Transcriptional and Translational Control Over Synthesis of Glutamic Dehydrogenases in Fungi

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To my parents for their most generous

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Abstract

Studies on the allosteric effects of various metabolites upon a few enzymes catalyzing reactions of the citric acid cycle and related pathways in Saprolegnia parasitica, Pythium debaryanum, Achlya sp. 1969 (all members of the class Oomycetes) revealed that the homologous enzymes had similar properties. Of the enzymes studied, NAD-specific glutamic dehydrogenase proved to be the most interesting. This enzyme was sensitive to catabolite repression by glucose and other metabolizable carbohydrates and inducible by glutamate and other amino acids directly metabolizable to glutamate. Because of the facility of growth and well-defined alternation of generations shown by Achlya, most of the biochemical studies were carried out on this organism.

When glutamic dehydrogenase synthesis was derepressed using glutamate as "inducer" after growth in a medium with glucose as the sole carbon source, the enzyme activity displayed a pattern which oscillated continuously between synthesis and degradation. When protein synthesis (followed by C^{14} amino acid incorporation) was studied under the same derepressed conditions, similar oscillating patterns were observed. RNA synthesis (followed by C^{14} uridine incorporation) on the other hand, continued to increase at a logarithmic rate. These synthetic patterns occurred under conditions where

growth (increase in dry weight) had ceased but differentiation of the mycelia to produce motile and non-motile spores was proceeding.

Experiments performed showed that the oscillating pattern was not an experimental artifact. First, synthesis of another enzyme closely coupled to glutamic dehydrogenase, NADP-specific isocitric dehydrogenase, did not show a corresponding oscillating pattern. The level of isocitric dehydrogenase activity remained constant and low throughout the derepression period. Secondly, upon analysis, fractions of derepressed cell-free extracts collected from a Sephadex G-200 column showed that the peak of enzyme activity coincided with a peak of newly-synthesized protein (C^{14}-amino acid activity). Thirdly, it was demonstrated that the enzyme was actually degraded during derepression. Prelabelled enzyme lost radioactivity (in part) during derepression. Evidently the protein is in a continuous state of turnover.

Kinetic studies performed on partially purified NADP-specific isocitric dehydrogenase in Achlya showed that this enzyme failed to catalyze the reductive carboxylation of α-ketoglutarate under all assay conditions used.

These results formed the basis for the postulation of a synthesis-degradation model as a coarse control over glutamic dehydrogenase synthesis in Achlya. Also a "hypothetical degrading enzyme" responsible for the degradation kinetics is postulated although no evidence for

such an enzyme could be obtained from in vitro studies.

The addition of 5-fluorouracil during conditions of derepression did not affect either the pattern of oscillation displayed by glutamic dehydrogenase activity or protein synthesis.

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List of Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
DTNB	5, 5 dithiobis-(nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetic acid
GDP	guanosine diphosphate
GMP	guanosine monophosphate
GTP	guanosine triphosphate
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
RNA	ribonucleic acid
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate

MUTANTS OF *E. coli*:

Lac i^-	regulator constitutive
Lac O^c	operator constitutive
i^{del1}	deletion of regulator gene
O^c_{67}	i and O genes deleted

Historical

I Regulation of Enzyme Synthesis in Microbial Systems

(a) Induction of Enzyme Synthesis

The essential kind of regulation for a catabolic enzyme is one which would prevent wasteful synthesis in the absence of substrate but allow for rapid synthesis in presence of the substrate. Induction is a control which satisfies these requirements. Consequently, it is found that most catabolic enzymes are inducible (1).

Most of our knowledge of how this system of induction operates comes from the work of Jacob and Monod (2) who formulated the "Operon Hypothesis" to explain adaptive control of the lactose-utilizing system of Escherichia coli. According to this model, structural genes for enzymes of degradation constitute a part of an operon (functional division of a unit of genes specifying related functions). Transcription of this information into m-RNA is governed by two genetic loci, the operator gene, "O" and the regulator gene "R". "R" specifies a repressor which by its interaction with inducer or operator locus determines the rate of enzyme synthesis. Attachment of this repressor to the "O" locus blocks transcription of the entire operon. If however, an inducer is present, it will combine with the repressor and modify it in such a manner so as to decrease it s affinity for the

operator locus. The operon is then derepressed and allows messenger production. Derepression results in the synthesis of all enzymes of the operon in a coordinate manner (3).

(b) Glucose Effect

Although induction is a very useful type of regulation, when the induced protein is excessive, it may become necessary to either limit the rate of synthesis of the inducible enzyme or to balance the synthesis by degradation. In degradation, energy is required, and it would be wasteful to form more of the catabolic enzyme than would suffice to saturate the central intermediary pathways. In order to accommodate this situation, a repression has been demonstrated for many catabolic systems and has been termed, "Glucose Effect".

i) Types of Glucose Effect

There are three defined types of glucose effect which have been observed in E. coli. One type was demonstrated by Loomis and Magasanik (4) in their studies of glucose-lactose diauxie (preferential utilization of glucose before lactose). When uninduced cells were exposed to glucose and to low concentrations of inducer (lactose) a permanent severe repression of β-galactosidase, required for hydrolysis of lactose, resulted. This repression was due to a reduction in the internal concentration of inducer caused by glucose probably by inter-

ference with inducer transport. A second type was observed by Moses and Prevost (5) and was called "transient repression". If microbial cultures were exposed to glucose after growth on another carbon source, synthesis of β-galactosidase was severely repressed for as long as one generation after which enzyme synthesis started at the reduced rate characteristic of glucose adapted cells. The third type results in a permanent weaker repression of β-galactosidase. This kind of repression has been termed catabolite repression (6) because, it is presumed, catabolites of glucose cause the repression. Tyler et al (7) have shown that transient repression is distinct from catabolite repression. Transient repression occured in a mutant insensitive to catabolite repression. Further, transient repression was established more rapidly than catabolite repression and was elicited by glucose analogues that were phosphorylated but not further catabolized by the cell. Thus, transient repression was not a consequence of the exclusion of inducer from the cell and did not require catabolism of the added compound.

ii) Environmental Conditions Producing Catabolite Repression

According to Magasanik's definition of catabolite repression (6), any environmental condition which would lead to an increase in the catabolic pool would lead to catabolite repression. This type of a

condition could result when anabolic activity is lower than catabolic activity. McFall and Magasanik (8) demonstrated catabolite repression in thymine-requiring mutants. Catabolite repression of β-galactosidase resulted when this mutant was starved of thymine in the presence of glycerol. If, however, glycerol was omitted from the medium, the enzyme became inducible. In the presence of glycerol, the catabolic pool would increase in size due to a decrease in catabolite exit. When glycerol was omitted, there was no entry or exit and thus no increase in the size of the catabolic pool.

iii) Mechanisms of Catabolite and Transient Repression

Nakada and Magasanik (9) have demonstrated that the formation of β-galactosidase m-RNA in E. coli is inhibited by glycerol in cells whose rate of protein synthesis has been reduced by the deprival of an amino acid or by addition of chloramphenicol (CM). This inhibition was specific for this m-RNA. It was shown that threonine-requiring cells exposed to the inducer in a glycerol-containing medium incorporated 5-fluorouracil into m-RNA, but produced neither active β-galactosidase nor a corresponding altered protein subsequent to the removal of inducer plus analogue and the addition of threonine. Further, Nakada and Magasanik (10) found that a state of catabolite repression does not affect the efficiency of translation of messenger molecules. This

conclusion resulted from the fact that induction in a medium deprived of both glycerol and threonine permitted the subsequent synthesis of β-galactosidase without inducer in a medium containing glycerol but no threonine.

In Saccharomyces carlsbergensis however, a different situation exists. Wijk (11) studied the effects of catabolite repression on β-glucosidase. This enzyme is induced by maltose and catabolite repressed in the presence of 1% glucose. Protoplasts were induced by maltose in a medium containing either 1% or 0.2% glucose (synthesis of this enzyme proceeds at a basal rate in 0.2% glucose). Upon removal of inducer and resuspension of protoplasts in a medium containing the lower glucose concentration, it was found that the same differential rate of enzyme synthesis was obtained whether 0.2% or 1% glucose was present during induction. Thus, glucose had no effect upon transcription of messenger. On the other hand, when protoplasts were induced in the presence of maltose and 0.2% glucose and resuspended in a medium of 0.2% glucose but with no maltose, β-glucosidase was synthesised. This synthesis was insensitive to actinomycin D. If, however, the medium into which the protoplasts were transferred contained 1% glucose, no enzyme was synthesised. Thus, it appears that catabolite repression acts on the translation rather than on transcription of β-glucosidase messenger in Saccharomyces carlsbergensis.

iv) Genetic Models

Much work has been done concerning the genetic basis for the various types of "Glucose Effects". Palmer and Moses (12) have demonstrated that transient repression of β-galactosidase in E. coli required the presence of a functional operator gene in the lactose operon. Total deletion of the operator gene (E. coli Lac mutant; O^c_{67}) abolished transient repression, even in the presence of a functional regulator gene. However, regulator constitutives (i^-) also show transient repression provided that the operator gene is functional. Catabolite repression, on the other hand, was not mediated via either the i gene or the O gene. Lac i^- mutants were as sensitive to catabolite repression as their induced parental wild-type strains. Further, glucose repressed constitutive enzyme synthesis in many O^c mutants. Tyler and Magasanik (13) however, found that the mutant O^c_{67} did in fact exhibit transient repression. They also found that transient repression was still manifested in strains carrying a single i^{-del1} mutation. Moses and Prevost (5) also observed transient repression in constitutive i^- strains. Thus, it seems that transient repression is not mediated by any known regulatory genes of the Lac operon. Mandelstam (14) provided further evidence to show that catabolite repression does not act through the lac i gene product. He was able to demonstrate that the repression produced by carbon compounds was

not reversed by methyl- β-D-thiogalactoside (a non-metabolizable inducer of the enzyme in wild-type *E. coli*). If a glucose catabolite reacted with the same aporepressor as Lac inducers, their effects should have been competitive.

Some recent work has suggested that the promotor region may be the site at which transient and catabolite repression are exerted. Pastan and Perlman (15) have isolated a mutant of the Lac promotor region in *E. coli* L-8 which has lost sensitivity to transient repression. Silverstone *et al* (16) have shown that a partial deletion in the Lac promotor region rendered the Lac operon insensitive to catabolite repression. They postulated that the deletion might remove the target site for a specific catabolite repressor protein. Such a repressor would interact with a small molecule, a product of glucose metabolism, and then combine with the target site to reduce messenger production by the Lac operon. Alternatively, catabolite repression of the Lac operon could be the result of a nonspecific catabolite interacting with the complex of DNA dependent RNA polymerase and the Lac specific promotor. This catabolite could thus reduce initiation of RNA synthesis by the polymerase.

Loomis and Magasanik (17) described mutants which were resistant to catabolite repression by glucose. They suggested that the gene affected (which they called the CR gene) was necessary for

catabolite repression in that it specified the apo-repressor involved in catabolite repression of the Lac operon. However, Rickenberg et al (18) found that this same mutant, although resistant to the repression effects of glucose was still sensitive to glucose-6-phosphate and mixtures of glucose and gluconic acid as catabolic repressors. These results strongly suggested that the mutation has in some manner affected either the formation or the maintenance of a steady state concentration of a derivative of glucose, the presumptive "effector" of catabolite repression.

Paigen and Williams (19) suggest another model for catabolite repression. In this model catabolite repression would act at the level of m-RNA synthesis but would not involve an apo-repressor. Burgess et al (20) discovered that RNA polymerase had two subunits; one containing enzyme activity, and the other determining substrate specificity. More than one form of the latter kind of subunit could exist so that each could recognize a different set of promotor regions. Catabolite repressible operons would then contain the same promotor sequence, showing a common species of RNA polymerase which would contain the control site for catabolite repression. Such a polymerase could be inhibited by a low molecular-weight metabolite, or may require cyclic AMP as a cofactor for activity. In the presence of 3'5'-AMP (cyclic AMP) the activity of this species of RNA polymerase would be

decreased and the transcription of catabolite repressible operons would be inhibited.

The experiments of several workers concerning the effect of cyclic AMP upon catabolite repression could add support for the model proposed by Paigen and Williams. Perlman and Pastan (21) found that cyclic AMP overcomes repression caused by glucose at the level of m-RNA production. Cyclic AMP failed to stimulate β-galactosidase production in cells in which m-RNA synthesis was arrested by inducer removal or proflavin addition. Thus, cyclic AMP appears to participate in the regulation of β-galactosidase m-RNA synthesis at the gene level. Further, Makman and Sutherland (22) reported that in the presence of glucose, cyclic AMP rapidly disappears from the cells and correspondingly appears in the medium. This suggested an effect of glucose both in preventing its formation within the cell and in stimulating its extrusion from the cell. Also, Pastan and Perlman (15) found that transient repression of the Lac operon was mediated by changes in the concentration of cyclic AMP.

v) Identity of Co-repressor

Several workers concentrated their efforts on the search for a catabolite co-repressor. Studies on the Lac system indicate that the i gene product is a protein (23) which arrests the synthesis of m-RNA

(24). From this evidence it is thought that the apo-repressor involved in catabolite repression is also a protein.

With respect to the identity of the co-repressor, Beggs and Rogers (25) found that galactose failed to exert a repressive effect on β-galactosidase in a galactose-negative mutant lacking the first two enzymes involved in galactose catabolism. Glucose completely repressed enzyme formation in this mutant. This same mutant, into which the genes for inducible galactose utilization had been introduced previously by transduction, again exhibited galactose repression. It was concluded that the galactose molecule itself is not the catabolite repressor of β -galactosidase, but that repression is exerted through some intermediate in galactose catabolism. The experiments of Loomis and Magasanik (26) with a hexose phosphate isomerase-negative mutant indicated that catabolite repression of the Lac operon did not require glucose, galactose, gluconate or their direct derivatives. Likewise, the compounds related to the citric acid cycle did not appear to be required for catabolite repression of the Lac operon, since glucose gave rise to strong repression in a mutant under conditions where the formation of citric acid cycle compounds was severely restricted. Therefore, they suggested that if catabolite repression of the Lac operon was affected by a single compound, this compound should be related to the pentoses and trioses of intermediary metabolism. Hsie and Rickenberg (27)

isolated a mutant which synthesised β-galactosidase and several other enzymes in a manner that displayed resistance to catabolite repression by glucose. In this mutant neither glucose nor gluconate, when added singly to the medium, inhibited the formation of β-galactosidase or tryptophanase. A mixture of glucose and gluconate or glucose-6-phosphate alone severely repressed the formation of the two enzymes. It was thus tentatively concluded that either glucose-6-phosphate or a close metabolic derivative caused the observed catabolite repression.

