THIAZOLIDINE-4-CARBOXYLIC ACID: PROPERTIES AND MODES OF ACTION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

Thiazolidine-4-carboxylic acid: properties and modes of action in the yeast *Saccharomyces cerevisiae*.

by James R. Paterniti, Jr.

Advisor: Professor Norman R. Eaton.

Cells of *Saccharomyces cerevisiae* showed differential growth inhibition when cultured on various carbon source in the presence of the proline analogue thiazolidine-4-carboxylic acid (TZ). On 0.5% yeast extract, 2% glucose and TZ (10 mg/ml) medium, growth lags from 8-10 hours were observed. After this initial period of inhibition recovery occurred, accompanied by slower rates of growth in exponential phase, and lowered cell titres in stationary phase. This growth inhibition could be largely reversed by the addition of proline to the medium. Similar patterns were observed in TZ-treated cultures using 0.5% acetate or 3% glycerol as the carbon source. By contrast, cell growth was totally inhibited on a medium containing 3% ethanol as the carbon source. This inhibition was not proline-reversible and appeared to have a different basis from that seen on other carbon sources. Indeed, TZ was shown to have two probable modes of action in yeast. It was found to be a noncompetitive inhibitor of yeast alcohol dehydrogenase. This inhibition could not be reversed by proline *in vitro*. TZ was also

found to be incorporated into protein.

In studies using radiolabeled analogue, it was found that TZ incorporation into protein was greatest during the lag phase of the growth cycle, and declined as cells progressed through the recovery phase. Thus the amount of TZ incorporation into protein could be related to the degree of growth inhibition observed. In an effort to explain the changes in TZ incorporation with the growth cycle, it was discovered that TZ entered the cell by way of a specific proline transport system. Changes in the activity of this system parallelled changes in both inhibition and incorporation patterns. Furthermore, changes in the activity of the proline permease system could occur under conditions of nitrogen starvation. Similar results have been reported in other fungal systems where nitrogen repression appears to control proline uptake (Kuznar, *et al.*, 1973; Arst & MacDonald, 1975).

The multidimensional aspects of TZ action were exploited in studies which used the analogue as a probe to select mutants in the proline system of yeast. One class of mutants were selected to be resistant to TZ on glucose and ethanol medium. Genetic and biochemical characterizations of a dominant mutant of this type showed that the mutation probably affected derepression of the proline uptake system.

Using a novel selection technique, mutants which were hypersensitive to TZ on glucose medium were obtained. These mutants were incapable of growth on glucose-TZ medium.

Characterization of one such recessive mutant suggested that the affected gene produced an altered proline permease. The relationship between both classes of mutants and the control of proline uptake in fungi was discussed.

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CHAPTER I

INTRODUCTION

Properties and Distribution of Amino Acid Analogues:

Many chemical substances cause growth inhibition in biological systems (for review see Webb, 1963). Of particular interest are classes of toxic amino acids whose efficacy in inhibiting growth is related to their ability to replace normal amino acids in cellular processes such as protein synthesis. The most effective of these amino acid analogues resembles its natural counterpart in size, shape and charge.

Structural changes which most often produce amino acid analogues are noted by Richmond (1962) to include: 1.) replacements in the skeleton such as substituting F for H as in p-fluorophenylalanine, a phenylalanine analogue; 2.) the replacement of phenyl by another ring structure such as by a pyridine ring as in 5-hydroxypyridine-2-ylalanine, a tyrosine analogue (Ravel *et al.*, 1965); 3.) replacement of one type of heterocyclic ring by another, as in β-1,2,4-triazol-3-ylalanine, a histidine analogue (Levin & Hartman, 1963). These types of structural changes often occur in nature, particularly in the higher plants and over 100 such effective analogues are known (for review see Fowden, 1964). In general, analogues of the larger amino acids are more frequent in nature than those of smaller amino acids. This is presumably because the relative size change which most re-

placements would cause is smaller for large amino acids than for small amino acids (e.g. methionine + CH_2 → ethionine, while, alanine won't substitute for glycine)(Richmond, 1962).

Modes of Action of Amino Acid Analogues:

In contrast to many other classes of inhibitors, amino acid analogues exhibit modes of action which have multidimensional aspects. Among these is their incorporation into biological molecules such as protein, in place of the normal amino acid. Such replacements can effect the stereochemical properties of protein and frequently lead to impairment or loss of biological activity (e.g. changing the structure of an enzyme's active site)(Fowden *et al.*, 1963). Proteins other than enzymes have been shown to be effected. Uitto and Prokop (1974) reported a decrease in the rate of collagen disposition in baby chick tendon cells cultures in the presence of proline analogues. The decrease is ascribed to an alteration in the tertiary structure of the collagen molecule by incorporation of the analogue, which rendered collagen more sensitive to protease degradation. In addition, analogue incorporation into non-protein molecules such as the β-alanine portion of pantothenic acid has been shown to occur (For review see Woolley, 1962).

There is much evidence to suggest that an amino acid analogue is taken into the cell by the transport system of

its normal counterpart. Numerous studies show competitive inhibition of both specific (Grenson *et al.*, 1966; Neale & Tristram, 1965), and general (Brockman, 1964; Eddy & Indge, 1962) amino acid uptake systems by analogues. Growth inhibition would be expected to be especially severe in cells which are auxotrophic for the amino acid involved.

The biosynthesis of amino acids is usually regulated by the end-product of the synthetic sequence (Calvo & Fink, 1971). Such regulation takes the form of end-product inhibition of the activity of the first enzyme in the synthetic sequence as well as repression of synthesis of the biosynthetic enzymes in the pathway. Reports of analogue-mediated "pseudo feedback inhibition" exist (Moyed, 1960), though proven instances of analogue-mediated repression are less apparent. This is because it is difficult to separate the effects of true repression of enzyme synthesis from a decrease in enzyme activity due to analogue incorporation (Wasmuth & Umbarger, 1973). This difficulty notwithstanding, 6-methyl-tryptophan has been reported to be a repressor of several enzymes involved in tryptophan biosynthesis (Lester & Yanofsky, 1961).

In addition to these general modes of action, amino acid analogues inhibit growth in other ways. Ethionine, when substituted for methionine can act as an ethyl donor in transferase-mediated metabolic reactions which normally involve the methyl group of methionine (Stekol, 1963). Such

a substitution has far-reaching biological consequences.

Azaserine shows an unusual mode of action in both eucaryote and procaryote systems. In addition to acting as an analogue of glutamine, azaserine causes cessation of growth by inhibiting formylglycineamide ribotide amidotransferase, which effectively blocks purine biosynthesis. The inhibition involves specific analogue binding to a single cysteine residue which cannot be reversed by glutamine (Levenberg *et al.*, 1957).

With certain exceptions, the effects of analogues can be reversed by their natural counterparts. This criterion of specific reversibility is often used to argue that the mode of action of an analogue involves its replacement of the natural amino acid. However, specific reversibility should be seen to constitute only partial proof, since examples which negate its validity have been reported (Fowden *et al.*, 1967).

The Importance of Amino Acid Analogues in Biochemical and Genetic Investigations:

From the previous discussion of the properties and modes of action of amino acid analogues, it is obvious that these molecules represent powerful tools for mechanistic investigations which focus on various aspects of cell metabolism and molecular function. Since analogues enter the cell via the uptake systems of their natural

counterparts, they can be used to study the requirements, specificity, and regulation of cellular transport systems. Two types of studies are usually undertaken: 1.) competition-kinetics studies between the analogue and the natural amino acid; 2.) genetic studies where analogue resistance is often used as a trait to select mutants which have definable lesions in properties relating to transport.

Amino acid analogues are often incorporated into protein. They can therefore be used to study the function of specific amino acid moeities in whole protein and to provide clues concerning the regulation of protein function. In addition, they have facilitated studies of the specificity of the protein synthetic apparatus for amino acids (Calender & Berg, 1966). Furthermore, selection of mutant cell lines which exhibit an altered response to an analogue may yield regulatory mutants affecting the pathways of amino acid biosynthesis. Such mutants are of prime importance in studies of the genetic regulation of biosynthesis (for review see Benveniste & Davies, 1973).

Finally, there is evidence to suggest that certain amino acid analogues are incorporated to differing extents by the protein synthetic apparatus of the eucaryote cytoplasm and mitochondrion (Wilkie, 1970). The phenomenon is thought to result from differences in the substrate specificities of the t-RNA- synthetases specific to each compartment. This phenomenon could be distinguished in the

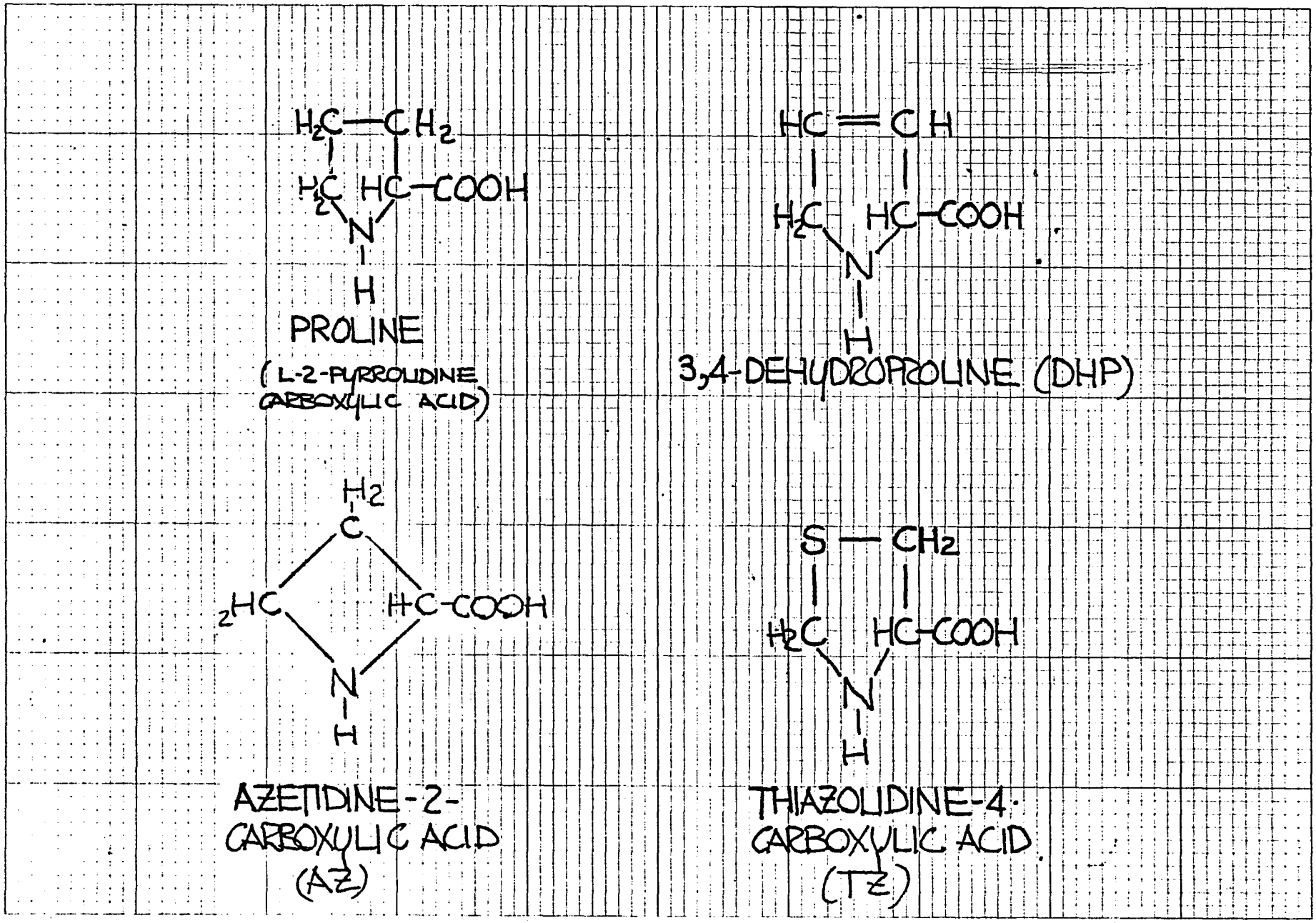
facultative anaerobe *Saccharomyces cerevisiae*, based on the ability of cells to grow on a fermentable carbon source in the presence of an analogue; the same or a smaller concentration of the analogue would inhibit growth on a non-fermentable carbon source. A system which allowed specific analogue incorporation into the products of mitochondrial protein synthesis would greatly facilitate the study of mitochondrial biogenesis. This approach would circumvent many of the objections raised against other methodologies for such studies (Schatz & Mason, 1974).

Proline and Its Analogues:

Although they have been recognized only recently, authentic analogues of proline have received much attention since they mimic a unique and important molecule. L-proline (Fig. 1) (L-2-pyrrolidine carboxylic acid) is the only imino acid which is normally incorporated by the protein synthetic apparatus of the cell (Steward *et al.*, 1958). Because of the disposition of the carboxylic acid and secondary amine groups in proline, its inclusion into a polypeptide chain causes disruption of the α-helical regions and results in bands in non-helical amino acid chains (Perutz *et al.*, 1965). Thus proline plays a singular role in determining protein structure.

Fig. 1. Proline and its analogues.

FIGURE 1


PROLINE
(L-2-PYRROLIDINE CARBOXYLIC ACID)
3,4-DEHYDROPROLINE (DHP)
AZETIDINE-2-CARBOXYLIC ACID (AZ)
THIAZOLIDINE-4-CARBOXYLIC ACID (TZ)

In contrast to most other amino acids, proline in virtually all cells is transported by an imino acid permease system, which has been shown by several criteria to be discrete, specific, and energy-requiring (Britten & McClure, 1962; Hirata *et al.*, 1974). In *Saccharomyces chevalieri* this transport system appears to be regulated by repression (Kuznar *et al.*, 1973); evidence for a similar system of control in *S. cerevisiae* will be presented here. Regulation of the enzymes of proline biosynthesis has been studied in *Escherichia coli* (Strecker, 1956; Baich & Pierson, 1965) and in fungi (Arst & MacDonald, 1975). The control appears to involve end-product inhibition of the first enzyme of the proline biosynthetic pathway in *E. coli*, and a complex control which includes induction as well as both carbon catabolite and nitrogen repression in fungi.

The putative analogues of proline include both the naturally occurring derivatives, azetidine-2-carboxylic acid (AZ), *cis* and *trans*(*allo*)-4-hydroxy-l-proline, l-pipecolic acid, *cis*-4-hydroxymethyl-l-proline and the chemically synthesized molecules, 3,4-dehydroproline (DHP), and thiazolidine-4-carboxylic acid (TZ)(Fowden *et al.*, 1967). Of these, AZ, which occurs in many species of the Liliacae (Fowden *et al.*, 1967), and the two chemically synthesized molecules (TZ and DHP) are proven analogues of proline. These molecules cause growth inhibition in a number of systems, and are terratogenic in developing sea urchin embryos

(Lallier, 1965). Figure 1 gives the chemical structures of these molecules.

The toxic action of these analogues can be attributed, at least in part, to changes in the stereochemical structure of the proteins in which they replace functionally essential proline residues. In particular, it has been shown that the growth of *E. coli* K-12 in DHP-containing medium, results in replacement by the analogue of 85% of the proline residues in alkaline phosphatase isolated from this organism (Fowden *et al.*, 1963). Although possessing full activity initially, the DHP-containing enzyme was less stable than the authentic enzyme and denatured rapidly upon standing. Moreover, incorporation of DHP into *E. coli* *B*-galactosidase resulted in a functionally inactive protein (Fowden *et al.*, 1963). Demonstrations of the incorporation of AZ and TZ (Fowden & Richmond, 1963; Vaughan *et al.*, 1974) or of a derivative (Unger & DeMoss, 1966) into plant and bacterial protein have been made. In addition, TZ has been shown to be incorporated into rat liver Bekhor *et al.*, 1965) and yeast (Paterniti *et al.*, 1975) cell protein. Peterson and Fowden (1965) reported that all three analogues are able to compete with proline for the prolyl-t-RNA- synthetase of *Phaseolus aureus* (mung bean) seedlings. Although to varying degrees, each analogue can mediate "pseudofeedback inhibition" of proline biosynthesis in *E. coli* (Tristram & Thurston, 1966), and Magaña-Schwencke *et al.*, (1973) have

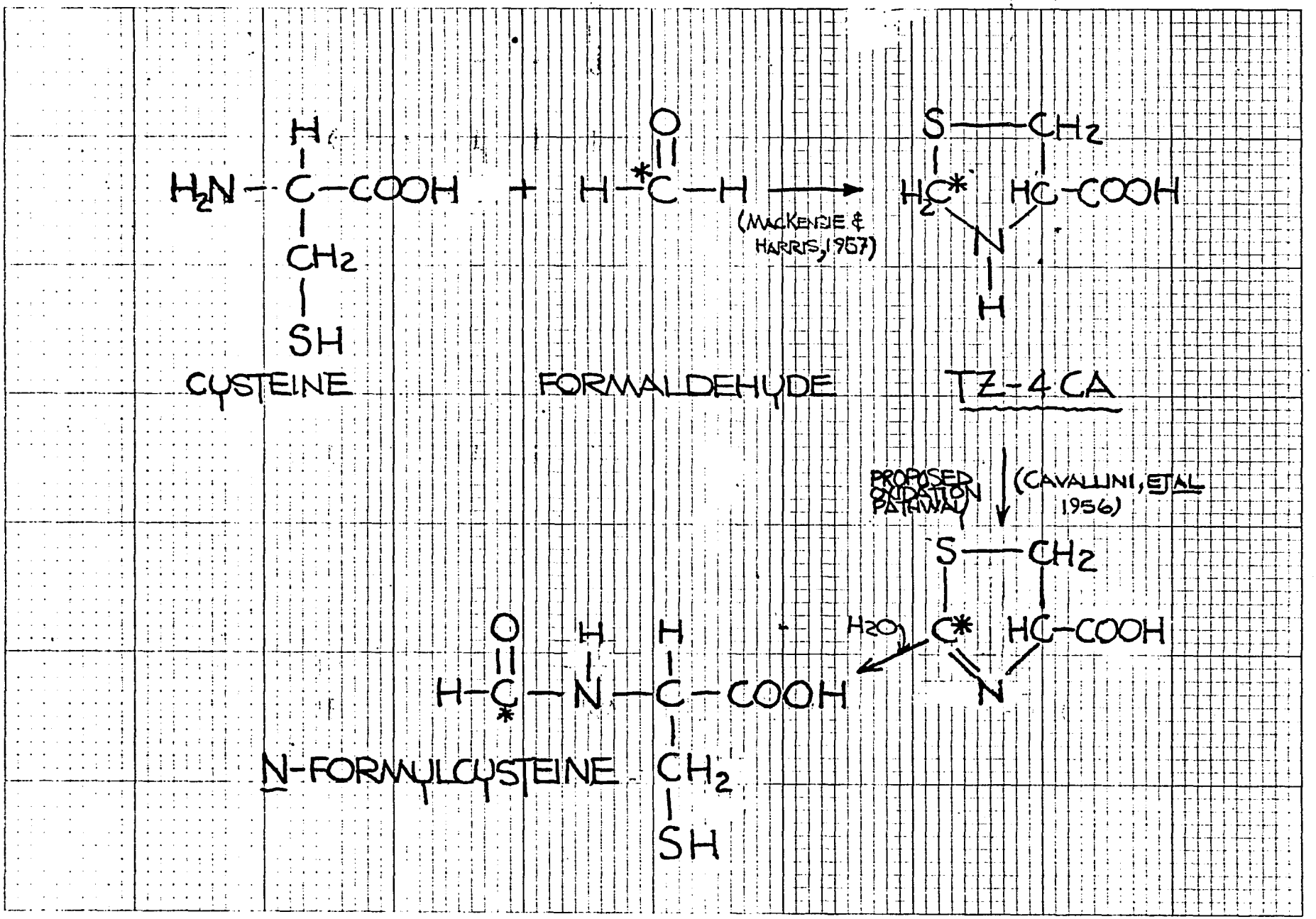
reported that TZ could compete with proline for uptake into *S. chevalieri*.

Thiazolidine-4-Carboxylic Acid:

TZ was first synthesized by Ratner and Clarke (1937). It is the product of the non-enzymatic condensation of equimolar amounts of L-cysteine and formaldehyde (Fig. 2) to yield the saturated imino acid in which *S* is present as the thioether (MacKenzie & Harris, 1957). As will be discussed at greater length, TZ manifests the ability to inhibit differentially the growth of yeast cells on fermentable and nonfermentable carbon sources. Moreover, evidence will be presented which shows that this differential inhibition does not result from exclusive incorporation into the mitochondrial compartment as was previously predicted (Wilkie, 1970). Rather it will be shown that the mode of action of the analogue depends upon the nature of the growth conditions, particularly the carbon source. This aspect of analogue inhibition has only recently been dealth with (Paterniti *et al.*, 1975). In addition, TZ will be shown to be an analogue which is suitable for use in selecting mutants which are altered with respect to the proline transport system.

Fig. 2. The synthesis and degradation of thiazolidine-4-carboxylic acid.

FIGURE 2.


CYSTEINE
FORMALDEHYDE
(MacKENZIE & HARRIS, 1957)
TZ-4CA
PROPOSED OXIDATION PATHWAY
(CAVALLINI, ET AL 1956)
H2O
N-FORMYLCYSTEINE

CHAPTER II

MATERIALS & METHODS

MEDIA - The media used for routine culture growth were 1% yeast extract (Difco), 2% peptone, 2% glucose (YEPD); or 0.5% yeast extract, 2% glucose (glucose medium). Differential growth inhibition studies were carried out in 0.5% yeast extract and one of the following carbon sources: 2% glucose (glucose medium), 3% glycerol (glycerol medium), 3% ethanol (ethanol medium), 3% glycerol and 2% ethanol (glycerol-ethanol medium), 0.5% sodium acetate (acetate medium). Media were prepared at double strength so that an equal volume of stock TZ solution (20 mg/ml or 75 mM) sterilized by filtration, an amino acid solution, or sterile distilled water could be added as required. Media used to identify strains carrying nutritional genetic markers (drop-out media), to select prototrophic colonies (synthetic minimal medium), and to sporulate diploid yeast strains (potassium acetate medium), were prepared as described elsewhere (Sherman *et al.*, 1970). Solid media were made by the addition of 2% agar to the broth component prior to autoclaving. When solid medium containing 10 mg/ml TZ was made, it was found necessary to substitute 0.1 M potassium phosphate buffer for distilled water in order to obtain medium with sufficient firmness for replica plating.

STRAINS - Table 1 lists all strains of *Saccharomyces* used in this study. Phenotypic listings reflect amino acid

and nitrogenous base requirements which were tested prior to use. When it differed from wild-type, strains were tested prior to use. When it differed from wild-type, strains were also classified with respect to their response to TZ. Specific allele designations were not verified. Nomenclature and symbols for markers follow the convention of the Carbondale Yeast Genetics Conference (von Borstal, 1963).

GROWTH EXPERIMENTS - Measurements of the extent of culture growth were routinely carried out in 6 ml test tube cultures. Cell number was usually determined by measuring the optical density of the culture at 590 nm on a Coleman Junior II spectrophotometer. This measurement was correlated with a standard curve of cell number vs. O.D. at 590 nm calibrated by triplicate hemocytometer counts. Determinations of viable cell number by dilution and plating of non-growing cultures were also made in certain cases. Growth study data reported were usually based on a minimum of two separate determinations.

PETITE INDUCTION - Petite (ρ^-) mutants of strain 1493-10C were induced by overnight growth in glucose medium containing 50 µg/ml acriflavine and selected by their inability to grow on glycerol medium (Slonimsky & Ephrussi, 1959). The petite character of these mutants was also verified by measurements of oxygen consumption, and cytochrome spectral patterns.

TABLE 1

- Yeast Strains -

Strain Designation	Nuclear Phenotype	Source
TZ Wild-type Strains		
1493-10C	α, ade, his_4	Hawthorne
1493-10C-p_1	α, ade, his_4	Acriflavine-induced petite mutant derived from 1493-10C
p_2	Same as 1493-10C-P_1	
p_3		
p_4		
p_5		
1412-4D	a, ade, met	Hawthorne
A364A	a, ade_1, ade_2, his_7, ura_1, try_1	Hartwell
clyl-1	a, ade_1, ade_2, his_7, ura_1, try_1, clyl-1	Temperature-sensitive cell lysis mutant derived from A364A
Z1EK-27D	α, lys_1	Mating-type tester strains, Blamire
103-1A	α, his_1	
103-1B	a, his_1	

TABLE 1 (con't.)

- Yeast Strains -

Strain Designation	Nuclear Phenotype	Source
TZ Mutants		
TZR1	a, ade_1, ade_2, his_7, ura_1, try_1, clyl-1, TZR	NTG-induced TZ resistant mutant derived from clyl-1
TZR4		
TZR6		
TZR12	Same as TZR1	
TZR17		
TZR25		
TZS12	a, ade_1, ade_2, his_7, ura_1, try_1, clyl-1, tzs	EMS-induced TZ hypersensitive mutant derived from clyl-1
TZS17	Same as TZS12	
TZS32		

SELECTION OF TZ RESISTANT MUTANTS - Wild-type strains of yeast were unable to grow on ethanol medium in the presence of 5 mg/ml of TZ. Strains resistant to 10 mg/ml of TZ on ethanol medium were selected after mutagenesis with *N*-methyl-*N'*-nitroso-*N*-nitroguanidine (NTG) (Fink & Lowenstein, 1969). Aliquots of a growing cell suspension were plated at a density of 10^7 cells/YEPD plate. Single crystals of NTG were placed in the center of each plate, and the plates incubated at 20C overnight. After mutagenesis, plates were replicated to the ethanol-TZ medium. In 5-7 days, small colonies appeared. These were subcultured and purified by streaking on ethanol-TZ medium. Mutants derived by this method were called "TZR"-series mutants.

The selection of mutants which were hypersensitive to TZ will be described in Chapter VII.

REAGENTS - Thiazolidine-4-carboxylic acid (Grade B) was obtained from Calbiochem in San Diego, California. [^{14}C]-TZ was synthesized according to the method of MacKenzie & Harris (1957). The condensation of equimolar amounts of L-cysteine and [^{14}C]-formaldehyde (Amersham/Searle) to form [^{14}C]-TZ (^{14}C-labeled at carbon #2) was carried out at room temperature. The radioactive yield was typically 40% of the original label. Purity of the twice-recrystallized product and that of the commercially obtained reagent were verified chromatographically using the system described below. The

specific activity of the [^{14}C]TZ, determined as described below, was 0.1 μC_i/mg. Cycloheximide (CHI) was obtained from Calbiochem. Chloramphenicol (CAP) was obtained from Parke-Davis. Erythromycin (ER) was obtained from Eli Lilly. Pyrazole was obtained from Eastman. [^{3}H]-proline was purchased from Amersham/Searle. All other chemicals were specified as reagent grade or better.

BIOCHEMICAL TECHNIQUES - Oxygen consumption of intact yeast cells of whole rat liver mitochondria was determined with a Rank oxygen electrode as described by Plummer (1971).

Alcohol dehydrogenase (ADH, E.C. 1.1.1.1) was assayed by modification of the method of Racker (1955). The procedural and statistical recommendations of Cleland (1967) were followed with respect to the conduct and evaluation of these experiments. Crude extracts of either strain 1493-10C or TZR-4-6A were the source of the enzyme. Cells used for extraction were grown to stationary phase culture in 1 liter of YEPD medium. The cells were harvested, washed with distilled water, 0.1 M potassium phosphate buffer pH 7.4, and finally resuspended in 60 ml of fresh buffer. The suspension was divided into 12 ml aliquots and frozen for subsequent use. After defrosting, the cells were broken in an Eaton press (Eaton, 1962) and the thawed suspension was centrifuged for 1 hour at 250,000 x g at 4C . The supernatant was removed without disturbing the lipid layer and a small portion was diluted 1:1000 with cold 0.01 M potassium

phosphate buffer pH 7.4 containing 0.1% bovine serum albumin (BSA), 10^{-3}M ethylenediamine tetraacetic acid (EDTA) and 10^{-3}M β-mercaptoethanol (BME). The addition of BSA, EDTA, and BME, to the dilution buffer was found to be necessary to maintan enzyme activity; extracts diluted with buffer lacking these reagents lost all enzyme activity within 1-2 hours. The crude enzyme preparation was further diluted to give suitable activity in assays. Total protein in extracts was determined by the microbiuret method (Zamenhoff, 1957) using BSA solution as a standard. All data for enzyme activities reported below were based on at least three determinations.

