I. ACTIVATION OF ASPARTATE TRANSCARBAMYLASE OF ESCHERICHIA COLI BY PURINE NUCLEOTIDES

II. REGULATION OF NITRITE REDUCTASE IN TOBACCO CELLS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

HANNA CHROBOCZEK KELKER

1969

This is to certify that the

thesis entitled

I. Activation of Aspartate Transcarbamylase of Escherichia Coli by Purine Nucleotides.

II. Regulation of Nitrite Reductase in Tobacco Cells.

presented by

Hanna Chroboczek Kelker

has been accepted towards fulfillment of the requirements for

Ph. D. **degree in** Botany / Plant Pathology

Robert S. Bandurski

Major professor

Date May 5, 1969

ABSTRACT

I. ACTIVATION OF ASPARTATE TRANSCARBAMYLASE OF ESCHERICHIA COLI BY PURINE NUCLEOTIDES

II. REGULATION OF NITRITE REDUCTASE IN TOBACCO CELLS

By

Hanna Chroboczek Kelker

I. The properties of an *in vitro* system from *Escherichia coli* were examined in which an increase in aspartate transcarbamylase activity occurred upon incubation with adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), phosphoenolpyruvate (PEP), amino acids and magnesium ions. The objective of this study was to ascertain whether protein synthesis or activation is responsible for the increase in enzyme activity.

Utilizing density labeling and subsequent density centrifugation it was demonstrated that the increase in aspartate transcarbamylase was not due to *de novo* synthesis of this enzyme or to extensive completion of preexisting peptides.

It was demonstrated by kinetic studies that incubation of *Escherichia coli* homogenate with ATP, GTP, PEP, amino acids and magnesium ions or of crystalline aspartate transcarbamylase with the same components, resulted in activation of the enzyme.

Aspartate transcarbamylase was activated in a synergistic manner by ATP and GTP in the presence of magnesium ions. Previously published studies showed that ATP is an activator and GTP is an inhibitor of this enzyme.

Magnesium ions modify the effect of purine nucleotides on aspartate transcarbamylase activity. GTP in equimolar concentration with magnesium ions had little effect on enzyme activity, while ATP and equimolar magnesium together were much better activator than ATP alone.

The activation of aspartate transcarbamylase by ATP and GTP in the presence of magnesium ions reported here might be of biological significance in equilibrating the pools of pyrimidine nucleotides and purine nucleotides of Escherichia coli.

II. The nitrate reductase activity of tobacco cells in liquid culture has been reported to be regulated by both its substrate, nitrate, and by end products of the nitrate assimilatory pathway, amino acids. The objective of this study was to determine if nitrite reductase, the next enzyme of the nitrate assimilatory pathway, is similarly regulated. It was also of interest to determine whether nitrate has to be converted to nitrite before it can cause an increase in nitrite reductase activity.

A modification of an assay of nitrite reductase activity utilizing methyl viologen reduced by sodium hydrosulfite was developed and the optimum conditions for

determination of nitrite reductase activity of tobacco cells were established.

Nitrite reductase activity increased simultaneously with the activity of nitrate reductase in tobacco cells grown on nitrate. It was demonstrated that tungstate, which inhibits the development of nitrate reductase activity in tobacco cells grown on nitrate, does not affect the development of nitrite reductase activity. It is possible, therefore, that nitrate is directly responsible for the increase in nitrite reductase activity. However, 7% of nitrate reductase activity of the control was detected in cells grown in the presence of tungstate; therefore, although the rate of nitrite formation must be greatly lowered, the possibility that nitrite reductase activity increases in response to nitrite, and not nitrate, cannot be excluded.

Addition of casein hydrolysate to medium containing nitrate inhibits the development of both nitrate reductase and nitrite reductase activities. The lower level of nitrite reductase activity in the presence of amino acids is apparently caused neither by an increase in the rate of decay nor by inhibition.

The activities of both enzymes change in a similar manner in conditions of "induction" by nitrate and "repression" by amino acids. However, nitrite reductase is the more stable of these two enzymes. Its activity decreases more slowly upon transfer of tobacco cells into nitrate-less medium.

I. ACTIVATION OF ASPARTATE TRANSCARBAMYLASE OF ESCHERICHIA COLI BY PURINE NUCLEOTIDES

II. REGULATION OF NITRITE REDUCTASE IN TOBACCO CELLS

By

Hanna Chroboczek Kelker

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

and MSU/AEC Plant Research Laboratory

1969

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation and gratitude to Professor R. S. Bandurski for his guidance and encouragement during the course of these studies. Appreciation is also expressed to Dr. P. Filner for help and guidance in the studies reported in Part II of this thesis. The author is also grateful to the members of the guidance committee, Dr. W. B. Drew, Dr. C. J. Pollard and Dr. J. A. Boezi. Helpful suggestions and discussions with Dr. J. E. Reimann, Dr. R. A. Hertel, Dr. J. E. Varner and Dr. N. E. Good are gratefully acknowledged.

* * * * *

TABLE OF CONTENTS

LIST OF TABLES

PART I

PART II

LIST OF FIGURES

PART I

PART I

ACTIVATION OF ASPARTATE TRANSCARBAMYLASE OF ESCHERICHIA COLI BY PURINE NUCLEOTIDES

INTRODUCTION

Aspartic acid transcarbamylase (ATCase) catalyzes the first reaction of pyrimidine biosynthesis:

$$\text{L-aspartate} + \text{carbamyl phosphate} \longrightarrow \text{N-carbamyl aspartate} + PO_4^{3-}$$

The functioning of this enzyme in Escherichia coli is under control by repression (Yates and Pardee, 1957) and by feedback inhibition by end products of the biosynthetic pathway, cytosine derivatives (Yates and Pardee, 1956; Gerhardt and Pardee, 1962).

Singh (1966) reported that if a cell free preparation of E. coli was incubated with ATP, GTP, PEP, magnesium ions and amino acids a twofold increase in the ATCase activity was observed. The cell free preparation was obtained from an E. coli strain selected for its ability to synthesize large quantities of ATCase in vivo and grown under conditions in which derepression of ATCase occurred. Protein synthesis in this mutant at the time of harvesting, therefore, would be directed towards selective synthesis of ATCase.

The requirements for the increase in ATCase activity resembled those for protein synthesis (Singh, 1966). This

system also was able to support some incorporation of (^{14}C)-amino acids into trichloroacetic acid (TCA) precipitable material. However, some of the observations indicated that the increase in ATCase activity might not result from synthesis of new enzyme molecules. These were:

a. the increase in enzyme activity was not absolutely dependent on amino acids,

b. incorporation of (^{14}C)amino acids into TCA precipitable material was small,

c. puromycin had no effect on the increase in ATCase activity while it caused a 90% inhibition of (^{14}C)leucine incorporation.

The purpose of this study was to learn what process is responsible for the increase in enzyme activity. The possibility that de novo synthesis of ATCase occurred was investigated by means of the density labeling technique (Hu et al., 1962). Although Singh (1966) had discounted the possibility that ATP, a known activator of ATCase (Gerhardt and Pardee, 1962), might be acting as an activator in this system, further studies were carried out to determine whether ATP or other components of the reaction mixture could be activating the enzyme. This was done by studying the effect of components of the incubation mixture on the kinetics of ATCase.

LITERATURE REVIEW

The functioning of biosynthetic pathways can be regulated by their respective end products. If end products of the biosynthetic pathway accumulate the flow of metabolites through this pathway can be stopped by cessation of synthesis of the enzymes involved in the formation of the end products, i.e., through repression. The functioning of the biosynthetic pathway can be stopped faster if the first enzyme or even enzymes of the entire pathway are inhibited by the end products, i.e., by feedback inhibition (Atkinson, 1965; Stadtman, 1966). Enzymes of a given pathway can be also a subject to activation by the end products of the related pathways, and thus operation of a given pathway can be adjusted to the whole metabolic network (Stadtman, 1966).

Aspartic acid transcarbamylase (ATCase) is the first enzyme of pyrimidine biosynthesis and therefore it is at a strategic point for the regulation of the flux of carbamyl phosphate and aspartic acid towards synthesis of pyrimidine nucleotides. The regularity role of ATCase in the control of pyrimidine biosynthesis in different organisms is discussed below with special consideration of E. coli ATCase and its properties.

Role of ATCase in Regulation of Pyrimidine Biosynthesis in E. coli

The first evidence for regulation of pyrimidine biosynthesis by feedback inhibition was reported for E. coli by Yates and Pardee (1956). They reported that E. coli ATCase is inhibited in vitro by the end product, cytidine 5'-phosphate (CMP). The effect of pyrimidine nucleotides on E. coli ATCase purified to crystallinity was reexamined by Gerhardt and Pardee (1962). The inhibition of this enzyme is specific for cytidine nucleotides and cytidine 5'-triphosphate (CTP) is the best inhibitor of the enzyme. Uridine derivatives have no effect on enzyme activity; deoxythymidine 5'-triphosphate is only slightly inhibitory. The role of cytidine derivatives as inhibitors of ATCase in vivo was demonstrated by Gerhardt and Pardee (1964). Addition of uracil or of CMP into the medium immediately prevented functioning of ATCase in E. coli. If 6-diazo-5-oxy-norleucine, an inhibitor of conversion of uridine triphosphate (UTP) to CTP was added to the medium along with uracil, no inhibition of ATCase was observed.

Purine nucleotides also affect ATCase activity (Gerhardt and Pardee, 1962). ATP activates the enzyme while GTP inhibits, although the inhibition is less than that observed for CTP. Gerhardt and Pardee (1962) postulated that the activation of ATCase by ATP might have biological importance in the equilibration of purine and pyrimidine pools of E. coli.

Yates and Pardee (1957) reported that the enzymes of pyrimidine biosynthesis in E. coli are also regulated by repression. The first three enzymes of pyrimidine biosynthesis, ATCase, dihydroorotase and dihydroorotic dehydrogenase became derepressed when E. coli was grown in medium lacking uracil. Derepression was especially pronounced in the case of ATCase where activity increased 500 fold due to de novo synthesis of this enzyme.

Properties of E. coli ATCase

ATCase of E. coli is one of the most extensively studied regulatory enzymes. It has been demonstrated by Gerhardt and Pardee (1962) that the dependence of reaction rate on aspartate concentration is sigmoid and, as recently demonstrated by Bethell et al. (1968), the saturation curve for carbamyl phosphate is also sigmoid. These characteristics indicate the existence of multiple binding sites and suggest that the binding of one molecule of substrate facilitates binding of another (Gerhardt, 1963).

The specific inhibitor, CTP, causes an increase in the apparent K_m for aspartate while the activator, ATP, causes a decrease in the apparent K_m. In both cases the V_{max} remained unchanged. These effects of nucleotides were measured at saturating concentrations of carbamyl phosphate. CTP and ATP had the same qualitative effect on the interaction between carbamyl phosphate binding sites when aspartate was saturating (Bethell et al., 1968).

The degree of interaction between aspartate binding sites can be altered. This has been demonstrated by the isolation of several mutants of E. coli in which dependence of reaction rate on aspartate concentration varies from hyperbolic to highly sigmoid. The saturation curve of ATCase for aspartate can also be altered by pH (Gerhardt and Pardee, 1964; Weitzman and Wilson, 1966). At pH 8.5, the optimum pH of the enzyme, the saturation curve of the enzyme is sigmoid. Lowering the pH to 6.0 leads to a change to a hyperbolic saturation curve (Gerhardt, 1963). At pH 10.2 ATCase exhibits another pH optimum and the saturation curve for aspartate becomes hyperbolic. The effect of high pH on decreasing the interaction between catalytic sites is fully reversible (Weitzman and Wilson, 1966).

The sigmoid kinetics of ATCase with respect to aspartate are abolished by short heating at 60°C, treatment with urea and mercurials (Gerhardt and Pardee, 1962). According to Gerhardt and Pardee (1962) the loss of sigmoid kinetics is accompanied by a loss of sensitivity of ATCase to CTP. To explain these results Gerhardt and Pardee (1964) postulated a model for the structure of ATCase in which the enzyme was constructed of subunits and interactions between aspartate binding sites located on different subunits accounted for sigmoid kinetics. Those interactions are strengthened by CTP and decreased by ATP. The native structure of ATCase is necessary for both interactions between substrate binding sites and CTP inhibition. Treatments with

heat, urea and mercurials dissociate ATCase into subunits and result in a change to hyperbolic kinetics and also loss of inhibition by CTP. However the results of Weitzman and Wilson (1966) indicate that interaction between subunits of ATCase is more complex since in their hands heat and urea treatment resulted in a loss of interaction between catalytic sites but not in a loss of inhibition by CTP.

The structure of ATCase is complex. Gerhardt and Schachman (1965) were able to demonstrate that in the presence of mercurials the native enzyme dissociates into two types of subunits: one with catalytic activity and another devoid of catalytic activity but able to bind the analog of the regulatory nucleotide, bromocytidine 5'-triphosphate (BrCTP). The subunits were named catalytic and regulatory, respectively. The molecular weight of the native enzyme and of the subunits were determined to be: $3.1 \cdot 10^5$ for the native enzyme, $9.4 \cdot 10^4$ for the catalytic subunit, and $3.4 \cdot 10^4$ for the regulatory subunit. Treatment of ATCase with 8 M urea (Weber, 1968) causes dissociation of the enzyme into two types of subunits. It appears that the catalytic subunit of Gerhardt and Schachman (1965) is further split by this treatment into two subunits since the molecular weight of the catalytic subunit reported by Weber (1968) is $4.7 \cdot 10^4$. The molecular weight of the regulatory subunit is $2.5 \cdot 10^4$ and this is in agreement with the value reported by Gerhardt and Schachman (1965). Only two amino-terminal amino acids are reported for ATCase-alanine for

the catalytic subunit and threonine for the regulatory subunit (Herve and Stark, 1967; Weber, 1968). The structure proposed for ATCase is therefore one consisting of four regulatory and four catalytic subunits with each subunit representing a single polypeptide.

The native ATCase has four aspartate binding sites (Changeux et al., 1968). Studies of binding of the substrate analog, succinate, confirmed conclusions from kinetic studies (Gerhardt and Pardee, 1962) that aspartate binding sites interact. Cooperativity of binding of succinate was exhibited only by the native enzyme and not by the subunits. According to Changeux et al. (1968) native ATCase binds four molecules of BrCTP. This is in contrast to the previous report by Gerhardt and Schachman (1965) of eight binding sites for CTP. Changeux et al. (1968) point out the possibility that there exist four additional sites with lower affinity for CTP. According to Changeux et al. (1968) ATP binds at the same site as CTP since in the presence of ATP the binding of CTP is diminished.

One of the models describing the action of regulatory enzymes has been proposed by Monod et al. (1963). This model postulates that the activity of these enzymes can be controlled by indirect interactions between distinct sites (allosteric sites) maintained by the protein molecule through conformational transitions. Monod et al. (1963) distinguished two types of allosteric interactions: interactions between identical ligands, termed homotropic, and

interactions between different ligands, termed heterotropic. The homotropic interactions between substrate binding sites are cooperative and they result in a sigmoidal plot of velocity versus substrate concentration. In heterotropic interactions substances unrelated structurally to the substrate, called allosteric effectors, cause a change in the apparent affinity of the substrate for the enzyme or in the turnover number (or both). Heterotropic interactions may result either in activation or inhibition of the enzyme and are either cooperative or antagonistic, respectively. The regulatory enzymes were postulated to be composed of identical subunits, designated as protomers. Each protomer binds one specific ligand. Allosteric enzymes can exist in a number of conformations differing in their affinity for the substrate. The most stable of these are symmetrical conformations. Cooperativity between ligand binding arises from coordinated transitions of all protomers to the conformational state for which the ligand has greatest affinity. The simplest model (Monod et al., 1965) postulates a pre-existing equilibrium of two conformational states of the allosteric enzymes differing in their affinity for the substrate and for the allosteric effectors.

The kinetic and structural properties of ATCase described above are compatible with this model. Some evidence is being accumulated that the homotropic and heterotropic interactions of ATCase are indeed maintained through conformational changes of ATCase.

Dratz and Calvin (1966) estimated from measurements of optical rotatory dispersion of ATCase that a helix-coil transition occurs upon addition of succinate and carbamyl phosphate while CTP causes a transition of the opposite sign. Further evidence that the binding of substrate and regulatory nucleotide results in a change of conformation of ATCase was reported by Gerhardt and Schachman (1968). The sedimentation coefficient of the native enzyme in the presence of succinate and carbamyl phosphate was decreased by 3.5% while in the presence of CTP the sedimentation coefficient was only slightly reduced. The change in conformation of ATCase was also evidenced by a sixfold increase in reactivity of sulfhydryl groups in the presence of carbamyl phosphate and succinate. The enhancement of sulfhydryl group reactivity was opposed by CTP. The results of the analysis of the data of Changeux et al. (1968) and of Gerhardt and Schachman (1968) by Changeux and Rubin (1968) indicate that one of the two conformational states of ATCase has little affinity for the substrate and high affinity for the regulatory nucleotide, CTP. This conformational state predominates in the absence of succinate. Another of the conformational states has a high affinity for succinate. These facts point out clearly that both cooperative and antagonistic effects are maintained through changes in protein conformation and that the change on addition of inhibitor is opposite and approximately equal to the effect of the substrate.

Regulatory Properties of ATCase from Other Organisms

Examination of properties of ATCase in a number of bacteria by Neumann and Jones (1964) revealed that this enzyme also has a regulatory function in bacteria other than *E. coli*. ATCase from *Aerobacter aerogenes* and *Serratia marcescens* is inhibited *in vitro* by both uridine monophosphate (UMP) and CMP. In *S. marcescens*, which is less closely related to *E. coli*, UMP is a better inhibitor of ATCase. Heating of ATCase from *S. marcescens* at 60°C for 5 minutes abolished the ability to be inhibited by nucleotides while the enzymatic activity remained unchanged. This suggests that the catalytic and regulatory sites are different in this system. ATCase from *Bacillus subtilis* is not inhibited by pyrimidines or purines. The functioning of ATCase in this organism is regulated by repression. ATCase from *Pseudomonas fluorescens* has a hyperbolic dependence of the reaction rate with respect to both aspartate and carbamyl phosphate. This enzyme is inhibited by UTP and to a lesser degree by ATP and GTP. In the presence of the inhibitor UTP the saturation curve of the enzyme with carbamyl phosphate becomes sigmoidal. Similarly, as in the case with *E. coli* ATCase, an increase in carbamyl phosphate causes a decrease in inhibition by UTP. It was not possible to achieve in this system a selective destruction of the inhibitory site by treatment with mercurials and heat.

ATCase is also an important enzyme in regulation of pyrimidine biosynthesis in eucaryotic organisms. In the yeast, Saccharomyces cereviseae, ATCase is repressed by uracil or its derivative (Kaplan et al., 1967). This enzyme is also subject to feedback inhibition. Of the nucleotides tested UTP was the strongest inhibitor while CTP, GTP and ATP had no effect on ATCase activity. However ATP antagonized inhibition of this enzyme by UTP. The in vivo counteraction by adenine of inhibition of ATCase by uracil was demonstrated previously by Burns (1966). Homotropic interactions are absent in this enzyme, since ATCase from yeast exhibits Michaelis-Menten kinetics with respect to both substrates. The existence of a regulatory site distinct from a catalytic site was inferred from the fact that inhibition by UTP is noncompetitive with respect to both substrates and from selective destruction of inhibition by UTP by brief heating at 60°C.