Dobrogosz (28) presented evidence that the mechanism responsible for catabolite repression of β-galactosidase formation in E. coli ML30 was dependent on the nature and efficiency of pyruvate dissimilatory reactions. Catabolite repression measured in relation to steady-state growth rates (29) indicated that the repression mechanism may in fact be a direct consequence of a cell's energy balance, as dictated by the production from pyruvate of "high-energy" molecules such as ATP or acetyl-coenzyme A. Dobrogosz (30, 31) presented data to indicate that a relationship may exist between catabolite repression and amino sugar metabolism in E. coli. N-acetyl-glucosamine (NAG) was found to be a potent catabolite repressor source and was shown to be capable of augmenting repression by glucose or gluconate. Assimilation of NAG is dependent on availability of acetyl-CoA and ATP. Results in a later study by Dobrogosz (32) provide direct evidence as to the identity of the co-repressor. NAG, at 10^{-5} to

10^{-4}M, produced severe catabolite repression of β-galactosidase synthesis in an *E. coli* culture that was normally unable to metabolize this compound beyond the acetylglucosamine-6-phosphate step. Furthermore, repression by NAG occurred under conditions that did not appear to involve any alteration either in carbon and energy metabolism or in growth of the organism. The repression occurred in a strain that was constitutive for β-galactosidase synthesis.

Thus, both induction and catabolite repression could be viewed as functioning cooperatively providing the organism with a highly sensitive and efficient control over the genes that are involved. The *i* gene system would be capable of turning the system on or off, whereas the catabolite repression system would govern or modulate the rate of transcription in response to the needs of the cell under varied growth circumstances (32).

II. Regulation of Enzyme Synthesis in Mammalian and Some Fungal Systems

Mammalian Systems:

The model of enzyme regulation in animal cells is not as clearly elucidated as that of bacterial cells. There is no evidence for the existence of operons in animal cells (33). There is considerable evidence however, that RNA metabolism is involved in enzyme regulation. Greengard et al (34) showed that actinomycin D, an inhibitor of RNA synthesis, abolished in vivo the rise induced by cortisone in the rat liver of tryptophan pyrrolase and tryosine-α-ketoglutarate transaminase. These observations indicated that the stimulation by cortisone of liver RNA synthesis is a necessary part of the enzyme induction process.

(a) Observations Concerning the Transcription Process:

i) DNA from Eukaryotes contains highly repetitive sequences.

ii) The main ultrastructural element of chromatin is an individual deoxynucleoprotein (DNP) molecule, containing one 2-stranded DNA and proteins.

iii) Histones determine the folding of DNP and specific restriction of transcription of the repeating DNA sequences (35).

iv) All RNA synthesis takes place in the nucleus

v) This RNA is DNA-like in base composition and most of it is confined to, and turning-over within, the nucleus

vi) This RNA is probably m-RNA and is active only in the cytoplasm

vii) Since m-RNA must be derived from nuclear DNA-like RNA (d-RNA), some portion of this material must be a precursor to cytoplasmic m-RNA (36)

viii) A large molecule of d-RNA is combined with a number of specific macroglobular protein particles, referred to as "informomers" and tightly packed along the DNA strand. Informomers participate in: (1) the removal of newly synthesized d-RNA from the DNA template; (2) m-RNA transport at least up to the nuclear membrane; (3) cleavage of giant d-RNA strands into shorter chains; and (4) the selection of d-RNA's to be transferred into the cytoplasm (net m-RNA) and to be destroyed in the cell nucleus.

(b) Induction and Repression

Studies concerning the regulation of synthesis of some hepatic enzymes were used as a model for furthering understanding of the mechanisms by which gene expression is regulated in mammalian cells (37).

The presence of an extensive and continual degradation, or turnover, of protein in rat liver (38, 39) as opposed to absence of demonstrable protein turnover in growing bacteria indicates the lack of a strict analogy between bacterial enzyme induction and changing enzyme levels in rat liver. The existence of turnover of enzymes in liver would allow

for mechanisms for altering the contents of enzymes by changes in rates of degradation as well as changes in rates of synthesis.

Kenney et al (37) induced tyrosine- α-ketoglutarate transaminase synthesis in livers of adrenalectomized rats with the protein hormones, insulin and glucagon. They found that the synthesis is elevated for a period of 2 or 3 hours and then returns to the basal rate, regardless of presence of inducing hormone. This suggested that these hormones elevate synthesis by making available a component which is present in limited supply and which survives for only a brief time once it is activated. Their data suggested a short half-life for the transaminase m-RNA and, therefore, they interpreted this induction response by postulating that insulin and glucagon make available an otherwise inactive store or pool of transaminase template. They further postulated that these hormones may effect the passage of a pool of m-RNA from the nucleus and into the cytoplasm. Enzyme synthesis would then be elevated as the excess m-RNA persists, and the induction could not be reinitiated until the pool is reformed at a constant low rate of RNA synthesis. Since actinomycin D blocked induction by insulin and glucagon, it too may have prevented the passage of RNA from the nucleus to the cytoplasm.

Tomkins (40) in working with "hepatoma tissue culture" postulated the existence of two genes regulating tyrosine α-ketoglutarate transaminase

synthesis: a structural gene and a regulatory gene. In this model the structural gene produces a relatively stable messenger which is translated into enzyme protein. The regulatory gene produces a labile inhibitor (R) which interfers both with accumulation of the messenger and with its translation. The inducer (a steroid hormone) is assumed to prevent "R" from inhibiting these two functions. Thus, the steroid would promote messenger accumulation. When the steroid is removed, tyrosine α-ketoglutarate transaminase synthesis slows rapidly, even though the messenger is stable. This is explained by assuming that "R", in the absence of steroid, quickly inhibits the translation of the message.

Tomkins also found that, in the presence of actinomycin D, the enhanced synthesis of tyrosine α-ketoglutarate transaminase became independent of the presence of the inducer. The model would account for this observation because "R" is labile and its production would be blocked by inhibitors of RNA synthesis. Thus, "R" decays, and the requirement for the hormone to antagonize "R" is likewise abolished.

Kenney's results (41) would add support to the conclusion that enzyme degradation, as well as synthesis, must be blocked when protein synthesis is stopped. He showed that the labelled enzyme (tyrosine α-ketoglutarate transaminase) is stable in the liver of rats treated with cycloheximide. He further suggested that transaminase

turnover may require the participation of a specific polypeptide, which could either catalyze or be a reactant in the process leading to the removal of the enzyme. Such a component would necessarily have an extremely short half-life, for transaminase removal ceases very quickly when protein synthesis is stopped. Also, tyrosine-transaminase was stable in actinomycin D-treated rats. This observation reflects a dual inhibition of both synthesis and removal of transaminase template instead of stability of transaminase template.

Pitot and Jost (42) studied the rate of synthesis and degradation of serine dehydratase by amino acids and glucose in rat liver. They found that the constant rate of enzyme synthesis induced by amino acids was the result of a decrease in enzyme degradation. During glucose repression, however, there was a complete inhibition of the rate of enzyme synthesis. Thus, one only sees degradation and, therefore, an apparent increase in the rate of degradation. They suggested that the translational control of serine dehydratase synthesis may be effected by glucose through its ability to prevent the release of the finished enzyme from the polysome complex.

Fungal System

Horowitz et al (43) postulated the existence of an unstable repressor which controlled the synthesis of tyrosine in Neurospora .

Lewis and Fincham (44) also suggested the existence of a

repressor controlling the synthesis of nitrate reductase in the Basidiomycete, Ustilago maydis. Nitrate reductase was induced by growth in a medium containing only nitrate as the nitrogen source. Ammonium ions repressed the enzyme and led to a rapid loss of activity. Cycloheximide prevented both induction of the enzyme and also ammonium induced loss of activity. When a culture growing in a minimal nitrate medium was transferred to a medium containing ammonium plus actinomycin D, nitrate reductase synthesis was slightly stimulated rather than repressed. These results suggested that the enzyme's inactivation is mediated by a specific protein which is induced by ammonium ions and whose function is to govern the level of nitrate reductase. Inhibition of m-RNA synthesis and protein synthesis would render nitrate reductase stable in ammonium minimal medium.

No satisfactory model has been proposed to explain the inducible enzyme systems of organisms other than bacteria.

III. Allosteric Regulation of Glutamic Dehydrogenases

(a) Bovine:

Glutamic dehydrogenase from bovine liver has been dealt with extensively by Colman and Frieden (45, 46), Frieden and Colman (47), Frieden (50), Yielding et al (51) and Yielding and Holt (48). Bovine liver glutamic dehydrogenase is nonspecific in the use of NAD^+ and $NADP^+$ as coenzymes. In addition to an active coenzyme site, the bovine liver enzyme has a highly specific purine nucleotide binding site (50). The coenzyme is thought to induce a change in the purine nucleotide-binding site so that, in the presence of coenzyme, glutamic dehydrogenase binds inhibitory purine nucleotides (GTP) tighter, and activators (ADP) weaker than does the enzyme in the absence of coenzyme (45, 48).

The active monomer of glutamic dehydrogenase has a molecular weight of 350, 000 and consists of six peptide chains each having a molecular weight of 50, 000 (46). Aggregation of the enzyme into tetramer form is concentration dependent. ADP and NADH facilitate the formation of hexamer rather than tetramer (46). ADP, which activates the enzyme in the presence of reduced pyridine nucleotides, binds preferentially to the polymer rather than to the monomer. NADH and NADPH alone behave in the same manner as ADP. GTP, an inhibitor, binds preferentially to the monomeric form (47). This may have some significance

to the cell since the enzyme has a concentration in vivo of approximately 1 mg per ml and is probably polymeric (47). Thus, purine and pyridine nucleotides can have a great influence upon the physical properties and activity of the enzyme in bovine liver.

(b) Neurospora:

Stachow and Sanwal (49) observed that NAD-specific glutamic dehydrogenase in Neurospora is inhibited by GTP and that this inhibition is alleviated in the presence of ATP.

(c) Thiobacillus:

The NAD-specific enzyme in Thiobacillus novellus exhibits a sigmoidal dependence of the initial velocity on NAD^+ and glutamate ligands. Upon addition of AMP and ADP, rate-concentration plots became hyperbolic and classical Michaelis-Menten kinetics was obeyed. There was a corresponding increase in Michaelis constants for ammonia, α-ketoglutarate and NADH (52).

(d) Blastocladiella:

Glutamic dehydrogenase in the unicellular water mould Blastocladiella emersonii is NAD-specific. There is no NADP-specific form of the enzyme in this organism. Glutamic dehydrogenase from this organism is oligomeric with a molecular weight of 230,000 ± 20,000 (53). Glutamic dehydrogenase is a regulatory enzyme in this organism

by virtue of the sigmoidal dependence of velocity on substrate concentration (53).

The table below is a composite of results from (53, 55, 56, 57, 58) on the regulatory properties of glutamic dehydrogenase from Blastocladiella.

Table I

Summary of response of Blastocladiella NAD-specific glutamic dehydrogenase to a variety of allosteric modulators.

A plus (+) sign denotes activation; a minus (-) sign, inhibition; (O) means no significant effect.

	NAD-specific Glutamic Dehydrogenase	
Modifier	Reductive Amination	Oxidative Deamination
Citrate	O	-
Isocitrate	O	-
Fumarate	O	-
Succinate	O	-
Fructose-1, 6-di PO_4	O	-
α-ketoglutarate	O	-
EDTA	O	-
AMP	+	+
ADP	+	+
ATP	-	-
GTP	-	-
Ca^{++}	+	-
Mn^{++}	+	-

In an acid pH isocitric dehydrogenase aggregates to inactive polymers and glutamic dehydrogenase is desensitized to all of its effectors. In an alkaline pH isocitric dehydrogenase forms active monomers and glutamic dehydrogenase is sensitized to regulation by a diversity of ligands.

It was also noted that Hg^{++} desensitizes glutamic dehydrogenase to all its effectors.

In the light of the above observations, LeJohn (55, 56, 57) presents a scheme for the regulation of glutamic and isocitric dehydrogenases. When citric acid cycle intermediates are abundant, large amounts of ATP are hydrolyzed to ADP + Pi, permitting an influx of Ca^{++}, Mg^{++} and Mn^{++} into the mitochondria in exchange for protons. The increasing pH and the accumulation of citrate and increasing AMP/ATP ratio would activate the isocitric dehydrogenase and inhibit the oxidative deamination of glutamate. (Glutamic dehydrogenase is located on the outer surface of the mitochondria and isocitric dehydrogenase inside.) Thus, the biosynthesis of glutamate could proceed and act as a "sink" for valuable organic acids. When the citric acid cycle intermediates are low, then the controls would be reversed, allowing glutamate to enter the citric acid cycle to supply intermediates.

A novel form of allosteric control in which NAD-specific glutamic

dehydrogenase obtained from fungi belonging to the subclass, Oömycetes, are activated by $NADP^+$ has been demonstrated (59, 60).

Table II*

Summary of response of Pythium debaryanum and Achlya sp NAD-specific glutamic dehydrogenases to a variety of allosteric modulators.

A plus (+) sign denotes activation; a minus (-) sign, inhibition; (0) means no significant effect.

Modifier	Pythium	Achlya
	Reductive Amination Reactions	
$NADP^+$	+	+
NADPH	+	+
P-enolpyruvate	+	+
GTP (GDP, GMP)	+	+[a]
CoA derivatives (short chain)[b]	+	+[a]
Palmitoyl-CoA	-	-
Oleyl-CoA	-	-
ATP (ADP)	+	0/(-)
AMP	-	(-)/0
3',5'-AMP	0[a]	0
3',5'-GMP	0[a]	0
Citrate (Isocitrate)	-	-
Dephospho-CoA	0	0
UTP (UDP, UMP)[‡]	+	+
UDP-Nucleotide Sugars[‡]	+	+
UDP-Amino Sugar[‡]	+	+

Modifier	Pythium	Achlya
	Oxidative Deamination Reactions	
$NADP^+$	0[a]	+
P-enolpyruvate	-	+
GTP (GDP, GMP)	0	-
CoA derivatives[b]	-	-
Palmitoyl-CoA	-	-
ATP (ADP)	0	-
AMP	-	-
Citrate	-	-

[a] Slight activating effect

[b] CoA; acetyl-CoA; acetoacetyl-CoA; malonyl-CoA; succinyl-CoA; n-propionyl-CoA; n-butyryl-CoA; n-hexyl-CoA; n-valeryl-CoA.

‡ Data taken from LeJohn, H. B. Biochem. Biophys. Res. Communs. 42, 538, 1971

* Taken from: LeJohn, H. B., Stevenson, R. M. and Meuser, R., J. Biol. Chem. 245, 5569, 1970.

A multivalent system of regulation upon NAD-specific glutamic dehydrogenase from the Oömycetes has been elucidated by LéJohn et al (59, 60). This kind of regulation has been explained as a means by which the enzyme can effect its amphibolic role in the cell. The activators serve in a dual capacity since they cumulatively antagonize the inhibitory effects of substrates (by α-ketoglutarate and NH_4^+) and other inhibitors (citrate, palmitoyl CoA).

Under energy rich conditions when the citric acid cycle operates as a biosynthetic unit, some activators unidirectionally stimulate the biosynthetic reaction of the enzyme and others inhibit the catabolic reaction unidirectionally.

On the other hand, since this enzyme is subject to catabolite repression, it may have evolved as a catabolic catalyst. If deamination reactions were allowed to cause an accumulation of α-ketoglutarate and NH_4^+, product inhibition would ensue. But, when this enzyme is to function in a biosynthetic capacity, multivalent control by the activators may supercede this inhibition by substrates.

