AN ABSTRACT OF THE THESIS OF

Ronald Eugene Nitsos (Name) for the M.S. (Degree) in Botany (Major)

Date thesis is presented May 12, 1966

Title The Effects of Univalent Cations on the Inductive Formation of Nitrate Reductase

Abstract approved Redacted for privacy
(Major professor)

An investigation was undertaken to determine whether potassium is indeed necessary for the *in vivo* synthesis of a specific protein. The effects of univalent cations on the inductive formation of nitrate reductase and on the level of activity of representative nonadaptive enzyme are presented.

Cultures of *Neurospora crassa* were grown in media without added univalent cations and the mycelia then transferred to induction media containing different univalent cations. The inductive formation of nitrate reductase was followed over a four-hour period. During the same period of incubation assays for the activity of the constitutive enzyme pyruvic kinase were conducted.

Results of nitrate reductase assays during the induction period indicate that the formation of this enzyme

is dependent upon an adequate concentration of potassium in the medium. Sodium, lithium, and ammonium could not substitute for potassium in the inductive formation of this enzyme. Rubidium was the only univalent cation besides potassium that partially functioned in the synthesis of nitrate reductase.

Studies of pyruvic kinase activity during the induction period indicate that its activity remained nearly constant. It appears that conditions were optimum for the rapid synthesis of nitrate reductase, but the rate of protein synthesis in general was not greatly changed during the induction period of nitrate reductase.

THE EFFECTS OF UNIVALENT CATIONS ON THE INDUCTIVE FORMATION OF NITRATE REDUCTASE

by

RONALD EUGENE NITSOS

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1966

APPROVED:

Redacted for privacy

Professor of Plant Physiology
In Charge of Major

Redacted for privacy

Chairman of Department of Botany

Redacted for privacy

Dean of Graduate School

Date thesis is presented May 12, 1966

Typed by Clistie F. Stoddard

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. Harold J. Evans for his encouragement and for his assistance during this investigation and preparation of this dissertation.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

THE EFFECTS OF UNIVALENT CATIONS ON THE INDUCTIVE FORMATION OF NITRATE REDUCTASE

INTRODUCTION

General Requirement for Potassium

Potassium (K^+) is necessary for the growth of most and perhaps all organisms. The first work showing the necessity of this cation for higher plant growth was conducted by Lucanus (74) in 1865, and by Birner and Lucanus (16) in 1866. The concentration of K^+ found in plants is greater than that of any other cation as shown by the extensive study of Beeson (11). Evans and Sorger (40) have cited evidence indicating that the concentrations of K^+ in 15 samples of normal dry leaves ranged from 1.7 to 2.8 percent, whereas in samples of K^+ deficient dry leaves the concentrations ranged from 0.8 to 1.2 percent. Analyses of 14 pasture species by Asher (8) showed K^+ concentrations of 0.1 N to 0.24 N in normal plants supplied with adequate K^+ and concentrations of 0.013 N to 0.17 N in deficient plants. The concentrations of K^+ in normal leaves is then approximately 0.05 N, while that in deficient leaves is about 0.02 N. Normal plant requirements for this cation are therefore quite large.

Relation of Potassium to Other Univalent Cations

Sodium (Na^+) has been shown to be an essential nutrient for certain microorganisms and several halophytic plants. Brownell (21) has demonstrated a nutritional requirement for Na^+ by Atriplex visicaria Heward ex Benth, and Williams (122) has shown a growth response to Na^+ by the halophyte Halogeton glomeratus (M. Bieb) C. A. Mey. According to Allen and Arnon (4) Na^+ is essential for growth of the blue-green alga Anabaena cylindrica Lemm. Woolley (125) could not show a specific requirement for Na^+ for the growth of Lycopersicon esculentum Mill.; however, the addition of one millimole of NaCl per liter of growth medium resulted in a 12 percent increase in dry weight. Considerable literature (10, 47, 48, 68, 84) is available providing evidence that Na^+ is beneficial to the growth of many higher plant species; nevertheless, it cannot substitute entirely for K^+. Thus it would appear that while some halophytic higher plant species require Na^+, the element functions in most species in a supplementary way when the K^+ supply is inadequate.

Evidence is available (47, 48, 91) that rubidium (Rb^+) may partially replace K^+ in the growth of several microorganisms and higher plants. Rahn (89) has shown that Rb^+ can partially substitute for K^+ in cultures of certain species of Aspergillus, Saccharomyces, and Mycobacterium. MacLeod and

Snell (76) showed that Rb^+ would partially replace K^+ in cultures of Streptococcus faecalis Anderson and Horder and Leuconostoc mesenteroides van Tieghem.

Lithium (Li^+) is of little nutritional value to higher plants and appears to be toxic. Edwards (35), and Aldrich, Vanselow and Bradford (3) have investigated the effects of Li^+ on the growth of corn root tips and citrus. No conclusive evidence was obtained showing a nutritional requirement for this cation. In the culture of citrus (3), Li^+ was reported to be toxic.

The ammonium ion (NH_4^+) is an active metabolite in the nitrogen metabolism of plants. In addition to this important role, this ion has been reported to function as a cofactor for specific enzymatic reactions (70, 105). In this context it plays an indirect role in the nutrition of plants and is considered here with the univalent cations. The physical properties of NH_4^+ including the radius of the ion and the charge are similar to those of other univalent cations such as Rb^+ and K^+, and thus offer an explanation for the capacity of the ion to substitute for K^+ in certain enzymatic reactions (61).

Association of Potassium with Specific Metabolic Processes

In a recent review, Evans and Sorger (40) have compiled the effects of K^+ deficiency in vivo and suggest that

this element functions in several different metabolic processes since numerous enzyme systems have been shown to require K^+ or other univalent cations. A deficiency of K^+ might be expected to affect overall metabolic processes in the tissues and to greatly reduce the activity of all K^+ requiring enzymes participating in the metabolic process in question.

A consistent consequence of K^+ deficiency in plants is the accumulation of soluble carbohydrates and reducing sugars (34, 113), and the inhibition of starch synthesis (85, 97). In higher plants carbohydrate utilization might be easily blocked as a result of the loss of activity of a necessary enzyme component of glycolysis such as pyruvic kinase, an enzyme shown to require K^+ or other univalent cations (39, 78). Metabolic blocks of this type could logically account for the observed accumulation of carbohydrates. Starch synthesis in plants and glycogen synthesis in animals are reported to be inhibited by K^+ deficiency (23, 88).

Potassium deficiency in certain microorganisms is also associated with a reduction in oxidative phosphorylation and photophosphorylation. According to Latzko (66, 67), oxygen and substrate dependent phosphate uptake, and light dependent photophosphorylation are dependent upon the presence of K^+ in the reaction medium.

The accumulation of amino acids and the failure to synthesize protein is another major metabolic symptom

associated with K^+ deficiency in plants. This deficiency symptom may be related to the observation of Webster (116) that the catalysis of the incorporation of amino acids into protein by extracts of Pisum sativum Linn. was dependent upon K^+. Several different partially purified enzyme systems known to be associated with the protein synthesizing mechanism have been reported to require K^+ for activity (29, 54, 72, 95, 96, 117). To date, however, the specific site or sites where limited K^+ causes a block in protein synthesis in vivo has not been identified.

Objectives of this Investigation

In this study it was decided to ascertain whether K^+ is indeed necessary for the synthesis of a specific protein. The effects of univalent cations on the in vivo adaptive formation of nitrate reductase and on the level of activity of a representative nonadaptive enzyme are presented.

LITERATURE REVIEW

Enzyme Induction

Every living organism continually synthesizes enzymes during its life span. The quantity of essential enzymes must increase in accordance with the increase in growth of the organism. During the stationary phase of growth, a continuous synthesis is necessary to replace those enzymes which have undergone destruction. Enzyme synthesis in the living organism has been shown to be under genetic and metabolic control.

The formation of enzymes is under the direction of the genetic make-up (genotype) of the organism (57). In 1945, Beadle (9), using the extensive information available from metabolic studies of mutant strains of Neurospora and other organisms, developed the "one-gene-one-enzyme" theory which proposes a separate gene for the formation of each enzyme. According to this theory, a specific enzyme can be synthesized only when its complementary gene is present. If the gene is absent or mutated, the active enzyme is not formed. The genotype of the organism would thus determine its enzyme complement and restrict the production of enzymes to those necessary for its metabolism. Chromosome maps of several microorganisms have shown that specific genes do control the synthesis of

numerous enzymes, including β-galactosidase (65) and tyrosinase (55).

In addition to genetic control, the synthesis of many enzymes by organisms also is controlled by the presence of certain metabolites (44). While the presence of a gene is required for the production of a specific enzyme, its presence alone does not assure that this particular enzyme will be formed. In many cases an inducer that is often the substrate for the enzyme may be required for enzyme formation (46, 63, 87). The action of the inducer is not related to the genetic information in the organism, but functions in some unknown manner as a stimulus to the synthesizing machinery. The formation of an enzyme in the presence of an inducer is brought about by the complete de novo synthesis of an enzyme molecule (57). The requirement for a substrate or similar molecules for the adaptive synthesis of β-galactosidase has been investigated in detail by Cohn (28), and Monod, Cohn-Bazire and Cohn (79).

Enzyme induction, then, involves the de novo synthesis of a specific enzymatically active protein from its constituent amino acids. This process is of great survival value to microorganisms in chemical competition for the nutrients in the environment. The fact that certain enzymes are adaptively formed provides a convenient means for the study of

many metabolic systems without disruption of the organism as an entity.

Role of Potassium in Amino Acid Synthesis

Amino acids have been shown to constitute the basic structural units of proteins. Extensive determinations of the amino acid components of different plant proteins have shown that they are made up of at least 20 different L-amino acids (110). Synthesis of these acids is accomplished by several complex pathways, some of which have been described in detail. The role of K^+ in some of the enzyme systems that participate in amino acid biosynthesis has been examined and its deficiency effects indicated.

One of the major pathways of amino acid formation is the reductive amination of certain keto acids (33, 118). Glutamate and aspartate, two amino acids that serve as precursors of other amino acids, are reported to be synthesized in this process (24, 118). Detailed investigations of the role of glutamate as a precursor in the synthesis of such amino acids as proline (108, 111, 112), leucine (2, 62), arginine (60), alanine (69), and histidine (80) have been reported. In addition to its formation by reductive amination of oxaloacetate, aspartate is also synthesized by the direct amination of fumaric acid involving the enzyme aspartase (123). Aspartate is also involved in the synthesis of arginine and

ornithine (60), as well as serving as a precursor for the synthesis of threonine and homoserine (1). Since the biosynthesis of keto acids may be a primary step in glutamate and aspartate formation, any alteration in keto acid synthesis would be expected to influence the synthesis of many amino acids. Potassium or certain other univalent cations are essential for the activity of pyruvic kinase (39). If K^+ deficiency were sufficiently severe to cause a block in the conversion of phosphoenolpyruvate to pyruvic acid in glycolysis, one would expect that keto acid biosynthesis would be impaired as an indirect effect of the deficiency.

A more direct role of K^+ in the synthesis of amino acids is indicated by the discovery of Paulus and Gray (86) that the catalysis of the synthesis of aspartyl phosphate by aspartokinase from certain microorganisms is dependent upon K^+. Since aspartyl phosphate synthesis is a necessary step in the biosynthesis of threonine and homoserine (18, 19), and possible isoleucine (2, 107) and lysine (1), it is apparent that many phases of amino acid metabolism probably could be influenced by K^+ deficiency. Furthermore, the enzymatic activation of methionine to form S-adenosylmethionine is reported to require K^+ (25, 81). S-adenosylmethionine plays an essential role in many transmethylation reactions as well as participating in the biosynthesis of cysteine (106).

The possible effects of K^+ deficiency on the intermediates in the synthesis of a variety of amino acids are too numerous to mention. Potassium is needed for the formation of carbamyl phosphate (77), pyruvic acid (39), and tetrahydrofolate (15). All these intermediates are important in the synthesis of certain amino acids.