ATCase from Neurospora crassa is also subject to feedback inhibition (Donachie, 1964). It is inhibited in vitro by uridine analogs (uridine, uridylic acid, UTP), thymidine and orotidylic acid but not by cytidine analogs. In contrast to E. coli ATCase the enzyme from Neurospora is inhibited by pyrimidines only at high aspartate concentrations. The concentration of carbamyl phosphate has no effect on inhibition by nucleotides. Activity of ATCase is also regulated by repression although the change in ATCase

activity upon withdrawal of exogenous pyrimidines is only threefold as compared with a 500-fold increase in E. coli.

Tentative evidence for feedback inhibition of ATCase from Chlorella was reported by Cole and Schmidt (1964). They found that the extracts of synchronous cultures of Chlorella contained heat stable inhibitors of the enzyme after one cell division.

No evidence for repression of ATCase in higher plants was reported. However the activity of this enzyme is changed with the physiological age of plant tissues (Stein and Cohen, 1965). Cotyledons of growing soybean seedlings contained lower activity than the shoot and the root. Activity of this enzyme is also highest in the growing tip of bean plant which contains the highest number of dividing cells. Feedback inhibition of ATCase from lettuce seedlings was reported by Neumann and Jones (1962). The lettuce enzyme is strongly inhibited by uridine derivatives with UMP being the best inhibitor. Cytidine derivatives were inhibitory only at high concentration and purine nucleotides had no effect on enzyme activity. The catalytic site is probably different from the regulatory site since UMP is a noncompetitive inhibitor with respect to both substrates. This is also supported by the fact, that ribose-5-phosphate relieves the inhibition by UMP. Ribose-5-phosphate does not affect catalytic activity and it likely acts by competing with UMP for the regulatory site.

No definite evidence is available with regard to regulation of ATCase in mammalian systems. As in plant systems, ATCase activity is highest in embryonic tissues (Kim and Cohen, 1965). ATCase activity was higher in the livers of fetal rats and guinea pigs than in the livers of adult animals. ATCase activity was also elevated in regenerating livers of rats. Evidence for the operation of feedback inhibition of ATCase in animal tissues is not available. Pyrimidine nucleotides had no effect on 140-fold purified enzyme from rat liver (Bresnick and Mossé, 1966). This enzyme had Michaelis-Menten kinetics with respect to both substrates. Curci and Donachie (1964) failed to detect an inhibitory effect of pyrimidine nucleotides on ATCase activity from rabbit reticulocytes.

Comparison of the properties of ATCase from different organisms (on the basis of available data) in Table 1 reveals that the E. coli enzyme is the only one in which homotropic interactions are detected with respect to both substrates. Cytidine derivatives are the best inhibitors of the E. coli enzyme and of two other representatives of the Enterobacteriaceae while in the other bacteria, fungi and higher plants uridine derivatives are the best inhibitors of ATCase. It appears that the activity of mammalian ATCase is not regulated by end products.

Table 1. Feedback inhibitors and some properties of ATCase from different sources

Source of the enzyme	Inhibitors	Activators	Allosteric substrates	Reference
E. coli	CTP, CDP, CMP deoxyCTP, deoxyCMP GTP	ATP deoxyATP	aspartate carbamyl phosphate	Gerhardt and Pardee (1962) Bethell *et al.* (1968)
S. marcescens	CMP, UMP	...	...	Neumann and Jones (1964)
A. aerogenes	CMP, UMP	...	...	Neumann and Jones (1964)
P. fluorescens	UTP, CTP ATP, GTP	...	carbamyl phosphate	Neumann and Jones (1964)
S. cereviseae	UTP	ATP antagonizes inhibition by UTP	...	Kaplan *et al.* (1967)
N. crassa	UTP, thymidine, orotidylic acid	...	...	Donachie (1964)
L. sativa	UMP, UTP, CMP	...	...	Neumann and Jones (1964)
Rat liver	...	...	...	Bresnick (1966)
Rabbit erythrocyte	...	...	...	Curci and Donachie (1964)

The regulatory site appears to be distinct from the catalytic site in all the systems discussed, but only in E. coli and P. fluorescens does the feedback inhibitor cause an increase in homotropic interactions. It is tempting to speculate that ATCase of eucaryotic organisms (discussed here are the fungi, lettuce and mammalian enzymes) resembles the subunit form of E. coli ATCase but is endowed with a regulatory site in the case of fungi and plant ATCase. However, a valid comparison of the properties of ATCase from various organisms will be possible only when the properties of this enzyme in many organisms have been better studied.

MATERIALS AND METHODS

I. Bacterial Strain

A strain of *E. coli* K_{12} 411-189 obtained from Dr. J. C. Gerhardt was used throughout this work. This mutant is diploid with respect to that portion of the genome (approximately one-third) containing the gene or genes coding for ATCase production. It has an absolute requirement for histidine and a partial requirement for leucine. Uracil is required for fast growth since the mutant has a defect in orotidylate decarboxylase and grows slowly in the absence of exogenous pyrimidines.

II. Growth Media

Growth medium MS-56 was used. It was prepared by adding the following compounds in grams per liter of distilled water: Na_2HPO_4, 4.4; KH_2PO_4, 2.6; $MgSO_4 \cdot 7\ H_2O$, 0.02; $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.014; $FeSO_4 \cdot 7\ H_2O$, 0.05; $(NH_4)_2\ SO_4 \cdot 4\ H_2O$, 4 and uracil, 0.008. This solution was autoclaved at 122°C and 15 psi for 20 minutes if the volume was 110 ml while 11 liter volumes were autoclaved for an hour. Sterile solutions of glucose and amino acids were added immediately prior to use

to make the final concentration in grams per liter: D-glucose, 2; L-leucine, 0.05; L-histidine, 0.07.

Solid medium was prepared by the addition of 2% agar to MS-56.

III. Maintenance, Growth of the Mutant and Preparation of the 30,000 x g Supernatant Solution of E. coli

The cultures of E. coli were maintained on MS-56 agar supplemented with 60 mg of uracil per liter. They were transferred every three months.

The inoculum for large volume cultures was prepared as follows: 110 ml of MS-56 media was inoculated from agar. The cells were grown at 37°C and aerated by swirling on a New Brunswick shaker. When Klett readings of the culture reached 160 (which corresponds to $2 \cdot 10^9$ cells per ml) 1.1 ml was transferred to 110 ml of MS-56 media. This culture was grown for approximately 12 hours, until Klett readings reached 160-190.

Large amounts of bacterial cells were prepared by growing E. coli at 37°C in 11 liters of MS-56 containing 0.6 ml of 1.66% Antifoam A (a silicone anti-foam agent obtained from Dow Corning Corporation). Three 110 ml flasks of the above described cultures were used as an inoculum. The medium was aerated under pressure with air sterilized by serial filtration through a charcoal filter and glass wool filter connected by sterile tubing with two immersion filters reaching to the bottom of the carboy. The cells were

grown for approximately four hours until the Klett readings reached 70. Growth of the culture was stopped by immersing the carboy in ice water for one hour. The cells were harvested by continuous flow centrifugation with the flow rate maintained at 600 ml per hour.

The harvested cells were suspended in 40 ml of 0.01 M phosphate buffer at pH 7.4 containing 0.06 M KCl and 0.14 M magnesium acetate. The suspension of cells was centrifuged for 20 minutes at 30,000 x g. The washed cells were suspended in 14 ml of the above buffer and passed through a precooled French Press Cell at 16,000 psi. The broken cells were centrifuged for 30 minutes at 30,000 x g. The supernatant solution was dialyzed for 4 hours against 2 liters of the above described buffer with one change of buffer. The dialyzed solution was used as a source of ATCase and was able to support the incorporation of amino acids into TCA precipitable material (Singh, 1966).

IV. Assay of ATCase Activity

The activity of ATCase was assayed by formation of carbamyl aspartate according to the method of Koritz and Cohen (1954) as modified by Gerhardt and Pardee (1962) for ATCase assay. The assay conditions unless otherwise specified were as follows: 3.6 mM carbamyl aspartate, 5 mM aspartate, 40 mM potassium phosphate buffer (pH 7.0) and the ATCase preparations were incubated in a volume of 0.5 ml for 30 minutes at 30°C. The reaction was stopped by addition of

2.5 ml of a cooled mixture of reagents a, b, and c mixed in a ratio of 3:1:1 before addition. The reagents were:

a. 66% H_2SO_4,

b. 22.5 mg of diacetyl monoxime dissolved per ml of water,

c. 114 mg of diphenyl-p-sulfonate dissolved in a 100 ml of 0.1 M HCl containing 0.4 g of detergent Atlas BRIJ.

The contents of the tubes were mixed and heated at 60°C for 30 minutes. Subsequently they were cooled in ice water and 0.5 ml of $K_2S_2O_8$ solution (2.5 mg/ml of water) was added. Color was developed during incubation at 30°C for 20 minutes and after cooling to 4°C the absorbancy was determined at 560 mμ against a reagent blank. The activity of ATCase was expressed as mμmoles of carbamyl aspartate synthesized per minute.

V. Assay of α-amylase Activity

The activity of α-amylase (obtained from Worthington) was assayed according to method of Shuster and Gifford (1962) as modified by Chrispeels and Varner (1967). A solution of potato starch was prepared before each assay. Native starch (150 mg) was boiled in 100 ml of a solution containing 600 mg of KH_2PO_4 and 200 μmoles of $CaCl_2$. After cooling the starch solution was centrifuged at 20,000 x g for 10 minutes. The clear supernatant was decanted and used for the assay. A

stock of iodine solution was prepared by dissolving 6 grams of KI and 600 mg of iodine in 100 ml of water. Before use one ml of this solution was diluted to 100 ml with 0.05 ml HCl.

An aliquot of the enzyme was diluted with water to make the reaction volume one ml. The reaction was started by adding one ml of starch solution and incubation was from 1 to 10 minutes at 30°C. The reaction was stopped by the addition of one ml of iodine reagent. Subsequently 5 ml of water were added to each tube and the absorbancy was read at 620 mμ. The decrease in absorbancy at 620 mμ as compared with the non enzymatic control was taken as a measure of α-amylase activity.

VI. Protein Determination

Protein content was determined according to Lowry et al. (1951). Egg albumin was used as a standard.

VII. Incubation of the E. coli Homogenate Leading to the Increase in ATCase Activity

Incubation of the E. coli homogenate which leads to an increase in ATCase activity was performed as described by Singh (1966). The "complete" system contained the following compounds in μmoles: potassium phosphate buffer (pH 7.4), 50; magnesium acetate, 60; ATP, 10; GTP, 10; PEP, 5; 50 μgrams of each of 20 amino acids, an aliquot of 30,000 x g supernatant solution of E. coli and enough distilled water

to make a volume of one ml. As a control an equal aliquot of 30,000 x g supernatant solution was incubated with enough of 0.01 M phosphate buffer (pH 7.4), containing 0.06 M KCl (designated standard buffer) to make a volume of one ml. Incubation was for 10 minutes at 37°C and it was terminated by placing the tubes in an ice bath. An aliquot of the control and the "complete" system was dialyzed at 4°C for 3 hours against 125 ml of standard buffer with two changes of buffer. Subsequently the dialyzed solution was diluted with the standard buffer and assayed for ATCase activity.

VII. Equilibrium Density Centrifugation of ATCase

1. Separation of (^{1}H)ATCase and (^{2}H)ATCase by equilibrium density centrifugation

   a. Preparation of 30,000 x g supernatant from cells grown on (^{2}H)water containing medium

(^{2}H)water was purified after previous use by boiling with charcoal, two distillations with $KMnO_4$, one from acidic and another from basic medium. The density of the distillate was 1.10 g/cm^3 while the density of the commercial 99% (^{2}H)water was 1.105 g/cm^3. MS-56 medium was prepared with the purified (^{2}H)water, the salts, glucose and amino acids were added as aqueous solutions. The total amount of water added was 8% of 1100 ml total volume. The autoclaved medium brought to 37°C was inoculated with E. coli grown in 33 ml of MS-56 containing (^{2}H)water. Growth of the cells,

harvesting, breakage and preparation of 30,000 x g supernatant were carried out as described in section III of Materials and Methods. The protein content of the dialyzed preparation was 4.92 mg/ml.

b. Preparation of the tubes for centrifugation

A stock solution of cesium formate of density 1.72 g/cm^3 was prepared by dissolving 9.6 grams of cesium formate in 6.4 ml of standard buffer. The volume of the stock solution needed for the gradient was calculated using the formula of Vinograd (1963):

$$V_{l,w} = (\rho_c - \rho_o)/(\rho_c - 0.997)$$

where $V_{l,w}$ is the volume of water used to dilute a stock solution of CsCl of density ρ_c to obtain one ml of a solution of the desired density ρ_o (ρ_o is here equal to 1.295 g/cm^3). The calculated volumes of stock solution and diluting buffer were multiplied by 4.5 to obtain the appropriate volumes adding to 4.5 ml of a final volume. The tubes were prepared for centrifugation as described below (see Table 2).

The contents of the tube were mixed with a capillary and 0.3 ml of paraffin oil was layered on top. The tubes were placed in a precooled SW-39L rotor and centrifuged in Spinco Ultracentrifuge for 67 hours at 39,000 rpm. Subsequently the tubes were punctured with a needle and one drop fractions were collected at 4°C. The refractive index of every fifth fraction was determined with a Bausch and Lomb Abbe-type refractometer. ATCase and α-amylase were assayed

Table 2. Content of the tubes for equilibrium density centrifugation of (^{2}H)ATCase and (^{1}H)ATCase

Component	Volume (ml)	Protein (mg)
(^{2}H)ATCase	0.08	0.394
(^{1}H)ATCase	0.02	0.500
α-amylase	0.005	0.095
standard buffer	2.4	...
cesium formate (1.70 g/cm^3)	1.91	...

in the remaining alternate fractions. The corresponding densities were determined with the aid of a standard curve. The standard curve was prepared by measuring the refractive index of a series of cesium formate solutions of known density. The densities were determined by weighing the cesium formate solutions in a 100 λ pipette.

2. Test for de novo synthesis of ATCase

Density labeling of preformed ATCase was achieved by growing E. coli on (^{2}H)water as described in section I of Materials and Methods. The density of the (^{2}H)water used was 1.08 g/cm^3. The protein content of the 30,000 x g supernatant was 4.18 g/cm^3.

a. Incubation of 30,000 x g supernatant solution from deuterated E. coli

A 0.1 ml aliquot of the dialyzed supernatant of E. coli was incubated with the components of the "complete"

system containing (^{1}H)amino acids, ATP, GTP, PEP, and magnesium ions as described in section VII of Materials and Methods. As a control 0.1 ml of the same preparation was incubated with 0.9 ml of standard buffer. After 3 hours of dialysis against standard buffer an aliquot was taken for assay of ATCase activity. The remaining solution was diluted with 1.5 ml of standard buffer and the measured volume of the diluted enzyme was taken for equilibrium density centrifugation.

b. Equilibrium density centrifugation

The solution of ATCase with the α-amylase marker in cesium formate of density 1.295 g/cm^{3} was prepared as described in Table 3.

Table 3. Content of the tubes prepared for equilibrium density centrifugation of (^{2}H)ATCase incubated with buffer (control) or with the complete incubation mixture

Component	Control		Complete	
	Volume (ml)	Protein (mg)	Volume (ml)	Protein (mg)
Diluted (^{2}H)ATCase	1.89	0.28	2.02	0.316
Cesium formate (1.70 g/cm^{3})	1.90	...	1.91	...
standard buffer	0.69	...	0.59	...
α-amylase	0.01	0.19	0.01	0.19

The tubes were covered with 0.3 ml of paraffin oil and centrifuged as described above. One drop fractions were collected. The refractive index of every fifth fraction was determined and α-amylase and ATCase were assayed in alternate fractions of those remaining.

IX. Studies of the Effect of Components of Incubation Mixture on ATCase Activity

1. ATCase from 30,000 x g supernatant of *E. coli* pretreated with the incubation mixture

An aliquot of the 30,000 x g supernatant of an *E. coli* homogenate containing 8 mg of protein was preincubated either with the buffer (control) or with the complete incubation mixture (complete) as described in section VII of Materials and Methods. Aliquots of the dialyzed solution were diluted to a final protein concentration of 0.08 mg/ml. Some of the samples were heated for 10 minutes at 60°C in 0.01 M phosphate buffer (pH 7.0) and subsequently cooled on ice. ATCase activity was assayed as described in section IV Materials and Methods, but the aspartate concentration was as indicated in Figure 3a.

Inhibition of ATCase by CTP was measured utilizing 2 mM CTP and the assay of the enzyme activity was conducted as described in Materials and Methods, section IV.

2. Crystalline ATCase pretreated with the incubation mixture

An aliquot of crystalline ATCase (obtained from Dr. J. C. Gerhardt) containing 2 μg of protein was preincubated with the buffer or complete incubation mixture as described in section VII of Materials and Methods. After incubation the enzyme was dialyzed against 20 mM phosphate buffer (pH 7.0), 2 mM mercaptoethanol and 0.2 mM ethylenediaminetetraacetic acid (EDTA). Aliquots of the diluted dialyzed solution containing 0.06 μg of protein were assayed for ATCase as described in section IV of Materials and Methods, but the aspartate concentration was as indicated in Figure 4.

3. Effect of GTP on activity of crystalline ATCase assayed in presence of ATP and magnesium ions

Crystalline ATCase (0.5 μg of protein) was incubated with 3.6 mM carbamyl phosphate, 5 mM aspartate, 0.1 mM EDTA, 40 mM MOPS (2-(N-morpholino)propanesulfonic acid, Dr. N. E. Good, unpublished) at pH 7.0, 2 mM ATP and 2 mM magnesium acetate. GTP concentration was as indicated in Figure 5.

4. Effect of ATP, GTP and magnesium ions on the dependence of reaction rate on aspartate concentration

Crystalline ATCase (0.08 μg of protein) was incubated with 3.6 mM carbamyl phosphate, 40 mM MOPS (pH 7.0), ATP, GTP and magnesium acetate (each 2 mM). Aspartate concentration was as indicated in Figure 6a.

5. Effect of GTP, and GTP plus magnesium ions on the activity of crude ATCase in the presence and absence of ATP

The 30,000 x g supernatant solution of E. coli (0.054 mg of protein) was incubated with 14 mM carbamyl phosphate (purified according to Gerhardt and Pardee, 1962), 40 mM phosphate buffer (pH 7.0) and 5 mM aspartate. ATP, when used, was 8 mM and magnesium acetate was equimolar to the indicated GTP concentration (Figure 7).

RESULTS

Separation of (^{1}H)ATCase and (^{2}H)ATCase by Equilibrium Density Centrifugation

(^{1}H)ATCase (obtained from E. coli grown on H_2O) and (^{2}H)ATCase (from E. coli grown on $(^2H)_2O$) were well resolved by equilibrium density centrifugation in a cesium formate gradient. As is shown in Figure 1 two major peaks of ATCase activity were observed which, while overlapping, differed in their density by 3.2%. The small middle peak at about fraction 60 is believed to be spurious since it did not appear in subsequent experiments.

The recovery of ATCase activity from the gradient was satisfactory. Approximately 105% of the activity of (^{2}H)ATCase was recovered in the heavier peak and 88% of the activity of (^{1}H)ATCase was recovered in the lighter peak.

