METABOLIC EFFECTS OF OXYGEN-TRANSFER IN

MICROBIAL CULTURE

by

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SYNOPSIS

The object of this work was to study some of the biochemical effects of various oxygen-transfer conditions in submerged cultures of a facultative anaerobe, Klebsiella aerogenes N.C.I.B. 418. A well-mixed laboratory fermenter with extensive instrumentation was used to reproduce and monitor some possible effects of 'cyclic aeration', a phenomenon experienced by microorganisms in most industrial fermentations.

Batch and continuous cultures were carried out both aerobically and anaerobically, using a synthetic glycerol-salts medium. 'Cyclic aeration' was simulated by 'intermittent aeration', air and nitrogen being supplied alternately to the fermenter over a five-minute cycle. The aerobic enzymes (glycerol kinase and glycerol-3-phosphate dehydrogenase) and anaerobic enzymes (glycerol dehydrogenase and dihydroxyacetone kinase) involved in the conversion of glycerol to dihydroxyacetone phosphate were assayed in each culture sample.

An aerobic→anaerobic transition, indicated most noticeably by a marked reduction of cell density, was found to take place rather sharply as the aeration time per cycle was progressively reduced. The transition point and the sharpness of the transition were dependent on oxygen-transfer efficiency during the aeration period, as well as on the aeration time per cycle, and resulted in reductions in the levels of the aerobic enzymes and increases in the levels of the anaerobic enzymes. Specific carbon dioxide production increased as 'aeration condition' was reduced to the point of fully anaerobic growth; its measurement was used, together with that of culture dissolved oxygen tension, to follow the progress of the transition. It is suggested that the determination of the complement of these enzymes could be used to assess the effectiveness of oxygen distribution in industrial fermenters.

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CHAPTER 1

INTRODUCTION

Biochemical engineering is a rapidly-developing discipline which uniquely combines a number of engineering fields (chemical, mechanical, electrical and electronic) with a wide range of scientific functions (biochemistry, microbiology, chemistry, metallurgy, etc.).

The inherent multi-disciplinary nature of biochemical engineering inevitably leads to various interactions between those disciplines, and these interactions may be detrimental to the overall performance of the system. This work studies one of these fundamental interactions.

The majority of biochemical engineering processes involve, in some way, the submerged culture of microorganisms. Choice of microorganism, by artificial or 'natural' selection and optimisation of its culture conditions for the particular process gives a wide range of possible biochemical processes. Thus, process conditions can be optimised to produce organic primary metabolites (e.g. citric acid, 2,3-butanediol), secondary metabolites (especially antibiotics), and enzymes (intra-cellular and extra-cellular), and can even be optimised to carry out highly stereo-specific chemical conversions (of steroids, for example), or simple metabolic oxidation of chemical pollutants in waste-treatment processes. The processes can also be optimised for the production of the microorganisms themselves; for their metabolic characteristics, as in the case of baker's yeast, or their nutritional benefits, as in the case of the 'single cell protein' processes, where a protein-rich 'biomass' is produced by essentially non-agricultural means.

The increased industrial utilisation of these various processes in recent years has led to the design and installation of larger culture vessels (fermenters) in order to meet demand and maintain economic

viability. Problems associated with mixing and aeration in large agitated vessels lead to inhomogeneity of the culture within the vessel, and therefore to fluctuations of nutrient (including oxygen) availability to individual microorganisms. Fluctuations of dissolved oxygen availability may be accentuated by hydrostatic effects in large fermenters; these effects are especially pronounced in the various 'air-lift' fermenter designs, where circulation is maintained between regions of differing dissolved oxygen tension.

Thus, 'cyclic aeration' is inherent to a greater or lesser extent in the aerobic operation of all large fermenters. Dissolved oxygen tension will vary between various regions within the fermenter; it may even reach critically low levels in certain regions of some fermenters, or merely fluctuate at levels constantly above critical in other fermenters. Since oxygen is a fundamentally important nutrient in aerobic fermentation processes, it might be expected that cyclic aeration could affect many characteristics of the microorganisms cultured in the fermenter. Evidence in support of this expectation has been found under operating conditions, but little work has been reported in the form of the effect of systematic imposition of 'cyclic aeration' on the biological behaviour of the cultured microorganisms.

The aim of this work is to quantify some effects of 'cyclic aeration' on the biochemistry of Klebsiella aerogenes grown in continuous submerged culture.

In order to study this interaction between a chemical engineering effect and its biochemical consequence, it is necessary to model 'cyclic aeration' in large fermenters as 'intermittent aeration' in a small laboratory fermenter. Although cyclic aeration would clearly occur in a random fashion, the effect would be expected to exhibit an average

cycle-time and dissolved oxygen fluctuation. For reasons of experimental reproducibility, dissolved oxygen variations are imposed regularly in the intermittently aerated model system.

A fermenter of 575 cm^3 working volume was used to culture K. aerogenes continuously in a synthetic glycerol-salts medium. Intermittent aeration was imposed by timer-controlled solenoid valves which controlled the supply of either air or oxygen-free nitrogen to the fermenter. The circulation rates over the range of impeller speeds used in this work were very high compared with the modelled circulation rates caused by the imposed variations of dissolved oxygen supply. Thus, mixing effects attributable to the vessel were insignificant compared to the intermittent-aeration effect. Whilst steady-state conditions were, of course, never reached over short time periods, when viewed over long periods, these conditions could be recognised. Cultures were harvested for biochemical determinations only when steady-state conditions of this type were apparent.

CHAPTER 2

LITERATURE REVIEW

A large number of papers have been published on the chemical engineering (especially heat- and mass-transfer) aspects of fermenter design. A natural extension of this work might be considered to involve the effects of various environmental influences on microbial cultures, since the design of the fermenter dictates many of the environmental influences that the culture undergoes.

An apparently little related area of investigation, in the realm of microbial biochemistry, involves the study of the fate of carbon energy-sources in microbial metabolism. Nevertheless, a number of environmental influences, including oxygen mass-transfer condition, are shown to affect carbon-source metabolism in fermentation systems.

This review therefore examines selected papers on oxygen mass-transfer in fermenters, and environmental influences, especially oxygen-availability, on microbial cultures. In addition, work on the metabolism of one particular carbon energy-source, glycerol, in a particular bacterium, Klebsiella aerogenes, is examined in detail within the wider context of bacterial glycerol metabolism. Some other metabolic studies on K. aerogenes are also covered.

2.1   Aeration and Mixing in Biological Reactors

2.1.1   Fermenter design

The most common form of reactor used for microbial culture is a stirred, baffled, cylindrical vessel. Mixing is mainly due to the action of one or more centrally-mounted rotary impellers, often of the multibladed-turbine design, in conjunction with the action of baffles attached to the inside wall of the vessel. Air is normally introduced through a sparger below the bottom impeller. This 'classical' fermenter design may provide adequate overall transfer of oxygen to the solution, but owing to complex mixing effects, distribution of dissolved oxygen (and other nutrients) may be less than perfect. (Blakebrough, 1972).

The mechanism by which oxygen is transferred from the air to solution within the fermenter has been the subject of a considerable amount of work. The processes governing this transfer have been more than adequately dealt with elsewhere (e.g. Calderbank, 1967) and will therefore not be covered here. Nevertheless, there appears to be little understanding of the effects of the various mechanical processes, designed to optimise heat and mass-transfer, on the physical and biochemical nature of the microorganisms grown within the fermenter. (Blakebrough, 1973).

A considerable amount of early work was carried out on the effects of various mechanical aspects of fermenter design on oxygen-transfer and distribution. Cooper et al. (1944) showed that oxygen-transfer rate is proportional to power input per unit volume in turbulent systems; Finn (1954) showed it to be proportional to the cube of impeller speed. Solomons and Perkin (1958) found that by placing the impeller and sparger at the bottom of the vessel, oxygen-transfer rates were reduced by 50%. They also found, however, that

if the sparger was placed at the bottom of the vessel and the impeller
left some distance above, then oxygen-transfer was considerably more
efficient than the situation where the sparger was directly below the
impeller.

The results of much of this type of early work were used in
the design of fermenters in use today.   Better understanding of the
behaviour of mixing fluids within these vessels has evolved relatively
recently.   Sinclair and Brown (1970) showed that some deviations from
ideal behaviour could be attributed to the presence of well-mixed zones,
with relatively poor interaction between zones.   Blakebrough (1972)
reported that in a baffled system, zoning occurs above and below each
impeller.   Thus, overall mixing within the fermenter is dependent on
interaction between zones.   It was also pointed out that this effect
is particularly important in continuous cultures, where a growth-limiting
nutrient may be added into only one zone, its overall distribution being
dependent on good mixing between zones.

Deviations of this type form ideal behaviour were earlier
attributed simply to 'apparatus effects' (Herbert et al. 1956),
demonstrating inadequate knowledge at that time.   Nevertheless, the
effect of insufficient bulk-mixing and short-term variations in dissolved-
oxygen tension on optimal metabolic rate have been recognised for some
time.   (Phillips and Johnson, 1961).   Indeed, the postulation that
variations in dissolved oxygen distribution might occur in fermenters
was made considerably earlier than this.   (Bartholomew et al. 1950).
Phillips and Johnson pointed out that the economically optimal metabolic
rate may be limited by the region of lowest aeration within the fermenter.
They added, however, that in cultures of Aerobacter aerogenes, oxygen-

-limitation did not appear to damage the cells. (<u>Aerobacter aerogenes</u> has now been reclassified under the genus <u>Klebsiella</u>).

The critical nature of dissolved-oxygen concentration in aerobic fermentations has been well-documented (Harrison, 1972a;Finn, 1967). However, work to determine experimentally the effects of dissolved oxygen variation on microorganisms is rare. This type of work has mainly been limited to studies of the effects of oxygen-tension on microorganisms. Carrizales (1974) studied respiratory and general macromolecular effects of intermittent oxygen-starvation in <u>K. aerogenes</u> cultures, using a small fermenter whose air and gaseous nitrogen supplies were controlled by timed solenoid valves. A very similar experimental approach was made by Soni and Ghose (1974) and by Mukhopadhyay and Ghose (1976). This type of equipment was also used in this work to study the effect of intermittent oxygen-starvation on more fundamental aspects of glycerol metabolism in <u>K. aerogenes</u>. The equipment is thereby designed to reproduce inequalities of dissolved-oxygen distribution in large fermenters.

2.1.2   Measurement of oxygen mass-transfer

The efficiency of oxygen mass-transfer is measured in terms of the oxygen mass-transfer coefficient, $K_L a$, which has units of $time^{-1}$. In an adequately aerated culture, $K_L a$ must be sufficient to meet the total oxygen demand of the microorganisms. Thus, the practical importance of $K_L a$ as a measurement of 'aeration-agitation intensity' is apparent. Its value is also affected by the physical and chemical properties of the culture, e.g. viscosity (Hattori <u>et al.</u>, 1972), dissolved salts, absolute temperature, etc. Tsao (1968) found that the surface-active properties of dissolved proteins may reduce $K_L a$ very significantly.

Measurement of $K_L a$ is universally accepted as an indicator of the overall dissolved-oxygen supply rate to microorganisms within the fermenter. A variety of techniques have therefore been suggested for $K_L a$ measurement.

Early methods measured the rate of oxygen solution by the use of oxygen-absorbing reagents. The method relies on the oxidation-rate of the reagent being limited only by the oxygen-transfer rate from the gas to the solution. Cooper et al. (1944) used the most common application of this technique, the cupric ion catalysed oxidation of sodium sulphite. This method has been widely studied and developed. (Linek and Tvrdik, 1971; Greenhalgh et al., 1975). Phillips and Johnson (1959) found the rate of oxygen absorption to be independent of sulphite concentration, providing that it was above 0.01M. However, the reliability of the method has been shown to depend upon particular conditions in the fermenter, and measurement is not made in an actual growing culture.

More recently, measurement of oxygen solution rate has been made using an enzyme system containing glucose oxidase. This is an extension of a microbiological method in which a bacterial culture converts glucose and oxygen to gluconic acid, the rate of acid formation being limited by the oxygen-transfer rate. (Tsao and Kempe, 1960) Replacement of the bacterium by the enzyme-system involved in this conversion permits experimental determination of $K_L a$ providing that basic kinetic data are known (Tsao, 1968; Hsieh et al., 1969).

Another method of measuring $K_L a$ in the absence of culture involves the use of a fast-response oxygen-electrode. After deoxygenation of the fermenter contents, a semi-logarithmic plot of

oxygen concentration (log) versus time (linear) during re-aeration yields the necessary data for calculation of $K_L a$ (Tsao, 1968).

Since $K_L a$ should be an indicator of overall oxygen-supply to the microorganisms in a culture, it is useful to use a $K_L a$ measurement method which involves a growing culture. An extension of the oxygen-electrode method, the dynamic measurement method involves the application of non steady-state kinetics. Oxygen tension is plotted as the air supply is cut and later restored. Thus, decrease in oxygen tension gives an indication of respiration rate, and the slope of the graph on re-aeration again yields essential data for $K_L a$ calculation. (Bandyopadhyay et al., 1967). The mathematical treatment involved in this method has been further developed by Fujio et al. (1973), who used an integrated form of the oxygen-balance equation. Páca and Grégr (1977) improved this treatment by introducing a correction-factor for the actual culture conditions. Páca and Grégr also modified a procedure for $K_L a$ determination by this method under oxygen-limited conditions.

Under steady-state continuous culture conditions, $K_L a$ can be easily measured by an oxygen-balance method (Pirt, 1975). Oxygen uptake rate is determined by volumetric oxygen gas analysis, and oxygen tension by the oxygen electrode. This method was used in this work, and is dealt with in more detail in the Theory section.

2.2   Environmental Influences on Microbial Cultures

It has been shown here that various aspects of fermenter design and operation are responsible for a few of the environmental influences to which a culture is subjected. Dissolved oxygen supply may be affected by both design and operation, whilst culture temperature and dilution rate are affected mainly by the operation of the fermenter. There are also a number of other environmental influences affecting the culture which are not normally attributable to fermenter-effects. They generally involve the physico-chemical properties of the culture medium, and include the chemical composition of the medium, culture pH, and osmotic pressure (Herbert, 1961).

2.2.1   Influence of medium composition and dilution rate

Providing that all essential components of a culture medium are present in sufficient quantity and are readily available, and that no growth inhibitors are present, then the medium will be capable of supporting unrestricted growth of microorganisms. However, the definition of a complete medium to achieve optimal metabolite production or maximum growth is quantitatively difficult, and in many processes this remains to be achieved (Pirt, 1975).

Assuming that one is concerned with a medium containing all the growth requirements of the organism, then growth rate in continuous culture will be dependent on dilution rate and a growth-limiting component. The growth-limiting component in a culture medium often has a great individual effect on the growing microorganisms since the chemical composition of the cell is primarily dependent on the growth rate, (Herbert, 1961). Light (1972) found that the protein content of yeast was 30% when cultures were nitrogen-limited, but 50% when they were

carbon-limited. Indeed the concentrations of enzymes may be affected by the nature of the growth-limiting component in the medium (Dean, 1972). Oxygen limitation, as will be seen later, may result in the induction of the enzymes of anaerobic metabolic pathways.

Dean (1972) additionally found that the concentrations of several enzymes varied with dilution rate in a series of variously-limited continuous cultures. Enzyme concentrations affected by dilution rate in this way may have direct consequences on metabolic pathways. For example, Tempest et al. (1967) found that in aerobic nitrogen-limited continuous cultures of K. aerogenes, large amounts of α-ketoglutarate were formed at very low dilution rates, but not at high dilution rates. Dilution rate also has a considerable effect on the chemical composition of cells since it directly determines growth rate. Herbert (1961) reported that the protein and nucleic acid composition of Aerobacter (Klebsiella)aerogenes varied with dilution rate over the range 0.1 to 0.8 h^{-1} in glycerol-limited cultures. There is a close relationship between growth-limiting nutrient and dilution rate since the maximum sustainable dilution rate is dictated by the concentration of that nutrient.

The presence of specific compounds in culture media may induce (or activate) or repress (or deactivate) particular enzymes; the enzymes are normally associated in some way with those compounds. An example that is particularly pertinent to this work was cited by Koch et al. (1964). They found that when E.coli cells were grown in the presence of glycerol or L-α-glycerophosphate, the enzymes glycerol kinase, L-α-glycerophosphate dehydrogenase and an L-α-glycerophosphate transport protein were induced.

2.2.2   Influence of culture pH and osmotic pressure

The main effects of pH in microbial culture are on metabolism (both primary and secondary), growth and macromolecular composition of the biomass.   Dissociation of acidic or basic compounds which may be inhibitory or essential to the microorganism will be affected by pH. .

Anaerobic metabolism is particularly sensitive to pH. Lactobacilli and streptococci produce mainly lactic acid at low pH, but acetic and formic acids at higher pH values (de Ley, 1962).   The higher pH therefore favours relatively greater production of acid equivalents, thus reducing pH.   This type of regulatory effect has been recognised in other cultures.   Secondary metabolism is also affected by pH.Rowley and Pirt (1972) showed that Aspergillus nidulans produces twenty times more melanin at pH 7.9 than at pH 7.0.

The major influence of pH on the macromolecular composition of microorganisms probably lies in its effect on their morphology, especially with respect to the structure of the cell-wall.   Pirt and Callow (1959) found a reduction in the hyphal length of Penicillium chrysogenum on raising the pH from 6 to 7.   The hyphae were considerably swollen at the higher pH.   Their conclusion was that the cell wall composition varies with the pH of the environment, leading to a weaker structure at higher pH values.   Other macromolecular compositions appear to be little affected by pH; Tempest and Hunter (1965) found little influence of pH on protein, carbohydrate, and nucleic acid in A. aerogenes.   Bacterial yield, however, was affected by pH value.

The cell-wall composition of certain microorganisms is also known to be affected by the osmotic pressure of the growth medium. Ellwood (1971) found that in phosphate-limited cultures of Bacillus

<u>subtilis</u> at pH 7.0, the major cell-wall polymer in medium containing less than 1% sodium chloride was teichuronic acid. However, on increasing the sodium chloride concentration to 6%, teichoic acid replaced the teichuronic acid.

High medium osmotic pressure may also dehydrate cells. (The effect of high osmotic pressure on cell wall composition may be designed to prevent this dehydration). Christian and Waltho (1964) found that a high sodium chloride content in the medium of <u>Staphylococcus aureus</u> halved the content of cellular water, and correspondingly increased the intracellular concentration of sodium chloride and amino acids.

2.2.3   <u>Influence of culture temperature</u>

All microorganisms are sensitive to temperature, since this affects the rates of cell reactions, the nature of metabolism, the nutritional requirements and the biomass composition (Pirt, 1975). Thus, whilst cellular reaction rates increase with increasing temperature, a point is reached where enzyme-catalysed reactions may no longer be possible owing to breakdown of protein structures. All microorganisms therefore have an optimal growth temperature. Thus, some enzymes may be innactivated at higher temperatures, resulting in new growth requirements for the microorganism. Ware (1951) found that <u>E. coli</u> requires glutamic acid and nicotinamide for growth at 44°C.

Temperature also has a direct effect on carbon energy-source requirement. Topiwala and Sinclair (1971) and Harrison and Loveless (1971a) found an increase in growth yield of <u>K. aerogenes</u> from glucose with decreasing temperature. They accounted for this by saying that maintenance energy increased with increasing temperature. The same

effect was reported by Brown and Rose (1969a) in glucose-limited cultures of C. utilis.

Secondary metabolism is affected by temperature, as it is by pH. Rowley and Pirt (1972), studying the effects of culture conditions on melanin formation by A. nidulans, found a two-fold increase in the production of this pigment between 23°C and 37°C in glucose-limited chemostat culture at 0.05 h^{-1}. (See also Section 2.2.2). The effect of temperature on the production of acid and gas by A. aerogenes has been examined by Greene and Jezeski (1954).

Temperature is known to exert a considerable effect on the macromolecular composition of microorganisms. Hunter and Rose (1972) studied the effects of temperature on the nucleic acid, protein, polysaccharide and lipid composition of Candida utilis and Saccharomyces cerevisiae grown in continuous culture. At a fixed dilution rate, the content of RNA was considerably reduced when the temperature was increased from 15°C to 30°C. (Data by Brown and Rose (1969a)). The effect was, however, also dependent on growth rate, apparently being most marked in chemostat cultures of C. utilis grown at 0.20 h^{-1}. A similar effect on the RNA content of A. aerogenes was found by Tempest and Hunter (1965).

The effect of temperature on protein composition, noted by Hunter and Rose (1972), was not as positive. The total protein of the yeasts they studied seemed to vary up or down, depending on the identity of the growth-limiting substrate. Alroy and Tannenbaum (1973), also working with continuous cultures of C. utilis, found that at constant dilution rate, the ratio of total nucleic acid to protein decreased with increasing temperature. This result may, however, be simply due to the effect of temperature on RNA content, as reported by Hunter and Rose (1972).

The formation of intracellular and extracellular polysaccharide by microorganisms is influenced by growth temperature. Tempest and Hunter (1965), working with glycerol-limited continuous cultures of A. aerogenes, found that more total cellular carbohydrate is synthesised at lower temperatures. However, they did not determine whether the distribution of cellular carbohydrate (i.e. cell wall or storage polysaccharide) was affected, or whether growth temperature equally affected all cellular carbohydrate. Brown and Rose (1969a),studying the effect of temperature on the total carbohydrate content of continuously grown C. utilis, found that when temperature was increased from 15°C to 30°C, the content decreased in glucose-limited cultures but increased in ammonia-limited cultures. (This is similar to the effect on yield and protein content in this organism found by these authors).

The most extensively observed temperature-induced effect on the macromolecular composition of microorganisms is on the composition of their lipids, especially fatty acid composition. (Hunter and Rose, 1972). The nature of this effect is invariably for cells to contain a larger proportion of unsaturated fatty acids (i.e. those with lower solidification temperature) at lower temperatures. (Farrell and Rose, 1967). Thus, Marr and Ingraham (1962) found that as the growth temperature of batch-cultured E. coli was increased from 10°C to 43°C, the content of unsaturated octadecanoic and hexadecanoic acids decreased, but that of palmitic acid correspondingly increased. There appeared to be a fairly smooth transition in this compositional variation between these two temperature extremes, indicating that membrane lipid structure may be continuously modified with change in temperature so as to maintain lipid function (Pirt, 1975).

2.2.4   Influence of dissolved oxygen availability..........

A large number of papers have been published on the effects on growing microorganisms of dissolved oxygen availability. Much of this work has studied the influence of dissolved oxygen tension (DOT) on respiration, metabolism, and enzyme concentrations.

2.2.4.1   .....on the fate of substrate carbon

A particularly interesting branch of this work concerns the effects of oxygen availability on product formation in facultative anaerobes. An early study in this area by Pirt (1957) determined the effect of oxygen supply rate on the fate of glucose carbon in growing cultures of Aerobacter cloacae. He found that under fully aerobic conditions, cell carbon and carbon dioxide accounted for most of the substrate carbon. However, anaerobically, glucose carbon was accounted for as cell carbon, carbon dioxide, acetic acid, formic acid, ethanol, butanediol and acetoin. With 'partially aerobic' metabolism, i.e. under oxygen-limited conditions, fermentation products were not obtained till a 'threshold dilution rate' was exceeded. A considerable amount of work on the relationship between DOT and the fate of substrate carbon has been published in the twenty years since the publication of Pirt's paper. Working on the optimisation of 2,3-butanediol production by A. aerogenes, Pirt and Callow (1958) found that 'partial aerobic' metabolism was necessary for reasonable production of this compound. Depriving the culture of oxygen caused a large increase in percentage conversion of sucrose to lactic acid. Butanediol production was approximately inversely proportional to oxygen transfer rate, as measured by the sulphite oxidation method. However, they found that a steady-state could not be produced anaerobically as the bacterial growth rate was too low.

Using continuous cultures of <u>Klebsiella aerogenes</u> growing at 0.2 h^{-1} with glucose in excess, Harrison (1965) examined the effect of various dissolved oxygen tensions at acid (6.0) and alkaline (7.4) pH on the metabolic products. At both pH values, the major product was pyruvate where the DOT exceeded 7 mm Hg; production of ethanol replaced pyruvate at low DOT. However, other major products were synthesised at low DOT: β-diol at the acid pH, or volatile acids at the alkaline pH. Regulation of the organism's environmental pH is thus evident at low DOT by diversion.of approximately half the glucose carbon to volatile acids at pH 7.4. Nevertheless, 20% of the glucose carbon was diverted to volatile acid production at the higher DOT.

Harrison and Pirt (1967) reported that in "limited oxygen" continuous cultures of <u>K. aerogenes</u> (i.e. under 5 mm Hg DOT) glucose carbon was accounted for as organisms, carbon dioxide, 2,3-butanediol, ethanol, acetic acid, formic acid and lactic acid. The actual proportions of each depended on oxygen-supply, pH, and whether the culture was nitrogen or carbon-limited. In fully oxygen-limited (i.e. under 0.1 mm Hg DOT) cultures, the levels of fermentation products increased, and there was a decrease in stored polysaccharides, leading to apparently lower dry cell weights. It was also reported here that, at pH 7.4 in anaerobic culture,thioclastic cleavage of pyruvate takes place and produces equimolar amounts of acetic and formic acids.

When an anaerobic culture of <u>K. aerogenes</u> was reaerated, Harrison and Loveless (1971,b) found that all the metabolic products built up in the anaerobic period were oxidised within three hours, and an aerobic steady-state was established after about eight hours. They observed that during the transition, glucose utilisation was

complete at all times; yield coefficients for glucose and oxygen were low, and respiration rate was high indicating a degree of uncoupling between growth and "energy-conserving" mechanisms. This led the authors to suggest that alternating anaerobic and aerobic cycles of growth could increase the overall rate of oxidation of glucose whilst maintaining low cell yields.

Harrison et al. (1969), studying the response of K. aerogenes to hyperbaric oxygen tensions, reported the production of a yellow flavin-like pigment at DOT values up to at least 450 mm Hg. Its precise identity, however, was not established. Moss et al. (1969) reported that the colour of C. utilis cultures changed from pink at high DOT to pale green at low DOT. No real explanation was offered.

The influence of dissolved oxygen availability on the fate of substrate carbon in microorganisms other than facultative anaerobes has also been extensively reported. A considerable amount of work with yeasts and moulds has been published:

Peterson et al. (1958) found that aeration had a strong influence on glycerol production by Torulopsis magnoliae. In addition, Stachl and Heumann (1963) found that in batch cultures of the brewer's yeast Saccharomyces cerevisiae, the yield of polyhydric alcohols was greatly increased by adequate aeration. Working with continuous cultures of this organism, Cowland and Maule (1966) found that growth rate was maximal and ester formation inhibited at low DOT. (0.2 to 2.3 mm Hg). At higher (8.4 to 74 mm Hg) levels of dissolved oxygen, growth was reduced, production of fermentation products reduced, but large quantities of acetoin and acetaldehyde were produced. Above 74 mm Hg DOT, ethanol production accounted for the majority of the glucose consumed.

Analysing the amino-acid pool of S. cerevisiae grown in
galactose-limited continuous cultures, Brown and Stanley (1972)
found the levels of several amino-acids in the pool to be considerably
affected by DOT. Generally, higher concentrations of these amino-
acids were found at low oxygen tensions; the effect was particularly
marked for aspartic acid, whose concentration of 7.2 mM at 75 mm Hg
oxygen tension was increased to 103.6 mM at 1 mm Hg. The concentrations
of some amino-acids, however, appeared unaffected. A similar inverse
relationship between DOT and lysine production has been reported in
continuous cultures of Brevibacterium (Beker et al. 1973).

Another yeast used extensively in studies of the effect of
DOT on the fate of substrate carbon is Candida utilis. Moss et al.(1969)
found that when well-aerated continuous cultures of this organism were
reduced to 1.2 μM dissolved oxygen, after ten hours, glucose consumption
had increased but cell production had decreased. The difference was
explained by high production of carbon dioxide. The same group, working
with continuous cultures of S. carlsbergensis (Moss et al., 1971)
found that when glucose was limited and oxygen abundant, respiration
predominated. However, when glucose was abundant ethanolic fermentation
predominated even when oxygen was also abundant.

Babij et al. (1969) found a strong relationship between
aeration intensity and the type of C_{18} fatty acids in C. utilis.
Higher levels of aeration appeared to induce the formation of polyunsaturated
fatty acids; the levels of stearic acid and oleic acid were decreased
whilst that of linolenic acid was increased. A similar effect on the
same organism was found by Brown and Rose (1969b). They also found
that reduction of dissolved oxygen tension from 5 mm Hg to under 1 mm Hg
decreased the ratio of $C_{18}:C_{16}$ fatty acids.

The production of secondary metabolites by moulds is known to be affected by dissolved oxygen tension. Early work on penicillin production by Wegrich and Shurter (1953) found that titres could be increased from 900 units.cm^{-3} to 2,000 units.cm^{-3} when agitation and aeration rates were increased. They interpreted the result by saying that good aeration is essential for high penicillin production. Brandl et al. (1966), working on the quantitative relationship between dissolved oxygen concentration and production of penicillin, bacitracin and gluconic acid, found that oxygen concentrations above 7 mg.l^{-1} inhibited bacitracin production; no inhibitory concentration of oxygen was found for the other two products' yield. Feren and Squires (1969), working on the production of another antibiotic, cephalosporin c, by the mould Cephalosporium, found the critical DOT of the mould (for respiration) to be 8 mm Hg. Maximum production of the antibiotic required a DOT of over 20 mm Hg.

Rowley and Pirt (1972) found that in cultures of A. nidulans, production of insoluble melanin was maximal at dissolved oxygen tensions over 30 mm Hg. Below this value, a soluble form of the pigment was secreted into the medium, reaching a maximum amount at 18 mm Hg. Carter and Bull (1971) found that DOT had no significant effect on mean length of hyphal segments and degree of branching in this organism. However, at DOT values below 3.5 mm Hg, free conidia appeared in the medium.

2.2.4.2   ......on amounts of catabolic enzymes

The amounts of many enzymes involved in the catabolism of carbon substrates have been found to vary with differing levels of dissolved oxygen in microbial cultures. Most work in this area

appears to have been carried out with cultures of facultatively anaerobic bacteria. However, some work with other organisms has been reported:

Zetelaki (1970) found that when the oxygen-transfer rate to a continuous culture of Aspergillus niger was increased, the activity of glucose oxidâse per gram of mycelium was increased. Carter and Bull (1969), working with cultures of Aspergillus nidulans, found that the activity of a number of enzymes involved in the glycolytic and hexose monophosphate pathways reached maximal values at dissolved oxygen tensions below 30 mm Hg.

Rickard et al. (1969) reported on the ability of the yeasts Candida utilis and Saccharomyces carlsbergensis cultured in oxygen-controlled environments to oxidise NADH. The results demonstrate a decrease in NADH oxidation rate of extracts for a decrease in the DOT experienced during growth. In most cases, NADH oxidation was activated by the addition of exogenous cytochrome c. Oura (1976) reported the effect of aeration intensity on the activities of enzymes of the glycolytic and pentose phosphate pathways in baker's yeast. The enzymes assayed in this work were hexokinase, alcohol dehydrogenase, phosphofructokinase, pyruvate decarboxylase and glucose-6-phosphate dehydrogenase. Activities were expressed graphically against the percentage oxygen (0-100%) in the fermenter gas-supply. The graphs are generally of complex form except for that of glucose-6-phosphate dehydrogenase, which shows a gradual increase in activity from 0 to 100% oxygen supply.

Research on the effect of DOT on the enzyme responsible for fixing nitrogen in Azotobacter species, nitrogenase, has been reported by a number of workers. It appears that Azotobacter chroococcum is

sensitive to excessive aeration when fixing molecular nitrogen, but not when fixed nitrogen (as NH_4^+) is available (Dalton and Postgate, 1969a).

In a 'follow-up' paper these authors (Dalton and Postgate, 1969b) suggested that the organism operated an oxygen-sensitive nitrogenase system in the presence of air by adopting a dual protective mechanism. This consisted of an enhanced cellular respiration rate as a means of excluding oxygen from the operating nitrogenase, and where this was inadequate, a conformational change in the enzyme complex took place such that the oxygen-sensitive sites became inaccessible to the oxygen molecule. Whilst this change was in operation, however, the enzyme could no longer fix nitrogen. Kelly (1969) found that cell-free nitrogenase preparations of A. chroococcum were insensitive to damage by oxygen, but when the complex was resolved into its enzyme components, oxygen-sensitivity was established.

The same pattern of oxygen-sensitivity has been found with cultures of A. vinelandii by Bulen et al. (1966). Nevertheless, this organism does require a minimal dissolved oxygen concentration of approximately 32 μM for effective operation of its oxidative phosphorylation system. (Ackrell and Jones, 1971). Below this value of dissolved oxygen, logarithmic growth was reported to cease. Nagai et al., (1971) also working with cultures of A. vinelandii, found the activity of the enzyme aldolase to be proportional to DOT and to rise to very high levels at high DOT. However, the activities of glyceraldehyde-3-phosphate dehydrogenase and isocitrate dehydrogenase were not affected.

The sensitivity to oxygen of two other aerobic nitrogen-fixing bacteria, Mycobacterium flavum and Derxia gummosa has been summarised by Hill et al. (1972). Generally, their responses are very similar to those of the Azotobacteraceae.

The transition from aerobic to anaerobic glucose utilisation in _Escherichia coli_ cultures and its effect on a number of enzymes has been thoroughly investigated by Thomas _et al._ (1972). The results suggest that the switch from aerobic to anaerobic metabolism commences well before the DOT reaches zero. Reduction of oxygen partial pressure below 28 mm Hg resulted firstly in an increase in the levels of respiratory enzymes [NADH oxidase (x 2.53), succinic dehydrogenase (x 1.4), cytochrome b_1 (x 3.91), cytochrome a_2 (x 2.45)] before the electron transport system collapsed, and the enzymes decreased in activity. Before the DOT reached zero, the change from respiration to fermentation was apparent from increases in the levels of fructose diphosphate aldolase, and glucose-6-phosphate and 6-phosphogluconate dehydrogenases, and a decrease in the level of 2-oxoglutarate dehydrogenase. When the DOT reached zero, cell dry weight, carbon dioxide formation and the level of isocitrate dehydrogenase all decreased, but acid production and phosphofructokinase activity increased. This latter enzyme from aerobic cultures was ATP-insensitive, but that from anaerobic cultures was ATP-sensitive.

In two consecutive papers on metabolic regulation in _E. coli_, Gray _et al._ (1966a, 1966b) examined the effect of aerobic and anaerobic growth on the localisation and type of enzymes. The group reported, in paper a, that aerobically-grown cells have many of the structural and enzymic properties of obligate aerobes, although the cytochromes are not typical. Anaerobically-grown cells retain many of the aerobic characteristics, including membrane-bound enzyme systems associated with the use of molecular oxygen. However, the anaerobic cells also form soluble enzymes similar to those of typical anaerobes. It is suggested that "this may afford the cells a considerable saving in

biosynthetic activity and enable rapid changes to take place under the influence of changing oxygen tension."

In paper b, the authors report that facultative anaerobes grown anaerobically and true anaerobes use the four-carbon dicarboxylic acid portion of the Krebs cycle in reverse for anaerobic electron transport. Under anaerobic conditions, energy is produced by glycolysis, and low but significant levels of Krebs cycle enzymes are still found.

Particularly relevant to the present work is the research which has been published on the effects of aerobic and anaerobic growth on the levels of the enzymes involved in glycerol dissimilation by Klebsiella aerogenes.

Lin et al. (1960), working on the effect of aerobic metabolism on the inducible glycerol dehydrogenase of Aerobacter aerogenes, gave evidence for inactivation of glycerol dehydrogenase by oxygen. They sampled cells for glycerol dehydrogenase activity throughout a batch culture; the activity started low and reached a maximum in the late log phase where DOT was lowest. Entry into the death-phase, after carbon-limitation was experienced, resulted in a sudden increase in DOT with a corresponding decrease in glycerol dehydrogenase activity. Evidence was also given for the irreversible destruction of this enzyme in aerobic culture, high levels only being built up in anaerobic culture. A dual aerobic-anaerobic metabolic route from glycerol to triose-phosphate was postulated in this paper. (See Section 2.3).

Neijssel et al. (1975) found, however, that active glycerol dehydrogenase was present in detectable amounts (approximately 10 units) in aerobic cultures of K. aerogenes grown on glycerol, providing that the culture was sulphate- or ammonia-limited. Glycerol kinase, however, was at immeasurably low levels (under 1 unit). When cultures were

glycerol-limited, aerobically grown cells contained high levels of
glycerol kinase (122 units) and low levels of glycerol dehydrogenase
(under 1 unit). Anaerobically grown cells contained high levels of
glycerol dehydrogenase (282 units), but low levels of glycerol kinase
(under 1 unit). (One unit of enzyme activity is defined as one
nanomole of substrate utilised per minute per mg. of protein).

Research on the effect of aerobic and anaerobic glycerol
metabolism on the levels of two other enzymes in K. aerogenes has also
been published. Ruch et al. (1974) found high levels of glycerol
kinase and glycerol-3-phosphate (G-3-P) dehydrogenase in aerobic
cultures grown on glycerol. Low levels of glycerol dehydrogenase and
dihydroxyacetone (DHA) kinase were found in these cultures. The
situation was reversed, however, in anaerobic cultures, although the
two aerobic enzymes did remain at significant levels. It is suggested
that the low levels of glycerol dehydrogenase in aerobically grown cells
may be the result of two kinds of control mechanism. In addition to
the energy-dependent inactivation when cells were shifted from anaerobic
to aerobic growth (Lin et al., 1960), specific repression of the gene
for glycerol dehydrogenase may also be taking place. DHA kinase, however,
is relatively stable after this transition. Ruch et al. also gave a
considerable amount of data on the effect of various carbon sources and
hydrogen acceptors on the levels of these enzymes in cells grown in
aerobic and anaerobic culture. The observations suggest the presence
of a regulatory control of the aerobic (G-3-P) pathway imposed by the
operation of the anaerobic (DHA) pathway.

In a paper on the genetic localisation of the enzymes of
aerobic and anaerobic glycerol catabolism in K. aerogenes (Ruch and Lin,
1975), a large number of assay results for the four enzymes is given.

Three genotypes of K. aerogenes and four different carbon sources were all used in both aerobic and anaerobic cultures. Wild-type (Strain 2103) cells grown on glycerol yielded the following assay results for the four glycerol related enzymes:

Aerobic - glycerol kinase, 380 units; G-3-P dehydrogenase, 100 units; glycerol dehydrogenase, under 1 unit; DHA kinase, 2 units.

Anaerobic - glycerol kinase, 3 units; G-3-P dehydrogenase, 8 units; glycerol dehydrogenase, 1,300 units; DHA kinase, 78 units.

(Enzyme activity units are defined as before). Thus, the major results are as reported previously, and further demonstrate two independent pathways of glycerol dissimilation; one aerobic, and the other anaerobic.

2.2.4.3   .....on amounts of Respiratory Enzymes

The effects of dissolved oxygen tension on the levels of cytochromes in a wide variety of microorganisms have been summarised by Harrison (1972 a). In all eukaryotes, including many microorganisms, the cytochromes transport electrons to the final electron acceptor, oxygen. The function of electron transport is to generate energy as ATP, the process being known as oxidative phosphorylation.

Early work on the influence of oxygen tension on cytochrome formation was carried out by Moss (1952) using continuous cultures of E. coli. Cytochromes a_1, a_2 and b_1 were all produced maximally in oxygen-limited cultures (0.1 atmospheres partial pressure of oxygen). When oxygen was in excess (0.75 atm.) cytochrome a_2 development was quicker, but reached a lower level than in the oxygen-limited cultures. Anaerobic cultures contained low levels of all three cytochromes.

Working with cultures of Aerobacter (Klebsiella) aerogenes, Moss (1956) found that cytochrome a_2 developed after aeration for several hours, but reached maximum levels when the dissolved oxygen

concentration was reduced from $10^{-3}M$ to $10^{-6}M$. The level of cytochrome b_1, however, was similar at all concentrations between these two values. Below $10^{-6}M$, levels of both cytochromes diminished to the anaerobic values. The author suggested that the diminished cytochrome a_2 content of highly oxygenated cells may be due to a lower requirement for cytochrome a_2 or a change-over of respiratory pathways to another system.

Also working with continuous cultures of K. aerogenes, Harrison et al. (1969) found that cytochrome a_1 and a_2 levels increased at low DOT, but cytochrome b_1 and b levels remained fairly constant. The same author (Harrison, 1972 b) found that the terminal oxidase (cytochrome a_2) level of K. aerogenes increased 200-fold on lowering the DOT from 5.3 mm Hg to under 0.4 mm Hg. Again, cytochromes b and o were little affected.

Thomas et al. (1972), investigating the transition from aerobic to anaerobic utilisation of glucose in E. coli, found that a decrease of oxygen partial pressure below 28 mm Hg firstly resulted in increases in the levels of cytochromes b_1 and a_2 before the electron transport system collapsed; they then decreased in activity together with other respiratory enzymes. Hino and Maeda (1966) found higher levels of cytochromes a_2, b_1 and a_1 in aerobically than anaerobically grown E. coli when the cells were not multiplying. Thus, the effect is unlikely to be due to selection, but may be due to control at the genetic level. Wimpenny and Cole (1967) reported that when E. coli was grown with nitrate as electron acceptor under anaerobic conditions, the cytochromes demonstrate the same pattern of induction as they do aerobically when oxygen is the electron acceptor.

Work on the effect of oxygen on the cytochrome levels of yeasts has been reported by the group led by Moss and Rickard. Moss et al.

(1969), working with continuous cultures of Candida utilis grown on glucose, found an inverse relationship between DOT and cytochrome concentration (notably cytochrome aa_3 and cytochrome oxidase). It is pointed out that oxygen represses cytochrome formation (Moss, 1956) and also inhibits haem synthetase (Porra and Jones, 1963). Moss et al. (1971) found that in continuous cultures of Saccharomyces carlsbergensis, high concentrations of glucose repressed cytochrome formation. The effect of oxygen concentration, when reduced from 188 to 3 μM, was to rapidly increase cytochrome levels. Cytochrome aa_3 concentration was highest at high DOT and low glucose concentration; when the glucose demand was satisfied, cytochrome aa_3 level was markedly reduced.

Rickard et al. (1971 a), working with batch cultures of S. cerevisiae and S. carlsbergensis, found that the concentrations of cytochromes a, b and c were dependent on oxygen concentration during growth, and also on initial glucose concentration. The cytochrome levels were maximal at low initial glucose concentrations for both species, and low oxygen concentration for S. cerevisiae, high oxygen concentration for S. carlsbergensis. The same group (Rickard et al., 1971 b), working with batch cultures of Candida utilis, reported similar results to those for the Saccharomyces species. Cytochromes a, b and c were usually decreased in concentration when glucose concentration was increased, the effect being exaggerated at low DOT. Generally, the change in cytochrome level with glucose concentration was also time-dependent; the effect was considerably more marked after 48 hours of culture than after 8 hours.

2.2.4.4 .....on the rate of respiration, respiratory oscillation

         and culture synchrony

Moss (1952) reported an early piece of work on the relationship
between oxygen tension and respiration in Escherichia coli. He found
an increase in respiration rate when this organism was grown in the
presence of oxygen, but reported that the respiratory activity of
aerobically and anaerobically grown cells did not show marked difference.
Hino and Maeda (1966) confirmed these observations, but showed that the
relatively small difference between aerobically and anaerobically grown
E. coli only applied when the substrate was glucose. The respiratory
activity of anaerobically grown cells was distinctly lower than that of
aerobically grown cells when formate, acetate, malate or succinate was
the substrate.

Much of the early work on the relationship between dissolved
oxygen concentration and respiratory activity appears to be rather
qualitative. However, a number of workers have found, when attempting
quantitative description of this relationship, that no simple rules can
be applied to all systems. It certainly appears that simple Michaelis-
Menten kinetics do not apply (Harrison, 1972 a), although a two-stage
Michaelis-Menten relationship has been demonstrated in a Pseudomonas
species. (Maclennan and Pirt, 1970). · Two different K_m values for
oxygen were observed; one at DOT values below 4.2 mm Hg (K_m = 0.2 mm Hg),
the other at higher DOT values (K_m = 1.2 mm Hg). Maclennan et al. (1971),
working with the same organism in methanol-limited continuous culture,
found that respiration rate increased as DOT decreased, to reach a
maximum at 3 mm Hg. It is suggested that for this organism to grow at
low DOT it requires more ATP, and satisfies this requirement by increasing
respiration rate in order to increase the rate of oxidative phosphorylation.

The response of respiration rate to DOT in a number of other microorganisms has also been shown to follow this seemingly anomalous pattern. Moss et al. (1969) found, in continuous cultures of C. utilis, that respiratory activity increased at low values of DOT providing that glucose was growth-limiting. The effect was not observed when glucose was in excess. Carter and Bull (1971) reported that continuously cultured Aspergillus nidulans exhibited increased potential respiration rate (i.e. respiration rate when exposed to excesses of oxygen and substrate) when grown at DOT values below 2 mm Hg. Batch-cultured Hemophilus parainfluenzae, an obligate aerobe, has been reported to show a similar pattern of response. (White, 1963).

Harrison and Loveless (1971 a), working with facultative anaerobes in chemostat culture, found higher respiration rates in cultures of E. coli and K. aerogenes where oxygen was limited than where oxygen was in excess. In a consecutive paper, these authors (Harrison and Loveless, 1971 b) found that when an anaerobic chemostat culture of K. aerogenes was converted to aerobic growth, both the in situ and potential respiration rates reached maxima at 2.5 hours after reaeration, and fell to steady values after 6 hours. They explained this as being due to a degree of uncoupling between growth and energy-conserving mechanisms.

Harrison and Pirt (1967) reported that decreasing the DOT in chemostat cultures of K. aerogenes below about 5 mm Hg resulted in increased respiration rates. When the DOT was increased to 5-10 mm Hg, the respiration rate decreased. It was suggested that decreasing the DOT to below 5 mm Hg acted as an uncoupler of oxidative phosphorylation. A decrease in respiration rate after an increase at low DOT was reported and said to be due to the build-up of an inhibitor's concentration.

Thus, oscillations of DOT and respiration rate took place in the transition area between excess-oxygen and limited-oxygen DOT values. The DOT range in this area was reported as 1-15 mm Hg. The period and amplitude of the oscillations varied with growth conditions.

Studying the biochemical basis of these oscillations, Harrison and Maitra (1969) found the ATP/ADP ratio in glucose-limited continuously cultured K. aerogenes to be constant above the critical DOT value. However, the ratio fell under oxygen-limited conditions, and reached a minimum in anaerobic cultures. Stimulated respiration rate, caused by lowering the DOT from 30 to 0 mm Hg for about 30 seconds, did not permanently affect the ATP level, indicating that a control system may operate to keep the ATP level constant. An explanation for this increased respiration rate during oxygen-starvation was also postulated: an alternative pathway for electrons to oxygen which by-passes one or more sites of oxidative phosphorylation functions, so causing a decreased ATP-production rate. A higher rate of oxidative phosphorylation is therefore required to keep the ATP level constant.

Degn and Harrison (1969) explained the oscillations of respiration rate in continuous cultures of K. aerogenes in a theoretical manner by analogy with the current in an electrolysis cell under potentiostatic conditions. The explanation is based on the experimentally established relationship between oxygen tension and culture respiration rate. Harrison (1972 b) reported that a pigment absorbing in the 500 nm region is transiently observed in chemostat cultures of K. aerogenes. It was suggested that this pigment might represent reactions which occur only at low oxygen tensions, and could possibly be involved in the mechanism for increasing respiration rates under oxygen-limiting conditions.

On applying an anaerobic shock to a controlled chemostat-culture of K. aerogenes, Harrison (1970) observed damped oscillations in pyridine nucleotide fluorescence (i.e. in NADH concentration) paralleled with oscillations of the same frequency in respiration rate. The actual frequency observed varied with the growth rate and culture conditions, but generally, lower frequencies were obtained under oxygen limitation. The oscillations were regarded by the author as a reflection of the regulatory system of the organism. After repeated anaerobic shocks, the number of oscillations increased but their damping decreased leading to undamped oscillations of pyridine nucleotide fluorescence. It was also noticed that if the glucose supply was cut, the oscillations stopped, but could be re-started by reconnecting the supply of glucose within about 10 minutes. If 15 minutes were permitted to lapse, the oscillations did not re-start until the culture experienced a further anaerobic shock.

It was also noticed by the author that the frequency of these oscillations changed with the metabolic state of the cells; they must, therefore, have intracellular origins. Moreover, when oscillations were stopped by cutting the glucose supply, they could be re-started by addition of succinate; a glycolytic intermediate was therefore probably not the main oscillator. A further important observation was that ATP level appeared to oscillate at the same frequency as pyridine nucleotide, indicating that oxidation reactions involved in the oscillations may be accompanied by phosphorylation. The phase relationships between oscillations in NADH and those in DOT were reported to be consistent with the control of respiration by NADH level. Furthermore, it was pointed out that the primary oscillator reaction probably involves feedback from the NADH or NADPH level, and lies off the main pathway

of glucose oxidation. No oscillations were detected when DOT was varied between 20 and 100 mm Hg, the range over which respiration rate and metabolism are independent of DOT. However, a little response was obtained at DOT values above the 10 mm Hg critical level, indicating that the cells were able to detect and react to DOT values above this critical level.

A rather interesting feature of this work concerned the observation that all the individual cells presumably oscillated in synchrony. It was pointed out that cells grown in chemostat culture possibly oscillate continuously, and the effect of the anaerobic shocks is to put them into synchrony. A mechanism for this synchronisation was postulated by the author. Dawson (1972), specifically working on continuously synchronised growth, reported that pulsed additions of a limiting nutrient to a chemostat culture at doubling-time intervals was found to synchronise the cell population. Under certain circumstances, therefore, pulsed aeration of a chemostat culture might be expected to induce culture population synchrony.

2.3   Utilisation of Glycerol by Bacteria

There are known to exist only two catabolic pathways by which glycerol is dissimilated in bacteria. Both involve two stages, a dehydrogenation and a phosphorylation, and differ only in the order in which these stages take place. Thus, in both cases the terminal product is dihydroxyacetone phosphate (DHAP), a compound which isomerises to glyceraldehyde phosphate, an intermediate of the Embden-Meyerhof glycolytic pathway. This apparently simple summary of all known modes of glycerol dissimilation in bacteria, however, wildly underestimates the complexity of the enzymology involved in the reactions.

In his very comprehensive review of bacterial glycerol dissimilation and regulation, Lin (1976) stressed the complexity of the enzymology of glycerol utilisation. He pointed out that certain organisms are able to utilise, in addition to glycerol, glycerol-3-phosphate (G-3-P) and/or dihydroxyacetone (DHA), the intermediates of the two pathways. Access of these compounds to the interior of the cell may require a unique permeation protein for each. Moreover, some bacteria are genetically equipped to exploit both pathways. They may therefore be capable of synthesising seven different enzymes, although environmental circumstances will usually dictate the necessity for only a portion of these. Lin (1976) also pointed out that considerable variations exist in the mechanisms of dehydrogenation. His review covers glycerol utilisation in the following bacteria: Bacillus subtilis, Escherichia coli K12, Gluconobacter oxydans, Halobacterium cutirubrum, Klebsiella aerogenes, Lactobacillus casei, Mycobacteria, Nocardia asteroides, Propionibacterium, Pseudomonas, Rhodopseudomonas, Salmonella typhimurium, Shigella flexneri, Staphylococcus aureus, and Streptococci. In this survey, however, attention will be focussed on

glycerol (and G-3-P and DHA) utilisation in two facultative anaerobes, E. coli.and K. aerogenes.

2.3.1   Entry of glycerol into cells

In all known cases, the permeation of glycerol into bacterial cells occurs by facilitated diffusion.   It has been argued that the intrinsic high permeability of phospholipid bilayers to glycerol, and the fact that maintenance of a physiologically significant concentration gradient of this subtrate may be energetically prohibitive, have left bacteria with little evolutionary incentive to develop an active transport system for the compound. (Lin, 1976; Lin, 1974).

In Bacillus subtilis, Saheb (1972 a) found that, normally, glycerol permeation is mediated by a carrier system which does not perform active transport, (i.e. against a concentration gradient). The author further found that glycerol kinase-negative mutants are unable to grow on glycerol, but could grow on G-3-P (Saheb, 1972 b). Thus, the phosphorylated intermediate can gain entry to the cell, even though it is unusual for phosphorylated species to pass through cell membranes.   (Hayashi et al., 1964).   The permeation is, therefore, catalysed by a G-3-P permease.   This enzyme is presumably inactivated by orthophosphate, since it has been reported that growth of B. subtilis on G-3-P can be strongly retarded by high concentrations of this ion. (Lindgren and Rutberg, 1974).

High permeability of E. coli cells to glycerol has been demonstrated by showing that osmotic pressure across the plasma membrane of the cells cannot be maintained when the cells are exposed to a hypertonic glycerol solution.   (Mager, et al., 1956).   More elaborate experiments, using similar principles, recorded rapid changes in turbidity

when cells were introduced to these solutions. The half-times of equilibration were 2 seconds for induced or constitutive cells (in the glycerol system), 10 seconds for non-induced cells, and 21 seconds for catabolite-repressed cells (Sanno et al., 1968). Thus, entry of glycerol into E. coli cannot be by simple diffusion since it is clearly inducible and presumably therefore expressed under the control of a gene.

Richey and Lin (1972) reported that this mechanism depends on the subsequent 'trapping' of the glycerol by an ATP-dependent phosphory-lation. They added, however, that the two gene products, the facilitor and the kinase, function independently. Hayashi and Lin (1965) also said that glycerol kinase is responsible for capture of glycerol from the medium, since mutants containing no kinase did not take up ^{14}C-glycerol. Nevertheless, these mutants can grow on L-α-glycerophosphate (Lin et al. 1962a). However, it seems possible that glycerol kinase-negative mutants would not be expected to take up significant amounts of glycerol even if the permeation is not directly controlled by the kinase. This is because glycerol would not be metabolised if no kinase enzyme were present, and if the compound is not transported against a concentration gradient, entry of glycerol would rapidly cease. Lin (1976) has reported that the absence of a concentrative mechanism for the cellular uptake of glycerol is indicated by the inability of all glycerol kinase-negative mutants to accumulate glycerol.

However, the ability to concentrate intracellular G-3-P by a mutant E. coli which is unable to metabolise the compound has also been reported by Lin (1976). Incubation of these mutant cells in 2 μM ^{14}C-G-3-P at 30°C resulted in a 1000-fold concentration of the substrate within 10 minutes. Further results, obtained using ^{32}P and ^{14}C isotopic labelling techniques, showed that neither cleavage nor exchange of the phosphoryl group occurs during the transport process.

Mutants defective in the active-transport enzyme, the G-3-P permease, cannot grow on G-3-P, but can still grow normally on glycerol and glucose-6-phosphate. (Hayashi et al., 1964).

Sanno et al.(1968) showed by the previously reported osmotic method that 1 M glycerol equilibrates across the membrane of induced K. aerogenes cells with a half-time of less than 2.5 seconds. It has also been shown that cells grown on glucose have an equivalent half-time of about 7 seconds (Ruch et al., 1974). Thus, as in E. coli, the permeation mechanism is inducible, although it is not yet known whether the two pathways in K. aerogenes (aerobic G-3-P and anaerobic DHA) share the same glycerol facilitator. However, dihydroxyacetone and glycerol may be sufficiently similar in structure (see Fig. 3.2) to share a facilitator protein (Lin, 1976). Ruch et al. (1974) showed that induced K. aerogenes cells are able to accumulate radioactive material when incubated with ^{14}C-G-3-P. Thus, entry of this material appears to be very similar to that in E. coli, i.e. active-transport catalysed by a G-3-P permease.

In summary, it appears that glycerol is facilitatively diffused into bacterial cells. Whilst requiring a facilitator protein, this is not an active-transport process since it does not operate against a concentration gradient. The process is, however, much more rapid than free diffusion, as indicated by entry half-time studies in induced and non-induced cells. Glycerol-3-phosphate is apparently actively transported into bacterial cells, as indicated by the concentration of this compound against a gradient by constitutive cells. The process requires a G-3-P permease enzyme.

2.3.2   Glycerol dissimilation

Studies on glycerol metabolism were originated by Magasanik and his co-workers   in the organism referred to as Aerobacter aerogenes, (i.e. Klebsiella aerogenes).   Magasanik et al. (1953) compared the dissimilation of glycerol in two strains of this organism; capsulated strain 1033 and acapsulated strain 1041.   Strain 1033 was found to dehydrogenate glycerol to dihydroxyacetone, whereas strain 1041 phosphorylated the substrate to L-α-glycerophosphate.   The key discovery of this early work was that strain 1033 does not require an exogenous hydrogen-acceptor, but strain 1041 requires oxygen for metabolism of glycerol.

Further studies by this group (Rush et al., 1957) confirmed these results and found pyruvate to be an intermediate in both strains for the complete oxidation of glycerol to carbon dioxide and water. Glycerol dehydrogenase was found to be inducible in strain 1033 but not in strain 1041.   Wiame et al. (1954) reported that in variants of Bacillus subtilis, glycerol is dehydrogenated to dihydroxyacetone. The latter is subsequently phosphorylated to DHAP, catalysed by the enzyme 'triokinase'.   They also found that the glycerol dehydrogenation reaction is reversible, and addition of excess DHA will cause a decrease in cell population as NADH is reoxidised.

