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THE UTILIZATION OF ORNITHINE IN *NEUROSPORA CRASSA*

by
Joan Barbara Nathan Karlin

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
1974

Doctoral Committee:

Professor Sally Allen, Co-Chairperson
Professor Rowland H. Davis, Co-Chairperson
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DEDICATION

In loving memory of my father,

Fred H. Nathan

ACKNOWLEDGMENTS

Though it is impossible to recognize all the individuals who encouraged me to pursue a postgraduate education and complete the requirements of the Doctor of Philosophy, I would like to take the opportunity to thank the following people whose support was instrumental.

First, I am indebted to my parents who stressed that the best education was only good enough. They made many sacrifices to insure that my sister and I grew up to be healthy, happy, and educated citizens. The philosophy and love of my family has been a great strength to me and I am very grateful to them.

Most important in the last few years has been the continued love and support of my husband, David. He has proven to be my best friend. His unselfish devotion has made it possible for me to simultaneously complete the thesis and begin working in Dr. Arthur Rubenstein's laboratory at the University of Chicago.

Dr. Rubenstein's patience with me, concern for my completing the thesis, and generosity are all greatly appreciated.

Special thanks goes to my research chairperson, Dr. Rowland H. Davis and all the people in his laboratory. The

help of everyone and special technical assistance from Sharon Bernhardt has contributed to the success of this work. It has been both stimulating and instructive to have been a part of Dr. Davis' research group. In the past few years his group has made several important findings regarding the regulation of the arginine pathway in Neurospora. Seeing the clarification of previous observations and the emergence of new hypotheses has been fun.

Dr. Davis also deserves special thanks for encouraging me during the more difficult periods of graduate school and my research. His interest in my career and his cooperation as well as the cooperation of my thesis committee are greatly appreciated.

TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF ILLUSTRATIONS viii

LIST OF APPENDICES ix

LIST OF ABBREVIATIONS x

CHAPTER ONE: INTRODUCTION 1

General Review
The Enzymes and Their Structural Genes of the Arginine Pathway in Neurospora
Location of Amino Acid Pools in Neurospora
Arginine Channeling
Ornithine Channeling
Objective and Rationale

CHAPTER TWO: MATERIALS AND METHODS 31

Materials
Growth and Sampling
Fractionation and Chromatography
Calculations

CHAPTER THREE: RESULTS 40

Incorporation of ^{14}C-ornithine into Polyamines
Incorporation of ^{14}C-ornithine into the Arginine Pathway
Catabolism of ^{14}C-ornithine to Glutamic-γ-semialdehyde
Turnover of ^{14}C-ornithine

CHAPTER FOUR: DISCUSSION 60

Polyamines
The Arginine Pathway

The Behavior of Ornithine
The Behavior of Citrulline and Arginine
The Proline Pathway

CHAPTER FIVE: CONCLUSIONS AND SPECULATIONS 72

APPENDIX 78

LITERATURE CITED 103

LIST OF TABLES

1. Polyamine Levels in Wild-Type Neurospora . . . 81

2. Recovery of Radioactivity from Samples During Fractionation 83

3. Recovery of Acid-Soluble Material from Na^+-Columns 85

4. Recovery of Polyamines from Na^+-Columns 86

5. Desalting Neutral and Acidic Amino Acids on H^+-Columns 90

LIST OF ILLUSTRATIONS

1. Diagram of the Structural Genes, Biochemical Steps and Compartments Involved in Arginine and Ornithine Metabolism in Neurospora Mycelia 13

2. Specific Radioactivity of the Total Intracellular Ornithine and New Polyamines . . . 41

3. Uptake of 10^{-7} M ^{14}C-L-ornithine from the Medium and Its Subsequent Metabolism 43

4. Specific Radioactivities of the Total Intracellular Ornithine, New Polyamines and New Citrulline after the Addition of ^{14}C-L-ornithine 47

5. Specific Radioactivities of Citrulline, Soluble Arginine and New-protein Arginine after the Addition of ^{14}C-L-ornithine . . . 52

6. Specific Radioactivities of Soluble Glutamic-γ-semialdehyde plus Soluble Proline, and New-protein Proline after the Addition of ^{14}C-L-ornithine 57

7. Representative Growth Curve of the Conidial Isolate of Strain 74A 79

8. High-Voltage Paper Electrophoresis at pH 5.3 of Na^+-column Fractions 87

9. Paper Chromatography (Phenol-H_2O-NH_3) of Neutral and Acidic Amino Acid Fractions . . 91

10. High-Voltage Paper Electrophoresis at pH 5.3 of Neutral and Acidic Amino Acid Fractions 94

11. Incorporation of Radioactivity into the Arginine Pathway 97

12. Incorporation of Radioactivity into the Proline Pathway 99

LIST OF APPENDICES

A. Growth Curve and Polyamine Concentrations . . . 78

B. Recovery and Identification of Radioactivity . 82

C. Raw Data for the Incorporation of Radioactivity into the Arginine and Proline Pathways 102

LIST OF ABBREVIATIONS

ARG -- L-arginine

ASA -- L-argininosuccinic acid

ASA-B -- anhydride B of argininosuccinic acid

ASA-C -- anhydride C of argininosuccinic acid

ASP -- L-aspartic acid

CIT -- L-citrulline

GLU -- L-glutamic acid

GSA -- Lglutamic-γ-semialdehyde

ORN -- L-ornithine

PRO -- L-proline

PUT -- putrescine

SPD -- spermidine

CHAPTER ONE

INTRODUCTION

General Review

During the last two decades evidence has accumulated which indicates that soluble compounds in procaryotes and eucaryotes are not uniformly distributed within cells (reviewed in Oaks and Bidwell, 1970). This concept, metabolic channeling, is based on the demonstration that: (a) a single compound derived from different routes often encounters different metabolic fates; and (b) exogenously supplied ^{14}C-labeled metabolites are not distributed homogenously within the cell (Oaks and Bidwell, 1970).

Bidwell, Barr and Steward (1964) have shown that in carrot explants, amino acids destined for protein synthesis were separated from amino acid storage pools and products of protein turnover. There is evidence that in rat liver the valine utilized in protein synthesis was separated from a valine storage pool (Mortimore, Woodside and Henry, 1972). In Rana pipiens oocytes it has been observed that ^{14}C-amino acids are preferentially localized in the nucleus, the cortex and in the animal pole cytoplasm (Merriam, 1969).

Only the evidence for the concept of metabolic channeling which has been derived from isotopic labeling studies

in fungi will be reviewed. Cowie and Walton (1956) studied the flow of carbon from sugar into free and protein amino acids in Candida utilis. Following a brief label with ^{14}C-fructose there was an exponential loss of radioactivity from the cold trichloroacetic acid pool. The majority of this loss was accounted for by an accumulation of radioactivity in the protein. In similar experiments the appearance of ^{14}C in individual amino acids of the protein was shown to correlate directly with the disappearance of radioactivity from the corresponding amino acids in the cold acid-soluble pool. These data were interpreted to show that amino acids incorporated into protein were randomly withdrawn from a single intracellular pool.

In competition experiments, the cells were first briefly labeled with ^{14}C-fructose and then transferred to a ^{12}C-fructose-minimal medium which was either unsupplemented or supplemented with a ^{12}C-amino acid mixture. No difference was detected in the decrease in radioactivity of the corresponding soluble amino acid pools in the two cultures. These results were interpreted as evidence for the presence of two functionally distinct amino acid pools which are of different origin: (1) The "internal" pool, derived from fructose, is a metabolically active pool from which protein amino acids are drawn; and (2) the "expandable" pool, formed in the presence of exogenous amino acids, does not equilibrate with the "internal" pool.

Cowie and McClure (1959) felt that more direct evi-

dence for these conclusions was obtained by following the fate of a brief pulse of carrier-free ^{14}C-glutamate. There was an exponential loss of the ^{14}C-glutamate from the cold trichoroacetic acid pool. The radioactivity was detected in protein as ^{14}C-glutamic acid, ^{14}C-arginine, and ^{14}C-proline. The rate of loss of radioactivity from the pool ^{14}C-glutamate was the same whether or not ^{12}C-arginine (1.0 mg/ml) was present in the medium after the pulse. However, the fact that ^{14}C-glutamate entered the "internal" pool does not appear to be consistent with their definitions of "internal" and "expandable" pools.

Cowie and McClure (1959) studied the characteristics of these two pools further. They showed that the "expandable" pool concentrated the exogenous amino acids from the medium. Increasing the concentration of exogenous ^{14}C-threonine led to an increase in the amount of ^{14}C-threonine in both the trichloroacetic acid soluble and precipitable fraction. Though over 90 percent of the radioactivity in the trichloroacetic acid soluble pool was ^{14}C-threonine, the radioactivity in the protein was equally distributed between isoleucine and threonine. Thus, a small fraction of the "expandable" pool threonine entered the "internal" pool, a metabolically active pool with respect to amino acid interconversion and protein synthesis.

Detection of some of the "expandable" pool ^{14}C-threonine entering the threonine "internal" pool is in contrast to the ^{14}C-fructose experiments where there was

no evidence for mixing of these two pools. However, the utilization of some of the ^{14}C-threonine for protein synthesis and amino acid interconversions is consistent with the results from the ^{14}C-glutamate experiment. The significance of the differences between the ^{14}C-fructose experiments and the ^{14}C-threonine and ^{14}C-glutamate experiments was not discussed by Cowie and McClure (1959).

In addition to concentrating exogenous amino acids, Cowie and McClure (1959) demonstrated that the "expandable" pool, unlike the "internal" pool, was more sensitive to osmotic shock. The "expandable" pool was 50 percent extractable with cold water. This was determinded by measuring the counts in cold water after the cells had been labeled with ^{14}C-threonine. On the other hand, the "internal" pool amino acids were relatively insensitive to osmotic shock as indicated by the fact that ^{14}C-fructose labeled cells lost only 3 to 8 percent of their pool material after exposure to cold water. Also the amino acids in the "expandable" pool, unlike those of the "internal" pool, exchanged more readily with exogenous amino acids.

The results of Cowie and Walton (1956), Cowie and McClure (1959) were further developed by Sims and Folkes (1964), who examined the incorporation of nitrogen into free and protein amino acids in Candida utilis grown on a minimal medium containing ^{15}N-ammonia. They showed that for some amino acids the entire intracellular pool

was randomly utilized for protein synthesis. However, for the amino acids aspartate, lysine, histidine, arginine, and ornithine, the metabolically active pools were only a fraction of the total intracellular pools. In contrast to Cowie, they demonstrated that even without supplementation, certain amino acids were distributed between two functionally distinct endogenous pools: the "metabolic" and "storage" pools.

Wiemken and Nurse (1973) localized the functionally distinct pools in Candida utilis. Treating logarithmic cells with cytochrome c under isotonic conditions disrupted the plasmalemma while the tonoplast (vacuolar membrane) was left intact. Only 10 to 20 percent of the amino acids were released from the cells. Following the treatment with cytochrome c, osmotic shock with water disrupted the vacuoles and released most of the remaining soluble amino acids. The vacuoles contained the majority of the basic, nitrogen-rich amino acids. Cells grown on arginine as the nitrogen source were pulse labeled with ^{14}C-(U)-arginine. This showed that the cytoplasmic arginine and glutamate had a faster turnover rate than did these same amino acids in the vacuolar extract. The difference in turnover rates of the cytoplasmic and vacuolar glutamate was confirmed in ^{14}C-(U)-glucose and ^{15}N-NH_4^+ pulse experiments with Candida grown on glucose and NH_4^+. In these experiments it was also

shown that the cytoplasmic glutamine had a more rapid turnover rate than did the vacuolar glutamine. Further, the turnover rate of the amide nitrogen of the cytoplasmic glutamine was greater than that of the vacuolar glutamine.

The difference in amino acid composition and turnover rates of the cytoplasmic and vacuolar pools demonstrates an extreme compartmentation of amino acid metabolism. Though the identification of a metabolically active cytoplasmic pool and a vacuolar storage pool is a crude simplification, it is in agreement with the kinetic data of Sims and Folkes (1964). The differences between the physical properties of Cowie and McClure's (1959) "internal" and "expandable" pools cannot be reconciled with those of the cytoplasmic and vacuolar pools as defined by Wiemken and Nurse (1973).

Nurse and Wiemken (1974) using synchronous cultures of Candida utilis investigated the organization of the amino acid pools as the cell progressed through the division cycle. Synchronous cells, grown on arginine as the nitrogen source, were pulse labeled with ^{14}C-(U)-arginine or ^{14}C-(U)-glucose at different stages of the cell cycle. It was shown that during the bud expansion phase the uptake and catabolism of ^{14}C-(U)-arginine (expressed in terms of culture volume) doubled abruptly. For the remainder of the cell cycle, i.e., during cell separation and until the next bud initiation, the accumulation and

catabolism of arginine remained constant per ml of culture. The protein content of these cells and the accumulation of radioactivity in the protein per ml of culture increased exponentially throughout the cell cycle. That the abrupt accumulation and catabolism of arginine in the cells during the bud expansion phase represented a period of increased amino acid biosynthesis was confirmed by the ^{14}C-(U)-glucose pulse experiments. The total radioactivity in the cells per ml of culture increased exponentially during the cell cycle, while the counts in the total amino acids per ml of culture increased abruptly only during the bud expansion phase. As in the ^{14}C-(U)-arginine experiments, the radioactivity in the amino acids remained constant during the remainder of the cell cycle. Analyses of the incorporation of radioactivity in individual amino acids had the same pattern of incorporation as did the total amino acid pool. Despite the abrupt increase in the formation of the amino acid pools, their rate of utilization for protein synthesis increased steadily throughout the cycle. It was also shown that amino acids derived from protein turnover contributed very little to the amino acid accumulation detected during bud expansion.

Nurse and Wiemken (1974) feel that these differences in amino acid pool sizes may be correlated with vacuolar morphology changes observed thus far only during the cell cycle of S. cerevisiae (Wiemken, Matile and Moor,

1970). During bud extension the vacuoles enlarge and coalesce, whereas later, shortly before the buds appear, they shrink and fragment to form numerous small vacuoles. The vacuoles of lag and stationary phase cells have been shown to contain enzymes such as RNAase, esterase and proteases (Matile, Wiemken and Guyer, 1971). In contrast there is decreased activity of the vacuolar enzymes in exponentially growing cells (Matile et al., 1971).

Earlier studies by Matchett and DeMoss (1964) showed that there are at least two functionally distinct pools of intracellular tryptophan in log phase mycelia of Neurospora. Endogenously derived tryptophan was preferentially utilized for protein synthesis. The pool was only a small fraction of the total intracellular tryptophan pool. Though some exogenously supplied tryptophan was used for protein synthesis, the majority was readily catabolized. The tryptophan pool which was the precursor for protein synthesis was called the "metabolic" pool and the non-metabolized endogenous tryptophan pool was referred to as the "expandable" pool. Tryptophan in the "internal" pool was thought to mediate repression of the biosynthetic enzymes while tryptophan in the "expandable" pool mediated induction of the catabolic enzymes (Matchett, Turner and Wiley, 1968).

Matchett and DeMoss' (1964) interpretation il-

lustrates the confusion in the literature about the definition of the terms "internal" and "expandable" pool. Based on the terminology of Cowie and Walton (1956), the "internal" pool is the metabolically active pool for protein synthesis. The "expandable" pool, formed only in the presence of exogenous amino acids, does not equilibrate with the "internal" pool. In contrast to Cowie, both Sims and Folkes (1964) and Matchett and DeMoss (1964) found evidence for two functionally distinct endogenous pools. Cowie did not report if the exogenous amino acids were catabolized.

Contrary to Matchett and DeMoss (1964), Halvorson and Cohen (1958) found that in Saccharomyces cerevisiae exogenously supplied ^{14}C-phenylalanine was preferentially incorporated into protein without mixing fully with the total intracellular pool. Similar results were obtained by Zalokar (1961) for the utilization of exogenous proline in Neurospora. Zalokar observed a lag in the incorporation of ^{14}C-proline (0.4 mg/ml) into protein. Since the lag was shorter than if the total intracellular proline pool (0.86 mg per g dry weight) was mixing with the entering label, Zalokar proposed that an "intermediary" or "internal" pool between 0.05 and 0.3 mg per g dry weight was mixing with the entering ^{14}C-proline prior to incorporation into protein. Experiments in which the temperature or the external concentration of

^{14}C-proline were varied were consistent with the existence of this "intermediary"-"internal" ^{12}C-proline pool. Mycelia were also pretreated with ^{12}C-proline prior to being fed ^{14}C-proline. The rapid appearance of radioactivity in protein was consistent with the assumption that exogenous ^{12}C-proline first entered the "intermediary"-"internal" pool and was then diverted to a reservoir pool, the "expandable" pool, where it no longer diluted the ^{14}C-proline as it entered protein.

Again, it can be seen that "internal" and "expandable" pools found by Zalokar (1961) do not behave the same way as those of Cowie and Walton (1956) or Matchett and DeMoss (1964). Cowie and Walton's (1956) original definitions, which resulted from studies on a specific organism under defined growth conditions, does not necessarily apply to other organisms under different conditions or at different developmental stages. However, this confusion in terminology should not obscure the important conclusion of all these studies: For some amino acids there is evidence that there is more than one distinct intracellular pool.

Currently known mechanisms responsible for channeling are (1) the regulatory and kinetic properties of enzymes, and (2) the physical compartmentation of metabolites and enzymes. The latter can be due to: (a) enzyme aggregates confining metabolites as "enzyme-

bound" intermediates and (b) organellar confinement of metabolites and enzymes (Davis, 1972). The studies in Neurospora on the channeling of carbamyl phosphate, an intermediate in pyrimidine and arginine biosynthesis, reviewed by Davis (1972), is an example of channeling achieved by compartmentation. These studies also illustrate that intracellular gradients of metabolites are established as a result of metabolite and enzyme localization (Davis, 1972).

Neurospora has also proven to be useful for evaluating the relative roles of kinetic and "spatial" factors as they affect the fate of arginine and ornithine in this organism. Both amino acids are involved in anabolic and catabolic pathways. The channeling mechanisms are of particular interest because it has been shown that wild-type Neurospora cells grown on minimal medium contain pools of arginine and ornithine (8 and 12 mM, respectively, in cell water) as well as significant levels of the catabolic enzymes, arginase and ornithine aminotransferase (Davis, Lawless and Port, 1970). In spite of the potential for catabolism of endogenous arginine and ornithine, little or none occurs *in vivo* (Srb, Fincham and Bonner, 1950; Davis, 1970). Yet exogenously supplied ornithine and arginine are catabolized *in vivo* (Srb et al., 1950; Abelson and Vogel, 1955; Vogel and Kopac, 1959; Davis, 1970).

