THE POOL IN RELATION TO PROTEIN SYNTHESIS, WITH SPECIAL REFERENCE TO *SCHIZOSACCHAROMYCES POMBE*

by

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Chapter 1

INTRODUCTION

Cells growing in a defined (minimal) medium containing only glucose, an inorganic nitrogen source and inorganic salts normally only produce amounts of precursors sufficient for the synthesis of their constituent macromolecules. That is, the precursors do not normally accumulate during growth of the culture. Moreover, if the cells are grown at a different rate under different nutrient conditions or temperature, this phenomenon is still observed, although the relative amount of different macromolecular components in the cell may have altered (Schaechter *et al.*, 1958; reviewed by Kjeldgaard, 1967), and the relative amounts of individual enzymes may have changed (Zabin, 1963). Such observations, alone or taken together with the known mechanisms of controlling the production of precursors by altering the synthesis and activity of enzymes, have been taken to indicate that cells control the production of precursors according to their requirements for synthesis of macromolecules (Kjeldgaard, 1967; Tristram, 1968; Engelberg, 1968). Consideration of whether such observations in fact imply the existence of control systems in cells is deferred to the discussion (Chapter 10).

In bacteria, if the medium is supplemented by a precursor, such as an amino acid, the synthesis of that amino acid may be

restricted so allowing utilization of the exogenously supplied material (Roberts et al., 1955). In a mutant blocked at one step in a biochemical pathway the intermediate immediately before the block may accumulate, but may not do so if the final product is supplied at a high concentration. The latter case is an example of 'end product inhibition' in which the activity of the first enzyme of the pathway is inhibited by the end-product of the pathway, which will in general be structurally unrelated to that enzyme's substrate (Umbarger, 1961). The former example is one of 'enzyme repression' in which generally the rate of synthesis of all the enzymes of the pathway is reduced. 'Enzyme induction' is an increase in the rate of synthesis of an enzyme, such as β-galactosidase, initiated by the addition of a new catabolite to the medium, in this case lactose or other structurally similar β-galactosides, and is generally accompanied by co-ordinate increases in other functionally related enzymes, in the present case those involved in the uptake of the catabolite. While early work (Umbarger, 1956) indicated end product inhibition in growing cells, this phenomenon has been studied largely in in vitro situations. Enzyme repression and induction have been investigated in detail only in systems where the addition of an external metabolite produces an effect on the level of enzymes detectable in the cells. In both these types of control it is a low molecular weight metabolite which initiates alteration in enzyme activity or synthesis.

Detailed hypotheses for both types of mechanisms have been put forward in recent years, and there is now much experimental evidence supporting these hypotheses. However, are these proposed mechanisms of control by low molecular weight metabolites those used by the cell for regulating its activities when growing in the presence of a single carbon source and in the absence of intermediary metabolites? We are not concerned here with what controls the overall rate at which proteins are synthesised. Even if that were determined there would still remain the problem of controlling the production of precursors to the particular requirements of growth. Clearly, limitations of essential components of the mechanism of protein synthesis could control the rate of protein synthesis generally. These factors have been considered and reviewed by Cohen (1966).

If we are to understand how the activity and synthesis of enzymes may be controlled by endogenous metabolites we must consider the proposed mechanisms for these controls in relation to the location of the control sites within the cell. The mechanism of protein synthesis as now understood was itself based on the observations of enzyme induction and repression which led Jacob and Monod to propose their 'repressor-operator' model for such controls of protein synthesis. A number of modifications and alterations have been developed and these have been reviewed many times, for example by Cohen (1966), and more recently by Richmond (1968) and Epstein and Beckwith

(1968). On this model the genome contains units (operons) of contiguous structural genes for functionally related enzymes, for example the genes specifying the enzymes of the histidine pathway in Salmonella typhimurium. Expression of the operon is under the control of another locus, the 'regulator' gene which specifies a cytoplasmic 'repressor' molecule. This repressor has steric specificity for a site (the 'operator' gene) which is near or part of the structural gene for the first enzyme in the operon and prevents the transcription of the structural gene for the enzyme. The repressor has another site which in inducible systems interacts with the inducer. This interaction with the inducer prevents the repressor from combining with the operator so allowing transcription of the operon. In repressible systems interaction with the operator only occurs if the second site has already interacted with the metabolite. The low molecular weight compounds causing induction or repression may be termed 'co-repressors'. On release of repression the levels of all the enzymes in the histidine operon of S. typhimurium rise to the same extent. This phenomenon has been termed 'co-ordinate repression' (Ames and Garry, 1959).

The Jacob-Monod hypothesis allows for control at the level of transcription (production of messenger RNA) or translation (synthesis of protein on the ribosome) during protein synthesis. Jacob and Monod (1961a) favoured control at the transcriptional level, but translational control models have been proposed

(Stent, 1964; Cline and Bock, 1966). On Cline and Bock's model structural alterations of partly formed polypeptides may be induced by interaction with the repressor or even directly with the co-repressor so as to prevent further translation of a polycistronic messenger (Cline and Bock, 1966). Structural genes for enzymes subject to both feed back inhibition and repression are frequently operator-proximal (Ames and Martin, 1964). Hence enzyme control by both types of mechanism may be due to the interaction between the enzyme and the end product of the pathway. Translational control mechanisms also allow alterations in the amount of enzyme in situations where the messenger RNA is stable. On Stent's model ribosomes on a messenger are supposed to release RNA from the DNA template as a consequence of peptide bond formation and translational movement of ribosomes along the messenger. There is therefore an obligate coupling between translation and transcription so that reduction in the overall rate of translation in a cell should result in a decrease in the overall rate of transcription. But this does not appear to be the case in E. coli (Forchhammer and Kjeldgaard, 1968).

Harris (1968) has reviewed the original evidence for the transcriptional model and concludes that later work lends greater support for translational control in eukaryotic cells and in bacteria. A protein has been isolated from E. coli inducible for β-galactosidase which binds with an inducer, isopropylthiogalactoside (Gilbert and Muller-Hill, 1966).

This protein was absent from non-inducible strains and has therefore been identified as the repressor. Moreover, this protein binds specifically with the DNA of the lac operon, and binding in this way is prevented by isopropylthiogalactoside (Gilbert and Muller-Hill, 1967). This provides strong support for transcriptional control of β-galactosidase in this case, but it remains to be shown that the repressor bound in this way does result in decreased production of the enzyme. A number of observations seem to favour translational control in bacteria, but the evidence is equivocal. For example, the enzymes of the lactose system in E. coli form an operon, but the enzymes are produced in different molar ratios (Zabin, 1963). They should be produced in equal molar amounts according to the Jacob-Monod hypothesis. In 'polarity' mutants, a mutation decreasing the production of an enzyme in an operon also decreases production of all the other operator-distal enzymes (Jacob and Monod, 1961b; Ames and Hartman, 1963). If the entire operon is transcribed in the form of one polycistronic messenger and this is translated on the ribosome from the operator end, then a mutation in one of the genes may cause the messenger to dissociate from the ribosome at the mutant codon and hence result in the phenomenon of polarity. Mutant codons of this type between cistrons of the same operon could also account for non-equimolar production of enzymes in the same operon. The operator-negative (O^c) mutants of Jacob and Monod (1961a) now appear not to have a mutation in the

operator locus, but in the first structural gene of the operon (Beckwith, 1964). They may therefore represent extreme cases of polar mutants. Operator-negative mutants of the lactose system in E. coli do not produce any lac messenger RNA (Attardi et al., 1963). The primary effect in polar mutants would therefore seem to be at the transcriptional level.

Although the 'operator-repressor' model of Jacob and Monod was originally proposed for inducible and repressible enzymes in bacteria, there is no evidence that this model should not hold for the synthesis of proteins even by higher organisms. In fact, messenger RNA for haemoglobin has recently been isolated and shown to synthesize in vitro a protein which produces the same chromatographic pattern of peptides as haemoglobin (Laycock and Hunt, 1969).

The reaction catalysed by enzymes thought to be involved in feed-back inhibition does not generally show first order Michaelis type kinetics, but typically the plot of reaction velocity versus substrate concentration is sigmoid. Similarly inhibition of enzyme activity does not show first order kinetics with variation in inhibitor concentration. This means that the enzyme is relatively insensitive to levels of substrate or inhibitor below a threshold level, but above this there is a range in which it is very much more sensitive. Since such enzymes may lose their sensitivity to inhibitors after certain treatments but still retain their catalytic activity, the inhibitor and substrate are thought to bind at

different sites to the enzyme (Monod *et al.*, 1963). Moreover, since the inhibitor need not be sterically related to the substrate, its interaction with the enzyme has been termed allosteric and such feed-back inhibition may therefore be called 'allosteric inhibition'. This subject has been reviewed by Atkinson (1966), Stadtman (1966) and more recently by Koshland and Neet (1968).

Where no distinction need be made between the co-repressors of enzyme synthesis and end-product inhibitors of enzyme activity we may refer to these low molecular compounds as 'controlling metabolites'.

In general induction is a phenomenon of catabolic pathways, while repression occurs in biosynthetic pathways. Induction is generally by means of the substrate of an enzyme, while repression is by means of the end-product of a pathway and affects the first enzyme of the pathway. The synthesis of amino acids has been widely shown to be controlled by the end-product amino acids which are precursors of proteins (reviewed by Tristram, 1968). Inducible enzymes are also often subject to regulation by other means, the synthesis of many inducible enzymes being repressed by glucose: the so-called 'glucose effect'. It has been shown that the mechanism of this repression may be as specific as end-product repression (McFall and Mandelstam, 1963) and the term 'catabolite repression' (Magasanik, 1961) has been proposed to describe the phenomenon. In general the end-products of catabolic pathways are not

obvious since the products seem to enter a pool of readily interconverted compounds. This may be the reason why specific controlling metabolites have not been found in many cases. The mechanism of control by catabolite repression has been assumed to be by genetic repression of the Jacob-Monod type. But there is as yet no clear evidence that catabolite repression is under genetic control (Loomis and Magasanik, 1964; see also review by Maas and McFall, 1964).

THE CONCEPT, SIGNIFICANCE AND FUNCTIONS OF THE POOL

If enzyme synthesis and activity are controlled by the mechanisms of induction, repression and end-product inhibition, then the 'pool' in the cell must assume considerable significance. We must, therefore, consider what is meant by the term 'pool', its possible functions and its organization within the cell. This thesis is largely restricted to consideration of the amino acids of the pool. The amino acids are of particular interest in view of the many studies on the involvement of amino acids in the regulation of biosynthetic pathways (reviewed by Stadtman, 1966; Richmond, 1968; Tristram, 1968). Aspartic acid, glycine and serine are also precursors of purine and pyrimidine synthesis and enzymes of these pathways are known to be affected by the amino acid precursors. Moreover, there is a considerable literature implicating amino acids in the control of RNA synthesis (reviewed by Kjelgaard, 1967). The essential functions of the protein amino acids in the cell

would appear to be acting as precursors of protein and RNA. Many more amino acids are now known, and the functions in intermediary metabolism of a large number are understood (Meister, 1964). Amino acids may act as respiratory substrates, but in organisms which grow in minimal media this must be limited. Pool amino acids may also be important in the osmoregulation of the cell, as has been suggested for bacteria (Kuczynski-Halmann et al., 1958).

In concept, the pool is generally taken as the low molecular weight compounds in the cell. The concept is essentially a simple physical one. The difficulty of employing such a concept as a definition of the pool lies in the manner in which so-called pool material is generally obtained. The basis of methods widely used are chemical and depend, for example, on ready solubility of pool material or precipitability of 'non-pool' material with acids such as 5% trichloro-acetic acid (TCA) or 5% perchloric acid (PCA). When acid extraction methods have been used the pool obtained has often been more explicitly termed the 'acid-soluble' or 'acid-extractable' pool. Consideration of cell ultrastructure indicates that chemical extraction methods must have rather complex effects on cell structure. Chemical treatment will in general disrupt cellular organization and release low molecular weight compounds. But what membranes of the cell are broken to release the low molecular weight materials within and what happens to the contents of membrane bound structures within the

cell such as mitochondria and the nucleus? Britten and McClure (1962) have proposed a definition of the pool which compromises the concept with the problems of extraction. Their definition is 'the total quantity of low molecular weight compounds that may be extracted under conditions such that the macromolecules are not degraded into low molecular weight sub-units'. Nevertheless, few workers have attempted to ascertain whether all the low molecular weight compounds have been removed from the material, or ascertained that macromolecules are not degraded. In cases where repeated extractions have been used it cannot be assumed that all the low molecular weight compounds have been removed, since another solvent may extract other compounds. Repeated extractions with different solvents could be necessary, but do not seem to have been attempted. Mechanisms of extracting pool material would seem to involve firstly some disruption of the membrane systems of the cell, and subsequently the solubilization of the low molecular weight compounds within. Both these stages have commonly been performed by a single solvent under one set of physical conditions. It does not, however, seem unreasonable to suppose that optimum conditions for the rupture of cell organelles should be different from those most suitable for complete removal of the pool from the cell debris.

In the yeast Candida utilis, two amino acid pools can be distinguished (Cowie and Walton, 1956; Cowie and McClure, 1959). The 'internal' pool is of fixed size, is maintained by

the cells when grown on minimal medium and is the immediate source of protein precursors. Amino acids in this pool are not sensitive to osmotic shock and do not exchange with exogenous amino acids. The 'expandable' or 'concentrating' pool is only formed during growth in media supplemented by amino acids. Amino acids in this pool are sensitive to osmotic shock and exchange rapidly with exogenous amino acids. Amino acids in the expandable pool cannot be utilized directly for protein synthesis, but have to pass into the internal pool. Interconversion of amino acids from 'parent' amino acids (glutamate, aspartate, and alanine) occurs only in the internal pool (Cowie and Walton, 1956; Cowie and McClure, 1959). The two pools can be extracted separately: the expandable pool alone is extracted with cold water, while cold trichloroacetic acid extracts both pools (Kempner and Cowie, 1960). A similar two-pool system for amino acids has also been found in Neurospora (Zalokar, 1961).

ULTRASTRUCTURE AND COMPARTMENTATION IN THE CELL

Consideration of endogenous control of enzyme synthesis and activity requires knowledge of the intracellular distribution of the enzymes and their controlling metabolites.

We may now consider what is known of the ultrastructure of S. pombe and other yeasts and the heterogeneity imposed by membrane-bound organelles on the distribution of enzymes and

their substrates. Maclean (1964) has demonstrated the presence of a plasmalemma just inside the cell wall of S. pombe and shown that it is in the form of a double membrane. The cytoplasm is packed with ribosomes, but a membranous system was evident with some regions forming closed systems designated 'vesicles'. There are also clear areas or 'holes' which were identified as lipid storage granules but labelled 'vacuoles'. A nucleus with a double nuclear membrane was also seen, but no mitochondria were discernable. The poor resolution within the cytoplasm seemed to be due to the presence of large numbers of ribosomes and difficulties in staining. These problems were largely overcome by Schmitter and Barker (1966) who employed RNase and snail gut enzyme treatment prior to fixation. These methods revealed the presence of definite mitochondria with cristae in exponential phase cells. In addition to the 'vacuoles' found by Maclean, sections showing mitochondria also revealed vesicles which contained some stained material and these were labelled 'granules' by Schmitter and Barker (1966).

The membranous structures of yeast have recently been reviewed by Marchant and Smith (1968). It is clear that yeasts generally contain vacuoles of the plant cell type which lack any definite content and are bounded by a single membrane: the tonoplast. These are classed as 'vacuoles' by Marchant and Smith. In addition generally there are also lipid filled storage granules and mitochondria. While some of the membrane-bound vesicular structures found by Maclean may be mitochondria,

as found by Schmitter and Barker, others may be equivalent to the 'granules' of Schmitter and Barker. We may conclude that liquid filled vacuoles of the plant cell type may occur in S. pombe. But generally these must be small though possibly numerous and not large and few in number as generally is the case in Saccharomyces (Marchant and Smith, 1968). The terms 'vacuole' and 'lipid storage vesicle' will be used in the present text in the sense of Marchant and Smith. We may, therefore, identify Maclean's 'vacuoles' as lipid storage vesicles and, tentatively, Schmitter and Barker's 'granules' as vacuoles, although some of Maclean's 'vesicles' may also fall into this category.

The structural heterogeneity of the cell imposes heterogeneities on the distribution of enzymes and their substrates which we must now consider in view of the simplicity of the pool concept. Relatively little work on enzyme cytology has been done on yeasts and in part this can be attributed to the difficulties in homogenising the cells without severely damaging cell organelles. We must therefore refer largely to work on organelles from other systems, much of which has recently been reviewed in book form (Enzyme Cytology, edited by D.B. Roodyn, Academic Press, 1967). The isolated preparations most frequently studied are those obtained by differential centrifugation of homogenates and we may consider the four basic fractions: nuclear, mitochondrial, microsomal and soluble, where soluble refers to the supernatant remaining after

sedimentation of all particulate material (Anderson and Green, 1967).

The degree of dependence of the many nuclear energy consuming reactions on the supply of ATP from the cytoplasm is uncertain. Certainly isolated nuclei can synthesize nucleoside triphosphates from nucleoside monophosphates (Osawa et al., 1957; McEwen et al., 1963a). The nucleus in fact appears to contain many of the enzymes and intermediates of nucleotide metabolism, some of them exclusively (Georgiev, 1967). Moreover, nuclei isolated in non-aqueous media contain some of the tricarboxylic acid cycle enzymes and intermediates and they can also catalyse catabolism via the hexose monophosphate shunt (McEwen et al., 1963b). The isolated nuclei are also able to incorporate amino acids into proteins on nuclear ribosomes in a manner that appears to be essentially similar to that of cytoplasmic protein synthesis (reviewed by Georgiev, 1967). Nuclei isolated in non-aqueous media are also known to catalyse the entire glycolytic sequence of reactions and to contain the low molecular weight metabolites of the pathway (McEwen et al., 1963b). Moreover, these enzymes appear to be in the nuclear sap, in the same relative proportions and in the same amounts on a total nitrogen basis as in the soluble phase of the cell (Roodyn, 1956). It has therefore been suggested (Siebert, 1961) that the nuclear sap is a continuation of the soluble phase of the cell into the nucleus. However, this evidence is merely for functional and structural similarity between the

nuclear sap and the soluble phase, not for actual continuity.

More conclusive evidence for continuity between the nuclear sap and the soluble phase of the cell should be available from studies on the permeability of nuclei. Unfortunately, studies of this nature are anomalous. For example, calf thymus nuclei retain acid-soluble (2% PCA) nucleotides on aqueous extraction (0.25M sucrose/0.003M calcium chloride) even though large amounts of nuclear proteins and nucleic acids may be lost (Osawa *et al.*, 1957). Release of the nuclear nucleotides and potassium ions may be achieved by addition of acetate to the aqueous nuclei but not by di- or tricarboxylic acids (the 'acetate effect', Osawa *et al.*, 1957). This has been explained by the observation that monocarboxylic acids will enter the acid-soluble pool of nuclei but di- and tricarboxylic acids will not (McEwen *et al.*, 1963c). But entry into the pool and release of pool material may be different processes. These results could be interpreted as indicating that the state of nucleotides within the nuclear pool is other than that of free solution.

The uptake of some nucleosides and amino acids appears to be sodium dependent and in the case of amino acids to be mediated by L-isomer specific permeases and to be dependent on energy from nuclear ATP synthesis (Allfrey *et al.*, 1961). Protein and nucleic acid synthesis are also sodium dependent and sodium has been considered to be important in controlling nuclear metabolism *in vivo* since many cells have high intra-

nuclear sodium concentrations. This sodium dependence of nuclei contrasts with the potassium dependence of cytoplasmic particles (Allfrey *et al.*, 1961) and may indicate a degree of separation between the nucleus and the soluble phase. Nuclei could obtain sodium and other compounds directly from the medium since infoldings of the plasmalemma extend to the nucleus in some cells (Palade, 1955). Many of these permeability studies seem at odds with the fact that nuclei appear to contain pores and *in vivo* must readily allow the passage of large molecules, such as RNA precursors of ribosomes, to the cytoplasm (Haguenau, 1958). The pores could, however, merely be depressed areas in the nuclear membrane.

Treatment with DNase has a marked effect on ATP synthesis and glycolysis in isolated nuclei: there is a great reduction in the activity of these pathways in contrast to isolated mitochondria which are not affected by DNase (McEwen *et al.*, 1963a). DNase treatment also reduces the tricarboxylic acid cycle but does not affect the hexose monophosphate shunt (McEwen *et al.*, 1963c). Addition of polyanions such as DNA, RNA or polyethylene sulphonate restores the activity lost on DNase treatment (McEwen *et al.*, 1963a, c). An explanation of this effect was that removal of DNA unmasks histones and these are known to inhibit nuclear metabolism. Added polyanions would probably combine with the histones and thereby make them ineffective as inhibitors (McEwen *et al.*, 1963a). But if this is the case why is the hexose monophosphate shunt not affected on DNase

treatment? These results could be interpreted as indicating a difference in the organization within the nuclear sap of the hexose monophosphate pathway. However, the possibility of more or less specific inhibition of enzyme activity by nuclear histones cannot be ignored.

The complex of enzymes and cofactors required for the tricarboxylic acid cycle (Krebs and Lowenstein, 1960) is present in functional form in mitochondria since in isolation they will oxidise pyruvate and cycle intermediates (Schneider and Hogeboom, 1956). However, many of the enzymes and some intermediates occur in the soluble fraction and, as we have seen, in the nucleus. The transaminases are also located in mitochondria, but again activity is also found in the soluble fraction. While some of these enzymes occur in both the mitochondrial and soluble fractions, it seems certain that the functional tricarboxylic acid cycle is in the mitochondria, for in general some key enzymes such as succinate dehydrogenase seem to be associated solely with mitochondria (Roodyn, 1967).

The mitochondrion is itself a spatially subdivided organelle, and it now seems that there are two fluid spaces: the 'inner' and 'outer' compartments which have been identified as the space between the two membranes and the inner matrix of the mitochondrion respectively (Klingenberg and Pfaff, 1966). These authors have also isolated many low molecular weight compounds from mitochondria, including nucleotides and free amino acids. 'Soluble' enzymes have been isolated and

assigned to one or other of the two spaces. However, the release of 'soluble' enzymes depends on their extractability in various media, and may not reflect their solubility in the fluids of the mitochondria. A more empirical classification according to ease of extraction has been proposed (Klingenberg and Pfaff, 1966). However, it seems that of the enzymes of the tricarboxylic acid cycle and amino acid biosynthetic pathways only succinate dehydrogenase and enzymes concerned with transport of metabolites are structurally bound to the mitochondrial membranes. The membranes also contain the enzyme of the electron transport system.

It is now becoming clear that in many cases where enzymes show a dual distribution in mitochondria and in the soluble fraction, the two enzymes are different proteins (isozymes). For example, glutamate dehydrogenase, a key enzyme in the formation of amino acids from tricarboxylic acid cycle intermediates, occurs in both fractions in yeast, but the two are isozymes (Stachow and Sanwal, 1964). Such partitioning of metabolic intermediates and isozymes will be of the utmost importance in considering the control of enzyme synthesis by metabolites, for it could allow control of enzymes in key locations without a detectable change in the overall level of the metabolite in the cell. In view of the degree of compartmentation in the cell, the significance of the existence of isozymes in the separate compartments is somewhat obscure. Morino et al. (1964) have shown that there are greater

immunological similarities in glutamic-oxaloacetic transaminases between the mitochondrial enzymes from different tissues of different mammalian species than between the enzymes in the mitochondrial and soluble fractions of the same tissue. This repetition of functionally similar, but structurally dissimilar, enzymes may result from the supposed evolutionary origin of mitochondria as symbionts.

A number of functions of yeast vacuoles have been deduced from their behaviour in intact cells without definite conclusions (reviewed by McClary, 1964). Recently Matile and Wiemken (1967) have isolated the prominent vacuole of Saccharomyces cerevisiae and concluded that it is the lysosome of the cell. Intact vacuoles were obtained by flotation from suspensions of lysed protoplasts in the presence of Ficoll. The isolated organelles were free of mitochondrial and soluble phase enzymes and surface features in electronmicrographs appeared identical to those in intact cells. The vacuolar sap was found to contain four hydrolytic enzymes, two acid endopeptidases, p-nitrophenylacetate-esterase and RNase, whose activity was 20-30 times higher than in the lysed protoplasts. Apart from a leucylaminopeptidase in the space between the plasmalemma and the cell wall (see Matile and Wiemken, 1967), no other structures in the cell were considered to contain hydrolytic enzymes. A distinctive layer between the cell wall and plasmalemma has been found in S. pombe (Maclean, 1964) and may be the location of some enzymes as suggested by Mitchison

and Creanor (1969).

Without doubt the soluble phase of the cell is complex and some form of compartmentation can exist (Anderson and Green, 1967). The existence of multi-enzyme complexes (Reed and Cox, 1966) would seem to effectively allow channelling of compounds along specific enzymatic pathways without intermediates becoming free in the cell. But the effectiveness of such channelling is not clear, for in some cases the loss of an intermediate in one pathway can in part be made up by supply from another pathway (Cotton and Gibson, 1965).

ENDOGENOUS REGULATION IN CELLS GROWING IN MINIMAL MEDIUM

There are few studies of control of enzyme synthesis and activity which demonstrate conclusively that endogenous levels of metabolites act as co-repressors or inhibitors of enzyme activity. Several studies indicate that this may be so, but some are quite inconclusive. For example, mutants of Salmonella typhimurium showing an alteration in the structure of the first enzyme of the histidine pathway rendering it non-susceptible to end-product inhibition still contain normal levels of the enzymes of the histidine pathway (Loper et al., 1964). These mutants excrete histidine. Hence it would appear that, in vivo, repression is insufficient to control histidine production and that end-product inhibition is of great importance. However, as mentioned above, this phenomenon is explicable as a single mutation effect on

translational control models (Cline and Bock, 1966).

It is a general phenomenon that only certain reactions in a metabolic sequence are involved in control either by feed-back inhibition or repression. The determination of the maximal catalytic capacities of the isolated enzymes will indicate potentially limiting enzymes. Similarly the detection of irreversible reactions under simulated *in vivo* conditions may indicate a control point in a sequence. However, it has to be shown that these possible regulation sites do in fact become rate limiting to the overall sequence *in vivo*. The enzyme with lowest catalytic capacity need not be the one under regulation in the cell.

Two methods have been used for the detection of control points and analysis of the mechanism of control in metabolic pathways *in vivo*. One uses the crossover theorem of Chance *et al.* (1958) and involves the determination of changes in the level of intermediates of the pathway during induced transitions from one steady state to another. The other method involves a comparison of the level of intermediates in the cell with those predicted from the equilibrium constants determined from the isolated enzymes of the pathway. This latter method is reviewed by Newsholme and Gevers (1967) and has been applied to studies of regulatory mechanisms in glycolysis of animal tissues. The former method has been extensively employed in investigations of glycolysis in yeasts. A transition from aerobic to anaerobic growth of intact cells

causes a train of damped sinusoidal oscillations in the level of reduced pyridine nucleotide in an inositol-requiring strain of Saccharomyces carlsbergensis. The oscillations are about the new steady state level. The glycolytic intermediates were also assayed and were also found to oscillate with a crossover point between glucose-6-phosphate and fructose-di-phosphate (Betz and Chance, 1964a, b). Similar results have been found in other yeasts by Hommes (1964a, b). Control points are difficult to establish in intact cells since experimental alterations in the level of many intermediates cannot be made. However, cell-free extracts of the glycolytic sequence have been studied (Chance et al., 1965). By particular attention to phase relationships of the oscillations in the concentration of the various intermediates and the effects of additional substrates and inhibitors the control mechanisms have been more fully elucidated. However, the oscillations of cell-free preparations are about twenty times slower than in vivo. But this may be attributable to the lower enzyme concentrations employed in the in vitro studies compared with the concentrations of the enzymes in the intact cells. Neither of these methods have been extensively used for pathways other than glycolysis and there are limitations to both methods. The equilibrium-constants method necessitates isolation of the enzymes and thus may alter the properties of the enzymes in their relation to the in vivo controlling mechanisms. The crossover theorem was proposed for location of the sites of

interaction of inhibitors with carriers of the electron transport chain where the concentrations of intermediates are fixed. Modification of the theorem for an enzyme sequence in which fluctuations of intermediates must occur if control exists necessitates some restrictive approximations (Chance et al., 1965). It should be noted that both these methods have assumed a simple pool for the intermediates examined. Nevertheless, studies of glycolysis by determinations of likely rate limiting enzymes and the two methods considered above do agree to a first approximation (reviewed by Scrutton and Utter, 1968). For glycolysis controls at the level of enzyme activity seem of paramount importance. Moreover, similar control of glycolysis is found in mature mammalian erythrocytes (reviewed by Rapaport, 1968) in which control by enzyme synthesis is not possible due to the absence of a nucleus.

Some studies have strongly indicated endogenous control in bacteria. Gorini (1958) has shown that for E. coli grown in limiting glucose supplemented by low levels of arginine there is no change in the amount of ornithine transcarbamylase (OTCase) assayable in extracts. In this case the production of endogenous arginine is limited by feed-back inhibition to complement exactly the arginine from the medium. But at high arginine concentrations, above those required for protein synthesis, the amounts of OTCase is reduced, so under these circumstances repression occurs. Gorini ~~and Maas~~ did not

eliminate the possibility that release from repression is contingent upon exogenously supplied arginine, or that this was a feature of the mutants used. Novick and Maas (1961) have attempted to demonstrate control by endogenously formed arginine in two ways. Firstly, in a "leaky" mutant in which the growth rate is limited by arginine, OTCase formation is 25 times higher than in the wild type. Secondly, in the wild type, limiting the endogenous production of arginine (by growth in an arginine-free medium) considerably increased the formation of OTCase. More recently Karlstrom and Gorini (1969) have shown that in an arginine auxotroph of E. coli strain K growing in a chemostat limited by arginine, a wide range of arginine pool sizes can be obtained. At long generation times the pool is low and the level of OTCase is high and may be higher than that for cells grown exponentially in minimal medium. Thus under normal conditions of growth OTCase is partially repressed.

In Aerobacter aerogenes grown on succinate as the carbon source, the enzyme histidine ammonia-lyase responsible for degradation of histidine, is induced by its product, urocanate (Schlesinger et al., 1965). Induction occurs more readily on addition of histidine than addition of urocanate to the medium. But mutants deficient in histidine ammonia-lyase respond less well to addition of histidine while induction by addition of urocanate is as strong as in the wild type. However, histidine enters the cells much more rapidly than urocanate so that

in wild type cells exogenous histidine is more readily converted to the intracellular inducer, urocanate, than in the mutant.

The most convincing evidence for the operation of end-product inhibition and control of enzyme synthesis by repression in eukaryotes is that of Donachie (1964a, b) for Neurospora crassa. In a pyrimidine auxotroph in which the block was after the enzyme aspartic transcarbamylase (ATCase), carbamyl aspartic acid (the product) accumulated only after exhaustion of exogenously supplied uridine. No carbamyl aspartic acid could be found intracellularly or in the medium when the mutant was grown in the presence of uridine, so end-product inhibition does occur. Moreover, in vitro, ATCase activity was inhibited by uridine derivatives. In the presence of exogenous pyrimidines, ATCase formation in the wild type was only slightly repressed. Removal of uridine from the medium of the auxotroph led to a rise of two to threefold in ATCase even when 20% growth was maintained by supplementing the medium with a poor pyrimidine source (dihydrouracil). In the wild type, ATCase must therefore be almost fully repressed (Donachie, 1964a). Heterokaryons containing different proportions of nuclei with and without an ATCase gene were prepared. Under conditions of full repression in heterokaryons ATCase activity was found to be directly proportional to the fraction of nuclei with a functional ATCase gene, i.e. proportional to gene concentration. But in heterokaryons growing in

unsupplemented minimal medium the amount of ATCase was not proportional to gene dose: reducing the gene dose to 50% of the maximum did not appreciably lower the amount of ATCase, but at lower gene doses the amount of ATCase was proportionally reduced. This indicates that lowering the ATCase results in immediate derepression so that ATCase is rate limiting for pyrimidine synthesis, and also that in the wild type just enough pyrimidine is made to ensure maximum repression (Donachie, 1964b).

There can be no doubt that regulation of transcription does occur in higher cells, but the mechanisms are not known. Clear examples are inactivation of one of the sex chromosomes in female mice (Evans et al., 1965) and the masking effects of histones to the action of RNA polymerase in various tissues (reviewed by Bonner et al., 1968). Changes in the pattern of protein synthesis which occur during embryonic development in frogs and sea urchins are accompanied by increases in template activity of the isolated chromatin. But these cases involve divisions of cells so that reorganisation of the histones may be occurring during the synthesis of new genomes. That histones act as repressors of gene activity in the manner of bacterial repressors seems unlikely since the binding of histones to DNA is strong and relatively non-specific. Bonner et al. (1968) maintain that specificity could be achieved by means of the RNA always associated with chromatin. However, the repressors must also specifically interact with the

co-repressor molecules. It has been reported (Dukes et al., 1966) that addition of cortisone to isolated liver nuclei from adrenalectomised rats does cause an increase in the rate of RNA synthesis, just as in intact animals. This does not occur in isolated chromatin and so may be mediated by some substance in the nucleoplasm. It has not been shown, however, that these cases of increased template activity involve the unmasking of genetic sites previously unavailable for transcription by RNA polymerase. They merely show alterations in the rate of transcription. Moreover, the RNA synthesised has never been shown to be biologically meaningful. Also, cases where increased template activity has been demonstrated have all involved incubation of the chromatin with added, usually bacterial, polymerase. This may transcribe sites unavailable for transcription by the RNA polymerase attached to the chromatin. Histones, which have been found in yeasts (Tonino and Rozijn, 1966), could play a similar role to that in other eukaryotic cells. In rat liver and sea urchins changes during the cell cycle have been found in the phosphate and thiol content of histones (Ord and Stocken, 1968). But such changes are unlikely to allow the expression of different genetic sites at all times through the cell cycle, as occurs in yeasts (see below).

The very specific sequences of puffs found in dipteran salivary gland chromosomes between and during successive moults indicate that transcription can be highly regulated in

eukaryotes. In isolated salivary gland nuclei the normal sequences of puffing patterns, including the distinctive puffs occurring at the end of the last larval instar of Chironomus, can be artificially induced (reviewed by Kroeger and Lezzi, 1966). Different groups of inorganic ions induce similar puffing patterns which may even be reversed by reversal of the ionic conditions. An important control feature for inducing or reversing the normal sequence of puffs appears to be the Na/K ratio. The induction of any one puff seems largely independent of other chromosomal regions since the normal sequence of puffs occurs after removal of other segments of the chromosomes. Moulting in whole animals is normally controlled by the hormone ecdysone. In Sciara coprophilia, treatment with cortisone (an ecdysone analogue) suppresses puff formation, yet normal development continues unarrested. On removal of cortisone at any stage, normal puffing patterns resume (Goodman et al., 1967). Puffing is therefore not essential for development and may not be mediated by ionic balances within the nucleus; moreover numerous other treatments are known to induce puffing patterns (Kroeger and Lezzi, 1966).

Recent work on the synthesis of enzymes in synchronous cultures has provided important information on regulation in growing cells. The work on bacteria has been reviewed by Donachie and Masters (1969), and work on bacteria and eukaryotes by Mitchison (1969). For inducible and repressible enzymes there are two well defined levels of enzyme which can

be determined, namely the basal level and the fully induced level. It should be noted that these levels are meaningful only for given growth conditions and could differ with conditions of growth. These levels of activity can generally only be determined with certainty by addition of supplements to the medium and are not meaningful for enzymes whose activity is unaffected by such means. It is argued (Donachie and Masters, 1969) that the amount of an enzyme in bacteria will in general be somewhere between the basal level and fully induced level. This amount of enzyme, maintained by the cell, has meaning for all enzymes and has been termed the "autogenous" level (Kuempel et al., 1965; Donachie and Masters, 1969). Enzymes have been assayed on lyophilised cells over several cell cycles in synchronous cultures of bacteria and yeasts. These assays measure enzyme activity and by implication the amount of enzyme present, since under the conditions of assay inhibitors will have been diluted out and excess substrate is provided. The changes in enzyme activity through the cell cycle have generally been accepted as measures of enzyme amount (Donachie and Masters, 1969; Mitchison, 1969). The autogenous amounts of enzymes measured in this manner generally show a stepwise increase at a certain stage in the cell cycle, the step occurring at approximately the same time in successive cell cycles.

Such stepwise increases occur in S. pombe in ornithine and aspartic transcarbamylases (Bostock et al., 1966), in tryphophan

synthetase (Robinson, 1966), and in alcohol and homoserine dehydrogenase (Robinson, 1969). The timing of all these steps does not appear to be restricted to any region of the cell cycle and most occur outside the period of DNA synthesis, since the 'S' phase lasts only about 20 minutes and occurs at 0.85 in the cell cycle (Bostock et al., 1966; Bostock, 1968). Halvorson and his colleagues have obtained similar results for enzymes in synchronous cultures of species of Saccharomyces. These workers have suggested that genes are only transcribable for limited periods of the cell cycle, and the genes on each chromosome are transcribed in order so that doubling in the amount of an enzyme corresponds to the time at which the structural gene is transcribed (Halvorson et al., 1966). This model has been termed 'linear reading' by Mitchison (1969), and requires that the order of enzyme steps is the same as the order of the corresponding structural genes on the chromosomes. Further evidence has been obtained suggesting that the order of enzyme steps does correspond to the positions of their structural genes (Tauro et al., 1968). However, this model as it stands cannot hold for S. pombe since some enzymes (sucrase and phosphatases) increase continuously through the cell cycle, and sucrase may be induced at all times in the cell cycle (Mitchison and Creanor, 1969).

An alternative model, which may be termed 'oscillatory repression' (Mitchison, 1969) has been proposed to explain the stepwise synthesis of enzymes (Goodwin, 1966). This model is

based on the fact that in a negative feed-back circuit stable oscillations are developed in the product (co-repressor) and the component that is regulated (enzyme). These oscillations become entrained to the cell division cycle by some other factor, which Goodwin (1966) assumes to be a small burst of messenger synthesis at the time of gene doubling. In this way only one oscillation occurs per cell cycle. This model fits all the data for the bacterial systems (Goodwin, 1966; Donachie and Masters, 1969), and could account for stepwise synthesis of enzymes in S. pombe.

The conditions of feed-back inhibition in vivo of these step enzymes is unknown. However, end-product inhibition and repression generally both have the same effect: a reduction in enzyme activity when the controlling metabolite is at a high concentration. These control mechanisms, therefore, tend to enhance one another and so we would expect end-product inhibition, if it occurs over the same concentration ranges, to follow the same pattern as repression. There should therefore be a single fluctuation in the controlling metabolite, the concentration being highest during the plateau phases of the enzyme step, and lowest during the rapid increase in enzyme amount. On the Goodwin model this situation is somewhat modified (see discussion, chapter 10).

In conclusion, we may say that the known mechanisms of control of enzyme synthesis and activity do probably operate in

cells growing in minimal medium. Other control mechanisms occur, such as catabolite repression, the mechanism of which is uncertain but may be similar to end-product repression. Control of transcription by histones in exponentially growing cells seems unlikely, but if it occurs is unlikely to be mediated by low molecular weight metabolites such as amino acids. Compartmentation of the cell may result in different concentrations of metabolites in different parts of the cell. Since enzymes are distributed non-uniformly between the various organelles of the cell, control by feed-back inhibition may be quite localised in the cell, but nevertheless significant to the metabolism of the whole cell. Nothing is known of the endogenous location of repressors. But clearly control of enzyme synthesis by repression and induction will depend on the location in the cell of repressors and co-repressors and may be greatly complicated by compartmentation. A further complication is the existence of isozymes which are differentially susceptible to end-product inhibitions. Their synthesis may also be under separate control. Estimates of enzyme activity in the cell may therefore give an incorrect estimate of the degree of inhibition or repression. Moreover, the concentration of a controlling metabolite may not simply reflect the level at which it controls an enzyme since such a metabolite may have functions other than that of a precursor of macromolecules or control of a pathway. The permeability of intracellular membranes may be important since particular controlling

metabolites could be transported within the cell without overall changes in its amount within the cell. In synchronous cultures of S. pombe the stepwise increases in enzymes could reflect substantial fluctuations in controlling metabolites. Control of biosynthetic pathways by protein amino acids through feed-back inhibition and repression has been widely demonstrated in many systems. Amino acids have also been implicated in the control of RNA synthesis.

Previous work on S. pombe relevant to the present thesis is considered in greater detail in the subsequent chapters as appropriate.

Chapter 2

GENERAL METHODS

ORGANISM, MEDIA, GROWTH, AND HARVESTING OF CELLS

All experiments were done using the fission yeast Schizosaccharomyces pombe (No. 132, National Collection of Yeast Cultures, Nutfield) which has been continuously cultured in this laboratory since 1953. In the summer of 1967 the cells became abnormal in that many of them became branched during exponential growth, and a fresh culture was obtained from the National Collection of Yeast Cultures.

The characteristic morphological features of this cell have been described in detail before (Mitchison, 1957), but essentially the cell is a cylinder 6 to 15 microns (μ) in length with a constant diameter of 3.5 μ. It has hemispherical ends. The cell wall is relatively thick. The cell grows only in length, generally at one end (Johnson, 1968), and at 12 to 15 μ a central transverse cell plate is formed, which later splits, the two new surfaces rounding off so that two similar cells are produced. Characteristically, a fission-scar in the form of a slight collar of cell wall material may be seen near that end of the cell at which a cell plate was last formed.

Cells were always cultured in liquid medium at 32°C unless otherwise stated. The mean generation time under these conditions was about two hours and forty minutes, but varied

between two hours fifteen minutes and two hours and fifty minutes. Stock cultures were maintained in 10 mls. of medium in Universal containers. Fresh stock cultures were prepared every two to fifteen days by transfer to a new container using a tungsten wire loop. Normal sterile procedures were followed when preparing fresh stock cultures and when inoculating large volumes of medium.

A minimal medium was used which contained inorganic salts, some vitamins, trace-elements, ammonium chloride as the nitrogen source, and glucose as the carbon source. The exact composition of this medium and its preparation is given in Appendix I. During the course of this work most experiments were done using a medium with additional phosphate. This new medium with additional phosphate is termed Edinburgh Minimal Medium 2 (EMM2), while the earlier medium is termed Edinburgh Minimal Medium 1 (EMM1). Some features of growth are not altered by the additional phosphate and may be referred to as occurring during growth in Edinburgh Minimal Medium (EMM) without the addition of a suffix.

All medium was sterilised before use by autoclaving for 10 to 25 minutes at 10 lbs. per square inch. Large volumes of medium were preheated and autoclaved for only 10 minutes and removed as soon as possible from the autoclave, as they tended to turn slightly brown, presumably due to caramelization of the glucose.

Cultures were generally grown in Pyrex Ehrlenmyer flasks,

stirred by means of magnetic stirrers and kept stopped by cotton wool bungs. The extent to which growth is due to anaerobic metabolism under these conditions is not known. But an attempt was made to keep conditions similar in all experiments by having the same relative volume of liquid in flasks of different size. The quantities used were:

800-1000 mls. in 2 litre flasks
400- 500 " " 1 " "
200- 250 " " ½ " "

The exception to these conditions were the large cultures (10 litres) grown up for making synchronous cultures, in which the medium surface exposed to air was relatively small. Vigorous stirring was used in these cultures such that a vortex was maintained. Unless otherwise stated, exponential cultures were prepared by inoculating fresh medium with cells which had been in stationary phase from 1 to 4 days. The size of the inoculum was generally 1/100th of the volume inoculated.

Cells were harvested by pelleting them in tubes or buckets in a centrifuge, by continuous-flow centrifugation (Christ continuous flow centrifuge), or by filtration. Centrifugation was in a MSE bench centrifuge at 1,500 g, or MSE Major or Mistral 4 L at up to 2,000 g. Filtration of small aliquots of cells was through 2 cm. diameter filters supported in a stainless-steel filter holder. Rapid filtration was achieved by means of a vacuum pump. Filters used were Oxoid Membrane Filters, 2 cm. diameter, Millipore PVC filters, or Whatman

glass fibre filters (Whatman GF/A 2.1 cm. diameter). The millipore filters were made by cutting large sheets into squares 21 mm. by 21 mm. Larger quantities of cells were collected on Whatman No. 50 filter papers 15 cm. diameter supported in a Millipore PVC Filter Holder or on a similar apparatus made in this laboratory which used Whatman No. 50 filters 32 cm. in diameter. These larger filter holders were used with a simple water pump.

COUNTING OF CELLS

The 'cell cycle' may be defined as the time between the formation of a cell and its subsequent division into two cells. This time may be taken as unity and any event in the cycle can then be positioned in time by a fraction of unity. The time of division is taken as zero or one. Since daughter cells do not separate immediately after cell plate formation, estimation of when cell division occurs and hence estimation of cell numbers in cultures of S. pombe requires some criterion of when a dividing cell becomes two cells. Under phase contrast and dark ground illumination, cell plate formation is seen to be preceded by the formation of a thin non-refractive plate. This becomes refractive, like the rest of the cell wall, and progressively thickens. The cells may be considered to be physiologically separated when the cell plate first becomes visible (Swann, 1962). Cell plate formation is followed by fission of the cell into two by splitting of the cell plate.

This commences as a furrow around the outside edge of the cell plate and when complete the two daughters, although usually still apparently stuck together, are generally no longer aligned. Mitchison (1957) has taken fission as the time of cell division and used this as the limits (zero and unity) of the cell cycle. This criterion has been used throughout this work also since it is in practice easier to determine and a more precise event in the life of the cell. Fission is more precise since the time from its commencement (furrow formation) to completion (when daughters have rounded ends) is less than 14 minutes, which is approximately one tenth of the cell cycle. Cell plate formation, however, commences at about 0.85 in the cell cycle (Mitchison, 1957, for cells grown in Wort Broth at 25°C; Bostock, 1968, for cells grown in EMM at 32°C) and is taken to end when fission begins and therefore occupies about one sixth of the cell cycle. Fission is also easier to determine since its commencement is clearly indicated by formation of a furrow around the cell while detailed examination of each cell is necessary to determine the presence or absence of a cell plate, however thin.

Three methods were used for the estimation of cell numbers: direct counting of aliquots of the culture in a Neubauer haemocytometer, determination of the optical density at a wavelength of 595 millimicrons, and by means of a Coulter Counter, model B.