Radioactivity of chromatographed samples was determined as described elsewhere (Stock & Rice, 1963). Radioactivity of samples on nitrocellulose filters was determined by counting the dry filters on the appropriate channel of a Nuclear Chicago Mark II scintillation counter using a toluene-POP-POPOP cocktail and either an external standards ratio program (^{3}H) or a channels ratio program (^{14}C) to correct for the effects of quenching. All samples were counted at an error level of 5% or less.

Glucose determinations were performed on duplicate samples of dilute culture medium using the coupled glucose oxidase-peroxidase assay (Lloyd & Whelan, 1969). A 100 µg/ml glucose solution, containing the same concentration of yeast extract as was present in the diluted culture samples, was

included in addition to the normal glucose standard to calibrate the assay system. All data points reported were the average of two separate determinations.

Preparation of rat liver mitochondria was carried out according to the procedure of Schnaitman and Greenawalt (1968).

Incorporation of (^{14}C)-TZ into protein was determined on cells of 1493-10C which were harvested from glucose medium at various times in the growth cycle. After washing, and prior to the addition of label, the cell concentration was adjusted to correspond to an optical density at 590 nm of .210. Aliquots of this suspension were allowed to equilibrate for 15 minutes at 30C in 1 ml of 0.1 M potassium phosphate buffer pH 7.0 in the presence or absence of cycloheximide (100 µg/ml), chloramphenicol or erythromycin (4 mg/ml)(see Chapter IV) or both. Labeled TZ was then added to a final concentration of 1 mg/ml. The samples were incubated at 30C for 30 minutes when the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA) followed by boiling for 10 minutes. The samples were filtered (HAWP02400, Millipore Corp.), and washed with eight 5 ml aliquots of 5% TCA and 95% ethanol prior to drying and counting. Control samples containing either no cells or dead cells were also included.

Uptake of [^{3}H]-proline or [^{14}C]TZ was usually determined on washed cell samples by incubation in 0.1 M potas-

sium phosphate buffer pH 7.4 containing either [^{14}C]TZ or [^{3}H]-proline (specific activity 80 $\mu C_i/\mu M$). At various times, 0.2 ml samples were withdrawn, filtered, and washed with four 20 ml aliquots of cold 0.1 M NaCl prior to drying and counting.

METABOLISM EXPERIMENTS - Labeling of cells was carried out in 6 ml of glucose medium containing 10 mg [^{14}C]TZ/ml for various lengths of time. Cells were harvested by centrifugation and washed extensively with distilled water. Further washing of 50 mg portions of cells was carried out on a Millipore filter until the effluent contained negligible radioactivity. The filter was placed in a 20 ml screw-top test tube and the cells were sequentially extracted by 10 minute agitations with 10 ml portions of each of the following: absolute ethanol; ethanol-ether (3:1); ethanol-ether (1:3); followed by two extractions with absolute ether. After the first ethanol extraction, the filter was free from cells and was discarded. The remaining ether was evaporated from the cells in a stream of air and the residue solubilized by addition of 0.5 ml of solubilizing solution (10 mM sodium phosphate pH 7.0, 1% sodium dodecyl sulfate (SDS), and 1% BME) followed by overnight incubation at room temperature (Weber & Osborn, 1969). The insoluble residue was pelleted by centrifugation at 10,000 x g for 20 minutes and the supernatant was carefully drawn off. This fraction represented an extract of whole cell protein. All fractions

were stored in the cold.

The solubilized protein fraction was hydrolyzed in ten times its volume of 6N HCl in sealed evacuated glass ampoules at 110C. After various hydrolysis times, the ampoules were opened and the contents evaporated to dryness at room temperature in a stream of air. The hydrolysate was redissolved in 0.5-1.5 ml of distilled water and stored in the cold.

Samples of the protein hydrolysated and reference samples were chromatographed on thin-layers of MN-300 cellulose (Brinkman Instruments) using butanol-water-glacial acetic acid (4:5:1) as the developing solvent. When a two-dimensional chromatographic system was employed, pyridine-water (4:1) was the second solvent system. After chromatography, amino and imino acids were detected by spraying the plates with ninhydrin (Oden & von Hofstein, 1954). Migration coefficients (Rf's) were the average of triplicate determinations and corresponded closely to the published values (Akhrem & Kuznetsova, 1964). Chromatographic patterns indicated that complete protein hydrolysis occurred within the first 24 hours of incubation.

CHAPTER III

RESULTS

Growth Studies on TZ-treated and Control Cells:

Unpublished preliminary studies by Wilkie and Eaton suggested that TZ inhibited the growth of susceptible yeast cells differentially. When exposed to 5 mg TZ/ml, cell growth was totally inhibited on a medium containing a non-fermentable carbon source. By contrast, after an initial lag which lasted from 8 to 10 hours, cell growth proceeded in the presence of 10 mg TZ/ml if the medium contained a fermentable carbon source, such as 2% glucose. Although parameters such as the concentration of TZ necessary to inhibit growth on ethanol medium were reproducible in any one strain, examples of strain-dependent variation were observed.

These findings were interpreted to suggest the possibility that the analogue might be incorporated differentially by the two protein synthetic systems of the yeast cell. Selective analogue incorporation into mitochondrial protein could result in a defective organelle and loss of the ability to metabolize non-fermentable substrates. By contrast, growth on fermentable substrates would be relatively uneffected, and could proceed at analogue concentrations higher than those necessary to inhibit growth on non-fermentable substrates. Based on this hypothesis, it should be expected that equivalent growth inhibition by TZ would be seen with

all non-fermentable substrates. Furthermore, incorporation studies using labeled TZ should demonstrate the selective analogue incorporation.

The Effect of TZ on Cells Grown in Glucose Medium:

A dose-response curve for strain 1493-10C, a representative susceptible strain, is shown in Figure 3. When present initially, TZ increased the length of the lag phase. The extent of the increase was directly related to the TZ concentration. In each case, the lag period was followed by an apparent recovery period, during which the rate of growth of the TZ-treated cell populations increased, although it never reached that seen in the untreated culture. As the TZ concentration increased, stationary phase was reached at progressively later times, accompanied by a decrease in final cell titre. This overall description was accurately reflected in the response of other yeast strains to TZ (Fig. 4).

There are several possible explanations for the extended lag phase exhibited by TZ-treated cells in glucose medium. Magaña-Schwencke and others (1973) have observed that proline analogues, including TZ can compete with proline for transport into *S. chevalieri*. If analogue concentrations were sufficiently high, this competition could effectively prevent the cell from accumulating the levels of proline required for optimum growth. The altered pool

Fig. 3. TZ dose-response curve for strain 1493-10C; glucose medium.

FIGURE 3

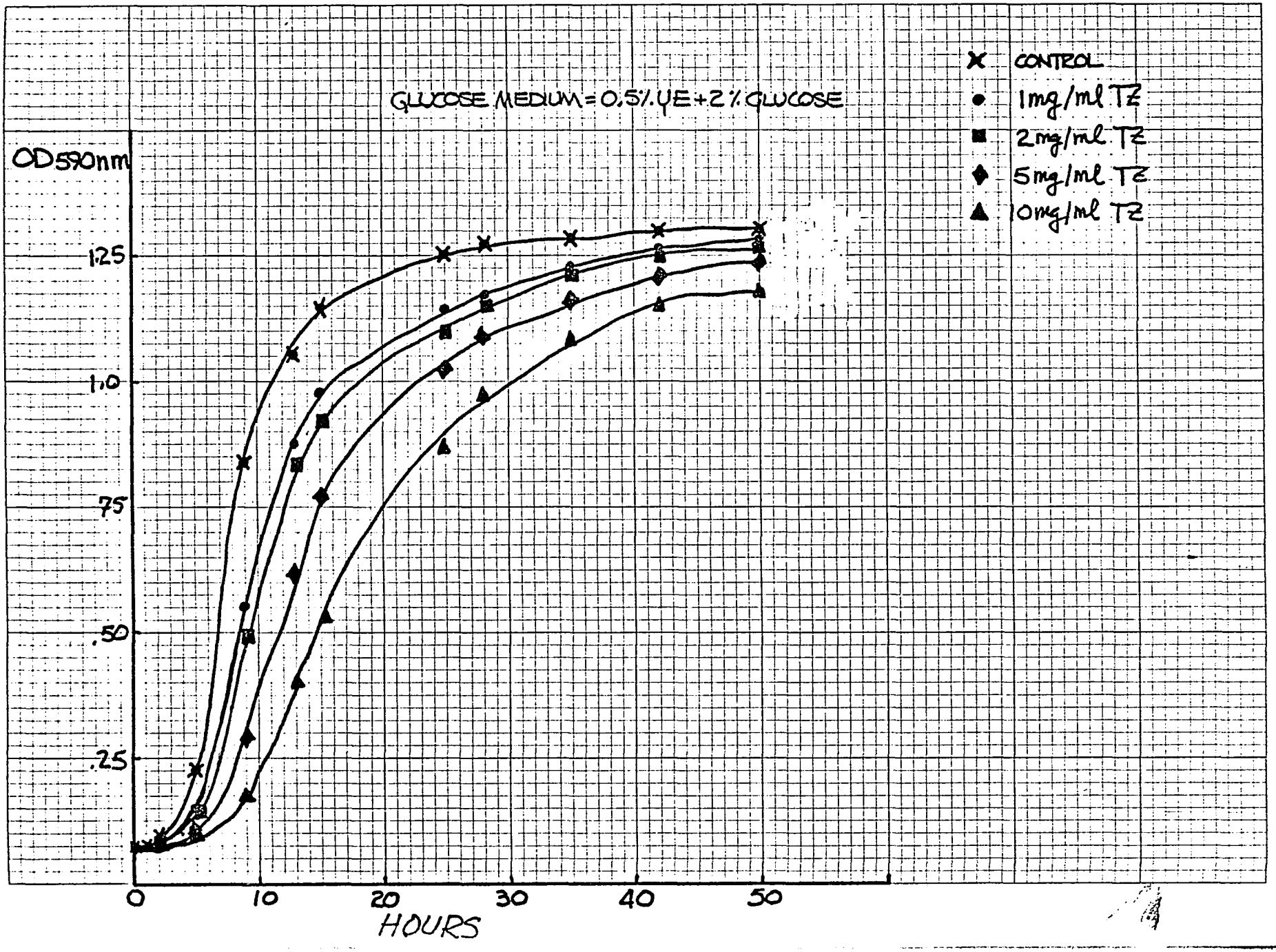
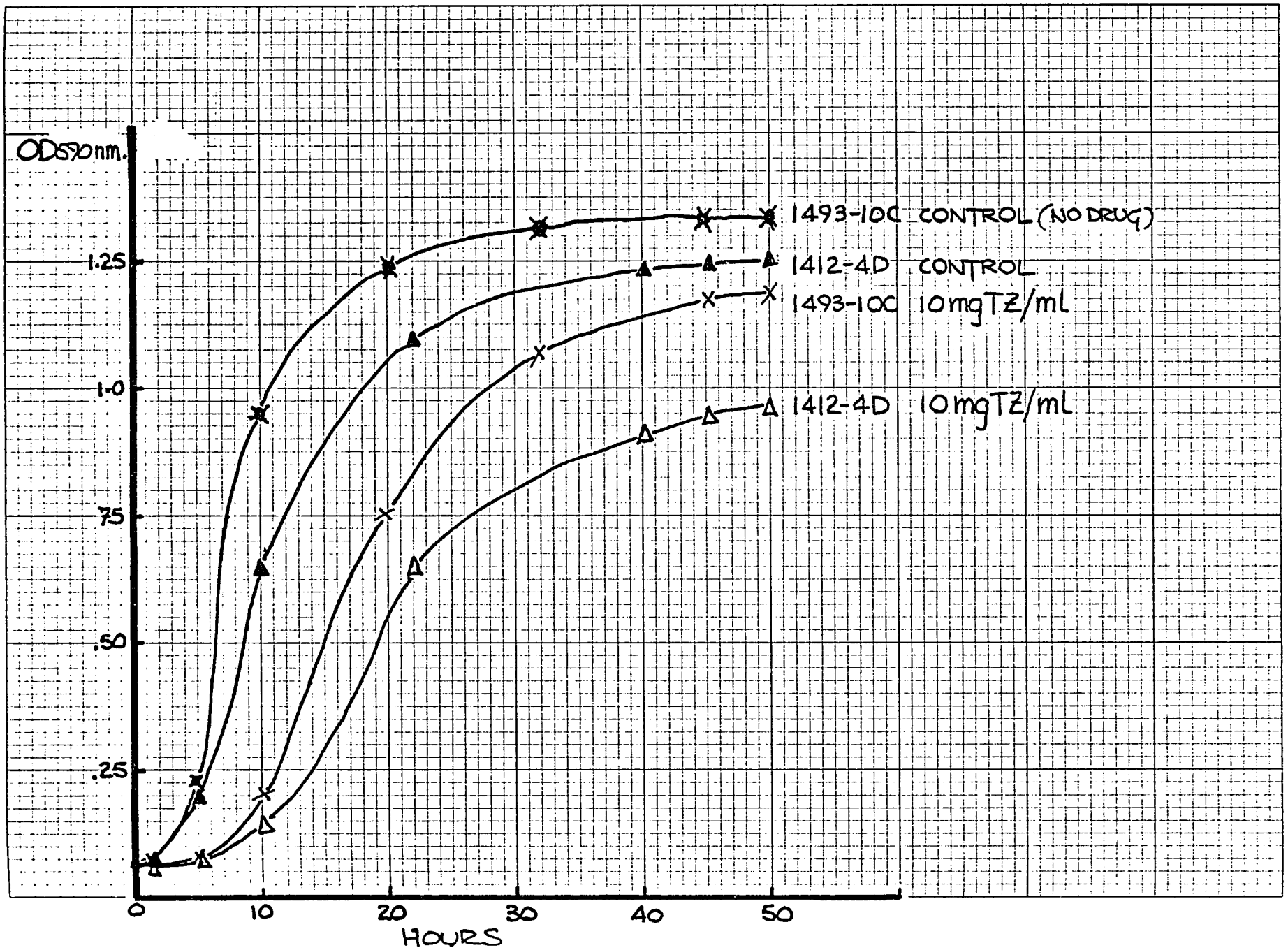

GLUCOSE MEDIUM = 0.5% YE + 2% GLUCOSE
CONTROL
1mg/ml TZ
2mg/ml TZ
5mg/ml TZ
10mg/ml TZ
OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS

Fig. 4. The response of different yeast strains to TZ on glucose medium.

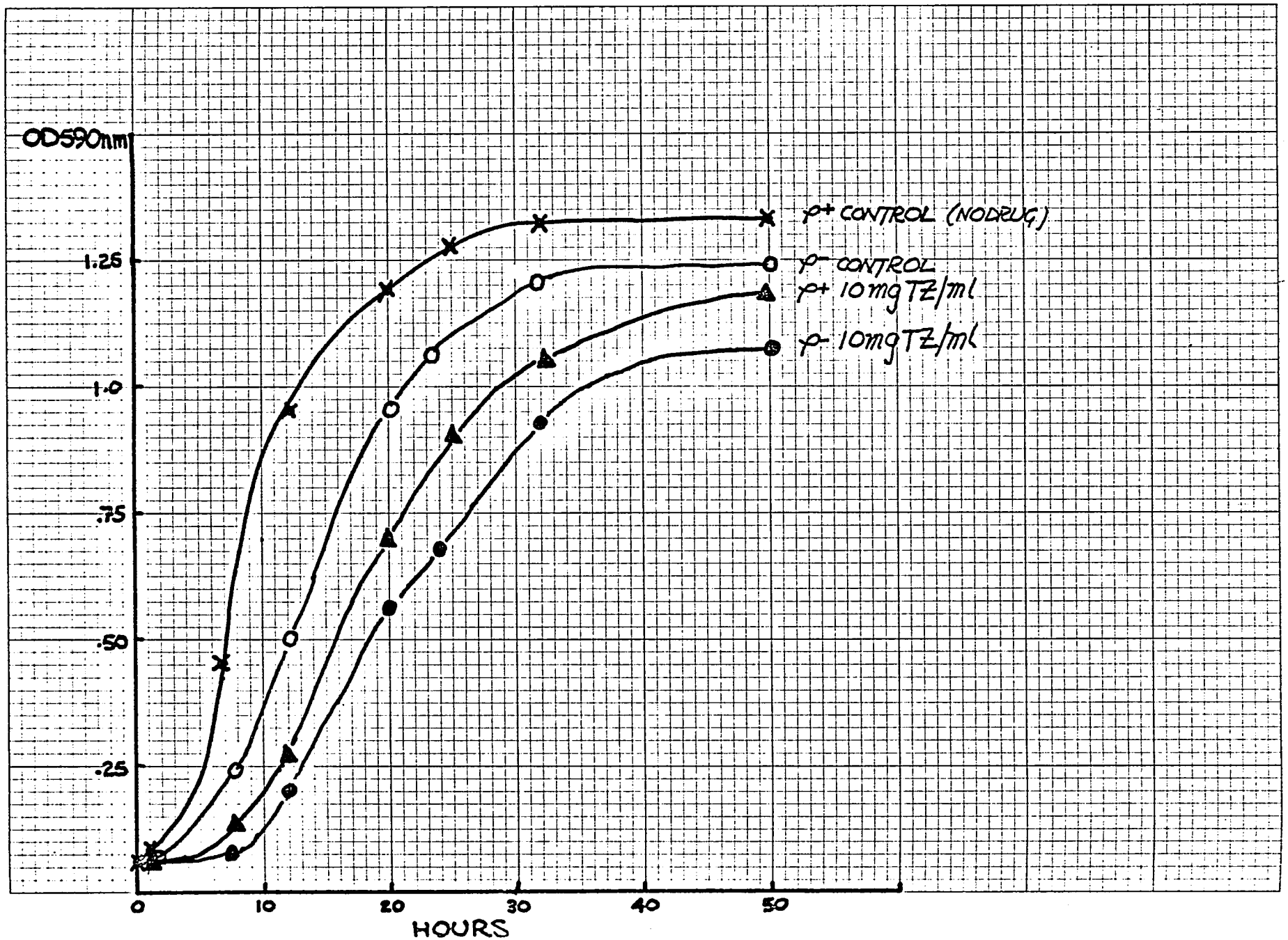
FIGURE 4


OD590nm.
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
1493-10C CONTROL (NO DRUG)
1412-4D CONTROL
1493-10C 10mgTZ/ml
1412-4D 10mgTZ/ml

size of this amino acid would be reflected in lower growth rates as a result of decreased rates of protein synthesis. In a related manner, TZ, though not incorporated into the products of cytoplasmic protein synthesis, could compete with proline for the cytoplasmic prolyl-t-RNA synthetase. Such antagonism has been postulated (Richmond, 1966) and is thought to be transient, since the intracellular concentration of unincorporated amino acid would increase and eventually outcompete the analogue. If such were the case, then it should be possible to demonstrate TZ uptake into the cell, though not its incorporation into protein. Linstead, Evans and Wilkie (1973) have suggested that the mitochondrial protein synthetic system contributes products of cellular substructures, notably membranes. Under these circumstances, production of faulty mitochondrial protein by virtures of analogue incorporation could have consequences for other cell functions. The "TZ-glucose lag" could thus be a manifestation of this relationship. One consequence of this hypothesis is the prediction that the TZ-glucose lag should not be seen in petite(ρ^-) cultures, since such cells lack mitochondrial protein synthesis. Figure 5 shows a typical response of acriflavine-induced petites (ρ^-) to TZ on glucose medium. This behavior, which was observed in five separately selected petites, indicated that the TZ-glucose lag did not depend for its expression on the presence of a functional mitochondrial protein synthetic system.

Fig. 5. The response of acriflavine-induced petites (ρ^-) of strain 1493-10C to 10 mg TZ/ml on glucose medium.

FIGURE 5


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
ρ+ CONTROL (NO DRUG)
ρ- CONTROL
ρ+ 10mg TZ/ml
ρ- 10mg TZ/ml

Alternative to the possible explanations of the TZ-glucose lag already discussed, is the hypothesis that the analogue was incorporated into cytoplasmically-synthesized protein. Evidence for this incorporation into rat liver cytoplasmic protein (Bekhor *et al.*, 1965) has already been mentioned. Such an hypothesis, is however inconsistent with the idea of selective analogue incorporation, and differential inhibition of growth by the analogue then appears anomalous.

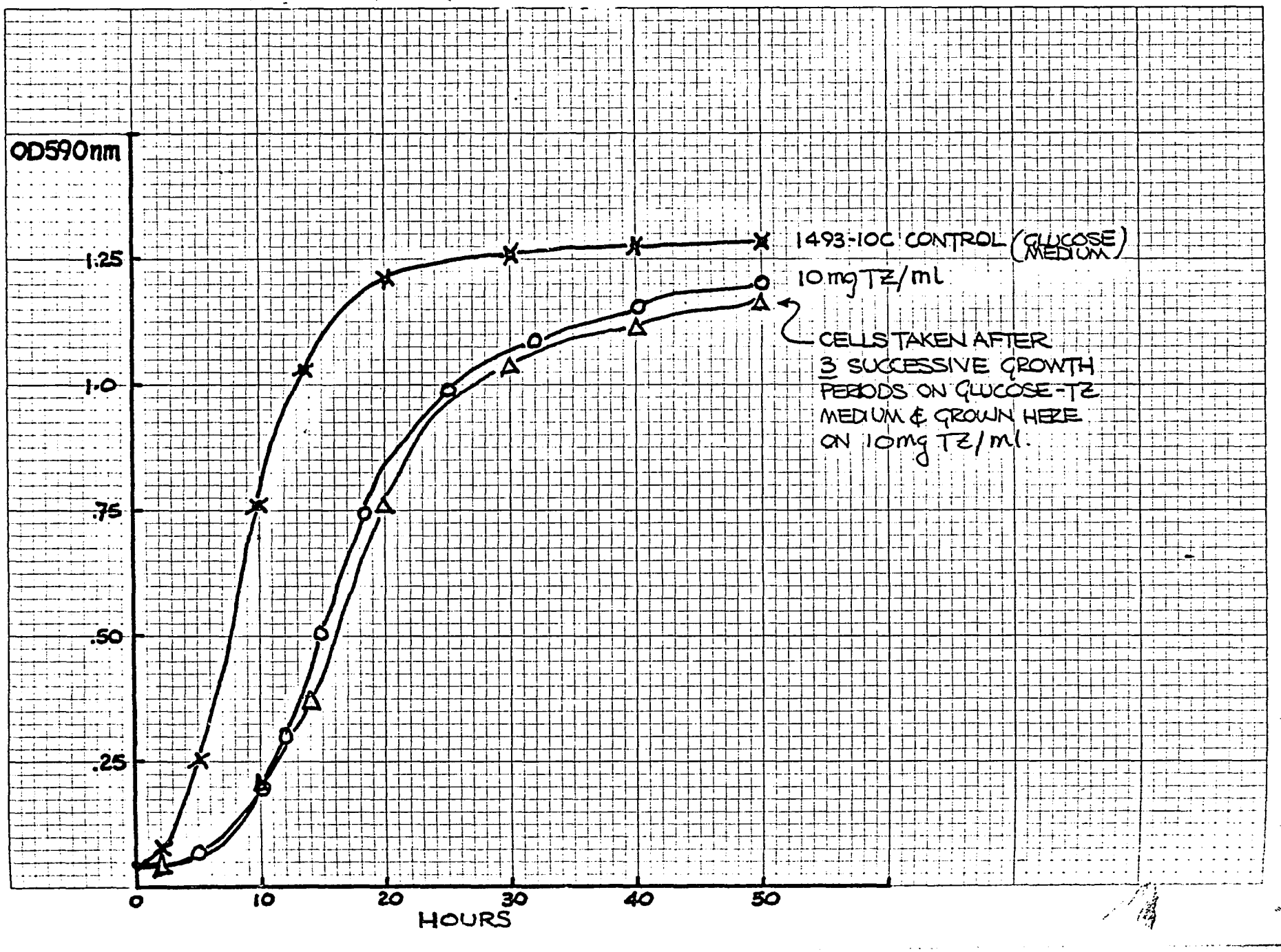
As mentioned previously, the lag period is followed by a recovery phase, during which the growth rate increased as cells actively divided. It can be demonstrated that recovery did not proceed by selection of cells which were resistant to the analogue. Serial transfers of glucose-TZ grown cells to fresh glucose-TZ medium, produced growth kinetic data which were identical to those of the original culture (Fig. 6). Significantly, cells which had recovered from the TZ-glucose lag were able to grow when inoculated into glycerol medium. This observation is not consistant with the hypothesis of growth inhibition by selective analogue incorporation into the mitochondrial compartment.

Richmond (1962) has observed that the effects of analogue action which rely on the ability of the analogue to replace the natural amino acid could be specifically reversed by that amino acid. Indeed, Unger and DeMoss (1966) have shown that TZ growth inhibition of *E. coli* cells was spe-

Fig. 6. The response of cells* to 10 mg TZ/ml after 3 successive growth periods in glucose-TZ medium.

* Unless otherwise stated, strain 1493-10C was used in all studies.

FIGURE 6


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
1493-10C CONTROL (GLUCOSE MEDIUM)
10 mg TZ/ml
CELLS TAKEN AFTER 3 SUCCESSIVE GROWTH PERIODS ON GLUCOSE-TZ MEDIUM & GROWN HERE ON 10mg TZ/ml.

cifically proline-reversible. Figure 7 shows the effect of proline on the TZ-glucose lag and subsequent recovery. Increasing concentrations of proline decreased the initial lag period and facilitated a more complete recovery. This effect was proline-specific, and other amino acids tested did not reverse the inhibition. This inhibition was in fact measurably increased by the presence of cysteine. This observation will be discussed more fully in Chapter VIII.

The Effect of TZ on Ethanol-Grown Cells:

Cell growth was totally inhibited on TZ-ethanol medium at one-half the concentration that still allowed growth on glucose (Fig. 8). This response was reproducible in any one strain, although as mentioned, minimum inhibitory concentrations varied between strains. Growth cessation was rapid and final; no recovery phase was observed even after 100 hours. However, cells were neither killed nor made petite by this treatment. Viable count data indicated that cells exposed to ethanol-TZ medium (5 mg TZ/ml) remained viable to the extent of 90% after 24 hours of exposure. In addition, the petite frequency of these same cells was determined by replica plating colonies onto glycerol medium and scoring the percentage of all colonies which were respiratory competent. The petite frequency (2-4%) of the TZ-treated cells was identical to that of untreated control cells. By contrast to the effects seen on glucose-TZ medium, the addition of as much as 100 mM proline to ethanol-

Fig. 7. The effect of amino acids on TZ inhibition of cells grown on glucose medium (1493-10C).