Table III*

Some probable relationships between anabolism and modulators of NAD-specific glutamic dehydrogenases of Oömycetes

Ligands	Biosynthetic and Related Reactions
AMP/ATP	(i) Adenylate control of amphibolic citric acid cycle activity (ii) NAD-kinase reaction
$NADP^+$/NADPH	Transhydrogenase couple of isocitric and glutamic dehydrogenase
Short and long chain acyl-CoA derivatives	Fatty acid biosynthesis and end product feedback effects
GTP	(i) Fatty acid biosynthesis (ii) Substrate level phosphorylation (iii) Gluconeogenesis via P-enolpyruvate
P-enolpyruvate	(i) Pyruvate metabolism (ii) Amino acid biosynthesis
Citrate	Citric acid cycle in biosynthesis
UTP	Amino Sugar Biosynthesis

*Data taken from LéJohn, H.B., Stevenson, R.M. and Meuser, R., J. Biol. Chem. 245, 5569, 1970.; and LéJohn, H.B. Biochem. Biophys. Res. Commun. 42, 538, 1971.

IV. Biology of the Aquatic Phycomycetes (61)

Saprolegnia parasitica, Achlya sp 1969 and Pythium debaryanum of the class Oomycetes which belongs to the major division Phycomycetes are of concern in this thesis. Saprolegnia and Achlya belong to the order Saprolegniales and Pythium belongs to the order Peronosporales.

Saprolegnia and Achlya are found most often in fresh water and are also abundant in moist soils. These two genera are characterized by two types of hyphae; first, the rhizoidal hyphae which enter the substratum; second, the mass of profusely branched, coencytic hyphae on the outside of the substratum, which forms the visible colony of the organism and on which the reproductive organs are formed.

The coencytic hyphae give rise to sporangia which are densely filled with protoplasm. A large number of nuclei stream into the cytoplasm of the sporangia from the somatic hyphae and these individual uninucleate cytoplasmic units then develop into zoospores. This pattern of asexual reproduction occurs in Achlya and Saprolegnia. It is this path which is followed in these organisms in the studies of this thesis.

When conditions are ripe for sexual reproduction, the somatic hyphae give rise to oogonia (female reproductive structures)

and antheridia (male reproductive structures). An oögonium is a thick-walled sphere containing many free oöspheres. The antheridia are smaller than the oögonia and are multinucleate. The antheridial nuclei fuse with the oöspheres in the oögonium to form a diploid zygote nucleus. After fertilization, a thick wall develops around each oösphere converting it to an oöspore. After a rest period, the oöspores are liberated from the disintegrated wall of the oögonium and then germinate.

Pythium mycelium consists of a slender coenocytic hyphae with cellulose walls. The sporangia remain attached to the hyphae and germinate in place. However, P. debaryanum does not have sporangia always remaining attached to the hyphae. The sporangia may be liberated and then germinate by germ tube.

When conditions are favorable, sexual reproduction takes place. After fertilization, the oösphere develops into a thick-walled, smooth structure which germinates after first undergoing a rest period.

Introduction to the Problem

Mitochondrial enzymes, which present an array of interesting controls, have been the subject of a number of recent investigations in those fungi belonging to the major division Phycomycetes (53-57). Initial studies were undertaken with the unicellular water-mould Blastocladiella emersonii (53-58), with NAD-linked glutamic dehydrogenase being particularly interesting since it directly links the metabolism of amino acids with the metabolism of organic acids and sugars. It should, therefore, be the subject of many metabolic controls. Indeed, glutamic dehydrogenase in this organism did demonstrate many unique kinetic and regulatory properties (53-57).

Studies on this enzyme were further extended to members of the class Oomycetes which include both unicellular and multicellular stages in their life cycle. NAD-linked glutamic dehydrogenases in the Oomycetes demonstrated the properties of a catabolic enzyme since they are inducible and subjected to "Glucose Effect" (59). At the same time, this enzyme must also function as a biosynthetic enzyme in order to meet the cell's requirements for amino acids because the NADP-linked glutamic dehydrogenase is absent among all organisms in the Phycomycetes[‡]. The amphibolic nature of this latter enzyme presented a rather perplexing situation. Since reducing power for

[‡] H. B. LeJohn, Nature (in the press), 1971.

cellular biosynthesis must be in the form of NADPH, some other means of obtaining NADPH had to be devised by the organism. It was found that glutamic dehydrogenase was closely coupled to an NADP-linked isocitric dehydrogenase (59, 60). The NAD-linked variety of isocitric dehydrogenase is absent in these fungi (Oomycetes). Therefore, it was suggested that the isocitric and glutamic dehydrogenases act cooperatively to supply the cell with both NADH and NADPH for biosynthesis as well as catabolism (59, 60).

Most systems of regulation in microorganisms have been concerned with the synthesis of proteins. However, in animal systems, regulation is looked upon as a control of the degradation rates as well as synthesis of certain proteins (37-39, 42, 65). It is shown in this thesis that glutamic dehydrogenase in Achlya sp. 1969, a member of the class Oomycetes, is probably regulated coarsely by a balance between degradation and synthesis and also finely controlled by at least 9 allosteric effectors.

Materials and Methods

I. Organisms

The fungi used for these experiments were obtained from American Type Culture Collection and Dr. J.S. Lovett, Purdue University, Indiana.

Fungi	Source
Achlya sp (1969)	Lovett
Saprolegnia parasitica	ATCC 11393
Pythium debaryanum	ATCC 9998

II. Growth of Organisms

(a) Carboy Cultures

All three organisms were grown in 3 litres of liquid medium under forced aeration for 2 days at 20°C. Inocula of Saprolegnia and Achlya originated from a dense population of spores released from 2 day-old stationary petri dish liquid cultures. Pythium was grown from shredded mycelial mats previously grown in small petri dishes.

Achlya was grown in 10 litres of liquid medium under conditions described above. The cell-free extract obtained from Achlya thus grown was used for the purpose of purification of NADP-specific isocitric dehydrogenase for kinetic studies.

(b) Petri Dish Cultures

Achlya was grown in stationary petri dish liquid cultures for 2 days at 20°C. Inoculum was obtained as described for carboy cultures.

III. Types of Media

(a) Induction Studies in Achlya, Saprolegnia and Pythium

The organisms were grown in carboys of the following composition:

i) PYG ---------- 1 g peptone, 1 g powdered yeast extract, 5 g glucose/litre distilled water

ii) PY Glut. ----- 1 g peptone, 1 g powdered yeast extract, 5 g glutamate/litre distilled water

(b) Derepression Studies in Achlya

Spores from a 48 hour old culture of Achlya were transferred to petri dish liquid G_2Y cultures where glutamic dehydrogenase activity was repressed. After 48 hours of growth, the spent G_2Y medium was removed by suction and the remaining mat washed twice with sterile distilled water. The induction medium (Glut. $_2$Y) was then added and the mats of Achlya were removed for enzyme analysis at regular intervals.

i) Repression medium (G_2Y) --- 5 g glucose and 0.5 g powdered yeast extract/litre of distilled water

ii) Induction medium (Glut. $_2$Y) --- 5 g glutamate and 0.5 g powdered yeast extract/litre distilled water

IV. Harvesting

All fungi were harvested by filtration on Whatman No. 1 filter paper, washed with buffer composed of 50 mM Tris-acetate, 10 mM phosphate and 1 mM EDTA at pH 7.3.

V. Cell-free Extracts

Mycelial mats were suspended in buffer of the above mentioned composition containing 10^{-4} M dithiothreitol. The mats were dispersed

uniformly with a mortar and pestle until a smooth suspension was obtained. The dispersed cell suspension was then sonically disrupted at 10°C until all hyphae were lysed.

Debris and unbroken cells were removed by centrifugation at 48,000 xg for 10 min at 4°C. The clear yellow supernatant was then assayed immediately for enzyme activity.

VI. Enzyme Assays

Enzyme assays were carried out in cell free extracts by measuring the rate of NAD^+ or $NADP^+$ reduction and NADH oxidation at 340 mμ in a Gilford model 2400 recording spectrophotometer in 3 ml silica cuvettes of 1 cm light path at 24°C. The only exception was citrate synthase in which case the rate which mercaptide ion complex formation between DTNB and CoASH was measured at 412 mμ. One unit of activity was defined as the concentration of enzyme which caused an O.D. change of .001 per min. Specific activity was expressed as units of enzyme activity per mg of protein. The method of Lowry et al (62) was used for protein determination.

Reaction Mixtures:

(1) NAD-specific Glutamic Dehydrogenase

α-ketoglutarate	6.6	mM
NADH	0.166	mM
$NADP^+$	0.33	mM
$(NH_4)_2SO_4$, pH 8	0.2	M
Tris-acetate buffer, pH 8	0.066	M
Distilled water ..... volume made up to..	3	ml

(2) Lactic dehydrogenase

NADH	0.166 mM
Pyruvate	10.0 mM
Tris-acetate buffer, pH 8	.066 M
Distilled water	volume made up to 3 ml

(3) Malic Enzyme

$NADP^+$	0.083 mM
$MnCl_2$	1.66 mM
Malate	10.0 mM
Tris-acetate buffer, pH 7.5	0.066 M
Distilled water	volume made up to 3 ml

(4) Citrate Synthase

Acetyl-S-CoA	0.033 mM
Oxaloacetate	0.033 mM
DTNB	0.066 mM
Tris-acetate buffer, pH 8	40 mM
Distilled water	volume made up to 3 ml

(5) NADP-specific Isocitric Dehydrogenase

Isocitrate	10.0 mM
$NADP^+$	0.083 mM
$MnCl_2$	1.66 mM
Tris-acetate buffer, pH 7.5	0.066 M
Distilled water	volume made up to 3 ml

VII. Enzymic Determination of Glucose

Achlya grown in a medium containing 2.5 g glucose and 0.5 g yeast extract per litre was filtered at regular intervals (details given in figure legend). The medium from which the cells were filtered was analysed for glucose content using glucostat (a prepared enzymatic

glucose reagent (Worthington Biochemical Corporation, Freehold, New Jersey).

VIII. Growth Curve for Achlya During a One-Generation Cycle

G_2Y cultures of Achlya were filtered at regular intervals as specified in legend to Fig. 2 using 0.45 μ millipore filters. These filters were previously dried to a constant weight. The mycelial mats together with the filter were also dried to a constant weight.

IX. Derepression of NAD-specific Glutamic Dehydrogenase in Achlya

Achlya was grown in 15 ml stationary petri dish G_2Y cultures for 48 hours. The G_2Y medium (in which the glutamic dehydrogenase would be repressed) was replaced by Glut.$_2Y$ medium in order to induce the synthesis of this enzyme. A set of control plates was run simultaneously in which the old G_2Y medium was replaced by fresh G_2Y medium. 3.1 μ Ci of C^{14} amino acid hydrolysate (or 2.5 μ Ci of C^{14} uracil) was added to each plate at the time of addition of the new medium. Each sample was duplicated (i.e. each time of sampling represents two mats of Achlya).

The cells were harvested at regular intervals and the mycelial mats, together with the filter were immediately placed on dry ice. The mats were kept on dry ice until completely frozen in order to

arrest further cellular reactions. The frozen mats were removed from the filter and suspended in buffer. Cell free extracts were then prepared as previously described under "Harvesting" and "Cell Free Extracts".

One hundred µl of cell-free extract was added to 100 µ l of ice-cold 10% TCA containing excess of unlabelled amino acids (or uracil) and kept in ice for 1/2 hour. Samples, taken at different times, were then filtered through millipore filters (0.45 µ) and washed with 10 ml casein hydrolysate (1 mg per ml of 5% TCA). The filter was air dried and dissolved in Bray's solution (64) of the following composition: 60 g naphthalene, 4 g 2, 5-diphenyloxazole, 0.2 g 1, 4-bis-2(5-phenyl-oxazolyl) benzene, 100 ml methanol, 20 ml ethylene glycol, 1 litre of p-dioxane. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer.

X. Derepression of Prelabelled NAD-specific Glutamic Dehydrogenase in Achlya

Mats of Achlya were grown from an inoculum of spores in 15 ml G_2Y liquid medium which contained 3.1 µ Ci of C^{14} amino acid hydrolysate. After 48 hours these mats were transferred to 15 ml Glut.$_2$Y medium which did not contain C^{14} amino acid hydrolysate. The mats were then treated as described in the previous section (IX) only in this case, the casein hydrolysate washings were collected (correction made for dilution) and counted as well as the TCA precipitate. All samples were

run in duplicate.

XI. Influence of 5-Fluorouracil (5-Fu) on the Derepression System of Achlya

Unlabelled 5-Fu (1 mg/ml medium) was added to 36 hour old G_2Y cultures of Achlya. These cultures (now designated as G_2Y-Fu) were further incubated for another 12 hours in order to allow the intracellular pool of uracil to be diluted by the 5-Fu. The same number of plates to which no 5-Fu was added were also incubated for the 48 hour period. At 48 hours, the G_2Y-Fu medium was replaced by Glut.$_2$Y medium at which time 3.1 μCi of C^{14} amino acid hydrolysate was added. At the same time, the G_2Y control medium was replaced by Glut.$_2$Y medium with no C^{14} amino acid hydrolysate addition. The mats were then prepared for counting as described in section IX.

Chemicals

Radioisotopes were obtained from the Radio Chemical Centre, Amersham, England. Other chemicals were obtained from the Sigma Chemical Company and P-L Biochemicals.

Results

I. A Study of Enzyme Control Systems in *Achlya*, *Pythium* and *Saprolegnia*

A study of control systems at the enzymic level was undertaken for the following Oömycetes: *Achlya*, *Saprolegnia* and *Pythium*.

Table IV summarizes these results. The only enzyme whose level of activity is significantly affected by growth of the organisms in glucose and glutamate is glutamic dehydrogenase. In each case, the activity of this enzyme was much lower when the organism was grown in the presence of glucose as compared to enzyme activity when grown in the presence of glutamate and absence of glucose. Further similarities among these three organisms were noted such as low pyruvate kinase and malic enzyme activities.

All three organisms respond similarly to these nutritional changes with respect to selected regulatory enzymes. Consequently, only one of these three organisms was studied in detail. For several reasons (ease of growth, handling, sporulation) *Achlya* was selected.

Table IV

The effect of different nutritional conditions on the amounts of different enzymes synthesized by Achlya, Saprolegnia and Pythium

Each organism was grown in a carboy culture for 48 hr in 3 litres of liquid medium and harvested. The cell free extract was analyzed for enzyme activity

Enzyme	Medium	Specific Activity* of Enzyme in Achlya	Specific Activity of Enzyme in Saprolegnia	Specific Activity of Enzyme in Pythium
Pyruvate kinase	PYG	60	71	87
	PYGlut.	47	40	73
	PY	47	40	84
NADP-specific	PYG	128	149	181
Isocitric	PYGlut.	115	183	206
Dehydrogenase	PY	233	149	300
NAD-specific	PYG	240	000	14
Glutamic	PYGlut.	905	651	766
Dehydrogenase	PY	1,082	597	1,113
Malic enzyme	PYG	35	30	58
	PYGlut.	36	36	53
	PY	35	42	72
Citrate synthase	PYG	175	598	491
	PYGlut.	210	342	400
	PY	323	480	631

*Specific activity was expressed as units of enzyme activity per mg of protein.

