

THE UNIVERSITY OF MANITOBA

CELLULAR REGULATION IN A WATER-MOULD:  
ALLOSTERIC EFFECTS ON GLUTAMATE DEHYDROGENASE ACTIVITY  
AND REGULATION BY CYTOKININS OF METABOLITE TRANSPORT  
AND MACROMOLECULAR SYNTHESIS

by

ROSELYNN MARGARET WEIR STEVENSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

October, 1974

CELLULAR REGULATION IN A WATER-MOULD:  
ALLOSTERIC EFFECTS ON GLUTAMATE DEHYDROGENASE ACTIVITY  
AND REGULATION BY CYTOKININS OF METABOLITE TRANSPORT  
AND MACROMOLECULAR SYNTHESIS

by

ROSELYNN MARGARET WEIR STEVENSON

A dissertation submitted to the Faculty of Graduate Studies of  
the University of Manitoba in partial fulfillment of the requirements  
of the degree of

DOCTOR OF PHILOSOPHY

© 1974

Permission has been granted to the LIBRARY OF THE UNIVER-  
SITY OF MANITOBA to lend or sell copies of this dissertation, to  
the NATIONAL LIBRARY OF CANADA to microfilm this  
dissertation and to lend or sell copies of the film, and UNIVERSITY  
MICROFILMS to publish an abstract of this dissertation.

The author reserves other publication rights, and neither the  
dissertation nor extensive extracts from it may be printed or other-  
wise reproduced without the author's written permission.



To my parents

## ACKNOWLEDGEMENTS

The guidance and patience of Dr. H.B.LéJohn during the course of these studies is most gratefully recognized. The excellent technical assistance of Renate Meuser is much appreciated, as is the counsel of Dr. I. Suzuki and other staff members.

The friendship of fellow students in the Department of Microbiology is valued. Particular thanks are due Swee Han Goh and Danny Singh for communicating their research results, and Linda Cameron for information regarding her studies, preparation of shocked cells, and use of Figure 56.

The financial support of a National Research Council of Canada scholarship has been appreciated.

## ABSTRACT

Purified NAD-specific glutamate dehydrogenases from two fungi, Achlya sp. (1969) and Pythium debaryanum, have been studied kinetically and some of their physical properties elucidated. An ordered binary-ternary kinetic mechanism prevailed with the order of substrate addition and release being identical with the order found for all NADP-specific glutamate dehydrogenases although NADP<sup>+</sup> is not a substrate of the enzyme. NADP<sup>+</sup> (or NADPH) acts as an allosteric activator of the enzyme, and it is proposed that this may reflect the evolution of the catalyst. In addition to NADP<sup>+</sup>, the enzymes are also allosterically activated by P-enol-pyruvate, short chain acyl-CoA derivatives, GTP, and ATP. These activators function cumulatively to antagonize the effects of the inhibitors, citrate, AMP, and long chain CoA derivatives. Pythium glutamate dehydrogenase, in particular, was shown to be sensitive to control by energy charge. The activators acted as unidirectional stimulants of the biosynthetic reaction, while some of them inhibited the catabolic reaction unidirectionally. The substrates of the biosynthetic reaction,  $\alpha$ -ketoglutarate and ammonia, are allosteric inhibitors. The activators modulated the enzyme against allosteric inhibition by its substrates. This multivalent control has been explained as a mechanism by which the enzyme effects its biosynthetic and catabolic roles in amphibolic reactions of the citric acid cycle.

A novel effect of pH on the degree of activation and inhibition was observed. The enzymes are most responsive at the pH minima, and least at the pH optimum. The relevance of these pH effects to the mitochondrial location of the enzymes is discussed. The purified glutamate dehydrogenases have a molecular weight of 225,000 daltons. They are susceptible to inactivation by hydrodynamic shear in sucrose density gradients at alkaline pH levels. Inactivation by sedimentation can be prevented by bound activators. The enzymes are extremely heat labile, denaturing rapidly above 37° C. The activators and inhibitors fail to stabilize the proteins against heat denaturation.

The initial rates of transport of five nucleosides and four nucleic acid bases into the germinated sporangiospores of Achlya were determined, and shown to be dependent upon pH, temperature, substrate concentration, and, in the case of nucleosides, metabolic energy. Competition studies demonstrated the existence of two categories of nucleoside import systems - a guanosine or purine nucleoside specific system, and a non-specific system that accepts both purine and pyrimidine nucleosides. From similar studies, it was determined that the nucleic acid bases are all transported by a common carrier that is not linked to metabolic energy. This import process appears to proceed by facilitated diffusion. Kinetics of nucleoside efflux support the concept that these metabolites are accumulated actively. A connection exists between  $Ca^{++}$  metabolism and transport of these precursors of nucleic acid biosynthesis as is demonstrated by the effects of

citrate and osmotic shock treatment, but the exact correlation is unelucidated.

Achlya has a unique purine binding receptor on the surface of the cell membrane that functions maximally when thiol groups are oxidized into disulfide bridges by  $\text{Ag}^+$ ,  $\text{I}_2$ , and mercurials. Interaction is specific for adenine and close analogues, with nucleosides, nucleotides and pyrimidine bases not reacting with it, and guanine reacting poorly. This receptor has been characterized as a cytokinin interaction locus, part of the  $\text{Ca}^{++}$  regulatory system which has been described for this organism.

Active transport of nucleosides and the L-isomers of the twenty common amino acids, with the exception of tryptophan, was deterred by cytokinin-related compounds. Tryptophan binding was enhanced by these reagents under the same conditions. From kinetic studies, it was determined that the cytokinins do not act at the site of amino acid entry into the cells. The  $V_{\text{max}}$  for transport either increased or decreased with cytokinin interaction, while the  $K_m$  was unaltered by this inhibition. The inhibition effect of cytokinins was immediate. The relative effectiveness of about thirty compounds with cytokinin activity was tested to assess possible application as a cytokinin bioassay procedure. The effect of the compounds over a range of pH values was also examined. Cytokinin-related compounds affected only those fungi belonging to the Oömycetes, and a relationship between plant pathogenicity and cytokinin response is implied.

Tryptophan and auxin (IAA) were shown to be interacting at a common site with cytokinin. It is suggested that this site of interaction is the  $\text{Ca}^{++}$ -binding glycopeptide found in Achlya. Radioactively labelled isopentenyladenine was found to bind rapidly to Achlya germlings, but apparently penetration occurs very slowly. This is consistent with a hormone-type effect mediated at the cell surface. Other cytokinin compounds and adenosine 3':5'-cyclic monophosphate (cyclic AMP) antagonized the binding of isopentenyladenine.

Isopentenyladenine added to Achlya germlings growing in glucose increased the specific activity of NAD-specific glutamate dehydrogenase to a level approaching that of cells grown with glutamate as a carbon source. However, an apparent increase in incorporation of nucleic acid precursors and amino acids into macromolecules was strongly inhibited by isopentenyladenine. Part of this inhibitory effect was attributed to the effect of cytokinins on active transport of metabolites, but an effect at the level of synthesis or degradation of macromolecules is also indicated. A possible mechanism is an effect of isopentenyladenine on RNase activity. Cyclic AMP effects on macromolecular synthesis and on cytokinin binding suggest it in some way may be involved in mediation of the response.

## TABLE OF CONTENTS

	PAGE
Acknowledgements .....	iii
Abstract .....	iv
Table of Contents .....	viii
List of Tables .....	xiii
List of Figures .....	xv
Abbreviations .....	xix
INTRODUCTION .....	1
HISTORICAL .....	5
Glutamate Dehydrogenase	
Allosteric Enzymes .....	6
Glutamate Dehydrogenase .....	9
Studies on the mammalian enzyme ....	10
Bacterial and fungal enzyme forms ..	16
Enzyme levels .....	24
Transport	
General .....	33
Types of Transport Systems .....	34
Sulphydryls in Transport Systems .....	36
Regulation of Transport .....	37
Amino Acid Transport .....	37
Nucleoside Transport .....	45
Cyclic AMP	
Cyclic AMP as a Cell Regulator .....	53
Cyclic AMP Enzymes	
Adenylyl cyclase .....	55
Phosphodiesterase .....	58
Protein kinases .....	59
Cyclic AMP Assays .....	62

TABLE OF CONTENTS CONTINUED

	PAGE
Effects of Calcium .....	63
Cyclic GMP .....	65
Cyclic AMP in the Bacteria .....	65
Cyclic AMP in Plant Systems .....	68
Occurence in plants .....	69
Cyclic AMP and plant hormones .....	74
Cyclic AMP in <u>Euglena</u> .....	79
Cyclic AMP in the Fungi .....	80
 Cytokinins	
General .....	93
Chemical Structure and Activity .....	94
Bioassays for Cytokinins .....	98
Cytokinins in Transfer RNA .....	99
Metabolism of Cytokinins .....	111
Cytokinesins .....	119
Cytokinin Action	
Plant hormone interactions .....	121
Senescence and synthesis effects ...	149
Genome interactions and RNA synthesis	152
Enzyme synthesis .....	153
Hormone receptors in moss .....	155
Plant pathogenesis .....	157
 MATERIALS AND METHODS	
Organisms and Growth	
A. Classification and Life Cycle .....	160
B. Media for Growth .....	164
C. Growth in Culture .....	166
Methods	
A. General Procedures	
1. Liquid scintillation counting ...	172
2. Protein determinations .....	172

TABLE OF CONTENTS CONTINUED

	PAGE
B. Enzyme Studies	
1. Purification procedures .....	173
2. Enzyme assays .....	175
3. Physical studies .....	176
C. Cyclic AMP Studies .....	179
D. Transport Kinetics	
1. Initial uptake rate .....	179
2. pH studies .....	181
3. Temperature studies .....	181
4. Efflux studies .....	181
5. Osmotically-shocked germlings ...	182
E. Studies with Cytokinins	
1. Transport studies .....	182
2. Induction studies .....	182
3. Protein and RNA synthesis .....	183
4. Protein and RNA degradation .....	184
Materials .....	185

RESULTS AND DISCUSSION

PART I: <u>Glutamate Dehydrogenase: Kinetics</u> <u>and Regulation</u> .....	190
Results:	
A. Enzyme Purification .....	190
B. pH Effect .....	192
C. Substrate and Kinetic Constants .....	196
D. Kinetic Mechanism-Product Inhibition..	206
E. Enzyme Effectors and Regulators .....	240
F. Physical Studies .....	287
Discussion:	
NADP-type Mechanism .....	298
NADP <sup>+</sup> Allosteric Regulation .....	301

TABLE OF CONTENTS CONTINUED

	PAGE
Nucleotide Balance Control .....	303
Multivalent Effects .....	305
Enzyme Control Mechanisms and Taxonomy ..	310
PART II: <u>Cyclic AMP Metabolism</u>	
Results and Discussion:	
Cyclic AMP Uptake .....	311
Cyclic AMP Enzymes .....	312
Cyclic AMP Assays .....	313
Possible Cyclic AMP Function .....	314
PART III: <u>Transport Systems for Purines,</u> <u>Pyrimidines and Nucleosides</u>	
Results:	
A. Characterization of Nucleic Acid	
Base Uptake Processes .....	315
B. Characterization of Nucleoside	
Transport Processes .....	322
C. Binding of Purine Bases .....	354
Discussion:	
Characterization of the Transport Systems.	
Purine Binding and Interactions with Ca <sup>++</sup> .	
Ca <sup>++</sup> in Transport .....	366
Purine Regulation of Ca <sup>++</sup> Transport .....	367
PART IV: <u>Cytokinin Effects on Transport</u>	
Results:	
A. Possible Transport Effects .....	371
B. Effects on Nucleoside Transport .....	374
C. Effects on Amino Acid Transport .....	374
D. Comparative Effectiveness of Cytokinins	389
E. Tryptophan Interactions .....	399

TABLE OF CONTENTS CONTINUED

	PAGE
F. Isopentenyladenine Transport and Binding .....	399
Discussion:	
Inhibition of Transport .....	411
Relative Effectiveness of Cytokinins .....	411
Tryptophan Stimulation and Interactions....	412
Implications .....	413
Part V: <u>Cytokinin Effects on Macromolecular</u> <u>Synthesis</u>	
Results:	
A. Effects on Induction of Glutamate Dehydrogenase .....	415
B. Protein and RNA Synthesis .....	418
C. Protein and RNA Synthesis and Degradation .....	424
D. Possible Cyclic AMP Interactions .....	428
Discussion:	
Enzyme Induction and Catabolite Repression .	432
Effects on Synthesis .....	433
CONCLUSIONS .....	435
REFERENCES .....	441

LIST OF TABLES

TABLE	PAGE
I Cytokinin compounds .....	96
II Comparison of different cytokinin bioassays .....	100
III Table of cytokinin effects .....	122
IV A summary of the purification of <u>Achlya</u> glutamate dehydrogenase .....	191
V A summary of the purification of <u>Pythium</u> glutamate dehydrogenase .....	193
VI Michealis and inhibition constants and $M_{0.5}$ values for <u>Pythium</u> and <u>Achlya</u> glutamate dehydrogenases .....	205
VII Predicted and observed product inhibition patterns of ordered binary-ternary and ternary-binary kinetic mechanisms according to Scheme 2 .....	222
VIII Product inhibition patterns for <u>Pythium</u> NAD-specific glutamate dehydrogenase in the presence and absence of allosteric activators .....	230
IX A summary of the response of <u>Pythium</u> and <u>Achlya</u> NAD -specific glutamate dehydrogenases to a variety of allosteric modulators .....	268
X Multivalent effects of positive modulators in the antagonism of AMP inhibition during the reductive amination of <u>Pythium</u> NAD-specific glutamate dehydrogenase.	281
XI Halftimes for heat denaturation of <u>Pythium</u> and <u>Achlya</u> NAD-specific glutamate dehydrogenases .....	296
XII Some probable relationships between anabolism and modulators of NAD-specific glutamate dehydrogenases of Oömycetes.....	309
XIII A summary of the $S_{0.5}$ values for transport of purines, pyrimidines, and nucleosides by germinated sporangiospores of <u>Achlya</u> .....	318
XIV Effects of various cytokinin compounds on amino acid uptake.	392

LIST OF TABLES CONTINUED

	PAGE
XV A summary of the relative inhibitory effects of various cytokinin-related compounds on the rate of active transport of methionine by germinated sporangiospores of <u>Achlya</u> .....	397
XVI Relative inhibition of the transport of the L-isomers of the 20 common amino acids into the germinated sporangiospores of <u>Achlya</u> by the cytokinin, isopentenyladenine.....	400

LIST OF FIGURES

FIGURE	PAGE
1. Asexual life cycle of <u>Achlya</u> .....	162
2. Optical density and protein content correlation .....	170
3. pH effect on GDH activity .....	194
4. NADP(H) activation of GDH .....	198
5. Oxidative deamination reaction of <u>Achlya</u> GDH .....	201
6. Reductive amination reaction of <u>Achlya</u> GDH .....	203
7. Oxidative deamination reaction of <u>Pythium</u> GDH .....	207
8. Rate-concentration plot for NADH for <u>Pythium</u> GDH .....	209
9. Rate-concentration plot for $\alpha$ -ketoglutarate for <u>Pythium</u> GDH .....	211
10. Rate-concentration plot for ammonia for <u>Pythium</u> GDH .....	213
11. Product inhibition studies without activator for <u>Pythium</u> GDH, $\text{NAD}^+$ varied substrate .....	216
12. Product inhibition studies without activator for <u>Pythium</u> GDH, glutamate varied substrate .....	218
13. Product inhibition studies with activator for <u>Pythium</u> GDH, $\text{NAD}^+$ varied substrate .....	226
14. Product inhibition studies with activator for <u>Pythium</u> GDH, glutamate varied substrate .....	228
15. Product inhibition by glutamate on <u>Pythium</u> GDH with $\text{NADP}^+$ ...	231
16. Product inhibition studies without activator for <u>Achlya</u> GDH, $\text{NAD}^+$ varied substrate .....	234
17. Product inhibition studies with activator for <u>Achlya</u> GDH, $\text{NAD}^+$ varied substrate .....	236
18. Product inhibition studies for <u>Achlya</u> GDH, glutamate varied substrate .....	238
19. Antagonism of ammonia inhibition by $\text{NADP}^+$ for <u>Pythium</u> GDH...	242

## LIST OF FIGURES CONTINUED

	PAGE
20. NADP <sup>+</sup> interactions with ammonia and $\alpha$ -ketoglutarate for <u>Pythium</u> GDH.....	244
21. Isocitrate inhibition of <u>Achlya</u> GDH.....	247
22. Lineweaver-Burk plots of citrate inhibition of <u>Achlya</u> GDH...	250
23. P-enolpyruvate activation of <u>Pythium</u> GDH .....	252
24. P-enolpyruvate antagonism of ammonia inhibition of <u>Pythium</u> GDH.....	255
25. Influence of ammonia on NADP <sup>+</sup> and P-enolpyruvate activation of <u>Achlya</u> GDH.....	257
26. Citrate inhibition of <u>Achlya</u> GDH with activators.....	259
27. Citrate inhibition of <u>Pythium</u> GDH.....	261
28. Rate-concentration plots for ammonia and $\alpha$ -ketoglutarate for <u>Pythium</u> GDH .....	265
29. Adenine nucleotide effects on <u>Achlya</u> GDH.....	270
30. 'Energy charge' effects on <u>Pythium</u> GDH, biosynthetic reaction.	272
31. 'Energy charge' effects on <u>Pythium</u> GDH, catabolic reaction...	274
32. Effects of CoA derivatives on <u>Pythium</u> GDH.....	278
33. Antagonism of citrate inhibition of <u>Pythium</u> GDH .....	283
34. Allosteric effectors of <u>Pythium</u> GDH at varied pHs.....	285
35. Zone sedimentation in sucrose density gradients of <u>Achlya</u> and <u>Pythium</u> GDHs.....	288
36. Thermal inactivation curve for <u>Pythium</u> GDH .....	292
37. Thermal decay plots for <u>Pythium</u> GDH at varied temperatures..	294
38. Rate-concentration plots for purine and pyrimidine uptake...	316
39. Inhibition of uracil transport .....	319
40. pH effects on purine and pyrimidine uptake .....	323
41. Temperature effects on purine and pyrimidine uptake .....	325
42. Rate-concentration plots for nucleoside uptake .....	327
43. Inhibition of nucleoside transport by metabolic poisons.....	330

## LIST OF FIGURES CONTINUED

	PAGE
44. Efflux of nucleosides .....	332
45. pH effects on nucleoside uptake.....	335
46. Temperature effects on nucleoside uptake .....	337
47. Competition kinetics, adenosine and thymidine uptake .....	340
48. Competition kinetics, cytidine and uridine uptake.....	342
49. Competition kinetics, guanosine uptake .....	344
50. Competition kinetics, inhibition by guanosine .....	346
51. Competition kinetics, inhibition by adenosine and inosine...	349
52. Citrate inhibition of nucleoside uptake .....	352
53. Stimulation of purine base binding by HgCl <sub>2</sub> .....	355
54. Stimulation of adenine binding to osmotically shocked cells by Ag <sup>+</sup> and I <sub>2</sub> .....	358
55. Time course of HgCl <sub>2</sub> activation on non-shocked and shocked cells .....	360
56. <sup>45</sup> Ca <sup>++</sup> release by purines .....	365
57. Purine effects on glutamate and aspartate uptake .....	372
58. Adenosine uptake, inhibition by isopentenyladenine and isopentenyladenosine .....	375
59. Isopentenyladenine inhibition of adenine and cytidine uptake.	377
60. Isopentenyladenine inhibition of methionine and isoleucine uptake .....	380
61. Time course of hexylaminopurine inhibition of histidine uptake.....	382
62. Lineweaver-Burk plots of isopentenyladenine inhibition of methionine and isoleucine uptake.....	385
63. Lineweaver-Burk plots of isopentenyladenine inhibition of arginine, histidine, leucine, lysine, phenylalanine and valine uptake.....	387
64. pH effects on isopentenyladenine inhibition of amino acid uptake.....	390
65. Effect of various cytokinin-related compounds on methionine uptake .....	395

LIST OF FIGURES CONTINUED

	PAGE
66. Enhanced tryptophan and IAA uptake by isopentenyladenine .....	402
67. Isopentenyladenine absorption .....	405
68. Isopentenyladenine binding to cells and filters .....	407
69. Inhibition of isopentenyladenine binding .....	409
70. Isopentenyladenine effects on glutamate dehydrogenase activity and amino acid incorporation .....	416
71. Isopentenyladenine effects on protein and RNA synthesis and precursor uptake.....	419
72. Isopentenyladenine and 2,4-Dnp effects on protein synthesis and precursor uptake .....	422
73. Isopentenyladenine effects on macromolecular synthesis and degradation .....	425
74. Cyclic AMP effects on isopentenyladenine inhibition of uracil incorporation .....	429

## ABBREVIATIONS

Ade	- adenine
Ado	- adenosine
AMP	- adenosine 5'-monophosphate
ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
BIS	- N,N'-methylene bisacrylamide
CCCCP	- carbonylcyanide-p-chlorophenyl hydrazone
Cyd	- cytidine
Cyt	- cytosine
Cyclic AMP	- adenosine 3':5'-cyclic monophosphate
Cyclic GMP	- guanosine 3':5'-cyclic monophosphate
DEAE	- diethylaminoethyl
DMSO	- dimethylsulfoxide
DNase	- deoxyribonuclease
2,4-Dnp	- 2,4-dinitrophenol
DTT	- dithiothreitol
EDTA	- ethylene diamine tetra-acetic acid
EGTA	- ethylene glycol-bis-(aminoethyl ether)-N,N'-tetra-acetic acid
GDH	- glutamate dehydrogenase
GTP	- guanosine 5'-triphosphate
Gua	- guanine
Guo	- guanosine
HAP	-hexylaminopurine
IPA, i <sup>6</sup> ade	- N <sup>6</sup> - ( $\Delta^2$ -isopentenyl)adenine
i <sup>6</sup> Ado	- N <sup>6</sup> - ( $\Delta^2$ -isopentenyl)adenosine
IAA	- indole-3-acetic acid (auxin)
mRNA	- messenger ribonucleic acid
NAD <sup>+</sup>	- nicotinamide adenine dinucleotide
NADH	- reduced nicotinamide adenine dinucleotide
NADP <sup>+</sup>	- nicotinamide adenine trinucleotide
NADPH	- reduced nicotinamide adenine dinucleotide
POPOP	- 1,4-Bis- [2-(5-phenyloxazolyl)] -benzene
PPO	- 2,5-diphenyloxazole

ABBREVIATIONS CONTINUED

PRPP	- phosphoribosyl pyrophosphate
RNase	- ribonuclease
rRNA	- ribosomal ribonucleic acid
S <sub>0.5</sub>	- substrate concentration giving half maximal reaction velocity
TEMED	- N,N,N',N'-tetramethylethylene diamine
Thd	- thymidine
Thy	- thymine
Tris	- trihydroxymethylaminomethane
Ura	- uracil
Urd	- uridine

I N T R O D U C T I O N

## INTRODUCTION

Regulation of cellular physiology presents a complex picture of interrelated controls, acting at many levels. At the level of an enzymatic reaction, there is immediate kinetic regulation caused by the availability of substrates and coenzymes, and by the physical environment, in terms of pH and temperature. Control over activity of an enzyme may also be exerted by allosteric interactions of metabolic products, or compounds whose relative levels reflect the energy and reducing power that is available to the cell. The presence or activity of the enzyme itself may be controlled by induction or repression of synthesis, or by the rate of degradation. These induction, repression, and degradation processes in turn are subject to control by factors reflecting overall cellular physiology, or by signals from the external environment. Such signals and factors can influence other processes besides specific enzyme reactions, including transport of metabolites, and the general rates of syntheses of nucleic acids and proteins.

The work reported in this thesis is an attempt to examine some of the regulatory processes which operate in the coenocytic fungus, Achlya. Particular emphasis is placed on the controls acting on the activity of a key enzyme, NAD-linked glutamate

dehydrogenase, and on the role of a group of plant hormones, the cytokinins, in regulation of some facets of cellular metabolism. Studies on the kinetic mechanism and allosteric regulation of the glutamate dehydrogenase are presented in the first section of the Results, and the relationships of the controls to overall metabolism are discussed. Induction of this enzyme under various growth conditions had been examined in this laboratory (Smaluck 1971). Subsequent studies of a possible role for cyclic AMP in the induction process, and in general metabolism (Cameron & LéJohn, unpublished results), provided two areas for further study. Results from examination of the first area of interest, calcium regulation, have been reported by Cameron and LéJohn (1972a). Some investigations into the second area, cyclic AMP metabolism, are briefly considered here.

As a result of looking at the uptake of exogenous cyclic AMP, it became of interest to determine how related nucleoside compounds were transported by these cells. Such studies were undertaken with the additional consideration that the availability of exogenous purines, and their uptake, might influence glutamate dehydrogenase regulation in some way, due to its role in purine biosynthesis and nitrogen metabolism. The characterization of the transport systems for nucleosides and for nucleic acid bases is presented in the third section of the Results. Transport processes for other compounds were also being examined, with particular emphasis on

calcium regulation. Clues suggesting that cytokinin played a role in transport regulation were detected in the studies of adenine and calcium transport and binding. A detailed study of the effects of cytokinins on the uptake of amino acids is presented here. The final section of the Results considers the possibility that cytokinins have effects on other aspects of cell metabolism besides active transport, and attempts were made to identify such actions. In summary, a variety of regulatory processes in Achlya are examined, and discussed in terms of the general physiology of the cell.

H I S T O R I C A L

## HISTORICAL

GLUTAMATE DEHYDROGENASEAllosteric Enzymes

Control of key reactions in the network of metabolic interactions has been found to be significant in overall regulation of metabolism. Enzymes catalyzing these key reactions frequently show complex reactions to various effectors, and the concept of allosterically regulated enzymes developed from studies of these interactions.

Allosteric enzymes consist of more than one polypeptide chain (subunits, or monomers), and they tend not to show simple Michaelis-Menten kinetic behaviour, but rather to give sigmoidal rate responses to substrate concentration. Such regulatory molecules may react atypically to substrate molecules if the substrate is also acting as an effector (homotropic enzymes); or they may respond to effectors other than the substrate, such as a metabolic end-product (heterotropic enzymes). In many cases the substrate is only one of several substances which produce varied responses by the enzyme, with complex end results. These basic concepts have been presented frequently in reviews (Stadtman 1966, Atkinson 1969)

and in textbooks (Lehninger 1970).

Monod, Changeux and Jacob (1963) proposed a model for allosteric enzyme behaviour, which consisted of polymeric enzymes, with one catalytic and one other site on each subunit. The term "allosteric" refers to this "other site". In this model, the enzyme was capable of existing in at least two conformational states, referred to as the "R" and the "T" states, and conversion between the states was a consequence of ligand binding at the allosteric sites. Considering these sites, in the case of homotropic enzymes, two sites for the substrate on each subunit are postulated, at least one of which is catalytic. Heterotropic enzymes would have a modulator site in addition to the catalytic site. In many cases, allosteric enzymes have been "desensitized" by either chemical or genetic modification, so that the modulator sites and activity are lost without loss of catalytic activity. Examples of this desensitization are considered in the examination of glutamate dehydrogenase.

The Monod, Changeux and Jacob model has been modified (Monod, Wyman & Changeux 1965, Rubin & Changeux 1966) and also varied by other workers (see Kirschner 1971, Atkinson 1969). However, the basic postulate of the mechanism of regulation involves conformational changes induced by binding of effectors, producing altered catalytic activity of the enzyme. While the classical model involved exclusive binding of substrate to the R state only,

Atkinson et al (1965) proposed that although the affinity of the two states differs, both may bind substrate. Koshland and co-workers (Koshland et al 1966, Kirtley & Koshland 1967, Conway & Koshland 1968) introduced two major variations in the model, the first being that binding of the first ligand could produce increased or decreased subsequent binding; and the second being that all subunits need not change simultaneously, thus a series of intermediate forms of the enzyme can occur. The kinetics of the molecular interactions of substrates and effectors with the enzyme form, and the characteristic patterns seen for various allosteric phenomena are dealt with in several comprehensive sources (Kirschner 1971, Plowman 1972), and will not be considered here.

The significance of regulatory enzymes is in their biological function, and the action of individual ligands on isolated enzymes must be considered in that light. A number of enzymes demonstrating regulatory responses in metabolism have been discussed by Stadtman (1966), and more recently by Sanwal (1970). Allosteric responses permit enzymes at key positions in metabolic sequences to respond to an overall end product of the sequence, or to multiple branches of a pathway, by feedback responses to molecules structurally different from the original substrate. Patterns of metabolic control involving such regulation are described in the two reviews mentioned above.

The allosteric effectors controlling an enzyme may be end products of a particular reaction sequence, and specific to it. However, allosteric enzymes will frequently also respond to signals by such ubiquitous compounds as ATP and AMP. Atkinson (1968)

proposed that the balance between the adenine nucleotide forms reflected the overall physiological state of the cell, and as such could act as a regulatory signal for enzymes involved in energy-utilizing or energy-producing reactions. Energy charge was thus formally defined in terms of concentrations as:

$$\frac{[ \text{ATP} ] + \frac{1}{2}[ \text{ADP} ]}{[ \text{ATP} ] + [ \text{ADP} ] + [ \text{AMP} ]}$$

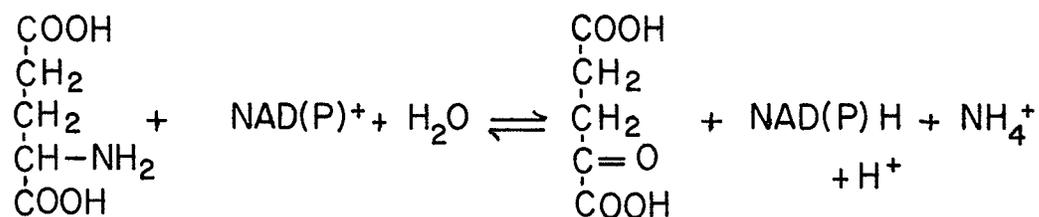
Enzymes of glycolysis and the citric acid cycle have been found to be responsive to this form of regulation.

A similar signal which can indicate overall physiological conditions is the balance of reduced coenzymes. Certain enzymes of Salmonella typhimurium have been reported to be allosterically inhibited by NADH (see Sanwal 1970), and regulatory controls involving the ratios of reduced to oxidized coenzymes, and the specificities of NAD and NADP mediated reactions have been proposed.

#### Glutamate Dehydrogenase

Glutamate dehydrogenase is a significant enzyme in the metabolism of organisms as the reaction catalyzed links the reactions of the glycolytic sequence and tricarboxylic acid cycle with nitrogen metabolism, through the transamination reactions that occur between amino acid forms. The reaction is the reversible

oxidative deamination of L-glutamate to  $\alpha$ -ketoglutarate and ammonia.



The various forms of the enzyme which occur have been extensively examined, from the point of view of physical structure, the interactions of substrates and effectors, their effects on kinetic properties, and also in terms of the metabolic regulation that controls this enzymatic process. The regulation can take place both as control of synthesis and turnover of the enzyme, and as immediate regulation of the kinetic process.

#### Studies on the mammalian enzyme

The enzyme from bovine liver mitochondria has been most extensively studied with regard to physical structure, molecular binding sites and interactions, and kinetic mechanism. These detailed studies provide a useful basis for examination and comparison with the forms from other species. The various aspects of the investigations have been reviewed at length by several authors (Goldin & Frieden 1971, Frieden 1970, Fisher 1970, 1973) and the basic findings will be only briefly mentioned here.

Kinetic studies of the glutamate dehydrogenase reaction mechanism of bovine liver enzyme have been used to try and determine if the order of addition of the substrates is ordered or random. This is of interest in the case of the reductive amination direction of the reaction, where there are three substrates involved,  $\alpha$ -ketoglutarate, ammonia, and NADH(or NADPH). Frieden (1959<sub>c</sub>) reported experimental kinetic data which agreed with a compulsory ordered mechanism, with NADPH binding first, followed by ammonium ion, and then  $\alpha$ -ketoglutarate. In his studies, NADPH was used as the coenzyme because at high concentrations the more reactive NADH produces complex kinetics and inhibition, apparently due to binding at a second, non-active site (Goldin & Frieden 1971).

Fahien and Strmecki (1969) found that in arsenate buffer rather than Tris-acetate used by Frieden, NADH did give linear plots, and in initial velocity studies with this co-enzyme, a sequential order of addition was found, with the binding of NADH being followed by  $\alpha$ -ketoglutarate, and then  $\text{NH}_4^+$ . With Tris buffer and NADPH the ammonium ion was found to be added before the  $\alpha$ -ketoglutarate, as in Frieden's studies. These workers suggested that the altered sequence in the presence of arsenate could be due to the Michaelis constants for  $\alpha$ -ketoglutarate and ammonium ions in that buffer (Fahien & Strmecki 1969). Engel and Dalziel (1970) reported on kinetic studies of both the NADH and NADPH reactions over a range of substrate concentrations, and proposed that a random order of substrate addition was more likely to satisfy the rate equation.

Silverstein and Sulebele (1973) used isotopic exchange to examine equilibrium kinetics for bovine glutamate dehydrogenase. On the basis of these studies, they proposed an "alternative order" mechanism of action, which they have defined as a branched sequence mechanism, in which the ligands may bind and dissociate in any order, with the rate of such binding and dissociation occurring both dependently and independently of other ligands.

A significant proportion of the studies on ligand binding effects on kinetic mechanism have been concerned with the effects of purine nucleotides, particularly adenosine and guanosine phosphates. Originally these compounds had been tested in an attempt to find a competitive inhibitor for the coenzymes,  $\text{NAD}^+$  and  $\text{NADP}^+$  (Frieden 1970). Wolff (1962) and Frieden (1962, 1963<sub>a</sub>) reported on the effects of these compounds on glutamate dehydrogenase, and Frieden (1963<sub>a</sub>) further showed that they acted at a site distinct from the active site. The effects of the guanosine compounds differ from the adenosine nucleotide forms, with guanosine and inosine nucleotides inhibiting, and adenosine forms other than ATP activating. The fact that the different purines have different effects correlates with differences observed in the binding of the compounds, in that it appears GTP and ADP only compete in part for the same site (Goldin & Frieden 1971). Cross and Fisher (1970) have interpreted this in terms of steric hinderance of binding to specific subsites, involving overlap of the ADP and GTP positions. Additional complex-

ities arise in kinetics and binding, in the form of different observed effects of the purines when NADH is used as a coenzyme rather than NADPH. Some of these may be a result of NADH binding to a second non-active site as was mentioned.

Fisher (1973) has reviewed in detail the studies on complexes formed between glutamate dehydrogenase and various substrates and modifiers. Specific chromophore signals altered due to ligand-enzyme interactions resulted in perturbations of the absorption spectra, which could be measured and interpreted. The results of such studies have been explained by Fisher et al (1970) in terms of the Ligand Exclusion Theory. Ligands were assumed to interact at two subsites on the enzyme surface, thus producing a changed spectral signal, or catalysis. Inhibitor and activator actions then are explained in terms of blocking or aiding of substrates binding to their subsites. Cooperative interactions could result if one ligand provided a subsite for a second ligand, and steric hindrance of binding at one set of subsites could result if subsites for another ligand intersected.

The Ligand Exclusion Model is one explanation of ligand interactions and the enzyme response to them. Alternatively, the conformational change model could be employed to explain the interactions. The models are not distinguished by kinetic studies, but the interactions described by Fisher (1973) in terms of ligand exclusion could equally well be interpreted as resulting from changes in sites

resulting from protein conformation changes in response to ligand binding.

Purine nucleotides have an additional effect on bovine glutamate dehydrogenase in addition to alterations in kinetic activity. Olson and Anfinsen (1953) reported that bovine enzyme undergoes reversible association to higher molecular weight forms, depending on protein concentration. Frieden (1959 a,b) found that this association was also affected by purine nucleotides. Proposals by Frieden (1959 a,b) and Tomkins, Yielding, and co-workers (1961, 1963, 1965) suggested that the dissociated form of the enzyme catalyzed the alanine dehydrogenase reaction, while the tetramer form of the enzyme had glutamate dehydrogenase activity. ADP acted to preserve the aggregated form of the enzyme, while GTP and steroids promoted disaggregation (Tomkins et al 1961). Subsequent studies, however, showed that instead of glutamate dehydrogenase activity being almost totally inhibited in the monomer state as Tomkins claimed, it did indeed use that substrate (Fisher et al 1962, Frieden 1963b) indicating that substrate specificity does not depend on aggregation. Bitensky, Yielding, and Tomkins (1965), in view of this information, proposed a modified scheme in which two conformational forms of the monomer occur, depending on which effectors are binding. Of the two forms only one substrate form would be capable of aggregation at certain protein concentrations. Frieden (1970) suggests that changes in the conformation of monomer

subunits in response to the ligands binding might result in the association changes, and as such the molecule is of interest in terms of the model of Monod, Wyman and Changeux (1965) for allosteric behaviour. Fisher (1973) again interprets such changes in monomer association in terms of the Ligand Exclusion model, with GTP stabilizing the monomer form by binding to subsites involved in protein-protein interactions.

Physical studies of the bovine liver enzyme indicate that the polymer form of the enzyme can be sub-divided into various distinct levels of organization (Fisher 1970). The monomer, or  $\beta$ - form in Fisher's designation, is a catalytically active group of six individually folded polypeptide chains. Each  $\beta$ -form is thought to consist of two  $\gamma$  subunits which can bind NADH but have no catalytic activity. At higher protein concentrations, three  $\beta$  subunits can aggregate to form the  $\alpha$  enzyme, with a total molecular weight of about 2 million daltons.

Unlike the bovine enzyme, the form isolated from chicken liver shows little tendency to aggregate, while the frog liver enzyme generally occurs as a dimer (Frieden 1962, Fahien et al 1965). These differences between the enzyme forms of different species of animals are also reflected in the relative reactivity with the two coenzymes. (Fahien et al 1965), and the extent of the effect of purine nucleotides (Frieden 1965). Further confirmation of differences were the immunological studies of Talal and Tomkins (1964).

Although the forms of the enzyme from various vertebrates showed differences, much more significant differences were found to occur between the animal enzymes and those of plants and microorganisms. Frieden (1965) found that these last non-animal enzymes were not affected by low concentrations of purine nucleotides, nor were they inhibited by zinc, apparently due to the lack of these specific binding sites. Of particular note in Frieden's studies was the discovery that all the non-animal enzymes are specific for just one of the two coenzyme forms used by the bovine enzyme. In addition, when an organism could use both coenzymes in the glutamate dehydrogenase reaction, two distinct forms of the enzyme could be isolated, as in the case of Neurospora crassa (Sanwal & Lata 1961) and yeast (DeCastro et al 1970). These findings raised interesting questions with regard to the mechanism of action of the enzyme forms, their regulation, and function in the organisms. Studies on these enzymes also have provided some perspectives in assessing the observed characteristics and metabolic role of the bovine form.

#### Bacterial and Fungal enzyme forms

Sanwal and Lata (1961) suggested that the occurrence in Neurospora crassa of two enzyme forms, each specific for a different coenzyme, reflected their separate role in catabolism and biosynthesis. Yeast also had been found to contain two glutamate dehydrogenases

(Holzer & Schneider 1957), as do a number of other fungi (see Casselton 1969). Studies of the regulation of the relative activities of the two enzymes (Sanwal & Lata 1962, Hollenberg et al 1970), to be discussed more fully below, indicated that the NAD-linked form functions in the catabolism of glutamate, while the NADP-linked enzyme synthesizes glutamate, providing the link between carbon and nitrogen metabolism. Studies of mutant forms of two-enzyme containing species also have helped to confirm this conclusion regarding enzyme roles. Mutants of Neurospora (Fincham 1957), Saccharomyces cerevisiae (Grenson & Hou 1972), and Aspergillus nidulans (Kinghorn & Pateman 1973) all show the effects of the specifically missing enzyme form in their growth responses to  $\text{NH}_4^+$  and glutamate.

Studies on bacteria which have only one unregulated form of the enzyme supported this concept between coenzyme specificity and metabolic role. The NAD-linked enzyme of the clostridia functions to provide  $\alpha$ -ketoglutarate for transamination and fermentation (see Goldin & Frieden 1971). On the other hand, the NADP-specific enzymes of E. coli (Halpern & Umbarger 1960) and of Nitrosomonas europaea (Hooper et al 1967) appear to function to form glutamate by ammonia incorporation. In these bacteria the catabolic or biosynthetic role of glutamate dehydrogenase appears to be integrated into overall control of cell metabolism by linking to other enzymes such as aspartase or transaminase, and by induction or repression of enzyme synthesis. In the fungi with two enzyme forms, differential

enzyme synthesis allows the organism to respond to the requirements for the catabolic or biosynthetic process.

However, in the lower fungi or Phycomycetes, only an NAD-linked glutamic dehydrogenase is found (LéJohn 1971). In these cases the more complex role required of this enzyme in mediating both catabolic and biosynthetic reactions is reflected in occurrence of a variety of allosteric controls. LéJohn (1971) has used the regulatory properties of glutamate dehydrogenase and other enzymes as one of the parameters in examination of the evolutionary relationships of fungi. Distribution of glutamate dehydrogenase regulatory forms were correlated with regulation of lactic and isocitric dehydrogenases, and with lysine pathways and cell wall composition in order to propose a scheme of relationships.

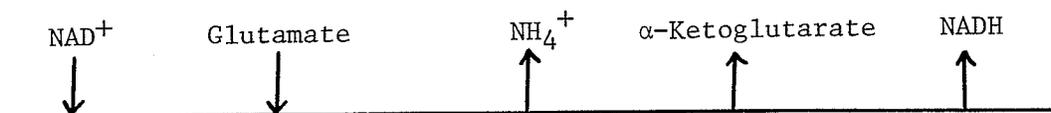
Three regulatory types of the NAD-linked glutamate enzyme were found in the Phycomycetes, while the higher fungi - Deuteromycetes, Ascomycetes, and Basidiomycetes - possess both the NAD- and NADP-linked forms. Type I of the forms found in the Phycomycetes occurs in the Chytridiales and most Mucorales, and is unregulated. The regulatory properties of the Type II enzyme, found in the Blastocladales, and in some Mucorales, are described below. The Type III regulatory pattern is found in enzymes of the Oomycetes and Hyphochytridiomycetes. Achlya sp. (1969) and Pythium debaryanum are members of the Oomycetes, and studies of the regulation of this enzyme will be presented in the "Results" section of this thesis.

Studies of the Type II glutamate dehydrogenase found in Blastocladiella emersonii are of significance with regard to both the mechanism of action of the controls and the physiological role of the enzyme which they reflect. The kinetic mechanism of this enzyme was found to be complex to distinguish as a result of various allosteric interactions producing non-Michaelian kinetic patterns. Thus hyperbolic saturation plots and biphasic double reciprocal plots were obtained at low substrate concentrations (LéJohn & Jackson 1968<sub>a</sub>). AMP was found to have a significant effect on these patterns, increasing the affinity of the enzyme for  $\text{NAD}^+$  and glutamate, with an accompanying decrease in affinity for NADH.

Initial velocity patterns of this enzyme were complex due to various concentrations and effector-dependent interactions, but seemed to indicate that the substrates are bound to the enzyme in an ordered sequence, rather than randomly. The manner in which initial velocity patterns may distinguish between random and ordered addition patterns was discussed by Frieden (1959<sub>c</sub>) with the beef liver glutamate dehydrogenase as an example.

LéJohn et al (1969) reported on subsequent product inhibition studies carried out to clarify the order of substrate addition and product release. These studies employed the methods described by Cleland (1963 a,b,c), based on predictions made from the complete steady-state equations for reaction rate. In the studies of the

Blastocladiella enzyme,  $\text{NAD}^+$  and  $\text{NADH}$  show competitive inhibition patterns with each other, indicating that they bind to the same enzyme form, presumably as the first substrates to bind to the free enzyme form. The reactions of the other substrates and products fit the following diagrammatic sequence:



One deviation was observed with  $\alpha$ -ketoglutarate in the inhibition of glutamate oxidation, where a cooperative interaction was observed, as defined by the  $[\text{S}]_{90} : [\text{S}]_{10}$  value by Kirtley and Koshland (1967). This indicated that  $\alpha$ -ketoglutarate is likely an allosteric inhibitor of the reaction, as is the case for the  $\text{NADP}$ -specific form of the enzyme from Neurospora crassa (West et al 1967).

Regulation of the  $\text{NAD}$ -specific glutamate dehydrogenase of the mitochondria of Blastocladiella has been found to occur by means of cations, particularly calcium and manganese (LéJohn 1968b), and by adenylates and guanylates (LéJohn & Jackson 1968a), and also by compounds connected with the tricarboxylic acid cycle and glycolysis, such as citrate, isocitrate, fructose-1,6-diphosphate, and fumarate (LéJohn 1968a). Of particular note in metabolism is the "unidirectional inhibition" of the reaction, by which the effect of citrate, isocitrate and related compounds, as well as  $\text{ATP}$  and  $\text{EDTA}$ , are exerted only against the oxidative deamination reaction (LéJohn

& Jackson 1968a). These substances were more inhibitory at higher pH values, only slightly effective at neutral pH, and ineffective below pH 6. Similarly it was found that enzyme activation by AMP was dependent on pH in the same manner. The model proposed in order to account for some of these regulatory effects took into account the mitochondrial location of the enzyme, and proposed that changes in pH accompanying the influx of cations in exchange for protons influenced the enzymes reactions with effectors. Under conditions of high energy supply, ATP would be hydrolysed, and the change in proton concentration would result in the inhibitors being better able to prevent the oxidative deamination of glutamate (LéJohn & Jackson 1968a).

The mitochondrial NAD-linked isocitric dehydrogenase of this organism has been found to be allosterically activated by citrate, AMP and ADP (LéJohn et al 1969). Under conditions where the glutamate dehydrogenase oxidative deamination reaction is inhibited, the isocitric dehydrogenase will produce  $\alpha$ -ketoglutarate, which will subsequently be reductively aminated. This is suggested to be acting as a "glutamate sink" for the storage of organic acids. Alterations in the overall cell energy would result in changes in pH, cations, adenylates, and organic acids, and result in oxidation of the glutamate.

The allosteric interactions of some of these effectors have been examined more closely in attempts to elucidate their actions.

Of particular interest have been the effects of adenylates. In kinetic studies (LéJohn & Jackson 1968a) the activator AMP was found to increase the affinity of the enzyme for  $\text{NAD}^+$  and glutamate, and to decrease the affinity for NADH. The kinetic patterns obtained for AMP activation with  $\text{NAD}^+$  as substrate were complex, showing cooperative interactions. ATP acts to reverse the activation, and appears to be competitive with AMP, perhaps for an allosteric site.

Previously it was noted that the effects of various inhibitors and activators were influenced by pH, and LéJohn (1968a) suggested that a desensitized form of the enzyme might occur at low pH. Sucrose gradient studies at various pH values demonstrated that under alkaline conditions the enzyme underwent irreversible dissociation to a monomer form (LéJohn et al 1969). Examining the possibility that ligand binding might preserve the active form, it was found that dissociation at pH 9 could only be prevented by the substrates  $\text{NAD}^+$  and NADH, and the activators AMP, ADP,  $\text{Ca}^{++}$ , and to some extent,  $\text{Mn}^{++}$ . Copper ion ( $\text{Cu}^{++}$ ) had been found to inhibit the reductive amination reaction without affecting the oxidative deamination reaction to any significant extent (LéJohn 1968b).  $\text{Cu}^{++}$  alone in sucrose gradients promoted dissociations at all pHs tested, but when added with activators and substrate, it inhibited completely only at acid pH (LéJohn et al 1969). Mercury ion ( $\text{Hg}^{++}$ ) also inhibited activity in a manner closely associated to pH effects,

and LéJohn and Jackson (1968 b) reported on the selective desensitization of the enzyme to AMP, ATP, and  $\text{NAD}^+$ , without simultaneous desensitization to citrate, EDTA, metabolites, or  $\text{Ca}^{++}$ . The drastically altered affinity of the enzyme for substrates and effectors at acid pH, and their ability to protect the enzyme from  $\text{Cu}^{++}$  and  $\text{Hg}^{++}$  inactivation in alkaline but not acid buffer (LéJohn et al 1969) supported the concept that the enzyme assumed one of two configurations depending upon pH. The effects of AMP,  $\text{Ca}^{++}$  and other allosteric ligands may be the result of modification of the protein structure, and a model of such interactions was proposed by LéJohn et al (1969) to account for observed negative and positive cooperativity effects.

Allosteric regulation has been found to affect glutamate dehydrogenases from other non-animal sources, including the enzymes from organisms possessing two coenzyme specific forms. Such regulation may provide a rapid physiological response prior to changes resulting from altered synthesis rates, or it may reflect a special physiological function performed by one of the enzyme forms.

Thiobacillus novellus, a facultative chemoautotroph, has both an NAD-linked and an NADP-linked glutamate dehydrogenase (LéJohn & McCrea 1968). AMP allosterically activates the NAD-linked form in the direction of  $\alpha$ -ketoglutarate formation, and LéJohn et al (1968) suggested that AMP could be acting as a signal pertaining to a requirement for NADH for use in ATP synthesis.

The NADP-specific enzyme of Neurospora crassa is subject to activation by some of the same effectors that affect the NAD-specific enzyme of Blastocladiella -  $\alpha$ -ketoglutarate, di- and polycarboxylic acids, and EDTA - as well as showing a similar pH response (West et al 1967). In this case a specific functional role for  $\alpha$ -ketoglutarate allosteric effects was proposed, involving a link with transaminases, and a channeling of  $\alpha$ -ketoglutarate into the tricarboxylic acid cycle. Some of the defective NADP-specific glutamate dehydrogenases found in mutants of Neurospora can be activated by heat or pre-incubation with substrate. West et al (1967) proposed that such mutants may occur due to lost or distorted allosteric controls resulting in the enzyme being held in an inactive form.

#### Enzyme levels

Induction: Studies of the two forms of glutamate dehydrogenase found in Neurospora crassa had been interpreted to mean that the nitrogen-source on which the organism was grown affected the relative amounts of the two forms of the enzymes (Sanwal & Lata, 1962). Exogenous glutamate, with ammonium nitrate also in the medium, decreased the NADP-form, and increased the NAD-activity. Urea, as a product of glutamate and ammonia, was suggested as the regulator for the concurrent synthesis of the enzyme forms. In cases

of increased enzyme activity in response to a stimulus, the question whether the effect is actually due to synthesis of new enzyme molecules rather than activation of pre-existing enzyme activity, or conversion of pre-existing precursors to active form is pertinent. Sanwal and Lata (1961) in their studies on Neurospora, demonstrated that the increased activity was indeed due to new synthesis, and proposed a genetic locus for urea action. Hutterman et al (1971) reported that changes in glutamate dehydrogenase levels during differentiation in Physarum polycephalum were due to de novo synthesis of the enzyme, a process under control at transcriptional and translational levels. The inducibility of the NADP-specific enzyme of Chlorella pyrenoidosa was examined by Talley et al (1972), and found to be dependent on both RNA and protein synthesis, implying a gene activation process is involved. These studies also indicated that the gene was available for transcription during the entire cell cycle and was not restricted to certain phases as reported for some mammalian genes (Tomkins et al 1969).

In yeast, Holzer (1966) reported that the NAD-specific enzyme was repressed by ammonia or a product, while the NADP-specific form was not. This would be expected if the NAD-specific enzyme was concerned with the catabolism of glutamate to ammonia and  $\alpha$ -keto-glutarate, while the NADP-form utilizes ammonia to produce glutamate. Bernhardt et al (1964) suggested that retarded feedback effects of ammonia on the regulatory system were responsible for the damped oscillation pattern of glutamate dehydrogenase production in carbon

and nitrogen starved cells transferred from ammonia to glutamate medium. This was observed only in starved cells where there is need to replenish nitrogen reserves before releasing ammonia into the medium.

Pateman (1969) employed mutants of Aspergillus nidulans to examine the effect of nitrate in increasing NADP-specific glutamate dehydrogenase activity. Mutants unable to convert nitrite or nitrate to ammonia (i.e. nitrate reductaseless) did not produce an increase in glutamate dehydrogenase. Nitrate alone was therefore not acting on induction. Urease-less mutants also were unable to lower the activity of glutamate dehydrogenase when urea could not be converted to ammonia. Thus the apparent effects of nitrate and urea are actually due to their conversion to ammonia. Small amounts of nitrate and ammonia added to glutamate result in high levels of glutamate dehydrogenase activity, compared with the low levels of the NADP-specific enzyme observed on glutamate alone. Pateman suggested that glutamate concentration has the true effector action in regulating synthesis of glutamate dehydrogenase. The "ammonia" effect thus would be due to increased glutamate synthesis by glutamate dehydrogenase at high ammonia levels. Increasing glutamate concentration would thus result in repression of enzyme synthesis.

Evidence for a role for NADP-glutamate dehydrogenase in the catabolism of nitrogen was provided by studies on an NADP-glutamate dehydrogenaseless mutant of Saccharomyces cerevisiae by Grenson and Hou (1972). This gdh-A mutant has lost the ammonia repression effect on the general amino acid permease, and on three other metabolic functions. The general permease likely functions to take up amino acids for use as a nitrogen source, so its control seems to be integrated with overall nitrogen catabolism. The ammonia effect on the NAD-linked glutamate dehydrogenase is not affected by this mutation, indicating another mechanism is involved in this case (Dubois et al 1973). Addition of glutamate to the gdh-A mutants so that the normal glutamate and amino acid pools are restored does not result in restoration of the ammonia controls, indicating that other components besides glutamate must be involved in regulation. Pateman et al (1973) proposed a model for ammonia regulation of the various uptake and enzymatic processes in Aspergillus, involving the NADP-glutamate dehydrogenase. Studies of ammonia-derepressed mutants indicated that there were two types of ammonia-regulated systems; first the ammonium uptake system, regulated by intracellular ammonia levels, and secondly, the systems for nitrate reductase, urea and glutamate uptake, regulated by extracellular ammonium concentrations. Only gdh-A mutants were derepressed for both types of system. Pateman proposed that NADP-glutamate dehydrogenase was located in a regulatory site in the

cell membrane where it can complex with only extracellular ammonium. This regulatory ammonium-enzyme complex would then regulate the control of urea and glutamate uptake, and nitrate reductase and other nitrogen-linked enzymes. Intracellular ammonium would form a second type of regulatory complex with glutamate dehydrogenase, possibly by reacting at a second ammonia site, and this form would regulate further ammonia uptake. Thus NADP-glutamate dehydrogenase would have a function as a regulatory protein.

Catabolite effects: Some conflicting reports occur regarding the influence of ammonia on synthesis of the two glutamate dehydrogenase forms. In yeast, reports by Holzer and co-workers (Hierholzer & Holzer 1963, Westphal & Holzer 1964) indicated that ammonia repressed NAD-glutamate dehydrogenase, and induced NADP-glutamate dehydrogenase. Similarly the NADP-form could be induced by ammonia in Chlorella (Talley et al 1972). In Neurospora, ammonia was suggested as a repressor of NADP-glutamate dehydrogenase, and an inducer of NAD-enzyme, (Stachow & Sanwal 1967, Barratt 1963).

Some of the complications arising in these studies may be due to the fact that the presence of sucrose can prevent the induction of NAD-glutamate dehydrogenase by ammonia (Strickland 1971). Under such conditions, the levels of the NADP-enzymes are

maintained. In other cases also, catabolites were found to affect the induction process. Polakis and Bartley (1965) reported that disappearance of sugars from the medium resulted in loss of activity of biosynthetic enzymes such as NADP-glutamate dehydrogenase in Saccharomyces cerevisiae. Tuveson et al (1967) also reported the requirement for sucrose for NADP-glutamate dehydrogenase induction in Neurospora, but indicated that the results did not directly support the reciprocal regulation of synthesis of the two enzyme forms as suggested by Sanwal and Lata (1962). A reciprocal relationship of synthesis in the presence of catabolites was observed by Kapoor and Grover (1970) in Neurospora crassa. The biodegradative enzyme, NAD-specific glutamate dehydrogenase, was repressed by sucrose and glucose, while the NADP-specific form was induced by increasing concentrations of the catabolites. Consistent with the biosynthetic role of the NADP-enzyme, the presence of glutamate in the growth medium overcame the catabolite induction effects.

Effects of glucose on the response of glutamate dehydrogenase enzyme levels to glutamate and ammonia have also been examined in Coprinus lagopus (Fawole & Casselton 1972) and in Aspergillus nidulans (Hynes 1974). In general, glucose was found to repress NAD-glutamate dehydrogenase production, preventing the increase seen when glutamate was the sole carbon and nitrogen source. In Coprinus, synthesis of the NADP-enzyme increases in response to

glucose, and Fawole and Casselton (1972) suggest this is actually a response to  $\alpha$ -ketoglutarate as a product of glucose metabolism. Hynes (1974) observed that in Aspergillus, growth on a medium containing glutamate resulted in rapid loss of the NADP-specific enzyme, but levels were maintained when the medium also contained glucose. Thus it appears that regulation of the two enzyme forms is affected both by nitrogen metabolism and by catabolite effects of carbon sources. As the glutamate dehydrogenase reaction is the link between the tricarboxylic acid cycle and amino acid metabolism, such interrelationships in regulation might be expected.

