

I declare that the work included in this Thesis is my own and that it has not been submitted for any other degree or professional qualification.

James Creanor

CONTENTS.

SUMMARY		p1
CHAPTER 1 -	<u>INTRODUCTION.</u>	p3
CHAPTER 2 -	<u>Materials and Methods.</u>	
	Organism.	p 13
	Media & growth of cultures.	p 13
	Optical density, cell number & cell plate index.	p 14
	Preparation of Synchronous Cultures.	p 15
	Preparation of induced synchronous cultures.	p 17
	Preparation of synchronous cultures using a zonal rotor.	p 17
	Oxygen uptake by the manometric method.	p 19
	CO ₂ evolution measured by the manometric method.	p 21
	Oxygen Uptake measured by a polar - ographic method.	p 22
	Assay for Hexokinase.	p 25
	Measurement of ¹⁴ CO ₂ evolution using a radiorespirometric method.	p 29
	Assay for ATP.	p 31
CHAPTER 3 -	<u>Oxygen uptake in cultures of S. pombe</u>	p 33
	Measurement of O ₂ uptake in asynchronous cultures using a manometric method.	p 33
	Oxygen Uptake in synchronous cultures using a manometric method.	p 37
	Polarographic measurement of O ₂ uptake in <u>S. pombe</u> .	p 43

Oxygen uptake in synchronous cultures.	p 43
Oxygen uptake of stationary cultures.	p 49

CHAPTER 4 -	<u>Oxygen Uptake in the presence of various inhibitors.</u>	p 51
	Oxygen uptake in a synchronous culture containing deoxyadenosine.	p 51
	Oxygen uptake in induced synchronous cultures.	p 54
	Oxygen uptake in the presence of cycloheximide.	p 60
	Oxygen uptake in the presence of 8 - hydroxyquinoline.	p 62
	Oxygen uptake in the presence of cyanide and azide.	p 63
	Oxygen uptake in the presence of erythromycin.	p 68

CHAPTER 5 -	<u>CO₂ evolution in asynchronous and synchronous cultures.</u>	p 70
	Introduction.	p 70
	Evolution of CO ₂ in asynchronous cultures.	p 73
	Evolution of CO ₂ in synchronous cultures.	p 77
	CO ₂ evolution in synchronous cultures prepared using sucrose gradients.	p 86
	Continuous measurement of CO ₂ evolution in a synchronous culture.	p 94
	CO ₂ evolution in cultures growing in yeast extract / glucose.	p 96
	CO ₂ evolution in strain 972 h ⁻ .	p 98

	CO ₂ evolution in synchronous cultures prepared on a zonal rotor.	p 102
	CO ₂ evolution in stationary cultures.	p 106
CHAPTER 6 -	<u>Evolution of CO₂ in cultures containing inhibitors.</u>	p 107
	Introduction.	p 107
	CO ₂ evolution in a synchronous culture containing deoxyadenosine.	p 110
	CO ₂ evolution in a synchronous culture containing mitomycin C.	p 112
	CO ₂ evolution in an induced synchronous culture.	p 114
	CO ₂ evolution in the presence of inhibitors of protein & RNA synthesis.	p 115
	Effect of iodoacetate on CO ₂ evolution and growth of <u>S. pombe</u> .	p 119
CHAPTER 7 -	<u>Measurement of CO₂ evolution by the radiorespirometric method.</u>	p 122
	Introduction.	p 122
	Estimation of flux through the path- way of glucose catabolism in <u>S. pombe</u> .	p 122
	Processing of glucose through the EMP pathway.	p 125
	¹⁴ CO ₂ evolution in exponential cultures.	p 128
	¹⁴ CO ₂ evolution in synchronous cultures of <u>S. pombe</u> .	p 128

CHAPTER 8 -	<u>Other experiments.</u>	p 136
	Introduction.	p 136
	Measurement of optical density in synchronous cultures	p 136
	Measurement of pH during the cell cycle of <u>S. pombe</u> .	p 141
	Production of ATP during the cell cycle of <u>S. pombe</u> .	p 142
	Measurement of hexokinase in asyn- chronous and synchronous cultures of <u>S. pombe</u> .	p 144
CHAPTER 9 -	<u>Discussion.</u>	p 152
	Part 1 : The biochemistry of the EMP pathway and of the Citric Acid Cycle.	p 153
	Part 2 : Respiration and fermentation during the cell cycle.	p 163
ACKNOWLEDGEMENTS		p 189
REFERENCES.		p 190
APPENDIX 1.		p 204

SUMMARY.

Oxygen uptake and CO_2 evolution were measured in synchronous cultures of the fission yeast Schizosaccharomyces pombe, growing in a minimal medium. The rate of oxygen uptake increased abruptly at the middle of the cycle and again at the end of the cycle. During the intervening periods the rate remained constant. Oxygen uptake was measured manometrically and polarographically.

The rate of CO_2 evolution doubled sharply at the time of nuclear division (0.75 of the way through the cycle). For the remainder of the cell cycle, the rate remained constant. Similar step-wise increases in the rate of CO_2 evolution could be induced by adding glucose to late exponential cultures. So various attempts were made to show that the steps in the rate of CO_2 evolution in synchronous cultures were not artefacts induced by the synchronisation technique.

The addition of DNA synthesis inhibitors and a nuclear division inhibitor to synchronous cultures had no effect on the patterns of oxygen uptake and CO_2 evolution. Similarly in an induced synchronous culture, in which DNA synthesis, nuclear division and cell division - but not "growth", were synchronised, oxygen uptake and CO_2 evolution showed a continuous increase and not a "synchronous" pattern.

Evolution of $^{14}\text{CO}_2$ was measured in synchronous cultures. Various technical problems made the interpretation of these results difficult but there is a similarity between the measurement of $^{14}\text{CO}_2$ evolution and CO_2 evolution measured manometrically in synchronous cultures

Experiments with protein and RNA synthesis inhibitors suggested either that an increase in oxygen uptake and CO_2 evolution was dependent on continued protein and RNA synthesis; or that in the absence of either protein or RNA synthesis, the demand for energy decreased and hence the flux through the energy yielding pathways which result in oxygen uptake and CO_2 evolution also decreased.

Other experiments showed that optical density increase doubled in a synchronous culture at about the same time that CO_2 evolution doubled in rate. Both hexokinase and ATP were shown to increase exponentially in synchronous cultures and are therefore not obvious regulators of, or obviously regulated by, oxygen uptake or CO_2 evolution.

The control of respiratory and fermentative activity is discussed and it is suggested that the levels of intermediates in the EMP pathway and the TCA cycle control the activities of these pathways.

CHAPTER 1.

Introduction.

The study of fermentation in yeast led to the beginning of Biochemistry in the second half of the nineteenth century. The pioneers of the study of fermentation, Pasteur, Buchner, Harden and Young were responsible for the concept of unity in the living world at the biochemical level, discovering, for example, that the metabolism of glucose by yeast was identical to that observed in muscle, except for the end-product.

While there is now almost complete understanding of the biochemistry of the pathways involved in the metabolism of glucose by yeast, information of how this is integrated into the life-cycle of the cells is more uncertain. This thesis represents a study of the relationship between the processes of energy production and the cell cycle of the fission yeast Schizosaccharomyces pombe.

The principal intention is to measure the uptake of oxygen and the evolution of carbon dioxide during the cell cycle and the life history of this yeast, thus obtaining a measure of the flux through the main energy-producing pathways of the cell. A secondary investigation is to alter the normal cell cycle by the use of inhibitors of nuclear division and DNA synthesis and to observe the effect of such alterations on oxygen uptake and CO₂ evolution. In other words, having established the pattern by which the energy metabolism of this yeast changes during its cell cycle, the question is asked of how committed the cell is to this pattern and how easily can the pattern be dissociated from other events constituting the cell cycle.

Glycolysis (The Embden - Meyerhof - Parnas Pathway).

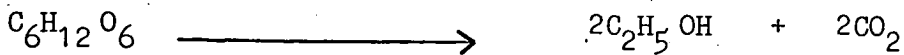
This pathway is involved with the metabolism of glucose and is of major importance whether cells are growing with or without oxygen. After glucose has been actively transported across the cell membrane (Cirillo. 1961), there are at least sixteen enzymes which are involved in the EMP pathway until pyruvate is formed. At this point, there are two alternative fates for the pyruvate; in the absence of oxygen, fermentation occurs and the pyruvate is converted to acetaldehyde and eventually to alcohol. Alternatively, pyruvate can be converted to Acetyl CoA and this enters the Citric Acid Cycle by condensation with oxaloacetate to form citrate.

Glycolysis, which will be taken here to include the fermentation part of the pathway, serves two main functions in the cell, as an energy source and as a provider of carbon skeletons.

There is much evidence that the EMP pathway is the major pathway of glucose oxidation in Saccharomyces cerevisiae (Blumenthal, Lewis & Weinhouse, 1954; Wang, Stern, Gilmour, Klumsoyr, Reed, Bialy, Christenson & Cheldelin, 1958; Chen, 1959). There are many other fungi which can utilise carbohydrates anaerobically, producing typical fermentation products. When Pasteur first published his memoirs on the fermentation of sugars, he showed that what was true for yeast was also applicable to other fungi. It has since been shown that other fungi are capable of growing purely by fermentative processes though they do require some oxygen. Aspergillus clavatus and certain other species have been shown to have a high capacity for alcoholic fermentation (Cochrane, 1958).

If one accepts for the moment that the EMP pathway is the major pathway of glucose catabolism, at least until the formation of pyruvate, then in some species and under some conditions, the next

stage of glucose catabolism is not executed to give the maximum energy supply. The Gay-Lussac equation for the alcoholic fermentation of glucose,



gives a change in free energy of about -56 kcal/mole. If this is compared with the figure for the complete combustion of glucose (-688 kcal/mole), it is obvious that fermentation is a much less economic process than respiration, since the latter results in the complete combustion of glucose. This prompted Pasteur (1876) to say that if yeast were supplied with air, they would cease to ferment. The yield of cells grown aerobically can be ten times greater than the yield when the growth is anaerobic; and the quantity of substrate assimilated for a given amount of cell growth is also much reduced when the growth is aerobic.

The Citric Acid Cycle.

The Citric Acid Cycle (also called the Tricarboxylic Acid Cycle) occupies a central place in the metabolism of a cell. As well as being a supplier of energy and carbon skeletons, it is the focus of the central pathways in the cell and its reactions and substrates play an important role in the biosynthesis of many important metabolites, including amino acids, purines, pyrimidines, long-chained fatty acids and porphyrins.

Krebs & Johnson (1937) first described the existence of this cycle and showed it to be present in a variety of organisms, but not in yeast or bacteria. There is now overwhelming evidence that this cycle exists in fungi in general and in yeast in particular. While it has been shown that organic acids of the TCA cycle are formed

in considerable amounts by certain fungi (Cochrane, 1958), such evidence suggests the interruption of a cyclic process rather than an operative TCA cycle (Niederpruem, 1965). There is also some nutritional evidence for the existence of a TCA cycle in fungi; some yeasts can utilise a variety of TCA cycle intermediates as sole carbon source (Barnett & Kornberg, 1960). It has also been shown that respiration and growth in Neurospora crassa are both inhibited by iodoacetate which operates in the middle of the Glycolytic Pathway at the level of triose phosphate dehydrogenase (Ryan, Tatum & Giese, 1944). The inhibition of respiration could be relieved by the addition of succinate while the inhibition of growth could be relieved by addition of succinate, malate, fumarate or pyruvate. All of these are intermediates of the TCA cycle and the reversal of the inhibition by them suggests the operation of a functional TCA cycle in this fungus.

There are several suggestions that the primary role of the TCA cycle in yeast is to provide intermediates for biosynthesis rather than to provide the cell with energy (Krebs, Gurin & Eggleston, 1952). It is still not possible to say that the energy supplied by other sources is sufficient to meet the demands of the cell in the absence of an energy contribution from the TCA cycle.

The biochemistry of the TCA cycle and of the electron transport chain in yeast has been well documented (Sols, Gancedo & De la Fuente, 1971) and a complete discussion of it does not belong here. In general, its function can be summarised by saying that for each mole of glucose which passes round the TCA cycle and along the electron transport chain, energy is accumulated by the cell in the form of 38 moles of the energy rich compound ATP. This latter compound is then utilised by the cell in a variety of anabolic reactions.

Comparision of the Respiration of *S. cerevisiae* and *S. pombe*.

A brief account of the Pasteur Effect and the "negative Pasteur Effect" must be considered before discussing the respiratory metabolism of these two yeasts. In the Pasteur Effect, there is an inhibition of fermentation by respiration accompanied by a slower rate of glucose utilisation in aerobic growth compared with anaerobic growth. There is a consistent increase in the level of hexose - mono - phosphates accompanying the Pasteur Effect and this is attributed to a feed - back control operating at the first irreversible step of the common Glycolytic pathway catalysed by the enzyme phosphofructokinase (Sols, 1968). The nature of the feed - back control could be ATP itself, which inhibits phosphofructokinase (Vinuela, Salas & Sols, 1963). This inhibition can be counteracted by competition from AMP (Sols & Salas, 1965 ; Ramaiah, Hathaway & Atkinson, 1964). While this is obviously a key factor in the control of glycolysis, it cannot by itself account for the Pasteur Effect since the level of ATP in anaerobically fermenting yeast does not increase when passing to aerobic conditions (Holzer & Witt, 1958). It has also been found that citrate operates a feed - back inhibition on phosphofructokinase and this has been suggested as a more likely candidate for the aerobic regulation of phosphofructokinase activity in yeast (Salas, Vinuela, Salas & Sols, 1965). The reason for this is that it has been argued that when oxygen is available for the oxidation of mitochondrial NADH, pyruvate is preferentially oxidised to Acetyl CoA since pyruvate oxidase has a much lower K_m than pyruvate decarboxylase, the latter leading to the production of alcohol. Acetyl CoA then enters the TCA cycle as citrate resulting in the potential to control the cycle at the level of its first irreversible enzyme. Thus glycolysis can be controlled by what can be regarded as its aerobic carbon end - product.

It is fairly obvious that feed-back regulation of glycolysis by ATP and citrate could prevent a maximal glycolytic rate while retaining optimal levels of ATP and citrate for biosynthesis.

The negative Pasteur Effect is the phenomenon where even under aerobic conditions, glucose can increase the rate of carbon dissimilation via the glycolytic pathway. This has also been referred to as aerobic fermentation and to glucose repression of respiration (DeDeken, 1966; Beck & von Meyenburg, 1968). This phenomenon has been explained by a regulatory mechanism analogous to the Crabtree Effect (Crabtree, 1929). Since all studies of the Pasteur Effect have been made on suspensions of cells, DeDeken (1966) has justifiably pointed out that one does not know if the Pasteur Effect plays an important role in the growth of cells. For example, during the exponential growth of S. carlsbergensis, the Pasteur Effect does not have an opportunity to work since the degradation of glucose proceeds entirely by fermentation. There are many theories as to which effect should operate and among these the most commonly advanced controlling factors are ADP levels (Belitzer, 1936; Chance & Hess, 1956; Coe, 1964) and P_i (Belitzer, 1936; Brin & Mc Kee, 1956; Bielka, 1963).

One can now examine the respiration of S. cerevisiae and S. pombe on the basis of the above discussion. Swanson & Clifton (1948) first showed that most of the aerobic growth of S. cerevisiae proceeded by fermentation. A quantitative study by Lemoigne, Aubert & Millet (1954) showed the presence of a diauxic growth pattern on high glucose concentrations; firstly a fast growth involving intense aerobic fermentation, followed by a slow growth involving the oxidation of the accumulated ethanol. At low glucose concentrations (5 - 10 mg/100 ml) there was no diauxic growth, suggesting that the pathway used for the oxidation of glucose might depend on the glucose

concentrations. Slonimski (1956) showed that at low concentrations of glucose (less than 6×10^{-3} M) the rate of adaptation to aerobic conditions increased as the glucose concentration increased. This is reasonable since the rate of aerobic fermentation of glucose is low at these concentrations and severely limits the energy supply. At glucose concentrations greater than 6×10^{-3} M, the rate of adaptation to aerobic conditions decreased as the glucose concentration increased. This work was later extended to growing cultures (Ephrussi, Slonimski, Yotsuyanagi & Tavlitzki, 1956), starting with cells which were fully adapted to aerobic conditions. They observed that the rate of aerobic fermentation increased sharply during exponential growth and at the same time the rate of oxygen uptake fell to a low value. A few generations before the end of the exponential phase, glucose no longer saturated the fermentation system and CO_2 evolution fell while oxygen uptake increased (respiratory adaptation). So it was concluded that a negative Pasteur Effect was an important part of the physiology of a yeast like S. cerevisiae growing in a high concentration of glucose.

In conclusion, one can say that S. cerevisiae exhibits a strong degree of respiratory inhibition by glucose, mediated at some stage of the glycolytic pathway by the levels of one or more intermediates.

DeDeken (1966) was unable to find any oxygen uptake in exponential cultures of S. pombe growing aerobically with glucose as carbon source and he concluded that the Respiratory Coefficient (CO_2/O_2) was infinite. He therefore deduced that this yeast was similar to S. cerevisiae in exhibiting a negative Pasteur Effect. There was not, however, strictly speaking, a repression of respiration by glucose - a peculiarity of the so-called "petite - negative"

species. This behaviour was confirmed in another petite - negative species, S. fragilis (Mc Clary & Bowers, 1968).

The respiratory metabolism of S. pombe has also been examined by Heslot, Goffeau & Louis (1970). Briefly, their findings were poor growth on non - fermentable substrates (pyruvate, acetate, lactate and glutamate), high fermentation rates of cultures growing aerobically on glucose ($RQ = 3.8 - 4.3$) but decreasing as the culture entered stationary phase ($RQ = 1.6$). This decrease in the RQ was caused by a reduction in the evolution of CO_2 rather than an increase in the uptake of oxygen. These values for the RQ are lower than those reported by DeDeken (1966) discussed above. Heslot et al (1970) also showed that the respiration of cells growing aerobically on glucose was strongly inhibited by 1 mM NaCN and by $1.66 \mu M$ Antimycin A, both of which are inhibitors of the electron transport chain (Slater, 1967 & 1973). Cells which were growing anaerobically had a low but significant oxygen uptake (when placed in an aerobic environment) but this oxygen uptake was not inhibited by Antimycin A and was only 33% inhibited by NaCN. This led to the suggestion that the oxygen uptake of cells growing anaerobically is required for something other than electron transport. The oxygen uptake of cells growing on glycerol was completely inhibited by the concentrations of the above inhibitors already discussed. Growth (and oxygen uptake) of S. pombe was not affected under any conditions by rotenone, suggesting that this yeast, like S. cerevisiae and S. carlsbergensis does not possess Site 1 of phosphorylation (Ohnishi, Sottocasa & Ernster, 1966). When cultures of S. pombe entered stationary phase, the uptake of oxygen was found to increase over a period of several hours by a factor of 1.6 . Unlike the experiments reported by DeDeken (1966), Heslot et al (1970) suggested that there was some degree of glucose repression of respiration in S. pombe but not at the same level

as was shown to operate in S. cerevisiae where the increase in the respiration rate after the exhaustion of glucose has been shown to be by a factor of about 20 (Ephrussi, Slonimski, Yotsuyanagi & Tavlitzki, 1956; Yotsuyanagi, 1962; Utter, Duell & Bernofsky, 1967).

Measurement of the Respiration Rate in Synchronous Cultures of Yeast.

A number of investigations into the oxygen consumption of a variety of synchronously dividing cell populations have been made, with a view to examining the energy metabolism of individual cells during their growth cycle. The situation in yeast is that the results produced have shown considerable variation, even within the same strain of certain species. The patterns of oxygen uptake which have been described show continuous increases in the rate, oscillations in rate once or twice per cell cycle, step-wise increases in the rate and increases in rate which are out of phase with the cell cycle. There are many such investigations into the respiration of S. cerevisiae (Nosoh & Takamiya, 1962; Scopes & Williamson, 1964; Kuenzi & Flechter, 1969; von Meyenburg, 1969; Wiemken, Matile & Moor, 1970; Cottrell & Avers, 1970, 1971; Greksak & Hanicova, 1973; Dharmalingam & Jayaraman, 1973). Investigations into the respiration of S. pombe are much less numerous (Osumi & Sando, 1969; Poole & Lloyd, 1973 a, 1974; Marchant, 1971).

The situation which has arisen as a result of these numerous investigations is a rather muddled one and results and problems of the above investigations will be considered in the Discussion at the end of this Thesis. At the moment one can say that the position requires some clarification and with the background of information in this laboratory of the cell cycle of S. pombe, it was hoped that

an investigation into the respiration of this yeast would go some way towards clarifying a complicated picture. There are also techniques available in S. pombe for distorting the cell cycle using various inhibitors which will be discussed later, techniques which are not readily available in S. cerevisiae.

Aims of this Thesis.

It is obvious that an investigation into the energy metabolism of a yeast would involve measuring the oxygen uptake of the cells. As well as doing this, it is intended to measure the evolution of CO₂ in synchronous cultures of S. pombe and it will be shown that in this way, a measure of the activity of the Glycolytic pathway is being made. Measurements of CO₂ evolution in synchronous cultures are few; one investigation showing a continuously increasing rate of evolution during the cell cycle of S. cerevisiae (Wiemken, Matile & Moor, 1970).

Considerable care will be taken in this work to establish that the pattern which is obtained for oxygen consumption and CO₂ evolution in this yeast are genuine cell cycle events and not artifacts introduced into the culture by the synchronisation method.

Oxygen uptake and CO₂ evolution will be measured in distorted cycles, using either inhibitors of DNA synthesis or of nuclear division. The same measurements will also be made in the presence of a variety of inhibitors, either in synchronous or in asynchronous cultures (e.g. protein, RNA, glycolysis, respiration, mitochondrial protein inhibitors).

CHAPTER 2.

Materials and methods.

Organism.

The fission yeast, Schizosaccharomyces pombe Lindner (1893) was used throughout this work. Lodder and Kreger-Van Rij (1952) classified this organism in the sub-family Endomycetoidea of the family Endomycetaceae, subclass Ascomycetes. Most of the experiments reported here were carried out on strain NCYC 132 (ATCC 24751), the "new strain" described by Mitchison & Creanor (1971b). This was obtained from the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey). Strain 972h⁻, which was used in a few experiments, was obtained from Professor U. Leupold, University of Bern.

The morphological characteristics of S. pombe are growth in length only of rod-shaped cells and division by the formation of a median septum, referred to as the "cell - plate". The youngest cells in a logarithmically growing culture of S. pombe are normally about 6 microns in length and 3.5 microns in width: cells at division are roughly 12 microns in length and the same width.

Media and Growth of Cultures.

Cultures were normally grown in a defined liquid medium, Edinburgh Minimal Medium (EMM 2) the constituents of which are shown in Appendix 1. This medium contains glucose as the carbon source, ammonium chloride as the nitrogen source, together with inorganic salts, trace elements and vitamins. Sodium acetate is used to buffer the initial pH of the medium at 5.5. The final cell number in this medium is 5×10^7 cells/ml and the generation time of an exponentially

growing culture is 2.5 hours at 32°C.

Stock cultures were maintained at 4°C on slopes of 2% Malt Extract Agar (Oxoid Ltd., Southwark Bridge Rd., London S.E.1). Cells were transferred from slopes into 10 ml EMM 2 in Universal Containers and grown to stationary phase at 32°C. These liquid cultures could then be used to inoculate experimental cultures for about a fortnight.

Experimental cultures were normally prepared by inoculating warm EMM 2 to a density of 2×10^4 cells/ml. These cultures were then grown up at 32°C for about 18 hours, either on an orbital shaker or in a shaking water bath. After growing for 18 hours at 32°C, the culture had attained a cell density of 3×10^6 cell/ml. If possible, the flasks used for growing the cultures were three times greater in volume than the volume of the medium.

Normal sterile techniques were employed in maintaining the stock cultures and in inoculating cultures for use the following day. Because of the acidity of the medium, it has never proved necessary to maintain sterile conditions for the duration of the experiments reported here.

Optical Density, Cell Number and Cell Plate Index.

A convenient estimate of the growth of a culture was obtained by measuring the optical density in a Unicam SP 600 Spectrophotometer. Using calibration curves of cell number against optical density, a reliable estimate of cell number in an asynchronous culture could be obtained rapidly.

Cell number was measured using a Coulter Counter (Model B) with a 70 micron aperture. A sample of 0.5 ml was removed from the culture and diluted first of all with 4 ml filtered electrolyte

(1% NaCl, 0.2% formaldehyde, in water). This sample was then sonicated for 90 seconds using an MSE 100 Watt Ultrasonic Disintegrator at a setting of 20 kc/ second and an amplitude of 2 microns. During the sonication, which was done in an ice bath, cell pairs and clumps were broken up. The sonicated sample was further diluted with filtered electrolyte to a cell density of $5 - 40 \times 10^3$ cells/ml. Two cell counts were then made on this sample using the Coulter Counter.

The cell plate index is the fraction of cells in a population showing cell plates at a particular time and is used as a measure of the degree of synchrony in synchronous cultures. This was determined using dark ground optics on a Zeiss Opton microscope and counting between 200 and 400 cells for each measurement. A cell was considered to be in the cell plate stage from the first appearance of the cell plate until it was just possible to see notches at the ends of the plate, indicating that cell division was taking place. The cell plate stage probably lasts for 15-20 minutes (Faed, 1959). It is probably correct to regard the beginning of the cell plate stage as the end of the cell cycle since the daughter cells are physiologically separate at this point, but the suggestion of Mitchison (1970) that cell separation should mark the beginning and end of the cycle is adopted here.

Preparation of Synchronous Cultures.

Synchronous cultures were usually prepared by the selection method of Mitchison & Vincent (1965). Four litre cultures, cell density $1 - 4 \times 10^6$ cells/ml, were harvested using a Whatman No 50 filter paper which was supported on a 15 cm Millipore holder. The cells were scraped from the paper using a thin spatula and resuspended

in 4ml of the original culture medium. The cell suspension was then shaken vigorously and layered onto 80 ml of either a 10-40% sucrose gradient or a $7\frac{1}{2}$ -30% lactose gradient in EMM 2 made with a gradient machine (Britten & Roberts, 1960) in a 100 ml centrifuge tube. The tube was then centrifuged in a swing-out head of a MSE 4L centrifuge until the top layer of the cells had moved about half way down the tube. The time and the speed of centrifuging varied with the gradient but an approximate figure was 5 minutes at 500 g at 25⁰C. The top layer of the cells was then removed using a 5 ml syringe and a long needle and added to warm medium to give the synchronous culture. The amount of cells removed from the top of the gradient normally gave 150 ml of synchronous culture at a cell density of 2×10^6 cells/ml, i.e. about 2% of the original culture. The culture was synchronous because the top layer of cells on the gradient was predominantly composed of the small cells at the beginning of the cycle. The culture was grown in a shaking water bath for the duration of the experiment which was normally until the end of the second synchronous division. To obtain larger synchronous cultures, the size of the starting culture was increased and more gradient tubes were used.

The effect of the synchronising procedure on subsequent cell growth was tested by proceeding through the technique till after the gradient had been centrifuged. The gradient was then thoroughly shaken and a sample, which contained cells at all stages of the cycle, was inoculated into fresh medium at the same cell concentration as was used in a synchronous culture.

Preparation of Induced Synchronous Cultures.

2' Deoxyadenosine, at a concentration of 2 mM, can be used to inhibit the synthesis of DNA in S. pombe and when applied in a pulse, can be used to prepare synchronous cultures (Mitchison & Creanor, 1971a). Cells were grown overnight to a concentration of 2×10^6 cells/ml. 2mM deoxyadenosine was added to the culture for a 3 hour pulse. At 3 hours, the culture was filtered, washed with warm medium and resuspended in fresh FMM 2. About $2\frac{1}{2}$ hours after resuspension, the culture underwent a synchronous division, followed by a second semi - synchronous division $1\frac{1}{2}$ hours later. Unlike the synchronous culture prepared by the selection of cells at the same stage of the cell cycle, cell size is not uniform in an induced synchronous culture. At the first synchronous division, cell size of the dividing cells was as variable as the total population in a randomly growing culture.

Deoxyadenosine was also added to a synchronous culture which had been prepared by the selection method. If the inhibitor was added to the synchronous culture about 30 minutes after preparation, the first synchronous division was not affected but the second synchronous division did not occur at the normal time. Eventually, the cells recovered from the effect of the inhibitor and began to divide about $6\frac{1}{2}$ hours after the deoxyadenosine was first added. Recovery appeared to vary between cells and when cell division began to occur, it did not do so synchronously.

Preparation of Synchronous Cultures using a Zonal Rotor.

Synchronous cultures can be prepared by separating cells according to size on a zonal rotor. One advantage of this method is that larger synchronous cultures can be obtained than is possible

on single tube gradients. However the time required to prepare a synchronous culture from a zonal rotor is about five times longer than the time required to prepare a synchronous culture using tube gradients.

This method has been applied to S.cerevisiae (Halvorson, Carter & Tauro, 1971), S.lactis (Carter & Halvorson, 1973) and S. pombe (Poole & Lloyd, 1973; Nurse, 1975; Mitchison & Carter, 1975).

A lactose gradient ($7\frac{1}{2}$ -30%) made up in EMM 2 was introduced into a MSE Type A Zonal Rotor while the rotor was running at 300 rpm. Exponential cultures were harvested as described earlier for tube separation. The harvested cells were scraped off the filter, resuspended in medium, introduced into the centre of the rotor using a 50 ml syringe and then overlaid with 30 ml distilled water to displace the cells from the central core. Once loaded, the rotor was accelerated to 2000 rpm when the migration of the cells could be seen visually. When the fastest migrating cells had gone about four-fifths the distance to the edge of the rotor, the centrifuge was decelerated to 300 rpm. The cells were removed from the rotor by pumping 40% sucrose into the outer edge to displace the cells from the centre. Since separation was according to size, the smallest cells were removed first. Later fractions contained progressively larger cells. Synchronous cultures of about 200 ml were required and to obtain this, the first two or three cell-containing fractions of 10 ml were pooled and inoculated into fresh medium.

In the technique described above, cells separate on the gradient according to their size and therefore according to their age. It has been more usual to use this method to separate cells

across the zonal rotor according to their stage in the cycle in order to follow cell cycle events without preparation of a synchronous culture. This technique has been applied to S. cerevisiae (Carter, Sebastian & Halvorson, 1971; Sebastian, Carter & Halvorson, 1971), S. lactis (Sebastian, Carter & Halvorson, 1971) and to S. pombe (Wells & James, 1972; Poole & Lloyd, 1973).

In this work it was not necessary to obtain fractions from an entire cell cycle spread across the rotor. Instead, an attempt was made to obtain fractions which were at least half-way through the cell cycle and to let such fractions grow synchronously. Normally, age fractionation on the zonal rotor has been carried out at 0°C to prevent changes occurring in the culture during the fractionation but in the present study, it was considered more important to minimise metabolic disturbance of the culture so collection of the cells, centrifugation and collection of the fractions were carried out at room temperature. In all, forty fractions were collected from the rotor, each consisting of 15 ml. Cultures were grown using, in one experiment, fractions 15 & 16 and in another experiment, fractions 19 & 20 were used.

Oxygen Uptake by the Manometric Method.

The instrument used, in the first instance, to measure oxygen uptake was the Warburg Manometer (Warburg, 1926) which was developed from the blood-gas manometer described by Barcroft & Haldane (1902). The principle of this manometer is that at constant temperature and volume, any changes in the amount of a gas can be measured by changes in its pressure.

A standard manometer and flask were used to measure oxygen uptake. Krebs's solution (Krebs, 1951) was used as manometric fluid

and consisted of 44 g anhydrous NaBr, 0.3 g Triton-X-100 and 0.3 g Evan 's Blue, all in 1 litre of water. This solution had a density of 1.033 at 20°C. During experiments to measure the uptake of oxygen, the flasks and manometers were immersed in a circular shaking water bath (Braun, Melsungen, West Germany) and shaken at 120 strokes/ minute. It was assumed that the temperature of the manometer, which was not immersed in the bath, did not differ significantly from that of the bath. The reference point in the closed side of the manometer was taken at 150 mm and the level of liquid in the closed side of the manometer was always adjusted to this point before recording the change in level in the open side of the manometer.

The technique used to follow the uptake of oxygen in a growing culture is described by Umbreit, Burris & Stauffer(1957) A sample of cells, normally 2 - 3 ml, was placed in a micro flask. The central well of the flask contained 0.2 ml of 20% KOH which was adsorbed on to a small piece of fluted filter paper. The flask was then ground on to the manometer using anhydrous lanolin and the apparatus placed in the shaking water bath and allowed to equilibrate for 15 minutes before beginning the measurements of oxygen uptake. The system was calibrated so that from the pressure changes observed, the amount of gas utilised could be measured. The method of calibrating the flask and the manometer is that described by Umbreit, Burris & Stauffer (1957).

Changes in the atmospheric pressure during an experiment were measured using a thermobarometer which consisted of a manometer and flask containing water. Pressure changes in the experimental flasks were corrected by adding or subtracting the change in the thermobarometer.

CO₂ Evolution Measured by the Manometric Method.

The method used for measuring the evolution of CO₂ in S. pombe was similar to that used for the manometric measurement of oxygen uptake except that KOH was not used to absorb the CO₂ evolved. The method used was a modification of the direct method of Warburg (Umbreit, Burris & Stauffer, 1957) which consisted of measuring the evolution of CO₂ without correcting for the uptake of oxygen. The Respiratory Coefficient (RQ) of an exponential culture of S. pombe growing on 1% glucose was shown to be about 10. This means that the rise in pressure caused by the evolution of CO₂ was ten times greater than the fall in pressure caused by the uptake of oxygen. The error in measuring the evolution of CO₂ without correcting for the uptake of oxygen was therefore ignored.

Between 2 and 4 ml of culture were placed in a 15 ml macro - flask which was then attached to a manometer. The volume used depended on the cell density of the culture. The apparatus was then placed in a Braun circular water bath and allowed to equilibrate for 10 - 15 minutes. The valve on the manometer was then closed and the increase in pressure caused by the evolution of CO₂ was measured over a 20 minute period. Changes in atmospheric pressure were corrected for using a thermobarometer. At the end of the 20 minutes, the flask was removed from the manometer and the contents discarded. A fresh sample of cells was then taken from the parent culture and the measurement was repeated. It was found advisable to make only a single measurement of the rate of CO₂ evolution from each sample : this will be discussed in Chapter 5.

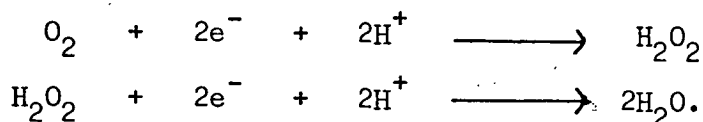
The manometer and flask were calibrated so that from the increase in pressure, the absolute amount of CO₂ evolved could be calculated. The method of calibration was similar to that used for

oxygen uptake.

An alternative method has been described for measuring the evolution of CO_2 in a single sample over a period of several hours (Zeuthen & Kramhøft, personal communication). The difference between this method and that described above is that less cells are used in the measurement. If a 1.5 ml sample of a culture containing 2×10^6 cells/ml is placed in a macro-flask, the evolution of CO_2 can be followed in this sample for about 4 hours. This technique was used to follow the evolution of CO_2 in asynchronous and synchronous cultures. From a graph showing the accumulation of CO_2 in a culture, tangents were drawn to the curve at intervals and a measure of the rate of CO_2 evolution was made. At the end of each experiment carried out using this technique, the cell density of the sample of culture in the Warburg flask was measured and compared with the cell density of the original culture which had been maintained in a shaking water bath during the course of the experiment. This, in some way, overcomes one objection to this procedure, that no control can be maintained on the cells while they are in the Warburg flask.

Oxygen Uptake measured by a Polarographic Method.

The polarographic method for measuring the uptake of oxygen utilises a Clark oxygen electrode. Oxygen diffuses through a thin "Teflon" membrane (0.0005 in.) and is reduced at a platinum surface immediately in contact with the membrane.



The other half-cell, composed of a Ag/AgCl electrode, is also incorporated in the base of the incubation vessel. The platinum

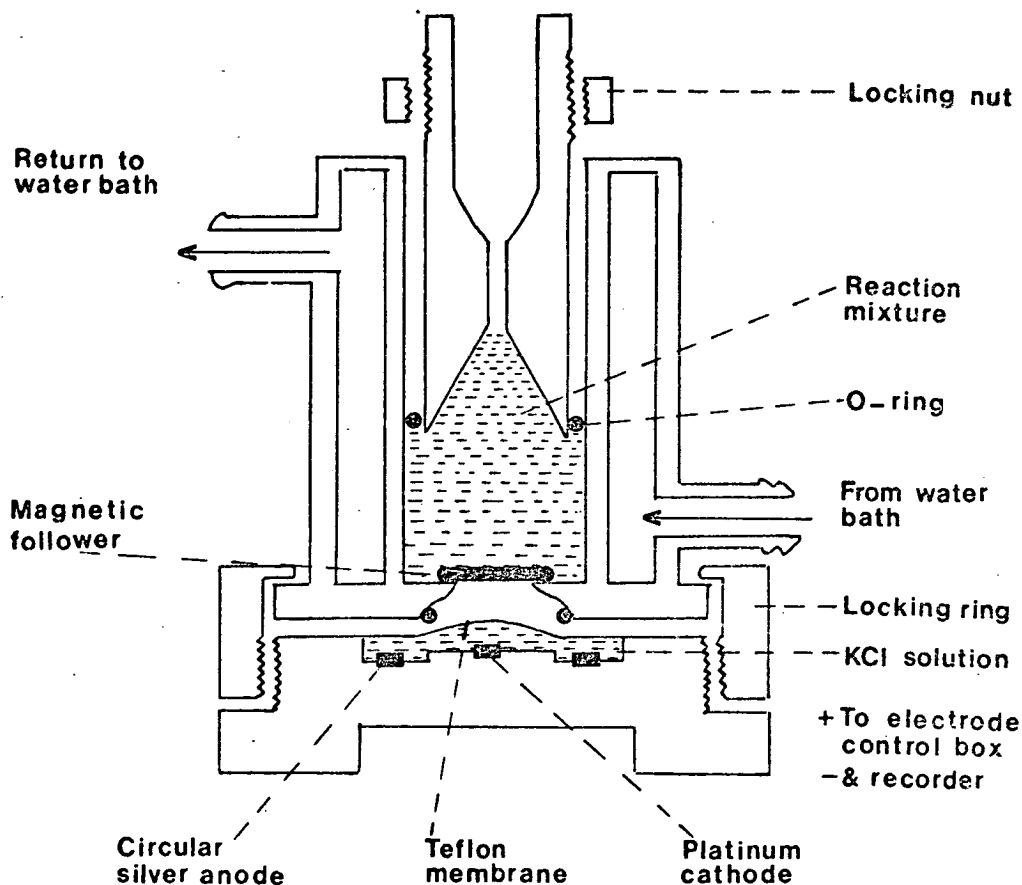


Fig 2 - 1. The Rank Oxygen Electrode.

The volume of the reaction mixture can be altered by changing the setting of the locking nut. Volumes varying from 1 ml to 5 ml can be used. The temperature of the circulating water was 33°C which maintained a temperature of 32°C in the chamber.

electrode is polarised at -0.6 v with respect to the Ag / AgCl and the current which flows under these circumstances is about 1 micro - amp in stirred, air-saturated water at 30°C. The current flowing is proportional to the partial pressure of oxygen in solution over a wide range.

A Rank Brothers Oxygen Electrode (Rank Brothers, Bottisham, Cambridge) was used to measure the dissolved oxygen concentration of solutions and hence the oxygen uptake of cultures of S. pombe. This apparatus is shown in Fig 2 - 1. The reaction chamber was made of perspex and this screwed down onto the electrode. The total volume of the reaction chamber was 5 ml. To measure oxygen consumption, a 3 ml sample containing at least 5×10^6 cells / ml was placed in the reaction vessel. Air was bubbled through this sample for 1 minute and 2 minutes later, the top was placed down onto the reaction chamber leaving no air space and taking care that no air bubbles were trapped in the reaction vessel. The culture was stirred throughout using a small magnetic follower. Additions could be made through the hole in the top using a No 21G syringe needle. The reaction chamber was kept at a constant temperature using a Grant water bath and a Grant circulating pump. A Rikadenki 2 Pen recorder, set at 1 mV full scale deflection, was used to measure the current flowing. From zero oxygen tension (achieved by adding a few crystals of sodium dithionite to EMM 2 in the reaction chamber) to air saturation fitted the full scale of the recorder. The concentration of salts in EMM 2 was sufficiently low that air saturation in EMM 2 was the same as air saturation in water. The solubility of oxygen in water was obtained from the International Critical Tables.

Two minutes after sealing the reaction chamber, the recorder was switched on and the fall in current was measured, thus giving a

measure of the oxygen consumption of the culture. In general, 6 - 10 minutes were required to establish the rate of oxygen uptake in a mid-exponential phase culture. This sample was then discarded and the procedure repeated using a further 3 ml sample from the experimental culture.

Assay for Hexokinase.

Hexokinase (E.C.2.7.1.1.) was assayed by a modification of the method of Darrow & Colowick (1962). Cells ($2-6 \times 10^6$) were collected on a Millipore filter (type BSWP), washed six times with distilled water and frozen. Samples could be stored in the deep freeze for several days. On thawing, 1 ml of the following reaction mixture was added to each sample :

0.1M Triethanolamine buffer, pH 7.6	4.78 ml
100 mg/ml glucose in the above buffer	4.48 ml
0.1 M Magnesium Chloride	0.75 ml
NADP (Sigma Chemicals, Ltd)	19 mg
Glucose-6-Phosphate Dehydrogenase (Boehringer & Sons)	17 μ L

The blank in this procedure was substrate without cells. This blank was chosen, rather than a blank with cells and without glucose-6-phosphate dehydrogenase, since the latter did not increase with increasing incubation time whereas the former did. This was probably caused by hexokinase contamination in the glucose-6-phosphate dehydrogenase. Leaving out the glucose from the incubation mixture gave a blank value which was constant irrespective of the incubation time.

Rather than follow the formation of NADPH on a recording spectrophotometer, a simple procedure was developed to measure the

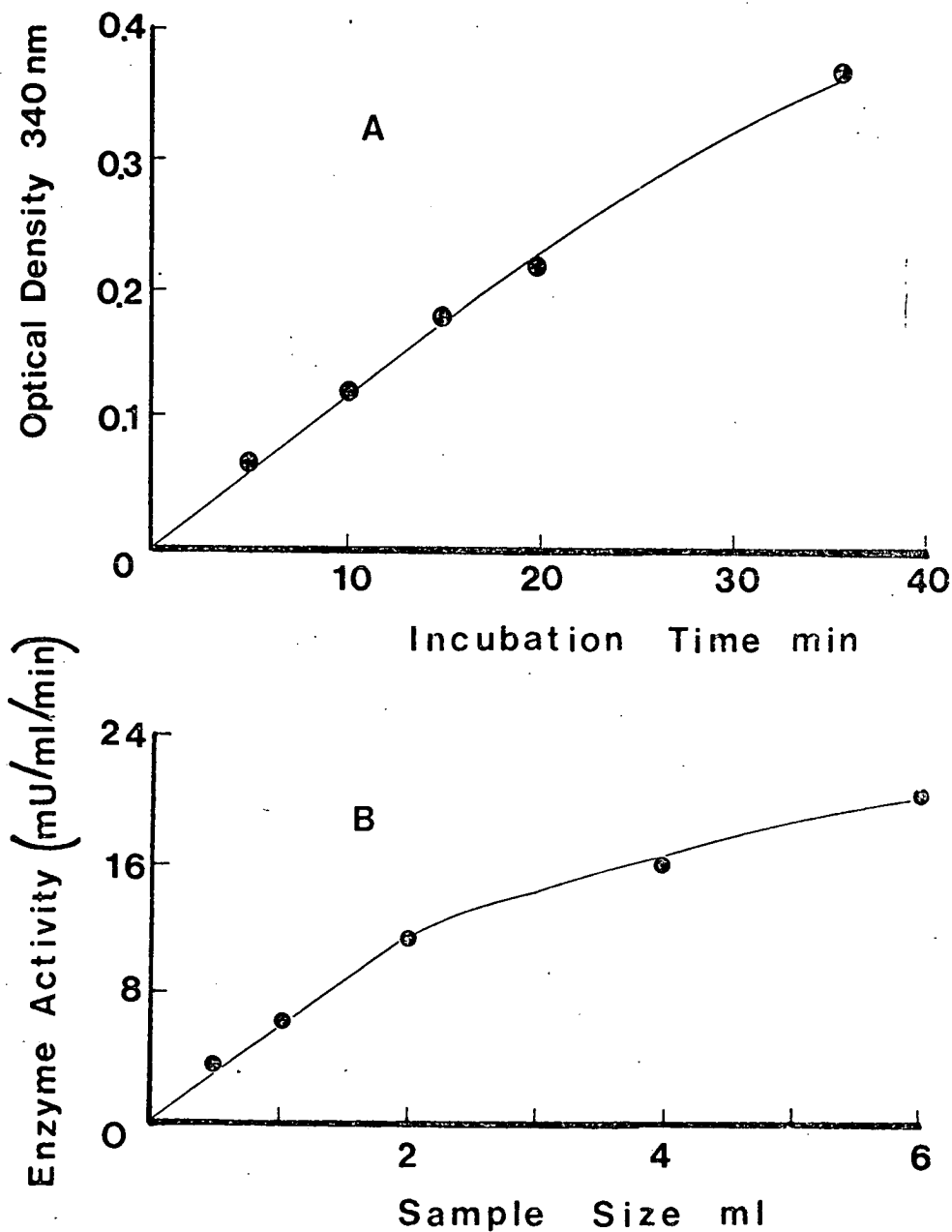


Fig 2 -2. Activity of Hexokinase with A. Increasing incubation time
B. Increasing sample size.

A. 0.5 ml samples of an exponential culture containing 3.5×10^6 cells/ml were assayed for hexokinase with various times of incubation.

B. Samples ranging from 0.5 ml to 6 ml were taken from the above culture. Samples were assayed for hexokinase using a 10 minute incubation time.

optical density at 340 nm of each sample after a constant incubation time (generally 10 - 20 minutes). This required that the reaction was stopped and that the NADPH which had been produced was not re - converted to NADP. To do this, 0.5 ml of 0.1 M EDTA was added to each sample. The effect of this was to chelate the magnesium from the reaction mixture and, since hexokinase and glucose-6-phosphate dehydrogenase both require magnesium, the reaction stopped. When this had been done, it was found that there was a gradual fall in the optical density at 340 nm, caused by the re-conversion of NADPH to NADP. To prevent this from happening, 0.5 ml of methanol was added to each sample at the same time as the EDTA. The optical density was then stable for at least an hour.

To obtain adequate enzyme activity, samples contained $2 - 6 \times 10^6$ cells. The linearity of the response of this range of cell concentrations is shown in Fig 2 - 2. The linearity of enzyme activity with incubation time is shown in the same figure. Provided that no more than 8×10^6 cells were incubated, a reasonably linear response with increasing cell concentration was obtained.

Hexokinase was assayed in synchronous cultures of S. pombe. These cultures were prepared by the method described above, using lactose gradients. Samples were collected in duplicate from the culture, washed six times and frozen overnight. On removal from the deep-freeze, all of the samples were assayed at the same time, setting up each sample 10 seconds apart and stopping the reaction at the same time interval. The time taken to read the optical density of the sixty or so samples from a synchronous culture was well within the hour for which it had been shown that the optical density of the NADH was stable.

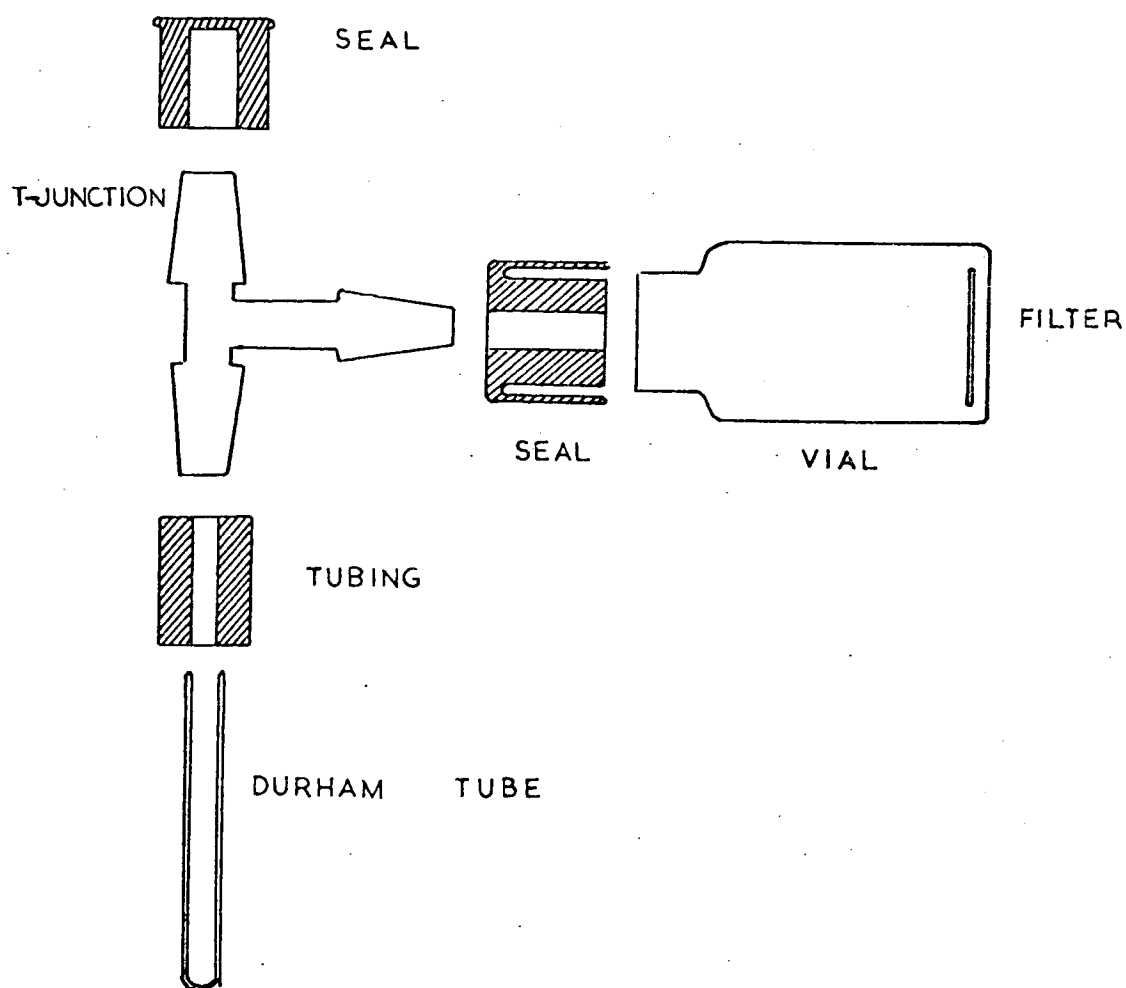


Fig 2 - 3. Apparatus used for the collection of $^{14}\text{CO}_2$ evolved from a growing culture.

See text for details.

Measurement of $^{14}\text{CO}_2$ Evolution using a Radiorespirometric Method.

The measurement of $^{14}\text{CO}_2$ evolution is a widely used technique for determining the activities of biochemical pathways using a specifically labelled precursor (Bloom & Stetten, 1953; Wang, Stern, Gilmour, Klumsoyr, Reed, Bialy, Christenson & Cheldelin, 1958). This method has also been used to measure the relative activities of the catabolic processes involved in the utilization of glucose by yeast (Duncombe, 1974).

D(U- ^{14}C) glucose, 284 mCi / mMole & 255 μCi / ml, D(1- ^{14}C) glucose, 60 mCi / mMole & 201 μCi / ml and D(6- ^{14}C) glucose, 59.6 mCi / mMole & 196 μCi / ml were obtained from the Radiochemical Centre, Amersham. The apparatus used for trapping the radioactive CO_2 was a modification (Sissons, 1976) of that described by Wohlhueter and Harper (1970). The apparatus is shown in Fig 2 - 3. The T - junction was made of polypropylene with an outside diameter of 7 - 9 millimetres. The long side - arm passed through a hole in a size 33 "subseal" turnover seal (William Freeman & Co, Barnsley, England) into a scintillation vial containing a 2.1 cm diameter Whatman GF/C glass fibre filter and 0.1 ml 1N KOH. The top side - arm was sealed by a size 29 "subseal" placed over the outside of the side - arm. 2cm of rubber tubing (4 mm bore) were placed over the outside of the bottom arm of the T - junction and onto this tubing was placed a Durham Fermentation tube (50mm X 6mm) containing the reaction mixture. The Durham tube was placed in a metal rack in a water bath at 32°C at a height such that the scintillation vial rested on the side of the water bath. The tubes were mixed on a vortex mixer every 5 minutes during the incubation and every 15 minutes during the subsequent collection of the radioactive CO_2 .

Experiments measuring the evolution of $^{14}\text{CO}_2$ were carried out

using the following three procedures :

1. 0.01 ml aliquots of radioactive glucose were placed in Durham fermentation tubes. 0.05 ml samples of cells were added to these tubes at intervals and after incubating for 10 - 15 minutes at 32°C, cell growth was stopped by injecting 0.1 ml 2 M perchloric acid into each tube through the top seal using a 1 ml disposable syringe and a 1 inch 25C disposable needle. The apparatus was then left in the water bath for a further 2 hours, mixing the contents of the Durham tube every 15 minutes. This ensured that all of the $^{14}\text{CO}_2$ was evolved and absorbed onto the KOH.