FIGURE 7

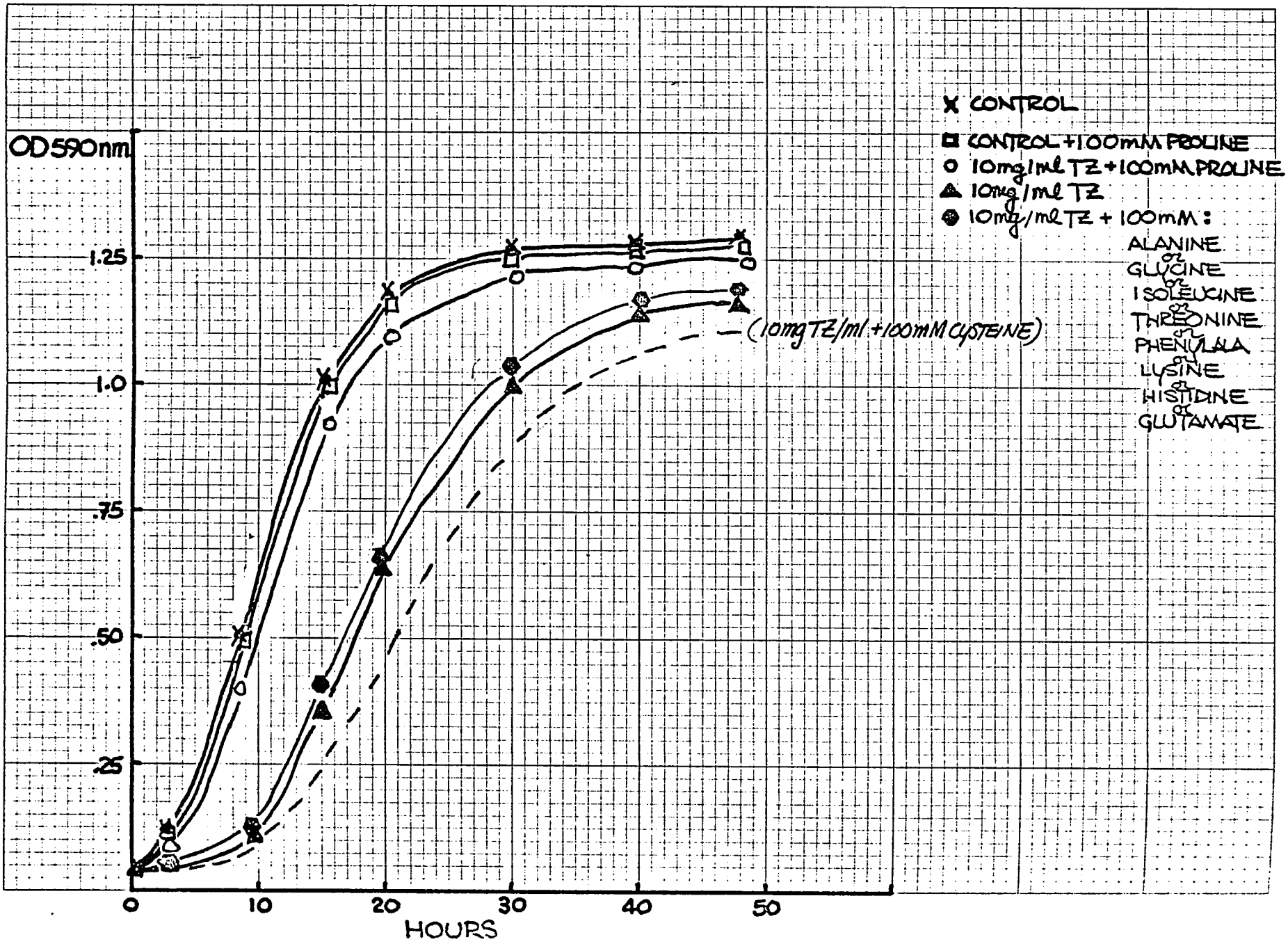
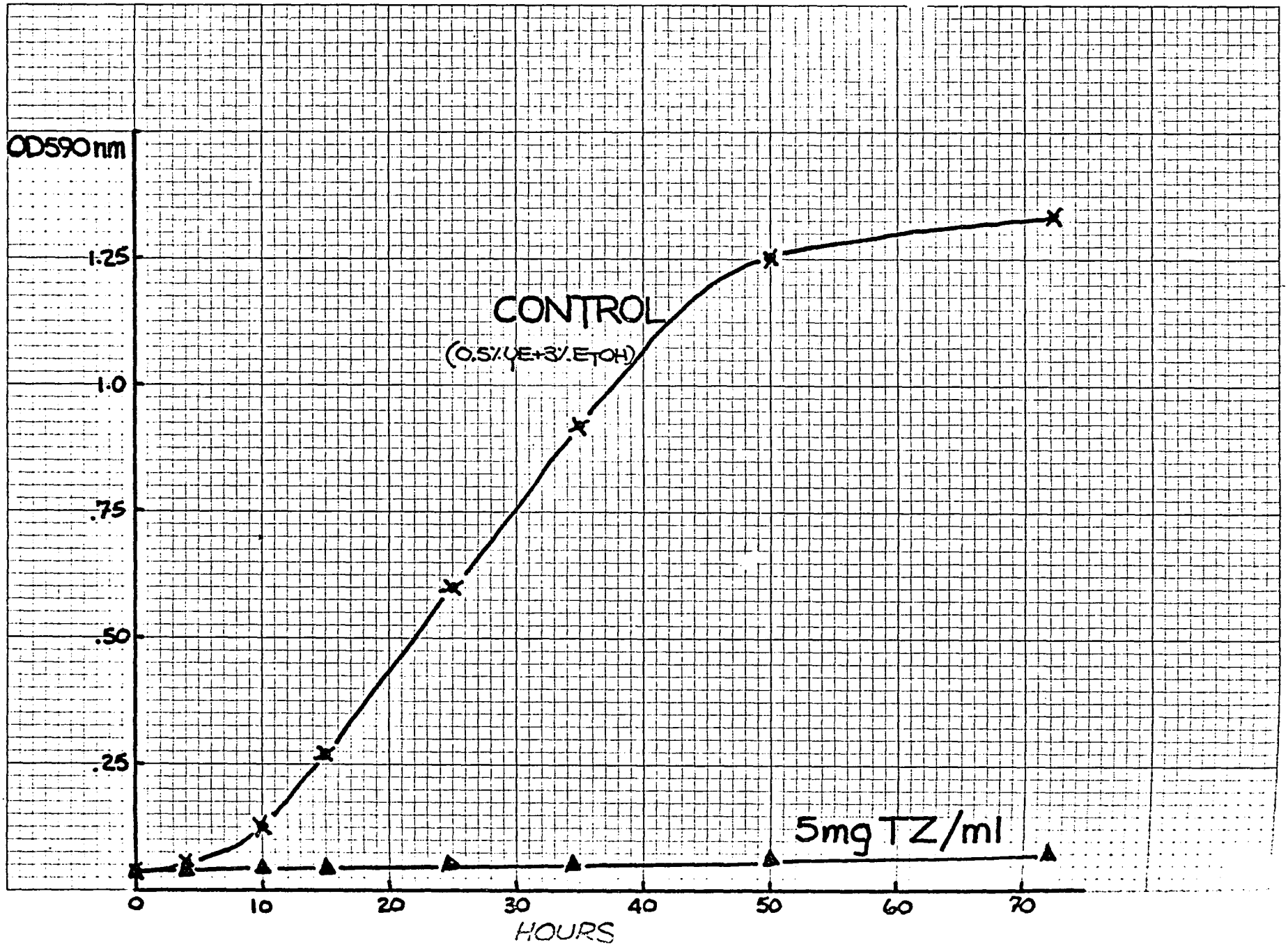

OD 590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
X CONTROL
CONTROL + 100mM PROLINE
10mg/ml TZ + 100mM PROLINE
10mg/ml TZ
10mg/ml TZ + 100mM:
ALANINE
GLYCINE
ISOLEUCINE
THREONINE
PHENYLALA
LYSINE
HISTIDINE
GLUTAMATE
(10mg TZ/ml + 100mM CYSTEINE)

Fig. 8. The response of cells to TZ on ethanol medium.

FIGURE 8


OD590 nm
1.25
1.0
.75
.50
.25
CONTROL
(0.5% YE+3% ETOH)
5mg TZ/ml
0
10
20
30
40
50
60
70
HOURS

TZ medium did not reverse growth inhibition (Fig. 9). However, the addition of 0.5% glucose to ethanol-TZ medium could bring about recovery after a considerable lag (Fig. 10). Cells rescued by glucose were found to be respiratory competent, and grew on glycerol medium. These results suggest that the TZ-mediated growth inhibition observed on glucose and ethanol media may have resulted by different mechanisms, one proline reversible, the other not.

The Effect of TZ on Cells Grown on Other Non-Fermentable Substrates:

In order to characterize further the response of yeast cells to this analogue, growth studies employing other non-fermentable substrates were undertaken. In contrast to the pattern of complete growth inhibition seen on ethanol-TZ medium, growth on glycerol-TZ medium proceeded after an initial lag, followed by recovery and a low stationary phase cell titre (Fig. 11). This pattern was similar to that obtained for treated cultures grown on glucose.

These results are inconsistant with those predicted from the original hypothesis of differential TZ inhibition by selective incorporation of the analogue. In addition, they have raised the question of whether glycerol metabolism, in the presence of TZ, proceeded in the same manner as in its absence. In this conclusion, mutants of Neurospora have been isolated which exhibited an alternate respiratory pathway which was cyanide-insensitive (Edwards

Fig. 9. The effect of proline on TZ inhibition of cells grown on ethanol medium.

FIGURE 9

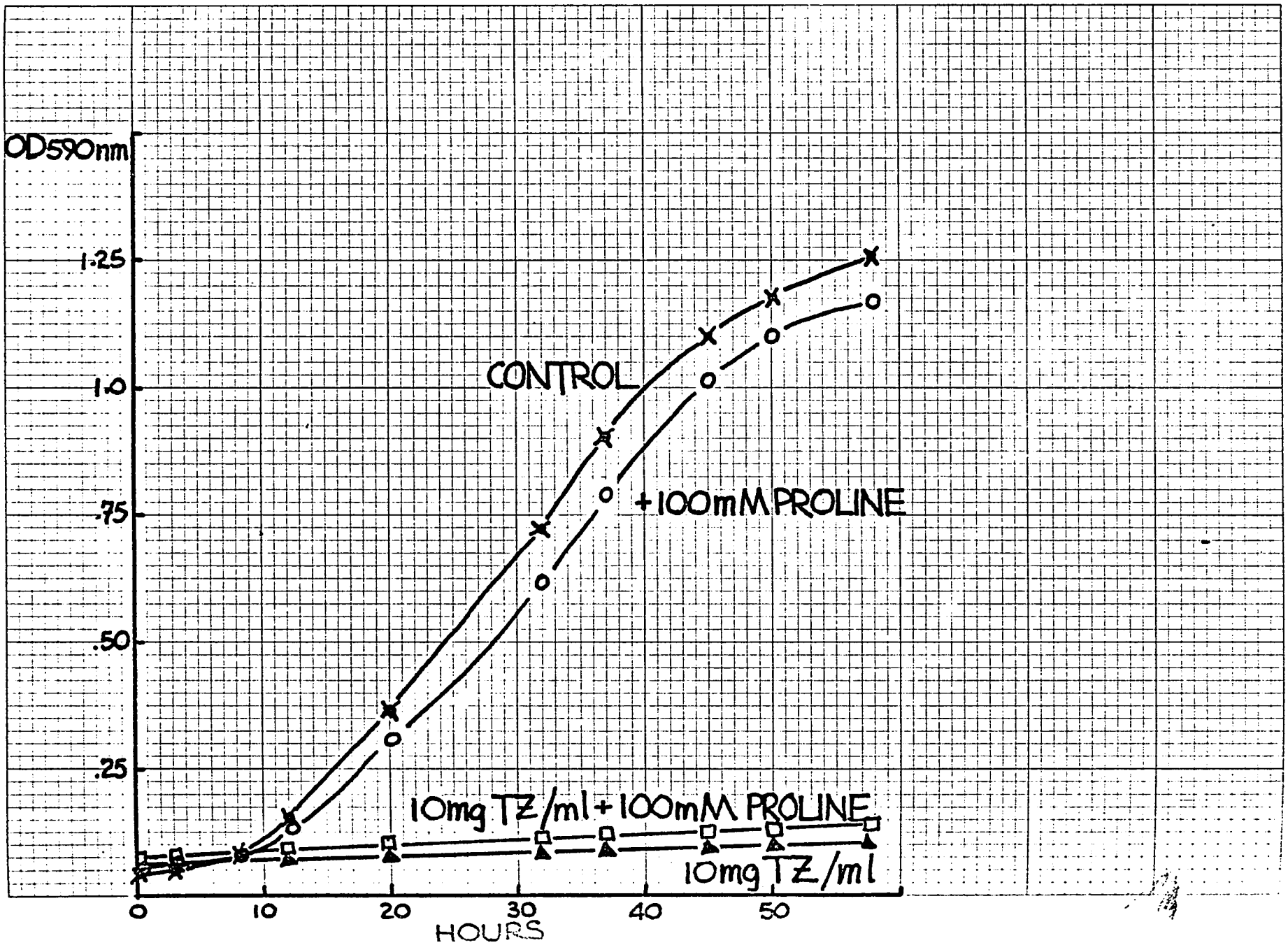

OD590nm
1.25
1.0
.75
.50
.25
CONTROL
+100mM PROLINE
10mg TZ/ml + 100mM PROLINE
10mg TZ/ml
0
10
20
30
40
50
HOURS

Fig. 10. Glucose-mediated recovery from TZ inhibition on ethanol medium. 0.5% glucose was added at times indicated by arrows.

FIGURE 10

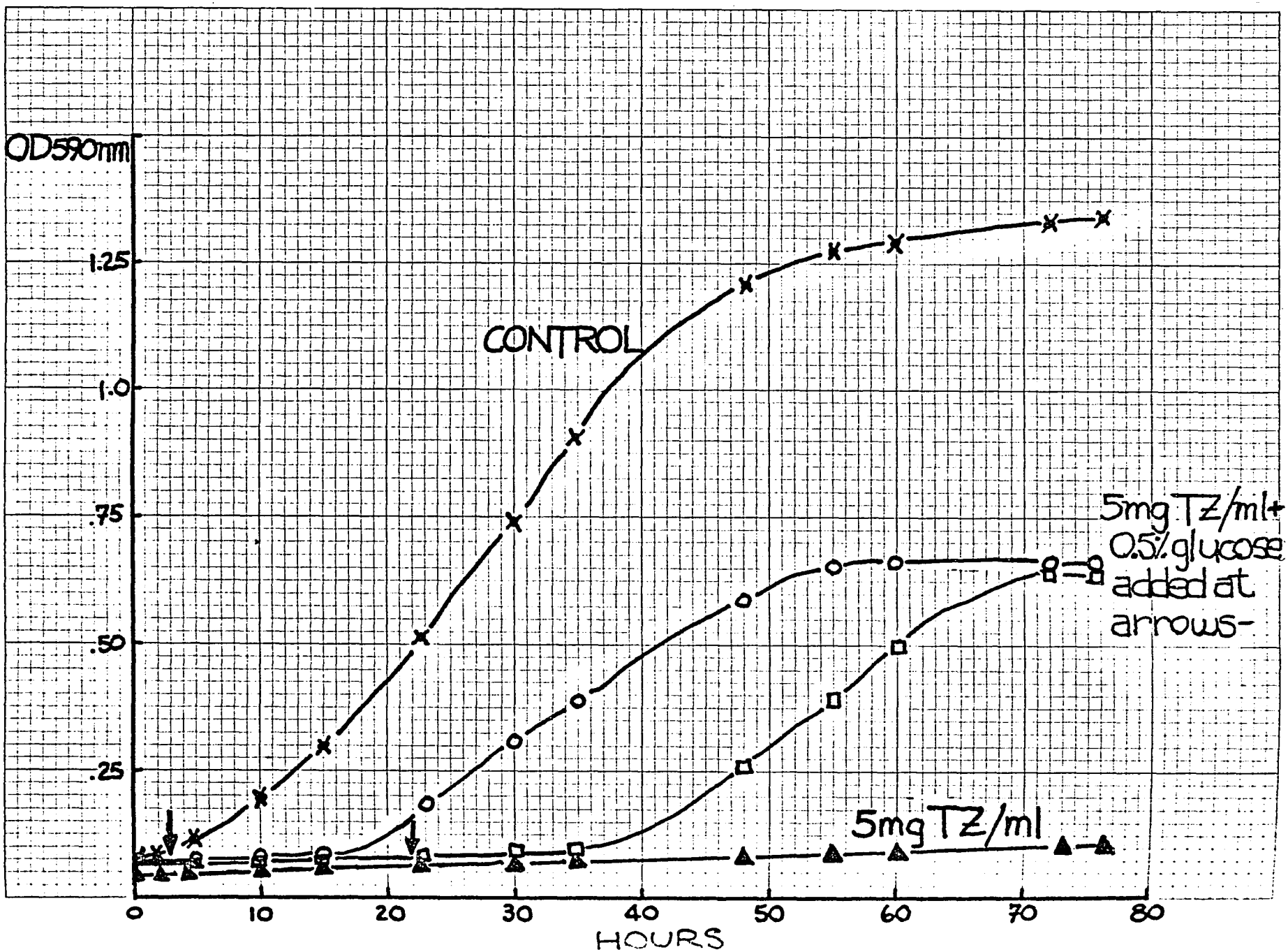
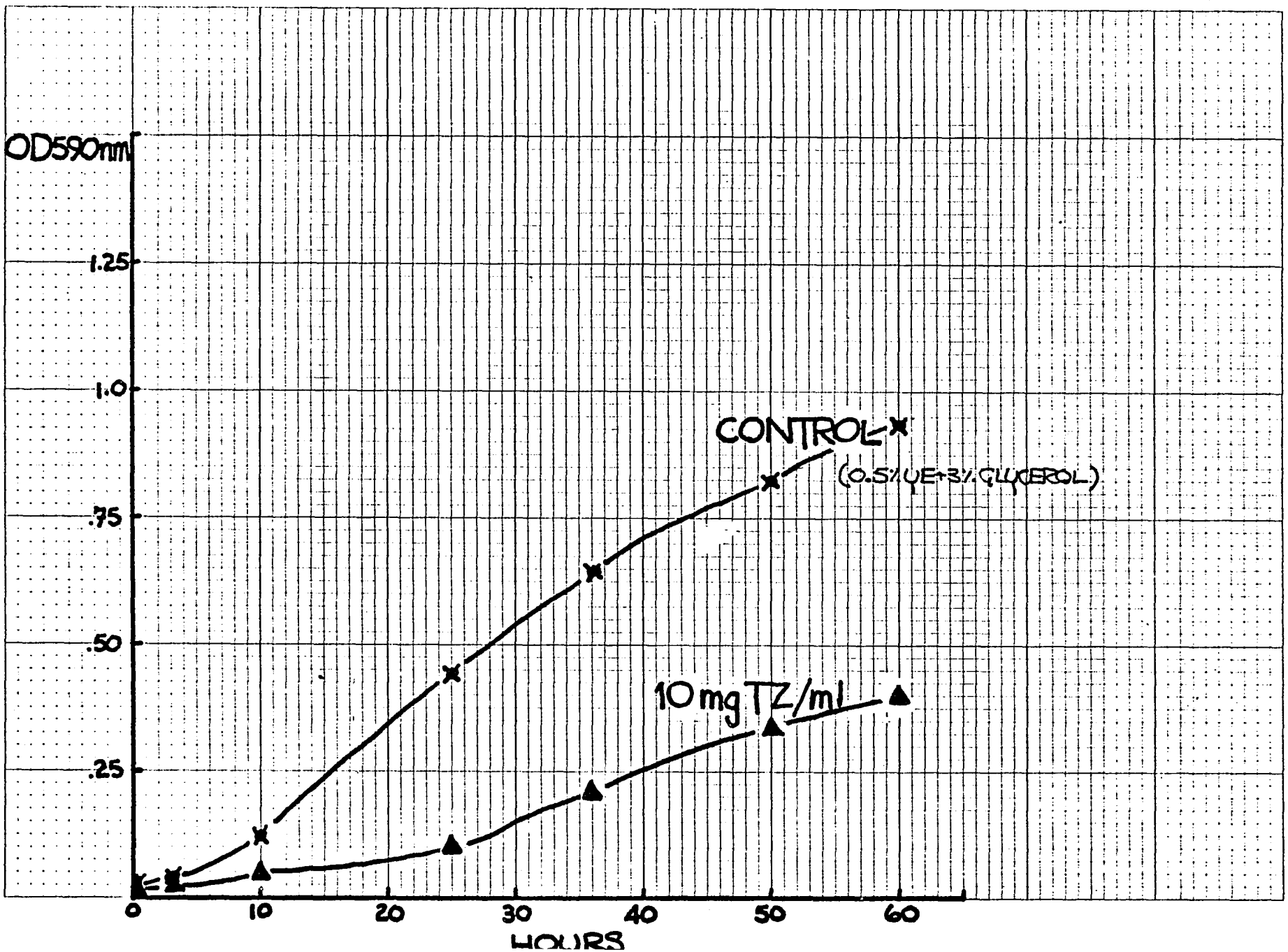

OD590nm
1.25
1.0
.75
.50
.25
CONTROL
5mg Tz/ml+
0.5% glucose
added at
arrows-
5mg Tz/ml
0
10
20
30
40
50
60
70
80
HOURS

Fig. 11. The response of yeast cells to TZ on glycerol medium.

FIGURE 11


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
HOURS
CONTROL
(0.5% YE + 3% GLYCEROL)
10 mg TZ/ml

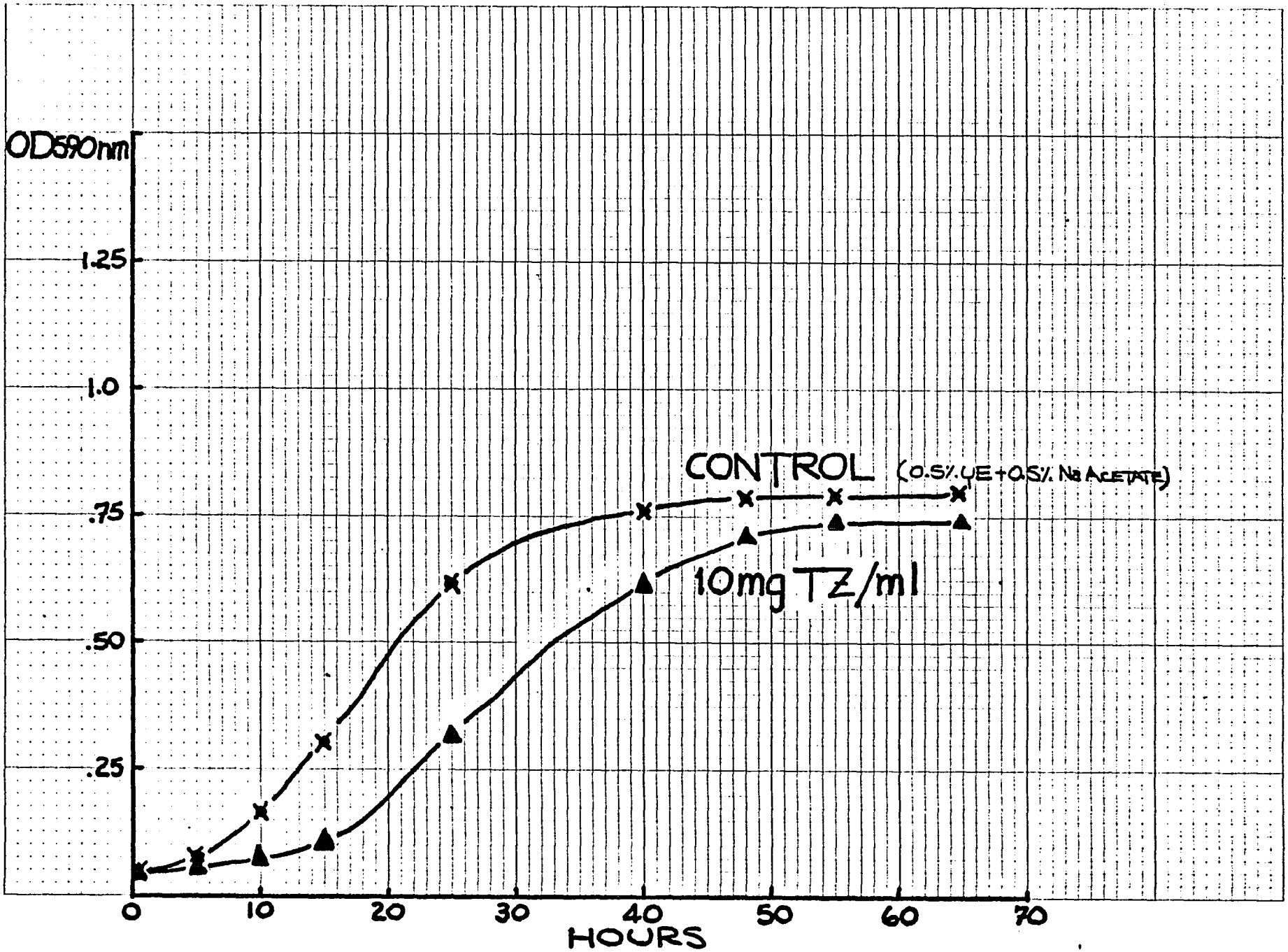
et al., 1974). This possibility was dismissed as inhibitors of an early step of glycerol utilization (30 mM NaF) in glycolysis, or of terminal electron transport (KCN), totally inhibited TZ-glycerol growth. Thus glycerol metabolism in TZ-treated cultures appeared to proceed by the same pathway as in untreated cultures. These results imply that the mitochondrial system was at least partially functional in TZ-treated cells.

The previous observations are strengthened by the results of growth studies on acetate-TZ medium. The growth patterns observed were similar to those on glycerol-TZ and glucose-TZ media (Fig. 12). In addition, acetate-TZ grown cells showed relatively a shorter lag period and a higher final cell titre than glycerol-TZ grown cells. Both of these parameters indicated a less severe response to TZ when cells were grown on acetate. In contrast to glycerol utilization, whose early steps usually involve enzymes of the glycolytic pathway, acetate oxidation is completely mitochondrial (Sols, *et al.*, 1970). Extensive cell growth on acetate medium argues strongly for a functional mitochondrial system.

The results of these growth studies established that cells showed repeatable patterns of response to TZ which depended upon the nature of the carbon source in the medium. Although varying in extent, an initial growth lag, followed by a period of recovery was chracteristically observed in

Fig. 12. The response of yeast cells to TZ on acetate medium.

FIGURE 12


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
70
HOURS
CONTROL (0.5% YE + 0.5% Na ACETATE)
10mg TZ/ml

TZ-treated cultures grown on glucose, glycerol, or acetate medium. These effects were at least partially reversible by proline in all cases, including petite cultures grown on glucose-TZ medium (Fig. 13). By contrast, the growth of cultures was completely inhibited by TZ on ethanol medium. This inhibition could not be reversed by the addition of either proline or other amino acids to the culture medium. Glucose added to the culture medium could relieve the inhibition, however this relief was relatively incomplete and was accomplished only after an extended lag period.

The effect of ethanol on the cellular response to TZ was elucidated by growth studies undertaken in which 2% ethanol was added to either glucose-TZ or glycerol-TZ medium (Figs. 14 & 15). Such additions increased the severity of the response of cells to TZ, distorting the glucose recovery phase, and completely inhibiting growth on glycerol-TZ medium. When ethanol was added to glucose medium without TZ, there was a measurable inhibition of growth (Fig. 14). It is likely that this effect was mediated at the level of cytoplasmic alcohol dehydrogenase. During glucose metabolism, this enzyme would normally reduce the acetaldehyde produced during glycolysis to ethanol. Elevated intracellular levels of ethanol would be expected to shift the reaction equilibrium away from ethanol production and thus interfere with NADH oxidation. Under conditions of catabolite repression, such effects should lower the rate of flux through the glycolytic

Fig. 13. The effect of proline on TZ inhibition of petite (p^-) cells of strain 1493-10C grown on glucose medium.

