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A study of the Physiology and Gas limited growth

of <u>Methylococcus capsulatus</u>

by

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SUMMARY

The results presented in this thesis have provided an
understanding of gas limited growth, as well as an insight
into the physiology of the methanotroph Methylococcus
capsulatus (Bath).   A theory of gas limited growth is
presented which explains the experimentally observed dilution
rate curves obtained during this investigation.   Measurement·
and calculations of yield data are described and the problems
encountered in these estimations are considered, particularly
the transient effect of pH additions on carbon dioxide production.
The maximum yield obtained was 0.65g. cells/g. of methane used.

The rate of gas utilisation by the organisms was proportional
to the partial pressure of the limiting gas entering the system.
Cultures of Methylococcus capsulatus (Bath) grown on air/methane
mixtures were oxygen-limited above 15% methane and methane limited
below 4% methane.   On occasions these cultures were sensitive to
sudden increases in oxygen availability and would completely
oxidise methane to carbon dioxide.   Antifoam additions lowered
the gas (oxygen) transfer rates.

An enclosed batch culture system was developed to enable
changes in the gas and liquid phases to be monitored during
growth.   Nutrient-limited growth in batch cultures (linear growth)
is described and typical growth curves presented.

0.1% v/v methanol did not inhibit growth of established
cultures of Methylococcus capsulatus (Bath) but did inhibit methane
oxidation.   Methanol was used in preference to methane (demon-
strated in batch and continuous culture) and was not detected in
culture supernatants.   Additions of 20 mM formate did not increase

the yield of the organism grown on methane and nitrate
mineral salts.

The problems of growth on three nitrogen sources are
discussed and the comparative yields on nitrate, ammonium
and dinitrogen were respectively 0.48, 0.52 and 0.45 g. cells/
g. of methane used.

Acetylene was found to be a potent specific inhibitor of
methane oxidation.   Ethane and ethanol inhibited methane-
uptake and were oxidised to acetaldehyde which accumulated in
the culture.

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Acknowledgements

Declaration

The material presented in this thesis is the result of original research conducted by myself, apart from help with the Fortran IV computer programme provided by Dan (?). None of it has been submitted for examination previously.

Abbreviations

AMS	ammonium mineral salts
ATP	adenosine triphosphate
D	dilution rate
DEAE	di-ethyl amino-ethyl
Dc	critical dilution rate
DCPIP	dichlorophenol indophenol
$\dfrac{dx_1}{dt}$	growth rate
f	flow rate
FAD	flavin adenine dinucleotide
FAO	Food and Agriculture Organisation
g	weight of limiting gas used
Ks	saturation constant
L	litres
M	maintenance respiration rate
MS	mineral salts
mg	milligram

n	no. of organisms after time t
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NMS	nitrate mineral salts
n_o	initial no. of organisms
O.D.	optical density
P	productivity
3PGA	3 phosphoglyceric acid
PMS	phenazine methosulphate
rpm	revolutions per minute
s	substrate concentration in culture
$\bar{s}$	steady state concentration of substrate in culture
SCP	single cell protein
S_R	substrate concentration in medium
t	time
t_d	doubling time
THF	tetra hydrofolate
V	culture volume
x	biomass concentration
$\bar{x}$	steady state biomass concentration
Y	yield coefficient
Y_{ATP}	ATP Yield
Y_{CH_4}	yield on methane
Y_{O_2}	yield on oxygen
z	number of generations

μ specific growth rate

μ_m maximum specific growth rate, or growth rate constant

One of the great debates at the present time is on the problem
of how to feed the ever increasing world population. An obvious
solution to this problem is to limit the growth of the population,
but this would involve much education in birth control methods, often
to illiterate populations. Some of this education would cut across
and conflict with many of the ideologies and religions in societies
throughout the world therefore this seemingly easy solution to the
world food shortage has many cultural and political problems to
overcome. Even if this solution were possible it would take many
years before any benefit could be obtained and should be viewed
as a long term solution to hunger.

"Some poor countries clearly have food shortages, but our
farmers (European) suffer from overproduction with the creation of
butter mountainsand wine lakes, and we know that if all the world's
food were distributed equitably and if none were fed unwastefully to
livestock there need be little hunger and no starvation for even
twice the present world population". (Mellanby, 1976). Once more
cultural and political fences have to be crossed to bring about the cure
suggested above.

This problem is man-made and as can be seen the solutions are
basically simple and need not necessarily involve new technology, but
changing the habits of many generations is a slow process, as there
are always pockets of resistance to change, even when that change is
for the better. New technology can soften this blow of change from
animal protein to vegetable protein or can provide less wasteful forms of
food for animals. Soya bean protein in its natural state is unpalateable,

but the protein can be extracted, spun into fibres and flavoured to represent the taste and texture of meat.

However all arable products are at the mercy of the climate, and of course there are usually several months between sowing and reaping of the crop. If a crop should fail then it could be another twelve months before that crop replanted could be harvested again. Micro-organisms could provide an answer to the problem of unreliability; growth of microbes in fermenters is not dependent on external factors to the system and a constant supply of biomass could be produced from industrial continuous fermenters.

In a system that has no environmental limitations on growth, micro-organisms can grow at a fixed maximum growth rate which is determined by a rate limiting step in the metabolism of the organism. As a consequence of this type of growth each organism can divide itself to form two where there was previously only one therefore the rate of increase of biomass can double (each new organism can grow as fast as the original). This type of growth (biomass doubling in a certain time and continuing over each similar time period) is called logarithmic or exponential growth. A simple example of exponential growth will provide an insight into the possibilities of micro-organisms yielding an ample source of biomass.

Consider a single cell of <u>Methylococcus</u> <u>capsulatus</u> (a methanotroph) growing on methane for two weeks (336 hr.). The maximum doubling time of this bacterium is about 2.4 hr.

Therefore in 336 hr the number of generations = $\frac{336}{2.4}$ = 140

From the equation of exponential growth

$$n = n^o 2^z$$ where n^o = initial number of bacteria

n = number of bacteria after time t

z = number of generations

So $n = 1 \times 2^{140}$

$= 10^{140} \log_{10} 2$

$= 1.4 \times 10^{42}$ cells after two weeks.

average mass of each cell = 10^{-12}g.

therefore cell yield in two weeks = 1.4×10^{30}g

= 230 x weight of the earth.

Of course the amount of biomass which can be produced will be limited by the size of the equipment used and the rate of supply and availability of the nutrients required.

In theory the growth of micro-organisms could provide sufficient biomass to feed all the world's population, but the quality of the biomass is a most important factor. It is unlikely that any single micro-organism could suffice as the sole source of food in the human diet. At present there is sufficient cereal production for the world's needs but many of these cereals are inefficiently converted to high protein products by feeding them to animals. If this requirement of cereals could be reduced by supplementing animal feeds with microbiologically produced food a greater residue of cereal would be available for human consumption. Proteins can also be extracted from the organism (single cell protein - S.C.P.) and used in the human diet (or the whole organism fed as

a protein source) as a high protein food in place of meat, again
releasing more cereal for human consumption by reducing the demand
for animal protein.

Bacteria, fungi and yeasts are all possible groups of micro-
organisms which could be used for S.C.P. production. Fungi are a less
likely proposition because of the nature of their growth, often
filamentous and growing in clumps. This makes fermentation (especially
continuous fermentation which is well adapted to the production of
biomass) technologically more difficult than the growth of individual
or budding cells of bacteria and yeasts.

Products from yeasts would be more readily accepted because of
its established use in baking and brewing as well as in commercial
market products e.g. marmite and yeast tablets etc. Bacterial products
are likely to meet greater resistance because of the unfamiliarity
of the organisms as a food stuff although widely used products, such
as yoghurt and cheese, contain a large number of bacteria directly
responsible for producing a texture and flavour acceptable to the
palate.

The most important consideration in the production of S.C.P. is
the carbon source on which the microbes are to be grown. Little benefit
would be derived if the carbon source could be consumed, as it is, rather
than it being converted to S.C.P. The main criteria for the feed stock
carbon source (which can account for 40% - 50% of the production costs)
are that it must be :-   cheap (possibly a waste product),

available in plentiful supplies
easily converted to S.C.P.
economically viable
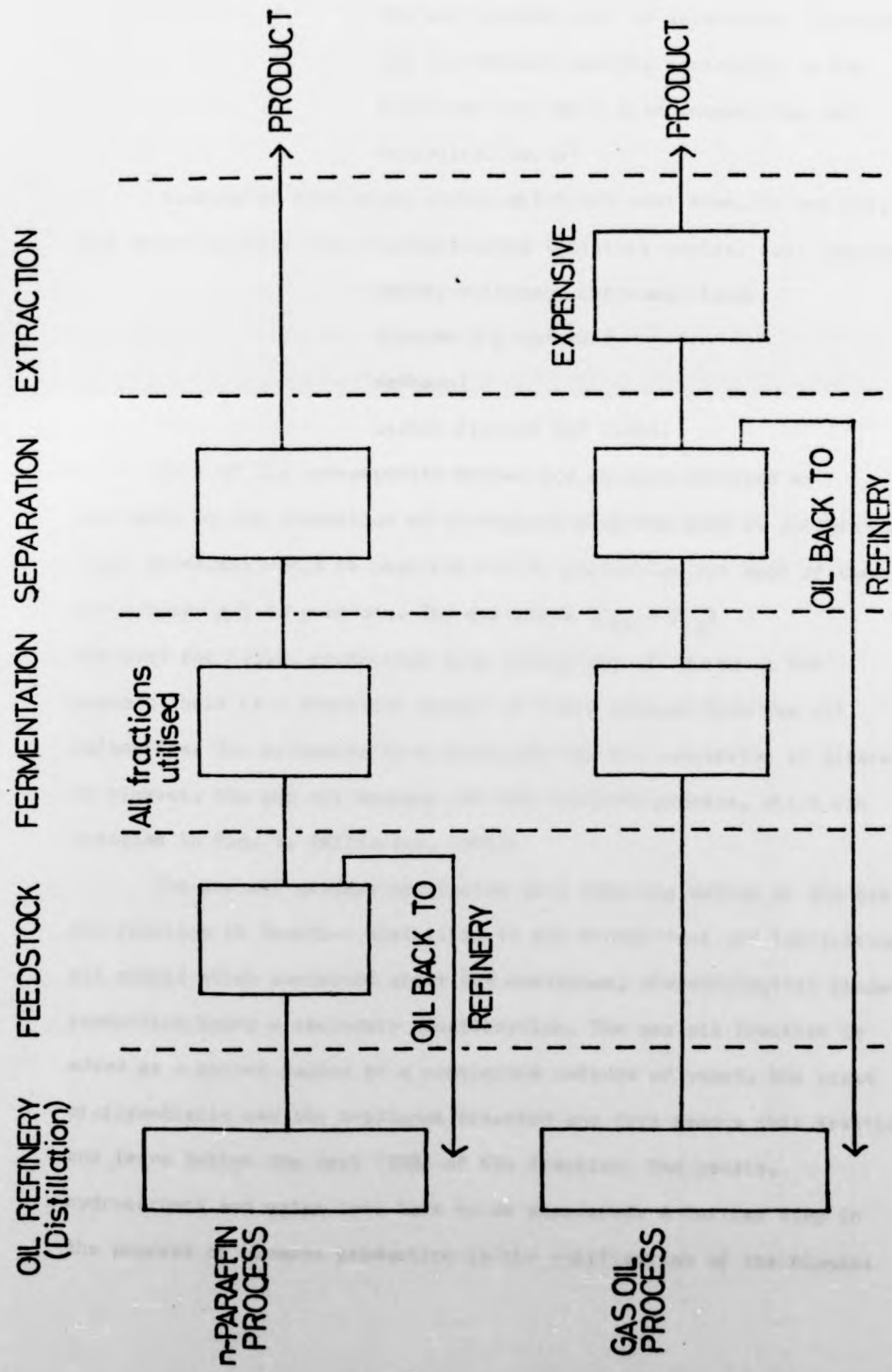
Fig. 1. Flow diagrams for yeast production form n-alkanes (Evans 1969)

the end product must be acceptable (non-toxic
and the protein quality conforming to FAO
standards for amino acid composition and
biological value)

Sources of feed stock carbon which can meet some, if not all,
the above criteria are :-carbohydrates (possibly wastes) e.g. vegetable
pulps, molasses, cornsteep liquer,

alkanes (C_1 upwards)

methanol

carbon dioxide and light.

Many of the carbohydrate wastes are already utilised as
nutrients in the production of biological products such as antibiotics.
These materials could be used for S.C.P. production but much of them
are already put to good use. The n-alkanes (C_{12} - C_{18})
are used for S.C.P. production from yeasts and of course at the
present there is a plentiful supply of these alkanes from the oil
refineries. Two processes were developed for the conversion of alkanes
to biomass, the gas oil process and the n-alkane process, which are
outlined in Fig. 1. (Wilkinson, 1971).

The gas oil process originated as a dewaxing method of the gas
oil fraction (a fraction distilling in the diesel fuel and lubricating
oil range) which contained about 10% n-alkanes, microbiological biomass
production being a secondary consideration. The gas oil fraction is
added as a carbon source to a continuous culture of yeast, the yeast
preferentially use the n-alkanes fraction and thus remove this fraction
and leave behind the rest (90%) of the fraction. The yeasts,
hydrocarbons and water then have to be separated. A further step in
the process of biomass production is the purification of the biomass

to remove the final traces of oil which are left after the initial
separation. This is done by an efficient but costly solvent
extraction process.

This gas oil process is run non-aseptically and the yeast
used in the process is kept the dominant organism by means of
controlling the environment (e.g. pH, temperature etc.) to favour
that particular yeast. There is of course the possibility that in
another part of the world a different organism might become dominant
or a toxigenic contaminant might enter the process which would cause
problems even if present in low numbers. This process, as a commercial
proposition has now been abandoned.

The n-alkane process differs from the gas oil process in that
the purified n-alkanes (up to C_{18}) are added to the culture which
is carbon limited and all the added hydrocarbons are utilised thus
doing away with the expensive solvent extraction procedure. However
set against this was the requirement for aseptic fermentations, and the
increased cost of the purified n-alkanes. It was later found unnecessary
to run the system aseptically.

The n-alkane process had more in its favour because more
parameters and quality can be tightly controlled e.g. carbon feedstock
content, biomass produced (mono-culture) and a product free from any
contaminating hydrocarbon fractions. The n-alkane process has been
adopted for S.C.P. production.

The final group of carbon sources to be considered are the C_1
compounds (methane, methanol and carbon dioxide). Methane and carbon
dioxide are end products of metabolism, methane from anaerobic slurry
fermentation and naturally in most freshwater environments and carbon

dioxide as one of the end products of respiration. Methane is also found in underground gas fields and is given off at oil fields. The methane from oil fields is more often than not flared off into the atmosphere, a negligent waste of energy, but up to now most of the major oil producing nations have not been in a position to utilise this gas industrially. It can only be hoped that these nations will be encouraged to consume natural gas by using it as a source of protein as well as in the petrochemical industry. Natural gas (mainly methane with traces of ethane) is tapped as an energy source in industrial nations and is not such a tempting proposition as a carbon source for S.C.P. production. The economy of the process would depend on the price commanded by methane (balanced by the price the biomass produced) in competition with other high protein products e.g. soya bean protein. In this context the price of methane would be the price for which the methane could be sold, not the actual cost of extraction. Methane is also a by-product of anaerobic slurry (sewage) fermentation at sewerage works. Even when these works are powered by this methane there is still excess. This is another possible source for S.C.P. production in the future.

Methanol, a petrochemical product of industry is used as an additive to petrol but there is excess capacity for its cheap production as a carbon feedstock for biomass production (as now being planned by ICI). Both bacteria and yeasts can grow on methanol so the choice of organisms to use is wider than for organisms using methane.

Many papers and reviews have discussed the possibility of producing S.C.P. from hydrocarbons (Wolnak et al. 1967; Hamer et al 1967; Ribbons, 1968; Norris 1968, Whittenbury, 1969 and 1971; Wilkinson 1971; Hamer and Norris 1971) and there has been some discussion as

to whether methane or methanol should be used as a C_1 carbon source
(Hamer and Norris 1971 and Cooney, 1975). Methane can now be cheaply
converted to methanol by a catalytic process, so the choice of
substrate is dependent on the practicability and economies involved
using the two carbon sources.

Methane as a carbon source for S.C.P. has several advantages
over other carbon sources (not methanol) in that it is :-

- available in nature in large quantities
- competitively priced
- often found in a high state of purity
- volatile, so leaves no toxic residues
- it has a restricted range of organisms that can utilise it
  (contamination is less of a problem).
- it is found in developing countries where it is often flared to waste.

these advantages of methane are also similar for methanol (can be
readily obtained in the pure state from industry). Methanol does have
advantages over methane :-

1) miscible with water whereas methane is a sparingly soluble
   gas but its solubility is similar to that of oxygen which both
   cultures require.
2) it is a liquid, therefore easier to handle, store and transport.
3) more oxidised than methane, so the organisms have a lower
   oxygen demand and produce less heat.
4) can be utilised by yeasts (only bacteria have been shown to
   utilise methane)

The problem with yeasts is that they contain less protein (45 - 55%) than bacteria (60 - 70%) and the quality (amino acid content) is also poorer. The choice seems to lie between bacteria grown on methane or methanol and this will probably depend on the availability and price of these two commodities at any particular time.

The final C_1 compound to be considered is carbon dioxide, it is readily available and can be utilised, with light as an energy source, by bacteria. A major problem with this sort of system for S.C.P. production in fermenters would be the technical difficulties of putting sufficient light energy into the culture to obtain dense enough concentrations of biomass to make the system economic plus also the economy of using light energy.

The topic of this thesis was to investigate the parameters which effect the growth of methanotrophs on methane and to estimate the effect of these parameters on yields, so methanotrophy will now be discussed in more detail.

METHANOTROPHY (METHANE-UTILISERS)

Methane utilisers are grouped under the heading of methylotrophs by the definition of Colby and Zatman (1972) who stated that "methylotrophy is the ability to grow non-autotrophically at the expense of carbon compounds containing no carbon-carbon bonds" e.g. methane, methanol, methylamine etc. This definition aptly describes the C_1 utilisers and states that if an organism can grow on a C_1 compound (other than carbon dioxide) it is a methylotroph; any other compounds which this organism can or cannot utilise as a carbon source is taken into consideration by the use of the terms 'facultative' or 'obligate'. Obligate methylotrophs can only grow on C_1 compounds whereas facultative methylotrophs can also grow on other compounds as well as C_1 molecules.

The methane utilisers stand out as a sub-group in the methylotrophs by their characteristic growth substrates and morphology, growing only on methane, methanol and dimethyl ether (two reports of other growth substrates are described in the next section) and all having an internal membrane system (Whittenbury et al , 1970). The one common factor is that they can grow on methane and by this criterion alone should be classified under their own name - methanotrophs. Those capable of growth on only methane and other C_1 compounds are obligate methanotrophs and those capable of using non-C_1 compounds, facultative methanotrophs.

Facultative methanotrophs

There have been two notable reports of facultative methanotrophs

the first was by Perry (1968) and named by him JOB 5 and assigned
to the genus Brevibacterium. This organism was first isolated
by Ooyama and Foster. (1965) on 2 methyl-butane. Also discussed in
less detail was 7E4 isolated on propane and assigned to the genus
Corynebacterium. Both these organisms would grow on methane and
higher n-alkanes. Brevibacterium JOB 5 grown on $C_1 - C_4$ n-alkanes
would oxidise all the n-alkanes $C_1 - C_{22}$ whereas cells grown up on
$C_5 - C_8$ n-alkanes would oxidise all the alkanes except propane,
which they oxidised poorly and no significant oxidation of either
ethane or methane. Methanol was oxidised poorly regardless of
growth substrate. This is an odd result as methane-utilisers
oxidise methanol quite readily (Reed, 1976), although the concentration
of methanol added might be quite critical, concentrations of methanol
between 0.01% to 0.5% v/v can inhibit growth. (Whittenbury et al 1970 b).

There are two criteria which must be satisfied before an
organism can be termed a facultative methanotroph :-

1) A pure culture - a mixed culture of a methanotroph and a
heterotroph (which can be difficult to separate) can mimic the
properties of a facultative methanotroph. The methanotroph being
the dominant organism when grown on methane and the heterotroph being
the dominant organism on organic compounds. There have been reports
of mixed cultures on methane growing better than pure cultures; this
could be explained by the contaminant removing an inhibitor or
producing a necessary supplement. Wilkinson et al (1974)
reported that a pure culture of a methanotroph grew very poorly on

methane whereas the same organism in a mixed culture could grow well on methane. It was shown that the methane utiliser when grown on methane excreted methanol and that concentrations in excess of 1.8 mM inhibited methane uptake, thus explaining the poor growth of the pure culture. One of the contaminants in the mixed culture was of the Hyphomicrobium genus and could grow on methanol. In continuous culture the Hyphomicrobium genus utilised the methanol produced and thus prevented an inhibition of the methanotroph. Additions of methanol to the culture inhibited the methane-utilising moeity until the methanol concentration was reduced by the contaminants enabling the methane to be utilised again. This is an example of a contaminant removing an inhibitor.

2) Pure chemicals - if poor quality chemicals are used growth may be due to the presence of contaminating compounds, e.g. the methane may contain ethane, propane etc. To verify that growth on methane is occuring growth must be related to the disappearance of methane.

Neither of these parameters were reported in any detail by Perry and the poor rates of methanol oxidation ($17 \mu l \ O_2$ / 90 min / 10 mg cells) is not borne out in any other methylotroph studied (Brown et al 1964; Patel and Hoare 1971; Reed, 1976). However, as it is pointed out, other organisms have low oxidation rates for methanol compared to those for other n-alcohols, but not methylotrophs.

The second report of a facultative methylotroph (Patt et al 1974) described elaborate procedures to prove purity of the culture and continuity between growth on methane and growth on organic compounds.

(Diluting to single cells before plating, replica plating, similar
DNA density gradients of cultures grown on methane and glucose).
The two criteria previously mentioned seemed to have been fairly
well satisfied.

Isolation of methanotrophs

The first well described methane-utilising micro-organisms
were by Sohngen (1906) and Kaserer(1906). Between these first
descriptions of methanotrophs and 1969 only three more types were
isolated and well described (Davis et al , 1964; Stocks and
McClesky, 1964; Foster and Davis, 1966 and Sohngens' organism was
also reisolated by Dworkin and Foster, 1956). This small number of
isolates does not reflect a sparse distribution of the organisms in
the environment, but inadequacies in the isolation techniques.
However these failings in procedure were overcome by Whittenbury
and fellow workers at Edinburgh (Whittenbury, 1969; Whittenbury et al
1970 b). The improvements in technique were to serial dilute and
spread plate the initial enrichment cultures after three days
incubation and frequently examine the plates microscopically rather
than continue growth through further enrichments. This examination
was necessary because of the slow growth of methane - utilisers, other
organisms would grow initially but stop growing after about 3 days, but
the methanotrophs did not appear until 5 to 7 days had elapsed. It was
found that isolation of these colonies was best performed when the
colonies were still fairly small (0.2 mm diameter). From these studies
it became evident that methane utilisers were ubiquitous and could
be found in most natural environments. Use of the above procedure

resulted in over 100 strains of methane - utilising bacteria being isolated.

It soon became apparent from studying these isolates that the methanotrophs could be split into two groups not only on the basis of cell shape and form of resting stage but more importantly by the internal membrane structure possessed by these organisms. The two groups were designated type 1 and type 11 . Type 1 organisms had bundles of disc shaped membrane vesicles, distributed throughout the cell, whereas type 11 organisms had a system of paired membranes running throughout the cell and often concentrated around the periphery (Whittenbury et al 1970 a and b).

The differences between the two types of methanotroph were not just structural; the two pathways of C_1 assimilation could be related to each of the groups. The ribulose monophosphate cycle of formaldehyde fixation was found in type 1 organisms. The serine pathway was used by the type 11 organisms for the fixation of the C_1 into cellular material (see later for details of pathways). Both pathways have been found in other methylotrophs.

PATHWAYS OF C_1 METABOLISM

1) C_1 oxidation

The pathway suggested by Dworkin and Foster (1956) -

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

is now generally accepted as the overall pathway of methane oxidation. This pathway was the logical conclusion from the results they obtained from resting cells of Pseudomonas methanica which would oxidise methanol, formaldehyde and formate to carbon dioxide. Further work by Leadbetter and Foster (1958) with the above organism and by Brown et al (1964) with Methanomonas methano-oxidans substantiated these results but they could only detect formate as an intermediate during the oxidation of these compounds. However, with the use of an aldehyde trapping agent, sodium sulphite, formaldehyde could be detected in the supernatant. Also methanol was shown to accumulate during methane oxidation if iodoacetate was used to inhibit the methanol dehydrogenase. However this effect could not be repeated by Higgins and Quayle (1970) or Hazeu (1975); in fact, iodoacetate inhibited methane oxidation to a greater extent than methanol oxidation.

a) Methane Oxidation

The first step in the oxidation of methane was for many years an unsolved mystery with only a few indications of the form of the oxidation that might occur, which led to several theories being put forward. Several reports indicated that the oxygen in methanol was derived from molecular oxygen. Leadbetter and Foster (1959) showed that O^{18} was incorporated into cellular material of Pseudomonas methanica to a greater extent if the organism is grown on methane as opposed to methanol. This might indicate a mixed function oxidase, similar to that

found in organisms that could oxidise higher alkanes by Peterson et al (1967) :-

$$CH_4 + O_2 + XH_2 \longrightarrow CH_3CH + H_2O + X$$
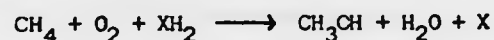
where XH_2 is a reduced co-enzyme. A dioxygenase reaction was postulated by Quayle (1972)

$$2CH_4 + O_2 \longrightarrow 2CH_3OH$$
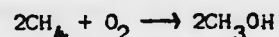
and a hydroxylase reaction by Whittenbury (1969) who based this on the result that a methanotroph had a 20% higher yield on methane than methanol suggesting that the initial oxidation step might be energy yielding

$$CH_4 + H_2O \longrightarrow CH_3OH + 2H.$$
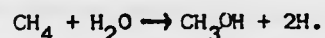
Leadbetter and Foster (1959) had only provided circumstantial evidence that molecular oxygen was incorporated into the methane molecule. However, Higgins and Quayle (1970), unable to demonstrate methanol accumulation with iodoacetate, accidentally found that high phosphate concentrations would cause methanol (up to 2.5 mM) to accumulate in methane respiring cells suspensions of Methylomonas methano-oxidans and Pseudomonas methanica . This enabled Higgins and Quayle (1970) to show that O^{18} was incorporated into this accumulated methanol in the predicted activity if molecular oxygen was used to oxidise methane.

Two other theories of the initial oxidation step are worth noting. Wilkinson (1971 and 1975), Davey (1971) and Thompson (1974) demonstrated that dimethyl ether could be oxidised, used as a substrate for growth and accumulated in the supernatants of suspensions of methane grown cells (although not consistently) of Methylomonas albus (BGB) and Methylomonas trichosporium(OB3b). This led them to postulate a

condensation reaction of two molecules of methane to yield dimethyl ether ($CH_3.O.CH_3$). The other theory was that of Hutchinson et al (1976) who postulated that formaldehyde could be produced from methane and oxygen by a free radical mechanism. There was evidence that methanotrophs were particularly resistant to mutation (Harwood et al 1972). This resistance to mutation was attributed to a very efficient repair mechanism because of highly mutagenic agents in the cell i.e. free radicals (Hutchinson et al, 1976) and that these free radicals were involved in methane oxidation.

Whole cell studies did not shed very much light on the mechanism of methane oxidation, other than an involvement with molecular oxygen. The first cell free evidence for the mechanism of methane oxidation came from Ribbons and Michalover (1970) who obtained methane stimulated $NADH_2$ disappearance and concomitant oxygen uptake with membrane particles of Methylococcus capsulatus strain Texas. Ferenci (1974) also demonstrated this with Pseudomonas methanica but neither study actually linked this phenomenon with methane disappearance nor with accumulation of a product. This was later done by Ribbons (1975) who showed methane disappearance and formation of formate as the product. Wadzinski and Ribbons (1975) postulated that formate was the product because of the activities of enzymes further down the pathway of C_1 metabolism in the crude extract used.

It was a different line of research that brought about an understanding of the mechanism in Methylosinas trichosporium (OB3b) Tonge et al (1974) studying the cytochromes of methylotrophs found high concentrations of a soluble carbon monoxide (CO) binding C-type

cytochrome. At the same time Hubley et al (1974) and Ferenci (1974)
had shown that whole cells of three strains of methanotrophs could
oxidise carbon monoxide to carbon dioxide with concomitant increase
in oxygen uptake; Ferenci also demonstrated cell free $NADH_2$
dependent oxidation of methane and carbon monoxide; this led Tonge et al
(1974) to suggest "that this CO-binding C-type cytochrome found in
such large quantities may well be involved with an oxidase or
oxygenase function in methane oxidation." Tonge et al (1975) had
established an assay system for the oxygenase and obtained a 38 fold
purification of this methane oxidising enzyme, a mono-oxygenase, from
crude extracts. The system was further purified and properties studied
by Tonge et al (1977) who found that the system consisted of three
sub-units; a soluble carbon monoxide binding cytochrome (104 fold
purification), a copper containing protein and a small protein (both
of which were purified 40 fold). The stoichiometry of the reaction
suggested a mono-oxygenase mechanism; the purified system utilised
ascorbate,or with partially purified methanol dehydrogenase, methanol
as electron donors but not $NAD(P)H_2$ which could only serve as an
electron donor in crude extracts. The immediate electron donor   is
the cytochrome C_{co} component as illustrated below from Tonge et al (1975).

$$CH_4 + O_2 \longrightarrow H_2O + CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

methane   methanol   formaldehyde NAD^+ $NADH_2$

mono-oxygenase   dehydrogenase dehydrogenase   formate

dehydrogenase

pteridine

$NADH_2$

cytochrome C_{co} XH_2
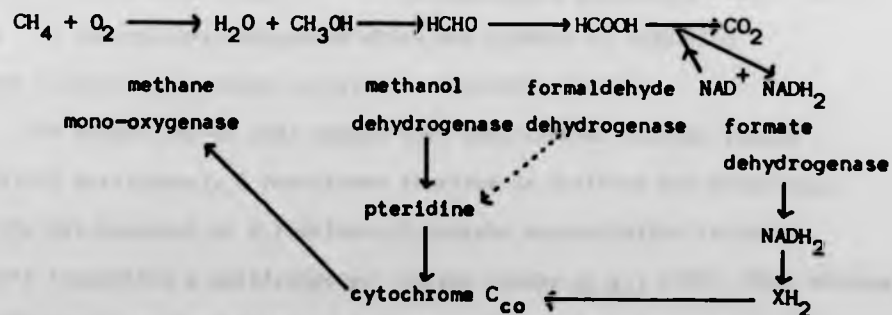
The cycle which generates electrons from methanol (and possibly formaldehyde) via a pteridine (Anthony and Zatman, 1967 a and b) was first put forward by Tonge et al (1975) and Higgins et al (1976) and verified by demonstrating methanol-driven methane oxidation in their purified system. This cycle was claimed to have consequences on the predicted yield values from methane (Y_{CH4}) of these cells as $NADH_2$ is not specifically required. This will be discussed later.

At the same time, in contrast to this cytochrome Cco-linked mono-oxygenase an $NAD(P)H_2$-dependent methane mono-oxygenase had been discovered in Methylomonas methanica (Colby et al, 1975) and from Methylococcus capsulatus strain Bath (Colby and Dalton, 1976). The enzyme from Methylococcus capsulatus strain Bath was soluble and differed from the other methane mono-oxygenases which have been described (all particulate). Further differences between this soluble enzyme and the others were found by Stirling and Dalton (1977) who looked at inhibitors of methane oxidation and compared Methylococcus capsulatus strain Bath with strain Texas. These results showed not only that strain Bath was different from strain Texas but also that Methylococcus capsulatus (Bath) showed a conflicting pattern of results from those obtained with Methylosinas trichosporium (Hubley et al. 1975). Colby et al (1977) found that the methane mono-oxygenase from Methylococcus capsulatus strain Bath. was a non-specific oxygenase which was capable of oxidising various substituted methane derivatives including methanol.

The properties of this enzyme have been further studied (Colby and Dalton pers.comm.). A non-linear increase in activity was noted when activity was measured as a function of protein concentration in crude extracts suggesting a multicomponent enzyme (Colby et al. 1976). DEAE cellulose chromatography resolved three components, named by them A,B and C.

A and C were absolutely required for activity, whereas fraction B
stimulated activity 2-4-fold depending on the extract. Fractions A and
B were relatively stable at $0^{o}C$. Fraction C was not stable at $0^{o}C$
and most of the activity would be lost over 24 hours, but addition
of sodium thioglycollate maintained activity during purification
procedure. This component was purified 80 fold and was shown to contain
iron (no copper was detected) and to have 1 mole of FAD per mole of
protein

b) Methanol and Formaldehyde oxidation

It is convenient to discuss both these C_1 compounds together
because it seems that they are oxidised by the same enzyme system in
methanotrophs as will be described below.

The first report of a methanol dehydrogenase was made by
Anthony and Zatman (1964) and described by them in a series of papers,
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that could be found for this enzyme was phenazine methosulphate (PMS)
which could be reoxidised by dichlorophenol indophenol (DCPIP) which
formed the basis of the spectrophotometric assay developed for this
enzyme; the enzyme was completely independent of nicotinamide nucleotides.
The enzyme also required ammonium ions for activity and it had a
pteridine prosthetic group.

A similar enzyme was found in Pseudomonas AM1 and an obligate
methanotroph Pseudomonas methanica by Johnson and Quayle (1964).
Other workers have also reported a PMS-linked, ammonium ion-requiring
methanol dehydrogenase in methylotrophs (Patel and Hoare (1971) and
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alcohols and aldehydes, perhaps most importantly that it could oxidise formaldehyde, being the product of methanol oxidation. Evidence that it was the same enzyme that could oxidise both methanol and formaldehyde came from two lines of investigation. 1)Patel and Felix (1976) and Goldberg (1976)   purified the enzyme from the organisms they were studying and found that the purified enzyme could still oxidise the two substrates. 2)Heptinstall and Quayle (1970) isolated a mutant of *Pseudomonas* AM1 which could not grow on methanol because of the loss of the methanol dehydrogenase activity; extracts from this organism also failed to oxidise formaldehyde by a PMS-linked, ammonium— requiring dehydrogenase.

The enzyme from *Methylococcus capsulatus* strain Texas and *Methylosinus trichosporium* (Patel and Felix, 1976) could only oxidise formaldehyde of the aldehydes tested. This was also the finding in *Methylococcus capsulatus* strain Bath. The enzymes from *Methylococcus capsulatus* (Texas) and *Pseudomonas methanica* were purified and found to have similar molecular weight, absorption spectra, pH optima, requirements of ammonium ions for activity and to have some shared antigenic determinants but different electrophoretic mobilities (Patel *et al*, 1972; Patel *et al* , 1973).

Wadzinski and Ribbons (1975) studied the distribution of methanol and formaldehyde oxidising enzymes in the cell. They found two types of oxidising systems; the PMS/DCPIP system was found in both the soluble and particulate fraction, and a methanol oxidase mainly associated with the membranes. This methanol oxidase could very well be the PMS/DCPIP enzyme linked to an electron transport system to oxygen.

Besides this PMS/DCPIP linked formaldehyde dehydrogenase several other types of systems have been found to oxidise formaldehyde in

methylotrophs. Three types appear to exist in Pseudomonas methanica ;
the PMS/DCPIP-linked enzyme, an NAD-linked dehydrogenase specific
for formaldehyde and requiring glutathione (Harrington and Kallio, 1960)
and a DCPIP-linked dehydrogenase with a broad specificity for
aliphatic aldehydes (Johnson and Quayle, 1964). An NAD-linked
dehydrogenase specific for formaldehyde but not requiring glutathione
was found in Pseudomonas M.S. (Kung and Wagner 1970) and in Bacterium
4B6 (Colby and Zatman, 1971).

The differing specificities of these enzymes for aldehydes
enabled some methylotrophs to oxidise primary alcohols to the fatty
acids (Leadbetter and Foster, 1960; several
organisms isolated by Hazeu, 1975), whereas Methylococcus capsulatus
(Bath and Texas) could oxidise methanol to its fatty acid (formate),
but all other primary alcohols only as far as the aldehyde (Patel and
Hoare, 1971 and in this thesis).

c) Formate Oxidation

Formate oxidation seems to be carried out by an NAD-linked
dehydrogenase which oxidises formate to carbon dioxide and produces
$NADH_2$ (Johnson and Quayle, 1964; Davey, 1971).

ASSIMILATION OF C_1 COMPOUNDS

There are two basic pathways by which reduced C_1 compounds can become incorporated into cellular material. Both these pathways effectively fix the C_1 at the formaldehyde level in the serine and ribulose monophosphate pathways. Perhaps it should be pointed out that not all the results have been obtained from one organism of each type and that the pathways were elucidated mainly by using facultative methylotrophs rather than methanotrophs. An advantage of the facultative methylotrophs is that genetic manipulation can be employed to obtain useful point mutations effecting the pathways and therefore growth requirements; whereas mutants of methanotrophs are difficult to obtain (Harwood et al, 1972) and the restricted range of carbon sources (only C_1) renders this type of investigation impossible. However it does seem that all methylotrophs fix C_1 compounds at the formaldehyde level and similarities in enzyme patterns and pulse chase experiments indicated that pathways are very similar.(Although each pathway with all the necessary enzymes should be verified for each organism).

1) Serine Pathway

The first evidence for this pathway was provided by isotopic label studies in intact cells. Kaneda and Roxburgh (1959) in methanol-grown Pseudomonas PRL-W4; Large et al (1961) in methanol-and formate-grown Pseudomonas AM1 and methanol-grown Hyphomicrobium vulgare; and Lawrence et al (1970) in methane-grown Methanomonas methano-oxidans. This evidence showed that label from methanol would appear at early times in malate/aspartate, serine and to a lesser extent in glycine; labelled

phosphate compounds appeared later. Evidence was also found that
carbon dioxide was fixed as well, $\left[^{14}C\right]$ from bicarbonate appeared in
glycine at early times, and suspensions that had 1% carbon dioxide
bubbled through them instead of air showed 50% less incorporation
of $\left[^{14}C\right]$ methanol. Large et al (1962 a) studied the distribution of
isotope in metabolites isolated from methanol-grown Pseudomonas
AM1 after incubation with $\left[^{14}C\right]$ methanol and $\left[^{14}C\right]$ bicarbonate. The
results indicated that the carboxyl group of glycine was derived from
bicarbonate; the hydroxy methyl group of serine came from methanol;
malate was formed from a carboxylation reaction of a C_3 fragment
derived from serine and also that a methylene C came from methanol.
The data can be summarised by the proposed following scheme

glycine   THF - Tetrahydrofolate

methylene - THF

THF

serine

hydroxypyruvate

$NADH_2$

NAD

glycerate

ATP

ADP

phosphoglycerate
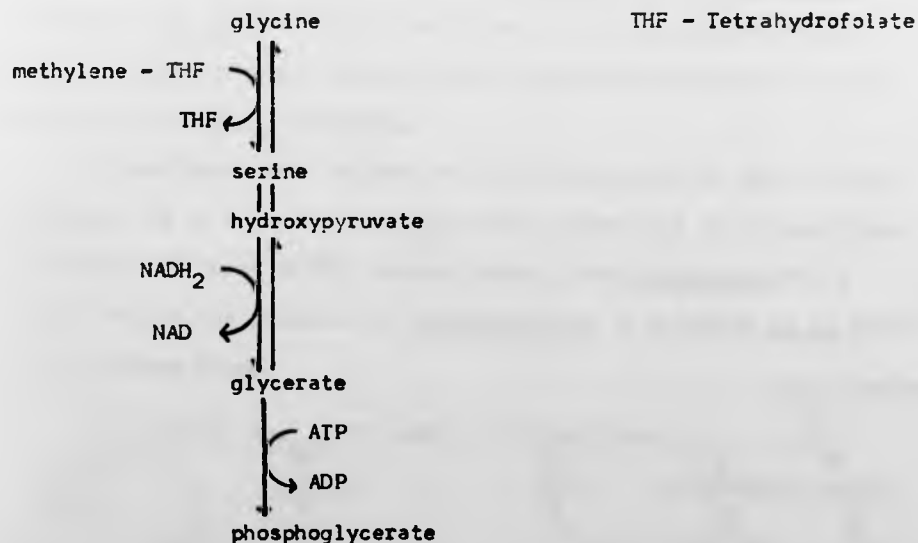
These label patterns and postulated pathways were also
supported by enzyme studies (Large and Quayle, 1963; Heptinstall and
Quayle, 1970; Blackmore and Quayle, 1970; Harder and Quayle, 1971 a).