A better understanding of this paradox has been acquired from the studies on the compartmentalization of the enzymes involved in arginine and ornithine metabolism and the localization of the amino acid pools.

The Enzymes and Their Structural Genes of the Arginine Pathway in Neurospora

The anabolic and catabolic pathways involved in arginine and ornithine metabolism in Neurospora are illustrated in Fig. 1.

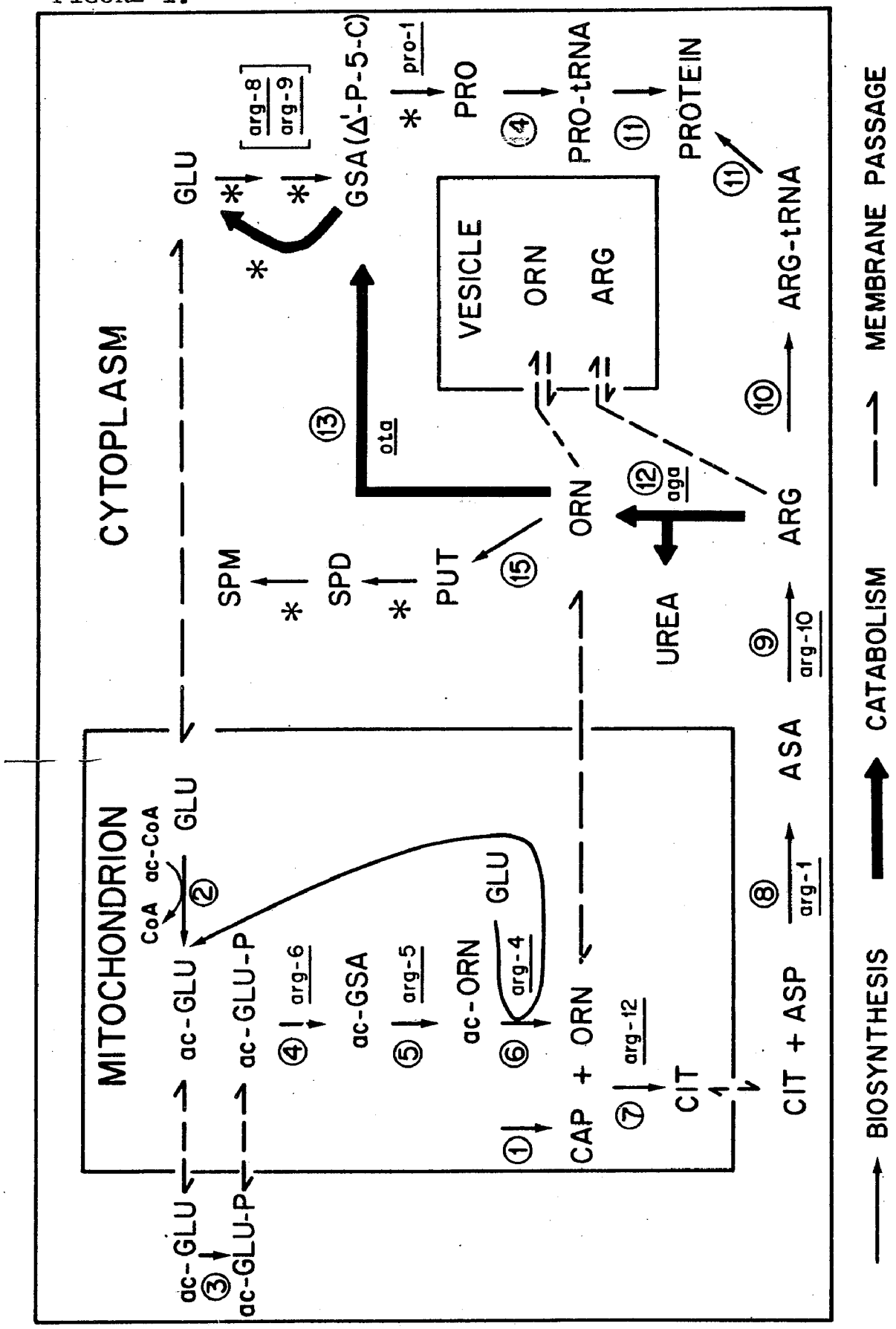
Arginine Biosynthesis

Glutamate is transformed via acetylated intermediates to ornithine. This set of reactions forms a cycle since the acetyl group is conserved in the transacetylase reaction. In this reaction, ornithine is liberated and acetylglutamate is regenerated (reviewed in Davis, 1967). Mutants for these reactions are known (Fig. 1). The growth requirements of such mutants (i.e., arg-4, arg-5, and arg-6), are satisfied by ornithine, citrulline, or arginine (reviewed in Davis, 1967). Citrulline is the product of the enzyme ornithine transcarbamylase which is specified by the arg-12 locus (Davis and Thwaites, 1963). Argininosuccinic acid synthetase and argininosuccinic acid lyase act in sequence to convert citrulline to arginine.

Beginning with glutamate, four of the enzymes involved in ornithine synthesis (i.e., N-acetylglutamate

Fig. 1.--Diagram of the structural genes, biochemical steps and cell compartments involved in arginine and ornithine metabolism in Neurospora mycelia. Enzymes: 1, carbamyl phosphate synthetase A (EC 2.7.2.5, arginine-specific); 2, N-acetylglutamate synthetase (EC 2.3.1.1); 3, N-acetyl-γ-glutamokinase; 4, N-acetylglutamic-γ-semialdehyde dehydrogenase; 5, acetylorinithine δ-transaminase (EC 2.6.1.11); 6, N^{α}-acetylornithine-glutamate transacetylase; 7, ornithine transcarbamylase (EC 2.1.3.3); 8, argininosuccinic acid synthetase (EC 6.3.4.5); 9, argininosuccinic acid lyase (EC 4.3.2.1); 10, arginyl-tRNA synthetase; 11, polypeptide formation; 12, arginase (EC 3.5.3.1); 13, ornithine aminotransferase (EC 2.6.1.13); 14, proline-tRNA synthetase; 15, ornithine decarboxylase (EC 4.1.1.17). Not localized is the enzyme, pyrroline-5-carboxylate oxidase, which oxidizes Δ^{1}-pyrroline-5-carboxylate to glutamate. Metabolites: GLU = L-glutamate; ac-GLU = N-acetyl-L-glutamate; ac-GLU-P = N-acetyl-γ-L-glutamyl phosphoric acid; ac-GSA = N-acetyl-L-glutamic-γ-semialdehyde; ac-ORN = N^{α}-acetyl-L-ornithine; ORN = L-ornithine; CAP = carbamyl phosphate; CIT = L-citrulline; ASP = L-aspartate; ASA = L-argininosuccinic acid; ARG = L-arginine; PUT = putrescine; SPD = spermidine; SPM = spermine; GSA = L-glutamic-γ-semialdehyde; Δ^{1}-P-5-C = Δ^{1}-pyrroline-5-carboxylate; PRO = L-proline

FIGURE 1.


CYTOPLASM
MITOCHONDRION
GLU
ac-CoA
CoA
ac-GLU
ac-GLU-P
ac-GSA
ac-ORN
CAP + ORN
CIT
CIT + ASP
ASA
ARG
UREA
ORN
PUT
SPD
SPM
GSA(Δ'-P-5-C)
PRO
PRO-tRNA
PROTEIN
ARG-tRNA
VESICLE
ORN
ARG
arg-8
arg-9
pro-1
arg-6
arg-5
arg-4
arg-12
arg-1
arg-10
ota
aga
BIOSYNTHESIS
CATABOLISM
MEMBRANE PASSAGE
* CELLULAR LOCALIZATION UNKNOWN

synthetase, N-acetylgutamic-γ-semialdehyde dehydrogenase, acetylornithine δ-transaminase [Cybis, unpublished] and N^{α}-acetylornithine-glutamate transacetylase [Weiss and Davis, 1973]) are part of the mitochondria. Located in the "soluble" fraction is one of the ornithine synthesizing enzymes, N-acetyl-γ-glutamokinase (Cybis, unpublished) and the last two enzymes of arginine synthesis (Weiss and Davis, 1973).

Polyamine Biosynthesis

Putrescine is synthesized from ornithine via ornithine decarboxylase (Davis et al., 1970). Putrescine and methionine are the precursors for the biosynthesis of spermidine and spermine (Green, 1957; Bachrach and Cohen, 1961). Neurospora mutants have not yet been isolated which lack the enzymes responsible for polyamine synthesis. Ornithine decarboxylase is found in the "soluble" fraction and is probably the only mode of synthesis of putrescine in Neurospora (Weiss and Davis, 1973).

Proline Biosynthesis

Glutamate also serves as a precursor of proline (Vogel and Bonner, 1954). It is reduced, possibly via glutamyl-γ-phosphate, to glutamic-γ-semialdehyde. Mutations occurring at either the arg-8 or arg-9 loci block the formation of glutamic-γ-semialdehyde (Vogel and Kopac, 1959). Glutamic-γ-semialdehyde cyclizes nonen-

zymically to Δ^1-pyrroline-5-carboxylate, which is subsequently reduced to proline. This last step is under the control of the pro-1 locus (Yura, 1959).

Arginine Catabolism

Two enzymes in this catabolic pathway have been more thoroughly investigated. The first, arginase (specified by the aga locus) is capable of hydrolyzing arginine to ornithine and urea (Mora, Tarrab and Bojalil, 1966; Davis et al., 1970). The second, ornithine aminotransferase, can convert ornithine to glutamic-γ-semialdehyde and its cyclized derivative (Vogel and Kopac, 1960). The ornithine aminotransferase structural gene, ota, is not linked to aga (Davis and Mora, 1968; Davis et al., 1970). A third enzyme, pyrroline-5-carboxylate oxidase, yields glutamate from glutamic-γ-semialdehyde, but has not been specifically described in Neurospora.

Both arginase and ornithine aminotransferase are found in the "soluble" fraction (Weiss and Davis, 1973).

Location of the Amino Acid Pools in Neurospora

Over 90 percent of the cellular arginine and ornithine pools in Neurospora crassa are found in an osmotically distinct organelle (Weiss, 1973). This organelle ("vesicle") has been isolated from exponential-phase, wild-type mycelia grown on Vogel's minimal medium

supplemented with 1.5 percent sucrose (Weiss, 1973). In the isolation procedure the mycelia are first treated with snail gut enzyme and then homogenized and fractionated in a 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 M sorbitol. The "vesicles" are separated from mitochondria on linear sucrose gradients (30 to 60 percent with a 65 percent sucrose cushion) in 10 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. Under these conditions the organelle has a mean density of 1.25 gm per cm^3 while the mitochondria have a mean density of 1.19 gm per cm^3 (Weiss, 1973). Recently it has been shown that in addition to the majority of the ornithine and arginine pools, most of the lysine and histidine are contained in these organelles (Weiss, unpublished). The vesicular content of other nitrogen-rich amino acids (i.e., glutamine and tryptophan) has not yet been determined. Only a small fraction of the neutral and acidic amino acids are found associated with purified "vesicles" (Weiss, unpublished). Electron micrographs of these organelle preparations reveal single membrane vesicles enclosing in some cases a dense interior. There is also a small amount of contamination by amorphous material and a few mitochondria (Weiss, unpublished). In the absence of full characterization, this organelle has been referred to as the "vesicle" (Weiss, 1973).

Kobr, Venderhaeghe, and Combepine (1969) have fractionated Neurospora crassa and isolated particles with a mean density of 1.222 gm per cm^3 on linear sucrose gradients. These particles, isolated from cells grown on Westergaard-Mitchell liquid medium with 1.5 percent acetate and 0.5 percent sucrose as the carbon source, contain isocitrate lyase, malate synthetase, malate dehydrogenase and possibly NAD-isocitrate dehydrogenase. These organelles, distinct from mitochondria, appear to be similar to the glyoxysomes of higher plants (Kobr et al., 1969).

Vacuoles have been isolated by flotation from lysed, germinating macroconidia of Neurospora crassa (Matile, 1971). The lysosomal nature of these vacuoles has been suggested by the high specific activity of hydrolyases in these organelles. These vacuoles are not seen in exponentially growing hyphae but they do reappear as the culture enters the stationary phase (Matile, 1971).

Because of the limited information on the "vesicle," it is premature to speculate as to whether the "vesicle" is related to either the microbody isolated by Kobr et al. (1969) or the lysosomal vacuole detected by Matile (1971).

Arginine Channeling

Two prominent features of the scheme in Fig. 1 are: (1) the compartmentation of the enzymes involved in arginine and ornithine metabolism and (2) the separation of most of the intracellular arginine and ornithine from their sites of synthesis and utilization. Biochemical studies using mutants have yielded further evidence that arginine and ornithine are compartmented.

In minimal-grown, logarithmic cultures of Neurospora the arginine pool is 8 mM in intracellular water. Under these steady-state conditions the intracellular arginine enters protein synthesis but not catabolism (Srb et al., 1950; Davis, 1970; Subramanian, Weiss and Davis, 1973), even though the catabolic enzymes are present at substantial levels (Davis et al., 1970). The absence of catabolism may be due to the fact that over 90 percent of the cellular arginine is in the vesicle (Weiss, 1973).

Subramanian et al. (1973) determined the metabolic fate and size of the cytoplasmic arginine pool as well as its relationship to that of the vesicular arginine pool. Minimal-grown logarithmic cultures were given a 2×10^{-7} M pulse of ^{14}C-arginine. The ^{14}C-arginine was taken up by the mycelia within the first three minutes. During the first two minutes the entering ^{14}C-arginine bypassed the resident ^{12}C-arginine pool and was fixed into protein via arginyl-tRNA. The ^{14}C-arginine not fixed into protein

entered the vesicle where it was selectively excluded from metabolism. This interpretation was based on the observation that the average specific radioactivity of the new protein arginine initially was 60-fold higher than the specific radioactivity of the total arginine pool. The average specific radioactivity of the new protein arginine declined rapidly and after two minutes was less than that of the total arginine pool. For the remaining two hours the specific radioactivity of the total arginine pool remained fairly constant. No ^{14}C-urea was detected throughout the experiment.

The fate of biosynthetic arginine (as opposed to externally supplied arginine), was tested by monitoring the uptake and utilization of a 2×10^{-7}M pulse of ^{14}C-ureido-citrulline (Subramanian et al., 1973). Based on the metabolism of the external ^{14}C-arginine pulse, it was hypothesized that the biosynthetic ^{14}C-guanidino-arginine would be used for protein synthesis. The labeled arginine not utilized for protein synthesis would be sequestered in the vesicle. It was, in fact, found that initially the ^{14}C-arginine bypassed the resident arginine pool and was fixed into protein; the remainder of the ^{14}C-arginine was withdrawn into the vesicle and thereafter substantially excluded from protein synthesis.

From the pulse experiments of Subramanian et al. (1973) it was calculated that the cytoplasmic arginine pool

was less than 5 percent of the total cellular pool. Therefore, the cytoplasmic arginine concentration is at most 0.5 mM in cell water, while the K_m of arginase for arginine is 5 mM (Subramanian et al., 1973). It appears that the vesicle, containing the majority of the intracellular arginine, may be adapted in part to minimize arginine catabolism when biosynthesis is the only source of this compound (Weiss, 1973).

Castañeda, Martuscelli, and Mora (1967) have shown that arginine catabolism does proceed when 1 mM arginine is present in the medium. There is a four-fold increase in the activity of the catabolic enzymes arginase and ornithine aminotransferase as well as an increase in the intracellular arginine concentration.

It appears that the regulation of arginine catabolism is more complex. An increase in the concentration of intracellular arginine as well as an increase in the activity of the catabolic enzymes is not always sufficient for arginine catabolism (Cañedo, Martuscelli, and Mora, 1967). It appears that the available nitrogen source is important in determining the metabolic fate of arginine (Cañedo et al., 1967). When L-citrulline is the sole nitrogen source in wild-type Neurospora, the mycelia become starved for nitrogen. Yet, the citrulline and arginine pools as well as the arginase, ornithine aminotransferase, and urease activities are increased. Sim-

ilarly, L-citrulline is neither a good nitrogen source nor proline source for the arg-8 mutant. However, exogenous arginine overcomes both nitrogen starvation and the proline requirement of the arg-8 mutant and nitrogen starvation of wild-type. The proline requirement of the arg-8 mutant is also satisfied when ammonium nitrate is added to the L-citrulline supplemented medium. It appears that ammonium nitrate facilitates the catabolism of the endogenous arginine, while in the absence of ammonium nitrate exogenously supplied arginine is preferentially catabolized (Cañedo et al., 1967).

The lack of arginine catabolism in cells grown on L-citrulline as the sole nitrogen source is further clarified by the studies of Mora, Salceda, and Sanchez (1972) on the regulation of arginase activity. They have shown both in vivo and in vitro that arginase activity is inhibited by L-citrulline, L-ornithine, and L-lysine. Consequently cells grown on L-citrulline have a high endogenous arginine pool which may feedback inhibit arginine synthesis (Mora et al., 1972). The inhibition of arginase by the elevated citrulline pool, together with the feedback inhibition of arginine biosynthesis allows the L-citrulline to be utilized efficiently as a source of arginine but not nitrogen (Mora et al., 1972). These studies do not explain how the presence of ammonium nitrate in the medium facilitates the catabolism of arginine derived from exogenous L-citrulline.

Ornithine Channeling

Previous studies have indicated that in Neurospora endogenous and exogenous ornithine do not have the same metabolic fates. Vogel and Bonner (1954) studied the nutritional requirements and accumulated intermediates in various mutants in order to determine the relationship of glutamate and ornithine to the arginine and proline pathways. Three classes of mutants were used (Fig. 1). (1) A proline auxotroph, pro-1, (2) An arginine auxotroph, arg-6, (3) Proline auxotrophs, arg-8 and arg-9. Nutritional studies with these mutants revealed that pro-1 growth requirements were satisfied only by proline. Arg-6 grew on ornithine, citrulline, and arginine but not on glutamic-γ-semialdehyde or proline. The proline auxotrophs, arg-8 and arg-9, could grow on glutamic-γ-semialdehyde, proline, ornithine, citrulline or arginine.

