REGULATION OF TYROSINE CATABOLISM IN *TETRAHYMENA PYRIFORMIS*

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Abstract

The metabolism of tyrosine and its regulation in Tetrahymena pyriformis was studied. Phenylalanine hydroxylase could not be assayed in extracts of the organisms but the conversion of phenylalanine to tyrosine was demonstrated *in vivo* and ^{14}C-labelled phenylalanine was incorporated into glycogen. The *in vivo* hydroxylation of phenylalanine to tyrosine was shown to be repressed in the presence of glucose in the growth medium.

The presence of a tyrosine-catabolizing pathway identical to that present in rat liver was demonstrated by the assay of all the enzymes involved and the conversion of homogentisic acid to fumarylacetoacetic acid by a semipurified preparation of homogentisate oxidase. ^{14}C-labelled tyrosine was incorporated into the glycogen of the organisms. All the enzymes of the pathway as well as the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase were repressed in the presence of glucose and of acetate in the growth medium. The repression was accompanied by a significant reduction in the incorporation of isotopic carbon from ^{14}C-tyrosine into glycogen. These results suggested that the pathway serves gluconeogenesis from tyrosine.

The repression by acetate was accompanied by the stimulation of the glyoxylate cycle, which serves gluconeogenesis from acetate, as evidenced by the elevated activity of the key enzyme of the cycle isocitrate lyase. Thus, there exists an inverse relationship between gluconeogenesis from acetate and gluconeogenesis from tyrosine in Tetrahymena.

The repression of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase by acetate suggested the existence of an alternate route for the conversion of acetate to carbohydrate which bypasses phosphoenolpyruvate. Strong evidence was obtained by the manometric assay of glyoxylate carboligase that the glycerate pathway might be that alternate route.

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ABBREVIATIONS

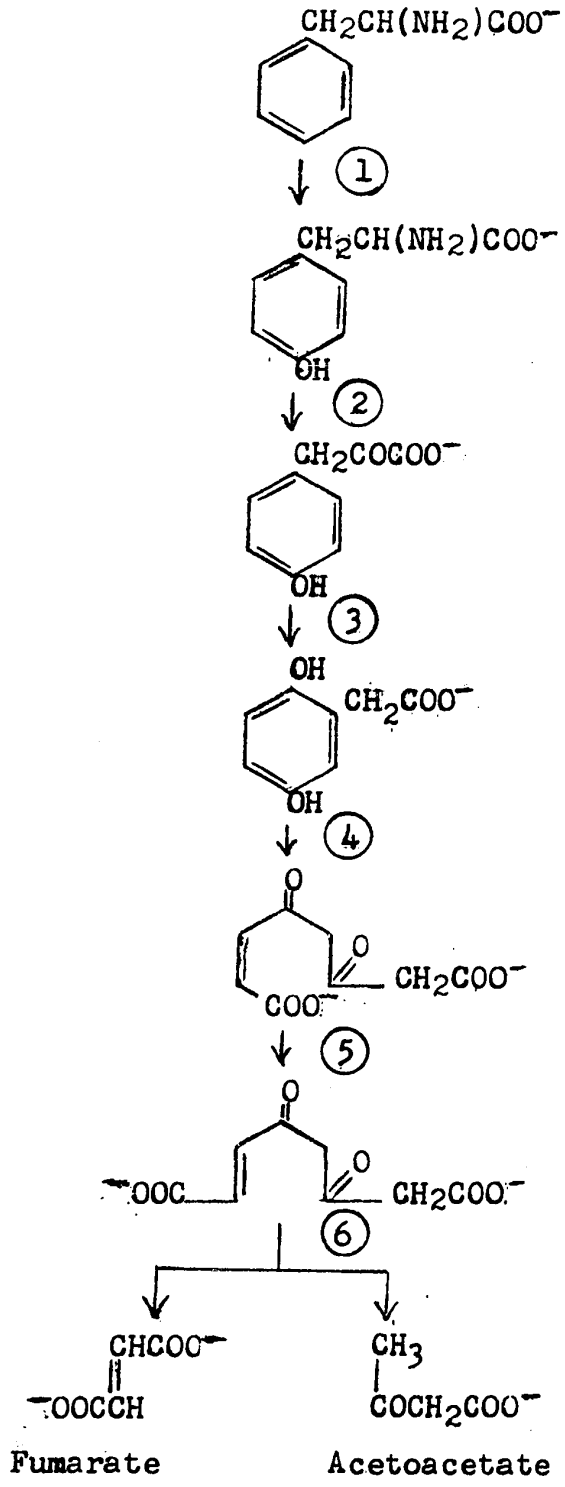
ADP	adenosine diphosphate
DNP	dinitrophenylhydrazine
dpm	disintegrations per minute
NAD^+	nicotinamide-adenine dinucleotide, oxidised
NADH	nicotinamide-adenine dinucleotide, reduced
$NADP^+$	nicotinamide-adenine trinucleotide, oxidised
NADPH	nicotinamide-adenine trinucleotide, reduced
PEPCK	phosphoenolpyruvate carboxykinase
phe	phenylalanine
TCA	trichloroacetic acid
TT	tyrosine transaminase
TPP	thiamine pyrophosphate
tyr	tyrosine

INTRODUCTION

Gluconeogenesis, the process converting noncarbohydrate precursors into carbohydrate, is carried out mainly by liver and kidney cells in mammals. Glycogen accumulation, the result of gluconeogenesis, is cyclic in the liver (Potter, et al., 1966; Lane and Mavrides, 1970). Since carbohydrate cannot be synthesized from lipids or fatty acids in higher animals, amino acids serve as the main carbon source in gluconeogenesis.

Phenylalanine and tyrosine are ketogenic and glucogenic amino acids (Krebs, 1964). The importance of these amino acids as precursors for carbohydrate synthesis is suggested by the finding that tyrosine transaminase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) activity rises in response to glucocorticoids (Lin and Knox, 1957). No other transaminase responds as quickly. If the stimulation of tyrosine transaminase by glucocorticoids is important metabolically, it may mean that these aromatic amino acids make a significant contribution to gluconeogenesis under certain conditions.

The major pathway of phenylalanine and tyrosine metabolism is their catabolism to fumarate and acetoacetate through the tyrosine-catabolizing pathway (Knox, 1955; scheme 1, pg. 2). Fumarate can then be converted to glucose through reverse glycolysis and acetoacetate can be used in the synthesis of fatty acids, or like fumarate can be used as an energy source and oxidised through the Krebs cycle.



① Phenylalanine Hydroxylase
② Tyrosine Transaminase
③ p-Hydroxyphenylpyruvate Oxidase
④ Homogentisate Oxidase
⑤ Maleylacetoacetate Isomerase
⑥ Fumarylacetoacetate Hydrolase

Scheme 1: The Route of Tyrosine and Phenylalanine Catabolism

Although there were indications as to the route of catabolism of these amino acids in the late 1800's and early 1900's, (Wolkow and Baumann, 1891; Neubauer, 1909) the pathway as we now know it was not completely elucidated until the 1950's (Knox and Edwards, 1955a; 1955b; Edwards and Knox, 1956). The pathway of phenylalanine catabolism begins with the hydroxylation of this amino acid to tyrosine. The process thereafter, from tyrosine to fumarate and acetoacetate, is the catabolism of tyrosine.

1. The Hydroxylation of Phenylalanine to Tyrosine

The hydroxylation of phenylalanine to tyrosine was postulated by Neubauer as early as 1909 (Neubauer, 1909). Shortly thereafter, Embden and Baldes (1913) observed an increase in tyrosine and acetoacetate in liver perfused with phenylalanine. The first direct evidence for the *in vivo* hydroxylation was presented by Moss and Shoenheimer (1940) who demonstrated the conversion of deuterium labelled phenylalanine to deuterium labelled tyrosine. This process was later shown to be irreversible (Grau and Steele, 1954).

Udenfriend and Cooper (1952), were able to assay the hydroxylation of phenylalanine to tyrosine in the presence of NAD^+ and O_2, by a soluble extract from rat liver. Preliminary studies of the reaction showed that the enzyme could be detected in the livers of several animals but not in any other tissue or organ. However, it has since been reported in the pancreas and kidney of mice (Tourian *et al.*, 1969).

The system was subsequently separated into two protein frac-

tions, both of which were required for enzyme activity (Mitoma, 1956) and was shown to require NADH and O_2, as well as a possible unidentified third cofactor (Kaufman, 1957). Kaufman (1958a) found that sheep liver was an excellent source for one of the protein fractions and was henceforth used in future studies. The two protein fractions were referred to as the sheep liver enzyme and the rat liver enzyme.

Upon further purification of these sytems, it was found necessary to add boiled liver extract to obtain hydroxylation activity (Kaufman, 1957). A cofactor was purified from the extract but its structure remained elusive for many years (Kaufman, 1958a; 1958b; Kaufman and Levenberg, 1959). It was found that tetrahydrofolic acid was an active cofactor in the hydroxylation system (Kaufman, 1958b), but the synthetic pteridines, 6-methyl and 6,7-dimethyl-2-amino-4-hydroxy-tetrahydropteridine were far more effective (Kaufman, 1959).

In 1963, it was found that there was a striking similiarity between a sepia pteridine and a reduced biopterin, pigments isolated from the eye of a mutant strain of Drosophila melanogaster, and the rat liver cofactor (Kaufman, 1963). It was shown conclusively, by spectral and chemical analysis and equal effectiveness as cofactors in the stimulation of the hydroxylation reaction, that the cofactor isolated from the rat liver extract was, in fact, 7,8-dihydrobiopterin (Kaufman, 1963). The reduced form of this, 7,8-tetrahydrobiopterine, was required in the hydroxylation reaction.

The sheep liver enzyme and NADPH were shown not to be directly involved in the hydroxylation reaction since they could be replaced by stoichiometric quantities of the cofactor (Kaufman, 1959). Thus their function was to recycle the cofactor which became oxidised during the hydroxylation of phenylalanine. The sheep liver enzyme was referred to as dihydropteridine reductase while the actual hydroxylating species, the rat liver enzyme, was called phenylalanine hydroxylase (L-phenylalanine, dihydrobiopterin: oxygen oxidoreductase, EC 1.14.3.1).

The activity of phenylalanine hydroxylase has been shown to vary in the rat according to age, sex and nutritional status, The activity increases with age in young male and female rats followed by a brief decline before the enzyme activity is stabilized. The level of activity, which is always found to be higher in male rats, declined rapidly in rats that were fasted, or fed on a protein-free diet. Addition of protein to the diet restored the activity but could not increase it beyond a certain basal level (Freedland et al., 1962).

2. The Route of Tyrosine Catabolism

Much of the early studies on the metabolism of tyrosine and phenylalanine were concerned with clinical studies on patients suffering from inborn errors in tyrosine metabolism which could be detected by abnormal levels of excretion products in the urine. The studies on alcaptonurea patients led Neubauer (1928) to postulate a route of tyrosine and phenylalanine metabolism leading

to homogentisate. Although parts of the original pathway have been disproved, the essential steps: phenylalanine ——→ tyrosine ——→ p-hydroxyphenylpyruvate ——→ homogentisate, have been substantiated.

In vivo, as well as in vitro, studies in the mammalian system have shown that phenylalanine and tyrosine give rise to acetoacetate. Edson (1935) in a study of acetoacetate formation from various amino acids, showed that phenylalanine, tyrosine, and leucine were the most ketogenic. This led to the postulation that the pathway was: phenylalanine ——→ tyrosine ——→ p-hydroxyphenylpyruvate ——→ homogentisate ——→ acetoacetate, which agreed with the earlier observations of Embden and Baldes (1913). Furthermore, Winnik et al. (1948) isolated radioactive acetoacetate after injection of ^{14}C-tyrosine into fasted rats. Most of the label was found in acetoacetate and none could be detected in other amino acids.

Tracer studies using ^{14}C-phenylalanine and ^{14}C-tyrosine labelled in specific carbons and incubated with rat liver slices confirmed that these amino acids gave rise to acetoacetate through a shift in the side chain of p-hydroxyphenylpyruvate from ring carbon 1 of tyrosine to ring carbon 2, concomitant with a hydroxylation and a decarboxylation to give homogentisate (Weinhouse and Millington, 1948; Schepartz and Gurin, 1949). Lerner (1949) showed that labelled phenylalanine, in addition to acetoacetate, gave rise to a second carbon fragment which he isolated and identified as malate. He proposed, however, that an intermediate pro-

duct could be an isomer of malate, namely fumarate.

No other intermediates of tyrosine and phenylalanine catabolism were isolated until Ravdin and Crandal (1951) demonstrated that homogentisate gave rise to a diketone structure, characterized as fumarylacetoacetate, which was subsequently hydrolyzed to fumarate and acetoacetate. Knox and Edwards (1955a) found still another intermediate between homogentisate and fumarylacetoacetate, which proved to be maleylacetoacetate, the cis isomer of fumarylacetoacetate, and were able to demonstrate the enzymatic isomerization of maleylacetoacetate to fumarylacetoacetate (Edwards and Knox, 1956). The complete pathway is shown in scheme 1, pg. 2.

All the tyrosine-catabolising enzymes have been assayed in the liver, which contains the highest activity, and the kidney. They are: tyrosine transaminase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5), p-hydroxyphenylpyruvate oxidase (p-hydroxyphenylpyruvate, ascorbate: oxygen oxidoreductase, EC 1.13.1.5), maleylacetoacetate isomerase (4-maleylacetoacetate cis-trans isomerase, EC 5.2.1.2), and fumarylacetoacetate hydrolase (4-fumarylacetoacetate fumaryl hydrolase, EC 3.7.1.2).

Indirect evidence for a transamination step in the oxidation of tyrosine was obtained from observations that the oxidation of tyrosine did not produce free ammonia, and required four moles of molecular oxygen per mole of tyrosine oxidised to acetoacetate and not five, as would be anticipated by a process involving oxidative deamination (Bernhein and Bernhein, 1934; La Du and

Greenberg, 1951; Schepartz, 1951). La Du and Greenberg (1951), using acetone powder extracts of rat liver, found that the oxidation of p-hydroxyphenylpyruvate required 3.5 atoms of oxygen and homogentisate required 2.0. Furthermore, the rate of tyrosine oxidation, but not that of p-hydroxyphenylpyruvate or homogentisate, was stimulated by the addition of oxaloacetate and α-ketoglutarate to the extract.

A specific transaminase for tyrosine was purified 100-fold from a soluble extract of dog liver and characterized by Canellakis and Cohen (1956a; 1956b). The enzyme contained copper, showed a low activity with other amino acids and required pyridoxal phosphate in catalytic quantities as well as α-ketoglutarate.

In mammalian systems, tyrosine transaminase has been reported in the mitochondria and cytosol (Miller and Litwack, 1969). The mitochondrial tyrosine transaminase, transaminates with α-ketoglutarate (100% activity), oxaloacetate (75% activity) and pyruvate (25% activity), (Miller and Litwack, 1969) whereas the soluble enzyme is specific for α-ketoglutarate (Canellakis and Cohen, 1956b).

The metabolic significance of the mitochondrial enzyme is not certain; it does not respond to the same stimuli as cytosol tyrosine transaminase (Litwack et al., 1963). Both forms of tyrosine transaminase are found in the liver, heart, skeletal muscle, brain and leucocytes (Fellman et al., 1969). However, the distribution of the enzyme between the cytosol and mitochondria varies. In the brain, for example, most of the enzyme

is mitochondrial (Miller and Litwack, 1969), whereas in the liver, the enzyme is mainly soluble (Ganellakis and Cohen, 1956a). Brain tyrosine transaminase has been implicated in the regulation of catecholamine biosynthesis by virtue of controlling tyrosine levels (Gibb and Webb, 1969). The liver tyrosine transaminase, on the other hand, has been studied from the viewpoint of its response to glucocorticoids.

Hepatic tyrosine transaminase can be greatly stimulated in rats by the injection of the glucocorticoid, hydrocortisone (Lin and Knox, 1957). The classical metabolic effect of glucocorticoids is glycogen formation from noncarbohydrate precursors such as amino acids (Levine, 1964). Injection of tyrosine was also found to elicit an increase in enzyme activity but not in adrenalectomized rats (Lin and Knox, 1957). The stimulation by tyrosine has, therefore, been considered to be an indirect effect due to the secretion of the glucocorticoid corticosterone, resulting from the stress of the injection (Kenney and Flora, 1961). Since tyrosine transaminase is intimately involved in the degradation of tyrosine, its stimulation by glucorticoids can be rationalized. However, the exact mechanism by which this stimulation is achieved is controversial and has been the subject for most of the investigations on this enzyme.

Kenney (1962), demonstrated that hydrocortisone administered to intact rats led to the de novo synthesis of tyrosine transaminase. He proposed that the primary mode of action of adrenal corticoids was to induce the synthesis of key enzymes, such as

tyrosine transaminase, involved in the supply of precursors for the synthesis of glycogen, and the secondary effect was glycogen synthesis. This hypothesis excluded the possibility of intermediary metabolites having an effect on the level of tyrosine transaminase. Ray et al. (1964), found that significant glycogen synthesis occurred in rats injected with hydrocortisone when protein synthesis was inhibited by actinomycin D. They proposed, therefore, that 'the primary mode of action of glucocorticoids was, in fact, to mobilize metabolites for glycogen synthesis' and secondarily 'to induce elevations of enzymes, possibly as a result of derepression of enzyme-forming systems caused by the alteration of the concentration of intermediates on the path to carbohydrate synthesis.' Tomkins et al. (1969), from studies on hepatoma cells in tissue culture, proposed a model for the action of hydrocortisone on the synthesis of tyrosine transaminase which involved the regulation of enzyme synthesis at the translational level. He proposed that the rate of transcription of 'mRNA' for tyrosine transaminase was constant under all conditions but that hydrocortisone, possibly through an effector molecule, inhibited the action of a cytoplasmic repressor which normally repressed the translation of the 'mRNA' into enzyme.

In vivo studies on hepatic tyrosine transaminase revealed that this enzyme is subject to a daily rhythm (Potter et al., 1966; Wurtman and Axelrod, 1967). The primary stimulus for initiation of the rhythm is the ingestion of dietary protein, the concentration of which determines the amplitude of the

rhythm (Potter et al., 1966; Wurtman et al., 1968; Lane and Mavrides, 1970).

In vivo corticosterone levels in the plasma are also subject to a daily rhythm which is unaffected by diet (Cohen et al., 1970) and preceeds the tyrosine transaminase rhythm by approximately four hours (Wurtman and Axelrod, 1967; Given et al., 1967). Although there is no direct proof that these two rhythms are coupled, there is evidence which supports a hormonal-dietary control of tyrosine transaminase in vivo. This is demonstrated by the finding that while the basal level of tyrosine transaminase activity in adrenalectomized and intact rats is the same (Rosen et al., 1963; Mavrides and Lane, 1969), the amplitude of the enzyme rhythm is reduced by 50% in adrenalectomized animals compared to the intact animals fed on the same diet (Mavrides and Lane, 1969). The effect of glucocorticoids is to enhance the effect of diet on tyrosine transaminase activity. Mavrides and Lane (1969), using adrenalectomized rats demonstrated a permissive role for the action of hydrocortisone which would explain this enhancement.

A metabolic significance of the tyrosine transaminase rhythm is implied by the studies of Lane and Mavrides (1970) who found that when gluconeogenesis was repressed, as may be indicated by the repression of a phosphoenolpyruvate carboxykinase daily rhythm (a pace-maker enzyme in gluconeogenesis) in rats fed a carbohydrate-rich diet, tyrosine transaminase was similiarly repressed. Under these conditions, the liver would not require

non-carbohydrate precursors, such as amino acids, for glycogen synthesis and hence a high activity in enzymes channelling these intermediates into glycogen would similiarly not be required.

In addition to glucocorticoids, other hormones have been found to influence hepatic tyrosine transaminase activity: glucagon (Greengard and Baker, 1966), insulin (Holten and Kenney, 1967), epinephrine in in vitro experiments (Wicks, 1968), growth hormone (Kenney, 1967) and cyclic 3',5'-AMP (Wicks et al., 1969).

Since tyrosine transaminase is the first enzyme in the pathway of tyrosine catabolism, it would be reasonable to expect that the control of this pathway might involve the regulation of tyrosine transaminase activity. This is even more plausible in light of the fact that tyrosine transaminase activity undergoes great changes in vivo within a relatively short period of time after the administration of hormones. However, there is no conclusive study demonstrating that this enzyme controls the catabolism of tyrosine. This is mainly due to the fact that tyrosine transaminase has been little studied in the context of tyrosine catabolism and only a very few studies have investigated the other four enzymes in the pathway. However, early studies on tyrosine oxidation implied that tyrosine transaminase is the rate-limiting step in this process.

La Du and Greenberg (1951) found, by comparing rates of oxygen uptake by acetone powder extracts incubated with tyrosine, p-hydroxyphenylpyruvate and homogentisate, that the rate of uptake of oxygen with tyrosine was the lowest, and concluded that

'the rate-limiting step in the series of reactions of tyrosine catabolism must precede p-hydroxyphenylpyruvate oxidation' under their experimental conditions. Knox and Le May-Knox (1951) later implicated this enzyme as the rate-controlling enzyme in tyrosine catabolism by showing that the rate of tyrosine oxidation was equivalent to the rate of tyrosine transamination. La Du et al. (1958) in a study of alcaptonurea patients, found that maleylacetoacetate isomerase and fumarylacetoacetate hydrolase activites were comparable to activities found in normal patients. Since the only known pathway for the formation of maleylacetoacetate is from the oxidation of homogentisate, it was concluded that these last two enzymes in the tyrosine oxidising pathway were not affected by levels of substrate and hence were not adaptive enzymes. Lin and Knox (1958), found that while tyrosine transaminase was stimulated five-fold, five hours after the intraperitoneal injection of hydrocortisone into rats, p-hydroxyphenylpyruvate oxidase activity did not change and homogentisate oxidase activity rose only approximately 22% over basal levels.

From these observations, it would appear that tyrosine transaminase has a regulating role in the catabolism of tyrosine. In the regulation of this enzyme in the mammalian system, it has been shown that dietary factors, as well as hormonal, play an important role. However, because of the complications imposed by an endocrine system, it is very difficult to distinguish between hormonal and dietary effects. A much simpler system, de-

void of hormonal effects, would be beneficial from this stand point.

3. Tetrahymena pyriformis as a System for the Study of the Regulation of Phenylalanine and Tyrosine Catabolism in Gluconeogenesis.

The ciliated protozoan, Tetrahymena pyriformis is a good system for the study of metabolite control of tyrosine transaminase activity. Despite the fact that protozoa are much lower on the evolutionary scale than the rat, they have many features in common with the rat liver cell. Kidder (1967) in fact, considers Tetrahymena to be a 'legitimate relative of the laboratory rat liver cells.'

Like liver cells, Tetrahymena are very glucogenic and may accumulate up to 23% of their dry weight in glycogen (Ryley, 1951). An analysis of the glycogen showed it to be very similiar to liver glycogen (Manners and Ryley, 1952). Tetrahymena are eucaryotic cells and contain an elaborate ultrastructure much like higher cells (Kidder, 1967). Furthermore, their nutritional requirements are similiar to those of the rat (Kidder, 1967). The main pathways such as the Krebs cycle, the Embden-Meyerhoff pathway, hexose monophosphate shunt, and terminal respiration dependent on the conventional flavoproteins and cytochromes have been reported (Seaman, 1955; Ryley, 1952). Haemoglobin is present (Keilin and Ryley, 1953), as well as the catecholamines, norepinephrine and epinephrine (Janakidevi et al., 1966a), serotonin (Janakidevi et al, 1966b) and 3'5' cyclic AMP phosphodiesterase activity (Blum, 1970). However, their function has not been

determined.

Major differences are also evident between Tetrahymena and liver cells. For example, these organisms can synthesize carbohydrates from fatty acids via an active glyoxylate cycle (Hogg and Kornberg, 1963), do not have a urea cycle (Hill and van Eys, 1965), can not synthesize all the required purines and pyrimidines (Kidder and Dewey, 1945; 1948), or thioctic acid (Elliott et al., 1962; Seaman, 1952), and synthesize benzoquinone rings via an abortive shikimic acid pathway (Miller, 1965).

Although the phylum protozoa contains at least 50,000 species, most of the biochemical studies have been carried out on only a few. This is due to the fact that very few protozoa have been obtained in axenic culture. Tetrahymena pyriformis was isolated by Lwoff in 1923 and thanks to the work of Dewey et al. (1950) can be grown in a chemically defined medium.

The pathway of phenylalanine and tyrosine catabolism has not hitherto been studied in Tetrahymena. However, nutritional experiments have shown that tyrosine is not an essential amino acid (Kidder and Dewey, 1951) and therefore, there is indirect evidence for the presence of phenylalanine hydroxylase. Furthermore, when Tetrahymena were incubated in the presence of various amino acids and the oxygen uptake was measured, it was found that the greatest stimulation in respiration was caused by phenylalanine and tyrosine indicating these amino acids were oxidised (Roth et al., 1954). Despite the fact that Roth and Eichel (1961) stated, without supporting evidence, that they failed in their attempts to demonstrate the existence of the enzymes of the

tyrosine oxidising pathway, Mavrides and D'Iorio (1969), assayed tyrosine transaminase in these organisms and demonstrated that regulatory mechanisms for this enzyme were operative. Phosphoenolpyruvate catboxykinase was assayed by Shrago and Shug (1966) and shown to be repressed by glucose as was tyrosine transaminase (Mavrides and D'Iorio, 1969). Both enzymes have been shown to increase in activity as the culture ages. Using the chemically defined medium of Dewey et al. (1950), Mavrides and D'Iorio (1969) found that the removal of acetate from the synthetic medium resulted in higher tyrosine transaminase activity than was observed in its presence, especially in the early part of the growth cycle (logarithmic or exponential phase) while the addition of glucose repressed the enzyme throughout the growth cycle whether acetate was present or not. Thus both acetate and glucose repressed tyrosine transaminase activity.

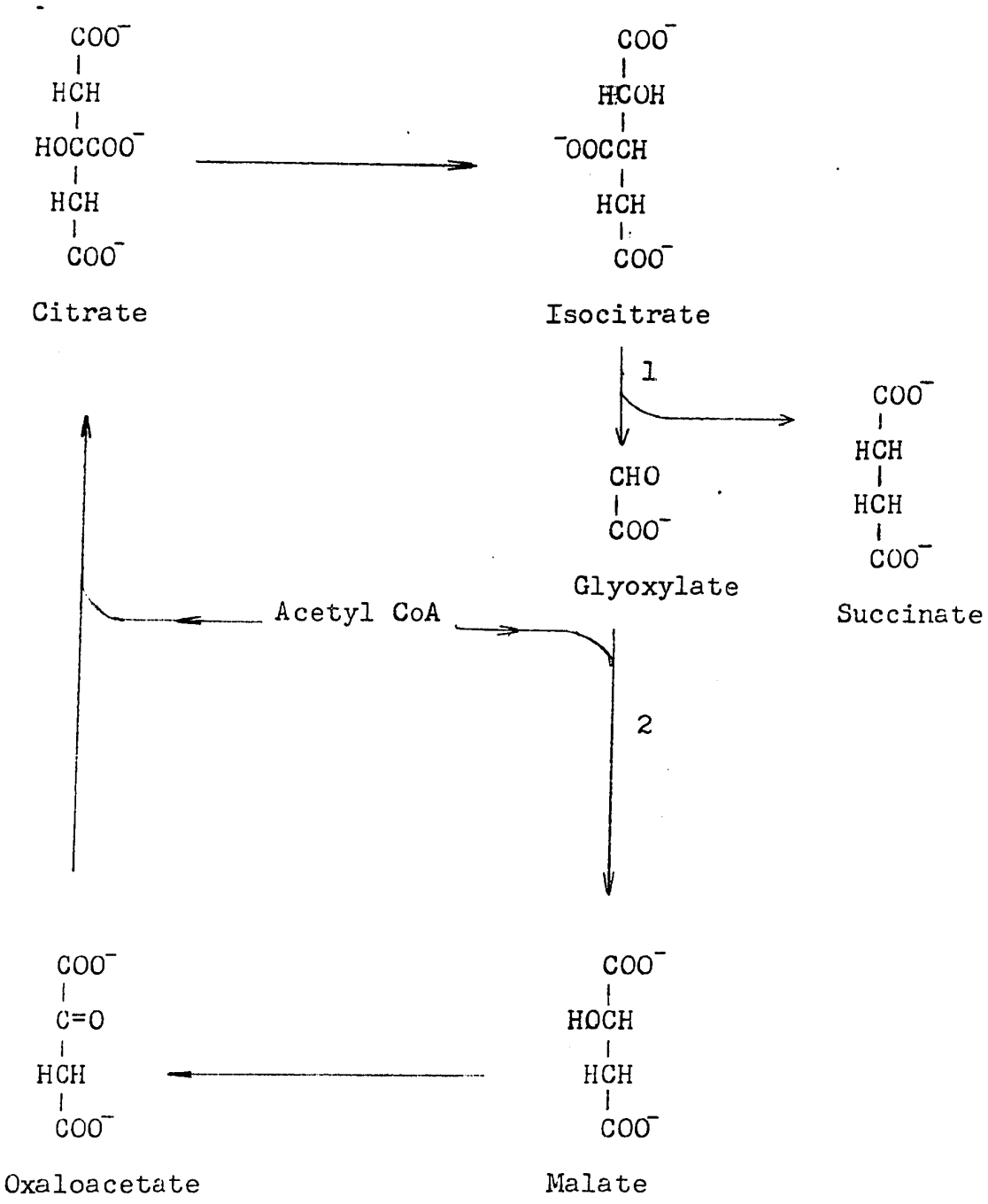
The major pathway of acetate metabolism in gluconeogenesis in Tetrahymena is the glyoxylate cycle which was reported in these organisams by Hogg and Kornberg (1963). The cycle is a bypass of the Krebs cycle and involves essentially the aldol cleavage of isocitrate to glyoxylate and succinate by isocitrate lyase (threo-D_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) followed by the condensation of one mole of the former with one mole of acetyl CoA catalyzed by malate synthase (L-malate glyoxylate-lyase (CoA acetylating), EC 4.1.3.2) to produce malate. Studies on these two enzymes in Tetrahymena, revealed that when the cycle was active, they were contained in specialized particles

known as peroxisomes or glyoxysomes (Hogg and Kornberg, 1963). Therefore, the operation of the glyoxylate cycle, which is an example of an anaplerotic sequence, may be viewed as an input of isocitrate from the mitochondria to the glyoxysomes and an output from the glyoxysomes to the mitochondria of succinate and malate (Muller, Hogg and de Duve, 1968; Scheme 2, pg. 17a).

The regulation of the glyoxylate cycle has not been elucidated, although reports have been published on the inhibition of isocitrate lyase by phosphoenolpyruvate (Kornberg, 1963; Ashworth and Kornberg, 1963). The compartmentation of the enzymes in a special structure is also a means of regulation, but the process by which this is accomplished is unknown.

Isocitrate lyase is considered to be the key enzyme in the glyoxylate cycle (Kornberg and Elsden, 1961); it is very responsive to external stimuli such as glucose (repression), and acetate (induction) (Hogg and Kornberg, 1963). Hogg and Kornberg (1963), as well as Levy and Scherbaum (1965a; 1965b), consider the operation of the cycle to be maximal during stationary phase growth of the culture. This was explained by the speculation that during this period of growth, intracellular lipids would be degraded providing acetate (acetyl CoA) for the synthesis of glycogen (Hogg and Kornberg, 1963).