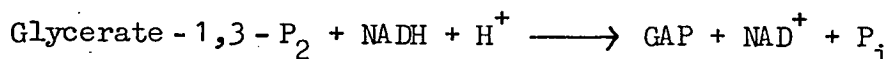
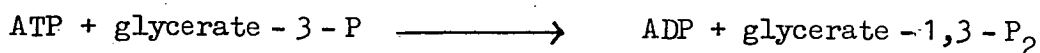
2. A quantity of radioactive glucose (normally about 0.1 ml) was added to a sample of culture (2 - 4 ml) which was then incubated in a water bath at 32°C. At intervals, 0.1 or 0.05 ml samples were removed and incubated in a Durham tube for 10 - 15 minutes while trapping the $^{14}\text{CO}_2$ evolved by the method described above.

3. Several 0.05 ml samples of a culture were placed in Durham tubes. 0.005 ml radioactive glucose were added to each sample as close to the same time as possible. The tubes were then sealed in the apparatus described above and incubated at 32°C for varying lengths of time. The reactions were stopped in each tube at the appropriate time by injecting 0.1 ml 2M PCA as described above.

After all of the $^{14}\text{CO}_2$ had been absorbed by the KOH, 10 ml 30% methoxyethanol and 0.5% butyl - PBD [2-(4' - tert - butylphenyl) - 5-(4'' - biphenyl) - 1,3,4, - oxadiazole] in toluene were added to each scintillation vial. Each sample was counted for 4 minutes as soon as possible after adding scintillant in a Packard Scintillation Counter, Model 3320.

Assay for ATP.

ATP was assayed using a Boehringer Biochemical Test Combination for ATP. The principal of this assay is as follows :-



The first reaction is catalysed by phosphoglycerokinase and the second by glyceraldehyde - 3 - phosphate dehydrogenase.

10 ml of an exponential culture containing at least 1.5×10^6 cells /ml were filtered on an Oxoid membrane filter (2 cm) which was immediately placed in a 25 ml beaker containing 1.8 ml distilled water at 100°C . The filter was left in the boiling water for 10 minutes to extract all of the ATP then it was left at room temperature for a further 10 minutes. The contents of the beaker were then transferred to a LP 3 (Luckham) tube which had been previously calibrated to hold 1 ml. The beaker was rinsed out twice with 0.1 ml distilled water and the total volume of the extract was made up to 1 ml. The tube was then centrifuged for 5 minutes at 2000 rpm using an MSE Mistral centrifuge and 0.8 ml supernatant removed for assay of ATP.

The materials used for the assay were as follows :-

Solution 1. 0.5 M triethanol amine buffer - pH 7.6

4 mM MgSO_4

6 mM glycerate - 3 - phosphate.

Solution 2. 2.5 mM NADH.

Solution 3. 7 mg / ml glyceraldehyde - 3 - phosphate dehydrogenase.

1 mg / ml phosphoglycerokinase

The reaction mixture was prepared by mixing 1 ml Solution 1, 0.1 ml Solution 2 and 0.4 ml cell extract. This was equilibrated for 6 minutes in a Unicam SP 800 spectrophotometer in a 1 cm path length cuvette. The OD_{340} was measured during the equilibration. To start the reaction, 0.01 Solution 3 was added, mixed well with a small paddle and the change in

OD₃₄₀ was measured for about 10 minutes. The fall in the optical density is a measure of the amount of ATP in the extract.

CHAPTER 3.

Oxygen Uptake in Cultures of *S. pombe*.

Measurement of Oxygen Uptake in Asynchronous Cultures using a Manometric Method.

Oxygen uptake was measured, in the first instance by a manometric method as described in Chapter 2. In this method, a constant volume was maintained in a sealed chamber and the amount of oxygen taken up was measured by changes of the pressure in the chamber.

The rates of oxygen uptake measured by the manometric method in a culture of *S. pombe* from mid-logarithmic phase until stationary phase are shown in Fig 3-1. There was gradual increase in the rate of oxygen uptake during exponential phase with a doubling time equal to the doubling time of cell numbers. When the culture entered stationary phase, the rate of oxygen uptake remained constant, in this experiment for at least five hours after growth had ceased. The nature of the oxygen uptake of stationary cultures will be discussed later in this Chapter.

The measurements of the rate of oxygen uptake shown in Fig 3-1 were achieved in a 20 minute period, using a culture which contained at least 20 million cells/ml. It was not possible to obtain a measure of the rate of oxygen uptake using a more dilute culture because of the relatively low amount of oxygen taken up during growth on glucose. In a culture as dense as this, nutrient levels, pH of the medium and oxygen solubility are going to change rapidly and any one of these could bring about a change in the pattern of oxygen uptake. For this reason, it was decided that experiments in which the uptake of oxygen was measured should be carried out, as far as possible, on cultures in which the cellular environment would change less. This involved using

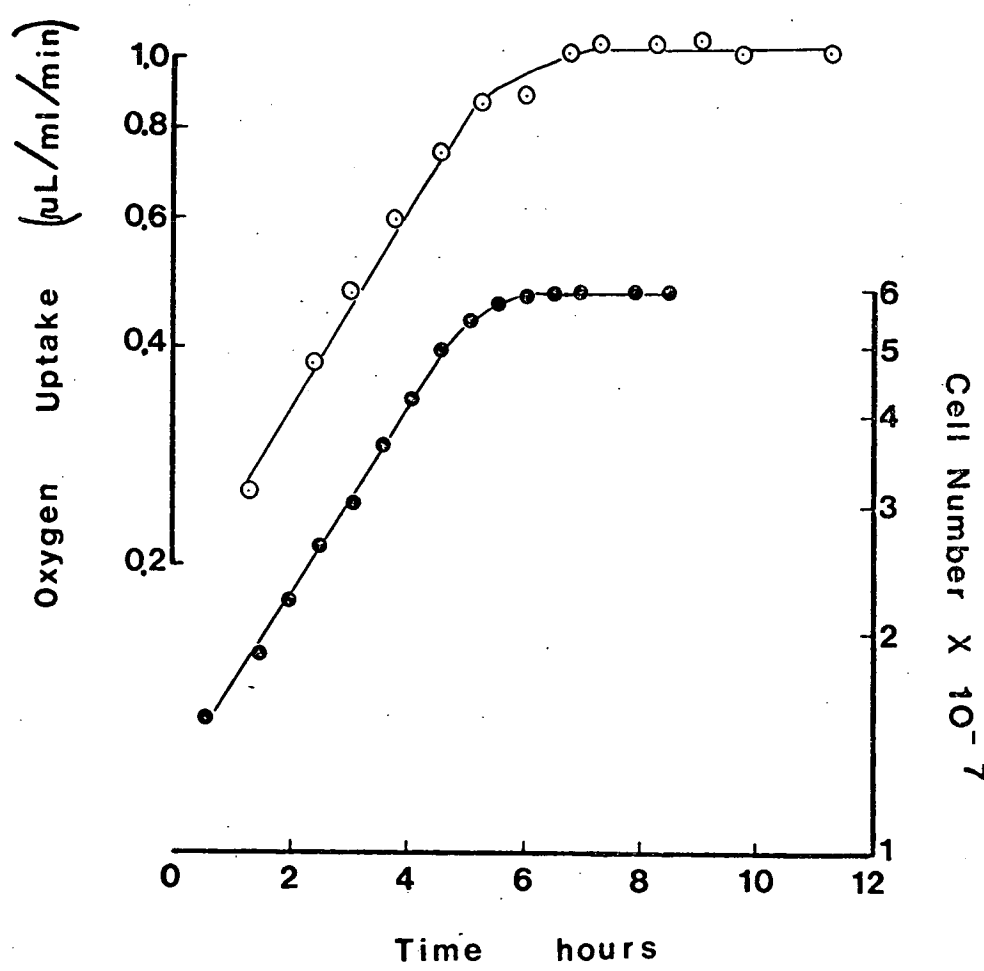


Fig 3 -1. Oxygen Uptake during the growth of an exponential culture of S. pombe.

Oxygen uptake was measured at intervals on 4 X 2 ml samples of culture for 40 minutes. After measurements, the samples were discarded.

○—○ Rate of oxygen uptake. ●—● Cell number.

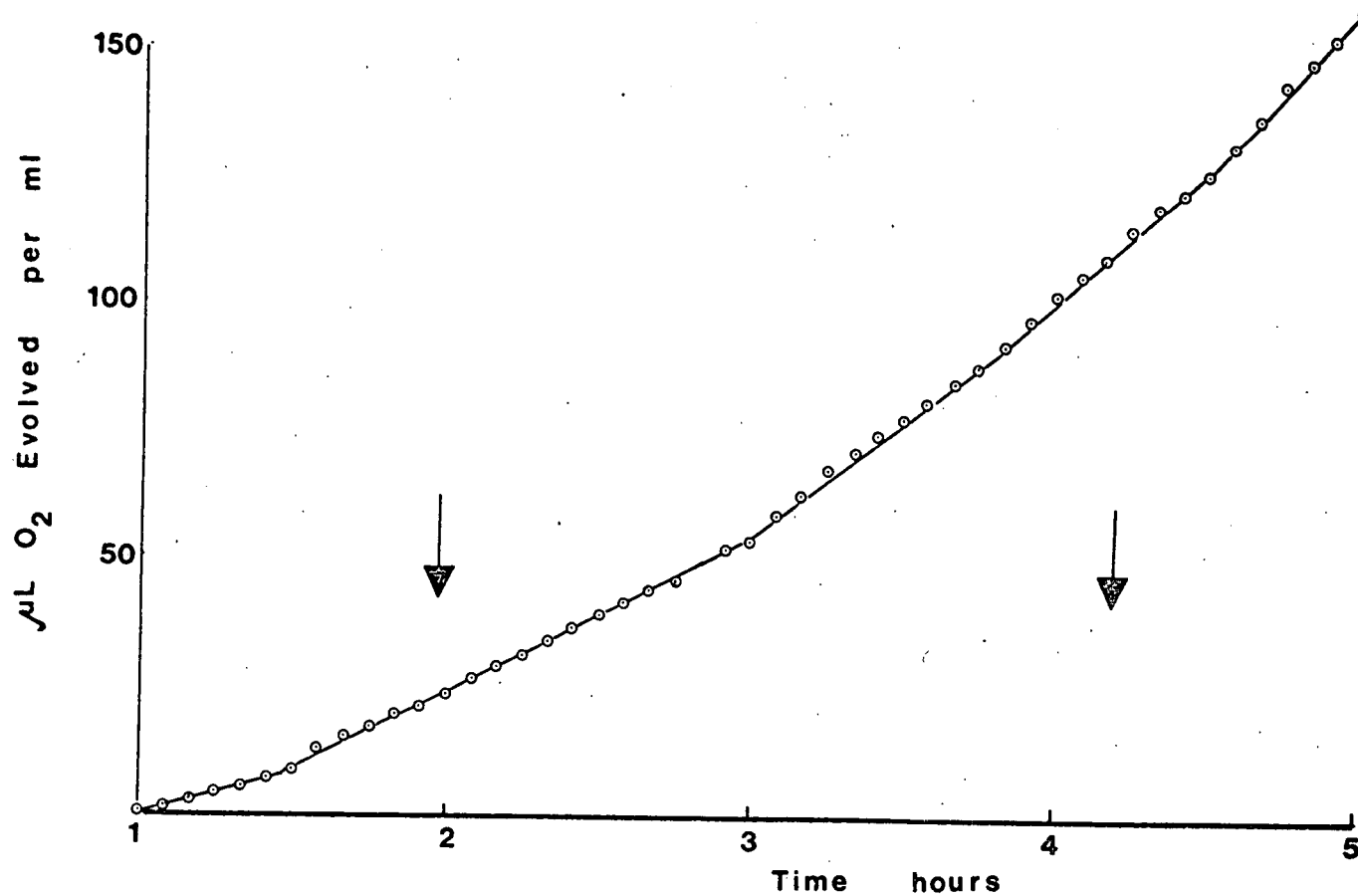


Fig 3-2. Oxygen uptake in a synchronous culture of S. pombe.

Oxygen uptake was measured every 5 minutes of 2 X 2 ml samples in micro-manometer flasks.

Arrows denote the times of the cell plate peaks during the first and second cycles.

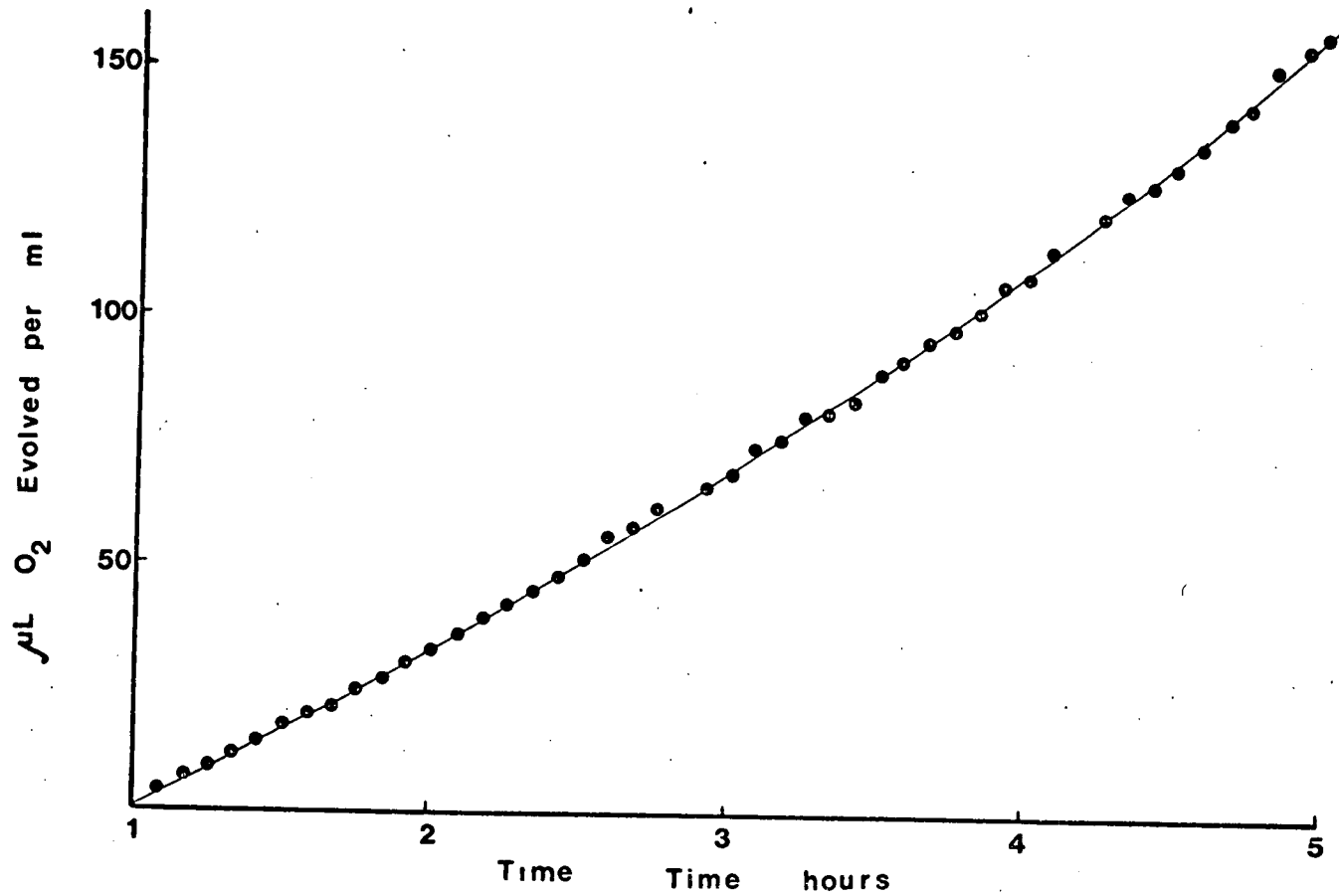


Fig 3-3. Oxygen uptake in an asynchronous control culture.

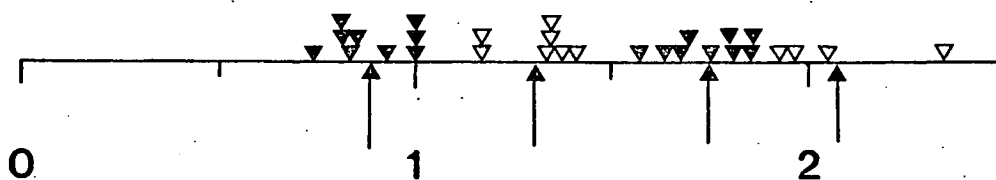
A control culture was prepared as described in the text. Oxygen uptake was measured on 2 X 2 ml samples every 5 minutes.

more dilute cultures and measuring the total amount of oxygen which was taken up by one sample continuously during at least two generation times. This method is less sensitive in measuring changes in rate but it is likely to be more reliable.

Oxygen Uptake in Synchronous Cultures using a Manometric Method.

A synchronous culture of S. pombe was prepared as described previously using a lactose gradient. Immediately after setting up the synchronous culture, 2 ml samples were placed in each of three micro manometer flasks (volumes approximately 5 ml). After attaching these flasks to the manometers, the apparatus was placed in a Braun water bath and left to equilibrate for 10 minutes. The taps on the manometers were then closed and the oxygen uptake measured on each sample for about 5 hours. The average oxygen uptake for the three cultures was then calculated and the results from one such experiment are shown in Fig 3 - 2. The results suggest that there are two changes in the rate of oxygen uptake during each cell cycle.

Oxygen uptake was measured on an asynchronous culture which had gone through the synchronisation technique as described in Chapter 2. The results of an experiment of this nature, shown in Fig 3 - 3, suggest that there is a smooth increase in the amount of oxygen taken up by this culture, with none of the sharp changes in rate which were observed in the synchronous cultures. The rate changes which were observed during the growth of the synchronous cultures are probably cell - cycle related events and not artifacts introduced by the synchronisation procedure. The possibility that the small cells which were removed from the top of the gradient were more susceptible to perturbations caused by the synchronisation procedure cannot be ruled



- 3-4. Cell cycle map of rate changes in O_2 uptake measured manometrically. Each arrow above the line represents a change in the rate of O_2 uptake in a synchronous culture. The data are divided up into 1st., 2nd., 3rd. and 4th rate changes measured during two cell cycles. In two experiments, rate changes were not seen near the beginning of the 2nd. cycle. The second rate changes measured in these two experiments are grouped with the 3rd. rate changes in the other experiments. Convincing rate changes at the start of the 3rd. cell cycle were only observed in 5 synchronous cultures.

▼ 1st. & 3rd. Rate changes. ▽ 2nd. & 4th. Rate changes.

Arrows below line represent means of each group of rate changes.

Experiment	1 st Change	2 nd Change	3 rd Change	4 th Change.
1	0	0.67	0.58	
2	0	0.39	0.51	
3	0	0.5	0.44	
4	0	0.35	0.4	0.36
5	0	0.52	0.36	0.35
6	0	0.77	0.39	
7	0	0.42	0.47	0.32
8	0	0.54	0.37	
9	0	0.54	0.48	
Mean		0.52	0.44	0.34
St. Dev ⁿ		0.133	0.07	0.021
St. Error		0.044	0.024	0.012

TABLE 3 - 1. Analysis of rate changes in oxygen uptake measured by the Warburg technique. The first rate change in each experiment is taken as Time 0; the rest of the rate changes during the two cell cycles are calculated in cell cycle time from the previous rate change.

out. If this were so, any such effect would be masked in the control cultures by the rest of the population which would not be affected in this way.

The collected results from measuring the oxygen uptake in 9 synchronous cultures are shown in the cell cycle map in Fig 3-4. There is considerable variation in the time at which the rate changes occurred in the various synchronous cultures and presenting the data in this way does not show any clear cell cycle pattern. It might be concluded that there is a group occurring near the end of the first cycle after inoculation of the synchronous culture, and two further groups in the first and second halves of the second cell cycle. The data was then presented in a different way to determine whether there was a significant similarity in the oxygen uptake patterns of the different cultures. The data which is shown in TABLE 3-1 shows the time intervals between the rate changes in individual experiments, taking the time of the first rate changes as zero in each case and expressing subsequent rate changes in cell cycle time after the preceeding rate change.

It is difficult to say precisely at what time during the cycle the changes in the rate of oxygen uptake occur. I would emphasise that the imprecise nature of these experiments makes the data shown in Fig 3-4 very dubious. However, the data collected in Table 3-1 do suggest that in the different experiments, while the exact timing of the rate changes in oxygen uptake vary during the cell cycle from experiment to experiment, there is always about half a cell cycle between the different rate changes in one experiment. This does not apply to the fourth rate change ; since, however, this was only seen in three experiments, and since considerable asynchrony had entered into the culture by this time, this observation is not considered important. Further discussion on the significance of this result is considered in Chapter 9.

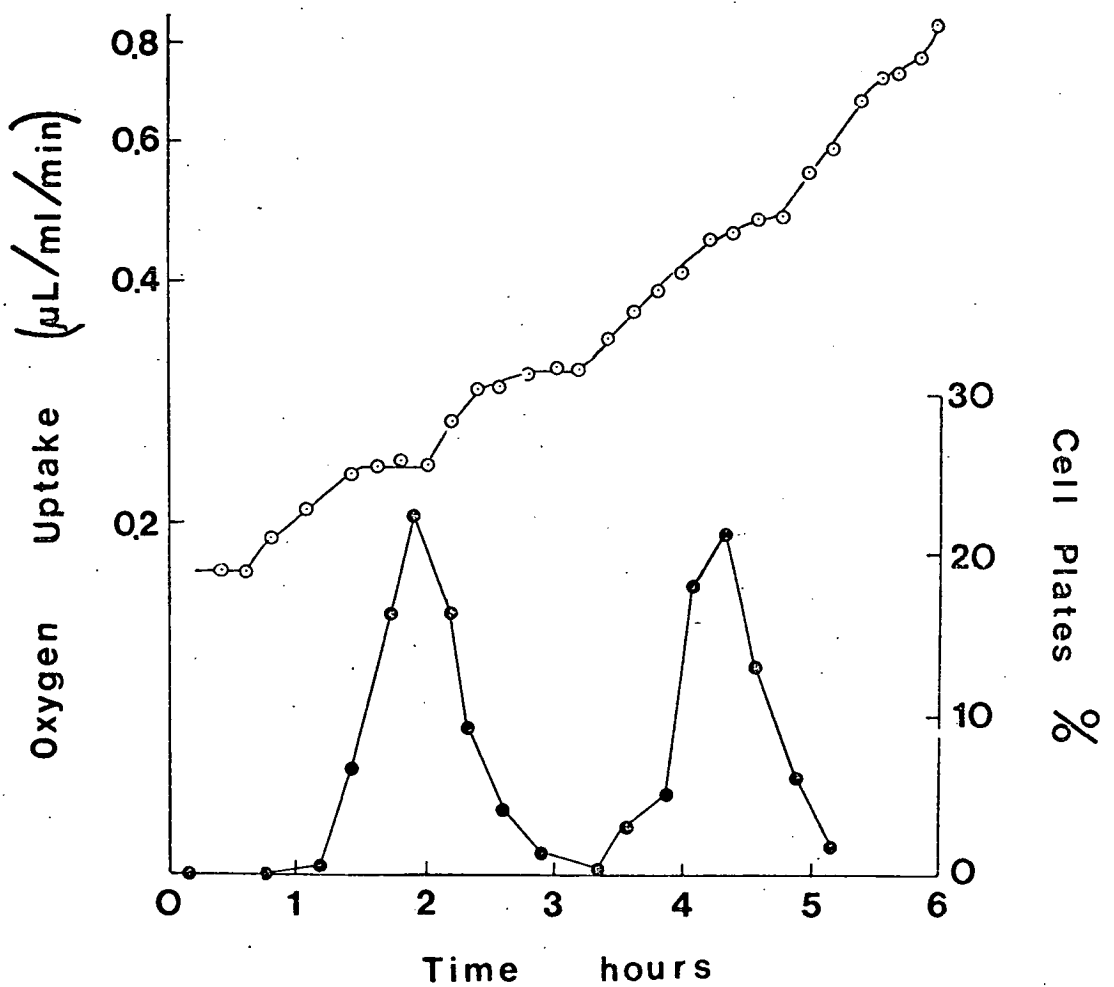


Fig 3-5. Oxygen uptake in a Synchronous Culture measured polarographically.

Oxygen uptake was measured every 12 minutes on 2 ml samples of a synchronous culture. Samples were discarded after each measurement.

○—○ Rate of Oxygen Uptake. ●—● % Cell Plates.

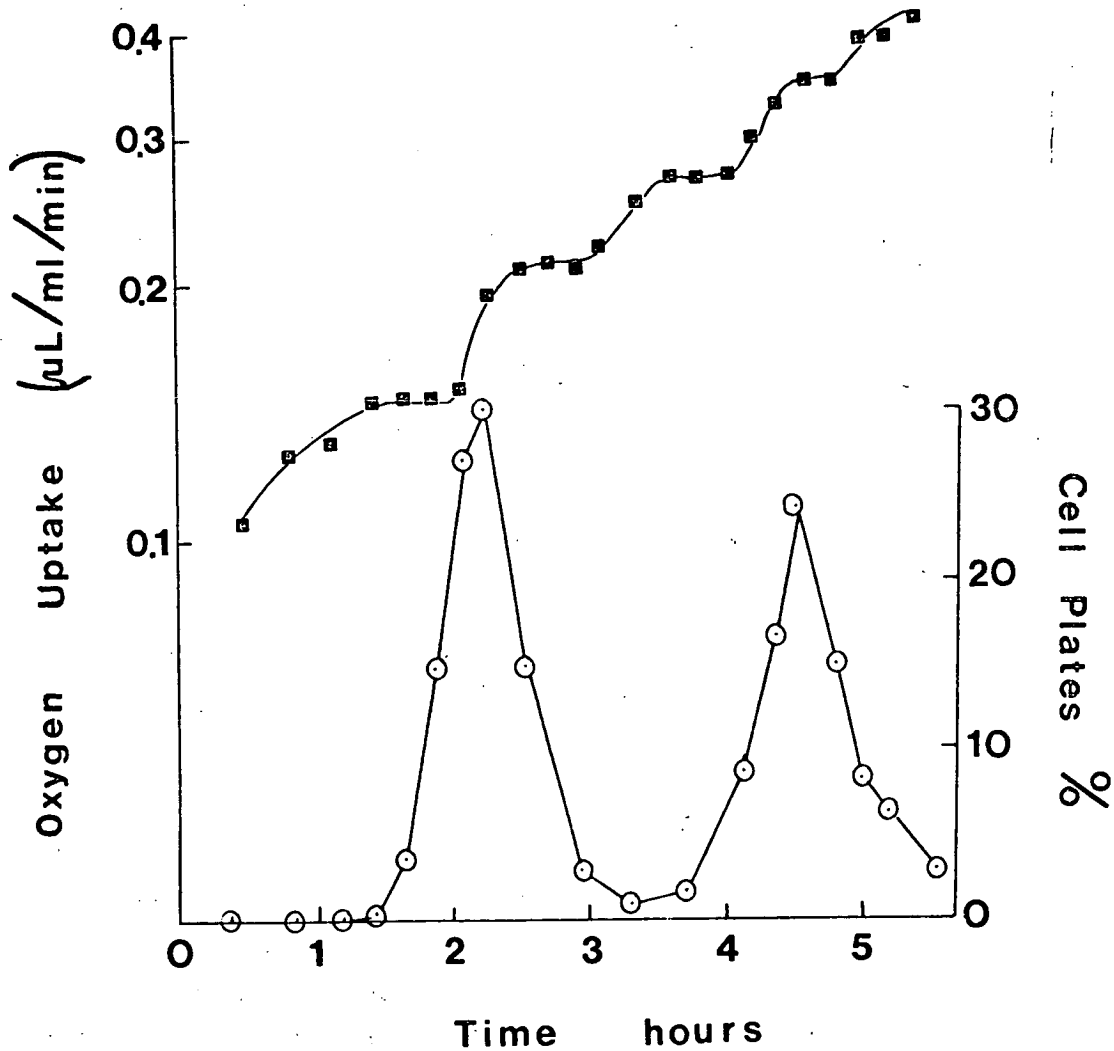


Fig 3 - 6. Oxygen Uptake in a Synchronous Culture measured polarographically.

Oxygen uptake was measured every 15 minutes on 2 ml samples.

After each measurement, the samples were discarded.

■ — ■ Rate of Oxygen Uptake.

○ — ○ % Cell Plates.

Polarographic Measurement of Oxygen Uptake in *S. pombe*.

Oxygen uptake was measured by the polarographic method which utilises the oxygen electrode described in Chapter 2. In this method, the current which flows through a culture is proportional to the amount of dissolved oxygen and the rate at which the current decreases is a measure of the rate of oxygen uptake in a growing culture. There are several advantages in this method which overcome some of the criticisms which have already been made of the manometric technique. In the first place, oxygen is measured in the presence of CO_2 . Secondly, this system is much more sensitive to changes in the uptake of oxygen and measurements of the rate of oxygen uptake could be made in a mid-exponential phase culture within 5 to 10 minutes. The concentration of cells which is required to achieve this is much less than that required in the manometric method so that the problem of changes in the environment of the cell during a short period of time is diminished in this case.

Oxygen Uptake in Synchronous Cultures.

Synchronous cultures were prepared using lactose gradients. The uptake of oxygen was measured on 3 ml samples from the culture, about once every 10 minutes as described in Chapter 2. The rates of oxygen uptake in two typical synchronous cultures are shown in Fig 3-5 and Fig 3-6. There were two periods during the cell cycle when the rate of oxygen uptake increased sharply. The extent of these changes in the rate were such that overall, the rate of oxygen uptake doubled once each cell cycle.

Asynchronous control cultures were prepared by the method previously described and the rate of oxygen uptake was measured on

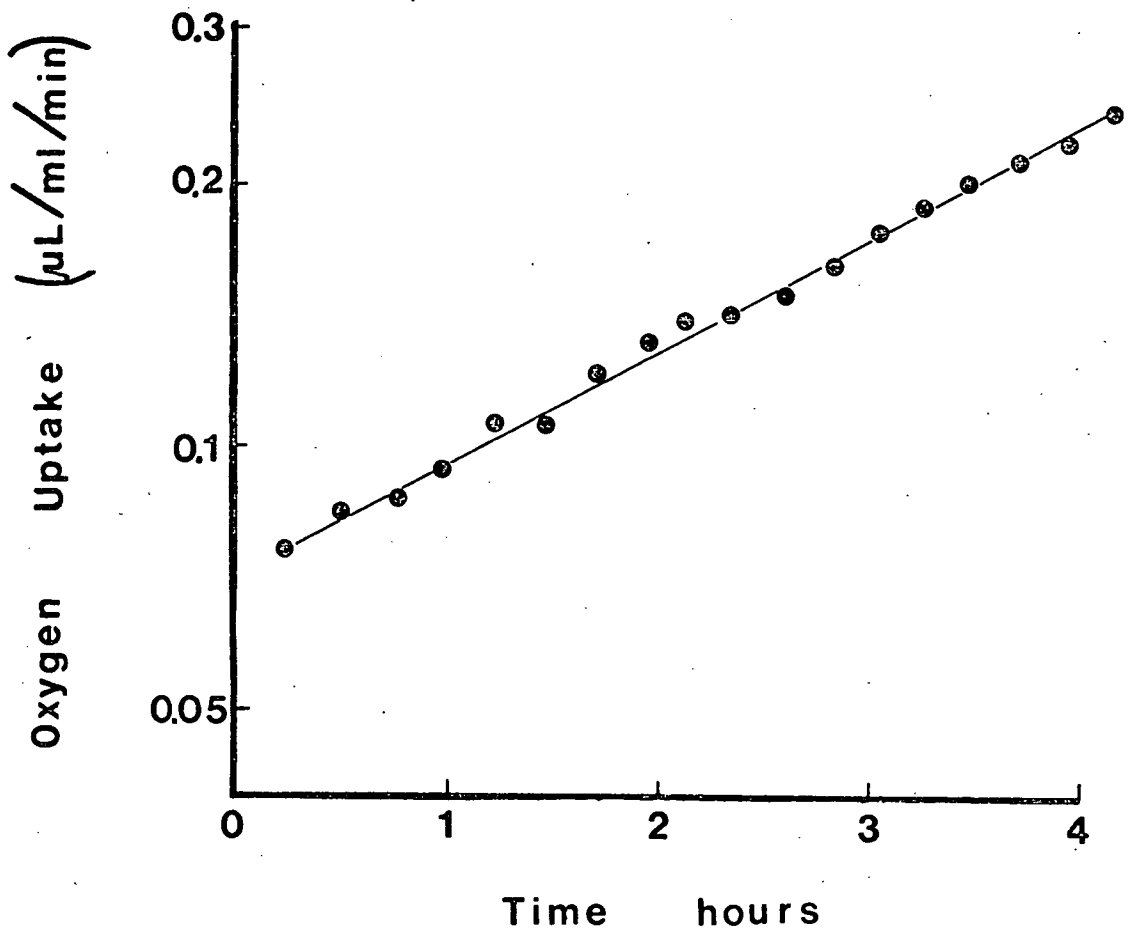


Fig 3 - 7. Oxygen Uptake in an Asynchronous Control Culture measured manometrically.

An asynchronous culture was prepared as described in the text. Oxygen uptake was measured at 15 minute intervals on 3 ml samples. After each measurement, samples were discarded.

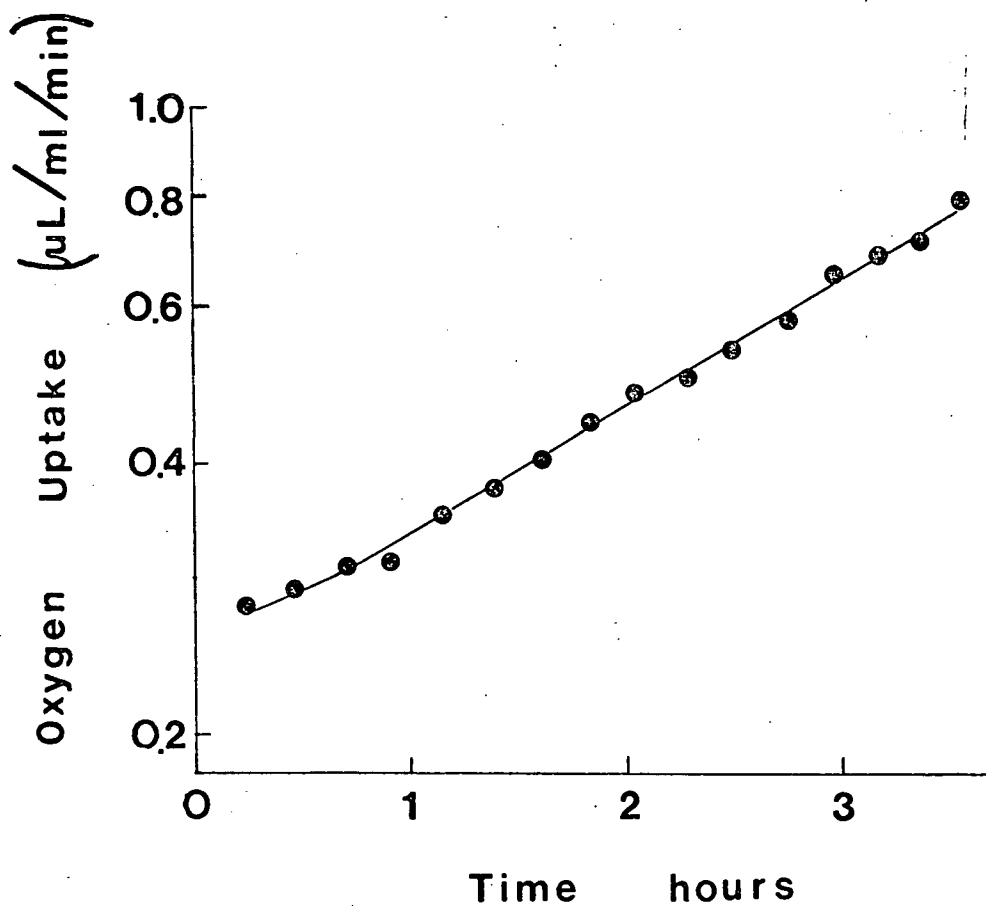


Fig 3 - 8. Oxygen Uptake in an Asynchronous Control Culture measured manometrically.

An asynchronous control culture was prepared as described in the text. Oxygen uptake was measured at 15 minute intervals on 3 ml samples. After each measurement, the samples were discarded.

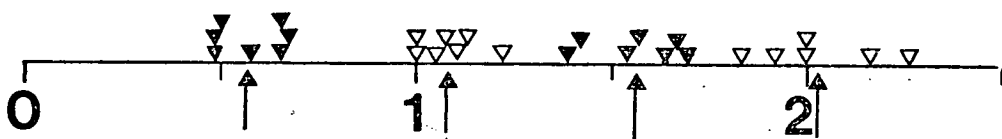


Fig 3 - 9. Cell Cycle map for rate changes in oxygen uptake measured using the oxygen electrode.

Each arrow above the line represents a change in the rate of oxygen uptake in one experiment.

Closed arrows represent rate changes in 1st cycle and half way through the second cycle.

Open arrows represent rate changes at the beginning of the second cycle and at the beginning of the third cycle.

Arrows below the line represent the means of first, second, third & fourth rate changes in all of the experiments.

Experiment	1 st Change	2 nd Change	3 rd Change	4 th Change
1	0	0.57	0.49	
2	0	0.56	0.65	0.57
3	0	0.57	0.43	
4	0	0.52	0.41	0.40
5	0	0.50	0.47	0.46
6	0	0.50	0.36	0.67
7	0	0.48	0.43	0.35
Mean		0.53	0.46	0.49
St. Dev ⁿ		0.04	0.093	0.13
St. Error		0.01	0.035	0.069

TABLE 3-2. Analysis of rate changes in oxygen uptake measured by the oxygen electrode. The first rate change in each experiment is taken as Time 0; the rest of the rate changes during the two cell cycles are calculated in cell cycle time from the previous rate change.

3 ml samples removed from the culture at intervals. The results from two of these experiments are shown in Fig 3-7 and Fig 3-8. The rate of oxygen uptake in both of these cultures showed a smooth increase with a doubling time equal to the doubling time of the culture. The sharp changes in the rate of oxygen uptake which were shown in the synchronous cultures can therefore be considered to be cell cycle events.

The collected results from measuring the rate of oxygen uptake in seven synchronous cultures are shown in the cell cycle in Fig 3-9. The results suggest that there is a consistent step in the rate of oxygen uptake mid-way through the first cell cycle after re-inoculation. There is then a second rate change occurring at the start of the second cell cycle and a further group at the middle of the second cycle. In some experiments, it was possible to see a further rate change at the beginning of the third cycle but, since the synchrony of the culture was deteriorating at this stage, it was not always possible to see this last rate change.

The time at which the rate changes occurred was then calculated in the same way as the data from the manometric experiments. The time of the first rate change was taken as zero and the data in TABLE 3-2 show the time intervals between the rate changes in individual experiments, expressing these time intervals in cycle time from the preceeding rate change. It is obvious from these data that there is a fairly constant time interval between the individual rate changes and this is very close to half of a cell cycle. This more or less agrees with the data obtained using the manometric method but is probably more reliable. There is also no significant difference between the intervals from the second to the third rate changes and from the third to the fourth, where the latter exist.

Oxygen uptake was measured in one other synchronous culture but I have not included this data in the cell cycle map shown in Fig 3-9. In this experiment, the rate of oxygen uptake changed mid-way through the first cycle, then again at 0, 0.26 and 0.62 of the way through the second cycle. In the second cycle, therefore, there was only about a quarter of a cycle between the times at which the rate changes in oxygen uptake occurred. This is unlike any of the other experiments which I have shown in Fig 3-9 and for this reason, I have not included this data in the cell cycle map

Oxygen Uptake of Stationary Cultures.

It was shown at the beginning of this Chapter how the rate of oxygen uptake was maintained at a high and constant level for many days after the culture had entered stationary phase. A brief attempt was made to determine the nature of the oxygen uptake of stationary cells.

A one day old stationary culture was filtered, washed and re-suspended in phthalate buffer at pH 4.5. It was assumed that this buffer would not have any detrimental effect on the cells since a modification of EMM2 has been developed in this laboratory which uses phthalate as buffer instead of acetate. Within 30 minutes after resuspension of the cells, the uptake of oxygen had fallen to 0. This suggests that there are no endogenous reserves of oxidisable material in the cells and that the high level of oxygen uptake in stationary cells is caused by a substrate in the medium.

Oxygen uptake of the washed cells was then measured in the presence of all of the components likely to be in stationary medium. The oxygen uptake with 0.1% ethanol (0.67 uL/ml/min) and 1% acetate (0.37 uL/ml/min) was similar to that seen in stationary cells (1.17 uL/ml/min). The oxygen uptake of stationary cells is probably the utilisation of these substrates.

So although S. pombe is able to oxidise these two substrates, it is unable to use them as carbon source. This information does raise the question of how much of the oxygen uptake observed in exponentially growing cells is caused by the oxidation of ethanol and acetate. Or, does the ability to oxidise these two compounds only originate when the culture has entered stationary phase and the oxygen uptake during exponential phase is caused by the oxidation of glucose?

CHAPTER 4

Oxygen Uptake in the presence of various Inhibitors.

It was shown in the previous Chapter how the rate of oxygen uptake varied during the cell cycle of S. pombe. At approximately the middle of the cell cycle the rate of oxygen uptake increased by approximately 50% then it remained constant until close to the start of the next cycle. The rate then increased again by about the same amount as before and again remained constant until mid-way through the next cycle when the pattern was repeated. In this Chapter, experiments will be described in which the cell cycle was distorted by the addition of an inhibitor of DNA synthesis and the effect of this inhibitor on the uptake of oxygen will be examined. Synchronous cultures will also be prepared using one of two DNA synthesis inhibitors in pulses and oxygen uptake will again be measured. Finally, the normal cell cycle will be distorted using a nuclear division inhibitor, mitomycin C, and oxygen uptake will be measured. In this way it is hoped to show the relationship between the events which constitute the normal cell cycle of S. pombe and the pattern which has been demonstrated for the uptake of oxygen during the normal cell cycle.

Oxygen Uptake in a Synchronous Culture containing Deoxyadenosine.

A synchronous culture was prepared using a lactose gradient as previously described. Forty minutes after preparation of the synchronous culture, deoxyadenosine was added to the culture to give a concentration of 2×10^{-3} molar and oxygen uptake was measured polarographically about every 15 minutes. The results, shown in Fig 4-1, show that the uptake of oxygen increased in the same periodic manner as was seen in a normal



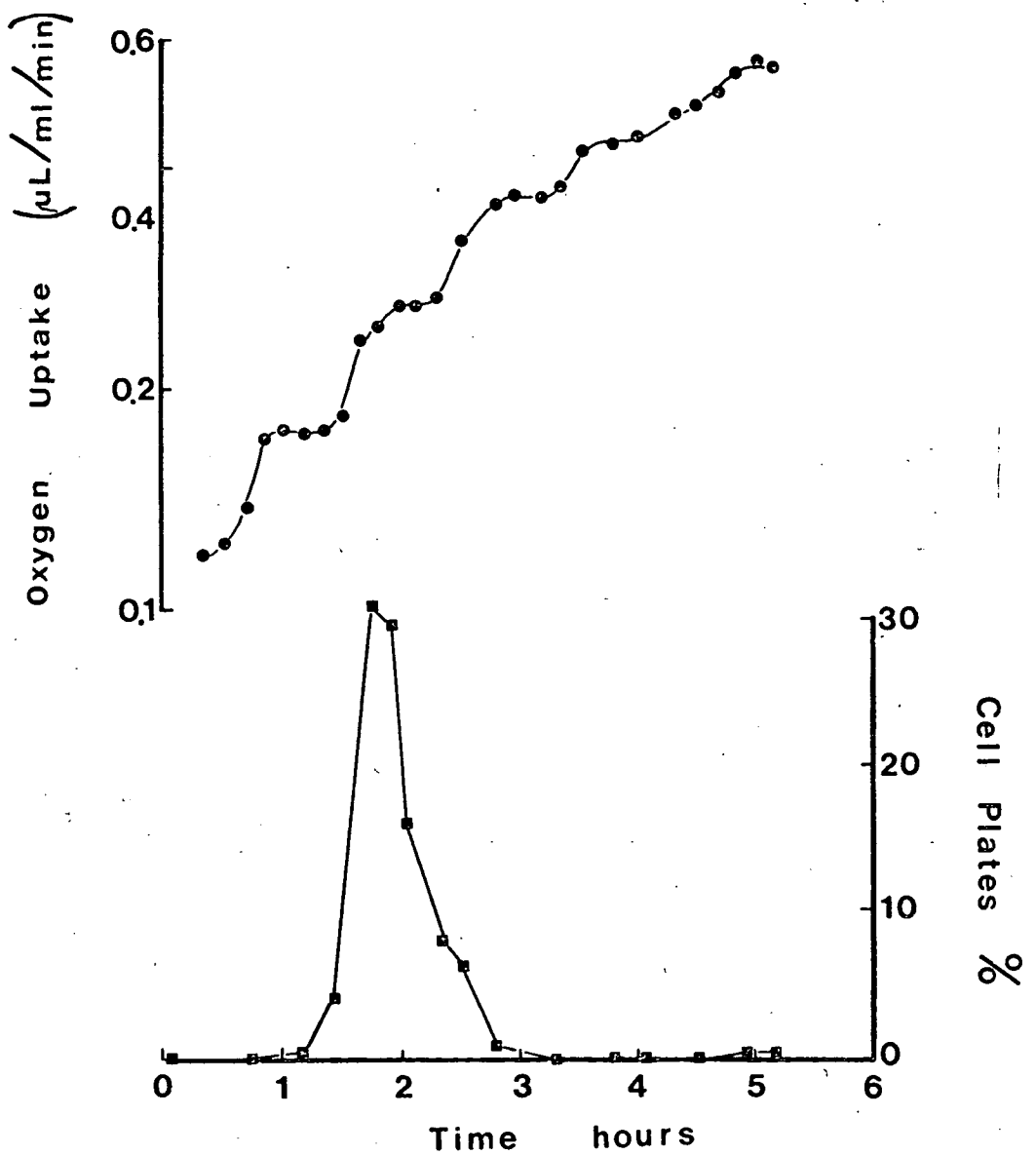


Fig 4-1. Oxygen Uptake in a synchronous culture containing deoxyadenosine.

A synchronous culture was prepared using a lactose gradient.

At 30 minutes, deoxyadenosine was added to the culture giving

2×10^{-3} molar. Oxygen uptake was measured at 10' intervals

on 1 ml samples using the oxygen electrode.

●—● Rate of oxygen uptake.

■—■ % Cell Plates.

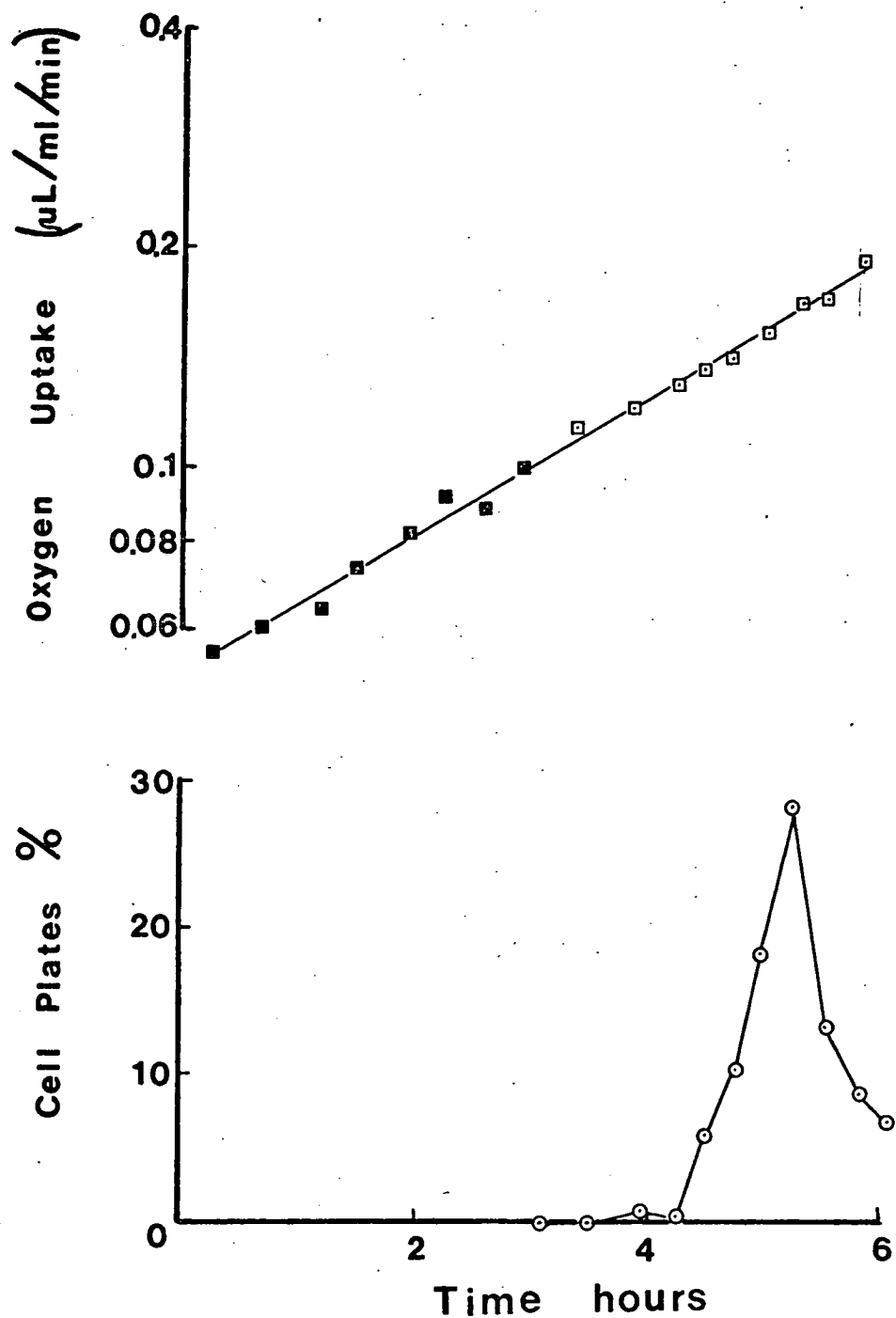


Fig 4-2. Oxygen uptake in a culture pulsed for 3 hours with Adr.

At time 0, Adr was added to a culture containing 3×10^6 cells / ml to give 2×10^{-3} molar. O_2 uptake was measured at intervals on 3 ml samples using the O_2 electrode. At 3 hours the culture was filtered & resuspended in fresh medium. O_2 uptake was measured for a further 3 hours.

■—■ O_2 uptake during pulse. □—□ O_2 uptake after pulse. ○—○ % Cell Plates

synchronous culture with a step in the rate in the middle of the first cycle, a further step at the time of the cell plate peak, followed by further steps in what would normally be the second cycle. The conclusion that one would draw from this experiment is that the oxygen uptake pattern which was seen in normal synchronous cultures is carried out independently of the synthesis of DNA.

Oxygen Uptake in Induced Synchronous Cultures.

There are two other ways of preparing synchronous cultures apart from the gradient method which has been used so far. One involves the addition of deoxyadenosine, the other the addition of hydroxyurea, both of which inhibit DNA synthesis in S. pombe (Mitchison & Creanor, 1971 a). When added for a 3 hour period to an exponentially growing culture and removed at the end of this time by filtration, the cells undergo a synchronous division 2 hours later, followed by a second, semi-synchronous division $1\frac{1}{2}$ hours later. Although cell division and DNA synthesis are synchronised in a culture of this nature, cells are not of uniform size as in a culture synchronised by the selection method and the cell size at division shows the same variability as is seen in a normal asynchronous culture. However, since all of the cells have been growing for at least one generation time without dividing, the smallest cells in an induced synchronous culture at the cell plate peak are as large as the biggest cells in an exponentially growing culture.

Oxygen uptake was first measured polarographically in a culture induced to divide synchronously using deoxyadenosine. The results, shown in Fig 4-2, show that there was a continuous increase in the rate of oxygen uptake during the pulse and afterwards when the culture was

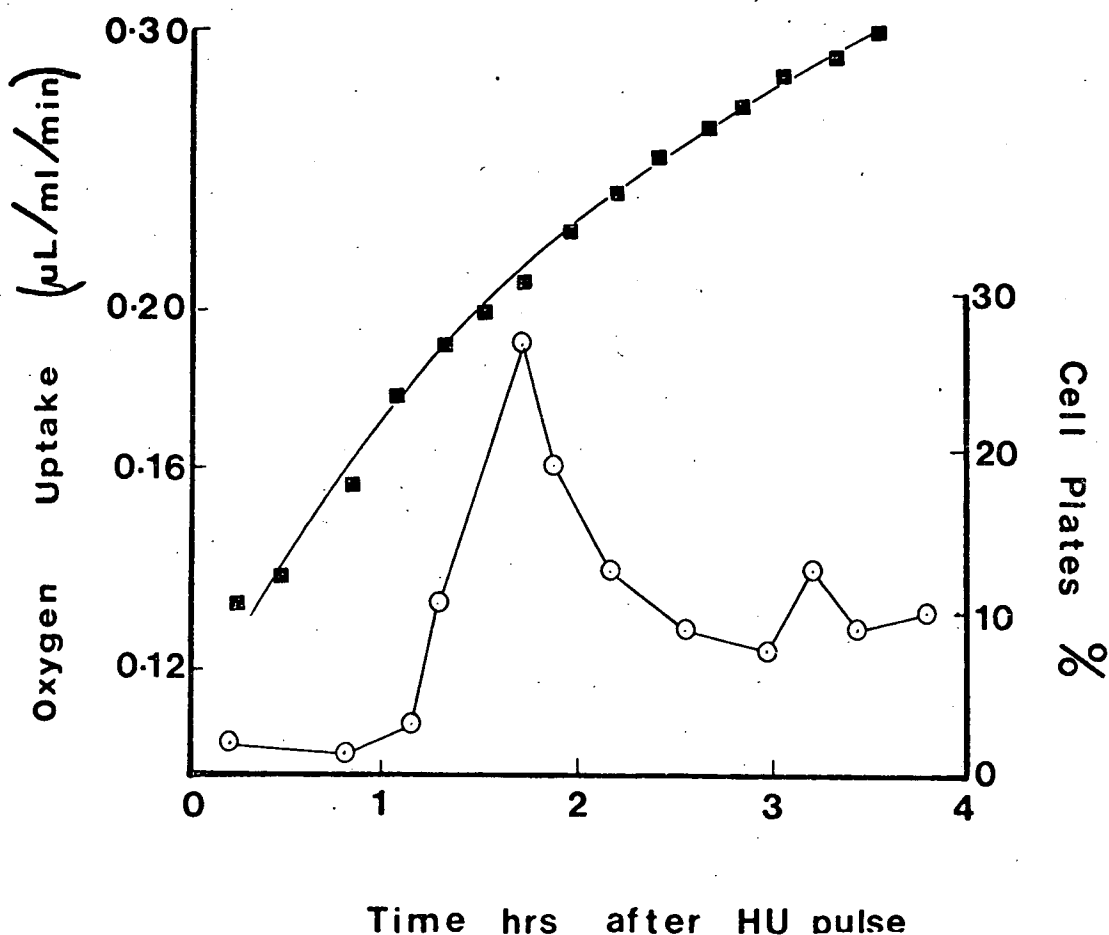


Fig 4-3. Oxygen Uptake after a 3 hour pulse of hydroxyurea.