Jacobs and van Demark (1960) compared the mechanism of glycerol oxidation in aerobically and anaerobically grown Streptococcus faecalis. Aerobic dissimilation was found to involve glycerol kinase, the resultant G-3-P being converted to DHAP by an FAD-stimulated oxidase. This enzyme reacts directly with oxygen, S. faecalis having no cytochrome system.   Anaerobic glycerol dissimilation depends on glycerol dehydrogenase working with DHA Kinase.   The dehydrogenation reaction

in this bacterium requires fumarate as the final hydrogen acceptor.
The authors further showed that aerobically-grown cells contain high
glycerol kinase and G-3-P oxidase activities, but low glycerol
dehydrogenase activity.   The reverse is true of anaerobically-grown
cells; a DHA kinase has not yet been demonstrated in vivo (Lin, 1976).
Thus, the reaction stages in this organism are basically the same as in
other bacteria examined here.   The enzymology involved, however, shows
some definite differences, mainly due to the absence of a cytochrome
system in the organism.   Thus alternative facilities for dealing with
reduced equivalents are required.

Lin and Magasanik (1960) found that an inducible NAD-linked
glycerol dehydrogenase from K. aerogenes was fairly heat-stable and had
maximum activity at pH 9.0.   They also found that the enzyme required
NH_4^+ and K^+ ions for activity; affinity for glycerol was greatly
increased by NH_4^+.   The enzyme was found to be inhibited by Zn^{2+} and
chelating agents.   Further studies on this enzyme, reported in a
consecutive paper (Lin et al. 1960) showed evidence that it is inactivated
by oxygen.   Thus, the presence of very low levels of glycerol
dehydrogenase activity in highly aerated cultures of K. aerogenes grown
on glycerol prompted the search for another enzyme which would
metabolise glycerol.   This led to the identification of a glycerol
kinase in this microorganism.

Zwaig and Lin (1966) found that fructose-1,6-diphosphate
(F-1,6-diP) is a feedback inhibitor of glycerol kinase in E. coli.
This may be important in preferential utilisation of glucose by this
organism since F-1,6-diP is an intermediate in glucose metabolism,
but not normally in glycerol metabolism.   They found that in wild-type
cells, glucose is effective in preventing glycerol utilisation.

Thus, when wild-type cells were inoculated into a medium containing a mixture of glucose and glycerol, classic diauxic growth occurred, with a lag period of about 1 hr. for adaptation to glycerol (Zwaig et al., 1970). The authors added that diauxie is no longer manifested if both repressor and feedback control of glycerol kinase are lifted by mutations. The activity of glycerol kinase was demonstrated to be rate-limiting in the metabolism of glycerol by E. coli; de-sensitisation of glycerol kinase to feedback inhibition was found to lead to faster growth than in the wild type, but may lead to possible substrate poisoning.

It has been found that, anaerobically, K. aerogenes can grow readily on glycerol, DHA or G-3-P as a sole source of carbon and energy. Glycerol and DHA can be utilised without exogenous hydrogen acceptors. Anaerobic growth on G-3-P, however, requires such acceptors (e.g. fumarate). Thus, it would appear that a G-3-P dehydrogenase may be produced anaerobically. The activity of this enzyme has been shown to be dependent on the presence of flavins, FAD and FMN. (Ruch et al., 1974). No stimulation of the G-3-P dehydrogenase enzyme from aerobically grown cells with flavins was found. It is therefore reasonable to assume that two separate enzymes operate for the aerobic and anaerobic dehydrogenation of glycerol.

Further evidence in support of this assumption arose from work on E. coli carried out by Kistler and Lin (1971). They found that a mutant of this organism, lacking normal L-α-glycerophosphate dehydrogenase necessary for aerobic growth on glycerol or L-α-glycerophosphate, can grow anaerobically using a soluble flavin-dependent L-α-GP dehydrogenase with fumarate or nitrate as exogenous hydrogen acceptor.

Double mutants lacking both dehydrogenases failed to show significant growth. Further work showed the product of the dehydrogenation reactions to be dihydroxyacetone phosphate (Kistler and Lin, 1972).

These various pieces of evidence for individual stages in the metabolism of glycerol, DHA and G-3-P in bacteria have recently been collated by two groups. Both focussed their attention on glycerol utilisation in Klebsiella aerogenes, and both have given a complete coverage of the parallel-pathway reaction schemes and their associated enzymology in this organism.

Ruch et al. (1974), in a paper on the regulation of glycerol catabolism in Klebsiella aerogenes, stated that this organism can metabolise glycerol by two linked pathways, one aerobic and the other anaerobic. The aerobic pathway involves phosphorylation of glycerol by an ATP-dependent kinase, to give glycerol-3-phosphate (G-3-P). The latter compound is then converted to dihydroxyacetone phosphate (DHAP) by dehydrogenases characteristic of flavoenzymes. In the anaerobic pathway, glycerol is converted to dihydroxyacetone (DHA) by an NAD-linked dehydrogenase, and the DHA is phosphorylated to DHAP by an ATP-dependent kinase. The presence of this latter pathway is said to allow K. aerogenes cells to grow anaerobically on glycerol in the absence of exogenous hydrogen acceptors. E. coli, lacking the enzymes of this pathway, is unable to do so. Evidence for the existence of separate kinase enzymes for glycerol and DHA is given in this paper.

The authors also present evidence on the genetic localisation of the operons for these pathways. The enzymes of the glp regulon, described for E. coli (Cozzarelli et al., 1968), are shown to be present

and inducible in K. aerogenes.   (These are the enzymes of the aerobic

G-3-P pathway).   Evidence of the separate regulation of the

expression of genes for the G-3-P and DHA pathways is also given.

Freedburg and Lin (1973) gave a detailed account of the control

mechanisms governing the expression of the members of the glp regulon

for glycerol and G-3-P in E. coli K12.

Ruch and Lin (1975) repeated the primary details of aerobic

and anaerobic glycerol metabolism in K. aerogenes that were reported

in the 1974 paper.   As in the 1974 paper, a diagrammatic representation

of the pathways is given, showing two G-3-P dehydrogenases, one

aerobic and the other, requiring flavins, anaerobic.   Two kinds of

constitutive mutants are reported to have been isolated; one affects

the aerobic and the other the anaerobic pathway.   Whilst it is known

that glycerol kinase and G-3-P dehydrogenase belong to separate operons

in E. coli, it was not possible to conclude from the results of this

work whether this is the case for glycerol dehydrogenase and DHA kinase

in K. aerogenes.

The second group reporting on the dual pathways of glycerol

metabolism in K. aerogenes is that of Neijssel et al. (1975).   The

organism reported to have been used by this group was strain NCIB 418,

the same strain as that used in the present work (see Experimental,

Section 5.1).   Thus, the results obtained by this group may be most

applicable to the research reported here.   The authors confirmed the

presence of the dual pathways reported previously.   However, no mention

is made of the existence of separate aerobic and anaerobic G-3-P

dehydrogenases in this organism.

The aerobic pathway involves a glycerol kinase with a high affinity for glycerol (apparent K_m is given as $1\text{-}2 \times 10^{-6}$M). The anaerobic pathway involves a glycerol dehydrogenase with a much lower affinity (apparent K_m of $2\text{-}4 \times 10^{-2}$ M). The simple aerobic/anaerobic differentiation of these two pathways, however, may not be fully justified. Evidence is given by these authors that whilst carbon-limited aerobically grown organisms use only the glycerol kinase pathway, aerobic sulphate- or ammonia-limited organisms apparently use only the glycerol dehydrogenase pathway. All anaerobic cultures used this latter pathway. Evidence for these conclusions is primarily given in the form of enzyme assay results of glycerol kinase and glycerol dehydrogenase for the variously-grown organisms.

However, a further piece of evidence concerned the glycerol oxidation kinetics of washed suspensions of aerobically-grown organisms. The glycerol-limited organisms displayed a high affinity for glycerol (apparent $K_m < 4 \times 10^{-4}$M), characteristic of glycerol kinase. The sulphate- or ammonia-limited organisms, however, had a much lower affinity for the substrate (apparent $K_m > 10^{-2}$M), characteristic of glycerol dehydrogenase. Thus, the rate of glycerol utilisation by cultures of K. aerogenes may be dependent on the kinetics of the first enzyme in the appropriate pathway. Unlike the other group (Ruch et al. 1974; Ruch and Lin, 1975), no assays of G-3-P dehydrogenase or DHA kinase, the second enzymes in the two pathways, were reported.

".... the demonstration of a DHA kinase, distinct from glycerol kinase, or the use of mutant analysis, may be important for the validation of the conclusion that strain NCIB 418 does depend on two separate pathways for the utilisation of glycerol." (Lin, 1976).

It is postulated that a critical factor in the modulation
between the two alternative pathways of glycerol metabolism may be
the intracellular concentration of the key intermediary metabolites, G-3-P
and DHA. Neijssel et al.(1975) suggest that the switch from the G-3-P
to the DHA pathway when glycerol is no longer limited may coarsely
regulate the rate of glycerol metabolism. Thus, the switch from the
high affinity (for glycerol) pathway to the low affinity pathway may
serve as a protective mechanism, avoiding the accumulation of
potentially-inhibitory metabolites.

The details of the dual pathways of glycerol metabolism in
K. aerogenes, derived from the literature quoted here, are explained
in Section 3.3 and displayed in diagrammatic form (Fig. 3.1).

2.3.3 Mutational manipulation of genes coding for related enzymes

Some work has been reported on the more 'artificial' methods
available for assurance of expression of selected genes coding for the
enzymes of glycerol metabolism. Thus, the required gene expressions
do not simply rely on a chemical species in the culture inducing the
production of these enzymes, as reported by Koch et al. (1964).

The techniques used to bring about the required changes in
genetic material are normally of two types. The intricacies of these
techniques are outside the scope of this work, but can be summarised
as follows: (a) Random mutation of existing genetic material by known
mutagenic agents (e.g. x-ray, nitrogen mustard, ethyl methane sulphonate)
followed by selective isolation of the required mutants in an appropriate
environment. (Lin et al., 1962b). (b) Selective transduction of
foreign genetic material into a host cell using a phage carrier.
Again, required mutants are selected in an appropriate environment.

Tanaka et al. (1967) working on the utilisation of mannitol, a compound bearing close structural similarity to glycerol, (but with a 6-carbon backbone),reported two pathways by which bacterial species dissimilate this compound. One involves phosphorylation by a phosphotransferase to mannitol-1-phosphate followed by dehydrogenation to fructose-6-phosphate. The other pathway, as in glycerol metabolism, involves the reverse of these two stages; dehydrogenation to fructose is followed by phosphorylation to fructose-6-phosphate. K. aerogenes is said to metabolise mannitol by the first reaction sequence. However, two genetic events in this organism (a loss mutation followed by constitutive mutation) can shift the mode of mannitol dissimilation to the other pathway. Thus, a phosphoenol pyruvate-dependent phospho-transferase is replaced by an NAD-linked dehydrogenase for utilisation of the substrate.

A similar enzyme replacement experiment, in E. coli K12, has been reported by St. Martin et al. (1977). This involves replacement of glycerol kinase by glycerol dehydrogenase using transduction techniques. As has been pointed out, both E. coli and K. aerogenes can grow anaerobically on glycerol, but E. coli requires an exogenous hydrogen acceptor. This study was intended to test whether the acquisition of an NAD-dependent glycerol dehydrogenase (at a sufficiently high level) by E. coli would enable it to grow anaerobically on glycerol with no exogenous hydrogen acceptors. It is reported that wild-type E. coli and glycerol kinase-negative mutants are able to grow on DHA. Therefore, only the one replacement should suffice to prove the point.

The original strain used in this work was a glycerol-negative mutant, strain 142, which has high levels of glycerol kinase.

Ethylmethane sulphonate mutagenesis, then alternate growth selection in appropriate media with progressively reduced glycerol concentration, and finally serial transfers into minimal medium containing 10 mM glycerol led to the production of strain 187, showing low glycerol kinase but high glycerol dehydrogenase activities. The high glycerol dehydrogenase trait of strain 187 is reported to have then been transduced in two steps by phage P1 into strain 204 which is impaired in the structural genes for glycerol kinase and aerobic G-3-P dehydrogenase, (as reported by Richey and Lin, 1972). The first transductant was selected on 100 mM glycerol, and then re-injected by the same phage preparation. The final transductant, strain 423, was selected on 10 mM glycerol.

The group reported that this final strain could not grow anaerobically on glycerol alone, but required exogenous nitrate. Fumarate did not function as hydrogen acceptor, NADH evidently being oxidised only by nitrate through the electron transport system of this mutant. The authors concluded that "the transfer of reducing equivalents from the substrate to NAD, instead of to a flavoprotein, as the first step of dehydrogenation reaction for glycerol dissimilation, cannot by itself account for the success of K. aerogenes 1033 to grow anaerobically on this three-carbon compound in the absence of an exogenous oxidant."

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CHAPTER 3

THEORY AND EXPERIMENTAL DESIGN

The theoretical basis upon which much of the experimental work was designed has been collated from a wide variety of sources. Although much of the work has been covered in the Literature Survey (Chapter 2), this section is intended to condense many of the reported facts which are particularly pertinent to this work into a useable form, and to show how they were adapted for experimental purposes.

3.1 Continuous Culture

3.1.1 Theory

It is essential to quantify the exact relationship between dilution rate and growth rate of microorganisms in continuous culture in order to fully appreciate the growth kinetics. Under the conditions of continuous culture, fresh medium is continuously introduced into the vessel at a constant rate. The culture volume is maintained at a constant value by simultaneous overflow of culture. The nett change in the concentration of organisms with time will therefore depend on the relative rates of growth and output of the culture.

Thus, change in biomass = growth - output.

i.e. $$\frac{dX}{dt} = \mu X - DX \tag{1}$$

where   X = cell density

t = time

μ = specific growth rate

D = dilution rate

and therefore $\frac{dX}{dt}$ = growth rate.

In a substrate-limited continuous culture, the value of μ is related to the concentration of the limiting nutrient (S) by Monod's adaptation of the Michaelis-Menten equation for enzyme kinetics (Monod, 1950). Thus:-

$$\mu = \mu_m \left(\frac{S}{K_S + S}\right) \qquad (2)$$

where μ_m = maximum specific growth rate, where no substrate is limiting.

K_S = the saturation constant, equal to the substrate concentration when $\mu = \mu_m/2$.

When steady-state conditions are established in the continuous culture, μ must equal D, since $\frac{dX}{dt} = 0$.

Thus, substituting in equation (2):-

$$D = \mu_m \left(\frac{S}{K_S + S}\right) \qquad (3)$$

For transient (or unsteady state) conditions, from equation (1):-

$$\frac{dX}{dt} = X \left[\mu_m\left(\frac{S}{K_S + S}\right) - D\right] \qquad (4)$$

The nett change of substrate concentration is given by another mass-balance equation:-

i.e. increase = input - output - $\frac{growth}{yield}$.

Thus:- $\frac{dS}{dt} = D.Sr - D.S. - \frac{\mu X}{Y} \qquad (5)$

where   Sr = substrate concentration in the fresh medium.

         Y = yield.

Thus, by combining equation (2) and equation (5):-

$$\frac{dS}{dt} = D (Sr-S) - \frac{\mu_m X}{Y} \left(\frac{S}{K_S + S}\right) \qquad (6)$$

Equations (4) and (6) completely define the behaviour of a continuous culture which obeys the fundamental growth relationships.

At steady-state, both $\frac{dX}{dt}$ and $\frac{dS}{dt}$ are zero, and substituting $\mu = D$:-

$$S = \frac{K_S . D}{\mu_m - D} \qquad (7)$$

By definition, X = Y (Sr-S), and therefore:-

$$X = Y\left[Sr - \frac{K_S . D}{\mu_m - D}\right]. \qquad (8)$$

If the values of the constants μ_m, K_S and Y are known for a given organism and growth medium, the steady-state concentrations of organisms and substrate in the continuous culture can be predicted for any value of dilution rate or inflowing concentration of the growth-limiting substrate.   This treatment has been shown to apply to the growth of <u>Aerobacter cloacae</u> (Herbert <u>et al</u>, 1956).

However, it has been shown that wall-growth within the culture vessel may lead to deviations from ideal behaviour.   (Luedeking, 1967). Topiwala and Hamer (1971) devised a mathematical model to predict such deviations at high dilution rate.   The general tendency is for wall-growth to extend finite biomass concentrations at dilution rates above

the maximum growth rate. Pirt (1975) reported that vigorous agitation often prevents wall-growth, although no perfect method for its prevention is known to date.

3.1.2   Experimental Design

3.1.2.1   Aerobic and intermittently aerated cultures

In order to investigate the relationships between carbon-limitation, oxygen-limitation and growth-limitation in continuous cultures of Klebsiella aerogenes, cultures were carried out at a series of impeller speeds over the full range of available dilution rates. Thus, from measurements of steady state cell density at each dilution rate and impeller speed, it was possible to construct a series of double substrate-limitation curves. The transition from carbon-limitation to oxygen-limitation as the dilution rate was increased beyond a threshold value at any particular impeller speed was recognisable mainly from the measurement of steady-state dissolved oxygen tension. Growth-limitation was recognised as a sharp reduction in cell density which accompanies a relatively slight increase in dilution rate. The same experiment was carried out in anaerobic continuous culture in order to determine the equivalent effects on the growth kinetics of the organism when no oxygen was available.

From the plots of cell density and dissolved oxygen tension against dilution rate at various impeller speeds, it was possible to select specific conditions of impeller speed and dilution rate to give known steady-state values of cell density and oxygen tension. Thus, selection of specific conditions to begin (fully aerobically) a series of intermittently aerated cultures was available. These conditions could be selected to represent a wide range of initial steady-state dissolved oxygen tensions. In practice, four intermittently aerated continuous cultures were performed in order to simulate the effects of

long cirulation times resulting from poor mixing, or poor aeration in a large fermenter. Conditions were available to select simulated poor mixing/poor aeration, poor mixing/good aeration, good mixing/poor aeration, and good mixing/good aeration. The well-mixed small fermenter could be considered as a completely homogeneous entity of about 10 cm equivalent spherical diameter circulating between regions of high and low oxygen-transfer in the large reactor. In all continuous cultures, cell density, dissolved oxygen tension, and the concentrations of carbon dioxide and oxygen in the exhaust gas were monitored.

3.1.2.2 Wall growth formation

A further series of experiments was undertaken to investigate the effects of agitation rate on the extent of wall growth at high dilution rates in aerobic continuous cultures of K. aerogenes. A film of biomass existed on the submerged surfaces within the fermenter at high dilution rate, preventing washout at dilution rates exceeding the maximum growth rate of the organism, owing to the attendant re-inoculation. The degree to which re-inoculation takes place is reflected in the culture cell density at dilution rates above D_c. (The critical dilution rate).

Assuming that all observed wall growth is sufficiently thin to allow fully aerobic metabolism by the cells involved, then the oxygen consumption of the entire culture is related to the total mass of cells. Thus, measurement of oxygen consumption at dilution rates in excess of D_c, and comparison with a plot of oxygen consumption vs. cell density where no wall growth occurred, allows estimation of the total mass of cells within the fermenter. (The plot of oxygen consumption vs. cell density can be derived from plots of oxygen consumption vs. dilution rate at a specific impeller speed, and cell density vs. dilution rate at the same impeller speed). Subtraction of the known mass of cells

in suspension (from cell density measurements) therefore permits estimation of the mass of cells adhering to static surfaces.

Experiments and calculations were carried out at a dilution rate greater than D_c (as determined in Section 6.1.3.2), and at four different impeller speeds. Thus, a plot of impeller speed vs. estimated mass of wall-growth cells was made. The method relies upon steady-state conditions being established, and aerobic metabolism by the wall-growth cells being possible.

3.2   Measurement of Oxygen Mass Transfer

3.2.1   Theory

In order to quantify the oxygen-transfer characteristics of the fermenter under different conditions of impeller speed and dilution rate, the calculation of the oxygen mass transfer coefficient, $K_L a$, is necessary. In the present work, this was determined by the oxygen balance method, the equation for which is:-

$$V_L \bar{N} = K_L a . V'(C^*-C).$$

- (McManamey, personal communication).

where:- V_L = volume of solution

$\bar{N}$ = rate of oxygen uptake

K_L = mass-transfer coefficient

a = interfacial area per unit volume of aerated solution

V' = total volume of aerated solution

C^* = solubility of oxygen in the solution.

C = concentration of oxygen in the solution

N.B. $V'-V_L$ = holdup volume

$\bar{N}$ is calculable from the oxygen balance as the air taken up as oxygen multiplied by the air flow rate.

C* is experimentally available

C is the proportion of C* given by measurement of dissolved oxygen.

Thus, $K_L a = \frac{V_L}{V'} \cdot \frac{\bar{N}}{(C^*-C)}$ (units of time^{-1})

This equation was used for all calculations of oxygen mass transfer by the steady-state oxygen balance method. Under known experimental conditions, however, many of the variables are replaced by fixed values in order to simplify the resultant calculations. This treatment naturally results in a highly empirical formula which only applies to specific fermenter conditions.

3.2.2   Experimental design

Experiments were performed on the laboratory fermenter to determine the ratio $\frac{V_L}{V'}$ under normal operating conditions of impeller speed and gas flow rate.

The solubility of oxygen in water was calculated from the empirical formula:- (Truesdale et al, 1955)

$$C_s = (14.161 + 0.00714\ T^2) - (0.3943\ T + 0.0000646\ T^3)$$

where C_s is the saturation concentration of oxygen in water (mg.l^{-1}) at T°C.

The use of this expression implies that the solubility of oxygen in the medium was equal to that in distilled water. Available evidence indicates this to not be unreasonable. Finn (1967) found the solubility of oxygen in water to be reduced by 15% in 0.5 M NaCl and 4% in 0.5 M H_2SO_4 at 25°C. The total concentration of ions (mainly sulphates and phosphates) in the culture medium used here was only about

0.1 M. Thus, a negligible reduction of oxygen solubility of ca.1-3% from that in distilled water is estimated, and may even be compensated for by a slight solubility increase caused by the 0.054 M glycerol in the medium. C* is thus calculated to be 6.86 mg O_2 l^{-1} at 37°C. (or 2.143 x 10^{-4} moles. l^{-1} oxygen). The actual concentration of oxygen in the solution, C, is directly proportional to this concentration, as indicated by the dissolved oxygen tension meter.

Air flow rate and the percentage of oxygen in the exhaust gas were also measured continuously under steady state conditions. From these measurements, and the calculated volume taken up by 1 mole of oxygen under the experimental conditions of temperature and pressure, the term $\dot{N}$ could be calculated.

Thus, under the normal operating conditions of V_L = 575 cm^3, V' = 625 cm^3, C* = 1.235 x 10^{-4} moles O_2, one mole of oxygen occupying 2.50 x 10^4 cm^3, and air flow rate of 575 $cm^3.min^{-1}$, K_La may be derived from the empirical relationship:-

$$K_La = \frac{1.713 \ (\% \ O_2)}{1.000 - (DOT \times 10^{-2})}$$

where   % O_2 = the percentage of oxygen in the exhaust gas.

DOT = the dissolved oxygen tension expressed as percentage of saturation.

In practice therefore, providing that the normal operating conditions quoted above are maintained, measurement of steady-state percentage oxygen in the exhaust gas and dissolved oxygen tension permits calculation of K_La by oxygen balance from this empirical relationship during continuous aeration. K_La has units of time^{-1}.

For conditions of intermittent aeration, it is convenient to define a 'time-averaged oxygen transfer coefficient', $(K_La)_0$. This is the value of K_La which would give a rate of oxygen transfer in a continuously aerated culture equal to the average rate at which oxygen is transferred under conditions of intermittent aeration. The value of $(K_La)_0$ is obtained by multiplying the fully-aerated K_La value by the fraction of the total cycle-time (0.2, 0.4, 0.6, 0.8 or 1.0) for which the culture is aerated. Owing to the inherent short-term unsteady-state of intermittently aerated cultures, $(\overset{\bullet}{K}_La)_0$ could not be determined directly, but 'time-averaging' is likely to result in reasonable approximations of oxygen transfer to the cultures.

3.3 Glycerol Metabolism in Klebsiella aerogenes 418

The information given here has been collated from a number of papers on this subject, especially those by Ruch et al. (1974), Ruch and Lin (1975) and Neijssel et al. (1975). The assay methods for the majority of enzymes involved in glycerol dissimilation in this organism are based on their known physiological biochemistry, and have been covered in a number of other papers (See Experimental Section 5.7).

3.3.1 Theory

3.3.1.1 Aerobic and anaerobic metabolic pathways

The aerobic and anaerobic pathways of glycerol metabolism in K. aerogenes 418 are shown diagrammatically in Figure 3.1. Figure 3.2 shows the structures of the compounds that are metabolically related to glycerol.

Aerobically, glycerol is phosphorylated by an ATP-dependent glycerol kinase (EC 2.7.1.30; ATP: glycerol 3-phosphotransferase).
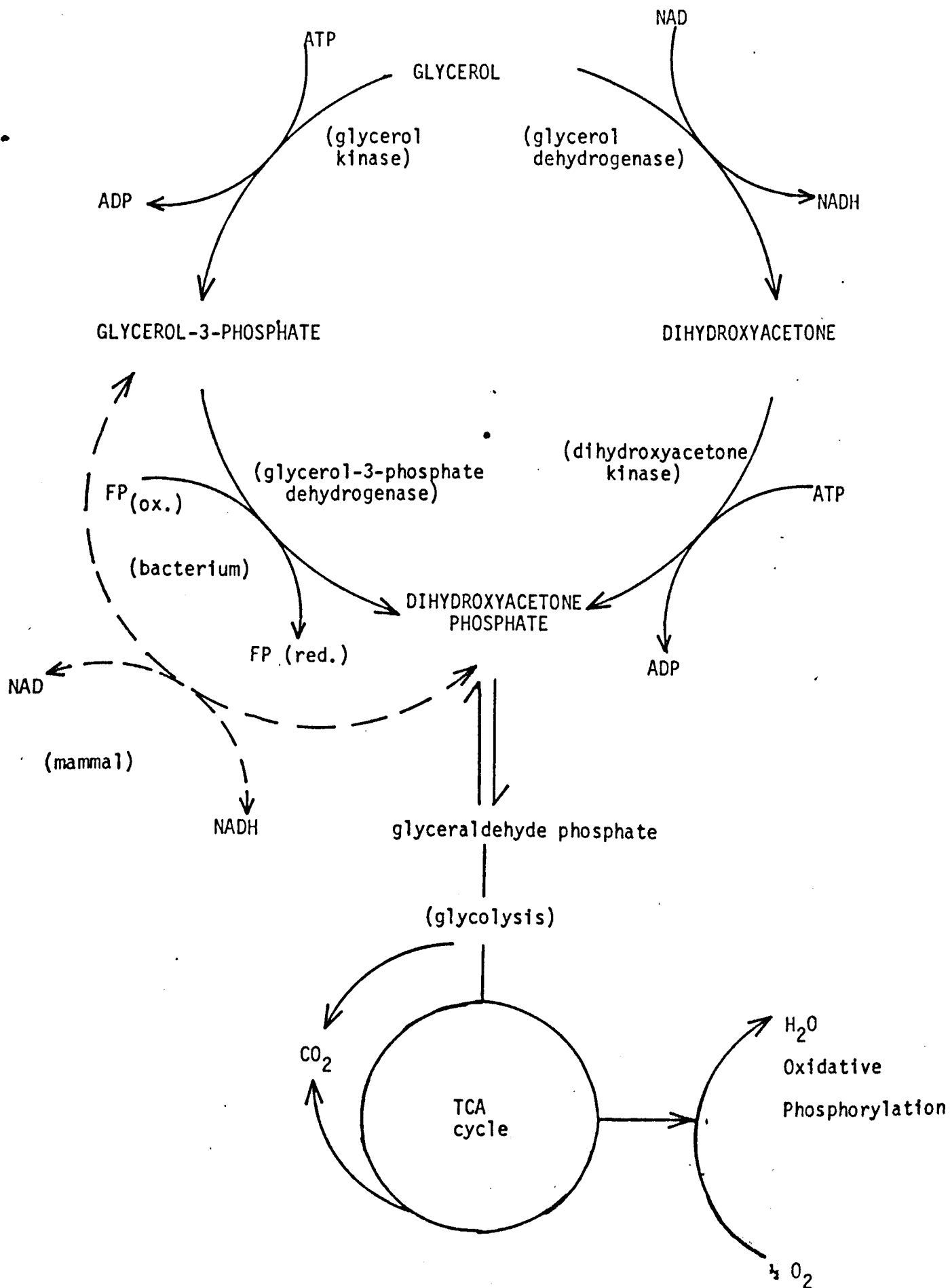
Figure 3.1   Pathways of Glycerol Metabolism
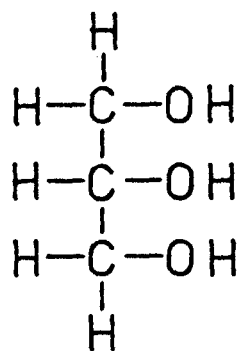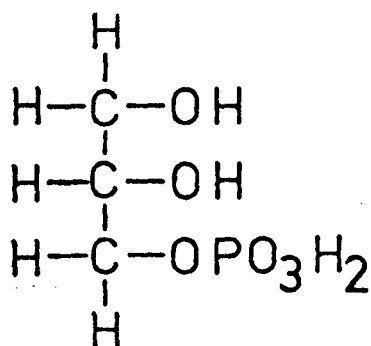
GLYCEROL

GLYCEROL-3-PHOSPHATE
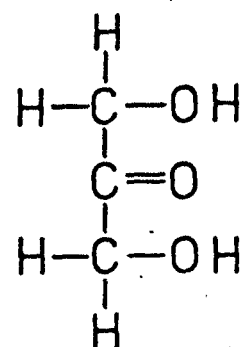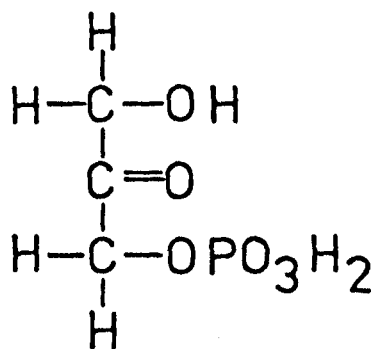
DIHYDROXYACETONE

DIHYDROXYACETONE PHOSPHATE
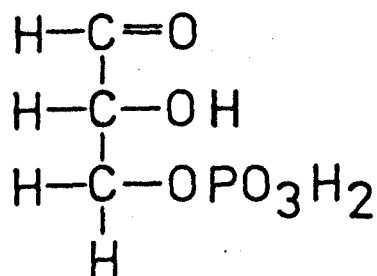
GLYCERALDEHYDE-3-PHOSPHATE

Fig. 3.2   Structural Formulae of Glycerol and Related Compounds

The resultant glycerol-3-phosphate is then dehydrogenated to dihydroxyacetone phosphate by a specific flavoprotein-linked glycerol-3--phosphate dehydrogenase (EC 1.1.99.5).

Anaerobically, glycerol is first dehydrogenated by an NAD-linked glycerol dehydrogenase (EC 1.1.1.6; Glycerol: NAD^+ 2-oxidoreductase). The resultant dihydroxyacetone is then phosphorylated by an ATP-dependent dihydroxyacetone kinase.

Thus, whether metabolism of the compound is aerobic or anaerobic, the common intermediate is dihydroxyacetone phosphate. This intermediate is then converted to D-glyceraldehyde-3-phosphate, an intermediate of the Embden-Meyerhof glycolytic pathway. Gluconeogenesis may also take place by reaction of dihydroxyacetone phosphate with D-glyceraldehyde-3-phosphate to give fructose-1,6-diphosphate, then fructose-6-phosphate, glucose-6-phosphate, and finally glucose. Normally, however, energy is yielded by glycolysis and (aerobically) the citric acid cycle and oxidative phosphorylation. Reduced NAD is presumably reoxidised by the reduction of metabolic intermediates in, for example, ethanol formation.

3.3.1.2   Assay of the enzymes of glycerol metabolism

The assay methods used for three of the four initial aerobic and anaerobic enzymes of glycerol metabolism in K. aerogenes 418 are based upon the reactions occurring in vivo. The fourth enzyme, glycerol-3-phosphate dehydrogenase, was assayed by linking its reaction with the reduction of a tetrazolium dye (MTT) via the mediator, phenazine methosulphate. (Kistler and Lin, 1971). Reduction of MTT was followed spectrophotometrically at 570 nm. A recorder, linked to the spectrophotometer, was arranged to plot absorbance against time,

the slope of the line being proportional to the rate of reduction of MTT
and hence to the activity of glycerol-3-phosphate dehydrogenase. Since
all enzymes are proteins, a reasonable standardisation method involves
expressing enzyme activity per unit weight of protein. Thus, in the
assay of this enzyme, activity is expressed as change of O.D. units per
minute per mg. protein.

Assays of glycerol kinase and glycerol dehydrogenase were
carried out consecutively on the same sample, the latter assay acting as
the baseline blank for the former. Since glycerol dehydrogenase is
an NAD-linked enzyme, and NADH absorbs strongly at 340 nm whereas NAD
does not, the reaction catalysed by this enzyme can be followed
spectrophotometrically at 340 nm. (Lin and Magasanik, 1960). From
the metabolic pathway chart (Figure 3.1), it can be seen that if the
enzyme is provided with glycerol and NAD, then the oxidation of glycerol
to dihydroxyacetone with the associated reduction of NAD may take place.
In practice, the cell extract containing the enzyme was buffered near the
optimum pH for the enzyme's activity. Glycerol was provided along with
NAD, reduction of the latter to NADH being plotted by the recorder as
increase in absorbance against time at a wavelength of 340 nm. Enzyme
activity could be expressed as μ.moles NAD reduced per minute per mg.
protein by converting change of O.D. units knowing the extinction
coefficient of NADH.

As has been pointed out, bacterial glycerol-3-phosphate
dehydrogenase is normally flavoprotein-linked. However, in mammalian
systems this enzyme is NAD-linked, and this property may be utilised
in the assays of glycerol kinase and dihydroxyacetone kinase in
K. aerogenes. Addition of a large excess of purified rabbit muscle
G-3-P dehydrogenase to an assay system therefore allows the oxidation

of glycerol-3-phosphate to dihydroxyacetone phosphate to be followed spectrophotometrically at 340 nm. With the enzyme in excess, the rate of reduction of NAD to NADH is dependent on the rate of provision of the substrate, glycerol-3-phosphate. The reaction is reversible, so the rate of oxidation of NADH to NAD is similarly dependent on the rate of provision of dihydroxyacetone phosphate.

Thus, in the assay of glycerol kinase, the cell extract was incubated with its substrate, glycerol, together with ATP, NAD, and rabbit G-3-P dehydrogenase. In practice, the glycerol dehydrogenase assay system was taken, and to it was added a large excess of rabbit G-3-P dehydrogenase. The system already contained equimolar concentrations of ATP and magnesium chloride, together with glycerol, NAD and cell extract in a buffered medium. (The pH optima of glycerol kinase and glycerol dehydrogenase are very close to each other). Thus, in addition to reduction of NAD by the action of glycerol dehydrogenase, the reduction of NAD as a direct result of the action of glycerol kinase was monitored, since the rate of provision of glycerol-3-phosphate depends on the activity of this latter enzyme. By simple subtraction of the gradient of the line attributed to glycerol dehydrogenase from that attributed to both enzymes, the activity of glycerol kinase was found, and similarly expressed as μ.moles NAD reduced per minute per mg. protein. This consecutive assay method is aided by the fact that samples which contain high glycerol kinase activity normally contain low glycerol dehydrogenase activity, and vice versa. Moreover, usage of the available substrates in both assays is negligible when compared with their overall concentrations.

The reversibility of the glycerol-3-phosphate ↔ dihydroxyacetone phosphate reaction, catalysed by the rabbit G-3-P dehydrogenase enzyme,

was used in the assay of dihydroxyacetone kinase. The cell extract was incubated with the substrate (dihydroxyacetone), NADH and ATP. Glycerol was also included as it is the preferred substrate of glycerol kinase, thus helping to eradicate difficulties caused by the dihydroxyacetone kinase activity of glycerol kinase, i.e. the active site of glycerol kinase may be 'saturated' with glycerol such that it does not phosphorylate dihydroxyacetone. This 'assay mix' was buffered at the pH optimum of the enzyme, and reduction in absorption against time at 340 nm was plotted. Thus, any reaction could be attributed to the reduction of dihydroxyacetone to glycerol, catalysed by glycerol dehydrogenase, and this served as the blank.

When rabbit G-3-P dehydrogenase was added, dihydroxyacetone phosphate provided by the action of the assayed enzyme could then be reduced to glycerol-3-phosphate, with the associated conversion of NADH to NAD and reduction of absorbance at 340 nm. Thus, subtraction of the blank line gradient from that obtained when an excess of the rabbit G-3-P dehydrogenase is present, allows calculation and expression of dihydroxyacetone kinase activity as μ.moles NADH oxidised per minute per mg. protein. Since high glycerol dehydrogenase and dihydroxyacetone kinase activities are normally found in the same sample, blank values can be as high as 50% of the total combined reaction rates.

It can be seen that, wherever possible, enzyme assays were based upon the actual reactions carried out in vivo. Thus, enzyme activities, not enzyme concentrations, were determined. This does not, however, apply to the assay of the enzymes of the cytochrome system. In this case, the absorption spectra of the enzymes themselves were determined, and the heights of specific absorbance peaks were measured. Thus, cytochrome concentrations could be expressed as picomoles per mg. protein,

although this gives little indication of the cytochromes' activities.

3.3.2   Experimental design

The assays of all four glycerol-related catabolic enzymes were carried out on all samples from the four intermittently aerated cultures. Cytochrome intensity spectra were measured in samples of cells grown aerobically and anaerobically.

Thus, the activities of the two aerobic and two anaerobic enzymes were measured in a large number of cell samples grown under a wide variety of aeration conditions.   These conditions ranged from fully aerated at high dissolved oxygen tension to fully anoxic, and included a number of 'modelled' systems.   For example, high aeration rate but poor mixing could be modelled by suitable selection of the intermittent aeration and culture conditions, and the aerobic and anaerobic enzymes in the resultant K. aerogenes cells could then by assayed.

From the results, it should be possible to correlate the oxygen-transfer condition under which a culture has been grown with its biochemical consequence in terms of the apparently preferred metabolic pathway.   Furthermore, the transition from aerobic to anaerobic glycerol metabolism in an intermittently aerated continuous culture as the aeration time per cycle is gradually reduced can be followed at the fundamental enzymatic level.   The trend of this transition may then be recognised.   If a sharp transition from aerobic to anaerobic metabolism is seen to occur as a result of a single step-change of aeration time per cycle, it may be possible to quantify the equivalent oxygen-transfer coefficient (or 'time-averaged' $(K_L a)_o$ value) at which this transition takes place.   Where the transition in enzyme levels is less abrupt, it may be possible to correlate the activity of an individual enzyme with the oxygen-transfer condition or 'time-averaged' dissolved oxygen tension.

CHAPTER 4

EQUIPMENT

The equipment used in this work was selected and constructed to facilitate highly-instrumented batch and continuous cultivation of microorganisms. Thus, the central feature was a fermentation unit with associated peripheral instruments for pH control, dissolved oxygen determination, and determination of oxygen and carbon dioxide in the exhaust gas stream. A multi-point recorder was included to continuously monitor these instruments' readings. Facilities for medium supply and spent-liquor collection were also included for continuous culture. In addition, air and oxygen-free nitrogen supplies were piped to the fermenter, and could be controlled by a solenoid-valve timer for intermittently-aerated cultures. (Plate 1).

A variety of equipment was used in the treatment and analysis of the bacterial cultures produced by the fermentation equipment. Cells were treated in a number of disruption and centrifugation units, and physical measurements were made with a wide variety of spectrophotometers.

4.1   Fermentation and Control Equipment

The fermentation unit, including its various in-built control equipment, pH control, and medium and gas supplies (and collection) are all that is required for controlled continuous culture. (Plate 2).

4.1.1   Fermentation unit

A bench-scale fermentation unit, the Bioflo Model C.30 (New Brunswick Scientific Co.Inc., New Brunswick, New Jersey, U.S.A.) was used for all fermenter cultures (Plate 3).

Oxygen
gas
analyser

Fermentation
Unit

Multi-
channel
recorder

Carbon
dioxide
gas
analyser

Control Equipment

Nitrogen and
CO_2/air gas
cylinders

Medium
supply
reservoir

Medium
pump

Spent
liquor
reservoir

Plate 1.   The Complete Culture and Gas Analysis Equipment

Plate 1.   The Complete Culture and Gas Analysis Equipment

Solenoid valve
timer

Dissolved
oxygen
analyser

Water-
trap
ice-
bath

Fermentation
Unit

Rotameters

pH
controller

Multi-channel
recorder

Ammonia
reservoir

Plate 2.   Fermentation and Control Equipment

Solenoid valve
timer

Dissolved
oxygen
analyser

pH
controller

Multi-channel
recorder

Rotameters

Ammonia
reservoir

Fermentation
Unit

Water-
trap
ice-
bath

Plate 2.   Fermentation and Control Equipment.

Exhaust gas

sampling

panel and manometer

Air and Medium
injector

Cooling
-water
constant
head

Hooded
sampler

Gas
sterilising
filters

Impeller
speed
controller

Probes

Gas-
liquid
separator

Solenoid
valve
(air)

Gas
supply

Fermention
Vessel

Manometer

Water
trap

Silica-
gel
gas
dryer

Temperature
Controller

Plate 3.   Fermentation Unit

Plate 3.   Fermentation Unit

Sampling Panel Type G S 96
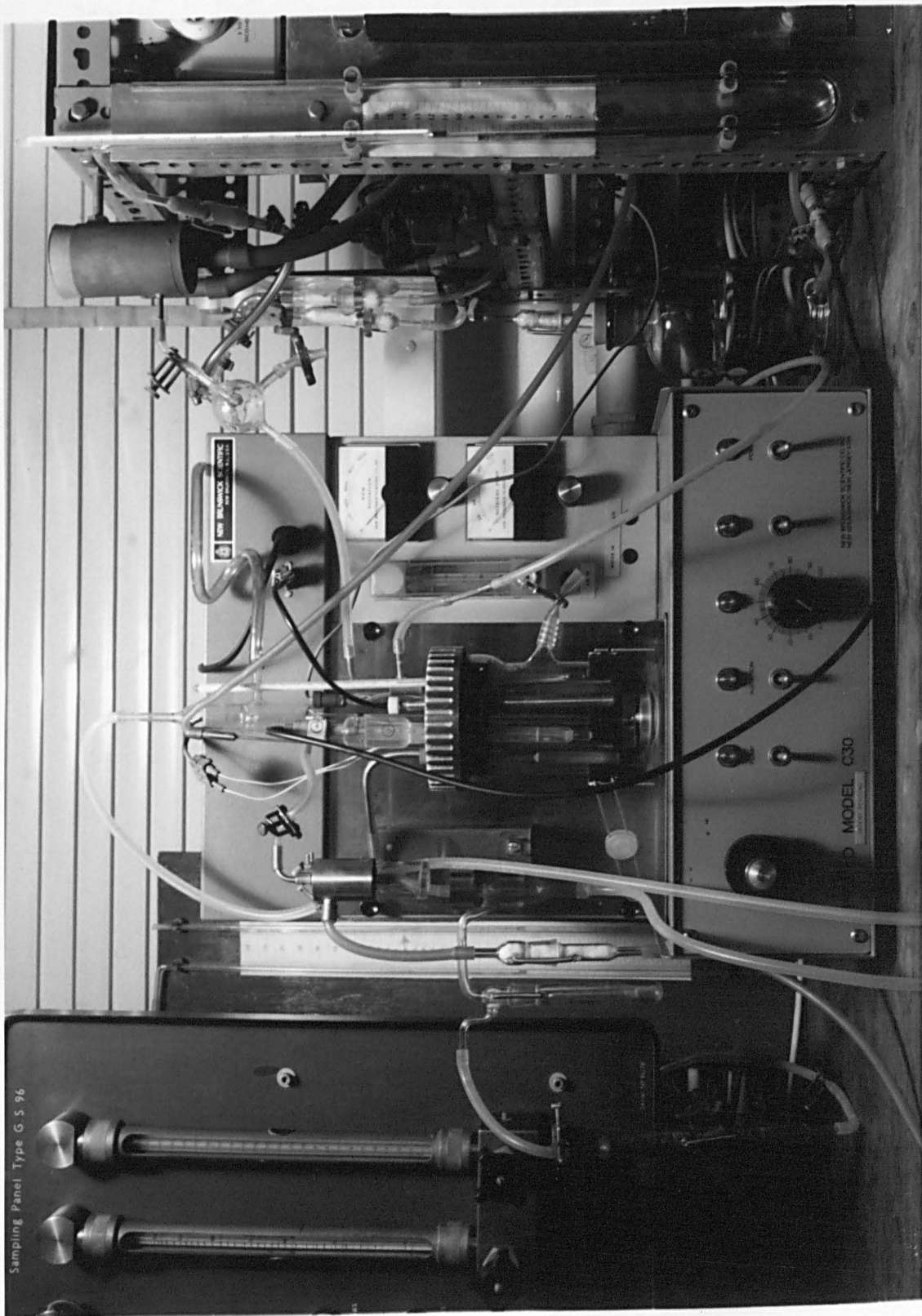
4.1.1.1   Fermentation vessel and culture

The fermentation vessel itself was constructed of heat-resistant glass, and designed for a working volume of 300 cm^3.   However, in this work, the weir-type overflow at this level was sealed, and the fermenter operated at its maximum volume of 575 cm^3.   Thus, fermenter head-space was kept to an absolute minimum (less than 5% total volume) to aid rapid aeration and deaeration by reducing back-mixing. The withdrawal of effluent gas and culture was achieved from the top-surface of the culture by entrainment of the liquid in the effluent gas stream through a stainless-steel tube (Herbert, Phipps and Tempest; 1965).   This tube extended vertically through the silicone-rubber fermenter-cap for about 10 cm, where it was turned through a right-angle to the horizontal, and led to a gas-liquid separator.

Culture samples were withdrawn from the fermenter by applying a negative pressure to a tube leading to the hooded-sampler.   Contamination was prevented by the presence of a glass-wool filter in the tube. Culture then flowed up a stainless-steel tube, through a silicone-rubber tube, and into a McCartney bottle.

4.1.1.2   Fermentation unit controls

Impeller-speed and temperature control were both contained within the fermentation unit.   A four-bladed impeller at the bottom of the vessel was magnetically coupled (6-pole) to a heavy-duty bottom-drive motor.   Impeller speed was continuously adjustable from 200 min^{-1} to 1000 min^{-1}, and was visibly checked on a meter calibrated in 20 min^{-1} units.

Culture temperature was checked by an accurate immersed mercury thermometer. A thermocouple in a stainless-steel pocket and temperature-controller in the fermentation unit governed the supply of power to a small 60 watt immersion heater in another pocket. The temperature controller was set using a calibrated dial on the front of. the unit, and checked with the thermometer. When necessary, the culture was cooled by passing cold water through an immersed cold-finger. The flow was controlled by a small constant-head device, and a visible drop-counter in the inlet-line.

In addition, air and medium pumps and controls were contained within the fermentation unit. However, these were not used in this work as (a) gas supplies were controlled by a solenoid valve timer, and (b) medium was pumped at dilution rates up to 1.40 h^{-1}, this being outside the range of the built-in pump for the higher fermenter volume.

4.1.2   Medium pump

A peristaltic flow inducer, Model MHRE 7 Mk. 3 (Watson Marlow Ltd., Falmouth, Cornwall) was used for pumping medium from the reservoir to the fermenter. The rate of rotation of the triple-rotor of this pump was continuously adjustable by a ten-turn control, giving, theoretically, one thousand possible settings.

In practice, using Silescol translucent silicone rubber tubing (Esco (Rubber) Ltd., London, W.1.) of bore 4.75 mm and wall-thickness 1.6 mm, the liquid flow rate was continuously adjustable from ca. 40 to ca. 820 cm^3 h^{-1}. Thus, with a culture capacity of 575 cm^3, dilution rates of between 0.07 h^{-1} and 1.4 h^{-1} were attainable. The pump was therefore calibrated such that any dilution rate could be selected simply by reference to the calibration graph and appropriately setting and locking the control.

The tube within the rotor was occasionally renewed by passing it a few centimeters through the tube-grips. This was to prevent fatigue-flattening under the stress conditions. In addition, it was lubricated with a smear of glycerol.

4.1.3   pH control

The culture pH was maintained at 6.5 by use of a Model RC pH recorder and controller (Analytical Measurements Ltd., Feltham, Middlesex). Upper and lower set-points were available, governing the supply of acid or alkali to the fermenter. In practice, however, only the lower set-point was used as all cultures continually produced acid. The controller also included a small strip-chart pH recorder. The pH controller received its signal from an autoclavable 20 cm Pye-Ingold combined pH electrode. (W.G. Pye Ltd., Cambridge). This electrode was tightly held in the silicone-rubber fermenter cap, and fed its signal to the controller via an autoclavable coaxial lead. The lower set-point of the controller governed a Micropump (Distillers Co.Ltd., Engineering Division, Epsom, Surrey) which supplied sterile 2M ammonia solution from a one-litre reservoir. The ammonia solution was obtained by aseptically adding 100 cm^3 of 0.880 ammonia to 900 cm^3 of sterile deionised water in the reservoir.

4.1.4   Gas supplies

The equipment was constructed so that air or oxygen-free nitrogen could be supplied to the fermenter. Under conditions of intermittent aeration these two gases were supplied alternately over a five-minute cycle, governed by the solenoid valve timer.

Gas supply-rates are expressed as specific gas flow-rates, and have units of min^{-1}. Specific gas flow-rate is defined as the volume of gas sparged into the fermenter as a fraction of the liquid volume in the fermenter per unit time. Under normal operation conditions, 1.0 min^{-1} is equivalent to 575 cm^3 min^{-1} gas flow-rate.

4.1.4.1 Gas Pre-treatment

Compressed air at 25 p.s.i., from the main line of the laboratory, was de-humidified and its pressure reduced by a regulator to 3 p.s.i.   It was then passed through a needle-valve and rotameter and either a manual or solenoid valve.   Sterilisation then took place by passage through a glass-wool packed filter.   White-spot grade oxygen-free nitrogen (British Oxygen Company, London W.6) was supplied from a standard pressure cylinder and treated in an identical, though separate, system to the air.

The two gas-lines met at a Y-junction downstream from the sterilising filters, and passed through a single flexible polythene tube to the air and medium injector.   This injector, constructed in heat-resistant glass in the Chemical Engineering Department glass-blowing workshop, was designed to allow simultaneous medium addition and gas-sparging at the centre of the impeller.   (Figure 4).   Medium entered at the top, and passed through two small orifices designed to prevent grow-back in the medium line.   The gas entered through a side-arm below the lower orifice.   Gas-supply pressure to the fermenter was checked on a mercury manometer connected, via a T-junction and sterilising filter, to the common flexible supply-line.

All gas-lines were constructed of copper or polythene tubing, depending on the pressure they were required to withstand.   Thus, oxygen-diffusion into the gas-supplies was effectively eliminated.

4.1.4.2 Solenoid valves and timer

The supply of air or nitrogen to the fermenter under conditions of intermittent aeration was governed by a pair of timer-controlled

Trist-Lucifer solenoid valves.   (Ronald Trist & Co.Ltd., Slough).

The timer unit, built in the Chemical Engineering Department electronic workshop, had two controls to time the opening of the two alternate solenoid valves.   Thus, as one timer completed its cycle, that valve was closed, the other opened, and the other timer then immediately started its cycle.   In this way, each valve could be set to open for any time from about five seconds to twelve minutes.   In practice, though, the total cycle-time of both valves was always fixed at five minutes.

4.2   On-Line Analysis

In order to gain instant information on the performance of a continuous culture, and necessary data for the calculation of oxygen-transfer and respiratory parameters, three determinations were made and recorded automatically and continuously.   The flow paths for the gas from the fermenter to the atmosphere via the analysers is shown in Figure 4.

4.2.1   Dissolved-oxygen analyser

Dissolved-oxygen tension was measured using a galvanic oxygen electrode which fed its signal to a dissolved-oxygen analyser which then fed the amplified signal to one channel of the multipoint recorder.

4.2.1.1   Oxygen electrode

Autoclavable oxygen electrodes, constructed in the Chemical Engineering Department workshops, were used for all measurements of dissolved-oxygen tension.   The electrodes operated galvanically by an electro-chemical reaction at a lead anode and silver cathode in acetate electrolyte.
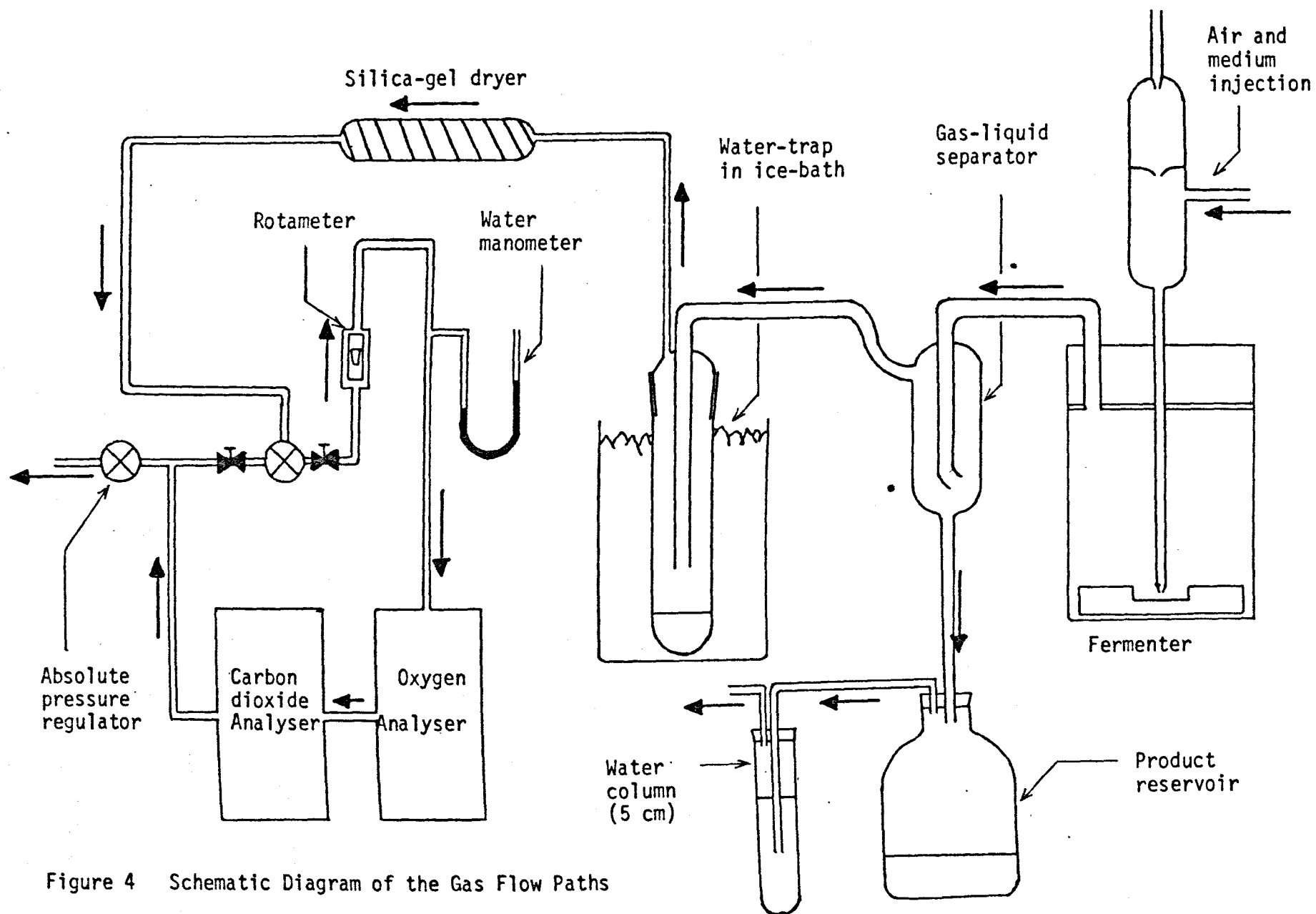
Figure 4   Schematic Diagram of the Gas Flow Paths

(Not drawn to scale)

The probes were designed and constructed along the lines of those by Elsworth (1972). The composition of the acetate electrolyte was also by Elsworth. (5.0 M Acetic acid, 0.5 M Sodium acetate, 0.1 M Lead acetate; pH ca. 3.0). Teflon and, later, polypropylene membranes both of 0.002 inch thickness were used on the probes. The response of the electrode is limited by diffusion of oxygen through the membrane, and polypropylene did appear to give a marginally improved response-time.

Elsworth reported that over the 0 to 0.21 atm. oxygen-tension range, the current from the electrode is proportional (±2%) to dissolved-oxygen tension. Thus, dissolved-oxygen measurement and recording is linear, no correction factor or calibration curve being required.