The fact that the glyoxylate cycle operates in the process of gluconeogenesis, led Mavrides and D'Iorio (1969) to speculate that a high transaminase activity observed during logarithmic growth of Tetrahymena in a medium not containing acetate, might



1 Isocitrate Lyase

2 Malate Synthase

Scheme 2: The Glyoxylate Cycle

be compensating for a decreased activity in the glyoxylate cycle. If the reports of Hogg and Kornberg (1963) and Levy and Scherbaum (1965a; 1965b), that the glyoxylate cycle was induced in stationary phase are correct, then it is difficult to explain why the repressive effect of acetate on tyrosine transaminase activity was observed in logarithmic phase.

It has been shown that in a species of Achromobacter, the addition of acetate to an exponentially growing population of cells resulted in the induction of isocitrate lyase (Rosenberger, 1962). Since the course of activity of this enzyme has never been studied in Tetrahymena, it appeared possible that the presence of acetate in a chemically defined medium might cause the operation of this cycle to be stimulated much sooner than had previously been suggested. If this were so, it might explain the difference in the course of tyrosine transaminase activity shown in a medium containing acetate compared to one lacking it.

Hence the relationship between the processes of tyrosine, phenylalanine and acetate metabolism in gluconeogenesis in Tetrahymena, presented itself as a very interesting topic of investigation. Of special interest would be the effect of 'diet' (supply of nutrients in the medium) on these processes.

RESEARCH AIMS

The regulation of the enzymes concerned with phenylalanine, tyrosine and acetate metabolism in the context of gluconeogenesis in the ciliated protozoan Tetrahymena pyriformis, strain W, was the main topic of the present research project.

CHAPTER 1

In vitro and *in vivo* studies on the hydroxylation of phenylalanine to tyrosine in *Tetrahymena pyriformis*.

PART I

In vitro studies on phenylalanine hydroxylase in Tetrahymena.

Introduction

Phenylalanine hydroxylase has never been assayed in Tetrahymena pyriformis. However, nutritional experiments by Kidder and Dewey (1951) have indicated that the tyrosine requirement of these organisms can be spared by phenylalanine. Thus, there is nutritional evidence for the presence of this enzyme in Tetrahymena.

In light of the fact that regulatory mechanisms are operative for tyrosine transaminase in these organisms (Mavrides and D'Iorio, 1969), it was of interest to determine whether a similiar control also existed for phenylalanine hydroxylase. If phenylalanine hydroxylase activity is not subject to metabolite control, then the hydroxylation of phenylalanine would be controlled, to a large extent, by the transamination of tyrosine. This is supported by the fact that the main pathway of phenylalanine metabolism begins by its conversion to tyrosine (at least in mammals; Knox, 1955).

The first approach to the problem of the control of phenylalanine hydroxylase activity in Tetrahymena pyriformis was to establish a workable assay for the enzyme and study its activity in Tetrahymena grown in various chemically defined media.

MATERIALS

Most chemicals were obtained from commercial outlets. The labelled phenylalanine - para-triated L-phenylalanine (specific activity 7.41 Ci/mmole) and ^{14}C-L-phenylalanine (uniformly labelled, specific activity 375 mCi/mmole) were obtained from New England Nuclear Chicago. Barium prephenate was generously supplied by Professor F. Gibson, University of Melbourne. Enzymes used in the determination of glucose - glucose oxidase (purified Type III) and peroxidase (from horseradish Type II), were obtained from Sigma Chemical Company. Isotone, the diluent used in the determination of cell counts with the Coulter Counter, was a product of Coulter Electronics of Canada. Proteose-peptone, yeast extract and thioglycollate were all purchased from Difco Laboratories. MN cellulose powder 300G was from Macherey, Nagel and Company.

GENERAL INFORMATION ON CULTURES OF TETRAHYMENA PYRIFORMIS

1. Stages of growth in cultures of Tetrahymena pyriformis

There are three main phases in the life cycle of a cell culture - logarithmic phase (log phase), stationary phase, and death phase.

The first stage is the logarithmic phase where the number of cells in the culture increases logarithmically. Log phase growth is representative of the period of time in which the culture conditions are optimal for growth. When factors such as pH, oxygen concentration, supply of nutrients or crowding become limiting, the culture passes through a post logarithmic phase (post log phase), into stationary phase. During stationary phase there is no net increase in the number of cells in the culture (the number of divisions equals the number of deaths). When growth conditons become severly limiting, the number of deaths exceeds the number of divisions and the culture enters into death phase.

The time required for a culture to complete each of these phases, depends upon the number of cells of the inoculum and the surface to volume ratio of the culture, as well as the choice of growth medium. For example, in the case of Tetrahymena, which is an aerobic organism, the span of each of these phases would be different in 100 ml of medium contained in a 300 ml flask compared to 100 ml of the same medium in a 500 ml flask.

2. General methods for the growth of Tetrahymena cultures

Media

1) Proteose-peptone

The medium consists of 2% proteose-peptone, which is a protein digest available commercially containing proteoses, peptones, and amino acids, and 0.1% yeast extract, which is the water soluble part of autolyzed yeast. The contents of the medium were dissolved in distilled water, neutralized with dilute ammonium hydroxide, and then autoclaved at 121° C for 20 minutes in an AMSCO autoclave.

2) Dewey, Parks, and Kidder's (1950) Basal medium

Stock solutions of the components of the medium were made and stored under a layer of toluene in polyethylene bottles at 4 °C. These components were labelled A, B, C, D, E, F, G, H, I. The medium contained 3%A, 2%B, 1%C, 1%D, 1%E, 1%F, 1%G, 1%H, and 0.1%I made up in distilled water and neutralized with dilute ammonium hydroxide. Autoclaving was done at 121 °C for 20 minutes.

The composition of the stock solutions is given on pgs. 25 and 26.

Stock Solutions of Ingredients of Basal Media

A

	g./l.
L-Arginine	4.0
L-Histidine	1.5
DL-Isoleucine	5.0
L-Leucine	5.0
L-Lysine	4.0
DL-Methionine	6.0
L-Phenylalanine	2.0
DL-Serine	8.0
L-Tryptophan	1.6
DL-Valine	1.0

B

DL-Alanine	5.5
L-Arginine	4.3
L-Aspartic acid	6.1
Glycine	0.5
L-Glutamic acid	11.65
L-Histidine	2.1
DL-Isoleucine	6.3
L-Leucine	9.7
L-Lysine	7.6
DL-Methionine	3.4
L-Phenylalanine	5.0
L-Proline	8.75
DL-Serine	7.7
DL-Threonine	8.8
L-Tryptophan	1.2
DL-Valine	6.6

C

	mg./l.
Ca pantothenate	10.0
Nicotinamide	10.0
Pyridoxine·HCl	100.0
Pyridoxamine·HCl	10.0
Pyridoxal·HCl	10.0
Riboflavin	10.0
Pteroylglutamic acid	1.0
Thiamine·HCl	100.0
Biotin (free acid)	0.05
Choline chloride	100.0

D

	g./l.
$MgSO_4 \cdot 7H_2O$	10.0
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	2.5
$MnCl_2 \cdot 4H_2O$	0.05
$ZnCl_2$	0.005

E

$CaCl_2 \cdot 2H_2O$	5.0
$CuCl_2 \cdot 2H_2O$	0.5
$FeCl_3 \cdot 6H_2O$	0.125

F

K_2HPO_4	100.0
KH_2PO_4	100.0

G

Sodium acetate	100.0

H	g./l.
Guanylic acid	3.0
Adenylic acid	2.0
Cytidylic acid	2.5
Uracil	1.0

I	mg./l.
Thioctic acid	4.0

3) Thioglycollate medium

A 2.98% solution of a commercially prepared dehydrated thioglyocllate medium was made up in distilled water. The mixture was dissolved by heating to boiling and then sterilized by autoclaving at 121°C for 20 minutes. The medium was stored in the dark at room temperature until used.

Growth of Tetrahymena cultures

1) Maintainance of stock cultures

The stock cultures were grown in 5 ml of proteose-peptone medium contained in 15 ml screw-cap culture tubes. Triplicate transfers were made every Tuesday and Friday. The first transfer was always saved for inoculating subsequent stock cultures and the second and third were used for inoculating parent cultures. All cultures were grown at 25°C ±1°C in a HOTPACK refrigerated incubator.

2) Parent cultures

Parent cultures were started with a 2% inoculum (V/V) from the stock cultures in 100 ml of proteose-peptone medium contained in 300 ml nepheloflasks. These were grown for 72 hours and then

used to inoculate the experimental cultures. This insured that the history of the inoculum was the same for each experiment.

3) Experimental cultures

Experimental cultures were started with a 2% inoculum (V/V) from 72 hour old parent cultures.

4) Sterility tests

Periodically, the purity of the Tetrahymena cultures was checked by inoculating 10 ml of thioglycollate medium (pg. 26) contained in 30 ml screw-cap culture tubes. These were incubated at 37 °C. Tetrahymena cells do not survive at this temperature, but any bacterial contamination would thrive. Bacterial growth in this medium can be detected visually after 2-3 days by the appearance of turbidity.

EXPERIMENTAL PROCEDURES

1) Guroff and Abramowitz's (1967) assay of phenylalanine hydroxylase

The assay required tritiated L-phenylalanine labelled in the para position only. During the hydroxylation reaction, the para-tritium migrates to the meta position with the subsequent release of one of the meta hydrogens (Guroff et al., 1966a; 1966b). The meta-labelled tyrosine is then reacted with N-iodosuccinimide which attacks the meta positions of tyrosine releasing tritium. The tritiated water thus formed can be recovered, free of the labelled substrate, by placing the assay mixture on an H^+ ion exchange column and eluting with water (Guroff and Abramowitz, 1967).

The enzyme assay contained in a total volume of 0.25 ml: 25 µmoles Tris buffer pH 7.3, 1 µmole unlabelled phenylalanine, 39,000 dpm para-tritiated L-phenylalanine, specific activity 7.41 Ci/mmole (purified as described in procedure 2, pg 29), 1 µmole NADH, 0.15 µmole pteridine cofactor (2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine), and enzyme. The reaction was initiated by the addition of the enzyme. Incubation of the assay was carried out at 37° C with shaking in an Eberbach water bath-shaker and the reaction was stopped by immersing the assay mixture in a water bath at 100°C for at least 1 minute. The incubation mixtures were then immediately cooled in crushed ice and 0.5 ml of 0.2N sodium acetate buffer, pH 5.5, was added. If the assay mixture was excessively cloudy at this point, it was spun down at 2,000xg in a refrigerated Serval centrifuge for 10-15

minutes at 0°C. To the cold supernatant, 0.2 ml of freshly prepared 1% aqueous solution of N-iodosuccinimide (purified as described in procedure 3, pg. 30) was added. The mixture was allowed to stand for five minutes at 0°C and then 0.05 ml of 30% trichloroacetic acid was added. This was centrifuged at 4,000xg for 10-15 minutes and the coloured supernatant was added to a Dowex 50 W-X8 ion exchange column prepared as follows: disposable Pasteur pipettes were fitted with cotton plugs and enough resin was added to form a column 4 cm high (the resin was conditioned according to procedure 4, pg. 30). After the resin had settled, 0.1 ml of a 1% slurry of activated charcoal was added to the top of the column which was then washed with 2 ml of distilled water before application of the sample. The columns were then eluted with 3 ml of distilled water. One-ml fractions were collected from the column in disposable scintillation vials containing 10 ml of Bray's (Bray, 1960) scintillation mixture (composition in procedure 6, pg. 31) and counted in a Nuclear Chicago or Beckman Scintillation Counter. Results were corrected for quenching and expressed as disintegrations per minute (dpm).

2) Purification of para-tritiated L-phenylalanine

Forty µcuries of para-triated L-phenylalanine, specific activity 7.41 Ci/mmole was spotted on Whatman 3MM chromatography paper. The chromatogram was developed by descending chromatography using an isopropanol:water:ammonia (80:10:10) solvent system. A standard chromatogram containing unlabelled phenylalanine

was developed at the same time and sprayed with ninhydrin (described in procedure 5, pg. 31) to locate the position of phenylalanine. The radioactive phenylalanine was eluted from the paper with slightly acidic distilled water and applied to a Dowex 50 W-X8 column contained in a disposable Pasteur pipette as described in procedure 1, pg. 28. The column was washed with 10 ml of distilled water and phenylalanine was eluted with 70 ml of 1N HCl collected in 5 ml fractions. The eluate was evaporated to dryness in a vacuum rotary evaporator and the residue was dissolved in an appropriate volume of 90% ethanol and stored at -20°C (Guroff and Abramowitz, 1967).

3) Purification of N-iodosuccinimide

N-iodosuccinimide was obtained commercially and recrystallized from hot dioxane with the addition of carbon tetrachloride to the cloud point. The crystals collected after the mixture had cooled were washed with carbon tetrachloride and stored at -20°C (Guroff and Abramowitz, 1967).

4) Procedure for conditioning Dowex 50 W-X8 resin

Dowex 50 W-X8 resin was conditioned in a batch operation process by washing successively with 2N HCl, 2N NaOH, and 2N HCl. The resin was washed with distilled water after each step until the washings had a neutral pH. The conditioned resin was stored in distilled water until used.

5) Detection of amino acids by the ninhydrin reaction

After drying, the chromatograms were sprayed with a ninhydrin solution composed of 0.2 grams ninhydrin/100 ml acetone and heated at 100°C for 2 minutes to develop the colour.

6) Composition of Bray's scintillation mixture

The Bray's scintillation mixture contained 4 grams 2,5-diphenyloxazole, 60 grams napthalene, 20 ml ethylene glycol, 100 ml methanol made up to 1 liter with dioxane (Bray, 1960).

7) Composition of Biosolv scintillation mixture

The mixture contained 0.4% 2,5-diphenyloxazole and 10% Biosolv BBS 3 (V/V) in toluene.

8) Composition of Toluene scintillation mixture

The scintillation mixture contained 4 grams 2,5-diphenyloxazole/liter toluene.

9) Procedure for harvesting cultures of Tetrahymena pyriformis

Tetrahymena cultures were centrifuged at 140xg for 5 minutes in a refrigerated Serval centrifuge set at 0°C. The cell pellet was washed twice in ice-cold isotonic saline and used for preparation of the cell extracts.

10) Preparation of cell extracts

Unless otherwise stated, the cell extracts were prepared by sonication of the washed cell pellet (procedure 9, pg. 31) in the appropriate buffer with a Biosonik III sonicator for

approximately 30 seconds. Viewing the sonicate under a low power microscope was routinely practised to insure that the sonication had completely disrupted the cells.

11) Assay of phenylalanine hydroxylase using ^{14}C L-phenylalanine

The assay mixture described by Guroff and Abramowitz (1967) (procedure 1, pg. 28), was used except that 50,000 dpm of uniformly labelled ^{14}C L-phenylalanine, specific activity 375 mCi/mmole, was substituted for para-tritiated L-phenylalanine. The incubation was stopped by boiling the assay mixture at 100°C in a water bath for at least 1 minute and an aliquot of the 27,000xg supernatant was spotted on Whatman #1 chromatography paper designed as follows: a large sheet of Whatman #1 paper 46x57 cm was cut into strips 2x50 cm which were left attached to a common head 7 cm wide and 2 cm strips were cut out between the strips. The head of the chromatogram was placed in a trough of a descending chromatography tank and weighted down with a glass rod and the paper was equilibrated with 100 ml of the developing solvent - isopropanol:water:ammonia (80:10:10) - placed in the bottom of the tank. Addition of the same solvent through an opening in the cover of the tank initiated the run, and the chromatograms were developed overnight. Standard chromatograms spotted with cold phenylalanine and tyrosine were run with each set of experiments and developed with ninhydrin (procedure 5, pg. 31). Once the run was completed, each experimental strip was cut into 1 cm-wide sections and counted in 10 ml of a toluene

scintillation mixture (procedure 8, pg. 31) in disposable scintillation vials, and counted in a Beckman scintillation counter. The results were corrected for quenching and expressed as disintegrations per minute (dpm). Histograms were plotted and the phenylalanine and tyrosine peaks were identified from their relative Rf values: the Rf of tyrosine relative to phenylalanine was 0.58.

12) Thin layer chromatography of the phenylalanine hydroxylase assay

Thin layer plates were prepared using MN cellulose 300G without binder. Fifteen grams of cellulose in 80 ml of distilled water was blended in a Waring blender and spread on glass plates 5x20 cm with a Desaga Heidelberg spreader using a thickness of 0.5 mm. The spotted plates were developed in a solvent system composed of tertiary amyl alchol:methanol:water:ammonia:2-butanone: acetone (50:5:15:5:20:10) (Heathcote and Haworth, 1969). The run was completed in approximately 5 hours at room temperature, after which time the plates were air-dried and 0.5 cm sections were scraped into disposable vials containing 10 ml of the Biosolv scintillation mixture (procedure 7, pg. 31) and counted in a Beckman scintillation counter. Counts were corrected for quenching and results were plotted in the form of histograms. Tyrosine and phenylalanine were identified by their relative Rf values obtained from running standards: the Rf of tyrosine relative to phenylalanine was 0.52.

13) Acetone powder of Tetrahymena cells

An aqueous suspension of washed Tetrahymena cells (procedure 9, pg. 31) was added slowly with vigorous stirring to 10 volumes of acetone cooled to -20°C. The cells were stirred briefly and allowed to settle. The supernatant was decanted and the cells were dried on a Buchner funnel and washed with 3 volumes of acetone at -20°C. After drying on the Buchner funnel, the cells were placed in a dessicator until used (procedure described by Gunsalus, 1955).

14) Procedure for lysing Tetrahymena cells with glycerol

Washed cells (procedure 9, pg. 31) were suspended in approximately 6 ml of 0.4M sucrose containing 0.1M Tris buffer pH 7.5 and 0.005M KCl, and left on ice for 10 minutes. The 18,000xg pellet was added to 2 ml of a mixture of 50% glycerol, 0.005M mercaptoethanol and 0.01M Tris buffer pH 7.4 and stirred for 2 hours at 4°C. This preparation was then assayed for enzyme activity (Mager and Lipmann, 1958).

15) Treatment of Tetrahymena cells with toluene for the in vitro assay of phenylalanine hydroxylase in intact cells

Washed cells (procedure 9, pg. 31) were suspended in 0.5M Tris buffer pH 7.3 and 0.4 µliters of toluene was added per ml of suspension (Gachelin, 1969). The cells were left for 20 minutes and then a 0.2 ml aliquot was used to assay phenylalanine hydroxylase (assay described in procedure 11, pg. 32). The total volume of the assay was increased to 0.5 ml.

16) Preparation of Pteridine Reductase from sheep liver

A 25% homogenate of sheep liver in 0.1M phosphate buffer pH 7.4 was centrifuged at 18,000xg for 45 minutes. The supernatant contained the pteridine reductase free of the phenylalanine hydroxylase and was used as a source for this enzyme (Kaufman, 1959).

17) The spectrophotometric assay of Prephenate dehydrogenase

Prephenate dehydrogenase (Prephenate: NAD(P) oxidoreductase (decarboxylating), EC 1.3.1.a) was assayed by the method of Cotton and Gibson (1965). The spectrophotometric assay depended upon an increase in absorption at 340 mmicrons due to the reduction of NAD^+. The assay, in a total volume of 1 ml, contained: 0.5 µmoles barium prephenate, 25 µmoles Tris buffer pH 8.2, 0.5 µmoles NAD^+ and enzyme. The blank did not contain barium prephenate and the reaction was initiated by addition of the enzyme. The reaction was followed at 25° C in a Unicam SP 800 spectrophotometer.

RESULTS

Experiment 1

Phenylalanine hydroxylase activity was assayed by the method of Guroff and Abramowitz (1967) (procedure 1, pg. 28). To insure that the assay worked, rat liver phenylalanine hydroxylase was assayed. A 25% homogenate of rat liver in 0.5M Tris buffer pH 7.3 was prepared and 0.1 ml of the 27,000xg supernatant was used to construct a progress curve.

The assay was successfully carried out using rat liver phenylalanine hydroxylase and a progress curve was established (figure 1-1., pg. 37).

Experiment 2

Once the assay method for phenylalanine hydroxylase had been established, it was tested using cell extracts of *Tetrahymena pyriformis*. Cells were grown for 72 hours and harvested (procedure 9, pg. 31). The cells were disrupted by freezing and thawing three times in 0.5M Tris buffer pH 7.3 and the 27,000xg supernatant was used for the assay of the enzyme. The construction of a progress curve from 0-40 minutes was attempted.

After a 40 minute incubation, a maximum of 800 dpm was recovered as tritiated water compared to the 39,000 dpm of para-tritiated phenylalanine added. There seemed to be very little assayable hydroxylase activity in the soluble cell extract.

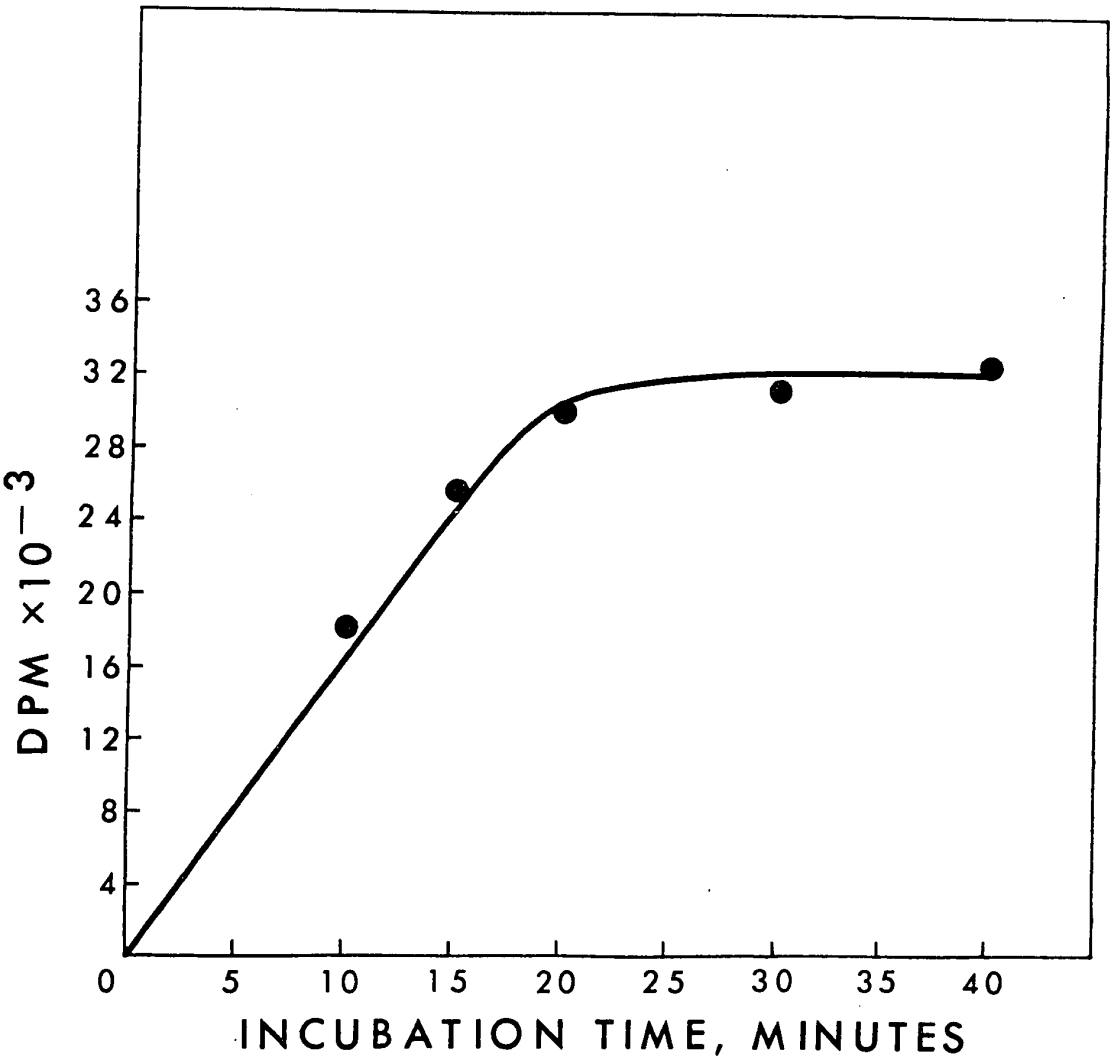


Figure 1-1: Progress curve for rat liver phenylalanine hydroxylase assayed according to the procedure of Guroff and Abramowitz (1967) described in Experiment 1, pg. 36.

Experiment 3

The next series of experiments were designed to test the effectiveness of different procedures for disrupting cells. It was considered possible that phenylalanine hydroxylase might be a particulate enzyme in Tetrahymena as in sheep liver (Kaufman, 1959). Therefore, the entire cell homogenate obtained by freezing and thawing the cells three times, as well as the 27,000xg supernatant, was assayed. This procedure of disruption was also tested in the presence of 1% deoxycholate.

Cells were disrupted by sonication using 120 sec, 60 sec, and 30 sec, sonication times. This procedure was also tested in the presence of 1% deoxycholate. To insure that the sonication did not inactivate the enzyme, a rat liver homogenate was sonicated and then assayed for phenylalanine hydroxylase activity.

Cell extracts were prepared in 0.5M Tris buffer pH 7.3, 0.5M phosphate buffer pH 7.3 and 0.25M sucrose.

The enzyme did not appear to be particulate or membrane bound since no increase in activity above the blank values was observed when the whole cell extract was tested or when cells were disrupted in the presence of 1% deoxycholate. No difference was observed by changing the buffer or disrupting the cells by sonication rather than freezing and thawing. Sonication of the rat liver homogenate did not alter phenylalanine hydroxylase activity.

Experiment 4

The next sequence of experiments explored the possibility that the conditions of the assay were not optimal.

Hence, the effectiveness of various cofactors was tested. Although Kaufman (1957) found that NADPH was a much better cofactor than NADH, both were tried. Substitution of the reduced nucleotides with 2.5 μmoles dithiothreitol or 2.5 μmoles glutathione was also tested as a nonenzymatic system for the regeneration of the pteridine cofactor (Bublitz, 1969). Cells were sonicated and both the 27,000xg soluble extract and the whole sonicate were used in the assays (procedure 1, pg. 28).

Inhibition of phenylalanine hydroxylase by metals has not been shown, however, 0.25 ml EDTA ranging in concentration from 0.01M - 0.002M, was added to the assay mixture.

Various pH were also investigated. A range of pH extending from 6.0 to 10.3 was tested using 0.5M imidazole buffer pH 6-7.6, 0.5M Tris buffer pH 7.6-8.55, 0.5M glycine buffer pH 8.55-10.3.

The effect of temperature was considered. Assays were carried out at 20°C, 25°C, and 37°C with shaking. The incubation period was one hour.

None of the conditions tested - pH, buffer, temperature, inhibition by metals, reducing cofactors, and regenerating systems for the pteridine cofactor - resulted in activity above blank values.

Experiment 5

The use of para-tritiated L-phenylalanine in the previous experiments, gave blank values which ranged from 600-800 dpm. For rat liver phenylalanine hydroxylase this represented less than 10% of the assay values, however, for Tetrahymena assays this was a very large blank. A different method of assaying phenylalanine hydroxylase in Tetrahymena extracts, was therefore tried in order to eliminate high blanks.

Uniformly labelled ^{14}C L-phenylalanine, specific activity 375 mCi/mmole, was used in place of para-tritiated phenylalanine and the reaction product was spotted on Whatman #1 paper (procedure 11, pg. 32). Tyrosine and phenylalanine were separated by descending chromatography and the chromatograms were analyzed. Rat liver phenylalalnine hydroxylase was assayed under the same conditions and used as a control to insure that the method worked.

Analysis of the Tetrahymena assay showed that all the radioactivity was recovered as phenylalanine. The rat liver assay gave two peaks, one corresponding to tyrosine the second to phenylalanine. The fact that rat liver phenylalanine hydroxylase could easily be assayed under the conditions of the assay led to the conclusion that the assay conditions were not at fault but, that phenylalanine hydroxylase in Tetrahymena was probably a very labile enzyme and was inactivated upon cell disruption.

Experiment 6

Different methods of preparing the Tetrahymena cell extracts were tried in hope of stabilizing the hydroxylase.

Acetone powders were, in the past, used to obtain a stable tyrosine-oxidising system (La Du and Greenberg, 1951) and therefore, an acetone powder extract of Tetrahymena pyriformis was prepared as described in procedure 13, pg. 34, and was assayed (as described in procedure 11, pg. 32). The reaction mixture was analyzed by a thin layer chromatography system which proved to be a fast and effecient method for separating phenylalanine and tyrosine (procedure 12, pg. 33), and analyzed.

A gentle method of disrupting cells is to lyse then with glycerol. Therefore, Tetrahymena cells were treated with glycerol as described in procedure 14, pg. 34, and the extract was assayed and analyzed as previously described (procedure 11, pg. 32).

Gachelin (1969), described an in vivo assay of the phosphotransferase system in E. coli which involved treating the intact cells of E. coli with toluene and then suspending them in the assay mixture. This treatment would alter the membrane permeability so that the components of the assay mixture would diffuse into the cells, react with the enzyme and the product could be analyzed. This procedure might offset any adverse effects upon disruption of the cell such as a loss of intracellular organization which might be important for enzyme activity. Therefore, this procedure (described in procedure 15, pg. 34) was tried on Tetrahymena cells and the assay was analyzed (procedure 11, pg. 32).

None of the procedures proved to be effective in stabilizing phenylalanine hydroxylase activity in Tetrahymena.

Experiment 7

The possibility that proteases realeased upon cell disruption might degrade phenylalanine hydroxylase was considered (Dickie and Liener, 1962). To test this possibility, Tetrahymena extract was added to rat liver exract and phenylalanine hydroxylase was assayed (procedure 11, pg. 32). Rat liver extract was assayed alone as a control.

The addition of the Tetrahymena extract to the rat liver extract did not alter the activity of the liver phenylalanine hydroxylase. Therefore, proteases from Tetrahymena did not degrade the rat liver enzyme, however, this experiment did not eliminate the possibility that the Tetrahymena enzyme might be susceptible to proteolytic attack.

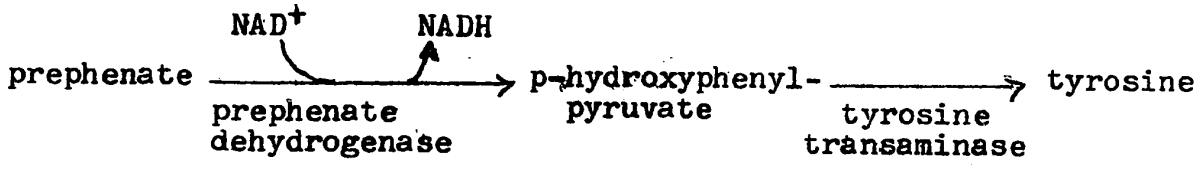
Experiment 8

It was also possible that the pteridine reductase was limiting in the assay. Therefore, this enzyme was obtained from sheep liver as described in procedure 16, pg. 35. Sheep liver extract was assayed for phenylalanine hydroxylase alone and in the presence of the Tetrahymena extract.

The addition of the sheep liver extract to Tetrahymena extract did not result in any hydroxylase activity. It was concluded that the reductase was not the limiting factor.

Experiment 9

Enzymes of the shikimic pathway are present in Tetrahymena and are used for the production of ubiquinone (Miller, 1965). Because nutritional studies by Kidder and Dewey (1951) showed that phenylalanine could relieve the tyrosine requirement of these organisms, it was suggested that the shikimic acid pathway was not operative for the synthesis of tyrosine. However, it might be possible to have both phenylalanine hydroxylase and the shikimic acid pathway operative in Tetrahymena for the formation of tyrosine. Therefore, a whole extract of Tetrahymena (procedure 10, pg. 31) was assayed for prephenate dehydrogenase, one of the enzymes in the shikimic acid pathway concerned with the biosynthesis of tyrosine (procedure 17, pg. 35). The reaction proceeds as follows:



Prephenate dehydrogenase activity could not be detected in extracts of Tetrahymena pyriformis. This, together with Kidder and Dewey's (1951) results, suggested that the shikimic acid pathway did not operate for the synthesis of tyrosine in this organism.

