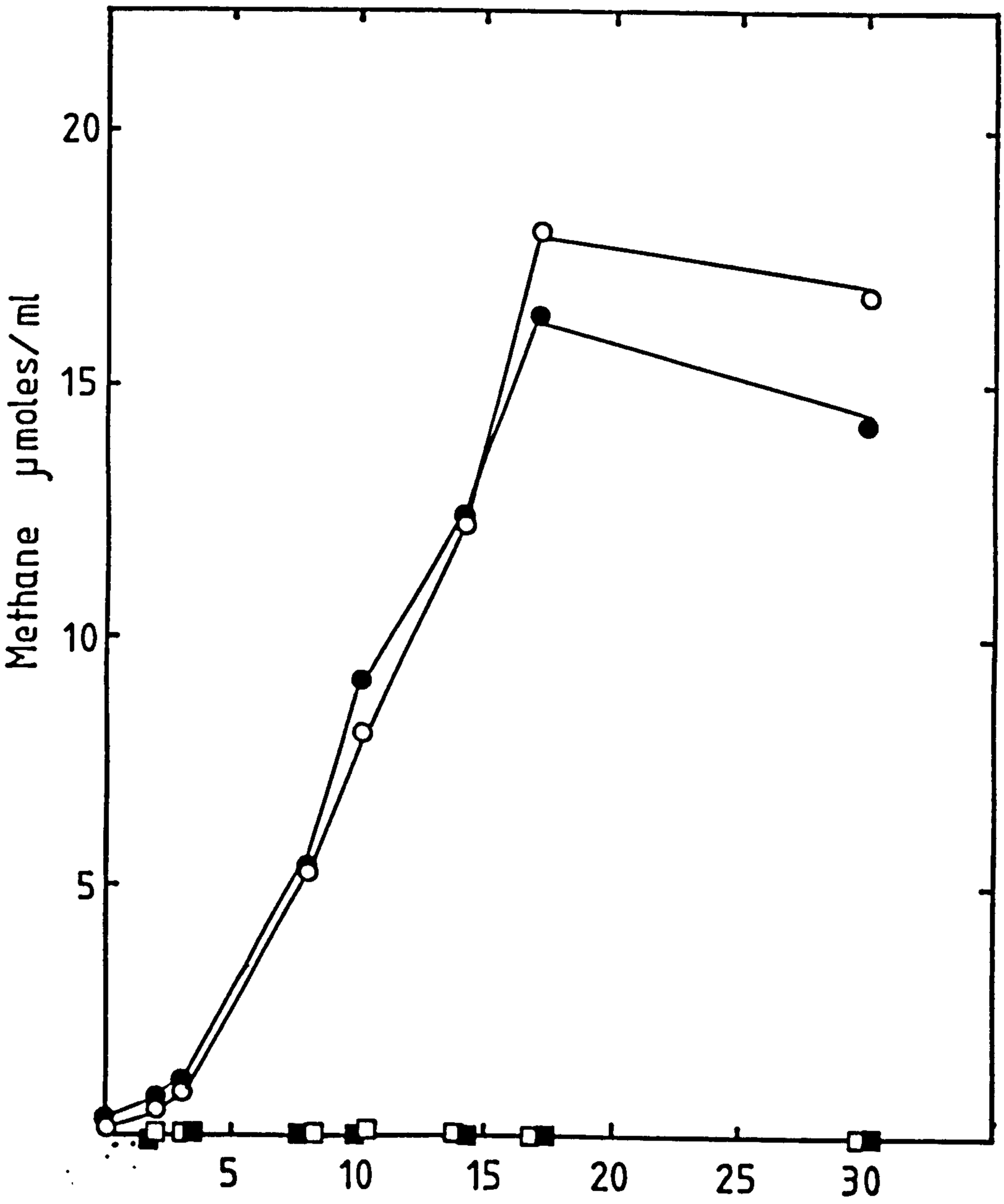
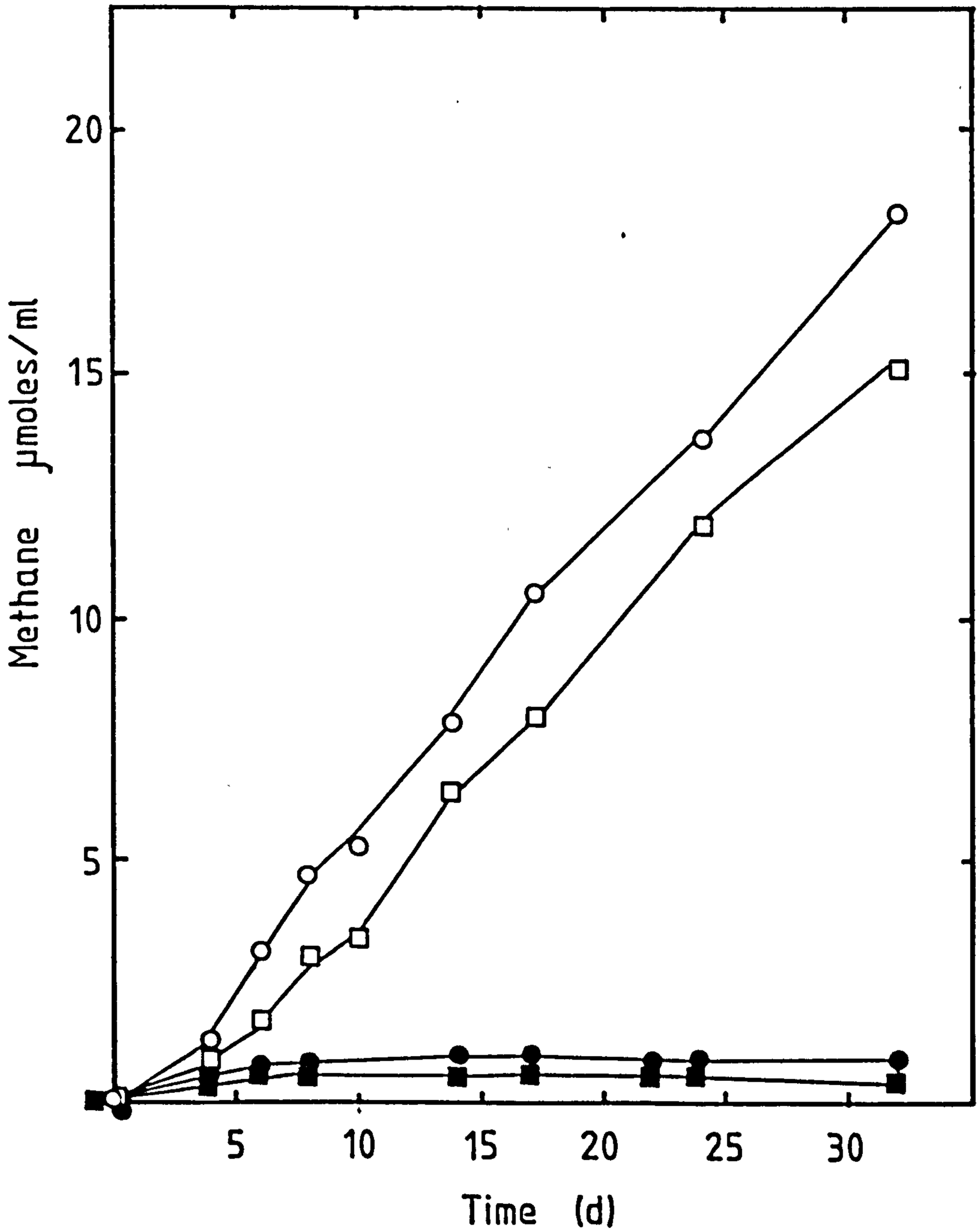

Methane µmoles/ml
20
15
10
5
5
10
15
20
25
30
Time (h)

Figure 55: Changes in concentration of methane during the closed culture cultivation of the isolated microbial association under an atmosphere of H_2 at 30°C in the presence of acetate (10 mM) in the absence (● - ●) and presence (○ - ○) of CO_2.