FIGURE 13

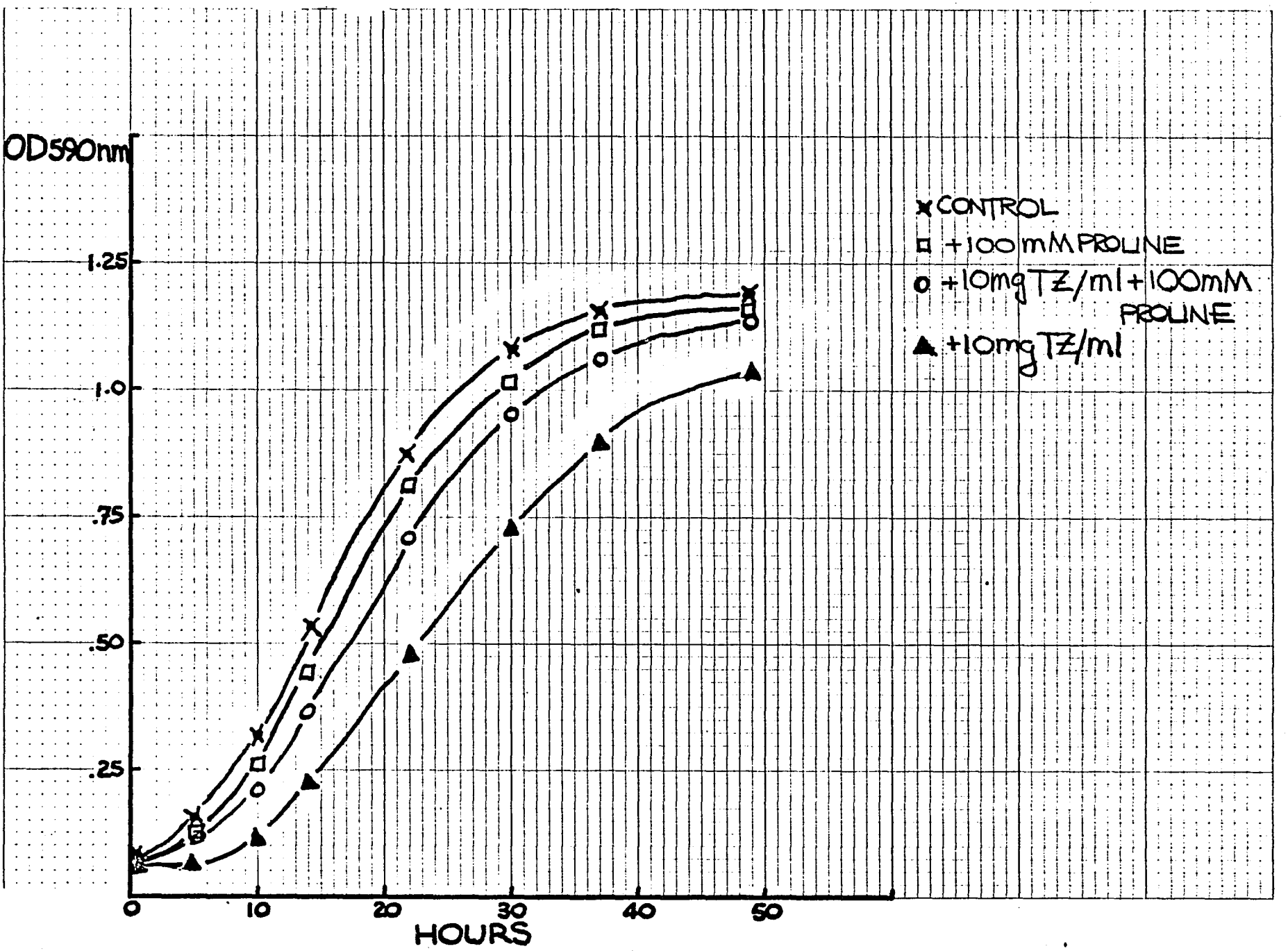

OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
CONTROL
+100 mM PROLINE
+10mg TZ/ml+100mM PROLINE
+10mg TZ/ml

Fig. 14. The effect of 2% ethanol on cell growth in glucose-TZ medium.

FIGURE 14

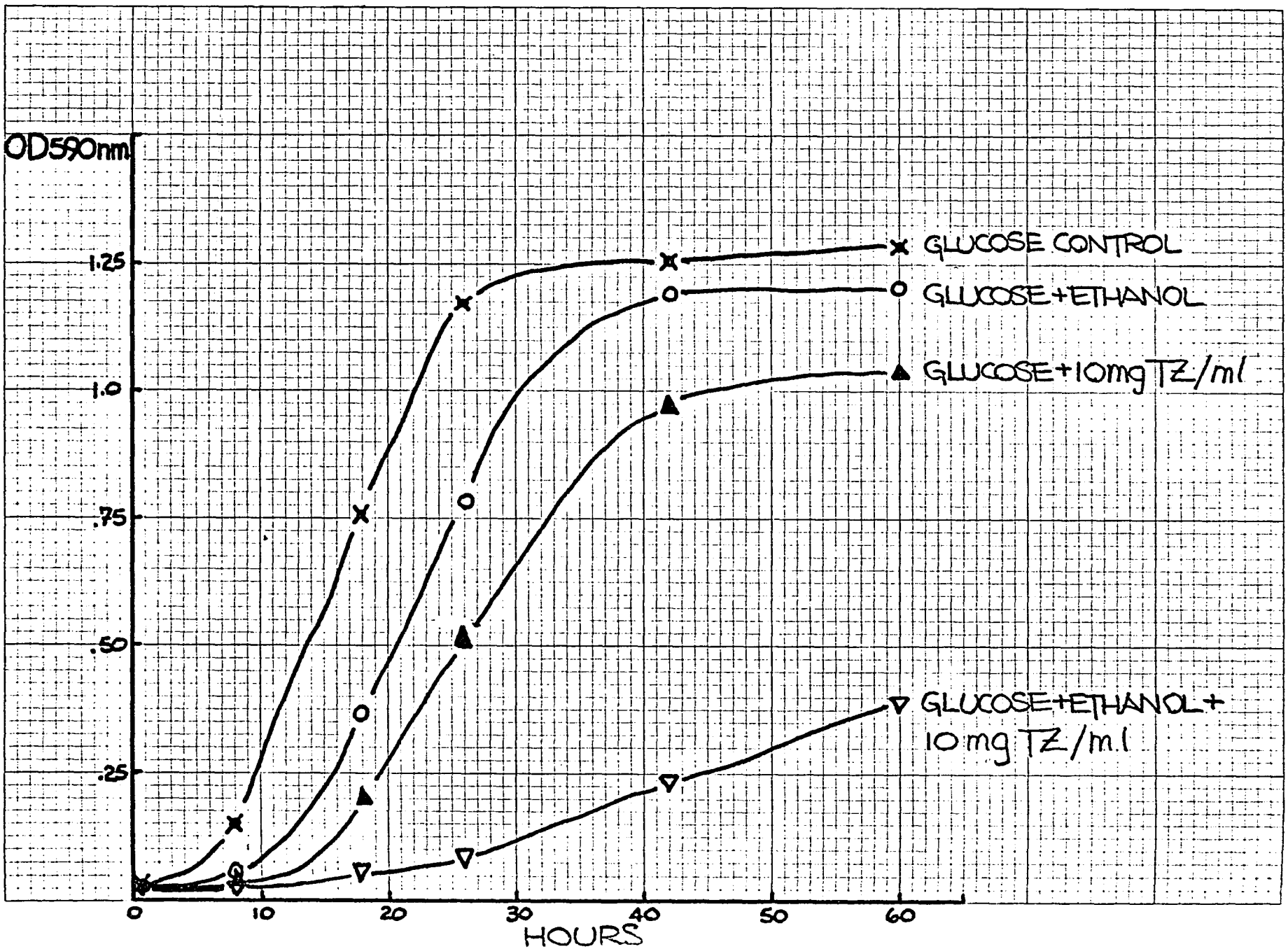
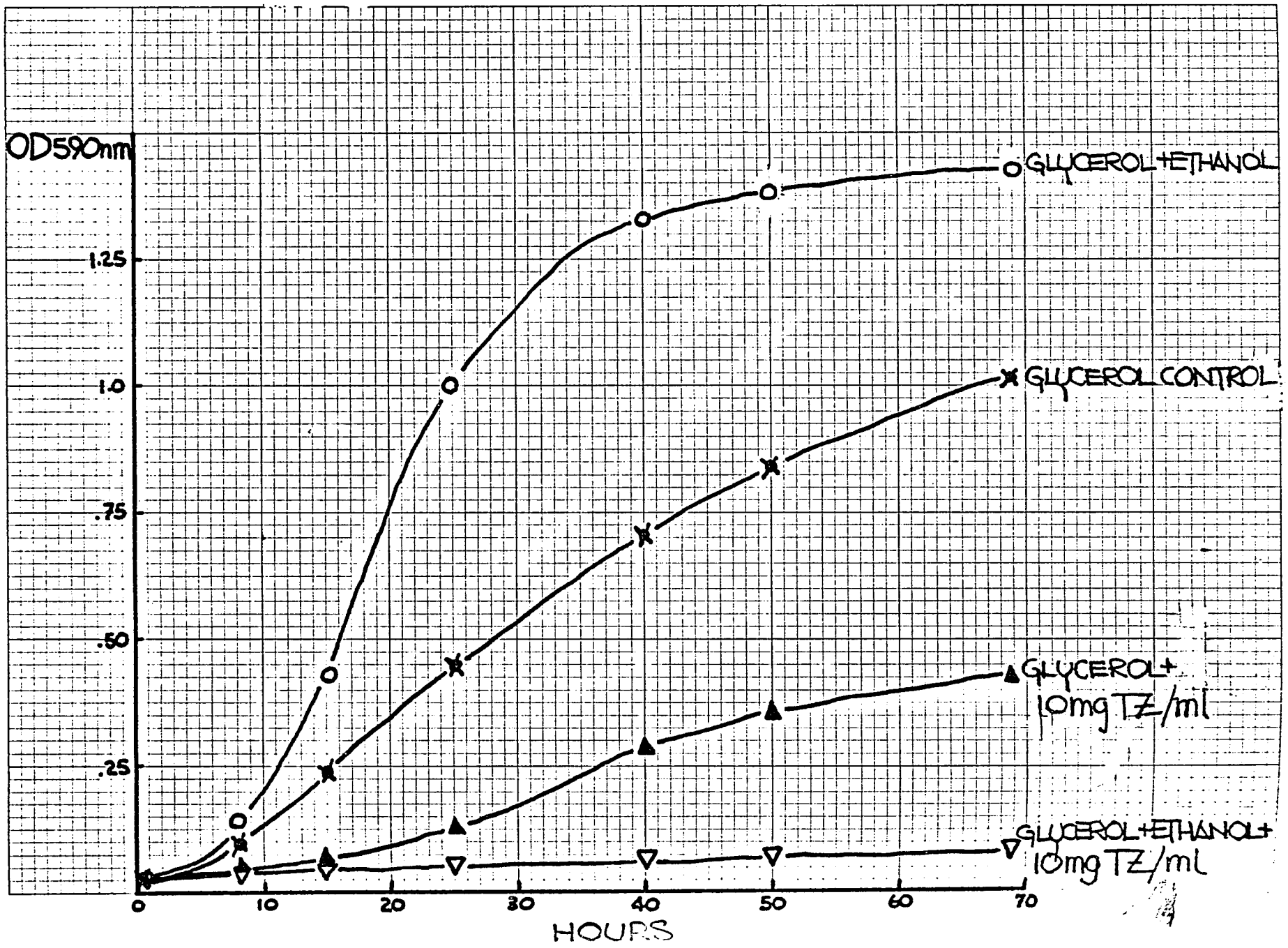

OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
HOURS
GLUCOSE CONTROL
GLUCOSE+ETHANOL
GLUCOSE+10mg TZ/ml
GLUCOSE+ETHANOL+
10 mg TZ/ml

Fig. 15. The effect of 2% ethanol on cell growth in glycerol-TZ medium.

FIGURE 15


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
70
HOURS
GLYCEROL+ETHANOL
GLYCEROL CONTROL
GLYCEROL+
10mg TZ/ml
GLYCEROL+ETHANOL+
10mg TZ/ml

pathway, resulting in slower rates of growth. If cells were not catabolite repressed, as in the case of growth on glycerol-ethanol medium, NADH produced glycolytically could be efficiently oxidized by the cytochrome system. Both substrates would be available to the mitochondrial system and rapid growth rates would be expected. Although such rapid growth was observed, total growth inhibition on glyceral-ethanol-TZ medium implied that ethanol was also interfering with glycerol utilization. This possibility will be discussed in a later section. If glucose-acetate-TZ or glycerol-acetate-TZ media were used, the severity of the TZ response was not dramatically increased. Rather, acetate actually stimulated growth on glycerol containing media, while showing a small but definite inhibition of glucose growth (Figs. 16 & 17).

These data indicate that the response of yeast cells to TZ is mediated under certain circumstances of growth by ethanol metabolism. These ethanol-dependent effects were not proline-reversible and thus differ from the initial TZ growth lag observed with other carbon sources.

As will be discussed below, the proline-reversible and irreversible components of TZ growth inhibition represent two different modes of action of the analogue. The proline-reversible component, recognized on glucose medium will be dealt with mechanistically in the following section.

Fig. 16. The effect of 0.5% acetate on cell growth in glucose-TZ medium.

FIGURE 16

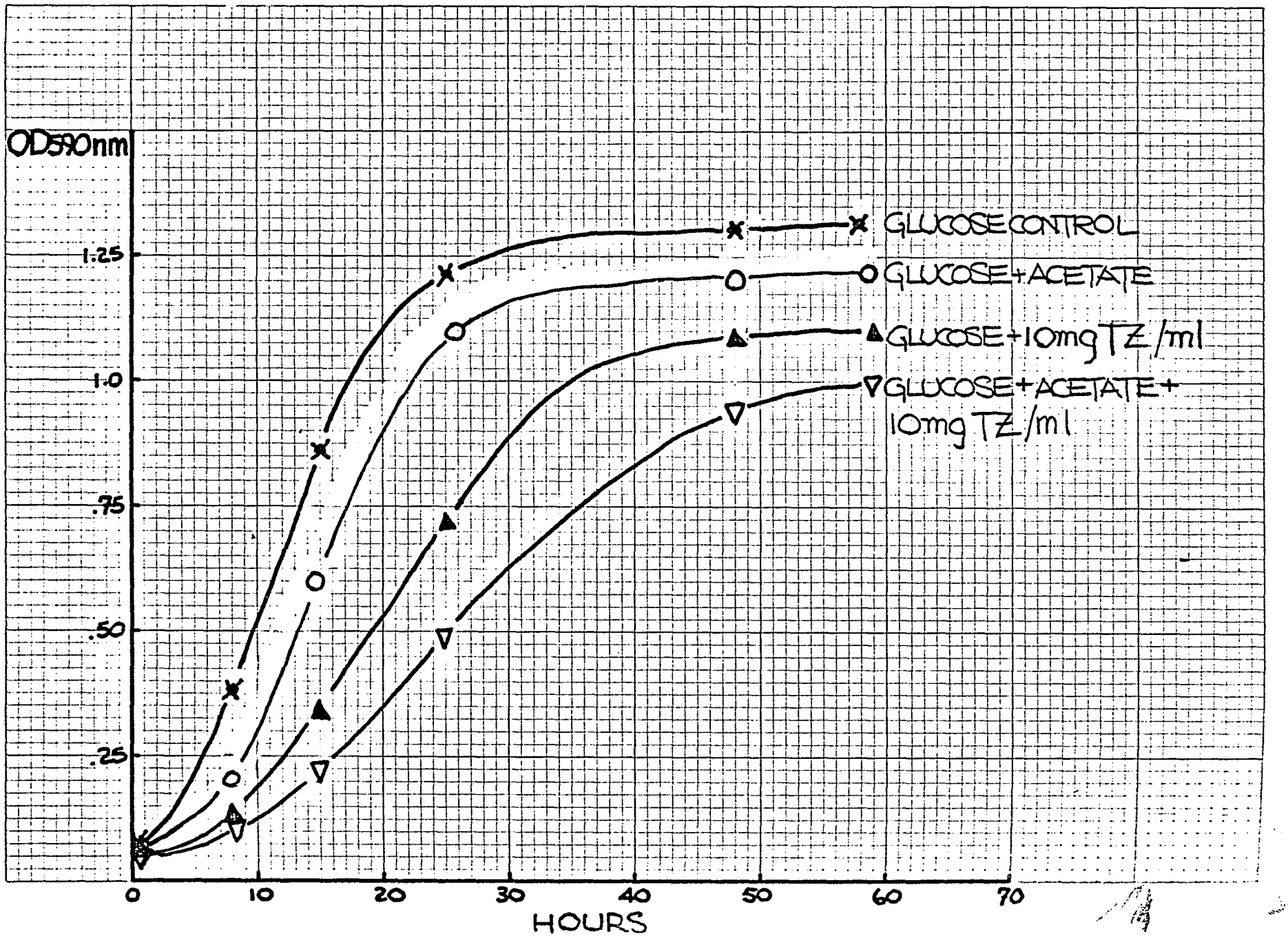
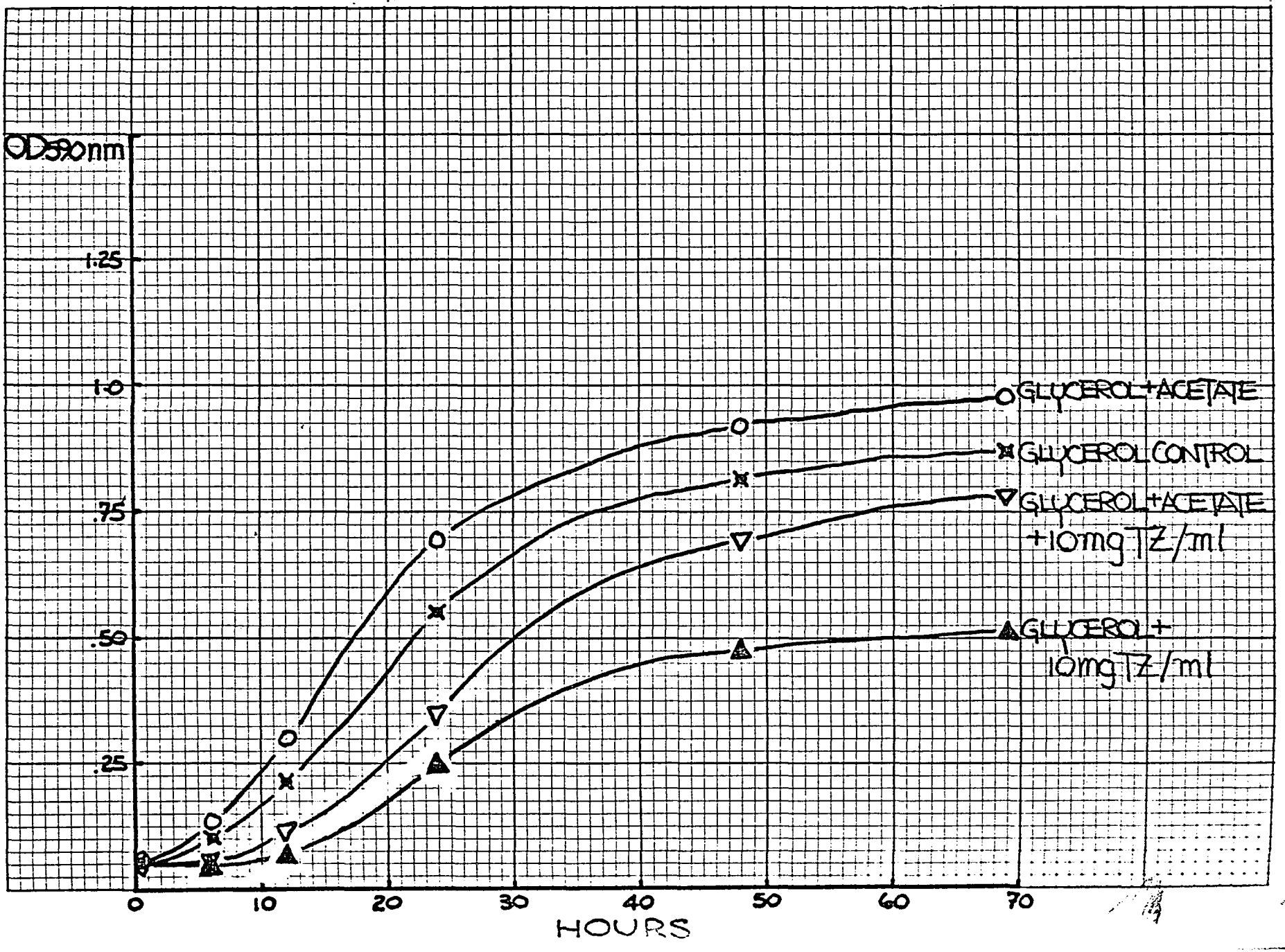

OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
70
HOURS
GLUCOSE CONTROL
GLUCOSE+ACETATE
GLUCOSE+10mg TZ/ml
GLUCOSE+ACETATE+
10mg TZ/ml

Fig. 17. The effect of 0.5% acetate on cell growth in glycerol-TZ medium.

FIGURE 17


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
70
HOURS
GLYCEROL+ACETATE
GLYCEROL CONTROL
GLYCEROL+ACETATE +10mg TZ/ml
GLYCEROL+ 10mg TZ/ml

CHAPTER IV

RESULTS

The Mechanism of Proline-Reversible Inhibition by TZ and Cellular Recovery Phenomena:

As reported by Unger and DeMoss (1966), inhibition of *E. coli* cell growth by TZ was attributed to the ability of the analogue to: 1.) mimic proline in its incorporation into cell protein; 2.) interfere with the utilization of proline in other metabolic processes. Prior to incorporation, TZ was converted into a closely related metabolic derivative, probably N-formylcysteine (Fig. 2). These experiments were carried out in a glucose-mineral salts medium, and the inhibition observed was specifically proline-reversible. Studies were therefore undertaken to establish whether or not TZ exhibited similar modes of action in glucose-grown cultures of yeast. Experiments which defined the ability of the analogue to be incorporated into protein are discussed here. Studies on the ability of the analogue to interfere with proline uptake will be presented subsequently.

Incorporation Patterns of [^{14}C]-Labeled TZ Into Strain 1493-10C:

If specific incorporation of TZ into proteins occurs, it should be possible to demonstrate it with radio-labeled analogue. The patterns of incorporation observed should be altered in a defineable manner by inhibitors of cytoplasmic and/or mitochondrial protein synthesis. As described in

Materials and Methods, glucose-grown cells harvested from early exponential, mid-exponential and early stationary phase were suspended at a constant cell density in 0.1 M potassium phosphate buffer pH 7.0 in the presence or absence of cycloheximide (CAP) and/or erythromycin (ER) or chloramphenicol (CAP). After an equilibration time of 15 minutes at 30C, [^{14}C]-TZ (specific activity 0.1 μC_i/mg) was added to a final concentration of 1 mg/ml and the cells were allowed to incubate for 30 minutes. The incorporation was stopped by addition of an equal volume of 10% TCA followed by boiling for 10 minutes. Cells were collected on filters, washed with 5% TCA then 95% ethanol, dried and assayed for [^{14}C]. The results of such studies are given in Table 2.

As can be seen from Exp. 1 in the table, values for incorporation in the presence of ER were higher than those in the control. Such spurious results were apparently the result of a complex which formed between TCA and erythromycin. This complex could not be completely removed from cells by the washing protocol employed in the experiments, and caused the green-blue color which cell suspensions took on after boiling with TCA. Independent studies confirmed this, and indicated that the amount of color formed was directly proportional to the erythromycin concentration. Substitution of chloramphenicol for erythromycin allowed this problem to be avoided.

The data from Exp. 2 (Table 2) indicated that [^{14}C]-TZ

TABLE 2

Incorporation of (^{14}C)-TZ Into Yeast Cells

The tables below reflect experimental values (CPM) for the incorporation of TZ per 1 x 10^6 cells. Incorporation studies were conducted as described in the text. Time designations in the tables refer to the age of the culture.

EXPERIMENT 1:

ADDITIONS	5 hours early log	9 hours mid log	14 hours late log
washed blank (no cells)	17	-	-
control	114	88	45
chi (20 µg/ml)	53	41	25
er (4 mg/ml)	253	284	236
chi and er	241	225	184

EXPERIMENT 2:

ADDITIONS	5 hours	9 hours	14 hours
washed blank	18	-	-
control	162(0)*	120(0)	108(0)
chi (20 µg/ml)	79(51)	72(40)	53(51)
cap (4 mg/ml)	147(9)	103(14)	88(21)
chi and cap	73(55)	70(42)	61(43)
boiled cells	53(65)	-	-

*Values in parentheses express the percentage of inhibition as compared with the control.

was incorporated into cells in a manner that was sensitive to inhibitors of both cytoplasmic and mitochondrial protein synthesis. In addition, the residual radioactivity which was not sensitive to either inhibitor, appeared to result from nonspecific binding of the label, since it was present to about the same extent in the boiled-cell preparations. These results indicated that TZ was incorporated into yeast cellular protein. Furthermore, incorporation into both cytoplasmic and mitochondrial compartments occurred. The pattern of incorporation roughly paralleled the relative amount of protein synthesis previously reported for the respective compartments (Schatz & Mason, 1974). No evidence of differential incorporation into either the cytoplasmic or mitochondrial compartment was observed.

It was also observed that the values for total incorporation per unit number of cells decreased as cells progressed through the growth cycle. These data suggest that the degree of growth inhibition seen could be related to the amount of TZ in cell protein. They also suggest that some mechanism might exist for altering the extent of TZ incorporation into protein. The nature of this mechanism has been investigated, and will be discussed below.

The previous study did not pose the question of the *form* in which TZ was incorporated into cellular protein. For this reason, studies were undertaken in which whole protein was extracted from cells grown in the presence of labeled ana-

logue. After acid hydrolysis, the amino acid residues were separated chromatographically and assayed for radioactivity. In this manner, data pertaining to the form of the analogue and the extent of its incorporation into protein was obtained. One and two-dimensional thin-layer chromatographic (TLC) systems were used to resolve the various amino acid residues of whole cell protein. These systems gave highly reproducible R_f values and ninhydrin reactions both for natural amino acids and TZ (Table 3). Chromatographic and radioactivity profiles for hydrolysates of whole cell protein and unhydrolyzed culture medium from cultures taken at two points in the growth cycle are shown in Figures 18 and 19.

From these data, several observations can be made: 1.) label derived from [^{14}C]-TZ was detected in the hydrolysate of whole protein from cells sampled during the glucose-TZ lag phase; 2.) this label differed from TZ in R_f value (.18 vs. .48) and in ninhydrin reaction product; 3.) in protein hydrolysates from cultures which have been allowed to recover from the glucose-TZ lag, no detectable radioactivity was present. Together these data argue that TZ, or a derivative, was incorporated into cellular protein during the lag phase of glucose-TZ growth.

Although the label migrated at an R_f which differed significantly from TZ and gave Ruhemann's purple (West, 1965) as its ninhydrin reaction rather than the yellow-brown

TABLE 3

R_f Values and Ninhydrin Reaction Colors for Amino Acids and TZ

Amino Acid or Analogue	R_f First*	R_f Second*	Ninhydrin
Alanine	.43/minor .34	.28	dark pink
Arginine	.35	.04	pink
Asparigine	.28	.12	grey-pink
Aspartic Acid	.35	.09	dark pink
Cysteine	.43/minor .20	.20	orange-pink
Glutamic Acid	.39	.16	pink w/ tail
Glutamine	.34	.17	dark pink
Glycine	.36	.14	pink-grey
Histidine	.30	.17	pink-grey
Isoleucine	.74	.52	pink
Leucine	.74	.53	pink w/ tail
Lysine	.28	.04	pink
Methionine	.59	.51	pink
Phenylalanine	.68	.56	lt. pink
Proline	.46	.31	yellow-brown
Serine	.36	.27	dark pink
Threonine	.41	.36	pink
Tryptophan	.58	.53	lt. pink
Tyrosine	.52	.50	lt. pink
Valine	.60	.46	dark pink
Thiazolidine-4-CA	.47	.56	yellow

*First solvent system: butanol:acetic acid:water (4:1:5)
Second solvent system: pyridine:water (4:1)
Support: MN-300 Cellulose
All R_f values reported have a sem. of ±5% or less.

Fig. 18 Chromatographic and radioactivity profiles for total cell protein hydrolysates, (A); & culture filtrates, (B); from a culture grown in the presence of 10 mg [^{14}C]-TZ/ml for 4 hr.

Legend to chromatographs:

(x) unidentified material at origin;
(o) TZ hydrolysis product (see text);
(1) cystine;
(2) asparigine, lysine;
(3) histidine;
(4) alanine, glutamine
(5) arginine, glycine, serine;
(6) glutamate
(7) alanine (major), cysteine, threonine;
(8) proline, TZ;
(9) valine;
(10) tyrosine;
(11) tryptophan;
(12) methionine;
(13) phenylalanine;
(14) isoleucine, leucine.