DISCUSSION

Attempts to assay phenylalanine hydroxylase in Tetrahymena pyriformis in vitro, failed. The results indicated that the enzyme was very labile in these organisms and lost its activity when the cells were disrupted. Supplementing the extract with a crude source of pteridine reductase, changing the conditions of the assay procedure described by Guroff and Abramowitz (1969), and testing various methods of disrupting the cells, proved fruitless.

It is known, however, that incubation of washed Tetrahymena cells with phenylalanine and tyrosine, increased the oxygen consumption 50-60% over controls (Roth et al., 1954; Roth and Eichel, 1961), indicating that these amino acids were oxidised. Despite these observations, attempts to demonstrate the oxidation of phenylalanine in cell-free homogentates of Tetrahymena, were unsuccessful (Roth et al., 1954).

It appears especially difficult to assay hydroxylation reactions in micro-organisms. Stanier (Stanier, 1955) has made the statement 'for many years we have been trying to demonstrate hydroxylation steps in bacterial systems but so far have had a total lack of success in finding extracts with enough activity to test.'

Although a demonstration of an in vitro hydroxylation of phenylalanine failed, it was rea onable to maintain that a phenylalanine hydroxylase activity was present in these organisms.

This view was strengthened by the failure to assay prephenate dehydrogenase in this organism. For this reason, experiments were designed to show that phenylalanine is indeed hydroxylated to tyrosine in Tetrahymena in vivo.

PART II

In vivo studies on the hydroxylation of phenylalanine to tyrosine in cultures of Tetrahymena pyriformis.

Introduction

Direct evidence for the hydroxylation of phenylalanine to tyrosine would be obtained by isolating isotopically labelled tyrosine from a culture of Tetrahymena grown in the presence of isotopically labelled phenylalanine. Furthermore, if phenylalanine is a precursor for glycogen synthesis during gluconeogenesis, then its rate of metabolism i.e., its rate of hydroxylation to tyrosine, should be diminished in the presence of glucose which represses gluconeogenesis (Levy, 1967). Therefore, in addition to demonstrating the in vivo hydroxylation of phenylalanine to tyrosine, attempts were also made to compare the in vivo metabolism of phenylalanine in Tetrahymena cultures grown in basal and basal plus 0.25% glucose and to determine whether phenylalanine was a precursor for glycogen synthesis.

EXPERIMENTAL PROCEDURES

18) Procedure for sterilizing ^{14}C-L-phenylalanine

An aliquot of the stock ^{14}C-L-phenylalanine (uniformly labelled, specific activity 375 mCi/mmole) stored in 1N HCl, was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in distilled water and sterilized by filtration through an ultra fine porosity fritted disc.

19) Extraction of Tetrahymena cells for analysis by paper chromatography in the demonstration of the in vivo hydroxylation of phenylalanine to tyrosine

The washed cell pellet (procedure 9, pg. 31) was sonicated for 2 minutes in distilled water at 30 second intervals and centrifuged at 4,000xg for 5 minutes. The supernatant was saved and the pellet was washed twice with distilled water. The washings and the supernatant were combined, deproteinized with 30% trichloroacetic acid and spun down once more at 4,000xg. The resulting supernatant was applied to a Dowex 50 W-X8 column, prepared as previously described (procedure 1, pg. 28). The column was washed with 20 ml of distilled water and the amino acids were eluted with 15 ml of 5N ammonium hydroxide. The eluant was evaporated to dryness in a vacuum rotary evaporator and the residue was dissolved in a small volume of 5N ammonium hydroxide and used to spot chromatograms.

20) Extraction and purification of glycogen

Two ml of 30% KOH was added to the cell pellet and the resulting mixture was heated in a boiling water bath for 20 minutes. To this was added 0.2 ml of saturated sodium sulphate and 2.5 ml of 95% ethanol. This was mixed thoroughly and allowed to stand on ice for 20 minutes before spinning down at 4,000xg for 10 minutes (Good et al., 1933). The glycogen pellet was washed twice with 55% ethanol and purified further by dialysis as follows: the glycogen was placed in dialysis tubing (0.719" in diameter, 1.21" flat width) and dialyzed against 3 liters of water (changed twice a day) stirred constantly with a magnetic stirrer (Hassid and Abraham, 1957).

21) Procedure for hydrolysis of purified glycogen

The purified glycogen (procedure 20), was precipitated from the dialysate by adding ethanol to a final concentration of 55%. The precipitated glycogen was dissolved in 1 ml of distilled water to which was added 1 ml of 0.6N HCl. This was placed in a 20 ml test tube topped with a glass ball and refluxed for 2.5 hours in a boiling water bath (Hassid and Abraham, 1957). This procedure proved to be 100% efficient in hydrolyzing the glycogen.

22) Two dimensional chromatography of the hydrolyzed glycogen

The sample (obtained as described in procedure 21), was applied to the lower left hand corner of Whatman #1 chromatograms were secured in a frame and placed in a chromatography

tank. Five hundred ml of a phenol:water:ammonia (438:61:1.6) system was used to develop the chromatogram in the first direction and the same volume of an isopropanol:ethyl acetate:water (7:1:2) system was used in the second direction. The first run took six hours and the second eight hours at room temperature. The chromatograms were dried overnight between each run.

23) Benzidine spray for locating sugars

The benzidine spray was composed of 1 gram benzidine·HCl, 20 ml glacial acetic acid, 20 ml distilled water, 8 grams trichloroacetic acid and 160 ml of 95% ethanol. The chromatograms were sprayed and heated in an oven at 110°C for 3-5 minutes. Sugars appeared as yellow spots.

24) Enzymatic assay of glucose by the glucose oxidase reaction

The method is described by Bergmeyer and Bernt (1963), and requires glucose oxidase and peroxidase. Peroxide produced from the oxidation of glucose by glucose oxidase is used by the peroxidase to oxidise ortho-dianisidine (3,3'-dimethoxybenzidine), the oxidised form of which can be measured spectophotometrically at 436 mmicrons and is proportional to the amount of glucose oxidised. The reaction proceeds as follows:

$$\text{glucose} \xrightarrow[O_2]{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$\text{o-dianisidine} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{oxidised o-dianisidine} + H_2O$$

A stock 0.12M sodium phosphate buffer-enzyme mixture, pH 7.0 was prepared as follows: 2.07 grams $Na_2HPO_4 \cdot 2H_2O$, 1.09 grams $NaH_2PO_4 \cdot 2H_2O$, 6 mg peroxidase and 38 mg glucose oxidase made up to 150 ml with distilled water. Fifty ml of this solution was added to 0.5 ml of a solution containing 10 ml of o-dianisidine.HCl in 2 ml of distilled water and stirred vigorously. Five ml of this solution was added to 0.2 ml of the test solution, mixed well and allowed to stand for 35 minutes at room temperature. The optical density was read at 436 mmicrons against a reagent blank and glucose was determined from a standard curve, The assay was sensitive enough to detect 2 μ grams of glucose in the assay mixture.

25) Procedure for determining isotopically labelled phenylalanine in the culture medium

From a 1-ml aliquot of the culture, 0,9 ml was added to 0.1 ml of 30% trichloroacetic acid to stop cell growth. This was spun down at 4,000xg and the cell pellet was washed once with 2 ml of isotonic saline. The combined washings and medium were made up to 10 ml volumetrically with distilled water. A 0.1 ml aliquot of this was counted in 10 ml of a 10% Biosolv scintillation mixture (procedure 7, pg. 31) and counted. The counts were corrected for quenching and the results were expressed as dpm.

26) Composition of modified Ringer buffer (Ryley, 1952)

The buffer contained 0.047M NaCl, 0.002M KCl. 0.001M $MgSO_4$, 0.012M potassium phosphate buffer pH 7.0. The buffer was pre-

pared and autoclaved at 120°C for 20 minutes.

27) Procedure for the extraction of Tetrahymena cells with perchloric acid

The frozen cell pellet was thawed in the presence of 1.3 volumes of 0.6N $HClO_4$ and sonicated for 5 minutes (30 second intervals) at 0°C. The sonicate was centrifuged at 4,000xg for 5 minutes and the pellet was extracted with 3x its volume of 0.33N $HClO_4$, twice. The supernatants were combined, brought to pH 3.5 with 30% KOH to precipitate $KClO_4$ and the salt was removed by centrifugation at 4,000xg for 5 minutes. The pellet was washed once with distilled water and the washing was added to the extract which was then lyophilized. All procedures were carried out at 0-4°C. The lyophilized residue was taken up in a small volume of ice cold distilled water and centrifuged to remove any traces of salt and the supernatant was used for subsequent analysis.

28) Analysis of the perchloric acid extract of Tetrahymena with a Beckman Amino Acid Analyzer, model 120B

A one-ml aliquot of the perchloric acid extract was added to one ml of 0.2N sodium citrate buffer pH 2.2 and applied to a 50 cm AA-15 resin (Beckman Custom Research Resin) column. The amino acids were eluted with 0.2N sodium citrate buffer pH 4.25 at 57°C with a flow rate of 34 ml/hour. Phenylalanine and tyrosine were identified by their retention times and co-chromatography. The quantity of amino acid was determined in μ moles by using the folmula of Spackman, Stein and Moore (1958):

$$\mu \text{ moles} = \frac{H \times W}{C}$$

where H is the height of the peak, W is the width of the peak at half the height, and C is the colour constant of the amino acid, determined by running standards.

29) Determination of dpm in tyrosine and phenylalanine in the perchloric acid extract of Tetrahymena cells

The same column, procedure for applying the sample, and method of elution was used in determining dpm in tyrosine and phenylalanine as previously described (procedure 28, pg. 52). The eluant from the column was passed through a Nuclear Chicago 4526 flow cell scintillation system and 0.5 ml fractions were collected using a LKB fraction collector. The fractions were counted in 10% Biosolv scintillation mixture (procedure 7, pg. 31) contained in disposable scintillation vials. Counts were corrected for quenching and expressed as dpm.

30) Procedure for determining cell counts

In order to determine the number of cells per ml of culture, a suitable aliquot of the culture was diluted to 50 ml with Isotone, a specially manufactured diluent for use in cell counting. The cells were counted in a Model B Coulter Counter using a 200 micron aperature. All counts were corrected for coincident loss.

RESULTS

Experiment 10

In order to demonstrate the *in vivo* hydroxylation of phenylalanine to tyrosine, cells were grown in 25 ml of basal medium contained in a 300-ml nephaloflask (procedure 3, pg. 27), for 24 hours. At this time, 1.5 μcuries of sterilized uniformly labelled ^{14}C-L-phenylalanine, specific activity 375 mCi/mmole (procedure 18, pg. 47), was added aseptically to the medium. The culture was allowed to grow for 6 hours in the presence of the label after which time, it was harvested (procedure 9, pg. 31). The cells were disrupted by sonication and the 4,000xg supernatant was used to obtain phenylalanine and tyrosine (procedure 19, pg. 47). The extract was spotted on Whatman #1 chromatography paper and analyzed by descending chromatography in an isopropanol:water:ammonia (8:1:1) solvent system. Similar extracts were analyzed by the same procedure using butanol:water:acetic acid (4:1:1) and phenol:ethanol:ammonia (5:4:1) as solvent systems. The chromatograms were analyzed (procedure 11, pg. 32) and the results were plotted in the form of histograms.

The hydroxylation of phenylalanine to tyrosine was successfully demonstrated in *Tetrahymena pyriformis*. Analysis of the TCA soluble amino acids by paper chromatography (fig. 1-2, pg. 54) gave two peaks of radioactivity, apart from that which remained at the origin, the positions of which corresponded to tyrosine and phenylalanine standards run at the same time. The Rf values of phenylalanine and tyrosine in three chromatography systems are presented in Table 1-1, pg. 55.

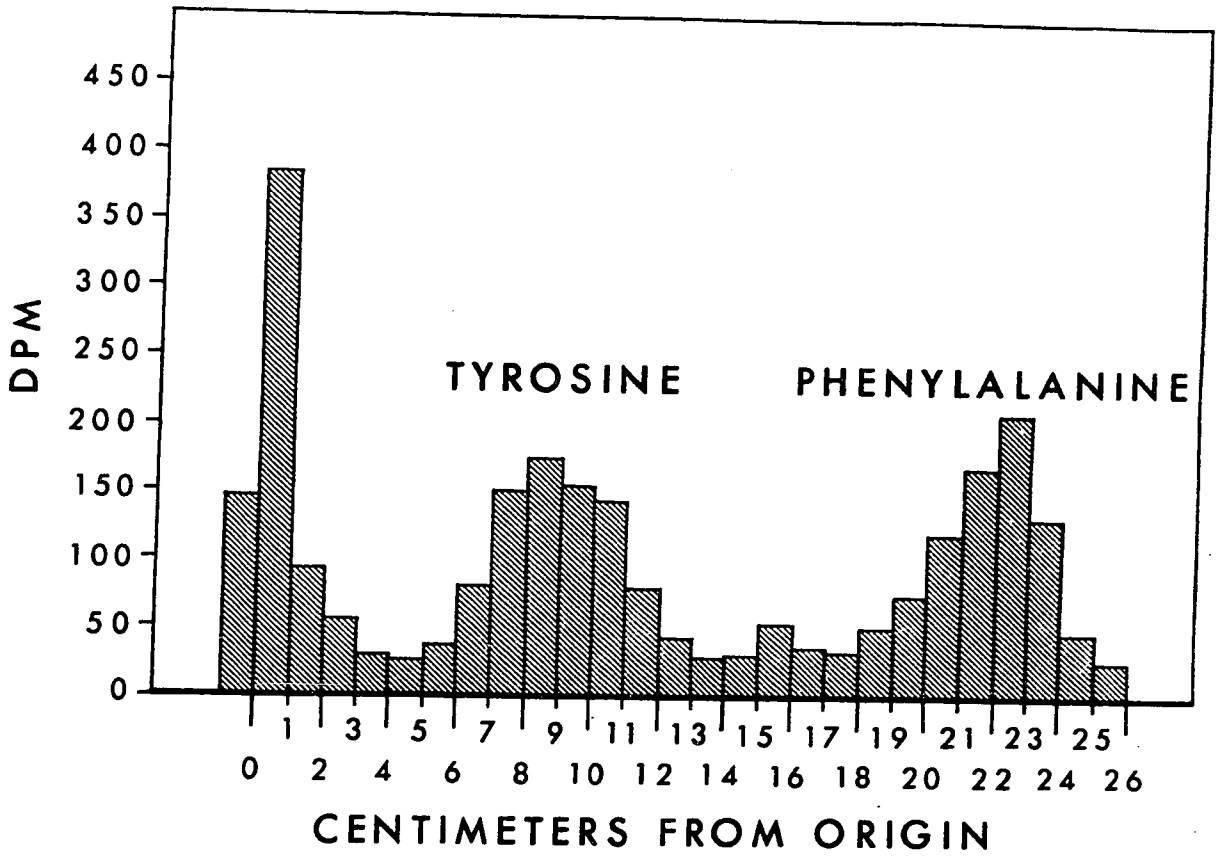


Figure 1-2: Paper chromatogram of the cell extract of a culture of Tetrahymena grown in a medium containing 14C-L-phenylalanine, demonstrating the in vivo hydroxylation of phenylalanine to tyrosine (exp. 10, pg. 53).

Table 1-1: One-dimensional descending paper chromatography of the cell extract from a culture of *Tetrahymena pyriformis* incubated in the presence of ^{14}C-L-phenylalanine. Results are from Experiment 10, pg. 53.

Solvent system	Standard Rf values		Experimental Rf values	
	Phe	Tyr	Phe	Tyr
isopropanol:H_2O:NH_3 (8:1:1)	0.85	0.36	0.79	0.38
BuOH:HAc:H_2O (4:1:1)	0.56	0.37	0.52	0.35
Phenol:EtOH:NH_3 (5:4:1)	0.79	0.48	0.72	0.43

Experiment 11

Once it had been shown that phenylalanine was hydroxylated to tyrosine, an experiment was designed to show that phenylalanine could be used as a carbon source in the synthesis of glycogen.

A 72 hour old culture of Tetrahymena grown in proteose-peptone medium (Procedure 3, pg. 27) was incubated in the presence of uniformly labelled ^{14}C-L-phenylalanine, specific activity 375 µcuries/mmole, (0.2 µcuries/ml culture) for 6 hours and harvested (procedure 9, pg. 31). Glycogen was extracted by the method of Good et al. (1933) and purified (procedure 20, pg. 48). This was then hydrolyzed (procedure 21, pg. 48) and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in a small volume of water and aliquots were spotted in the left hand corner of Whatman #1 chromatography paper (10"x10"). Three chromatograms were spotted - two with the hydrolysate, one with the hydrolysate plus a glucose standard and the chromatograms were developed by two-dimensional chromatography (procedure 22, pg. 48). After the runs had been completed, the standard chromatogram (hydrolysate plus glucose) was sprayed with a benzidine spray (procedure 23, pg. 49) to determine the position of glucose, and the corresponding areas on the two unsprayed chromatograms were circled. The areas were cut out from the chromatograms and one was counted in 10 ml of a toluene scintillation mixture (procedure 8, pg. 31) contained in a scintillation vial, while the other was eluted. The eluate was analyzed for

glucose by the glucose oxidase reaction (procedure 24, pg. 49). The specific activity of glucose could be determined from these results, and expressed as dpm/μmole glucose.

Radioactive carbons from phenylalanine were isolated in glucose obtained by the hydrolysis of purified glycogen. The specific activity of the isolated glucose was 8,473 dpm/μmole.

Experiment 12

In order to determine whether the addition of glucose to a culture of Tetrahymena had any affect on the rate of utilization of phenylalanine, the uptake of labelled phenylalanine from the culture medium was compared in Tetrahymena cultures grown in basal and basal plus 0.25% glucose media.

Cells were grown in 25 ml of these two media contained in 300 ml nephaloflasks (procedure 3, pg. 27). After 22 hours of growth (log phase), 2.5 μcuries of sterilized, uniformly labelled ^{14}C-L-phenylalanine (specific activity 375 μcuries/mmole; procedure 18, pg. 47), was added aseptically to each culture. One-ml aliquots were removed aseptically every hour from 1-8 hours and 17-24 hours after the addition of the label to the culture. The ^{14}C-L-phenylalanine remaining in each medium was determined and expressed as % dpm remaining/ml culture medium (procedure 25, pg. 50).

The results are presented in fig. 1-3. The data indicates that the rate of uptake of phenylalanine is altered by the addition of glucose to a culture of Tetrahymena. The rate of uptake

in basal plus glucose compared to basal was calculated from the slopes in fig. 1-3, pg. 59. The ratio of these slopes (basal plus glucose/basal) was 0.659.

Experiment 13

Experiment 12, pg. 57, showed that glucose decreased the rate of uptake of phenylalanine by Tetrahymena cells. To dissociate the effect of glucose on the transport of phenylalanine across the cell membrane, and its effect on phenylalanine hydroxylase activity, washed cells grown in the presence and absence of glucose, were incubated in Ringer's buffer in the presence of ^{14}C-L-phenylalanine.

It was of interest to determine whether glucose had an effect on the ratio of tyrosine to phenylalanine in the amino acid pool of the cells i.e., whether glucose would decrease the rate of hydroxylation so that the tyrosine/phenylalanine ratio in the basal plus glucose culture would be lower than in the basal culture. Furthermore, if glucose did repress hydroxylase activity, it might be possible to show this effect in vivo by determining the ratio dpm tyrosine/dpm phenylalanine for both cultures and comparing them.

Hence, cells were grown in 500 ml of basal and basal plus 0.25% glucose media for 51 hours (post log phase) (procedure 3, pg. 27). The cells were harvested (procedure 9, pg. 31) and washed three times with a modified Ringer's buffer pH 7.0 (procedure 26, pg. 50). Cell counts were taken in a Model B Coulter Counter (procedure 30, pg. 52) and the volumes of the washed cells

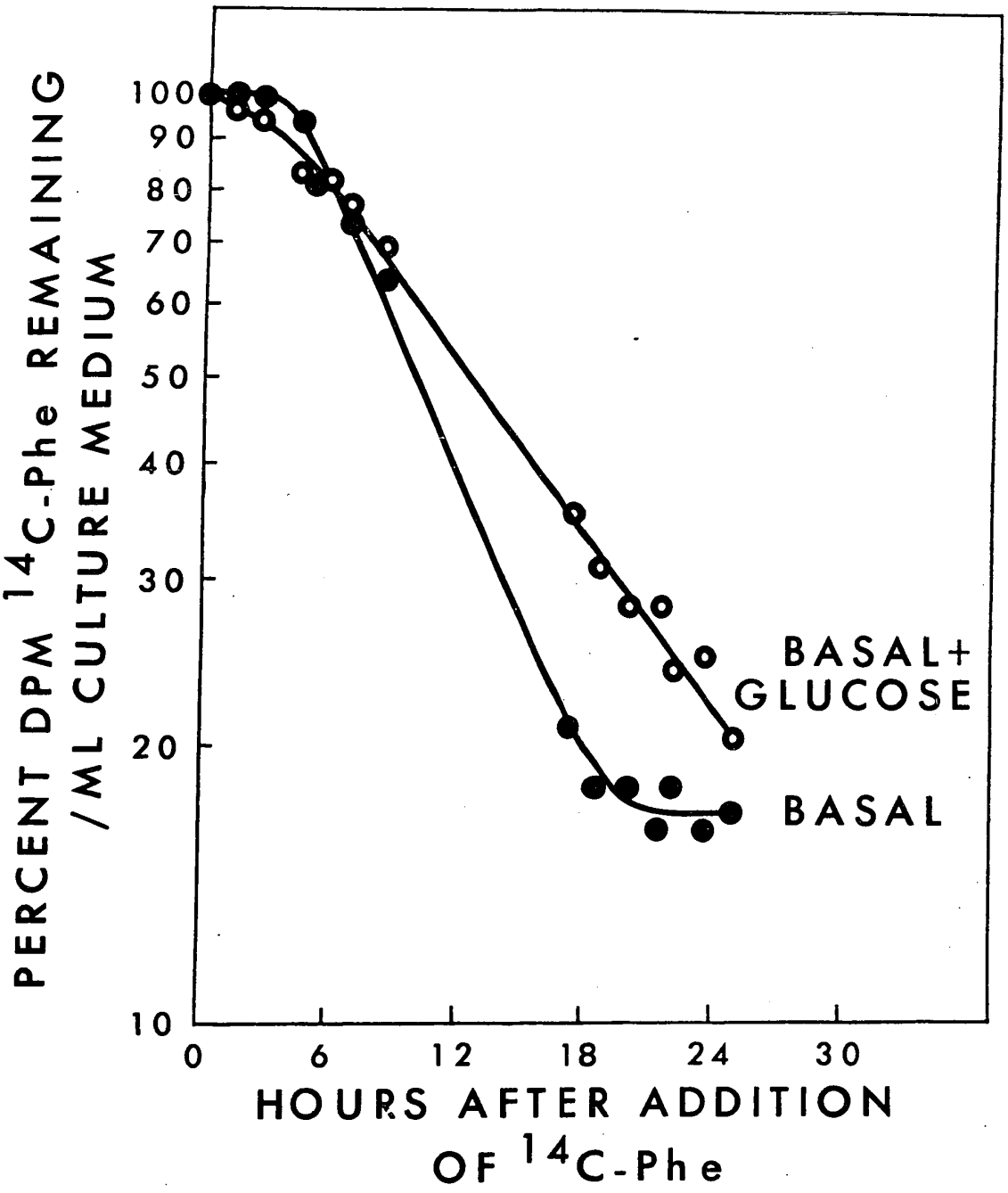


Figure 1-3: The rate of uptake of ^{14}C-L-phenylalanine from a basal and a basal plus 0.25% glucose culture media by Tetrahymena pyriformis cells. Labelled phenylalanine was added to 22 hour old cultures (exp. 12, pg. 57).

adjusted so that there were approximately 2,000,000 cells per ml of Ringer's buffer. A total volume of 9 ml of cells from each medium were incubated in the presence of 0.45μ curies of ^{14}C-L-phenylalanine, uniformly labelled, specific activity 375 μcuries/mmole, for 30 minutes. During this time the cells formed characteristic colonies and exhibited good motility. At the end of the incubation, the cells were centrifuged at 140xg for 5 minutes and washed three times with buffer. The cell pellet was frozen in liquid nitrogen and extracted with $HClO_4$ acid (procedure 27, pg. 51). The extract was lyophilized, taken up in a small volume of distilled water and analyzed in an amino acid analyzer to determine the number of μ moles of phenylalanine and tyrosine present in the extract (procedure 28, pg. 51). A second aliquot was run on the same column and the eluate was collected in 0.5 ml fractions with a LKB fraction collector and counted (procedure 29, pg. 52).

The results of the experiment are presented in Tables 1-2 (pg. 60) and 1-3 (pg. 61). As can be seen in Table 1-2, glucose did not affect the ratio of tyrosine/phenylalanine in the amino acid pool i.e. the ratio for the basal plus glucose culture was 1.10 and for the basal culture was 1.04 . The rate of hydroxylation, however, was slightly different (Table 1-3, pg. 61). The ratio of dpm tyrosine/dpm phenylalanine was 0.57 for the basal culture and 0.468 for the basal plus glucose culture, The rate of hydroxylation in the presence of glucose was 82% of that in its absence. Since the amino acids in the amino acid pool were

Table 1-2: Results from experiment 13 (pg. 58) showing the dpm tyrosine, dpm phenylalanine as well as μ moles phenylalanine and tyrosine in the cell extracts of two Tetrahymena cultures, grown in the presence and absence of glucose. From these results, the specific activity of these amino acids was calculated.

Culture	moles tyr/ ml extract	moles phe/ ml extract	moles tyr/ moles phe/ ml extract	dpm tyr/ ml extract	dpm phe/ ml extract	SA* tyr	SA* phe
Basal	0.0314	0.0303	1.04	17,102	30,019	544,650	990,726
Basal plus glucose	0.0345	0.0313	1.10	13,153	28,085	381,800	897,858

*SA specific activity expressed as dpm/μmole

Table 1-3: Ratio of the dpm tyr/dpm phe and SA tyr/SA phe obtained from experiment 13, pg. 58, as an index of the hydroxylation reaction. The relative rate of hydroxylation for the basal plus glucose culture to the basal culture was calculated from both sets of results.

Culture	SA tyr/SA phe*	Per cent of basal	dpm tyr/dpm phe	Per cent of basal
Basal	0.550	100.0	0.570	100.0
Basal plus glucose	0.425	77.3	0.468	82.1

*SA specific activity expressed as dpm/ μmole

the same for both cultures, it was also valid to use the ratio of the specific activity (SA) of these amino acids as an index of the hydroxylation rate. For basal, SA tyrosine/SA phenylalanine was 0.55, and for basal plus glucose was 0.425. From these data the rate of hydroxylation in the presence of glucose was 77% of that in its absence.

Therefore, it appears that there was a slight repression (28-33%) of the hydroxylation of phenylalanine to tyrosine in the presence of glucose.

DISSCUSSION

An *in vivo* hydroxylation of phenylalanine to tyrosine was demonstrated in cultures of *Tetrahymena pyriformis* (fig. 1-2, pg. 54). Moreover, carbons from phenylalanine were shown to be used for the synthesis of glycogen, thus indicating that phenylalanine could serve as a precursor for glycogen synthesis in the process of gluconeogenesis. Furthermore, it was found that in the presence of glucose, cultures of *Tetrahymena* showed a reduced rate of phenylalanine uptake from the culture medium (fig. 1-3, pg. 59). If phenylalanine was a precursor for glycogen synthesis in gluconeogenesis, then a reduced rate in the metabolism of this amino acid by cultures grown in the presence of glucose, which represses gluconeogenesis (Levy, 1967), would be anticipated. It could not however, be ruled out that glucose competed with phenylalanine for transport into the cells and hence reduced its rate of uptake. Thus, the observed effect could be interpreted as a decreased transport across the cell membrane, rather than an effect of glucose on the metabolism of this amino acid. By quantitating the *in vivo* hydroxylation of phenylalanine to tyrosine in washed cells, it was shown that in cells grown for 51 hours in the presence of glucose, there was a 28-33% reduction in the hydroxylation compared to the control grown in the absence of glucose. The ratio of these amino acids in the amino acid pools of these two cultures, however, did not vary (Table 1-2, 1-3, pgs. 61, 62).

Therefore, the hydroxylation of phenylalanine to tyrosine *in vivo*, appears to be repressed by glucose. Compared to tyrosine transaminase activity which is repressed several fold after 51 hours gorwth of *Tetrahymena* in basal plus glucose medium (Mavrides and D'Iorio, 1969), the repression of phenylalanine hydroxylase (28-33%) was only slight. However, it is difficult to draw a comparison between the repression of these two enzymes since in one instance, tyrosine transaminase, we are discussing the repression of enzyme activity by changes in enzyme specific activity, and in the other case, phenylalanine hydroxylase, we are studying the actual conversion of substrate to product. A more valid comparison of these two steps would be to study the *in vivo* conversion of tyrosine to p-hydroxyphenylpyruvate in the presence and absence of glucose and determine the degree of repression.

SUMMARY FOR CHAPTER 1

1) Attempts to assay phenylalanine hydroxylase in Tetrahymena, were unsuccessful, however, the in vivo hydroxylation of isotopically labelled phenylalanine to tyrosine was demonstrated.

2) Carbons from ^{14}C-labelled phenylalanine were isolated in purified glycogen, indicating a possible role of this amino acid in gluconeogenesis.

3) Cells grown in the presence of glucose showed a reduced rate of uptake of phenylalanine from the culture medium compared to a control culture grown in the absence of glucose.

4) The in vivo hydroxylation of phenylalanine to tyrosine was shown to be reduced 28-33% in a culture grown in the presence of glucose.

CHAPTER 2

Studies on the regulation of the tyrosine catabolizing pathway and the incorporation of carbons from tyrosine into glycogen in *Tetrahymena pyriformis* during gluconeogenesis.

INTRODUCTION

The demonstration of the hydroxylation of phenylalanine to tyrosine, and the incorporation of phenylalanine carbons into glycogen (Chapter 1), as well as the presence of tyrosine transaminase (Mavrides and D'Iorio, 1969) in Tetrahymena, suggested that the catabolism of phenylalanine and tyrosine followed the same route as was observed in higher animals (scheme 1, pg. 2).

Mavrides and D'Iorio (1969) found that tyrosine transaminase activity was repressed by the presence of acetate and glucose in the culture medium of Tetrahymena. Therefore, it was of interest to establish the pathway of tyrosine catabolism in Tetrahymena pyriformis (Strain W) and examine the effect of acetate and glucose on the activity of the enzymes of the pathway and flow of carbon from tyrosine to glycogen.

In studies with rat liver, it was found that a pace-maker enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), and tyrosine transaminase, followed the same pattern of activity and responded to the same stimuli (Lane and Mavrides, 1970). It appeared that a similar relationship might exist in Tetrahymena. Shrago and Shug (1966) studied the pattern of activity of PEPCK in Tetrahymena grown in a proteose-peptone medium and demonstrated a repression of this enzyme's activity when glucose was added to the culture medium. Similar results were reported for tyrosine transaminase activity (Mavrides and D'Iorio, 1969).