4.2.1.2   Dissolved oxygen meter

A New Brunswick Model D.0.40 dissolved-oxygen analyser was used in this work. The function of this analyser was simply to convert the signal from the oxygen electrode to a useable form. Thus, the meter was calibrated to read % saturation with respect to the uninoculated medium. This reading was fed directly to one channel of the recorder.

4.2.2   Gas analysers

The effluent gas stream, together with overflow culture passed out of the fermenter through a draught tube which lead to a gas-liquid separator. The separator, constructed from Pyrex glass in the Chemical Engineering Department glass-blowing workshops, was designed to direct the effluent-flow down a silicone-rubber tube to the collection reservoir. However, a small back-pressure, provided by a 5 cm. column of aqueous disinfectant sealed to the collection reservoir, forced a proportion of the gas from the separator into a connected water-trap in an ice-water

bath. The gas then passed through a cotton-wool filter and small silica-gel dryer to the gas analysers' sampling panel. The volume of this gas-treatment equipment was kept to an absolute minimum in order to maintain fast response under conditions of intermittent-aeration. (Fig.4).

The type G.S. 96 sampling panel (Servomex Controls, Crowborough, Sussex) was included to supply a constant 100 $cm^3.min^{-1}$ of gas at constant pressure to the analysers. Excess gas was vented to the atmosphere via an absolute-pressure regulator. Gas-flow to the analysers was controlled by a needle-valve, and monitored on the panel's rotameter. Its pressure was measured by a water manometer.

4.2.2.1   Oxygen gas analyser

The volume percentage of oxygen in the exhaust gas was measured by means of a D.C.L-Servomex Type 83 oxygen-analyser. (Servomex Controls Ltd.).

Operation of the analyser is dependent on the paramagnetic property of oxygen. The analysis cell is enclosed by a magnetic field, and within this cell, a small magnetically-susceptible glass dumbell is suspended. The position of the dumbell is governed by the paramagnetic nature of the surrounding gas and a balancing force imparted on the suspension mechanism. Thus the current required to maintain the dumbell in a constant position is measured and displayed on the meter by a null-balance mechanism. Hence, the current is directly proportional to the oxygen concentration, and the meter's response is therefore linear. No correction factor or calibration curve was required, the analyser simply being set at 0% and 19.95% oxygen with appropriate calibration gases.

The range of percentage oxygen gas concentration fed to one channel of the multipoint recorder could be selected as 0-20% or 1.0-21%, or any 5% 'band' within the 0-25% range.

4.2.2.2   Carbon dioxide gas analyser

The volume percentage of carbon dioxide in the exhaust gas was measured using an infra-red gas analyser (I.R.G.A.) Model SB 2.   (Sir Howard Grubb Parsons & Co.Ltd., Newcastle-upon-Tyne).

The property of the carbon dioxide molecule to absorb radiation of infra-red wavelengths is utilised in the principle of operation of this instrument.   The gas, passing from the oxygen analyser, passes through a 5 mm path-length optical cell.   An infra-red beam passes through this cell, and another beam through an optically identical system with carbon dioxide-free gas in the reference cell.   The difference in energy between the two beams is then measured by a sensitive detection system, this difference being proportional to the energy absorbed in the sample cell.   The energy difference is converted and displayed on a meter, and is representative of the number of carbon dioxide (or other infra-red absorbing) molecules in the sample cell.   Thus, the measurement of carbon dioxide depends not only on the proportion of this gas, but also the gas pressure.   Precautions were taken to remove water vapour, which also absorbs infra-red radiation, from the exhaust gas.

The meter-reading was found not to be linearly dependent on carbon dioxide concentration in the sample-gas.   Thus, calibration as outlined in Section 5.6.4.1 was carried out and a calibration curve constructed.   (Fig. 5.6.5).   The signal from the meter was fed into one channel of the multipoint recorder.

4.2.3   Data collection and storage

Dissolved oxygen tension, oxygen gas and carbon dioxide gas concentrations were all continuously recorded on a Watanabe Model MC 641 multi-channel recorder.   (Manufactured under license and supplied by Environmental Equipments Ltd., Wokingham, Berkshire).

Each channel of this instrument governed a different coloured ink pen, these pens being offset from each other by 5 mm.   Thus, cross-over facilities and hence full chart-width was available for each pen. Ease of chart-reading was facilitated by z-fold chart paper.   Moreover, continuous operation was aided by the availability of very low chart speeds.

Increased resolution of features was made possible by decreasing the mV range of the parameter being measured, and increasing the chart speed.   Thus, fine-structure analysis of dissolved-oxygen peaks under conditions of intermittent aeration was made by this method.

4.3   Analytical Equipment

A number of analytical procedures were carried out on culture samples removed from the fermenter.   These analyses invariably involved the use of one of a number of spectrophotometers.   Pre-treatment of cultures by a variety of techniques (e.g. centrifugation, cell disruption, etc.) was usually necessary before a spectrophotometric analysis procedure could be carried out.   This "ancillary" equipment is therefore included in this section.

4.3.1   Spectrophotometry

Absorbance measurements made at visible and near-U.V. wavelengths were usually made using a Pye-Unicam SP 600 Series II spectrophotometer.   (Pye-Unicam Ltd., Cambridge).   Wavelength and filter-type were manually set on this instrument, ease of operation for individual samples being its main advantage.   This instrument was therefore used for routine cell density measurements, as well as single absorbance measurements in protein determinations, etc. Pye-Unicam glass cells were employed for absorbance measurements in the visible spectrum.

The glycerol-related enzyme assays were carried out using a Pye-Unicam SP 500 Series II visible-U.V. spectrophotometer.   Wavelength was set manually, with automatic changeover from tungsten lamp (in the visible spectrum) to deuterium lamp (in the ultra-violet).   The instrument was used essentially for measurement of change in absorbance with time at a fixed wavelength in an enzyme-assay system.   It was therefore linked to a Pye-Unicam AR 45 linear-logarithmic recorder which plotted absorbance (up to 1 decade on the logarithmic function) using the set chart-speed as a time-base.   Silica-quartz cuvettes (Frost Instruments Ltd., Wokingham, Berkshire) were used to contain the assay systems; they were maintained thermostatically at 37°C by a Pye-Unicam SP 507 constant temperature cell holder with water supplied at constant temperature from a small Grant SB 1 water-bath.   (Grant Instruments Ltd., Cambridge).

Cytochrome concentration measurements were made using a Perkin-Elmer 356 double-beam spectrophotometer.   (Perkin-Elmer Ltd., Beaconsfield, Bucks.).   Wavelengths were set equal on each beam, and the instrument used to automatically scan two samples simultaneously

between two selected wavelengths, normally in the visible spectrum.
The output from one channel minus that from the other was plotted on
a Perkin-Elmer recorder. The instrument had cryogenic facilities for
obtaining low-temperature spectra in liquid nitrogen. (at - 196°C).

More conventional scanning of the visible-U.V. absorption-
spectrum of a sample was carried out using a Pye-Unicam SP 1800
ultraviolet spectrophotometer and AR 25 recorder. This single-beam
instrument was used to determine the absorption spectra of culture
sample supernatants in silica-quartz cuvettes.

Fluorescence spectrophotometry was carried out on a culture
sample supernatant in order to gain information on the nature of a
fluorescent compound apparently excreted into the medium. The
instrument used for this analysis was a Perkin-Elmer Fluorescence
Spectrophotometer 204. Excitation and fluorescence spectra were scanned
manually. The sample was contained in a silica-quartz cuvette.

4.3.2   Centrifugation

Separation of components of different densities in a mixture
is most easily carried out by enhanced gravitational forces in a
centrifuge. The method was normally used for separation of bacterial
cells from their suspending medium, although it was also occasionally
used for separation of intra-cellular components following cell
disruption.

An M.S.E. High Speed 18 refrigerated centrifuge (M.S.E.
Scientific Instruments Ltd., Crawley, Sussex) was used routinely for
this purpose. The bowl of the centrifuge was maintained thermostatically
at the set temperature, normally 0°C. Two rotors were used on this

instrument, choice of rotor depending on the size of sample to be centrifuged. At the maximum speed of 18,000 r.p.m., the 6 x 100 cm^3 rotor developed 40,000 G. at its periphery, and the 16 x 15 cm^3 rotor, 38,000 G. Bowl-temperature and rotor-speed were both set manually and maintained automatically, as was centrifugation time.

For quick centrifugation procedures not requiring high G-forces or thermostatic operation (as in precipitate separation), an M.S.E. Super Minor centrifuge was used. Using four buckets each, of 4 x 15 cm^3 capacity, the maximum G-force available from this bench-scale machine approached 3,000 G. M.S.E. polypropylene capped centrifuge tubes were used in both centrifuges.

4.3.3 Cell disruption

Disruption of K. aerogenes cells was normally carried out by sonication of the bacterial suspensions. For this purpose, a Dawe Type 7530 A Soniprobe sonicator was used. (Dawe Instruments Ltd., London, W.3). Eight energy-levels were available on this instrument, each level being tunable for optimal energy output. A stainless-steel probe with titanium tip transmitted the energy from the unit into the sample suspension. Cooling of the sample in an ice-water bath was normally necessary owing to the relatively large amount of energy dissipated throughout the suspension, which had a minimal volume of about 30 cm^3.

In addition to sonication, which acts by creating "cavitation" within the liquid, cell disruption was occasionally carried out by techniques where the sample was subjected to very high rates of shear. The advantage of these techniques was that small volumes of very thick cell suspensions could be disrupted. (These could not be disrupted by sonication). For this procedure, a Model 512 compressed air-driven

disrupter (Stanstead Fluid Power Ltd., Stanstead, Essex) or small
French press was used.

4.3.4   Other ancillary equipment

A number of pieces of laboratory equipment were routinely used
in this work.   In enzyme assay procedures, small volumes of liquids
were dispensed by a Pipetman P200 adjustable microlitre pipette
(Techmate Ltd., Wheathampstead, Herts.).   This micro-pipette was fully
adjustable to dispense any volume from 10 to 200 μl from disposable tips.

A Stanton Unimatic CL 5D balance (Stanton Instruments Ltd.,
London, S.W.17) was used for accurate laboratory weighing.   This
instrument, especially useful for weighing very small amounts of
biochemicals, was used with dessicant in the balance-chamber.   The
sensitivity of the balance was 0.01 mg. (10^{-5} g).

A Dynacap pH-meter (W.G. Pye Ltd., Cambridge) was used for
routine pH measurement.   This meter had the advantage of range-selection
such that 0-10, 4-14, or any two pH units could be selected for full
scale deflection.   Thus, accurate measurement of pH between pH 6.0 and
pH 8.0 was available by selection of the 6-8 range and calibration with
a pH 7.0 buffer.

4.4   Chemicals and Biochemicals

Chemicals used in the culture media were normally of high
(or analytical) grade purity.   The basic inorganic chemicals were
obtained from B.D.H. Chemicals Ltd., Poole, Dorset and from Fisons
Laboratory Reagents, Loughborough, Leicestershire.   These included
ammonium sulphate;   sodium and potassium phosphates;   iron, magnesium,
sodium and calcium chlorides;   iron, manganese, zinc and copper sulphates;

boric acid, sodium hydroxide, and ammonium molybdate. Glycerol was obtained in 5-litre Winchesters as an analytical-grade reagent as were concentrated hydrochloric acid and 0.880 ammonium hydroxide.

Organic chemicals, e.g. citric acid, acetylacetone, acetate salts, etc. were obtained from the same companies. Nutrient agar was obtained from Oxoid Ltd. Folin-Ciocalteau reagent and sodium potassium tartrate, used in the Folin method for determination of protein, were obtained from Fisons Analytical Reagents Ltd. Sodium arsenite and potassium cyanide were also obtained from these suppliers and stored in a locked poison cupboard.

A number of chemicals and biochemicals were required for enzyme assay work. These were often obtained in very small amounts (as little as 25 mg) and most were heat-labile. Suitable storage facilities at 4°C and -20°C (both dessicated) were therefore used where necessary.

Sigma Chemical Co.Ltd., Kingston-on-Thames, Surrey, supplied the following:- Adenosine-5'-triphosphate, bovine serum albumin, dihydroxyacetone, glycine, hydrazine dihydrochloride, MTT tetrazolium dye, phenazine methosulphate, DL-α-glycerophosphate, triton X-100 and Sigma 7-9 tris buffer.

Boehringer Corporation Ltd., Lewes, Sussex, supplied:- β-nicotinamide adenine dinucleotide (oxidised and reduced forms), flavin adenine dinucleotide, flavin mononucleotide, and the rabbit-muscle enzyme glycerol-3-phosphate dehydrogenase.

Compressed gases, 'white-spot' grade oxygen-free nitrogen and the mixture 5% carbon dioxide: 95% air, were obtained from the British Oxygen Company Ltd., London W.6. 'Silcolapse' food-grade silicone antifoam was obtained from I.C.I., Billingham.

CHAPTER 5

EXPERIMENTAL

5.1 Stock Cultures

Klebsiella aerogenes N.C.I.B. 418 was the sole bacterial species used in the experimental work. Stock cultures of this facultative anaerobe were maintained at 4°C on nutrient-agar slopes, and sub-cultured monthly. No deterioration in viability was apparent over the period of this work.

5.2 Culture Medium

A synthetic medium, adapted from that used by Tempest, Hunter and Sykes (1965) and Carrizales (1974), was used for all cultures in the fermenter.

Glycerol, the sole carbon and energy source, was present in the medium at a final concentration of 5.0 g.l^{-1} (0.054 M).

Other medium-constituents were:-

Citric acid: 0.001 M

Ammonium sulphate: 0.05 M

Potassium dihydrogen orthophosphate: 0.045 M

Disodium hydrogen orthophosphate: 0.005 M

Magnesium chloride: 0.00125 M

Calcium chloride: 0.0001 M

Ferric chloride: 0.0001 M

In addition, micronutrients containing a number of essential ions were included in the medium. Used by Wase (1965), the following amounts were made up to 100 cm^3 with distilled water, and maintained in solution at low pH by the inclusion of about 10 cm^3 of concentrated hydrochloric acid.

$FeSO_4.7H_2O$:   0.25 g

Boric acid   :   0.10 g

Ammonium molybdate: 0.02 g

$MnSO_4.4H_2O$:   0.04 g

$ZnSO_4.4H_2O$:   0.04 g

$CuSO_4.5H_2O$ • :   0.45 g

0.5 cm^3 of this solution was included in every litre of medium.

In practice all medium constituents except for the glycerol, ammonium sulphate and potassium dihydrogen orthophosphate were made up in bulk to 120 times the required final concentration. The concentrate was autoclaved and maintained aseptic, and when required, it was diluted with deionised water and the other three constituents added.

Antifoam was used in all cultures; 0.2 cm^3 of a 20% emulsion of food-grade silicone antifoam was included in each litre of medium.

The pH of the complete medium was adjusted to 6.5 by the addition of 4 M sodium hydroxide solution before the volume was finally made up to the required level.

5.3   Sterilisation

All equipment and media required to be sterile was autoclaved at 121°C and 15 p.s.i. (gauge steam-pressure).

The complete medium was sterilised in 12-litre batches for 40 minutes. Similarly, the fermenter (on its demountable tray), together with probes, attached tubes, and filters, was autoclaved for 40 minutes. After cooling, tube connections were made aseptically, and medium pumped into the fermenter.

Shake-flask cultures, required for fermenter inoculum, were autoclaved for 15 minutes. 500 cm^3 'dimpled' conical flasks containing 50 cm^3 of the complete medium were normally used. Prior to sterilisation, they were fitted with porous polypropylene plugs and wrapped with aluminium foil to prevent plug-wetting.

Glassware, such as McCartney bottles and pipettes, required to be sterile, was either autoclaved for 15 minutes, or left overnight in an oven at 200°C.

5.4   Preparation of Equipment for Culture

Following sterilisation and re-installation, a number of procedures were followed before the equipment could be used for culture.

5.4.1   Probe calibrations

Sterilised medium was agitated and aerated, usually overnight, in order to detect contamination within the system. If, as was invariably the case, no contamination was observed, the fermenter was de-oxygenated with oxygen-free nitrogen, and the dissolved-oxygen meter set at zero. The fermenter was then re-aerated under the particular conditions of temperature, pressure, gas flow-rate and impeller speed to be used. When the dissolved-oxygen reading reached a steady value, it was set at 100% saturation.

A small sample of medium was withdrawn from the fermenter and its pH accurately measured. The pH controller was then set at this value, and allowed to re-adjust to pH 6.5 when necessary. This adjustment was made automatically by the addition of sterile 2 M ammonia solution. Slightly high-pH media were not adjusted since acid is produced by the growing culture which then rapidly reduces the pH.

The medium-temperature was maintained at 37.0°C in all cultures. The temperature controller was adjusted to maintain this temperature reading on an accurate immersed mercury thermometer.

5.4.2   Gas-analyser calibrations

Both oxygen·and carbon dioxide gases were determined on a % volume basis in the exhaust gas from the fermenter.   Both analysers were calibrated before a batch culture, and daily during continuous use. After warming-up, the analysers were flushed with oxygen-free nitrogen and their zero-points set.   They were then flushed with a 5.0% CO_2 - 95.0% air mixture.   When a steady meter reading was obtained, the carbon dioxide analyser was set on full-scale deflection at its maximum sensitivity setting.   Similarly, the oxygen analyser was set to read 19.95%.

Calibration settings for dissolved oxygen, gaseous carbon dioxide and gaseous oxygen were checked and adjusted on the multi-point recorder.   Full calibration of the carbon dioxide gas analyser is described in Section 5.6.4.1.

5.5   Culture Techniques

Batch cultures were carried out both in shake-flasks and in the fermenter;   continuous cultures were carried out using the fermenter. Cultures were carried out aerobically and anaerobically in both types of equipment.

5.5.1   Batch cultures

5.5.1.1   Shake-flask cultures

The simplest type of batch culture was that carried out in a

simple aerobic shake-flask.   For this purpose, standard 500 cm^3 'Pyrex'
conical flasks with four 'dimples' near the base were used.   These
normally contained 50 cm^3 of medium, and were each fitted with a porous
polypropylene plug prior to sterilisation.   Inoculation of the flask
was performed aseptically by taking a small amount of surface culture
from an agar-slope using a platinum loop, and washing this into the
medium.   The flask was then clamped into the shaker, and shaken at
30°C and 250 cycles. min^{-1} for at least 12 hours.   Simple flask
cultures of this type were normally used as inocula for fermenter
cultures.

A more elaborate shake-flask design was used to grow anaerobic
cultures.   (Fig. 5.5).   In order to pass a gas (in this case, oxygen-
free nitrogen) through the shake flask, it was found necessary to pre-wet
the gas to prevent evaporation of the culture.   This was performed by
first passing the gas from the cylinder through a sterilising filter
and then through a sintered-glass aerator in a 250 cm^3 conical flask
containing sterile medium.   The gas then passed through an identical
sinter and flask (used for the culture), and finally to the atmosphere
via a disinfectant-trap.   All tubes were made of polythene, and all
joints in the glassware were of the ground-glass type.   Both flasks
were partially-immersed in a water-bath at 37°C and shaken at 100
cycles min^{-1}.   Thus, entry of oxygen to the flasks was effectively
eliminated, whilst mixing and temperature control were maintained.
Samples were withdrawn using a sterile syringe attached to a hypodermic
needle in a serum cap fitted in a small side-arm.   Using this equipment
in parallel with an identical layout through which air was passed,
growth of aerobic and anaerobic cultures was compared at an elementary
level.

250 cm^3
Conical flask with
ground flass fittings

1 cm^3 syringe with
'Luer' fitting to
wide-bore hypodermic
needle

Sintered-glass
aerator

Sterilising
filter

Disinfectant
trap

Atmosphere

Serum cap

Culture flask

Gas-wetting flask

Oxygen-free
Nitrogen

- 87 -
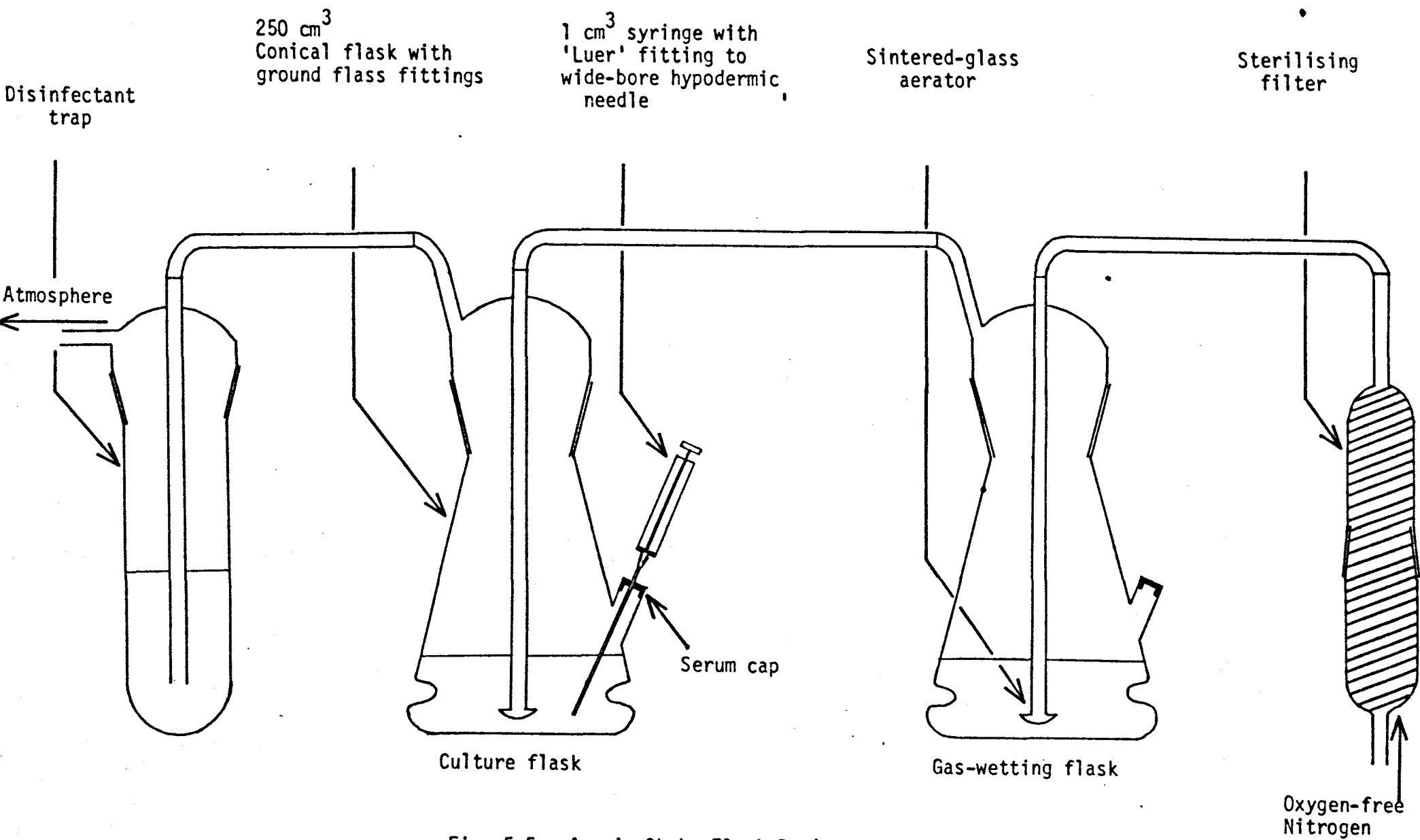
Fig. 5.5   Anoxic Shake Flask Design

5.5.1.2 Fermenter batch-cultures

Batch cultures were carried out in the fermenter to determine the parameters of carbon and oxygen limitations under the range of operating conditions available. In addition, the technique was used to determine the maximum growth-rate where no individual nutrient was limiting.

The major use of batch cultures, however, was simply as a preliminary to continuous cultures. For this purpose, it was found feasible to operate the fermenter continuously at a low dilution rate ($0.10 \ h^{-1}$) whilst the batch-growth was taking place. This procedure abolished the need to observe the culture and switch on the medium-pump at the appropriate point.

Batch cultures were also carried out in the fermenter anaerobically. These cultures were designed to investigate the nature of anaerobic batch growth and its maximum rate. Depletion of glycerol during these cultures was also followed.

All cultures in the fermenter were inoculated with 50 cm^3 of culture grown in the aerobic shake-flasks. The culture was aseptically transferred to a small glass bottle designed to contain 50 cm^3 and fit the hooded culture-sampler. The inoculum was then 'blown' into the fermenter aseptically by application of air-pressure through the sampler sterilising-filter.

5.5.2   Continuous cultures

Continuous cultures were carried out aerobically, anaerobically, and under conditions of intermittent aeration.

Aerobically, a series of continuous cultures was carried out over the range of dilution-rates from 0.10 h^{-1} to 1.40 h^{-1} at four different impeller speeds (700, 800, 900 and 1000 min^{-1}). All other conditions for these cultures were maintained constant. Thus, the relationship between carbon (glycerol) limitation and oxygen limitation under four differing conditions of oxygen transfer was studied. Cell density and dissolved-oxygen tension were both monitored at each steady state condition of dilution rate and impeller speed. Thus, a series of double-substrate (C-O) limitation curves was obtained, to give invaluable information for the design of intermittent-aeration cultures.

From these curves, four conditions of impeller speed and dilution rate were selected as starting-points for intermittent-aeration cultures. These were designed to give a range of fully-aerobic steady-state dissolved-oxygen tensions. For each culture, a total cycle time of five minutes was maintained. Thus, under conditions of intermittent aeration, the total aeration plus deaeration time was always five minutes. During each culture, the aeration time was progressively reduced in discrete one-minute steps, the deaeration time was accordingly increased, and the culture allowed to reach new steady-state conditions. Each intermittently aerated culture was therefore grown under six conditions of aeration, from fully aerobic to fully anaerobic.

Dissolved-oxygen tension and carbon dioxide and oxygen in the exhaust gas were all continuously monitored and recorded for all intermittent-aeration cultures. (The gases used, air and oxygen-free nitrogen, were sparged at 1.0 min^{-1}). In addition, the culture cell-density was monitored at each steady-state condition. Thus, information on the bacterial population and its respiratory

behaviour as a function of aeration time per cycle at each of four initial (aerobic) oxygen-transfer conditions was obtained. Additionally, culture was harvested from the overflow at each steady-state condition in order to provide samples for enzymatic analysis.

Fully-anaerobic continuous cultures were carried out in order to provide information on the effect of dilution rate on cell density under these conditions. The utilisation of the two primary catabolites of glycerol was tested both in aerobic and anaerobic continuous cultures.

5.5.3   Culture harvest technique

In order to obtain the required relatively large bacterial samples for enzymatic analysis, it was necessary to harvest culture from the fermenter overflow during continuous operation rather than remove samples directly. This technique had the added advantage of not interfering in any way with steady-state conditions.

The procedure was as follows:-

I   Approximately 300 cm^3 of culture was collected in an ice-cooled vessel against the slight pressure required to divert some of the exhaust gas through the gas analysers. The cold (approx. 5°C) liquor was then centrifuged for 30 minutes at 40,000 g using a refrigerated centrifuge (M.S.E. High Speed 18) set at 0°C.

II   The supernatant was discarded, a small sample having been retained for analysis. The cell pellet was then thoroughly resuspended in an ice-cold buffered isotonic solution (0.075 M NaCl + 0.050 M phosphate buffer, pH 7.0). Centrifugation was then repeated as above.

III   The second supernatant was discarded, and the washed cell pellet frozen and stored at -20°C in the polypropylene centrifuge tube.

Culture samples treated in this way were found to be completely stable during several months' storage.

5.6   Analytical Techniques

A number of techniques were employed to gain information on the consequences of substrate utilisation in growing batch and continuous cultures.

5.6.1   Dry cell weight - optical density calibration

Accurate analysis of substrate conversion to cellular material is available by the measurement of dry cell weight. However, the routine use of dry cell weight for cell density measurement is not acceptable since it is both laborious and time-consuming. Fortunately, there exists an identifiable relationship, usually linear, between dry cell weight and the turbidity of a bacterial culture. Thus, measurement of turbidity (in the form of optical density) at a suitable wavelength in the visible spectrum gives direct knowledge of the culture's dry cell weight per unit volume (or cell density).

Thus, a calibration graph of cell density vs. optical density was required. The information needed to construct this graph was obtained as follows:-

I   10.0 cm^3 samples were removed from the fermenter during the progression of a batch culture. The optical density of each sample was measured at 650 nm (against water) using the Pye-Unicam SP 600 II spectrophotometer.

Although this procedure was found to be satisfactory, no samples were removed after the culture had entered the stationary phase. (Hadjipetrou et al., 1964, found that the turbidity: dry cell weight ratio fell by 8% in the stationary phase of K. aerogenes batch culture).

II   Each sample was centrifuged for 30 minutes at 38,000 g (M.S.E. High Speed 18 centrifuge, set at 0°C). The supernatants were discarded, and the cell pellets resuspended in 10.0 cm^3 of ice-cold 0.1 M phosphate buffer, pH 6.5.

III   Centrifugation was repeated as above, the supernatants discarded, and the cell pellets resuspended in 10.0 cm^3 of ice-cold deionised water. Centrifugation was then again repeated, and the supernatants discarded.

III   The washed cell pellets were then thoroughly resuspended in 10.0 cm^3 of deionised water and poured onto a pre-weighed clock-glass. The glasses were dried in an oven at 100°C for 15 hours, and then placed in a desiccator to cool.

IV   Each clock-glass, with its dried cell-cake was weighed to 0.01 mg in a desiccated-chamber balance.   (Stanton CL5D).

The information provided by this procedure permitted the construction of a calibration graph for the determination of cell density (as dry cell weight) from a culture's optical density. (Fig. 5.6.1). The procedure had the advantage of directly comparing the dry cell weight with the optical density of a culture determined under actual experimental conditions. Thus, cell densities of all cultures were determined by this graph from their optical densities, measured directly on removal from the fermenter.
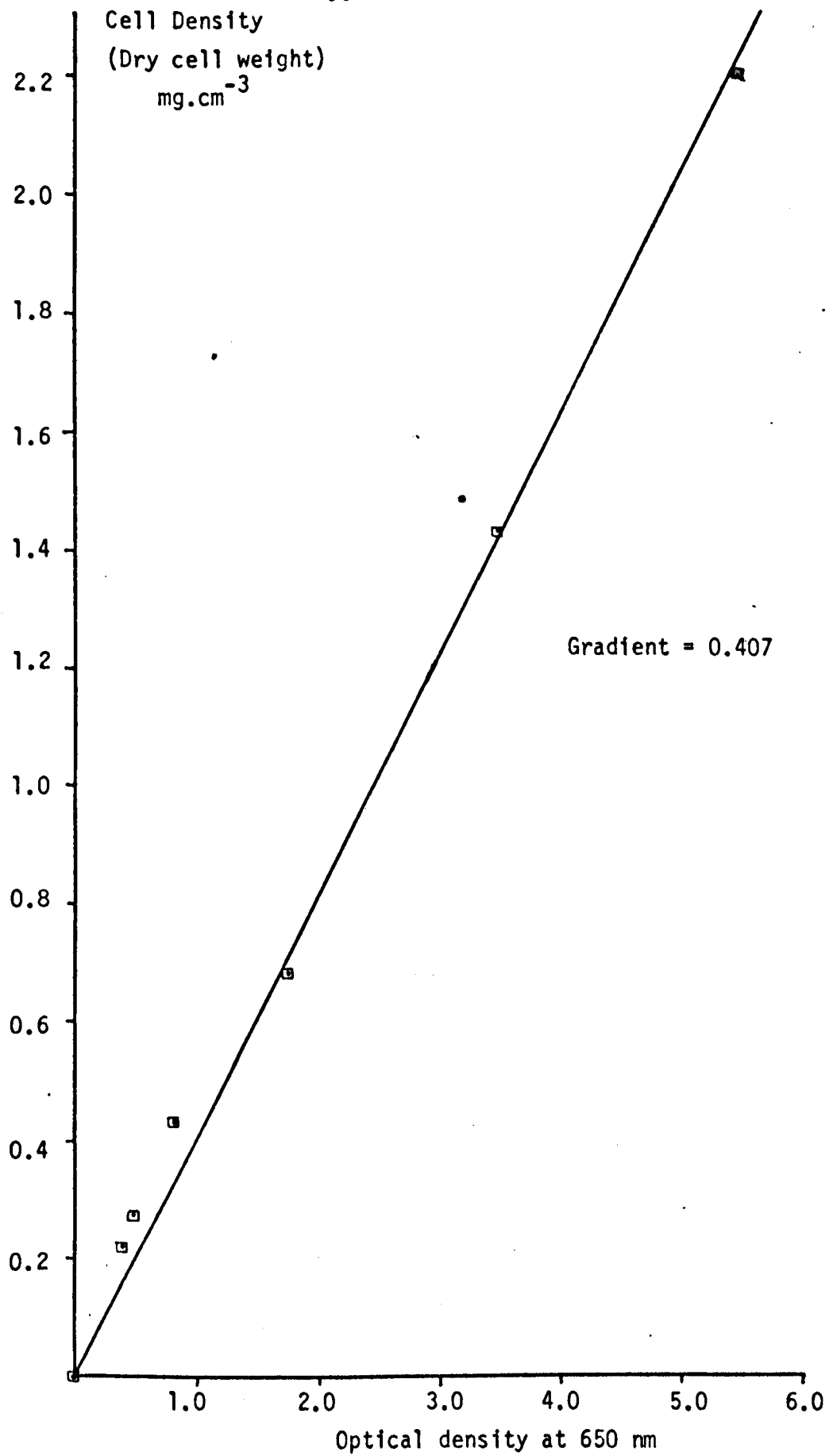
Figure 5.6.1   Optical Density - Cell Density Calibration Graph

5.6.2 Glycerol determination

The utilisation of glycerol was monitored in many cultures by its determination in the culture supernatant. The technique used was an adaptation of a colorimetric determination of formaldehyde. (Nash 1953). The method has been used by Jones (1968) and Carrizales (1974).

The glycerol is first oxidised to formaldehyde by periodic acid and sodium arsenite solution. The formaldehyde then undergoes the Hantzsch reaction by reacting with acetylacetone in the presence of excess ammonium ions to give the yellow chromophore diacetyldihydrolutidine (DDL), which is measured spectrophotometrically at its absorption peak at 420 nm.

5.6.2.1 Reagents

The reagents used in this determination were as follows:-

Solution A:-   0.015 M periodic acid; neutralised to pH 7.5 with NH_4OH, and buffered in 0.1 M phosphate.

Solution B:-   0.25 M sodium arsenite; neutralised to pH 7.0 with HCl.

Solution C:-   0.02 M acetylacetone (pentane 2:4 dione) and 0.05 M acetic acid in 1 M ammonium acetate solution.

A standard glycerol solution was used for the preparation of a calibration graph. Ten solutions were prepared in the range 2.50-25.0 $\mu g.cm^{-3}$ glycerol.

5.6.2.2 Procedure

I 1.0 cm^3 of a suitably-diluted sample was taken in a test-tube.

1.0 cm^3 of solution A was added, mixed, and left at room temperature for 15 minutes.

II   1.0 cm^3 of solution B was added, mixed, and left for a further 5 minutes.

2.0 cm^3 of solution C was then added.

III   The test-tubes were heated in a boiling-water bath for exactly 5 minutes. They were then rapidly cooled under a running tap.

IV   The optical density of each solution was determined at 420 nm against a reagent blank, using the Pye-Unicam SP 600 II spectrophotometer.

In practice, supernatant samples were first routinely diluted 200-fold. The feedstock glycerol concentration of 5.0g.l^{-1} then corresponds to the maximum point on the calibration graph (Fig. 5.6.2). The points on this graph deviate remarkably little from the straight line despite the low concentrations of glycerol involved, demonstrating the sensitivity of the method.

5.6.3   Protein determination

Two methods, 'Biuret' and 'Folin', were available for the determination of protein. Choice of method depended on: (a) the type of sample (whole cells or solution), (b) the amount of protein expected in the sample, (c) the sensitivity or accuracy required, and (d) the ease of the method for routine analysis.

In practice, the Folin method, being a very sensitive but practically vulnerable determination procedure, was used only to determine protein concentration in samples used for cytochrome assay. The Biuret method, although much less sensitive, was found to be a reasonably accurate and robust method for the determination of protein at 'normal' physiological concentrations. Moreover, the Biuret method was found to be considerably less fraught with the interference problems
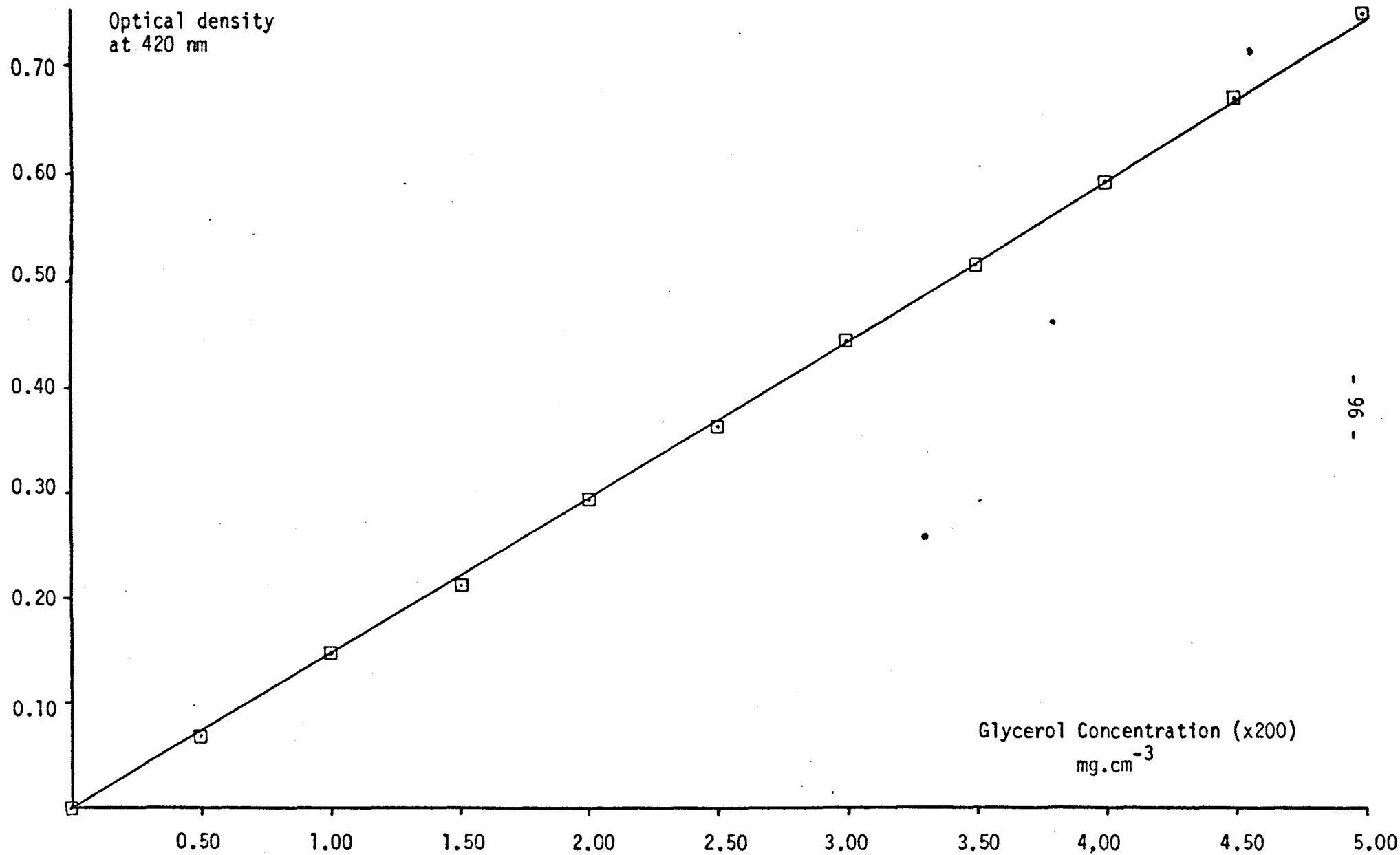
Fig. 5.6.2   Glycerol Determination Calibration Graph

associated with the Folin method, due to the latter's sensitivity to other chemical species.

5.6.3.1 The Biuret method

The procedure used was identical to that of Herbert, Phipps · and Strange (1971). 'The method includes, as a preliminary stage, incubation at 100°C in 1 molar sodium hydroxide. Although intended primarily for the disruption of cell-walls for measurement of intra-cellular protein, this preliminary stage was found to be useful for the solubilisation of suspended or precipitated proteins, so ensuring complete determination.

The biuret reaction between proteins (or peptides) and alkaline Cu^{2+} yields a purple complex which can be measured spectrophotometrically.

5.6.3.1.1 Reagents

I 3 M sodium hydroxide solution.

II 2.5% (w/v) $CuSO_4.5\ H_2O$

Standard protein solutions were prepared using bovine serum albumin at 1.0, 2.0, 3.0 and 4.0 mg. cm^{-3}.

5.6.3.1.2 Procedure

I 2.0 cm^3 of each sample was pipetted into a test-tube and 1.0 cm^3 of 3 M sodium hydroxide added. The tube contents were mixed and heated in a boiling-water bath for 5 minutes.

II The test-tubes were cooled, and 1.0 cm^3 of 2.5% $CuSO_4$ solution was added.

III   They were vortex-mixed, allowed to stand at room temperature for 5 minutes, and then centrifuged (bench-top centrifuge) for about 10 minutes.

IV   The optical density of each supernatant was determined at 555 nm against a reagent blank (Pye-Unicam SP 600 II spectrophotometer).

The standard solutions' points on the calibration graph (Fig. 5.6.3) fit an almost perfect straight line, indicating the general reliability of the method.

5.6.3.2 The Folin method

This method (Cole, J. A., 1976) determines small amounts of soluble proteins accurately. Samples are treated with a reagent containing sodium hydroxide, sodium carbonate, copper sulphate and sodium potassium tartrate, before adding the Folin-Ciocalteau reagent. The latter is essentially a phosphotungstic-phosphomolybdic acid solution which is reduced to 'molybdenum-blue' by proteins (and other species) and can thus be determined colorimetrically. The reaction is almost entirely due to tyrosine and tryptophan, other amino-acids having little effect.

5.6.3.2.1 Reagents

Solution A:-   2% Na_2CO_3 in 0.1 M NaOH solution

Solution B_1:-   1% $CuSO_4$ solution

Solution B_2:-   2% Na-K-Tartrate

Folin-Ciocalteau reagent:-   dilute each cm^3 with 0.7 cm^3 of distilled water.

Also, for calibration purposes, a 1.0 $mg.cm^{-3}$ solution of B.S.A. was prepared.
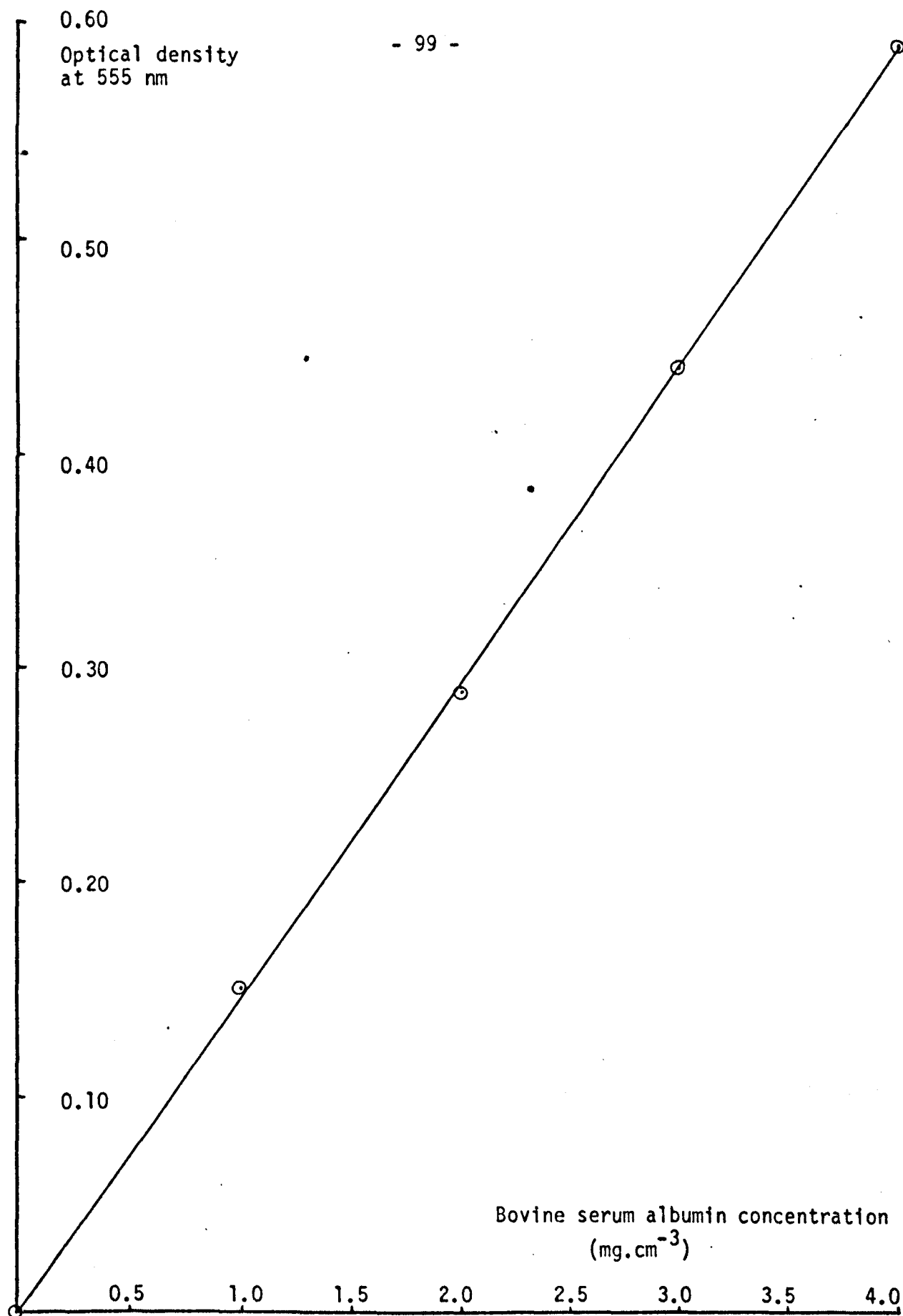
Figure 5.6.3   Protein Determination (Biuret Method)
Calibration Graph

5.6.3.2.2 Procedure

I Immediately before starting, equal volumes of solutions B_1 and B_2 were mixed. All test-tubes contained 0.6 cm^3 of sample which had been diluted so that they contained less than 1.0 $mg.cm^{-3}$ protein. Duplicate sample tubes were prepared, containing 100 μl of diluted sample + 0.50 cm^3 water and 50 μl sample + 0.55 cm^3 water. (For calibration, the 1.0 $mg.cm^{-3}$ B.S.A. standard solution was taken at 25,50,75 and 100 μl, with suitable dilution to 0.60 cm^3).

II 50 cm^3 of solution A was mixed with 1.0 cm^3 of solution B, and 3.0 cm^3 of the mixture added to each sample. They were vortex-mixed, and left to stand for exactly 10 minutes.

III 0.30 cm^3 of the diluted Folin-Ciocalteau reagent was then added to each tube, vortex mixed, and left to stand for 30 minutes.

IV Optical density at 750 nm was determined against the reagent blank. (Pye-Unicam SP 600 II).

The calibration graph obtained (Fig. 5.6.4) was found to exhibit a slight curvature.

5.6.4 Gas analysis

Aerobic culture of microorganisms requires oxygen and produces carbon dioxide. These two chemical species, found in the gaseous form under normal conditions, are therefore central to respiratory processes.

Thus, determination of these two gases in the fermenter effluent stream gives a great deal of information on the extent to which respiration is being employed in the oxidation of the carbon source. Moreover, anaerobic cultures of K. aerogenes, whilst obviously using no oxygen, produce proportionately (per unit mass of cells) more carbon dioxide than aerobic cultures.
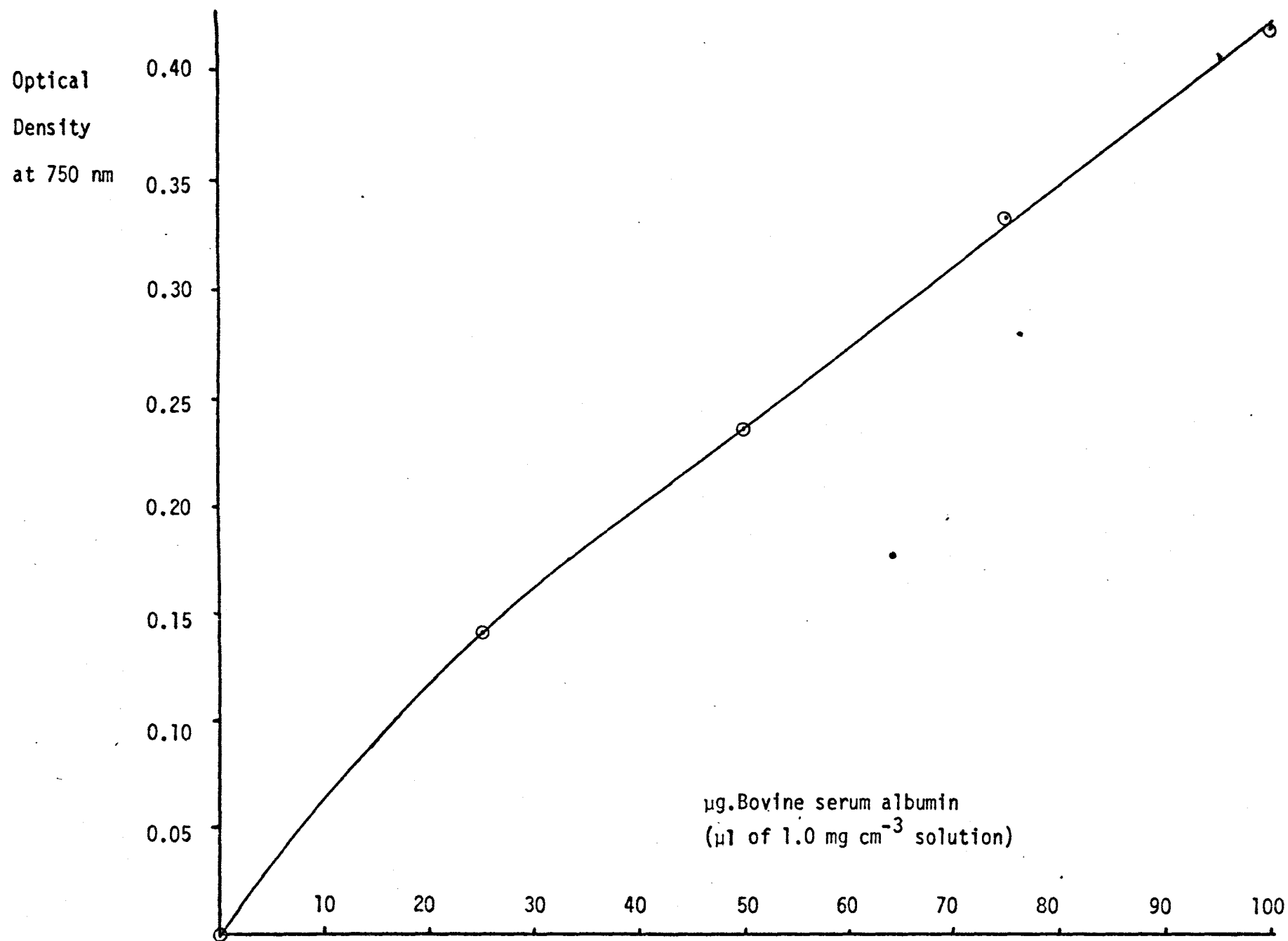
Figure 5.6.4   Protein Determination (Folin Method) Calibration Graph

Dissolved-oxygen determination of the microbial culture yields considerable information on the carbon limitation - oxygen limitation balance within the culture.

Gaseous carbon dioxide and oxygen in the fermenter exhaust gas and dissolved oxygen in the culture were, therefore, all continually measured and recorded in all fermenter cultures. 100 cm^3 min^{-1} exhaust gas was diverted through the oxygen gas and carbon dioxide gas analysers, as shown in the 'Equipment' section. (See Fig. 4).

5.6.4.1 Carbon dioxide gas analysis

Carbon dioxide was measured by an instrument with depends on the principle that this gas absorbs radiation of infra-red frequency. The instrument was calibrated using mixtures of 95% air/5% CO_2 and oxygen-free nitrogen. Thus, the ratio of $O_2:CO_2$ concentration was always 4:1, but total amounts of each gas varied between the mixtures. By determination of oxygen in the gas, the carbon dioxide concentration was known, and its corresponding meter-reading used to construct a calibration curve. (Fig. 5.6.5). This curve was used for all determinations of carbon dioxide from the meter (or recorder) reading. A daily calibration check was carried out during continuous monitoring, as outlined in Section 5.4.2.

5.6.4.2 Oxygen gas analysis

Oxygen in the exhaust gas was measured by an instrument which relies on the paramagnetic property of the oxygen molecule. The signal strength fed to the meter then depends on the oxygen concentration in the analysis-cell. Thus, the response of the instrument was
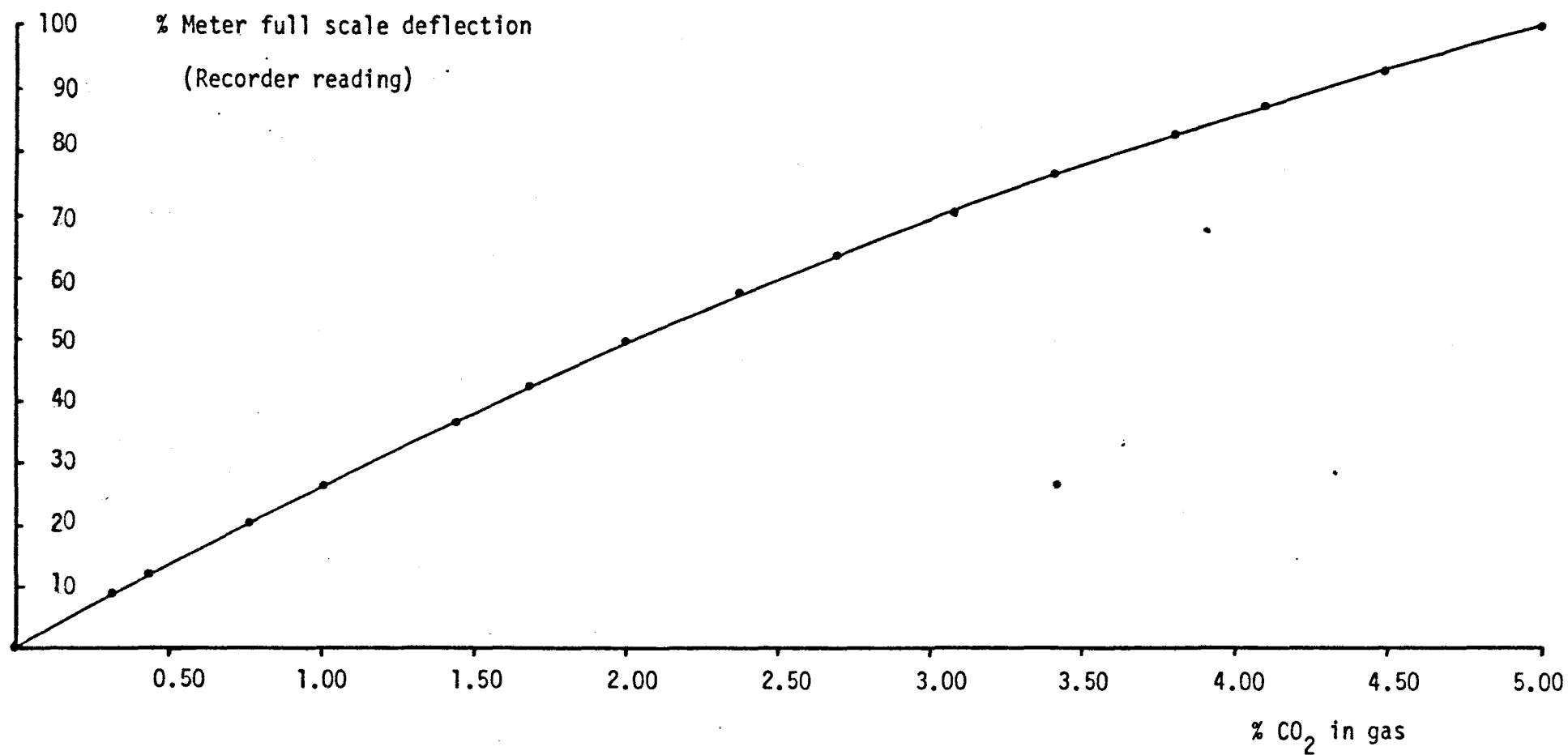
Figure 5.6.5   Carbon dioxide Meter Calibration Curve

linear over its entire 0-25% (by volume) range.