These studies verified the existance of enzymes which were involved in the above scheme (the serine pathway). Pseudomonas AM1 was shown to have a THF formylase and a methylene THF dehydrogenase (Large and Quayle, 1963). Besides these enzymes, Large et al (1962 b) had found and purified a phosphoenol pyruvate carboxylase in Pseudomonas AM1, which accounted for the carbon dioxide fixation step. Mutant studies of lesions in the serine pathway by Heptinstall and Quayle (1970) and Harder and Quayle (1971 a and b) also confirmed this pathway of C_1 incorporation in Pseudomonas AM1. A key enzyme in this pathway was hydroxypyruvate reductase which converts hydroxypyruvate to glycerate.

A similar pathway was found in Methanomonas methano-oxidans by Lawrence et al (1970) who also looked for the key enzyme of the serine pathway in other obligate methanotrophs and found that it was restricted to type 11 organisms.

A problem of this pathway was the regeneration of the C_2 units to accept the C_1 molecule. The icl-serine pathway was first postulated by Bellion and Hersh (1972) in methylamine grown Pseudomonas MA. A slight variant was proposed for Hyphomicrobium X by Harder et al (1973) which is shown below.
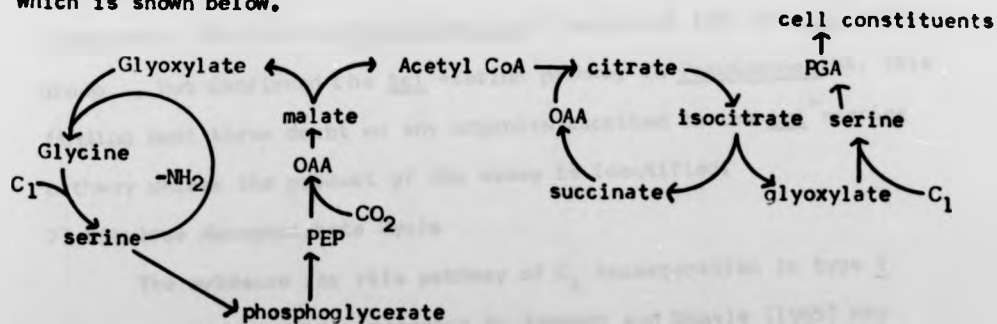
The serine pathway provides malate from glycine, a reduced C_1

unit and carbon dioxide. The malate is activated to malyl-CoA by
malate thiokinase which·is cleaved to glyoxylate and acetyl CoA
by malyl CoA lyase. The acetyl CoA is oxidised to glyoxylate via part
of the tricarboxylic acid cycle and cleaving isocitrate by isocitrate
lyase to succinate and glyoxylate (an essential enzyme for this
pathway, hence _icl_ -serine pathway). The glyoxylate can then be used
for net synthesis of cell constituents as a C_2 unit was regenerated
at the malyl CoA lyase step to accept a further C_1 unit to repeat the
sequence.

Not all serine pathway organisms have isocitrate lyase these
include _Pseudomonas_ AM1. The actual means of oxidising acetyl CoA to
glyoxylate is unknown but it may involve glycollate (Dunstan _et al_, 1972)
This icl^- - serine pathway has been assigned to more organisms than
was originally thought, because of a misinterpretation of the isocitrate
lyase assay (Attwood and Harder, 1977) which involved trapping
glyoxylate as glyoxylate phenylhydrazone, which is measured at 324 nm.
Unfortunately this assay is not specific and oxoglutarate phenylhydrazone
also gives the same colour. Attwood and Harder have found that what had
been taken as icl^+ were infact icl^- as the activity initially
ascribed to the isocitrate lyase was actually measuring oxoglutarate
appearance. This led to _Hyphomicrobium_ X being put into the icl^--serine
group , but confirmed the icl^+-serine pathway in _Pseudomonas_ MA. This
finding must throw doubt on any organism ascribed to the icl^+-serine
pathway unless the product of the assay is identified.

2) Ribulose Monophosphate Cycle

The evidence for this pathway of C_1 incorporation in type 1
methanotrophs was first presented by Johnson and Quayle (1965) who
showed that in methane and methanol grown _Pseudomonas methanica_
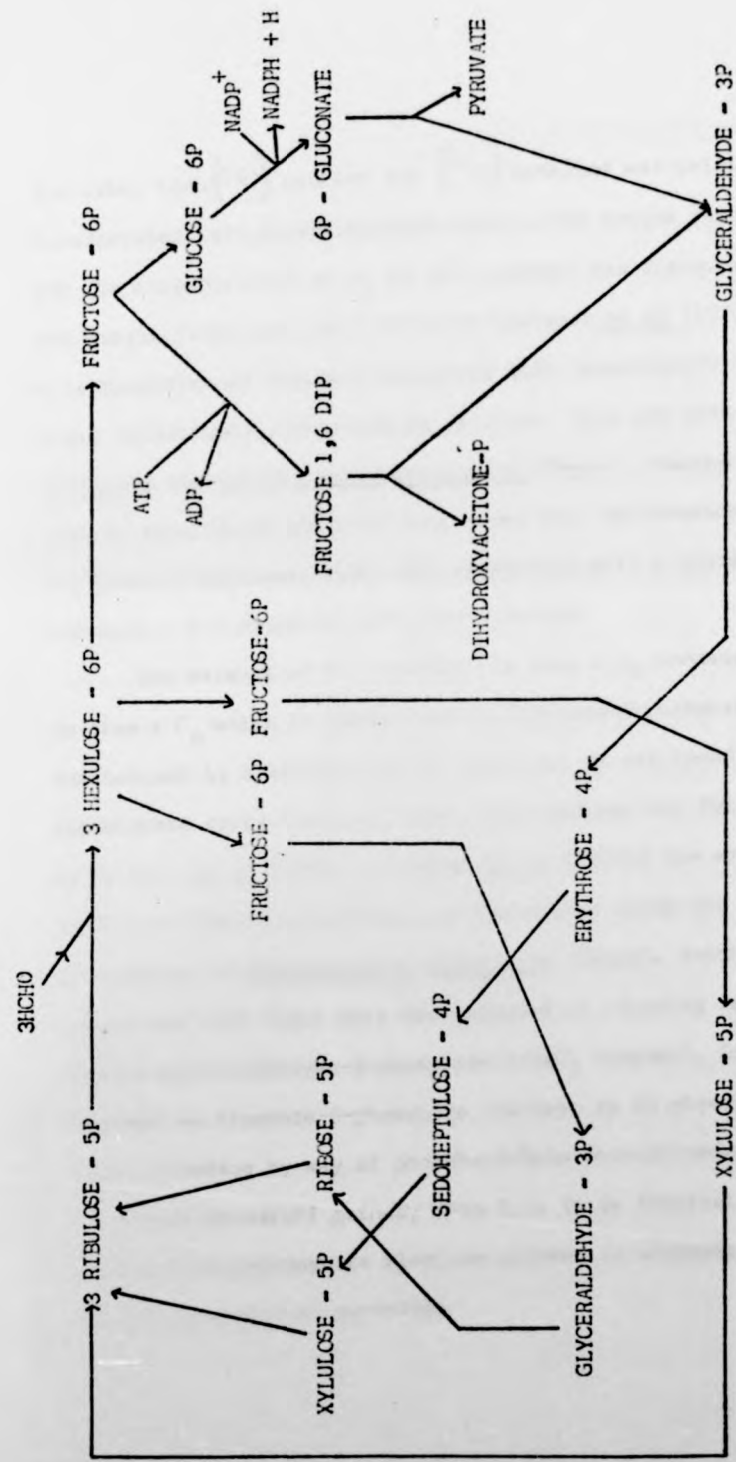
Fig. 2. The ribulose monophosphate cycle of formaldehyde fixation

the label from $\left[^{14}C\right]$ methane and $\left[^{14}C\right]$ methanol was initially
incorporated into phosphorylated sugars. The enzyme responsible
for the incorporation of C_1 in this pathway was discovered by Kemp
and Quayle (1966 and 1967) who with Lawrence et al (1970) demonstrated
a condensation of ribose-5-phosphate with formaldehyde to give a
sugar tentatively identified as allulose this was with Pseudomonas
methanica and Methylococcus capsulatus (Texas). However further
work by Kemp (1972 and 1974) has shown that the product is D-erythro-
L-glycero 3 hexulose, which was consistent with a condensation of
ribulose - 5 - phosphate with formaldehyde.

The essence of this pathway is that a C_5 condenses with a C_1
to give a C_6 which is isomerised to fructose-6-phosphate and then is
metabolised by a similar set of reactions as are found in the ribulose
diphosphate cycle (Quayle, 1972). This pathway was further elucidated
by Ferenci et al (1974) and Strøm et al (1974); the work of Kemp
(1972 and 1974) was verified and D-allulose could not be metabolised
by extracts of Methylococcus capsulatus (Texas). Another point to
emerge was that there were two pathways of cleaving fructose-6-phosphate
to give gylceraldehyde-3-phosphate and a C_3 fragment. One involving the
cleavage by fructose-6-phosphate aldolase as in glycolysis and the
other, cleavage by way of phospho-2-keto-deoxygluconate aldolase as in
the Entner-Douderoff pathway (Fig 2.). It is interesting to note that
the fructose-6-phosphate aldolase pathway is energetically more favourable
for the formation of pyruvate.

CONTINUOUS CULTURE OF METHANOTROPHS

The possibility of producing S.C.P. from methane was suggested by Silverman (1964) although the concept of using micro-organisms (yeasts) as a source of food had been suggested and attempted on several occassions from 1910 onwards (Hamer & Norris, 1971). Since then many reviews have discussed the possibility of producing S.C.P. from methane or methanol (Whittenbury, 1969 and 1971; Wilkinson, 1971; Hamer and Norris 1971).

The first reports of yields obtained by growth on methane were in mixed batch culture systems. Hamer et al (1967) studied the growth of a mixed culture on methane with particular considerations of the gas phase above the culture and the explosive risks involved. They concluded that safe operation can be achieved and wastage reduced by recycling the gas phase. Other reports on cell yields on batch culture were by Iandolo and Klass (1967); Vary and Johnson (1967); Wolnak et al (1967); Silverman and Ooyama (1968); Whittenbury et al (1970 b).

Reports of continuous culture of methanotrophs began about 1970; the yield data and stoichiometries of substrate utilisation are tabulated in Table 1. along with the references. The observation by Harwood and Pirt (1972) of growth of Methylococcus capsulatus (Texas) in batch and continuous culture indicated that growth conditions could be very important in obtaining satisfactory yields. They found that under methane-limited continuous culture the yield was 1.01 g. cells/g. methane used and 0.29 g. cells/g. oxygen used, but under oxygen-limited conditions the yields were 0.3 g. cells/g. methane used and 0.41 g. cells /g. oxygen used. Under oxygen limited conditions it appeared that incomplete oxidation of methane

was occuring and methanol was accumulating in the culture (75% of methane carbon was unaccounted for in terms of cells and carbon dioxide). They

also reported a curious result that Amberlite CG-120 and ground glass stimulated growth and gave higher densities of cultures grown up in their presence the reason proposed for this result was that the additive removed some inhibiter of growth that accumulated in the culture. Also, along these lines Weaver and Dugan (1972) reported that small clay particles increased methane oxidation in cultures of methanotrophs. Wilkinson and Harrison (1973) studied the affinities of two components of a mixed culture for methane and methanol. The two organisms studied were a methane utilising Pseudomonas and a Hyphomicrobium species isolated from the mixed culture. The pure culture of the Hyphomicrobium species had an affinity for methanol four orders of magnitude lower than that of the methanotroph cultuie and the affinity of the methanotroph would probably cause an accumulation of inhibitory levels of this substrate. This was the reason they gave for non-growth cf the pure methanotroph on methane. This mixed culture was further studied by Wilkinson et al (1974) who demonstrated that the Hyphomicrobium species could indeed remove inhibitory levels of methanol to allow the methanotroph to grow. This they demonstrated in a continuous culture to which they added methanol this addition (as low as 0.06 g.L^{-1}) completely inhibited methane oxidation, the Hyphomicrobium population increased and reduced the methanol concentration allowing the methanotroph to start to grow on methane again. An initial concentration of 1.8 mM methanol inhibited methane oxidation. They also presented a mathematical model for the interactions of this system which fitted the results reasonably well

ORGANISM	Y_{CH_4}	Y_{O_2}	STOICHIOMETRIES $O_2 : CO_2$	% CARBON RECOVERY	CULTURE	REFERENCE
MIXED	1.11	0.25	2.23:		BATCH	HAMER et al (1967)
MIXED	0.5-0.7	.08-.23			BATCH CH_4lim	VARY & JOHNSON (1967)
MIXED	0.70	0.23	1.44:		BATCH O_2lim.	VARY & JOHNSON (1967)
METHANOMONAS METHANO-OXIDANS						
MIXED	1.01	0.36			BATCH	SILVERMAN & OOYAMA (1968)
PURE METHANOTROPHS						
MIXED	1.0-1.1		1.0-1.1:		BATCH	WHITTENBURY ET AL (1970)
MIXED	0.90	0.26	1.7:		C.C.	BEWERSDORFF & DOSTALEK(1971)
MIXED	0.62	0.22	1.44:		C.C.	SHEEHAN & JOHNSON(1971)
METHYLOCOCCUS CAPSULATUS(TEXAS)						
MIXED	1.01	0.29	1.8±0.36	103	C.C.CH_4Lim.	HARWOOD & PIRT (1972)
METHYLOCOCCUS CAPSULATUS(TEXAS)						
MIXED	0.31	0.51	0.3: .05	SEE TEXT	C.C.O_2lim.	HARWOOD & PIRT (1972)
MIXED	0.99	0.40	1.24±0.45	110	C.C.CH_4lim	WILKINSON ET AL (1974)
MIXED	0.80	0.24	1.71±0.58	105	C.C.O_2lim	WILKINSON ET AL (1974)

TABLE 1. Reported Yields and Stoichiometries of methanotrophs grown on methane.

C.C. = Continuous Culture.

lim = limited.

except at the lower dilution rates (0.06 - 0.08). Wilkinson and Hamer (1974) also studied the effect of wall growth on a mixed methane utilising bacterial population in batch and continuous culture systems. In the continuous culture system the wall growth was beneficial to the culture because it helped to stabilize the culture close to the normal washout point (D_c) but no enhancement of productivity was observed. Hamer et al (1975) discussed S.C.P. production from methane and presented a mathematical model for a double gas limited culture (if this is possible to achieve) as well as the equation $P = Dx = $ constant for a gas limited culture. This equation was also put forward by Sheehan and Johnson (1971). If the phenomenon of self inhibition is common to methanotrophs [Eroshin, et al (1968) showed that many metabolites inhibit growth of Methylococcus capsulatus (Texas)] it is probable that high cell densities can only be obtained by using defined mixed cultures or using a system in which the inhibition products can be removed (Hamer et al 1975)

Table 1 shows some of the yields, stoichiometries and carbon recoveries reported in the literature. It is difficult to make any critical comments on the array of values obtained. The wide range observed could obviously be because of strain differences and different cultivation techniques. Also the many pitfalls and practical difficulties (as will be emphasised in this thesis) of obtaining reliable carbon balances with a gaseous substrate may add to the variations observed in the results.

This wide variation in yield data has made theoretical estimation of yields rather difficult to explain. However van Dijken and Harder (1975) have made an attempt at predicting yields in methylotrophs. They

viewed growth on C_1 compounds as a two stage process 1) the formation
of 3 phospho-glyerate from C_1 units and 2) the synthesis of cell
material from 3PGA (for which they assume a Y_{ATP} of 10.5).Considering
each oxidation step of the C_1 compounds, methane to methanol required
$NADH_2$, methanol to formaldehyde could only produce two ATP's,
formaldehyde to formate up to 3 ATP's and formate to carbon dioxide
produced $NADH_2$ from which they assumed 3 molecules of ATP could
be generated. They tabulated all the possibilities i.e. methanol/
formaldehyde could give 0,1 or 2 ATP molecules, formaldehyde/formate
could give 0,1,2 or 3 and formate/carbon dioxide could yield 3 ATP
molecules. The two pathways of C_1 fixation were considered (viz.
serine pathway and ribulose monophosphate pathway) and the energy
required or produced for these pathways. From the amount of cells
produced, the number of moles of methanol required for the necessary
carbon and number of moles of ATP were calculated (taking into account
the requirements of each of the two pathways). Then from the $NADH_2$
required for the synthesis of cellular material enough additional
methanol was oxidised to carbon dioxide to produce this $NADH_2$. The
number of steps producing reduced coenzymes could then be calculated
and the number of moles of ATP produced could be calculated (using
all the possibilities from just 3 to 8 moles of ATP from a mole of
methanol oxidised to carbon dioxide) From this the number of moles of
ATP produced were taken away from the number required(calculated from
Y_{ATP}). The difference was then made up by oxidising sufficient methanol
to carbon dioxide to provide the necessary amount of ATP to balance the
equation. The yield can then be calculated from the weight of cells

produced divided by the weight of methanol consumed. The maximum yield that could be obtained from methanol was 0.73 (as opposed to 0.3 - 0.5 from growth studies). If the oxidation of methane to methanol neither produces nor requires energy then the yield on methane would be twice that on methanol (i.e. molecular weight of methane is half that of methanol). These figures were for the more efficient ribulose monophosphate pathway, the yields from the serine pathway were about 20% less efficient.

One interesting factor which was pointed out was that _Methylococcus capsulatus_ which appeared to have a mixed function oxidase for the oxidation of methane requiring $NADH_2$, could only produce this $NADH_2$ by oxidising formate to carbon dioxide, so for every molecule of methane converted to methanol a molecule of carbon dioxide would be produced. In other words no carbon could be fixed. To overcome this problem a reverse electron flow was postulated to provide the necessary $NADH_2$, this production of $NADH_2$ requiring 3 ATP molecules per molecule of $NADH_2$. This was then fitted into the scheme for predicting yields. The highest theoretical yield on methane, using reverse electron flow was 0.91, without reverse electron flow it was 0.75.

Since this publication several results have thrown new light on the problem. Tonge et al (1975 and 1977) have postulated from experimental results that the electrons from methanol (and possibly formaldehyde) dehydrogenase can drive the methane mono-oxygenase in _Methylosinus trichosporium_ , thus doing away with the absolute requirement for $NADH_2$ ($NADH_2$ could only be used as a donor for the methane mono-oxygenase in crude extracts) and so this organism does

not require reverse electron flow however what the actual donor is
in vivo is unknown.

Colby and Dalton (1976) have shown that Methylococcus capsulatus
(Bath) appears to require $NADH_2$ for its mono-oxygenase this again
seemed to require reverse electron flow. However D. Stirling (pers.comm.)
has found an NAD-linked formaldehyde dehydrogenase which could provide
the necessary $NADH_2$ without invoking reverse electron flow. However
H. Dalton (in press) has demonstrated that a reverse electron flow
mechanism can function in cell free extracts of Methylococcus capsulatus
(Bath).

The theoretical discussion of yields can at the present time
only predict a possible maximum yield. The yield that is of interest
to the industrialist is the amount of biomass that can be produced
from one ton of substrate put into the fermenter. So, although the
theoretical yields are of interest it is the actual yields achieved
during fermentation that is important.

MATERIALS AND METHODS

Organisms

The organisms used in this study isolated by Whittenbury et al (1970 b) were 1) Methylococcus capsulatus (Bath strain) which was the principal organism used because of industrial considerations, 2) Methylomonas albus (BG8) which represented type 1 organisms, 3) Methylosinus trichosporium (OB3 b) which represented type 11 organisms. The nomenclature and strain numbers used were those of Whittenbury et al (1970 b)

Inorganic and Organic Chemicals

All chemicals used were of 'Analar' quality, if obtainable, and were purchased from the following manufactures, British Drug Houses Ltd., Hopkins and Williams Ltd., Fisons Ltd., and Sigma Chemical Co. Ltd.

Gases

Biologically produced methane (97 - 99% methane rest carbon dioxide) was obtained initially from the Greater London Council, Mogden Works, Middx., and in the latter part of the study from British Oxygen Co. Ltd. Commercially pure ethane, oxygen, nitrogen, air, carbon dioxide and helium were purchased from British Oxygen Co. Ltd.

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MINERAL SALTS MEDIUM (M.S.)

Mg SO_4 .	1 g . L^{-1}
Ca $Cl_2.H_2O$	0.27 g. L^{-1}
Fe EDTA	4 mg. L^{-1}
5% phosphate solution	20 ml. L^{-1}
T.E. Solution	1 ml. L^{-1}

Nitrate mineral salts (NMS) - MS + 1g.L^{-1} KNO_3

Ammonium mineral salts (AMS) - MS + 0.5g.L^{-1} NH_4Cl.

5% Phosphate Solution

KH_2PO_4	13 g.L^{-1}
Na_2HPO_4	16.6g.L^{-1}

Trace Elements (T.E.) Solution — g.L^{-1}

$CuSO_4.5H_2O$	0.2
Fe $SO_4.7H_2O$	0.5
Zn $SO_4.7H_2O$	0.4
H_3BO_4	0.015
Co $Cl_3.6H_2O$	0.05
$Na_2MoO_4.2H_2O$	0.5
EDTA	0.25
Mn $Cl_2.4H_2O$	0.02
Ni $Cl_2.6H_2O$	0.01.

Table 2. Basal salts media and Trace element solution.

media (1.5g Bacto Difco agar / 100 ml.), the basic media described above were used; however for continuous culture with high optical densities of bacteria (above approx. $O.D_{540}$ 5) additional nitrogen sources were added so they would be slightly in excess in the culture. All batch growth media were adjusted to pH 6.8 prior to autoclaving and the addition of a sterile 5% phosphate buffer made afterwards (2ml / 100 ml. media). As the pH of continuous cultures changed quite rapidly in the culture pot it was found unnecessary to pH the media as frequent pH additions had to be made to the culture to maintain a constant pH. The slightly acidic nature of the media had little effect on this pH correcting system.

Maintenance of Cultures

Stock cultures were kept on agar slopes in test tubes (with cotton wool plugs or plastic caps to allow gas circulation) and incubated in domestic "Tupperware" polythene containers (1L. in volume). These vessels had lids with airtight seals and a port sealed with an airtight cap. The containers were gassed by partially filling a football bladder with methane and adding the gas through the ports in the lid. The stocks were sub-cultured at least every two weeks (any longer, viability might be lost and / or fungal contamination could be a problem). Methylococcus capsulatus was grown and sub-cultured at 45°C all other organisms were kept at 30°C.

Shake Flask Culture

Inocula for continuous culture and the closed batch system were obtained from shake flask cultures in 250 ml. "Quickfit" flasks (neck size 19/26) containing 10 - 25 ml. of media. The flasks were dry

heat sterilized and sterile media added aseptically, the flasks
were either inoculated from slope cultures and the cotton wool
plugs replaced by sterile 'Suba Seals' (size 37) or inoculated from
other flask cultures when the liquid inocula were added via a
sterile syringe through the 'Suba Seals'. 40 ml. of the gas
phase was removed from the flasks and 40 ml. of methane inserted by
using a 50 ml. syringe.

Gas Analyses

The routine assays of oxygen, nitrogen, methane and carbon
dioxide were carried out on a Pye Unicam 104 Katharometer.
Unfortunately it was not possible to assay all four gases on one
chromatographic column. The use of two columns, Porapak R and
Molecular Sieve (mesh size 60 - 80) enabled all the gases to be
separated and analysed. The Porapak column separating air (oxygen
nitrogen and argon), methane and carbon dioxide, the Molecular
Sieve separating oxygen, nitrogen and methane (absorbing carbon
dioxide in the stationary phase). The columns were heated to $50^{\circ}C$ with
a flow rate of 30 ml / min of helium as the carrier gas. The results
were recorded on a Servoscribe potentiometer recorder (type RE 540.20)
with a mechanical integrator attachment (see appendix 1   for
standardisation of gas chromatograph). Acetylene and ethylene were
also assayed using this system.

A Pye Unicam FID was used to detect and assay methanol,
ethanol, acetaldehyde, acetylene and ethylene. Porapak R was used
to detect acetylene and ethylene at $60^{\circ}C$, the other compounds were

assayed at 120°C, for both assays 30 ml. / min of nitrogen were
used as the carrier gas.

The Batch System

To keep going a continuous culture requires a great deal
of effort, merely to maintain a "healthy" pure culture, sterilise
media and checking all the systems are functioning correctly. There are
many reasons why a run may have to be stopped; if a culture must be
maintained in a pure state contamination would require that the
system be stripped down, cleaned, autoclaved and inoculated, the
whole process requires several days to establish a steady-state. One
of the most frustrating ends to a run is mechanical or electrical
failure which may manifest itself as :- loss of pH control, stirrer
gland breakdown, blockage of the gas flow line into the fermenter,
rupture of tubing at a pumping point or even autoclave failure with
the resulting lack of media.

Any of the above factors can halt a series of experiments
resulting in a loss of many days work. To prevent the loss of a
culture through experimental design an enclosed batch system was
developed to estimate the effect of any change in environment imposed
on the culture. To obtain useful results, with respect to yields, a
completely enclosed system had to be set up to monitor the changes in
gas composition and cultural conditions, without any loss from the
system. This system proved more difficult to perfect than anticipated
because of 1) the size of the system, 2) the need for leak proof joints,
3) smooth circulation of the gas phase, 4) correct alignment of each
part of the system and 5) the necessary calculation involved.
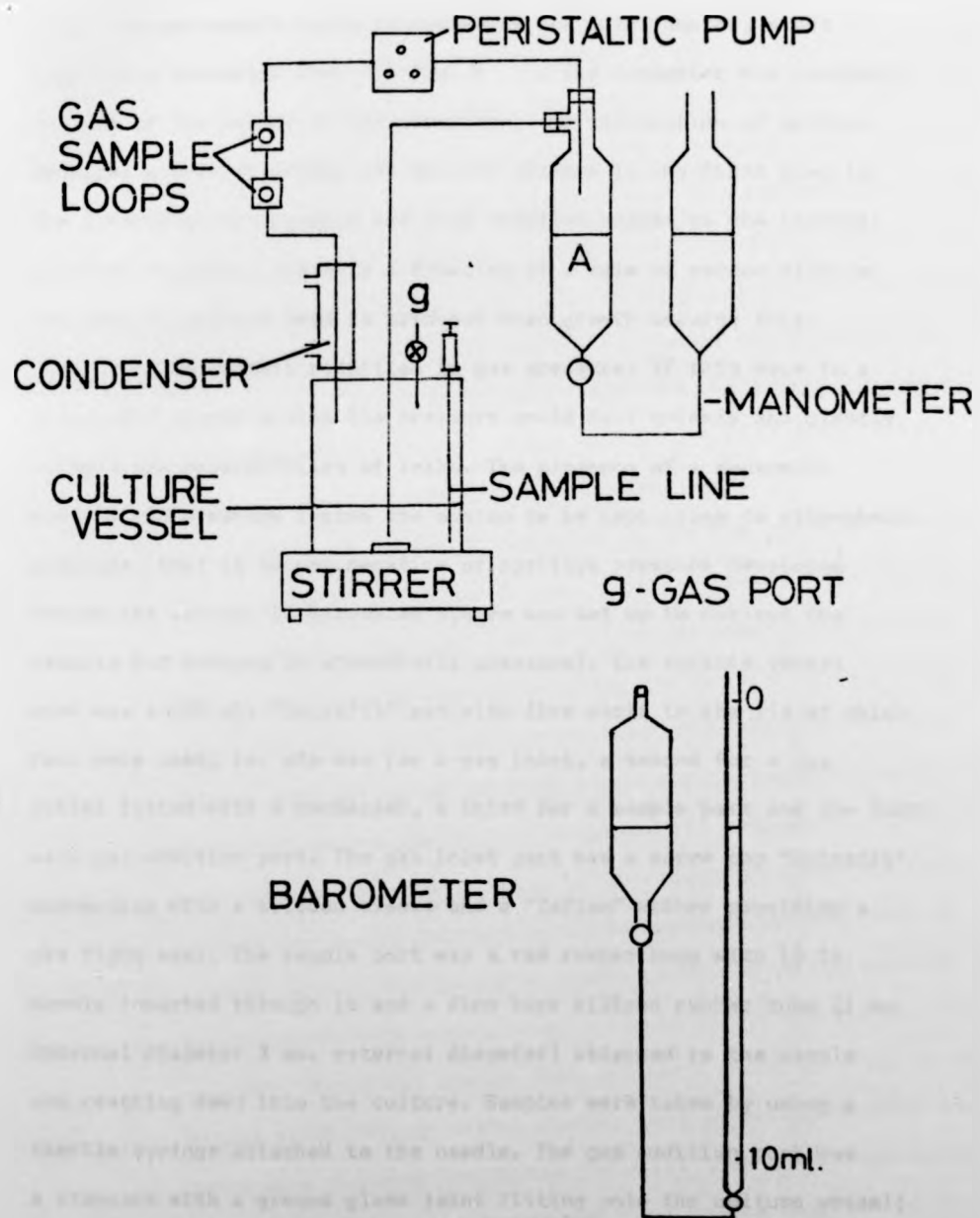
Fig. 3. Closed Batch System and Barometer.

The system consisted of a culture vessel with a culture sample
port, two gas sample loops connected to the Katharometer, a gas
pump and a manometer arm, see fig. 3   . The manometer was necessary
because of the nature of the substrate; the utilization of methane
requires a mole of oxygen per mole of methane in the first step in
the incorporation sequence and also requires oxygen as the terminal
electron acceptor, but only a fraction of a mole of carbon dioxide
per mole of methane used is produced when growth occurs; this
results in an overall reduction in gas pressure. If this were in a
completely closed system the pressure would fall quickly and greatly
enhance the possibilities of leaks. The presence of a manometer
enabled the pressure inside the system to be kept close to atmospheric
pressure that is no net negative or positive pressure developed
inside the system. (A barometer system was set up to correct the
results for changes in atmospheric pressure). The culture vessel
used was a 500 ml. "Quickfit" pot with five ports in the lid of which
four were used, ie. one was for a gas inlet, a second for a gas
outlet fitted with a condenser, a third for a sample port and the forth
as a gas addition port. The gas inlet port was a screw cap "Quickfit"
connection with a silicon sleeve and a "Teflon" washer providing a
gas tight seal. The sample port was a red rubber bung with $1\frac{1}{2}$ in.
needle inserted through it and a fine bore silicon rubber tube (1 mm.
internal diameter 3 mm. external diameter) attached to the needle
and reaching down into the culture. Samples were taken by using a
sterile syringe attached to the needle. The gas addition port was
a stopcock with a ground glass joint fitting onto the culture vessel;

gas injections were made through a piece of clamped tubing put
on the other side.

The whole of the culture vessel could be autoclaved, the inlets
and outlets being plugged with cotton wool to maintain asepsis. The
recycling gas left the culture vessel via a condenser thus ensuring
removal of water vapour (the culture vessel was immersed in a water
bath, usually at 45°C for M.C.) otherwise water would condense
throughout the system. The most convenient place for the gas sample
ports was immediately after the culture vessel and as excessive
water vapour or water in the gas sample was detrimental to the
chromatographic technique, it was essential to fit a condenser
between the culture vessel and the sample ports.

The sampling system was a Pye series 104 gas sampling valve
(Cat. No. 12654) which had 0.5 ml. sample loops, one for each column
on the gas chromatograph, thus enabling gas samples to be taken
without breaking the enclosed system. The gas was circulated by the
use of a Watson-Marlow peristaltic pump (MHRE 200) using Tygon
tubing. This tubing was changed frequently (after every second or
third run) as the peristaltic action of the pump caused the tubing
to split and for this to occur in the middle of a run was disastrous.
The gas was pumped into the manometer system and from there to the
culture vessel. The manometer system consisted of two parallel side
separating funnels connected by a length of silicon tubing, the
manometer fluid was approx. 0.1N sulphuric acid, in order to prevent
excess solubility of carbon dioxide (see later). However it was
found when checking the system for leaks that the carbon dioxide
did disappear from the system and there was an increase in the volume

of nitrogen; methane also disappeared but at a veryslow rate. The loss was minimised by opening the stopcock tap on the system's manometer arm (A) when the total volume of the system was being measured and allowing the system to equilibrate to atmospheric pressure. This procedure became necessary once the culture was actively growing and the higher partial pressures of carbon dioxide were generated.

The system was kept as close to atmospheric pressure as possible in order to minimise the possibility of gas leaks. However because the gas volume of the enclosed batch system was about 1300 ml. changes in atmospheric pressure could adversely upset the results. To correct for this a barometer system was set up (Fig 3) consisting of a 100 ml. separating funnel connected to a 10 ml. (100 cm in length) burrette by silicon tubing, the total volume of the system up to the 0 mark on the burrette was 170 ml. Into this system 40 ml. of distilled water was added giving an air space in the separating funnel of 120 - 130 ml. Changes in this air space due to changes in atmospheric pressure and temperature were monitored and the batch system volume appropriately corrected. In this type of system it was important to obtain a balance between culture volume and gas volume so that any changes which occured were large enough to be accurately measured but not too rapid so as to prevent measurements of rates being obtained ie.if the culture volume was too large changes in optical density were small or if the gas phase was too large changes in gas partial pressures were small, with the resulting loss of accuracy. If the gas phase was too small the gases were consumed rapidly and became severely limiting to growth before any phase of growth could be measured. In this system a 100 ml. culture was used with an initial gas phase of about 1200 ml.

The main problem with an enclosed batch system was the prevention of leakage (or exchange) of gases with the atmosphere. Leaks were minimised by keeping glass tubing to glass tubing connections to a minimum. Originally silicon tubing was used in the peristaltic pump and to join glass tubes together, however this type of tubing was found unsuitable because it was too permeable to gases. The final system had only three connections, one was at the pump using Tygon tubing (6.0 mm internal, 7.6 mm external diameter) and the others were between the manometer and culture vessel and between the condenser and the sample loops for which thick wall red rubber tubing was used. (These joints were found necessary to give some degree of flexibility to the system).

This batch culture system enabled the growth of the culture and consequential gas changes to be monitored from lag to stationary phase of growth. The changes that occured when additions were made to the culture in linear (gas limited) growth could be followed. The calculations involved were quite lengthy and a programme for a Compucorp Statistician was devised to shorten the length of time spent on working out the results (see appendix IV)

The calculations involved in obtaining the ratios of the gases in the system were identical to those used for the continuous culture results (see later). From these ratios it was possible to work out the volume of each gas from the total volume of gas in the system. This total volume was obtained by adjusting the manometer liquid menisci to the same level (to adjust to atmospheric pressure) and reading the volume of liquid in flask A. Thus the total volume

in the system at any given time was :-

$$1365 - a = b$$ where a was the volume of liquid in flask A.
(the total volume of the system was 1365 ml.)

the volume of any gas in the system was :-

$$b \times P_p \text{ gas}$$ where P_p = partial pressure.

If these values were compared to the volumes of each gas at the start of the experiment, the volume of each gas used or produced, as a consequence of growth and metabolism, can be followed.

However there were two problems involved with calculating the gas volumes :- 1) about 40% of the gas phase was at $45^{\circ}C$ and the rest at room temperature. Overcoming this the system was considered as two parts, a part where (1) the gas space was at $45^{\circ}C$ in a constant volume and (2) gas space at room temperature which decreases in volume as growth occurs. The total volume of the system was worked out as though all the system was at room temperature, thus the volume of the system when working was actually larger than the calculated volume ie.

a) Actual volume = volume of gas at $45^{\circ}C$ + volume of gas at room temperature

b) Calculated volume = volume of gas at $45^{\circ}C$ corrected to room temperature + volume of gas at room temperature

Subtracting b) from a)

Actual volume - calculated volume = volume of gas at $45^{\circ}C$ - volume of gas at $45^{\circ}C$ corrected to room temperature.

The volume of gas at 45°C corrected to room temperature could be calculated by assuming all of the culture vessel to be at 45°C and using the gas laws. e.g. $\dfrac{V_1}{V_2} = \dfrac{T_1}{T_2}$ where V = volume,

              T = temperature ($^{\circ}$A),

              1 is under one set of conditions,

              2 is under another set of conditions

A simple way to check on the actual volume difference was to set the system going at room temperature (including culture vessel) and then to raise the temperature of the culture vessel to 45°C (the working temperature) and note the difference in volume which occurs. This method not only corrected for the culture vessel gas being at 45°C but also corrected for any increase in water vapour pressure and any rise in temperature of the gas in the rest of the system as a consequence of the hotter gas in the culture vessel. (The condenser cooled this gas to a certain extent). The actual difference observed was approximately 80 ml. (noted before every run). So the actual working volume of the system had to be reduced by the value obtained before the volume of the gases in the system can be calculated.

2) The functioning of the gas sample ports required helium to be passed through the loops when a sample was taken. So when the gas atmosphere was passed through the loops again the helium, previously in the loops, was now in the enclosed batch system. This volume was assumed to be 1 ml. at each sample(ie. each loop having a volume of 0.5 ml.) and not to be of any significance in the calculations. During runs with the system it was noted that the nitrogen content

increased with time, this apparent increase in nitrogen was finally
traced to the fact that the helium in the chromatography
columns of the Katharometer was under pressure (due to the back
pressure of the columns) and that every time a set of gas samples
were taken not 1 ml. of helium was added to the system (to compensate
for the 1 ml. of gas removed) but 1.6 mls. were added, thus increasing
the volume of the enclosed system and as helium was not detected
this increase in volume was added to the gases in the system in
proportion to their partial pressures. So after each gas reading the
system volume had to be decreased by $(1.6 - 1)$ml. Corrections could
be made for the volumes of the gases removed for the samples, but
because 1 ml.of helium is left in the system, uncorrected, the volume
of the system is larger than if the sample had just been removed. So,
in effect the calculated volume of each of the gases in the system are
slightly larger than they actually are, thus compensating to some
degree for the gas removed. In addition the partial pressures of the
important gases(oxygen, methane and carbon dioxide) are only 0.2 at
a maximum (or 0.1 averaged over a run) and therefore the total loss
over a run of thirty samples would only be 3 ml.

Corrections for removals or additions to the culture (e.g. for
$O.D_{540}$ estimations, substrate additons) were also made. Finally the
volumes of all the gases were converted to STP when necessary.

Continuous Culture Apparatus

An L.H. Engineering 5L. (3.5 L. culture volume) was used for the
most part of this study, although later on a 3.0L. (2.0-2.5L. culture
volume) was also used. The apparatus had temperature, pH and antifoam
controls as well as meters for stirring, r.p.m and oxygen tension. The
nature of the study to be undertaken (yield data) required constant

gas flows for both air and methane, and sampling vessels, with ports, for the gas phase assays. The apparatus had to be set up with this in mind, enabling samples to be taken and gas flows measured without interrupting the system as a whole.

Stirring

The impeller shaft was driven from above and was connected to the motor by a system of belts and pulleys. The shaft itself was direct drive and the impellers were attached to a shaft which passed through a water and gas tight coupling to outside the fermenter. (Problems are listed at the end of this section)

pH control

Initially probes were used with external reference electrodes and sealed central electrolyte space, however these electrodes did not fully withstand autoclaving and failed to function after the second or third autoclaving. Activion electrodes were used later on and proved less problematic following autoclaving. An E.I.L pH meter with alarm settings was used, the alarm when activated switched on a pump via a Crouzet timer which could be set for pumping cycles from 0 to 3 minutes in duration.