Hydroxyurea was added to an exponential culture containing 2×10^6 cells/ml to give a concentration of 1.1×10^{-2} molar. 3 hours later the culture was filtered and resuspended in fresh medium (Time 0). Oxygen uptake was measured on 3 ml samples every 15 minutes using the oxygen electrode.

■—■ Rate of oxygen uptake. ○—○ % Cell Plates.

dividing synchronously. This suggests that although the culture is dividing synchronously, undergoing nuclear division and DNA synthesis synchronously, the pattern of oxygen uptake does not show a "synchronous" behaviour. One would say, therefore, that this experiment supports that described in Fig 4-1 by demonstrating that in a culture which consists of cells synchronised with respect to DNA synthesis and cell division only, the pattern of oxygen uptake is not synchronised.

A similar result, shown in Fig 4-3, was obtained when a culture was synchronised using 1.1×10^{-2} molar hydroxyurea. Here again, although the cells were dividing synchronously and making DNA synchronously, oxygen uptake increased in a continuous manner and did not show the periodic pattern which was seen in a normal synchronous culture. It appears that a culture growing in the presence of hydroxyurea is inhibited with respect to growth and this is reflected in the falling rate of oxygen uptake in the culture, even after the pulse. This has been noted before (Mitchison & Creanor, 1971 a) and is one of the reasons why deoxyadenosine is usually chosen as a DNA synthesis inhibitor. Nevertheless, this experiment also suggests that the pattern of oxygen uptake in synchronous cultures is independent of the synthesis of DNA.

One further experiment was carried out to confirm the independence of the oxygen uptake pattern from DNA synthesis, nuclear division and cell division. Mitomycin C has been reported to inhibit DNA synthesis in micro-organisms and in tumour cells by becoming covalently bound to the DNA (Kersten & Kersten, 1969). In S. cerevisiae, cell division was shown to be inhibited by mitomycin C at a concentration of 0.4 mg/ml but RNA and protein synthesis were not affected during the treatment (Williamson & Scopes, 1962). Similar results have been found with S. pombe. At a concentration of 0.1 mg/ml, cell division was inhibited

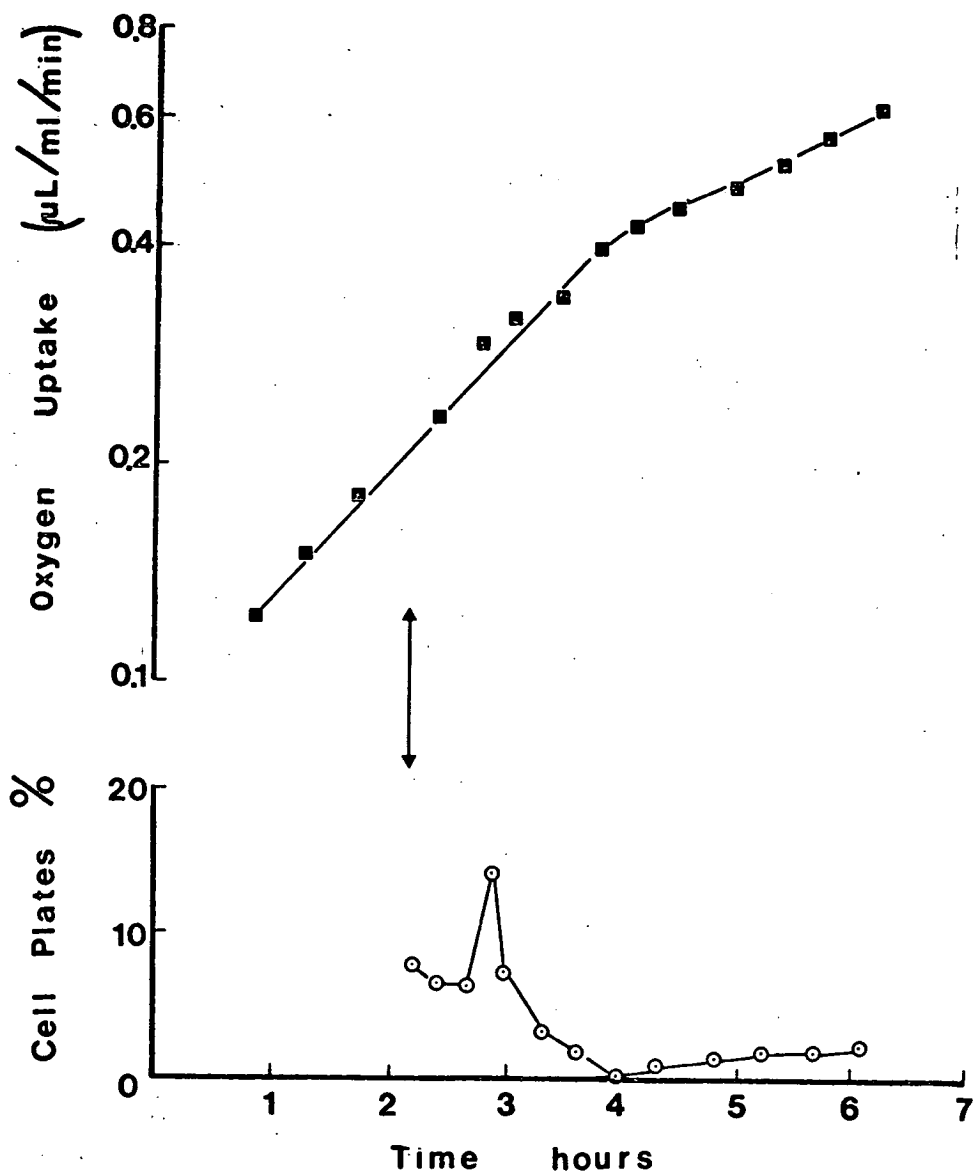


Fig 4 - 4. Oxygen uptake in a culture containing Mitomycin C.

Oxygen uptake was measured on an exponential culture at 30 minute intervals using the O_2 electrode. After 130 minutes, mitomycin C was added to the culture to give 0.1 mg/ml. O_2 uptake was measured for a further 4 hours.

■—■ Rate of oxygen uptake. ○—○ % Cell Plates.

Arrow denotes time of addition of mitomycin C.

but neither RNA or protein synthesis were affected. By adding mitomycin C at various times to a synchronous culture, it was concluded that inhibition of DNA synthesis and of cell division was indirect and that the primary action of the antibiotic was an inhibition of nuclear division (Robinson, 1972). When added to a synchronous culture shortly after preparation, nuclear division, which normally occurs at 0.75 of the way through the cell cycle, was inhibited and the cells were neither able to divide or to make DNA. This situation is different to that seen with deoxyadenosine since in the latter case the cells are arrested in G₁, after nuclear division has occurred but before DNA has been synthesised. In the case of mitomycin C, however, the cells are in the G₂ state.

Oxygen uptake was examined in an asynchronous culture containing mitomycin C. This experiment is similar to that described in Fig 4-2 and Fig 4-3 above involving pulses of deoxyadenosine and hydroxyurea. In this case, however, cells which had not undergone nuclear division when the inhibitor was added were unable to do so with the result that a short time after the addition of the mitomycin C there was no further cell division in the culture. Rather than remove the inhibitor after 3 hours as above, oxygen uptake was measured polarographically for a total of 6 hours in the continued presence of mitomycin C. It can be seen in Fig 4-4 that the rate of oxygen uptake continued to increase throughout the duration of the treatment. This again suggests that an increase in the rate of oxygen uptake is independent of nuclear division, DNA synthesis and cell division. It should be noted that in this experiment, the increase in the rate of oxygen uptake began to fall off after about 2 hours in the presence of the mitomycin C and that the inhibition of cell division was not complete. It will be shown later that the evolution of CO₂ is also inhibited eventually in the presence

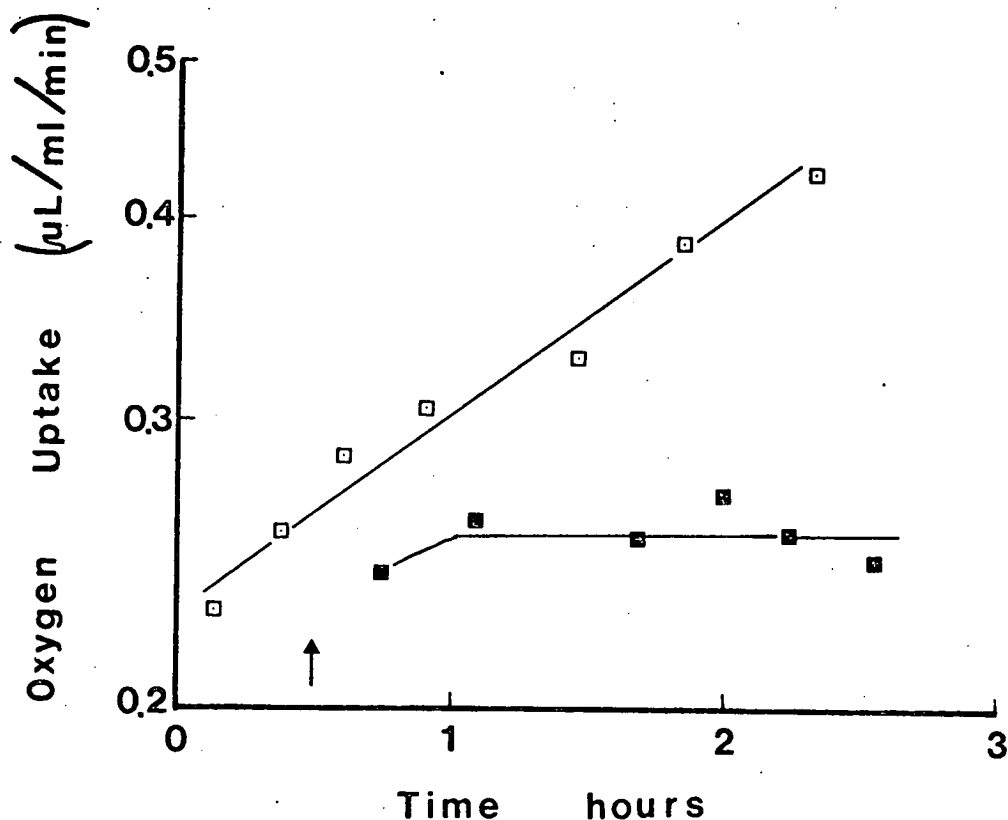


Fig 4-5. Oxygen uptake in an exponential culture containing cycloheximide.

Oxygen uptake was measured, using the O_2 electrode, on 3 ml samples of an exponential culture containing 6×10^6 cells/ml. After 30', cycloheximide was added to part of the culture to give 0.1 mg/ml. Oxygen uptake was measured on this culture at intervals for a further 2 hours.

□—□ Oxygen uptake in the untreated culture.

■—■ Oxygen uptake in the presence of cycloheximide.

Arrow denotes time of addition of cycloheximide.

of mitomycin C and I suggest that these effects are caused by some other inhibition by mitomycin C; for example the flux through the EMP pathway might be inhibited by this antibiotic.

Oxygen Uptake in the presence of Cycloheximide.

Cycloheximide is a potent inhibitor of protein synthesis in eukaryotic cells and is considered to act by stopping the elongation of polypeptide chains (Siegel & Sisler, 1964, 1965). It has also been shown to inhibit initiation of polypeptide chains in some mammalian systems (Lin, Mosteller & Hardesty, 1966). Cycloheximide has been used to inhibit protein synthesis in S. pombe (Mitchison & Creanor, 1969) and enzyme activity has been measured in cultures containing the inhibitor (Mitchison & Creanor, 1969, 1971 b). The effect of cycloheximide on oxygen uptake in S. pombe was investigated to test whether an increase in the rate of oxygen uptake is dependent on concomitant protein synthesis.

Cycloheximide, at a concentration of 0.1 mg/ml has been shown to inhibit leucine incorporation in S. pombe by 92% within 10 minutes. (Mitchison & Creanor, 1969). Oxygen uptake was measured in a culture containing this concentration of cycloheximide and in a control untreated culture at the same time. The results of this experiment, shown in Fig 4-5, indicate that almost immediately after the addition of the cycloheximide, there is no further increase in the rate of oxygen uptake. There is also no decrease in the rate in the presence of the inhibitor suggesting that the components of the oxygen uptake system are stable, or that they are being stabilised in the presence of the cycloheximide.

This result could mean one of two things. Either the increasing rate of oxygen uptake which is seen in normally growing cultures is dependent on protein synthesis; or the inhibition of protein synthesis

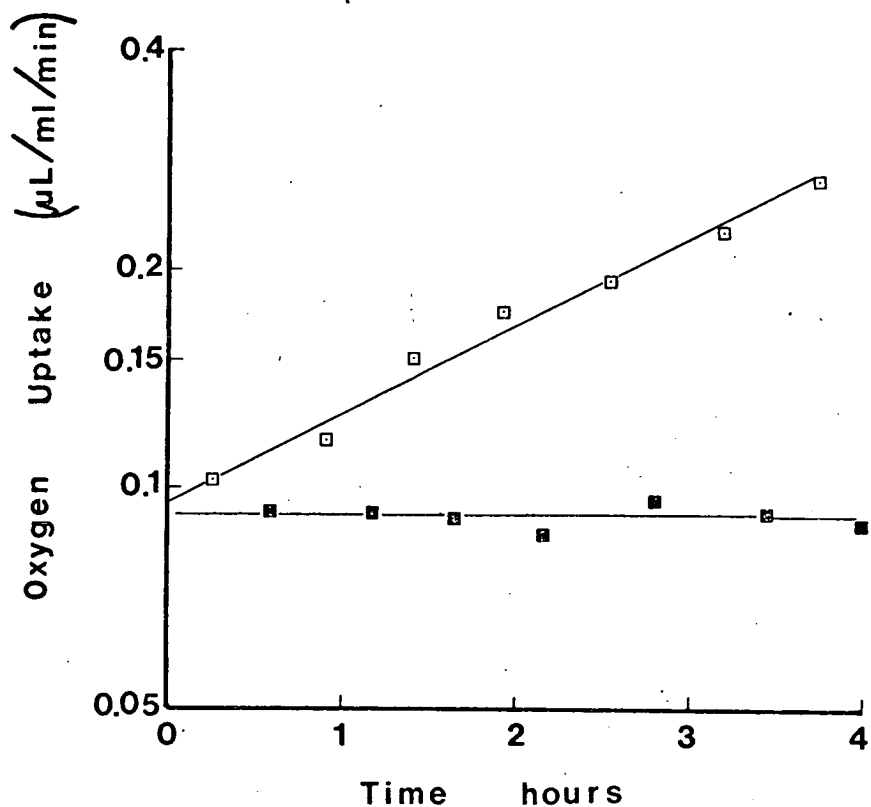


Fig 4 - 6. Oxygen uptake in an exponential culture containing hydroxy-quinoline.

At Time 0, 8-hydroxyquinoline was added to part of an exponential culture containing 3×10^6 cells/ml at a concentration of 0.05 mg/ml. Oxygen uptake was measured on 3 ml samples of this culture and on 3 ml samples of the untreated culture using the oxygen electrode.

□—□ Rate of oxygen uptake in the untreated culture.

■—■ Rate of oxygen uptake in the presence of hydroxyquinoline.

in an exponentially growing culture (and consequently of RNA synthesis, DNA synthesis and cell division) leads to a decrease in the energy requirements of the cell and this operates a feed-back inhibition at one of the steps in the oxygen uptake system. It is not possible to distinguish between either of these possibilities at the moment. The first of these possibilities has, however, one interesting implication. If an increase in the rate of oxygen uptake is dependent on protein synthesis, cycloheximide inhibits cytoplasmic protein synthesis and even at this concentration has no effect on mitochondrial protein synthesis.

One might conclude that oxygen uptake, which is a mitochondrial function, is dependent on nuclear protein synthesis. It is, of course, well known that most of the mitochondrial proteins are synthesised from nuclear genes and cytoplasmic ribosomes (Schatz & Mason, 1974) and this result might afford a physiological demonstration of the need for nuclear protein synthesis to change the activity of a mitochondrial function. It will be suggested later in this Chapter that an increase in the rate of oxygen uptake in S. pombe is also dependent on mitochondrial protein synthesis.

Oxygen Uptake in the presence of 8-Hydroxyquinoline.

The synthesis of RNA can be inhibited in S. pombe using 8-hydroxyquinoline. (Fraser & Creanor, 1974). This inhibition operates by the chelation of manganese and magnesium in the nucleus of the cell, thus rendering the RNA polymerase inactive (Fraser & Creanor, 1975).

Oxygen uptake was measured in an exponential culture in the presence of 0.05 mg/ml hydroxyquinoline. The results of this experiment are shown in Fig 4-6. Immediately on adding the inhibitor, there is no

further increase in the rate of oxygen uptake and , as with cycloheximide, neither is there any fall in the rate after the addition of the hydroxyquinoline. The conclusion from this result is that any increase in the rate of oxygen uptake is dependent on RNA synthesis. The fact that there is no rise at all after the addition of the inhibitor suggests instability in the RNA molecules which are required for the synthesis of new components of the oxygen uptake system. The components themselves are stable since there is no decrease in the rate of oxygen uptake after the addition of the hydroxyquinoline. Unlike cycloheximide, it is not known whether the hydroxyquinoline is affecting nuclear RNA synthesis alone or whether there is also an inhibition of mitochondrial RNA synthesis.

The argument which was used in the section dealing with the effect of cycloheximide on oxygen uptake might also be applied in this case. In the absence of RNA synthesis, protein synthesis and DNA replication are eventually inhibited. There may, therefore be a decrease in the energy requirements of the cell and this operates a feed-back inhibition at one of the steps in the oxygen uptake system. As was stated for cycloheximide inhibition, it is not possible to distinguish between this possibility just discussed and the possibility that oxygen uptake does not increase because of the inhibition of RNA synthesis itself.

Oxygen Uptake in the presence of Cyanide and Azide.

Sodium cyanide is an inhibitor of cytochrome oxidase (Slater, 1967) and it has been reported to inhibit oxygen uptake in S. pombe (Heslot et al, 1970). This is taken to show that site 111 is present. Sodium azide on the other hand may have several sites of action

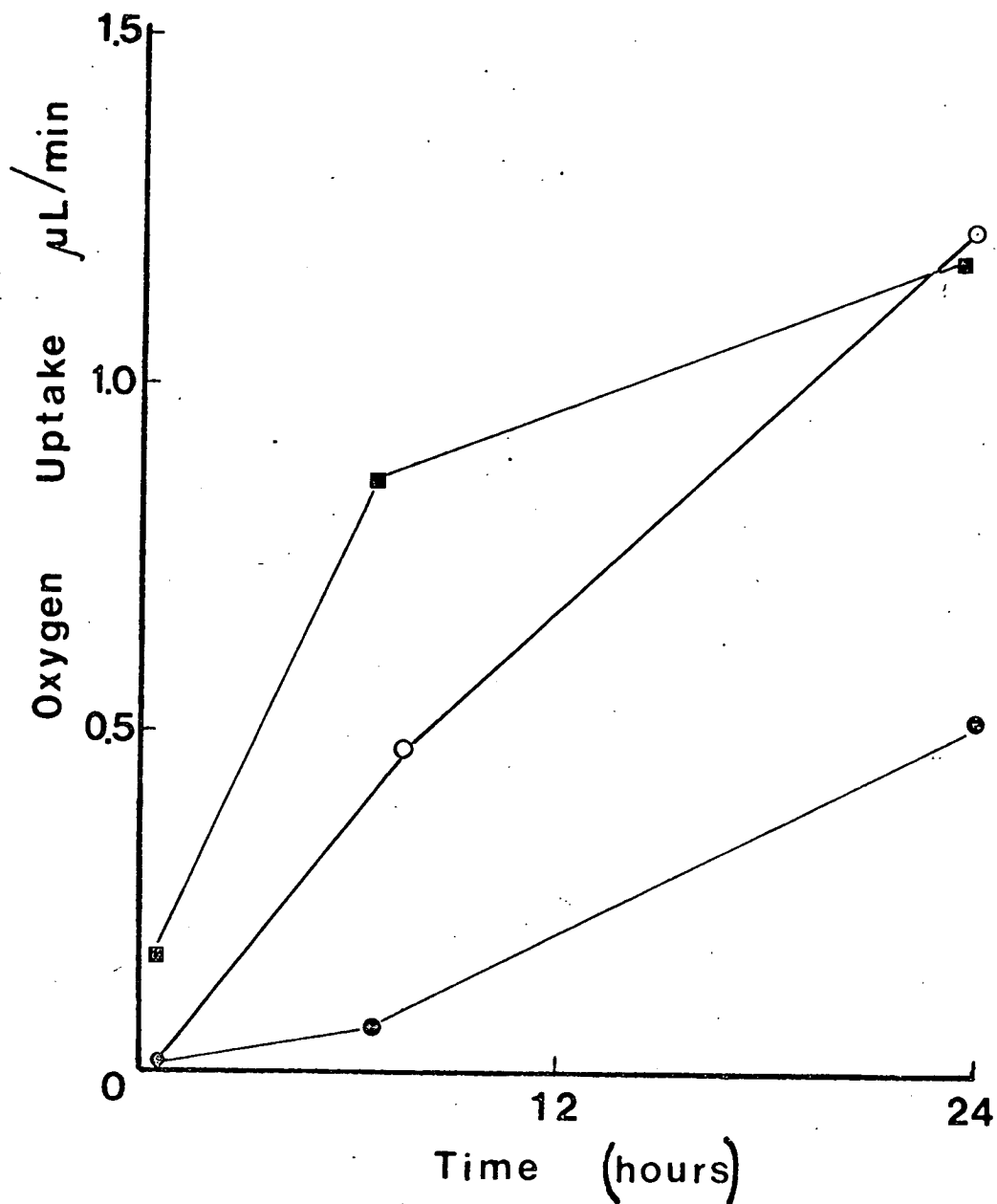


Fig 4-7. Oxygen Uptake in a culture containing cyanide or azide.

At time 0. sodium cyanide and sodium azide were added to samples from an exponential culture containing 8×10^6 cells /ml, to give concentrations of 0.05 and 0.01 mg/ml respectively. Oxygen uptake was measured several times over the next 24 hours on an untreated culture and on the two cultures containing the inhibitors.

- Oxygen Uptake in the untreated culture.
- Oxygen uptake in the presence of cyanide.
- Oxygen uptake in the presence of azide.

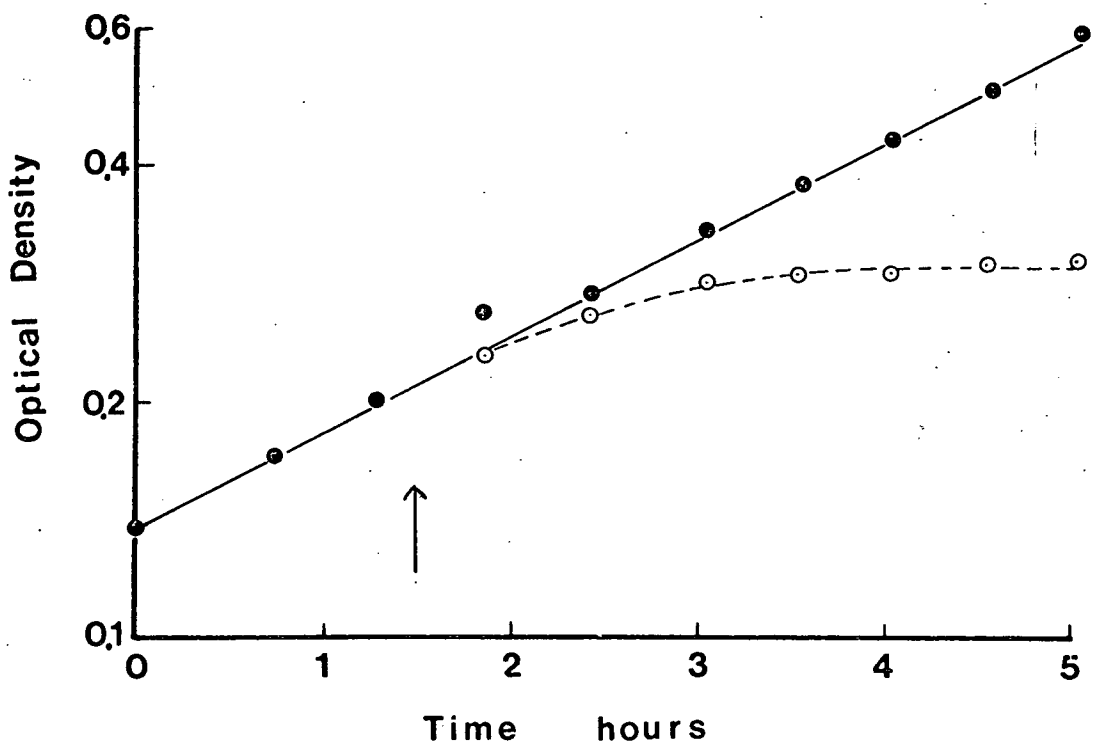


Fig 4 - 8. Optical density increase in a culture containing Sodium Cyanide. Optical density was measured at intervals in an exponential culture containing 2×10^6 cells/ml at time 0. At 1 hour 50', sodium cyanide was added to part of the culture at a concentration of 0.05 mg/ml. Optical density was then measured for a further $3\frac{1}{2}$ hours on both cultures.

- Optical Density in the untreated culture.
- Optical Density in the presence of Sodium Cyanide.

Arrow denotes time of addition of Sodium Cyanide.

depending on its concentration (Slater, 1967) and it has been reported to act like an uncoupler when added to the growth medium of S. pombe (Heslot et al, 1970).

At concentrations of 0.05 mg/ml sodium cyanide and 0.01 mg/ml sodium azide, respiration of exponentially growing cells is strongly inhibited. Inhibition by cyanide is instantly reversible. After several hours in the presence of either of these inhibitors, oxygen uptake is resumed and at the end of the exponential phase, the oxygen uptake in the cultures containing either sodium cyanide or sodium azide is as high as the oxygen uptake of the control culture. The results from such an experiment with sodium cyanide are shown in Fig 4-7. It would appear that the inhibitor is being exhausted from the medium in these experiments since addition of further cyanide again causes almost complete inhibition of oxygen uptake. Also if the cells are filtered from the medium and the medium inoculated with cells which have not been treated previously with cyanide, there is no inhibition of oxygen uptake. Whether the cells themselves have taken up the cyanide and complexed it with other molecules, or whether the cyanide is actually broken down by the cells, cannot be determined at this time.

If the same concentration of NaCN is added to a culture at low cell density (2×10^6 cells/ml), the cells do not recover from the effect of the inhibitor and respiration does not begin again. In this type of experiment, shown in Fig 4-8, growth is strongly inhibited after about $2\frac{1}{2}$ hours and the final cell density is about $1/10^{\text{th}}$ that of the control. This experiment might mean that in the absence of oxygen uptake cell growth is unable to continue, suggesting that the low level of respiration in S. pombe is vital for the growth of the cells. On the other hand, cyanide and azide affect the activity of many enzymes

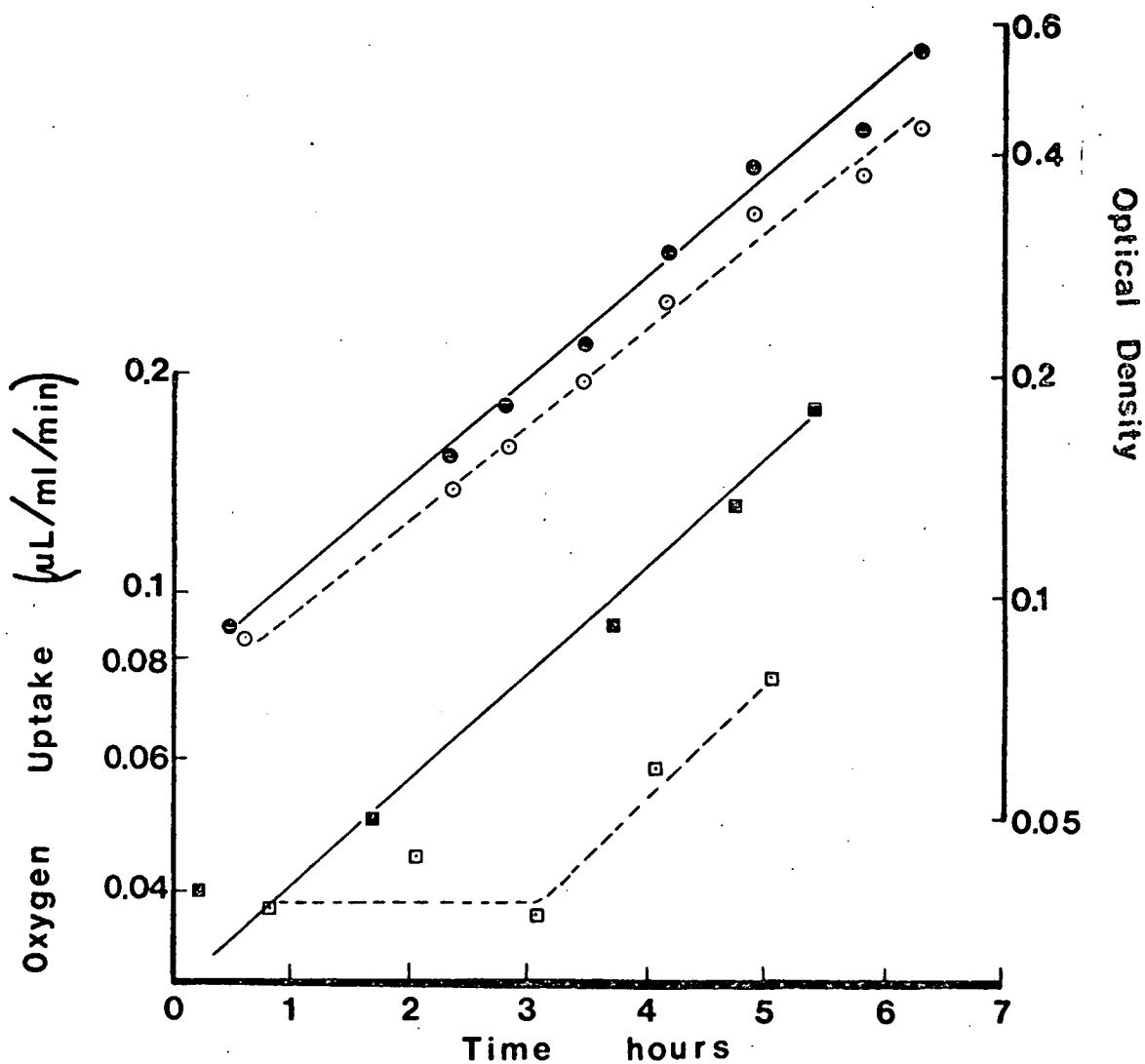


Fig 4-9. Optical Density & O₂ uptake in a culture containing erythromycin.

At time 0, erythromycin was added to part of an exponential culture containing 1×10^6 cells/ml at a concentration of 0.05 mg/ml. O₂ uptake was measured on both cultures for 6 hours.

- Optical density of control. ○—○ Optical density + erythromycin.
- O₂ uptake of control. □—□ O₂ uptake + erythromycin.

(Dixon & Webb, 1964) and it is possible that growth is inhibited, not because of the inhibition of respiration but because of the inhibition of some other enzymes in the cell.

Attempts to inhibit respiration with antimycin A were not successful. Inhibition of oxygen uptake was not obtained using the concentration of antimycin A which has been reported to inhibit respiration in S. pombe by 85% (Heslot et al, 1970). It was only when this inhibitor was added at 10 times the concentration that 50% inhibition of oxygen uptake was obtained. But it is possible that this concentration of antimycin A was affecting the functioning of the oxygen electrode since oxygen uptake was inhibited in every culture measured subsequently. This inhibition of oxygen uptake only disappeared when the electrode had been washed for 24 hours. This effect was not investigated at the lower concentration of antimycin used by Heslot et al (1970), neither was the precise nature of this effect examined.

Oxygen Uptake in the presence of Erythromycin.

Oxygen uptake was measured in the presence of erythromycin which has been reported as an inhibitor of mitochondrial protein synthesis (Thomas & Wilkie, 1968). At a concentration of 0.01 - 0.05 mg/ml, erythromycin immediately prevented further increase in the rate of oxygen uptake in an exponential culture of S. pombe, as shown in Fig 4-9. Unfortunately, about $1\frac{1}{2}$ hours after the addition of the inhibitor, the rate of oxygen consumption again began to increase, due probably to a recovery of the cells. In other experiments it was shown that increasing the concentration of the inhibitor did not prolong the period of inhibition and that the respiration in cultures at the end of the exponential phase was similar to that of untreated cultures.

Using less dense cultures did not improve the time of inhibition with erythromycin as it did with cyanide. However, there was no apparent inhibition of cell growth during the period when the culture was not experiencing an increase in the respiration rate. Though the period when inhibition was complete was short, there was no deviation in the optical density of the treated culture from that of the control (Fig 4-9). One can perhaps conclude on the basis of this rather inconclusive evidence that the level of respiration maintained during the period in which further increase in the rate was inhibited, was sufficient to maintain the growth of the culture at a normal level. How long this situation would persist is impossible to say using this inhibitor.

CHAPTER 5

Carbon Dioxide Evolution in Asynchronous and Synchronous Cultures.

Introduction.

One conclusion from the results reported in the previous Chapter is that fission yeast is capable, to some extent, of growing without uptake of oxygen. This is consistent with the fact that most yeasts are facultative aerobes and means that a measurement of oxygen uptake in synchronous cultures of S. pombe is giving only a partial answer to the question of energy production during the cell cycle.

The experiments described in this Chapter are concerned with the evolution of CO_2 in synchronous cultures of S. pombe prepared by a selection technique and involving the use of lactose gradients. The validity of the results obtained was tested by measuring the rates of evolution of CO_2 in asynchronous control cultures. Single measurements of the rate were made on each sample removed from the cultures.

It was later discovered that a similar investigation into the rate of CO_2 evolution in S. pombe (strain 972h⁻) was being carried out in the Carlsberg Laboratory (Zeuthen & Kramhøft, per. comm.) using a complete medium and continuously monitoring the production of CO_2 in a sample of synchronous culture during two synchronous divisions. A few experiments were therefore carried out using the same medium and the same technique of measuring the rate of CO_2 evolution as described by the above workers. CO_2 evolution was also measured on strain 972h⁻ and the results compared with those obtained using strain 132.

Finally, an attempt was made to prepare synchronous cultures starting with cells which were already half way through the cycle and to measure the rates of evolution on these cultures.

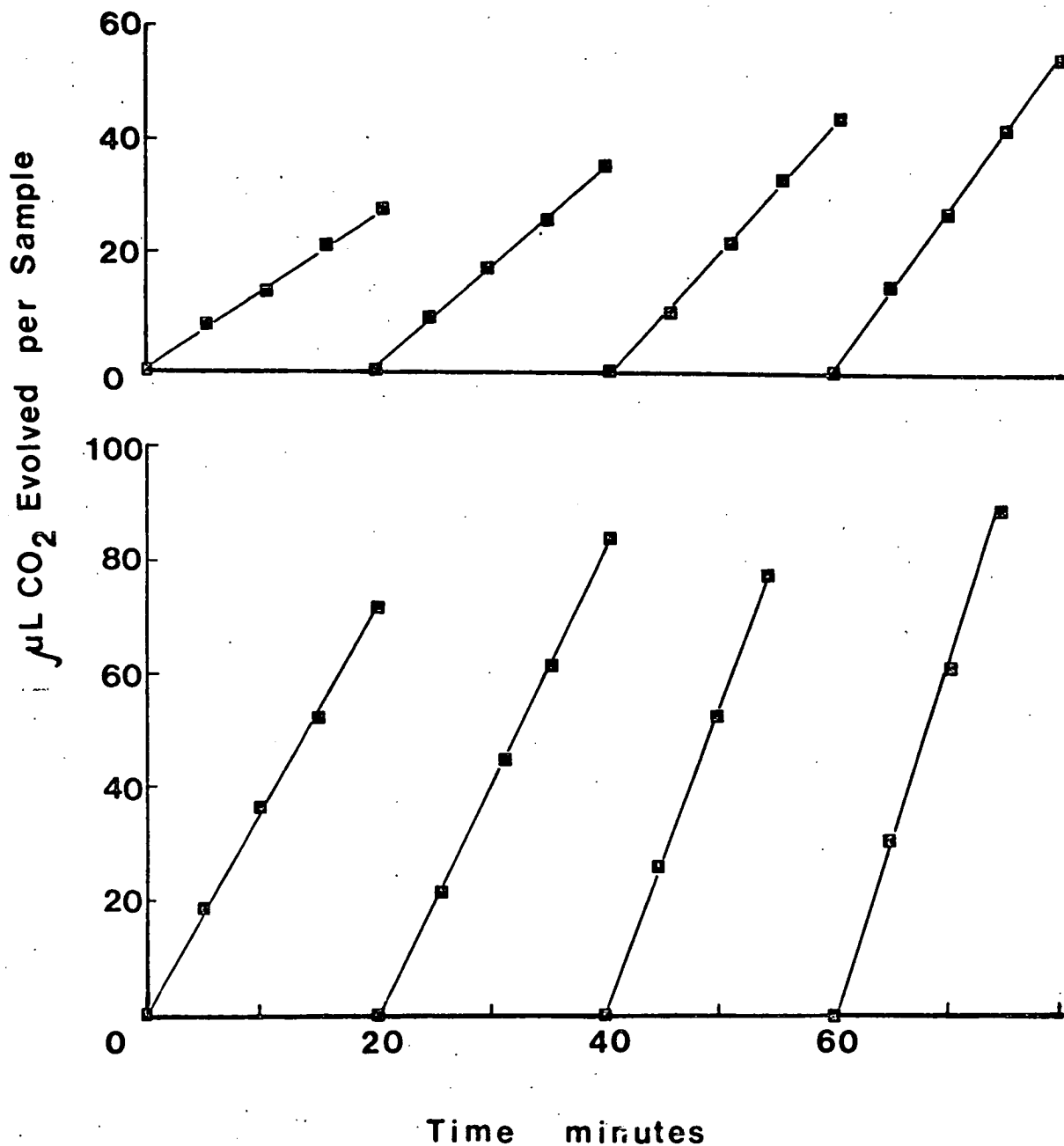


Fig 5 -1. CO₂ evolution in an exponentially growing culture.

CO₂ evolution was measured for 20 minute periods on 4 ml samples removed at intervals from the culture. At the end of each measurement, the sample was discarded.

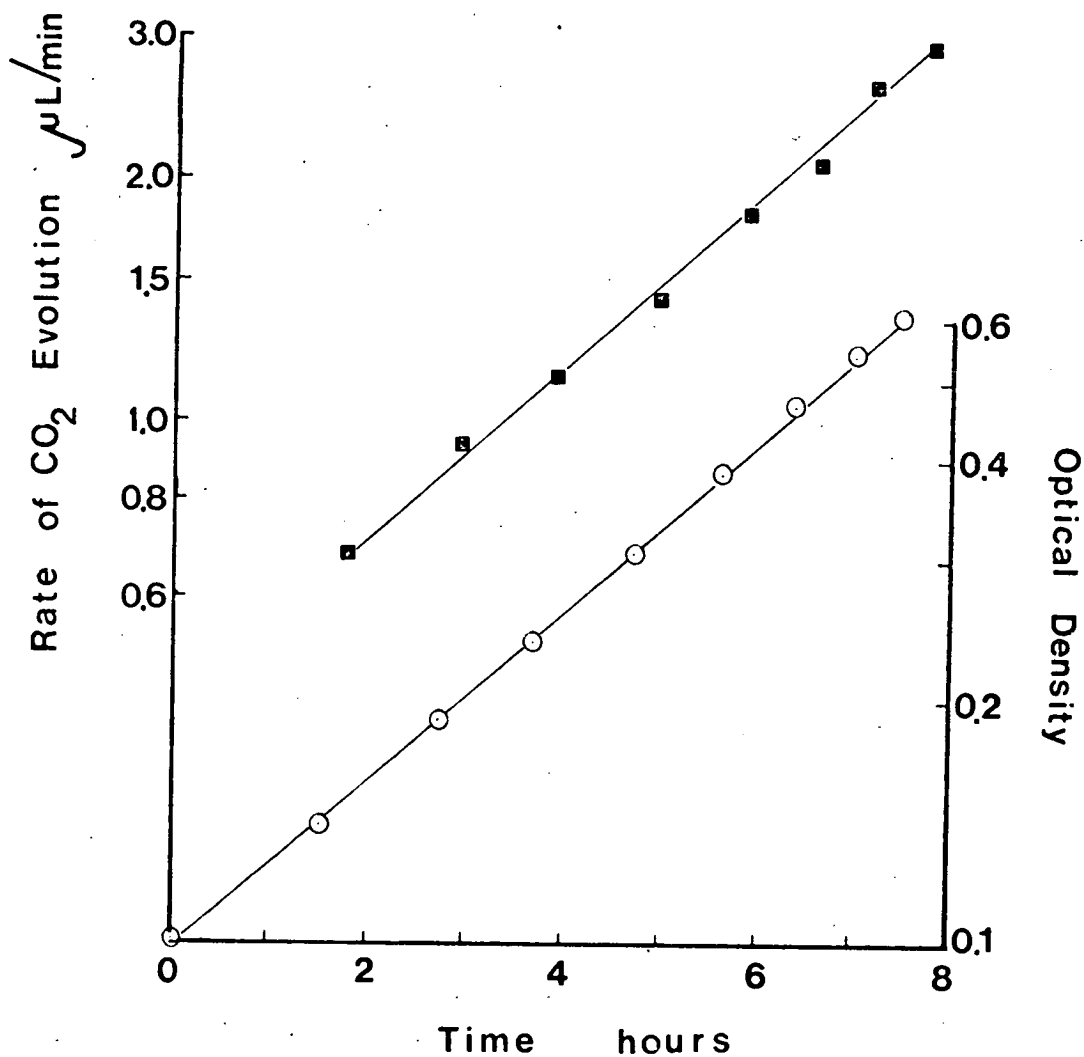


Fig 5 -2. The rate of CO₂ evolution during the exponential phase.

Rates of CO₂ evolution were calculated from each of the measurements shown in Fig 5 -1.

- — ■ Rate of CO₂ evolution.
- — ○ Optical Density measured at 595 nm.

Evolution of CO₂ in Asynchronous Cultures.

The method used to measure the rate of evolution of CO₂ was a modification of the direct method of Warburg (Umbreit, Burris & Stauffer, 1957). The evolution of CO₂ in samples removed from an exponential culture is shown in Fig 5- 1. Each sample was measured for a 20 minute period after which it was discarded. The rate of evolution of CO₂ in each of these samples is shown in Fig 5- 2. There was a continuous rise in the rate of evolution of CO₂ during this phase and the doubling time of this rise was similar to the doubling time of the optical density of the culture which is shown in the same figure. The culture was therefore considered to be in a balanced state with respect to this parameter according to the definition of Campbell (1957) which states that growth is balanced over a time interval if, during that interval, every extensive property of the growing system increases by the same factor. Changes in the rate of evolution of CO₂ when the culture entered stationary phase will be discussed later.

Only one measurement of CO₂ evolution was made on each sample removed from the parent culture. If the tap on the manometer was simply opened to release the pressure then closed again to make a second measurement of the rate of CO₂ evolution, this rate was invariably found to be lower than the first measurement. If this was repeated again, the third measurement of the rate was lower still. This suggests that the CO₂ in the Warburg flask was in some way toxic to the culture.

This does raise the possibility that during the first measurement of the rate, the CO₂ being evolved was already having a toxic effect on the culture and was leading to a low estimate of the rate. This was thought to be unlikely for two reasons. In the first place, the lines shown in Fig 5- 1 do not show any fall off

Fig 5 - 3A. Continuous monitoring of CO₂ evolution in an exponential culture of *S. pombe*.

A sample of 3ml of exponential culture containing 1.5×10^6 cells/ml was placed in a Warburg flask and CO₂ evolution was measured for 4 hours. At the same time, a 1.5 ml sample of the same culture was placed in another flask and the CO₂ evolved by this sample was measured for 4 hours.

▼—▼ CO₂ evolution in the 1.5 ml sample.

▽—▽ CO₂ evolution in the 3 ml sample.

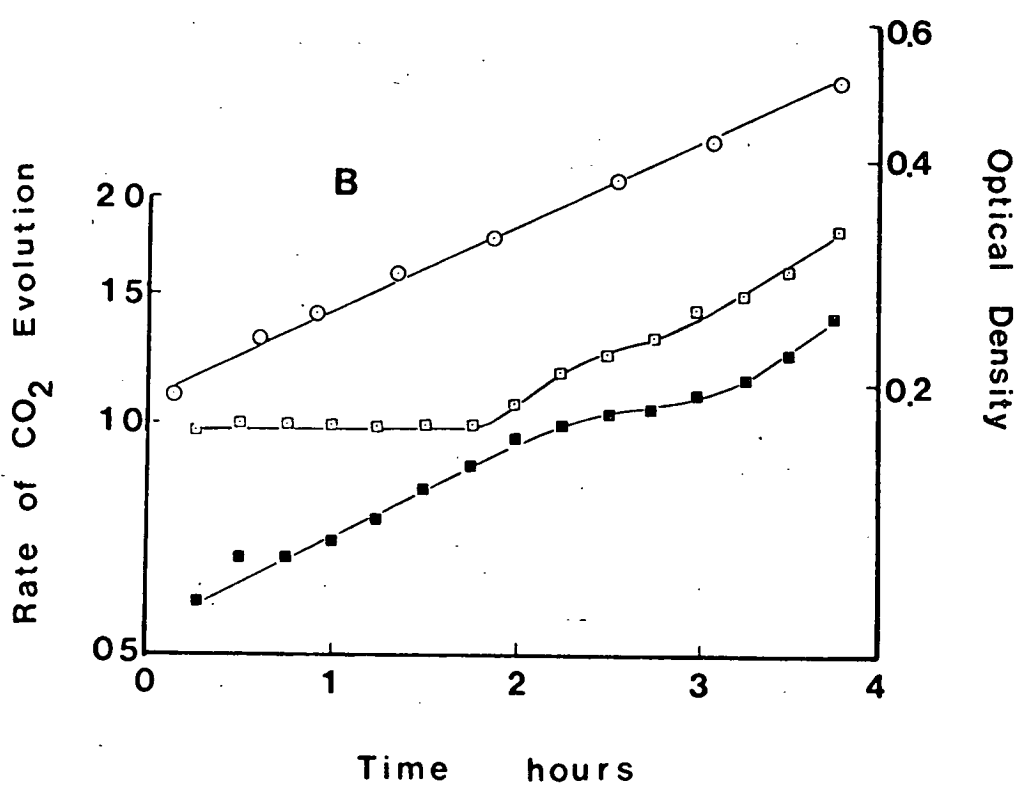
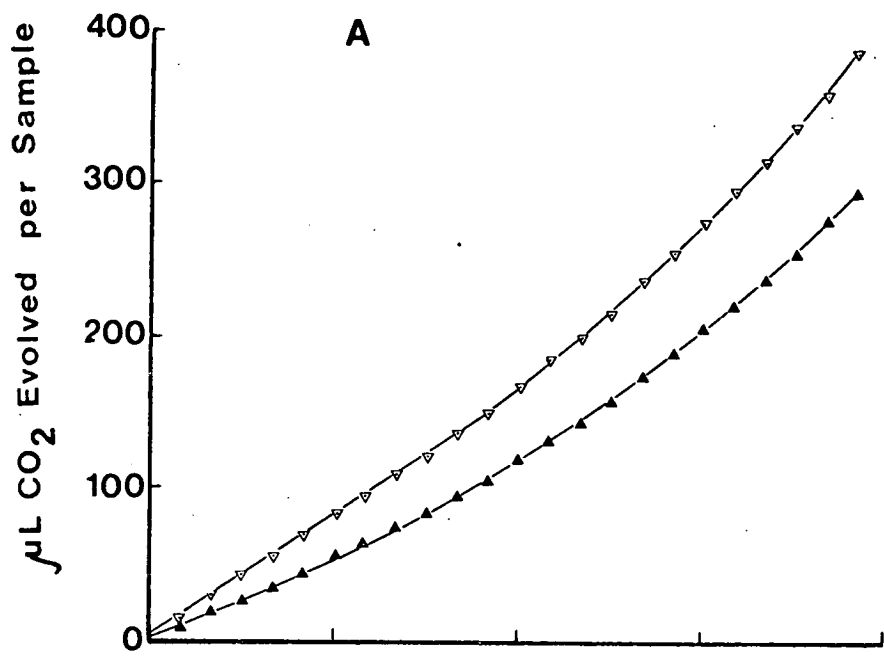
Fig 5 - 3B. Rates of CO₂ evolution obtained from continuous measurements of CO₂ evolved from different sizes of samples.

Using the data shown in Fig 5 - 3A, the rates of CO₂ evolution were calculated at intervals by drawing tangents to the two curves.

○—○ Optical Density of the original culture grown in a water bath during the period of measurement.

■—■ Rate of CO₂ evolution in the 1.5 ml sample.

□—□ Rate of CO₂ evolution in the 3 ml sample.



with time. Secondly, when the rate of CO_2 evolution was measured on samples four times greater than those normally measured, the rate was invariably four times greater. This suggests that the larger CO_2 output by the larger sample was not itself inhibiting the evolution of CO_2 , at least during the first measurement. It was concluded, therefore, that the measurement made during the first 20 minutes was indeed a true measurement of the rate of CO_2 evolution but that no more than one measurement should be made on each sample.

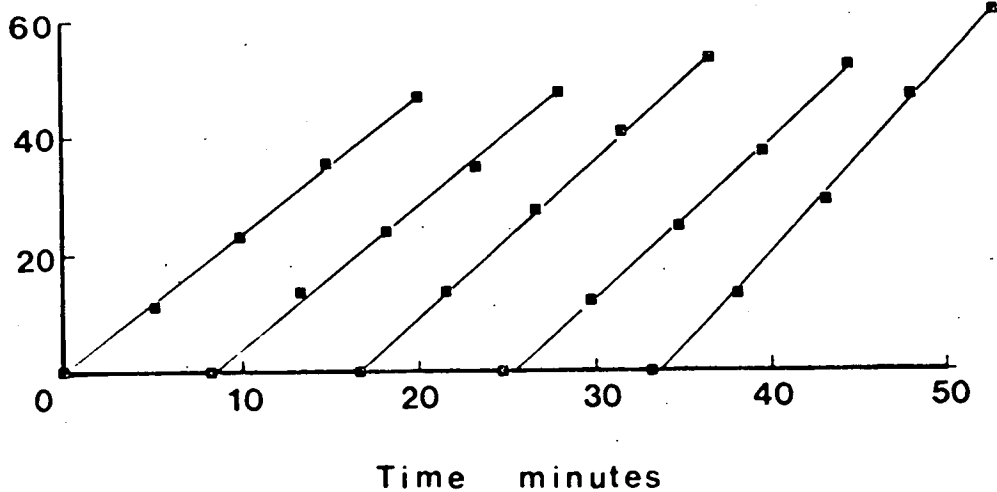
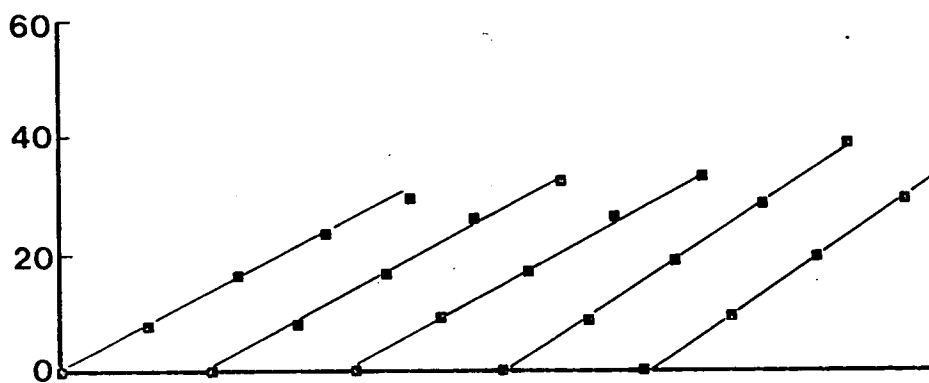
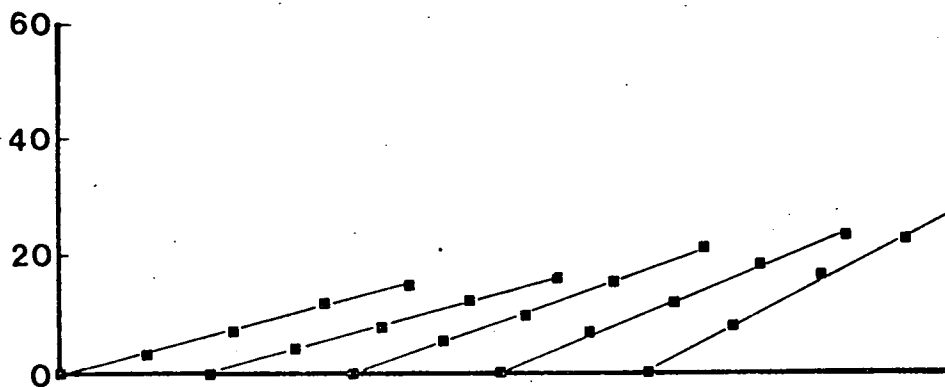
Using a 1.5 ml sample of a culture of S. pombe containing 2×10^6 cells/ml, Zeuthen & Kramhøft (personal communication) have shown that CO_2 evolution could be measured continuously on this sample for about 4 hours. Since this involved half the number of cells as were used in the procedure described above, it follows that less CO_2 was evolved and inhibition of the culture by the CO_2 was either avoided or greatly reduced.