Whilst the output to the recorder was normally 0-20% or 1-21%, it was possible to select a 5% 'band' within this range for more accurate analysis. Thus, for fully-aerated cultures, the recorder was set to read 16-21% O_2. Again, daily calibration is dealt with in Section 5.4.2.

5.6.4.3 Dissolved oxygen analysis

Dissolved oxygen was monitored and recorded continuously in all aerobic and intermittently-aerated fermenter cultures. As detailed in the 'Equipment' section, the output from the probe to the meter (and thence recorder) is linear in response to dissolved oxygen tension. Thus, dissolved oxygen tension was expressed as percentage saturation with respect to the uninoculated medium.

By application of Henry's law, which states:-

$$C_s = H\ p_g$$

where C_s = saturation concentration of oxygen,

   H   is   Henry's constant,

and p_g = partial pressure of oxygen in the gas phase, dissolved oxygen tension could be expressed as a partial pressure in mm mercury.

Calibration of the dissolved oxygen meter is covered in Section 5.4.1.

5.7   Enzyme Assays

In order to gain detailed information on the mode of glycerol energy-source utilisation by the culture, under a number of aeration conditions, four basic enzyme assays were routinely carried out.

As detailed in the 'Theory' section, there are two aerobic and two anaerobic enzymes·which convert glycerol to the common intermediate, dihydroxyacetone phosphate, in K. aerogenes 418.   In fully-aerobic cultures, glycerol kinase and glycerol-3-phosphate dehydrogenase bring about this conversion; their anaerobic counterparts are glycerol dehydrogenase and dihydroxyacetone kinase.   These were, therefore, the four enzymes assayed in all intermittently-aerated cultures.   (From fully aerobic to fully anaerobic growth).   At the opposite end of the electron-transfer chain, cytochromes were assayed in fully aerobic and fully anaerobic cultures.

5.7.1   Sample preparation for Glycerol-related enzyme assays

A standard procedure was developed for the conversion of a frozen washed cell-pellet, which had been stored since harvesting at -20°C, into a cold, clarified, and physiologically-buffered solution of the soluble cellular material suitable for the assay of the four basic glycerol-related enzymes.

This procedure was therefore used identically on all samples required for assay:-

I   The frozen washed cell pellet was removed from the deep-freeze and allowed to just thaw in an ice-bath at 0°C.   It was then mixed with a glass rod, and resuspended to an appropriate volume in a small glass beaker with ice-cold deionised water.   (The actual volume of resuspension depended on pellet-size, but was 30-60 cm^3).

II   Surrounded by an ice-water jacket at 0°C, the suspension was disrupted by sonication for nine minutes.   Disruption by sonication of this type (at maximum energy-level) produces quite a large amount of energy which is dissipated as heat.   Three 'cooling rests' were therefore employed, where the probe was removed from the suspension and they were both allowed to cool.   The actual procedure was therefore:- (a)   Sonication (setting 8) - 3 minutes

(b)   'Cooling rest' - 1 minute

(c)   Sonication - 2 minutes

(d)   'Cooling rest' - 1 minute

(e)   Sonication - 2 minutes

(f)   'Cooling rest' - 1 minute

(g)   Sonication - 2 minutes

III   A sample of the sonicate was drawn into a sterile syringe of 20 cm^3 capacity, and expressed (at up to 75 ps.i.) through a disposable filter of 0.22 μm pore size (Millex filter, Millipore Corporation, Bedford, Mass.).   Gross cell debris and unbroken cells were thereby removed, and the filtrate appeared as a clear liquid which was rapidly returned to 0°C.

IV   Exactly 2.0 cm^3 of the filtrate was pipetted into a test-tube, and stored at -20°C for protein analysis.

V   The remaining filtrate had ca. 5 $mg.cm^{-3}$ bovine serum albumin added for protection against proteolysis.

The B.S.A. acts by (a) stabilising existing proteins, and (b) providing a large excess (at least 2.5 times) concentration of protein as an alternative substrate for any proteolytic enzymes.

This stabilised filtrate containing 0.3 to 2.1 mg.cm^{-3} of microbial protein, was used for the enzyme assays.   It was found that this method of filtration and B.S.A. stabilisation considerably reduced loss of enzymic activity when compared to conventional centrifugation.   This was probably due to the much shorter time of sample treatment as well as the stabilisation.

All four enzyme assays were carried out on an individual sample before a new sample was prepared.   Although this involved considerable practical difficulties, in that all four assay systems had to be available at the same time, the entire process from sample preparation to completion of four assays was carried out in about one hour..   Thus, sample stability was ensured to be better than in the situation where a number of samples are prepared together.   The procedure had the added advantage that all four assays were carried out on the same sample, justifying considerably more reliance on comparisons of those four assay results than might otherwise be reasonable.

5.7.2   Glycerol kinase and glycerol dehydrogenase assays

As detailed in the 'Theory' section 3.3.1.1, glycerol is first aerobically phosphorylated by glycerol kinase or anaerobically dehydrogenated by glycerol dehydrogenase.   A method was developed here to assay those two enzymes simultaneously in a single sample.   The glycerol kinase assay was based on that developed by Freedburg and Lin (1973); the glycerol dehydrogenase assay being developed from that of Lin and Magasanik (1960).   The two were combined in such a way that the dehydrogenase assay serves as the blank for the kinase assay. Addition of rabbit G-3-P dehydrogenase to that system then assays both

the dehydrogenase and the kinase, and the latter activity is simply found by subtraction.

5.7.2.1 Reagents

The basic assay mix contained:-

20 mM Adenosine triphosphate (ATP)

20 mM Magnesium chloride

1.0 mM Nicotinamide adenine dinucleotide (NAD)

in   0.25 M Glycine and 0.25 M Hydrazine buffer, pH 9.5

(All at final assay concentrations).

In practice, 100 cm^3 of buffer was prepared by dissolving 1.88 g of glycine and 2.63 g hydrazine.2 HCl in ca. 60 cm^3 deionised water.   About 20 cm^3 of 3 M NaOH was required to adjust the pH to exactly 9.5, and the volume was made up to 100 cm^3 with deionised water.

For 25.0 cm^3 of assay mix, 0.461 g ATP, 0.153 g $MgCl_2.6H_2O$, and 0.025 g NAD were dissolved in the buffer, and made up to 25.0 cm^3. Further dilution to the required final assay concentrations was made with the other assay components:-

0.10 M Glycerol

(Used as a 3 M stock solution, containing 2.76 g glycerol dissolved to 10 cm^3 in deionised water).

100 µg rabbit glycerol-3-phosphate dehydrogenase. (G-3-P dehydrogenase; commercially available 10.0 mg.cm^{-3} crystal-suspension was diluted ten-fold with deionised water).

All components of the assay-system were made freshly each day, and kept near 0°C.

5.7.2.2 <u>Procedure</u>

The equipment used in the enzyme assays is detailed in the Equipment section. 1 cm light-path silica-quartz cuvettes were contained within a thermostatic cuvette-holder at 37°C in the Pye-Unicam SP 500 UV-Visible spectrophotometer linked to the linear-logarithmic recorder·(Logarithmic operation).

I.   2.00 cm^3 of the basic assay mix was pipetted into the cuvette.

Cell extract and deionised water to a total volume of 0.80 cm^3 were added and mixed. (The cell extract volume varied from 10 μl to 200 μl, depending on the sample).

II.   Absorbance at 340 nm, using the deuterium lamp, was plotted on the logarithmic recorder for 2 minutes at 20 $sec.cm^{-1}$. The gradient of this straight line, invariably zero, was taken as the blank activity for glycerol dehydrogenase. (Line 1).

III.   100 μl of the 3 M glycerol solution was added to the assay system, the cuvette contents were thoroughly mixed and returned to 37°C.

IV.   Absorbance at 340 nm was again plotted for 2 minutes at 20 $sec.cm^{-1}$ (Line 2).

Increase in absorbance with time, measured as a straight-line gradient, was attributed to glycerol dehydrogenase activity.

V.   100 μg (i.e. 100 μl of a 1.0 $mg.cm^{-3}$ suspension) of rabbit G-3-P dehydrogenase was rapidly added to the system and mixed.

VI.   Absorbance at 340 nm was again plotted for 2 minutes at 20 $sec.cm^{-1}$ (Line 3).

The activity of glycerol dehydrogenase in the sample was found by subtracting the gradient of line 1 from that of line 2. Similarly, glycerol kinase activity was found by subtracting the gradient of line 2 from that of line 3.

Knowing the concentration of protein in the sample, the sample volume, and the extinction coefficient of NADH, enzyme activities were expressed as μmoles NAD reduced per minute per mg. protein.

5.7.3 Dihydroxyacetone kinase assay

A method was developed and optimised here for the assay of dihydroxyacetone kinase (DHA-kinase) by spectrophotometrically following the linked oxidation of NADH. (See Results Section 6.4.3). Thus, the instrumentation and much of the procedure was identical to the glycerol kinase - glycerol dehydrogenase assays, although decrease in absorption with time at 340 nm was followed.

5.7.3.1 Reagents

The basic assay mix contained:-

20 mM Magnesium chloride

0.50 mM Reduced nicotinamide adenine dinucleotide (NADH)

10 mM Glycerol

7.5 mM Dihydroxyacetone (DHA)

in 0.1 M tris-HCl buffer, pH 8.0. (All final assay concentrations). In practice, buffer was prepared by dissolving 3.63 g tris in deionised water, adjusting to pH 8.0 with dilute HCl, and making up to 100 cm^3 with deionised water.

To prepare 25.0 cm^3 of basic assay mix, 26.6 mg NADH, 69.1 mg glycerol, 50.7 mg DHA, and 1.22 g $MgCl_2.6H_2O$ were dissolved and made up to that volume in the buffer. The quoted concentrations were finally achieved by dilution with the other assay components:-

20 mM Adenosine triphosphate (ATP) (To make a stock solution, 0.9213 g ATP was dissolved and made up to 5.0 cm^3 with deionised water).

100 μg rabbit glycerol-3-phosphate dehydrogenase (G-3-P dehydrogenase). (As before, commercially available 10.0 $mg.cm^{-3}$ crystal-suspension was diluted ten-fold with deionised water).

All components of the assay system were made freshly each day, and kept near 0°C.

5.7.3.2 Procedure

The instrumental set-up was identical to that used for the glycerol kinase - glycerol dehydrogenase assays.

I.   1.00 cm^3 of the basic assay mix was pipetted into the silica cuvette. To this was added 200 μl of the ATP solution and cell extract and deionised water (Normally 200 μl of cell extract and 1.50 cm^3 of water). The components were thoroughly mixed, and allowed to reach 37°C.

II. Absorbance at 340 nm was plotted on the logarithmic recorder for 2 minutes at 20 $sec.cm^{-1}$. The gradient of this line was taken as the blank activity for DHA-kinase.

III. 100 μg (i.e. 100 μl of a 1.0 $mg.cm^{-3}$ suspension) of rabbit G-3-P dehydrogenase was rapidly added and mixed. The cuvette was returned to 37°C.

IV. Absorbance at 340 nm was again plotted for 2 minutes at 20 sec.cm^{-1}.

The difference between the second and first gradient was taken as being due to dihydroxyacetone kinase activity. The enzyme activity was expressed as μ.moles NADH oxidised per minute per mg.protein.

5.7.4 Glycerol-3-phosphate dehydrogenase assay

The method used for the assay of G-3-P dehydrogenase was based on that by Kistler and Lin (1971).

In K. aerogenes, this enzyme is not NAD-linked, and therefore spectrophotometric monitoring of NAD reduction cannot be employed in the assay. However, a 'non-physiological' system was used, where the reaction catalysed by the enzyme is linked to the reduction of a tetrazolium dye. The reaction was therefore followed spectrophotometrically (or colorimetrically) at the reduced dye's absorption peak of 570 nm. The equipment used was identical to that used for the other glycerol-related enzyme assays, although a tungsten lamp was used at this wavelength in the visible spectrum. A more detailed account of the theory of this assay appears in the 'Theory' section 3.3.1.2.

5.7.4.1 Reagents

The reaction mixture was prepared so that the 3.0 cm^3 contained:-

10.0 μ moles potassium cyanide

100 μg MTT dye

[3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide].

600 μg. PMS mediator

[Phenazine methosulphate]

100 μ moles DL-α-Glycerophosphate in 100 mM phosphate buffer, pH 7.5, containing 0.2% (v/v) Triton X-100 (Included to improve the solubility of the reduced form of the tetrazolium acceptor-dye).

In practice, the solutions were made up as follows:-

Solution A:- 0.033 M KCN

• 0.333 M Phosphate buffer, pH 7.5.

(52 g 1^{-1} NaH_2PO_4 and 47.3 g.1^{-1} Na_2H PO_4 mixed to give pH 7.5, containing 2.17 g KCN).

Solution B:- 1.00 M DL-α-glycerophosphate.

(1.6204 g made up to 5.0 cm^3 with deionised water).

Solution C:- 1.00 mg MTT

6.00 mg PMS

60 μl Triton X 100

(made up to 10.0 cm^3 with deionised water).

In addition, 3 x 10^{-6} moles of flaving mononucleotide (FMN) and 3 x 10^{-8} moles of flavin adenine dinucleotide (FAD) were included in certain assays, though they were not routinely used.

Solution C was rapidly made up and stored in the dark (by wrapping foil round the bottle) as it was found to be extremely light-sensitive. Flavin solution and Solution B were prepared freshly each day, but the others were reasonably stable. All solutions were stored under refrigeration.

5.7.4.2 Procedure

The spectrophotometer wavelength was set at 570 nm. (Tungsten lamp). Otherwise, the equipment was set up exactly as before.

I.   0.90 cm^3 of Solution A was pipetted into the cuvette.

To this was added cell extract and deionised water to a combined volume of 1.00 cm^3 (cell extract volume was normally 10-100 μl, depending on the individual sample).

1.00 cm^3 of Solution C was added, and the cuvette-contents · mixed and returned to 37°C.

II.   Absorbance at 570 nm was plotted for 2 minutes at 20 sec.cm^{-1} The gradient of the resultant line, mainly due to the photo-sensitivity of the dyes, was taken as the reaction blank.

III. 100 μl of Solution B (the enzyme's substrate) was added, mixed, and the cuvette returned ot its thermostatic holder.

IV.   Absorbance at 570 nm was again plotted for 2 minutes at 20 sec.cm^{-1}.

The gradient of the first line was subtracted from that of the second, and the difference attributed to glycerol-3-phosphate dehydrogenase activity.   Since the tetrazolium dye is a non-physiological reactant, enzyme activity was simply expressed as change of O.D. units per minute per mg. protein.

5.7.5   Cytochrome assay

The concentration of cytochromes, especially cytochrome b, in the cells was measured spectrophotometrically.   Thus, absorption peaks in the visible spectrum of a concentrated cell extract could be attributed to various cytochromes.   Quantitatively, the peak at 560 nm (α-peak of cytochrome b) was measured using a double-beam spectrophotometer.

A cell pellet of aerobically or anaerobically grown K. aerogenes was resuspended in 0.05 M phosphate buffer, pH 7.5 at 4°C.   This thick suspension was disrupted by two passages through a French press, and

used without any further separation procedure. The extract was poured
into two cuvettes; to one were added a few crystals of dithionite
(a reducing agent), and to the other a few crystals of potassium
ferricyanide (an oxidising agent).

Reduced versus oxidised spectra were carried out from 660 nm
to 490 nm. The peak heights were measured, and knowing the extinction
coefficient of the cytochrome and the protein concentration in the
extract (determined by the Folin method), cytochrome concentrations
were calculated and expressed as picomoles per mg. protein.

For purely qualitative determination, low-temperature spectra
were also carried out. An accessory sample-holder containing liquid
nitrogen at -196°C was used for this purpose. The advantage of
low-temperature spectra was found to be in sharpening the resolution of
existing peaks. However, they cannot be used for quantitative
determination due to random light-scattering by the ice crystals.

CHAPTER 6

RESULTS

Results are presented of work carried out with aerobic, anaerobic and intermittently-aerated cultures of Klebsiella aerogenes N.C.I.B. 418 grown on glycerol as sole source of carbon and energy. Assays of the enzymes of aerobic and anaerobic glycerol metabolism in the bacteria grown under these various conditions are also presented.

6.1   Aerobic Cultures

Aerobic cultures were carried out under a wide variety of conditions in shake-flasks, and in the fermenter. Both batch and continuous cultures were carried out aerobically in the fermenter.

6.1.1   Shake-flask cultures

50 cm^3 aerobic shake-flask cultures were normally routinely prepared for fermenter inocula. The simple growth kinetics of these cultures was investigated by measurement of optical density (650 nm) at specific time intervals. The results, expressed as a semi-logarithmic plot of cell density against time are shown in Fig. 6.1.1. (The initial glycerol concentration was 5.0 g l^{-1}).

It can be seen that there is initially a rapid rise in cell density, probably caused by distribution of the cell-conglomerate from the platinum loop. Up to about 6 hours from inoculation there is a lag, but growth rate accelerates from 6-8 hours. After 8 hours from inoculation, growth appears to be logarithmic. The terminal cell density of about 2.65 mg.cm^{-3} (dry cell weight) is reached about 12 hours after inoculation, the culture probably then being glycerol-limited.

The relationship between specific growth rate (μ) and doubling time (t_d) of a microbial batch culture is given by the equation:-

$$t_d = \frac{\log_e 2}{\mu} = \frac{0.693}{\mu}$$

- (Pirt, 1975)

The minimum doubling time of the aerobic shake-flask culture shown in Fig. 6.1.1 is 48 minutes. Thus, $\mu = \frac{0.693}{0.80}$ h^{-1}. The specific growth rate of this culture is therefore ca. 0.87 h^{-1}. This value is not widely different from those obtained from growth-rate measurements in fermenter batch cultures.
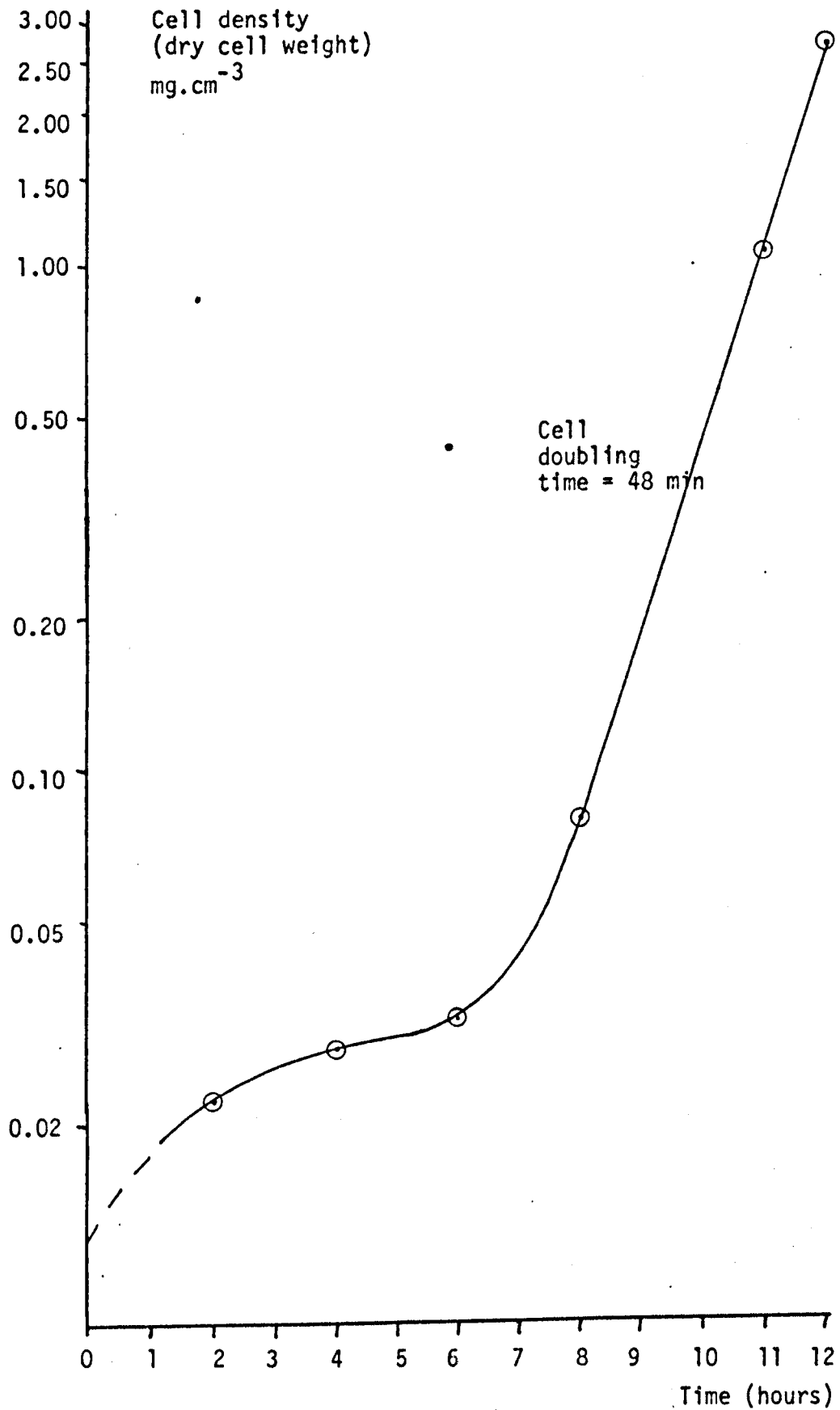
Fig. 6.1.1   Semi-logarithmic Growth Curve
(Shake Flask Aerobic Culture)

6.1.2   Fermenter batch cultures

Aerobic batch cultures in the fermenter were routinely carried out as a preliminary to continuous cultures. More detailed analyses of growth kinetics and respiration were however made, together with investigations of the effects of various impeller speeds.

6.1.2.1   A fully instrumented batch culture

In order to obtain as much information as possible using the available analytical techniques, an aerobic batch culture was carried out with determinations of cell density, residual glycerol and dissolved oxygen tension in the culture, and of oxygen and carbon dioxide in the exhaust gas. The medium was that used in all cultures, containing $5.0 \, g.1^{-1}$ glycerol. The impeller speed was $700 \, min^{-1}$, and air flow rate $1.0 \, min.^{-1}$

Measurements were continuously recorded, or made at half-hourly intervals. Table 6.1.1 shows these measurements as a function of time and they are plotted in Fig. 6.1.2.

A semi-logarithmic plot of cell density against time reveals the doubling time (t_d) during the logarithmic phase of growth to be 46 minutes (Fig. 6.1.3). This is equivalent to a specific growth rate (μ) of $0.90 \, h^{-1}$.

Fig. 6.1.2 shows that the rise in cell density and the fall of residual glycerol concentration fit virtually complementary curves. A similar relationship applies to the rise of carbon dioxide and fall of oxygen concentrations in the exhaust gas. Dissolved oxygen tension (DOT) falls to a low level (24% saturation) at the point of carbon limitation, this being symptomatic of the low $(700 \, min^{-1})$ impeller speed. Smoothness of the curves is evident, probably indicating that substrate

Table 6.1.1   Analysis of an Aerobic Batch Culture

Time from inoculation (Hours)	Cell density (D.C.W mg.cm.$^{-3}$)	DOT (% of saturation)	Residual Glycerol (mg.cm^{-3})	% Exhaust Gas	
				Carbon Dioxide	Oxygen
Medium	0.00	100	5.05	0.00	20.95
0.0	0.18	100	4.72	0.00	20.8
0.5	0.17	99.5	4.72	0.01	20.8
1.0	0.18	99.0	4.79	0.02	20.7
1.5	0.21	98.0	4.72	0.03	20.5
2.0	0.27	96.5	4.86	0.10	20.5
2.5	0.37	93.5	4.52	0.18	20.3
3.0	0.57	89.5	4.21	0.24	20.2
3.5	0.85	84.0	3.71	0.38	20.1
4.0	1.32	73.5	2.88	0.64	19.9
4.5	2.09	52.0	1.64	1.08	19.2
5.0	3.32	24.0	0.027	1.60	18.2

Figure 6.1.2   A Fully Instrumented Aerobic Batch Culture in the Fermenter
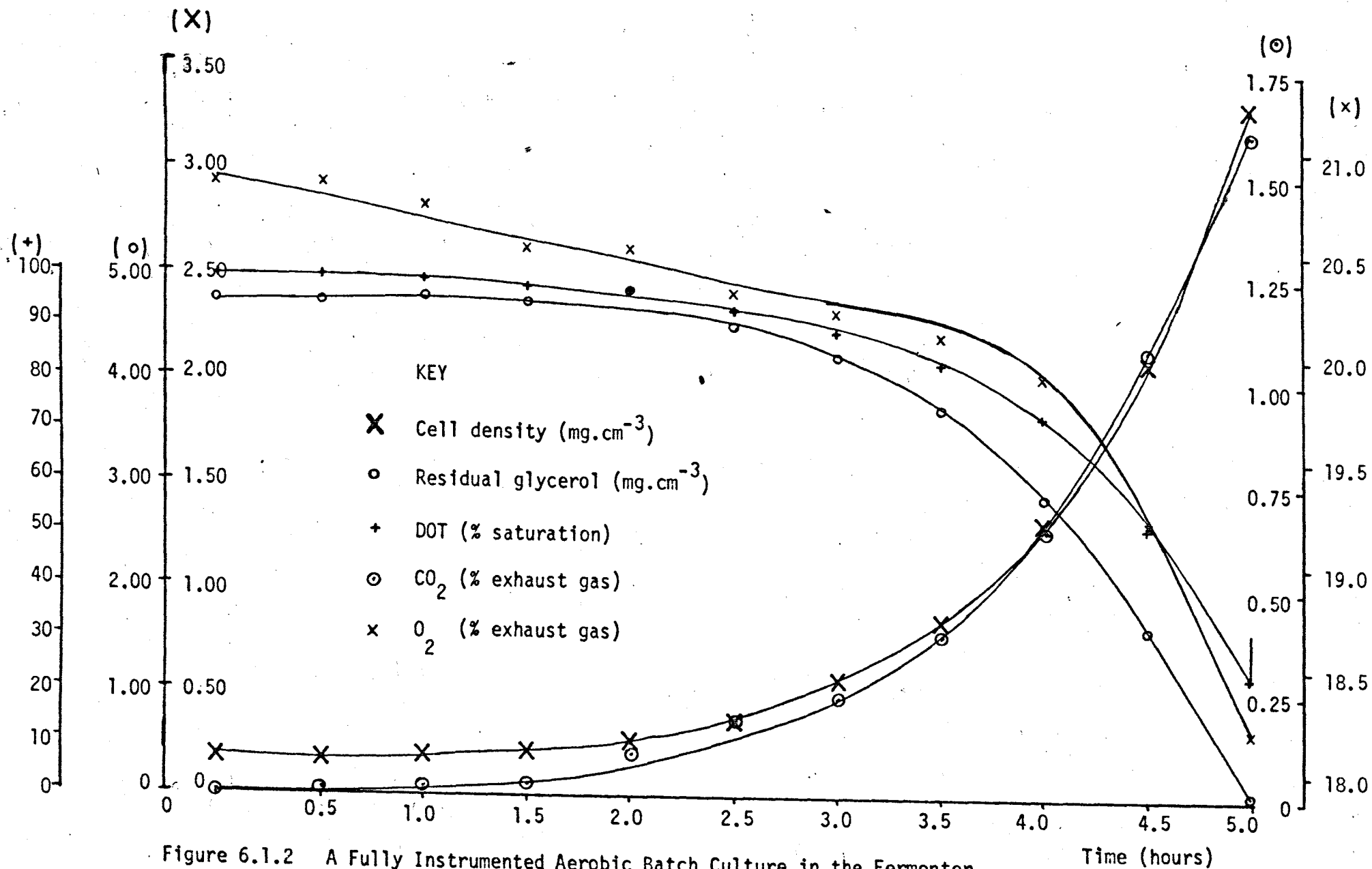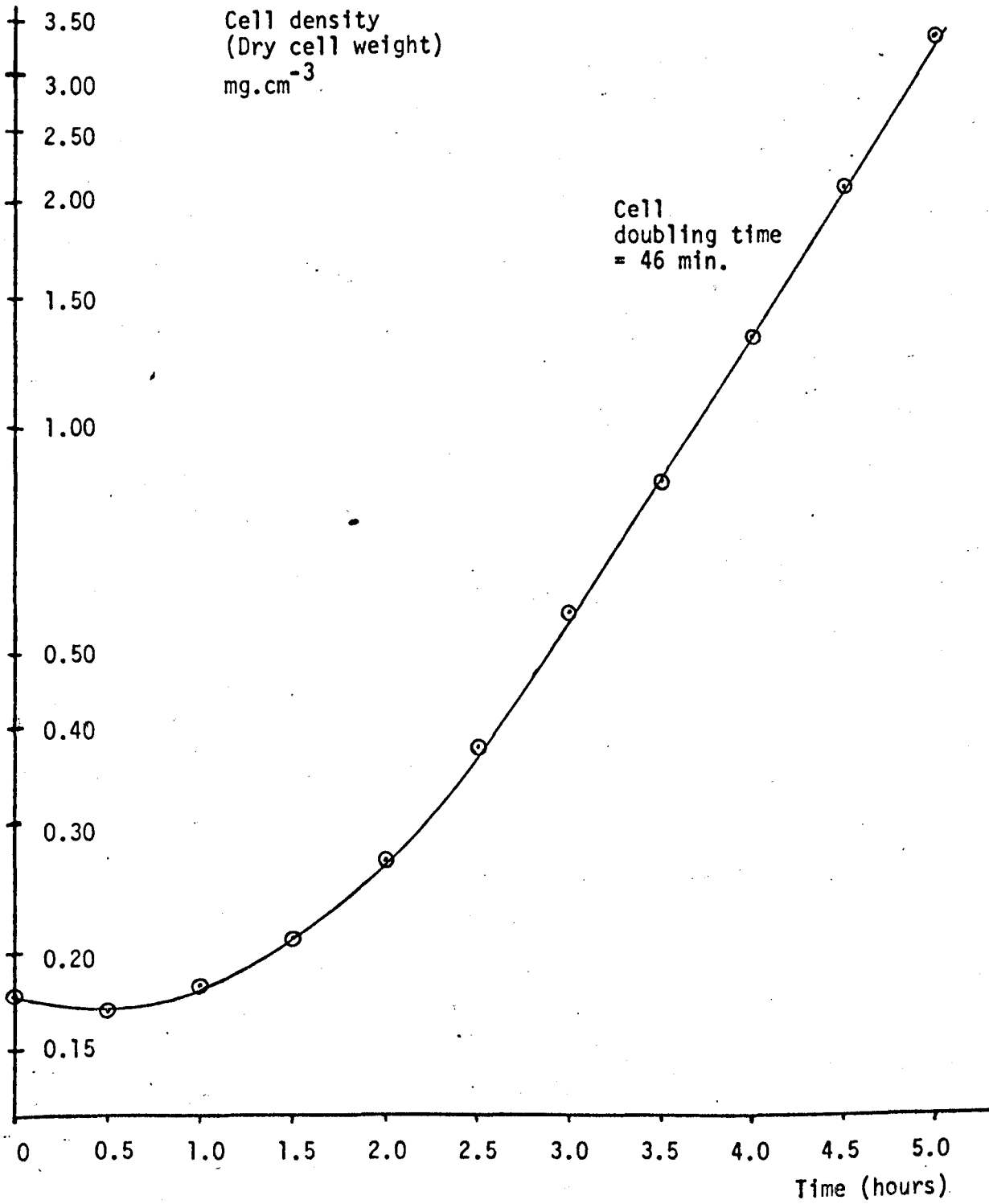
Fig. 6.1.3   Semi-logarithmic Growth Curve
             (Fermenter Aerobic Culture)

utilisation and growth process were carried out without interruption, and were only limited by the organism's maximum growth rate under these conditions.

The results verified that the highest cell density is obtained when residual (supernatant) glycerol concentration just reaches zero, indicating that only carbon-limitation prevents further growth. Carbon-limitation at exactly 5.0 hours from the time of inoculation was observed by an immediate rise in dissolved oxygen tension, which left a sharp peak on the recorder chart. Glycerol concentration at this point was found to be virtually zero (0.027 mg.cm^{-3}). Similar peaks in the traces of carbon dioxide and oxygen concentrations in the exhaust gas were found at 5.0 hours.

The results of one typical aerobic batch culture are reported here. Except for residual glycerol concentration, all these measurements were routinely made or recorded during batch cultures, as a preliminary stage to fully-instrumented continuous cultures. The results of other batch cultures were found to be in close agreement with the typical results given here.

6.1.2.2 Effect of impeller speed

A series of aerobic batch cultures was carried out at four different impeller speeds, and hence four different oxygen-transfer coefficients. The object of the experiment was to determine if differing oxygen-transfer conditions in the fermenter had any effect on the batch growth kinetics of the organism if DOT was maintained above the critical level.

Media (containing 5.0 g.l^{-1} glycerol), pH, temperature, and all other culture conditions were identical in all cultures. The

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impeller speeds used were 700 min^{-1}, 800 min^{-1}, 900 min^{-1} and 1000 min^{-1}. The results of cell doubling time, specific growth rate, terminal (carbon-limited) cell density and terminal dissolved oxygen tension are shown in Table 6.1.2:-

Table 6.1.2   Batch cultures at Different Impeller Speeds

Impeller Speed (min.$^{-1}$)	Cell Doubling-Time (min.) t_d	Specific Growth Rate (h^{-1}) μ	(Terminal) Cell Density (mg.cm^{-3})	DOT (% satn.)
700	48	0.87	3.26	26
800	47	0.88	2.97	58
900	50	0.83	3.03	78
1000	46	0.90	3.30	81

It can be seen that there is no clear relationship between the growth kinetics of a batch culture and impeller speed within this range. Similarly, the terminal cell density when the substrate concentration reaches a limiting value appears unrelated to impeller speed. However, the differences in oxygen-transfer efficiencies among the four cultures are evident from the terminal dissolved oxygen tensions.

The clear trend is for DOT to reach lower values at the lower impeller speeds. The difference in terminal DOT between successive impeller speeds diminishes as the speed increases. However, it can be seen that at none of the impeller speeds investigated does DOT reach zero or a limiting value, the lowest (26% satn. at 700 min^{-1}) being well above this value. Thus, impeller speeds maintaining dissolved oxygen tensions above the critical level appear to have no obvious

effect on the growth kinetics of batch cultures of K. aerogenes.

6.1.3   Continuous cultures

As in aerobic batch culture, the main variable whose effects were investigated in continuous culture was impeller speed. After early experiments to verify that a glycerol concentration of 5 g.l^{-1} in the medium was optimal for studies of oxygen and carbon-limitation, a series of continuous cultures at four different impeller speeds was carried out at dilution rates up to the washout value. The effects of impeller speed on oxygen transfer coefficient ($K_L a$) and wall growth formation were also investigated.

6.1.3.1   Determination of a suitable glycerol concentration for further studies

Two continuous cultures were carried out, one with medium containing 5 g.l^{-1} glycerol, the other 10 g.l^{-1}. Air was passed into the fermenter at a rate of 0.63 min^{-1}. All other aspects of the cultures were identical, including the impeller speed of 850 min.$^{-1}$, chosen as the arithmetic mean of the four speeds intended for further studies.

As in all continuous cultures of this type, steady-state dissolved oxygen tension fell as dilution rate was increased. The values of steady-state DOT at each dilution rate are shown for each medium glycerol concentration in Fig. 6.1.4. A straight line is fitted to the points in both cases.

Assuming that oxygen limitation occurs when the steady-state DOT value falls to near zero, it can be seen that this limitation occurs at a considerably higher dilution rate in the culture provided with 5 g.l^{-1} glycerol then in that with 10 g.l^{-1} glycerol in the
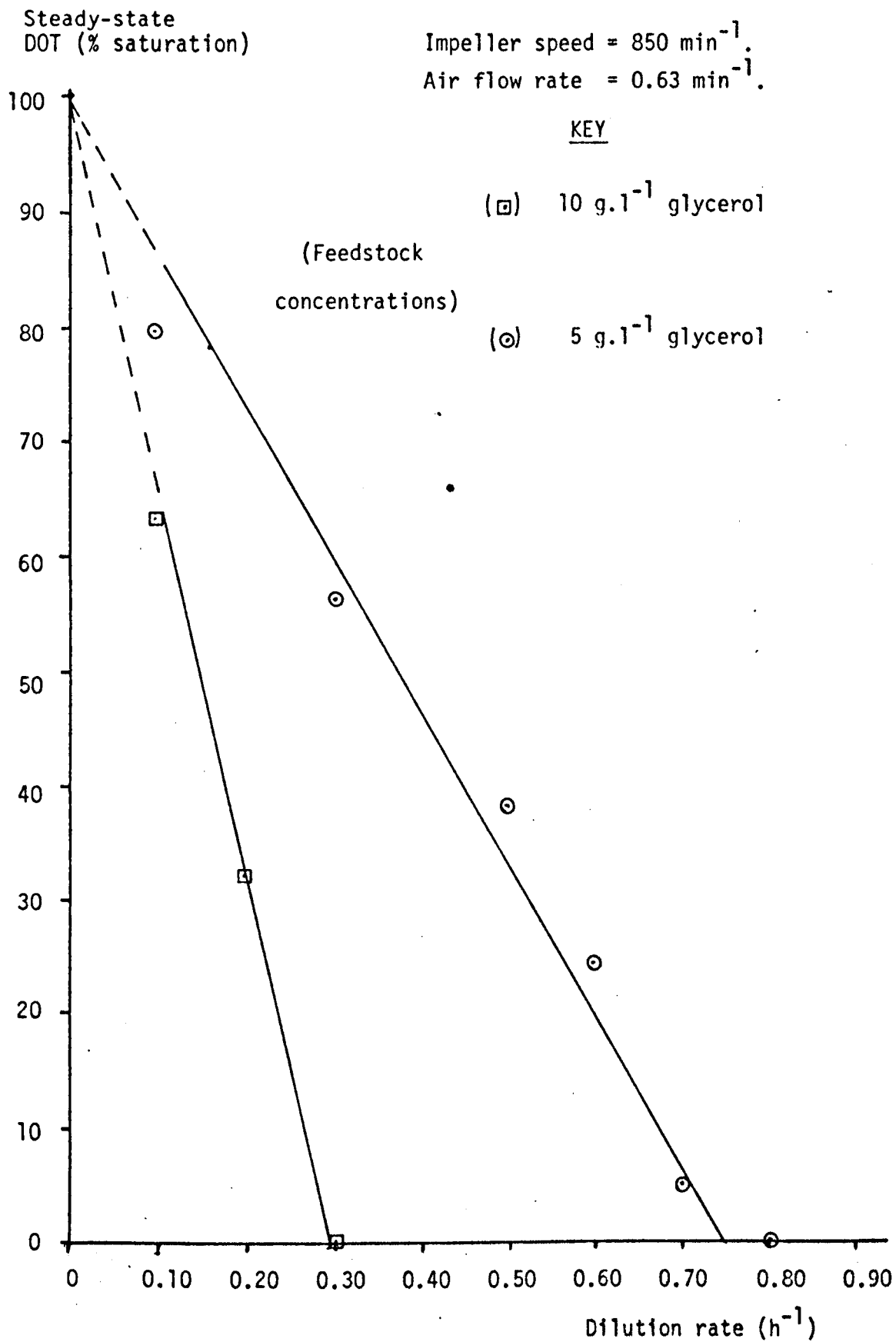
Figure 6.1.4   Effect of Dilution Rate on Steady-State DOT at two
Feedstock Glycerol Concentrations

medium.   Extrapolation of the lines to the x-axis shows zero DOT to
be found at:-

D = ca. 0.75 h^{-1} for medium containing 5 $g.l^{-1}$ glycerol

D = ca. 0.30 h^{-1} for medium containing 10 $g.l^{-1}$ glycerol

Thus, for studies of the transition from carbon-limitation to
oxygen-limitation with increasing dilution rate in continuous cultures,
10 $g.l^{-1}$ glycerol is too high a medium concentration since oxygen-
limitation occurs at low dilution rate.   Media containing 5 $g.l^{-1}$
glycerol seem to be better suited for these studies, but should also be
combined with a higher air flow rate of 1.0 $min.^{-1}$.   These conditions
of glycerol concentration and air flow rate were therefore used in all
further continuous cultures of K. aerogenes.

6.1.3.2   Effects of Impeller speed

Continuous cultures were carried out at impeller speeds of
700 $min.^{-1}$, 800 $min.^{-1}$, 900 $min.^{-1}$, and 1000 $min.^{-1}$ over the range of
dilution rates from 0.10 h^{-1} to 1.40 h^{-1}. ($>D_c$).   Cell density and
steady-state DOT were measured at all conditions of impeller speed and
dilution rate.   In addition, oxygen and carbon dioxide concentrations
in the exhaust gas were determined under all conditions.

The plots of cell density and steady-state DOT against
dilution rate for all four impeller speeds are shown in Fig. 6.1.5.

The medium contained 5.0 $g.l^{-1}$ glycerol, and the culture was
maintained at pH 6.5 and 37.0°C.   Air was sparged at 1.0 min^{-1}.

The gradients of the DOT lines can be seen to become
increasingly negative as impeller speed is reduced.   Moreover, the
difference between the gradients of successive cultures becomes greater
at the lower impeller speeds.   (This effect parallels the difference
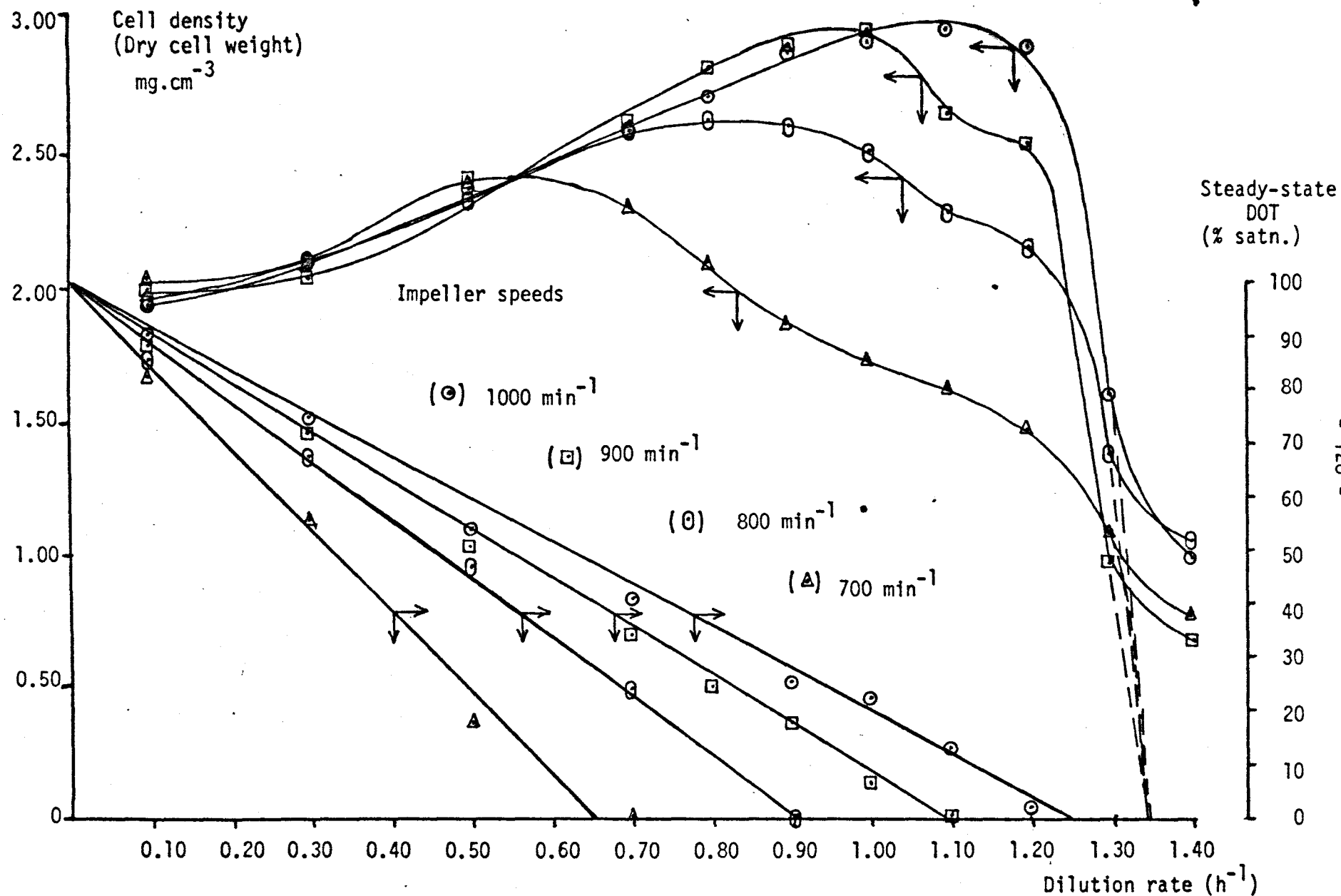
Figure 6.1.5   Cell Density and DOT vs. Dilution Rate at a Series of Impeller Speeds

in dissolved oxygen tension peak heights in batch cultures at these four impeller speeds, as shown in Section 6.1.2.2).

The cell density vs. dilution rate curves show classical double (C-O) substrate limitation at a series of oxygen-transfer coefficients. At low (≤ 0.5 h^{-1}) dilution rates, where steady-state DOT is above the critical level, the curves follow each other closely. (Again, this reflects the results of batch-growth kinetics at DOT values above critical at a series of impeller speeds, as shown in Section 6.1.2.2). However, above D = 0.5 h^{-1}, the curves can be seen to differ owing to the onset of oxygen limitation. The dilution rate at which maximum cell density is found increases with increasing impeller speed and can be seen to be a little lower than the dilution rate at which steady-state DOT falls to zero. Thus, above a dilution rate which is peculiar to each impeller speed (and oxygen-transfer coefficient), carbon limitation is replaced by oxygen limitation. Increase of residual glycerol concentration from immeasurably low levels was also found at dilution rates close to these. These observations are summarised in Table 6.1.3:-

Table 6.1.3   Transition from Carbon-Limitation to Oxygen-Limitation

Impeller Speed (min^{-1})	Approx. Dilution Rate (h^{-1}) at which:		
	Maximum Cell Density is found	DOT falls to zero	Residual Glycerol Concentration rises above zero
700	0.55	0.65	0.70
800	0.80	0.90	0.80
900	0.95	1.10	1.10
1000	1.10	1.25	1.20

The extent to which oxygen limitation affects each culture can be seen from Fig. 6.1.5. The effect is considerably more marked at 700 min^{-1} than at 800 min^{-1}., which in turn is more marked than at 900 min^{-1}. Again, the relative differences diminish at higher impeller speeds. At an impeller speed of 1000 min^{-1}, growth-rate limitation intervenes at a dilution rate lower than that at which oxygen limitation would occur. Thus, oxygen limitation is just avoided at all dilution rates under these conditions.

At dilution rates in excess of 1.20 h^{-1}, all cultures suffered drastic reduction of cell density. However, cell densities did not fall appreciably at a dilution rate of 1.40 h^{-1}; considerable wall-growth was noticed in the cultures at this dilution rate. Thus, it appeared that re-inoculation from wall-growth was preventing complete washout. (See Section 6.1.3.4). In order to determine the critical dilution rate, D_c, in the absence of reinoculation, it was therefore necessary to extrapolate the rapidly-falling cell density curve to the x-axis for all cultures. D_c appears to be independent of impeller speed, at ca. 1.35 h^{-1}. This is equivalent to a cell doubling time of ca. 30.8 minutes.

Measurements of oxygen and carbon dioxide concentrations in the exhaust gas as a function of dilution rate were also made for each culture. Fig. 6.1.6 shows oxygen consumption (as a percentage of the air supplied) plotted against dilution rate for each impeller speed. Fig. 6.1.7 shows similar plots of carbon dioxide (as a percentage of the exhaust gas) production vs. dilution rate.

Again all plots closely follow each other up to D = ca. 0.5 h^{-1}. Above this dilution rate, however, total culture oxygen consumption and CO_2 production at 700 min^{-1} begin to reach plateaux. Similar plateaux are found at 800 min^{-1}, but at higher levels. At impeller
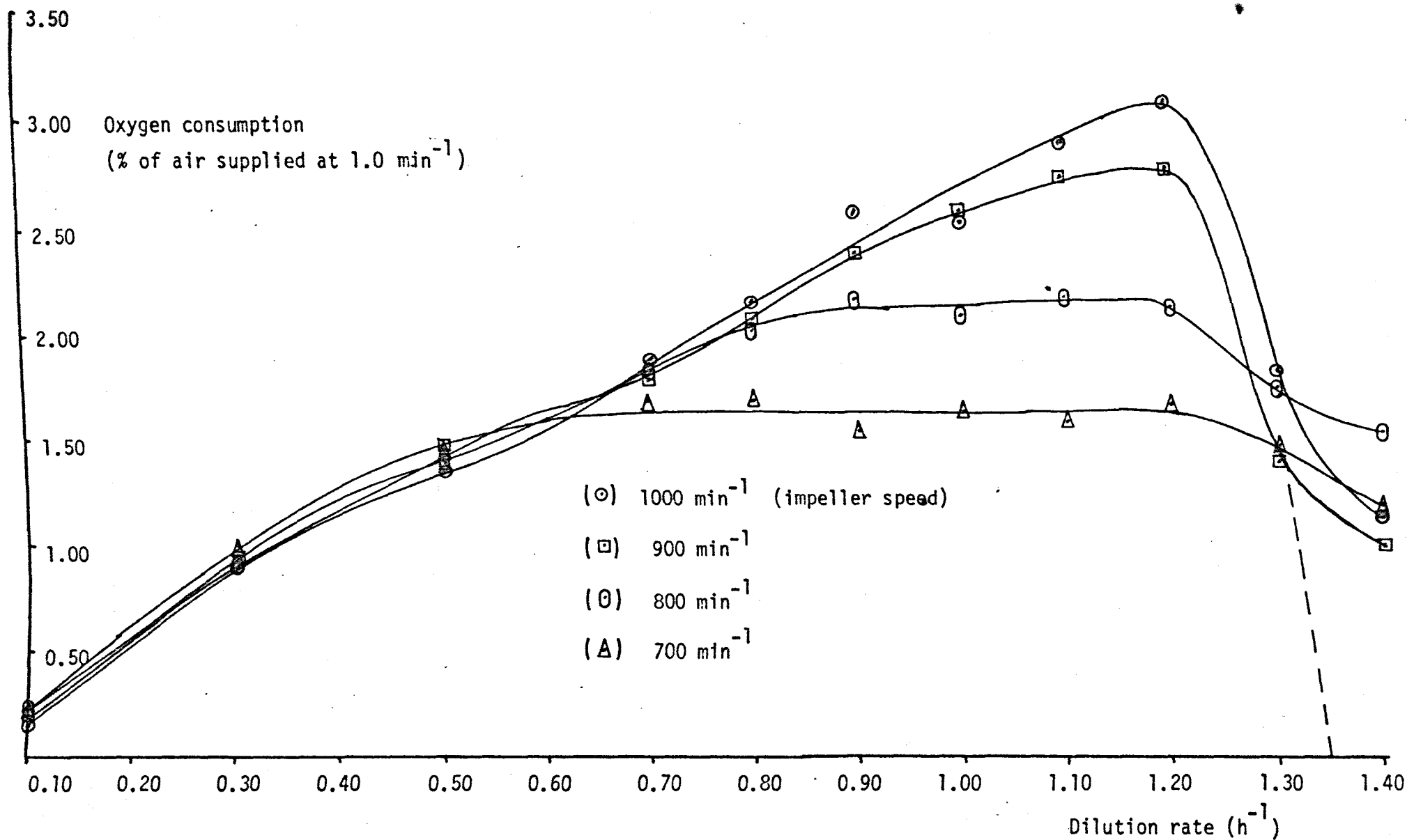
Figure 6.1.6   Oxygen Consumption vs. Dilution Rate at a Series of Impeller Speeds.
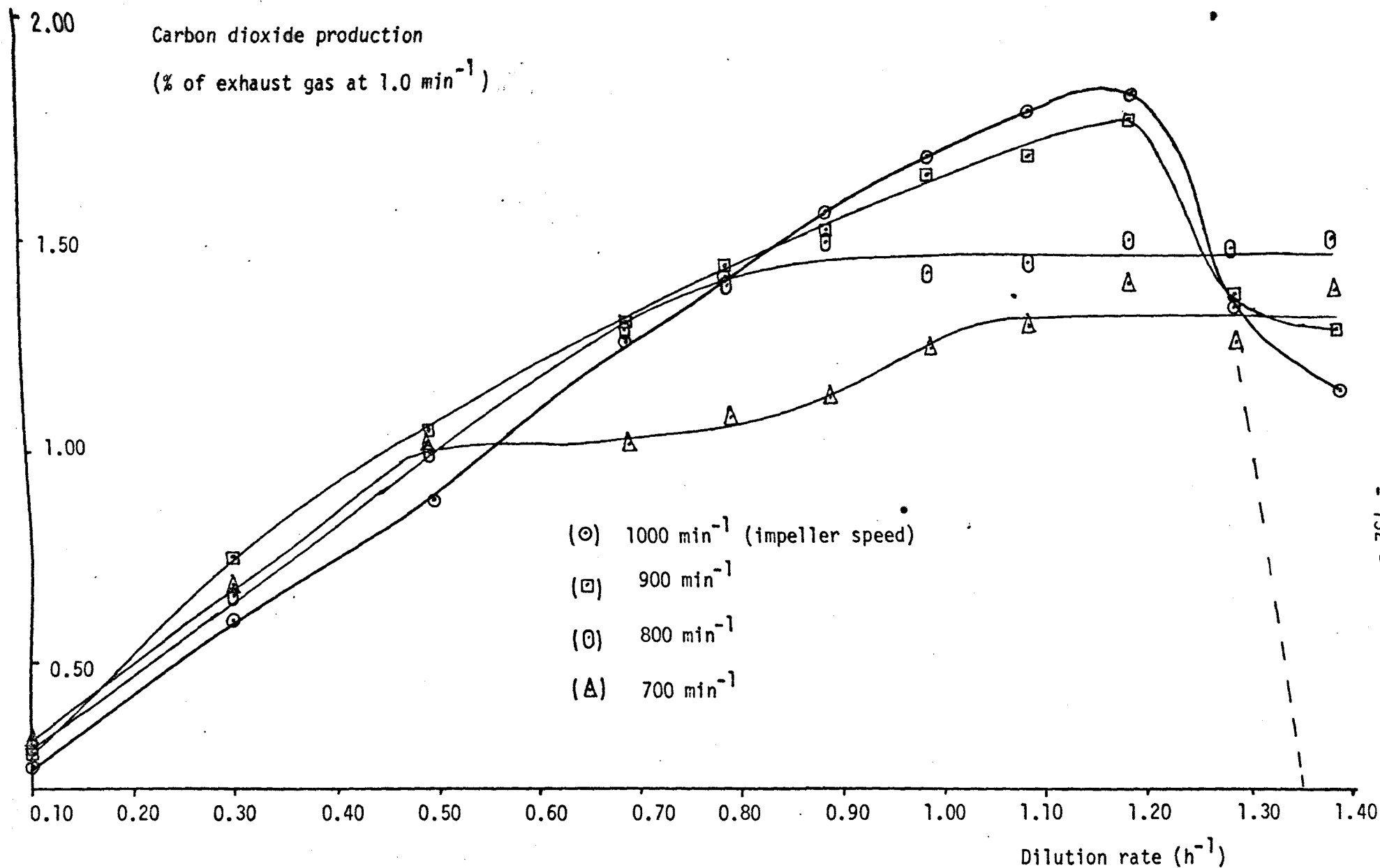
Figure 6.1.7   Carbon dioxide Production vs. Dilution Rate at a Series of Impeller Speeds.

speeds of 900 and 1000 min^{-1}, however, oxygen consumption and CO_2 production continue to increase with dilution rate,and reach peaks at $D = 1.20 \ h^{-1}$.

An empirical measurement of respiration rate is made by division of total culture oxygen consumption (as a percentage of the air supplied) by the culture cell density. Since all other conditions are constant, the figures obtained from such calculations are directly related to the true respiration rate (expressed as m.mole O_2 consumed. g dry weight$^{-1}.h^{-1}$) by a constant factor. Table 6.1.4 shows calculations of 'respiration rate' at three example dilution rates:-

Table 6.1.4   Empirically-Derived 'Respiration Rates'

Dilution Rate (h^{-1})	Impeller Speed (min^{-1})	Oxygen Consumption (% Air Supplied)	Culture cell Density (mg.cm^{-3})	'Respiration Rate' (% O_2/cell density)
0.50	700	1.45	2.43	0.60
0.50	800	1.35	2.33	0.58
0.50	900	1.55	2.37	0.65
0.50	1000	1.35	2.35	0.57
0.80	700	1.65	2.10	0.79
0.80	800	2.05	2.63	0.78
0.80	900	2.10	2.69	0.78
0.80	1000	2.15	2.81	0.77
1.10	700	1.65	1.64	1.01
1.10	800	2.15	2.30	0.93
1.10	900	2.75	2.63	1.05
1.10	1000	2.95	2.97	0.99

Thus, at any particular dilution rate, it appears that respiration rate is independent of impeller speed, although the two variables involved in the calculation (oxygen consumption and cell density) are both affected by impeller speed at dilution rates in excess of 0.50 h^{-1}. However, as might be expected, respiration rate does increase with dilution rate.