On NMS medium the pH became alkaline and hydrochloric acid was used as the pH addition, the strength of the acid used depended on the density of the culture, usually the strength was 0.5M although 1M acid was sometimes used. During growth on AMS the pH dropped quickly and the strength of the potassium hydroxide used as pH addition was always 1M. The pH change on MS medium was acidic but was slow compared to that on AMS, so less concentrated alkali

was used (0.5M), except when cultures were changed from AMS to MS and then the potassium hydroxide concentration was kept at 1M.

Temperature

The temperature was controlled by a Churchill circulator, the temperature inside the fermenter being recorded by a thermocouple probe. The circulating water from the "Churchill" was also used to cool the drive shaft couplings in the fermenter head plate and then entered into a wide stainless steel U-tube passing down to the bottom of the culture and back out again through the top of the fermenter. The "Churchill" could either heat the circulating water or cool it by means of cold water from the mains supply running through a coil in the water reservoir.

Antifoam

Foam detection was undertaken by means of a probe inserted through the head plate of the fermenter to which the live terminal was connected, the fermenter head plate formed the other side of the electrical circuit. The probe was isolated from any electrical connection to the fermenter. The plate on the end of the probe was held about 2 cm. above the surface of the culture. In a no foam situation there is no completion of the foam detection circuit, but if the culture foamed to a level sufficient to touch the probe, the circuit is completed by the current passing through the culture to the metal of the fermenter pot. The completion of this circuit switches on the antifoam pump controlled by a Crouzet timer enabling additions of antifoam solution to be made on three minute cycles, pumping for between 0 to 3 minutes at a time. The antifoam solution used was 0.25% v/v of antifoam A emulsion (30% silicone) in distilled water.

Oxygen Tension Measurement

The oxygen tension in the culture was measured with a
Western Biological oxygen probe connected to an L.H. Engineering
meter. The membrane on the probe   was changed after every run of
more than two weeks.

Medium Flow and Measurement

Fresh medium was added to the culture by means of a Watson Marlow
peristaltic pump (MHRE 200) with silicon tubing of 1mm internal
diameter and 1mm. thick walls. The tubing was moved along periodically
to prevent excess wear and the possibility of a split in the tubing.

To obtain an approximate flow rate of medium a 10ml. pipette
was placed by means of a T-junction into the medium line between the
pump and the fermenter, the pipette usually clamped off. To take
a measurement the tubing between the fermenter and the pipette was
clamped off and the clamp removed from the tubing to the pipette. The
rate of medium flow was measured from the length of time to pump a
known volume of medium using the pipette to measure the volume. After
the reading had been taken the medium in the pipette was allowed to
run into the fermenter and then the tubing to the pipette clamped
again. A more convenient and more accurate estimation of medium flow,
taking into account antifoam and pH additions, was to record the
volume of spent medium in the waste pot at known time intervals. This
method of calculating the dilution rate also measures rates over longer
periods of time than the pipette method thus giving a more accurate
overall dilution rate.

Medium left the culture via an overflow tube inserted into
the culture from the base plate. This tube could be altered in height

to change the working volume of the fermenter, if necessary.

Culture Sampling

Two methods of sampling were used, both utilising sampling hoods :- 1) Samples were taken from the overflow line by diverting the spent culture into the sampling vessel, this could take around 10 minutes or longer at low dilution rates.

2) A better method was to have a metal tube passing through the head plate into the culture, enabling about 75% of the culture to be removed if required. The metal tube was connected to the sample hood by silicon tubing with two clamps, one at each end of the tube. When samples were taken the clamp nearest the fermenter was removed and used to clamp the gas outlet line, the overflow line was held closed with thumb and forefinger and then the second clip opened enabling the gas pressure to build up in the fermenter to force the culture into the sample bottle. The first sample taken was discarded because it contained material which had previously been in the tubing and not representative of the culture. The sample bottle was removed and a fresh sterile bottle put in its place and the procedure repeated to obtain the material for analysis.

Gas Stream

The measurement of growth yields required constant flows of gas. Air was obtained from an air pump (Charles Austen Pumps Ltd.) model F65, a bleed valve prevented undue back pressure building up between the flowstat and the pump. Methane was obtained from a gas cylinder at a pressure of 10 p.s.i. on the line. The air and methane were passed
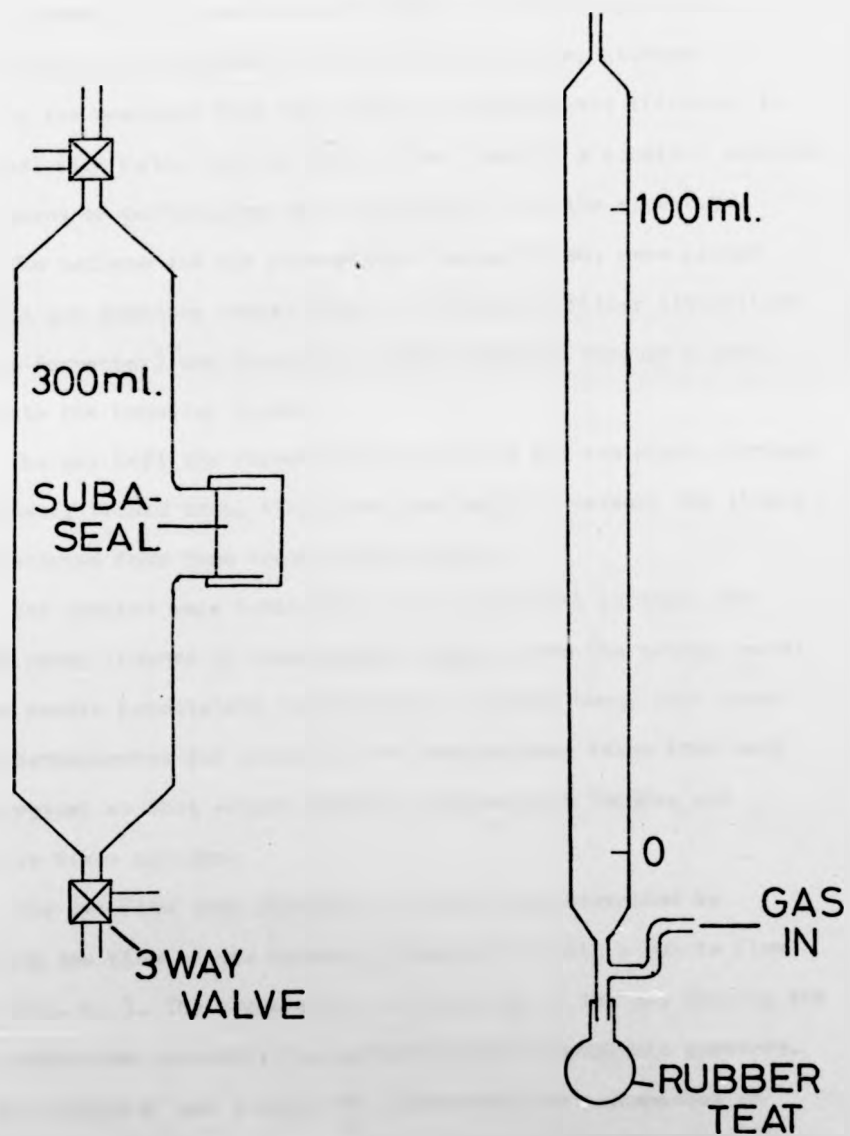
Fig. 4. Gas Sample Vessel and Bubble Flow Meter

through Platon Flowstats and gap meters before being mixed. The flowstats gave fairly good constant flows of gas, the gap meter floats giving an estimation of the rate of gas flow, although sometimes the measured flow rate could be considerably different to the actual flow rate. However keeping the float in a constant position gave a means of controlling any fluctuations from the flowstats.

The methane and air stream after being joined, were passed through a gas sampling vessel (fig. 4   ), then a filter (sterilized with the fermenter) and finally into the fermenter through a port underneath the impeller blades.

The gas left the fermenter at a port in the top plate, through a condenser, liquid trap, filter and gas sampling vessel. The liquid trap prevented foam from reaching the filter.

Gas samples were taken with a 1ml. gas tight syringe. The syringe being flushed 10 times before removal from the sample vessel and the needle immediately inserted into a rubber bung, then taken to the Katharometer for analysis. Two samples were taken from each sample vessel so that assays could be made on both Porapak and Molecular Sieve columns.

The gas flow rate through the system was determined by measuring the flow of gas leaving the fermenter with a bubble flow meter (fig. 4.). The temperature and pressure of the gas leaving the system were also recorded, the pressure being atmospheric pressure. From the volume of gas leaving the system and the percentages of nitrogen in the inflowing and outflowing gas streams the inflowing gas rate could be calculated. The procedure used to calculate the volume of gases used or produced by the culture is described in the results and discussion section (section 1b).

Mechanical Problems Encountered During Continuous Culture

The main problem of continuous culture is not usually the culture itself but the many mechanical and electrical pieces of apparatus required to keep the cultural conditions constant. Some of the problems encountered are listed below.

pH Control System. - pH probes would fail to function efficiently.
The pH meter was affected by vibrations from the air pump, this pump was removed from the fermenter housing.
The pH probe was earthed to the framework of the fermenter (it should not have been).
The tubing at the pH addition pump would block, leak or allow pH addition to the culture when the pump was not turning.

Autoclaving   Tubing often came off connectors if not securely fastened on.
20 L. flasks would shatter.
The bungs in the 20 L. bottles would come out.
Plastic connectors would disintegrate, especially on the pH addition line.

Temperature Control Few problems, the temperature would normally be kept at $45^{\circ}C$ without any requirement for cooling water. However in hot weather the culture

temperature would increase, so cooling water
was passed through the circulator.
Circulator seized.

DOT measuring   Probes have a finite life (about 12 months) and
system   could fail to function during a run.

Gas supply   Gas leaks were a major problem, especially when
setting up a system for the first time.
Gas inlet would block.
Overflow tube would become partially blocked and
cause the culture volume to vary. Removal of the
hood over the outlet cured this problem.

Dry Weight Estimations

The concentration of biomass in terms of dry weight was
initially estimated by the use of two methods :-

1). Acid washed glass centrifuge tubes were weighed   to
constant weight after being heated at $80^{o}C$. Known volumes of culture
were added to these tubes and the cells spun down. The supernatant
was discarded and then the tube and pellet was weighed to constant
weight again after being heated at $80^{o}C$.

2). A known volume of culture was filtered through a
preweighed 'Millipore' filter (heated at $80^{o}C$ before weighing).
The filter with the cells was weighed to constant weight at $80^{o}C$.

These two methods gave similar results, and so the filter
method was used as the routine method of dry weight estimations.

this method was simpler (no acid washing or centrifuging and easier
to handle.)

Fig. 5. shows the optical density at 540 nm. measured on
a Pye Unicam S.P.500 plotted against the concentration of organism
(mg. dry weight. ml^{-1}).
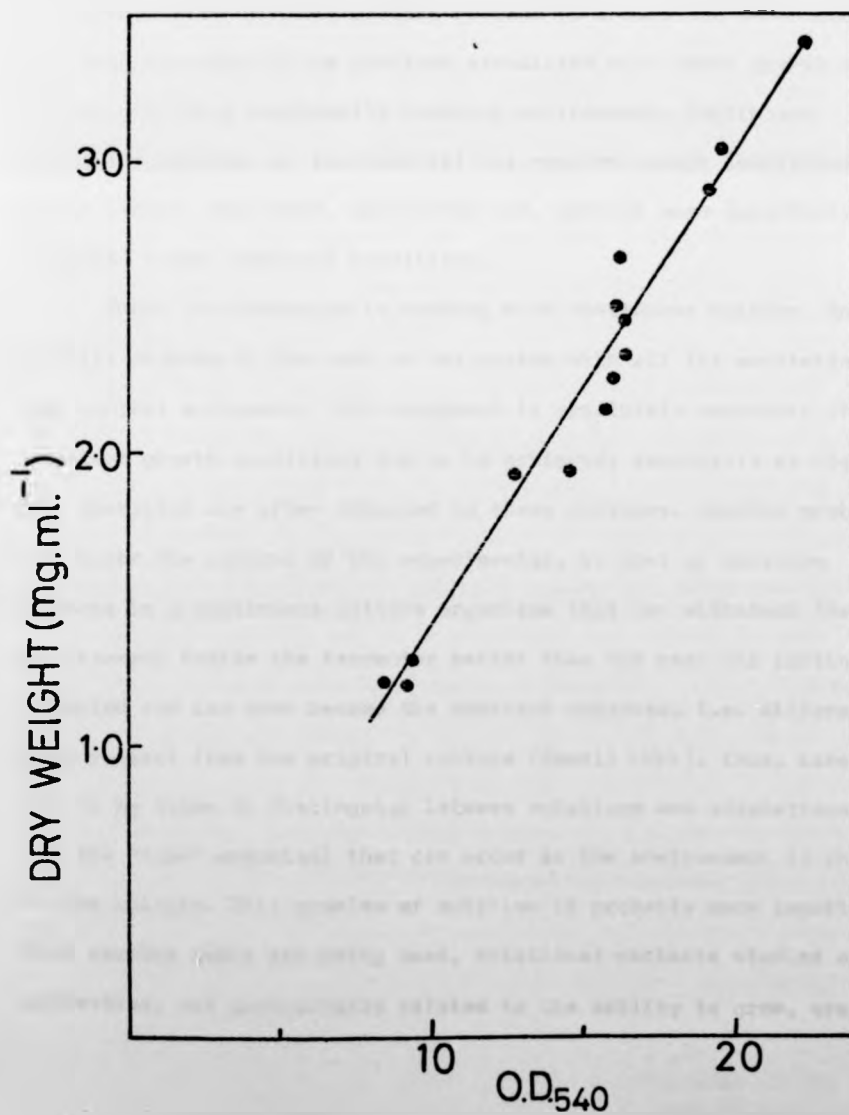
Fig.5. Calibration of $O.D_{540}$ readings for Dry Weight of Methylococcus
capsulatus in mg.ml^{-1}

RESULTS AND DISCUSSION

1. CONTINUOUS CULTURE - A MEANS OF SINGLE CELL PROTEIN PRODUCTION

Continuous culture has been used as a tool in microbiology since about 1950 (Novick and Szilard, 1950, Monod, 1950.). The introduction of this method of culturing has been one of the major advances in the study of micro-organisms. The ability to culture micro-organisms for long periods of time in a constant environment has overcome many of the problems associated with batch growth and its accompanying continually changing environment. Continuous culture (chemostat or turbidostat) has enabled growth conditions to be closely monitored, quantified and, perhaps more importantly, repeated under identical conditions.

There are drawbacks to working with continuous culture. An initial problem is the cost of the system with all its monitoring and control equipment. This equipment is absolutely necessary if constant growth conditions are to be achieved, especially as high cell densities are often obtained in these cultures. Another problem, not under the control of the experimenter, is that of mutation because in a continuous culture organisms that can withstand the environment inside the fermenter better than the rest are continually selected and can soon become the dominant organism, i.e. different in some respect from the original culture (Powell 1958). Thus, care has to be taken to distinguish between mutations and adaptations (of the "same" organism) that can occur as the environment is changed in the culture. This problem of mutation is probably more important when complex media are being used, mutational variants studied or properties, not particularly related to the ability to grow, are being

selected e.g. fermentation products, secondary metabolites etc.

For single cell protein (S.C.P.) production the effect of
mutations is not such a problem, in that any mutations which do
occur and become incorporated into the culture as a whole will
probably make the culture better suited to their environment
(fermentation conditions). The problem will be important if mutational
changes alter the end product i.e. toxicity or quality, or the
organisms become less efficient at converting their substrates to
biomass.

In many respects continuous culture is ideally suited for S.C.P.
production, its advantages in cost terms over batch cultures is many
fold and, of course, the product, because of the controlled
environment, will be consistent.

The project undertaken for this thesis was to investigate the
factors which affect the efficiency of biomass production from methane.
This study was involved with the techniques of cultivation and the
effects of changes in the environment on efficiency of growth of
methanotrophs. The continuous culture system, the measurement and
calculation of results had to be developed and tested. To have
consistency between results it was only possible, in the time available,
to study closely one organism; the choice of this organism was very
important. Obviously the organism to study would be one which could
be used commercially, taking into account all the parameters that
an industrialist would require for single cell protein production.
Not only would the efficiency of conversion of substrate (methane)
to biomass be of importance but also the organism should be able to :-

- grow reasonably quickly,

- withstand the environmental conditions inside an
  industrial fermenter,

- be relatively easy to grow,

- easy to harvest,

- as little extracellular material as possible,

- have a high growth temperature,

- and finally, and most importantly the end product
  must be acceptable as a source of single cell protein.

The reason for a high growth temperature is that cooling of
industrial fermentations is always a problem. Organisms produce
heat as they grow and in large industrial fermenters this heat has
to be removed. In small laboratory fermenters the surface area to
volume ratio is usually sufficiently large   to allow any heat
developed to be dissipated away to the surrounding environment. In the
case of growth on methane the cooling problem is of even greater
significance because of the reduced nature of the substrate and the
amount of oxidation necessary to oxidise the carbon source to the level
of cellular material and to carbon dioxide for energy production with
its related heat production. For these reasons the choice of organism
to study was <u>Methylococcus capsulatus</u> (Bath strain) its main advantage
over other types of methanotrophs was its ability to grow at $45^{\circ}C$.

To study the efficiency of growth it is necessary to be able
to obtain values for amounts of substrates used and biomass produced.
This held many problems in that the substrate was a gas and much less
easy to handle than the conventional dissolved substrate in the medium.
Part (a) in this chapter deals with the efficiency determinations and
Part (b) with the gas limited growth of methanotrophs leading to a
different type of continuous culture (from the chemostat or the
turbidostat) which required a new theory to predict changes in steady
state cell densities as the dilution rate was varied.

1.a) THE PROBLEM OF EFFICIENCY CALCULATIONS

There are several ways of defining the efficiency with which an organism can convert a particular substrate to biomass. Perhaps the most common definition used is known as the yield constant (Y) where :-

$$Y = \frac{\text{weight of bacteria formed}}{\text{weight of substrate used}}$$

this definition is used by the industrialist where the price of the substrate is always given per unit weight so that the yield of biomass can always be equated to the cost of raw materials however when theoretical growth yields are being considered the yield constant is sufficient for comparison of different organisms growing on the same substrate but is not particularly applicable to comparison of yields on different substrates. A more useful definition for theoretical studies is the molar growth yield (Y_m)

$$Y_m = \frac{\text{weight of cells produced}}{\text{mole of substrate used}}$$

which gives the amount of cells that can be expected to be produced from one mole of the substrate. This definition does have inadequacies when substrates with different numbers of carbon atoms are being considered, when another yield constant, the carbon growth yield (Y_c) can be used.

$$Y_c = \frac{\text{weight of carbon in bacteria produced}}{\text{weight of carbon used}}$$

Throughout this thesis any references to yields will be of the yield constant unless stated otherwise.

MEDIUM IN

('C' SOURCE)

GAS OUT (UNUSED 'C' SOURCE,

CO_2 AND VOLATILE

PRODUCTS)

GAS IN

('C' SOURCE AND CO_2)

OVERFLOW

(UNUSED 'C' SOURCE, CELLS AND

DISSOLVED PRODUCTS)
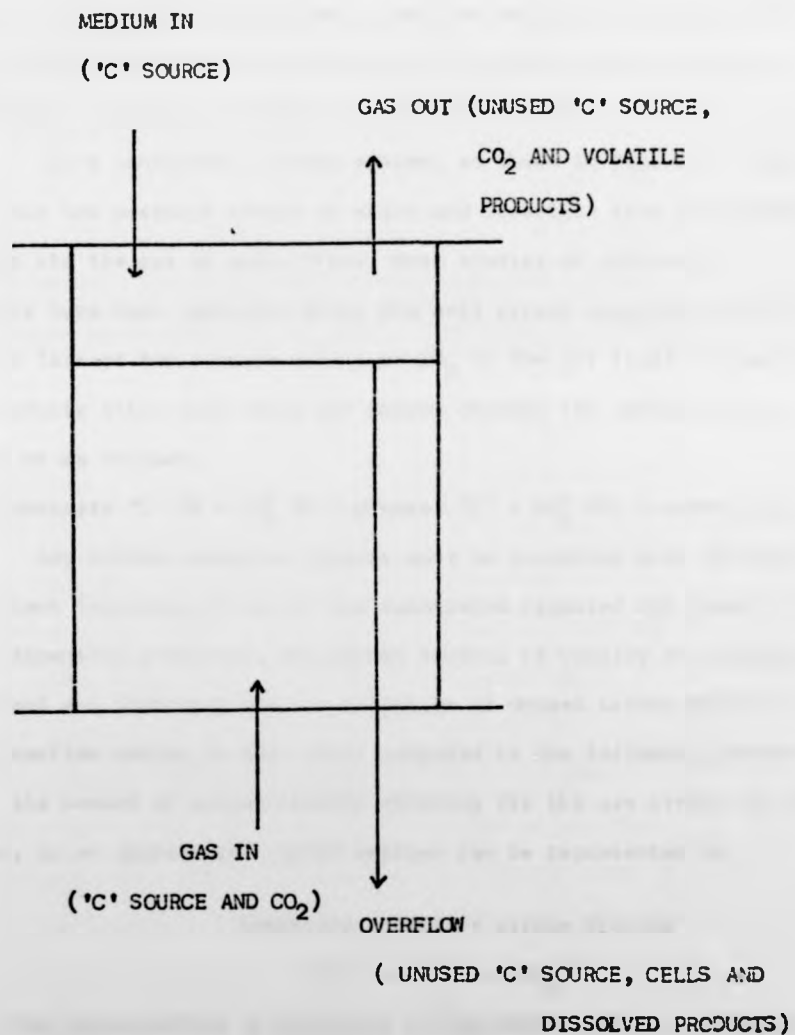
Fig. 1.1. Routes of carbon in and out of a fermenter

Whichever definition is used, a method has to be devised for actually measuring the efficiency. Besides arriving at a figure for the yield, obtaining a carbon balance is also desirable so that all the carbon entering the system can be accounted for.

In a continuous culture system, as shown in fig. 1.1. there are only two possible routes of entry and departure from the chemostat, either via the gas or media flows. Most studies of continuous culture have been conducted where the only carbon entering is in the medium (except for a small amount of CO_2 in the air flow). Assuming no products other than cells and carbon dioxide the carbon balance would be as follows.

Substrate 'C' IN + CO_2^c IN = Biomass 'C' + CO_2^c OUT + unused substrate 'C'

Any system producing biomass must be concerned with the most efficient (economical) use of the substrates required for growth. The most expensive substrate, the carbon source, is usually the limiting nutrient and therefore the concentration of unused carbon source in the overflow medium is very small compared to the inflowing concentration ;

the amount of carbon dioxide entering via the gas stream is also small, so an approximate carbon balance can be represented by :-

Substrate = cells + carbon dioxide

$$S^c F = x^c F + CO_2^c$$

The concentration of substrate (S) is determined by the amount added to the medium in preparation or more accurately by assaying for the particular substrate from the medium line. The cellular carbon is present in the cells (dry weight) leaving the culture and gaseous carbon dioxide can be estimated by either having a known steady flow

of gas through the system and assaying the effluent gas stream for
the partial pressure of carbon dioxide or simply to bubble the
effluent gas through a carbon dioxide trapping agent for a set time
and then assaying for the amount of trapped carbon dioxide. The
inflowing gas could be scrubbed of carbon dioxide before entering the
system.

However, when the carbon source is a gas, as with methane,
the problem of obtaining a carbon balance becomes much more complex.
Not only is the sampling more arduous but also consistent gas flow
regulators are required which give constant rates of gas flow over
long periods of time. Even when the culture is methane limited, not all
the methane is consumed and a considerable portion of the entering
methane leaves via the gas outlet port. This is true of the small
laboratory scale fermenters used in this study; on the other hand
large tower fermenters, as would be used by industry, would consume
most, if not all, the entering methane. Before any attempt can be
made   to measure a carbon balance for growth on methane in
continuous cultures constant flows of gases are essential. The
gases can be mixed before entering the culture or added seperately.
For a small laboratory fermenter it is desirable to mix the gases
before they enter the culture, although this limits the proportion of
gases which can be added because of the explosive nature of methane /
air mixtures between 5% and 15% methane in air. Adding the gases
seperately has the problem of estimating how much of each gas has
entered the system per unit time. Which would mean putting great
reliance on the gas flowstats without a simple accurate check of the rates

concerned. The gap meters do give some idea of the flow rates but the floats jump up and down the tube, especially at low flow rates making accurate control difficult.

Assuming no products other than cells and carbon dioxide, a carbon balance for growth on methane would be :-

$$CH_4 \ ^C \ IN + CO_2^C \ IN = biomass \ 'C' + CO_2^C \ OUT + UNUSED \ CH_4^C$$

This equation is similar to the one for a dissolved carbon source only now the unused carbon source is not small and the carbon dioxide in the inflow gas can be significant as the methane used contained contaminating carbon dioxide (1 - 2 % carbon dioxide). The inflowing carbon dioxide could be scrubbed out but this would require repeated changes of the scrubbing agent during long runs on the fermenter. However as samples have to be taken to assay for the content of the inflowing gas (nitrogen, oxygen and methane), and as a consequence the partial pressure of carbon dioxide is also determined, the assay for incoming carbon dioxide is not inconvenient.

To obtain the value for the efficiency with which an organism can convert methane into biomass (and obtain a carbon balance) samples have to be taken of the inflowing and outflowing gases as well as determining the amount of biomass leaving the system via the overflow. The procedure for this is described in the following section.

Fig.1.2. FLOW DIAGRAM FOR ANALYSIS OF GAS SAMPLES
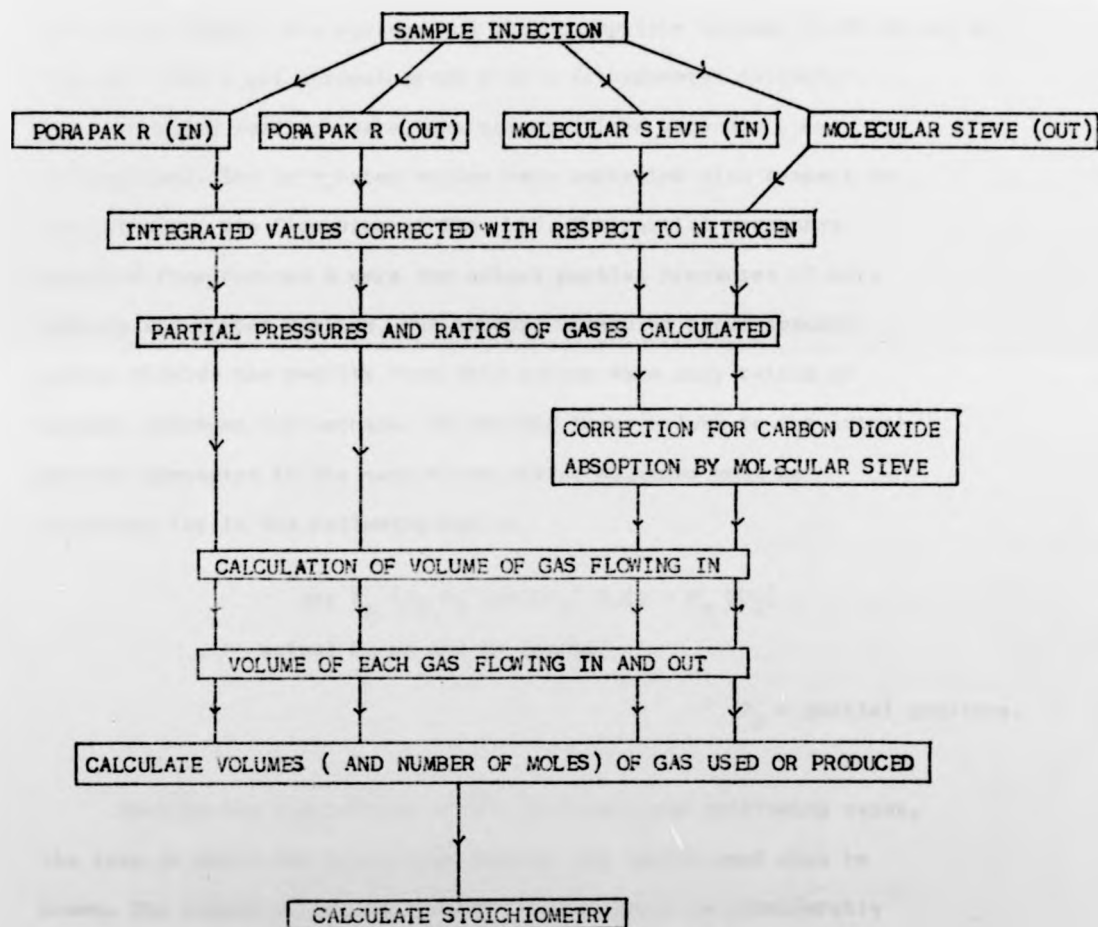
Samples were taken from the inflowing and outflowing gas lines
with a gas tight 1 ml. syringe and the appropriate volumes (0.35 to 0.5 ml)
injected into a gas chromatograph with a Katharometer dectector.
The integrated readings were then computed (see appendix 1 for
calibration). The integrated values were corrected with respect to
the nitrogen, see flow diagram fig. 1.2. The partial pressures
obtained from Porapak R were the actual partial pressures of air,
methane and carbon dioxide, but because molecular sieve absorbs
carbon dioxide the results from this column were only ratios of
oxygen, nitrogen and methane. To correct these values to the actual
partial pressures in the sample the ratios obtained must be
corrected for in the following way :-

$$\text{gas } P_p \ (O_2, N_2 \text{ and } CH_4) \ X \ (1 - P_p \ CO_2)$$
$$= \text{ actual } P_p \text{ of gas in sample.}$$

$$P_p = \text{partial pressure.}$$

Besides the composition of the inflowing and outflowing gases,
the rate at which the gases flow through the system must also be
known. The volume of gas leaving the system could be considerably
different from that entering, because the volume of gas consumed
(methane and oxygen) was three times greater than the gas produced
(carbon dioxide). The inflowing volume was not easy to measure
accurately, the gap meter floats often were not steady and exact
readings were not possible from the scales on the gap meter. An
inline bubble meter was installed between the gas sampling vessel
and the bacterial filter. This bubble meter gave reasonable results
but was awkward to use, the bubbles moved jerkily as the gas was
forced into the bottom of the culture and, of course, the gas would

be under pressure from being forced into the culture. To overcome
these problems it was realised that the volume of gas flowing
through the system could be calculated by measuring the rate at
which the gases left the fermenter with a bubble flow meter and
correcting to standard temperature and pressure. The flow of gas out
was steadier than the flow of gas in. Knowing the volume and composition
of the gas flow out it was possible to calculate the inflow gas
rate by assuming the nitrogen flowing through the system to be
constant (neither used nor produced) and use it as an internal
standard. The volume of nitrogen flowing out per unit time must
equal the volume flowing in per unit time and as the composition
of the inflowing gas was known the total volume of gas entering the
system can be calculated as follows :-

'X' ml.min.$^{-1}$ gas out of a partial pressure 'a' of nitrogen.

Therefore $X \times a = b$ where b = volume of nitrogen flowing
through the system per minute.

If 'b' ml.min^{-1} of nitrogen flowing in and the partial
pressure of nitrogen entering is 'c' then :-

$$\frac{b}{c} = y \quad \text{where } y = \text{total volume of gas entering} \\ \text{the system}$$

Then substituting for 'b' from the previous equation

$$y = \frac{ax}{c}$$

However the assumption that nitrogen is neither used nor
produced has to be treated with caution; the organisms could be
utilising dinitrogen as their nitrogen source, although the amount
consumed in a laboratory fermenter (2 - 4 l. culture volume) at cell

densities of $1 - 4$ $g.L^{-1}$ is small compared to the total amount of gaseous nitrogen passing through the system. Even if a culture is fixing dinitrogen, corrections can be made by knowing how much dinitrogen is being fixed (i.e. percentage nitrogen in the dry biomass). Also, especially with mixed cultures, denitrification could be occuring.

The volume of each gas used or produced can now be calculated from the volume of gases flowing in and out and their respective gas composition e.g. for methane

$$\text{methane} \quad \text{in} \quad d = y \ P_p \ CH_4 \quad \text{in}$$
$$\text{methane} \quad \text{out} \quad e = x \ P_p \ CH_4 \quad \text{out}$$

Therefore   volume of methane consumed $= d-e$.

The volumes of each gas used or produced per hour was converted to the number of $mmoles.hr^{-1}$. 22.4 L. of oxygen and methane and 22.3 L. of CO_2 are 1 mole. From these values the gas stoichiometries are obtained by dividing the number of $mmoles. \ hr^{-1}$ of oxygen used and the number of $mmoles.hr^{-1}$ of carbon dioxide produced by the number of $mmoles.hr^{-1}$ of methane consumed. The stoichiometry of growth gives the number of molecules of oxygen used or carbon dioxide produced per molecule of methane consumed. The weight of each gas used or produced can easily be calculated from the number of mmoles used or produced per hour.

The calculations involved were mathematically simple but numerous; to work out one set of results (even with a calculator) took several minutes, also about ten sets of readings were taken per steady state. To lessen the length of time used in doing the calculations a computer programme was devised using Fortran $\underline{IV}$ computer language.

The programme is given in appendix 11

Although the programme saved time, the computer centre
was a distance from the department and delivery and collection of
the programme and results was inconvenient. Fortunately the
department acquired a programmeable desk top calculator
(Compucorp Statistician). This had the advantage of being available
at any time and results could be calculated immediately they were
obtained. So a programme was devised for this calculator and is
also given in appendix 111

Output Carbon Dioxide Fluctuations

Gas samples taken from the input line gave constant gas
partial pressures; however the output samples varied with respect
to the partial pressure of carbon dioxide. These variations could be
accounted for by the pH additions.

The solubility of carbon dioxide as such is not dependent on
pH, but dissolved carbon dioxide reacts with water to give bicarbonate
and carbonate ions in a dynamic equilibrium expressed by the following
equations :-

$$CO_2 \text{ gas} \rightleftharpoons CO_2 \text{ dissolved} + H_2O \rightleftharpoons HCO_3^- + H^+$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$$
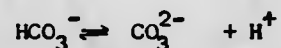
The balances of these equations are not only dependent on the
partial pressure of carbon dioxide but also on the pH of the
solution in which it is dissolved. High pH draws the equilibrium
towards carbonate, low pH towards carbon dioxide. Any rise in pH will
allow the culture medium to absorb more carbon dioxide as its dissolved
products, conversely a fall in pH will push these products out of
solution as the gas.
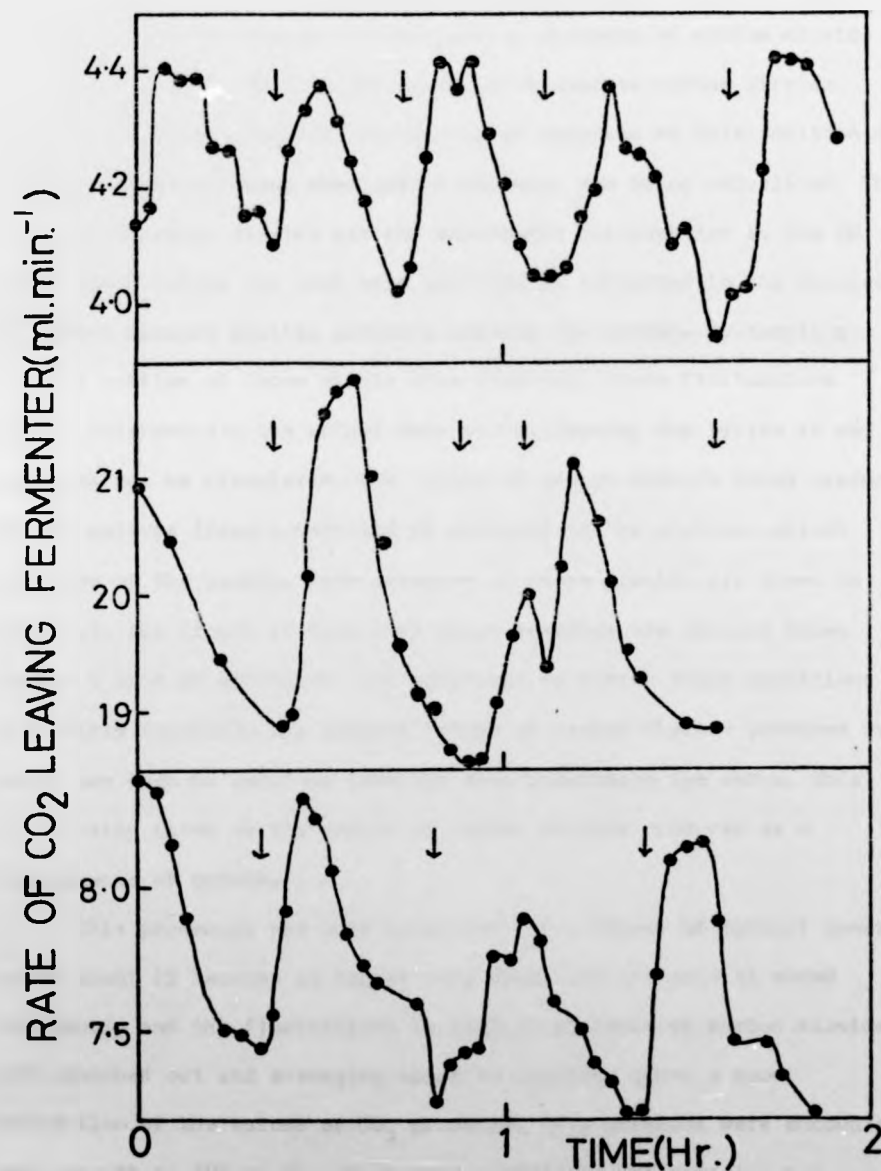
Fig. 1.3. Examples of fluctuations in carbon dioxide partial pressure in exhaust gas from fermenter.

On NMS the culture pH rises due to the utilisation of the
nitrate ion; to maintain a constant pH hydrochloric acid is added
via the pH control system. As the partial pressure of carbon dioxide
is usually between 3% - 7% the amount of dissolved carbon dioxide
(as its dissolved products) forced out of solution by this addition of
acid can be significant when carbon balances are being calculated. This
release of carbon dioxide gas and subsequent reabsorbtion as the pH
rises again before the next acid addition is reflected in the changes
of carbon dioxide partial pressure leaving the system. By sampling
the gas outflow at three minute time intervals these fluctuations
can be followed and the actual rate of CO_2 leaving the system at any
one time can be calculated. The volume of carbon dioxide being produced
by the culture (from growth and pH changes) can be plotted against
the time of the sample. Some examples of these results are shown in
fig. 1.3. The length of time over which readings are usually taken
is for 3 or 4 pH additions. (pH additions in steady state conditions
are fairly regular). The average volume of carbon dioxide produced by the
cells can then be obtained from the area underneath the curve, this
value being taken as the amount of carbon dioxide produced as a
consequence of growth.

This procedure was only necessary for cultures of optical densities
below about 12 because at higher cell densities the acid is added
frequently and the fluctuations in partial pressure of carbon dioxide
are smoothed out and averaging about 10 readings gives a good
reflection of the volume of CO_2 produced. Less problems were encountered
with growth on AMS as the pH changed rapidly as the ammonium ion
was removed and frequent alkali additions were made. On nitrogen
free media the pH of the culture only changed slowly and alkali additions
were infrequent and considerable time apart.

b) Theory of Bacterial Growth

Bacteria multiply by means of binary fission, (one organism divides to give two viable organisms which can also undergo binary fission). This type of growth when the divisions occur at regular intervals is called exponential growth i.e.

$$n_o \longrightarrow n_o \times 2 \longrightarrow n_o \times 4 \longrightarrow n_o \times 8 \qquad \text{where } n_o = \text{initial}$$

number of organisms.

or $\quad n = n_o 2^z \qquad$ (1) $\qquad z$ = number of generation

$$z = \frac{t}{td} \quad \frac{time}{doubling \ time}$$

n = number of bacteria after time t.