Degradative controls: Hynes (1974) has reported the rapid loss of NADP-glutamate dehydrogenase from Aspergillus when glucose is absent from the medium. As protein synthesis inhibitors prevented this loss of activity, he suggested that an inactivating protein, such as a protease, is present in the absence of glucose. In the yeast Candida utilis inactivation of glutamine synthetase and NAD-specific glutamate dehydrogenase have been observed to occur in response to changes in the nitrogen compounds available (Ferguson & Sims 1971). The difference in the coenzyme specificity of the enzyme affected by the inactivation process may be indicative of different regulatory controls on yeast enzymes, or perhaps the cytoplasmic location of the yeast enzymes compared with the mitochondrial site in other fungi.

Studies by Smaluck (1971) on the derepression of the single NAD-glutamate dehydrogenase of Achlya by glutamate, showed that an oscillatory pattern of synthesis and degradation occurred. Each succeeding period of degradation was followed by a phase of synthesis from a higher starting point. A model for the control of NAD-glutamate dehydrogenase induction was proposed, involving relative rates of synthesis and degradation being controlled by a signal consisting of the ratio of reduced to oxidized nucleotide coenzymes.

Studies of the stability of glutamate dehydrogenases from animal sources have also indicated that degradation may play a role in control of enzyme levels. Balinsky et al (1970) reported that the enzyme isolated from frog and tadpole liver was unstable. Beef enzyme has also been found to be unstable with latent proteolytic activity (Cassman & Schachman 1971). Eisenkraft and Veeger (1970) have examined this instability in terms of the state of association of the enzyme, and the influence of allosteric effectors on this. Similar studies on allosteric interactions and the form of the enzyme have been mentioned previously for the studies on NAD-glutamate dehydrogenase of Blastocladiella emersonii by LéJohn et al (1969).

Degradation of these enzymes, and the role of effectors in varying the susceptibility of the molecules to degradation may

well have a function in regulation of enzyme levels. Schimke and Doyle (1970) have reviewed the regulation of enzymes in animal cells, and discussed the role of degradation in such regulation. For degradation to act in control of enzyme levels, the degradation process itself must come under some form of regulation. How such control is exerted is not clear, for animal or other systems.

### TRANSPORT

The existence of transport systems allows cells greater benefit from the uptake of ions and metabolites from the external milieu than would the entry of the same substances by free diffusion. Carrier molecules and energy-linked uptake processes allow uptake at a faster rate, and against concentration gradients. The specificity of the systems allows for the uptake of required molecules in preference to others available. Regulatory effects connected with the transport systems can both control the extent of uptake to meet cellular requirements, and also a signal for alterations in cellular metabolism to respond to changes in the availability of substances in the environment.

The vast field of transport studies, including associated consideration of membrane structure, has been extensively reviewed. Recent reviews have by necessity been restricted to treating only a specific area or approach to transport, such as studies on transport of one metabolite, (Oxender 1972, Ring 1970), on the transport processes of a specific organism (Scarborough 1973), on specific components of the transport systems (Pardee 1968), or on energy relationships (Mitchell 1966).

### Types of transport systems

Although some molecules may enter cells by free diffusion processes, a significant portion of biological transport takes place by mediated processes. Kinetically these systems are characterized by saturation with increased substrate concentration, specificity for the structure of the transported molecule, and susceptibility to competitive inhibition. The concept of carrier molecules in the cell membrane that can specifically combine with certain molecules and translocate them across the membrane has been used as a model to account for these characteristics (see Stein 1967).

Facilitated diffusion systems are a form of mediated transport in which the driving force is provided by a concentration or electrochemical gradient across the cell membrane. Stein (1967) had dealt with the kinetics of this process extensively, and also with two phenomena connected with it. Exchange diffusion is a consequence of a postulated carrier-substrate complex that is more mobile than the carrier alone, so that preloading cells with one substrate will increase the rate at which a second substrate can be taken up, if it shares the same uptake system, due to a faster return of the carrier to the external membrane surface when it is free (Lin 1971). In counterflow, a substrate is driven out against its own concentration gradient by the movement of another substrate down its gradient. This can be observed in energy-deprived cells,

such as those treated with metabolic poisons (Stein 1967).

Active transport processes differ from facilitated diffusion in that there is a direct link to energy reactions of the cell; metabolic energy is consumed as substrates are moved against an electrochemical gradient. Such systems may involve direct ATP expenditure such as in driving the  $\text{Na}^+ - \text{K}^+$  pump, or the uptake process may be linked to systems such as the pump, as occurs in the case of uptake of amino acids and sugars. Mitchell (1966) used the concepts of such linked transport and energy processes to explain the input of energy to transport in the chemio-osmotic theory.

An alternate mechanism is the direct expenditure of high energy compounds to phosphorylate molecules during the transport process, as is seen to occur in bacterial transport of sugars (Kaback 1970). Transport reactions such as the  $\text{Na}^+ - \text{K}^+$  ATPase and the vectorial phosphorylation of sugars distinctly involve enzymatic reactions in the transport processes. The similarity of transport systems to enzymes in terms of their specificities, and kinetic responses suggests that these systems might be viewed as special cases of enzyme function. Similarities to regulation of enzyme processes are also seen in the regulation of transport processes.

### Sulfhydryls in transport processes

Sulfhydryl groups are frequently contributors to the active sites of enzymes, and their importance in the function and structure of membranes has been reviewed by Rothstein (1970). Chemical probes can be used to obtain information regarding active components of proteins and other molecules, and the use of such techniques and the problems involved in interpretation of the results are discussed in that review.

Of particular interest are studies involving mercaptide formation, as a result of treatment with  $\text{HgCl}_2$ , or organic mercurials. The formation of the complexes is easily reversible by addition of another sulfhydryl agent, such as cysteine, and normal function may be restored if denaturation has not occurred. Organic mercurials, such as thimerosal (merthiolate), differ from inorganic  $\text{HgCl}_2$  by being monofunctional (reacting with only one ligand); by their different solubilities in water and lipid; and by their larger molecular size, which may result in slower penetration to internal sulfhydryl groups and consequent slower action (Webb 1966). Kinetics of mercurial inhibition may be complex, due to possible interactions at the active site, at sites unrelated to function, and at sites which indirectly affect function by structural modifications. Sulfhydryl groups are of interest in studies of transport as they have been demonstrated to affect sugar and cation transport, ATPase activity, and hormone binding in a number of systems (see Rothstein 1970).

### Regulation of transport

As with enzymes, regulation of metabolite uptake can occur at two levels; the first being an immediate feedback effect on an existing transport system, and the second being a slower response at the gene level, resulting in induction or repression of the synthesis of a specific transport system. Direct immediate effects on the activity of a transport system may be the result of action of a high internal concentration of the substrate or a metabolic product of it, or the response to other regulatory molecules involved in cell metabolism which interact in an allosteric fashion. Regulation at the genetic level is illustrated by the classic case of the induction of the lactose permease of E. coli in response to induction by  $\beta$ -galactoside or its analogs.

### Amino Acid Transport

#### Transport systems

Transport systems operating for amino acid uptake are of particular note because of the importance of these compounds in metabolism, and because of the structural variation among the amino acid groups. General studies of the uptake of amino acids have focused on the variety and specificity of the uptake systems,

their kinetics, regulation, energy-link for the process, and physical components of the systems. Uptake by animal cells has been reviewed recently by Heinz (1972) and will not be considered here. The systems found in bacteria have also been studied in detail and reviewed with respect to the methodology of determining initial uptake rates by filtration assays, and associated problems (Oxender, 1972).

Determination of the specific transport systems involved in amino acid uptake has involved examination of the kinetics of the uptake process and the competitive inhibition of uptake; studies on transport mutants; and physical isolation of some components of the systems, such as the transport proteins. The systems found in different species of bacteria differ to some extent in their number and specificity, but variation is also seen with different conditions of growth or induction. Based on studies of E. coli and Salmonella typhimurium, the transport systems were found to be specific for the following groups of amino acids: a) glycine, alanine, serine; b) proline; c) valine, leucine, isoleucine; d) phenylalanine, tryptophan, tyrosine; e) lysine, arginine; f) histidine; g) methionine; and h) glutamate, aspartate.

The general pattern of specific permeases found in the yeast Saccharomyces cerevisiae is very similar to the permeases found in bacteria (Grenson 1973). Studies of competition kinetics and mutants by Grenson and co-workers established the existence of

specific permeases for lysine (Grenson 1966), arginine (Grenson et al 1966), histidine (Crabeel & Grenson 1970), methionine (Gits & Grenson 1967), and dicarboxylic amino acids (Joiris & Grenson 1969). Multiple permeases are of physiological significance in allowing differential regulation of uptake of amino acids to meet specific anabolic and catabolic requirements. Operation of a single permease for all amino acids could result in one amino acid, present at high concentration, or having high affinity for the uptake system, blocking uptake of other potentially valuable amino acids. In addition, the presence of genetically different systems can be of significance in mutants impaired in the transport system for one amino acid group (Grenson 1966).

Besides the specific permeases in yeast, there is also a general permease, capable of transporting most of the amino acids (Grenson et al 1970). This system apparently acts to take up amino acids to be used in catabolism, and is the major uptake system for amino acids to be used in catabolism, and is the major uptake system for amino acids under conditions of nitrogen starvation (Surdin et al 1965). The conditions of growth used in early studies may explain why it was first thought that amino acid uptake in fungi was non-specific (see Grenson et al 1966). A similar general uptake system is found to operate in nitrogen-starved Penicillium chrysogenum (Benko et al 1967).

Scarborough (1973) has reviewed the amino acid systems found in Neurospora crassa, grouping them as AA I for aromatic and neutral amino acids, AA II for neutral and basic amino acids, AA III and AA IV for basic and acidic forms respectively, and AA V for methionine. In Penicillium chrysogenum evidence has been presented for systems for methionine (Benko et al 1967), acidic and basic amino acids (Hunter & Segal 1971), and cysteine and cystine (Skye & Segal 1970). It has been suggested that the methionine-specific transport system, which operates in addition to the methionine uptake by other systems, may have the role of scavenging sulfur, under conditions of sulfur-starvation, based on studies of Neurospora (Pall 1971) and Penicillium (Benko et al 1967). In Saccharomyces cerevisiae, methionine is also taken up by two distinct systems (Gits & Grenson 1967), indicating a similar function may occur there.

The specificity of amino acid transport in Achlya germlings has been examined by Singh (1974) using kinetic methods. This organism also apparently has two systems involved in methionine uptake, one of which is unique to methionine alone. There are eight other groups of permease systems in this organism similar to those of the previously discussed species. These are: (a) glutamate-aspartate; (b) the LIV (Leucine, isoleucine, valine); (c) PTT (phenylalanine, tyrosine, tryptophan); (d) HLA (histidine, lysine, arginine); (e) cysteine; (f) serine-threonine; (g) proline; and (h) asparagine - glutamine.

### Regulation of amino acid transport

Few amino acid transport systems occur entirely on the basis of induction, and Ring (1970) points out the significance of constitutive amino acid uptake systems in allowing immediate uptake and utilization of amino acids that may occur in the medium. Regulatory controls at the genetic level are, however, found in some microorganisms, and these may allow for increased uptake of certain amino acids when they are present at high levels in the environment. In particular, induction of scavenger permeases may increase under starvation conditions (Benko et al 1967, Surbin et al 1965). Regulatory controls acting at the genome level are also likely to be responsible for changes in transport systems observed with increasing age in Neurospora mycelium (Thwaites & Pendyala 1969, Pall 1969). Induction and repression effects have also been reported for some of the specific permeases of Saccharomyces cerevisiae (Grenson 1973), and for uptake processes in Candida utilis (Jones & Wild 1973).

Specific feedback control of the uptake system for histidine was observed in yeast by Crabeel and Grenson (1970). The effect of preloading cells with a particular amino acid on subsequent transport has been found to be specific for that particular amino acid (Grenson 1973). Grenson suggested (Grenson 1973, Crabeel & Grenson 1970) that feed-back control is a regulatory mechanism to

prevent excessive uptake of external amino acids, while an exit process functions to regulate the size of internal pools.

Inhibition of protein synthesis in bacteria does not appear to affect the uptake processes for amino acids (Oxender 1972), but in yeast and fungi, inhibitors of protein and RNA synthesis rapidly inhibit uptake (see Ring 1970). Grenson et al (1968) proposed that this was due to feedback inhibition from the increased intracellular amino acid pools when synthesis is blocked. Feedback effects involving transinhibition of amino acid transport in Penicillium chrysogenum and Streptomyces hydrogenans have been described by Hackette et al (1970) and Ring et al (1970). (Transinhibition refers to the direct effects of intracellular substrate on the permease, rather than effects of metabolites of the substrate). An alternate hypothesis for the effects of protein synthesis inhibitors on transport was that the transport permeases have a rapid rate of turnover, and so are lost when resynthesis is blocked (Elas & Rosenberg 1967, Holden & Utech 1967). These proposals were supported by experiments by Gross & Ring (1969).

Recent studies on Achlya (Cameron & LéJohn 1972 a, b) and Penicillium chrysogenum (Hunter et al 1973) have indicated that the effect of cycloheximide on transport is closely related to the role of  $Ca^{++}$  in the membrane and in transport processes. In

Achlya, although amino acid transport takes place without exogenous  $\text{Ca}^{++}$ , it was found that removal of membrane calcium by chelation or arresting calcium transport by citrate or EGTA, resulted in an immediate stoppage of amino acid transport. Addition of calcium to these deprived cells enhanced uptake of amino acids by 100%. Cycloheximide, on the other hand, inhibits RNA and protein synthesis, but amino acid transport proceeds as long as  $\text{Ca}^{++}$  transport continues. Thus the role of cycloheximide in blocking protein synthesis, and the amino acid uptake process could be separated (Cameron & LéJohn 1972 a). It was proposed that calcium is involved in various aspects of cell metabolism, including amino acid transport, membrane structure, and protein synthesis. Further studies of the role of calcium in the transport processes, using the selective inhibitor of  $\text{Ca}^{++}$  activated  $\text{Mg}^{++}$  - ATPase, ruthenium red, demonstrated the specificity of the calcium requirement in amino acid uptake (Cameron & LéJohn 1972 b). A role in linking amino acid transport with protein synthesis was suggested for calcium by these authors. Some further aspects of regulation of amino acid transport in Achlya will be discussed in Results .

Hunter and Segal (1973<sub>a</sub>) examined the cycloheximide-induced decay of amino acid transport in Penicillium chrysogenum, and the interaction of cycloheximide effects with transinhibition effects on uptake. In order to explain the actions of a wide variety of

inhibitors, a proton or charge gradient was proposed to be involved in energizing transport processes (Hunter & Segal 1973 b). These authors considered that the effects of calcium described for Achlya might be related to this gradient. Further studies on P. chrysogenum demonstrated that in this organism also, calcium was involved in amino acid transport processes, and in the effect of cycloheximide on these processes (Hunter et al 1973). Cycloheximide produced an immediate inhibition of L-leucine uptake in nitrogen- or carbon-starved cells, particularly in phosphate buffers. Added calcium reversed the cycloheximide inhibition (less successfully with increasing phosphate concentrations), but did not alter the inhibition of protein synthesis. These results support the proposal of a calcium-link in amino acid transport made by Cameron and LéJohn (1972b). Cycloheximide was also found to promote a continual uptake of calcium by the mycelium (Hunter et al 1973), and these authors suggested that cycloheximide labilizes the membrane bound calcium. In the actual transport process, the role of calcium has not been precisely defined, whether it is involved in energy processes by way of ATPase or charge gradient, or whether its importance is in the structure of the membrane or its components. However, studies of regulatory processes in amino acids transport require consideration of how calcium may be involved in such regulation.

### Nucleoside transport

In a number of organisms examined, nucleic acid bases and their derivatives are found to be transported by different mechanisms. In some cases transport systems are specific for a particular form of purine or pyrimidine; in many cases there is a close regulatory link to subsequent metabolism. Examination of the systems operating in different organisms gives some idea of the possible mechanisms and regulatory processes involved.

In animal cells, it appears that uptake occurs by facilitated diffusion (Jacquez 1962). The kinetics of the uptake process for nucleosides in human erythrocytes, studied by Oliver and Paterson (1971), showed characteristic behaviour of a mediated, facilitated diffusion system, including saturability, competitive inhibition, counterflow, and accelerative efflux. Uridine, thymidine, and inosine appeared to use the same transport system, which was distinct from those used by free bases and free sugars. Studies on chemical specificity of the system demonstrated that the uptake process was more sensitive to alterations in the sugar moiety than the base, although both deoxyribosides and ribosides could be transported (Cass & Paterson 1972). Uridine and thymidine were not phosphorylated during the uptake process.

Although the results of accelerative diffusion experiments in the erythrocytes indicated that uridine and thymidine had a

common uptake mechanism, shared by inosine, studies on other mammalian cells indicate that different systems are involved (Mizel & Wilson 1972). Nucleoside transport processes in mammalian cells have been examined with particular consideration of their regulation, because of their involvement in studies of DNA and RNA synthesis regulation. Uptake of the radioactive nucleic acid precursors has been found to be affected by some tested agents. Mizel and Wilson (1972) reported that colchicine, an antimetabolic agent, specifically inhibited nucleoside uptake by human and hamster cell lines. It had no effect on synthetic processes in the cells, nor was its effect related to other effects on the microtubules. The apparent site of action was on membrane components of the different nucleoside transport systems; components which are separate for each system but similar in function. No effect was observed in E. coli cells treated with colchicine.

Gallo and Whang-Peng (1971) found that one of the effects of the hypermodified nucleoside, isopentenyladenosine, on phytohaemagglutinin-stimulated human lymphocytes was a rapid inhibition of uridine uptake. These workers proposed a cyclic AMP role in the overall effects of isopentenyladenosine on these cells. In other cases cyclic AMP has been found to affect cell proliferation, and Hauschka et al (1972) proposed that such effects might be mediated by cyclic-AMP induced limitation of metabolite uptake.

Their studies on Chinese Hamster cells demonstrated a rapid inhibition of the uptake of thymidine and uridine in response to dibutyryl-cyclic AMP, which they attributed to changes in either the membrane or specific carriers. A second effect, more slowly expressed, on the action of thymidine kinase, could affect both the transport and incorporation events. Benedetto and Cassone (1974), in connection with the cyclic AMP effects, reported that fairly high concentrations of theophylline also could inhibit uptake by HeLa and 37RC cell lines.

Regulation of uptake processes in animal cells must be assessed in terms of the activity of enzymes involved in subsequent metabolism such as thymidine and uridine kinases, and phosphoribosyl transferases. Such enzymes act to trap nucleosides and bases that do not so easily efflux, and their activity and synthesis depends upon regulation by intracellular pools, and other physiological factors.

In some bacteria, the subsequent metabolism of purine compounds is an integral part of the uptake process, so that a single energy input is involved in transport and the first metabolic conversion. Berlin and Stadtman (1966) had observed that the activity of purine phosphoribosyltransferase was proportional to purine uptake, and suggested a role for the enzyme in regulation of uptake. In a series of papers Hochstadt-Ozer and Stadtman (1971 a,b,c,) reported that the uptake of adenine by E. coli whole cells

and membrane vesicles was PRPP dependent, and accompanied by conversion of adenine to AMP. Removal of the enzyme, purine phosphoribosyl transferase, from membrane vesicles by osmotic shock resulted in loss of uptake activity. Subsequent studies (Hochstadt-Ozer 1972) showed that nucleosides were transported by a two-step process involving first conversion to the free base by nucleoside phosphorylase, followed by uptake by the same phosphoribosylation mechanism involved in base transport. Uptake of adenine and adenosine was mediated by adenine phosphoribosyltransferase, while guanine, hypoxanthine, guanosine and inosine were substrates for a 6-hydroxypurine phosphoribosyltransferase. The location of the phosphorylase and transferase enzymes, and the kinetics of uptake suggests that these enzymes may interact in the overall transport and regulatory processes.

Uptake of purines by the E. coli system has been demonstrated to be sensitive to end-product inhibition by purine nucleotides, and to repression by a purine nucleotide derivative (Hochstadt-Ozer & Stadtman 1971 a). Phosphoribosyltransferase activity and PRPP-dependent transport have been found to be inhibited by guanosine tetraphosphate, a compound which accumulates under conditions of amino acid starvation (Hochstadt-Ozer & Cashel 1972). As guanosine and adenosine nucleotides do not accumulate in cells under those physiological conditions, this factor may be involved in an important regulatory process. Pyrimidine uptake in E. coli

has not been examined in the same extensive manner as the uptake of purines. The importance of ATP and GTP and energy compounds, and the purine basis of coenzyme compounds probably has resulted in the greater emphasis on purine uptake studies. This same distinction in functional roles may also have resulted in differences in the uptake processes and regulation of uptake for the two classes of nucleic acid precursors.

Uptake of adenine and adenosine by Salmonella typhimurium apparently is the same or very similar to the E. coli system (Hoffmeyer & Neuhard 1971). However the uptake process for adenosine in Micrococcus sodonensis is different, reflecting a bacteriostatic effect of adenosine on growth of this organism (Pickard et al 1974). The phosphorylase-phosphoribosyl transferase system did not appear to operate in M. sodonensis, probably due to lack of phosphorylase activity. Adenosine deaminase activity in membrane preparations appeared to be involved in uptake and accumulation of exogenous adenosine in the form of inosine, but the mechanism which takes up inosine - or adenosine at high concentrations- is not known.

The uptake processes for nucleosides and bases have been examined in Neurospora crassa and several yeasts. In the yeasts, particular stress has been placed on the regulatory roles of intracellular pools in limiting further uptake. Adenine uptake by Schizosaccharomyces pombe appeared to be highly specific based on

competition kinetics with purine nucleosides and nucleotides (Cummins & Mitchison 1967). Adenine taken up is accumulated in an intracellular pool, mainly in the form of inosine and AMP, suggesting a close link with the nucleoside pyrophosphorylases. This pool appears to exert regulatory control on transport, de novo synthesis, and intracellular purine conversions. A second pool of inosine was found to fill up rapidly from the main pool, and to retain its labelled substances over a long period. The function of this second pool was not clear, and it did not seem to exert regulatory influence on the metabolism of the purines.

Guanine uptake in this same yeast species was examined by Pourquie (1970) who suggested that the uptake of adenine and guanine was mediated by at least two separate systems, sensitive to different purine compounds. Adenine and hypoxanthine competitively inhibited guanine uptake, likely by action at the transport site, and also resulted in feedback inhibition following pre-filling. Pourquie and Heslot (1971) used mutant strains deficient in different steps of de novo purine synthesis to examine the intracellular purine metabolism of Schizosaccharomyces pombe. Exogenous purine mononucleotides could not support growth of mutants unable to synthesize AMP, GMP, IMP, indicating that these compounds must be degraded prior to uptake. Nucleosides can support the growth of purine requiring strains as well as the free bases can, but as they do not compete with the purines for uptake, it is likely that they

are taken up by different systems than the bases, and are not degraded prior to uptake. Growth responses of various mutants to guanine and guanosine led to the conclusion that pyrophosphorylation of guanine is a required step in utilization of the exogenous compounds, with intracellular guanosine being first converted to guanine. Unlike E. coli however, phosphoribosyl transferase activity is independent of the uptake process, which is likely regulated by intracellular pool concentrations.

It had also been demonstrated for Saccharomyces cerevisiae that the uptake and phosphoribosylation of pyrimidines were separate steps (Grenson 1969). In addition, these studies showed that uptake of cytosine, uracil and uridine was by distinct systems. Polak and Grenson (1973) further showed that in both Saccharomyces cerevisiae and Candida albicans adenine, cytosine, and hypoxanthine shared a common transport system, each competitively inhibiting the uptake of the others in kinetic studies. This argues against the occurrence of a transport system involving vectorial action of a phosphoribosyl transferase. Pickering and Woods (1972) examined purine uptake in Saccharomyces cerevisiae and reported the occurrence of at least two purine permeases, one accepting adenine and hypoxanthine, and the other, guanine and hypoxanthine. Mutations affecting transport processes were found to occur without significant differences in the phosphoribosyl transferase activities of the mutants, indicating

that utilization of exogenous purine bases is a two step process. These studies also indicated regulation of purine uptake by the intracellular purine pool.

In Neurospora crassa subsequent metabolism and intracellular pools also appear to be involved in uptake regulation (Schiltz & Terry 1970). Two systems were indicated as being involved in nucleoside uptake; one specific for purine nucleosides, and the other a common system for both purine and pyrimidine nucleosides. These systems were inhibited by NaCN and 2,4-dinitrophenol. Changes in the transport processes were observed at different stages of development, the alterations being cycloheximide-sensitive.

## CYCLIC AMP

Cyclic AMP as a Cell Regulator

Adenosine-3'5' monophosphate (cAMP) was originally referred to as a "second messenger" because of its intracellular mediation of the effects of hormones, or first messengers, which circulate in the blood stream of mammals until they reach a "target cell". The concept of intracellular mediation of hormone effects by cyclic AMP was developed by E. W. Sutherland as a result of studies on the degradation of a storage product, glycogen, in the liver and muscle of animals (see Robison et al 1971). Two hormones, epinephrine and glucagon, were known to stimulate glycogenolysis; further studies established that the activity of the enzyme phosphorylase was the control point for the hormone stimulation. Phosphorylase activity was found to be increased by the phosphorylation of a serine residue in an inactive form of phosphorylase, phosphorylase b. The enzyme responsible for transferring a phosphate group of ATP to phosphorylase b, phosphorylase b kinase, was itself activated by a phosphorylation reaction, involving a third enzyme, protein kinase. Two facts linked cyclic AMP to this system. First, the activity of the protein kinase enzyme starting the sequence of

activations was found to be dependent on cyclic AMP; and second, the hormone acting on the target cell was found to increase the level of cyclic AMP within the cell. Sutherland thus proposed that the concentration of cyclic AMP within the cell increased as a result of hormone action on a membrane-bound enzyme, adenylyl cyclase, and that the increased cyclic AMP mediated the hormone effect by activation of various processes or enzymes within the cell (see Pastan & Perlman 1971, Jost & Rickenberg 1971, Walsh et al 1970).

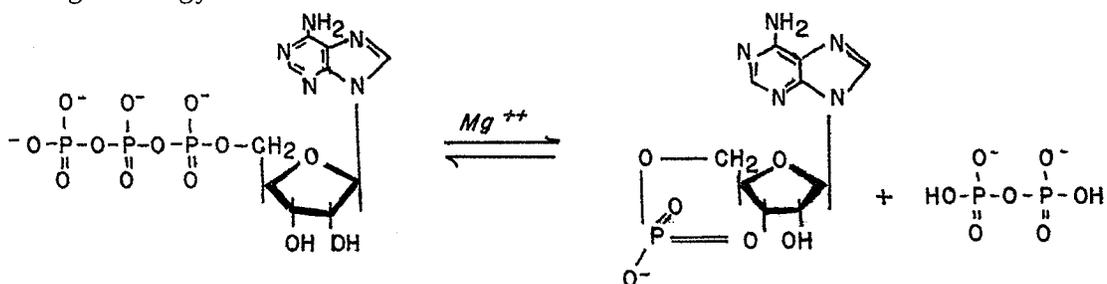
Other hormones acting in other tissues and reactions also showed cyclic AMP involvement, and Sutherland developed criteria which could be used to determine if a hormone effect was mediated by cyclic AMP. In such a cyclic AMP mediated process, the hormone should increase the levels of cyclic AMP in the tissue, the adenylyl cyclase activity should be increased in the presence of the hormone, and finally, cyclic AMP added to the tissue should cause the same physiological effects as addition of the hormone (see Pastan & Perlman 1971).

Cyclic AMP has been shown to be involved in many events in animal cells, including response to other hormones than those of the glycogen system, neurotransmitter function, histone phosphorylation, permeation and secretion regulation, muscle contraction, and control of cellular morphology, proliferation, and transformation (see Pastan & Perlman 1971, Robison et al 1971). In light of these

varied effects, the mechanisms by which cyclic AMP levels are controlled are of importance, as are the mechanisms by which a specific effect can be produced.

### Cyclic AMP enzymes

Adenylyl cyclase: Sutherland's group first described the reactions and properties of the enzymes adenylyl cyclase (Sutherland, Rall & Menon 1962) and phosphodiesterase (Sutherland & Rall 1958). Cyclic AMP is synthesized by adenylyl cyclase, with formation of a high energy bond as follows:



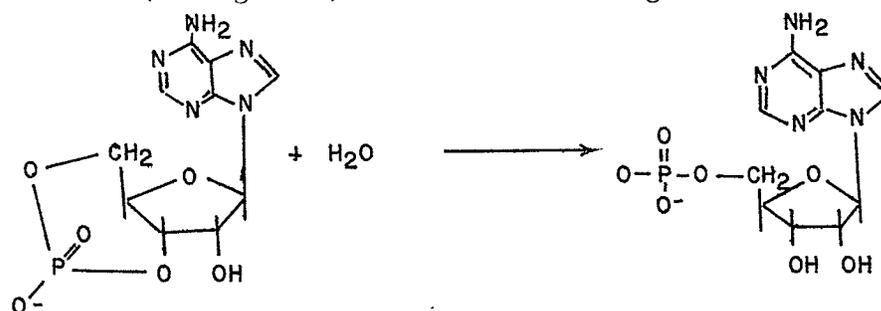
Studies on cyclic AMP production show that the free energy of the 3' bond is about 1.6 kcal greater than the energy available from hydrolysis of the  $\gamma$ - $\beta$  bond of ATP (Greengard & Kuo 1970). Thus the reaction synthesizing cyclic AMP should be reversible, which has been confirmed, and cyclic AMP should be highly reactive under certain conditions. Unlike other high energy compounds, however, cyclic AMP is a relatively stable molecule (Posternak 1971). The

fate of the pyrophosphate split from ATP in the reaction under cellular conditions is not known. It may be accepted by some intracellular compounds, or acted on by pyrophosphatase. The only cofactor required for the reaction in most cases is magnesium, which may be replaced by manganese. Calcium not only cannot replace magnesium, but also inhibits activity (Robison et al 1970). Other specific effectors may act on the enzymes of specific tissues. Hormones which increase cyclic AMP levels in vivo also are found to stimulate. Fluoride stimulates adenylyl cyclase activity of most mammalian enzymes, and a mechanism for this stimulation involving displacement of phosphate from an inactive phosphorylated form of the enzyme has been proposed by Constantopoulos and Najjar (1973).

Hormones which increase cyclic AMP levels in vivo are also found to stimulate adenylyl cyclase in broken cell preparations of the tissue. The enzyme is almost always associated with particulate fractions, usually the plasma membrane (Davoren & Sutherland 1963). This is important in considering its role in response to hormone interactions at the cell surface. Solubilization of mammalian adenylyl cyclase by detergent treatments cause a loss or reduction in its sensitivity to hormone action (Robison et al 1970). Due to its close association with the cell membrane, and its lability, the enzyme has not been well characterized by size or protein structure.

Birnbaumer (1973) has reviewed experiments on isolated membrane-bound adenylyl cyclase systems, which have provided information regarding the effects of hormone signals on activity of the adenylyl cyclases. The hormone receptor sites located on the outer surface of target cells are bifunctional in that they receive a specific hormone signal and produce a response to it. The cellular response to hormone signals is reversible, and requires sustained interaction of the hormone and receptor to maintain it. Experimental evidence described by Birnbaumer has shown that tissues responding to more than one hormone possess different receptors specific for each hormone, but all the hormone interactions result in activation of the same adenylyl cyclase component. Thus the coupling or linking of the hormone-receptor interaction to the adenylyl cyclase activity is of importance in the interaction of hormones with cells. The mechanism of this coupling process is not known, but it has been determined that there is an active response of the hormone-receptor involving a number of interactions by different purine compounds, such as GTP, AMP, and adenosine; cations; and phospholipid components of the membrane. Modulation of the hormone response can be affected by factors affecting this coupling, and by the overall metabolism of the cell itself.

Phosphodiesterase: Degradation of cyclic AMP by the action of phosphodiesterase involves specific hydrolysis of the 3'5'-diester bond (Cheung 1970) as in the following reaction:



The fact that specific inhibitors of phosphodiesterase, such as the methylxanthines, caffeine and theophylline, act to increase cellular cyclic AMP levels suggests that phosphodiesterase is involved in in vivo regulation of cyclic AMP levels. As the enzyme occurs in the same tissues as adenylyl cyclase, and as it is a stable enzyme with a relative activity much greater than that of adenylyl cyclase (see Cheung 1970), then for cyclic AMP to accumulate in the cell degradation activity must be controlled. This may work by compartmentalization of the enzymes of synthesis and degradation, or cellular conditions which favour adenylyl cyclase activity may inhibit phosphodiesterase long enough to build up cyclic AMP levels.

Purification procedures carried out on the phosphodiesterase activity indicated that multiple forms of the enzyme occurred in some tissues. These various activities were associated with enzyme fractions with different  $K_m$  values, different molecular weights,

and different inhibition characteristics (Thompson & Appleman 1971). These variations may be a result of different subunit structures for the enzyme. Purification of phosphodiesterase from rat brain tissue and Escherichia coli has shown that one or more components are involved in enzyme structure, and removal of such a component results in activity loss. Addition of the component, found to be a protein, re-activates the enzyme (Cheung 1971). As phosphodiesterase reacts to a wide variety of inhibitors, such as methylxanthines, ATP, pyrophosphate, citrate, organic acids, and stimulators, such as ammonium ion and imidazole, then the interaction with regulatory sub-units is a subject for considerable speculation (see Cheung 1971).

Protein kinases: The major characteristics of adenylyl cyclase and phosphodiesterase are the same regardless of their source. How then can cyclic AMP levels result in the message of a specific hormone producing a specific physiological change in a cell or tissue? Cyclic AMP -dependent protein kinases similar to those mediating hormone action in the glycogen system are found in many tissues, and it was postulated that these enzymes provided the link to specific reactions. Kuo & Greengard (1969) examined protein kinases from a wide variety of sources, to determine if the specificity of a cyclic AMP response depended on the type of kinase activated, and if the multiple protein kinases in a tissue mediated different responses to different stimuli. Such studies have not yet provided clear answers to these questions. The kinases of fifteen different types of bovine tissue had many properties in

common, but they also showed variations which could be instrumental in determining reaction specificity (Greengard & Kuo 1970). Some tissues appeared to have multiple kinase activities. In some cases distinct cyclic AMP and cyclic GMP stimulated kinase activities could be detected in the same tissue. One of the kinases of lobster tail is activated by high cyclic AMP and low cyclic GMP, while the second kinase reacts under the reverse conditions (Kuo & Greengard 1970a).

Protein kinase activity is routinely tested by assaying an extracts' ability to phosphorylate some relatively non-specific substrate, such as casein or histone. During purification steps however, the ability to react with one substrate may be lost while reactions with the other are undiminished. This would suggest that there is some difference in substrate specificity among the kinases in the cell (Greengard & Kuo 1970). Cyclic AMP dependence of the kinase activity of rat skeletal muscle could be reduced or eliminated during DEAE-column chromatography (Reimann, et al 1971). A model has been proposed which could account for different peaks of activity detected during purification and the different substrate specificities involved in cyclic AMP activation of protein kinase activity (Reimann et al 1971, Tao et al 1970, Gill & Garren 1971). Cyclic AMP activation of protein kinase appears to produce a decrease in the sedimentation coefficient of the enzyme suggesting a dissociation is involved (Garren et al 1971). The model proposes

that the protein kinase consists of a catalytic subunit (C) and a cyclic AMP receptor, regulatory subunit (R). Binding of cyclic AMP to R would cause dissociation of the cyclic AMP-R complex, leaving an active catalytic subunit. Strong evidence for this model is the fact that activation of the kinase produced a decrease in the sedimentation coefficient, indicating a dissociation occurred, and the fact that added receptor protein could reverse the cyclic AMP activation. Further separation studies indicated that several different R subunits might associate with the C subunit (Thompson & Appleman 1971, Corbin & Brostrom 1971), which could account for different enzyme characteristics. A fraction (F) that inhibits cyclic AMP-dependent protein kinase while stimulating cyclic GMP-dependent activity has also been reported (Kuo & Greengard 1969).

This model could explain how an increase in intracellular cyclic AMP concentration could activate a particular protein within the cell. However, control over cell reactions also requires that the kinase respond as rapidly to a drop in cyclic AMP levels. In the model described, cyclic AMP must be removed from the R subunit in order that it might reassociate with the catalytic subunit. This might be achieved if the cyclic AMP bound to the subunit was very susceptible to phosphodiesterase action. However, experiments showed that in actual fact the phosphodiesterase activity of bovine muscle was inhibited by the addition of purified protein kinase to

the reaction mixture (O'Dea et al 1971). Inhibition was found to be due to the inability of phosphodiesterase to hydrolyze the bound species of cyclic AMP, so cyclic AMP apparently must dissociate from the complex to be hydrolyzed. As the concentration of free cyclic AMP decreases as a result of hydrolysis, cyclic AMP must dissociate from the complex to maintain an equilibrium. As the free concentration decreases, then the amount of bound cyclic AMP will also decrease, and thus so will the amount of active enzyme subunit.

#### Cyclic AMP assays

The effect of cyclic AMP on protein kinase, and the binding of cyclic AMP by a regulatory subunit of this enzyme, has been used as the basis for some cyclic AMP assay methods. Kuo and Greengard (1970b) used activation of the histone phosphorylation reaction of protein kinases as an assay method, while Gilman (1970) and Walton and Garren (1970) used the competition between endogenous cyclic AMP and added radioactive cyclic AMP for binding to protein kinase molecules as a measure of cyclic AMP in tissue preparations. Competition for binding of endogenous protein preparations has also been used by Brown et al (1971) with extracts from bovine adrenals, and by Sy and Richter (1972b) with yeast binding protein. Competition for binding of a prepared antibody is the basis of the radioimmunoassay of Steiner et al (1969).

Conversion of cyclic AMP to its adenine nucleotide derivatives, and subsequent coupling to enzymatic reactions (Goldberg et al 1972) or to the firefly luminescence reaction (Johnson 1972) have also been used as assays. Purification of cyclic AMP from an extract and subsequent physical identification has also been described (Brooker 1972, Krishna 1968). Konijn (1970) used Dictyostelium attraction as a bioassay of cyclic AMP, while changing levels of cyclic AMP in other tissues have been monitored by pre-labelling with radioactive adenine, and determining changes in the specific activity of isolated cyclic AMP (Kebabian et al 1972).

#### Effects of calcium

The importance of calcium ion and its requirement for many cellular responses to cyclic AMP has been documented by Rasmussen (1970), who proposed that this ion is an integral part of the basic cyclic AMP control system. By linking cyclic AMP and calcium, as two interrelated intracellular messengers, the regulation system can be extended in scope, to include reactions of systems in which calcium has a significant effect. Such reactions include  $\text{Ca}^{++}$ -activated ATPase function, membrane changes between the resting or calcium-associated state and the active or calcium-dissociated state, secretion events, and muscle and microtubule functions (see Rasmussen 1970). The model proposed for operation of the adenylyl cyclase control system is essentially the same whether the cell excitation occurs by a chemical signal, as in the case of hormones, or by

electrical impulses, as in the case of nervous system function. Excitation produces increased adenylyl cyclase activity, and thus increased intracellular cyclic AMP levels. The increased cyclic AMP levels activates protein kinases which in turn phosphorylate various cellular proteins, as well as possibly having other effects such as allosteric enzyme activation. The calcium concentration in the cell also increases in response to the excitation event, by cyclic AMP mobilization of intracellular calcium, and by the direct effect of the primary stimulus on the cell membrane and the  $\text{Ca}^{++}$  - ATPase pump. Increased calcium concentrations may act on cellular reactions, either of the newly phosphorylated proteins, or of other independent proteins. It may also produce a feedback effect on adenylyl cyclase.

The diverse response of cells to this basic regulatory system are explained in terms of levels or orders of specificity, involving the type of signal, and its reception; the characteristics of the specific cell type responding; and the manner in which the cell responds to the signal. The significance of calcium in response to hormone signals has been described by Rasmussen (1970), and particular note can be made of the cases in which hormone action may produce increased cyclic AMP levels, but no physiological effect is observed due to lack of calcium. The importance of calcium in cyclic AMP responses in the lower organisms will be discussed later in connection with the slime

molds, where both cyclic AMP and  $\text{Ca}^{++}$  are implicated as extra-cellular messengers.

### Cyclic GMP

Cyclic GMP has been found to occur in all living tissues, usually at a concentration one-tenth to one-fiftieth that of cyclic AMP in the tissue (see Hardman 1971). Hormone-associated changes in cyclic GMP levels have been demonstrated in some cells, and the effects of cyclic AMP on some protein kinases has been noted earlier (Kuo & Greengard 1970a). Workers in Goldberg's laboratory (Hadden et al 1972) have proposed that the opposing effects of the two nucleotides observed in certain systems, such as muscle contraction-relaxation, proliferation-contact inhibition of cells, and glycogen synthesis-breakdown, are examples of a regulation mechanism for "bidirectional controlled" systems. An example of such regulation was observed in mouse fibroblasts, where it was seen that a variety of cyclic AMP-induced effects could be overcome by the addition of cyclic GMP (Kram & Tomkins 1973).

### Cyclic AMP in the bacteria

The demonstration of cyclic AMP occurrence in E. coli by Makman and Sutherland (1965) extended the possible scope of the messenger function of this molecule to include the bacteria as

well as mammalian tissues. Those studies showed that the level of cyclic AMP in the bacterial cell reflected the energy level of the cell. High concentrations of cyclic AMP were found in starved cells, but the level rapidly dropped with addition of glucose to the medium.

The presence of glucose prevents the expression of genes for certain inducible enzymes - the phenomena known as transient repression or catabolite repression (Magasanik 1961). Thus the effect of cyclic AMP on these systems was examined. It was found that addition of cyclic AMP could prevent the repression of inducible enzymes such as  $\beta$ -galactosidase by glucose. The action of cyclic AMP was specific for these enzymes as total RNA and protein synthesis were not stimulated and only the production of inducible enzymes increased (Perlman & Pastan 1968). Evidence that confirmed the involvement of cyclic AMP levels in regulation of inducible enzyme production came from studies involving adenylyl cyclase-deficient mutants of E. coli (Perlman & Pastan 1969). Non-lactose-fermenting mutants were screened for the presence of adenylyl cyclase and cyclic AMP in the cell. Addition of cyclic AMP to mutants in which neither was detected was found to permit the synthesis of the lactose-utilizing enzymes. Other inducible enzyme deficiencies also occurred in these particular mutants, indicating a general control over induction by cyclic AMP. Naturally occurring revertants of these mutants, able to grow on lactose, were found to have regained adenylyl cyclase activity (Pastan & Perlman 1970).

The lac system has been well characterized with regard to controls, and thus was a useful experimental system for the study of cyclic AMP action on induction of enzymes. For enzyme productions a purified cell-free lac system was found to require the presence of cyclic AMP and a cyclic AMP receptor protein in addition to the specific lac repressor and inducer. The requirement for the cyclic AMP receptor protein (CRP) (Emmer et al 1970) or the Catabolite Gene Activator Protein (CAP) (Zubay et al 1970) was demonstrated in mutants unable to produce the lac enzyme despite normal cyclic AMP production. Preparations of a protein with high affinity for cyclic AMP from other cells could be added to the extracts of mutant cells, with a resulting normal enzyme production (Perlman & Pastan 1969). That cyclic AMP acted on the transcription process was indicated by the specific increase in lac mRNA in the stimulated systems. Further investigations with the cell-free system using the antibiotic rifampicin, a specific inhibitor of the initiation process of transcription, demonstrated that the cyclic AMP effect involved initiation. Mutants altered in the lac promoter site showed reduced or lost sensitivity to cyclic AMP (Perlman et al 1969). The requirement for an intact promoter site for cyclic AMP action is consistent with its involvement in initiation as the promoter is the binding site for RNA polymerase, so that CRP binding to the promoter site of DNA would aid the binding of RNA polymerase. Interaction of cyclic AMP with CRP increases its affinity for DNA,

and thus increases RNA polymerase binding (de Crombrughe et al 1971). By influencing the efficiency of initiation, the concentration of cyclic AMP in the cell sets the maximum rate for operon response to its specific inducer. As cyclic AMP levels are related to the energy state of the cell, then cyclic AMP in this system acts as a "second messenger" by controlling the amount of enzyme to be synthesized in response to a primary, specific inducer signal for the operon.

In an analogous fashion, the bacterial cell's cyclic AMP level can be used by infecting phage particles in order to determine if the energy reserves of the host cell are sufficient to support the lytic growth cycle. High cyclic AMP levels, indicating low energy or glucose concentrations, appear to direct the RNA polymerase to transcribe the phage lysogenic genes. Adenyl cyclase deficient mutants of Salmonella typhimurium were found to be more readily lysed by phage P22 in response to low cyclic AMP levels in the cell. Addition of cyclic AMP reversed this effect in mutants (Hong et al 1971).

#### Cyclic AMP in plant systems

Cyclic AMP has been demonstrated to occur in bacterial and animal cells, and to play a regulatory role in a variety of reactions in these cells. Examination of plants produced greater difficulties

in demonstrating the occurrence of the compound and providing evidence of its function. In examining a possible connection between plant hormones and cyclic AMP function, the complex interactions of the plant hormones themselves make the role of cyclic AMP difficult to assess. To consider in some detail the function of cyclic AMP in plants and fungi, some of the evidence regarding the occurrence and action of the compound is considered here.

#### Occurrence in plants

In early studies of cyclic AMP and adenylyl cyclase distribution, activity could not be demonstrated in plant tissues. (Sutherland et al 1962). Neither could cyclic AMP activated protein kinases be detected in the surveys by Kuo and Greengard (1969). However, occurrence of these enzymes and cyclic AMP was not completely ruled out, as the assay systems used might not have been suitable for detection. Several workers have described the incorporation of radioactive adenine into compounds behaving similarly to cyclic AMP in chromatographic systems. Such evidence was presented for the occurrence of cyclic AMP synthesis in lettuce seedlings (Narayanan et al 1970, Pradet et al 1971), in Avena coleoptiles (Salomon & Mascarenhas 1971), and barley aleurone layers (Pollard 1970). Such evidence is subject to various criticisms, and presents problems in assessing actual levels. Thus various other methods have been used to attempt to determine cyclic

AMP concentrations in plant tissues.

The results of the lettuce seed assays were re-examined by Raymond, Narayanan and Pradet (1973), using both the protein kinase activation assay and the bioluminescence assay. Both assays gave similar results for the lettuce seeds and for tissue of higher plants tested, generally being in the range of 33 to 235 pmoles per mg nitrogen. Salomon and Mascarenhas (1972) also have re-examined the results for Avena coleoptiles, using Steiner's antibody binding assay. They reported that the concentration of cyclic AMP detected increased rapidly on auxin treatment. Brown and Newton (1973) examined the levels in Phaseolus vulgaris, using a series of extensive chromatographic and electrophoretic procedures to purify cyclic AMP from extracts, followed by spectrophotometric concentration determinations. They reported initial levels of 3 to 9 nmoles per gram of dry weight in six day old plants, but found that the levels dropped greatly as the plants matured.

In other cases also, the levels of cyclic AMP detected have been found to vary with certain treatments or conditions. In Avena etioplasts, Wellburn et al (1973) reported that the beef binding protein assay showed levels of greater than 80 pmoles per mg protein, with the levels increasing upon illumination. Bachofen (1973) used the same assay to demonstrate a gradient of concentration, decreasing from the tip down the coleoptile of maize seedlings. Radioimmunoassays of Jerusalem artichoke tubers demonstrated a drop from 60 pmoles per gram of fresh tissue to 25 pmols per gram with the onset of sprouting (Giannattasio et al 1974). The Gilman assay of soybean

callus tissue and Avena coleoptiles was reported to show levels of 10 to 20 pmols per gram of tissue (Brewin & Northcote 1973 a). However, the levels were found to be significantly affected by the constituents of the soybean callus medium, by subculturing procedures, and by treatments with auxins and cytokinins. Intracellular levels of cyclic AMP in tobacco pith tissues were found to be auxin controlled, when examined by the radioimmunoassay and the cyclic AMP-protein kinase activation test (Lundeen et al 1973). The levels were related to other auxin-regulated cellular events, such as cell-enlargement and DNA replication, but the relationships between the events and cyclic AMP concentrations and auxin are not known. Kinetin was in this case reported to have little effect on cyclic AMP levels. Cyclic GMP levels in these tissues were also measured, and found to remain essentially constant.

Certain other reports still indicate failure to find significant levels of cyclic AMP in plants. Alvarez et al (1974) reported that levels detected by the Gilman and the Wastila kinase binding assays were insignificant, or close to the sensitivity limit of the assay.

Direct comparisons of the levels of cyclic AMP found in the various tissues is not easy, as the various reports express values in terms of dry weight, wet weight, protein or nitrogen content of the tissues. Also from indications in some assays, the age of the plant, the specific tissue tested, the growth conditions and

growth hormones, and the species of the plant will all produce variations in the cyclic AMP content compared with other assays. Some such factors may also account for failure to detect the compound in some cases.

Another area investigated for possible evidence that cyclic AMP levels may function in plant regulation, are studies to detect the presence of enzymes which can control the production and degradation of the substance. Despite the earlier failure to detect adenylyl cyclase (Sutherland et al 1962), some subsequent reports indicate that it does occur in plants. Wood et al (1972) demonstrated cyclic AMP synthesis activity associated with the membrane and cell wall fractions of both normal and tumour cells of Vinca rosea. Adenylyl cyclase activity in maize coleoptile preparations was reported to be enhanced by indoleacetic acid (Janistyn 1972). The activity in Jerusalem artichoke tubers was demonstrated to be sensitive only to enhancement by gibberellic acid, not to other plant hormones or NaF (Giannattasio & Macchia 1973). Alvarez (1970) reported finding a soluble, NaF-activated adenylyl cyclase activity in barley seeds, the synthesis and secretion of which was under control of gibberellins and abscisic acid. It was, however, later reported that the presence of fluoride in the assay system of barley seed extracts could produce adenosine 5' phosphofluoridate, which may interfere in the assay (Alvarez et al 1974). Authentic cyclic AMP production was not detected in barley seeds.

Cyclic AMP phosphodiesterase activity was reported in barley seeds and carrot leaves (Pollard & Venere 1970, Pollard 1971), and in pea seedlings (Lin & Varner 1972). The pea seedling enzyme could hydrolyze both the 2'3'-cyclic AMP and the 3'5'-AMP forms, producing the 3'-nucleotide rather than the 5'-form produced by animal enzymes. Similarly, the enzyme activity detected in barley seeds was demonstrated to be non-specific (Vandepeute et al 1973), and this has suggested that the major function of this enzyme is in RNA degradation rather than specific cyclic AMP regulation. The product of the phosphodiesterase activity in Vincia rosea cells however, was 5'AMP (Wood et al 1972). Electrofocusing and inhibitor experiments suggest that two cyclic AMP phosphodiesterases may occur in these tissues.

Cyclic AMP phosphodiesterase activity was reported to occur in soybean callus (Brewin & Northcote 1973b), and in Jerusalem artichoke tubers (Giannattasio et al 1974). The activity of this last enzyme was found to increase with breaking of dormancy, correlating with altered cyclic AMP levels. Both these reports suggest that failure to detect distinct hormone-type effects from exogenous cyclic AMP may be due to extensive degradation of the compound either before or after its entry. Shimoyama et al (1972b) reported a cyclic AMP phosphodiesterase inhibitor in potato, and a nicotinamide effect on activity of the enzyme (Shimoyama et al 1972a).

Cyclic AMP and plant hormones

Despite problems with the assay systems, and doubts about enzyme specificity, there does appear to be reasonable evidence to indicate that cyclic AMP occurs in plant tissues. The attempts to demonstrate a significant function or effect, analogous to the roles of cyclic AMP in animals and bacteria, have met with equal or greater obstacles than those found in attempts to demonstrate the presence of the compound.

A suggestion of a link between plant hormone action and cyclic AMP came from the altered levels or enzyme activities found upon treatment of various tissues with auxins ( Brown & Newton 1973, Salomon & Mascarenhas 1971, Janistyn 1972, Azhar & Krishna Murti 1971), gibberellins (Pollard 1970, Giannattasio & Macchia 1973), and cytokinins (Brewin & Northcote 1973a). Sutherland's concept of the cyclic AMP second messenger, and his criteria for cyclic AMP involvement in a response, require that cyclic AMP mimic the effect of the hormone. In the plant systems studied, this has proven to be a difficult condition to meet, either because of differing and interlocking hormone effects, or because of degradation problems with exogenously supplied cyclic AMP.

One of the most frequently cited examples of possible cyclic AMP involvement in plant hormone action is the gibberellin-induced  $\alpha$ -amylase release observed in barley endosperm. Secretion of such hydrolytic enzymes was also found to be enhanced by cyclic AMP ( Dufus & Dufus 1969 , Galsky & Lippincott 1969). However,

studies by Keates (1973) indicated that gibberellic acid does not significantly increase the cyclic AMP content of barley aleurone layers, and he has stated that the cyclic AMP levels found at optimal gibberellic acid concentration are too low to be acting as a hormone amplification mechanism. Cyclic AMP also had been implicated in auxin and gibberellin effects of cell expansion in Jerusalem artichoke (Kamisaka 1972), and in gibberellin promotion of hypocotyl elongation in lettuce seedlings (Kamisaka et al 1972). However, Kamisaka and co-workers have subsequently demonstrated that cyclic AMP does not directly substitute for these hormones, and appears to have a different effect or mechanism. Cyclic AMP did not act like gibberellic acid in induction of lettuce seed germination, but acted synergistically at sub-optimal concentrations of the hormone (Kamisaka & Masuda 1971). Similarly, in the expansion of artichoke tuber slices, cyclic AMP has a potentiating or enhancing effect with auxin, and an additive effect with gibberellic acid and kinetin, but no growth-promoting effect alone (Kamisaka et al 1973). In elongation studies of Avena coleoptile segments, Cline et al (1974) reported that cyclic AMP and dibutyryl cyclic AMP had little or no effect compared with auxin, despite reports by Salomon and Mascarenhas (1971) that auxin treatment increased cyclic AMP synthesis, suggesting a hormone response.

Hartung (1973) suggested that sugars played an important role in this elongation process due to cell turgor, and that

cyclic AMP might act as a second messenger of auxin or gibberellin by inducing certain invertases and permeases.

In a number of other systems, cyclic AMP has been reported to have an effect like that of plant hormones. Exogenous cyclic AMP was reported to have an auxin-like effect in delaying petiole abscission in Coleus (Salomon & Mascarenhas 1971) and in stimulating tryptophan oxygenase synthesis (Azar & Krishna Murti 1971). Goldthwaite (1974) reported that cyclic AMP, dibutyryl cyclic AMP, and theophylline mimic the effect of gibberellin in senescence inhibition in leaf discs of Rumex obtusifolius. Other nucleotides, particularly 3'AMP, were also effective, and a feedback effect on adenylyl cyclase or phosphodiesterase was proposed. In view of the results of careful study of similar effects, these reports of cyclic AMP mimicing hormone effects in total, must be regarded with some caution.

The effects and possible function of cyclic AMP may be made more difficult to judge if the event being examined is affected by a number of control systems. Rast et al (1973) reported that cyclic AMP acted to stimulate betacyanin synthesis in etiolated Amaranthus paniculatus seedlings, but did not completely replace phytochrome. These workers concluded that cyclic AMP could replace the absolute requirement for light in betacyanin synthesis. In connection with light effects, Janistyn (1972) reported an increase in cyclic AMP levels following irradiation with red light, and

Wellburn et al (1973) observed similar increases with illumination of Avena etioplasts. A complex system for control of chlorophyll synthesis involving phytochromes, gibberellins, and cyclic AMP has been proposed by Holm and Miller (1972).

The manner in which cyclic AMP could act as a hormone mediator, either as a second messenger or as a potentiating factor, remains unclear. Cyclic AMP appears not to act by activation of protein kinases of plants (Kuo & Greengard 1969 , Keates 1973), although the protein kinases in yeasts, which do contain endogenous cyclic AMP, also are not cAMP activated (Sy & Richter 1972 a). Some evidence involving required synthesis for cyclic AMP effects in plants has been reported. Metabolic inhibitors such as 6-methylpurine, cycloheximide, and abscisic acid, depressed the gibberellin and cyclic AMP induction of acid phosphatase in barley endosperm (Gilbert & Galsky 1972). For the artichoke tuber expansion system, the possibility has been raised that cyclic AMP might act to make the tissue more sensitive to auxin effects in a manner similar to gibberellic acid, by stimulating RNA synthesis (Kamisaka et al 1973). A cyclic AMP stimulation of RNA synthesis has been reported for Avena chromatin, duplicating the auxin-stimulated synthesis in coleoptile sections (Salomon & Mascarenhas 1971, 1972). In other species, cyclic AMP has been shown to stimulate rRNA synthesis in isolated chloroplasts of Euglena gracilis (Keirns et al 1973, Carritt & Eisenstadt 1973), and in Dictyostelium

discoideum (Farrell & De Toma 1973).