Role of Potassium in Protein Synthesis

The following is a list of the ingredients which are known to be required for the cell-free synthesis of a finished polypeptide: amino acyl-soluble ribonucleic acid (amino acids, adenosine triphosphate, soluble ribonucleic acid, activating enzymes), polysomes (ribosomes, messenger ribonucleic acid, magnesium ions), guanosine triphosphate, enzyme fractions, and thiol groups.

Nucleic Acid Components and Their Role

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the major types of nucleic acids known to function in protein synthesis. Both of these are found in animals, plants, and microorganisms. DNA is present in the nucleus or nuclear region while RNA occurs predominately in the cytoplasm. The structures of these two types of nucleic acids are quite similar with the main exception being that deoxyribose is the sugar in DNA nucleotides whereas ribose is the

sugar component of RNA.

The roles of these two nucleic acids, however, are quite different. DNA comprises the genetic material of the chromosomes whose discrete portions, the genes, determine the hereditary characteristics of the organism. DNA functions in directing the synthesis of particular types of RNA which determine the amino acid sequence in proteins (103). There are at least three distinct types of RNA present in organisms. The first type, ribosomal RNA, is formed of ribonucleoprotein and concentrated into particles called ribosomes. Ribosomes serve as the active site for amino acid incorporation into protein and also serve as structural supports for messenger RNA (52). Messenger RNA (MRNA) is the transcriber of protein synthesis in that it relates the genetic information of DNA. It is synthesized on a DNA template and remains as a single stranded polymer. MRNA, in association with ribosomes, functions as a template and, as such, regulates the joining together of amino acids in a genetically determined pattern (20). Transfer or soluble RNA (SRNA) is a low molecular weight RNA which functions as an acceptor of activated amino acids and as a carrier of them to the sites of protein synthesis on the MRNA templates (53). SRNA also is single stranded and probably is formed on a DNA template. A distinguishing characteristic of it is the presence of additional bases in its structure, different from those normally present

in RNA (98).

Considerable work on the roles of DNA and RNA in the synthesis of proteins has been conducted, and excellent reviews are available which give a much better insight into their functions than could be accomplished in this report (26, 41, 56, 75, 94, 124). Since DNA functions as a director in RNA synthesis, any disruption of this continuity would be expected to cause serious consequences in an organism. The possible role of K^+ in the formation and role of DNA and RNA in the process of protein synthesis will be considered here.

The pyrimidine components found in DNA and RNA are cytosine, thymine, and uracil. Synthesis of orotic acid, the basic analogue of these nucleotides, involves the condensation of carbamyl phosphate and aspartate (90). A definite requirement of K^+ or other univalent cations for the activity of carbamyl phosphate synthetase has been established (77). The indirect role of K^+ for aspartate formation has been discussed in the previous section. Deficiencies of K^+ in either of these two synthetic systems could result in a restriction of the formation of the pyrimidine components of the nucleotide molecules, and thus limit both DNA and RNA formation.

Purines associated with the structures of DNA and RNA are adenine and guanine. Their biosynthesis takes place through a complicated process involving glutamate, glycine, tetrahydrofolic acid, and aspartate to yield the basic

structure inosinic acid (22). Tetrahydrofolic acid serves as a methyl donor for the formation of the purines, and the activation of this complex has been shown to require K^+ (15, 49). In relation to its function, S-adenosylmethionine often serves as the source of the methyl group, and a K^+ requirement for the synthesis of this compound has been discussed (25, 81). A deficiency of K^+ therefore could affect both purine and pyrimidine biosynthesis. Such effects of K^+ deficiency would be expected to influence the formation of both DNA and RNA, and ultimately the synthesis of proteins.

Even though nucleic acids are required for protein synthesis, K^+ deficiency seems to have a greater effect on protein synthesis than nucleic acid synthesis. Ennis and Lubin (36) have studied a mutant strain of *E. coli* having a defective K^+-transport system and reported that the incorporation of C^{14}-guanine into RNA was only partially reduced (30-40 percent) under K^+ deficiency. In contrast, the incorporation of C^{14}-leucine into protein was almost completely inhibited under the conditions of K^+ deficiency. They feel that their results indicate that K^+ has a more prominent role in protein synthesis *per se* than in the actual synthesis of nucleic acids.

Amino Acid Activation

Amino acid activation is a two-step process involving a separate enzyme for each amino acid (12). The enzyme functions by catalyzing the activation of the carboxyl group of the amino acid by adenosine triphosphate (ATP). The products are pyrophosphate and an amino acyl-adenosine monophosphate (AMP) complex that remains bound to the enzyme. The reaction is reversible as indicated by studies using radioactive labeled pyrophosphate (31, 51). ATP is the only nucleotide triphosphate that will effectively function in this reaction (13). The activating enzymes appear to be fairly specific in respect to the amino acids activated; however, some enzymes function with more than one amino acid (64).

The last step in the activation process is a reaction of the amino acyl-AMP-enzyme complex with an SRNA molecule to form the amino acyl-SRNA. The enzyme is liberated, and AMP is a product. The amino acid becomes attached through its carboxyl group to the 2' or 3' hydroxyl group of the ribose moiety of the terminal adenosine group of SRNA (126). Each type of SRNA exhibits considerable but not absolute specificity toward amino acids (14), and more than one SRNA type may exist for each amino acid (121).

The amino acid activation reactions have been shown to require Mg^{++} and a thiol group in addition to ATP. The

activation of tyrosine in cell free extracts appears to function more effectively in a medium containing K^+ than in one containing Na^+ (95). Holley et al. (54), however, concluded that K^+ deficiency in rats had little effect on the activity of this enzyme. In regards to the thiol requirement, Davie, Koningsberger and Lipmann (30) showed that p-chloromercuribenzoate inhibited the activating enzymes. In many in vitro systems, glutathione functions as the thiol group by maintaining a reduced state of thiols presumably on the surface of the activating enzymes.

It is interesting to note that glutathione contains peptide bonds. These bonds are formed by mechanisms different from those involved in the synthesis of proteins. Activating enzymes are not required, and SRNA is not necessary for glutathione synthesis (109). The formation of glutathione, nevertheless, has been shown to require Mg^{++}, ATP, and K^+ (119). The substitution of other univalent cations for K^+ cannot be demonstrated (99).

Binding of MRNA and Amino Acyl-SRNA to Ribosomes

When ribosomes are isolated from bacteria or rabbit reticulocytes in media with a Mg^{++} concentration of 1.0 mM, particles with sedimentation constants of 70S to 80S are obtained (115). The ribosomes most active in the synthesis of proteins are those having sedimentation constants greater

than 80S (45, 92). By the use of mild conditions for the preparation of ribosome fractions, it was shown that fractions heavier than 80S apparently resulted from an attachment of ribosomes to MRNA (114). This finding contributed to the hypothesis that ribosomes move along the MRNA strand synthesizing a polypeptide chain as they progress. At the end of the MRNA chain, the ribosomes become detached and the polypeptide chain is released. The attachment of the MRNA to the ribosomes, therefore, is believed to be an important step in the protein synthesizing apparatus. According to Lubin (72), K^+ is important in the binding of MRNA to the ribosomes from E. coli. In a cell-free preparation from rabbit reticulocytes, Schweet et al. (96) have shown that 0.07 M K^+ in addition to Mg^{++} is necessary for the nonenzymatic binding of polyuridylic acid (poly-U) to ribosomes. This reaction was shown to be reversible at low Mg^{++} concentrations. According to Schweet et al. (96), K^+ and Mg^{++} are important in maintaining the integrity and structural configuration of the complex.

The next step in protein synthesis involves the binding of amino acyl-SRNA to the MRNA-ribosome complex. This attachment is postulated to involve an interaction of the triplet code of MRNA with SRNA. Additional forces, however, are believed to function in the nonspecific binding of amino acyl-SRNA to the MRNA-ribosome complex. The existence of an amino acyl-SRNA-MRNA-ribosome complex has been observed in

many cases (17). Its role as an obligatory intermediate in protein synthesis was established by Nakamoto et al. (82) utilizing poly-U as the messenger, and ribosomes and extracts from E. coli. In their studies, preincubation of poly-U, amino acyl-SRNA, ribosomes, and either NH_4^+ or K^+ resulted in the formation of an amino acyl-SRNA-MRNA-ribosome complex which could be used immediately in the synthesis of a polypeptide. Work by Lubin and Ennis (73) also has provided evidence for a requirement of K^+ or NH_4^+ in the transfer of the amino acyl-SRNA to the ribosomes. According to Arlinghaus et al. (5), the binding of amino acyl-SRNA to the complex of MRNA and ribosomes from rabbit reticulocytes requires K^+, GTP, and two soluble enzyme fractions. Conclusions consistent with these have been reported by Spyrides (104). In this case NH_4^+ was superior to K^+ as the univalent cation activator. Glutathione was not required for the binding of amino acyl-SRNA to the MRNA-ribosome complex, but it is reported to be necessary for the final reactions of protein synthesis. It seems clear, then, that both the enzymatic and nonenzymatic binding of amino acyl-SRNA to the ribosomes is an important reaction in protein synthesis that requires either K^+ or NH_4^+ as a cofactor.

While GTP has been shown to be necessary for protein synthesis, its exact role in this process is still somewhat vague. Conway and Lipmann (29) have established a close

correlation between the activity of the poly-U synthesizing system and the GTPase activity in extracts of E. coli. Both systems show a response to NH_4^+ or K^+ and were inhibited by either Na^+ or Li^+. It has been known for some time that two enzyme fractions function in this amino acyl-SRNA transfer to polypeptides (17). Utilizing rabbit reticulocytes, Arlinghaus, Shaeffer and Schweet (6, 7) have shown an association of the GTPase activity with one of the enzyme fractions, designated TF-1. This extract catalyzes the transfer of the amino acyl-SRNA to the ribosome, and this process is known to require K^+.

Formation of the Peptide Bond and RNA Turnover

The exact mechanism of the formation of the peptide bond itself is still one of the major unsolved problems of protein synthesis. After the amino acyl-SRNAs are transferred to the MRNA-ribosome complex, the amino acids are joined together in a linear fashion to the C-terminal end of the growing polypeptide (120). Once the amino acids are condensed, the SRNA is released and can then function in the transfer of other amino acids (53). Upon completion, the polypeptide and the ribosomes are released from the MRNA.

Another factor that may be indirectly involved in protein synthesis is the formation and degradation of RNA in the organism. Since Ennis and Lubin (36) have established

that the effects of K^+ deficiency on RNA synthesis is less than the effect on protein synthesis, the possibilities of K^+ playing a key role in RNA synthesis seems remote. The degradation of RNA, however, has been associated with a K^+ requirement. Spahr and Schlessinger (102) have described a phosphodiesterase which degrades RNA. The activity of this enzyme is dependent on K^+. In a series of experiments utilizing the K^+ deficient mutant of E. coli, Ennis and Lubin (37, 38) have shown that RNA synthesized during K^+ deficiency is stable in a medium containing Na^+ but is rapidly degraded in one containing K^+. They attribute this degradation to the possible presence of the phosphodiesterase described by Spahr and Schlessinger (102). In addition, they have shown that K^+ depleted cells returned to a medium containing adequate K^+ initiate protein synthesis more rapidly than RNA synthesis. These results support the theory that the inhibition of protein synthesis by K^+ deficiency lies within the peptide bond synthesizing apparatus. The effects of K^+ deficiency on the synthesis of precursors and cofactors seems to be indirect.

MATERIALS AND METHODS

Cultural Methods

A wild strain of Neurospora crassa, kindly supplied by Dr. Ronald Cameron, was used in all of the experiments. The organism was cultured in a series of different media. The chemicals used in the preparation of the various media were reagent grade, and all solutions were prepared in glass distilled water. Glassware and other vessels utilized were acid washed with 3 N HCl and thoroughly rinsed with distilled water before use.