For good repeatable results from haemocytometer counts,

it was necessary to count at least two chambers which had been filled by drops from aliquots taken separately from the culture. Counts were made within 15 minutes, otherwise the sample was kept at 4°C and counted within 24 hours. Optical density determinations were made in 1 cm. light path cuvettes in a Unicam SP500 or SP600 spectrophotometer. The numbers of cells per ml. of culture was then determined by a calibration curve of optical density at 595 millimicrons against haemocytometer counts obtained from growing cultures. Coulter counts were determined on aliquots diluted with 1.0% saline containing 2.0% formalin. This electrolyte was filtered before use and a lower threshold setting of 4 was used to eliminate any counts from remaining particulate contamination. A 70 micron orifice tube was used. The multiplier settings used were: 1/amplification at 1 and 1/aperture current at 1. Coulter counting necessitates separation of cell pairs in which fission has commenced without breakage of any cells. This was achieved by sonication of the sample (usually 1 ml. plus 1 ml. of electrolyte) in a 20 ml. MSE tube held in ice using a MSE 100 Watt Ultrasonic Disintegrator at a frequency setting of 20 kilocycles per second and amplitude of 2 microns. A $\frac{3}{8}$" diameter titanium vibrator probe was used, immersed not more than $\frac{1}{8}$" into the sample to be sonicated. Sonication for 90 seconds separated cell pairs without appreciable cell breakage.

SYNCHRONOUS CULTURES

Synchronously dividing cultures of S. pombe were prepared by the method of Mitchison and Vincent (1965). Cultures of 4 to 10 litres were grown to between 2 and 8 million cells per ml. in 10 litre Pyrex flasks and vigorously stirred by means of a magnetic stirrer. The cells were harvested and loaded on to the top of linear density gradients (10% - 40%) with up to 10^{10} cells per gradient. These gradients were 75 ml. in volume and were made in 100 ml. MSE centrifuge tubes using a gradient machine. The gradients were made from 10% and 40% solutions of glucose or sucrose in EMM. The gradients were equilibrated to 25°C before use and after loading with cells were centrifuged at 1,300 g. (1,500 r.p.m., MSE Mistral 4 L) for between 4 and 11 minutes. The 'cloud' of cells immediately above the main band of cells on the gradient was removed by means of a 5 ml. syringe fitted with a large needle bent over at right angles near the tip. These cells were inoculated into fresh EMM to a concentration between 0.8 and 2 million cells per ml. The culture was kept stirred continuously by means of a magnetic stirrer throughout the subsequent experiment. Non-synchronous control cultures were obtained by using a sample from a vigorously shaken gradient in which cells had been centrifuged.

Synchronous cultures were monitored for adequate synchrony and for timing of the cell cycle by determinations of cell concentration at frequent intervals, or by monitoring the percentage of cells showing cell plates. The latter method

simply entailed drying down small samples of the culture on microscope slides on a hot plate. When dried, the crystallised medium was washed off to leave the cells stuck to the slide. The cells were then stained to show up the cell plate by firstly smearing with Indian ink containing 4% formalin and, secondly, when the ink had dried, staining the cytoplasm of the cells by a solution of crystal violet. The percentage of cells showing cell plates was easily determined from such slides by counting.

The time between divisions in synchronous cultures varied from 2 hours 15 minutes to 2 hours 50 minutes. Occasionally cultures proved to be non-synchronous or had a mean generation time in excess of 3 hours. Experiments with such cultures have been discounted.

DRY WEIGHT DETERMINATION

Samples of cells for dry weight determination were filtered through tared 2.1 cm. Whatman GF/A filters. Samples between 10 and 80 ml. were taken from cultures with 25×10^6 to 1×10^6 cells/ml. respectively. The cells were washed on the filter with at least 80 ml. ice-cold water. The filter was then removed and dried immediately by placing it directly under a Phillips 250 watt infra-red lamp supported 9" above. After 90 seconds the filter was removed and kept in a clean numbered plastic petri dish at room temperature until re-weighed. For each series of weight determinations at least two control

filters were used. These were treated exactly like the other filters except that, instead of filtering cells through them, an aliquot of fresh medium was filtered.

The weights of filters before and after filtering cells were determined by means of a Cahn Gram Electrobalance using the 10 mg. range. The Whatman GF/A filters weighed between 16.5 and 18.0 mg., so, in order to obtain the sensitivity of the 10 mg. range, two tares were made from aluminium foil, one weighing about 18 mg. and the other about 16 mg. The 18 mg. tare was placed and always remained in the control stirrup of the beam. The 16 mg. tare was placed in the working stirrup to set the balance to zero and the 10 mg. standard added to it to set the 10 mg. limit. The standard and the 16 mg. tare were both removed and replaced by a filter to be weighed. The difference in weight before and after filtration of a sample gave the weight of cells in the sample. This weight was corrected for the change in weight of the control filters. The control filters were found to lose between 0.7 and 1.0% of their initial weight.

Drying periods of more than 90 seconds under the infra-red lamp and desiccation up to 24 hours produced no significant decrease in weight of the cells. The estimates of dry weight have therefore been taken as estimates of the weight of the cells dried to constant weight.

CHEMICAL ASSAYS

Fractionation of Cells. The assay of various cell fractions such as total protein and RNA is normally performed on separate extracts of the cells. RNA is commonly estimated from hydrolysed extracts obtained by acid or alkaline treatment on plant and animal tissues (Hutchison and Munro, 1961). Protein in animal tissues is widely estimated from dilute alkali extracts by the method of Lowry et al. (1951), while in plant tissues total protein is commonly derived from determinations of total nitrogen after extraction of lipid materials. For the purposes of this work it seemed desirable to estimate protein by the Lowry method since estimates from total nitrogen would include nitrogen from cell wall materials (Ingram, 1955), and the complete separation of protein and cell wall material is not easy. The method of Lowry is for the determination of protein in solution or for proteins isolated by precipitation from solution. The difficulty of extracting the protein from cells with a substantial cell wall was overcome by estimating protein in the fractions remaining after extraction of RNA. Both the Schmidt-Thannhauser and Schneider extraction methods were used, but were slightly modified so that protein and RNA could be estimated on the same samples of cells. These fractionation procedures are outlined diagrammatically in Table 1. With larger samples of cells the quantities in the extraction procedures were increased proportionately. Pool material was not always extracted with cold PCA as shown (see

Chapter 3). With the Schmidt-Thannhauser procedure, cells were digested for 2-3 hours with 1 N sodium hydroxide at 32°C. A sticky mass of cells remained and complete cell walls could be detected on microscopic examination, but less than 1% showed any refractive content. Protein material was precipitated by addition of an equal volume of 10% PCA. Complete precipitation was only achieved after cooling to 0°C. After centrifugation the pellet was washed twice with absolute alcohol and dried. The residue was digested overnight at 32°C with 0.2 N sodium hydroxide containing 2% deoxycholate (DOC). A modified Lowry procedure was then used to allow direct estimation of protein in this material.

The Schneider procedure involved digestion of the cells after the pool extraction with 5% PCA for 15 minutes in a water bath at 70°C. After digestion, samples were cooled to 0°C to allow complete precipitation of protein material, and then treated essentially in the same manner as for the Schmidt-Thannhauser procedure (see Table 1). Occasionally a lipid extraction procedure was inserted either after extraction of the pool or after acid or alkali digestion. This procedure (Hutchison and Munro, 1961) was a series of successive washings in:

ethanol
ethanol-chloroform (3:1) (twice)
ethanol-ether (3:1)
ether

Occasionally the pellet of material obtained after extraction

of the pool was freeze-dried prior to removal of RNA. Where this was not done, no more than 0.05 ml. of water was left after the final wash. This was achieved by careful removal using a fine-tipped pipette attached to a water suction pump.

Eaton's (1962) mechanical method for total cell rupture was also used. Ten ml. of cell suspension at a concentration of up to 10^8 cells/ml. were pipetted into the press previously cooled in a carbon-dioxide/ethanol freezing mixture. The piston was inserted above the cells and the entire press placed in a hydraulic press. After about five minutes, when the suspension was completely frozen, pressure was applied up to 10,000 lb./in.^{2}. Within a minute or two a proportion of the sample was forced explosively through the orifice into the collecting tube, due to liquefication under pressure. With decrease in pressure the sample froze again. Pressure was re-applied until at 3-4,000 lb./in.^{2} a further aliquot was forced through the orifice. This process was repeated four to six times before the entire sample had been forced into the collecting tube.

Microscopic examination showed that at least 95% of cells were ruptured by this method. If the cell suspension was frozen and then thawed before passing it through the press, no intact nuclei could be observed. It therefore seemed likely that this method caused disruption of all membranous structures within the cell.

Protein. For this work a method for the estimation of total cell protein was desired.

The method of Lowry et al. (1951) measures tyrosine and tryptophan in addition to the copper complex formed with protein in alkaline solution of normality not greater than 0.1. It was found essential to ensure that cell samples used for estimation of protein were exceedingly well washed since EMM gives a positive Lowry reaction, which is due to the vitamins. For a given volume this reaction is about fifty times greater for medium from a culture at 2×10^6 cells/ml. than for the protein in the cells of that volume. During the early part of this work protein was estimated on extracts of cold PCA precipitated material obtained by incubation overnight at 32°C with 0.1 N sodium hydroxide without deoxycholate. Reagents used were:

A. 0.20 N sodium hydroxide
B. 4% sodium carbonate
C. 2% sodium potassium tartrate
D. 1% copper sulphate
E. Folin-Ciocalteu reagent

A copper alkali solution was prepared fresh as required by mixing 100 ml. of A. with 100 ml. of B. To this was added 2 ml. of C. and 2 ml. of D. in that order. Folin-Ciocalteu reagent (Hopkin and Williams Ltd., Folin-Ciocalteu for phosphatase) was diluted three fold prior to use. To the protein sample of 1 ml., including the remaining cell wall debris, was added 5.0 ml. of the copper alkali solution. This was mixed thoroughly and left at room temperature for 10-30 minutes

before adding 0.5 ml. of diluted Folin-Ciocalteu reagent. This reagent was added as rapidly as possible and mixed thoroughly immediately. These samples were then set aside for ten minutes out of direct sunlight. Their absorbence at a wavelength of 740 mu was then determined against a sample blank of 0.1 N sodium hydroxide as reference. A standard aqueous solution of bovine serum albumin was prepared and stored in a deep freeze. Aliquots of this were made up to 1 ml. with water and treated exactly like the samples above, except that a water blank was used as reference. A calibration line of amount of protein versus absorbence at 740 mu was constructed and the amount of protein in the unknown determined from this. This method gave results with a standard error of ± 14% on replicates (12) from the same culture. This is an unsatisfactorily high error.

A likely contributory cause of poor results with the above method seemed to be incomplete removal of protein from the acid precipitate. Extraction of protein by higher concentrations of alkali up to 0.5 N gave slightly higher mean values for protein, but with the same standard error. Addition of deoxycholate at 2% to the alkali considerably reduced the standard error (± 9%) and resulted in maximum estimates by the Lowry method in 0.2 N sodium hydroxide. Deoxycholate had no effect on the absorbence values for standard protein solutions. The Lowry procedure was modified to account for the higher alkalinity (0.2 N) of the samples by reducing the normality of

component A. above to 0.10. The mean amount of protein in cells estimated by this modified procedure was 15% higher than the mean estimated by the previous method without deoxycholate. However, the formation of maximum colour and its subsequent decay were both much more rapid than given by the standard Lowry procedure. Figure 1 shows that colour remained at a maximum from 8 to 30 minutes only and thereafter declined relatively rapidly, retaining only 83% of the maximum colour after 3 hours. With this method the absorbence was always determined between 10 and 30 minutes from the time of addition of the Folin-Ciocalteu reagent.

It remains uncertain that estimates of protein determined in this way measure the total protein of the cell. The amount of protein removed by washing the acid precipitate with alcohol is negligible. Estimates of protein on material which had not been alcohol washed gave mean values 3% higher than estimates on material which had been alcohol washed or subjected to the full lipid extraction procedure. This difference was not found to be statistically significant, and fell within the limits of error of the assay method. Estimates of total nitrogen on the acid precipitates left only 8% of the nitrogen unaccounted for by protein. The 8% unaccounted for may be cell wall material possibly of a non-protein nature.

Nitrogen. Samples for total nitrogen determination were first digested to the inorganic state. Samples were heated in hard

glass test tubes with 0.4 ml. of 'digest acid'. This acid was prepared by mixing the following three components:

100 ml. concentrated sulphuric acid
100 ml. saturated potassium hydrogen sulphate
0.2 gm. sodium selenate

Heating was at about 320°C in a sand tray containing electrically heated elements and was continued for at least one hour after the samples became clear. When cool, the samples were diluted with distilled water to 10 ml. and 1 ml. aliquots removed for determination of nitrogen content. These samples were mixed with 2 ml. 0.5 N sodium hydroxide and 1 ml. Nessler reagent and after 30 minutes in the dark the absorption at 410 millimicrons was determined in a spectrophotometer.

The Nessler reagent was prepared in 2 litre quantities in the following manner. Firstly, 100 gm. potassium iodide was dissolved in 100 ml. water. To this was added saturated mercuric chloride until a permanent red precipitate was formed. Four hundred ml. of 5 N sodium hydroxide was then added and the solution made up to 2 litres with distilled water. This was allowed to stand for several weeks and the clear supernatant removed as required.

A calibration curve of absorption at 410 millimicrons against the amount of nitrogen was determined with each batch of unknowns using a standard solution of ammonium sulphate containing 56 micrograms of nitrogen per ml. Aliquots of this standard were digested and assayed in the same manner as the samples of unknowns.

Pool Amino Acids (∝-amino group). The total quantity of amino acids in pool samples was determined by the method of Cocking and Yemm (1954) for ∝-amino groups. The method involves the oxidative de-amination of the amino acids with the formation of ammonia which then reduces ninhydrin to a purple-coloured compound in acid solution. A pH of 5 for the final assay solution is essential and clearly the sample must be initially ammonia-free for accurate estimates of the total quantity of amino acid. Ammonia-free samples were obtained by repeated evaporation of the samples to dryness over a boiling water bath in the presence of a drop of 1 N sodium hydroxide. The residue was finally taken up in a known volume of distilled water (generally 1.5 ml.) and a 1 ml. aliquot of this used for assay. In those cases where the pool material was obtained by acid extraction, the pH of the 1 ml. sample was adjusted to 5 by titrating with 0.5 N NaOH using methyl red indicator. One ml. of the final solution was used for the assay, and the amount of amino acid material in the original sample computed from the volumetric determinations.

The reagents used were:

0.2M citrate buffer (21.00 gm. citric acid plus 200 ml. 1.0 N NaOH diluted to 500 ml. with distilled water)

5% ninhydrin in 2-methoxy-ethanol

0.0002M potassium cyanide in 2-methoxy-ethanol

The citrate buffer and ninhydrin solutions were prepared fresh as required. The cyanide solution was prepared from a stock

solution of 0.01M aqueous potassium cyanide which was diluted 50 fold with 2-methoxy-ethanol. 0.5 ml. of the citrate buffer was added to the 1 ml. samples, followed by 0.2 ml. of the ninhydrin solution. The material was mixed well by shaking and then heated for 15 minutes in a boiling water bath. The samples were then cooled by immersion in ice-cold water and their absorption at 570 millimicrons determined in a spectrophotometer. A series of standards was assayed with each batch of unknowns. The standard used was leucine, and a calibration curve was determined for each experiment. Results were generally expressed in micromoles of leucine and no account was taken of the different extent to which different amino acids react with ninhydrin.

The colour remained reasonably stable after 30 minutes, but when large numbers of samples were assayed, the reaction mixtures were heated in the boiling water bath in order, cooled, and subsequently their absorption determined in the same order.

Ribonucleic Acid. Estimates of the amount of ribonucleic acid (RNA) in acid or alkali extracts were made from the absorption of the material at 260 mu. The absorbence readings were converted to amount of RNA on the assumption that 31 ugm. of the hydrolysed RNA per ml. gave an extinction of 1.0. Since S. pombe contains 100-150 times as much RNA as DNA (Mitchison and Lark, 1962; Bostock, 1969), at least 99% of the absorption

at 260 mu will represent absorption due to RNA.

Ornithine Transcarbamylase. Ornithine transcarbamylase (OTCase, EC 2.1.3.3) was estimated by the method of Gerhart and Pardee (1962) on cells which had been washed, freeze-dried and resuspended in 0.2 ml. water. The assay mixture contained:

I. 0.20 ml. cell suspension

II. 0.10 ml. freshly prepared carbamyl phosphate, 4 mg./ml.

III. 0.10 ml. of 0.03M L-ornithine

IV. 0.10 ml. of 1.0M glycine buffer pH 9.5

Reagents used were:

A. 66 ml. conc. sulphuric acid added to 34 ml. water at 0°C

B. 22.5 mg./ml. diacetylmonoxime

C. Diphenylamine-P-sulphonate solution containing:

1) 114 mg. disodium diphenylamine sulphonate
2) 10 ml. 1 N hydrochloric acid
3) 1.0 ml. solution Atlas "BRIJ 35", 50 gm. in 100 ml. water
4) 89 ml. water

D. 2.5 mg./ml. $K_2S_2O_8$

Components A, B and C were mixed in the ratio 3:1:1 by volume just prior to use. This mixture is termed the '3:1:1 mix'.

The assay mixture was incubated at 28°C and initiated by addition of component I. After 30 minutes 2.50 ml. of ice-cold 3:1:1 mix was added, and the entire mixture thoroughly mixed and kept at 0°C until the next step. Then the samples were heated at 60°C for 30 minutes. After equilibration for

several minutes at room temperature, 0.50 ml. of D was added, and the samples well shaken. The absorbence at a wavelength of 560 millimicrons was determined.

The absorption spectrum of the colour formed is shown in figure 3. The colour formed was unstable and was found to reach a maximum 45 minutes after addition of component D (see figure 2). Component D was therefore added to samples in order at known time intervals, and the absorbence of the samples was determined in the same order commencing 45 minutes after preparation of the first sample. Samples for assay were diluted, if necessary, to give a final cell concentration below 6×10^6 cells/ml., for, as shown in figure 4, the assay is not linear at higher concentrations.

The carbamyl phosphate used (Sigma Chemicals) was found to give a very high blank reading probably due to impurities of urea. The carbamyl phosphate was therefore made up with water to 25 mg./ml. and an equal volume of ice-cold 95% ethanol added with continuous stirring. After 30 minutes at $0^{\circ}C$ the precipitate was filtered, washed with 95% ethanol and dried over conc. sulphuric acid at $0^{\circ}C$. This material was taken up in water at $0^{\circ}C$ and used within two hours.

Carbohydrate. Carbohydrate material in the pool was estimated by the anthrone method as described by Mokrasch (1954). The reagent used was a solution of anthrone in sulphuric acid prepared as follows. One gram of anthrone was dissolved in

one litre of sulphuric acid obtained by adding conc. analar sulphuric acid to 290 ml. water. Six ml. of this reagent was added to pool samples of 1 ml. by means of an ARH automatic pipetting unit used without the automatic filling fitment. In this way the anthrone reagent was added very rapidly and accurately. The samples were then heated for 40 minutes at 80°C in a water bath, then cooled and the absorption at 460 mu determined by means of a spectrophotometer. Calibration was by means of a standard solution of glucose.

RADIO-ISOTOPE TECHNIQUES

Uniformly labelled ^{14}C glucose and amino acids were obtained from the Radiochemical Centre, Amersham. They were generally diluted with water so that convenient volumes could be used for each experiment. All materials were stored in a deep-freeze to avoid bacterial contamination. The activities of the materials used were as follows:

Glucose	230 m Ci/mM
L-lysine monohydrochloride	25 m Ci/millatom of C labelled at a concentration of 10 μ Ci/ml.
L-arginine monohydrochloride	
L-alanine	
L-leucine	
L-serine	

Labelled cells were collected on 2 cm. diameter filters (Oxoid membranes or Millipore filters), washed and dried. The filters were then either mounted on planchets and counted in

an IDL low background β-counter, or placed in scintillation vials with butyl PBD (Ciba Ltd.) at 5 gm./litre analar toluene and counted in a Packard 2002 TriCarb Scintillation Spectrometer. The efficiency of counting with the IDL β-counter was about 3%, while the efficiency of the scintillation method was about 65% for ^{14}C.

STATISTICAL TREATMENTS

The variance (s^2) of a set of (n) values was calculated in the usual manner:

$$s^2 = \frac{\Sigma(x - \bar{x})}{n}$$

where $\bar{x}$ is the mean, the standard deviation (s) being taken as the square root of the variance. The mean of a set of values has been quoted together with the standard error (s.e.) in the form

$$\bar{x} \pm \text{s.e.}$$

where

$$\text{s.e.} = \frac{s}{\sqrt{n}}$$

A figure in brackets after the standard error denotes the number of estimates (n) from which the mean and standard error were calculated.

The significance of the difference of the mean from a small sample ($\bar{x}$) from a hypothetical mean (u) was tested by means of the 't' test where

$$t = \sqrt{n}.(\bar{x} - \mu)/s$$

the degrees of freedom being n - 1. The probability of the

difference being due to chance was then determined from the position of t on the t-table in Fisher and Yates' tables.

Regressions were calculated in the normal way, the regression coefficient (b) being calculated from

$$b = \frac{\Sigma(x - \bar{x})(y - \bar{y})}{\Sigma(x - \bar{x})^2}$$

The variance of the deviations of the ordinates (s^2y) from the regression line was calculated from the formula:

$$s^2y = \frac{1}{n - 2}\left[\Sigma(y - \bar{y})^2 - \frac{\{\Sigma(x - \bar{x})(y - \bar{y})\}^2}{(x - \bar{x})^2}\right]$$

Data from synchronous cultures over three division cycles were on occasion tested for their goodness of fit to a segmented curve (three straight lines) or a smooth curve (polynomial of fifth order, or an exponential) using the method of Williams (1969). The analysis involves consideration of the residual sum of squares remaining after fitting the data to the different models. The ratio of these residual sums of squares is used to decide whether the data best fits the smooth model, the segmented model, or whether no definite allocation can be made. The analysis is computerised.

TABLE 1

SCHMIDT-THANNHAUSER FRACTIONATION

Washed sample of cells (3-10 x 10^6 cells)
1-3 ml. ice-cold 5% PCA for 8-25'
Centrifuge 0°C 3' at 1,000 g.

SUPERNATANT → POOL

PELLET
Wash 2 x ice-cold water (freeze-dry)
Add 1.0 ml. 1N NaOH
After 2-3 hrs. at 32°C add 1.0 ml. 10% PCA
Cool to 0°C
Centrifuge 0°C 10' at 1,000 g.

SUPERNATANT → RNA

PELLET
Wash 2x ab. alcohol. Dry.
Take up in 1.0 ml. 0.2N NaOH plus 2% DOC
Leave overnight at 32°C
→ PROTEIN

SCHNEIDER FRACTIONATION

Washed sample of cells (3-10 x 10^6 cells)
1-3 ml. ice-cold 5% PCA for 8-25'
Centrifuge 0°C 3' at 1,000 g.

SUPERNATANT → POOL

PELLET
Wash 2 x ice-cold water (freeze-dry)
Digest 15' at 70°C with 5% PCA
Cool to 0°C
Centrifuge 0°C 10' at 1,000 g.

SUPERNATANT → RNA

PELLET
Wash 2x ab. alcohol. Dry.
Take up in 1.0 ml. 0.2N NaOH plus 2% DOC
Leave overnight at 32°C
→ PROTEIN

FIGURE 1

TIME COURSE OF COLOUR FORMATION AND DECAY
IN LOWRY DETERMINATIONS OF PROTEIN

Taken from a continuous recording from a
Beckman spectrophotometer reading at 740 mu

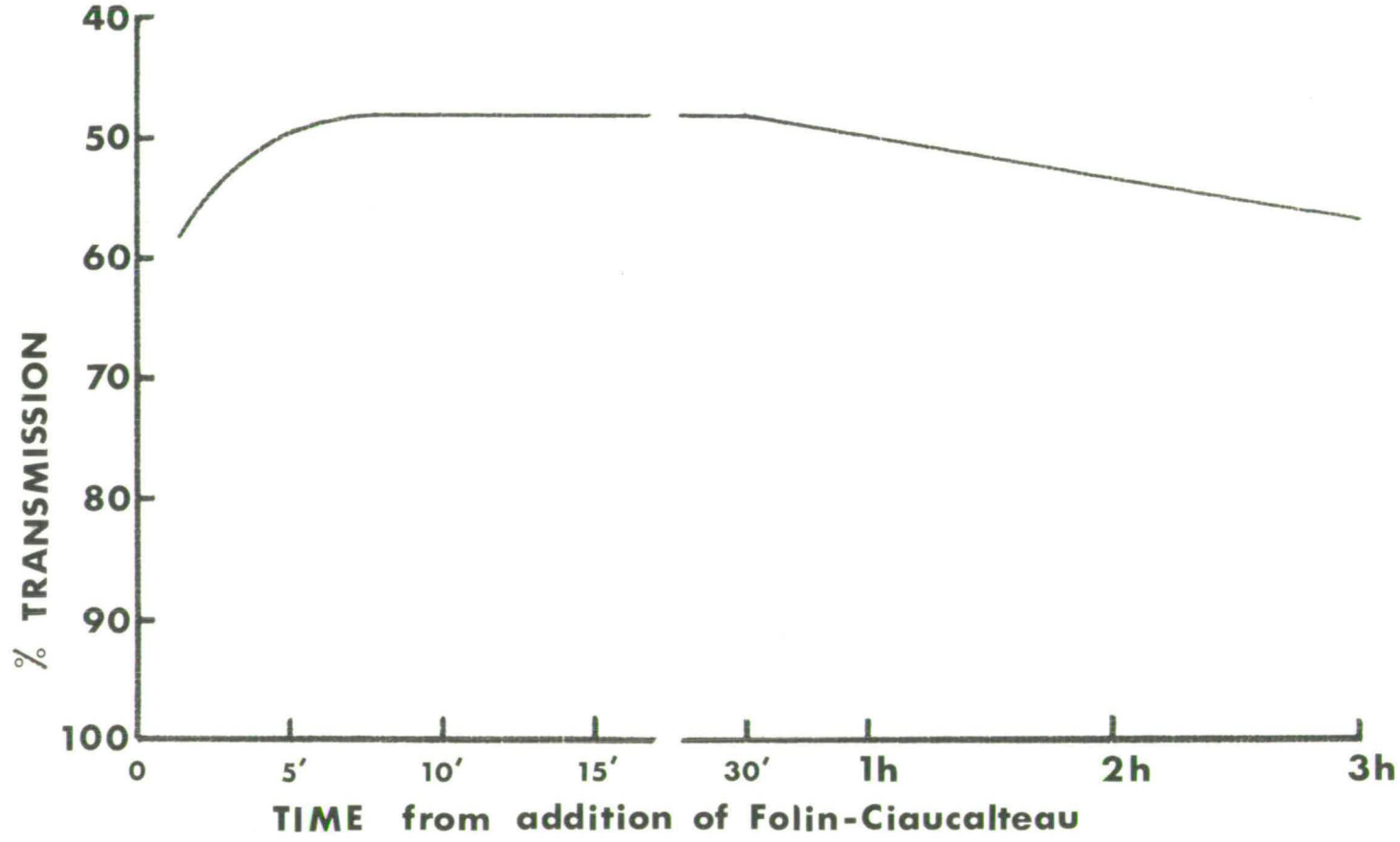

40
50
60
70
80
90
100
% TRANSMISSION
0
5'
10'
15'
30'
1h
2h
3h
TIME from addition of Folin-Ciaucalteau

FIGURE 2

TIME COURSE OF COLOUR DEVELOPMENT IN ASSAY FOR ORNITHINE TRANSCARBAMYLASE

FIGURE 3

ABSORPTION SPECTRUM OF COLOUR FORMED IN ASSAY FOR ORNITHINE TRANSCARBAMYLASE

FIGURE 4

ASSAY FOR ORNITHINE TRANSCARBAMYLASE WITH INCREASING AMOUNTS OF CELLS

Aliquots of cells from 0.01 ml. to 0.20 ml. were taken from a washed suspension of cells at 80 x 10^6 cells/ml. Above 0.08 ml., i.e. 6.4 x 10^6 cells, the assay gives too low an estimate of the enzyme.

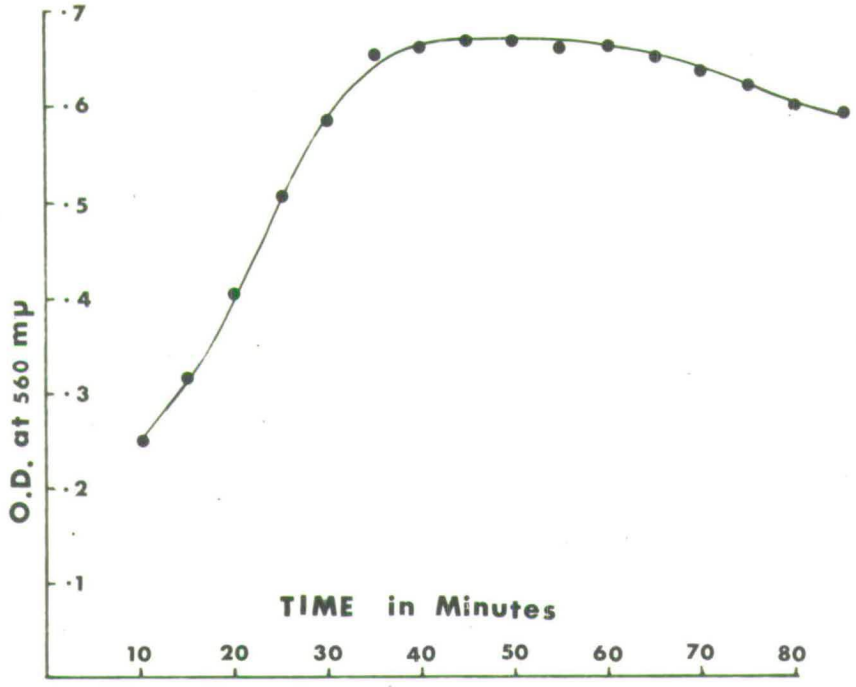


FIGURE 2

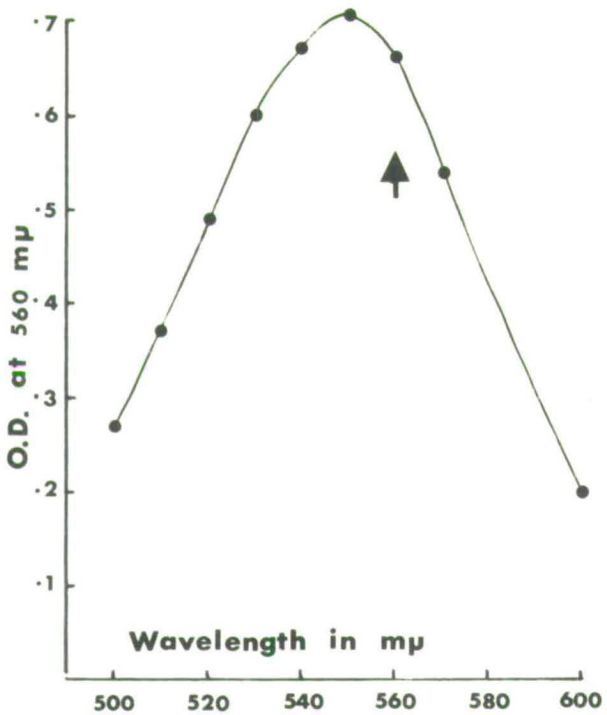


FIGURE 3

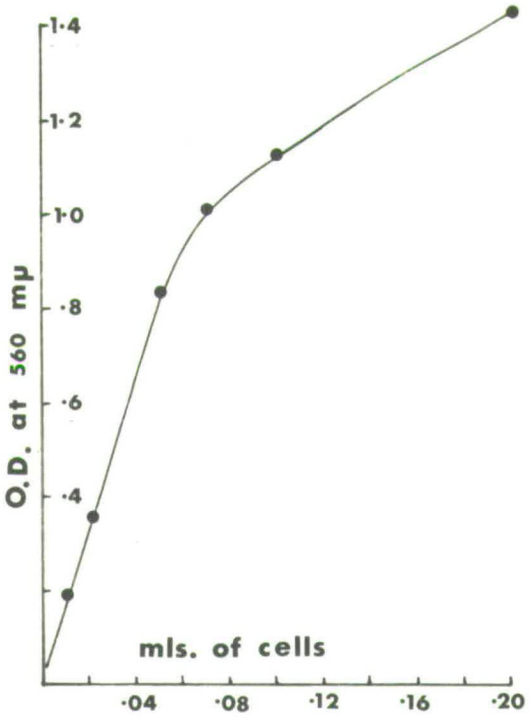


FIGURE 4

Chapter 3

SEPARATION AND ASSAY OF POOL AMINO ACIDS

At the time this work was begun there was no indication of how many different amino acids were present in the pool or how diverse their relative amounts. For the present work the main interest was in the amino acids found in proteins, since fluctuations in these could be related to amino acid biosynthetic enzymes. It therefore seemed desirable in the first instance to find a method whereby all or most of the protein amino acids in the pool could be separated and assayed. If a suitable method of synchronisation for S. pombe were developed, a very complete picture of variations in amino acid pool components during the cell cycle could then be determined. On the assumption that the behaviour of the amino acid pool was similar to that of the total pool during the cell cycle, large percentage changes were predicted. The total pool has been estimated to vary from 9 to 23% of the total dry mass (Mitchison and Cummins, 1964) in the cell cycle. Thus, on the above assumption, variations of about 100% in the amount of amino acids were predicted.

PAPER CHROMATOGRAPHY

With the possibility of large fluctuations of amino acids during the cell cycle the first obvious method of separation was paper chromatography. One-dimensional methods seemed to

have definite advantages when considering the assay of the individual amino acids. For, with a two-dimensional method, a separate paper would be required for each pool sample with the consequent difficulty of comparing quantitatively the amounts of amino acids separated at different times on different papers. The method of Franglen (1963) seemed most suitable since it claimed to separate adequately all the protein amino acids with an error of only ± 3%. The method uses four sequential one-dimensional runs with solvents containing slightly different proportions of n-butanol, acetic acid, and water. The amounts of individual amino acids are assayed after ninhydrin staining, by means of a Joyce-Loebl Chromoscan Densitometer. Using standard Whatman papers 46 cm. by 57 cm., this method would easily allow 20 samples to be separated on a single paper, including samples of standards for identification and calibration.

Pool samples were obtained by the freeze-thawing method (Chapter 4). Generally the washed cells were frozen in 0.6 ml. of water and up to 0.5 ml. of the supernatant obtained on thawing was spotted on a spotting line drawn parallel to and 11 cm. from one of the shorter ends of a sheet of Whatman 3 MM paper. The spotting line was drawn nearest to the end of the paper at which machining commenced so that solvent movement was in the direction of the machining of the paper. Samples were spotted 2 cm. apart with continuous drying of the paper by means of a hand hair-drier so that spots did not exceed 0.4 cm.

in diameter. Fine-tipped lambda pipettes made by Camlab were found most suitable. A finishing line was also drawn on the paper 27 cm. from the spotting line.

A Shandon Chromatank 500 was used for descending chromatography with two sheets of paper inserted even when only one paper had spots on it. The papers were allowed to equilibrate overnight with about 100 ml. of solvent in the bottom. Next morning 100 ml. of solvent were added to the trough and this was allowed to develop along the papers until the front reached the finishing line. The chromatogram was then removed and dried rapidly using a small electric heater with a fan. The same procedure was followed with the subsequent solvents.

All chromatography, including preparation of solvents, was carried out in a warm room at 28°C. The four solvents contained varying quantities of acetic acid and were prepared as follows. Glacial acetic acid was diluted with water to give a 40% solution (v/v). This was added to n-butanol to give the four solutions containing various percentages (v/v) of the diluted acetic acid:

I. 30%
II. 35%
III. 37.5%
IV. 40%

The papers were stained by dipping in the cadmium-ninhydrin reagent of Atfield and Morris (1961). This reagent was prepared by dissolving 0.050 g. cadmium acetate in a mixture of 5.0 ml. water plus 1.0 ml. glacial acetic acid. 50 ml.

acetone were added and 5.00 g. ninhydrin dissolved in this mixture. This reagent was prepared fresh as required. The chromatograms were cut into strips just large enough to fit into the sliding specimen holder of the chromoscan. These strips were cleared by soaking in paraffin oil and drained. The chromoscan was arranged for densitometry measurement by reflectance, using a non-diffusing white background, a 0.5 mm. wide slit, and a blue-green filter (Joyce-Loebl No. 5-023). The length of the slit was cut down to 5 mm. and the chromatogram strips scanned to include the centres of each spot.

In Franglen's method the chromatograms were cut into horizontal strips each containing the standards and unknowns of one amino acid. This method proved unsatisfactory for a number of reasons. Firstly, pool material from S. pombe did not show complete separation, particularly in the region of the acidic and neutral amino acids. An equimolar mixture of standard amino acids gave much better separation, but still there were two sets of amino acids which remained inadequately resolved, viz. lysine, histidine, arginine, and serine, glycine. Secondly, the solvent front was curved so that it was difficult to cut the chromatograms into strips and scan them. These two factors compounded to make the quantitative estimation of most of the amino acids in pool samples impossible, since a parallel base line scan, free of absorption from the amino acid spots, could not be made. The only suitable method of scanning seemed to be in the direction of solvent movement. The papers

were therefore cut into vertical strips so that all the amino acids of one sample were on one strip.

Lack of resolution in the pool samples seemed most likely to have been due to excess of some amino acid which streaked and hence merged with adjacent amino acids, or some other material in the sample, such as large amounts of salts resulting in general streaking of the spots. The latter seemed least likely since valine, phenyl-alanine and the leucine/isoleucine spots generally showed no streaking. The most likely amino acid in excess seemed to be glutamic acid since streaking was most pronounced amongst the middle spots. This point was further investigated by preparing two-dimensional chromatograms of pool material.

The two solvents used for two-dimensional separation of pool amino acids were saturated phenol/water (3:1 v/v) and n-butanol/acetic acid/water (90:10:29 v/v) in that order. The same apparatus, type of paper and colour development techniques were used as for the one-dimensional method above. From such chromatograms it was clear that glutamic acid was the largest component of the amino acid pool. Two non-protein amino acids were also identified: ornithine and citrulline. Some protein amino acids could not be seen; these were tryptophan, methionine, and tyrosine. With larger pool samples there was streaking due to glutamate, so the presence of small amounts of these amino acids could not be established. The intensity of colour in the glutamic acid spot was about four times greater

than that for any other amino acid.

HIGH VOLTAGE ELECTROPHORESIS

To overcome some of the difficulties arising from the paper chromatography, high voltage electrophoresis methods were investigated. An E. Miles 10kV Electrophoresis apparatus taking 46 cm. x 57 cm. Whatman papers with water-cooled plates was used. This apparatus was housed in a teaching laboratory in the Department of Biochemistry. Two electrolytes were used: pyridine/acetic acid at pH 5.2 and formic/acetic acid at pH 2.0. These were prepared from analar reagents in 10 litre quantities in the manner indicated in the table below, and the pH values checked before use.

Electrolyte	Method of Preparation
Formic/acetic acid pH 2.0	205 ml. 90% formic acid; 744 ml. glacial acetic acid made up to 10 litres with distilled water
Pyridine/acetic acid pH 5.2	204 ml. pyridine; 95 ml. glacial acetic acid made up to 10 litres with distilled water

Using formic/acetic acid, a spotting line was drawn 10 cm. from one of the shorter ends of a sheet of Whatman No. 1 paper. Samples were spotted on this line exactly as for the paper chromatography method above. When the spots were dry, the paper was transferred to the electrophoresis apparatus and wetted with electrolyte on both sides of the spotting line.

Care was taken to ensure that the two fronts of electrolyte reached the spotting line at the same time. This was achieved by having a glass rod immediately under the spotting line until the two fronts had almost met. Seven kV was applied for 30 minutes. This was sufficient to move lysine (the fastest moving amino acid in this electrolyte) about 25 cm. from the spotting line. The paper was rapidly dried, sealed in a polythene bag and later stained by dipping in cadmium/ninhydrin as for paper chromatography. The position of each amino acid was determined by means of standard mixtures as for paper chromatography.

Using pyridine/acetic acid, a spotting line was drawn centrally across a sheet of Whatman No. 1 paper parallel to the shorter sides. Spotting and subsequent electrophoresis was as with formic/acetic acid. This method separates the basic amino acids towards the cathode, the acidic amino acids towards the anode, with the neutral amino acids left unresolved near the spotting line, but slightly towards the cathode.

The proline spot in the pyridine/acetic system showed up as a yellow spot only after several weeks storage of the papers. This system resolves adequately lysine, histidine, arginine, glutamic acid, and aspartic acid. The spots obtained by these high-voltage electrophoresis methods were very much smaller (less than 8 mm.) than those formed by paper chromatography, and it was possible to scan strips including the same amino acid of different samples. The standard error

from 8 spots with equal amounts of lysine was ± 11%. Unfortunately it was not easy to cut and scan strips all the way across the papers since the spots separated in a 'W' shaped array. This was probably due to uneven cooling of the paper during electrophoresis and could not be corrected.

The application of these two methods left the neutral amino acids unresolved. However, with the separation of aspartic and glutamic acid from the neutrals in the pyridine/acetic system, a better resolution of the neutrals seemed possible by cutting out such a spot of neutral amino acids and using it as a sample spot in the formic/acetic system. Strips of these neutral amino acids were prepared and subjected to electrophoresis in the formic/acetic system, but poor resolution was achieved due to streaking. Several methods of applying the strips of neutrals to the new papers were tried. These methods were simply laying the strip of neutrals out on the spotting line or hand-sewing them on using the usual wetting procedure. Wetting of the strip of neutrals before placing it on the new paper was also tried.

At this time the total amino acid pool was determined by the Cocking and Yemm method in synchronous cultures. The pattern of increase in pool amino acids through the cell cycle appeared to be approximately exponential (see Chapter 7). If fluctuations existed they were considered to be less than 10% and so the high-voltage electrophoretic techniques were considered inadequate.

AUTO ANALYSIS

Pool samples were prepared as for the other methods above, except that prior to freezing known standards were added to the samples of cells.

Chromatographic separation was by means of a Technicon single column Autoanalyser using the method of Spackman, Moore and Stein (1958), or the faster two column method (Spackman, 1964) using an Evans Autoanalyser (Evans Electroselenium Ltd., Halstead, Essex).

With the single column method L-nor-leucine was used as a standard. 0.2 μ moles of nor-leucine were added to the sample of cells in 1 ml. distilled water prior to extraction of the pool by freeze-thawing. After freeze-thawing up to 0.8 ml. of the supernatant were removed and 0.2 N hydrochloric acid added to make the acidity of the sample 0.1 N. This sample was then loaded on to the autoanalyser column. Each amino acid was estimated by measuring the area under the appropriate peak and dividing by the area under the nor-leucine peak, which was taken to represent 0.2 u moles. In this way the total amount of amino acids in the original sample were estimated directly without having to take account of volume errors in transferring the sample on to the analyser column.

With the Evans autoanalyser, two internal standards were used. These were DL-nor-leucine and L-2-amino-3-guanidopropionic acid, 0.2 μ moles of each per sample. About 0.4 ml. of the pool extract from each sample was placed on each of the two

columns. To avoid having to adjust the sample to an acid pH, the pool was extracted by freeze-thawing in the analyser sample application buffer. This was 0.2 M sodium citrate at pH 2.2. The amino acid pool extracted in this way was identical to that obtained by freeze-thawing in water (see Chapter 4).

With the single column method, cirtulline and proline were usually found to be unresolved. In cases where the citrulline peak (estimated from the scan at 570 mμ) was situated directly over the proline peak (estimated from the scan at 440 mμ) both citrulline and proline could be estimated (H. Kascer, personal communication). The areas under the peaks for the 570 mμ and 440 mμ scans (A_{570}, A_{440}) were determined and the areas for citrulline and proline (A_{cit}, A_{pro}) estimated from the following relationships:

$$A_{cit} = 1.04\ A_{570} - 0.20\ A_{440}$$

$$A_{pro} = 4.87\ A_{440} - 0.92\ A_{570}$$

With the two column method proline and citrulline were generally only partially resolved, so that estimates of proline were not possible. Estimates of citrulline were, however, made, but could occasionally include proline material and so be too high. This error must, however, be small since the amount of proline in pool samples of S. pombe is only about 14% of the amount of citrulline (see Chapter 4, Table 6).

The change from using the Technicon analyser to the Evans analyser occurred at about the time cells were first grown on

EMM2, so that all analyses with the Technicon analyser were for cells grown on EMM1.

The error of autoanalyser methods is generally quoted to be about 3%, but this point is considered further in Chapter 7.

Chapter 4

EXTRACTION AND COMPOSITION OF POOL MATERIAL

A number of different procedures have been used for the extraction from cells of material which has been termed 'pool' material. Generally these procedures have not been examined for their efficiency in removing the low molecular weight compounds and rely on precipitation of macromolecules or disruption of cellular organization to separate the pool material. If Britten and McClure's (1962) definition of the pool (see Chapter 1) is used, a number of questions should be borne in mind in an investigation of the procedures followed in extracting pool material. Washing of the cells is generally a prerequisite for extracting pool material. All material physiologically outside the cell must be removed from the outside of the cells, so that none will remain and be included with the pool material. A number of situations arise: the pool in question may be one of material, such as glucose or an amino acid supplement, obtained by simply transporting the compound from the medium into the cell. In these cases washing the cells free of exogenous material before extracting the pool is all important. Material from the medium must pass through the cell wall before being transported into the cell, and within the cell wall may exist in a special state and may not be readily removed, e.g. the Donnan free space and apparent free space of plant cells (Briggs et al., 1961). There is

less of a problem if the pool is of compounds synthesised by the cell and not derived simply by uptake from the medium, e.g. the amino acid pool of S. pombe grown on EMM. In such cases prior washing will only be necessary if the medium contains materials which interfere with the identification or quantification of pool material once it has been extracted. Pool material may be lost on washing with water, as in some bacteria (Britten, 1956) so the osmolarity of the washing solution may also be important.

Having adequately washed the cells, the extraction procedure used should be sufficient to remove only low molecular weight compounds and should not allow interconversion of the low molecular weight materials. Most extraction procedures result in precipitation of proteins and so probably minimise enzymatic hydrolysis or interconversion of pool compounds. However, labile pool components will be lost in most extraction procedures and little can be done to avoid this unless special procedures known to prevent their breakdown are used. Different extraction procedures have been used principally for convenience in estimating particular pool components. But if different procedures give essentially similar pools, confidence in the efficiency of all the procedures would be greatly increased. Comparisons of pool material obtained by different methods are rare. Hancock (1958) made a detailed comparison of material obtained by nine different pool extraction procedures on Staphylococcus aureus. Similar

quantities of amino acid, inorganic phosphate, and 260 mμ absorbing material were obtained by all the methods.

A difficult point to decide is the maximum molecular weight permissible for inclusion in the pool. In considering the relation of the amino acid pool to protein synthesis, it should not be ignored that the cell may make peptides or nucleotide peptides of low molecular weight. Whether the materials of this nature extracted from yeasts (Davies and Harris, 1960a,b; Davies, Harris and Neal, 1961; Millen and Saltmarsh-Andrew, 1965) have a significant role in the metabolism of the cell is not clear. They are obtained by prolonged treatment with cold aqueous ethanol and cold TCA and it is not clear that breakdown products of high molecular weight compounds do not contribute to these compounds. It is stated (Harris and Neal, 1960) that the "advantage of employing the dual extraction procedure rather than direct extraction of total nucleotides in aqueous trichloro-acetic acid or perchloric acid as commonly carried out is manifest, because the ethanolic solvent is more selective and the interpretation of the ion-exchange chromatograms is therefore simplified." While this method may lead to less confusion because fewer compounds separate out on ion-exchange chromatography, this cannot be taken to mean that it is a better method for extracting pool material.