(■ - ■) and (□ - □) denote zero controls in the absence and presence of CO_2.


Methane µmoles/ml
0
5
10
15
20
5
10
15
20
25
30
Time (d)

methane evolution from the culture increased at an accelerated rate of 1.06 μ mol ml^{-1} d^{-1} to reach 18.0 μ mol ml^{-1} by day 32. Hence, although the presence of acetate had only a slight effect on the rate, an elevated terminal concentration of methane resulted. The exact role of acetate in this experiment was hard to determine as very little acetate was utilised with the concentration decreasing from 10.26 mM at day 4 to 9.45 mM by day 32. One possibility was that the acetate was used biosynthetically by the H_2 + CO_2 utilising methanogens, and in fact this could have been the case as the culture methane increased to 128.7 μg ml^{-1} an increase of 34.9 μg ml^{-1} compared with H_2 + CO_2 alone (Table 14). It would, therefore, appear that acetate metabolism to methane was inhibited by the presence of H_2, which was in fact shown in the experiment where very little methane was formed in the presence of acetate + H_2 (0.88 μ mol ml^{-1}) when the acetate concentration increased slightly from 11.35 to 11.83 mM.

Inhibition of acetate metabolism to methane in the presence of H_2 was confirmed by the use of labelled acetate (Figure 56).

The experimental procedure used was the same as that detailed earlier (4.7) with 2 ml of culture taken from the five-stage chemostat (Vessel A) and incubated with 2 μ Ci ml^{-1} [U-^{14}C], [1-^{14}C], [2-^{14}C] acetate and [^{14}C] HCO_3^-. Headspace gas samples were taken at regular intervals and specific activities of labelled CH_4/CH_4 (μ Ci μ mol^{-1} ml^{-1}) determined.

Comparison of Figures 56 and 48 clearly shows that the

Table 14: Effect of H_2 on acetate catabolism to CH_4 by the isolated microbial association. Culture parameters after 30 and 32 days incubation for the O.F.N. and H_2 headspace cultures respectively.

		Methane[1]	Acetate[2]	Methane Origin[3]	Culture Dry Wt[4]
1.	BMS $-CO_2$ + OFN	0	ND	-	ND
2.	BMS $-CO_2$ + OFN + acetate	16.5	ND	acetate	ND
3.	BMS $-CO_2$ + H_2			-	47.2
4.	BMS $-CO_2$ + H_2 + acetate	0.88	11.83	-	26.7
5.	BMS + CO_2 + OFN	0	ND	-	ND
X 6.	BMS + CO_2 + $O_F^{\wedge}N$ + acetate	18.0	ND	acetate	ND
7.	BMS + CO_2 + H_2	15	0	H_2/CO_2	93.8
8.	BMS + CO_2 + H_2 + acetate	18.0	9.46	H_2/CO_2	128.7

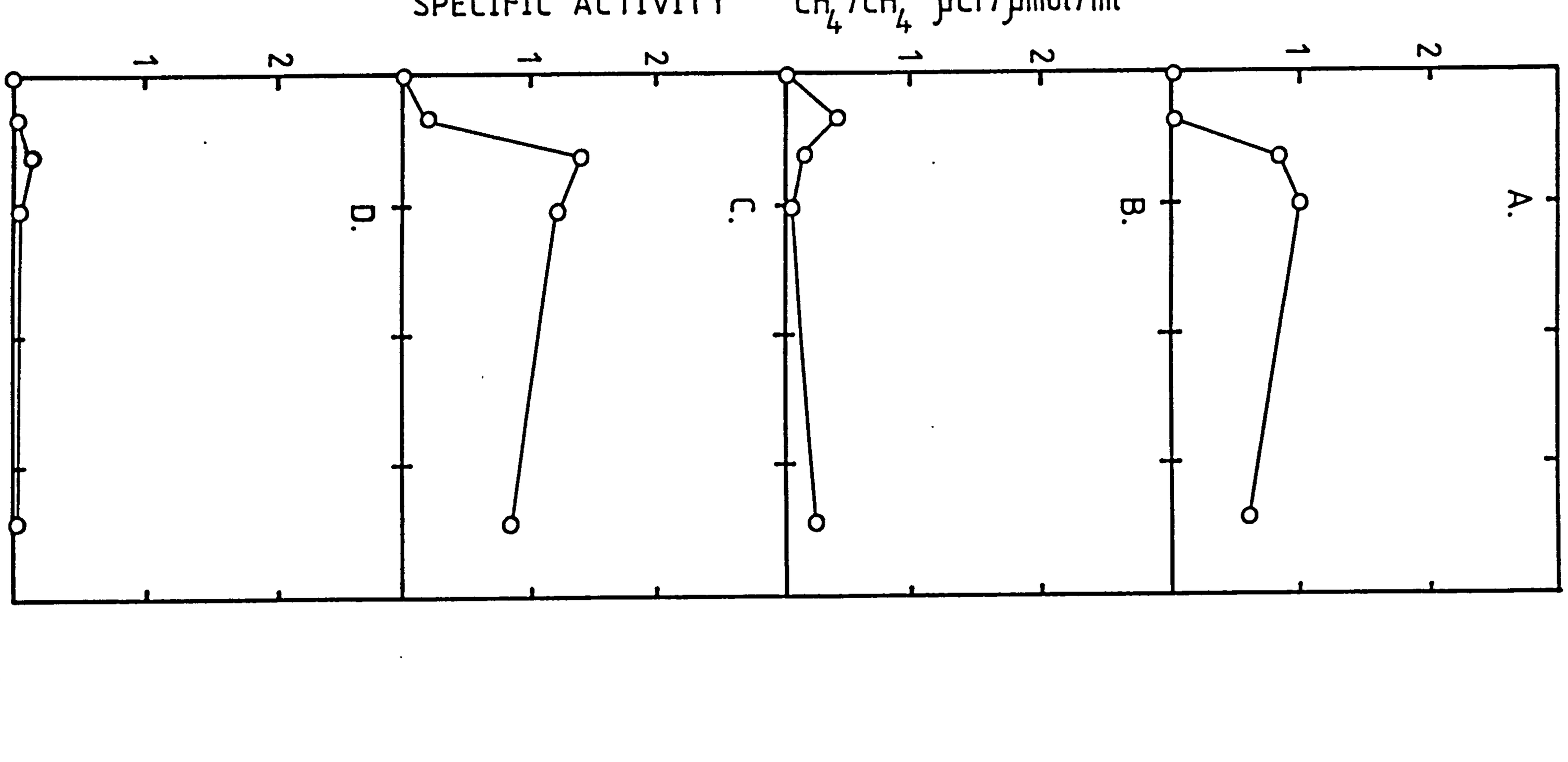
1 µmol ml^{-1}

2 µmol ml^{-1}

3 conjectural

4 µg ml^{-1}

Figure 56: Changes in culture headspace ^{14}C-CH_4/CH_4 specific activities during the anoxic closed culture cultivation of the isolated microbial association under an atmosphere of H_2 in the presence of U-^{14}C Acetate (A), 1-^{14}C Acetate (B), 2-^{14}C Acetate (C) and ^{14}C-HCO_3^- (D).