Media

The composition of the three types of media employed in this investigation are summarized in Table I. Fries' medium has been described by Ryan et al. (93). The concentrations of the micronutrients used in all the media are the same as those present in Fries' medium. Slant cultures used to grow the organism for spore production contained Fries' medium with 15 g agar added per liter of solution. The Standard medium and the Induction medium are modifications of an inorganic nutrient solution described by Evans (39). The Standard medium has neither NO_3^- nor K^+ added while the level K^+ was varied in the Induction medium by the addition of the desired amounts of K_2SO_4. The pH of the Standard medium was

TABLE I. Composition of the various media used in this study.

Nutrients	Concentration of Nutrient in Medium		
	Fries'	Standard	Induction
Na-Tartrate	1.03 g/l		
NaCl	0.10 g/l		
$NaNO_3$	3.00 g/l		
KH_2PO_4	3.00 g/l		
$MgSO_4$	0.50 g/l		0.50 g/l
$CaCl_2 \cdot 2H_2O$	0.10 g/l	0.07 g/l	
$MgHPO_4 \cdot 3H_2O$		2.09 g/l	1.40 g/l
$CaSO_4$		0.13 g/l	
L-Glutamic Acid		5.15 g/l	
$Ca(NO_3)_2 \cdot 4H_2O$			1.42 g/l
Sucrose	20.00 g/l	20.00 g/l	20.00 g/l
Biotin*	10.0 µg/l	10.0 µg/l	10.00 µg/l
Micronutrients*			
$Na_2B_4O_7 \cdot 10H_2O$	89 µg/l	89 µg/l	89 µg/l
$CuCl_2 \cdot 2H_2O$	270 µg/l	270 µg/l	270 µg/l
$MnCl_2 \cdot 2H_2O$	72 µg/l	72 µg/l	72 µg/l
$(NH_4)_6Mo_7O_{24}$	7 µg/l	7 µg/l	7 µg/l
$ZnCl_2$	5 mg/l	5 mg/l	5 mg/l
FeEDDHA (6% Fe)	3 mg/l	3 mg/l	3 mg/l

* Added as solutions

adjusted to 4.5 with solid $Ca(OH)_2$. The pH of the Induction medium also was adjusted to 4.5 using HCl. The different media were autoclaved at 15 pounds pressure for 15 minutes and cooled prior to inoculation.

Growth Conditions

For the growth curves, the Standard medium (Table I) was used to which was added K_2SO_4 in the appropriate concentrations. Erlenmeyer flasks (500 ml) containing 200 ml of the Standard medium were inoculated from conidial suspensions. The flasks were incubated in the dark at 30° C and agitated daily to inhibit sporulation. At 24-hour intervals flasks were removed and the mycelia collected on a Buchner funnel using Whatman No. 1 filter paper. The mats were washed three times with distilled water and dried in an oven at 78° C for 24 hours. Dry weights were determined using a Mettler analytical balance, type H15.

When larger quantities of mycelia were desired, 2800 ml Fernback flasks each containing one liter of the Standard medium were used. Inoculated flasks were incubated in the dark at 30° C for six days with daily agitation. Mats were harvested by squeezing the mycelium and culture liquid through cheesecloth. The mycelial mats were then washed three times with distilled water.

Induction Conditions

The washed mats were divided into approximately equal sections and resuspended in liter flasks containing 330 ml of the Induction medium (Table I). Flasks were incubated on a Gyrotory shaker at 30° C in the dark. At hourly intervals samples were removed, filtered, and washed as described previously. Immediately after washing, the mats were weighed, wrapped in aluminum foil, and placed in powdered dry ice for quick freezing.

Assay Procedures

Preparation of Extract

All operations, unless otherwise stated, were carried out at 0 to 4° C. After freezing, mats were transferred to a cold mortar and pestle containing approximately 0.5 g of acid washed sand. The mats were quickly ground for two to three minutes until a fine powder was obtained, and cold buffer solution (43) was added at the rate of 10 ml per g of tissue. The buffer solution contained the following concentrations of components: Tris-hydroxymethylaminomethan (Tris) buffer, 0.1 M; ethylenediamine tetraacetic acid (EDTA), 3×10^{-4} M; and cysteine, 0.01 M. The pH of the buffer solution was adjusted to 7.8 with HCl. The slurry of buffer and mycelium was ground for an additional minute, decanted, and

centrifuged for 15 minutes at 20,000 x g at 4° C in a Servall refrigerated centrifuge. The supernatant was then decanted and placed in an ice bath until used.

Nitrate Reductase Assay

The enzyme activity was measured using a slight modification of the procedure described by Hageman and Flescher (43). The complete assay mixture in a volume of 1.0 ml contained the following final concentrations of components: sodium pyrophosphate (pH 7.0), 4×10^{-2} M; KNO_3, 1×10^{-2} M; reduced nicotinamide adenine dinucleotide phosphate (NADPH), 3×10^{-4} M; extract, 0.2 ml containing approximately one mg of protein. The reaction was initiated by the addition of the enzyme. The blank contained all components except NADPH. The assay mixture was allowed to incubate for 10 minutes at 27° C, and the reaction stopped by the addition of 0.2 ml of 1.0 M zinc acetate followed by 3.0 ml of 95% ethanol. The solution was thoroughly mixed and then centrifuged for five minutes at approximately 2,000 x g using an International desk model centrifuge. The clear supernatant was decanted, and to it was added 1.0 ml each of a solution of 1% sulfanilamide in 3.2 N HCl and a solution of 0.02% aqueous N-1-naphthyl ethylenediamine dihydrochloride. The color was allowed to develop for 10 minutes. Optical density was determined at 540 mμ using a Beckman DU spectrophotometer

with the slit adjustment at 0.03 mm.

Pyruvic Kinase Assay

The same enzyme extract used for the nitrate reductase assay also was used for the pyruvic kinase assay except that the extract was diluted 1:25 with the buffer solution. The procedure used is a modification of that of Kachmar and Boyer (59). The assay mixture of 1.0 ml contained the following final concentrations of constituents: the cyclohexylammonium salt of phosphoenolpyruvate, 1.5×10^{-3} M; Tris-adenosine diphosphate (Tris-ADP), 2.5×10^{-3} M; Tris buffer (pH 7.0), 5×10^{-2} M; $MgCl_2$, 1×10^{-2} M; KCl, 5×10^{-2} M; Tris-MoO_3 (pH 7.0), 1.25×10^{-3} M; and 0.2 ml of the extract containing approximately 0.05 mg of protein. Blanks contained all components except Tris-ADP. The reaction was initiated by the addition of the enzyme, and the mixture incubated for 10 minutes at 37° C. The reaction was stopped by the addition of 1.0 ml of a 0.0125% solution of 2,4-dinitrophenylhydrazine followed by 7.0 ml of 0.43 N NaOH. The color was allowed to develop for 10 minutes, and the samples were then centrifuged for 10 minutes at approximately 2,000 x g in an International desk model centrifuge. The optical density was determined at 525 mμ on the Beckman DU spectrophotometer with the slit adjusted to 0.03 mm.

Other Assays

Protein was determined using the Folin technique as described by Lowry et al. (71). Optical density measurements were determined at 660 mμ on a Bausch and Lomb 340 colorimeter. Potassium concentrations were determined using the flame photometric method as described by Johnson and Ulrich (58). Percent transmission was determined at 770 mμ using a flame photometer attachment on the Beckman DU spectrophotometer.

RESULTS

Growth Responses to Potassium

Since Fries' medium is normally used for the culture of Neurospora, a comparison of the growth of the organism on the Standard medium with that on Fries' medium was necessary. The results presented in Figure 1 show that the organism grew equally well on either medium. The rates of growth and total yields of dry matter at intervals throughout the growth period were similar.

In order to determine a K^+ concentration which would yield an adequate quantity of mycelium needed for the induction experiments, yet not furnish sufficient K^+ to interfere with the experiments, a preliminary investigation was conducted utilizing various levels of K^+ in the growth medium (Figure 2). As shown by the data, the concentrations of K^+ in the medium had a striking effect on the growth of the organism. The rate of growth increased by a factor of approximately two when the K^+ concentration was increased from no K^+ (0 K^+) to 0.125 mM K^+. This doubling of the rate with the doubling of the K^+ concentration was evident for concentrations up to 0.5 mM K^+. Concentrations of K^+ greater than 0.5 mM resulted in no further marked increase of rates of growth. As shown by the data in Figure 2, considerable

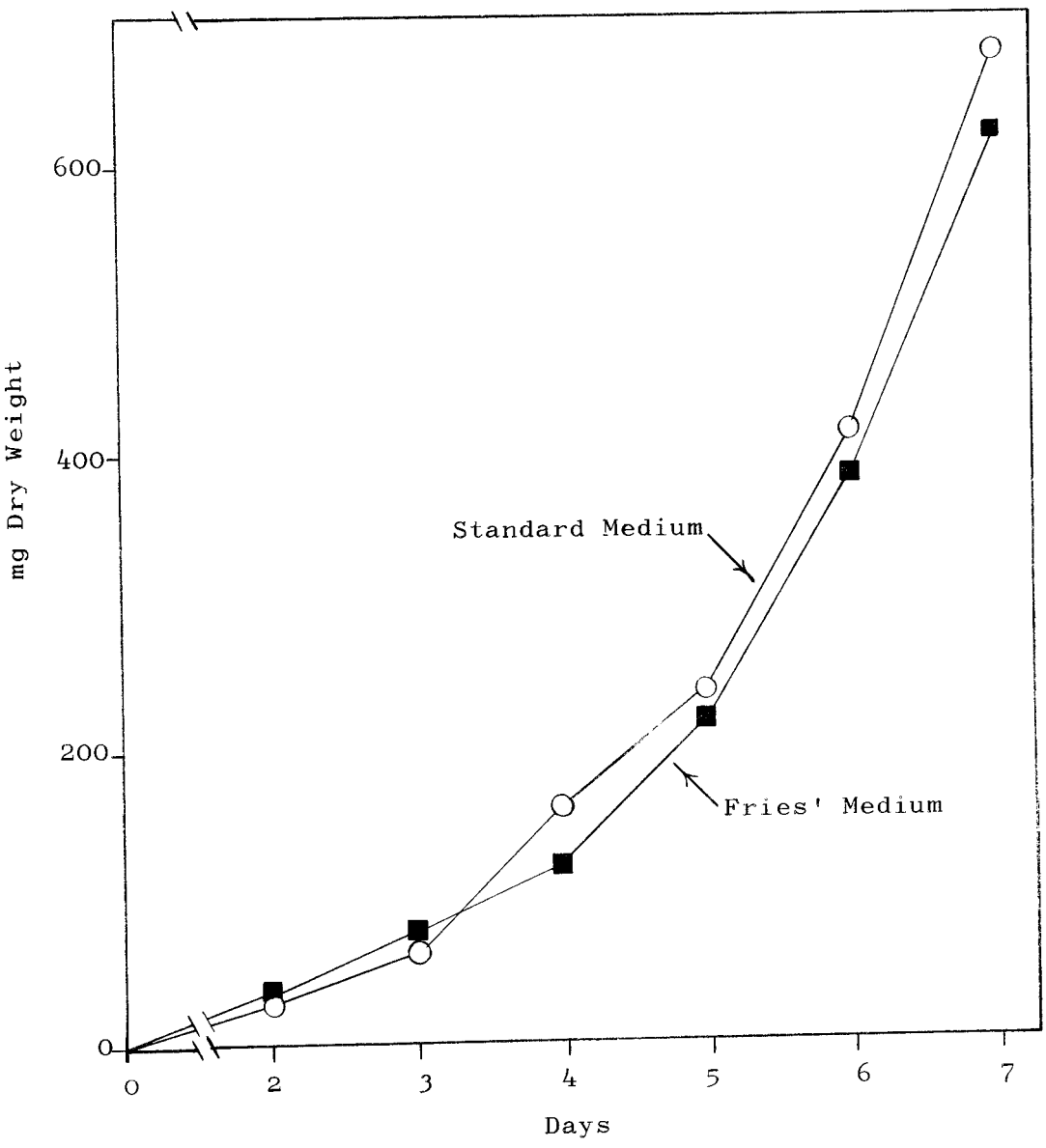

FIG. 1. Comparison of the effects of Fries' and Standard media on the growth of Neurospora. Cultures were incubated at 30° C in the dark in Erlenmeyer flasks (500 ml) each containing 330 ml of the indicated medium. Each medium contained 2.5 mM K^+. Points are means of three determinations.