A detailed study of the course of tyrosine transaminase activity (Mavrides and D'Iorio, 1969), established that there was a drop in enzyme activity during log phase in all growth media, followed by a rise. A similar detailed study has not been reported for PEPCK activity. Hence, it was decided to investigate whether this pattern was also true for this enzyme. Furthermore, if it could be established that both enzyme activities dropped, then it would be possible to determine whether the synthesis of both enzymes was initiated at the same time or independently. Evidence for a coupling of the regulation of PEPCK and tyrosine transaminase activities in the mammalian system, was presented by Mavrides and Lane (1970), who demonstrated that PEPCK activity increased just prior to an increase in tyrosine transaminase activity during the course of the daily rhythms of these two enzymes.

MATERIALS

Uniformly labelled ^{14}C-L-tyrosine, specific activity 300 mCi/mmole, was obtained from New England Nuclear Chicago. Fumarylacetoacetate was prepared enzymatically as described in procedure 7b, pg. 7 . Tautomerase (p-hydroxyphenylpyruvate-enol-ketotautomerase) from pig kidney grade II, used in the assay of tyrosine transaminase and p-hydroxyphenylpyruvate oxidase and malic acid dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) from pig heart, used in the assay of phosphoenolpyruvate carboxykinase, were obtained from Sigma Chemical Company.

EXPERIMENTAL PROCEDURES

Enzyme Assays

1) Tyrosine transaminase

Tyrosine transaminase catalyzes the formation of p-hydroxyphenylpyruvate from tyrosine. The reaction was measured using a Unicam SP. 800 spectrophotometer by an increase in absorbance at 310 mmicrons due to the formation of an enol-borate complex with p-hydroxyphenylpyruvate. Three-ml cuvettes were used and the reaction was assayed at 25°C. The activity of the enzyme was expressed as µmoles p-hydroxyphenylpyruvate formed $min^{-1}mg\ protein^{-1}$.

The assay was described by Lin et al. (1958) and contained in three ml: 12 µmoles tyrosine, 80 µmoles α-ketoglutarate (neutralized with KOH), 10 µmoles diethyldithiocarbamic acid, 0.125 µmoles pyridoxal phosphate, 300 µmoles borate-phosphate buffer pH 7.8 (0.42 M borate, 0.17 M phosphate). The blank was prepared by omitting tyrosine. An extinction coeffecient of 9850 $M^{-1}cm^{-1}$ was used for the enol-borate complex.

2) p-Hydroxyphenylpyruvate oxidase

p-Hydroxyphenylpyruvate oxidase catalyzes the formation of homogentisate from p-hydroxyphenylpyruvate. The enzyme was assayed by following the consumption of p-hydroxyphenylpyruvate which was complexed with borate buffer and therefore absorbed at 310 mmicrons.

The assay, described by Lin et al. (1958), was carried out

in a 1-ml cuvette at 25°C and in a volume of 1 ml contained: 80 µ moles borate-phosphate buffer pH 7.8 (0.17M phosphate, 0.42M borate), 0.28 µ moles p-hydroxyphenylpyruvate, 0.033 K units of tautomerase. The assay mixture was incubated for 10 minutes before the addition of enzyme to allow the formation of the p-hydroxyphenylpyruvate borate complex to reach a steady level which was recorded as a constant absorption at 310 mmicrons. The blank was prepared without p-hydroxyphenylpyruvate. Enzyme activity was expressed as µ moles p-hydroxyphenylpyruvate consumed min^{-1} mg $protein^{-1}$, calculated using an extinction coeffecient of 9850 $M^{-1} cm^{-1}$ for the enol-borate complex.

3) Homogentisate oxidase

Homogentisate oxidase catalyzes the transformation of homogentisate to maleylacetoacetate. The assay was followed spectrophotometrically by an increase in optical density at 330 mmicrons due to the formation of maleylacetoacetate which absorbs at this wavelength.

The assay was described by Edwards and Knox (1955) and contained in a volume of 1 ml: 100 µ moles phosphate buffer pH 7.2, 6.0 µ moles homogentisate, 4.5 µmoles ascorbate (neutralized by NaOH just before use). The reaction was carried out at 25° C and enzyme activity was expressed as µmoles maleylacetoacetate formed min^{-1}mg $protein^{-1}$, calculated using an extinction coeffecient of 13,500 $M^{-1}cm^{-1}$ for maleylacetoacetate.

4) Maleylacetoacetate isomerase

The assay of this enzyme was based on following a decrease in absorption at 330 mmicrons due to the conversion of maleylacetoacetate through fumarylacetoacetate to fumarate and acetoacetate. The system, therefore, required the presence of maleylacetoacetate isomerase and fumarylacetoacetate hydrolase.

The assay was coupled to the assay of homogentisate oxidase. Once the formation of maleylacetoacetate from homogentisate by homogentisate oxidase had been completed, 1 µmole of the cofactor for the isomerase, glutathione, was added in a total volume of 0.01 ml. This procedure overcame the difficulties encountered using a crude preparation of enzymatically formed maleylacetoacetate contaminated with homogentisate (Knox and Edwards, 1955b) and was much simpler. Maleylacetoacetate can only be stored for a very short period of time due to its isomerisation to fumarylacetoacetate (5 days).

The reaction was carried out at 25° C in a volume of 1 ml. Enzyme activity was expressed as µmoles maleylacetoacetate consumed $min^{-1} mg\ protein^{-1}$ using an extinction coeffecient of 13,500 $M^{-1} cm^{-1}$ for maleylacetoacetate.

5) Fumarylacetoacetate hydrolase

This enzyme catalyzes the conversion of fumarylacetoacetate to fumarate and acetoacetate. The reaction can be followed spectrophotometrically at 330 mmicrons as described by Edwards and Knox (1955). Fumarylacetoacetate is not available commer-

cially and must be prepared enzymatically by the incubation of a semipurified preparation of homogentisate oxidase with homogentisate (Ravdin and Crandal, 1951) as described in procedure 7a,b, pgs. 75, 76. The fumarylacetoacetate was isolated as the crude silver salt. The same preparation was used in all the assays of fumarylacetoacetate hydrolase in this study.

The assay contained, in a total volume of 1 ml: 100 µ moles phosphate buffer pH 7.2, enough fumarylacetoacetate preparation to give an absorption of 1.5 optical density units. The reaction was carried out at 25°C and the blank was prepared with the omission of substrate. Enzyme activity was expressed as µ moles fumarylacetoacetate hydrolyzed min^{-1} $mg\ protein^{-1}$ using an extinction coeffecient of 13,500 $M^{-1}cm^{-1}$ for fumarylacetoacetate.

6) Phosphoenolpyruvate carboxykinase

Phosphoenolpyruvate carboxykinase catalyzes the reversible formation of phosphoenolpyruvate from oxalacetate. The conversion can be assayed spectrophotometrically at 340 mmicrons by the addition of malate dehydrogenase and NADH, which converts oxalacetate to malate with the concomitant oxidation of NADH, to the assay mixture. In order that the consumption of NADH could be observed by an increase in optical density at 340 mmicrons, the position of the experimental and blank cuvettes in the spectrophotometer were interchanged. This automatically corrected for any endogenous oxidation of NADH by the enzyme preparation.

The assay, described by Chang and Lane (1966), contained in

a total volume of 3 ml: 150 μ moles potassium bicarbonate, 300 μ moles imidazole buffer pH 6.6, 20 μ moles sodium floride, 3.75 μ moles ADP, 3.0 μ moles manganese chloride, 6 μ moles glutathione, 0.45 μ moles NADH, 10 μ molar units malate dehydrogenase (1 μ molar unit converts 1 μ mole substrate per minute at pH 7.5, at 25°C), and 3.75 μ moles phosphoenolpyruvate. The blank was prepared by omitting ADP. The assay mixture was preincubated for 10 min. at 25°C and the reaction was started by the addition of phosphoenol-pyruvate. Enzyme activity was expressed as μ moles NADH consummed min^{-1} $mg\ protein^{-1}$ using an extinction coeffecient of 6,220 $M^{-1}cm^{-1}$ for NADH.

7) Enzymatic preparation of fumarylacetoacetate for use in the assay of fumarylacetoacetate hydrolase

a) Partial purification of homogentisate oxidase from rat liver

All procedures were carried out at 0°C. Fifteen grams of rat liver was homogenized by hand in two volumes of Lechninger's buffer pH 7.7 (Lechninger, 1945). The homogenate was centrifuged at 8,000xg for 10 minutes to remove the bulky precipitate and then recentrifuged for 1 hour at 16,000xg. The 16,000xg supernatant was adjusted to pH 7.0 and enough 95% ethanol was added dropwise with constant stirring at 0°C to obtain a final concentration of 32% ethanol. This was then spun down at 10,000xg for 10 minutes and the supernatant, containing maleylacetoacetate isomerase and fumarylacetoacetate hydrolase, was discarded. The precipitate was washed once with 32% ethanol and resuspended in

one-half the volume of distilled water which had been adjusted to pH 7.5. The suspension was then placed in a dialysis tubing (0.719" diameter, 1.21" flat width) and dialyzed for 2 hours at 2-4°C against distilled water with constant stirring with a magnetic stirrer. This enzyme preparation is reportedly stable for several months if frozen at -10°C (Ravdin and Crandal, 1951). Upon thawing, the suspension was centrifuged at 17,000xg for 10 minutes, and the pellet was discarded. The supernatant contained the homogentisate oxidase activity and was used to prepare fumarylacetoacetate.

b) Enzymatic preparation of fumarylacetoacetate

Fumarylacetoacetate was obtained as the silver salt according to the method of Ravdin and Crandal (1951). The mixture was composed of 5 ml of 0.08M sodium carbonate, 5 ml of 0.064M homogentisate and 10 ml of the partially purified rat liver homogentisate oxidase. This was placed in a 125 ml Erlenmeyer flask fitted with a rubber stopper with two pieces of glass tubing. One piece of tubing was attached to a gas cylinder containing 95% oxygen-5% carbon dioxide, the other served as an air outlet so that a continuous stream of oxygen could be passed through the flask during the incubation. The contents of the flask were flushed with oxygen for 6 minutes at room temperature with shaking and then incubated at 38°C in a metabolic shaker for 40 minutes with a continuous passage of oxygen through the flask.

At the end of the incubation, 0.14 volumes of ice-cold 50%

trichloroacetic acid was added to the flask and this was allowed to stand on ice for 30 minutes in order to chemically isomerize maleylacetoacetate, the product of homogentisate oxidation, to fumarylacetoacetate. This was then centrifuged for 10 minutes at 17,000xg and 0.11 volume of a 20% solution of silver nitrate was added to the supernatant. A silver 'proteinate' precipitate was removed by centrifuging at 17,000xg for 10 minutes and ice-cold 4M ammonium hydroxide was added dropwise to the supernatant which was stirred constantly with a magnetic stirrer. As the pH rose, a dark precipitate formed, however, beyond pH 5.5 no further precipitation occurred. The mixture was allowed to stand on ice for 1 hour and was then centrifuged at 17,000xg for 10 minutes. The resulting black precipitate was washed three times with 95% ethanol to remove any traces of silver nitrate, then once with absolute alcohol, and finally with ether. The washed residue was stored overnight in the dark in a dessicator over phosphorous pentoxide at room temperature. The crude silver salt is reported to be stable under these conditions (Ravdin and Crandal, 1951).

When the residue had dried, absorption spectra were taken in 0.1N HCl (pH 1.0) and 0.1N NaOH (pH 13.0) that were characteristic of fumarylacetoacetate i.e. λ max.= 317 mmicrons at pH 1.0, λ max.= 348 mmicrons at pH 13.0.

8) Purification of homogentisate oxidase from Tetrahymena pyriformis and the enzymatic preparation of fumarylacetoacetate

The same procedure was used to purify homogentisate oxidase free of maleylacetoacetate isomerase and fumarylacetoacetate hydrolase from Tetrahymena as was described previously (procedure 7a, pg. 75). For this purpose, 3.5 liters of cells were grown in proteose-peptone medium for 72 hours and harvested (procedure 3, pg. 27). The homogentisate oxidase preparation was shown to be free of isomerase activity and was used for the enzymatic preparation of fumarylacetoacetate as described in procedure 7b, pg. 76. The crude silver salt of fumarylacetoacetate was isolated and gave a very poor spectrum. Therefore, the purification of the compound was carried beyond the steps described for the isolation of fumarylacetoacetate from rat liver as follows (Ravdin and Crandal, 1951).

After the crude salt had dried, 100 mg was weighed out and suspended in 10 ml of distilled water, chilled on ice and 7.5 ml of 0.2N HNO_3 was added dropwise while stirring with a magnetic stirrer. The black precipitate was removed by centrifuging at 8,000xg for 10 minutes and was discarded. To the clear supernatant was added 0.25 ml of saturated NaCl to precipitate the silver ions. Silver chloride was removed by centrifuging at 8,000xg for 10 minutes. The clear supernatant was extracted continuously with 2 volumes of peroxide-free ether for 1 hour by placing the solution in a ground stoppered bottle and laying the bottle on its side in a metabolic shaker. This was then set at a very low

speed so that there was a constant renewal of solvent at the interphase. After the extraction was finished, the ether layer was evaporated down to dryness with a flow of nitrogen at room temperature. The solid was taken up in a small volume of 15% ethanol in ether (V/V) and fumarylacetoacetate was precipitated with petroleum ether while cooling in a solution of ethanol and ice. The yellow-brown precipitate was dried in the dark at room temperature over phosphorous pentoxide for 5 days. At this stage, fumarylacetoacetate is reportedly at least 95% pure (Ravdin and Crandal, 1951). Spectra were taken at pH 1.0 (0.1N HCl) and pH 13.0 (0.1N NaOH).

9) Extraction and purification of ^{14}C-labelled glycogen from cultures of Tetrahymena pyriformis

Glycogen is insoluble in 55% ethanol and therefore, in order to minimize possible losses of glycogen, cells were spun down at 4,300xg for 5 minutes in a mixture of 55% ethanol and all washings of the cell pellet were done in 55% ethanol. Glycogen was extracted by the method of Good et al. (1933), and dialysed (procedure 20, pg. 48). After dialysis, an aliquot was counted in 10 ml of a 10% Biosolv scintillation mixture (procedure 7, pg. 31) contained in disposable scintillation vials.

10) Preparation of the Tetrahymena cell extract for use in the enzyme assays

The washed cell pellet was suspended in 0.2M phosphate buffer pH 7.2 and sonicated using a Bronwill Biosonik III son-

icator. The sonicate was always viewed under a low power microscope to insure that the cells were completely disrupted. The 27,000xg supernatant, obtained after centrifuging for 15 minutes, was used in all the enzyme assays.

11) Protein determination

Protein was determined using the method of Lowry et al. (1951).

RESULTS

Experiment 1

A drop in tyrosine transaminase activity during logarithmic growth of Tetrahymena has been observed (Mavrides and D'Iorio, 1969). It was decided, therefore, to study both phosphoenolpyruvate carboxykinase and tyrosine transaminase activities in detail during this part of the growth phase in order to determine whether both enzymes followed the same course of activity.

One hundred ml of a culture of Tetrahymena pyriformis in proteose-peptone medium was inoculated (procedure 3, pg. 27). Twenty-ml aliquots were removed aseptically every 2 hours between 24 and 32 hours growth of the culture. Five ml of the aliquot was used to determine cell counts (procedure 30, pg. 52), and the remaining 15 ml was harvested (procedure 9, pg. 31) and used for the preparation of the enzyme extract (procedure 10, pg. 79). Protein was determined (procedure 11, pg. 80), and mg protein per 10^6 cells was calculated. The enzyme assays for phosphoenolpyruvate carboxykinase and tyrosine transaminase were performed as described in procedures 1 and 6, pgs. 71 and 74.

The activity of both phosphoenolpyruvate carboxykinase and tyrosine transaminase decreased linearly with the same slope in logarithmic phase (fig. 2-1, pg. 82). A comparison of the slopes of the enzyme activities with the slope of the cell growth in fig. 2-1, i.e. the slope of phosphoenolpyruvate carboxykinase activity was-0.85, for tyrosine transaminase was-0.84, and for cell growth

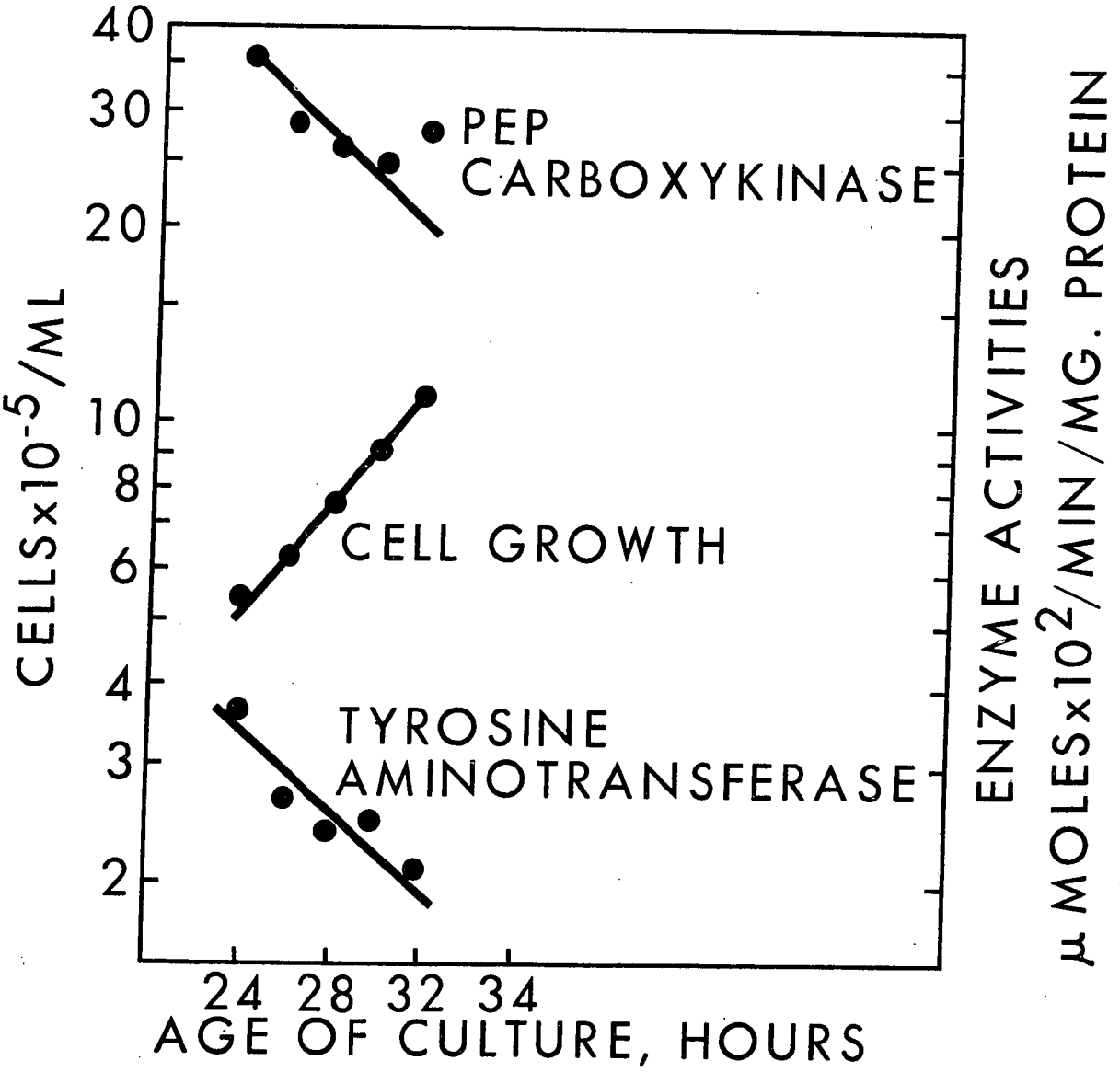


Figure 2-1: Pattern of tyrosine transaminase and phosphoenol-pyruvate carboxykinase activity during logarithmic growth of Tetrahymena cells (exp. 1, pg. 81).

was 1.05, shows that the decrease in enzyme activities is not strictly a dilution of existing enzyme by cell growth. There appears to be some synthesis, although minimal, of these two enzymes during this time of growth. The mg protein per 10^6 cells was constant throughout the course of the experiment (Table 2-1, pg. 84). Therefore, the observed changes were not due to a dilution effect caused by a change in the concentration of intracellular protein.

Experiment 2

Figure 2-1 (pg. 82), demonstrates that both phosphoenolpyruvate carboxykinase and tyrosine transaminase decreased in activity in logarithmic phase. It is known that both these enzymes have high activity in stationary phase (Shrago and Shug, 1966; Mavrides and D'Iorio, 1969). Therefore, by following the pattern of enzyme activity from logarithmic to stationary phase, it would be possible to determine whether phosphoenolpyruvate and tyrosine transaminase activities started to increase at the same time or independently. A single point in experiment 1 (pg. 81) at 32 hours growth for phosphoenolpyruvate carboxykinase activity (fig. 2-1, pg. 82) indicated that phosphoenolpyruvate carboxykinase did in fact increase in activity prior to an increase in tyrosine transaminase activity.

The experimental design was the same as experiment 1 except that 10 ml aliquots of the culture were removed aseptically every 2 hours from 28-34 hours and at 38 hours growth. Five ml was

Table 2-1: Protein content per 10^6 cells determined for experiments 1, 2, and 5. The results represent the mean ± standard error of the individual determinations made for the culture at the time of the enzyme assays.

Experiment number		mg protein/10^6 cells mean ± standard error
1		1.88 ± 0.057
2		1.94 ± 0.047
5 part 1:	Basal	1.15 ± 0.110
	Basal + glucose	1.37 ± 0.090
part 2:	Basal	1.11 ± 0.160
	Basal − acetate	0.94 ± 0.079

used to determine cell counts (procedure 30, pg. 52) and the remaining 5 ml was used to prepare the enzyme extract (cells were harvested and extracted according to procedures 9 and 10 pgs. 31, 79).

A plot of total phosphoenolpyruvate carboxykinase and tyrosine transaminase activity per 5 ml of a Tetrahymena culture sampled between 28-38 hours of growth, shows that phosphoenolpyruvate carboxykinase activity increased 2 hours prior to an increase in tyrosine transaminase activity (fig. 2-2, pg. 86). Two hours represents approximately one-third of the generation time of these organisms (the generation time is approximately 6-7 hours) and is, therefore, a significant difference. The mg protein per 10^6 cells was constant throughout the course of the experiment (Table 2-1, pg. 84).

Experiment 3

Evidence for the existence of the tyrosine-catabolizing pathway in Tetrahymena was indirect. Therefore, assays were established for the remaining four enzymes in the pathway: p-hydroxyphenylpyruvate oxidase, homogentisate oxidase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase.

Various volumes and ages of cultures grown in proteose-peptone medium were used to determine the presence of the tyrosine-catabolizing enzymes. Cells were harvested (procedure 9, pg. 31) and cell extracts were prepared (procedure 10, pg. 79). All assays were carried out spectrophotometrically in a Unicam SP.800 spectrophotometer at 25°C as described under

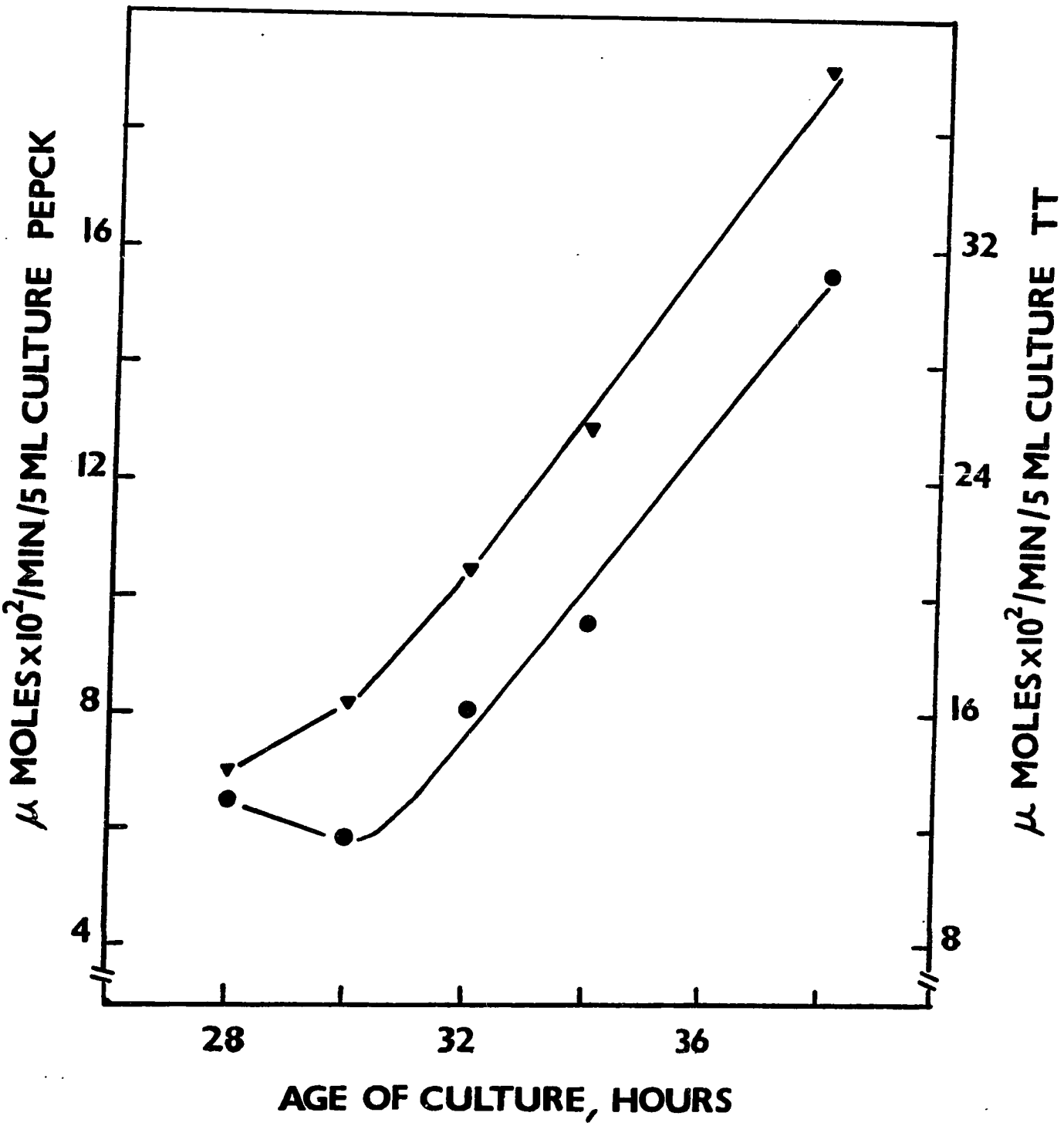


Figure 2-2: Increase in phosphoenolpyruvate carboxykinase (▲) and tyrosine transaminase (●) activities in a Tetrahymena culture during log and post log phases of growth. Activity is expressed as total activity in 5 ml of culture (exp. 2, pg. 83).

experimental procedures: p-hydroxyphenylpyruvate oxidase procedure 2, pg. 71, homogentisate oxidase procedure 3, pg. 72, maleylacetoacetate isomerase procedure 4, pg. 73, fumarylacetoacetate hydrolase procedure 5, pg. 73. Each assay was tested for lineariy with enzyme concentration.

All four enzymes were assayed in Tetrahymena pyriformis proving the existence of the tyrosine-catabolizing pathway in these organisms. A plot of enzyme concentration versus v_o (initial velocity), was linear for each enzyme assayed (fig. 2-3, pg. 88).

Experiment 4

The tyrosine-catabolizing pathway was also detected in Tetrahymena by partially purifying homogentisate oxidase from these organisms and enzymatically forming fumarylacetoacetate.

Seven, three-liter Delong culture flasks containing 500 ml of a proteose-peptone medium were grown for 72 hours (procedure 3, pg. 27). The cells were harvested (procedure 9, pg. 31) and sonicated in Lechninger's buffer pH 7.7 (Lechninger, 1945). Homogentisate oxidase was purified by the procedure 8 described on pg. 78. Fumarylacetoacetate was then prepared enzymatically using this preparation and purified (procedure 8, pg. 78).

Homogentisate oxidase was purified from Tetrahymena pyriformis and used to prepare fumarylacetoacetate from homogentisate. The purified fumarylacetoacetate had a characteristic absorption spectra: pH 1.0 λ max = 317 mmicrons, pH 13.0 λ max = 348 mmicrons (fig. 2-4, pg. 89).

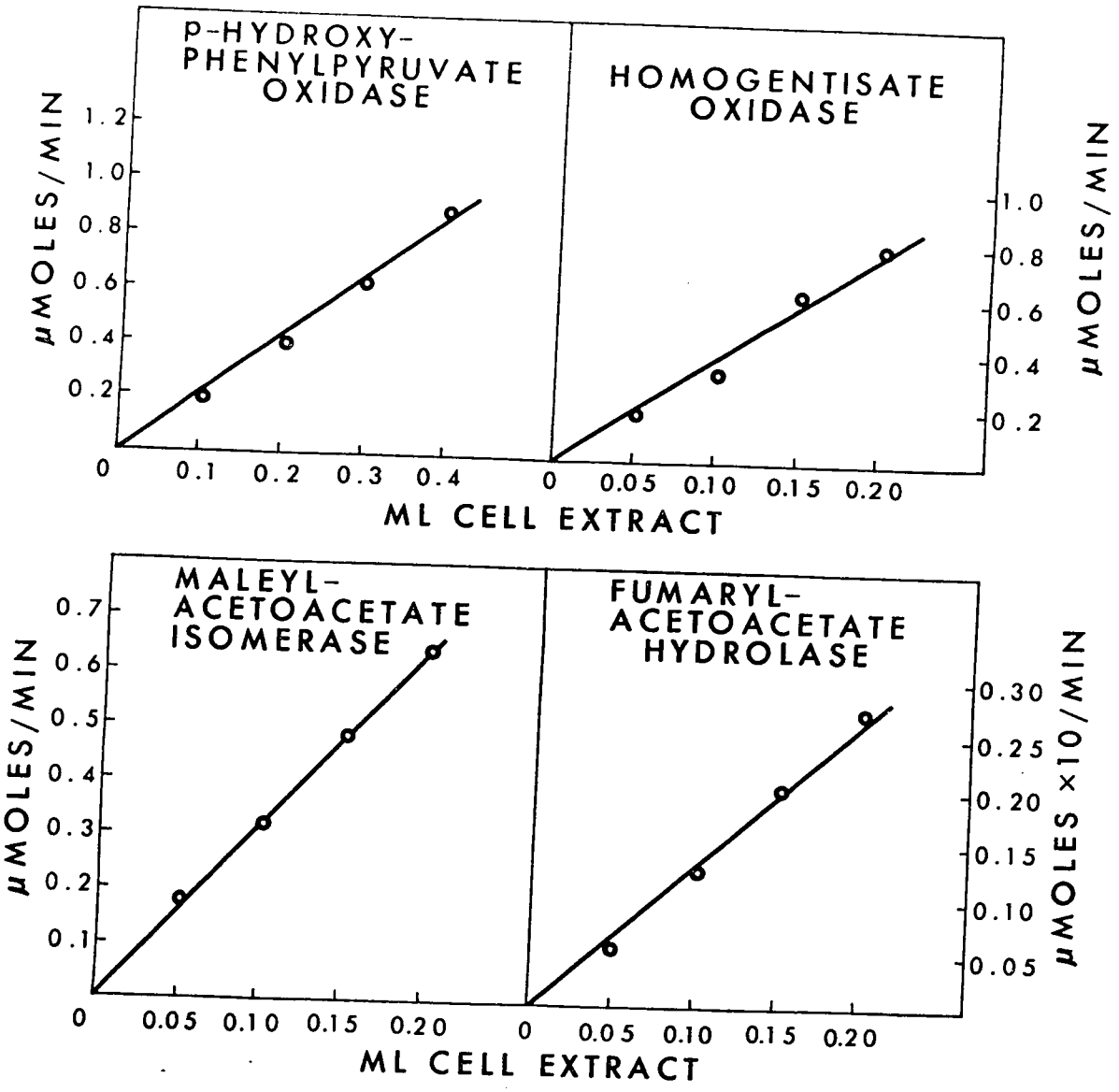


Figure 2-3: Assay of enzymes in the tyrosine-catabolizing pathway in cell extracts of Tetrahymena pyriformis (exp. 3, pg. 85).