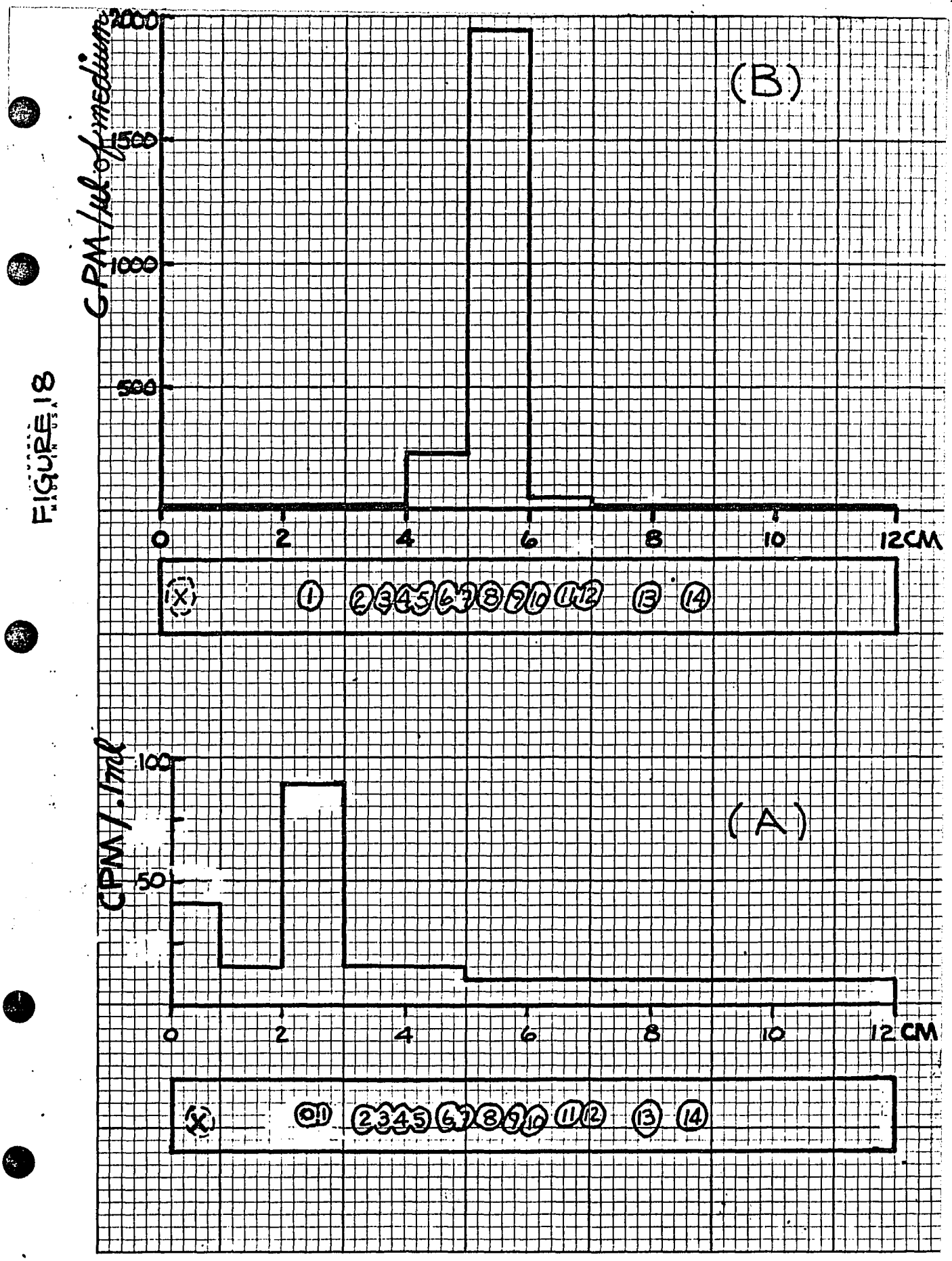

CPM/ul of medium
2000
1500
1000
500
(B)
0
2
4
6
8
10
12CM
(X)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
CPM/.1ml
100
50
(A)
0
2
4
6
8
10
12 CM
(X)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 18

Fig. 19. Chromatographic and radioactivity profiles for total cell protein hydrolysates from a culture grown in the presence of 10 mg [^{14}C]-TZ/ml for 16 hours.

Legend to chromatographs:

As in Fig. 18.

FIGURE 19

color which is indicative for imino acids (Schroeder, 1968), evidence for the *cellular* conversion of TZ to a derivative was not compelling. Alterations in the chromatographic properties of the labeled compound could reflect destruction of TZ caused by the hydrolysis procedure. The acid hydrolysis procedure followed here is known to destroy the proline analogue azedtidine-2-CA (Baum *et al.*, 1975) and the strongly oxidizing environment could also alter TZ. In order to ascertain whether or not the alterations observed were artifacts of the hydrolysis regimen, labeled TZ was subjected to this regimen, then co-chromatographed with unlabeled authentic analogue. The results (Fig. 20A) indicated that the hydrolysis regimen itself produced alterations in R_f value and ninhydrin product equivalent to those seen initially. Furthermore, material showing the same altered chromatographic behavior could be isolated from TZ-glucose culture medium which had been stored for 2 to 3 weeks (Fig. 20B). By contrast, a stock TZ solution showed only the normal TZ spot even after 6 months storage at room temperature (Fig. 20C). Such observations do not rigorously exclude the cellular conversion of TZ to a derivative. It should be pointed out that in the cell protein hydrolysates, *all* of the detectable radioactivity which migrated from the origin was present as the TZ derivative. By contrast, a *mixture* of the derivative and TZ was seen in all other cases.

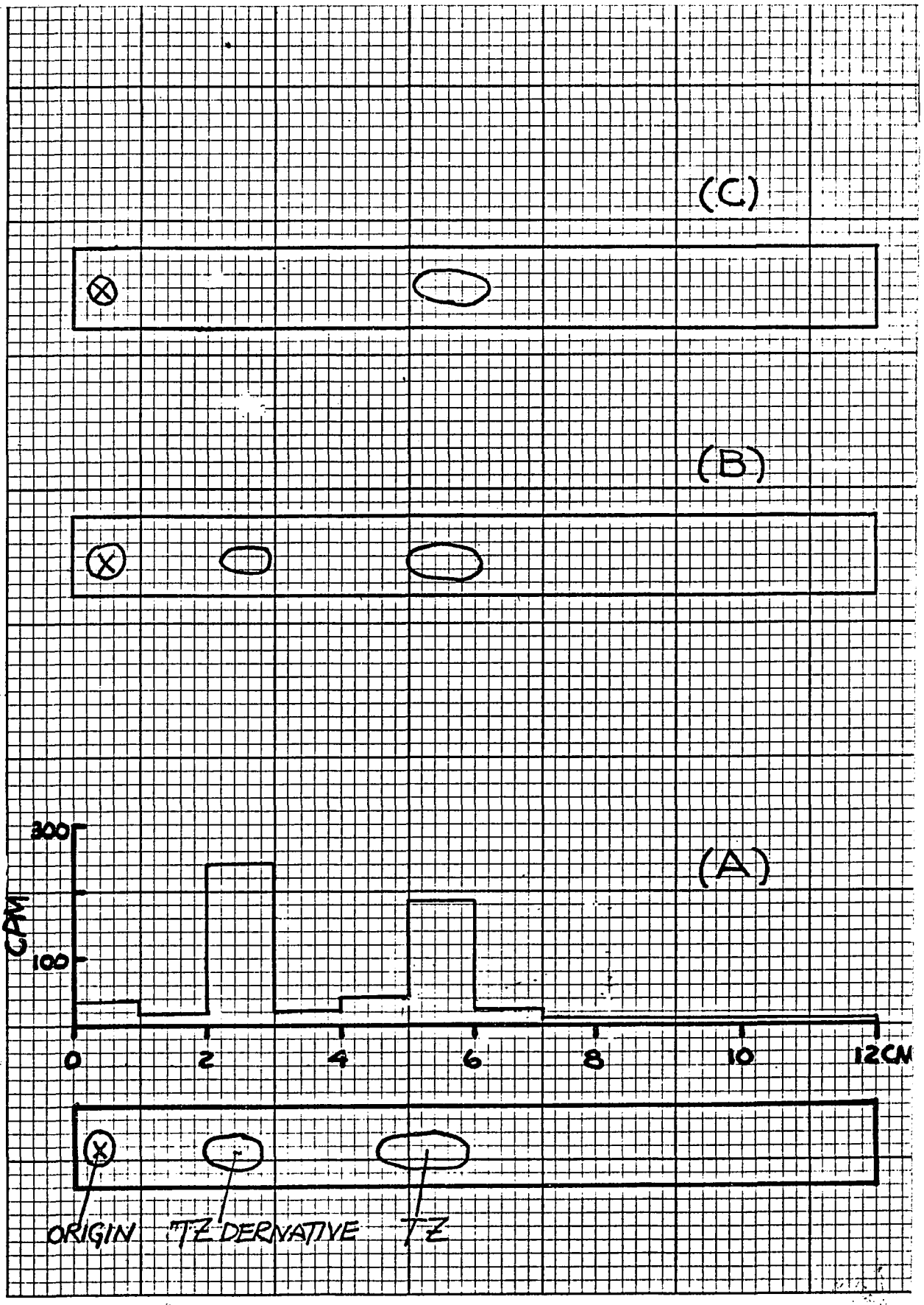
The identity of the derivative is unknown. However, its

Fig. 20. Chromatogaphic and radioactivity profiles of TZ and derivatives.

(A). ^{14}C-labeled TZ was subjected to the hydrolysis regimen used to prepare protein hydrolysates and chromatographed with authentic cold TZ as previously described.

(B). Glucose-TZ medium which had been stored at room temperature for 2-3 weeks. For the sake of clarity, spots present in glucose medium alone have been ommitted.

FIGURE 20
A, B & C



R_f value (0.18 ± 0.01) is very close to that of the minor component of cysteine (R_f 0.20 ± 0.01) (Table 3). In addition, the shift in ninhydrin reaction from yellow-brown to Ruhemann's purple indicated that the derivative was not an imino acid. The conversion of TZ to *N*-formylcysteine was postulated to procced via an oxidation reaction (Fig. 2) which results in the opening of the thiazole ring and the formation of a substituted cysteine molecule (Cavallini *et al.*,1956). Such considerations, in the absence of definitive evidence, are at least not inconsistent with the view that TZ is present in yeast cell protein as *N*-formyl-cysteine.

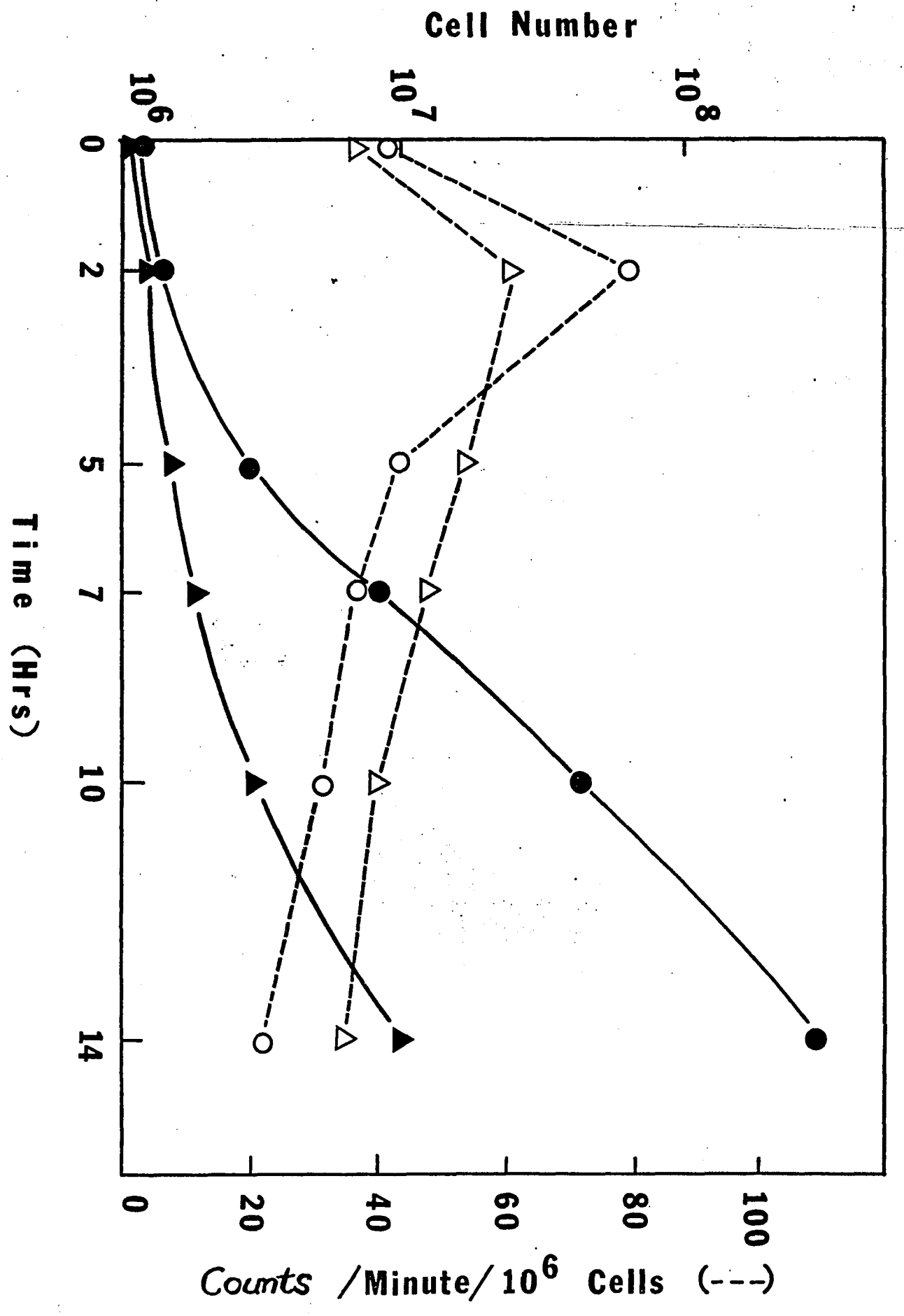
These data combined with those of the previous study argue strongly for the incorporation of TZ, or a derivative, into cell protein. They indicate further, that the extent of this incorporation was related to the degree of growth inhibition observed.

The Mechanism of Recovery From the TZ-Glucose Lag:

If the degree of TZ growth inhibition on glucose medium is related to the intracellular concentrations of TZ, then the existence of a mechanism for the alteration of these intracellular levels must be postulated. A likely mechanism to accomplish this alteration would mediate changes in the extent of TZ uptake with the growth cycle. Such a system would allow uptake on a *per cell* basis to be greatest near the beginning of the growth cycle, during the TZ lag, and

Fig. 21. Uptake of (^{14}C)-TZ at various points in the growth cycle.

Cells of strain 1493-10C were inoculated into glucose medium (o) and glucose-TZ medium (Δ). At times indicated, samples were removed, washed and resuspended at a constant cell number in 100 mM potassium phosphate buffer, pH 7.0. After equilibration, (^{14}C)-TZ was added to a final concentration of 1 mg/ml and the incubation was allowed to proceed for 10 minutes. Samples were removed, filtered, and washed extensively with ice-cold 0.1 M naCl. After drying, the filters were counted as described.


Cell Number
10^6
10^7
10^8
0
2
5
7
10
14
Time (Hrs)
0
20
40
60
80
100
Counts /Minute/ 10^6 Cells (---)

to decline as recovery occurred. In this manner, higher concentrations of analogue would be available initially to compete with proline for incorporation into protein. As the concentration declined, less incorporation would occur. In experiments designed to measure *per cell* uptake of [^{14}C]-labeled TZ as a function of culture age, the postulated pattern was observed (Fig. 21). After a lag of about 1-2 hours, TZ uptake rapidly increased to a peak value, then decreased progressively. The same pattern was observed in cells *pre-grown* in unlabeled TZ and in this case, the decreased uptake mirrored recovery. The remarkably similar uptake patterns of TZ-grown cells, and cells not previously exposed to TZ, indicated that the general pattern of uptake did not depend upon prior exposure to the analogue. Furthermore, since very little growth occurred during the first few hours, TZ uptake was not tightly coupled to cell growth. Thus recovery from growth inhibition on glucose-TZ medium was related to a progressive decrease in TZ uptake.

If recovery were due to a mechanism which decreased intracellular concentrations of TZ by restricting the entry of the analogue, then interference with this mechanism should be reflected in altered growth patterns. Dimethylsulfoxide (DMSO) is known to alter the permeability of the yeast cell membrane (Adams, 1972) and thus should interfere with recovery by limiting the ability of the cell to exclude the

Fig. 22. The effect of dimethyl sulfoxide treatment on the ability of cells to recovery from TZ inhibition on glucose medium.

Ice-cold, filter sterilized DMSO was added to chilled cell suspensions to the final concentrations indicated in the figures at the start of the growth experiments.

FIGURE 22.

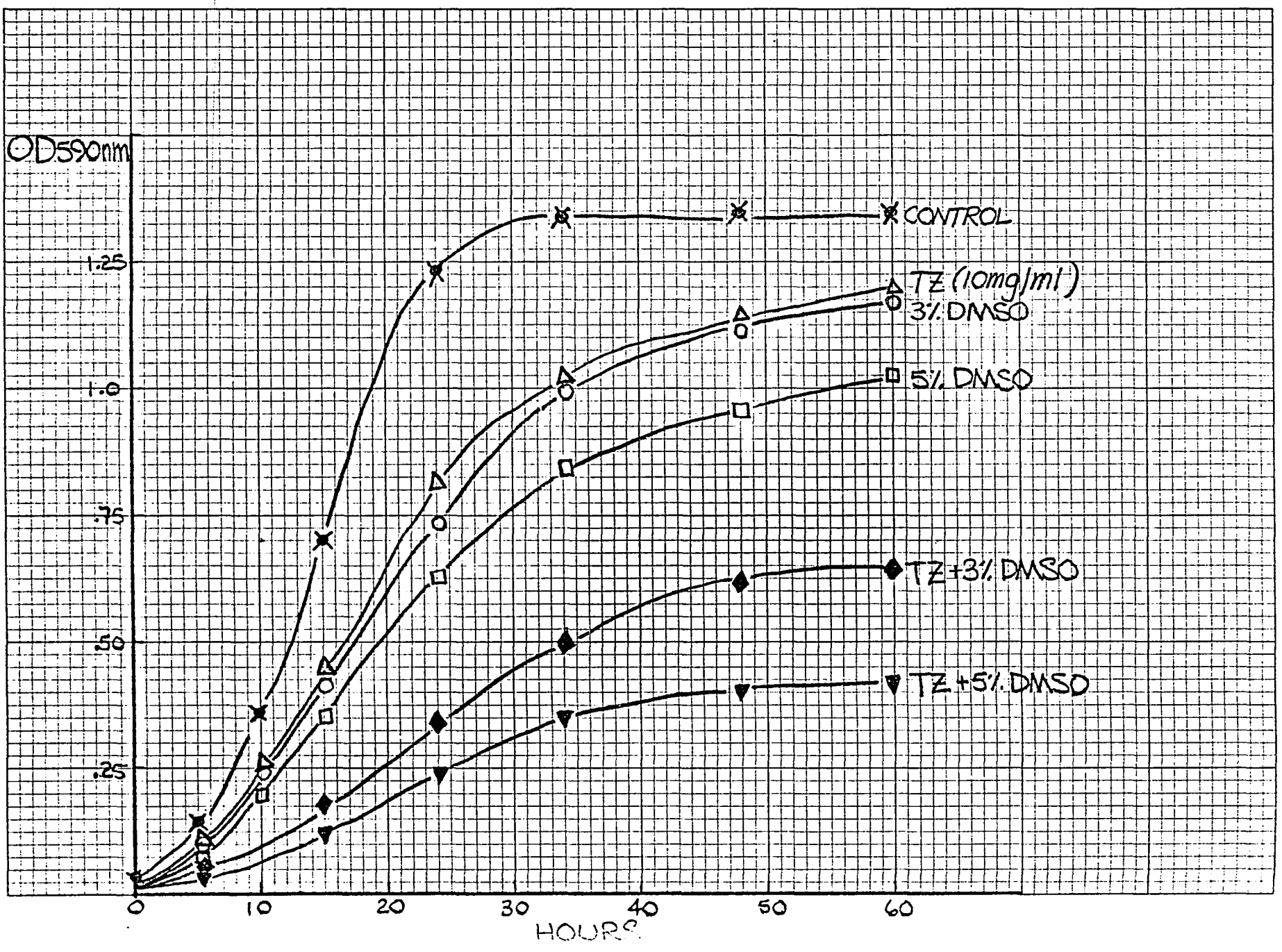
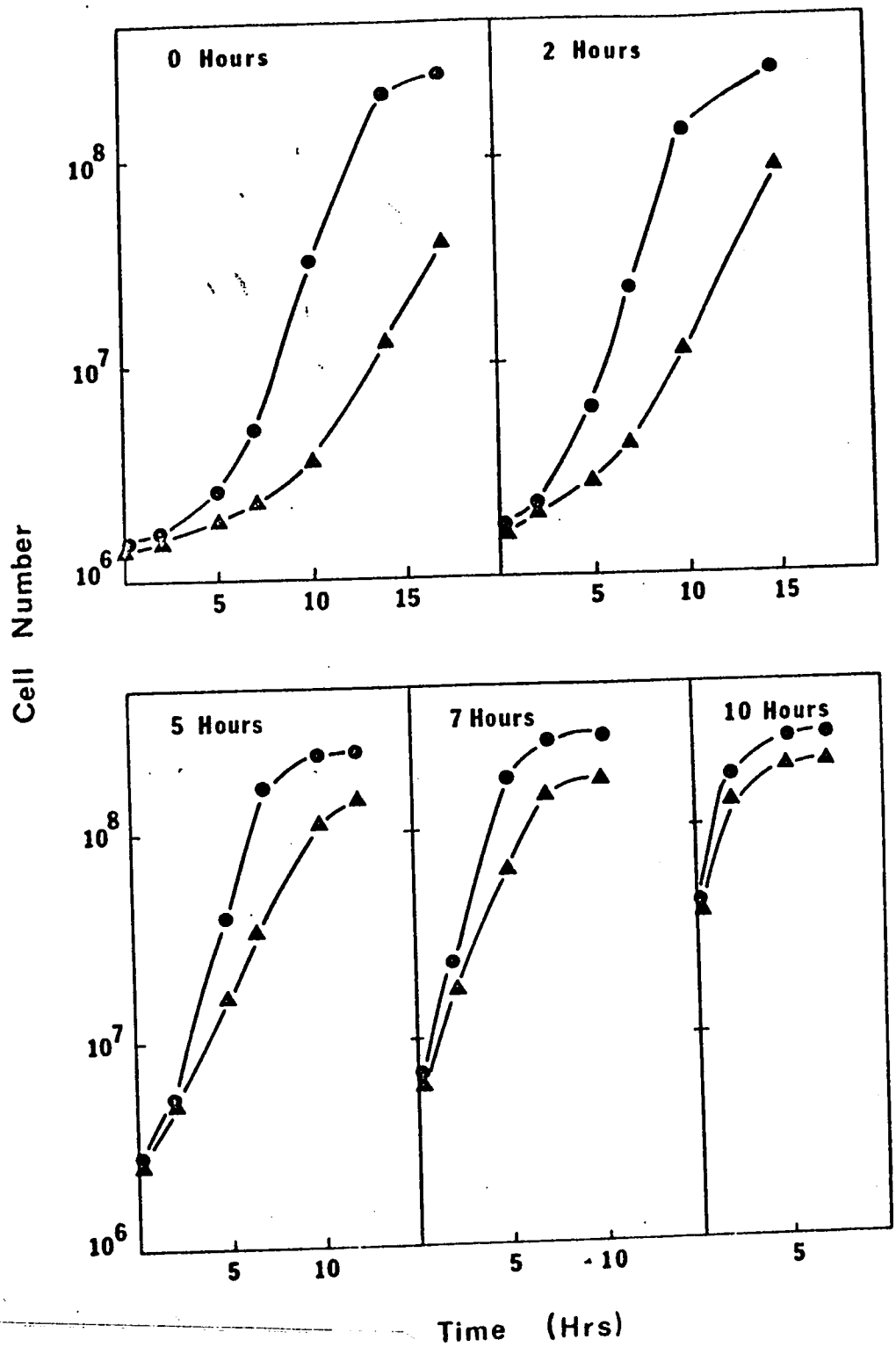

OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
60
HOURS
CONTROL
TZ (10mg/ml)
3% DMSO
5% DMSO
TZ+3% DMSO
TZ+5% DMSO

Fig. 23. "Shift Experiments".

TZ (10 mg/ml), was added to the medium of cultures grown on glucose medium for times indicated at the top of each box. (Δ) represent TZ-treated cultures; (o) are controls.


0 Hours
2 Hours
5 Hours
7 Hours
10 Hours
Cell Number
10^8
10^7
10^6
5
10
15
Time (Hrs)

figure

analogue. DMSO-treated cells showed a much more severe response to TZ on glucose medium than did untreated controls (Fig. 22). The enhancement of inhibition was directly related to the DMSO concentration (and presumably to the DMSO-induced increase in membrane permeability).

Finally, if recovery were dependent on a progressive decrease in the ability to take up TZ (which did not depend upon prior exposure to the analogue) then cells should be less susceptible to the effects of TZ as they progressed through the growth cycle. The results of studies in which aliquots of a growing culture received TZ treatments at increasingly later times in the growth cycle is shown in Figure 23. The greatest TZ-induced response, in terms of the length of the resulting lag and the depression in final cell titre was seen when TZ was added before the culture was 5 hours old. As cell progressed through the growth cycle, a decreased TZ response was observed. The addition of TZ to a culture pre-grown for 10 hours caused almost no detectable difference in either the rate of extent of culture growth. The degree of this decreased response is correlated with the decline in the amount of TZ uptake.

In summary, these studies suggest that: (1) TZ or a derivative was incorporated into yeast cell protein; (2) incorporation into protein was greatest during the glucose lag period and declined during recovery phase; (3) recovery from the lag period was linked to the ability of cells to exclude

the analogue; (4) the control of analogue exclusion did not depend upon prior exposure to the analogue, and was not tightly coupled to cell division.

TZ has been shown to be taken into *S. chevalieri*, a racial variant of *S. cerevisiae* (Bicknell & Douglass, 1970) by way of a specific imino acid transport system (Magaña-Schwencke *et al.*, 1973). The relationship of analogue uptake patterns to proline transport will be presented in Chapter VI.

CHAPTER V

The Mechanism of Proline-Irreversible Inhibition by TZ:

As previously demonstrated, the growth of yeast cells on ethanol medium was completely inhibited by TZ in a proline-irreversible manner. By contrast, TZ inhibition observed on glucose, as well as non-fermentable carbon sources (other than ethanol) was largely proline-reversible. The addition of ethanol to other types of culture media increased the severity of the TZ response. These observations suggested that TZ was mediating a second mode of inhibition; distinct from its incorporation into protein, and related to ethanol metabolism. Evidence for a second mode of TZ inhibition has also been reported in *E. coli* (Unger & DeMoss, 1966). Kinetic analysis of TZ inhibition patterns produced a second component which could be distinguished from inhibition caused by analogue incorporation into protein. The mechanism of this inhibition was not investigated.

The Effect of TZ on Ethanol Metabolism:

In order to elucidate the nature of proline-irreversible TZ inhibition in yeast, the rate of oxygen consumption, with ethanol as substrate, was measured in cells pre-grown in glycerol-TZ medium. This rate was found to be less than 30% of the rate seen when an equal number of glycerol-grown cells were used under identical conditions. Although this observation further suggested that growth inhibition seen on ethanol-

TZ medium resulted from a block in ethanol metabolism, such studies could not identify the site of the block. Attention was then focused on specific components of the yeast alcohol metabolism pathway.