The doubling time is the time taken for the concentration of organisms to double.

The growth of a culture can be treated as a first order chemical reaction, the rate of growth being proportional to the concentration of biomass.

i.e.

$$A \longrightarrow B$$

$$\frac{dA}{dt} = -k \ A \qquad\qquad k - \text{rate constant}$$
$$A - \text{concentration of substrate.}$$

or for bacterial growth

rate of change of biomass = $\dfrac{dx_1}{dt} = \mu x$ $\qquad\qquad x_1$ - biomass concentration

μ - specific growth rate

The rate in this case is positive because the product is the same as the substrate, so biomass concentration increases.

Therefore $\mu = \dfrac{dx_1}{dt} \cdot \dfrac{1}{x}$ - exponential growth equation - (2)

also $\mu = \dfrac{d \,(\log e\ x)}{dt}$

let x increase from x_1 to x_2 in time t_1 to t_2.

Then $\mu = \dfrac{\log e\ \dfrac{x_2}{x_1}}{(t_2 - t_1)}$

if $(t_2 - t_1)$ = td (doubling time) then $x_2 = 2. x_1$

so $\mu = \dfrac{\log e\ \ 2}{td}$ - (3)

If the culture has an excess of all requirements then μ and td are constants. However what happens when one of the substrates is almost consumed ? Obviously growth must stop eventually. In continuous culture the concentration of limiting substrate is very low; Monod (1942) showed that at low concentrations of substrate there is a relationship between the specific growth rate and the concentration of the limiting growth substrate expressed by the following equation :-

$$\mu = \mu_m \ \ \left(\frac{s}{K_s + s}\right) \qquad - (4)$$

Where μ_m is the growth rate constant or maximum specific growth rate, s is the substrate concentration in the culture and K_s, the saturation constant which is the concentration of s which gives $\frac{1}{2} \mu_m$. The K_s values are usually low (0.2m M for glucose) compared to the

concentration of

substrate in the inflowing medium however the concentration of
limiting substrate in the culture is low and in the order of the
K_s value.

The relationship between the initial substrate concentration
and the amount of growth which can occur before this substrate is
exhausted was investigated by Monod (1942) who found it to be
linear :-

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate consumed}} = Y \qquad (5)$$

Y - Yield coefficient

from this equation it is possible to relate growth rate to the rate
of substrate utilisation :-

$$\frac{dx_1}{dt} = - Y \frac{ds}{dt} \qquad (6)$$

These values of Y, K_s and μ_m, which are constants for any
particular set of conditions, fitted into equations 2, 4 and 6
adequately describe batch growth and these same constants can be
used in the theory of continuous culture.

1b) Theory of Continuous Culture

The theory of continuous culture described below is that of Herbert et al (1956) who based their ideas on those of Monod (1950) and Novick and Szilard (1950). The points of the theory which are particularly relevent to biomass production will be emphasized.

The residence times of bacteria in a culture to which media is being added and removed at the same rate are determined by the dilution rate (D) where :-

$$D = \frac{f}{V} \qquad (7)$$

f is the flow rate of medium through the system and V is the culture volume.

Consider a continuous culture to which medium is being added that contains all the required substrates in excess except for one, the limiting substrate (oxygen also being in excess for aerobic growth.). The conditions which can be altered in this type of system are the limiting substrate concentration, medium flow rate and culture volume. Any theory must be able to predict the consequences of any changes in these parameters.

Consider changes in biomass concentration :- any change in biomass concentration is determined by the rate of biomass production and the rate at which the culture is removed

change in biomass in culture vessel	=	amount of biomass production due to growth	−	amount of biomass leaving by outflow from system.

$$\frac{dx_2}{dt} V = \frac{dx_1}{dt} V - fx$$

$\frac{dx_2}{dt}$ is the actual change in biomass concentration in the fermenter.

dividing through by V.

$$\frac{dx_2}{dt} = \frac{dx_1}{dt} - \frac{f x}{V} \qquad\qquad \text{but } \frac{f}{V} = D$$

Therefore $\frac{dx_2}{dt} = \frac{dx_1}{dt} - Dx$

also from equation 2 $\qquad \frac{dx_1}{dt} = \mu x$

So $\qquad \frac{dx_2}{dt} = \mu x - Dx$

$$\frac{dx_2}{dt} = x (\mu - D) \qquad\qquad (8)$$

So if μ is greater than D, $\frac{dx_2}{dt}$ will be positive i.e. the culture density will increase, but if D is greater than μ then $\frac{dx_2}{dt}$ will be negative and the organism concentration will fall until eventually the culture density will be zero. This situation is called wash out when the bacteria cannot maintain themselves in the prevailing cultural conditions. In a steady state situation where all the parameters are constant (including x) then $\frac{dx}{dt} = 0$.

$$0 = x \ (\mu - D)$$

therefore $\qquad \mu = D.$

So in a steady state situation the dilution rate is numerically equal to the specific growth rate. However these relationships do not give any indication of what dilution rates can be obtained or what the organism concentration will be for any particular set of conditions.

Considering changes in substrate concentration:- The rate at which the limiting substrate concentration(s) in the culture changes is dependent upon the rate of supply of the substrate, the rate at which this nutrient is consumed by the organisms as they grow and the rate at which unused substrate leaves the fermenter pot i.e.

change in amount of substrate $\quad = $ input - output - consumption in the culture

$$\frac{ds}{dt}. \ V \quad = \ fs_R - fs - \frac{dx_1}{dt} . \ V . \ \frac{1}{Y} \qquad (9a)$$

From equation 6.

$\frac{ds}{dt}$ is the change in substrate concentration in the culture and s_R the concentration of substrate in the inflowing medium divide through by V.

$$\frac{ds}{dt} = Ds_R - Ds - \frac{\mu x}{Y} \qquad \left(\frac{dx}{dt} = \mu x \right) \qquad (9b)$$

Equations 8 and 9 both contain μ which itself is dependent upon s (equation 4). So μ can be substituted for   in each of these equations.

From 8. $\dfrac{dx_2}{dt} = x \left[\mu_m \cdot \dfrac{s}{(Ks + s)} - D \right]$ (10)

From 9. $\dfrac{ds}{dt} = D (s_R - s) - \dfrac{\mu_m x}{Y} \cdot \dfrac{s}{(K_s + s)}$ (11)

These two equations define the behaviour of a continuous culture based on the fundamental growth equations 2, 4 and 6. In a steady state situation $\dfrac{dx_2}{dt}$ and $\dfrac{ds}{dt}$ are equal to zero. So solving these equations as follows can predict the changes in x and s of a continuous culture in terms of Y, μ_m and K_s if D and s_R are changed by the experimenter.

Taking equation 10

$$\dfrac{dx_2}{dt} = x \left[\mu_m \cdot \dfrac{s}{(K_s + s)} - D \right]$$

but $\dfrac{dx_2}{dt} = 0$ in a steady state

so $0 = x \left[\mu_m \cdot \dfrac{s}{(K_s + s)} - D \right]$

therefore $\mu_m \cdot \dfrac{s}{(K_s + s)} = D$ i.e. either x must be zero, which is not possible or $\mu_m \cdot \dfrac{s}{(K_s + s)} - D = 0$.

$$\mu_m s = DK_s + Ds$$

$$DK_s = \mu_m s - Ds$$

$$DK_s = s (\mu_m - D)$$

Therefore $\quad s = \dfrac{DK_s}{(\mu_m - D)}$ $\hspace{2cm}$ (12)

Taking equation 9 (i.e. not substituting for μ)

$$\frac{ds}{dt} = D (s_R - s) - \frac{\mu x}{Y}$$

but $\qquad \dfrac{ds}{dt} = 0$ in a steady state so

$$D (s_R - s) = \frac{\mu x}{Y}$$

so $\qquad s_R - s = \dfrac{x}{Y}$

or $\qquad x = Y (s_R - s)$

substituting for s from equation 12

$$x = Y (s_R - \frac{K_s D}{(\mu_m - D)}) \hspace{1.5cm} (13)$$

These last two equations (12 and 13) define what concentrations of substrate and organisms can be expected in any steady state, using only the fundamental growth constants (K_s, μ_m and Y), s_R and D which

are set by the experimenter. Changes in culture volume will affect
the dilution rate if the flow rate of medium is kept constant and so
the values for a new steady state with a change in volume can be
determined by the change in the dilution rate which occurs.

What happens if the dilution rate is slowly increased ? All
the steady state values can be worked out from equations 12 and 13,
but in a steady state $\mu = D$ and μ has a maximum value (μ_m) which is
determined by some rate limiting step in the metabolism of the
organism. So if D is increased above μ_m, then from equation 8 the rate
of change of organism concentration ($\frac{dx_2}{dt}$) is negative and the
organism concentration will fall to zero (washout). The value of
D that is numerically equal to μ_m or the maximum dilution rate at
which a steady state can be obtained is called the critical dilution
rate (D_c).

Herbert et al (1956) plotted out theoretical curves for $\bar{x}$ and $\bar{s}$
(the $^-$ represents steady state values) from their equations. They
also plotted a curve for productivity from the fermenter; productivity
(P) is the amount of organisms leaving the fermenter per unit time :-

$$P = fx \quad (14) - \text{actual weight of organism per unit time}$$
$$\text{or} \quad P = Dx \quad (15) \text{ which is the weight of organisms per unit time}$$
$$\text{per culture volume.}$$

This curve for productivity is of prime importance for biomass
production. These curves are shown in fig.1.4.

x,s & P

S

X

P

D
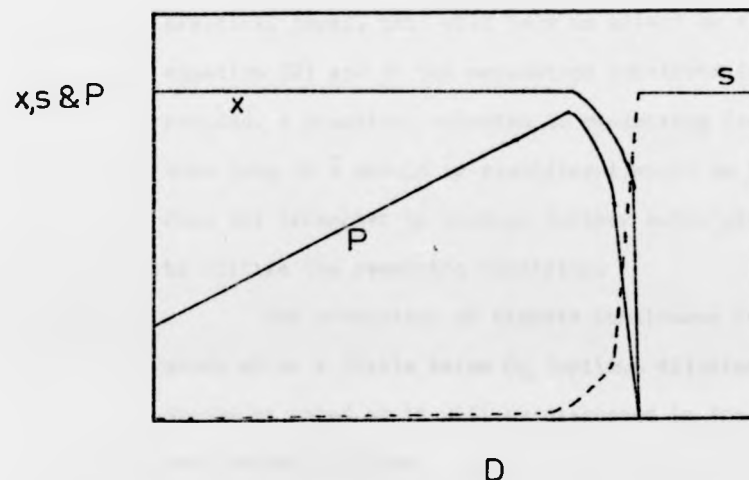
Fig.1.4. Theoretical dilution rate and productivity curves from a
continuous culture (Herbert et al 1956)

As can be seen from this figure organism concentration and
substrate concentration changes very little up to the washout
situation. The productivity increases as the dilution rate is
increased and reaches a maximum just before washout occurs, so the
optimum dilution rate for biomass production would appear to be
just below the critical dilution rate. But the optimum dilution
rate for utilizing the substrate would be at zero dilution rate
when all the available substrate will be consumed, because increasing
the dilution rate also increases the concentration of substrate in
the culture supernatant (equation 12). However the actual concentration
of substrate in the culture is usually very low, many fold less than
that in the inflowing media, except close to the critical dilution
rate. This loss, in terms of substrate washed out, can be minimised

by increasing the input concentration of substrate to the highest
practical level, this will have no effect on $\bar{s}$ (dependent on D,
equation 12) and so the percentage substrate lost can be further
reduced. A practical solution to minimising (and even removing)
this loss, if $\bar{s}$ should be significant, would be to allow the effluent
from the fermenter to undergo further batch growth, in another vessel,
to utilize the remaining substrate.

For production of biomass continuous cultures should be
grown at or a little below D_m (optimum dilution rate). This point
should be noted as it will be discussed in the next section on
gas limited cultures.

The preceeding section gave a good basic theory for continuous
culture. However it soon became apparent that deviations from the
predicted theory could and did occur. The main deviations from the
predicted results concerned two assumptions commonly made but not
always holding true. The first assumption is, that perfect mixing
must occur in the fermenter i.e. each drop of medium entering the
vessel is evenly distributed throughout the fermenter instantaneously.
The lack of perfect mixing leads to steady states being obtained above
the critical dilution rates, shown below.
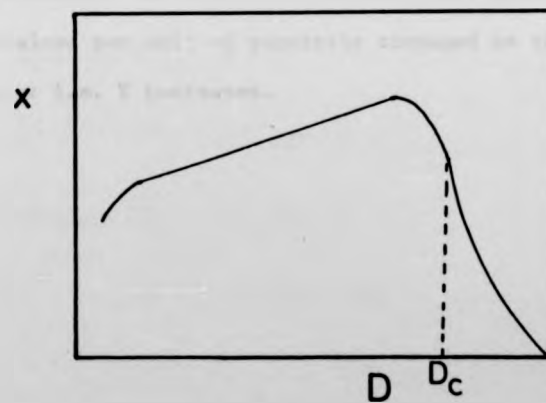
The reason for these steady states is that the imperfect mixing in
particular parts of the culture allows the effective dilution rate
to be lower than the overall dilution rate i.e. an effective dilution
rate below D_c and so allowing growth.

The second assumption that the yield coefficient (Y) is a
constant at all dilution rates also does not hold true. It was
found   with some cultures that as the dilution rate was increased
the biomass concentration also increased, showing that Y must be
increasing i.e. more biomass from the same amount of substrate. A
reason put forward to explain this was that organisms require a
certain amount of energy for purely maintainance purposes (organising
and keeping the cellular machinery functional); this is called
maintainance energy (M) (Pirt, 1965). A bacterium that is not
growing has to keep itself viable and 100% of the energy consumed
will be in the form of this maintainance energy. However as the
organism starts to grow the proportion of energy consumed as M
becomes smaller. This energy (it is assumed) is still required when
the cell is growing but it is a constant amount so as the organisms grow
at faster rates the proportion of the substrate required for
maintainance energy becomes smaller, so more of the energy derived
from the substrate can be channelled into cell growth and more cells
can be obtained per unit of substrate consumed as the culture
grows faster i.e. Y increases.

stream

Theory of a Gas Limited Continuous Culture

A continuous culture limited by a gas in the gas phase can neither be described as a chemostat nor a turbidostat. The limiting substrate is not in the medium nor is the organism density controlled by varying the medium flow rate. To work with such a system it soon became obvious that a theory had to be worked out before any attempt could be made at planning experiments. The main difference between a chemostat (the theory explained in the previous section) and a gas limited culture is that in a chemostat the medium flow rate (i.e. dilution rate) is proportional to the rate of supply of the limiting substrate whereas in the gas limited system the rate of supply of the limiting substrate remains constant what ever the dilution rate.

Cultures limited by a gas can still be expected to behave in accordance with the basic growth equations already mentioned. However care must be taken not to use equations containing (or derived from) s_R (limiting substrate concentration in inflowing medium) as this parameter has no relevance to gas limited cultures. Equations already derived will be used in this section where they have any relevance to understanding gas limited cultures.

from equation 7, $D = \dfrac{f}{V}$

therefore $f = DV$

also from equation 13 $P = xf$

so $P = xDV.$ (16)
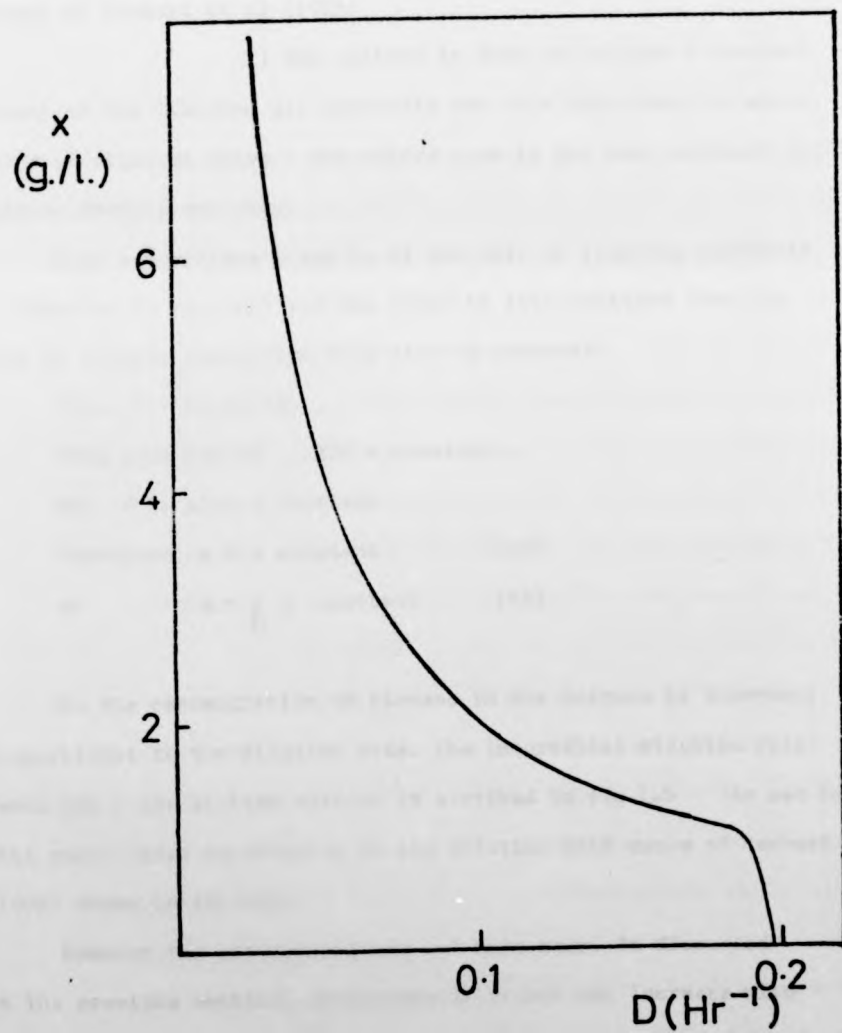
stream

Fig. 1.5. Theoretical dilution rate curve for a gas limited culture

Assumptions :- 1) The characteristics of the organism do
not change with changes in dilution rate i.e. no storage products,
no change in efficiency. This assumption is also used in the basic
theory of Herbert et al (1956)

2) The culture is able to utilize a constant
amount of the limiting gas substrate per unit time over the whole
range of dilution rates - the volume used is the same although the
culture density may vary.

From assumptions 1 and 2, if the rate of limiting substrate
utilisation is constant and the yield is also constant then the
rate of biomass production will also be constant.

i.e.   P = constant

from equation 16 xDV = constant

but   V is also a constant

therefore $x D$ = constant   (17)

or $x = \dfrac{1}{D}$. constant   (18)

So the concentration of biomass in the culture is inversely
proportional to the dilution rate. The theoretical dilution rate
curve for a gas limited culture is sketched in fig.1.5   As can be seen
this curve bears no relation to the dilution rate curve of Herbert et al
(1956) shown in fig 1.4.

However the assumptions may not hold true. As discussed
in the previous section, efficiency of growth may increase with
increases in dilution rate, imperfect mixing may occur and, not relevent
to the chemostat, the amount of limiting gas consumed may not be
constant over a wide range of dilution rates and consequential changes
in organism concentration.

The gas uptake rate in a fermenter can be represented by the following equation :-

$$N = K_L a \, (C_s - C_L)$$

    N - gas uptake rate

    $K_L a$ - gas transfer coefficient

    C_s - gas partial pressure in input

    C_L - dissolved gas tension

As is discussed in section 3(a) for a gas limited culture the dissolved gas concentration is very small compared to C_s so the equation becomes

$$N = K_L a \, C_s$$

Obviously this would hold true if every molecule of gas entering the liquid phase were consumed immediately; this could be the case in a straight forward chemical reaction but in biological systems the rate of gas utilisation will depend on the properties of the enzymes concerned, especially the affinity of these enzymes for their substrates (K_m - for an enzyme, K_s for the organism as a whole).

With the above equation in mind let us consider assumption 2. In a small laboratory fermenter even though a gas is limiting the growth of a culture and the effective concentration of that gas is practically zero in the culture itself, there can still be a considerable amount of that limiting gas leaving with the exhaust gas. So in effect there is still an additional potential source of limiting gas to the culture if it can be utilized. To understand this problem it is necessary to consider all the factors which effect the transfer of a gas from the gas phase to the organism. These factors are listed below :-

    1) Partial pressure of limiting gas in inflowing gas stream

2) Partial pressure of limiting gas in outflowing gas stream

3) the rate of gas utilisation by each organism

4) the growth rate

5) cell density - the culture grows up to a density that utilizes (nearly) all the limiting substrate

6) gas transfer rate a) gas phase to liquid (dissolved) phase

                      b) from dissolved phase across the

bacterial cell wall and to the site(s) of gas metabolism.

7) the affinity of the organism for the substrate.

Consider two steady states in a shift up situation with a doubling in dilution rate   fig 1.6. below   represents unit volume of the culture and G is the weight of limiting gas used.

PARAMETERS   G, D, x   G , 2D $x/2$

RATE OF GAS   a   2a
USED PER ORGANISM

$$S = \frac{DK_s}{(\mu_m - D)} \qquad\qquad \frac{2DK_s}{(\mu_m - 2D)}$$
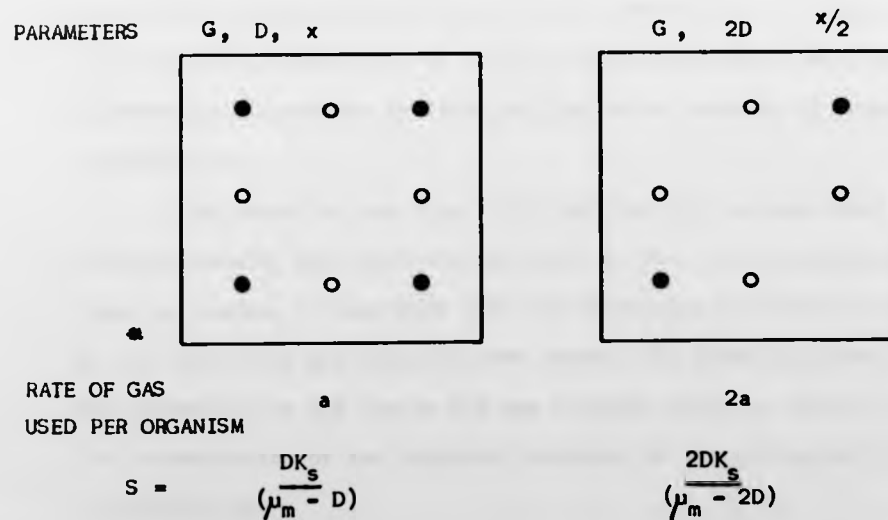
Fig.1.6. Represents unit volume of culture for a doubling in dilution rate.

The only physical change to the culture is that the medium

● - represents micro-organisms

○ - represents gas bubbles

flow rate is doubled so the partial pressure and volume of the inflowing gases are the same from one dilution rate to the other. From equation 18 a doubling in dilution rate will mean a halving of the organism concentration if no change in the amount of gas used occurs, so the rate of gas uptake by each organism is doubled. This doubling of gas utilization rate is absolutely necessary for an organism to grow at twice the previous rate. Supposing the less dense culture cannot obtain as much gas from the supernatant as the denser culture. This would seem a reasonable assumption; the closer the organisms are together the more gas they should be able to remove by creating a greater gas transfer gradient. A direct consequence of this drop in gas utilisation rate would be a lowering in the amount of gas used and a consequent drop in the productivity at the higher dilution rate. However let us consider the factors which will be affected by an increase in dilution rate and a lowering of organism concentration.

The amount of gas used by the culture will be dependent on the gas transfer rate from the gas phase to the liquid (dissolved) phase. This, of course, is dependent upon the difference in partial pressure of the gas in the gas and dissolved phases. The lower the dissolved gas concentration the faster the gas transfer from the gaseous phase. The concentration of the limiting substrate in the culture(s) is defined by equation 12

i.e. $\qquad s = \dfrac{DK_s}{(\mu_m - D)}$

As can be seen from this an increase in D will bring about an increase in s, so from a theoretical standpoint the dissolved gas

concentration will be higher at the faster dilution rate. This value
of s for gas limited cultures must only be an average value because
one can envisage that if there is still some gas in the gas phase
then the concentration of dissolved gas next to the gas bubbles
must be higher than at the site of metabolism because there is a
continual passage of the gas from the higher concentration in the
gas phase to the sites of metabolism where the gas is consumed. This
movement can be considered as the passage of the gas molecules down a
concentration gradient. The denser the culture is, i.e. the closer
the organisms are together, the greater the effective rate at which
the gas can be removed and thus the steeper the concentration gradient.
The average value will still be set by equation 12, but now more
molecules are passing down this so called concentration gradient. Of
course weighed against this is the fact that in the denser culture the
rate of gas utilization by each organism is half that of the less
dense culture. So, is the denser, slower growing culture with a lower
limiting substrate concentration in the culture and a greater transfer
gradient going to be able to use significantly more of the limiting gas
than the faster growing culture ? The changes which occur in s are
likely to be very small indeed, in terms of gas concentration, and
so this might not have any significant effect on the gas transfer rate.
This is also true of the increase in the gas transfer gradient because
in any steady state the culture grows up to a density that utilizes
nearly all the limiting substrate available and to extract any more
from the now depleted gas phase might take a considerable change in
the rates concerned.

However if a denser culture does use more gas it will be necessary to account for this in any theory on gas limited cultures because, of course, as the dilution rate increases the productivity will decline, not due to any fall in efficiency of growth but because less gas is being used. In a chemostat, more often than not, as the dilution rate is increased the efficiency of growth also increases. If this happened in a gas limited culture it could very well be masked by a fall in the amount of gas used. Measurements of gas consumption would show up this discrepency.

So using assumption 1 (constant efficiency) and equation 16

$$P = xDV$$

and equation 5

$$Y = \frac{\text{wt. of bacteria formed}}{\text{wt. of substrate consumed}}$$

$$Y = \frac{P}{G}$$

where G is the weight of limiting gas used per unit time

therefore $P = YG$

so $xDV = YG$ (19)

$$\frac{xD}{G} = \frac{Y}{V} = \text{const}$$ (20),   Y and V are constants

therefore $\frac{x}{G} = \frac{1}{D} \times \text{const} \left(\frac{Y}{V}\right)$ (21)

If $\frac{x}{G}$ is plotted agains $\frac{1}{D}$ any deviation from a straight line would indicate that the yield is changing, the volume of the culture should be a constant.

The theory so far has only dealt with general predictions as to the behaviour of the culture as the dilution rate is changed. A more precise estimation of the parameters is given by expanding the theory of Herbert et al (1956) to cover a gas limited culture. The prediction of substrate concentration made in equation 12 will still hold true for a gas limited culture i.e.

$$S = \frac{K_s D}{(\mu_m - D)}$$

But equation 13 for the estimation of x will not hold true as the equation contains the term S_R which is not applicable to a gas limited culture. However if equation 9a is considered in the light of gas limited culture an equation for the estimation of x can be derived. Equation 9a comes from the equation of substrate utilisation rates i.e.

rate of substrate = rate of substrate - rate of substrate - rate of
used   supplied   utilised   unused substrate
                                                        removed from
                                                        the culture

in the case of a chemostat, equation 9a

$$\frac{dsV}{dt} = f s_R - \frac{dx_1. V.1}{dt \quad Y} - f s$$

However in a gas limited culture the rate of limiting substrate supply is constant and is represented by G. So equation 9a can be rewritten to apply to a gas limited culture as follows

$$\frac{ds.V}{dt} = \frac{G - dx_1}{dt} . \frac{V.1}{Y} - fs$$

dividing through by V and substituting μx for $\frac{dx_1}{dt}$

$$\frac{ds}{dt} = \frac{G}{V} - \frac{\mu x}{Y} - Ds$$

in a steady state situation $\frac{ds}{dt} = 0$, so

$$\frac{\mu x}{Y} = \frac{G}{V} - Ds$$

$$x = \frac{Y}{\mu} \left(\frac{G}{V} - Ds \right)$$

also in a steady state $\mu = D$, so

$$x = \frac{Y}{D} \left(\frac{G}{V} - Ds \right)$$

dividing the bracket by D

$$x = Y \left(\frac{G}{DV} - s \right)$$

but $DV = f$ from equation 7

$$x = Y \left(\frac{G}{f} - s \right)$$

and substituting s from equation 13

$$x = Y \left(\frac{G}{f} - \frac{K_s D}{(\mu_m - D)} \right) \qquad (23)$$

However because K_s is very small compared to G and for practical purposes over most of the dilution rate curve the factor $\dfrac{K_s D}{(\mu_m - D)}$ will be insignificant and equation 23 will become

$$x = \frac{YG}{f} = \frac{YG}{DV}$$

This, of course, is the same as equation 21. This relationship will hold true for most dilution rates except those very close to the critical dilution rate when $\dfrac{K_s D}{(\mu_m - D)}$ will have a significant value i.e. $(\mu_m - D)$ becomes very small.

Perhaps one of the most important differences between a chemostat and a gas limited continuous culture as far as biomass production is concerned is that the former has an optimal dilution rate (D_m) for biomass production, whereas in the latter system the productivity is constant up to a point near the washout situation and is only dependent on the input rate of the limiting gas. Although, if efficiency does change with growth rate, there will be an optimum dilution rate. But the change in productivity will not be so dramatic as in a chemostat. This constant productivity of a gas limited culture does have advantages over the chemostat in that the dilution rate can be set to obtain the cells in the optimum conditions for harvesting (i.e. low dilution rate - high biomass concentration and less throughput of medium, or a dilution rate which is optimal for protein content of the cells.)

RESULTS FROM GAS LIMITED CONTINUOUS CULTURES

To investigate the effect of dilution rate on gas limited
cultures two dilution rate curves were obtained, one on NMS the
other on AMS. The culture grown on NMS was in the 3.5l culture
fermenter which limited the practicability of increasing the dilution
rate much above 0.13 which would require about 11 L. of medium
per day. However the run on AMS was in a 2.3 L. culture fermenter
and this enabled dilution rates up to 0.27 to be obtained. The
maximum growth rate was determined by increasing the dilution rate
to 0.39 for several hours and the decline in culture density followed.

For each culture steady states were obtained at each dilution
rate studied. The results obtained were computed as described in the
preceeding sections. Fig. 1.7 shows the three types of curves that
can be plotted from gas limited cultures for the two runs which were
undertaken. The plots of dilution rate (D) agains culture density (x)
did follow fairly closely to the predicted curves as did the plots of
$^1/D$ against x. However it should be noted that this curve for the
AMS culture (2.5) does suggest that as the dilution rate was increased
the culture density was becoming lower than expected. These curves
should go through the origin but by linear regression analysis the
points at which the curves cross the Y-axis were slightly greater than
zero although possibly not significantly above zero. However the final
analysis of these results, plotting $^1/D$ against $^x/g$ did give a truer
picture of what was actually happening to the culture as the dilution
rate was increased. Perhaps not so obvious in the NMS culture because
of the lower dilution rates used, but as can be seen the point where
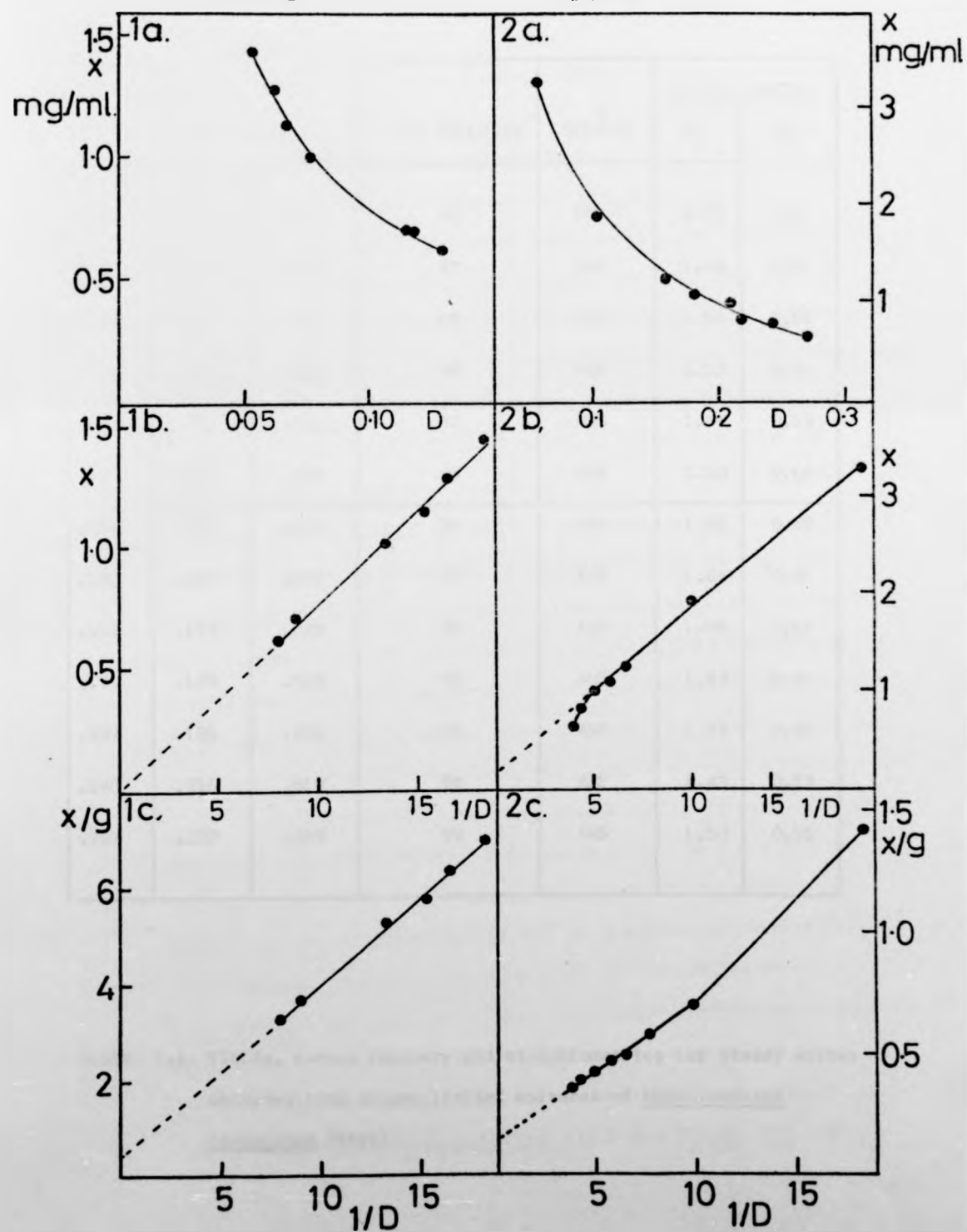
Fig. 1.7. Dilution rate curves from oxygen limited cultures of
Methylococcus capsulatus (Bath) on NMS and AMS media

D	YO_2	Y_{CH_4}	% C RECOVERY	N SOURCE	STOICHIOMETRY	
					O_2	CO_2
.055	.136	.414	95	NMS	1.52	0.67
.061	.138	.398	87	NMS	1.44	0.61
.066	.135	.413	93	NMS	1.54	0.66
.076	.142	.435	94	NMS	1.53	0.65
.115	.149	.421	87	NMS	1.41	0.59
.129	.150	.450	94	NMS	1.50	0.64
.055	.179	.544	94	AMS	1.52	0.58
.103	.167	.546	97	AMS	1.64	0.61
.158	.178	.549	92	AMS	1.54	0.56
.179	.188	.563	93	AMS	1.49	0.55
.207	.185	.606	94	AMS	1.54	0.56
.240	.216	.619	94	AMS	1.43	0.53
.267	.220	.659	99	AMS	1.50	0.55

Table. 1.1. Yields, carbon recovery and stoichiometries for steady states obtained from oxygen limited cultures of _Methylococcus capsulatus_ (Bath)

the curve crosses the Y-axis is now further from zero. The results
from the AMS culture are much clearer, the line is not linear over
the whole range and as the dilution rate increases, the density of
culture per unit of limiting gas becomes higher than expected. The
reason for these results is that as the dilution rate increased, the
amount of gas used decreased; thus giving a lower culture density
than is expected at the higher dilution rates (2b), but at the same
time the yield per gram of substrate was increasing as the dilution rate
was increased thus counterbalancing this lower cell density
because of the lower gas utilisation rates. This is revealed in
plot 2c. where the amount of gas being consumed is considered and
the only reason now for a deviation from linearity is a change in
yield (Y). The curve would only be expected to pass through the
origin if Y was a constant. This might be taken to be the case in
plots 1b and 2b and so it is essential in gas limited cultures to
actually determine the amount of limiting gas being consumed. The plot
of $\frac{1}{D}$ against $\frac{x}{g}$ would also remove any effect due to changes in gas
flow rates or stirring speeds over the range of dilution rates used.

Table 1.1. actually shows the yields at the different dilution
rates and as could be inferred from graphs 1c and 2c the yields do
actually increase as the dilution rate increases. The differences
between the two nitrogen sources will be discussed in section 4.

Reports of maximum doubling times for methanotrophs
(Whittenbury, 1969) of about 3 hours would give a critical dilution rate of
about0.22h.$^{-1}$, with this value in mind the rates of medium required
could be coped with using a culture volume of 2.3L . However, as it
turned out, Methylococcus capsulatus would grow faster than was

expected which again led to problems of providing sufficient
medium for dilution rates in excess of 0.27. However it is possible
to estimate μ_m (maximum specific growth rate) by increasing the
dilution rate above D_c (critical dilution rate) and following the
rate of decline of the culture density. The μ_m can be calculated
from this rate from the following equation

$$\ln x = (\mu_m - D) t + \ln x_0$$

lnx – log of O.D at time t

$\ln x_0$ – log of initial O.D

where the slope of a plot of ln x against t would be equal to
$(\mu_m - D)$. The results from this experiment were analysed by linear
regression analysis and gave a correlation coefficient of 0.998 and a
slope of -0.0729. So from above, the μ_m was calculated to be
0.321 h^{-1}.   (D= 0.394 h^{-1})

2. THE CLOSED BATCH SYSTEM

The batch system was developed as a test bed for changes
in environment which were to be imposed on the continuous culture
systems and also for comparison between methanotrophs. Although
batch cultures are notorious for giving inconsistant results
because of the continuously changing environment, it is possible
to draw useful information from such experiments especially when
inhibition of growth occurs. This aspect of inhibition of growth which
can manifest itself in batch systems should be considered when
continuous culture experiments are planned to prevent a premature
termination of a fermenter run.