In these mutants the effectiveness of different amino acids as growth supplements had been observed (Srb et al., 1950). Citrulline was a more effective growth factor than ornithine for the arg-6 mutant. In contrast, arg-8 and arg-9 grew better on exogenous ornithine than on exogenous citrulline. Independent evidence for the differential utilization of exogenous and endogenous ornithine was obtained from tracer experiments in wild-type Neurospora (Abelson and Vogel, 1955; Vogel and Kopac, 1959). When 2- ^{14}C-ornithine (3 µg/ml) was added to the

medium the incorporation into protein proline relative to protein arginine was almost 50 percent higher than the incorporation obtained when the medium was supplemented with 300 µg/ml of 2- ^{14}C-ornithine.

At that time it was not yet known whether in Neurospora ornithine, like proline, was derived from glutamic-γ-semialdehyde, or if ornithine was derived via acetylated intermediates. Based on the differential utilization of exogenously supplied ornithine by the arginine and proline pathways, Vogel and Bonner (1954) originally concluded that endogenous ornithine, though capable of acting as an arginine precursor, is not available for proline synthesis. In order to explain this conclusion they proposed the concept of channeling of ornithine in Neurospora. The important feature of this concept is that the enzymes involved in the proline pathway are physically separated from those in the ornithine pathway.

In order to better understand the different, though not mutually exclusive, metabolic fates of exogenous and endogenous ornithine, the effects of the arg-12^{s} mutation on the proline and arginine pathways were studied. Arg-12^{s} itself creates no nutritional requirement, even though the level of ornithine transcarbamylase activity is reduced 40-50 fold and the K_m for ornithine is increased 5-10 fold (Davis, 1962a, Davis 1962b). When the arg-12^{s} mutant is grown on minimal medium, the arginine pool is

one-tenth of that found in wild-type; yet the mutant exhibits normal growth rates. This shows that the endogenous ornithine supply can support the required rate of arginine synthesis (Davis, 1962a, Davis, 1968).

Davis (1968) showed that when arg-12^{S} is combined with arg-8 or arg-9, the proline requirement imposed by the latter mutants is eliminated. Studying it further he showed that in the arg-8, arg-12^{S} double mutant the glutamic-γ-semialdehyde used for proline biosynthesis was derived from ornithine catabolism via ornithine aminotransferase (Fig. 1). When catabolism was mutationally blocked in the triple mutant, arg-8, arg-12^{S}, ota, there was no growth on minimal medium or on medium supplemented with ornithine or arginine. However, the growth requirement was satisfied by proline.

Davis (1968) felt that the arg-12^{S} mutation increased the availability (via catabolism) of endogenous ornithine for proline biosynthesis. This could be due in part to the two-fold elevation of the ornithine pools on minimal medium characteristic of strains carrying the arg-12^{S} mutation, rather than to increased activity of the ornithine aminotransferase (Davis, 1968). (Both arg-12^{S} and arg-8, arg-12^{S} had only slightly induced levels of ornithine aminotransferase.) The increased ornithine pools could be a consequence of a regulatory response to the decreased arginine concentrations (Davis, 1968).

Though the arg-12^{s} mutation does increase the availability (via catabolism) of endogenous ornithine for proline biosynthesis, Davis (1968) presented evidence that even in wild-type a small amount of endogenous ornithine is catabolized. The genetic background influences the degree of leakiness of an arg-8 or arg-9 mutant, and some strains, after a long lag period, grow quite well on minimal medium. When ornithine catabolism was mutationally blocked in arg-8, the double mutant, arg-8, ota, did not grow on minimal medium or medium supplemented with ornithine or arginine. However, proline did satisfy the growth requirements of the double mutant. These results substantiate the assumption that normally some endogenous ornithine is converted to glutamic-γ-semialdehyde via ornithine aminotransferase. This rate of catabolism, however, is not sufficient for wild-type growth.

Davis (1968) showed that arg-12^{s} also affects the utilization of exogenous ornithine by mutants unable to synthesize ornithine (Fig. 1). Single mutants, such as arg-5, can use exogenous ornithine at less than 10^{-3}M in the medium for arginine synthesis. However, when arg-5 is combined with arg-12^{s}, the double mutant, arg-5, arg-12^{s}, is incapable of growth on exogenous ornithine unless its concentration is 0.1 M or greater, and even then growth is poor.

Exogenously supplied ornithine can be more ef-

ficiently transformed to arginine in strains carrying the arg-5 and arg-12^{s} genotype if catabolism is blocked by a third mutation, ota, which imposes an ornithine aminotransferase deficiency (Davis, 1968). The triple mutant, arg-5, arg-12^{s}, ota, is able to utilize low concentrations of exogenous ornithine for arginine synthesis even more efficiently than the arg-5 single mutant. In the triple mutants, growth on ornithine causes much higher intracellular ornithine concentrations than in other arg-12^{s} strains grown on ornithine.

Based on the effects of arg-12^{s} on mutants of the proline and arginine pathways, it was difficult to determine whether the elevated ornithine pools were overcoming a concentration or locational requirement, or both (Davis, 1968).

Objective and Rationale

Further clarification of the channeling of ornithine in Neurospora is needed. Taking into consideration the nutritional studies with mutants, the multiple intracellular sites of ornithine utilization, and the localization of the ornithine pool, certain hypotheses can be made about the fate of endogenous ornithine:

1. The total ornithine synthesized under steady-state conditions should be the sum of the total amount of cellular arginine (i.e., citrulline and argininosuccinic acid pool and protein

arginine), polyamines and ornithine. This is 263 nanomoles per mg dry weight per generation.

2. About 4/5 of the ornithine synthesized in the mitochondria should be transformed to citrulline.
3. The remaining ornithine (approximately 1/5) should depart from the mitochondria and should enter the cytoplasm where it has several fates:
   a. Approximately 1/2 of the cytoplasmic ornithine should be converted to putrescine.
   b. The majority of the non-metabolized ornithine should be sequestered by the vesicle.
   c. Little catabolism should occur because the cytoplasmic concentration is not sufficiently high.

Monitoring the uptake and utilization of a 10^{-7}M pulse of uniformly labeled ^{14}C-L-ornithine in wild-type Neurospora is a way of testing these hypotheses. It is assumed that initially the ^{14}C-ornithine which is taken up through the cell membrane first enters the cytoplasm where it freely mixes with endogenous cytoplasmic (non-organellar) ornithine and is utilized by ornithine decarboxylase. Determination of the specific radioactivity of the newly synthesized polyamines immediately following the pulse of

^{14}C-ornithine is a means of estimating the "cytoplasmic" ornithine pool exposed to ornithine decarboxylase, a cytoplasmic enzyme. If there is no endogenous cytoplasmic ornithine pool, the initial specific radioactivity of the newly synthesized polyamines will approach the specific radioactivity of the added ^{14}C-ornithine. If there is a cytoplasmic ornithine pool, which is only a fraction of the total ornithine pool, then the initial specific radioactivity of the newly synthesized polyamines will be greater than that of the total cellular ornithine pool, but significantly less than that of the added ^{14}C-ornithine. According to the hypotheses it is expected that there will be a small cytoplasmic ornithine pool.

The ^{14}C-ornithine not utilized for putrescine synthesis has three other possible fates: (1) Some of the ^{14}C-ornithine may be catabolized to glutamic-γ-semialdehyde which is then converted to proline and/or glutamic acid. From such data the flux through ornithine aminotransferase can be estimated. Because it is anticipated that there is only a small cytoplasmic ornithine pool, it is expected that the flux of ^{14}C-ornithine through ornithine aminotransferase will be small. (2) Some of the ^{14}C-ornithine may enter the mitochondria and be transformed to citrulline. A comparison of the specific radioactivity of the newly synthesized citrulline to that of the total ornithine pool and to that of the newly syn-

thesized polyamines is a means of determining if there is a mitochondrial ornithine pool. According to the hypotheses it is expected that the mitochondrial ornithine pool is small and turns over rapidly. (3) The ^{14}C-ornithine which is not immediately metabolized is expected to be sequestered from the cytoplasm in the vesicle.

Monitoring the specific radioactivity of the newly synthesized polymaines over longer intervals after the administration of ^{14}C-ornithine will reveal if, and approximately how much, vesicular ornithine is released into the cytoplasm. It is expected that endogenous ornithine from the mitochondria, rather than the ornithine from the "vesicle," is the main source of ornithine for putrescine synthesis. Therefore at these later times the specific radioactivity of the newly synthesized polyamines should be lower than the specific radioactivity of the total cellular ornithine pool. It is also possible to determine if vesicular ornithine is catabolized and whether it enters the mitochondria.

CHAPTER TWO

MATERIALS AND METHODS

Materials

A conidial isolate from the wild-type strain, 74A (R. H. Davis, personal collection) was used throughout this investigation. Vogel minimal medium N (Vogel, 1964) supplemented with 1.5 percent sucrose was used for growth. Putrescine and spermidine were purchased from Sigma Chemical Co. The uniformly labeled ^{14}C-L-ornithine (203 mCi/mmole) was purchased from New England Nuclear Corp. The ^{14}C-ornithine was purified before use by column chromatography, and the degree of purity was further determined by high voltage electrophoresis.

All radioactivity measurements were made on a Beckman CPM-100 liquid scintillation counter with the quench standardized. A modification of the Toluene/Triton X-100 (2:1 v/v) liquid scintillant was used (Turner, 1972). The toluene contained 0.5 percent w/v of 2,5-diphenyloxazole (PPO). Reagent grade Triton X-100 was obtained from Research Products International.

Growth and Sampling

The 74A strain was grown exponentially from a conidial inoculum (1.0×10^6 conidia per ml) in 1,000-ml cultures of

minimal medium with forced air at 25 C. The cultures were used when they had attained a dry weight of 0.30 to 0.40 mg per ml. Dry weights were determined by harvesting and acetone-drying 30 to 40 ml of culture.

In the experiments ^{14}C-ornithine was added to a final concentration of about 0.1 μM (43,000 counts per min [cpm] per ml). At frequent intervals (15 sec intervals for the first 6 minutes, followed by 1 min, 2 min, 3 min, and 30 min intervals), 3-ml and 10-ml samples were simultaneously withdrawn from the cultures. The 3-ml samples were analyzed for the uptake of the radioactivity from the medium and the 10-ml samples were analyzed for the metabolism of the ^{14}C-ornithine. The uptake of the isotope by the mycelia was determined by the following method: The 3-ml harvests were pipetted initially into a solution of unlabeled ornithine at 4 C, giving a final concentration of 10 mM unlabeled ornithine. No further uptake or exchange of the isotope took place under these conditions. The mycelia were later collected by suction on Gelman GA-6 cellulose acetate membrane filters (0.45 μ pore size) and washed with cold 10 mM ornithine. The filters with the mycelia were first air-dried and then counted. Aliquots of the filtrate containing the growth medium were counted for the purpose of verifying the percentage of ^{14}C-ornithine in the mycelia.

The 10-ml samples for determining the metabolism

of the ^{14}C-ornithine were initially pipetted into a trichloroacetic acid solution at 4 C giving a final concentration of 5 percent acid. Later the acid-insoluble material was separated from the acid-soluble fraction by spinning for 5 min at top speed in a clinical centrifuge. The acid-insoluble material was extracted once more with 5 percent trichloroacetic acid and this supernatant was combined with the first. This combined extract will be referred to as the acid-soluble pool. The acid-soluble pool was extracted four times with ethyl ether, evaporated at 60 C to near dryness, redissolved and used for column chromatography.

Protein was isolated from the trichloroacetic acid-extracted residue by a modified procedure of Roberts, Abelson, Cowie, and Bolton (1955). The incubation of the residue in 5 percent trichloroacetic acid at 90 C for one hour was omitted in order to retain all the ^{14}C-proline and ^{14}C-arginine present in the protein and aa-tRNA (Cowie and Walton, 1956). The protein was hydrolyzed overnight in 6N HCl in evacuated tubes at 110 C. The hydrolysates were evaporated, redissolved in water and filtered, evaporated again, and redissolved in 6N HCl or water for chromatography. Measurements of the radioactivity during the isolation procedure established that the radioactivity was associated with the protein fraction and that during hydrolysis it was all liberated as soluble,

chromatographically identifiable proline and arginine.

Fractionation and Chromatography

The amino acids in the acid-soluble pool were initially separated on analytical grade Dowex-50 x 8 (Na^+-form, 200-400 mesh) columns (0.7 by 15 cm). The amino acids were eluted first with 50 ml of 0.116 M Na_3 citrate (pH 5.3), followed by 40 ml of 0.30 M Na_3 citrate (pH 5.3) and finally 30 ml of 0.2 N NaOH. The first 20 ml of the 0.116 M Na_3 citrate buffer contained proline, glutamic-γ-semialdehyde, glutamate, argininosuccinic acid and citrulline. Ornithine emerged in the second 20 ml of 0.116 M Na_3 citrate buffer. Arginine was found in the second and third 10 ml fraction of the 0.30 M Na_3 citrate buffer. Putrescine and spermidine were displaced by the first 20 ml of the 0.2 N NaOH. The method of Subramanian et al. (1973) was used to purify the proline and arginine in the protein hydrolysates. Over 90 percent of the radioactivity applied to the Na^+-columns was recovered (Table 3, Appendix B). The recovery of the labeled polyamines (which constitute only 3 percent of the total acid-soluble radioactivity) was improved from 73 percent to 95 percent by the addition of 1 μmole of unlabeled putrescine and 2 μmoles of unlabeled spermidine to the acid-soluble material prior to column chromatography (Table 4, Appendix B).

Fractions containing the neutral and acidic amino

acids were desalted on the Dowex-50 x 8 columns in the H^+-form and were eluted with successive additions of 10 ml of 0.1 N HCl, 30 ml of 1.0 M piperidine, 10 ml of 1.0 M NaCl and 10 ml of 0.2 N NaOH (Table 5, Appendix B). Over 82 percent of the amino acids emerged in the second 10 ml fraction of piperidine This fraction was later analyzed for the percentage of radioactivity in each amino acid. Because the third 10 ml fraction of 1.0 M piperidine contained 8 percent of the radioactivity and had the same distribution of amino acids as the previous one, it was discarded. The salts emerged in the 0.2 N NaOH fraction. The ornithine, arginine and polyamine fractions were desalted according to the method of Subramanian et al. (1973). The amino acid fractions were evaporated to dryness and redissolved in water or concentrated HCl and used for chromatography and electrophoresis.

Proline, citrulline, argininosuccinic acid and glutamic acid isolated at the front of the Na^+-columns and desalted on the H^+-columns (above) were further purified by paper chromatography in phenol:water (100:20 v/v) in an NH_3 atmosphere, and on high voltage paper electrophoresis using pyridine:acetic acid:water (6.1: 2.72 : 391 v/v/v) at pH 5.3 (Figs. 9 and 10, Appendix B). This system did not distinguish argininosuccinic acid from glutamic-γ-semialdehyde. (The identity of the other amino acids and polyamines was also frequently checked in the

high voltage electrophoresis system [Fig. 8, Appendix B].) Radioactivity was detected by an Actigraph III paper strip scanner (Nuclear-Chicago Corp.) and the amino acids by a ninhydrin spray. At least 90 percent of each radioactive compound was indistinguishable from a corresponding unlabeled standard.

Concentrations of the amino acids in the acid-soluble pool and protein were estimated colorimetrically: arginine by the method of Van Pilsum, Martin, Kito, and Hess (1956); proline and glutamic-γ-semialdehyde and ornithine by the method of Chinard (1952). The polyamine concentration in the acid-soluble pool was determined by David R. Morris (by the method in Morris, Koffron and Okstein, 1969) at several stages during logarithmic growth (Table 1, Appendix A). It was shown that the concentration of polyamines per mg dry weight remained constant and this value was used for all future determinations of the polyamine concentration.

Calculations

The counts in each neutral and acidic amino acid were determined from the percentage of the total radioactivity detected in these compounds after resolution by paper chromatography and electrophoresis. Determinations with a mixture of ^{14}C-proline, ^{14}C-citrulline and ^{14}C-glutamate standards showed that there was no appreciable

loss of these amino acids during paper chromatography (Table 5, Appendix B).

Knowing the percentage of isotope in the mycelia, the counts in the intracellular ornithine were computed from the counts isolated in the ornithine fraction on the Na^+-columns.

The specific radioactivity, counts per min per nmole (cpm/nmole), of the acid-soluble pool compounds was calculated from the raw counts (or the best-fit curve for the counts) and the calculated average nmoles of the corresponding compound present in the total intracellular pool. Direct determinations of the specific radioactivity of the soluble ornithine and arginine isolated from the mycelia agreed within 16 percent of the calculated value.

For a given interval the specific radioactivity of the ornithine entering the following newly synthesized compounds was calculated: polyamines; citrulline; protein arginine and protein proline. Certain parameters are important in such computations. The doubling time (150 min) (Fig. 7, Appendix A) of the mycelia and the concentration of the compounds (per unit dry weight) remained constant during exponential growth. Therefore, at each interval the increase in the nanomoles of polyamines, citrulline and its respective derivatives could be calculated according to the formula: $\Delta X = X_o(e^{kt}-1)$

where X_o was the concentration of the compound in a 10-ml volume of culture at the beginning of the interval. For a 150 min doubling time the value of k was 0.0046/min. From the determination of radioactivity in each fraction the increase in radioactivity (Δcpm per 10-ml culture) was obtained. Thus for each interval the average specific radioactivity of a new compound was computed by dividing the increase in counts (Δcpm) by the increase in nmoles (ΔX).

For determining the specific radioactivity of the newly synthesized polyamines the radioactivity data was corrected for the loss of one molecule of $^{14}CO_2$ from the uniformly labeled ^{14}C-L-ornithine molecule during putrescine synthesis. Determinations in vitro with E. coli ornithine decarboxylase and ^{14}C-U-L-ornithine confirmed that one molecule of $^{14}CO_2$ was released for every molecule of putrescine formed. This shows that the $^{14}CO_2$ had the same specific activity as the other ^{14}C-carbons in the ^{14}C-U-L-ornithine molecule (Weiss and Scott, unpublished). The Δcpm for each interval were computed from the best-fit curve for the corrected cpm data. The Δnmoles for each interval in a 10-ml sample was calculated for the concentration of total polyamines which included putrescine (0.6 nmoles per mg dry weight); spermidine (21 nmoles per mg dry weight); and spermine (0.2 nmoles per mg dry weight) (Table 1, Appendix, A).

In calculating the specific radioactivity of the newly synthesized citrulline the counts found in the citrulline, argininosuccinic acid, soluble and protein arginine were included. For each time point the counts appearing in each of these compounds was totaled and from the best-fit curve the Δ cpm for each interval was computed. The calculation of the Δnmoles for each interval was based on the sum of the concentrations in the 10-ml sample of citrulline plus argininosuccinic acid (together 3 nmoles per mg dry weight [Davis, unpublished]); soluble arginine (27 nmoles per mg dry weight) and protein arginine (179 nmoles per mg dry weight).