CO_2 evolution was therefore measured using this procedure for 4 hours on a 1.5 ml sample and on a 3 ml sample of an exponential culture of S. pombe containing 2×10^6 cells / ml. The results, shown in Fig 5- 3A, indicate that the amount of CO_2 evolved in the smaller sample increased at a faster rate than the amount of CO_2 evolved in the larger sample. Measurements of the rate of CO_2 evolution were made at intervals along these curves and the values obtained are shown in Fig 5- 3B along with the optical density of the original culture. This clearly indicates that the rate of CO_2 evolution was strongly inhibited in the larger sample, whereas the rate of CO_2 evolution in the smaller sample doubled in a time comparable to the doubling time of the optical density of the culture, at least for the first 2 hours. At the end of the 4 hour period, 0.25 ml was

Fig 5 - 4. CO_2 evolution in a Synchronous Culture of *S. pombe*.

A synchronous culture was prepared using a lactose gradient. At 20 minute intervals a 4 ml sample was placed in a Warburg flask and CO_2 evolution was measured over a 20 minute period. After each period, the contents of the flask were discarded and the procedure repeated.

$\mu\text{L CO}_2$ Evolved per Sample



removed from each of the Warburg flasks and the cell number of these samples was compared with the cell number of the original culture which had been left to grow in a shaking water bath during the course of the experiment. The cell density in the smaller sample (6.9×10^6) was similar to the cell density in the original culture (7.4×10^6) whereas the cell density in the larger sample was much lower (3.2×10^6).

This method would require further investigation before one was fully convinced that this was a legitimate measure of the CO_2 evolution in a culture of S. pombe. The results described above do suggest that there is a possibility of inhibition both of cell growth and of CO_2 evolution during continuous monitoring of CO_2 in a Warburg flask. The above experiment was also carried out some time after the other experiments described in this Chapter because of the parallel investigations which were being carried out in the Carlsberg Laboratory using this continuous monitoring technique. At the moment, the above method of continuous CO_2 monitoring is thought to have some problems and the method which was used in the bulk of the experiments reported in this Chapter is possibly a more satisfactory one.

Evolution of CO_2 in Synchronous Cultures.

Synchronous cultures of S. pombe were prepared by the selection method described in Chapter 2 and using lactose gradients. Using two manometers and flasks, it was possible to obtain a measure of the rate of evolution of CO_2 at 20 minute intervals during the growth of a synchronous culture. The evolution of CO_2 in samples removed from a synchronous culture are shown in Fig 5- 4. The rates of evolution of CO_2 were calculated from each of these lines and the results

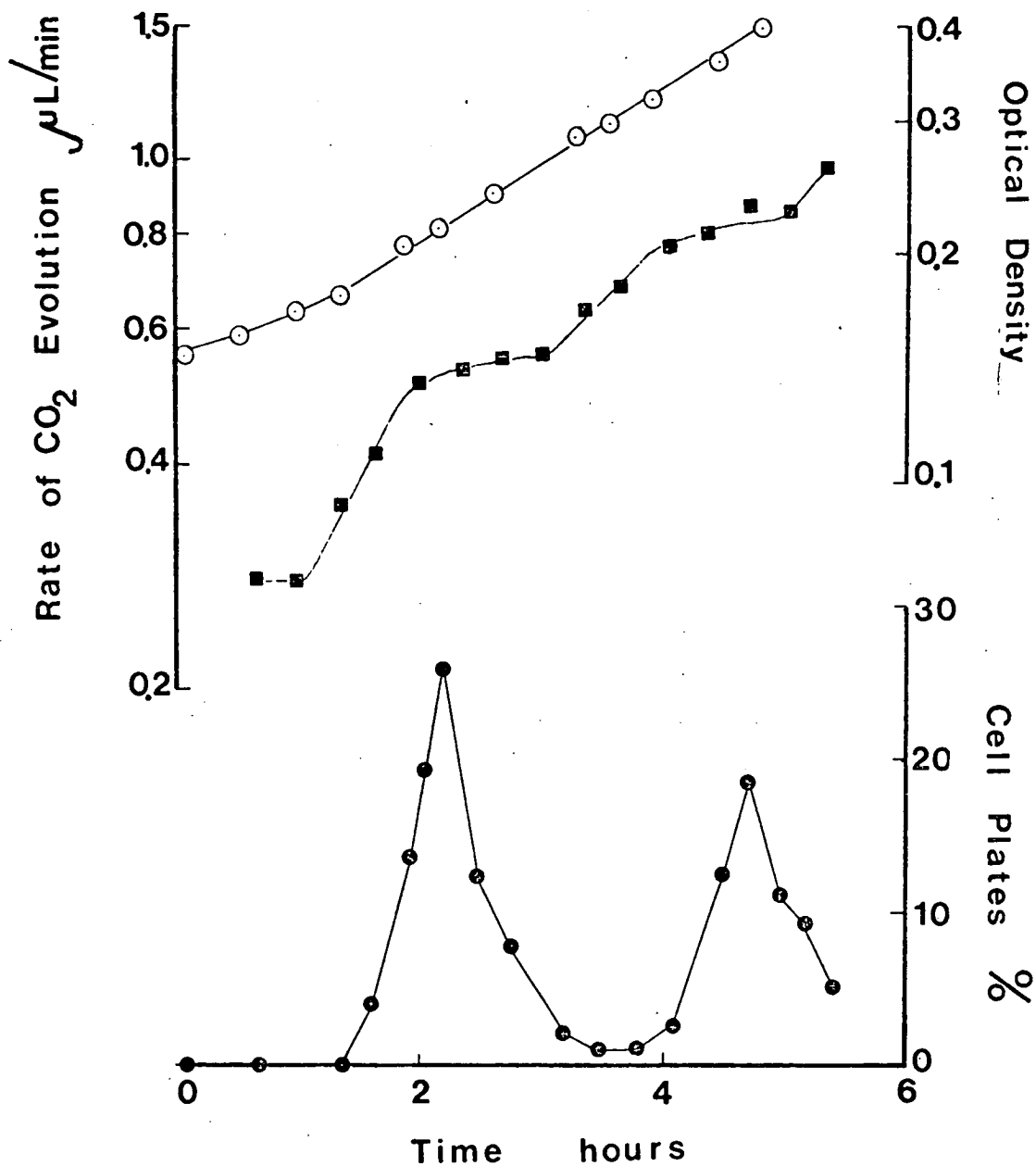


Fig 5 - 5. CO₂ evolution in a synchronous culture of S. pombe.

Each measurement of the rate of CO₂ evolution was obtained by measuring the CO₂ evolved from a 4 ml sample over a 20' period as shown in Fig 5 - 4. After each measurement the sample was discarded.

○—○ Optical Density. ●—● % Cell Plates.
 ■—■ Rate of CO₂ evolution.

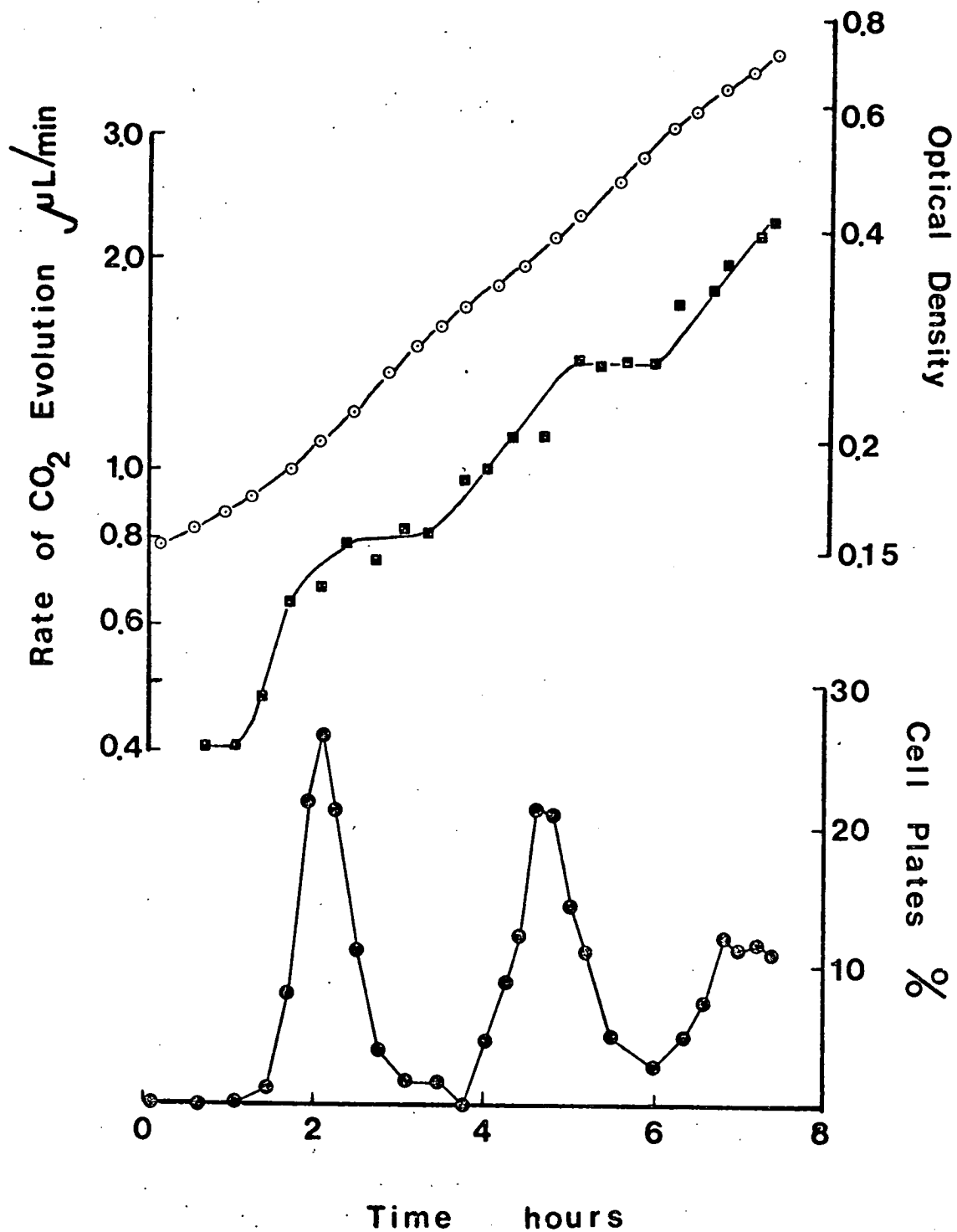


Fig 5 - 6. CO₂ evolution in a synchronous culture of S. pombe.

Each measurement of the rate of CO₂ evolution was obtained by measuring CO₂ evolved by a 3 ml sample over a 20' period.

○—○ Optical Density. ●—● % Cell Plates.
 ■—■ Rate of CO₂ evolution.

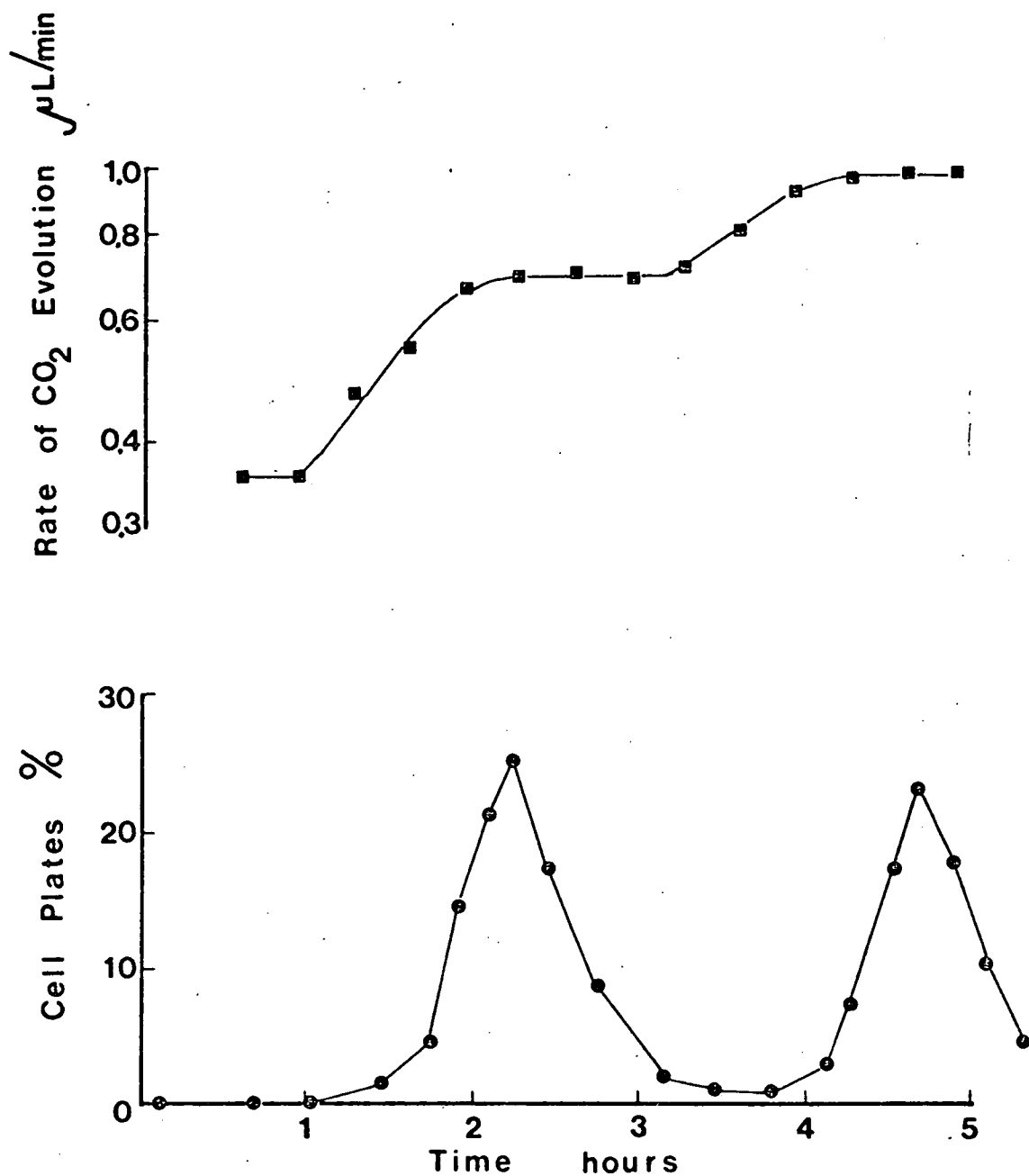


Fig 5 - 7. CO₂ evolution in a synchronous culture of S. pombe.

Each measurement of the rate of CO₂ evolution was made by measuring the CO₂ evolved from a 3 ml sample over a 20 minute period.

■ — ■ Rate of CO₂ evolution.

● — ● % Cell Plates.

Experiment	First Rate Change		Second Rate Change	
	Time	% Change	Time	% Change
1	0.69	100	0.90	50
2	0.82	100	0.74	62
3	0.79	72	0.59	85
4	0.77	107	0.56	65
5	0.72	109	0.54	70
6	0.68	100	0.51	48
7	0.76	100	0.69	66
8	0.78	105	0.79	73
9	0.83	93	0.73	90

TABLE 5 - 1.

Analysis of rate changes in the evolution of CO₂ in synchronous cultures

The time at which the rate of CO₂ evolution altered during two cell cycles is shown in cell cycle time. The extent of the change in rate is also given for rate changes in both cycles.

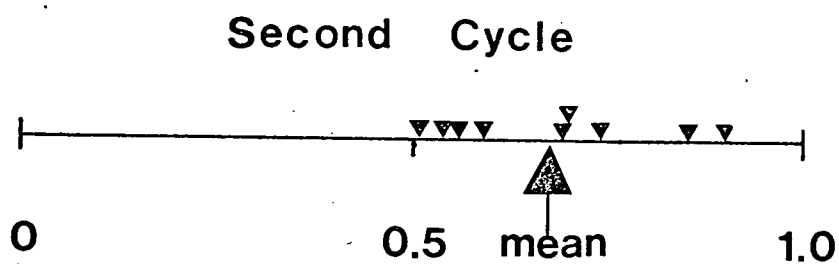
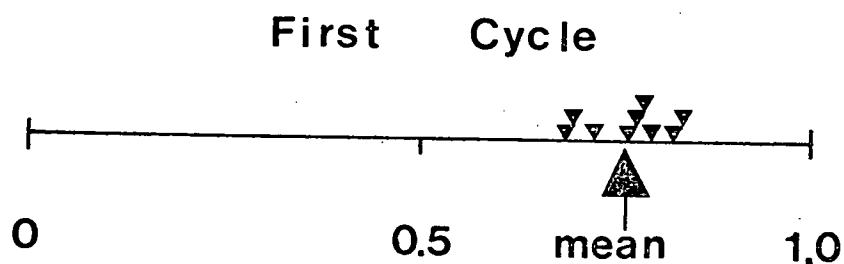


Fig 5 - 8. Cell Cycle map of rate changes in the evolution of CO_2 in synchronous cultures.

Each arrow represents a single determination of the time at which the rate change in CO_2 evolution occurred.

Mean in 1st Cycle = 0.76

Mean in 2nd Cycle = 0.67

obtained by doing this in three typical synchronous cultures are shown in Fig 5 - 5, Fig 5 - 6 & Fig 5 - 7. The rate of CO_2 evolution remained constant for most of the cell cycle and increased sharply towards the end of the cycle. In the first cycle after setting up the synchronous culture, the rate of CO_2 evolution invariably doubled; in the second cycle, the increase in the rate was sometimes less than a doubling. The times at which the changes in the rate of CO_2 evolution occurred in nine synchronous cultures and the extent by which the rate increased is shown in TABLE 5 - 1. In two of the nine cultures, the extent of the rise in the second cycle was little more than 50%. This underdoubling in the second cycle has been observed in measuring enzymes in S. pombe and has been thought to be due to changing conditions during the growth of the culture (Mitchison & Creanor, 1969).

The results from measuring the rates of CO_2 evolution in nine synchronous cultures are shown in the cell cycle map in Fig 5 - 8. In the first cycle, the time for the mid-point of the rate change was at 0.76 of the cycle and in the second cycle, the time was at 0.67. There was no significant difference between the time of the rate changes in the first and second cycles (Student's T - test).

The increase in the rate of CO_2 evolution occurred over a significant portion of the cell cycle. This was mainly due to the imperfect synchrony and it is likely that the change in the rate of CO_2 evolution is a relatively abrupt process in individual cells. The time taken for the rate of CO_2 evolution to double in a synchronous culture (about 60 minutes) is similar for the time reported for either the DNA or cell numbers (60 minutes) to double in synchronous cultures (Mitchison & Creanor, 1971b). Each of these are short processes in individual cells.

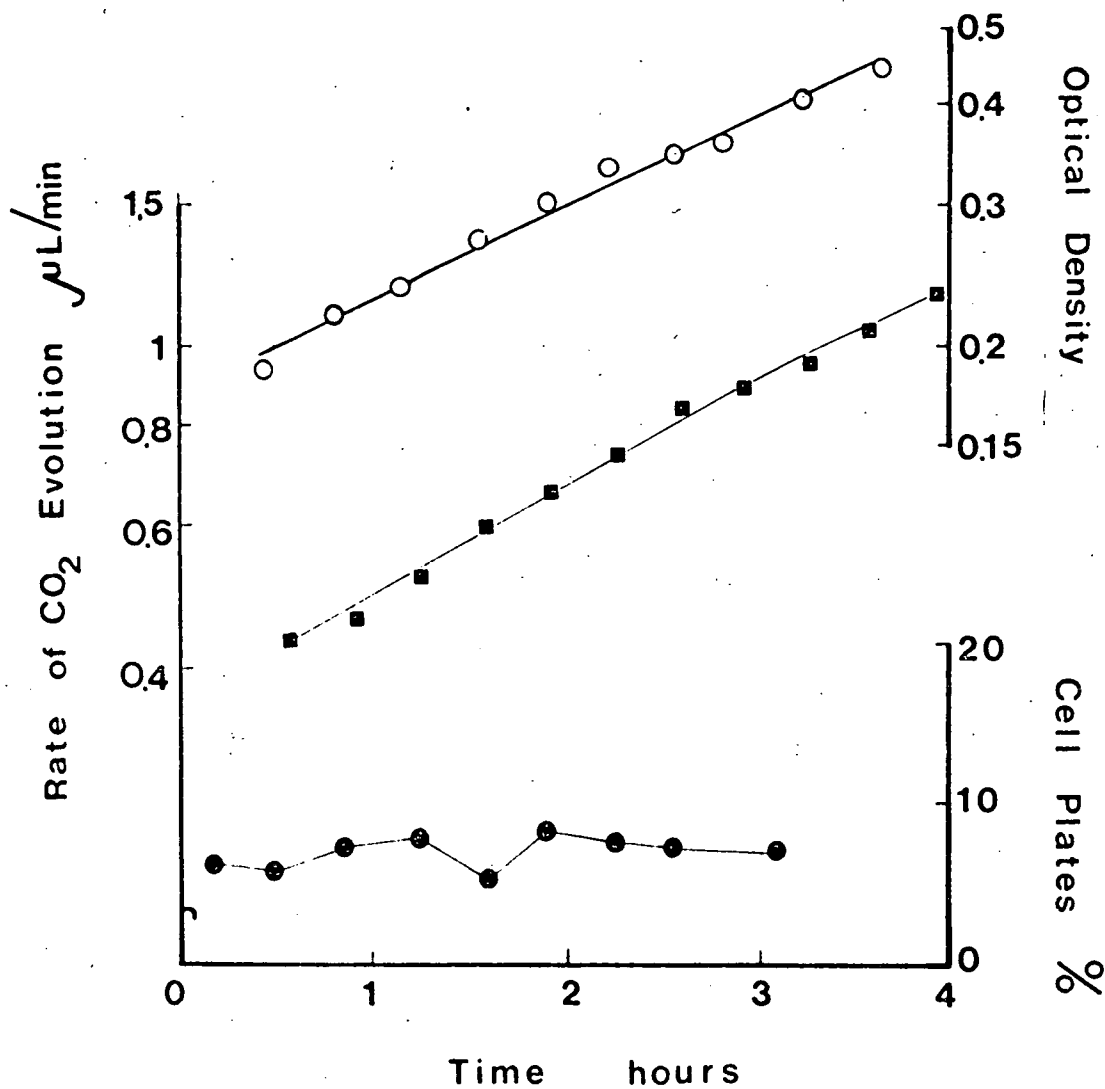


Fig 5 - 9. CO₂ evolution in an asynchronous control culture of S. pombe.

The control culture was prepared by shaking up a lactose gradient and inoculating a sample of cells into fresh medium. Each measurement of the rate of CO₂ evolution was made by measuring the CO₂ evolved from a 3ml sample over a period of 20 minutes.

○—○ Optical Density.

■—■ Rate of CO₂ evolution.

●—● % Cell Plates.

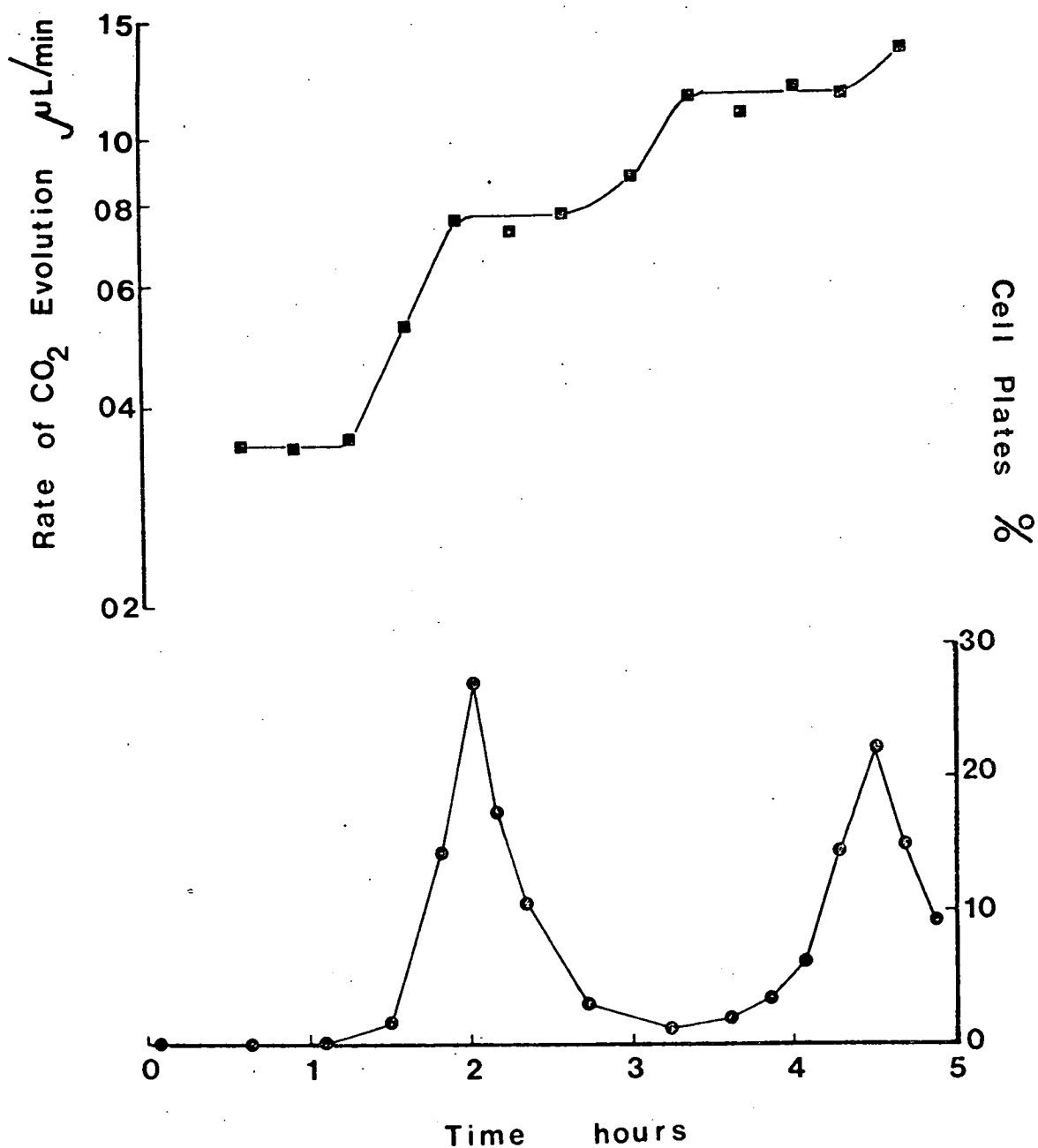


Fig 5-10. CO₂ evolution in a synchronous culture prepared using a sucrose gradient.

Each measurement of the rate of CO₂ evolution was made by measuring the CO₂ evolved from a 3 ml sample over a 20 minute period.

■—■ Rate of CO₂ evolution.

●—● % Cell Plates

It is possible that the results obtained by measurements of CO_2 evolution in synchronous cultures do not reflect cell cycle events but are simply perturbations caused by the synchronisation method. This possibility was tested by collecting cells and centrifuging them on a lactose gradient as for the preparation of a synchronous culture. Instead of removing the small cells from the top of the gradient, the tube was thoroughly shaken and some of the cells were inoculated into fresh medium at the same cell density as in the synchronous cultures. CO_2 evolution was then examined in this culture and the rates of evolution are shown in Fig 5 - 9. There was a continuous increase in the rate of CO_2 evolution and there were no signs of the abrupt steps which were observed in the synchronous cultures. At first sight, therefore, the periodic changes in the rate of CO_2 evolution observed in the synchronous cultures could be concluded to represent real, cell cycle events.

CO_2 Evolution in Synchronous Cultures prepared using Sucrose Gradients.

The synchronous and asynchronous control cultures which have been described above were all prepared using lactose gradients, on the assumption that since lactose is not metabolised by S. pombe, it would be a more suitable gradient material than, for example, glucose or sucrose. This assumption was tested by preparing a synchronous culture using a sucrose gradient and measuring the rate of CO_2 evolution as shown in Fig 5 - 10. There are some differences between this culture and those which were prepared using lactose gradients. The time taken for the first rise in the rate of CO_2 evolution to occur was significantly shorter in the synchronous culture prepared using sucrose as the gradient material (30 mins) than it was in any of the synchronous cultures prepared using lactose

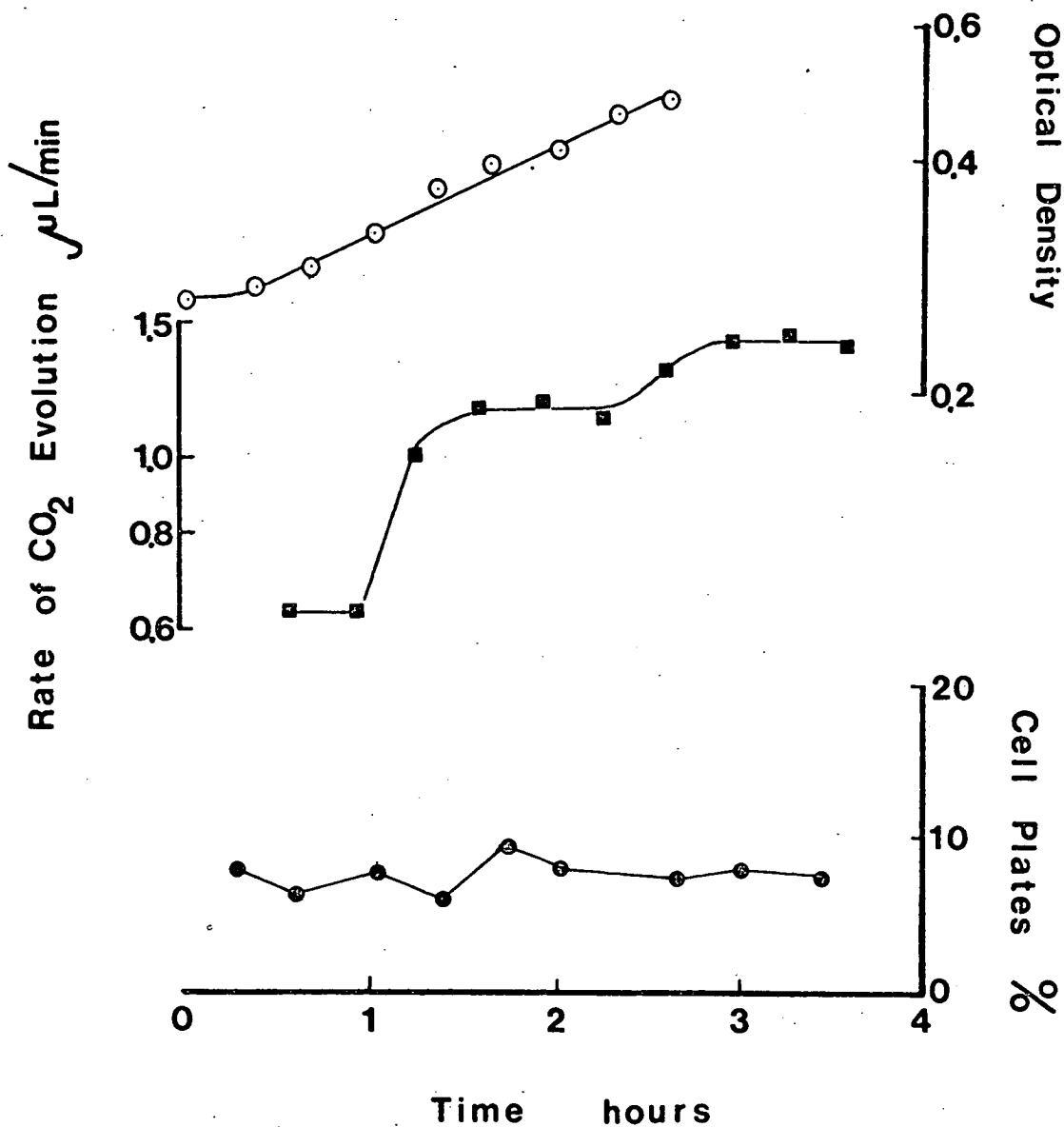


Fig 5 - 11. CO₂ evolution in an asynchronous control culture prepared using a sucrose gradient.

Each measurement of the rate of CO₂ evolution was made by measuring the CO₂ evolved from a 3ml sample over a 20' period.

○—○ Optical Density. ■—■ Rate of CO₂ evolution.
 ●—● % Cell Plates.

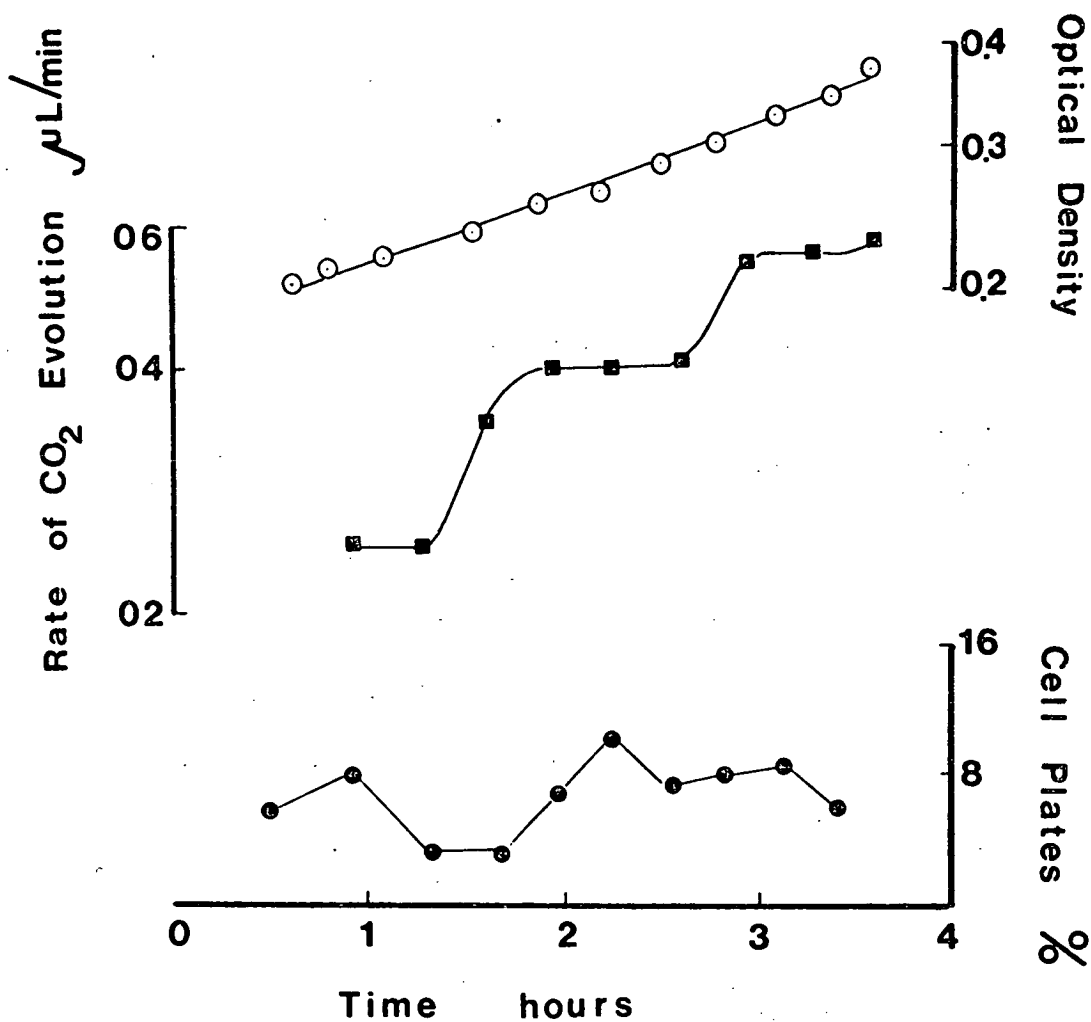


Fig 5 - 12. CO₂ evolution in a culture resuspended in fresh medium.

A late log phase culture containing 2×10^7 cells/ml was resuspended in fresh medium to a cell density of 2×10^6 cells/ml. CO₂ evolution was measured for 20' periods on 4 ml samples as described previously.

○—○ Optical Density.

■—■ Rate of CO₂ evolution.

●—● % Cell Plates.

gradients (average time 68 minutes) although cell division appeared to be no more synchronous in the sucrose synchronous culture. The second rise in the rate of CO_2 evolution in the sucrose synchronous culture occurred at 0.39 of the way through the second cycle, which was much earlier in the cycle than any of the cultures prepared using lactose gradients (Fig 5 - 8).

The rate of CO_2 evolution was therefore examined in an asynchronous control culture prepared using a sucrose gradient and the results are shown in Fig 5 - 11. The rate of CO_2 evolution increased in a step-wise manner about 1 hour after re-inoculation of the culture. There was a further step-wise increase in the rate about $1\frac{1}{2}$ hours later, the same time interval as was seen in the rate of CO_2 evolution in a synchronous culture prepared using sucrose gradients. This result then, is identical to that obtained from the sucrose synchronous culture described above. The conclusion from these two experiments is that the step-wise increase in the rate of CO_2 evolution is a direct result of the sucrose gradient.

The evolution of CO_2 is therefore a cellular process which can be induced to show an abnormal pattern of increase by allowing the culture to spend a short time on a sucrose gradient. Two further types of experiment indicate that periodic changes in the rate of CO_2 evolution may be induced by changing the environment of the cells. If a culture containing 1×10^7 cells/ml was resuspended in fresh medium at a cell density of 3×10^6 cells/ml, the rate of CO_2 evolution, shown in Fig 5 - 12, showed a step-wise pattern of increase after resuspension. The extent of the rises and their periodicity depended on the density of the culture before resuspension; the more the culture density was above about 8×10^6 cells/ml, the greater the periodic rise in the rate of CO_2 evolution after resuspension

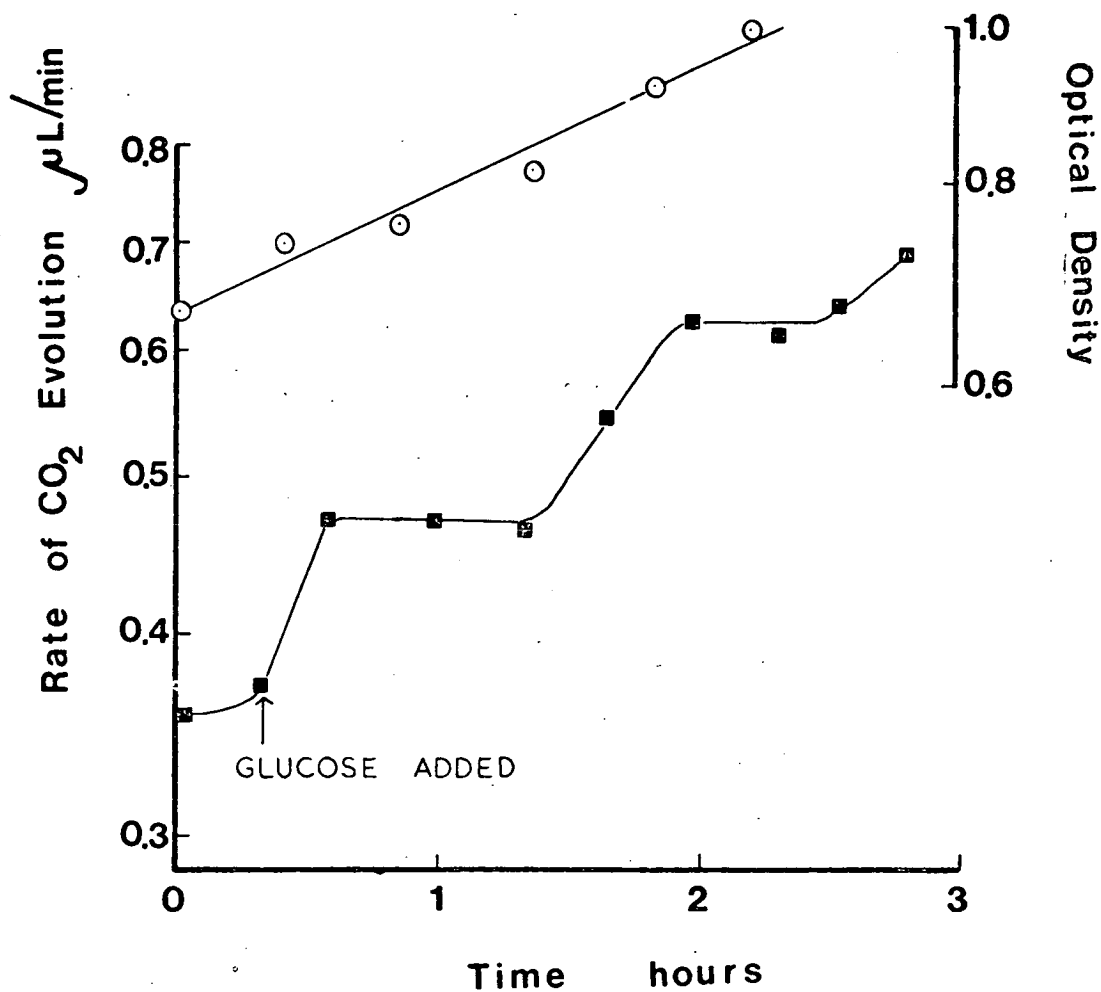


Fig 5 -13. CO₂ evolution in a culture given an additional 1% glucose.

A log phase culture containing 1×10^7 cells/ml was given an additional 1% glucose at the time shown. The rate of CO₂ evolution was then measured on 2 ml samples of this culture by measuring the CO₂ evolved over a 20 minute period.

○—○ Optical Density.

■—■ Rate of CO₂ evolution.

and the longer the period between the first and the second rises. If, however, the culture contained around 3×10^6 cells/ml before resuspension in fresh medium, the rate of evolution of CO_2 showed the same continuous pattern of increase as it did before resuspension. Exponential cultures used to prepare synchronous cultures were not used at cell densities higher than 3×10^6 cells/ml.

A similar step-wise increase in the rate of CO_2 evolution was observed if 1% glucose was added to a culture containing around 1×10^7 cells/ml, as shown in Fig 5-13. This effect was not observed if the culture contained around 3×10^6 cells/ml before addition of glucose.

One possible conclusion from these results obtained by resuspending a high density culture in fresh medium or by adding glucose to it, is that periodic increases in the rate of CO_2 evolution were induced by glucose starvation followed by feeding. It is also possible that this situation was being mimicked when cells were concentrated and placed on a sucrose gradient, thus producing the periodic increase in the rate of CO_2 evolution which was observed in the synchronous culture and in the asynchronous control cultures which were prepared using sucrose gradients. This effect did not appear to occur when an asynchronous control culture was prepared using a lactose gradient.

The possibility must also be considered that the small cells which were removed from the top of the lactose gradients to prepare the synchronous cultures showed periodic increases in the rate of CO_2 evolution, similar to those increases shown by the entire cell population when sucrose was used as the gradient material. The possibility of such a cell cycle specific perturbation will be discussed later in this Chapter.

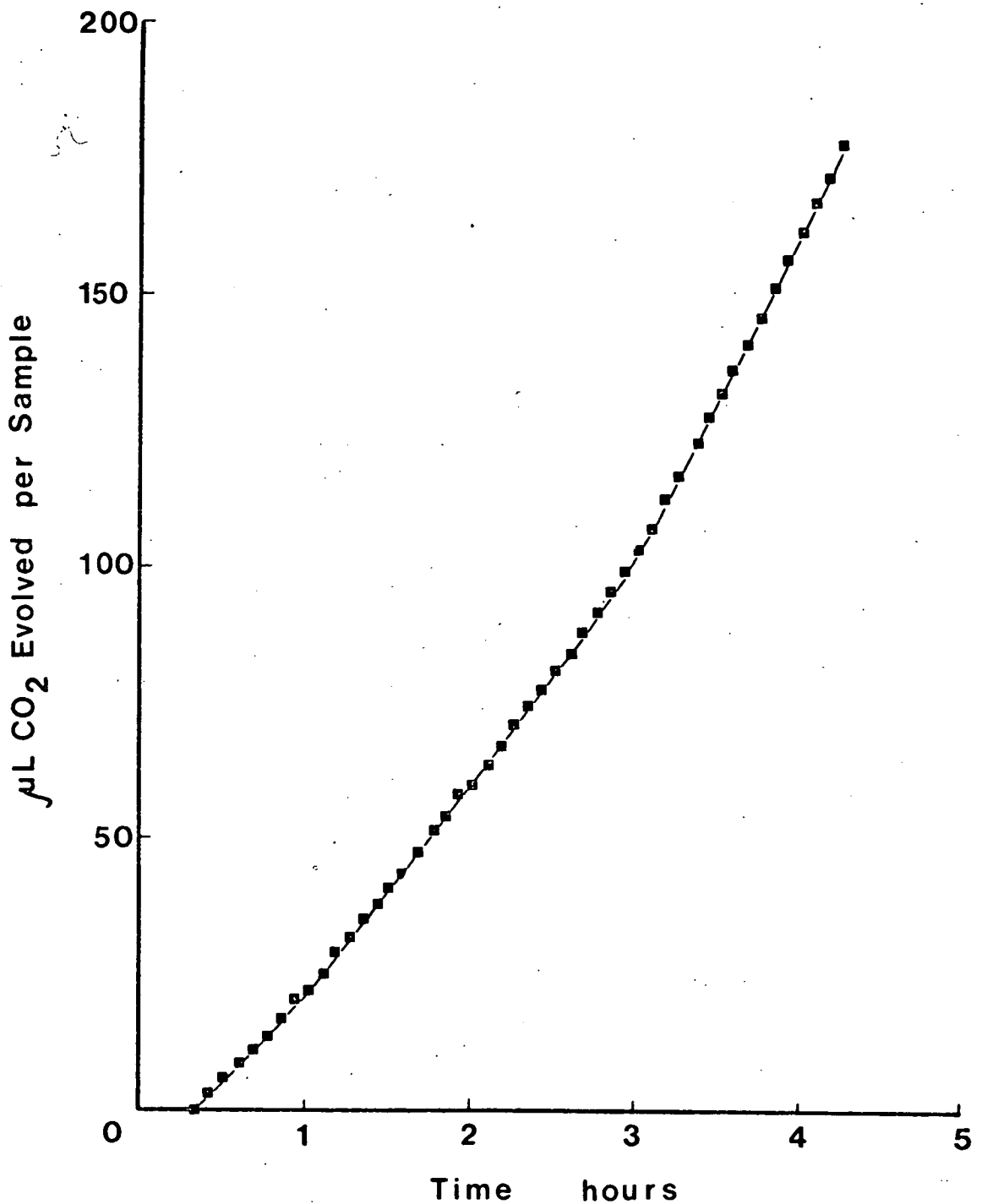


Fig 5 -14. Continuous monitoring of CO₂ evolution in a synchronous culture.

5 minutes after preparing a synchronous culture, 2 X 2 ml samples were placed in two Warburg flasks and CO₂ evolved was measured over a $4\frac{1}{2}$ hour period, taking readings every 5 minutes and averaging the accumulation of CO₂.

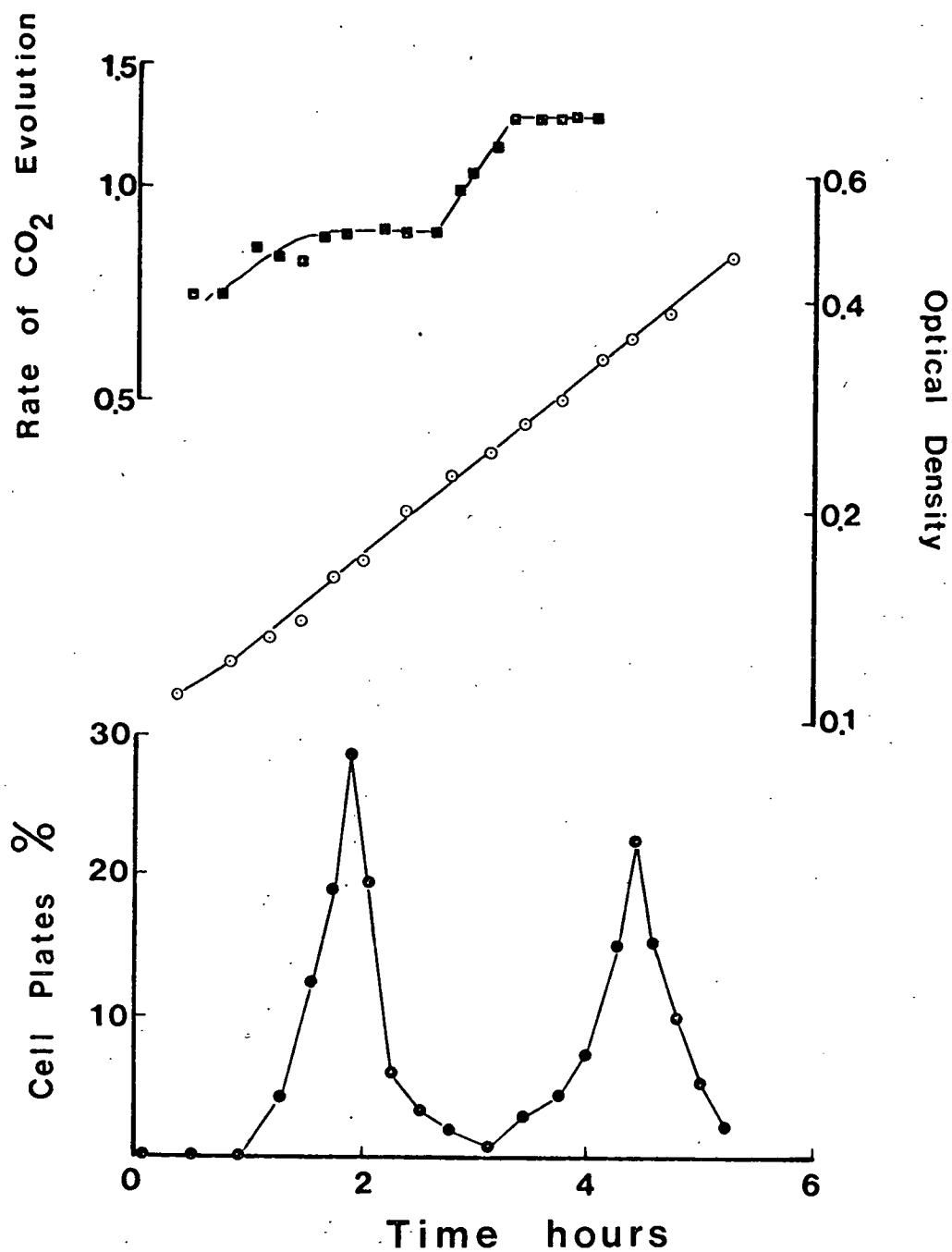


Fig 5-15. Rate of CO₂ evolution in a synchronous culture by continuous measurement of CO₂ evolved by one sample over a prolonged time. The rate of CO₂ evolution was estimated at intervals from the data shown in Fig 5-14 by drawing tangents to the curve. Cell plates & optical density were measured in the original culture.

■—■ Rate of CO₂ evolution. ○—○ Optical Density.
 ●—● % Cell Plates.

Continuous Measurement of CO₂ Evolution in a Synchronous Culture.

It has been shown that CO₂ evolution can be measured continuously on one sample, provided that the sample is sufficiently small so that cell division and CO₂ evolution are not inhibited by the CO₂ itself. A synchronous culture was prepared using a lactose gradient. The cell density of the synchronous culture was 1.5×10^6 cells/ml. CO₂ evolution was measured on two samples of 2ml for a four hour period and the amount of CO₂ accumulated during this time is shown in Fig 5 - 14. The rate of CO₂ accumulation was measured at intervals from this graph and this is shown in Fig 5 - 15, along with the optical density and the cell plate index of the synchronous culture.

The measurements of the rate of CO₂ evolution show an increase during the first cycle, followed by a second, and more pronounced, step - wise increase during the second cycle. The method is not sufficiently sensitive to show accurately the position and the extent of the rate increase during the first cycle. However, the increase in rate during the second cycle was considerably less than a doubling and, during the four hours of measurement, the rate of CO₂ evolution increased by a mere 78%.

Continuous measurement of CO₂ evolution in an asynchronous culture, shown earlier in Fig 5 - 3, suggests that in small samples, a valid measure of the rate of evolution could be obtained for at least a two hour period. There is, however, in Fig 5 - 3, a suggestion that the rate of CO₂ evolution is no longer increasing with the same doubling time as the optical density of the culture. It seems possible, therefore, that the evolution of CO₂ was being inhibited in both samples in the synchronous culture shown in Fig 5 - 15.