A role for cyclic AMP in regulation of cell division processes in plant cells has been proposed by Wood, Braun and co-workers. Phosphodiesterase activity in normal and crown gall Vinca rosea tissues was inhibited by cytokinesins, or cell division factors (Wood et al 1972). A degradation-resistant form of cyclic AMP, 8-bromo-adenosine 3'5' cyclic monophosphate, was found to replace the requirements for either a cytokinin or cytokinesin in tobacco pith tissue, suggesting that the effects of these hormones were cyclic AMP-mediated (Wood & Braun 1973). Dekhuijzen and Overeem (1972) had reported that dibutyryl-cyclic AMP could replace cytokinin for soybean callus growth, but attributed its action to the N<sup>6</sup>-butyryl side chain, as the ribosyl 3'5' cyclic monophosphate group was not required for butyryladenine to function. Use of either 8-bromo-cyclic AMP, or cyclic AMP with theophylline, acted to induce tracheary element differentiation in lettuce discs, mimicing the effect of  $\beta$ -indoleacetic acid and kinetin (Basile et al 1973). A relationship was observed between intracellular levels of cyclic AMP and cell division events in tobacco pith tissues induced to divide by treatment with both auxin and kinetin (Lundeen et al 1973). Such studies suggested that cyclic AMP is involved in regulation of cell division of plant cells, acting as a promoter or suppressor in a manner reflecting environmental as well as cellular events. In another

study, examination of cyclic AMP levels in normal and crown gall tissues showed no correlation between the levels and growth rates (Drlica et al 1974), unlike the suppressed synthesis observed in transformed mammalian cells. However, it was suggested that small transitory changes could remain undetected in the asynchronously dividing cells. Another report has indicated that exogenous cyclic AMP can inhibit the development of crown gall tumours induced in bean leaves (Babula & Galsky 1974).

#### Cyclic AMP in *Euglena*

The possibility that cyclic AMP might be involved in the switch between heterotrophic and photosynthetic modes of growth in *Euglena gracilis* has been considered in view of other observed relationships between cyclic AMP and light (e.g. betacyanin production, photoreceptors in the retina). Keirns et al (1973) reported the occurrence of a cellular, membrane-associated adenylyl cyclase, a soluble phosphodiesterase in the chloroplast, and a cyclic-AMP-stimulated protein kinase, as well as high intracellular levels of cyclic AMP, all of which suggest that cyclic AMP has a function in this organism. These workers have proposed that cyclic AMP is involved in the development of chloroplasts, despite the failure to demonstrate altered cyclic AMP levels with light-dark treatments, or medium changes, and failure of exogenous cyclic AMP to produce

chlorophyll development. The 40% stimulation in rRNA synthesis seen in isolated chloroplasts suggested that the cyclic AMP target might be the transcription process (Carritt & Eisenstadt 1973).

#### Cyclic AMP in the Fungi

The occurrence of cyclic AMP and the related enzymes of cyclic AMP metabolism has been reported for a variety of fungi. In addition, exogenous cyclic AMP has been demonstrated to have effect on the metabolism and morphogenesis of various species of fungi.

The classic case of cyclic AMP involvement in regulation in the fungi occurs in the aggregation and differentiation of the slime molds, Dictyostelium discoideum and Physarum polycephalum (Konijn 1972). In some slime molds, cyclic AMP plays the role of extracellular messenger or hormone, in order to induce aggregation of amoeboid cells. This chemotactic activity occurs only in species in which aggregation operates over a wide territory. Cyclic AMP also is found in species which do not respond to it by aggregation, indicating that it may function intracellularly in these species.

The amoebae of Dictyostelium discoideum were known to aggregate in chemotactic response to acrasin, secreted by clumps of cells or aggregation centres of amoebae, where differentiation occurred to produce a stalked fruiting body (see Bonner 1971). The

amoeba were also known to contain an acrasin-destroying enzyme. Studies of acrasin identified it as cyclic AMP, and the acrasinase was shown to be phosphodiesterase (Konijn et al 1967). Exogenous cyclic AMP in experimental systems inhibited the synthesis of cyclic AMP from radioactive precursors (Mason et al 1971). Thus a feedback system could operate during aggregation so that when one amoeba begins to secrete cyclic AMP, secretion by neighbouring cells will be inhibited, allowing centre formation. A related species of slime mold, Physarum polycephalum, was also shown to secrete cyclic AMP.

If amoebae aggregation depends on cyclic AMP, the slime molds must have protection against exogenous cyclic AMP sources interfering with cell regulatory mechanisms. Control may be maintained by the secretion into the medium of a phosphodiesterase that hydrolyzes the cyclic AMP (Riedel & Gerisch 1971, Murray et al 1971) . Physarum has been shown to contain two distinct phosphodiesterase activities (Chang 1968) with different properties. One appears to be particulate, probably involved with control of intracellular cyclic AMP levels. The other enzyme, which has considerably greater stability, is released into the growth medium for extra-cellular function. Dictyostelium also releases an extracellular phosphodiesterase, and recent studies have suggested that an inhibitor of phosphodiesterase is released prior to aggregation ( Riedel et al 1973 ) . Mutants lacking this inhibitor do not aggregate, suggesting that development might be triggered by the

increased extracellular cyclic AMP levels resulting from phosphodiesterase inhibition (Riedel & Gerisch 1971).

Calcium ion was found to play a major role in the aggregation of Dictyostelium discoideum. If  $\text{Ca}^{++}$  was absent, cyclic AMP could be produced, but no aggregation would occur. Between  $10^{-4}$  and  $10^{-6}$  M  $\text{Ca}^{++}$  concentration (varied by an EGTA buffer system), aggregation occurred as a function of calcium concentration (Mason et al 1971). It was observed that addition of cyclic AMP to the cells resulted in an outflow of labelled calcium ions from the cells (Chi & Francis 1971). Considering the relationship between cyclic AMP, calcium efflux, and aggregation movement, it is possible that the sudden change in calcium ion concentration within the cells might result in changes in the calcium-stimulated contractile systems involved in amoeboid movement. The direction of cell movement thus could be changed by calcium ion concentration changes. A role for a protein kinase in mediating the change in calcium ion concentrations could also be considered in such a model.

In addition to its role in aggregation, cyclic AMP apparently also acts as a trigger for differentiation after aggregation. Bonner (1970) showed that cyclic AMP caused isolated, unaggregated amoeba to develop the thick cellulose walls and large vacuoles characteristic of the stalk cells of the fruiting body. Differentiation into stalk cells uses up almost all the

cell reserves in a period of high catabolic activity; cyclic AMP might act by stimulation of this catabolic activity.

Studies of developmental control in the slime molds have examined the occurrence of specific enzyme activities, and how their appearance correlates with the overall developmental program (see Francis 1969, Wright 1966). Nestle and Sussman (1972) have looked at the effect of cyclic AMP on this developmental process, particularly with regard to production of UDPG pyrophosphorylase and UDP galactose-4-epimerase, two enzymes involved in the production of fruiting bodies. Addition of cyclic AMP altered the patterns of production of these enzymes, and when added at a critical period, resulted in aberrant fruiting body structures formed.

Farrell and De Toma (1973) observed that cyclic AMP produced an increased capacity for RNA synthesis in nuclei isolated from aggregation stage Dictyostelium discoideum. AMP was also found to stimulate this synthesis, but it was not effective in producing differentiation in isolated cells (Bonner 1970). Overall, these observations may relate to a requirement for a balance of the external nucleotides to be maintained by phosphodiesterase activity.

In Physarum polycephalum, protein kinase activity affected by cyclic AMP has been reported, suggesting that the same mechanism of action of cyclic AMP may operate in these organisms as in animal cells. Kuehn (1971) reported that two activities could be isolated, one cyclic AMP-activated and the other inhibited by cyclic AMP

concentrations greater than  $10^{-9}$  M. At that time he suggested that the two oppositely regulated enzymes could operate in the cell if they were separated by compartmentalization, the activated form being associated with cellular components, and the more readily soluble inhibited form being on the cell surface. Further studies showed, however, that the effect of cyclic AMP on the protein kinase activity depended on the stage of the cell cycle (Kuehn 1972), the inhibition being maximal in the G2 phase. The kinase activity became independent of cyclic AMP effects by mid-S phase, with inhibition control returning by the end of mitosis. This author suggested that as protein kinase levels remain the same during the cell cycle (in the absence of cyclic AMP), then the different cyclic AMP effects on activity may depend on interaction of cyclic AMP and some unidentified factors, rather than on changes in the levels of two separate kinases.

Early studies on yeast cells and extracts had shown that cyclic AMP, as well as other nucleotides, enhanced oscillatory cycles of  $\text{NAD}^+$  reduction and oxidation (Chance & Schoener 1964). Low levels of cyclic AMP were reported to occur in whole cells of Saccharomyces carlsbergensis ( $3 \times 10^{-2}$   $\mu\text{moles/kg}$  wet weight) (Cheung 1966). In cell extracts the cyclic AMP levels apparently stayed essentially constant throughout the course of the oscillations in the pyridine nucleotides (Cheung 1966). The exact site of cyclic AMP action on the oscillatory mechanism is not known (Chance et al 1965).

In Saccharomyces cerevisiae and Saccharomyces carlsbergensis cyclic AMP appears to play a role similar to its function in catabolite repression in bacteria. Fang and Butow (1970) found that glucose repression of respiratory enzymes in S. cerevisiae protoplasts could be reversed by cyclic AMP, but not specifically, as 5'AMP also had the same effect. Tsuboi et al (1972) reported that cyclic AMP and dibutyryl cyclic AMP, but not other adenine nucleotides, could reverse glucose-repression of sporulation. When added to medium not enriched with glucose, no effect on sporulation was seen. Phosphodiesterase inhibitors also acted to reverse the glucose repression (Tsuboi & Yanagishima 1973). The effects of theophylline and caffeine were observed to differ and Tsuboi and Yanagishima suggested that the phosphodiesterase of S. cerevisiae is theophylline-sensitive like these of the bacteria, rather than caffeine-sensitive like the animal forms. Caffeine may be acting directly on the sporulation effect. Overall, cyclic AMP could act on sporulation through promotion of respiratory adaptation, but it might equally play a direct role in the mechanism that controls the change from vegetative growth to sporulation.

van Wijk and Konijn (1971) used a Dictyostelium attraction bioassay to examine the intracellular concentrations of cyclic AMP in Saccharomyces carlsbergensis grown on different carbon sources. Levels were higher in cells which show a lower degree of catabolite repression, and levels increased as glucose-grown

cells were adapted to growth on galactose or maltose. These studies suggested that cyclic AMP could play a role in a regulatory system sensitive to catabolite repression, similar to the situation in E. coli.

Other evidence for cyclic AMP presence and function in yeast was presented by Sy and Richter (1972, a, b) from studies on Saccharomyces fragilis. A protein of molecular weight 24,000 daltons was isolated from the cytoplasm of cells, with an estimated binding constant of 5 nM for cyclic AMP at pH 7.4. Similar proteins were demonstrated in other Saccharomyces strains, activity being relatively constant, although slightly lower at high glucose concentrations. This binding protein was used to assay intracellular levels of cyclic AMP in Saccharomyces fragilis, by an adaptation of the Gilman procedure (Sy & Richter 1972 b). Intracellular levels in high glucose medium were low and constant over a 30 hour period. Cells in low glucose medium, or in lactate, showed an increase, plateauing as the cells entered the stationary growth phase. These authors suggested a role for cyclic AMP in catabolite repression, particularly as changes in adenylyl cyclase activity (see below) and cyclic AMP levels correlated with changes in carbon source and aeration.

Working with mutants of a Saccharomyces strain in which invertase production was insensitive to glucose repression, Montencourt et al (1973) did report there slightly higher steady state levels of cyclic AMP in cells grown in low-glucose medium, as

measured by the Gilman assay. However, there was no apparent correlation between the cyclic AMP levels and the sensitivity of invertase production to hexose, indicating that the role of cyclic AMP in catabolite control in yeast is not yet clear.

In Saccharomyces cerevisiae, the reserve carbohydrate trehalose can be rapidly degraded following production of the enzyme trehalase. Intracellular cyclic AMP levels were found to peak during the lag phase immediately proceeding trehalose degradation, and in vitro, cyclic AMP was found to activate a cryptic form of trehalase found in cell extracts, (van der Plaats 1974). The activation mechanism which apparently did not require protein synthesis is not known, but it was suggested by van der Plaats (1974) that regulation of the utilization of trehalose during the cell cycle might depend on cyclic AMP signals. The cyclic AMP peak returns to the basal level when trehalose degradation has ceased; this decay is not prevented by addition of theophylline.

Adenylyl cyclase activity was found to be associated with the membrane of Saccharomyces fragilis, in both whole cells and spheroplasts (Sy & Richter 1972 b). Activity was stimulated by fluoride, and inhibited by pyrophosphate. Saccharomyces cerevisiae and S. carlsbergensis showed low or no activity in these assays, which the authors proposed did not show better activity. Londesborough & Nurminen (1972) reported adenylyl cyclase activity in Saccharomyces cerevisiae which was  $Mn^{++}$ -dependent, rather than  $Mg^{++}$ -dependent like the enzyme in S. fragilis.

Cyclic AMP phosphodiesterase activity had also been reported in yeast; Speziale and van Wijk (1971) found  $Mn^{++}$ -dependent activity in Saccharomyces fragilis.

Sy and Richter (1972a) reported that the binding protein used in the cyclic AMP assays did not appear to function as an inhibitor of phosphodiesterase, nor as an enzyme itself. They proposed that its function might instead be related to a catabolite repression role of cyclic AMP. Recently, however, Takai et al (1974) have isolated a cyclic AMP-dependent protein kinase from baker's yeast, and they proposed that cyclic AMP in yeast acts as a regulator of phosphorylation reactions, rather than as a bacterial-like catabolite gene activator. The properties of this protein are distinct, but similar to cyclic-AMP dependent protein kinases found in mammalian cells, with the catalytic and regulatory subunits of the rat and yeast enzymes being capable of producing "hybrid" forms.

Enzymes of cyclic AMP metabolism and evidences of control have also been found in the Ascomycetes. Flawia and Torres (1972a) reported the activity of adenylyl cyclase as a membrane-bound enzyme in Neurospora crassa. The enzyme was not activated by NaF, and the activity was  $Mn^{++}$ -dependent rather than  $Mg^{++}$ -dependent. The substrate of the Neurospora enzyme was identified as a  $Mn^{++}$ -ATP complex, which gave a sigmoid activity response (Flawia & Torres 1972 b).  $Mn^{++}$  activation at low concentration of the enzyme would correlate with the two sites for divalent cations in animal enzymes. These authors suggest that intracellular cyclic AMP levels are

related to changes in the  $Mn^{++}$ -ATP ratio; and that the position of the enzyme on the cell surface indicates that it can respond to environmental signals.

Scott and Solomon (1973) demonstrated the presence of cyclic AMP phosphodiesterase activity in Neurospora crassa, and suggested that its characteristics indicated its closer relationship to animal than bacterial forms. This would be consistent with the general similarity of cyclic AMP control of glycogen metabolism in Neurospora with that of higher organisms. In the presence of  $Mg^{++}$ -ATP, cyclic AMP promoted conversion of glycogen phosphorylase to an active form (Tellez-Inon & Torres 1970), in contrast to the allosteric regulation of glycogen synthetase observed in other fungi. At sub-optimal divalent cation concentration, Glucagon has been found to produce a response in Neurospora similar to that in animal cells; increasing the rate of glycogenolysis. Decreased glycogen synthetase activity and increased glycogen phosphorylase activity may be mediated by the observed activation of membrane-bound adenylyl cyclase by glucagon (Flawia & Torres 1972 c). Insulin inhibition of this enzyme is counteracted by glucagon (Flawia & Torres 1973). As a result of these observations, it has been suggested that certain proteins, such as hormone receptors of fungi and animals, may have evolved from a common ancestral gene.

The effect of cyclic AMP on regulation of citrate fermentation and growth in Aspgerillus niger was studied by Wold and Suzuki (1973 a, b). Addition of cyclic AMP was found to stimulate the rate of

citrate synthesis and sucrose utilization. These authors suggested that abnormal cyclic AMP metabolism might produce loss of glycolysis control, so that excess citrate is produced. Zinc ions were also observed to be involved in the action of cyclic AMP in regulation of growth, and Wold (1974) suggested that the two are partners in regulation in Aspergillus. Cyclic AMP and cyclic GMP both promoted the clumping of conidia, resulting in pellets of mycelia, when grown in agitated submerged liquid cultures (Wold & Suzuki 1973 b). Parallels were drawn between cyclic AMP promotion of "adhesiveness" of conidia and germlings, and the effects of cyclic AMP in cell surface reactions in mammalian cell systems. Enzymes of cyclic AMP metabolism were also demonstrated to occur in Aspergillus niger (Wold 1974), including adenylyl cyclase, cyclic AMP binding proteins, and two phosphodiesterases, one extra-cellular, the other intra-cellular.

Morphogenesis effects of cyclic AMP are also observed in the Basidiomycetes and Zygomycetes. These are of some interest in view of similar morphogenec effects in the slime molds. Uno and Ishikawa (1973 a) demonstrated that cyclic AMP and AMP could induce formation of fruiting bodies in monokaryotic mycelium of Coprinus macrorrhizus. Cyclic AMP binding activity from cell-free extracts and the active Fruiting Inducing Substances (FIS), cyclic AMP and AMP, could produce fruiting bodies in inducible strains of Coprinus macrorrhizus. Studies of the enzymes of cyclic AMP metabolism in this organism

demonstrated the presence of a very labile adenylyl cyclase activity, which increased prior to cell aggregation or fruiting body formation (Uno & Ishikawa 1973 b). A phosphodiesterase producing 5'AMP was characterized, and its inhibitors, caffeine, theophylline, and 3'AMP, were found to be effective inducers of fruiting bodies. Strains with no cyclic AMP enzymes or binding proteins, and no FIS fraction, were unable to produce fruiting bodies. Multiple protein kinase activities were reported in Coprinus mycelium (Uno & Ishikawa 1974), with both cyclic AMP-inhibition and stimulation observed. The regulatory effects on protein kinases found to occur at concentrations of cyclic AMP that are found in cells imply that phosphorylation reactions may be involved in fruiting body formation, but there is no further evidence regarding a precise mechanism.

In contrast, in another Basidiomycete, Schizophyllum commune, Schwalb (1974) found that addition of 1 mM cyclic AMP resulted in fruiting body formation being either blocked or abnormal in expression. Dibutyryl cyclic AMP produced the same effect as cyclic AMP, while cyclic GMP has no effect at the same levels. However, these results must also be considered in connection with the influence of environmental conditions on cyclic AMP effects.

In the Zygomycete, Mucor racemosus, cyclic AMP appears to be involved in the shift between yeast-like and hyphal morphology which is induced by growth conditions (Larsen & Sypherd 1974). Yeast-like growth occurs when fermentative activity is favoured, and hyphal morphology is induced by factors such as aeration and glucose concentration that allow respiratory activity. Dibutyryl

cyclic AMP inhibits the yeast to hyphae shift normally accompanying aeration, and can induce a hyphae to yeast change. (Cyclic AMP or butyrate alone did not affect the process). Induction of the hyphal growth was accompanied by a decrease in intracellular cyclic AMP levels, as measured by the Walton and Garren binding protein assay. Overall, cyclic AMP does appear to play a role in morphogenesis control in this organism, with effects being influenced by growth conditions and metabolic activities.

Cyclic AMP has also been implicated in the inhibition of perithecal initials formation in the imperfect fungus Monacrosporium doedyciodes (Galsky et al 1972), antagonizing the promotional effect of 6-methylpurine and 8-azoguanine. These workers proposed a control mechanism at the level of RNA synthesis.

## CYTOKININS

The cytokinins are a group of compounds, mainly N<sup>6</sup>-substituted adenine derivatives, named for their action in promoting cell division (cytokinesis) in plant tissues. In studies of in vitro culture of plant pith tissue, it was observed that a supply of auxin was necessary for cell enlargement, and an additional factor was required to induce cell division (Jablonski & Skoog 1954). This requirement could be satisfied by addition of coconut milk, malt extract, yeast extract, or autoclaved DNA. Subsequently, Miller and co-workers (Miller et al 1955, 1956) reported the isolation and characterization of kinetin from autoclaved DNA.

The general term "cytokinin" was proposed by Skoog et al (1965) and has since been used to include the various compounds with similar cell division promoting activity. Fox (1969) has discussed the sources of other terms which have been used in the literature at various times, including "kinin", "kinetenoid", "phytokinin" and "phytocytomine". Reviews of cytokinins and their action have been prepared at various times, including those of Miller (1961), Srivastava (1967), Letham (1967, 1969), Helgeson (1968), Fox (1969), Skoog and Armstrong (1970), Kende (1971), and Hall (1973).

### Chemical Structure and Activity

Using the effect of cytokinins on plant tissue growth as a test, Skoog's group in Wisconsin extensively studied the relationship between the chemical structure of a compound and its biological effectiveness (Skoog & Leonard 1967, Skoog & Armstrong 1970). In general, greatest activity was shown by N<sup>6</sup>-substituted adenine compounds. Alterations to the structure of the adenine ring itself greatly reduced or eliminated activity. For example, the guanine analogue of kinetin, furfurylguanine (2-furfurylamine-6-hydroxypurine), and benzylguanine are both inactive (van Eyk & Veldstra 1966). Skoog and Leonard (1967) suggested that the activity of purines substituted at the 1,3, or 9 positions could be due to conversions to the active 6-position derivative. As reviewed by Fox (1969), it was found that for activity, the 1-position of the adenine ring must be free. Substitutions at the 2 or 3 positions may also result in inactivity, but to a lesser extent than for a blocked 1-position.

A large number of N<sup>6</sup>-substituent groups will give the compound equal or better activity than the furfuryl group of kinetin. Activity produced by a non-polar side group increased with the chain length of the group, up to a maximum of five carbon units, and then declined. The presence of a double bond at any position in the side chain enhanced activity, as do polar groups (Skoog &

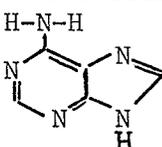
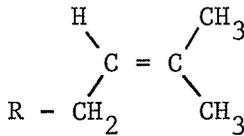
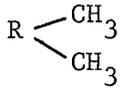
Miller 1957, Skoog & Armstrong 1970). Substitution of a benzene ring for the furfuryl moiety of kinetin gives a more effective compound, N<sup>6</sup>-benzylaminopurine (Miller 1967). In general, the effect of substituent groups on side chains was to increase activity if they tended to make the molecule more planar, and decrease activity if the molecule became less planar (Skoog & Armstrong 1970).

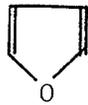
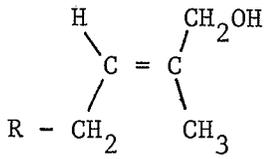
Certain compounds without the substituted-adenine structure have also been found to have similar biological effects as the cytokinin adenine compounds, and these are generally considered in this group. Examples of these are diphenylurea, and benzimidazole. The reason for their activity is not known, although it has been suggested that the phenyl ureas and the urideopurine derivatives form a class of compounds distinct from the cytokinins, but with similar activities (Skoog & Armstrong 1970, Hall et al 1972). Table I lists the chemical structures of a variety of cytokinin compounds, particularly those of interest in these studies.

Chemical synthesis of structural analogues of cytokinin-active compounds has produced a number of compounds with antagonistic action in bioassays, which have been referred to as "anti-cytokinins" (Skoog et al 1973). The substances were of a group characterized by the compound 3-methyl-7-(3-methylbutylamino)pyraxolo-[4,3-d]-pyrimidine (Hecht et al 1971a). The chemical action was examined in detail (Skoog et al 1973) by comparisons between a large variety of compounds. The requirement for a side chain in a position comparable to the N<sup>6</sup>-substituent of cytokinins,

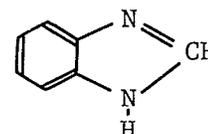
Table I

CYTOKININ COMPOUNDS

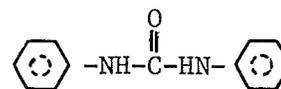
NAME	ABBREV.	STRUCTURE
adenine		
6-amino-3-dimethylallyl purine (triacanthine)		( <sup>3</sup> N-isomer of isopentenyladenine)
6-benzylaminopurine (benzyladenine)	BAP	R - CH <sub>2</sub> - 
6-benzylmercaptapurine	BMP	(S-atom replaces 6-amino group)
6-butoxypurine	BxP	R - O-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>
6-butylaminopurine		R - CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>
6-(γ,γ-dimethylallyl)amino- purine <u>or</u> N <sup>6</sup> -(Δ <sup>2</sup> -isopentenyl) adenine	i <sup>6</sup> ade, IPA	
6-(γ,γ-dimethylallyl)amino- purine, <u>or</u> N <sup>6</sup> -(Δ <sup>2</sup> -isopentenyl) adenine	i <sup>6</sup> Ado, IPAdo	
6,6-dimethylaminopurine	DMAP	R 
6-heptylmercaptapurine	HpMP	
6-hexylaminopurine	HAP	R - (CH <sub>2</sub> ) <sub>5</sub> - CH <sub>3</sub>
6-hexylmercaptapurine	HMP	

6-histaminopurine	HiP, HisAP	$R - C_3H_3N_2CH_2CH_2NH_2$
6-furfurylaminopurine (kinetin)	Kn, FAde	$R - CH_2 - $ 
6-furfurylaminopurine riboside (kinetin riboside)		
6-methylaminopurine	MAP, MeAP	$R - CH_3$
6-(trans-4-hydroxy-3-methyl- but-2-enyl)amino purine, (zeatin)	Zea	
6-propylaminopurine		$R - CH_2CH_2CH_3$
6-propylmercaptapurine	PMP	$R - S-CH_2CH_2CH_3$

Benzimidazole



N'N'-diphenylurea



and the relative effectiveness of variants of this side chain suggests a specific interaction-site competition between the cytokinins and antagonists. Some of the effects of the antagonist compounds, such as their relationships to the diphenylurea compounds, and reactions in different bioassay systems, raise questions regarding their action, but in general these studies provide a useful approach to examining cytokinins' mechanism of action.

#### Bioassays for Cytokinins

Bioassays of cytokinin activity reflect the biological effects of the compounds on different facets of cell metabolism, and on different organisms. Following the discovery of the cell-division promoting effect of cytokinins, the first bioassays related the influence of varied concentrations of the compounds to the weight of soybean or tobacco tissue grown in vitro over a period of two to four weeks (Murashige & Skoog 1962, Miller 1963). Fox (1969) discussed the merits of the soybean callus assay compared with the test using tobacco tissue. The soybean system shows a linear response to kinetin concentration over a wider concentration range than does tobacco tissue, but the latter is less sensitive to toxic substances found in tissue extracts.

Certain desirable characteristics of a cytokinin assay can be deduced from this comparison. The assay used should show a linear response, and show the response over a wide concentration range. The sensitivity of the assay should be such that it can

detect the levels of the compounds occurring in biological materials, and it should be specific for compounds that do have biological effects. Substances found in the test samples should not significantly antagonize the assay. In addition the test should be relatively easy to perform, and should be completed in as short a time as possible. This last requirement for speed of assay has been the major drawback to use of the cell division assay, as well as for many other tests.

The various other methods which have been used to assay cytokinins have been listed in Table II, with references and assessments. The variety of approaches to bioassay of cytokinins reflect the many effects which have been detected and attributed to the presence of cytokinins in different systems. The original definition of a cytokinin was, and still is, in terms of a cell division-promoting activity. As the fundamental mechanism or basis of cytokinin effects is elucidated, the definition, and the bioassay procedures, will likely be modified in to meet the new concepts.

#### Cytokinins in Transfer RNA

A significant occurrence of cytokinin molecules is in the structure of transfer RNA of all species. Studies by Holley et al (1965) on the sequence of yeast alanyl-transfer RNA showed that nine of the 77 nucleotide positions in that structure were occupied by modified

TABLE II

COMPARISON OF DIFFERENT CYTOKININ BIOASSAYS

TEST SYSTEM & REFERENCES	PARAMETER OBSERVED	BIOASSAY TIME	SENSITIVITY & SPECIFICITY	ADVANTAGES & DISADVANTAGES
<u>Cell Division Tests</u>				
(a) Tobacco pith (1)	Fresh and dry weight of callus increase linearly, within certain limits, with log increase in cytokinin concentration in culture medium.	2-4 wks	For some cytokinins, linear relation between callus plug weight and 0.1 nM to 1 $\mu$ M cytokinin. Auxin required for effect.	Extreme sensitivity makes it ideal for pure cytokinins. Growth period very long, and must be aseptic. Problem with explant uniformity.
(b) Soybean callus (2)	as above	as above, but with a broader "window" of indication in 48 hr	as above, but with a broader "window" of sensitivity, up to 100 $\mu$ M.	Sensitivity and more consistent linear response make it useful for both pure and impure preparations. Disadvantages as above.
(c) Carrot phloem (3,4,5)	Weight and number of cells in phloem explants grown with cytokinins increase with log cytokinin concentration.	2 -3 wk, indication in 7-10 days	As sensitive as above, but less specific than earlier callus assays.	Material readily available and easily stored. Can be made sterile. Thiamine, glycine, nicotinic acid inhibit. Adenine has effect.

Senescence Tests

Richmond-Lang Effect on chlorophyll degradation (6,7)	Chlorophyll is extracted from test material incubated with cytokinin in the dark. Chloro- phyll measured spectrally. (Leaves used include <u>Xanthium</u> , corn, tobacco, Chinese cabbage, and radish)	48 hr	Linear relation between concentration of cyto- kinin and chlorophyll remaining. Detects as low as 0.1 $\mu$ M, and as high as 5-50 $\mu$ M non- quantitatively. Lacks specificity.	Rapid, therefore sterility not a problem. Leaves must be prepared in dim light prior to use. Seasonal changes can affect leaf responses. Simple test to perform. Interference from purines and high sugar concentrations.
same (8)	Same test using detached oat or wheat seedling blades.	as above	as above	Secondary advantage is that only a small drop of cytokinin is required.

#### Cell Enlargement Tests

(a) Leaf disc expansion with radish leaves (9)	Cell enlargement of immature leaves aged in the dark for 24 hr. Discs are cut out and floated in test solution for 18 hr in the light. Discs then blotted dry and weighed.	18 hr	Cell weight increases with increase in cyto- kinin concentration. Sensitivity similar to callus assays but limit is 1 nM.	Rapid, therefore need not be sterile. Seasonal changes can affect leaves. Special leaf preparation required.
(b) Leaf discs of etiolated bean seedlings (2,10,11)	Same as above, but using discs from 7-9 day old dark-grown bean leaves with a main lateral vein. Discs floated in the dark. Increases in diameter or weight measured.	48 hr	Less sensitive than the radish system. Compares with chlorophyll retent- ion assay in sensitivity.	Gibberellins, red light, and cobalt may interfere. May be useful to investigate interrelationships of effects

(c) Radish cotyledon enlargement (12,13)	Radish cotyledons excised immediately after germination and soaked in test solution in the light. Cotyledon weight used as measure of size increase.	3 days	Weight increase is essentially a linear function of log cytokinin concentration. Sensitivity as for callus assays.	Seeds easily germinated, and can be surface sterilized. Relatively rapid and specific. Cotyledons require preparation.
(d) Pea stem section enlargement (14)	4mm stem sections of 6 day old seedling stems put in test solution. Length and diameters of sections are compared before and after.	24 hr	Increase in size is sensitive over a wide range.	Uses effects on cell division. Rapid. Sucrose, IAA, gibberellins, cobalt have effects. Response varies with sections used. Time-consuming measurements.

#### Germination Tests

Lettuce seeds (15,16,2)	Seeds soaked in test solution in the dark for 8 hr then transferred to filter paper wet with the solution for 24 -60 hr. Germination percentage scored.	32-68 hr	Germination in absence or presence of red light measured. Sensitivity poor, with permeability problems. Detects 1 nM cytokinin in some cases.	Requires high concentration and large amounts of cytokinin. Red light and gibberellins will affect. May have sterility problem.
Lettuce seed embryos (17)	Excised embryos in cytokinin plus abscisic acid solution are tested for cytokinin overcoming inhibition by abscisic acid	46 hr	Relatively sensitive with linear response.	Depends on double effect of two hormones. Must consider penetration and endogenous levels in embryos.

Frond Expansion Test

(a) Water lily ( <u>Lemna minor</u> ) ( 3,18)	Single plant in basal medium, incubated in the light for test period. Plant weighed, and frond number and area determined.	12 days	Frond number or weight increase relate to log cytokinin concentration. Not sensitive; lowest detection at about 0.25 µM.	Long test period. Low sensitivity and poor selectivity. Extraneous factors influence system.
---	---	---------	---	---

(b) <u>Spirodela</u> (3)	Cytokinins satisfy the non- photosynthetic red light requirement for growth. Grown in dark; other conditions as above.	14 days	as above	as above
-----------------------------	--	---------	----------	----------

Differentiation Tests

(a) Moss buds in <u>Tortella</u> <u>caespitosa</u> (19,20), <u>Pohlia</u> <u>nutans</u> (21), & <u>Funaria hygrometrica</u> (22,23)	Cytokinin induces bud forma- tion in moss <u>proteonemata</u> . Buds are counted before and after exposure to test solution.	48 hr	Number of buds increases logarithmically with cytokinin concentration. Broad specificity and fairly sensitive. Detects at 10 nM levels; linear correlation to 100 µM.	All-or-nothing response easy to measure. Rapid and selective for purine compounds. Somewhat tedious, and some non-purine active compounds not effective.
--	--	-------	---	--

(b) Tobacco pith buds (2)	Cytokinins promote adventit- ious budding from tobacco stem slabs. Incubation with- out auxin leads to bud form- ation in 12 days; with auxin and cytokinin buds appear much later (5 wks).	less than 12 days	Not very sensitive; trace amounts of auxin inhibit buds. Adenine has some effect. Linear correlation with cyto- kinin concentration when system works.	Auxin antagonism. Certain cytokinin concentrations inhibit, therefore not quantitative. Sterility problems.
---------------------------------	---	----------------------	--	---

(c) Apical dominance release ( 24)	Application of substance to lateral bud at second node of 6-day old pea plants with apical bud intact releases apical dominance effect. Bud cut off and weighed after 3 days; compared to control bud.	3 days	Reasonably specific for adenine cytokinins, no effect with some other compounds. Only moderately sensitive.	Fairly rapid. Auxin may affect results.
------------------------------------	--	--------	---	---

Metabolic Synthesis Tests

(a) Nitrate reductase induction (25)	Increased induction of nitrate reductase activity in <u>Agrostemma githago</u> embryos by nitrate when cytokinins present.	30-60 min	Activity of enzyme increases with cytokinin between 10 nM and 100 $\mu$ M	Very rapid response, but not assessed for suitability as an assay.
(b) Betacyanin (pigment) production (26, 27, 28)	Cytokinins induce betacyanin production in <u>Agrostemma</u> seedling cotyledons and hypocotyls when incubated in the dark with tyrosine. Pigment extracted and measured spectrally.	18-72 hr	Broad specificity. Extracted pigment increases in optical density with the log of cytokinin concentration from 1 nM to 10 $\mu$ M.	Can measure small amounts of pigment pure or in extracts. Rapid. Disadvantages are in manipulation of seeds, and processing of extracts leading to error.
(c) Deoxyisoflavone synthesis ( 29)	Induction of deoxyisoflavone correlates with soybean growth in culture. Callus is chopped finely and incubated in liquid medium, then ethanol or acid extracted. Optical density read.	45 hr	Reasonably sensitive to cytokinins. Correlates with growth of soybean.	Quantitative measure of effect. Auxin may affect synthesis. May be measuring a secondary hormone effect. Deoxyisoflavone is alkali-sensitive.

Metabolite Transport and Binding Tests

(a) Inhibition of active uptake by Achlya (30)  
Cytokinins inhibit the transport of amino acids, sugars, and nucleosides in germinated spores. Membrane filter assay used to measure effect on uptake.  
Decrease in uptake with increased cytokinin levels between 0.1  $\mu$ M to 1mM. Selective for cytokinin bases; ribosides not active.

(b) Enhanced uptake and binding of auxin and tryptophan to Achlya germlings (30)  
Cytokinins enhance auxin and tryptophan binding to a cell wall glycopeptide. Can measure with filter assay as above.  
Specificity as above. Increases in binding with concentrations from 0.1 nM to 1 mM. As above. Interaction of auxin and cytokinin can be assessed.

---

REFERENCES:

- 1) Murashige, T. & Skoog, F. (1962) Plant Physiol. **15**:473-497
- 2) Miller, C.O. (1963) Modern Methods in Plant Analysis **6**: 194-202
- 3) Letham, D. (1967) Planta **74**: 228-242
- 4) Caplin, S. & Steward, F. (1952) Ann. Bot. **16**:219-234, and (1948) Science **108**:655-657
- 5) Steward, F. & Shantz, E.M. (1959) Ann. Rev. Plant Physiol. **10**: 379-404
- 6) Osborne, D.J. & McCalla, D.R. (1961) Plant Physiol. **36**: 219-221
- 7) Kende, H. (1964) Science **145**: 1066-1067
- 8) Gunning, B. & Barkley, W. (1963) Nature **199**: 262-265

- 9) Kuraishi, S. (1959) Science Papers, College of General Education, Univ. Tokyo 9: 67-104 (cited by Miller, in reference (2), p.198)
- 10) Scott, R.A. & Liverman, J.L. (1956) Plant Physiol. 31: 321-322
- 11) Miller, C.O. (1956) Plant Physiol. 31: 318-319
- 12) Letham, D. (1968) in Biochemistry and Physiology of Plant Growth Substances (F.Wightman & G.Setterfield, eds.), Runge Press, Ottawa, pp. 19-31
- 13) Letham, D. (1971) Plant Physiol. 25: 391-396
- 14) Sommer, N.F. (1961) Plant Physiol. 14: 741-749
- 15) Skinner, C.G., J.R.Claybrook, F.D.Talbert & W.Shive (1957) Plant Physiol. 32:117-120, and Skinner, C.G. & W.Shive (1957) Plant Physiol. 32: 500-501
- 16) Miller, C.O. (1958) Plant Physiol. 33: 115-117
- 17) Black, M., J.D.Bewley & D.Fountain (1974) Planta 117: 145-152
- 18) Loeffler, J.E. & J.van Overbeek (1964) in Regulateurs naturels de la croissance vegetale, C.N.R.S., Paris, pp.77-82 and Letham, D. (1961) Nature 191: 1119-1120
- 19) Gorton, B.S., C.G.Skinner & R.E.Eakin (1957) Arch.Biochem.Biophys. 66: 493-496
- 20) Brandes, H. & H.Kende (1968) Plant Physiol. 43: 827-837
- 21) Mitra, G.C. & A.Allsop (1959) Phytomorph. 9: 64-71
- 22) Valadon, L.R.G. & R.S.Mummary (1971) Physiol. Plant. 24: 232-234
- 23) Hahn, H. & M.Bopp (1968) Planta 83: 115-118
- 24) Thimann, K. & T.Sachs (1966) Amer.J.Bot. 53: 731-739
- 25) Kende, H., H.Hahn & S.E.Kays (1971) Plant Physiol. 48: 702-706
- 26) Kohler, K.H. & K.Conrad (1966) Biologische Rundsch. 4: 36-40
- 27) Bigot, C. (1968) Compt.Rend.Acad.Sci. (Paris) 266: 349-352
- 28) Biddington, N.L. & T.H.Thomas (1973) Planta 111:183-186
- 29) Miller, C.O. (1969) Planta 87: 26-35
- 30) LéJohn, H.B. (manuscript submitted)

forms of the four common nucleosides. The types of modification, their occurrence, and their possible functions have been dealt with extensively by Hall (1971). Evidence indicates that there is an increase in the numbers of modified nucleosides per transfer RNA molecule with increasing evolutionary complexity, and it has been suggested that this may reflect increasing complexity of regulatory controls involving transfer RNA molecules (Hall 1971).

In general, the positions of modified components in the nucleotide sequence of transfer RNA molecules indicate that they are of importance in determining the secondary structure, forming the loops and bends between the paired regions (Zachau et al 1966), (Hall 1971). Of particular interest in relation to cytokinins is the fact that in all known transfer RNA sequences, the base adjacent to the 3' end of the anticodon is a purine, and frequently a substituted purine (Letham 1973). Cytokinin activity was found to be associated with species of transfer RNA corresponding to codons beginning with U (Skoog et al 1966, Armstrong et al 1969). These species of transfer RNA (for cysteine, serine and tyrosine) have been found to contain the cytokinin,  $N^6-(\Delta^2\text{-isopentenyl})$  adenosine, or, in the case of E. coli, a further modification,  $N^6-(\Delta^2\text{-isopentenyl})\text{-2-methylthioadenosine}$  (Burrows et al 1968). In transfer RNA species for codons beginning with the letter A, the compound  $N\text{-}[9\text{-}(\beta\text{-D-ribofuranosyl})\text{purin-6-yl carbamoyl}]$  threonine is found and probably has an analogous function (Ishikura et al

1969). This compound was thought to have no cytokinin-like growth promoting ability, but Dyson et al (1972) proposed that this could be the result of transport problems, and demonstrated that analogues without the polar carboxylic group were transported and showed good activity in bioassays. The different structure of this compound, and a different biological effect seen in the budding of tobacco callus tissue (McDonald et al 1971), suggests that it might be one of a different class of compounds from the adenine cytokinins, as mentioned earlier.

The presence of these modified purines in the 3' anticodon position is of functional significance, as the absence of the modifying group, or its chemical modifications in vitro, adversely affects the ability of the transfer RNA molecules to function in protein synthesis (Fittler & Hall 1966, Gefter & Russell 1969, Faulkner & Uziel 1971). The evidence of these three groups was that absence or alteration of the modified nucleoside did not alter the ability of the transfer RNA to become charged with an aminoacyl group, but did interfere with its subsequent binding to the ribosome-messenger RNA complex. The iodine modification of E. coli transfer RNA<sup>phe</sup> described by Faulkner & Uziel (1971) is reversible, with a restoration of function in poly-phenylalanine synthesis.

A model for the role of the modified 3' anticodon purine in transfer RNA structure has been proposed by Fuller & Hodgson (1967), who suggest that the alkylated purine, occurring at the start of

the anticodon triplet, prevents reading of any other triplet. In addition, the base stacking of the nucleosides in the anti-codon loop is maximized in such a way as to give the 5' base - the "Wobble Base" of Crick's hypothesis (Crick 1966) - the maximum amount of conformational flexibility. In support of this concept Ghosh & Ghosh (1970) have reported that the removal of the base Y (a modified guanosine) does affect the response to various codons. Their results are in conflict with the studies previously mentioned, in that ribosome-mRNA complex formation with transfer RNA is not affected.

Further suggestion of a role for cytokinins in interactions with the ribosome was the demonstration by Berridge et al (1970) that the ability of various free cytokinins to bind to purified ribosomes of Chinese cabbage was related to their biological cytokinin activity.

As the presence of cytokinin in the transfer RNA molecule markedly affects function of that molecule, the possibility that cytokinin activity is mediated through the presence of the compounds in transfer RNA has been considered by several workers. Exogenous cytokinins could perhaps induce the synthesis or activation of specific cytokinin-containing transfer RNAs. Such a mechanism implies that the exogenous cytokinin becomes part of the molecule, and in support of this Fox (1966) reported the finding of radioactively labelled benzyladenine in soluble RNA fractions. Burrows et al (1971) also indicated that 6-benzyladenosine occurred in the transfer RNA of tobacco callus grown on 6-benzyladenine.

Other workers however, have not been able to substantiate the direct incorporation of cytokinins into transfer RNA. Kende & Tavares (1968) demonstrated that radioactive 6-benzylamino-9-methyl-purine is still biologically active in the soybean callus assay, despite the masking of the 9-position which would prevent its incorporation into RNA. Similar results were reported by Richmond et al (1970) and Bezemer-Sybrandy & Veldstra (1971). Fox et al (1971) suggested that these results could be explained by biological removal of the masking group. However, Elliot & Murray (1972) reported that the amount of detectable incorporation of 6-benzyl-adenine into transfer RNA, including non-specific and transbenzylation reactions, was too small to account for cytokinin effects. Also, a mechanism such as that proposed by Fox does not account for the cytokinin effects of such compounds as N-N'-disubstituted ureas, which are unlikely to be incorporated into transfer RNA, or the failure of Kende & Tavares (1968) to find a cytokinin auxotrophic mutant of E. coli.

It thus appears unlikely that exogenous cytokinins act directly by their incorporation into transfer RNA molecules and subsequent function. The cytokinins occurring as an important unit of the transfer RNA now appear to result from the subsequent modification of specific adenosine residues in the preformed molecule (i.e. synthesis at the macromolecular level). Mevalonic acid has been demonstrated to be the precursor of the isopentenyl group of

isopentenyladenosine residues (Peterofsky 1968, Fittler et al 1968, Kline et al 1969). Isopentenyladenosine occurs in the transfer RNA of tobacco tissue cultures, despite the fact that the tissue requires an exogenous cytokinin source for growth (Chen & Hall 1969). This indicates that the effect of cytokinins on growth appears to be distinct from the synthesis of transfer RNA.

Possible connection between transfer RNA and cytokinin action by mechanisms other than direct incorporation has also been considered. Such proposals suggest that in some way cytokinins alter the patterns of isoaccepting transfer RNA species which occur in a tissue, changes which have been observed to occur with growth and differentiation (Sueoka & Sueoka 1971).

Other workers suggest that cytokinins could act to control degradation of certain isoaccepting transfer RNA species. It has been proposed (Anderson & Cherry 1969, Rijven & Parkash 1971 and Cherry & Anderson 1972) that specific nucleases can bind at the cytokinin groups of certain transfer RNA molecules, and break the primary structure, destroying function. Added cytokinins could protect these species of transfer RNA by binding to the nucleases and inhibiting their action.

#### Metabolism of Cytokinins

Hall and co-workers have examined the role of the transfer RNA component N<sup>6</sup>-isopentenyladenosine, in differentiation and

maturation processes in a number of biological systems (Hall 1970, Dyson & Hall 1972, Hall et al 1972 ) . As mentioned previously, the cytokinin components of transfer RNA arise by modification of nucleosides in preformed transfer RNA. During degradation and catabolism of transfer RNA, the modified constituents are released , along with the other reusable nucleotides. The fate of these modified forms is of interest, in view of their biological activity. If the effects of the cytokinins observed in many systems reflect natural regulatory processes, then it would be expected that controls exist to regulate the levels of cytokinins in the cell. This would be of particular importance if cellular cytokinins resulted solely or largely from transfer RNA degradation.

One such possible degradation mechanism described by Hall's group (Hall et al 1971 ) is the enzyme activity of adenosine aminohydrolase, as isolated from chicken bone marrow. Isopentenyladenosine is converted to inosine at one-fiftieth the rate that the enzyme converts adenosine. Isopentenyladenosine and a number of other analogues are competitive inhibitors of the adenosine reaction. The enzyme has been reported as occurring in the bone marrow of rabbits and humans, but not other animal tissues (Hall & Mintsoulis 1973, Hall et al 1971). In plants, an analogous enzyme found in cultured tobacco callus degrades isopentenyladenosine to adenosine rather than inosine (Pačes et al 1971). Recently a similar cytokinin oxidase from corn kernels has been characterized (Whitty & Hall 1974).

Another enzyme implicated in isopentenyladenosine catabolism is xanthine oxidase. McLennan and Pater (1973 a) suggested that following the enzymatic conversion of isopentenyladenosine to isopentenyladenine in mammalian cells and Lactobacillus acidophilus, or to N<sup>6</sup>-(3-hydroxy-3-methylbutyl)-adenine in pea seedlings (McLennan et al 1968), these metabolic products were oxidized, first to the 8-hydroxy- and then the 2,8-dihydroxy- derivatives. Isopentenyladenosine was not metabolized by xanthine oxidase. McLennan & Pater (1973 b) suggest that as different tissues and organisms metabolize isopentenyladenosine in different fashions, then the variety of effects seen for the compound may reflect the variety of derivatives produced in the different systems. They suggest that the 5' monophosphate ester of isopentenyladenosine, likely produced by adenosine kinase activity, may be the active form in chicken liver and kidney, and in some cell cultures. Such an argument leads to consideration of what the active forms of cytokinins are, whether they originate from transfer RNA catabolism or exogenous sources.

Fox and co-workers have made extensive studies of the metabolic products of 6-benzyladenine in tobacco and soybean tissues, with regard to possible incorporation into transfer RNA. They report that the bulk of radioactively labelled benzyladenine was destroyed in the tissue by processes which cleaved the side chain from the purine ring, allowing the purine to be scavenged (Fox & Chen 1967, 1968). The benzyl side chain in soybean tissue is

apparently metabolized to a compound similar to benzoic acid (Fox et al 1972). Studies indicated that the remaining twenty to twenty-five percent of the labelled benzyladenine taken up was converted to a stable, long-lived derivative. During incubations of about two hours, some of the benzyladenine was converted to benzyladenosine monophosphate and to benzyladenosine (Dyson et al 1972, Fox et al 1972). Over a longer period these two compounds disappear, as does the rest of the benzyladenine, with the concurrent appearance of the long-lived derivative, suggesting that these two compounds may be precursors. This persistent metabolite was later identified as 6-benzylamino-7-glucofuranosylpurine (Deleuze et al 1972, Fox et al 1973). It is active as a cytokinin, and, when added back to cells as a cytokinin source, it was stable and unchanged after thirty days. Similar compounds have been found in tobacco tissue, potato tubers, and lupin seedlings (Fox et al 1973), and it is likely that the most prominent metabolite of zeatin in radish seedlings is the 7-glucoside form (Parker et al 1972). If indeed this is an active long-lived cytokinin form, then questions arise as to its mechanism of action, its eventual fate, and the regulation of levels of the compound in the cell. Particularly, if this compound is so stable, how can the levels respond to changing conditions?

In other situations it does not appear so certain that the glucoside compound is the active form. Some other reactions considered to be cytokinin effects take place too rapidly to allow

for the formation of this compound. The production and function of the 7-glucoside form may just be part of a specific form of cytokinin-controlled growth regulation, or just one of the tissue-specific metabolic products that have a specific function in this case.

In further consideration of the requirements for a riboside or other sugar form to produce the active cytokinin form, it should also be noted that various pyrazolo-[4,3d] pyrimidine analogs of cytokinins, which show both positive and negative cytokinin action, are unlikely to carry a sugar moiety at the analogous position to the cytokinins, as there is a carbon rather than a nitrogen atom at that position (Hecht et al 1971b, Leonard et al 1969).

Dorée, Terrine, and Guern (1972) have examined the uptake and metabolism of kinetin and some other cytokinin compounds by cultured cells of Acer pseudoplatanus (sycamore). Their model of the kinetin uptake process links the rate of uptake very closely with its subsequent metabolism in the cells. Time course studies of uptake show a saturation process, but they do not extrapolate to zero time. This was taken to indicate the occurrence of peripheral binding sites at the cell surface, which are immediately saturated. No competition by adenine for this binding was observed, suggesting that the sites are distinct from those for actual uptake, where competitive inhibition of kinetin uptake is exerted by other purine compounds. Two rates of uptake were observed in the Acer cells; an initial rapid rate, and a subsequent slower rate becoming stabilized after an hour. The rate of kinetin influx remains constant, with

the second slower rate resulting from a gradual increase in efflux from the cell, as was demonstrated by studies of the rate of uptake in preloaded cells compared with untreated cells. Further studies showed that only a small fraction of the total labelled kinetin taken up was involved in the outward efflux. Two phase absorption curves were also found for propylaminopurine and benzylaminopurine. Chemical structure was seen to influence both the  $V_{\max}$  and apparent affinity constants for the uptake system (Terrine et al 1972).

Free kinetin does not seem to be accumulated as such in the cells (Terrine et al 1972). A major part of the kinetin taken up was found to be converted into nucleotides, including kinetin riboside, and labelled adenylic, guanylic, and inosinic nucleosides which had apparently had the furfuryl group removed during metabolism. The possible enzyme mechanism(s) involved in kinetin metabolism has been considered. Kinetin may be acted upon by a purine phosphoribosyl transferase during the process of transport, as this enzyme activity has been found to be high in Acer pseudoplatanus cells (Sadorge et al 1970). Kinetin riboside also could be shown to be formed from kinetin and ribose-1-phosphate by the action of nucleoside phosphorylase (Dorée & Guern 1967).

The rate of degradation of the kinetin nucleoside in the cells by removal of the furfuryl substituent group is suggested as limiting the rate of continuing uptake of exogenous kinetin (Dorée et al 1972a). This degradation to adenosine, guanosine and inosine

compounds could be mediated by enzymes such as adenosine amino-hydrolase (Terrine et al 1969). This enzyme, already mentioned as a candidate for transfer RNA catabolism, is of broad specificity and has been isolated from various sources. Terrine et al (1972) used this enzyme to examine the rates of cytokinin nucleoside breakdown in vitro. The  $V_{max}$  of degradation seemed to be related to the structure more than the size of the N<sup>6</sup>-substituent group. In addition, there appeared to be a reciprocal relationship between biological activity and reaction rate with the most active compounds being degraded most slowly. Uptake and subsequent metabolism of 6-methyladenine has been observed to be different than for other compounds examined, largely because the methyl group is not retained for a long period (Dorée & Guern 1973). It may be worth noting that this compound has been reported to have slight activity as a cytokinin antagonist when screened in tobacco callus bioassays (see Hecht et al 1971a) and the removal of the methyl group may be producing an adenine antagonism effect, such as reported in some other situations (Torrey 1962, Fries 1960). It is not clear on the basis of the current studies on Acer pseudoplatanus whether the enzymes metabolizing cytokinin compounds directly control the rate of uptake, or whether their substrate sites just have similar specificities. However, it does appear that in the case of Acer pseudoplatanus cells the intracellular pool is involved in regulating continued uptake, and degradation reaction rates should influence this through pool size effects.

The eventual degradation of kinetin to nucleic acid purine components agrees with the earlier studies of MacCalla et al (1962), who examined the fate of benzyladenine after long periods of incubation in Xanthium pennsylvanicum and Phaseolus vulgaris, finding that the riboside was a major metabolite, along with other purine ribosides.

Further studies of Dorée (1973) showed that the synthesis of the N<sup>6</sup>-substituted nucleotides does not proceed through a two step reaction catalyzed by a nucleoside phosphorylase and an adenosine kinase, but rather through direct transfer of a 5-phosphoribosylpyrophosphate group. This reaction has been demonstrated using adenine, methylaminopurine, benzyladenine, kinetin, and propylaminopurine as substrates (Sadorge et al 1972). In Acer pseudoplatanus these monophosphate compounds are not further phosphorylated, probably because there is no enzyme system to catalyze the reaction (Dorée & Terrine 1972). Dorée & Guern (1973) suggest that instead of the N<sup>6</sup>-side group being removed by adenosine deaminase as had been proposed earlier (Terrine et al 1972, Hall et al 1971), it is in fact removed from the N<sup>6</sup>-substituted monophosphate by AMP deaminase. This enzyme, from rabbit skeletal muscle, converts these compounds to IMP at a rate that depends on the nature of the substituent group (Dorée & Terrine 1972). It is proposed that the action of this enzyme in degrading the pool of N<sup>6</sup>-substituted nucleoside monophosphates regulated the continued uptake of exogenous cytokinin molecules (Dorée & Guern 1973), so that the intracellular pool of N<sup>6</sup>-substituted compounds reaches

a constant value, followed by an uptake during which the uptake of each molecule is balanced by the removal of one substituent side chain.

These same workers have also examined the uptake and metabolism of cytokinin compounds in Schizosaccharomyces pombe. In these cells, the N<sup>6</sup>- side chain of molecules taken up is oxidatively removed, producing hypoxanthine, and its riboside or nucleotide. The major part of the metabolites inside the cell consist of these compounds (Dorée et al 1972 b.), unlike the pools of kinetin and its metabolites found in Acer pseudoplatanus, which act to control the further uptake. In Schizosaccharomyces the size of the internal N<sup>6</sup>-substituted base pool is controlled by a degradation process acting on the base form (Guern et al 1972). This system may utilize the adenosine aminohydrolase enzyme activity, or xanthine oxidase, in the process of degradation.

### Cytokinesins

Various studies mentioned have suggested that cytokinins are metabolized into active forms. Another proposal regarding the active forms of cytokinins and their mechanism of action is that of Wood & Braun (1967), who suggest that the N<sup>6</sup>- substituted purine cytokinins activate systems in the plant cells that produce chemically different molecules that are the real cell-division promoting factors (CDF). One such compound isolated from Vinca

rosea crown gall tumour cells has been identified as a 3,7-alkyl-2-alkylthio-6-purinone, with an attached glucose moiety (Wood 1970). The glucose residue suggests that it cannot arise directly from RNA as cytokinins possibly could. This compound is produced continuously in the tumour tissues, which divide uncontrollably and do not require kinetin as a growth factor. In normal cells it is produced only in response to exogenous application of cytokinin. CDF isolated from tumour tissue will replace completely the kinetin requirement of normal tissues in culture (Wood & Braun 1967).