For computing the specific radioactivity of the newly synthesized protein arginine and proline only the raw data on the radioactivity of these amino acids in the protein and the new molecules added per unit protein were used. In this calculation protein turnover was neglected because the measurements were made over such a short period of time and the cells were in logarithmic growth.

CHAPTER THREE

RESULTS

After addition of ^{14}C-ornithine to the medium, it was rapidly taken up by the mycelia, and this rate decreased appreciably between three and six minutes (Figs. 2 and 3). Within six minutes 95 percent of the isotope was present in the mycelia.

An analysis of the label in the intracellular pool indicates that some of the entering ^{14}C-ornithine was being metabolized and that the metabolism continued at a slower rate after uptake of the isotope was completed (Fig. 3). Fifty percent of the entering ^{14}C-ornithine was simultaneously utilized by three enzymes: (1) ornithine decarboxylase, as measured by ^{14}C-polyamines; (2) ornithine carbamyltransferase, as measured by ^{14}C-citrulline and subsequent ^{14}C derivatives of citrulline; and (3) ornithine aminotransferase, as measured by ^{14}C-glutamic-γ-semialdehyde, soluble ^{14}C-proline and protein ^{14}C-proline.

Incorporation of ^{14}C-Ornithine into Polyamines

The recovery of polyamines from the Na^+-columns (see Methods) was better controlled for in the experiment in Fig. 2 than in the one shown in Fig. 3. During the

Fig. 2.--(Main Axes): Specific radioactivity of the total intracellular ornithine and new polyamines. Points representing intracellular ornithine are plotted at the sampling times indicated on the abscissa. Points representing the new polyamines are plotted at the mid-point of the individual successive intervals. (Inset): Uptake of 10^{-7} M ^{14}C-ornithine from the medium and its subsequent metabolism via ornithine decarboxylase (ODCase). The line describing each curve is derived from the raw cpm for each point.

FIGURE 2.

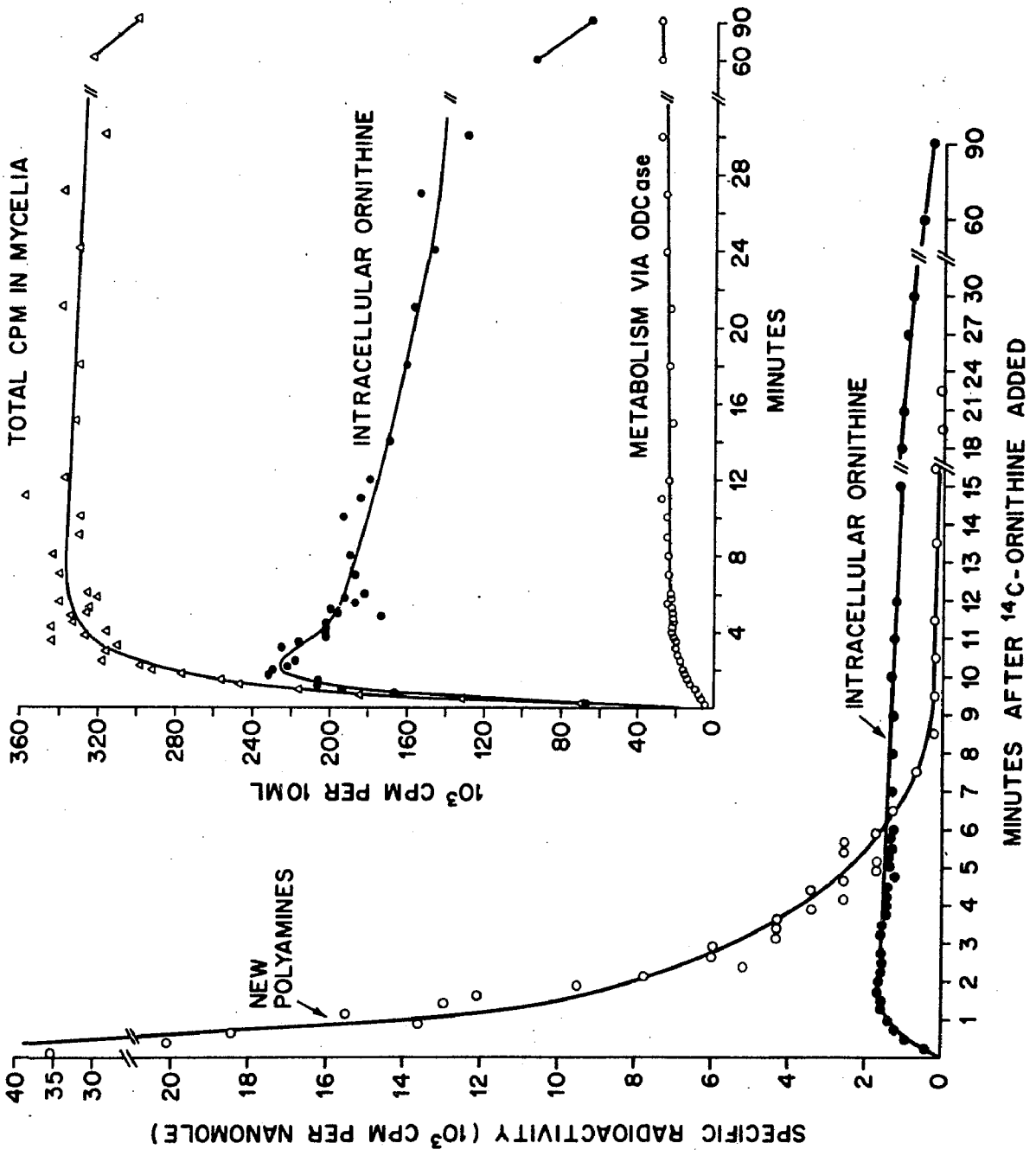
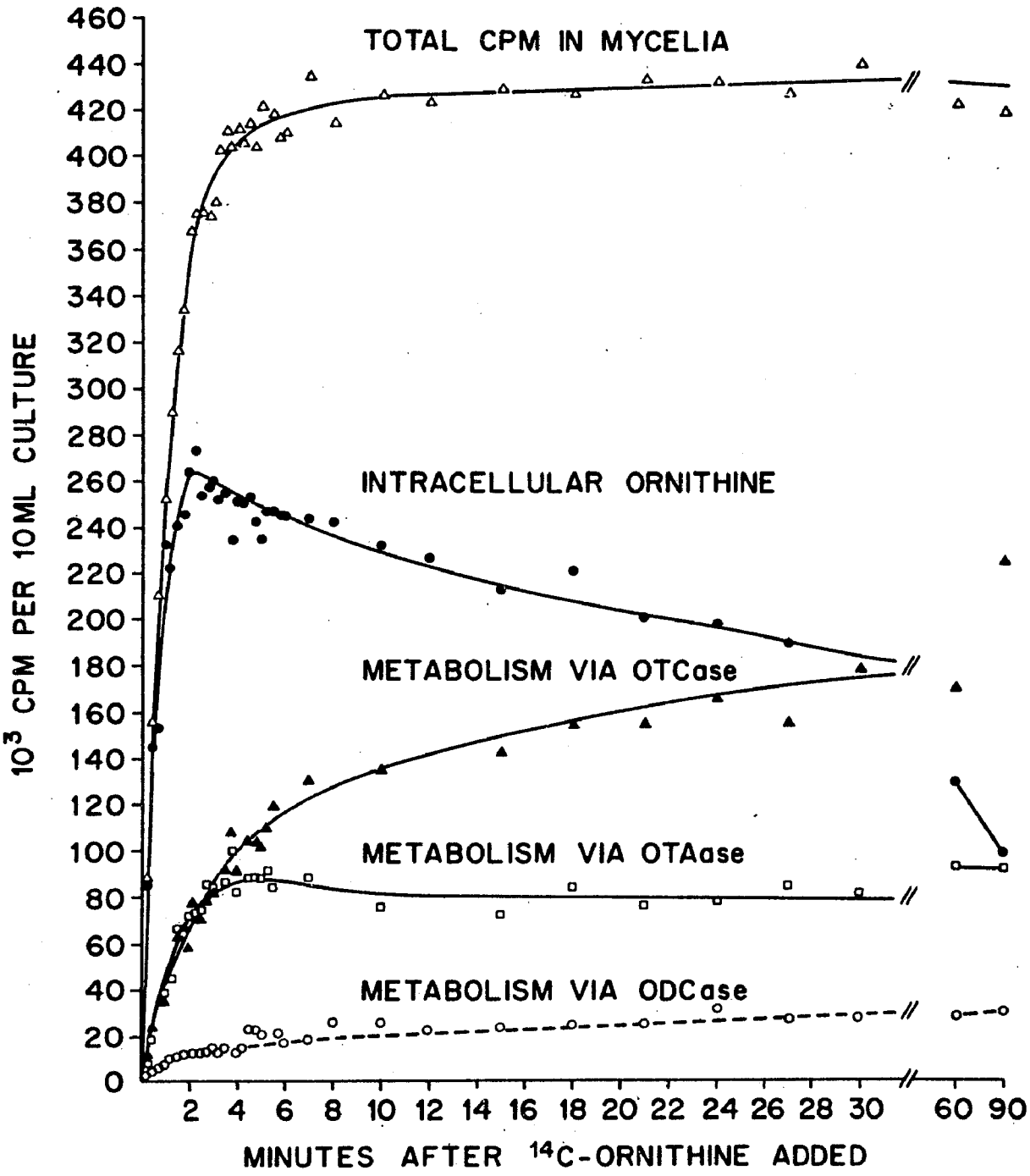

TOTAL CPM IN MYCELIA
INTRACELLULAR ORNITHINE
METABOLISM VIA ODCase
MINUTES
10³ CPM PER 10ML
NEW POLYAMINES
INTRACELLULAR ORNITHINE
SPECIFIC RADIOACTIVITY (10³ CPM PER NANOMOLE)
MINUTES AFTER ¹⁴C-ORNITHINE ADDED

Fig. 3.--Uptake of 10^{-7} M ^{14}C-L-ornithine from the medium and its subsequent metabolism via ornithine decarboxylase (ODCase); via ornithine aminotransferase (OTAase); via ornithine transcarbamylase (OTCase). The line describing each curve is derived from the raw cpm for each point.

FIGURE 3.


TOTAL CPM IN MYCELIA
INTRACELLULAR ORNITHINE
METABOLISM VIA OTCase
METABOLISM VIA OTAase
METABOLISM VIA ODCase
10^3 CPM PER 10ML CULTURE
MINUTES AFTER ^{14}C-ORNITHINE ADDED
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 60 90

first six minutes the pattern of incorporation of ^{14}C-ornithine into polyamines paralleled that of the uptake of the label. However, once the uptake of the isotope was completed, the intracellular ^{14}C-ornithine entered the polyamines only slowly (Fig. 2, inset).

A comparison of the specific radioactivities of the total ornithine pool and the new polyamines is shown on the main axes of Fig. 2. During the first 15-sec interval the average specific radioactivity of ornithine incorporated into polyamines (35.9×10^3 cpm/nmole) is greater than that of the total intracellular ornithine pool (0.24×10^3 cpm/nmole) and less than the specific radioactivity of the external ^{14}C-ornithine (451×10^3 cpm/nmole). Further analyses of these data give an estimate of the intracellular ornithine pool available for polyamine synthesis. During this 15-sec interval the average specific radioactivity of the total cellular ornithine pool is 150-fold less than that of the polyamines. This comparison indicates that only 0.67 percent of the total cellular ornithine pool was available for polyamine synthesis and it was preferentially labeled. This value is probably an over-estimate of the steady-state conditions since it disregards the significant flux through the pool during this interval.

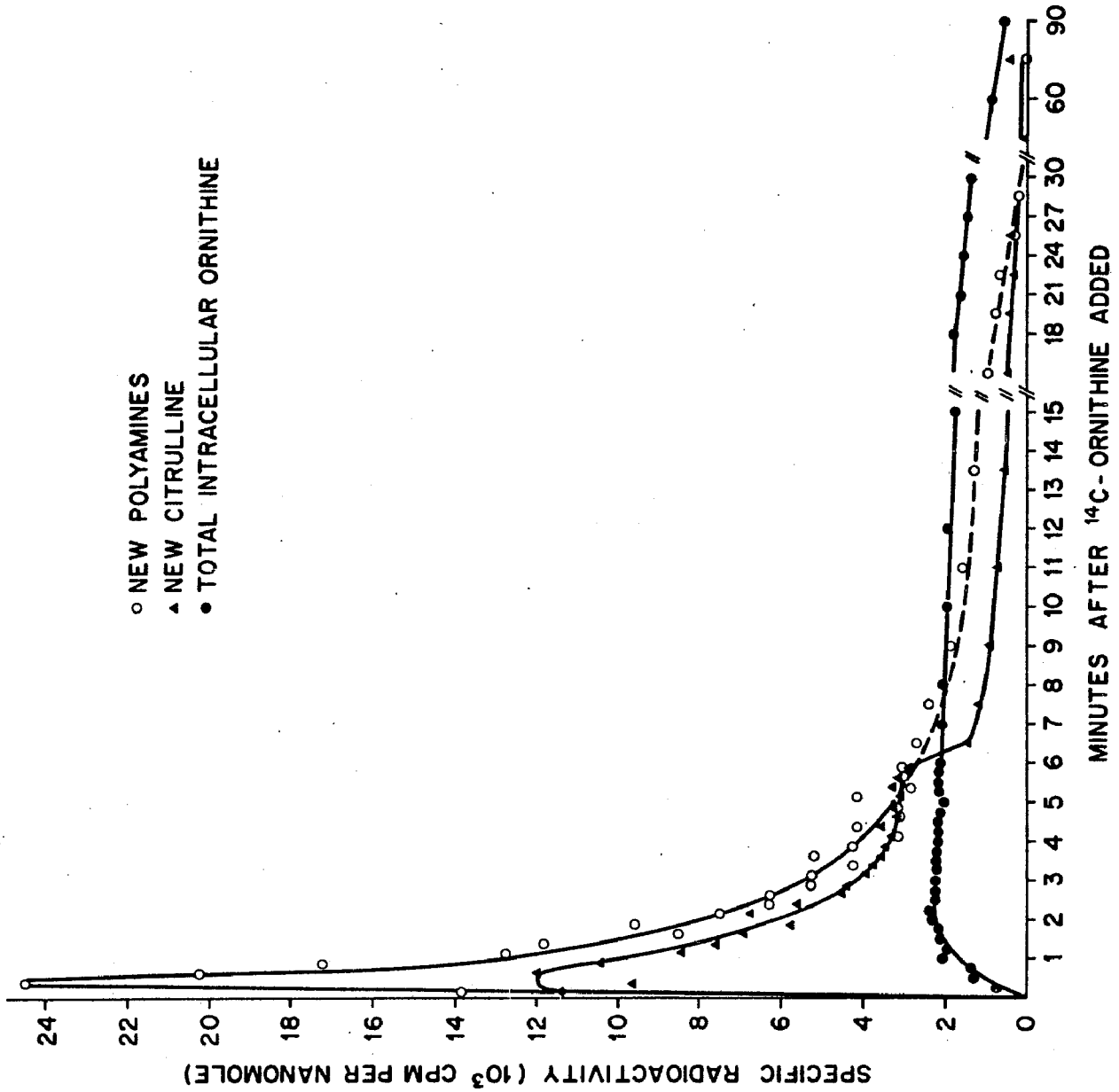
A comparison of the specific radioactivity of the new polyamines to that of the external ^{14}C-ornithine

reveals that during the first 15-sec interval the isotope was diluted almost 13-fold. During this interval the amount of ^{14}C-ornithine removed from the medium by the cells in a 10-ml sample was 0.154 nmoles (69.4×10^3 cpm/ 451×10^3 cpm/nmole). Upon entering the cytoplasm the ^{14}C-ornithine mixed with almost 12 parts (1.8 nmoles) of unlabeled ornithine. Therefore, in a 10-ml sample the unlabeled ornithine pool accessible to ornithine decarboxylase (1.8 nmoles) is only 1.3 percent of the total intracellular ornithine pool (140 nmoles). Though this value, 1.3 percent, is again an over-estimate because flux is disregarded, it is similar to the 0.67 percent determined from the comparison of the specific radioactivity of the new polyamines to that of the total ornithine.

In spite of the difficulties in determining the radioactivity entering polyamines in the experiment in Fig. 3, it can be seen that the pattern of incorporation of radioactivity into polyamines (Figs. 3 and 4) is very similar to that found in the experiment illustrated in Fig. 2. A comparison during the first 15-sec interval of the average specific radioactivity of the new polyamines (13.8×10^3 cpm/nmole) to that of the total ornithine pool (0.38×10^3 cpm/nmole) reveals that the specific radioactivity of the total ornithine pool is 36-fold less than that of the new polyamines (Fig. 4).

Fig.4.--Specific radioactivities of the total intracellular ornithine, new polyamines and new citrulline after the addition of ^{14}C-L-ornithine. Points representing intracellular ornithine are plotted at the sampling times indicated on the abscissa. Points representing the new polyamines and new citrulline are plotted at the mid-point of the individual successive intervals.

FIGURE 4.


○ NEW POLYAMINES
▲ NEW CITRULLINE
● TOTAL INTRACELLULAR ORNITHINE
SPECIFIC RADIOACTIVITY (10^3 CPM PER NANOMOLE)
0
2
4
6
8
10
12
14
16
18
20
22
24
MINUTES AFTER ^{14}C-ORNITHINE ADDED
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 18 21 24 27 30 60 90

This indicates that approximately 2.8 percent of the total ornithine pool was available for polyamine synthesis.

A comparison of the specific radioactivity of the new polyamines to that of the external ^{14}C-ornithine shows that during the first 15-sec interval the isotope was diluted almost 32-fold. During this interval the amount of ^{14}C-ornithine removed from the medium by the cells in the 10-ml sample was 0.191 nmoles (86. x 10^3 cpm / 451 x 10^3 cpm/nmole). Upon entering the cytoplasm the ^{14}C-ornithine was diluted by almost 31 parts (6 nmoles) of unlabeled ornithine. Therefore, in the 10-ml sample the unlabeled ornithine pool accessible to ornithine decarboxylase (6 nmoles) is only 5.3 percent of the total endogenous ornithine pool (114 nmoles). Though flux is disregarded in both calculations in this experiment (Fig. 4), it is estimated that between 2.8 and 5.3 percent of the endogenous ornithine pool is used for polyamine synthesis.