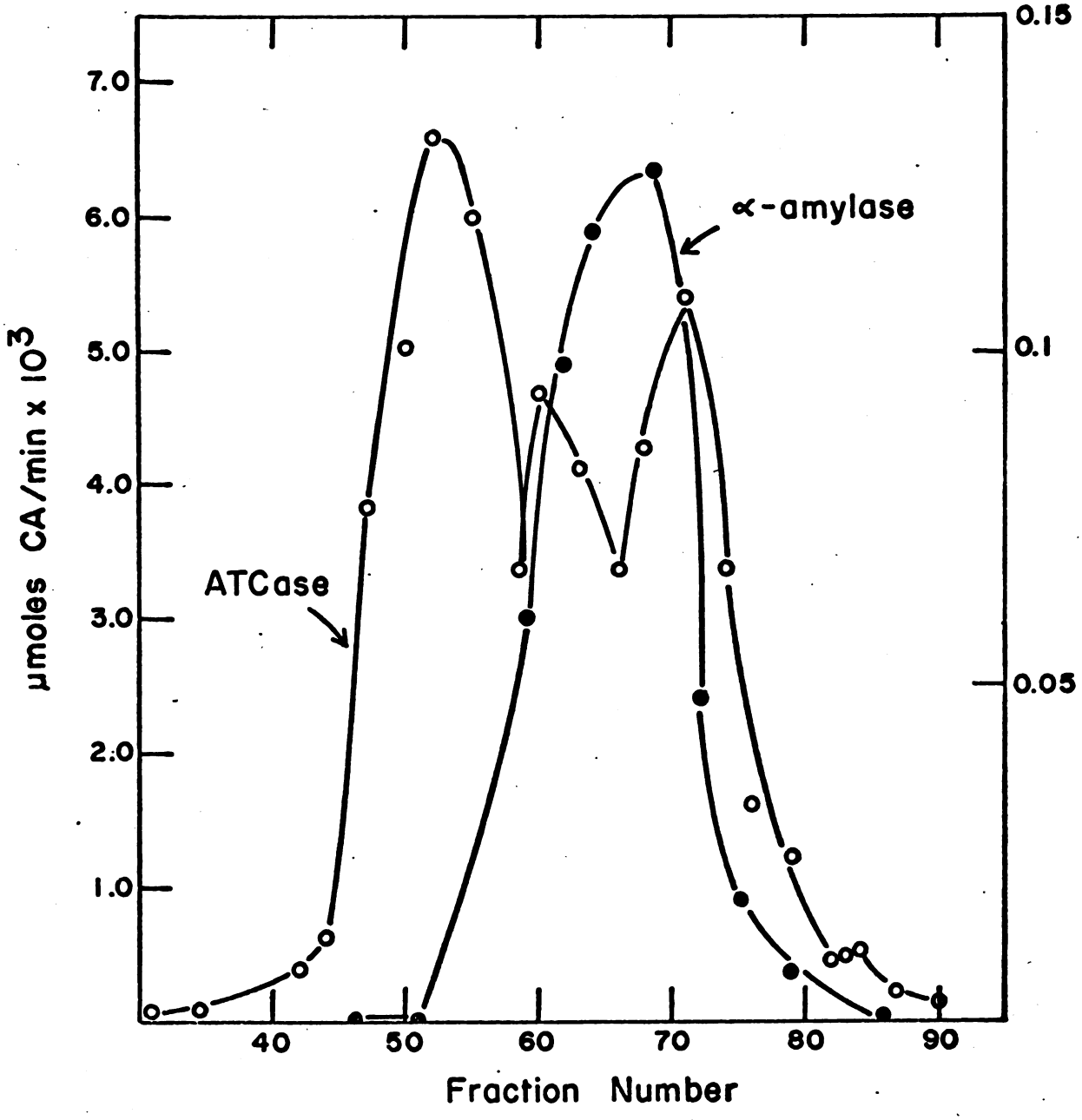
Test for de novo Synthesis of ATCase

Density labeling and equilibrium density centrifugation was introduced by Hu et al. (1962) as a test for de novo synthesis of β-galactosidase. This technique, as described by Filner and Varner (1967) was utilized in this study as means of detecting newly synthesized ATCase.

Figure 1. Distribution of ATCase activity from *E. coli* grown on (^{2}H)water and from *E. coli* grown on (^{1}H)water after centrifugation on cesium formate gradient.

The direction of decreasing density is from left to right.

Abbreviations used: CA = N-carbamyl aspartate, Δ A 620/min = decrease in absorbancy at 620 mμ taken as a measure of α-amylase activity.


0.15
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.1
0.05
μmoles CA/min x 10³
ΔA 620/min
ATCase
∝-amylase
40
50
60
70
80
90
Fraction Number

For this purpose 30,000 x g supernatant solution obtained from E. coli grown on (^{2}H)water and therefore containing (^{2}H)ATCase was incubated under conditions in which the increase in ATCase activity occurs and then dialyzed for 3 hours (as described in Materials and Methods, section VII). (^{1}H)amino acids were utilized as a component of the incubation mixture so that newly formed ATCase would be of different density. Subsequent separation of proteins differing in their density by equilibrium density centrifugation enables one to detect newly synthesized enzyme.

As demonstrated in Table 4 the activity of ATCase in a supernatant solution prepared from E. coli grown on (^{2}H)-water was increased by 100% upon incubation with the complete incubation mixture, containing (^{1}H)amino acids, ATP, GTP, magnesium ions and PEP as compared with the control. This is in agreement with the report of Singh (1966).

Table 4. Activity of (^{2}H)ATCase after preincubation; recovery of ATCase activity from the gradient

	μmoles of carbamyl aspartate/min x 10^3		
System	In 1 ml of incubation mixture	Put on the gradient	Recovered from the gradient
Control	55.7	30.9	36.4 (109%)
Complete	113.0	78.6	40.3 (51%)

Subsequent equilibrium density centrifugation of the preincubated (^{2}H)ATCase revealed that the distribution of ATCase activity on the gradient remained the same relative to the α-amylase marker regardless of whether the enzyme was preincubated with the buffer or with the complete incubation mixture (Figure 2a and b). Since there was no change in the density or in the symmetry of the peak of ATCase upon incubation with (^{1}H)amino acids neither appreciable finishing of preexisting polypeptide chains nor of de novo synthesis of ATCase occurred in this system.

The equilibrium position of (^{2}H)ATCase peak was at lower density, as compared with the α-amylase marker, than in Figure 1. This could be explained by the fact, that the density of (^{2}H)water used in this experiment was 1.08 g/cm^3, as compared with 1.10 g/cm^3 in the previous experiment.

Recovery of ATCase activity from the gradient was complete for the control (ATCase preincubated with buffer). However only half the activity was recovered after centifugation of ATCase preincubated with the complete incubation mixture. This experiment has been repeated with similar results.

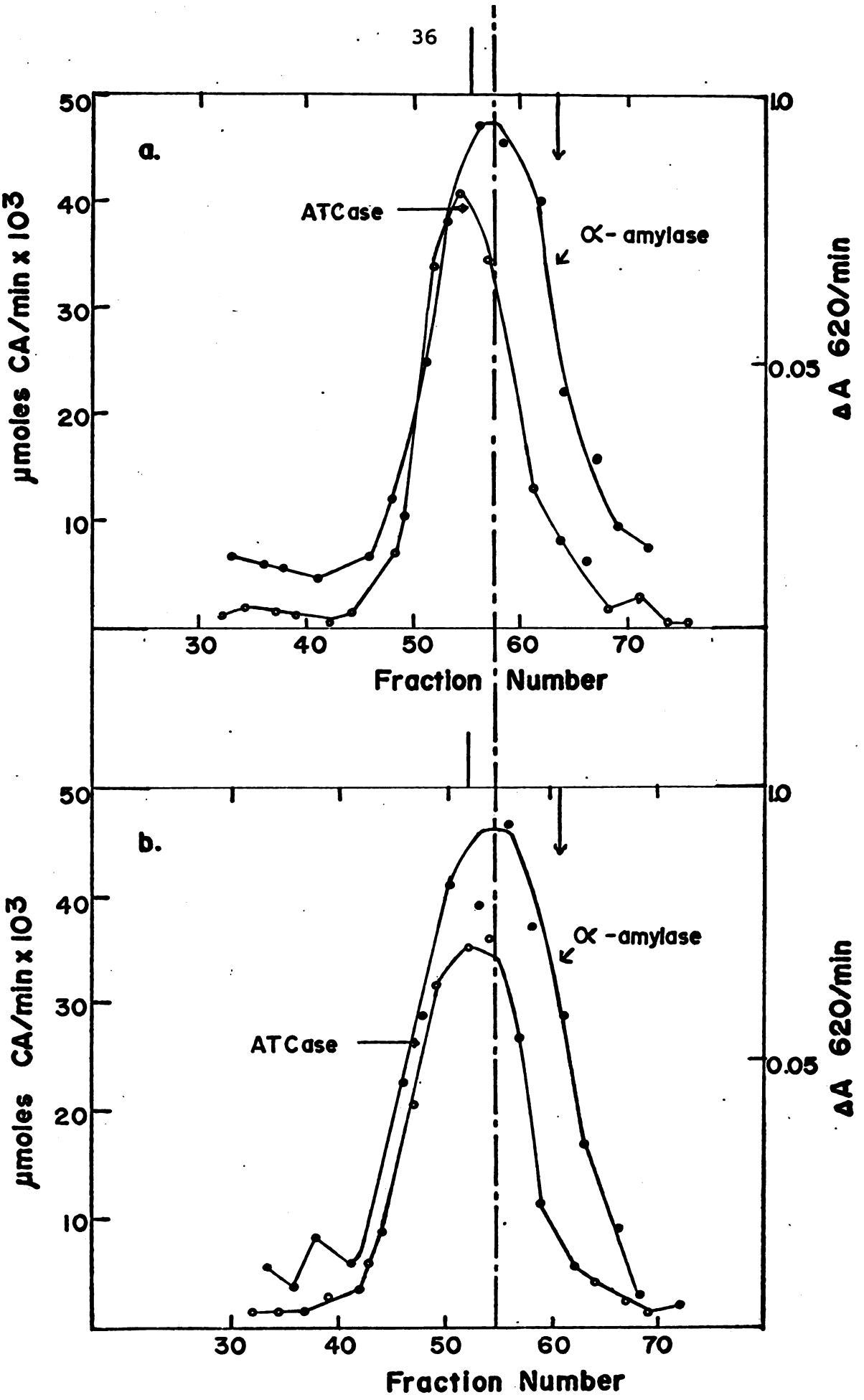
Previous results indicated that the recovery of both (^{1}H)ATCase and (^{2}H)ATCase from cesium formate gradient is good. Therefore, it can not be argued that the preferential loss of (^{1}H)ATCase was responsible for this poor recovery. It is possible that if ATCase became activated by the undialyzed components of the incubation mixture (dialysis

Figure 2. Distribution of ATCase activity from E. coli grown on (^{2}H)water after centrifugation on cesium formate gradient.

a. Enzyme preincubated with standard buffer.

b. Enzyme preincubated with the complete incubation mixture.

The arrows indicate the equilibrium position of (^{1}H)ATCase.


a.
ATCase
α- amylase
μmoles CA/min x 10³
ΔA 620/min
Fraction Number
b.
α -amylase
ATCase
μmoles CA/min x 10³
ΔA 620/min
Fraction Number

was only for three hours) the loss of ATCase activity would be due to the dissociation of activating compounds during prolonged centrifugation.

Effect of Pretreatment with the Complete Incubation Mixture on the Properties of ATCase from the 30,000 x g Supernatant Solution

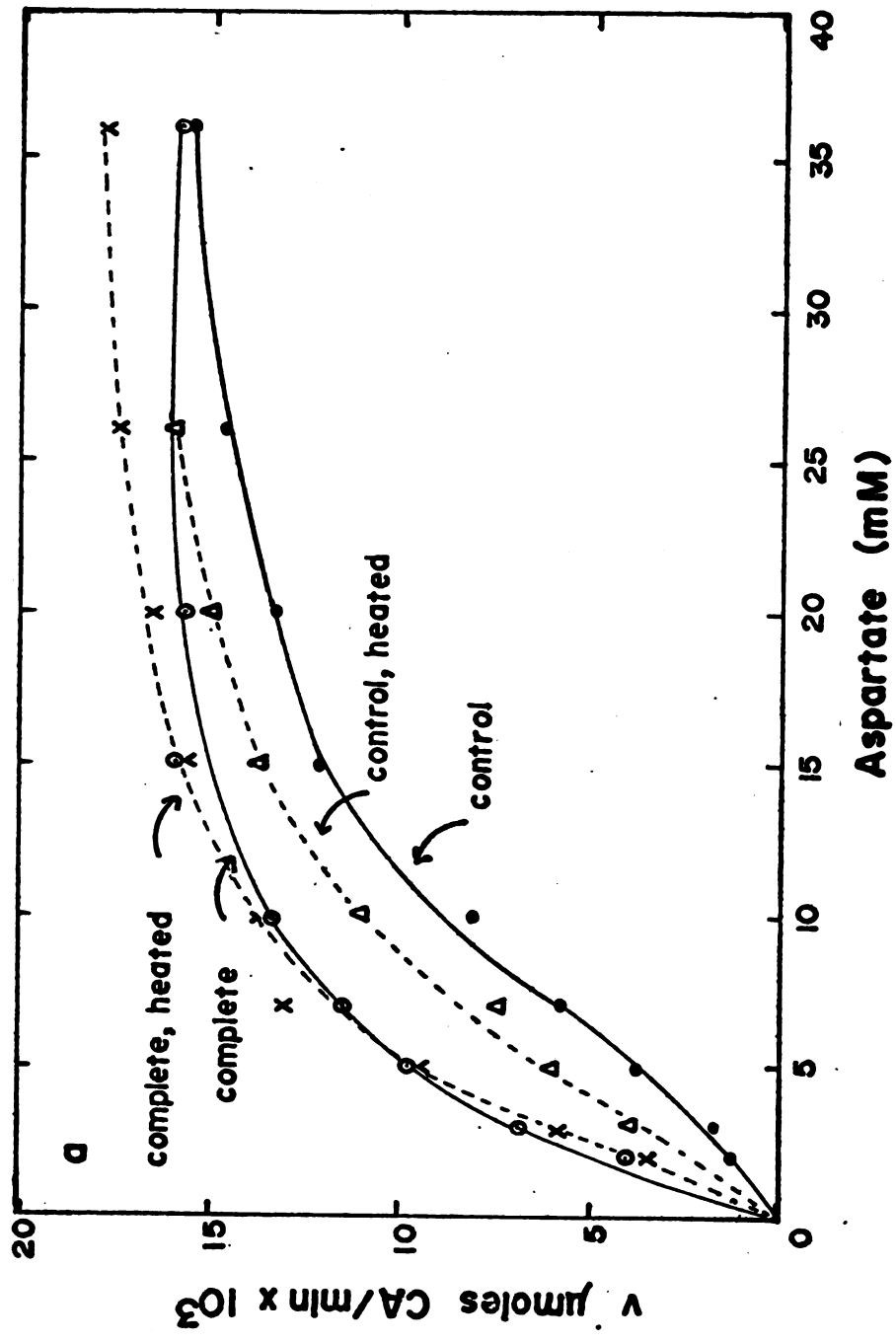
The kinetic properties of ATCase in this system were studied to examine the possibility of whether the enzyme became activated upon pretreatment with ATP, GTP, PEP, magnesium ions and amino acids (experimental details as in Materials and Methods, section IX, 1).

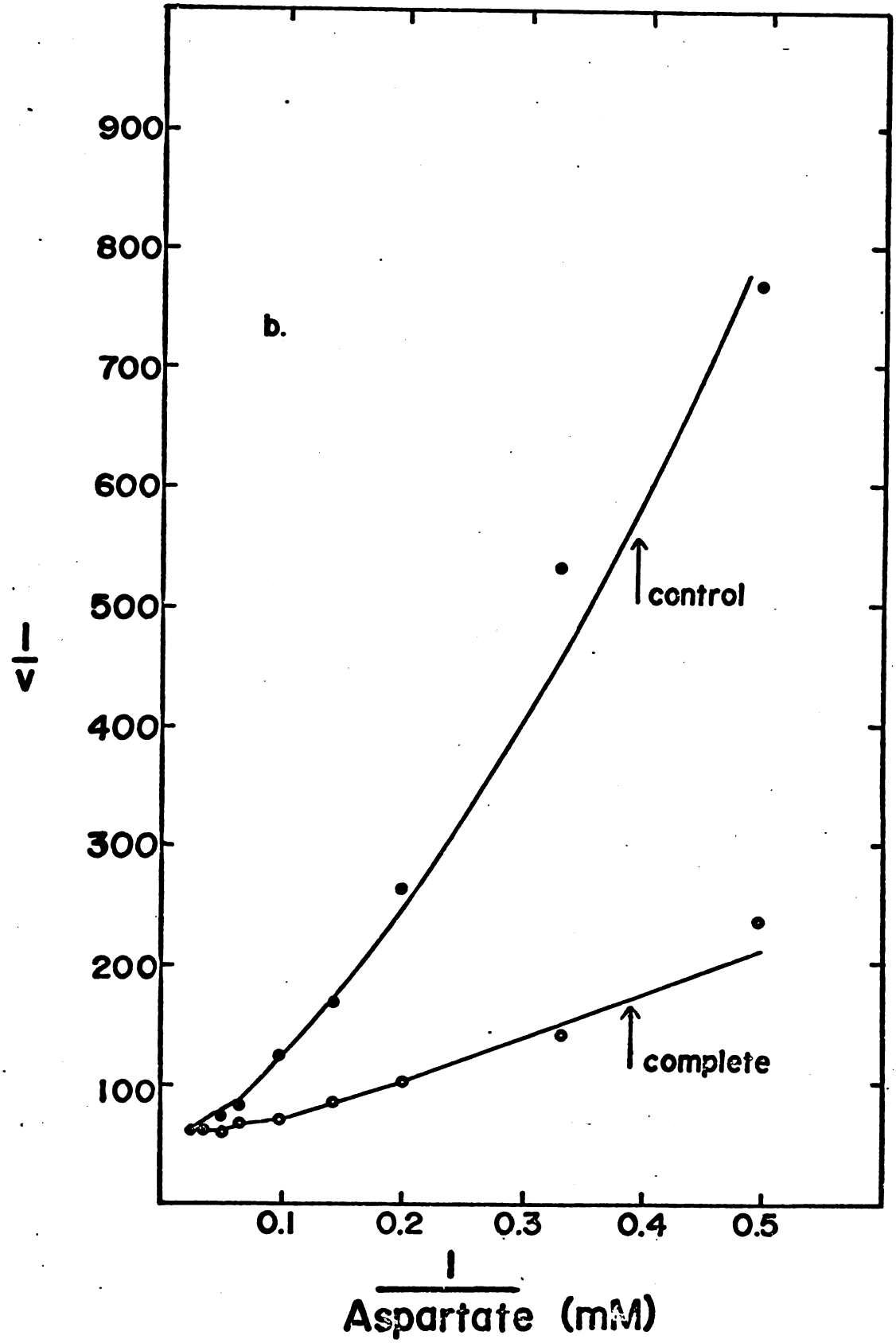
The dependence of reaction velocity on aspartate concentration was changed from a sigmoidal to a hyperbolic function as a result of preincubation (Figure 3a). The difference in reaction rate of the control and of ATCase pretreated with the complete incubation mixture was greatest at low aspartate concentrations. At saturating concentrations the difference in reaction rates almost disappeared. The increase in ATCase activity reported by Singh (1966) and in this study was measured at 5 mM aspartate, the concentration at which the difference in the reaction rate was a 100%. The Lineweaver-Burke plot of the control and of ATCase pretreated with the complete incubation mixture shows that preincubation leads to a change from an upward curving to a nearly linear plot (Figure 3b). These changes in the kinetics of ATCase indicate that the enzyme became activated as a result of preincubation.

Figure 3a. Dependence of the reaction rate of ATCase of 30,000 x g supernatant solution of *E. coli* on aspartate concentration.