A cyclic AMP-mediated mechanism of cytokinin action on cell division through cytokinesin synthesis was proposed by Wood, Lin & Braun (1972) when it was found that the purinone CDFs were inhibitors of cyclic AMP analog, could replace the cell division promoting activity of cytokinesin and cytokinin, suggesting that the regulation of cell division may be controlled by cyclic AMP levels in the cells (Wood & Braun 1973, Basile et al 1973). Supporting this, a correlation between intracellular cyclic AMP levels and the cell cycle has been described for tobacco pith tissues (Lundeen, Wood & Braun 1973).

It is of interest that the purinone CDF is effective in the soybean callus and barley leaf senescence tests of cytokinin activity, but not in the Funaria moss-bud production assay (Wood et al 1969). As the substituted urea compounds that show cytokinin activity in tissue culture tests are also ineffective in moss-bud test (Valadon & Mummery 1971), this suggests that not all cytokinin

effects are mediated in exactly the same manner. The significance of metabolism into a different "active form" may also vary in different systems.

#### Cytokinin action

The variety of bioassay methods listed in Table II demonstrates that the effects of cytokinins are themselves variable. Discussion of the active forms of the compounds has also suggested that different systems may respond by different mechanisms. In addition, the overall response frequently is strongly influenced by factors such as levels of other growth regulators, environment, and physiological state of the material. A general survey of the effects attributed to cytokinin action has been made in Table III. Examination of some of these responses may be useful in trying to elucidate the cytokinin mechanism of action. However, besides the complexities introduced by interacting factors, there is also a danger that attempts to postulate a unified mechanism of action may be an over-simplification.

#### Plant hormone interactions

Besides the cytokinins, plant hormones include the auxins, the gibberellins, ethylene, and plant growth inhibitors such as

Table III: TABLE OF CYTOKININ EFFECTS

GENERAL EFFECT	SYSTEM	SPECIFIC EFFECT	CYTOKININ SPECIFICITY	LITERATURE REFERENCE
<u>I. In Plants</u>				
<u>CELL GROWTH</u>				
expansion	bean leaf	size expansion	K, BAP	Scott, R. A. & J.L. Liverman (1956) Plant Physiol. <u>31</u> 321-322.
	sunflower hypocotyls	increased fresh and dry weight, without elongation	K	de Ropp, R.S. (1956) Plant Physiol. <u>31</u> 253-254.
	leaf discs	expansion promoted	BAP	Loercher, L. & J.L. Liverman (1959) Plant Physiol. <u>34</u> Suppl. xv
	radish leaf discs	expansion promoted	general	Kuraishi, S. (1959) Sci. Papers Coll. Gen. Educ. Univ. Tokyo <u>9</u> 67-104.
	<u>Impatiens</u> immature petals (excised)	accelerated expansion	K (ethionine)	Klein, A.D. & C.W. Hagen, Jr. (1961) Plant Physiol. <u>36</u> 1-9.
	bean seedling cuttings	stimulation of treated whole leaves; inhibition of untreated leaves	BAP	Leopold, A.C. & M. Kawase (1964) Amer. J. Bot. <u>51</u> 294-298.
	pumpkin cotyledons (excised)	expansion accelerated	K	Banerji, D. & M.M. Laloraya (1965) Naturwiss. <u>52</u> 349-350.

<u>Lemna minor</u> fronds	enlargement of fronds in light	K (coconut milk)	Loeffler, J.E. & J. van Overbeek (1964) in <u>Rég. Nat. Croiss. Vég.</u> pp. 77-82.
"	frond area increase stimulated at low (10 <sup>-6</sup> M) concentra- tions; inhibited at high concentrations	K	Tasser de Jong, J.G. & H. Veldstra (1971) <u>Plant Physiol.</u> 24 235-238.
growth inhibition	tomato hypocotyls	K (TAA; GA stim.)	Pegg, G.F. (1962) <u>Ann. Bot.</u> 26 219-232.
	soybean hypocotyls	K	Vanderhoef, L.N. & J.L. Key (1968) <u>Plant &amp; Cell Physiol.</u> 9 235-238.
	pea stem segments	Z	Witham, F.H. & C.O. Miller (1965) <u>Plant Physiol.</u> 18 1007-1017.
MITOSIS/DIVISION			
stimulated	unorganized tobacco tissue	K	Skoog, F. & C.O. Miller (1957) <u>Symp. Soc. Exptl. Biol.</u> 11 118-131.
	radicles of ungerminated lettuce seeds	K, BAP, Benzylthio- purine	Haber, A.H. & H.J. Luippold (1960) <u>Physiol. Plant.</u> 13 298-307.
	mature pea root tissue	K	Torrey, J.G. (1961) <u>Exptl. Cell Res.</u> 23 281-299.

inhibited	intact <u>Allium cepa</u> roots	inhibited mitosis	K	McManus, M.A. (1960) Nature <u>185</u> 44-45.
	pea leaf discs	inhibition in light	K	Humphries, E.C. & A.N. Wheeler (1960) J. Exptl. Bot. <u>11</u> 81-85.
	tobacco pith tissue	increased mitosis and cell division	K	Das, N.K., K. Patau & F. Skoog (1956) Physiol. Plant <u>9</u> 640-651.
		cell division promoted	Z	Miller, C.O. (1965) Planta <u>54</u> 1052-1058.

#### DIFFERENTIATION

root develop- ment	<u>Isatis</u> <u>tinctoria</u> & <u>Convolvulus</u> <u>arvensis</u>	enhanced shoot regeneration from root segments	K	Danckwardt-Ulliesrom, C. (1957) Physiol. Plant. <u>10</u> 794-797 and Torrey, J.G. (1958) Plant Physiol. <u>33</u> 258-263.
	primary pea root sec- tions	lateral root induction (IAA required)	K (ade reversal)	Torrey, J.G. (1962) Physiol. Plant. <u>15</u> 177-185.
	bean hypocotyl, and rooted primary de- tached leaves	inhibition by effect on cell type produced	K	Humphries, E.C. & W. Maciejewska- Potapczyk (1960) Ann. Bot. <u>24</u> 311-316 and Humphries, E.C. & G.N. Thorne (1964) Ann. Bot. <u>28</u> 391-400.
	<u>Begonia</u> leaf cuttings	inhibition at high concentration, promotion at low concentration	endog.	Heide, O.M. (1965) Physiol. Plant. <u>17</u> 789-804.

shoot, root, and bud growth	Tobacco callus	concentration- dependent effects on root and bud growth; auxin- ratio effect	K, BAP, 16ade	Skoog, F. & C.O. Miller (1957) Symp. Soc. Exptl. Biol. <u>11</u> 118-131. and Hamzi, H.Q. & F. Skoog (1964) PNAS (USA) <u>51</u> 76-83.
	<u>Saintpaula</u> <u>ionantha</u> cuttings	budding increased	K	Plummer, T.H. & A.C. Leopold (1957) Proc. Amer. Soc. Hort. <u>70</u> 442-444.
	<u>Begonia</u> leaf discs	induced shoots; repressed roots	K	Schraudolf, H. & J. Reinert (1959) Nature <u>184</u> 465-466.
abscission	<u>Phaseolus</u>	retarded when applied to abscission zone; enhanced when applied elsewhere	K	Osborne, D.J. & S.E. Moss (1963) Nature <u>200</u> 1299-1301.
	<u>Phaseolus</u> leaves	promotion at low concentration; inhibition at high	K	Chatterjee, S.K. & A.C. Leopold (1964) Plant Physiol. <u>39</u> 334-337.
apical dominance	lateral buds, pea stem (and other sources)	release of lateral buds from effect of apical bud or auxin	K	Wickson, M. & K.V. Thimann (1958) Plant Physiol. <u>11</u> 62-74. and Sachs, T. & K.V. Thimann (1964) Nature <u>201</u> 939-940.
tracheary element differen- tiation	pea stem segments	functional xylem induced	K	Sorokin, H.P. & K.V. Thimann (1964) Protoplasmia <u>59</u> 326-
	tobacco tissue	induced tracheids	K	Bergmann, L. (1964) Planta <u>62</u> 221-254.
	mature lettuce parenchyma discs	tracheary element formation induced	K	Dalessandro, G. & L.W. Roberts (1971) Amer. J. Bot. <u>58</u> 378-385 and Torrey, J.G., D.E. Fosket & P.K. Hepler (1971) Amer. Sci. <u>59</u> 338-352.

special structures	tobacco pith tissue	chloroplast maturation induced	K	Stetler, D.A. & W.M. Laetsch (1965) Science <u>149</u> 1387-1388.
	<u>Datura innoxia</u> pollen grains	induction of haploid anther-producing embryos increased	general (other hormones, Fe-chelators)	Sopory, S.K. & S.C. Maheshwari (1973) Z. Pflanzenphysiol. <u>69</u> 97-99.
	tobacco roots	pseudonodule formation induced in root cortex	K (IAA)	Arora, N., F. Skoog & O.N. Allen (1959) Amer. J. Bot. <u>46</u> 610-613.
	<u>Solanum tuberosum</u>	tuber initiation induced	K (auxin antag.)	Palmer, C. E. & O.E. Smith (1969) Plant & Cell Physiol. <u>10</u> 657-664.
Fruit production	plum & apple fruitlets	fruit and seed development	endog.	Letham, D. (1963) New Zeal. J. Bot. <u>1</u> 336-350.
	figs	parthenocarpy induced	SD 8339 (synthetic)	Crane, J.C. & J. van Overbeek (1965) Science <u>147</u> 1468-1469.
	grapes	increased fruit size, induction of fruit set	BAP, der.; endog.	Weaver, R.J. & J. van Overbeek & R.M. Pool (1965) Nature <u>206</u> 952-953.
Flowering	<u>Cichorium intybus</u>	overcomes cold-requirement for induction	K (Vit E)	Michniewicz, M. & A. Kamińska (1964) Naturwiss. <u>51</u> 295-296.

<u>Perilla &amp; Pharbitis</u>	overcomes short-day requirement	K (ade)	Butenko, R.G. M.K. Chailakhyan (1961) Proc. Acad. Sci. USSR (Bot.) <u>141</u> 196-199.
<u>Arabidopsis thaliana</u>	overcomes long-day requirement	K (Vit E)	Michniewicz, M. & A. Kamińska (1965) Naturwiss. <u>52</u> 623.
intact plants, tomato and pea	application to roots, inhibited flowering in tomato; accelerated in pea	K	Wittwer, S.H. & R.R. Dedolph (1963) Amer. J. Bot. <u>50</u> 330-336.

---

SYNTHESIS

<u>Lemna minor</u>	<u>starch</u> increased at high concentration; decreased at low	BAP, K	Hillman, W.S. (1957) Science <u>126</u> 165-166 and Tasser de Jong J.G. & H. Veldstra (1971) Plant Physiol. <u>24</u> 235-238.
detached <u>Impatiens</u> petals	anthocyanin production increased	K	Klein, A.O. & C.W. Hagen, Jr. (1961) Plant. Physiol. <u>36</u> 1-9.
tobacco tissue	<u>lignin</u> synthesis activated	K	Bergmann, L. (1964) Planta <u>62</u> 221-254.
tobacco tissue	<u>Thiamine</u> synthesis activated	K	Digby, J. & F. Skoog (1966) Plant Physiol. <u>41</u> 647-652 and Linsmaier, E.M. & F. Skoog (1964) Planta <u>62</u> 146-154
soybean tissue	<u>deoxyisoflavone</u> synthesis stimulated	general	Miller, C.O. (1969) Planta <u>87</u> 26-35.

<u>Amaranthus</u> seedlings	Betacyanin production increased	general	Kohler, K.H. & Conrad, K. (1966) Biologische Rundschau 4 36-40.
cucumber cotyledons	chlorophyll increased (in light)	BAP	Fletcher, R.A. & D. McCullagh (1971) Can. J. Bot. 51 1347-1354.
dicotyledons	cell wall metabolism altered, with failure of cells to separate in agitated culture	BAP	Halperin, W. & S. Minocha (1973) Can. J. Bot. 51 1347-1354.
leaves of intact plants	patterns of lipid fatty acids and lipid- bound sugars altered	K, Z	Kull, U. & R. Büxenstein (1974) Phytochem. 13 39-44.

#### DORMANCY AND GERMINATION

1. Seed Germination a) promoted	bean seeds	promotion	K, BAP, PhenylAP, HexylAP	Miller, C.O. (1956) Plant Physiol. 31 318-319.
	lettuce seeds	stimulation	K	Haber, A.H. & H.J. Luippold (1960) Plant Physiol. 35 168-173; 486-494.
	beech, hazel, rowan seeds	stimulation; but not for intact seeds	K (thiourea, GA)	Frankland, B. (1961) Nature 192 678-679.

b) dormancy release	apple seedlings	induced dormancy break	K	Pieniazek, J. (1964) Acta. Agrobot. <u>26</u> 157-169.
	<u>Xanthium</u> seeds	dormancy of "upper" seed broken, with RNA synthesis involved	K	Khan, A.A. (1966) Physiol. Plant. <u>19</u> 869-874.
	pear embryos	dormancy release	K (GA)	Khan, A.A. (1972) Plant growth Substances 1970, pp 207-215.
c) reversal of germination inhibition	irradiated lettuce seed	stimulation of recovery of germination	K	Haber, A.H. & H.J. Luippold (1960) Plant Physiol. <u>35</u> 168-173.
	<u>Xanthium</u> seeds	reversal of coumarin and xanthaline inhibition (light requirement)	K (red light)	Khan, A.A. & N.E. Tolbert (1965) Plant Physiol. <u>18</u> 41-43.
	lettuce seeds	reversal of far-red light inhibition (light required)	K	ibid
	lettuce seeds	reversal of (+) abscissin induced inhibition	K	Sankhla, N. & D. Sankhla (1968) Physiol. Plant <u>21</u> 190-195.

2. Bud effects	apple buds	development promoted	K, BAP	Chvojka, L., K. Vereš & J. Kosel (1961) <u>Biol. Plant.</u> <u>3</u> 140-147 and Engelbrecht, L. & K. Mothes (1962) <u>Naturwiss.</u> <u>49</u> 427.
	"	dormancy broken; O <sub>2</sub> consumption increased	BAP	Chvojka, L., M. Trávníček & M. Zakourilova (1962) <u>Biol. Plantarum</u> <u>4</u> 203-206.
	<u>Vitis vinifera</u> buds	breaks rest	K	Weaver, R.J. (1963) <u>Nature</u> <u>198</u> 207-208.
	trees, terminal buds	stimulate growth at early rest, and after rest only	K (GA)	Leike, H. (1967) <u>Flora (abt.A)</u> <u>158</u> 351-362.
3. Effect on Rest Period	potato tubers	decrease in content of growth inhibitor; rapid break in rest period	K, Z (purines)	Hemberg, T. (1970) <u>Physiol. Plant.</u> <u>23</u> 850-858. and Dutta, T.R. & D.M.K. Kaley (1968) <u>Ind. J. Plant. Physiol.</u> <u>11</u> 88-94.
SENESCENCE EFFECTS				
Retardation of symptoms	detached <u>Xanthium</u> Leaves	chlorophyll and protein loss retarded	K	Richmond, A. & A. Lang (1957) <u>Science</u> <u>125</u> 650-651.
	<u>Xanthium</u> leaf discs (etiolated)	yellowing delayed, RNA and protein levels maintained	K	Osborne, D.J. (1962) <u>Plant Physiol.</u> <u>37</u> 595-602.
	<u>Brassica</u> leaf discs	protein levels maintained	K	Kuraishi, S. (1968) <u>Physiol. Plant.</u> <u>21</u> 78-83.

intact bean plants	leaf senescence retarded	BAP	Fletcher, R.A. (1969) <u>Planta</u> 89 1-8.
corn seedling leaves	chlorophyll and protein preserved (senescence enhanced at low concentrations)	BAP	Tavares, J. & H. Kende (1970) <u>Phytochem.</u> 9 1763-1770.
barley seedlings (etiolated)	retarded protein and chlorophyll loss	K	Stobart, A.K., P.R. Shewry & D.R. Thomas (1972) <u>Phytochem.</u> 11 571-577.
oat leaf sections	chlorophyll retained	K, BAP [Z, i <sup>6</sup> ade ineffective]	Varga, A. & J. Bruinsma (1973) <u>Planta</u> 111 91-93.

Effects on Transport

promotion & mobilization	detached tobacco leaves	cell-to-cell directed transport of glycine to application site	K	Mothes, K., L. Engelbrecht & O. Kulaeva (1959) <u>Flora</u> 147 445-464.
	excised leaves in dark	induced transport	K	Mothes, K. & L. Engelbrecht (1961) <u>Phytochem.</u> 1 58-62.
	detached oat leaves	glycine and phosphate attracted to application site	K	Gunning, B.E.S. & W.K. Barkley (1963) <u>Nature</u> 199 262-265.
	corn leaves	phosphate mobilization to application site	K	Müller, K. & A.C. Leopold (1966) <u>Planta</u> 68 167-185; 186-205.

DEGRADATION AND SYNTHESIS PROCESSES (Normal, and effects in Senescence)

RNA levels	onion root nuclei	rapid increase in RNA	K	Guttman, R. (1957) J. Biophys. Biochem. Cytol. <u>3</u> 129-131.
	excised soybean hypocotyl	inhibition of synthesis, especially rRNA	K	Vanderhoeft, L.N. & J.L. Key (1968) Plant & Cell Physiol. <u>9</u> 343-351.
	peanut cotyledons	all types RNA increased, no DNA increase	BAP	Carpenter, M.B. & J. Cherry (1966) Biochim. Biophys. Acta <u>114</u> 640-642.
	barley leaves, tobacco pith culture	mRNA synthesis increased, or preserved	K	Srivastava, B.K.S. (1967) Ann. N.Y. Acad. Sci. <u>144</u> 260-278.
DNA levels	tobacco pith	rapid increase in DNA content	K (auxin)	Patau, K., N.K. Das, & F. Skoog (1957) Physiol. Plant <u>10</u> 949-966.
	onion root tips	decrease in DNA, RNA doubled	K	Jensen, W.A., E.G. Pollock, P. Healey & M. Ashton (1964) Exptl. Cell Res. <u>33</u> 523-530.
Effects on RNase	excised barley leaves	reduced activity	K	Srivastava, B.I.S. & G. Ware (1965) Plant Physiol. <u>40</u> 62-64.
	tobacco leaves	decreased activity	K (protein synthesis inhib.)	Bagi, G. & G.L. Farkas (1968) Experientia <u>24</u> 397-398

detached oat leaves	blockage of increased RNase I activity	K	Udvardy, J., G.L. Farkas & E. Marrè (1969) <i>Plant &amp; Cell Physiol.</i> <u>10</u> 375-386.
barley leaf discs	counteraction of increase in RNase and Proteinase activities	K, BAP	Atkin, R.K. & B.I.S. Srivastava (1969) <i>Physiol. Plant.</i> <u>22</u> 742-750.
darkened oat leaves	inhibition of RNase increase	K	Shibaoka, H. & K.V. Thimann (1970) <i>Plant Physiol.</i> <u>46</u> 212-220.
<u>Pisum sativum</u> epicotyls, microsomal fractions	abolishes IAA-induced increase in activity (synergistic effect)	BAP	Birmingham, B.C. & G.A. MacLachlan (1972) <i>Plant Physiol.</i> <u>49</u> 371-375.
<hr/>			
detached wheat leaves (senescing)	levels maintained, with increase in ER	K	Shaw, M. & M. Manocha (1965) <i>Can. J. Bot.</i> <u>43</u> 747-755.
excised tobacco leaves	rRNA preserved	K	Srivastava, B.I.S. (1967) <i>Ann. N.Y. Acad. Sci.</i> <u>144</u> 260-278.
excised barley leaves	rRNA and ribosomes preserved	K	Srivastava, B.I.S. & C. Arglebe (1968) <i>Physiol. Plant.</i> <u>21</u> 851-857.
Chinese cabbage leaves	rRNA, ribosomes preserved	K	Berridge, M.V. & R.K. Ralph (1969) <i>Biochim. Biophys. Acta</i> <u>182</u> 266-269.

Ribosomes;  
levels

Ribosomes; function	isolated ribosomes	cytokinin binding	K	Berridge, M.V., R.K. Ralph & D. Letham (1970) <u>Biochem. J.</u> <u>119</u> 75-84.
	pumpkin cotyledon	numbers and activity increased, enzymes not affected.	BAP	Kliachko, N.L., Yakoleva, L.A. & Kulaeva, O.N. (1973) <u>Dokl. Nauk SSSR</u> <u>211</u> 1235-1238 <u>Acad.</u> (English edition, pp 126-128)
Transcription & genome interaction	dormant pear embryos	increased activity of chromatin-bound polymerase	K	Khan, A.A. (1972) <u>Plant Growth Substances</u> 1970 pp. 207-215.
	extracts of tobacco, pea soybean cells	interaction with genetic material	K	Matthyse, A.G. & M. Abrams (1970) <u>Biochim. Biophys. Acta</u> 199 511-518. and Matthyse, A.G. & C. Phillips (1969) <u>Proc. Nat. Acad. Sci. (U.S.)</u> <u>63</u> 897-903.
Protein	tomato fruit locule plastids	increased amino acid incorporation	K	Davies, J. N. & E.C. Cocking (1967) <u>Biochem. J.</u> <u>104</u> 23-33.
	isolated tobacco chloroplasts	stimulation of syn- thesis (age-dependent effect)	BAP	Kulaeva, O.N. & E.G. Romanko (1967) <u>Dokl. Nauk. Acad. SSSR (bot.)</u> <u>177</u> 464-467 (Eng. trans. 175-177)
	mitochon- dria of <u>Vigna</u> seedlings	increase in protein specific activity	K	Bhattacharyya, J. & S.C. Roy (1969) <u>BBRC</u> <u>35</u> 606-610.

<u>Tropaeolum majus</u> detached leaves	decreased degradation, 8 levels are higher	K	Mizrahi, Y., J. Amir & A.E. Richmond (1970) <u>New Phytol.</u> 69 355-361.
darkened oat leaves	inhibition of proteolysis	K	Shibaoka, H. & K.V. Thimann (1970) <u>Plant Physiol.</u> 46 212-220.
corn seedling leaves	inhibition of protein breakdown	BAP	Tavares, J. & H. Kende (1970) <u>Phytochem.</u> 9 1763-1770.
soybean chloroplasts	increased amino acid incorporation in older leaf chloroplasts; no response in those of younger leaves	K	Marchetti, S.E. & F.J. Baron (1971) <u>Adv. Frontiers of Plant Sciences</u> 28 397-404.
tobacco cultures	inhibition of uracil and leucine incorporation	K	Nudel, V. & E.S. Bamberger (1971) <u>Plant Physiol.</u> 47 400-403.
<u>Lemna minor</u>	alterations in rates of synthesis and degradation, influenced by medium	BAP	Trewavas, A. (1972) <u>Plant Physiol.</u> 49 47-51.

---

EFFECTS ON ENZYMES

Induction and Synthesis	barley roots	K	Steinhart, C., J. D. Mann & S.H. Mudd (1964) <u>Plant Physiol.</u> 39 1030-1038.
	tyramine methylpherase; specific increase in this and not four other enzymes		

bean leaves	enhanced phosphate movement to application site	K (IAA req.)	Seth, A.K. & P.F. Wareing (1967) J. Exptl. Bot. <u>18</u> 65-77.
No mobilization			
bean leaves	no observed increase in phosphorus uptake and translocation	K	Resnick, M.E. & E.R. Montaldi (1968) Biol. Prod. Veg. <u>5</u> 99-111.
whole bean plants, senescing	no mobilization of $^{14}\text{C}$ and $^{32}\text{P}$ compounds in the whole plant	BAP	Adedipe, N.O. & R.A. Fletcher (1970) J. Exptl. Bot. <u>21</u> 969-974.
oat leaves	integrity maintained	K	Shibaoka, H. & K.V. Thimann (1970) Plant Physiol. <u>46</u> 212-220.
tobacco chloroplasts	increased leucine permeability, leading to increased incorporation	K	Richmond, A.E., B. Sachs & D.J. Osborne (1970) Physiol. Plant. <u>24</u> 176-180.
sunflower leaf discs, and cotyledons	increased uptake of $\text{K}^+$ , $\text{Rb}^+$ , $\text{Li}^+$ , but not $\text{Na}^+$	K	Ilan, I. (1971) Physiol. Plant. <u>25</u> 230-233 and Ilan, I., T. Gilad & L. Reinhold (1971) <i>ibid</i> <u>24</u> 337-341.
bean primary leaves	$\text{Na}^+$ absorption	BAP	Jacoby, B. & J. Dagan (1970) Physiol. Plant. <u>23</u> 397-403.
bean leaves	$^{14}\text{C}$ assimilates transported	BAP	Fletcher, R.A., G.A. Hofstra & N.O. Adedipe (1970) Physiol. Plant. <u>23</u> 1144-1148.

<u>Phaseolus</u> <u>hypocotyl</u> <u>discs</u>	<u><math>\alpha</math>-amylase</u> ; synthesis increased	K	Clum, H.H. (1967) <u>Plant Physiol.</u> <u>42</u> 568-572
peanut & squash seeds	<u>isocitrate lyase</u> ; partial replacement of germination stimulus for induction	BAP	Penner, D. & F.M. Ashton (1967) <u>Biochim. Biophys. Acta</u> <u>148</u> 481-485
squash seedlings	<u>proteinnase</u> ; effect of embryonic axis in induction replaced	BAP, K, Phenyl AP, BAPder.	Penner, D. & F.M. Ashton (1967) <u>Plant Physiol.</u> <u>42</u> 791-796
<u>Agrostemma</u> embryos	<u>nitrate reductase</u> ; increase in activity with nitrate induction	BAP	Kende, H., H. Hahn & S.E. Kays (1971) <u>Plant Physiol.</u> <u>48</u> 702-706 and Kende, H. & T. Shen (1972) <u>Biochim. Biophys. Acta</u> <u>286</u> 118-125
"	<u>nitrate reductase</u> ; <u>de</u> <u>novo synthesis</u> increased	BAP	Hirschberg, K., G. Hubner & H. Borriess (1972) <u>Planta</u> <u>108</u> 333-337
excised pea roots	<u>nitrate reductase</u> ; decrease in synthesis (tissue specific?)	K	Sahulka, J. (1972) <u>Biol. Plant.</u> <u>14</u> 330-336
excised pea root	<u>glutamate</u> <u>dehydrogenase</u> ; antagonism of IAA- promoted synthesis	K	ibid
carrot callus	<u>glutamate</u> <u>dehydrogenase</u> ; concentration-dependent effect	K	Werner, D. & D. Goglin (1970) <u>Planta</u> <u>91</u> 155-164

activity	(general)	<u>respiratory kinases;</u> inhibited	BAP	Tuli, V., D.R. Dilley & S.H. Wittwer (1964) Science <u>146</u> 1477-1479
tobacco callus		<u>hexose monophosphate</u> <u>shunt enzymes;</u> specific decrease with decrease in growth rates	K	Scott, K.J., J. Paly & H.H. Smith (1964) Plant Physiol. <u>39</u> 709-711
soybean callus; tobacco leaves		<u>adenine phosphoribosyl</u> <u>transferase;</u> inhibited	K	Nicholls, P.B. & A.W. Murray (1968) Plant Physiol. <u>43</u> 645-648
detached wheat leaves		<u>acid phosphatase,</u> <u>lipase, esterase,</u> <u>RNase;</u> levels altered	K	Sodek, L. & S.T.C. Wright (1969) Phytochem. <u>8</u> 1629-1640
tobacco leaf		<u>aminoacyl sRNA</u> <u>synthetase;</u> increased activity	K	Anderson, J.W. & K.S. Rowan (1966) Biochem. J. <u>101</u> 15-18
Chinese cabbage nuclei & chloroplast extracts; tobacco & carrot nuclei		<u>protein kinases;</u> activity inhibited	K	Ralph, R.K., M.V. Berridge & D. Letham (1972) Biochem. J. <u>130</u> 901-911

wheat  
seedling  
leaves

5' (3')-ribonucleotide  
phosphohydrolyase  
inhibited non-  
competitively (cyclic  
nucleotides competitive)

general for  
ribosides

Polya, G. (1974) PNAS 70  
1299-1303

distribution

lentil  
embryonic  
axis

peroxidase isozymes;  
altered distribution  
patterns

K  
(IAA, ABA)

Gaspar, T., A.A. Khan & D. Fries  
(1973) Plant Physiol. 51 146-149

#### EFFECTS WITH LIGHT

Replacement of  
light requirement

bean  
leaves  
lettuce  
seeds

effects similar to  
red light

K, BAP,  
PheAP,  
HexAP

Miller, C.O. (1956) Plant Physiol.  
31 318-319

Amaranthus  
seedlings

betacyanin synthesis

Piattelli, M., M. Giudici de Nicola  
& V. Castrogiovanni (1969) Phytochem.  
8 731-736

tobacco  
leaves

nitrate reductase  
synthesis, sub-  
stitution for light  
requirement (absolute?)

Lips, S.H. & N. Roth-Bejerano (1969)  
Science 166 ;09-110

Lemna minor

substitution for non-  
photosynthetic light  
in growth and starch  
production

BAP, K

Tasser de Jong, J.G. & H. Veldstra  
(1971) Plant Physiol. 24 239-241

synergistic

lettuce  
seeds

with light, germination  
is promoted; little  
effect in dark

K

Leff, J. (1964) Plant Physiol. 39  
299-303

altered effect	bean leaf discs	expansion promoted in the dark; inhibited in light	K	Humphries, E.C. & A.N. Wheeler (1960) J. Exptl. Bot. <u>11</u> 81-85 and Humphries, E.C. (1958) Nature <u>181</u> 1081-1082
----------------	-----------------	--	---	---

---

CYCLIC AMP EFFECTS

levels	soybean callus	levels increased with treatment	K	Brewin, N.J. & D.H. Northcote (1973) J. Exptl. Bot. <u>24</u> 881-888
	tobacco tissue	addition to cultures results in cell division and correlated cAMP level changes	K	Lundeen, C.V., H.N. Wood & A.C. Braun (1973) Differentiation <u>1</u> 255-260

---

RESPIRATION AND TRANSPIRATION

Stomatal opening	barley leaves	transpiration and opening enhanced in detached mature leaves, but not young leaves	K (GA)	Livné, A. & Y. Vaadia (1965) Physiol. Plant. <u>18</u> 658-664
	mature primary barley leaves	increased rate of opening and CO <sub>2</sub> intake	K	Meidner, H. (1967) J. Exptl. Bot. <u>18</u> 556-561
respiration	tobacco leaves	inhibition	K	Sugiura, M. (1963) Bot. Mag. Tokyo <u>76</u> 359-362
	soybean callus	inhibition; concentration dependent effect; adenine reversal	Z, K	Moore, T.S., Jr., & C.O. Miller (1972) Plant Physiol. <u>50</u> 594-598

II. Plant Diseases

Fungal

<u>Taphrina deformans</u> in peach leaves	production of IAA- and kinetin-like substances implicated in neoplastic growth induction	endog.	Sommer, N.F. (1961) <u>Physiol. Plant.</u> <u>14</u> 460-469
<u>Erysiphae</u> (powdery mildew)	development on cucumber leaves checked completely	K	Dekker, J. (1963) <u>Nature</u> <u>197</u> 1027-1028
<u>Uromyces</u> , & <u>Botrytis</u>	no effect	K	ibid
<u>Helminthosporium</u> in excised oat leaves	synergistic enhancement of toxic effect of vitorin toxin	K	Luke, H.H. & H. Wheeler (1966) <u>Phytopath.</u> <u>56</u> 138-139
<u>Verticillium albo-atrum</u> on potato roots	decrease in pectolytic enzyme of the fungus	K, BAP	Patil, S.S. & A.E. Dimond (1968) <u>Phytopath.</u> <u>58</u> 868-869
<u>Agrobacterium tumefaciens</u> infecting <u>Vinca rosea</u>	Crown Gall Tumor; implied in tumor induction	endog.	Wood, H.V. & A.C. Braun (1967) <u>Ann. N.Y. Acad. Sci.</u> <u>144</u> 244-250
<u>Corynebacterium fascians</u>	Fasciation disease; symptoms duplicated	K, BAP, PheAP, PheUreas	Thimann, K.V. & T. Sachs (1966) <u>Amer. J. Bot.</u> <u>53</u> 731-739 and Klämbt D., G. Thies & F. Skoog (1966) <u>Proc. Nat. Acad. Sci. (U.S.)</u> <u>56</u> 52-59

Viral

Tobacco Mosaic, in tobacco leaves	lesions reduced; virus production stimulated	K, KR, BAP, ipAdo	Milo, G.E. & B.I.S. Strivastava (1969) <u>Virol.</u> 38 26-31
Tobacco Mosaic, in bean leaves	inhibition of lesions and viral production	"	ibid
Turnip Yellow Mosaic, in Chinese cabbage	viral protein production maintained, but intact particles reduced	K	Berridge, M.V. & R.K. Ralph (1969) <u>Biochim. Biophys. Acta</u> 182 266-269
Tomato Spotted Wilt, on petunia leaves	number and size of lesions reduced	K	Selman, I.W. (1964) <u>Ann. Appl. Biol.</u> 53 67-76
Tobacco Mosaic, in tobacco leaf discs	viral production inhibited	K	Kiraly, Z. & J. Szirmai (1964) <u>Virol.</u> 23 286-288
Tomato aucuba mosaic	viral production stimulated in detached leaves, in attached leaves either stimulated or inhibited, dependent on age, and concentration	K	Daft, M.J. (1965) <u>Ann. Appl. Biol.</u> 55 51-56

III. In Lower Plants, Fungi, Micro-organisms

MOSSES	<u>Tortella caespitosa</u>	budding induced	general	Gorton, B.S., C.G. Skinner & R.E. Eakin (1957) Arch. Biochem. Biophys. <u>66</u> 493-
	<u>Funaria hygrometrica</u>	budding induced; target cell interaction	BAP	Brandes, H. & H. Kende (1968) Plant Physiol. <u>43</u> 827-837
FERNS	<u>Marsilea drummondii</u> (water fern)	induction of copious root growth in aseptic culture	K	Allsop, A. & A. Szweykowska (1960) Nature <u>186</u> 813-814
ALGAE	<u>Spirogyra longata</u>	promoted cell division; increased elongation	K	Olszewska, M.J. (1958) Bull. Soc. Sci. Lettres Lodz. Classe III <u>9</u> No. 1 1
	marine algae (mixed)	both stimulation and inhibition of growth in culture	endog.	Bentley-Mowat, J.A. & S.M. Reid (1968) Ann. Bot. <u>32</u> 23-32
	<u>Volvox</u>	possible role in growth and spheroid development	endog.	Van Staden, J. & C.M. Breen (1973) Pl. Sci. Lett. <u>1</u> 325-330
FUNGI	<u>Melampsora lini</u>	required in medium for aerial mycelium development in culture of rust-infected flax leaves	coconut milk	Turel, F.L.M. & G.A. Ledingham (1957) Can. J. Micro. <u>3</u> 813-819
	<u>Saccharomyces</u> with IAA, greatly slowed growth rate		K	Kennell, D. (1960) Exptl. Cell. Res. <u>21</u> 19-33

<u>Neurospora</u> <u>crassa</u> (infertile strains)	increased perithecial production in crosses	K	Lee, B.O. (1961) Nature <u>192</u> 288.
<u>Acetabularia</u> <u>mediterranea</u>	regeneration of frag- ments inhibited	K	Brachet, J. (1958) Exptl. Cell Res. <u>14</u> 650-651
"	concentration- dependent effect on stem formation	K	Zetsche, K. (1963) Planta <u>59</u> 624-634
<u>Acetabularia</u> <u>crenulata</u>	differentiation af- fected; shorter stalk, cap for- mation stimulated	K	Spencer, T. (1968) Nature <u>217</u> 62-64
<u>Bruceella</u> <u>abortus</u> , pneumococcus	selective effect in change in population from non-virulent to virulent	K	Braun, W., W. Firshein & J. Whallen (1957) Science <u>125</u> 445-447
<u>E. coli</u>	cell division rate increases	K	Kennell, D. (1960) Exptl. Cell Res. <u>21</u> 19-33
<u>Bacillus</u> <u>megatrium</u> , <u>Agrobacterium</u> <u>tumefaciens</u>	growth stimulated; no change in morphology	K	Maruzzella, J.C. & J.G. Garner (1963) Nature <u>200</u> 385.

BACTERIA

<u>E. coli</u> ,	some growth stimulation	K	Maruzzella, J. C. & J. G. Garner (1963) Nature 200 385.
<u>S. aureus</u> ,			
<u>Erwinia</u>			
<u>carotovora</u>			
<u>Cl. michae-</u>	inhibition of growth	K	Maruzzella, J. C. & J. G. Garner (1963) Nature 200 385.
<u>gensis</u>			
<u>Cl. thermo-</u>	cellulose digestion	K	Quinn, L.Y., R.P. Oates & T.S. Beers (1963) J.Bact. 86 1359.
<u>cellum</u>	permitted without added yeast extract		

---

IV. Animals

LOWER ANIMALS

<u>Paramecium</u>	generation time short-	K	Guttman, R. & A. Back (1960) Science 131 986-987
<u>caudatum</u>	ened especially at sub-optimal conditions		
Planaria	irreversible altera-	K	Henderson, T. H., C. G. Skinner & R. E. Eakin (1962) Plant Physiol. 37 552-555.
	tion in differentiated tissues via xanthine oxidase effect	& analogs	
Hydra	regeneration affected (inhibition)	6-sub.- purines (ade)	Skinner, C. G., W. Shive, R. G. Ham, D. C. Fitzgerald Jr., & R. E. Eakin (1956) J. Amer. Chem. Soc. 78 5097-5100.

---

HIGHER ANIMALS

Whole Animals	high rate of cell division induced on injection	K	Ogawa, Y., Y. Abe & K. Fujioka (1957) Nature 180 485-486.
rats with Yoshida tumor cell transplant			

mice	radioprotective effect for survival seen on injection	ipAdo	Prasad, N., S. C. Bushong & H.L. Barton (1971) <u>Experientia</u> <u>27</u> 1332-1333.
mice immunized with sheep red blood cells	inhibition of immune response	ipAdo	Diamanstein, T., B. Wagner & A. S. Bhargava (1971) <u>FEBS Lett.</u> <u>15</u> 225-228.
Cell Cultures			
skin cultures	retarded outgrowth of epithelium; no effect on cells of carcinoma or cystic disease	K	Orr, M. F. & B. McSwain (1960) <u>Cancer Res.</u> <u>20</u> 1362-1364.
<u>Triturus viridescens</u>	stimulated mitosis in mucosa of alimentary tract	K	Buckley, W. B., E. R. Witkus & C. A. Berger (1962) <u>Nature</u> <u>194</u> 1200-1201.
Sarcoma-180 cells	cytotoxic, growth inhibition	ipAdo	Grace, J. T., M. T. Hakala, R. H. Hall & J. Blakeslee (1967) <u>Proc. Amer. Assoc. Cancer Res.</u> <u>8</u> 23.
human and rat cells; PHA stimulated	mitosis inhibited at $10^{-5}M$ ; stimulated at $10^{-7}M$	ipAdo	Gallo, R. C., J. Whang-Peng & S. Perry (1969) <u>Science</u> <u>165</u> 400-402.
Gastroin-testinal mucosa, lymphoid tissues	inhibition of growth, <u>in vitro</u> and <u>in vivo</u>	ipAdo	Suk, D., C. L. Simpson & E. Mihich (1970) <u>Cancer Res.</u> <u>30</u> 1429-1436.

rat spleen cells PHA stimulated	inhibition, via effect on tRNA methylase	ipAdo	Hacker, B. & T. L. Feldbush (1971) Cancer <u>27</u> 1384-1387.
human lymphocytes PHA stimulated	inhibition of DNA and RNA synthesis (immunosuppression)	ipAdo	Hacker, B. & T. L. Feldbush (1971) Cancer <u>27</u> 1384-1387.
derived cell line, RP 6410	inhibition at $3 \times 10^{-6}M$ , not at $2 \times 10^{-6}M$	ipAdo	Rathbone, M. P. & R. H. Hall (1972) Cancer Res. <u>32</u> 1647-1650.

#### V. General Effects

tRNA function	<u>E. coli</u> tyr-tRNA	altered base in anticodon region affects ribosome binding	ms <sup>2</sup> ipa	Gefter, M. L. & R. L. Russell (1969) J. Mol. Biol. <u>39</u> 145-157.
tRNA enzymes	soybean cotyledons	altered Leu-tRNA and synthetase function	BAP	Anderson, M. B. & J. H. Cherry (1969) Proc. Nat. Acad. Sci. (U.S.) <u>62</u> 202-209.
	<u>E. coli</u> & calf spleen enzymes	inhibition of tRNA methylases	ribosides of K, BAP, Z	Wainfan, E. & B. Landesberg (1971) FEBS Lett. <u>19</u> 144-148.

Abbreviations used for specificity: K, kinetin; BAP, benzylaminopurine; Z, zeatin; HexAP, hexylaminopurine; PheAP, phenylaminopurine; i<sup>6</sup>ade, isopentenyladenine; ipAdo, isopentenyladenosine; ms<sup>2</sup>ipa, isopentenyl-2-methylthioadenosine; ade, adenine; IAA, indole acetic acid; GA, gibberellic acid; ABA, abscisic acid. "Endog." refers to uncharacterized endogenous cytokinin compounds. "General" indicated an effect common to most cytokinins tested. Notations in brackets refer to factors interfering or producing similar effects.

abscisic acid (see Hill 1973). Each of these groups are characterized by their chemical form, and the biological effects attributed to its members. In the whole plant or organism the concentration of the other substances, and their distribution, will, however, determine how an added substance such as cytokinin will affect the system.

An example of such complex interactions is seen in the growth of tobacco pith cells in tissue culture, which requires the presence of both an auxin and a cytokinin in the medium. Auxin alone resulted in cell enlargement without division; kinetin alone produced no growth (Skoog & Miller 1957). Modifications in the auxin to kinetin ratio resulted in varying responses of growth and differentiation, with roots being produced at low kinetin concentrations. As the kinetin levels were increased, a progression through large-celled undifferentiated callus, leafy bud-like outgrowths on callus, and small-celled undifferentiated callus was observed. Interactions between cytokinins and auxins are also seen in the apical dominance effect in stems, where increased cytokinin levels relieve the auxin-mediated inhibition of axillary bud development (Wickson & Thimann 1958).

Some of the other effects associated primarily with cytokinin action which have been examined in some detail are of interest for considering mechanisms of action. This is particularly so for studies of cytokinin effects on macromolecular synthesis and degradation, where the action of cytokinins can be observed on biochemical processes.

Senescence and synthesis effects

Detached leaves undergo a rapid aging process, with loss of chlorophyll and protein. Richmond and Lang (1957) reported on the effect of kinetin in delaying the appearance of senescence in detached Xanthium leaves, with a slowing in the rate of protein level decline. Mothes and Engelbrecht (1961) described the effect of kinetin in attracting metabolites to areas of application on an excised leaf, and suggested that increased synthesis could be taking place at these locations due to the availability of metabolites. Osborne (1962) reported that kinetin treatment prevented the decline in nucleic acid, protein and chlorophyll levels associated with senescence, and suggested that the stimulation of synthesis or maintenance of levels was the essential feature of kinetin action against senescence. In particular, a primary action at the level of mRNA synthesis was favoured (Osborne 1965). Studies involving increased incorporation of precursors as indications of cytokinin enhanced synthesis have, however, been criticized on the basis that the rate of incorporation can be affected by pool sizes, or the rate of concomitant degradation (Tavares & Kende 1970, Kende 1971, Trewavas 1972).

Tavares and Kende (1970) examined the effect of benzyladenine on protein synthesis and degradation in corn leaf discs. Prelabelled protein in the discs showed slower breakdown in the presence of cytokinin, while the specific activity of the protein

did not change, indicating no great enhancement of synthesis of new, unlabelled protein due to cytokinin. This supports the conclusions of Kuraishi (1968) that the rate of protein degradation is the factor affected by kinetin in discs of senescing Brassica leaves. Similarly, increased levels of DNase, RNase, and peptidase were reported to occur in senescing barley leaves, and kinetin was found to somewhat suppress these increases (Balz 1966, Atkin & Strivastava 1969). Other evidence also indicates that RNase activity increases with senescence, and that cytokinins influence this increased activity (Shibaoka & Thimann 1970, Dove 1972). These results all suggest that cytokinins act by prevention of degradation rather than promotion of synthesis in senescing leaves.

The effects of cytokinins on senescing leaves has been studied extensively by Thimann and co-workers, in order to try and determine the mechanism of cytokinin action. The amino acid, L-serine was found to promote the loss of chlorophyll in leaf assays, counteracting the effects of cytokinin, auxin, or adenine. (Shibaoka & Thimann 1970). Proteolysis in the leaves was promoted by serine, and it was suggested by these authors that this might be due to the presence of serine at the active site of proteolytic enzymes. In terms of the action of kinetin in this system, Thimann, Shibaoka and Martin (1972) suggested that it might promote the synthesis of a protein component that specifically inhibited proteinase formation. In this model, serine should antagonize the kinetin by enhanced proteinase formation, with that enzyme able to hydrolyze the kinetin-induced protein. To investigate this model, Martin and

Thimann (1972 a, b) examined the formation of protease in senescing oat leaves. Two proteinases of leaves showed an increase in activity during senescence; an increase that was enhanced by serine and opposed by kinetin and by cycloheximide. The serine enhancement of senescence particularly in the presence of kinetin, and the senescence effect itself, was prevented by cycloheximide, thus linking protein synthesis to the expression of senescence effects. These studies supported the concept of serine being incorporated into a proteolytic enzyme, or perhaps acting as an inducer of the enzyme, and of the acting of cytokinins in preventing the formation of the enzyme and thus retarding senescence (Martin & Thimann 1972 b). These authors stressed the importance of interactions between cytokinins and amino acids, and reported on studies of the involvement of serine, arginine, cysteine, alanine and glycine in senescence effects. These various antagonistic actions appear to work in a complex of levels, which may reflect multiple regulatory controls on the overall process.

Studies by Trewavas (1972 ) , on protein synthesis and degradation rates in Lemna minor indicate that turnover rate constants in general, and in the presence of benzyladenine, are affected by the composition of the medium. In sucrose-mineral salts medium, benzyladenine increases the rate constant of synthesis; in water, it alters only the rate constant of degradation, reducing it. In water, then, the effect is in agreement with the concept that in senescing leaves cytokinins act by reducing the degradation rate. Of interest in these studies on L. minor is the fact that

the greatest effect on growth rate, as measured by frond number, results from the omission of calcium from the medium. All mineral deficiencies, especially nitrate, calcium, and sulfate, cause reduced synthesis rates and increased degradation rates. This may be of significance at the molecular level of cytokinin action, particularly in view of the effects observed for cytokinins on calcium transport in Achlya (LéJohn & Cameron 1973).

#### Genome interactions and RNA synthesis

While the senescence studies do link maintained levels of RNA to the prevention of RNase activity, in other cases there appears to be actual increases in synthesis. For several plant hormone groups there is evidence to implicate hormone-genome interactions in alterations in RNA synthesis rates or patterns. Increases in RNA synthesis in response to gibberellins have been reported in pea nuclei (Johri & Varner 1968) and in response to auxins in coleoptiles of Avena and rice (Biswas & Sen 1959) and coconut endosperm nuclei (Mondal et al 1972). Guilfoyle & Hanson (1974) reported that RNA synthesis stimulation by synthetic auxin is due to the activation of ribosomal RNA polymerase to produce longer RNA chains.

Further examination of these responses have shown that the hormone effect is mediated by a reactive protein which appears to produce specific binding with the chromatin template in an in vitro

system (Matthysse 1970, Matthysse & Phillips 1969, Mondal et al 1972, Johri & Varner 1968). A similar protein factor from chromatin of peas was found to interact with kinetin or with zeatin, to allow stimulation of RNA synthesis in isolated systems (Matthysse & Abrams 1970). These workers reported that addition of this factor allowed stimulation of RNA synthesis by kinetin, from both purified DNA as well as chromatin template. It was suggested that the protein-hormone complex must recognize some part of the DNA and then act to facilitate some process of RNA synthesis. This proposal of Matthysse and Abrams (1970) is a similar one to that made later by Mondal et al (1972) to account for the action of auxin on RNA synthesis.

#### Enzyme synthesis

The induction of the synthesis of enzyme in response to cytokinins is of interest as resembles the hormone-signaled responses in animal cells. Some of the enzymes induction processes observed in response to plant hormones have already been mentioned in connection with cyclic AMP effects in plants.

Cytokinins have been demonstrated to affect the enzymes of nitrogen metabolism in plants, a fact of interest in view of the studies on glutamate dehydrogenase in Achlya. Nitrate reductase activity in plants is induced by nitrate concentrations, and influenced by environmental factors such as light intensity (Beever

& Hageman 1969). Borriss (1967) reported that cytokinins, but not gibberellins or auxin, increased nitrate reductase activity in embryos of Agrostemma githago. It had been thought that light was a requirement for nitrate reductase synthesis, and Lips and Roth-Bejerano (1969) reported that cytokinin replaced light in the process in tobacco leaves. Light now appears to be related to induction by general effects on the protein synthesis machinery (see Knypl 1973), but this was an indication of different modes of action by two compounds affecting enzyme activity. Kende et al (1971) showed that the effects of benzyladenine and nitrate on Agrostemma githago were additive and distinct from each other. Both in the Agrostemma system and in studies on cucumber cotyledons, the inhibitory effect of cycloheximide was much greater on the cytokinin-induced activity than on that induced by nitrate. Although it is possible that the induction process could involve activation of a pre-existing nitrate reductase, it appears that there is de novo enzyme synthesis in response to nitrate in tobacco tissue (Zielke & Filner 1971) and to cytokinins in Agrostemma embryos (Hirschberg et al 1972). Overall protein synthesis was examined by Kende and Shen (1972) in this last system, and it was found that the induced enzyme activity was not correlated with, and thus creditable to, an overall increase in general protein synthesis. It is not clear whether the cytokinins are specifically acting at the gene transcription level, as suggested by Knypl (1973), but the rapid response to cytokinins observed in such systems as Agrostemma provide useful studies of cytokinin effects at the biochemical level.

In contrast to these studies of nitrate reductase, another study of nitrogen-linked enzymes reported by Sahulka (1972) suggests that in excised pea roots kinetin acts to decrease nitrate reductase activity. In this same study, kinetin was found to act as an antagonist of IAA-promoted glutamate dehydrogenase activity, but to have no effect alone. In carrot callus, kinetin increased glutamate dehydrogenase activity at low concentrations, but decreased it at concentrations unfavourable for growth (Werner & Goglin 1970). Sahulka (1972) suggested that cytokinin effects on nitrogen-linked enzyme activities may be a contributing factor in the inhibition of root growth by kinetin. However, he also has pointed out that growth regulator actions on enzyme activities vary with the enzyme examined, the incubation period of the cytokinin with the tissue, and the combinations of growth regulators used.

#### Hormone receptors in moss

A clearly defined model has been proposed for the mechanism of action of hormones on animal cells, as was described in considering cyclic AMP effects. The interaction of cytokinins with moss cells may provide a model system for studies of plant hormone mechanisms. Cytokinins have been shown to induce or enhance bud formation in mosses (Gorton et al 1957, Bopp 1968) and endogenous cytokinins have been demonstrated to occur in these organisms (Bauer 1966, Klein 1967, Beutelmann 1973).

Brandes and Kende (1968) prepared autoradiographs of Funaria hygrometrica protonemata exposed to radioactive benzyladenine, which showed an accumulation of the cytokinin in "target" cells. These, they proposed, could possess binding sites that made the cells responsive to the hormone. Existence of such sites could explain the mechanism by which cells dedifferentiate and lose sensitivity to cytokinins (Bopp & Diekmann 1967). Sensitivity thus requires prior formation of specific sensitive sites or cells. The action of the hormone requires that it be present during a critical period of time; washing off the hormone at different stages resulted in varying degrees of reversal of budding (Brandes & Kende 1968). The intracellular effects that eventually result in bud production have not yet been established. Presumably these involve effects at the biochemical level, and some studies suggest gene activation effects, with alterations in the RNA produced (Schneider et al 1969).

Recent reports by Menon and Lal (1974) suggest that kinetin is not the major factor in regulation of bud induction in Physcomitrium pyriforme, and that in fact a wide variety of environmental and other factors, including abscisic acid, are interacting to select from the various possible morphogenetic responses. Indeed, a response is seldom the result of a single isolated stimulus, and there have been other reports of moss differentiation being affected by various growth hormones (Maltzahn 1959, Szweykowska 1962, Johri & Desai 1973, Sood & Chopra 1973).

Plant pathogenesis

Disease symptoms in plants may be the result of host growth responses to altered hormonal balances resulting from pathogenic attack. Two considerations need to be taken into account in relating the effects of a pathogen to cytokinins. The first is that, as in normal growth, the observed effects of cytokinins on a plant involves their overall interactions with other types of plant hormones. The second consideration is that an observed alteration in the level of cytokinins in infected tissues, such as reported for rust-infected bean leaves (Király et al 1967), may a reflection of host response to infection as much as a pathogen-produced cause of the disease symptoms.

There are some cases in which disease symptoms are shown to be related to the presence of cytokinins, or are duplicated by the application of cytokinins. Kinetin treatment produces the symptoms of "fasciation disease", which is a result of Corynebacterium fascians infection (Thimann & Sachs 1966). It was reported that isopentenyladenine was produced by this organism (Helgeson & Leonard 1966, Klämbt et al 1966) , but subsequent indications were that it may be the result of transfer RNA hydrolysis during isolation procedures ( Rathbone & Hall 1972).

Isopentenyladenine was also reported to occur in the bacterium Agrobacterium tumefaciens (see Kende 1971) which is capable of initiating crown gall tumour growth by means of a "Tumour Inducing

Principle". The mechanism of action of T.I.P. is not known. Johnson et al (1970) examined the possibility that the ability of tumour cells to produce cytokinins resulted in more cytokinin groups in the transfer RNA of infected cells, with a resulting rapid growth rate in tumours. However, the 4S RNA fractions of normal and tumour cells showed no detectable differences when used in bioassays, supporting the earlier conclusion that cytokinin-induced growth responses are not mediated by the transfer RNA molecules.

Cytokinins produced by parasites such as fungi (Shaw & Samborski 1956) or insect larvae (Engelbrecht 1971) appear to be responsible for the mobilization of nutrients to "green islands" formed on leaves at the infection point. Thimann and Sachs (1966) have suggested that this might be the "evolutionary sense" for cytokinin production by parasites. Root infections by mycorrhizal fungi may also be related to the demonstrated production of cytokinins by such organisms as Rhizopogon roseolus and Amanita rubescens (Miller 1967).

M A T E R I A L S   A N D   M E T H O D S

## MATERIALS AND METHODS

ORGANISMS AND GROWTHA. Classification and Life Cycle

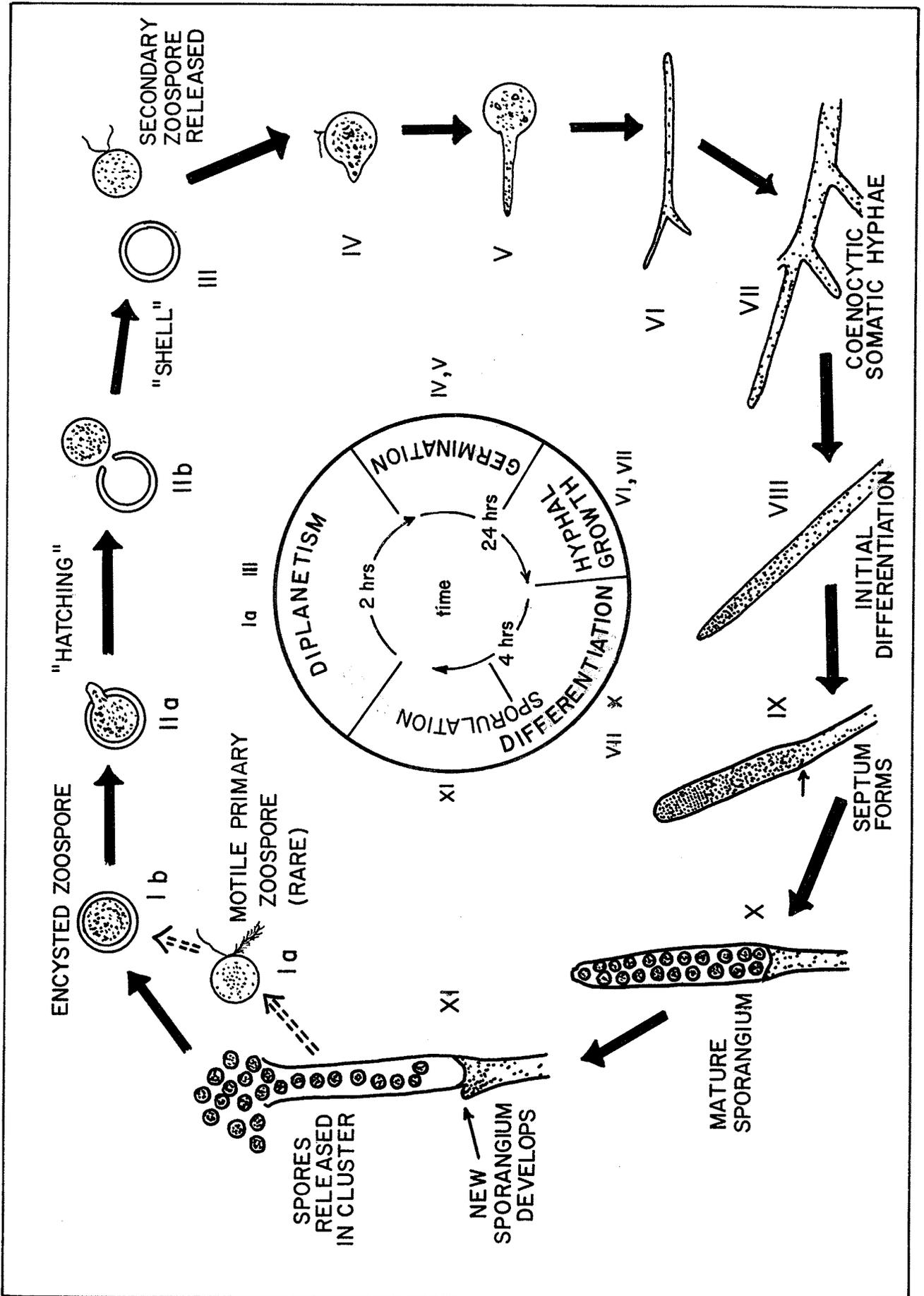
Most of the studies reported here were carried out on a species of water mould, Achlya, which is further described below. Detailed studies of the glutamate dehydrogenase found in Pythium debaryanum were also performed, and are reported along with the results for the Achlya enzyme. Both these organisms belong to the Oömycetes.