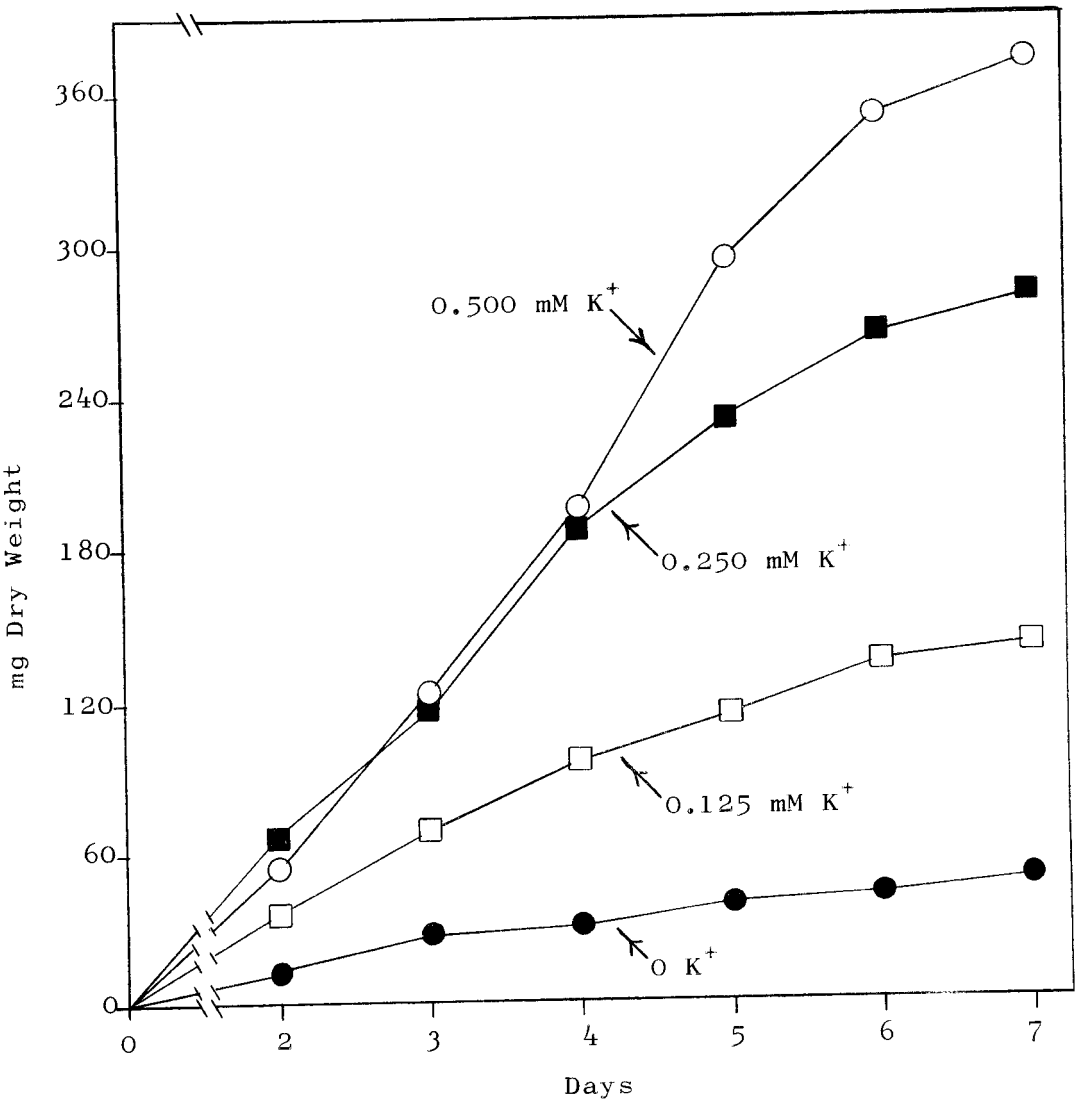

FIG. 2. Effects of K^+ concentrations on the growth of Neurospora. The organism was cultured in Erlenmeyer flasks (500 ml) containing 330 ml of the Standard medium plus the levels of K^+ indicated above. Cultures were incubated in the dark at 30° C. Each point is the mean of three determinations.

growth of the organism occurred in the medium to which no K^+ was added. An analysis of the medium by flame photometric procedures showed a K^+ concentration of 0.01 mM. This may be attributed to contamination by the reagents utilized. This concentration is considerably below the 1.0 mM concentration considered to be the minimum level of K^+ producing optimum growth in microorganisms (27).

To determine the effect of the level of K^+ in the culture medium used for Neurospora on the rates of the adaptive formation of nitrate reductase, experiments were conducted in which the organism was grown in Standard medium with different levels of K^+, then transferred to the Induction medium where the adaptive formation of the enzyme was studied. In one culture, no K^+ (0 K^+) was added to the Standard medium, and to the other 0.125 mM K^+ was added. The results (Figure 3-A) show that the mycelium previously cultured in 0.125 mM K^+ and then incubated in Induction medium with 2.5 mM K^+ synthesized nitrate reductase at a rapid rate. When Neurospora mycelium previously grown with 0.125 mM K^+ was placed in the Induction medium without added K^+, nitrate reductase also was adaptively formed at a rapid rate (Figure 3-B). Apparently sufficient K^+ was transferred from the Standard medium to maintain the adaptive synthesis of the enzyme. The mycelium used for the experiment represented by Curves C and D in Figure 3 was cultured in a Standard medium

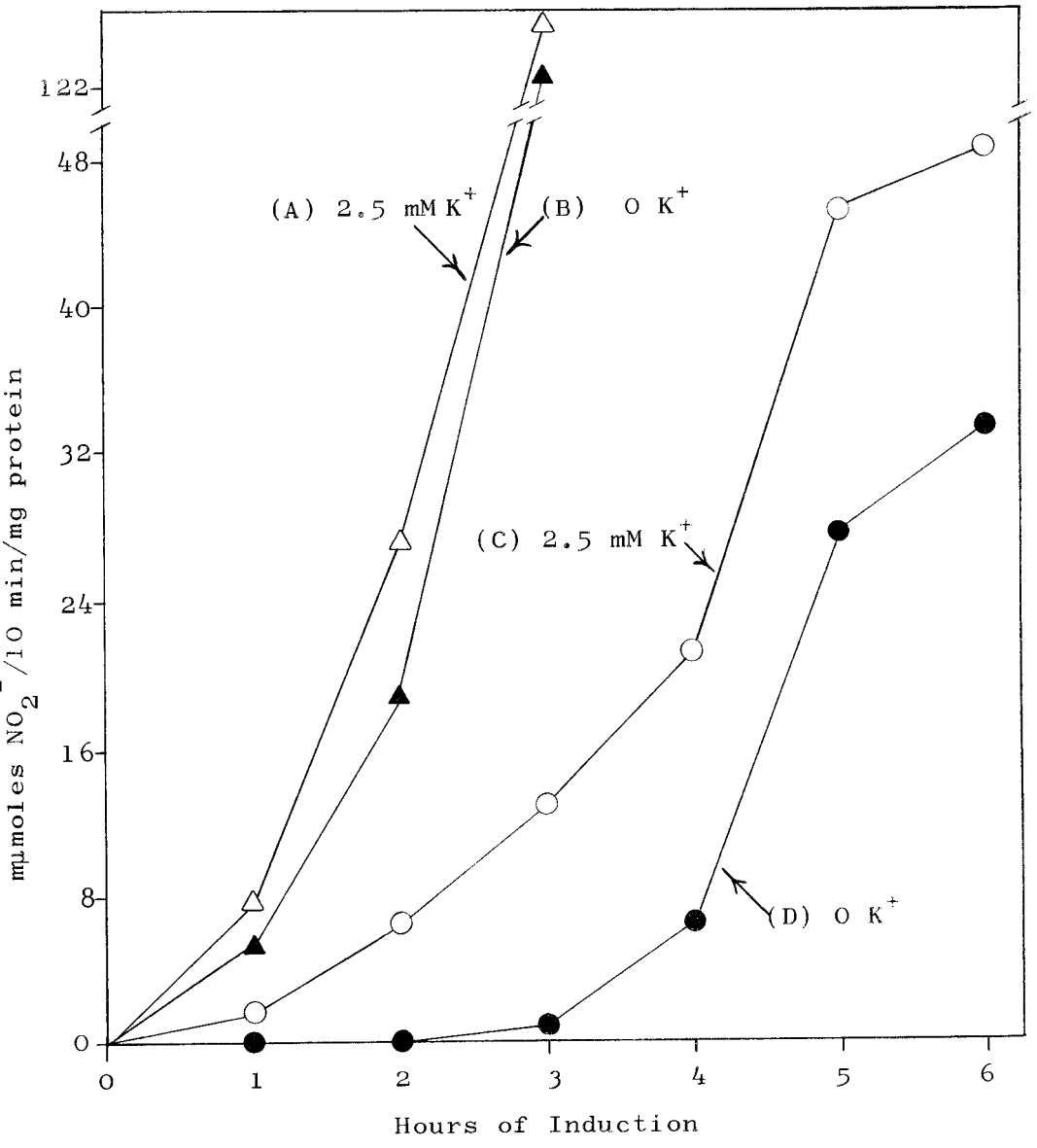

FIG. 3. Adaptive formation of nitrate reductase in Neurospora. The mycelium used in the induction experiments shown by curves A and B was grown in the Standard medium containing 0.125 mM K^+ while that used in the experiments represented by curves C and D was grown in the Standard medium having no K^+ added. The organism was grown for 5 days in the Standard medium at 30° C in the dark, and then transferred to Induction medium containing the levels of K^+ as indicated in the graphs. Induction and assay procedures used are those described under Materials and Methods.

lacking added K^+. When this mycelium was transferred to an Induction medium also lacking added K^+ (Figure 3-D), it is apparent that nitrate reductase formed at a very slow rate during the first four hours of incubation. This rate increased sharply after this four-hour period. When a comparable sample of mycelium previously cultured without added K^+ was transferred to the Induction medium containing 2.5 mM K^+, the adaptive formation of nitrate reductase was initiated within one hour (Figure 3-C). This rate increased steadily and rapidly up to the four-hour period of incubation after which time the rate of synthesis increased sharply. It is clear that the presence of K^+ in either the Standard or Induction medium has a striking effect on the formation of nitrate reductase.