One important difference between batch and continuous culture
is that in a batch culture the concentration of a utilisable
substrate is declining and must be initially high to give a reasonable
amount of growth. If this substrate is also an inhibitor e.g. methanol
for methanotrophs, only low concentrations of the compound can be
added at any time, thus limiting the initial concentration of substrate
and consequently the amount of possible growth. In continuous
culture if this substrate is the limiting factor then the actual
concentration of that substrate in the culture will be very low, but the
concentration in the incoming medium can be high (many times the concentration
that would inhibit growth). The substrate is utilised immediately it
enters the culture and never reaches inhibitory levels (unless some
other factor becomes limiting in the system). An example of this is
growth on methanol by Methylococcus capsulatus ; in batch culture
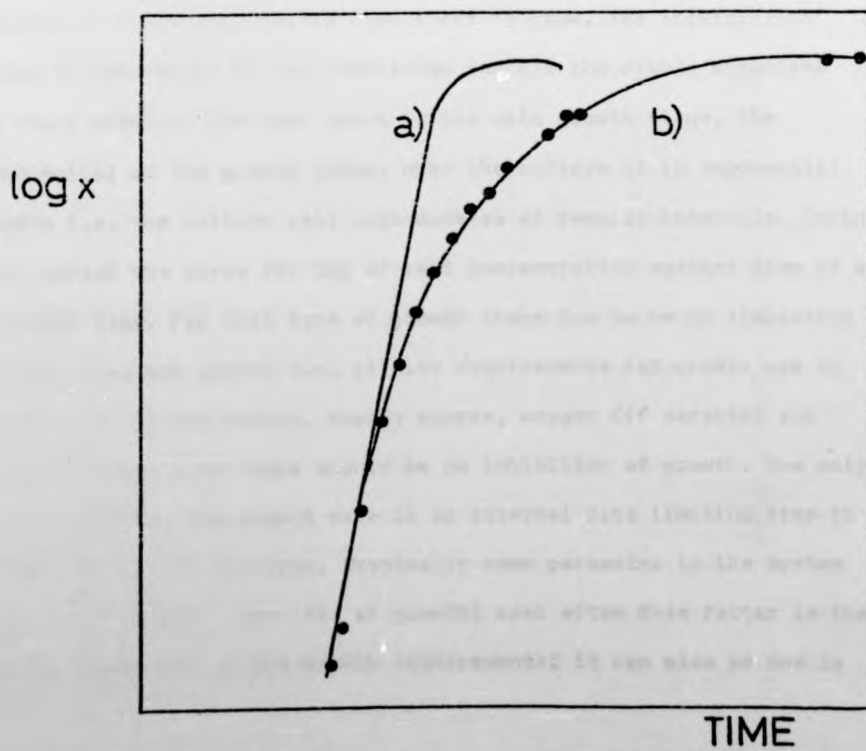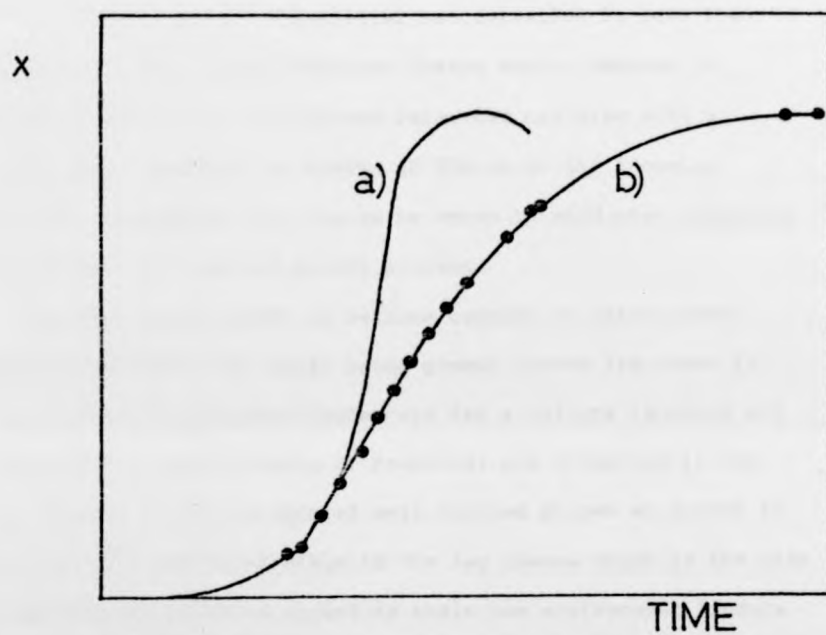
Fig.2.1. Batch growth curve   a) Theoretical curve of exponential growth
b) Gas limited batch growth curve.

growth will only occur if the initial concentration is less than
25 mM. and even then growth does not always occur. However an
established culture in a continuous fermenter can grow with a
concentration of methanol in excess of 250 mM in the incoming
medium. This phenomenon must always be borne in mind when comparing
effects in the two types of growth systems.

How does batch growth on methane compare to batch growth
on other substrates ? The usual batch growth curves are shown in
Fig. 2.1 (curves (a)); these figures are for a culture in which all
the substrates (except oxygen, if required) are dissolved in the
medium. There are usually several well defined phases of growth in
a batch culture. The first stage is the lag phase, which is the time
taken for the organisms to adjust to their new environment (induce
enzymes if necessary) before they start to grow. The acceleration
phase follows which is the time taken for all the viable organisms
to start growing. The next phase is the main growth stage, the
exponential or log growth phase, when the culture is in exponential
growth i.e. the culture cell mass doubles at regular intervals. During
this period the curve for log of cell concentration against time is a
straight line. For this type of growth there has to be no limitation
on the organisms growth i.e. all its requirements for growth are in
excess e.g. carbon source, energy source, oxygen (if aerobic) and
mineral salts also there should be no inhibition of growth. The only
factor limiting the growth rate is an internal rate limiting step in the
metabolism of the bacteria. Eventually some parameter in the system
will start to limit the rate of growth; most often this factor is the
exhaustion of one of the growth requirements; it can also be due to

an accumulation of a toxic growth product or inhibition by a
change in pH.

This slowing down of growth or deceleration phase heralds the
end of growth or the stationary phase. Once growth has stopped the
cells may begin to lyse; a drop in the optical density of the culture
would indicate this. Also in the latter stages of the growth cycle some
organisms may produce their resting stages e.g. spores or cysts.

Batch growth on methane follows a similar course of events as
described above but with one major difference(Fig. 2.1 curve (b)).
Methanotrophs have a very high oxygen demand (about 3.5 molecules
of oxygen are required for every molecule of carbon fixed) and
because oxygen is not very soluble the rate at which oxygen can
pass from the gaseous phase to the site of metabolism soon becomes
limiting as the culture grows.

The initial partial pressure of methane in air is usually about 0.20;
partial pressures between 0.05 and 0.15 are explosive mixtures and so
the concentration of methane must be kept outside this explosive range.
To start with less than 0.05 partial pressure of methane would mean an
almost immediate limitation due to the low methane concentration and
also very little growth would be possible. With 0.2 partial pressure
of methane in air the oxygen is only 0.17, so extra oxygen was added
to the system so that oxygen did not become severely limiting too
early in the growth cycle.

On inoculation of the culture both methane and oxygen are in
excess, the culture not being very dense. So the batch growth curve
is as described earlier however as the culture grows and the total
biomass increases the oxygen demand also increases. The initial part
of the growth curve does exhibit exponential growth, but this does not
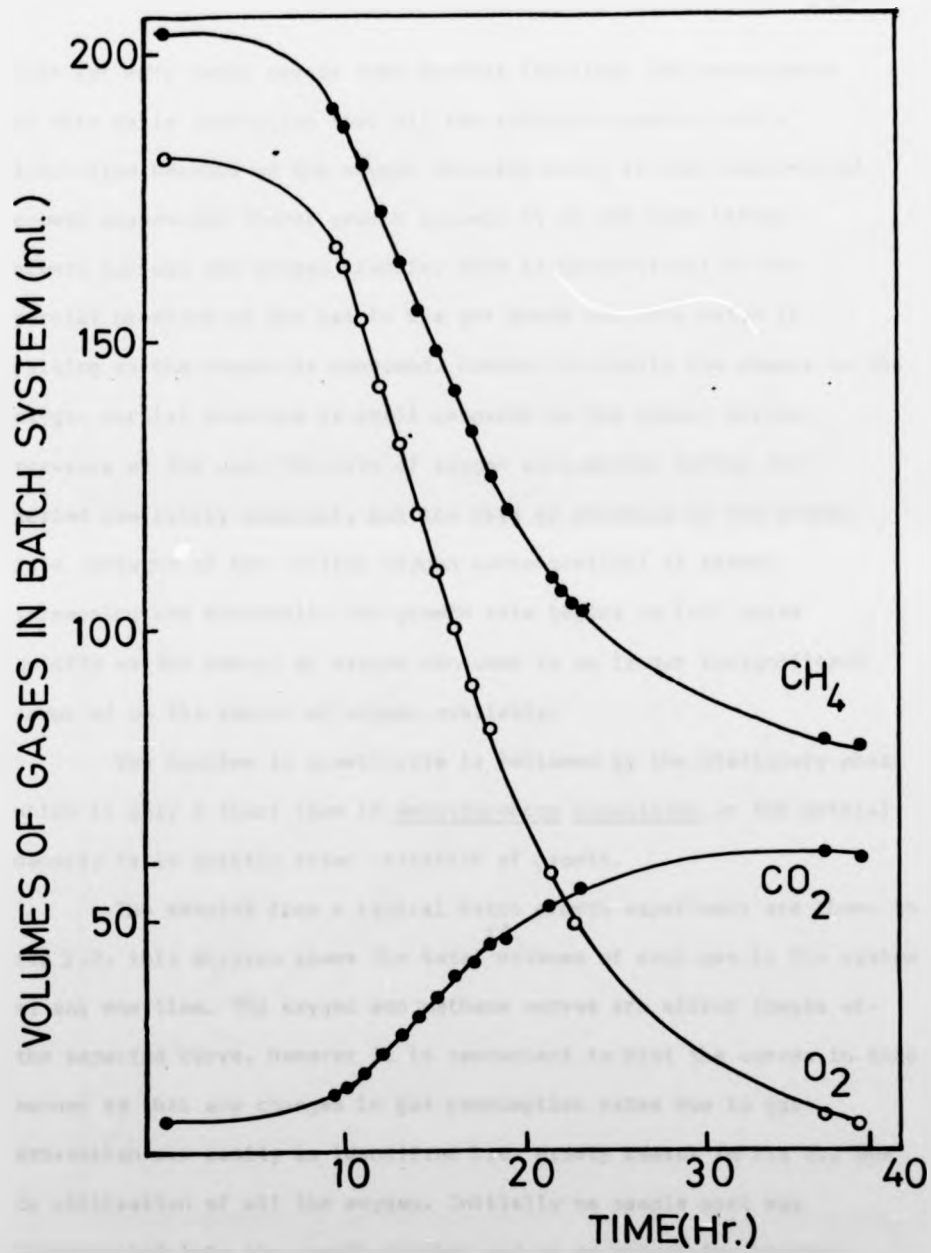
Fig. 2.2. Typical gas limited batch growth curve (volumes of gases
in the closed batch system) of _Methylococcus capsulatus_ (Bath)

last for very long; oxygen soon becomes limiting. The consequence
of this early limitation (not all the substrate used up but a
limitation because of the oxygen transfer rate) is that exponential
growth ceases and linear growth ensues. It is not true linear
growth because the oxygen transfer rate is proportional to the
partial pressure of the gas in the gas phase and this value is
falling as the oxygen is consumed. However initially the change in the
oxygen partial pressure is small compared to the actual partial
pressure of the gas. The rate of oxygen consumption during this
period was fairly constant, but the rate of decrease of the growth
rate (because of the falling oxygen concentration) is slowly
increasing and eventually the growth rate begins to fall quite
quickly as the amount of oxygen consumed is no longer insignificant
compared to the amount of oxygen available.

The decline in growth rate is followed by the stationary phase
which is only a short time in Methylococcus capsulatus as the optical
density falls quickly after cessation of growth.

The results from a typical batch growth experiment are shown in
fig 2.2, this diagram shows the total volumes of each gas in the system
at any one time. The oxygen and methane curves are mirror images of
the expected curve. However it is convenient to plot the curves in this
manner so that any changes in gas consumption rates due to gas
exhaustion can easily be identified i.e. growth ceases in fig 2.2 due
to utilisation of all the oxygen. Initially no sample port was
incorporated into the growth chamber and so no values for biomass
concentration were possible during this experiment. Cell density
determinations were required in some experiments and for these a
sample port was incorporated into the growth chamber. Frequent
sampling from the culture would significantly alter the volume of
the culture and also optical density readings (0.6 ml. culture
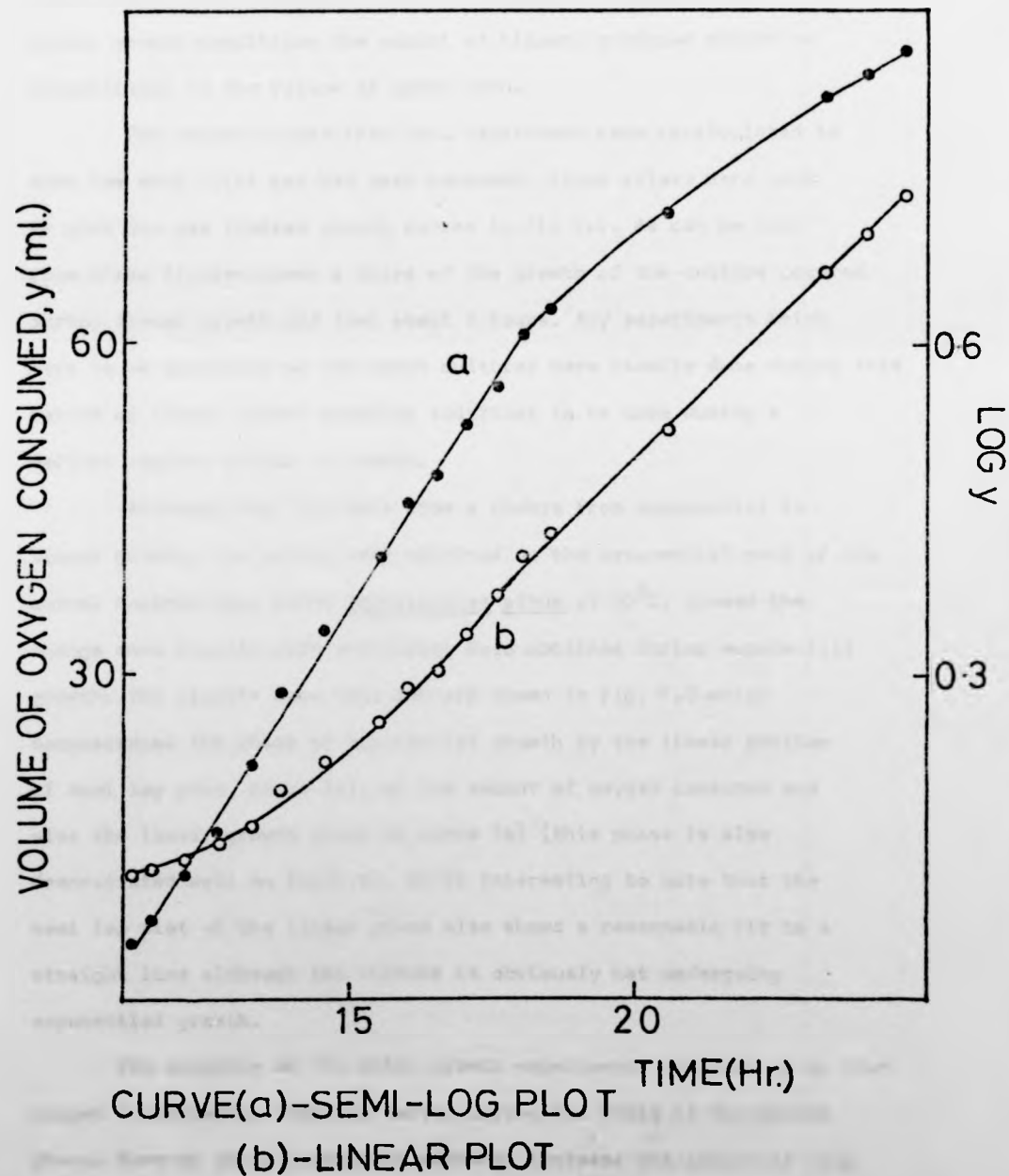
Fig. 2.3. Batch growth curve (oxygen used) showing change from exponential

to linear (gas limited) growth of _Methylomonas_ _albus_ at 30°C

+ 2.4 ml. diluent) were not particularly consistent and gave poor curves (see later) compared to the gas readings. Anyway under normal growth conditions the amount of biomass produced should be proportional to the volume of gases used.

The oxygen values from this experiment were recalculated to show how much total gas had been consumed, these values were used to plot the gas limited growth curves in fig 2.1. As can be seen from these figures about a third of the growth of the culture occured during linear growth and took about 6 hours. Any experiments which were to be performed on the batch cultures were usually done during this period of linear growth enabling additions to be made during a defined regular period of growth.

Although fig. 2.2 does show a change from exponential to linear growth, few points were obtained on the exponential part of the curve. Another run, using Methylomonas albus at $30^{\circ}C$, showed the change more clearly with sufficient data obtained during exponential growth. The results from this run are shown in fig. 2.3 which demonstrates the phase of exponential growth by the linear portion of semi log plot, curve (a), of the amount of oxygen consumed and also the linear growth phase on curve (b) (this phase is also demonstrated well on fig 2.2). It is interesting to note that the semi log plot of the linear phase also shows a reasonable fit to a straight line although the culture is obviously not undergoing exponential growth.

The majority of the batch growth experiments were set up so that oxygen would be the limiting factor during the whole of the growth phase. However extra oxygen was added to increase the length of time of the linear growth phase. If the amount of oxygen added was such
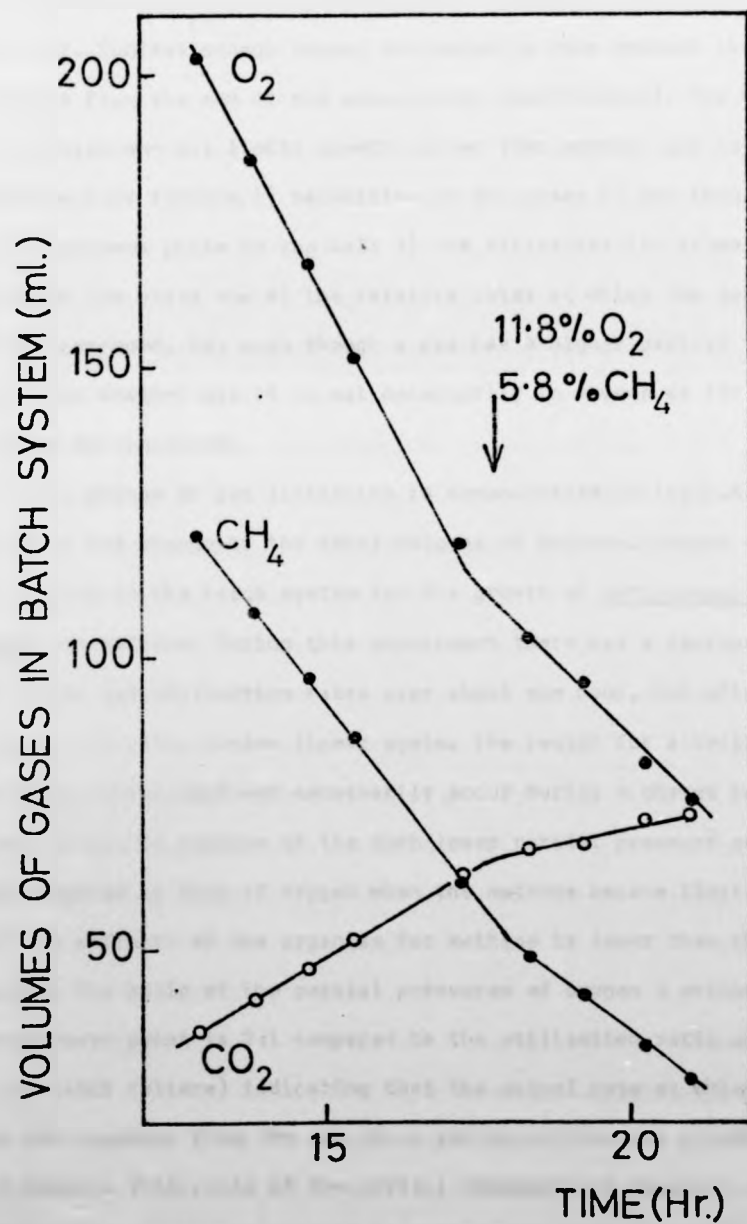
Fig. 2.4. Volumes of O_2, CH_4 and CO_2 in the batch system showing

change in gas limitation (O_2 to CH_4) of a culture of

Methylococcus capsulatus (Bath)

that the methane would be exhausted before all the oxygen was consumed
then at some point during the growth cycle a change in gas limitations
should occur. (unless enough oxygen was added to make methane the
limiting gas from the end of the exponential growth phase). The actual
levels at which one gas limits growth rather than another gas is
dependent on four factors, 1) solubility of the gases 2) gas transfer
rates from gaseous phase to the cell 3) the affinities (Ks values) of the
organism for the gases and 4) the relative rates at which the gases
need to be consumed. So, even though a gas has a higher partial
pressure than another gas it is not necessarily in excess as far as
the culture is concerned.

This change of gas limitation is demonstrated in fig 2.4. The
figure shows the change in the total volumes of methane, oxygen and
carbon dioxide in the batch system for the growth of Methylococcus
capsulatus on methane. During this experiment there was a decrease
of 50% in the gas utilisation rates over about one hour, but after
this change the rates became linear again. The reason for a decline
in the rates, which need not necessarily occur during a change in
gas limitations, is because of the much lower partial pressure of
methane compared to that of oxygen when the methane became limiting
or that the affinity of the organism for methane is lower than that
for oxygen. The ratio of the partial pressures of oxygen : methane at
the change over point is 2:1 compared to the utilisation ratio of
1.4:1 (in batch culture) indicating that the actual rate at which
methane can transfer from the gas phase and be utilised is greater than
that of oxygen. This ratio of the partial pressures of oxygen to methane
which determines whether a culture is methane or oxygen limited is very
important when limitations are being considered for continuous culture.

3. GENERAL PARAMETERS OF GROWTH

a) Gas Limitation

It is essential to define the limiting substrate of any
continuous culture before attempts can be made at interpreting
results obtained with such a system. The limiting substrate is
usually contrived to be a dissolved nutrient entering the culture
with the medium. More often than not this limiting substrate is the
carbon source but major constituents of the mineral salts complement
(sulphate ions, magnesium ions, nitrogen source) can also be
employed as the limiting substrate. However limitation by a
mineral salt often leads to imbalances in the metabolism of the
organism. Ideally for S.C.P. production all the nutrients should
be utilised as economically as possible, especially the carbon source
as this constituent is usually the most expensive component. As
discussed in section 1. the most expensive substrate is optimally
made to be the limiting nutrient in continuous culture, as the
concentration of a limiting substrate in the culture (and therefore
the effluent) is usually very low. To verify that a particular
substrate is limiting in a continuous culture, the concentration of
that substrate can either be decreased or increased and there should be,
as a consequence of this change, a proportionate decrease or increase
in the cell density of the culture.

For a culture that is limited by a constituent in the medium
the results are usually unambiguous unless, on increasing the concentration
of the limiting substrate, another substrate should become limiting
when the expected increase in cell density would not occur. This
also holds true in a gas limited culture with only one gaseous

substrate. However in a situation where two of the substrates are
in the gas phase it is not just the amount of each gas available
to the culture but also the ratio of the gases in the gas phase that
will determine the limiting substrate. Increasing the total volume
of gas passing through the system, but not changing the ratio of
these gases will only indicate whether a gas is limiting the
system or a constituent in the medium. To decide which gas is
limiting the system it is necessary to change the rate of addition
of one of the gases, thereby changing the partial pressure of that
gas in the input gas stream and therefore changing the amount of
that particular gas available to the culture. However just by
changing the rate of addition of one gas, the rate at which the
total gas flows through the system will be changed i.e. the more
gas being passed through a system the less efficient the system will
be at removing any particular gas from the mixture. To overcome this
problem the total volume of gas entering the system can be kept
constant and the partial pressures of the gases altered by changing
all the individual gas flow rates.

Another possible way of checking if a culture is gas limited
would be to use the theory of gas limited cultures presented in
section 1b). If the dilution rate is increased in a culture limited
by carbon in the medium there should be very little change in
the density of the culture (possibly an increase) unless the critical
dilution rate is exceeded whence the culture would wash out.
However if a gas is limiting the culture then the cell density should
fall in accordance with equation :-

$$Dx = constant$$
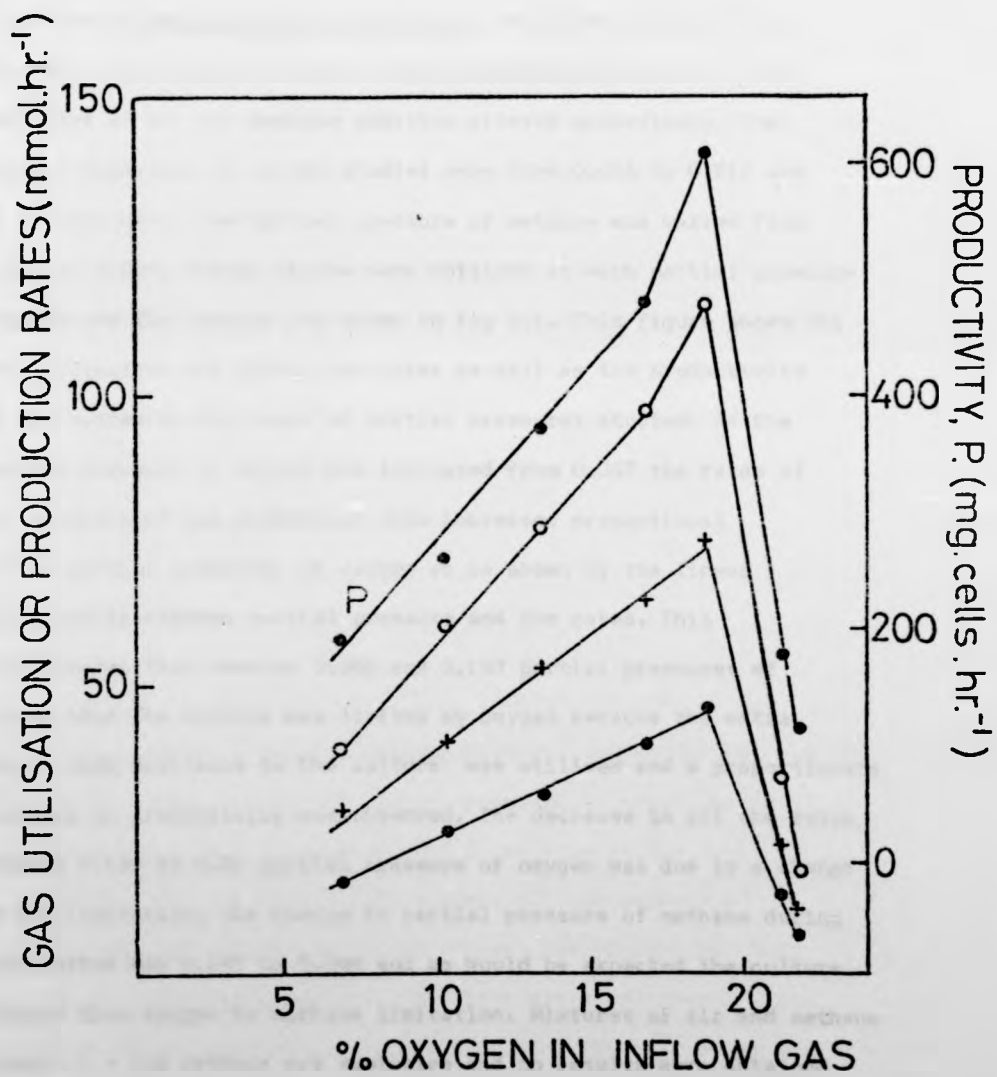
Fig. 3.1. Effect of oxygen partial pressure on a gas limited
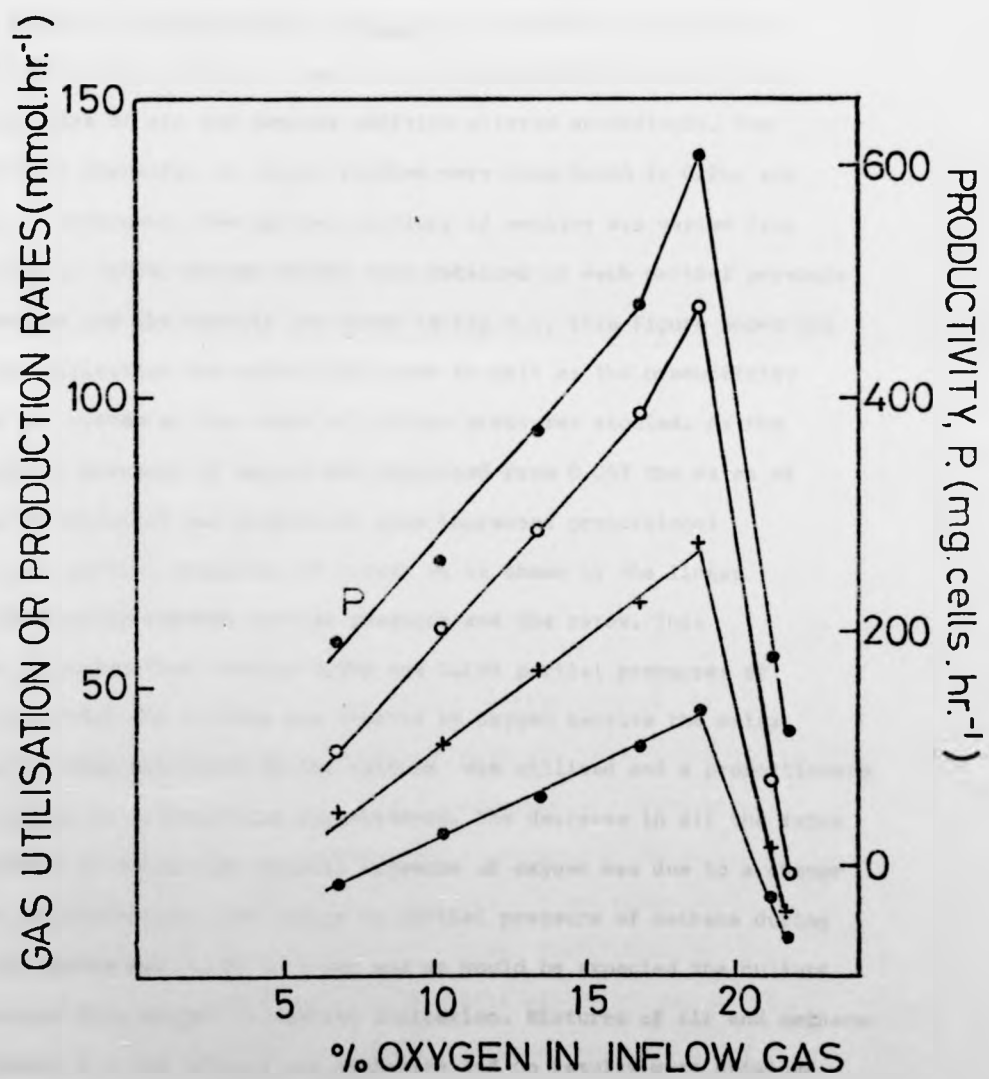
culture of _Methylococcus capsulatus_ (Bath)

Fig. 3.1. Effect of oxygen partial pressure on a gas limited
culture of Methylococcus capsulatus (Bath)

The effect of changing the gas partial pressures but keeping constant
the total volume of gas entering the system was carried out with
a culture of Methylococcus capsulatus . The total volume of gas
entering the system was kept at approximately 360 ml.min $^{-1}$ and
the rates of air and methane addition altered accordingly. The
partial pressures of oxygen studied were from 0.066 to 0.216 and
as a consequence the partial pressure of methane was varied from
0.695 to 0.024. Steady states were obtained at each partial pressure
studied and the results are shown in fig 3.1. This figure shows the
gas utilisation and production rates as well as the productivity
of the system at the range of partial pressures studied. As the
partial pressure of oxygen was increased from 0.067 the rates of
gas utilisation and production also increased proportional
to the partial pressures of oxygen as is shown by the linear
relationship between partial pressure and the rates. This
demonstrates that between 0.066 and 0.187 partial pressures of
oxygen that the culture was limited by oxygen because the extra
oxygen made available to the culture was utilised and a proportionate
increase in productivity was observed. The decrease in all the rates
between 0.187 to 0.21 partial pressure of oxygen was due to a change
in gas limitation; the change in partial pressure of methane during
this period was 0.146 to 0.044 and as would be expected the culture
changed from oxygen to methane limitation. Mixtures of air and methane
between 5 - 15% methane are explosive and no results were obtained
intentionally between these limits; unfortunately it is in this
'black box' that the change occurs between oxygen and methane
limitation.

The equation usually quoted to define gas transfer in a fermenter is :-

$$R = K_L a \ (C_s - C_L)$$

R = gas uptake rate

$K_L a$ = gas transfer coefficient

C_s = gas partial pressure in gas phase

C_L = dissolved gas tension

$K_L a$ is a constant if the stirring speed, culture volume and volume of gas entering the culture are not changed. In an oxygen limited culture the dissolved oxygen tension (C_L) is usually very low, undetectable with the oxygen probe and meter used and so C_L is insignificant compared to C_s therefore

$$C_s - C_L \simeq C_s$$

and $\quad R = K_L a \ C_s$

From this equation, in an oxygen limited culture, if only the partial pressure of oxygen is changed then the oxygen uptake rate (R) should be proportional to the partial pressure of oxygen entering the system (C_s). This is demonstrated to be true in Fig 3.1.

The $K_L a$ for oxygen was 5.8 mmoles. h^{-1} per percent of inflowing gas between 6.7 and 16.7% oxygen. The highest partial pressure of oxygen under oxygen limitation was 0.187 the $K_L a$ for this value showed a disproportionate increase, the rate being 6.1 mmoles.h^{-1} per percent oxygen in. Two possible reasons for this are 1) as more gases were consumed the volume of gas leaving the system was decreasing thus giving a greater residence time to the gases in the culture and therefore more efficient use of the gases available would be expected. 2) The density of the culture was

increasing and this may also have led to more of the limiting gas
being utilised.

During all these steady states the conditions e.g. temperature,
stirring speed, volume of gas entering the culture etc. were kept
constant. So, from the $K_L a$ values it should be possible to estimate
the change over point with respect to gas limitation. For methane the
$K_L a$ value was 4.95 mmoles.h^{-1} per percent methane in. This value is
lower than that for oxygen (5.9) which would seem to imply that the
rate at which oxygen can exchange from the gas phase to the site
of metabolism is greater than that for methane. But the actual
affinities of the organism (i.e. K_s) for the gases will be an
important factor in deciding the rate at which the gases will be
consumed. From the literature the affinity of methylotrophs for
methane (K_s) are 2.6 x 10^{-5}M Harrison (1973) and 1.9 x 10^{-5}M Wilkinson
and Harrison (1973). The K_m for the methane monooxygenase in crude
extracts of Methylococcus capsulatus (Bath) was found to ъ
1.6 x 10^{-4}M (Colby et al , 1977). The K_s values for oxygen uptake
obtained with other organisms have been as low as 1.34 x 10^{-6}M
(Torula utilis - Johnson, 1967). However it was found that both K_s
and maximum oxygen uptake rate varied with dissolved oxygen tension
so that simple Michaelis-Menton Kinetics did not apply. If the affinity
for methane is less than that for oxygen it would not be surprising
that the $K_L a$ value for oxygen is higher than that for methane.
However it would be interesting to determine the actual point at
which the changeover between oxygen and methane limitation did occur.
This can be estimated by extrapolating the curves into the 5% - 15%
methane range by using the $K_L a$ values obtained. Doing this the
changeover point should occur at 14.53% CH_4. The rates obtained at
18.7% oxygen (14.64% methane) were possibly very close to a double

gas limitation situation. In fact the rate for methane utilisation was slightly greater than the $K_L a$ values obtained would indicate, but at the same point the oxygen utilisation rate was also higher than the $K_L a$ values obtained from the lower partial pressures would indicate. It is possible that if the K_s for methane uptake rate compared to that of oxygen is higher that this is giving an apparent transfer rate for methane lower than that for oxygen; and so it could be that as the partial pressure of methane increases the $K_L a$ for methane transfer might also increase.

A question is raised that what partial pressure of a gas should be used to determine the actual partial pressure of that gas as far as the culture is concerned ? Should it be the partial pressure of that gas in the inflowing gas or the partial pressure of that gas in the outflowing gas or some value between the two? Obviously in the culture there will be bubbles of gas which have just been formed from fresh gas entering system, "exhausted" bubbles of gas being given up to the head space in the fermenter and a spectrum of bubbles with different partial pressures of gases between the two extremes. The bubbles being given up to the head space should also have this same spectrum if perfect mixing is occuring in the fermenter. So the partial pressure of the gases in the head space should also be the average partial pressure of the gases in the culture i.e. every bubble has an equal chance of "escaping" from the culture to the head space be it a newly formed bubble or one that has been in the fermenter for sometime. However the incoming gas enters from the bottom of the culture and the bubbles must reach the top before they can be vented to the head space. Also as the impeller blades are directly over

the gas entry port, any bubbles will first be flung out to the side
of the fermenter before they can reach the top. So the partial
pressure of the utilisable gases in the head space are probably
lower than the average for the bubbles in the culture. The taller
the fermenter the more so this will be true. The $K_L a$ values for
oxygen and methane were compared using the input and output partial
pressures of the gases, these are shown in table 3.1.

LIMITING GAS	% LIMITING GAS IN	% LIMITING GAS OUT	FOR LIMITING GAS	
			$K_L a$ FROM INPUT %	$K_L a$ FROM OUTPUT %
O_2	6.67	2.89	5.74	13.24
O_2	10.15	4.37	5.88	13.66
O_2	13.31	5.91	5.73	12.90
O_2	16.73	7.79	5.75	12.35
O_2	18.7	7.93	6.11	14.41
CH_4	4.40	2.37	4.98	9.24
CH_4	2.23	1.11	4.91	9.87

TABLE 3.1   Effect of input or output partial pressure of gas on the $K_L a$ value
$K_L a$-(mmole. h^{-1} per percent in or out)

As can be seen from the table the $K_L a$ values calculated from
output percentage were just over twice those from the input percentage
but by comparing the two sets of figures no change was observed in the
$K_L a$ values as the partial pressure of the gases were altered. This
was not surprising if the relationship $R = K_L a C_s$ holds true. Consider
two steady states in one the partial pressure of the limiting gas (2b)
is twice that of the other (b) and that the total volume of gas
entering the system in a time (t) is 'V'. From table 3.2 it can be

seen that as the partial pressure of the limiting gas is doubled, the
rate of utilisation doubles, as would be expected but also the amount
of gas not being consumed also doubles.

AMOUNT OF LIMITING GAS IN	RATE OF GAS UTILISATION	AMOUNT OF LIMITING GAS USED	AMOUNT OF LIMITING GAS UNUSED
bv	$K_L ab$	$K_L abt$	$bv - K_L abt$
2bv	$K_L a2b$	$2K_L abt$	$2bv - 2K_L abt$ $= 2(bv - K_L abt)$

TABLE 3.2. Effect of doubling amount of limiting 'gas in' on amount of
limiting gas leaving the fermenter.

So, it was not expected that the $K_L a$ values calculated from output
would change as the partial pressure of input limiting gas was altered.
There are two reasons, already mentioned, why the $K_L a$ value might
increase as the partial pressure of oxygen is increased (higher
culture densities and longer resident times of the gases in the
culture) However this is not demonstrated from the results except for
the highest partial pressure of oxygen used for an oxygen limited
culture.

The use of input or output partial pressures to determine the
$K_L a$ value of gas limited cultures does not seem to have much effect
on the constant nature of this parameter. However, from a practical
stand point, the input partial pressure has advantages over the output.
One is that a culture is set up at a predetermined partial pressure
of the limiting gas (the output value will not be known until a steady
state is obtained) and it might be beneficial to know what gas transfer

rate to expect. Another reason for using input partial pressures is that
the output partial pressure is smaller than the input (could be very
small) and this would lead to small inaccuracies in the estimation of
output partial pressures giving larger errors in $K_L a$ estimations
when the gas utilisation rate is divided by the partial pressure.

Effect of Increased Oxygen Availability

The inocula for the continuous culture were grown up in
250 ml. flasks. This starter culture (25 ml) was inoculated into
3.5L. of medium in the fermenter and allowed to batch up until a
reasonably dense culture (O.D.~5) was obtained; the medium flow
was then switched on. During this batching up process it was
noted that if too high a stirring speed or oxygen partial pressure
was used then growth was very slow. To avoid this slow growth the
oxygen partial pressure and stirring rate were only slowly increased
during this batching up process. The dissolved oxygen tension
measuring system, an L.H. engineering meter and probe, helped a
great deal in deciding when to increase the gas supply to the
culture i.e. when the dissolved oxygen tension was approaching
zero.