This value (2.8 to 5.3 percent) of the endogenous ornithine available for polyamine synthesis is larger than the estimated available pool (0.67 to 1.2 percent) in the experiment in Fig. 2. Despite the inaccuracies in the calculations, the results of both experiments indicate that prior to use in the ornithine decarboxylase reaction the ^{14}C-ornithine entering the cytoplasm initially mixed with a very small fraction of the un-

labeled intracellular ornithine.

For the first six minutes the average specific radioactivity of the new polyamines continued to decline in successive 15-sec intervals, although it remained greater than that of the total ornithine pool. After six minutes the specific radioactivity of the ornithine pool remained higher than that of the new polyamines (Fig. 2). This indicates that the remaining ^{14}C-ornithine was actually excluded from polyamine synthesis.

Incorporation of ^{14}C-Ornithine into the Arginine Pathway

The utilization of ^{14}C-ornithine for citrulline synthesis was measured by the appearance of ^{14}C in citrulline and its anabolic derivatives (Figs. 3 and 4). After a peak at 30 seconds the ^{14}C-ornithine entering citrulline decreased regularly. For the first six minutes the average specific radioactivity of the new citrulline was lower than that of the new polyamines but greater than that of the total intracellular ornithine pool. This implies that the entire cellular ornithine pool is not available to ornithine transcarbamylase, but that ornithine transcarbamylase has access to a larger unlabeled ornithine pool than does ornithine decarboxylase. After the first six minutes the average specific radioactivity of the new citrulline was less than that of the cellular ornithine and at all

times was lower than that of the new polyamines.

Once the counts in citrulline and argininosuccinic acid entered the arginine pool and protein arginine, the behavior of these pool amino acids could be analyzed with respect to that of the protein arginine. In Fig. 5, a comparison of the specific radioactivities of citrulline plus argininosuccinic acid, soluble arginine and new-protein arginine reveals that citrulline plus argininosuccinic acid had the highest specific radioactivity throughout. After 3 minutes the specific radioactivity of the citrulline and argininosuccinic acid declined because of replacement from less radioactive, endogenous ornithine. The decline in specific radioactivity confirms the impression imparted by the raw data (Fig. 3), which suggests that ^{14}C-ornithine, though sequestered, continues to enter citrulline synthesis at a slow rate.

Following the increase in the specific radioactivity in the citrulline and argininosuccinic acid was the rapid increase in the average specific radioactivity of the new-protein arginine. The latter was followed by an increase in the specific radioactivity of the pool arginine. Until 12 minutes the average specific radioactivity of the new-protein arginine was greater than that of the pool arginine. This shows that the labeled arginine, made from the ^{14}C-ornithine, was selectively used for protein synthesis and largely by-

Fig. 5.--Specific radioactivities of citrulline, soluble arginine and new-protein arginine after the addition of ^{14}C-L-ornithine. New-protein arginine determinations are plotted at the mid-points of the individual successive intervals. Points representing soluble compounds are the specific radioactivities determined for the sampling times indicated on the abscissa.

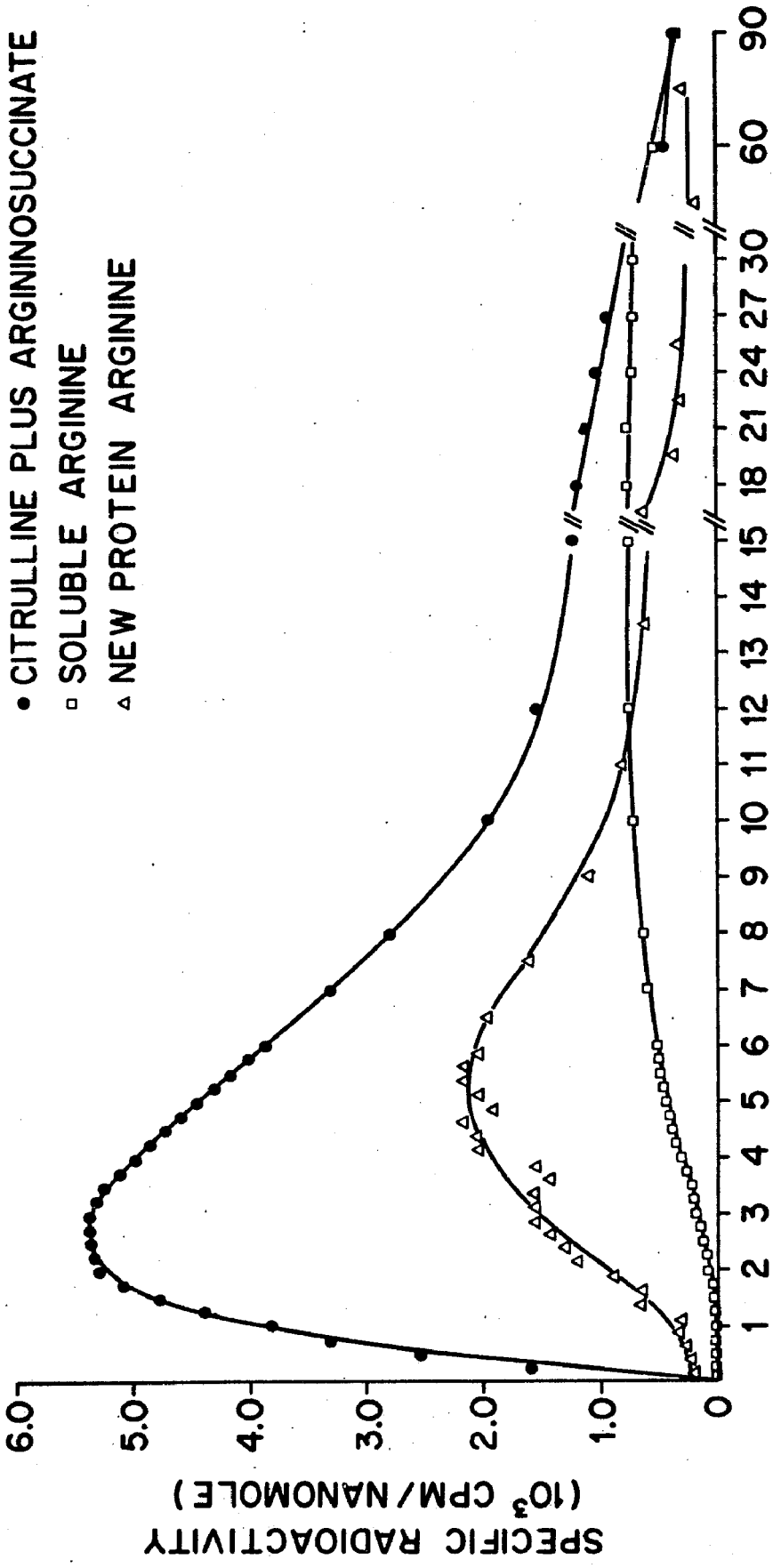

• CITRULLINE PLUS ARGININOSUCCINATE
□ SOLUBLE ARGININE
△ NEW PROTEIN ARGININE
SPECIFIC RADIOACTIVITY
(10³ CPM/NANOMOLE)
6.0
5.0
4.0
3.0
2.0
1.0
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 18 21 24 27 30 60 90
MINUTES AFTER ¹⁴C-ORNITHINE ADDED

FIGURE 5.

passed the bulk of the unlabeled arginine. After 12 minutes the pattern changes and the average specific radioactivity of the new-protein arginine, while continuing to decrease, was less than that of the soluble arginine. This indicates that by 12 minutes the remaining labeled arginine is sequestered from protein synthesis and that the new protein is made from unlabeled precursors. This is not completely consistent with the observation that the specific radioactivity of the citrulline plus argininosuccinic acid remained greater than that of the pool and protein arginine throughout the experiment. There are two possible errors in the determination of the specific radioactivity of the citrulline plus argininosuccinic acid pools: (1) The concentration of these amino acids, as determined by the Koritz and Cohen (1954) colorimetric procedure for carbamyl amino acids, could be too low. (2) The radioactivity in citrulline and argininosuccinic acid may have been over-estimated. However, if the specific radioactivity of citrulline plus argininosuccinic acid is correct, then these data suggest that part of these pools are sequestered from arginine biosynthesis.

Catabolism of ^{14}C-Ornithine to Glutamic-γ-Semialdehyde

Catabolism of ornithine via ornithine aminotransferase (Fig. 1) is detected only during the first

3 min of the experiment (Fig. 3). The size of the ornithine pool available for catabolism cannot be determined because the rate of formation of glutamic-γ-semialdehyde via ornithine aminotransferase is not known. However, using certain assumptions, the flux (nanomoles of ornithine per mg dry weight per 15-sec interval), through ornithine aminotransferase can be estimated. Because both ornithine aminotransferase and ornithine decarboxylase are localized in the "soluble" fraction, it is assumed that both these enzymes have access to the same cytoplasmic ornithine pool. Therefore, in this calculation it is assumed that during each 15-sec interval the average specific radioactivity of the cytoplasmic ornithine pool is the same as that of the newly synthesized polyamines. Consequently the nanomoles of ornithine catabolized per 15-sec interval is computed by dividing the total Δcpm entering glutamic-γ-semialdehyde, soluble proline, and protein proline by the specific radioactivity of the newly synthesized polyamines in that same interval. With 12 measurements during the first 3 minutes of the experiment, the flux averages at 0.18 $\pm$ 0.05 nanomoles of ornithine per mg dry weight per 15 sec interval. This flux is 50 percent of that required to maintain the demand for pool and protein proline levels characteristic of wild-type growth.

Once the counts have entered the proline pathway one can calculate how the total proline pool behaves with

respect to the protein proline. In Fig. 6 a comparison of the specific radioactivity of the soluble proline to the new-protein proline reveals that the pattern of incorporation is very similar. The specific radioactivity peaks at 3 minutes and declines thereafter. The fact that the average specific radioactivity of the new-protein proline is less than that of the pool proline could indicate that the catabolically derived proline (labeled) is in part sequestered from the proline available by another route for protein synthesis. Because it is possible that there was an error in the determination of the counts present in the soluble proline we are not certain about the interpretation of the data. If the radioactivity in the soluble proline was overestimated, then it is possible that the entire soluble proline pool is randomly utilized for protein synthesis.

Turnover of ^{14}C-Ornithine

The data for the utilization of the ^{14}C-ornithine for polyamine and citrulline synthesis indicate that the entire cellular ornithine pool is not a direct intermediate between its own synthesis and that of these other compounds. This result can be analyzed more quantitatively. Since in wild-type Neurospora ornithine catabolism is neither substantial nor is it the major route of glutamic-γ-semialdehyde synthesis (Davis, 1968), only the ornithine needed for the polyamine and arginine pathway is

Fig. 6.--Specific radioactivities of soluble glutamic-γ-semialdehyde plus soluble proline, and new-protein proline after the addition of ^{14}C-L-ornithine. New-protein proline determinations are plotted at the mid-points of the individual successive intervals. Points representing the soluble compounds are the specific radioactivities determined for the sampling times indicated on the abscissa.

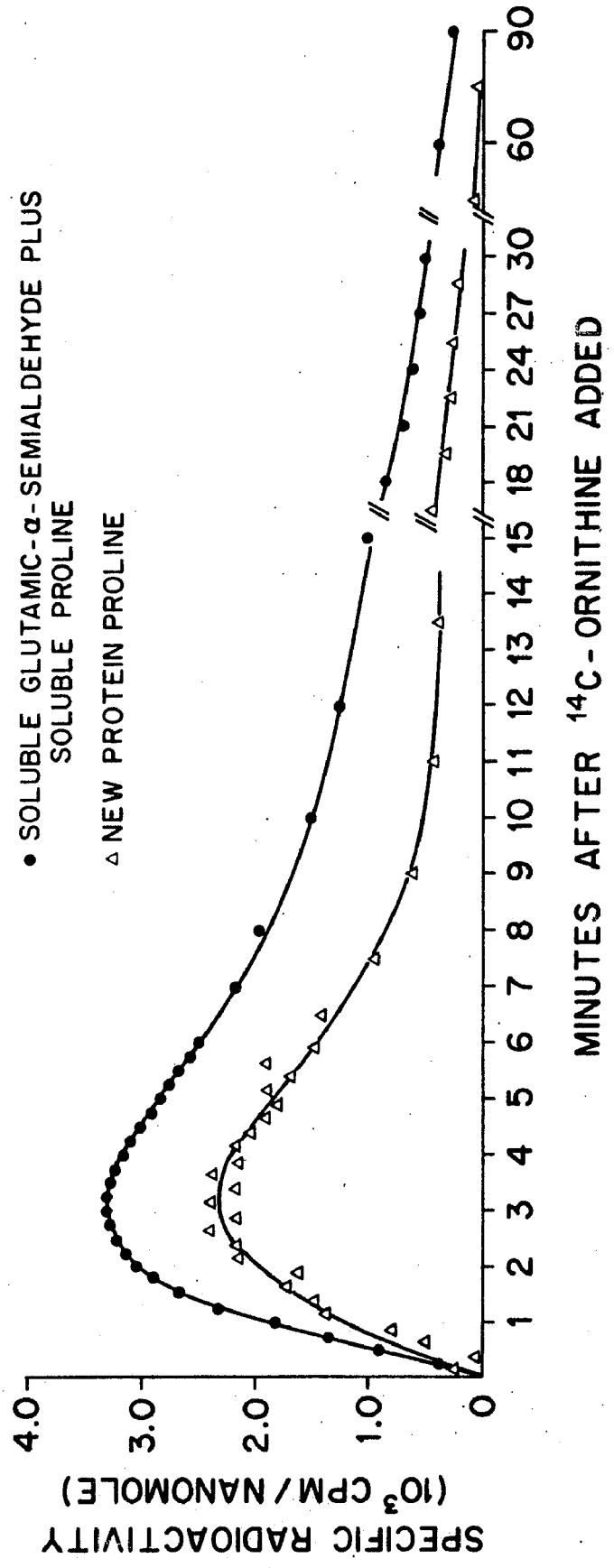

• SOLUBLE GLUTAMIC-α-SEMIALDEHYDE PLUS SOLUBLE PROLINE
△ NEW PROTEIN PROLINE
SPECIFIC RADIOACTIVITY (10³ CPM / NANOMOLE)
4.0
3.0
2.0
1.0
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 18 21 24 27 30 60 90
MINUTES AFTER ¹⁴C-ORNITHINE ADDED

FIGURE 6.

considered. The ornithine pool would have to turn over about four times per half generation in order to maintain the required flux into polyamines, the arginine pathway, and the ornithine pool. If the entire ornithine pool was randomly used, the counts remaining in ornithine after a half generation should be $(1/2)^4$ or 0.0625 of that at the beginning. At two minutes there were about 260,000 cpm in the cellular ornithine pool per 10-ml sample. After 75 minutes only 16,000 cpm per 10-ml sample are expected in the total ornithine pool; instead there were 8-fold as many (122,000 cpm).

CHAPTER FOUR

DISCUSSION

In these experiments the size of the cytoplasmic ornithine pool was estimated and the compartmental behavior of ornithine metabolism was studied. This was accomplished by following the rate of uptake and utilization of a 10^{-7} M pulse of uniformly labeled ^{14}C-L-ornithine. Polyamine synthesis was used as a criterion to determine the size of the cytoplasmic ornithine pool. The presence of radioactivity in arginine biosynthesis revealed the ability of cytoplasmic ornithine to enter mitochondria. The catabolic fate of cytoplasmic ornithine was monitored by the appearance of counts in the proline pathway. Ornithine sequestration was analyzed by comparing the average specific radioactivity of new polyamines and new citrulline to that of the total intracellular ornithine pool.

Polyamines

Because polyamine synthesis was used as a marker for determination of the size of the cytoplasmic ornithine pool, it is important to ascertain if the entering ^{14}C-ornithine significantly increased this pool and consequently the flux through ornithine decarboxylase. In

the first 15 sec interval (Fig. 2 inset) the amount of ^{14}C-ornithine (0.154 nmoles per 10 ml culture) removed from the medium was greater than at any other time. Yet a comparison during this interval of the specific radioactivity of the external ornithine to that of the new polyamines reveals that each entering ^{14}C-ornithine molecule was diluted by 12 endogenous ^{12}C-ornithine molecules. Thus it appears that the rate of entry of ^{14}C-ornithine into the mycelia did not significantly alter the size of the endogenous cytoplasmic pool. However, in determining the size of the cytoplasmic ornithine pool the flux of endogenous ornithine through the cytoplasm was also disregarded. It is possible that this assumption may not be entirely valid. The reason for this is that a comparison of the average specific radioactivity of the new polyamines to that of the total ornithine pool for the first 30-sec interval, instead of the first 15-sec interval, of the experiment gives a larger value for the cytoplasmic ornithine pool. In the experiment in Fig. 2 the increase in the value of the cytoplasmic ornithine pool is from 0.67 to 1.67 percent and in the experiment in Fig. 4 the increase is from 2.8 to 3.6 percent.

In spite of the evidence which suggests that in the mycelia there is significant flux of ornithine, its rate cannot be determined. Therefore, the <u>exact</u> size of the cytoplasmic ornithine pool cannot be determined and

the calculated cytoplasmic pool is a maximal estimate. Recognizing these limitations, the important conclusions from the analyses of the incorporation of the ^{14}C-ornithine into polyamines are: (1) the cytoplasmic ornithine pool is a small fraction (i.e., at the most, 5 percent) of the total endogenous ornithine pool; (2) the majority of the endogenous ornithine pool is metabolically inactive. This ornithine may be sequestered in the "vesicle" or distributed between the "vesicle" and other cellular components. This is in agreement with the fractionation experiments on wild-type Neurospora (Weiss, 1973), which, after accounting for amino acid losses during the isolation procedure, showed that more than 90 percent of both the ornithine and arginine pools were in the "vesicle."

The Arginine Pathway

The Behavior of Ornithine

Similar to the new polyamines, for the first six minutes of the experiment the specific radioactivity of the new citrulline was greater than that of the total ornithine. However, throughout the experiment the specific radioactivity of the new citrulline was less than that of the new polyamines (Fig. 4). During the first 15-sec interval the average specific radioactivity of the new citrulline was 30-fold greater than that of

the total ornithine (Fig. 4). This shows that in the same experiment, the ^{14}C-ornithine metabolized to citrulline was diluted by an endogenous ornithine pool (3.3 percent of the total) which was larger than the ^{12}C-ornithine pool (2.8 percent) available for polyamine synthesis (Fig. 4). Presumably, the additional ornithine represents a small, mitochondrial ornithine pool. Dilution of the cytoplasmic ornithine by a mitochondrial ornithine pool is consistent with the localization of ornithine transacetylase (Weiss, 1973) and ornithine transcarbamylase in the mitochondria (Bernhardt and Davis, 1972; Weiss, 1973).