1. Achlya: Achlya species, (order, Saprolegniales) generally are found in fresh water and soil, with some forms causing root and seed rot in higher plants (Webster et al 1970). The species of Achlya used in these studies has been designated Achlya sp. (1969), and was obtained from Dr. J. S. Lovett, Purdue University, U.S.A. This is a homothallic species, distinguished by profuse sporulation when grown at 24°C in stationary culture for 30 hours. This feature made it particularly amenable for study.

Hyphal growth can occur in two forms; a rhizoidal form entering the substratum, or a coenocytic branched form on top of the substratum. The sporangia and sexual reproductive organs would generally appear on the latter form (Alexopoulos 1962). The sexual cycle in which oögonia and antheridia form on the hyphae has been extensively studied, particularly with regard to the steroid hormone signals involved in the regulation of the process (Barksdale 1969). The studies described here are restricted to the asexual cycle, during which the coenocytic hyphae produce sporangia and zoospores.

The stages of the asexual life cycle have been examined under the culture conditions used in this laboratory, and described by Cameron and LéJohn (1972 a). The developmental pattern is presented in Figure 1. Spores released from the sporangium are generally encysted (Stage I b), and only rarely is a motile primary zoospore (I a) observed to be released with the cysts. A few minutes after release, the cysts "hatch" by forming a vesicle into which the protoplasm of the encysted zoospore is rapidly emptied (II a and II b). A motile secondary zoospore is formed; this is kidney-shaped, with flagella in the lateral depression (III). The process of producing two successive spore stages without an intervening vegetative phase is referred to as diplanetism (see Snell & Dick 1971). During germination, a definite germ tube is produced (IV), followed by elongation of the tube (V), and production of a

Figure 1: A schematic summary of the asexual life cycle of the strain of Achlya used in these studies. The times indicated for each stage (inner circle) represent growth under laboratory conditions. The scales used in illustrating the different stages vary.



second germ tube at the opposite pole of the spore. This hyphal tube elongates and branches (VI to VII), but remains coenocytic, devoid of cross septae. During differentiation, protoplasm and nuclei stream to the tips of hyphae (VIII), and a septum forms about 150-200  $\mu\text{m}$  from the tip (IX) to form a cell which matures into a sporangium (X). During maturation a series of protoplasmic cleavages occur, with each nucleus in the sporangium enclosed in a cyst. These clustered cysts are released at the tip of the sporangium, and a new differentiated structure may begin to form within the emptied sporangium (XI).

2. Pythium: Pythium debaryanum, (order, Peronosporales), was obtained from the American Type Culture Collection (ATCC 9998), and maintained on agar slants. The hyphae of this organism are also coenocytic, but the species did not produce asexual spores with the same ease as did the previously described Achlya species. A sexual life cycle also occurs in Pythium (see Alexopoulos 1962).

#### B. Media for Growth

The following media and buffers were the most commonly used during the course of these studies.

##### 1. G<sub>2</sub>Y Medium:

5.0 g glucose

0.5 g yeast extract

..... to 1 litre with tap  
distilled H<sub>2</sub>O

This medium was used as a liquid medium in plates, carboys, and Roux flasks, or with added agar (20 g/l) as slants.

2. GT<sub>2</sub>Y Medium:

5.0 g glutamate (monosodium)

0.5 g yeast extract

..... to 1 litre with  
tap-distilled H<sub>2</sub>O

3. PY Medium:

1.0 g peptone

1.0 g yeast extract

..... to 1 litre with  
tap-distilled H<sub>2</sub>O

PYG and PYGT media have, in addition, 5 g/l glucose or glutamate respectively.

4. Czapek Dox Medium (CZ):

This is a commercial medium from Oxoid Ltd., London, England, containing per litre: NaNO<sub>3</sub> (2.0 g), KCl (0.5 g), Magnesium glycerophosphate (0.5 g), FeSO<sub>4</sub> (0.01 g), K<sub>2</sub>SO<sub>4</sub> (0.35 g), Sucrose (30.0 g). The pH is 6.8 approximately.

5. Defined Medium (Achlya)

This medium was prepared according to the procedure described by Barksdale (1963). The components are: sodium glutamate (500 mg), L-cystine (1 ml of a 20 mg/ml HCl solution), KH<sub>2</sub>PO<sub>4</sub> (1.5 mM), KCl (2.0 mM), MgSO<sub>4</sub> (0.5 mM), CaCl<sub>2</sub> (0.5 mM), sodium EDTA (10 mg), Tris-base (1.2 g), glucose (500 mg), and 10 ml of Metal Mix 4; in 1 litre Millipore distilled water. Metal Mix 4 consists of 100 ml freshly

prepared solution containing 100 mg sulfosalicylic acid and 200 mg of a ground mixture of  $\text{Fe}(\text{NH}_4)_2 \cdot (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  (28.9 g),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , (8.8 g), and  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (3.1 g). The glucose was autoclaved separately from the other components and was added prior to use, as was the  $\text{CaCl}_2$  on some occasions. The mixture was adjusted to pH 6.8 prior to sterilizing.

6. General Buffer: (for enzyme purification procedures)

50 mM Tris-acetate  
 10 mM Potassium Phosphate (mono and di-basic)  
 1 mM EDTA  
 ..... at pH 7.3

Dithiothreitol (DTT) was added just prior to use when required ( $10^{-4}$  M).

7. Transport Buffer:

5 mM Tris-acetate (pH 7.0)  
 1 mM NaCl  
 1 mM KCl  
 5 mM glucose

In transport studies, a double strength buffer was used to suspend the cells, and the final concentration of the buffer when added to an equal volume of assay mixture was as given above. This concentration was also used as the rinse buffer.

C. Growth in Culture

1. Plate cultures: Achlya hyphal mats were grown in 20 ml volumes of liquid medium in 9 cm diameter plastic petri dishes. In  $\text{G}_2\text{Y}$  medium, hyphal mats would show good sporulation within 48 hours at  $20^\circ$  to  $24^\circ$  C. Inoculum for the plates was 2 ml of a spore sus-

pension, prepared by shaking week-old hyphal mats from G<sub>2</sub>Y plates in flasks of sterile distilled water (approximately three mats per 100 ml). Week-old mats were used in this process as they did not fragment and could be easily removed from the spore suspension. Pythium cultures were carried in much the same manner, using shredded mycelial mats from 3-day old cultures growing in CZ medium rather than spores as the inoculum.

2. Carboys: To obtain large volumes of hyphal material for enzyme purification procedures, cultures were grown in carboys under forced aeration. These carboys contained between 5 and 10 litres of medium with a few drops of antifoam A (Dow Chemical Co.) to reduce foaming. The medium in the carboys could be G<sub>2</sub>Y, but for isolation of glutamate dehydrogenase either PY or PYGT medium was used to induce higher levels of the enzyme. An inoculum for Achlya consisted of either spores or 2-day old mats, and for Pythium, shredded mats. Carboys were grown for two days at 20° C, after which the cellular material was collected by filtering with suction onto Whatman No. 1 filter paper discs, and washing with the General Buffer. Mycelial mats were then weighed, and frozen at -20° C if not used immediately.

3. Large-scale germling preparations: For studies of transport and cytokinin effects, large scale spore production was obtained by growing Achlya mats from 10 ml spore inoculum added to 90 ml G<sub>2</sub>Y medium in Roux flasks. After incubation for two days

at 24° C, the flasks were shaken vigorously by hand (approximately 30 sec) to liberate spores from the hyphal material. Contents of the bottles were poured aseptically through eight layers of cheesecloth, into a 2.8 litre Fernbach flask. To provide the optimal surface area, five to ten Roux flask cultures were collected in each Fernbach flask. Five Roux flasks will provide about 140 ml of germling suspension at the density used in transport studies.

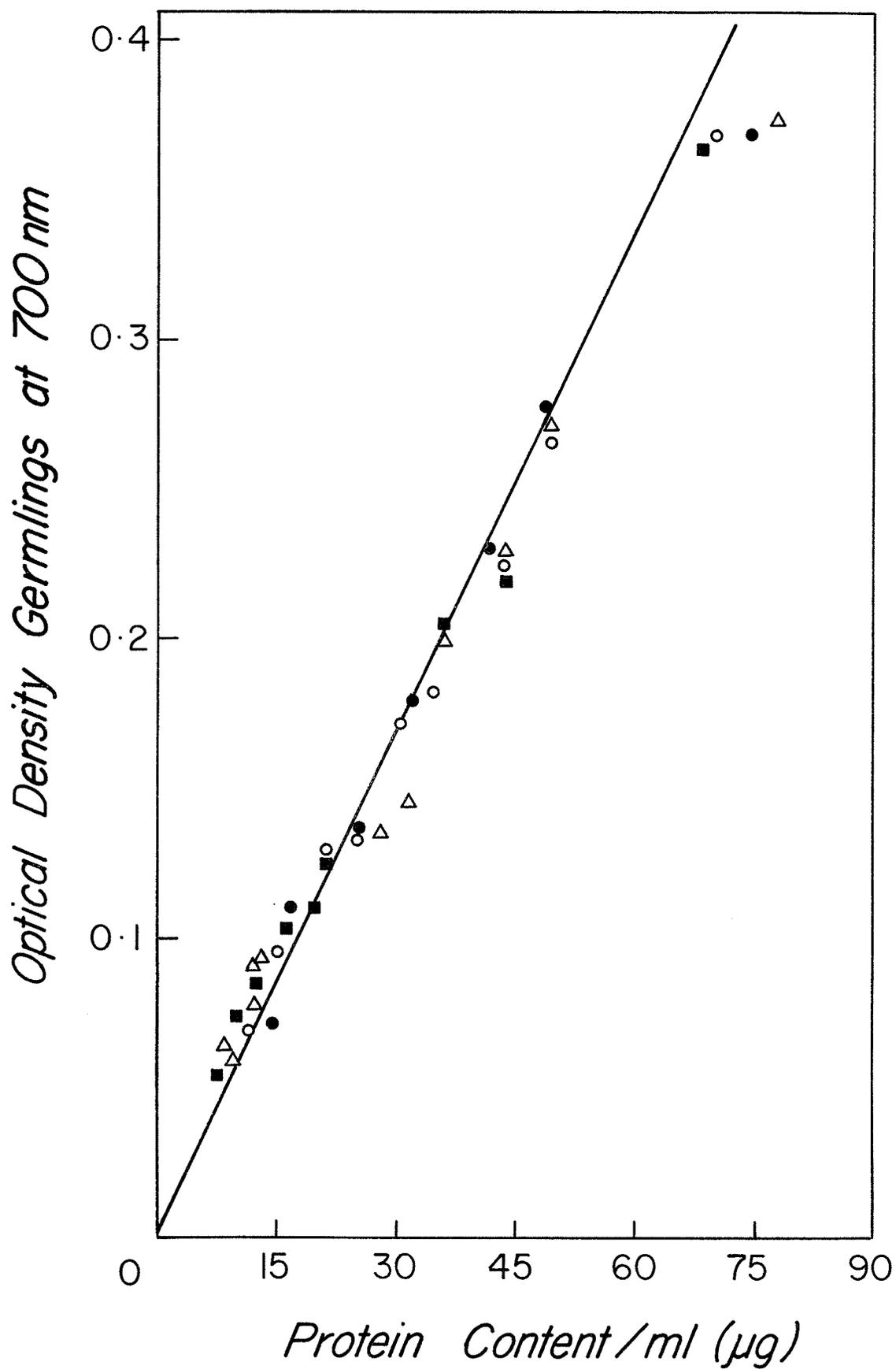
The spore suspension in spent medium was diluted by the addition of 250 ml sterile water to the spore suspension from 5 Roux flask cultures. The cheesecloth and trapped mycelia were discarded, and the Fernbach flask was covered with a sterile cotton plug. The spores were allowed to germinate by stationary incubation for 15 hours at 11° C. During this period, the residual metabolites in the spent medium provided nutrients for spore germination. Each spore produced a single long germination tube (germling) of about 150  $\mu\text{m}$  in length. Germination under these conditions was usually better than 80% (Cameron & LéJohn 1972a).

Just prior to use, the germlings were collected by filtering the suspension through a nylon mesh cloth, 61  $\mu$  pore size, obtained from Henry Simon Ltd., Stockport, England. Alternatively, 12 layers of cheesecloth is adequate for collecting these germlings. After rinsing the germlings with water or fresh medium, the germlings were resuspended in the appropriate medium or buffer. The suspension was stirred using a magnetic stirrer, then refiltered through one layer of cheesecloth to remove large clumps of cells.

Stirring was continued during the course of experiments.

Direct counts of germlings in the suspension are not practical for obtaining standardized germling concentrations prior to assays. In order to prepare uniform suspensions, a calibration curve relating the linear relationship between optical density and protein content was prepared and used routinely. The correlation was made with protein content because viability is about 80% of the cell number. The standard curve for this correlation was prepared by measuring the optical density of various germling suspensions at  $A_{700}$ , using a Gilford recording spectrophotometer, then measuring the soluble protein concentration of cell extracts prepared from the cell suspensions by the Lowry (1951) procedure. To prepare these extracts, cells were filtered through  $0.45 \mu\text{m}$  membrane filters, frozen in  $\text{N}_2$ , triturated and suspended in 1 ml of 10 mM Tris-acetate (pH 7.0) buffer (pH 7.0) before sonication (Insonator Model 1000, Ultrasonic Systems Inc., N. Y.), and centrifugation to remove cell debris. The standard curve obtained is shown in Figure 2. Generally, suspensions to be used in transport studies were diluted to an optical density of  $0.20 \pm 0.01$ .

Figure 2: Relationship between protein content of germinated sporangiospores of Achlya and optical density at 700 nm. Each experimental symbol represents an average of three determinations and the different symbols represent analyses performed on independent cell populations prepared over a 2-week period.



## METHODS

A. General Procedures

1. Liquid scintillation counting: In the studies involving radioactive tracers, liquid scintillation counting techniques were employed. Small volumes of liquid samples, collected cells, scrapings or cuttings from chromatograms, or most commonly, membrane filters with trapped samples, were placed in glass scintillation vials. Ten ml counting fluid, prepared according to Bray (1960) was added. The composition of this scintillation fluid was: naphthalene, scintillation grade (60 g/l), PPO (4 g/l), POPOP (0.2 g/l), ethylene glycol (20 ml/l), methanol (100 ml/l), and p-dioxane to 1 litre. Radioactivity was determined with the use of a Packard Tri-Carb, or, in later work, in a Beckman Model LS 230 liquid scintillation spectrometer, with external standard corrections.

2. Protein determinations: For most general procedures, protein content was determined by using the method of Lowry et al (1951), with bovine serum albumin as the standard.

For determination of protein concentration in highly purified enzyme preparations, the absorbance ratio at  $A_{260}$  and  $A_{280}$  was

used, using a Gilford 2400 spectrophotometer with a 1 ml quartz cuvette of 1 cm light path. Protein concentration was determined from this ratio using the method of Warburg and Christian (1942).

B. Enzyme Studies (Glutamate Dehydrogenase)

1. Purification procedures:

(a) Cell extracts: Mycelial mats of Achlya or Pythium were suspended in General Buffer, using 3 ml of buffer per gram of cells (wet weight). The cells were soaked in the buffer and dispersed uniformly with a chilled mortar and pestle. Frozen mats were broken with the mortar and pestle prior to the addition of buffer. The suspensions obtained in this manner were blended at 5° C for two minutes in a Waring blender to produce a thick paste of homogenized cells. The paste was sonically disrupted, using a Raytheon 10 kc sonic oscillator with water jacket at 10° C. Suitable breakage was obtained by sonicating small volumes to liquid consistency (approximately one minute), followed by sonication of a 50 to 70 ml volume for 15 minutes. Microscopic examination showed breakage to be better than 95%.

Debris and unbroken cells were removed by centrifugation at 48,000 x g for 10 minutes at 4° C in a Sorvall Model RC 2B centrifuge. The clear yellow supernatant was stored at -20° C overnight.

(b) DEAE-Cellulose columns: The thawed crude extract was recentrifuged at 48,000 x g for 15 minutes to remove denatured proteins and other insolubles. Crude extract, 50 to 75 ml, was absorbed directly onto a DEAE-cellulose column (40 x 2.5 cm) which had been prepared with cellulose equilibrated in the homogenizing buffer. The Achlya enzyme was eluted by running the column with the same buffer containing 10 mM KCl. For Pythium, a linear KCl gradient of 0 to 0.75 M was required to elute activity. Fractions were collected in the cold, and those fractions showing high activity were pooled.

(c) Ammonium sulfate precipitation: The enzyme protein was precipitated from the pooled fractions by slowly adding powdered ammonium sulfate to 0.6 saturation, and stirring in an ice bath. The pellet collected by centrifugation at 48,000 x g was redissolved in a small volume of homogenizing buffer. The enzyme was desalted by passage over Sephadex G-25 beads. To remove traces of malic dehydrogenase, the enzyme can be re-chromatographed through carboxymethyl-cellulose, although this step can generally be omitted.

(d) Sephadex filtration: The enzyme was filtered through Sephadex G-200 beads, using General Buffer to elute. For stability of the Achlya enzyme, 1 mM phosphoenolpyruvate was added to the buffer; this was not required with the Pythium enzyme. The enzyme

was collected by 0.6 saturation with ammonium sulfate, redissolved as before, and stored frozen at  $-20^{\circ}$  C. In some cases the few tubes containing the peak of activity were collected separately from the "shoulder" tubes in an attempt to increase homogeneity. Activity of these fractions was frequently high enough to be used directly in studies without collection of the protein by precipitation.

2. Enzyme assays: Assays of sample activities, and kinetic studies, were carried out using a Gilford Model 2400 recording spectrophotometer at  $24^{\circ}$  C, with a 3 ml silica cuvette of 1 cm light path. Activities were computed from the change in optical density during reduction of  $\text{NAD}^{+}$  or oxidation of NADH. One unit of enzyme activity represents an optical density change of 0.001 per minute at 340 nm. Specific activities are expressed as units per mg protein.

Kinetic data was treated according to the formulations of Cleland (1967); the nomenclature of the reaction mechanism and the description of kinetic constants are those of Cleland. Linear kinetic plots were fitted by eye.

The standard assay mixtures are as given below. All are in a total volume of 3 ml of distilled  $\text{H}_2\text{O}$ .

Glutamate dehydrogenase: (reductive amination reaction)

Achlya

10 mM  $\alpha$ -ketoglutarate, pH 8  
 0.167 mM NADH  
 0.167 mM NADP<sup>+</sup>  
 0.2 M (NH<sub>4</sub>)SO<sub>4</sub>  
 67 mM Tris-acetate, pH 8

Pythium

6.67 mM  $\alpha$ -ketoglutarate, pH 8  
 0.167 mM NADH  
 0.167 mM NADP<sup>+</sup>  
 50 mM (NH<sub>4</sub>)SO<sub>4</sub>  
 67 mM Tris-acetate, pH 8

Glutamate dehydrogenase: (oxidative deamination reaction)

33 mM glutamate  
 2.67 mM NADH  
 67 mM Tris-acetate, pH 9

33 mM glutamate  
 1.33 mM NADH  
 67 mM Tris-acetate, pH 9

D(-) Lactate dehydrogenase:

10 mM pyruvate  
 0.167 mM NADH  
 67 mM Tris-acetate, pH 8

1.67 mM pyruvate  
 0.167 mM NADH  
 67 mM Tris-acetate, pH 8

Isocitrate dehydrogenase, NADP-specific

33 mM isocitrate  
 0.167 mM NADP<sup>+</sup>  
 1.67 mM MnCl<sub>2</sub>  
 67 mM Tris-acetate, pH 8

(same)

Malic dehydrogenase

0.167 mM oxalacetate  
 0.05 mM NADH  
 67 mM Tris-acetate, pH 8

(same)

3. Physical Studies:

(a) Polyacrylamide gel electrophoresis: Polyacrylamide disc gel electrophoresis was carried out using the general procedures described by Davis (1964). Stock solutions for a pH 8.0 gel were

as follows:

Solution A:	1 N HCl	48 ml
	Tris base	6.85 g
	TEMED	0.46 ml
	Distilled H <sub>2</sub> O .....	to 100 ml
Solution B:	1 N HCl	48 ml
	Tris base	4.95 g
	TEMED	0.46 ml
	Distilled H <sub>2</sub> O .....	to 100 ml
Solution C:	Acrylamide	30 g
	BIS	0.8 g
	Distilled H <sub>2</sub> O .....	to 100 ml
Solution D:	Acrylamide	10 g
	BIS	0.8 g
	Distilled H <sub>2</sub> O .....	to 100 ml
Solution E:	Riboflavin	4 mg/ 100 ml

The lower gel was prepared by mixing equal volumes of freshly prepared ammonium persulfate (0.14 g/100 ml H<sub>2</sub>O) solution, and a mixture containing one part solution A, two parts solution C, and one part distilled water. A stacking gel of composition one part solution B, two parts solution D, one part solution E, and four parts water was prepared.

Samples of 50 to 100  $\mu$ l enzyme preparation were placed on top of the gel tubes, using a 40% sucrose solution to increase density. Bromphenol blue (0.05% in H<sub>2</sub>O) was used as tracking dye. Electrophoresis was carried out with a Tris-barbital buffer, pH 7,

containing 5.52 g barbital diethylbarbituric acid and 1 g Tris base per litre. Gels were run in an anionic system, with a current of approximately 5 ma/tube, in a chamber with cooling jacket. Electrophoresis was completed when the tracking dye reached a position 1 cm from the lower end of the gel.

Gels were removed and stained for protein and enzyme activity. A 1% Coomassie brilliant blue solution, diluted 1:40 with 10% Trichloroacetic acid was used to stain proteins. After 2 hours, the gels were destained overnight in 7% acetic acid. For enzyme activity stains, the gels were incubated in the dark for 30 minutes at room temperature in a mixture containing 0.1 M Tris-acetate-HCl buffer, pH 9.0, sodium glutamate (845 mg),  $\text{NAD}^+$  (1.933 mg), phenazine methosulfate (2 mg), and nitroblue tetrazolium dye (40 mg) per 100 ml volume (LéJohn et al 1968).

(b) Sucrose density gradients: Zone sedimentation in sucrose density gradients was conducted according to the method of Martin and Ames (1961). Five ml linear gradients of 5 to 20% (w/v) sucrose were prepared in cellulose nitrate tubes. The sucrose solutions were prepared in the General Buffer. Effectors, as required, were added to the sucrose solutions prior to making the gradients. The pH of the gradients was altered by changing the buffer pH.

Approximately 1000 units of enzyme activity were applied to each gradient, but the volume added never exceeded 100  $\mu\text{l}$ .

Centrifugation was for 15 hours at 30,000 rpm with an SW 50.1 swinging bucket rotor in a Spinco Model L2-65B ultracentrifuge at 4° C. The gradients were collected in fractions of 10 drops, and 50  $\mu$ l samples were assayed for activity and protein.

(c) Thermal denaturation studies: Studies of the thermal properties of enzymes were carried out on small samples of enzyme, partially purified, and on crude extracts. The samples were incubated at various temperatures for the required periods of time, using water and ice baths. Calculations for thermal studies were made as described by Hsu et al (1965).

#### C. Cyclic AMP Studies

The methods used in these studies are described in Part II of the results section.

#### D. Transport Kinetics

1. Initial uptake rate: The quantity of radioactive material taken up as a function of time was taken to represent a measurement of initial reaction rate. This measurement was made during the period when a linear relationship existed between time and uptake.

Germling suspensions were prepared in the double strength Transport Buffer, as described earlier, and allowed to pre-equilibrate for 15 minutes prior to the start of an assay. Tubes were prepared containing in a total volume of 1 ml water the radioactive material to be taken up and the effectors being tested. A stock solution of radioisotope and non-radioactive carrier was prepared for use in these assay tubes, generally of 5 mM final concentrations of carrier and containing between 3000 and 5000 counts per minute per  $\mu$ l. The substrate concentrations in the assay tubes were varied by adding suitable aliquots of this solution.

The reactions were started by adding 1 ml of cell suspension to each assay tube at timed intervals (0.20 min), and mixing rapidly on a Vortex mixer. Cells were allowed to concentrate the metabolite for a set time, generally 10 minutes. The reaction was stopped by filtering the tube contents through 0.45  $\mu$ m pore size membrane filters, using a 30-chamber Millipore manifold attached to vacuum pumps. The filters had been pre-soaked in the Transport Buffer, and the sample on the filter was rapidly washed with three successive 5 ml volumes of the same buffer, at room temperature. Washed filters were transferred to scintillation vials. Uptake of the substrate was calculated from the radioactivity retained on the filter, using the ratio of non-radioactive carrier concentration to specific radioactivity in the stock substrate solution to estimate the nmoles taken up per ml of cells per minute.

2. pH studies: For studies of uptake at varied pH values, germling suspensions were prepared in modified Transport Buffer with Tris-acetate reduced to 1 mM, while the other components were held constant. One ml of this suspension was added to assay tubes containing 25 mM Tris-acetate at the specified pH, along with the substrate. Assays were then conducted as before.

3. Temperature studies: Prior to initiating the reaction, assay tubes, and individual tubes containing 1 ml of germling suspension were pre-equilibrated for 10 minutes in water baths of different temperatures. The reaction was then started by mixing the contents of the two tubes, and the assay tubes were incubated for a further 10 minutes prior to filtering.

4. Efflux studies: The rate of metabolite exit was measured on germlings pre-loaded with the radioactive metabolite for 30 min in the presence of cycloheximide (10  $\mu$ g/ml). The germlings were collected and washed by filtering through 61  $\mu$ m nylon mesh, then resuspended in non-radioactive buffer with cycloheximide. Additions of metabolic energy uncouplers such as CCCP or 2,4-Dnp, and of excess metabolites were made as required. One ml samples were taken at frequent intervals, filtered, washed, and the residual radioactivity in the cells determined.

5. Osmotically-shocked germlings: Osmotically-shocked germlings differed from the germlings used in other transport studies in having been prepared from freshly germinated spores (2 hr at 28° C) in G<sub>2</sub>Y. The osmotic shock procedure was the general method of Heppel (1971) as employed by LéJohn and Cameron (1973) and LéJohn et al (1974). Shocked cells, devoid of a component of the Ca<sup>++</sup> transport system, were used in transport studies in the same manner as the normal germlings.

#### E. Studies with Cytokinins

1. Transport studies: Studies of the effects of cytokinins on transport of metabolites were carried out in the same manner described above. Cytokinins were added to the assay mixtures in the form of a small volume (0.5% final concentration) of DMSO. DMSO was also added to all controls.

2. Induction studies: For studies of the induction of glutamate dehydrogenase, germlings from an overnight spore suspension were resuspended in a buffered yeast extract solution (2 mM Tris-acetate, pH 7 and 0.5 g/l yeast extract) for pre-labelling procedures. The cells were incubated with algal <sup>14</sup>C-protein hydrolysate for 30 minutes with stirring, then refiltered, washed, and resuspended in the same medium without radioactive label.

Glucose or glutamate solutions were added to individual volumes of cell suspension to a final concentration of 5% (w/v).

Samples of the cells were collected at specified intervals by membrane filtration, washed with General Buffer, and frozen after removal from the filter. Cell extracts were made by adding a small volume of the same buffer, and breaking the cells using a sonic probe (Insonator Model 1000, Ultrasonic Systems Inc., New York) for 30 seconds. The extracts were centrifuged (5 min., 700 x g), and the supernatants tested immediately for glutamate dehydrogenase activity. Extract samples were also precipitated with equal volumes of ice-cold trichloroacetic acid for 30 min, then membrane filtered on Millipore 0.45  $\mu$ m filters and washed with cold 5% trichloroacetic acid containing unlabelled casamino acids. The filters were then counted for radioactivity. Protein content of the extracts was determined using the method of Lowry et al (1951).

3. Protein and RNA synthesis: For studies of cytokinin effects on protein and RNA synthesis, the germlings were resuspended in the defined medium of Barksdale (1963). Radioactive label was added to the cells at the same time as isopentenyladenine solution or DMSO. The suspensions were incubated with stirring, and at 15 minute intervals, samples of one ml volume were removed and added to equal volumes of 10% trichloroacetic acid, then incubated on

ice for 30 min before filtration. When transport ability of the cells was to be measured, volumes of the cytokinin-treated and the untreated cells were kept unlabelled, and at the same intervals samples were removed to test transport rates.

4. Protein and RNA degradation: To examine turnover of proteins and nucleic acids in the presence of isopentenyladenine, germlings were suspended in the defined medium and prelabelled with either  $^{14}\text{C}$ -protein hydrolysate or  $^3\text{H}$ -uracil for 30 minutes. The cells were then collected on nylon mesh (61  $\mu\text{m}$  pore size), washed with Transport Buffer, and resuspended in the same buffer. Additions of isopentenyladenine and cycloheximide were made as required at that time. Cycloheximide was used at a concentration of 28  $\mu\text{M}$  to completely block protein synthesis and partially block RNA synthesis (Cameron & LéJohn 1972a). One ml samples were taken at intervals, precipitated in cold trichloroacetic acid, and treated as above.

## MATERIALS

Chemicals

Most biochemicals used were obtained from Sigma Chemical Company, St. Louis, Missouri. Exceptions were: CoA, acetyl CoA, acetoacetyl-CoA, succinyl-CoA, palmitoyl-CoA, and oleyl-CoA which were purchased from P-L Biochemicals; dithiothreitol, from Calbiochem; nitoblue tetrazolium dye and phenazine methosulfate from Mann; and IAA from Nutritional Biochemicals. All the cytokinin compounds were purchased from Sigma. Media and other general chemical were of laboratory quality and purchased from commercial sources.

Enzymes

Commercial preparations of enzymes were obtained as follows; porcine heart malate dehydrogenase and NADP-isocitrate dehydrogenase from Boehringer; and rabbit skeletal muscle pyruvate kinase and lactate dehydrogenase from Sigma.

Other materials

DEAE-cellulose (medium mesh) was obtained from Sigma; Sephadex gels and equipment from Pharmacia; thin layer Silica Gel from Brinkmann Instruments Canada Ltd., and gel electrophoresis supplies from Canalco Industrial Corp. Chemicals and vials for liquid scintillation counting were obtained from Kent Laboratories (Fraser Medical Supplies Ltd.), Vancouver, B.C. Membrane filters (0.45  $\mu$ ) were obtained from both Millipore Corp., and Gellman Instrument Co.

The majority of the radioactive isotopes were purchased from Amersham/Searle. Exceptions are noted in the following list of radioisotopes and their specific radioactivities.

Nucleic acid compounds:

[G-<sup>3</sup>H] adenosine (12.1 Ci/mmole); [G-<sup>3</sup>H] cytidine (2.5 Ci/mmole); [G-<sup>3</sup>H] uridine (6.4 Ci/mmole); [methyl-<sup>3</sup>H] thymidine (27 Ci/mmole); [8-<sup>3</sup>H] guanosine (11 Ci/mmole); [2-<sup>3</sup>H] adenine (20 Ci/mmole); [2-<sup>14</sup>C] cytosine sulphate (59 Ci/mmole); [6-<sup>3</sup>H] uracil (25 Ci/mmole); [2-<sup>14</sup>C] thymine (60 mCi/mmole); [2-<sup>14</sup>C] xanthine (48 mCi/mmol) (Schwartz-Mann); [2-<sup>3</sup>H] adenosine-5'-monophosphoric acid, ammonium salt (21 Ci/mmol); [5-<sup>3</sup>H] cytidine-5'-monophosphoric acid, ammonium salt (22.8 Ci/mmol); [8-<sup>3</sup>H] guanosine-5'-monophosphoric acid, ammonium salt (5 Ci/mmol), and [8-<sup>3</sup>H] adenosine-3',5'-cyclic monophosphoric acid, ammonium salt (20.7 Ci/mmole).

[<sup>3</sup>H-(6)]-uridine, (1 mCi/0.025 ng) used in Part V was obtained from New England Nuclear.

Amino acids: (All L-optical isomers unless noted otherwise).

alanine [<sup>3</sup>H(G)] (1.1 Ci/mmole)  
arginine [5-<sup>3</sup>H]monohydrochloride (22 Ci/mmole)  
asparagine [<sup>3</sup>H(G)] (100 mCi/mmole)  
aspartic acid [<sup>3</sup>H(G)] (178 mCi/mmole)  
cysteine [<sup>14</sup>C(U)]hydrochloride (38.6 mCi/mmole)  
glycine[-2-<sup>3</sup>H] (2 Ci/mmole)  
glutamine [<sup>14</sup>-C(U)] (48 mCi/mmole)  
glutamic acid [<sup>14</sup>-C(U)] (10 μCi/mmole)

histidine  $[-2,5-^3\text{H}]$  (58 Ci/mmole)  
 isoleucine  $[-4,5-^3\text{H}(n)]$  (17.7 Ci/mmole)  
 leucine  $[-4,5-^3\text{H}]$  (Ci/mmole)  
 methionine  $[\text{methyl-}^3\text{H}]$  (100 mCi/mmole)  
 3-phenylalanine  $[\text{ring-4-}^3\text{H}]$  (58 Ci/mmole)  
 proline  $[-^3\text{H}(G)]$  (178 mCi/mmole)  
 serine  $[-3-^3\text{H}]$  (500 mCi/mmole)  
 threonine  $[^{14}\text{-C}(U)]$  (228 mCi/mmole)  
 tryptophan  $[-^3\text{H}(G)]$  (3.1 Ci/mmole) (New England Nuclear)  
 tyrosine  $[-3,5-^3\text{H}]$  (40 Ci/mmole)  
 valine  $[-2,3-^3\text{H}(n)]$  (31.6 Ci/mmole)

#### Others

$[^{14}\text{C-U}]$ -protein hydrolysate, containing 15 amino acids  
 (52 mCi/mA),  $^{45}\text{CaCl}_2$  as  $\text{CaCl}_2$  in aqueous solution, and 3-indolyl-  
 acetic acid- $[2-^{14}\text{C}]$ , (55 mCi/ mmole).

#### Isopentenyladenine label

$\text{N}^6$ -isopentenyladenine- $[G-^3\text{H}]$  hydrochloride was custom  
 labelled by Amersham/Searle by catalytic exchange in an aqueous  
 medium with a platinum catalyst. Labile tritium was removed and  
 the radioisotope was supplied as a crude aqueous solution (57 mCi/  
 25 ml). This extract was further purified by lyophilizing and  
 chromatographing aliquots of the concentrate on Whatman No. 1 paper  
 with a 2-propanol: conc.  $\text{NH}_4\text{OH}$ :  $\text{H}_2\text{O}$  (7:1:2) solvent system. Ultra-  
 violet absorbing areas co-chromatographing with authentic isopentenyl-  
 adenine were cut out and eluted with water. The eluted label was

lyophilized in small volumes and stored as a dried powder at 4°C. Prior to lyophilization the concentration of isopentenyadenine in the solution was determined by measuring the absorbance at 275 nm and comparing with isopentenyadenine solutions of known concentration. Specific activity of the radioisotope was calculated as 8.6  $\mu\text{Ci}/\mu\text{mol}$  (Batch 2; Nov. 1972.)

RESULTS AND DISCUSSION

## PART I

## GLUTAMATE DEHYDROGENASE : KINETICS AND REGULATION

## RESULTS

A. Enzyme Purification

1. Achlya enzyme: The purification procedure for this enzyme is summarized in Table IV. The final preparation was judged to be almost pure by two criteria. (a) During sedimentation in sucrose density gradients under conditions that ensure stability of the enzyme, the protein and activity peaks were coincident. (b) Filtration of the enzyme through Sephadex G-200 beads in columns resolves only one coincident band of protein and enzyme activity. Analysis for purity in the Spinco Model E analytical ultra-centrifuge was difficult because the enzyme dissociated easily even with effectors in the buffer. The enzyme was also unstable at alkaline pH. Consequently polyacrylamide gel electrophoreses which were carried out at pH 8 produced a diffuse protein band that did not migrate significantly from the cathode. Activity staining also appeared very close to the stacking gel.

Table IV

A Summary of the Purification of  
Achlya Glutamic Dehydrogenase

Step	Vol (ml)	Units	Protein (mg)	Sp. Act	Purification
Centrifuged crude extract	70	2,072,000	1932	1072	-
DEAE-cellulose	110	1,724,800	128.2	9400	9
Ammonium sulfate (0.6 saturation)	10	1,950,000	65.0	30,000	28
CM-cellulose	75	540,000	41.0	13,170	12
Ammonium sulfate (0.6 saturation)	5	550,000	8.0	68,000	63
G-200 Sephadex	30	460,000	4.25	108,200	100
Ammonium sulfate (0.55 saturation)	4	448,000	3.51	127,600	118

Enzyme stored frozen or in the cold without some ammonium sulfate inactivates readily. As little as 10 mM ammonium sulfate is adequate to protect the Achlya enzyme from deterioration on storage at  $-20^{\circ}\text{C}$ . The instability of the enzyme isolated from Saprolegnia, a closely related species, has been found to be even more unstable, requiring  $\text{NADP}^{+}$  and P-enolpyruvate in the storage solution.

2. Pythium enzyme: The slightly modified procedure for the preparation of the Pythium enzyme is summarized in Table V. This enzyme was considerably more stable than the Achlya form, requiring little or no ammonium sulfate for stabilization. Over 40% recovery of the enzyme can be obtained at the final step; the lower yield for the Achlya form may be due to the relative instability of that protein.

#### B. pH Effect

The effect of hydrogen ion concentration on the reductive amination and oxidative deamination reactions was studied using Tris-acetate buffers with the standard assay mixture. As shown in Figure 3 a and b, the reactions of the Achlya enzyme have an optimum of pH 8.0, while the Pythium enzyme's optimal pH is more alkaline. Addition of the effectors  $\text{NADP}^{+}$  and P-enolpyruvate to the Pythium enzyme did not significantly alter the pH response. The interaction of the various effectors of the enzymes and their effects on pH optima are discussed later.

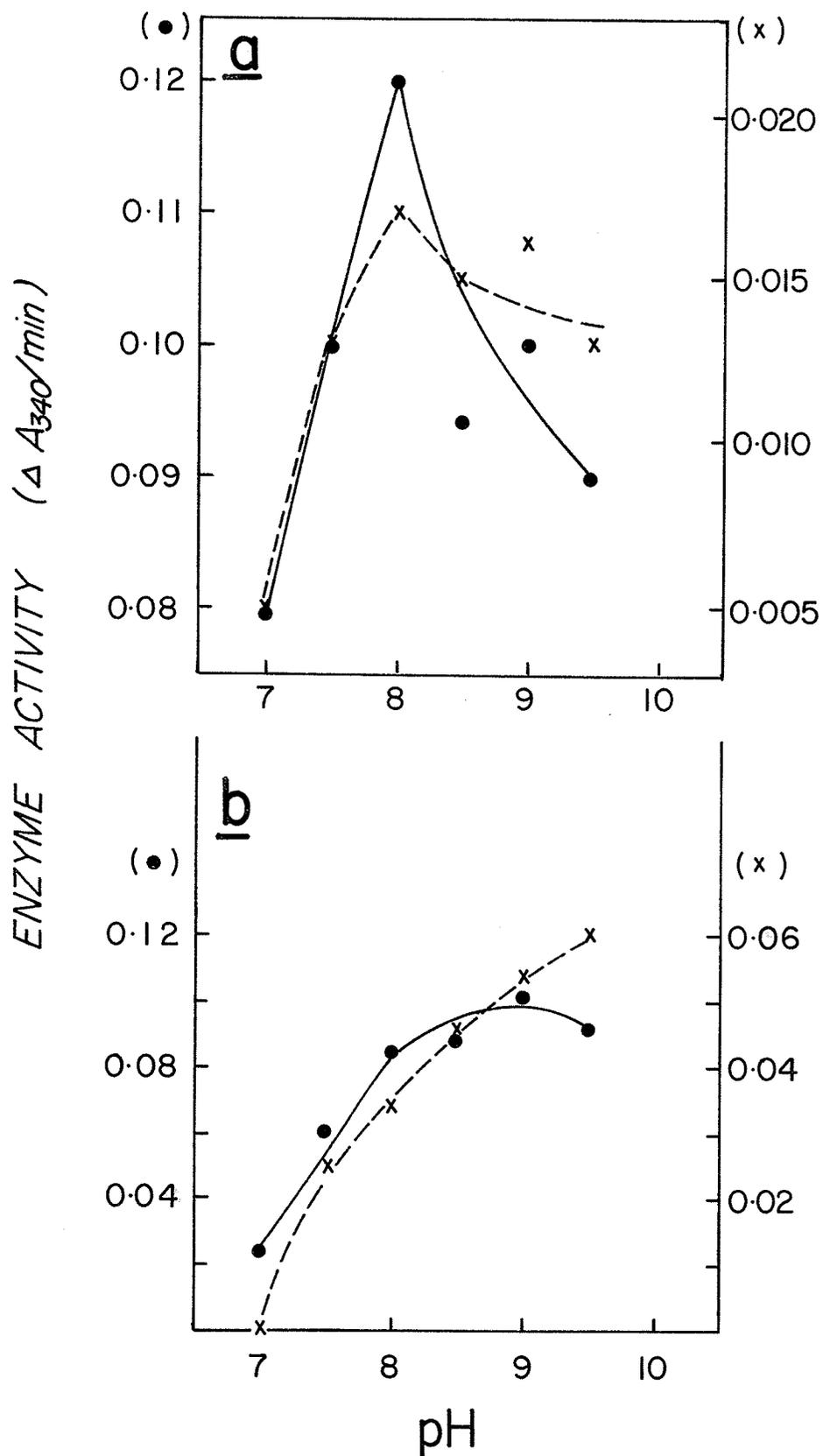
Table V

A Summary of the Purification of  
Pythium Glutamic Dehydrogenase

Step	Vol (ml)	Units	Protein (mg)	Sp. Act.	Purifi- cation
Centrifuged crude extract	110	3,870,000	2,090	1,900	-
DEAE-cellulose	80	2,560,000	200	13,000	6.8
Ammonium sulfate (0.6 saturation)	5	2,450,000	57	43,000	22.6
G-200 Sephadex	13.5	3,400,000	9	340,000	179.0
Ammonium sulfate (0.6 saturation)	2	1,900,000	5.6	333,333	176.0

Only the central core of tubes containing highly active fractions were collected at all chromatographic steps.

Figure 3: Effect of pH on the glutamate dehydrogenase reductive amination ( ● ) and oxidative deamination ( × ) reactions of Achlya (a) and Pythium (b). Tris-acetate buffers were used with other reagents as for the standard assays.



### C. Substrate and Kinetic Constants

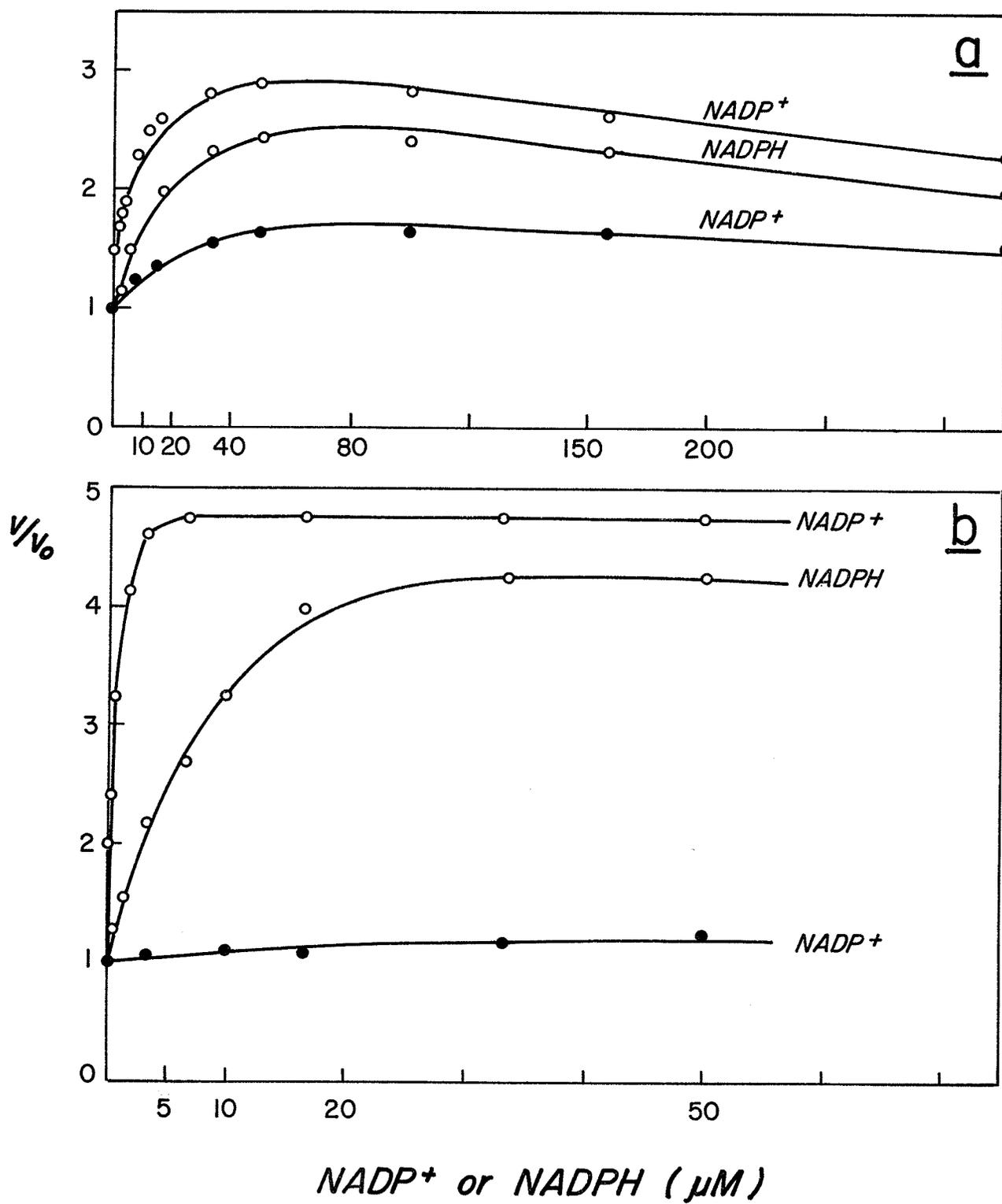
1. NADP<sup>+</sup> as Effector: It was determined that activity of the glutamate dehydrogenases required NAD<sup>+</sup> (or NADH) as substrate, and that NADP<sup>+</sup> (or NADPH) cannot substitute. However, it was also found that NADP<sup>+</sup> and NADPH strongly activated the reactions of the NAD-specific glutamate dehydrogenases of Achlya and Pythium, as well as those of related species such as Saprolegnia, Phytophthora, and Rhizidiomyces.

Analyses were carried out to determine whether NADP<sup>+</sup> and NADPH were being consumed in the reactions, since glutamate dehydrogenases from a large number of organisms can catalyse the reduction of both NAD<sup>+</sup> and NADP<sup>+</sup>. A reaction system consisting of 0.133 mM NAD<sup>+</sup>, 0.0167 mM NADP<sup>+</sup>, 16.67 mM glutamate, 67 mM Tris-acetate pH 8 and 5 µg of purified Pythium glutamate dehydrogenase in 3 ml volume was incubated at 25°C for 30 min and reduction of the coenzyme (NAD<sup>+</sup>) was followed spectrophotometrically. At intervals, 10 µl of the reaction mixture was added to 1 ml of reaction mixture containing 1 mM Mn<sup>++</sup>, 1 mM isocitrate, and 0.05 µg porcine heart NADP - specific isocitric dehydrogenase, and the reduction of NADP<sup>+</sup> was determined. The same procedure was followed, substituting NADPH for NADP<sup>+</sup>. For the reverse reaction, the reaction system consisted of 0.0167 mM NADH, 0.0167 mM NADP<sup>+</sup>, 6.67 mM α-ketoglutarate, 10 mM ammonia, 67 mM Tris-acetate (pH 8.0) and 2 µg of purified Pythium enzyme. Analysis for residual NADP<sup>+</sup> was conducted with isocitrate dehydrogenase as

before. In all cases  $\text{NADP}^+$  was not converted to NADPH. Other control studies showed that the porcine heart isocitrate dehydrogenase is specific for  $\text{NADP}^+$ , and the glutamate dehydrogenase reaction is specific for  $\text{NAD}^+$  or NADH.

The effect of  $\text{NADP}^+$  and NADPH on both the oxidative deamination and reductive amination reactions was examined. The effects of increasing concentrations of the activators are shown in Figure 4 for the Achlya enzyme (Fig. 4 a) and for the Pythium enzyme (Fig. 4 b), represented as the ratio of activity with activator present ( $v$ ) to the activity with no activator ( $v_0$ ). The relative degree of activation by  $\text{NADP}^+$  attained during the reductive amination reaction was considerably larger than that reached in the oxidative deamination process. The difference in effects on the two reactions is quite pronounced in the case of the Pythium enzyme, with the oxidative reaction hardly stimulated, while there was a 5-fold activation in the reductive reaction. NADPH is less effective than  $\text{NADP}^+$  as a stimulant, but it produces similar effects on the reactions as  $\text{NADP}^+$ . These observations suggest that (i)  $\text{NADP}^+$  is probably the actual activator, which NADPH mimics, and (ii) the control by  $\text{NADP}^+$  is unidirectionally oriented in favour of the reductive step. The nature of the  $\text{NADP}^+$  activation required that subsequent studies of the kinetic behaviour of the enzymes be examined in both the presence and absence of effector. Other modulators also influence the reactions and will be discussed later.

Figure 4: Activation of glutamate dehydrogenase from Achlya  
(a) Pythium (b) by  $\text{NADP}^+$  and NADPH during the  
oxidative deamination ( ● ) and reductive amina-  
tion ( ○ ) reactions.  $v$  is the reaction rate  
with activator, and  $v_0$  the rate without activator.



2. Determination of Michaelis Constants: A series of kinetic analyses were carried out on Pythium and Achlya enzymes to estimate their true Michaelis constants. One substrate was held fixed at several different levels, while another was varied. The Lineweaver-Burk double reciprocal rate concentration plots of these studies are shown in Figures 5 to 10, and the Michaelis constants are given in Table VI for both enzymes. Because of acute inhibition of the enzymes, particularly the Pythium catalyst, by relatively low concentrations of the substrates ammonia and  $\alpha$ -ketoglutarate in the absence of activator, true  $K_m$  values could only be obtained for glutamate and  $NAD^+$ .

Saturation curves and double reciprocal plots for the oxidative deamination reaction of the Achlya enzyme are shown in Figure 5, and for the reductive amination reaction in Figure 6, as determined both with and without  $NADP^+$ . The enzyme from this species was less sensitive to the poisonous effects of substrates, and consequently was utilized to study the nature of substrate-activator interactions. The double reciprocal plots in Figures 5 and 6 show that the  $NADP^+$  binding site does not coincide with any of the catalytic sites, with non-competitive interaction between activator and substrate being displayed. It is not, however, possible to discern whether different enzyme forms are involved. The results also indicate that  $NADP^+$  does not greatly enhance the affinity of the enzyme for its substrates, but does increase the maximal velocity ( $V_{max}$ ) of the reaction.

Figure 5: Saturation curves for the oxidative deamination reaction of Achlya glutamate dehydrogenase in the presence (●) and absence (○) of  $\text{NADP}^+$ . Insets: Double reciprocal rate-concentration plots. Assay system consisted in (a), of 1.33 mM  $\text{NAD}^+$ , 67 mM Tris-acetate, pH 9 and 5  $\mu\text{g}$  enzyme protein; in (b), of 33.3 mM glutamate, 67 mM Tris-acetate, pH 9 and 10  $\mu\text{g}$  enzyme.  $\text{NADP}^+$  saturating at 0.167 mM.

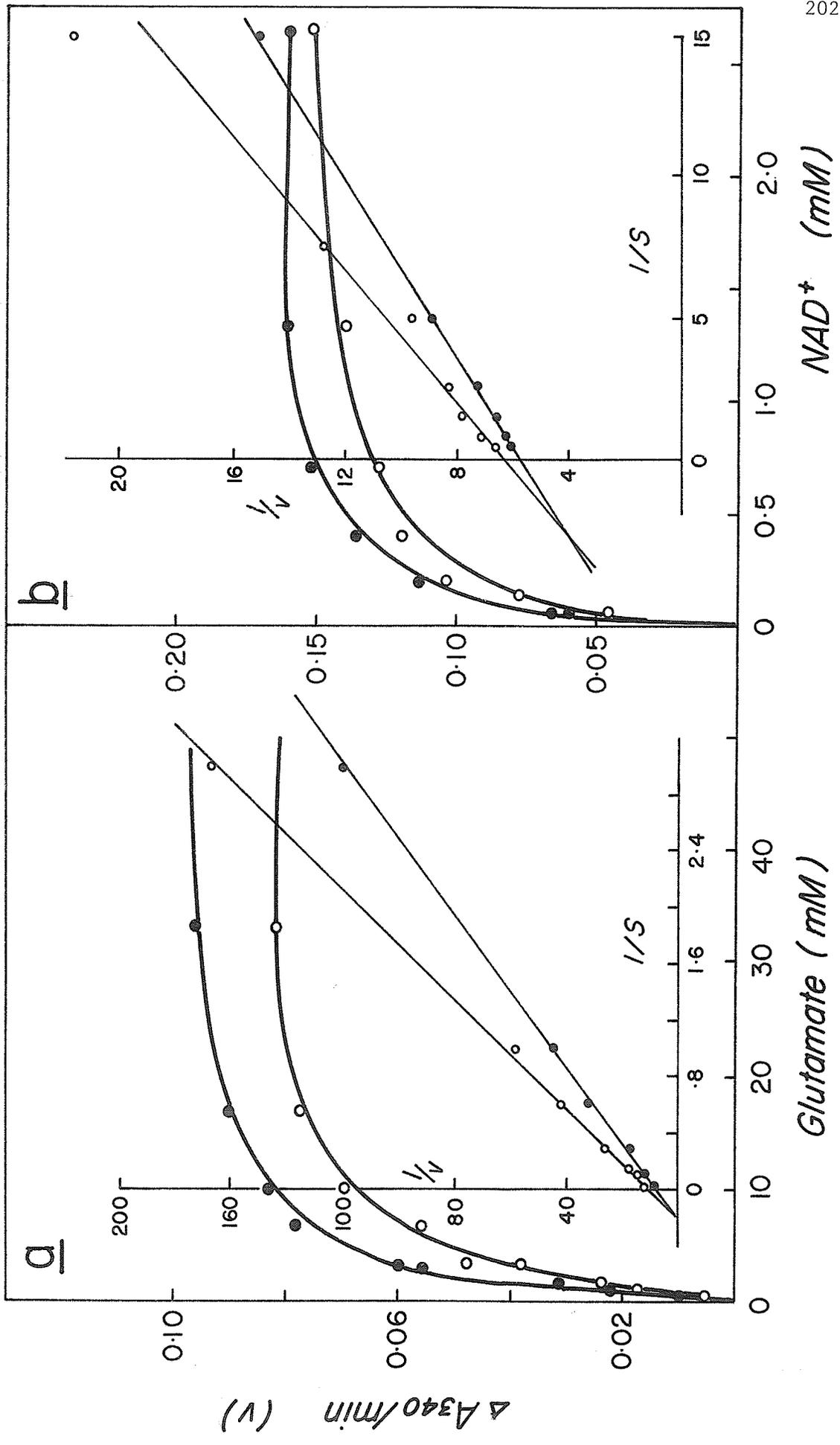


Figure 6: Saturation curves for the reductive amination reaction of Achlya glutamate dehydrogenase in the presence (●) and absence (○) of  $\text{NADP}^+$ . Insets: Double reciprocal rate-concentration plots. Assay system consisted in (a), of 100 mM  $\text{NH}_4^+$ , 0.167 mM NADH,  $\alpha$ -ketoglutarate varied, 67 mM Tris-acetate pH 8; in (b) 6.67 mM  $\alpha$ -ketoglutarate and NADH varied 4  $\mu\text{g}$  enzyme and 0.167 mM  $\text{NADP}^+$  were used in both (a) and (b).