II. Influence of Amino Acids, Organic Acids and Carbohydrates on Enzyme Control Systems in Achlya

(a) The results in Table V show that glutamic dehydrogenase, lactic dehydrogenase and malic enzyme appear to be catabolic enzymes in this organism as their activities are low when grown in the presence of carbohydrates and high when grown with amino acids as carbon source. This effect was more pronounced for glutamic dehydrogenase as compared to lactic dehydrogenase and malic enzyme.

Activities of NADP-specific isocitric dehydrogenase and citrate synthase are not greatly affected by the presence of carbohydrates as compared to their activities in amino acid containing medium. Thus, NADP-specific isocitric dehydrogenase and citrate synthase are probably biosynthetic enzymes in Achlya.

Galactose, lactose, succinate, fumarate and malate do not appear to be metabolized by Achlya (LeJohn, unpublished data) and this could explain why the activities of the enzymes are not markedly altered when these compounds were provided as carbon-sources.

In the light of these observations (Tables IV and V) and of those made by others in this laboratory (59, 60) it was decided to confine the scope of the remainder of this thesis to NAD-specific glutamic dehydrogenase and NADP-specific isocitric dehydrogenase in Achlya.

Table V

Influence of amino acids, organic acids and carbohydrates on enzyme synthesis in Achlya

Achlya was grown in 100 ml shake cultures for 48 hr. After harvesting, the cell free extracts were analyzed for enzyme activity

Substrate	Specific Activity Glutamic Dehydrogenase	Specific Activity Lactic Dehydrogenase	Specific Activity Malic Enzyme	Specific Activity Citrate Synthase	Specific Activity Isocitric Dehydrogenase
20 mM Glucose	568	34	8	568	268
" " Sucrose	810	27	54	1,310	459
" " Fructose	2,500	270	47	1,175	540
" " Xylose	1,785	164	26	364	362
" " Arabinose	1,192	108	23	275	271
" " Galactose	3,559	288	84	983	678
" " Lactose	1,360	104	28	864	304
" " Glycerol	2,578	222	58	718	406
5 mM Succinate	4,014	408	169	2,112	915
" " Fumarate	2,643	115	83	2,011	632
" " Malate	4,051	206	133	2,974	818
1.6 mM Lactate	4,634	365	61	1,484	658
5 mM Pyruvate	2,375	200	100	171	475
" " Alanine	6,401	253	155	1,915	915
" " Glutamate	4,011	230	109	1,313	329
" " Aspartate	7,017	333	136	2,824	1,052
" " Glycine	11,951	438	122	5,914	2,438
" " Serine	5,416	666	177	3,875	1,875

(b) Response of NADP-specific isocitric dehydrogenase and NAD-specific glutamic dehydrogenase from Achlya to varying glucose or α-methyl-D-glucoside and glutamate concentrations

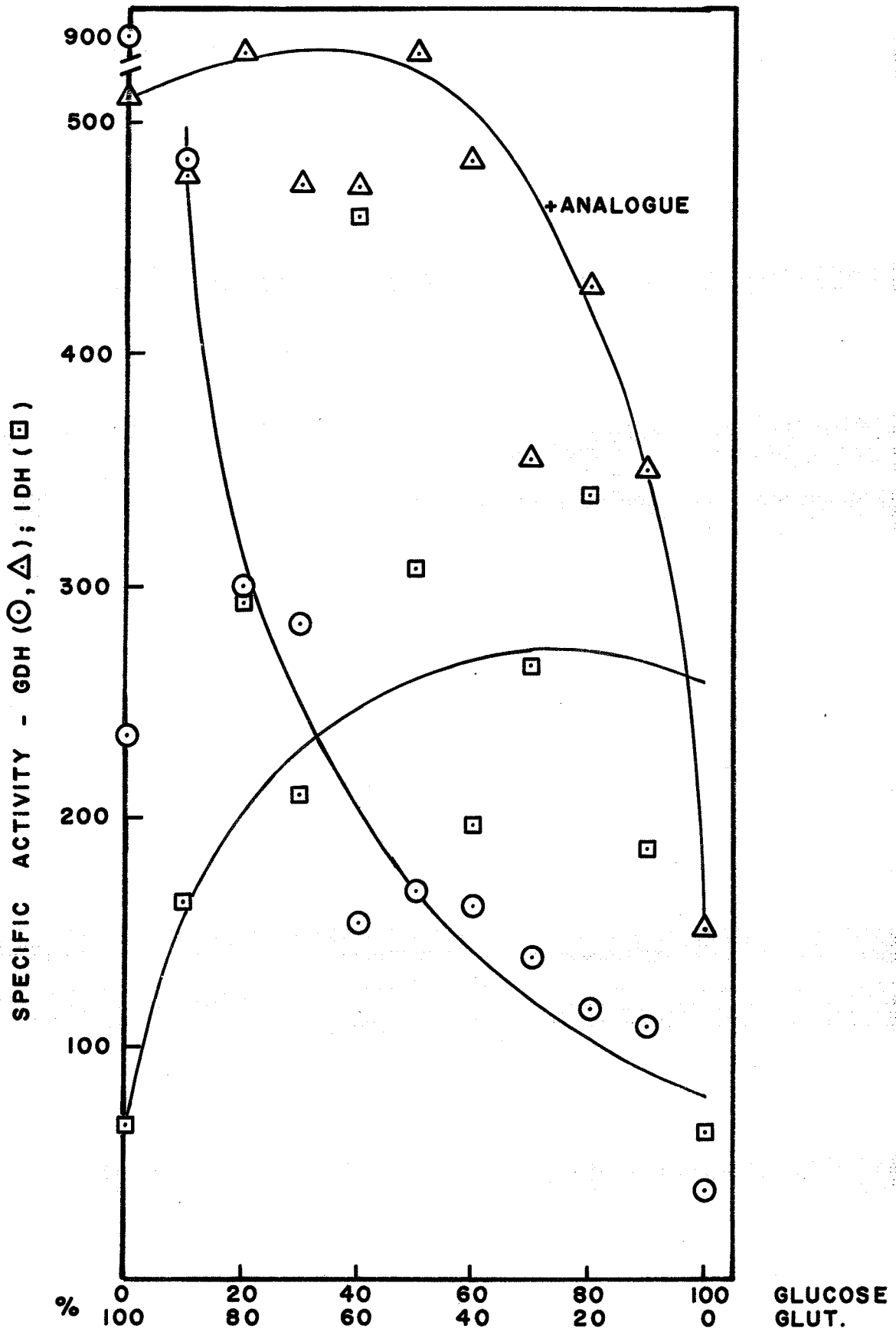
With increasing glucose and decreasing glutamate concentrations, glutamate dehydrogenase activity decreases whereas, isocitric dehydrogenase activity increases (Fig. 1). Glutamate dehydrogenase activity remained unaffected by increasing α-methyl-D-glucoside and decreasing glutamate concentrations up to a concentration of 0.25% α-methyl-D-glucoside. At greater concentrations of α-methyl-D-glucoside, glutamate dehydrogenase activity decreased. Evidently, the analogue does not appear to be efficiently metabolized by the organism. At greater concentrations it may be slowly metabolized or the repressive effect observed may be due to indirect effects on other cellular functions.

III. Derepression of Glutamic Dehydrogenase by Glutamate in Achlya

Derepression experiments were conducted on 48 hour cultures since, at this time most of the glucose has been utilized (Table VI). Achlya at this time is at the stationary phase of growth (Fig. 2).

Upon addition of glutamate following the repression of glutamic dehydrogenase activity through "Glucose Effect", glutamic dehydrogenase is induced in a unique pattern reminiscent of dampened sinusoidal oscillation. This pattern of oscillation appeared as a period of synthesis followed by a period of degradation of newly-synthesized

Fig. 1 Analysis of cell free extracts for NAD-specific glutamic dehydrogenase activity after growth of Achlya for 48 hr in the presence of varying concentrations of glucose or α-methyl-D-glucoside plus glutamate. Achlya was grown from an inoculum of spores in 100 ml liquid shake cultures.


900
500
400
300
200
100
SPECIFIC ACTIVITY - GDH (⊙, △); IDH (⊡)
+ANALOGUE
%
0 20 40 60 80 100
100 80 60 40 20 0
GLUCOSE
GLUT.

material. Degradation is not complete and is rapidly overcome by synthesis, each time at a higher starting point. This parallels the pattern of C^{14} amino acid incorporation into protein which was recorded at the same time as analysis of enzyme activity.

From Fig. 3 the value of the rate constant "k" for protein synthesis was calculated as 0.021.

The set of controls in which the mats of Achlya were exposed to fresh G_2Y medium during the period of induction showed a low constant level of glutamic dehydrogenase activity (Fig. 4). Also, the pattern of protein synthesis appeared as a rise to a plateau.

Since these observations could be mere artifacts, several experiments were carried out to eliminate this possibility.

(a) Table VII indicates that NADP-specific isocitric dehydrogenase activity remains approximately constant during conditions of derepression. Glutamic dehydrogenase on the other hand continues to show the same pattern of oscillation.

(b) An experiment was carried out in order to find out what happens to the preformed glutamic dehydrogenase during the period of induction. This was done by determining the amount of radioactivity lost from prelabelled glutamic dehydrogenase at different times of induction. Radioactivity due to C^{14} amino acids incorporation was determined both in the TCA precipitate and in the TCA filtrate (i. e. TCA-Casein hydrolysate washing as described in method in section X).

Table VI

Glucose Utilization by Achlya

Time (hours)	% Glucose remaining in the medium
0	100
24	88
48	33.2
72	25.2

Fig. 2 Growth curve for Achlya in which dry weight of mycelial mat is plotted against time.

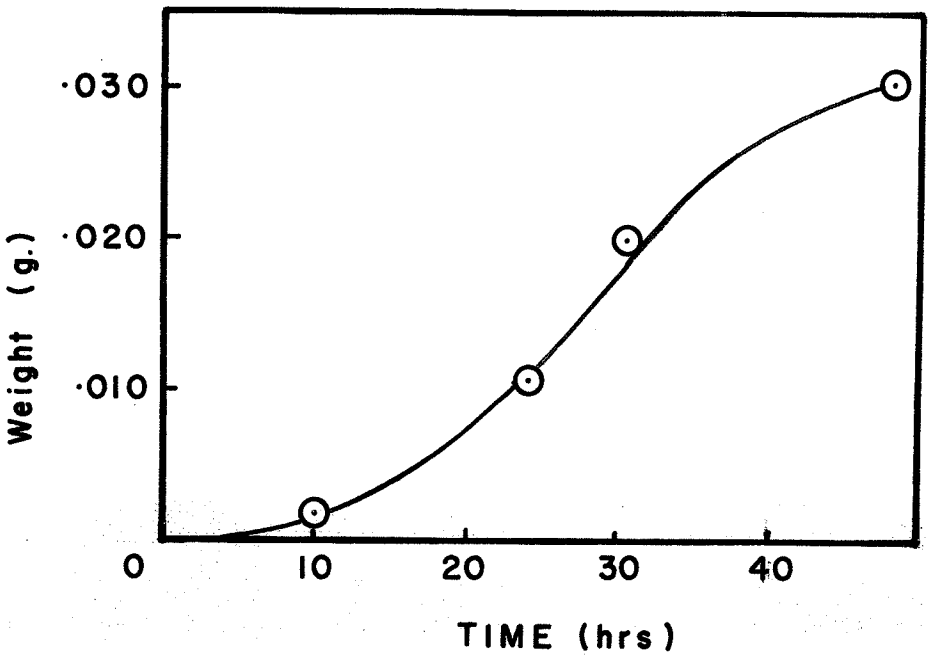
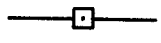

·030
·020
·010
0
10
20
30
40
Weight (g.)
TIME (hrs)

Fig. 3 Derepression of NAD-specific glutamic dehydrogenase by glutamate in Achlya

Relative increase (percent) of glutamic dehydrogenase specific activity during derepression.



Relative increase in C^{14} amino acid incorporation into protein during derepression.

Inset - semi-log plot of C^{14} amino acid incorporation into protein against time.

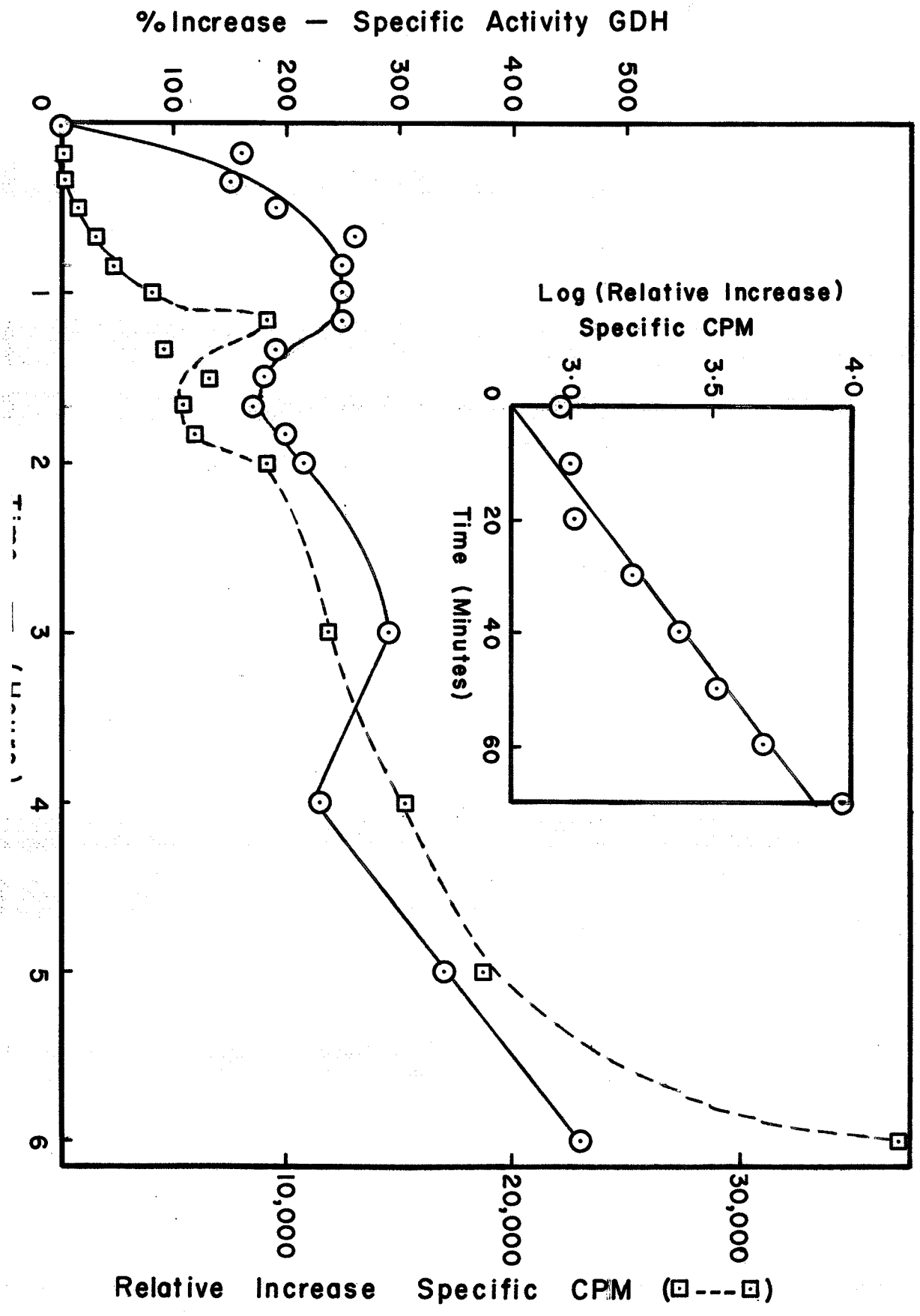
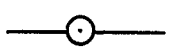

% Increase — Specific Activity GDH
100
200
300
400
500
0
1
2
3
4
5
6
Log (Relative Increase)
Specific CPM
3·0
3·5
4·0
0
20
40
60
Time (Minutes)
10,000
20,000
30,000
Relative Increase Specific CPM (⊡---⊡)

Fig. 4 Repression of NAD-specific glutamic dehydrogenase by glucose in Achlya

Glutamic dehydrogenase specific activity during repression.