As in the calculation of the cytoplasmic ornithine pool, the absolute size of the mitochondrial ornithine pool cannot be determined. These experiments do show, however, that the mitochondrial ornithine pool is similar in size to that of the cytoplasmic pool. This further confirms Weiss' results (1973) that the majority of the large, intracellular, ornithine pool is not in the mitochondria but rather in the "vesicle."

After the initial burst of counts into polyamines and citrulline there is a rapid decrease in the specific radioactivity of both these compounds (Figs. 2 [main axes] and 4). (The shoulder between 4 and 6 min in the citrulline curve [Fig. 4] is probably due to an experimental error.) After six minutes the average specific

radioactivity of the new polyamines and new citrulline was less than the specific radioactivity of the total ornithine pool, which remained fairly constant. This is again in accord with the enzyme localization experiments (Weiss, 1973), which show that the mitochondrion is the source of endogenous ornithine synthesis. The decline in the specific radioactivity of the new polyamines and the incorporation of ^{14}C-ornithine into the arginine pathway reveals that in vivo the mitochondrial membrane is permeable to ornithine, both in its exit and its entry. These results, however, do not elucidate the route by which endogenous ornithine leaves the mitochondria and enters the "vesicle" or the reverse (Fig. 1).

The flux of cytoplasmic ornithine (nanomoles of ornithine per mg dry weight per 15-sec interval) through the mitochondrial membrane can be estimated. Again it is assumed that during each 15-sec interval the average specific radioactivity of the cytoplasmic ornithine pool is the same as that of the newly synthesized polyamines. Consequently the amount (nanomoles) of ornithine entering the mitochondria per 15-sec interval is computed by dividing the sum of the counts in citrulline and its derivatives by the specific radioactivity of the newly synthesized polyamines in the same interval. With 18 measurements during the first 4-1/2 minutes of the

experiment, the flux of cytoplasmic ornithine entering the mitochondria averages at 0.190 ± 0.05 nanomoles of ornithine per mg dry weight per 15-sec interval. Because the ^{14}C-ornithine entering the cell did not increase the cytoplasmic ornithine pool significantly, the estimated flux of cytoplasmic ornithine into mitochondria is presumably the normal rate characteristic of wild-type mycelia grown on minimal medium. This flux is 54 percent of the calculated demand for pool and protein arginine during growth of wild-type mycelia.

The data from these experiments, together with the enzyme and pool localization studies, may explain why an arg-5 is able to grow on exogenous ornithine but the double mutant, arg-5, arg-12^{s}, grows very poorly even on high levels of exogenous ornithine (Davis, 1968). Though the majority of the ornithine which enters the mycelia is sequestered in the vesicle, it is possible that continuous entry of exogenous ornithine elevates the cytoplasmic ornithine pool. This in turn increases the flux of ornithine through the mitochondrial membrane. Because arg-5 has a normal ornithine transcarbamylase, this additional flux of cytoplasmic ornithine is sufficient to support wild-type growth. However, this flux is not sufficient to support the needs of the double mutant, arg-5, arg-12^{s}, which has an ornithine transcarbamylase with a ten-fold increased K_m for ornithine.

The observation that the arg-12^{s} mutant is prototrophic with only two-fold elevated levels of cellular ornithine (Davis, 1968), may be due more precisely to a high local concentration of ornithine in the mitochondria of the arg-12^{s} strain. The reasoning behind this conjecture is that the lower arginine pool in the arg-12^{s} probably relieves the feedback inhibition of N-acetyl-γ-glutamokinase (Fig. 1) and consequently the rate of ornithine synthesis is increased. Because ornithine synthesis occurs in the mitochondria, the aberrant ornithine transcarbamylase of the single mutant, arg-12^{s}, has access to this elevated mitochondrial pool and the cytoplasmic enzymes and "vesicle" compete for the ornithine which escapes the mitochondria.

This scheme of ornithine utilization may also explain why arg-12^{s} suppresses an arg-8 or arg-9 mutant (Fig. 1) (Davis, 1962b; Davis, 1968). The increased ornithine pools in arg-12^{s} reflect not only a decreased utilization of ornithine by ornithine transcarbamylase but also a decrease in the feedback inhibition of the N-acetyl-γ-glutamokinase by arginine (Fig. 1). Thus, biosynthetic ornithine may spill out into the cytoplasm at a sufficient rate to support substantial catabolism. This agrees with the observation that the arg-8, arg-12^{s} double mutant has wild-type growth rates and that the endogenous ornithine pools in both arg-12^{s} and arg-8, arg-12^{s} are two-fold greater than in wild-type cells

grown in minimal medium (Davis, 1968).

The finding that over 95 percent of the cellular ornithine pool is a metabolically dead pool, sequestered in the vesicle, is consistent with the kinetic analysis of ^{15}N ammonia assimilation in Candida utilis (Sims and Folkes, 1964). It was shown that at least 97 percent of the ornithine (derived from glutamate) served as a storage pool while the remainder was the metabolically active pool involved in arginine synthesis.

The Behavior of Citrulline and Arginine

The endogenously derived ^{14}C-arginine initially was efficiently used for protein synthesis and bypassed the bulk of the ^{12}C-arginine pool (Fig. 5). The non-metabolized ^{14}C-arginine was sequestered. Analogous to using polyamine synthesis as a criterion for the cytoplasmic ornithine pool, arginine incorporated into protein can be used in a diagnostic fashion for the cytoplasmic arginine pool (Fig. 1). A comparison during the first 15-sec interval of the specific radioactivity of the total intracellular arginine pool (2.1 cpm/nmole) to that of the new-protein arginine (197 cpm/nmole) reveals that about 1 percent of the ^{12}C-arginine pool is cytoplasmic. Due to the low specific radioactivities of the pool and new-protein arginine the cytoplasmic arginine pool cannot be accurately estimated. In spite

of this, the calculation indicates the presence of a small, metabolically active, cytoplasmic arginine pool. This is consistent with Weiss' result (1973) that over 90 percent of the arginine pool is in the "vesicle." It is assumed that the metabolically inactive pools of both arginine and ornithine reside within the same "vesicle."

At the later times in the experiment (Fig. 5) the specific radioactivity of the citrulline and argininosuccinic acid pools were greater than that of the arginine pool and new-protein arginine. As indicated in the Results the interpretation is not clear. It is either due to experimental error or sequestration of part of the citrulline and/or argininosuccinic acid pools.

These results on the fate of endogenously derived ^{14}C-arginine are consistent with the results of Subramanian et al. (1973). Both experiments show that there are two functionally distinct pools of arginine. The calculations showed that at most, 5 percent of the total arginine pool is in the cytoplasmic fraction and is the intermediate for protein synthesis. The majority of the arginine pool (also derived from endogenous arginine) is a storage pool, presumably located in the "vesicle."

However, throughout our experiment the specific

radioactivity of the citrulline and argininosuccinic acid remained higher than that of the pool and new protein arginine. As previously mentioned it is not certain if this is due to experimental error or sequestration of the citrulline and/or argininosuccinic acid pools. Subramanian, et al. (1973) also observed that 6 to 7 percent of the isotope in the citrulline and argininosuccinic acid disappeared more slowly after the first 20 min. At that time this residual radioactivity was attributed to possible derivatives. In order to reconcile both sets of observation it appears that a more accurate determination of the argininosuccinic acid pool and of the radioactivity in the citrulline and argininosuccinic acid pools is necessary.

The present results and those of Subramanian et al. (1973) are in agreement with the behavior of the soluble arginine in Candida utilis (Sims and Folkes, 1963; Wiemken and Nurse, 1973). Sims and Folkes' kinetic analysis of ^{15}N ammonia assimilation into pool and protein arginine showed that the specific radioactivity of protein arginine was much larger than that of pool arginine. These data indicated that though all the cellular arginine was derived from glutamate, the cell contained more than one arginine pool. The simplest model was that one pool was the true metabolic pool supplying arginine for protein synthesis and that the second

pool, a spatially separated, storage pool, was derived from the first and increased at the same rate as did growth.

Wiemken and Nurse (1973) have isolated and characterized the amino acid pools located in the cytoplasm and vacuoles of Candida utilis (see Introduction). Pulse labeling experiments indicate that a rapidly metabolized amino acid pool is located in the cytoplasm. Only 2-3 percent of the total soluble ornithine and arginine are contained in the cytoplasmic extract while the remaining 97-98 percent is in the vacuolar extracts. The vacuole contains a high proportion of the basic, nitrogen-rich, amino acids which show a slow turnover rate, indicative of a storage function.

The Proline Pathway

The calculated average flux of ornithine through ornithine aminotransferase was 0.18 $\pm$ 0.05 nanomoles ornithine per mg dry weight per 15-sec interval. This flux occurred only while the ^{14}C-ornithine entered the cytoplasm (Fig. 3). This can be due to the fact that later in the experiment the specific radioactivity of the ornithine pool available for catabolism was too small to be detected. Yet during this later period the cytoplasmic ^{14}C-ornithine pool continued to be utilized for polyamine synthesis (Fig. 3). This rapid cessation of ornithine catabolism may be an indication of further compartmentation of the

ornithine pool and enzymes in the "soluble" fraction.

The pattern of incorporation of radioactivity into pool and new protein proline was very similar (Fig. 6). However, the specific radioactivity of the pool proline remained greater than that of the new protein proline throughout the experiment. Because of the small size of the cellular proline pool and the errors in calculating the radioactivity in the soluble proline, it is difficult to determine if one or more metabolically distinct proline pools exists within the mycelia.

Zalokar (1961) followed the incorporation of ^{14}C-proline into Neurospora mycelia. He found evidence for an "intermediate"-"internal" pool used for protein synthesis and an "expandable" reservoir pool which he felt could possibly be distributed between the "vesicles" of the endoplasmic reticulum in the protoplasts and/or the "vacuoles" of older cells. Since in Zalokar's experiments the growth conditions are not comparable to ours, this issue can best be clarified by monitoring the utilization of a pulse of ^{14}C-proline in logarithmically growing wild-type mycelia in liquid culture.

CHAPTER FIVE

CONCLUSIONS AND SPECULATIONS

The results of these experiments are consistent with the results of Subramanian et al. (1973), Weiss (1973), and Weiss and Davis (1973). From these experiments the following conclusions can be drawn about the compartmental behavior of ornithine. The cytoplasm contains a very small fraction (at the most 5 percent) of the ornithine pool. This pool turns over rapidly and supports the polyamine requirements of the cell. Though some of this cytoplasmic ornithine is catabolized, this rate of catabolism is not sufficient to support the proline requirements characteristic of wild-type growth. The proline demands normally are met by the conversion of glutamate to proline (Fig. 1). The cytoplasmic ornithine can enter the mitochondria but this flux of cytoplasmic ornithine through the mitochondria is not adequate for the arginine demands of wild-type cells. However, the ornithine which is synthesized in mitochondria is more than adequate to satisfy the requirements of the arginine pathway. The majority (95 percent) of the ornithine pool is an inactive pool which is sequestered in the vesicle. This vesicular ornithine does

exchange with the ornithine in the cytoplasm and that in mitochondria.

Similar to the behavior of ornithine, it has been confirmed that in the cytoplasm there is a small (at the most 5 percent) metabolically active, arginine pool. This cytoplasmic arginine pool turns over rapidly with respect to protein synthesis but is not capable of supporting catabolism. As in the case of ornithine, the majority (95 percent) of the arginine is an inactive pool sequestered in the "vesicle."

As previously mentioned, Weiss (unpublished) has shown that in addition to containing the majority of the arginine and ornithine pools, the majority of the pools of the other basic, nitrogen-rich amino acids (lysine and histidine) are also located in the "vesicle." The compartmentation of amino acid pools is not unique to Neurospora. Wiemken and Nurse (1973) have shown that the majority of the basic, nitrogen-rich amino acids are located in the vacuole of Candida utilis.

In order to understand the regulation of the compartmental behavior of arginine and ornithine in Neurospora, it is necessary to study the physiological role of the "vesicle" with respect to its influence on the distribution of amino acids between the "vesicle," cytoplasm, and other organelles. Thus far, it appears that the "vesicle" is a means of finely tuning the cytoplasmic concentration of these amino acids.

With respect to the regulation of ornithine metabolism, some easy and very interesting experiments could be done. It would be nice to know if the "vesicle" has a limited capacity for each basic amino acid or a finite capacity for basic amino acids in general. One way to answer this question would be to grow wild-type mycelia on arginine and see if "vesicular" ornithine is dumped into the cytoplasm. This could be ascertained by first giving wild-type mycelia (grown on minimal medium) a 10^{-7} M pulse of uniformly labeled ^{14}C-L-ornithine. Once the ^{14}C-ornithine has equilibrated in the mycelia, ^{12}C-arginine would be added to the medium. Again polyamine synthesis could be used as a marker for the cytoplasmic ornithine pool. If the "vesicular" ornithine were dumped into the cytoplasm, it would be expected that the new polyamines would continue to incorporate radioactivity and that the radioactivity would disappear at a faster rate from the total ornithine pool than in the cells grown on minimal medium throughout the experiment (Figs. 2 and 4). If in the presence of exogenous arginine, "vesicular" ornithine is dumped into the cytoplasm, it would be interesting to monitor the catabolic fate of the ornithine as well as its utilization by the arginine pathway.

In another experiment the capacity of the "vesicle" to sequester an elevated ornithine pool, when

the total arginine pool is very reduced, could be determined. It could be tested whether or not the endogenous cytoplasmic ornithine pool is elevated in arg-12^{s} strains which still have the ability to synthesize ornithine. As in the case of wild-type, the uptake and utilization of a 10^{-7} M pulse of uniformly labeled ^{14}C-L-ornithine can be monitored in an arg-12^{s} single mutant grown on minimal medium. The size of the metabolically active ornithine pool and the degree of sequestration of the ornithine in arg-12^{s} could be compared to those values already determined for wild-type mycelia. Further information could be obtained by monitoring the incorporation of ^{14}C-ornithine into the arginine pathway. It would be very interesting to know if, and how much, of the endogenous arginine is sequestered from protein synthesis because the total arginine pool is significantly reduced in the arg-12^{s}. Because of the elevated total ornithine pool and the reduced levels of endogenous arginine in the arg-12^{s}, the behavior of the ^{14}C-ornithine in this strain could give further insight as to how the "vesicle" regulates the metabolism of these amino acids.

In order to differentiate if the cytoplasmic ornithine pool available for polyamine synthesis is the same as that available for ornithine catabolism (via ornithine aminotransferase), it would be interesting to monitor the uptake and utilization of a 10^{-7} M pulse of

uniformly labeled ^{14}C-L-ornithine in the double mutant, arg-8, arg-12^{s}, grown on minimal medium. Because ornithine catabolism is the only source of glutamic-γ-semialdehyde in these cells, the specific radioactivity of the newly synthesized glutamic-γ-semialdehyde could be compared to that of the total ornithine pool as well as to that of the new polyamines. Finding that the specific radioactivity of the new glutamic-γ-semialdehyde is the same or different than that of the new polyamines would be helpful in further understanding the compartmental behavior of ornithine metabolism.

In both the proposed arg-12^{s} and arg-8, arg-12^{s} experiments it would also be very useful to analyze the behavior of the glutamic-γ-semialdehyde and pool proline with respect to that of the new-protein proline.

Acquiring more information about the role of the "vesicle" in regulating the compartmental behavior of ornithine metabolism is only the beginning. The regulation of the metabolism of the basic, nitrogen-rich amino acids is probably a very complex phenomena with the "vesicle" having an instrumental role.

Davis (unpublished) has evidence that Neurospora supplemented with ornithine leads to a dramatic decrease of the arginine pool and a smaller decrease of the lysine pool; while supplementation with arginine significantly diminishes the ornithine and citrulline pools as well as

the lysine pool. Addition of lysine to medium decreases both the ornithine and arginine pool with an increase in the citrulline pool. In yeast, supplementation with lysine results in a smaller arginine pool and repression of the arginine biosynthetic enzymes (Bechet, Grenson, and Wiame, 1970). It is possible that the additional lysine confines the arginine to the cytoplasm. Despite the decrease in the total arginine pool, the new steady-state arginine concentration in the cytoplasmic compartment may have increased. The latter may feedback inhibit the cytoplasmic enzyme of ornithine synthesis, N-acetyl-γ-glutamokinase. (Cybis, unpublished, has shown that this is the only enzyme in the arginine pathway which is sensitive to feedback inhibition.) The increased cytoplasmic arginine pool could also affect the amount of arginyl-t-RNA responsible for the repression of the arginine biosynthetic enzymes. Catabolism of the cytoplasmic arginine may be prevented by the inhibition of arginase by the elevated citrulline and lysine pools (Mora et al., 1972).

In order to better understand the regulatory role of the "vesicle," in addition to physiological experiments and further characterization of the enzyme content of the "vesicle," it will be helpful to isolate "vesicular" mutants and derepression mutants of the ornithine and arginine pathways.

APPENDIX A

GROWTH CURVE AND POLYAMINE CONCENTRATION

The strain used in these studies, a conidial isolate of strain 74A, has a 150 min doubling time at 25 C (Fig. 7) during exponential growth.

Samples of mycelia at different stages of log growth were analyzed for their polyamine content by Dr. David R. Morris (by the method in Morris et al., 1969) (Table 1). The major polyamine in Neurospora is spermidine (21 nmoles per mg dry weight). Traces of putrescine (0.6 nmoles per mg dry weight) and spermine (0.2 nmoles per mg dry weight) are also present. The total polyamine pool (average 22 nmoles per mg dry weight) remains constant during exponential growth.

Fig. 7.--Representative growth curve of the conidial isolate of strain 74A.

FIGURE 7.