Pool Extraction Methods. Since TCA has been used

extensively for extraction of pool material in S. pombe, this method was first investigated. A comparison was also made between immediate extraction of the cells and extraction after freezing. Replicate samples were taken from a culture at 3×10^6 cells/ml. These samples were washed with distilled water three times and finally all but about 0.05 ml. of water removed after centrifugation. Half the samples were frozen rapidly in a carbon dioxide/ethanol freezing mixture and after 40 minutes were thawed at 0°C and then treated exactly like the unfrozen samples.

Separate samples were incubated in an ice-water bath for times varying from one minute to 4 hours and 40 minutes with 4.2 ml. of either 5% TCA or distilled water. The cell material remaining was centrifuged and 1 ml. aliquots of the supernatant removed. Two of these were assayed for total nitrogen and two for pool amino acids. Figures 5 and 6 show the time course of extraction from the means of these assays.

Pool material was extracted by 10% PCA in a similar experiment and the ultraviolet-absorbing material obtained was estimated by the absorption at a wavelength of 260 mμ. The time course of extraction of this material is shown in Figure 7.

It is clear from these experiments that a constant amount of pool material is removed by extraction for periods of 15 to 30 minutes. Moreover, simple freeze-thawing of the cells removed as much material as TCA or PCA extraction for 15 to 30 minutes on unfrozen cells. This method seemed highly

convenient, particularly for estimating total ninhydrin positive material, since neutralisation of the extracting agent is unnecessary.

The time course of extraction of amino acids by TCA at different strengths has also been investigated. With TCA at more than 15%, charring of the cells occurs with extraction periods of more than 20 minutes and none of these methods yielded more material than did 5% TCA.

The amounts of amino acid material and 260 mμ absorbing material extracted by several different extraction procedures on equal aliquots of washed cells are given in Tables 2 and 3. The amounts are in arbitrary units and are the means of triplicate estimates. Clearly all these methods give essentially similar amounts of amino acids and 260 mμ absorbing material.

The effect of extraction by 25% ethanol, 10% PCA, boiling water, and repeated freeze-thawing on cells which had been frozen-thawed and washed on membrane filters was tested. The second extraction procedure released no further measurable amounts of amino acid material or 260 mμ absorbing material. The absorption spectra in the ultra-violet region of pool material obtained by freeze-thawing and by cold 10% PCA extraction for 15 minutes were characteristic of those of nucleotides (~~Yoas and Vincent, 1960~~ Beaven et al., 1955). For convenience the 260 mμ absorbing material is termed 'nucleotide material', but its true composition is unknown.

Washing. The effect of washing cells on the amount of pool material obtained was investigated by collecting equal aliquots of cells on membrane filters and washing three times with 20 ml. aliquots of ice-cold glucose, lactose, or sodium chloride at various molarities. Pool material was then extracted by freeze-thawing. The results are given in Tables 4 and 5.

If cells are washed in a solution of a lower molarity than their content, pool material could be lost by diffusion. The osmolarity of S. pombe has been determined from the behaviour of protoplasts obtained by removal of the cell wall, and is estimated to be about 0.9 (Duffus, per. comm.). This figure is similar to that obtained for other yeasts (Conway and Armstrong, 1961). Clearly, essentially similar amounts of material are obtained in all cases in the above experiments, so that a water soluble pool of the form found by Britten (1956) cannot exist. Suspending cells in ice-cold water for 20 minutes before filtering and washing was also found to cause no reduction in the amount of pool amino acid and 260 mu absorbing material (Tables 2 and 3).

Changes in the pool during exponential growth. Changes in the total amino acid pool and nucleotide pool (260 mμ absorbing material) during exponential growth in EMM1 are shown in Figure 8. The amount of material per cell remains more or less constant during exponential growth. After 14 x 10^6

cells/ml. (when growth rate decreases) the total amino acid pool rises slightly while the nucleotide pool drops markedly. The constancy of these pool components during exponential growth in EMM1 is perhaps surprising since other cellular components decrease (see Chapter 5). Since dry mass and volume of cells decrease during, so-called, exponential growth, the pool of nucleotides and amino acids must increase as percentages of total mass and volume.

In EMM2 the amount of pool amino acid and nucleotides also remain constant (Figure 9). In this case they are also constant relative to volume, mass and other cellular components, since in EMM2 there is balanced growth (see Chapter 5).

Autoanalyses of pool amino acids through exponential growth were not carried out. An attempt was however made to see if the relative proportions of amino acids varied during exponential growth in EMM1 and EMM2. Ten pool samples were prepared, by freeze-thawing, from cultures between 1×10^6 and 10×10^6 cells/ml. Equal aliquots were separated by paper electrophoresis using both electrolyte systems (see Chapter 3). Examination of the stained chromatograms showed no obvious major variations in the composition of the amino acid pool for cells grown in EMM2. For cells grown in EMM1 there appeared to be an increase in basic amino acids (ornithine, lysine, and arginine) during exponential growth.

Amino acid pool components. The amounts of amino acids

in pool material obtained by freeze-thawing of water washed cells grown to 3 x 10^6 cells/ml. in EMM1 are given in Table 6, together with the percentage that each component forms of the total. Some distinctive features are immediately obvious. There is an excess of glutamic acid over all other components and, apart from ornithine and citrulline, the amino acid peaks that could be estimated represented protein amino acids. With the Technicon Analyser, protein amino acids that could not be detected were: asparagine, glutamine, and tryptophan. The chromatogram was remarkably free of unknown peaks. The three unknowns regularly found in analyses with the Technicon Analyser were denoted X, P and Q, and their relative positions are as indicated in Table 6. Trace quantities of argino-succinic acid were also noted.

The large excess of glutamic acid means that estimates of total amino acids are largely estimates of the glutamic acid pool. If the behaviour of this component is different from the majority of the other amino acids, important changes in minor components could be overlooked. Table 6 also gives the amount of amino acids in pool material obtained by freeze-thawing of cells grown to 3 x 10^6 cells/ml. in EMM1, but washed with 1M lactose. The composition of the amino acid pool is essentially the same as that from water washed cells, so water washing does not selectively reduce the amount of the minor pool components.

Using an Evans analyser, the amounts of amino acids in

pool material obtained by freeze-thawing in water, freeze-thawing in 0.2M citrate buffer (the application buffer for the Evans analyser) and cold 5% TCA extraction were as given in Table 7. It is clear that the three extraction methods give essentially the same total amounts of pool amino acid, as already indicated by assays of total ninhydrin positive material by the Cocking and Yemm method. Also the three methods give essentially the same amounts of each amino acid.

The 570 mμ scan for a typical chromatogram from the Evans analyser is shown in Figures 10 and 11. Two unknown peaks were always present. These have been denoted X and Y. The unknown X probably corresponds to the similarly labelled unknown found in the Technicon chromatograms, since its position relative to aspartic acid is the same. The unknown Y could correspond to Q found in the Technicon chromatograms, but the positions are not identical. There was no trace of an unknown peak corresponding to P. Tryptophan was again not detected on the Evans chromatograms. If present it should have given a peak before that for aspartic acid and also before the unknown X. Since X disappears entirely on hydrolysis (see below) it could not itself have been tryptophan. Ornithine could not be detected as a separate peak on the Evans chromatograms. But the presence of this amino acid in pool material from all three extraction methods was established by paper electrophoresis (pH 2.0, see Chapter 3) of aliquots taken from the pool extracts (samples 1, 2 and 3, Table 7). Again

asparagine and glutamine could not be detected. Valine and cystine were not estimated from chromatograms obtained with the Evans analyser, for, as can be seen from Figure 10, an unequivocal baseline cannot be drawn because of the buffer change occurring just before these peaks. It is estimated that the amount of amino acids ignored by these omissions, including proline, cannot exceed 5%.

Amino acids found in pool material after hydrolysis at 105°C for 18 hours are also given in Table 7. The pool material was in this case obtained by freeze-thawing. Since there is no substantial increase in the amino acid components, pool material obtained in this manner must be free of protein. The unknowns X and Y, which disappear on hydrolysis, are probably small peptides.

The intracellular concentrations of pool amino acids are given in Table 8.

Other pool components. Two additional components of the pool were investigated in an attempt to account for the total mass of the pool: these additional components were organic acids and pool carbohydrates.

A qualitative and rough quantitative estimate of non-volatile organic acids was made in the following manner. Pool material extracted by freeze-thawing from 600 ml. of cells grown to 3 x 10^6 cells/ml. was made up to 20 ml. with water and passed through a Dowex 1 x 10, 200-400 mesh ion exchange column.

The resin was converted to the formate form from the chloride form in which it was obtained, and an 8 cm. packed column prepared. The column was washed with 400 ml. of water. The organic acids were then eluted with 20 ml. of 6 N formic acid. The eluate was collected and evaporated to dryness in a crucible heated in a boiling water bath. The residue was taken up in 50 ulitres of water and spotted on to a sheet of Whatman No. 1 chromatography paper together with standards of citric, malic, succinic, and fumaric acids. The acids were separated by one-dimensional descending chromatography for 16 hours using a tertiary amyl alcohol/formic/water solvent (3:1:3 v/v). Five hundred ml. of the solvent was shaken in a separating funnel and the lower layer was run into the bottom of a Shandon 500 Chromatank, and the upper layer was placed in the trough of the tank. After development the paper was air-dried and any traces of formic acid then removed by steaming in an autoclave at 5 lbs. per square inch for 30 minutes, with the vent tap just open to allow escape of the steam. The paper was then sprayed with a 0.04% solution of bromo-cresol purple in 50% ethanol. On exposing the papers to dilute ammonia fumes the spots stood out yellow against a purple background. The positions and sizes of the spots were quickly marked as the purple background faded very quickly. The presence of the four acids (citric, malic, succinic and fumaric) was confirmed by reference to the positions of the standards on the paper. The total quantity of these acids was roughly estimated by

reference to the colour intensity relative to the standards, as 100 µg. Thus the amount of organic acid per cell must be approximately 0.05 µµg.

The total quantity of pool carbohydrate material expressed as glucose was determined by the anthrone method as described in Chapter 2. The mean amount in exponential phase cells grown in EMM2 was found to be 0.925 µµg. per cell.

The mean composition of pool material in exponential phase cells grown in EMM2 is summarised in Table 9. The large amount of pool material unaccounted for may be inorganic salts.

It is clear from the results described in this chapter that several quite different methods of extraction give essentially the same amounts of pool materials. This allows different techniques to be used to suit different situations. Thus freeze-thawing has been used where low pHs introduce difficulties, such as in the assay of amino acids, while acid extraction has been used where rapid extraction is necessary with the possibility of collecting the cells on filters, such as in labelling experiments. The reason why different extraction procedures yield essentially the same pool material is attributed to the cell wall. It seems likely that the cell wall acts as a molecular sieve in S. pombe allowing release of low molecular weight materials once the plasmalemma and other cell membranes are ruptured, but retaining macromolecular components. Certainly no proteins have been found to be released after freeze-thawing. This is essentially the

method used for preparing cells for enzyme assays (Bostock et al., 1966; Mitchison and Creanor, 1969) and in no case has enzyme activity been found in the supernatant material after this treatment. The cell debris must be present during enzyme assays if any detectable reaction is to occur (J.G. Creanor and A.R. Robinson, per. comm.).

TABLE 2

AMOUNT OF POOL AMINO ACIDS REMOVED BY DIFFERENT EXTRACTION METHODS

Extraction Method	Time	Temp.	Amount
Water	5'	100°C	0.256
25% ethanol	15'	0°C	0.257
5% TCA	15'	0°C	0.267
10% PCA	15'	0°C	0.263
Freeze-thawing in:			
Water			0.262
0.02M acetate buffer pH 4.9			0.255
0.2M citrate buffer pH 2.0			0.259
1M sodium chloride			0.257
Water	20'	0°C	0.003

TABLE 3

AMOUNT OF POOL 260 mμ ABSORBING MATERIAL REMOVED BY DIFFERENT EXTRACTION METHODS

Extraction Method	Time	Temp.	Amount
Water	5'	100°C	0.425
25% ethanol	15'	0°C	0.430
5% TCA	15'	0°C	0.416
10% PCA	15'	0°C	0.424
Freeze-thawing in:			
Water			0.440
0.02M acetate buffer pH 4.9			0.429
0.2M citrate buffer pH 2.0			0.433
1M sodium chloride			0.436
Water	20'	0°C	0.002

TABLE 4

AMOUNT OF POOL AMINO ACID OBTAINED AFTER WASHING CELLS WITH SOLUTIONS OF VARIOUS MOLARITIES

Molarity	Glucose	Lactose	Sodium chloride
Water	.310	.325	.324
0.01	.323	.332	.316
0.1	.316	.320	.322
0.2	.316	.318	.329
0.3	.302	.325	.332
0.4	.324	.320	.318
0.5	.330	.330	.304
0.6	.322	.311	.324
0.7	.325	.316	.324
0.8	.306	.309	.321
0.9	.311	.306	.316
1.0	.316	.322	.312

Pool material obtained by freeze-thawing

TABLE 5

AMOUNT OF POOL 260 mμ ABSORBING MATERIAL OBTAINED AFTER WASHING CELLS WITH SOLUTIONS OF VARIOUS MOLARITIES

Molarity	Glucose	Lactose	Sodium chloride
Water	.372	.370	.380
0.01	.370	.374	.370
0.1	.364	.376	.372
0.2	.378	.380	.368
0.3	.362	.372	.375
0.4	.371	.377	.372
0.5	.372	.368	.377
0.6	.364	.378	.364
0.7	.366	.370	.372
0.8	.374	.364	.372
0.9	.370	.360	.369
1.0	.374	.372	.370

Pool material obtained by freeze-thawing

TABLE 6

POOL AMINO ACIDS

Amino acid	umoles of amino acid: Washed: Water	Washed: 1M Lactose	% of Total (water washed)
X	0.0899	0.0870	2.79
Aspartic acid	0.1288	0.1302	4.00
Threonine	0.1346	0.1320	4.18
Serine	0.1803	0.1792	5.60
P	0.0249	0.0252	0.77
Glutamic acid	1.1915	1.2003	37.02
Citrulline	0.1393	0.1400	4.33
Proline	0.0189	0.0160	0.59
Q	0.0910	0.1020	2.83
Glycine	0.1516	0.1470	4.71
Alanine	0.3450	0.3440	10.72
Methionine	0.0088	0.0080	0.27
Isoleucine	0.0115	0.0120	0.36
Leucine	0.0182	0.0180	0.57
Tyrosine	0.0120	0.0116	0.37
Phenylalanine	0.0306	0.0312	0.95
Ornithine	0.0665	0.0660	2.07
Lysine	0.2118	0.2160	6.58
Histidine	0.0416	0.0402	1.29
Arginine	0.3219	0.3240	10.00
Total	3.2187	3.2299	100.00

Samples: 220 ml. culture at 3×10^6 cells/ml. grown in EMM1.

Pool material obtained by freeze-thawing.

Analysed by Technicon autoanalyser.

TABLE 7

POOL AMINO ACIDS OBTAINED BY DIFFERENT METHODS

Amino acid	umoles of amino acid					
	1	%	2	3	4	5
X	0.1645	4.27	0.1602	0.1720	0.1760	absent
Aspartic acid	0.2290	5.95	0.2140	0.2090	0.2240	0.2350
Threonine	0.2102	5.46	0.2222	0.1908	0.2240	0.2100
Serine	0.2310	6.00	0.2470	0.2500	0.2410	0.2376
Glutamic acid	1.2670	32.89	1.2520	1.2700	1.2870	1.2902
Citrulline	0.1990	5.17	0.1842	0.1802	0.1950	0.2012
Y	0.0210	0.55	0.0240	0.0200	0.0260	absent
Glycine	0.1440	3.74	0.1500	0.1530	0.1452	0.1420
Alanine	0.3960	10.28	0.4160	0.4200	0.4220	0.3889
Methionine	0.0100	0.26	0.0121	0.0104	0.0090	0.0098
Isoleucine	0.0191	0.50	0.0220	0.0180	0.0216	0.0197
Leucine	0.0251	0.65	0.0247	0.0260	0.0242	0.0248
Tyrosine	0.0200	0.52	0.0180	0.0192	0.0208	0.0210
Phenylalanine	0.0517	1.34	0.0526	0.0500	0.0492	0.0520
Lysine	0.3210	8.33	0.3290	0.3198	0.3200	0.3402
Histidine	0.0643	1.67	0.0602	0.0656	0.0647	0.0580
Arginine	0.4590	11.92	0.4492	0.4520	0.4380	0.4620
Total	3.8519		3.8374	3.8260	3.8877	3.6924

1. Pool material obtained by freeze-thawing in water.
2. Pool material obtained by freeze-thawing in 0.2M citrate buffer.
3. Pool material obtained by 5% TCA extraction, 15 minutes at 0°C.
4. Dialysate from cells ruptured by Eaton press.
5. Hydrolysed pool material obtained by freeze-thawing.

Samples: 200 ml. culture at 4 x 10^6 cells/ml.

Cells washed with water, grown in EMM2.

Analysed by Evans autoanalyser.

TABLE 8

AMOUNT AND CONCENTRATION OF POOL AMINO ACIDS

Data from Table 7, sample 1.

The mean volume of S. pombe has been taken as 148 μ^3, the value during the constant volume phase (Mitchison, 1957).

Amino acid	% Composition	uM amino acid per cell x 10^{-9}	Concentration in uM per ml.
X	4.27	0.2101	1.420
Aspartic	5.95	0.2928	1.978
Threonine	5.46	0.2687	1.816
Serine	6.00	0.2953	1.995
Glutamic	32.89	1.6185	10.936
Citrulline	5.17	0.2544	1.719
Y	0.55	0.0271	0.183
Glycine	3.74	0.1840	1.243
Alanine	10.28	0.5059	3.418
Methionine	0.26	0.0128	0.086
Isoleucine	0.50	0.0246	0.166
Leucine	0.65	0.0320	0.216
Tyrosine	0.52	0.0256	0.173
Phenylalanine	1.34	0.0659	0.445
Lysine	8.33	0.4099	2.770
Histidine	1.67	0.0822	0.555
Arginine	11.92	0.5866	3.964
Total		4.8964	

TABLE 9

MEAN COMPOSITION OF POOL MATERIAL FOR EXPONENTIAL PHASE CELLS GROWN IN EMM2

Pool Component	Amount in µµg./cell
Total weight	3.737
Amino acids	0.721
Nucleotide'	0.065
Carbohydrate	0.925
Organic acids	0.050
	1.761

Total pool weight accounted for = 47%

FIGURE 5

TIME COURSE OF POOL NITROGEN EXTRACTION BY COLD 5% TCA

Solid circles: Frozen cells, TCA extracted

Open circles: Unfrozen cells, TCA extracted

Solid squares: Unfrozen cells, water control

Open squares: Frozen cells, water control

FIGURE 6

TIME COURSE OF POOL AMINO ACID EXTRACTION BY COLD 5% TCA

Solid circles: Frozen cells, TCA extracted

Open circles: Unfrozen cells, TCA extracted

Solid squares: Unfrozen cells, water control

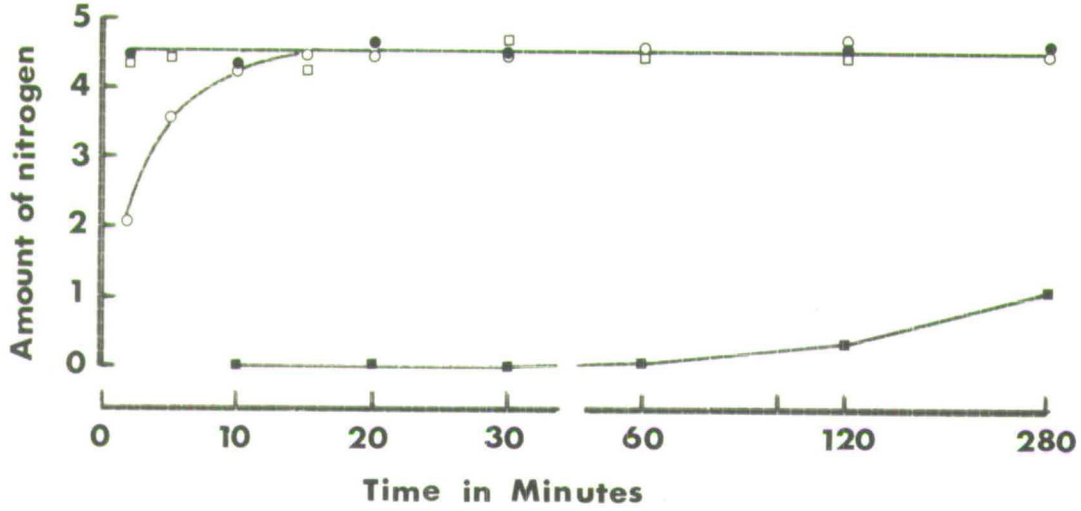
Open squares: Frozen cells, water control

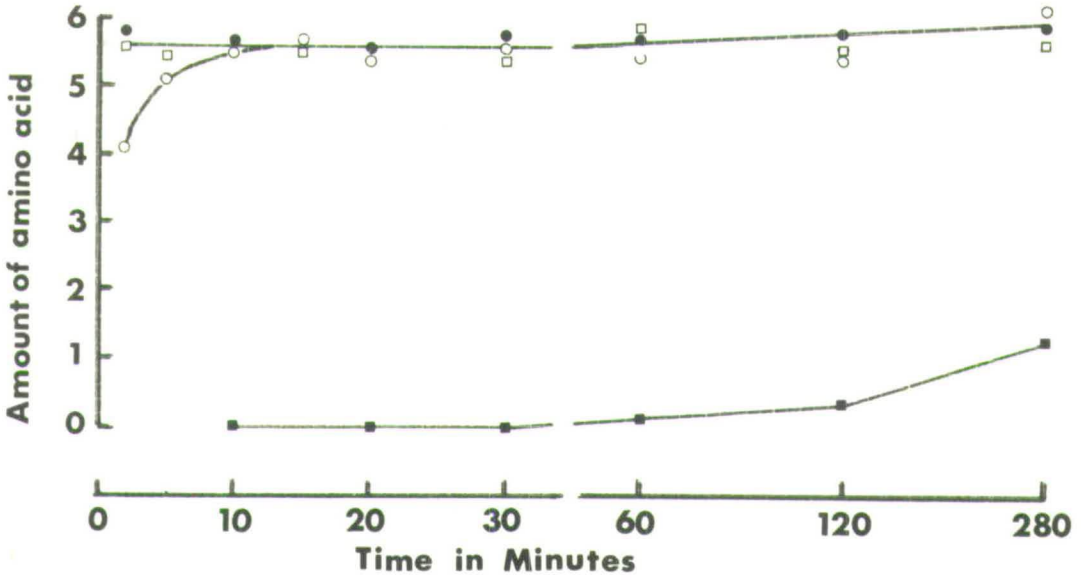
FIGURE 7

TIME COURSE OF EXTRACTION OF POOL UV-ABSORBING MATERIAL

Solid circles: Frozen cells, extracted with cold 5% PCA, O.D. 260 mμ

Open circles: Unfrozen cells, extracted with cold 5% PCA, O.D. 260 mμ


Amount of nitrogen
0
1
2
3
4
5
0
10
20
30
60
120
280
Time in Minutes


Amount of amino acid
0
1
2
3
4
5
6
0
10
20
30
60
120
280
Time in Minutes

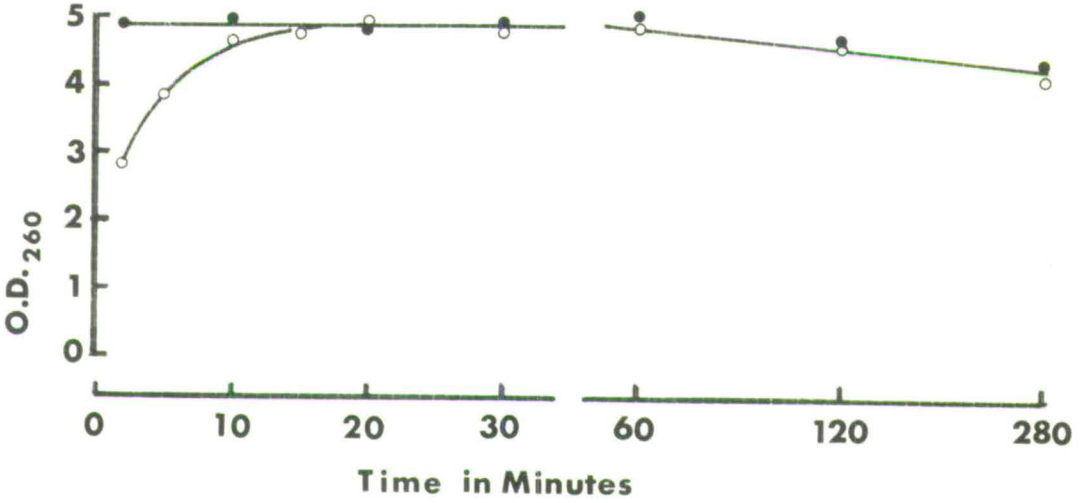
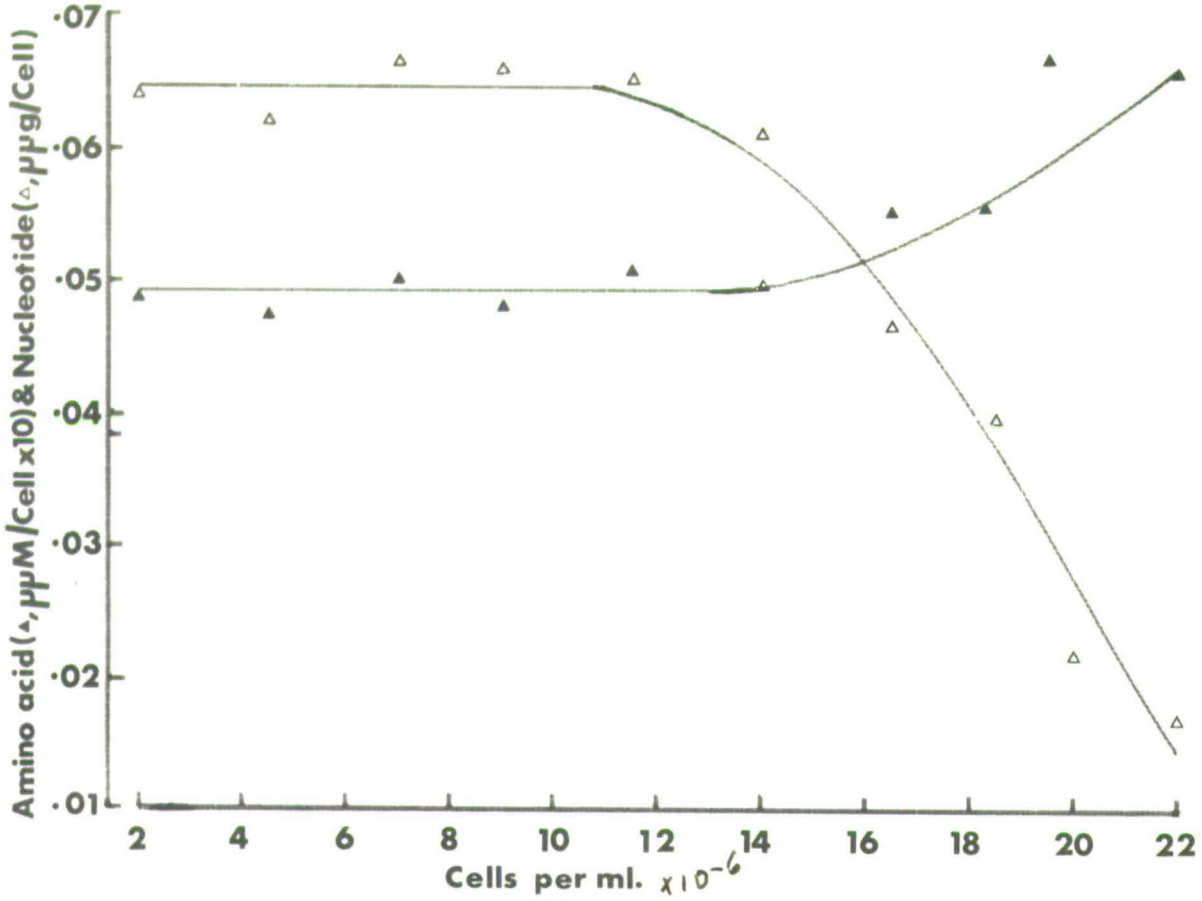

O.D.260
0
1
2
3
4
5
0
10
20
30
60
120
280
Time in Minutes

FIGURE 8

CHANGES IN POOL AMINO ACID AND NUCLEOTIDE MATERIAL DURING GROWTH IN EMM1

FIGURE 9

CHANGES IN POOL AMINO ACID AND NUCLEOTIDE MATERIAL DURING GROWTH IN EMM2


Amino acid(▲,μμM/Cell x10)& Nucleotide(△,μμg/Cell)
·07
·06
·05
·04
·03
·02
·01
2
4
6
8
10
12
14
16
18
20
22
Cells per ml. x10⁻⁶

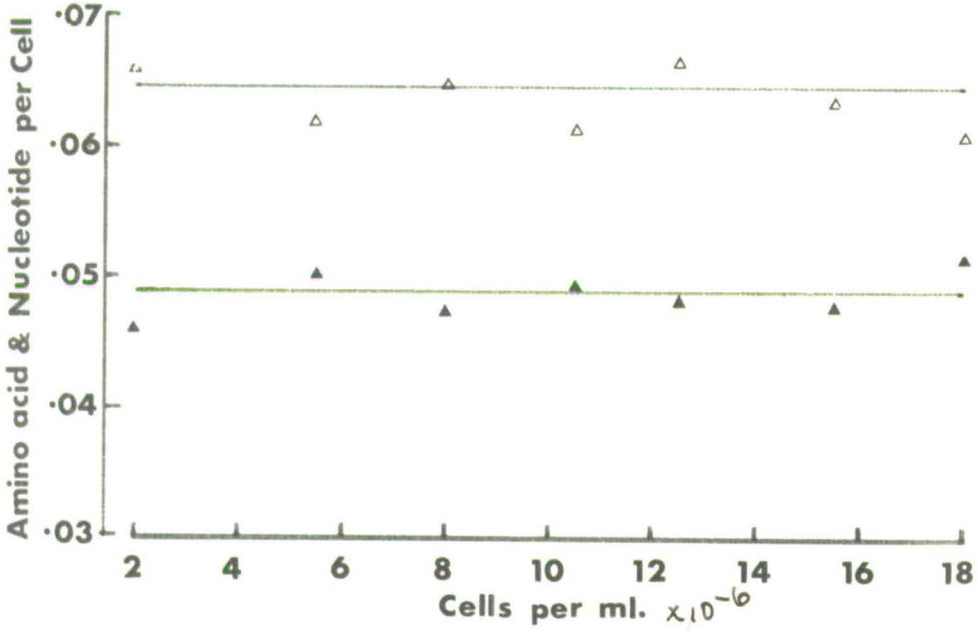

Amino acid & Nucleotide per Cell
·07
·06
·05
·04
·03
2
4
6
8
10
12
14
16
18
Cells per ml. x10⁻⁶

FIGURE 10

CHROMATOGRAM TRACE OF ACIDIC AND NEUTRAL AMINO ACIDS
FROM THE POOL OF CELLS GROWN IN EMM2
ANALYSIS BY EVANS AUTOANALYSER, ACCELERATED METHOD

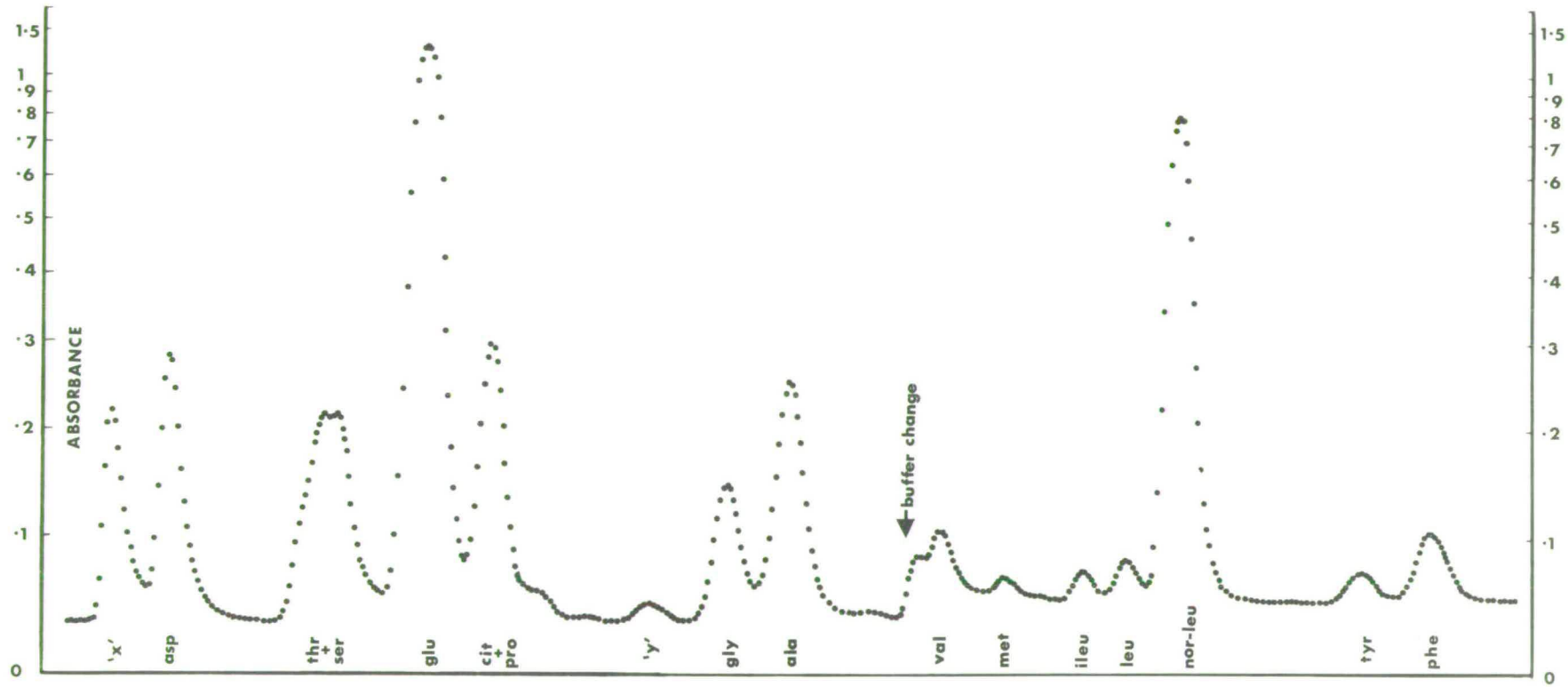
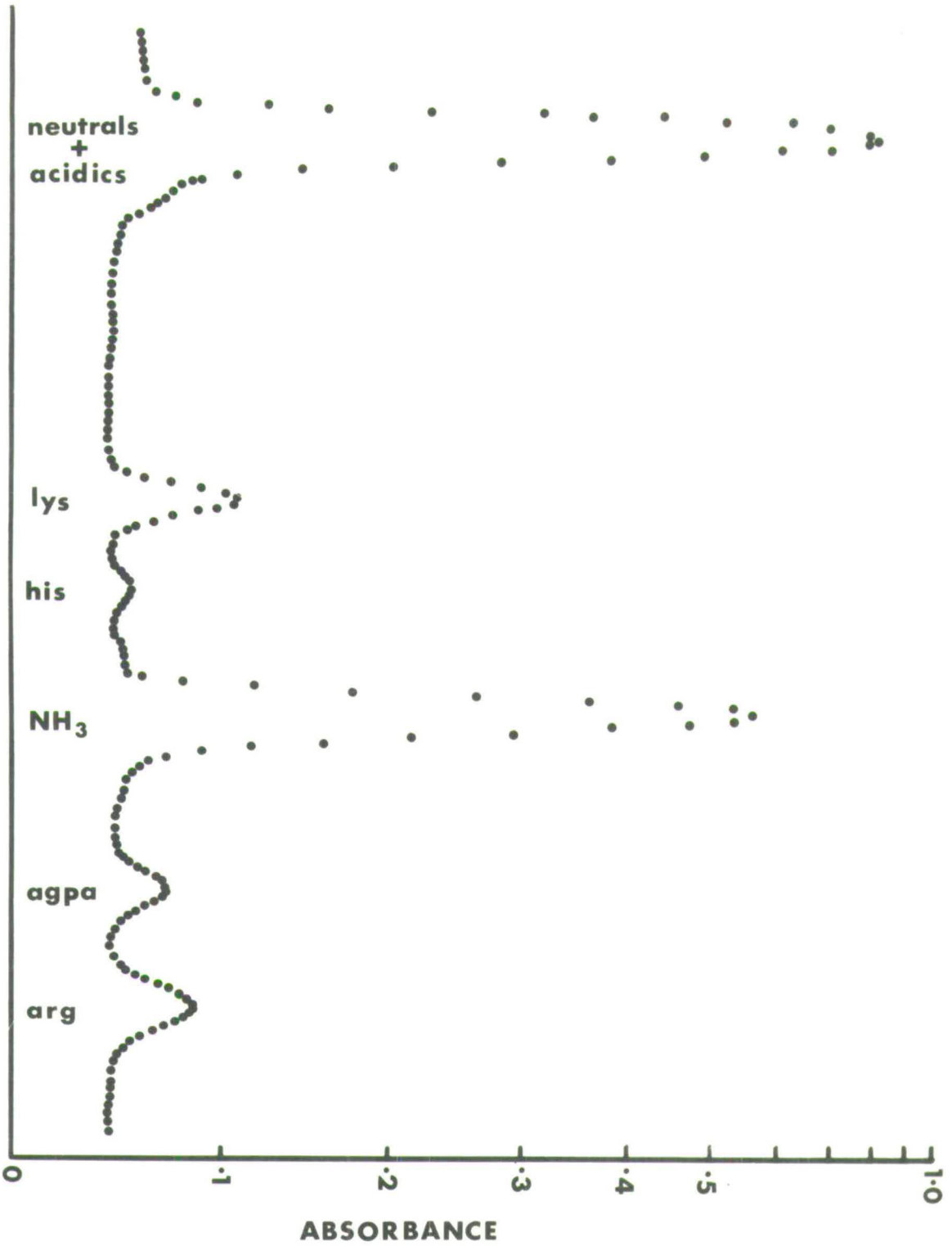

ABSORBANCE
1·5
1
·9
·8
·7
·6
·5
·4
·3
·2
·1
0
'x'
asp
thr + ser
glu
cit + pro
'y'
gly
ala
buffer change
val
met
ileu
leu
nor-leu
tyr
phe

FIGURE 11

CHROMATOGRAM TRACE OF BASIC AMINO ACIDS FROM
THE POOL OF CELLS GROWN IN EMM2
ANALYSIS BY EVANS AUTOANALYSER, ACCELERATED METHOD


neutrals
+
acidics
lys
his
NH3
agpa
arg
0
·1
·2
·3
·4
·5
1·0
ABSORBANCE

Chapter 5

THE SYNTHESIS OF PROTEIN AND RNA IN BATCH CULTURES

GROWTH IN EMM1

The number of cells/ml. during growth in EMM1 shows an exponential increase up to about 10 x 10^6 cells/ml. (Figure 12). Thereafter the rate of increase in cell numbers decreases and eventually the cells stop dividing. The stationary phase concentration in the experiment shown in Figure 12 was just over 20 x 10^6 cells/ml. But from a number of experiments it has been found that the stationary phase concentration varies from 20 to 25 x 10^6 cells/ml. The patterns of increase in protein, RNA and total dry weight are also shown in Figure 12, from which it is clear that these components do not all increase at the same rate after about 4 x 10^6 cells/ml. Moreover, the absolute amounts of RNA and protein appear to decrease sometime after this stage. There cannot, therefore, be balanced growth through much of the growth period in which the concentration of cells increases exponentially. To a certain extent the decreases in amount per cell of proteins and RNA (Figure 13) during exponential growth correlated with the decrease in mean cell length that occurs throughout most of the exponential phase. But plotting the data of this experiment on a per cell basis (Figure 13) shows that even before 4 x 10^6 cells/ml. there is unbalanced growth, in this case the amount of protein per cell apparently

increasing at a time when RNA is more or less constant (up to 3×10^6 cells/ml.) or decreasing (after 3×10^6 cells/ml.). For comparative purposes such data must be related to the stage in bulk culture. Figure 14 shows the amounts of the various components plotted against cell concentration. The data for RNA agrees with the figures given by Mitchison and Lark (1962) for S. pombe also grown in EMM1 at 32°C. The deviation of their data for the amount of RNA per cell from the values given here can be attributed to the fact that each of their determinations was for a separate culture.

The absence of balanced growth is by no means a unique feature. It occurs in S. pombe in other media and other micro-organisms (Johnson, 1968). In the case of S. pombe the lack of balanced growth cannot be simply attributed to the progressive decrease in cell length through batch culture, since the amounts of RNA and protein per cell decrease at different rates, and at some stages of growth their relative amounts alter in opposite directions.

The levels of RNA and protein in stationary phase were found to be more or less constant: RNA = 0.66 ± 0.10 µµg., protein = 2.30 ± 0.28 µµg. These low levels together with the comparatively high levels during early exponential phase imply rapid synthesis at some stage after inoculation. Studies of so-called 'exponential' phase cells of S. pombe have generally referred to cultures at above 10^6 cells/ml. For growing cultures obtained by diluting stationary cultures

one hundred fold, there will be about two generation times before the cells reach 10^6 cells (see Table 10). The pattern of growth after inoculation and before 10^6 cells/ml. was therefore investigated.

GROWTH ON RE-INOCULATION

On re-inoculating stationary phase cells into fresh medium, there was always a lag phase before the number of cells/ml. began to increase. The first division was found to be semi-synchronous, but synchrony was lost after the first division and the cell concentration increased constantly thereafter at the mean exponential phase rate. The length of the lag phase varied between 2 and 6 hours, and correlated positively with the age of the stationary phase culture used as inoculum. There was always a lag of at least two hours, and for a three day old stationary phase culture the lag was about 6 hours. The pattern of increase of RNA and protein per cell in such a culture is shown in Figure 15, together with the increase in cell length during the lag phase. The amounts of RNA and protein in the stationary phase cells used as inoculum are also shown. It is clear that during the lag phase and first semi-synchronous division there are abnormally large increases on a per cell basis of protein and RNA. The amount of RNA increases over tenfold, while the amount of protein more than doubles. The early exponential amounts of RNA and protein per cell (Figure 13) are therefore regenerated during

the lag phase. It is interesting to note that the rate of RNA synthesis during the lag phase is greater than the mean rate for exponential phase cells, firstly because exponential phase cells less than double their amount of RNA per cell, and, secondly, because the doubling time of RNA is just under two hours during lag phase (Figure 15), and therefore well under the mean generation time.

Considerable time was spent in attempts to find the cause of unbalanced growth in S. pombe and to rectify this situation. This meant that much of the early work on pool amino acids and protein synthesis was done on cells in EMM1 growing in an unbalanced state. It should be noted that unbalanced growth is a feature even of cells grown in a complex medium. Johnson (1968) has shown that for S. pombe grown in MEB there is a progressive decrease in cell length and glucose content per cell during 'exponential' growth, with a re-generation of the early exponential values in the lag phase. Moreover, Mitchison (1957) found that the rate of dry mass increase in single cells never quite doubled in the second cell cycle. The absence of balanced growth is unlikely to be due solely to a low level of the carbon source since MEB is rich in peptides, amino acids and fermentable sugars other than glucose (see Appendix I). More or less balanced growth during batch culture up to 8×10^6 cells/ml. was eventually achieved by increasing thirty fold the amount of phosphate in the medium (EMM2). This effect of phosphate was discovered by Bostock

(1968) during investigations into DNA synthesis in S. pombe. Before considering growth in this higher-phosphate medium, the effects of limiting sulphur and glucose on the growth of S. pombe are considered.

SULPHUR AND GLUCOSE LIMITATION

The nature of the unbalanced growth in EMM1 is such that the cells tend towards the stationary phase state before the rate of cell division decreases. The rationale of the experiments designed to find the cause of unbalanced growth was therefore to find what factors limited growth and resulted in their becoming stationary. It is in fact clear that some factor limits growth in EMM since yields of over 40 x 10^6 cells/ml. can be obtained with growth on MEB.

The pattern of growth in a culture without added sulphate is striking. As shown in Figure 16, the growth of such a culture, as monitored by optical density, falls abruptly at one point. Two hours before this sharp fall in growth rate, the mean cell length begins to decrease, that is, growth in length of the cells appears to cease, but division continues. At the time when the growth rate rapidly drops, the cells appear identical to stationary phase cells.

If cells are grown in medium with low levels of sulphate, the final yields of cells at the end of the growth phase is less than normal, as shown in Figure 17. The size of the stationary phase cells in all cultures were just like normal

stationary phase cells. The final yield of cells does not appear to be affected until the level of sulphur in the medium drops below 3 mg./litre. Since the normal amount of sulphur (in the form of sodium sulphate) in EMM is 6.4 mg./litre, cessation in growth cannot be due to lack of sulphur. The fact that quite appreciable growth can occur in the absence of sulphur, as sodium sulphate, can be attributed to impurities of sulphur in other constituents of the medium and the sulphur present in the inoculum.

The final cell concentration in cultures grown at various levels of glucose, above and below the normal amount in EMM (10 gm./litre) are shown in Figure 18. It appears that glucose levels above the normal do result in higher cell concentrations, but not appreciably higher. An increase of 2½ times over the normal amount of glucose only increases the cell concentration from 24 to 30 x 10^6 cells/ml., i.e. by about 20%. Glucose deficiency therefore seems unlikely to be the sole factor causing the cells to enter the stationary phase. Moreover, the mean cell length of the cells in the stationary phase <u>decreases</u> with increase in the level of glucose in the medium in which the cells were grown (Figure 18). It is therefore unlikely that growth of cells in EMM is unbalanced due to limiting glucose. If it were, one would anticipate an <u>increase</u> in the mean cell length at the end of the growth phase with increases in the level of glucose.

GROWTH IN EMM2

The amounts of RNA and protein per cell during growth in EMM2 are shown in Figure 19, together with the changes in cell length. Increase in cell number is exponential up to 15 x 10^6 cells/ml. compared with 10 x 10^6 cells/ml. for EMM1, but there is balanced growth at least up until 8 x 10^6 cells/ml. Thereafter the amount of protein and RNA per cell drops, as does the mean cell length. Bostock (1968) has shown that the amount of DNA per cell also remains constant up to 8 x 10^6 cells/ml., but that thereafter the amount per cell also drops.