The results of this section are discussed in further detail in Chapter 7.

6.1.3.3   Determination of oxygen-transfer coefficients at four impeller speeds'

As outlined in the Theory and Experimental Design Chapter (Section 3.2), the oxygen-transfer coefficient, $K_L a$, can be found in steady-state continuous culture by an oxygen-balance method. Thus, by knowing the steady-state values of percentage air consumed as oxygen ($\%$ O_2) and dissolved oxygen tension (DOT), and providing that the culture conditions are as outlined in Section 3.2, the oxygen-transfer coefficient may be calculated from the empirical relationship:-

$$K_L a = \frac{1.713 \ (\% \ O_2)}{1.000 - (DOT \times 10^{-2})} \quad min^{-1}$$

The oxygen-transfer coefficients of the four continuous cultures carried out at impeller speeds of 700 min^{-1}, 800 min^{-1}, 900 min^{-1} and 1000 min^{-1} were determined by this method. Thus, variation of $K_L a$ with impeller speed at a single dilution rate, and with dilution rate at a single impeller speed may be checked.

Using the empirical formula given above, the $K_L a$ values given in Table 6.1.5 were calculated at each impeller speed and every dilution rate investigated. All $K_L a$ values have units of minutes^{-1}. It can be seen that at D = 0.10 and 0.30 h^{-1} and at D = 1.30 and 1.40 h^{-1}, $K_L a$ values determined in this way may vary widely from those within the central range of values. Mean values are therefore given over the range of dilution rates 0.50 to 1.20 h^{-1} at each impeller speed. The general trend is, of course for higher $K_L a$ values at higher impeller speeds. Wide variations in $K_L a$ at high and low dilution rates may be due to inaccuracies in the measurement of dissolved oxygen tension and oxygen concentration in the exhaust gas. Despite these untypical results, it is obvious that there is, as expected, no relationship between dilution rate and $K_L a$.

Increase of $K_L a$ with increase of impeller speed does not appear to be linear, high differences being found between 700 and 800 min^{-1} and between 900 and 1000 min^{-1}. (Both differences are ca. 1.0-1.2 min^{-1}). Between 800 and 900 min^{-1} impeller speeds, however, $K_L a$ increases by only ca. 0.5 min^{-1}.

6.1.3.4 Effect of impeller speed on wall-growth formation

As reported in Section 6.1.3.2, re-inoculation from wall-growth at high dilution rate (>D_c) prevented washout in all four continuous cultures. An investigation of the extent of wall growth at D = 1.40 h^{-1} and at impeller speeds of 700, 800, 900, and 1000 min^{-1} was therefore instigated. As outlined in the Theory and Experimental Design Chapter (Section 3.1.2.2), wall-growth cells were considered to be using oxygen at the same specific rate as suspended cells. The total mass (dry cell weight) of wall growth cells was therefore

TABLE 6.1.5 $K_L a$ Values at each Growth Condition of Four Continuous Cultures

Dilution Rate (h^{-1})	Impeller Speed (min^{-1})			
	700	800	900	1000
0.10	2.02	2.08	7.62	2.57
0.30	3.80	5.62	5.12	6.18
^0.50	3.13	4.45	4.58	4.98
0.70	2.91	4.55	4.20	5.47
0.80	2.91	-	4.16	-
0.90	2.66	3.77	4.42	5.94
1.00	2.83	3.65	4.55	5.61
1.10	2.74	3.84	4.71	5.72
v1.20	2.91	3.72	4.80	5.41
1.30	2.49	3.06	2.47	3.62
1.40	2.06	2.71	1.76	2.27
Mean value over indicated Range (0.5-1.2)	2.87	4.00	4.49	5.52

(all min.$^{-1}$)

determined from measurements of the total oxygen consumption and suspended cell density of the culture.

Thus, comparison of total oxygen consumption by the culture at $D = 1.40$ h^{-1} with a plot of oxygen usage vs. dilution rate (Fig. 6.1.6) gives a dilution rate and hence cell density(from Fig. 6.1.5) at which a culture with no wall-growth is consuming the same amount of oxygen. Subtraction of the actual suspended cell density from the derived figure, and multiplication by the culture volume gives the total dry cell weight of wall-growth (i.e. unsuspended) K. aerogenes cells.

The results of these calculations for the four impeller speeds are shown in Table 6.1.6. Total dry weight of wall-growth cells is plotted against impeller speed in Fig. 6.1.8(a). The plot is seen to exhibit a smooth curve, wall-growth decreasing with increasing impeller speed. The smoothness of the curve may indicate general reliability of the method even though speculative assumptions were made in its derivation. If the curve of total wall-growth vs. impeller speed is optimistically extrapolated to the x- and y-axes, where wall growth and impeller speed are (respectively) zero (Fig. 6.1.8(b)), it may be speculated that below about 400 min^{-1}, wall growth reaches a constant level, and is independent of impeller speed. Moreover, the acceleration in wall-growth depletion at higher impeller speeds appears to reach the ultimate level of zero wall-growth at ca. 1400 min^{-1}. These figures should be treated with great caution, however, since there is no direct evidence to substantiate them.

Table 6.1.6   Wall-Growth Formation at $D = 1.40$ h^{-1} and Four Impeller

Speeds

Impeller Speed (min^{-1})	700	800	900	1000
Dilution Rate at which total O_2 - consumption is the same as at 1.40 h^{-1}	0.39	0.59	0.30	0.40
Cell Density at the above dilution rate $(mg.cm^{-3})$	2.27	2.46	2.04	2.22
Cell density at $D = 1.40$ h^{-1} $(mg.cm^{-3})$	0.81	1.04	0.69	0.99
Effective 'cell density' of wall-growth $(mg.cm^{-3})$	1.46	1.42	1.35	1.23
Total dry weight of wall-growth cells (mg)	840	817	776	707
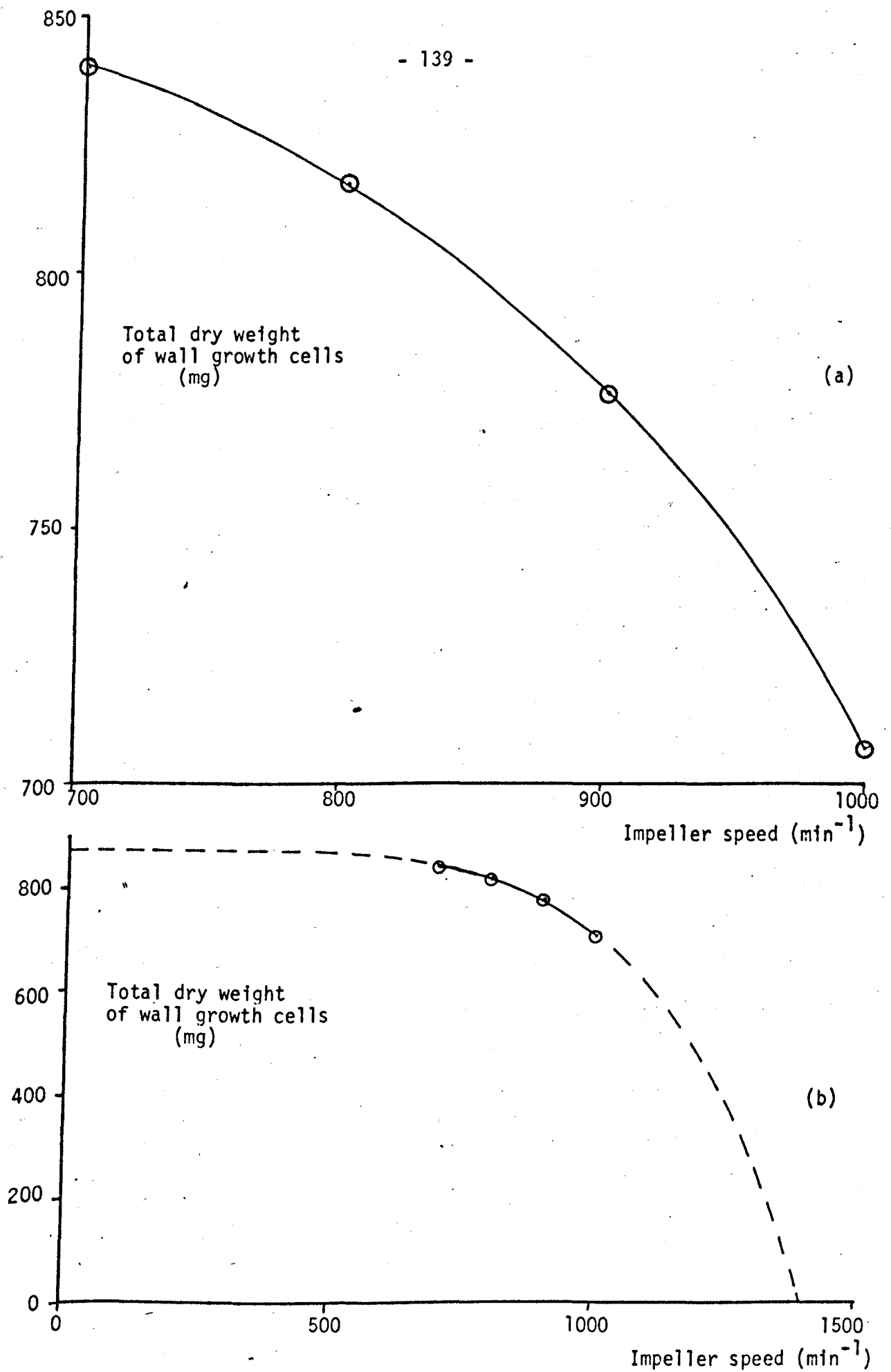
Figure 6.1.8   Effect of Impeller Speed on Total Wall Growth.

6.2   Anaerobic Cultures

Anaerobic cultures were carried out in specially designed shake-flasks and in the fermenter, where both batch and continuous anaerobic cultures were grown.   Intermittently aerated cultures normally ended (at zero aeration time per cycle) with a continuous anaerobic culture, but these will be dealt with in Section 6.3.

6.2.1   Shake-flask cultures

In order to determine whether anaerobic growth of K. aerogenes 418 on glycerol-salts medium with no added hydrogen acceptors was possible, simple flask cultures were grown in the apparatus described in Section 5.5.1(Fig. 5.5).   The medium used for these cultures was exactly the same as for all other cultures, containing 5.0 mg.cm^{-3} glycerol.

Sterile, pre-wetted oxygen-free nitrogen (containing less than 0.1 ppm oxygen) was passed through a sintered glass aerator in the culture flask.   Mixing was further provided by shaking the culture at 100 cycles.min^{-1} in a water-bath maintained thermostatically at 37°C. Samples were withdrawn, normally at hourly intervals, through a serum cap fitted with a sterile wide-bore hypodermic needle, into a sterile 1.0 cm^3 syringe.   A new sterile syringe was then aseptically fitted.

Normally, two cultures were carried out simultaneously; one aerobic, the other anaerobic.   Thus, basic comparison was made of the simple growth kinetics of aerobic and anaerobic cultures, grown under otherwise identical conditions.   (Air was supplied in exactly the same manner to the aerobic culture).   It was soon found that high flow rates of nitrogen to the anaerobic culture flask (1.0-1.5 min^{-1}) prevented batch growth.   This has been said to be due to 'stripping' of dissolved carbon dioxide below the minimum level required for growth (Neijssel,

personal communication). For this reason, nitrogen flow rates of 0.2 min^{-1} were used in anaerobic batch cultures.

In practice, 20 cm^3 of aerobically-grown inoculum was injected into 80 cm^3 of sterile medium in both the aerobic and anaerobic flasks. Thus, total culture volume in both 250 cm^3 flasks was 100 cm^3. The results of two typical cultures of this type are shown plotted (cell density vs. time) in Fig. 6.2.1.

The high inoculum concentration (0.54 $mg.cm^{-3}$ at the start of the culture) aided immediate growth of the aerobic culture with no obvious lag phase. Logarithmic growth of this culture was then sustained at a low rate for 3-4 hours, up to the point of carbon limitation. Gradual decay for the following 4 hours was then exhibited. The cell doubling time during the period of logarithmic growth was 2.35 h, equivalent to a specific growth rate of ca. 0.3 h^{-1}. The peak cell density of 1.505 $mg.cm^{-3}$ (dry cell weight) is rather lower than the expected cell density of ca. 2.65 $mg.cm^{-3}$, as found in aerobic shake-flask cultures grown in an orbital-shaker.

Anaerobic growth, however, exhibited a lag period of 3-4 hours. Growth then occurred over a period of 2-3 hours. The growth rate was low, and reached a peak cell density of only 0.98 $mg.cm^{-3}$, from an inoculum + medium cell density of 0.58 $mg.cm^{-3}$. Decay over the following two hours then took place. Residual glycerol concentration after eight hours growth was found to be at immeasurably low levels in both cultures. These two typical aerobic and anaerobic cultures in this apparatus show obvious differences between the growth kinetics of aerobically and anaerobically grown K. aerogenes, and are discussed in further detail in Chapter 7.
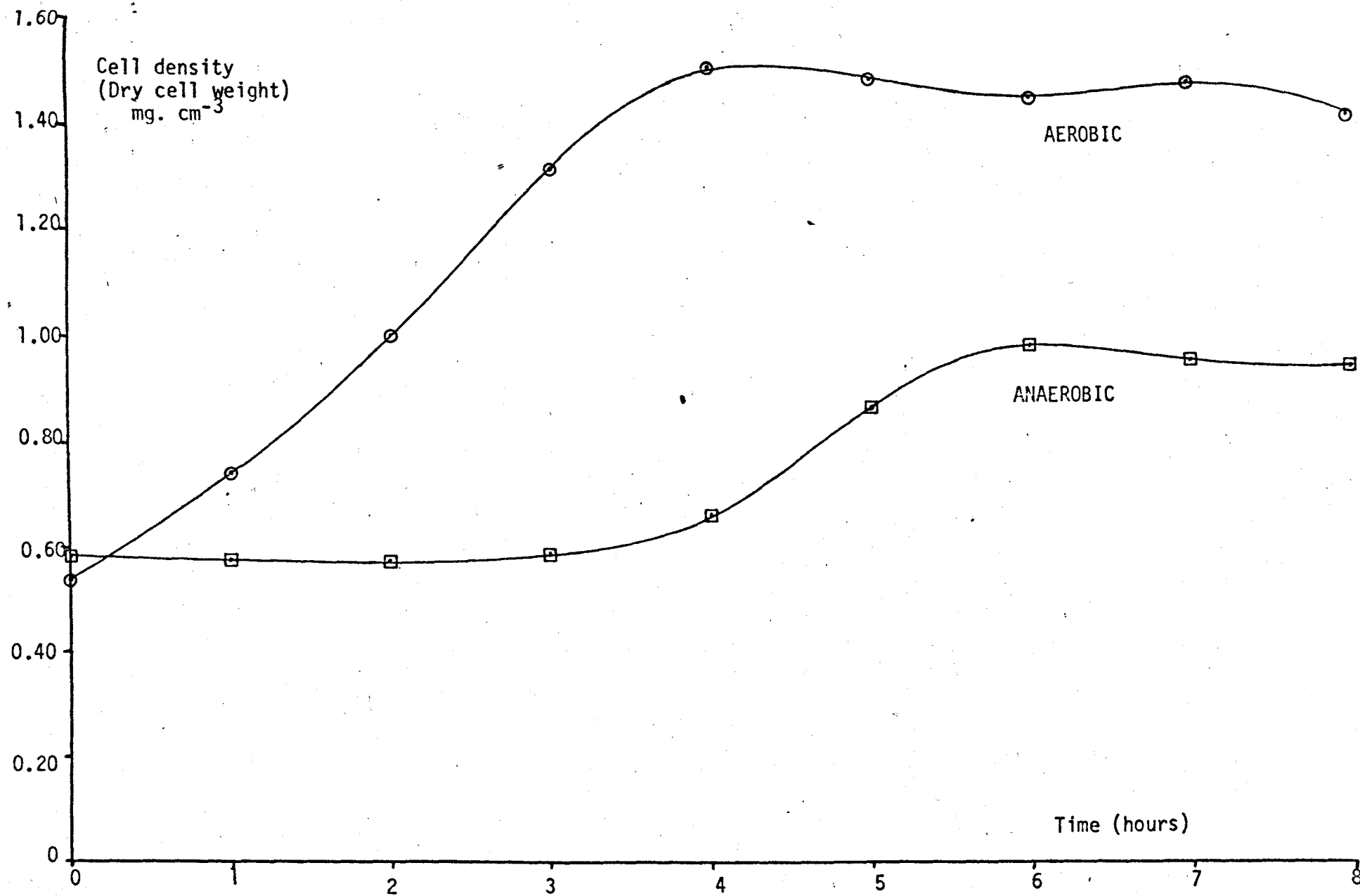
Figure 6.2.1   Simultaneous Aerobic and Anaerobic Shake Flask Cultures

6.2.2   Fermenter batch culture

Knowing anaerobic culture of K. aerogenes 418 on glycerol-salts medium to be feasible, more detailed study of substrate utilisation and growth kinetics was carried out in the more closely controlled environment of the fermenter.

The fermenter, containing 5.0 mg.cm^{-3} glycerol medium controlled at 37°C and pH 6.5, was purged with 0.2 min^{-1} oxygen-free nitrogen (115 cm^3 min^{-1}), and agitated at an impeller speed of 600 min^{-1}. Approximately 50 cm^3 of aerobically-grown inoculum was introduced to the fermenter (at 0 hours); a typical anaerobic batch growth curve is shown in Fig. 6.2.2.   Residual glycerol in the culture supernatant was determined in each sample removed for cell density determination.

From the plot of cell density vs. time, it can be seen that following a lag of under 4 hours, growth accelerates over about 3 hours, and rises linearly for about 1.5 hours.   Correspondingly, residual glycerol concentration falls linearly over the first 3 hours of growth, and then falls very rapidly over the following 2 hours.   If the growth curve is plotted semi-logarithmically, no linear portion of the plot is obvious.

However, semi-logarithmic plot of cell density vs. time reveals a maximum gradient corresponding to a doubling time (t_d) of ca. 1.17 hours.   This is equivalent to a maximum specific growth rate (μ_{max}) of ca. 0.59 h^{-1} for an anaerobic batch culture grown under the quoted conditions.

6.2.3   Continuous culture

A continuous anaerobic culture was grown over a range of dilution rates.   Culture cell density, residual glycerol concentration and carbon dioxide concentration
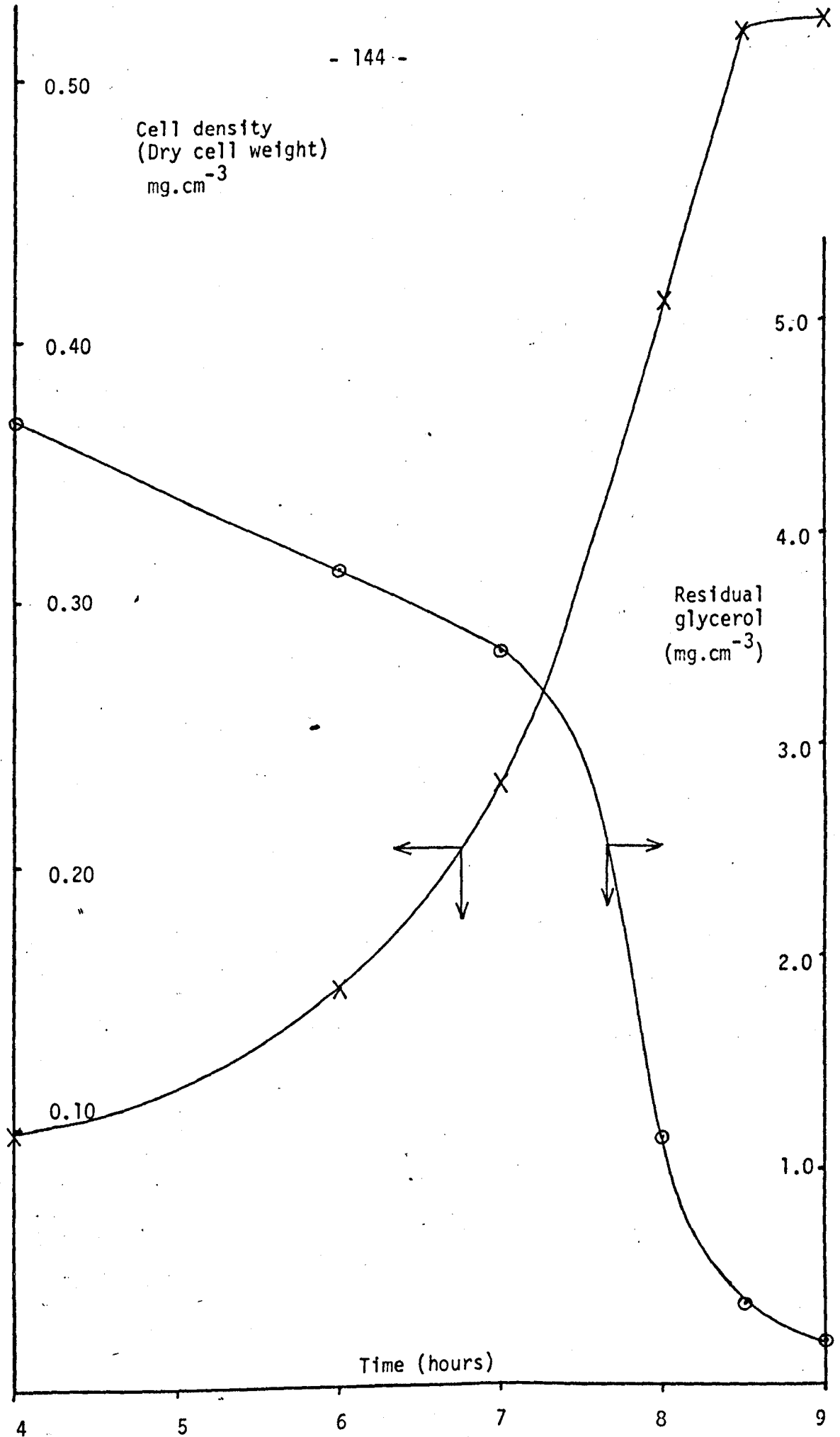
Figure 6.2.2   Batch Growth Curve with Glycerol Determination
(Fermenter Anaerobic Culture)

in the exhaust gas were all monitored at dilution rates from 0.10 h^{-1} to 0.90 h^{-1}. Unfortunately, the low impeller speed of 600 min^{-1} permitted the formation of a great deal of wall growth at higher dilution rates, and re-inoculation of the culture prevented washout, making it very difficult to determine the critical dilution rate (D_c). It should be noted that since silicone rubber tubing was used for all liquid-transfer, the oxygen-pervious nature of this tubing may have permitted the introduction of a small amount of dissolved oxygen to the culture.

The results of this experiment are given in Table 6.2.1:-

Table 6.2.1   Continuous Anaerobic Culture

Dilution Rate (h^{-1})	Cell Density $(mg.cm^{-3})$	Residual Glycerol Concentration $(mg.cm^{-3})$	CO_2 Production $(cm^3.min^{-1})$
0.10	0.31	0.23	-
0.30	0.32	0.47	-
0.50	0.36	0.59	2.84
0.70	0.28	0.88	3.25
0.80 (W.G.)	0.30	-	-
0.90 (W.G.)	0.28	0.74	-

W.G. = Wall Growth

Washout appears to begin at D = 0.70 h^{-1}, but wall growth at dilution rates above this prevents the determination of D_c. The residual glycerol concentration in the culture supernatant is well above zero at all dilution rates, and tends to rise with dilution rate.

Cell densities are very low compared with the equivalent values in aerobic culture. For example, at $D = 0.50$ h^{-1}, the anaerobic culture cell density is only ca. 15% of the equivalent aerobic value.

Carbon dioxide production by the culture was monitored at two dilution rates. In order to gain a more accurate understanding of CO_2 production, values should be corrected for the cell density of the culture at constant volume:-

Dilution Rate (h^{-1}) (D)	$\dfrac{CO_2 \text{ production}}{\text{cell density}}$ ratio (C)	$\dfrac{CO_2 \text{ production/cell density}}{\text{Dilution rate}}$ $(\frac{C}{D})$
0.50	7.89	15.8
0.70	11.61	16.6

Thus, it appears that carbon dioxide production per unit mass of cells may be directly related to dilution rate, the ratios $\frac{C}{D}$ being similar at the two dilution rates examined.

Further study of continuous anaerobic cultures is made in Section 6.3, since most intermittently-aerated continuous cultures ended at zero aeration time per cycle.

6.2.4 Anaerobic/aerobic transitions

In order to gain information on the processes involved when an anaerobic culture is aerated, and when an aerobic culture is rendered anoxic, these conversions were made whilst dissolved oxygen tension and oxygen and carbon dioxide concentrations in the exhaust gas were recorded.

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Conversions were made to continuous cultures which were growing under otherwise steady-state conditions at dilution rates of 0.10 h^{-1}.

Fig. 6.2.3 shows the effects on DOT, and O_2 and CO_2 concentrations in the exhaust gas when 1.0 min^{-1} air is replaced by 0.2 min^{-1} oxygen-free nitrogen at the zero time point. (Adapted from the chart-trace of a multipoint recorder).

DOT falls first rapidly, then more slowly, to reach zero after ca. 0.8 hours, where it remains. Similarly, as dissolved oxygen is stripped from the culture, and oxygen is purged from the gas-lines, the oxygen content of the exhaust gas rapidly falls to zero (ca. 0.2 hours), where it remains. The output of carbon dioxide from the culture is considerably more informative: From an initial concentration of 0.31%, CO_2 first rises rapidly to reach a peak of 0.68% after only 0.1 hours. (This is presumably due to the lower gas flow-rate purging CO_2 from the system at a higher concentration). CO_2 concentration then falls gradually, to reach a minimum of ca. 0.12% after ca. 1.5 hours, and then gradually rises. The rise in CO_2 output, presumably due to adaptation by the culture, continues slowly up to ca. 5 hours, but then accelerates to reach a peak at 7.0 hours. Slight overshoot in this peak is evident, CO_2 concentration just reaching 1.00% before falling back to a steady-state value of 0.85% after 8 hours from the original conversion. This general response of CO_2 output from the culture when this conversion is made was found to be reproducible.

Fig. 6.2.4 shows the corresponding effects on DOT and gas concentrations when 0.2 min^{-1} oxygen-free nitrogen was replaced by 1.0 min^{-1} air (Adapted from a chart-trace).

DOT rose rapidly from zero to reach 70% saturation in 0.1 hours, and peaked at 84% saturation after 0.5 hours. It then gradually
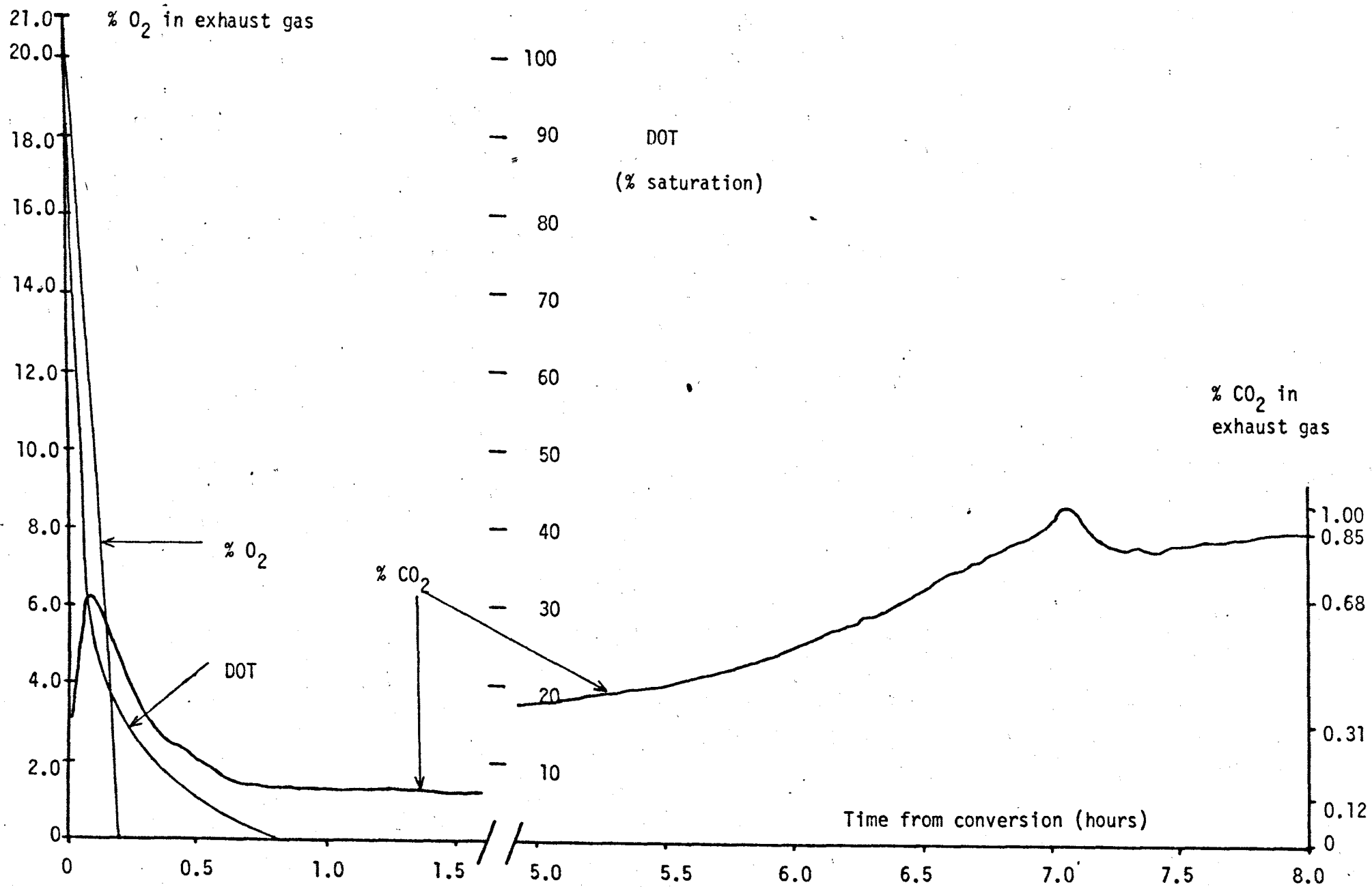
Figure 6.2.3   Gas Traces following an Aerobic/Anoxic Conversion
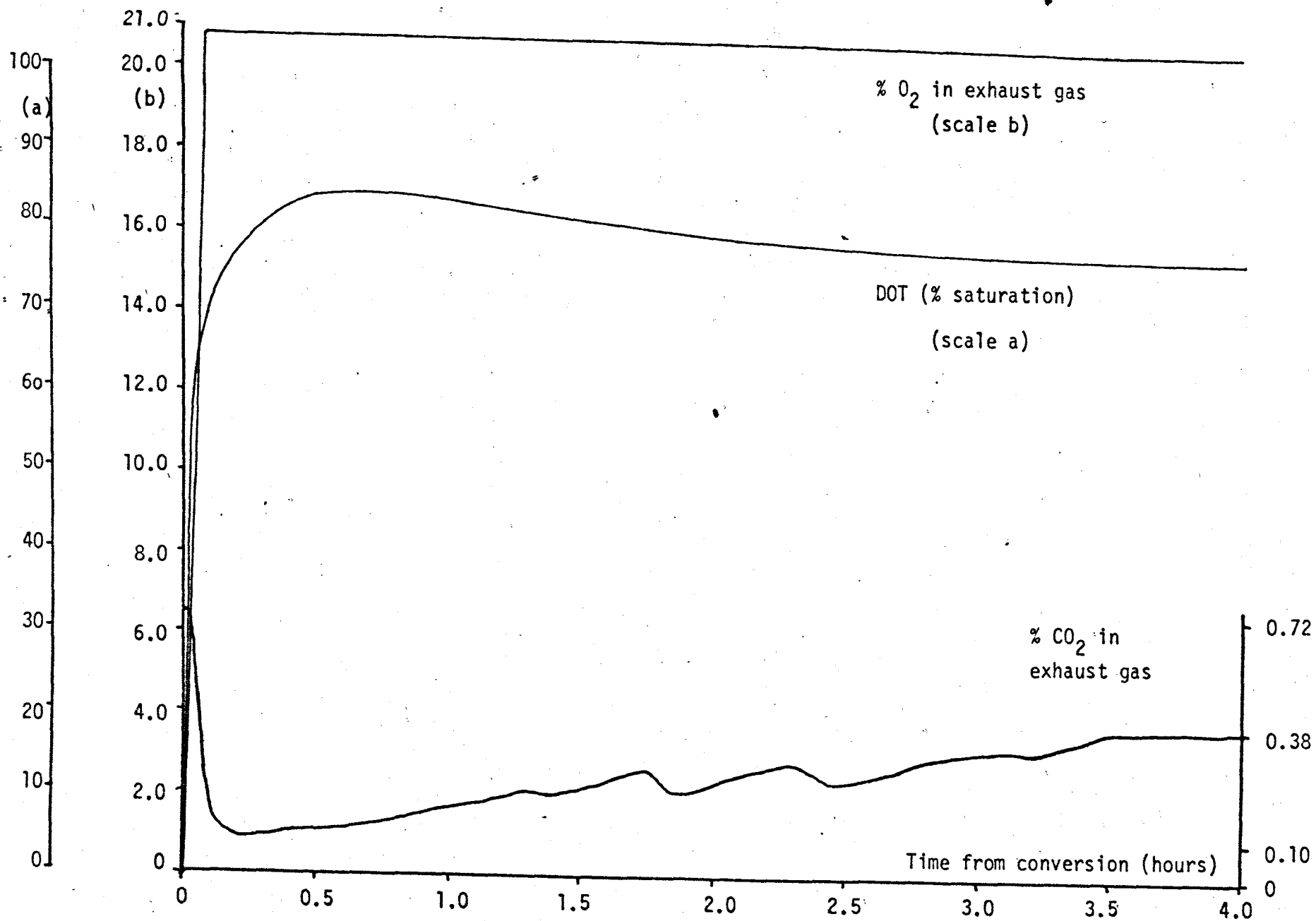
Figure 6.2.4   Gas Traces Following on Anaerobic/Aerated Conversion

decreased to reach a steady-state value of 77% after a total of 3.5 hours from conversion. % O_2 in the exhaust gas rose in a similarly rapid manner from zero to 20.8% in 0.1 hours. It then slowly fell to a steady-state value of 20.5% after a total of 3.5 hours. % CO_2 in the exhaust gas fell rapidly in the first 0.1 hours following the conversion from 0.72% to a minimum of 0.10%. (The speed of this reduction was probably aided by the increased gas flow rate). CO_2 then gradually rose to a steady-state value of 0.38%, again after a total of 3.5 hours from the original conversion.

It certainly appeared that the culture was capable of taking up oxygen as soon as the conversion was made, since DOT did not rise to 100% saturation, and % O_2 did not rise to 20.95%. Moreover, the conversion in this direction (anaerobic to aerobic) appeared to be undertaken more readily than in the other direction (aerobic to anaerobic). The time taken to reach a new steady-state was only 3.5 hours, compared with the 8.0 hours required to establish the anaerobic culture at the same dilution rate (0.10 h^{-1}).

6.2.5   Alternative substrate utilisation by anaerobic and aerobic
         cultures

As explained in the Theory and Experimental Design Section 3.3.1.1, glycerol is metabolised by K. aerogenes 418 by two different pathways leading to the common intermediate, dihydroxyacetone phosphate. Both pathways involve two stages, and therefore each includes a different intermediate. In the aerobic pathway, the intermediate is glycerol-3--phosphate (G-3-P); the anaerobic intermediate is dihydroxyacetone (DHA). (See Fig. 3.1).

The object of this series of experiments was to expose carbon-limited aerobic and anaerobic cultures of K. aerogenes to the intermediates (G-3-P and DHA), and to judge the response of the culture to the alternative substrate. In the case of anaerobic cultures, judgement was made by recording the CO_2 output from the culture before, during and after exposure. % CO_2 output in the exhaust gas was similarly recorded for aerobic cultures; the response of dissolved oxygen tension was additionally recorded.

In all four experiments (two anaerobic, two aerobic), the cultures were grown continuously on media containing 5 mg.cm^{-3} glycerol at a dilution rate of 0.50 h^{-1}. When steady-state growth had been apparent for at least 24 hours, the medium pump was switched off whilst recording % CO_2 output and DOT. When the former reading reached zero, and the latter (in the case-of aerobic cultures) 100% saturation, G-3-P or DHA solution was added to the culture to a final culture concentration of 4 mM. Recording then continued until the readings returned to the 'starved' levels.

Fig. 6.2.5 (adapted from the chart-traces) shows the effect on % CO_2 output of adding G-3-P or DHA to two anaerobic cultures in the described manner.

Addition of G-3-P results in a very small increase in carbon dioxide evolution by the culture. The maximum height of the peak, after ca. 0.3 hours from introduction of the compound is 0.08% CO_2. CO_2 evolution then slowly decays, to return to zero after a further hour.

By contrast, addition of an equimolar amount of DHA results in a much more rapid rise in CO_2 evolution, reaching a consirably higher peak of 1.05% CO_2 after 0.25-0.30 hours from the addition. Again, resumption of zero CO_2 evolution requires approximately one further hour.
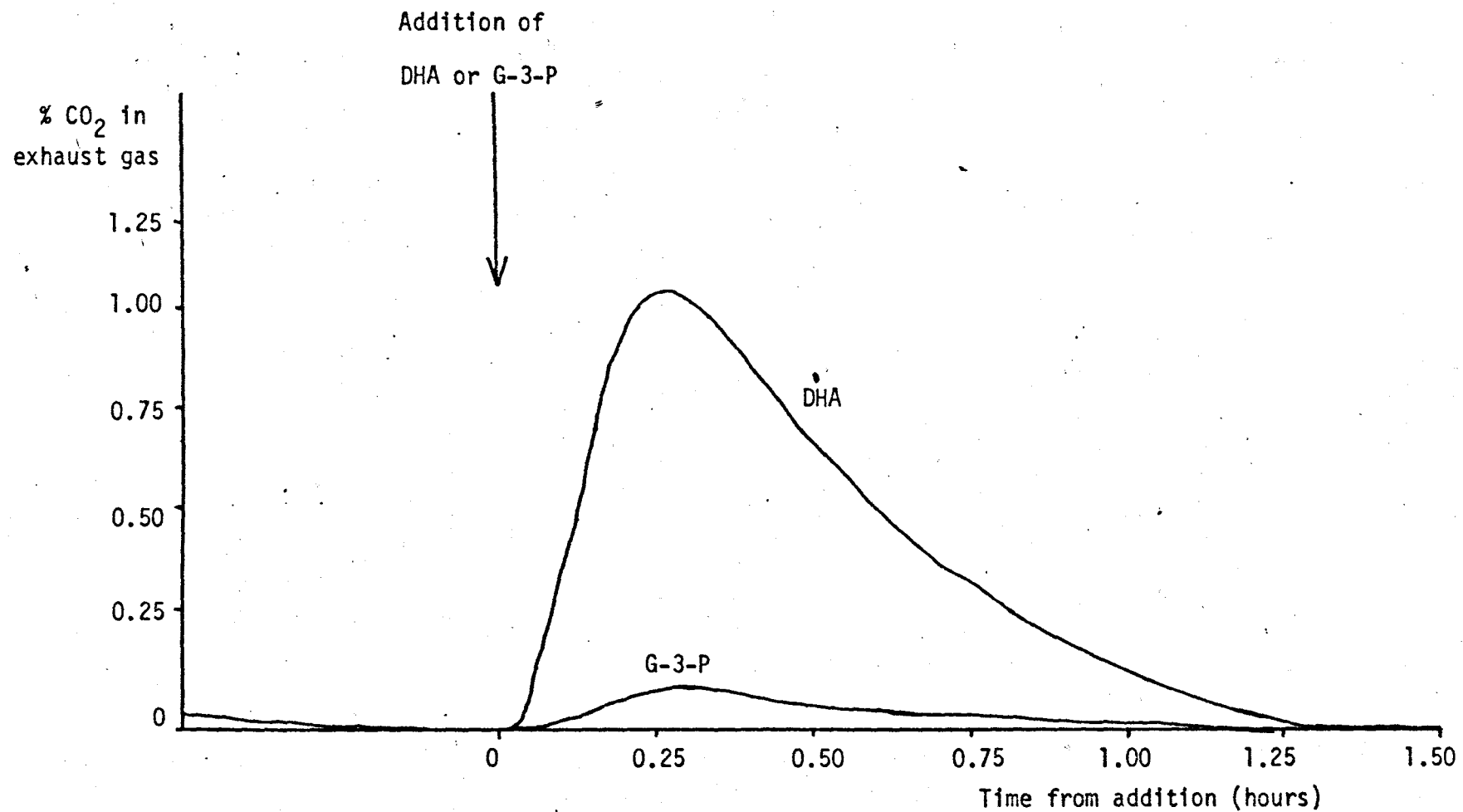
Figure 6.2.5   Effect of the Addition of Dihydroxyacetone or Glycerol-3-phosphate to Starved Anaerobic Cultures

The best measurement of substrate-suitability of these two intermediates for the anaerobic culture is probably made by estimation of the area under the CO_2-peak. From Fig. 6.2.5, it is obvious that this area is very much greater (ca. 15 times) for DHA than for G-3-P in the anaerobic culture.

Fig. 6.2.6 (again adapted from chart-traces) shows the effect on % CO_2 in the exhaust gas and DOT (% saturation) of adding DHA or G-3-P to two aerobic cultures.

The response of the culture to addition of DHA appears complex. CO_2 evolution rises to ca. 0.28% after ca. 0.2 hours, where it remains for a further 0.1 hours, and then increases to a new peak of ca. 0.38% at 0.50-0.55 hours after the addition. CO_2 evolution then falls rapidly, to reach zero once more after a total of ca. 0.9 hours. The response of dissolved oxygen tension to the introduction of DHA is complementary, falling to a plateau at 68% saturation between 0.2 and 0.3 hours from the addition, and then falling further to 65% satn. at ca. 0.5 hours. Thus, a 'double-response' is evident, the two parts of the response taking different amounts of time to take place.

Addition of G-3-P to the 'starved' aerobic culture results in single peaks of CO_2 evolution and DOT reduction. % CO_2 in the exhaust gas reaches the peak of 0.28% after 0.15 hours, and decays to zero after a total of 0.5 hours from the introduction of the compound. A similar, though again slightly quicker response (due to 'inertia' in the exhaust gas analysis system) in DOT reduction is found. A minimum of 79% satn. at ca. 0.08 hours occurs as a single peak which increases back to 100% satn. after a total of 0.5 hours.

It therefore appears that K. aerogenes grown aerobically on glycerol is able to utilise both dihydroxyacetone and glycerol-3-phosphate.
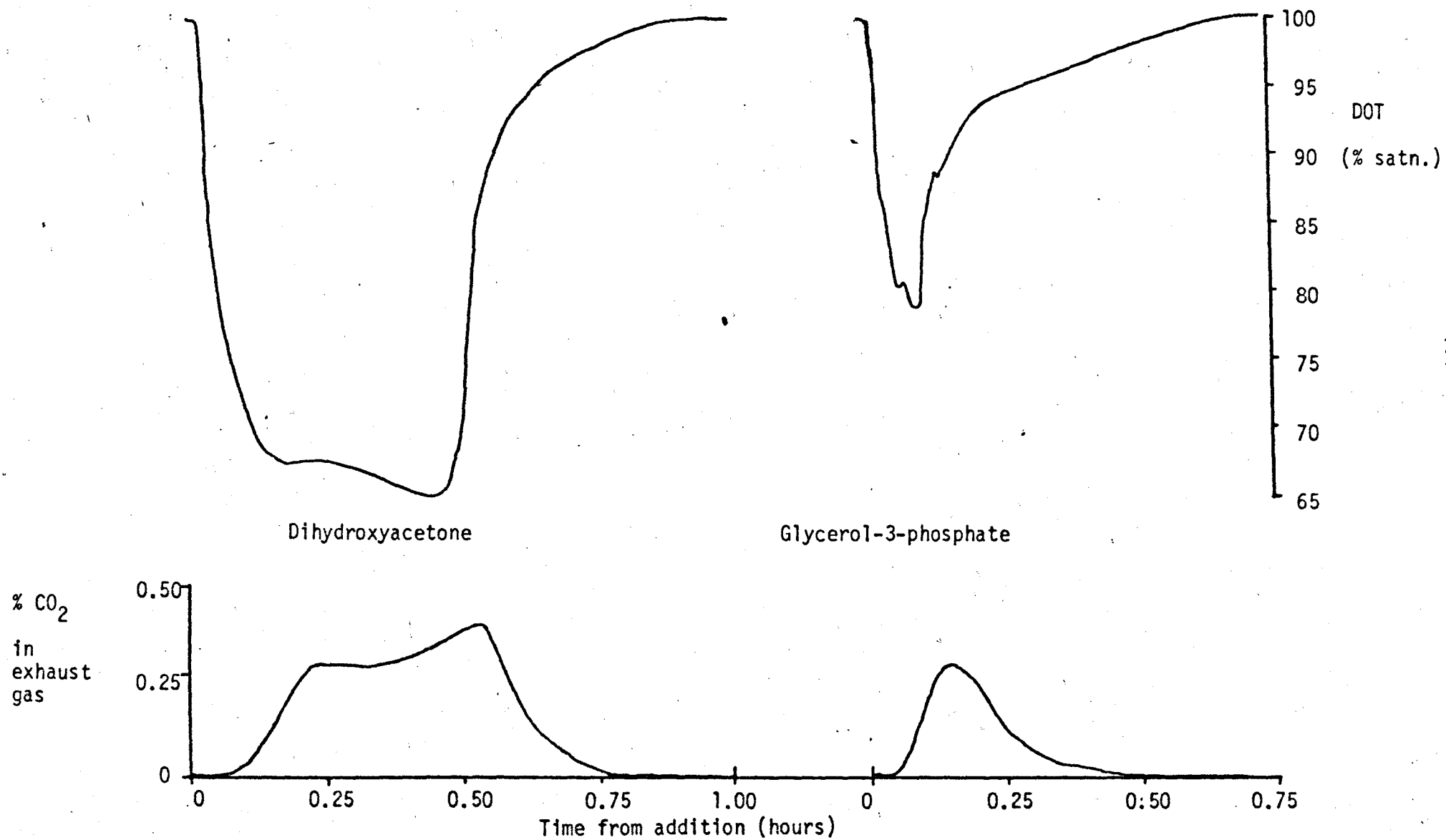
Figure 6.2.6   Effect of the Addition of DHA or G-3-P to Starved Aerobic Cultures

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The area under the % CO_2 trace for DHA, however, is approximately three times that under the trace for G-3-P.   Utilisation of DHA by the aerobic culture does not appear to involve a simple single-stage process, unlike utilisation of G-3-P (or glycerol).   K. aerogenes grown anaerobically on glycerol is able to utilise extracellular dihydroxyacetone, but utilisation of extracellular glycerol-3-phosphate appears to take place only to a very limited extent.   The small peak may even be due to impurities (e.g. glycerol) in the commercial reagent. Further discussion of these results is presented in Chapter 7.

6.3   'Intermittent Aeration' Cultures

6.3.1   Introduction

A series of four intermittently aerated continuous cultures of K. aerogenes 418 was carried out in order to simulate mixing and aeration effects in submerged culture. As explained in the Theory and Experimental Design section 3.1.2.1, the range of conditions chosen was such that all possibilities from good aeration/good mixing through poor aeration/good mixing and good aeration/poor mixing to poor aeration/poor mixing were simulated.

A starting point (i.e. fully aerobic) dissolved oxygen tension was chosen for each intermittently aerated (IA) culture. For IA culture 1, a fully aerobic DOT of ca. 75% saturation was required. By reference to the DOT vs. dilution rate plots for aerobic continuous cultures at four different impeller speeds (Fig. 6.1.5), an impeller speed of 900 min^{-1} and a dilution rate of 0.30 h^{-1} were selected, to give the required DOT for this culture. For IA culture 2, a fully aerobic DOT of ca. 45% saturation was required, and an impeller speed of 800 min^{-1} and dilution rate of 0.50 h^{-1} were accordingly selected. Similarly, for IA culture 3, an aerobic DOT of ca. 15% saturation was provided by an impeller speed of 700 min^{-1} and dilution rate of 0.55 h^{-1}. Finally, for IA culture 4, an oxygen-limited aerobic culture was required, with steady-state DOT of zero. This was provided again by an impeller speed of 700 min^{-1}, but a dilution rate of 0.70 h^{-1}.

Avoiding the use of the vessel's maximum impeller speed of 1000 min^{-1}, the wide range of dilution rates (0.30 h^{-1} to 0.70 h^{-1}) was unavoidable for the required range of aerobic dissolved oxygen tensions, as is apparent from Fig. 6.1.5. (Use of 1000 min^{-1} impeller speed for long-term continuous cultures was undesirable

because of unpredictable vibration).   Dilution rate must therefore be
borne in mind when interpreting the results of these experiments.

Each intermittently aerated culture was begun aerobically and
with uninterrupted aeration (i.e. 1.0 min^{-1} air).   A five-minute cycle
was used in all experiments, aeration being provided for an integral number
of minutes per cycle, varying from zero to five.   For the remaining
period of the cycle, the fermenter was flushed with oxygen-free nitrogen,
also at 1.0 min^{-1}.   The fully aerobic cultures were maintained at
steady-state conditions until intermittent aeration was required.
Aeration was then provided for four minutes and de-aeration for one
minute per cycle.   When a 'steady-state' had become established, and
the necessary measurements made and samples taken, a new cycle of
intermittent aeration was imposed, (three minutes of aeration, two of
deaeration per cycle).   The process was repeated through two minutes
aeration, three minutes deaeration, and one minute aeration, four
minutes deaeration per cycle.   Finally, fully anaerobic conditions were
established, where there were five minutes of deaeration per five minute
cycle.

When a change of aeration time per cycle was made, a new
'steady-state' was sought.   Although conditions could never be described
as truly 'steady-state' over the short five minute cycle time period,
when viewed over a longer period it was found possible to determine that,
eventually, various measured parameters (e.g. DOT) varied over a fixed
range and in a regular manner.   When this condition was reached
measurements of carbon dioxide and oxygen concentrations in the exhaust
gas, and dissolved oxygen tension in the culture, were made.   Moreover,
normally about 300 cm^3 of culture was harvested from the overflow, and

treated for storage as described in Section 5.5.3. Continuous recording (on a multipoint recorder) of % CO_2, % O_2 and DOT, together with sample removal for cell density measurement, aided the recognition of the culture's adaptation to new conditions, and the establishment of steady-state conditions.

For IA cultures, therefore, measurements of CO_2 and O_2 concentrations in the exhaust gas and DOT are presented here as the range over which these parameters varied under 'steady-state' conditions. Nevertheless, the relatively small range of variation of CO_2 concentration in the exhaust gas normally allowed the measurement of a mean CO_2 output by the culture. Cell density measurements were made under steady-state conditions, and therefore the ratio CO_2 concentration: cell density was calculable under most conditions. This measurement, effectively of CO_2 output per unit mass of cells, was found to be very useful for the recognition of the onset of anaerobic metabolism, since the ratio was considerably greater in anaerobic than in aerobic cultures.

Since four cultures were carried out, each under six different aeration conditions, a system of nomenclature for the 24 possible culture/ aeration condition combinations was required. Thus, for each combination, the impeller speed (min^{-1})/dilution rate (h^{-1})/aeration-deaeration time per cycle is presented in the results.

e.g. 800/0.50/3-2, 900/0.30/5-0 (fully aerobic), 700/0.55/0-5 (fully anaerobic), 700/0.70/4-1, etc.

Each of the four cultures was continued up to the point of fully anaerobic growth, or , in the case of IA culture 4 (700/0.70), until the culture washed out.   (See Section 6.3.5).

Owing to the breakdown of a deep-freeze which contained a number of the cell samples from the four cultures required for enzyme assays, it was necessary to replicate a number of the culture conditions in order to replace the samples. At the same time, a dissolved oxygen probe with a high-permeability polypropylene membrane became available. Trial usage of this probe under several of the culture conditions proved it to have far superior response characteristics to the old (Teflon-membraned) probe. It was therefore decided to repeat the entire series of cultures using the fast-response dissolved oxygen probe.

Whilst all measurements except the measured DOT range were comparable at each attempt at a particular culture condition, only the results of the improved second attempt are reported here, although examples of the results' reproducibility are given. Whilst general reproducibility is demonstrated, it is felt that it would be detrimental to the reliability of the results if any attempts at 'averaging' were made. Moreover, the improved equipment and technique used to derive the second set of results render the first set, apart from its comparative uses, redundant. Similarly, the enzyme assay results given in Section 6.4.4. were derived by an improved technique; the particular culture conditions refer to those of the second set of four cultures, the cells having been harvested whilst the quoted steady-state measurements were being made.

6.3.2   Intermittent aeration culture 1
         (900/0.30/5-0 - 0-5)
         This culture, with the highest initial (aerobic) dissolved oxygen tension and highest oxygen-transfer coefficient was intended to simulate the effects of good aeration. Intermittent aeration therefore

simulated varying degrees of mixing in a basically well aerated culture.
(i.e. oxygen-transfer during the aeration-period was efficient).

900/0.30/5-0

Steady-state conditions in the initial fully aerobic culture
were readily established.   Dissolved oxygen tension, as predicted, was
high, at about 78% saturation.   Owing to the low dilution rate and
carbon-limited conditions, relative O_2 consumption and the CO_2
concentration in the exhaust gas were both low.   (0.7% and 0.60%
respectively).

900/0.30/4-1

Steady-state at this aeration condition was rapidly reached
in both cultures, and no adaptation process appeared evident (from the
gas-traces).   Dissolved oxygen tension did not appear to reach zero
during the periods of deaeration.   However, allowing for the finite
response time of the DOT probe, it is possible that DOT fell to zero
for a short time in each cycle.   A slight increase of cell density over
the fully aerated condition appeared to be maintained.

900/0.30/3-2

Steady-state was again reached quickly (i.e. within 24 hours),
although some adaptation did appear to take place since the height of
the DOT peaks was greater than at the previous aeration condition.
Moreover, this increase of DOT peak height took place gradually over
ca. 20 hours.   In addition, a shoulder on the aeration side of the
peak developed over the period of this adaptation, and remained when

steady-state conditions were reached.   DOT fell to zero during the deaeration period.   A slight reduction of cell density was observed, together with a slight increase in CO_2 output over the fully-aerated level.   Thus, CO_2 production per unit mass of cells increased slightly.

900/0.30/2-3

Adaptation to these conditions was apparent from the gas-traces. The DOT peaks were reduced in height, and an inflection appeared at approximately 60% of the peak height.   The range of CO_2 concentration in the exhaust gas became very wide (100% variation) between the two parts of each cycle, but was maintained in this manner at the steady-state conditions.   Significantly, the culture cell density was maintained at the aerobic level even though the period of deaeration was longer than that of aeration.

An interesting development at this aeration condition was the production of a yellow-green pigment in the culture supernatant.   The production was maintained throughout the steady-state period, and was therefore not a transient effect;   moreover, its production was reproduced at the second attempt.   None of the other twenty aeration conditions of intermittently aerated cultures produced this pigment in such noticeable amounts.   Identification of the pigment's chemical identity was attempted, and is described in Section 6.5.2.