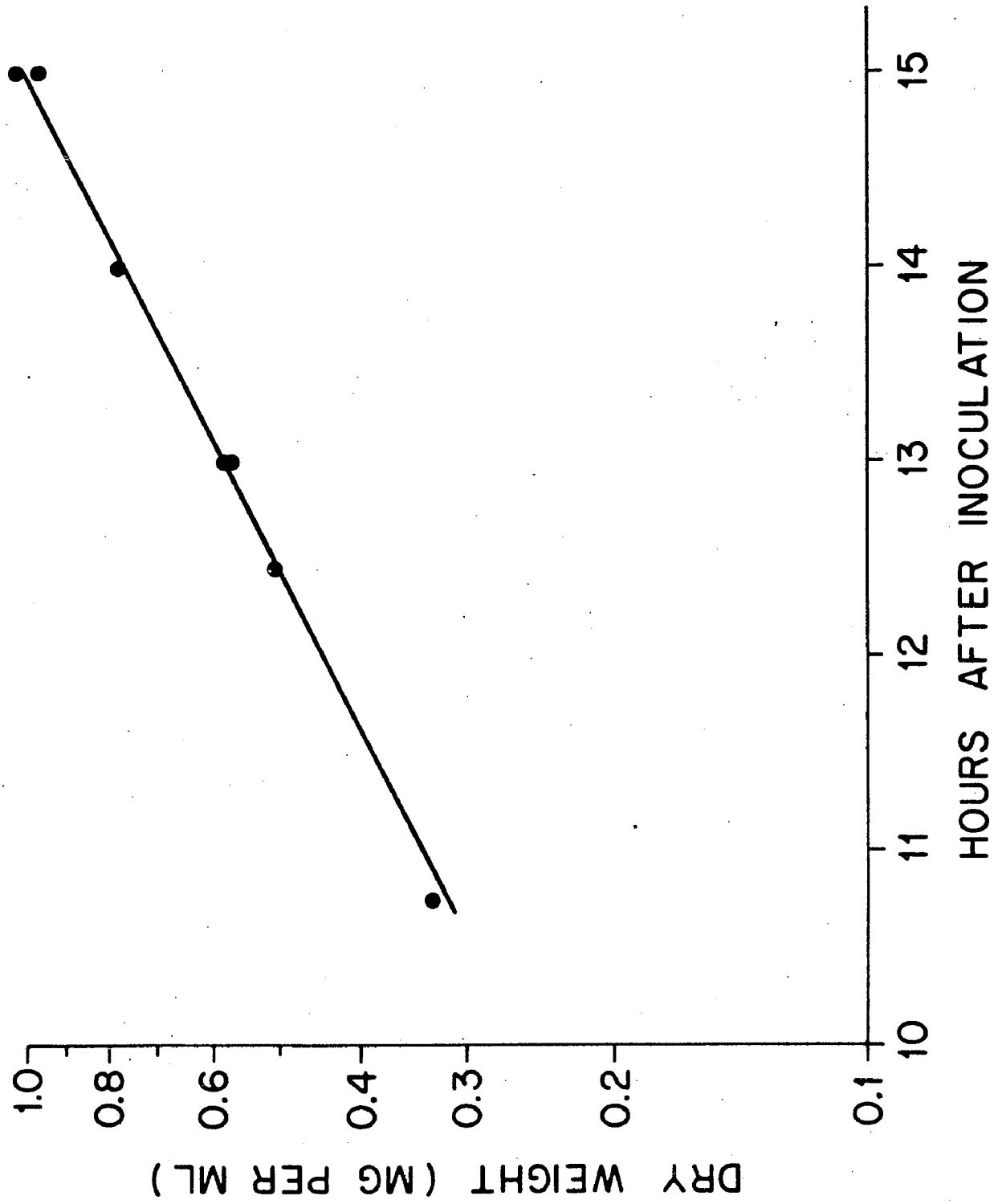

DRY WEIGHT (MG PER ML)
1.0
0.8
0.6
0.4
0.3
0.2
0.1
10
11
12
13
14
15
HOURS AFTER INOCULATION

TABLE 1.--Polyamine Levels in Wild-Type Neurospora

Time After Innoculation (Hours)	Dry Weight (mg)	Polyamines (nmoles/mg)		
		Putrescine	Spermidine	Spermine
11:05	18.0	0.5	19	0.2
11:13	19.4	0.3	21	0.2
11:19	18.3	0.4	17	0.2
11:29	16.4	0.5	20	0.2
13:30	16.2	0.6	22	0.1
13:35	15.7	0.7	20	0.3
13:49	16.0	0.7	23	0.2
14:30	18.7	0.6	22	0.2
14:40	15.8	0.7	22	0.1
14:47	16.0	0.6	21	0.1
14:51	17.1	1.0	21	0.2
	Average:	0.6 $\pm$ 0.04	21 $\pm$ 0.5	0.2 $\pm$ 0.02

APPENDIX B

RECOVERY AND IDENTIFICATION OF RADIOACTIVITY AFTER CELL FRACTIONATION

The recovery of radioactivity of five samples, taken from a culture at different times after a ^{14}C-ornithine pulse was added, is illustrated in Table 2. Of the radioactivity added (433,000 cpm per 10 ml of culture) 94 to 98 percent is recovered during fractionation of the mycelia and medium (Table 2, a-d). Less than 1 percent of the radioactivity was lost from the acid-insoluble material during the ethanol-ether and ether washes (Table 2, b). Solubilization of the acid-insoluble material was complete (a) as indicated by the very low counts detected in the remaining residue (Table 2, c) and (b) by the identity of label released as free arginine of free proline.

In computing the uptake of ^{14}C-ornithine the total radioactivity present in the mycelia and medium was determined (Table 2). The decrease with time in the total counts relative to the counts added can be due to quenching. It is possible that the geometry of the mycelia pad did quench some of the radioactivity while there was no quenching in the medium or column sample fractions.

Because between 92 and 95 percent of the radio-

TABLE 2.--Recovery of Radioactivity from Samples During Fractionation

Sample Time	4.25 min	6.25 min	12.25 min	18.25 min	30.25 min
Total counts added	433,000	433,000	433,000	433,000	433,000
	Total Counts per Minute				
^{14}C-ornithine in Mycelia plus Medium [a]	385,000	383,000	383,000	371,000	379,000
Fractionation of Mycelia plus Medium					
a) Acid-soluble pool recovery from Na^+-column[b]	384,000	369,000	337,000	311,000	278,000
b) Ethanol-ether plus ether washes of acid-insoluble extract	2,900	4,000	4,400	4,200	6,100
c) Residue after hydrolysis	300	300	500	500	600
d) Hydrolysate recovery from Na^+-column[b]	36,000	51,500	80,600	102,000	123,000
Total Counts	423,000	425,000	423,000	419,000	408,000
% Recovery (i.e., of total counts added)	98%	98%	98%	97%	94%

[a]Values from uptake of ^{14}C-ornithine determinations (see Methods).

[b]Sum of all the Fractions from Na^+-columns.

activity in the acid-soluble pool was recovered from the Dowex-50 x 8 (Na^+-form) columns (Table 3), selective loss of a given amino acid was not considered initially. Results of previous experiments using ^{14}C-ornithine, ^{14}C-arginine and ^{12}C-putrescine indicated that greater than 95 percent of these molecules were recovered from the columns. However, as indicated in Fig. 3 the counts detected in the polyamine fraction did not yield a smooth curve with respect to time. Only 2-3 percent of the radioactivity in the acid-soluble pool is in the polyamines (Figs. 2 and 3 and Table 3). Therefore, a comparison was made of the recovery of ^{14}C-putrescine and ^{14}C-spermidine added to unlabeled acid-soluble pool with and without additional ^{12}C-putrescine and ^{12}C-spermidine as carrier (Table 4). Greater than 95 percent of the putrescine was recovered whether or not additional ^{12}C-polyamines were present in the extract (Table 4). However, the recovery of spermidine was improved from 73 percent to 94 percent by the additional carrier. Because spermidine is the major polyamine in wild-type Neurospora (Table 1), 1 µmole of ^{12}C-putrescine and 2 µmoles of ^{12}C-spermidine were added to each acid-soluble extract in the experiment in Fig. 2.

The purity of labeled compounds in fractions containing ornithine, arginine, and polyamines (Table 3) was checked by high-voltage paper electrophoresis at pH 5.3. The ornithine fraction is free of contamination (Fig. 8b). No polyamines or ornithine were detected in the arginine

Table 3.--Recovery of Acid-Soluble Material from Na^+-Columns

Sample Time		4.25 min	6.25 min	12.25 min
			Total CPM	
Acid-Soluble Pool		420,000	400,000	355,000
Fraction	Amino Acid			
A + B	Neutral + Acidic	106,000	84,000	46,600
C + D	Ornithine	244,000	236,000	220,000
E + F[a]	Blank	988	988	988
G + H	Arginine	23,400	37,200	55,400
I[a]	Blank	202	202	202
J + K	Polyamines	9,860	10,400	13,700
Total		384,000	369,000	337,000
% Recovery		92%	92%	95%

[a]Average counts from six columns.

TABLE 4.--Recovery of Polyamines from Na^+-Columns

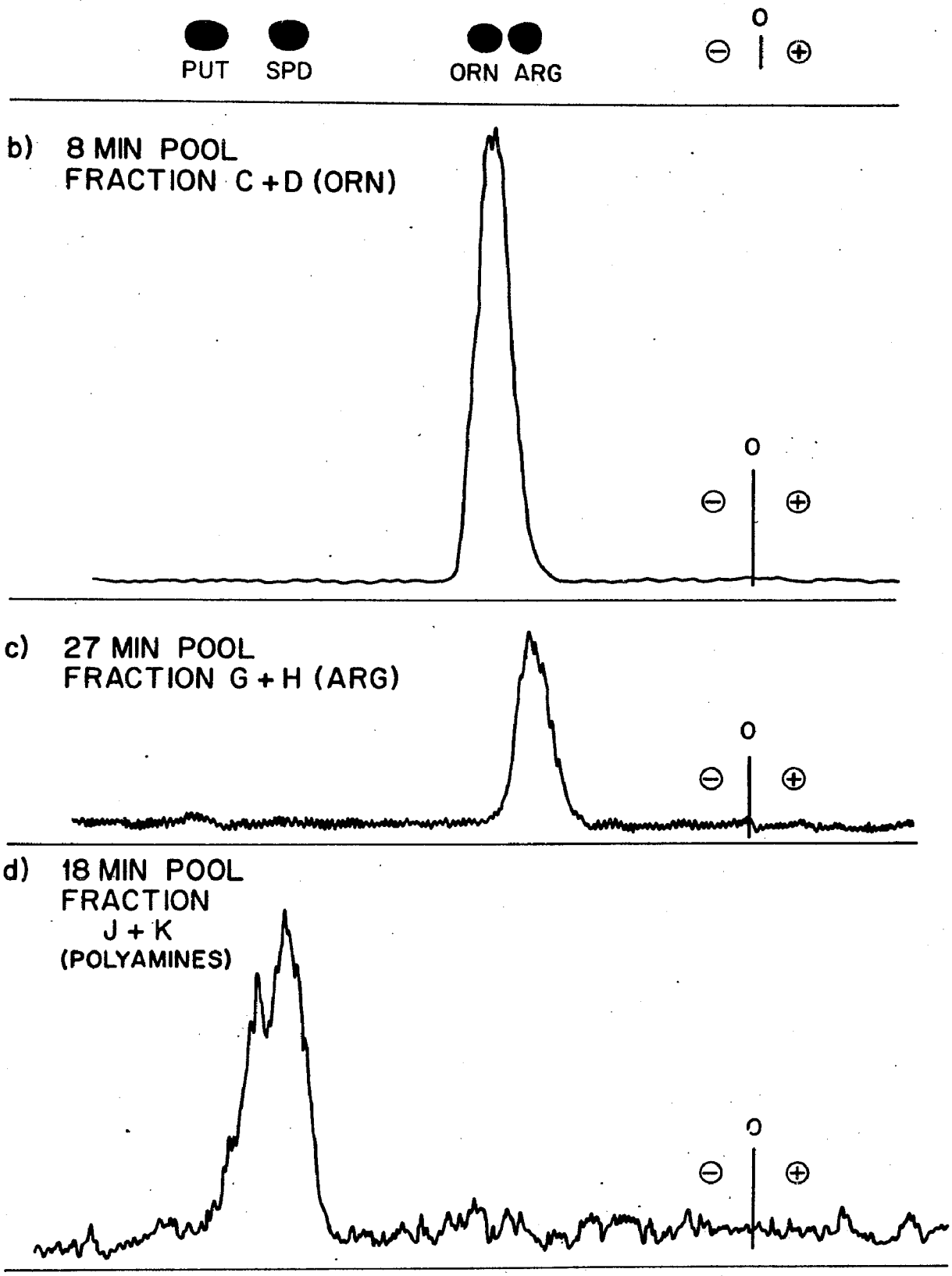
	Total CPM			
	A*		B*	
	PUT	SPD	PUT	SPD
Counts added	149,000	136,000	149,000	136,000
Counts Recovered				
Column 1	133,000	104,000	166,000	125,000
Column 2	144,000	102,000	168,000	130,000
Column 3	184,000	96,500	169,000	128,000
Column 4	124,000	102,000	169,000	120,000
Column 5	. . . .	98,800	. . . .	130,000
Column 6	. . . .	97,500	. . . .	127,000
Ave Recovery per Column	146,000	100,000	168,000	127,000
% Recovery	98%	73%	113%	94%

*A: ^{14}C-PUT or ^{14}C-SPD was added to acid-soluble extract after ether extraction and applied to four and six individual columns, respectively.

*B: Same as "A" except 1µmole ^{12}C-PUT and 2µmoles ^{12}C-SPD were added to each sample prior to fractionation on Na^+-column.

Fig. 8.--High-voltage paper electrophoresis at pH 5.3 of Na^+-column fractions. O = origin; + = annode; - = cathode; a) migration of ^{12}C-arginine, ^{12}C-ornithine, ^{12}C-spermidine, and ^{12}C-putrescine as detected by ninhydrin. b-d) Actigraph III scan of radioactivity in Na^+-column fractions.

FIGURE 8.


a) ^{12}C-AMINO ACID MIXTURE
PUT
SPD
ORN
ARG
O
⊖
⊕
b) 8 MIN POOL
FRACTION C + D (ORN)
c) 27 MIN POOL
FRACTION G + H (ARG)
d) 18 MIN POOL
FRACTION
J + K
(POLYAMINES)

fraction (Fig. 8c). The polyamine fraction, also free of contamination, contained radioactive spermidine (Fig. 8d). Because of the small amount of radioactivity in the polyamine fraction, it was not possible to show that very early in the experiment the radioactivity initially entered the putrescine.

Since fractions A and B (Table 3) contained amino acids of both the arginine and proline pathways, it was necessary to fractionate these amino acids further. Fractions A and B were combined and desalted on Dowex-50 (H^+-form) columns. The recovery of a standard mixture of ^{12}C- and ^{14}C-amino acids was compared to that of an experimental sample (Table 5). 100 percent of the radioactivity was recovered from the standard mixture and 89 percent from the sample (Table 5). An important feature of this desalting procedure was that there was no selective elution of any of the ^{14}C-amino acids. The distribution of radioactivity in the two piperidine fractions was identical to that in the mixture of standards applied to the columns (Table 5). Therefore, only the second 1.0 M piperidine fraction (containing over 80 percent of the radioactivity) was used for analyses of the distribution of radioactivity among the neutral and acidic amino acids.

Asparate, glutamate, anhydrides of argininosuccinic acid, citrulline and proline could be separated by paper chromatography using phenol:water (100:20) in an ammonia atmosphere (Fig. 9a). There were two difficulties.

TABLE 5.--Desalting Neutral and Acidic Amino Acids on H^+-Columns

	Total CPM	
	Standard[a]	Experimental[b]
Counts Added	140,000	54,700
Eluting Solvent		
amino acids in 0.116 M Na citrate pH 2.0	1,300	
0.1 N HCl	700	
1.0 M piperidine	200	1,600
1.0 M piperidine	117,000[c]	39,500
1.0 M piperidine	11,600[c]	2,400
1.0 M NaCl	6,100	2,300
6.0 N HCl	4,400	2,900
Total	141,000	48,700
% recovery	101%	89%

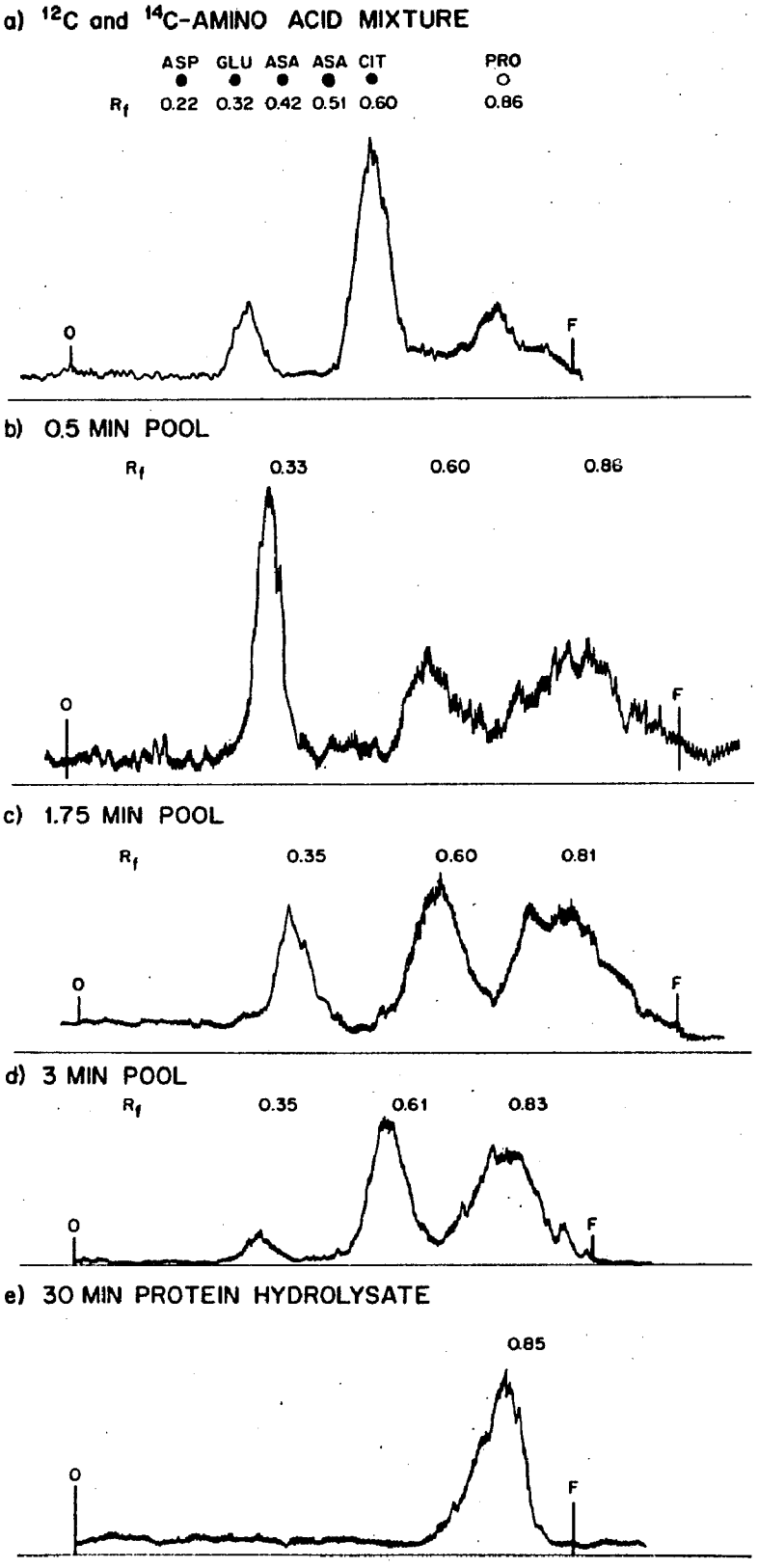
[a]Standard amino acid mixture contained the following ^{12}C-amino acids: glu (0.13 mg); asp (0.13 mg); cit (0.44 mg); pro (0.44 mg); asa (0.65 mg). The distrubution of radioactivity was: 59% ^{14}C-cit; 28% ^{14}C-pro; 13% ^{14}C-glu.