The mean composition of cells during exponential growth in EMM2 is summarised in Table 11. The total dry weight of the pool was obtained by the method described in Chapter 8. The dry weight of the cell wall was obtained by weighing the cell after extracting the cytoplasm using the extraction procedure of Johnson (1965). Cytoplasmic material is removed by digesting with 17.5% (w/w) NaOH at 90°C for 30 minutes prior to filtering and washing the "ghosts" that remain.

Two further experiments are described in this chapter although their relevance to the overall work will be considered only in the general discussion (Chapter 10). These experiments concern the effect of malonate and of high osmotic pressure on aspects of growth.

THE EFFECT OF MALONATE ON GROWTH

It is clear from the experiment shown diagrammatically in Figure 20 that malonate (added at time indicated by arrow 1) depresses the growth of cells and that this effect is reversible by succinate (added at the time indicated by arrow 2). Succinate was added in the form of sodium succinate and this was adjusted to pH 4.6 with sodium hydroxide before addition to the culture.

THE EFFECT OF HIGH OSMOTIC PRESSURE ON THE AMINO ACID POOL

S. pombe will grow in EMM2 with the addition of 2M sorbitol. The generation time is increased to over 6 hours and there is a considerable lag phase before exponential growth commences. But the osmotic pressure of this medium is over 30 times greater than that of normal EMM2 so that if amino acids were important in osmoregulation we would anticipate a corresponding increase in the size of the amino acid pool. The composition of the amino acid pool of cells grown to 3.6×10^6 cells/ml. in EMM2 with 2M sorbitol was determined and the result is given in Table 12. It is clear that there is no significant increase in the total amount of pool amino acid material per cell, nor is the composition of the pool greatly altered from that in normal EMM2 (see Chapter 4). It has in fact recently been demonstrated that in Saccharomyces the most important osmotic component of the pool is trehalose and amino acids are not involved (K. Ikeda and P. Ottolenghi, per. comm.).

TABLE 10

Schematic growth in cell numbers of S. pombe throughout batch culture for an inoculation dilution of 1 to 100 from a stationary phase culture at 20×10^6 cells/ml.

Generation	Time (hours) from inoculation to end of generation	Cells/ml. $\times 10^{-6}$	
Lag	2	0.2	
1	4½	0.4	
2	7	0.8	
3	9½	1.6	
4	12	3.2	
5	14½	6.4	
6	17	12.8	
7	19½	25.6	Stationary

TABLE 11

MEAN COMPOSITION OF CELLS IN EXPONENTIAL GROWTH IN EMM2

Component	Amount in µµg./cell
Total weight	27.05
Protein	7.85
RNA	3.74
DNA	0.037
Cell wall	8.12
Pool	3.74
	23.49

Total weight accounted for = 87%

Data for DNA from Bostock (1968)

TABLE 12

THE AMINO ACID POOL OF CELLS GROWN IN 2M SORBITOL

Cells grown in EMM2 with 2M sorbitol. Sample for analysis filtered, washed with 2M sorbitol and the pool extracted by freeze-thawing.

Amino acid	µmoles amino acid	% Composition
X	0.1483	4.26
Aspartic acid	0.2118	6.08
Threonine	0.1907	5.48
Serine	0.2120	6.09
Glutamic acid	1.1965	34.37
Citrulline	0.1825	5.24
Y	0.0212	0.61
Glycine	0.1306	3.75
Alanine	0.3637	10.45
Methionine	0.0092	0.26
Isoleucine	0.0177	0.51
Leucine	0.0229	0.66
Tyrosine	0.0187	0.54
Phenylalanine	0.0480	1.38
Lysine	0.2930	8.42
Histidine	0.0600	1.72
Arginine	0.3540	10.17
	3.4808	

Samples analysed: 200 ml. culture at 3.6×10^6 cells/ml.

Total amount amino acid per cell = 4.8344 µmoles.

FIGURE 12

CHANGES IN DRY WEIGHT, PROTEIN AND RNA DURING GROWTH IN EMM1 INTO STATIONARY PHASE

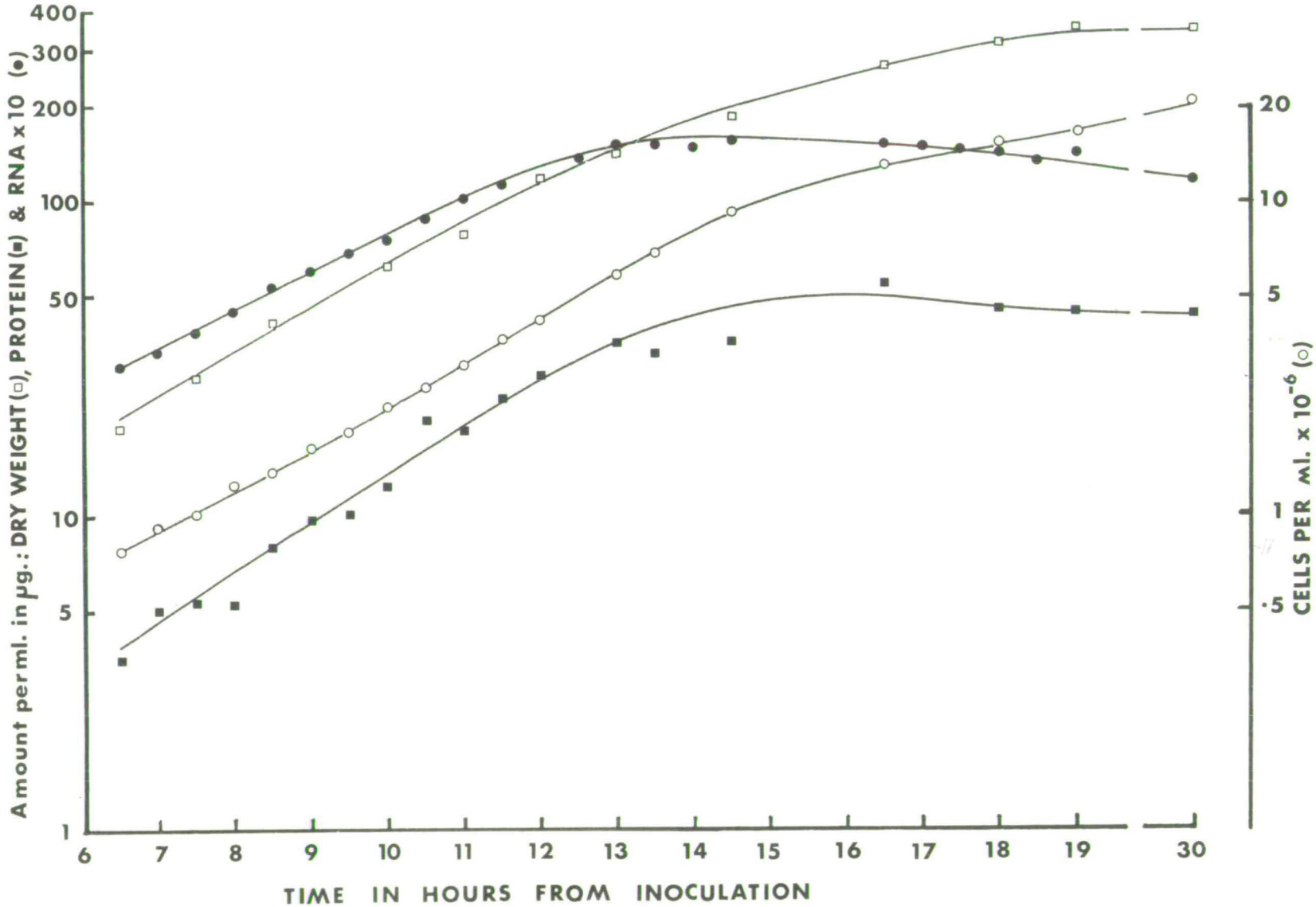

Amount per ml. in μg.: DRY WEIGHT (□), PROTEIN (■) & RNA x 10 (●)
CELLS PER ml. x 10⁻⁶ (○)
400
300
200
100
50
10
5
1
20
10
5
1
·5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
30
TIME IN HOURS FROM INOCULATION

FIGURE 13

CHANGES PER CELL IN DRY WEIGHT, PROTEIN AND RNA DURING GROWTH IN EMM1 INTO STATIONARY PHASE

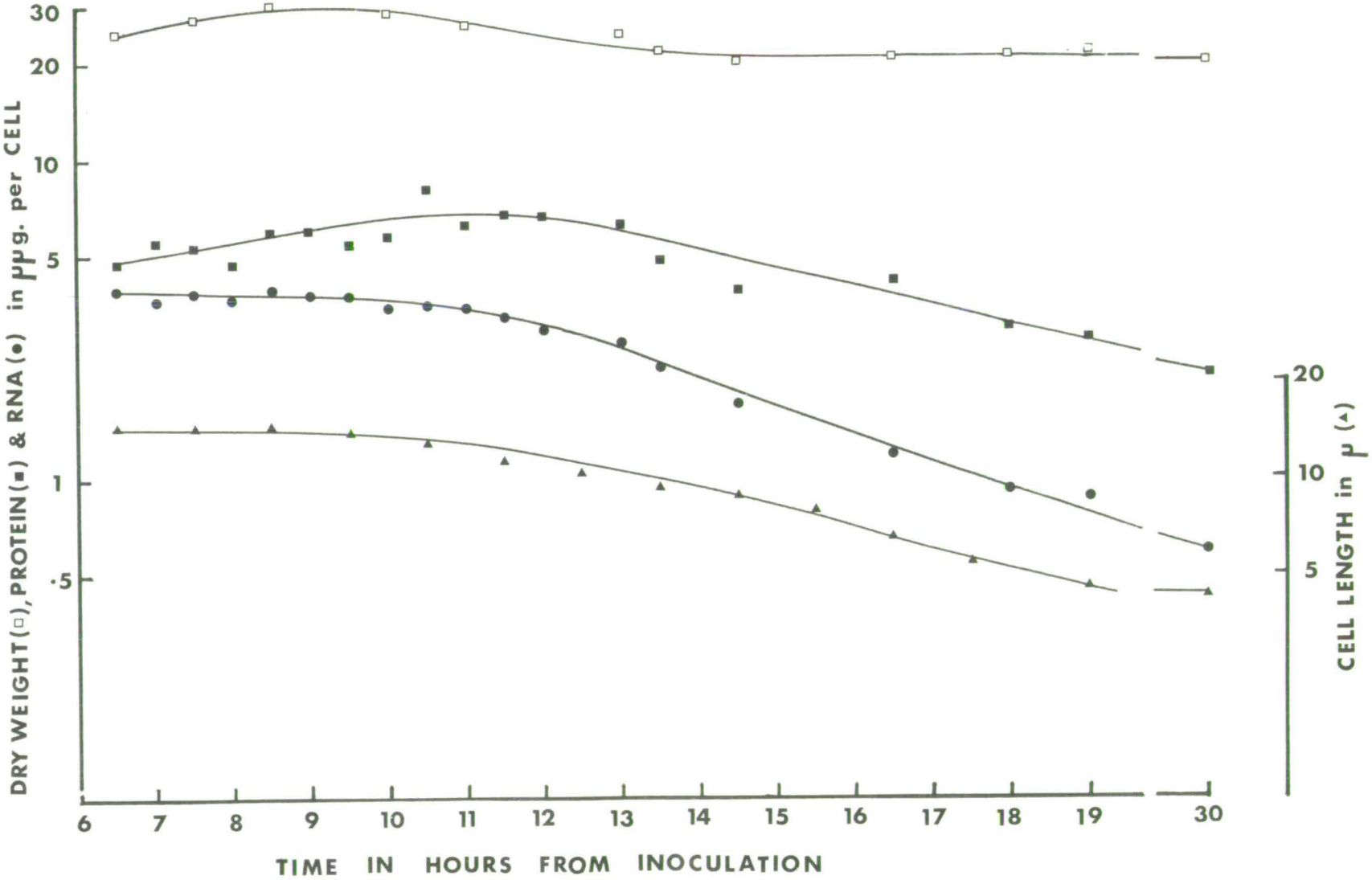

30
20
10
5
1
·5
DRY WEIGHT (□), PROTEIN (■) & RNA (●) in μμg. per CELL
20
10
5
CELL LENGTH in μ (▲)
6
7
8
9
10
11
12
13
14
15
16
17
18
19
30
TIME IN HOURS FROM INOCULATION

FIGURE 14

DRY WEIGHT, PROTEIN, RNA AND CELL LENGTH
AT VARIOUS STAGES OF GROWTH IN EMM1

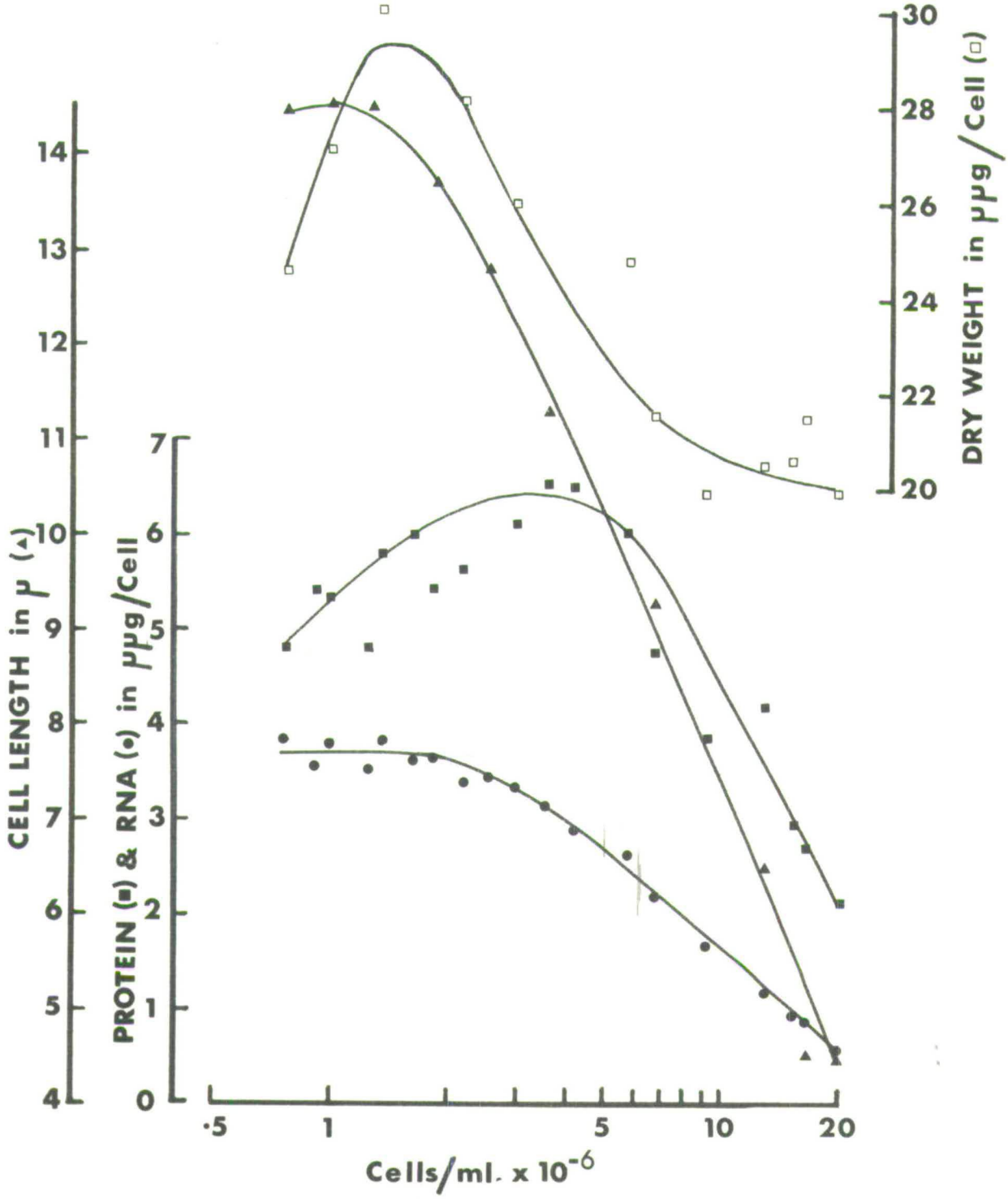

CELL LENGTH in μ (▲)
PROTEIN (■) & RNA (●) in μμg/Cell
DRY WEIGHT in μμg/Cell (□)
Cells/ml. x 10⁻⁶
·5
1
5
10
20
14
13
12
11
10
9
8
7
6
5
4
7
6
5
4
3
2
1
0
30
28
26
24
22
20

FIGURE 15

CHANGES PER CELL IN PROTEIN, RNA AND CELL LENGTH ON RE-INOCULATING 3 DAY OLD STATIONARY CELLS INTO FRESH EMM1

The amount of protein and RNA and the cell length of the stationary phase cells are indicated at the stage marked by an arrow 'S'.

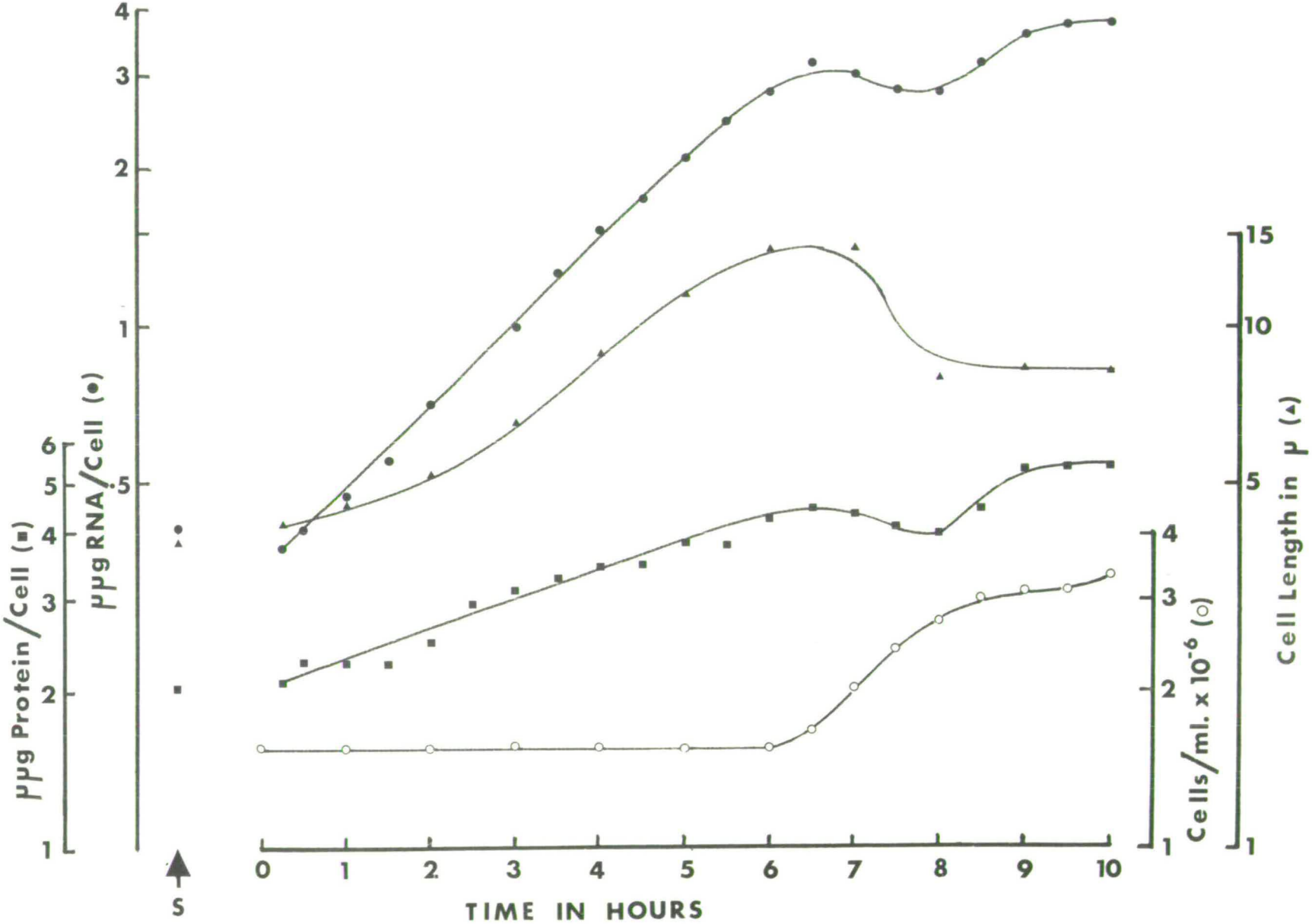

μμg Protein/Cell (■)
μμg RNA/Cell (●)
Cells/ml. x 10⁻⁶ (○)
Cell Length in μ (▲)
TIME IN HOURS
S
0
1
2
3
4
5
6
7
8
9
10

FIGURE 16

THE EFFECT OF LIMITING SULPHATE ON GROWTH IN EMM1

Squares: Culture grown in medium without sodium sulphate

Circles: Control culture grown in normal EMM1

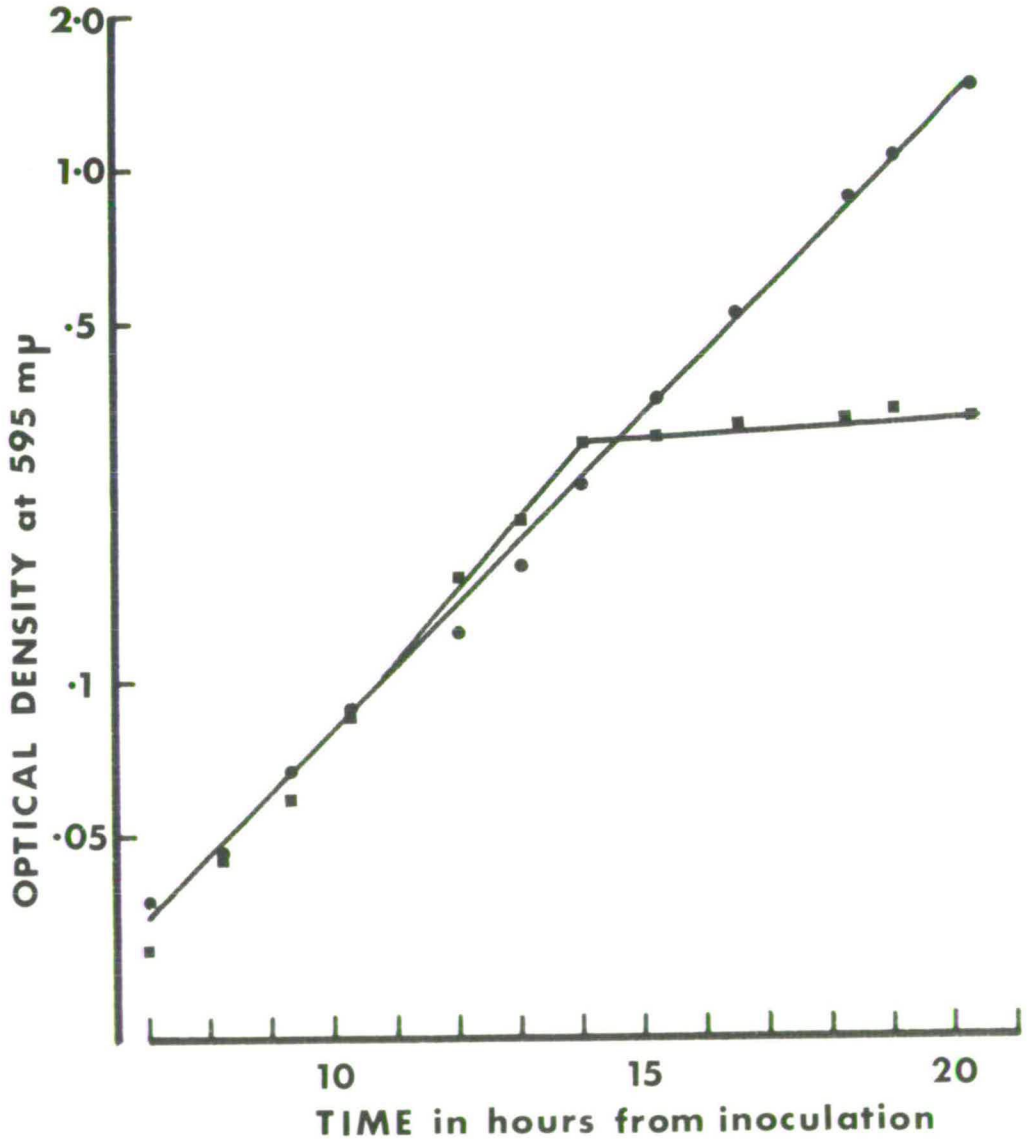

2·0
1·0
·5
·1
·05
OPTICAL DENSITY at 595 mμ
10
15
20
TIME in hours from inoculation

FIGURE 17

THE YIELD, IN DRY WEIGHT, OF CELLS GROWN IN EMM1
WITH VARIOUS LEVELS OF SULPHUR

The normal level of sulphur in EMM1 is 6.4 mg./ml. Only concentrations of sulphur below the normal level were tested.

The cells were grown to the stationary phase before harvesting, and 20 ml. aliquots weighed on tared filters.

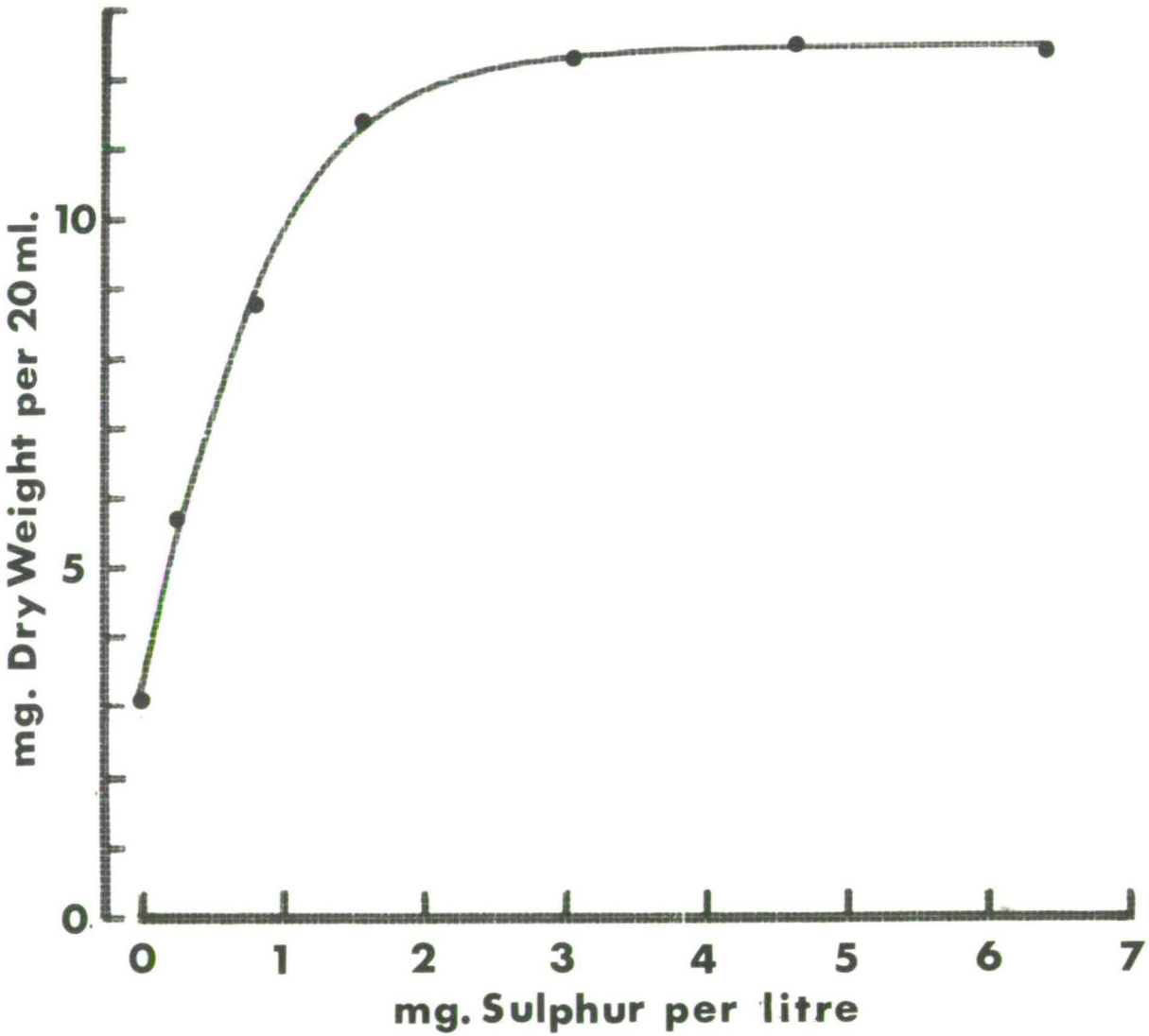

mg. Dry Weight per 20 ml.
10
5
0
0
1
2
3
4
5
6
7
mg. Sulphur per litre

FIGURE 18

STATIONARY PHASE CELL LENGTH AND CONCENTRATION RESULTING FROM GROWTH IN EMM1 WITH VARIOUS AMOUNTS OF GLUCOSE UP TO 2½ TIMES THE NORMAL AMOUNT

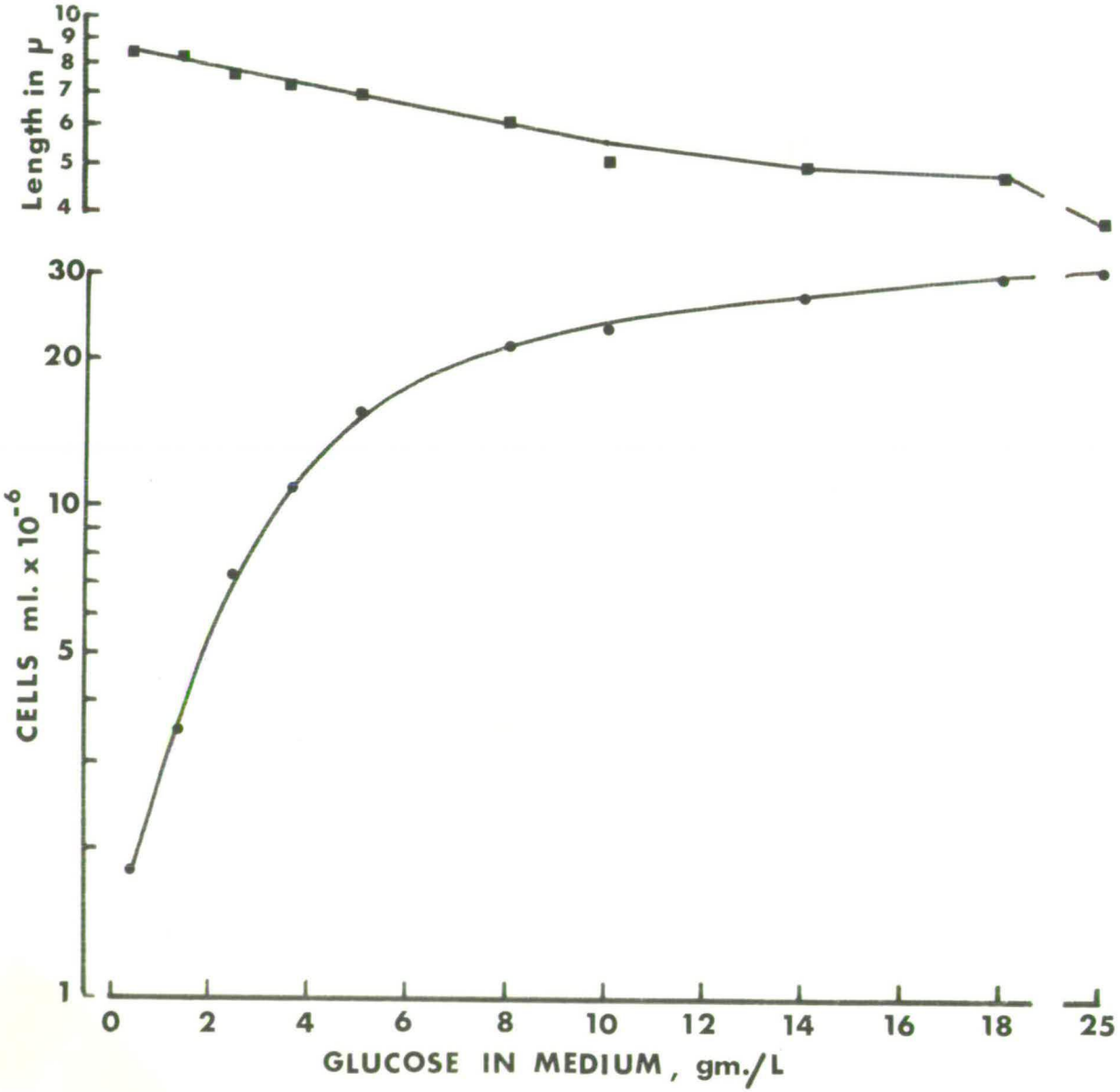

Length in μ
10
9
8
7
6
5
4
CELLS ml. x 10⁻⁶
30
20
10
5
1
0
2
4
6
8
10
12
14
16
18
25
GLUCOSE IN MEDIUM, gm./L

FIGURE 19

AMOUNT PER CELL OF PROTEIN AND RNA, AND MEAN CELL LENGTH AT VARIOUS STAGES OF GROWTH IN EMM2

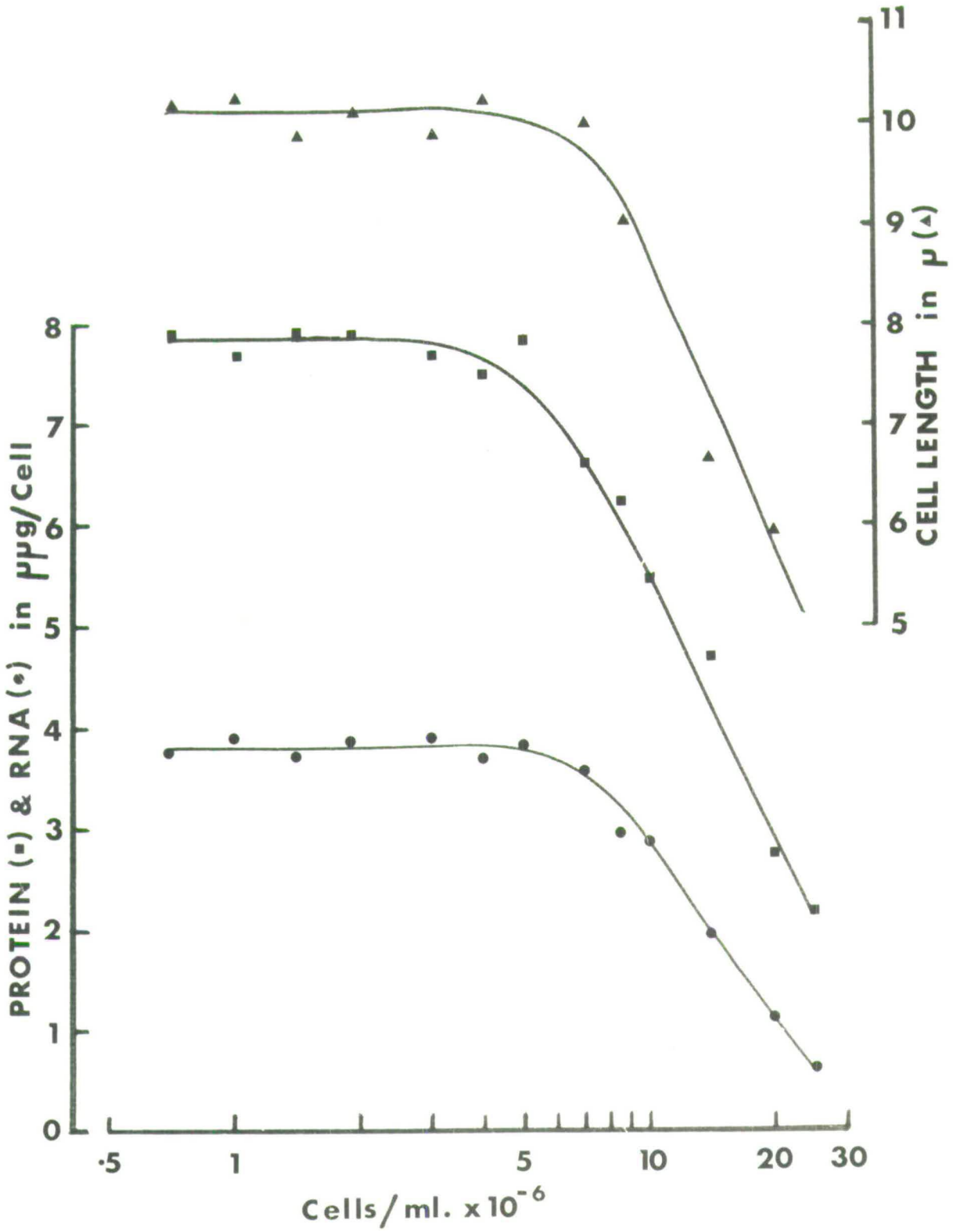

PROTEIN (▪) & RNA (•) in μμg/Cell
CELL LENGTH in μ (▲)
Cells/ml. x 10⁻⁶
0
1
2
3
4
5
6
7
8
5
6
7
8
9
10
11
·5
1
5
10
20
30

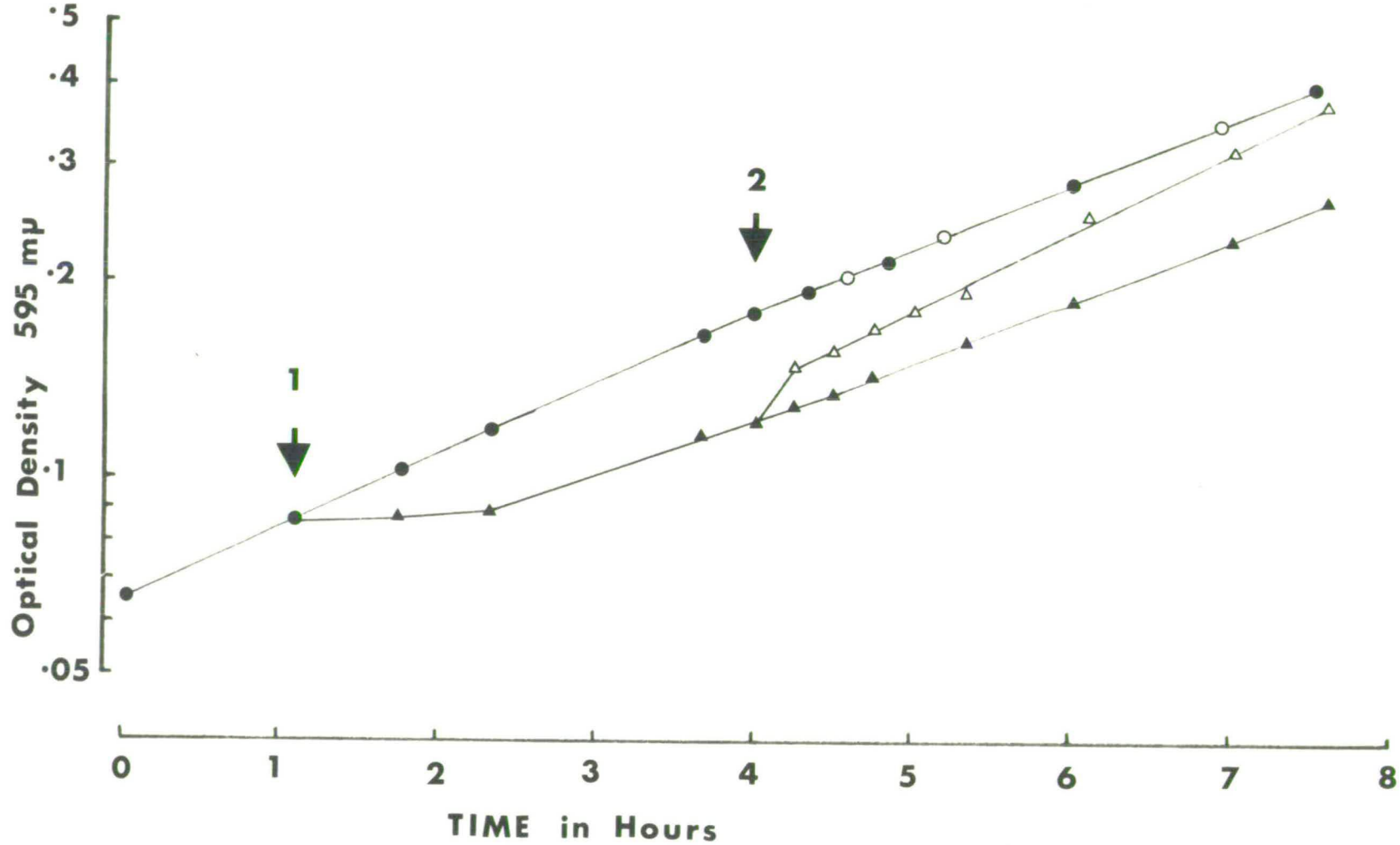
FIGURE 20

GROWTH OF CELLS IN EMM1 SHOWING THE EFFECT OF MALONATE AND SUCCINATE

Growth of an untreated aliquot of the culture is shown by the solid circles.

At the time indicated by the arrow 1, the culture was divided and malonate (2×10^{-3} M) was added to one half (solid triangles).

At the time indicated by the arrow 2, each of the two cultures were again divided and succinate (5×10^{-3} M) added to one of each (open triangles, open circles).


·5
·4
·3
·2
·1
·05
Optical Density 595 mμ
1
2
0
1
2
3
4
5
6
7
8
TIME in Hours

Chapter 6

EXPERIMENTS WITH RADIOACTIVELY LABELLED POOL COMPONENTS

The fate of carbon-containing pool compounds in the cell can be followed by brief immersion of the cells in ^{14}C-glucose. However, in EMM in which glucose is the sole carbon source, the specific activity of added labelled glucose is so greatly reduced that negligible activity is found in the cells even after labelling for two hours. This problem was overcome by labelling in EMM2 prepared without glucose. This medium lacking glucose will be termed glucose-less EMM2. Cells grown in normal EMM2 were centrifuged at 32°C, the supernatant decanted and the cells then resuspended in the same volume of glucose-less EMM2 at 32°C to which ^{14}C-glucose had been added. The uptake of label and its incorporation into cold TCA insoluble compounds is shown in Figure 21. Between 2 and 6 minutes from the time of labelling the percentage of counts in the pool was over 82%.

If the pool is labelled in this way for a five-minute period and the cells then resuspended in the original medium, there is a rapid disappearance of counts from the pool, with appearance of the label in cold TCA insoluble material as shown in Figure 22. We may conclude that the carbon-containing pool materials are largely precursors of macromolecules. There are, however, two features of this experiment that require

clarification. Firstly, why does the total count in the cells decrease after resuspending labelled cells in EMM2? Secondly, does suspending cells for five minutes in glucose-less medium cause any loss of pool material? These points are now considered.

The loss of total counts from the cells in the above experiment could be due to loss as carbon dioxide or in part to loss by exchange from a 'free space' in the cells (Briggs et al., 1961). If the loss is due to exchange from a free space, it should not occur on resuspending briefly labelled cells in water instead of fresh EMM. But, as shown by the data in Table 13, there is no significant difference in the total counts in cells incubated in water or EMM2 up to two hours after labelling of the pool. Moreover, as shown below, significant amounts of carbon dioxide are lost from exponential cells and the loss of total counts is attributed to this. Resuspending exponential phase cells in water at 32°C for five minutes revealed no significant difference in the pool amino acid, 260 mμ absorbing material, or in total pool weight (see Table 14). The procedure for rapid labelling of the pool does not therefore cause any gross alteration in the existing pool.

While brief immersion experiments of the above type will reveal the immediate fate of pool carbon compounds, overall changes in the distribution of carbon in the cells can be answered by prolonged growth in labelled medium so that

uniform labelling is achieved. Figure 23 shows the distribution of carbon in cell components during growth in EMM2. The results essentially confirm the chemical assays of Chapter 5 and show that growth is balanced in EMM2 up to at least 8×10^6 cells/ml. The mean percentage of cellular carbon in the pool during exponential growth was 10.1% and increased to 13.6% in the stationary phase. The total counts in the cells plus medium were obtained by drying aliquots of the culture on planchets so that any dissolved carbon dioxide would be lost. It is clear from Figure 23 that the rate of loss of counts from the total culture is higher during stationary phase than during the exponential phase. There is an increase in this loss from 40,000 counts/hour/3 ml. aliquot in exponential phase to 62,000 counts/hour/3 ml. aliquot in the stationary phase.

Replicate cultures of 10 ml. were grown in tightly stoppered Universal containers to 5×10^6 cells/ml. (exponential phase) and to 24×10^6 cells/ml. (stationary phase). Five ml. of saturated barium hydroxide solution was injected by means of a hypodermic needle into each of the cultures through the rubber washer in the lid. This was made possible by using lids with a small hole drilled in the top. The containers were then well shaken and subsequently aliquots removed, dried on planchets and counted. In this way over 97% of the original ^{14}C-glucose counts could be accounted for. The loss of counts in the experiment shown in Figure 22 is therefore most likely due to the loss of carbon dioxide.

The flow into macromolecules of precursors synthesized from glucose is not affected by addition of precursors to the medium. This was shown in the following manner. Two equal aliquots from a culture at 3 x 10^6 cells/ml. were labelled by suspending the cells for five minutes in glucose-less medium with added ^{14}C-glucose. One of these aliquots (C) contained in addition 0.1 mg./ml. of eight different amino acids. After the five-minute labelling period the other aliquot was divided into two and the cells resuspended in either EMM2 with the addition of the eight amino acids (A) or without added amino acids (B). As shown in Figure 24, addition of amino acids to the medium after the labelling period has no detectable effect on the flow of existing labelled pool material into cold TCA insoluble material. However, addition of amino acids during the labelling period (C) markedly reduces the subsequent incorporation of ^{14}C into macromolecular material. This competitive effect must occur before or at the time the amino acids enter the pool which contains the immediate precursors of macromolecules since amino acids already in this pool are not affected.

Further evidence for the presence of a separate pool for amino acids derived from the medium was obtained by use of labelled amino acids. As shown in Figure 25, the uptake of an added amino acid is very rapid, the pool reaching a constant size after about twenty minutes. If further non-labelled amino acid is added at this time, uptake of label practically

ceases, but the amount of labelled material in the pool remains constant. Unlike pool material synthesized from glucose, amino acid derived directly from the medium is not on the main pathway of macromolecular synthesis and is 'diluted' by addition of further quantities of the amino acid to the medium. If only trace quantities of a labelled amino acid are added to the medium, uptake of up to 50% of all the material occurs within two hours, as shown in Figure 26. No further labelled amino acid is removed from the medium, even after several hours. Under these circumstances the pool first increases in size and then, as the rate of total uptake begins to fall off, the labelled pool material becomes exhausted. Similar results to those obtained with lysine were obtained with ^{14}C uniformly labelled arginine, alanine and leucine. Serine appeared not to be accumulated by S. pombe, as no counts could be found in cells even after two hours incubating with ^{14}C serine, uniformly labelled. It was also established that uptake of amino acids from the medium required the presence of glucose and did not occur at $4^{o}C$.