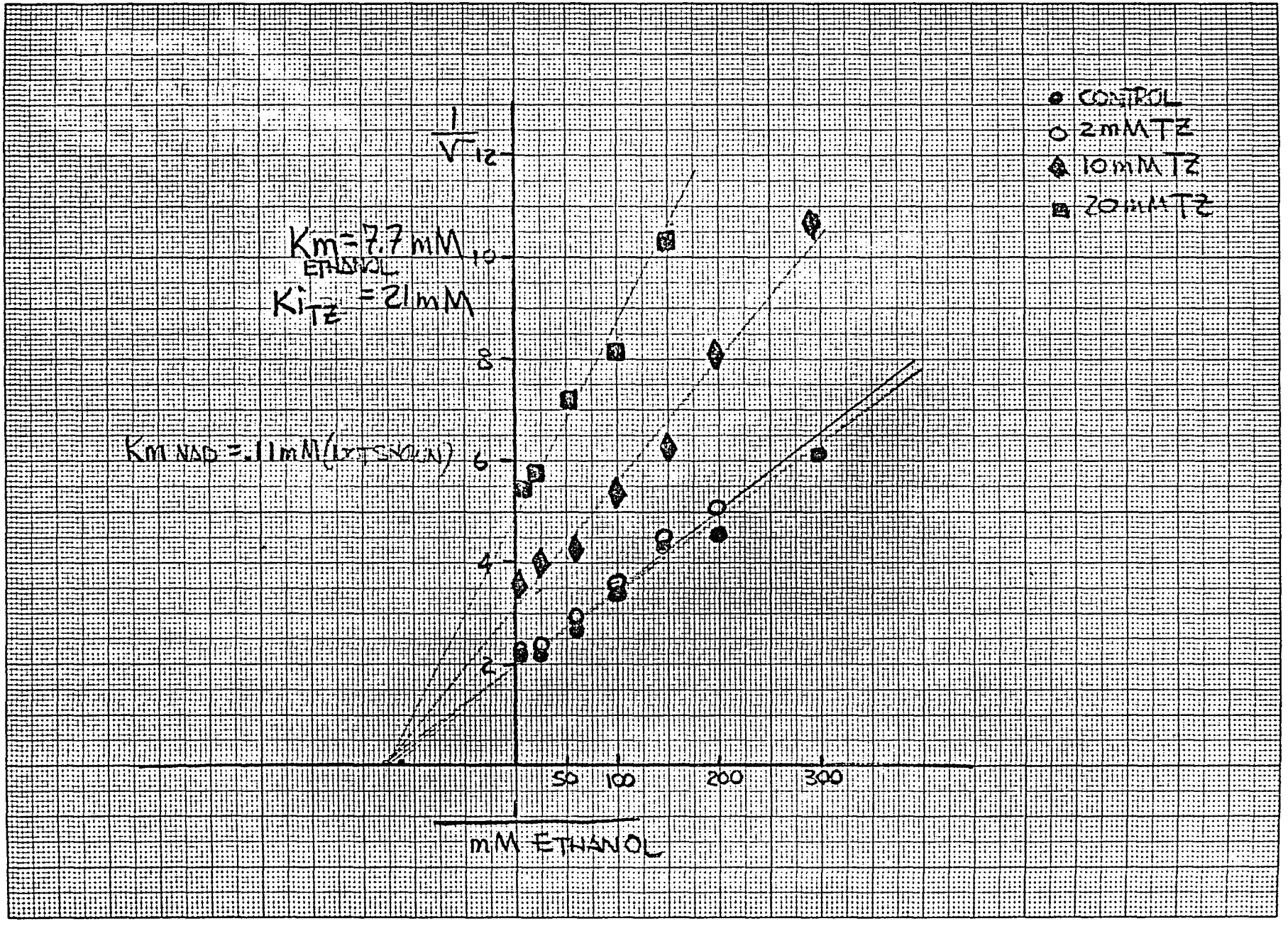
Alcohol Dehydrogenase:

The initial step in ethanol metabolism is its oxidation to acetaldehyde by alcohol dehydrogenase (ADH: E.C. 1.1.1.1). This enzyme has been studied in a variety of organisms (Racker, 1950; Jornvall, 1973). In yeast, three specific forms of ADH have been identified (M. Ciriacy, personal communication). The predominant form is a constitutive cytoplasmic enzyme, which is thought to function primarily in the reduction of acetaldehyde to ethanol during glycolysis. The utilization of ethanol as a substrate is thought to involve two glucose-repressible forms of ADH, one cytoplasmic, the other mitochondrial.

A preparation containing ADH activity was made from extracts of strain 1493-10C, as described in *Materials and Methods*. When prepared in buffer containing BME, BSA, and EDTA, the crude extracts were stable for five hours or longer. In such preparations, the apparent K_m for ethanol was 7.7 mM, and that for NAD was 1.1×10^{-4}M (Fig. 24). These values agree well with the published literature (Hayes & Velick, 1954). The addition of TZ to the assay medium resulted in the non-competitive inhibition of ADH with an apparent K_i of 20 mM (Fig. 24). This inhibition could not be

Fig. 24. Lineweaver-Burk plot of alcohol dehydrogenase from strain 1493-10C.

Crude enzyme extracts were prepared and stabilized as described in *Materials and Methods*. ADH was assayed by the method of Racker (1955) in an assay system containing: 85 µM sodium phosphate buffer, pH 6.6; 20 µM NAD; 25 µM Ethanol; 10-50 µg of crude enzyme extract in a total volume of 1.4 ml at 30C. Under these conditions, the decrease in absorbance at 340 nm was linear with time and protein concentration.

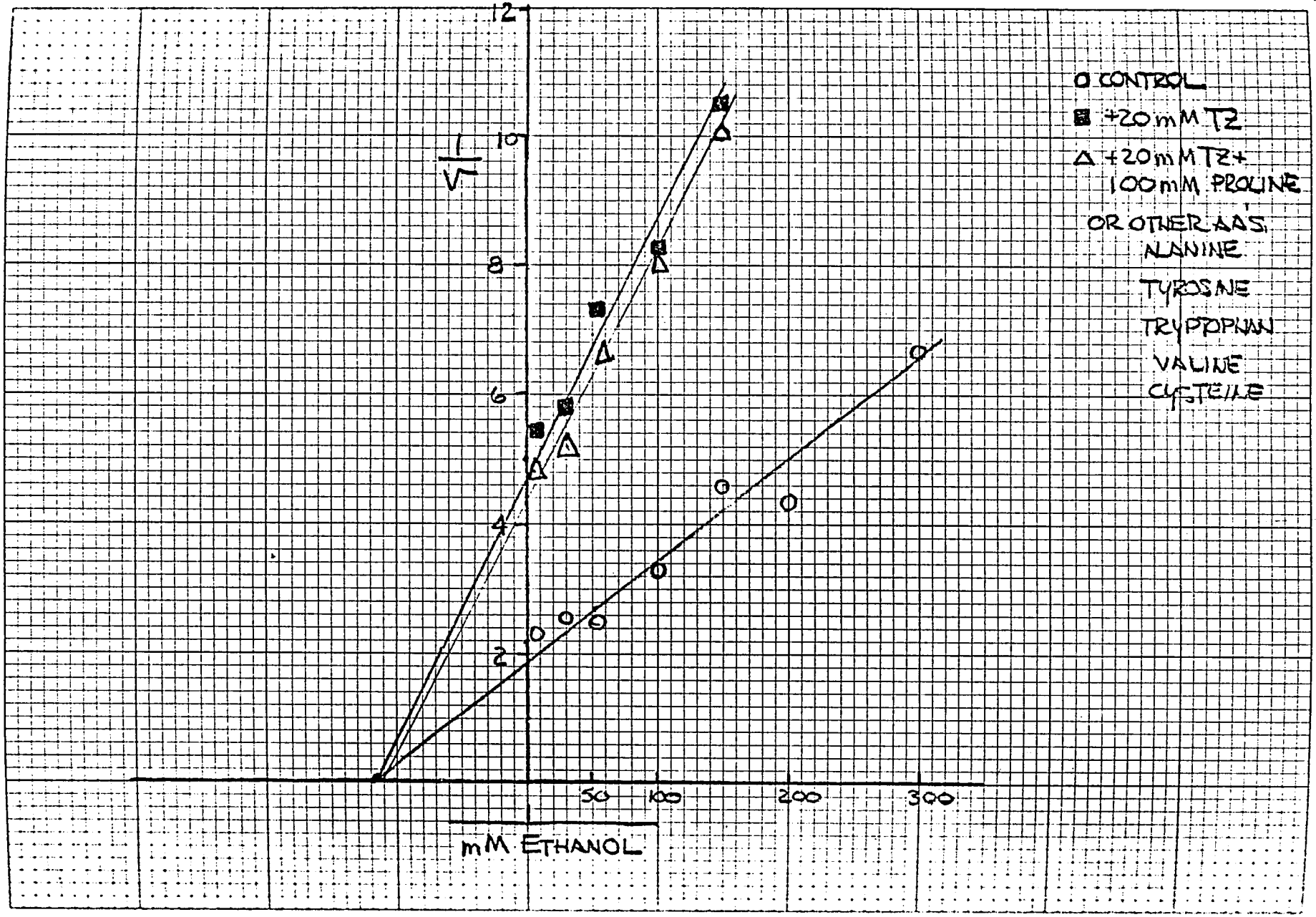


reversed by the addition of either proline or other amino acids at concentrations of 100 mM (Fig. 25). It is interesting that TZ is structurally similar to the pyrazole derivatives which have been shown to be potent inhibitors of ADH (Reynier, 1969; Dahlbom *et al.*, 1974).

In summary, the data presented strongly suggest that TZ inhibits the growth of cells on ethanol-containing medium by specifically interfering with the utilization of ethanol as an energy source. The mechanism of this interference involves the non-competitive inhibition of ADH by TZ in a proline-irreversible manner.

Fig. 25. Lineweaver-Burk plot showing non-competitive inhibition of ADH which cannot be reversed by proline or other amino acids.

Assay conditions were as described in the legend to Figure 24.


12
10
8
6
4
2
1/V
50
100
200
300
mM ETHANOL
○ CONTROL
■ +20mM TZ
△ +20mM TZ+
100mM PROLINE
OR OTHER AA'S.
ALANINE
TYROSINE
TRYPTOPHAN
VALINE
CYSTEINE

CHAPTER VI

The Proline Transport System in *S. Cerevisiae*:

In a previous section, evidence was presented which showed that recovery from the TZ-glucose lag was brought about concurrently with changes in the pattern of analogue uptake. Such changes resulted in a progressive exclusion of the analogue from the cell. The relationship between this pattern and imino acid uptake will be discussed below along with general observations concerning the proline transport system in *S. cerevisiae*.

Specific proline transport systems have been described in bacteria (Hirata *et al.*, 1971), algae (McNamer & Stewart, 1973), fungi (Magaña-Schwencke *et al.*, 1973), and higher mammals (Holtapple *et al.*, 1973). Studies in *S. chevalieri* (Magaña-Schwencke *et al.*, 1968) indicated that TZ was a competitive inhibitor of proline uptake, suggesting that TZ and proline entered the cell by the same uptake system. This transport system was highly specific for imino acids, and other amino acids were unable to compete with proline for transport. In addition, the activity of this transport system was controlled by the nitrogen levels in the medium. Placed in nitrogen-free buffer (66 mM potassium phosphate, pH 5.5) containing a carbon source (0.1 M glucose), cells increased their ability to transport proline by several thousand-fold within 3-4 hours. This increase required

energy, was specific for proline, and was sensitive to inhibitors of transcription and translation. Furthermore, with the selective use of inhibitors, the transcription-dependent events could be partially isolated from the translational events, which they preceded. From these observations, it was concluded that N-starvation was probably mediating its effect by derepression of a specific proline transport system. A similar system of control had been suggested for leucine uptake in *E. coli* (Inui & Akedo, 1965). Observations reported on the proline transport system of *S. chevalieri* (Schwencke *et al.*, 1969; Magaña-Schwencke *et al.*, 1969; Kuznar *et al.*, 1973) formed the basis for the studies described below.

Characteristics of the Proline Transport System in *S. Cerevisiae*:

Studies using [^{3}H]-proline have indicated that the uptake of proline into the cell followed the same pattern as that observed for TZ (Fig. 26 A). Proline uptake peaked within 1-2 hours after inoculation of the culture, and declined to a maintenance level thereafter. In an attempt to optimize proline uptake, 25 mM glucose was added to the incubation medium. The addition of glucose resulted in a decrease of 10% in the amount of proline uptake measured (Fig. 26B). Glucose-dependent inhibition of amino acid uptake (Glutamate) has been observed in crab nerve (Evans, 1973). In yeast, the inhibition has been postulated

Fig. 26. (A). The uptake of (^{3}H)-proline at various points in the growth cycle. (B). The effect of 25 mM glucose on proline uptake.

Uptake experiments were performed on glucose-grown cells as described in the legend for figure 21 using (^{3}H)-proline at a concentration of 35 µM.

In (B), 25 mM glucose was added to the medium in which the uptake experiments were performed.

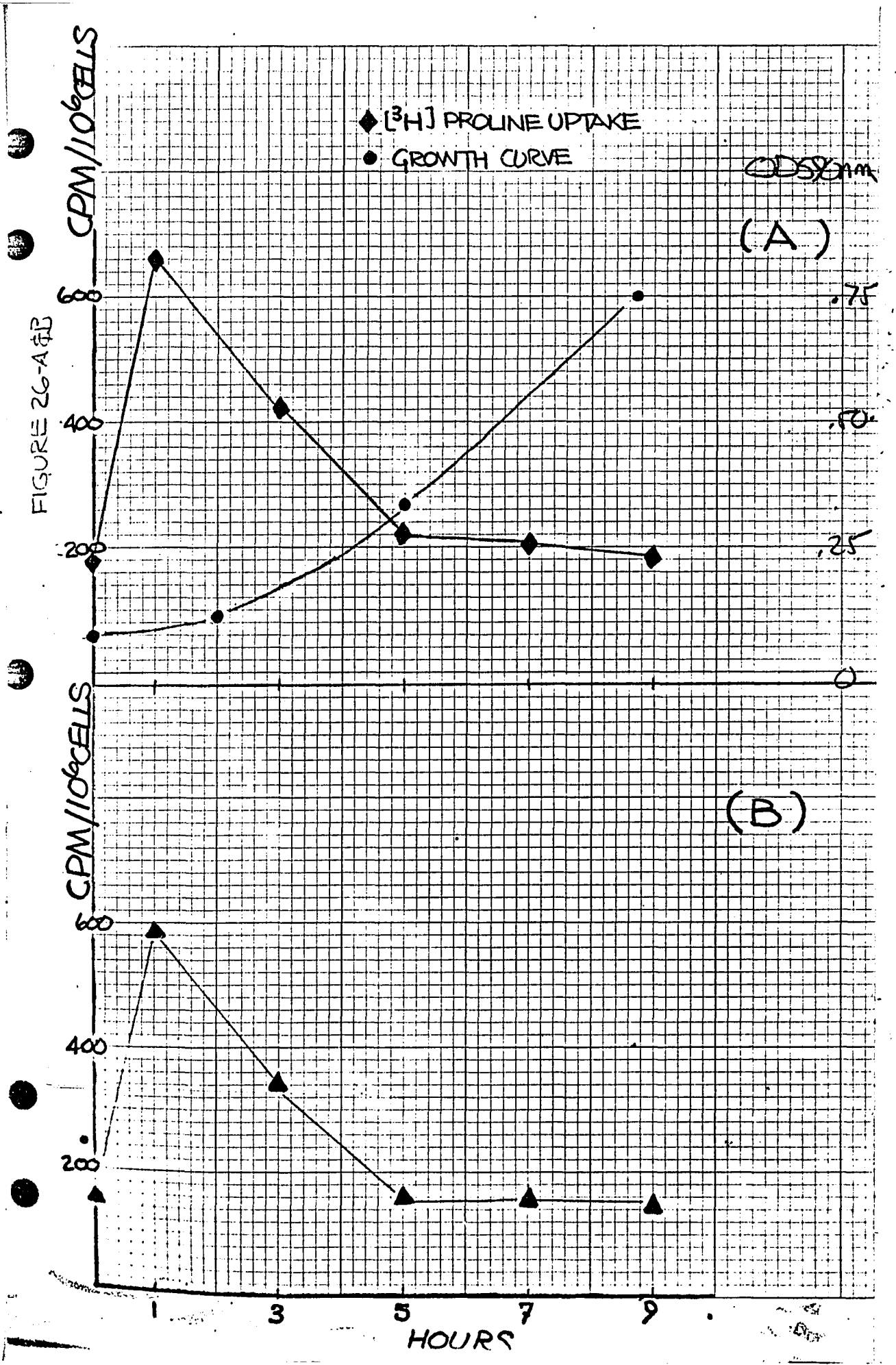

[3H] PROLINE UPTAKE
GROWTH CURVE
OD 550 nm
(A)
.75
.50
.25
0
CPM/10⁶CELLS
600
400
200
(B)
CPM/10⁶CELLS
600
400
200
1
3
5
7
9
HOURS

FIGURE 26-A&B

to result from non-specific interference by hexoses with other transport systems (Vollbrecht & Radler, 1974).

The apparent affinity constant (K_m) for the uptake of proline at 30C was 30 µM (Fig. 27). Other amino acids showed no ability to compete with proline for uptake. TZ was observed to be a competitive inhibitor of proline uptake with an apparent inhibitor constant (K_i) of 40 µM (Fig. 27). These values were very similar to those reported for *S. chevalieri* (K_m proline = 25 µM: Magaña-Schwencke *et al.*, 1968), and indicated that the proline transport system showed a high affinity for the analogue.

Thus, changes in TZ uptake during the growth cycle reflected changes in the activity of the proline transport system. The finding of cyclic changes in the activity of this system contrasts with results of investigations in *E. coli* which showed that proline transport remained essentially constant throughout the growth cycle (Britten & McClure, 1962). However, proline uptake in *Chlorella* showed cyclic fluctuations in activity which were correlated with the levels of endogenous carbohydrate pools (McNamer & Stewart, 1973). Furthermore, Arst and Mac Donald (1975) have reported that activity in one of the proline permease systems of *Aspergillus nidulans* was repressed by high nitrogen levels, and showed sensitivity to carbon catabolite repression.

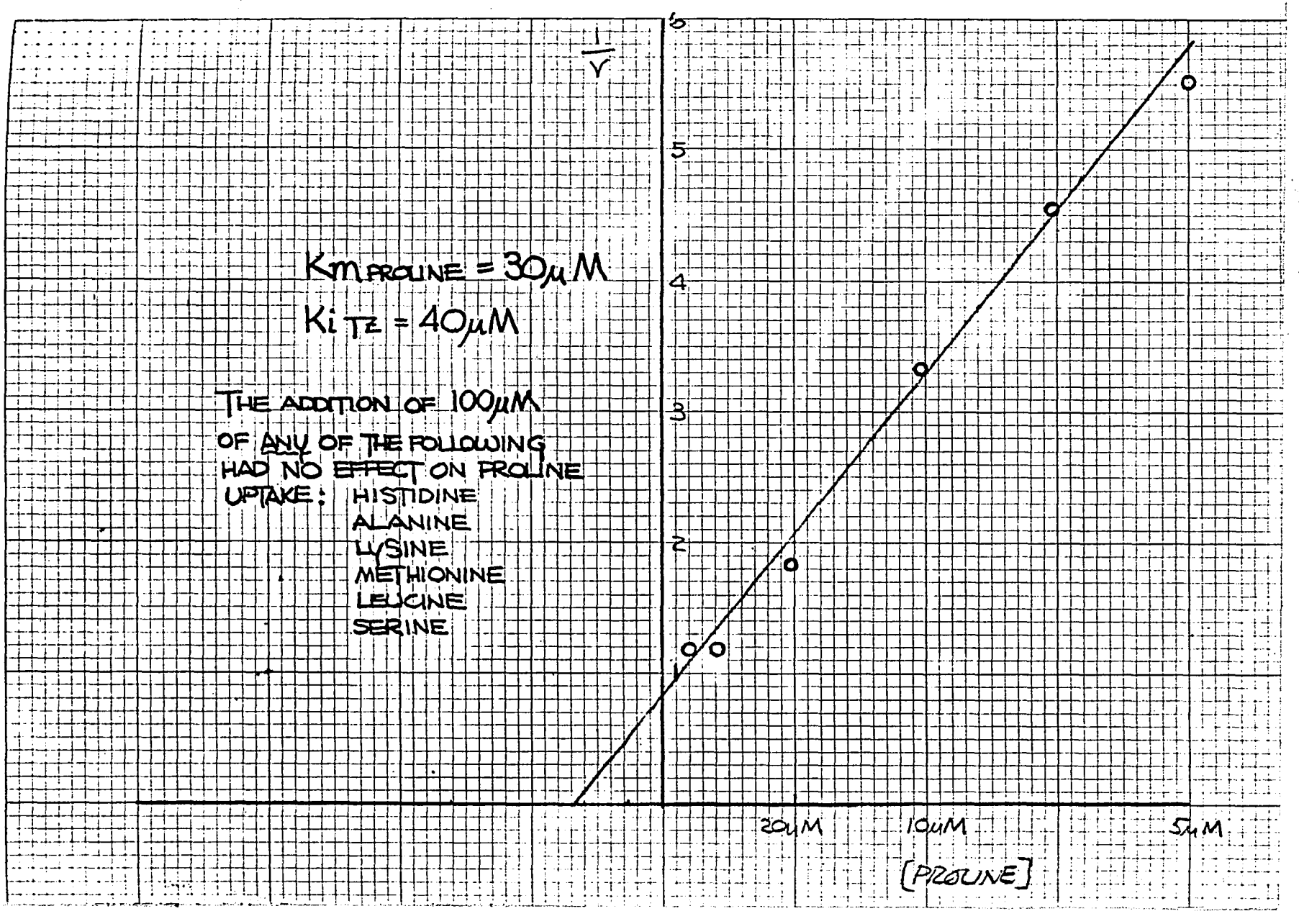
As previously discussed, the activity of the proline transport system in *S. chevalieri* (Kuznar *et al.*, 1973) was

Fig. 27. Lineweaver-Burk plot for proline uptake at 30C.

LINEWEAVER-BURK PLOT FOR PROLINE UPTAKE AT 30°C.

46 0700

FIGURE 27



$K_{m\,PROLINE} = 30\mu M$

$K_i\,TZ = 40\mu M$

THE ADDITION OF 100μM OF ANY OF THE FOLLOWING HAD NO EFFECT ON PROLINE UPTAKE:

HISTIDINE
ALANINE
LYSINE
METHIONINE
LEUCINE
SERINE

sensitive to nitrogen levels. A similar mechanism could mediate the cyclic fluctuations in proline transport activity seen in *S. cerevisiae*. In this way, partial derepression of the proline transport system would occur in stationary phase as nitrogen became limiting. Elaboration of the transport system could not take place because of the depleted pool of metabolized carbon, which is characteristic of stationary phase cultures. Following adaptation to fresh medium, the elaboration of the uptake system would proceed and an increased rate of proline uptake would be seen. Nitrogen in the culture medium would repress further increases in activity, and proline transport would begin to decline to a maintenance level, as dilution of the permease system (perhaps by growth and protein turnover) occurred.

Evidence of such a system of control would be established by studies which demonstrated increased proline uptake upon nitrogen starvation. Such an increase should require the presence of a carbon source, and should be proline-specific as well as sensitive to inhibitors of protein synthesis. In addition, severe nitrogen starvation might be expected to produce a greater degree of derepression with different decay kinetics from those observed in culture medium.

Table 4 describes the changes in the rates of proline uptake observed upon nitrogen starvation. When glucose was present in the starvation buffer, the rate of azide-sensitive

TABLE 4

Uptake Patterns of (^{3}H)-proline During Nitrogen Starvation

Time hrs.	*Rate of uptake pM/min/10^6 cells	Minus Glucose	Plus 5mM NaN_3	Plus 10µg/ml cycloheximide
0	0.5	-	0.08	0.38
0.5	0.40	0.45	-	-
1	0.90	-	-	-
2	2.60	0.70	-	-
3	6.40	-	-	0.28
4	5.90	1.20	-	-
5	6.10	-	0.10	-

*Twice-washed stationary phase cells were suspended in 66 mM potassium phosphate buffer, pH 5.5 with 100 mM glucose at a density of 5 x 10^7 cells/ml. At various intervals, samples were removed, washed, and resuspended in 0.1 M phosphate buffer pH 7.4 at a constant cell number. After equilibration at 30C, labeled proline was added to a final concentration of 35 M. Samples were removed at regular intervals, filtered, washed, and counted as previously described.

proline uptake increased 12-fold within 3 hours and remained at this elevated level for at least 2 hours. Omission of glucose from the starvation buffer largely prevented this increase, and cycloheximide treatment blocked it entirely. The possibility of concommitent increases in the uptake of other amino acids was not investigated. Kuznar *et al.*, (1973) have reported that no cycloheximide-sensitive increase in the transport of *other* amino acids occurred during nitrogen starvation in *S. chevalieri*.

The activity of the proline permease system in *S. cerevisiae* thus appeared to be regulated in a manner similar to that of *S. chevalieri*. Although differing dramatically in extent, the proline transport system in *S. cerevisiae* responded to the same conditions which produced an increase of several thousand-fold in *S. chevalieri*. The operation of such a regulatory system is sufficient to explain the cyclic fluctuations in proline uptake observed during normal cell growth.

CHAPTER VII

Selection and Characterization of TZ-Resistant and TZ-Hypersensitive Mutants:

In an effort to provide an experimental framework for the confirmation and extension of these studies, genetic investigations into the cellular response to TZ were initiated. The paradigm for these investigations involved the selection of mutant cell lines which displayed an altered response to TZ.

Isolation and Characterization of TZ-Resistant (TZr) Mutants:

The complete inhibition of growth on ethanol-TZ medium has provided a powerful selection mechanism for the isolation of TZ-resistant mutants. As described in Chapter II, mutants were selected from NTG-treated cells of strain cly1-1 (see below) based on their ability to grow on solid ethanol-TZ-medium. Thirty-six such mutants were selected, of which six showed high levels of resistance to TZ on ethanol medium (Fig. 28). The ability to respire ethanol during TZ growth was also measured in several of these mutants and found to be close to that of untreated cells (Table 5). In every case, mutants resistant to TZ on ethanol medium showed an equivalent increase in resistance to TZ when grown on glucose (Fig. 29). High-level resistance mutants crossed to a wild-type strain produced diploids which were either partially or fully TZ-resistant when compared to the haploid

Fig. 28. Growth curves for TZR mutants on ethanol-TZ medium.

Mutants derived from clyl-1 as described in *Materials and Methods*.