[b]Neutral and acidic amino acids (fractions A and B from Na^+;columns) of 1.75 min acid-soluble extract.

[c]Distribution of radioactivity was: 59% ^{14}C-cit; 28% ^{14}C-pro; 13% ^{14}C-glu.

Fig. 9.--Paper chromatography (Phenol - H_2O - NH_3) of neutral and acidic amino acid fractions. O = origin; F = solvent front. a) Migration of ^{12}C-amino acids as detected by ninhydrin. Actigraph III scan of radioactivity of ^{14}C-amino acids. b-d) Actigraph III scan of radioactivity in pool samples. e) Actigraph III scan of radioactivity in protein hydrolysate sample.

FIGURE 9. PAPER CHROMATOGRAPHY (PHENOL - H_2O - NH_3) NEUTRAL AND ACIDIC FRACTIONS



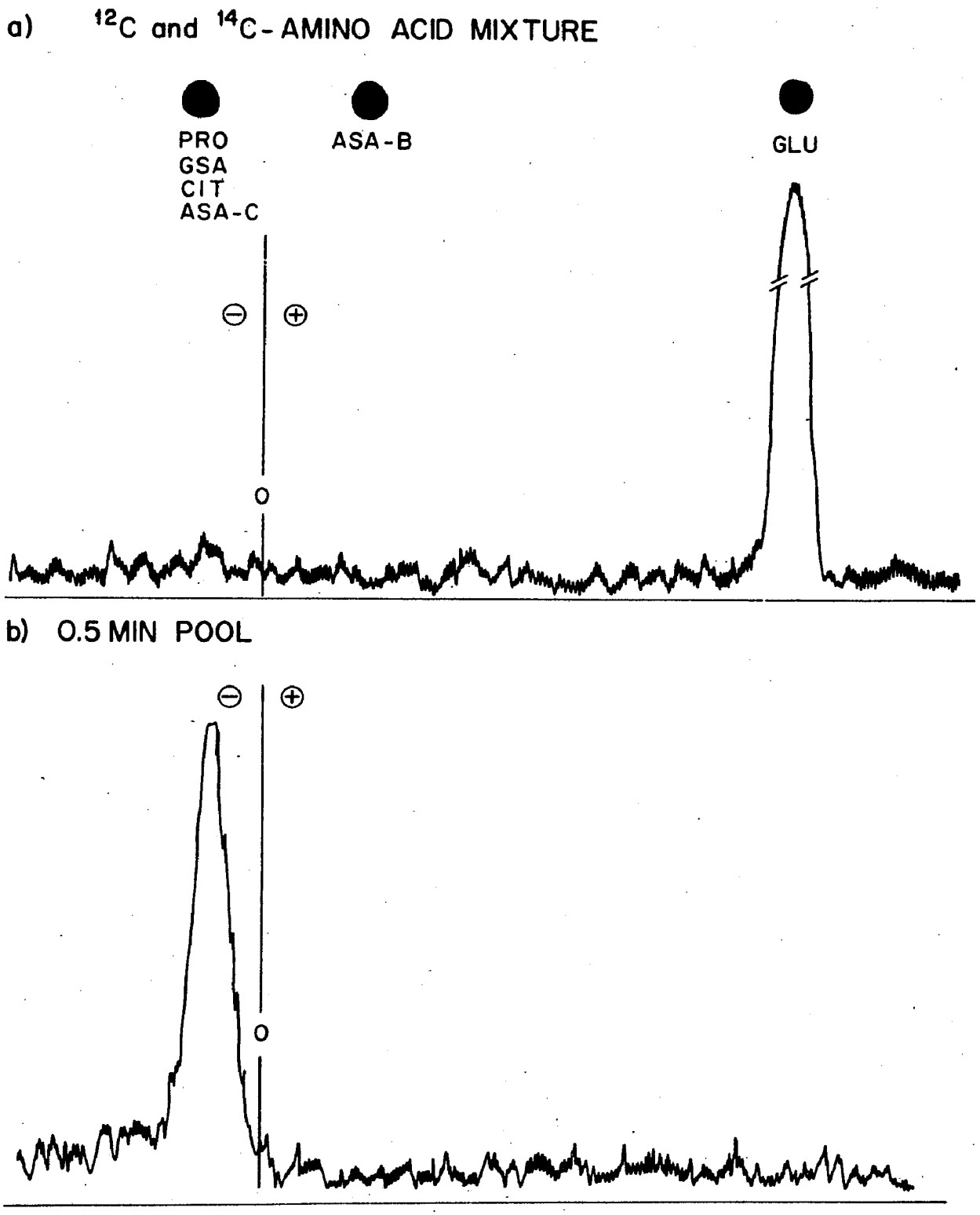
(1) Argininosuccinate (free acid) probably has the same R_f value (0.32) as glutamate (Westall, 1960). (2) Δ^1-pyrroline-5-carboxylate (Fig. 1) gave two spots in this chromatography system. (The Δ^1-pyrroline-5-carboxylate, made by Dr. Strecker, was not over 4 years old.) One spot, R_f 0.42, is identical to that of one of the argininosuccinate anhydrides (Fig. 9a). The other spot, R_f 0.57, could not be easily distinguished from citrulline, R_f 0.60 (Fig. 9a). Using high-voltage paper electrophoresis at pH 5.3, glutamate could be separated from argininosuccinic acid and its two anhydrides (Fig. 10a). However, Δ^1-pyrroline-5-carboxylate and anhydride C of argininosuccinic acid behaved identically in this system (Fig. 10a). Therefore, there was some difficulty in determining the percentage of counts entering the arginine and proline pathways.

The resolution of radioactivity in neutral and acidic amino acids of the acid-soluble extract of the 0.5 min, 1.75 min, and 3 min samples is illustrated in Fig. 9. There was no difficulty in resolving citrulline from proline (Fig. 9b-d). High-voltage electrophoresis was done to determine if the peak with an R_f 0.33-0.35 was argininosuccinic acid or glutamate. As indicated in Fig. 10b no radioactivity was detected in the glutamate position. The inability to detect argininosuccinic acid on the electrophoretogram could have been due to the

Fig. 10.--High-voltage paper electrophoresis at pH 5.3 of neutral and acidic amino acid fractions. O = origin; + = anode; - = cathode. a) Migration of ^{12}C-amino acids as detected by ninhydrin. Actigraph III scan of radioactivity in ^{14}C-glutamate. b) Actigraph III scan of radioactivity in pool sample.

FIGURE 10.

HVE pH 5.3 NEUTRAL AND ACIDIC FRACTION



fact that it was in the anhydride C form (Fig. 10a). There also was no detectable radioactivity in glutamate in the neutral and acidic fractions from the protein hydrolysates (Fig. 9e). Therefore, it was assumed that the peak with an R_f of 0.33-0.35 on the chromatograms of the pool samples was argininosuccinic acid.

The amount of labeled material present at R_f 0.33-0.35, relative to the citrulline and proline peaks, decreased with time (Fig. 9). Thus, an identification error will affect the initial values of the radioactivity in citrulline plus argininosuccinic acid (Fig. 11) and in the soluble proline plus glutamic-γ-semialdehyde (Fig. 12). If the labeled material at R_f 0.33-0.35 was glutamate and not argininosuccinic acid, then during the first minute the radioactivity of the citrulline plus argininosuccinic acid (Fig. 11) is 45 percent too high, decreasing to 20 percent too high. After the first two minutes the labeled material at R_f 0.33-0.35 only constitutes 5-15 percent of the total radioactivity in the neutral and acidic fraction.

It is impossible in this system to determine the contribution of Δ^1-pyrroline-5-carboxylate to the citrulline peak. Consequently, it cannot be determined if the radioactivity in citrulline plus argininosuccinic acid (Fig. 11) was overestimated and the radioactivity in the soluble proline plus glutamic-γ-semialdehyde (Fig. 12)

Fig. 11.--Incorporation of radioactivity into the arginine pathway. The line describing each curve is derived from the raw cpm for each point.

FIGURE 11.

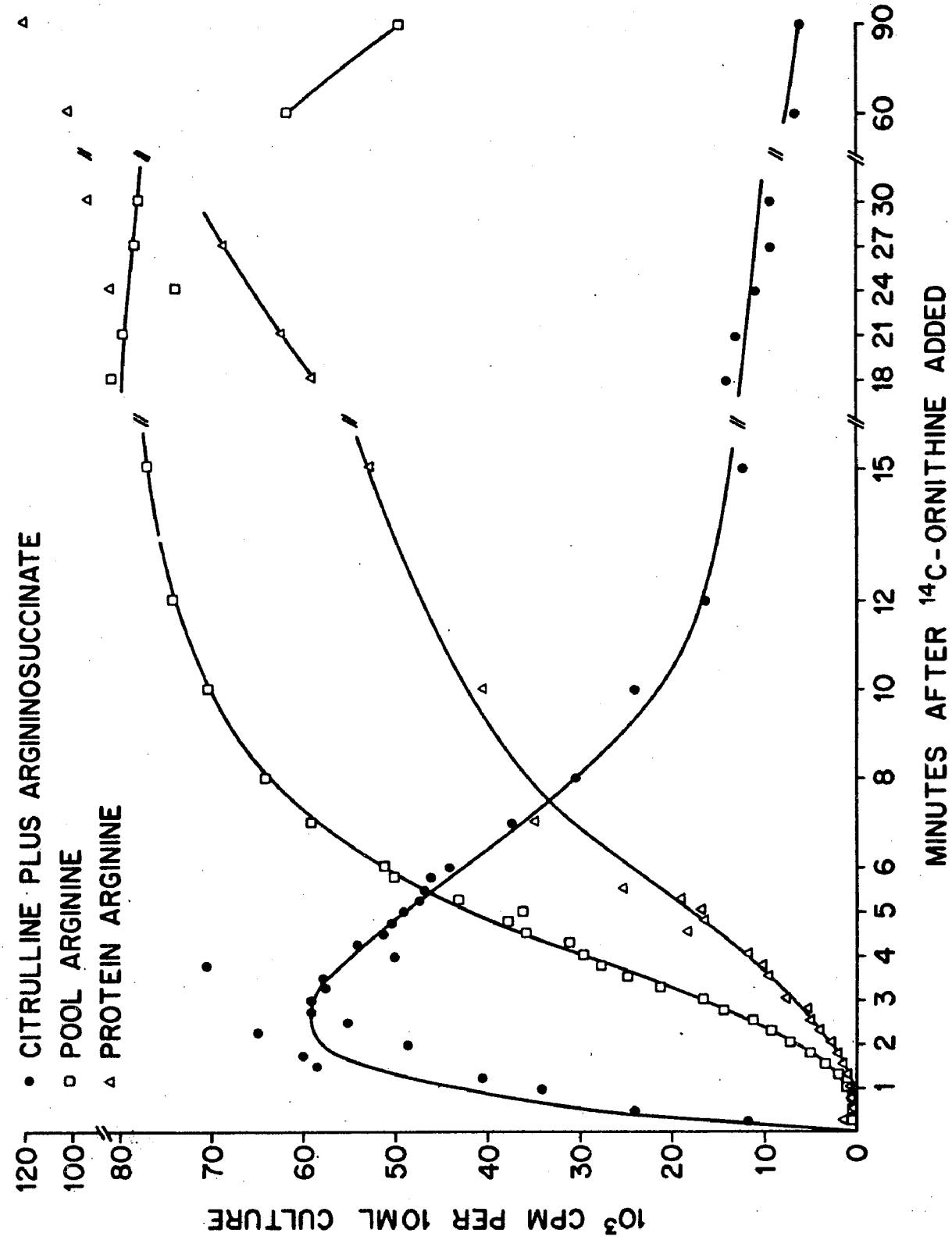
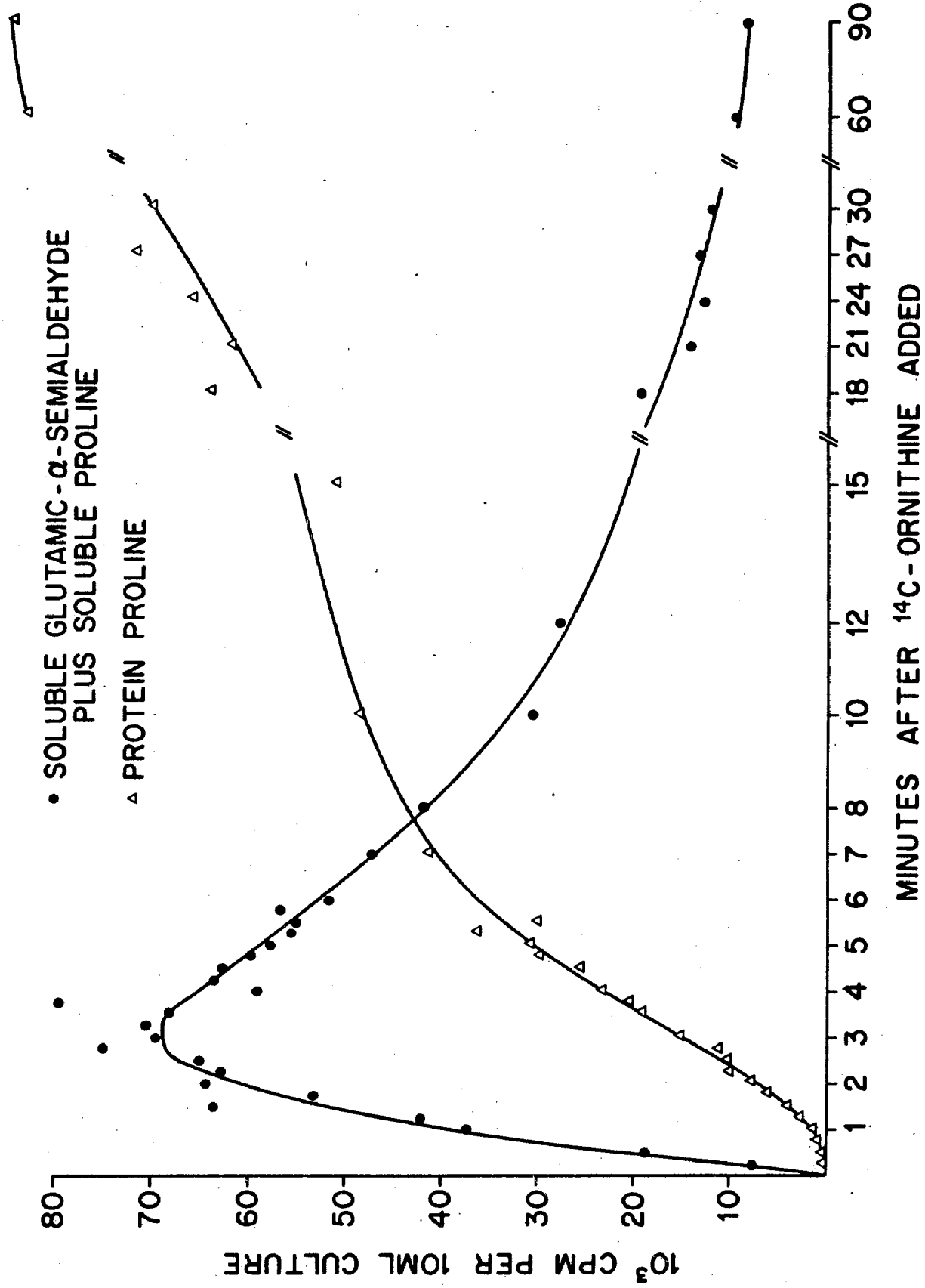

• CITRULLINE PLUS ARGININOSUCCINATE
□ POOL ARGININE
△ PROTEIN ARGININE
10^3 CPM PER 10ML CULTURE
MINUTES AFTER ^{14}C-ORNITHINE ADDED

Fig. 12.--Incorporation of radioactivity into the proline pathway. The line describing each curve is derived from the raw cpm for each point.

FIGURE 12.


• SOLUBLE GLUTAMIC-α-SEMIALDEHYDE PLUS SOLUBLE PROLINE
▵ PROTEIN PROLINE
10³ CPM PER 10ML CULTURE
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 10 12 15 18 21 24 27 30 60 90
MINUTES AFTER ¹⁴C-ORNITHINE ADDED

was underestimated. These errors together with the possible misclassification of the R_f 0.33-0.35 peak could partially explain the result that in the latter part of the experiment the specific radioactivity of the citrulline plus argininosuccinate was greater than that of the total arginine pool (Fig. 6). As previously indicated the radioactivity in soluble and protein arginine (Fig. 11) and protein proline (Fig. 12) was easily obtained.

APPENDIX C

RAW DATA FOR THE INCORPORATION OF RADIOACTIVITY INTO THE ARGININE AND PROLINE PATHWAYS

As already indicated in Appendix B, there may be an error in the determination of the radioactivity in the citrulline plus argininosuccinic acid (Fig. 11) and glutamic-γ-semialdehyde plus soluble proline (Fig. 12). However, there were no amino acid identification problems in determining the radioactivity in the soluble and protein arginine (Fig. 11) and protein proline (Fig. 12). From the best-fit curve for the radioactivity data in Figs. 11 and 12, the specific radioactivity of the given compounds was calculated. The specific radioactivity data for the compounds in Figs. 11 and 12 is shown in Figs. 5 and 6 respectively.

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