SPECIFIC ACTIVITY $^{14}CH_4/CH_4$ µCi/µmol/ml
1
2
A.
B.
C.
D.
10
20
30
Time (h)

presence of H_2 had a significant effect on the evolution of ^{14}C-CH_4. Addition of U-^{14}C acetate resulted in an initial increase in the specific activity to 1 $\mu Ci \mu mol^{-1}$ ml^{-1} after 10 hours after which a decrease was observed. With 1-^{14}C acetate (Figure 56B) there was an initial increase in the specific activity to 0.4 $\mu Ci\ \mu mol\ ml^{-1}$ after which a decrease and finally a slight increase to 0.3 $\mu Ci\ \mu mol^{-1}$ ml^{-1} by the end of the experiment. When methyl-labelled acetate was added (Figure 56C) the pattern of specific activity was similar to that with universal-labelled acetate except in this case the maximum activity (after 7 hours) was 1.4 $\mu Ci\ \mu mol^{-1}$ ml^{-1}. The results presented here confirmed that H_2 did have an effect on acetate catabolism to methane, although the inhibitory effect was not apparent immediately (only after approximately 7 to 10 hours). The explanation for this delay was not immediately apparent although it could have been due to the poor solubility of H_2.

4.10 Summary

The results of this series of batch culture experiments led to the following conclusions :

1. The microbial association isolated in the multi-stage chemostat degraded hexanoate initially by a syntrophic co-culture between (A), a β-oxidiser and a sulphate-reducing bacterium in the presence of sulphate, and (B) a β-oxidiser and an acetogen/methanogen when sulphate was absent (or depleted);

2. The acetate produced by B-oxidation was metabolised by a methanogen;

3. Although both acetogenic and methanogenic activity was observed with the culture growing on H_2 + CO_2, and fluoroacetate inhibited methanogenesis, it was difficult to conclude for certain the interactions between these two groups. However, the results did suggest that conversion of H_2 and CO_2 to methane proceeded via homoacetogenesis and susbequent methanogenic conversion of the acetate to methane;

4. Acetate conversion to methane by the methanogenic component of the microbial association was via the classic aceticlastic route; and

5. Acetate metabolism to methane was completely inhibited in the presence of H_2, although if CO_2 was available then methane could be producedfrom reduction of CO_2. The mechanism of inhibition could not be determined from the experiment although it would appear to have been time-dependent, that is, the earlier H_2 was added to the acetate catabolising culture the more complete the inhibition.

5. General Discussion

One of the major problems encountered when investigating the complex interspecies interactions of terminal anoxic catabolism is compartmentalisation of the species under study without disturbing the complete association. The multi-stage chemostat, especially when subjected to a non-constant dilution rate (the 3-vesel system), proved to satisfy this criterion.

Under a constant dilution rate regime in the presence of 1.4 mM influent sulphate, all the major metabolic events, namely, hexanoate catabolism, sulphate reduction, acetogenesis and methanogenesis, occurred in the first vessel. This was also apparent when the influent sulphate concentration was increased to 5 mM, which suggested that at these two concentrations, there was considerable overlap of species activities domains or localisation around habitat domains. When the influent sulphate was increased to 10 mM evidence of partial displacement of the methanogens was found, although hexanoate was still completely dissimilated in the first vessel.

As hexanoate was dissimilated, butyrate, propionate and acetate were detected. Butyrate and acetate were the expected intermediates if hexanoate was degraded by β-oxidation (the "common" method of fatty acid metabolism). The appearance and subsequent disappearance of propionate at the two lower influent sulphate concentrations was unexpected and not readily explainable, especially with the hindsight of subsequent batch culture experiments.

The role of sulphate-reduction to the integrity of the

microbial association was demonstrated by the shift in metabolic intermediates on increasing the influent sulphate concentration. This was probably facilitated by the increased H_2-utilising capacity (both thermodynamically and kinetically) of the sulphate-reducing bacteria compared with the methanogens and homoacetogens. Increased H_2-removal could allow both greater metabllism of hexanoate and butyrate to acetate and subsequent metabolism of the latter to methane. It was apparent that the sulphate-reducing bacteria became limited by carbon and/or electron donor sources since in the presence of 5 and 10 mM influent sulphate residual sulphate was measured in the vessels; a situation similar to results found in the natural environment. By introducing a non-constant dilution rate regime, the methanogenic bacteria were displaced from the first vessel ($D = 0.05\ h^{-1}$) and localised in the second vessel which was subjected to a dilution rate of $0.015\ h^{-1}$. Thus, it would appear that either the methanogens had a $D_{crit} < 0.05$ and $> 0.015\ h^{-1}$ or that the methanogens were competitively displaced by the sulphate-reducing bacteria, whose activity was confined to vessel A. The outcome of this displacement, compounded by sulphate limitation in vessel A, resulted in the presence of residual substrate. Hence, reduction ofCO_2 (to methane) became the major electron sink for hexanoate oxidation in vessels B and C.

From the results of the studies with the two multi-stage chemostats, it was evident that four groups of bacteria (H_2-producing acetogenic, sulphate-reducing, homoacetogenic and

methanogenic) constituted the interacting microbial association. In the presence of excess sulphate, the sulphate-reducing bacteria were the major H_2 sinks of H_2 formed from β-oxidation of volatile fatty acids. This interaction constituted a first tier association. The second tier was that of interaction between acetate formation, by the H_2-producing acetogens, and acetate dissimilation by methanogenic bacteria. When sulphate was limited, sulphate reduction was replaced by homoacetogenesis/methanogenesis as the route of H_2-removal.

From a series of closed culture experiments, initiated to further investigate the microbial interactions involved in the metabolism of hexanoic acid by the isolated microbial association, two key areas were identified : (a) metabolism of hexanoic acid and its putative breakdown products, and, more specifically (b) methanogenesis from acetate.

Although reported studies with batch cultures, studying hexanoate dissimilation, involved the use of co-cultures, of a proton-reducing acetogen bacterium together with a H_2-utilising methanogen or sulphate reducer, and were characterised by acetate accumulation, this feature was not observed in this study when acetate was metabolised ot methane.

Although sulphate was found to have a dramatic effect on the metabolism of hexanoic acid in the multi-stage chemostat models, similar, but less dramatic, results were found in batch culture with significant changes in the stoichiometry of the reaction apparent

where increased rates of butyrate dissimilation and acetate formation, and reduced terminal methane concentrations, were coincident with a residual sulphate decrease of 5 mM. Since methane was generated from acetate even in the presence of sulphate, it may be assumed that sulphate-reducing activity was confined to H_2-removal.