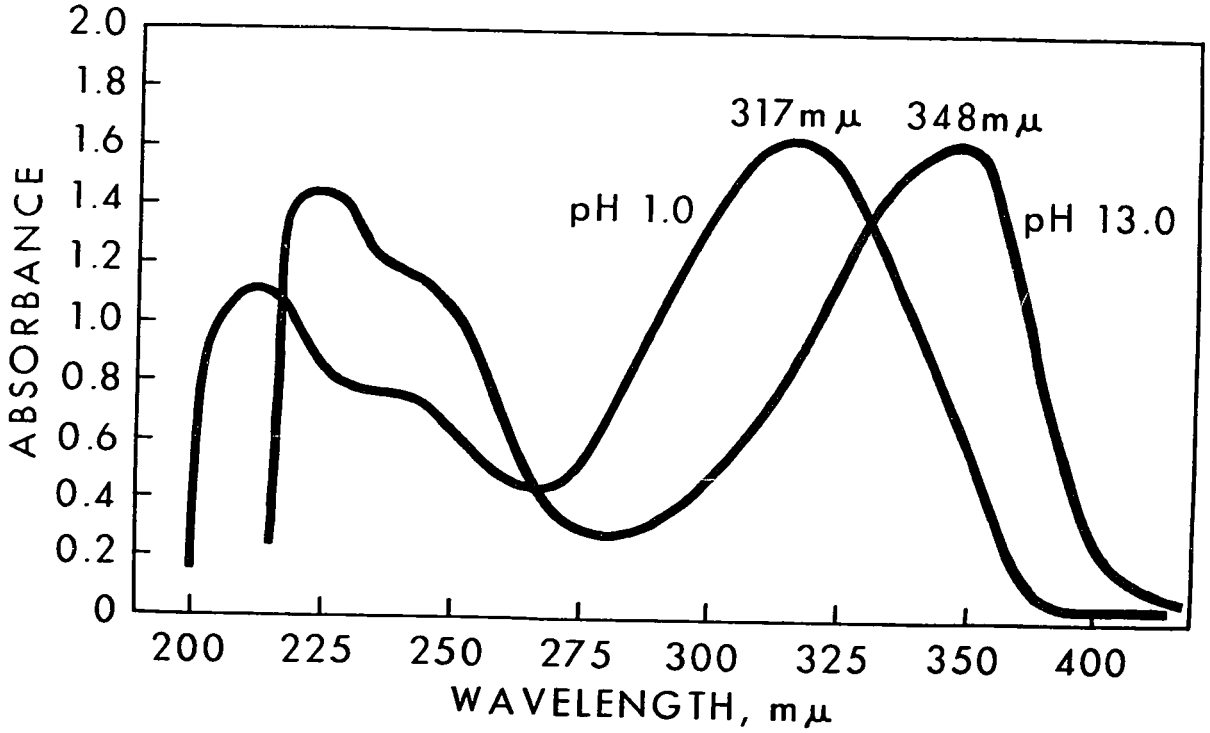


Figure 2-4: Absorption spectra at pH 1.0 and pH 13.0 of fumarylacetoacetate prepared enzymatically with a semi-purified preparation of homogentisate oxidase obtained from Tetrahymena pyriformis (exp. 4, pg. 87).

This corresponded well to the spectra observed by Ravdin and Crandal (1951).

Experiment 5

Once the operation of the tyrosine-catabolizing pathway in Tetrahymena had been established (experiments 3 and 4, pgs. 85, 87), the effects of glucose and acetate on the activity of the tyrosine-catabolizing enzymes and phosphoenolpyruvate carboxykinase were investigated.

The experiment was divided into two parts. In part I, cells were grown in basal medium and in basal medium supplemented with 0.25% glucose. In part II, cells were grown in basal medium and in basal medium lacking acetate. The composition of the basal medium is described on pg. 24 (medium #2).

To obtain an adequate number of cells for the assay of the 5 enzymes of the pathway and phosphoenolpyurvate carboxykinase, from the same culture at one time, it was necessary to harvest a 200 ml culture for each of the 5 determinations made in the experiment between 24 and 72 hours growth of the cultures. Therefore, 5 basal and 5 basal plus 0.25% glucose cultures were required for part I of the experiment, and 5 basal and 5 basal minus acetate cultures for part II. The two parts of the experiment were carried out at different times, however, all 10 flasks of each part were inoculated at the same time from the same parent culture (procedure 3, pg. 27). Five-ml aliquots were taken from each culture at the time of harvesting (procedure 9, pg. 31), and used to obtain cell counts (procedure 30, pg. 52). The re-

mainder of the culture was used to prepare the enzyme extract (procedure 10, pg. 79). Enzyme assays were carried out as described in procedures 1-6, pgs. 71-75. Protein was determined (procedure 11, pg. 80) and mg protein per 10^6 cells was calculated.

A plot of the activities of the tyrosine-catabolizing enzymes and phosphoenolpyruvate carboxykinase from cultures of Tetrahymena grown in basal and basal plus 0.25% glucose is presented in fig. 2-5a,b, pgs. 92, 93, and from basal and basal minus acetate fig. 2-6a,b, pgs. 94, 95. All enzyme activities were repressed in the presence of glucose and acetate. The most prominent repression by glucose was observed in stationary phase (72 hours growth), whereas the greatest repression by acetate was observed in logarithmic and post-logarithmic phases (log phase 24-32 hours growth; post log phase 48-55 hours growth). In stationary phase the enzyme activities in basal and basal minus acetate media tended to converge. The repression of phosphoenolpyruvate carboxykinase activity by acetate appears to be minimal (fig. 2-6b, pg. 95). However, the course of acetate repression of this enzyme and tyrosine transaminase can be seen more clearly in fig. 2-7, pg. 96, where per cent repression by acetate is plotted versus the age of the culture. The maximum repression was observed at approximately 48 hours growth, after which time it decreased.

Using the data from this experiment, plots were constructed relating phosphoenolpyruvate carboxykinase activity to the activity of each enzyme of the tyrosine-catabolizing pathway. The specific activities of phosphoenolpyruvate and an enzyme in the tyrosine

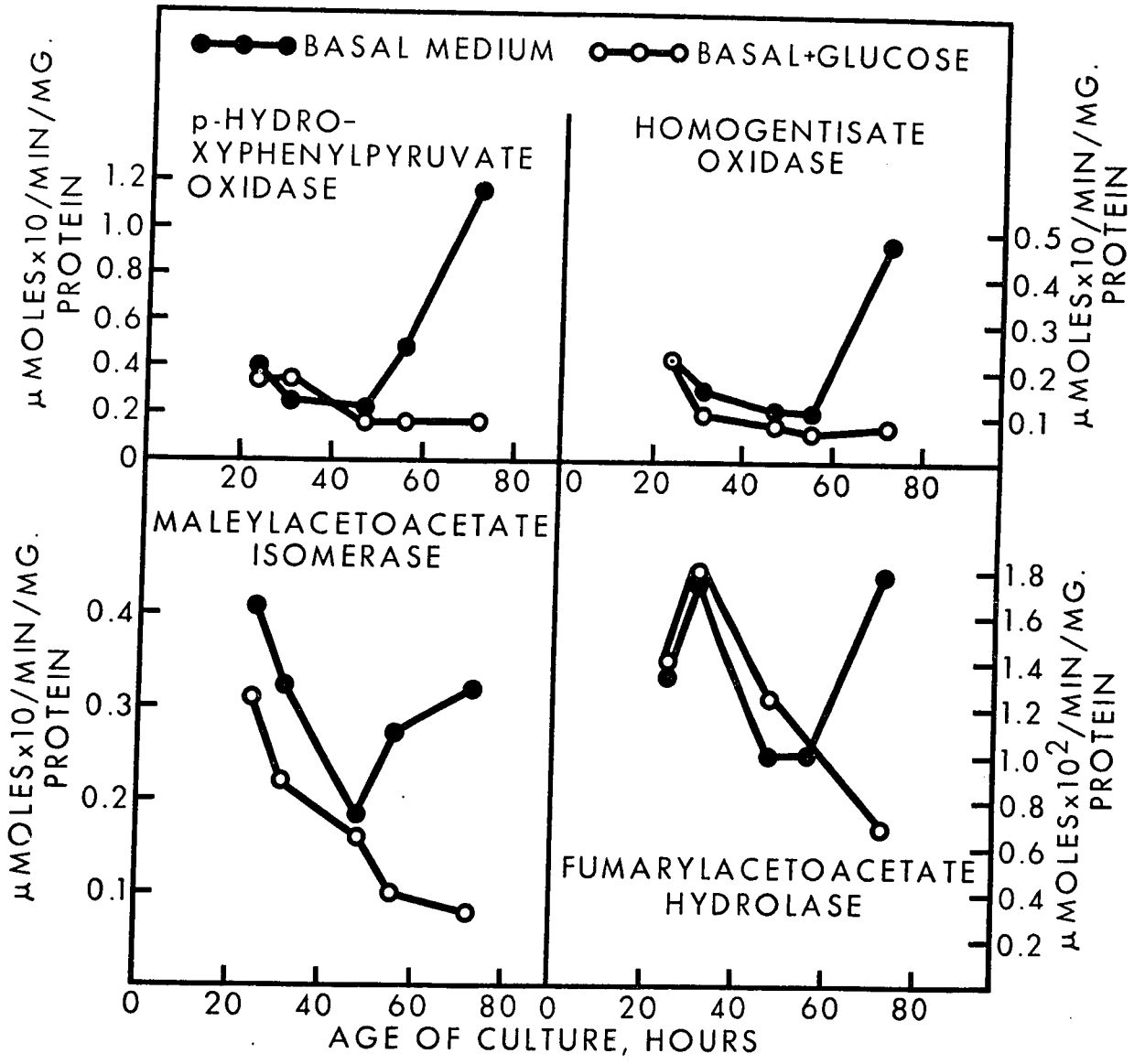


Figure 2-5a: Repression of the activities of four enzymes in the tyrosine-catabolizing pathway in cultures of Tetrahymena pyriformis grown in basal medium containing acetate and glucose compared to cultures grown in basal medium containing acetate (exp. 5, part I, pg. 90).

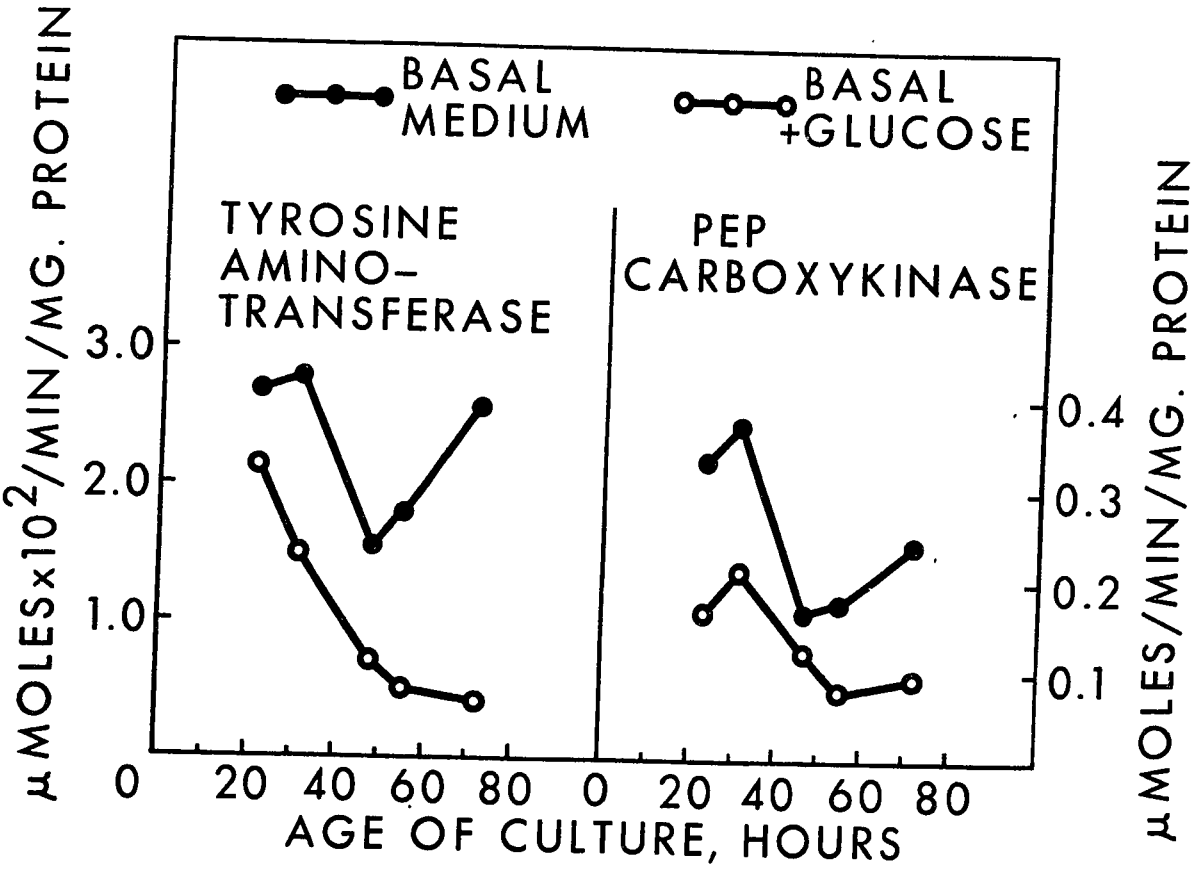


Figure 2-5b: Repression of tyrosine transaminase and phosphoenol-pyruvate carboxykinase activities by glucose in Tetrahymena pyriformis. The activities are compared in cells grown in a basal medium containing acetate, in the presence and absence of glucose (exp. 5, part 1, pg. 90).

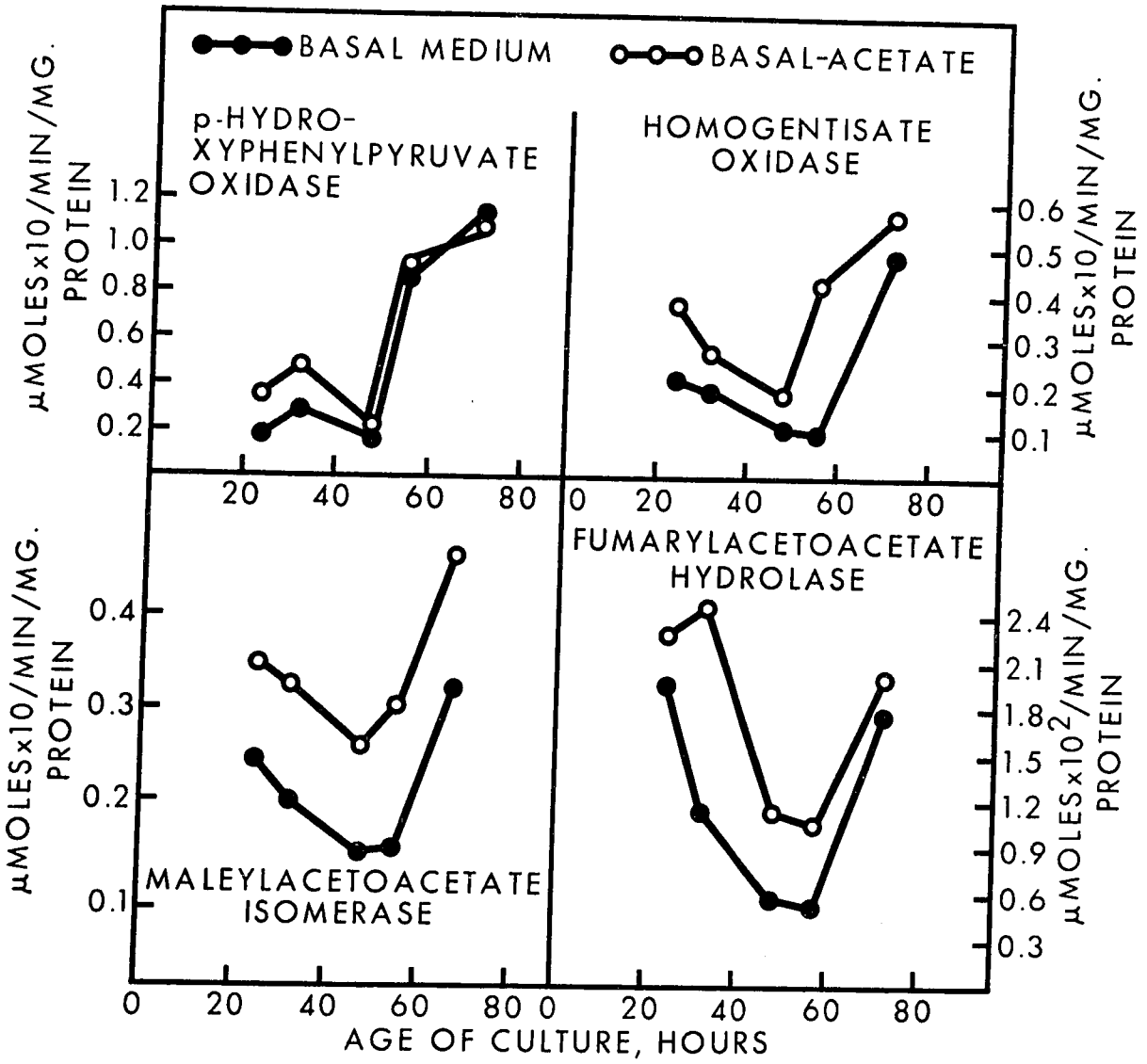


Figure 2-6a: Repression of the activities of four enzymes in the tyrosine-catabolizing pathway in cultures of Tetrahymena pyriformis grown in basal medium containing acetate compared to cultures grown in basal medium lacking acetate (exp. 5, part II, pg. 90).

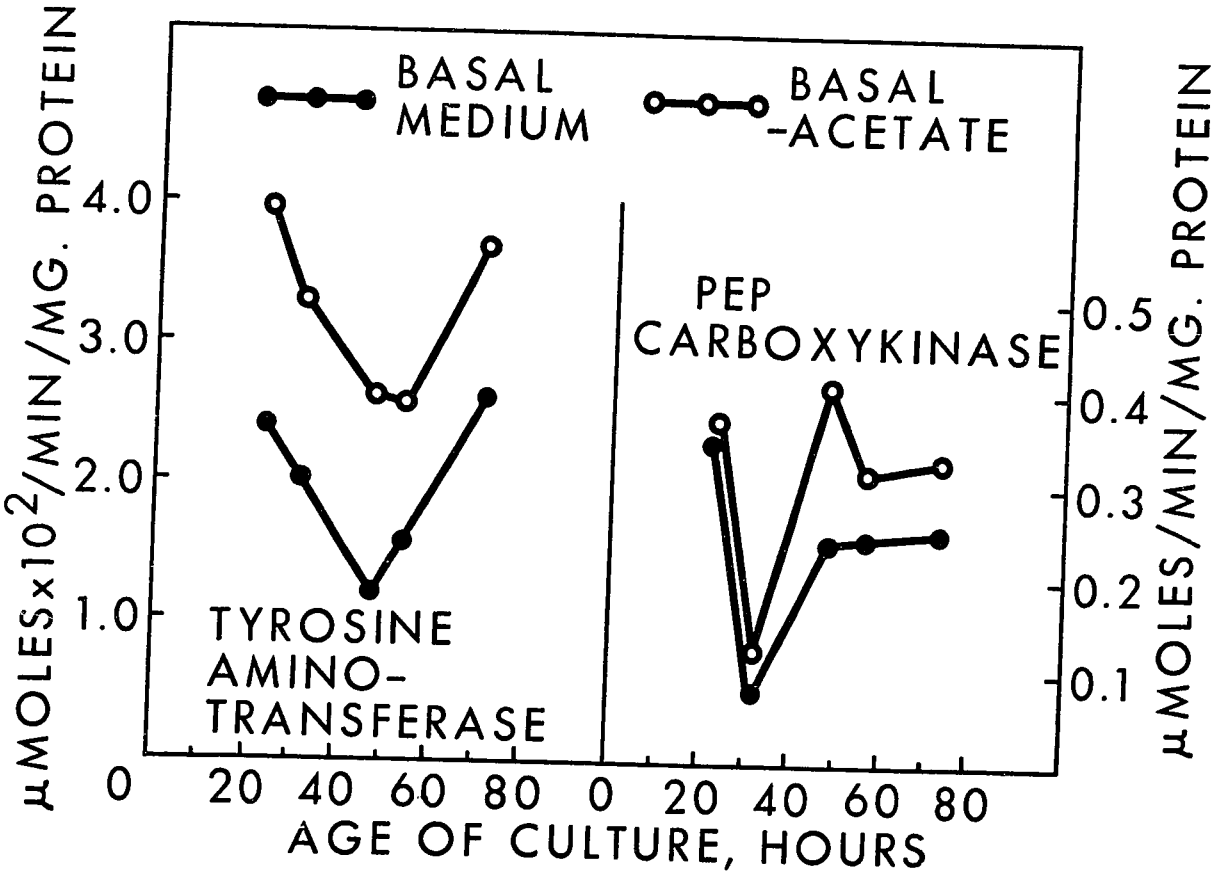


Figure 2-6b: Repression of tyrosine transaminase and phosphoenol-pyruvate carboxykinase activities by acetate in Tetrahymena pyriformis. The activities are compared to cultures grown in a basal medium lacking acetate (exp. 5, part II, pg. 90).

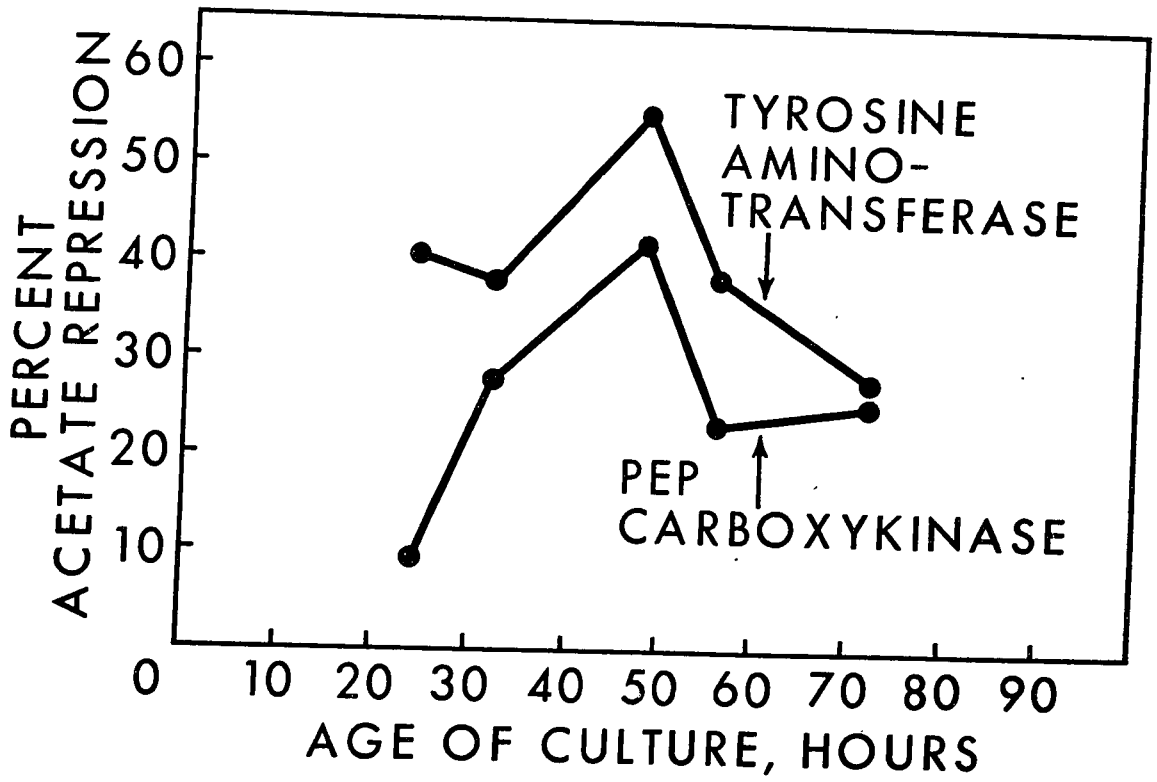


Figure 2-7: The pattern of repression of tyrosine transaminase and phosphoenolpyruvate carboxykinase by acetate, plotted as % repression calculated from activity observed in basal/basal-acetate media. The point of maximum repression for both enzymes occurred in post log phase (around 48 hours, exp. 5, part II, pg. 90).

catabolizing pathway at a given time were plotted against each other (figs. 2-8a,b, pgs. 98, 99). The only plot for which a straight line passing through the origin could be obtained by a least square fit was the plot of tyrosine transaminase activity against phosphoenolpyruvate carboxykinase. The mg protein per 10^6 cells did not vary significantly throughout the course of the experiment (Table 2-1. pg. 84).

Experiment 6

The metabolic significance of the changes in activity of the tyrosine-catabolizing enzymes observed when Tetrahymena were grown in the presence or absence of glucose or acetate was next investigated.

Three 500-ml culture flasks containing 200 ml of basal, basal minus acetate and basal plus 0.25% glucose were inoculated (procedure 3, pg. 27) and allowed to grow for 27 hours. At this time 100 ml of culture was drawn aseptically from each flask. The cells were harvested and washed with sterilized Ryley's ringer phosphate buffer pH 7.0 (described in procedure 26, pg. 50) and then suspended in a small volume of buffer. An aliquot of the cell suspension was taken to determine cell counts (procedure 30, pg. 52) and the volumes of the three suspensions was adjusted so that each contained the same number of cells per ml (for 27 hours growth this was 200,000 cells/ml and for 72 hours growth 2,000,0000/ml). A one-ml aliquot from each adjusted cell suspension was then incubated with 1 μcurie of uniformly labelled ^{14}C-L-tyrosine (specific activity 300 mCi/mmole), for 1 hour

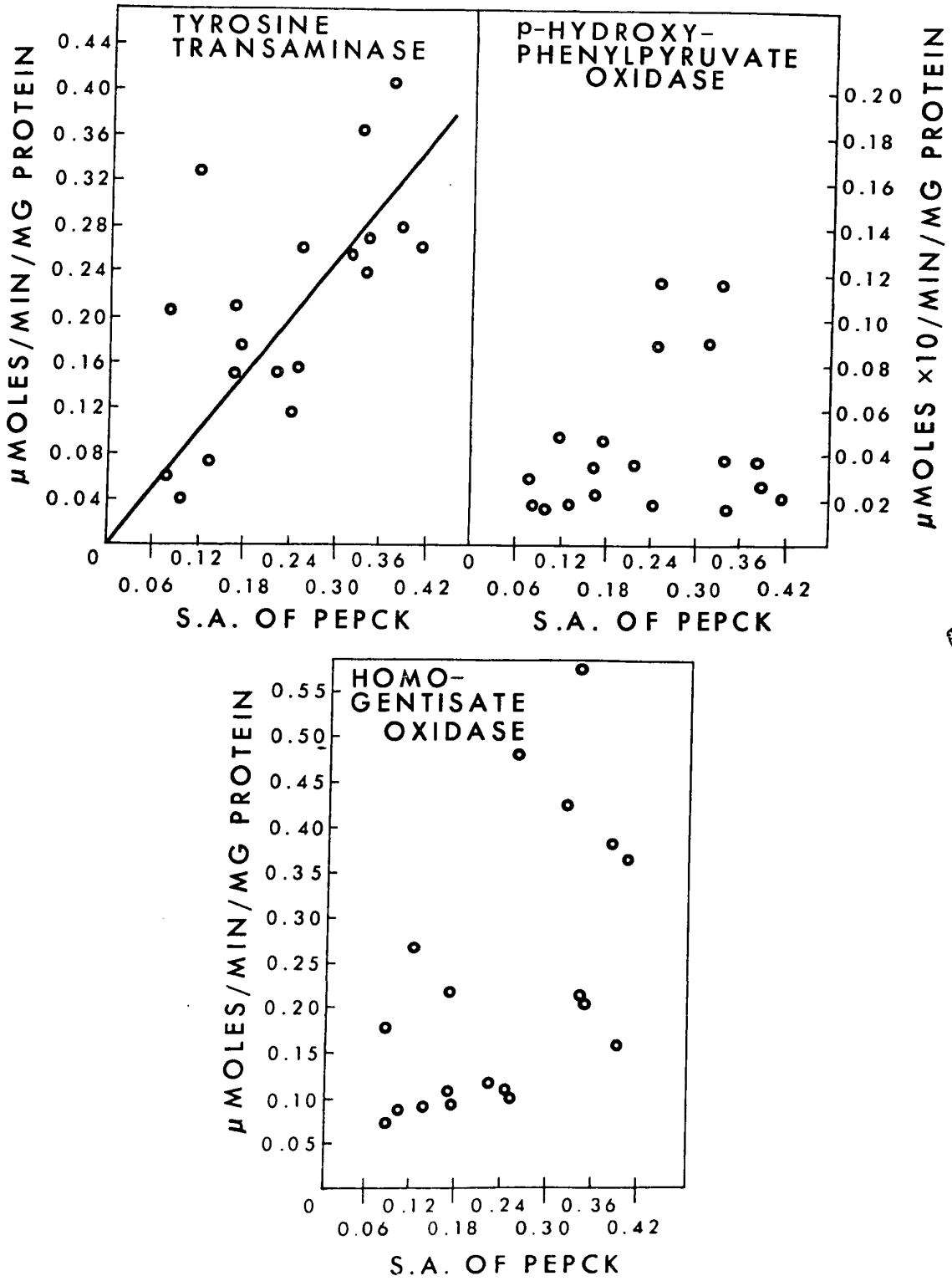


Figure 2-8a: Scatter plots of the specific activity of phosphoenolpyruvate carboxykinase at various times in the life of a Tetrahymena culture, against the corresponding specific activities of tyrosine transaminase, p-hydroxyphenylpyruvate oxidase and homogentisate oxidase. The data was analyzed using the least square fit formulae (exp. 5, pg. 90).