Table VI

Michaelis and Inhibition Constants and  $M_{0.5}$  Values  
for Pythium and Achlya Glutamate Dehydrogenases

Constant	<u>Achlya</u>		<u>Pythium</u>	
	(+)NADP <sup>+</sup>	(-)NADP <sup>+</sup>	(+)NADP <sup>+</sup>	(-)NADP <sup>+</sup>
K (NADH)	$3.1 \times 10^{-5}$	$5.5 \times 10^{-5}$	$6.3 \times 10^{-5}$	$6.7 \times 10^{-4}$
K (NAD <sup>+</sup> )	$1.7 \times 10^{-4}$	$6.1 \times 10^{-4}$	$8.0 \times 10^{-5}$	$9.5 \times 10^{-5}$
K ( $\alpha$ -ketoglutarate)	$1.0 \times 10^{-3}$	$3.3 \times 10^{-3}$	$1.67 \times 10^{-4}$	$3.9 \times 10^{-3}$
K (ammonia)	$1.03 \times 10^{-2}$	$2.6 \times 10^{-2}$	$5.8 \times 10^{-3}$	$1.1 \times 10^{-2}$
K (glutamate)	$6.55 \times 10^{-3}$	$1.5 \times 10^{-2}$	$1.0 \times 10^{-3}$	$1.18 \times 10^{-3}$
$K_i$ (ammonia)	$1.5 \times 10^{-2}$	$3.6 \times 10^{-2}$	$5.55 \times 10^{-3}$	$1.1 \times 10^{-2}$
$K_i$ (NADH)	$1.4 \times 10^{-5}$	$2.7 \times 10^{-5}$	$8.5 \times 10^{-5}$	$4.0 \times 10^{-5}$
$K_i$ (glutamate)	-	-	$8.3 \times 10^{-2}$	-
Ligand	$M_{0.5}$			
	Reductive amination	Oxidative deamination	Reductive amination	Oxidative deamination
Citrate (isocitrate)	$2.5 \times 10^{-4}$	$1.33 \times 10^{-5}$	$1.65 \times 10^{-2}$	$3.3 \times 10^{-4}$
P-enolpyruvate	$6.0 \times 10^{-6}$	-	-	-
NADP <sup>+</sup>	$3.3 \times 10^{-7}$	-	$3.3 \times 10^{-6}$	$1.6 \times 10^{-5}$
NADPH	$5.5 \times 10^{-6}$	-	$1.0 \times 10^{-5}$	-

(all values expressed as M )

The double reciprocal rate concentration plots for the oxidative deamination reaction of the Pythium enzyme are shown in Figure 7. The plots are linear, and intersect to the left of the  $v^{-1}$  axis, which, according to Cleland's terminology, indicates that the substrates  $\text{NAD}^+$  and glutamate must bind to the enzyme before any product is released (i.e. sequential rather than ping pong mechanism). The substrates for the reductive amination reaction of glutamate dehydrogenase, except  $\text{NADH}$ , are extremely inhibitory. Rate-concentration plots for all three substrates are presented in Figures 8, 9 and 10, in the presence and absence of  $\text{NADP}^+$ .  $\text{NADP}^+$  served not only as an activator of the reaction, but also as an antagonist of substrate inhibition. The double reciprocal plots in the presence of  $\text{NADP}^+$  are not typically Michaelian in form. They are biphasic, characterized by two distinct slopes to the curve. Examination of the curves obtained through substrate inhibition indicated that the process may be co-operative in nature.

#### D. Kinetic Mechanism - Product Inhibition Studies

Product inhibition studies were carried out in order to elucidate the kinetic mechanism of the reaction. As it was of interest to determine if the activators of the reaction altered the kinetic mechanism, the kinetic patterns were studied both with

Figure 7: Lineweaver-Burk representation of the oxidative deamination reaction catalyzed by Pythium NAD-specific glutamate dehydrogenase in the absence of activator(s). (a) Glutamate as varied ligand,  $\text{NAD}^+$  held fixed at several different concentrations as follows: (  $\circ$  ), 0.067 mM; (  $\square$  ), 0.133 mM; (  $\bullet$  ) 0.266 mM; (  $\triangle$  ), 0.40 mM; (  $\blacktriangledown$  ), 2.66 mM. (b)  $\text{NAD}^+$  as varied substrate, glutamate held fixed at several different concentrations as follows: (  $\circ$  ), 1.66 mM; (  $\square$  ), 3.33 mM; (  $\bullet$  ), 5 mM; (  $\triangle$  ), 6.66 mM; (  $\blacktriangledown$  ) 16.67 mM. Assay was conducted in 67 mM Tris-acetate, pH 9 with 10  $\mu\text{g}$  enzyme. Inset: (a) and (b): - replots of intercepts against fixed substrate concentrations.

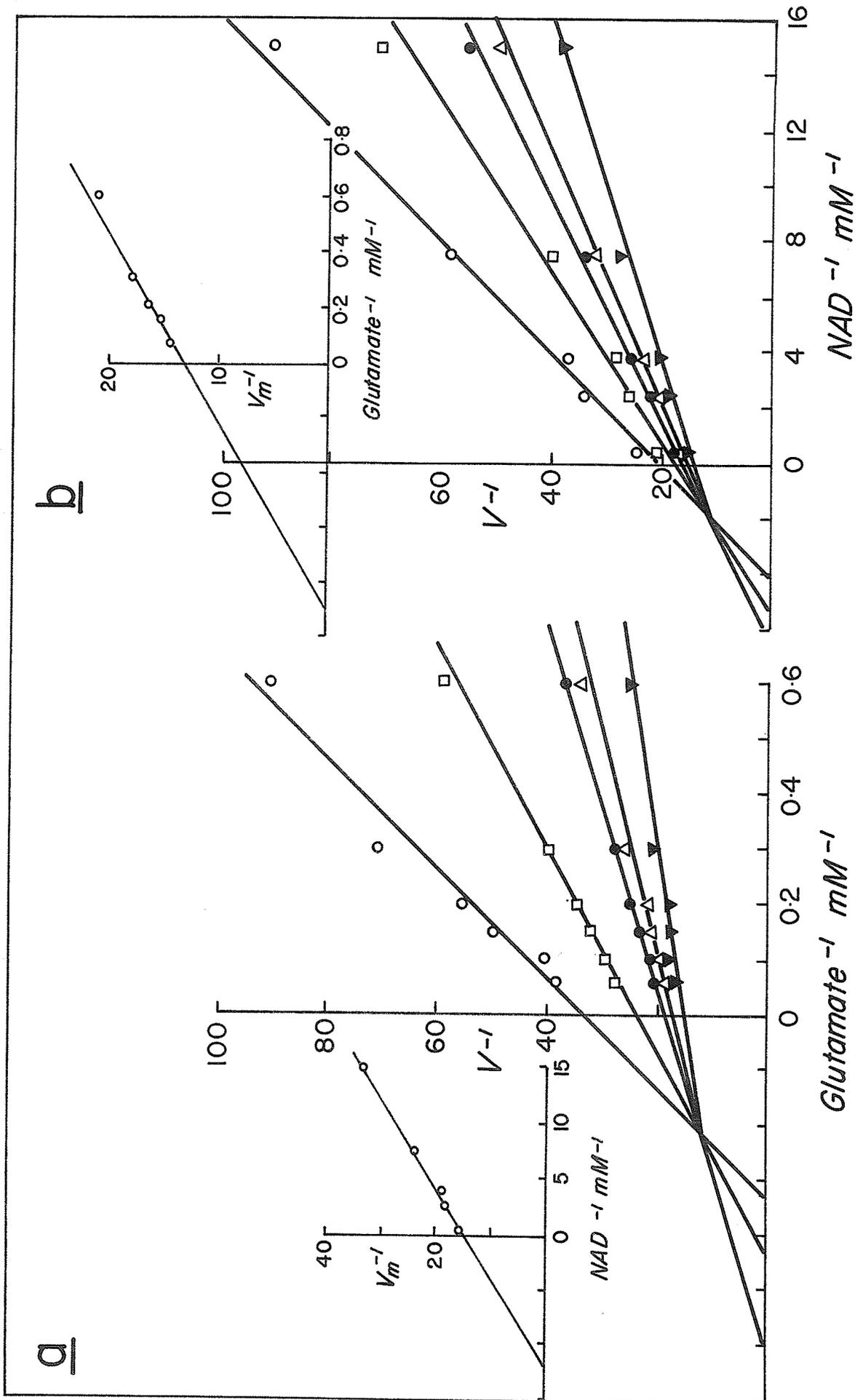


Figure 8: Rate-concentration plot with NADH as variable substrate of Pythium glutamate dehydrogenase in the absence of activators (  $\square$  ); in the presence of P-enolpyruvate (  $\triangle$  ) and NADP<sup>+</sup> (  $\circ$  ). Inset. Double-reciprocal plot.

Assay system: 6.67 mM  $\alpha$ -ketoglutarate;  
20 mM NH<sub>4</sub><sup>+</sup>; 67 mM Tris-acetate, pH 8;  
2  $\mu$ g enzyme, P-enolpyruvate at 0.25 mM;  
16.67  $\mu$ M NADP<sup>+</sup>.

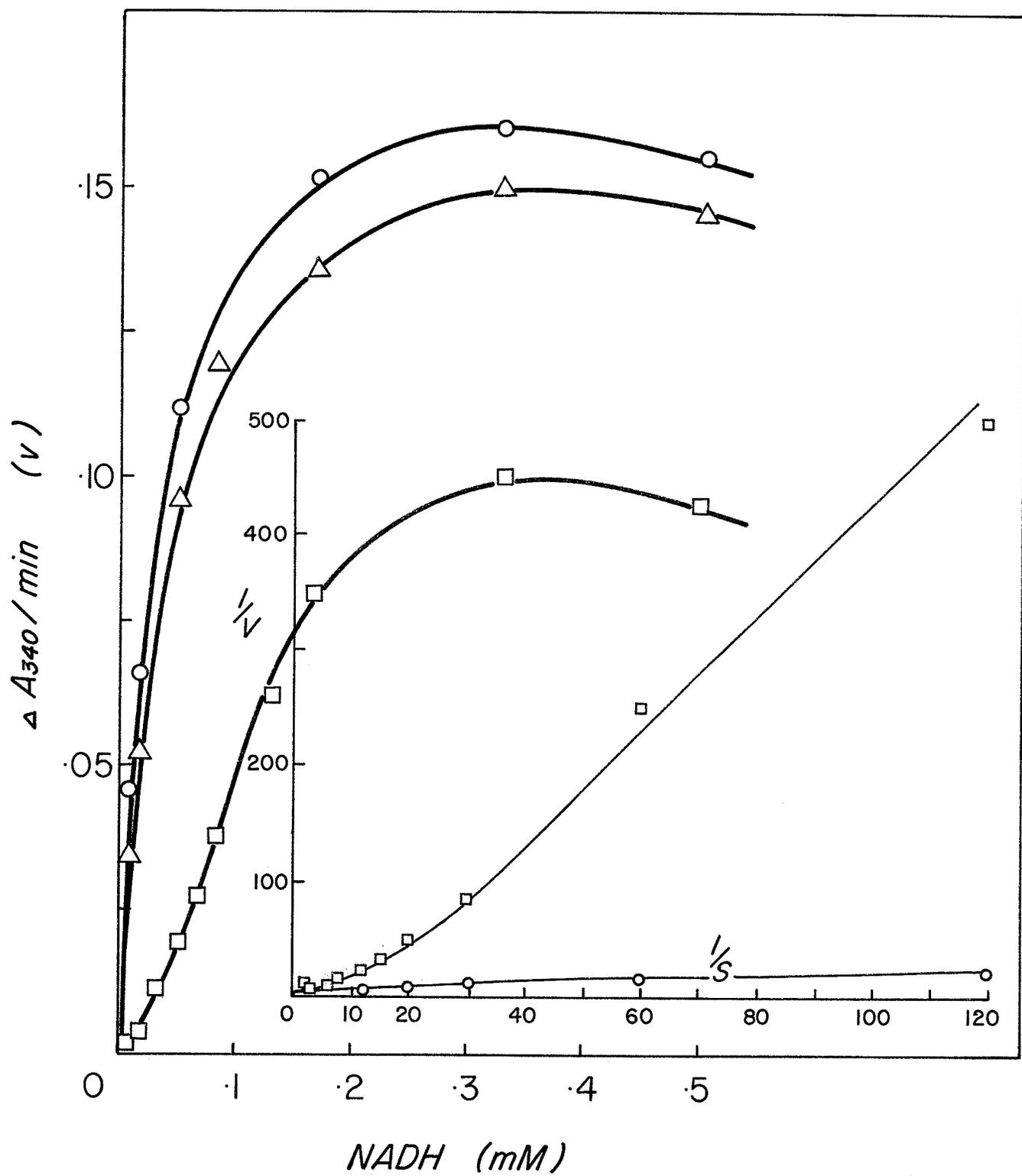


Figure 9: Rate-concentration plot with  $\alpha$ -ketoglutarate as variable substrate in the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase in the presence of  $\text{NADP}^+$  (  $\square$  ) and in the absence of  $\text{NADP}^+$  (  $\circ$  ). Inset: Double reciprocal plot. Assay system as for Fig. 8 with NADH at 0.167 mM.

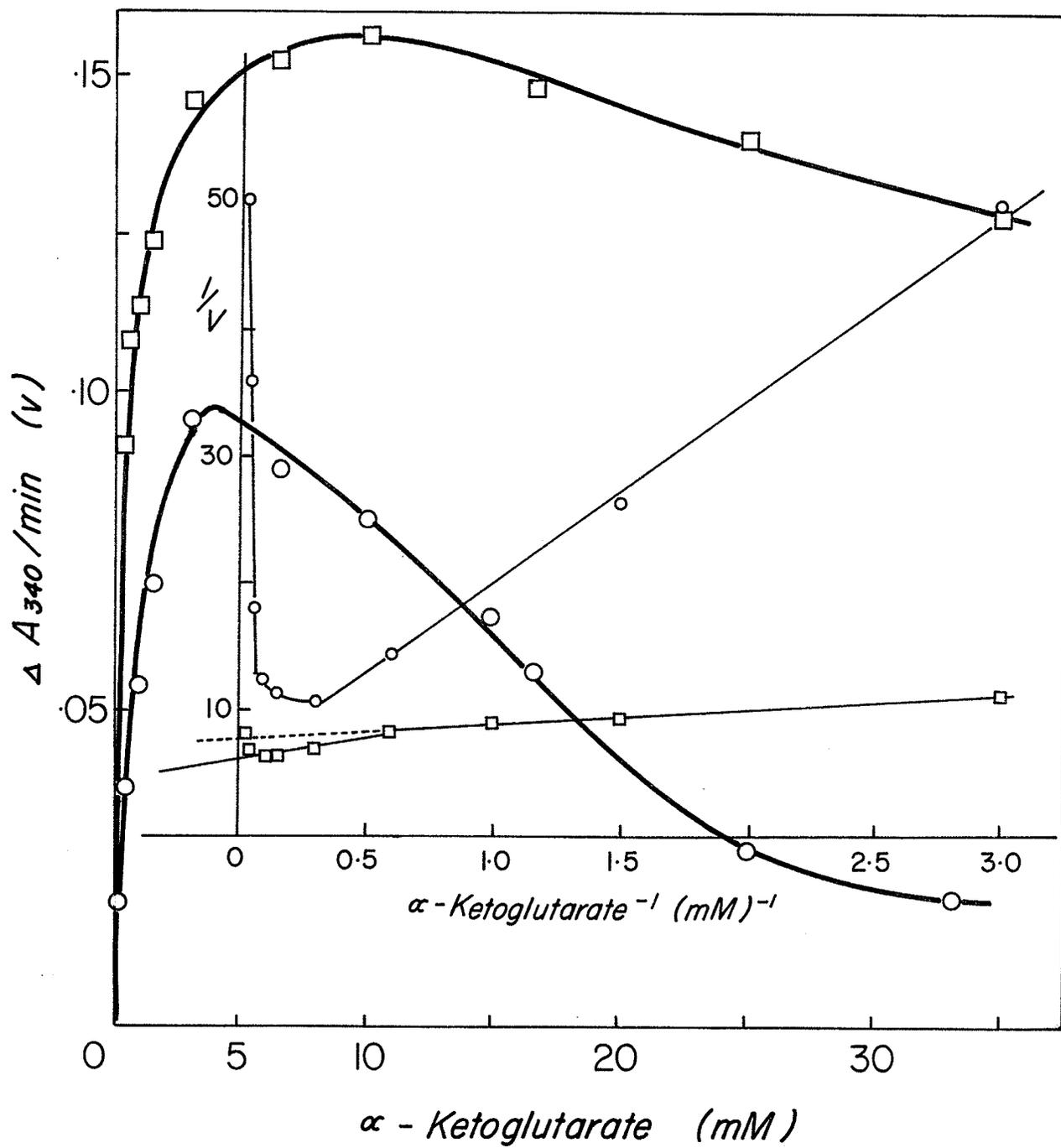
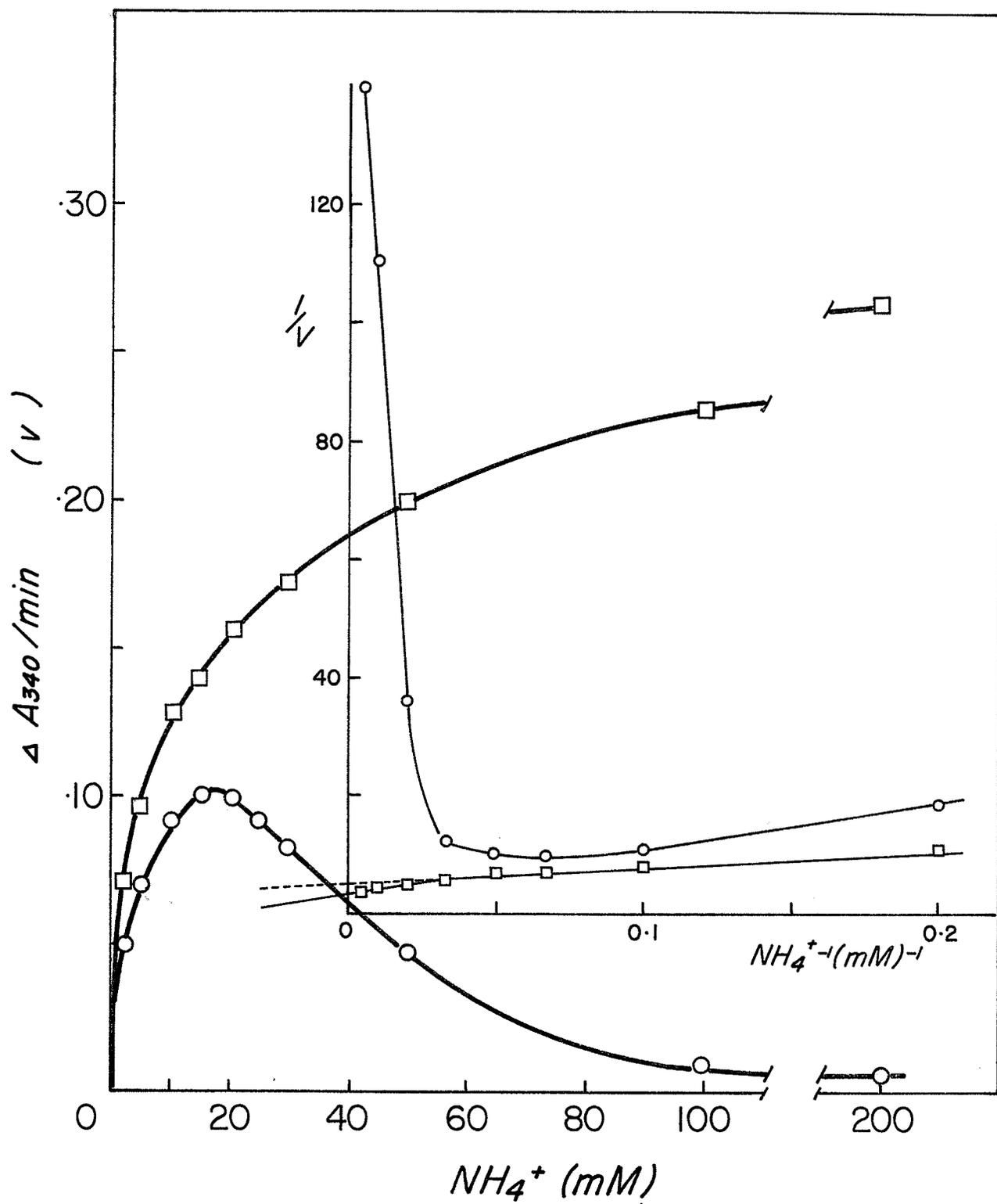
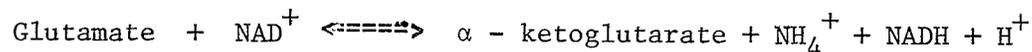


Figure 10: Rate-concentration plot with  $\text{NH}_4^+$  as variable substrate in the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase in the presence of  $\text{NADP}^+$  (  $\square$  ) and in the absence of  $\text{NADP}^+$  (  $\circ$  ). Inset: Double reciprocal rate-concentration plot. Assay system as for Fig. 8 with NADH at 0.167 mM.



and without effectors. For the reaction:



the concentration of  $\text{H}^+$  may be ignored at the fixed pH value used in the assays.

1. Pythium enzyme: without activators: For the Pythium glutamate dehydrogenase, double reciprocal rate-concentration plots were linear for the oxidative deamination (Figure 7), but non-linear for the reductive amination reaction (Figures 8, 9, and 10). Studies were therefore made on the oxidative reaction, using glutamate and  $\text{NAD}^+$  as substrates, and ammonia,  $\alpha$ -ketoglutarate and NADH as product inhibitors. The results of these combinations are shown in Figures 11 and 12 for studies without activators. In Figure 11 a, ammonia was the inhibitor, glutamate held at fixed saturating level and  $\text{NAD}^+$  varied over a wide concentration range. The primary plots ( $v^{-1}$  against substrate  $^{-1}$ ) were linear, uncompetitive (i.e. lines essentially parallel) at different ammonia levels. The secondary plot, intercept or slope against inhibitor concentration, was non-linear, as indicated by the broken line. In Figures 11 b and c, NADH and  $\alpha$ -ketoglutarate were used as inhibitors, respectively. The primary and secondary plots were linear. The inhibition pattern was competitive for NADH inhibition (plots intersecting at the  $v^{-1}$  axis), and non-competitive for  $\alpha$ -ketoglutarate (plots intersecting to the left of the  $v^{-1}$  axis). Figures 12 a, b and c represent primary and secondary plots for the same three products as inhibitors

Figure 11: Lineweaver-Burk representation of product inhibition studies of the oxidative deamination reaction catalyzed by Pythium NAD-specific glutamate dehydrogenase in the absence of activator(s).  $\text{NAD}^+$  as varied ligand.

(a) Ammonia as inhibitor at the following concentrations: (  $\circ$  ), 15 mM; (  $\square$  ), 10 mM; (  $\triangle$  ), 5 mM; (  $\nabla$  ), 0 mM. Inset: replot of intercepts against ammonia concentrations

(b) NADH as inhibitor at the following concentrations: (  $\circ$  ), 0.167 mM; (  $\square$  ), 0.083 mM; (  $\triangle$  ), 0 mM. Inset: replot of slopes against NADH concentrations.

(c)  $\alpha$ -Ketoglutarate as inhibitor at the following concentrations: (  $\circ$  ), 6.66 mM; (  $\square$  ), 3.33 mM; (  $\triangle$  ), 1.67 mM; (  $\nabla$  ), 0 mM. Inset: replot of intercepts against  $\alpha$ -ketoglutarate concentrations.

Assay condition as for Fig. 7.

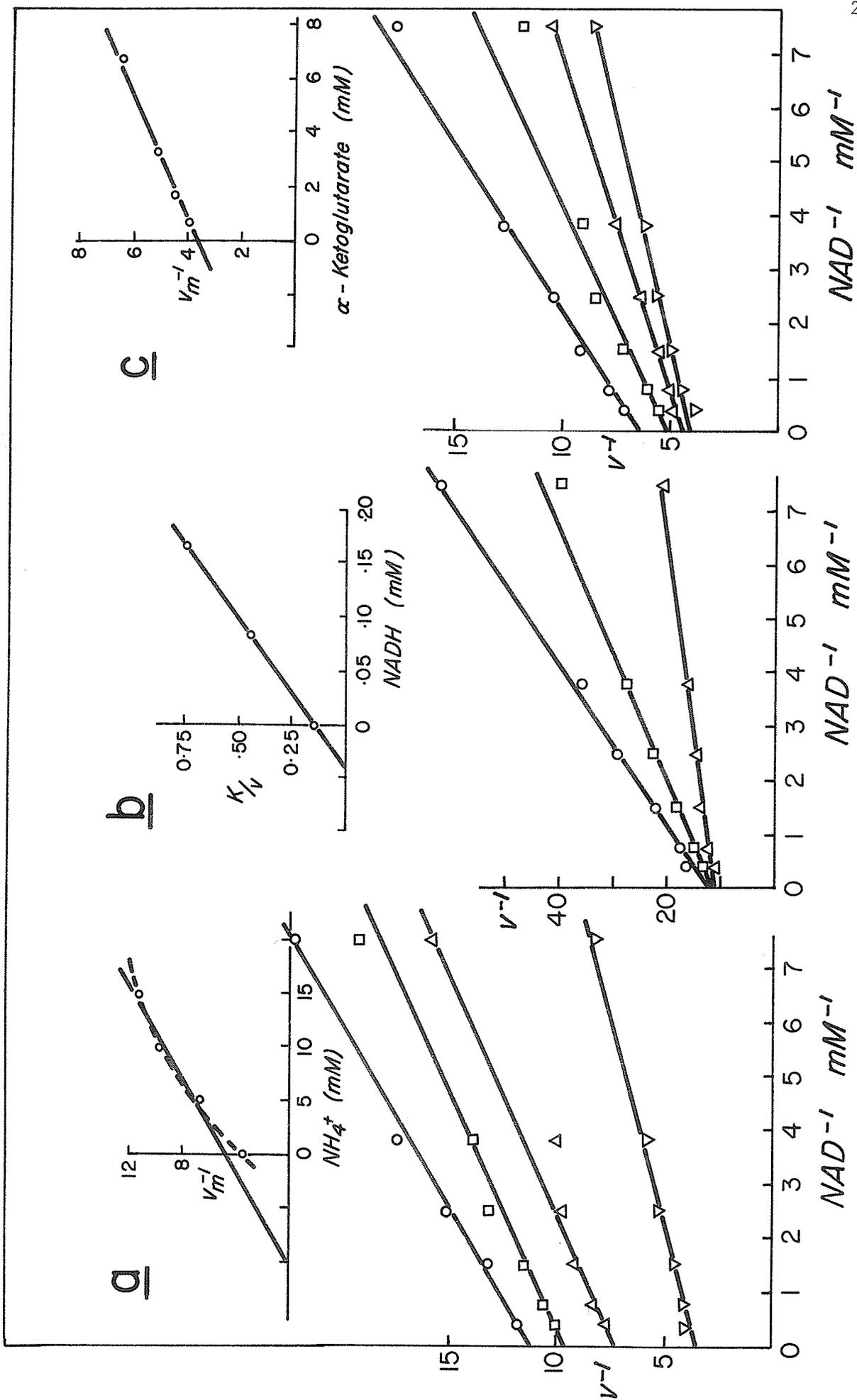
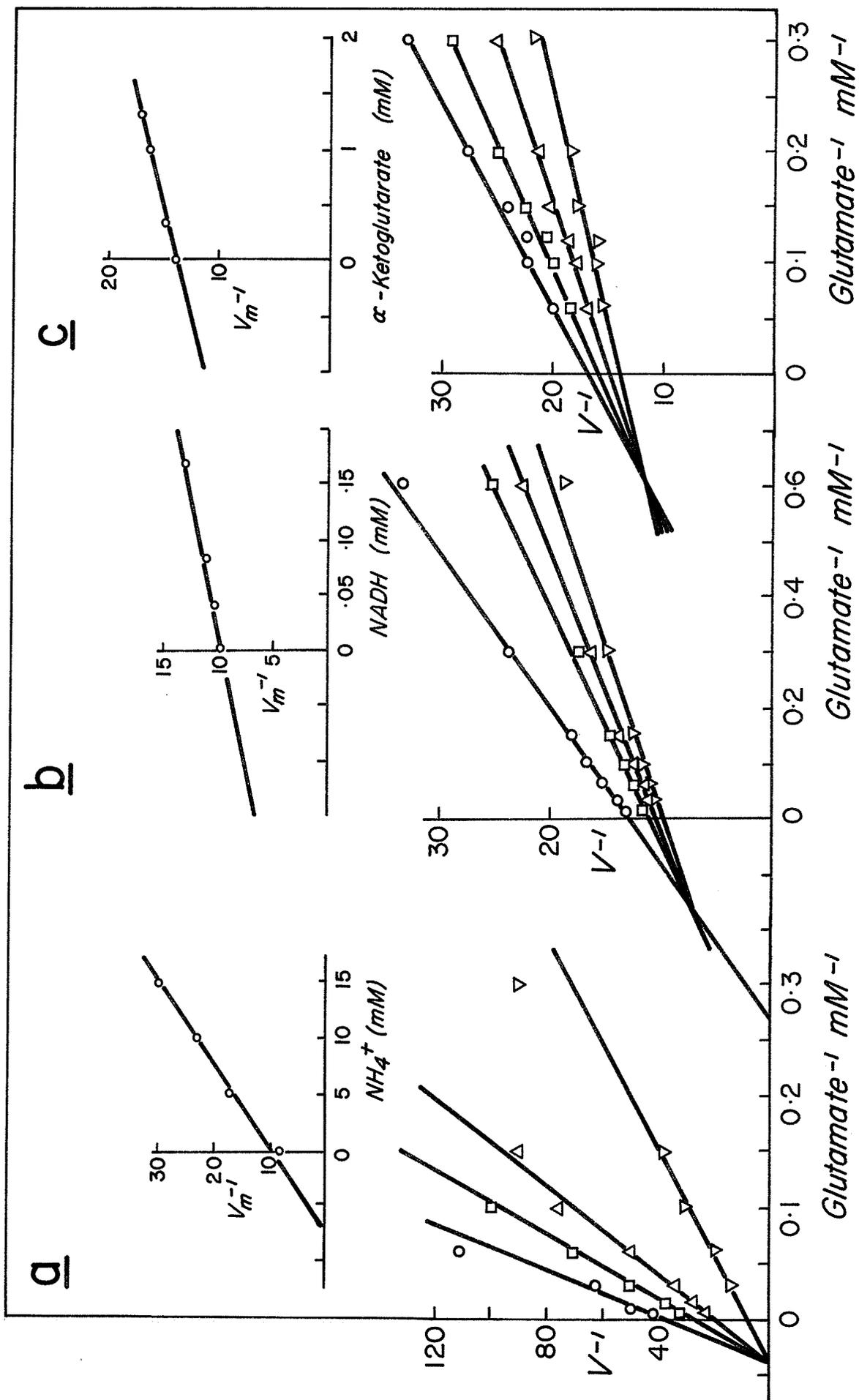


Figure 12: Lineweaver-Burk representation of product inhibition studies of the oxidative deamination reaction catalyzed by Pythium NAD-specific glutamate dehydrogenase in the absence of activator(s). Glutamate as varied ligand. (a) Ammonia as inhibitor at the following concentrations: (  $\circ$  ), 15 mM; (  $\square$  ), 10 mM; (  $\triangle$  ), 5 mM; (  $\nabla$  ), 0 mM. Inset: replot of intercepts against ammonia concentrations. (b) NADH as inhibitor at the following concentrations: (  $\circ$  ), 0.167 mM; (  $\square$  ), 0.083 mM; (  $\triangle$  ), 0.042 mM; (  $\nabla$  ), 0 mM. Inset: replot of intercepts against NADH concentrations. (c)  $\alpha$ -ketoglutarate as inhibitor at the following concentrations: (  $\circ$  ), 1.33 mM; (  $\square$  ), 1 mM; (  $\triangle$  ), 0.33 mM; (  $\nabla$  ), 0 mM. Inset: replot of intercepts against  $\alpha$ -ketoglutarate concentrations. Assay condition as for Fig. 7.



with glutamate as the varied substrate and  $\text{NAD}^+$  held at a fixed saturating level. The patterns elicited were as follows:

(Fig. 12 a) ammonia and glutamate, non-competitive with glutamate  $K_m$  unchanged; (Fig. 12 b) NADH and glutamate, non-competitive with a  $K_m$  change for glutamate; and (Fig. 12 c)  $\alpha$ -ketoglutarate and glutamate, also non-competitive, with a change in the  $K_m$  for glutamate. Secondary plots were all linear.

As  $\text{NAD}^+$  and NADH are competitive (Fig. 11 b), they therefore should bind to the same enzyme form, probably the free enzyme. The non-competitive inhibition patterns for the other products indicates that they do not bind to the same enzyme forms. These kinetic studies of product inhibition employ the procedures of Cleland (1967) to determine kinetic mechanism. The algebraic treatment of the steady-state rate equation for the ordered binary-ternary ("Ordered Bi-Ter") reaction of glutamate dehydrogenase was previously discussed by LéJohn et al (1968) for the enzymes of Thiobacillus novellus. The steady-state rate equation in terms of Cleland's kinetic constants is as follows:

$$v = V_1 \left( AB - \frac{PQR}{K_{eq}} \right) / \left( K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_bK_qP}{K_pK_{iq}} + \frac{K_{ia}K_bR}{K_{ir}} + \frac{K_bAP}{K_{ip}} + \frac{K_{ia}K_bK_rPQ}{K_pK_{iq}K_{ir}} + \frac{K_aBR}{K_{ir}} + \frac{K_{ia}K_bQR}{K_{iq}K_{ir}} + \frac{ABQ}{K_{iq}} + \frac{ABP}{K_{iq}} + \frac{K_{ia}K_bPQR}{K_pK_{iq}K_{ir}} + \frac{K_rK_bAPQ}{K_pK_{iq}K_{ir}} + \frac{ABQ}{K_{iq}} + \frac{K_{ia}K_bBQR}{K_{ib}K_{iq}K_{ir}} + \frac{ABPQ}{K_{ip}K_{iq}} + \frac{K_{ia}K_bBPQR}{K_pK_{ib}K_{iq}K_{ir}} \right) \quad (1)$$

A and B represent substrates; P, Q and R the three products in the Bi-Ter reaction. By setting two of the three products at zero concentration, and removing their terms from the steady-state equation as not applicable under initial velocity conditions, an equation is obtained predicting the effect of inhibition by the third product. Done in turn for each of the products, this treatment provides predictions of the product inhibition kinetic patterns under conditions where different substrates are fixed or varied. The following equations represent the forms for the different types of inhibition:

$$\text{linear competitive} \quad v = VS / K ( 1 + I/K_i) S \quad (2)$$

$$\text{linear uncompetitive} \quad v = VS / K + S ( 1 + I/K_i) \quad (3)$$

$$\text{linear non-competitive} \quad v = VS / K ( 1 + I/K_{iS}) + S(1 + I/K_{ii}) \quad (4)$$

(see Cleland 1967).

A summary of the predicted product inhibition patterns obtained by this procedure is shown in Table VII with the observed patterns for the Pythium enzyme. These predictions were made using the following designation of terms for substrates and product inhibitors on the ordered Bi-Ter reaction; A,  $\text{NAD}^+$ ; B, glutamate; P,  $\alpha$ -ketoglutarate; Q,  $\text{NH}_4^+$ ; and R, NADH. A line diagram of this mechanism would appear

TABLE VII

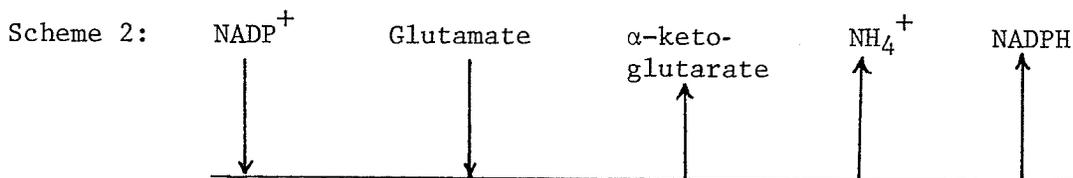
Predicted and observed product inhibition patterns of ordered binary-ternary and ternary-binary kinetic mechanisms according to Scheme 2.

Binary-ternary: Varied Substrate	Inhibitor					
	<u>NADH</u>		<u>Ammonia</u>		<u><math>\alpha</math>-ketoglutarate</u>	
	Pred.	<u>Pythium</u>	<u>Achllya</u>	Pred.	<u>Pythium</u>	<u>Achllya</u>
NAD <sup>+</sup>	C	C	C	UC	UC	NC
Glutamate	NC	NC	NC	UC	NC*	NC
<hr/>						
Ternary-binary: Varied Substrate	<u>Glutamate</u>					
	<u>NADH</u>		<u>Pythium</u>		<u>Pythium</u>	
	Pred.	<u>Pythium</u>	<u>Pythium</u>	Pred.	<u>Pythium</u>	<u>Pythium</u>
NADH	C	C	C	UC $\rightarrow$ NI	UC	UC
$\alpha$ -ketoglutarate	UC	UC	UC	NC	NC	NC
Ammonia	UC	UC	UC	UC $\rightarrow$ NI	UC	UC

Abbreviations used: C, competitive; NC, noncompetitive; UC, uncompetitive.  
 (\*) indicates a deviation from the theoretical pattern.



and is referred to as the "NADP-type" mechanism.



In this case the order of release of  $\alpha$ -ketoglutarate and ammonia are reversed with respect to Scheme 1 order.

The predictions summarized in Table VII were made using product designation of the NADP-type mechanism, substituting  $\text{NAD}^+$  ( $\text{NADH}$ ) for the  $\text{NADP}^+$  ( $\text{NADPH}$ ) coenzyme. In comparing the predicted and observed product inhibition patterns in this table, there is a single deviation from all predictions. Ammonia served as a non-competitive inhibitor of glutamate binding when, theoretically, it should be an uncompetitive inhibitor. If predictions made according to the NAD-type mechanism were used instead of the NADP-type pattern, then  $\alpha$ -ketoglutarate should be an uncompetitive inhibitor of  $\text{NAD}^+$  and glutamate. These predictions were not satisfied except when  $\text{NH}_4^+$  was used as an inhibitor of glutamate binding (Figure 12 a).

The results, therefore do not completely define the kinetic mechanism. Only the order of  $\text{NAD}^+$ ,  $\text{NADH}$ , and glutamate binding can be determined unequivocally. Release of ammonia could proceed by either the NAD-type or NADP-type mechanism, although the NAD-

type scheme is less likely. Product inhibition studies with allosteric activators were carried out in an attempt to resolve the mechanism order.

2. Pythium enzyme : with activators: With  $\text{NADP}^+$  at saturating concentration (0.167 mM), the product inhibition studies of Figures 11 and 12 were repeated, and these results are presented in Figures 13 and 14. The product inhibition patterns obtained are identical to those obtained without activators, but the inhibition constants were increased by a factor of 2. Other modulators of the oxidative deamination reaction, GTP, P-enolpyruvate, and acyl CoA derivatives (discussed later), did not effect any change in the product inhibition pattern, when used singly or in multiple combinations. A summary of the varied product inhibition patterns obtained with all the activators is given in Table VIII. The results were still equivocal.

An alternative approach was taken to resolve the question. An experiment was conducted to determine the order of substrate binding to the enzyme in the reductive amination reaction. This involved product inhibition studies of this reaction in the presence of an activator. Figure 15 illustrates the product inhibition patterns obtained when glutamate was used as inhibitor. With  $\alpha$ -ketoglutarate as the varied substrate,  $\text{NADP}^+$  saturating at 50  $\mu\text{M}$  concentration, and ammonia held fixed at a high level of 100 mM, the product inhibition pattern obtained with glutamate inhibiting was

Figure 13: Product inhibition in the presence of activator.

Same conditions as for Fig. 11. Note that (b) and (c) are interposed. In Fig. 11 (c), the symbols represent rates at the following concentrations of NADH: (  $\circ$  ), 15 mM; (  $\square$  ), 10 mM; (  $\triangle$  ) 5 mM; (  $\nabla$  ), 0 mM.  $\text{NADP}^+$  present throughout at 0.166 mM. Assay condition as for Fig. 7.

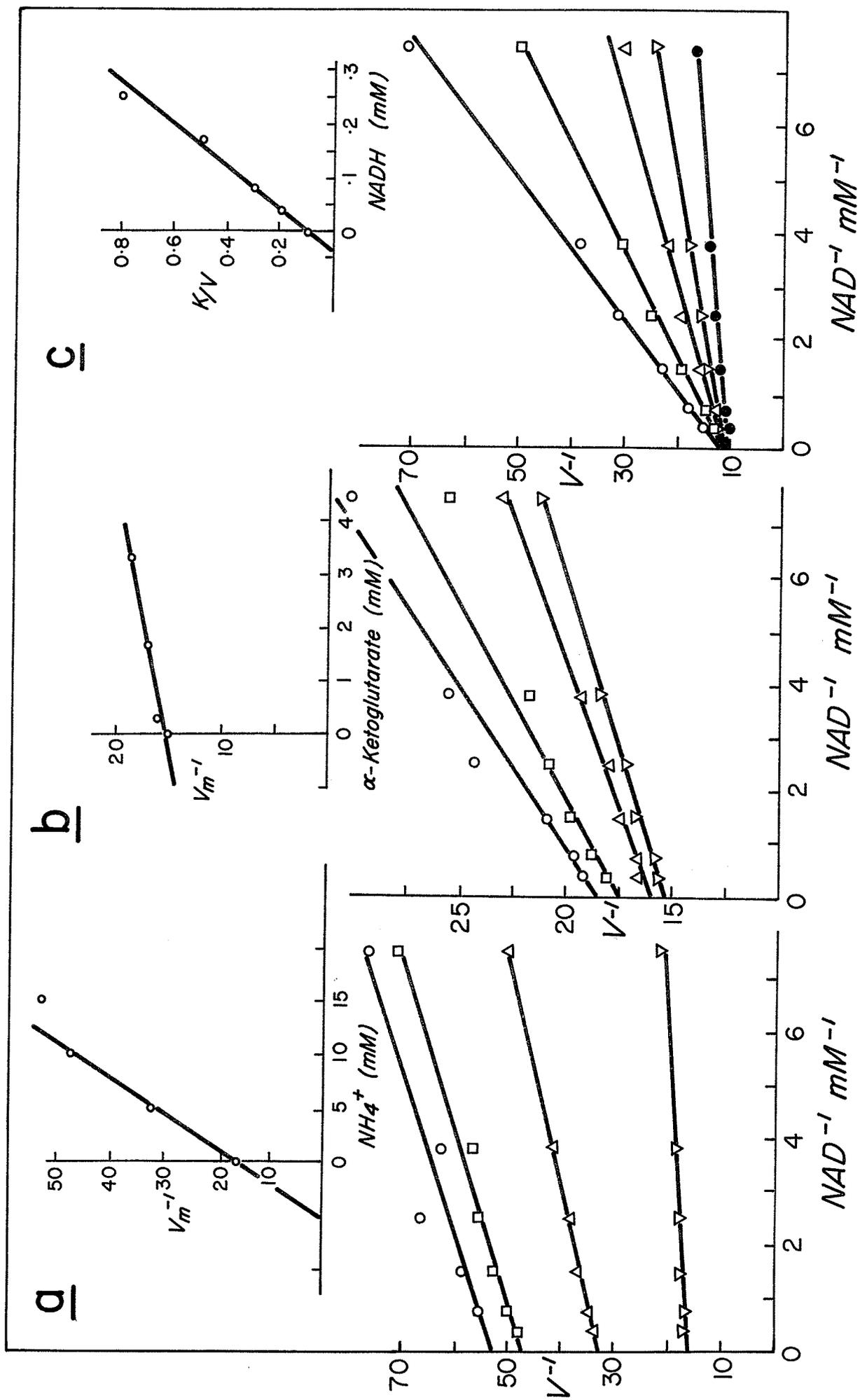


Figure 14: Product inhibition in the presence of activator.

Same conditions as for Fig. 12 except that in Fig. 14 (b) (  $\circ$  ) is 0.25 mM; (  $\square$  ) 0.167 mM; (  $\triangle$  ), 0.083 mM; (  $\nabla$  ), 0.042 mM; (  $\bullet$  ), 0 mM NADH.  $\text{NADP}^+$  present throughout at 0.166 mM. Assay condition as for Fig. 7.

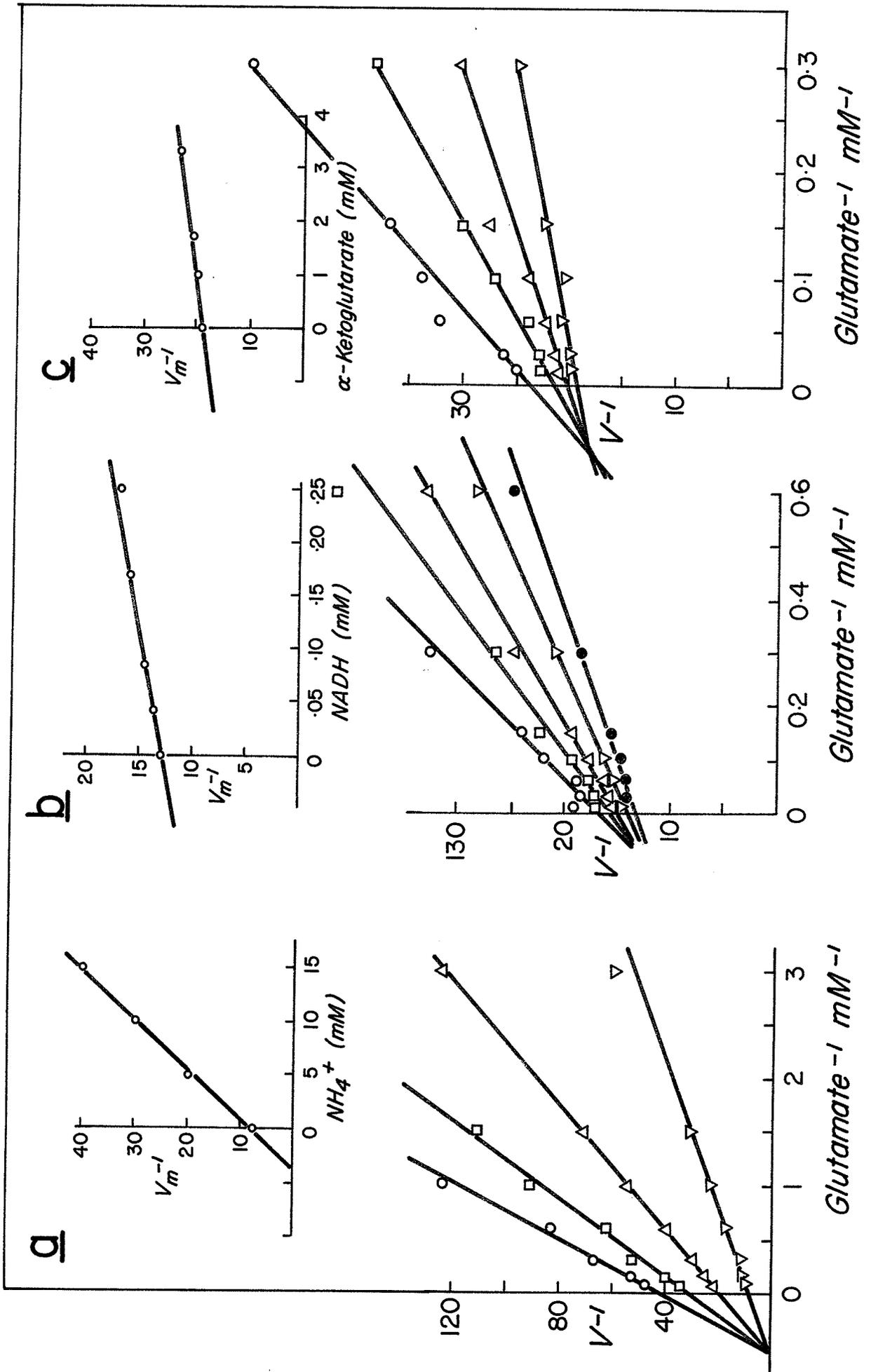


Table VIII

Product inhibition patterns for Pythium NAD-specific glutamic dehydrogenase in the presence and absence of allosteric activators.

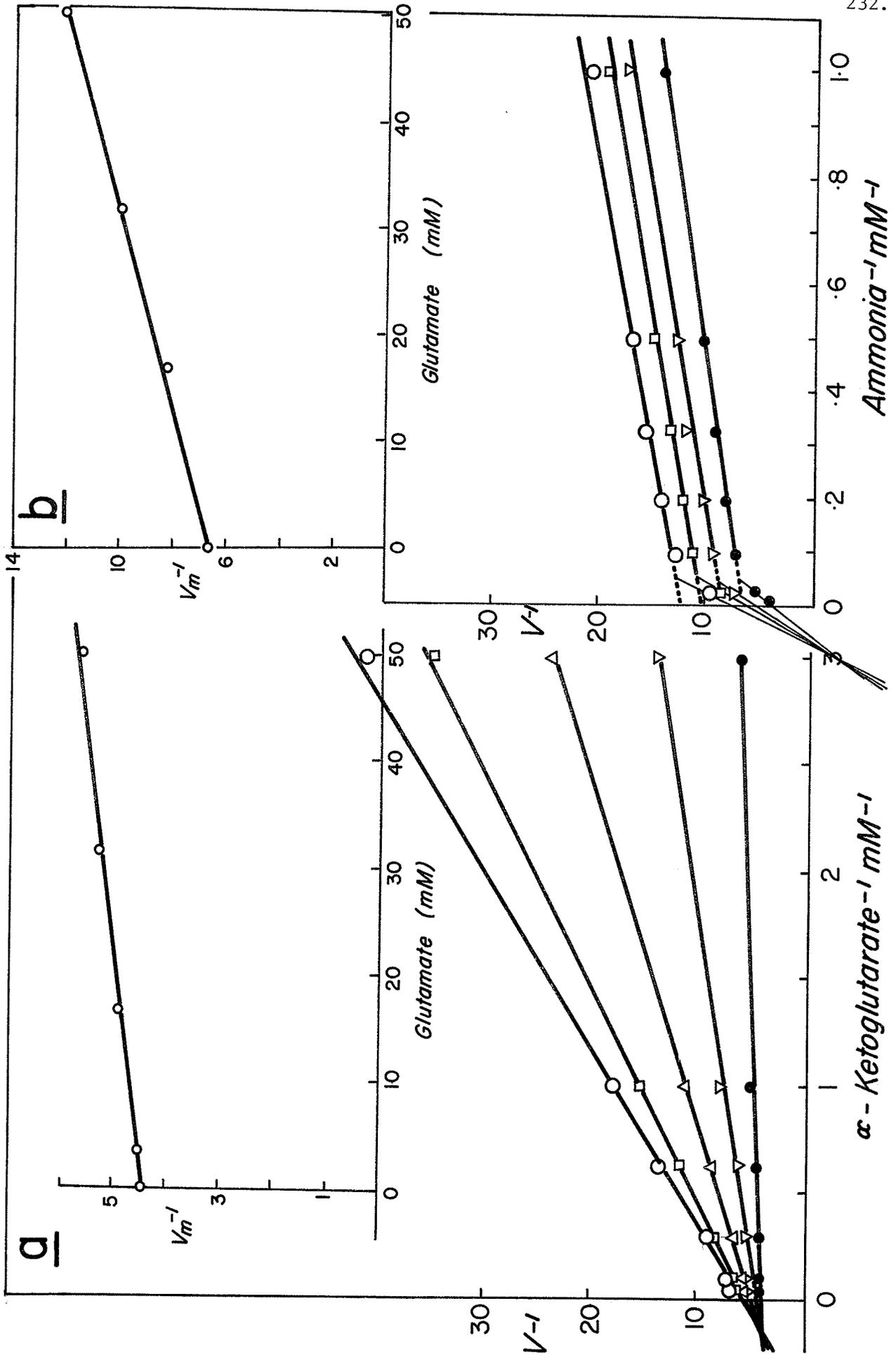
Activators(s)	Varied substrate					
	NAD <sup>+</sup>			Glutamate		
	Inhibitor			Inhibitor		
	$\alpha$ -Kg	NH <sub>4</sub> <sup>+</sup>	NADH	$\alpha$ -Kg	NH <sub>4</sub> <sup>+</sup>	NADH
None	NC	UC	C	NC	NC	NC
(All five activators)	NC	UC	C	NC	NC	NC
Any of five activators*	NC	UC	C	NC	NC	NC

\*GTP, Acetyl CoA, P-enolpyruvate, NADP<sup>+</sup>, ATP.

$\alpha$ -Kg,  $\alpha$ -ketoglutarate; NH<sub>4</sub><sup>+</sup>, Ammonia.

NC, noncompetitive; UC, uncompetitive; C, competitive. All activators were used at saturating concentrations of 0.333 mM except ATP which was 3 mM.

Figure 15: Product inhibition of the reductive amination, catalyzed by Pythium NAD-specific glutamate dehydrogenase, by glutamate in the presence of NADP<sup>+</sup>. (a)  $\alpha$ -ketoglutarate as varied ligand. Glutamate as inhibitor at the following concentrations: (  $\circ$  ), 50 mM; (  $\square$  ), 33.33 mM; (  $\triangle$  ), 16.67 mM; (  $\nabla$  ), 3.33 mM; (  $\bullet$  ), 0 mM. Inset: replot of parallel lines intercepts against glutamate concentrations. Assay system contained 16.66 mM  $\alpha$ -ketoglutarate (replacing ammonia as second fixed ligand) and other reactants as outlined for Fig. 15 (a). 2  $\mu$ g enzyme was used.



non-competitive (Figure 15 a). The secondary plot was linear and the inhibition constant estimated very high (Table VI). When ammonia was used as the varied substrate, and glutamate as inhibitor (Figure 15 b), primary plots were linear and uncompetitive, and the secondary plots were also linear, with a correspondingly high  $K_i$  for glutamate. This observation satisfied the predictions of the kinetic mechanism of a ternary-binary ordered sequential reaction outlined in Table VII. The mechanism is further considered in the "Discussion" section.

3. Achlya enzyme: Prior to the intensive studies of product inhibition patterns of the Pythium enzyme, similar studies had been carried out using the Achlya catalyst. These product inhibition patterns are presented in Figures 16, 17 and 18. With  $\text{NAD}^+$  as the varied substrate, NADH shows competitive inhibition patterns, both in the absence (Figure 16 a) and presence (Figure 17 a) of  $\text{NADP}^+$  as activator.  $\alpha$ -Ketoglutarate inhibition with  $\text{NAD}^+$  as varied substrate (Figure 16 b and 17 b), and some studies of varied glutamate with the inhibitors NADH (Figure 18 a),  $\text{NH}_4^+$  (Figure 18 b), and  $\alpha$ -ketoglutarate (Figure 18 c and d) showed similar patterns to those of the Pythium enzyme. These results are also summarized in Table VII.

The pattern for  $\text{NH}_4^+$  inhibition differs from the Pythium results and from the predicted pattern. However, as previously

Figure 16: Lineweaver-Burk representation of product inhibition studies of the oxidative deamination reaction catalyzed by Achlya NAD-specific glutamate dehydrogenase in the absence of activators.  $\text{NAD}^+$  as varied ligand, glutamate at 0.02 mM. (a) NADH as inhibitor at the following concentrations: (  $\circ$  ), 0.067 mM; (  $\square$  ), 0.033 mM; (  $\triangle$  ), 0 mM. (b)  $\alpha$ -ketoglutarate as inhibitor at the following concentrations: (  $\square$  ), 1.67 mM; (  $\triangle$  ), 0.33 mM; (  $\circ$  ), 0 mM. (c) Ammonia as inhibitor at the following concentrations: (  $\nabla$  ), 50 mM; (  $\circ$  ), 30 mM; (  $\square$  ), 10 mM; (  $\triangle$  ), 0 mM. Insets: replots of slopes (a) or intercepts (b, c) against inhibitor concentration.