If CO₂ evolution was itself being inhibited by the CO₂

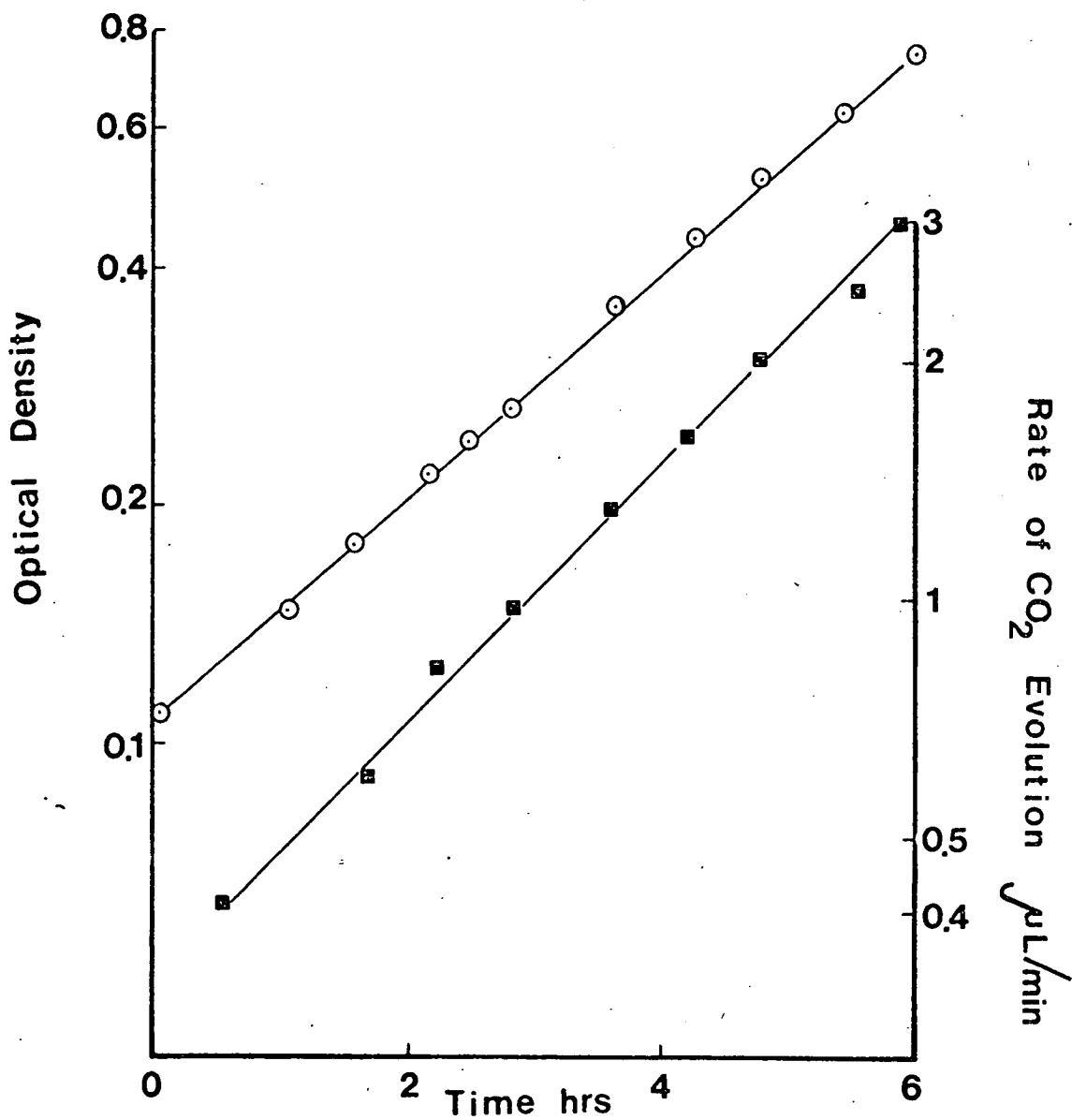


Fig 5 - 16. CO₂ evolution in S. pombe growing on yeast extract + 1% glucose.

The rate of CO₂ evolution was measured by following the CO₂ evolved by a 3 ml sample over a 20 minute period.

- Optical Density at 595 nm.
- Rate of CO₂ evolution.

evolved in the Warburg flasks, some degree of inhibition of cell division should also occur. At the cell plate peak, the cell number in a synchronous culture is changing rapidly and even a small inhibition of cell division should show up as a large decrease in the cell number of the inhibited sample compared with a normally growing synchronous culture. Cell counts were therefore made on samples removed from each of the Warburg flasks and these counts (3.3×10^6 and 3.9×10^6 cells/ml) were less than the cell count of the original culture (4.9×10^6 cells/ml) which had been growing in a shaking water bath during the course of the experiment. So enough CO_2 was evolved from both samples in the Warburg flasks to inhibit cell division in the synchronous culture.

This result was obtained in only one synchronous culture as a repeat of the technique which was being used to measure the evolution of CO_2 in the Carlsberg Laboratory. From this result, however, some inherent flaws in this technique seemed to emerge and it seems probable that CO_2 evolution in S. pombe cannot be monitored continuously and reliably in Warburg flasks over an extended period.

CO_2 Evolution in Cultures growing in Yeast Extract /Glucose.

It was stated at the beginning of this Chapter that experiments were being carried out in the Carlsberg Laboratory using a complete medium. The evolution of CO_2 was therefore measured in complete medium consisting of 0.5% yeast extract (Difco), 1 or 3% glucose and 0.2 g KH_2PO_4 . The pattern of CO_2 evolution did not alter when either 1 or 3% glucose was used in the medium.

Rates of CO_2 evolution in an asynchronous culture growing in the medium described above are shown in Fig 5 - 16. Growth, as

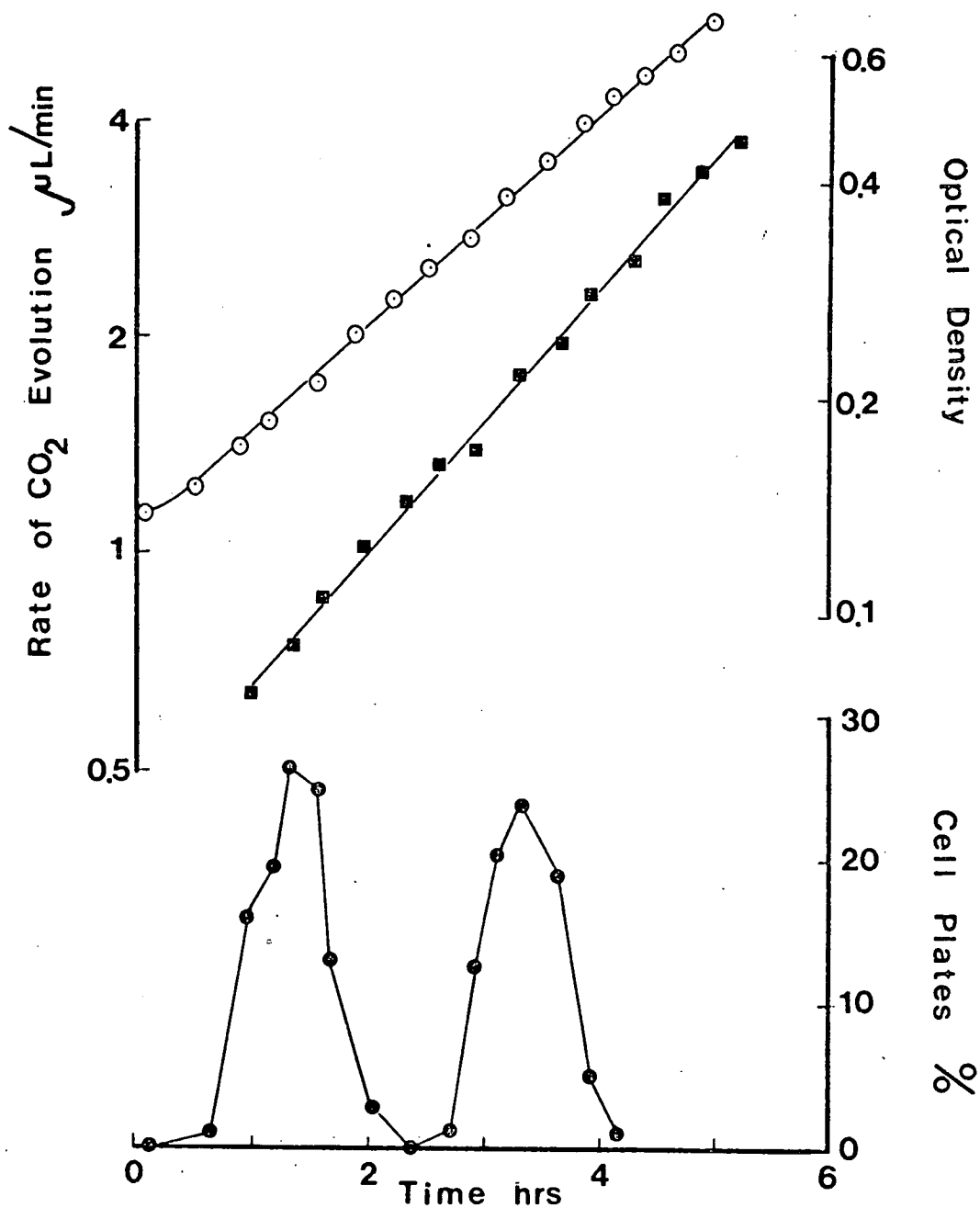


Fig 5-17. CO₂ evolution in a synchronous culture growing in yeast extract.

A synchronous culture was prepared using a lactose gradient made up in yeast extract medium. CO₂ evolution was measured on 3 ml samples over a 20 minute period after which the sample was discarded.

○—○ Optical Density.

■—■ Rate of CO₂ evolution.

●—● % Cell Plates.

measured by the increase in optical density of the culture was faster (120 minutes) in this medium than in EMM2 (140 - 150 minutes) and the rate of CO_2 evolution increased faster than the growth of the culture (90 minutes). This compares with a doubling time of the rate of CO_2 evolution in EMM2 of 150 minutes. The cultures growing in yeast extract /glucose did not therefore appear to be in a balanced state with respect to the parameters of CO_2 evolution and optical density; a balanced culture, as defined earlier, is one in which, during a given interval of time, every extensive property of the system increases by the same factor. (Campbell, 1957).

CO_2 evolution was measured in a synchronous culture growing on yeast extract and 3% glucose and prepared using a lactose gradient. The results of this experiment are shown in Fig 5-17 and it is clear that the pattern observed is different to that shown by the synchronous cultures grown in minimal medium. In the culture growing on yeast extract, there was a continuous increase in the rate of CO_2 evolution during two synchronous cell divisions and, once again, the doubling time of the culture growth, as measured by the increase in optical density (120 minutes) was slower than the doubling time for the rate of CO_2 evolution (100 minutes).

CO_2 Evolution in Strain 972 h⁻.

The experiments which have so far been described in this Chapter were done using S. pombe strain NCYC 132. A different strain was then examined to see whether the same pattern of CO_2 evolution was observed in synchronous cultures. Strain 972 h⁻ was grown in the same medium EMM2 as previously described. Synchronous cultures were prepared by the same method and using lactose gradients. The evolution of CO_2 was measured by the same modification of the direct

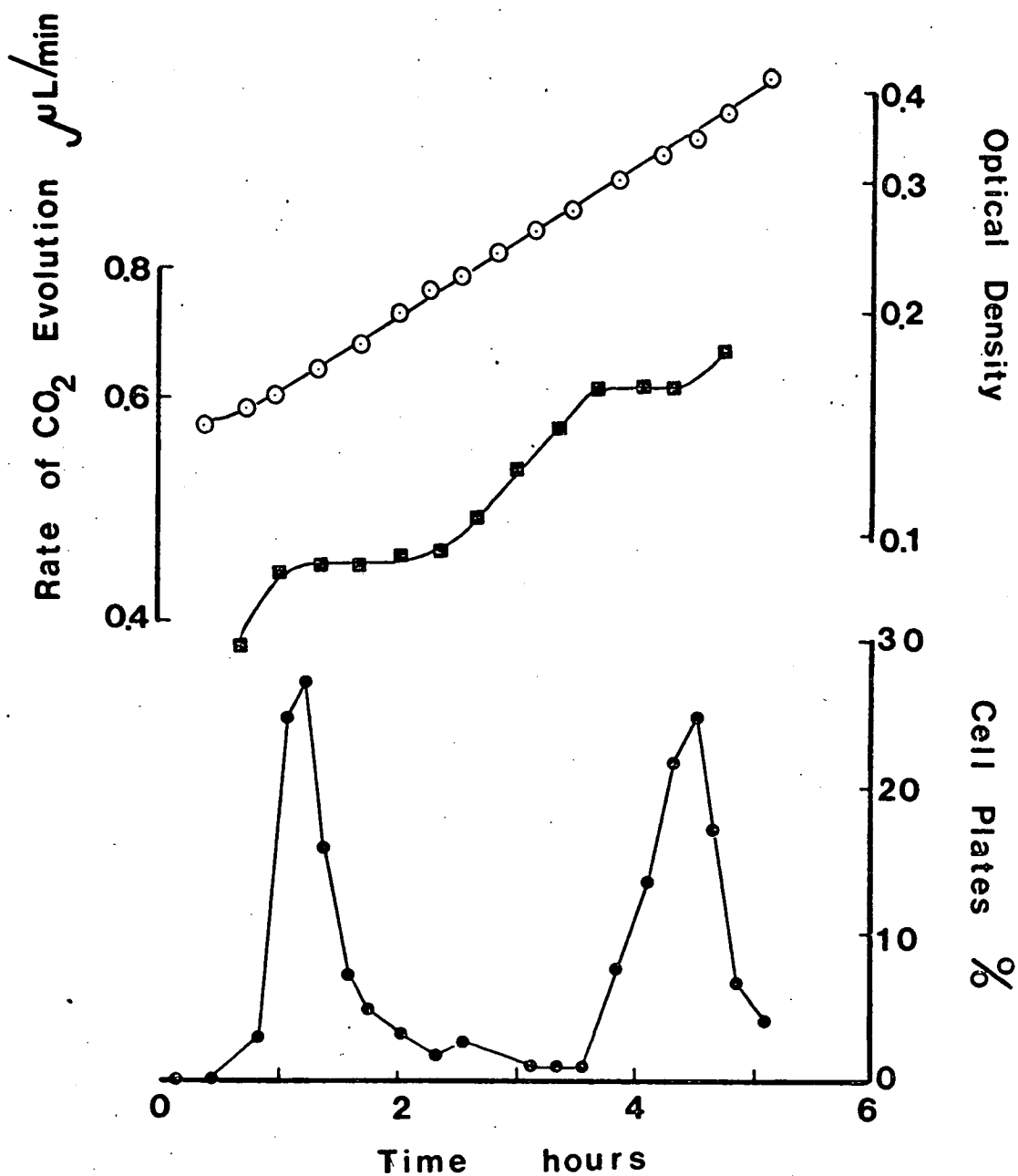


Fig 5-18. CO₂ Evolution in a Synchronous Culture of *S. pombe*, strain 972 h⁻.

A synchronous culture of 972 h⁻ was prepared in EMM2 using a lactose gradient. The rate of CO₂ evolution was measured on 3 ml samples over a 20 minute period. After each measurement the sample was discarded.

○—○ Optical Density.

■—■ Rate of CO₂ Evolution.

●—● % Cell Plates.

method of Warburg, since the RQ of this strain is also about 10.

The rates of evolution of CO_2 in a synchronous culture of strain 972 h⁻ are shown in Fig 5 - 18. There was a doubling in the rate of CO_2 evolution at a discrete time during the second half of the cell cycle. There were several differences, however, between the results for the two strains. The peak of dividing cells occurred in 972 h⁻ at about 1 hour after the cells had been removed from the top of the gradient. This was in contrast to the synchronous cultures of strain 132 when the peak of dividing cells occurred at about 2 hours. In the case of 972 h⁻, the cells at the top of the gradient which were removed to prepare the synchronous culture were not cells at the beginning of the cell cycle but cells which had reached about half way through the cycle. A possible explanation for this is that cells of strain 972 h⁻ do not separate immediately after division but remain attached for some time. A cell pair on a gradient would behave, therefore, like one large cell since the separation of a culture on a gradient of this nature is by size difference. In an attempt to overcome this, immediately after collection, the cell suspension was sonicated for 5 seconds at a setting of 20 kc/sec and an amplitude of 2 microns using an MSE Ultrasonic disintegrator as previously described for the preparation of samples for cell counting. The suspension was then placed on top of the gradient and centrifuged as before. This procedure failed on two counts. The cells which were removed from the top of the gradient to prepare the synchronous culture were still cells which were about half way through the cycle. Moreover, growth of these cells (as measured by increase in optical density) was impaired for a considerable period after re-inoculation, in some cases there was no increase in optical density for 80 minutes. Abnormally low increases in the protein

content per cell have also been measured in synchronous cultures of 972 h⁻ which had been subjected to sonication before centrifuging the cell concentrate on a gradient (P.N.Nurse, personal communication).

Both strains, therefore, showed a doubling in the rate of CO₂ evolution at approximately the same time during the cell cycle. More work would have to be done with strain 972 h⁻ to establish the exact timing of the rate change. In the case of strain 132, cells at the start of the cycle were removed from the top of the gradient to prepare the synchronous culture; in the case of strain 972 h⁻, cells from half way through the cycle were removed from the top of the gradient. One could account for the results with the synchronous and asynchronous cultures of strain 132 by postulating that only the smallest cells in an exponential culture were perturbed during the synchronisation procedure. This perturbation is such that a change in the rate of CO₂ evolution occurred in such small cells about 1 hour after reinoculation into fresh medium and again 2 hours later. This accounts for the results in the synchronous cultures in which the smallest cells in an exponential culture are removed from the rest of the population and it also accounts for the results obtained with the asynchronous control cultures. In the latter, cells at all stages of the cycle are removed and reinoculated into fresh medium. Since one is postulating that only the smallest cells in a population are going to be perturbed by the synchronisation procedure, such a control culture would contain a predominant number of cells which were unaffected by the treatment they had just undergone and would not show periodic increases in the rate of CO₂ evolution. This argument presumes that periodic changes in the rate of CO₂ evolution seen in synchronous cultures are purely artifacts and that the true pattern is something different (possibly continuous).

This is the ultimate criticism which can be made of any measurement which has been made in a synchronous culture and which demonstrates a periodic change in any event. An assumption which has always been made in the preparation of synchronous cultures by a selection method is that the technique does not affect cells at one stage during the cell cycle any differently from cells at another stage i.e. that a cell cycle specific perturbation has not been introduced.

Measurements of the rate of CO_2 evolution in strain 972 h⁻ to some extent refute this criticism. In this case, cells at a different stage of the cell cycle were selected and produced what appears to be a change in the rate of CO_2 evolution at the same stage of the cycle as strain 132. Therefore, it seems likely that the doubling in the rate of CO_2 evolution observed in S. pombe is a genuine cell cycle event.

It would be more convincing, however, to show that a doubling in the rate of CO_2 evolution occurred in strain 132, irrespective of the cycle stage of cells at the start of the synchronous culture. An attempt to demonstrate this will be discussed in the next section.

CO_2 Evolution in Synchronous Cultures prepared on a Zonal Rotor.

An attempt was made to prepare synchronous cultures of S. pombe using inocula at different stages of the cell cycle. Ideally, synchronous cultures would be prepared by separating the smallest cells in a population from the rest and by separating cells which were half way through the cycle from the rest of the population, measuring the rate of CO_2 evolution in both of these cultures and comparing the

times at which steps, if any, occurred in the rates of evolution of CO_2 . Such an experiment would have several advantages. In the first place, if steps occurred in the rates of evolution of CO_2 in both cultures at the same absolute time after re-inoculation, then these steps would occur at different times during the cell cycles of both cultures. It would then seem that the steps in the rate of evolution of CO_2 were artifacts caused by the synchronisation technique. If, however, the steps occurred only in the synchronous culture prepared by selection of the smallest cells, it would be likely that the steps in the rate of CO_2 evolution observed in this synchronous culture were also produced by a synchronisation artifact. If, finally, the steps in the rate of CO_2 evolution occurred at the same cycle time in both cultures, but at different absolute times after re-inoculation, the evidence would be greatly strengthened that the steps which have been described in the rate of CO_2 evolution are cell cycle events happening in normally growing individual cells and are not a result of the synchronisation treatment.

Attempts to separate an entire cell cycle by centrifuging a culture of S. pombe on a gradient in a tube have never been successful. This method has only proved suitable for separating the small cells from the rest of the population, samples from lower down the gradient containing cells at all stages of the cycle (Mitchison & Creanor, unpublished observations). Since this method has been applied successfully to Saccharomyces cerevisiae, it is mysterious why it should not also be applicable to S. pombe. It has been shown, however, that S. pombe can be separated according to size on a zonal rotor (Wells & James, 1972; Poole & Lloyd, 1973 a & b; Mitchison & Carter, 1975). This method was used in an attempt to prepare synchronous cultures of S. pombe in which cells at the start of the cycle and cells

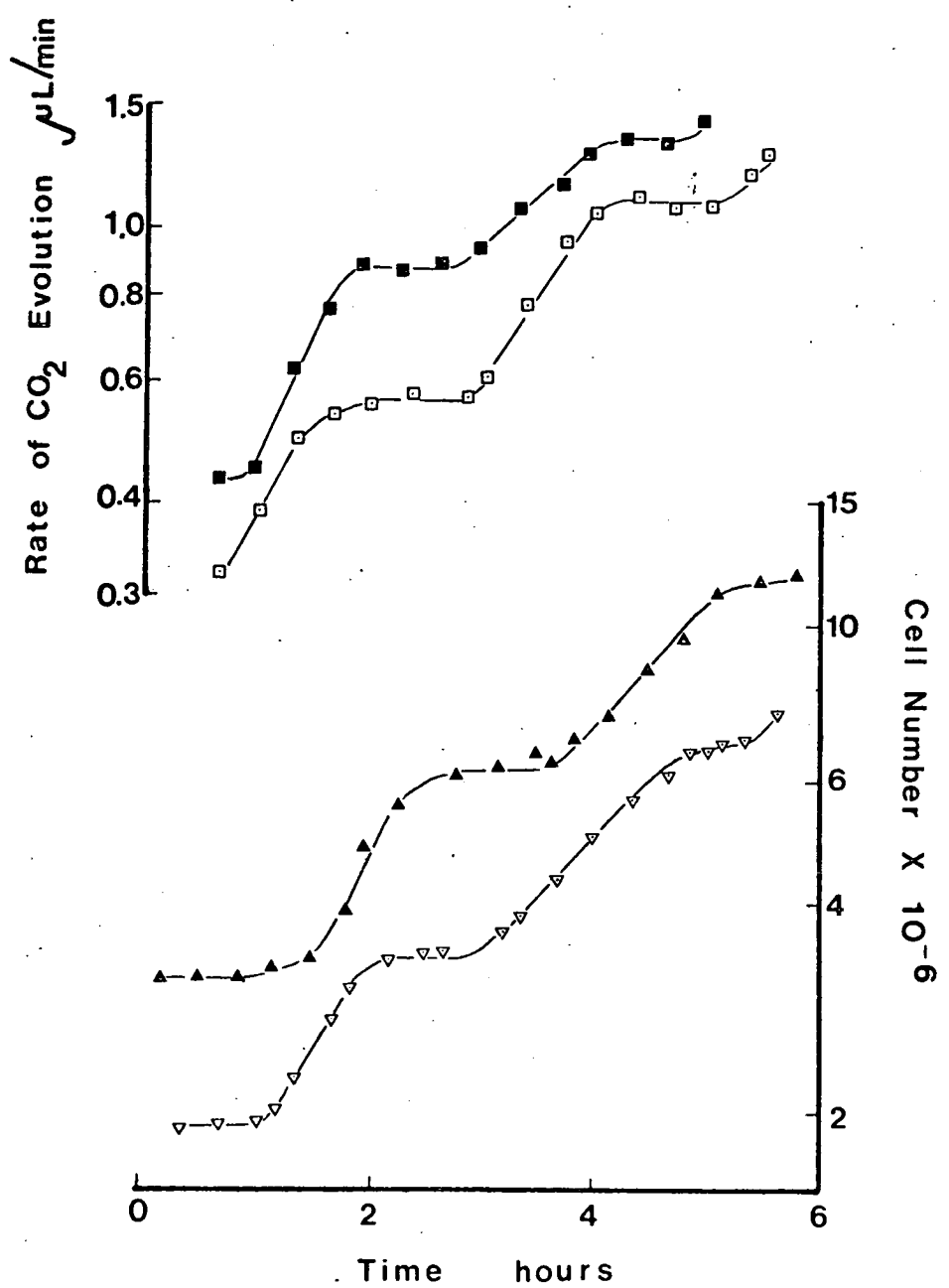


Fig 5-19. CO₂ evolution in synchronous cultures from the zonal rotor.

CO₂ evolution was measured on 3 ml samples of two synchronous cultures, one prepared by selecting small cells from the top of the rotor, the other from cells half way down the rotor.

- CO₂ evolution from middle cut.
- CO₂ evolution from top cut.
- ▽—▽ Cell number from middle cut.
- ▼—▼ Cell number from top cut.

half way through the cycle were used to prepare the synchronous culture.

The first attempt to prepare two synchronous cultures using cells at different stages of the cell cycle is shown in Fig 5 - 19. CO_2 evolution was measured on both cultures and in both there was the characteristic doubling in the rate of CO_2 evolution. Several comments should be made about these results. In the first place, there was considerable delay after re - inoculation of both cultures before the optical density began to increase. This occurred over a much longer period than in synchronous cultures prepared from tube gradients. This could be accounted for by the increased time which the cells spent on the zonal gradient. The cells usually spent no longer than 6 minutes on a tube gradient. In the case of the zonal separation, at least 20 minutes were spent by the cells on the gradient before they could be re - inoculated into fresh medium. Secondly, although larger sized cells were presumed to be removed from a position half way down the gradient, the time at which these cells divided was a mere 10 - 20 minutes before the time at which the smaller cells divided. The synchrony in the culture which contained the larger cells was also poor and it was therefore difficult to show that the step in the rate of CO_2 evolution in the culture which contained the larger cells was significantly different from the time at which the step in the rate of CO_2 evolution occurred in the culture made from the small cells.

Further attempts to make synchronous cultures using the zonal rotor and separating cells which were at least half way through the cycle have not proved successful. When fractions lower than those described in Fig 5 - 19 were removed from the zonal rotor, the cultures set up using these cells were found to be more or less asynchronous.

It would seem therefore, that it is not possible to separate a culture according to stage in the cycle and then to grow these cells to make a synchronous culture, either using gradients in tubes or using the zonal rotor. Although a cell cycle has been spread across a zonal rotor (using cells and gradient at 0°C), it may be a more stringent test of the degree of separation to attempt then to grow individual fractions as a synchronous culture. It seemed, therefore, that with S. pombe, only the fractions containing the smallest cells of a population could be separated on a zonal rotor and used to grow a synchronous culture.

CO₂ Evolution in Stationary Cultures.

It was shown in Chapter 3 how the oxygen uptake remained at a constant level when a culture entered stationary phase and that this situation continued for many days afterwards. Both ethanol and acetate, which are present in the medium of a stationary culture, were being oxidised in this situation.

Immediately a culture entered stationary phase, the rate of CO₂ evolution decreased until it became difficult to measure using the technique which has been employed throughout this Chapter. As the evolution of CO₂ decreased and the rate of oxygen uptake remained constant, the ratio of gas taken up (oxygen) to gas expelled (CO₂) approached unity. Finally, several days after the cultures entered stationary phase, the RQ was 1. The meaning of this result is not at all clear. Presumably as much CO₂ is evolved as oxygen is taken up. This must mean a net increase in ATP. Does this ATP serve to maintain the cell in a viable state? Or does the cell make poly-phosphates in stationary phase? It is possible that both of these possibilities are correct. There is some evidence that poly-phosphate granules increase during stationary phase (J.W. May, per. comm).

Evolution of CO₂ in Cultures containing Inhibitors.Introduction.

It has been established that the rate of evolution of CO₂ increased periodically during the cell cycle of S. pombe growing in minimal medium. The relationship between this event with other periodic events during the cell cycle of S. pombe was then examined.

A cell cycle map for S. pombe is shown in Fig 6- 1. There are three well characterised periodic events during the cell cycle of S. pombe which can be inhibited without affecting the growth of the culture. DNA synthesis, which normally occurs at the end of the cycle, can be inhibited using 2' Deoxyadenosine (Mitchison & Creanor, 1971 a & b); nuclear division, which normally occurs at 0.75 of the cycle (Bostock, 1969) can be inhibited using Mitomycin C (Robinson, 1972); cell division at the end of the cycle can also be inhibited using Mitomycin C but this is probably a secondary effect of the inhibition of nuclear division. The evolution of CO₂ was examined in synchronous cultures in which one or more of the above periodic events had been inhibited.

A method was described in Chapter 2 for the preparation of synchronous cultures by blocking DNA synthesis using deoxyadenosine for a three hour period and then releasing the block. In such a synchronous culture, only cell division and DNA synthesis were synchronised. This has been described as "Induction Synchrony" since all of the cells in a population were induced to divide at the same time. CO₂ evolution was examined in a synchronous culture which had been prepared by this method.

The evolution of CO₂ was also examined in cultures which contained inhibitors of RNA synthesis, protein synthesis and glycolysis to show how far increases in CO₂ evolution were dependent on continued operation of these functions.

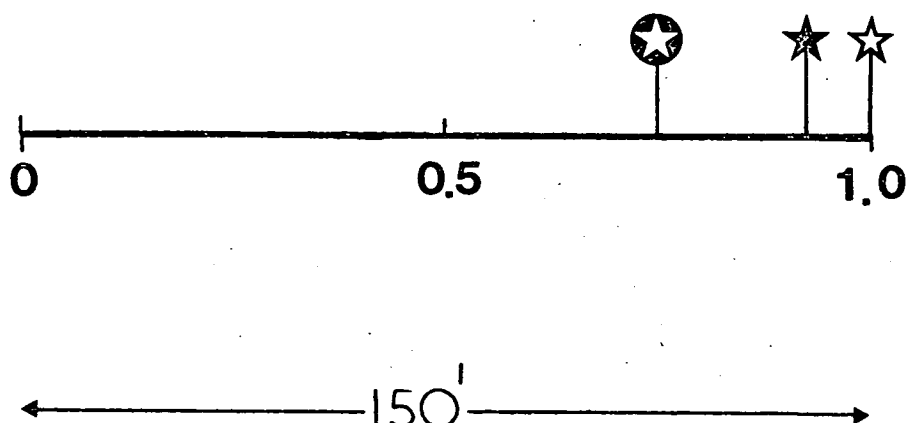


Fig 6-1. Cell Cycle map for S. pombe growing in EMM2 at 32°C.

The generation time is 150 minutes.

Relative positions of mid-points of nuclear division	⊙
Cell plate formation	⊠
DNA synthesis	△

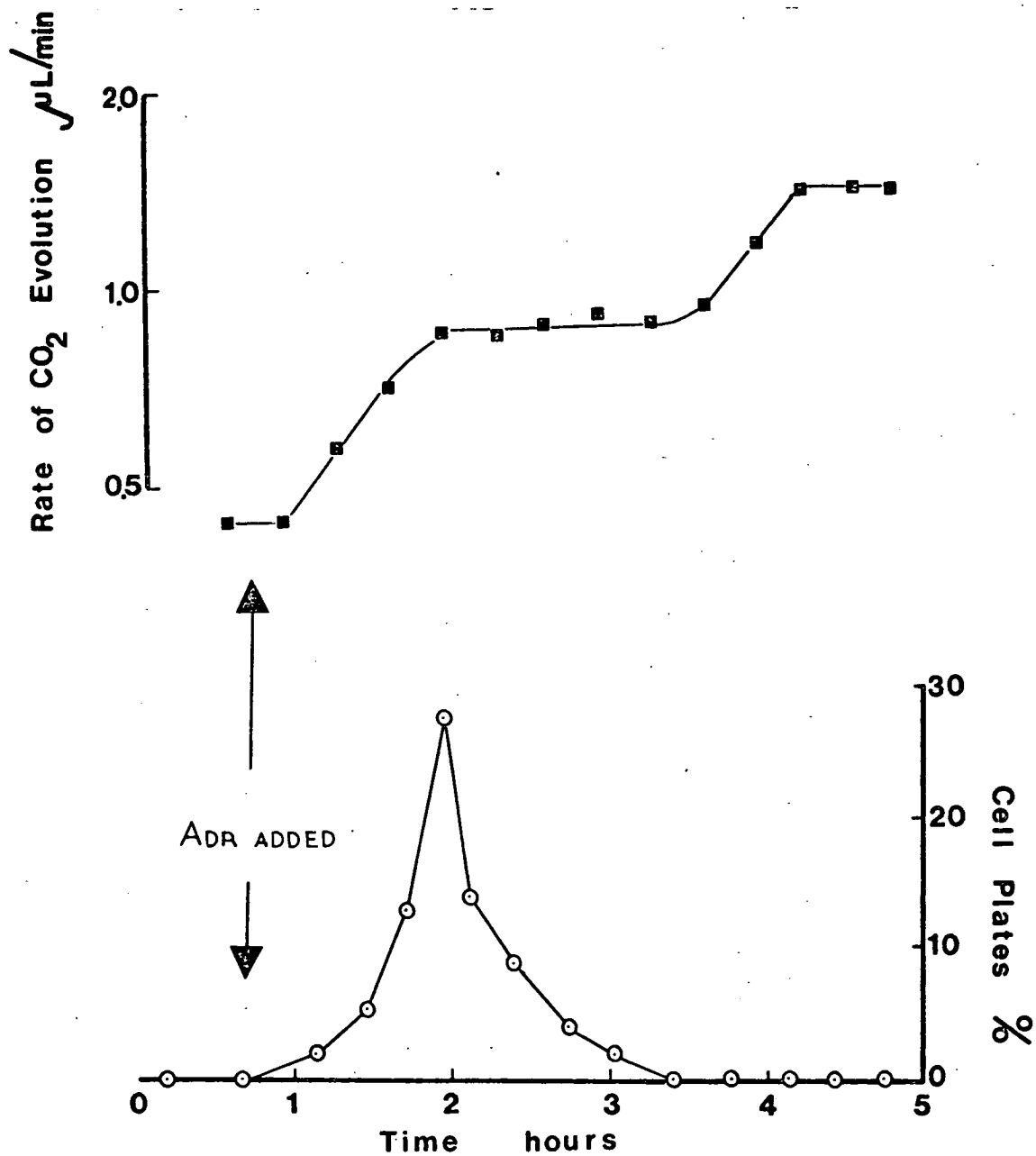


Fig 6-2. CO₂ evolution in a synchronous culture + deoxyadenosine.

Deoxyadenosine added to the culture at 40 minutes. CO₂ evolution was measured once on 2 ml samples for 20' at 20 minute intervals.

■—■ Rate of CO₂ evolution.

○—○ % Cell Plates.

110
CO₂ Evolution in a Synchronous Culture containing Deoxyadenosine.

A synchronous culture of S. pombe was prepared using a lactose gradient as previously described. 40 minutes after setting up the culture, 2 mM deoxyadenosine (Adr) was added. The rate of evolution of CO₂ was measured before and after the addition of the inhibitor and is shown in Fig 6- 2. There was a doubling in the rate of CO₂ evolution at 88 minutes after setting up the synchronous culture, at the same time as the doubling in the rate occurred in untreated cultures (Fig 5- 8). There was a further increase in the rate of CO₂ evolution (by 70%) occurring at 235 minutes. A step - wise increase in the rate would also be seen in an untreated synchronous culture, both at this time and by approximately this amount.

From the time of addition of the Adr, there is no further synthesis of DNA in the synchronous culture but, since each cell has two copies of the DNA at the time of addition of the inhibitor, the first synchronous division is unaffected (Mitchison & Creanor, 1971a). Further cell division is inhibited either until the inhibitor is removed or until the cells slowly recover from the effect of the Adr over a period of 5 - 6 hours. This experiment shows, therefore, that the doubling in the rate of CO₂ evolution which was shown in synchronous cultures of S. pombe is also seen in synchronous cultures in which the synthesis of DNA, and consequently cell division, had been inhibited. There is, however, little or no inhibition of growth in synchronous cultures which contain Adr so that after six hours in the presence of the inhibitor, cells are about twice as long as they normally are when cell division occurs. (Mitchison & Creanor, 1971a).

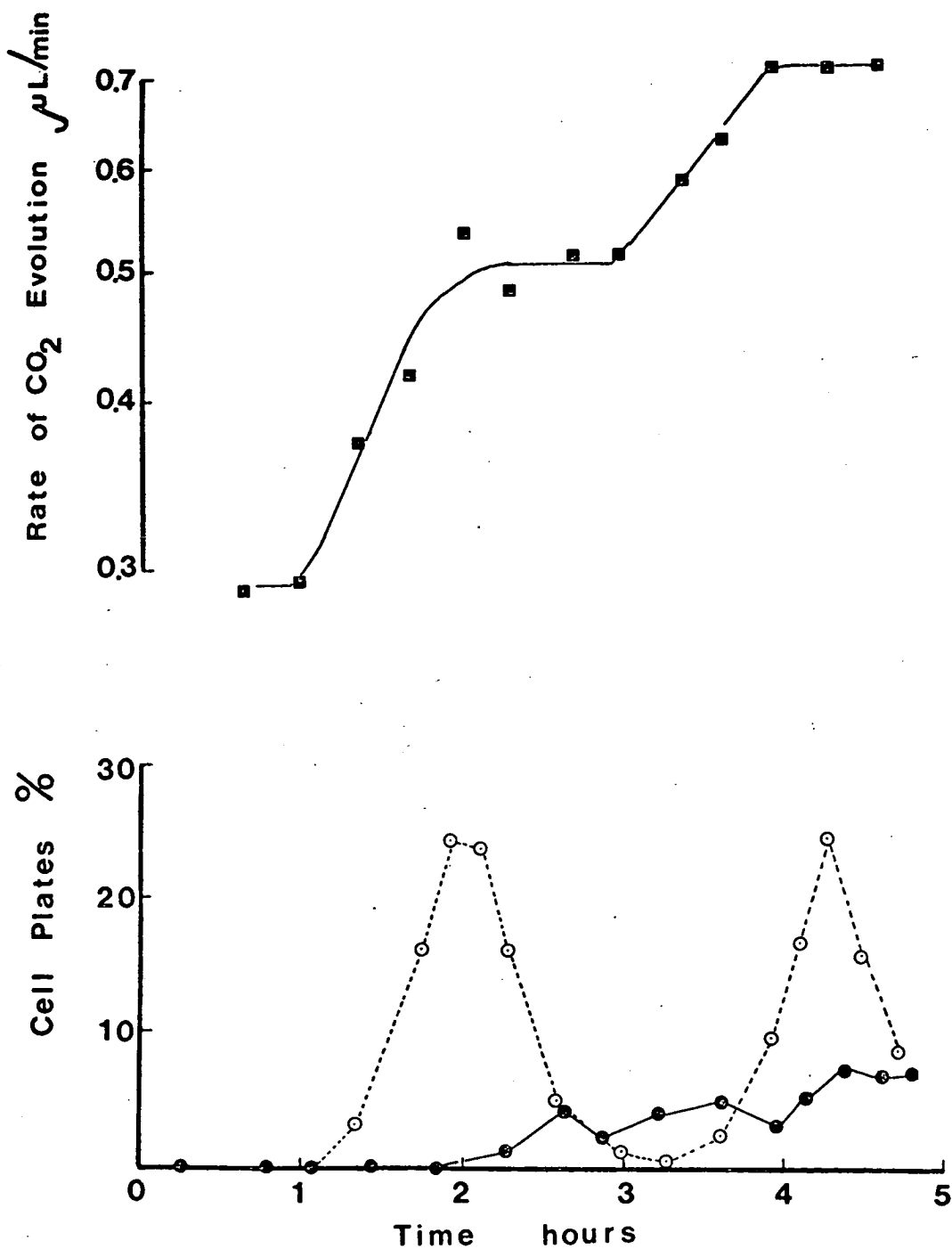


Fig 6-3. CO₂ evolution in a synchronous culture containing mitomycin C.

Mitomycin C was added to the culture at 5 minutes. CO₂ evolution was measured once on 3 ml samples for a 20' period.

- Rate of CO₂ evolution. ○—○ % Cell Plates in untreated culture.
 ●—● % Cell Plates in culture containing mitomycin C.

112

CO₂ Evolution in a Synchronous Culture containing Mitomycin C.

A synchronous culture of S. pombe was prepared as before using a lactose gradient. 10 minutes after preparation, mitomycin C was added to the culture to give 0.1 mg/ml. Immediately after the addition of the inhibitor, CO₂ evolution was measured on 3 ml samples at 20 minute intervals for 5 hours.

The rate of evolution of CO₂ showed a similar pattern in this culture, shown in Fig 6-3, to that observed in untreated synchronous cultures. At 90 minutes, the rate of CO₂ evolution increased sharply by 80%. The rate then remained constant until 2 hours later when it again increased in a step-wise manner, but this time the extent of the increase was 40%.

Mitomycin C inhibits nuclear division in S. pombe (Robinson, 1972). When added to a synchronous culture shortly after preparation, the cells are unable to undergo nuclear division during the first cycle and therefore cell division and DNA synthesis are indirectly inhibited. There were, however, some cells which did escape from the effect of the inhibitor and these cells began to divide at about 2 hours after the addition of the mitomycin C. There was no synchrony of division and those cells which did divide seemed to do so rather slowly. It was estimated that at 5 hours after the addition of the mitomycin C, about 20% of the cells had divided or were in the process of dividing.

This experiment shows, therefore, that an approximate doubling of the rate of CO₂ evolution occurred in a synchronous culture in which nuclear division had been directly inhibited using mitomycin C. As a result of this inhibition of nuclear division, cell division did not occur in this culture. A conclusion is that the increase in the rate of CO₂ evolution is independent of nuclear

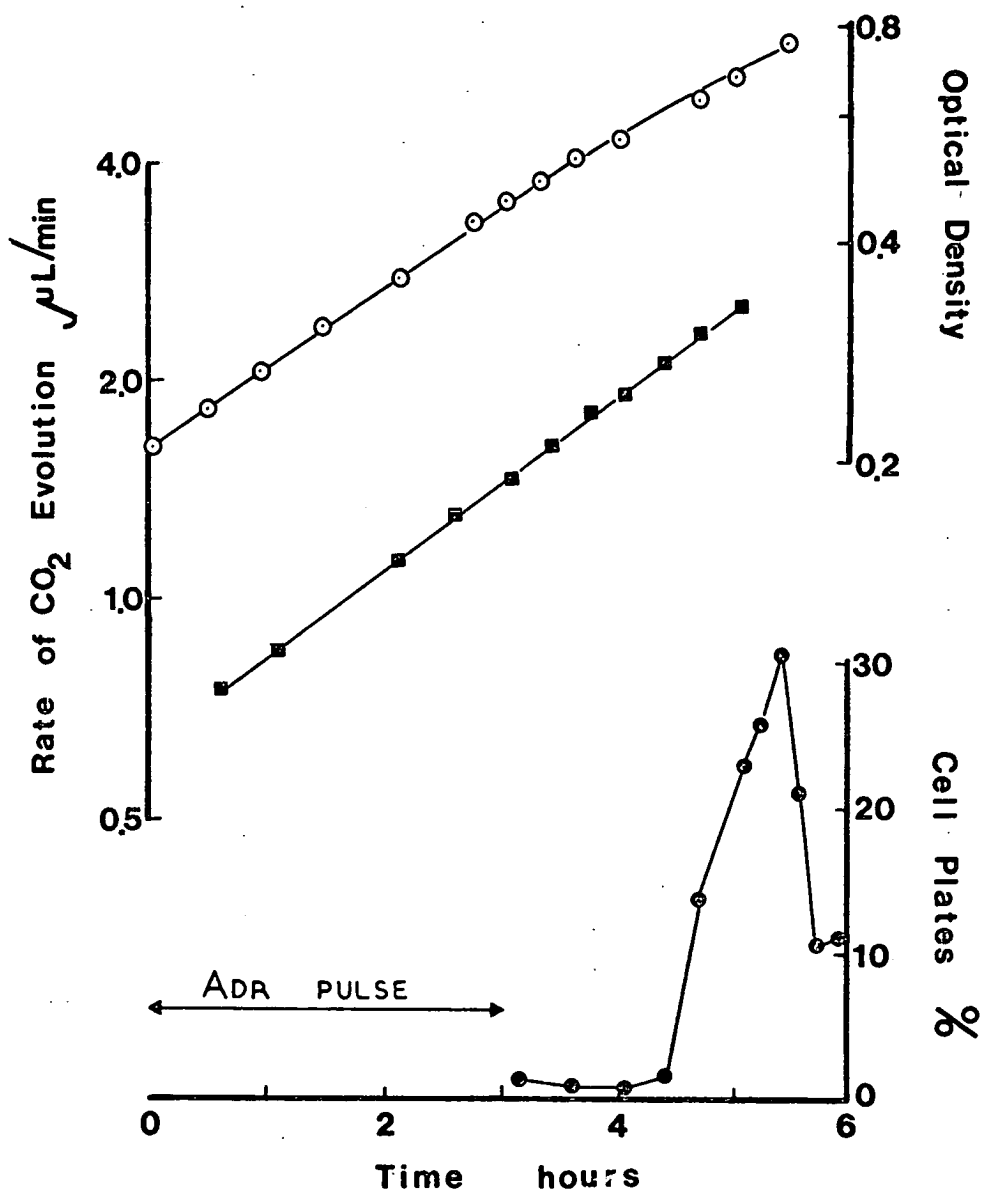


Fig 6-4. CO₂ evolution in a culture synchronised with a pulse of ADR.
 2 mM deoxyadenosine added to an exponential culture at time 0 and removed at 3 hours by filtration. The cells were resuspended in medium without inhibitor. CO₂ evolution was measured on 2 ml samples over a 20 minute period.

○—○ Optical Density. ■—■ Rate of CO₂ evolution.
 ●—● % Cell Plates.

114
division, cell division and DNA synthesis.

There was a distinct under - doubling in the rate of CO_2 evolution in what would have been the second cycle in this synchronous culture containing mitomycin C. While this has also been observed in untreated synchronous cultures, the extent of the rise has never been as small as this. This could be an indirect effect of the mitomycin C in S. pombe acting as an inhibitor of the processes leading to the production of CO_2 . Alternatively, inhibition of nuclear division, DNA synthesis and cell division could reduce the demand for ATP in the cell and thus the flux through the Glycolytic Pathway and the production of CO_2 could be similarly reduced.

CO_2 Evolution in an Induced Synchronous Culture.

2 mM deoxyadenosine was added to an exponential culture of S. pombe containing 2×10^6 cells /ml. The rate of CO_2 evolution was measured on 2 ml samples of this culture for three hours, as shown in Fig 6-4. At the end of this time, the culture was filtered, washed with warm EMM 2 and resuspended in the same volume of warm fresh medium. Measurements of the rate of CO_2 evolution were continued on this culture for a further $2\frac{1}{2}$ hours. The optical density of the culture was measured throughout the experiment and the cell plate index was measured during the period after the Adr pulse.

During the Adr pulse, the cell plate index remained at 8% (Mitchison & Creanor, 1971a) which is the value of the cell plate index in a normal asynchronous culture. Towards the end of the pulse, the cell plate index dropped to about zero where it remained until $1\frac{1}{2}$ hours after the inhibitor had been removed. The cells then went through a synchronous cell division at 140 minutes after the Adr had been removed. During the Adr pulse and after its removal, the

rate of CO₂ evolution and the optical density of the culture both showed a steady increase, with a doubling time of approximately 140 minutes.

This result is consistent with the result obtained in a synchronous culture containing a DNA synthesis inhibitor. In a selection synchronous culture, inhibition of DNA synthesis had no effect on the pattern observed for the rate of evolution of CO₂. In the case of the induced synchronous culture, synchronisation with respect to DNA synthesis did not give rise to a corresponding synchronisation in the pattern of CO₂ evolution. It can be concluded, therefore, that an increase in the rate of CO₂ evolution during the cell cycle of S. pombe is independent of DNA synthesis during that cell cycle or the previous cell cycle.

CO₂ Evolution in the presence of Inhibitors of Protein & RNA Synthesis.

CO₂ evolution was examined in cultures of S. pombe in which either protein synthesis or RNA synthesis has been inhibited. The antibiotic cycloheximide is a potent inhibitor of protein synthesis in many eukaryotic systems and is thought to act at the ribosomal level by preventing the transfer of amino acids from t-RNA to the growing polypeptide chain (Siegel & Sisler, 1965). There is also evidence that cycloheximide inhibits protein synthesis in S. pombe (Mitchison & Creanor, 1969). The synthesis of RNA has been inhibited in S. pombe using 8-hydroxyquinoline (Fraser & Creanor, 1974). This inhibitor is thought to act by chelation of magnesium and manganese from the RNA polymerase, thus preventing its action (Fraser & Creanor, 1975).

The rate of evolution of CO₂ was measured in exponential cultures of S. pombe which contained one or other of the above inhibitors.

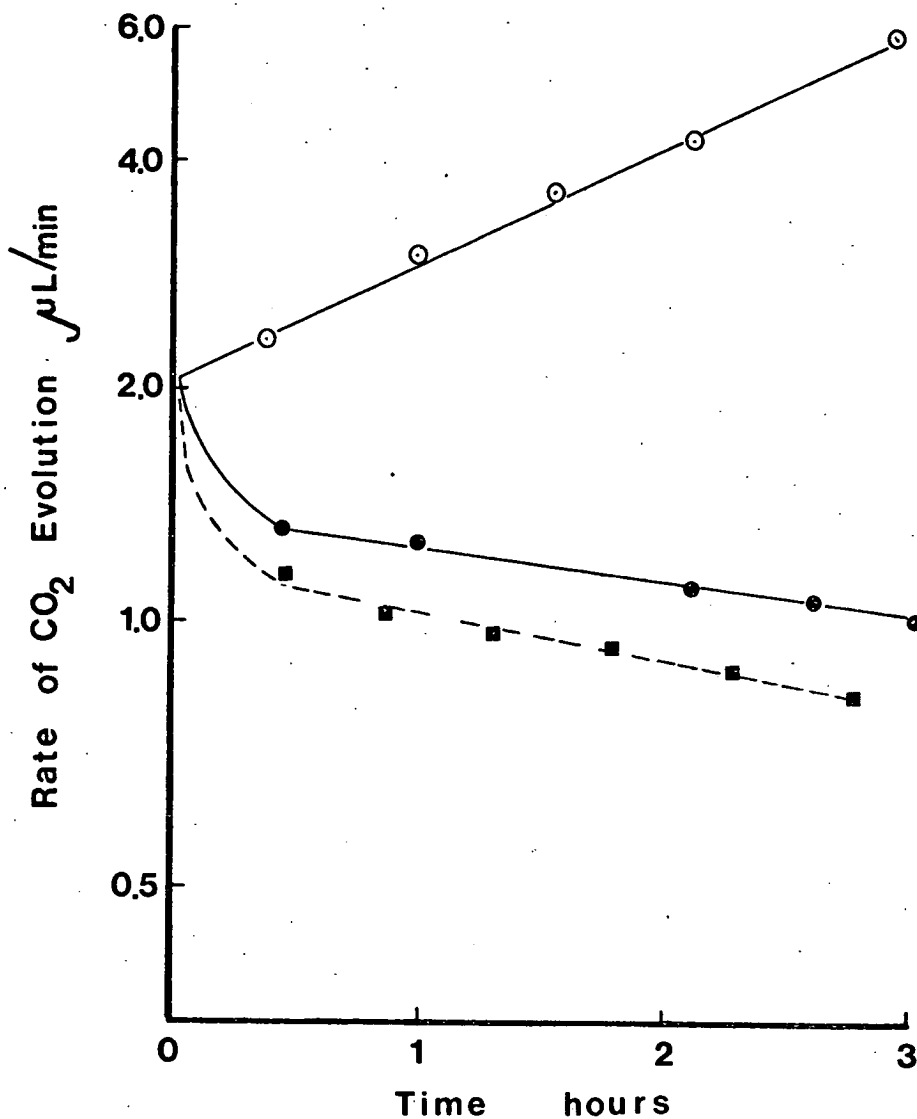


Fig 6-5. CO₂ evolution in cultures containing 0.1 mg/ml cycloheximide or 0.05 mg/ml 8-hydroxyquinoline.

Cycloheximide or hydroxyquinoline was added at time 0 to two samples removed from an exponential culture. CO₂ evolution was measured on 3 ml samples removed from the untreated culture and from each culture containing an inhibitor.

- CO₂ evolution in the untreated culture.
- CO₂ evolution in culture + cycloheximide.
- CO₂ evolution in culture + hydroxyquinoline.

An exponential culture was divided into three parts. To one part was added cycloheximide to give a concentration of 0.1 mg/ml, which causes complete inhibition of protein synthesis in S. pombe within a few minutes (Mitchison & Creanor, 1969). Hydroxyquinoline was added to the second part of the culture to give a concentration of 0.05 mg/ml. This concentration gives more or less complete and instantaneous inhibition of RNA synthesis (Fraser & Creanor, 1974). The third part of the culture was used as a control. CO₂ evolution was then measured on each of these cultures and the combined results are shown in Fig 6 - 5. In the cultures which contained either cycloheximide or hydroxyquinoline, immediately after the addition of the inhibitor, the rate of CO₂ evolution fell and this fall continued for the duration of the experiment.

The most obvious conclusion from this experiment is that both RNA and protein synthesis are required for an increase (and for maintenance) in the rate of evolution of CO₂. It could be argued that in the absence of either protein or RNA synthesis, the components of the pathway involved in the production of CO₂ begin to decay and the flux through the pathway decreases. This, however, is unlikely because of the shape of the decay curve. It seems possible that inhibition of either RNA synthesis or protein synthesis would drastically reduce the energy requirements of the cell. Previously it has been shown that the major source of energy in S. pombe is the oxidation of glucose by way of the Embden - Meyerhof - Parnas pathway, and this pathway is also the source of most of the CO₂ evolved by S. pombe. It is reasonable to imagine a reduction in the energy requirement of the cell leading to a reduction in the amount of glucose utilized and hence a reduction in the amount of CO₂ evolved.