900/0.30/1-4

On transition to 1-4 aeration, the DOT inflection point of the previous culture condition became the new DOT peak.   The height of the peak then increased as adaptation to these conditions proceeded. Fig. 6.3.1, adapted from the multipoint chart-trace, shows this
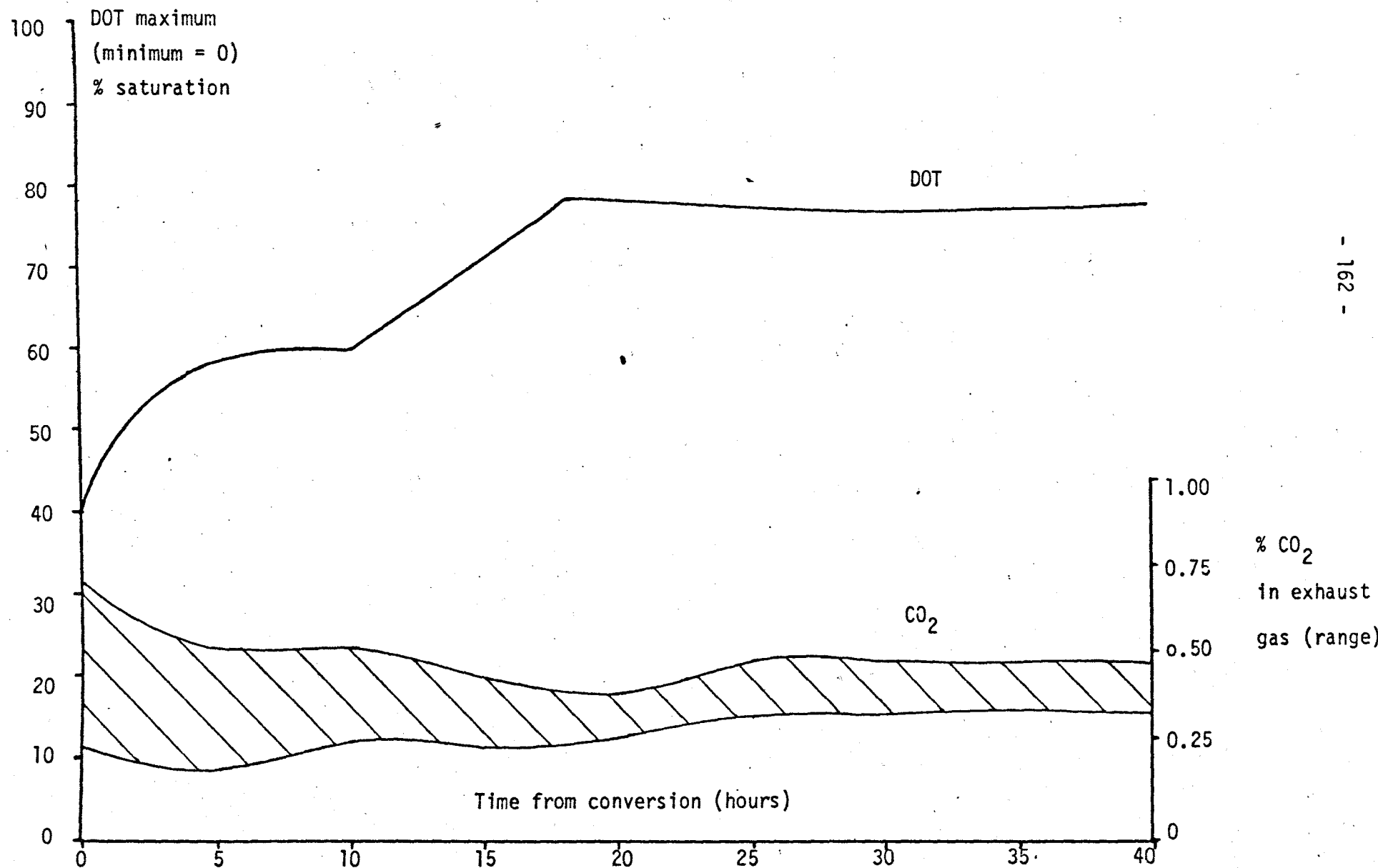
Figure 6.3.1   Response of Culture DOT Range and CO_2 Production Range to the 900/0.30/2-3→1-4 Conversion

adaptation of the culture and the eventual establishment of steady-state conditions of both DOT range and % CO_2 range. The time permitted to lapse from first changing the condition to actually harvesting the culture was ca. 40 hours. Measurement of cell density under steady-state conditions (and simple observation of the culture) revealed that an essentially aerobic → anaerobic transition had taken place. Cell density fell from 2.08 mg.cm^{-3} (2-3) to 0.53 mg.cm^{-3} (1-4). Fig. 6.3.1 shows this transition to have apparently taken place in two stages which were separated by a plateau of the DOT peaks at 7-10 hours from the change of conditions. It can also be seen that the CO_2 output from the culture continued to change considerably after the time when DOT range had reached a steady-state.

900/0.30/0-5

Adaptation to this aeration condition took place rapidly. Culture cell density approximately halved whilst total CO_2 output remained at a similar level to that of the previous condition.

Thus, whilst maintaining the impeller speed at 900 min^{-1}, and dilution rate at 0.30 h^{-1}, the cultures were progressively reduced by integral stages of aeration condition from fully aerobic to fully anaerobic growth. Measurements were made at each steady-state as culture was simultaneously harvested from the overflow for enzyme assays. A number of adaptation processes were noted between successive steady-states, but only in one case could the adaptation be said to have involved a transition. This transition, from 'essentially' aerobic to 'essentially' anaerobic growth took place when the aeration condition was converted from 900/0.30/2-3 to 900/0.30/1-4.

Table 6.3.1   Steady-State Results for IA Culture 1   (900/0.30)

Aeration Condition	Cell Density $(mg.cm^{-3})$	DOT Range (% saturation; as measured)	% O_2 in Exhaust gas (Range)	CO_2 in Exhaust gas. (% F.S.D. of meter) (Range)	Mean % CO_2 in exhaust gas	$\dfrac{\% CO_2}{\text{cell density}}$
5-0	2.08	78	20.3	16.5	0.60	0.290
4-1	2.26	3-75	5.5-20.1	17-19.5	0.68	0.302
3-2	2.04	1-79	1.1-19.7	14-21.5	0.66	0.324
2-3	2.08	0-67	0.1-18.5	12-25	0.68	0.327
1-4	0.53	0-77	0-15.3	10-13	0.40	0.762
0-5	0.24	0	0	12.5	0.45	1.84
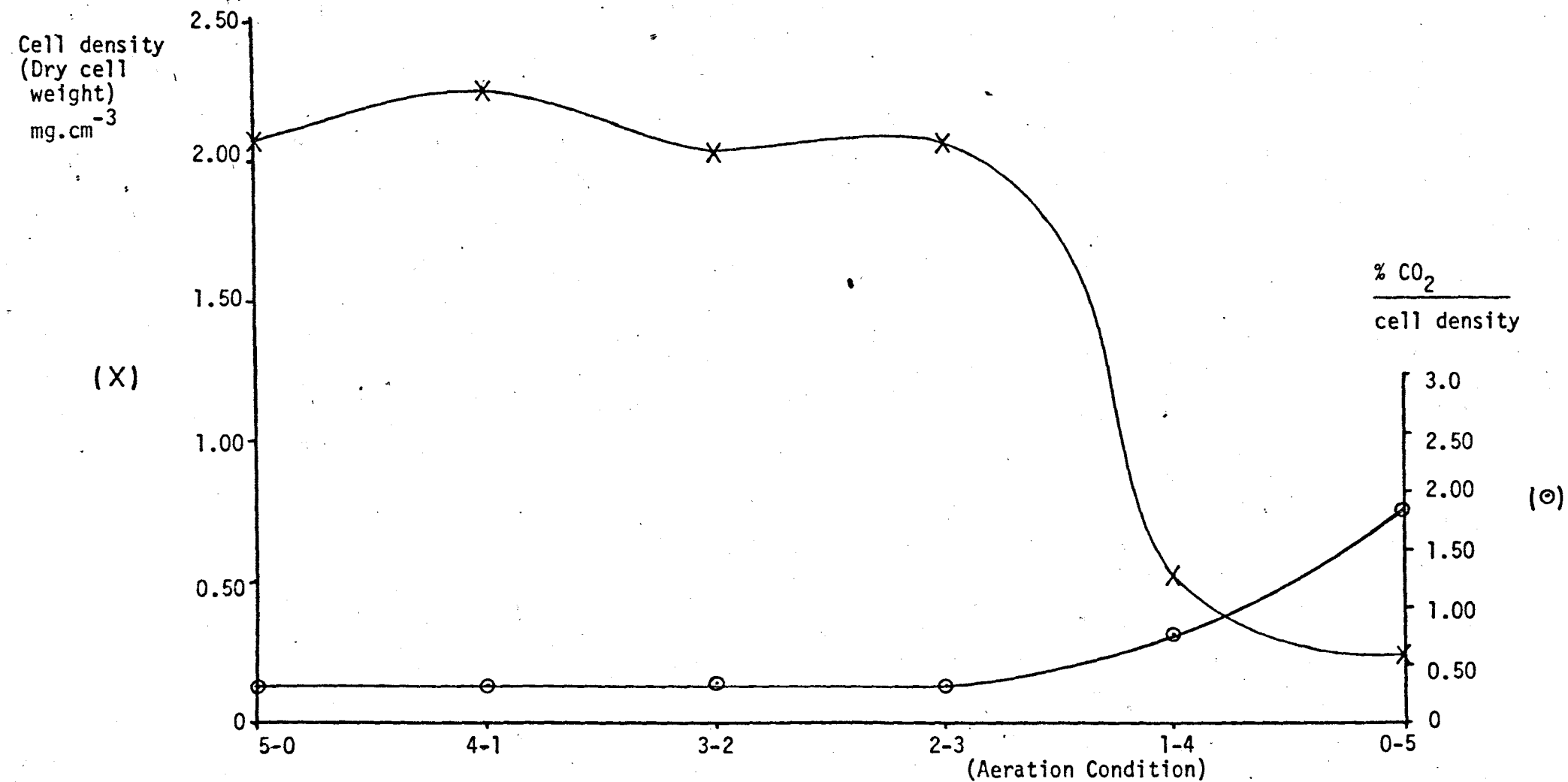
Figure 6.3.2   Intermittent Aeration Culture 1.   (900/0.30) Plots of Cell Density and CO_2 Production

vs. Aeration Condition.

Results for one culture at each steady-state are given in
Table 6.3.1.   Cell density and % CO_2/cell density are plotted against
aeration time per cycle in Fig. 6.3.2.   Steady cell densities up to
900/0.30/2-3 can be seen, followed by the transition at 900/0.30/1-4,
and fully anaerobic culture at 900/0.30/0-5.   Increase of CO_2
production per unit mass of cells (i.e. % CO_2/cell density) can be seen
when the culture undergoes the transition.   Further discussion of these
results in context with those of the other IA cultures is included in
Chapter 7.   Discussion of the different types of DOT peak response
(e.g. shoulder, inflection, double-peak, etc) is also included in that
Chapter.

6.3.3   Intermittent aeration culture 2
(800/0.50/5-0 - 0-5)

This culture had a lower impeller speed and higher dilution rate
than those of IA culture 1.   Thus, the fully aerated (5-0) DOT was, as
predicted, considerably lower, at ca. 45% saturation.   Like IA culture
3, this culture simulates 'intermediate' qualities of aeration and good
to poor mixing, although this culture is considerably better aerated
than culture 3.

800/0.50/5-0

Steady-state conditions, with DOT at 46% saturation and cell
density at 2.30 mg.cm^{-3}, were readily established. CO_2 output was
considerably higher than for the previous culture (0.90% cf. 0.60%),
probably due to the higher dilution rate.

800/0.50/4-1

Following conversion to this condition, DOT did not appear to reach zero during deaeration, and an inflection on the aeration side of the peak was immediately established. The fully aerated (5-0) cell density was maintained at the steady-state (after ca. 20 hours), though CO_2-production increased slightly. No recognisable adaptation process took place, the gas-traces at steady-state appearing very much as they did immediately after the conversion.

800/0.50/3-2

Again, no real adaptation process was apparent from the gas traces. However, immediately following conversion, the baseline for the DOT peaks fell to 1% satn., which, allowing for the lag of probe response, was probably actually zero. Moreover, a 'double-peak' DOT response was immediately established and maintained under steady-state conditions. When the solenoid valve timer switched-on the air supply following the two minutes of deaeration, DOT immediately rose by ca. 1.5%, and then fell back slowly to the baseline. Just before the timer switched-on the nitrogen supply, DOT began to rise very rapidly, to be restrained at a sharp peak of ca. 14% saturation by deaeration. (This and other DOT responses are described and discussed in more detail in Chapter 7). CO_2 production remained at a similar level to 4-1, although the amplitude of the response was considerably increased. Cell density was maintained at the fully aerated level of 2.30 mg.cm^{-3} under the rapidly-attained steady-state conditions.

800/0.50/2-3

A true adaptation to those conditions was obvious from the gas traces. On conversion, the second peak of the 3-2 double-peak DOT

response was lost, leaving the first peak and a plateau.   However, as adaptation took place, both parts of the DOT response increased in magnitude.   The latter rose more rapidly than the former, until a single broad peak of 10-12% saturation height was established after about 2.5 hours.   Following some 12 hours of this DOT response, the DOT peaks began to increase successively in height.   A transitional process was then apparent, where DOT range rose for a further 12 hours, to peak at 0-54% satn. range.   Following this slight overshoot, steady-state conditions of DOT range 0-47% satn. were established after a total of approximately 36 hours from conversion.   (Harvesting was carried out after 44 hours).   Both stages of this transition can be seen in Fig. 6.3.3 (Traced from the chart-trace).

An aerobic → anaerobic transition was also evident from cell density measurement, which fell to 0.83 $mg.cm^{-3}$ at the steady-state. However, this is certainly higher than the fully anaerobic level. Moreover, CO_2 production also fell from the 3-2 level, resulting in little change of CO_2 production per unit mass of cells.   Thus, an 'intermediate' area between aerobic and anaerobic metabolism is apparent under these conditions.

800/0.50/1-4

On conversion to this aeration condition, the range of DOT response was temporarily (for ca. 5 hours) reduced, but recovered to the previous level.   The amplitude of CO_2 production response decreased during this period, and remained as such at the rapidly-attained steady-state.   It was therefore apparent that slight adaptation to this aeration condition had taken place, exemplified by a considerable reduction of cell density to a steady-state level of 0.28 $mg.cm^{-3}$.
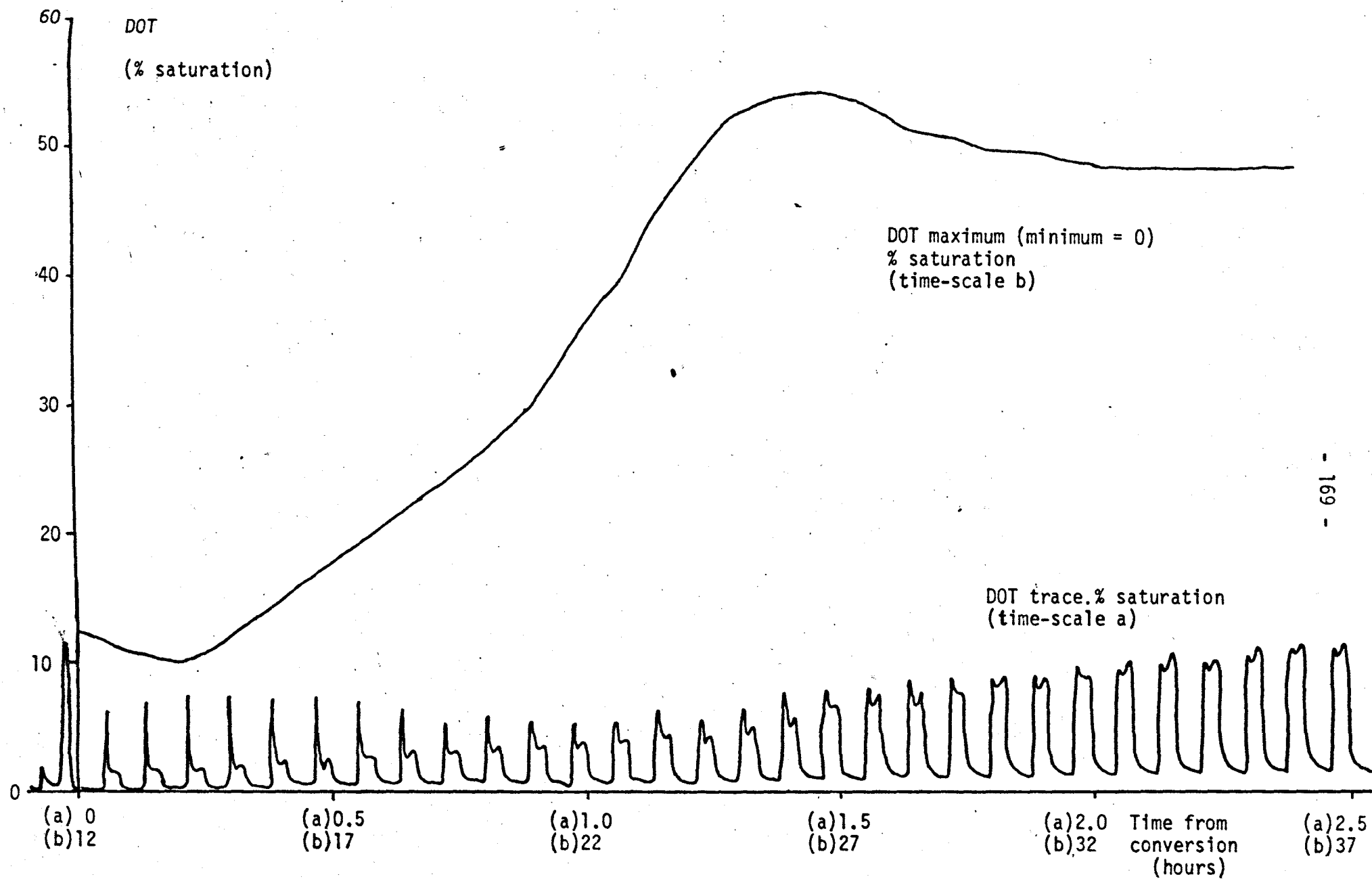
Figure 6.3.3   Response of Culture DOT to the 800/0.50/3-2→2-3 Conversion

Furthermore, the ratio % CO_2/cell density increased by over three times, indicating considerably more anaerobic metabolism than at the previous condition.

800/0.50/0-5

Steady-state conditions at this anaerobic condition were rapidly established. Cell density again fell considerably, although CO_2 production increased slightly compared with the previous aeration condition.

The results of one 800/0.50 IA culture over the full range of aeration conditions are given in Table 6.3.2. Cell density and. % CO_2/ cell density are plotted against aeration condition in Fig. 6.3.4. The plot shows a more gradual transition of aerobic to anaerobic metabolism. The main difference between this culture and IA culture 1, however, is the low cell density at the 2-3 aeration condition, this being symptomatic of the lower oxygen-transfer coefficient during the aeration period.

6.3.4 Intermittent aeration culture 3
(700/0.55/5-0 - 0-5)

A lower impeller speed and slightly higher dilution rate than IA culture 2 resulted in a lower oxygen-transfer coefficient and (fully aerated) DOT in the two cultures carried out under these conditions. Thus, 'poor to intermediate' qualities of aeration were simulated, together with good to poor mixing.

TABLE 6.3.2　　Steady-State Results for IA Culture 2 (800/0.50)

Aeration Condition	Cell Density $(mg.cm^{-3})$	DOT Range (% saturation; as measured)	% O_2 in Exhaust gas (Range)	CO_2 in Exhaust gas. (% meter F.S.D.) (Range)	Mean % CO_2 in exhaust gas	$\dfrac{\% \ CO_2}{cell \ density}$
5-0	2.30	46	19.75	24	0.90	0.391
4-1	2.30	4-36	4-19.7	23-27	0.95	0.413
3-2	2.30	0-14	1-19.0	23-32	1.05	0.457
2-3	0.83	0-47	0.2-19.2	11-16	0.48	0.582
1-4	0.28	0-44	0-15.8	14-16	0.55	1.96
0-5	0.12	0	0	17	0.63	5.16
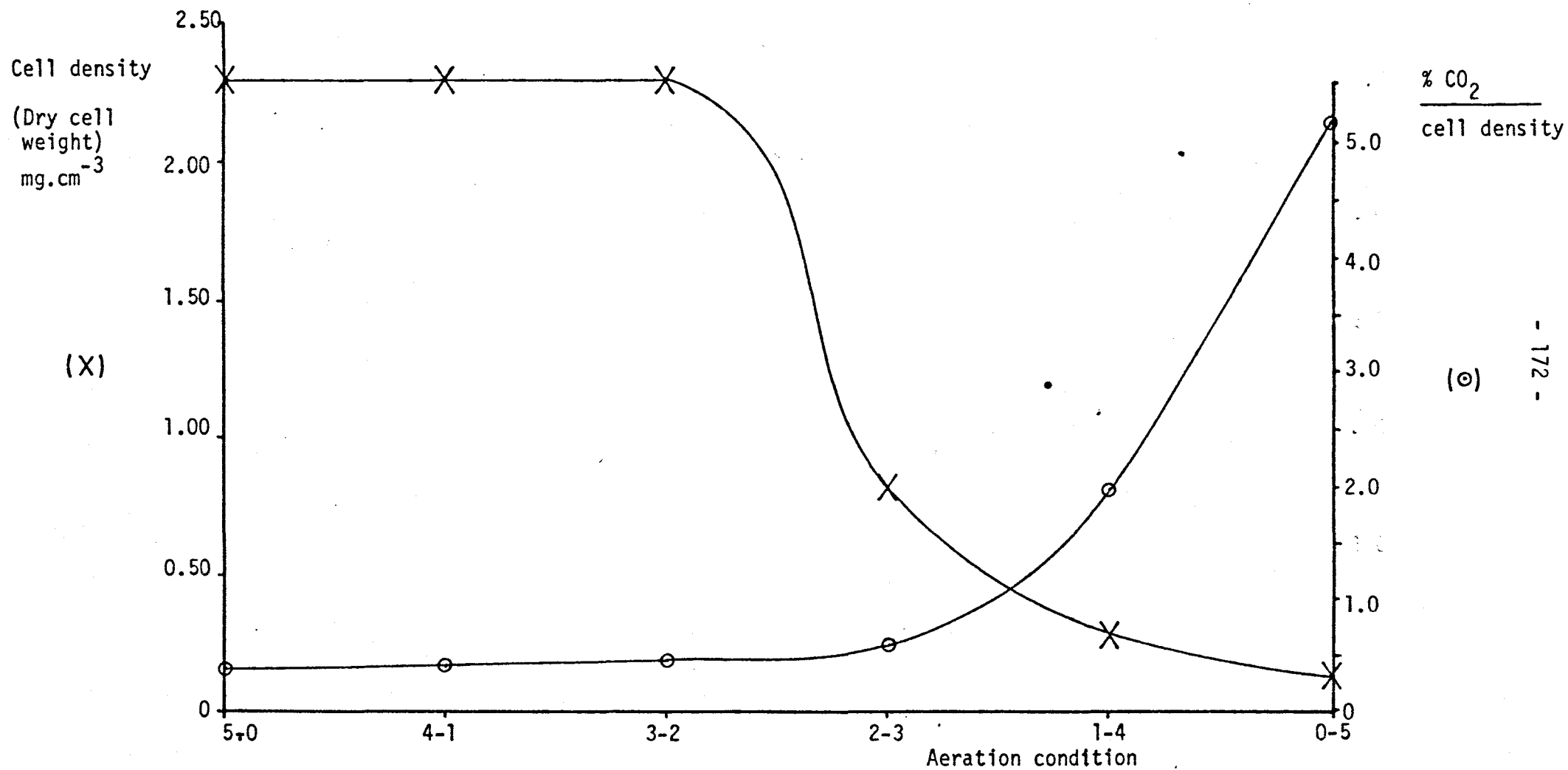
Figure 6.3.4   Intermittent Aeration Culture 2 (800/0.50)

Plots of Cell Density and CO_2 Production vs. Aeration Condition

700/0.55/5-0

The predicted low initial DOT was 16% saturation, and the cell density was 2.49 mg.cm^{-3} (Dry cell weight).

700/0.55/4-1

On conversion to this condition, DOT immediately fell to zero, where it stayed. However, when the steady-state was established, small increases of DOT every time the air was switched on were noticed. These slight peaks, however, fell back to zero almost immediately, and were probably simply due to rapid admission of oxygen to an unprepared culture. No real adaptation to these conditions was apparent, and a new steady-state was rapidly attained. Both cell density and CO_2 production were slightly lower than in the fully aerated culture.

700/0.55/3-2

Immediately on conversion, CO_2 production fell, as did the minimum point of the % O_2 in the exhaust gas. However, adaptation to this aeration condition was apparent, CO_2 production eventually recovering and even slightly exceeding the previous value. Moreover, oscillation of the DOT value began to increase, until it reached 0-18% satn., and then fell back. At the eventual steady-state, which required ca. 40 hours to be fully established, DOT showed the 'double-peak' response (as at 800/0.50/3-2) over a small 0-3% saturation range. Cell density was slightly decreased, but remained at a level typical of a fully aerobic culture.

700/0.55/2-3

The small DOT range increased immediately on conversion to 2-3 aeration. This increase continued for approximately 9 hours

to 0-30% satn., overshot,and then fell back very slowly to reach a steady-state range of 0-12% satn. after a total of ca. 40 hours had elapsed from conversion. (CO_2 output reached a steady-state somewhat earlier, at about 25 hours). The DOT peak response also changed from a double peak to a single broad peak, with a zero base-line. This behaviour, in conjunction with a large reduction in cell density (to 0.48 mg.cm^{-3}) is typical of the fundamental change from aerobic to anaerobic metabolism. The transition, as seen from the recorder trace, was very similar to that for 800/0.50/2-3, as shown in Fig. 6.3.3.

700/0.55/1-4

Adaptation to this condition was not apparent, DOT and % CO_2 traces being hardly affected in magnitude, only in fine-structure, i.e. the DOT peak height was identical to that for 2-3 aeration, but the peak was considerably sharper; the amplitude of CO_2 oscillation fell considerably, but the mean CO_2 concentration in the exhaust gas remained at a very similar level. Nevertheless, cell density fell by over 50%, resulting in a considerably higher production of CO_2 per unit mass of cells. Steady-state was attained rapidly, and culture harvest carried out within 24 hours of the conversion of aeration condition.

700/0.55/0-5

Immediately on conversion to fully anaerobic conditions CO_2 production increased, but then fell back to reach a steady-state value after only 2 hours. This value was, in fact, very similar to those of the 2-3 and 1-4 aeration conditions, but since cell density again fell considerably, CO_2 production per unit mass of cells was

Table 6.3.3   Steady-State Results for IA Culture 3   (700/0.55)

Aeration condition	Cell density ($mg.cm^{-3}$)	DOT Range (% saturation; as measured)	% O_2 in Exhaust gas (Range)	CO_2 in Exhaust gas (% meter F.S.D.) (Range)	Mean % CO_2 in. Exhaust gas	$\dfrac{\% \ CO_2}{Cell \ density}$
5-0	2.49	16	19.5	28	1.08	0.432
4-1	2.32	0-1.5	4-19.2	23-28	0.98	0.423
3-2	2.20	0-3.0	1-18.4	25-31	1.08	0.491
2-3	0.48	0-12	0.2-18.9	17-22	0.72	1.51
1-4	0.20	0-12	0-16	18-20	0.70	3.59
0-5	0.13	0	0	18.5	0.68	5.38

Figure 6.3.5   Intermittent Aeration Culture 3 (700/0.55)
Plots of Cell Density and CO_2 Production vs. Aeration Condition

once again increased. A steady-state was attained rapidly, with no real adaptation being obvious, and harvesting was carried out within 24 hours.

The results of one 700/0.55 IA culture over the full aeration-condition range are given in Table 6.3.3. Cell density and % CO_2/cell density vs. aeration time per cycle plots are given in Fig. 6.3.5. These plots show the transition to anaerobic metabolism to have taken place at a higher aeration time per cycle than that for IA culture 2. Moreover, an even lower cell density at the 2-3 aeration condition was exhibited, again indicating the poorer oxygen-transfer conditions.

6.3.5   Intermittent aeration culture 4
(700/0.70/5-0 - 3-2)

The two cultures grown under the worst conditions of oxygen transfer were both found to wash out when converted from 3-2 to 2-3 aeration. One assumes, therefore, that an aerobic → anaerobic transition took place at this conversion, but the dilution rate of 0.70 h^{-1} was too high to maintain a continuous anaerobic culture, i.e. D_c for an anaerobic culture grown under these conditions is probably under 0.70 h^{-1}.

Nevertheless, poor aeration, indicated by oxygen-limited conditions even under continuous aeration, was simulated together with varying qualities of mixing.

700/0.70/5-0

At the steady-state, DOT was zero, as predicted. Thus, oxygen-limited conditions were established under continuous aeration. The CO_2 production of 1.20% was high, indicative of the relatively

high dilution rate. Similarly, high consumption of oxygen (the concentration in the air was reduced by 1.7%) was noted. Although the cell density was at the fully aerobic level, the oxygen-limited conditions appeared to give rise to a pale grey coloration of the culture, which is more typical of anaerobic cultures. Moreover, CO_2 output seemed to be considerably more pH-sensitive under these oxygen-limited conditions.

700/0.70/4-1

Steady-state was rapidly attained on conversion to this aeration condition. Adaptation was, however, apparent since DOT rose from zero, and actually exhibited a double-peak response under steady-state conditions. The second (higher) peak reached 10% saturation, which would not be expected under oxygen-limited conditions. Furthermore, the appearance of the culture took on the more typically aerobic pale yellow coloration. Cell density fell slightly from the fully aerated level, but CO_2 production was slightly increased.

700/0.70/3-2

An adaptational response was found when the culture was converted to this aeration condition. The DOT response changed from that of a relatively small double peak to a broad single peak which reached 32% satn. Again, the transition between these two response types, which may represent an aerobic → anaerobic transition, took on the typical form illustrated in Fig. 6.3.3. (cf. 800/0.50/2-3 and 700/0.55/2-3). This transition was further illustrated by a reduction of cell density, which reached a steady-state 1.41 mg.cm^{-3}, and was therefore above typical anaerobic levels. This 'intermediate' cell

Table 6.3.4   Steady-State Results for IA Culture 4   (700/0.70)

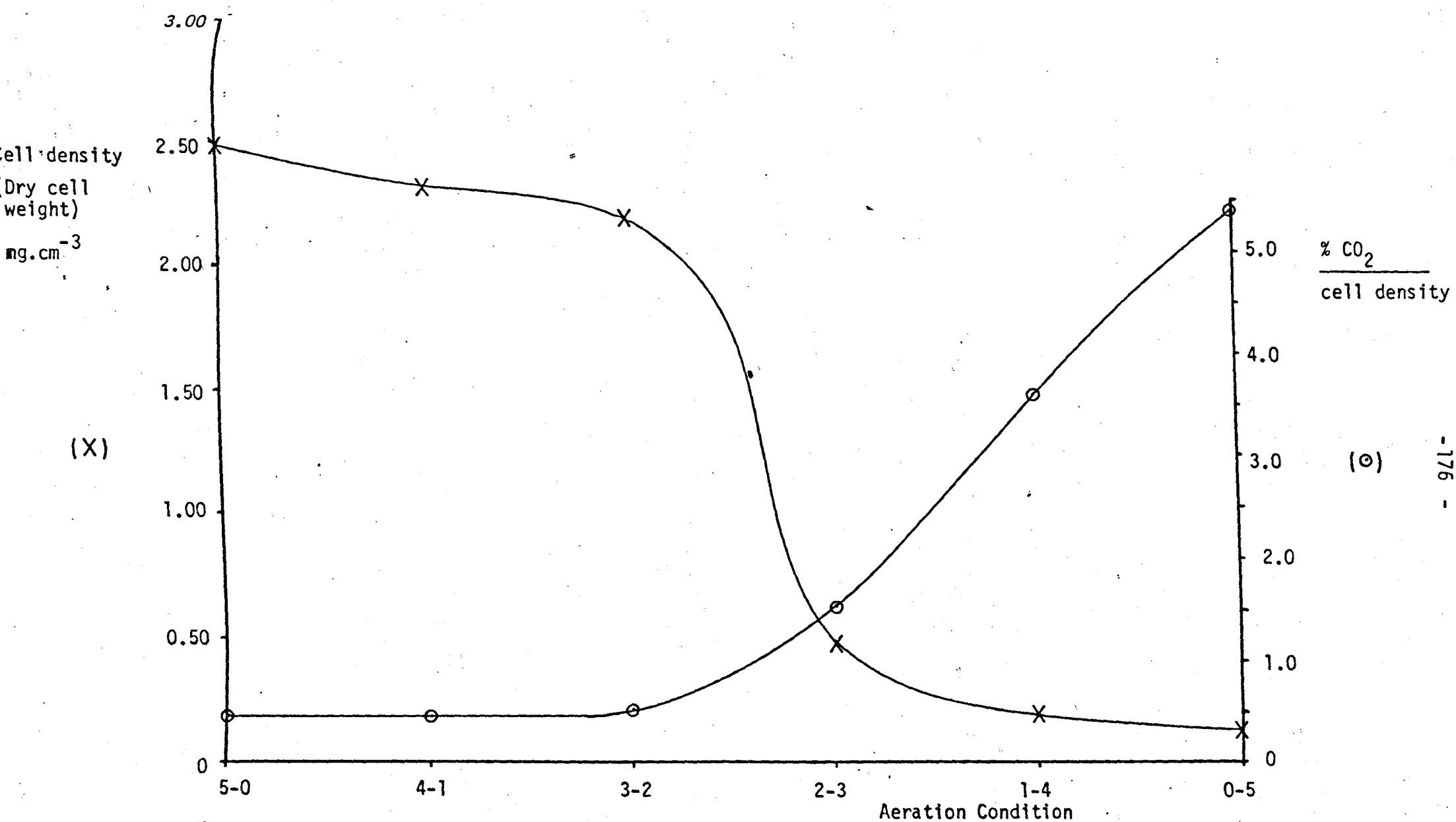| Aeration Condition	Cell density $(mg.cm^{-3})$	DOT Range (% saturation; as measured)	% O_2 in Exhaust gas (Range)	CO_2 in Exhaust gas (% meter F.S.D) (Range)	Mean % CO_2 in Exhaust gas	$\dfrac{\% \ CO_2}{cell \ density}$
5-0	2.36	0	19.25	31	1.20	0.509
4-1	2.26	0-10	4.6-18.9	30.5-35.5	1.29	0.570
3-2	1.41	0-32	0.6-19.2	18.5-27	0.86	0.612
(2-3)	(0.085)	-	-	(22)	(0.82)	(ca. 5)
(1-4)	0	-	-	-		
(0-5)	0	-	-	-		

Figure 6.3.6   Intermittent Aeration Culture 4 (700/0.70)
Plots of Cell Density and CO_2 Production vs. Aeration Condition

density level was found to be reproducible. CO_2 production also fell, such that the % CO_2/cell density ratio was barely altered, (cf. 800/0.50/ 2-3), and metabolism might therefore be crudely considered to be 'partly aerobic - partly anaerobic'.

700/0.70/2-3, 1-4, 0-5

Conversion to the 2-3 aeration condition under these poor oxygen-transfer conditions resulted in a very low cell density of ca. 0.085 mg.cm^{-3}. Although CO_2 production remained at a measurable level, it is likely that much of this was due to wall-growth bacteria.

Thus, anaerobic growth at this aeration condition could not be maintained at the dilution rate of 0.70 h^{-1}, and washout therefore occurred. Nevertheless, an approximation for % CO_2/cell density is made.

The results for the available steady-state conditions of one of these cultures are given in Table 6.3.4. Cell density and % CO_2/ cell density are plotted vs. aeration condition in Fig. 6.3.6. Cell density can be seen to fall considerably at the 3-2 aeration condition, and fall to a very low level at the 2-3 condition. Estimated CO_2 production per unit mass of cells reaches a high level at this latter aeration condition, as normally found in anaerobic cultures of low cell density. However, due to this very low cell density, harvest of cells at 700/0.70/2-3 would not have yielded sufficient material for enzymatic assay, and was therefore not attempted.

6.3.6   Calculation of the oxygen-transfer coefficients for
         intermittent aeration cultures

Although direct calculation of K_La for intermittently aerated

cultures is not possible, as outlined in the Theory and Experimental Design section, it is possible to calculate a 'time-averaged' value of K_La for oxygen, $(K_La)_o$. Since oxygen-transfer during the aeration periods is likely to be of the same efficiency as that of an equivalent fully aerated culture, and oxygen-transfer to the culture fluid during deaeration is zero, then multiplication of the fully aerated K_La by the proportion of time for which the culture is aerated yields this time-averaged $(K_La)_o$ value.

As shown previously (Section 3.2.2), under the conditions used in all fully aerated continuous cultures in the fermenter, K_La may be calculated by oxygen balance from the empirical relationship:-

$$K_La = \frac{1.713 \, (\% \, O_2)}{1.000 - (DOT \times 10^{-2})} \qquad min^{-1}$$

Using the data given in Tables 6.3.1, 6.3.2, 6.3.3 and 6.3.4, K_La values may be calculated for the four continuously aerated cultures from the above equation. Intermittently aerated culture $(K_La)_o$ values may then be calculated by proportion, as in Table 6.3.5:-

Table 6.3.5 'Time-averaged' $(K_La)_o$ values for IA cultures (min^{-1})

Culture Conditions	5-0	4-1	3-2	2-3	1-4	0-5
900/0.30	5.45	4.36	3.27	2.18	1.09	0
800/0.50	3.97	3.18	2.38	1.59	0.79	0
700/0.55	3.06	2.45	1.84	1.22	0.61	0
700/0.7	3.00	2.40	1.80	1.20	0.60	0

From examination of Figures 6.3.2, 6.3.4, 6.3.5 and 6.3.6, and by reference to Table 6.3.5, it is possible to determine the approximate $(K_L a)_0$ value below which the aerobic → anaerobic transition took place. The 700/0.70/3-2 culture may probably be described as 'essentially aerobic', whilst the 800/0.50/2-3 culture is probably best described as 'essentially anaerobic'. The $(K_L a)_0$ value of the former was 1.80 min^{-1}, that of the latter 1.59 min^{-1}. Thus, the transitional value of $(K_L a)_0$ for these culture conditions is 1.70 ± 0.10 min^{-1}.

6.4   Assay of the Enzymes of Glycerol Metabolism

The theory behind the methods used for the assay of the four enzymes of glycerol metabolism is outlined in the Theory and Experimental Design section 3.3.1.   The reactions catalysed by these enzymes are also described in that section (Fig. 3.1).   The procedures followed in these assays are described in detail in Experimental Section 5.7.

In the cases of the glycerol kinase, glycerol dehydrogenase and glycerol-3-phosphate dehydrogenase assays, the experimental methods were taken from the papers quoted in Section 5.7.   However, each of these assay methods required adaptation, modification and optimisation to suit the particular requirements of the experimental situations; the optimisation of these assay methods is described here.   The dihydroxyacetone kinase assay was developed here from first principles, the method being based on the known physiological reactions in Fig. 3.1. The derivation and optimisation of an assay method for DHA kinase are also described here, together with some kinetic and inhibitory studies of the enzyme.

Assays of these four enzymes of glycerol metabolism in K. aerogenes were carried out on all harvested cell samples from the intermittently aerated cultures.   The results of these assays for all 4 enzymes at all 21 culture/aeration conditions are presented here.

6.4.1   Optimisation of the glycerol kinase and glycerol dehydrogenase
          assay methods

The glycerol kinase assay was based on that developed by Freedburg and Lin (1973), the glycerol dehydrogenase assay being based on that by Lin and Magasanik (1960).   Since many of the components of the two assay systems were identical, a method was developed to assay

both enzymes in the same vessel, the glycerol dehydrogenase assay acting as the blank for the glycerol kinase assay. This combined system also relied on the fact that the pH optima of the two enzymes are very close.

Thus, by reference to Fig. 3.1, it can be seen that if all assay components except glycerol and the mammalian (rabbit) G-3-P dehydrogenase were included, any reaction (measured as an increase in absorption at 340 nm) would not be due to the action of glycerol kinase or glycerol dehydrogenase. This therefore served as the overall reaction blank. Inclusion of glycerol, however, allowed reduction of NAD linked to glycerol dehydrogenase to take place, and therefore acted as the assay of this enzyme.

All components used in the assay of glycerol dehydrogenase resulted in a perfectly functioning assay system over a wide range of activities, and no modification was therefore deemed necessary. Addition of the rabbit G-3-P dehydrogenase in an 'excess' amount to the system then permitted the assays of both glycerol dehydrogenase and glycerol kinase, as explained in the Experimental Section 5.7 and in Theory and Experimental Design. However, the exact amount of rabbit G-3-P dehydrogenase necessary to provide an 'excess' was not known, and a series of assays was therefore carried out in order to determine this amount.

An aerobic cell extract, known to have a high glycerol kinase activity, was used in the experiment. Assays of glycerol kinase were carried out using 100 μl of this extract, together with the standard assay mix, and 20, 40, 60, 80 or 100 μg of the rabbit G-3-P dehydrogenase. (In the form of a crystal suspension in 3 M $(NH_4)_2SO_4$, containing 10 mg.cm^{-3} of the enzyme). The results of the experiment, in the form of the measured slope known to be due to the reduction of NAD, are

shown in Table 6.4.1:-

Table 6.4.1   Effect of the Amount of Rabbit G-3-P Dehydrogenase on

the Reaction Rate of the Glycerol Kinase Assay System

μg Rabbit G-3-P Dehydrogenase in Assay System (in 3.0 cm^3)	Measured Rate of Absorbance Increase at 340 nm (O.D. units min^{-1})
20	0.150
40	0.220
60	0.345
80	0.350
100	0.348

A further experiment involved doubling the concentration of NAD in the assay system, but was not found to increase the maximum measured rate of NAD reduction.

From the results, it can be seen that the maximum rate of NAD reduction in an active glycerol kinase assay system was provided by a minimum concentration of 60 μg of rabbit G-3-P dehydrogenase in 3.0 cm^3 of assay mix.   100 μg of this enzyme was therefore used in all glycerol kinase assays, which were as described in Experimental Section 5.7.2.

6.4.2   Optimisation of the glycerol-3-phosphate dehydrogenase assay method

The method used for the assay of this enzyme was based on that by Kistler and Lin (1971).   As explained in the Experimental Section

5.7.4, and in the Theory and Experimental Design chapter, the reaction
catalysed by this enzyme is linked to the reduction of a tetrazolium dye
in this assay.   The reduced form of the dye absorbs more strongly at
570 nm than the oxidised form, and its reduction, due to action of the
enzyme, is therefore followed spectrophotometrically.   This type of
assay was reported to be necessary due to the fact that the bacterial
enzyme is not NAD-linked.   This was tested by replacement of the PMS-MTT
dye system by 1 mM NAD, and following the absorbance at 340 nm.

It has been reported (e.g. Lin, 1976) that in addition to an
aerobic G-3-P dehydrogenase, there may also exist an anaerobic G-3-P
dehydrogenase in many bacteria.   Either or both of these enzymes may
be dependent on the flavins, flavin adenine dinucleotide (FAD) and
flavin mononucleotide (FMN).   Thus, assays of G-3-P dehydrogenase
activity in aerobic and anaerobic cell extracts in both the presence
and absence of added flavins were carried out.   In the assay systems
in which flavins were included, FMN and FAD were added to give final
concentrations of 10^{-6} moles cm^{-3} and 10^{-8} moles cm^{-3} respectively.
The results of these experiments are given in Table 6.4.2:-

Table 6.4.2 Assays of G-3-P dehydrogenase Activity in the Presence
and Absence of Flavins

(a) Containing 1 mM NAD in place of PMS, MTT and Triton X100.

µl cell extract	Added Flavins?	Corrected Rate of Absorbance Increase at 340 nm (O.D. units min^{-1})
25 (aerobic)	no	- 0.005
25 (aerobic)	yes	- 0.003

(b)   Containing PMS, MTT and Triton X100

μl Cell Extract	Added Flavins?	Corrected Rate of Absorbance Increase at 570 nm (O.D. units. min^{-1})
25 (aerobic)	No	0.120
25 (aerobic)	Yes	0.092
100 (aerobic)	No	0.498
100 (aerobic)	Yes	0.340
100 (anaerobic)	No	0.007
100 (anaerobic)	Yes	0.006

From these results, it is concluded that the G-3-P dehydrogenase in K. aerogenes is not NAD-linked, and cannot therefore be assayed by following absorbance increase at 340 nm in the presence of NAD.   Moreover, the presence of flavins at the quoted concentrations (as used by Kistler and Lin) was not found to increase the activity of G-3-P dehydrogenase in either aerobic or anaerobic cell extracts.   In fact, addition of flavins was repeatedly found to decrease G-3-P dehydrogenase activities in aerobic cell extracts. (By 20-30%).

Very low G-3-P dehydrogenase activity was found in extracts of anaerobically grown cells, whether flavins were present or not. Since the organism exhibits an alternative pathway for anaerobic glycerol metabolism, an anaerobic G-3-P dehydrogenase is probably unnecessary. In summary, high activities of G-3-P dehydrogenase were found only in the extracts of aerobically grown cells of K. aerogenes. This activity was found not to be dependent on the presence of added FAD and FMN, but was directly related to the amount of cell extract in the assay system. The enzyme was found not to be NAD-linked, and

existed at only very low activities in anaerobic cell extracts.
Thus, all subsequent assays of G-3-P dehydrogenase were carried out
according to the method of Kistler and Lin (1971), but no flavins
were included in the assay systems.   The actual method used is given
in Experimental Section 5.7.4.

6.4.3   Derivation and optimisation of an assay for the enzyme:
         Dihydroxyacetone Kinase

6.4.3.1   Introduction and derivation

The only methods for the assay of this enzyme that have been
published to date involve related, but indirect, physical measurements
of processes connected to the enzyme's activity:-

(a) Use of the pH-stat to measure acid production caused by
phosphorylation of the substrate.   (Lin et al., 1960).

(b) Measurement of the amount of labelled phosphorylated
material deposited on a DEAE-cellulose filter after ATP, the extract
containing the enzyme and ^{14}C-dihydroxyacetone were incubated together.
(Ruch et al., 1974; Richey and Lin, 1972).

A method was therefore sought for the assay of this enzyme
using the spectrophotometric equipment used for the other three
glycerol-related enzyme assays.   Examination of Fig. 3.1 shows that
dihydroxyacetone (DHA) might be converted to glycerol-3-phosphate
(G-3-P) via dihydroxyacetone phosphate (DHAP).   Fig. 6.4.1 has been
adapted from Fig. 3.1 to show this reaction scheme more clearly.
When DHA is phosphorylated to DHAP, the reduction of DHAP to G-3-P is
possible.   With an excess of the rabbit G-3-P dehydrogenase included,
the latter reaction involves the oxidation of NADH, and may be followed
by a decrease in absorption at 340 nm with time.
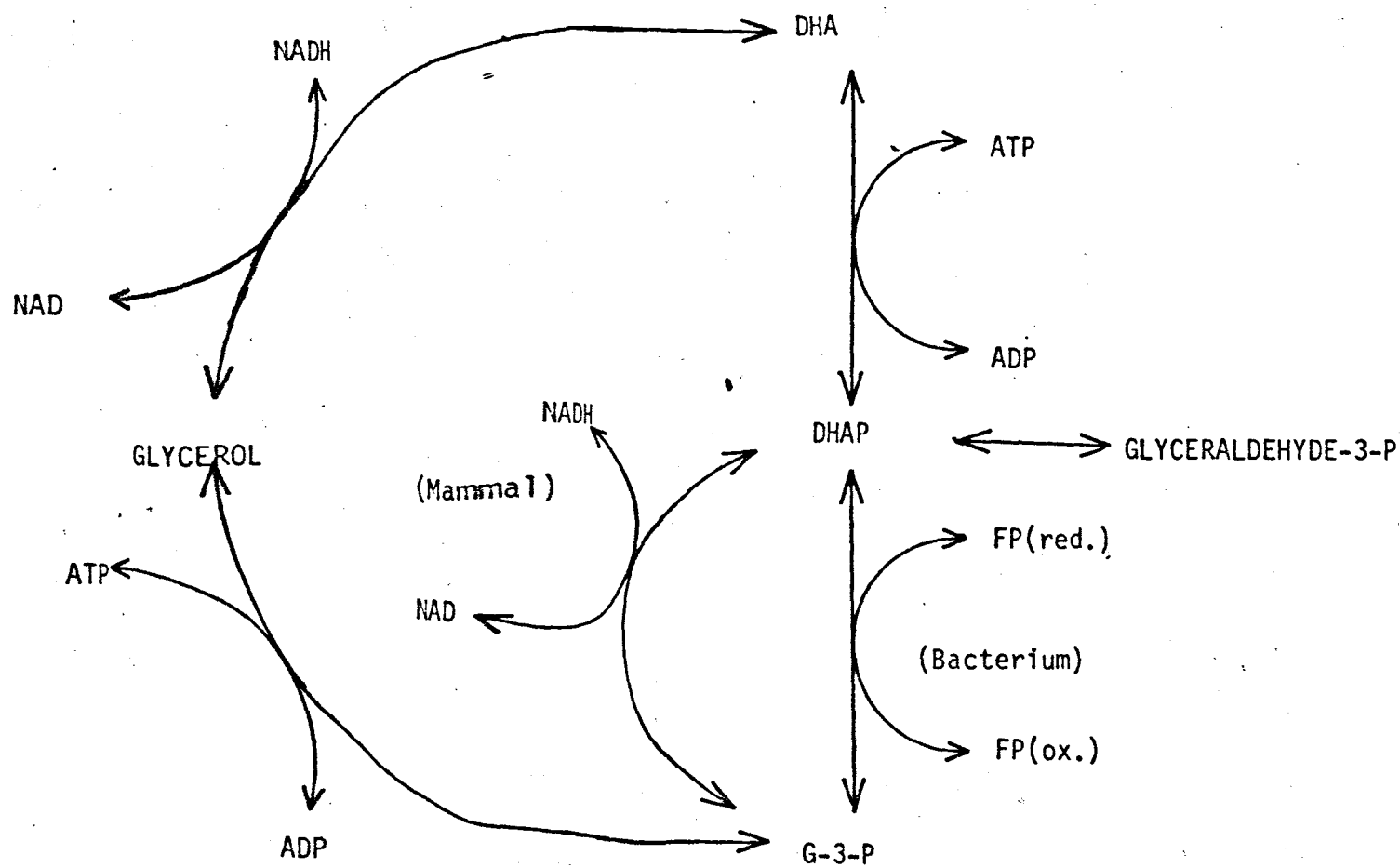
Figure 6.4.1   Reaction Pathways Involved in the Dihydroxyacetone Kinase Assay

Kinetic studies of G-3-P dehydrogenase have shown it to catalyse both the forward and back reactions readily. Furthermore, diversion of DHAP to glyceraldehyde-3-phosphate has been shown to be unfavoured, since triose phosphate isomerase catalyses this reaction in favour of DHAP by a ratio of approximately 20:1 (Under the conditions subsequently used in the assay) (Wren, E.A. Personal Communication). The reduction of DHA to glycerol by glycerol dehydrogenase would be a major competing reaction. However, exclusion of ATP or rabbit G-3-P dehydrogenase in the assay-blank would allow the competing reaction to continue; addition of the excluded reaction component, and subtraction of the first reaction rate from the second would then permit allowance for this competing reaction in the final assay result. These theories were all tested in trial assays.

6.4.3.2   Optimisation of pH and NADH concentration

Following a series of initial trial assays which demonstrated the feasibility of the assay method, the optimisation of these two variables in the assay system was carried out in a series of experiments.

pH

A pH of 7.5 was used in early trial assays, but was actually found to result in an increase of O.D. at 340 nm when the ATP and cell extract were mixed in the cuvette. This was found to be due to a precipitation reaction, the extent of which depended on the amount of cell extract added. The precipitation was completely prevented by raising the pH value of the assay system by only 0.5 units, to 8.0. The pH optima of the reactions attributed to DHA kinase in extracts from both aerobically and anaerobically grown cells were determined.

The results of these experiments are shown in Table 6.4.3:-

Table 6.4.3   Rates of Reactions attributed to DHA kinase at a Series

of pH Values

| pH | Corrected Reaction Rates (O.D. units.min^{-1}) | |
	50 µl Anaerobic Cell Extract	50 µl Aerobic Cell Extract
7.5	0	0.022
8.0	0.120	0.336
8.5	0.082	0.552
9.0	0.048	0.288
9.5	0.040	0.236

From these results, it appears that the optimum pH of the reaction attributed to the anaerobic enzyme is 8.0; that attributed to the aerobic enzyme is 8.5.   Later work showed these to probably be two separate enzymes, and only the anaerobic enzyme to be a true dihydroxyacetone kinase.

NADH concentration

A series of DHA kinase assays of extracts of anaerobically grown cells were carried out at different NADH concentrations.   The results are given in Table 6.4.4.

Table 6.4.4   Rates of Reactions attributed to DHA kinase at a

Series of NADH Concentrations

NADH concentration (mM)	0.10	0.20	0.30	0.40	0.50	0.60	0.70
Relative Reaction Rate at 340 nm (50 μl anaerobic cell extract)	0.6	4	7.5	9	10	7.5	6.5

The optimum concentration of NADH in the DHA kinase assay thus appears to be 0.50 mM, and was therefore used in all subsequent assays of this enzyme.   This is rather a high concentration of NADH, and does therefore provide an optically dense solution at 340 nm.   A wide instrument slit-width was therefore found necessary.

6.4.3.3   Studies of the enzyme and optimisation of the substrate

concentration

The high reaction rates attributed to an aerobic enzyme during the pH optimisation were unexpected, even though the aerobic cell extracts contained considerably (ca. 5 times) more protein than the anaerobic extracts.   The different pH optima of the aerobic and anaerobic enzymes is evidence of them being two separate enzymes.

The inclusion of 10 mM glycerol in the assay system was found to totally inhibit the reaction carried out by aerobic cell extracts. The anaerobic reaction, however, was merely partly inhibited.   This discovery lead to an experiment designed to find the nature of the inhibition of this enzyme by glycerol.   The optimum concentration of the enzyme's substrate, dihydroxyacetone, in the assay system was also found from this experiment.

Assays of DHA kinase, using 50 μl of anaerobic cell extract, were carried out using a series of eight DHA concentrations. A further series of assays at four of these DHA concentrations were also carried out, but 10 mM glycerol was included in the reaction system. The reaction rates at each substrate concentration, with and without 10 mM glycerol, are given in Table 6.4.5:-

Table 6.4.5   DHA kinase Assays at a Series of Substrate Concentrations

(a)   With no added glycerol.

DHA concentration (mM) [S]	$\frac{1}{[S]}$	Reaction Rate (O.D. units min^{-1}) (V)	$\frac{1}{V}$
1.25	0.800	0.022	45.45
2.50	0.400	0.034	29.41
3.75	0.267	0.042	23.81
5.00	0.200	0.046	21.74
6.25	0.160	0.054	18.52
7.50	0.133	0.056	17.86
8.75	0.114	0.050	20.00
10.00	0.100	0.048	20.83

(b)   With 10 mM glycerol in the Assay system.

DHA concentration (mM) [S]	$\frac{1}{[S]}$	Reaction Rate (O.D. units min^{-1}) (V)	$\frac{1}{V}$
1.25	0.800	0.0092	108.7
3.75	0.267	0.020	50.00
6.25	0.160	0.031	32.26
8.75	0.114	0.035	28.57

The Michaelis-Menten equation for an enzyme-substrate reaction is:-

$$V = V_{max} \frac{[S]}{K_m + [S]}$$

where V = velocity of the enzyme-catalysed reaction.

V_{max} = maximum reaction velocity

$[S]$ = substrate concentration

K_m = Michaelis-Menten constant, the value of $[S]$ when $V = \frac{V_{max}}{2}$

Lineweaver and Burke (1934) re-arranged the Michaelis-Menten equation to give:-

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

Thus, a plot of $\frac{1}{[S]}$ as ordinate against $\frac{1}{V}$ gives a straight line of intercept $\frac{1}{V_{max}}$ on the y-axis, and $-\frac{1}{K_m}$ on the x-axis.

The Lineweaver-Burke plot was carried out for the DHA kinase assay results given in Table 6.4.5, both with and without 10 mM glycerol. Figure 6.4.2 shows these two plots. V_{max} was found to have the same value whether the enzyme was inhibited with 10 mM glycerol or not. The value found from Fig. 6.4.2, for V_{max} is 0.080 O.D. min^{-1}. K_m, however is altered by glycerol inhibition. With no glycerol, the value of K_m for DHA kinase is 3.33 mM; with 10 mM glycerol, K_m = 10.0 mM.
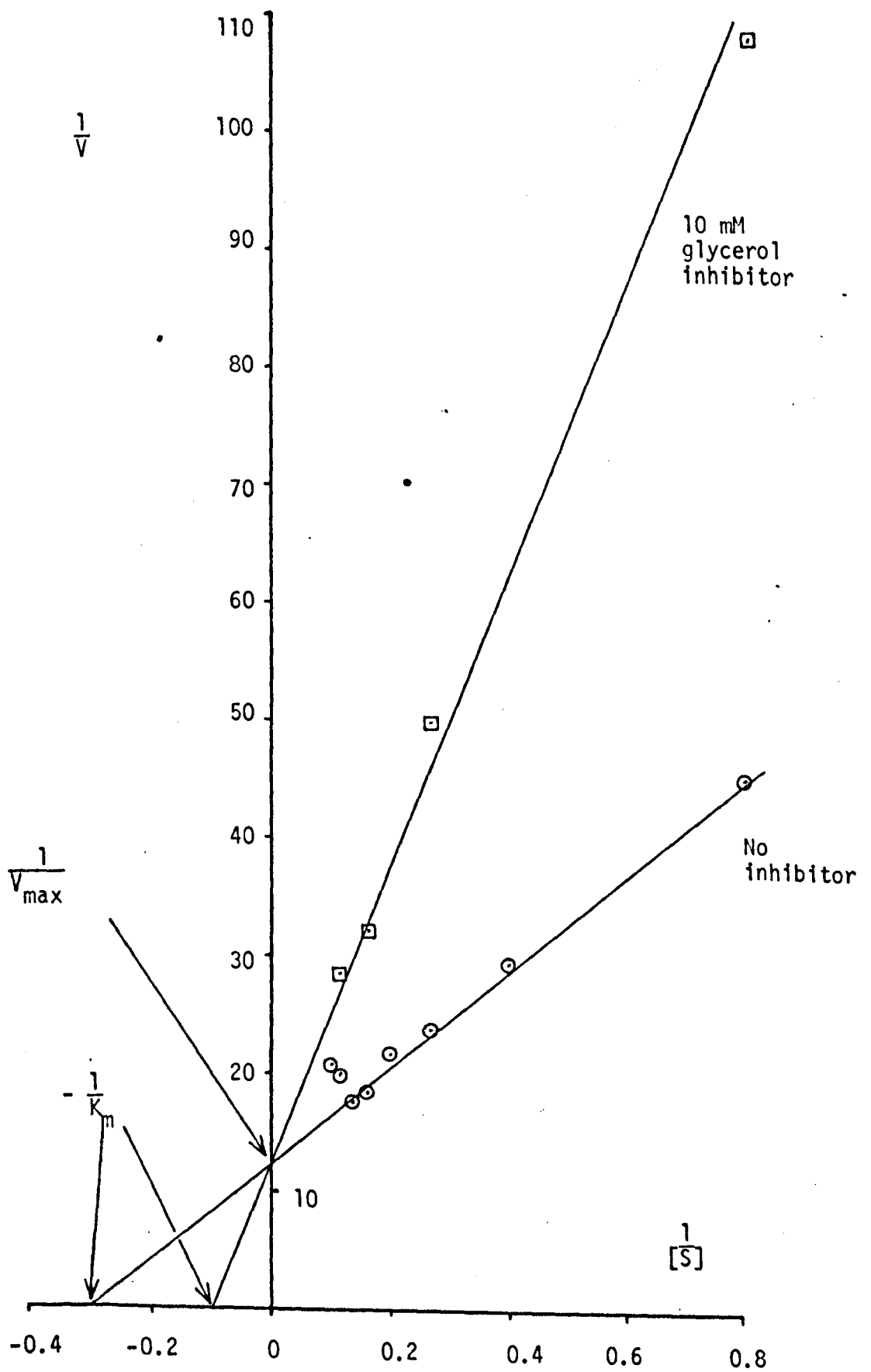
Figure 6.4.2   Lineweaver - Burke Plot of Dihydroxyacetone Kinase

Assay Results

Thus, since V_{max} is unaltered by the presence of the inhibitor but K_m is increased three-fold, inhibition of dihydroxyacetone kinase by glycerol is 'competitive'. It also appears that the enzyme may be inhibited by high concentrations of its substrate, DHA, since concentrations in excess of 7.50 mM lead to a reduction of the reaction rate (from Table 6.4.5). This also shows as two 'out of line' points at low $\frac{1}{[S]}$ values on the Lineweaver-Burke plot. For this reason, 7.50 mM DHA was used in all subsequent DHA kinase assays.