ATCase was pretreated with buffer (control) or with complete incubation mixture (complete); complete and control were heated at 60°C for 10 min.

b. A double reciprocal plot of complete and control.


v μmoles CA/min x 10³
Aspartate (mM)
a
complete, heated
complete
control, heated
control


b.
900
800
700
600
500
400
300
200
100
1/V
control
complete
0.1
0.2
0.3
0.4
0.5
1/Aspartate (mM)

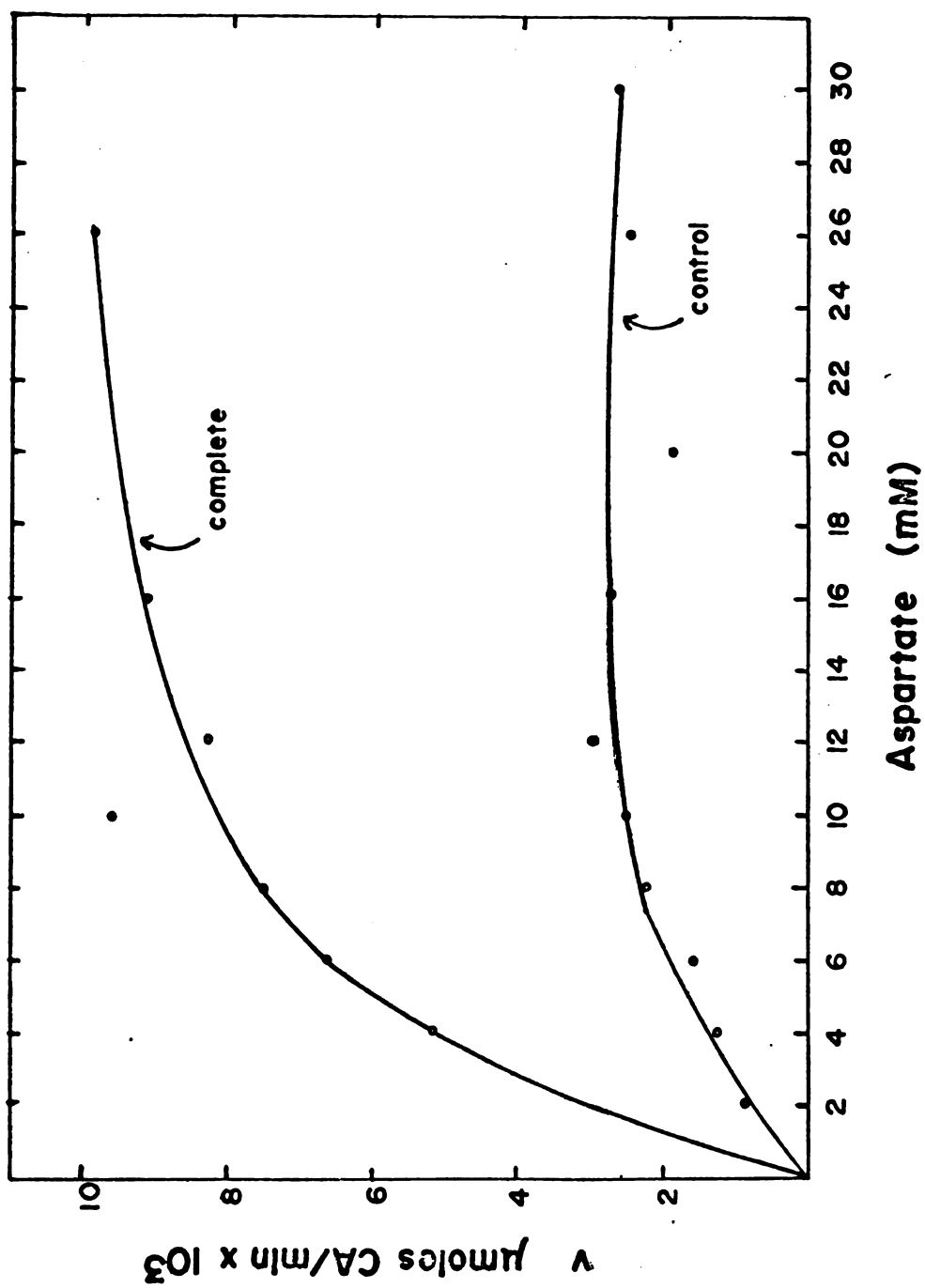
Heating at 60°C for 10 minutes, a treatment which causes a change from sigmoidal to hyperbolic kinetics of crystalline ATCase (Gerhardt and Pardee, 1962; Weitzman and Wilson, 1966), increased the reaction rate of the control sample of ATCase by 50% (measured at 5 mM aspartate) but heating had little effect on the reaction rate of ATCase pretreated with nucleotides and other components of the incubation mixture. Also, the inhibition of ATCase by 2 mM CTP measured at 5 mM aspartate was increased from 45 to 75% as a result of pretreatment.

Effect of Components of the Incubation Mixture on Crystalline ATCase

As is shown in Figure 4, treatment of crystalline E. coli ATCase with the complete incubation mixture followed by three hours of dialysis prior to the assay (as described in Materials and Methods, section IX, 2) caused approximately a threefold increase in the observed V_{max} as compared with the control. The same increase in the V_{max} was observed when crystalline ATCase was preincubated with only ATP, GTP and magnesium acetate. The V_{max} was increased from 3.5 to 10.2 μmoles of CA/min x 10^3 when ATCase was incubated with ATP, GTP and magnesium acetate. If GTP and magnesium acetate, or ATP and magnesium acetate were omitted, the observed V_{max} were 6.4 and 5.4 μmoles CA/min x 10^3, respectively.

Figure 4. Dependence of the reaction rate of crystalline ATCase of E. coli on aspartate concentration.

ATCase was pretreated with buffer (control), or with complete incubation mixture (complete).


v μmoles CA/min x 10³
2
4
6
8
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Aspartate (mM)
complete
control

The simultaneous requirement for ATP, GTP and magnesium ions was confirmed by the studies of the effect of these compounds on ATCase activity when they were added directly to the assay mixture. As is shown in Figure 5 the activity of ATCase measured at 5 mM aspartate (as described in Materials and Methods, section IX, 3) in the presence of 2 mM ATP and 2 mM magnesium acetate was stimulated by GTP. ATP, GTP and magnesium acetate (Materials and Methods, section IX, 4) caused a decrease in the K_m for aspartate but only a small change in the V_{max} (Figure 6a and b).

The dependence of the reaction rate of the crystalline enzyme on aspartate concentration was less sigmoidal than in the case of the crude enzyme preparation. Also the properties of the crystalline ATCase changed upon storage. After 8 days of storage at 4°C the increase in V_{max} after pretreatment with ATP, GTP and magnesium ions was not threefold but twofold. Aging of the enzyme limited the attempts to obtain a firm value for the apparent K_m for aspartate in the presence of ATP, GTP and magnesium ions.

ATCase was reported previously to be a stable enzyme. Gerhardt and Holoubek (1967) reported that the properties of ATCase did not change upon prolonged storage at 4°C. A change in the properties of ATCase was reported by Bethell *et al.* (1968) but this occurred only after 2 years of storage at -20°C.

Figure 5. Activation of crystalline ATCase by GTP in the presence of ATP and magnesium acetate (both 2 mM).

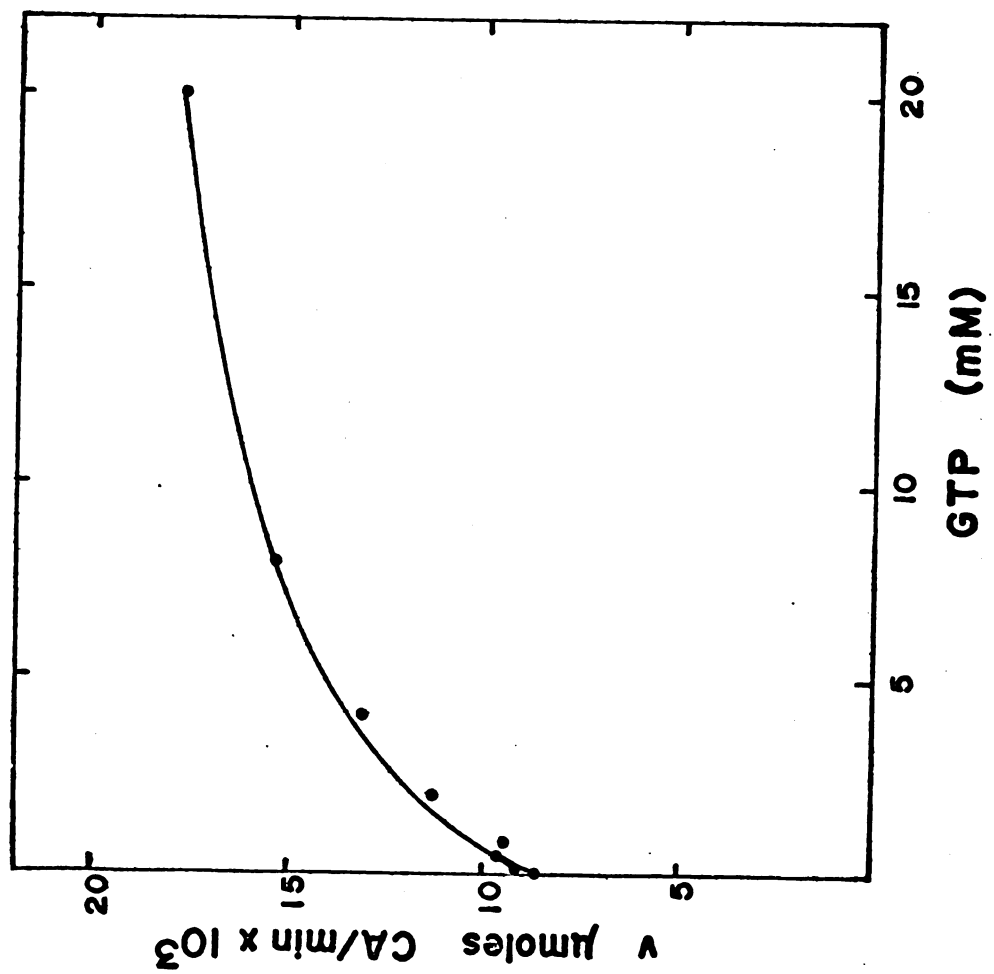
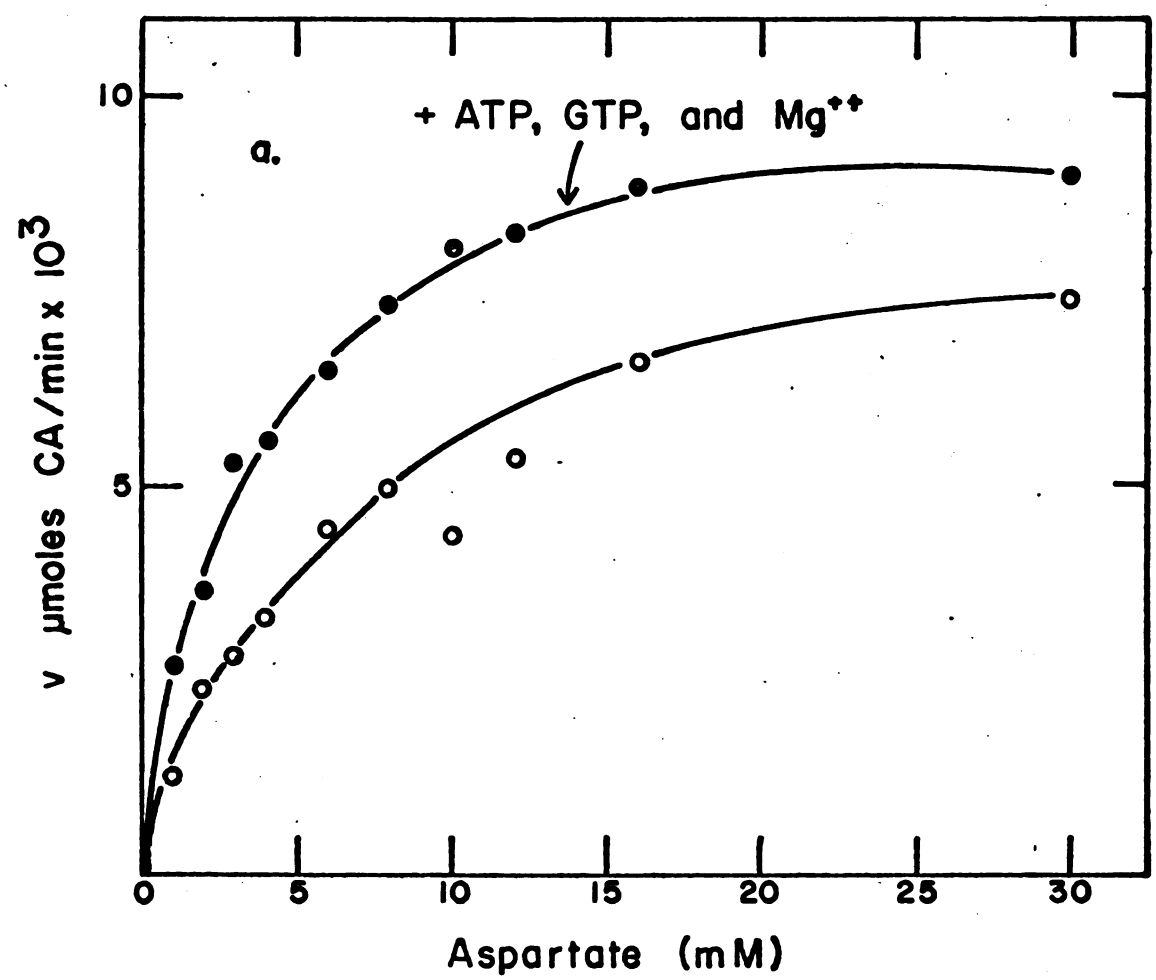
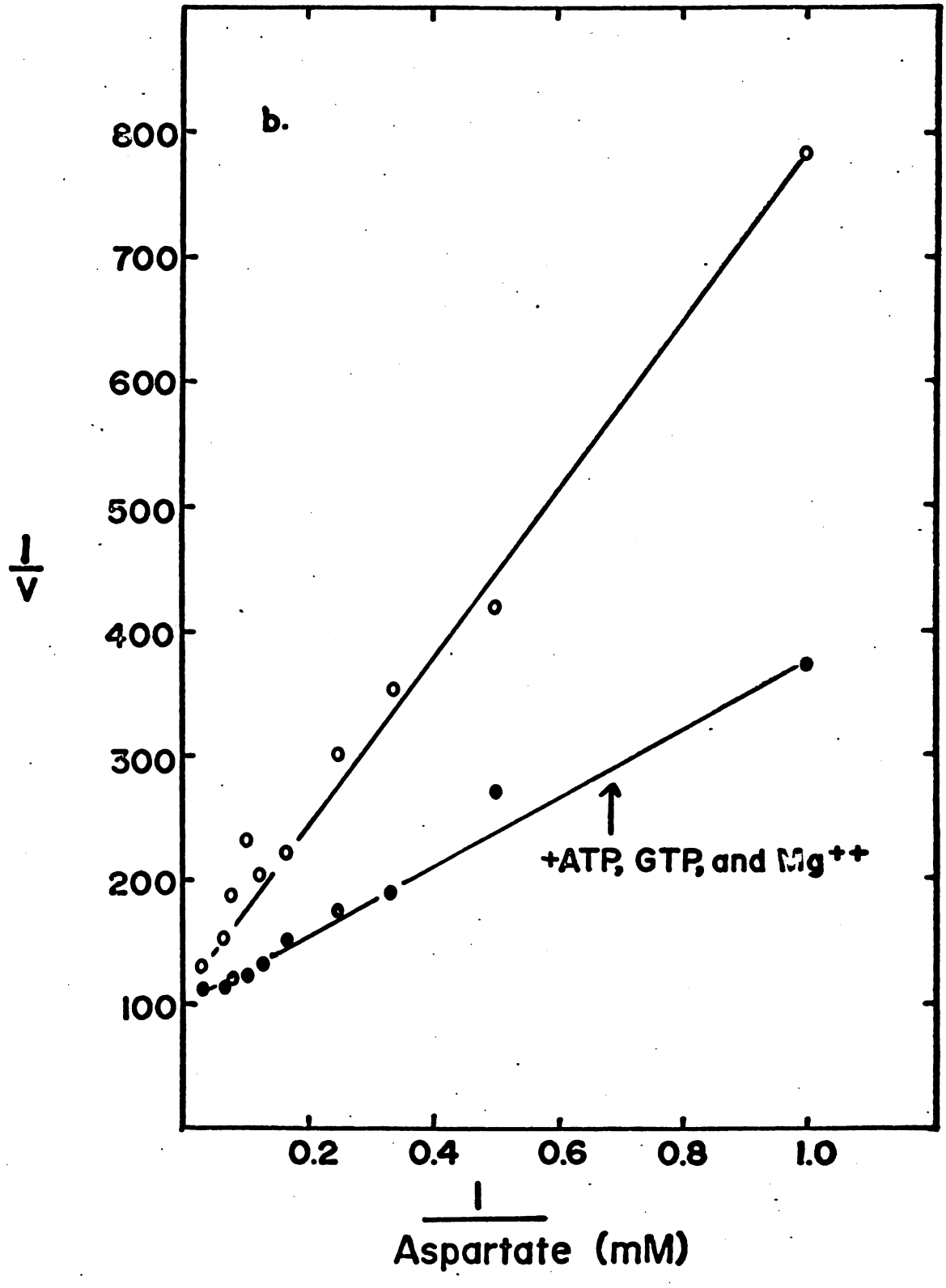

V μmoles CA/min x 10³
20
15
10
5
5
10
15
20
GTP (mM)

Figure 6a. Dependence of reaction rate of crystalline ATCase on aspartate concentration in the presence and absence of ATP, GTP and magnesium acetate (each at 2 mM).

b. A double reciprocal plot of the same data.


a.
+ ATP, GTP, and Mg++
v µmoles CA/min x 10^3
10
5
0
5
10
15
20
25
30
Aspartate (mM)


b.
800
700
600
500
400
300
200
100
1/V
0.2
0.4
0.6
0.8
1.0
1/Aspartate (mM)
+ATP, GTP, and Mg++