C^{14} amino acid incorporation into protein during repression

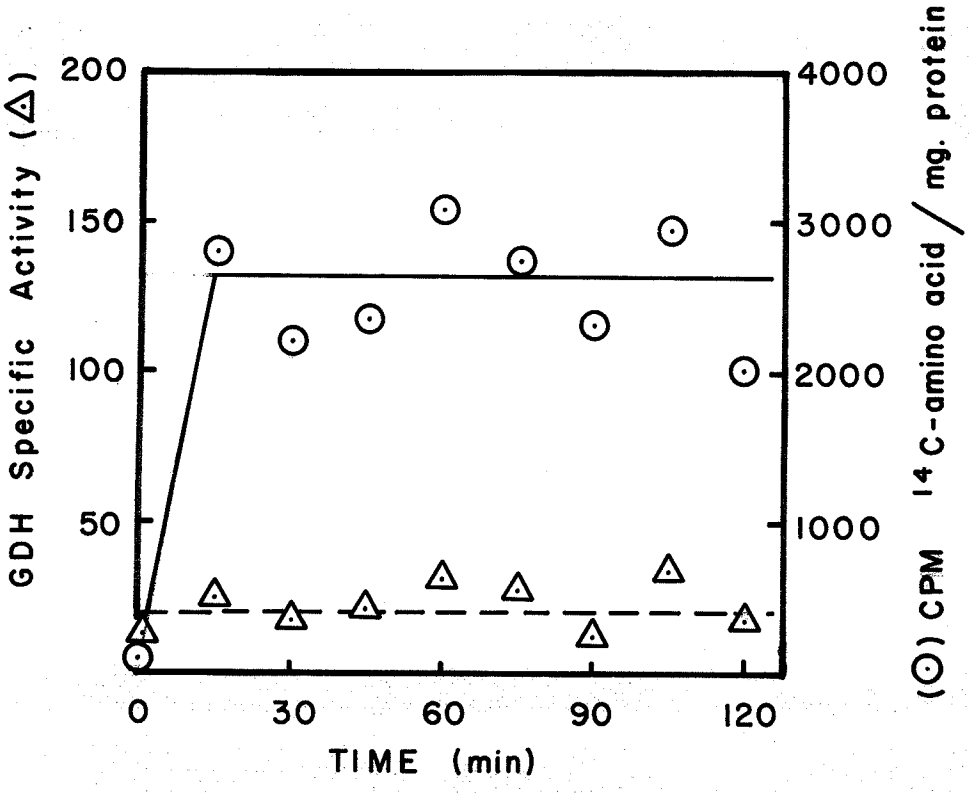

GDH Specific Activity (△)
200
150
100
50
4000
3000
2000
1000
(⊙) CPM ¹⁴C-amino acid / mg. protein
0
30
60
90
120
TIME (min)

Table VII

A comparison of Glutamic and Isocitric dehydrogenase activities in Achlya under derepressed conditions

Time (min)	Specific Activity Glutamic dehydrogenase	Specific Activity Isocitric dehydrogenase	Ratio $\frac{\text{GDH}}{\text{IDH}}$
0	320	102	3.0
15	445	116	3.7
30	578	124	4.5
45	576	122	4.7
60	589	108	5.5
75	545	113	4.8
90	495	110	4.5
105	628	102	6.1
120	779	131	6.1

The radioactivity detected in the filtrate would result from TCA soluble materials, probably oligopeptides (i.e. degraded protein) in this case. From Fig. 5 it is shown that the radioactivity in the TCA precipitate and filtrate follows the same pattern of oscillations as the induced enzyme i.e. periods of synthesis followed by phases of degradation. The peaks of radioactivity detected in the filtrate coincided with the low points in the oscillating pattern detected in the precipitate. In other words, when the radioactivity due to degraded protein is at its highest point, the radioactivity due to larger and more complete proteins is at its lowest point. Thus, according to this observation, it would seem that preformed enzyme decays and is synthesized in the same manner as the newly-synthesized enzyme.

(c) The objective of the next experiment was to see if glutamic dehydrogenase is actually synthesized (rather than activated) during derepression. After a derepression and labelling (C^{14} amino acid hydrolysate) period of one hour, the cell free extract was passed through a 30 cm x 1.5 cm sephadex G200 column (Fig. 6). The glutamic dehydrogenase specific activity elution profile coincided with the radioactive material of high molecular weight. Those radioactive peaks (of low molecular weight) lacking enzyme activity could represent either degraded glutamic dehydrogenase or incompleted enzymes in synthesis or other proteins.

Fig. 5 Loss of C^{14} label in prelabelled glutamic dehydrogenase during derepression in Achlya

Radioactivity lost from the enzyme and other proteins during derepression.

Radioactivity retained in the enzyme and other proteins during derepression.

Specific activity of the enzyme during the period of derepression.

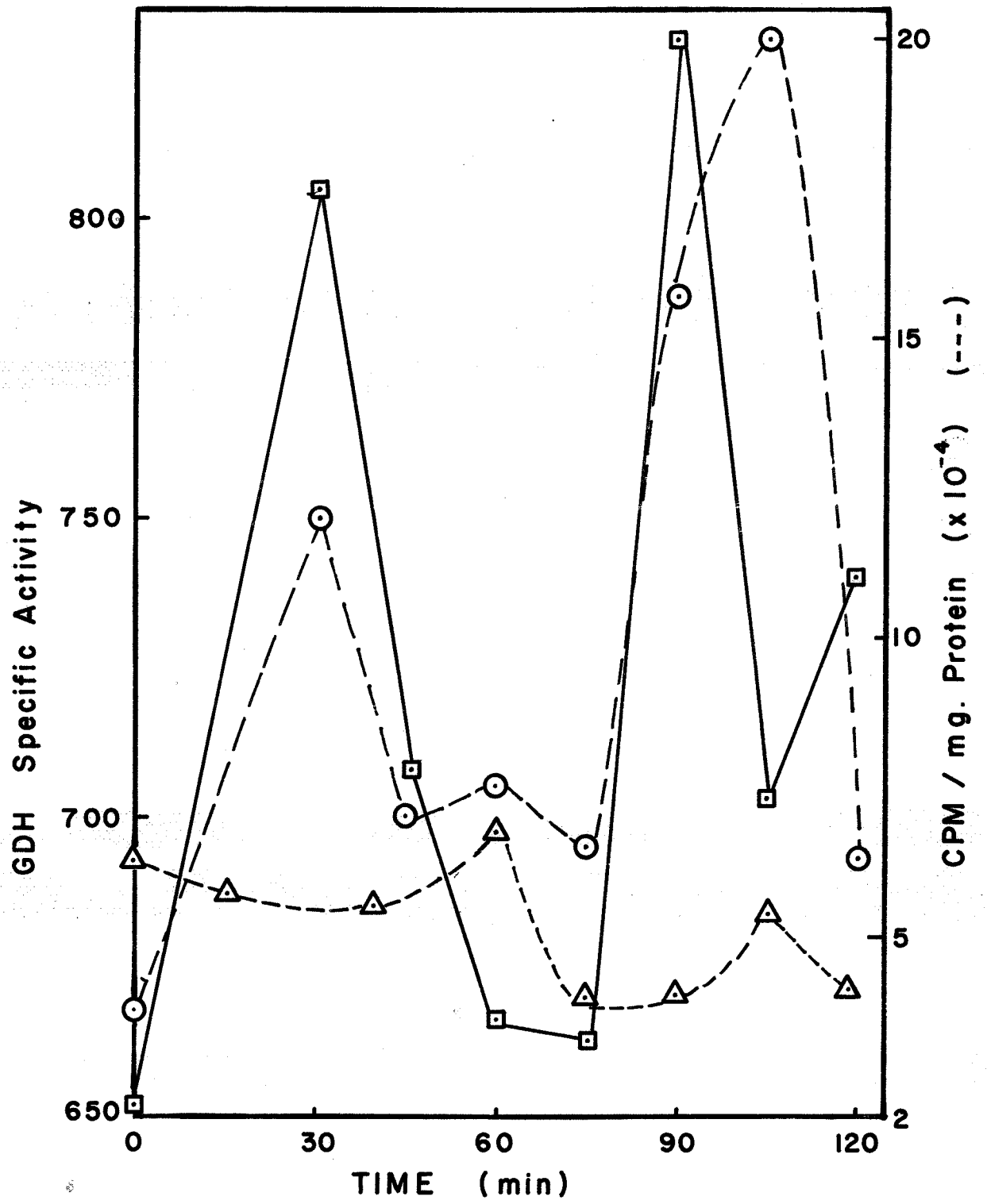
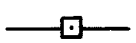
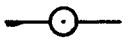

GDH Specific Activity
800
750
700
650
0
30
60
90
120
TIME (min)
20
15
10
5
2
CPM / mg. Protein (x 10⁻⁴) (---)

Fig. 6 Elution profile of derepressed glutamic dehydrogenase from a sephadex G-200 column

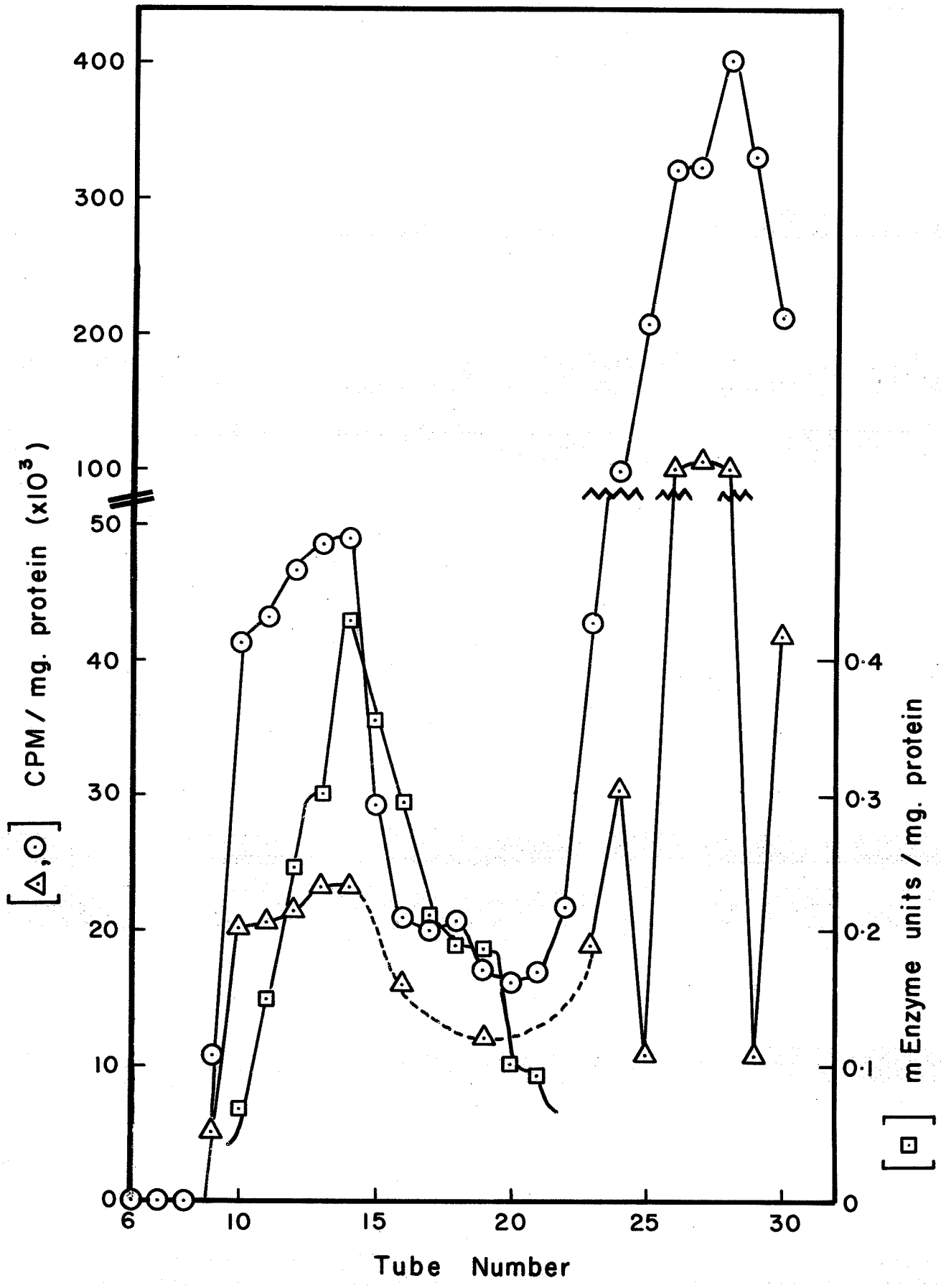


Specific activity of glutamic dehydrogenase in 100 µl of eluent.



C^{14} amino acid incorporation into protein as detected in 100 µ l of eluent.

C^{14} amino acid incorporation into protein as detected in a TCA precipitate of 100 µ l of eluent.


400
300
200
100
50
40
30
20
10
0
6
10
15
20
25
30
Tube Number
[Δ,⊙] CPM / mg. protein (x10³)
[⊡] m Enzyme units / mg. protein
0·4
0·3
0·2
0·1
0

IV. RNA Synthesis during Derepression of Glutamic Dehydrogenase by Glutamate

C^{14} uridine incorporation continued unabated at a logarithmic rate throughout the period of derepression (Fig. 7). However, the pattern of oscillations previously described for glutamic dehydrogenase specific activity remained undisturbed during this time of derepression. The first order rate constant for RNA synthesis (k_{RNA}) was 0.027 and this value is comparable to the rate constant for protein synthesis ($k_{Protein}$) which is 0.021. Therefore it appears that protein synthesis and RNA synthesis may be coupled during the period of derepression.

V. Influence of 5-Fluorouracil (5-Fu) on the Derepression System of Achlya

As shown in Table VIII, 5-Fu had no effect upon the typical pattern of oscillation displayed by glutamic dehydrogenase specific activity and C^{14} amino acid incorporation pattern. However, the relative increases in specific activities of the enzyme and C^{14} amino acid incorporation were about the same in 5-Fu free and 5-Fu-treated cultures. This is a surprising result since the objective of this experiment was to see if this analogue (5-Fu) would produce a faulty m-RNA which would eventually cause fraudulent enzyme synthesis.