On occasions it was noticed that when the stirring rate was
increased not only did the dissolved oxygen tension increase but also the
pH would drop from 6.8 down to as low as 6.4 (pH changes on NMS rises)

During these increases in stirring speed gas
readings were taken to estimate the gas utilisation rates. On
occasions stoichiometries of these gas utilisation rates indicated
that all the methane was being converted to carbon dioxide
(O_2:CH_4:CO_2, 2.0:1.0:1.0). It would seem from these results that
Methylococcus _capsulatus_ did not particularly relish high dissolved
oxygen tensions (DOT) and in an effort to decrease this DOT as much
of the oxygen as possible was removed by "burning" methane to carbon
dioxide and in the process creating a greater demand for oxygen.

TIME (h)	O.D	%CH$_4$ IN	VOLUME OF GAS USED OR PRODUCED			STOICHIOMETRY		
			O$_2$	CH$_4$	CO$_2$	O$_2$	CH$_4$	CO$_2$
0	9.45	18.7	27.27	18.40	11.66	1.48 :	1 :	0.63
1.8		4.68	16.73	8.99	6.95	1.86 :	1 :	0.77
2.7		4.74	15.91	8.82	6.86	1.79 :	1 :	0.77
5.3	7.50	4.75	14.19	8.17	6.55	1.74 :	1 :	0.80
6.5		4.77	12.96	7.43	6.53	1.74 :	1 :	0.98
7.3	6.65	4.62	13.35	6.45	5.94	2.07 :	1 :	0.92
12.0	5.37	4.65	9.61	4.46	4.48	2.15 :	1 :	1.00
25.0	2.80	4.60	6.49	4.33	3.17	1.50 :	1 :	0.73
27.3	2.62	4.54	6.67	3.98	3.02	1.68 :	1 :	0.76
30.0	2.52	4.61	6.58	4.60	3.05	1.43 :	1 :	0.66
31.8	2.62	4.55	6.80	4.65	2.95	1.46 :	1 :	0.63

Table 3.3. Effect of increasing oxygen availability on a culture of
Methylococcus capsulatus (Bath) by changing from oxygen
to methane limitation.

From this 'burning' situation the culture slowly begins to fix
carbon again and stoichiometries return to normal.

This phenomenon is probably best demonstrated by the results
presented in Table 3.2 which gives the gas utilisation and production
rates from a culture in a steady state which was changed from oxygen
limitation to methane limitation. The culture took several hours (7)
to reach the situation when all the methane was oxidised to carbon
dioxide but in the meantime the amounts of oxygen used and carbon
dioxide produced compared to methane are considerably higher than
before the change or 30 hours after. This experiment also showed that
a denser culture can utilise more of a limiting gas than the less
dense culture obtained at an O.D of 2.6

However this 'burning' situation was not always observed as
can be seen from Fig.3.2. This figure shows the effect of increased
stirring rate on gas utilisation rates. The culture was in a steady
state and then the stirring speed was increased from 300 r.p.m. to
600 r.p.m. It is interesting to note that cells can immediately
increase their gas utilisation rates by 100%, showing that although
the culture was gas limited the cells could still utilise more gas
than was available. This result throws some doubt on the interpretation
of oxygen utilisation rates obtained by removing the cells from a
culture and obtaining rates of oxygen utilisation in an oxygen
electrode and then relating these rates to the conditions in the culture.
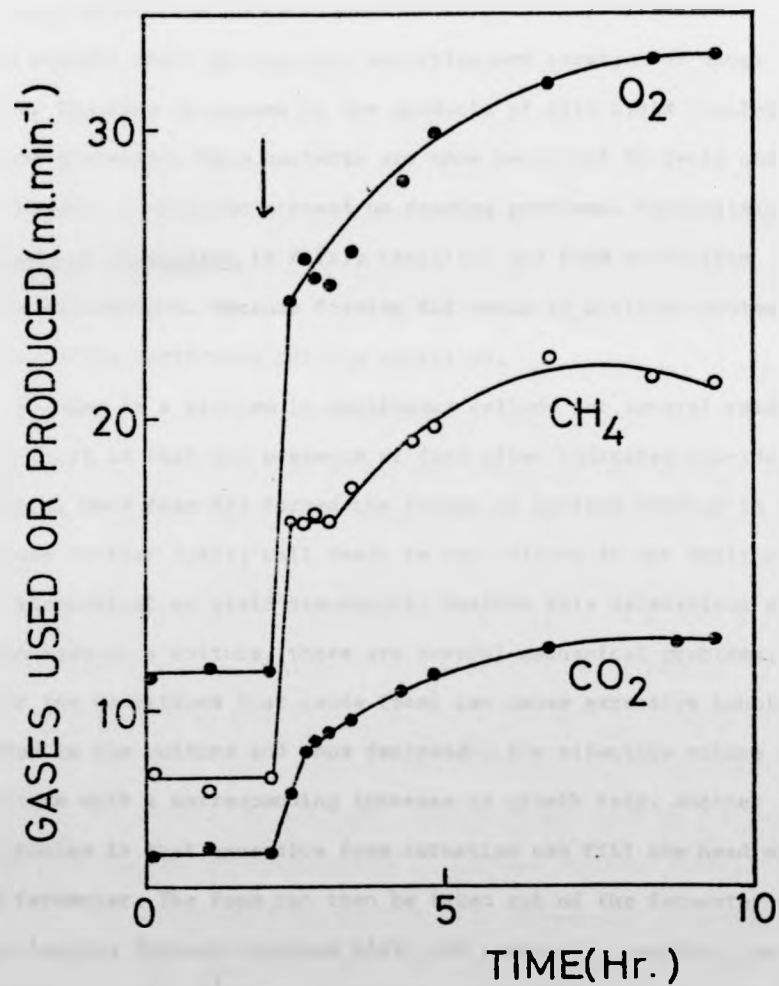
Fig. 3.2. Effect of increasing stirring speed on a culture of
Methylococcus Capsulatus (Bath)

b) Foaming and the Effect of Antifoam Addition

Foam production in continuous culture is an ever present
problem brought about by vigorous agitation and aeration of dense
cultures. The foam is caused by the products of cell lysis (nucleic
acids and proteins). Some bacteria are more resistant to lysis and
under 'normal' conditions present no foaming problems. Fortunately
Methylococcus capsulatus is fairly resilient and foam production
was only intermitent. Because foaming did occur an antifoam system
was used in the continuous culture apparatus.

Foaming is a problem in continuous culture for several reasons,
not the least is that the presence of foam often indicates non-ideal
conditions. Once foam has formed the forces of surface tension in the
foam cause further lysis; cell death in the culture is not desirable
from a theoretical or yield standpoint. Besides this deleterious effect
foam can have on a culture, there are several mechanical problems;
foam (or the conditions that cause foam) can cause excessive bubble
formation in the culture and thus decrease the effective volume of
the culture with a corresponding increase in growth rate. Another
major problem is that excessive foam formation can fill the head space
in the fermenter. The foam can then be taken out of the fermenter with
the gas leaving through the head plate and eventually reaching the
bacteriological filter(there to prevent contamination via the gas
outlet from the atmosphere. The foam reaching the filter will wet it
and make it much less efficient as a bacteriological filter. Also
the wet filter (and cells) will retard the rate of gas flow that can
pass through it and result in a pressure building up inside the fermenter.

There are two methods of controlling foam formation, both using foam detection devices as described in the materials and methods section :-

1) Mechanical foam breaker - this can be similar to the impeller blades in the culture but placed above the culture. In the foaming situation the blades are turned to mechanically break up the foam. This system was not available on the fermenters used in this study.

2) Antifoam addition - perhaps not as desirable as the above technique but probably able to cope better with heavy foaming. In the foaming situation an antifoam solution (described in materials and methods section) is pumped into the culture, the antifoam solution decreasing surface tension and effectively bursting (and preventing formation of) bubbles causing the foam. This system was used during this study.

The antifoam addition technique is less desirable than the mechanical foam breakers because an addition of an essentially unnecessary compound is made to the culture. The antifoam might have an effect on the cells or be metabolised by them; but perhaps more important is the effect the antifoam solution might have on the gas transfer rate because of the decrease in surface tension. This is probably not too important when the gas is in excess, as is usually the case in a carbon limited culture but could present problems in gas limited cultures.

It had been noticed on several occassions when the culture was being batched up that addition of antifoam solution to a foaming culture with a high dissolved oxygen tension would result in an immediate lowering of this oxygen tension (as well as reduction in the amount of foam). This could have serious consequencies in a gas limited continuous culture if this effect was because of a reduction in

oxygen transfer rate. Comparisons between steady states, some of
which had antifoam added and others not, would be distorted if
the $K_L a$ (transfer coefficient) is different from one steady state
to the other because of antifoam additions. To investigate the
effect of antifoam solution addition an oxygen limited steady state
culture of Methylococcus capsulatus was set up and to this culture
was added 10 ml. of a 1% antifoam solution. The results are given
in Table 3.4.

GAS UTILISATION OR PRODUCTION RATES $ML.MIN^{-1}$			ANTIFOAM ADDITION
O_2	CH_4	CO_2	
39.4	27.7	15.8	BEFORE
29.5	21.3	11.8	AFTER

TABLE 3.4. Effect of antifoam on gas utilisation rates

As can be seen the antifoam addition decreased the gas utilisation
rates. Presumably the gas transfer rate(s) are decreased by the
addition of antifoam, in this case a final concentration of
0.003% gave a 25% reduction in the rate of gas consumption and
production. This reduction was maintained for two hours after the
addition of antifoam (for the length of time readings were taken)
and it took 24 hours before the rates were back to those obtained
before the addition.

Care must be taken in the use of chemical antifoam agents
added to gas limited cultures, and the effects of antifoam addition
must be considered when results are compared. Small repetitive
additions of antifoam solution to cultures to prevent excessive foam

production could very well decrease the gas transfer rates. So a decrease in gas utilisation rates on changing conditions may not be due directly to the change in the environment but to a change in antifoam addition rate.

To minimise the effect of the antifoam agent it was found that a 0.2% solution was adequate to control foaming in cultures of Methylococcus capsulatus. Usually no antifoam additions were required for cultures of Methylococcus capsulatus .

3c. Effect of Temperature and pH on Cultures of Methylococcus capsulatus

No thorough investigation was carried out into the effect of pH on cultures of Methylococcus capsulatus. However failures of the pH controlling system did throw some light on the effects of pH. Low pH's (below 6.5) would cause the culture to foam slightly and the cells to clump together, this could be seen under the microscope. Also the pH's above 7.0 caused the culture to foam, and it was noticed during the batching up process when the pH controlling system was not switched on (to allow the pH probe to stabilise) that pH's above 7.0 would be detrimental to the oxygen uptake rate of the culture. This was demonstrated when the pH controlling system was switched on and the pH brought down to 6.8, on occasions this was done whilst there was a high DOT and on addition of the acid there was an immediate fall in the DOT.

The effect of temperature was studied more closely. The temperature of the culture was increased or decreased until growth ceased or washout occured. The highest temperature at which steady states could be obtained was $50^{\circ}C$ and this was only possible if the temperature was increased in small increments. Increasing the temperature from $45^{\circ}C$ to $50^{\circ}C$ caused the culture to washout $(D = 0.1)$, however if the culture temperature was increased from $45^{\circ}C$ to $48^{\circ}C$ and then to $50^{\circ}C$ a steady state was obtained at the higher temperature. A further increase of $2^{\circ}C$ to $52^{\circ}C$ caused washout to occur and even after several days of batching at $52^{\circ}C$ no growth could be measured and no significant production of carbon dioxide could be detected. The lowest temperature at which a steady state was obtained was $33^{\circ}C$ $(D = 0.05)$, washout

occured if the temperature was dropped to $31^{\circ}C$, lower dilution
rates were not tried at $31^{\circ}C$.

Temperature changes did not have much effect on the
stoichiometries; only at $34^{\circ}C$ and $50^{\circ}C$ could any change be detected.
At $34^{\circ}C$ the stoichiometry was 1.72 : 1 : 0.65 (oxygen: methane:
carbon dioxide) and at $50^{\circ}C$ it was 1.60:1:0.70
as opposed to 1.55 - 1.60 : 1 : 0.58 - 0.61 between these temperatures.
It seems from these results that Methylococcus capsulatus has a
wide optimum temperature range between $35^{\circ}C$ to $48^{\circ}C$.

4. NITROGEN METABOLISM

Methylotrophs are capable of utilising a wide variety of
nitrogen sources e.g. ammonium ion, nitrate ion, dinitrogen, amino
acids, etc. Whittenbury et al, 1970), Eroshin et al, (1968).
For the purposes of S.C.P. production amino acids, as a source of
nitrogen, would be too expensive. This leaves three possibilities :-

> 1. Ammonium ion '
>
> 2. Nitrate ion
>
> 3. Dinitrogen.

Nitrogen can enter cell metabolism by two reactions, both
requiring the nitrogen to be in the form of ammonia.

1. α-Keto-glutarate + NH_3 + $NADH_2 \longrightarrow$ glutamate + NAD + H_2O

glutamate dehydrogenase

2.   glutamate + ATP + $NH_3 \longrightarrow$ glutamine + ADP + P_i + H_2O

glutamine synthetase

The amino and amide groups are transferred to other compounds
by transaminase reactions.

The efficiency considerations of growth on the above three
nitrogen sources will depend on how much energy is required to form
ammonia from them. Obviously, nitrogen in the form of ammonium ion
requires no conversion and so no energy is required.

Nitrate ions are reduced to ammonia by two reactions via
nitrite using nitrate and nitrite reductases both these reactions
require $NADPH_2$. Dinitrogen probably requires the highest input of
energy (reducing power) to provide ammonia. The enzyme nitrogenase
converts dinitrogen to ammonia by several steps on this one enzyme

complex. The intermediates have not been isolated and the only detected product is ammonia. This reaction requires the input of 1.5 molecules of $NADPH_2$ per molecule of ammonia produced and between 2-10 molecules of ATP (these are the range of values that have been reported in the literature, Benemand and Valentine, 1972).

From the above discussion it would seem that ammonium ions should give the highest yields because less energy would be required for fixing the nitrogen; nitrate would give a lower yield and dinitrogen the lowest.

Besides considering the yields on various nitrogen sources another important factor in nitrogen metabolism is that nitrogen is a major constituent of the cell (after carbon and oxygen) and in continuous culture high cell densities are obtained, which require quantities of nitrogen in excess of that normally added to the mineral salts medium. Most continuous cultures of methanotrophs are carried out under gaseous limitation, but of course care must be taken to ensure that the added nitrogen source is not exhausted or else the culture could become nitrogen limited. (This would be reflected in a rise in dissolved oxygen tension in an oxygen limited culture). However several methanotrophs can fix dinitrogen, which is in the gas phase, and it seems probable (unless high oxygen tensions prevent it) that if all the added nitrogen source is consumed then the nitrogenase would become derepressed and the culture would then be utilising two different nitrogen sources at the same time. The parameters of growth would be more complex, and if the cell density is varied from one steady state to another, without a change in added nitrogen source, the amount of nitrogen obtained from dinitrogen will

be varying as well. Thus more than one parameter would be changing from one steady state to another, which is undesirable if meaningful results are to be obtained without having to consider the effect of two parameters.

To overcome this problem excess nitrogen source should be present in the culture, · in amounts that will always be in excess (not always possible because of inhibitory effects) or estimations of cell density can be made and the concentration of added nitrogen in the medium varied so that the nitrogen source is only slightly in excess.

Investigation of the nitrogen source is necessary not only from an efficiency point of view but also to decide which nitrogen source should be routinely used and in what concentrations.

Effect of Different Nitrogen Sources

It soon became apparent that cultures grown on ammonium chloride became acidic and required additions of potassium hydroxide (1M) to maintain the pH at 6.8, whereas growth using potassium nitrate as the nitrogen source caused the culture to become alkaline and hydrochloric acid (0.5 - 1.0 M) had to be added to control the pH. Growth on dinitrogen resulted in a slight fall in pH and potassium hydroxide (0.5 M) was used as the pH addition.

The closed batch system was used to compare the different nitrogen sources as well as the continuous culture apparatus. Growth on each nitrogen source will be discussed separately and then yields on these nitrogen sources considered together.

1) Potassium Nitrate

Growth on potassium nitrate, as mentioned above,

caused the culture to become alkaline. In continuous culture 0.5 M hydrochloric acid was usually used as pH addition, but during batch growth the increase was only slight and no pH control was required. The result from batch growth is shown in the typical batch growth curve (section 2 p.106) and described in the text.

To obtain an estimate of how much growth a particular concentration of potassium nitrate would support, 250 ml. erlmyer flasks containing 9 ml. of medium and different concentrations of potassium nitrate (0.01 - 0.12%) were set up. The flasks were flushed with argon and 40 ml. of oxygen and 40 ml. of methane added to each flask (40 ml. of gas phase was removed before each addition). The flasks were flushed with argon to remove dinitrogen from the flasks and thus prevent fixation of dinitrogen by the cells. An inoculum was grown up in a 250 ml. flask with 0.02% potassium nitrate in the medium. The low level of nitrate would all be utilised and none would be added to the flasks on inoculation.

After inoculation growth was followed by measuring the density of the culture in the side arm of the flask with an Eel nephelometer. The flasks were reflushed and oxygen and methane added again throughout the growth phase. When growth had stopped (no change in nephelometer reading) the culture density was estimated at 540 nm. on a Pye Unicam S.P.500, the pH and tests for nitrate and nitrite were also carried out. The results were as would be expected up to 0.08% potassium nitrate concentration, but above this concentration no growth was observed.

The lack of growth above 0.08% potassium nitrate did not correlate with growth which normally occured on NMS (0.1% potassium nitrate)
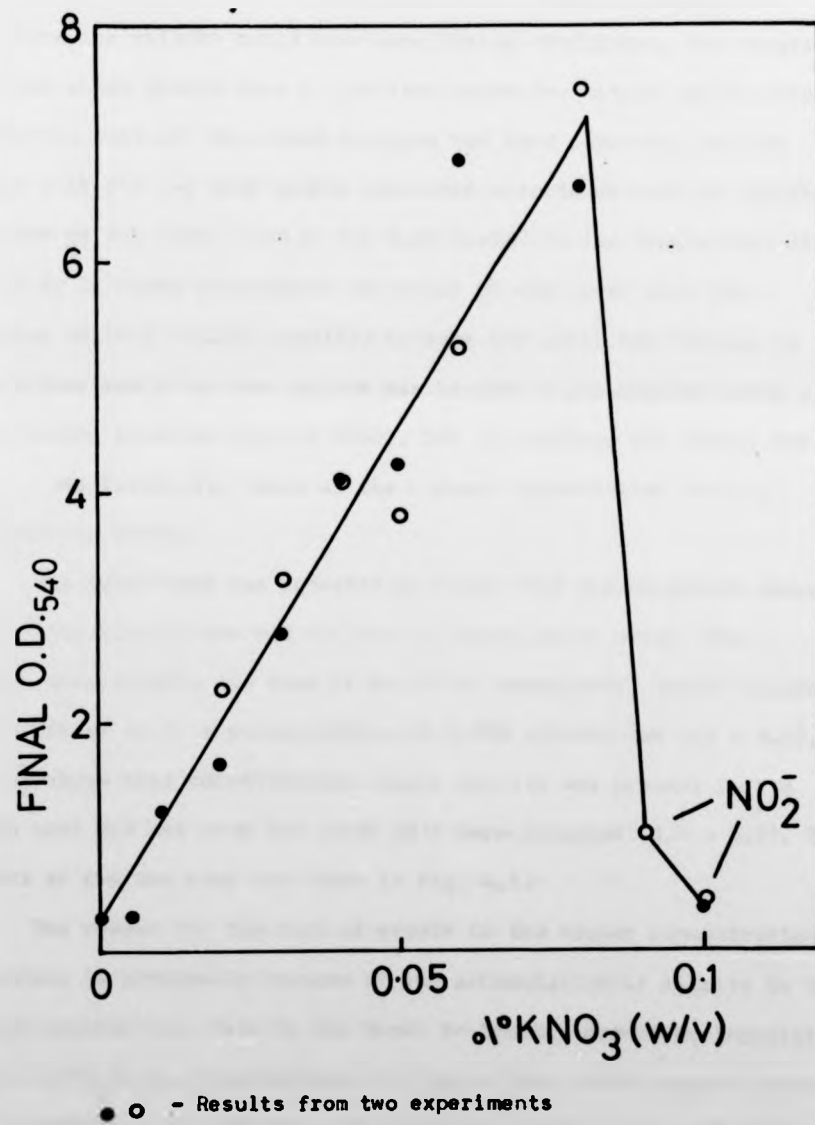
Fig. 4.1. Effect of different concentrations of potassium nitrate on
final cell densities of batch cultures of _Methylococcus
capsulatus_ (Bath)

however the inoculum in this experiment was lacking nitrate and
therefore the culture could have been fixing dinitrogen. The flasks
that had shown growth gave no positive tests for nitrate or nitrite
indicating that all the added nitrogen had been consumed, but the
flasks that did not show growth contained nitrite as well as nitrate.
Also the pH was lower (5.0 in the 0.1% flask) in the flasks that did
not grow. In these experiments the final pH was lower than the
starting pH (6.2 - 6.6), possibly because the cells had started to
lyse before the pH of the culture was tested. A calibration curve of
nephelometer readings against SP500, 540 nm readings was drawn, the final
O.D. of the flasks was taken as the highest nephelometer reading
converted to SP500.

The experiment was repeated to check that the no growth above
0.08% potassium nitrate was not due to experimental error. The
results were broadly the same as the first experiment, growth occured
in the flasks up to a concentration of 0.08% nitrate (pH 6.2 - 6.6),
but not above this concentration. Again nitrite was present in the
flasks that did not grow and lower pH's were observed (4.6 - 5.2). The
results of the two runs are shown in Fig. 4.1.

The reason for the lack of growth in the higher concentrations
of nitrate is presumably because of the accumulation of nitrite in the
culture supernatant. Nitrite was shown to inhibit growth by inoculating
flasks containing concentrations of nitrate that would support growth
but in addition concentrations of potassium nitrite between 0.05% and
0.2%, these flasks did not show any growth. Why should these flasks
accumulate nitrite ? Perhaps cells from a medium lacking any nitrate,
exposed to sudden excesses of this nitrogen source rapidly convert some

nitrate to nitrite (as they must to fix the nitrogen) but are unable to utilise the amounts of nitrite produced. The point where growth occurs is when the cells can remove the nitrite formed before inhibitory concentrations can accumulate.

This effect of nitrite accumulation in cultures using nitrate could be a problem in continuous culture where the concentration of excess nitrate can vary greatly from one steady state to another, although the changes are usually gradual and the cells are not exposed to sudden changes in nitrate concentration. In fact the accumulation of nitrite in nitrate grown continuous cultures did not present a serious problem; under non-adverse conditions nitrite was never detected in culture supernatants. However on some occasions when the cultures did start to washout nitrite was detected in the supernatants; the appearance of this nitrite was probably not the cause of the washout situation but was rather a consequence of some other adverse environmental factor acting on the culture.

2) Ammonium Chloride.

Growth on ammonium chloride was not as simple as that on nitrate. In the closed batch system the pH fell rapidly and growth was inhibited by the low pH (4.4) before any of the gases were completely consumed. Also in continuous culture pH additions were more frequent and therefore more potassium hydroxide was added to cultures grown on ammonium than acid was added to nitrate grown cultures. Experiments to determine the amount of growth on different concentrations of ammonium chloride were not performed as growth would have been inhibited by the lowering of the pH before all the nitrogen source could have been consumed. However estimations could be made by comparing

the amount of nitrogen present in AMS medium and that of NMS medium.

Ammonium ions have been reported to inhibit growth of methylotrophs (Whittenbury et al. 1970), a reason given for this inhibition is that the ammonium ion is a competitive inhibitor of methane mono-oxygenase, the ammonium ion having a similar structure to that of the methane molecule. Indeed the methane mono-oxygenase has been demonstrated to oxidise ammonium to hydroxylamine and that the ammonium ion is a competitive inhibitor of this enzyme (H. Dalton in press). The reports of inhibition of growth by excess ammonium could be due to the competition mentioned above but also if ammonium (or ammonia) is being oxidised to nitrite (whole cells oxidise ammonium to nitrite and nitrate), then it could be the accumulation of these oxidation products that was preventing further growth.

Whereas large excesses of nitrate had no effect on the growth of Methylococcus capsulatus (Bath), excesses of ammonium could have, so media had to be made up with concentrations of ammonium chloride that would not give much excess ammonium in the culture. However enough must be added so that the ammonium does not become "limiting" (i.e. if all the ammonium was consumed, the culture might begin to fix dinitrogen) or else even more complications arise. It was found that even excesses of 0.04% ammonium chloride caused small concentrations of nitrite (0.06 mM) and larger concentrations of nitrate (4.4 mM) to accumulate in the culture supernatant. This apparently non-functional metabolism of ammonium, a wasteful process in terms of energy, must give lower yields than could be expected from growth on ammonium. A greater problem is that the excess ammonium will inhibit methane oxidation and the oxidation products, especially nitrite, could also inhibit growth. Also if all the ammonium is consumed and dinitrogen

fixation occurs, then the amount of nitrogen derived from dinitrogen
will vary with changes in culture density. Whether or not excess
or "limiting" ammonium chloride is used, every new steady state will
have two parameters changed :-

1) The change which is brought about by the parameter being studied
2) Variation of dinitrogen fixation or effect of different
   excess concentrations of ammonium.

It would be a difficult task to keep a constant excess of
ammonium ions or a constant amount of dinitrogen fixation when
various other parameters are being studied. It would however be
possible when all the parameters have been optimised for S.C.P.
production because no changes in cell density would be expected and
obviously from an economic point of view a system which utilised all
the added nitrogen source would be less wasteful. A gain in this
sort of system is that some of the cellular nitrogen will be derived
from the essentially free dinitrogen in the air. Excess ammonium might
have advantages of higher yields on the carbon source but problems
could arise from nitrite accumulation.

3) Dinitrogen

There is a paradox with aerobic dinitrogen
fixing organisms, in that the enzyme which converts dinitrogen to
ammonium, nitrogenase, is sensitive to oxygen and is inhibited by it,
but of course the organism requires oxygen for growth. So one
necessary substrate is an inhibitor of the fixation of another
essential substrate (if no other nitrogen source is available, other
than dinitrogen). Oxygen is not only required as the terminal electron

acceptor but also in the oxidation of methane to methanol by the
methane mono-oxygenase. Perhaps the nature of the gaseous
carbon-source, necessitating a limitation by a gas, which can
be oxygen, is helpful in the growth of dinitrogen fixing
methanotrophs. A low concentration of oxygen in the culture
whilst providing oxygen for growth.

Batching up cultures under dinitrogen-fixing conditions was
difficult because the initial concentration of oxygen was always
high. Low partial pressures of oxygen and slow stirring speeds could
be used, but this would mean only slow growth. However, if dinitrogen-
fixing cultures are required it is better to grow the cultures up on
ammonium or nitrate and then add M.S. medium when the culture has
grown sufficiently to be able to keep the oxygen tension down. In
practice changing cultures from nitrate or ammonium to dinitrogen-
fixing conditions (on methane) did not prove too difficult, because
the change over from nitrate or ammonium-excess to dinitrogen-
fixing conditions was gradual and the dissolved oxygen tension (DOT)
was always low (being the limiting substrate its concentration in the
culture was always close to zero).

Growth on methanol, using dinitrogen as the nitrogen-source,
was more difficult because methanol inhibited growth on dinitrogen.
To overcome this the culture has to be run methanol-limited, but this
leaves the problems of excess oxygen, which has been overcome in this
laboratory (H. Dalton pers. comm.) by using an oxygen controller to
keep the concentration of oxygen in the culture low by means of

varying the stirring speed (i.e. if the oxygen tension goes above a
set value a lower stirring speed is automatically used to prevent
inhibition by the excess oxygen).

Choice of Nitrogen Source

The choice of nitrogen source for studies of gas limited
continuous cultures was self evident from the foregoing discussion.
Growth on ammonium had problems of inhibition by excess ammonium,
accumulation of nitrite and nitrate, and 'limiting' ammonium could
not be used because of the varying levels of dinitrogen fixation
as the cell densities changed. The sensitivity of dinitrogen fixing
cultures to oxygen and the introduction of a third gaseous substrate
ruled out using dinitrogen as the routinely used nitrogen source.
Nitrate as a nitrogen source presented few problems, although possibly
less efficient than ammonium grown cultures. For these reasons nitrate
was routinely used as the nitrogen source.

Yields on Different Nitrogen Sources

Continuous cultures of Methylococcus capsulatus have been
grown on all three nitrogen sources and the average yields obtained
at dilution rates of about $0.1.h^{-1}$ are shown in Table 4.1.

NITROGEN SOURCE	AMMONIUM CHLORIDE	POTASSIUM NITRATE	DINITROGEN
Y_{CH_4}	0.52	0.43	0.45
Y_{O_2}	0.16	0.17	0.14
STOICHIOMETRIES. $O_2 : CH_4 : CO_2$	1.59 : 1 : 0.57	1.51 : 1 : 0.62	1.63 : 1 : 0.64

Table 4.1. Yields and Stoichiometries obtained on three different
nitrogen sources

The yield data from methane (Y_{CH_4}) were as expected, ammonium giving the highest yield and dinitrogen the lowest. However the yields from oxygen (Y_{O_2}) did not follow the same pattern. Nitrate grown cultures gave the higher yields from oxygen and dinitrogen the lowest. The reason for this is that ammonium ions can be oxidised to nitrite and nitrate, these reactions require molecular oxygen and so these cultures will utilise more oxygen per gram of cells because some of the oxygen consumed is not used for growth. The stoichiometries also show this effect, in that more oxygen is consumed per molecule of methane used. It was noted that ammonium-'limited'cultures (i.e. no detectable ammonium in the supernatant) gave lower values for oxygen (1.5 - 1.56) than the cultures with excess ammonium (1.54 - 1.64). This is because ammonium 'limited' cultures do not accumulate any oxidation products of the nitrogen source whereas ammonium excess cultures do accumulate these products. As mentioned earlier the ammonium grown cultures gave a wider range of values for stoichiometries, presumably because of the different effects of excess ammonium or ammonium limited cultures.

(The yield data from the dilution rate experiment for growth on nitrate in section 1 was not representative because these were some of the first results obtained and the gas analysis had not been perfected.)

5. EFFECT OF OTHER METABOLISABLE COMPOUNDS ON CULTURES OF Methylococcus capsulatus (Bath)

1.) Effect of Methanol on Methane-utilising Methylococcus capsulatus

The reports of Harwood and Pirt (1972), and Wilkinson et al (1974) of methanol accumulation in cultures of methanotrophs growing on methane were disturbing from an efficiency point of view. Methane that could be used for biomass production was being channelled into a by-product, (so reducing efficiency) that may also be toxic according to Wilkinson et al (1974). Harwood and Pirt (1972) reported methanol-accumulation in oxygen limited cultures of Methylococcus capsulatus (the Foster and Davies or Texas strain) growing on methane in continuous culture although this accumulation did not appear to inhibit growth it drastically reduced the efficiency of biomass production. The type 11 organism studied by Wilkinson et al (1974) grew very poorly on methane and this poor growth was attributed to the toxic effect of the accumulated methanol. Cultures of Methylococcus capsulatus (Bath) were frequently sampled and assayed for methanol in the supernatant but no significant accumulation of methanol was detected. This failure to find methanol accumulating in cultures of Methylococcus capsulatus (Bath), indicated that this organism differed in some respect from the organisms for which methanol accumulation has been observed.

The closed batch system was ideal for studying the effect of methanol on cultures of Methylococcus capsulatus (Bath), by enabling methanol to be added to a culture growing on methane. Using this system the effect of methanol concentration on methane uptake rate and growth was studied. The system was set up with methane (as the sole carbon source) and NMS medium. The fermenter chamber was inoculated
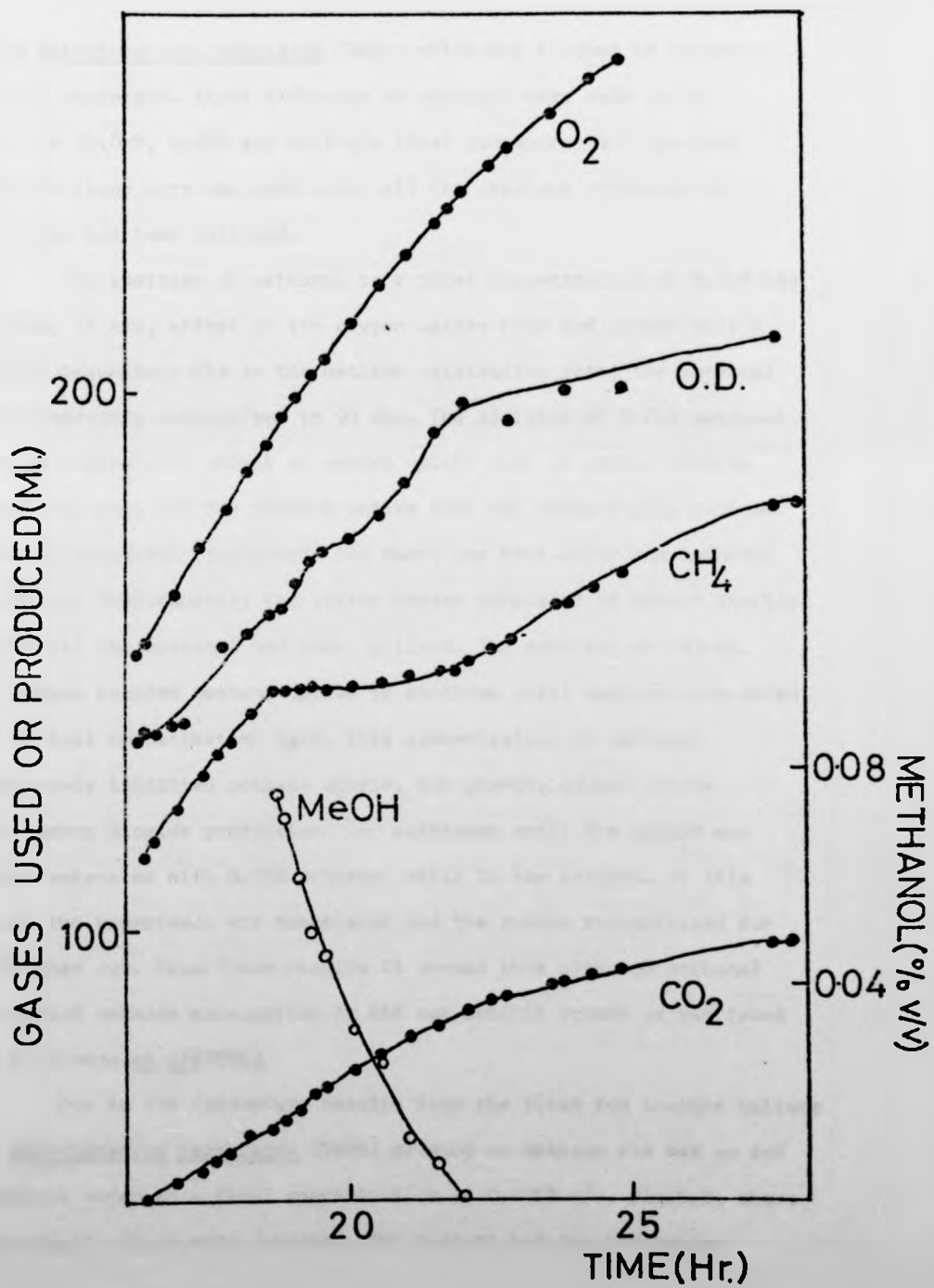
Fig. 5.1. Effect of methanol on a batch culture of

Methylococcus capsulatus (Bath) growing on methane

with Methylococcus capsulatus (Bath) which was allowed to commence
growth overnight. Three additions of methanol were made to the
culture (0.01%, 0.05% and 0.1% v/v final concentration) the last
two additions were not made until all the previous additions of
methanol had been utilised.

The addition of methanol to a final concentration of 0.01% had
little, if any, effect on the oxygen uptake rate and caused only a
slight transitory dip in the methane utilisation rate; the methanol
was completely metabolised in 90 min. The addition of 0.05% methanol
had no appreciable effect on oxygen uptake rate or carbon dioxide
evolution rate but the methane uptake rate was dramatically reduced
(almost completely inhibited) for about one hour after the methanol
addition. Unfortunately the system became exhausted of oxygen shortly
after all the methanol had been utilised. The addition of 100 ml.
of oxygen enabled methane uptake to continue until methanol was added
to a final concentration 0.1%. This concentration of methanol
completely inhibited methane uptake, but growth, oxygen uptake
and carbon dioxide production   continued until the oxygen was
again exhausted with 0.02% methanol still in the culture. At this
point the experiment was terminated and the system resterilised for
a further run. From these results it seemed that although methanol
inhibited methane consumption it did not inhibit growth as was found
by Wilkinson et al(1974).

Due to the incomplete results from the first run another culture
of Methylococcus capsulatus (Bath) growing on methane was set up and
methanol added to a final concentration of 0.075% v/v. Fig.5.1. shows
the results which were obtained. The culture had reached oxygen

limitation and was growing at a rate proportional to the oxygen
concentration; on addition of methanol (0.075% v/v final concentration)
methane consumption was completely inhibited for 90 min. Methane
consumption restarted when the methanol concentration in the culture
had fallen to 0.03%, but remained depressed until all the methanol
had been utilised, this can be seen from the increase in the rate of
methane disappearance after all the methanol had been utilised.
There was a burst of carbon dioxide production (3 ml.) soon after
the addition of the methanol, this burst of carbon dioxide production
was in addition to the constant rate of gas production before and
after addition of the methanol. This phenomenon was also reported by
Wilkinson et al (1974). There was no discernable effect on the oxygen
uptake rate when the methanol was added.

The addition of methanol to an oxygen limited batch culture
of Methylococcus capsulatus had no effect on oxygen uptake rate but
completely inhibited methane oxidation. From the fact that there was no
change in the oxygen uptake rate it seems that the cells rapidly adapt
(if adaption is necessary) to the new carbon source and no inhibition
in uptake of the limiting nutrient was observed. On addition of the
methanol the burst of carbon dioxide production indicated that a
sudden 'large' concentration of carbon source (methanol) precipitated
a rapid oxidation of this carbon source to carbon dioxide, but that
a balanced metabolism was obtained shortly after this increase in
oxidative activity. Although the optical density readings are not very
satisfactory they do seem to show a slight decline in the growth rate
when the methanol was added, presumeably because more methanol was

being converted to carbon dioxide than happens in 'normal' growth.
After this initial decline in growth rate the rate increased again
after about 15 min., this time to a faster rate than on methane.
This increased rate of growth on methanol is to be expected in an
oxygen limited culture because growth on methane requires more
oxygen per mole of methane utilised (mono-oxygenase) than does
methanol. This faster rate continued for about 40 min. and then
declined at the time when methane was beginning to be utilised
again. However, from this decline, the growth rate increased once
more indicating that as the methane began to be consumed an imbalance
in the metabolism occured which was overcome as soon as the methanol
disappeared. The optical density readings did not rise very much
after all the methanol had been consumed, but oxygen and methane
were still consumed. No explanation can be given for this part of
the curve.

At the time this experiment was performed it was assumed that
methanol was metabolised by a methanol dehydrogenase and that all
the oxygen used in growth on methane (vis. 1 molecule for oxidation
of methane + 0.4 molecules as a terminal electron acceptor) could
be used solely as an electron acceptor for growth on methanol. However
Colby et al (1977) have shown that methanol can also be oxidised by
the methane mono-oxygenase in vitro ; it is possible therefore that
some or all of the methanol was utilised via the mono-oxygenase
pathway which would have consequences on the yield from methanol. This
will be further discussed in the conclusion.