Although the removal of H_2 to facilitate oxidation of volatile fatty acids is well documented, the possible role of acetate removal in volatile fatty acid dissimilation is not fully resolved.

In the absence of sulphate, both acetate and methane were formed from H_2/CO_2, which confirmed homo-acetogenic activity within the microbial association. Whether the methanogens and homo-acetogens were competing for or both equally utilising H_2/CO_2, was not resolved. However, another possibility did exist that the homoacetogens produced acetate exclusively from H_2/CO_2 to the exclusion of methanogenesis, whilst the latter generated CH_4 from the acetate produced. This possibility was supported by the observation that the presence of fluoro-acetate significantly inhibited methanogenesis from H_2/CO_2. The results obtained from the use of this inhibitor, however, were not conclusive. Although the microbial association was found to metabolise hexanoate and butyrate, propionate was not dissimilated. The reason for this, particularly in view of the observed propionate dissimilation in the multi-stage chemostat, was hard to explain.

Acetate metabolism by the methanogenic component of the microbial association was found to be "conventional", namely, the aceticlastic mechanism.

The critical role of H_2 in methanogenesis from acetate was clearly shown in the time supplementation studies when it was seen that H_2 first inhibited and then promoted methanogenesis. The results, in fact, suggested that metabolism of acetate by methanogens was regulated by H_2 or a product of H_2 metabolism. Consideration of the energetics of the two pathways offers a possible explanation, since methanogenesis from H_2/CO_2 yields considerably more free energy than methanogenesis from acetate.

In summation : a microbial association was isolated from landfill by use of a multi-stage chemostat. The association was found to contain the four groups of bacteria as outlined above. Although experiments with batch cultures tended to confirm the conclusions drawn from the multi-stage chemostat studies, some anomalies, namely propionate formation and utilisation, the concentrations of residual acetate, especially in the low influent sulphate systems, and the presence of residual sulphate, particularly with the system subjected to 5 mM influent sulphate, remain unresolved.

The multi-stage chemostat did, however, prove to be a useful model for studying the landfill ecosystem, which is characterised by overlapping niches and sequential usage of transient concentrations

of electron acceptors, although complementary closed culture studies were necessitated.

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7.1 APPENDIX 1

Experimental design; optimisation of the culture volume : headspace ratio

The possibility of end product inhibition in methane fermentations could originate from partial pressures of CH_4 and/or CO_2 although previous work by Hansson & Molin (1981) showed that CO_2 and not CH_4 was inhibitory. for example, at a partial pressure of 1 bar of CO_2 acetate utilisation by methanogens was inhibited by approximately 60% (Hansson & Molin, 1981). The workers also found that CO_2 only significantly effected degradation of acetate and propionate but not with butyrate and glucose.

The exact mechanism of this inhibition is hard to ascertain as methane concentrations up to 1 bar had no substantial inhibitory effects, although the possibility exists that the inhibitory effect could have been connected with solubility of the gases which is high for CO_2 and low for CH_4. CO_2 solubility could also have effected the pH of the culture which in turn could have had an effect on the catabolism of methanogenic substrates. CO_2 in solution may be found in any one of 3 forms : free CO_2, HCO_3^- and CO_3^{2-} according to the pH. Hence the addition of CO_2 would tend to make the solution more acidic and conversely removal of CO_2 would render the solution alkaline. However, the amount of free CO_2 present in excess of equilibrium CO_2 is sometimes called 'aggressive CO_2' as it is able to react with alkaline carbonates or metals. This could be envisaged as having an effect on anaerobic batch cultures as gaseous products would naturally accumulate in the headspace and thus increase the

pressure inside the vessel. The effect of 'aggressive CO_2' on metabolic processes is, however, unknown.

An experiment was initiated to investigate the effect of culture headspace : liquid volume on the methanogenic degradation of hexanoic acid.

Experimental, results and discussion

50 ml aliquots of basic mineral salts medium (B) were dispensed into triplicate flasks of increasing volume thus providing headspace : culture volume ratios from 0.5 to 11.0. Each flask was inoculated with 3 ml of culture and supplemented with 5 mM hexanoate and 20 mM sulphate. The flasks were incubated in the dark stationary at 30°C and culture headspace was analysed for methane at regular intervals.

After 3 days incubation (Figure 57) the methane had reached concentration of 0.06 μ moles ml^{-1} at a ratio of 0.5. The concentration then gradually increased to 0.257 μ moles ml^{-1} at the highest ratio (11.0). Hence at this stage the general trend was that the higher the ratio the more methane was produced.

After 7 days incubation at the smallest ratio (0.5) the methane concentration had increased to 5.5 μ moles ml^{-1} (Figure 58). The methane gradually decreased to 5.0 μ moles ml^{-1} at a ratio of 1.6 and then abruptly peaked at a ratio of 2.0 with 6.2 μ moles ml^{-1} CH_4 detected. The CH_4 concentration then gradually decreased to 3.7 u moles ml^{-1} at a ratio of 5.0 and 0.5 μ moles ml^{-1} at a ratio of 11.0. Thus the trend which was noted 4 days previously had changed

Figure 57: Methane production after 3 days incubation in the presence of 5 mM hexanoate under different headspace:culture volume ratios.