These results demonstrate the existence of a separate pool for amino acids derived from the medium and that this material passes into the pool of amino acids synthesized from glucose before incorporation into macromolecules, as found in Candida utilis by Cowie and Walton (1956). These two pools will be named the 'internal' and 'expandable' pools as suggested by Cowie and Walton (1956). Evidence for the

presence of an expandable pool of amino acids in S. pombe grown in Malt Extract Broth is presented in Chapter 8.

The results of certain experiments reported in this chapter are apparently anomalous. For the experiments shown in Figures 24 and 25 are essentially the same, but the results appear different. Figure 25 shows that addition of excess of an amino acid markedly reduces the transfer of that amino acid in the existing pool into macromolecules. Figure 24, on the other hand, shows that addition of excess non-radioactive amino acids after labelling the pool, has no significant effect on the transfer of pre-labelled pool material into macromolecules (Figure 24, A). The explanation of this must be that an amino acid in the expandable pool is readily 'diluted' by further additions of that amino acid to the medium while the internal pool material is not affected, and that the flux of amino acid from the expandable pool into macromolecules must be very small compared with that derived from endogenously synthesized amino acid.

TABLE 13

COMPARISON OF THE RETENTION OF LABELLED POOL MATERIAL IN CELLS INCUBATED IN WATER AND EMM2

Cells were suspended in glucose-less EMM2 with ^{14}C-glucose for 6 minutes. Equal aliquots were then filtered and resuspended in either water or EMM2 both at 32°C. 3 ml. samples were removed at intervals, filtered and washed with water before counting.

Time in minutes	Counts per 3 ml. sample	
	Cells incubated in water	Cells incubated in EMM2
5	176	182
15	158	160
30	160	150
60	148	155
120	154	158

TABLE 14

THE EFFECT OF POOL COMPONENTS OF SUSPENDING CELLS IN WATER AT 32°C FOR FIVE MINUTES BEFORE EXTRACTING THE POOL

Cells were harvested at 4.8×10^6 cells/ml., filtered and rapidly washed once on the filters with 20 ml. ice-cold water. The pool was then extracted immediately by freeze-thawing in water, or after suspending the cells for 5 minutes in water at 32°C. In the latter case the cells were filtered after the 5 minutes at 32°C, resuspended in ice-cold water and then frozen.

	Pool extracted directly	Pool extracted after 5' in water at 32°C
Dry weight of pool (% total cell dry weight)	13.4	13.2
Pool amino acid	0.432	0.430
Pool 260 mμ absorbing material	0.232	0.240

Pool dry weight determined as described in Chapter 8.

Amount of pool amino acid and 260 mμ absorbing material in arbitrary units.

FIGURE 21

THE TOTAL UPTAKE (CIRCLES) AND INCORPORATION (SQUARES) OF ^{14}C-GLUCOSE BY CELLS IN GLUCOSE-LESS EMM2

The dotted line is the difference between the total uptake and incorporation, and represents the pool.

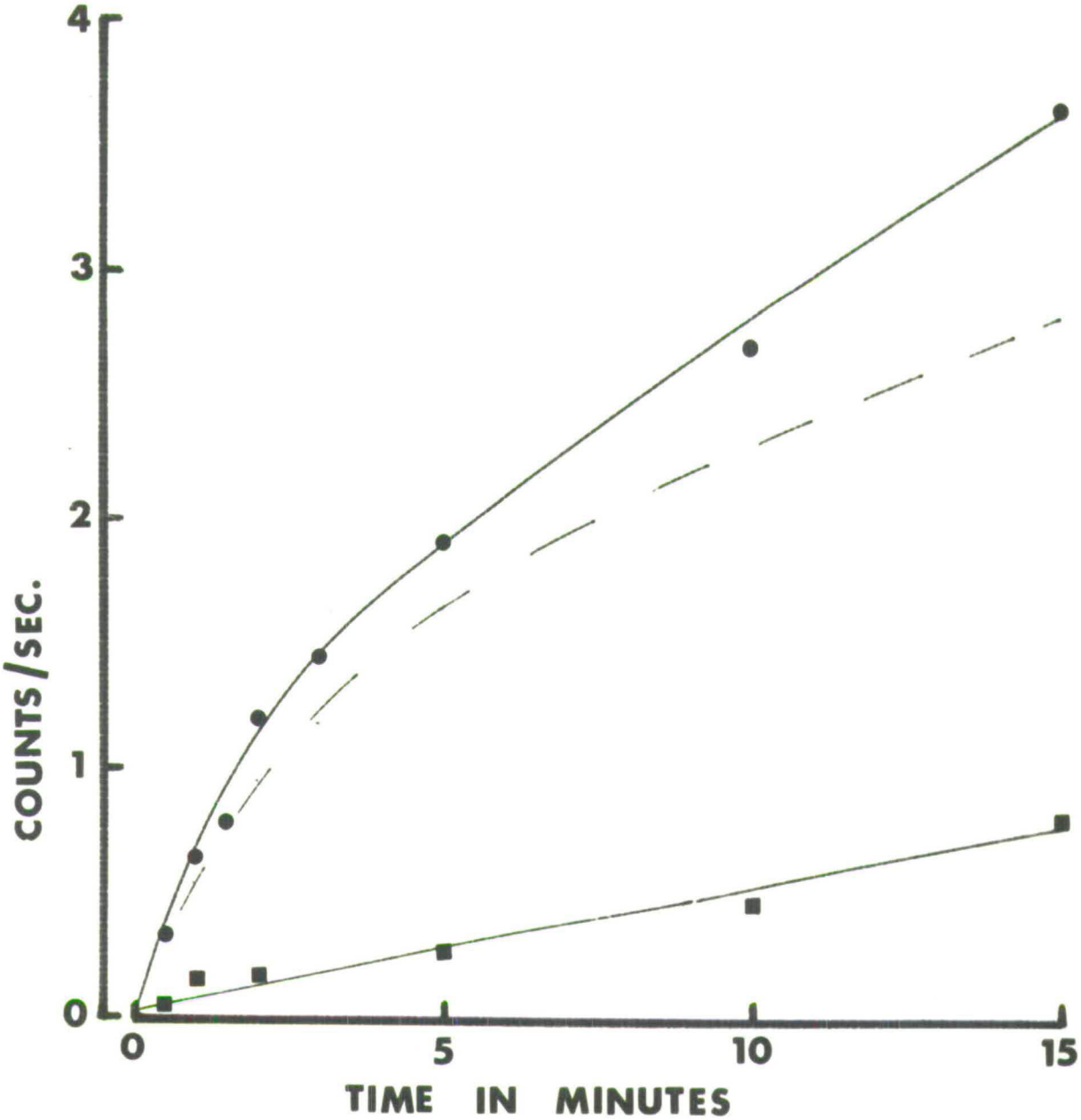

4
3
2
1
0
COUNTS/SEC.
0
5
10
15
TIME IN MINUTES

FIGURE 22

TRANSFER OF ^{14}C FROM THE POOL (DOTTED LINE)
TO MACROMOLECULES (SQUARES)

Exponential cells were labelled with ^{14}C by immersion in glucose-less EMM2 for 5 minutes with ^{14}C-glucose added. The cells were then transferred to non-radioactive EMM2 and samples removed for estimation of total uptake (circles) and incorporation into TCA insoluble material (squares).

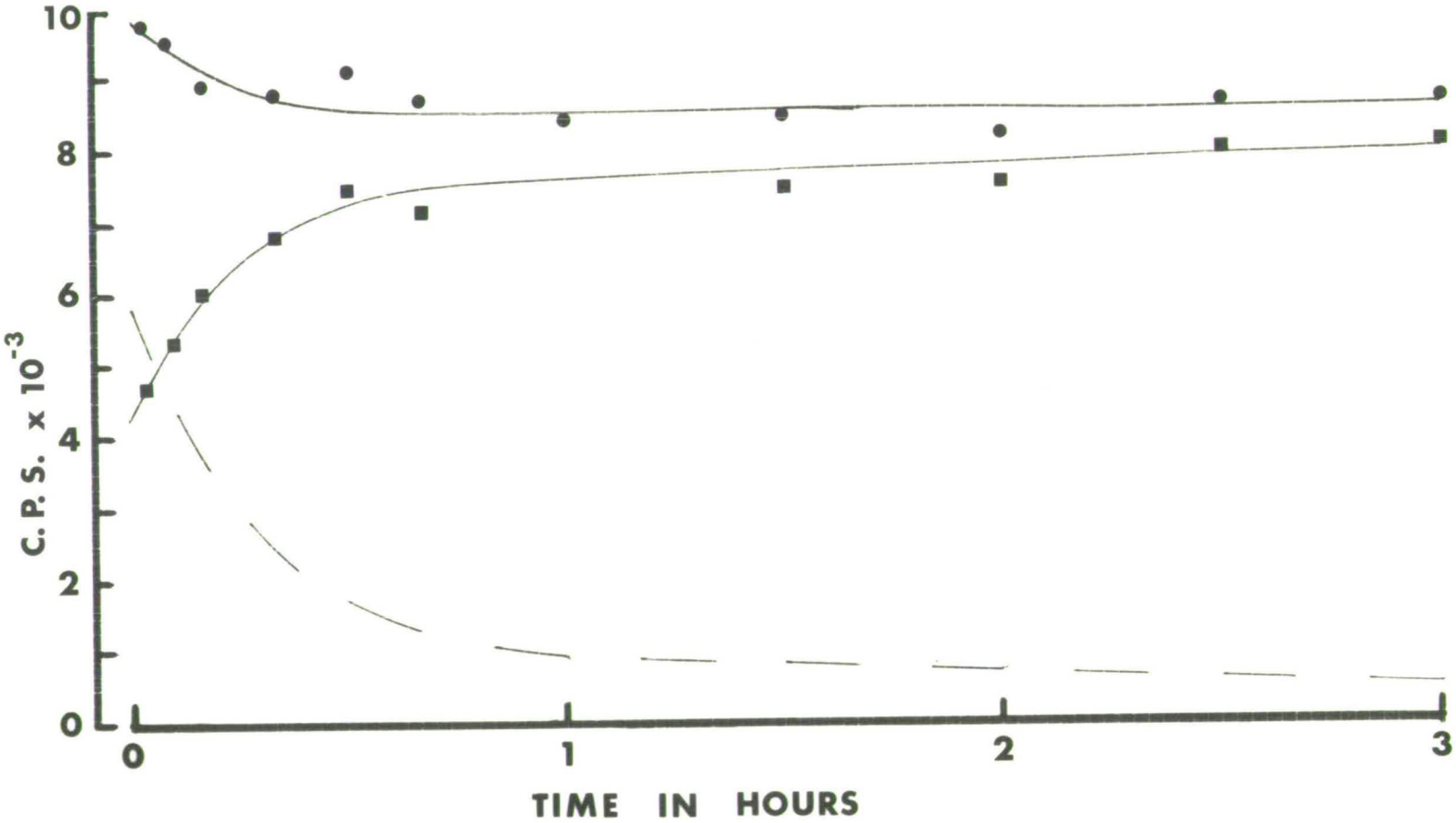

10
8
6
4
2
0
C.P.S. x 10^-3
0
1
2
3
TIME IN HOURS

FIGURE 23

DISTRIBUTION OF ^{14}C IN CELLS GROWING IN EMM2

Culture grown up from a small inoculum (0.1 ml. stationary phase into 100 ml.) with ^{14}C-glucose. At time zero the cell concentration was 3×10^6 cells/ml. and at 8 hours, about 8×10^6 cells/ml.

Open squares: Total activity in culture (cells and medium)

Solid circles: Total activity in cells

Solid squares: Activity in cells less the pool

Solid triangles: Activity in protein fraction of cell

Open triangles: Activity in RNA fraction of cells

Fractionation procedure: Schmidt-Tannhauser.

Sample sizes: 3 ml., filtered and washed on membrane filters.

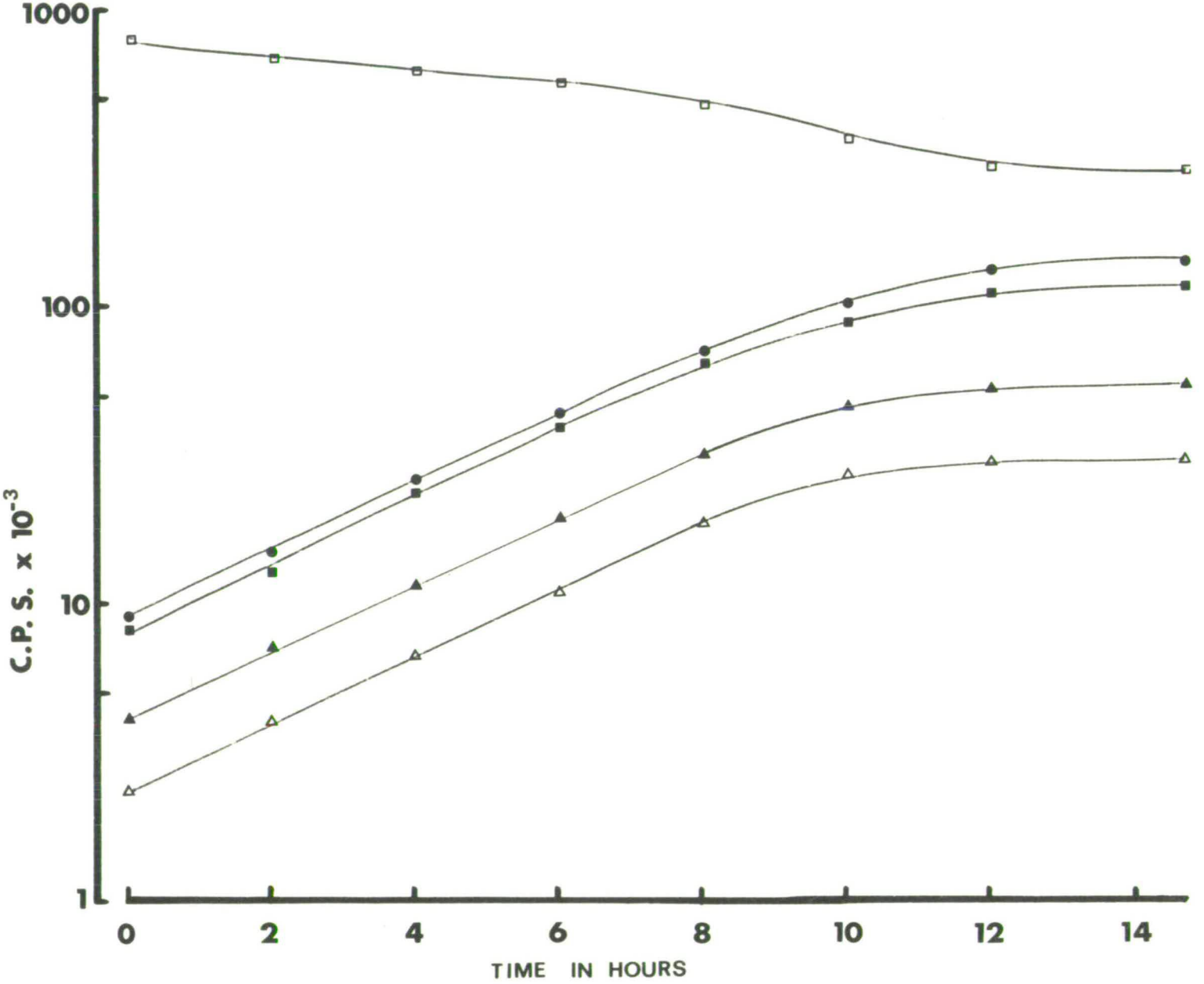

1000
100
10
1
C.P.S. x 10^{-3}
0
2
4
6
8
10
12
14
TIME IN HOURS

FIGURE 24

THE COMPETITIVE EFFECT OF AMINO ACID SUPPLEMENTS DURING BUT NOT AFTER THE LABELLING OF POOL COMPONENTS WITH ^{14}C FROM GLUCOSE

Cells labelled by 5 minutes immersion in glucose-less EMM2 with added ^{14}C-glucose.

A. Labelled with ^{14}C in glucose-less EMM2. Resuspended in EMM2 plus amino acids (solid squares).

B. Labelled with ^{14}C in glucose-less EMM2. Resuspended in EMM2 without addition of amino acids (open squares).

C. Labelled with ^{14}C in glucose-less EMM2 together with ^{12}C amino acids. Resuspended in EMM2 without addition of amino acids (solid triangles).

Solid circles indicate total uptake for A and B.

Amino acid supplement used was 0.1 mg./ml. of each of:

Alanine	Glycine
Arginine	Leucine
Aspartic acid	Lysine
Glutamic acid	Threonine

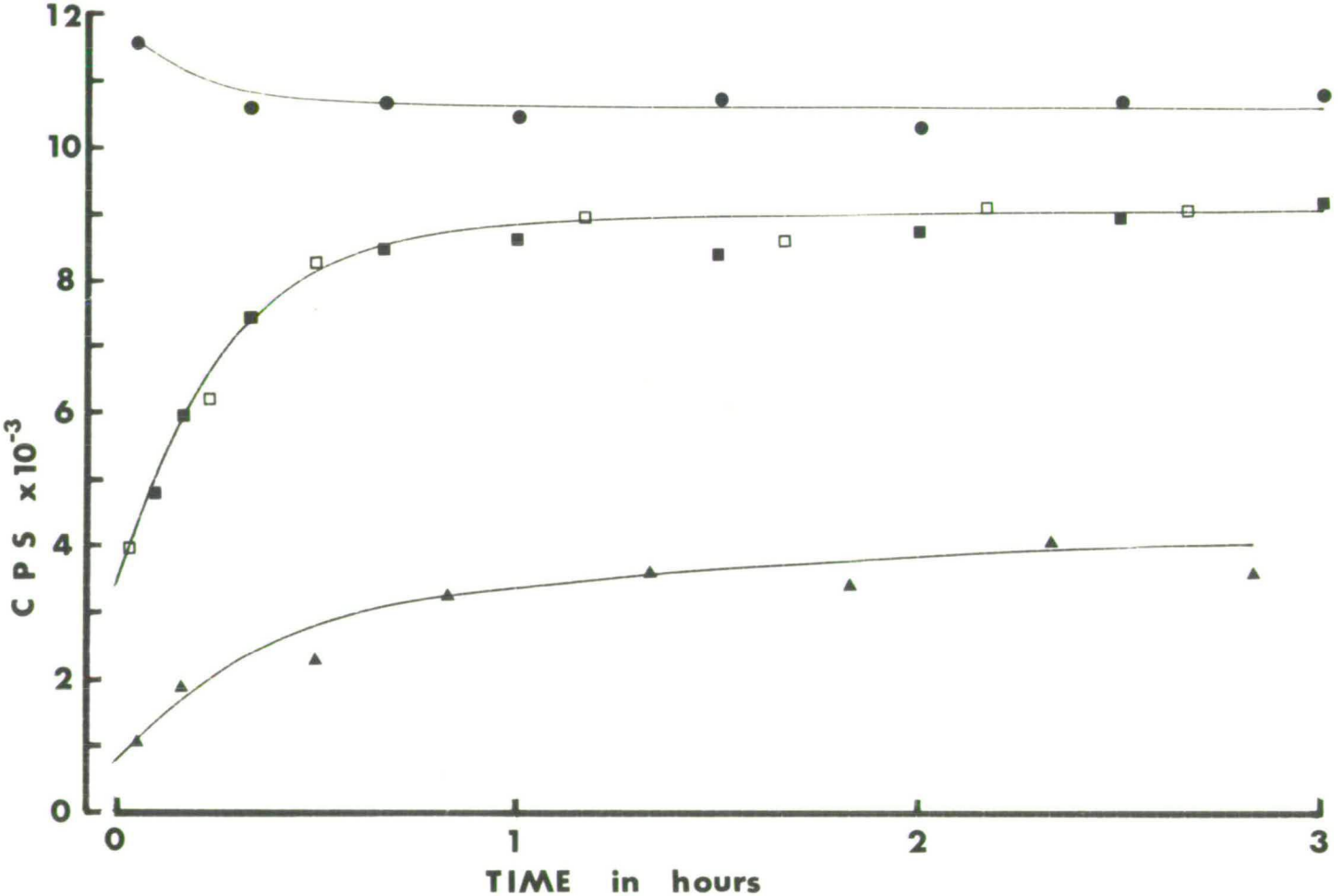

12
10
8
6
4
2
0
CPS x10-3
0
1
2
3
TIME in hours

FIGURE 25

UPTAKE AND INCORPORATION OF ^{14}C-LYSINE
BEFORE AND AFTER ADDITION OF
NON-RADIOACTIVE LYSINE

The culture was divided into two after 20 minutes (indicated by arrow) and non-radioactive lysine added to one half of the culture (open symbols). ^{14}C-lysine with carrier to a concentration of 2 µg./ml. was added at time zero.

Solid circles: Total uptake of lysine.
Solid squares: Incorporation into acid insoluble material.
Open circles: Total uptake after addition of non-radioactive lysine to the medium (20 µg./ml.).
Open squares: Incorporation into acid insoluble material after addition of non-radioactive lysine to the medium.

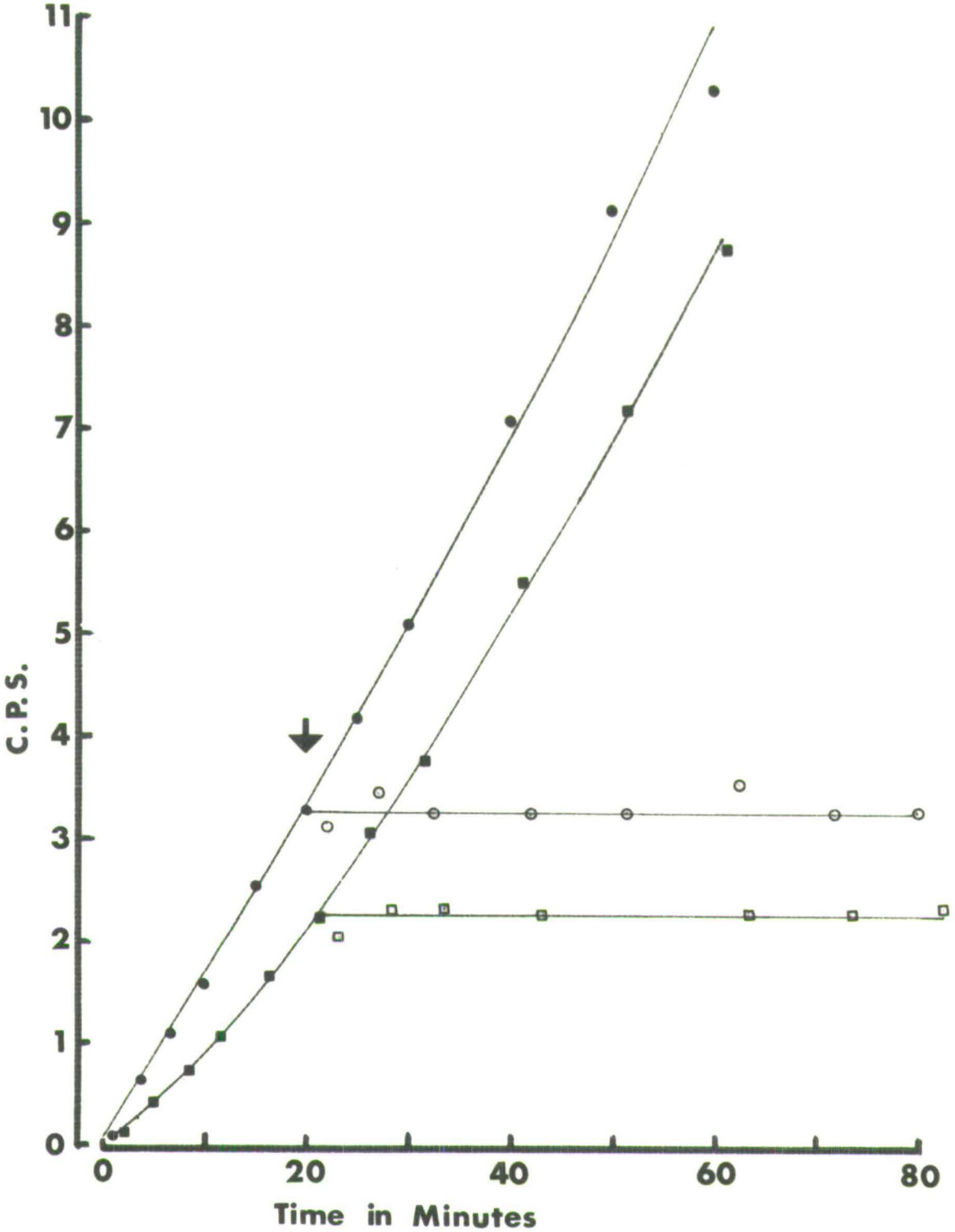

11
10
9
8
7
6
5
4
3
2
1
0
C.P.S.
0
20
40
60
80
Time in Minutes

FIGURE 26

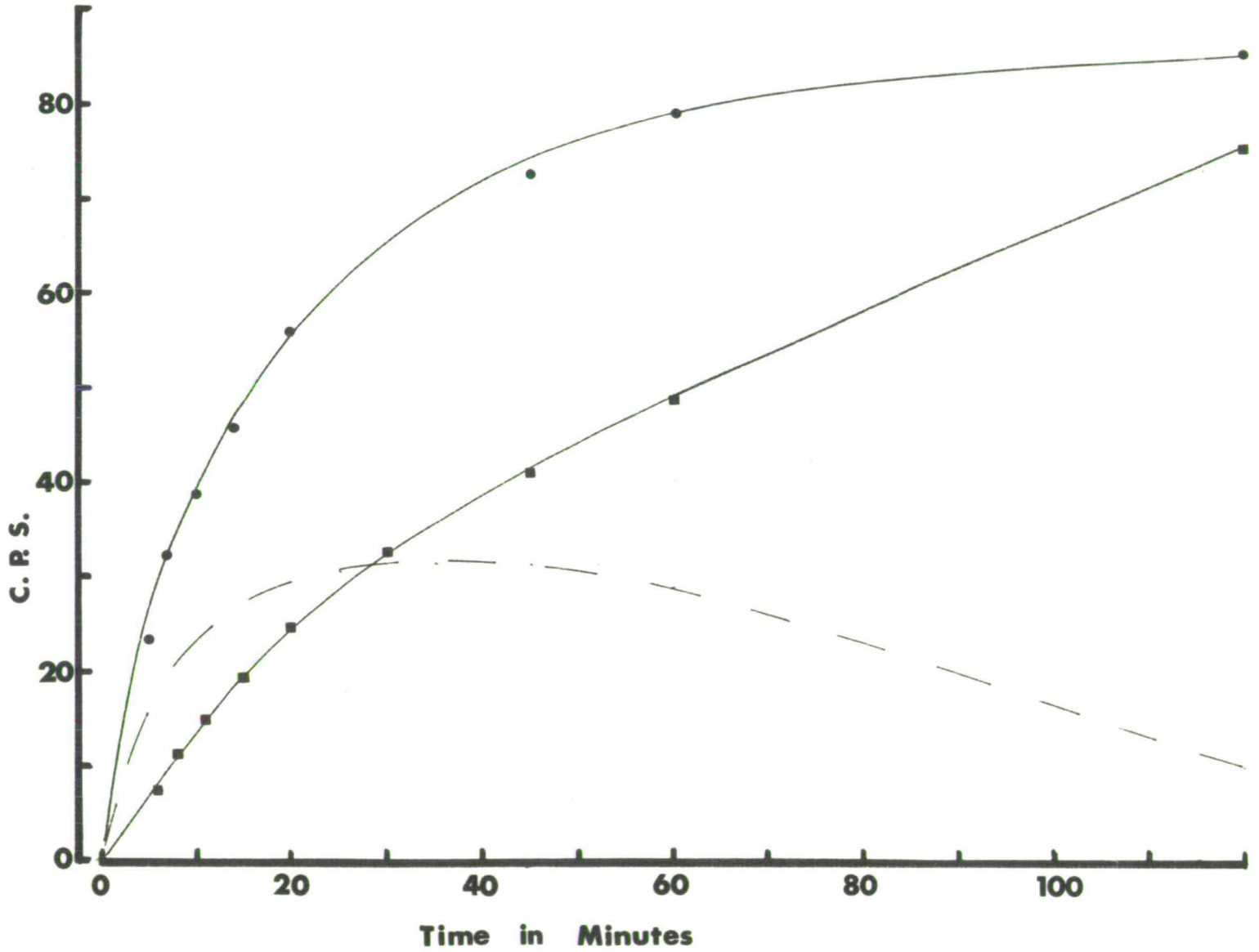
THE UPTAKE AND INCORPORATION OF
CARRIER FREE ^{14}C-LYSINE

Solid circles: Total uptake into cells.

Solid squares: Incorporation into acid insoluble material.

Dotted line indicates the pool, obtained by difference.

The ^{14}C-lysine added at time zero was not diluted with non-radioactive lysine. After 100 minutes half the total activity in the medium had been removed.


80
60
40
20
0
C. R. S.
0
20
40
60
80
100
Time in Minutes

Chapter 7

THE POOL, PROTEIN AND RNA IN SYNCHRONOUS CULTURES

Prior to the development of a method for obtaining synchronous cultures of S. pombe (Mitchison and Vincent, 1965), all work on the pattern of synthesis of macromolecular and pool components during the cell cycle was necessarily restricted to investigation on single cells. The pattern of total dry mass increase was conclusively determined by interferometry measurements on single growing cells (Mitchison, 1957). Determinations of macromolecular (Mitchison and Walker, 1959; Mitchison and Lark, 1962; Mitchison and Wilbur, 1962; Johnson, 1965) and pool (Mitchison and Cummins, 1964; Cummins and Mitchison, 1964; Mitchison, Cummins, Gross and Creanor, 1969) components through the cell cycle involved experimental errors which precluded definitive determinations of the pattern of synthesis. The methods and results of most of this work has been reviewed by Mitchison (1963a,b). The technique for determining the pattern of synthesis of macromolecular components (Mitchison and Wilbur, 1962) was to label exponential phase cells for a short time (generally 22 minutes; 10% of a generation time at 25°C) with a precursor. The cells were then fixed and autoradiographs prepared. From these, grain counts were made within a rectangle surrounding the cell, this rectangle being 10 μ x 20 μ for cells between 7 and 16 μ in length. The grain count is a measure of the amount of radioactive precursor

incorporated during the pulse and is a measure of the rate of synthesis of the macromolecule in that cell (Mitchison and Wilbur, 1962). Timing in the cell cycle relied on the basic feature of growth in S. pombe; that growth of the cell occurs essentially only in length and that the rate of this growth through the majority of the cell cycle is exponential (Mitchison, 1957; Swann, 1962). Cell length was converted into fractions of a generation time using the conversion graph of Mitchison and Walker (1959). In this way it was possible to get an average curve of the rate of synthesis at different points in the cell cycle (Mitchison, 1963a). The scatter in the data for this type of experiment is large (Mitchison and Wilbur, 1962), but can largely be attributed to known causes of variation, such as inaccuracies in timing in the cell cycle from length determinations (Gill, 1965), the limited number of grains that can be resolved over an autoradiograph of a cell as small as S. pombe, and the random nature of radioactive decay (Mitchison, 1963a,b). These autoradiographic techniques did, however, show that the amount of protein and carbohydrate (Mitchison and Wilbur, 1962) and RNA (Mitchison and Lark, 1962) through the cell cycle did not increase linearly with a rate change at the time of division, as occurs in total dry mass (Mitchison, 1957). More accurate determinations of the pattern of synthesis of protein and RNA and of pool components have therefore been attempted using synchronous cultures.

As we have seen (Chapter 5), the growth of S. pombe is

markedly affected by the level of phosphate in minimal medium. Before the discovery of the effect of higher phosphate (Bostock, 1968), a number of experiments were done on synchronous cultures grown in EMM1, but subsequently all work was done on cells grown in EMM2. The earlier determinations are reported briefly first.

CULTURES GROWN IN EMM1

Figure 27 shows the pattern of synthesis of protein and RNA in a synchronous culture together with the increase in pool amino acid and nucleotide material over more than two synchronous divisions. Five such experiments gave essentially the same results. Clearly, as in batch cultures, the amount of RNA and protein per cell decreases in successive generations. The amount of RNA and protein per cell varied between experiments, but the amounts were found to correspond to the stage in 'exponential' growth from which the synchronous cultures were derived. These experiments show that to a first approximation the pattern of increase in RNA, protein, pool amino acid and nucleotides was exponential, although RNA and protein did not quite double in each generation. There is certainly no major rise in the middle of the cell cycle of the two pool fractions, as Mitchison and Cummins (1964) have shown for the total pool.

The determination of the composition of the amino acid pool necessitates large synchronous cultures, since approximately 4×10^8 cells per sample are required for satisfactory

analysis of the amino acids (Chapter 4). For this reason, sampling was restricted to one cell cycle in a synchronous culture grown in EMM1, and the size of the sample progressively reduced. The results of such an experiment are shown in Figures 28 and 29. The composition of the pool is similar to that of pool material from an exponential culture grown in EMM1 (Chapter 4, Table 6), except that the levels of threonine and serine are considerably lower (10% of exponential phase levels). This is curious since this synchronous culture was obtained from exponential phase cells harvested at 3×10^6 cells/ml., which is the same cell concentration as that used for the batch culture analyses.

It is clear from Figures 28 and 29 that there are no substantial fluctuations in the amino acid pool components. Proline is the only component that shows a definite fluctuation (Figure 29). But since this amino acid is present in very low amounts and is estimated indirectly (see Chapter 3) too much weight should not be given to this result. The total amino acid in the pool (shown in Figure 28) shows a very smooth exponential increase and is in agreement with the estimates of total ninhydrin positive material in the pool (Figure 27).

CULTURES GROWN IN EMM2

Figure 30 shows the pattern of synthesis of protein, RNA and pool amino acid and nucleotide material in a synchronous culture grown in EMM2. Unlike synchronous cultures in EMM1,

there is no apparent decrease in the rate of synthesis of protein and RNA during growth up to 10 x 10^6 cells/ml. These determinations were repeated at least five times, with similar results, but the assays were not always done simultaneously in the same synchronous culture. Total cellular nitrogen, after removal of the pool, was also estimated. Two of these experiments are shown in Figure 31.

The patterns of increase of the pool components and total RNA and nitrogen were concluded to be exponential, since regression analyses on the logarithms of the data showed no residual error unaccounted for by the error in the determinations themselves. This was shown by a comparison of the variance of the deviations of the data from the regression line (s^2y, Chapter 2) with the variance of the assay method, after transforming s^2y in the manner outlined later in this chapter. In every case the variances were shown to be equivalent by means of a variance ratio (F) test at the 5% significance level (using the table giving the 2.5% points in "Biometrika Tables for Statisticians", since we are concerned with a two-tailed test). Some doubt existed with the Lowry determinations for protein, since the error of the method is high (see Chapter 2). Some experiments seemed to indicate three steps per cell cycle in total protein, as in the experiment shown in Figure 32.

An accurate determination of the pattern of protein synthesis was considered impossible by means of the Lowry method, for, as shown in Appendix II, the maximum differences

between three possible models of synthesis (linear, exponential and quadratic) are smaller than the error of the Lowry method. The maximum difference between an exponential increase and a quadratic increase is only 2.73%, while the maximum differences between these patterns and a linear increase are 5.62% and 8.33% respectively (see Appendix II). An estimate of the pattern of protein synthesis was therefore obtained by determining the rate of protein synthesis in synchronous cultures. Such determinations should more readily distinguish between exponential, quadratic and linear synthesis since the rates of synthesis for these models would be exponential, linear and constant respectively. The change in the rate of protein synthesis in two synchronous cultures is shown in Figure 33. The rate of protein synthesis appears to increase linearly in each cell cycle. This experiment was repeated four times on synchronous cultures and three times on asynchronous control cultures. The data was tested for best fit to a smooth model (exponential increase) or segmented model (three straight lines) by the method of Williams (1969) (see Chapter 2). The computer analyses gave no discrimination for one of the experiments on a synchronous culture, and this experiment has therefore been ignored. The other three experiments on synchronous cultures all best fitted the segmented model, while all three control experiments best fitted the smooth model. These six results form a contingency table whose probability, using Fisher's exact test, is $3!3!3!3!/6!3!3! = \frac{1}{20}$, i.e. 5%. We may

therefore conclude that the pattern of protein synthesis in synchronous cultures is quadratic with a significance level of $P = 0.05$. The exact curve of synthesis might differ slightly from this pattern since there is a slight downward curvature in the rate of protein synthesis which can be clearly seen in the second cell cycle of the experiments shown in Figure 33.

Pool amino acids were determined in two synchronous cultures grown in EMM2 in the same manner as the experiment in EMM1 described above, except that the analyses were done with an Evans autoanalyser. The results of one of the EMM2 experiments are shown graphically in Figure 34. The mean percentage composition of the pool has been calculated and is given in Table 15. Comparison with Table 7, Chapter 4, shows that the mean composition in this synchronous culture and the composition for asynchronously grown cells are essentially the same.

From Figure 34 it is apparent that all the pool components except alanine increase more or less exponentially throughout the synchronous culture. Alanine, which was low during the first two cell cycles, suddenly increases rapidly at the beginning of the third cycle (see Figure 34). It would appear that the mean exponential amount of alanine is restored rapidly at this time.

It is now necessary to consider whether the irregularities in the increases of the amino acids deviate significantly from an exponential pattern. The regression co-efficients (b) and the variance of the deviations (s^2y) of the data for each amino

acid were calculated (see Chapter 2) and the results are given in Table 15. The abscissa values used in the calculation of b and s^2y were the positions in the cell cycle (see Figure 34) taken to be between 0 and 1 in the first cycle, 1 and 2 in the second cycle and 2 and 3 in the third cycle. Two features of this data were considered. Firstly, whether the regression co-efficients deviate significantly from 0.3010 ($\log_{10}2$) which is the expected value if the amino acids double in amount during one cell cycle. Secondly, the extent to which the estimates of each amino acid deviated from the regression line. The first point is easily tested by means of a 't' test of the form given in Table 15. The value of t for each regression co-efficient is given in Table 15. In no case is the difference between the observed and expected regression co-efficient significant at the 5% level.

It is clear from the variances (s^2y) in Table 15 that the variances are greater the lower the mean amount of amino acid analysed, as also found by Spackman (1967), using the accelerated two column method of autoanalysis. A direct comparison of the deviations of the amino acid data from the regression lines with the quoted errors of the analysis method is not possible since the regressions were calculated on the logarithms of the data. However, the variance of the data before taking logarithms can be estimated since, if

$$y = \log_e x$$

$$\text{then var } x = \bar{x}^2 \text{ var } y$$

$$\text{i.e. var } x = (2.3\,\bar{x})^2 \text{ var } y \quad \text{if}$$

logarithms to base 10 have been taken (Kendall and Stuart, "The Advanced Theory of Statistics", vol. 1, p. 232). Taking 'x' as the data before logarithms were taken, the largest variance for experiment 1 (Table 15) is 0.05147 where $\bar{x} = 12$. Hence var $x = (2.3 \times 12)^2 \cdot 0.05147 = 39.2$. Thus the standard error is $39.2/17 = 0.368$. The largest percentage standard error is therefore $0.368 \times 100/12 = 3.1\%$.

The data for amino acid determinations in the second synchronous culture in EMM2 is given in Table 16. This data is essentially similar to that obtained in the first experiment (Table 15) and therefore confirms the conclusion drawn from the first experiment.

The introduction to this chapter was largely concerned with previous work on single cells of S. pombe. There have been some studies on other yeasts including synchronous cultures. Sando (1963) has determined the pattern of RNA and protein synthesis in synchronous cultures of S. pombe using a different synchronization procedure to that employed in the present work. He claims that protein synthesis is almost linear throughout the cell cycle and that RNA synthesis is greatest during the first part of the cell cycle. However, these results are by no means conclusive since only four samples per hour were taken and the cultures are peculiar in that the periodic increase in cell concentration never constitutes a doubling in cell number. Johnson (1965) has estimated the pattern of synthesis of cell wall material in S. pombe from autoradiographic experiments,

and concluded that the cell wall increases exponentially in amount in cells grown in MEB.

Williamson and Scopes have determined the pattern of synthesis of protein and RNA in Saccharomyces cerevisiae. RNA synthesis appeared to cease just before cleavage and to resume only after an appreciable delay and then to proceed at a more or less constant rate (Williamson and Scopes, 1960). Protein synthesis was continuous throughout the division cycle but the total amount of nitrogen per cell increased discontinuously (Williamson and Scopes, 1961). This was interpreted as indicating a fluctuation in nitrogen containing pool material during the cell cycle. Pool amino acids in synchronous cultures of yeasts have not been investigated, but Dawson (1965) has shown that in Candida utilis the spectrum of amino acids in the pool alters with the rate of growth and the nature of the carbon source in minimal media unsupplemented by amino acids. Dawson suggested that the free amino acid pool reflects the pattern of feedback conditions that exist at any given growth rate on a particular carbon source and that 6-C and 3-C catabolic intermediates were used preferentially at fast growth rates while 3-C and 2-C intermediates were utilized at slower growth rates.

TABLE 15

ANALYTICAL DATA FOR POOL AMINO ACID DETERMINATIONS FROM SYNCHRONOUS CULTURE, EMM2, EXPERIMENT 1

(17 Samples, see Figure)

The data was expressed in nM and transformed by taking logarithms to base 10. The calculated slopes of the regression lines and the variances are for the data transformed in this way. The mean amount for each amino acid is the mean calculated from the amounts estimated from the chromatograms, and the percentage composition is derived from these means.

Amino acid	Regression coefficient (b)	t	Mean amount of amino acid analysed, in nM	% composition	Variance of the deviations from the regression line (s^2y)
Glutamic	0.3393	0.6684	482	36.41	0.01746
Arginine	0.2998	0.1970	154	11.63	0.01980
Alanine	0.3220	0.3230	100	7.55	0.02250
Lysine	0.3045	0.0495	112	8.46	0.02661
Serine	0.3204	0.4470	92	6.95	0.02002
Aspartic	0.2909	0.3380	78	5.89	0.00475
Threonine	0.2880	0.2460	52	3.93	0.01480
Citrulline	0.3005	0.0730	72	5.44	0.02501
X	0.3400	0.9475	55	4.15	0.00900
Glycine	0.3328	1.1749	41	3.10	0.00389
Histidine	0.3102	0.1080	21	1.59	0.03880
Phenylalanine	0.3384	0.4710	19	1.46	0.03352
Leucine	0.2894	0.1040	12	0.91	0.05147
Y	0.3127	0.1224	9	0.68	0.04852
Tyrosine	0.3060	0.0518	10	0.76	0.04954
Isoleucine	0.2909	0.1131	8	0.60	0.04240
Methionine	0.3220	0.2334	4	0.30	0.04300

The value of t was calculated from the formula:

$$t = \frac{b - \beta}{s^2 y} \cdot \sqrt{(x - \bar{x})^2}$$

where β is the expected regression coefficient on the basis of exact doubling in the cell cycle, i.e. $\beta = \log_{10} 2 = 0.3010$.
The d.f. = n - 2, which is 15 in this case.
(Formula from Bailey: Statistical methods in Biology, E.U.P., 1959, p. 98.)

TABLE 16

ANALYTICAL DATA FOR POOL AMINO ACID DETERMINATIONS FROM A SYNCHRONOUS CULTURE, EMM2, EXPERIMENT 2

(12 Samples)

See Table 15 for explanation of data

Amino acid	Regression coefficient (b)	t	Mean amount of amino acid analysed, in nM	% composition	s^2_y
Glutamic	0.3247	0.5167	506	37.51	0.02024
Arginine	0.2846	0.3257	159	11.74	0.02436
Alanine	0.3401	0.8646	109	8.06	0.01967
Lysine	0.3103	0.1898	108	7.98	0.02306
Serine	0.3272	0.5393	95	7.03	0.02267
Aspartic	0.3006	0.0881	76	5.66	0.01979
Threonine	0.2798	0.4838	54	4.01	0.02006
Citrulline	0.3400	0.7745	68	5.04	0.02437
X	0.3267	0.4942	54	4.00	0.02599
Glycine	0.3309	0.6246	47	3.51	0.02202
Histidine	0.2907	0.1961	23	1.72	0.02651
Phenylalanine	0.2889	0.2195	19	1.44	0.02919
Leucine	0.3312	0.4891	12	0.85	0.03663
Y	0.3033	0.3213	8	0.57	0.04923
Tyrosine	0.3291	0.1331	9	0.66	0.04458
Isoleucine	0.2827	0.2869	6	0.47	0.03909
Methionine	0.3347	0.5057	3	0.22	0.04267

Values of 't' calculated as in Table 15, but in this case d.f. = 10, since the number of samples was 12.

FIGURE 27

THE SYNTHESIS OF PROTEIN, RNA AND POOL AMINO ACID AND NUCLEOTIDE MATERIAL IN AN EMM1 SYNCHRONOUS CULTURE

Open squares: Cell concentrations, obtained by haemocytometer counts.

Solid squares: Total protein.

Solid circles: Total RNA.

Open triangles: Pool nucleotide (OD-260) material.

Solid triangles: Pool amino acid.

Synchronous culture: 600 ml., obtained from 10 litres of cells harvested at 3×10^6 cells/ml. separated on a glucose gradient.

Samples (10 ml.) collected and washed on PVC Millipore filters.

Pool material obtained by freeze-thawing.