Effects of Univalent Cations on Enzyme Induction

Results of representative experiments showing the effects of a series of different univalent cations in the Induction media on the adaptive formation of nitrate reductase are presented in Figures 4 and 5. In these experiments, the mycelia were grown in the Standard media with no K^+ added, and then were transferred to Induction media containing 2.5 mM concentrations of the univalent cations indicated in the legend of Figures 4 and 5. The rate of formation of the enzyme was slow during the first hour of induction in all

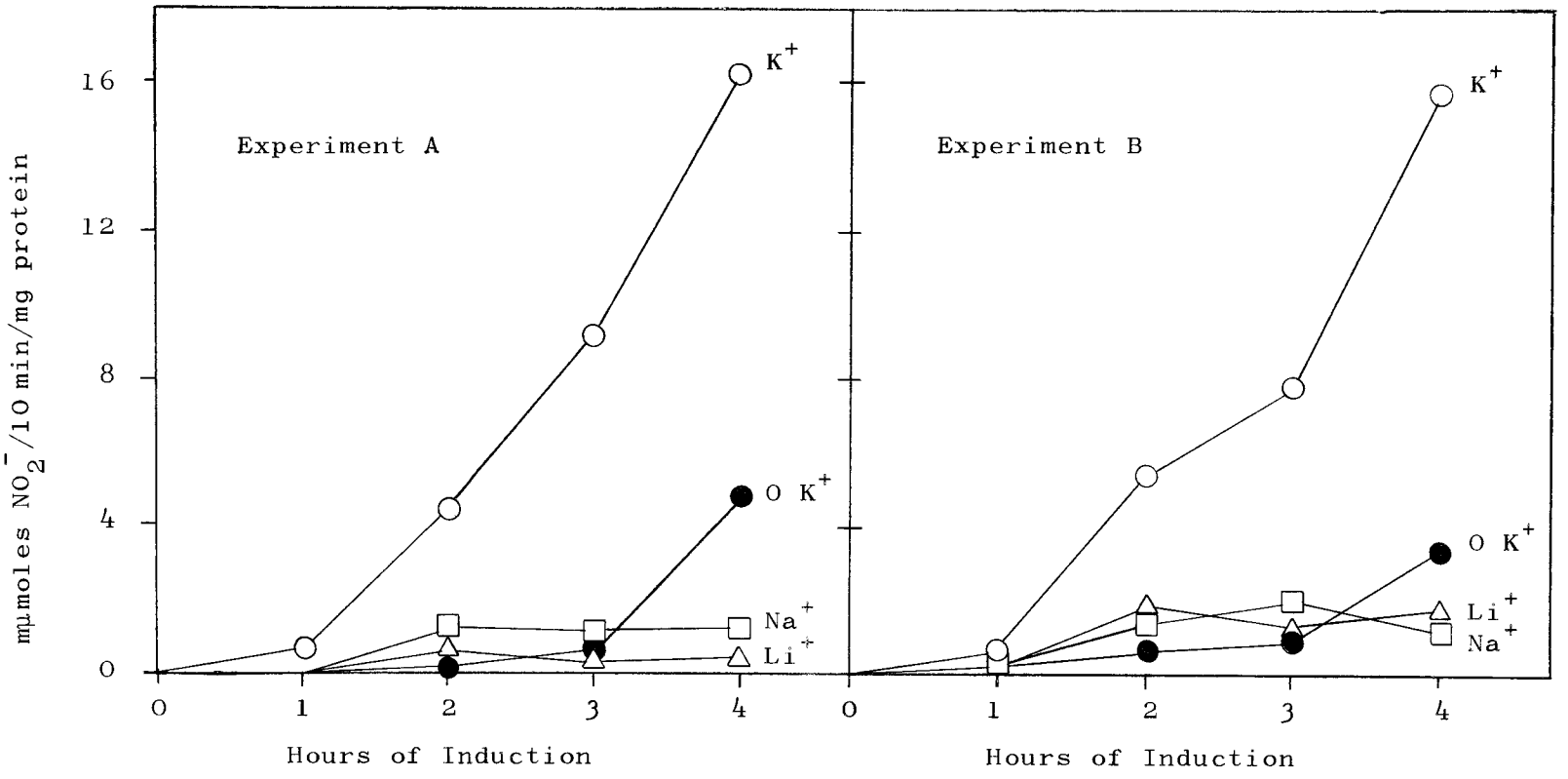

FIG. 4. A comparison of the effectiveness of K^+, Na^+, and Li^+ as cations for the adaptive formation of nitrate reductase. Cultures were incubated in the dark at 30° C on a Gyrotory shaker. The following univalent cation salts were added to the Induction medium (Materials and Methods) to yield a concentration of 2.5 mM of the univalent cation: ○—○ K_2SO_4; □—□ Na_2SO_4; △—△ Li_2SO_4; ●—● None added (0 K^+).

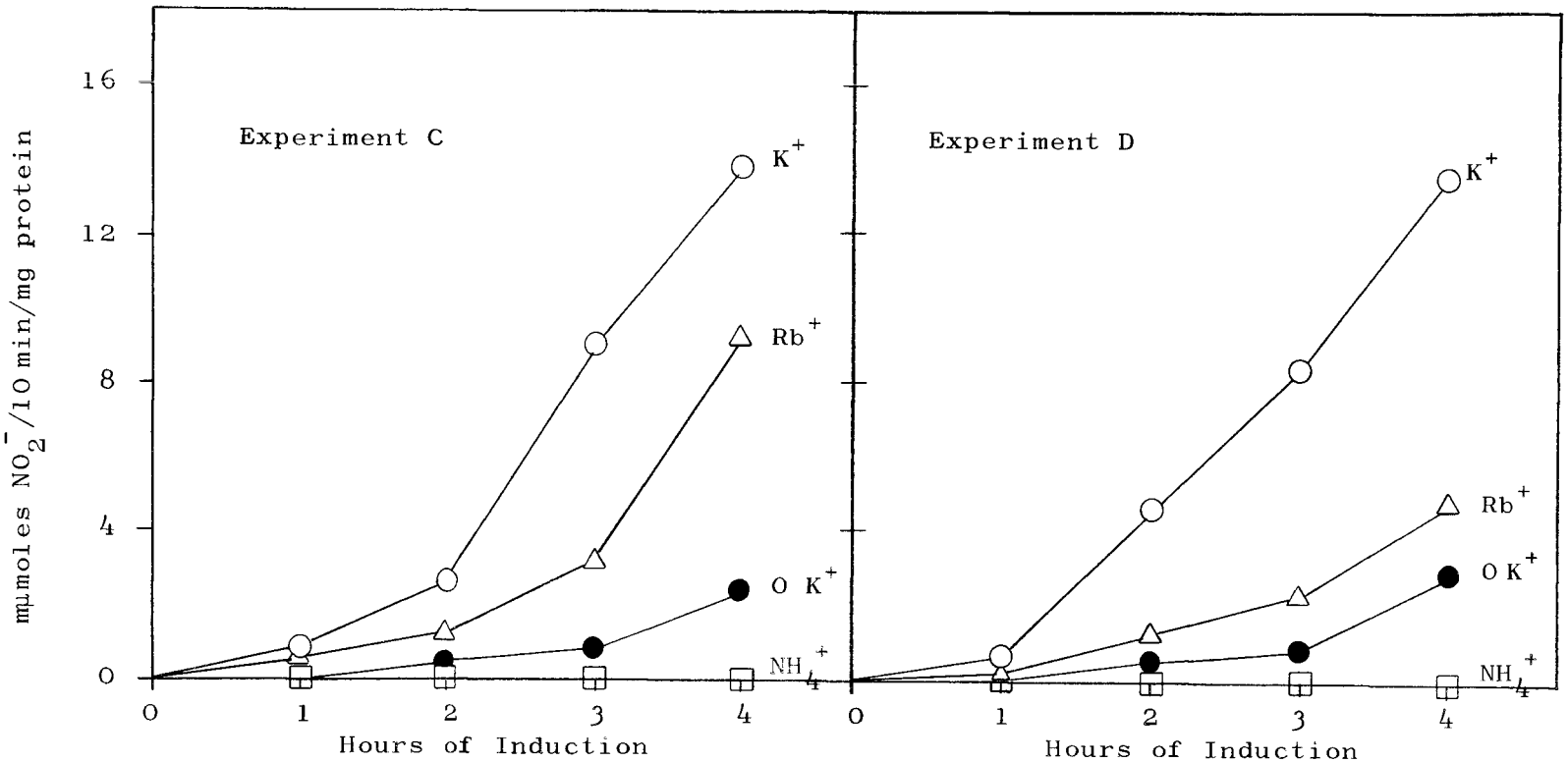

FIG. 5. A comparison of the effectiveness of K^+, Rb^+, and NH_4^+ as cations for the adaptive formation of nitrate reductase. Cultures were incubated in the dark at 30° on a Gyrotory shaker. The following univalent cation salts were added to the Induction medium (Materials and Methods) to yield a concentration of 2.5 mM of the univalent cation: ○——○ K_2SO_4; △——△ Rb_2SO_4; □——□ $(NH_4)_2SO_4$; ●——● None added (0 K^+).

cultures. In the cultures containing K^+, the rate of nitrate reductase formation increased at an almost linear rate for the period between one and three hours. When K^+ was lacking in the Induction medium, nitrate reductase formation was slow for the first three hours of incubation, but increased rapidly after this three-hour period. The experiments in which different univalent cations were substituted for K^+ in the Induction medium show that neither Na^+ nor Li^+ were effective univalent cations for the adaptive formation of nitrate reductase (Figure 4). In some experiments Li^+ not only failed to stimulate but also inhibited the inductive formation of the enzyme. When Rb^+ was substituted for K^+ in the Induction medium (Figure 5), the adaptive formation of nitrate reductase in the mycelia occurred at a rate approximately one-half of that observed for mycelia in the Induction medium containing K^+. The addition of NH_4^+ to the Induction medium (Figure 5) resulted in a complete inhibition of the inductive synthesis of the enzyme. The inhibitory effect of NH_4^+ on the adaptive formation of nitrate reductase has been reported previously by Sorger (100, 101).

The data presented in Table II show the K^+ concentrations in the mycelia used for the various induction experiments. Since K^+ was not added to the medium used for the initial culturing of the organism, the amount of K^+ detected

TABLE II. Flame photometric analysis for K^+ present in the mycelia prior to induction. Values are means of three replicates. Experiments A, B, C, and D are reported in Figures 4 and 5.

Experiment	mg K^+ Per g Dry Matter
A	1.6
B	7.2
C	0.4
D	0.4

in the mycelia must have been derived from that present as contaminants in the reagents used for the preparation of the nutrient solutions. As shown by the data in Table II, the K^+ content of the mycelia used in Experiment B of Figure 4 contained 7.2 mg of K^+ per g of dry matter. This concentration is much greater than that detected in the mycelium used for other experiments and must have been derived from some unknown contamination. The amount of K^+ transferred to the Induction medium (Experiment B, Figure 4) apparently was insufficient to meet the requirements for the adaptive formation of the enzyme as shown by the fact that the addition of K^+ markedly stimulated enzyme formation.

In Table III data are presented showing the K^+ content of the Induction media to which was added the indicated

TABLE III. Flame photometric analysis for K^+ present in the Induction medium (Materials and Methods) containing 2.5 mM concentrations of the indicated univalent cation salt as reported in Figures 4 and 5.

Univalent Cation Salt Added To Induction Medium	K^+ Concentration in Medium	
	mg K^+/l	Millimolar K^+
None	0.8	0.020
K_2SO_4	102.0	2.550
Na_2SO_4	10.0	0.250
Li_2SO_4	0.2	0.001
Rb_2SO_4	0.1	0.001
$(NH_4)_2SO_4$	0.1	0.001

univalent cation salts. These results indicate that Na_2SO_4 must have been contaminated with K^+. As shown by the data in Figure 4, this concentration was not sufficient for the rapid induction of nitrate reductase.

Effects of Univalent Cations on a Nonadaptive Enzyme

In studying the effects of the different univalent cations on the inductive formation of nitrate reductase, it was considered necessary to determine if there was any effect of the added cations on a representative constitutive enzyme in the organism. The activity of pyruvic kinase was determined, therefore, during the period of inductive formation of

nitrate reductase. Figures 6 and 7 illustrate typical results obtained in the study of the activity of pyruvic kinase during the induction period. There was no consistent trend in the results presented in Figure 6 and 7. In Experiment A, Figure 6, there was no appreciable decrease in the activity of the enzyme during the induction period, with the exception that the addition of Li^+ appeared to result in reduced enzyme activity. In Experiment B, Figure 6, there is some evidence that pyruvic kinase activity in the mycelia decreased slightly during the nitrate reductase induction period. No specific effect of the addition of either K^+, Na^+, or Li^+ was apparent. In one experiment the addition of Rb^+ or NH_4^+ appeared to cause significant reduction in activity of the enzyme during the induction period. However, these effects were not consistently obtained.