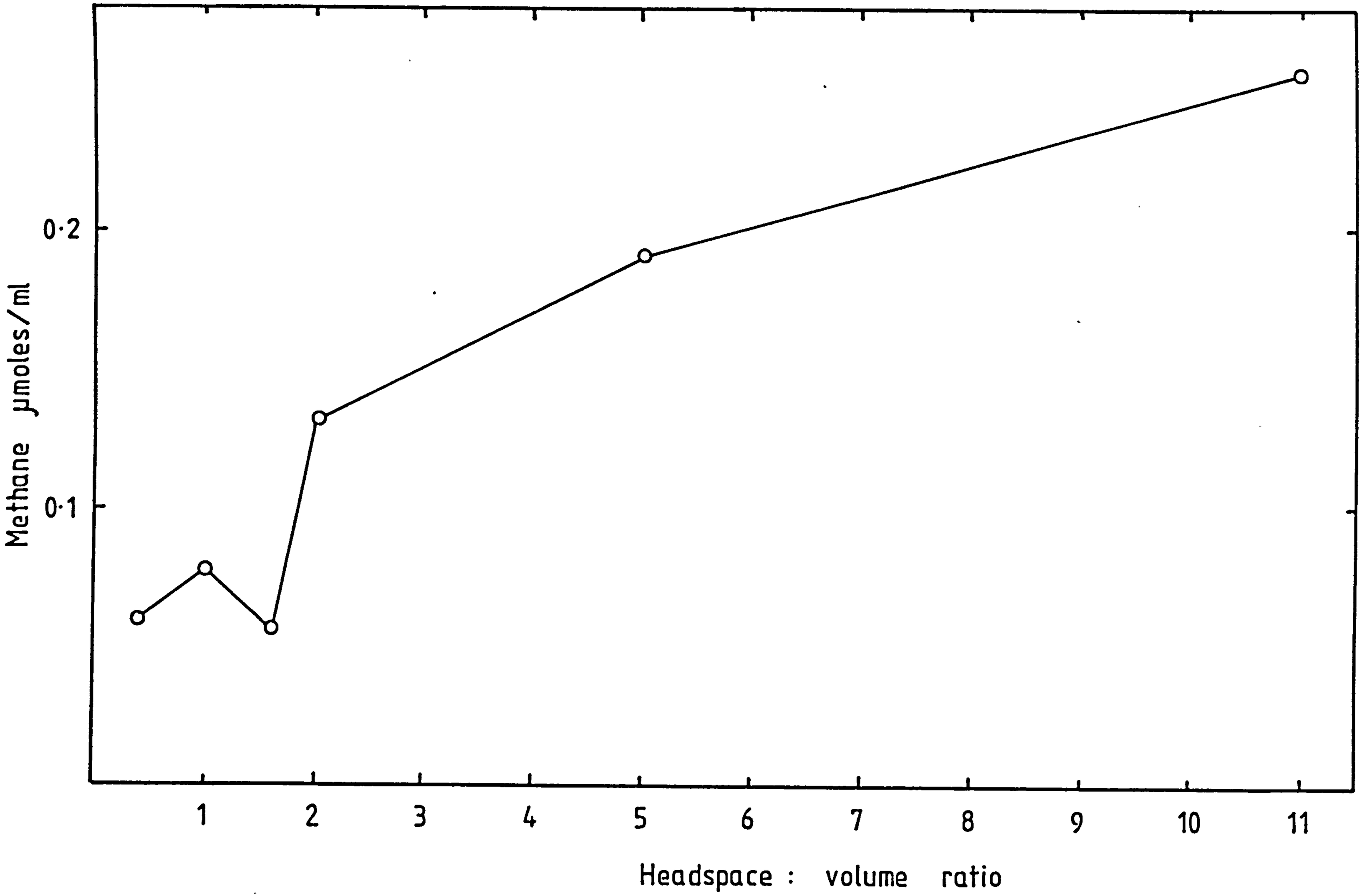
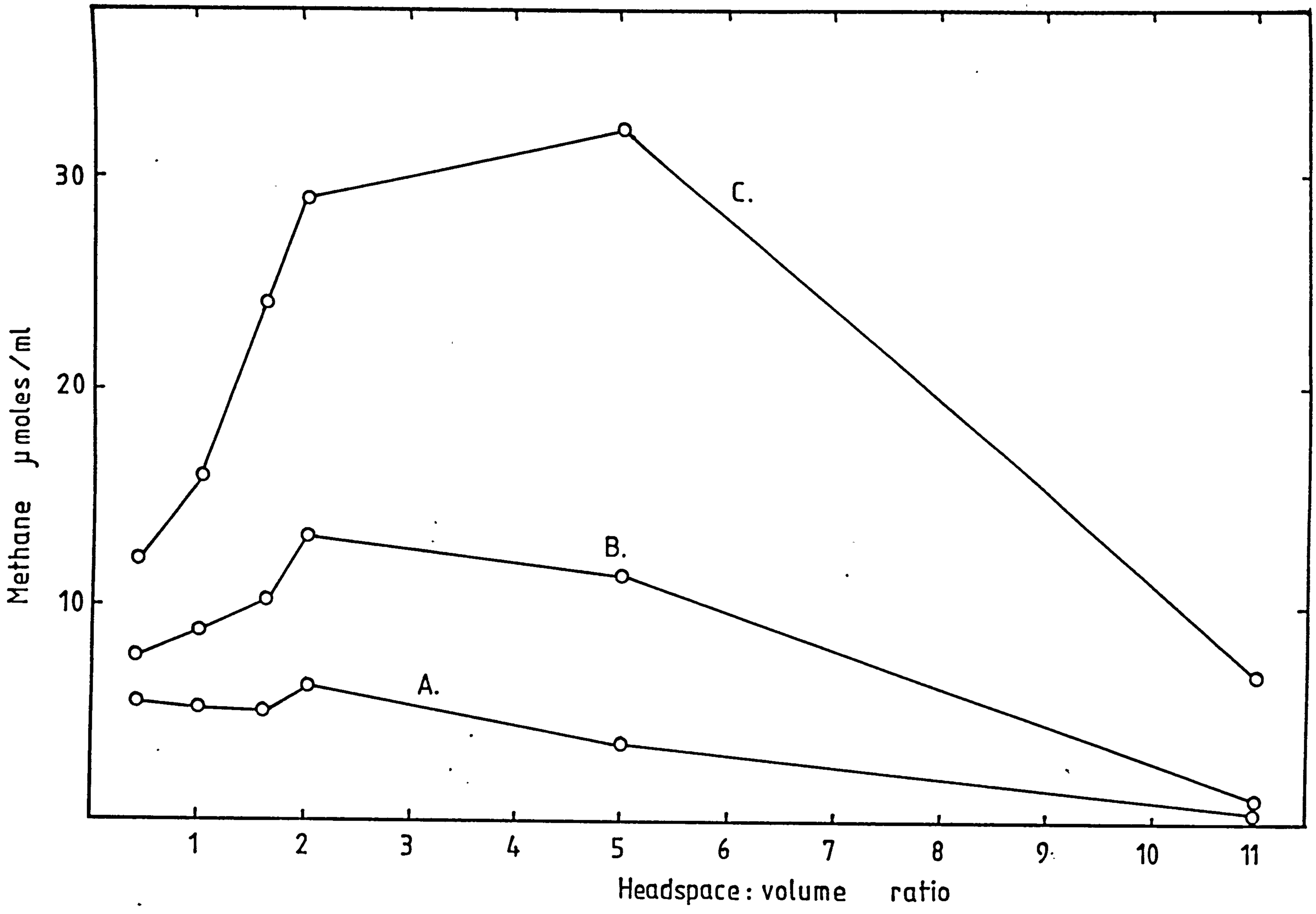

Methane µmoles/ml
0·2
0·1
1
2
3
4
5
6
7
8
9
10
11
Headspace : volume ratio

Figure 58: Methane production after 7(A), 10(B), and 15(C) days incubation in the presence of 5 mM hexanoate under different headspace:culture volume ratios.


Methane µmoles/ml
30
20
10
C.
B.
A.
1
2
3
4
5
6
7
8
9
10
11
Headspace : volume ratio

and there appeared to be a peak in methane production at a headspace:culture ratio of 2.0.

After 3 further days incubation this same trend was observed with 7.6 μ moles ml^{-1} CH_4 detected at a ratio of 0.5, 13.2 μ moles ml^{-1} at 2.0 and 1.2 μ moles ml^{-1} at a ratio of 11.0.

By day 15 (C) the methane concentration in the lowest ratio (0.5) was 12.0 μ moles ml^{-1} which subsequently increased linearly at a rate of 21.0 μ moles ml^{-1} unit $ratio^{-1}$ to give a concentration of 29.0 μ moles ml^{-1} with a ratio of 2.0. The methane concentration increased slightly to 32.1 μ moles ml^{-1} when the headspace:culture ratio was raised to 5.0. This concentration, however, subsequently decreased to 7.0 μ moles ml^{-1} with the highest ratio (11.0).