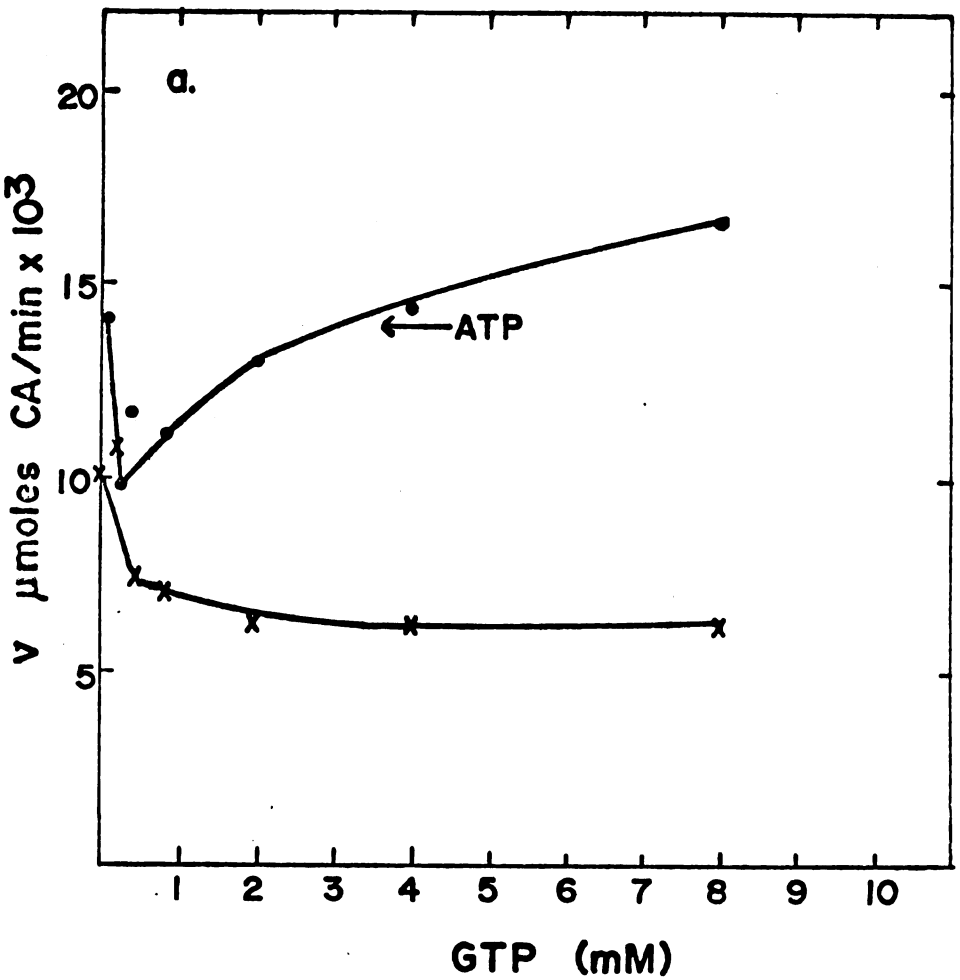
Studies of the Effect of Purine Nucleotides on the Activity of ATCase from the 30,000 x g Supernatant Solution of E. coli

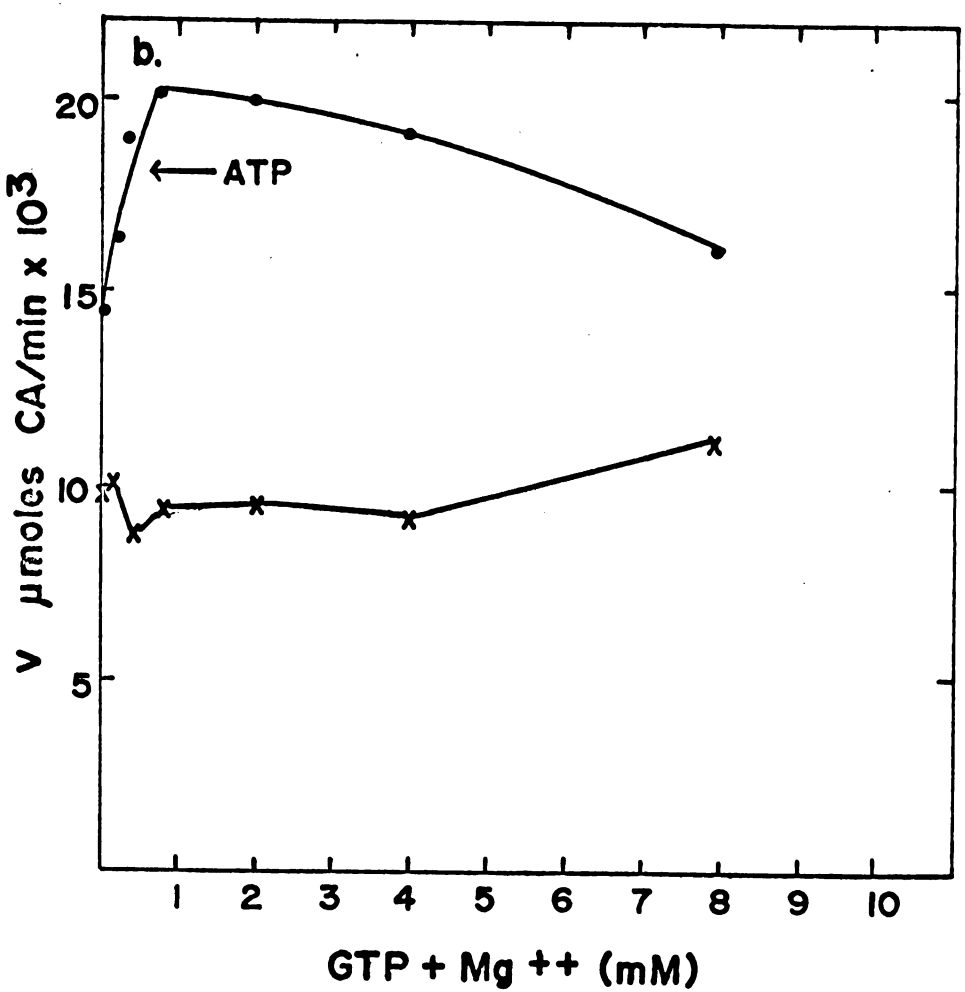
These experiments were performed with the objective of gaining a better understanding of the activation of ATCase by ATP, GTP and magnesium ions. These results are summarized below.

ATP and magnesium ions at equimolar concentrations activate ATCase more than ATP itself; ATP activates ATCase by 25%, while ATP and magnesium ions, both at 2 mM, activate the enzyme by 70% (ATCase activity was assayed at 5 mM aspartate, as described in Materials and Methods, section IX, 5).

GTP alone is an inhibitor of ATCase (Figure 7a). The inhibitory effect of GTP is modified by magnesium ions. As is shown in Figure 7b GTP and magnesium ions at equimolar concentration have no effect on ATCase activity. In the presence of ATP, GTP is inhibitory at low concentrations but it is slightly stimulatory above 4 mM (Figure 7a). GTP and magnesium ions in the presence of ATP activate the enzyme at low concentrations.

Figure 7. The effect of GTP (a), or GTP and magnesium ions (b) on ATCase activity of 30,000 x g supernatant of *E. coli* in the presence and absence of 8 mM ATP.


a.
20
15
10
5
v µmoles CA/min x 10³
ATP
1 2 3 4 5 6 7 8 9 10
GTP (mM)


b.
20
15
10
5
v µmoles CA/min x 10³
ATP
1 2 3 4 5 6 7 8 9 10
GTP + Mg ++ (mM)

DISCUSSION

The increase in ATCase activity under the conditions described by Singh (1966) and measured at 5 mM aspartate was confirmed in this study. Examination of the properties of this system by density labeling and subsequent equilibrium density centrifugation revealed that the synthesis of new molecules of ATCase or finishing of preexisting peptides did not cause the increase in ATCase activity. These results, along with the results of kinetic experiments, clearly point out that ATCase became activated during incubation with the complete incubation mixture.

The change in the dependence of reaction velocity on aspartate from sigmoidal to hyperbolic suggests that the interaction between the aspartate binding sites was decreased as a result of pretreatment with the complete incubation mixture. Heat treatment, which is known to abolish homotropic interactions of the native enzyme (Gerhardt and Pardee, 1962; Weitzman and Wilson, 1966), did not greatly change the activity of the enzyme pretreated with the complete incubation mixture. This could be interpreted according to the model of Monod *et al.* (1965) that during preincubation a shift of equilibrium occurred towards the conformation of the enzyme having higher affinity for the substrate.

Study of the activation of crystalline ATCase by components of the incubation mixture demonstrated that the components involved in activation in this system were ATP, GTP and magnesium ions. The effect of these compounds on ATCase activity has been studied previously. ATP activates ATCase while GTP inhibits the enzyme (Gerhardt and Pardee, 1962) and magnesium ions have been found to be inhibitory (Kleppe and Spaeren, 1966). However, the synergistic activation of ATCase by ATP and GTP in the presence of magnesium ions has not been reported before.

Preincubation of the crystalline enzyme with ATP, GTP and magnesium ions causes a threefold increase in the V_{max}. This increase was not reproduced when ATP, GTP and magnesium ions were added directly to the assay. It is possible that pretreatment of the enzyme with nucleotides and magnesium has a different effect on ATCase than the addition of the same compounds to the assay mixture. However, it is also possible that the observed discrepancy was caused by a change in the properties of the enzyme on storage.

Crystalline ATCase was observed to respond differently to the purine nucleotides than the crude enzyme. Pretreatment with purine nucleotides and magnesium caused an increase in the V_{max} of the crystalline enzyme and a different response was obtained when ATP was added directly to the assay mixture. ATP at saturating concentration activated the crystalline enzyme by 100% (Gerhardt and Pardee, 1962)

while the crude enzyme became activated by 25% at saturating concentrations of ATP. The observed difference could be caused by interfering substances present in the crude cell homogenate, or possibly by the degradation of nucleoside triphosphates in the crude extract during 10 minutes of incubation. However, it is also possible that the observed difference could be caused by an alteration of the properties of ATCase during growth and derepression. The mutant utilized in this study was harvested in a partially derepressed state while the crystalline enzyme preparation was obtained from an E. coli which had been completely derepressed for ATCase activity (Gerhardt and Holoubek, 1967). It is possible, therefore, that derepression of ATCase is associated with the increase in the sensitivity of this enzyme to regulatory nucleotides. A change in the degree of feedback inhibition of ATCase associated with the derepression of this enzyme was observed in S. cerevisieae by Kaplan et al. (1967).

Studies of the effect of purine nucleotides on ATCase activity revealed that their effect is modified by magnesium ions. The effect of magnesium ions and magnesium-nucleotide ligands was studied by Kleppe and Spaeren (1966). They reported that ATP and magnesium ions in equimolar concentrations was a few per cent better as an activator than ATP alone. The inhibitory effect of CTP was slightly decreased in the presence of equimolar magnesium.

The modifying effects of magnesium ions described here are much more pronounced. ATP and equimolar magnesium act as a much better activator than ATP itself. A preliminary experiment designed to test for the competition of ATP and ATP-Mg for the same site did not give a clear answer as to whether the ATP-Mg binds at the same site as ATP but it also did not indicate that ATP-Mg is the true ligand. GTP, an inhibitor of ATCase, did not affect ATCase activity in the presence of equimolar magnesium ions. It is possible that GTP-Mg does not bind to ATCase or even if bound does not affect the interaction between aspartate binding sites.

ATP and GTP in the presence of magnesium ions become synergistic activators of ATCase (Figure 7b). The synergistic effect of ATP and GTP in presence of magnesium ions resembles to a degree a cooperative feedback inhibition (Stadtman, 1966) of the first enzyme of purine biosynthesis, glutamine phosphoribosylpyrophosphate amido transferase of A. aerogenes. This enzyme is synergistically inhibited by 6-hydroxypurine and 6-aminopurine derivatives. Nierlich and Magasanik (1965) postulated that both nucleotides combine with the enzyme at different sites which, although different, can interact to produce an apparent synergistic inhibition of enzyme activity.

A similar model, although highly speculative, can be constructed for activation of ATCase by ATP, GTP and magnesium ions. It is possible that two sites binding ATP and

GTP can interact in the presence of magnesium ions and influence each others binding.

The existence of two sites for the regulatory nucleotides is not in disagreement with the known facts about binding of regulatory compounds to ATCase. Gerhardt and Pardee (1962) on the basis of kinetic evidence postulated that ATP, CTP and GTP compete for the same regulatory site. Data of Changeux et al. (1968) indicate that ATP and CTP apparently bind at the same site. No data were reported to substantiate hypothesis that GTP binds at the same site.

The biological significance of the activation of ATCase by ATP was discussed previously by Gerhardt and Pardee (1962). The activation of ATCase by ATP would lead to an increase in pyrimidine biosynthesis if the buildup of the ATP pool occurred and to an eventual balanced ratio of pyrimidines and purines necessary for nucleic acid synthesis. The activation of ATCase by ATP and GTP in the presence of magnesium ions would provide an even more efficient mechanism for equilibration of pyrimidine and purine pools when both purines are present in the cell in abundance. It is possible that dATP and dGTP exhibit a similar synergistic effect on ATCase activity. This possibility was not tested here but appears feasible since dATP and dGTP have the same effect on ATCase activity as ATP and GTP, respectively.

Activation of ATCase by purines has not been reported in systems other than E. coli. However, in yeast

(*S. cerevisieae*) ATP was found to counteract the inhibition of ATCase by pyrimidines both *in vivo* (Burns, 1966) and *in vitro* (Kaplan *et al.*, 1967). A meaningful comparison of regulatory properties of ATCase in *E. coli* and in other systems is not possible, since the *E. coli* enzyme is the most extensively studied. It appears that *E. coli* ATCase is uniquely well designed for its regulatory function not only by feedback inhibition, but also by feedback activation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Atkinson, D.E. 1965. Biological feedback control at the molecular level. Science, 150, 851.

Bethell, M.R., K.E. Smith, J.E. White and M.E. Jones. 1968. Carbamyl phosphate: An allosteric substrate for aspartate transcarbamylase of Escherichia coli. Proc. Nat. Acad. Sci., U.S., 60, 1442.

Bresnick, E., and H. Mossé. 1966. Aspartate transcarbamylase from rat liver. Biochem. J., 101, 63.

Burns, V.W. 1966. Regulation of pyrimidine biosynthesis and its strong coupling to the purine system. Biophys. J., 6, 787.

Changeux, J.-P., J.C. Gerhardt and H.K. Shachman. 1968. Allosteric interactions in aspartate transcarbamylase. I. Binding of specific ligands to the native enzyme and its isolated subunits. Biochemistry, 7, 531.

Changeux, J.-P., and M.M. Rubin. 1968. Allosteric interactions in aspartate transcarbamylase. III. Interpretation of experimental data in terms of the model of Monod, Wyman and Changeux. Biochemistry, 7, 553.

Chrispeels, M.J., and J.E. Varner. 1967. Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol., 42, 398.

Cole, F.E., and R.R. Schmidt. 1964. Control of aspartate transcarbamylase activity during synchronous growth of Chlorella pyrenoidosa. Biochem. Biophys. Acta, 90, 616.

Curci, M.R., and W.D. Donachie. 1964. An attempt to find pyrimidine inhibitors of a mammalian aspartate carbamoyltransferase. Biochem. Biophys. Acta, 85, 338.

Donachie, W.D. 1964. Regulation of pyrimidine biosynthesis in Neurospora crassa. I. End-product inhibition and repression of aspartate transcarbamoylase. Biochem. Biophys. Acta, 82, 284.

Dratz, E.A., and M. Calvin. 1966. Substrate- and inhibitor-induced changes in the optical rotatory dispersion of aspartate transcarbamylase. Nature, 211, 497.

Filner, P., and J.E. Varner. 1967. A test for de novo synthesis of enzymes: density labeling with H_2O^{18} of barley α-amylase induced by gibberellic acid. Proc. Nat. Acad. Sci., U.S., 58, 1520.

Gerhardt, J.C., and A.B. Pardee. 1962. The enzymology of control by feedback inhibition. J. Biol. Chem., 237, 891.

Gerhardt, J.C. 1963. The effect of the feedback inhibitor, CTP, on subunit interactions in aspartate transcarbamylase. Cold Spring Harbor Symp. Quant. Biol., Vol. 28, 491.

Gerhardt, J.C., and A.B. Pardee. 1964. Aspartate transcarbamylase, an enzyme designed for feedback inhibition. Fed. Proc., 23, 727.

Gerhardt, J.C., and H.K. Schachman. 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. Biochemistry, 6, 1054.

Gerhardt, J.C., and H. Holoubek. 1967. The purification of aspartate transcarbamylase of Escherichia coli and separation of its protein subunits. J. Biol. Chem., 242, 2886.

Gerhardt, J.C., and H.K. Schachman. 1968. Allosteric interactions in aspartate transcarbamylase. II. Evidence for different conformational states of the protein in the presence and absence of specific ligands. Biochemistry, 7, 538.

Herve, G.L., and G.R. Stark. 1967. Aspartate transcarbamylase. Amino-terminal analyses and peptide maps of the subunits. Biochemistry, 6, 3743.

Hu, A.S.L., R.M. Bock and H.O. Halvorson. 1962. Separation of labeled from unlabeled proteins by equilibrium density gradient sedimentation. Anal. Biochem., 4, 489.

Kaplan, J.G., M. Duphil and F. Lacroute. 1967. A study of the aspartate transcarbamylase activity of yeast. Arch. Biochem. Biophys., 119, 541.

Kleppe, K., and U. Spaeren. 1966. Aspartate transcarbamylase from Escherichia coli. II. Interaction of metal ions with substrates, inhibitors and activators. Biochem. Biophys. Acta, 128, 199.

Kim, S., and P.P. Cohen. 1965. Transcarbamylase activity in fetal liver and in liver of partially hepatectomized parabiotic rats. Arch. Biochem. Biophys., 109, 421.

Koritz, S.B., and P.P. Cohen. 1954. Colorimetric determination of carbamylamino amino acids and related compounds. J. Biol. Chem., 209, 145.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 263.

Monod, J., J.-P. Changeux and F. Jacob. 1963. Allosteric proteins and cellular control system. J. Mol. Biol., 6, 306.

Monod, J., J. Wyman and J.-P. Changeux. 1965. On the nature of allosteric transitions: A plausible model. J. Mol. Biol., 12, 88.

Neumann, J., and M.E. Jones. 1962. Aspartate transcarbamylase from lettuce seedlings: case of end product inhibition. Nature, 195, 709.

Neumann, J., and M.E. Jones. 1964. End-product inhibition of aspartate transcarbamylase in various species. Arch. Biochem. Biophys., 104, 438.

Nierlich, D.P., and B. Magasanik. 1965. Regulation of purine ribonucleotide synthesis by end product inhibition. The effect of adenine and guanine ribonucleotides on the 5'-phosphoribosylpyrophosphate amidotransferase of Aerobacter aerogenes. J. Biol. Chem., 240, 358.

Shuster, L., and R.H. Gifford. 1962. Changes in 3'-nucleosidase during germination of wheat embryos. Arch. Biochem. Biophys., 96, 534.

Singh, R.M.M. 1966. An in vitro increase in aspartyltranscarbamylase activity. Biochem. Biophys. Res. Commun., 22, 684.

Stadtman, E.R. 1966. Allosteric regulation of enzyme activity. In: Advances in Enzymology. Vol. 28. Interscience Publishers, New York, p. 41.

Stein, L.I., and P.P. Cohen. 1965. Correlation of growth and aspartate transcarbamylase activity in higher plants. Arch. Biochem. Biophys., 109, 429.

Vinograd, J. 1963. Sedimentation equilibrium in buoyant density gradient. In: Methods in Enzymology. Vol. VI. Ed. by S.P. Colowick and N.O. Kaplan. Academic Press, New York, p. 855.

Weber, K. 1968. Aspartate transcarbamylase from Escherichia coli. Characterization of the polypeptide chains by molecular weight, amino acid composition and amino-terminal residues. J. Biol. Chem., 243, 543.

Weitzman, P.D., and I.B. Wilson. 1966. Studies on aspartate transcarbamylase and its allosteric interaction. J. Biol. Chem., 241, 5481.

Yates, R.A., and A.B. Pardee. 1956. Pyrimidine biosynthesis in Escherichia coli. J. Biol. Chem., 221, 743.

Yates, R.A., and A.B. Pardee. 1957. Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem., 227, 677.