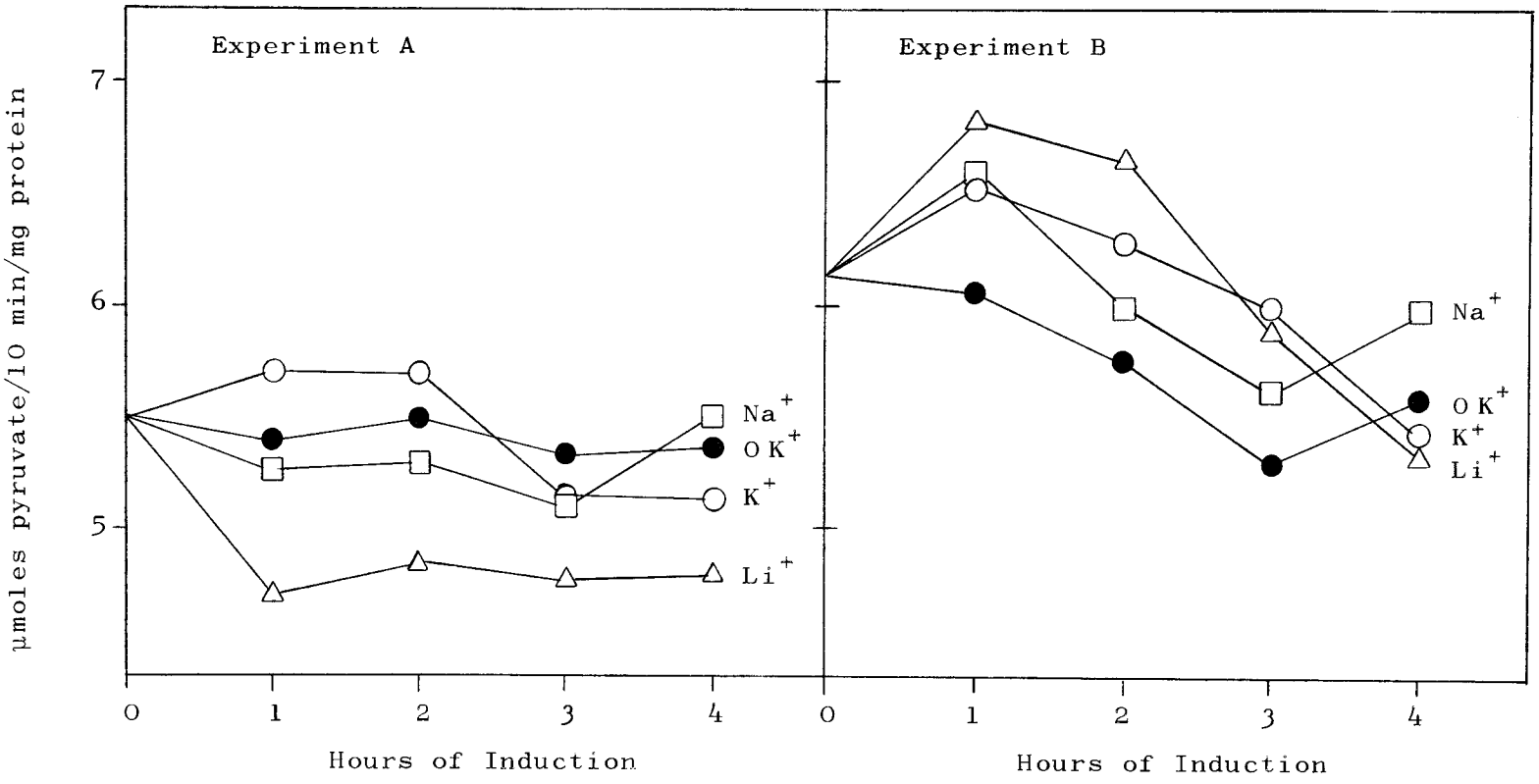

FIG. 6. A comparison of the effects of K^+, Na^+, and Li^+ on the activity of the non-adaptive enzyme pyruvic kinase during the period of adaptive formation of nitrate reductase. Cultures were incubated in the dark at 30° C on a Gyrotory shaker. The following univalent cation salts were added to the Induction medium (Materials and Methods) to yield a concentration of 2.5 mM of the univalent cation: ○—○ K_2SO_4; □—□ Na_2SO_4; △—△ Li_2SO_4; ●—● None added (0 K^+).

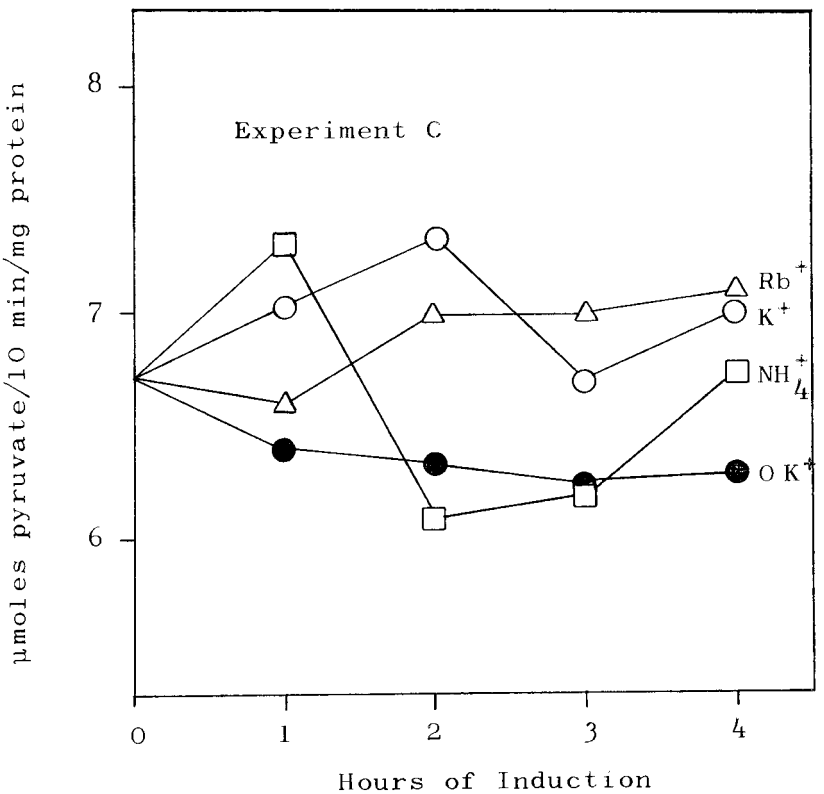

FIG. 7. A comparison of the effects of K^+, Rb^+, and NH_4^+ on the activity of the nonadaptive enzyme pyruvic kinase during the period of adaptive formation of nitrate reductase. Cultures were incubated in the dark at 30° C on a Gyrotory shaker. The following univalent cation salts were added to the Induction medium (Materials and Methods) to yield a concentration of 2.5 mM of the univalent cation: ○—○ K_2SO_4; △—△ Rb_2SO_4; □—□ $(NH_4)_2SO_4$; ●—● None Added (0 K^+).

DISCUSSION

Effects of Potassium in the Growth Medium on the Adaptive Formation of Nitrate Reductase

When Neurospora was cultured in the Standard medium containing 0.125 mM K^+ and then incubated in the Induction medium containing no added K^+, the rate of nitrate reductase formation was nearly equal to that of a comparable mycelium mat induced in 2.5 mM K^+ (Figure 3, A and B). These results may be interpreted on the basis of the assumption that the organism accumulated a sufficient quantity of K^+ from the growth medium. This accumulation by the organism permitted a rapid rate of enzyme formation in the Induction medium. Accumulation of K^+ in actively growing cells is known to occur in many organisms (42). It seems evident from these results that relatively low levels of K^+ are needed in tissues for rapid formation of the enzyme.

When K^+ was omitted in both the Standard and Induction media, the rate of synthesis of nitrate reductase was quite slow initially, but increased appreciably after three hours of incubation (Figure 3-D). This increase was found consistently in all experiments where the mycelium was grown and induced on media lacking added K^+. The reasons for this behavior is not clear. One might speculate that during the initial three-hour period the mycelium had accumulated

sufficient K^+ from impurities in the medium to function in the adaptive formation of the enzyme. Since an analysis of the mycelium prior to induction (Table II) shows a low K^+ concentration of approximately 1.0 mg K^+ per g dry tissue, it seems reasonable to suggest that the organism was able to adjust to the low K^+ environment and, after a period of three hours, synthesize nitrate reductase under conditions where the K^+ concentration was at a very low level.

Another consideration concerns the fact that NH_4^+ can substitute for K^+ in protein synthesis (73). During normal growth conditions, some breakdown of organic constituents would be expected to occur in the organism. If NH_4^+ can function in the synthesis of nitrate reductase, one might postulate that NH_4^+ ions resulting from protein breakdown during the induction period might be sufficient for the inductive formation of this enzyme. The level of NH_4^+ would have to be present in low concentrations since it has been established that relatively high concentrations of NH_4^+ inhibit the inductive formation of nitrate reductase (100, 101).

Univalent Cations and Protein Synthesis

Since adaptive formation of nitrate reductase is reported to involve protein synthesis, one would expect that

any univalent cation requirement for protein synthesis also would be exhibited for the adaptive formation of nitrate reductase. The results presented in Figures 4 and 5 show clearly that nitrate reductase formation is dependent upon an adequate concentration of K^+ in the Induction medium. The effects of K^+ on the synthesis of the protein nitrate reductase in *in vivo* systems are in agreement with those results of Webster (116) who reported that K^+ was needed for the incorporation of labeled amino acids into protein by pea extracts.

In a schematic outline of the protein synthesizing process already discussed in the Literature Review, K^+ is reported to function in the following steps: tyrosine activation, nonenzymatic binding of MRNA to ribosomes, amino acyl-SRNA binding to ribosomes, and possibly in MRNA turnover. From the types of experiments conducted in this investigation it is not possible to speculate whether the block or blocks in the synthesis of nitrate reductase are related directly to the above mentioned sites in protein synthesis or indirectly to some essential metabolic step related to protein synthesis. On the basis of the results presented by Ennis and Lubin (36), K^+ deficiency would appear to have a greater effect on protein synthesis *per se* than on the snythesis of RNA.

As shown by the data in Figures 4 and 5, K^+ not only is required for the induction of the enzyme, but is highly specific in its function. In these and other experiments, Li^+ would not replace K^+ to any appreciable extent as a univalent cation for enzyme induction. The results are consistent with the report (3) that Li^+ has no nutritional value for plants or microorganisms. It is also apparent from the data in Figure 4 that the addition of Na^+ to the Induction medium failed to stimulate the synthesis of nitrate reductase. Sodium has been reported to be beneficial for the growth of certain organisms, but from the data presented here and that presented by Webster (116), it has little value as a cofactor for protein synthesis. An interesting observation in this study is that neither Na^+ nor Li^+ functions effectively as a cofactor for the induction of nitrate reductase. In contrast, when K^+ was omitted in the Induction medium, nitrate reductase formation appeared to be initiated after the three-hour period. It would appear that the addition of either Na^+ or Li^+ to the medium has completely inhibited protein synthesis whereas the omission of added univalent cations resulted in enzyme formation after the lapse of three hours. An adequate explanation of this behavior is not available at present.

When Rb^+ was used as the added cation for the induction of nitrate reductase, the enzyme was formed at a rate

approximately one-half that obtained when K^+ was used. Rubidium is known to partially substitute for K^+ as a nutrient for certain microorganisms (76, 89). The partial substitution of Rb^+ for K^+ can be extended to specific metabolic reactions as indicated by the capacity of Rb^+ to replace K^+ as a univalent cation for acetic thiokinase activity in in vitro studies (50). An explanation for this replacement may be related to the fact that Rb^+ and K^+ have similar ionic radii whereas the atomic radii of Na^+ and Li^+ are much larger (61).

When NH_4^+ was substituted for K^+ in the Induction medium, a complete inhibition of the formation of nitrate reductase was observed (Figure 5). Ammonium is known to inhibit the inductive formation of this enzyme (100, 101). Even though NH_4^+ is known to be superior to K^+ as an activator for certain individual reactions of in vitro protein synthesis (73, 82), concentrations of NH_4^+ used in this investigation were completely ineffective in the synthesis of nitrate reductase. It seems possible that NH_4^+ may function in one of the reactions of protein synthesis, but may inhibit other processes, thus ultimately inhibiting the whole protein synthesizing process.