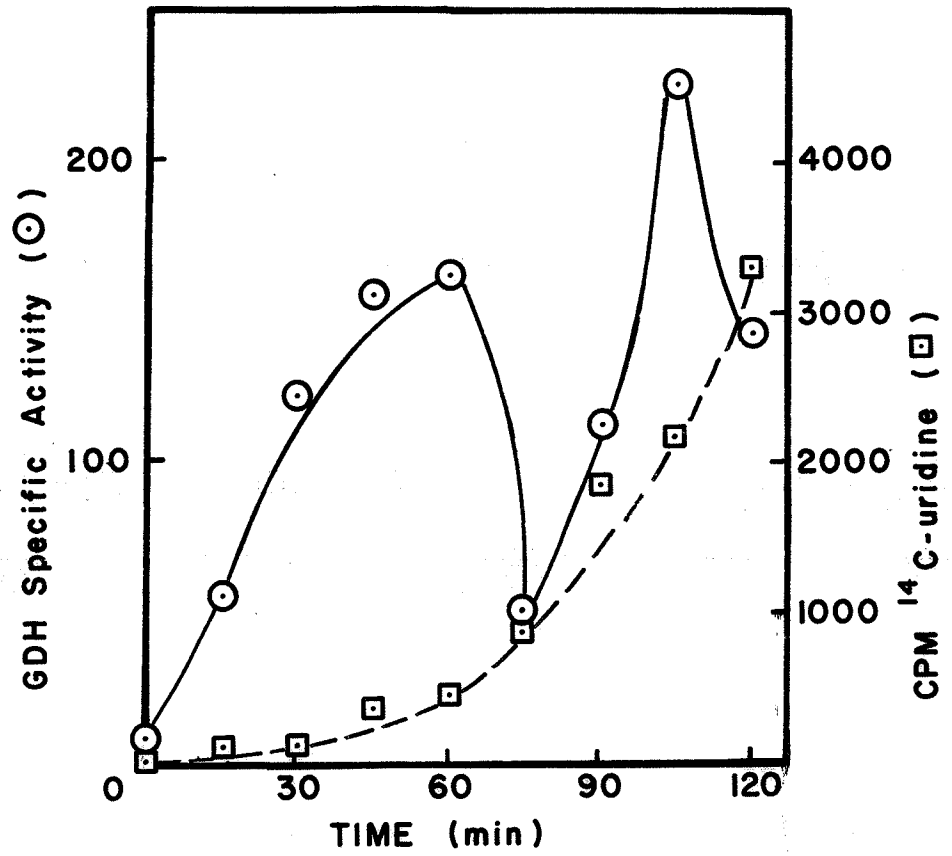
Fig. 7 RNA Synthesis during derepression of glutamic dehydrogenase in Achlya

(a) ⊙——⊙ Glutamic dehydrogenase specific activity during derepression.

C^{14} uridine incorporation into RNA during derepression.

(b) Log plot of C^{14} uridine incorporation into protein

⊡- - - -⊡


GDH Specific Activity (⊙)
CPM ^{14}C-uridine (⊡)
200
100
0
4000
3000
2000
1000
30
60
90
120
TIME (min)

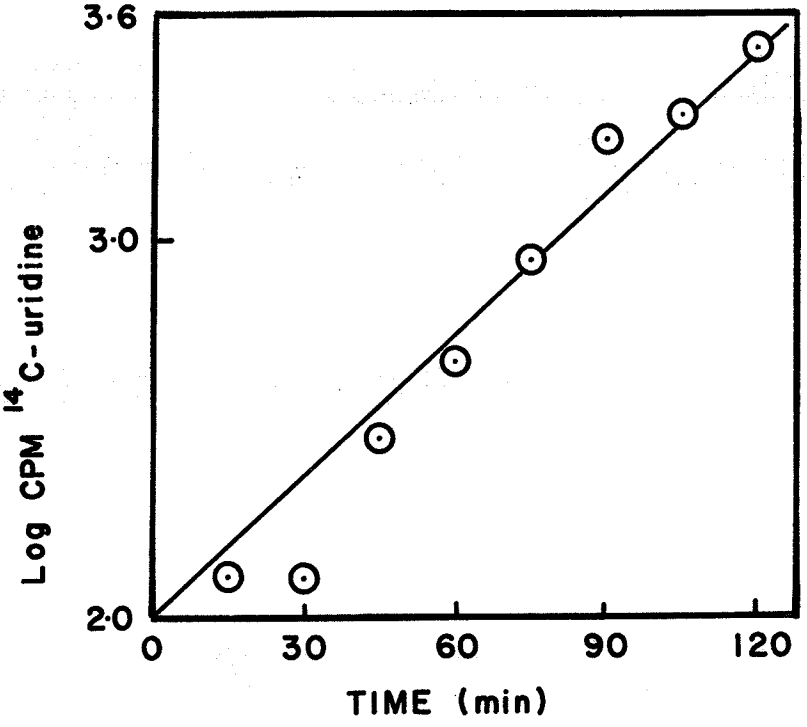

Log CPM ^{14}C-uridine
3·6
3·0
2·0
0
30
60
90
120
TIME (min)

Table VIII

Effect of 5-Fluorouracil on the derepression of Glutamic dehydrogenase in Achlya

Time (min)	Specific Activity Glutamic dehydrogenase	counts/min/mg protein of C^{14} amino acid incorporation	Specific Activity Glutamic dehydrogenase	counts/min/mg protein of C^{14} amino acid incorporation
	+ 5-Fluorouracil		-5-Fluorouracil	
0	111	388	77	74
30	121	4,100	63	3,628
60	176	7,100	114	9,657
90	130	5,200	94	7,139
120	224	20,232	138	9,409

VI. Kinetic Studies on NADP-specific Isocitric dehydrogenase from Achlya

(a) Purification of NADP-specific isocitric dehydrogenase

A summary of the purification procedure is given in Table IX. Cell free extract was obtained as described under "Materials and Methods".

Chilled acetone was added to the cell free extract in a dropwise manner to 45% saturation at 4°. The sample was then centrifuged and the precipitate discarded. Acetone was then added in the same manner to the supernatant to 60% saturation. The sample was then centrifuged. Enzyme activity remained in the precipitate which was resuspended in 40 ml harvesting buffer (as described in "Materials and Methods). The dissolved precipitate was adsorbed directly on a DEAE-cellulose column (40 x 2.5 cm). The cellulose was previously equilibrated with harvesting buffer. The enzyme was eluted from the column using the same buffer containing 0.1 M KCl and collected in 5 ml portions. The enzyme was recovered from the fractions by ammonium sulfate precipitation (75% saturation), dissolved in harvesting buffer, and further purified by filtering through Sephadex G-200 column (100 x 2.5 cm) using the same buffers with 0.1 M KCl added. The enzyme was recovered by ammonium sulfate precipitation (75% saturation), dissolved in a small volume of harvesting buffer containing 20% glycerol. The enzyme was stored at -20°C and is relatively stable in this condition for several weeks.

Table IX

Purification of NADP-specific isocitric dehydrogenase

Step	Protein (mg/ml)	Total volume (ml)	Total protein (mg)	Total enzyme activity	Specific Activity of enzyme	Purification
Crude Extract	8.8	2,250	19,800	3,586,400	181	
Acetone precipitation (45%)	2.7	3,935	10,554	2,773,000	263	2
Acetone Precipitation (60%)	9.6	37	355	762,650	2,147	12
DEAE-Cellulose	0.25	230	57	303,600	5,299	29
Ammonium sulfate (0.75% saturation) and dialysis	4.5	6	27	171,600	6,363	35
Sephadex G-200	0.14	79	11	82,160	7,469	44
Ammonium sulfate (0.75% saturation)	1.25	3.9	5	50,132	10,201	57

(b) Determination of Michaelis Constants

When $NADP^+$ was used as the varied substrate and isocitrate added as the second substrate, a family of straight lines was obtained and these have been plotted in Lineweaver-Burk form. These lines intersected to the left of the v^{-1} axis. Similarly when isocitrate was used as the varied substrate and $NADP^+$ as second substrate, a family of straight lines intersecting to the left of the v^{-1} axis was obtained. These results are presented in Fig. 8. These results specify an ordered sequence for the reaction.

This enzyme does not appear to catalyze the reverse reaction. Attempts to reverse this reaction at different pH values, different substrates and enzyme concentrations and different buffers under initial velocity conditions met with failure.

(c) Product Inhibition Studies

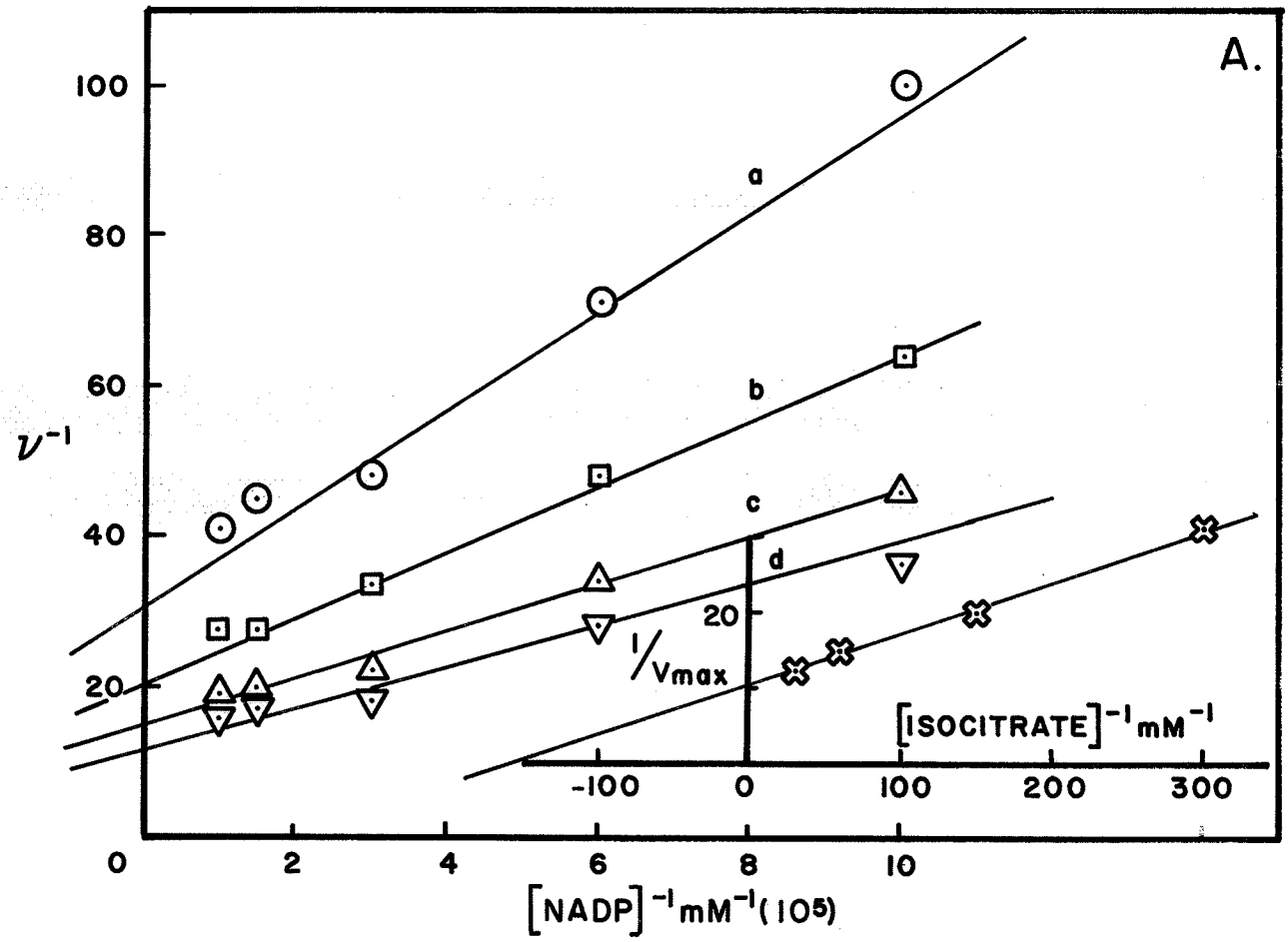
Since the reaction is ordered and essentially irreversible the kinetic mechanism could be a Theorell-Chance Mechanism in which no central complex is formed. The steady state rate equation for a Theorell-Chance kinetic mechanism has been developed by Cleland (63).

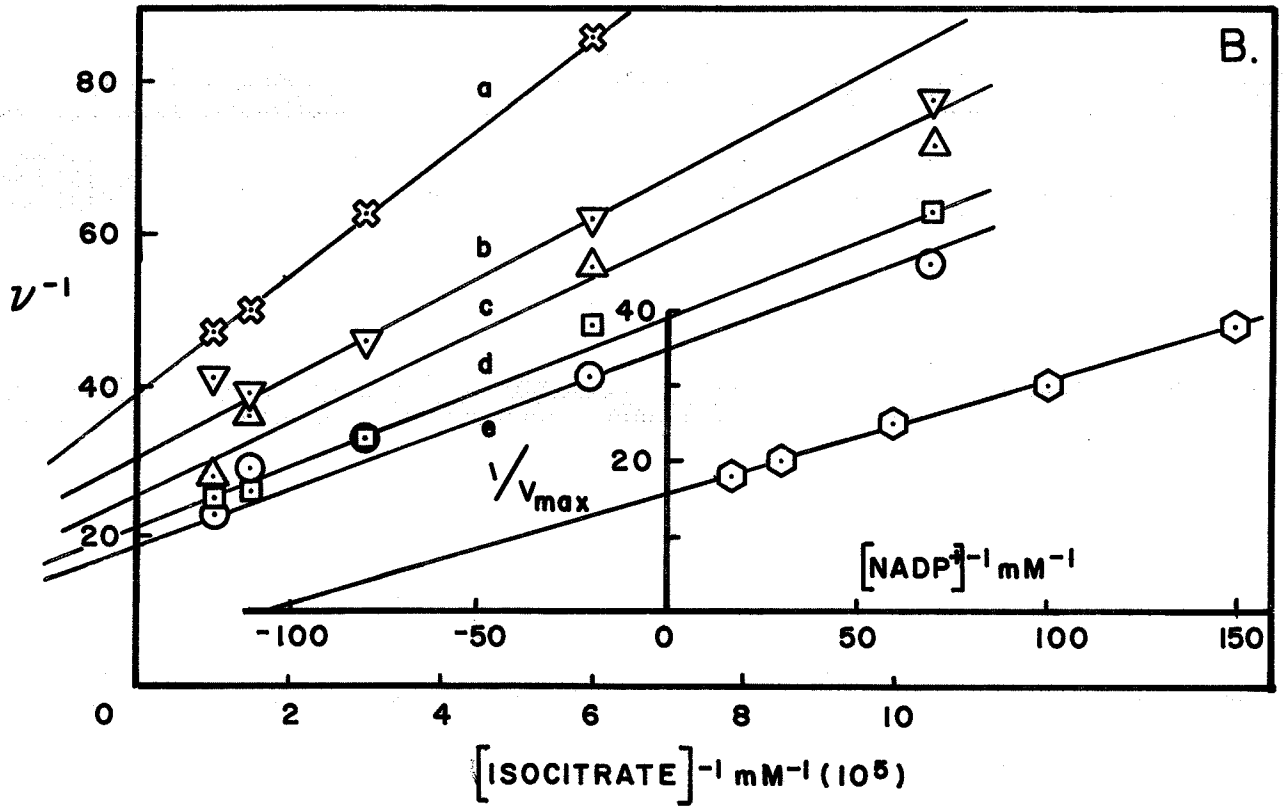
Fig. 8 (A) Double reciprocal plots of velocity against $NADP^+$ concentration at several fixed levels of isocitrate. Assays were conducted in 0.2 M Tris-HCl buffer, pH 7.5. The concentrations of isocitrate were: (a) .0033 mM; (b) .0067 mM; (c) .0167 mM; (d) .033 mM.