The inhibition of methane oxidation by methanol can be explained
by this dual specificity of the methane mono-oxygenase for methane and
methanol; despite the apparent K_m's obtained by Colby et al (1977)
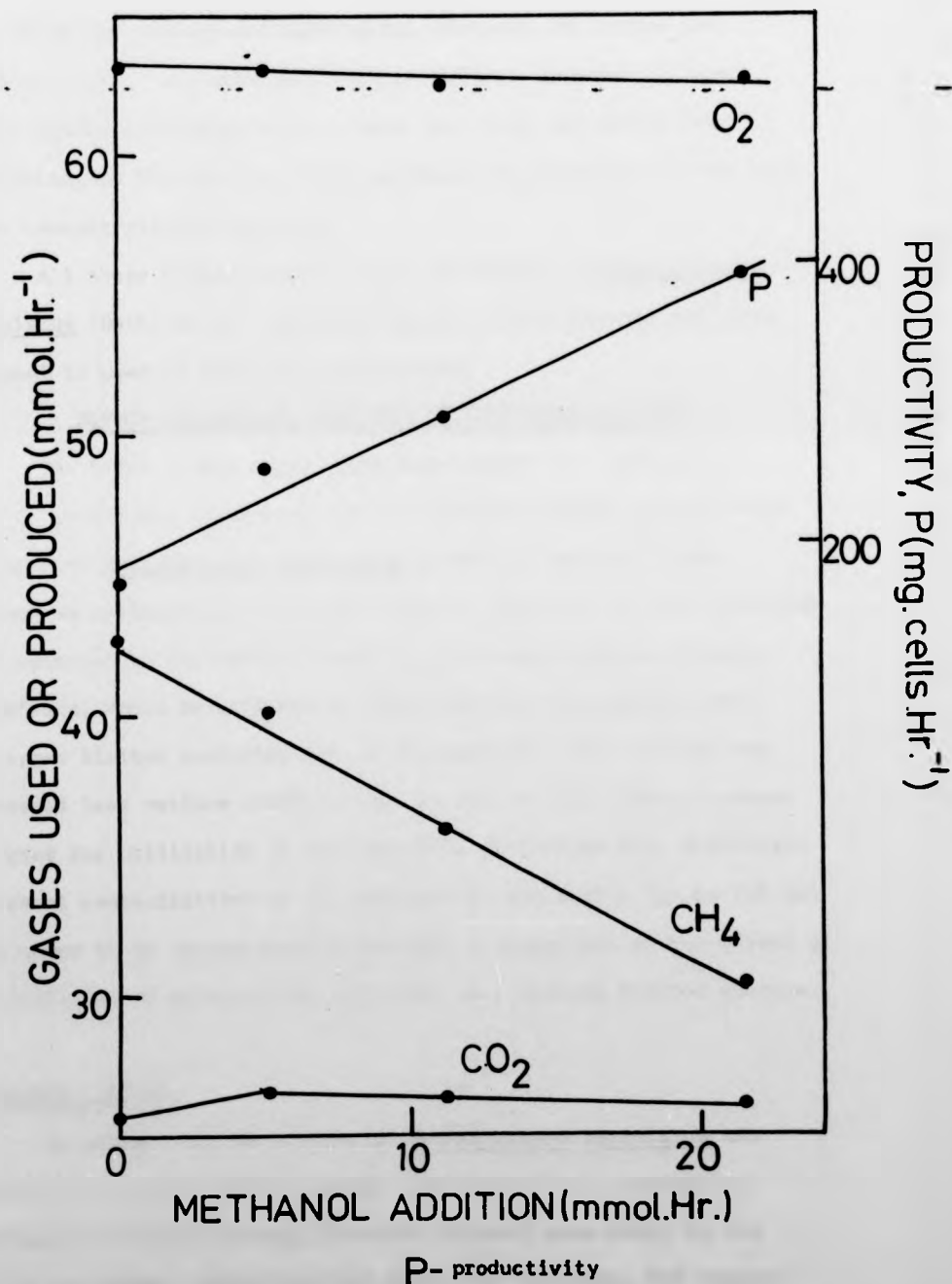
Fig. 5.2. Effect of methanol addition to an oxygen limited

culture of Methylococcus capsulatus (Bath).

of 0.16 mM for methane and 0.64 mM for methanol. Of course the concentration of the sparingly soluble methane compared to that of the miscible methanol will be very low, thus explaining the inhibition, by the methanol being oxidised in preference to the much lower concentration of methane.

All these results show is that the growth of Methylococcus capsulatus (Bath) is not inhibited by up to 0.1% methanol and that methanol is used in preference to methane.

EFFECT OF METHANOL ADDITION TO CONTINUOUS CULTURE

The batch growth experiments have shown that methanol is used in preference to methane and it therefore seemed unlikely that cultures of Methylococcus capsulatus growing on methane would accumulate methanol in continuous culture. From this it was predicted that methanol in the medium, added to continuous cultures growing on methane, would be utilised in preference to the methane. Also in oxygen limited cultures, that as the methanol concentration was increased less methane would be used as more of the limiting oxygen used for utilisation of the methanol. To confirm this prediction different concentrations of the methanol in the medium (up to 100 mM) were added to an oxygen limited culture. A comparison of the effect of the additions of methanol was also made to a methane limited culture.

a) OXYGEN LIMITED

An oxygen limited culture of Methylococcus capsulatus was set up and a steady state obtained. To this culture, growing on methane, increasing concentrations of methanol were added to the medium and steady states obtained after each increase. The results from this set of steady states are shown in Fig. 5.2. A rate of supply of 22 mmol. methanol.h^{-1} corresponded to a concentration of methanol in
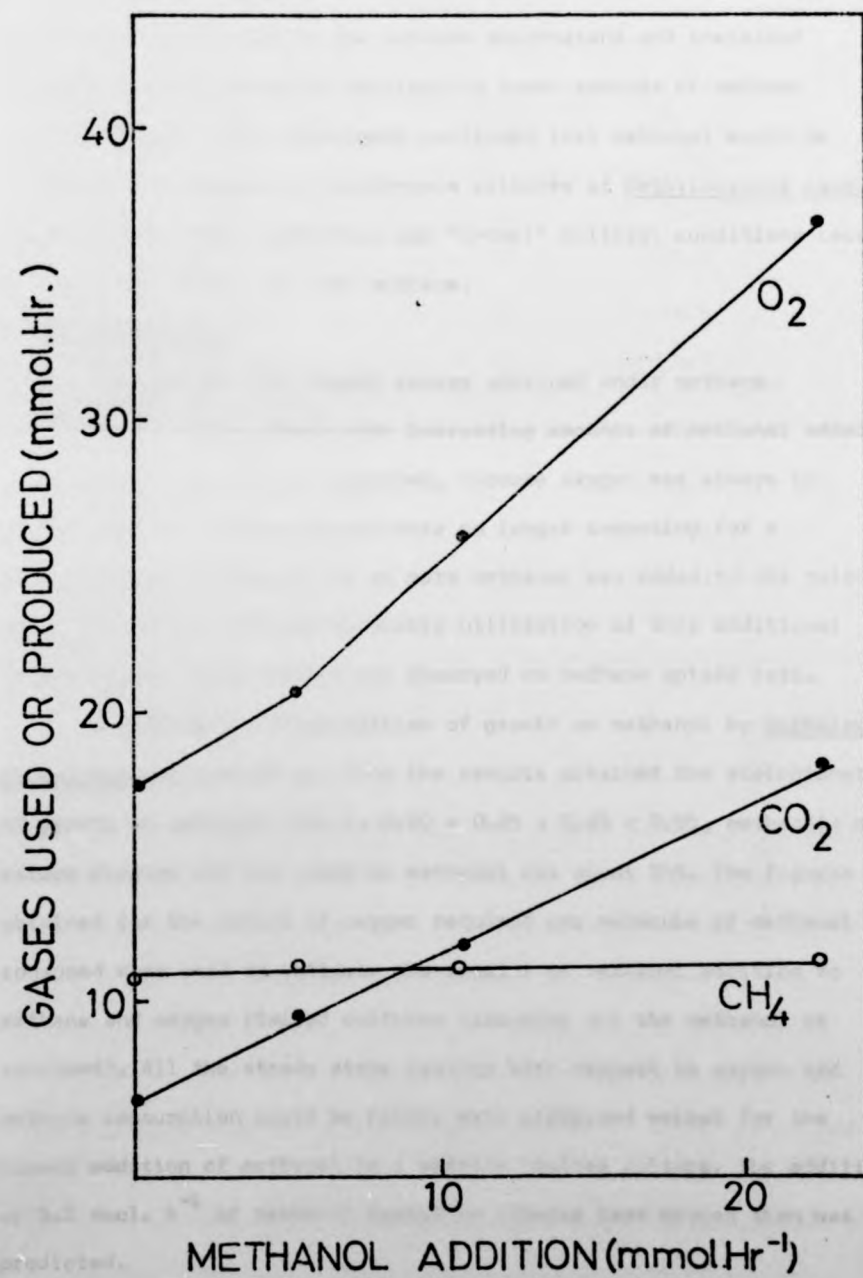
Fig. 5.3. Effect of methanol addition to a methane limited

culture of Methylococcus capsulatus (Bath)

the inflow medium of 100mM. As was expected all the methanol was utilised;
none could be detected in the culture supernatant and increased
concentrations of methanol resulted in lower amounts of methane
being consumed. This experiment confirmed that methanol would be
unlikely to accumulate in continuous cultures of Methylococcus capsulatus
(Bath) under oxygen limitation and "normal" cultural conditions because
of its preferential use over methane.

b) METHANE-LIMITED

The results from steady states obtained under methane-
(or carbon) limited growth with increasing amounts of methanol added
are shown in Fig. 5.3. As expected, because oxygen was always in
excess, the two carbon sources were no longer competing for a
limited supply of oxygen. So as more methanol was added to the culture
extra oxygen was consumed to enable utilisation of this additional
carbon source and no effect was observed on methane uptake rate.

A preliminary investigation of growth on methanol by Methylococcus
capsulatus was undertaken. From the results obtained the stoichiometries
of growth on methanol were 1: 0.80 - 0.85 : 0.45 - 0.55, methanol: oxygen :
carbon dioxide and the yield on methanol was about 35%. The figures
obtained for the amount of oxygen required per molecule of methanol
consumed were used to estimate the results of methanol addition to
methane and oxygen limited cultures (assuming all the methanol is
consumed). All the steady state results with respect to oxygen and
methane consumption could be fairly well predicted except for the
lowest addition of methanol to a methane limited culture. The addition
of 5.2 mmol. h^{-1} of methanol seemed to require less oxygen than was
predicted.

Table 5.1. shows the yield with respect to oxygen and the

percentage of carbon entering the cells.

LIMITATION	METHANOL ADDITION mmol.h^{-1}	% CARBON INTO CELLS	YO$_2$ (%)
CH$_4$	0	41	19.2
	5.2	38	22.9
	10.6	38	24.3
	22.5	37	25.4
O$_2$	0	28	14.2
	5.1	30.7	16.2
	11.2	30.6	17.3
	25.6	31.7	19.8

TABLE 5.1. Effect of methanol addition to a methane grown culture of Methylococcus capsulatus

As would be expected because growth on methanol is more efficient with respect to oxygen utilisation the yield on oxygen increases as more methanol is added to both oxygen and methane limited cultures. Also because methanol was utilised more efficiently than methane it was expected that as the methanol concentration was increased so the percentage of carbon entering the cells should also increase. This was only true in the oxygen limited culture; in the methane limited culture the percentage of carbon entering the cells decreased as the amount of methanol was increased, an unexpected result. A possible explanation for this result was that the carbon recovery decreased as the methanol increased (from 101% for methane alone to 89% for 22.5 mmol.h^{-1} methanol) this would indicate that some extracellular

material was being produced. Unfortunately no assays of the
culture supernatant were undertaken.

LIMITATION OF CULTURE	[HCOOH] IN MEDIA (mM)	$O.D_{540}$	VOL. OF GASES USED OR PRODUCED ml/min.		
			O_2	CH_4	CO_2
CH_4	0	3.16	7.05	5.11	2.95
	20	3.14	7.40	5.22	4.32
O_2	0	7.44	25.94	18.39	11.37
	22	6.72	25.57	17.33	12.97

Table 5.2. Effect of formate additions to oxygen and methane limited

cultures of Methylococcus capsulatus (Bath)

2) The Effect of Formate on a Continuous Culture of _Methylococcus capsulatus_

growing on Methane

The production and utilisation of reducing power was discussed in the introduction to this thesis. At the time of these experiments there was a possibility that cells grown on methane would be short of reducing power, especially $NADH_2$, hence the postulation of reverse electron flow by van Dijken & Harder(1975) to provide this necessary $NADH_2$ (for the methane mono-oxygenase). To test whether _Methylococcus capsulatus_ was lacking in $NADH_2$, formate was added to cultures growing on methane. The formate could be oxidised to carbon dioxide by formate dehydrogenase with the consequent production of $NADH_2$; this extra $NADH_2$ allowing more of the methane being metabolised to be fixed at the formaldehyde step instead of being oxidised through to carbon dioxide to provide reducing power.

About 20 mM formate was added to both oxygen and methane limited cultures of _Methylococcus capsulatus_ and steady states obtained; the results are shown in Table 52. As can be seen from the optical density readings, before and after formate was added, there was no increase in yield although more carbon was being consumed. Although the culture supernatant was not assayed for formate, the increased carbon dioxide production confirmed that the formate was being oxidised. The increased carbon dioxide in the methane limited culture accounted for 95% of the added formate, and 85% in the oxygen limited culture. In both cases it appeared that additional oxygen was required as terminal electron acceptor for the extra reducing power produced. In the methane limited

culture more oxygen was used and in the oxygen limited culture less oxygen was therefore available for methane oxidation and growth, with a consequent reduction in the amount of methane used and a drop in the yield.

These results indicated that Methylococcus capsulatus growing on methane was not suffering from a lack of reducing power, as the production of additional $NADH_2$ had no effect on the yield. In the light of recent findings on the pathway of C_1 metabolism this result is not particularly surprising. D. Stirling (pers.comm) has found an NAD linked formaldehyde dehydrogenase in the strain of Methylococcus used for this study, thus enabling two $NADH_2$ to be produced per molecule of carbon dioxide produced from methane. Although the methane mono-oxygenase from Methylococcus capsulatus does appear to require NADH, (Colby and Dalton, 1976) other methanotrophs may use a different electron donor as has been postulated for Methylosinus trichosporium (Tonge et al 1977). This organism can apparently use electrons generated from methanol oxidation to drive methane oxidation. Although this purified system did not require $NADH_2$ this might not be the case in whole cells.

Also when this organism is grown on excess ammonium ions, some of the ammonium ions are oxidised and nitrite and nitrate accumulate in the culture. This oxidation is a reducing power requiring process and any organism lacking in reducing power is unlikely to undertake this apparently pointless oxidation. Some methanotrophs accumulate poly β hydroxy-butyrate which, again, would be unlikely to occur in organisms lack reducing power.

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The results from formate additions might not be as clear cut as first seemed, because D.E.F Harrison (personal communication) has added formate to methylotrophs growing on methanol and again no increase in yield was observed. In fact as the formate concentration was increased the yield started to fall.

The results obtained from formate addition to the batch culture system were not reproducible. The addition of formate to a final concentration of 0.1% gave an initial increase in the rate of carbon dioxide production and methane consumption but the rates returned to those before the addition after 1½ hours. The addition of formate to a final concentration of 0.05% had the effect of reducing carbon dioxide production rate and had no discernable effect on methane uptake. In both cases the effect on the rate of oxygen uptake (the limiting substrate) was minimal and only a slight "hiccup" was noted on the rate when the formate was added.

3) Effect of Acetylene and Ethylene on Methane and Methanol Utilising

Methylococcus capsulatus

Methylotrophs can utilise many different sources of
nitrogen (Whittenbury et al, 1970) including nitrate ions, ammonium
ions and dinitrogen. To define a steady state obtained in continuous
culture it is necessary to know what nitrogen source is being used.
If the added nitrogen (nitrate or ammonium) was completely consumed
then the culture was likely to start fixing dinitrogen from the gas
phase. The enzyme which reduces dinitrogen to ammonia, nitrogenase, is under
genetic control in that when an organism has no need for a nitrogenase,
none of it is synthesised. However to be sure that a culture is fixing
dinitrogen it is necessary to show N_2^{15} incorporation into cellular
material. This is a tedious procedure and it has been found that
another means of establishing dinitrogen fixation was to assay for the
nitrogenase using the acetylene reduction test, (Dilworth, 1966).
This assay measures the capacity of the nitrogenase to reduce acetylene
to ethylene if it is provided with a supply of energy.

It was realised that some methylotrophs could probably fix
dinitrogen by their ability to grow on nitrogen free media, but little
success was achieved using the acetylene reduction test. Whittenbury
et al (1970) screened all their strains for acetylene reduction in
ammonium starved cultures, but could only find one organism,
Methylosinus trichosporium PG, which would actively reduce acetylene.

The continuous cultures used in this study sometimes consumed
all the added nitrogen source, so it was necessary to determine if
these cultures were fixing dinitrogen. It was believed that a quick
test would be to introduce acetylene into the inflowing gas stream
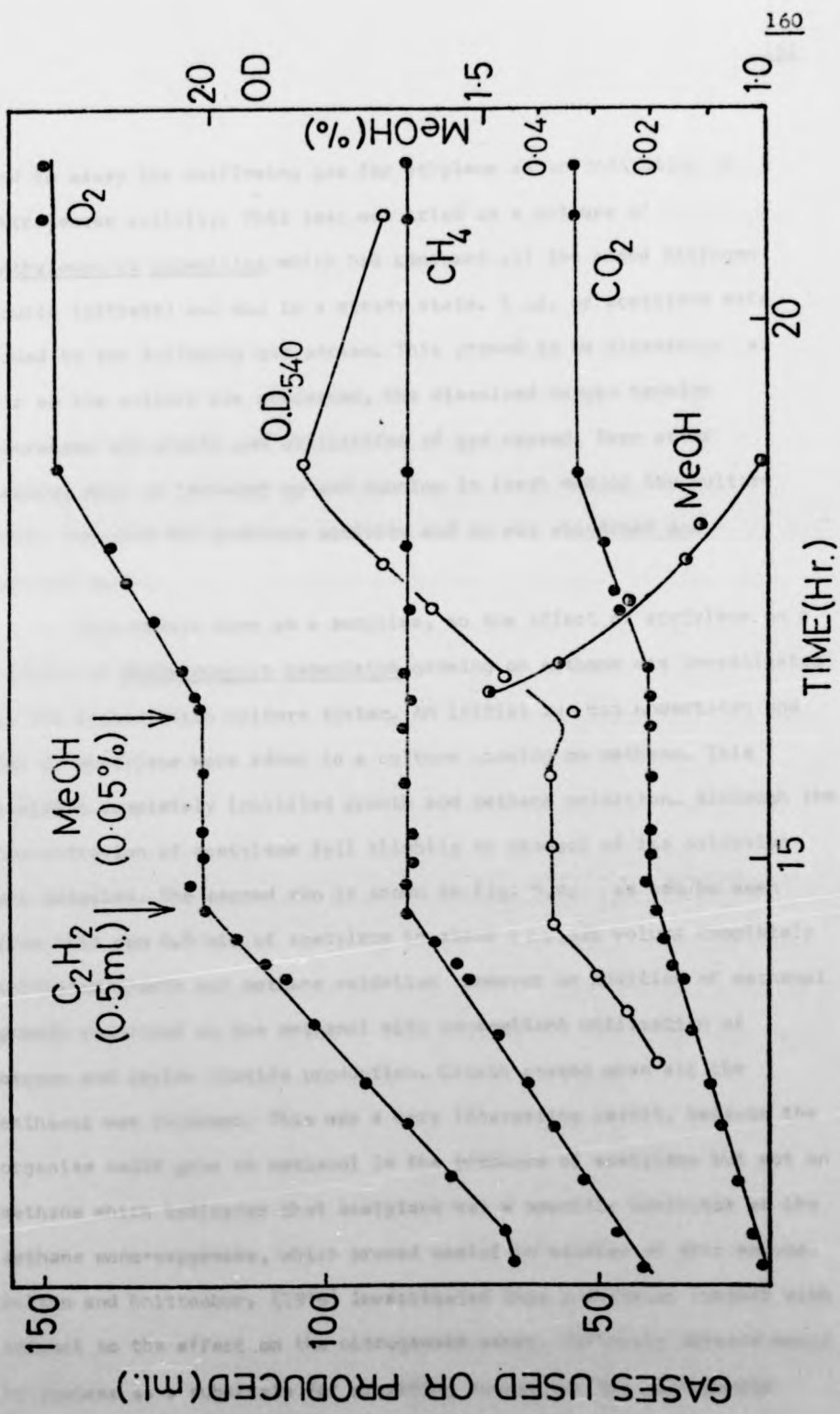
Fig. 5.4. Effect of adding acetylene and thanmethanol to a culture of *Methylococcus capsulatus* (Bath) growing on methane.

and to assay the outflowing gas for ethylene as an indication of nitrogenase activity. This test was tried on a culture of Methylococcus capsulatus which had consumed all the added nitrogen source (nitrate) and was in a steady state. 5 ml. of acetylene were added to the inflowing gas stream. This proved to be disastrous as far as the culture was concerned, the dissolved oxygen tension increased and growth and utilisation of gas ceased. Even after several days of batching up and running in fresh medium the culture never regained its previous activity and so was abandoned and started again.

This result came as a surprise, so the effect of acetylene on a culture of Methylococcus capsulatus growing on methane was investigated in the closed batch culture system. An initial run was undertaken and 3ml of acetylene were added to a culture growing on methane. This addition completely inhibited growth and methane oxidation. Although the concentration of acetylene fell slightly no product of its oxidation was detected. The second run is shown in Fig. 5.4. as can be seen from this run 0.5 ml. of acetylene in about 1 L. gas volume completely inhibited growth and methane oxidation however on addition of methanol growth continued on the methanol with concomitant utilisation of oxygen and carbon dioxide production. Growth ceased when all the methanol was consumed. This was a very interesting result, because the organism could grow on methanol in the presence of acetylene but not on methane which indicated that acetylene was a specific inhibitor of the methane mono-oxygenase, which proved useful in studies of this enzyme. Dalton and Whittenbury (1976) investigated this phenomenon further with respect to the effect on the nitrogenase assay. Obviously methane would be useless as a substrate for providing energy for the nitrogenase

activity and this led the above workers to look for other compounds
which could be used by the cell to provide energy for the nitrogenase.
They found several compounds that could be used in the nitrogenase
assay in whole cells e.g. methanol, formate, hydrogen etc.

De Bont et al (1974) had postulated that negative results in
the acetylene reduction test on methane grown methanotrophs was
because   the ethylene produced was oxidised by the
methanotroph.   (N_2^{15} incorporation was positive). However, from this
study, it is likely that it was the inhibition of the methane
mono-oxygenase by the acetylene that prevented positive results being
obtained, as the same organism, grown on methanol, would give positive
results for nitrogenase activity.

To complete this study of the effect of the substrate and product
of the acetylene reduction test on methane grown cells, cultures growing
on methane had 30 ml. and 3 ml. of ethylene added to them. The 30 ml.
addition (3% final concentration) completely inhibited growth and
methane utilisation whereas 3 ml of ethylene (0.3% final concentration)
gave only a 50% inhibition of the gas utilisation rates. In these cases
a product of ethylene oxidation was detected in liquid samples injected
into the flame ionisation detector chromatograph, this product was later
identified as ethylene oxide by Colby and Dalton (1977).
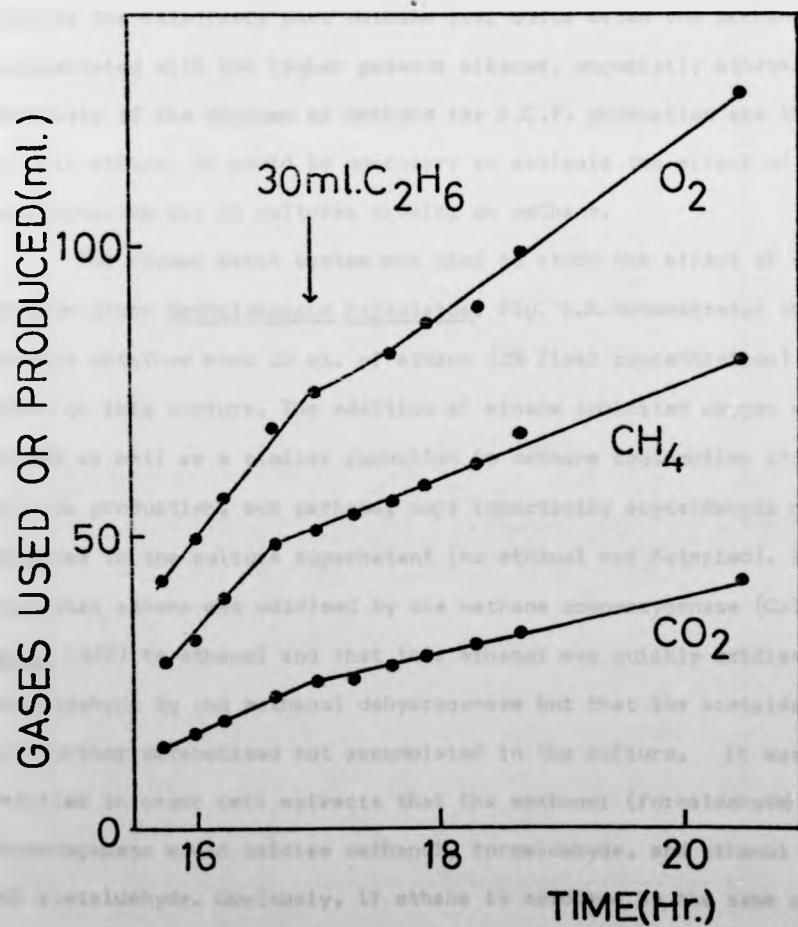
Fig. 5.5. Effect of ethane on a batch culture of

<u>Methylococcus</u> <u>capsulatus</u> (Bath) growing on

methane.

4) The Effect of Ethane and Ethanol on Methane Utilising Methylococcus capsulatus

Methane as a carbon source for S.C.P. production could be obtained
from anaerobic fermentation of sewage, oil wells and natural gas fields,
the latter two probably the most plentiful supply at the present time.
Unfortunately some   sources of methane (other than sewage) do not
provide the relatively pure methane gas. Quite often the methane is
contaminated with the higher gaseous alkanes, especially ethane.
Obviously if the sources of methane for S.C.P. production are likely to
contain ethane, it would be necessary to estimate the effect of this
contaminating gas on cultures growing on methane.

The closed batch system was used to study the effect of ethane on
methane grown Methylococcus capsulatus. Fig. 5.5. demonstrates the
results obtained when 30 ml. of ethane (3% final concentration) were
added to this culture. The addition of ethane inhibited oxygen utilisation
by 50% as well as a similar reduction in methane consumption and carbon
dioxide production, but perhaps, more importantly acetaldehyde was
detected in the culture supernatant (no ethanol was detected). It would
seem that ethane was oxidised by the methane mono-oxygenase (Colby
et al 1977) to ethanol and that this ethanol was quickly oxidised to
acetaldehyde by the methanol dehydrogenase but that the acetaldehyde was
not further metabolised but accumulated in the culture.   It was later
verified in crude cell extracts that the methanol (formaldehyde)
dehydrogenase would oxidise methanol, formaldehyde, and ethanol but
not acetaldehyde. Obviously, if ethane is oxidised by the same enzyme as
the methane, competition would occur at the active site. The results
showed that ethane not only inhibited methane oxidation but also the
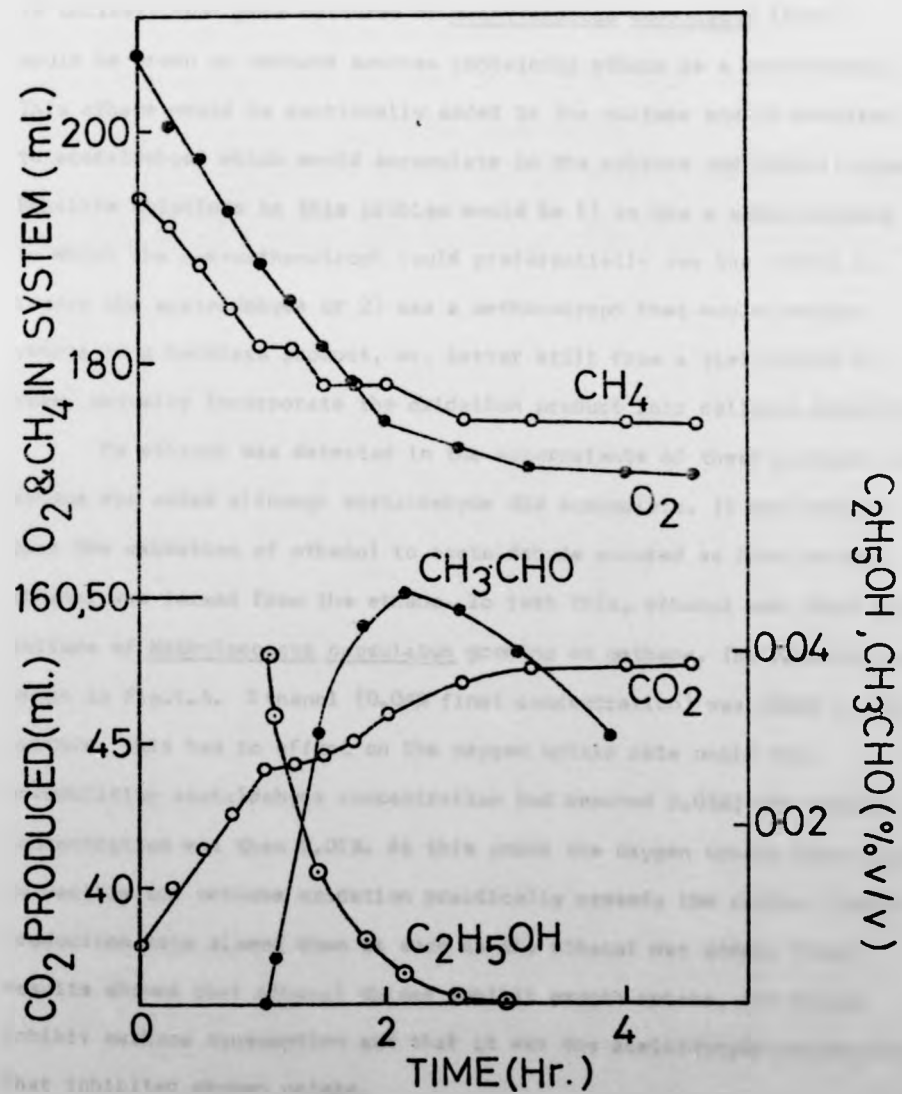
Fig. 5.6. Effect of ethanol on a batch culture of Methylococcus capsulatus (Bath) growing on methane.

oxygen uptake rate indicating that the ethane was blocking the active site to a certain extent, probably being metabolised more slowly than methane. The accumulation of acetaldehyde, a potent inhibitor of bacterial growth, might also reduce the gas utilisation rates. So it is unlikely that pure cultures of Methylococcus capsulatus (Bath) could be grown on methane sources containing ethane as a contaminant. This ethane would be continually added to the culture and be oxidised to acetaldehyde which would accumulate in the culture and inhibit growth. Possible solutions to this problem would be 1) to use a mixed culture in which the non-methanotroph could preferentially use the ethane or remove the acetaldehyde or 2) use a methanotroph that would oxidise ethane to a harmless product, or, better still from a yield point of view, actually incorporate the oxidation product into cellular material.

No ethanol was detected in the supernatants of these cultures when ethane was added although acetaldehyde did accumulate. It was assumed that the oxidation of ethanol to acetaldehyde occured as fast as the ethanol was formed from the ethane. To test this, ethanol was added to a culture of Methylococcus capsulatus growing on methane. The results are shown in Fig.5.6. Ethanol (0.04% final concentration) was added to the culture, this had no effect on the oxygen uptake rate until the accumulating acetaldehyde concentration had reached 0.03%; the ethanol concentration was then 0.01%. At this point the oxygen uptake rate started to decline and methane oxidation practically ceased; the carbon dioxide production rate slowed down as soon as the ethanol was added. These results showed that ethanol did not inhibit oxygen uptake, but it did inhibit methane consumption and that it was the acetaldehyde accumulation that inhibited oxygen uptake.

An interesting observation during this experiment was that
after the ethanol was added a small amount of methanol was detected
in the culture supernatant, and that this remained for about 1 hour.
At the time of this experiment the mechanism of methane mono-oxygenase
was not fully established and this accumulation of methanol was an
indication that methanol may very well be an intermediate in the pathway
of methane oxidation as has been shown subsequently.

6. GROWTH OF OTHER METHANOTROPHS

Although Methylococcus capsulatus was the organism studied
in detail for this thesis, two other organisms were grown up in
the closed batch and continuous culture systems and one other
organism in the closed batch system. Methylosinus trichosporium (OB3b),
Methylomonas albus (BG8) and Pseudomonas methanica (P.M.) were
grown up in the closed batch system at 30°C. Only Pseudomonas
methanica grew as well as Methylococcus capsulatus the other two
organisms requiring in excess of 60 hours for completion of a growth
cycle.

The results from the closed batch system with respect to final
O.D readings and volumes of gases used or produced were not particularly
reproducible and so few conclusions can be drawn from these results.
However table 6.1 shows the stoichiometries obtained with the four
organisms studied.

ORGANISM	STOICHIOMETRY		
	O_2 :	CH_4 :	CO_2
Methylococcus capsulatus	1.33 - 1.41 :	1 :	0.39 - 0.48
Methylosinus trichosporium	1.42 :	1 :	0.42
Methylomonas albus	1.1 - 1.3 :	1 :	0.30 - 0.37
Pseudomonas methanica	1.37 :	1 :	0.38

Table 6.1. The stoichiometries of growth of four species of
methanotrophs in the closed batch culture system.

The results for _Methylococcus capsulatus_ appear to show that less oxygen is used or carbon dioxide produced per molecule of methane in the batch system as opposed to the continuous culture system. This could be because of methane being lost from the system, but the rate of methane lost would not have been significant. The loss of carbon dioxide (which disappeared the fastest in control runs) could account for some if not all of the lower value obtained. As can be seen from Table 6.1 the results are fairly similar, except for one difference, that is the stoichiometry for oxygen from _Methylomas albus_ (1.1 - 1.3) which is lower than for the other organisms.

Two attempts were made to grow _Methylomonas albus_ in continuous culture and one attempt at _Methylosinus trichosporium_ (terminated prematurely because of accidental running in of the pH addition and killing the culture). The batch growth of these organisms was slow and no success could be achieved at obtaining steady states at a dilution rate of 0.05; the cultures always washed out.

SUMMARY AND CONCLUSION

The results presented in this thesis have provided an understanding of gas limited growth as well as some insight into the physiology of the methanotroph Methylococcus capsulatus (strain Bath). These results will be summed up in the order that they were presented in the results and discussion section unless results from different sections are drawn together; these will be discussed under the appropriate heading.

Gas Limited Growth

A continuous culture based on gas-limited growth can neither be described as a chemostat nor a turbidostat, and required a new concept to understand the different parameters involved, although most of the established equations of bacterial growth could still be applied. In some respects the theory of gas limited growth (providing a limiting nutrient at a constant rate despite changes in dilution rate) can be applied to light limited cultures of phototrophs, although quantification of the amount of limiting light available to a culture (considering ambient light and density of culture) is a much more complex task than estimations of gas utilisation rates.

It is essential for any continuous culture that the limiting nutrient should be known. This was defined in cultures of Methylococcus capsulatus (Bath) by keeping the total rate of supply of gas constant but varying the partial pressures of the individual gases in the mixture. The results from this experiment showed that oxygen was the limiting nutrient down to partial pressures of oxygen of 0.15 whereas partial pressures of 0.02 and 0.04 for methane gave methane limited cultures (this was for methane/air mixtures).

The dilution rate curve (D against x) for a gas limited culture
was completely different from that obtained from chemostat cultures.
The curve itself, other than providing evidence that the limiting
nutrient was being supplied at a constant rate, could not give
much information as regards the efficiency of growth. If all the
parameters remained constant over a wide range of dilution rates
then a plot of $1/D$ against x could yield useful information about
the culture. However, although most of the parameters could be kept
constant, the effect of increasing the dilution rate was to decrease
the density of the culture. So two parameters were varying and this
had consequences on the gas utilisation rates, in that less dense
cultures could not use as much limiting gas as denser cultures. This
was demonstrated by the results obtained from the dilution rate
experiments as well as from the experiment of increasing the amount
of oxygen available to the culture by changing from an oxygen to a
methane limited culture. These experiments showed that denser cultures
could use more of the limiting gas; at higher dilution rates (lower
cell densities)gas utilisation rates were lower and as the density
of culture declined in the methane limited culture the rate of gas
utilisation also decreased.

The inability of less dense cultures to use as much gas as the
denser cultures meant that (if the yield is constant) the productivity
of the system would decline with increasing dilution rate because of
lower gas utilisation rates and not because of a fall in the yield.
Organisms often become more efficient at higher dilution rates and so
two opposing factors are having an effect on the productivity of the
system. The plot $1/D$ against x   is not capable of distinguishing

between these two factors and interpretations from this curve are difficult. However a plot of $1/D$ against X/g does overcome this problem as the X/g figure takes into consideration the amount of limiting gas being used, so that the curve can now reflect unambiguously any changes in Y which might be occuring.

The results showed (Fig 1.7) that the manipulation of the results by the above procedure did provide a useful diagrammatic way of expressing the results from a dilution rate curve. From these curves and the actual yields obtained it appears that Methylococcus capsulatus (Bath) does increase in efficiency of substrate utilisation (oxygen and methane) as the growth rate is increased. The increase was about 10% for an increase in dilution rate from 0.055 to $0.13.hr^{-1}$ of a nitrate grown culture and about 22% for an increase in dilution rate from 0.055 to $0.26hr^{-1}$ for an ammonium grown culture. The ammonium grown culture did not show very much increase in efficiency until the dilution rate was increased above 0.15 hr.$^{-1}$.

The Batch Culture

The batch culture did not provide very much useful information on yields as the results obtained were inconsistent, but it was an extremely useful tool for studying inhibition effects and co-oxidation phenomena.. The batch growth curve for Methylococcus capsulatus was similar to the exponential growth curves obtained for other organisms but it differed in one major aspect. The period of exponential growth only lasted a short time and gas limited growth occured for most of growth cycle. The effect of gas limited growth was to give a period of almost linear growth. The point to emerge

from this study was that these gas limited batch cultures did not appear to differ much from the 'normal' batch growth curves on first inspection and on some occasions linear and semi-log plots would both give reasonable straight lines although the culture was obviously in a linear growth phase.

Three other organisms were studied in the batch system, Pseudomonas methanica, methylosinus trichosporium and Methylomonas albus all grown at $30^{\circ}C$. Only Pseudomonas methanica grew as well as Methylococcus capsulatus (Bath) and completed its growth cycle in 40 hours whereas the growth cycles for the other organisms took in excess of 60 hours. The stoichiometries of growth for all four organisms were fairly similar except that Methylomonas albus seemed to require less oxygen per mole of methane consumed than did the other organisms.

Growth of Methylococcus capsulatus in Continuous Culture

Methylococcus capsulatus (Bath) proved to be a good organism to grow in continuous culture, as not much difficulty was encountered in growing the organism. It produced very little if any foam and no wall growth occured. It had a broad range of growth temperatures ($33^{\circ}C - 50^{\circ}C$) and the yield seemed to be fairly constant between $35^{\circ}C$ and $48^{\circ}C$; temperatures outside this range resulted in lower yields being obtained. The maximum growth temperature was $50^{\circ}C$; no growth or carbon dioxide production could be detected at $52^{\circ}C$ and $33^{\circ}C$ was the lowest temperature at which steady states could be obtained ($D = 0.05 \ h^{-1}$).

The rates of gas utilisation were proportional to the partial pressure of the limiting gas in the incoming (and outflowing) gas phase.

The K_La for oxygen appeared to be 17% higher than for methane, although this lower value for methane could have been because of the low partial pressures of methane used (0.02 and 0.04). Higher partial pressures of methane may give higher K_La values if the K_s value for methane is relatively high compared to that for oxygen.