The results were surprising, insofar as methane production was inhibited at the highest ratio (11.0) when one would have expected the concentration/ml culture to increase due to absence of inhibition by gaseous products. An explanation for this inhibition is hard to tender,although one possibility could be that redox conditions within the high ratio flasks were unsatisfactory, and, therefore, somewhat inhibitory for growth of the strict anaerobes within the microbial association. This possibility is illustrated in Figure 58 where at the high ratio the depth of culture is of importance since this could affect the redox gradient in the culture. On the other hand it was expected that the low ratios would be slightly inhibitory and in fact the methane concentration in the lowest ratio (0.5) was 62.6% less than that found in the 5.0 ratio

flask.

The results then suggested that when lower ratios are used in experiments, then the higher pressures of gaseous end products which result are inhibitory. Whether the inhibitory product in this instance was methane or carbon dioxide or a mixture of the two, is hard to ascertain. However interpretation of the results recommend that when batch culture experiments are initiated they should have a headspace:culture ratio between 2.0 and 5.0.

7.2

APPENDIX 2

Analysis of V.F.A. by Gas Liquid Chromatography

Volatile fatty acids are important intermediates in the degradation of organic material (Zeikus, 1983b). Although significant quantities are formed in anaerobic metabolic processes, the intermediates are usually converted to the terminal products CH_4 and CO_2.

The analysis of V.F.A. can prove to be a useful indicator of metabolic activity in anoxic environments. For example, the V.F.A. concentration in sludge digesters has been recognised as an important operational parameter as a sudden rapid production generally indicates impending failure (Andrews, Cole & Pearson, 1964).

Total volatile fatty acids can be determined colourimetrically or by titration. However, with both these methods individual V.F.A. are not qualified or quantified.

High performance liquid chromatographic separation and quantification of V.F.A. have been reported by amongst others, Ehrlich et al. (1981). The main disadvantage of H.P.L.C., however, is the protracted assay times and poor detector responses.

Ion-exclusion chromatography, although sensitive,requires a vacuum distillation stage and also most runs can take a substantial time interval. For example, Parkes & Taylor (1983b) reported an assay time of ~ 45 minutes for hexanoate.

The majority of methods reported in the literature involve G.L.C. analysis although resolution of free fatty acids by gas liquid chromatography has been limited by : (1) adsorption of the acids in the column; and (2) postulated molecular association of the acids in a vapour state.

With G.L.C. methods, however, adsorption is recognised as the more significant problem since this can lead to "tailing" of the peaks, irregular shaped peaks and 'ghosting'. 'Ghosting' occurs when a solution more polar than the acids being analysed is injected subsequent to a solution of free fatty acids. The result is that acids previously adsorbed in the column are eluted. 'Ghosting' is dependent on the column packing, acid nature, injector type, nature of the "ghost" eluter and injection sequence (Van Eenaeme et al., 1974).

Due to these difficulties, V.F.A. are often first converted to their esters prior to analysis (Martin & Swinehart, 1968).

Four main methods have been used for overcoming the problems associated with analysing V.F.A. by G.L.C; (1) use of a highly polar liquid phase; modification of the (2) liquid phase; or (3) the inert support; or (4) the carrier gas (Henderson & Steedman, 1982).

According to Henderson & Steedman (1982) use of polar liquid phases has had little success, although modification of the liquid phase has been widely used. Characteristically this has involved the addition of a non-volatile acid to the liquid phase for the purpose of suppressing hydrogen-bonding and the resulting

dimerisation. Phosphoric acid has been the most commonly used additive, for example, Dees & Moss (1979) used 15% SP 1200 + 1% H_3PO_4 to separate C_2 to C_4 acids, although the peaks "tailed" badly and acetic acid eluted on the "tail" of the solvent.

Porous polymers have been used both with and without modification. For example, Henderson & Steedman (1982) used Poropak Q with oxalic acid to facilitate separation of C_2 to C_6 acids successfully.

Ackman & Burgher (1963) reported that if the free fatty acids were analysed in the continually polar atmosphere of formic acid the problems with adsorption would be minimised.

Despite these limitations the analysis by G.L.C. still has the obvious advantages of sensitivity, speed of analysis and resolution.

Three methods of volatile fatty acid analysis were examined. In the first, an ether extraction step was used prior to separation on a column of 15% SP 1200 + 1% H_3PO_4 on Chromosorb W.A.W. In the second, the samples were acidified with formic acid prior toinjection onto 10% F.F.A.P. on Chromosorb G.A.W. Finally, the acidification and direct injection technique was repeated with neopentyl glycol sebacate on Anakrome polyester as the column support material.

Experimental, results and discussion

Method 1

In this method all manipulations were made in ice baths. 1 ml of culture supernatant was added to approximately 0.4 g NaCl

followed by 0.2 ml of 6M HCl and 1 ml of diethyl ether. The samples were shaken in the sealed vial and allowed to stand overnight at +4°C. 2 µl of the ether layer were then injected into the chromatograph.

A glass column (length 2 m, external diameter, 4 mm) was filled with 15% SP 1220 + 1% H_3PO_3 on acid washed Chromosorb W.A.W. (mesh size 100 - 120) (Phase Separations Ltd.). The flow rate of the carrier gas (Argoshield 5) was set at 40 ml min^{-1} with the temperatures of the column, detector and injector at 150°, 200° and 200°C respectively.

Acetate, propionate, butyrate and hexanoate were prepared as standards (10 mM) as above.

A typical G.L.C. trace obtained by this method is shown in Figure 59. A large solvent peak (ether) with a retention time of 0.56 min was followed by the V.F.A. peaks, acetate, propionate and butyrate which were detected after retention times of 1.85, 2.25 and 3.01 min respectively. Unfortunately these peaks fell on the solvent tail and hence the results would be difficult to quantify accurately. Hexanoate (6.59 min) gave a poor response and as a consequence a badly shaped peak was obtained.