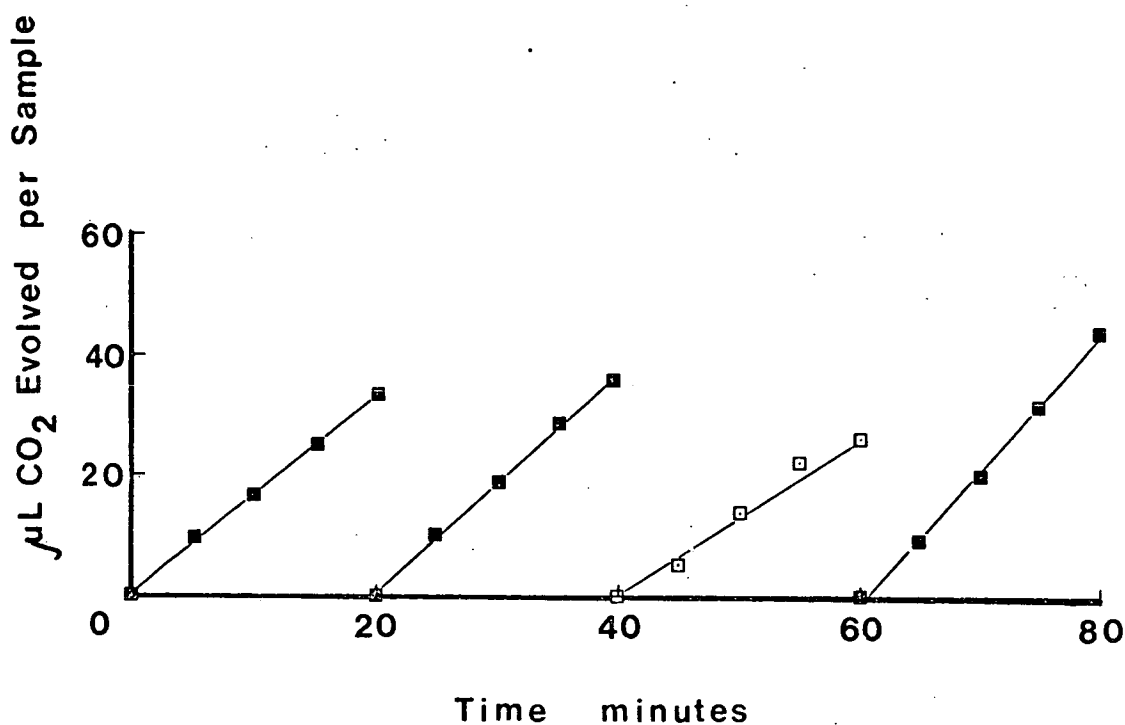


Fig 6-6. CO₂ evolution in an exponential culture before, during and after a 15 minute pulse of 0.1 mg /ml cycloheximide.

CO₂ evolution was measured for 20 minutes on 3 ml samples removed from an exponential culture. At 25 minutes, 0.1 mg / ml cycloheximide was added to the culture and CO₂ evolution was measured on a 3 ml sample. At 40 minutes the culture was filtered, washed and resuspended in fresh medium and CO₂ evolution was measured on a further 3 ml sample.

- CO₂ Evolution in the absence of cycloheximide.
- CO₂ Evolution in the presence of cycloheximide.

The stability of the components of the pathway involved in the production of CO_2 can be tested more simply by applying a pulse of either inhibitor to a culture of S. pombe and measuring the rate of CO_2 evolution before, during and after the pulse. This experiment had to be carried out using cycloheximide since it has not been possible to reverse the action of hydroxyquinoline. When added for 15 minutes to an exponential phase culture of S. pombe, the evolution of CO_2 was shown to fall by the same amount as was shown in Fig 6-5. Immediately on removing the cycloheximide by filtration, the rate of CO_2 evolution, as illustrated in Fig 6-6, returned to the same rate as before the addition of the inhibitor. This result suggests, therefore, that the fall in the rate of CO_2 evolution in cultures containing cycloheximide was caused by a fall in the energy requirements of the cell and that immediately after the inhibitor was removed, the demand for energy increased, producing an increase in the rate of CO_2 evolution.

Effect of Iodoacetate on CO_2 Evolution and Growth of S. pombe.

Iodoacetate is an inhibitor of the Embden - Meyerhof - Parnas pathway (Rafter, 1957). It acts at the eighth step of the pathway, preventing the conversion of glyceraldehyde - 3 - P to 1,3, diphosphoglycerate by the enzyme glyceraldehyde - P - dehydrogenase.

The effect of this inhibitor on CO_2 evolution in S. pombe is shown in Fig 6-7. When added to a log phase culture containing 3×10^6 cells/ml, CO_2 evolution was immediately and completely inhibited by 4×10^{-3} M iodoacetate. Inhibition appeared to be all or nothing; slightly lower concentrations of the inhibitor had little or no effect on CO_2 evolution. Iodoacetate at 4×10^{-3} M, which caused complete inhibition of CO_2 evolution in a culture

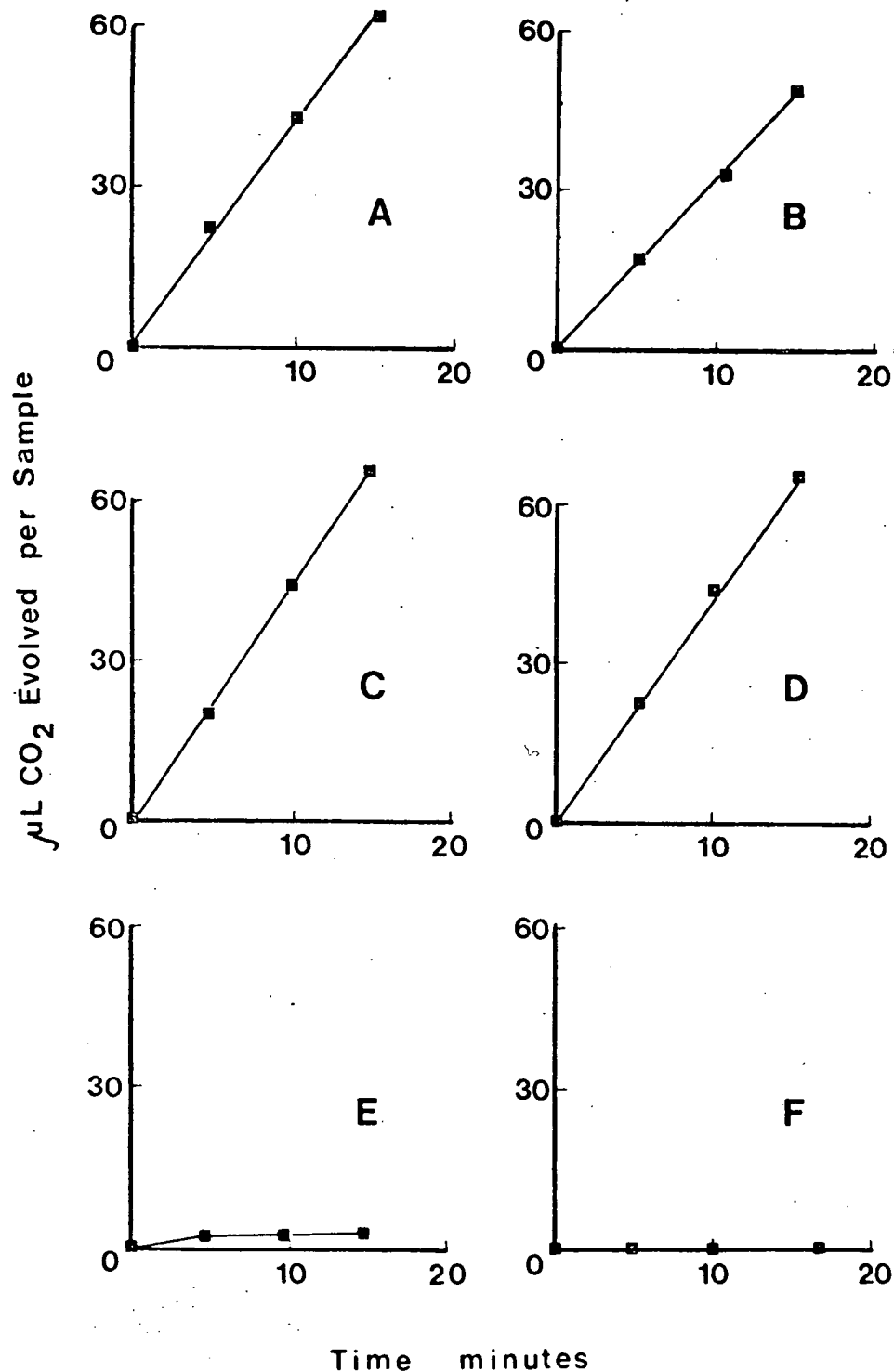


Fig 6-7. CO₂ evolution in cultures containing Iodoacetate.

CO₂ evolution was measured in cultures containing :-

- A. 6×10^{-5} molar; B. 1×10^{-4} molar; C. control.
 D. 1×10^{-3} molar; E. 2×10^{-3} molar; F. 4×10^{-3} molar
 iodoacetate.

containing 3×10^6 cells/ml, had no effect in a culture which contained 4.5×10^6 cells/ml. To cause complete inhibition of CO_2 evolution in a culture at this cell density, it was necessary to add iodoacetate at a concentration of 1×10^{-2} M.

If glycolysis is completely inhibited in S. pombe, it follows that the supply of most, if not all, of the energy of the cell is lost. This would mean that in the presence of an inhibitory concentration of iodoacetate, growth of the culture would very soon be strongly inhibited. This was found to be the case. In the culture discussed above, addition of 4×10^{-3} M iodoacetate not only caused complete inhibition of CO_2 evolution but, from the time the iodoacetate was added, there was no measureable increase in either the optical density or the cell number of the culture. A further implication of this result is that there are no readily available energy reserves in S. pombe; inhibition of energy supply caused an immediate inhibition of growth.

CHAPTER 7.

Measurement of CO₂ Evolution by the Radiorespirometric Method

Introduction.

CO₂ evolution was measured using this technique for two reasons. In the first place, using glucose labelled in different positions, it is possible to measure the activity of each of the pathways along which glucose can be oxidised (Bloom & Stetten, 1953). Secondly, the pattern of CO₂ evolution can be measured in synchronous and in asynchronous cultures using this technique.

Estimation of Flux through the Pathways of Glucose Catabolism in *S. pombe*.

There are three possible pathways for the catabolism of glucose in yeast (Bloom & Stetten, 1953 : Wang et al, 1958 : Blumenthal et al, 1954), the Embden - Meyerhof - Parnas Pathway (EMP), the Tricarboxylic Acid Cycle (TCA) and the Hexose Monophosphate Pathway (HMP).

To measure the activities of the three pathways, 0.01 ml each of D(U - ¹⁴C) glucose, D(1 - ¹⁴C) glucose and D(6 - ¹⁴C) glucose were added to three 1 ml samples of an exponential culture containing 1.5×10^6 cells/ml. The evolution of ¹⁴CO₂ was measured on 0.1 ml samples of these cultures. Changes in cell number were followed on the untreated culture during the course of the experiment. The results of this experiment are shown in TABLE 7 - 1, and these confirm the fermentative nature of *S. pombe* growing on 1 % glucose as carbon source. During most of the exponential phase, 90 % of the glucose taken up by the cell is metabolised by the EMP pathway until pyruvate is formed, at which point it is converted to acetaldehyde then to ethanol with the evolution of CO₂. During the early stages of exponential

Cell No. X 10^{-6} /ml	Embden - Meyerhoff - Parnas Pathway.	Pentose Cycle.	TCA Cycle.
2.34	91.8%	4.3%	3.8%
4.0	88.9%	1.9%	9.2%
9.5	86.4%	2.3%	11.3%
21	82.3%	0	17.7%
45	84.6%	0	15.4%

TABLE 7 - 1. Percentage of CO_2 evolved from the EMP pathway, The pentose pathway & the TCA Cycle in S. pombe.

Details of the experiment are given in the text. Samples of culture were incubated in the presence of $\text{D}(\text{U}-^{14}\text{C})$ glucose, $\text{D}(1-^{14}\text{C})$ glucose & $\text{D}(6-^{14}\text{C})$ glucose. CO_2 evolution was measured in 0.1 ml samples of these three cultures at intervals.

Calculation of the percentage activity of each of the three pathways was based on the following assumptions :-

- $\text{D}(\text{U}-^{14}\text{C})$ glucose : $^{14}\text{CO}_2$ evolved in all three pathways.
- $\text{D}(1-^{14}\text{C})$ glucose : $^{14}\text{CO}_2$ evolved in TCA Cycle & Pentose Cycle.
- $\text{D}(6-^{14}\text{C})$ glucose : $^{14}\text{CO}_2$ evolved in the TCA Cycle.

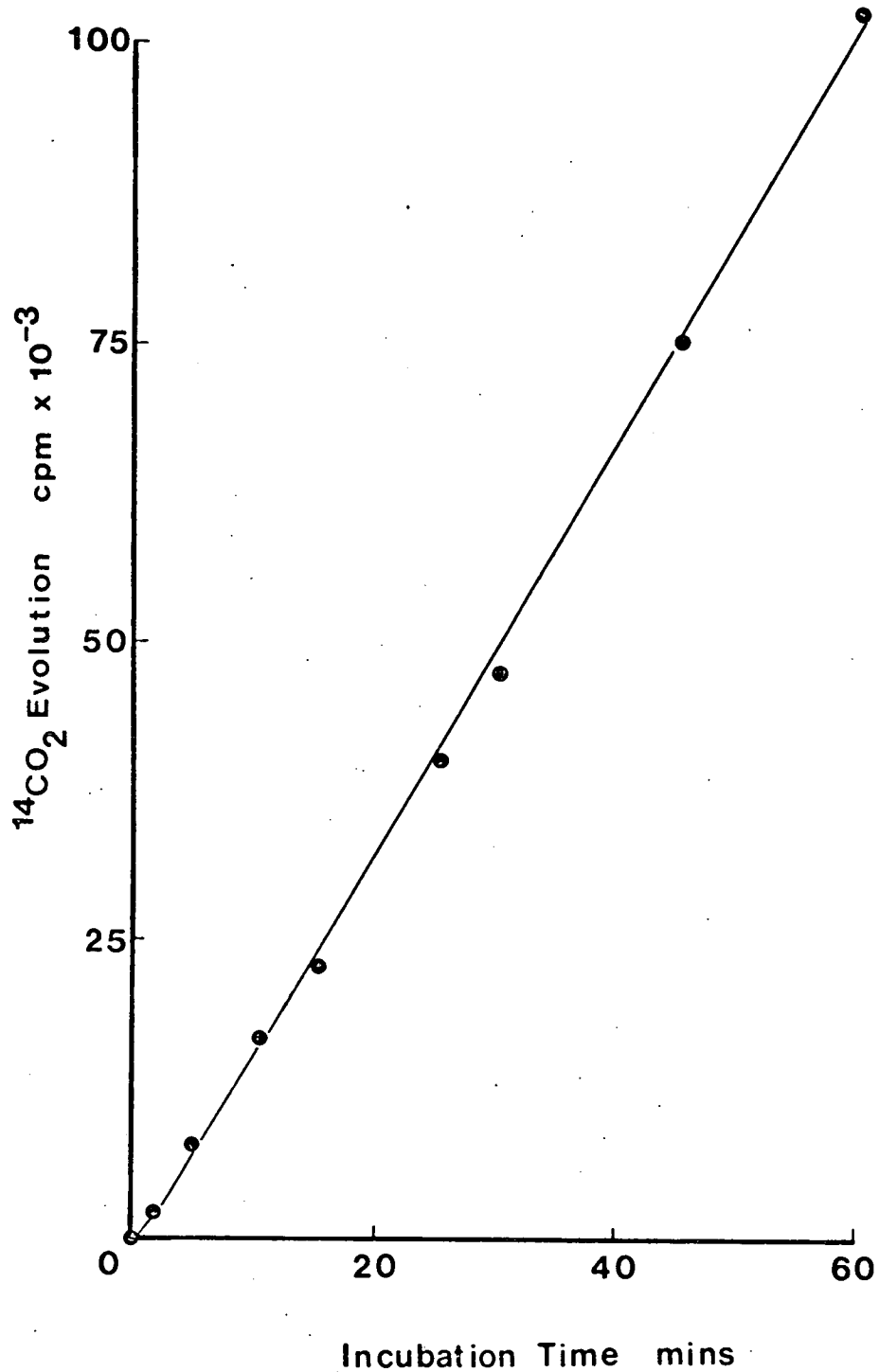


Fig 7 -1. $^{14}\text{CO}_2$ evolution with various incubation times.

At time 0, 10 samples of 0.1 ml from an exponential culture were added to 10 aliquots of 0.01 ml D(U- ^{14}C)glucose. The evolution of $^{14}\text{CO}_2$ was followed for varying lengths of time in these samples as previously described. Reactions were stopped by the injection of 0.1 ml 2 molar perchloric acid.

growth, the remaining 10% of the glucose taken up by the cell is metabolised fairly equally between the HMP and the TCA cycle. It is not clear why the activity of the HMP should fall to zero during the later stages of exponential growth.

Processing of glucose through the EMP Pathway.

To test whether glucose was catabolised rapidly through the EMP pathway or whether there was a large pool of intermediates in the pathway, D(U- ^{14}C) glucose was added to ten 0.1 ml samples of a log phase culture. These samples were incubated for periods between 2 minutes and an hour at 32°C and the radioactive CO_2 evolved during these times was measured. The results of this experiment, shown in Fig 7-1, show that the graph of radioactivity against time passes through the origin, within the limits of measurement. If there was a pool of intermediates of a considerable size, there would have been a delay of some period before the intermediate pools were filled and steady state labelling was reached. One assumes, therefore, that there is little, if any, delay between the entry of glucose into the cell and its final conversion to CO_2 and alcohol.

One further experiment was carried out to confirm this result. A culture was grown in the presence of D(U- ^{14}C) glucose for two generation times. At the end of this time, $^{14}\text{CO}_2$ was evolved at a rate giving 10,000 counts/min. The culture was filtered, washed and resuspended in fresh medium and $^{14}\text{CO}_2$ evolution was measured for periods varying from 2 minutes after resuspension to 1 hour after resuspension. The activity of $^{14}\text{CO}_2$ evolved was 100 counts per minute in the first two minutes after resuspension and 88 counts per minute in the first hour after resuspension. This means that almost immediately on removal of the radioactive glucose, a small amount of $^{14}\text{CO}_2$ was

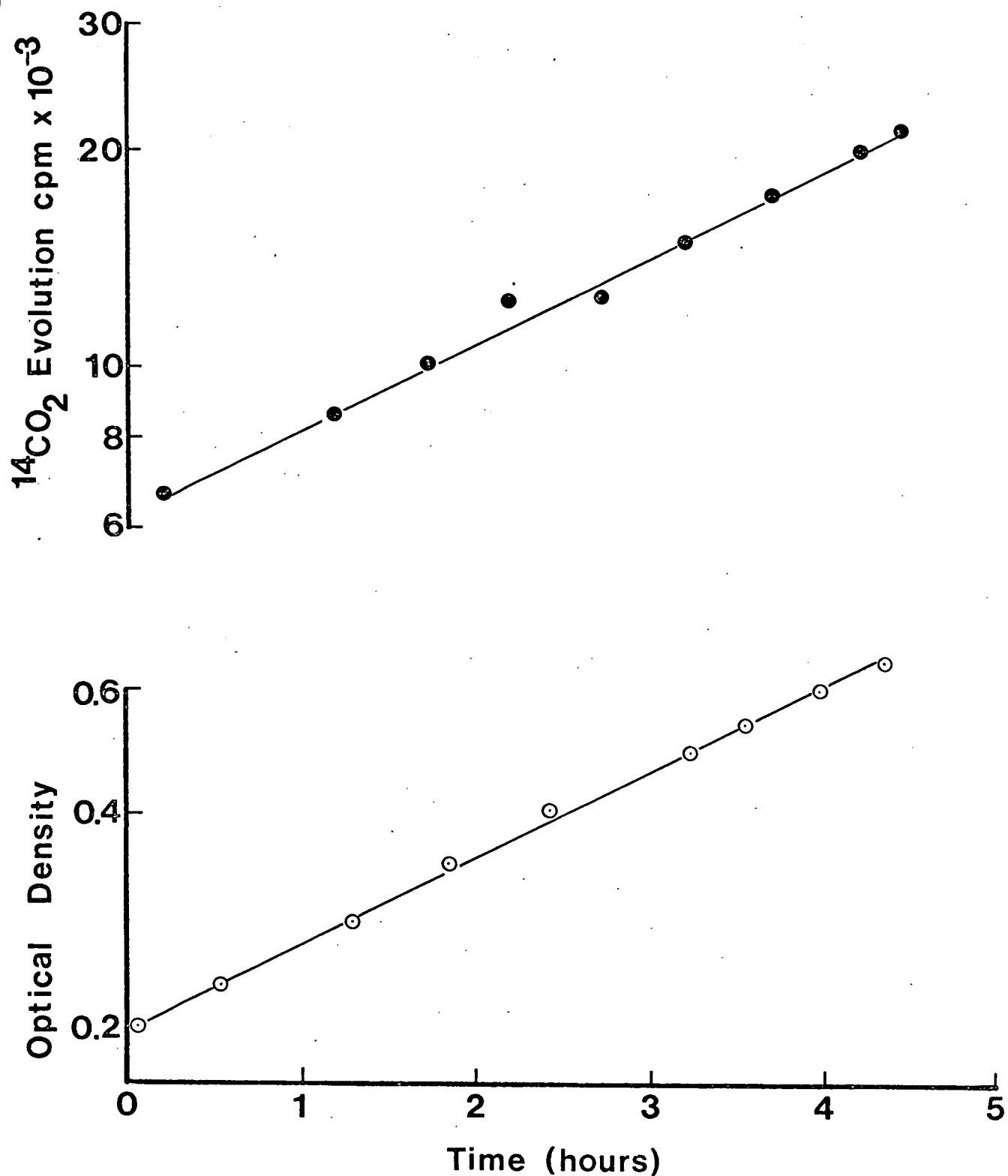


Fig 7 - $^{14}\text{CO}_2$ evolution during exponential growth of S. pombe.

At intervals during a 5 hour period, 0.1 ml culture was added to 0.01 ml D(U- ^{14}C)glucose & $^{14}\text{CO}_2$ evolution was measured for 20'.

●—● Rate of $^{14}\text{CO}_2$ evolution. ○—○ Optical density.

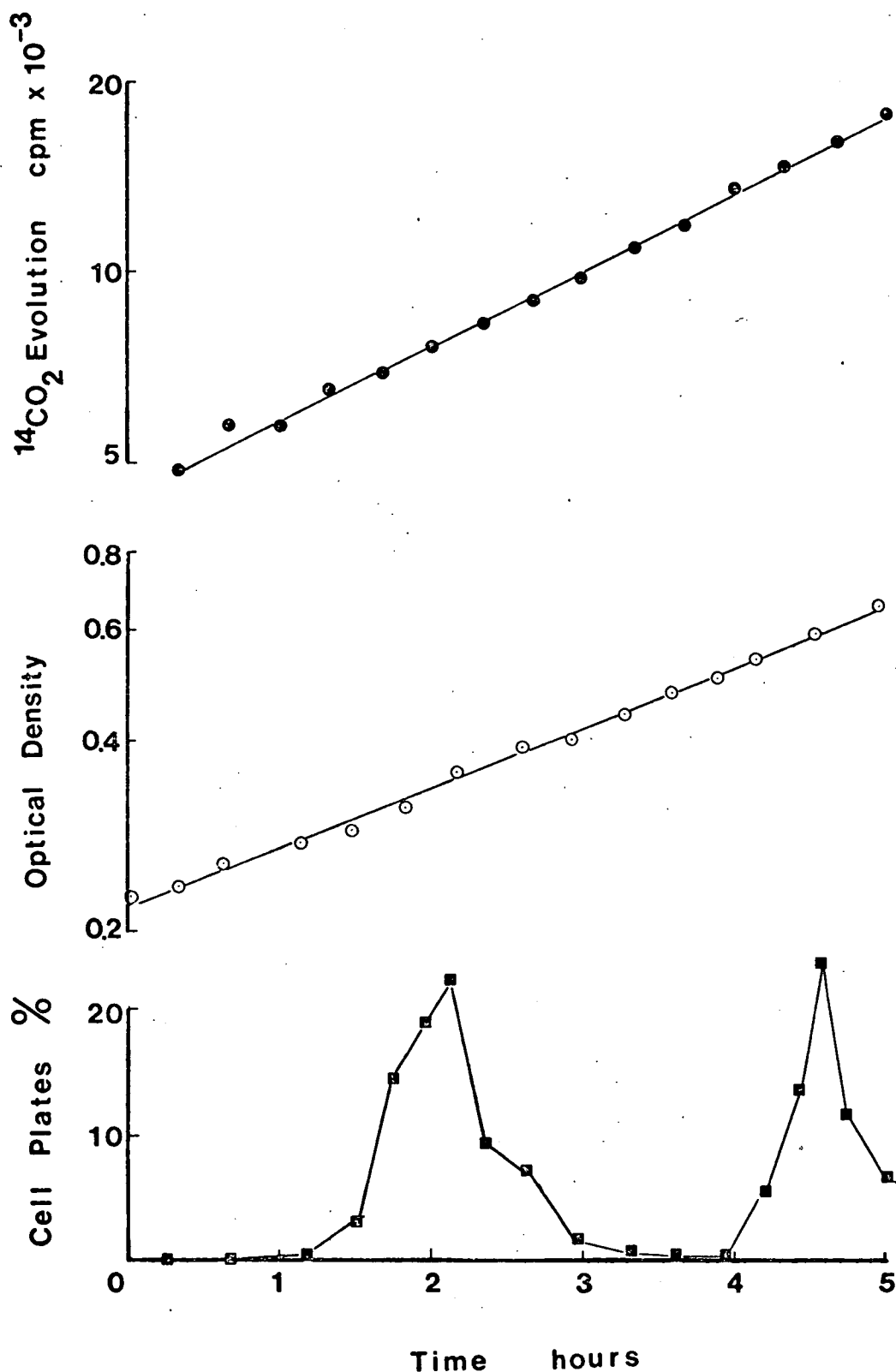


Fig 7 - 3. $^{14}\text{CO}_2$ evolution in synchronous culture of S. pombe.

At 20' intervals, 0.1 ml samples of culture were added to 0.01 ml D(U- ^{14}C)glucose & $^{14}\text{CO}_2$ evolution was measured for 20' periods.

●—● Rate of $^{14}\text{CO}_2$ evolution.

○—○ Optical Density.

■—■ % Cell Plates.

evolved and no more $^{14}\text{CO}_2$ was evolved during the next hour. This supports the conclusion from Fig 7 - 1 that there is little or no pool of glucose intermediates in the EMP pathway

$^{14}\text{CO}_2$ Evolution in Exponential Cultures.

The evolution of $^{14}\text{CO}_2$ was then measured during the growth of an exponential culture of S. pombe. 0.1 ml samples of culture were added to 0.01 ml D(U- ^{14}C)glucose at intervals over a 5 hour period. After 20 minutes incubation at 32°C during which time $^{14}\text{CO}_2$ was evolved and trapped, 0.2 ml 2 molar perchloric acid was injected into the sample and $^{14}\text{CO}_2$ was trapped and measured as described in Chapter 2. $^{14}\text{CO}_2$ evolved and the optical density of the culture are shown in Fig 7 - 2. The evolution of $^{14}\text{CO}_2$ doubled in rate in 154 minutes while the doubling time for the optical density of the cultures was 150 minutes. This would seem, therefore, to be a reliable measure for the evolution of CO_2 in an exponential culture of S. pombe.

$^{14}\text{CO}_2$ Evolution in Synchronous Cultures of S. pombe.

A synchronous culture was prepared using a lactose gradient as previously described. D(U- ^{14}C)glucose [284 mCi / mMole, 255 uCi / ml] was diluted with an equal part of distilled water and fifteen 0.01 ml aliquots were dispensed into Durham Fermentation tubes. At 20 minute intervals, 0.1 ml samples of the synchronous culture were added to the radioactive glucose samples and $^{14}\text{CO}_2$ evolution was measured for 20 minutes. The reactions were stopped by the addition of perchloric acid as previously described. The results of this experiment are shown in Fig 7 - 3; the rate of CO_2 evolution measured by this technique increased continuously during the two synchronous divisions. CO_2

evolution increased at a faster rate than the optical density of the culture. This could be accounted for by the fact that the synchronous culture was consuming glucose during its growth while a constant amount of radioactive glucose was used throughout the experiment. This means that the specific activity of the radioactive glucose was rising constantly during the course of the experiment. This experiment was repeated several times and produced similar results.

This experiment does not confirm the results obtained by measuring the evolution of CO_2 manometrically. In the latter case, the rate of CO_2 evolution was shown to double sharply over a short time. Since the synchronous cultures were produced using identical techniques, the differences between the results must lie in what is being measured and not in any intrinsic differences between the cultures.

There are several possibilities for the difference between the result obtained in the synchronous culture described above and the result obtained by the manometric measurement of CO_2 evolution.

1. The contributions of the minor pathways may vary widely during the cell cycle, thus affecting the time taken to metabolise the glucose fully to CO_2 .
2. The time taken for a glucose molecule to pass along the EMP pathway might vary according to the cell cycle stage of the cell.
3. There may be a pool of metabolic intermediate (or intermediates), the size of which varies during the course of the cell cycle. Such a pool might fill slowly during part of the cycle and empty rapidly at one point during the cycle.

The experiments described in TABLE 7 - 1 and in Fig 7 - 1 make all of these possibilities unlikely. Further, the experiment in which radioactive glucose was filtered from a culture and the culture was resuspended in fresh medium showed that no $^{14}\text{CO}_2$ was evolved from the

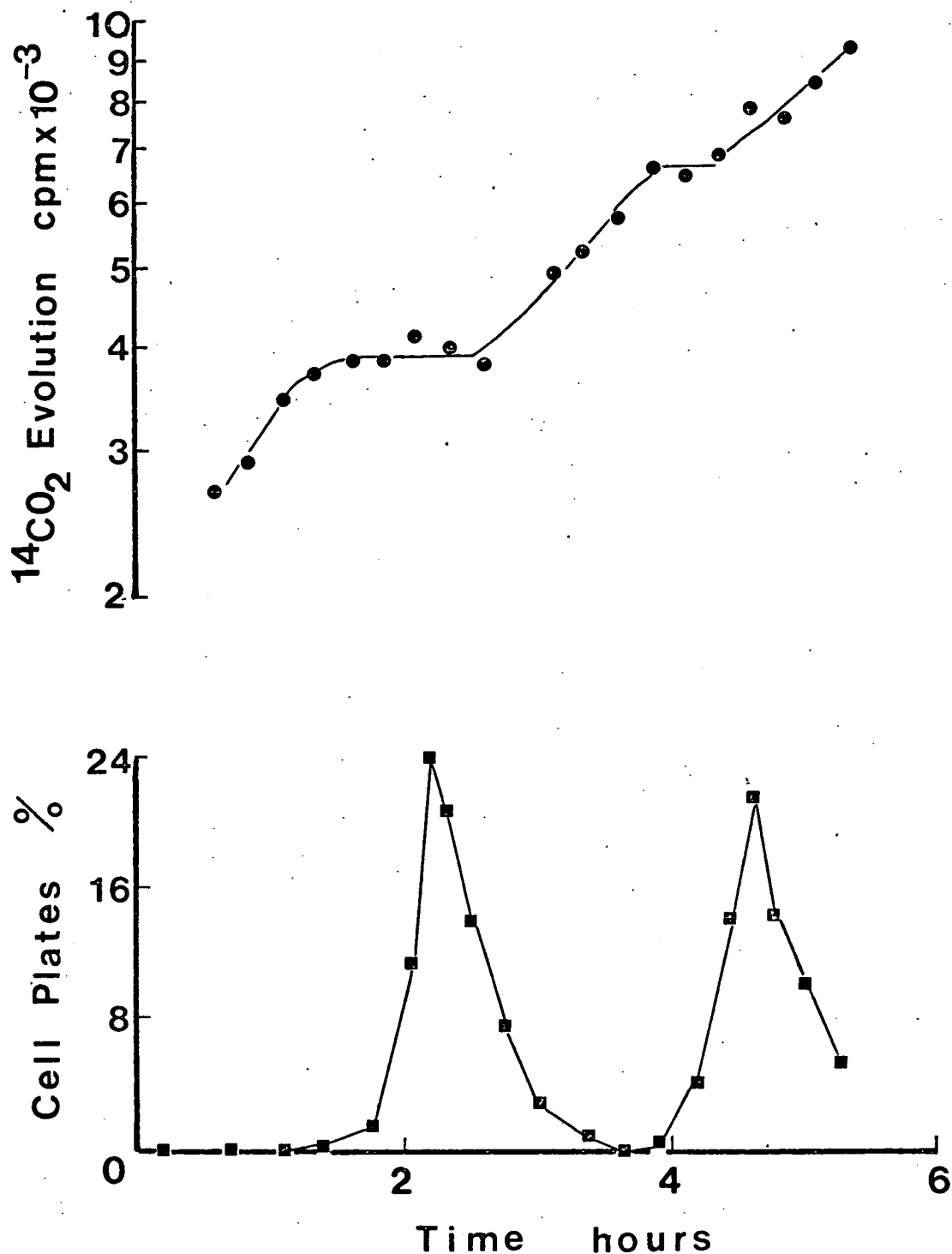


Fig 7-4. $^{14}\text{CO}_2$ evolution in a synchronous culture of S. pombe.

A synchronous culture was prepared using a lactose gradient as previously described. 5 minutes after preparation, 0.1 ml D(U- ^{14}C)glucose was added to 3 ml of the culture. Starting 25' later, $^{14}\text{CO}_2$ evolution was measured for 12 minutes on 0.1 ml samples from the culture containing the radioactive glucose. $^{14}\text{CO}_2$ was trapped and measured as previously described.

●—● Rate of $^{14}\text{CO}_2$ evolution.

■—■ % Cell Plates.

cells and supported the conclusion that there were little or no intermediates at significant concentration in the EMP pathway.

It was decided, however, to test the possibility that a pulse of radioactive glucose applied to the cells did not, during the subsequent 10 - 15 minutes, give a true measure of CO_2 evolution. In other words, steady state labelling was not obtained during the period of the pulse. The results obtained in an asynchronous culture and shown in Fig 7 - 1 suggest that steady state labelling was obtained in a short time. However, there may be cell cycle variations in the time of glucose processing which would not be seen in an asynchronous culture.

The evolution of CO_2 was therefore measured in a synchronous culture when it was likely that steady state conditions had been achieved. The most satisfactory way of doing this was thought to be by adding the radioactive glucose at the start of the experiment to a sample of the synchronous culture and then removing aliquots at intervals and measuring the CO_2 evolved. A synchronous culture was prepared as described previously using a lactose gradient. Five minutes after preparing the synchronous culture, 0.1 ml $\text{D}(\text{U}-^{14}\text{C})$ glucose was added to a 3 ml sample of the synchronous culture. Measurement of $^{14}\text{CO}_2$ evolution was commenced 25 minutes later when 0.1 ml samples were removed and $^{14}\text{CO}_2$ evolution was measured for 12 minutes as previously described. The results of this experiment, shown in Fig 7 - 4, clearly show that the rate of $^{14}\text{CO}_2$ evolution increased periodically during the cell cycle, in a similar way to the measurements of CO_2 evolution made by the manometric method. The first change in the rate of $^{14}\text{CO}_2$ evolution occurred too soon after re - inoculation of the synchronous culture for the timing of the step to be measured precisely; the second rate change, however, occurred at 0.37 of the way through the second

cycle, much earlier than the time of the rate change measured by the manometric method. Repeats of this experiment gave variable times for the second rate change and also variable data.

In the experiment discussed above, cells had been growing in the presence of radioactive glucose from shortly after setting up the synchronous culture. When samples were removed from the culture and placed in fermentation tubes, a certain amount of $^{14}\text{CO}_2$ would be in solution, the amount depending on the pH of the culture and the time that the culture had been growing in the presence of the radioactive glucose. It was then shown that after growing for 2 hours in the presence of radioactive glucose, 50% of the counts obtained by measuring $^{14}\text{CO}_2$ evolution were already in the medium before starting the experiment. This means that experiments in which $^{14}\text{CO}_2$ evolution was measured in the continuous presence of radioactive glucose must be regarded with a considerable degree of suspicion. The variations in the data and in the time at which the rate change occurred, could be accounted for by the fact that the background count, attributed to CO_2 dissolved in the medium, could amount to 50% of the total counts and this background could well be variable. For example, the actual pipetting of the sample into the fermentation tube could drive off some of the $^{14}\text{CO}_2$ already dissolved, and this could be done with variable amount of force.

The results from measuring the evolution of $^{14}\text{CO}_2$ are rather indecisive. It has been shown in a superficial way that there are no pools of Glucose intermediates of major significance. Neither are there large amounts of glucose retained by the cell. Yet measuring the evolution of $^{14}\text{CO}_2$ during short pulses of glucose gave results unlike those obtained by the manometric method and by continuous

	1 st Reading (8')	2 nd Reading (25')
CONTROL	500	645
2 M PCA	520	668
2 M HCl	546	688

TABLE 7 - 2. Alcohol estimation after incubation with 2 M perchloric acid and 2 M hydrochloric acid.

Alcohol was estimated using SIGMA kit No 330.

3 ml Tris buffer, pH 8.5 was added to a vial containing ADH and NAD. 0.1 ml 2% TCA was then added.

1 ml of this solution was placed in each of three spectrophotometer cells and the OD_{340} was measured. To one cuvette was added 0.167 ml 0.072% ethanol; to the second was added the same amount of alcohol which had been previously treated for 2 hours at 32°C with 2 M PCA; to the third was added the same amount of alcohol after 2 hours at 32°C with 2 M HCl.

The optical density at 340 nm of each solution was measured after 8 minutes and again after 25 minutes when the reaction was complete. The initial OD_{340} was subtracted from each measurement.

labelling with glucose (although there are technical difficulties in the latter method which make interpretation difficult).

The final investigation which was carried out was to test whether the method was giving a totally erroneous measure of $^{14}\text{CO}_2$ evolution either in pulses or by continuous labelling. The one possibility which was tested was the fate of the alcohol in the fermentation tube during measurement of $^{14}\text{CO}_2$ evolution. The addition of $\text{D}(\text{U}-^{14}\text{C})$ glucose to a culture, as well as producing $^{14}\text{CO}_2$ will also produce labelled ethanol. For every molecule of CO_2 produced, one molecule of ethanol will be formed, but this will have twice the number of counts as the two molecules of $^{14}\text{CO}_2$ since there are two carbon atoms. It was thought possible that after the addition of 2 M perchloric acid to the sample in the fermentation tube, the alcohol may have been oxidised to acetic acid or even to CO_2 . This was difficult to test directly. However, 2 M perchloric acid was added to a sample of medium (EMM2) containing 0.1% alcohol, in a fermentation tube. This was treated similarly to the samples in which $^{14}\text{CO}_2$ evolution was measured; the sample was incubated at 32°C for about 2 hours, after which the alcohol concentration was measured using an alcohol determination kit (Sigma Chemical Co. Kit No 330). At the same time the alcohol level after treatment with 2 M HCl was also measured.

The results of this experiment together with details of the assay are shown in TABLE 7-2. It is quite clear that there is no fall in the amount of alcohol either after treatment with perchloric acid or after treatment with hydrochloric acid. So the results in which $^{14}\text{CO}_2$ evolution was described are not invalid because of decomposition of the alcohol during collection of the gas.

It is hoped to investigate this problem further at some future time. This could best be done by examining in detail the kinetics of

$^{14}\text{CO}_2$ evolution at different times during the cell cycle.

CHAPTER 8.

Other Experiments.

Introduction.

It has been established that the uptake of oxygen and the evolution of CO_2 increase discontinuously during the cell cycle of S. pombe. An attempt was made to determine whether other growth parameters showed a similar pattern of changing rates during the cell cycle and to ascertain the mechanism by which the change in the oxygen consumption and the CO_2 evolution patterns was brought about.

Measurement of Optical Density in Synchronous Cultures.

A widely used measure of cell growth is the optical density of a culture which is thought to be most closely related to the dry mass of a cell (Mitchison, 1970). In an exponentially growing culture of S. pombe, it has been shown previously on several occasions that the optical density of the culture increased with a doubling time which was more or less identical to the doubling time of cell numbers. On entering stationary phase, a culture showed no further increase in optical density. When the growth of a culture was stopped, by removing glucose for instance, the optical density increase also stopped immediately.

The optical density increase has been measured frequently in the synchronous cultures which have been described previously in this work. A collection of results from measuring the optical density in 7 different synchronous cultures is shown in Fig 8 - 1. All of these synchronous cultures were prepared using lactose gradients.

There is a suggestion in all of these experiments that the

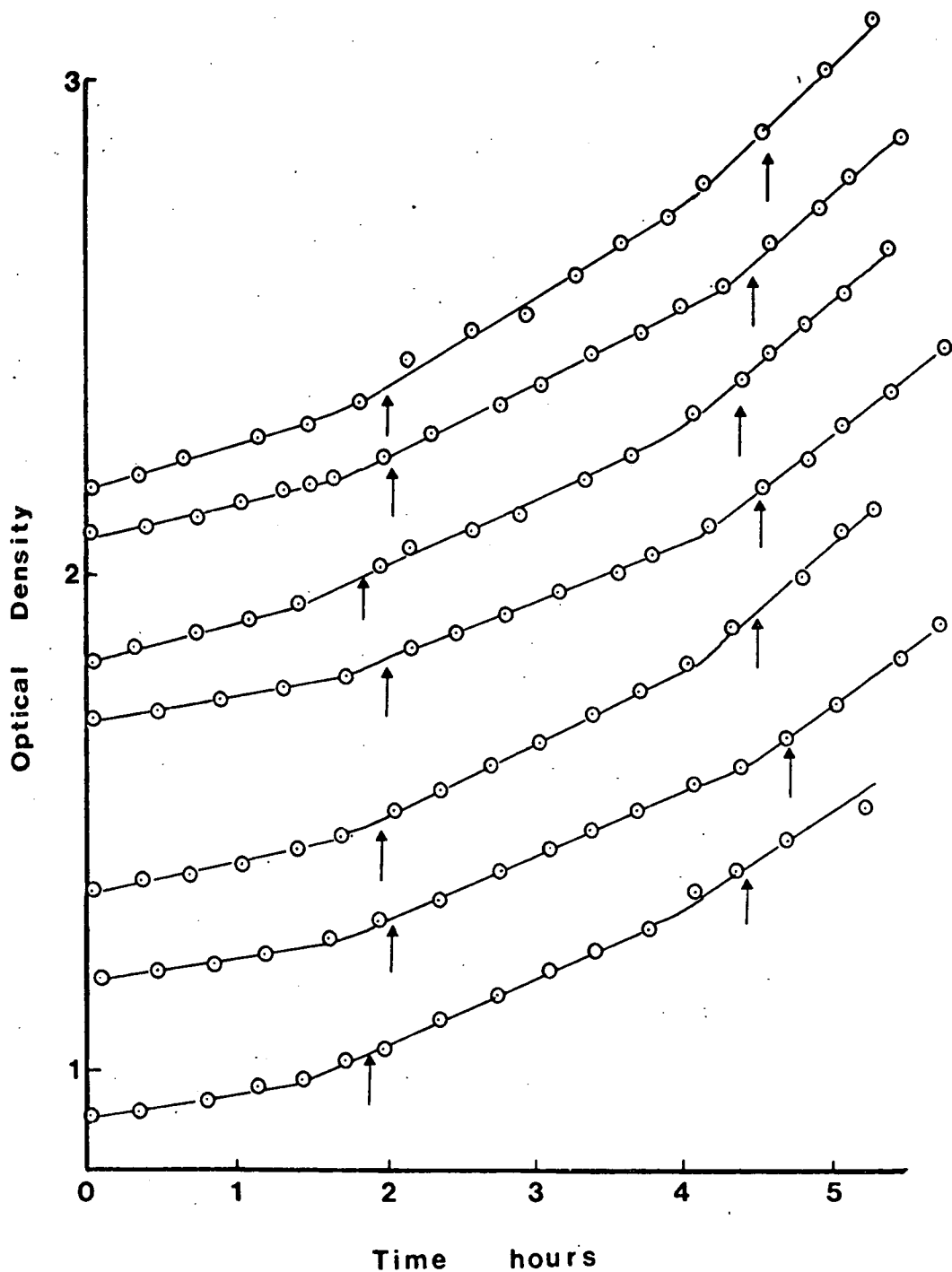


Fig 8-1. Optical Density in Synchronous cultures of *S. pombe*.

Each curve represents optical density measurements in one synchronous culture. All cultures were prepared using lactose gradients and optical density was measured using a Unicam SP 800.

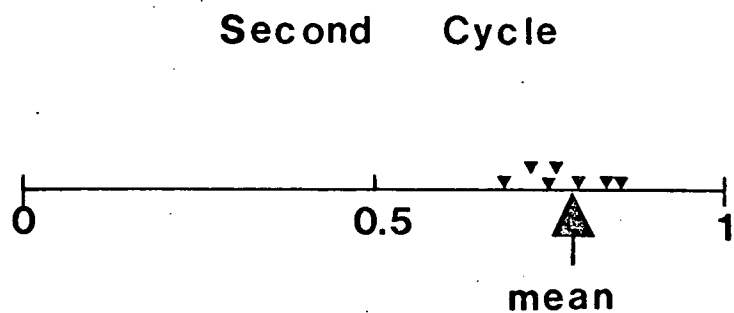
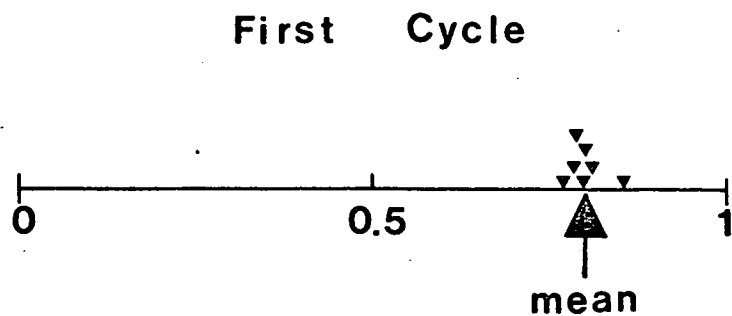


Fig 8-2. Timing of rate changes in optical density increase in synchronous cultures of S. pombe.

The timing of each rate change is derived from the data shown in Fig 8-1.

First cycle : Mean = 0.80

Second Cycle : Mean = 0.77

optical density of the cultures increased in a linear manner until a short time before the first cell plate peak, at which time the rate doubled fairly sharply. The rate then appeared to remain roughly constant during the next two hours or so until it again appeared to double, a short time before the second cell plate peak. The time at which the doubling in rate occurred during each cycle was estimated roughly and this data is shown in the cell cycle map in Fig 8-2.

Optical density was not measured in great detail and all of the data which are presented above were made in synchronous cultures which had been prepared for some other reason. One fact which does emerge however, is that the time at which the rate change in optical density occurred in synchronous cultures was only 0.05 of a cycle later than the time at which the change in the rate of CO_2 evolution occurred (Fig 5-8). The differences between these two timings are not statistically significant. This fact could be purely coincidental: it could also mean that a doubling in the flux through the Embden-Meyerhof-Parnas pathway (and therefore in the energy supplied by this pathway) gives rise to a doubling in the metabolic activity of the cell and this results in a doubling in the rate of optical density increase.

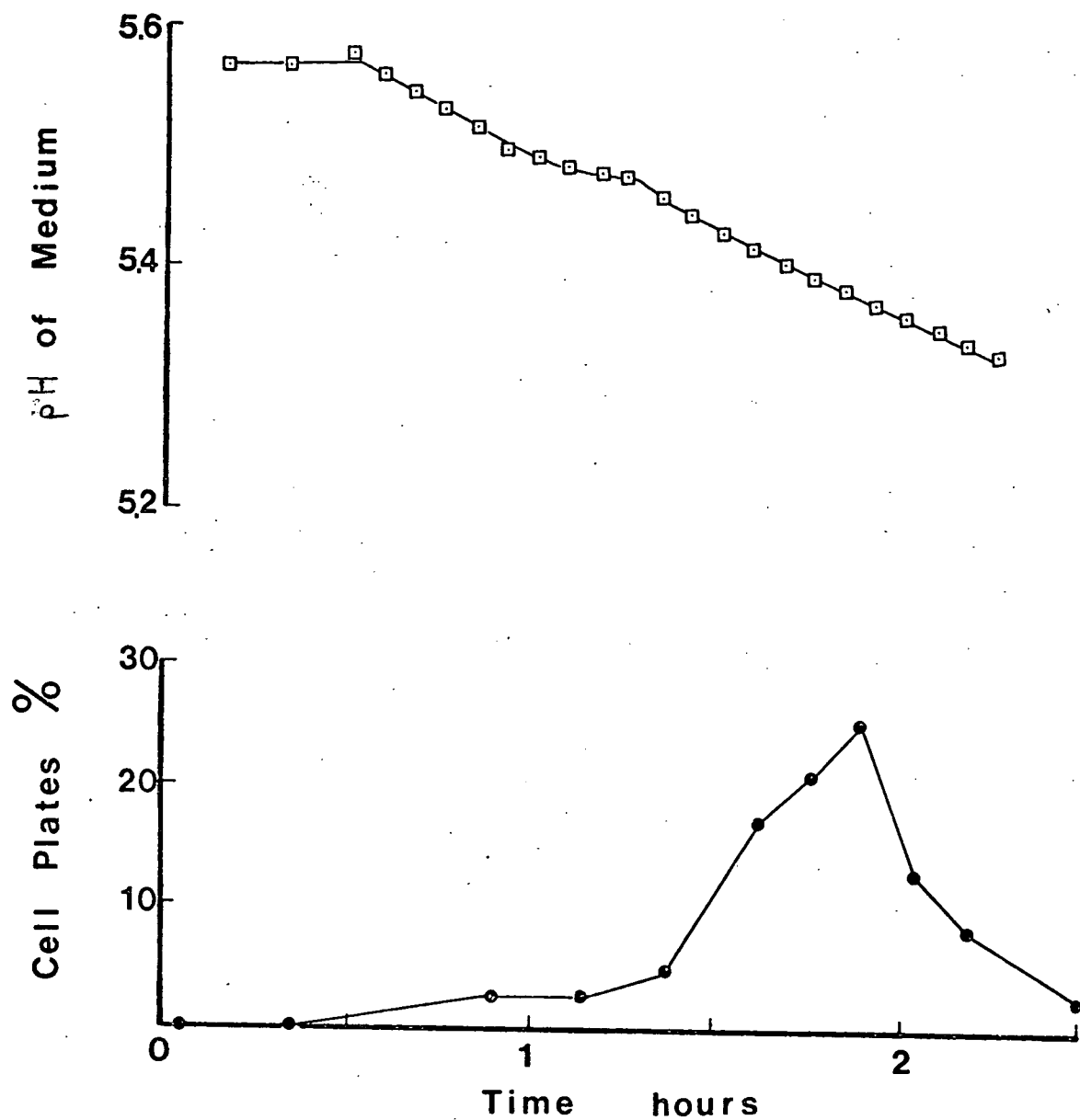


Fig 8 - 3. Measurement of pH during the first cycle in a synchronous culture.

A synchronous culture was prepared using a lactose gradient and inoculated to a cell density of 4.5×10^6 cells/ml.

The pH of the culture was measured at 5' intervals, keeping the culture in a water bath at 32°C during the measurement.

□ — □ pH of culture.

● — ● % Cell Plates.

Measurement of pH during the Cell Cycle of *S. pombe*.

Not all of the CO_2 evolved during the growth of *S. pombe* passes into the gas phase. A certain amount of the gas will remain in solution so that there is an equilibrium between the liquid and the gas phases. The constancy of the equilibrium depends on an unchanging environment in which the cells grow. During the measurement of CO_2 evolution in a Warburg flask, it has been assumed that in the twenty minute period required to measure the rate of evolution of CO_2 , there was no significant change in the medium to alter the equilibrium between the liquid and the gas phases.

One parameter which might strongly affect the solubility of CO_2 is the pH of the medium. If there was a sudden fall in the pH of the medium during the cell cycle of *S. pombe*, less CO_2 would remain in solution and more would be driven off into the gas phase. This could produce a pattern for the rate of CO_2 evolution similar to that observed in synchronous cultures. While this may seem unlikely, it was decided to measure the change in pH which occurred during the growth of a synchronous culture of *S. pombe*. A synchronous culture was prepared using a lactose gradient and to a cell density of 5×10^6 cells/ml. This was higher than the cell density normally employed in synchronous cultures but it was assumed that changes in pH might be small and a reliable measure would be obtained using a dense culture. The culture was grown in a water bath at 32°C and a pH electrode was placed in it. The culture was stirred using an under-water magnetic stirrer and the pH was measured at 5 - 10 minute intervals.

The pH measurements made on this culture during the first cycle after reinoculation are shown in Fig 8 - 3. There was a continuous fall in the pH of the medium but there was no evidence of a dramatic change at any particular stage of the cell cycle. This experiment does

142
however, corroborate the pattern which was observed for CO₂ evolution in the synchronous cultures. At the time when the rate of CO₂ evolution doubled in the first cycle after re-inoculation of the synchronous culture, the pH of the medium began to fall more rapidly. It is impossible to say whether the rate change in CO₂ evolution caused the accelerated fall in the pH of the medium or vice-versa. Nevertheless, the result of measuring the pH of the medium during the cell cycle of S. pombe is consistent with a doubling in the rate of CO₂ evolution towards the end of the cycle.