Relation of Univalent Cations to Constitutive Enzymes

In an effort to determine whether the addition of univalent cations to the Induction medium had an effect on a representative constitutive enzyme in the mycelium, pyruvic kinase was assayed at periods during the induction process. As shown by the results in Figures 6 and 7, only a very slight decrease if any occurred in the activity of this enzyme during the induction period. The addition of a series of different univalent cations to the Induction media had relatively little effect on the activity of this enzyme. In comparing the activities of pyruvic kinase and nitrate reductase during the induction period, it appears that nitrate reductase was rapidly synthesized while pyruvic kinase remained at a nearly constant level. From these results and from the protein content of extracts, it would appear that the conditions were optimum for the rapid synthesis of nitrate reductase, but the rate of protein synthesis in general was not greatly increased. The addition of Na^+, Li^+, Rb^+, or NH_4^+ possibly may have influenced the stability of pyruvic kinase, thus accounting for the slight decrease in its activity.

SUMMARY

An investigation was conducted to determine the effects of univalent cations on the *in vivo* synthesis of a specific protein by *Neurospora crassa*. The effects of univalent cations on the inductive formation of nitrate reductase and on the level of activity of a representative nonadaptive enzyme were determined. The results of these experiments may be summarized as follows:

1. The inductive formation of nitrate reductase has a specific requirement for the presence of an adequate concentration of K^+ in the Induction medium.

2. Rubidium could partially substitute for K^+ in the Induction medium for the adaptive formation of nitrate reductase, but Na^+, Li^+, and NH_4^+ either failed to stimulate or inhibited the formation of this enzyme.

3. Pyruvic kinase activity throughout the induction period was not greatly changed when any of the univalent cations were present in the Induction medium.

4. It would appear from the results that K^+ is required for the rapid synthesis of nitrate reductase, and that overall protein synthesis in general was nearly constant during the formation of nitrate reductase.

BIBLIOGRAPHY

1. Abelson, Phillip. Amino acid biosynthesis in Escherichia coli: Isotopic competition with C^{14}-glucose. Journal of Biological Chemistry 206:335-343. 1954.

2. Adelberg, Edward A. The biosynthesis of isoleucine, valine, and leucine. In: A Symposium on Amino Acid Metabolism, ed. by William D. McElroy and H. Bently Glass. Baltimore, John Hopkins Press, 1955. p. 419-430.

3. Aldrich, D. G., A. P. Vanselow and G. R. Bradford. Lithium toxicity in citrus. Soil Science 71:291-295. 1951.

4. Allen, M. B. and Daniel I. Arnon. Studies on nitrogen-fixing blue-green algae. II. The sodium requirement of Anabaena cylindrica. Physiologia Plantarum 8:653-659. 1955.

5. Arlinghaus, Ralph, Gabriel Favelukes and Richard Schweet. A ribosome-bound intermediate in polypeptide synthesis. Biochemical and Biophysical Research Communications 11: 92-96. 1963.

6. Arlinghaus, Ralph, Joseph Shaeffer and Richard Schweet. Enzymatic stages in amino acid transfer to protein. Federation Proceedings 23:165. 1964.

7. _____ Mechanism of peptide bond formation in polypeptide synthesis. Proceedings of the National Academy of Sciences 51:1291-1299. 1964.

8. Asher, D. I. Potassium uptake by pasture plants as affected by root development and the potassium concentrations in the root environment. Ph.D. thesis. Nedlands, University of Western Australia, 1964. n.p.

9. Beadle, G. W. Biochemical genetics. Chemical Reviews 37:15-96. 1945.

10. Bear, Firman E. (ed.) Sodium symposium. Soil Science 76:1-96. 1953.

11. Beeson, Kenneth C. The mineral composition of crops with particular reference to the soils in which they were grown. Washington, 1941. 164 p. (Department of Agriculture. Miscellaneous Publication 369)

12. Berg, Paul. Specificity in protein synthesis. Annual Review of Biochemistry 30:293-324. 1961.

13. Berg, Paul et al. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. I. The mechanism of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid formation. Journal of Biological Chemistry 236: 1726-1734. 1961.

14. Bergman, Fred H., Paul Berg and M. Dieckmann. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. II. The preparation of leucyl-, valyl-, isoleucyl-, and methionyl ribonucleic acid synthetases from Escherichia coli. Journal of Biological Chemistry 236:1735-1740. 1961.

15. Bertino, J. R. Activation of tetrahydrofolate dehydrogenase by certain cations. Biochimica et Biophysica Acta 58:377-380. 1962.

16. Birner, H. and B. Lucanus. Wasserculturversuche mit Hafer. Die Landwirtschaftlichen Versuchs-Stationen 8:128-177. 1866.

17. Bishop, John O. and Richard S. Schweet. Participation of two enzyme fractions in the transfer of amino acids from amino acyl-RNA to reticulocyte ribosomes. Biochimica et Biophysica Acta 54:617-619. 1961.

18. Black, Simon and Nancy M. Gray. Enzymatic phosphorylation of l-aspartate. Journal of the American Chemical Society 75:2271-2272. 1953.

19. Black, Simon and Nancy G. Wright. β-aspartokinase and β-aspartyl phosphate. Journal of Biological Chemistry 213:27-38. 1955.

20. Brenner, Sydney. RNA, ribosomes, and protein synthesis. Cold Spring Harbor Symposia on Quantitative Biology 26: 101-110. 1961.

21. Brownell, P. F. Sodium as an essential micronutrient element for a higher plant (Atriplex vesicaria). Plant Physiology 40:460-468. 1965.

22. Buchanan, John M. and Standish C. Hartman. Enzymic reactions in the synthesis of purines. Advances in Enzymology 21:199-261. 1959.

23. Buchanan, John M., A. Baird Hastings and Frances B. Nesbett. The effect of the ionic environment on the synthesis of glycogen from glucose in rat liver slices. Journal of Biological Chemistry 180:435-445. 1949.

24. Bulen, William A. The isolation and characterization of glutamic dehydrogenase from corn leaves. Archives of Biochemistry and Biophysics 63:173-183. 1956.

25. Cantoni, G. L. S-adenosylmethionine: A new intermediate formed enzymatically from L-methionine and adenosine triphosphate. Journal of Biological Chemistry 204:403-416. 1953.

26. Chargaff, Erwin and J. N. Davidson (eds.) The nucleic acids. New York, Academic Press, 1955. 3 vols.

27. Cochrane, Vincent W. Physiology of fungi. New York, Wiley, 1958. 524 p.

28. Cohn, Melvin. Contributions of studies on the β-galactosidase of Escherichia coli to our understanding of enzyme synthesis. Bacteriological Reviews 21:140-168. 1957.

29. Conway, Thomas W. and Fritz Lipmann. Characterization of ribosome-linked guanosine triphosphate in Escherichia coli extracts. Proceedings of the National Academy of Sciences 52:1462-1469. 1964.

30. Davie, Earl, Victor V. Koningsberger and Fritz Lipmann. The isolation of a tryptophan-activating enzyme from pancreas. Archives of Biochemistry and Biophysics 65: 21-38. 1956.

31. DeMoss, J. A. and G. David Novelli. An amino acid dependent exchange between inorganic pyrophosphate and ATP in microbial extracts. Biochimica et Biophysica Acta 18:592-593. 1955.

32. Dintzis, Howard M. Assembly of the peptide chains of hemoglobin. Proceedings of the National Academy of Sciences 47:247-261. 1961.

33. Downes, Helen R. The chemistry of living cells. 2d ed. New York, Harper and Row, 1962. 645 p.

34. Eaton, Scott V. Effects of potassium deficiency on growth and metabolism of sunflower plants. Botanical Gazette 114:165-180. 1952.

35. Edwards, John K. Cytological studies of toxicity in meristem cells of roots of Zea mays. II. The effects of lithium chloride. Proceedings of the South Dakota Academy of Science 21:65-67. 1941.

36. Ennis, Herbert L. and Martin Lubin. Dissociation of ribonucleic acid and protein synthesis in bacteria deprived of potassium. Biochimica et Biophysica Acta 50:399-402. 1961.

37. _____ Pre-ribosomal particles formed in potassium-depleted cells. Biochimica et Biophysica Acta 95:605-623. 1965.

38. _____ Selective synthesis of ribosomal protein during recovery from unbalanced growth. Biochimia et Biophysica Acta 95:624-633. 1965.

39. Evans, Harold J. Effect of potassium and other univalent cations on activity of pyruvic kinase in Pisum sativium. Plant Physiology 38:397-402. 1963.

40. Evans, Harold J. and George J. Sorger. Role of mineral elements with emphasis on the univalent cations. Annual Review of Plant Physiology 17:1966 (in press)

41. Frisch, Leonora (ed.) Synthesis and structure of macromolecules. In: Cold Spring Harbor Symposia on Quantitative Biology, Long Island, 1963. 610 p.

42. Giese, Arthur. Cell Physiology. 2d ed. Philadelphia, W. B. Saunders, 1962. 592 p.

43. Hageman, R. H. and Donna Flescher. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiology 35:700-708. 1960.

44. Halvorson, Harlyn O. The induced synthesis of proteins. Advances in Enzymology 22:99-156. 1960.

45. Hardesty, Boyd et al. Hemoglobin and polyphenylalanine synthesis with reticulocyte ribosomes. Cold Spring Harbor Symposia on Quantitative Biology 28:215-222. 1963.

46. Haughton, Barbara G. and H. K. King. Induced formation of leucine decarboxylase in Proteus vulgaris. The Biochemical Journal 80:268-277. 1961.

47. Hewitt, E. J. Mineral nutrition of plants in culture media. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic Press, 1963. p. 97-133.

48. _____ The essential nutrient elements: Requirements and interactions in plants. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic Press, 1963. p. 137-360.

49. Hiatt, A. J. Formic acid activation in plants. II. Activation of formyltetrahydrofolate synthesis by magnesium, potassium, and univalent cations. Plant Physiology 40:189-193. 1965.

50. Hiatt, A. J. and Harold J. Evans. Influence of certain cations on activity of acetic thiokinase from spinach leaves. Plant Physiology 35:673-677. 1960.

51. Hoagland, Mahlon B. An enzymic mechanism for amino acid activation in animal tissue. Biochimica et Biophysica Acta 16:288-289. 1955.

52. _____ The relationship of nucleic acids and protein synthesis as revealed by studies in cell free systems. In: The nucleic acids, ed. by E. Chargaff and J. N. Davidson. Vol. 3. New York, Academic Press, 1960. p. 349-408.

53. Hoagland, Mahlon B. and Lucy T. Comly. Interaction of soluble ribonucleic acid and microsomes. Proceedings of the National Academy of Sciences 46:1554-1563. 1960.

54. Holley, Robert W. et al. Partial purification of the threonine- and tyrosine-activating enzymes from rat liver, and the effect of potassium on the activity of the tyrosine enzyme. Journal of Biological Chemistry 236:197-199. 1961.

55. Horowitz, N. H. et al. Genetic determination and enzymatic induction of tyrosinase in Neurospora. Journal of Molecular Biology 2:96-104. 1960.

56. Ingram, Vernon M. The biosynthesis of macromolecules. New York, W. A. Benjamin, Inc., 1965. 223 p.

57. Jacob, Francois and Jacques Monod. Genetic regulatory mechanisms in the synthesis of proteins. Journal of Molecular Biology 3:318-356. 1961.

58. Johnson, Clarence M. and Albert Ulrich. Analytical methods for use in plant analysis. Sacramento, 1959. p. 25-78. (California. Agriculture Experiment Station. Bulletin 766)

59. Kachmar, J. F. and P. D. Boyer. Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic kinase. Journal of Biological Chemistry 200:669-682. 1953.