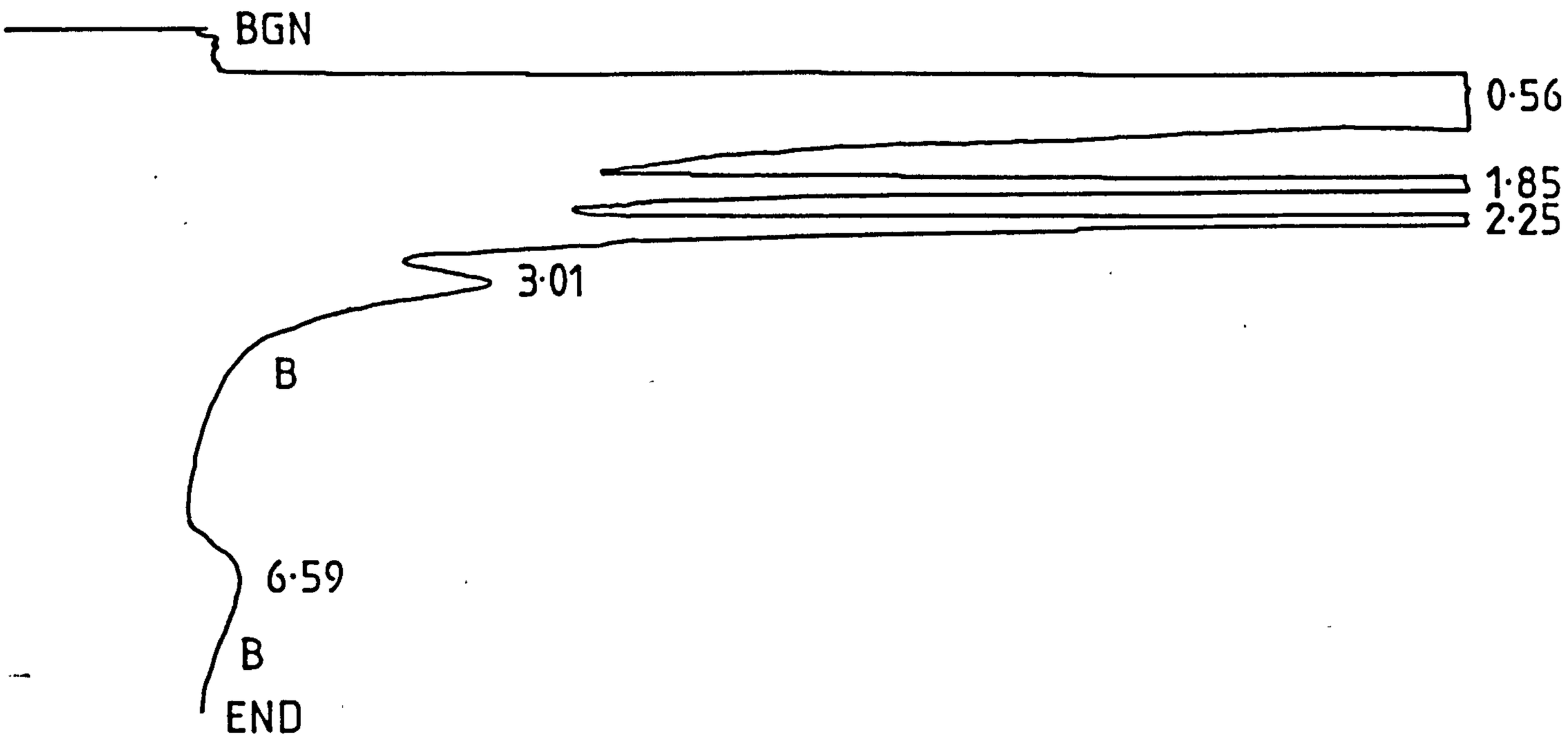
As a consequence of the above, and following the development of methods 2 and 3, this method was abandoned.

Figure 59: Separation of a sample which contained acetate, propionate, butyrate and hexanoate at retention times of 1.85, 2.25, 3.01 and 6.59 min respectively. The solvent peak, ether, eluted after only 0.56 min.

BGN = Begin.

END = End.

B = Basepoints


BGN
0·56
1·85
2·25
3·01
B
6·59
B
END

Method 2

Samples, 0.9 ml of the culture supernatant, were acidifed with 0.1ml of formic acid 'Aristar' (B.D.H.). 1 µl of this solution was then directly injected onto the column. The column, 10% F.F.A.P. on Chromosorb G.A.W. (80-100 mesh) (Phase Separations Ltd.) was maintained at 150°C. The injector and detector temperatures were both 200°C. The carrier gas (O.F.N.) flow rate was set at 40 ml min^{-1}.

Before commencing analysis, 10 µl of 10% formic acid was injected onto the column and the baseline allowed to stabilise.

A standard mixture of 10mM of each of acetate, propionate, butyrate, valerate, methyl-butyrate and hexanoate were effectively resolved by Method 2. Acetate was eluted after 2.74 min and hexanoate after 11.96 min. although better resolution was obtained compared with method 1, the assay time was much longer.

From the results it can be seen that sharp peaks were obtained with very little tailing.

The peak at 3.91 min was unidentified and was associated with 10% formic acid chemical contaminants.

The method was calibrated with acetate, propionate and butyrate, Table 15 and Figure 60. The correlation coefficients of each of the V.F.A. standard curves was greater than 0.99 with the intercept close to the origin; acetate, -0.315, propionate, -0.3537 and butyrate, -0.157.

Table 15: Statistical analysis of calibration curves for the V.F.A.,acetate, propionate and butyrate giving the correlation coefficient, the slope, the intercept with the y-axis and the integrator (y axis) results with the V.F.A. concentration set at 10 mM (x axis).

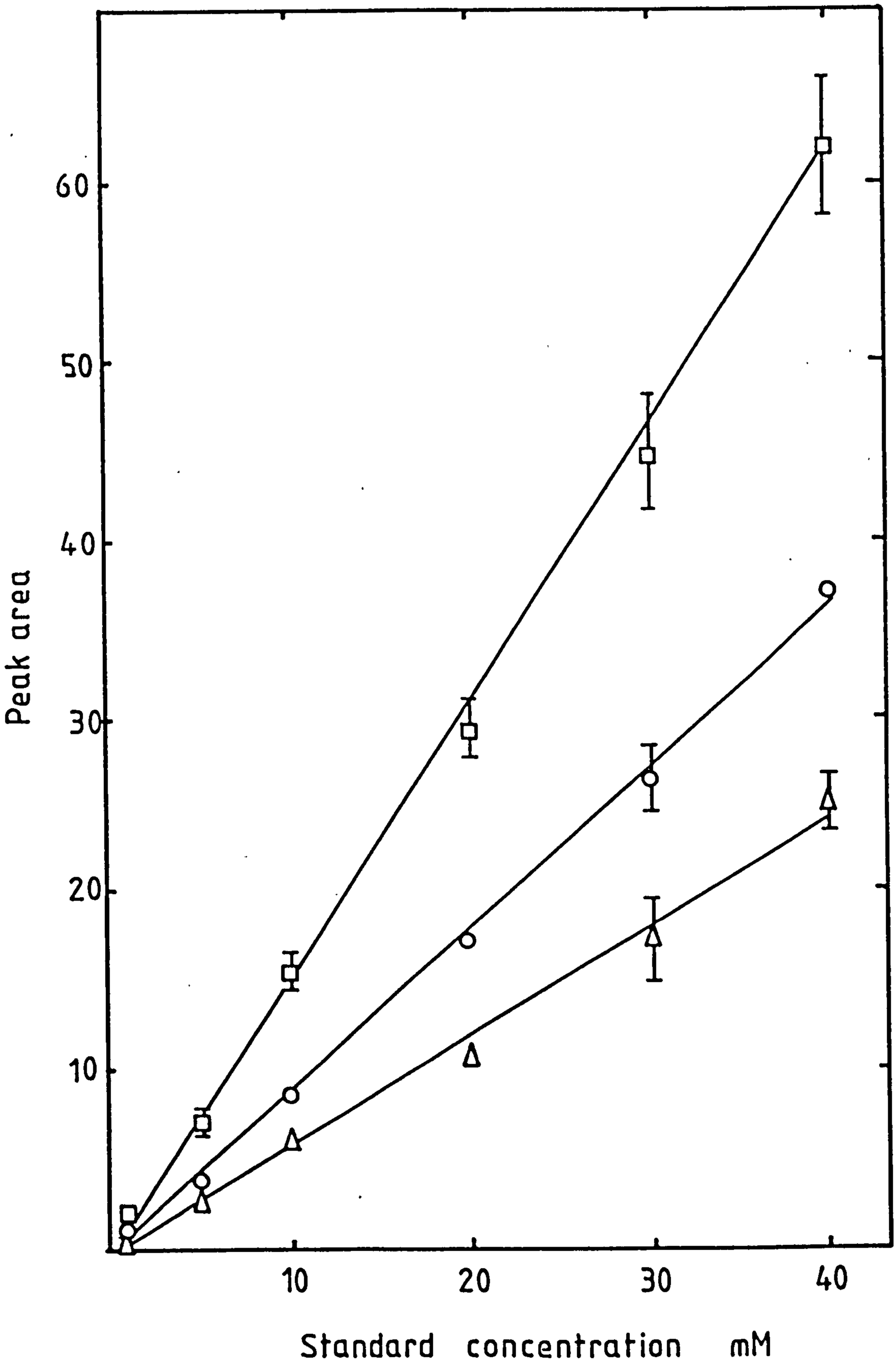
	Acetate	Propionate	Butyrate
correlation coefficient	0.996	0.999	0.999
slope	0.612	0.92	1.528
intercept with y-axis	-0.315	-0.3537	-0.157
x = 10	y = 5.804	y = 8.85	y = 15.125