Inset: replot of intercepts from which the Michaelis constant for isocitrate is obtained.

(B) Double reciprocal plots of velocity against isocitrate concentration at several fixed levels of $NADP^+$. Assays were conducted in 0.2 M Tris-HCl buffer, pH 7.5. The concentrations $NADP^+$ were: (a) .0067 mM; (b) .010 mM; (c) .0167 mM; (d) .033 mM; (e) .0667 mM.

Inset: replot of intercepts from which the Michaelis constant for $NADP^+$ is obtained.


A.
100
80
60
40
20
v^{-1}
a
b
c
d
20
$1/V_{max}$
[ISOCITRATE]$^{-1}$ mM^{-1}
-100
0
100
200
300
0
2
4
6
8
10
[NADP]$^{-1}$ mM^{-1} (10^5)


B.
80
60
40
20
v^{-1}
a
b
c
d
e
40
20
$1/V_{max}$
[NADP]$^{-1}$ mM^{-1}
-100
-50
0
50
100
150
0
2
4
6
8
10
[ISOCITRATE]$^{-1}$ mM^{-1} (10^5)

Using the King-Altman method[‡] the steady-state rate equation for an ordered Theorell-Chance reaction involving two reactants and three products is as follows:

$$v = V_1V_2\,AB - \frac{V_1V_2PQR}{k_{eq}} \quad V_1\,\frac{PQR}{k_{eq}} + \frac{k_rPQ}{k_{eq}} + \frac{k_rAPQ}{k_{ia}k_{eq}} + \frac{k_pBRQ}{k_{ib}k_{eq}} + \frac{k_pRQ}{k_{eq}} \quad \ldots\ldots$$

$$\ldots\ldots + \frac{k_{ip}k_qR}{k_{eq}} + \frac{k_qPR}{k_{eq}} + \frac{k_{ir}k_{ip}k_q}{k_{eq}} + V_2k_aB + \frac{V_2k_{ia}k_bP}{k_{ip}} + V_2k_bA \ldots\ldots$$

$$\ldots\ldots \frac{V_2k_bAP}{k_{ip}} + \frac{V_2k_ak_rk_pABQ}{k_{ir}k_{ia}k_{ip}k_q} + V_2AB + \frac{V_2k_aRB}{k_{ir}} \qquad \ldots\ldots(1)$$

The Haldane relationship is

$$K_{eq} = \frac{k_{ir}k_{ip}k_q}{k_{ia}k_b} \left[V_1/V_2\right]^n \qquad \ldots\ldots(2)$$

where n = 1.

When either P, Q or R=0, the second term in the numerator of the rate equation (1) above cancels out and using the Haldane relationship, equation (1) reduces to

$$\frac{1}{v} = \frac{PQR\,k_{ia}k_b}{V\,ABk_{ir}k_{ip}k_q} + \frac{PQ\,k_rk_{ia}k_b}{k_{ir}k_{ip}k_qABV} + \frac{PQ\,k_rk_b}{k_{ir}k_{ip}k_qBV} + \frac{RQ\,k_pk_{ia}k_b}{A\,k_{ib}k_{ir}k_{ip}k_qV}$$

$$+ \frac{RQ\,k_pk_{ia}k_b}{k_{ir}k_{ip}k_qABV} + \frac{R\,k_qk_{ia}k_b}{k_{ir}k_qABV} + \frac{PR\,k_{ia}k_b}{k_{ir}k_{ip}ABV} + \frac{k_{ia}k_b}{ABV} + \frac{k_a}{AV} +$$

$$\frac{k_{ia}k_bP}{k_{ip}ABV} + \frac{k_bP}{k_{ip}BV} + \frac{k_ak_rQ\,k_p}{k_{ir}k_{ia}k_{ip}k_qV} + \frac{1}{V} + \frac{k_aR}{k_{ir}AV} + \frac{k_b}{BV} \qquad \ldots\ldots(3)$$

[‡]King, E.L., and Altman, C., J. Phys. Chem. 60, 1375 (1956)

In product inhibition, if the reaction is inhibited by any one of the products, α-ketoglutarate, HCO_3^- or NADPH, Equation 1 can be used to predict what P, Q and R may be. When R is used as inhibitor and P and Q are zero concentration under initial velocity conditions, then Equation 1 can be modified to

$$\frac{1}{v} = \frac{1}{B}\left\{\frac{k_q k_{ia} k_b R}{k_{ir} k_q AV} + \frac{k_{ia} k_b}{AV} + \frac{k_b}{V}\right\} + \left\{\frac{k_a}{AV} + \frac{k_a R}{k_{ir} AV} + \frac{1}{V}\right\} \quad \ldots\ldots(4)$$

When substrate A is varied and substrate B is saturating, the product R affects slope only. If substrate B is varied and substrate A is saturating, the product R affects both intercept and slope.

When R and P are at zero concentration and Q is used as inhibitor, Equation 1 is modified to

$$\frac{1}{v} = \frac{1}{B}\left\{\frac{k_{ia} k_b}{AV} + \frac{k_b}{V}\right\} + \frac{1}{V}\left\{\frac{k_a}{A} + \frac{k_a k_r k_p Q}{k_{ir} k_{ia} k_{ip} k_q} + 1\right\} \quad \ldots\ldots\ldots (5)$$

When either A or B is saturating, Q affects intercept terms only.

When R and Q are at zero concentration and P is used as inhibitor, Equation 1 is modified to

$$\frac{1}{v} = \frac{1}{A}\left\{\frac{k_{ia} k_b}{BV} + \frac{k_a}{V} + \frac{k_{ia} k_b P}{k_{ip} BV}\right\} + \left\{\frac{k_b P}{k_{ip} BV} + \frac{k_b}{BV} + \frac{1}{V}\right\} \quad \ldots\ldots\ldots (6)$$

When A is saturating and substrate B is varied, product P affects slope only.

The following table is a summary of the experimental results of the product inhibition studies.

Table X

Product inhibition studies on NADP-specific isocitric dehydrogenase from Achlya sp 1969.

(a) Isocitrate at saturating concentration and $NADP^+$ is varied. (Fig. 10)

Product Inhibitor	Inhibition Pattern
HCO_3^-	uncompetitive
NADPH	competitive

(b) $NADP^+$ at saturating concentration and isocitrate is varied. (Fig. 9)

Product Inhibitor	Inhibition Pattern
HCO_3	non-competitive
NADPH	non-competitive
α-ketoglutarate	competitive

The theoretical predictions support the experimental data if $NADP^+$ is substrate A, isocitrate is substrate B, α-ketoglutarate is product P, HCO_3^- is Q and NADPH is product R.

The complete reaction occurs by an obligatory ordered sequence by a Theorell-Chance kinetic mechanism schematized as follows:

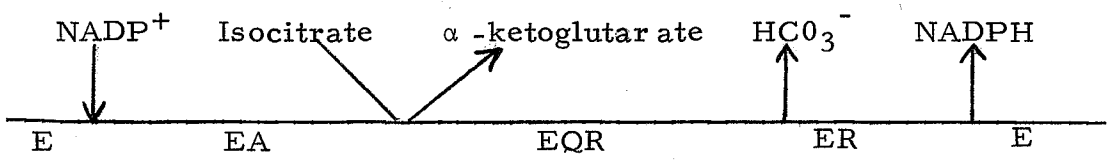


Fig. 9 Product inhibition of NADP-specific isocitric dehydrogenase with isocitrate as the varied substrate. $NADP^+$ was held at a high constant concentration of 1mM. Assays were carried out in 0.2 M Tris-HCl buffer, pH 7.5 in the presence of 3.33 mM $MnCl_2$.

A) Product inhibition by HCO_3^-; concentrations of HCO_3^- used were: (a) 100 mM; (b) 66.67 mM (c) 16.67 mM; (d) 0 mM.

B) Product inhibition by NADPH; concentrations of NADPH used were: (a) 0.1 mM; (b) 0.067 mM; (c) 0.0167 mM; (d) 0 mM.

C) Product inhibition by α-ketoglutarate; concentrations of α-ketoglutarate used were; (a) 16.67 mM; (b) 3.33 mM; (c) 1.67 mM; (d) 0 mM.

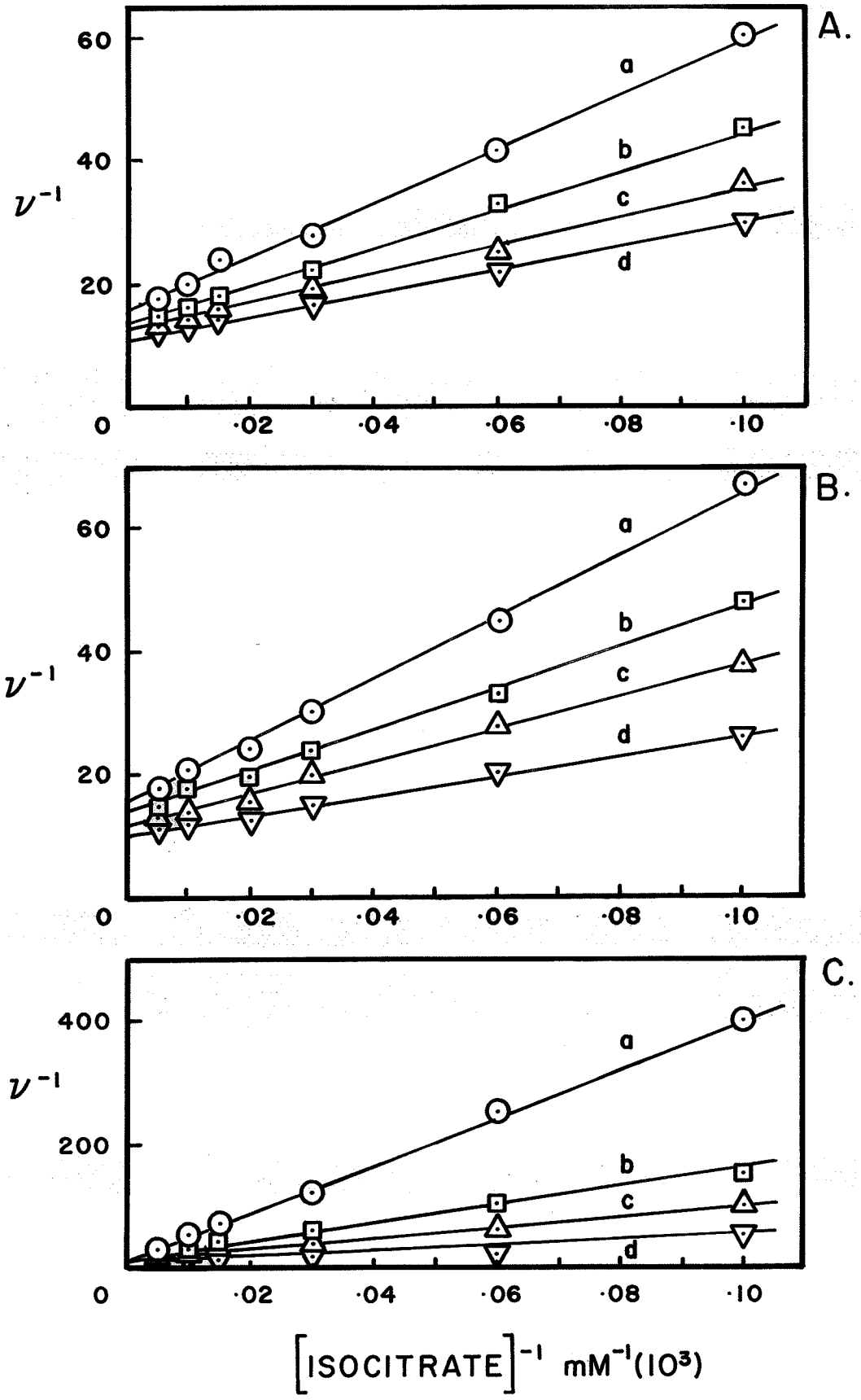
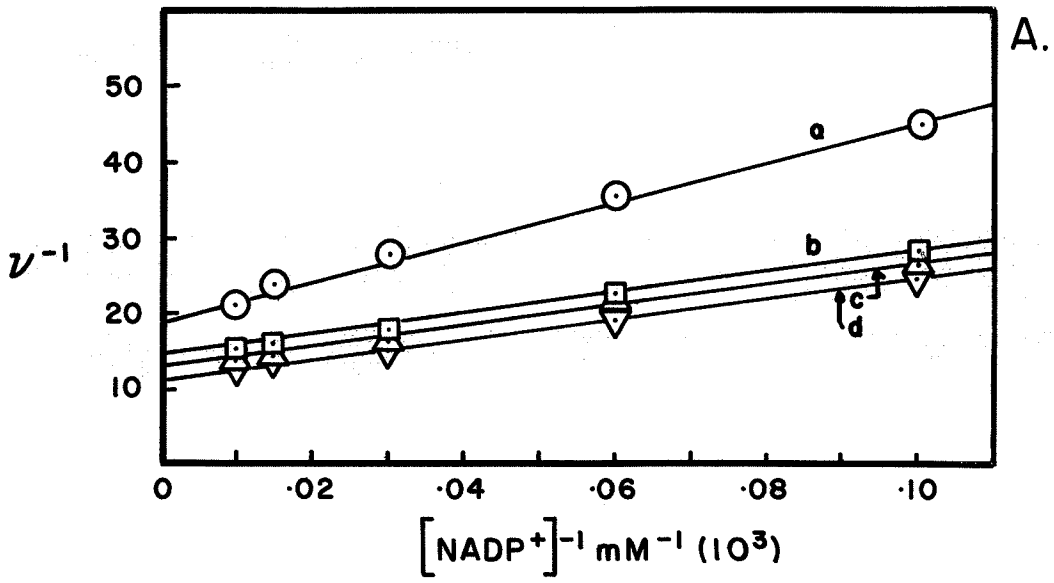
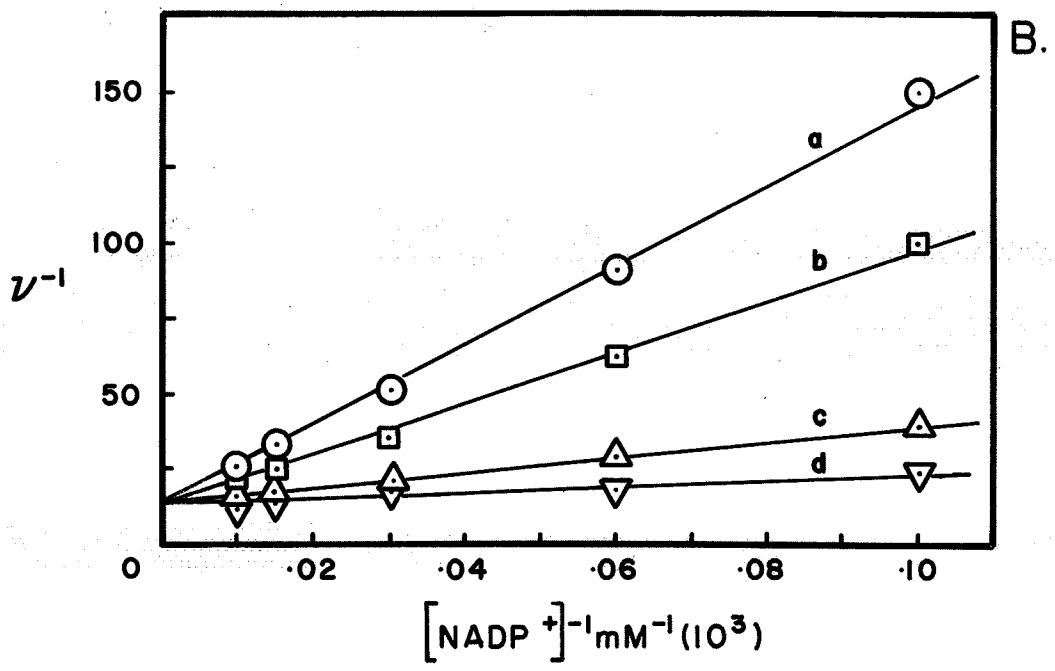