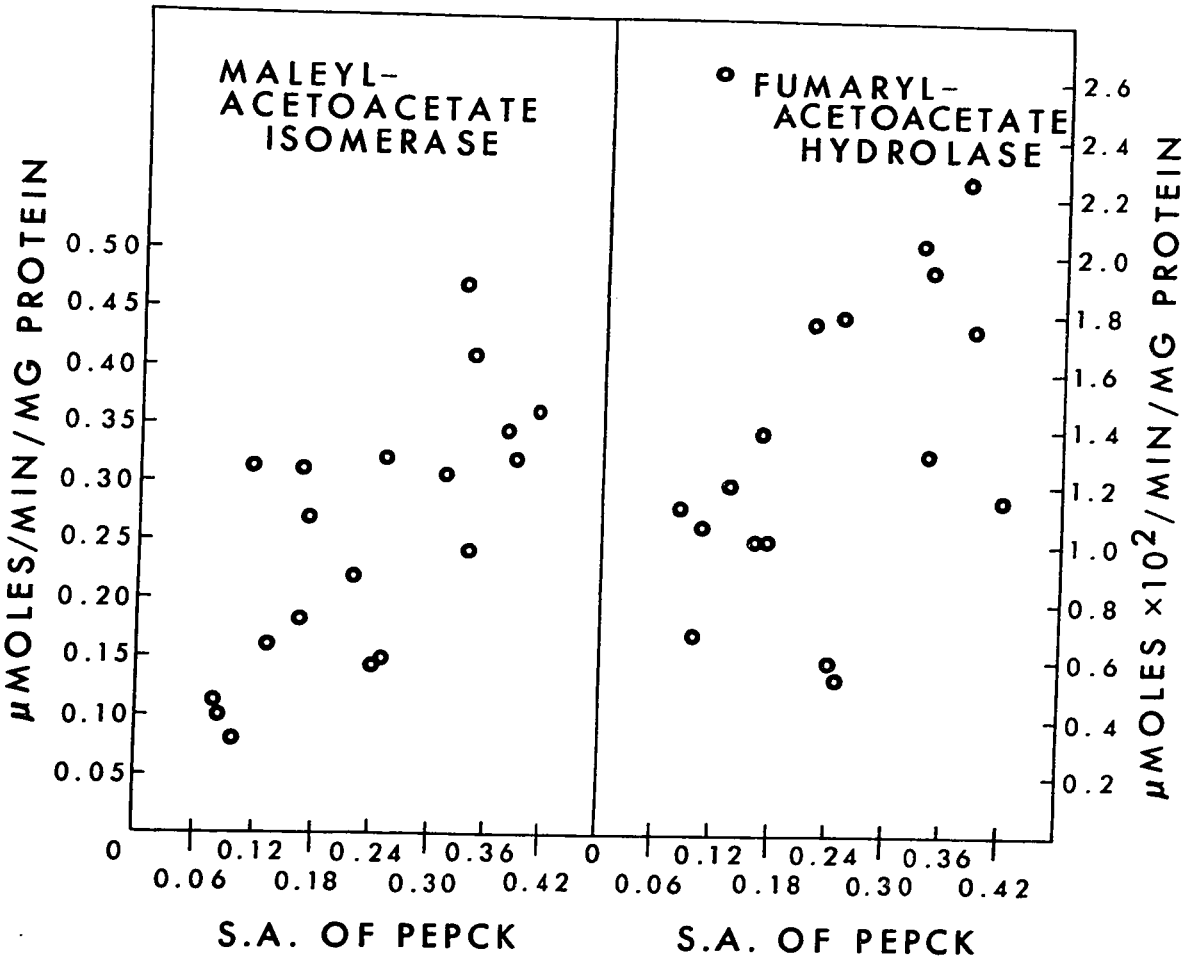


Figure 2-8b: Scatter plots relating the specific activities of maleylacetoacetate isomerase and fumarylacetoacetate hydrolase to the corresponding specific activities of phosphoenolpyruvate carboxykinase (exp. 5, pg. 90).

and glycogen was isolated as described in procedure 9, pg. 79. The remaining 100 ml of the three cultures was allowed to grow to 72 hours at which time the cultures were in early stationary phase and glycogen isolation was repeated. Results were expressed as disintegrations per minute (dpm) tyrosine incorporated into glycogen per 10^6 cells per hour.

The results are presented in Table 2-2, pg. 101. These results, as well as the results of experiment 5 (fig. 2-5a,b, and fig. 2-6a,b, pgs. 92-95), demonstrate that a change in the activity of the tyrosine-catabolizing pathway, as determined by changes in enzyme activity, corresponded to similiar changes in the incorporation of tyrosine carbons into glycogen. Hence, a decrease in activity of the tyrosine-catabolizing pathway in the basal medium compared to the basal minus acetate, was manifested by a lower incorporation of tyrosine carbons into glycogen. The repression by acetate on the flow of tyrosine carbons to glycogen was the same at 27 hours and 72 hours growth while there was a significant increase in the repression of glucose from 27 to 72 hours (at 27 hours the repression was 32.7% and at 72 hours was 58%).

Table 2-2: The incorporation of ^{14}C-L-tyrosine into glycogen by Tetrahymena cells grown in basal, basal minus acetate and basal plus 0.25% glucose in log and stationary phase. The experimental procedure is described in experiment 6, pg. 97.

Age of culture	Phase of growth	Medium	dpm tyrosine ineorporated into glycogen per 10^6 cells per hour	% of basal
27	log	basal	174,335	100.0
		basal minus acetate	322,525	185.0
		basal plus 0.25% glucose	134,790	77.3
72	stationary	basal	34,339	100.0
		basal minus acetate	64,583	188.0
		basal plus 0.25% glucose	14,441	42.0

DISCUSSION

The time courses of tyrosine transaminase and phosphoenolpyruvate carboxykinase activities in Tetrahymena are very similiar. Both enzymes decreased in activity during log phase at the same rate (fig. 2-1, pg. 82) and increased in activity in late log and post log phases (fig. 2-2, pg. 86). An increase in phosphoenolpyruvate carboxykinase activity towards the end of log phase preceded an increase in tyrosine transaminase activity by 2 hours (fig. 2-2, pg. 86). The same sequential rise in the two enzyme activities was also shown in rat liver where the rise of carboxykinase preceded the rise of the transaminase in the daily rhythms of the two enzymes (Lane and Mavrides, 1970). Both enzymes were repressed by glucose and acetate in a similiar fashion in Tetrahymena (figs. 2-5b, 2-6b, pgs. 94, 95). Furthermore, the plots presented in fig. 2-8a,b (pgs. 98, 99) show that tyrosine transaminase was the only enzyme in the tyrosine-catabolizing pathway which demonstrated a close relationship to phosphoenolpyruvate carboxykinase activity. It would seem, therefore, that both enzymes respond to similiar stimuli. Hence, it can be inferred that their functions may be linked metabolically in the process of gluconeogenesis. Evidence for this was obtained by showing that the carbon flow from tyrosine to glycogen varied in accordance with the activities of these two enzymes (Table 2-2, pg. 101.

The presence of the tyrosine-catabolizing pathway in Tetrahymena was clearly established by assaying the enzymes in the pathway (fig. 2-3, pg. 88). Furthermore, one of the enzymes,

homogentsiate oxidase was purified and used to prepare enzymatically an intermediate of the pathway, fumarylacetoacetate, which was characterised by its absorption spectra at pH 1.0 and pH 13.0 (fig. 2-4, pg. 89).

It would appear that regulatory mechanisms are operative for the control of this pathway in Tetrahymena. All enzyme activities were repressed when glucose or acetate was present in the culture medium.

It was shown that all these changes in the activity of the pathway were reflected in corresponding changes in the flow of tyrosine carbon atoms to glyoogen (Table 2-2, pg. 101). Therefore, the effect of acetate and glucose on the functioning of this pathway can be viewed in the context of an effect on gluconeogenesis from tyrosine. Glucose is known to repress gluconeogenesis in Tetrahymena, thus, not only was the activity of the tyrosine-catabolizing pathway repressed in its presence but the activity of phosphoenolpyruvate carboxykinase, a pace-maker enzyme in gluconeogenesis, was also repressed (fig. 2-5b, pg. 93). The effect of acetate on the activity of the tyrosine-catabolizing pathway and phosphoenolpyruvate carboxykinase was more difficult to explain. Shrago, Brech and Templeton (1967), noted that the addition of acetate to a culture of Tetrahymena grown in proteose-peptone for 72 hours, repressed phosphoenolpyruvate carboxykinase activity by approximately 16%. They, however, did not view this as a significant effect and concluded that the carboxykinase activity was not affected by acetate. The repres-

sion by acetate shown in fig. 2-7 (pg. 96), however, was substantial: the repression at 48 hours growth was 40%, and at 56-72 hours was 25%. This pattern has been shown by other experiments to be reproducible.

Mavrides and D'Iorio (1969), speculated that the repression of tyrosine transaminase activity by acetate was compensated for by an increased glyoxylate cycle activity. The effect of acetate on phosphoenolpyruvate carboxykinase activity, however, is not as easily rationalized. The question arises as to why phosphoenolpyruvate carboxykinase activity should be affected by acetate. Whether carbons for glycogen synthesis originate from acetate or amino acids, they must be channeled through phosphoenolpyruvate (Muller et al., 1968). Thus the repression of phosphoenolpyruvate carboxykinase by acetate appears to be incompatible with the presence of an active glyoxylate cycle channeling acetate to glycogen.

The repression of the tyrosine-catabolizing pathway by acetate, suggested there was a relationship between gluconeogenesis from amino acids (tyrosine, and phenylalanine, at least), and gluconeogenesis from acetate. The pathway which is most concerned with acetate metabolism in Tetrahymena and serves the carbon flow from acetate to glycogen, is the glyoxylate cycle (Hogg and Kornberg, 1963). The key enzyme in the glyoxylate cycle is isocitrate lyase (Kornberg and Elsden, 1961). Therefore, the next area of investigation was the relationship between isocitrate lyase, tyrosine transaminase, and phosphoenolpyruvate carboxykinase activities.

SUMMARY FOR CHAPTER 2

1) A close relationship was demonstrated between the activities of phosphoenolpyruvate carboxykinase and tyrosine transaminase.

2) The presence of the tyrosine-catabolizing pathway in Tetrahymena pyriformis was established.

3) The presence of acetate and glucose in the culture medium of Tetrahymena was shown to repress the activity of all the enzymes in the tyrosine-catabolizing pathway and phosphoenolpyruvate carboxykinase.

4) The metabolic significance of the pathway was established by showing that repression of the activities of the tyrosine-catabolizing enzymes was accompanied by a reduction in the incorporation of isotopically labelled tyrosine into glycogen.

CHAPTER 3

Studies on the activities of isocitrate lyase, tyrosine transaminase and phosphoenolpyruvate carboxykinase and their regulation in *Tetrahymena pyriformis*.

INTRODUCTION

The repressive effect of acetate on the tyrosine-catabolizing enzymes (figs. 2-6a,b, pgs. 94, 95), suggested a relationship between the metabolism of acetate and tyrosine in the process of gluconeogenesis. In these organisms, acetate is converted to glycogen via the glyoxylate cycle (Hogg and Kornberg, 1963: Scheme 2, pg. 17a). In fact, the addition of acetate to cultures of E. coli (Kornberg, 1963), Achromobacter (Rosenberger, 1962; Kornberg et al., 1964), and Tetrahymena pyriformis (Hogg and Kornberg, 1963), induces the formation of the key enzyme in the glyoxylate cycle, isocitrate lyase. Levy (1967), showed that isocitrate lyase activity increased in shaken cultures of Tetrahymena pyriformis when stationary phase was induced through anaerobiosis by reducing the surface to volume ratio of the culture and eliminating shaking of an exponentially growing population of cells. The assumption was made, that this represented a true stationary phase and hence the conculsion was drawn that the glyoxylate cycle was most active in stationary phase. Stationary phase, however, is normally determined by a variety of factors, one of which may be anaerobiosis. Other factors, such as a limiting supply of nutrients, pH changes, and a build-up of toxic products in the medium, must also be considered. Therefore, it can be argued that Levy's (1967) induced stationary phase did not represent a normal stationary phase.

The repressive effect of acetate on the activity of the tyrosine-catabolizing system was most pronounced in log phase,

and was relieved as the culture entered stationary phase (figs. 2-6a,b, pgs. 94, 95). A comparison of the patterns of tyrosine transaminase and isocitrate lyase activities in Tetrahymena pyriformis grown in a defined medium containing acetate should serve as a useful system for the study of acetate and tyrosine metabolism. From the results of the previous chapter, it appeared likely that acetate, when present in the medium, might be used preferentially for the synthesis of glycogen. The increased flow of carbon from tyrosine to glycogen in the absence of acetate might, therefore, be compensating for a reduced supply of carbon from acetate for glycogen synthesis.

If an inverse relationship between the activities of isocitrate lyase and tyrosine transaminase existed, as was suggested by the findings in Chapter 2, addition of acetate to the culture medium of Tetrahymena when cells were in stationary phase and tyrosine transaminase activity was high, should result in a repression of tyrosine transaminase and stimulation of isocitrate lyase activities.

The repressive effect of acetate on the activity of phosphoenolpyruvate carboxykinase, also deserved closer study. Therefore, the relationship between the activities of phosphoenolpyruvate carboxykinase, tyrosine transaminase, and isocitrate lyase, was also investigated.

EXPERIMENTAL PROCEDURES

1) Preparation of cell extract for use in enzyme assays

Cell extracts were prepared immediately before their use from washed cells (procedure 9, pg. 31) due to the instability of isocitrate lyase (Levy and Wasmuth, 1970). Cells were sonicated in ice-cold 0.25M sucrose with a Biosonik III sonicator. The disruption of the cells was checked with a low power microscope. The whole sonicate was used to assay isocitrate lyase and the 27,000xg supernatant was used for the assay of tyrosine transaminase and phosphoenolpyruvate carboxykinase. All preparations were carried out at 0-4 °C.

2) Protein determinations

Protein was determined for the whole sonicate to calculate the specific activity of isocitrate lyase and for the 27,000xg supernatant for the calculation of the specific activities of tyrosine transaminase and phosphoenolpyruvate carboxykinase by the method of Lowry et al. (1951).

Enzyme assays

3) Isocitrate lyase

Isocitrate lyase catalyzes the reversible formation of glyoxylate and succinate from L_s-isocitrate.

The principle of the assay depends upon an increase in optical density at 324 mmicrons due to the formation of glyoxylate phenyl-

hydrazone. The assay method was described by Dixon and Kornberg (1959) and contained in one ml: 67 μ moles potassium phosphate buffer pH 6.85, 5 μ moles magnesium chloride, 3.3 μ moles phenylhydrazine·HCl, 2 μ moles cysteine·HCl and 1.7 μ moles L_s-isocitrate. Isocitrate was omitted from the blank. The reaction was started by the addition of Ls-isocitrate to the experimental cuvette after a steady optical density reading was obtained at 324 mmicrons. Specific activities were expressed as μ moles glyoxylate formed min^{-1} mg $protein^{-1}$ using an extinction coefficient of 17,000 $M^{-1}cm^{-1}$ for the hydrazone.

RESULTS

Experiment 1

The first experiment was designed to determine the course of tyrosine transaminase and isocitrate lyase activities during logarithmic and stationary phase of Tetrahymena pyriformis grown in three different media: basal, basal minus acetate, and basal plus 0.25% glucose (composition of the basal medium is described on pg. 24 (medium #2).

Five hundred ml cultures of each medium were grown in 3-liter Delong culture flasks (procedure 3, pg. 27). Tyrosine transaminase and phosphoenolpyruvate carboxykinase were assayed every 3 hours in log phase and approximately every 24 hours thereafter. Twenty-five ml aliquots were drawn aseptically for each point. Five ml of the aliquot was used to determine cell counts (procedure 30, pg. 52) and the remaining 20 ml was harvested (procedure 9, pg. 31) and used to prepare the cell extract (procedure 1, pg. 109) for use in the enzyme assays.

Isocitrate lyase is a particulate enzyme and, therefore, 0.1 ml of the whole sonicate (procedure 1, pg. 109) was used to assay the enzyme in 1-ml cuvettes as described in procedure 3, pg. 109. Tyrosine transaminase was assayed in 3-ml cuvettes as described in procedure 1, pg. 71, using 0.1 ml of the 27,000xg supernatant of the cell extract. The mg protein per 10^6 cells was calculated throughout the course of the experiment.

The pattern of isocitrate lyase activity in basal, basal

minus acetate, and basal plus 0.25% glucose, is presented in fig. 3-1, pg. 113. The same pattern of activity was observed in all three media, i.e., the greatest activity was found in log phase and the lowest was found in stationary phase. Under the conditions of this experiment, the cells were still in log phase at 33 hours growth.

The pattern of tyrosine transaminase activity in these three media was not the same (fig. 3-2, pg. 114). Tyrosine transaminase reached a very high level of activity during log phase in basal minus acetate medium, whereas in basal medium it did not increase significantly until much later in stationary phase (approximately 80 hours growth). In both basal and basal minus acetate media, the characteristic drop in tyrosine transaminase activity during log phase was observed. In the presence of glucose (0.25%), the enzyme activity was repressed. The results for tyrosine transaminase activity corresponded to those reported by Mavrides and D'Iorio (1969).

A comparison of figs. 3-1 (pg. 113) and 3-2 (pg. 114) shows that when isocitrate lyase activity was high during log phase in basal medium, tyrosine transaminase was low and, when the lyase activity dropped during stationary phase, tyrosine transaminase activity rose. In basal minus acetate medium, the lyase activity was considerably lower compared to the basal medium (less than 50% of the activity in basal medium) whereas tyrosine transaminase activity was considerably higher in basal minus acetate medium (approximately 100% increase in activity compared

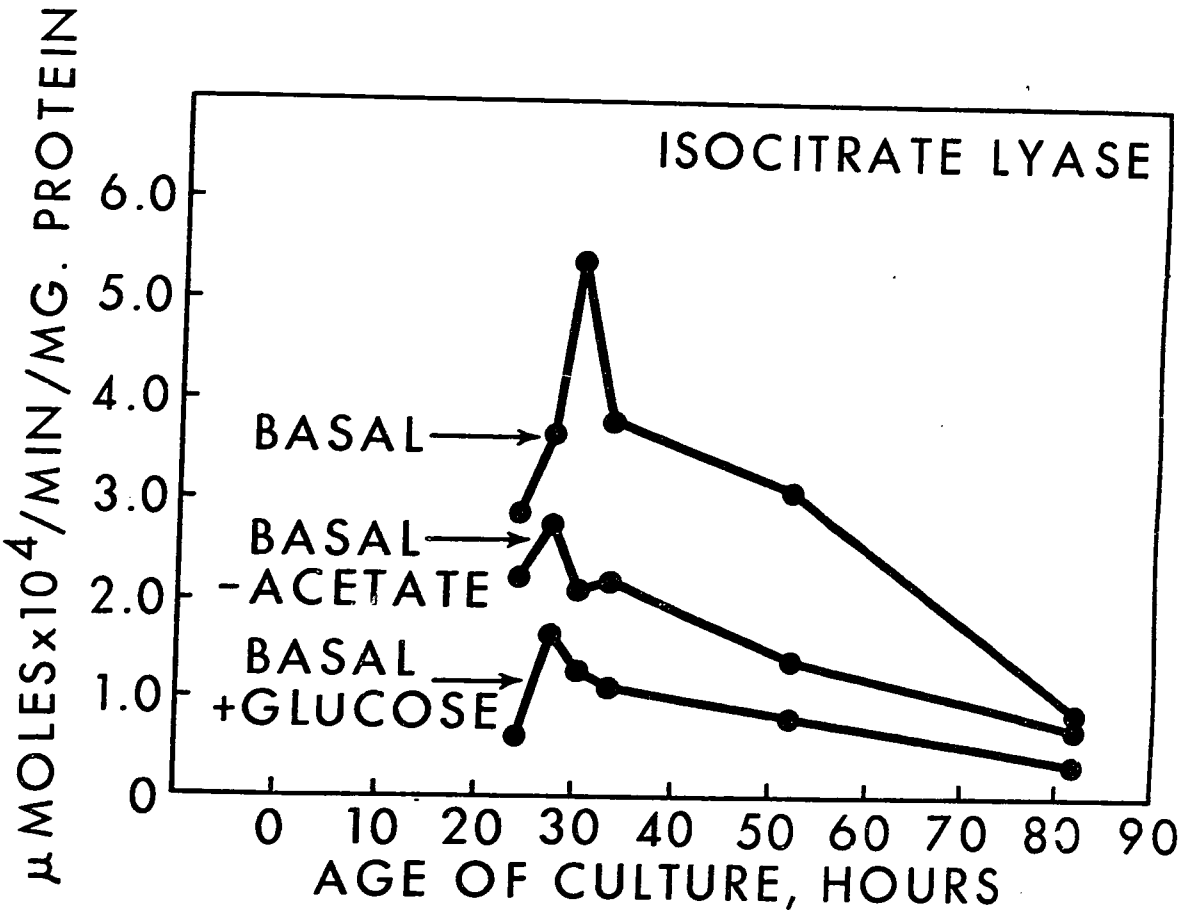


Figure 3-1: Pattern of isocitrate lyase activity in cultures of Tetrahymena pyriformis grown in a basal medium containing acetate, a basal medium lacking acetate, and a basal medium containing acetate and 0.25% glucose (exp. 1, pg. 111).

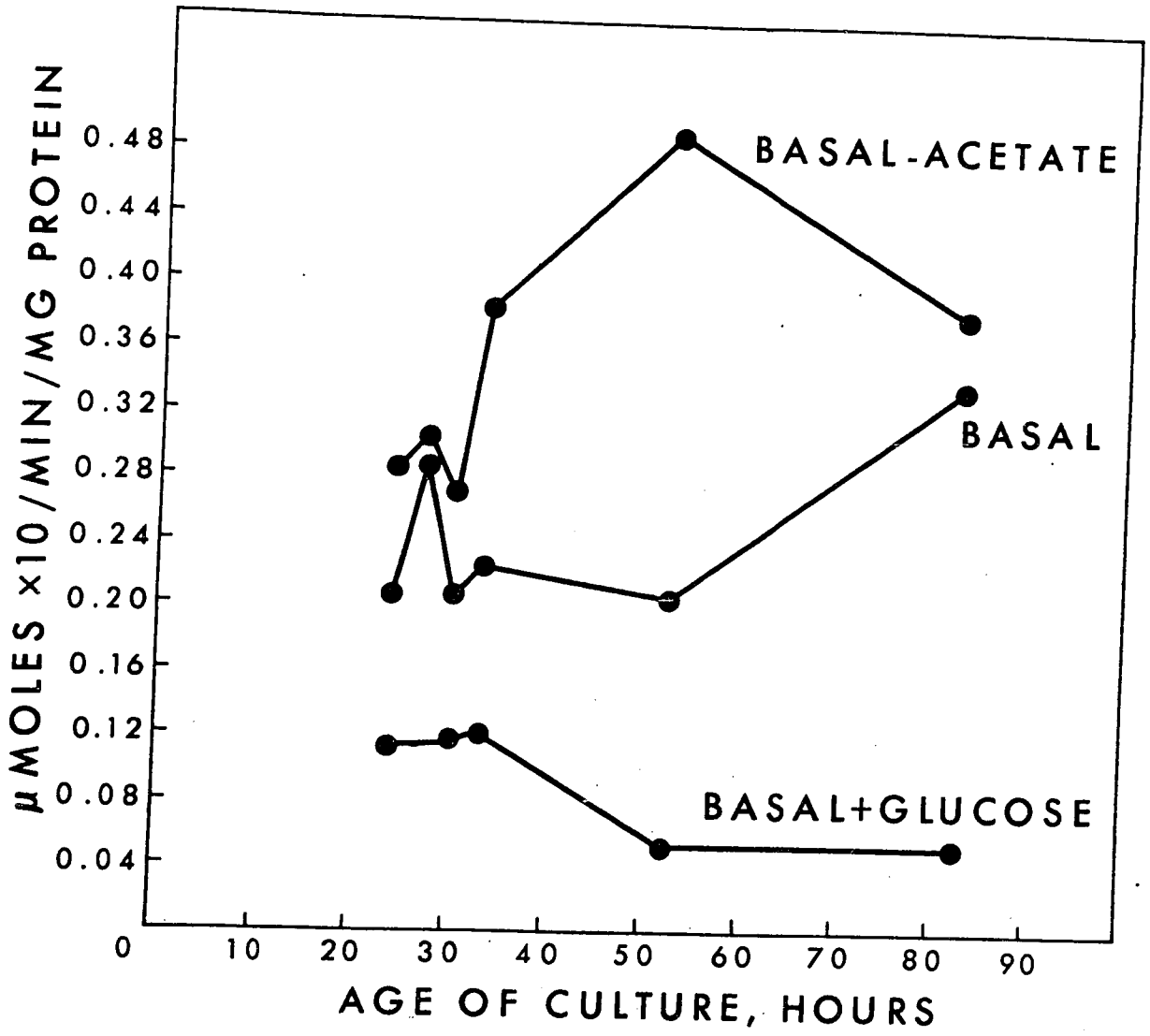


Figure 3-2: Pattern of tyrosine transaminase activity in cultures of Tetrahymena pyriformis grown in a basal medium containing acetate, a basal medium lacking acetate, and a basal medium containing acetate and 0.25% glucose (exp. 1, pg. 111).

to the basal medium).

The protein content per 10^6 cells was constant throughout the course of the experiment (Table 3-1, pg. 116).

Experiment 2

Most of the studies by other investigators (Hogg and Kornberg, 1963; Levy, 1967; Levy and Scherbaum, 1965a, 1965b) on isocitrate lyase activity in Tetrahymena, have been done using a proteose-peptone medium for growth of the cells. Therefore, isocitrate lyase was assayed in cultures grown in proteose-peptone and basal medium to determine whether the pattern of enzyme activity was the same for both cultures. It was conceivable that in a proteose-peptone medium, the glyoxylate cycle would be more active in stationary phase than in log phase as has been reported (Hogg and Kornberg, 1963).

The experimental procedure was exactly the same as described in experiment 1, pg. 112, except that only isocitrate lyase activity was assayed.

The pattern of enzyme activity was the same for both cultures in log phase where a peak in activity was observed. However, a high level of activity was maintained in the proteose-peptone culture after the initial burst, whereas a rapid decline of activity occurred in the basal medium (fig. 3-3, pg. 117).

Table 3-1: Protein content per 10^6 cells determined for experiments 1 and 2. The results represent the mean ±standard error of individual determinations made throughout the course of the experiments.

Experiment number	mg protein/10^6 cells mean ± standard error
1 basal	0.612± 0.042
basal minus acetate	0.605± 0.051
basal plus 0.25% glucose	0.725± 0.095
2 basal (control)	0.923± 0.074
basal	0.716± 0.052

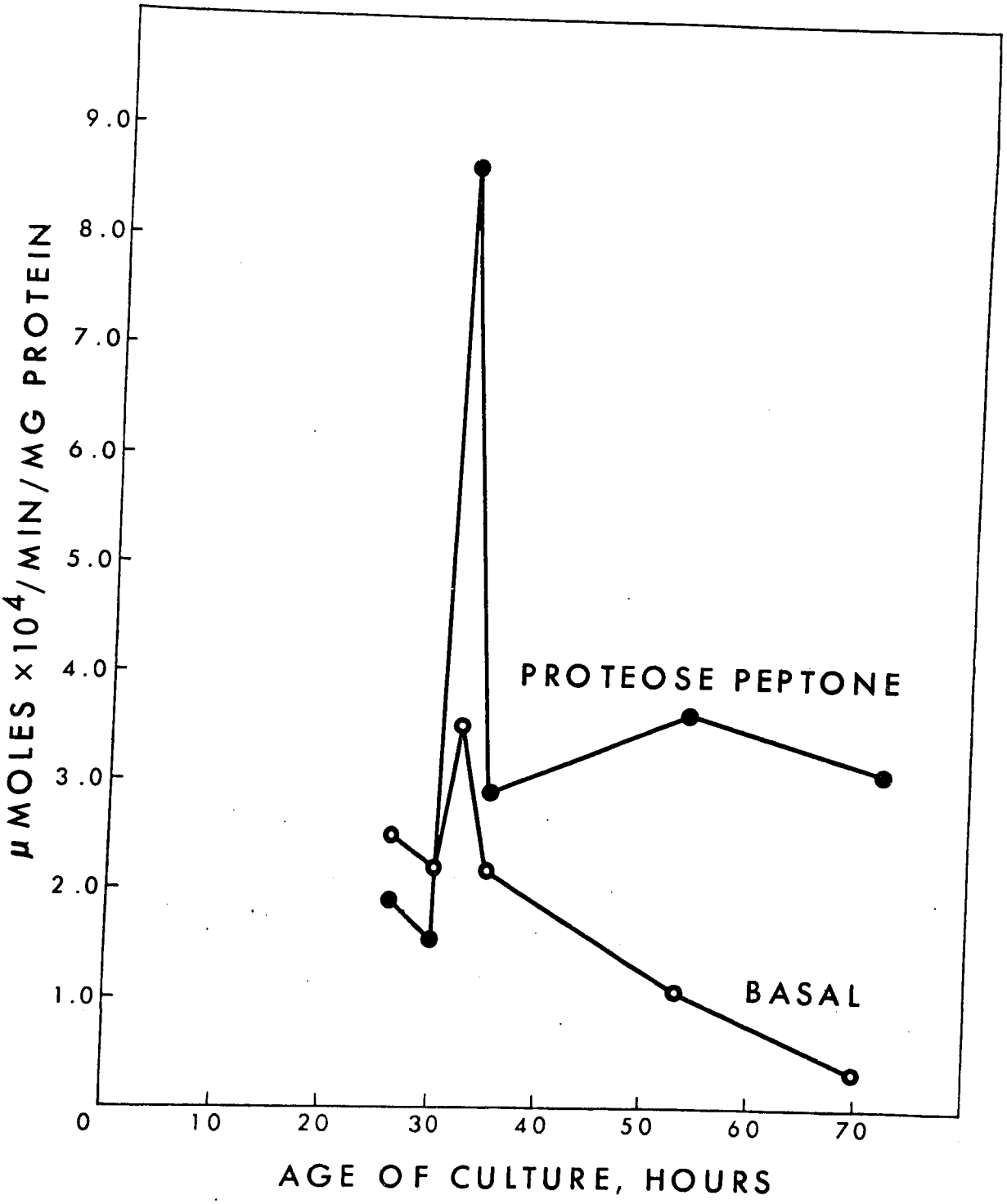


Figure 3-3: Comparison of the course of isocitrate lyase activity in cultures of Tetrahymena grown in a basal and a proteose-peptone medium (exp. 2, pg. 115).

Experiment 3

From the results of experiment 1, it was obvious that isocitrate lyase and tyrosine transaminase activities were inversely related i.e., when tyrosine transaminase was high, isocitrate lyase was low. Since phosphoenolpyruvate carboxykinase activity followed the same pattern as tyrosine transaminase (figs. 2-5b, 2-6b, pgs. 93, 95), it followed that phosphoenolpyruvate carboxykinase activity would also be inversely related to the lyase activity.

Hence, the next logical step was to assay phosphoenolpyruvate carboxykinase, in addition to tyrosine transaminase and isocitrate lyase. Furthermore, since the removal of acetate from the basal medium allowed more tyrosine transaminase synthesis, it was reasoned that a second addition of acetate during cell growth to the basal medium, when tyrosine transaminase activity was high and isocitrate lyase was low, should result in the induction of lyase and the repression of tyrosine transaminase.

In this experiment, therefore, cells were grown in 2 flasks (3 l.) each containing 500 ml of basal medium (procedure 3, pg. 27). One culture was designated as a control, to which acetate would not be added. Twenty-five ml aliquots were drawn aseptically from both cultures at approximately 24 hour intervals before the addition of acetate (500 mg/400 mls culture medium). Twenty ml was harvested (procedure 9, pg. 31) and used for the preparation of the cell extract (procedure 1, pg. 109), and the remaining 5 ml was used to determine cell counts (procedure 30, pg. 52).

Isocitrate lyase (procedure 3, pg. 109), phosphoenolpyruvate carboxykinase (procedure 6, pg. 74), and tyrosine transaminase (procedure 1, pg. 71) were assayed every 24 hours to determine the point at which isocitrate lyase activity was low and tyrosine transaminase activity was high (97 hours growth). At this point 500 mg of acetate in solution was added to the experimental culture (500 mg acetate/400 ml culture medium). Twenty-five ml aliquots were then drawn 3 hours and 6 hours after the addition and all three enzymes were assayed. A final assay was taken at 28 hours after the addition of acetate (124 hours of growth). Protein content per 10^6 cells was determined for both cultures throughout the course of the experiment.