Figure 61: Volatile fatty acid standard curves using Method 2.

Acetate

Propionate

butyrate


60
50
40
30
20
10
Peak area
10
20
30
40
Standard concentration mM

As a consequence of this accuracy this method was used routinely with little loss in precision.

Method 3

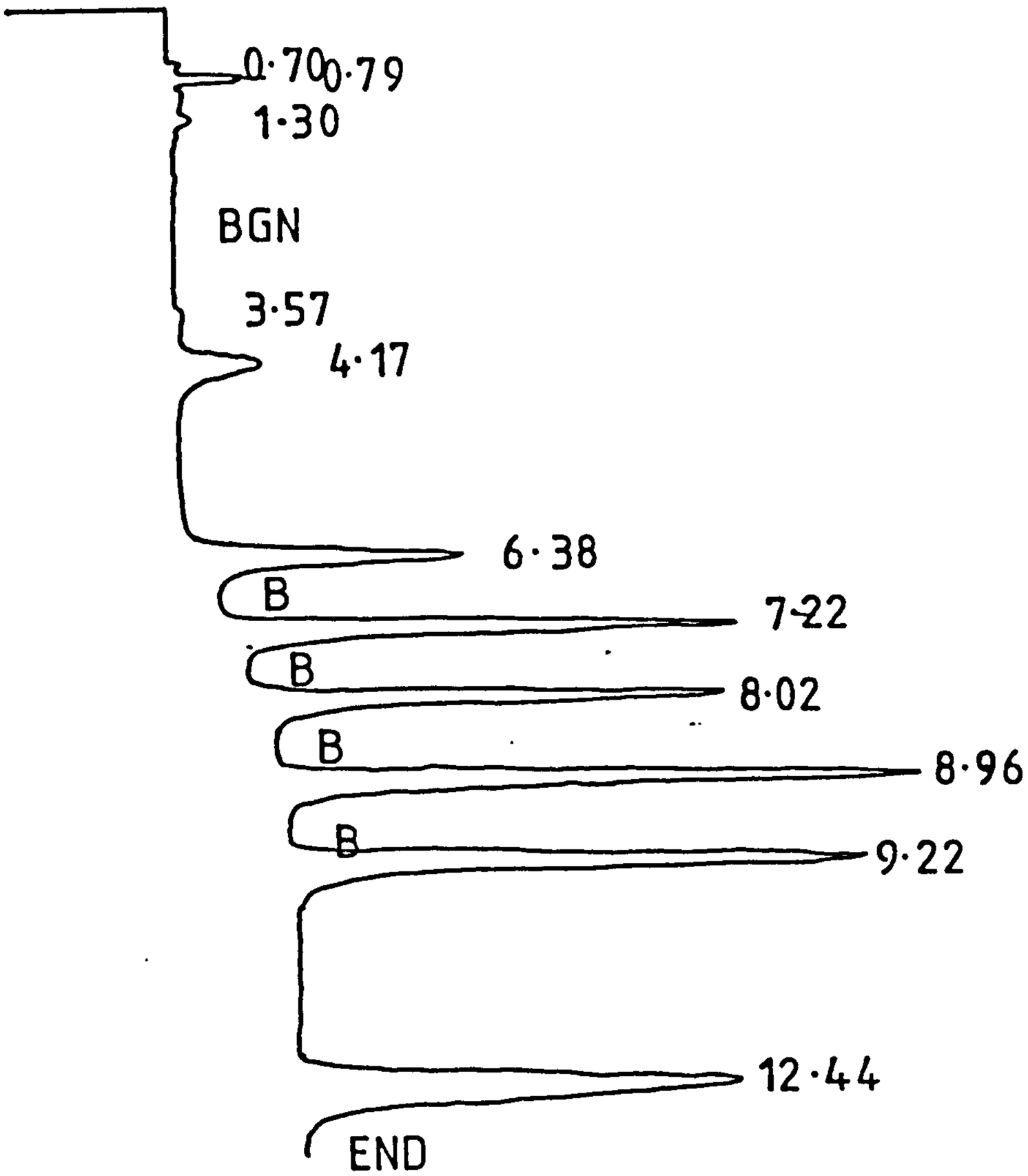
The method was similar to method 2 above, with the exception that the column packing material was 5% Neopentyl glycol Sebacate + 10% H_3PO_4 on Anakrome Polyester (80-100 mesh) (Phase Separations Ltd.).

The column was operated under temperature programming conditions with the initial oven temperature set at 100°C for 5 min. The oven temperature was then increased to 150°C at a rate of 40°C min^{-1} with the increase in the base line compensated for by forcing base points.

Method 3 was developed to negate the unidentified peak at 3.91 in method 2. A standard trace is shown in Figure 61. Acetate was eluted after 4.17 min at the lower temperature (100°C). The temperature of the oven was then increased facilitating release of the other acids. Forced base points (B) compensated for the increase in base line due to the increase in temperature.

The initial lower temperature facilitated effective separation of acetate from other unknown compounds (probably from the formic acid).

Figure 62: G.L.C. trace of 10 mM standard mixture of acetate (4.17 min), propionate (6.38 min), iso-butyrate (7.22 min), butyrate (8.02 min), valerate (8.96 min), methyl butyrate (9.22 min) and hexanoate (12.44 min). The peaks recorded at 0.70, 0.79, 1.30 and 3.57 min are associated with the formic acid. The method (3) is detailed in the text and involved temperature programming with the baseline corrected by forced basepoints (B).


0·70
0·79
1·30
BGN
3·57
4·17
6·38
B
7·22
B
8·02
B
8·96
B
9·22
12·44
END

As already mentioned, the major advantage of this method compared with method 2 was that no interfering peaks were recorded. The overall run time was approximately the same, although one disadvantage of this method was that the oven had to return from 130°C to 100°C which took about 5 min.

Summary

Of the three methods developed, method 3 proved to be the most satisfactory for the analysis of V.F.A. Although the technique was sensitive and reproducible for the V.F.A. greater than acetate, a major limitation was that formic acid could not be detected. However, this was a limitation of flame ionisation detection and not the chromatography.