However, the results also suggest that substrate inhibition may not be demonstrated unless considerably higher DHA concentrations are included in the assay system if 10 mM glycerol is included as a competitive inhibitor. Since this concentration of glycerol was included in all assays in order to inhibit the glycerol kinase, it seems that, in retrospect, a higher DHA concentration than 7.50 mM should perhaps be included in the DHA kinase assay system.

Competitive inhibition of DHA kinase by glycerol suggests that the chemical structures of DHA and glycerol may be sufficiently similar (Fig. 3.2) to allow them to be bound to the active site of the same enzyme. The converse may also apply; apparent glycerol kinase activities were found in anaerobic cell extracts, but may be due to DHA kinase using glycerol as an alternative substrate. Total inhibition of apparent high DHA kinase activity in extracts of aerobic cells by the inclusion of 10 mM glycerol was found. Thus, it seems possible that glycerol kinase may phosphorylate DHA when glycerol is not available; inclusion of glycerol may, by providing the preferred substrate for the enzyme, prevent phosphorylation of DHA by glycerol kinase. The price paid, however, is partial (competitive) inhibition of the DHA kinase enzyme.

Using concentrations of 20 mM each for ATP and Mg^{2+}, and
100 µg of the rabbit G-3-P dehydrogenase in a total assay system volume
of 3.0 cm^3, the assay method for dihydroxyacetone kinase described in
Experimental section 5.7.3 was developed from the optimisation results
given here.

6.4.4 Assays of glycerol-related enzymes in cell samples from the intermittently aerated cultures

All four enzymes involved in the first two stages of aerobic
and anaerobic glycerol metabolism in K. aerogenes 418 (see Fig. 3.1)
were assayed in all cell samples from the four intermittently aerated
cultures.   The experimental methods used for the assay of these enzymes
(glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol
dehydrogenase, and dihydroxyacetone kinase) are detailed in Experimental
Section 5.7.

Culture samples were harvested (from the fermenter overflow)
and stored as described in Experimental Section 5.5.3.   Intermittently
aerated cultures at any particular culture/aeration condition were
allowed to stabilise to a 'steady-state' (as defined in Section 6.3.1),
and remain in this state for several generations, before the culture was
harvested.   These techniques were intended to assure preservation of
enzyme activities in the stored cells which were representative of the
activities in the steady-state IA cultured cells actually in the
fermenter.   Any loss of enzyme activity was hopefully distributed
evenly throughout all the relevant enzymes.   Stored cell samples were
treated to make an extract suitable for enzyme assay by the method
explained in Experimental Section 5.7.1.   Again, this was intended to
maintain a constant enzyme complement.   Since the method was used

universally, any activity losses should again have been evenly distributed.

Thus all intervening procedures between the intermittently aerated culture and the assay of the four relevant enzymes were designed for the derivation of results that were representative of the levels of these enzymes when the cells were harvested. As each IA culture was-repeated, so each enzyme assay was repeated. The results given here are of enzyme assays of the samples whose culture data are given in Section 6.3, since the cells were grown using perfectly operating equipment, and the improved extract stabilisation techniques of filtration and B.S.A. addition were employed only with the cells from the second set of cultures. As with the culture data, general reproducibility was demonstrated, although the first set of assay results are redundant and 'averaging' may be detrimental to accuracy. All enzyme assays were carried out according to the methods described in Experimental Section 5.7, and, with one exception, were expressed as μ.moles NAD(H) reduced (or oxidised) per minute per mg protein. The exception was the G-3-P dehydrogenase assay, which was not NAD-linked, and was therefore expressed as O.D. units change per minute per mg protein.

In order to express enzyme activities in this way, for each assay system , knowledge of the following is required:-

(a) Volume of cell extract used in each assay.

(b) Concentration of protein in the extract.

(c) Rate of reaction measured as O.D. units change per minute.

(d) Extinction coefficient of NADH.

(a) and (d) were known, (c) was measured, and (b) was determined by the Biuret method, described in Experimental section 5.6.3.1. Protein concentrations varied widely, being highest in the aerobic cell extracts

and lowest in the anaerobic cell extracts. The protein concentrations of the samples whose enzyme assay results are given here are listed in Table 6.4.6.

In order to convert O.D. units change $min^{-1}.mg$ $protein^{-1}$ to μ moles $NADH.min^{-1}.mg$ $protein^{-1}$, the extinction coefficient of NADH was required. The value used was 6.22×10^3 for a molar solution across a 1 cm light path (Wren, E.A., Personal communication).

Thus, 1 $m.mole.cm^{-3}$ has an O.D. of 6.22, and 1 μ mole in 3.0 cm^3 has an O.D. of $\frac{6.22}{3}$ across the 1 cm light path. The conversion factor of O.D. $units.min^{-1}.mg$ $protein^{-1}$ to μ moles $NADH.min^{-1}.mg$ $protein^{-1}$ is therefore $\frac{3}{6.22}$, or 0.482.

The specific activity of each of the four enzymes in the 21 samples is given in Table 6.4.7, 6.4.8, 6.4.9 and 6.4.10.

The activity of each enzyme for a particular IA culture is plotted against aeration time per cycle in Figs. 6.4.3, 6.4.4, 6.4.5 and 6.4.6. Thus, the trends in the content of individual enzymes within the cells can be seen as the aeration time per cycle is progressively reduced. In addition, the differences in content of an individual enzyme at any particular aeration time per cycle among the four IA cultures can be seen. The general trends are as one might expect: decreasing levels of aerobic enzymes and increasing levels of anaerobic enzymes as the aeration time per cycle is progressively decreased.

The aerobic enzymes, glycerol kinase and G-3-P dehydrogenase are generally present at higher levels in the better aerated cultures. (i.e. IA cultures 1 and 2). Moreover, especially in IA culture 1 (900/0.30) they appear to reduce linearly from the maximum (5-0) value

TABLE 6.4.6   Cell Extract Protein Concentrations (Biuret)

Culture Sample	Protein concentration $(mg.cm^{-3})$
IA Culture 1:-	
900/0.30/5-0	1.715
900/0.30/4-1	1.960
900/0.30/3-2	1.613
900/0.30/2-3	1.497
900/0.30/1-4	1.449
900/0.30/0-5	0.429
IA Culture 2:-	
800/0.50/5-0	1.860
800/0.50/4-1	1.866
800/0.50/3-2	1.868
800/0.50/2-3	1.383
800/0.50/1-4	0.558
800/0.50/0-5	0.358
IA Culture 3:-	
700/0.55/5-0	2.018
700/0.55/4-1	2.058
700/0.55/3-2	2.135
700/0.55/2-3	0.938
700/0.55/1-4	0.460
700/0.55/0-5	0.365
IA Culture 4:-	
700/0.70/5-0	1.409
700/0.70/4-1	1.722
700/0.70/3-2	1.361

Tables 6.4.7-10   Specific Enzyme Activities in Samples from IA

Cultures 1, 2, 3 and 4

Table 6.4.7   Glycerol kinase activities

(1 Unit ≡ 1μ mole NAD reduced.min^{-1} mg $protein^{-1}$)

Culture Sample	Specific Activity (Units)	Culture Sample	Specific Activity (Units)
IA Culture 1:-		IA Culture 3:-	
900/0.30/5-0	12.6	700/0.55/5-0	4.54
900/0.30/4-1	10.1	700/0.55/4-1	0.819
900/0.30/3-2	7.61	700/0.55/3-2	0.947
900/0.30/2-3	4.44	700/0.55/2-3	0.350
900/0.30/1-4	0.238	700/0.55/1-4	0.671
900/0.30/0-5	0.781	700/0.55/0-5	1.15
IA Culture 2:-		IA Culture 4:-	
800/0.50/5-0	4.72	700/0.70/5-0	1.92
800/0.50/4-1	4.54	700/0.70/4-1	2.55
800/0.50/3-2	1.58	700/0.70/3-2	1.35
800/0.50/2-3	0.310		
800/0.50/1-4	0.471		
800/0.50/0-5	1.17		

These results are plotted in Fig. 6.4.3.
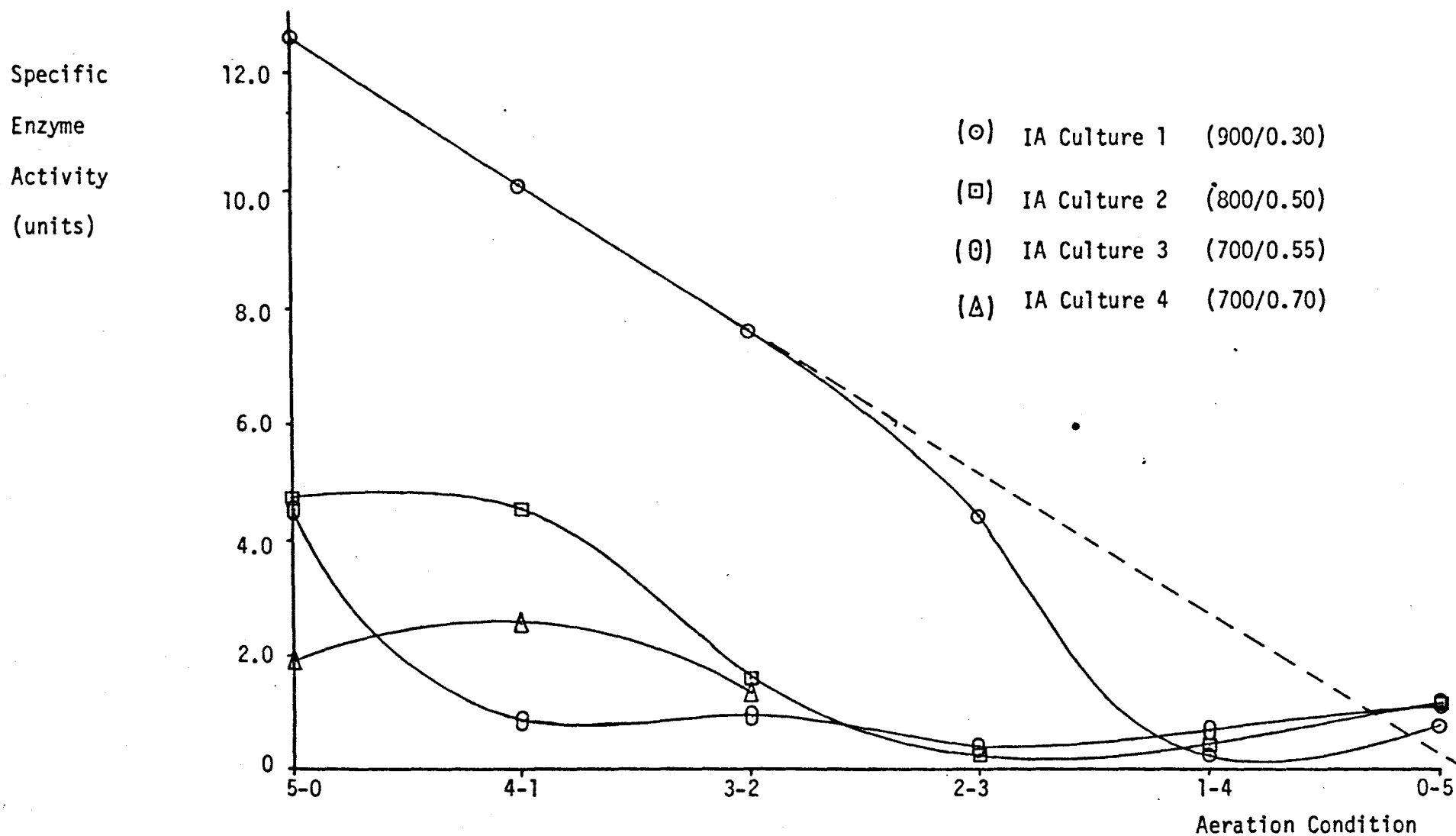
Figure 6.4.3   Glycerol Kinase Activities of Samples from Intermittent Aeration Cultures

Table 6.4.8   Glycerol-3-phosphate dehydrogenase activities

(1 unit ≡ 1 O.D. unit change min^{-1} mg $protein^{-1}$)

Culture Sample	Specific Activity (Units)	Culture Sample	Specific Activity (Units)
IA Culture 1:-		IA Culture 3:-	
900/0.30/5-0	3.66	700/0.55/5-0	2.67
900/0.30/4-1	3.11	700/0.55/4-1	1.12
900/0.30/3-2	1.99	700/0.55/3-2	1.17
900/0.30/2-3	1.53	700/0.55/2-3	1.23
900/0.30/1-4	0.297	700/0.55/1-4	1.37
900/0.30/0-5	0.303	700/0.55/0-5	1.01
IA Culture 2:-		IA Culture 4:-	
800/0.50/5-0	3.82	700/0.70/5-0	1.42
800/0.50/4-1	3.01	700/0.70/4-1	2.21
800/0.50/3-2	1.83	700/0.70/3-2	1.18
800/0.50/2-3	1.07		
800/0.50/1-4	0.878		
800/0.50/0-5	0.652		

These results are plotted in Fig. 6.4.4

Figure 6.4.4   Glycerol-3-Phosphate Dehydrogenase Activities of Samples from Intermittent Aeration Cultures

Table 6.4.9   Glycerol dehydrogenase activities

1 Unit $\equiv$ 1μ mole NAD reduced min^{-1} mg protein^{-1})

Culture Sample	Specific Activity (Units)	Culture Sample	Specific Activity (Units)
1A Culture 1:-		1A Culture 3:-	
900/0.30/5-0	0	700/0.55/5-0	0
900/0.30/4-1	0	700/0.55/4-1	0
900/0.30/3-2	0	700/0.55/3-2	0
900/0.30/2-3	0	700/0.55/2-3	0.075
900/0.30/1-4	0.057	700/0.55/1-4	0.246
900/0.30/0-5	0.388	700/0.55/0-5	0.482
1A Culture 2:-		1A Culture 4:-	
800/0.50/5-0	0	700/0.70/5-0	0
800/0.50/4-1	0	700/0.70/4-1	0
800/0.50/3-2	0	700/0.70/3-2	0.012
800/0.50/2-3	0.031		
800/0.50/1-4	0.177		
800/0.50/0.5	0.411		

These results are plotted in Fig. 6.4.5.
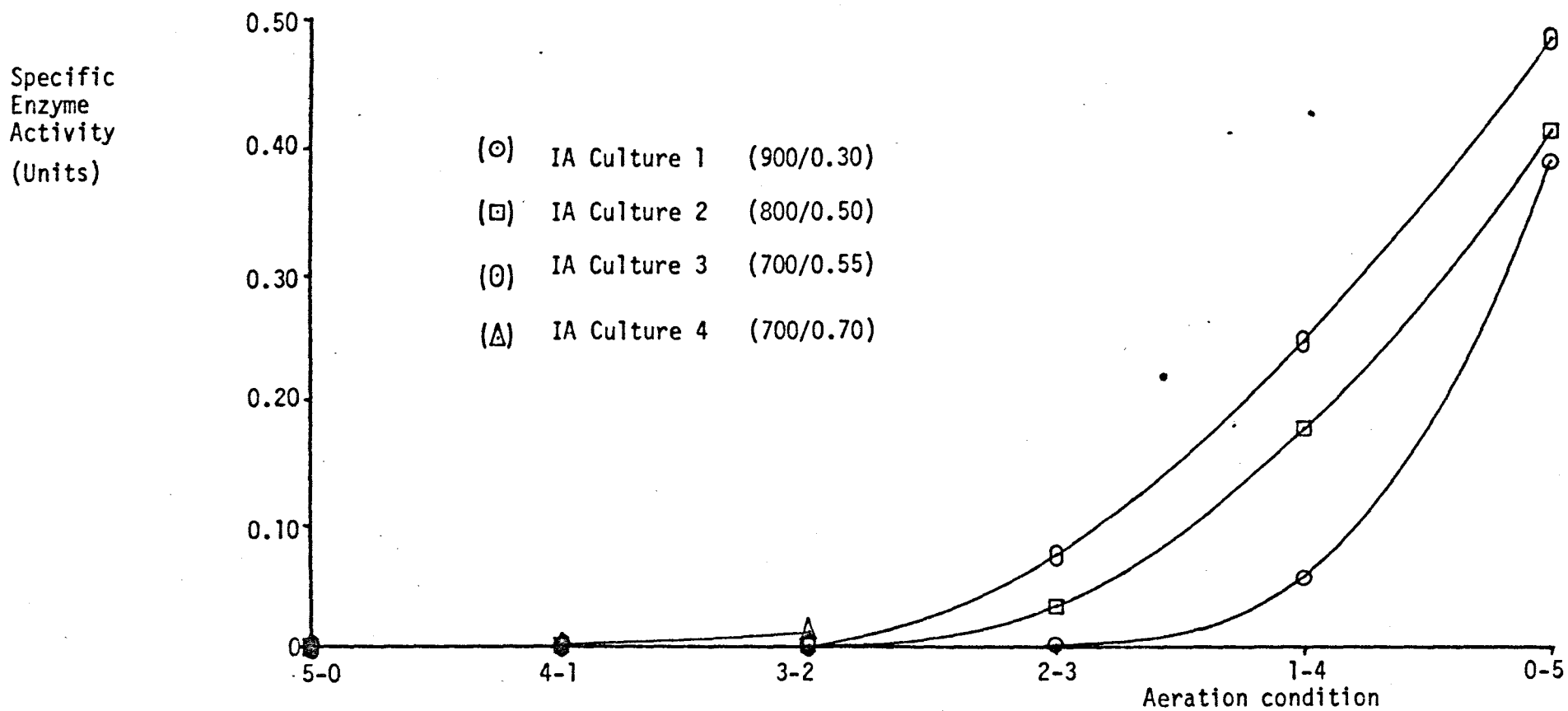
Figure 6.4.5   Glycerol Dehydrogenase Activities of Samples from Intermittent Aeration Cultures

Table 6.4.10   Dihydroxyacetone kinase activities

(1 Unit $\equiv$ 1μ mole NADH oxidised. min^{-1} mg $protein^{-1}$)

Culture Sample	Specific Activity (Units)	Culture Sample	Specific Activity (Units)
IA Culture 1:-		IA Culture 3:-	
900/0.30/5-0	0	700/0.55/5-0	0
900/0.30/4-1	0	700/0.55/4-1	0.025
900/0.30/3-2	0	700/0.55/3-2	0.010
900/0.30/2-3	0	700/0.55/2-3	0.181
900/0.30/1-4	0.045	700/0.55/1-4	0.614
900/0.30/0-5	0.798	700/0.55/0-5	0.899
IA Culture 2:-		IA Culture 4:-	
800/0.50/5-0	0	700/0.70/5-0	0.110
800/0.50/4-1	0	700/0.70/4-1	0.018
800/0.50/3-2	0	700/0.70/3-2	0.503
800/0.50/2-3	0.168		
800/0.50/1-4	0.428		
800/0.50/0-5	0.866		

These results are plotted in Fig. 6.4.6
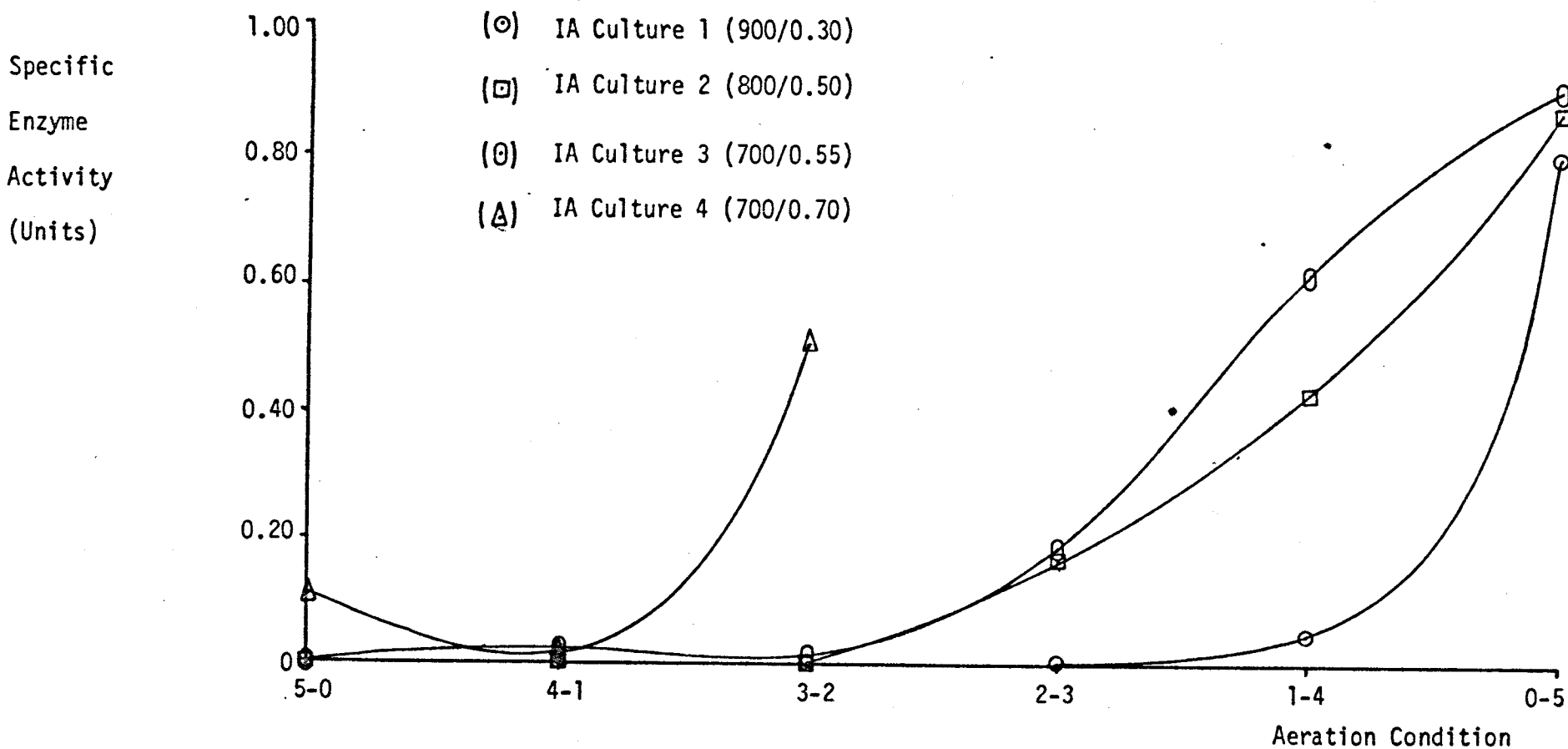
Figure 6.4.6   Dihydroxyacetone Kinase Activities of Samples from Intermittent Aeration Cultures

to the aerobic → anaerobic transition. This seems true for the G-3-P dehydrogenase levels of both IA culture 1 and culture 2, as well as the glycerol kinase level of IA culture 1. After the aerobic → anaerobic transition, the levels of aerobic enzymes remain at fairly stable low levels, although all glycerol kinase activities increase slightly in the fully anaerobic (0-5) cultures. The levels of aerobic enzymes in IA culture 4 (700/0.70) increase on the cultures' conversion from 5-0 to 4-1 aeration, then decrease again at 3-2 aeration. This effect was not seen in any of the other three cultures.

The levels of the anaerobic enzymes, glycerol dehydrogenase and DHA kinase remain at or near zero at the aeration conditions when aerobic metabolism is evident. For IA culture 4, however, there is a small positive DHA kinase activity at the fully aerated (5-0) condition which reduces on conversion to 4-1 aeration. The orders (with respect to aeration time per cycle) in which the anaerobic enzyme levels increase depend entirely on the aeration efficiency of the individual cultures, although all reach similar levels of these enzymes in the fully anaerobic state. Thus, the anaerobic enzyme levels of IA culture 4 rise at a lower aeration time per cycle than those of IA culture 3, etc., and the levels of individual enzymes are higher for the more poorly-aerated cultures at a single aeration condition. Further discussion of these results is presented in Chapter 7.

6.5   Other Analytical Procedures

In addition to the assay of glycerol-related enzymes, the identification and assay of respiratory enzymes was carried out. Cytochromes were identified, and their concentrations were measured, in both aerobically and anaerobically grown K. aerogenes 418 cells. This work is reported here.

During the work on intermittently aerated cultures, a yellow-green pigment was produced in noticeable amounts only at one particular culture/aeration condition.   The production of this pigment by IA culture 1 (at 900/0.30/2-3 aeration) was found to be maintained in continuous culture, and to be reproducible.   Its production did not occur in such noticeable amounts in any of the other twenty culture/aeration conditions.   The attempted identification of this pigment is reported here.

6.5.1   Identification and assay of cytochromes

This work was intended to find qualitative and quantitative differences of cytochrome type and concentration between cells which had been grown aerobically and those which had been grown anaerobically.

In order to prepare a sample for cytochrome analysis, the procedure described in Experimental section 5.7.5. was followed. The protein content of quantitative samples was determined by the Folin method, as described in Experimental section 5.6.3.2.   Reduced versus oxidised spectra were carried out from 660 nm to 490 nm wavelengths, using a double-beam instrument with λ_1 set equal to λ_2.   For qualitative work, the absorption peaks were resolved in much finer detail using a cryostatic sample holder at the temperature of liquid

nitrogen (-196°C). However, random light-scattering by ice-crystals
prevents the use of this technique for quantitative purposes.

Fig. 6.5.1 shows the traced room temperature cytochrome spectra
of an aerobic and an anaerobic cell extract. (Reduced vs. oxidised).
The most prominent absorption peak in both spectra is the α-peak of
cytochrome b (at 560 nm). The smaller β-peak of this cytochrome is
displayed more prominently in the aerobic extract (at 530 nm). In
addition to the cytochrome b peaks, the anaerobic extract displays a
slight cytochrome d peak (at 625 nm), and 'hints' at cytochrome a_1
(at 600 nm). The aerobic extract shows no peaks for these two
cytochromes.

Fig. 6.5.2 shows the traced low-temperature spectrum of the
anaerobic cell extract. A broad peak at 625 nm, indicating the presence
of cytochrome d in anaerobic K. aerogenes, is shown. The peak for
cytochrome a_1 is not shown in any better detail than at room temperature,
and may therefore be an instrumental artefact. The main improvement
at -196°C, however, is in the resolution of the cytochrome b α-peak.
The peak appears as a doublet, at 556 nm and 559 nm, exhibiting
cytochromes b_{556} and b_{559}. An improved resolution slow scan from
520 nm to 580 nm (at 3.3 times the sensitivity) is overlaid, showing the
doublet in even finer detail, and a strong cytochrome b β-peak at 530 nm.
The low-temperature spectrum of the aerobic cell extract did not show
significantly improved resolution, but displayed a single α-peak for
cytochrome b at 557 nm.

Quantitative determination of cytochrome concentrations in
the aerobic and anaerobic cell extracts was made using the ambient
temperature spectra (Fig. 6.5.1). The heights of the α-peaks for
cytochrome b of each extract were measured, bearing in mind that the

0.01

Absorbancy
Increment

0.03

Absorbancy
Increment

Anaerobic
Cell Extract

Aerobic
Cell Extract

500   520   540   560   580   600   620   640   660

Wavelength (nm)
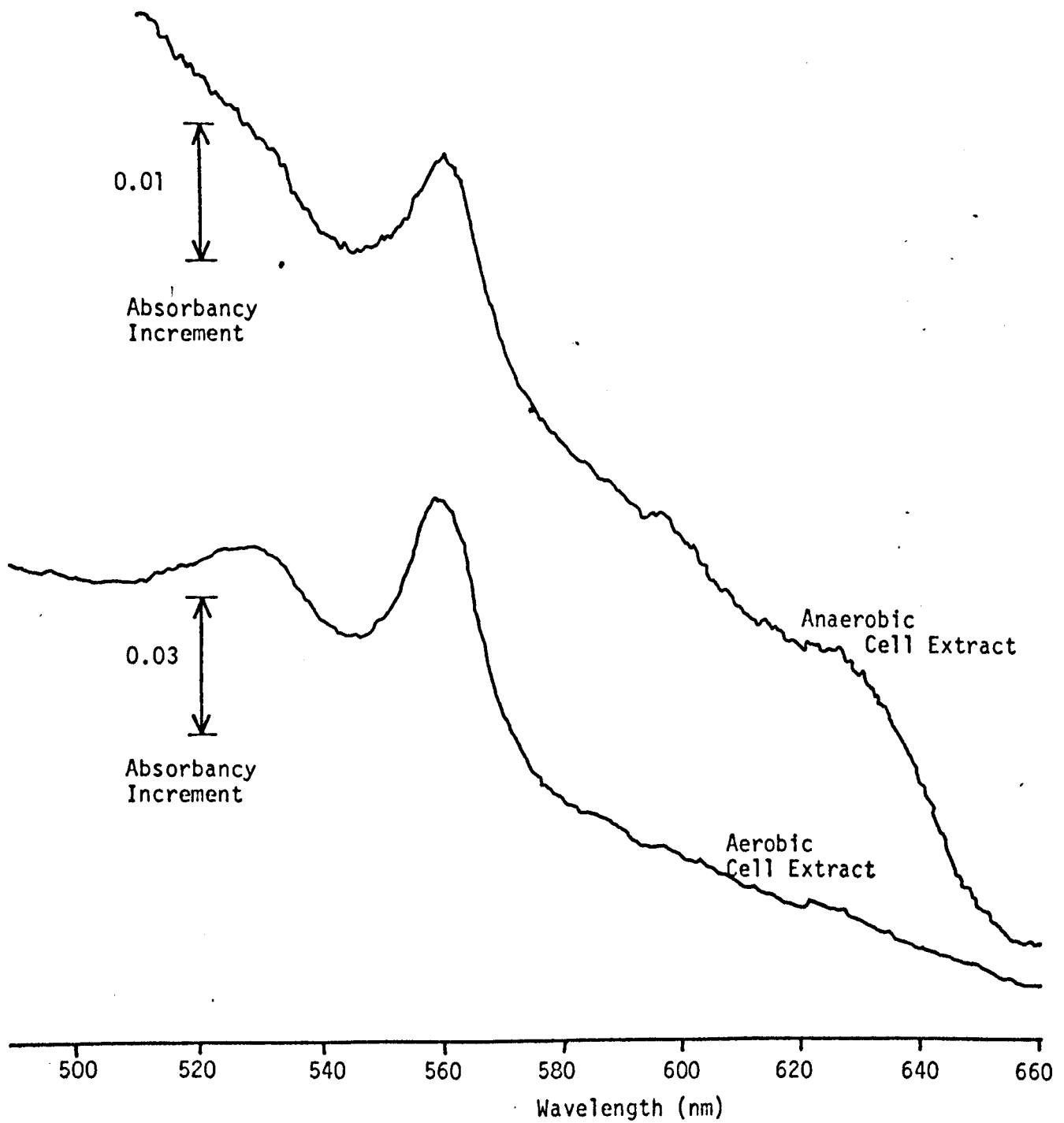
Figure 6.5.1   Ambient Temperature Cytochrome Spectra of Aerobic and

Anaerobic Cell Extracts (Reduced vs. Oxidised)
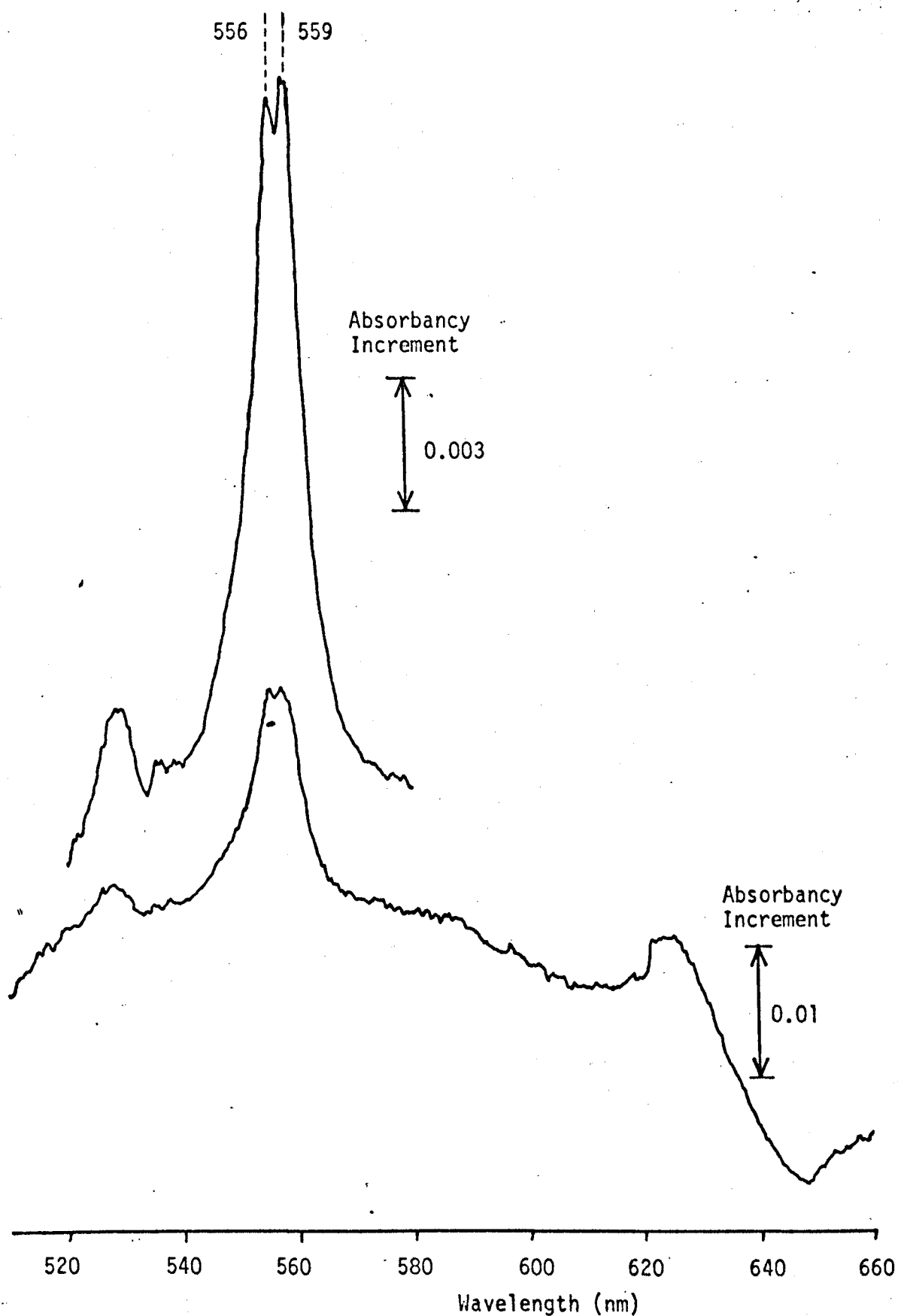
Figure 6.5.2   Low Temperature (-196°C) Cytochrome Spectrum of the
Anaerobic Cell Extract (Reduced vs. Oxidised)

spectrum of the anaerobic extract was carried out at a sensitivity setting of 3.0 times that of the aerobic extract. (i.e. the anaerobic peak heights are exaggerated by a factor of 3.0).

Knowing the instrument full scale deflection to be 0.1 A (anaerobic), and 0.3 A (aerobic), where A $\equiv$ O.D. units, drawing a suitable baseline and measurement of the α-peak heights yields:-

α-peak height of aerobic extract = 0.0473 O.D. units

α-peak height of anaerobic extract = 0.0130 O.D. units

The extinction coefficient of a 1 mM solution of cytochrome b across this path length = 20.0 O.D. units. The protein concentrations of each cell extract, determined by the Folin method were:

aerobic cell extract:- 26.7 mg.cm^{-3}

anaerobic cell extract:- 12.0 mg.cm^{-3}

A 1mM solution $\equiv$ 1 μmole.cm^{-3} $\equiv$ 10^6 p.moles.cm^{-3}. Hence, the concentrations of cytochrome b in the extracts are:

aerobic cell extract:- $\frac{0.0473}{20}$ x 10^6 p.moles.cm^{-3}

= 2365 p.moles.cm^{-3}

= 88.6 p.moles.mg protein^{-1}

anaerobic cell extract:- $\frac{0.0130}{20}$ x 10^6 p.moles.cm^{-3}

= 650 p.moles.cm^{-3}

= 54.2 p.moles.mg protein^{-1}

Thus, the ratio of cytochrome b concentration in the aerobic:anaerobic cells was 1.63. The growth conditions of the aerobic and anaerobic

cells should be noted: <u>K. aerogenes</u> cells were cultured continuously at a dilution rate of 0.30 h^{-1} and impeller speed of 900 min^{-1}, and provided with either 1.0 min^{-1} air or 0.2 min^{-1} oxygen-free nitrogen.

The variations in the levels of the four glycerol-related enzymes between aerobically and anaerobically grown cells (reported in Section 6.4.4) are obviously not repeated in the levels of cytochrome b in these cells. Whilst the concentration of cytochrome b in cells grown under 900/0.30/5-0 conditions is 1.63 times that of cells grown under 900/0.30/0-5 conditions, the corresponding ratio of glycerol kinase activities in cells grown under the same conditions being 16.1:1. These results are further discussed in Chapter 7.

6.5.2   Identification of an excreted pigment

The yellow-green pigment which was excreted by the cells into the medium under intermittently aerated (900/0.30/2-3) conditions of continuous culture was subjected to UV-visible spectroscopic analysis and spectrofluorimetric analysis.

Firstly, however, concentration of the pigment was necessary, and this was attempted by two different methods. The culture harvested from the fermenter under the quoted culture/aeration conditions was first centrifuged for 0.5 hours at 40,000 G. The resultant supernatant, containing the pigment, was poured off, and concentration of the pigment attempted.

Solvent extraction of the pigment into four different organic solvents at both alkaline and acid pH values was tried. Samples of the supernatant were shaken with 0.1 volumes of chloroform, diethyl ether, n-hexane and toluene at pH 4.5 and at pH 9.5. In no case did the the pigment pass into the organic phase.

A 150 cm^3 sample of the supernatant was freeze-dried to completion, the process taking about 4 hours. The dried solids were then redissolved to 10% of the original volume (i.e. 15 cm^3). The bright yellow solution was then centrigued for 1.5 hours at 40,000 G to remove remaining suspended solids. A ten-fold concentration of the pigment was thus successfully carried out by this method.

A U.V-visible spectrum of the pigment solution (requiring dilution) was carried out from 200 nm to 600 nm wavelengths. Absorption peaks were found at 207 nm and (most noticeably) at 258 nm, with an absorption minimum between these two peaks at 236 nm. Shoulders on the decreasing absorption baseline were found at 420 nm and 473 nm. There was no other prominent feature on this spectrum.

It was discovered that the culture supernatant fluoresced when subjected to U.V irradiation from a hand-held lamp. Further study, using the concentrated pigment solution, showed a strong 'blue fluorescence', i.e. U.V excitation leading to strong emission in the blue sector of the optical spectrum. This discovery prompted more quantitative work on the fluorescence properties of the pigment. The information in Table 6.5.1 was obtained by manual scanning of the fluorescence spectrum of the pigment solution:-

Table 6.5.1   Fluorescence Data of Pigment Solution

Fluorescence	λ Excitation (nm)	λ Fluorescence (nm)	Effect of dithionite reduction or peroxide oxidation
Strong	365	440	No effect
(½ strength)	(410)	(485)	(No effect)
Weak	465	520	Abolished by reduction No effect by oxidation

These data indicate that there are strong broad excitation
and fluorescence peaks at 365 nm and 440 nm respectively. The widths
of the peaks are indicated by the wavelengths at which they are at
half-strength. Weak excitation and fluorescence peaks at 465 nm and
520 nm (respectively), which are abolished by dithionite reduction but
unaffected by peroxide oxidation, may be due to the presence of oxidised
flavin.

The strong fluorescence of the pigment at 440 nm indicates
that it may be a member of the pteridine group of compounds. A member
of this group, pterin (2-amino, 4-hydroxy pteridine) has an excitation
peak at 360 nm, and a corresponding fluorescence peak at 440 nm.
(Wren, E.A., personal communication). These wavelengths are very close
to (or even coincidental with) those observed in the fluorescence
spectrum of the pigment. No other compound which exhibits these
particular excitation and fluorescence wavelengths can be found.

Harrison et al. (1969) reported the production of a yellow
flavin-like pigment in cultures of K. aerogenes which were subjected
to hyperbaric oxygen tensions. He also observed (Harrison, 1972b)
the transient observation of a pigment absorbing in the 500 nm region
in chemostat cultures of K. aerogenes. Moss et al. (1969) reported a
colour change in C. utilis cultures from pink at high DOT to pale green
at low DOT. None of these pigments was, however, identified.

CHAPTER 7

DISCUSSION

The results of the experimental work, presented in Chapter 6, promote discussion of several aspects of aerobic, anaerobic and intermittently aerated culture of K. aerogenes. All cultures utilised glycerol as the sole source of carbon and energy; discussion is therefore particularly focussed on the effects of oxygen-transfer efficiency on the organism's utilisation of this compound. The effects of oxygen-transfer on the levels of the aerobic and anaerobic enzymes of glycerol metabolism are given special attention. Moreover, the utilisation of the two primary metabolites of glycerol catabolism by aerobic and anaerobic cultures is discussed.

The basic variable in considering oxygen mass-transfer in fully aerated cultures was impeller speed. Discussion of the relationship between impeller speed and double-substrate (carbon and oxygen) limitation is presented. The effect of impeller speed on the formation of wall growth is also discussed.

7.1   Aerobic Cultures

Aerobic batch and continuous cultures were carried out in the fermenter under a variety of oxygen-transfer conditions.   With full aeration, maintained at 1.0 min^{-1}, oxygen-transfer efficiency was controlled solely by adjustment of impeller speed.

7.1.1   Effect of oxygen-transfer efficiency on carbon and oxygen limitation

The data in Table 6.1.2 shows the effects of different impeller speeds on the growth rate, and final cell density, in four aerobic batch cultures. ' Dissolved oxygen tension at the point of glycerol limitation was also recorded; in no case did it reach zero.   Impeller speed (and therefore oxygen-transfer efficiency) appears to be unrelated to the growth kinetics and peak cell density.   Since DOT, even at the lowest impeller speed of 700 min^{-1}, remains well above critical levels, this result is not surprising.   Thus, the maximum specific growth rate (μ_{max}) of between 0.83 h^{-1} and 0.90 h^{-1} is reached in all four batch cultures.

The growth kinetics of the cultures grown in aerobic shake flasks show similar results.   Fig. 6.1.1 shows a typical growth curve of a culture shaken in baffled flasks at 37°C and 250 cycles.min^{-1} in an orbital shaker.   The specific growth rate of this culture during the period of logarithmic growth is 0.87 h^{-1}, and is therefore within the range of growth rates found in fermenter batch cultures.   However, when cultures were grown aerobically in the water-bath apparatus, designed for aerobic or anaerobic cultures (Fig. 5.5), specific growth rates of only ca. 0.3 h^{-1} were found;   logarithmic growth at this low rate was maintained for 3-4 hours. (Fig. 6.2.1).   Moreover, the peak

cell density of ca.1.51 mg.cm^{-3} was considerably lower than the 2.65 mg.cm^{-3} found in cultures grown in the orbital shaker. Both types of shake flasks (i.e. orbital shaker and water-bath)included baffle-indentations ('dimples'); however orbital-shaker flasks contained a liquid volume of 50 cm^3 in a flask volume of 500 cm^3 whilst water-bath flasks contained a volume of 100 cm^3 in a 250 cm^3 flask volume. In addition, the orbital shaker agitated the flasks in a circular manner at 250 cycles.min^{-1}, whereas the water bath agitated in a reciprocal manner at only 100 cycles min^{-1}. Access of air to the cultures was by different mechanisms. In orbital-shaker flasks, air simply diffused through porous polypropylene plugs, whilst in the water-bath flasks, air was sparged directly into the liquid (at 1.0 min^{-1}) through sintered-glass aerators. Removal of dissolved carbon dioxide presumably took place by these mechanisms.

Thus, whilst it was not possible to monitor dissolved oxygen tension in either of the aerobic shake flask cultures, it is reasonable to speculate that oxygen-transfer in the orbital-shaker flasks is considerably more efficient than in the water-bath flasks. The low growth-rate of the water-bath cultures was probably due to early oxygen limitation, a situation which did not apply to the orbital-shaker cultures. The low cell density of water-bath cultures is also symptomatic of oxygen limitation; cell density reductions were also found in oxygen-limited continuous cultures. Growth ceased when glycerol (i.e. carbon) limitation was experienced in both types of shake flask culture. If the oxygen-transfer rate is very low, as in the water-bath cultures, then μ_{max} is considerably reduced, and the yield of cells is also reduced. In the orbital shaker cultures, oxygen transfer is maintained above critical levels and oxygen

limitation is avoided. Efficient removal of waste CO_2 allows unhindered growth at the μ_{max} of 0.83-0.90 h^{-1}, with a high bacterial yield.

This situation also applies to the fermenter batch cultures at the chosen impeller speeds of 700, 800, 900 and 1000 min^{-1}. Fig. 6.1.2 shows the growth curve of such a batch culture at an impeller speed of 700 min^{-1}. μ_{max} is 0.90 h^{-1} during the period of logarithmic growth; DOT falls to 24% saturation at the point of carbon limitation. Residual glycerol (in the culture supernatant) and the percentages of oxygen and CO_2 in the exhaust gas are also plotted. All five curves are smooth, indicating smooth progression of substrate utilisation and product (including cellular) formation. Oxygen limitation, which would be primarily indicated by reduction of DOT to values close to zero, is not evident, and growth therefore occurs at the apparent maximum value for the organism under these conditions.

In continuous culture, control of oxygen or carbon limitation is possible by adjustment not only of impeller speed, but also of dilution rate. It is immediately apparent from Fig. 6.1.5 that the critical dilution rate (D_c) of four aerobic continuous cultures (1.35 h^{-1}) is considerably greater (ca. 50%) than the maximum growth rate (μ_{max} = 0.90 h^{-1}) of corresponding batch cultures. No full explanation for this can be offered using the available evidence. However, it may be reasonable to speculate that 'forced' high growth rates at high dilution rate (>0.90 h^{-1}) may be possible if there are no substrate or product inhibitions. Control of the mechanism governing high growth rate would probably then be at the genetic level by, for example, increases in the levels of DNA-and RNA-replicating enzymes. It should be noted here that whilst wall-growth prevented washout at D = 1.40 h^{-1}, wall-growth was not apparent at lower dilution rates, especially if carbon

limitation was still apparent. Thus, maintenance of high cell density at dilution rates in excess of μ_{max} cannot be attributed to re-inoculation from wall-growth. Like μ_{max}, D_c is not affected by impeller speed within the range of 700-1000 min^{-1}.

However, impeller speed does affect the transitional dilution rate at which carbon-limited growth becomes oxygen-limited. Table 6.1.3 shows the approximate dilution rates of four continuous cultures (at 700, 800, 900 and 1000 min^{-1}) at which the transition took place. Three values are given at each impeller speed, corresponding to three different ways of measuring this transitional dilution rate; the values at zero DOT and peak cell density were derived from Fig. 6.1.5. The increased efficiency of oxygen-transfer at the higher impeller speeds is evident from the fact that DOT falls to zero at an increasing dilution rate as the impeller speed is increased, i.e. at higher impeller speeds, more oxygen is transferred into the solution, and therefore more 'substrate carbon' can be oxidised in a given amount of time. Thus, in the situation where double the substrate concentration was included in the medium and all other variables were maintained constant, DOT fell to zero at 0.30 h^{-1} instead of the normal 0.75 h^{-1}, as shown in Fig. 6.1.4.

This phenomenon of the $K_L a$ effect on the carbon-limited/ oxygen-limited transitional dilution rate in continuous culture is totally responsible for the shapes of the double-substrate limitation curves shown in Fig. 6.1.5. This series of plots of cell density against dilution rate at four different impeller speeds show the effect of oxygen transfer efficiency on bacterial yield at any given dilution rate. Up to 0.5 h^{-1}, the curves are, within the limits of experimental

reproducibility, identical. However, as DOT at the lowest impeller speed (700 min^{-1}) falls close to the critical level, cell density reaches a peak value, and slowly falls as dilution rate is increased, since oxygen is limited. Cell density falls to a level which can be sustained on the limited amount of dissolved oxygen available. As dilution rate is increased towards D_c, the classical double substrate-limited cell density curve is exhibited, as seen in Fig. 6.1.5.

At higher impeller speeds (800 min^{-1} and 900 min^{-1}), onset of oxygen-limited conditions with increasing dilution rate is delayed, since larger amounts of substrate carbon can be oxidised with the available dissolved oxygen. The effect of oxygen limitation on the cell density vs. dilution rate curve thus becomes less obtrusive as the impeller speed is increased. At an impeller speed of 1000 min^{-1}, it appears that oxygen limitation may be just avoided at virtually all dilution rates up to D_c. Peak cell density at this impeller speed is reached at $D = 1.1\text{-}1.2 \ h^{-1}$. DOT falls to zero at $D = 1.25 \ h^{-1}$, but growth-rate limitation above $D = 1.2 \ h^{-1}$ appears to prevent oxygen limitation. Thus, a classical carbon-limited cell density vs. dilution rate curve is exhibited, with peak cell density at a dilution rate close to D_c.

Oxygen-transfer efficiency (i.e. impeller speed) has no effect on D_c, which is unaltered at the extrapolated value of 1.35 h^{-1}. Since impeller speed has been shown not to affect maximum growth rate (μ_{max}) in batch cultures, it is not surprising to find that it has no effect on the critical dilution rate (D_c).

The differential effect of oxygen transfer on cell density at the four impeller speeds is also reflected in the plots of oxygen consumption and CO_2 production vs. dilution rate. Both of these plots

(Figs. 6.1.6 and 6.1.7) follow similar patterns to each other. In each case, up to a dilution rate of 0.5 h^{-1}, oxygen consumption and CO_2 production are independent of impeller speed, as is the case for cell density. At higher dilution rates, however, the effect of oxygen limitation at an impeller speed of 700 min^{-1} and later at 800 min^{-1}, becomes apparent. At 700 min^{-1}, oxygen consumption remains constant between D = 0.6 h^{-1} and 1.2 h^{-1}; at 800 min^{-1}, it is constant between D = 0.8 h^{-1} and 1.2 h^{-1}. This finding reflects the fact that under oxygen-limited conditions, the oxygen solution-rate is fixed and limiting. Thus, the constant oxygen consumption indicates that the culture is consuming all the dissolved oxygen that is made available by oxygen-transfer processes. This type of plateau in the oxygen consumption vs. dilution rate curve is less evident at an impeller speed of 900 min^{-1}, and non-existent at 1000 min^{-1}. Again, this reflects the fact that oxygen limitation only becomes evident at high dilution rate at 900 min^{-1}, and is avoided completely at 1000 min^{-1}.

Carbon dioxide production when plotted against dilution rate (Fig. 6.1.7) also produces plateaux at the two lower impeller speeds. These are not, however, as marked as those for oxygen consumption, but increase slightly at the higher dilution rates. These increases probably indicate slightly increased CO_2 production under oxygen-limited conditions; greatly increased CO_2 production of anaerobic cultures is reported in Section 6.3. Owing to heavy wall-growth at D = 1.30 h^{-1} and 1.40 h^{-1}, washout is prevented, and neither oxygen consumption nor CO_2 production falls to zero. As with cell density, graphs may be extrapolated to zero at D = 1.35 h^{-1}.

Whilst true respiration rates under each condition of $K_L a$ and dilution rate have not been calculated, a number of example 'respiration rates' have been empirically derived by simply dividing the oxygen consumption by the cell density at any point; they are therefore directly proportional to the true respiration rates. These are displayed for each impeller speed at three example dilution rates (0.50, 0.80 and 1.10 h^{-1}) in Table 6.1.4. The 'respiration rates' vary little with impeller speed at any particular dilution rate, even though both cell density and oxygen consumption may vary widely, especially at higher dilution rates. Thus, it appears that culture respiration rate may depend only on dilution rate, even under oxygen-limited conditions. Cell density may therefore be controlled by oxygen consumption (and therefore oxygen solution rate under oxygen-limited conditions); control of respiration rate to a fixed value at a fixed dilution rate would then follow.

7.1.2   Wall-growth formation

In addition to its effects on $K_L a$ values, the results of Section 6.1.3.4 show that impeller speed may have a notable effect on the extent to which wall-growth can become established in bacterial cultures. Wall-growth was formed at high dilution rates in all cultures, and consequently prevented complete washout. It was found feasible to calculate the extent of wall growth from its oxygen consumption; the calculation is itemised in Table 6.1.6. The plots of wall-growth at D = 1.40 h^{-1} vs. impeller speed are shown in Fig. 6.1.8. A smooth 'convex' curve is exhibited which is extrapolated, albeit with uncertainty, to both axes in graph b.

At low impeller speeds (i.e. below 500 min^{-1}), the extent of wall growth may reach a constant high level. This extent would probably be controlled by oxygen-limited conditions, which would permit only a limited total cellular growth. Indeed, it may be reasonable to suppose that for this reason, wall-growth would actually decrease at very low impeller speeds since the resultant low $K_L a$ values would severely limit the total growth possible. Nevertheless, this hypothesis was not tested, and wall-growth was investigated only at the four impeller speeds used in all other cultures.

Between impeller speeds of 700 min^{-1} and 1000 min^{-1}, total wall growth, as calculated in Section 6.1.3.4, fell at an increasing rate as the impeller speed was increased. The most obvious explanation for this phenomenon is one based simply on the mechanical (turbulence) effect produced by the impeller. At higher impeller speeds, turbulence is increased, and probably 'washes' increasing numbers of cells off the submerged surfaces. In addition, since the velocity of a cell must fall to zero for it to become attached to a submerged surface, higher impeller speeds (and therefore higher culture 'swirl-speeds') would result in lower numbers of cells becoming attached. The extrapolated plot implies that above an impeller speed of 1000 min^{-1}, the accelerated reduction of total wall-growth would continue, and eventually lead to zero wall-growth. The fermenter used in this study, however, had an impeller speed maximum of 1000 min^{-1}, and the hypothesis could not, therefore, be tested.

7.2   Anaerobic Cultures

The results of Section 6.2 show that the anaerobic cultivation of K. aerogenes on glycerol as sole source of carbon and energy is quite feasible.   Experiments were carried out on both batch (shake-flasks and fermenter) and continuous anaerobic cultures.   The transitions from aerobic to anaerobic (and vice versa) growth were examined instrumentally, especially by following the CO_2 output of the cultures.   Similar techniques were used to test the utilisation of the two primary glycerol metabolites in both aerobic and anaerobic cultures.

7.2.1   Batch and continuous culture

Initially, the shake-flask apparatus shown in Fig. 5.5 was designed and used to demonstrate the feasibility of anaerobic culture in the usual glycerol-salts medium, containing 5.0 mg.cm^{-3} of glycerol. Fig. 6.2.1 shows the growth curves of an aerobic and an anaerobic culture grown simultaneously in two sets of apparatus.   Samples were aseptically withdrawn at hourly intervals, and their optical densities were measured.   Logarithmic growth (at a low rate) of the aerobic culture has been discussed in Section 7.1.1.   Although μ_{max} was only 0.3 h^{-1}, the logarithmic phase started immediately, with no apparent lag phase.