PART II

REGULATION OF NITRITE REDUCTASE IN TOBACCO CELLS

INTRODUCTION

Assimilatory reduction of nitrate in plants is carried out by two enzymes, nitrate reductase and nitrite reductase:

$$NO_3^- \xrightarrow[\text{nitrate reductase}]{} NO_2^- \xrightarrow[\text{nitrite redutase}]{} NH_3$$

Nitrate reductase catalyzes the reduction of nitrate to nitrite. The subsequent reduction of nitrite to ammonia, which involves a six electron change, is catalyzed by nitrite reductase. A vast amount of evidence has been accumulated demonstrating that nitrate reductase is an adaptive enzyme. The activity of this enzyme appears in plants grown on nitrate as a nitrogen source and not in plants grown on ammonia or amino acids (Candela et al., 1957; Afridi and Hewitt, 1962; Beevers et al., 1965). The increase in nitrate reductase activity in response to nitrate can be abolished by inhibitors of protein synthesis (Afridi and Hewitt, 1965; Beevers et al., 1965) which suggests that the increase in nitrate reductase activity is due to synthesis of enzyme rather than activation. However, activation could also conceivably depend upon protein synthesis. It has been demonstrated in two systems that the increase in nitrate reductase activity in the presence of nitrate is accompanied by

an increase in nitrite reductase activity (Ingle et al., 1966; Stewart, 1968; Joy, 1968).

The studies reported in this thesis were carried out to examine the regulation of nitrite reductase in tobacco cells. Experiments were designed to answer the following questions:

1. Is the increase in nitrite reductase activity associated with the increase in nitrate reductase activity in tobacco cells growing on nitrate? The increase in nitrate reductase activity in tobacco cells in response to nitrate was demonstrated previously by Filner (1966).
2. Do amino acids, which act as repressors of nitrate reductase activity in tobacco cells (Filner, 1966), also control the activity of nitrite reductase?
3. How is the regulation of nitrite reductase activity linked to the regulation of nitrate reductase activity? In particular, can nitrate cause an increase in both enzyme activities or does it have to be converted to nitrite before it can cause an increase in nitrite reductase activity?

In order to study the regulation of nitrite reductase activity in tobacco cells it was necessary to develop a simple assay for enzyme activity and to establish optimum conditions for determination of nitrite reductase activity.

To gain an understanding of the relationship between the processes which cause an increase in the activities of both enzymes the kinetics of appearance of activities of nitrate reductase and nitrite reductase were examined in tobacco cells grown on a nitrate containing medium. The end product regulation of nitrite reductase was studied by following the effect of amino acids on the increase of activity of nitrite reductase and of nitrate reductase in tobacco cells grown on nitrate containing medium.

The question of whether nitrate has to be reduced to nitrite before it can cause an increase in nitrite reductase activity in tobacco cells was studied utilizing tungsten. Tungsten is in the same group of elements as molybdenum and consequently may act as competitive inhibitor of molybdenum functions. Keeler and Varner (1958) showed a competitive inhibition of molybdenum by tungstate in the growth of Azotobacter. Both (^{185}W)tungstate and (^{99}Mo)molybdate were found to be incorporated into molybdo- and tungstoproteins. Tungstate inhibits the appearance of nitrate reductase activity in tobacco cells (Heimer et al., unpublished) and therefore provides a valuable technique for the study of appearance of nitrite reductase activity in the absence of nitrate reductase activity.

LITERATURE REVIEW

Properties of Nitrite Reductase from Plants

It is known that nitrite is metabolized rapidly by green plants in the light (Vanecko and Varner, 1955; Kessler, 1955). One of the first reports of nitrite reductase activity in an *in vitro* system was by Huzisige and Satoh (1961). The authors demonstrated that nitrite was metabolized by a soluble enzyme from spinach in the presence of an illuminated grana preparation. Subsequently, Sanderson and Cocking (1964) demonstrated, in a similar system isolated from tomato leaves, that the disappearance of nitrite is accompanied by a quantitative accumulation of ammonia.

It was later demonstrated that the reduction of nitrite can be carried out by plant enzymes in darkness in the presence of an appropriate reducing agent. Hageman *et al.* (1962) reported a quantitative reduction of nitrite to ammonia using nitrite reductase isolated from *Cucurbita pepo*. The reducing agent affective as the electron donor for this enzyme could be either palladized asbestos under hydrogen or reduced nicotinamide adenine dinucleotide phosphate (NADPH) with catalytic amounts of benzyl viologen.

In Clostridium pasteurianum ferredoxin participates in the reduction of nitrite to ammonia and can be replaced by methyl viologen, a dye of similar oxidoreductive potential (Mortenson et al., 1962). Plant nitrite reductase can also utilize reduced ferredoxin in addition to viologen dye as an electron donor (Losada et al., 1963; Hewitt and Betts, 1963). Reduction of nitrite by NADPH in a chloroplast extract in darkness is ferredoxin dependent (Losada et al., 1963). Ferredoxin can also serve as an electron donor if it is reduced in the light in the presence of a spinach grana preparation, or if it is reduced in darkness by hydrogen gas and hydrogenase of C. pasteurianum. The reduction of nitrite mediated by ferredoxin in light is accompanied by O_2 evolution and formation of high energy bonds of ATP (Paneque et al., 1964).

Nitrite reductase has been purified from several plants. The best studied of these systems is nitrite reductase from spinach (Ramirez et al., 1966; Joy and Hageman, 1966; Shin and Oda, 1966; Hewitt et al., 1968). Huzisige et al. (1963) reported that a preparation of nitrite reductase from spinach of specific activity 0.45 μmoles NO_2^-/min per mg protein showed the absorption spectrum of a flavoprotein. However, preparations of nitrite reductase from spinach of higher purity : 0.98 μmoles NO_2^-/min per mg of protein (Shin and Oda, 1966) and 3.25 μmoles NO_2^-/min per mg of protein (Ramirez et al., 1966) did not contain a flavin component. NADPH can be utilized as an electron donor for

the enzyme only if the reaction mixture is supplemented with ferredoxin and NADP reductase (Ramirez et al., 1966; Joy and Hageman, 1966).

Hydroxylamine reductase activity was found to be associated with the purified nitrite reductase (Betts and Hewitt, 1966; Hewitt et al., 1968). Hydroxylamine is one of the postulated intermediates in the reduction of nitrite to ammonia. It has been demonstrated that nitrite reductase from C. pepo reduced hydroxylamine slower than nitrite with either reduced benzyl viologen or reduced ferredoxin as electron donor (Hageman et al., 1962; Betts and Hewitt, 1966). Also hydroxylamine did not inhibit reduction of nitrite by the enzyme from C. pepo (Creswell et al., 1965). Lazzarini and Atkinson (1961) utilizing E. coli nitrite reductase showed that $(^{15}N)NO_2$ can be reduced to ammonia without dilution by a hydroxylamine pool. Therefore, free hydroxylamine is not an intermediate in the reduction of nitrite to ammonia.

The presence of a sulfhydryl protecting agent in the extraction medium was beneficial for extraction of nitrite reductase in an active state. The optimum concentration of cysteine required for the extraction of this enzyme from tomato leaves is 10^{-3} M (Sanderson and Cocking, 1964). The enzyme from maize had the highest activity if extracted with cysteine at concentrations between 10^{-3} M and 10^{-2} M (Joy and Hageman, 1966).

Measurements of the effect of pH on nitrite reductase activity indicate that the pH optimum is dependent upon the assay utilized. Spinach nitrite reductase exhibited the highest activity between 7.1 and 7.8 if the assay was conducted with ferredoxin reduced by hydrosulfite (Ramirez et al., 1966). A similar pH optimum for this enzyme was established if benzyl viologen reduced by sodium hydrosulfite was utilized as an electron donor but the pH optimum was between 6.0 and 7.0 if the reducing agent was NADPH (Joy and Hageman, 1966). In no case was a sharp pH optimum for nitrite reductase observed.

The intracellular location of nitrite reductase has been shown to be within the chloroplasts in green leaves (Ritenour et al., 1966; Ramirez et al., 1966). However, nitrite reductase activity has also been found in roots of tomato (Sanderson and Cocking, 1964), roots of barley (Miflin, 1967) and in etiolated radish cotyledons (Ingle et al., 1966).

Regulation of Nitrite Reductase and Nitrate Reductase

Nitrate reductase isolated from soybean was described as a flavoprotein by Evans and Nason (1953). Fluorometric analysis indicated that this enzyme contained flavin adenine dinucleotide (FAD) and that the boiled enzyme was active with D-amino acid oxidase specific for FAD. Nitrate reductase is able to utilize reduced FAD or flavin

mononucleotide (FMN) as electron donors (Paneque et al., 1965; Schrader et al., 1968; Maretzki and Dela Cruz, 1967). Plant nitrate reductase could also be coupled to pyridine nucleotides. Soybean nitrate reductase can utilize both nicotinamide adenine dinucleotide (NADH) and NADPH (Evans and Nason, 1953) while the enzyme from other plants is specific for NADH (Beevers et al., 1964). Molybdenum was demonstrated as a component of nitrate reductase from fungi (Nicholas and Nason, 1954). There is no unequivocal evidence that molybdenum is a component of purified plant nitrate reductase but it has been shown to be associated with soybean nitrate reductase during four purification steps (Evans and Hall, 1955). Molybdenum deficient plants grow better on ammonia than on nitrate (Agarwala, 1952) and molybdenum was demonstrated to be essential for utilization of nitrate by intact plants (Spencer and Wood, 1954). Furthermore, molybdenum is necessary for induction of nitrate reductase in cauliflower leaves (Afridi and Hewitt, 1962).

Nitrate reductase activity appears in higher plants in the presence of nitrate (as reviewed by Kessler, 1964). It was demonstrated that in the presence of nitrate an increase in activities of both nitrate reductase and nitrite reductase occurs in radish cotlyledons (Ingle et al., 1966) and in Lemna (Joy, 1968; Stewart, 1968). Activities of these two enzymes started to increase in a sequential manner, i.e., the activity of nitrate reductase increased first and then after several hours lag nitrite reductase activity

started to increase. Ingle et al. (1966) postulated that nitrate causes an increase in the activity of nitrate reductase while nitrite reductase increases in response to nitrite, which was produced as a result of nitrate reductase action. Nitrite causes an increase in the activities of both enzymes in radish cotyledons (Ingle et al., 1966).

Neither ammonia nor amino acids caused repression of the activities of both enzymes in radish cotyledons (Ingle et al., 1966). In Lemna ammonia causes repression of the activities of both enzymes. Repression of nitrate reductase and nitrite reductase does not occur in a coordinate manner since the ratio of nitrite reductase to nitrate reductase activities decreases with increasing amounts of ammonia added to the medium (Sims et al., 1968).

Nitrate assimilatory enzymes in the yeast, Candida utilis, are isolated as a large complex, designated the "nitrosome" (Sims et al., 1968). Both enzymes are induced by nitrate and nitrite and they are repressed completely by ammonia and partially by glutamic acid. The ratio of activities associated with nitrosome is constant regardless of growth conditions. This constant ratio of activities is characteristic of enzymes regulated in a coordinate manner.

Pateman et al. (1967) demonstrated that nitrate assimilation in Aspergillus is under the control of three genes. These genes are not linked and are as follows: a regulator gene, a structural gene for nitrate reductase

and a structural gene for nitrite reductase. Mutants in the regulatory gene loci either have constitutive nitrate reductase and nitrite reductase or lack both enzymes. Nitrate and nitrite can induce nitrate reductase and nitrite reductase. Pateman et al. (1967) demonstrated unequivocally that nitrate directly induces nitrite reductase since mutants lacking nitrate reductase activity can induce nitrite reductase in response to nitrate.

MATERIALS AND METHODS

Tobacco Cell Lines

Tobacco cells grown in liquid culture were utilized as an experimental material. The cell line utilized throughout most of this study was the XD cell line isolated from tobacco stems by Filner (1965). This cell line was selected for its ability to grow on a defined liquid medium, M-1D, described below. Another cell line utilized was the R line which was selected from the XD line for its ability to grow on M-1D in the presence of the inhibitory amino acid, threonine (Heimer, unpublished).

Growth Media

Liquid medium M-1D described by Filner (1965), contained the following compounds in moles x 10^{-5} in a liter of distilled water: $Ca(NO_3)_2 \cdot 4\ H_2O$, 84.8; KNO_3, 79.1; $NaH_2PO_4 \cdot\ H_2O$, 11.9; $NaSO_4$, 141.0; $MgSO_4 \cdot 7\ H_2O$, 146.0; KCl, 87.1; $FeC_6H_5O_7 \cdot 3\ H_2O$, 0.67; $MnSO_4 \cdot 4\ H_2O$, 2.2; $ZnSO_4 \cdot 7\ H_2O$, 0.52; H_3BO_3, 2.4; KI, 0.45; nicotinic acid, 0.41; pyridoxine·HCl, 0.049; thiamine·HCl, 0.03; 2,4-dichlorophenoxy acetic acid, 0.23; sucrose, 5,840. The pH of this solution was adjusted to 6.5 before autoclaving.

Nitrate-less M-1D was prepared by substituting the chlorides of potassium and calcium for nitrates. M-1D supplemented with tungstate was prepared by dissolving $Na_2WO_4 \cdot 2\ H_2O$ in M-1D prior to sterilization. Casein hydrolysate solution was prepared by dissolving vitamin free casein hydrolysate (purchased from Difco) in distilled water in a concentration of 0.1 g per ml. The medium supplemented with casein hydrolysate was prepared by adding a sterilized solution of casein hydrolysate to the previously sterilized medium.

Maintenance and Growth of Cells

Tobacco cells were grown in 500 ml aliquots of M-1D. Stock cultures were transferred every 12 days to a new batch of medium by adding 25 ml of 12 day old parent culture to 500 ml of M-1D. The cells were grown on a shaker with a horizontal displacement of 4 inches at 80 cycles per minute at $28^{o}C$.

The growth experiments were started by inoculating media with cells from a 12 day old culture as described above. The cells were grown for the desired period of time and then harvested.

Harvesting of the Cells and Preparation of the Tobacco Cell Homogenate for the Enzyme Assays

The cells from one liter of culture were harvested after the desired period of growth by filtration on Whatman paper No. 1 and their fresh weight was determined. The cells were suspended in tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.5) containing 10^{-3} M cysteine (5 ml of buffer was used for 1 gram of cells) and homogenized at 4°C with forty strokes of a motor driven teflon-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 20 minutes. The supernatant solution was used as a source of nitrite reductase activity.

Nitrate reductase was assayed in the fraction precipitable by 50 per cent saturated ammonium sulfate. This fraction was prepared as follows: an aliquot of the 10,000 x g supernatant solution of tobacco cell homogenate was added to an equal volume of saturated ammonium sulfate (pH 7.5) containing 10^{-3} M cysteine. After 1 hour at 4°C this solution was centrifuged at 20,000 x g for 20 minutes. The precipitate was suspended to one-fifth of the original volume of the cell extract in 0.01 M phosphate buffer at pH 7.5 containing 10^{-3} M cysteine. An aliquot of this solution was assayed for nitrate reductase activity.

Assay of Nitrate Reductase Activity

Nitrate reductase activity was determined by a modification of the method of Paneque et al. (1965). The composition of the incubation mixture was as follows: 50 μmoles of phosphate buffer (pH 7.5), 10 μmoles of KNO_3, 200 μmoles of FMN, 2.3 μmoles of sodium hydrosulfite (added in 0.05 ml of 0.095 M $NaHCO_3$), an aliquot of the enzyme extract and enough water to make the final volume 1 ml. After the addition of enzyme extract the reaction mixture was incubated at 25°C for 15 minutes. The reaction was stopped by stirring on a Vortex mixer until the FMN was completely oxidized. The nitrite formed was determined by the colorimetric method of Snell and Snell (1949). To the contents of the tubes 1 ml of 1% sulfanilamide in 3 M HCl was added followed by 1 ml of 0.02% N-1-naphtylethylenediamine dihydrochloride. The tubes were centrifuged in a clinical centrifuge for 5 minutes. The absorbancy of the supernatant at 540 mμ was determined and compared to a zero time control. The activity of nitrate reductase was expressed as mμmoles of nitrite formed during one hour per gram of fresh weight of cells.

Assay of Nitrite Reductase Activity

Nitrite reductase was assayed according to the modified assay of Ramirez et al. (1966). The assay was conducted in the following manner:

The reaction mixture was prepared by adding 100 μmoles of phosphate buffer (pH 7.5), 2 μmoles of methyl viologen, 5 μmoles of KNO_2, and enough distilled water to make the final volume 3 ml. The tubes were flushed with nitrogen and covered with rubber stoppers. An aliquot of the cell homogenate was then added and the reaction was started by adding 40 μmoles of sodium hydrosulfite in 0.4 ml of 0.29 M $NaHCO_3$ (the sodium hydrosulfite solution was prepared immediately before the assay). The tubes were incubated at 30°C for the desired period of time. The reaction was stopped by shaking the tubes vigorously on a Vortex mixer until methyl viologen was completely oxidized. An aliquot of the reaction mixture was taken for nitrite determination. It was diluted 30-fold with distilled water and 1 ml of 1% sulfanilamide in 3 M HCl was added followed by 1 ml of 0.02% N-1-naphtylethylenediamine dihydrochloride. After 10 minutes the absorbancy was determined at 540 mμ. Controls from which cell homogenate was omitted were done with each assay. The average absorbancy at 540 mμ of those controls was taken as a measure of initial absorbancy of nitrite under the conditions of the assay. The decrease in the absorbancy at 540 mμ was taken as a measure of nitrite reductase activity. The activity of nitrite reductase was expressed as μmoles of nitrite utilized per hour per gram of cells.

Determination of Protein Content

Protein content of the 10,000 x g supernatant solution of tobacco cell homogenate was determined in the fraction precipitable by 10% trichloroacetic acid (TCA). To 0.5 ml of the 10,000 x g supernatant solution an equal volume of 20% TCA was added. After 12 hours enough 10% TCA was added to make the final volume 10 ml. The tubes were centrifuged in the clinical centrifuge at 3,000 rpm for 10 minutes. The precipitate was resuspended in 10 ml of 95% ethanol and subsequently centrifuged as described above. The supernatant solution was decanted and the pellet was dried in the air stream. The pellet was dissolved in 1 ml of 1.0 M NaOH and its protein content was determined by the procedure of Lowry *et al.* (1951). Bovine serum albumin dissolved in 1.0 M NaOH at a concentration of 1 mg/ml was used as a standard.

RESULTS

Properties of Nitrite Reductase of Tobacco Cells

One of the objectives of this study was the development of a reliable and simple assay for the nitrite reductase of tobacco cells. The assays, utilizing benzyl viologen or methyl viologen reduced by sodium hydrosulfite as electron donors, were considered especially suitable since they can be carried out aerobically.

The assay of nitrite reductase activity was first carried out using benzyl viologen reduced by sodium hydrosulfite as the electron donor, as described by Joy and Hageman (1966). Nitrite reductase activity could be detected using this assay. However, this assay did not prove satisfactory. A number of different concentrations of both benzyl viologen and sodium hydrosulfite were tested, but in no case was it possible to determine a ratio of concentrations of these two reagents which gave reproducible conditions of reduction of benzyl viologen.

The assay of nitrite reductase activity, utilizing methyl viologen reduced by sodium hydrosulfite as an electron donor (Ramirez et al., 1966), was subsequently utilized.

As demonstrated in Table 1, the activity of nitrite reductase, measured as disappearance of nitrite, was not detected in the absence of hydrosulfite. An increase in the amount of sodium hydrosulfite up to 20 μmoles in the assay caused an increase in the amount of nitrite utilized.

Table 1. The effect of varying concentrations of sodium hydrosulfite on the activity of nitrite reductase from tobacco cells*

System	Sodium hydrosulfite μmoles	NO_2^- reduced μmoles/h per g of cells
Complete	0	0.0
	5	5.6
	10	14.0
	20	13.5
	30	16.4
	40	18.5
	50	15.5
Complete minus cell homogenate	30	0.0
	40	0.5

*The complete system contained in a final volume of 3 ml the following: 100 μmoles of Tris·HCl (pH 7.5), 4 μmoles of KNO_2, 2 μmoles of methyl viologen and 0.8 ml of homogenate of 2 day old tobacco cells. Tobacco cell homogenate was prepared by homogenizing 1 g of tobacco cells in 2 ml of 0.1 M Tris·HCl (pH 7.5) containing 10^{-3} M cysteine. Hydrosulfite solution was prepared by dissolving 100 μmoles of $Na_2S_2O_4$ per ml of 0.29 M $NaHCO_3$ and was added as indicated.