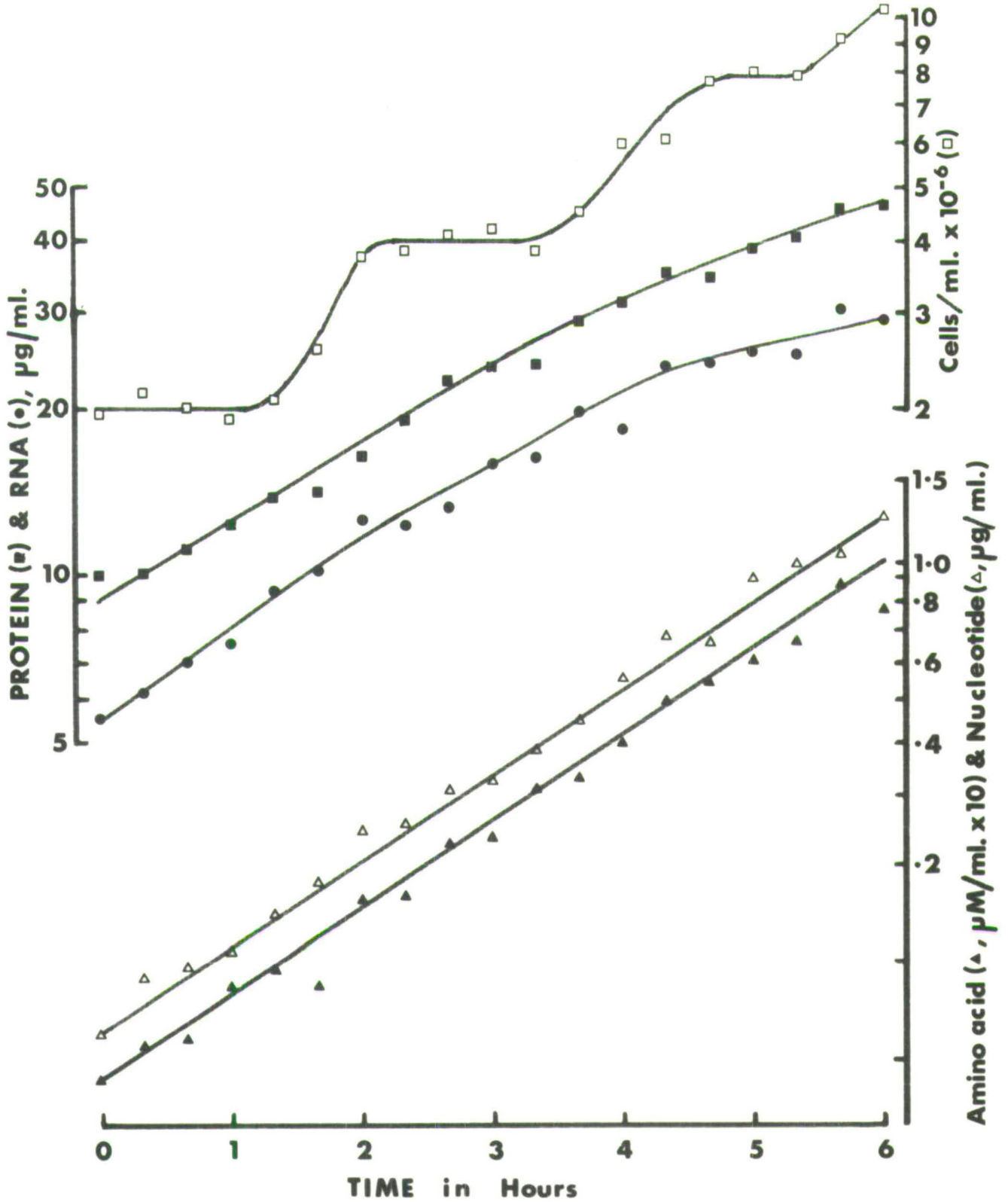

PROTEIN (■) & RNA (●), µg/ml.
50
40
30
20
10
5
Cells/ml. x 10⁻⁶ (□)
10
9
8
7
6
5
4
3
2
Amino acid (▲, µM/ml. x 10) & Nucleotide (△, µg/ml.)
1·5
1·0
·8
·6
·4
·2
0
1
2
3
4
5
6
TIME in Hours

FIGURE 28

CHANGES IN POOL AMINO ACIDS IN AN
EMM1 SYNCHRONOUS CULTURE

For times and sizes of samples and timing in the cell cycle, see Figure 29.

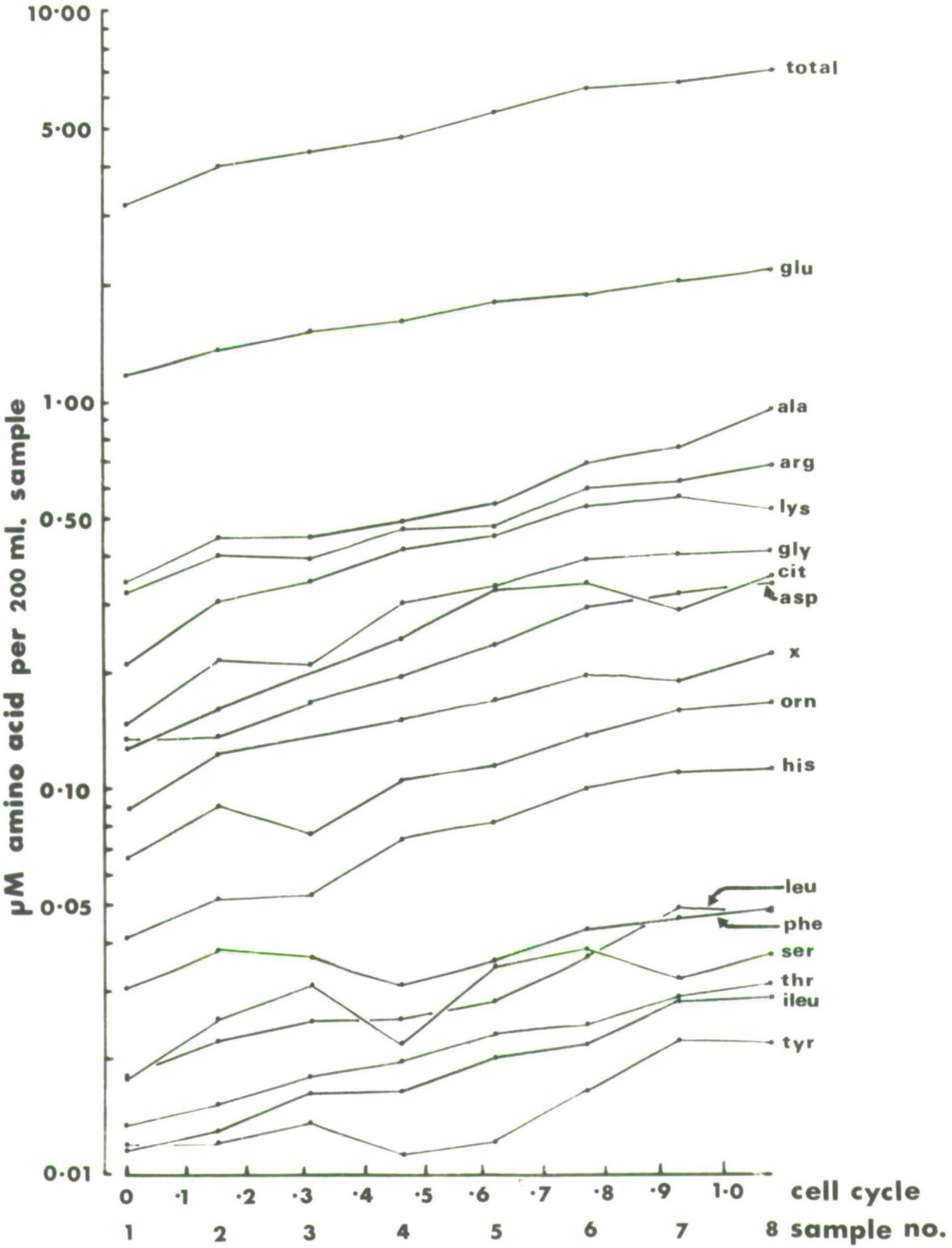

10·00
5·00
1·00
0·50
0·10
0·05
0·01
μM amino acid per 200 ml. sample
total
glu
ala
arg
lys
gly
cit
asp
x
orn
his
leu
phe
ser
thr
ileu
tyr
0
·1
·2
·3
·4
·5
·6
·7
·8
·9
1·0
cell cycle
1
2
3
4
5
6
7
8 sample no.

FIGURE 29

CHANGES IN CELL CONCENTRATION IN A SYNCHRONOUS CULTURE GROWN IN EMM1 SHOWING THE TIMES AT WHICH 8 SAMPLES WERE TAKEN FOR ANALYSIS OF POOL AMINO ACIDS. THE CHANGES PER 200 ml. SAMPLE OF TWO OF THE POOL AMINO ACIDS ARE ALSO SHOWN

Changes in the other pool amino acids are shown in Figure 28. Cell counts were by haemocytometer. Times 0 and 1 in the cell cycle were taken from the midpoint of the step increase in cell concentration.

Sizes of sample for amino acid analysis:

Nos. 1 and 2, 200 ml.
3 and 4, 180 ml.
5 and 6, 160 ml.
7 and 8, 140 ml.

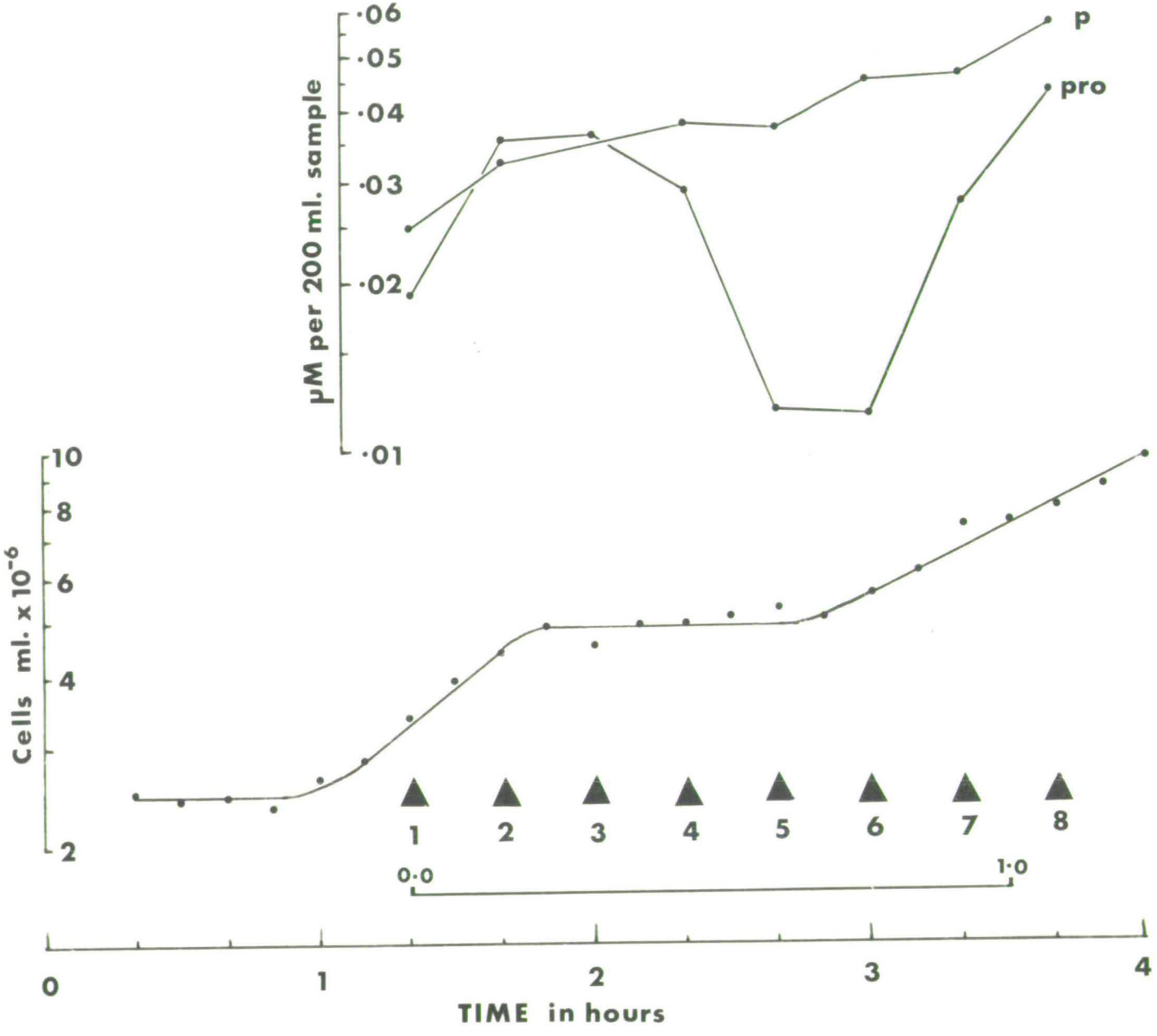

·06
·05
·04
·03
·02
·01
µM per 200 ml. sample
p
pro
10
8
6
4
2
Cells ml. x 10⁻⁶
1
2
3
4
5
6
7
8
0·0
1·0
0
1
2
3
4
TIME in hours

FIGURE 30

CHANGES IN TOTAL PROTEIN, RNA AND POOL
AMINO ACID AND NUCLEOTIDE MATERIAL
IN AN EMM2 SYNCHRONOUS CULTURE

Cell concentrations determined by haemocytometer counts.
10 ml. aliquots of cells collected and washed on PVC Millipore filters.
Pool material obtained by freeze-thawing.

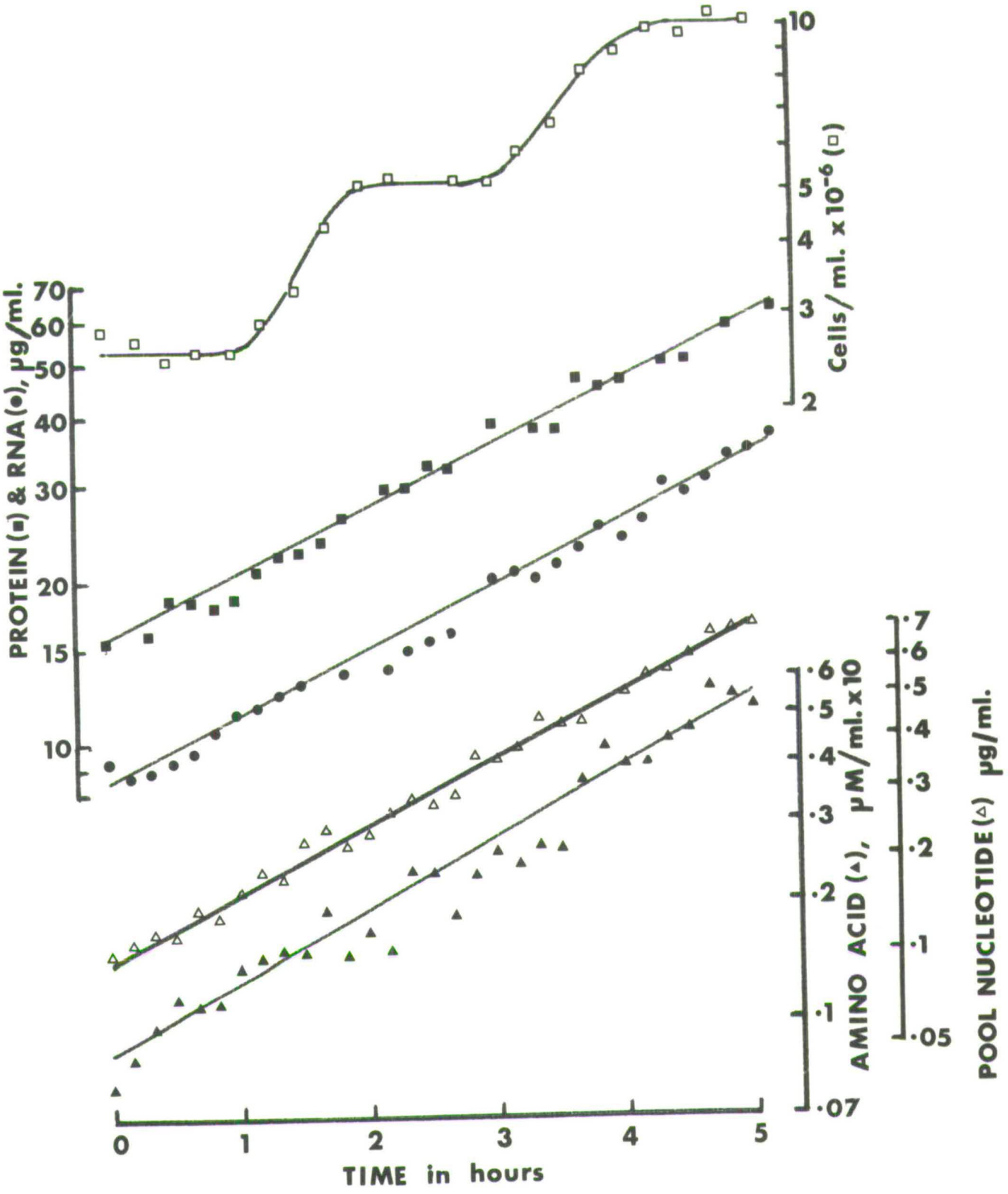

PROTEIN (■) & RNA (●), µg/ml.
70
60
50
40
30
20
15
10
Cells / ml. x 10⁻⁶ (□)
10
5
4
3
2
AMINO ACID (▲), µM/ml. x 10
·6
·5
·4
·3
·2
·1
·07
POOL NUCLEOTIDE (△) µg/ml.
·7
·6
·5
·4
·3
·2
·1
·05
0
1
2
3
4
5
TIME in hours

FIGURE 31

TOTAL NITROGEN ESTIMATED IN EMM2
SYNCHRONOUS CULTURES

Nitrogen was estimated on cell material after removal of the pool by freeze-thawing.
The arrows indicate the positions of the cell plate peaks in the synchronous cultures.

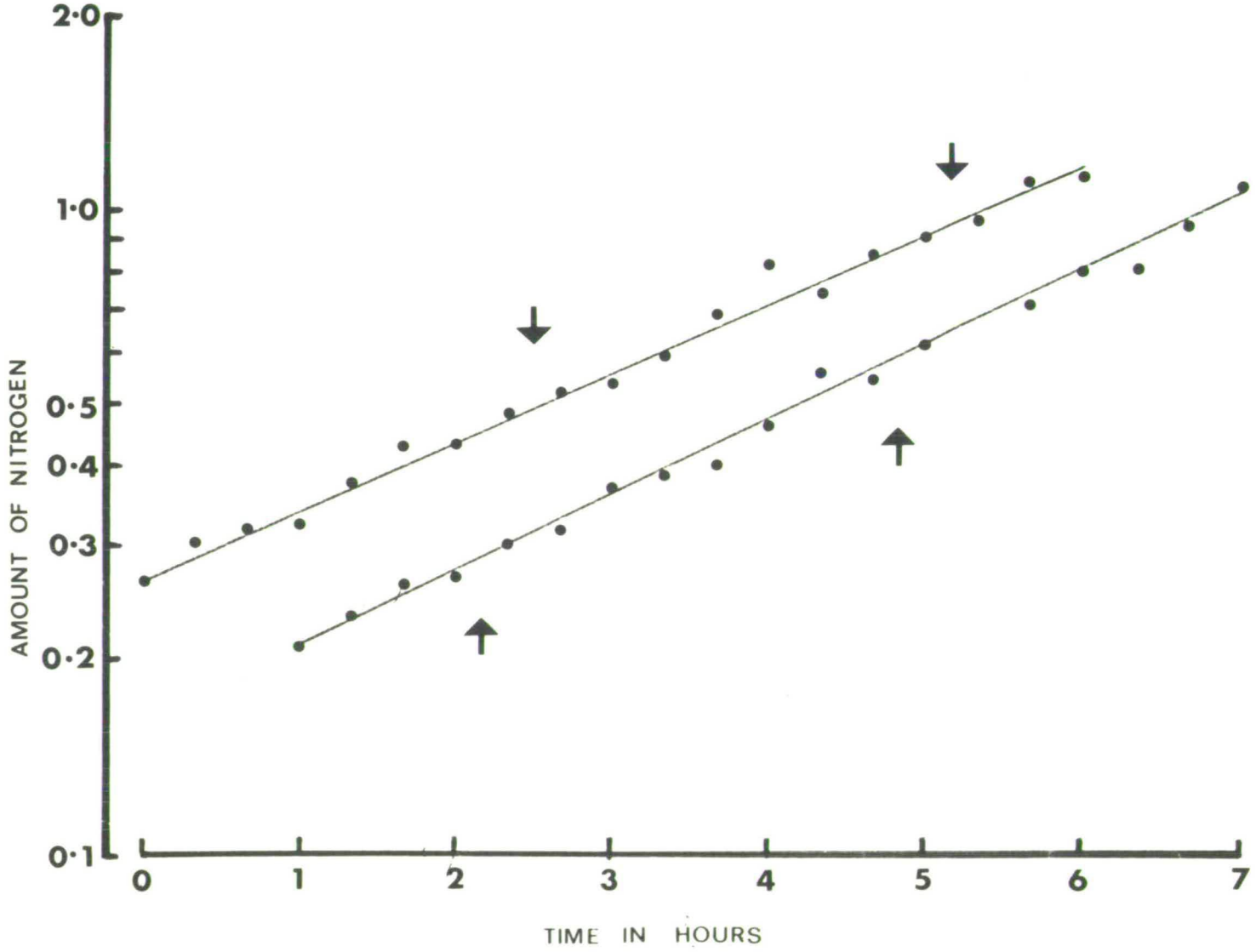

2·0
1·0
0·5
0·4
0·3
0·2
0·1
AMOUNT OF NITROGEN
0
1
2
3
4
5
6
7
TIME IN HOURS

FIGURE 32

CHANGES IN TOTAL PROTEIN IN A SYNCHRONOUS CULTURE GROWN IN EMM2

Note that three steps can be seen in the synthesis of protein in each cell cycle in this experiment.

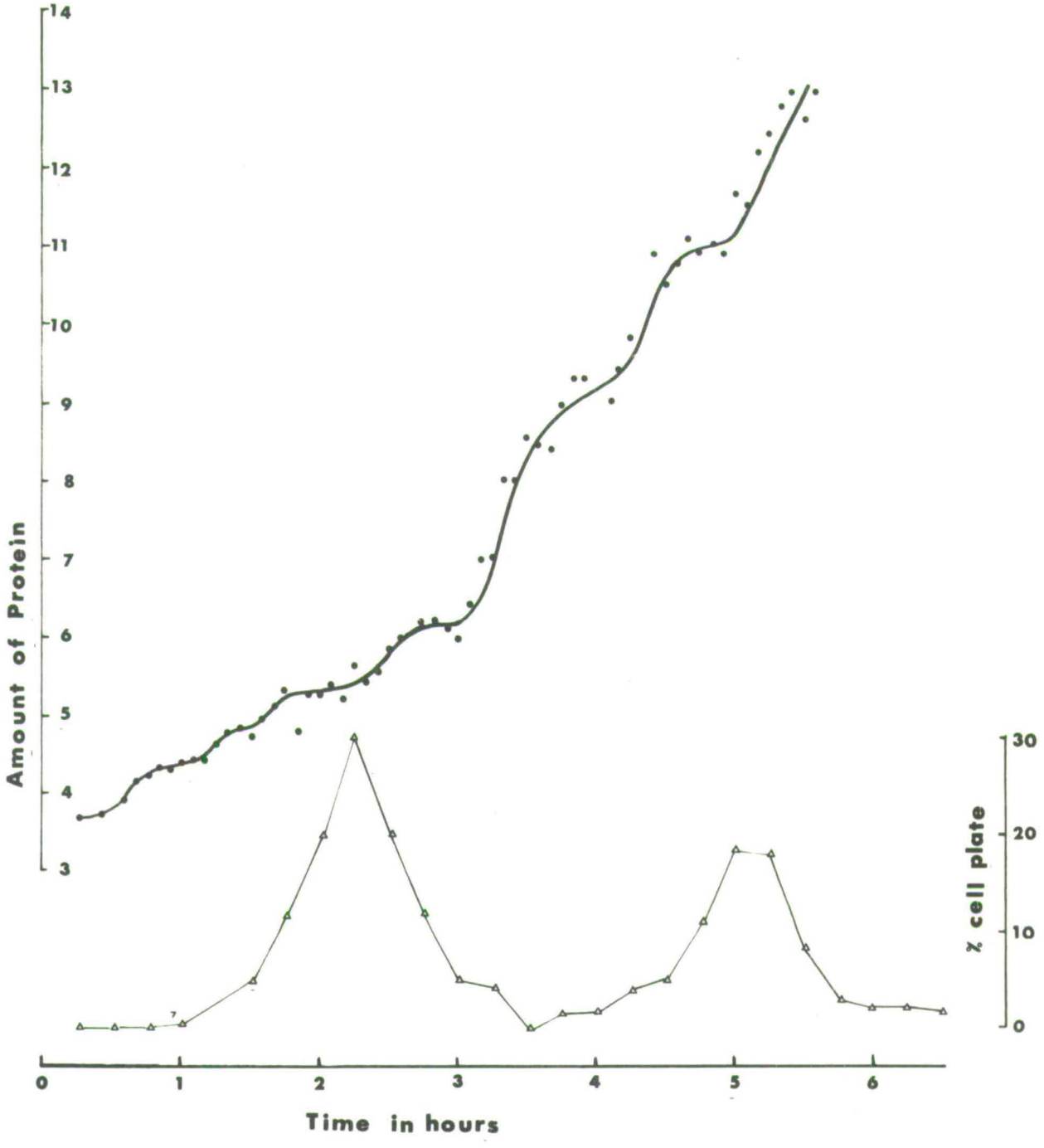
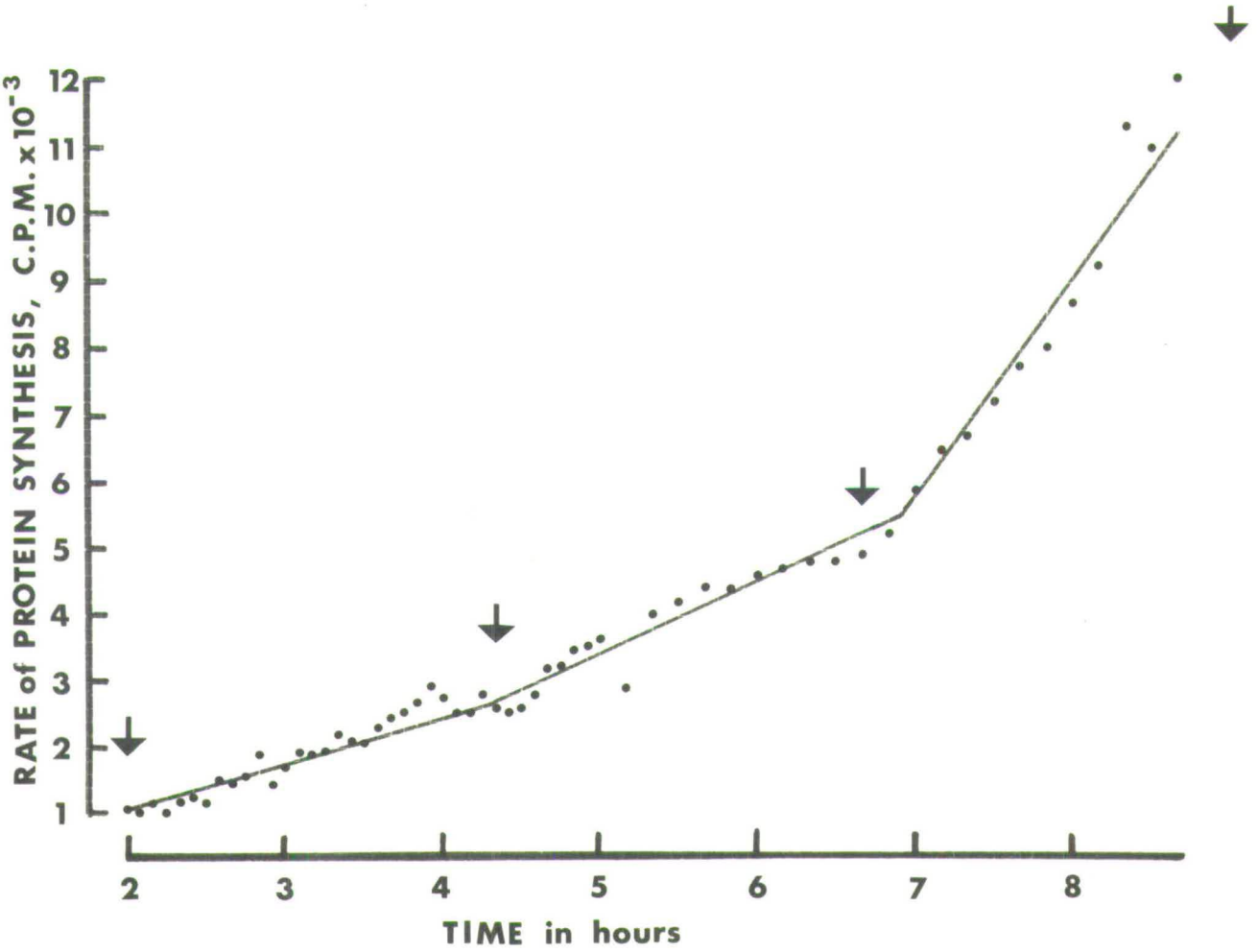

Amount of Protein
14
13
12
11
10
9
8
7
6
5
4
3
% cell plate
30
20
10
0
0
1
2
3
4
5
6
Time in hours

FIGURE 33

RATE OF PROTEIN SYNTHESIS IN EMM2
SYNCHRONOUS CULTURES

1 ml. aliquots were removed every five minutes and incubated for 12 minutes at 32°C with 1 µg./ml. lysine containing ^{14}C-lysine. 1 ml. of ice-cold 10% TCA was then added and after 15 to 30 minutes the sample was filtered, washed with 5% TCA and water. The cells were then dried on the filter and later put into vials for counting in a scintillation counter.

The arrows indicate the times of cell plate peaks in the synchronous cultures.


RATE of PROTEIN SYNTHESIS, C.P.M. x 10⁻³
12
11
10
9
8
7
6
5
4
3
2
1
2
3
4
5
6
7
8
TIME in hours

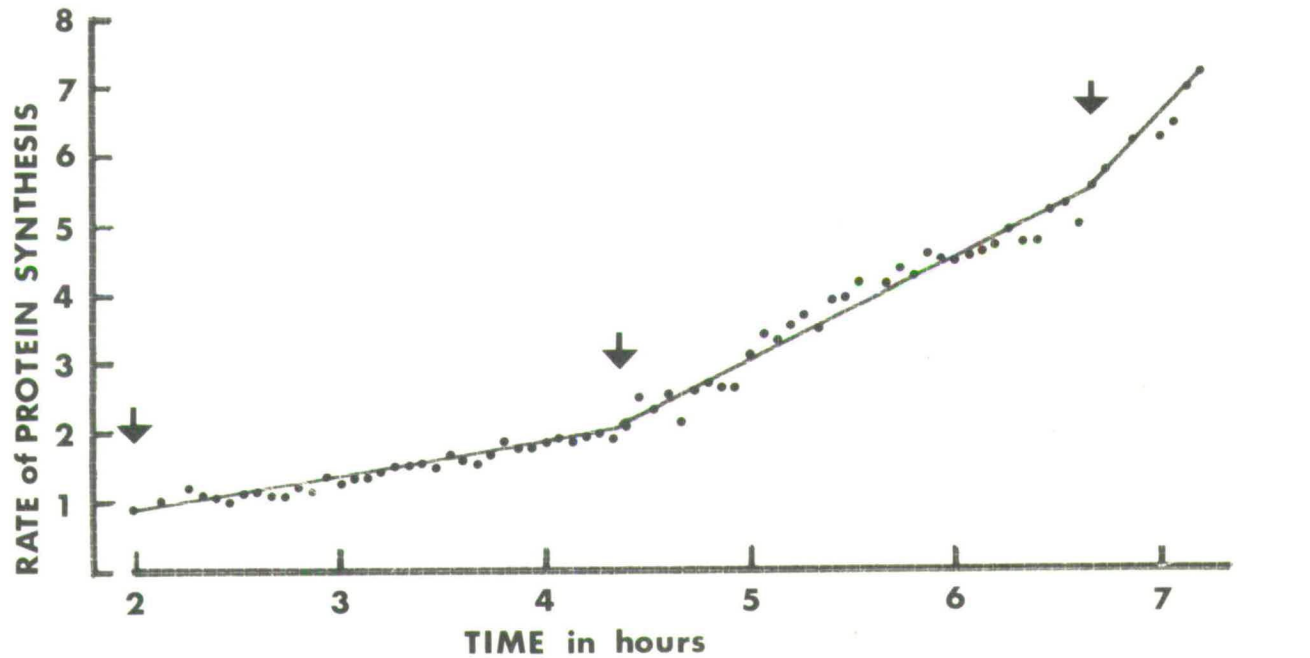
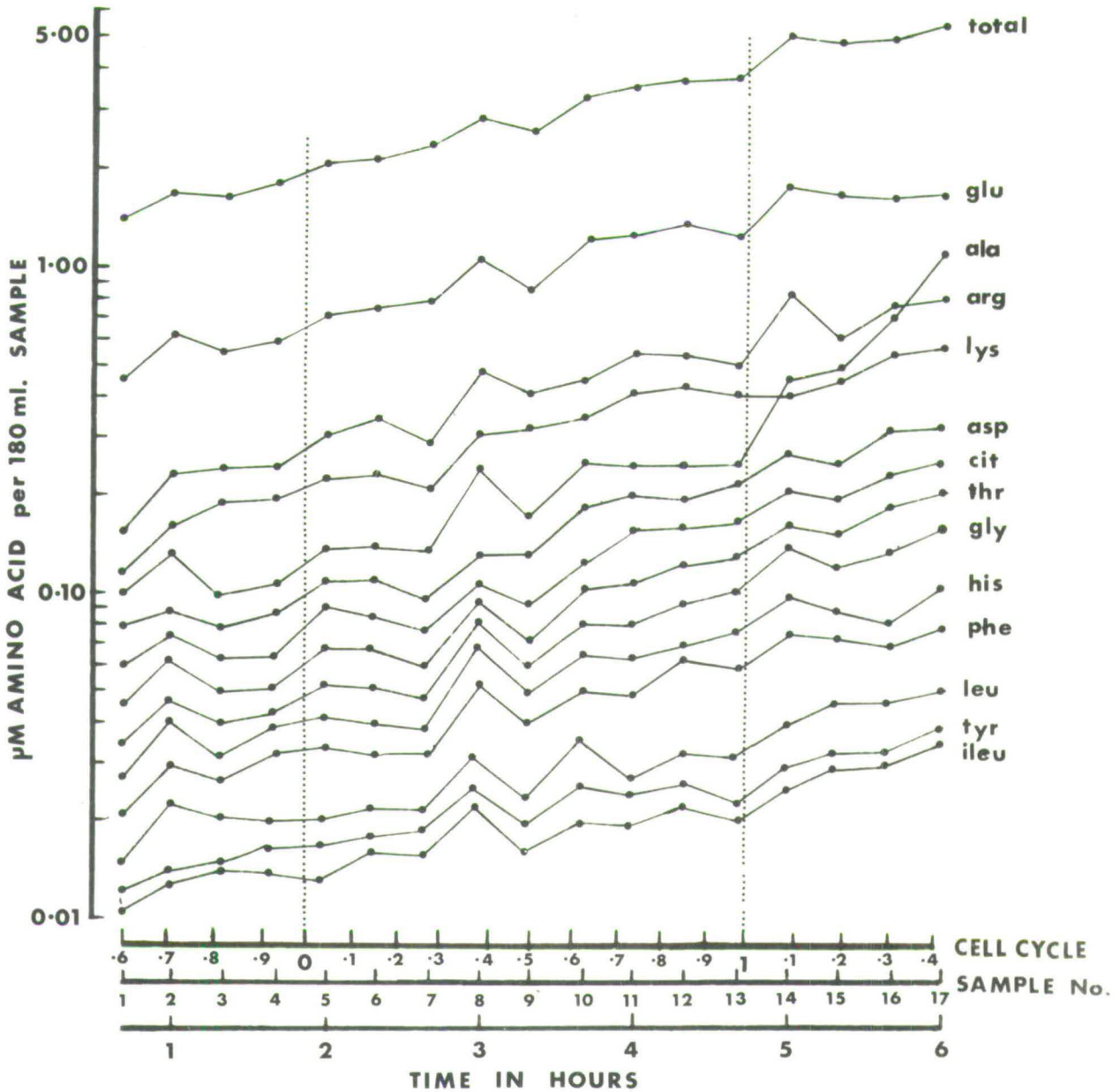

RATE of PROTEIN SYNTHESIS
8
7
6
5
4
3
2
1
2
3
4
5
6
7
TIME in hours

FIGURE 34

CHANGES IN POOL AMINO ACIDS IN AN EMM2
SYNCHRONOUS CULTURE
(Experiment 1, 17 samples)

Timing in the cell cycle was from estimates of cell numbers in the culture (haemocytometer counts), taking the mid-point of the step in cell numbers as 0 and 1 in the cell cycle.

Synchronous culture: 1.6 litres obtained from 20 litres harvested at 5×10^6 cells/ml.


5·00
1·00
0·10
0·01
μM AMINO ACID per 180 ml. SAMPLE
total
glu
ala
arg
lys
asp
cit
thr
gly
his
phe
leu
tyr
ileu
·6 ·7 ·8 ·9 0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 1 ·1 ·2 ·3 ·4
CELL CYCLE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
SAMPLE No.
1 2 3 4 5 6
TIME IN HOURS

Chapter 8

TOTAL DRY WEIGHT AND THE TOTAL POOL IN SYNCHRONOUS CULTURES

It is clear from the results of Chapter 7 that the synthesis of the macromolecules measured (protein and RNA) closely fit exponential or quadratic patterns of increase throughout the cell cycle. Also, the pool constituents measured (amino acids and nucleotides) increase exponentially with no indication of a fluctuation such as Mitchison (1957) inferred from the linear increase in mass of single cells. As we have seen (Chapter 7), the patterns of synthesis of macromolecules as determined by earlier workers is in agreement with the present work, but the pool data is at variance with the dry mass determinations of Mitchison and Cummins (1964) which confirmed the existence of a fluctuating pool by interferometry measurements of whole and acid extracted cells.

There are differences in the conditions of growth used in earlier work by Mitchison and his colleagues, while the present work has all been done at 32°C in EMM. Mitchison's earliest work on S. pombe was for cells grown at 25°C in Malt Extract Broth (MEB, see Appendix I) while the work confirming the existence of a fluctuating pool (Mitchison and Cummins, 1964) was done at 32°C, but still using MEB. While the pattern of dry mass increase has been shown not to change between 23°C and 32°C for cells grown in MEB (Mitchison, Kinghorn and Hawkins, 1963) this does not necessarily mean that there is a fluctuating

pool in cells growing at 25°C. The pattern of synthesis of RNA in the cell cycle was determined spectrophotometrically by Mitchison and Walker (1959) for cells grown at 28°C in MEB. In this case pool material was removed by a 10 minute extraction with 0.02M acetate buffer, pH 4.9, while in the work of Mitchison and Cummins pool material was removed by 5% TCA after freezing. The acid extraction method could not be used by Mitchison and Walker due to absorption by the acid in the ultraviolet, but it was not shown that similar amounts of pool material were removed by the two methods. It now appears that similar quantities of 260 mμ absorbing material (and amino acids) are removed by these methods (Chapter 4). Mitchison and Lark (1962) determined the pattern of RNA synthesis from autoradiographs of pulse labelled cells grown at 32°C in EMM1 and at 25°C in MEB. The determinations of protein and carbohydrate by Mitchison and Wilbur (1962) using the same techniques were only done in MEB grown cells and at temperatures of 25°C or below. In view of these differences it seemed desirable to re-determine the pattern of dry mass increase during the cell cycle using synchronous cultures in EMM and to determine if there was a fluctuation in the total pool material under these circumstances.

Aliquots of 20 ml. were removed at ten minute intervals from synchronous cultures in EMM2 and the dry weight of the cells determined as described in Chapter 2. This experiment was repeated six times: the results from one such experiment

are shown in Figure 35. The maximum difference between an exponential increase and a linear increase in cell mass during the cell cycle is only 5.7% (see Appendix II). A computerised method for analysing the data from these experiments was therefore used. The method was that of Williams (1969) (see Chapter 2). The data for five of the experiments best fitted the smooth model (exponential increase) while the data for one experiment best fitted the segmented model (three straight lines). In this type of analysis experiments showing no discrimination between the smooth and segmented models (see Chapter 2) may be rejected. In fact, none of the experiments gave no discrimination. We may assume that the probability of the analysis confirming the smooth model or the segmented model is $\frac{1}{2}$ in both cases. On this assumption the chance of getting five results favouring the smooth model and one favouring the segmented model is given by the first two terms of the binomial $(x + y)^6$ where x and y = $\frac{1}{2}$. These two terms are $(\frac{1}{2})^6 + 5(\frac{1}{2})^5.(\frac{1}{2}) = 6/64 = 0.094$. Clearly the assumption that the experiments fit the two models with equal probability must be wrong. The experiments, therefore, show that total cell dry weight in synchronous cultures grown in EMM2 increases exponentially with a significance level of P = 0.09.

In each of the above six experiments and in an asynchronous control culture the weight of the pool was determined in the following manner. The cells on the filters were frozen and thawed and then washed with ice-cold water and dried as before.

The weight of the cellular material remaining was determined and hence the amount of material lost by this procedure was calculated as a percentage of the original total dry weight of the cells. This method of extracting pool material was found to remove the same amount of material as freeze-thawing before filtering the cells and allowed an estimate of the total pool from the same sample as that used for total dry weight. Determinations of total pool weight, amino acid, 260 mμ absorbing material and carbohydrate on five replicate aliquots for each component showed no significant differences in the amounts of the pool material obtained by the two methods (Table 17).

The pool dry weight in synchronous cultures determined by the above method was not found to fluctuate during the cell cycle. The mean values together with the standard errors for all the determinations from each experiment are given in Table 18. In each experiment the variation is less than 8% and is not significantly different from that found in the control culture.

It is therefore clear that for cells grown in EMM there is no significant fluctuation in the total weight of pool material and that the pattern of total cell dry weight increases exponentially during the cell cycle. However, for growth in MEB, total cell mass may increase linearly under conditions where there is evidence for a fluctuation in the dry mass of the ~~cell~~ pool during the cell cycle (Mitchison and

Cummins, 1964). It has been supposed that this fluctuation in the mass of the pool accounts for the linear increase in total cell mass while macromolecular components of the cell increase at increasing rates during the cell cycle (Mitchison and Cummins, 1964; Mitchison, 1963a,b). This possibility was investigated by growing cells in MEB and differentially extracting the 'expandable' pool by the osmotic method of Kempner and Cowie (1960). The 'internal' pool may be estimated from the difference between the total pool, obtained by freeze-thawing, and the 'expandable' pool. It is clear from the data of Table 19 that there is an 'expandable' pool for cells grown in MEB and that this represents 6.5% of the total cell mass while the 'internal' pool represents 12.3%; slightly less than the pool in cells grown on EMM. As shown in Chapter 6 no carbon-containing material is lost from cells grown in EMM after the osmotic treatment which extracts the 'expandable' pool. Also, this treatment caused no reduction in the amount of pool amino acid in cells grown in EMM (see "Washing", Chapter 4). It therefore seems reasonable to suggest that the material in the 'expandable' pool of cells grown in MEB represents amino acids accumulated from the medium (see Appendix I for composition of MEB). This was investigated by growing cells in MEB to 8×10^6 cells/ml. and extracting the 'expandable' pool or both pools by the above methods, from 100 ml. aliquots of the culture. The pool amino acids were analysed and the compositions are given in Table 20. The

composition of amino acids in the internal pool is very similar to that of the amino acid pool in cells grown in EMM2 (see Chapter 4, Table 7). The total pool material gave three small unidentified peaks eluting before 'x' and one peak eluting before tyrosine. These were not entirely extracted with the expandable pool. Also, there was a considerable amount of proline in the expandable pool (which was estimated as 'citrulline' in Table 20) but this was largely absent from the internal pool, as is the case for the pool from cells grown in EMM2 (see Chapter 4). The total amount of amino acid and the dry weight of this material per cell in the two pools are also given in Table 20. The dry weight values were calculated on the basis of all the pool amino acids being glutamic acid (M.W. = 147.13). Taking the dry weight of MEB grown cells as 27.48 µµg. (see Table 19) the total dry weight of material in the expandable pool is 6.5 x 27.48/100 = 1.786 and of this the amino acids estimated in Table 20 comprise 1.3039 x 100/1.786 = 73%. The majority of material in the expandable pool is therefore amino acid.

It is clear that the two pools obtained by the extraction procedures outlined above are similar to those obtained by Kempner and Cowie (1960). We may further assume that the internal pool which is comparable to the pool found in cells grown in unsupplemented media (EMM) does not fluctuate during the cell cycle, while the expandable pool may fluctuate. The extreme limits for the size of the total pool would then be

approximately 12.3% (no expandable pool) and 25.3% (twice the mean expandable pool). These extremes are in close agreement with the figures found by Mitchison and Cummins (1964): 9% and 23%.

If the fluctuation in the pool material found by Mitchison and Cummins (1964) is due to a fluctuation in only the expandable pool formed during growth in MEB then the material in the expandable pool must be eliminated by the cell during the second half of the cell cycle. This curious situation may have its explanation in the phenomenon of shock excretion (Lewis and Phaff, 1964). Lewis and Phaff found that in species of Saccharomyces growing in complex media the size of the nitrogenous pool was proportional to the amount of nitrogenous material in the medium and that this pool material was lost rapidly by the cells on suspending them in glucose without amino acids. The nitrogenous material was re-absorbed within two hours. This phenomenon was independent of the osmotic pressure of the suspending medium, but required the presence of a fermentable sugar. Inhibition of adenosine triphosphate synthesis did not prevent shock release but inhibition of the entry or breakdown of glucose did (Lewis and Phaff, 1965). The phenomenon was therefore considered to result from changes in the state of the cell contingent upon the continuous flow of a fermentable sugar into the cell. The rate of uptake of glucose does in fact appear to change during the cell cycle of budding yeast (Kotyk and Kleinzeller, 1963).

TABLE 17

COMPARISON OF POOL MATERIAL OBTAINED BY FREEZE-THAWING BEFORE AND AFTER FILTRATION OF THE CELLS

	Method 1 Freeze-thawing cells before filtration	Method 2 Freeze-thawing cells on the filters
Total pool weight as a percentage of total cell weight	13.95	13.66
Pool amino acid	0.294	0.312
260 mμ absorbing material	0.408	0.397
Pool carbohydrate	0.247	0.251

In method 1 pool material was separated from cell debris, after freeze-thawing, by filtration. Normally this separation is simply achieved by centrifugation. In method 2 cells were filtered and dried on the filters before freeze-thawing.

Estimates are means of five replicate assays and, apart from dry weight, are in arbitrary units.

TABLE 18

TOTAL DRY WEIGHT OF THE POOL IN SYNCHRONOUS CULTURES AND AN ASYNCHRONOUS CONTROL CULTURE

The mean percentage dry weight and the standard errors are given for each culture, together with the number of dry weight determinations for each culture.