The pattern of phosphoenolpyruvate carboxykinase, tyrosine transaminase, and isocitrate lyase activities during growth of Tetrahymena pyriformis in basal medium is given in fig. 3-4, pg. 120. This data was taken from the control flask in the experiment. In this experiment, 24 hours growth represented log phase, 50 hours represented early post log phase, and stationary phase was reached by 80 hours.

Fig. 3-4, pg. 120 establishes that an inverse relationship exists between tyrosine transaminase and phosphoenolpyurvate carboxykinase activities and isocitrate lyase. Furthermore, it can be inferred that in basal medium, the glyoxylate cycle is most active in log and post log phases whereas tyrosine transaminase and phosphoenolpyruvate carboxykinase are most active

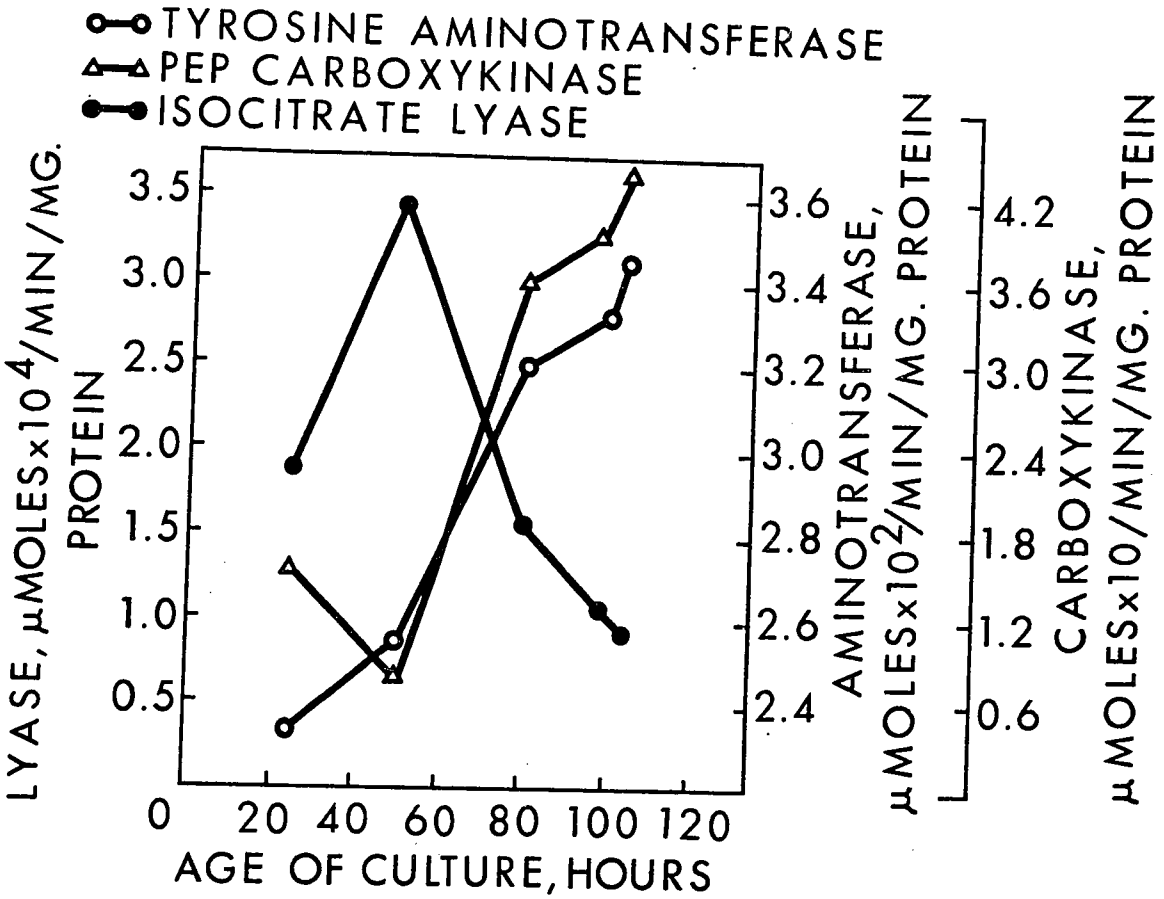


Figure 3-4: Demonstration of an inverse relationship between phosphoenolpyruvate carboxykinase and tyrosine transaminase activities and isocitrate lyase activity in Tetrahymena pyriformis. Results are taken from the control flask in exp. 3, pg. 118.

active in stationary phase indicating that the tyrosine-catabolizing pathway is most active at this time.

Acetate was added to the experimental flask when the culture was 97 hours old (stationary phase). At this time tyrosine transaminase activity was high and isocitrate lyase activity was low (fig. 3-4, pg. 120). Subsequent assays showed that within 3 hours, there was a 50-60% increase in lyase activity over the control flask (fig. 3-5, pg. 122), and a 30% drop in tyrosine transaminase activity (fig. 3-6, pg. 123). Twenty-eight hours later, there was a 260% increase in lyase activity over the control and a 50% drop in tyrosine transaminase activity. The pattern of phosphoenolpyruvate carboxykinase activity did not change upon the addition of acetate at this stage of growth (fig. 3-7, pg. 124).

Twenty-eight hours after the addition of acetate to the experimental flask, there was a 20% increase in cell population over the control which was still in stationary phase (124 hours growth), proving that the acetate was metabolised.

The protein content per 10^6 cells did not vary significantly during the course of the experiment (Table 3-1, pg. 116).

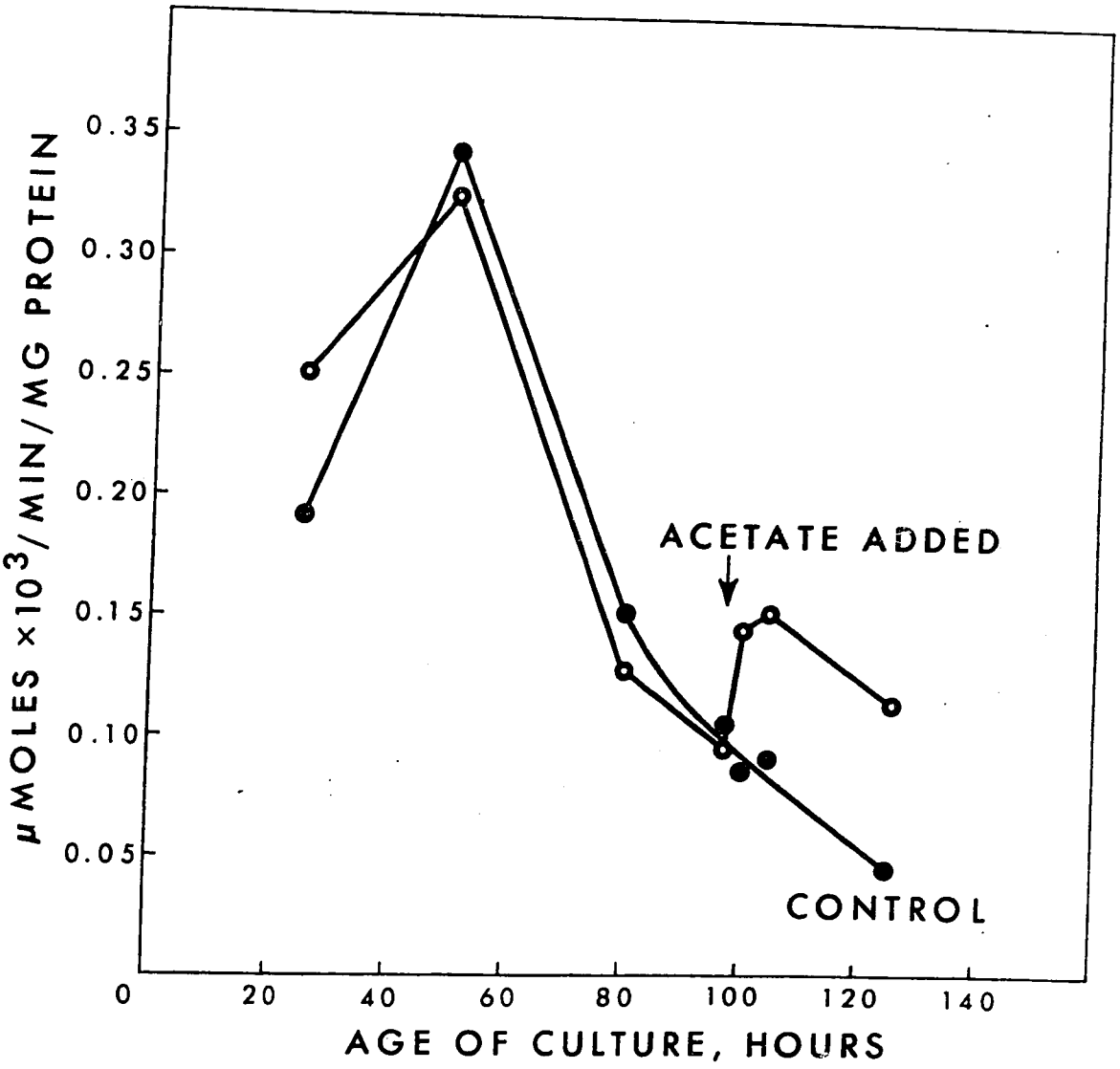


Figure 3-5: The induction of isocitrate lyase by the addition of acetate (500 mg acetate/400 ml culture) to a stationary culture of Tetrahymena pyriformis grown in basal medium. The pattern of activity is compared to a control culture to which acetate was not added (exp. 3, pg. 118).

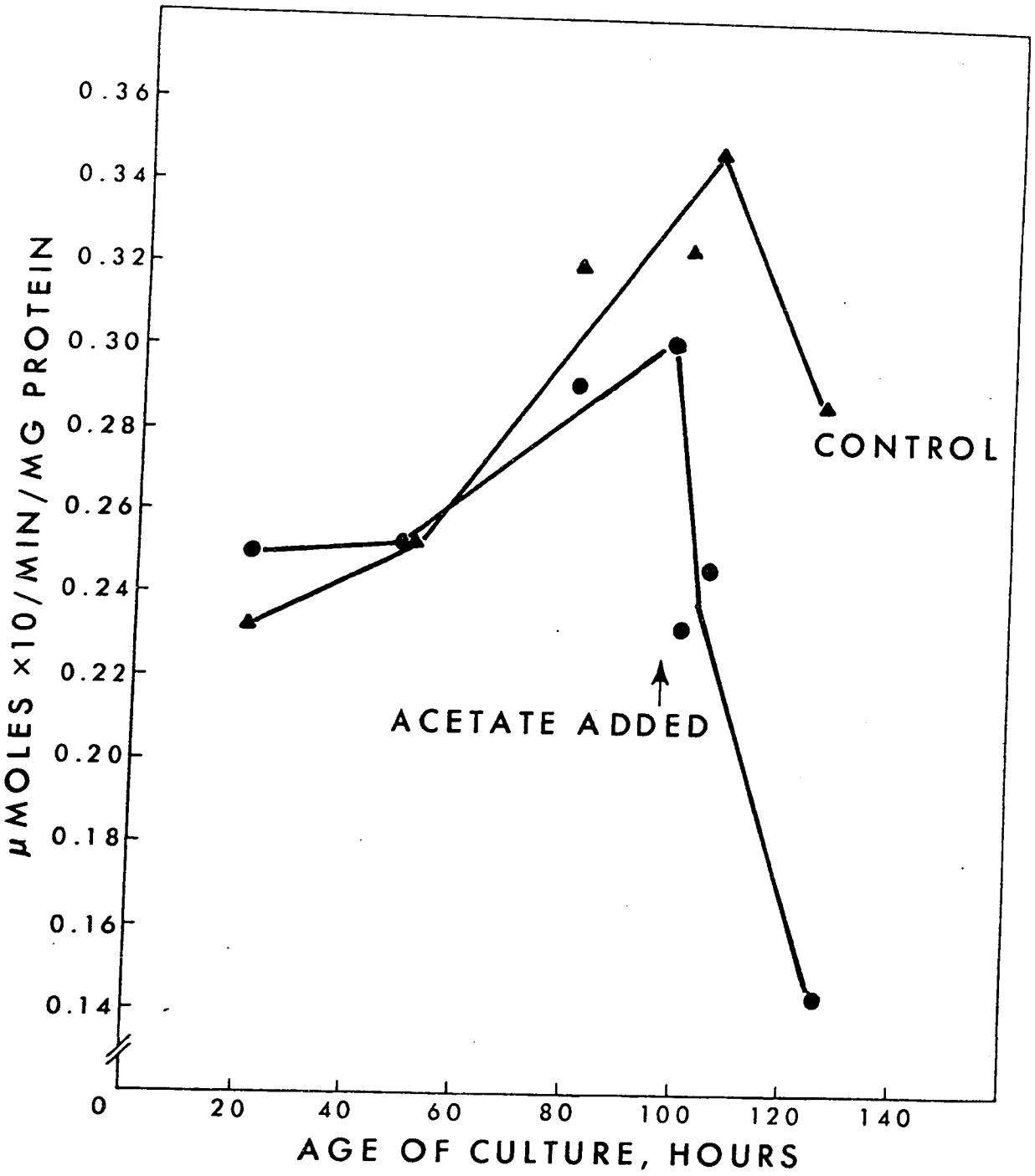


Figure 3-6: The repression of tyrosine transaminase by the addition of acetate (500 mg acetate/400 ml culture) during stationary phase to a culture of Tetrahymena pyriformis grown in basal medium. The pattern of activity is compared to a control culture to which acetate was not added (exp. 3, pg. 118).

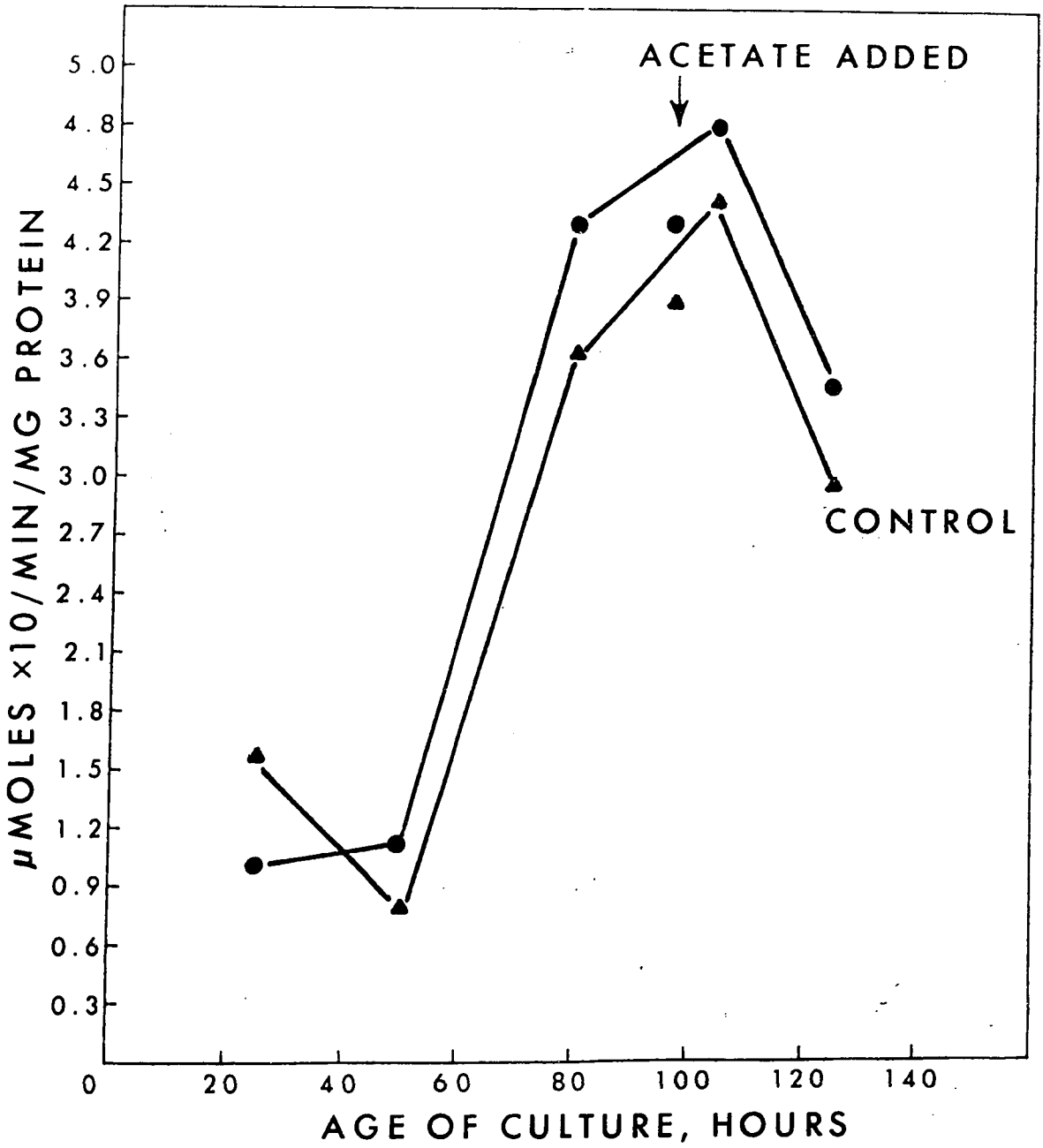


Figure 3-7: The pattern of phosphoenolpyruvate carboxykinase in basal medium during the growth of Tetrahymena pyriformis. Acetate (500 mg acetate/400 ml culture) was added to the experimental flask at 97 hours growth (exp. 3, pg. 118).

DISCUSSION

The inverse relationship between the activities of isocitrate lyase and tyrosine transaminase in three different nutritional environments (figs. 3-1, 3-2, pgs. 113, 114), suggested an interpretation for the repression of tyrosine transaminase by acetate. This relationship lends credence to the hypothesis that acetate is actively converted to glycogen while it is present in the medium, after which time other carbon sources, such as tyrosine and phenylalanine would be used. In agreement with previous findings (Mavrides and D'Iorio, 1969; Levy, 1967), both enzymes were repressed in the presence of glucose (figs. 3-1, 3-2, pgs. 113, 114).

However, the pattern of isocitrate lyase activity shown in fig. 3-1 (pg. 113), does not agree with reports that the lyase activity is highest in stationary phase (Levy, 1967; Levy and Scherbaum, 1965a, 1965b; Hogg and Kornberg, 1963). Most work reported on isocitrate lyase activity in Tetrahymena by other investigators was done with cells grown in proteose-peptone medium. The assay of lyase activity in cells grown in proteose-peptone medium, resulted in the same pattern of activity as that shown in fig. 3-1, pg. 113, with the exception that a high activity was maintained after the initial burst had subsided (fig. 3-3, pg. 117). This peak in activity in log phase was observed under all the tested conditions. Chua and Ronkin (1966), reported a high concentration of acetyl CoA in log phase cultures of Tetrahymena

which rapidly declined as the culture entered post log and stationary phases. This experimental finding was related to fatty acid synthesis in these organisms. However, it is possible that some of the acetyl CoA is converted to glycogen, especially since it is known that acetate (acetyl CoA) stimulates lyase activity (Hogg and Kornberg, 1963). This would supply a reasonable explanation as to why the lyase activity increased during this phase of growth even when acetate is absent from the medium (fig. 3-1, pg. 113).

Analysis of the pattern of tyrosine transaminase and lyase activities in the basal and basal minus acetate media, demonstrates the convergence of the two enzyme activities in the two media as the cultures age, as was previously shown for tyrosine transaminase by Mavrides and D'Iorio (1969). Figs. 3-1, and 3-2 (pgs. 113, 114), show that at 52 hours of growth the ratio of lyase activities in the basal and basal minus acetate media is 2.2, and that of tyrosine transaminase activities is 0.41. At 82 hours of growth the ratio of lyase is 1.03, and of tyrosine transaminase is 0.88. Hence, as the lyase activities in basal and basal minus acetate tend towards the same value, so do the tyrosine transaminase activities. If one assumes that the changes in enzyme activities reflect the respective flow of carbons from tyrosine and acetate to glycogen, then these enzyme patterns suggest that as the concentration of acetate in the basal medium approaches zero, the lyase activities in the two media converge towards the same value. When the flow of acetate carbons is the

same, that is very low, in basal and basal minus acetate media (i.e., 82 hours growth in figs. 3-1, 3-2, pgs. 113, 114), the flow of tyrosine carbons is likewise the same in the two media, and hence, the tyrosine transaminase activities are comparable.

The inverse relationship of lyase and transaminase is again apparent in figs. 3-5, and 3-6 (pgs. 122, 123) where the addition of acetate repressed tyrosine transaminase activity and induced isocitrate lyase within three hours. Phosphoenolpyruvate carboxykinase activity was unaffected by the addition of acetate (fig. 3-7, pg. 124). However, at this stage the cells were rather old and may have become relatively unresponsive to nutritional stimuli as is suggested by the fact that the lyase was stimulated to only 50% of its peak activity in log phase (fig. 3-5, pg. 122). This is further supported by the observation that phosphoenolpyruvate carboxykinase activity started to decrease in both the control and the experimental cultures between 104 and 124 hours growth (fig. 3-7, pg. 124).

The demonstration of an inverse relationship between phosphoenolpyruvate carboxykinase and lyase activities was very interesting although not unexpected since it had previously been shown that tyrosine transaminase and phosphoenolpyruvate carboxykinase have similiar patterns of activity (Chapter 2). If carbons from the glyoxylate cycle make an important contribution to gluconeogenesis, then it is difficult to rationalize why the key enzyme of the glyoxylate cycle, isocitrate lyase and a pacemaker enzyme in gluconeogenesis, phosphoenolpyruvate carboxy-

kinase, have opposite patterns of activity (fig. 3-4, pg. 120). Furthermore, since acetate carbons contribute to the synthesis of glycogen, why should acetate repress phosphoenolpyruvate carboxykinase activity (fig. 2-7, pg. 96)?

These facts suggested that a route, not requiring phosphoenolpyruvate carboxykinase activity, existed in Tetrahymena for the conversion of acetate to glycogen. This was the subject of the next series of investigations.

SUMMARY FOR CHAPTER 3

1) The course of enzyme activity of isocitrate lyase in growing Tetrahymena cells was established. The maximum activity was observed in logarithmic and post logarithmic phases of growth, followed by a rapid decline in activity during stationary phase. As previously reported, the activity of isocitrate lyase was high in cultures grown in the presence of acetate, and was repressed in cultures grown in the presence of glucose.

2) An inverse relationship between phosphoenolpyruvate carboxykinase and tyrosine transaminase activities and isocitrate lyase activity was demonstrated.

3) The repression of tyrosine transaminase activity and the induction of isocitrate lyase activity was shown to occur simultaneously after the addition of acetate to a culture of Tetrahymena in stationary phase.

CHAPTER 4

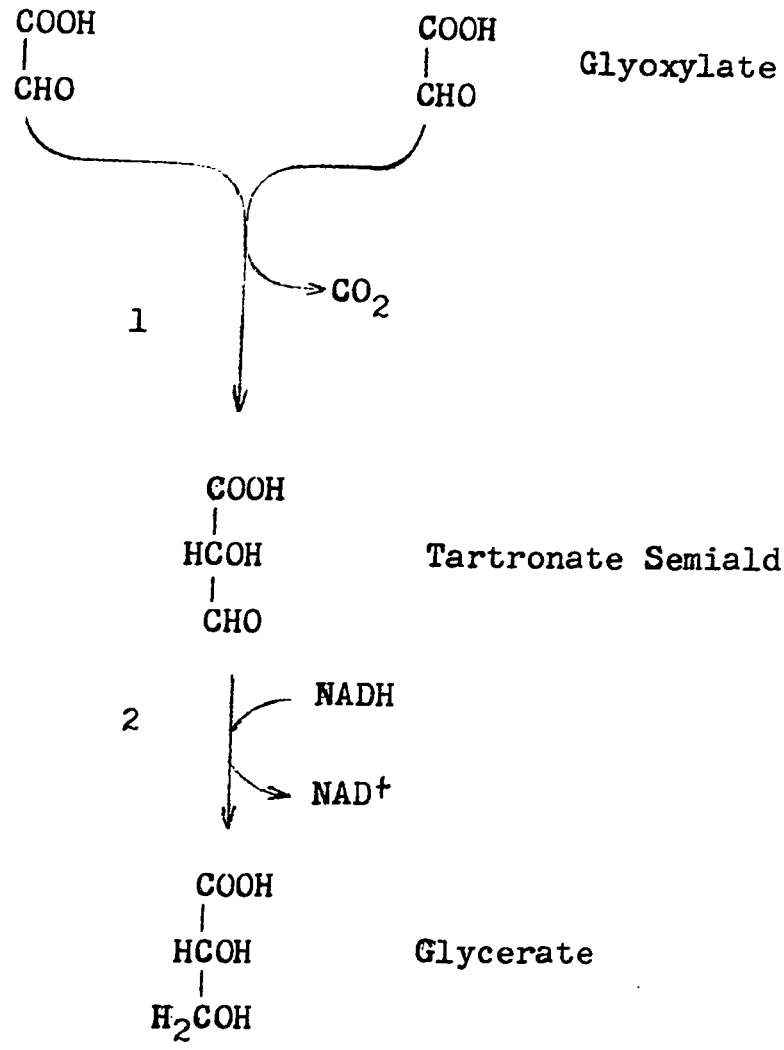
The glycerate pathway. A study on an alternate route of acetate metabolism for gluconeogenesis.

INTRODUCTION

It has been demonstrated that there is an inverse relationship between the activities of isocitrate lyase and phosphoenolpyruvate carboxykinase in Tetrahymena (fig. 3-4, pg. 120), and that acetate represses phosphoenolpyruvate carboxykinase activity (fig. 2-7, pg. 96). This led to the hypothesis that an alternate route of acetate metabolism leading to glycogen, circumventing the need for phosphoenolpyruvate carboxykinase but requiring lyase activity, might exist. These criteria were satisfied by the glycerate pathway (Kornberg, 1961).

This pathway is an alternate route for the metabolism of glyoxylate, one of the cleavage products of isocitrate (scheme 3, pg. 132). In this pathway, two molecules of glyoxylate condense to form tartronate semialdehyde and carbon dioxide through the action of glyoxylate carboligase (glyoxylate carboxy-lyase (dimerizing and reducing), EC 4.1.1.b). The reaction requires thiamine pyrophosphate (TPP) and magnesium ions (Krakow et al., 1961). The tartronate semialdehyde is then reduced by tartronate semialdehyde reductase (D-glycerate: NAD(P) oxidoreductase, EC 1.1.1.60) and NADH to glycerate (Gotto and Kornberg, 1961a; 1961b). Glycerate can then be phosphorylated to glycerate-3P, and further metabolised to glucose.

Although this pathway is not known in mammalian systems, it has been assayed in Pseudomonas (Kornberg and Gotto, 1961), sporulating Bacillus cereus (Megraw and Beers, 1963), and a mutant



1 Glyoxylate Carboligase
2 Tartronate Semialdehyde Reductase

Scheme 3: The Glycerate Pathway

strain of E. coli (Kornberg and Sadler, 1960). Hence, the next experiments were designed to test the possibility that the glycerate pathway was also operative in Tetrahymena pyriformis (strain W).

EXPERIMENTAL PROCEDURES

1) Assay of a glyoxylate dependent NADH oxidation by cell extracts of Tetrahymena pyriformis

The assay was described by Kornberg and Gotto (1961). The reaction proceedes as shown in scheme 3, pg. 132. The disappearand of NADH was measured spectrophotometrically at 340 mmicrons at 25°C in a 1-ml cuvette containing: 33 µmoles of potassium phosphate buffer pH 7.2, 3.3 µmoles magnesium chloride, 0.17 µmoles thiamine pyrophosphate, 0.13 µmoles NADH, 2.2. µmoles glyoxylate. In order to compensate for the oxidation of NADH by the cell extract, NADH was added to both the blank and experimental cuvettes. Their positions in the spectrophotometer were reversed so that a glyoxylate dependent oxidation of NADH could be followed by an increase in optical density at 340 mmicrons. The reaction was initiated by the addition of glyoxylate to the experimental cuvette. Results were expressed in terms of µmoles NADH oxidised using an extinction coeffecient of 6,220 $M^{-1}cm^{-1}$ for NADH.

2) Manometric assay of glyoxylate carboligase

The procedure was described by Kornberg and Gotto (1961). The assay measured the evolution of carbon dioxide resulting from the condensation of two molecules of glyoxylate to tartronate semialdehyde (scheme 3, pg. 132).

The total volume of the assay, contained in a single sidearm Warburg-manometer flask, was 1.9 ml. The main compartment

of the flask contained 40 µmoles potassium phosphate buffer pH 7.2, 10 µmoles magnesium chloride, 0.5 µmoles thiamine pyrophosphate. The side-arm contained 30 µmoles of glyoxylate in a total volume of 0.1 ml. The flasks were gassed with O_2-free N_2 for 2 minutes and preincubated at 30°C with shaking at a speed of 63 oscillations per minute, for 10 minutes. A thermobarometer containing 1.9 ml of phosphate buffer was monitored throughout the incubation time. After the base readings had stabilized, the reaction was started by tipping the contents of the side-arm into the main compartment. The reaction was followed by measuring changes in pressure due to the evolution of carbon dioxide. The flasks were calibrated and the results were expressed in µl carbon dioxide evolved.

3) Formation of a dinitrophenylhydrazine derivative of the reaction product in the glyoxylate carboligase assay mixture

At the end of the incubation, 0.5 ml of 6N HCl was added to the reaction flask containing 0.5 ml of Tetrahymena extract and the control flask incubated in the absence of glyoxylate. The deproteinized assay mixtures were centrifuged at 12,000xg for 10 minutes and the supernatants were saved. An excess of 0.5% solution of dinitrophenylhydrazine·HCl in 2N HCl was added to the supernatants. After the dinitrophenylhydrazine derivative had crystallized, an aliquot from each mixture was removed and centrifuged. The crystals were dissolved in 0.1N NaOH and UV spectra were taken against a blank containing a dinitrophenylhydrazine derivative of glyoxylate made by adding pure glyoxylate to a saturated solution of dinitrophenylhydrazine·HCl.

RESULTS

Experiment 1

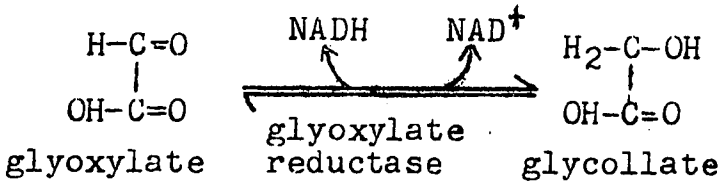
The conversion of glyoxylate to glycerate via the glycerate pathway involves the consumption of NADH (scheme 3, pg. 132), due to the reduction of tartronate semialdehyde to glycerate by tartronate semialdehyde reductase. Ideally, this could be measured by the addition of tartronate semialdehyde to an assay mixture containing NADH. However, the semialdehyde is not available commercially due to its instability and must, therefore, be prepared chemically or enzymatically just before its use (Kornberg and Gotto, 1966).

A glyoxylate-dependent oxidation of NADH was used by Kornberg and Gotto (1961) as a means of assaying the glycerate pathway and as a first approach this seemed a reasonable and simple method. Certainly if this pathway were present, an oxidation of NADH dependent on the presence of glyoxylate could be demonstrated. Another possible reaction is the reduction of glyoxylate to glycollate by glyoxylate reductase (glycollate: NAD^+ oxidoreductase, EC 1.1.1.26). However, Muller *et al.* (1968), could not detect this enzyme in *Tetrahymena pyriformis* (Strain E). Therefore, the demonstration of a glyoxylate-dependent oxidation of NADH by extracts of *Tetrahymena*, appeared to be a good system for determining the presence of the glycerate pathway.