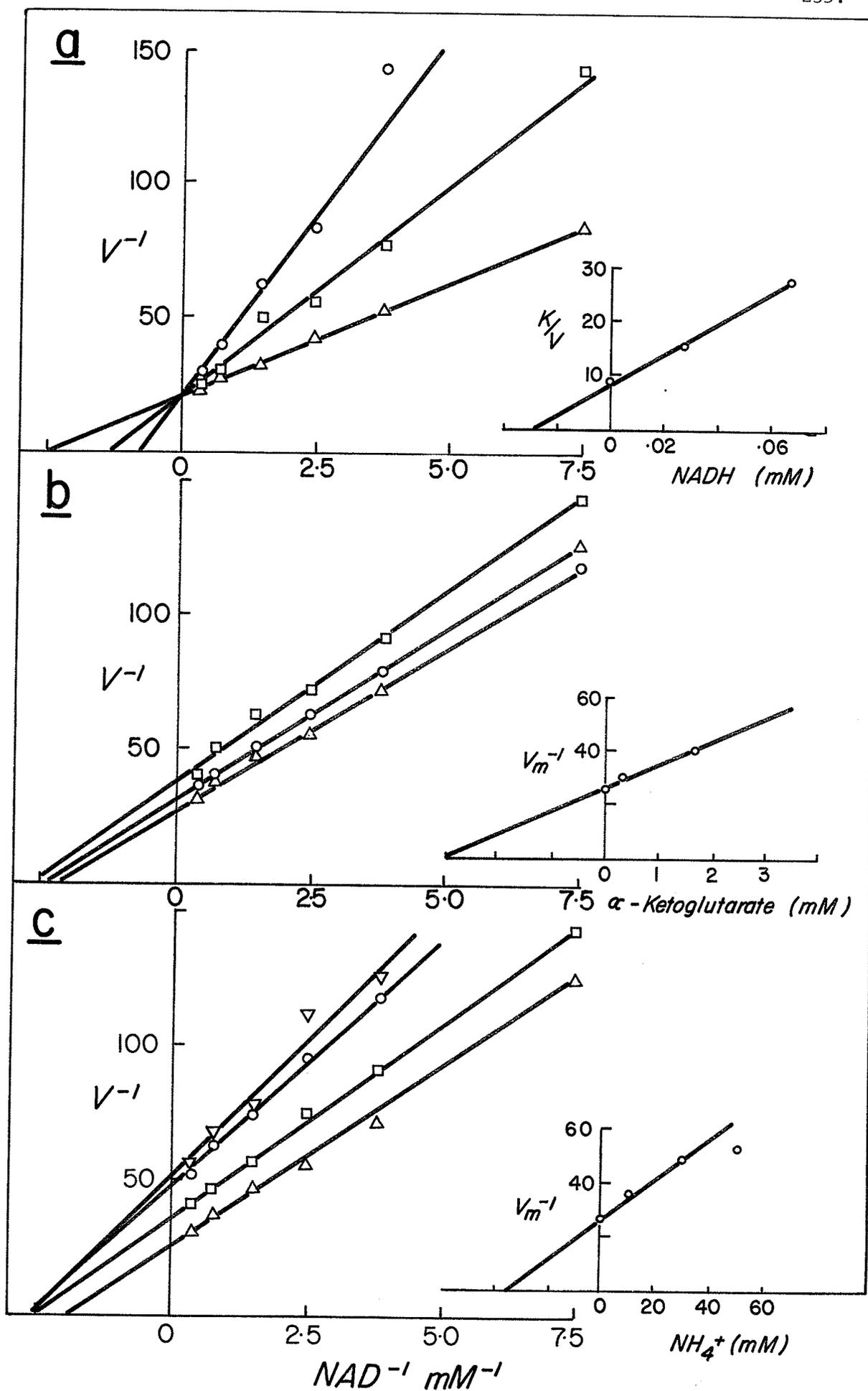


Figure 17: Lineweaver-Burk representation of product inhibition studies of the oxidative deamination reaction catalyzed by Achlya NAD-specific glutamate dehydrogenase with  $\text{NADP}^+$  (0.33 mM).  $\text{NAD}^+$  as varied ligand. Glutamate at 66.7 mM. (a) NADH as inhibitor at the following concentrations: ( $\nabla$ ), 0.067 mM; ( $\bullet$ ), 0.50 mM; ( $\triangle$ ), 0.033 mM; ( $\square$ ) 0.016 mM; ( $\circ$ ) 0 mM. (b)  $\alpha$ -Ketoglutarate as inhibitor at the following concentrations; ( $\nabla$ ), 6.67 mM; ( $\bullet$ ), 3.33 mM; ( $\times$ ), 1.67 mM; ( $\triangle$ ), 1.0 mM; ( $\square$ ), 0.67 mM; ( $\circ$ ) 0.33 mM. (c) Ammonia as inhibitor at the following concentrations; ( $\nabla$ ) 50 mM; ( $\triangle$ ), 20 mM; ( $\square$ ), 10 mM; ( $\circ$ ), 0 mM. Insets: replots of slope (a) or intercept (b, c) against inhibitor concentration.

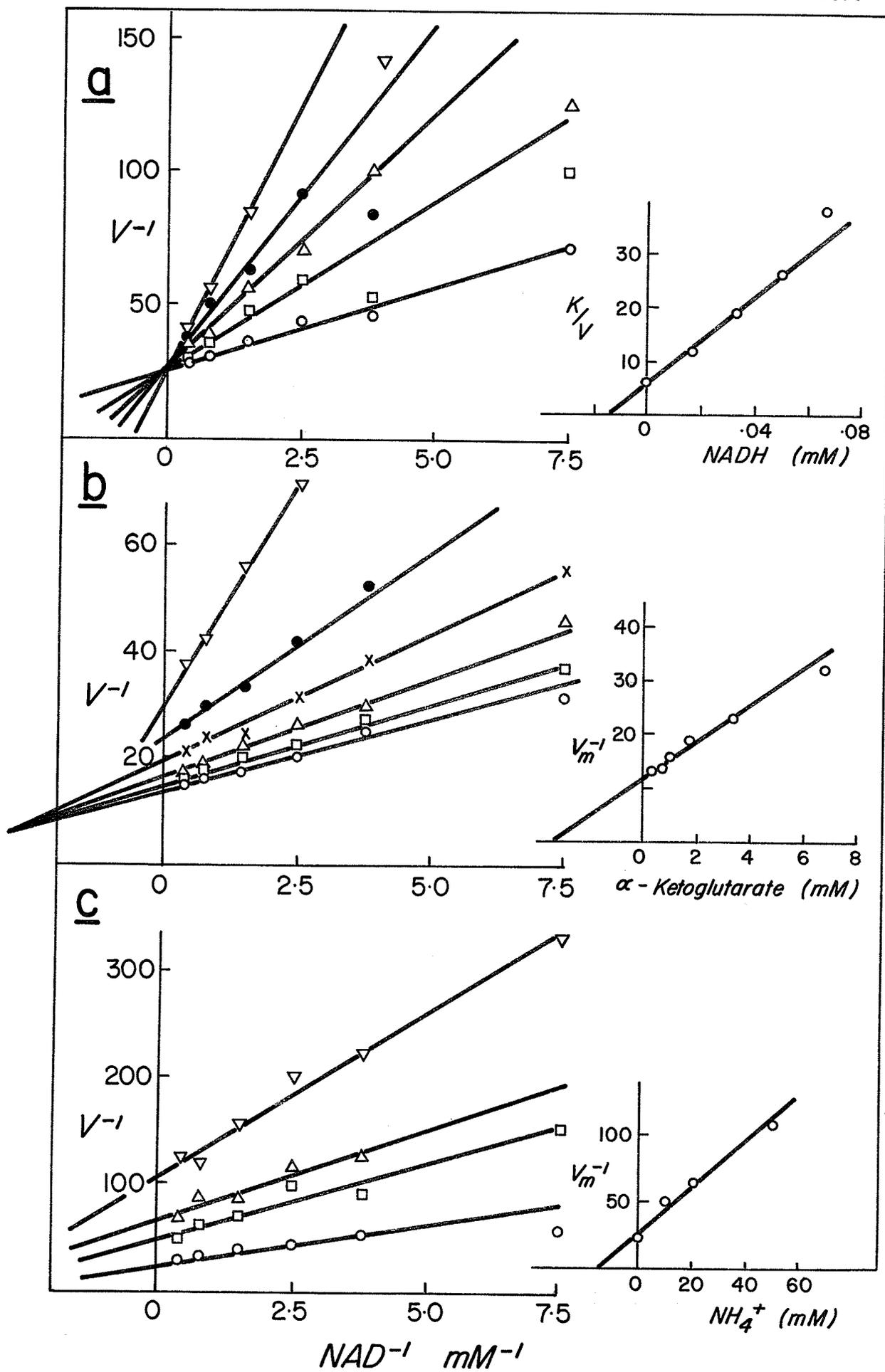
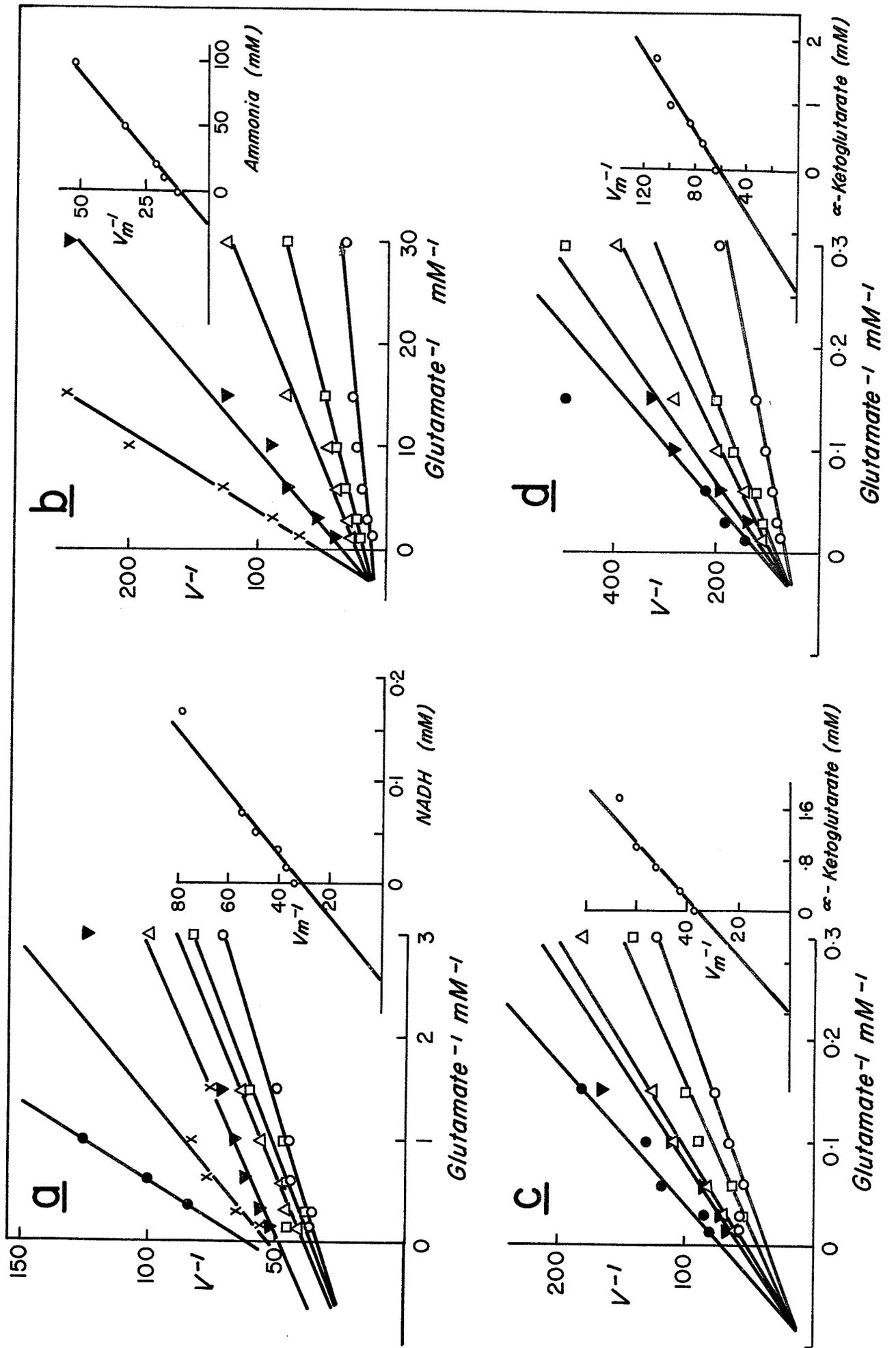


Figure 18: Lineweaver-Burk representation of product inhibition studies of the oxidative deamination reaction catalyzed by *Achlya* glutamate dehydrogenase, with glutamate as varied ligand. (a) NADH inhibition with 0.33 mM  $\text{NADP}^+$  as activator. NADH concentrations as follows; ( ● ), 0.167 mM; ( × ), 0.067 mM; ( ▼ ), 0.05 mM; ( △ ), 0.33 mM; ( □ ), 0.0167 mM; ( ○ ), 0 mM.

(b) Ammonia inhibition with  $\text{NADP}^+$ . Ammonia concentrations as follows; ( × ), 100 mM; ( ▼ ), 50 mM; ( △ ), 20 mM; ( □ ), 10 mM, ( ○ ), 0 mM.

(c)  $\alpha$ -ketoglutarate inhibition of desalted enzyme with 0.33 mM  $\text{NADP}^+$ .  $\alpha$ -Ketoglutarate concentrations as follows; ( ○ ), 1.67 mM; ( ▼ ), 1.0 mM; ( △ ), 0.67 mM; ( □ ), 0.33 mM; ( ○ ), 0 mM.

(d)  $\alpha$ -Ketoglutarate inhibition of desalted enzyme without  $\text{NADP}^+$  concentrations as for (d) above.



mentioned, the Achlya enzyme preparation requires ammonium sulfate to protect it from deterioration. This presents problems in determining accurate product inhibition patterns. The studies of  $\alpha$ -ketoglutarate inhibition of the reaction with glutamate as varied substrate shown in Figure 18 c and d were carried out on enzyme from which the ammonium sulfate had been removed by passage through a small Sephadex G-25 column. The desalted enzyme, however, showed rapid loss in activity in even a short period, making kinetic assays difficult. As the Pythium enzyme preparations did not present the problems associated with high ammonia concentrations, the detailed product inhibition studies to resolve the reaction order were carried out on that enzyme. The results obtained from the Achlya form indicate that the kinetic mechanism operating in the two species is essentially the same.

#### E. Enzyme Effectors and Regulators

1. NADP(H) Effects: The effects of  $\text{NADP}^+$  and NADPH in activating the reactions of the glutamate dehydrogenases of both Achlya and Pythium were described previously. The summary of  $S_{0.5}$  and  $M_{0.5}$  values for ligands interacting with the enzymes, with and without  $\text{NADP}^+$  were presented in Table VI, where  $S_{0.5}$  represents the affinity constants, and  $M_{0.5}$  the activation constants. The degree of activation is arbitrary as it depends on the concentrations of the substrates,  $\text{NH}_4^+$ , and  $\alpha$ -ketoglutarate, which also function as allosteric inhibitors.

For the Pythium enzyme, it was seen that ammonia and  $\alpha$ -ketoglutarate were the principal substrate inhibitors in the reductive amination reaction (Figures 9 and 10). Achlya glutamate dehydrogenase was less susceptible to ammonia toxicity. The maximum reaction velocity of the enzyme is difficult to estimate because the substrate is apparently continuously inhibiting the enzyme as the substrate level increases. When  $\text{NADP}^+$  was added, substrate inhibition was reversed, as was seen in Figures 9 and 10. The results of a more detailed study of the antagonism of  $\text{NH}_4^+$  inhibition by  $\text{NADP}^+$  in the reductive amination reaction catalyzed by the Pythium enzyme is shown in Figure 19. Varied levels of the activator were used over a fixed range of ammonia concentrations. When the  $\text{NADP}^+$  concentration was increased to 4 times  $M_{0.5}$ , the inhibition was completely overcome. The interaction of  $\alpha$ -ketoglutarate, the second substrate inhibitor in this Pythium reaction, with ammonia and  $\text{NADP}^+$  was also examined. Saturation curves for  $\alpha$ -ketoglutarate in the absence of  $\text{NADP}^+$  and at several different ammonia levels are shown in Figure 20. Ammonia concentration varied from 20 mM, the optimum at which there is no perceptible inhibition, to 100 mM, at which concentration the inhibition was nearly 100%. At all levels of  $\alpha$ -ketoglutarate, ammonia was inhibitory when used above 20 mM. Upon addition of  $\text{NADP}^+$ , the enzyme was released from ammonia inhibition. Under these conditions ammonia concentration as high as 100 mM was non-

Figure 19: Antagonism of  $\text{NH}_4^+$  inhibition by  $\text{NADP}^+$  in the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase. Assay components: 6.67 mM  $\alpha$ -ketoglutarate; 0.167 mM NADH; 67 mM Tris-acetate, pH 8; 2  $\mu\text{g}$  enzyme;  $\text{NADP}^+$  as specified.

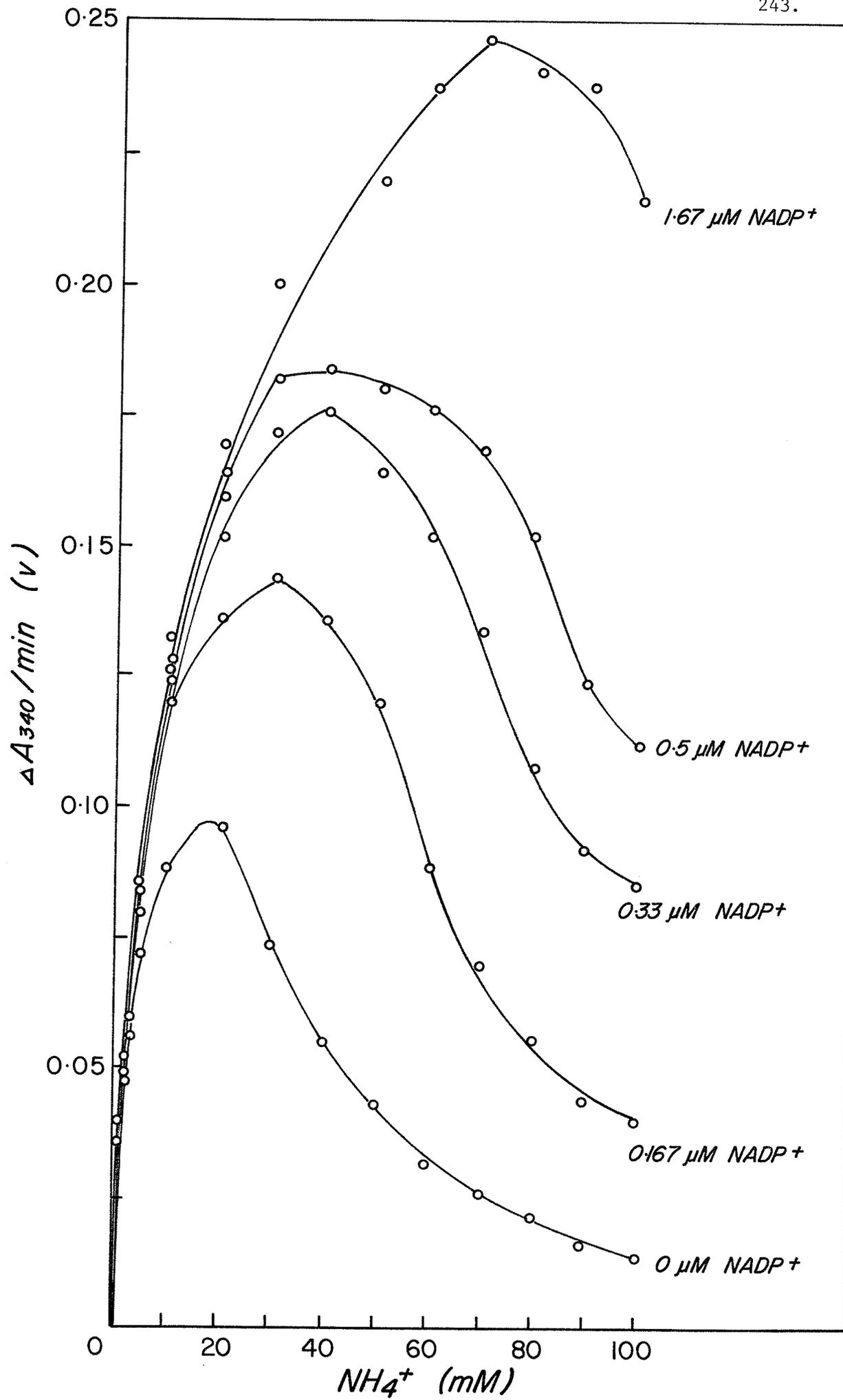
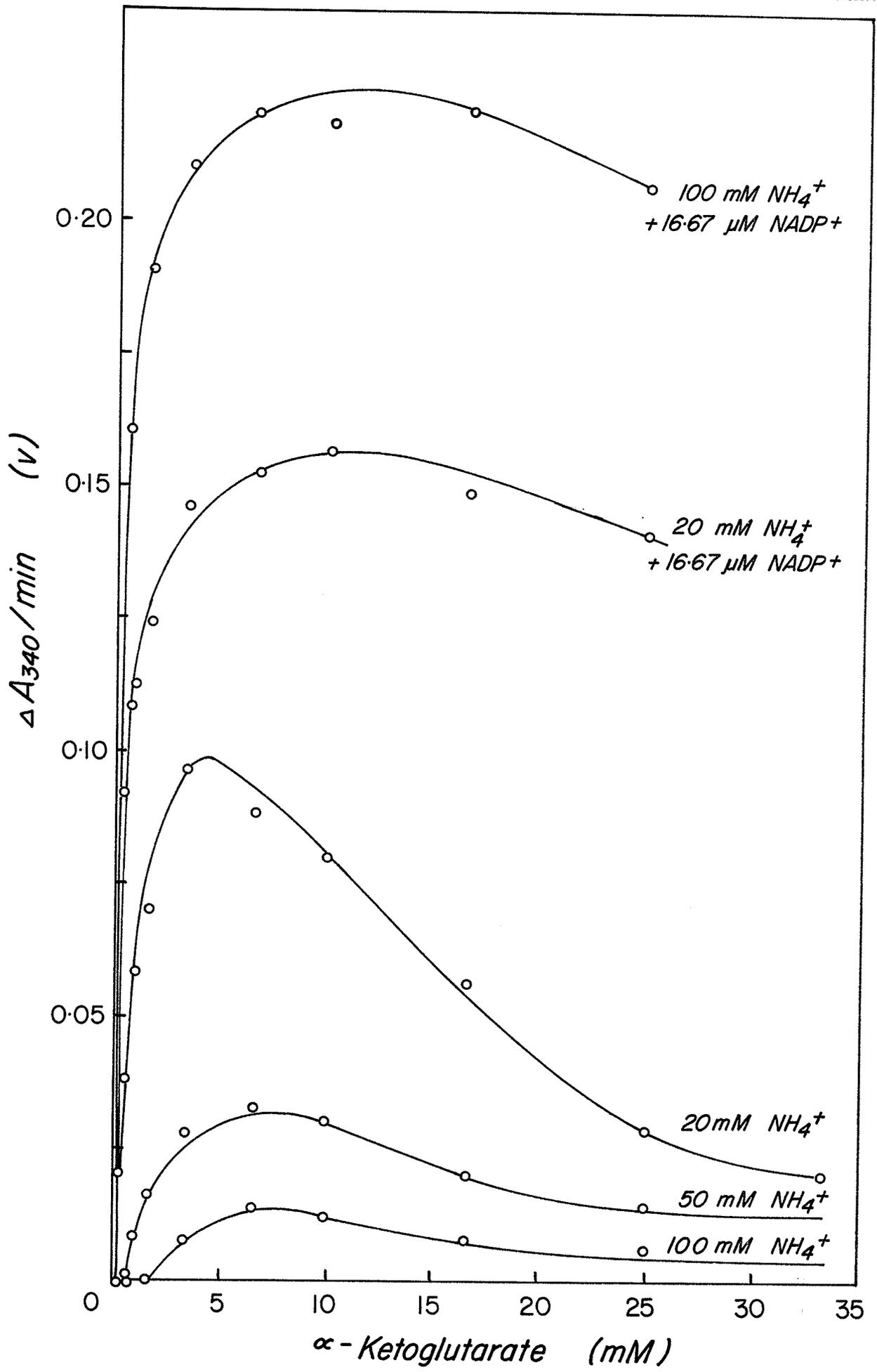


Figure 20: Influence of  $\text{NADP}^+$  on the interactions between the substrate-inhibitors,  $\text{NH}_4^+$  and  $\alpha$ -ketoglutarate in the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase. Assay components: 0.167 mM NADH; 67 mM Tris-acetate, pH 8; 2  $\mu\text{g}$  enzyme; other reactants specified in the figure.



inhibitory. There was a corresponding increase in  $V_{\max}$  and affinity for  $\alpha$ -ketoglutarate and ammonia.

2. Citrate and Isocitrate Effects: Evidence was already available to show that glucose metabolism leads to the repression of glutamate dehydrogenase synthesis (Smaluck 1971). During screening for effectors that might cause inhibition of glutamate dehydrogenase activity, citrate and isocitrate were found to be strong poisons of the activity of this enzyme. Both are equivalent in their effect. As for all other ligands, the kinetics of citrate action on the Pythium and Achlya catalysts showed some quantitative differences. The  $M_{0.5}$  values for citrate are given in Table VI. The oxidative deamination reaction was quite susceptible to citrate effects. For the Achlya enzyme, the  $M_{0.5}$  for citrate in the oxidative deamination catalysts without activators was estimated as  $3.3 \times 10^{-4}M$ . In the reductive amination reaction,  $M_{0.5}$  was calculated as  $1.65 \times 10^{-2}M$ . This is 50-fold greater than the oxidative deamination reaction value. Both values were determined at pH 8.0.

The interaction of  $NADP^+$  with this inhibition was examined for both the Achlya and Pythium enzymes. For the Achlya form, Figure 21 shows the effect of isocitrate on the oxidative deamination reaction, in the absence (Fig. 21 a) and the presence (Fig. 21 b) of  $NADP^+$ . The effects of citrate and  $NADP^+$  on the Achlya oxidative deamination and reductive deamination reaction are presented in

Figure 21: Isocitrate inhibition of Achlya glutamate dehydrogenase oxidative deamination reaction. (a) shows inhibition with no  $\text{NADP}^+$ , while (b) demonstrates antagonism by 0.33 mM  $\text{NADP}^+$ . The results are presented as a modified Hill plot, where  $\bar{v}$  is the velocity of the reaction, and  $\bar{V}$  is the maximal velocity. The slope,  $\underline{n}$ , reflects the molecules of ligand bound. Assay conditions were 50 mM glutamate, 2.66 mM  $\text{NAD}^+$ , and 67 mM Tris-acetate, pH 8.

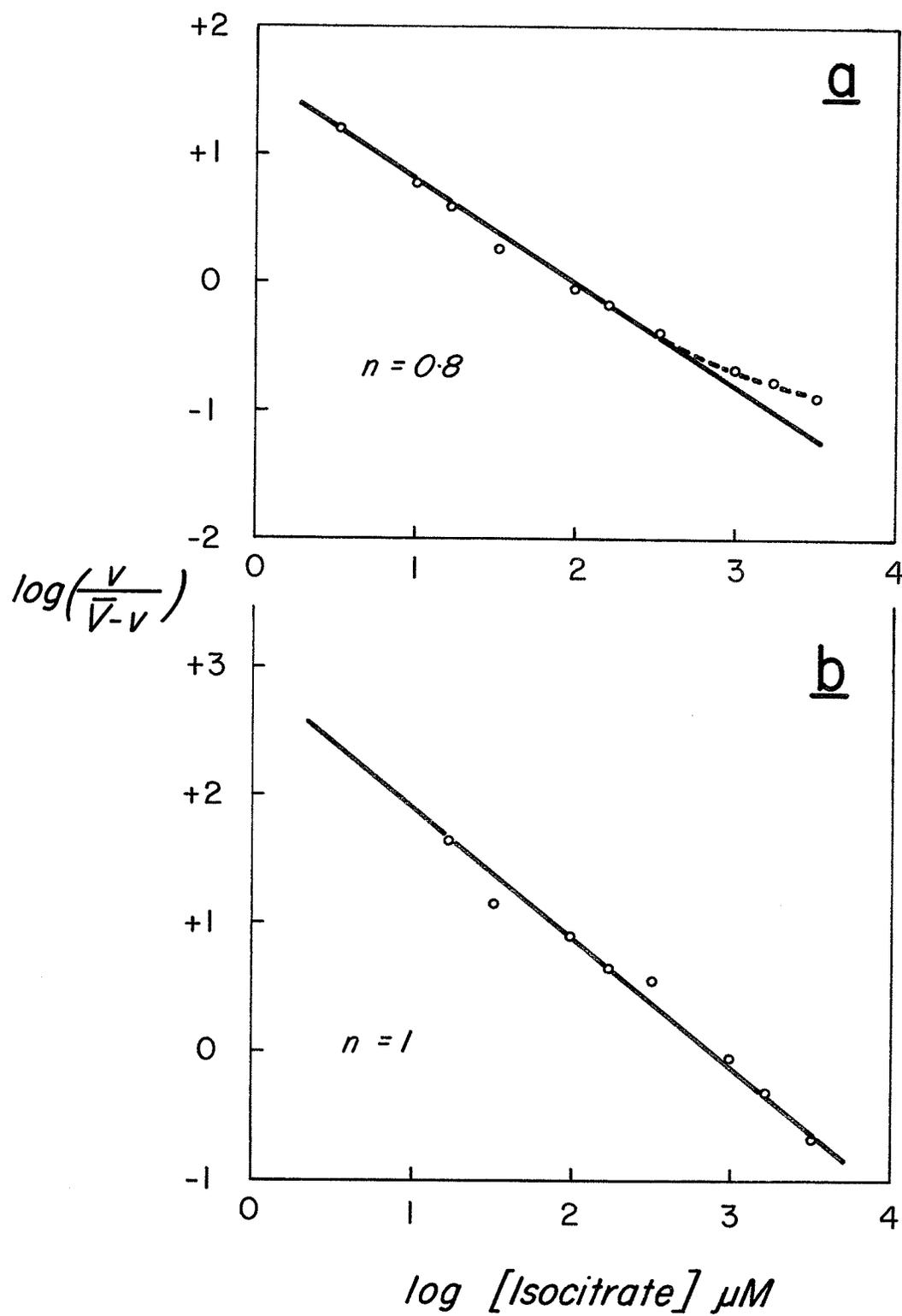


Figure 22 a and b, with glutamate and  $\alpha$ -ketoglutarate as the varied substrates.

3. P-enolpyruvate effects: It was found that  $\text{NADP}^+$  could not reverse the inhibitory effects of citrate completely. The possible involvement of a second activator in the system, which could act cumulatively with  $\text{NADP}^+$  to overcome citrate inhibition completely, was confirmed when P-enolpyruvate was found to be an activator. This was unexpected as P-enolpyruvate is a precursor of citrate in metabolism. The effector role of P-enolpyruvate was confirmed by determining the residual level of P-enolpyruvate in the reaction mixture in a pyruvate kinase-lactate dehydrogenase coupled assay system. The method described by Czok and Eckert (1963) was used. No P-enolpyruvate had been utilized at the completion of the glutamate dehydrogenase reaction.

In Figure 23, results of the activation of the reductive amination reaction of Pythium glutamate dehydrogenase are shown, for P-enolpyruvate alone, and in combination with saturating levels ( $500 K_m$ ) of  $\text{NADP}^+$ . In addition to being an activator, P-enolpyruvate was inhibitory at relatively high concentrations.  $\text{NADP}^+$  (0.167 mM) failed to relieve the inhibitory effects of P-enolpyruvate. Activation by P-enolpyruvate, like  $\text{NADP}^+$ , was unidirectional. The oxidative deamination reaction was not stimulated, even at extremely low concentrations of P-enolpyruvate. P-enolpyruvate resembled  $\text{NADP}^+$  in its antagonism of ammonia inhibition of the

Figure 22: Lineweaver-Burk representation of citrate inhibition of the oxidative deamination (a) and reductive amination (b) reactions catalyzed by Achlya glutamate dehydrogenase. Reaction conditions for (a) were 4 mM  $\text{NAD}^+$ , and 67 mM Tris-acetate pH 8. Additions were as follows; (●), no addition; (○), 0.3 mM  $\text{NADP}^+$ ; (□), 1 mM citrate and 0.3 mM  $\text{NADP}^+$ ; (△), 3.3 mM citrate and 0.3 mM  $\text{NADP}^+$ . Reaction conditions for (b) were 0.5 M ammonia, 0.167 mM NADH, and 100 mM Tris-acetate, pH 7. Additions were as follows; (●), no additions; (○), 0.33 mM  $\text{NADP}^+$ ; (△), 10 mM citrate; (□) 10 mM citrate and 0.33 mM  $\text{NADP}^+$ .

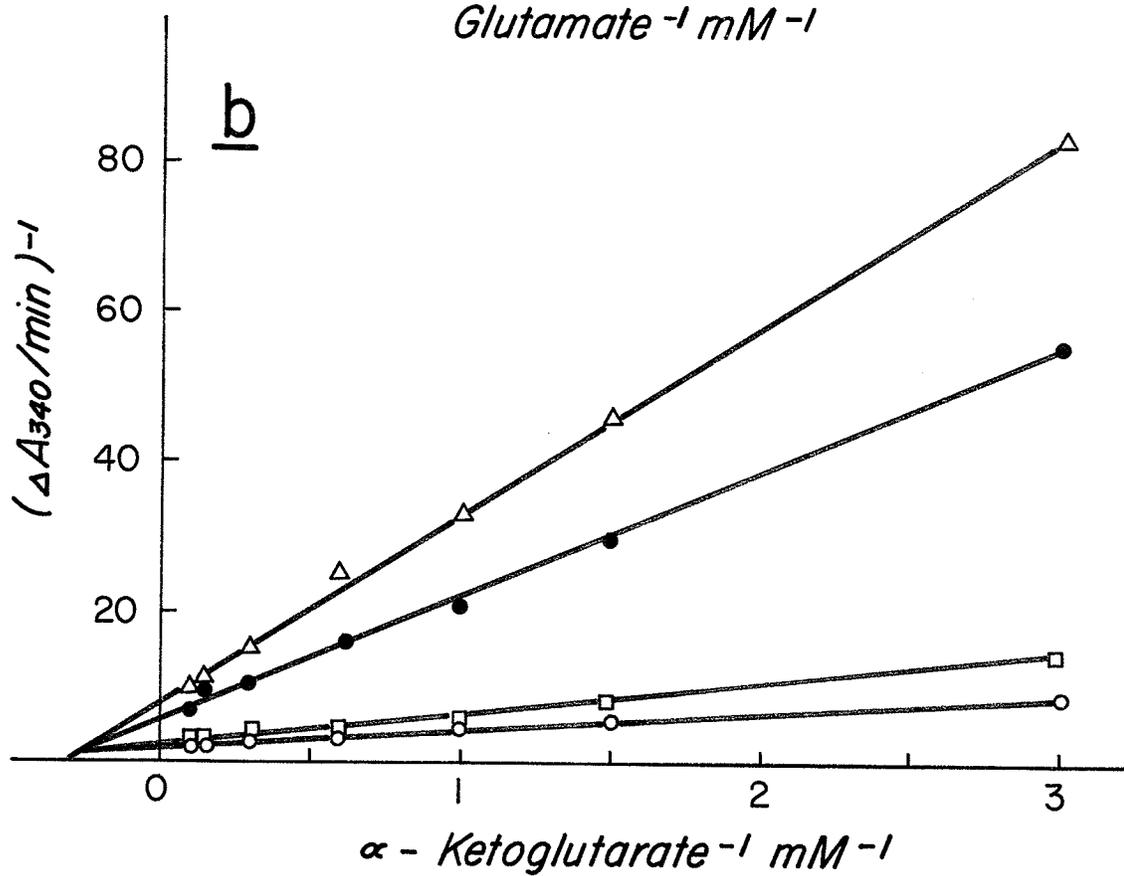
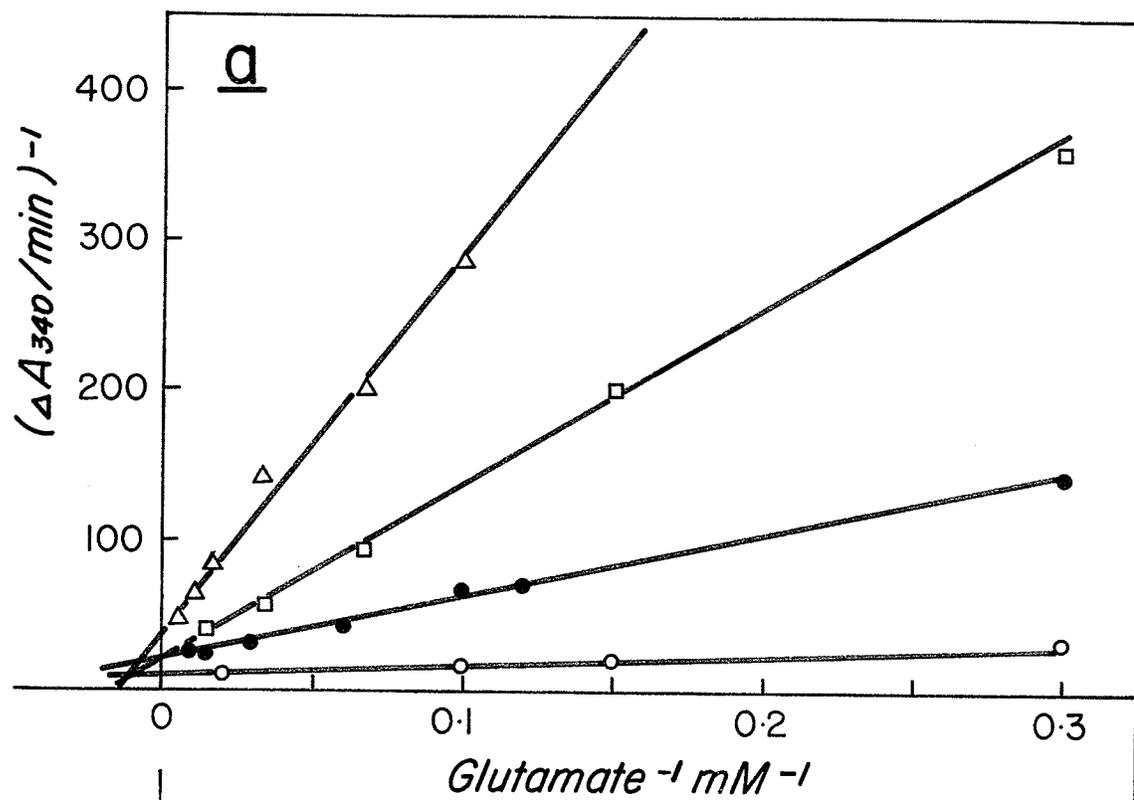
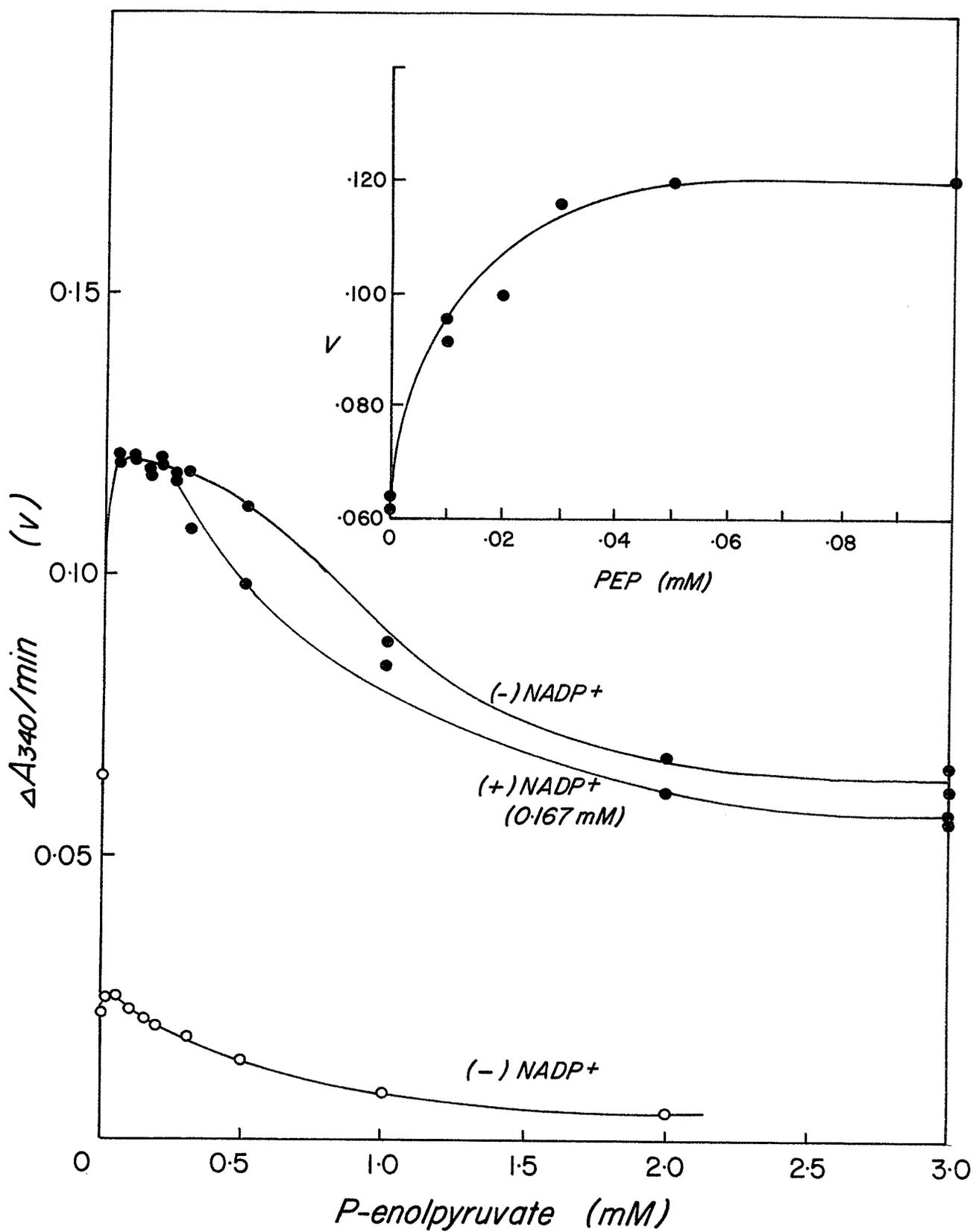


Figure 23: Activation of Pythium glutamate dehydrogenase by P-enolpyruvate in the reductive amination reaction (●) and in the oxidative deamination reaction (○). Inset: Enlargement of the saturation curve at low P-enolpyruvate levels.



Pythium reductive amination reaction (Fig. 24). Significant differences are seen in the influence of P-enolpyruvate on the Achlya glutamate dehydrogenase, compared with the Pythium effects described above. As shown in Figure 25, P-enolpyruvate could not activate the reductive amination reaction of the Achlya enzyme effectively above 50 mM ammonia concentration, whereas  $\text{NADP}^+$  can.

4. Interactions: The studies on interactions of  $\text{NADP}^+$  and P-enolpyruvate with citrate inhibition of the Achlya catalyst are shown in Figure 26. Individually the activators partially relieved citrate inhibition. Pairwise combinations of the activators produced a multivalent effect. Both completely reversed citrate inhibition. This is shown more clearly with the data obtained for the Pythium catalyst (Fig. 27 a, b). Because of the inefficacy of P-enolpyruvate as an activator of the oxidative deamination reaction of Pythium enzyme, the multivalent feature was studied in detail on the reductive reaction.

The reductive amination reaction of Pythium enzyme is 50% inhibited with  $2.5 \times 10^{-4}$  M citrate. In the presence of  $\text{NADP}^+$ , the enzyme showed more resistance to citrate inhibition, and the  $M_{0.5}$  was increased to  $1.6 \times 10^{-2}$  M. P-enolpyruvate was considerably less effective in this regard. When  $\text{NADP}^+$  was used at completely saturating concentrations ( $500 K_m$ ), inhibition by citrate above 20 mM levels was still manifested. However, combination of P-enolpyruvate and  $\text{NADP}^+$  was adequate to remove citrate inhibition

Figure 24: Antagonism of  $\text{NH}_4^+$  inhibition by P-enolpyruvate in the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase. Assay components: as in Fig. 19, P-enolpyruvate as specified.

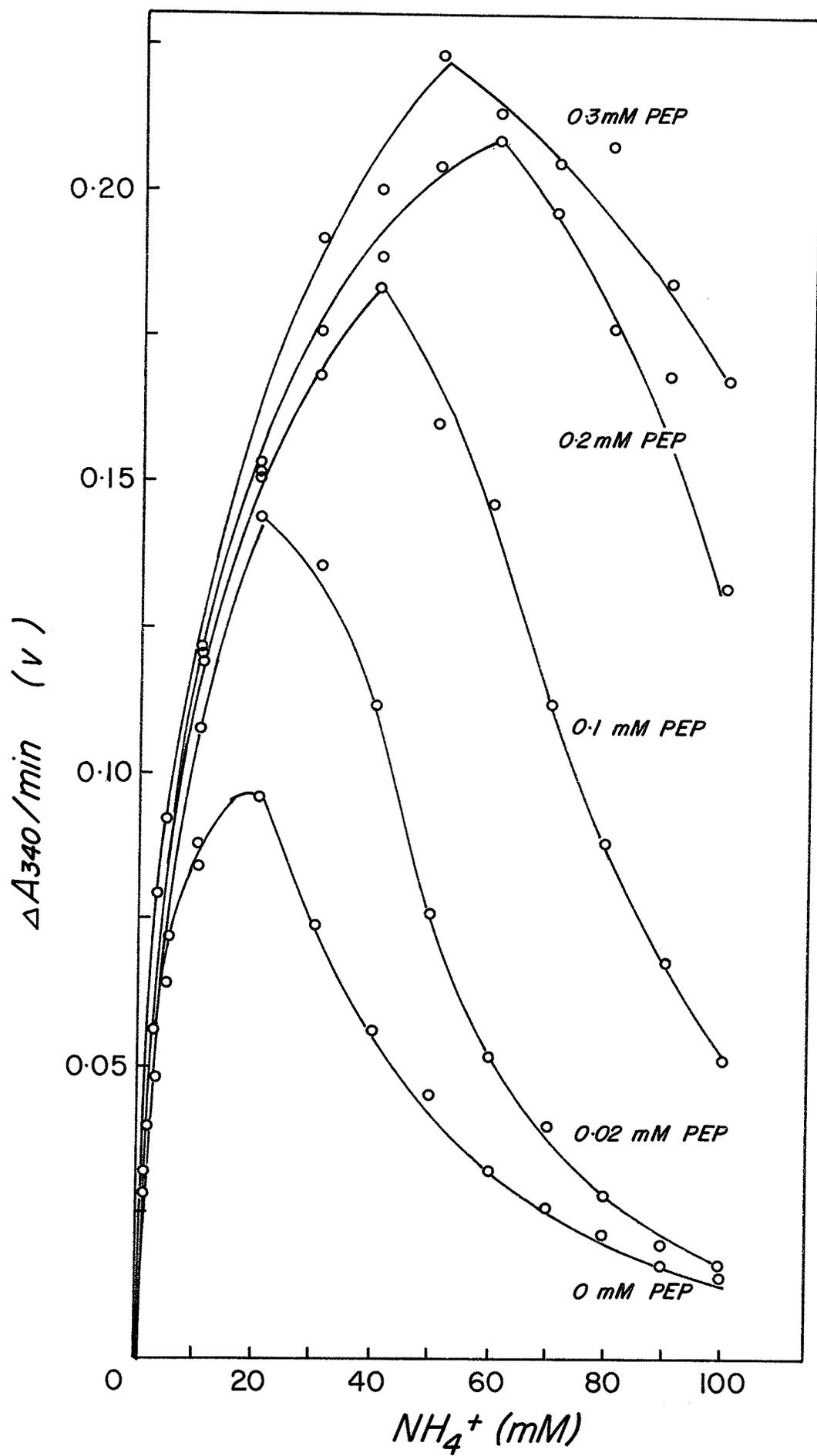


Figure 25: Influence of  $\text{NH}_4^+$  on the activation by  $\text{NADP}^+$  (  $\Delta$  ) and P-enolpyruvate (  $\square$  ) in the reductive amination reaction catalyzed by Achlya glutamate dehydrogenase.

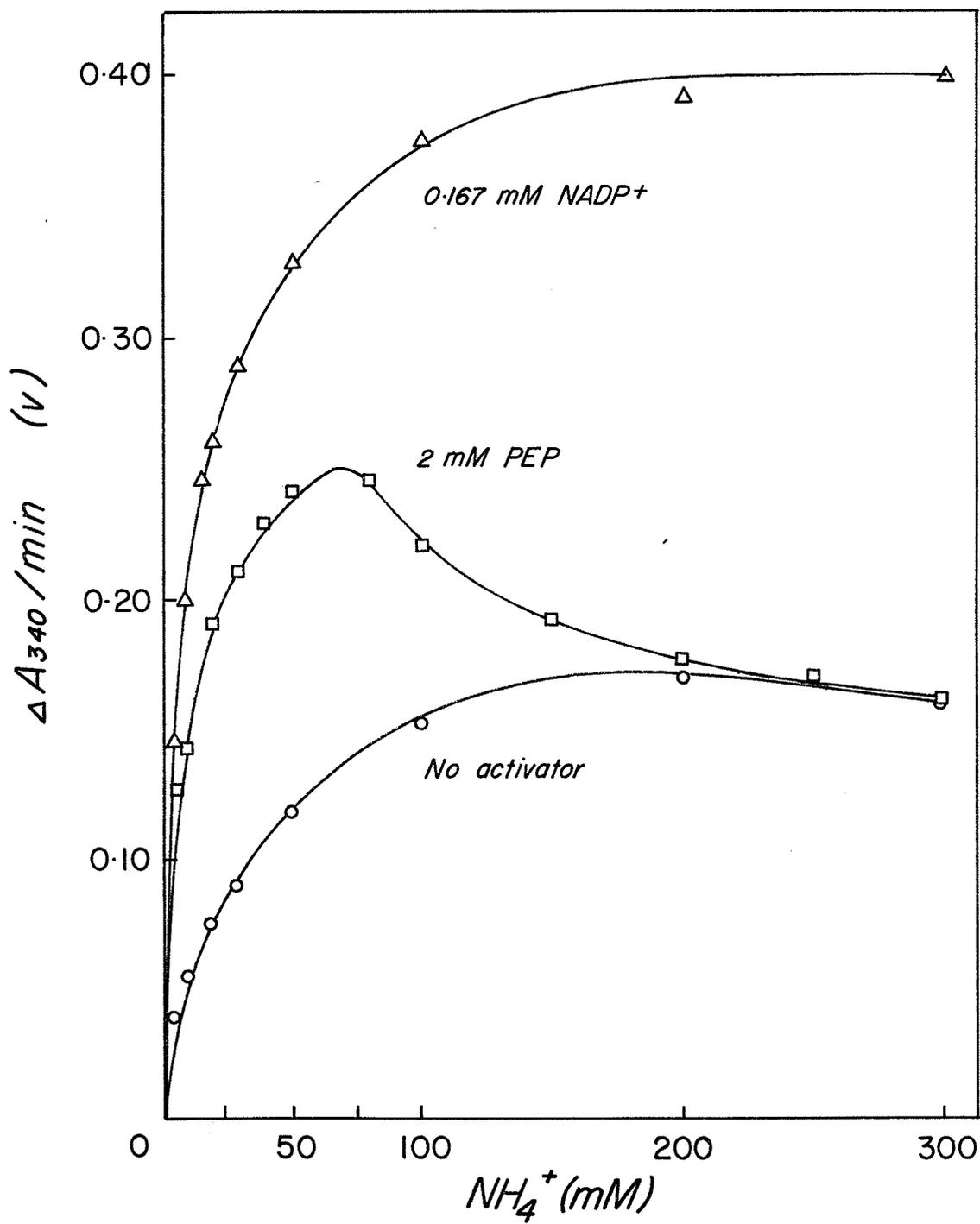


Figure 26: Inhibition by citrate of the reductive amination ( ● ) and oxidative deamination ( ○ ) reactions of glutamate dehydrogenase from Achlya. Substrates were used at saturating levels.  $\text{NADP}^+$  and P-enolpyruvate when used together, were at the individual concentrations indicated on the graph.

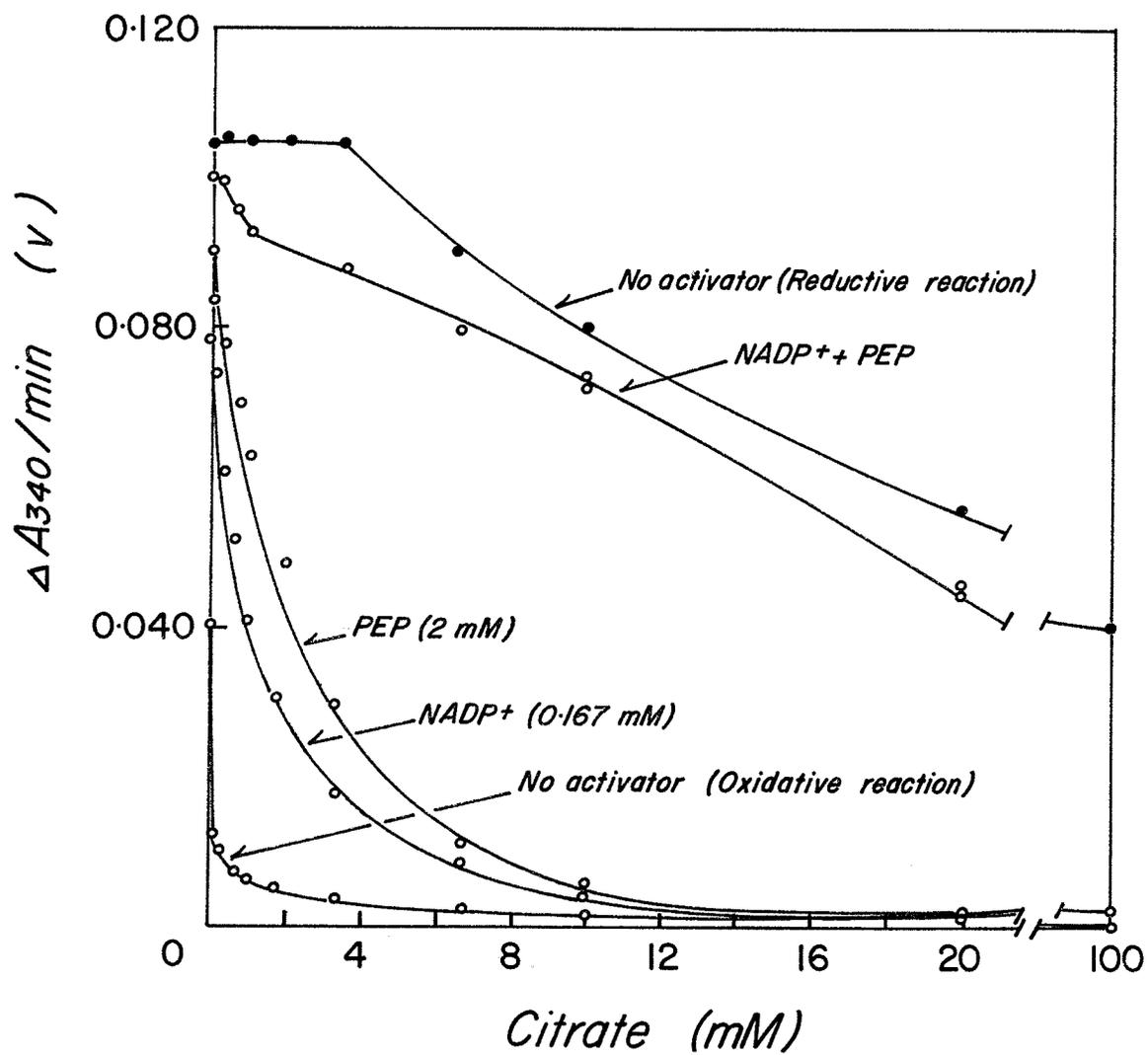
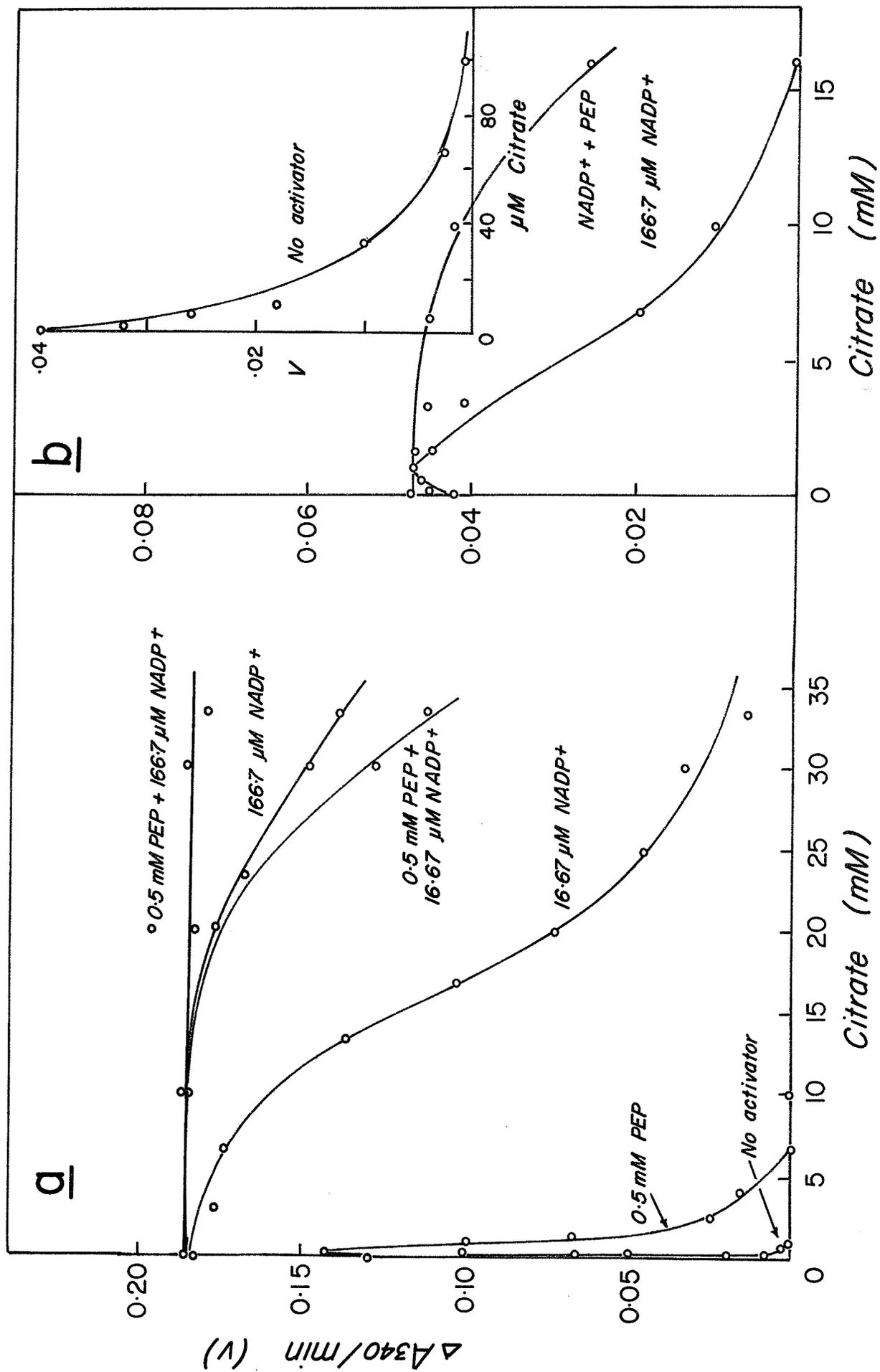


Figure 27: Inhibition by citrate of the (a) reductive amination (b) oxidative deamination reactions catalyzed by Pythium glutamate dehydrogenase. In (b), inset represents the curve for citrate inhibition without activator. The outer graph, the curve for antagonism by  $\text{NADP}^+$  (0.167 mM) and by  $\text{NADP}^+$  (0.167 mM) with P-enolpyruvate (0.5 mM). Substrates were used at saturating levels.



completely. Citrate inhibition of the oxidative deamination reaction of Pythium glutamate dehydrogenase was more severe. The reaction was 50% inhibited by  $1.33 \times 10^{-5}$  M citrate in the absence of activators. Although the activators do not stimulate this reaction to any great extent, they still act as multivalent antagonists against citrate inhibition (Fig. 27 b).