Although Methylococcus capsulatus requires oxygen for growth, on occasions it appeared that the culture did not relish sudden exposure to high dissolved oxygen tension (D.O.T) and responded by completely oxidising methane to carbon dioxide and consuming two molecules of oxygen for every molecule of methane used. This result was seen as an effort by the culture to reduce the D.O.T. by utilising as much oxygen as possible. This effect would not be desirable in S.C.P. production where changes in D.O.T. might occur in large industrial fermenters. Another interesting feature of this organism is that its capability of utilising the limiting substrate (oxygen) was about twice the actual rate at which it was using it.

When gas limited continuous cultures are being run care should be exercised about using antifoam agents, as the addition of these compounds can reduce the gas (oxygen) transfer rates and thus alter steady state values.

As an organism for S.C.P. production Methylococcus capsulatus does have several features in its favour: high growth temperature, reasonable tolerance to pH changes, not very much lysis in cultures grown for this study & can be pelleted by centrifugation fairly easily. One interesting possibility of concentrating these cells would be to cause the culture to foam and then collect the foam. The reason for this

is that on one occasion when the culture foamed, the foam that
had collected and subsided in the liquid trap on the outlet line
had concentrated the culture approximately ten fold, whether or not
this stripping out of the cells could be utilised in harvesting
the cells is unknown. Against these advantages are the possible
adverse effects of changes in D.O.T. and the yields obtained which
will be compared to other published data.

The yields on methane reported in the literature vary from
0.3 - 1.11 obviously the conditions of culturing and strain
differences will have much to do with this wide variation however
it is possible that the many difficulties involved in obtaining
reliable values for the rates of gas utilisation or production
might also account for some of the variations.

The results from this thesis have shown that several readings
are required before some idea of the actual rates of gas utilisation
and production can be obtained. At slow gas flow rates the pH additions
can have considerable effect on the amount of carbon dioxide
apparently being produced and to obtain a true value readings have
to be taken over 3 or 4 pH additions.

The highest yield obtained during this study was 0.65g.dry
weight/g. methane consumed. This value appears to be on the low side
comparing it to reported yield values from other organisms. So for S.C.P.
production this organism might be at a disadvantage as far as yields
are concerned, although complete optimisation of conditions has not
been accomplished. Of course, perhaps the most important factor will
be the quality of the product, but this consideration was beyond the
scope of this study.

If this organism were used for S.C.P. production, what nitrogen source should be used ? From the results on the different nitrogen sources it would seem that ammonium would give the highest yield with respect to both methane and oxygen if the optimum conditions are employed. But the amount of ammonium added will have to be tightly controlled to prevent excess ammonium in the supernatant which could cause problems with nitrite accumulation or inhibition of methane oxidation as well as a fall in the yield with respect to oxygen. Probably the best way to grow this organism would be ammonium "limited" so that part of cells nitrogen came from dinitrogen, this might lower the yield slightly but would remove the complications of having excess ammonium in the culture.

The Effect of Metabolisable Growth and Non - Growth Substrates

Methanol and Formate

The effect of metabolisable substrates was interesting from several points of view perhaps a metabolisable substrate (other than methane) might increase the yield, or inhibit growth or cause another compound to accumulate in the supernatant. The reports of Harwood and Pirt (1972) and Wilkinson et al (1974) of methanol accumulation in cultures of methanotrophs growing on methane was disturbing from a yield point of view as well as the possible inhibitery effect of the metabolite (Wilkinson et al, 1974). Culture supernatants of Methylococcus capsulatus (Bath) when analysed never showed any significant accumulation of this product. However it was interesting to know what effect methanol would have on a culture grown on methane. This was initially done in the closed

batch system. The results from this experiment showed that methanol inhibited methane uptake but did not inhibit growth; it seemed that the methanol was used in preference to the methane. The reason for this is the high concentration of methanol in the culture as opposed to the sparingly soluble gas, and indeed if this methanol could be oxidised by the methane mono-oxygenase then the inhibition of methane uptake would be caused by competitive inhibition on the methane mono-oxygenase.

The accumulation of methanol in cultures of Methylococcus capsulatus (Bath) was unlikely because of its preferential use over the methane. To check this out increasing concentrations of methanol (in the medium)   added to both oxygen and methane limited cultures of this organism growing on methane. As was expected in the oxygen limited culture the methanol was used in preference to the methane; the amount of methane utilised decreased as the concentration of methanol was increased. Whereas, again as expected, in the methane limited culture (excess oxygen) no decrease in methane uptake was noted but now more oxygen was consumed to accomodate utilisation and growth on the added methanol. In this situation the two carbon sources are not competing for a limiting amount of oxygen.

The stoichiometries of methanol utilisation were interesting in that they were not what would be expected from studying the results from growth on methane. The stoichiometry of oxygen utilisation for methane grown cells is 1.45 - 1.6 oxygen : 1 methane, as one mole of oxygen is required to oxidise 1 mole of methane to methanol then 0.45 - 0.6 moles of oxygen are required for growth and utilisation of this methanol. So it was expected that growth on methanol would require between 0.45 - 0.6 moles of oxygen per mole of methanol,

however the figures obtained were about 0.8. This indicated that more oxygen was being used as terminal electron acceptor than was expected. Results since then (Colby et al , 1977) have shown that the methane mono-oxygenase can oxidise methanol. This could explain the higher oxygen requirement than was expected, in that some of the methanol might be oxidised by the methane mono-oxygenase (which has since been shown to be present in methanol grown cultures). The results of Whittenbury et al (1970 b) of stoichiometries of methanol utilisation 1.1 oxygen : 1 methanol could be explained by this phenomenon, and also the low yields which they obtained on this substrate. Indeed cultures grown on methanol in the closed batch system have shown similar results.

A culture was grown up on three aliquots of methanol (2.5 mmol. each addition), the first addition was used to start the culture growing, the other two additions were not made until all the previous addition had been utilised. The results are shown in Table 2.

Methanol added (mmole.)	Amount of gases used or produced. (mmole.)		$O.D_{540}$ (after all methanol consumed)	Stoichiometry O_2 : MEOH: CO_2
	O_2	CO_2		
2.5	1.62	0.95	0.76	0.65 : 1 : 0.38
2.5	2.15	1.02	1.42	0.86 : 1 : 0.41
2.5	2.27	1.00	1.92	0.91 : 1 : 0.40

Table.3.   Growth of Methylococcus capsulatus on methanol

This table shows the amount of gases used or produced for each aliquot of methanol added. There was no difference between the amounts of

carbon dioxide produced, but as the culture became denser more
oxygen was consumed per aliquot of methanol from 0.65 to 0.91
moles of oxygen per mole of methanol. It is difficult to explain
why a sparse culture should utilise less oxygen per mole of methanol
than a denser culture because it would be expected that the ratio
of any enzyme activities would be the same (perhaps not so true for
the first aliquot when the cells were used as an inoculum) so the
pathways of methanol metabolism should be similar and therefore
utilise similar amounts of oxygen. The results do show that the
denser cultures used more oxygen per aliquot of methanol presumably
because some of the methanol was being oxidised by the methane
mono-oxygenase. This did not seem to have much effect on the amount
of biomass produced, the second aliquot giving a similar increase in
O.D. as the first. However the increase in O.D. from the final aliquot
was lower than the first two. Another possible explanation of this
apparent higher oxygen requirement for growth on methanol could be
that growth on methanol requires more oxygen as terminal electron
acceptor than for growth on methane.

This apparent oxidation of methanol by the methane mono-oxygenase
would indicate that there is "spare" $NADH_2$ in the cells grown on
methanol, but what about cells grown on methane ? Van Dijken and
Harder (1975) have postulated that Methylococcus capsulatus must have
reverse electron flow to provide the necessary $NADH_2$ to drive the
methane mono-oxygenase. At that time the NAD dependent formaldehyde
dehydrogenase (D.Stirling pers. comm.) had not been discovered in this
organism, so a possible test to determine if this organism was
lacking $NADH_2$ was to add formate to cultures grown on methane.
This formate should be oxidised to carbon dioxide with the concomitant

production of $NADH_2$, thus relieving the requirement for reverse
electron flow and should allow more of the methane entering the
cells metabolism to be channelled into cellular material as less
should now have to be oxidised to carbon dioxide to provide
sufficient reducing power ($NADH_2$) for the cells metabolism.

The results from these experiments have been contradictory;
results present in this thesis from both oxygen and methane limited cultures
(nitrate) of _Methylococcus capsulatus_ (Bath) indicated that the addition
of 20 mM formate had no beneficial effect on yields and if anything
lowered the productivity because the formate oxidation appeared to consume
more oxygen, presumably as a terminal electron acceptor. This result
indicated that oxidation of formate with production of $NADH_2$ was of no
benefit to cells. However a preliminary study on the addition of formate
to ammonium grown cells showed an increase in O.D. on addition of the
formate; no satisfactory gas utilisation rates were obtained from this
experiment. Also Reed (1976) has shown that formate additions did
increase yield.

Acetylene and Ethylene

An understanding of the effect of acetylene and ethylene on
cultures of _Methylococcus capsulatus_ (Bath) was necessary because
of the involvement of these two compounds in the acetylene reduction
test for nitrogenase activity. The most striking result from these
experiments was that acetylene was a potent inhibitor of methane
oxidation but that concentrations of acetylene that completely
inhibited methane oxidation did not affect growth on methanol,
indicating that acetylene was a specific inhibitor of the first step
of methane oxidation i.e. methane to methanol by the methane mono-
oxygenase. This finding has proved useful in oxidation studies to

determine whether or not the methane mono-oxygenase was involved
in a particular oxidation when crude extracts were used (Colby et al
1977). This inhibition of the methane oxidation by acetylene explains
the negative results from the acetylene reduction test performed
on methanotrophic cultures which were apparently fixing dinitrogen;
these cultures had only methane as a source of electrons which
would be required by the nitrogenase for activity. This problem
was solved by Dalton & Whittenbury (1976) who used electron donors
other than methane e.g. methanol, formate etc.

Ethylene, the product of the acetylene reduction test,
inhibited methane (50 % inhibition by ·3%ethylene)and was also
oxidised by Methylococcus capsulatus (Bath) to ethylene oxide. This
oxidation of ethylene was put forward by de Bont and Mulder (1974)
as a reason for the negative results from the acetylene reduction
test, but was later retracted by them (1976) when they found that
acetylene, besides inhibiting methane oxidation, also inhibited
ethylene oxidation. The methane mono-oxygenase from Methylococcus
capsulatus (Bath) has been shown to oxidise ethylene to ethylene
oxide by Colby et al (1977)

Ethane and Ethanol

Some natural sources of methane contain contaminating amounts
of ethane and so it was important to determine the effect of ethane
on cultures of Methylococcus capsulatus grown on methane. The
results from these experiments were not very promising from an
S.C.P. production stand point as the ethane inhibited methane oxidation
and the product of ethane oxidation, acetaldehyde, accumulated in
the culture. No ethanol was detected in the culture supernatant.
Ethane is presumeably oxidised via the methane mono-oxygen (Colby
and Dalton, 1977) to ethanol and this ethanol must be immediately
oxidised to acetaldehyde. To verify that ethanol was converted to

acetaldehyde, ethanol was added to a culture of Methylococcus capsulatus growing on methane. The ethanol did inhibit the oxidation of methane but it was probably the acetaldehyde that inhibited oxygen uptake; the ethanol was rapidly converted to acetaldehyde. An interesting observation during this experiment was the accumulation of a low concentration of methanol on addition of the ethanol; this methanol was presumably formed from oxidation of methane to methanol which was probably competitively inhibited by the ethanol on the active site of the methanol (alcohol) dehydrogenase. This accumulation was a further indication that methane was oxidised to methanol and that it was the probable pathway of methane metabolism.

This effect of ethane on methane grown cells is a problem which will have to be overcome if sources of methane with contaminating ethane are to be used. Two possibilities of overcoming this problem would be to use a methanotroph that can convert ethane to a non-toxic product or use a mixed culture, the non-methanotroph utilising ethane or products of ethane oxidation.

A Final Comment

It is possible to produce S.C.P. from methane, but whether or not the economy of the process will enable this means of S.C.P. production to become a commercial proposition will be determined by the relative market prices of methane and other high protein food sources (e.g. soya bean and fish meal). Methylococcus capsulatus has some properties in its favour as a source of S.C.P. (high growth temperature, few problems of cultivation). However its yield, which appears to be lower than published data for other

organisms (although optimisation has not been completed) and
its response to ethane are possible adverse factors for an organism
which is to be used for S.C.P. production.

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APPENDIX 1

CALIBRATION OF KATHAROMETER

The gas analysis technique was not satisfactory when this study was begun for two reasons. 1) The oxygen and nitrogen peaks were not separated this made estimations of these gases difficult and 2) quantitative data could not be satisfactorily obtained; this was not found out until several attempts at calibration had been attempted over several weeks. The first problem was easily corrected; the column was repacked and conditioned in an oven at $200^{\circ}C$ with a flow rate of helium of 100 ml. min^{-1}. If the temperature of conditioning is too low the oxygen and nitrogen peaks were not separated.

The second problem was not so quickly resolved; several attempts were made at calibrating the system to obtain quantitative results from the peak heights as it was believed this could be done. Unfortunately no system could be developed, of course the amount of each gas present is proportional to the area under the peaks and not just the peak height. To do this a servoscribe with an integrater attachment was eventually acquired. The arrival of this instrument considerably reduced the problem of gas analysis and quantitative data could now be obtained from the integration values. However not every gas gave the same response with respect to the area under the peak. It was found that, for example, 50% nitrogen would give 1000 units under the peak whereas 50% methane would only give 900 units under the peak for methane . So these values had to be corrected.

These correction factors were obtained when the Katharometer was calibrated, but calibration presented a problem. How should the gases be calibrated ? Commercially prepared and analysed gas mixtures were obtained but these were found unsatisfactory. Mixtures of gases could be obtained by injecting a known volume of gas into a sealed container and analysing the gas mixture in the container. This method was not very satisfactory, although approximate values could be obtained (injecting gas increased the pressure, so the same volume that is to be added could be removed before sample is added). Two other methods that were used were 1) to mix two predetermined and measured flows of gas and analyse the gas in a vessel through which this mixture has been passed for sufficient time for complete equilibration (this was known to have occured when consistent readings were obtained). This method was quite useful however occasionally one of the gas flow rates would change from the beginning to the end making the results obtained useless. The second method which had fewer pitfalls was to use a 0.5 ml. sample loop with the sample injector, this enabled consistent additions of exactly the same volume of gas. Pure gases were passed through these loops and when atmospheric pressure was obtained the sample was injected into the column.

Nitrogen was arbitarily selected as the standard gas and its correction was designated as 1.0 (nitrogen is separated on both columns) From the nitrogen values obtained correction factors could be calculated for all the other gases (i.e.   oxygen 1.04-1.08, methane 1.07-1.16, carbon dioxide 0.82-0.87). These correction factors when multiplied by the integrated values obtained from one sample would give the corrected integrated readings which were now proportional to the partial pressures

of the gases in that sample. The partial pressures of the gas
obtained from Poropak R were simple to calculate

i.e.
$$P_p\,CH_4 = \frac{CH_4{}^I}{AIR^I + CH_4{}^I + CO_2{}^I}$$

I = corrected integrater reading

For the actual partial pressures of the gases from Molecular
Sieve the values obtained for a sample of the same gas phase had to
be used from Poropak R because the Molecular Sieve absorbed carbon dioxide.

i.e.
$$P_p\,O_2 = \frac{O_2{}^I}{O_2{}^I + N_2{}^I + CH_4{}^I + \left[\left(\dfrac{O_2{}^I + N_2{}^I}{AIR^I} \right) \times CO_2{}^I \right]}$$

This is the value the carbon dioxide would have been if it
could have been assayed.

Another way to correct for this lack of CO_2 is to multiply
the ratios of the gases by the partial pressure of the three added
together to give a correct partial pressure of each individual gas
in the mixture

i.e.
$$P_p\,O_2 = \frac{O_2{}^I}{O_2{}^I + N_2{}^I + CH_4{}^I} \, (1 - P_p CO_2)$$

APPENDIX 11

FORTRAN IV programme for analysis of gas readings from continuous fermenter.

```
** ICL 4120 DES1 SYSTEM  :  VOL 50


.&JOB;LS/R013/SS;




&OPTIONS;


&FORTRAN;L;
    1*
    2*          DIMENSION ELEMT(100,12), PERCT(100,13), CORECT(100,12)
    3*          REAL RUNNO, CH4CA, CH4CB, O2C, CO2C, N2, O2, SUM1, SUM2, SUM3,
    4*         1SUM4, CON,ARGON
    5*          INTEGER DATE(5), TEST

    6* C--READ IN DATA

    7*          READ(7,10) DATE, RUNNO, TEST
    8*       10 FORMAT(5A4,F5.2,I3)
   10*          READ(7,20) CH4CA,CH4CB,O2C,CO2C,N2,O2,CON,ARGON
   11*       20 FORMAT(F6.3,F6.3,F6.2,F6.2,F6.3,F5.3,F5.2,F5.4)
   12*          READ(7,30) ((ELEMT(K,J),J=1,12),K=1,TEST)
   13*       30 FORMAT(12F6.2)
   14*
   15*
   16* C--PRINT HEADINGS
   17*
   18
   19*          WRITE(2,100)
   20*      100 FORMAT(1H1//63X,13HSTOICHIOMETRY)
   21*          WRITE(2,110) DATE
   22*      110 FORMAT(/6X,11HS H STANLEY,40X,24HUNCORRECTED INTEGRATOR E'S,
   23*         132X,5A4)
   24*          WRITE(2,500)
   25*      500 FORMAT(/17X,6HOXYGEN,13X,8HNITROGEN,11X,11HMETHANE (B),12X,
   26*         13HAIR,14X,11HMETHANE (A),8X,14HCARBON DIOXIDE)
   27*          WRITE(2,510)
   28*      510 FORMAT(14X,2HIN,7X,3HOUT,8X,2HIN,7X,3HOUT,8X,2HIN,7X,3HOUT,
   29*         18X,2HIN,7X,3HOUT,5X,2HIN,7X,3HOUT,9X,2HIN,7X,3HOUT)
   30*          DO 920 K=1,TEST
   31*          WRITE(2,120)K,(ELEMT(K,J),J=1,12)
   32*      120 FORMAT(5X,I3,12F10.2)
   33*      920 CONTINUE
   34*          WRITE(2,100)
   35*          WRITE(2,130) DATE
   36*      130 FORMAT(/6X,11HS H STANLEY,39X,24HCORRECTED INTEGRATOR E'S,
   37*         133X,5A4)
   38*          WRITE(2,500)
   39*          WRITE(2,510)
   40*
   41*
   42* C--CORRECT INPUTS
   43*
   44*
   45*          DO 900 M = 1,TEST
   46*          ELEMT(M,1) = ELEMT(M,1) * O2C
   47*          IF(O2C.EQ.0.) ELEMT(M,1)=0.
```

```
 48*          ELEMT(M,2) = ELEMT(M,2) * O2C
 49*          IF(O2C.EQ.0.) ELEMT(M,2)=0.
 50*          ELEMT(M,5) = ELEMT(M,5) * CH4CB
 51*          IF(CH4CB.EQ.0.) ELEMT(M,5)=0.
 52*          ELEMT(M,6) = ELEMT(M,6) * CH4CB
 53*          IF(CH4CB.EQ.0.) ELEMT(M,6)=0.
 54*          ELEMT(M,9) = ELEMT(M,9) * CH4CA
 55*          IF(CH4CA.EQ.0.) ELEMT(M,9)=0.
 56*          ELEMT(M,10) = ELEMT(M,10) * CH4CA
 57*          IF(CH4CA.EQ.0.) ELEMT(M,10)=0.
 58*          ELEMT(M,11) = ELEMT(M,11) * CO2C
 59*          IF(CO2C.EQ.0.) ELEMT(M,11)=0.
 60*          ELEMT(M,12) = ELEMT(M,12) * CO2C
 61*          IF(CO2C.EQ.0.) ELEMT(M,12)=0.
 62*      900 CONTINUE
 63*
 64* C--PRINT CORRECTED INTEGRATOR E'S
 65*          DO 930 K=1,TEST
 66*          WRITE(2,120)K,(ELEMT(K,J),J=1,12)
 67*      930 CONTINUE
 68*
 69* C--CALCULATE UNCORRECTED PERCENTAGES
 70*
 71*
 72* C--PRINT UNCORRECTED PERCENTAGES
 73*
 74*          WRITE(2,530)
 75*      530 FORMAT(1H1//54X,13HSTOICHIOMETRY)
 76*          WRITE(2,140) DATE
 77*      140 FORMAT(6X,11HS M STANLEY,34X,30HUNCORRECTED SAMPLE PERCENTAGES,
 78*         121X,5A4)
 79*          WRITE(2,540)
 80*      540 FORMAT(/14X,6HOXYGEN,9X,8HNITROGEN,7X,11HMETHANE (B),9X,
 81*         13HAIR,6X,11HMETHANE (A),4X,14HCARBON DIOXIDE)
 82*          WRITE(2,560)
 83*      560 FORMAT(12X,2HIN,5X,3HOUT,6X,2HIN,5X,3HOUT,6X,2HIN,5X,3HOUT,
 84*         16X,2HIN,5X,3HOUT,6X,2HIN,5X,3HOUT,6X,2HIN,6X,3HOUT)
 85* C
 86*          DO 206 M=1,TEST
 87*          SUM2 = ELEMT(M,2) + ELEMT(M,4) + ELEMT(M,6)
 88*          SUM3 = ELEMT(M,7) + ELEMT(M,9) + ELEMT(M,11)
 89*          SUM4 = ELEMT(M,8) + ELEMT(M,10) + ELEMT(M,12)
 90*          SUM1 = ELEMT(M,1) + ELEMT(M,3) + ELEMT(M,5)
 91*          IF (SUM1.LT.1.0) GO TO 11
 92* C
 93* C
 94*        8 PERCT(M,1) = (ELEMT(M,1)/SUM1) * 100.
 95*          PERCT(M,3) = (ELEMT(M,3)/SUM1) * 100.
 96*          PERCT(M,5) = (ELEMT(M,5)/SUM1) * 100.
 97*       13 PERCT(M,2) = (ELEMT(M,2)/SUM2) * 100.
 98*          PERCT(M,4) = (ELEMT(M,4)/SUM2) * 100.
 99*          PERCT(M,6) = (ELEMT(M,6)/SUM2) * 100.
100*       16 PERCT(M,7) = (ELEMT(M,7)/SUM3) * 100.
101*          PERCT(M,9) = (ELEMT(M,9)/SUM3) * 100.
102*          PERCT(M,11) = (ELEMT(M,11)/SUM3) * 100.
103*       19 PERCT(M,8) = (ELEMT(M,8)/SUM4) * 100.
104*          PERCT(M,10) = (ELEMT(M,10)/SUM4) * 100.
105*          PERCT(M,12) = (ELEMT(M,12)/SUM4) * 100.
106*          GO TO 905
107*       11 PERCT(M,1)=0.
```

```
108*          PERCT(M,3)=0.
109*          PERCT(M,5)=0.
110*          GO TO 13
111*     905 CONTINUE
112*          WRITE(2,150)M,(PERCT(M,J),J=1,12)
113*     150 FORMAT(5X,I3,12F8.3)
114*     906 CONTINUE
115* C
116* C
117* C--CORRECTING SAMPLE PERCENTAGES
118* C
119*          DO 1001 M=1,TEST
120*          PERCT(M,1)=(PERCT(M,1)*(100.-PERCT(M,11)))/100.
121*          IF(PERCT(M,1).EQ.0.) PERCT(M,1)= PERCT(M,7)*O2
122*          IF((PERCT(M,7).EQ.0.).OR.(O2.EQ.0.))PERCT(M,1)=0
123*          PERCT(M,3)=(PERCT(M,3)*(100.-PERCT(M,11)))/100.
124*          IF(PERCT(M,3).EQ.0.) PERCT(M,3)= PERCT(M,7)*N2
125*          IF((PERCT(M,7).EQ.0.).OR.(N2.EQ.0.))PERCT(M,3)=0
126*          PERCT(M,5)=(PERCT(M,5)*(100.-PERCT(M,11)))/100.
127*          IF(PERCT(M,5).EQ.0.) PERCT(M,5)= PERCT(M,9)
128*          IF(PERCT(M,9).EQ.0)PERCT(M,5)=0
129*          PERCT(M,2)=(PERCT(M,2)*(100.-PERCT(M,12)))/100.
130*          PERCT(M,4)=(PERCT(M,4)*(100.-PERCT(M,12)))/100.
131*          PERCT(M,6)=(PERCT(M,6)*(100.-PERCT(M,12)))/100.
132* C
133* C--CALCULATE C-OUT
134* C
135*          PERCT(M,13) = PERCT(M,3)/PERCT(M,4)
136*          IF(PERCT(M,3).EQ.0.) PERCT(M,13)=0.
137*          IF(PERCT(M,4).EQ.0.) PERCT(M,13)=0.
138*    1001 CONTINUE
139* C
140* C--PRINT CORRECTED PERCENTAGES
141* C
142*          WRITE(2,530)
143*          WRITE(2,700) DATE
144*     700 FORMAT(6X,11HS H STANLEY,34X,28HCORRECTED SAMPLE PERCENTAGES,
145*         123X,5A4)
146*          WRITE(2,710)
147*     710 FORMAT(/14X,6HOXYGEN,9X,8HNITROGEN,7X,11HMETHANE (B),9X,
148*         13HAIR,8X,11HMETHANE (A),4X,14HCARBON DIOXIDE,6X,5HC-OUT)
149*          WRITE(2,560)
150*          DO 1002 K=1,TEST
151*          WRITE(2,720)K,(PERCT(K,J),J=1,13)
152*     720 FORMAT(5X,I3,12F8.3,4X,F8.3)
153*    1002 CONTINUE
154* C
155* C CORRECTING OXYGEN PERCENTAGES FOR ARGON IN OXYGEN PEAK
156* C
157*          DO 1003 M=1,TEST
158*          PERCT(M,1)=PERCT(M,1)-(PERCT(M,3)*ARGON)
159*          PERCT(M,2)=PERCT(M,2)-(PERCT(M,4)*ARGON)
160*    1003 CONTINUE
161*          WRITE(2,530)
162*          WRITE(2,700) DATE
163*          WRITE(2,710)
164*          WRITE(2,560)
165*          DO 1004 K=1,TEST
166*          WRITE(2,720)K,(PERCT(K,J),J=1,13)
167*    1004 CONTINUE
```

```
168*  C--CORRECTING PCT'S BY FACTOR C-OUT
169*  C
170*        DO 915 M = 1,TEST
171*        CORECT(M,1) = PERCT(M,13) * PERCT(M,2)
172*        CORECT(M,2) = PERCT(M,13) * PERCT(M,6)
173*        CORECT(M,3) = PERCT(M,13) * PERCT(M,10)
174*        CORECT(M,4) = PERCT(M,13) * PERCT(M,12)
175*  C
176*  C--CALCULATING VOLUMES OF GAS USED AND PRODUCED
177*  C
178*        CORECT(M,5) = CON * (PERCT(M,1)-CORECT(M,1))
179*        CORECT(M,6) = CON * (PERCT(M,5)-CORECT(M,2))
180*        CORECT(M,7) = CON * (PERCT(M,9)-CORECT(M,3))
181*        CORECT(M,8) = CON * (CORECT(M,4)-PERCT(M,11))
182*  C
183*  C--CALCULATE AVERAGE METHANE VALUE
184*  C
185*        CORECT(M,9) = (CORECT(M,6) + CORECT(M,7))/2.
186*        IF(CORECT(M,6)+CORECT(M,7).EQ.0.) CORECT(M,9)=0.
187*  C
188*  C--CALCULATE STOICS
189*  C
190*        CORECT(M,10) = 1.0
191*        CORECT(M,11) = CORECT(M,5)/CORECT(M,9)
192*        IF(CORECT(M,5).EQ.0.) CORECT(M,11)=1.
193*        IF(CORECT(M,9).EQ.0.) CORECT(M,11)=0.
194*        CORECT(M,12) = CORECT(M,8)/CORECT(M,9)
195*        IF(CORECT(M,8).EQ.0.) CORECT(M,12)=0.
196*        IF(CORECT(M,9).EQ.0.) CORECT(M,12)=0.
197*   915 CONTINUE
198*  C
199*  C--PRINT CALCULATED VOLUMES OF GASES
200*  C
201*        WRITE(2,550)
202*   550 FORMAT(1H1//55X,13HSTOICHIOMETRY)
203*        WRITE(2,160) DATE
204*   160 FORMAT(/4X,11HS H STANLEY,92X,5A4)
205*        WRITE(2,170)
206*   170 FORMAT(/15X,20HCORRECTED GAS OUTPUT,24X,21HGASES USED & PRODUCED,
207*       122X,13HSTOICHIOMETRY)
208*        WRITE(2,600)
209*   600 FORMAT(/9X,6HOXYGEN,3X,6HMETH B,3X,6HMETH A,4X,3HCO2,9X,
210*       15HOXYGEN,3X,6HMETH B,3X,6HMETH A,4X,3HCO2,5X,6HMET AV,7X,
211*       13HCH4,5X,6HOXYGEN,4X,3HCO2)
212*        DO 1000 K=1,TEST
213*        WRITE(2,180)K,(CORECT(K,J),J=1,12)
214*   180 FORMAT(2X,I3,F9.3,F9.3,F9.3,F9.3,4X,F9.3,F9.3,F9.3,F9.3,F9.3,
215*       17X,F4.1,1X,F9.3,F9.3)
216*  1000 CONTINUE
217*  C
218*        WRITE(2,190)
219*   190 FORMAT(1H1//10X,5HFINIS)
220*        STOP
221*        END
```

APPENDIX <u>111</u>

<u>COMPUCORP PROGRAMME FOR GAS UTILISATION AND GAS PRODUCTION RATES IN</u>
<u>THE CONTINUOUS FERMENTER</u>

This programme enables gas utilisation and production rates to
be estimated from gas samples taken from input and output gas flow
lines (two samples from each, one onto Porapak A the other on
Molecular Sieve). The procedure is explained in the materials and
methods section.

The programme below is written as it should be entered into
the Compucorp Statistician.

Variable data (correction factors) are put into stores to be
recalled during the programme.

$St_n 1 - C_a^{CH4}$ correction factor for methane on column a (Porapak R)

$St_n 2 - C_b^{CH4}$ correction factor for methane on column b (Molecular Sieve)

$St_n 3 - C^{O_2}$ correction factor for oxygen

$St_n 3 - C^{CO_2}$ correction factor for carbon dioxide

Values that are entered during the programme are indicated by
the step in the programme. (int - refers to integrater value off chart)

See Compucorp instruction book for programme details

HALT

P.E. - Atmospheric pressure, P

$\bullet\atop\bullet$ (mm.Hg.)

HALT

P.E. - Gas sample temperature, T

$\bullet\atop\bullet$ ($^{\circ}$A)

HALT

P.E. - Time for 100 ml. gas at P and T.

x

2155 - To correct to STP

-

HALT

P.E.

=

St_{nn} 60 - volume gas out corrected to
STP.$(ml.min^{-1})$

HALT

176

P.E. - AIR IN (int)

St_{nn}01 - AIR^{I}

HALT

P.E. - CH_4IN (int)

x Rcl_n 1

=

St_{nn}02 CH_4^{I}

HALT

P.E. - CO_2 IN (int)

x Rcl 4

=

St_{nn} 03 - CO_2^{I}

ADV

HALT

Br_{nn} 46

Rcl_{nn} 05

P.E. - P_p AIR IN (a)

St_{nn} 08

Rcl 06

P.E. - P_p CH_4 IN (a)

St_{nn} 09

Rcl_{nn} 07

St_{nn} 10

P.E. - P_p CO_2IN

176

HALT

P.E. - AIR OUT (int)

St_{nn} 01

HALT

P.E. - CH_4 OUT (int)

x Rcl_n 1

=

St_{nn} 03

ADV

Br_{nn} 46

$Rcl_{nn}05$	[	P.E.-N_2 OUT (int)
$St_{nn}11$	l	$St_{nn}02$
P.E. - P_p AIR OUT	-	HALT
Rcl_{nn} 06	Rcl_{nn} 10	P.E. CH_4 OUT (int)
St_{nn} 12	]	x
P.E. - $P_p CH_4$ OUT	$St_{nn}20$	$Rcl_{n}2$
Rcl_{nn} 07	=	=
St_{nn} 13	P.E. - $P_p O_2$ IN	St_{nn} 03
P.E. - $P_p CO_2$ OUT	St_{nn} 14	ADV
176	Rcl_{nn} 06	Br_{nn} 46
HALT	x	Rcl 05
P.E. - O_2 IN (int)	Rcl_{nn} 20	x
x	=	[
Rcl 3	P.E - $P_p N_2$ IN	l
=	St_{nn} 15	-
St_{nn} 01	Rcl_{nn} 07	$Rcl_{nn}13$
HALT	x	]
P.E - N_2 IN (int)	Rcl_{nn} 20	$St_{nn}21$
St_{nn} 02	=	=
HALT	P.E.-$P_p CH_4$ IN(b)	P.E.-$P_p O_2$ OUT
P.E.-CH_4 IN (int)	St_{nn} 16	$St_{nn}17$
x	176	Rcl_{nn} 06
Rcl_n 2	HALT	x
=	P.E.-O_2 OUT (int)	Rcl_{nn} 21
St_{nn} 03	x	=
ADV	$Rcl_n 3$	P.E-$P_p N_2$ OUT
Br_{nn} 46	=	St_{nn} 18
Rcl_{nn} 05	St_{nn} 01	Rcl_{nn} 07
x	HALT	x

Rcl_{nn} 21

=

P.E.-P_pCH_4OUT (b)

St_{nn} 19

176

Rcl_{nn}18

x

Rcl_{nn} 60

÷

Rcl_{nn} 15

=

St_{nn} 22

P.E.-VOLUME IN (ml.min^{-1})

176

Rcl_{nn} 09

x

Rcl_{nn}22

-

[

Rcl_{nn} 12

x

Rcl_{nn} 60

]

=

P.E.-VOLUME CH$_4$ USED (a)

(ml.min^{-1})

St_{nn} 23

Rcl_{nn} 16

x

Rcl_{nn} 22

-

[

Rcl_{nn} 19

x

Rcl_{nn} 60

]

=

P.E.-VOLUME CH$_4$ USED (b)

(ml.min^{-1})

+

Rcl_{nn} 23

÷

2

=

P.E

St_{nn} 26

Rcl_{nn} 13

x

Rcl_{nn} 60

-

[

Rcl_{nn} 10

x

Rcl_{nn} 22

]

=

P.E.-VOLUME CO$_2$ PRODUCED

(ml.min^{-1})

St_{nn}24

Rcl_{nn} 14

x

Rcl_{nn}

22

-

[

Rcl_{nn} 17

x

Rcl_{nn} 60

]

=

P.E.-VOLUME O$_2$ USED

(ml.min^{-1})

St_{nn} 25

176

Rcl_{nn} 26

÷

22.4

x

60

=

P.E.-(mmol.CH$_4$ USED.h^{-1})

St_{nn} 27

Rcl_{nn} 24

÷

22.26

x

60

=

P.E.-(mmol.CO_2 PRODUCED.h^{-1})

St_{nn} 28

Rcl_{nn} 25

$\overset{=}{\cdot}$

22.4

$\times$

60

$=$

P.E.(mmol.O_2 USED.h^{-1})

St_{nn} 29

ADV

Rcl_{nn} 29

$\overset{=}{\cdot}$

Rcl_{nn} 27

$=$

P.E.-STOIC.FOR O_2

Rcl_{nn} 29

$\div$

Rcl_{nn} 27

$=$

P.E.

176

ADV

ADV

Br_{nn} 00

*460 — POSITION IN PROGRAMME

Rcl_{nn} 01

$\overset{=}{\cdot}$

[

Rcl_{nn} 01

+

Rcl_{nn} 02

+

Rcl_{nn} 03

]

St_{nn} 04

$=$

St_{nn} 05

Rcl_{nn} 02

$\overset{=}{\cdot}$

Rcl_{nn} 04

$=$

St_{nn} 06

Rcl_{nn} 03

$\overset{=}{\cdot}$

Rcl_{nn} 04

$=$

St_{nn} 07

Resume

It should be noted that a sub-routine is used for repeated
sequences of calculations of working out the proportion of the
three gases in each sample. If this was not done the number of
steps in the programme would have been greater than the machine
could handle.

APPENDIX IV

COMPUCORP PROGRAMME TO ESTIMATE VOLUMES OF GASES IN THE CLOSED BATCH SYSTEM

This programme calculates the volumes of the gases in the closed gas system for any two simultaneous (almost) gas samples, one into Porapak R the other onto Molecular Sieve. See section 2 of results and discussion for details of procedure.

The variable constants are put into stores to be recalled during the programme. (int-refers to integrater values off the chart)

$St_n 1 - C^{CO_2}$ correction factor for carbon dioxide

$St_n 2 - C^{O_2}$ correction factor for oxygen

$St_n 4 -$ volume correction for batch system (batch at 45^oC, rest

                                                    at room temperature)

$St_n 5 -$ initial volume of barometer

$St_n 6 - C^{CH_4}_a$ correction factor for methane on columns a (Porapak)

$St_n 7 - C^{CH_4}_b$ correction factor for methane on columns b (Molecular Sieve)

$St_n 8 -$ Total volume of system

$St_{nn} 60 -$ Date

See compucorp instruction book for programming details

HALT	–	P.E.-manometer volume
Rcl_{nn} 60	Rcl_n 4	(of liquid)
P.E.- Date	–	x
HALT	HALT	Rcl_n 5
[177]	P.E. – culture volume	$\div$
Rcl_n 8	–	[
	HALT	144

\-

HALT

P.E.- Barometer (height of meniscus

]

=

St_{nn} 20

P.E - gas volume of system

[176]

HALT

P.E - AIR (int)

St_{nn} 01

HALT

P.E.-CH_4 a (int)

x

Rcl_n 6

=

St_{nn} 02

HALT

P.E. - CO_2 (int)

x

Rcl_n 1

=

St_{nn} 03

ADV

HALT

P.E - O_2 (int)

x

Rcl_n 2

=

St_{nn} 04

HALT

P.E. - N_2 (int)

St_{nn} 05

HALT

P.E.-CH_4 b (int)

x

Rcl_n 7

=

St_{nn} 06

[176]

Rcl_{nn} 01

+

Rcl_{nn} 02

+

Rcl_{nn} 03

=

St_{nn} 07

Rcl_{nn} 01

$\div$

Rcl_{nn} 07

=

P.E - P_p AIR

x

Rcl_{nn} 20

=

P.E.- volume of air

ADV

Rcl_{nn} 02

$\vdots$

Rcl_{nn} 07

=

P.E. -P_pCH_4 a

x

Rcl_{nn} 20

=

P.E - volume of CH_4 a

St_{nn} 09

ADV

Rcl_{nn} 03

$\div$

Rcl_{nn} 07

=

P.E - P_p CO_2

x

Rcl_{nn} 20

=

P.E - volume of CO_2

[176]

Rcl_{nn} 04

+

Rcl_{nn} 05

$\div$

Rcl_{nn} 01

x

Rcl$_{nn}$ 03

+

Rcl$_{nn}$ 04

+

Rcl$_{nn}$ 05

+

Rcl$_{nn}$ 06

=

St$_{nn}$ 08

Rcl$_{nn}$ 04

$\div$

Rcl$_{nn}$ 03

=

P.E. - P$_p$ O$_2$

x

Rcl$_{nn}$ 20

=

P.E. volume of O$_2$

ADV

Rcl$_{nn}$ 05

$\div$

Rcl$_{nn}$ 08

=

P.E. P$_p$N$_2$

x

Rcl$_{nn}$ 20

=

P.E. volume of N$_2$

ADV

Rcl$_{nn}$ 06

$\div$

Rcl$_{nn}$ 08

=

P.E. - P$_p$ CH$_4$ b.

x

Rcl$_{nn}$ 20

=

P.E. - volume of CH$_4$ b

+

Rcl$_{nn}$ 09

$\div$

2

=

P.E. Average volume of CH$_4$

[176]

[176]

Br$_{nn}$ 00