		Total weight of pool as a percentage of total cell weight
Synchronous cultures	1	13.61 ± 0.91 (60)
	2	13.82 ± 1.10 (62)
	3	13.50 ± 1.08 (58)
	4	13.73 ± 0.98 (65)
	5	13.48 ± 1.07 (68)
	6	13.52 ± 1.11 (60)
Control culture		13.53 ± 0.99 (62)

TABLE 19

THE DRY WEIGHT OF THE 'INTERNAL' AND 'EXPANDABLE' POOLS FOR CELLS GROWN ON MALT EXTRACT BROTH

Replicate 25 ml. aliquots were taken from a culture at 8×10^6 cells/ml. These were weighed before or after pool extraction. For total dry weight the cells were filtered on Whatman GF/A filters and washed with EMM2 at 32°C. Extraction of the 'expandable' pool was by suspending the cells in 40 ml. ice-cold water for 20 minutes. Both pools were extracted together by freeze-thawing.

	mass of cells in mg.	mass/cell in µµg.
Total mass of cells	5.494 ± 0.142 (5)	27.48
Mass less expandable pool	5.137 ± 0.134 (5)	24.37
Mass less both pools	4.463 ± 0.137 (5)	22.32

Size of both pools = 100 x (5.495 - 4.463)/5.495 = 18.8%

Size of expandable pool = 100 x (5.495 - 5.137)/5.495 = 6.5%

Size of internal pool = 18.8 - 6.5 = 12.3%

TABLE 20

THE AMINO ACID COMPOSITION OF THE 'INTERNAL' AND 'EXPANDABLE' POOLS OF CELLS GROWN IN MEB

Samples analysed: 100 ml. aliquots from MEB culture at 8×10^6 cells/ml. Extraction of the 'expandable' pool was by suspending the filtered and washed cells in 40 ml. ice-cold water for 20 minutes. The total pool ('internal' and 'expandable') was extracted by freeze-thawing.

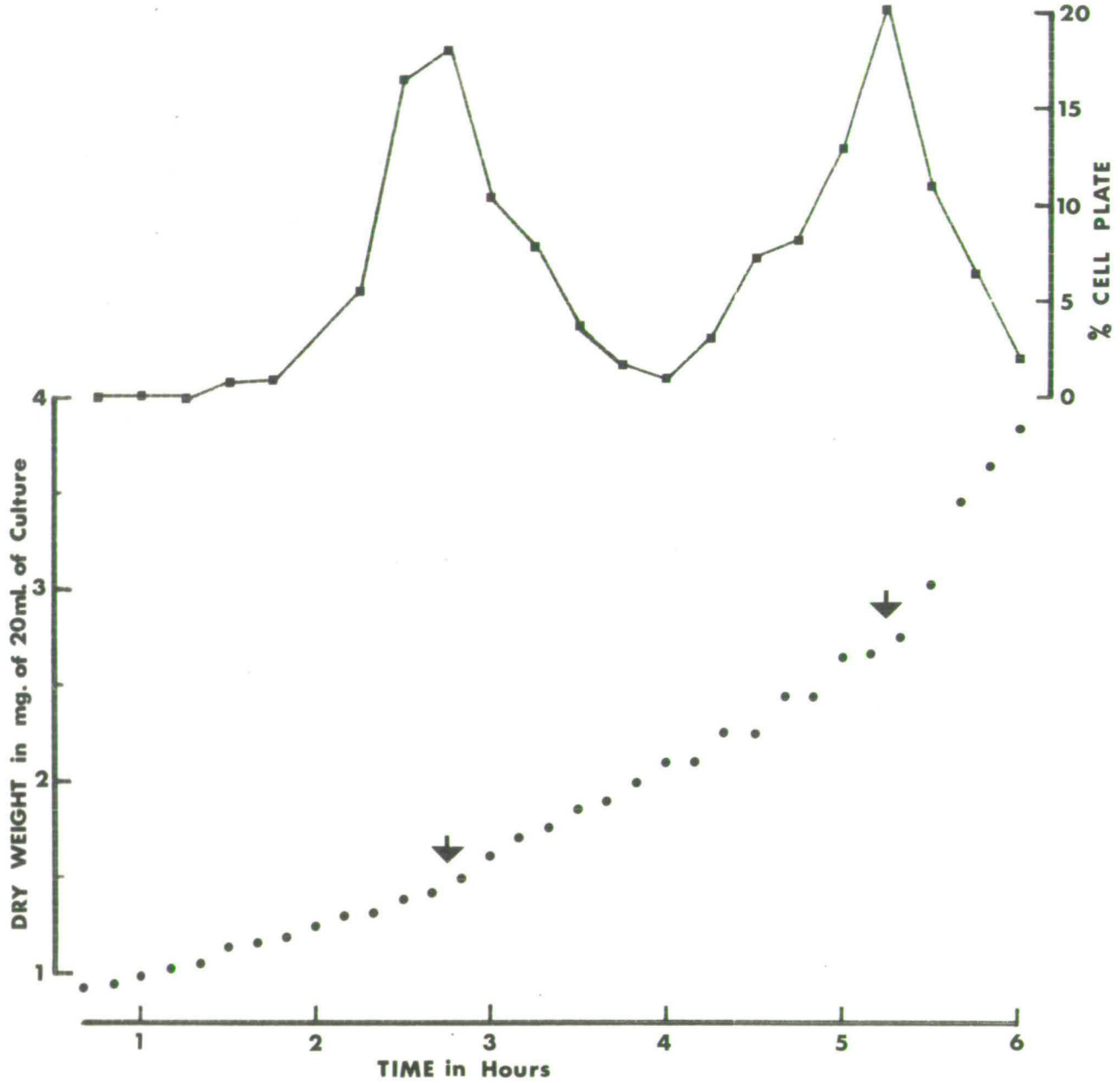
	Expandable pool		Total pool		Internal pool (by difference)	
	µmoles	%	µmoles	%	µmoles	%
X	0.0851	1.20	0.2933	2.63	0.2082	5.10
Aspartic	0.7664	10.81	1.0211	9.14	0.2547	6.24
Threonine	0.4396	6.20	0.6531	5.85	0.2135	5.23
Serine	0.8153	11.50	1.0525	9.42	0.2372	5.81
Glutamic	1.5066	21.25	2.7595	24.70	1.2529	30.69
Citrulline	0.0424	0.60	0.2393	2.14	0.1968	4.82
Y	0.0071	0.10	0.0316	0.28	0.0245	0.60
Glycine	1.1932	16.83	1.3247	11.86	0.1315	3.22
Alanine	1.6519	23.30	2.0532	18.38	0.4013	9.83
Methionine	0.0142	0.20	0.0183	0.16	0.0041	0.10
Isoleucine	0.0142	0.20	0.0505	0.45	0.0363	0.89
Leucine	0.0149	0.21	0.0443	0.40	0.0294	0.72
Tyrosine	0.0000	0.00	0.0176	0.16	0.0176	0.43
Phenylalanine	0.0000	0.00	0.0637	0.57	0.0637	1.56
Lysine	0.2623	3.70	0.6436	5.76	0.3813	9.34
Histidine	0.1843	2.60	0.2578	2.31	0.0735	1.80
Arginine	0.0922	1.30	0.6482	5.80	0.5560	13.62
Total	7.0898		11.1723		4.0825	
Amount/cell in µM x 10^{-9}	8.8622		13.9654		5.1031	
Dry weight/ cell, µµg.	1.3039		2.0547		0.7508	

Analysed by Evans autoanalyser.

FIGURE 35

CHANGES IN TOTAL CELL DRY WEIGHT IN AN EMM2 SYNCHRONOUS CULTURE

The top graph shows changes in percentage cell plate, the peaks being indicated by arrows on the lower graph of cell dry weight.


DRY WEIGHT in mg. of 20 mL of Culture
1
2
3
4
TIME in Hours
1
2
3
4
5
6
% CELL PLATE
0
5
10
15
20

Chapter 9

THE RELATIONSHIP OF THE AMINO ACID POOL TO PROTEIN SYNTHESIS

The analyses of pool amino acid components in synchronous cultures (Chapter 7) have shown no significant fluctuations during the cell cycle. Yet all enzymes assayed to date in S. pombe have shown a discontinuous increase during the cell cycle, typically this pattern being a stepwise increase or a change in rate (Mitchison, 1969). One could presume that small fluctuations (about 10% or less) in an amino acid pool component is sufficient to alter the pattern of an enzyme in the cell cycle. If one is to invoke fluctuations of more than 10% as the causative agents determining such enzyme patterns then one must also invoke some degree of complexity in the organization of the amino acid pools which would allow relative fluctuations at certain sites in the cell. This could be achieved, for example, by spatial compartmentation of amino acids within the cell. Mitochondria generally contain the majority of the enzymes involved in amino acid biosynthesis and mitochondrial-like structures have been found in S. pombe (Schmitter and Barker, 1966), but have not yet been studied biochemically. Amino acid pool material obtained by freeze-thawing and acid extraction almost certainly includes material from mitochondria and nuclei (see Chapter 4). Thus any fluctuations in the amino acid pools within these organelles

will have been included in the amino acid pool analyses in synchronous cultures. Since these fluctuations were negligible, the relative amounts of amino acids within the mitochondria and nuclei must be very small compared to the cytoplasm if there are to be marked fluctuations within the organelles.

This chapter is concerned with an attempt to analyse the mitochondrial pool of amino acids and experimental uncoupling of protein synthesis from the total amino acid pool.

Mitochondrial amino acids. Mitochondria were isolated from cells broken by means of the Eaton press (see Chapter 2). To minimise rupture of the mitochondria the cell suspension was prepared in 1M sorbitol containing 20% glycerol and the cells were harvested in exponential phase (6×10^6 cells/ml.) and ruptured immediately without prior freezing. 6×10^{10} cells ruptured in this manner were layered on to the top of a sorbitol step gradient of 15 ml. consisting of equal volumes of 2M, 3M and 4M sorbitol all made up in EMM2. This method has been used by Duffus (1969) for separation of nuclei in S. pombe. From assays of succinic dehydrogenase the mitochondria appear to be largely in the top, 2M sorbitol fraction after centrifugation for 20 minutes at 25,000 r.p.m. in a Spico SW25 rotor (Duffus, per. comm.). This material was collected and washed twice with 0.25M sucrose with centrifugation at 8,000 g. after the first wash and 5,000 g. after the second wash.

One-fifth of the pellet was used for electron microscopic examination. Electron micrographs were prepared by fixing the material in 5% glutaraldehyde, dehydrating with acetone, staining with 1% uranyl acetate and embedding in Vestapol. The sections (shown in Figure 36) were stained with aqueous lead citrate and examined in a Siemkens electron microscope. There is some damage, but cristae are clearly visible and the mitochondria are the same size as those found in sections of whole cells by Schmitter and Barker (1966). The inner and outer membranes show the characteristic separation of sucrose isolated mitochondria (Klingenberg and Pfaff, 1965).

Four-fifths of the mitochondrial pellet were frozen and later thawed and the supernatant analysed for amino acids. The level of all amino acids in this mitochondrial pool was extremely low, but the relative amounts of each amino acid seemed to be approximately the same as in the total pool. The amino acid composition was not calculated as most of the peaks were too small for satisfactory estimation. These results are taken to indicate that the mitochondrial amino acid pool is largely lost during the isolation procedure. This is most unfortunate since the usual method of isolating mitochondria in yeast, involving prior preparation of protoplasts (by snail-gut enzymes) before rupture of the cells, is not feasible for large numbers of cells of S. pombe (Duffus, per. comm.).

Experimental uncoupling of protein synthesis and pool amino acids. Although no correlations have been found between the synthesis of proteins and amino acid pool components, we may still attempt to find causal relationships between pool components and the synthesis of proteins by attempting to uncouple the two events. Such experimental manipulation would be necessary even if correlations had been found between fluctuations in pool components and appropriate enzymes in the cell cycle. For the existence of such a correlation would not alone establish that the two events were causally related. Experimental uncoupling could, however, merely indicate that the amino acid pool constituted a precursor pool, which has already been established (Chapter 6). For, if protein synthesis is specifically blocked, one would anticipate that the levels of the protein precursors would increase rapidly. The type of uncoupling that would be most informative would be one in which the growth of the cell was appropriately altered without killing the cell and in which the duration and position of the DNA synthetic period was known. The reason for the latter requirement is that we are making no assumptions as to the mechanism whereby a fluctuation in a pool amino acid may cause an alteration in the rate of synthesis of a particular enzyme. The mechanism could be at the ~~genetic~~ transcriptional or translational levels. Since ultimately the pattern of protein synthesis in the cell cycle is genetically determined, it would seem desirable not to alter the time at which the

genetic material doubles, or at least to know what alterations accompanied the uncoupling treatment.

We may consider two types of experiment which could point to causal relationships between the amino acid pool and the pattern of protein synthesis during the cell cycle:

1) We may alter the pool in some way and observe the concomitant pattern of protein synthesis, or
2) we may alter the pattern of protein synthesis and observe the behaviour of the pool.

These two types of experiment may concern the relationship of particular proteins such as specific enzymes, or they may concern the total amino acid pool and the total or major fractions of the protein.

Consider the first type of experiment. It is in fact very difficult to alter the amino acid pool of S. pombe, as we have already seen (Chapters 4 and 6). One method known to cause the loss of the amino acid pool in Saccharomyces cervisiae is ultraviolet (uv) irradiation (Swenson and Dott, 1961). However, the dose required to cause pool loss is lethal (B.F. Gill, per. comm.) and so of dubious use. Moreover, at such doses the irradiation will almost certainly have more than one effect, for even at considerably lower levels of irradiation which cause division delay in S. pombe there are complex changes in uv sensitivity with two major phases in the cell cycle at which cells are most sensitive to uv (Swann, 1962; Gill, 1965). This complexity in the response to uv

irradiation is unfortunate since at the doses used by Gill there is no effect on the overall rate of growth, only a delay in division.

We may, however, consider the more specific alteration of a single amino acid in the pool in relation to protein synthesis. Arginine is known to be an inhibitor of ornithine transcarbamylase (OTCase) in several cases (Stadtman, 1966). Since the activity of OTCase in the cell cycle doubles sharply near the middle of the cell cycle (Bostock et al., 1966) we might expect that the addition of arginine to the medium of an exponential culture of S. pombe would lower the level of OTCase. This was not found to be the case over a four-hour period, after addition of arginine at five different levels in the medium (see Figure 37). The fact that OTCase activity increases sharply at one stage in the cell cycle could be interpreted as meaning that the enzyme can only be made at this stage. Addition of an inhibitor may therefore only have an effect when cells reach this stage. But the experiment under consideration was continued for almost two generation times and so a definite effect should have been observed if arginine has an inhibitory effect.

It is still possible to argue that arginine has an effect on OTCase but that this is such that the mean amount per cell remains the same. For example, the stepwise increase in OTCase during synchronous growth could be lost in the presence of arginine, but the mean amount averaged over the cell cycle

remain as before. If this is so it seems possible from the above experiments that any level of arginine between 1 and 100 µgm. will have this effect. Arginine was therefore added to give 60 µgm./ml. at the beginning of a synchronous culture in EMM2 and 1 ml. samples removed at 10 minute intervals for assay of OTCase. The result is shown in Figure 38 and clearly indicates that the pattern of OTCase is essentially unaltered from that in cultures without arginine (Bostock et al., 1966).

Other amino acid biosynthetic enzymes assayed in S. pombe are also step enzymes which are unaltered in amount and pattern of synthesis by addition of amino acids to the medium (Robinson, 1966, and per comm.). In the case of tryptophan synthetase, Robinson (1966) also tested for permeability of the cells to tryptophan: labelled tryptophan was found to enter the pool, but there was negligible incorporation of labelled tryptophan into protein.

We may now consider the second type of experiment listed above which would indicate a causal relationship between the amino acid pool and the synthesis of proteins. Three agents for altering the pattern of protein synthesis were considered: chloramphenicol, actidione (cycloheximide), and 2-phenyl ethanol. Chloramphenicol has no apparent effect on the growth of S. pombe as monitored by optical density even at levels up to the limits of solubility for the agent (2.5 mgm./ml. at 25°C). Moreover, estimates of the amount of protein in chloramphenicol-treated and control cultures showed no

difference in amount after 1, 3 and 5 hours. It is concluded that in S. pombe chloramphenicol is unable to enter the cell.

Actidione has a quite marked effect on the growth of S. pombe at concentrations above 20 µgm./ml. As shown in Figure 39 there is a sharp cessation in the synthesis of protein after a delay of about 10 minutes and the increase in OTCase activity also ceases abruptly. RNA synthesis continues for somewhat longer, but ceases after about 65 minutes. These changes are accompanied by a rapid increase in the amounts of pool amino acids (Figure 40). However, after actidione treatment there is no growth or division of cells for at least 14 hours. Although there may be division thereafter, the cells ultimately die and cannot be maintained by subculturing into fresh medium, even without actidione. This experiment shows that actidione does not satisfy the requirements outlined above. It does, however, provide further evidence that the endogenously synthesized amino acid pool is largely a pool of precursors for synthesis of macromolecules and confirms similar findings by Hancock (1960) for Staphylococcus aureus.

Bostock (1968) has investigated the effect of 2-phenyl ethanol (PE) on the growth and synthesis of DNA in S. pombe. There is no effect on growth at concentrations of PE below 0.1%, but at 0.4% and above virtually all growth is stopped. At 0.2% PE the growth rate is reduced: the doubling time is about 4 hours 15 minutes. The cells grow into stationary phase at a lower concentration than normal, but the cells can

be maintained indefinitely in EMM2 plus 0.2% PE by subculturing. From DNA determinations in synchronous cultures with 0.2% PE Bostock concluded that the S and G2 phases were of the same absolute duration as in normal EMM2 grown cultures. The increase in the generation time was due to a greatly extended G1 phase which normally is almost absent in S. pombe.

The effects of PE on protein and pool amino acids in synchronous cultures were determined in the following manner. Synchronous cultures were prepared from 20 litre cultures grown to between 2 and 5 x 10^6 cells/ml. in EMM2 plus 0.2% PE. The cells were separated on glucose gradients made up as normally but with the addition of 0.2% PE. The top layer of cells were inoculated into fresh EMM2 plus 0.2% PE. Protein and pool amino acids were assayed. The results of two such experiments are given in Figure 41, and the results of a control culture in Figure 42.

It is clear that, while the mean amount of protein and pool amino acids are not greatly altered, the pattern of protein synthesis is quite different from cultures grown in the absence of PE. There are plateau periods in the cell cycle during which the total amount of protein does not increase. The period during which protein synthesis does occur occupies about 2 hours 20 minutes and so is of the same duration as in normal cultures. Moreover, as far as the accuracy of the data allows, the rate of protein synthesis during the synthetic period is also exponential. There is one curious feature in

the protein data. While in the first synchronous cycle the period of protein synthesis is more or less in the middle of the cell cycle, this period becomes progressively earlier in the subsequent two cycles. The protein synthesis cycle appears to be slightly out of step with the cell division cycle.

While the pattern of protein synthesis in synchronous PE treated cultures appears markedly different from normal synchronous cultures, this is not the case for the total amino acid pool. Total pool amino acids increase exponentially throughout the cell cycle as in untreated synchronous cultures. Detailed analyses of the composition of the amino acid pool throughout the cell cycle have not been done for PE treated synchronous cultures. Three samples for autoanalysis were, however, taken at regularly spaced intervals in one experiment at the stages indicated by the numbered arrows in Figure 41. The pool analyses are given in Table 21 and show no marked differences in the composition of the amino acid pool. It seems reasonable to infer that, as in untreated cells, there are no marked fluctuations in the amino acid components of the pool throughout the cell cycle in PE treated cells.

The primary effect of 2-phenyl ethanol is not clear. In *E. coli* the most sensitive synthetic process appears to be induced enzyme synthesis, although protein synthesis generally and RNA synthesis are affected at quite low concentrations (Rozenkranz *et al.*, 1965). 2-Phenyl ethanol has been shown to inhibit the synthesis of RNA, DNA and protein in *Neurospora*

and to inhibit the uptake of amino acids and, partially, the uptake of glucose (Lester, 1965). Whatever the effect of 2-phenyl ethanol on S. pombe it would appear that total protein synthesis can be uncoupled from the synthesis of pool amino acids without inhibiting the synthesis of DNA, although the generation time is increased by the insertion of a G1 phase.

TABLE 21

ANALYSIS OF AMINO ACIDS IN SAMPLES FROM A SYNCHRONOUS CULTURE GROWN IN 0.2% 2-PHENYL ETHANOL

Samples of 220 ml., 180 ml. and 150 ml. were removed at the times indicated by the arrows numbered 1, 2 and 3 respectively (see Figure 41). The cell concentration was 2.6×10^6 cells/ml. at the time the first sample was removed. The pool compositions given below have been calculated for a sample volume of 220 ml. in each case.

	1		2		3	
Amino acid	µmoles	%	µmoles	%	µmoles	%
X	0.1472	5.20	0.1432	4.87	0.1479	5.13
Aspartic acid	0.1726	6.10	0.1729	5.88	0.1779	6.17
Threonine	0.1534	5.42	0.1588	5.40	0.1517	5.26
Serine	0.1772	6.26	0.1755	5.97	0.1816	6.30
Glutamic acid	0.9702	34.28	0.9817	33.39	0.9321	32.33
Citrulline	0.1477	5.22	0.1502	5.11	0.1623	5.63
Y	0.0154	0.54	0.0123	0.42	0.0138	0.48
Glycine	0.1002	3.54	0.1079	3.67	0.1087	3.77
Alanine	0.2550	9.01	0.3005	10.22	0.3123	10.83
Methionine	0.0096	0.34	0.0079	0.27	0.0084	0.29
Isoleucine	0.0125	0.44	0.0150	0.51	0.0167	0.58
Leucine	0.0204	0.72	0.0194	0.66	0.0196	0.68
Tyrosine	0.0136	0.48	0.0162	0.55	0.0153	0.53
Phenylalanine	0.0425	1.50	0.0376	1.28	0.0424	1.47
Lysine	0.2383	8.42	0.2346	7.98	0.2321	8.05
Histidine	0.0430	1.52	0.0506	1.72	0.0496	1.72
Arginine	0.3116	11.01	0.3558	12.10	0.3108	10.78
Total	2.8304		2.9401		2.8832	

FIGURE 36

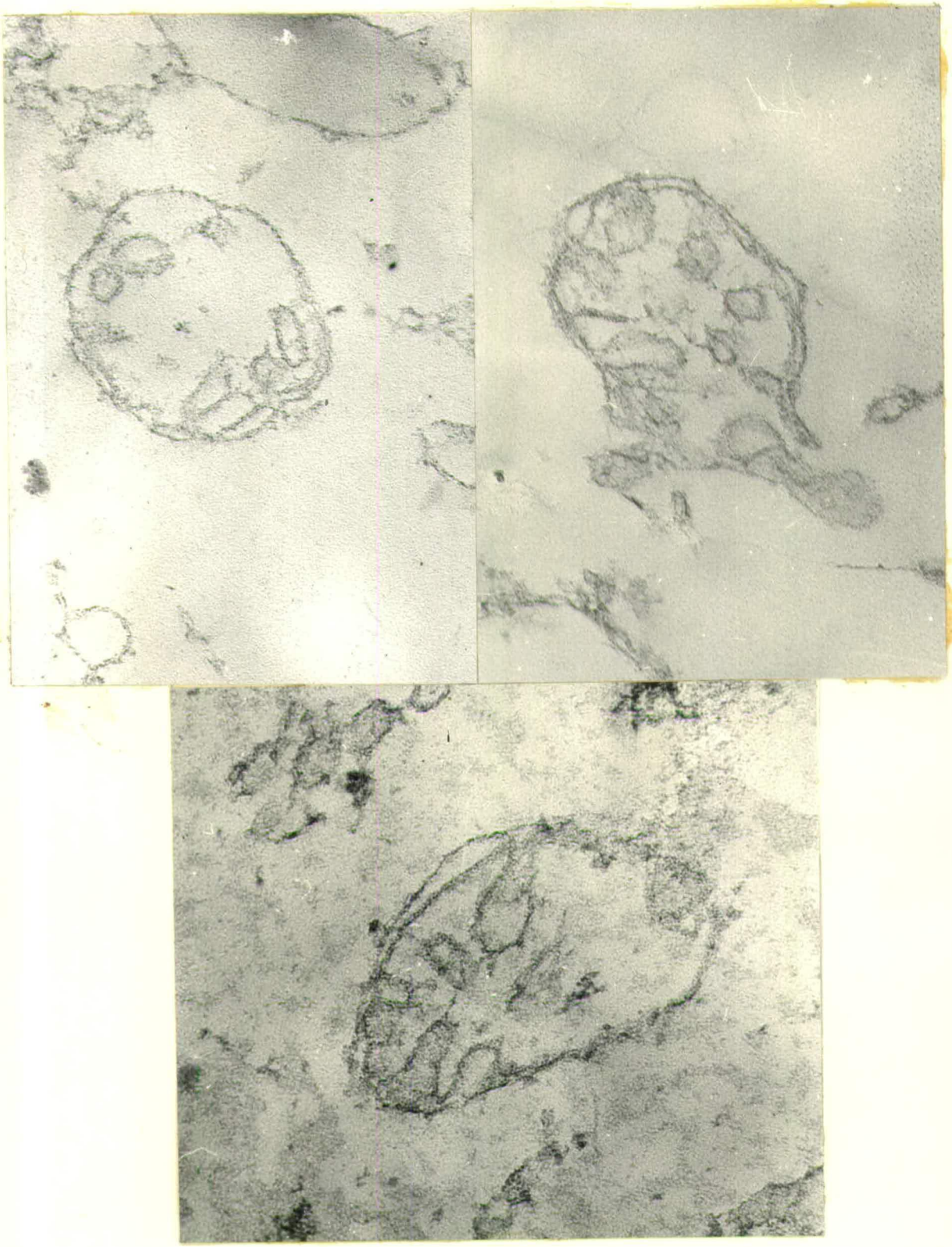
ELECTRON MICROGRAPHS OF MITOCHONDRIA

Plates were at a magnification of 28,000.

Scale as indicated.

Material was fixed with 5% glutaraldehyde, dehydrated with acetone, stained with 1% uranyl acetate, and embedded in Vestapol.

Sections stained with aqueous lead citrate.



2,000 Å

FIGURE 37

THE EFFECT OF ARGININE ON THE SYNTHESIS OF ORNITHINE TRANSCARBAMYLASE

After one hour, the culture was divided into five, and arginine added to each at a different concentration. OTCase assays before splitting the culture (indicated by arrow) are shown by the open triangles.

Solid circles: arginine at 1 µgm./ml.

Open circles: arginine at 25 µgm./ml.

Solid squares: arginine at 50 µgm./ml.

Open squares: arginine at 75 µgm./ml.

Solid triangles: arginine at 150 µgm./ml.

Culture grown in EMM2. Cell concentration at zero time was 2×10^6 cells/ml.

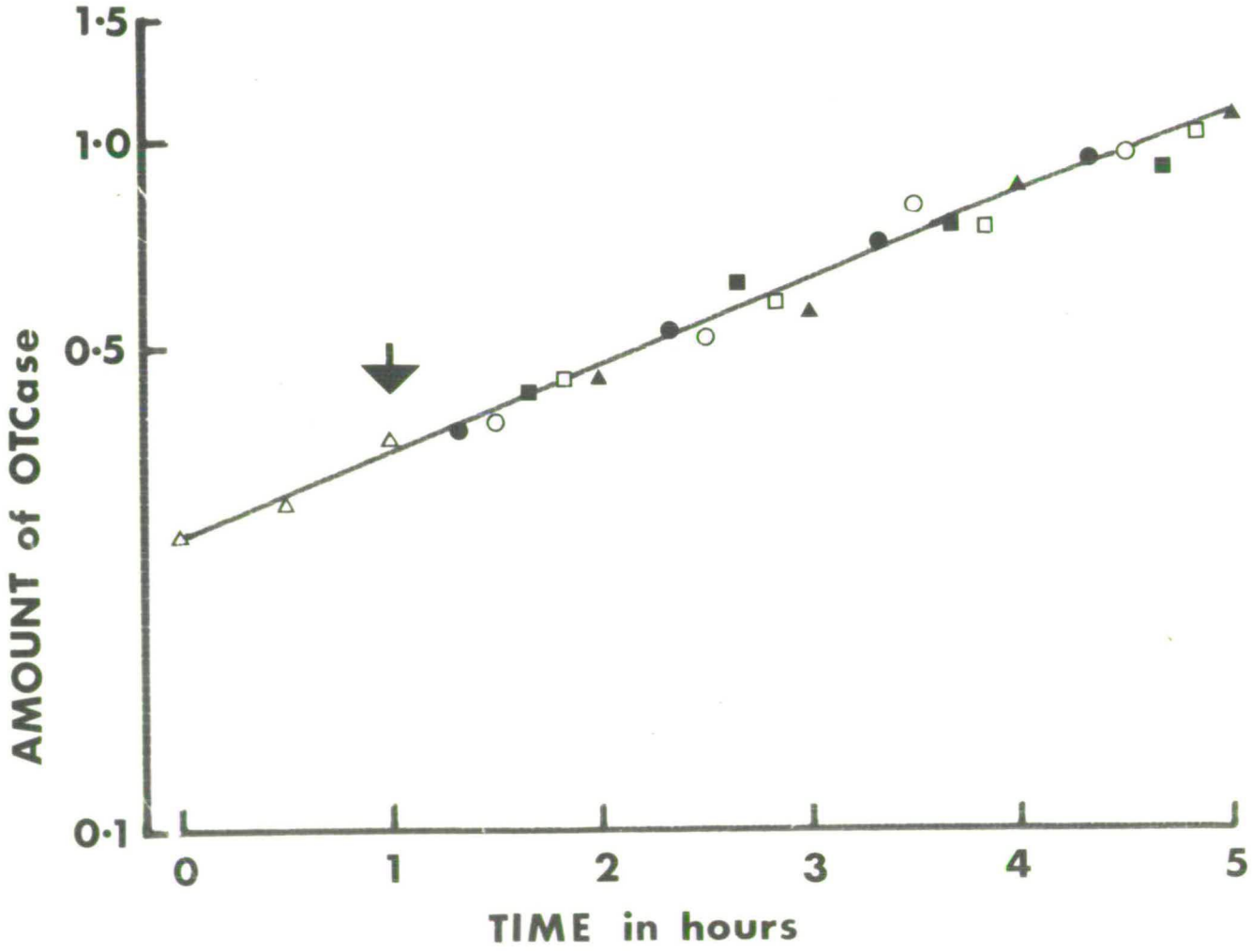

1·5
1·0
0·5
0·1
AMOUNT of OTCase
0
1
2
3
4
5
TIME in hours

FIGURE 38

SYNTHESIS OF ORNITHINE TRANSCARBAMYLASE
IN AN EMM2 SYNCHRONOUS CULTURE

The times of the cell plate peaks in the synchronous culture are indicated by arrows.

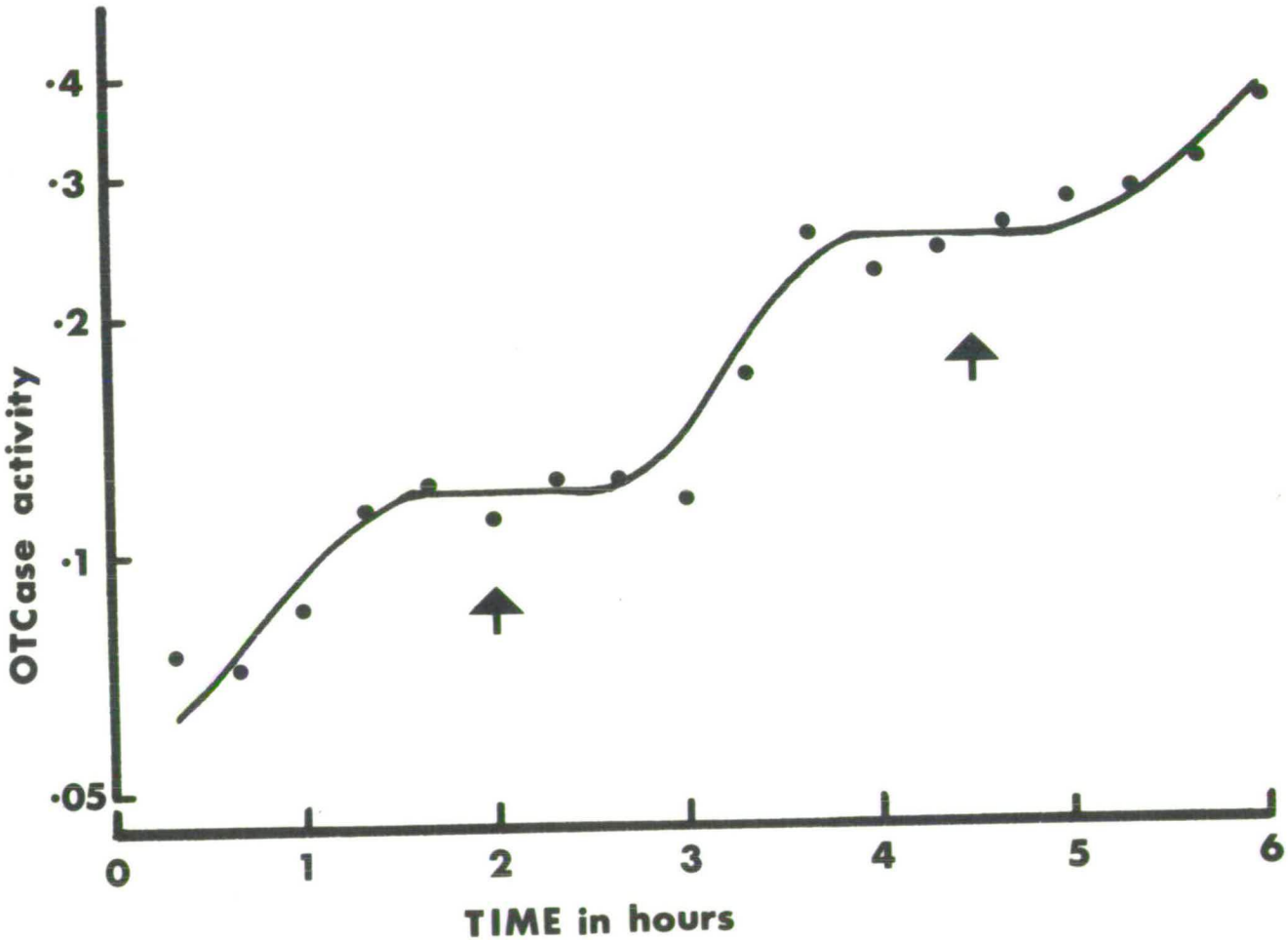

·4
·3
·2
·1
·05
OTCase activity
0
1
2
3
4
5
6
TIME in hours

FIGURE 39

CHANGES IN AMOUNT OF RNA, PROTEIN AND ORNITHINE TRANSCARBAMYLASE AFTER ADDITION OF ACTIDIONE TO AN EXPONENTIAL CULTURE GROWN IN EMM2

The culture was at a cell concentration of 10^6 cells/ml. at time zero. Actidione was added after one hour (arrows in figure) to a concentration of 20 µg./ml.

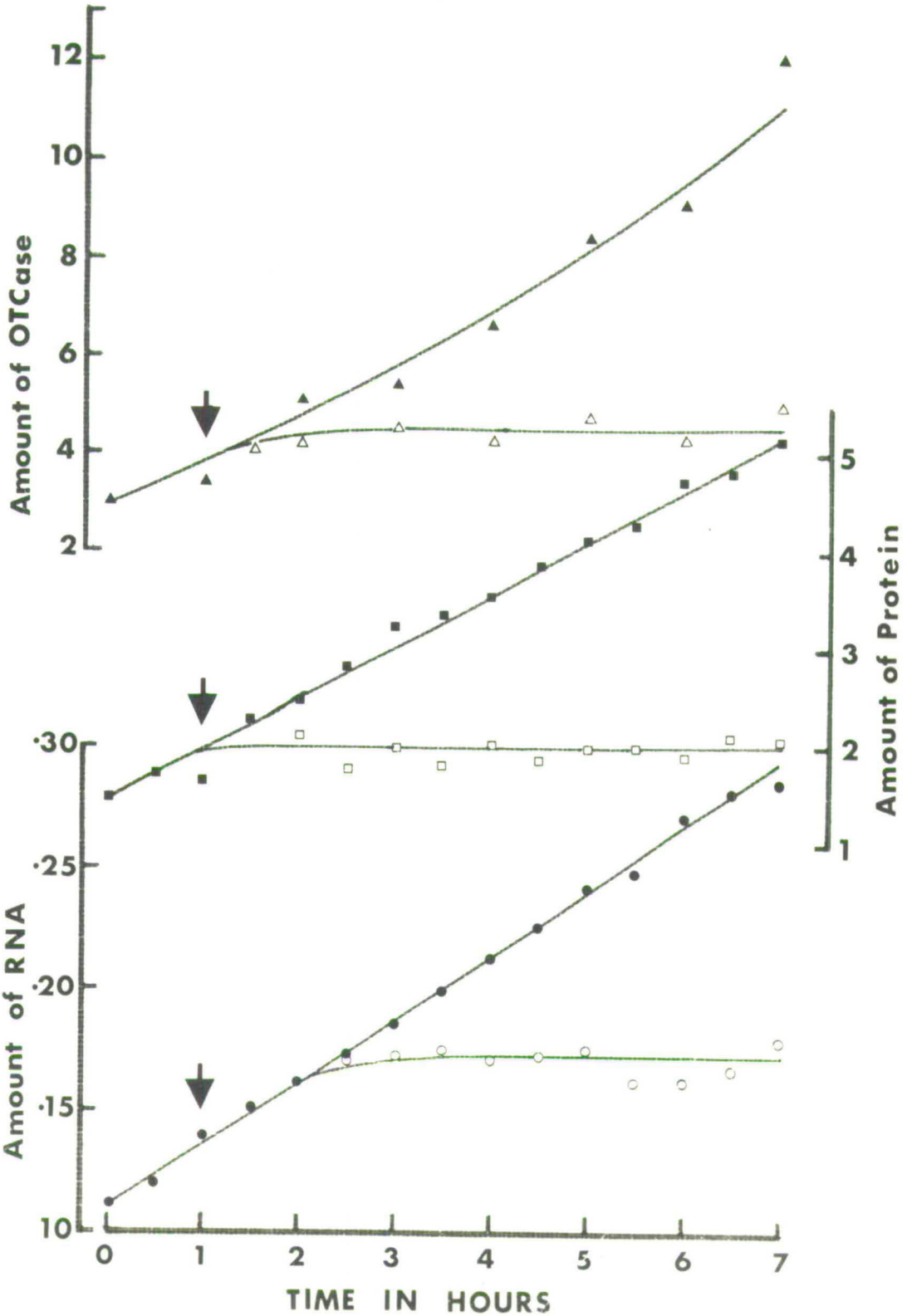

Amount of OTCase
12
10
8
6
4
2
Amount of RNA
·30
·25
·20
·15
10
Amount of Protein
5
4
3
2
1
0
1
2
3
4
5
6
7
TIME IN HOURS

FIGURE 40

THE AMOUNT OF POOL AMINO ACID AFTER ADDITION OF ACTIDIONE (20 µmg./ml.)

Actidione was added at the time indicated by the arrow. The cell concentration at zero time was 1.8×10^6 cells/ml.

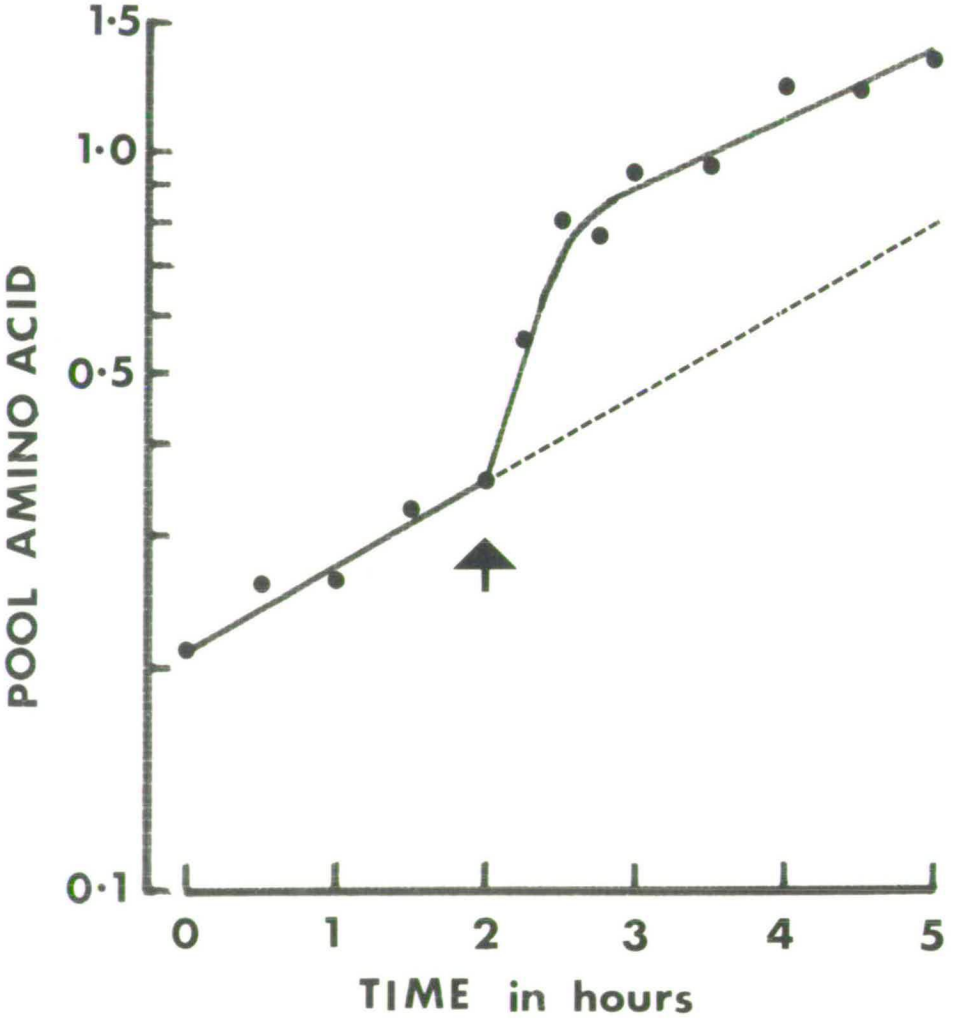

1·5
1·0
0·5
0·1
POOL AMINO ACID
0
1
2
3
4
5
TIME in hours

FIGURE 41

TWO EXPERIMENTS SHOWING THE CHANGES
IN TOTAL PROTEIN AND POOL AMINO ACID
DURING SYNCHRONOUS GROWTH IN EMM2
PLUS 0.2% PE

The three numbered arrows indicate the times at which samples were taken in one of the experiments, for analysis of the amino acid components (see Table 21).
The un-numbered arrows indicate the times of the peaks in percentage cell plate.

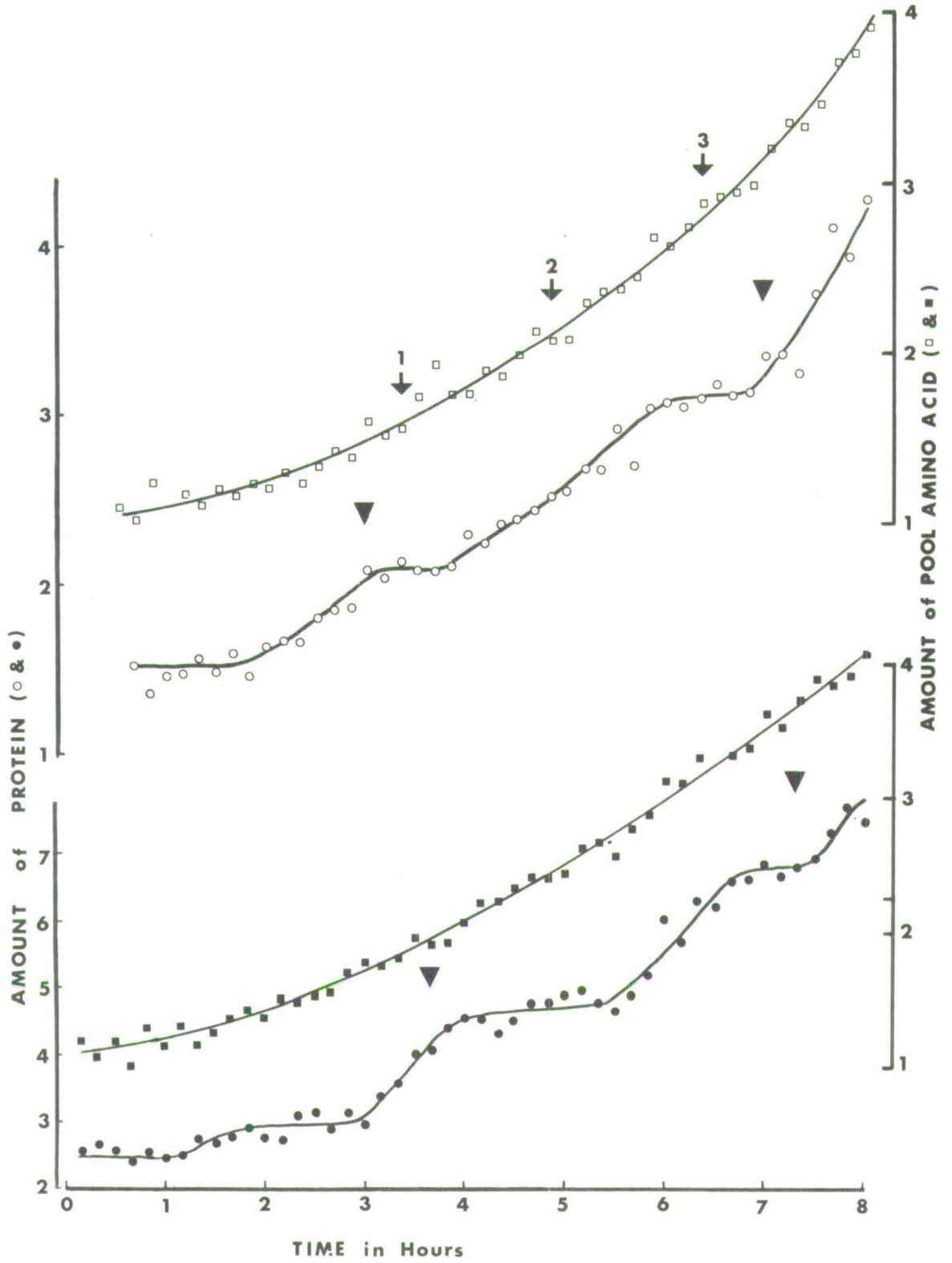
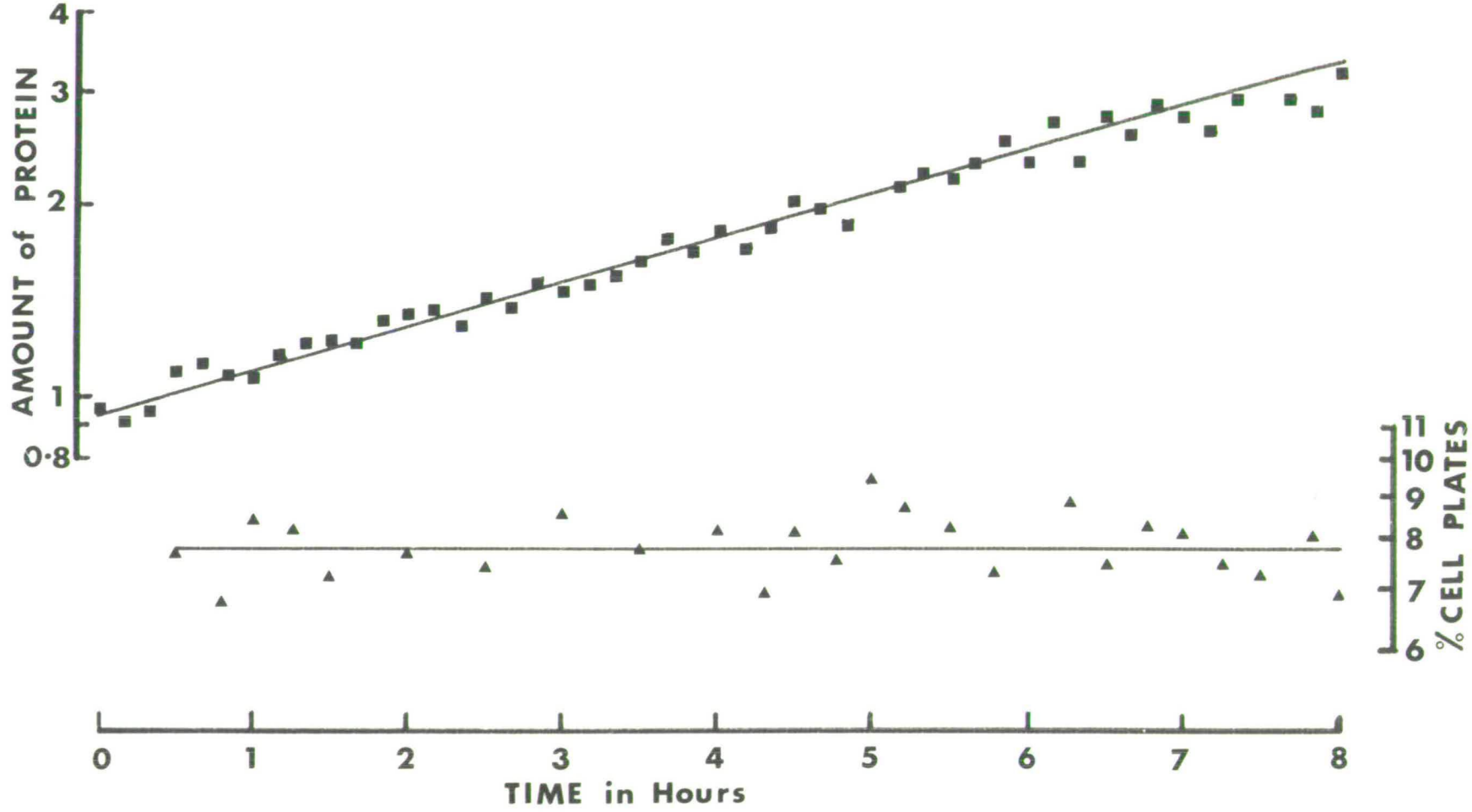

AMOUNT of PROTEIN (○ & ●)
AMOUNT of POOL AMINO ACID (□ & ■)
TIME in Hours
1
2
3
0
1
2
3
4
5
6
7
8

FIGURE 42

CHANGES IN TOTAL PROTEIN IN AN ASYNCHRONOUS CONTROL CULTURE GROWN IN EMM2 PLUS 0.2% PE

The percentage cell plate through the culture is also shown.