60. Kasting, R. and C. C. Delwiche. Ornithine, citrulline, arginine interconversion in higher plants. Plant Physiology 30(Supplement):28. 1955.

61. Kayne, F. J. and C. H. Suelter. Effects of temperature, substrate, and activating cations on the conformations of pyruvic kinase in aqueous solutions. Journal of the American Chemical Society 87:897-900. 1965.

62. Knox, W. Eugene and E. J. Behrman. Amino acid metabolism. Annual Review of Biochemistry 28:223-256. 1959.

63. Kogut, Margot, M. R. Pollock and E. J. Tridgell. Purification of penicillin-induced penicillinase of Bacillus cereus NRRL569: A comparison of its properties with those of a similarly purified penicillinase produced spontaneously by a constitutive mutant strain. The Biochemical Journal 62:391-401. 1956.

64. Krishnaswamy, P. R. and Alton Meister. Enzymatic synthesis and reactions of amino acyl adenylates. Journal of Biological Chemistry 235:408-415. 1960.

65. Landman, Otto E. Properties and induction of β-galactosidase in Bacillus megaterium. Biochimica et Biophysica Acta 23:558-569. 1957.

66. Latzko, E. Zur Rolle des K^+ bei der Oxydativen Phosphorylierung. Biochimica et Biophysica Acta 47:350-357. 1961.

67. Latzko, E. and K. L. Mechsner. Bedeutung der Alkalionen for die Intensität der Lichtphosphorylierung bei Chlorella vulgaris. Naturwissenschaften 45:247-248. 1958.

68. Leonard, Chester D. and Firman E. Bear. Sodium as a fertilizer for New Jersey soils. New Brunswick, 1950. 24 p. (New Jersey. Agriculture Experiment Station. Bulletin 752)

69. Leonard, Mary Jane Kreko and R. H. Burris. A survey of transaminases in plants. Journal of Biological Chemistry 170:701-709. 1947.

70. Lin, Edmund C. and Boris Magasanik. The activation of glycerol dehydrogenase from Aerobacter aerogenes by monovalent cations. Journal of Biological Chemistry 235:1820-1823. 1960.

71. Lowry, Oliver et al. Protein measurements with the Folin phenol reagent. Journal of Biological Chemistry 193:265-275. 1951.

72. Lubin, Martin. A priming reaction in protein synthesis. Biochimica et Biophysica Acta 72:345-348. 1963.

73. Lubin, Martin and Herbert L. Ennis. On the role of intracellular potassium in protein synthesis. Biochimica et Biophysica Acta 80:614-663. 1964.

74. Lucanus, B. Versuche über die Erziehung einiger Landpflanzen in wässriger Lösung. Die Landwirtschaftlichen Versuchs-Stationen 7:363-371. 1865.

75. Luck, J. Murry (ed.) Annual review of biochemistry. Vol. 31. Palo Alto, Annual Reviews, Inc., 1962. 731 p.

76. MacLeod, Robert A. and Esmond E. Snell. The effect of related ions on the potassium requirement of lactic acid bacteria. Journal of Biological Chemistry 176:39-52. 1948.

77. Marshall, Margaret, R. L. Metzenberg and Phillip P. Cohen. Physical and kinetic properties of carbamyl phosphate synthetase from frog liver. Journal of Biological Chemistry 236:2229-2237. 1961.

78. Miller, Gene and Harold J. Evans. The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiology 32:346-354. 1957.

79. Monod, Jacques, Germaine Cohn-Bazire et Melvin Cohn. Sur la biosynthese de la β-galactosidase (lactose) chez Escherichia coli. La specificite de l'induction. Biochimica et Biophysica Acta 7:585-599. 1951.

80. Moyed, H. S. and Boris Magasanik. The biosynthesis of the imidazole ring of histidine. Journal of Biological Chemistry 23:149-153. 1960.

81. Mudd, S. Harvey and G. L. Cantoni. Activation of methionine for transmethylation. III. The methionine-activating enzyme of bakers yeast. Journal of Biological Chemistry 231:481-492. 1958.

82. Nakamoto, Tokumasa et al. Formation of peptide bonds. I. Peptide formation from amino acyl-SRNA. Cold Spring Harbor Symposia on Quantitative Biology 28:227-231. 1963.

83. Nason, Alvin and H. J. Evans. Triphosphopyridine nucleotide-nitrate reductase in Neurospora. Journal of Biological Chemistry 202:655-673. 1953.

84. Nicholas, D. J. D. Inorganic nutrient nutrition of microorganisms. In: Plant physiology, ed. by F. C. Steward. Vol. 3. New York, Academic Press, 1963. p. 363-447.

85. Nightingale, G. T., L. G. Schermerhorn and W. R. Robbins. Some effects of potassium deficiency on the histological structure and nitrogenous and carbohydrate constituents of plants. New Brunswick. 1930. 36 p. (New Jersey. Agriculture Experiment Station. Bulletin 499)

86. Paulus, Henry and Edith Gray. Multivalent feedback inhibition of aspartokinase in Bacillus polymyxa. Journal of Biological Chemistry 239:PC4008-4009. 1964.

87. Pollock, M. R. Induced formation of enzymes. In: The enzymes, ed. by Paul D. Boyer, Henry Lardy and Karl Myback. 2d ed. Vol. 1. New York, Academic Press, 1959. p. 619-680.

88. Pulver, R. and F. Verzar. Connexion between carbohydrate and potassium metabolism in the yeast cell. Nature 145:823-824. 1940.

89. Rahn, Otto. Substitutes for potassium in metabolism of the lowest fungi. Journal of Bacteriology 32:393-399. 1936.

90. Reichard, Peter. The enzymic synthesis of pyrimidines. Advances in Enzymology 21:263-294. 1959.

91. Richards, F. J. Physiological studies in plant nutrition. XI. The effect on growth of rubidium with low potassium supply, and modification of this effect by other nutrients. Part 2. The effect on dry-weight distribution, net assimilation rate, tillering, etc. Annals of Botany, n.s. 8:323-356. Oct. 1944.

92. Riselbrough, R. W., A. Tissières and J. D. Watson. Messenger-RNA attachment to active ribosomes. Proceedings of the National Academy of Sciences 48:430-436. 1962.

93. Ryan, Francis J., G. W. Beadle and E. I. Tatum. The tube method of measuring the growth rate of Neurospora. American Journal of Botany 30:784-799. 1943.

94. Schweet, Richard and John Bishop. Protein synthesis in relation to gene action. In: Molecular genetics, ed. by J. Herbert Taylor. New York, Academic Press, 1963. p. 353-404.

95. Schweet, Richard S., Robert Holley and Esther H. Allen. Amino acid activation in hog pancreas. Archives of Biochemistry and Biophysics 71:311-325. 1957.

96. Schweet, Richard et al. Studies on hemoglobin synthesis. Medicine 43:731-745. 1964.

97. Sideris, C. P. and H. Y. Young. Effects of potassium on chlorophyll, acidity, ascorbic acid, and carbohydrates of Ananas comosus (L.) Merr. Plant Physiology 20:649-670. 1945.

98. Singer, Maxine F. and Giulio L. Cantoni. Studies on soluble ribonucleic acid of rabbit liver. Terminal groups and nucleotide composition. Biochimica et Biophysica Acta 39:182-183. 1960.

99. Snoke, John E., Sam Yanari and Konrad Bloch. Synthesis of glutathione from γ-glutamylcysteine. Journal of Biological Chemistry 201:573-586. 1953.

100. Sorger, George J. TPNH-cytochrome c reductase and nitrate reductase in mutant and wild type Neurospora and Aspergillus. Biochemical and Biophysical Research Communications 12:396-401. 1963.

101. _____ Simultaneous induction and repression of nitrate reductase and TPNH cytochrome c reductase in Neurospora crassa. Biochimica et Biophysica Acta 99: 234-245. 1965.

102. Spahr, P. F. and D. Schlessinger. Breakdown of messenger ribonucleic acid by potassium-activated phosphodiesterase from Escherichia coli. Journal of Biological Chemistry 238:PC2251-2253. 1963.

103. Spiegelman, S. The relation of informational RNA to DNA. Cold Spring Harbor Symposia on Quantitative Biology 26:75-90. 1961.

104. Spyrides, George J. The effect of univalent cations on the binding of SRNA to the template-ribosome complex. Proceedings of the National Academy of Sciences 51: 1220-1226. 1964.

105. Stadtman, E. R. The purification and properties of phosphotransacetylase. Journal of Biological Chemistry 196:527-534. 1952.

106. Stekol, Jacob A. Synthetic pathways of methionine, cysteine, and threonine. In: A Symposium on Amino Acid Metabolism, ed. by William D. McElroy and H. Bently Glass. Baltimore, John Hopkins Press, 1955. p. 509-556.

107. Strassman, Murry et al. The biosynthesis of isoleucine. Journal of the American Chemical Society 78:228-232. 1956.

108. Strecker, Harold J. The interconversion of glutamic acid and proline. Journal of Biological Chemistry 235:3218-3223. 1960.

109. Strumeyer, David H. and Konrad Bloch. Some properties of γ-glutamylcysteine synthetase. Journal of Biological Chemistry 235:PC27. 1960.

110. Tristram, G. R. The amino acid composition of proteins. In: The proteins, ed. by Han Neurath and Kenneth Bailey. Vol. 1. New York, Academic Press, 1953. p. 181-233.

111. Vogel, Henry J. and David Bonner. On the glutamate-proline-ornithine interrelation in Neurospora crassa. Proceedings of the National Academy of Sciences 49:315-331. 1940.

112. Vogel, Henry J. and Bernard D. Davis. Glutamic γ-semialdehyde and Δ-pyrroline-5-carboxylic acid intermediates in the biosynthesis of proline. Journal of the American Chemical Society 74:109-112. 1952.

113. Wall, Monroe E. The role of potassium in plants. II. Effect of varying amounts of potassium on the growth status and metabolism of tomato plants. Soil Science 49:315-331. 1940.

114. Warner, Jonathan R., Alexander Rich and Cecil E. Hall. Electron microscope studies of ribosomal clusters synthesizing hemoglobin. Science 138:1399-1403. 1962.

115. Watson, J. D. Involvement of RNA in the synthesis of proteins. Science 140:17-26. 1963.

116. Webster, George C. Effects of monovalent cations on the incorporation of amino acids into protein. Biochimica et Biophysica Acta 20:565-566. 1956.

117. _____ Factors required for amino acid incorporation by disrupted ribonucleoprotein particles. Archives of Biochemistry and Biophysics 70:622-624. 1957.

118. _____ Nitrogen metabolism in plants. New York, Row, Peterson and Co., 1959. 152 p.

119. Webster, George C. and J. E. Varner. Peptide-bond synthesis in higher plants. II. Studies on the mechanism of synthesis of γ-glutamylcysteine. Archives of Biochemistry and Biophysics 52:22-32. 1954.

120. Webster, Robert E., Dean L. Engelhardt and Norton D. Zinder. In vitro protein synthesis: Chain initiation. Proceedings of the National Academy of Sciences 55:155-161. 1966.

121. Weisblum, Bernard, Seymour Benzer and Robert Holley. A physical basis for degeneracy in the amino acid code. Proceedings of the National Academy of Sciences 48: 1449-1454. 1962.

122. Williams, M. Coburn. Effect of sodium and potassium salts on growth and oxalate content of Halogeton. Plant Physiology 35:500-504. 1960.

123. Williams, Virginia R. and Russell T. McIntyre. Preparation and partial purification of the aspartase of Bacterium cadaveris. Journal of Biological Chemistry 217:467-477. 1955.

124. Wiseman, Alan. Organization for protein biosynthesis. New York, American Elsevier, 1965. 217 p.

125. Woolley, Joseph T. Sodium and silicon as nutrients for the tomato plant. Plant Physiology 32:317-320. 1957.

126. Zachau, Hans George, George Acs and Fritz Lipmann. Isolation of adenosine amino acid esters from a ribonuclease digest of soluble liver ribonucleic acid. Proceedings of the National Academy of Sciences 44: 885-889. 1958.