Hydrosulfite in a 20 fold excess over methyl viologen did not cause inhibition of nitrite reductase activity and nitrite reductase activity was dependent upon methyl viologen as an electron donor (Table 2).

Table 2. The effect of heating the tobacco cell homogenate and the requirement for methyl viologen for nitrite reductase activity*

System	NO_2 reduced μmoles/h per g of cells
Complete	8.9
minus methyl viologen	1.1
Complete, cell homogenate heated at 100°C for 10 minutes	0.2

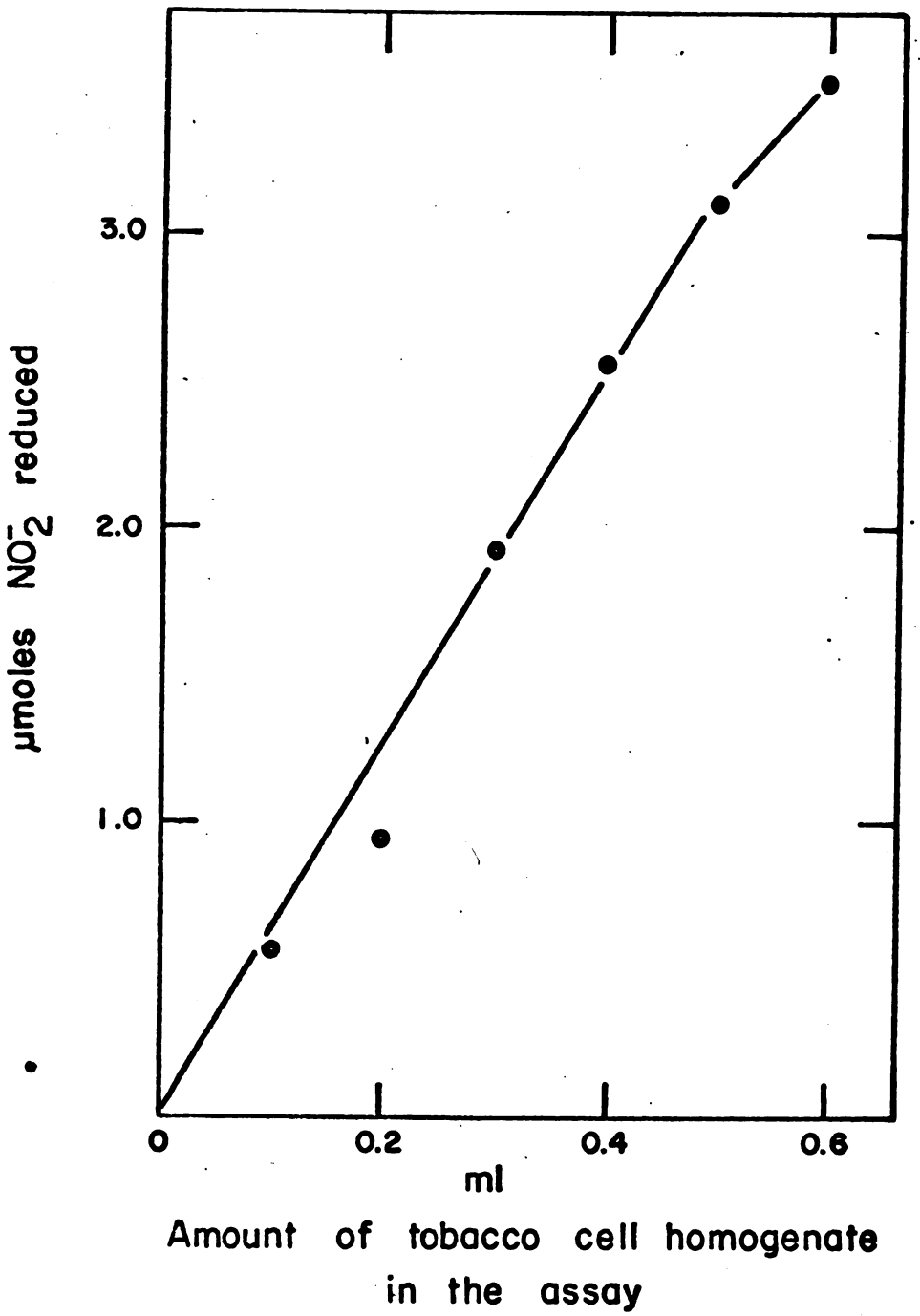
*The complete system contained in a final volume of 3 ml the following: 100 μmoles of Tris·HCl (pH 7.5), 4 μmoles of KNO_2, 2 μmoles of methyl viologen, 40 μmoles of sodium hydrosulfite added in 0.4 ml of 0.29 M $NaHCO_3$ and 0.8 ml of homogenate of 5 day old tobacco cells. Tobacco cell homogenate was prepared by homogenizing 1 g of tobacco cells in 2 ml of 0.1 M Tris·HCl (pH 7.5) containing 10^{-3} M cysteine.

The activity observed in the absence of methyl viologen is within limits of experimental error. The fact that no disappearance of nitrite was detected in the absence of tobacco cell homogenate (Table 1) indicates that no chemical reduction of nitrite occurred under the conditions of the assay. The nitrite reductase activity of the tobacco cell homogenate was destroyed by boiling (Table 2).

The activity of nitrite reductase was linear with incubation time during 1 hour. As demonstrated in Figure 1, nitrite reductase activity was also linear with the amount of tobacco cell homogenate. The estimation of nitrite reductase activity was more accurate if the decrease in the nitrite content was higher than 10% of the initial value,

Figure 1. Dependence of nitrite reductase activity on the concentration of tobacco cell homogenate.

Nitrite reductase was assayed as described in Materials and Methods. The incubation time was 14 minutes. The homogenate of 3½ day old tobacco cells was added as indicated. Nitrite reductase activity is expressed as μmoles of nitrite disappeared from the incubation mixture under conditions of the assay.


3.0
2.0
1.0
µmoles NO_2^- reduced
0
0.2
0.4
0.6
ml
Amount of tobacco cell homogenate in the assay

For routine estimations of nitrite reductase activity the time of incubation and the amount of enzyme extract were appropriately adjusted so that the decrease in nitrite content during the assay was at least 30%.

Nitrite reductase of tobacco cells does not demonstrate a sharp pH optimum but it appears to be approximately 7.5. A variety of buffers were found to affect the activity of nitrite reductase. In the standard conditions of the assay and at pH 7.5 the nitrite reductase activity was the highest in phosphate buffer, while in Tris and Hepps (N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid, Dr. N. E. Good, unpublished) the rate was 33% and 50% lower, respectively.

The dependence of nitrite reductase activity from tobacco cells on nitrite concentration was studied. The increase in nitrite concentration up to 1.6 mM caused an increase in the reaction rate but nitrite was inhibitory above 2 mM. The inhibition of nitrite reductase activity of illuminated grana preparation from tomato by nitrite at concentrations above 0.25 mM was reported by Sanderson and Cocking (1964). Substrate inhibition of purified nitrite reductase from spinach was not observed (Ramirez et al., 1966).

Nitrite reductase from tobacco cells resembles other plant nitrite reductases described in the literature. Its activity is dependent upon reduced viologens which can substitute for ferredoxin, a likely physiological electron for

nitrite reductase. Addition of cysteine at 10^{-3} M causes an increase in the activity of nitrite reductase extracted from tobacco cells. The pH optimum of this enzyme is approximately 7.5. An important characteristic of nitrite reductase that was not demonstrated in the present study is a quantitative relationship between nitrite utilized and ammonia formed. Preliminary experiments indicate that ammonia was formed upon incubation of tobacco cell homogenate in the conditions of this assay. However, the stoichiometric relationship remains to be determined.

Changes of Activity of Nitrate Reductase with Age of Culture Tobacco Cells

Parent cultures of tobacco cells grown on M-1D do not contain any nitrate reductase activity but do have detectable levels of nitrite reductase activity. The activities of both enzymes increased simultaneously after 12 day old tobacco cells of XD and R line were inoculated into fresh M-1D (Figure 2a and b). The increase in the activity of both enzymes was observed after 2 hours. No apparent lag in the appearance of nitrite reductase was observed as compared to nitrate reductase.

Tobacco cells inoculated into M-1D deplete the medium of nitrate after 10 days (Filner, 1966). Figure 3a demonstrates that, following inoculation, the total content of soluble protein increases, reaches a peak after 8 days of growth, and subsequently declines. The activities of nitrate

Figure 2. Appearance of nitrite and nitrate reductase activity after transfer of 12 day old parent culture into M-1D.

a. XD cell line.

b. R cell line.

Activities of both enzymes were assayed as described in Materials and Methods.

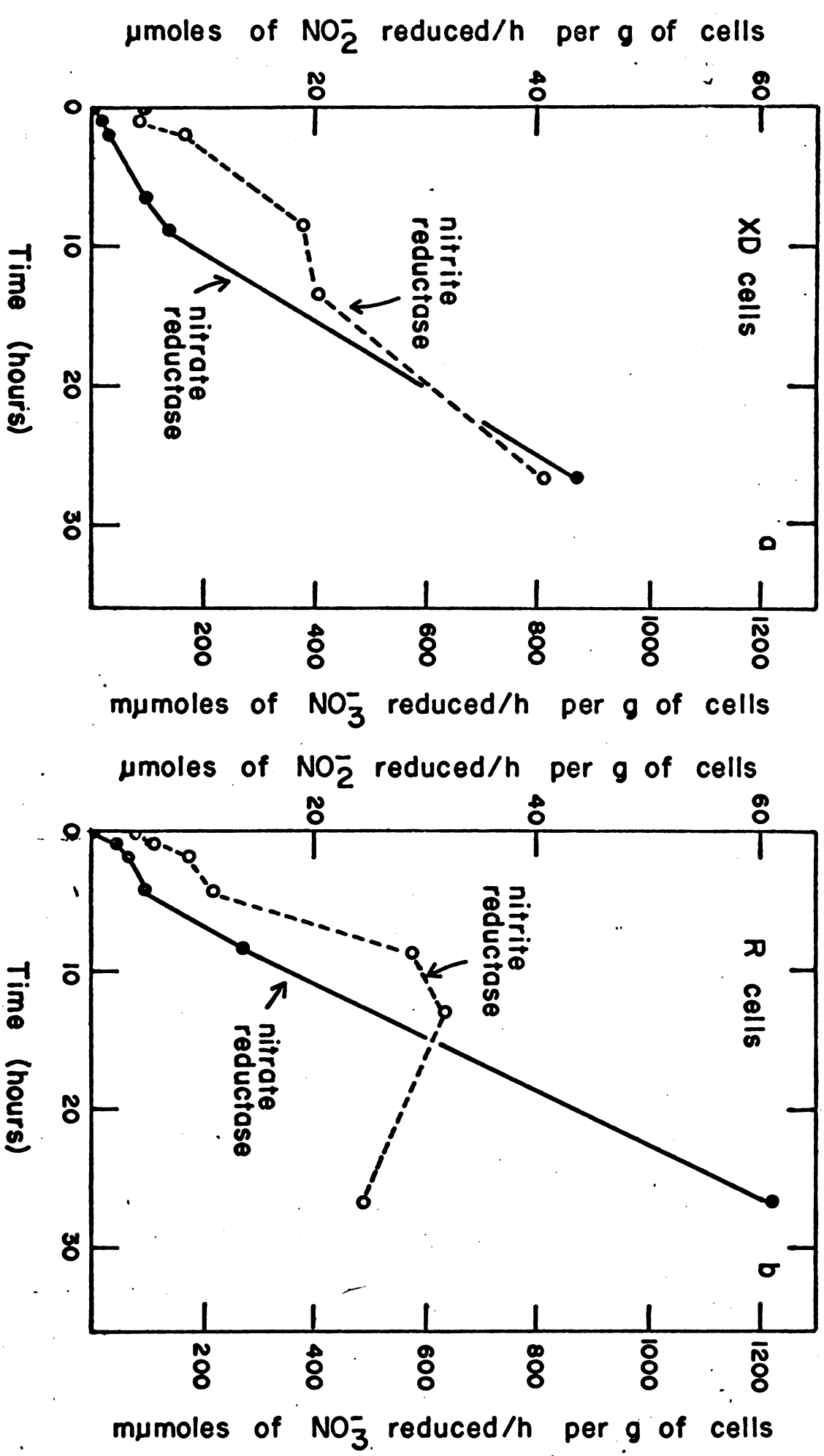

µmoles of NO_2^- reduced/h per g of cells
20
40
60
0
10
20
30
Time (hours)
nitrite reductase
nitrate reductase
XD cells
a
200
400
600
800
1000
1200
mµmoles of NO_3^- reduced/h per g of cells
µmoles of NO_2^- reduced/h per g of cells
20
40
60
0
10
20
30
Time (hours)
nitrite reductase
nitrate reductase
R cells
b
200
400
600
800
1000
1200
mµmoles of NO_3^- reduced/h per g of cells

Figure 3. Changes in the activity of nitrite reductase and nitrate reductase with age of tobacco cells.

a. Activity is expressed per liter of culture.

b. Activity is expressed per milligram of protein.

Ten day old XD cells were inoculated into M-1D and harvested as indicated. Activities of nitrate reductase and nitrite reductase were determined as described in Materials and Methods.

a
b
protein
nitrite reductase
nitrate reductase
Time (days)
µmoles of NO_2^- reduced/h per liter of culture
µmoles of NO_3^- reduced/h per liter of culture
mg protein/liter of culture
µmoles of NO_2^- reduced/h per mg protein
µmoles of NO_3^- reduced/h per mg protein

reductase and nitrite reductase (expressed per liter of culture) reach a peak after 4 and 7 days of growth, respectively. The disappearance of the activities of both enzymes from the culture is faster than that of total protein. This is especially true in the case of nitrate reductase, which declines to a undetectable level after 12 days of growth, while some nitrite reductase activity was still detectable in 15 day old tobacco cells. The specific activities of the two enzymes increased during the first 2½ days of growth on M-1D and during subsequent growth the specific activity of nitrite reductase declined at a slower rate than that of nitrate reductase (Figure 3b).

This increase in the activity of nitrite reductase was observed only in tobacco cells grown in medium containing nitrate as the only nitrogen source. If tobacco cells were grown for 15 days in medium containing casein hydrolysate and nitrate, the activity of nitrite reductase was negligible at all times. The absence of nitrite reductase activity in cells grown on medium supplemented with casein hydrolysate apparently was not caused by inhibition of nitrite reductase by amino acids, since the nitrite reductase activity of the mixture of extracts of tobacco cells grown on M-1D and grown on M-1D supplemented with amino acids was equal to the sum of activities of nitrite reductase in the two extracts.

The Effect of Casein Hydrolysate on the Formation of Nitrate Reductase and Nitrite Reductase

Casein hydrolysate inhibits the formation of nitrate reductase in tobacco cells grown on medium containing nitrate (Filner, 1966). It was therefore of interest to determine whether the formation of nitrite reductase is similarly affected. To test this, 12 day old XD cells and 14 day old R cells were grown for 48 hours on M-1D supplemented with different concentrations of casein hydrolysate. The activities of nitrate reductase and nitrite reductase were then estimated in the homogenates.

As demonstrated in Figure 4a and b, casein hydrolysate inhibits the appearance of both enzymes in R and XD cell lines in a strikingly similar manner. The inhibitory effect of casein hydrolysate was not proportional to its concentration in the medium. Addition of casein hydrolysate at a concentration of 0.1 gram/liter caused 75% inhibition of nitrate reductase activity and 70% inhibition of nitrite reductase activity in XD cells. Raising the concentration of casein hydrolysate 10 fold caused only a few per cent higher inhibition of both enzymes.

The R cell line was selected from the XD line for its ability to grow on nitrate containing medium in presence of 10^{-4} M threonine, an amino acid which inhibits growth apparently by preventing the development of nitrate reductase activity. Both the nitrate accumulation and the

Figure 4. The effect of casein hydrolysate on the formation of nitrate reductase and nitrite reductase activity.

a. Twelve day old cells of the XD line were inoculated into M-1D containing casein hydrolysate as indicated and harvested after 48 hours.

b. Fourteen day old cells of the R cell line were inoculated into M-1D containing casein hydrolysate as indicated and harvested after 48 hours.

Activity of nitrate reductase was assayed as described in Materials and Methods. The reaction mixture for determination of nitrite reductase activity included in a final volume of 3 ml 100 μmoles of Tris·HCl (pH 7.5), 4 μmoles of KNO_2, 2 μmoles of methyl viologen and 40 μmoles of sodium hydrosulfite, and tobacco cell homogenate prepared in 5 ml per gram of tissue of Tris·HCl (pH 7.5) containing 10^{-3} M cysteine.

a
XD line
nitrite reductase
nitrate reductase
Casein Hydrolysate (g/liter)
μmoles of NO2 reduced/h per g of cells
μmoles of NO3 reduced/h per g of cells
b
R line
nitrite reductase
nitrate reductase
Casein Hydrolysate (g/liter)
μmoles of NO2 reduced/h per g of cells
μmoles of NO3 reduced/h per g of cells

activity of nitrate reductase are higher in R cells than in the XD cells (Heimer, unpublished). As demonstrated in Figure 4, the activity of nitrite reductase is also higher in the R cells.

The ratio of activities of nitrate reductase and nitrite reductase was not constant in XD and in R lines of tobacco cells grown in the media supplemented with different amounts of casein hydrolysate. This indicates that regulation of nitrate reductase and nitrite reductase is not executed in a coordinate manner.

Stability of Nitrate Reductase and Nitrite Reductase in vivo

Knowledge of the stabilities of both nitrate reductase and nitrite reductase is important for comparing the levels of their activities in different growth conditions and consequently in evaluating the means of their regulation. The effect of casein hydrolysate on the stability of nitrite reductase was also estimated to ascertain whether the decrease in the activity of nitrite reductase in tobacco cells grown in the presence of casein hydrolysate is caused by a decrease in the formation of the enzyme or by an increase in its breakdown.

The stability of both enzymes was examined in the following experiment. A 12 day old parent culture of tobacco cells was subcultured into M-1D for 24 hours. The cells were then transferred sterilely to nitrate-less M-1D

or nitrate-less M-1D supplemented with 3.0 g/liter of casein hydrolysate. As demonstrated in Table 3, the activity of nitrite reductase increased during the first 8 hours after transfer into nitrate-less medium and after an additional 16 hours only a 16% decrease in nitrite reductase activity from its highest value was observed.

Table 3. Activity of nitrate reductase and nitrite reductase after transfer of induced cells to nitrate-less M-1D, or nitrate-less M-1D supplemented with casein hydrolysate*

Time after transfer (hours)	Nitrate reductase mμmoles NO_3^-/h per g of cells		Nitrite reductase μmoles of NO_2^-/h per g of cells	
	NO_3^--less	NO_3^--less + casein hydr.	NO_3^--less	NO_3^--less + casein hydr.
0	320		21.15	
4	350	320	23.4	32.7
8	260	325	33.8	27.9
12	375	255	29.7	33.6
24	42	150	28.5	32.7

*Twelve day old cells of the XD line were subcultured into M-1D for 24 hours. Subsequently they were transferred sterilely to nitrate-less M-1D, or nitrate-less M-1D supplemented with casein hydrolysate at 3.0/g liter. Activities of both enzymes were assayed as described in Materials and Methods.