Production of ATP during the Cell Cycle of S. pombe.

The net gain to S. pombe by the passage of one molecule of glucose along the EMP pathway is the production of energy in the form of the energy-rich bonds of two molecules of ATP. This high energy compound is then used by the cell in a variety of anabolic pathways, in the synthesis of proteins and nucleic acids for instance. The continued production of ATP and its regulation is therefore of paramount importance to the growth and division of a cell.

It has been shown that a measurement of CO₂ evolution in S. pombe gives a measure of the flux through the EMP Pathway and that this pathway is the source of a large part of the ATP produced by the cell. Measurements were therefore made of the ATP content during the cell cycle of S. pombe to determine whether this was related to the measurements of the flux through the EMP pathway. It is more or less axiomatic that ATP must be produced at double the rate if the flux through the EMP pathway were to double at a particular stage of the cell cycle, but it remains to be seen whether or not this is reflected in the ATP content of the cells.

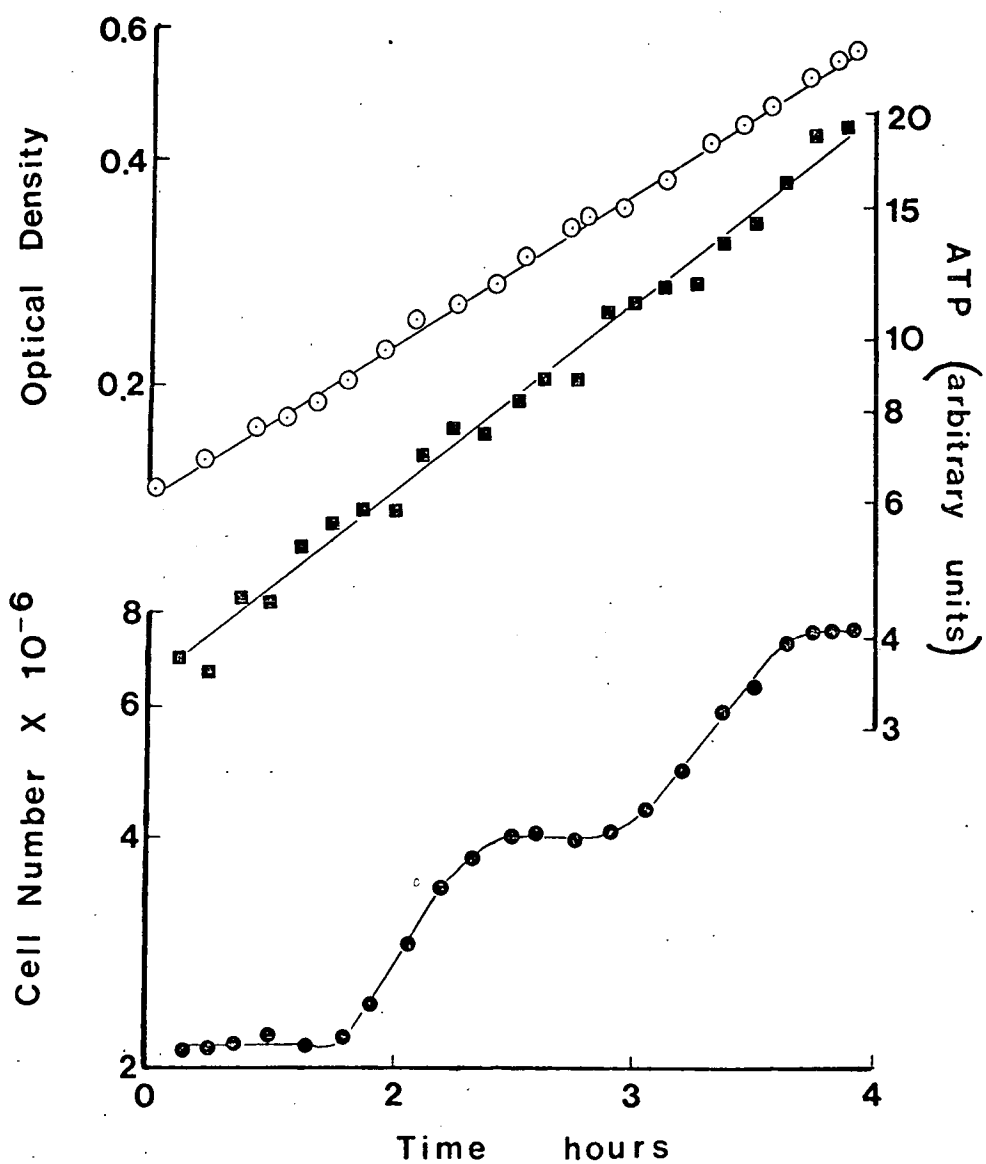


Fig 8 - 4. ATP content of cells during the growth of a synchronous culture. A synchronous culture was prepared using a lactose gradient. The ATP content of 10 ml samples from the culture was measured at 15 minute intervals.

○—○ Optical Density.

■—■ ATP content of 10 ml samples.

●—● Cell Number.

A synchronous culture was prepared using a lactose gradient. Samples of 10 ml were removed from the culture every 15 minutes, collected by filtration and placed in a boiling water sample for 10 minutes. The ATP was extracted and measured as described in Chapter 2.

Estimates of the ATP content of S. pombe during two cell cycles are shown in Fig 8-4. The amount of ATP per sample appears to increase exponentially during the cycle but it is not possible to say from the data whether the increase in ATP is in the form of several waves per cycle. There is, however, no sharp doubling in the amount at any one stage of the cell cycle.

This is not a particularly accurate technique for measuring the ATP content of cells. It is also open to the criticism that during the collection of the samples and before they have been inactivated by boiling, the ATP content has altered by turnover.

The work in this section on ATP production in S. pombe was carried out in collaboration with Dr. J.W. May, Monash University, Melbourne, Australia.

Measurement of Hexokinase in Asynchronous & Synchronous Cultures of S.pombe.

The processing of a metabolite along a metabolic pathway to its end product is carried out by enzymes. One way, therefore, in which the flux through a pathway could be controlled is by altering the amount of the enzymes which carry out each step in the pathway. A more rapid control could, however, be exerted by altering the activity of the enzymes of the pathway by the addition or the removal of substrates. Thus, if the activity of all of the enzymes of a pathway doubled, this could lead to a doubling in the flux through the pathway. A modification

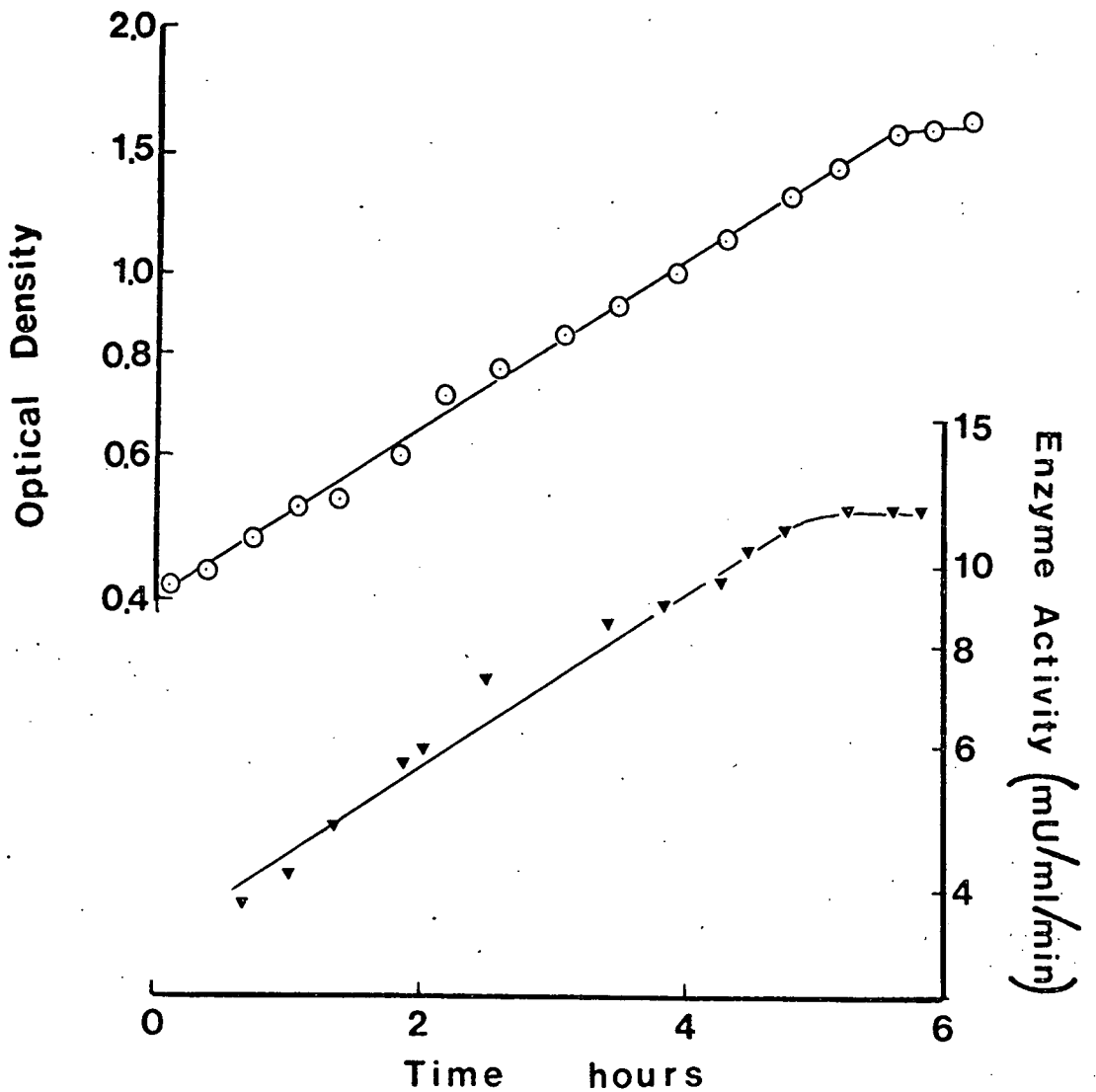


Fig 8-5. Hexokinase activity during exponential growth of S. pombe. Enzyme activity was measured every 20' on 0.2 ml samples removed from an exponentially growing culture. Samples were washed four times with distilled water, stored in the deep freeze and assayed the following day.

○—○ Optical Density. ▼—▼ Enzyme activity/sample.

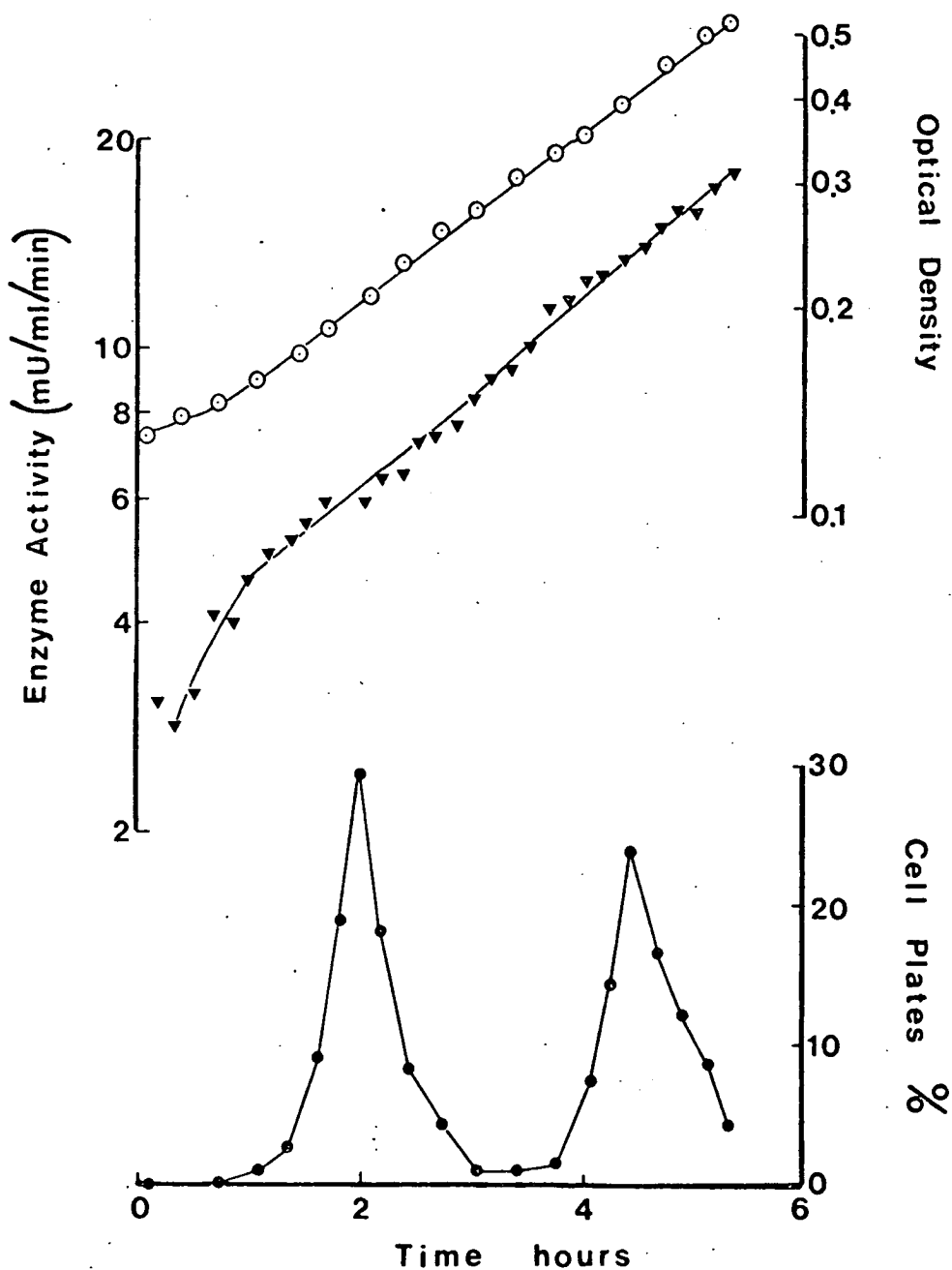


Fig 8-6. Hexokinase activity in a synchronous culture of S. pombe.

Enzyme activity was measured every 10' on two 1 ml samples removed from the culture. Samples were stored in the deep freeze & assayed the following day.

○—○ Optical Density.

▼—▼ Enzyme activity (mean of 2)

●—● % Cell Plates.

of this theory would be that the activity of a pathway could be controlled by one, rate-limiting enzyme. If all of the enzymes of a pathway were present in excess with the exception of one, a doubling in the activity of this "pace-maker" enzyme could result in a doubling in the flux through the pathway.

The evolution of CO_2 occurs at the second last step of the EMP pathway. There are at least sixteen enzymes involved in the oxidation of glucose to ethanol and CO_2 in the EMP pathway. There may also be one or more enzymes involved in the uptake of glucose into the cell before the first enzyme of the pathway, hexokinase, catalyses the phosphorylation of glucose to glucose-6-phosphate. There are therefore many possible sites at which the flux through this pathway could be regulated.

Measurement of the activity of hexokinase is quite straight forward as was shown in Chapter 2. For this reason, it was decided to measure the activity of this enzyme during the cell cycle of S. pombe. The activity of hexokinase was first measured in an exponentially growing culture of S. pombe. As shown in Fig 8-5, the activity of this enzyme increased during log phase with a doubling time of approximately $2\frac{1}{2}$ hours. When the culture entered stationary phase, there was no further increase in the activity of the enzyme.

A synchronous culture of S. pombe was prepared as described previously using a lactose gradient. Two samples of 0.5 ml were removed from the culture every 10 minutes, washed six times with distilled water and frozen. The following day, the samples were removed from the deep freeze and assayed for hexokinase as described in Chapter 2. The results from one synchronous culture are shown in Fig 8-6 and they suggest that the activity of hexokinase is increasing in a roughly exponential manner during the two cell cycles with a doubling time similar to the doubling time of the culture. There is, however, a

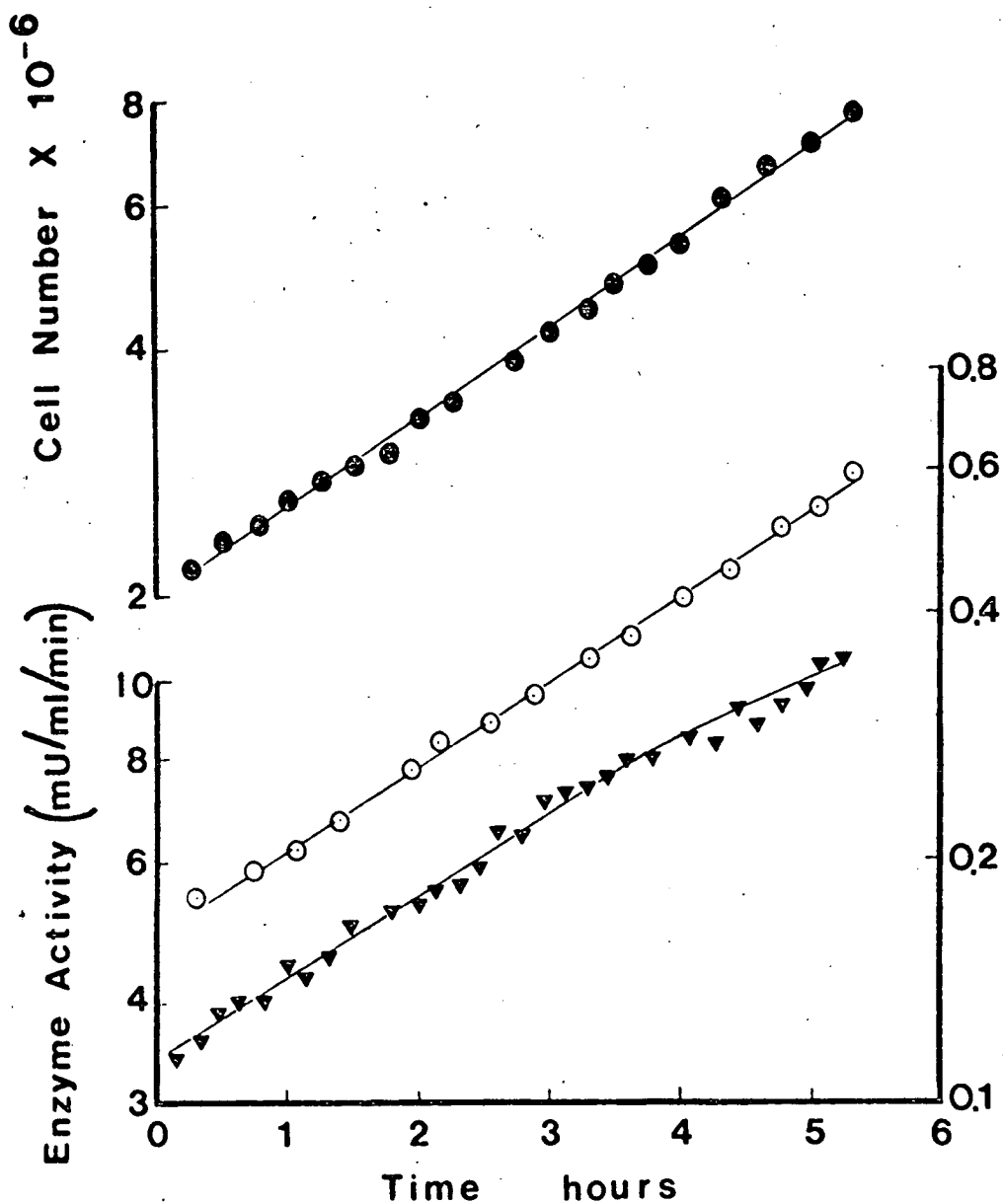


Fig 8-7. Hexokinase activity in an asynchronous control culture.

A control culture was prepared as previously described. Enzyme activity was measured every 10' on two 0.5 ml samples removed from the culture. Samples were kept frozen & assayed next day.

●—● Cell Number. ○—○ Optical density.
 ▼—▼ Enzyme activity (mean of 2)

rapid rise in activity immediately on reinoculation, a possible effect of the synchronisation procedure.

The activity of hexokinase in a control culture, prepared from a shaken gradient as previously described, is shown in Fig 8-7. The activity of the enzyme increased continuously for about 5 hours after inoculation of the culture.

Several points should be made about these experiments. There was a rapid rise in the activity of hexokinase shortly after inoculation of the synchronous culture. This is quite possibly an effect of the synchronisation procedure but it is obvious that there was not a similar rise in the activity of the enzyme in the asynchronous control culture. So it is possible that the small cells which were removed to prepare the synchronous culture were uniquely affected by the synchronisation procedure and this produced an initial perturbation in the activity of hexokinase.

The activity of hexokinase is obviously not periodic in the same way that the rate of evolution of CO_2 increased periodically during the cell cycle. There may be minor fluctuations in both the synchronous culture (apart from the initial perturbation) and in the control culture but repeats of these experiments did not produce any clear pattern. This was the only enzyme in the glycolytic pathway which was examined in this work and the significance of this result will be discussed in the final Chapter. It does appear that this enzyme is not the site at which the activity of the glycolytic pathway is controlled.

Two other experiments were carried out in order to characterise this enzyme more fully in S. pombe. Addition of cycloheximide to eukaryotic cells rapidly causes protein synthesis to cease. The activity

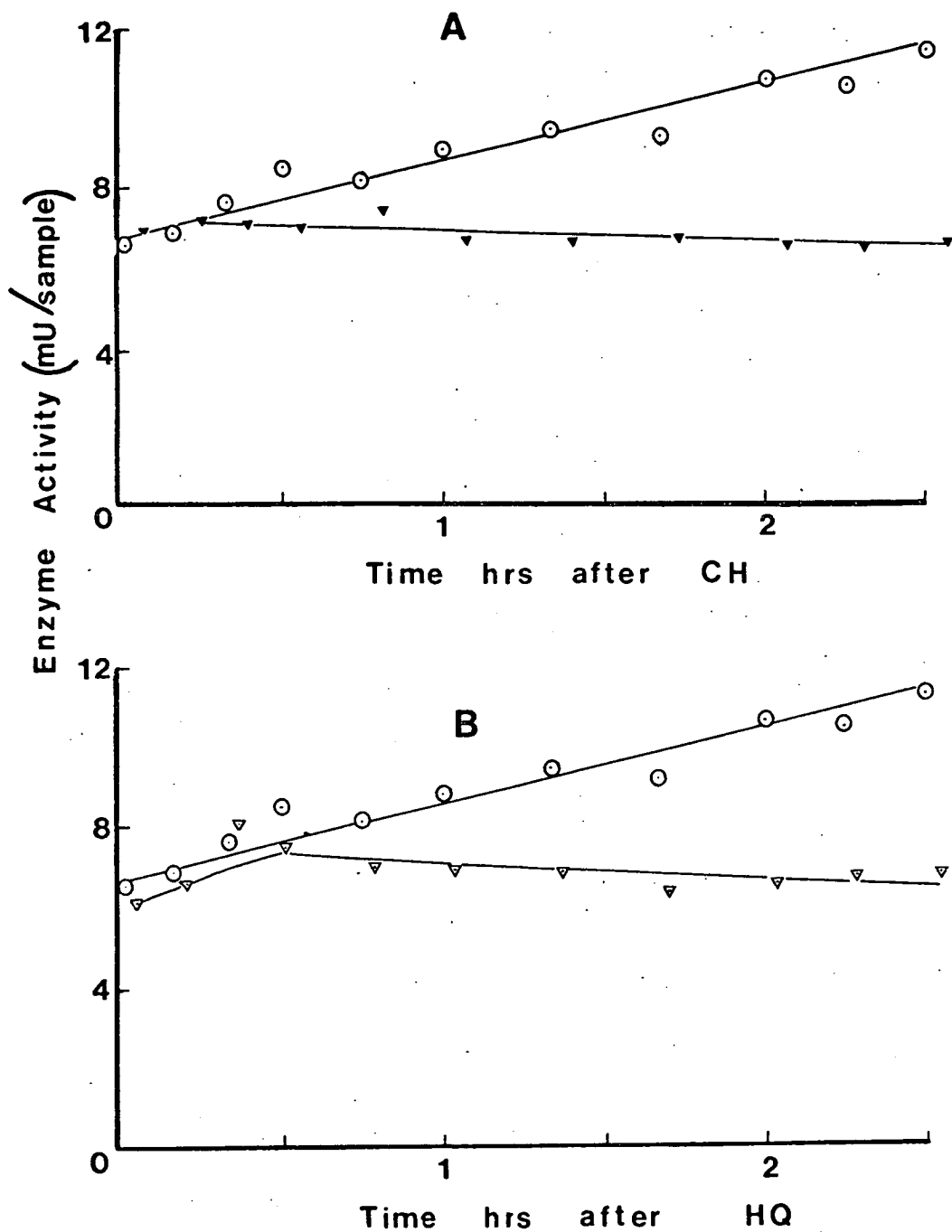


Fig 8-8 Hexokinase activity in cultures containing cycloheximide (0.1 mg /ml) or hydroxyquinoline (0.05 mg/ml).

At time 0, hydroxyquinoline or cycloheximide was added to part of an exponential culture containing 4.5×10^6 cells/ml. 0.5 ml samples were taken from these & from the original culture.

○—○ HK in control. ▼—▼ HK + Cy. Hex. ▼—▼ HK + 8-HQ.

of several enzymes has been measured in cultures of S. pombe which contain cycloheximide in order to obtain an estimate of the delay between the synthesis of the enzyme protein and its final assembly into an active enzyme (Mitchison & Creanor, 1969). This has been referred to as the "precursor delay". The activity of hexokinase was measured in exponentially growing cultures of S. pombe containing cycloheximide and the results, shown in Fig 8 - 8A, suggest that there is a delay of about 15 minutes from the addition of the inhibitor until the activity of the hexokinase remained constant.

It is now possible to examine the activity of enzymes in S. pombe in conditions where the synthesis of RNA has been specifically inhibited (Fraser & Creanor, 1974). An experiment was therefore carried out in which hydroxyquinoline was added to an exponentially growing culture of S. pombe and samples were removed to assay the activity of hexokinase. The effect of the hydroxyquinoline was to completely inhibit RNA synthesis within a few minutes of its addition. The results of this experiment, shown in Fig 8 - 8B, show that the activity of hexokinase continued to rise for about 30 minutes after the addition of the inhibitor. The simplest interpretation of this result is that the mRNA of this enzyme is unstable and, allowing for the precursor delay, has a half-life of about 5 minutes (Creanor, May & Mitchison, 1975). It has been shown, however, that the average mRNA population of S. pombe is more stable than hypothetical control molecules, either RNA or protein, which stimulate protein synthesis at the ribosomal level (Fraser, 1975). It may be, therefore, that the apparent cessation of hexokinase synthesis is caused by the decay of one or more control molecules rather than by the decay of the mRNA.

CHAPTER 9.

Discussion.

Introduction.

The primary investigation that has been carried out in this work has been to examine what happens to glucose after it has been taken up by Schizosaccharomyces pombe by examining the rates of CO₂ evolution and oxygen uptake, and thus the sources of energy, precursors and reducing power have been examined during the cell cycle.

This discussion will be divided into two parts. In the first, what has been learned of the biochemistry of the pathways of glucose assimilation (respiration and fermentation) will be discussed. The respiration and fermentation patterns of S. pombe will then be compared with other yeasts, in particular S. cerevisiae. In the second part of this discussion, the changes in the patterns of fermentation and respiration during the normal cell cycle will be discussed and related to other cell cycle studies. While this has formed the major part of this work, some attention will also be given to the patterns of respiration and fermentation in synchronous cultures growing in the presence of inhibitors and in cultures which have been synchronised by using pulses of these same inhibitors.

The Biochemistry of the EMP Pathway and of the Citric Acid Cycle.

Glucose, when it is transported into the cell, passes along the various steps of the EMP pathway until pyruvate is formed. At this point, pyruvate can either enter the Citric Acid Cycle by way of Acetyl-CoA or it can be decarboxylated to acetaldehyde and eventually to alcohol. Either of these alternatives results in a gain of energy by the cell. Respiration, by way of the Citric Acid Cycle and the Electron Transport Chain results in the formation of 38 moles of ATP from each mole of glucose taken up by the cell; fermentation on the other hand, leads to the production of 2 moles of ATP for each mole of glucose taken up by the cell. So respiration is much more efficient in the production of useable energy than fermentation.

It is quite clear that in exponential cultures of S. pombe growing on 1% glucose as carbon source, at least 90% of the glucose taken up by the cell is fermented to ethanol and CO₂. The Respiratory Coefficient (RQ) in this yeast has been reported to range from four to infinity (DeDeken, 1966; Heslot, Goffeau & Louis, 1970). A value of infinity was reported by DeDeken (1966) who was unable to detect any oxygen uptake. I have found that the RQ of S. pombe is about 10 in either strain 132 or strain 972 h⁻, a figure similar to that obtained by Kramhøft & Zeuthen (personal communication).

S. pombe therefore utilises glucose by fermentation rather than by respiration, even though the latter is a much more efficient energy producer. Since the cell does grow in this way, there has to be some advantage in this mode of growth. One way of looking at this situation is to consider the yield of ATP which comes from the functioning of the EMP pathway and the Citric Acid Cycle. 90% of the glucose which is

taken up by S. pombe is processed by fermentation, and each mole of glucose leads to the production of 1.8 moles of ATP. 5% of the glucose assimilated proceeds along the Citric Acid Cycle and for each mole of glucose taken up by the cell, 1.8 moles of ATP are produced. In other words, the low level of respiration produces for the cell exactly the same amount of energy as the much higher level of fermentation.

It has been claimed that the "cost" to the cell in maintaining a separate mitochondrial system is greater than the net product that would be obtained by transcribing and translating the entire mitochondrial genome (Borst & Grivel, 1973). In spite of this, a mitochondrial system is maintained by S. pombe and it is possible that if the cell is growing in sufficient levels of glucose to supply all the ATP required for growth by the fermentation process, the functioning of the Citric Acid Cycle could serve simply for the supply of carbon skeletons for the synthesis of proteins and amino acids (Krebs, Gurin & Eggleston, 1952) the demand for which might not be met by the functioning of the EMP pathway alone.

Cells are in fact able to grow completely anaerobically but the final yield of cells is much less (up to a quarter less) than when the culture is growing aerobically. This is consistent with the fact that glucose is utilised much more efficiently as far as ATP production is concerned in aerobic cultures, in fact twenty times the amount of glucose is required to produce the same amount of ATP anaerobically as aerobically. When a culture of S. pombe was grown anaerobically, the final cell number of the culture was about 1×10^7 cells/ml; when the culture was grown aerobically using the same medium, the final cell number was 5×10^7 cells/ml.

It was not possible to confirm the anaerobic growth pattern using inhibitors. The experiment with sodium cyanide showed that growth

was inhibited about $1\frac{1}{2}$ hours after the addition of the inhibitor (Fig 4-8). It is very likely, however, that the addition of the inhibitor was affecting a number of enzymes apart from merely inhibiting the final stage of the Electron Transport Chain: a list of enzymes inhibited by low concentrations of NaCN is presented by Dixon & Webb (1964) and among these enzymes are some of the EMP pathway and of the Citric Acid Cycle. So it is not possible to conclude that growth is inhibited in the absence of oxygen uptake. The fact that cultures can grow anaerobically makes this conclusion extremely unlikely, but it is possible that growth is much slower in the absence of oxygen uptake. This result does emphasise the dangers in overinterpretation of results obtained by the use of inhibitors.

It was not possible to measure the growth of a culture in the absence of oxygen uptake caused by the addition of Antimycin A. Only 50% inhibition of oxygen uptake was obtained in S. pombe strain 132 using $20\mu\text{M}$ antimycin A, a concentration of inhibitor which was more than 10 times greater than the concentration which was reported to give 90% inhibition of respiration in strain 972 h⁻ (Heslot et al, 1970). While the different strains may be reacting differently to the inhibitor, it is also possible that this concentration of antimycin A was affecting the working of the oxygen electrode, as was discussed in Chapter 4. This effect has not been reported previously and, in my opinion, it casts some doubts on any measurements of oxygen uptake made in the presence of this inhibitor. Investigation into the precise nature of this effect was not made.

S. pombe will grow on non-fermentable carbon sources though it does so at a slower rate. The slower rate may be due to the fact that penetration of the carbon source may be slower than that of glucose or to the fact that enzymes required for the metabolism of other carbon

sources may exist in small amounts and may not be inducible. Under these circumstances, not only the supply of ATP may be limiting, but also the supply of carbon skeletons could limit growth rate. I have examined the growth of S. pombe in only one non-fermentable carbon source, glycerol. This is a relatively poor substrate for strain 132 but a better substrate for strain 972h⁻. Allowing for differences in generation time, the oxygen uptake in glycerol grown cells is 5 times greater than that in cells grown on glucose as carbon source. This confirms observations by Heslot et al (1970) that there is some degree of glucose repression of respiration in this yeast, as was discussed in Chapter 1.

Effect of Inhibitors of Protein Synthesis on Oxygen Uptake & CO₂ Evolution.

The dependence of oxygen uptake and CO₂ evolution on protein synthesis in S. pombe was tested by using the inhibitors cycloheximide, which inhibits protein synthesis on 80S ribosomes, and erythromycin, which inhibits mitochondrial protein synthesis. Both of these inhibitors appear to act quickly and are reversible. Their effect on the growth and respiration of S. pombe are, however, quite different.

The action of cycloheximide on S. pombe has been discussed in Chapters 4 and 6. The increase in the rate of CO₂ evolution in an exponential culture was shown to be immediately inhibited after the addition of 0.1 mg/ml cycloheximide (Fig 6-5), and the rate of CO₂ evolution itself was shown to decrease over an extended period after the addition of the inhibitor. It was suggested that this fall in the rate was due to a decrease in the utilisation of ATP in cultures in which protein synthesis was directly inhibited and the synthesis of RNA and DNA were also inhibited as a result of the inhibition of protein

synthesis. Cycloheximide is reversible in S. pombe and, on removal of the inhibitor, the rate of CO₂ evolution returned to the rate which had been achieved in the culture before the addition of the cycloheximide. This not only supports the concept that a fall in the utilisation of energy was responsible for the decrease in the rate of CO₂ evolution, but it also suggests that the components of the EMP pathway are probably stable under these conditions.

The effect of cycloheximide on oxygen uptake in S. pombe was also examined. Shortly after addition of the inhibitor, the rate of oxygen uptake stopped increasing and remained at a constant level for a considerable time (Fig 4-5). In this case, therefore, there does not appear to be a similar feed-back operating to inhibit the uptake of oxygen as was seen in the evolution of CO₂ using the same inhibitor. While there is no further increase in the rate of oxygen consumption in the absence of protein synthesis, neither did the rate fall in these circumstances. It was discussed earlier (Chapter 3), however, that S. pombe, while it does not grow on either ethanol or acetate as carbon source, is still capable of oxidising these substrates. In the presence of cycloheximide, it is possible that it is not glucose which is being oxidised but one of these other compounds. Investigation of this was not carried out in detail in the present study.

There is considerable evidence that mitochondrial protein synthesis is not inhibited in the presence of cycloheximide and also that mitochondrial DNA synthesis will continue for several rounds of replication in S. cerevisiae in the presence of cycloheximide while nuclear DNA replication stops immediately (Grossman, Goldring & Marmur, 1969). It has been suggested that there may be a pool of proteins which is sufficient to initiate one or more rounds of mitochondrial DNA replication, assuming that initiation is by the same mechanism as

operates in bacteria (Kjeldgaard, 1967) through the action of a specific initiator protein. The results with cycloheximide and oxygen uptake suggest that for an increase in oxygen uptake to occur, a process which is largely mitochondrial, cytoplasmic protein synthesis must occur.

Oxygen uptake was also examined in the presence of erythromycin. The effect of adding this inhibitor was to inhibit further increase in the rate of oxygen uptake immediately, without causing any decrease in the rate (Fig 4-9). The effect of this inhibitor was fairly short-lived and after about $1\frac{1}{2}$ hours, the rate of oxygen uptake again began to increase. There was not, however, during the period when the rate of oxygen uptake did not increase, any inhibition of growth of the culture and in the continued presence of erythromycin cultures reached the same stationary cell concentration as untreated cultures. The period when the rate of oxygen uptake was inhibited could not be prolonged by increasing the concentration of erythromycin. The final conclusion from this experiment is that an increase in the rate of oxygen consumption also depends on the synthesis of mitochondrial proteins (i.e. protein made on the mitochondrial protein synthesising machinery) as well as on the synthesis of cytoplasmically produced proteins.

Effect of RNA Synthesis Inhibition on Oxygen Uptake & CO₂ Evolution.

Both oxygen uptake and CO₂ evolution were examined in cultures containing 0.05 mg/ml 8-hydroxyquinoline which at this concentration rapidly and completely inhibits the synthesis of all RNA in S. pombe with the exception of 4S and 5S (Fraser & Creanor, 1974). The action of this inhibitor is not reversible.

When added to an exponentially growing culture of S. pombe, 8-hydroxyquinoline completely and immediately prevented further increase in the rate of oxygen uptake (Fig 4-6) without at the same time causing any fall in the rate. It is not possible to say whether this inhibitor was affecting nuclear RNA and mitochondrial RNA at the same time. I would conclude tentatively that an increase in the rate of oxygen consumption was dependent on concomitant synthesis of RNA, but one cannot differentiate between a dependence of oxygen uptake on nuclear or mitochondrial RNA synthesis. It is also possible that a decrease in the utilisation of ATP occurs in this situation, as was suggested for the cycloheximide result, and that either alcohol or acetate, both of which are present in the medium, are being oxidised.

The evolution of CO_2 in the presence of hydroxyquinoline showed a similar pattern to that observed in the presence of cycloheximide. Immediately on addition of the inhibitor, the rate of CO_2 evolution decreased and continued to do so for the duration of the experiment (Fig 6-5). A similar argument can be applied in this case; inhibition of RNA synthesis leads to a decrease in the utilisation of ATP and this leads to a decrease in the rate of CO_2 evolution through the operation of a feed-back inhibition at one of the steps of the EMP pathway. It is not known whether the components of the EMP pathway are being degraded in the presence of hydroxyquinoline since it has not proved possible to reverse the action of this inhibitor in S. pombe.

CONCLUSIONS.

It has been shown that the Respiratory Coefficient of S. pombe growing on glucose is about 10, meaning that most of the glucose taken

up by the cell is assimilated by the EMP pathway and finally oxidised to ethanol and CO_2 . This situation is similar to that found in other yeasts and has previously been demonstrated for S. pombe. Anaerobic growth could not be demonstrated by the use of inhibitors of oxygen uptake, presumably because of side-effects of the inhibitors.

Inhibition of transcription and of translation leads to a rapid effect on oxygen uptake and CO_2 evolution. If the utilisation of energy is reduced in these two situations, then it is reasonable that the supply of energy will also be discontinued. The nature of the oxygen uptake in the presence of either cycloheximide or hydroxyquinoline is rather equivocal since there are several other compounds which can be oxidised by the cell without leading to cell growth.

The dependency of respiration on protein synthesis, either cytoplasmic or mitochondrial, is a much more difficult problem since it is not at all clear at this time how large a part the protein synthesising capacity of the mitochondria plays in the general metabolism of the cell. It is quite clear that most mitochondrial components are coded for by nuclear genes (Vary, Edwards & Stewart, 1969; Mahler & Perlman, 1971; Schatz & Mason, 1974). There is no evidence, however, that mRNA transcribed in one system is translated in another (Borst, 1972). Most mitochondrial proteins are coded for by nuclear genes, translated on cytoplasmic ribosomes and then transported into the mitochondria (Roodyn, Reis & Work, 1961; Beattie, Basford & Koritz, 1966, 1967; Groot, Rouslin & Schatz, 1972). Much of the evidence for this comes from the petite mutants of S. cerevisiae in which most, if not all, of the mitochondrial DNA is lost (Mounolou, Jacob & Slonimski, 1966; Goldring, Grossman, Krupnick, Cryer & Marmur, 1970; Nagley & Linnane, 1970) and there is no functional mitochondrial protein synthesising machinery. All of the proteins present in such mitochondria

are therefore made on the cytoplasmic machinery and they include the enzymes of the Citric Acid Cycle, ferrochelatase, cytochrome c, cytochrome b_2 , mitochondrial F_1 -ATPase, mitochondrial polypeptide elongation factors and three sub-units of cytochrome oxidase. The outer mitochondrial membrane and an apparently normal inner membrane are also present. This evidence suggests that the bulk of the mitochondrial membranes and most of the enzymes are produced on nuclear genes by the cytoplasmic system.

It is more difficult to argue that the absence of a protein in petites, for example, suggests that the protein is mitochondrial in origin (Schatz & Mason, 1974). For example, a protein may be missing because it is normally made on mitochondrial ribosomes though it is coded for by the nucleus; or it may be made in the cytoplasm and may require a mitochondrial factor for assembly in the mitochondria; also the loss of activity may not necessarily mean the loss of a protein. It is equally unsatisfactory to demonstrate the mitochondrial origin of a protein by the use of inhibitors since such a procedure is open to the same sorts of objections as have just been discussed.

Inhibition of either cytoplasmic protein synthesis or mitochondrial protein synthesis has been shown to prevent further increase in the rate of oxygen uptake. The simplest explanation of these results would be to say that both cytoplasmic and mitochondrial protein synthesis are required for an increase in the rate of oxygen uptake to occur. Since it has been clearly demonstrated that the bulk of the mitochondrial proteins are nuclear in origin, the result with cycloheximide is probably a reasonable one. But it does appear at present that no more than twelve polypeptides are coded for by the mitochondrial DNA and synthesised by the mitochondrial machinery. Three of these are associated with cytochrome oxidase, four with oligomycin-sensitive

ATPase and one with cytochrome b. The four other polypeptides are still unaccounted for. So one could argue that it is the synthesis of one or more of these components which is controlling the uptake of oxygen and that in the absence of their synthesis, no increase in the rate of oxygen uptake can occur. So, while the combined mitochondrial translation products account for about 5 - 15% of the mitochondrial mass (Schatz & Mason, 1974) the continued synthesis of one or all of these components is essential for the continued increase in the rate of oxygen uptake in S. pombe.

The results with cycloheximide and erythromycin on oxygen uptake in S. pombe could possibly be accounted for by a feed-back system operating at some stage of the EMP pathway, the Citric Acid Cycle or the Electron Transport Chain. This would be similar to the scheme proposed to account for the reduction in the rate of CO₂ evolution in the presence of cycloheximide or hydroxyquinoline. This would seem to me to be a more reasonable proposition since the operation of a feed-back inhibition on the EMP pathway, which has been proposed, would eventually affect the entry of substrate into the Citric Acid Cycle and the flow through the Electron Transport Chain. However, there is no evidence at present to support the concept that protein components of the pathways are limiting, or the concept that a feed-back inhibition operates to control the flux through one or other of these three pathways.

PART 2.

Respiration and Fermentation during the Cell Cycle.

The uptake of oxygen and the evolution of CO_2 have been examined during the cell cycle of S. pombe. Both energy production and carbon skeletons are obviously required for cell growth and the present study looks at how the energy production processes are involved in growth by examining oxygen consumption and CO_2 evolution in a synchronously growing and dividing culture.

It has been discussed how S. pombe exhibits a degree of glucose repression of respiration and also a repression of respiration by fermentation. A measure of oxygen uptake would therefore give an incomplete answer when one is examining the energy metabolism of the cell since a major part of glucose catabolism is by way of the EMP pathway and fermentation. Since it has been demonstrated that 90% of the glucose taken up by the cell is assimilated in this way, it follows that a measure of the evolution of CO_2 is also a measure of the flux through the EMP pathway and of the activity of fermentation. For this reason, the evolution of CO_2 has been examined in synchronously growing cultures of S. pombe so that combined with the data for oxygen uptake, a more complete picture of the energy metabolism of this cell might be obtained.

The question was then asked of how committed the cell is to a particular pattern of respiration and fermentation. It is possible, for example, to disrupt the progress of the cell through the normal cell cycle and in so doing, to look at the patterns of respiration and fermentation in cultures of this nature. This was done by preventing the synthesis of DNA in synchronous cultures of S. pombe and by inhibiting nuclear division, thus indirectly blocking cell division.

In all of these cases, the uptake of oxygen and the evolution of CO_2 were measured and the patterns obtained were compared to the patterns which had been obtained in normal synchronous cultures.

Several other cell cycle parameters were also measured and these were correlated with the patterns of respiration and fermentation during the cell cycle.

Oxygen Uptake and CO_2 Evolution.

The experiments in Chapters 3 and 5 describe measurements of the rates of oxygen uptake and CO_2 evolution during the normal cell cycle of S. pombe. It has been discussed earlier how these two phenomena are probably equally important as far as the energy supply to the cell is concerned.

Experiments measuring the oxygen uptake during the cell cycle of a normally growing synchronous culture show that there are two stages during the cell cycle when the rate of oxygen uptake increased. These measurements were carried out using a manometric technique and also a polarographic method. The results obtained by the manometric method were imprecise because of the low oxygen uptake in S. pombe. The polarographic method, using an oxygen electrode, was much more reliable and this data suggested that there was one change in the rate of oxygen consumption in the middle of the cycle, followed by a second at the end of the cycle or near the start of the next cycle. During the intervening periods the rate of oxygen uptake remained constant. Each change in the rate was an increase of about 50%, with the result that overall the rate doubled during each cell cycle.

The rate of oxygen uptake was also measured in asynchronous control cultures prepared, as described in Chapter 2, by carrying out the synchronisation procedure until the end of the centrifugation, then

S. cerevisiae.

<u>REFERENCE</u>	<u>MEDIUM</u>	<u>SYNCHRONISATION</u>	<u>PATTERN OF O₂</u>	<u>CONTROLS.</u>
h & akamiya	Minimal	Centrifugation & high temperature	1 oscillation / cycle.	-
es & illiamson	Complete	Feeding / starvation.	1 step / cycle.	-
zi & Fiechter	Minimal	Starvation / feeding.	1 oscillation / cycle.	-
Meyenburg	Minimal	Shift in dilution rate & gradients.	1 peak / cycle.	0
ken et al.	Complete.	Equilibrium centrifugation.	1 peak / cycle.	0
rell & Avers.	Complete.	Feeding / starvation.	Continuous increase.	-
sák & anicova	Complete.	Long periods of centrifugation.	1 step / cycle.	0
malingam yaraman.	Complete.	Feeding / starvation.	1 peak / cycle.	-

S. pombe.

<u>REFERENCE</u>	<u>MEDIUM</u>	<u>SYNCHRONISATION</u>	<u>PATTERN OF O₂</u>	<u>CONTROLS.</u>
hant.	Complete	Feeding / starvation.	2 steps / cycle.	-
i & Sando.	?	Feeding / starvation.	1 step / cycle.	-
e & Lloyd.	Minimal	Zonal centrifugation.	2 peaks / cycle.	0

TABLE 9-1. Summary of investigations into the oxygen uptake of
synchronous cultures of S. cerevisiae & S. pombe.

References are given in detail in the text. In the column
designated "CONTROLS", (-) means that control cultures
could not be made using the synchronisation technique
employed: (0) means that controls were not carried out.

instead of removing the small cells at the top of the gradient, the contents of the tube were mixed and a sample of cells at all stages of the cell cycle were inoculated into fresh medium. The purpose of this procedure was to test whether the results obtained in synchronous cultures might have been caused by the synchronisation treatment itself. However, such control cultures indicated that the rate of oxygen uptake increased continuously, suggesting that the results obtained in the synchronous cultures represented real, cell cycle - specific changes in the rate of oxygen uptake.

The increases in the rate of oxygen uptake at two points during the cell cycle have not been demonstrated before in synchronous cultures of yeast. There are, however, certain similarities between this result and other results which have been reported for the oxygen uptake of synchronous cultures of yeast. Previous reports of the uptake of oxygen in synchronous cultures of S. pombe and S. cerevisiae are summarised in TABLE 9-1. This table gives the cell type, the medium used, the synchronisation procedure adopted and indicates whether or not control cultures testing the effect of the synchronisation treatments could be and were examined. In some synchronisation methods, the end result is to induce all of the cells in a culture to divide synchronously. Obviously it is not possible to test the effect of a synchronisation treatment of this nature on a culture which contains cells at all stages of the cell cycle, since the procedure only produces cells at one specific stage of the cycle.

Most investigations into the uptake of oxygen in synchronous cultures of yeast have been carried out in S. cerevisiae and a variety of techniques have been used in the preparation of synchronous cultures. There is also a wide variation in the patterns which have been reported for the uptake of oxygen in synchronous cultures of this species. The

rate of oxygen uptake was shown to increase in a series of sharp steps, with the rate constant during the intervening periods in synchronous cultures of S. cerevisiae growing in a semi-defined medium and synchronised by a feeding - starvation method (Scopes & Williamson, 1964). A similar result was reported for the oxygen uptake of the same yeast synchronised by ficol gradients and growing in a complex medium (Greksák & Hanicová, 1973). However, using the same yeast and the same methods, Cottrell & Avers (1970, 1971) reported a continuous increase in the rate of oxygen uptake in synchronous cultures. Another investigation, utilising a starvation method of synchronisation and growing the cells in a semi-defined medium, produced a peak pattern, with a maximum in the rate of oxygen uptake once per cycle (Dharmalingam & Jayaraman, 1973). This pattern was shown in the cultures if glucose was used as carbon source, but not with maltose as the carbon source. Kuenzi

and Flechter (1969) synchronised S. cerevisiae by growing the cells in a chemostat with glucose as the rate limiting nutrient. After growing the cells for six generations, the medium supply was interrupted for seven hours, during which time the cells grew on the accumulated ethenol. Seven hours later fresh medium was supplied to the culture which then underwent a synchronous division. The oxygen uptake during the synchronous division oscillated in rate with a maximum once per cycle.

An elaborate technique was employed by Nosoh & Takamiya (1962) to prepare synchronous cultures of S. cerevisiae. The cells were grown in a minimal medium and 15 ml of packed cell volume was subjected to about fifteen short, low speed centrifugations. The resultant cells were held at a temperature of 40°C for 1 hour and then inoculated into fresh medium, at a concentration of between 10^6 and 10^8 cells/ml. In this situation, the rate of oxygen uptake was again shown to oscillate,

probably at the end of G1 and at the start of the budding stage. This investigation was only carried on for one cycle after preparation of the synchronous culture.

Von Meyenburg (1969) has examined the respiration of S. cerevisiae in cultures growing under glucose limitation and synchronised by a shift - up or a shift - down of the dilution rate. Under these conditions, there was an increase in the oxygen uptake about 20 minutes before the bud initiation stage. The higher rate was maintained for about an hour, at which time the rate (expressed per gram of dry weight) fell to the same value as before the increase and remained at this level for the rest of the cycle. Similar results were obtained at different growth rates, but the slower the generation time, the less marked were the changes in respiration. When cells were grown in 3% glucose and synchronised by the density gradient method (Wiemken, Matile & Moor, 1970) a maximum in the rate of oxygen uptake was found at the time of initial budding.

Using a complex medium and synchronising the cells by equilibrium gradient centrifugation, a maximum in the rate of oxygen uptake was found at the time of initial budding (Wiemken et al, 1970).

The overall result is that two step - wise patterns have been obtained, one continuously increasing pattern and five oscillatory patterns. There is at present no way to account for these different patterns. It may be that the different synchronous cultures contain mitochondrial populations having different degrees of synchrony (Cottrell & Avers, 1971). The history of the cells prior to synchronisation may be important (Kimball, Caspersson, Svensson & Carlson, 1959) and it is obvious that the history of many of these synchronous cultures prior to synchronisation was very different. It was shown in Chapter 5 how the pattern of CO₂ evolution in S. pombe could easily be disturbed

in a number of ways. This was not investigated for oxygen uptake but it is conceivable that the same situation might exist. In which case, one can examine the above patterns and regard with some degree of suspicion any synchronisation method which would be likely to cause perturbation in CO_2 evolution and possibly in oxygen uptake. Using this criterion, any synchronous culture prepared by a starvation method would be most likely to produce abnormal patterns in the cycles just after reinoculation into growth medium. In fact it has been shown that growth in terms of volume and mass lacked balance (did not double) in the first cycle after reinoculation but this was a temporary phenomenon (Scopes & Williamson, 1964). Neither was the pattern of oxygen uptake in phase with the cell cycle in synchronous cultures prepared by this technique (Dharmalingam & Jayaraman, 1973).

Considering next the synchronous cultures prepared by a selection technique in complex medium. It was shown in Chapter 5 how the pattern of CO_2 evolution changed from a discontinuous one in minimal medium to an imbalanced pattern in complex medium. Oxygen uptake was not measured under similar conditions but it is possible that a continuous pattern might have been obtained in complex medium. So the pattern of respiration and fermentation might depend very much on the medium in which the cells are growing.

The greatest imponderable during the preparation of synchronous cultures is how much the parameters measured during subsequent growth of the cultures are merely disturbances caused by the synchronisation technique. The investigations shown in TABLE 9-1 do not show a single case in which the effects of the synchronisation treatment were examined in an asynchronously growing culture. In some cases, it was not possible to prepare control cultures as discussed earlier. The investigations in which synchronous cultures were prepared using

isopycnic gradient centrifugation could, however, be criticised for lack of controls. The technique employed was to chill the cells to 0°C, then centrifuge for 40 minutes on a dextrin gradient (von Meyenburg, 1969; Wiemken et al, 1970). One would really like to see what the effect of this sort of treatment would be on an asynchronously growing culture. The technique of Nosoh & Takamiya (1962) described earlier used cells which had been through a variety of treatments, any of which might be expected to perturb subsequent growth of the cells.

I would conclude from this discussion on the patterns of oxygen uptake made in S. cerevisiae that there are reasons why each and every pattern could be regarded with some degree of suspicion. While it is true that starvation methods of synchronisation cannot be used to produce control cultures to test the effect of the treatment, such methods of synchronisation should be avoided when one is examining a cell cycle parameter intimately connected with the metabolism of the cell and likely to be influenced by the prior history of the culture.