Consequently, a 44 hour-old culture of *Tetrahymena* grown in 200 ml of basal medium (procedure 3, pg. 27) was harvested

(procedure 9, pg. 31). The washed cell pellet was sonicated in 0.25M sucrose and the whole sonicate was used for the enzymatic assay described in procedure 1, pg. 134.

A glyoxylate-dependent NADH oxidation by extracts of Tetrahymena was demonstrated (fig. 4-1, pg. 138). However, it was found that the omission of the cofactors for glyoxylate carboligase, thiamine pyrophosphate and magnesium chloride, did not alter the rate of the reaction. It therefore appears that unlike Tetrahymena strain E, strain W contains glyoxylate reductase, making the spectrophotometric method an unsuitable assay for the presence of the glycerate pathway. The reduction of glyoxylate to glycollate proceeds as follows:



Experiment 2

A second method of determining the presence of the glycerate pathway would be to assay for glyoxylate carboligase. According to scheme 1, pg. 132, this enzyme, in the presence of thiamine pyrophosphate and magnesium ions, catalyzes the condensation of 2 molecules of glyoxylate to tartronate semialdehyde and carbon dioxide. The activity of this enzyme may, therefore, be assayed manometrically by measuring the evolution of carbon dioxide.

A manometric assay was set up according to the procedure

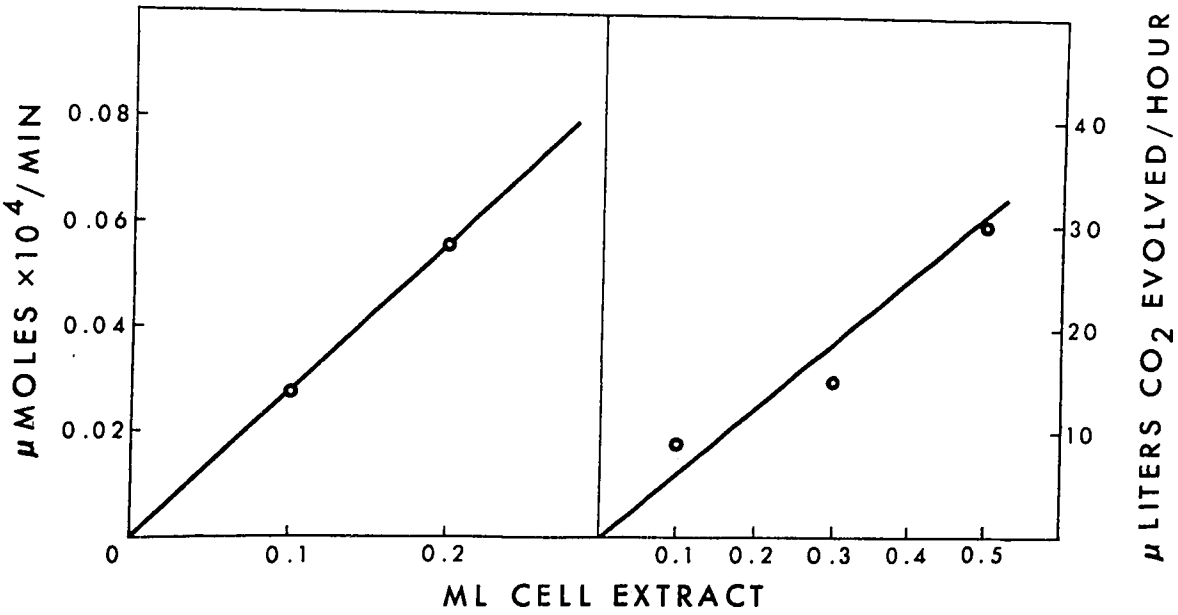


Figure 4-1 (left): Demonstration of a glyoxylate-dependent oxidation of NADH by a cell-free extract of Tetrahymena pyriformis (exp. 1, pg. 136).

Figure 4-3 (right): Plot of v_o (initial velocity) versus enzyme concentration for the manometric assay of glyoxylate carboligase in Tetrahymena (exp. 2, pg. 137).

2, pg. 134. Tetrahymena cells were grown for 44 hours in 200 ml of basal medium (procedure 3, pg. 27) and harvested (procedure 9, pg. 31). The cells were sonicated in 0.25M sucrose and the whole extract was used in the enzyme assay. Three concentrations of enzyme were assayed, 0.5 ml, 0.3 ml and 0.1 ml, and two controls were prepared using 0.3 ml of extract. One control was prepared without the substrate, glyoxylate, and the second was prepared without the cofactors thiamine pyrophosphate and magnesium ions.

Kornberg and Gotto (1961), characterized the product of the glyoxylate carboligase reaction as tartronate semialdehyde. A dinitrophenylhydrazine derivative of this compound was reported to absorb strongly at 560-570 mmicrons under alkaline conditions.

Therefore, a dinitrophenylhydrazine derivative of the reaction flasks containing 0.5 ml of Tetrahymena extract and the control incubated in the absence of glyoxylate were prepared (procedure 3, pg. 135). Ultra violet spectra of the alkaline derivative were taken against a blank containing a pure dinitrophenylhydrazine derivative of glyoxylate. Enough glyoxylate derivative was added to the blank to balance the absorption of this compound in the experimental cuvette.

The assay of glyoxylate carboligase, using three concentrations of Tetrahymena extract is presented in fig. 4-2, pg. 140. The reaction is shown to be linear with enzyme concentration within experimental error (fig. 4-3, pg. 138). A plot of the reaction in the control flasks compared to an experimental flask containing the same concentration of Tetrahymena extract in

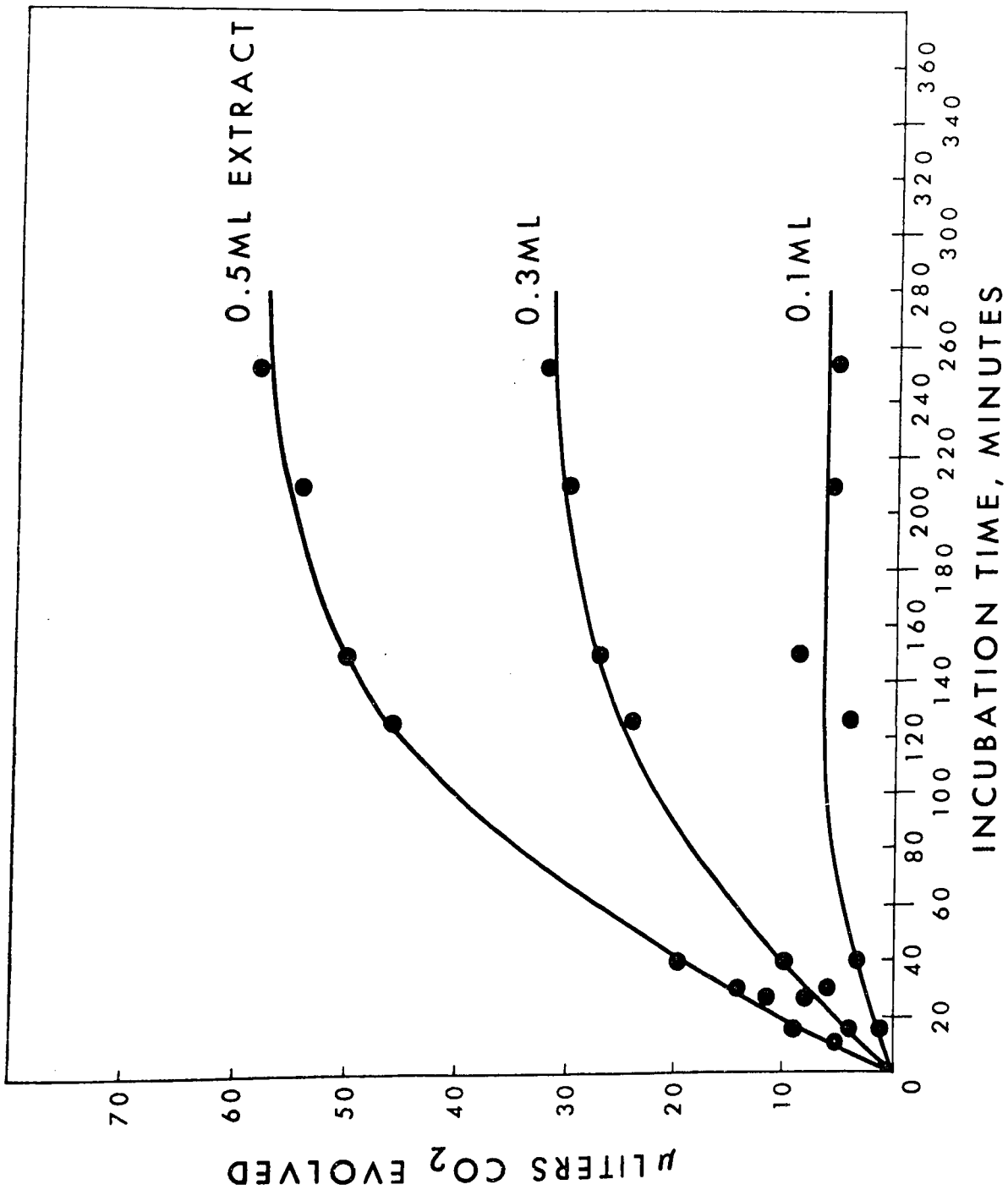


Figure 4-2: Manometric assay of glyoxylate carboligase using three concentrations of a Tetrahymena cell extract (exp. 2, pg. 137).

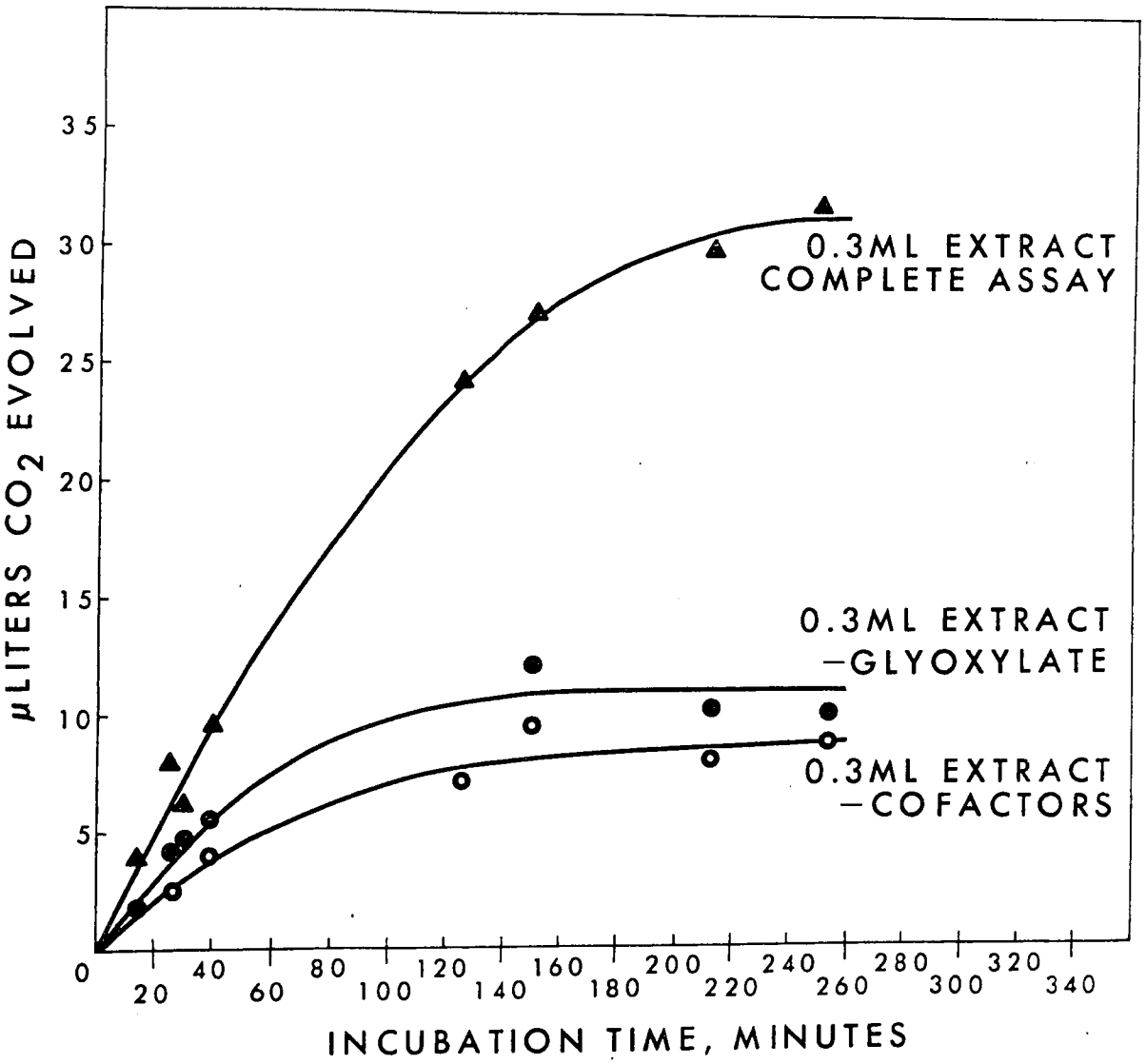


Figure 4-4: Control flasks in the assay of glyoxylate carboligase compared to the experimental flask. One control lacked the cofactors for the reaction, thiamine tyrophosphate and Mg ions, the second control lacked the substrate, glyoxylate. All three flasks contained 0.3 ml of Tetrahymena cell extract (exp. 2, pg. 137).

fig. 4-4 (pg. 141), illustrates that the reaction was dependent upon the cofactors for glyoxylate carboligase, thiamine pyrophosphate and magnesium ions, as well as glyoxylate.

The UV spectra of the dinitrophenylhydrazine derivative of the experimental (0.5 ml of extract) and a control flask (incubated in the absence of glyoxylate) is given in fig. 4-5 (pg. 143). A peak absorbing at 560-570 mmicrons was observed in the experimental flask but not in the control. This corresponded to the reported absorption of the dinitrophenylhydrazine derivative of tartronate semialdehyde given as 560-570 mmicrons (Kornberg and Gotto, 1961).

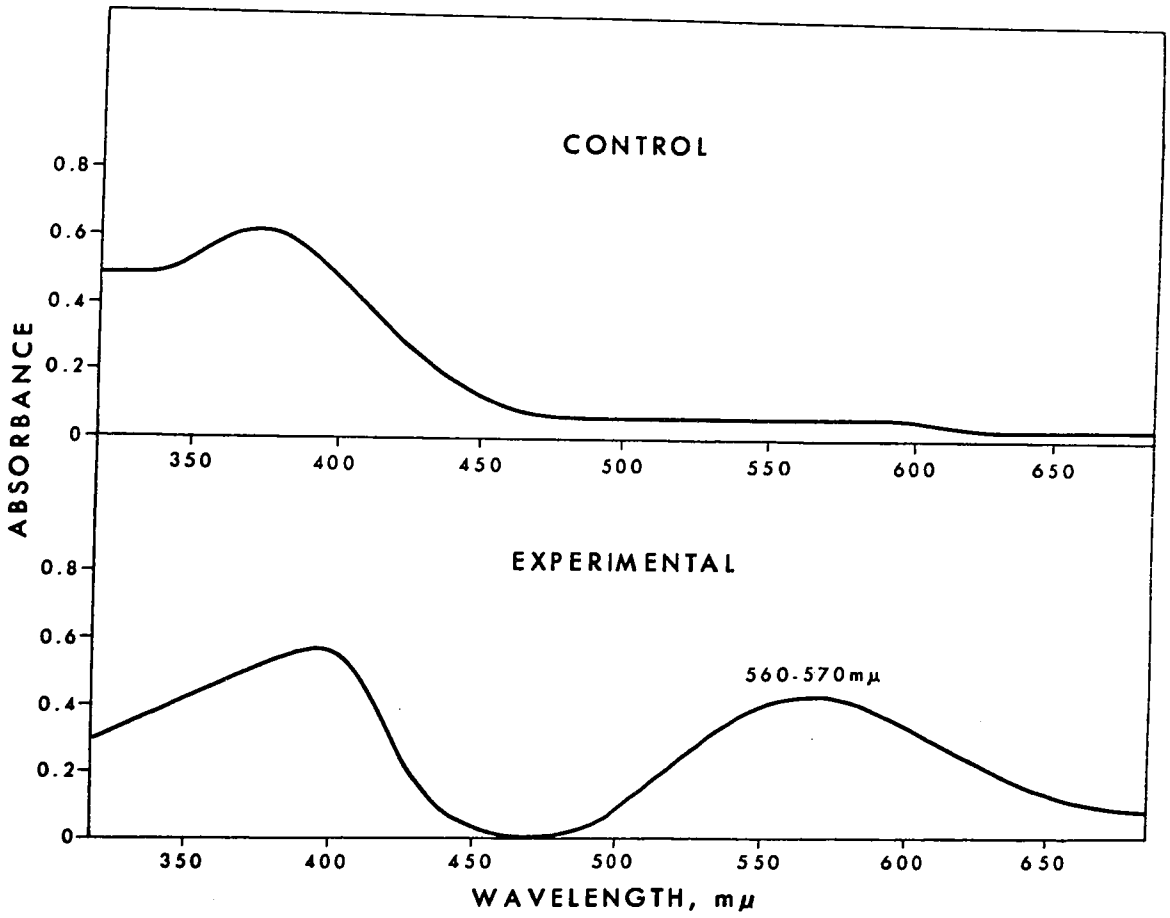


Figure 4-5: UV spectra of the crystals isolated after the addition of dinitrophenylhydrazine to a control and an experimental flask used in the assay of glyoxylate carboligase. The peak observed in the experimental flask at 560-570 mmicrons, corresponded to the reported maximum for the DNP-derivative of tartronate semialdehyde (exp. 2, pg. 137).

DISCUSSION

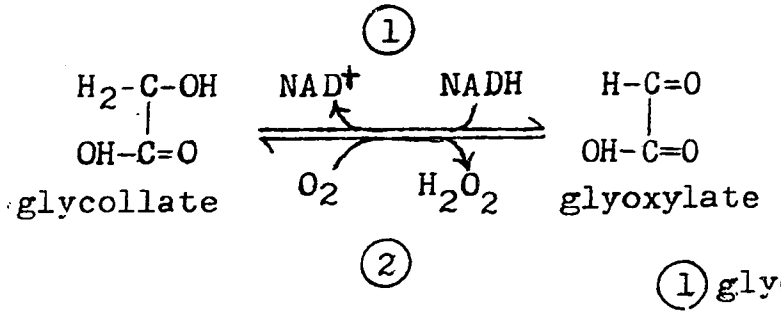
The possibility that the glycerate pathway is present in Tetrahymena pyriformis, strain W, is supported by the successful assay of glyoxylate carboligase and the spectral characterisation of the reaction product, tartronate semialdehyde (figs. 4-3, 4-4, pgs. 138, 141). More conclusive evidence must be obtained, however, before the glycerate pathway is firmly established, such as the assay of tartronate semialdehyde reductase and demonstration of the *in vitro* formation of glycerate from glyoxylate.

The significance of this pathway in gluconeogenesis has not been established. Theoretically, it presents an alternate route for the conversion of glyoxylate to carbohydrate which circumvents the well-established flow of carbons through phosphoenolpyruvate. If the glycerate pathway is present, high phosphoenolpyruvate carboxykinase activity would not be required and the observations in Chapter 3 on the inverse relationship between phosphoenolpyruvate carboxykinase and isocitrate lyase activities (fig. 3-4, pg. 120), and in Chapter 2 on the repression of the former activity by acetate (fig. 2-7, pg. 96), would be explained.

This would not be the first instance where a carbon flow to glycogen has been described which did not require phosphoenolpyruvate carboxykinase. An interesting study by Veneziale *et al.* (1970) provided evidence for the conversion of pyruvate to glucose in perfused liver when phosphoenolpyruvate carboxykinase

activity was inhibited by quinoline. The conversion proceeded at 60% of the rate observed in the control (without inhibitor). Tracer studies, using labelled pyruvate, revealed that the specific activity of 3-phosphoglycerate was approximately the same as lactate, higher than malate and twice as great as phosphoenolpyruvate. In addition an unknown intermediate was isolated but could not be identified. The possibility that this intermediate could be tartronate semialdehyde was not investigated. These findings indicate that perhaps a pathway related to the glycerate pathway is functional in the mammalian system which does not require phosphoenolpyruvate as an intermediate and can serve the needs of gluconeogenesis.

The presence of glyoxylate reductase in Tetrahymena strain W was unexpected due to the reported absence of this enzyme in strain E (Muller et al., 1968; fig. 4-1, pg. 138). However, a glycollate oxidase (glycollate: oxygen oxidoreductase, EC 1.1.3.1) activity has been reported in the peroxisomes of Tetrahymena and a functional system employing the combined action of these two enzymes has been described as a route for the oxidation of extramitochondrial NADH (de Duve and Baudhuin, 1966). The scheme is as follows:



(1) glyoxylate reductase

(2) glycollate oxidase

Therefore, it is possible that such a system is operative in Tetrahymena.

SUMMARY FOR CHAPTER 4

1) Glyoxylate reductase was assayed in Tetrahymena indicating a possible mechanism for the oxidation of extramitochondrial NADH by the coupling of this enzymes action with that of glycollate oxidase.

2) Strong evidence was obtained for the presence of the glycerate pathway in Tetrahymena by assaying for glyoxylate carboligase and characterising the product of this enzyme reaction as tartronate semialdehyde, an intermediate in the glycerate pathway. This pathway was viewed as a route for the conversion of acetate to glycogen which would not require phosphoenolpyruvate carboxykinase.

GENERAL DISCUSSION AND SUMMARY

Although attempts to assay phenylalanine hydroxylase in cell-free extracts of Tetrahymena pyriformis, strain W, were unsuccessful, it was shown that phenylalanine could be hydroxylated to tyrosine in vivo (fig. 1-2, Chapter 1).

Phenylalanine and tyrosine are classified as ketogenic and glucogenic amino acids in the mammalian system (Butts et al., 1938; 1949; Edson, 1935). Hence, it was suspected that they could be used as carbon sources for the synthesis of glycogen in gluconeogenesis in Tetrahymena. This was proven by isolating isotopically labelled glucose, obtained from the hydrolysis of glycogen purified from Tetrahymena grown in the presence of isotopically labelled phenylalanine (Chapter 1). In addition, glucose, which represses gluconeogenesis (Levy, 1967), reduced the in vivo hydroxylation of phenylalanine to tyrosine (Table 1-3, Chapter 1).

The assay of tyrosine transaminase in Tetrahymena and the demonstration of the repression of this enzyme activity by glucose (Mavrides and D'Iorio, 1969), in addition to the above findings, strongly suggested that the pathway of tyrosine and phenylalanine catabolism in Tetrahymena, was similiar to the mammalian system (scheme 1, Chapter 1). This was proven in this study by demonstrating the presence of p-hydroxyphenylpyruvate oxidase, homogentisate oxidase, maleylacetoacetate isomerase, and fumarylacetoacetate hydrolase which, together with tyrosine

transaminase, comprise the tyrosine-catabolizing pathway (fig. 2-3, Chapter 2). In addition, homogentisate oxidase was partially purified from Tetrahymena and used to prepare fumarylacetoacetate, which was characterized spectrally (fig. 2-4, Chapter 2).

The regulation of the activities of all the enzymes in the tyrosine-catabolizing pathway, as well as the activity of a pacemaker enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase, was studied. The presence of acetate or glucose in a chemically defined medium, repressed all the enzymes. Repression by acetate occurred during log and post-log phases (figs. 2-5a,b, Chapter 2), whereas glucose repressed maximally in stationary phase (figs. 2-6a,b, Chapter 2). The repression of phosphoenolpyruvate carboxykinase activity by acetate (fig. 2-7, Chapter 2) was not easily explained since acetate is a precursor for glycogen synthesis in glyconeogenesis in Tetrahymena (Hogg and Kornberg, 1963), and hence, should not repress the activity of a key enzyme in this process. Repression of enzyme activities by glucose was in keeping with the observation that glucose represses gluconeogenesis (Levy, 1967).

It was interesting that the repression caused by acetate was a relatively short-term effect. A detailed study of the course of tyrosine transaminase activity by Mavrides and D'Iorio (1969), demonstrated that the repression of this enzyme in a medium containing acetate, was relieved as the culture aged, and eventually the activity was equal to that found in a culture

grown in the absence of acetate. Clearly, the metabolism of acetate by these cultures had an effect on the metabolism of tyrosine. This was substantiated by showing that in the absence of acetate, a culture of Tetrahymena incorporated almost twice the amount of tyrosine carbons into glycogen as was observed in a control grown in the presence of acetate (Table 2-1, Chapter 2).

It has been widely accepted, on the basis of very few experiments, that the conversion of acetate to glycogen is most significant during stationary phase of growth. This conversion is accomplished through the glyoxylate cycle which is controlled by the activity of isocitrate lyase (Hogg and Kornberg, 1963). A detailed study of the pattern of activity of this enzyme during the growth cycle of Tetrahymena has never been reported. In fact, the method of studying this enzyme in Tetrahymena was to compare the activity of a point in log phase to a point in stationary phase (Hogg and Kornberg, 1963). Other experimentors have studied the activity of this enzyme in an artifically induced stationary phase (Levy and Scherbaum, 1965a; 1965b). It can be argued, however, that this method did not reproduce the natural course of events in a growing culture.

When studied, it was found that the activity of isocitrate lyase was actually higher in log and post-log phases than in stationary phase (figs. 3-1, 3-3, Chapter 3). In fact, in Tetrahymena grown in a chemically defined medium, isocitrate lyase peaked in log phase and decreased precipitously thereafter (fig. 3-1, Chapter 3). The course of lyase activity was the same in

the presence or absence of glucose or acetate, only the range varied. Hence, even in the absence of acetate, the lyase activity peaked in log phase. This might be explained by the results of Chua and Ronkin (1966), who showed that the concentration of acetyl CoA in cultures of Tetrahymena grown in a proteose-peptone medium was very high in log phase and decreased rapidly as the culture entered into stationary phase. Since it has been shown that acetate induces isocitrate lyase (Hogg and Kornberg, 1963), it might be inferred that this peak in activity, which was also observed in proteose-peptone medium (fig. 3-3, Chapter 3), might be related to the endogenous concentration of acetyl CoA.

If the pattern of lyase activity can be interpreted to mean that acetate is converted to glycogen early in the life cycle of Tetrahymena, then the effect of acetate on the flow of tyrosine carbons to glycogen could be explained on the basis that in the presence of acetate, less carbon flow from tyrosine would be required to supply the needs of gluconeogenesis as originally suggested by Mavrides and D'Iorio (1969).

A comparison of lyase and transaminase activities in a basal medium containing acetate to one lacking acetate demonstrated this point. When the ratio of lyase activities in the presence and absence of acetate was 2.2, the ratio of transaminase activities was 0.41. Later when the ratio for lyase was 1.03, the ratio for transaminase was 0.88 (figs. 3-1, 3-2, Chapter 3). Thus, as the activity of lyase in these two media tended towards the same value, so did the activities of tyrosine

transaminase. This could be interpreted to mean that as the acetate concentration was reduced in the basal medium (acetate present), the requirement of the culture for tyrosine carbons approached that of a culture of the same age grown in the absence of acetate.

A closer study of the relationship between these two enzymes revealed that the addition of acetate to a Tetrahymena culture in stationary phase at a point when tyrosine transaminase activity was high (fig. 3-6, Chapter 3), and isocitrate lyase was low (fig. 3-5, Chapter 3), resulted in the repression of tyrosine transaminase and induction of isocitrate lyase. The pattern of activities for tyrosine transaminase and phosphoenolpyruvate carboxykinase was the mirror image of the pattern of lyase activity (fig. 3-4, Chapter 3). Thus, an inverse relationship existed between tyrosine transaminase and lyase, and phosphoenolpyruvate carboxykinase and lyase, while tyrosine transaminase and phosphoenolpyruvate carboxykinase displayed the same pattern of activity.

A close relationship between the courses of activities of tyrosine transaminase and phosphoenolpyruvate carboxykinase was observed throughout all the experiments. Both tyrosine transaminase and phosphoenolpyruvate carboxykinase decreased in activity at the same rate during log phase (fig. 2-1, Chapter 2), and increased in activity in post log phase (fig. 2-2, Chapter 2). Phosphoenolpyruvate carboxykinase activity rose just prior to an increase in tyrosine transaminase activity as was also observed in rat liver by Lane and Mavrides (1969). Both activities were

repressed by acetate and glucose (figs. 2-5b, 2-6b, Chapter 2). Furthermore, of all the enzymes in the tyrosine-catabolizing pathway, only tyrosine transaminase demonstrated a significant relationship to phosphoenolpyruvate carboxykinase activity (figs. 2-8a,b, Chapter 2). This signified that the remaining 4 enzymes involved in tyrosine catabolism did not respond as quickly as the first enzyme (tyrosine transaminase) did to nutritional changes in the growth media.

These factors, together with the demonstration that the effect of changes in the activity of tyrosine transaminase was a corresponding change in the flow of tyrosine carbons to glycogen, established that tyrosine transaminase and phosphoenolpyruvate carboxykinase were linked metabolically in the process of gluconeogenesis in Tetrahymena and demonstrated a functional significance of the changes in tyrosine transaminase activity.

The relationship between isocitrate lyase and phosphoenolpyruvate carboxykinase activities, appeared to be paradoxical. Not only were their courses of activity mirror images, but a known inducer of isocitrate lyase, acetate, repressed phosphoenolpyruvate carboxykinase activity (fig. 2-7, Chapter 2). The results suggested that an alternate route of acetate metabolism in Tetrahymena leading to glycogen synthesis and bypassing phosphoenolpyruvate, might exist.

The glycerate pathway might be this alternate route. Through this pathway, two molecules of glyoxylate are converted via an intermediate, tartronate semialdehyde, to carbon dioxide and gly-

cerate. Upon phosphorylation the glycerate can enter the Embden-Meyerhoff pathway and be converted to glycogen.

Preliminary attempts to demonstrate the presence of this pathway in Tetrahymena were successful. Glyoxylate carboligase, which catalyzes the condensation of two molecules of glyoxylate to tartronate semialdehyde and carbon dioxide, was assayed manometrically (figs. 4-2, 4-4, Chapter 4) and a dinitrophenylhydrazine derivative of the reaction product was shown to have the same spectral characteristics reported for the dinitrophenylhydrazine derivative of tartronate semialdehyde (fig. 4-5, Chapter 4). Hence, a route of acetate metabolism in Tetrahymena for the purposes of gluconeogenesis may indeed not require phosphoenolpyruvate carboxykinase activity thus explaining the dissimiliarities between the pattern of this enzyme's activity and that of isocitrate lyase.

In Tetrahymena, all the enzymes of the tyrosine-catabolizing system are susceptible to metabolite control (figs. 2-5a,b, 2-6a,b, Chapter 2), and undergo significant changes in activity unlike the enzymes of the mammalian pathway (La Du, 1966). Gluconeogenesis from phenylalanine and tyrosine occurs in Tetrahymena (Chapter 1; Table 2-2, Chapter 2), as does gluconeogenesis from acetate (Levy and Scherbaum, 1965). However, the initial steps of these two processes, as refleced in the activities of the enzymes of the tyrosine-catabolizing route and in the activity of isocitrate lyase, appear to occur, to a major extent, as mutually exclusive events. The present study in Tetrahymena,

demonstrates the regulation of these events by an interplay of metabolites regulating enzyme activities.

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