The anaerobic culture, however, was found to exhibit a lag phase of 3-4 hours before any growth occurred.   This lag was probably due to the fact that an aerobically grown inoculum was introduced into an anoxic environment.   Thus, adaptation of the cells to this environment was required before any substrate utilisation and growth could take place. During this lag phase, therefore, the two enzymes of anaerobic glycerol metabolism (glycerol dehydrogenase and DHA kinase) were probably induced.

The culture cell density did not increase greatly during the growth period of only 2-3 hours, and was followed by gradual decay.

After 8 hours, the residual glycerol concentration in each culture supernatant was measured, and found to be immeasurably low. Thus, carbon limitation was the most likely cause of growth-cessation in both cultures. The yield of aerobic cells at the growth peak was 1.505 mg.cm^{-3}, and that of anaerobic cells was 0.98 mg.cm^{-3}. The lower yield of anaerobic cells reflects the less efficient pathways of anaerobic metabolism, resulting in a reduced yield of ATP per unit mass of carbon source. Complete oxidation of glycerol to carbon dioxide and water is not possible since oxidative phosphorylation cannot function in the absence of an exogenous electron acceptor. Thus, the ATP which is yielded aerobically during oxidative phosphorylation is not available in anaerobic metabolism.

It was found that anaerobically-produced organic compounds, some of which are formed in the re-oxidation of reduced NAD, may be metabolised by aeration of the static anaerobic cultures. This effect was found when anaerobic cultures which had passed their growth peak were re-aerated, and their cell densities were found to increase. The phenomenon is a good indicator of the more 'complete' nature of aerobic glycerol metabolism which yields more available energy (as ATP), and thus permits more cellular growth. Similar results were reported by Harrison and Loveless (1971 b).

Since the anaerobic production of organic acids obviously results in a reduction of the culture pH in shake-flask cultures, it was thought desirable to control the pH to 6.5. Thus, cultures were carried out anaerobically in the fermenter, as described in Section 6.2.2.

A typical anaerobic growth curve with residual glycerol analysis is shown in Fig. 6.2.2, and exhibits linear, not logarithmic, growth. Again, the use of aerobically-grown inocula resulted in lag-phases of ca. 4 hours.

Whilst a semi-logarithmic plot of the growth curve did not yield a straight line, the maximum gradient showed μ_{max} to be ca. 0.6 h^{-1}. This maximum growth rate is lower than that of aerobic cultures, and again, is symptomatic of the lower energy-yield of anaerobic metabolism. This is also reflected in the peak cell density of only about 0.52 mg.cm^{-3}. This value of cell density is much more representative of typical anaerobic cultures than that obtained in the shake-flask apparatus, since the inoculum concentration used in the fermenter culture was only about $\frac{1}{6}$ of that used in the shake-flask. Thus, five-fold growth was found in fermenter cultures, whereas cell density increased by only about 70% in shake-flask cultures.

Residual (supernatant) glycerol concentration fell in a fashion which was complementary to the increase of cell density. Nevertheless, it did not fall completely to zero, even at the growth-peak. This may be due to interference in the glycerol determination by other organic species in the culture supernatant. Since the determination effectively involves measurement of formaldehyde concentration, it is reasonable to suppose that other aldehydes (and alcohols) may interfere, giving apparent values which are above zero.

Continuous anaerobic culture of K. aerogenes on 5.0 mg.cm^{-3} glycerol was found to be rather difficult under the conditions used. Since silicone rubber tubing, which is known to be pervious to oxygen, was used for medium addition, it is possible that a small quantity of dissolved oxygen was introduced into the fermenter in the medium.

Wall-growth, resulting in culture re-inoculation, was heavy in the continuous culture, especially at the higher dilution rates. Thus, accurate determination of D_c was not possible, but was estimated from the available evidence to be about 0.7 h^{-1}.

Again, apparent residual glycerol concentrations were somewhat above zero at all dilution rates (Table 6.2.1). Since glycerol was most likely to be growth-limiting, interference in the glycerol determination procedure by anaerobic metabolites is the most obvious explanation of these results. Incomplete oxidation of glycerol, which results in the production of these organic metabolites, also results in inefficient energy conversion. Thus, cell densities are low compared with those of equivalent aerobic cultures at the same dilution rates; at D = 0.50 h^{-1}, the anaerobic culture cell density is only about 15% of the equivalent aerobic value.

Carbon dioxide production by the culture was monitored at two dilution rates, 0.50 and 0.70 h^{-1}. The production of CO_2 was high, especially when the relatively low culture cell density is taken into account. It was found feasible to follow the onset of anaerobic metabolism in the intermittently aerated cultures by measurement of the CO_2 production: cell density ratio, which is considerably greater in anaerobic than in aerobic cultures. This ratio appeared also to be directly related to dilution rate in continuous anaerobic cultures at 0.50 and 0.70 h^{-1}. This may indicate glycerol limitation, since the production of CO_2 by the culture appeared to depend on the rate of provision of glycerol.

7.2.2   Use of the equipment to study anaerobic/aerobic transitions
          and substrate utilisation

The full fermentation and gas-analysis equipment was used to
determine the effects of converting aerobic cultures to the anoxic
condition and anaerobic cultures to the aerated condition.   Constant-
monitoring of CO_2 production was especially useful for this work, and
was also used to determine the substrate suitability of DHA and G-3-P
for aerobic and anaerobic cultures.

The details of the anoxic/aerated conversions, with respect to
CO_2 production, oxygen consumption, and culture dissolved oxygen tension,
are described in Section 6.2.4.   Fig. 6.2.3 shows these three gas
traces following an aerobic→anoxic conversion;   Fig. 6.2.4 shows them
following an anaerobic→aerated conversion.   It is immediately obvious
that oxygen consumption and DOT are relatively uninformative in both
conversions, but that CO_2 production does yield a considerable amount
of information.

Adaptation to steady-state anaerobic growth by an aerobic
culture growing under the particular conditions of dilution rate and
gas flow rates quoted in Section 6.2.4 takes approximately 8 hours.
There is a net increase of CO_2 production by the culture over this period,
although cell density falls considerably.   Between 0.5 hours and 7 hours
from the conversion, the rise of CO_2 production is gradual.   A peak
is reached at 7 hours, indicating a small degree of 'overshoot' in the
adaptation mechanism.   Nevertheless, at no time did CO_2 production fall
to zero, and therefore metabolism of glycerol was probably never
completely prevented by this conversion.   A likely combination of small
residual concentrations of the anaerobic enzymes of glycerol metabolism
in the aerobic cells and rapid induction or activation of these enzymes

in the anoxic conditions was probably responsible for continuation of some CO_2 production.

Similarly, when an anaerobic culture was aerated, CO_2 production fell rapidly at first, but not to zero. It then gradually increased to reach a new steady-state, at a lower level than in the anaerobic culture after less than 4 hours. During this period, cell density increased considerably, to reach the expected aerobic level. The gradual rise of CO_2 production was not completely smooth, but included several small peaks and troughs; these may be indicative of control mechanisms operating during the transition from anaerobic to aerobic growth, but no real evidence to substantiate this is available.

Oxygen appeared to be consumed as soon as the anaerobic→aerobic conversion was made, since neither DOT nor % O_2 in the exhaust gas rose to their maximum values. Thus oxidative phosphorylation operated virtually immediately following the conversion; the enzymes involved in respiration, especially those of the cytochrome system were presumably constitutive in the anaerobic cells. Complete substrate oxidation was therefore possible as soon as oxygen was available; this may have aided the rapid transition time of under 4 hours, compared to the 8 hours required to establish an anaerobic culture when the opposite conversion was made.

Harrison and Loveless (1971 b) also found rapid adaptation to aerobic metabolism of an anaerobic culture when it was reaerated. Gray et al. (1966 a) reported that anaerobically-grown E. coli retained many of the aerobic characteristics, including membrane-bound enzyme systems associated with the use of molecular oxygen; if this retention is generally applicable to facultative anaerobes, it may adequately explain these observations.

The effect on culture CO_2 production (and DOT in aerobic cultures) of adding dihydroxyacetone and glycerol-3-phosphate to both aerobic and anaerobic cultures is described in detail in Section 6.2.5. Fig. 6.2.5 shows the CO_2 production of two starved anaerobic cultures before and after the addition of equimolar quantities of DHA and G-3-P Fig. 6.2.6 additionally shows the effect on DOT of these additions to two starved aerobic cultures.

The results shown in these two figures indicate that K. aerogenes which has been anaerobically cultured on glycerol is capable of transporting DHA into the cell and metabolising it immediately. G-3-P, however, is not readily metabolised by an identical culture. The total amount of CO_2 produced by the metabolism of DHA was approximately 15 times that produced by the metabolism of G-3-P. Fig. 3.1 shows DHA to be the anaerobic intermediate of glycerol metabolism, whereas G-3-P is the aerobic intermediate. Thus, high levels of DHA kinase in the anaerobic cells allow rapid and complete metabolism of DHA by anaerobic cultures. However, G-3-P dehydrogenase levels in anaerobic cultures are low, and G-3-P is probably therefore unsuitable as a substrate for these cultures. These results are thus additional evidence for the pathways of glycerol metabolism shown in Fig. 3.1.

Both DHA and G-3-P can be metabolised by starved aerobic cultures, since the addition of either results in immediate increases of culture CO_2 production and decreases of DOT. The metabolism of G-3-P, indicated by simple peaks of CO_2 production and DOT, demonstrates that this phosphorylated species may be transported into the cell. However, the CO_2 production peak caused by the metabolism of DHA is not simple, but appears to consist of two peaks with a relatively static area dividing the two.(The DOT response is complementary). The reasons for

this 'double-response' to DHA by the starved aerobic culture are not obvious. Theoretically, one might expect DHA to be metabolised only to a very limited extent, since it is the anaerobic intermediate. However, it is known that glycerol kinase, which is present in large amounts in these aerobic cells, may act as a DHA kinase (Lin 1976; Hayashi and Lin, 1967). Thus, DHA is probably phosphorylated to DHAP by glycerol kinase, and metabolism of the compound is therefore possible.

7.2.3   Cytochromes in anaerobic and aerobic cell extracts

The identification and assay of the cytochromes in anaerobic and aerobic cell extracts is reported in Section 6.5.1. The room temperature cytochrome spectra of both cell extracts, shown in Fig. 6.5.1, appear rather similar. The most prominent absorption peak in both spectra is the α-peak of cytochrome b at 560 nm. Quantitative determination of cytochrome b, however, shows the concentration of this cytochrome in aerobic cells to be ca. 1.63 times that in the anaerobic cells. Since the cytochromes are the respiratory enzymes which catalyse the reactions of oxidative phosphorylation, one might expect the ratio of aerobic cytochrome concentration to anaerobic cytochrome concentration to be considerably greater than this. The high concentration of presumably 'redundant' cytochrome may be partly responsible for the ability of anaerobic cells to consume oxygen as soon as the culture is aerated, as discussed earlier; Gray et al. (1966 a) found membrane-bound enzymes associated with the use of molecular oxygen in anaerobically-grown E. coli.

There is also more detail in the anaerobic cytochrome spectrum, with a definite peak, representative of cytochrome d, at 625 nm. The slight peak at 600 nm, representative of cytochrome a_1, may be an

instrumental artefact, since its resolution is not improved in the low temperature spectrum. The discovery of extra cytochromes in the anaerobic cell extract which are not in the aerobic extract is surprising. It is possible that the lower sensitivity of the aerobic cytochrome spectrum may have resulted in their failure to appear, but. that they are present in both cell extracts. Nevertheless, the reason for an apparently measurable concentration of cytochrome d in the anaerobic cell extract is not immediately obvious. The presence of an alternative electron acceptor to oxygen in the culture might explain this phenomenon, but this is unlikely to be the case. The very small quantities of dissolved oxygen which were inevitably introduced into the continuous culture may have been responsible for induction of these cytochromes.

The low temperature spectrum of the anaerobic cell extract splits the cytochrome b α-peak to reveal the presence of cytochromes b_{556} and b_{559}. This improved resolution was not shown in the low temperature spectrum of the aerobic cell extract, and this may be indicative of only one cytochrome b in this extract. Again, the reasons for this are not apparent, but the overall concentration of cytochrome b in the aerobic cell extract was greater. The ratio of glycerol kinase activities in aerobic:anaerobic cell extracts grown under identical conditions, however, is ten times that of the ratio of cytochrome b concentrations. Thus, respiratory enzymes are probably not repressed or deactivated as dramatically as aerobic substrate-linked enzymes in the anaerobic cells.

7.3   'Intermittent Aeration' Cultures

Intermittently aerated (IA) cultures were designed to reproduce, on a laboratory equipment scale, the effects of various aeration and mixing conditions which might be experienced by cultures in large fermenters.   Measurements of cell density, oxygen consumption, CO_2 production and dissolved oxygen tension were made at each IA condition, as reported in Section 6.3.   In addition, samples of K. aerogenes cells were removed, and the activities of two aerobic and two anaerobic enzymes of glycerol metabolism were assayed at each IA condition, as reported in Section 6.4.   Thus, a degree of insight into the mode of metabolism of the 5.0 $mg.cm^{-3}$ of glycerol which was provided in the medium of all IA continuous cultures was available.

The operation and nomenclature of IA cultures is explained in Section 6.3.   Four IA cultures were carried out at impeller speed/ dilution rate values of 1) 900/0.30, 2) 800/0.50, 3) 700/0.55, 4) 700/0.70. The first three cultures were carried out over the full (5-0→0-5) aeration-deaeration range;   the fourth was carried out over the 5-0→3-2 range.

7.3.1   Cell density and carbon dioxide production of IA cultures

Measurements of cell density and CO_2 production were made at each of the 21 'steady-state' IA conditions. CO_2 production divided by cell density was calculated, to give an indication of CO_2 production per unit mass of cells.   Thus, the plots of cell density and CO_2 production/cell density vs. aeration condition, shown in Figs. 6.3.2, 6.3.4, 6.3.5 and 6.3.6 were made available.

The trend in all of these plots is for a sudden reduction of cell density at a single step-reduction of aeration time per cycle.

A slight decrease of cell density with decreasing aeration condition following this sudden reduction is also noticeable. CO_2 production per unit mass of cells increases slightly when the sudden cell density reduction takes place, but increases much more markedly during the following smaller reduction of cell density. Cell density prior to the sudden reduction tends to be fairly constant. These general trends are not so noticeable at the low $(K_L a)_o$ conditions of IA culture 4. Thus, all four cultures appear to be able to withstand one minute step-reductions of aeration in the five minute cycle up to a definite limit. When this limit is passed, drastic reduction of cell density is accompanied by a slight increase of CO_2 production per unit mass of cells. Further step-reductions of aeration condition lead to further, though less dramatic, reductions of cell density, and greater increases of CO_2 production.

Reduction of cell density in these cultures indicates general transition from aerobic to anaerobic metabolism; increase of CO_2 production is a further indicator. It therefore appears that the major transition between these two metabolic states occurs at a specific step-reduction of aeration condition. Further reliance on anaerobic metabolism then follows as the aeration condition is further reduced. Table 6.3.5 gives the time-averaged oxygen-transfer coefficient, $(K_L a)_o$, values for all 24 possible IA culture conditions. As explained in Section 6.3.6, the approximate value of $(K_L a)_o$ at which the aerobic/anaerobic transition took place was between 1.59 min^{-1} and 1.80 min^{-1} in all cultures. The transitional $(K_L a)_o$ value of 1.7 $\pm$ 0.1 min^{-1} is therefore given. The fact that the major aerobic/anaerobic transition took place in all four cultures when the $(K_L a)_o$ fell below this value indicates that a degree of continuity exists between all

24 possible IA culture conditions.

The four cell density vs. aeration condition curves are simultaneously plotted in Fig. 7.3.1. From this composite graph it is apparent that the major aerobic/anaerobic transition takes place at the highest aeration condition with the lowest $(K_L a)_0$ culture (IA culture 4), and all subsequent transitions in the other three cultures take place in order of $(K_L a)_0$. Thus, the transition for IA culture 1 takes place at the lowest aeration condition. This result .is not unexpected, but does serve to demonstrate the relationship between the four cultures. Furthermore, the fundamental importance of oxygen-transfer coefficient in dictating the major aerobic/anaerobic transition is well demonstrated.

The CO_2 production of each unit mass of cells can be seen, from the four IA culture plots, to be a good indicator of the culture's increasing anaerobic utilisation of glycerol after the major aerobic/anaerobic transition has taken place. The ultimate CO_2 production in the fully anaerobic cultures appears to be dependent on the dilution rate of the culture; this finding in continuous anaerobic culture is discussed in Section 7.2.1.

A composite graph of the four CO_2 production vs. aeration condition curves is shown in Fig. 7.3.2.

The order in which the CO_2 production plots rise, with respect to decreasing aeration condition, is the same as that for the reduction of cell density. Thus, CO_2 production of the lowest $(K_L a)_0$ culture (IA culture 4) rises first, followed by the expected order of the other three cultures. This trend further indicates the importance of relative oxygen-transfer efficiencies in dictating the order in which the four cultures undergo the transition from aerobic to anaerobic metabolism.
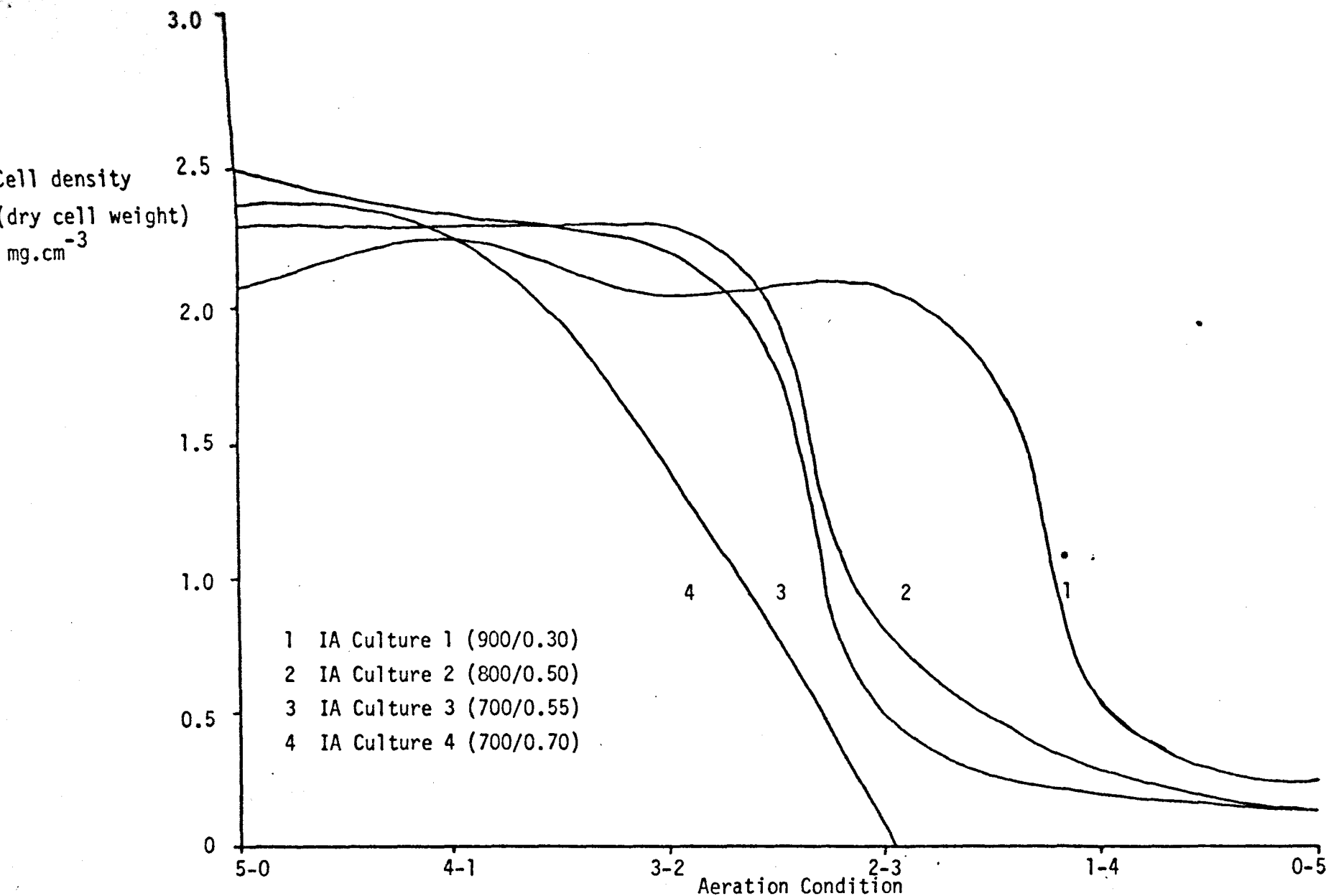
Figure 7.3.1   Cell Density vs. Aeration Condition Plots for all Four Intermittent Aeration Cultures
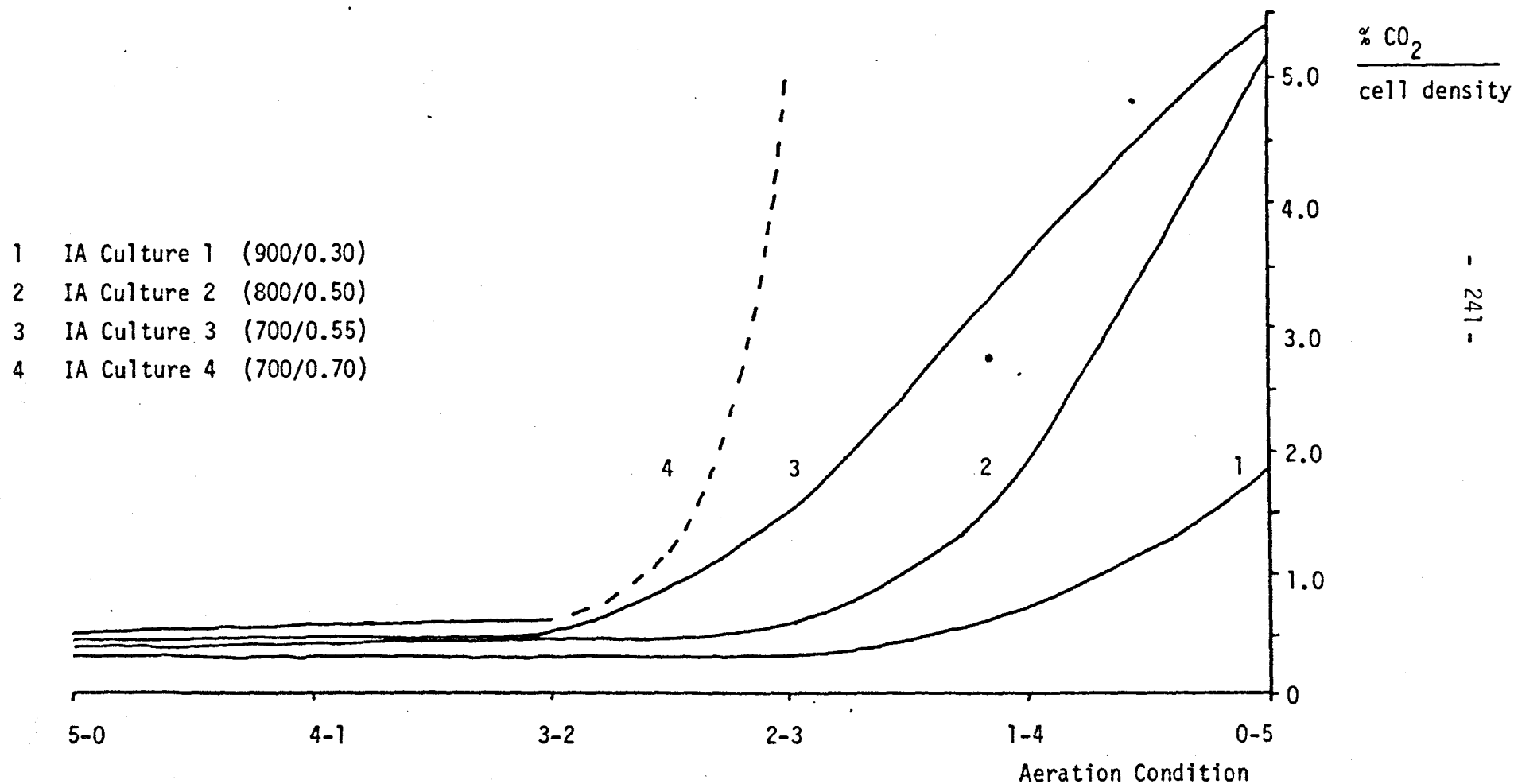
Figure 7.3.2 CO_2 Production vs. Aeration Condition Plots for all Four Intermittent Aeration Cultures

1   IA Culture 1   (900/0.30)
2   IA Culture 2   (800/0.50)
3   IA Culture 3   (700/0.55)
4   IA Culture 4   (700/0.70)

CO_2 production (per unit mass of cells) appears to be a good indicator of the 'degree' of anaerobic metabolism, providing that the culture dilution rate is taken into account. Comparison of the composite graph (Fig. 7.3.2) with the plots of dihydroxyacetone kinase activities vs. aeration condition for the four cultures (Fig. 6.4.6) shows the two figures to follow very similar patterns. Thus, the trends in 'degree' of anaerobic metabolism (as measured by CO_2 production) appear to correlate quite well with the trends in the activity of a substrate-related anaerobic enzyme (DHA kinase). This correlation does not work as well for glycerol dehydrogenase, but does serve to demonstrate the relationship between anaerobic metabolism and the activity of an anaerobic enzyme.

The combination of cell density and CO_2 production measurements for any particular IA culture condition (at the steady-state) is thus very useful in the determination of 'degree' of anaerobic metabolism. The trends of these values as aeration time per cycle is reduced therefore indicates the 'onset' of anaerobic metabolism. Where intermediate 'degrees' of anaerobic metabolism are apparent, the question of whether all cells are metabolising glycerol partially aerobically and partially anaerobically, or whether some cells are metabolising glycerol fully aerobically and the remainder fully anaerobically, arises. This question cannot be answered from the available evidence; the truth may even be a combination of these two extremes.

7.3.2   Use of dissolved oxygen tension measurement in IA cultures
The values of dissolved oxygen tension (DOT) given in the results of the IA cultures in Section 6.3 were normally in the form of a DOT range, since the four conditions of intermittent aeration in

each culture resulted in oscillation of DOT. However, unlike

culture CO_2 production, it was not possible to determine a mean value

of DOT from the oscillation. Nevertheless, the response of the DOT

trace to a change of aeration condition of a culture proved to be useful

for following the progress from one steady-state to the next. This is

especially true where a major aerobic/anaerobic transition took place,

since both the range of the oscillation and the shape of individual

DOT peaks were found to vary with time. Indeed, the shapes of the

final DOT peaks under steady-state conditions were found to depend

upon particular culture conditions.

Fig. 6.3.1 shows the response of both DOT and CO_2 production

ranges during the 40 hours following the conversion of a 900/0.30/2-3

culture to the 1-4 aeration condition. (Fig. 6.3.2 shows that the

major aerobic/anaerobic transition took place following this conversion).

The height of the DOT peaks is shown, their corresponding minima being

zero. This trend of DOT maximum with time indicates the adaptation of

the culture to the new aeration condition. Adaptation appears to take

place in two stages followed by a period of over 20 hours, during which

the DOT peak maxima settled to the steady-state value of 77% saturation.

The range of total CO_2 production by the culture is often additionally

useful for following the progress of adaptation. In the particular

case shown in Fig. 6.3.1, CO_2 production range reduced considerably as

the adaptation proceeded, and reached a steady-state mean value of 0.40%

of the exhaust gas after 40 hours. The use of these two traces in

conjunction with cell density measurements was found to be very informative

in following adaptations of this type, and recognising the stability

of a new steady-state.

Investigation of the change of DOT peak shape with time during an adaptation process was also made. A tracing of the actual DOT trace in the 2.5 hours immediately following the conversion of a 800/0.50/3-2 culture to the 2-3 aeration condition is shown in Fig. 6.3.3. DOT peak maxima (where the minima are zero) from 12 to 37 hours from the conversion are also shown. As soon as the conversion was made, the 'double-peak' response was lost, to be replaced after 2.5 hours by a single broad peak. This peak increased between 14 and 27 hours from the conversion, and reached a final steady-state range of 0-47% saturation after about 36 hours. Thus, not only was the initial change of DOT peak shape immediately after conversion followed, but also the subsequent gradual increase of DOT peak height during the major aerobic/anaerobic transition was recorded.

The significance of the height and shape of individual DOT peaks recorded in IA cultures now becomes apparent. The height of individual DOT peaks probably indicates the metabolic requirement for the culture to take up oxygen during the aeration period. Thus, high DOT peaks indicate that the culture is consuming a relatively small proportion of the dissolved oxygen that is available to it. Conversely, small DOT peaks indicate the culture's high requirement for oxygen. The increase of DOT peak height during a major aerobic/anaerobic transition therefore demonstrates the culture's diminishing requirement for oxygen; induction of the enzymes of anaerobic glycerol metabolism reduces the culture's dependency on oxygen as the final electron acceptor. It is therefore quite feasible to monitor the time-dependent induction of anaerobic metabolism in cultures of this type by recording the DOT response throughout the transition.

Examination of the DOT traces in all 14 intermittently aerated
conditions used in the four IA cultures revealed that they could be
categorised into four basic DOT response-types.   When a steady-state
was reached at any particular IA culture condition, the DOT response
was found to be completely reproducible from one five-minute cycle to·the
next.   A specific response-type was normally found to occur in either
a predominantly aerobic or a predominantly anaerobic culture, although
one type, the single broad peak, was typical of the 'intermediate
metabolism' cultures.   Although the pattern of DOT response is classifiable
in this way, its magnitude is not, and therefore individual steady-state
peak heights depend on the specific culture conditions, as explained
earlier.

The four basic DOT response-types are shown in Fig. 7.3.3;
they were obtained by increasing the chart speed (and, in some cases,
reducing the DOT full-scale range) in order to magnify the response on
both the time and DOT axes.   Fig. 7.3.3 was obtained by tracing typical
examples of the responses from the chart-paper when IA culture conditions
were at a steady-state.   It is possible to put forward an explanation
for each of the response-types based on the manner in which the cultures
may be metabolising glycerol throughout the five-minute cycle time:-

Type 1:- Double-peak response.
This response-type is found under steady-state conditions of
the following cultures.

$700/0.70/4-1$ (Cell density $= 2.26$ mg.cm^{-3})

$700/0.55/3-2$ (Cell density $= 2.20$ mg.cm^{-3})·

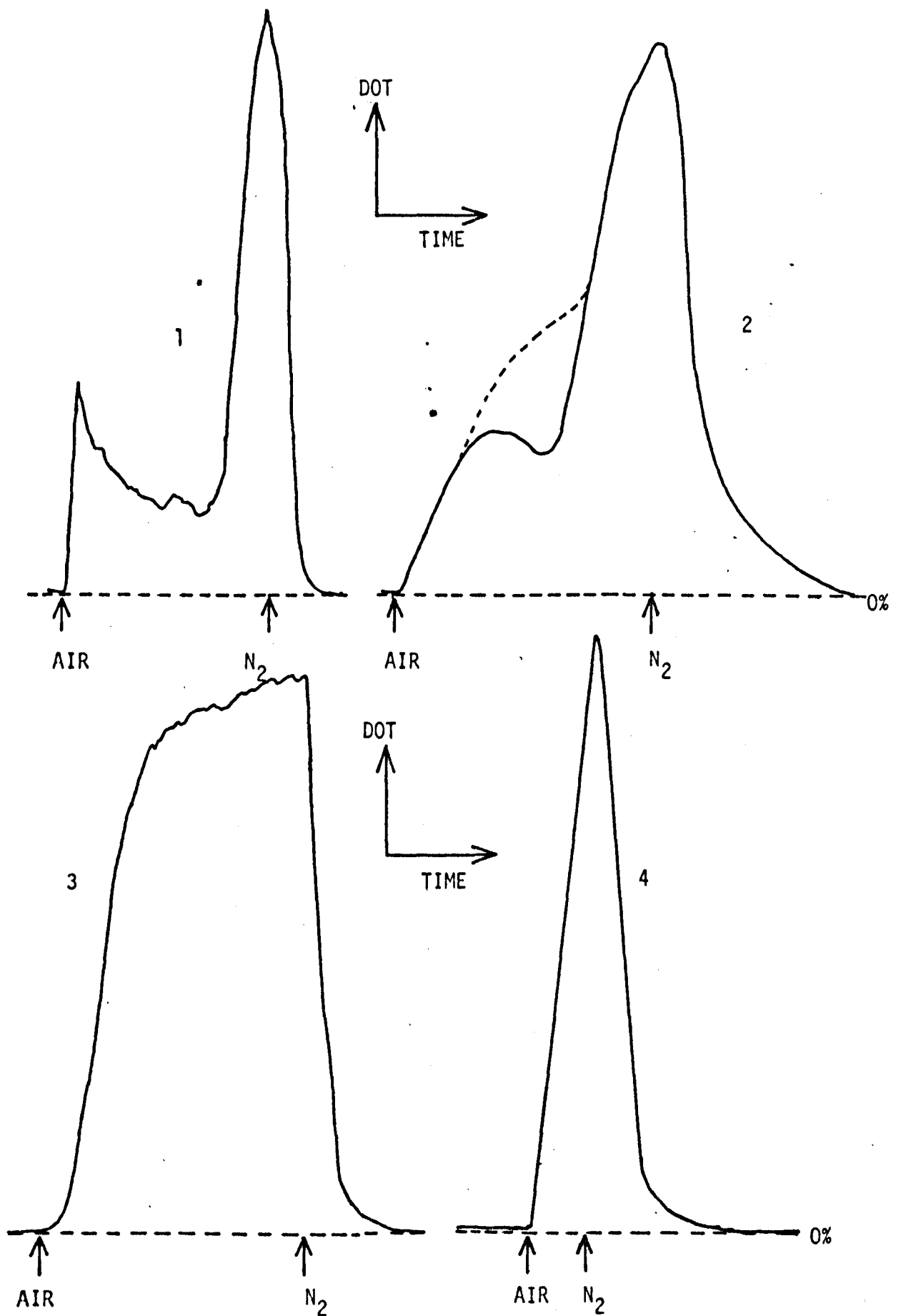$800/0.50/3-2$ (Cell density $= 2.30$ mg.cm^{-3})

Figure 7.3.3   The Four Basic Types of Dissolved Oxygen Tension Response
in all IA Cultures

These cell densities are all indicative of aerobic cultures.

When aeration commences, DOT rises immediately, but the culture consumes dissolved oxygen at an increasing rate during the first few seconds of aeration. Thus, when the rate of oxygen consumption balances the rate of its supply, a sharp DOT peak is formed. DOT then reduces as oxygen demand continues to increase; the bacteria may be oxidising the metabolic intermediates (which were accumulated when no oxygen was available) during·this period, but also metabolising an increasing amount of the excess glycerol. A high rate of oxygen-uptake (and glycerol metabolism) continues, forming a trough in the DOT response.

As the culture becomes glycerol-limited, DOT again increases as relatively little dissolved oxygen is required; DOT may then form a small second peak before deaeration commences (e.g. at 700/0.70/4-1), indicating that metabolism of the continuously-supplied glycerol takes place at low DOT. Alternatively (at the two other culture conditions) the DOT peak may still be increasing when it is abbreviated by deaeration before reaching a steady-state value. (A small (ca. 10 seconds) delay in the introduction of nitrogen into the fermenter was found to result in a considerably higher second DOT peak in these two cultures). As the oxygen-free nitrogen purges the culture, DOT falls to zero until aeration re-commences,and the cycle is repeated.

This type of response therefore indicates that the culture receives sufficient oxygen during the aeration period to maintain aerobic metabolism, with overall glycerol limitation. The high cell densities of these cultures are also indicative of efficient aerobic metabolism.

Type 2:- Single peak response, with an inflection or shoulder.

This response-type is found under steady-state conditions of the following cultures.

800/0.50/4-1   (Cell density = 2.30 $mg.cm^{-3}$)

900/0.30/2-3   (Cell density = 2.08 $mg.cm^{-3}$)

900/0.30/3-2   (Cell density = 2.04 $mg.cm^{-3}$)

These cell densities are again all indicative of aerobic cultures. Indeed, the response-type is very similar to the double-peak, although the wide level portion between the two DOT peaks is absent. Moreover, the second peak is so much larger than the first that the first is probably better classified as an inflection (in the 900/0.30/3-2 culture condition, the inflection is replaced by a shoulder, as shown in Fig. 7.3.3). The DOT response range (i.e. peak heights) is always much higher than the double-peak in this response-type, since oxygen-transfer conditions are generally more efficient.

When aeration commences, a rapid DOT rise results, but increasing oxygen consumption by the culture results in the DOT inflection (or shoulder). Rapid oxidation of metabolic intermediates proceeds for a relatively short period, and the culture then metabolises excess glycerol. DOT then increases towards the fully aerated level when the excess glycerol is metabolised, and forms a relatively broad (and high) peak whilst metabolism of the glycerol in the added medium proceeds. DOT is once more reduced to zero when deaeration commences, and the cycle is repeated.

This response-type indicates that the culture is probably receiving sufficient oxygen throughout the cycle to maintain fully aerobic metabolism and glycerol limitation; this is also indicated by the high cell densities.

<u>Type 3</u>:- Single broad peak.

This response-type is found under steady-state conditions of the following cultures.

700/0.70/3-2   (Cell density = 1.41 mg.cm^{-3})

700/0.55/2-3   (Cell density = 0.48 mg.cm^{-3})

800/0.50/2-3   (Cell density = 0.83 mg.cm^{-3})

These cell densities are indicative of the 'intermediate' areas between fully aerobic and fully anaerobic metabolism.   This response-type is thus found in predominantly anaerobic cultures which maintain a cell density above the expected fully anaerobic level.   The trace is similar to that of type 2, although there is only one definite peak.

When aeration commences, DOT rises immediately and rapidly. However, dissolved oxygen is not removed from solution as quickly as in the two previous response-types, and no first peak or inflection is therefore formed.   Some aerobic metabolism of metabolic intermediates and glycerol does, however, occur, since a DOT plateau is formed at high DOT.   Gradual reduction in the concentration of aerobic substrates may be indicated by the slight increase of DOT which is always found at the broad peak, since decreasing amounts of oxygen are required.   Not all excess glycerol is metabolised aerobically, and DOT is reduced by deaeration.   The cycle is then repeated.

Both aerobic and anaerobic metabolism of glycerol appears to take place in these cultures.   Oxygen limitation is indicated by low cell densities, although they are maintained above normal anaerobic levels.

<u>Type 4</u>:- Single sharp peak.

This response-type is found under steady-state conditions
of the following cultures.

700/0.55/1-4   (Cell density = 0.20 mg.cm^{-3})

800/0.50/1-4   (Cell density = 0.28 mg.cm^{-3})

900/0.30/1-4   (Cell density = 0.53 mg.cm^{-3})

(DOT peak heights are (respectively) 12, 44 and 77% saturation).
These cell densities, with one exception, are indicative of anaerobic
metabolism.   The higher 900/0.30/1-4 cell density probably indicates
efficient oxygen-transfer during the one-minute aeration time per cycle.

Aeration simply results in the production of a sharp DOT peak,
the height of which depends on the efficiency of oxygen-transfer during
the single minute of aeration.   This peak type is thus observed in
essentially anaerobic cultures which are aerated for one minute of the
total cycle time.   It seems likely, however, that some oxygen may be
consumed by the cultures, and a degree of aerobic metabolism may therefore
still exist, i.e. cell density values are greater than those of the fully
anaerobic cultures, and CO_2 production is smaller.   Thus, it is likely
that oxidative phosphorylation may take place during the short period
that oxygen is available.

Two other DOT responses, at 700/0.55/4-1 and 900/0.30/4-1, were
found.   In the former case, DOT remained at zero, although small (ca.
2% satn.) transient increases on re-aeration were noticed; aerobic
metabolism was apparent.   In the latter case, DOT remained at a baseline
of 75% saturation, although the single minute of deaeration resulted in
a single sharp (inverted) DOT peak down to 3% satn.

Thus, each of the four basic types of DOT response can be
theoretically explained by the way in which the glycerol carbon-source

is possibly being metabolised; this is fundamentally controlled by aeration of the fermenter. It is not surprising, therefore, that any individual DOT response-type can be further classified as being typical of 'essentially' aerobic or 'essentially' anaerobic metabolism. The major aerobic/anaerobic transition which occurs on the conversion from one specific IA culture condition to the next often occurs with a specific change of DOT response-type. For example, a double-peak response is always converted to a single broad peak response, by a single step-reduction of aeration condition, as is shown in Fig. 6.3.3.

The reproduction of identical DOT responses in steady-state IA cultures, and the possible explanation for these in terms of the progress of glycerol metabolism throughout the five-minute cycle time, suggests that all the individual bacterial cells in the culture are behaving in a similar manner at any point in time. If this were not the case, one might expect the random behaviour of the microbial population to lead to a loss of fine-structure of the DOT response-types, and might even lead to simple undamped oscillation of DOT when the culture is intermittently aerated. Thus, one effect of intermittent aeration in these cultures may be to synchronise the metabolism of the cells; the fundamental importance of oxygen-availability to the cells and the regular manner in which oxygen is made available may lead to synchrony in the uptake of oxygen by the cells, and thus synchrony in their substrate metabolism.

The imposed variations in dissolved oxygen availability takes place over a five-minute cycle time. The doubling time (t_d) of the four IA cultures ranges from 2.3 hours (138 min.) at D = 0.30 h^{-1} to 0.99 hours (59.4 min.) at D = 0.70 h^{-1}. The large difference between

cycle time and doubling time of the culture probably leads to asynchronous cell division since 12 to 28 cycles lapse between successive cell divisions. Nevertheless, when cells divide, they may do so at a specific point in the five-minute cycle; if this were the case, between 12 and 28 specific points of cell division would exist in each culture doubling time. Thus, depending on the dilution rate of the particular IA culture, $\frac{1}{12}$ th to $\frac{1}{28}$ th of the total bacterial population would divide at a specific point in each five-minute cycle. The results given do not, however, provide sufficient evidence to substantiate this hypothesis.

If the imposition of intermittent aeration is completely valid for modelling the effects of cyclic aeration in large vessels, then one would expect that microorganisms circulating throughout the vessel might experience DOT variations which were not simply oscillating, but which would follow specific DOT profiles. On the other hand, specific DOT responses may be an artefact of this model caused by the rigidity of the aeration-deaeration cycle; the more random DOT variations experienced by microorganisms in the real situation may lead to their experiencing relatively simple DOT fluctuations. In either case, the inhomogeneity of the culture in the large vessel would prevent the metabolic synchrony which the model system seems to exhibit, although synchrony within small areas of the large vessel might exist temporarily.

7.4   The Activities of the Enzymes of Glycerol Metabolism

The activities of all four enzymes involved in the first two stages of aerobic and anaerobic glycerol metabolism in K. aerogenes were determined in all 21 cell samples from the four intermittently aerated cultures. These results are given in Section 6.4.4. Glycerol kinase (the primary aerobic enzyme) specific activities are listed in Table 6.4.7, and plotted against aeration condition for each culture in Fig. 6.4.3. Similarly, Table 6.4.8 and Fig. 6.4.4 display glycerol-3--phosphate dehydrogenase (the secondary aerobic enzyme) specific activities; Table 6.4.9 and Fig. 6.4.5 display glycerol dehydrogenase (the primary anaerobic enzyme) specific activities; Table 6.4.10 and Fig. 6.4.6 display dihydroxyacetone kinase (the secondary anaerobic enzyme) specific activities. The reactions which are catalysed by these four enzymes are diagrammatically displayed in Fig. 3.1.

The general trends are for the two aerobic enzymes to decrease in activity and the two anaerobic enzymes to increase in activity as the aeration condition is progressively reduced. It is apparent, however, that the levels of the aerobic enzymes do not fall to zero in the anaerobic cultures whereas the levels of the anaerobic enzymes in the fully aerobic cultures are generally zero. This is further evidence of the constitutive nature of the aerobic enzymes in cells which have been cultured fully anaerobically, and partially explains the observed rapid transition from anaerobic to aerobic metabolism when anaerobic cultures are aerated, as explained in Section 7.2.2. The converse transition from aerobic to anaerobic metabolism is seen to take place less readily.

The particularly efficient oxygen-transfer conditions of IA culture 1 appear to give rise to high glycerol kinase activities in

samples cultured at the first four aeration conditions. Furthermore, these activities reduce linearly with decreasing aeration condition whilst predominantly aerobic (glycerol-limited) metabolism is still evident. The linear extension of this activity reduction is shown in Fig. 6.4.3, and can be seen to pass very nearly through zero at the fully anoxic (0-5) condition. This may be good evidence of dissolved oxygen sensitive production of glycerol kinase as the 'average' amount of oxygen dissolved in the culture [and $(K_L a)_o$ value] reduces linearly with reducing aeration condition. i.e. high DOT leads to high glycerol kinase activity.

The glycerol kinase activities of the samples of the other cultures are all considerably lower, and this may be indicative of their poorer oxygen-transfer conditions which permit onset of oxygen limitation at relatively high aeration conditions. An interesting example is the 700/0.55/4-1 sample, which shows a very low glycerol kinase activity. Examination of this sample's DOT response shows that it exhibited an untypical steady-state zero DOT, with small transient peaks on reaeration. This again is good evidence of DOT sensitivity of glycerol kinase synthesis in these cultures. However, it is not possible to say whether it is the expression or activation of the enzyme, or both, that is increased with increasing DOT.

G-3-P dehydrogenase appears to exhibit similar DOT sensitivity to glycerol kinase. Relatively high levels of this enzyme were found at the higher aeration conditions of IA cultures 1 and 2. Reduction of G-3-P dehydrogenase activity with reducing aeration condition appears to take place linearly; linear regression analysis of the activities of G-3-P dehydrogenase in these two cultures are shown as broken lines

in Fig. 6.4.4. The line for IA culture 1 can be seen to pass very close to both the fully aerated (5-0) activity point and zero activity under fully anoxic (0-5) conditions. The line for IA culture 2 does not lie quite as close to the theoretical line. Thus, apparent sensitivity to DOT may be exhibited by G-3-P dehydrogenase in a similar manner to that exhibited by glycerol kinase synthesis.

Generally, low activities of both of these enzymes were found in the oxygen-limited and anaerobic cells. However, slight increases of glycerol kinase activities in the fully anaerobic (0-5) cells compared to the cells grown under corresponding 1-4 aeration conditions can be seen. Studies of DHA kinase, reported in Section 6.4.3.3, show that this anaerobic enzyme may act as a glycerol kinase, and glycerol kinase may act as a DHA kinase. Thus, apparently increased levels of glycerol kinase in the fully anaerobic cells are likely to be due to the higher levels of DHA kinase in these cells acting as glycerol kinase, and so giving positive assay results. The relationship between glycerol and DHA as potential substrates for DHA kinase, including the action of glycerol as a competitive inhibitor of DHA kinase, is discussed in Section 6.4.3.3.

Whilst the trend of the aerobic enzyme activities was normally to reduce with reducing aeration condition, this trend did not apply to the poorly aerated IA culture 4 (700/0.70). The low activities of glycerol kinase and G-3-P dehydrogenase at the fully aerated condition of this culture reflect the steady-state zero culture DOT. However, instead of reducing when the aeration condition was converted to 4-1 operation, the activities of both aerobic enzymes increased. This was accompanied by the formation of a double-peak DOT response of 0-10%

saturation magnitude, as reported in Section 6.3.5. Thus, periods
of positive DOT were accompanied by increases (of 33% and 55%
respectively) in the activities of glycerol kinase and G-3-P
dehydrogenase. Moreover, observation of the culture revealed that
it had taken on a more typically 'aerobic' appearance at the 4-1
aeration condition, a pale yellow colour of the culture having replaced
pale grey under the fully aerated, though oxygen-limited, conditions.

A small DHA kinase activity in the 700/0.70/5-0 sample was
reduced to virtually zero when converted to 4-1 aeration. This
result was not, however, shown in the glycerol dehydrogenase assays.
It thus appears that the 'degree' to which aerobic metabolism was evident
in the fully aerated IA culture 4 was relatively small, reflecting poor
oxygen-transfer conditions; decreasing the aeration condition (to 4-1)
appeared to actually increase the aerobic 'activity' from the evidence
of enzymatic and culture DOT measurements, as well as that of the
culture's appearance. However, further decrease of the aeration
condition to the 3-2 level again resulted in decreased activities of
the aerobic enzymes, and considerably increased the activities of the
anaerobic enzymes. A considerable cell density reduction indicated
that a major aerobic/anaerobic transition had taken place, and the DOT
response was of the single broad peak type.

The activities of the two anaerobic enzymes, glycerol
dehydrogenase and DHA kinase, were found to increase in an
'accelerating' manner as aeration condition was reduced in IA cultures
1, 2 and 3. The order in which the anaerobic enzyme activities of the
three cultures increase, with respect to decreasing aeration condition,
reflects their relative oxygen-transfer efficiencies. Thus, the
activities in IA culture 3 cells increased first, and those of culture 1

last.   Indeed, the high cell density of the 900/0.30/2-3 sample
was reflected in zero activities of both anaerobic enzymes.   The
activities of these two enzymes in the three fully anaerobic culture
samples were very similar.

The curved 'accelerating' increases of glycerol dehydrogenase
and DHA kinase with decreasing aeration condition probably indicates
the culture's rapidly increasing dependence on anaerobic metabolism of
glycerol as the aeration period decreases.   The evidence available does
not show whether the synthesis of these enzymes are sensitive to dissolved
oxygen, though the similarity between DHA kinase and glycerol kinase may
extend to DOT sensitive production of both enzymes.

In summary, there are positive correlations between the extent
to which intermittent aeration is imposed (i.e. deaeration time per cycle)
upon cultures of K. aerogenes, using glycerol as the sole source of
carbon and energy, and the intracellular activities of the two enzymes
involved in anaerobic glycerol metabolism.   (Glycerol dehydrogenase and
dihydroxyacetone kinase.)   There are positive correlations between
aeration condition (i.e. aeration time per cycle) and the intracellular
activities of the two enzymes involved in aerobic glycerol metabolism.
(Glycerol kinase and G-3-P dehydrogenase.)   When carbon limitation and
aerobic glycerol metabolism are evident, these latter correlations appear
to be quite strong, as indicated by the linear enzyme activity/aeration
condition relationship.

Thus, whilst dissolved oxygen availability in the culture may
remain above the critical level for the major part of the cycle time,
a considerable effect may be exerted on the levels of aerobic enzymes.
This effect may exist when no effect on cell density or CO_2 production

can be seen. Assay of the activities of the intracellular enzymes thus provides a sensitive method for the detection of biochemical effects caused by dissolved oxygen variation which do not show up in standard 'physical' measurements.

This sensitivity could possibly provide a useful tool to investigate, using a biological rather than physico-chemical technique, the oxygen-transfer characteristics of any fermenter. In the operation of this technique, K. aerogenes would be grown in a glycerol-salts medium under steady-state conditions. Cells would then be harvested, and the intracellular activities of the four glycerol-related enzymes determined. Comparison of these activities with those in cells grown under standard oxygen-transfer, but otherwise identical, conditions, might give some insight into the oxygen-transfer efficiency to a growing culture in that particular vessel.

CHAPTER 8

CONCLUSIONS

8.1   The use of a well-mixed laboratory scale fermenter with a minimal head space, combined with solenoid-valve control of air and nitrogen supplies, was found to be adequate for the imposition of intermittent aeration in cultures of <u>Klebsiella aerogenes</u> grown continuously on a glycerol-salts medium.   The use of a dissolved oxygen analyser, gaseous oxygen analyser and gaseous carbon dioxide analyser all linked to a multi-channel recorder was found to be very useful in the study of the effects of aerobic, anaerobic and intermittently aerated culture of this organism.   Further study of the biochemical effects of aerobic, anaerobic and intermittently aerated culture was found to be possible by the assay of glycerol kinase, glycerol-3- -phosphate dehydrogenase, glycerol dehydrogenase and dihydroxyacetone kinase activities in cell samples from these cultures.

8.2   The use of intermittent aeration in a small fermenter to model the effects of cyclic aeration in a large fermenter seems valid. Nevertheless, the rigid aeration-deaeration cycle, which may result in specific DOT response-types and whole-culture metabolic synchrony in the model, does not perfectly reproduce the more random DOT variations in a large fermenter;   results of this type may, therefore, only apply to the intermittently aerated model.   The model may, however, be more successful in reproducing the biochemical, especially enzymatic, effects of cyclic aeration.   If this is the case, the use of this system in investigating the oxygen-transfer characteristics of a fermenter by assay of the enzymes of aerobic and anaerobic glycerol metabolism in <u>K. aerogenes</u> grown in the fermenter might be possible.

8.3   There are positive correlations between aeration condition and the intracellular activities of glycerol kinase and glycerol-3- -phosphate dehydrogenase in cells cultured under conditions of intermittent aeration.   Conversely, there are negative correlations between aeration condition and the intracellular activities of glycerol dehydrogenase and dihydroxyacetone kinase in these cells.   The linear relationship between glycerol kinase and G-3-P dehydrogenase activities and aeration condition in efficiently oxygenated cultures may indicate dissolved oxygen sensitive synthesis of these enzymes.

8.4   The highest activities of aerobic enzymes and the lowest activities of anaerobic enzymes at any particular aeration condition generally tend to exist in cells which are grown in the more efficient oxygen-transfer conditions.   Thus, as aeration condition is reduced, the enzyme complements which are typical of anaerobic glycerol metabolism appear at high aeration condition in cells grown under the least efficient oxygen-transfer conditions, but only at low aeration condition in cells grown under the most efficient oxygen-transfer conditions.

8.5   Bacteria grown under conditions of intermittent aeration normally have significant levels of both aerobic and anaerobic enzymes. However, a marked change of enzyme complement takes place between two successive aeration conditions, and is related to a major transition between aerobic and anaerobic glycerol metabolism.   Nevertheless, normally, the levels of aerobic enzymes diminish and those of anaerobic enzymes increase as the aeration condition is further reduced after this

transition; increasing 'degrees' of anaerobic glycerol metabolism are therefore evident up to the point of fully anaerobic growth.

8.6   The major aerobic/anaerobic transition is accompanied by a considerable reduction of culture cell density. A slight increase of carbon dioxide production per unit mass of cells accompanies this transition, and further increases as aeration condition is further reduced. Thus anaerobic cultures produce considerably more carbon dioxide than aerobic cultures, although the absolute amount depends on the dilution rate.

8.7   A yellow-green pigment is excreted into the surrounding medium by cells which are grown under specific conditions of intermittent aeration. The pigment, which may be a member of the pteridine group, is produced continuously under steady-state culture conditions with an impeller speed of 900 min^{-1}, a dilution rate of 0.30 h^{-1}, and 2 minutes aeration - 3 minutes deaeration in each cycle.

8.8   The enzyme dihydroxyacetone kinase can be assayed by linking its reaction to that of the NAD-linked mammalian glycerol-3-phosphate dehydrogenase in an in vitro assay system. Rate of reaction, which is proportional to enzyme activity, is measured by recording the reduction of absorbance (at 340 nm) with time. This decrease of absorbance is caused by the oxidation of NADH to NAD as the linked reaction, which depends on the activity of DHA kinase, proceeds.

DHA kinase is competitively inhibited by glycerol, and may be able to utilise this compound as an alternative substrate. Similarly, glycerol kinase may be able to utilise DHA.

CHAPTER 9

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Appendix

NOMENCLATURE

a	interfacial area per unit volume of aerated solution (cm^{-1})
C	concentration of oxygen in the solution (gl^{-1})
C^*	solubility of oxygen in the solution (gl^{-1})
C_s	saturation concentration of oxygen (gl^{-1})
D	dilution rate (h^{-1})
D_c	critical dilution rate (h^{-1})
DOT	dissolved oxygen tension (as % saturation)
H	Henry's constant ($g\ cm^{-1}\ min^{-2}$)
K_L	mass-transfer coefficient ($cm\ min^{-1}$)
$K_L a$	overall mass-transfer coefficient (min^{-1})
$(K_L a)_o$	time-averaged oxygen-transfer coefficient defined on page 55 (min^{-1})
K_m	Michaelis-Menten constant (gl^{-1})
K_S	saturation constant (gl^{-1})
λ	wavelength (nm)
$\overline{N}$	rate of oxygen uptake ($gl^{-1}\ min^{-1}$)
P_g	partial pressure of oxygen in gas phase (mm Hg)
$[S]$	substrate concentration (gl^{-1})
t	time (min.)
t_d	cell doubling-time (min.)
T	temperature (^oC)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
V	velocity of the enzyme-catalysed reaction as defined in the text
V_{max}	maximum velocity of the enzyme-catalysed reaction as defined in the text
V'	total volume of aerated solution (cm^3)
V_L	volume of solution (cm^3)
X	cell density (mg. cm^{-3} dry cell weight)