The situation in S. pombe is equally unclear and three different investigations have produced three different patterns. Firstly, it was shown that the oxygen uptake rate increased in a single step at the time of nuclear division while during the remainder of the cycle the rate remained constant (Osumi & Sando, 1969; Osumi, Masuzawa & Sando, 1968). These measurements were made on cells which had been synchronised by a starvation method (Sando, 1963) in which the cycles after synchronisation are shorter than normal and the cell number did not double. Measurements of the rate of oxygen uptake were made rather far apart for this result to be very convincing.

Using the same method of synchronisation, Marchant(1971) showed two steps in the rate of oxygen uptake per cycle, one occurring at about

the middle of the cycle, the other at the end of the cycle. In this investigation, however, the first cycle was twice as long as a normal cell cycle and measurements were not carried on sufficiently long to determine whether or not the pattern was repeated in the second cell cycle.

A more thorough investigation into the respiratory activity of S. pombe growing on either glucose or glycerol as carbon source was made by Poole & Lloyd (1973 a, 1974). Synchronous cultures were made using a zonal rotor, as already described (Chapter 2), using sucrose as the gradient material and growing the cells in a minimal medium. While this may be an admissible choice of gradient material when the cells are growing on glucose as carbon source, it is slightly disturbing that sucrose should be used as gradient material when the cells are growing on glycerol as carbon source. Sucrase in S. pombe is an external enzyme (Mitchison & Creanor, 1969) and it must be assumed that during the centrifugation procedure, which can last up to twenty minutes, a considerable proportion of the sucrose must have been converted to glucose, thus complicating the position as to which carbon source was being utilised at the start of the synchronous culture.

Poole & Lloyd (1973 a) found that in glucose grown cells, oxygen uptake increased exponentially overall in a synchronous culture but rose to maxima twice per cycle. In some experiments, oscillations of smaller amplitude occurred at the minima of oxygen uptake. When the cells were grown in glycerol as carbon source, oxygen uptake increased in rate twice per cycle and remained constant in the intervening periods (Poole & Lloyd, 1974). The timings of the peaks in glucose grown cells and of the steps in glycerol grown cells were the same, one occurring at the time of cell division and the other in the middle of the cell cycle. Poole & Lloyd concluded that there were two components

of the oxygen uptake system in S. pombe, one increasing at all times during the cell cycle and the other oscillating twice during the cycle. The component which increased at all times during the cycle was not easily uncoupled and was selectively blocked by inhibitors of the electron transport chain (cyanide and antimycin A). The other component was easily uncoupled and could only be inhibited by high concentrations of cyanide and antimycin A. The periodicity of the oxygen uptake was thought to be caused by the synthesis of rate limiting factors.

These three investigations into the oxygen uptake of S. pombe were carried out in two different ways. In the first two discussed above, the starvation method of synchronisation was used and the criticisms which have been applied to measurements of respiration in S. cerevisiae synchronised by starvation methods can also be applied here. The investigation of Poole & Lloyd (1973 a) is more rigorous but it lacks controls to test the effects of their synchronisation procedure or the precision of their measurements. The separation of cells using a zonal rotor takes at least 20 minutes and during this time the culture might be subjected to all sorts of perturbations. It is also difficult to regard the existence of peaks, which depend on a single point, as being very convincing.

There are similarities between some of the results discussed above and the results for oxygen uptake presented in this thesis. Two investigations into the uptake of oxygen during the cell cycle of S. pombe have shown changes in rate, either steps or peaks, twice per cycle at the same time during the cycle as the steps in oxygen uptake which I have presented (Poole & Lloyd, 1973 a ; Marchant, 1971). The differences between these and other results could well be accounted for by differences in the medium, differences in the synchronisation technique and, in some cases, lack of convincing data.

The purpose of the above comparisons and discussion was not to show that each and every result obtained by measuring the uptake of oxygen in S. pombe and S. cerevisiae has so far been wrong. What I would like to emphasise is that most results are correct, although the interpretations might be optimistic, for the particular conditions used at the time, whether medium or synchronisation technique. It does seem obvious that a parameter such as the uptake of oxygen, which is measuring the energy metabolism of the cell, is likely to be extremely dependent on the condition of the cell and that condition is likely to be strongly influenced by the conditions of the culture both during the growth of the synchronous culture and, equally, during the growth of the culture before synchronisation. Until it is possible to find out what brings about the changes in oxygen uptake during the cell cycle and that this influences oxygen uptake differently in different conditions, then there is no other satisfactory explanation for this series of investigations producing such widely differing results.

Investigations into the evolution of CO_2 have been made much less frequently. Two investigations have shown that the rate of evolution of CO_2 increased exponentially during the cell cycle of S. cerevisiae growing on a complex medium (von Meyenburg, 1969 ; Wiemken et al, 1970). This result is not inconsistent with the result which I have shown for S. pombe growing in yeast extract (Fig 5-17).

Before discussing the implications of the patterns which have been demonstrated for oxygen uptake and CO_2 evolution in S. pombe, a brief mention will be made of results obtained in other systems.

There has been a long history of measurements of respiration in dividing eggs. Much of the early work was done by Zeuthen using a variety of vertebrate and invertebrate eggs (Zeuthen, 1946, 1955 ; Holter & Zeuthen, 1957 ; Frydenberg & Zeuthen, 1960), and the pattern is one in which oxygen uptake falls during mitosis and rises at the time of cell cleavage. Other investigations showed this rhythm in some eggs but not in others (Scholander, Claff, Sveinsson & Scholander, 1952 ; Scholander, Leivestad & Sundness, 1958). The size of the fluctuations in oxygen uptake are, however, very small and if the splitting of the chromosomes and the cell requires appreciable amounts of energy, this does not manifest itself by large increases in the respiration. It has been suggested, however, that energy could be drawn from a pool which had been built up during the whole cycle (Epel, 1963).

A similar pattern but with greater troughs at mitosis and higher peaks at cell division has been described in other cell systems. In HeLa cells oxygen uptake at mitosis is one half of the peak value during the S period (Robbins & Morrill, 1969) ; there is a sharp drop in respiration of Lilium at mitosis (Erickson, 1947) ; respiration in Chlorella also shows an oscillatory pattern but there are two troughs, both occurring after cell division (Curnett & Schmidt, 1964) ; Trillium also shows a drop in respiration at the time of mitosis (Stern & Kirk, 1948) ; synchronised cultures of Astasia longa have also been shown to have a decreased respiration rate at the time of mitosis (James, 1965 ; Wilson & James, 1966) ; in Tetrahymena, Zeuthen (1953) has shown that the respiration rate per cell (measured on single cells) increased linearly through the cycle until just before division when the rate stopped increasing or even decreased slightly ; Løvlie, however, showed that while in some cells the respiration rate increased linearly throughout the cycle, in other cells the increase was exponential

(Lövlie, 1963). The respiration rate of Tetrahymena, synchronised by heat shocks has also been shown to decrease at the time of cell division (Padilla, Cameron & Elrod, 1966). In Chlamydomonas reinhardtii, the fusion of many small mitochondria during the cell cycle to form one giant mitochondria coincides with a decrease in the rate of oxygen uptake (Osafune, Mihara, Hase, Ohkuro, 1972)

It has been suggested that the fall in oxygen uptake at mitosis is caused by the stoppage of macromolecular synthesis (Robbins & Morrill, 1969). Most of the cells discussed above cease to make RNA at that time and in some there is also a reduction in protein synthesis. This would also account for the differences between most of the other systems mentioned above and yeast and Tetrahymena. In the latter two cells, there is no decline in the rate of RNA or protein synthesis at division (Mitchison, 1971). It may be possible to conclude then that the rate of macromolecular synthesis in most cells is of major importance in controlling the respiratory rate during the cell cycle.

The evolution of CO_2 in synchronous cultures of S. pombe showed a doubling in rate at the time of nuclear division while during the rest of the cycle the rate remained constant (Fig 5 - 8). Asynchronous cultures showed a continuously increasing rate of CO_2 evolution, suggesting that a real cell cycle event was being measured.

Such a simple conclusion may not be completely correct, however, since it was shown that it was very easy to introduce periodic increases in the rate of CO_2 evolution by taking dense cultures and simply adding glucose to them (Fig 5 - 13). Or, if asynchronous control cultures were made using sucrose gradients, the rate of CO_2 evolution increased in a periodic manner, similar to that shown by the synchronous cultures

prepared using a lactose gradient (Fig 5-11). It was concluded, therefore, that the flux through the glycolytic pathway, as measured by the rate of evolution of CO_2 , could easily be disturbed by subjecting the cells to a variety of fairly mild treatments.

Because of these observations, considerable care was taken to ensure that the patterns obtained by measuring the rate of CO_2 evolution in synchronous cultures of S. pombe reflected the pattern which would be seen in normally growing cells and was not an artefact induced by the synchronisation technique. Since the asynchronous control culture prepared using a lactose gradient showed a continuously increasing rate of CO_2 evolution, it is possible that the smallest cells in the population were induced by the synchronisation procedure to produce a step pattern in the rate of CO_2 evolution. This would therefore be a cell cycle - specific artefact. It should be pointed out that such a criticism could be made of any measurement of a periodic event made using a synchronous culture. The induction of cell cycle - specific artefacts is difficult to investigate and there is no evidence of how frequent these are. However, the evolution of CO_2 does seem to be a parameter which is extremely sensitive to the treatment of the culture prior to synchronisation and therefore merits a considerable amount of attention.

One piece of evidence which suggests that this is not a cell cycle - specific artefact introduced by the synchronisation procedure is that cells of strain 972 h⁻, taken fortuitously from the top of the gradient when they were half way through the cell cycle, also showed a doubling in the rate of CO_2 evolution at the same time during the cycle as was seen in strain 132 when small cells were taken from the top of the gradient. It is unsatisfactory, of course, to use measurements made on one strain of cells as a control for measurements made on

another strain, but since in other respects these two strains have a similar behaviour (in RQ, time of DNA synthesis, mean generation time, time of nuclear division, cell size etc.) it is an indication that the measurements of CO₂ evolution made on synchronous cultures of strain 132 reflect the pattern of CO₂ evolution during the normal cell cycle.

The experiment to test conclusively for the presence of cell cycle specific artefacts could not be done. Ideally, one would have prepared a synchronous culture by separating cells which were mid-way through the cycle from the rest of the population, similar to the experiment reported for strain 972 h⁻. It proved impossible to do this, either using tube gradients or using the zonal rotor. The latter method has been used to separate the cell cycle of S. pombe across the rotor (Wells & James, 1972 ; Poole & Lloyd, 1973 b ; Mitchison & Carter, 1975). This has been discussed in detail in Chapter 5. It only remains to say that there are no examples of the use of this technique in S. pombe to separate other than the small cells from the rest of the population and then to grow these cells as a synchronous culture. To do this may require a degree of separation superior to that which has been described for separating the cell cycle across a zonal rotor.

Further evidence that the evolution of CO₂ is greatly affected by the conditions of the culture both before and after the synchronisation procedure comes from the experiments which were carried out in yeast extract / glucose medium. In this case, it was shown that the rate of CO₂ evolution increased continuously during the cell cycle and the steps which were seen in minimal medium were not observed (Fig 5 - 17). It was also shown that the increase in the rate of CO₂ evolution was faster than the generation time of the culture, measured by the increase in optical density. This was taken to indicate a lack of balance with respect to this parameter (CO₂ evolution). It is not clear why the

pattern of CO_2 evolution should alter by changing the growth medium; it has, however, been shown to be a measurement which is easily perturbed.

It was not possible to obtain reliable measurements of CO_2 evolution using the radiorespirometric technique. Various technical difficulties which were not solved during the course of this study arose. Firstly, pulses of radioactive glucose gave a result in a synchronous culture which was different to that obtained by the manometric technique. This problem is one that will be pursued in the future.

Continuous exposure of a culture to radioactive glucose produced a step-wise increase in the rate of CO_2 evolution; the timing of this step was, however, variable and the data was unreliable. This was found to be due to the presence of $^{14}\text{CO}_2$ dissolved in the medium during the growth of the culture and evolved during the measurement of the $^{14}\text{CO}_2$. This problem was not pursued any further in the present study. However, one is left with the situation that one radiorespirometric measurement of CO_2 evolution did, within limits, produce the same result as the Warburg method. So some confirmation of the manometric measurements has been obtained.

The question of glucose uptake and carbon skeleton utilization is one which merits some investigation in the future. Although it is not suitable in its present form for studying the evolution of $^{14}\text{CO}_2$, the radiorespirometric method described in this Thesis should have an application in an investigation into the kinetics of glucose utilization.

What is the significance of a double step in the rate of oxygen uptake and a single step in the rate of evolution of CO_2 during the cell cycle of S. pombe? It has been postulated that rate limiting factors, increasing in activity periodically during the cell cycle, are responsible for periodic increases in the rate of oxygen uptake (Poole & Lloyd, 1973a). This may well be the simplest (and the correct) explanation for this phenomenon. It does, however, assume that the pathways leading to the uptake of oxygen are working at the maximum rate and that further increases in the flux are dependent on the synthesis of these rate limiting factors. This will be discussed in the section dealing with measurement of respiratory enzymes during the cell cycle.

It seems equally likely that the levels of the intermediates in the pathways of glucose degradation could themselves control the rate of flux through the pathways. The simplest model would be that the level of citrate controls the activity of the Citric Acid Cycle and of the EMP pathway, upon which the activity of the electron transport chain depends. Fluctuations in the amount of citrate during the cell cycle would cause variations in the activity of one of the key enzymes of the EMP pathway (say phosphofructokinase) and hence variations in the flux through the EMP pathway and the Citric Acid Cycle. It was discussed in Chapter 1 how the level of citrate (and ATP) have been shown to affect the activity of phosphofructokinase, the first irreversible enzyme of the common glycolytic pathway. It may be a more realistic control for the cell to regulate the activity of its metabolic pathways according to its needs, rather than simply to double the flux once or twice per cycle as the activity of rate limiting enzymes doubled. One could visualize this operating through an increased demand for carbon skeletons at particular stages in the cycle, leading

to a depletion in either the level of citrate or of ATP, thus relieving the repression on phosphofructokinase. An increased flux through the EMP pathway and through the Citric Acid Cycle would result and eventually would be seen as an increase in the rate of CO_2 evolution and of oxygen uptake. The pathways would then be controlled directly according to the needs of the cell, operating a finer control than might be possible by altering the amount of the "rate limiting enzymes".

Another level of control has to be invoked to explain why the rate of CO_2 evolution changes once per cycle while that of oxygen uptake increases at two points during the cell cycle. It is possible, at this time, to imagine changes in the demand for whatever these two pathways are supplying. In the case of CO_2 evolution, one is dealing with the flux through the EMP pathway mainly, and with the production of energy. So one could imagine an increase in energy utilisation at the stage of the cycle where the rate of CO_2 evolution doubled. The flux through the EMP pathway, the Citric Acid Cycle and the electron transport chain will all influence the uptake of oxygen. How is it then that there are two changes in the rate of oxygen uptake while there is only one change in the rate of CO_2 evolution, and the change in the rate of CO_2 evolution does not occur at the same time during the cell cycle as either of the oxygen uptake changes? A possible way in which this might be brought about has been suggested by Holzer (1961). He has suggested that when oxygen is available for the oxidation of mitochondrial NADH, pyruvate is preferentially oxidised to Acetyl - CoA rather than decarboxylated to acetaldehyde, since pyruvate oxidase has a much lower K_m than pyruvate decarboxylase. This clearly would give a method for the control of the Citric Acid Cycle and of the electron transport chain at the expense of the fermentation pathway and might well be the explanation for the two pathways

having different patterns during the cell cycle. It is also possible that while the EMP pathway is mainly supplying energy to the cell, the Citric Acid Cycle is responsible for the maintenance of an adequate supply of carbon skeletons to the cell, as was suggested in Chapter 1. It is then necessary to have an increase in the demand for carbon skeletons happening at a different time during the cell cycle from the increase in the demand for energy to account for the difference between the patterns of CO₂ evolution and of oxygen uptake. There is, however, no information as to how and why the demand for carbon skeletons should increase at the middle and at the end of the cycle.

Activities of Respiratory Enzymes in Yeast.

The activities of both respiratory enzymes and of mitochondrial enzymes have been measured in synchronous cultures of both S. cerevisiae and S. pombe. Cottrell & Avers (1971) have shown that cytochrome c oxidase and malate dehydrogenase both show single step-wise increases in activity during the cell cycle of S. cerevisiae in conditions where the uptake of oxygen was showing an exponential increase. A similar step-wise increase in the activities of cytochrome c oxidase and of succinate dehydrogenase has been reported in the same yeast (Greksák & Hanicová, 1973). Cytochrome c oxidase and succinate cytochrome c reductase have also been shown to increase in a step-wise manner during the cell cycle of S. cerevisiae (von Meyenburg, 1969) at the same time as the uptake of oxygen was shown to oscillate with a maximum in rate once per cycle. The activities of cytochromes aa₃, b and c have also been investigated during the cell cycle of S. cerevisiae (Cottrell, Rabinowitz & Getz, 1975) and all of these were shown to increase continuously during the cycle with approximate

doublings in rate during successive cycles. It does not seem, therefore, that patterns of oxygen uptake in S. cerevisiae correlate with any of the respiratory enzymes. Perhaps such a correlation does not exist. Or perhaps the wrong enzymes have so far been measured and that the activity of the pathways leading to the uptake of oxygen is controlled by the activity of one or more enzymes.

An investigation into respiratory enzymes in S. pombe has been carried out by Poole & Lloyd (1973 b). They have shown that in synchronous cultures growing on glucose as carbon source in a synthetic medium and synchronised by gradient centrifugation, two enzymes, succinate dehydrogenase and NADH - cytochrome c reductase, both show peaks in activity at two points during the cell cycle. Cytochrome c oxidase, on the other hand, showed one peak in activity in the middle of each cycle. The difficulty about this data is that once again, peaks are defined by single points and the inherent variability of the enzyme assays is not discussed. A conclusion drawn from these results was that different enzymes of the same membrane system (cytochrome c oxidase & succinate dehydrogenase of the inner mitochondrial membrane) were not synthesised, or activated, at the same time during the cell cycle, suggesting that the stoichiometry of the individual components in a membrane system may vary at different times during the cell cycle. It was also concluded that a factor other than the activity of any of the enzymes measured was responsible for controlling the overall respiration rate since the peaks in oxygen uptake did not occur at the same time during the cycle as the peaks in activity of any of the enzymes.

So, in S. pombe, it has also proved impossible to find an enzyme which demonstrates the same pattern during the cell cycle as the oxygen uptake. It seems that the activity of either respiration or fermentation

is not controlled by the activity of the first enzyme of the EMP pathway, hexokinase (Chapter 8). This will be discussed later in this Chapter.

A complicated interplay of many factors is probably involved when the respiratory and fermentative activity of a cell changes during the cycle. The discussion so far in this Chapter has suggested that there may be other ways of controlling the activity of these two functions without necessarily involving the synthesis of rate-limiting enzymes.

Oxygen Uptake & CO₂ Evolution in Distorted Cell Cycles.

The uptake of oxygen and the evolution of CO₂ have been examined in asynchronous and synchronous cultures containing inhibitors of DNA synthesis or nuclear division. The same measurements were made on cultures which had been induced to divide synchronously by the application of a pulse of a DNA inhibitor. In this way, I have examined the dependency of the patterns which have been observed for oxygen uptake and CO₂ evolution on other periodic events during the normal cell cycle.

The results of the experiments in which the uptake of oxygen was measured in cultures containing deoxyadenosine or hydroxyurea (to inhibit DNA synthesis) or mitomycin C (to inhibit nuclear division) can be summarised by saying that in all of these cases there was little or no effect on the increase in the rate of oxygen uptake (Chapter 4). A similar result was shown for the evolution of CO₂ in the presence of these inhibitors (Chapter 6) in that the rate of CO₂ evolution continued to increase in the same way as was seen in the absence of these inhibitors.

Two periodic increases in the rate of oxygen uptake were also seen in a synchronous culture in the presence of deoxyadenosine (Fig 4-1): a doubling in the rate of CO_2 evolution was shown in synchronous cultures containing deoxyadenosine (Fig 6-2) or mitomycin C (Fig 6-3). In other words, a periodic doubling in the rates of either CO_2 evolution or of oxygen uptake was seen in synchronous cultures in which the synthesis of DNA or nuclear division were directly inhibited.

Synchronous cultures were also prepared using pulses of DNA inhibitors. These showed a continuous increase in the rate of CO_2 evolution (Fig 6-4) and in the uptake of oxygen (Figs 4-2 & 4-3). So in synchronous cultures of this nature, the uptake of oxygen and the evolution of CO_2 did not show the "synchronous pattern" which was seen in the normal synchronous cultures.

The existence of a DNA / Division Cycle and of a Growth Cycle has been postulated in S. pombe (Mitchison & Creanor, 1971 a). While there are numerous instances of periodic events occurring during the cell cycle of S. pombe (Mitchison, 1957; Bostock, Donachie, Masters & Mitchison, 1966; Mitchison & Creanor, 1969, 1971 b; Wain & Staatz, 1973; Fraser & Moreno, 1976; Staatz, 1976) none of these events have been shown to be associated with the growth cycle and the existence of the latter has relied on the fact that growth of S. pombe will continue in a more or less unaffected manner in the absence of DNA synthesis, nuclear division or cell division. However, some years ago it was reported that several enzymes increased periodically during the cell cycle of S. pombe (Bostock et al, 1966). These same enzymes were examined in induced synchronous cultures and were shown to be continuous (Sissons, Mitchison & Creanor, 1973) and it was concluded that the doubling in the activity of these enzymes was an event associated with the growth cycle. More careful examination of these enzymes has shown

that the step-wise increases in activity were perturbations induced by the synchronisation technique and that when measured carefully, these enzymes all showed continuous increases during the cell cycle (cited in Mitchison, 1976). So there are still no satisfactory markers for the growth cycle of S. pombe.

This investigation has shown, however, that there are periodic events occurring during the cell cycle of S. pombe which are still present in the absence of the main events of the DNA / division cycle. The existence of a growth cycle is still a possibility in this organism. No matter what controls the rates of oxygen uptake and CO_2 evolution in this yeast (enzyme levels or fluctuations in intermediates), this has to be altering in a periodic manner during the cell cycle in order to bring about the periodic changes in CO_2 evolution and oxygen uptake. This periodic alteration must still occur in the absence of the events of the DNA / division cycle and is not synchronised when only DNA synthesis, nuclear division and cell division are synchronised.

Measurement of other events during the Cell Cycle of S. pombe.

In Chapter 8, measurement of other events which might be associated with the growth of S. pombe were made. This was done in an attempt to show at least a temporal, if not a causal, relationship with the patterns which had been demonstrated for oxygen uptake and CO_2 evolution.

The optical density of a culture has been used throughout this work as an overall measure of the growth of a culture, without any real comprehension of what growth parameter the optical density is measuring. However, its main advantage is that it is quick and easy to do. It has been suggested that this measurement is most closely

related to the dry mass of a cell (Mitchison, 1970) so that a measurement of the optical density during the growth of a synchronous culture is giving some information on the growth pattern of the cell. The results obtained (Fig 8-2) suggest that the rate of increase of the optical density in a synchronous culture doubled very close to the time when the rate of CO_2 evolution was found to double. It is possible that this is purely coincidental and that the two events are totally unrelated. However, it is also possible that the doubling in the rate of CO_2 evolution is a signal for the general metabolism of the cell to double and that this is reflected in a change in the rate of optical density increase.

Attempts to find step-wise increases in the activity of hexokinase and in the amount of ATP, corresponding to the pattern shown by either the uptake of oxygen or the evolution of CO_2 , have not proved successful. The inability to find a step-wise increase in the activity of hexokinase is not surprising in view of the rarity of step enzymes in S. pombe (Mitchison, 1976). It has been discussed previously that a more fruitful approach to finding the effectors for the patterns shown by oxygen uptake and CO_2 evolution might be an examination of the intermediates of the EMP pathway, the level of citrate and the activity of phosphofructokinase, which enzyme is more likely to control the activity of the EMP pathway and respiration, if indeed these are controlled by enzyme activity.

A convincing pattern for the ATP content of synchronous cultures of S. pombe was not demonstrated. If, however, one is dealing with two sources of ATP in the cell, fermentation and respiration, and the contribution from these two sources is more or less equal, then the total picture might be rather complicated and give what appears to

be a continuous pattern, similar to that shown (Fig 8-4). There is also no information on the turn-over of ATP during the cell cycle and, although the rate of production might change periodically during the cell cycle, the rate of utilization might also change periodically; in fact this might be the prime effector for the patterns seen in oxygen uptake and CO_2 evolution. If this were the case, then an examination of the ATP content of cells would give a completely meaningless picture.

Further Work.

I would like to establish rigorously that the rate change pattern shown for CO_2 evolution in particular was a genuine cell cycle event and not a synchronisation artefact. This would be done if it were possible to produce a synchronous culture which consisted at the start of cells which were at a stage of the cycle other than the beginning. Ideally, one would like to obtain cells which were half way through the cycle. This could possibly be done by further investigation using the zonal rotor and I hope to do this.

It has been stated frequently that the levels of intermediates might influence the flux through the EMP pathway and the activity of phosphofructokinase. This is obviously a question that has to be answered. It has been impossible in this work and in other studies of this kind, to relate the respiratory pattern to the activity of any of the enzymes involved in the pathways of energy production. Perhaps measuring more enzymes would eventually produce an answer. It has been argued, however, that a finer control over the activity of the pathways producing energy would be possible if the flux through the pathways was regulated according to the needs of the cell. There is some evidence that the mechanism for achieving this exists. Alterations in the amount

of citrate or ATP can modulate the activity of phosphofructokinase. It seems important, therefore, to examine the level of citrate and the activity of phosphofructokinase during the cell cycle.

ACKNOWLEDGEMENTS.

I wish to express my thanks and apologies to the many people for whom the time that I have spent on this Thesis has been a traumatic experience.

Firstly, my supervisor and friend, Professor J.M. Mitchison has shown a remarkable degree of patience and given me considerable advice and encouragement as my obsession with this work increased daily.

Dr P.A.G. Wilson lent me the Warburg manometers and the Rikadenki recorder, both of which were invaluable during the course of this work. I am grateful to him for all his help and interest.

Most of my colleagues over the past four years have in various ways made this study easier. I have had most help from Dr R.S.S. Fraser particularly in the discussion of "synchronous artefacts", and I thank him for this and for being an efficient pre-filtration device for the first attempts at each Chapter. When the Mathematics became more complicated than additions and subtractions, I was grateful always to be able to get advice from Dr P.A. Fantes.

I apologise to Mr D. Cremer for making his life difficult over the "pictures" and I am grateful to him for doing his usual first class job. I also thank Mrs Yvonne Bisset for help with the drawing of many of the graphs and with correction of final copies.

Finally, my wife and family have had to live with my problems, night and day. Most of the credit for this work belongs to them and it is because of them that it may have any merits at all.

References.

- Barcroft, J. & Haldane, H.A. (1902). A method of estimating the oxygen and carbonic acid in small quantities of blood. J. Physiol., Lond 28 : 232 - 240.
- Barnett, J.A. & Kornberg, H.L. (1960). The utilization by yeast of the Tricarboxylic Acid Cycle. J. gen. Microbiol. 23 : 65 - 82.
- Beattie, D.S., Basford, R.E. & Koritz, S.B. (1966). Studies on the biosynthesis of mitochondrial protein components. Biochemistry, N.Y. 5 : 926 - 930.
- Beattie, D.S., Basford, R.E. & Koritz, S.B. (1967). The inner membrane as the site of the in Vitro incorporation of [^{14}C] leucine into mitochondrial protein. Biochemistry, N.Y. 6 : 3099 - 3106.
- Beck, C. & von Meyenburg, H.K. (1968). Enzyme pattern and aerobic growth of Saccharomyces cerevisiae under various degrees of glucose limitation. J. Bacteriol. 96 : 479 - 486.
- Belitzer, W.A. (1936). Über die umgekehrte Pasteursche Reaktion (On the Reverse Pasteur Reaction). Biochem. Z. 283 : 339 - 342.
- Bielka, H. (1963). Stoffwechsel virus induzierter Leukämischer Leukocyten 1V. Crabtree Effekt. Z. Krebsforsch. 65 : 196 - 200.
- Bloom, B. & Stetten, DeW. Jr. (1953) Pathways of glucose catabolism. J. Am. chem. Soc. (Amer). 75 : 5446.
- Blumenthal, H.J., Lewis, K.F., Weinhouse, S. (1954). An estimation of pathways of glucose catabolism in yeast. J. Am. chem. Soc. (Amer). 76 : 6093 - 6097.
- Borst, P. (1972). Mitochondrial Nucleic Acids. Annu. Rev. Biochem. 41 : 333 - 376.
- Borst, P. & Grivel, L.A. (1973). Mitochondrial Nucleic Acids. Biochimie. 55 : 801 - 804.

- Bostock, C.J. (1968). Nucleic Acid synthesis in the growth cycle of the fission yeast Schizosaccharomyces pombe and other organisms. Ph.D. Thesis, University of Edinburgh.
- Bostock, C.J., Donachie, W.D., Masters, M. & Mitchison, J.M. (1966). Synthesis of enzymes and DNA in synchronous cultures of Schizosaccharomyces pombe. Nature, Lond. 210 : 808 - 810.
- Brin, N. & McKee, R.W. (1956). The inhibition of respiration by glucose, fructose and mannose in the Ehrlich Mouse Ascites T mor. Cancer Res. 16 : 364 - 368
- Britten, R.J. & Roberts, R.B. (1960). High resolution density gradient sedimentation analysis. Science. 131 : 32 - 33.
- Campbell, A. (1957). Synchronisation of cell division. Bacteriol. Rev. 21 : 263 - 272.
- Carter, B.L.A. & Halvorson, H.O. (1973). An evaluation of the oscillatory repression model of periodic enzyme synthesis in yeast. Expl. Cell Res. 76 : 152 - 158.
- Carter, B.L.A., Sebastian, J. & Halvorson, H.O. (1971). The regulation of the synthesis of arginine catabolizing enzymes during the cell cycle in Saccharomyces cerevisiae. Adv. Enzyme Regul. 9 : 253 - 263.
- Chance, B. & Hess, B. (1956). On the control of metabolism in Ascites tumor cells. Ann. N.Y. Acad. Sci. 63 : 1008 - 1016.
- Chen, S.L. (1959). Carbohydrate assimilation in actively growing yeast, Saccharomyces cerevisiae. 1. Metabolic pathways for [^{14}C] glucose utilization by yeast during aerobic fermentation. Biochim. biophys. Acta. 32 : 470 - 479.
- Cirillo, V.P. (1961). Sugar transport in micro - organisms. A. Rev. Microbiol. 15 : 197 - 218.
- Cochrane, V.W. (1958). Physiology of Fungi. 524pp. Wiley, N.Y.

- Coe, E.L. (1964). The reversal of the Crabtree Effect by Iodoacetate (Ehrlich ascites tumor cells). Biochim. biophys. Acta. 93 : 209 - 211).
- Cottrell, S.F. & Avers, C.J. (1970). Evidence of mitochondrial synchrony in synchronous cultures of yeast. Biochim. Biophys. Res. Commun. 38 : 750 - 757.
- Cottrell, S.F. & Avers, C.J. (1971). Mitochondrial Activities in synchronous cultures of Bakers' Yeast. In "Autonomy and Biogenesis of Mitochondria and chloroplasts". pp 481 - 491. Ed. by N.K. Boardman, A.W. Linnane & R.M. Smillie. North-Holland Publishing Co: Amsterdam & London.
- Cottrell, S.F., Rabinowitz, M. & Getz, G.S. (1975). Cytochrome synthesis in synchronous cultures of the yeast Saccharomyces cerevisiae. J. biol. Chem. 250 : 4087 - 4094.
- Crabtree, H.G. (1929). LX1. Observations on the carbohydrate metabolism of tumours. Biochem. J. 23 : 536 - 545.
- Creanor, J., May, J.W. & Mitchison, J.M. (1975). The effect of 8-hydroxyquinoline on enzyme synthesis in the fission yeast Schizosaccharomyces pombe. Eur. J. Biochem. 60 : 487 - 493.
- Curnett, S.G. & Schmidt, R.R. (1964). Possible mechanisms controlling the intracellular level of inorganic polyphosphate during synchronous growth of Chlorella pyrenoidosa. Endogenous respiration. Expl. Cell Res. 36 : 102 - 110.
- Darrow, R.A. & Colowick, S.P. (1962). Hexokinase from Bakers' Yeast. in "Methods in Enzymology" Vol 5. pp 226 - 235. Ed by Colowick, S.P. & Kaplan, N.O.
- De Deken, R.H. (1966). The Crabtree effect and its relation to the petite mutation. J. Gen. Microbiol. 44 : 149 - 167.

- Dharmalingam, K. & Jayaraman, J. (1973). Mitochondriogenesis in synchronous cultures of yeast. 1. Oscillatory pattern of respiration. Arch. Biochem. Biophys. 157 : 197 - 202.
- Dixon, M. & Webb, E.C. (1964). The Enzymes. Longmans Publishing Co.
- Duncombe, W.G. (1974). Short-term radiorespirometry of cell suspensions. Biochem. J. 144 : 487 - 496.
- Epel, D. (1963). The effects of carbon monoxide inhibition on ATP level and the rate of mitosis in the sea urchin egg. J. Cell Biol. 17 : 315 - 319.
- Ephrussi, B., Slonimski, P.P., Yotsuyanagi, Y. & Tavlitzki, J. (1956). Variations physiologiques et cytologiques de la levure au cours du cycle de la croissance aérobie. C.r. Trav. Lab. Carlsberg Ser. physiol. 26 : 87 - 102
- Erickson, R.O. (1947)..Respiration of developing anthers. Nature, Lond. 159 : 275 - 276.
- Faed, M.J.W. (1959). Division and growth relationships in single cells. Ph.D. Thesis. University of Edinburgh.
- Fraser, R.S.S. (1975). Turnover of polyadenylated RNA in fission yeast : evidence for the control of protein synthesis at the translational level. Eur. J. Biochem. 60 : 477 - 486.
- Fraser, R.S.S. & Creanor, J. (1974). Rapid and selective inhibition of RNA synthesis in yeast by 8-Hydroxyquinoline. Eur. J. Biochem. 46 : 67 - 73.
- Fraser, R.S.S. & Creanor, J. (1975). The mechanism of inhibition of Ribonucleic Acid synthesis by 8-hydroxyquinoline and the anti-biotic lomofungin. Biochem. J. 147 : 401 - 410.
- Fraser, R.S.S. & Moreno, F. (1976). Rates of synthesis of poly-adenylated messenger RNA and ribosomal RNA during the cell cycle of Schizosaccharomyces pombe. J. Cell Sci. In the press.

- Frydenberg, O. & Zeuthen, E. (1960). Oxygen uptake and carbon dioxide output related to the mitotic rhythm in the cleaving eggs of Dendraster excentricus and Urechis caupo. C. r. Trav. Lab. Carlsberg. 31 : 423 - 455.
- Goldring, E.S., Grossman, L.I., Krupnick, D., Cryer, D.R. & Marmur, J. (1970). The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during the induction with Ethidium Bromide. J. molec. Biol. 52 : 323 - 335.
- Greksák, M. & Hanicová, M. (1973). Activities of succinate dehydrogenase and cytochrome oxidase in synchronous aerobic cultures of aerobically and anaerobically grown yeast. Biologia (Bratislava). 28 : 425 - 433.
- Groot, G.S.P., Rouslin, W. & Schatz, G. (1972). Promitochondria of anaerobically grown yeast. VII. Effect of oxygen on promitochondrial protein synthesis. J. biol. Chem. 247 : 1735 - 1742.
- Grossman, L.I., Goldring, E.S. & Marmur, J. (1969). Preferential synthesis of yeast mitochondrial DNA in the absence of protein synthesis. J. molec. Biol. 46 : 367 - 376.
- Halvorson, H.O., Carter, B.L.A. & Tauro, P. (1971). Synthesis of enzymes during the cell cycle. Adv. microb. Physiol. 6 : 47 - 106.
- Heslot, H., Goffeau, A. & Louis, C. (1970). Respiratory metabolism of a "petite negative" yeast Schizosaccharomyces pombe 972 h⁻. J. Bacteriol. 104 : 473 - 481.
- Holter, H. & Zeuthen, E. (1957). Dynamics of early echinoderm development, as observed by phase contrast microscopy and correlated with respiration measurements. Pubbl. Staz. zool. Napoli. 29 : 285 - 306.
- Holzer, H. (1961). Regulation of carbohydrate metabolism by Enzyme competition. Cold Spring Harbor Symp. Quant. Biol. 26 : 277 - 288.

- Holzer, H. & Witt, I. (1958). Die ersten Stoffwechselveränderungen nach NH_4^+ -Ionen zu Glucose oxydierenden Hefezellen. Biochem. Z. 330 : 545 - 554.
- James, T.W. (1965). Dynamic respirometry of division synchronised Astasia longa. Expl. Cell Res. 38 : 439 - 453.
- Kersten, H. & Kersten, W. (1969). Inhibitors - tools in Cell Research. p 11. Ed. by Bucher, T. & Sies, H. Springer - Verlag, Berlin.
- Kimball, R.F., Caspersson, T.O., Svensson, G. & Carlson, L. (1959). Quantitative studies on paramecium aurelia. 1. Growth in total dry weight measured by the scanning interference microscope and X-ray absorption methods. Expl. Cell Res. 17 : 160 - 172.
- Kjeldgaard, N.O. (1967). Regulation of nucleic acid and protein formation in bacteria. Adv. microb. Physiol. 1 : 39 - 95.
- Krebs, H.A. (1951). Improved manometric fluid. Biochem. J. 48 : 240 - 241.
- Krebs, H.A. & Johnson, W.A. (1937). Role of citric acid in intermediate metabolism in animal tissues. Enzymologia. 4 : 148 - 156.
- Krebs, H.A., Gurin, S. & Eggleston, L.V. (1952). The pathway of oxidation of acetate in bakers' yeast. Biochem. J. 51 : 614 - 628.
- Kuenzi, M.T. & Fiechter, A. (1969). Changes in carbohydrate composition during the budding cycle of Saccharomyces cerevisiae. Arch. Mikrobiol. 64 : 396 - 407.
- Lemoigne, M., Aubert, J.P. & Millet, J. (1954). La production d'alcool et la rendement de croissance de la levure de boulangerie cultivée en aérobiose. Annals Inst. Pasteur, Paris. 87 : 427
- Lin, S.-Y., Mosteller, R.D. & Hardesty, B. (1966). The mechanism of sodium fluoride and cycloheximide inhibition of haemoglobin biosynthesis in the cell-free reticulocyte system. J. molec. Biol. 21 : 51 - 69.

- Lindner, P. (1893). Wochschr. Brau. 10 : 1298.
- Lodder, J. & Kreger-van Rij, N.J.W. (1952). The yeast. A taxonomic study. Amsterdam : North Holland Pub. Co.
- Lovlie, A. (1963). Growth in mass and respiration rate during the cell cycle of Tetrahymena pyriformis. C. r. Trav. Lab. Carlsberg. 33 : 377 - 413.
- McClary, D.O. & Bowers, W.D. jr. (1968). Mitochondrial changes accompanying acriflavine - induced petite mutation in Saccharomyces fragilis. J. Ultrastruct. Res. 25 : 34 - 45.
- Mahler, H.R. & Perlman, P.S. (1971). Mitochondriogenesis analyzed by blocks on mitochondrial transcription and translation. Biochemistry, N.Y. 10 : 2979 - 2990.
- Marchant, R. (1971). The initiation of cell wall synthesis in para-synchronous cultures of Schizosaccharomyces pombe. Arch. Mikrobiol. 78 : 205 - 213.
- Meyenburg, H.K. von (1969). Energetics of the budding cycle of Saccharomyces cerevisiae during glucose limited aerobic growth. Arch. Mikrobiol. 66 : 289 - 303.
- Mitchison, J.M. (1957). The growth of single cells. 1. Schizosaccharomyces pombe. Expl. Cell Res. 13 : 244 - 262.
- Mitchison, J.M. (1970). Physiological and cytological methods for Schizosaccharomyces pombe. In Methods in Cell Physiology. Vol 4, pp 131 - 165. Ed by D.M. Prescott. New York & London, Acad. Press.
- Mitchison, J.M. (1971). The Biology of the Cell Cycle. Cambridge University Press.
- Mitchison, J.M. (1976). Enzyme Synthesis during the Cell Cycle. In press.
- Mitchison, J.M. & Carter, B.L.A. (1975). Cell Cycle Analysis. In Methods in Cell Biology. Vol X1, pp 201 - 219. Ed by D.M. Prescott. New York & London, Academic Press.

- Mitchison, J.M. & Creanor, J. (1969). Linear synthesis of sucrase and phosphatases during the cell cycle of Schizosaccharomyces pombe. J. Cell Sci. 5 : 373 - 391.
- Mitchison, J.M. & Creanor, J. (1971a). Induction synchrony in the fission yeast Schizosaccharomyces pombe. Expl. Cell Res. 67 : 368 - 374.
- Mitchison, J.M. & Creanor, J. (1971b). Further measurements of DNA synthesis and enzyme potential during the cell cycle of the fission yeast Schizosaccharomyces pombe. Expl. Cell Res. 69 : 244 - 247.
- Mitchison, J.M. & Vincent, W.S. (1965). Preparation of synchronous cultures by sedimentation. Nature, Lond. 205 : 987 - 989.
- Mounolou, J.C., Jacob, H. & Slonimski, P.P. (1966). Mitochondrial DNA from yeast "petite" mutants : specific changes in buoyant density corresponding to different cytoplasmic mutations. Biochem. biophys. Res. Commun. 24 : 218 - 224.
- Nagley, P. & Linnane, A.W. (1970). Mitochondrial DNA - deficient petite mutations of yeast. Biochem. biophys. Res. Commun. 39 : 989 - 996
- Niederpreum, D.J. (1965). Tricarboxylic Acid Cycle. In The Fungi pp 269 - 300. Ed by G.C. Ainsworth & A.S. Sussman. New York & London, Acad. Press.
- Nurse, P. (1975). Genetic Control of cell size at cell division in yeast. Nature, Lond. 256 : 547 - 551.
- Nosoh, Y. & Takamiya, A. (1962). Synchronisation of the budding cycle in yeast cells, and the effect of carbon monoxide and nitrogen deficiency on the synchrony. Plant Cell Physiol. (Tokyo) 3 : 53 - 66.

- Ohnishi, T., Sottocasa, G. & Ernster, L. (1966). Current approaches to the mechanism of energy - coupling in the respiratory chain. Studies with yeast mitochondria. Bull. Soc. Chim. Biol. 48 : 1189 - 1203.
- Osafune, T., Mihara, S., Hase, E. & Ohkuro, I. (1972). Electron microscope studies on the vegetative cellular life of Chlamydomonas reinhardi Dangeard in Synchronous culture : 1. Some characteristics of changes in sub - cellular structures during the cell cycle, especially in the formation of giant mitochondria. Plant Cell Physiol. (Tokyo). 13 : 211 - 227.
- Osumi, M. & Sando, N. (1969). Division of yeast mitochondria in synchronous cultures. J. Electron Microsc., Chiba Cy. 18 : 47 - 56.
- Osumi, M., Masuzawa, E. & Sando, N. (1968). Mitochondrial formation in synchronous cultures of Schizosaccharomyces pombe. Japan Women's Univ. J. 15 : 33 - 41.
- Padilla, G.M., Cameron, I.L. & Elrod, L.H. (1966). The physiology of repetitively synchronised Tetrahymena. In Cell Synchrony, pp269 - 288. Ed by I.L. Cameron & G.M. Padilla. New York & Lond. Academic Press.
- Pasteur, L. (1876). Etudes sur la Bière. Gauthieur - Villars éd., Paris.
- Poole, R.K. & Lloyd, D. (1973a). Respiratory Oscillations and heat evolution in Synchronously dividing cultures of the fission yeast Schizosaccharomyces pombe 972 h⁻. J. gen. Microbiol. 77 : 209 - 220.
- Poole, R.K. & Lloyd, D. (1973b). Oscillations of enzyme activities during the cell cycle of a glucose - depressed fission yeast, Schizosaccharomyces pombe 972 h⁻. Biochem. J. 136 : 195 - 207.
- Poole, R.K. & Lloyd, D. (1974). Changes in respiratory activities during the cell cycle of the fission yeast Schizosaccharomyces pombe 972h⁻ growing in the presence of glycerol. Biochem. J. 144 : 141 - 148.

- Rafter, G.W. (1957). The reaction of o - iodobenzoate with 3 - phosph - glyceraldehyde dehydrogenase. Arch. Biochem. Biophys. 67 : 267 - 271.
- Ramaiah, A., Hathaway, J.A. & Atkinson, D.E. (1964). Adenylate as a metabolic regulator. Effect on yeast phosphofructokinase kinetics. J. biol. Chem. 239 : 3619 - 3622.
- Robbins, E. & Morrill, G.A. (1969). Oxygen uptake during the HeLa cell life cycle and its correlation with macromolecular synthesis. J. Cell Biol. 43 : 629 - 633.
- Robinson, A.A. (1972). The relationship of enzymes to the cell cycle with particular reference to Schizosaccharomyces pombe. Ph.D. Thesis. University of Edinburgh.
- Roodyn, D.B., Reis, P.J. & Work, T.S. (1961). Protein synthesis in mitochondria. Requirement for the incorporation of radioactive amino acids into mitochondrial protein. Biochem. J. 80 : 9 - 21.
- Ryan, F.J., Tatum, E.L. & Giese, A.C. (1944). The four - carbon respiratory system and growth of the mold Neurospora. J. cell. comp. Physiol. 23 : 83 - 94.
- Salas, M.L., Viñuela, E., Salas, M. & Sols, A. (1965). Citrate inhibition of phosphofructokinase and the Pasteur Effect. Biochem. biophys. Res. Commun. 19 : 371 - 376.
- Sando, N. (1963). Biochemical studies on the synchronised culture of Schizosaccharomyces pombe. J. gen. appl. Microbiol., Tokyo 9 : 233 - 241.
- Schatz, G. & Mason, T.L. (1974). Mitochondrial Protein Synthesis. A. Rev. Biochem. 43 : 51 - 87.
- Scopes, A.W. & Williamson, D.H. (1964). The growth and oxygen uptake of synchronously dividing cultures of Saccharomyces cerevisiae. Expl. Cell Res. 35 : 361 - 371.

- Siegel, M.R. & Sisler, H.D. (1964). Site of action of cycloheximide in cells of Saccharomyces pastorianus. 11. The nature of inhibition of protein synthesis in a cell-free system. Biochim. biophys. Acta. 87 : 83 - 89.
- Siegel, M.R. & Sisler, H.D. (1965). Site of action of cycloheximide in cells of Saccharomyces pastorianus. 111. Further studies on the mechanism of resistance in Saccharomyces species. Biochim. biophys. Acta. 103 : 558 - 567.
- Sissons, C.H. (1976). Improved technique for accurate and convenient assay of biological reactions liberating $^{14}\text{CO}_2$. Analyt. Biochem. 70 : 454 - 462.
- Sissons, C.H., Mitchison, J.M. & Creanor, J. (1973). Enzyme synthesis and potential during induction synchrony in the fission yeast Schizosaccharomyces pombe. Expl. Cell Res. 82 : 63 - 72.
- Slater, E.C. (1967). Application of inhibitors and uncouplers for a study of oxidative phosphorylation. In Methods in Enzymology, Vol 10, pp. 48 - 57. Ed. by R.N. Estabrook & M.E. Pullman. Acad. Press: New York & Lond.
- Slater, E.C. (1973). The mechanism of action of the respiratory inhibitor, antimycin. Biochim. biophys. Acta. 301 : 129 - 154.
- Slonimski, P.P. (1956). Adaptation respiratoire : developpement du système hémoprotéique induit par l'oxygène. Proc. 3rd Int. Congr. Biochem. pp. 242.
- Sols, A. (1968). Regulation of Carbohydrate transport and metabolism in yeast. In Aspects of yeast metabolism. pp. 47 - 70. Ed by A. K. Mills. Blackwell Scientific Publ; Oxford & Edinburgh.
- Sols, A. & Salas, M.L. (1965). Phosphofructokinase. 111. Yeast. In Methods in Enzymology, Vol 9, pp. 436 - 442. Ed. by S.P. Colowick & N.O. Kaplan.

- Sols, A., Gancedo, C. & De la Fuente, G. (1971). Energy yielding metabolism in yeasts. In The Yeasts, Vol 2, pp. 271 - 307.
Ed. by A.H. Rose & J.S. Harrison.. Acad. Press; New York & Lond.
- Staatz, W.D. (1976). Some aspects of RNA synthesis during the cell cycle of the fission yeast Schizosaccharomyces pombe.
Ph.D. Thesis. University of Edinburgh.
- Stern, H. & Kirk, P.L. (1948). The oxygen consumption of the microspores of Trillium in relation to the mitotic cycle.
J. gen. Physiol. 31 : 243 - 248.
- Swanson, W.H. & Clifton, C.E. (1948). Growth and assimilation in cultures of Saccharomyces cerevisiae. J. Bacteriol. 56 : 115
- Thomas, D.Y. & Wilkie, D. (1968). Inhibition of mitochondrial protein synthesis in yeast by erythromycin : cytoplasmic and nuclear factors controlling resistance. Genet. Res. 11 : 33 - 41.
- Utter, M.F., Duell, E.A. & Bernofsky, C. (1967). Alterations in the respiratory enzymes of the mitochondria of growing and resting yeast. In Aspects of yeast metabolism, pp. 197 - 215. Ed by A.K. Mills. Blackwell Scientific Publ. Oxford & Edinburgh.
- Umbreit, W.W., Burris, R.H. & Stauffer, J.F. (1957). Manometric Techniques. Burgess Publ. Co., Minneapolis.
- Vary, M.J., Edwards, C.L. & Stewart, P.R. (1969). The biogenesis of mitochondria. 1X. Formation of the soluble mitochondrial enzymes malate dehydrogenase and fumarase in Saccharomyces cerevisiae.
Arch. Biochem. Biophys. 130 : 235 - 243.
- Viñuela, A., Salas, M.L. & Sols, A. (1963). End-product inhibition of yeast phosphofructokinase by ATP. Biochem. biophys. Res. Commun. 12 : 140 - 145.

- Wain, W.H. & Staatz, W.D. (1973). Rates of synthesis of ribosomal protein and total ribonucleic acid through the cell cycle of the fission yeast Schizosaccharomyces pombe. Expl. Cell Res. 81 : 269 - 278.
- Wang, C.H., Stern, I., Gilmour, C.M., Klumsoyr, S., Reed, D.J. Bialy, J.J. Christensen, B.E. & Cheldelin, V.H. (1958). Comparative study of glucose catabolism by the radiorespirometric method. J. Bacteriol. 76 : 207 - 216.
- Warburg, O. (1926). Über den Stoffwechsel der Tumoren. Springer, Berlin. (English translation by F. Dickens, 1930. Constable, Lond.)
- Wells, J.R. & James, T.W. (1972). Cell cycle analysis by culture fractionation. Expl. Cell Res. 75 : 465 - 474.
- Wiemken, A., Matile, P. & Moor, H. (1970). Vacuolar dynamics in synchronously budding yeast. Arch. Mikrobiol. 70 : 89 - 103.
- Williamson, D. H. & Scopes, A.W. (1962). Cell division and the synthesis of macromolecules in synchronously dividing cultures of Saccharomyces cerevisiae. Proc. Int. Union Physiol. Sci. 1 : 758
- Wilson, B.W. & James, T.W. (1966). Energetics and the synchronized cell cycle. In Cell Synchrony - Studies in biosynthetic regulation. pp. 236 - 255. Ed by I.L. Cameron & G.M. Padilla. Academic Press ; New York & Lond.
- Wohlhueter, R.M. & Harper, A.E. (1970). Coinduction of rat liver branched chain - ketoacid dehydrogenase activities. J. biol. Chem. 245 : 2391 - 2401.
- Yotsuyanagi, Y. (1962). Études sur le chondriome de la levure. 1. Variation de l'ultrastructure du chondriome au cours du cycle de la croissance aérobie. J. Ultrastruct. Res. 7 : 121 - 140.
- Zeuthen, E. (1946). Oxygen uptake during mitosis. Experiments on the eggs of the frog Rana platyrrhina. C.r. Trav. Lab. Carlsberg 25 : 191 - 228.

Zeuthen, E. (1953). Growth as related to the cell cycle in single -
cell cultures of Tetrahymena pyriformis. J. Embryol. exp. Morph.
1 : 239 - 249.

Zeuthen, E. (1955). Mitotic respiratory rhythms in single eggs of
Psammechinus miliaris and of Ciona intestinalis.
Biol. Bull. mar. Biol. Lab. Woods Hole. 108 : 366 - 385.

APPENDIX 1.

Edinburgh Minimal Medium. (EMM 2)

EMM 2 contains in 1 litre distilled water :-

Carbon source	glucose	10g
Nitrogen source.	NH_4Cl	5g
Phosphate source.	NaH_2PO_4	0.3g

SALTS.

buffer -	Sodium acetate	1g
	KCl	1g
	MgCl_2	0.5g
	Na_2SO_4	0.01g
	CaCl_2	0.01g

VITAMINS

Inositol	10mg
Nicotinic Acid	10mg
Calcium pantothenate	1mg
Biotin	0.01mg

TRACE ELEMENTS

H_3BO_3	0.5mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.4mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.4mg
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.2mg
$\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$	0.16mg
KI	0.1mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.04mg
Citric Acid.	1mg

Salts were stored in separate solutions at 20X strength; vitamins & trace were kept at 1000X strength. Stock solutions were kept at 4°C with a few drops of a preservative (2 pts chlorobutane, 1 pt chlorobenzene 1 pt dichloroethane). This preservative was boiled off before use.