A.
B.
C.
a
b
c
d
60
40
20
0
400
200
·02
·04
·06
·08
·10
ν^{-1}
$[\text{ISOCITRATE}]^{-1}$ $mM^{-1}(10^3)$

Fig. 10 Product inhibition of NADP-specific isocitric dehydrogenase with $NADP^+$ as the varied substrate. Isocitrate was held at a constant concentration of 0.1 mM. Assays were carried out in 0.2 M Tris-HCl buffer, pH 7.5 in the presence of 3.33 mM $MnCl_2$.

A) Product inhibition by HCO_3^-; concentrations of HCO_3^- used were: (a) 100 mM; (b) 50 mM; (c) 33.33 mM; (d) 16.66 mM and 0 mM.

B) Product inhibition by NADPH; concentrations of NADPH used were: (a) 0.133 mM; (b) 0.100 mM; (c) 0.0167 mM; (d) 0 mM.


A.
50
40
30
20
10
v^{-1}
a
b
c
d
0
·02
·04
·06
·08
·10
$[NADP^+]^{-1}$ mM^{-1} (10^3)


B.
150
100
50
0
v^{-1}
a
b
c
d
·02
·04
·06
·08
·10
$[NADP^+]^{-1}$mM^{-1}(10^3)

Discussion

Because of the absence of NADP-specific glutamic dehydrogenase in Achlya sp 1969, the NAD-linked enzyme must serve both the anabolic and catabolic needs of the cell. Since this enzyme is amphibolic, it must be very carefully controlled by the cell. Indeed, this enzyme has been found to be allosterically controlled by several effectors (59, 60) which dictate the direction of the reversible reaction catalysed by this variety of glutamic dehydrogenase. This enzyme is also genetically controlled as it is subject to catabolite repression (59). The co-repressor may be some metabolic product of glucose since several carbohydrates (Table V) did repress this enzyme while amino acids and organic acids of the citric acid cycle did not. It is not likely that glucose itself is a co-repressor since α-methyl-glucoside, an analogue of glucose, did not repress glutamic dehydrogenase except at very high concentrations (Fig. 1) which could be due to toxic effects. The cells do not grow with this analogue as substrate.

Under conditions of derepression, yet another type of control upon this enzyme was revealed. When glutamic dehydrogenase was derepressed by glutamate, an oscillating pattern of activity resulted i.e. a period of synthesis followed by a phase of degradation. This degradation is not complete and is rapidly overcome by synthesis each time at a higher starting point. This pattern paralleled C^{14}-amino

acid incorporation into proteins followed over the same period of derepression (Fig. 3).

In order to validate such observations, several experiments were carried out. First, the activity of another closely coupled enzyme NADP-specific isocitric dehydrogenase was followed along with the glutamic dehydrogenase during the derepression conditions (Table VII). It was found that while glutamic dehydrogenase continued to follow its usual oscillatory pattern, isocitric dehydrogenase activity remained approximately constant. This result strongly suggests that this unusual pattern of enzyme induction (NAD-specific glutamic dehydrogenase) was real and not an artifact. Secondly, passage of cell free extract from Achlya labelled during a derepression period of one hour through Sephadex G-200 column (Fig. 6) resolved the nature of the newly-synthesized protein which was largely glutamic dehydrogenase. Thus it would appear that actual synthesis of glutamic dehydrogenase did occur when derepressed by glutamate.

Fig. 5 shows the loss of C^{14} label in prelabelled glutamic dehydrogenase from Achlya during derepression. It was noted that there was a direct relationship between the loss of radioactivity from pre-synthesized material and loss of enzyme activity. These results would indicate that: (a) newly synthesized enzyme is rapidly degraded even as synthesis continues and (b) preformed enzyme also turns over

during derepression.

RNA synthesis was also followed during the period of derepression by glutamate in Achlya. It was observed that the synthesis of RNA increased at a logarithmic rate upon addition of glutamate (Fig. 7). The value for the first order rate constant "k" obtained from the log plot (Fig. 3) was practically identical to the value of the first order rate constant for protein synthesis. These results signify that RNA synthesis and protein synthesis are probably coupled. It also appears that a translational control may dictate whether synthesis or degradation prevails.

Glutamic dehydrogenase as studied by Balinsky et al (65) in in vitro frog liver suspensions was also unstable. They observed that it had a much more rapid turn over rate than that of the bulk of mitochondrial proteins. They suggested that a rapid rate of turn over may be characteristic of a number of mitochondrial enzymes and that the mitochondria are not static and that some components are being replaced continuously during the life-time of these organelles. Glutamic dehydrogenase in Achlya, being a mitochondrial enzyme would certainly support this concept.

Various other workers conducting experiments performed with mammalian systems (37, 40, 41, 42, 65) have also reported an induction of enzyme synthesis and a subsequent degradation. Interestingly, Tschudy et al (66) found

that injection of estradiol to ovariectomized rats produced oscillations in the levels of hepatic δ-aminolevulinic acid synthetase and dehydratase. They suggested that this phenomenon of oscillatory changes in level may occur among a number of inducible enzymes when the control cycle is perturbed by various factors such as nutritional changes. All of these studies point to the fact that controls upon enzyme activity may be levelled at degradation as well as synthesis.

Thus, a possible picture of NAD-specific glutamic dehydrogenase in Achlya begins to emerge as a mitochondrial enzyme which is inducible and unstable and which because of an abrupt environmental change undergoes rapid oscillatory changes in level until a new steady-state is attained. The environmental change is a shift from a condition in which the enzyme is repressed (in glucose containing medium) to a condition in which the enzyme is derepressed (in glutamate containing medium). When the spent G_2Y was replenished by fresh G_2Y, glutamate dehydrogenase activity remained at a constant low level and protein synthesis increased rapidly to reach a plateau (Fig. 4). But when the environment was drastically altered, by induction with glutamate, the imbalance in the nutritional status of the cell is reflected in the periodic synthesis-degradation of inducible proteins.

In the studies referred to above (37,40,41, 42, 65), involvement of a second protein controlling the degradation of the induced enzyme

when it was induced to a certain critical level, was suggested. Shapiro and Stadtman (67) reviewed observations that had been made on the regulation of glutamine metabolism in E. coli. Glutamine synthetase is modulated in response to variations in the supply of nitrogen. Certain critical α-ketoglutarate:glutamine ratios activates/or inactives a deadenylating enzyme complex which, in turn, activate or inactivate glutamine synthetase correspondingly.

In the light of these observations it is conceivable that similar activating and inactivating systems may exist in Achlya. Perhaps a second protein (at the moment, hypothetical) regulated by critical levels of some compound(s) could regulate glutamic dehydrogenase activity in Achlya.

Since isocitric dehydrogenase and glutamic dehydrogenase were found to be closely coupled (59, 60), it is possible that isocitric dehydrogenase may also be involved in this control. Fig. 1 indicates that isocitric dehydrogenase and glutamic dehydrogenase respond in an opposite manner to increasing glucose and glutamate concentrations. While glutamic dehydrogenase is subject to the glucose effect, isocitric dehydrogenase activity increases. Glutamic dehydrogenase is induced by increasing the time of exposure to glutamate while isocitric dehydrogenase activity remains at a constant low level (Table VII). The close coupling of these two enzymes is suggested because only the NAD-specific

glutamic dehydrogenase and the NADP-specific isocitric dehydrogenase exist in Achlya (59, 60). The alternative dehydrogenases (NADP-linked glutamic dehydrogenase and NAD-linked isocitric dehydrogenase) are non-existent. Thus, the two enzymes must cooperate in order to control the balance of oxidized and reduced $NADP^+$ and NAD^+ levels (59, 60).

The work of Kapoor and Grover (68) would add support to this line of reasoning. They found that in Neurospora which possesses both NAD^+ and $NADP^+$ specific glutamic dehydrogenases, the NAD- specific glutamic dehydrogenase is subject to repression by sucrose and glucose. $NADP^+$ specific glutamic dehydrogenase, on the other hand, is induced by increasing concentrations of the catabolite. These data suggested to them that a reciprocal relationship existed between these two enzymes during synthesis in the presence of catabolites.

Thus, on the basis of the observations made in this laboratory, together with the observations made by others mentioned in this discussion, a model for the control of NAD^+ specific glutamic dehydrogenase in Achlya is suggested. During conditions of derepression (Fig. 3), greater amounts of glutamic dehydrogenase are induced with increasing time of exposure to glutamate. This would mean that increasing amounts of NAD^+ are reduced by the following reaction:

$$\text{glutamate} + NAD^+ \longrightarrow \alpha\text{-ketoglutarate} + NH_3^+ + NADH \quad E_q^n \qquad (1)$$

Kinetic studies on $NADP^+$-specific isocitric dehydrogenase in Achlya revealed that the reductive carboxylation of α-ketoglutarate was not catalyzed by this enzyme. Thus, the reaction catalyzed by isocitric dehydrogenase appears to proceed in one direction only:

$$\text{Isocitrate} + NADP^+ \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + NADPH \qquad Eq^n\ (2)$$

Since NADP-specific isocitric dehydrogenase appears to be irreversible, the supply of $NADP^+$ is crucial in the equilibrium. If isocitric dehydrogenase can not regenerate the necessary $NADP^+$ by reversal of the reaction, regeneration may occur through coupling with the glutamic dehydrogenase. $NADP^+$ may be replenished at the expense of glutamate, NAD^+ and NADPH by the following reaction:

$$\text{Isocitrate} + NH_4^+ + NADP^+ + NADH \rightleftharpoons NAD^+ + NADPH + \text{glutamate} + CO_2$$

$$Eq^n\ (1) + Eq^n\ (2)$$

Under conditions of derepression, if excess glutamic dehydrogenase is produced, the pyridine nucleotide balance may be offset to such an extent that degradation is necessary to bring about readjustment. It is noteworthy that $NADP^+$ is an allosteric activator of the glutamic dehydrogenase (59). Degradation could be accomplished by a "hypothetical degrading enzyme" which could exist either in the active or inactive form. This enzyme may be activated or inhibited when a critical ratio of reduced to oxidized nucleotide balance is reached in the

cell. This "hypothetical degrading enzyme", in its active form would inactivate glutamic dehydrogenase by degrading it in part. As soon as the balance of nucleotides was restored to its normal level, the "hypothetical degrading enzyme" would be inactivated and glutamic dehydrogenase activity restored by further synthesis.

Thus, glutamic dehydrogenase in Achlya can be pictured as an enzyme which is continually being synthesized and degraded. At some phases of the life cycle of the organism, as dictated by the levels of oxidized and reduced pyridine nucleotides, the rate of synthesis may exceed the rate of degradation and an increase of enzyme activity would be observed. At other times, the rate of degradation may exceed the rate of synthesis and a decrease in enzyme activity may be seen.

An experiment with the metabolic inhibitor, 5-Fu raises some interesting speculations about the proposed model. When 5-Fu was added along with glutamate in the usual derepression experiment, glutamic dehydrogenase induction and C^{14} amino acid incorporation patterns remained unaffected. The expected result would be a decrease in glutamic dehydrogenase activity due to synthesis of a faulty message since 5-Fu would normally be incorporated in place of uracil into m-RNA. However, the cell may either have neutralized the toxic effect of this analogue, or the 5-Fu may not have been able to

enter the cell at all. This is not likely because 5-Fu inhibits the cells from growing or developing further when added at any stage of development prior to sporulation. Alternatively, the messenger RNA for glutamic dehydrogenase may be stable and thus would not be affected by 5-Fu.

Thus, since NAD-specific glutamic dehydrogenase is amphibolic in Achlya due to the absence of NADP-linked enzyme, glutamic dehydrogenase is regulated by a need to maintain a nucleotide balance. This is accomplished during conditions of derepression by controlling the balance of degradation to synthesis.

This enzyme is known also to be allosterically controlled (59, 60). Activators: short chain acyl CoA derivatives, ATP, GTP, UTP, PEP, $NADP^+$. Inhibitors: citrate, AMP, long chain acyl CoA derivatives. Under energy rich conditions when glucose is being actively metabolized, glutamic dehydrogenase activity is at a low level and the citric acid cycle is operating as a biosynthetic unit. The allosteric activators act cumulatively to antagonize the effects of the inhibitors and unidirectionally stimulate the biosynthetic reaction. (For detailed explanation see; "Allosteric Regulation of Glutamic Dehydrogenase" in the Historical section).

Thus, NAD-specific glutamic dehydrogenase is regulated by both a coarse control ("Glucose Effect") and by several fine controls in the form of allosteric effectors. What then, is the physiological

significance of developing such extensive and elaborate controls on the glutamic dehydrogenases from these organisms? When glutamic dehydrogenase acts as a catabolic enzyme the NH_4^+ resulting from this reaction would be utilized in transamination reactions. NH_4^+ is an allosteric inhibitor of the enzyme. Under these conditions, glutamic dehydrogenase activity would remain at a low level (i.e. during active glucose catabolism). If, however, glutamate is the energy source, an excessive and probably toxic amounts of NH_4^+ will be produced unless the cell could efficiently transaminate all of this NH_4^+ into other amino acids. For this reason, this feed-back inhibitory effect by the end-product (ammonia) is effectively antagonized by the diverse allosteric modifiers found. Thus, this enzyme must be very carefully controlled by several regulatory systems in order to prevent accumulation of NH_4^+.

References

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