Addition of casein hydrolysate to the nitrate-less medium did not cause an increase in the disappearance of nitrite reductase activity from the cells. On the contrary, after the initial increase in nitrite reductase during the 4 hours after transfer of the cells to the medium supplemented with casein hydrolysate enzyme activity remained virtually unchanged.

Nitrate reductase is less stable under the same conditions. Twenty-four hours after transfer of tobacco cells to nitrate-less M-1D nitrate reductase activity decreased by 87% from its highest value. Casein hydrolysate does not increase the decay rate of nitrate reductase in tobacco cells.

Effect of Tungstate and Casein Hydrolysate on the Formation of Nitrate Reductase and Nitrite Reductase

Tungstate causes a decrease of nitrate reductase activity in tobacco cells grown on M-1D, probably by competing with molybdate and thus causing formation of a nonfunctional enzyme (Heimer et al., unpublished). Therefore, the use of tungstate makes it possible to determine whether nitrite formation is necessary for the induction of nitrite reductase, or whether nitrate can induce this enzyme directly.

Table 4 demonstrates that tungstate at $2 \cdot 10^{-5}$ M and $4 \cdot 10^{-5}$ M causes respectively, 82 and 92% inhibition of nitrate reductase activity. Tungstate at $2 \cdot 10^{-5}$ M has no

Table 4. The effect of tungstate and casein hydrolysate on the formation of nitrate reductase and nitrite reductase*

Growth medium	Nitrate reductase $m\mu moles\ NO_3^-/h$ per g of cells	Nitrite reductase $\mu moles\ NO_2^-/h$ per g of cells
M-1D	528	29.7
M-1D + $2 \cdot 10^{-5}$ M tungstate	95	35.7
M-1D + $4 \cdot 10^{-5}$ M tungstate	40	24.9
M-1D + casein hydrolysate	84	10.0
M-1D + $2 \cdot 10^{-5}$ M tungstate + casein hydrolysate	16	15.3
M-1D + $4 \cdot 10^{-5}$ M tungstate + casein hydrolysate	10	11.7

*Twelve day old tobacco cells of XD line were subcultured into growth media described in Table 4. Casein hydrolysate, when used, was at 3.0 g/liter. The cells were harvested after 24 hours of growth and the activities of nitrate reductase and nitrite reductase were assayed as described in Materials and Methods.

effect on the activity of nitrite reductase, while at $4 \cdot 10^{-5}$ M it causes a 17% decrease in the activity of nitrite reductase.

Addition of casein hydrolysate to M-1D has a greater effect on nitrate reductase activity than the addition of tungstate. Casein hydrolysate at 3.0 g/liter causes 84% inhibition of nitrate reductase activity and 66% of nitrite reductase activity.

The fact that tungstate has little or no effect on the activity of nitrite reductase does not prove that nitrate is the inducer of nitrite reductase. The low level of nitrate reductase observed in the cells grown in these conditions may provide enough nitrite to induce fully nitrite reductase. However, when the same level of nitrate reductase is present in casein hydrolysate grown cells, nitrite reductase activity is reduced to one-third as compared with its level in the cells grown on M-1D. One of the effects of the addition of casein hydrolysate to M-1D is a decrease in the accumulation of nitrate by tobacco cells, while tungstate does not affect nitrate accumulation by tobacco cells (Heimer et al., unpublished). It appears, therefore, that there is a better correlation between nitrate content of the cells and the activity of nitrite reductase than between the activities of nitrate reductase and nitrite reductase. However, since casein hydrolysate lowers the nitrate content and decreases the activity of nitrate reductase, it also causes a decrease in nitrite formation. The question of whether nitrate or nitrite is the inducer of nitrite reductase remains open.

DISCUSSION

A reproducible and convenient assay was developed for nitrite reductase of tobacco cells by modification of a method of Ramirez et al. (1966). The reaction rate measured by this assay is proportionate to both time and amount of cell homogenate in the assay. Therefore, this assay is suitable for quantitative studies of changes in nitrite reductase activity with the physiological state of the cultured cells. The nitrite reductase activity estimated in tobacco cells utilizing this assay is higher than that of nitrate reductase in tobacco cells. The ratio of the activities of the two enzymes vary, depending on the physiological state of the cells, but nitrite reductase activities are at least 40 fold higher. It appears that tobacco cells have a very efficient mechanism for metabolizing nitrite which is toxic.

The nitrite reductase of tobacco cells has been found to be an adaptive enzyme. The appearance of this enzyme activity in tobacco cells depends on the nitrogen source present in the medium. Nitrite reductase activity is not detected in cells grown on a medium containing amino acids as the sole nitrogen source but it is observed in

cells grown on nitrate containing medium. It is tempting to speculate that nitrite reductase synthesis is induced in the presence of nitrate, but it is not possible to distinguish on the basis of available data whether nitrate causes activation of the preexisting enzyme or its de novo synthesis. It is also possible that nitrite reductase is derepressed upon utilization of repressive amino acids after the transfer of the cells to the fresh medium.

The increase in nitrite reductase and nitrate reductase activities occurs simultaneously in tobacco cells grown on nitrate containing medium and continues roughly until the bulk of the nitrate is utilized from the medium. This suggests that the mechanism governing the appearance of the two enzymes is similar.

When the activities of both enzymes were followed in tobacco cells during 16 days of growth nitrite reductase activity persisted after nitrate reductase decreased to an undetectable level. The difference in the levels of activities of the two enzymes in older cells can be explained by the observation that nitrite reductase is more stable than nitrate reductase upon transfer of induced cells to nitrate free medium. The difference in the stability of the two enzymes has been observed also in other plants. The decay rate of nitrate reductase in the absence of substrate is twice as fast as that of nitrite reductase in the wheat seedlings (Schrader et al., 1968). Nitrite reductase in radish cotyledons is also more stable than nitrate reductase

(Ingle et al., 1966). The fact that the first enzyme of assimilatory nitrate reduction is especially labile might be of biological importance for the efficient regulation of nitrate assimilation.

The rate of nitrite reductase formation decreases if amino acids are added to growth medium containing nitrate. The decrease in nitrite reductase activity could be caused by inhibition of the enzyme by amino acids. However, there was no decrease in nitrite reductase activity in a mixture of homogenates of cells grown on nitrate and cells grown on nitrate plus casein hydrolysate indicating that an effective concentration of inhibitor is not present in cells grown in the presence of amino acids. The possibility that amino acids act by increasing the rate of decay of nitrite reductase has to be excluded since, as demonstrated in this study, amino acids do not enhance the decay of nitrite reductase activity. It appears, therefore, that amino acids act by preventing synthesis or activation of nitrite reductase.

Data reported in this study do not provide a full understanding of regulation of nitrate reductase and nitrite reductase in tobacco cells. Studies of regulation of metabolic pathways in bacteria were advanced by combining data on enzyme activities in conditions of induction and repression, and genetic studies. These data made it possible to distinguish several regulatory systems. It can not be assumed in the absence of any conclusive evidence that the

same regulatory mechanisms operate in bacteria and in higher plants, but it might be informative to compare the regulation of nitrate assimilatory enzymes with the different mechanisms of regulation of synthesis of bacterial enzymes described below:

1. The enzymes of a metabolic pathway, i.e., enzymes responsible for utilization of exogenous lactose in E. coli, are coded for by a cluster of structural genes and are under control by regulator molecules coded for by distinct regulatory gene. This cluster of genes and the associated operator region is designated "operon" (Jacob and Monod, 1961). The genes of the operon are expressed in a coordinate manner, i.e., the ratio of the amount of any enzyme to the amount of another enzyme in the same operon remains constant regardless of its induction or repression. The activities of the enzymes of a given operon increase simultaneously; however, it has been demonstrated that the genes of lactose operon appear in temporal sequence (Alpers and Tomkins, 1966). In certain conditions the genes of the histidine operon are translated in a sequential manner (Berberich et al., 1967).

2. Enzymes of a metabolic pathway are coded for by structural genes not adjacent to each other. Such a system consists of several operons and is under control of a single regulatory gene, and has been designated "regulon" (Maas and Clark, 1964). The genes of the regulon show similar, but

not coordinated, expression as described for arginine biosynthetic enzymes by Maas and Clark (1964).

3. Enzymes of a metabolic pathway are induced coordinately in groups, as in the pathway of degradation of mandelate (Stevenson and Mandelstam, 1965; Mandelstam and Jacoby, 1965). In this pathway the first three enzymes are coordinately induced by mandelate, the fourth enzyme is induced by its substrate and its product, in turn, coordinately induces a group of remaining enzymes. A considerable lag (40 minutes) is observed between the appearance of the enzymes of the first and the third operon. This mode of induction is termed "sequential." Each of the three operons can be repressed. The product of the last enzyme of each operon can repress the enzymes of this operon and also independently, the enzymes of the operons coding for enzymes catalyzing earlier steps in the mandelate pathway. This type of repression is called "multisensitive."

No information is available with respect to the linkage of the genes coding for nitrate reductase and nitrite reductase in plants. It has been demonstrated that nitrite reductase is a chloroplast enzyme while nitrate reductase has been characterized as a soluble enzyme (Ritenour et al., 1967). However, localization of the enzyme in cell organelles does not exclude the possibility that it is coded for by nuclear genetic material. Utilizing genetic methods it has been demonstrated that a plant

mitochondrial enzyme is coded for by nuclear genes (Longo and Scandalios, 1969).

The fact that ratios of nitrate reductase to nitrite reductase activity in tobacco cells grown on media containing different amounts of amino acids is not constant indicates that the regulation of these two enzymes in tobacco cells, unlike yeast (Sims et al., 1968), is not coordinate.

The fact that there is no lag in the appearance of nitrite reductase activity in cells grown on nitrate does not support the idea advanced by Ingle et al. (1966) that the induction of nitrate reductase and nitrite reductase is sequential. Also inconsistent with both the sequential model of induction and the postulate that nitrite and not nitrate is the inducer of nitrite reductase activity is the fact that tungstate had little effect on the formation of nitrite reductase activity, while it caused a considerable decrease in the activity of nitrate reductase. Nitrate reductase and nitrite reductase were induced by nitrite in radish cotyledons (Ingle et al., 1966) and nitrite induces nitrate reductase in cauliflower (Candela et al., 1957). Nitrite, therefore, may be an inducer of both enzymes in higher plants. However, nitrate might be contaminated with sufficient nitrate or it may be oxidized to nitrate either in the medium or by the plant.

The nitrate assimilatory enzymes were induced in yeast by both nitrate and nitrite (Sims et al., 1968) and

in Aspergillus (Pateman et al., 1967). Regulation of nitrate and nitrite reductase is under control of a regulatory gene in Aspergillus, but structural genes for nitrate reductase and nitrite reductase are not linked (Pateman et al., 1967). There is no evidence for the existence of the regulatory genes of nitrate assimilation in higher plants. If such genes exist they will be difficult to demonstrate due to the difficulties of obtaining single gene mutations in higher plants.

BIBLIOGRAPHY

BIBLIOGRAPHY

Afridi, M.M.R., and E.J. Hewitt. 1962. Induction and stability of nitrate reductase in tissues of higher plants. Life Sci., 1, 287.

Afridi, M.M.R., and E.J. Hewitt. 1965. The inducible formation of nitrate reductase in higher plants. II. Effect of environmental factors, antimetabolites and amino acids on induction. J. Exp. Bot., 16, 628.

Agarwala, S.C. 1952. Relation of nitrogen supply to the molybdenum requirement of cauliflower grown in sand culture. Nature, 169, 1099.

Alpers, D.H., and G.M. Tomkins. 1966. Sequential transcription of the genes of lactose operon and its regulation by protein synthesis. J. Biol. Chem., 241, 4434.

Beevers, L., D. Flesher and R.H. Hageman. 1964. Studies on the pyridine nucleotide specificity of nitrate reductase in higher plants and its relationship to sulfhydryl level. Biochem. Biophys. Acta, 89, 453.

Beevers, L., L.E. Schrader, D. Flesher and R.H. Hageman. 1965. Role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol., 40, 691.

Berberich, M.A., J.S. Kovach and R.F. Goldberger. 1967. Chain initiation in a polycistronic message: sequential versus simultaneous derepression of the enzymes for histidine biosynthesis in Salmonella typhimurium. Proc. Nat. Acad. Sci., U.S., 57, 1857.

Betts, G.F., and E.J. Hewitt. 1966. Photosynthetic nitrite reductase and the significance of hydroxylamine in nitrite reduction in plants. Nature, 210, 1327.

Candela, M.I., E.G. Fisher and E.J. Hewitt. 1957. Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiol., 32, 280.

Creswell, C.F., R.H. Hageman, E.J. Hewitt and D.P. Hucklesby. 1965. The reduction of nitrate, nitrite and hydroxylamine to ammonia by enzymes from Cucurbita pepo L., in the presence of reduced benzyl viologen as electron donor. Biochem. J., 94, 40.

Evans, H.J., and A. Nason. 1953. Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiol., 28, 233.

Evans, H.J., and N.S. Hall. 1955. Association of molybdenum with nitrate reductase from soybean leaves. Science, 122, 922.

Filner, P. 1965. Semi-conservative replication of DNA in a higher plant cell. Expt. Cell Res., 39, 33.

Filner, P. 1966. Regulation of nitrate reductase in cultured tobacco cells. Biochem. Biophys. Acta, 118, 299.

Hageman, R.H., C.F. Creswell and E.J. Hewitt. 1962. Reduction of nitrate, nitrite and hydroxylamine to ammonia by enzymes extracted from higher plants. Nature, 193, 247.

Heimer, Y.M., J.L. Wray and P. Filner. Unpublished. The effect of tungstate on nitrate assimilation in higher plant tissues.

Hewitt, E.J., and G.F. Betts. 1963. Reduction of nitrite and hydroxylamine by ferredoxin and chloroplasts grana from Cucurbita pepo. Biochem. J., 89, 20p.

Hewitt, E.J., D.P. Hucklesby and G.F. Betts. 1968. Nitrite and hydroxylamine in inorganic nitrogen metabolism with reference principally to higher plants. In: Recent Aspects of Nitrogen Metabolism in Plants. Ed. by E.J. Hewitt and C.V. Cutting. Academic Press. New York, p. 47.

Huzisige, H., and K. Satoh. 1961. Photosynthetic nitrite reductase. I. Partial purification and properties of the enzyme from spinach leaves. Botan. Mag., 74, 178.

Huzisige, H., K. Satoh, K. Tanaka and T. Hayasida. 1963. Photosynthetic nitrite reductase. II. Further purification and biochemical properties of the enzyme. Plant Cell Physiol., 4, 307.

Ingle, J., K.W. Joy and R.H. Hageman. 1966. The regulation of activity of the enzymes involved in the assimilation of nitrate by higher plants. Biochem. J., 100, 577.

Jacob, F., and J. Monod. 1961. On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol., Vol. 26, 193.

Joy, K.W., and R.H. Hageman. 1966. The purification and properties of nitrite reductase from higher plants, and its dependence on ferredoxin. Biochem. J., 100, 263.

Joy, K.W. 1968. Enzymes of nitrate assimilation in Lemna. Plant Physiol., 43, S-8.

Keeler, R.F., and J.E. Varner. 1958. The metabolism of molybdate and tungstate in Azotobacter. In: Trace Elements. Academic Press, p. 297.

Kessler, E. 1955. Role of photochemical processes in the reduction of nitrate by green Algae. Nature, 176, 1069.

Kessler, E. 1964. Nitrate assimilation by plants. Ann. Rev. Plant Physiol., 15, 57.

Lazzarini, R.A., and D.E. Atkinson. 1961. A triphospho-pyridine nucleotide-specific nitrite reductase from Escherichia coli. J. Biol. Chem., 236, 3330.

Longo, G.P., and J.G. Scandalios. 1969. Nuclear gene control of mitochondrial malic dehydrogenase in maize. Proc. Nat. Acad. Sci., (in press).

Losada, M., A. Paneque, J.M. Ramirez and F.F. Del Campo. 1963. Mechanism of nitrite reduction in chloroplasts. Biochem. Biophys. Res. Commun., 10, 298.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 263.

Maas, W.K., and A.J. Clark. 1964. Studies on the mechanism of repression of arginine biosynthesis in Escherichia coli. II. Dominance of repressibility in diploids. J. Mol. Biol., 8, 365.

Mandelstam, J., and G.A. Jacoby. 1965. Induction and multi-sensitive end-product repression of the enzymic pathway degrading mandelate in Pseudomonas fluorescens. Biochem. J., 94, 569.

Maretzki, A., and A. Dela Cruz. 1967. Nitrate reductase in sugarcane tissues. Plant Cell. Physiol., 8, 65.

Miflin, B.J. 1967. Distribution of nitrate and nitrite reductase in barley. Nature, 214, 1133.

Mortenson, L.E., R.C. Valentine and J.E. Carnahan. 1962. An electron transport factor from Clostridium pastuerianum. Biochem. Biophys. Res. Commun., 7, 448.

Nicholas, D.J.D., and A. Nason. 1954. Molybdenum as an electron carrier in nitrate reductase action. Arch. Biochem. Biophys., 51, 310.

Paneque, A., J.M. Ramirez, F.F. Del Campo and M. Losada. 1964. Light and dark reduction of nitrite in a reconstituted enzymic system. J. Biol. Chem., 237, 1737.

Paneque, A., F.F. Del Campo, J.M. Ramirez and M. Losada. 1965. Flavin nucleotide nitrate reductase from spinach. Biochem. Biophys. Acta, 109, 79.

Pateman, J.A., B.M. Rever, and D.J. Cove. 1967. Genetic and biochemical studies of nitrite reduction in Aspergillus nidulans. Biochem. J., 104, 103.

Ramirez, J.M., F.F. Del Campo, A. Paneque and M. Losada. 1966. Ferredoxin nitrite reductase from spinach. Biochem. Biophys. Acta, 118, 58.

Ritenour, G.L., K.W. Joy, J. Bunning and R.H. Hageman. 1967. Intracellular localization of nitrate reductase, nitrite reductase and glutamic acid dehydrogenase in green leaf tissue. Plant Physiol., 42, 233.

Sanderson, G.W., and E.C. Cocking. 1964. Enzymic assimilation of nitrate in tomato plants. II. Reduction of nitrite to ammonia. Plant Physiol., 39, 423.

Schrader, L.E., G.L. Ritenour, G.L. Eilrich and R.H. Hageman. 1968. Some characteristics of nitrate reductase from higher plants. Plant Physiol., 43, 930.

Shin, M., and Y. Oda. 1966. Photosynthetic nitrite reductase from spinach. Plant Cell Physiol., 7, 643.

Sims, A.P., B.F. Folkes and A.H. Bussey. 1968. Mechanisms involved in the regulation of nitrogen assimilation in microorganisms and plants. In: Recent Aspects of Nitrogen Metabolism in Plants. Ed. by E.J. Hewitt and C.V. Cutting. Academic Press. New York, p. 91.

Snell, F.D., and C.T. Snell. 1949. Colorimetric Methods of Analysis. D. Van Nostrand Company. New York, p. 804.

Spencer, D., and J. G. Wood. 1954. The role of molybdenum in nitrate reduction in higher plants. Austr. J. Biol. Sci., 7, 425.

Stevenson, I.L., and J. Mandelstam. 1965. Induction and multi-sensitive end-product repression in two converging pathways degrading aromatic substances in Pseudomonas. Biochem. J., 96, 354.

Stewart, G.R. 1968. The effect of cyclohexamide on the reduction of nitrate and nitrite reductase in Lemna minor. Phytochemistry, 7, 1139.

Vanecko, S., and J.E. Varner. 1955. Studies of nitrite metabolism in higher plants. Plant Physiol., 30, 388.