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FIGURE 28

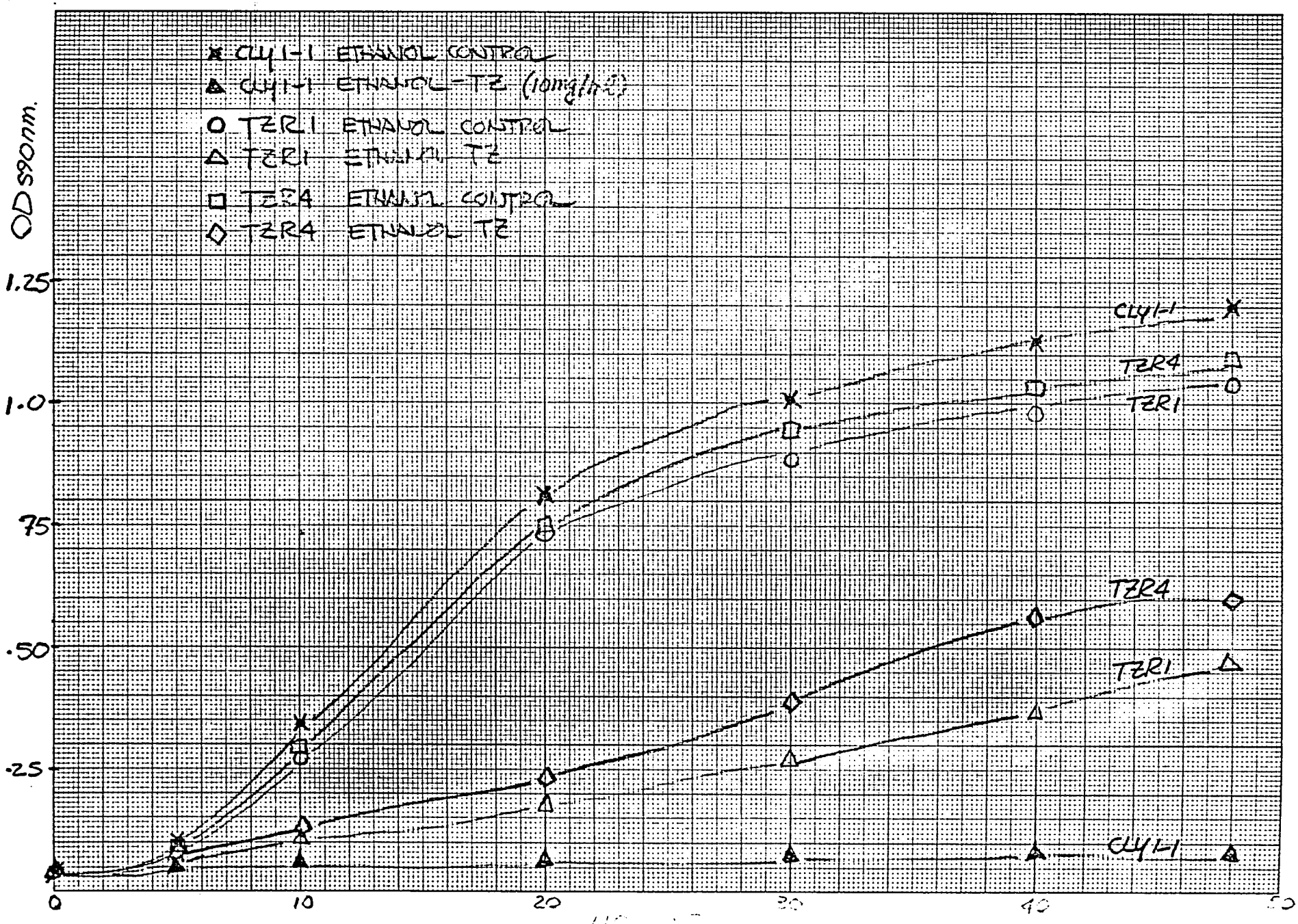


TABLE 5

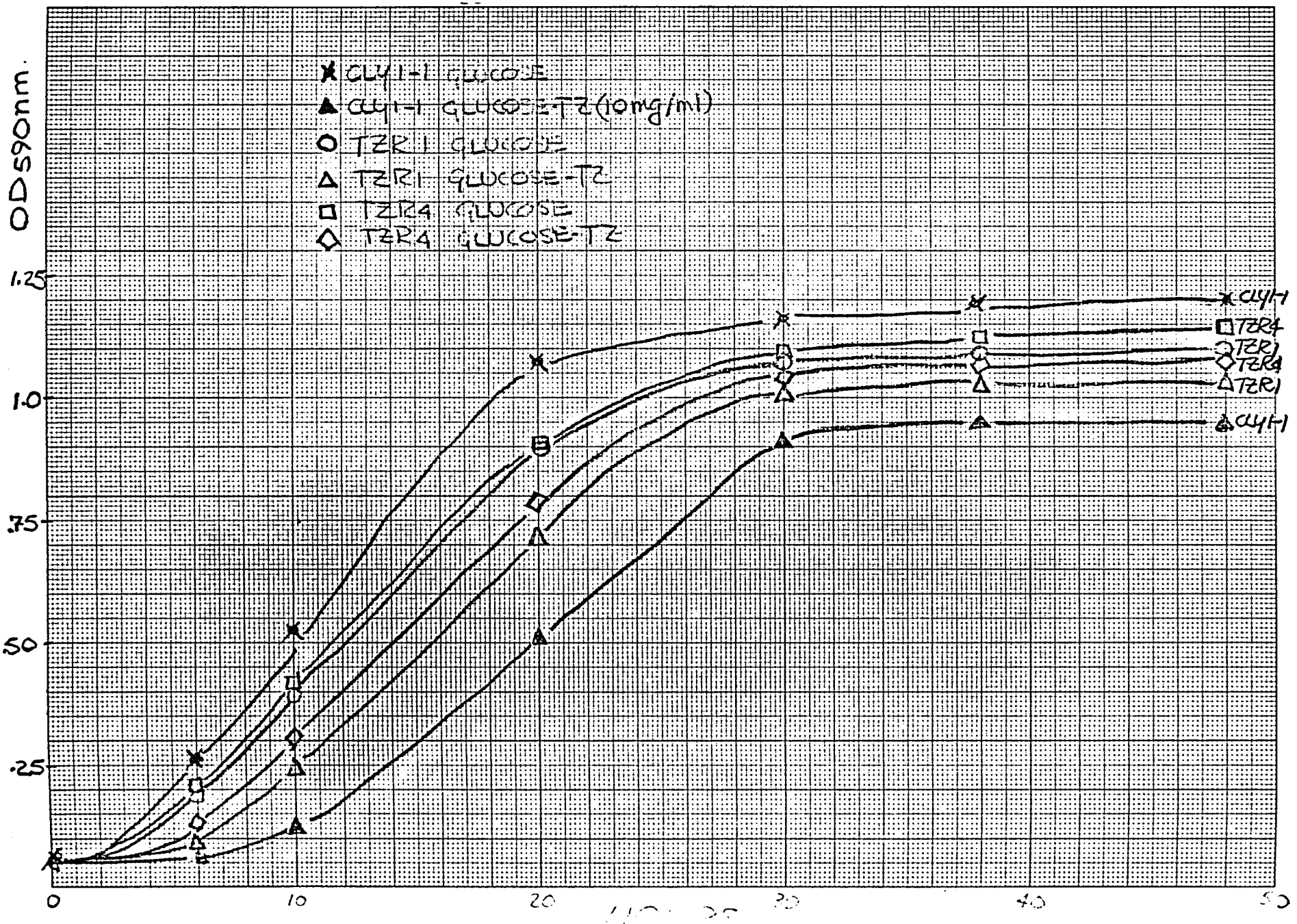
Relative Rates of Ethanol Oxidation Measured in Wild-type and TZR (TZ-resistant) Mutants

Strain	Growth Medium	Rate of Oxygen consumption $\mu l/min/10^7$ cells
cly1-1	glycerol	.75
cly1-1	glycerol-TZ	.20
TZR1	glycerol	.60
TZR1	glycerol-TZ	.47
TZR4	glycerol	.63
TZR4	glycerol-TZ	.52

For these studies, strains were pregrown in 0.5% yeast extract (YE), +3% glycerol (glycerol medium) in the presence or absence of 10 mg TZ/ml. After washing the cells were suspended in 20 mM phosphate buffer, pH 7.4 in a Rank oxygen electrode. After equilibration and measurement of the endogenous respiratory rate, ethanol was added to a final concentration of 20 mM.

Fig. 29. Growth curves for TZR mutants on glucose-TZ medium.

FIGURE 29


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
CLY1-1 GLUCOSE
CLY1-1 GLUCOSE-TZ(10mg/ml)
TZR1 GLUCOSE
TZR1 GLUCOSE-TZ
TZR4 GLUCOSE
TZR4 GLUCOSE-TZ
CLY1-1
TZR4
TZR1
TZR4
TZR1
CLY1-1

resistant parent. When sporulated, diploids from all crosses showed a segregation pattern which was consistant with the hypothesis that a single gene mutation in each mutant conferred TZ-resistance. The segregation of all other genetic markers including mating type was as expected and was independent of the particular TZR mutation (Table 6). When resistant spores of the opposite mating type, derived from the same mutant were crossed, the diploid showed levels of resistance identical to those of the heterozygous diploid for all mutants, indicating that in fact all of the TZR mutations were dominant (Fig. 30). When crossed in all possible pairwise combinations, diploids showing approximately the same levels of TZ resistance as the parents were formed. Difficulties with low sporulation frequency and poor spore viability prevented allele testing of these mutants.

The TZR-4 Mutant:

The data presented above, particularly that which indicated that TZR mutants selected on ethanol-TZ medium were also highly resistant on glucose medium, are most consonant with the hypothesis that TZR mutants are altered in TZ uptake. Other mechanisms of ethanol-TZ resistance such as that resulting from the production of altered ADH molecules which are insensitive to TZ would not be expected to decrease the glucose-TZ response so drastically. Furthermore, kinetic studies, on the ADH from TZR-4-6A, showed that

TABLE 6

Segregation Patterns From Crosses Between

TZ-resistant (TZR) Mutants and

Wild-type Strain Z1EK-27D

Cross (Z1EK-27D x)	#Tetrads Analysed	*Mating Type	*"cly" Gene	*HIS	*LYS	*TZR
R1	12	12	12	12	11	11
R4	15	15	14	15	14	15
R6	13	13	13	12	12	12
R12	12	11	11	10	10	10
R17	10	10	9	10	9	9
R25	11	11	10	11	10	11

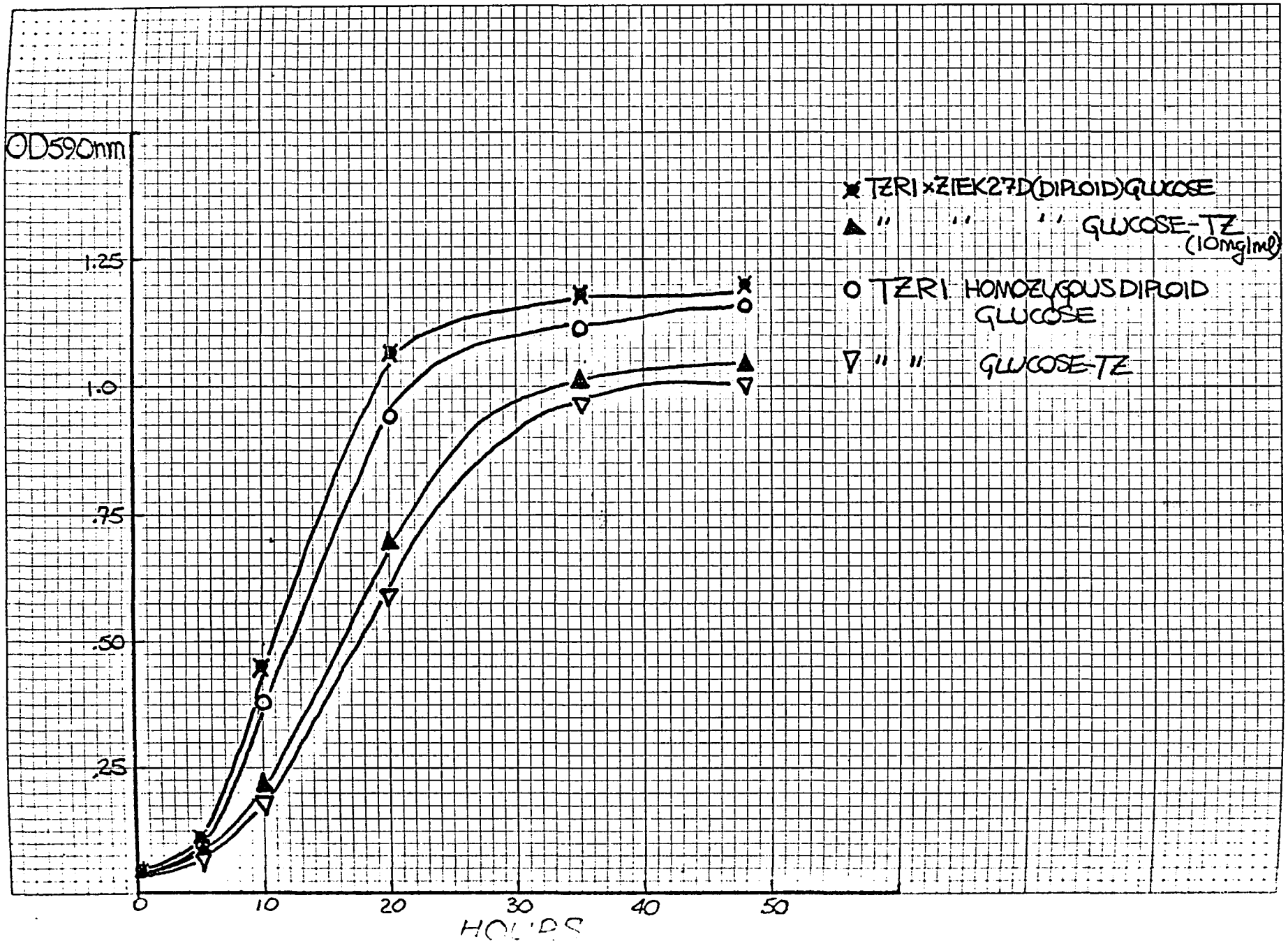
*Numbers indicate the tetrads which gave a normal (2:2) segregation pattern for the marker concerned.

**TZR (resistance to TZ) was determined by growing the progeny of each spore on both ethanol and ethanol-TZ medium for 48 hours. The final cell titres were then compared to those obtained for the haploid resistant mutant.

Fig. 30. Growth curves for heterozygous and homozygous TZ-resistant diploid on glucose medium.

Heterozygous diploids were formed between haploid TZ-resistant mutants and ZlEK-27D, a wild-type strain.

FIGURE 30


OD590nm
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
TZR1 x ZIEK27D (DIPLOID) GLUCOSE
" " " GLUCOSE-TZ (10mg/ml)
TZR1 HOMOZYGOUS DIPLOID GLUCOSE
" " GLUCOSE-TZ

the enzyme was TZ-inhibitable to the same extent as the enzyme isolated from the wild-type parent (Table 7). The over-production of proline, which is the basis of a dehydroproline (DHP) resistant mutant of *E. coli* (Baich & Pierson, 1965) would only be expected to prevent ethanol-TZ inhibition if it blocked initial TZ uptake, since proline itself does not reverse ADH inhibition.

TZ-resistance at the transport level could result from changes in the realtive affinity of the permease system for proline and TZ. Mutants of *E. coli* have been reported which cannot take up azetidine 2-carboxylic acid (AZ) (Neale & Tristram, 1965), although they retain the ability to accumulate proline and are sensitive to DHP. It is clear from this that changes in the pattern of control of imino acid uptake which prevented the accumulation of effective levels of TZ would also result in cellular resistance.

Uptake studies using [^{14}C]-TZ and similar studies using [^{3}H]-proline (Figs. 31 & 32) have indicated that TZR4, when compared to wild-type, showed a decreased ability to take up either TZ or proline. The initial and maintenance levels of uptake were identical in the mutant and wild-type for both proline and TZ on glucose medium. The major difference between the uptake patterns of TZR4 and cly1-1 was the absence of a spike of uptake in the mutant which occurred in the wild-type strain at about 4 hours. These data suggest that the function of the gene affected in the TZR4 mutant

TABLE 7

Kinetic Studies on the Alcohol Dehydrogenase Prepared From Wild-type and TZ-resistant Strains

Strain	Km (Ethanol)	Km (NAD)	Ki (TZ)	Effect of proline
clyl-1	8.0 mM	.15 mM	25 mM	nil
TZR4-6A	8.5 mM	.12 mM	25 mM	nil

Preparation of crude extracts and assay of alcohol dehydrogenase were conducted as described in Materials and Methods and the legend to figure 24.

Fig. 31. Uptake of (^{14}C)TZ by parental wild-type and a TZ-resistant mutant.

Experiments were performed as described in the legend to Figure 21.

[14C]-TZ UPTAKE CLY1-1 & TZRA(6A)
Insulin
CLY1-1
TZRA(6A)
CPM/10^7 CELLS
600
400
200
0
10
20
30
HOURS
EUGENE DIETZGEN CO.
MADE IN U.S.A.
NO. 341-M DIETZGEN GRAPH PAPER
MILLIMETER

Fig. 32. Uptake of (^{3}H)-proline by parental wild-type and a TZ-resistant mutant.

[3H]-PROLINE UPTAKE CCY 1-1 & TZR46A
0.5μCi/ml
CPM/10⁷CELLS
8,000
6,000
4,000
2,000
CCY 1-1
TZR46A
0
10
20
30
HOURS
EUGENE DIETZGEN CO.
MADE IN U.S.A.
NO. 341-M DIETZGEN GRAPH PAPER
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concerns the derepression of the proline uptake system. The alternate hypothesis that the mutation lowered the affinity of the proline transport system for both imino acids is not rigorously excluded. However, such a "permease" mutation would be expected to be *recessive* in the diploid condition. In this connection, *dominant* regulatory mutations affecting derepression of proline permease and proline biosynthetic systems in *Aspergillus* have recently been reported (Arst & MacDonald, 1975). Further attempts to characterize TZR4 as either a "derepression negative" (regulatory gene mutation) or a permease mutant (structural gene mutation have not been made.

Isolation and Characterization of TZ-Hypersensitive (TZS) Mutants:

The importance of selecting mutants which were unable to recover from growth inhibition by TZ on glucose medium is clear. Since recovery normally involved changes in the activity of the proline uptake system, a likely mechanism for hypersensitivity would involve genetic changes which produce alterations in the specificity or pattern of imino acid uptake. The ability to select such mutants would provide a tool for the extension of studies into imino acid uptake and metabolism in yeast. Unfortunately, the hypersensitive phenotype is manifested by a lack of growth in much the same way as is auxotrophy. The cryptic nature of non-growing mutants in growing cell populations has tra-

ditionally made their selection difficult. Enrichment techniques, such as nystatin treatment (Thouvenot & Bourgeois, 1971) are logistically cumbersome and, in our hands, unreliable. The recent introduction of "suicide strains" (Littlewood, 1972), that is, strains which kill themselves if allowed to grow under the appropriate conditions, has greatly facilitated the selection of various types of conditionally non-growing mutants. The utilization of one such "suicide strain" has produced specific TZ-hypersensitive mutants as a general selection system for other classes of non-growing mutants.

Clyl-1 (formerly popl-1) was derived by Hartwell's group (Hartwell, 1967) from strain A364A. This strain is temperature-sensitive for viability as a result of a single, recessive nuclear gene mutation. Incubation of the mutant under growth conditions at the restrictive temperature (35C) caused abnormal division figures to be formed. Continued incubation caused the cells to lyse. Cells grown at the permissive temperature (25C) appeared normal. It was postulated that the defect involved the synthesis of an abnormal cell wall (Hartwell, 1971). Lethality profiles for clyl-1 under growth and non-growth conditions are shown in Table 8. Cell lysis occurred at the restrictive temperature only when growth conditions were provided.

In order to select TZS mutants, cells of clyl-1 were mutagenized with ethylmethanesulfonate as described else-

TABLE 8

Lethality Profiles for cly1-1 Under Growth and Non-growth Conditions

TIME	YEPD (Growth) 25C.	YEPD (Growth) 35C.	SM (Non-growth) 25C.	SM (Non-growth) 35C.
0	192	203	145	140
3	297	244	144	143
21	2500+	99	147	136

Identical cultures in growth (YEPD) and non-growth medium (SM) were incubated at 25C. and 35C. At the times indicated, samples were removed, diluted and plated on YEPD plates which were incubated at 25C. Data shown represents the average of three plates for each determination.

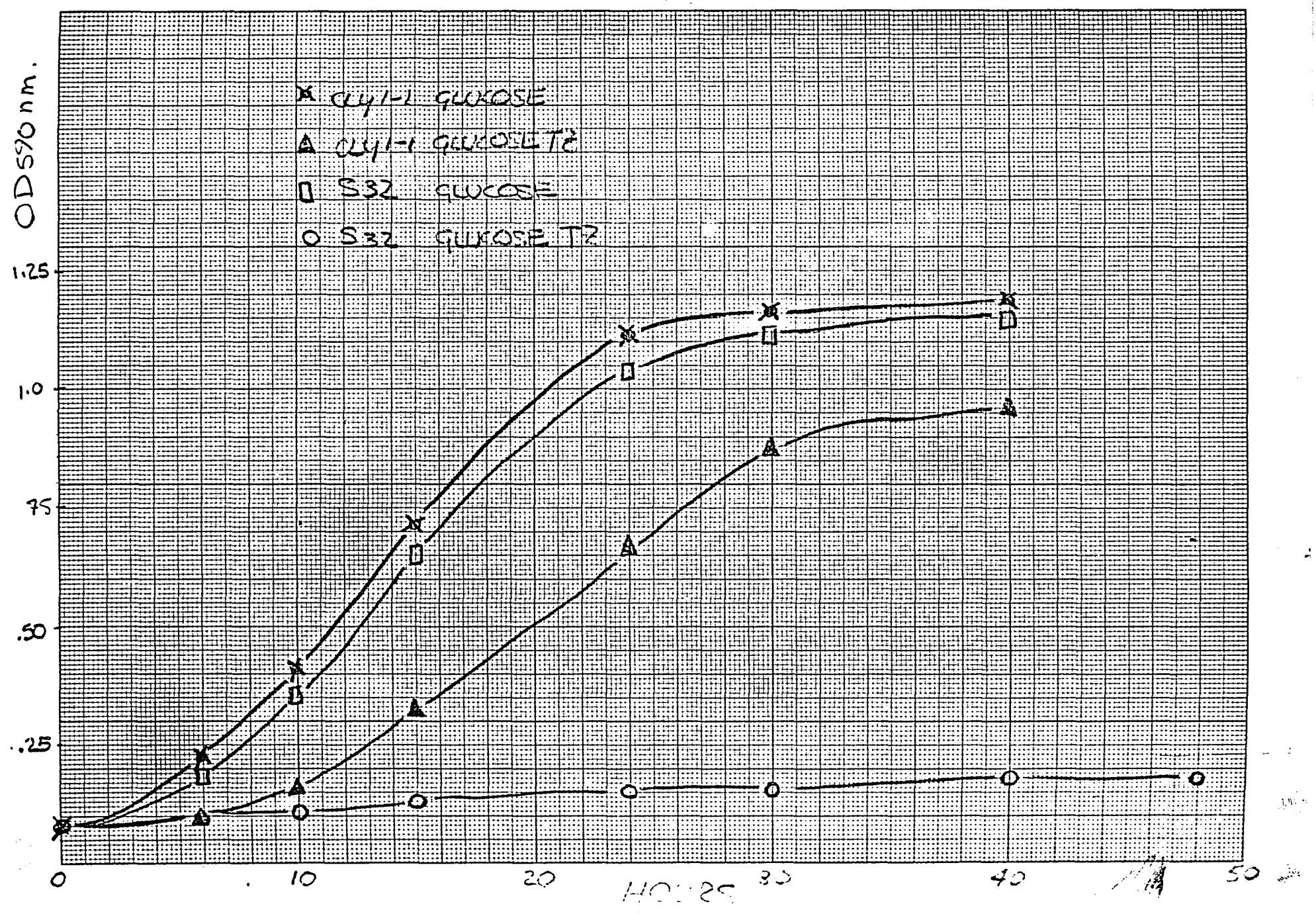
where (Sherman *et al.*, 1970) and suspended in glucose-TZ medium (10 mg TZ/ml) at a density of 1 x 10^6 cells/ml at 35C. Samples were removed at various times during a 72 hour period, plated on YEPD medium and incubated at 25C. After 7-10 days, small colonies appeared which were purified by streaking on YEPD. YEPD master-plates prepared from the purified colonies were replicated to glucose-TZ medium to identify possible hypersensitive mutants. Although 40 independently arising mutants were originally identified, most were unstable, and only three proved suitable for further analysis.

The three stable TZS mutants were almost completely sensitive to TZ on glucose medium (Fig. 33). Their growth was also totally inhibited on ethanol-TZ medium. Diploids made between a wild-type strain and any of the mutants showed a wild-type response to the analogue on glucose-TZ medium, which indicated that the hypersensitive phenotype was recessive to wild-type.

Initial studies on the genetic segregation of the TZS trait were hampered by extremely poor spore viability and abnormal segregation patterns in crosses. Hartwell, in personal communications, has acknowledged encountering the same difficulty with this strain. He has suggested that another cell-lysis strain, cly8, be used for future mutant isolations. After repeated attempts, genetic analysis of the TZS mutants was finally accomplished. Segregation

Fig. 33. Growth curves for parental wild-type and TZ-sensitive mutant, TZS32 on glucose medium.

FIGURE 33


OD 590 nm.
1.25
1.0
.75
.50
.25
0
10
20
30
40
50
HOURS
X cyl-1 GLUCOSE
A cyl-1 GLUCOSE T2
□ S32 GLUCOSE
○ S32 GLUCOSE T2

data (Table 9) indicated that each mutant carried two unlinked genes, either of which caused TZ-hypersensitivity. These data were reconfirmed by complementation analysis of each mutant. Spores from tetrads showing 4:0 (hypersensitive: wild-type) segregation were crossed among themselves and the diploids were tested for TZ hypersensitivity. The diploids were sporulated and the segregation patterns of the TZS traits was analysed. In all cases, the data were consistant with the original observation that each stable TZS isolate was indeed a double mutant.

Complementation analysis between all possible mutant combinations (Table 10) indicated that each double mutant had one locus in common. Thus, at least four complementation groups, which have been designated mtt-1, mtt-2, mtt-3, and mtt-4 (mtt: mutant type), can be identified with the TZS phenotype.

Among the possible mechanisms of TZ-hypersensitivity is proline auxotrophy. If the required intracellular levels of proline cannot be maintained as the activity of the proline transport system declines, then recovery will probably not occur. Proline auxotrophic mutants are known in bacterial systems (Curtiss, 1965). However, with the exception of a single report by Thouvenot and Bourgeois (1971), such mutants have not been identified in yeast. Ambitious attempts by other workers to isolate proline auxotrophic mutants have been to date unsuccessful (G. Fink, personal

TABLE 9

Segregation Patterns From Crosses Between TZ-hypersensitive Mutants (TZS) and Wild-type Strains Z1EK-27D

Cross (Z1EK27D x)	#Tetrads Analysed	*Mating Type	"Cly" Gene	HIS	LYS	TZS**
S12	12	12	11	12	11	2 (2:2) 7 (3:1) 3 (4:0)
S17	13	13	11	12	11	3 (2:2) 8 (3:1) 2 (4:0)
S32	16	15	13	15	13	3 (2:2) 10 (3:1) 3 (4:0)

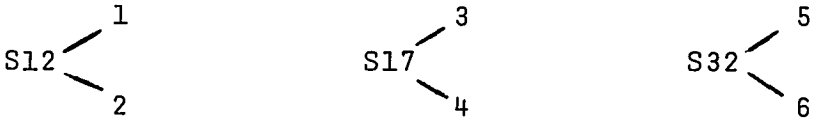
*Numbers indicate the tetrads which gave the expected (2:2) segregation pattern for the marker concerned.

** TZS (hypersensitivity to TZ) was determined by growing the progeny of each spore on both glucose and glucose-TZ medium for 48 hours. The final cell titres were then compared to those obtained for the haploid hypersensitive mutants. The data are tabulated to show the distribution of the tetrads among the various ascal types. All ratios are given as hypersensitive:wt.

TABLE 10

Complementation Analysis of Crosses Between TZ-hypersensitive (TZS) Mutants

The analysis in Table 9 showed that each stable TZS isolate was a double mutant. The specific genetic content of the spores from 4:0 (sens:wt) asci with respect to TZS mutations was determined, and the isolated single mutants were crossed in all possible pairwise combinations. The single mutations are arbitrarily numbered as indicated.



Complementation Diagram

	1	2	3	4	5	6
1	-	+	-	+	+	-
2		-	+	+	+	+
3			-	+	+	-
4				-	+	+
5					-	+
6						-

communication). When examined for proline auxotrophy by replication to two different proline-less media (a complete synthetic and supplemented minimal medium were used), no meiotic segregant of any TZS mutant showed a growth requirement which could be relieved by the addition of proline to the culture medium. In addition, the effects of TZ were not reversible by proline in many cases. As will be discussed below, the growth test for proline auxotrophy would be seriously deficient if applied to only the double mutants, since the identification of a proline auxotroph in a proline uptake-negative background could not be made.