AMOUNT of PROTEIN
4
3
2
1
0·8
% CELL PLATES
11
10
9
8
7
6
0
1
2
3
4
5
6
7
8
TIME in Hours

Chapter 10

DISCUSSION

The majority of the experimental work reported in the preceding chapters was discussed in relation to previous work on S. pombe and the relevant literature in the appropriate chapters. It remains, however, to consider those parts of the work relevant to the control of protein synthesis in relation to the phenomenon of stepwise enzyme synthesis found in S. pombe, other yeasts, and bacteria. This general discussion is therefore arranged so as to consider the evidence for the mechanism of synthesis of amino acids, the location of the pool, the meaning of 'control' in cell growth and the properties and nature of these controls. An analysis of the theories proposed for the control of stepwise enzyme synthesis is also given in relation to the information provided by the present work. The in vivo existence of feedback controls of enzyme synthesis of the Jacob-Monod type is also considered together with recent evidence showing the complexity of regulatory phenomena in some pathways.

The tricarboxylic acid cycle (Krebs and Lowenstein, 1960) is fundamentally a mechanism whereby pyruvate is oxidatively decarboxylated and is generally the respiratory pathway of yeasts. The primary intermediates of carbohydrate metabolism utilized for the synthesis of amino acids are pyruvate, α-ketoglutarate and oxaloacetate for the synthesis of alanine,

glutamate and aspartate respectively. The majority of the other amino acids are obtained by transamination with these three amino acids, mainly glutamate and aspartate (Meister, 1964). The removal of oxaloacetate and ∝-ketoglutarate from the tricarboxylic acid cycle for amino acid synthesis necessitates their replenishment in the cycle. This appears to be due either to the formation of a C_4 acid by carbon dioxide fixation with pyruvate (Work et al., 1941), or the operation of the glyoxylate bypass (Kornberg and Krebs, 1957), which is essentially an anabolic sequence increasing the amount of malate and hence other cycle intermediates. These mechanisms allow utilization of cycle intermediates without hindering the functioning of the tricarboxylic acid cycle as a respiratory pathway.

Clearly, the tricarboxylic acid cycle is of fundamental importance for the synthesis of amino acids. While there is no detailed evidence for the existence of the cycle and associated amino acid biosynthetic pathways in S. pombe, work on other yeasts would indicate that these reaction sequences do exist and are largely located in mitochondria (Vitols and Linnane, 1961). Isolated mitochondria from Neurospora have been shown to produce several amino acids (Kinsey and Wagner, 1966). Three experimental findings support the notion that S. pombe has a tricarboxylic acid cycle:

1) Four of the organic acid intermediates have been detected by paper chromatography: citrate, malate, succinate

and fumarate (see Chapter 4). The presence of citrate is strong evidence for the existence of one of the enzymes of the tricarboxylic acid cycle. For the only known method of producing citrate from glucose is by the reaction between oxaloacetate and acetyl-CoA catalysed by condensing enzyme (Gilvarg and Davis, 1956). This is the mode of entry into the cycle of acetyl-CoA formed from glucose via pyruvate in the glycolytic sequence.

2) Mitochondria have been found by electron microscopy (Schmitter and Barker, 1966), and have been isolated (see Chapter 9).

3) Malonate causes a decrease in growth rate, which is reversible by addition of succinate (see Chapter 5). Malonate is a specific inhibitor of succinic dehydrogenase which is competitively reversed by succinate. This indicates the presence of succinic dehydrogenase in S. pombe, whose presence in the miotchondrial fraction of ruptured cells has been confirmed by direct assay (Duffus, per. comm.).

As mentioned in the opening paragraph of this thesis, it is sometimes accepted that the observation of balanced growth of cells in minimal media under different regimes of growth indicates that the molecular composition of the cells is controlled. A model of cell growth displaying these phenomena but not involving feedback control mechanisms is outlined in Appendix III. The demonstration of feedback control of enzyme synthesis or activity, by addition of supplements to

the medium or by the behaviour of isolated enzymes *in vitro*, does not prove that these mechanisms are used by cells in other circumstances. Of course the demonstration of such control mechanisms does make their existence *in vivo* in cells growing in minimal media entirely possible. It is perhaps forgotten by those who argue that balanced growth implies feedback controls, that this would require the simultaneous evolution of enzymes and the supposed control systems. One can accept the existence of feedback control mechanisms in cells from considerations of the inadequacies of the simplistic model (Appendix III). The model does not account for the particular amount of each molecular component of cells, nor does it allow 'adaptation' in the sense described below. We will, therefore, consider these two limitations of the simplistic model together with what is meant by 'control'. Firstly, in the model (Appendix III) vast excesses of any number of components could occur and would require unnecessary but unavoidable expenditure of energy since each molecular component of the cell doubles in amount in each cell cycle. Nevertheless, it is important to realise that considerations of enzyme sequences clearly show features which may be considered as 'controls' but which are simply consequences of the enzyme sequences themselves and the law of mass action (Chance, 1961; Kascer, 1957, 1963; Rosen, 1968). For an open system (see Kascer, 1957), in the steady state, the flux at each step in an enzyme sequence will be the same. The final product of such a sequence, say an amino

acid, may be incorporated into proteins and hence enter another state within the cell so that there will be an irreversible reaction maintaining a flux in one direction. An alteration of any intermediate in the system will result in alteration in the flux at each step in the sequence, but these will settle down so that finally the system is as before and the net flux through the sequence is identical to that before the disturbance. In particular, wide fluctuations in the amount of the initial metabolite (the carbon source in the medium) will be buffered out and will not radically alter the flux through the pathway. Also, wide variations in the activity of an enzyme catalysing the production of the pathway's end product need not alter the size of the end product pool, as argued theoretically and shown to be the case in the arginine pathway of Neurospora by Kascer (1963). Of course, when the final product of the sequence is one of the enzymes determining the flux at one step in the sequence, a disturbance may cause a temporary decrease in the rate of production of that enzyme so that a lower amount of the enzyme will be maintained when the system settles down. This will have resulted simply from the kinetic organization of enzyme sequences (Kascer, 1963). The cause of the disturbance is a separate matter. Enzyme induction or repression could be the cause and would result in a temporary increase in rate of synthesis of the enzyme. A new level of enzyme would then be maintained even by the previous rate of synthesis. A return to a lower amount of

enzyme per cell could, however, be achieved by 'diluting' out the enzyme through growth. An important type of control in which the activities of enzymes remain unaltered can occur in branched enzyme sequences. If the enzymes of two sequences with a common precursor have different affinities for the common substrate over different concentration ranges, a simple switch mechanism is possible (Chance, 1961; Rosen, 1968) and this type of inherent control does seem to regulate the extent of glycolysis and respiration in yeasts (Holzer, 1961, 1968). These phenomena clearly show features of control. But the term 'control' should perhaps be restricted to cases of feedback control. The use of the term 'control' will henceforth be restricted to feedback control, while 'control' due solely to the properties of enzyme sequences will be termed 'inherent control'.

Control of a pathway is only effective if the enzyme controlled is rate limiting for the pathway. The activity of some enzymes in a pathway may be reduced by as much as 97% without any effect on the overall growth rate, as demonstrated for enzymes of the arginine pathway (Kascer and Burns, 1968). It is therefore clear that the amounts of many enzymes may not be regulated at all exactly to the cell's requirements at any one time. But the meaning of a rate limiting, or controlling enzyme in an enzyme sequence is not immediately obvious, due to the inherent controls within such systems. For each enzyme in a multi-enzyme system there will be a 'sensitivity co-

efficient' C (Kascer and Burns, 1968) which is defined as the ratio of the percentage change in the flux through the pathway to the percentage change in enzyme activity causing the change. The maximum possible value of C is 1, for then a 1% change in an enzyme causes a 1% change in the associated flux so that that enzyme will be totally rate limiting. Clearly, in any given pathway, changes in the amount or activity of the enzyme with the highest calue of C will cause the greatest change in the flux through that pathway. Decreases in the amounts of enzymes with low C values will have almost negligible effects. However, as shown by Kascer and Burns (1968), the sensitivity coefficients of enzymes in a multi-enzyme system are not independent and the value of C for any enzyme may change over the range of its activity. We may modify the simplistic model (Appendix III) by including feedback control mechanisms. These would tend to regulate the flux through all pathways of the system and make it 'adaptable'. These feedback controls could not alter the C values of enzymes. But in the event of one enzyme becoming rate limiting (e.g. through a decrease in activity induced by a mutation or a depletion of a supplement to the medium) the cell could increase the amount of that enzyme by reducing the production of other enzymes (with low C values). In this way a change which might otherwise have drastically reduced the growth rate of the cell would be limited through "optimal allocation of enzymes" (Burns, 1969).

There may also be forms of inherent control in the

synthesis of macromolecules generally. The interdependence of RNA and protein synthesis may alone cause a change in the relative amounts of these materials in a cell when the growth rate is changed and this interdependence may also determine the amounts of these materials in cells when they achieve balanced growth after inoculation into fresh medium (Koch, 1961). This type of interdependence has been demonstrated in cells (Kennell and Magasanik, 1962). However, changes in cellular components with alteration in the growth rate cannot always be attributable to this simple form of inherent control (Neidhardt and Fraenkel, 1961). But the growth rate of cells seems to be largely determined by their capacity to produce protein (Schaechter *et al.*, 1958). Hence the production of additional protein should lower the growth rate, and this has been found to be the case in mutants constitutive for β-galactosidase (Horiuchi *et al.*, 1962).

These arguments show that the phenomenon of balanced growth cannot be taken to indicate that feedback controls must operate in the cell. Nevertheless, we can argue that feedback controls exist, on the basis of cellular economy.

Before considering the relationship of pool amino acids to protein synthesis, we must consider the composition of the amino acid pool. We may use telonomic arguments (see Davis, 1961) to consider why pools of amino acids exist in cells and why they have the composition they do. It should first be made clear that pools of precursors in cells necessitates an expenditure of

energy which from a telonomic point of view may be uneconomic for the cell, unless of course the pool fulfils some necessary function within the cell. It is clear from the experiment in which cells were grown in a high molarity of sorbitol that the amino acid pool in S. pombe is not an essential factor in the osmoregulation of the cell (Chapter 5). As argued in Appendix III, if a pool component is large and the cell is growing in a balanced fashion, then the flux of material into the pool must be considerably higher than the efflux from that pool. Hence in the absence of feedback control, the enzyme whose activity causes the flux into the pool must be present in greater amount than if the pool were small. Such considerations could account for the fact that the numerous amino acid intermediates involved in the amino acid biosynthetic pathways have not been easily detected in micro-organisms (Holden, 1962a). But why then are there readily detectable pools of the protein amino acids, particularly of glutamic acid, and, in the case of S. pombe at least, also of citrulline and ornithine? The pools of the protein amino acids could be accounted for on the basis of growth media or by postulating that they are necessary for feedback control systems of the amino acid biosynthetic enzymes. It is quite possible that the levels of nutrients used in the culture media of micro-organisms is very considerably higher than those present in the environment in which the cells evolved. It is a feature of the synthesis of enzymes (including mechanisms for the uptake of nutrients from

the medium) that there is always a basal level of activity. Thus in the presence of 'excessive' nutrients there could be a flux through the enzyme pathways which could not be limited by the cell. In this case the levels of the end product pools would necessarily be relatively high.

The almost universal finding that the pool of glutamate in cells is high may be for a reason additional to that presented above for protein amino acids in general. It seems only logical to assume, as expounded by Horowitz (1965), that the evolution of biochemical pathways was backwards, that is, the final enzyme producing a compound utilized in the synthesis of, say, protein evolved first and only subsequently were enzymes evolved that produced the substrate of the final enzyme, and so on successively backwards along the pathway. At the same time it would clearly be economically advantageous to cut off the synthesis of these biosynthetic enzymes in the presence of gratuitous end products (amino acids in the case under consideration). We may therefore postulate feedback control of the production of enzymes in evolving biochemical pathways. But when several pathways eventually have a common precursor such as glutamate, in the case of amino acids, then several identical enzymes (isozymes) may have been formed for the production of the common precursor. If this were not the case, i.e. only one such enzyme existed but feedback controls did exist, then addition of excess of any one amino acid would cut off the synthesis of all the other amino acids. But on a

telonomic basis the maintenance of several isoenzymes is costly. An alternative, more economical system would be to maintain a relatively large pool of the common precursor (glutamate) in some way, which by its size would be buffered against temporary sudden effluxes. Control points at some later stage along biosynthetic pathways from glutamate may then exist and allow different fluxes along different pathways. In fact, only two glutamic dehydrogenases are found in yeasts (Kaplan, 1963) and Neurospora (Stachow and Sanwal, 1964). Both enzymes favour the formation of glutamate in vitro, but in Neurospora one is diphosphopyridine nucleotide (DPN) and the other triphosphopyridine nucleotide (TPN) specific. But the activity of the DPN enzyme is inhibited by purine nucleotides (Stachow and Sanwal, 1964) which suggests that this enzyme is primarily involved in providing the precursor of glutamine which gives rise to purine nucleotides (Buchanan and Hartman, 1959).

The high levels of citrulline and ornithine in the pool is most likely due to the presence of a urea cycle (Krebs, 1952) in which the urea is utilized for the synthesis of carbamyl phosphate, thereby retaining nitrogen in the system (Metzenberg, 1957; Buchanan and Hartman, 1959).

The state and intracellular location of pool amino acids is not clear (Holden, 1962b; Heinz, 1962; see also Chapter 1). Kinetically the expandable pool in Candida utilis appears as an obligate step in the passage of amino acids from the medium into the internal pool where they are utilized as

precursors of proteins (Cowie and McClure, 1959). Pool material is viewed by Cowie and McClure as being complexed with macromolecular components, probably proteins, so that they may be metabolically inactive until they are released. Physical compartmentation is not invoked by Cowie and McClure but material in the expandable pool is not considered to be in free solution (see Heinz, 1962). The two amino acid pools in Neurospora are rather different, for on addition of an amino acid (proline) to the medium there is a delay in incorporation into protein which is much smaller than the theoretical delay for the size of the pool determined in extracts (Zalokar, 1961). In this case exogenous amino acid is considered to pass through the 'internal' pool into the 'expandable' pool where the amino acid is accumulated. Zalokar suggests that exogenously added amino acid enters the soluble phase of the cell first (this is the 'internal' pool) and that the 'expandable' pool consists either of amino acids adsorbed on to some cell components or stored in separate liquid compartments such as vacuoles. Whether pool components are osmotically active in bacteria has been investigated by Mitchell and Moyle (1956) and Mitchell (1959). The observation that under different sucrose solutions water was taken up by cells in amounts which were predicted by the total solutes extracted by cold TCA indicated that the pool material was in a free state. Whether amino acids were involved in this process is not clear. But Conway and Armstrong (1961) have found in yeasts that less than half

the total osmolarity of the cells can be accounted for by the inorganic ions, organic acids and amino acids which they assayed in pool extracts. Also, in S. pombe the total dry weight of the pool has not been accounted for (see Chapter 4). However, since amino acids in S. pombe are not involved in osmoregulation it is possible that they are never osmotically active but are bound as suggested by Cowie and McClure. Adsorption sites for cations have been demonstrated in yeast cells (Giles and McKay, 1965). The kinetic interrelationships of the two pools in S. pombe (Chapter 6) are similar to those found in Candida by Cowie and McClure.

In considering whether a pool constituent controls the synthesis of enzymes involved in the biosynthesis of that pool component we need to know the levels of both the pool component and the enzyme. We may then consider whether there is a causal relationship between the two. But we are immediately faced with the curious observation that the synthesis of many enzymes is periodic in the cell cycle of bacteria and yeasts (Mitchison, 1969; Donachie and Masters, 1969). This would seem to indicate that the concentrations of co-repressors oscillate. But it is not sufficient to suppose that the instantaneous level of enzyme in these situations should correlate with the degree of repression (or de-repression) caused by the co-repressors at that moment. Such a system would not maintain periodic synthesis of enzymes and so cannot alone provide an explanation of the observations. Goodwin's

model (Goodwin, 1966) overcomes these problems since it claims to allow undamped oscillations to be maintained. The basis of the model is that since there will always be a lag (albeit rather short) between a change in the concentration of a co-repressor and an alteration in the rate of synthesis of an enzyme on the Jacob-Monod model, oscillations in the concentration of co-repressor and rate of enzyme synthesis can occur (Goodwin, 1963). The fact that generally only one step in enzyme synthesis occurs in each cell cycle is accounted for on Goodwin's model by the periodic small bursts of messenger RNA synthesis that occurs during DNA synthesis (Hanawalt and Wax, 1964): these bursts 'entrain' the steps in enzyme synthesis to the cell cycle (Goodwin, 1966).

A very simple mechanism for stepwise synthesis of enzymes is possible when the enzyme is unstable (Donachie, 1965). However, the enzymes showing this pattern of synthesis in S. pombe all seem to be stable (Robinson, per. comm.; for OTCase, see Chapter 9). The model suggested by Goodwin could be considered to account for periodic enzyme synthesis in S. pombe, as well as bacteria (Donachie and Masters, 1969; Goodwin, 1966) under certain conditions. It is necessary to examine what these conditions are. But first of all it must be pointed out that the model proposed by Goodwin can only be taken as a suggestion and his claimed proof by computer simulations has been proved to be false (Griffith, 1968). The rate of messenger RNA production (and hence rate of enzyme synthesis)

is taken to be proportional to a function of co-repressor concentration (x) of the form

$$\frac{K_1}{K_2 + x^n}$$

where K_1, K_2 are constants and 'n' is the power of repression (Goodwin, 1966; Griffith, 1968, in which 'm' is used for 'n'). If 'n' is small then a large fluctuation in the concentration of co-repressor will be necessary to cause a comparable change in the rate of enzyme synthesis, but if 'n' is large, a small fluctuation in co-repressor concentration will cause a large alteration in the rate of synthesis of enzyme. Goodwin (1966) only considered the case n = 1 and, as shown by Griffith (1968), this can never produce stable oscillations in the components of the feedback circuit. In fact the minimum power of repression ('n') that will result in oscillations is 8, and such high powers of repression were considered unlikely in practice by Griffith (1968). Goodwin (1966) shows that his model makes certain predictions. One is that under conditions of continuous growth, as in a chemostat, oscillations should be maintained indefinitely. This situation has not yet been shown to occur, and a demonstration of its occurrence would not prove the theory, since under chemostat conditions such oscillation could occur by other means (Degn and Harrison, 1969). Another prediction is that cells must interact (e.g. through their co-repressors) if they are to maintain synchrony with simultaneous oscillations in the components of the cells. This could only

be investigated in chemostats with the same limitations cited above. Finally, on the important question of entrainment, Goodwin has not considered the size of the burst in messenger RNA synthesis at the time of DNA synthesis necessary to cause entrainment, and also he has not shown that oscillations in co-repressor concentration and rates of enzyme synthesis could occur at times relatively far removed from the period of DNA synthesis, as occurs in S. pombe.

The 'linear reading' theory (Mitchison, 1969) put forward by Halvorson and his colleagues (Halvorson et al., 1966) accounts for enzyme steps on the basis that the structural genes are transcribed linearly along each chromosome, the period at which each gene can be transcribed in the cell cycle being limited. Thus no controls are postulated for each enzyme separately, the only control necessary being linear transcription. A likely mechanism for this is the enzyme which transcribes the DNA into messenger RNA. The enzyme could travel along each chromosome with obligatory sequential transcription (Halvorson et al., 1966). Transcription periods could be limited for a number of reasons: metabolite added via the medium may not affect the feedback controls in the cell, or the majority of enzymes in yeasts could be fully repressed under the conditions of growth utilized (Chapman and Bartley, 1967). It has been argued (Mitchison, 1969) that a demonstration of inducibility for an enzyme at all times in the cell cycle would disprove the linear reading 'theory'. This

situation has been demonstrated in S. pombe for sucrase, and acid and alkaline phosphatases (Mitchison and Creanor, 1969). We have, however, merely to propose that there is one mechanism of transcription that ensures that all loci are transcribed in each cell cycle but that additional transcription of any site can occur at any time by a separate mechanism if conditions of de-repression exist (Tauro et al., 1968). Hanawalt and Wax (1964) have in fact proposed that transcription under conditions of full repression is different from that under conditions of de-repression. They propose that under conditions of full repression transcription may be coupled directly to DNA replication. If this were the case, synthesis of enzymes could be periodic even under conditions of full repression. Moreover, this would provide an explanation for periodic synthesis of constitutive enzymes. A consequence of DNA-linked transcription would be that under conditions of de-repression there would be an additional small step in enzyme synthesis. But these additional steps would almost certainly be too small to detect in view of the errors in estimates of enzymes: a fivefold increase in amount of enzyme over the basal level would obscure the other step. A further consequence of DNA-linked transcription would be that unless delays can occur between transcription and translation all such steps should occur during the period of DNA replication or within a few minutes of the S period. In one case such a delay does not occur: Rotheman and Coleman (1968) have shown that there

is a delay of only a few minutes between transcription and translation for alkaline phosphatase in a strain of E. coli under conditions of both repression and de-repression. To date, synthesis of enzymes under conditions of full repression appears to be non-periodic in bacteria (reviewed by Donachie and Masters, 1969) and yeasts (Mitchison and Creanor, 1969). However, in few cases has the same enzyme been measured under conditions of full repression and de-repression, and the results are not decisive (Donachie and Masters, 1969).

A number of observations lead one to suspect that in S. pombe, as in Neurospora, some, if not the majority of biosynthetic enzymes, are fully repressed. Although amino acids added to the medium are known to enter the cell (Chapter 6; Robinson, 1966) the synthesis of amino acid biosynthetic enzymes are not affected (Robinson, 1966, and per. comm.; Chapter 9). But supplements of amino acids clearly are not utilized extensively as precursors of proteins (Chapter 6). They may be used as respiratory substrates. This observation would be the one expected if the amino acid biosynthetic enzymes were all repressed and sufficient amino acids were formed from glucose for maximal growth.

Enzyme induction appears essentially to be of an either-or nature. But the rate at which enzyme synthesis proceeds under conditions of induction is proportional to the concentration of inducer used (Pardee and Prestige, 1961). The precise kinetics is not well known for most enzymes, but since the

interactions of co-repressors with specific repressors is likely to be of an allosteric nature (Monod *et al.*, 1963) we may consider the kinetics of allosteric inhibition as a model for the kinetics of enzyme production by co-repressors. Enzyme steps in synchronous cultures generally show an approximate doubling in amount over a period of about one tenth of the cell cycle. The problem is therefore to estimate the change in pool concentration that would cause a 100% change in the amount of an enzyme during such a brief period. Clearly, a step in amount of enzyme could be generated by a period of synthesis, over a tenth of the cell cycle, ten times greater than the mean rate averaged over the whole cycle. The smallest deviation in amount of amino acid from an exponential increase that could be reliably detected in the present investigation is estimated to be about 6%. However, a fluctuation of this amount would not automatically *generate* oscillations in the rate of enzyme synthesis and co-repressor concentration. For this to occur, the fluctuation in co-repressor concentration must be only about 2% *or less* (Griffith, 1968), and such high co-operative effects have not yet been reported for allosteric effectors of feedback inhibition. Hence it is not possible to say that the pool fluctuations found are too large for oscillatory repression to be the explanation of stepwise enzyme synthesis. It is unlikely that pool estimates of sufficient accuracy could be made to decide satisfactorily whether oscillatory repression is the

cause of periodic enzyme synthesis. However, if pool oscillations greater than those predicted by the theory were found to occur, the periodic synthesis could be attributed to the pool material, but some other explanation of the pool oscillations would then be necessary. It also seems unlikely that sufficiently exact estimates of the increase in the rate of enzyme synthesis during the step will be possible. For, while in any one cell the period of doubling may be much shorter than one tenth of the cell cycle, between cell variations would not allow sharper doubling times to be detected in synchronous cultures.

The results of Chapter 7 show that all the pool amino acids assayed increase exponentially through the cell cycle. While there are no significant deviations from this pattern, the rate of volume increase is not uniform through the cell cycle. Mitchison (1957) found an exponential increase in volume during the first three quarters of the cell cycle, and thereafter the volume remained constant. As a result the concentrations of pool amino acids will fluctuate with a maximum at 0 or 1 and a minimum at 0.75 in the cell cycle. The amount of this fluctuation can be calculated: if there were no fluctuation in concentration, then all the amino acids would have doubled in amount by 0.75. In fact, by 0.75 the amount has increased from 1 at time 0 to $_e 0.75 \log^2 = 1.68$. The maximum fluctuation in concentration of amino acids is therefore $100/(2-1.68) = 16\%$. Without doubt such large

fluctuations could account for stepwise synthesis of enzymes by oscillatory repression. However, this is on the assumption that the amino acids, and water in the cell, remain distributed between the various cell fractions in the same relative manner at all stages in the cell cycle. If there really are fluctuations in the concentrations of amino acids during the cell cycle, these fluctuations would all be essentially the same, with a minimum concentration at 0.75. On any model involving feedback regulation of enzyme synthesis we would expect all enzymes controlled in this way by amino acids to step at approximately the same time. In fact, since some of these enzymes may be repressed and others de-repressed by amino acids, we might anticipate two groups of step enzymes in the cell cycle: one group near the time the pool concentrations are lowest (0.75), and another when the pool concentrations are highest (0 or 1). Too few amino acid biosynthetic enzymes have been assayed to date in S. pombe to say whether this is generally true or not, but some of the enzymes assayed are well outside these periods (Bostock et al., 1966; Robinson, 1966). It is therefore necessary to consider what explanations of steps in enzyme synthesis are possible in the absence of detectable fluctuations in the concentrations of pool amino acids.

There are four obvious ways in which we could explain why fluctuations in amino acids do not occur, despite fluctuations in the rate of synthesis of enzymes which the amino acids may

regulate.

1) The free amino acids may not be the real co-repressors. There is evidence indicating that conditions favouring activation of amino acids (binding to transfer RNA) are necessary for repression to occur in bacteria (Schlessinger and Magasanik, 1964; Eidlic and Neidhardt, 1965) and in Neurospora (Nazario, 1967b). It has therefore been suggested that amino acids bound to the appropriate transfer RNAs are the co-repressors (reviewed by Neidhardt, 1966). However, there are cases of amino acid analogues which are not activated which still cause repression (Ravel, White and Shive, 1965).

2) Control may be effected by the co-factor rather than the amino acid substrate of enzymes involved in amino acid biosynthetic pathways. In branched pathways in which a co-factor or one of the two substrates of a reaction is common to two pathways, there are generally separate systems for the synthesis of the common component, each separately controlled. This means that if the flux through one pathway decreases, the flux through the other pathway need not also be decreased. Control of both pathways is therefore likely to be by means of the common substrate or co-factor. This situation is known to occur in the synthesis of arginine and pyrimidines in Saccharomyces (Lacroute et al., 1965). Carbamyl phosphate is a substrate of ornithine transcarbamylase in the arginine pathway and a substrate of aspartic transcarbamylase in the pyrimidine pathway. In both cases there is feedback

repression by the end products of the synthesis of carbamyl phosphate and not the amino acid substrates of the first reactions in the pathways. Two separate mechanisms for the synthesis of carbamyl phosphate were inferred from the existence of two groups of mutants in which synthesis of carbamyl phosphate was controlled by pyrimidines in one case and arginine in the other (Lacroute et al., 1965).

Whether such control by the non-amino acid component of enzyme reactions is a frequent phenomenon in amino acid biosynthetic pathways is not clear. It is, however, likely to occur in some other pathways since other amino acids, such as glycine, are involved in the synthesis of precursors of nucleic acids. Moreover, amino acids are interconvertible and many are synthesized from the same precursor, such as the "glutamate family" of amino acids from glutamate. Control by co-factors could therefore be widespread.

3) Pool amino acids may occur in separate compartments within the cell. In this case there could be large fluctuations in one small compartment controlling the synthesis of an enzyme, but this fluctuation may not be detectable in total pool extracts. The phenomenon of compartmentation of enzymes and pool compounds was reviewed in Chapter 1. We are here concerned with how this compartmentation in the cell will influence feedback control systems in the cell. There is considerable evidence indicating that the biogenesis of mitochondria is to some extent independent of the rest of the cell

(Roodyn and Wilkie, 1968). Mitochondria of yeasts are now known to contain DNA which is quite distinct from the nuclear DNA (Moustacchi and Williamson, 1966; Corneo et al., 1966). Moreover, yeast mitochondria contain distinctive ribosomes (Rogers et al., 1967), some tRNAs and amino acyl tRNA synthetases. However, the outer membrane appears to be synthesized in the cytoplasm under nuclear control while some of the proteins of the inner membrane and enzymes of the matrix appear to be synthesized within the mitochondria from cistrons in mitochondrial DNA (Roodyn and Wilkie, 1968). For enzymes specified and synthesized within mitochondria, all the relevant elements of the control circuit could be within the mitochondria. In this case the only pool of significance would be the mitochondrial pool. Many of the enzymes involved in amino acid biosynthesis are located in mitochondria, but they may not be synthesized there. Some enzymes of the inner membrane such as cytochrome c are known to be synthesized in the cytoplasm under nuclear control (Freeman et al., 1967). For such enzymes the relevant controlling pool will be outside the mitochondria. Clearly some aspects of mitochondrial biosynthesis are under nuclear control (Gibor and Granick, 1964; Roodyn and Wilkie, 1968) and thus an extra element may be involved in the circuit controlling the synthesis of even those enzymes specified by mitochondrial DNA.

In cases of enzyme induction, the most significant portion of the pool will be that within the nucleus, for, on the Jacob-

Monod model, the inducer must combine with and remove the repressor situated on the operator. For repression the significant portion of the pool need not be in the nucleus, but is likely to be where the repressor molecules are situated. The intracellular location of repressors is entirely unknown. For translational control the significant portion of the pool would presumably be in the cell sap for both induction and repression.

4) The intracellular distribution of amino acids may alter during the cell cycle without deviation from an exponential increase in the overall amounts. In this way very large fluctuations could occur within organelles such as mitochondria, without any fluctuations in the total cellular amounts of amino acids. Ion transport systems occur in mitochondrial (Chappell and Crofts, 1966) and nuclear (Georgiev, 1967) membranes, and these could provide the mechanisms for controlled alterations in the levels of amino acids at specific stages in the cell cycle. However unlikely this suggestion may seem, it must be remembered that if a fluctuation in the end pool of an amino acid pathway were found and this was larger than predicted by the oscillatory repression theory, then there must be some explanation for the fluctuation other than periodicity in synthesis of the enzyme controlling the flux in the pathway.

The amino acid pathway studied in greatest detail *in vivo* in an organism closely related to *S. pombe* is probably the arginine pathway of *Neurospora*. As we have seen from the work of Donachie (1964a,b; reviewed in Chapter 1), the synthesis of OTCase is maximally repressed in exponential cultures. Addition of arginine to the medium does not decrease the level of the enzyme. However, increases in the level of OTCase have been found in mutants (of the arginino-succinase and argininosuccinate synthetase loci) and in these the arginine pool is lower than in the wild type. In these mutants, increases in the size of the arginine pool (by addition of citrulline or arginine to the medium) decreases the level of OTCase proportionately. It therefore seems that there is a control system for OTCase which could be due to repression by arginine but that this is fully repressed in exponential growth. However, several pool components of the pathway are altered in the mutants and increase differentially on addition of supplements to the medium. Moreover, there are situations in which the arginine pool remains almost constant in size while the citrulline pool varies and correlates inversely with the level of OTCase. Citrulline would therefore appear to be the controlling factor in some circumstances. Hence it cannot be stated unequivocally that control of OTCase is by the arginine pool in *Neurospora*: many, if not all, the pool components in the pathway may be involved (Ilse B. Barthelmess, per. comm.). Furthermore, Nazario (1967a) has

shown in Neurospora mutants, which are deficient in argininosuccinase and accumulate argininosuccinate, that OTCase is derepressed even in the presence of high mycelial pools of arginine. Nazario (1967b) has shown that arginyl-tRNA synthetase in Neurospora is inhibited by argininosuccinate and has shown that, under conditions of argininosuccinate accumulation, the pool of arginyl-tRNA is less fully charged than normally. These results were interpreted by Nazario (1967b) on the basis that arginyl-tRNA is the co-repressor.

The complexities in the control of the arginine pathway may in part be due to the involvement of its intermediates in other pathways of intermediary metabolism, in particular the urea cycle and the biosynthesis of carbamyl phosphate which is itself a substrate of OTCase. It may be that in other pathways of amino acid biosynthesis these complexities would not be found and the existence of feedback controls of the Jacob-Monod type could be readily verified. The fact that the arginine pathway generates one of the substrates (carbamyl phosphate) of the bimolecular reaction catalysed by OTCase, makes it impossible to predict whether pool components will have a positive or negative effect on the pathway, without knowing the level of each pool component and the kinetic relationships between them (Kascer and Burns, 1968; Rosen, 1968). Moreover, for a bimolecular reaction which is rate limiting in a pathway, one of its substrates must be rate limiting (Chance, 1961) and if this substrate is to be rate

limiting at all levels of enzyme activity, the other substrate would most likely be near saturation level. It is not generally known if this situation exists *in vivo* for pathways thought to be controlled by feedback repression.

The significance of stepwise synthesis of enzymes during the cell cycle is obscure. Since all enzymes in anabolic pathways assayed to date in *S. pombe* and *Saccharomyces cerevisiae* show this pattern of synthesis, and the total protein increases continuously during the cell cycle, it seems quite likely that the majority of anabolic enzymes will show stepwise synthesis. For some enzymes periodic synthesis may be clearly related to their function. For example, thymidine kinase synthesis has been found to occur just before DNA synthesis (Littlefield, 1966). Since RNA synthesis is continuous throughout the cell cycle stepwise synthesis of an enzyme, such as aspartic transcarbamylase producing RNA precursors, is obscure. Turnover of protein and RNA may provide a means of biochemical differentiation in the absence of growth, but in growing cultures of yeasts this turnover is exceedingly small (Halvorson, 1958). However, the activities of protein degradative enzymes have been shown to increase at the end of the cell cycle in yeasts (Sylven *et al.*, 1959) and may have significance for this period of the cell cycle. Significant protein turnover at the end of the cell cycle has also been found in bacteria (Nishi and Kogoma, 1965).

The significance, but not the mechanism, of periodic

enzyme synthesis could be seen to lie in the properties of loosely coupled events (Engelberg, 1968). In a system in which each cellular component must double in every generation and the synthesis of many of the components is regulated, loose-coupling could ensure that the state at any one time recurs periodically. During each cell cycle there is a sequence of steps representing the synthesis of different enzymes. These steps need not occur in a precise order in each cell cycle, but loose-coupling could ensure that the order is repeated approximately (Engelberg, 1968). Without doubt enzyme pathways <u>are</u> loosely coupled, if only through the network of their intermediates. This rationalization could not, however, account for co-ordination of events proceeding in parallel rather than in series. The major synthetic events that occur in parallel during the cell cycle are synthesis of protein in general, and RNA synthesis. As in bacteria,(Stent and Brenner, 1961) the synthesis of RNA in yeasts (Ycas and Brawerman, 1957) is dependent on adequate supply of amino acids. In bacteria this 'stringency' has been shown to be due to a genetic regulatory locus which in certain mutants is lost (Stent and Brenner, 1961). There therefore appears to be a specific genetic mechanism ensuring co-ordinate synthesis in parallel. Lack of an amino acid does not now appear to limit merely the production of RNA precursors, but seems to result in a limitation in energy metabolism generally (Irr and Gallant, 1969).

An important aspect of feedback repression that has been largely ignored is the gene dose and repressor concentration in the cell. While gene dose alone may not be so significant under conditions of de-repression, the amount of an enzyme has been found to be directly proportional to gene dose under conditions of full repression (Donachie, 1964a,b). Some early work on the kinetics of production of the β-galactosidase repressor was interpreted as indicating relatively weak binding of the repressor with the operator combined with growth-instability of the repressor (Sadler and Novick, 1965). However, the data has also been interpreted in terms of a growth-stable repressor with tight-binding of the repressor and very few molecules of repressor per cell (Koch, 1967), as demonstrated to be the case for β-galactosidase by Gilbert and Muller-Hill (1967). Novick's group now agree with this interpretation and account for the apparent growth-instability of the repressor by showing that the phenomenon of induction of β-galactosidase without inducer at high temperatures only occurs if DNA synthesis takes place, i.e. the number of operators relative to repressors increases (Barbour, Gross and Novick, 1968). The existence of so few repressor molecules per cell (not more than twice the number of operators) may have important consequences for the synthesis of enzymes in the cell cycle. For if transcription is sequential along each chromosome, then, since regulator genes are not generally adjacent to the loci they control, there will be cyclic

variations in the relative amount of repressors and operators. In this way periodic enzyme synthesis could occur in the absence of pool fluctuations and solely as a result of the organization of the genome.

Without doubt lack of knowledge of the organization and distribution of pool compounds throughout the cell is a major difficulty in our understanding of the role of pool materials as co-repressors. Equally, the distribution and synthesis of repressor molecules is poorly understood. Although the genetic basis of feedback regulatory mechanisms is clear, the detailed physiology of the process is not. Very little is at present known of the elements in the feedback circuits controlling the synthesis of enzymes apart from the conditions which bring the circuit into operation. While enzyme cytology progressed rapidly with the development of techniques for the isolation of cell organelles, the desire to relate observations on low molecular weight materials to the pool concept seems to have hindered progress in understanding the regulatory functions of the pool materials.

Appendix I

CULTURE MEDIA

Edinburgh Minimal Medium 1 (EMM1) is a modification of the minimal medium used by Leupold, U., Arch. Jul. Klaus-Stiftung, Zurich, 30, 506 (1955).

Per 1000 ml. distilled water:

(A)	C source	Glucose	10 g.
(B)	N source	NH_4Cl	5 g.
(C)	Salts	Sodium acetate (buffer)	1 g.
		KCl	1 g.
		$MgCl_2$	0.5 g.
		NaH_2PO_4	10 mg.
		Na_2SO_4	10 mg.
		$CaCl_2$	10 mg.
(D)	Vitamins	Inositol	10 mg.
		Nicotinic acid	10 mg.
		Calcium pantothenate	1 mg.
		Biotin	10 mg.
(E)	Trace elements	H_3BO_3	500 ug.
		$MnSO_4.H_2O$	400 ug.
		$ZnSO_4.7H_2O$	400 ug.
		$FeCl_3.6H_2O$	200 ug.
		$H_2MoO_4.H_2O$	160 ug.
		KI	100 ug.
		$CuSO_4.5H_2O$	40 ug.
		Citric acid	1000 ug.

The pH of the freshly prepared medium is about 5.3, but drops to about 4.5 when the cells reach stationary phase.

Edinburgh Minimal Medium 2 (EMM2) is the same as EMM1 except that the phosphate salt is increased thirty-fold to 300 mg./l. The phosphate is in this case added to the final solution before sterilization.

Malt Extract Broth (MEB) is a 2% solution (w/v) of Oxoid Malt Extract Broth (CM 57) which consists of Malt Extract and Mycological Peptone. The former is mainly maltose and is free of glucose. The latter is free from fermentable carbohydrates and is an enzymatic hydrolysate of proteinaceous materials containing polypeptides, dipeptides and amino acids (from the Oxoid Handbook).

Appendix II

MAXIMUM DIFFERENCES BETWEEN LINEAR, EXPONENTIAL AND QUADRATIC INCREASES DURING THE CELL CYCLE

Consider the equations for a line, an exponential and a quadratic curve:

$$y = px + c$$
$$y = ae^{bx}$$
$$y = lx^2 + mx + n$$

where p, c, a, b, l, m and n are constants. Suppose y increases from 1 to 2, while x increases from 0 to 1, i.e. some cell component (y) doubles during a cell cycle (x, from 0 to 1). Then these curves must satisfy the points (0, 1) and (1, 2). Hence we obtain the constants p = 1, c = 1, a = 1 and b = log 2. The quadratic under consideration (Chapter 7) is the one whose differential is a line whose slope is the same as the line through (o, 1) and (1, 2), i.e. y = 2lx + m must have the same slope as y = x + 1. Hence $l = \frac{1}{2}$. Also, since the quadratic must pass through the two points, we obtain $m = \frac{1}{2}$ and n = 1. The equations thus become:

$$y = x + 1 \quad \ldots\ldots\ldots\ldots\ldots 1$$
$$y = e^{x\log 2} \quad \ldots\ldots\ldots\ldots\ldots 2$$
$$y = \frac{x^2}{2} + \frac{x}{2} + 1 \quad \ldots\ldots\ldots\ldots\ldots 3$$

Maximum Difference (y') between line and exponential:

$$y' = x + 1 - e^{x\log 2} \quad \text{from 1 and 2 above.}$$

For a maximum $dy'/dx = 0$

$$\text{i.e.} \quad 1 - \log 2 \cdot e^{x \log 2} = 0$$

$$\text{i.e.} \quad x = \frac{\log (1/\log 2)}{\log 2} = 0.530$$

At this value $y' = 1.530 - e^{0.367}$

$$= 1.530 - 1.444$$

$$\text{maximum} = 0.086$$

Hence the maximum percentage difference between the line and the exponential is:

$$\frac{0.086}{1.530} \times 100 = \underline{5.62\%}$$

Maximum Difference (y') between line and quadratic:

$$y' = x + 1 - \frac{x^2}{2} - \frac{x}{2} - 1$$

$$= \frac{x}{2} - \frac{x^2}{2}$$

$dy'/dx = \frac{1}{2} - x = 0$ for a maximum

$$\text{i.e.} \quad x = \tfrac{1}{2}$$

At this value $y' = \frac{1}{4} - \frac{1}{8} = \frac{1}{8}$

Hence the maximum percentage difference between the line and the quadratic is:

$$\frac{100}{8 \times 1.5} = \underline{8.33\%}$$

Maximum Difference between Exponential and Quadratic

The maximum differences calculated above occur at very nearly the same stage in the cell cycle, i.e. at 0.530 and

0.500. Hence the maximum difference between the exponential and the quadratic is approximately the difference between the two maximum differences, i.e. 8.33 - 5.60 = 2.73%.

Appendix III

A SIMPLISTIC MODEL OF CELL GROWTH IN WHICH CONTROL OF THE PRODUCTION OF CELLULAR COMPONENTS IS UNNECESSARY FOR BALANCED GROWTH

As molecular biology is now understood, the 'determinants' for the synthesis of macromolecules or the macromolecules themselves are primary gene products: in the case of proteins, including enzymes such as those involved in the synthesis of carbohydrates, the determinants are messenger RNAs, while other cistrons of the genetic material specify directly the synthesis of other species of RNA, such as ribosomal RNA and transfer RNAs. It is now well documented that the genetic material generally doubles in each cell cycle in a most exact manner, and for fixed conditions of growth it doubles at a specific time in the cell cycle. It follows that, for given growth conditions, if the production of the 'determinants' is the same in each cell cycle, then the macromolecular composition of the cell will be the same in each cell generation, unless the conditions of growth change with growth. Under different conditions of growth the total amount and relative composition of the determinants may be different, but again the macromolecular composition of the cell will remain constant in successive generations, although the relative amounts of the macromolecules may be different from the previous conditions of growth.

Consider now the low molecular weight pool materials in cells. It is clear that if the flux (F_1) of material into a pool (P) is greater than the flux (F_2) out of the pool, then P will tend to increase. It will not, however, increase indefinitely because in the course of growth a cell must also increase the amount of the pool material, as well as all its macromolecules. Thus F_1 must always be greater than F_2, if a pool is to exist at all. We may consider two components in F_1: one required to maintain F_2 (F_1', say) and the other to allow that increase in P necessary for growth of this component with overall growth of the cell (F_1'', say). If F_1 happens to be very much larger than F_2 this does not mean that P will increase indefinitely with the result that unbalanced growth ensues. Instead, P will merely increase to that size consistent with production of P by F_1''.

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