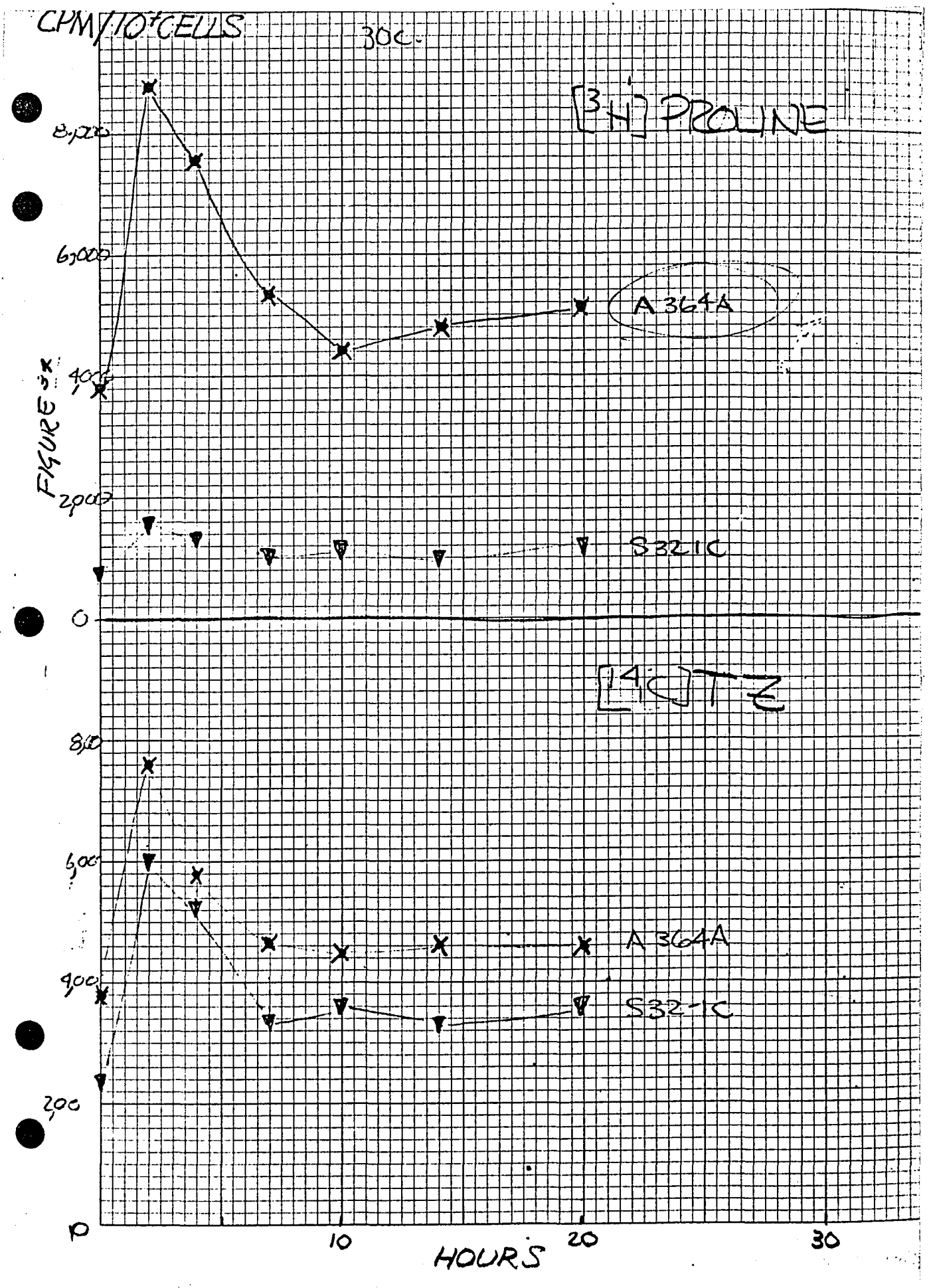
The Activity of the Proline Uptake System In TZS 32-1C:

The patterns of radio-labeled proline and TZ uptake were examined in this strain which carries a TZ sensitivity-confering mutation at the mtt-1 locus (Figs. 34 A & B). The data suggest that proline uptake in the mutant was seriously depressed at all points in the growth cycle when compared to the wild-type parent. By contrast, *TZ* uptake proceeded in the mutant at nearly wild-type levels, and showed the expected changes in activity during the growth cycle. The decreased proline uptake during both spike and maintenance periods indicated that the ability of the proline uptake system to transport its natural substrate had been impaired.

A likely hypothesis to explain these data assumes that mtt-1 is the site of the structural gene for the proline permease protein. Since a permease is thought to interact di-

Fig. 34. Uptake of (^{14}C)-TZ (A); and (^{3}H)-proline by wild-type and a TZ-hypersensitive mutant, S32-1C.

Uptake experiments were performed as described in the legend to figure 21.


CPM/10⁴CELLS
30C
[3H] PROLINE
8,000
6,000
4,000
2,000
0
8,00
6,00
4,00
2,00
A364A
S321C
[14C]TZ
A364A
S32-1C
FIGURE 3x
0
10
20
30
HOURS

rectly with the substrate to be transported (Lin, 1970), changes in the primary structure of this protein could alter its affinity for specific imino acids, while leaving the pattern of formation of the permease during the growth cycle unaltered. Such a condition would not be expected to be proline-reversible, and should appear recessive to wild-type in the diploid condition.

CHAPTER VIII

DISCUSSION

A. TZ Inhibition Patterns on Various Fermentable and Non-fermentable Carbon Sources:

Strains of *Saccharomyces* grown in the presence of TZ, on certain carbon sources (glucose, glycerol, acetate), showed transient growth inhibition followed by a period of partial recovery. This inhibition was specifically proline-reversible and did not depend for its expression on a functional mitochondrial system. (Studies not reported here, have demonstrated that TZ is neither an inhibitor of electron transport, nor an uncoupler of oxidative phosphorylation in preparations of whole mitochondria from rat liver.) The degree of growth inhibition was shown to depend upon the analogue concentration, the nature of the carbon sources in the growth medium, and the point in the growth cycle at which the analogue was added to the culture. In addition, studies reported elsewhere (Paterniti, *et al.*, 1975) have shown that cells which were glucose-repressed by pregrowing in 10% glucose-containing medium, were incapable of growth when transferred to acetate-TZ medium. Under conditions where de-repressed cells were used, recovery and growth normally occurred. Such studies indicated that the state of catabolite repression of the culture is also a factor in the response of cells to the analogue.

Growth studies in which ethanol was added to TZ-contain-

ing medium, either as the sole carbon source, or as part of a mixture, gave patterns which reflected severe inhibition by the analogue from which recovery usually did not occur. This inhibition was not proline-reversible, although it was partially glucose reversible. The nature of this inhibition strongly suggested that its mechanism was different from that seen on other carbon sources.

Under certain experimental conditions, ethanol was shown to interfere with the utilization of other carbon sources such as glucose or glycerol. In the case of glucose, it seems likely that if high concentrations of ethanol were present intracellularly, the overall rate of acetaldehyde reduction in glycolysis would be lowered. Such an effect would result from a shift in the reaction equilibrium away from ethanol production, which would be expected to interfere with NADH oxidation, and therefore with total glycolytic activity. This effect would be most pronounced under conditions where glucose repressed the formation of mitochondrial enzymes which might reoxidize NADH. The catabolite repression of mitochondrial respiratory chain enzymes by glucose would also prevent rapid ethanol utilization and thus prolong its inhibitory effect. Under such circumstances, pathways such as the hexose monophosphate shunt might serve to produce some energy from glucose. However, in the absence of a fully functioning respiratory chain, this contribution would necessarily be small.

When ethanol was added to glycerol medium in the absence of TZ, growth rates were obtained which were greater than those seen with either carbon source alone. Such results are not surprising when one considers that glycerol is relatively troublesome to metabolize for energy, but furnishes an excellent starting material for carbohydrate and lipid biosynthesis. The opposite situation obtains for ethanol. The combination of these two carbon sources should provide conditions for rapid and extensive culture growth.

By contrast, the nature of the interaction of these carbon sources in the presence of TZ is less clear. There are at least two conceptual ways of viewing the total growth inhibition which occurs on glycerol-ethanol-TZ medium. It is possible that for the purpose of *energy production*, when ethanol is present in the medium cells do not "see" glycerol, which may be channeled primarily into biosynthetic pathways. Under conditions where ethanol metabolism is blocked (e.g. by TZ), no growth will occur. Such a hypothesis can be tested by metabolism experiments in which CO_2 production from labeled glycerol is determined in the presence and absence of ethanol.

Alternatively, ethanol may be an obligatory intermediate in glycerol metabolism, perhaps required for oxidation of the NADH formed when glycerol enters the glycolysis pathway. In the presence of TZ, ADH is not active, and ethanol would not be formed from glycerol. Some interesting evi-

dence for a link between glycerol and ethanol metabolism comes from studies in our laboratory on alcohol dehydrogenase mutants of yeast obtained through the kind generosity of Dr. Michael Ciriacy of Freiburg University, West Germany. Such mutants lack all but trace amounts of the constitutive cytoplasmic ADH. In addition, they lack all detectable mitochondrial ADH, and produce either an altered or inactive repressible cytoplasmic enzyme. One such mutant, 16-2B, which synthesizes only an altered but active repressible cytoplasmic ADH, is able to grow on either glucose or acetate, but is incapable of growth on *either* glycerol or ethanol medium. Further studies on ADH mutants and their revertants may be helpful in clarifying the relationships between the metabolism of these carbon sources.

B. Probable Modes of Action of TZ In *S. Cerevisiae:* Proline Reversible:

The data obtained from growth studies indicated that TZ probably exhibited two different modes of action in yeast. In its role as an analogue of proline, TZ or a derivative was shown to be incorporated into cell protein. Although the specific replacement of proline residues in amino acid chains by TZ has not been demonstrated, radioactivity from [^{14}C]TZ was recovered after hydrolysis and chromatography of an extract of total cell protein. TZ incorporation was sensitive to inhibitors of cytoplasmic and mitochondrial protein synthesis, and no evidence for the

differential incorporation of TZ into these compartments was found.

Radioactivity from [^{14}C]TZ-labeled proteins is always recovered from a region of the chromatograph which does not have the R_f of TZ. It is unclear whether such results are mere artifacts or if they represent the actual form in which TZ is incorporated into protein. In this context, Baum *et. al.*, (1975) have reported the alteration of another proline analogue, azetidine-2-carboxylic acid (AZ) during its recovery from rabbit reticulocyte hemoglobin. The alteration was shown to be an artifact of the acid hydrolysis procedure. Hydrolysates obtained after BaOH treatment contained unaltered AZ. Although the results of this study are not directly comparable to the present study, the use of alternate hydrolysis protocols would be an important part of any further effort to determine the form in which TZ is incorporated into protein.

The amount of labeled TZ which could be incorporated into cell protein was found to depend upon the stage in the growth cycle at which the label was introduced. In general, incorporation was greatest in lag phase cultures and declined as the cycle progressed. The pattern of TZ incorporation could be directly related to the degree of TZ inhibition observed, and this suggested that a mechanism for the regulation of TZ incorporation existed. It was reasoned tht one level at which this control could be exerted was the entry of the

analogue into the cell.

When the level of analogue uptake was examined in relation to the growth cycle, fluctuations in uptake activity, which paralleled both TZ incorporation and TZ inhibition patterns were observed. It was found that prior exposure to the analogue did not significantly alter this uptake pattern. However, when the pattern of uptake was perturbed, e.g. by permeabilizing cells with DMSO, the kinetics and extent of recovery from analogue inhibition were profoundly altered. This relationship between uptake and inhibition patterns was reinforced by experiments in which TZ was added to cultures at progressively later times in the growth cycle, with progressively less inhibitory effect. Taken together, these data argued that recovery from TZ inhibition involved exclusion of the analogue from the cell, and that this discrimination occurred at the level of uptake.

In the light of the foregoing observations, as well as those which showed that the effects of TZ on glucose medium were specifically proline reversible, studies were undertaken to determine if TZ was carried into cells by way of a proline permease system. Reports on the proline uptake system of the closely related ascomycete, *S. chevalieri* were used as a basis for these studies (Magaña-Schwencke *et al.*, 1968; Kuznar *et al.*, 1973). Proline uptake in *S. cerevisiae* was shown to exhibit the same fluctuations in activity as were observed for the uptake of TZ. Further-

more, TZ could compete specifically with proline for uptake while amino acids could not. In some experiments, cysteine seemed to have had a slight stimulatory effect on both proline and TZ uptake. It is possible that this effect explains the increase in TZ inhibition seen when cysteine was present in culture medium (see page 30).

In general, the proline uptake system of *S. cerevisiae* showed similar properties to that in *S. chevalieri*. In addition to similar substrate specificities, uptake in both systems was azide-sensitive and the apparent affinity constants for substrates were almost identical. Furthermore, nitrogen repression has an important role in the regulation of proline uptake in both organisms, although the extent of the changes in activity which accompanied derepression was different. Nitrogen repression control of proline uptake is not unique to these fungi, and it is seen in combination with catabolite repression and induction in *Aspergillus* (Arst & MacDonald, 1975).

C. Probable Modes of Action of TZ in *S. Cerevisiae:* Proline Irreversible:

As previously discussed, under the experimental conditions employed (10 mg TZ/ml, 100 mM proline), TZ inhibition on ethanol medium was not proline reversible. This indicated that even in the presence of high concentrations of proline (100 mM), TZ could enter cells. (This same conclusion is reinforced by examination of the apparent affinity

constants for proline (25 μM), and TZ (40 μM). The analogue was shown to inhibit yeast alcohol dehydrogenase in a non-competitive manner, which was not proline reversible *in vitro*. On glucose medium the inhibition of ADH itself would not be expected to block cell division, since ADH-less or pyrazole-treated strains of yeast grow well on glucose medium (unpublished observations). Furthermore, if proline were present in glucose-TZ medium. additional competition with the analogue would be seen at the prolyl-t-RNA synthetase as has been reported for TZ by Peterson and Fowden (1965) in mung-bean seedlings. By contrast, ADH inhibition would cause complete growth cessation when ethanol was the sole carbon source.

D. Genetic Aspects:

From the considerations already presented, it is clear that mutations which produced an altered response to TZ could occur in any of a number of genes which controlled diverse cellular activities. Viewed in these terms, the analogue represents a potentially powerful tool for the selection of mutants in the proline uptake, biosynthetic, or regulatory systems, as well as mutants of the ADH enzymes and the protein synthesis system. Examples of this are TZ-resistant and hypersensitive mutants which have been isolated and characterized.

The TZ-resistant strain (TZR-4) selected for genetic and biochemical studies arose from a single dominant muta-

tion which probably affected the derepression of the proline uptake system. By contrast, the TZ-hypersensitive strain (TZS 32-1C) carried a recessive mutation which probably altered the substrate specificities of the proline permease.

Mutants with similar genetic and biochemical properties have been reported in the proline "operon" of *Aspergillus* (Arst & MacDonald, 1975). One class of mutants map in a regulatory region between a tightly-linked proline permease structural gene and the putative structural gene for a common subunit of proline oxidase and Δ'-pyrroline-5-carboxylic acid dehydrogenase. These *cis*-dominant mutations affect the derepression of the sturctural genes of the "operon". In addition, recessive mutations have been characterized which map in the structural gene regions of this system.

The proline system in *Aspergillus* represents the first demonstration of an operon-type regulatory complex in a eukaryote. By contrast, the nature of the genetic control of proline transport and biosynthesis in yeast is completely unclear. In this regard, studies on the functional relationships between various TZ-resistant and TZ-hypersensitive mutants may prove particularly promising.

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase (E.C. 1.1.1.1.)
AZ	azetidine-2-carboxylic acid (proline analogue)
BME	β-mercaptoethanol
BSA	bovine serum albumin
CAP	chloramphenicol
CHX	cycloheximide
DHP	3,4-dehydro proline (proline analogue)
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetra-acetic acid
ER	erythromycin
R_f	retardation factor
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TLC	thin-layer chromatography
TZ	thiazolidine-4-carboxylic acid (proline analogue)
TZR	TZ-resistant
TZS	TZ-hypersensitive

LITERATURE CITED

Adams, B. G. 1972. Method for decryptification of α-glucosidase in yeast with dimethyl sulfoxide. Anal. Biochem. 45:137-146.

Akhrem, A. A., and A. I. Kuznetsova. 1964. Thin-layer chromatography; a practical laboratory handbook. Daniel Davey & Co., New York.

Arst, H. N., and D. W. MacDonald. 1975. A gene cluster in *Aspergillus nidulans* with an internally located *cis*-acting regulatory region. Nature 254:26-31.

Baich, A., and D. J. Peirson. 1965. Control of proline synthesis in *Escherichia coli*. BBA 104:397-404.

Baum, B. J., L. S. Johnson, C. Franzblau, and R. F. Troxler. 1975. L-azetidine-2-carboxylic acid incorporation into hemoglobin in rabbit reticulocytes *in vitro*. J. Biol. Chem. 250:1464-1471.

Bekhor, I. J., Z. Mohseni, and L. A. Bavetta. 1965. Inhibition of ^{14}C-proline incorporation into rat liver ribosomes by thiazolidine-4-carboxylic acid in a cell-free system. Proc. Soc. Exptl. Biol. Med. 119:765-769.

Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. Ann. Rev. Biochem. 42:471-506.

Berg, P. 1961. Specificity in protein synthesis. Ann. Rev. Biochem. 30:293-324.

Bicknell, J. N., and H. C. Douglas. 1970. Nucleic acid homologues among species of *Saccharomyces*. J. Bacteriol. 101:505-512.

Britten, R. J., and F. T. McClure. 1962. The amino acid pool in *Escherichia coli*. Bacteriol. Rev. 26:292-335.

Brockman, H. E. 1964. Effects of amino acids on the utilization of tryptophan and indole for growth by a mutant of *Neurospora crassa*. J. Gen. Microbiol. 34:31-41.

Calender, R., and P. Berg. 1966. The catalytic properties of tyrosyl ribonucleic acid synthetases from *Escherichia coli Bacillus subtilis*. Biochem. 5:1690-1695.

Calvo, J. M., and G. R. Fink. 1971. Regulation of biosynthetic pathways in bacteria and fungi. Ann. Rev. Biochem. 40:943-968.

Cavallini, D., C. DeMarco, B. Mondov, and F. Trasarti. 1956. Studies on the metabolism of thiazolidine carboxylic acid by rat liver homogenate. Biochim. Biophys. Acta 22:558-564.

Cleland, W. W. 1967. The statistical analysis of enzyme kinetic data. Adv. Enzymol. 29:1-32.

Curtiss, R. 1965. Chromosomal abberations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. 89:28-40.

Dahlbon, R., B. Tolf, A. Akeson, G. Lundquist, and H. Theorell. 1974. On the inhibitory power of some further pyrazole derivatives on horse liver alcohol dehydrogenase. Biochem.

Biophys. Res. Commun. 57:549-553.

Eaton, N. R. 1962. New press for disruption of microorganisms. J. Bacteriol. 83:1359-1360.

Eddy, A. A., and K. J. Indge. 1962. The dependence of amino acid transport in yeast on both energy supply and intracellular potassium ions. Biochem. J. 82: 15-16.

Evans, P. D. 1973. The uptake of l-glutamate by the peripheral nerves of the crab, *Carcinus maenus*. Biochim. Biophys. Acta 311:302-313.

Fink, G. R., and R. Lowenstein. 1969. Simplified method for testing mutagens in *Saccharomyces*. J. Bacteriol. 100:1126-1127.

Fowden, L., S. Neale, and H. Tristram. 1963. Effect of 3,4-dehydro-L-proline on growth and protein synthesis. Nature 199:35-38.

Fowden, L., and M. H. Richmond. 1963. Replacemnt of proline by azetidine-2-carboxylic acid during biosynthesis of protein. Biochim. Biophys. Acta 71:459-461.

Fowden, L. 1964. The chemistry and metabolism of recently isolated amino acids. Ann. Rev. Biochem. 33:173-204.

Fowden, L., D. Lewis, and H. Tristram. 1967. Toxic amino acids: their action as antimetabolites. Adv. Enzymol. 29:89-161.

Grenson, M., M. Mousset, J. M. Wiame, and J. Bechet. 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine-

transporting system. Biochim. Biophys. Acta 127: 325-338.

Hartwell, L. H. 1967. Macromolecular synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. 93: 1662-1670.

Hayes, J. E., and S. F. Velick. 1954. Yeast alcohol dehydrogenase: molecular weight, coenzyme binding, and reaction equilibria. J. Biol. Chem. 207:225-244.

Hirata, H., A. Asano, and A. F. Brodie. 1971. Respiratory dependent transport of proline by electron transport particles from *Mycobacterium phlei*. Biochem. Biophys. Res. Commun. 44:368-374.

Hirata, H., F. C. Kosmakos, and A. F. Brodie. 1974. Active transport of proline in membrane preparations from *Mycobacterium phlei*. J. Biol. Chem. 249:6965-6970.

Holtzapple, P., M. Genel, C. Rea, and S. Segal. 1973. Metabolism and uptake of l-proline by human kidney cortex. Pediat. Res. 7:818-825.

Inui, Y., and H. Akedo. 1965. Amino acid uptake by *Escherichia coli* grown in the presence of amino acids: evidence for repressibility of amino acid uptake. Biochim. Biophys. Acta 94:143-152.

Jornvall, H. 1973. Partial similarities between yeast and liver alcohol dehydrogenases. Proc. Natl. Acad. Sci. 70:2295-2298.

Kuznar, J., J. Schwencke, and N. Magaña-Schwencke. 1973. Sarcosine and amino acid uptake in *Saccharomyces*

chevalieri: derepression by nitrogen starvation. Biochim. Biophys. Acta 318:273-280.

Lallier, R. 1965. Effects de différents analogues de la proline sur le développement de l'oeuf de l'oursin *Paracentrotus lividus*. Exptl. Cell. Res. 40:630-636.

Lester, G. and C. Yanofsky. 1961. Influence of 3-methylanthroanilic acid and anthanilic acids on the formation of tryptophan synthetase in *Escherichia coli*. J. Bact. 81:81-89.

Lavenberg, B., I. Melnick, and J. M. Buchanan. 1957. Biosynthesis of the purines. XV. The effect of aza-L-serine and 6-diazo-5-oxo-L-norleucine on inosinic acid biosynthesis *de novo*. J. Biol. Chem. 225:163-176.

Levin, A. P., and P. E. Hartman. 1963. Action of a histidine analogue, 1,2,4-triazole-3-alanine in *Salmonella typhimurium*. J. Bacteriol. 86:820-828.

Lin, E. C. C. 1970. The genetics of bacterial transport systems. Ann. Rev. Genetics 4:225.

Linstead, D., I. Evans, and D. Wilkie. 1973. Mitochondrial genetic factors in the cellular response to chlorimipramine in *Saccharomyces cerevisiae*. *In* International Conference on the biogenesis of mitochondria, Academic Press Inc., New York.

Littlewood, B. w. 1972. A method for obtaining antibiotic-sensitive mutants in *Saccharomyces cerevisiae*. Genetics 71:305-308.

Lloyd, J. B., and W. J. Whelan. 1969. An improved method for enzymic determination of glucose in the presence of maltose. Anal. Biochem. 30:467-472.

MacKenzie, C. and J. Harris. 1957. N-formyl cysteine synthesis in mitochondria from formaldehyde and l-cysteine via thiazolidine carboxylic acid. J. Biol. Chem. 227:393-406.

Magaña-Schwencke, N., and J. Schwencke. 1968. A proline transport system in *Saccharomyces chevalieri*. Biochim. Biophys. Acta 173:313-323.

Magaña-Schwencke, N., and J. Schwencke, and J. Kuznar. 1973. Imino acid transport in yeast: the uptake of sarcosine. Biochim. Biophys. Acta 318:281-288.

McNamer, A. D., and C. R. Stewart. 1973. Proline uptake and utilization by *Chlorella pyrenoidosa*. Plant Physiol. 52:561-564.

Mortimer, R. K., and D. C. Hawthorne. 1969. Yeast genetics. In: The Yeasts, vol. 1: Editors, A. H. Rose and J. S. Harrison, Academic Press Inc., New York.

Moyed, H. S. 1960. False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. J. Biol. Chem. 235:1098-1102.

Neale, S., and H. Tristram. 1965. Loss of ability to concentrate proline in *Escherichia coli:* basis of resistance to proline analogues. J. Gen. Microbiol. 39:vii-viii.

Oden, S., and B. von Hofsten. 1954. Detection of fingerprints by the ninhydrin method. Nature (London) 173: 449-450.

Paterniti, Jr., J. R., D. Wilkie, and N. R. Eaton. 1975. Selective inhibition of growth by the proline analogue thiazolidine-4-carboxylic acid and its probable mode of action in *Saccharomyces cerevisiae*. Antimicrobial Agents & Chemother. 7:25-31.

Perutz, M. F., J. C. Kendrew, and H. C. Watson. 1965. Structure and function of haemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. J. Molec. Biol. 13:669-678.

Peterson, P. J., and L. Fowden. 1965. Purification, properties and comparative specificities of the enzyme prolyl-transfer ribonucleic acid synthetase from *Phaseolus aureus* and *Polygonatum multiflorum*. Biochem. J. 97:112-124.

Plummer, D. T. 1971. An introduction to practical biochemistry. McGraw-Hill Ltd., London.

Racker, E. 1955. Alcohol dehydrogenase from baker's yeast p. 500-503. *In* S. Colowick and N. O. Kaplan (Eds.), Methods in Enzymology, vol. 1. Academic Press Inc., New York.

Ratner, S., and H. T. Clark. 1937. The action of formaldehyde upon cysteine. J. Am. Chem. Soc. 59:200-203.

Ravel, J. M., M. N. White, and W. Shive. 1965. Activation of tyrosine analogues in relation to enzyme repression.

Biochem. Biophys. Res. Comm. 20:352-359.

Reynier, M. 1969. Pyrazole inhibition and kinetic studies of ethanol and retino oxidation catalyzed by rat liver alcohol dehydrogenase. Acta Chem. Scand. 23:1119-1129.

Richmond, M. H. 1962. The effect of amino acid analogues on growth and protein synthesis in microorganisms. Bacteriol. Rev. 26:398-420.

Richmond, M. H. 1966. *In:* Biochemical Studies of Antimicrobial Drugs. 16th Sympos. Soc. Gen. Microbiol. London, Cambridge University Press.

Rosen, B. P. 1973. Basic amino acid transport in *Escherichia coli:* properties of canavanne-resistant mutants. J. Bacteriol. 116:627-635.

Schatz, G., and T. L. Mason. 1974. The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. 43:51-87.

Schnaitman, C., and J. W. Greenawalt. 1968. Enzymatic properties of the inner and outer membranes from rat liver mitochondria. J. Cell Biol. 38:158-175.

Schroeder, W. A. 1968. The primary structure of proteins. p. 51. Harper and Row. New York.

Schwencke, J., and N. Magaña-Schwencke. 1969. A proline transport system in *Saccharomyces chevalieri*. Biochim. Biophys. Acta 173:313-323.

Sherman, F., G. R. Fink, and H. B. Lukins. 1970. Methods in Yeast Genetics. Cold Spring Harbor Laboratory for Quantitative Biology. Cold Spring Harbor, New York.

Slonimski, P. O. and B. Ephrussi. 1959. Action de i'acriflavine sur les levures. V. Le system des cytochromes des mutants 'petite colonie'. Ann. Inst. Pasteur (Paris) 77:47-60.

Sols. A., C. Gancedo, and G. Delafuente. 1969. *In:* The Yeasts, vol. 2. Editors, A. H. Rose and J. S. Harrison. Academic Press Inc., New York.

Stekol, J. A. 1963. Biochemical basis for ethionine effects on tissues. Adv. Enzymol. 25:369-393.

Steward, F. C., J. K. Pollard, A. A. Patchett, and B. Witkop. 1958. The effects of selected nitrogen compounds on the growth of plant tissue cultures. Biochim. Biophys. Acta 28:308-317.

Stock, R., and C. B. F. Rice. 1963. Chromatographic Methods. Science Paperbacks, London.

Strecker, H. J. 1956. The interconversion of glutamic acid and proline. J. Biol. Chem. 225:825-834.

Thouvenot, D. R. and C. M. Bourgeois. 1971. Optimisation de la selection de mutants de *Saccharomyces cerevisiae* par nystatine. Ann. Inst. Pasteur 120:617-625.

Tristram, H. and C. F. Thurston. 1966. Control of proline biosynthesis by proline and proline analogues. Nature 212:74-75.

Uitto, J. and D. Prockop. 1974. Incorporation of proline analogues into collagen polypeptides. Biochim. Biophys. Acta 336:234-251.

Unger, L. and R. D. DeMoss. 1966. Action of a proline analogue, l-thiazolidine-4-carboxylic acid, in *Escherichia coli*. J. Bacteriol. 91:1556-1563.

Unger, L. and R. D. DeMoss. 1966. Metabolism of a proline analogue, l-thiazolidine-4-carboxylic acid, by *Escherichia coli*. J. Bacteriol. 91:1564-1569.

Vaughan, D. and E. Cusens. 1974. Effects of hydroxyproline on the growth of excised root segments of *Pisum sativum* under asceptic conditions. Planta. 112:243-252.

Vollbrecht, D. and F. Radler, 1974. The formation of higher alcohols by amino acid auxotrophic mutants of *Saccharomyces serevisiae*. Arch. Mikrobiol. 94:351-358.

von Borstal, R. C., ed. 1963. Carbondale yeast genetics conference. Microbial Genetics Bull. 19 (suppl.).

Wasmuth, J. J. and H. E. Umbarger. 1973. Participation of branched-chain amino acid analogues in multivalent repression. J. Bacteriol. 116:562-570.

Webb, J. L. 1963. Enzyme and Metabolic Inhibitors. Academic Press, Inc. New York.

Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

West, R. 1965. Sigfried Ruhemann and the discovery of ninhydrin. J. Chem. Educ. 42:386-392.

Wilkie, D. 1970. Selective inhibition of mitochondrial synthesis in *Saccharomyces cerevisiae* by canavanine. J. Mol. Biol. 47:107-113.

Woolley, D. W. 1962. A Study of Antimetabolites. John Wiley & Sons, Inc. New York.

Zamenhoff, D. 1958. *In:* Colowick, S. P. and N. O. Kaplan. Methods in Enzymology, vol. 3. Academic Press, New York.