5. Sigmoid Inhibition: An enzyme that displays sigmoidal kinetics will satisfy the Michaelis-Menten equation with the following modification:

$$v/V_{\max} = (S)^n / [K' + (S)^n] \quad (5)$$

where  $S$  is the ligand concentration, and  $n$  is the interaction coefficient. Koshland et al (1966) have proposed a little used device that can discriminate between cooperative and non-cooperative protein-ligand interaction. They suggested that by determining the cooperativity index ( $R_S$ ), which is the ratio of the substrate concentration necessary to give 90% saturation to the concentration that gives 10% saturation, any deviation from the Michaelis-Menten hyperbolic relationship can easily be determined.  $R_S$  should be 81 for all cases that follow Michaelian kinetics. At  $(S)_{90}$  and  $(S)_{10}$ , Equation (5) can be written in the form of two simultaneous equations as follows:

$$0.9 = (S)_{0.9}^n / [K' + (S)_{0.9}^n] \quad (6)$$

and 
$$0.1 = (S)_{0.1}^n / [K' + (S)_{0.1}^n] \quad (7)$$

which can be reduced to:

$$[ (S)_{90} / (S)_{10} ]^{\underline{n}} = 81 \quad (8)$$

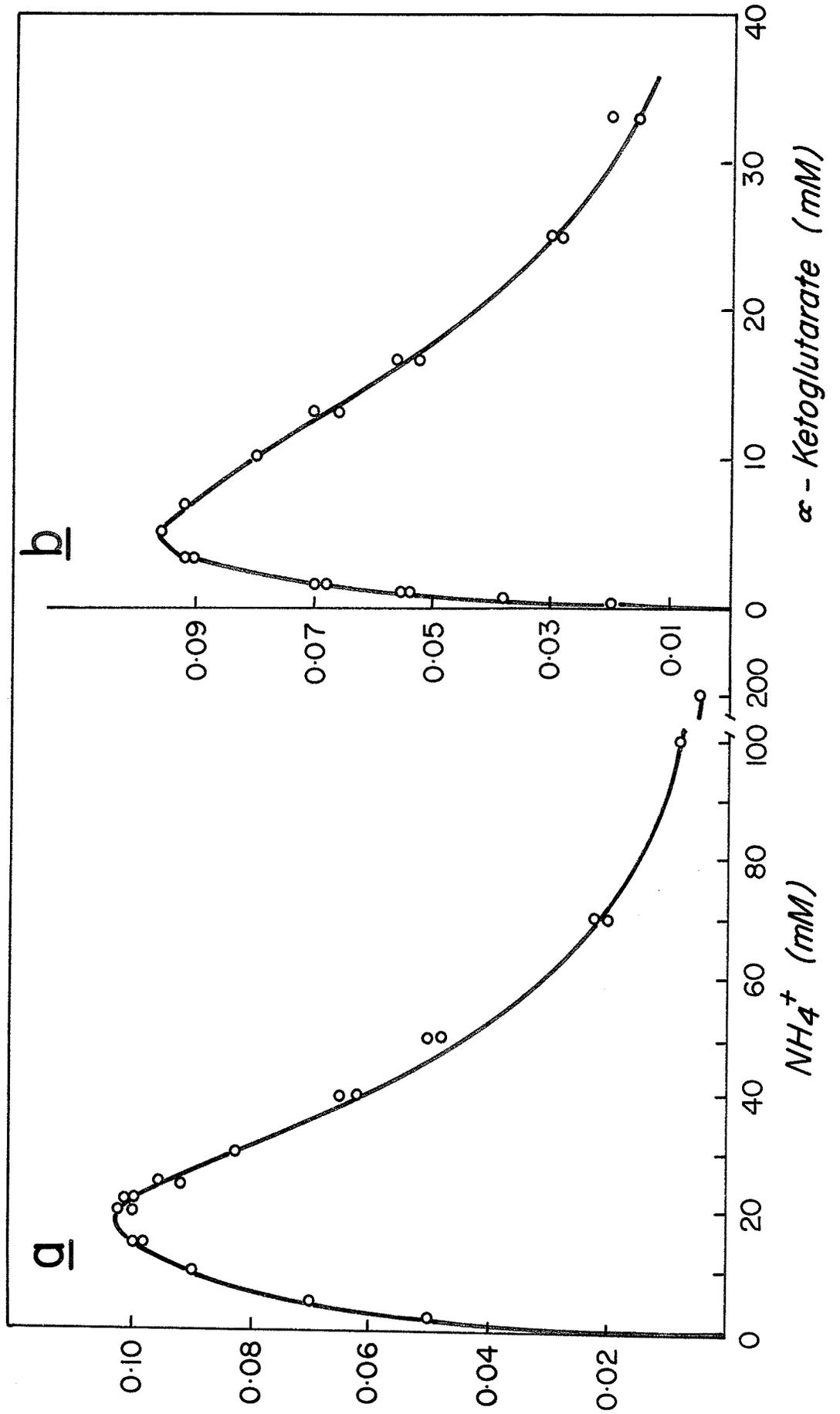
From Equation (8), one could determine the nature of interaction between ligand and protein and evaluate the interaction coefficient,  $\underline{n}$ , which is only an approximate indication of the number of substrate-binding sites involved in the interaction. When a substrate acts as an allosteric inhibitor, theoretically, it should be possible to analyze the saturation curve on the same basis, by using Equation (9):

$$[ (SI)_{90} / (SI)_{10} ]^{\underline{n}} = 81 \quad (9)$$

Most substrate inhibition curves would give  $\underline{n}$  values greater than 1 when Equation (5) is used. Other diagnostic procedures would be required to confirm whether or not true cooperativity is involved. This approach has some value only when modulators activate an enzyme at high substrate concentration ranges.

The computational procedure followed was to select as zero inhibitor concentration the concentration of substrate at which there is neither an increase nor a decrease in the reaction rate when the substrate level was increased further by at least 10%. The concentration of substrate required to reduce this optimal rate by 10% was taken to represent  $(SI)_{10}$ , and the concentration that reduced the optimal rate by 90% was taken as  $(SI)_{90}$ . Such analysis of the data of Figure 28 a in which ammonia acted as a substrate and an inhibitor of the reductive amination reaction

Figure 28: Rate-concentration plots for (a)  $\text{NH}_4^+$ ;  
(b)  $\alpha$ -ketoglutarate as variable substrate in the biosynthetic reaction catalyzed by Pythium NAD-specific glutamate dehydrogenase. Reactants used were as follows: in (a) 6.67 mM  $\alpha$ -ketoglutarate, 0.167 mM NADH, 66.67 mM Tris-acetate, pH 8, 2  $\mu\text{g}$  enzyme; in (b) 20 mM  $\text{NH}_4^+$  and other components as in (a), except that  $\alpha$ -ketoglutarate concentration was varied as indicated.



of Pythium glutamate dehydrogenase showed that the interaction of the inhibitor with the enzyme may be positive cooperative.

$(SI)_{90}/(SI)_{10}$  was estimated as 3, and the  $n$  value computed as 4. A similar calculation done for  $\alpha$ -ketoglutarate as an inhibitor and substrate (Figure 28 b) gave a  $(SI)_{90}/(SI)_{10}$  value of 3.72 and  $n$  of 3.3.

6. Allosteric activators: The effects of  $NADP^+$ , NADPH, and P-enolpyruvate as allosteric activators of the glutamate dehydrogenases of Achlya and Pythium were described previously. A survey of other possible modulators showed that short chain fatty acid coenzyme A esters (CoA, acetyl-CoA, acetoacetyl-CoA, succinyl-CoA, n-butyryl-CoA, n-propionyl-CoA, malonyl-CoA, n-hexyl-CoA and n-valeryl-CoA) and the guanylates (GTP, GDP and GMP) activate these enzymes. ATP as an activator is discussed below in terms of adenylate control. Cyclic nucleotides (cyclic AMP and cyclic GMP) had slight stimulatory effect on the biosynthetic reaction, but inhibited the catabolic process. The effects of the various modulators on the oxidative and reductive reactions of the two enzyme species are summarized in Table IX. Activation by all effectors was more pronounced on the Pythium catalyst than the Achlya form. Besides quantitative differences in the influence of activators on the two enzymes, the effects of adenylates and P-enolpyruvate markedly differed between the two species. Pythium

Table IX

A summary of the response of Pythium and Achlya NAD-specific glutamic dehydrogenases to a variety of allosteric modulators. A plus (+) sign denotes activation; a minus (-) sign, inhibition; (0) means no significant effect.

<u>Modifier</u>	Reductive Amination Reactions of,	
	<u>Pythium</u>	<u>Achlya</u>
NADP <sup>+</sup>	+	+
NADPH	+	+
P-enolpyruvate	+	+
GTP (GDP, GMP)	+	+ *
CoA derivatives (short chain) <sup>a</sup>	+	+ *
Palmitoyl CoA	-	-
Oleyl CoA	-	-
ATP (ADP)	+	0
AMP	-	-
3'5'AMP	0 *	0
3'5'GMP	0 *	0
Citrate (Isocitrate)	-	-
Dephospho-CoA	0	0
<u>Modifier</u>	Oxidative Deamination Reactions of,	
	<u>Pythium</u>	<u>Achlya</u>
NADP <sup>+</sup>	0 *	+
P-enolpyruvate	-	+
GTP (GDP, GMP)	0	-
CoA derivatives <sup>a</sup>	-	-
Palmitoyl CoA	-	-
ATP (ADP)	-	-
AMP	-	-
Citrate	-	-

<sup>a</sup>CoA; acetyl CoA; acetoacetyl CoA; malonyl CoA; succinyl CoA; n-propionyl CoA; n-butyryl CoA; n-hexyl CoA; n-valeryl CoA.

\*Slight activating effect.

glutamate dehydrogenase was inhibited by P-enolpyruvate when the catabolic reaction was catalyzed, whereas Achlya glutamate dehydrogenase was activated. ATP inhibited the catabolic reaction of the Achlya enzyme but not that of Pythium. Other than these subtle modifications, the two enzymes displayed very similar properties despite very different affinities for the ligands interacting with them (Table VI).

7. Response to "Energy Charge": The NAD-specific glutamate dehydrogenase of Achlya is relatively insensitive to modulation by adenylates. The oxidative deamination (catabolic) reaction is inhibited 65% by AMP and 22% by ATP at 5 mM concentrations (Fig. 29 a). The reductive amination (biosynthetic) reaction is unaffected by similar concentrations of AMP and ATP (Fig. 29 b). On the other hand, glutamate dehydrogenase from Pythium was found to be extremely sensitive to the influence of adenylates. ATP functioned as a mild activator, and AMP as a strong inhibitor of the biosynthetic and catabolic reactions (Figs. 30 and 31). In these experiments, addition of adenylate kinase was omitted. According to the formulations of Atkinson (1968), the response of Pythium glutamate dehydrogenase to varied proportions of AMP, ADP, and ATP in catalyzing the catabolic and biosynthetic reactions was of the energy-utilizing (U-) type. In the absence of ADP, as in these experiments, absolute energy charge values were not obtained. The interest was in observing the general pattern of response of the enzyme to adenylates. Although ADP, like ATP, activates the

Figure 29: Effects of adenine nucleotides on the (a) catabolic, (b) biosynthetic reaction of Achlya NAD-specific glutamate dehydrogenase. The reaction rate  $v_e$  and  $v_o$  represent activities in the presence and absence of an effector. Effectors used were (●), ATP; and (⊙), AMP. Assays were conducted at pH 8 with substrates at the standard assay concentrations.

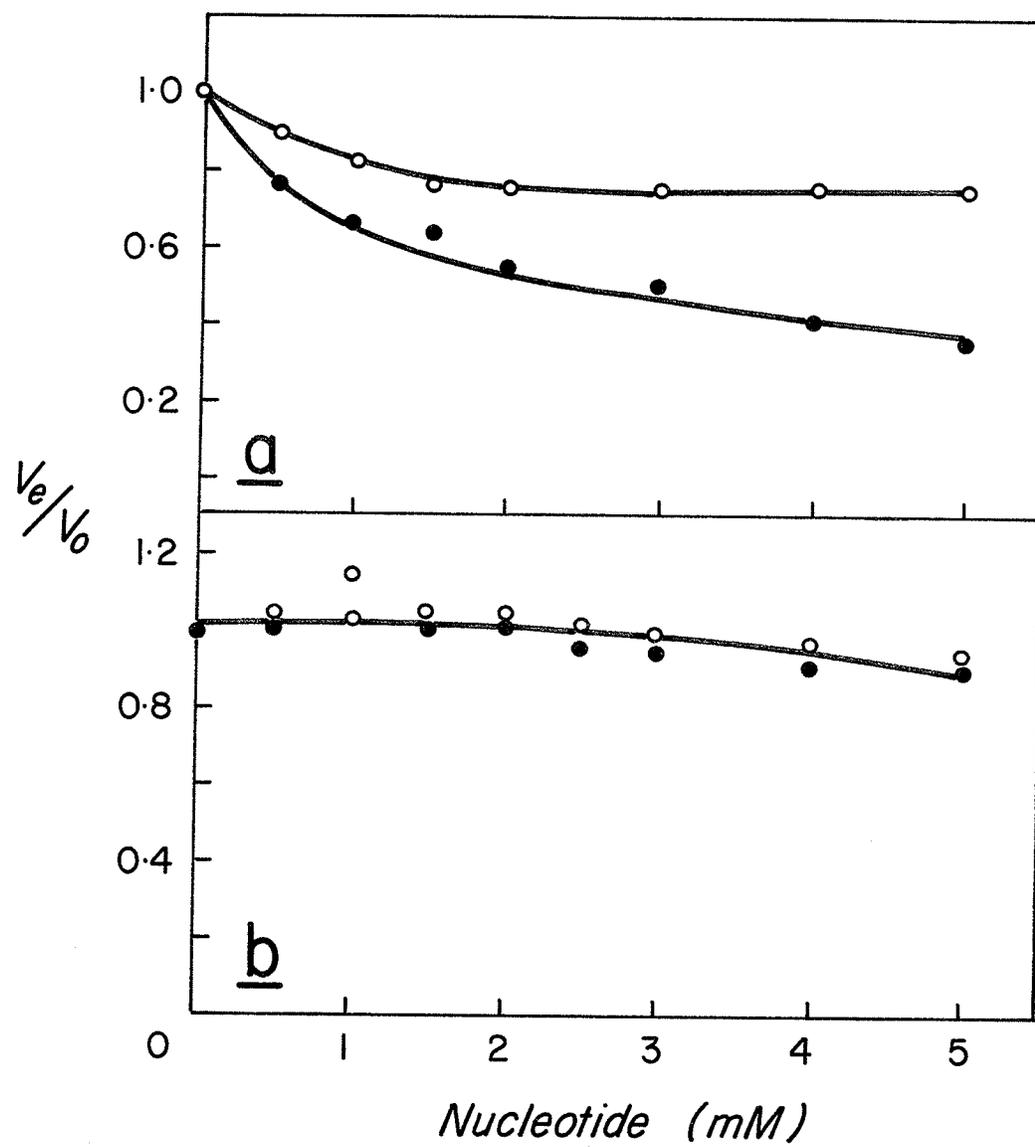


Figure 30: Effects of 'energy charge' on the biosynthetic reaction of Pythium glutamate dehydrogenase. Activators were used at the following concentrations, 0.167 mM (GTP and CoA); 0.5 mM, (P-enolpyruvate), 0.0167 mM ( $\text{NADP}^+$ ). The concentrations of ATP and AMP used are specified. The addition of adenylate kinase to bring ATP and AMP into equilibrium with ADP was omitted since 'energy charge' depends, essentially, on ATP and AMP levels.

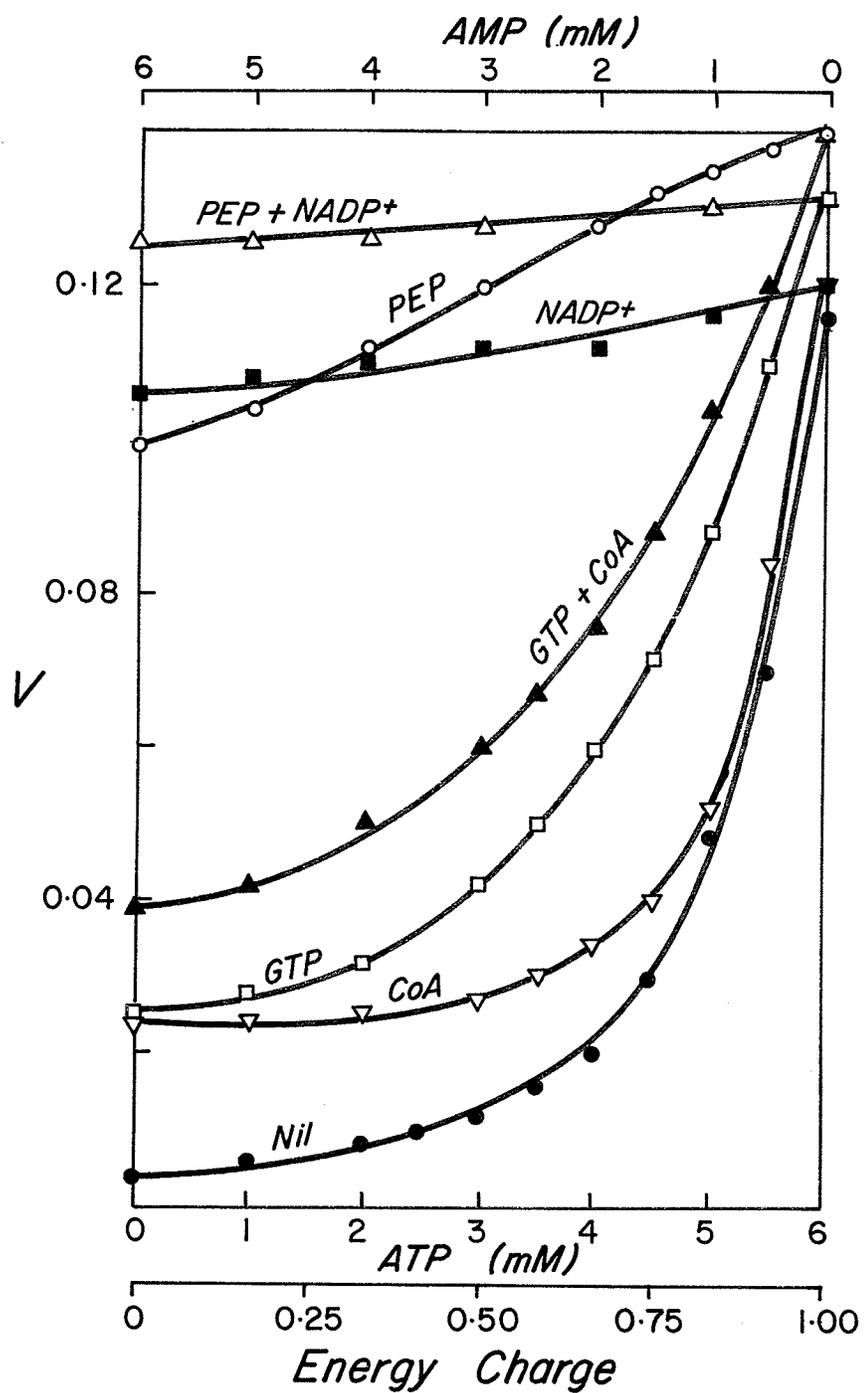
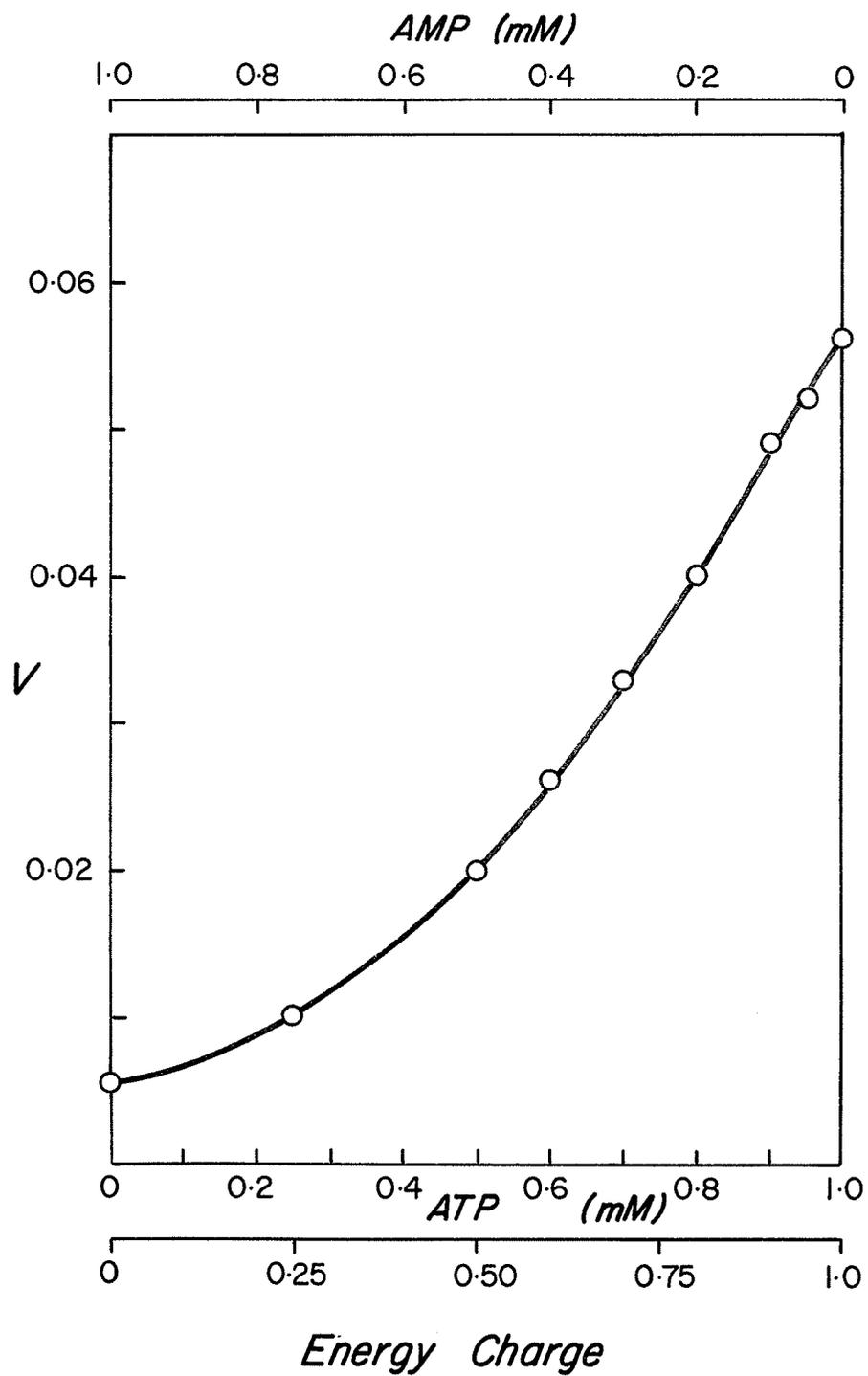


Figure 31: Effects of energy charge on the catabolic reaction of Pythium glutamate dehydrogenase. No activators were used.



enzyme slightly, the "energy charge" values given in Figures 30 and 31 are only apparent.

One point of significance is the disproportionately large difference in adenylate concentrations required to control the biosynthetic and catabolic reactions. The catabolic reaction was completely inhibited by 1 mM AMP, whereas the biosynthetic reaction required 6 mM AMP for effective control. Therefore, at other than equilibrium concentrations, the adenylate control system would operate unidirectionally, as previously defined for the glutamate dehydrogenase of Blastocladiella emersonii (LéJohn 1968a). Although the catabolic reaction may be completely inhibited at adenylic acid levels above 1 mM, the biosynthetic reaction would be operative, albeit at a reduced efficiency. With ATP present, the difference would be more marked because the adenylate effect is a non-linear function of total adenylates, not of single components.

The results shown in Figure 30 demonstrate that only P-enolpyruvate and  $\text{NADP}^+$  can independently release Pythium glutamate dehydrogenase from AMP inhibition. The other activators, GTP, CoA and derivatives, were incapable of doing this even when combined.

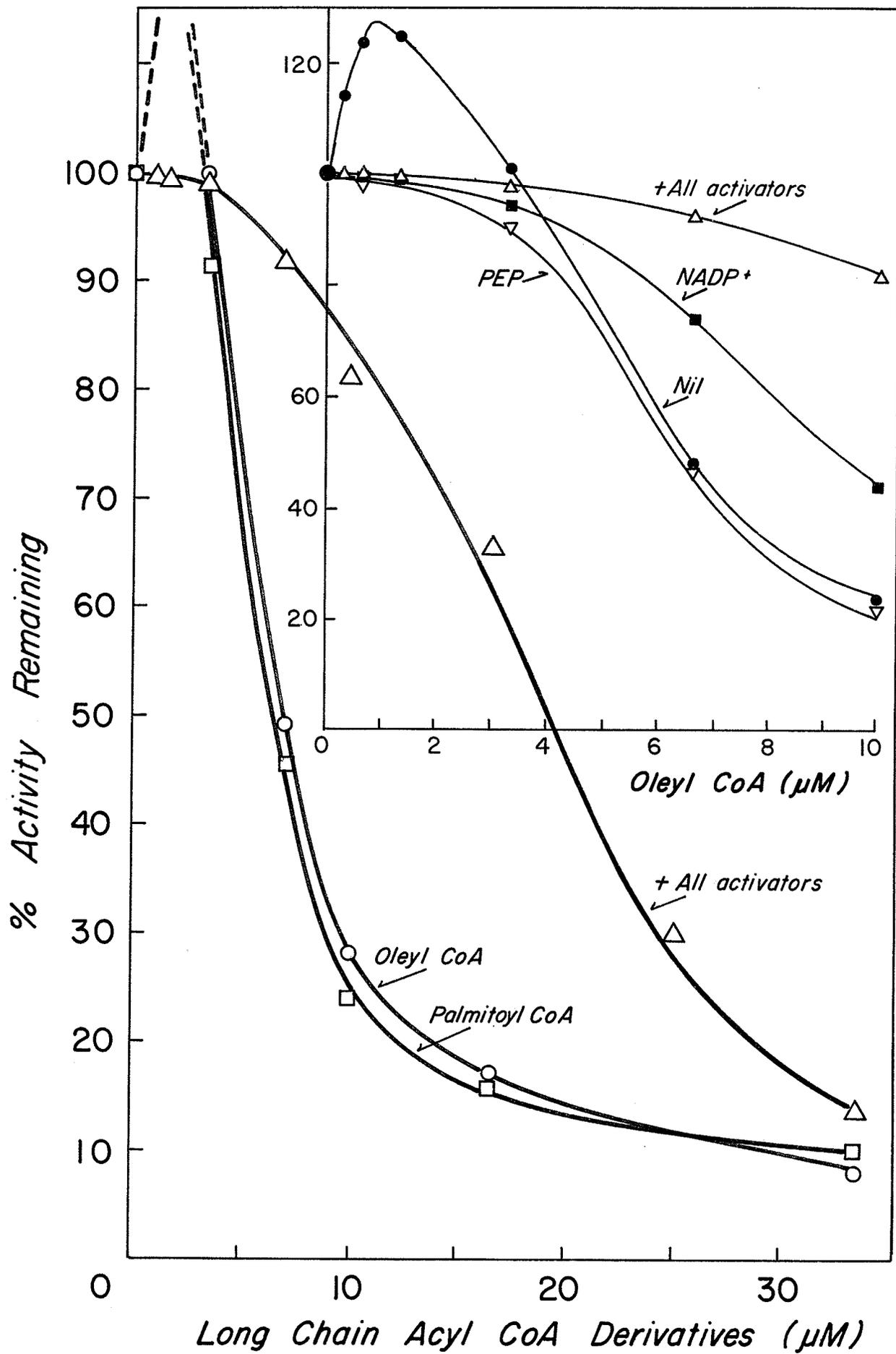
8. Allosteric Inhibitors: In addition to citrate and AMP long chain fatty acid-CoA esters were found to be inhibitors of these glutamate dehydrogenases (Fig. 32). The two esters studied in

some detail were palmitoyl-CoA and oleyl-CoA. At very low concentrations, the esters activated the enzyme slightly (Fig. 32 inset). This may be caused by the presence of traces of free CoA present in the commercial preparation of the esters. Alternatively, the ester may be acting as a competitive inhibitor with one of the substrates that inhibit the enzyme. At higher concentrations, the ester would bind at its own inhibitor site as well.

Single activators did not antagonize the inhibition by the long chain CoA esters, with only  $\text{NADP}^+$  showing a slight effect. In Figure 32, inset, it is shown that significant protection against oleyl-CoA inhibition occurred when all activators are present. At  $10 \mu\text{M}$  oleyl-CoA concentration, the enzyme was 80% inhibited in the absence of activators, but only 20% inhibited in their presence. Significant inhibition always occurred at oleyl-CoA levels above  $10 \mu\text{M}$ , whether activators were present or not. It is possible that in this range the coenzyme may inhibit by detergent action. Such inhibition, which would result in irreversible protein denaturation, has been discussed by Taketa and Pogell (1966).

9. Multivalent effects: Although the activators of glutamate dehydrogenase are phosphorylated structures, their forms are so different that a clear picture cannot be obtained regarding the functional groups of importance in the activation processes. Phosphate is unlikely to be the single important factor, as AMP is

Figure 32: Inhibition of the biosynthetic reaction catalyzed by Pythium glutamate dehydrogenase by palmitoyl and oleyl CoA and antagonism by the activators, GTP, CoA, P-enolpyruvate and  $\text{NADP}^+$  against oleyl CoA inhibition. Inset. An enlargement of the curves at low oleyl CoA concentrations. The concentrations of activators used are given in Fig. 30 legend. The expression (All Activators) represents the four activators given above.



an inhibitor, and several other phosphorylated compounds have no influence. An alternative interpretation is that multiple sites may be involved in the activation process. Tests were done to determine if there was any cumulative action of the activators, using their ability to antagonize inhibition by citrate, AMP, and long chain fatty acid-CoA derivatives as a test model. The reason for this approach was that while all the activators except ATP can easily reverse the allosteric inhibition by substrates, the non-substrate inhibitors were more toxic and resisted antagonism by single activators.

The effects of activators used singly and in combination to antagonize AMP effects on Pythium glutamate dehydrogenase are shown in Table X. Only  $\text{NADP}^+$  and P-enolpyruvate could independently overcome most of the inhibition, while GTP, CoA (or acetyl-CoA) did not antagonize AMP inhibition significantly. In combination, GTP and CoA were no better than CoA alone. In contrast,  $\text{NADP}^+$  and P-enolpyruvate acted cumulatively and antagonized AMP completely. Although P-enolpyruvate was cumulative with either GTP or CoA,  $\text{NADP}^+$  did not show any cumulative property with these two compounds. It would appear that  $\text{NADP}^+$ , GTP and CoA were interacting at the same or closely related sites. P-enolpyruvate definitely has a distinct site. However, because of the relatively small differences in percentage deinhibition elicited when CoA and GTP were used, it became necessary to estimate the number of activator sites by using other inhibitors.

Table X

Multivalent effects of positive modulators in the antagonism of AMP inhibition during the reductive amination of Pythium NAD-specific glutamic dehydrogenase. Assays were carried out in a basic reaction mixture of 6 mM AMP, 0.167 mM NADH, 6.67 mM  $\alpha$ -ketoglutarate, 20 mM  $\text{NH}_4^+$ , 66.67 mM Tris-acetate, pH 8, and 4  $\mu\text{g}$  of enzyme in 3 ml volume. Activators, when used, were at the following concentrations:  $\text{NADP}^+$ , 0.0167 mM; P-enolpyruvate, 0.5 mM; GTP, 0.33 mM; CoA, 0.167 mM.

Ligands (s)	% Inhibition
None	100
$\text{NADP}^+$	22
P-enolpyruvate	12
CoA	66
Acetyl CoA	68
GTP	75
GTP + CoA	68
$\text{NADP}^+$ + CoA	18
$\text{NADP}^+$ + GTP	20
$\text{NADP}^+$ + CoA + GTP	20
$\text{NADP}^+$ + P-enolpyruvate	0
P-enolpyruvate + CoA	6
P-enolpyruvate + GTP	2
P-enolpyruvate + CoA + GTP	10
P-enolpyruvate + $\text{NADP}^+$ + GTP + CoA	0

All of the activators were tested for their ability to release the enzyme from citrate inhibition. Studies of GTP, ATP, and acyl-CoA derivatives effects were compared with the cumulative antagonism already demonstrated for  $\text{NADP}^+$  and P-enolpyruvate (Fig. 27). Although ATP is an activator (based on the adenylate control hypothesis), it failed to antagonize citrate. At 3 mM concentration it produced values nearly coincident with the control plot in Figure 33. GTP, CoA and P-enolpyruvate were weakly antagonistic to citrate. Only  $\text{NADP}^+$  showed a significant antagonistic effect. GTP and CoA had some cumulative ability, but they were considerably less effective than  $\text{NADP}^+$  alone. When P-enolpyruvate and either GTP or CoA were used, their cumulative effect did not quite match that of  $\text{NADP}^+$ . All three combined were as effective as  $\text{NADP}^+$  alone. When all four activators were used, complete antagonism of citrate inhibition occurred (Fig. 33). From these results, it appears that all of the activators have separate binding sites.

10. Effect on pH response: As the NAD-specific glutamate dehydrogenases of these fungi are located in part in the mitochondria, and are subject to the pH fluctuations occurring in those organelles, the effects of pH on the enzyme are of interest, particularly in relation to the allosteric modulator actions. Such studies have been previously reported for the enzyme of Blastocladiella emersonii (LéJohn et al 1969, LéJohn and Jackson 1968a). The effects of pH on the multiple modulators of the Pythium enzyme are shown in Figure 34.

Figure 33: Antagonism of citrate inhibition by activators of the biosynthetic reaction catalyzed by Pythium glutamate dehydrogenase. Activators used were at the concentrations indicated for Fig. 30. The values for ATP (3 mM) as antagonist were nearly coincident with the control plot and so have been omitted.

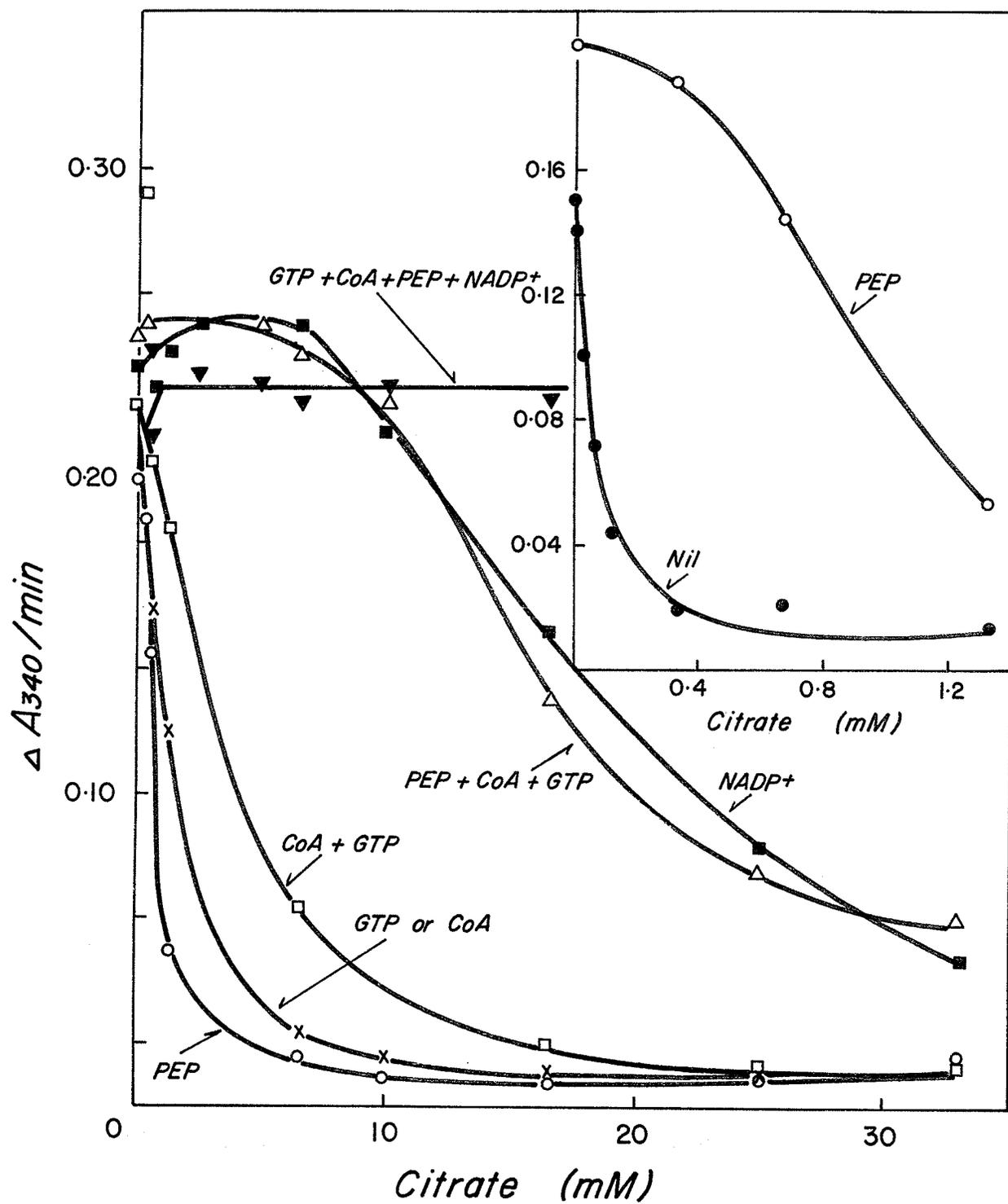
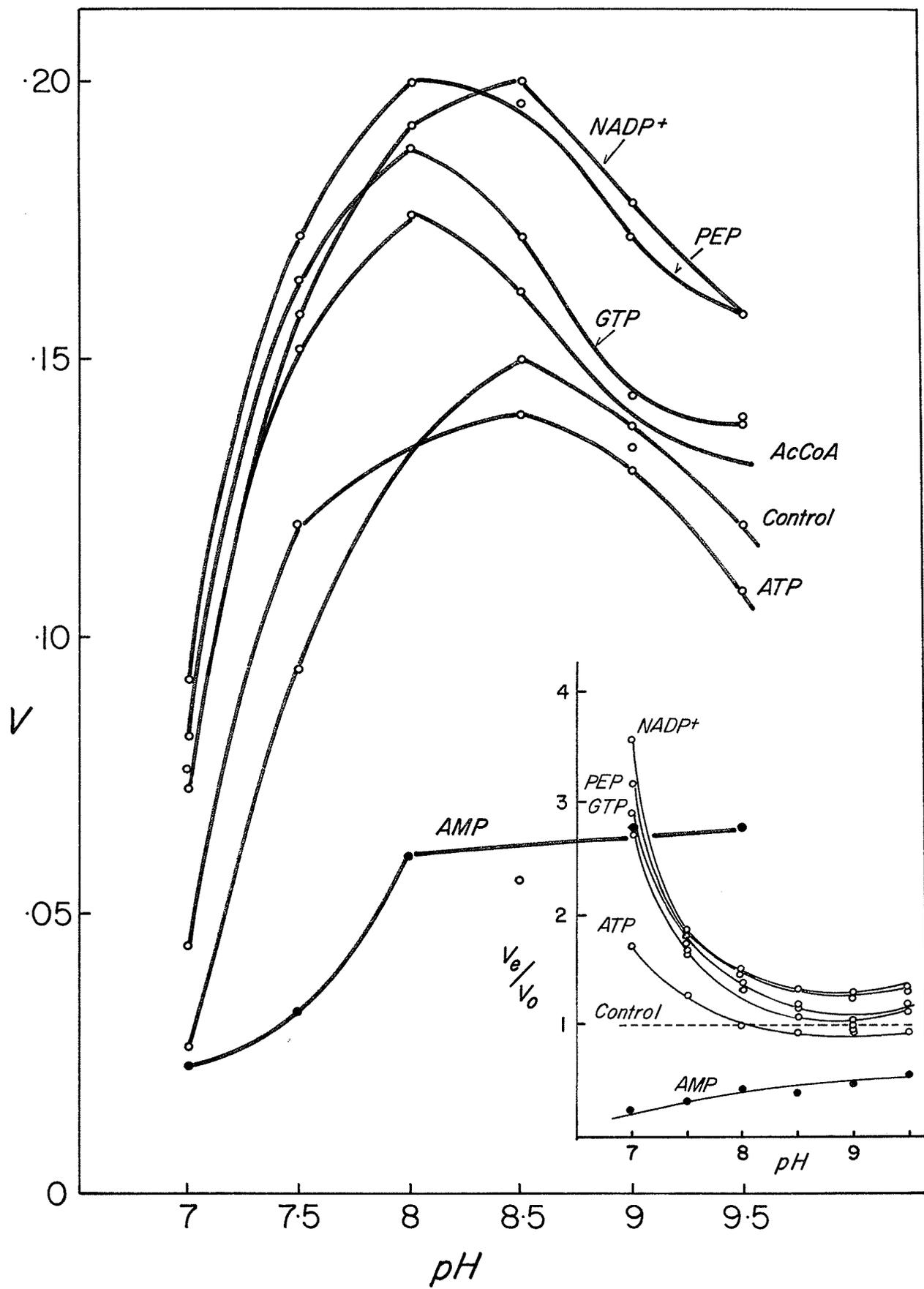


Figure 34: Influence of allosteric activators and inhibitor on the rate of the reductive amination reaction catalyzed by Pythium glutamate dehydrogenase at different pH values. The reaction system consisted of 20 mM  $\text{NH}_4^+$ , 0.167 mM NADH; 6.67 mM  $\alpha$ -ketoglutarate, 67 mM Tris-acetate at the pH values indicated. The effectors were at the following concentrations: GTP, Acetyl CoA, (0.33 mM); P-enolpyruvate, (0.5 mM);  $\text{NADP}^+$ , (16.66 mM); ATP, (2 mM); AMP, (2 mM).

Inset. The same data expressed as relative degree of activation and inhibition by effectors at the different pH levels.  $\underline{v}_0$ , is rate in the absence of effector;  $\underline{v}_e$ , is rate in the presence of an effector.



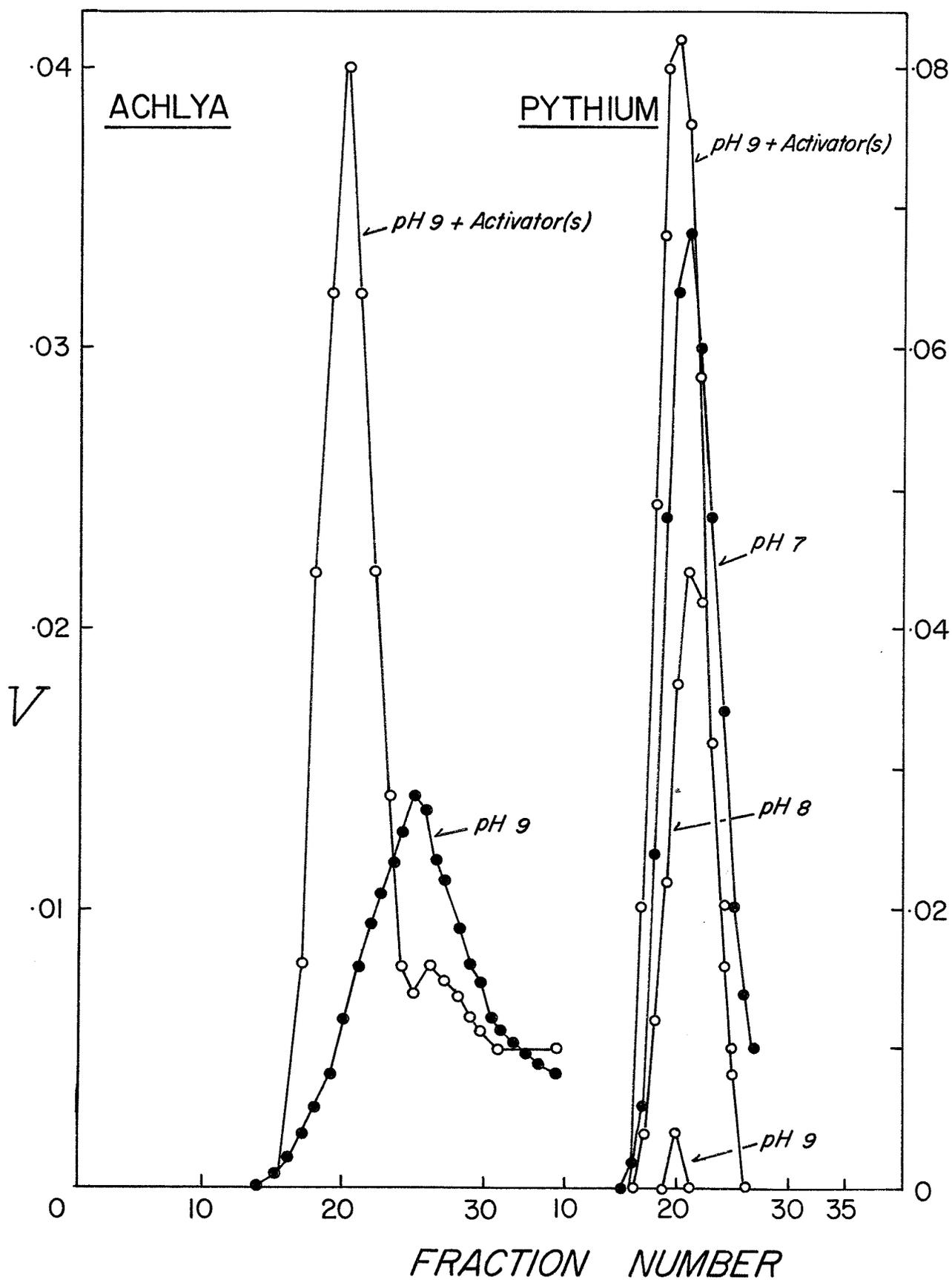
To minimize complications of substrate inhibition which occur in the range in which the modulators are most effective - the modifiers were studied at non-inhibitory substrate levels. In the absence of activators the pH optimum was 8.5. When activators were added singly to the reaction, the pH optimum observed was 8.0, except in the case of P-enolpyruvate, for which there was no change.

One novel feature was the relative degree of activation of the enzyme by the activators at different pH values.  $\text{NADP}^+$  activation at pH 7 was over 3.5-fold, but only 1.5-fold at pH 8.0. The same pattern was observed for all the other activators although the relative degree of activation differed (Fig. 34). ATP was the least effective activator. The influence of the inhibitor, AMP, was similar to the effect shown by the activators; the enzyme being less inhibited at pH 8 than at pH 7. Evidently at pH 7, which is not the optimum in vitro, the enzyme is in the state most receptive to modulation. Since the activators are relatively ineffective in the oxidative deamination reaction, a study of pH activation in this direction was not pursued in detail.

## F. Physical Studies

1. Zone sedimentation: Studies of the behaviour of the purified glutamate dehydrogenases in sucrose density gradients are shown in Figure 35. At pH 9, the enzymes were unstable during centrifugation. Enzymes stored in the cold at pH 9 during the

Figure 35: Zone sedimentation in sucrose density gradients of Achlya and Pythium glutamate dehydrogenase at pH 7, 8, and 9; and at pH 9 in the presence of the activators as indicated on the graph. On the graph, the notation activators represents 0.1 mM NADP<sup>+</sup>, 1 mM P-enolpyruvate, used individually, or in combination in the sucrose density gradients. The results given are from one experiment involving one activator. The data for the other centrifugations involving different activators are similar to the values presented and so have not been included. Each gradient contained 20 µg enzyme. 50 µl of 140 µl samples assayed in the standard assay mixtures. Internal marker, porcine heart malate dehydrogenase (mol. wt. 67,000) peaks at Tube 31. Due to traces of ammonia in the enzyme from Achlya (necessary for stability) some activity was recovered at pH 9.



period of the centrifugation lost very little activity, indicating that these enzymes are sensitive to hydrodynamic shear at alkaline pH values, as is the case with the Blastocladiella enzyme (LeJohn et al 1969).

At pH 8, over 50% of the enzymatic activity was lost during centrifugation, but at pH 7 there was little deterioration (Fig. 35). Enzyme activity could not be recovered upon incubating the fractions with activator in the cold. The sensitivity of the enzymes to pH and hydrodynamic shear was utilized as a method of studying the binding properties of the modifiers in the enzyme. Figure 35 shows the stabilization of the enzyme against inactivation during centrifugation at pH 9 by the presence of activators. Citrate did not protect the enzyme as the activators did.

Porcine heart malate dehydrogenase (mol wt 67,000) was used as an internal marker. All the glutamate dehydrogenases have approximately the same molecular weight of 225,000 daltons. Binding of activators did not alter this size appreciably. Inactivation at pH 9 can be accounted for in part, by dissociation of the protein.

2. Thermal properties: The glutamate dehydrogenases of Achlya and Pythium show extreme heat lability, not characteristic of other enzymes of these same organisms (including citrate synthetase, NADP-specific isocitrate dehydrogenase, D(-)lactate

dehydrogenase, and malate enzyme). The heat stability of the Pythium catalyst is shown in Fig. 36. The enzyme is fully active at 30°C. Above this temperature it is extremely labile, being completely inactivated within 20 min at 40°C. The first order rate constant,  $k$ , and the half-life,  $t_{1/2}$ , for heat denaturation were computed from the results of Figure 37. The values for both species are given in Table XI. When  $\log k$  was plotted against the reciprocal of the absolute temperature, a straight line was obtained (inset to Figure 37), in agreement with the Arrhenius formula of:

$$\log k = - ( H_a / 2.303 R ) \frac{1}{T} + \log s$$

where  $H_a$  is the heat of inactivation for denaturation,  $R$  is the gas constant, and  $s$  a constant. The  $H_a$  values for Achlya and Pythium glutamate dehydrogenases are also given in Table XI.

All attempts to stabilize the purified enzymes against thermal inactivation at these relatively low temperatures by incubation with activating or inhibiting ligands were unsuccessful. Crude extracts of Achlya lost activity when stored frozen for short periods during enzyme purification. Activity could however be recovered from cell mats frozen and subsequently extracted. Studies of the loss of activity in samples of crude extract held at 30°C for 3 hrs were carried out, with various substrates and activators added to the extracts. A loss of 80% of the original activity was observed at 30°C, compared with a 40% drop in crude extract kept in an ice bath

Figure 36: Thermal inactivation curve of Pythium glutamate dehydrogenase. Partially purified enzyme at a protein concentration of 5 mg per ml was incubated for 3 min at the specified temperature, then chilled for 2 min in an ice-salt bath. An aliquot was then used to assay the reductive amination reaction. All substrates and  $\text{NADP}^+$  were held at saturating concentrations. The results are expressed as per cent residual activity as a function of temperature.

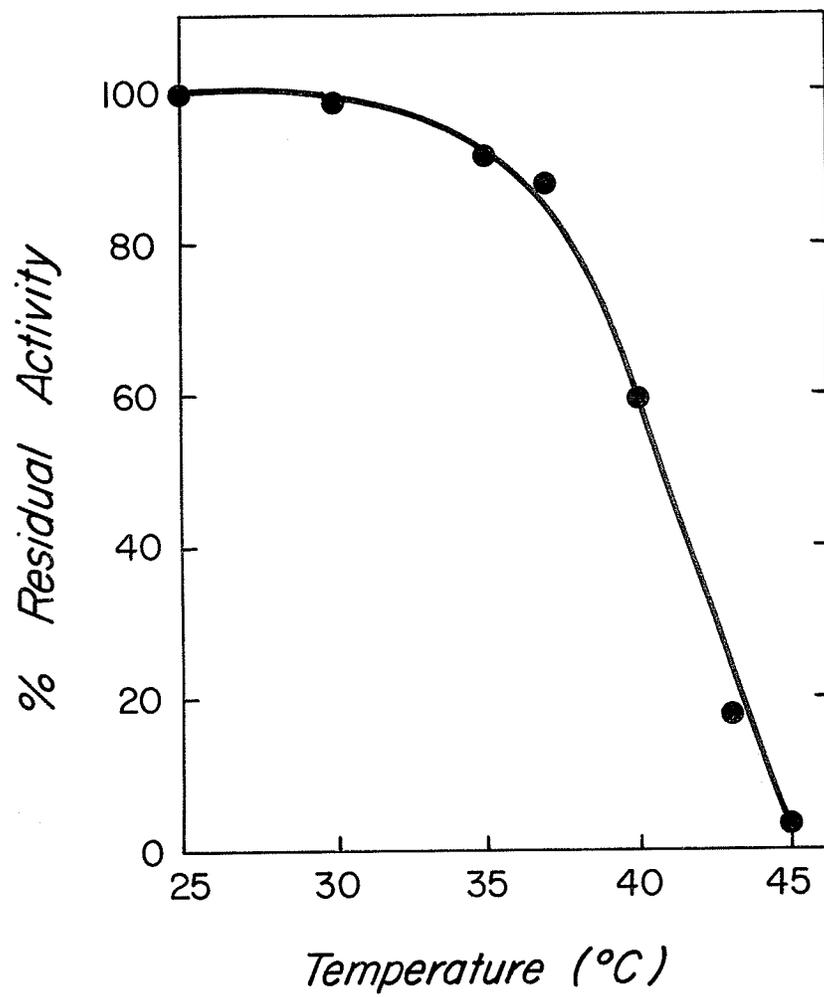


Figure 37: Thermal decay plots of Pythium glutamate dehydrogenase at several different temperatures. (  $\circ$  ),  $30^{\circ}$ ; (  $\triangle$  ),  $35^{\circ}$ ; (  $\square$  ),  $37^{\circ}$ ; (  $\nabla$  ),  $40^{\circ}$ . Inset. Arrhenius plot of the data.

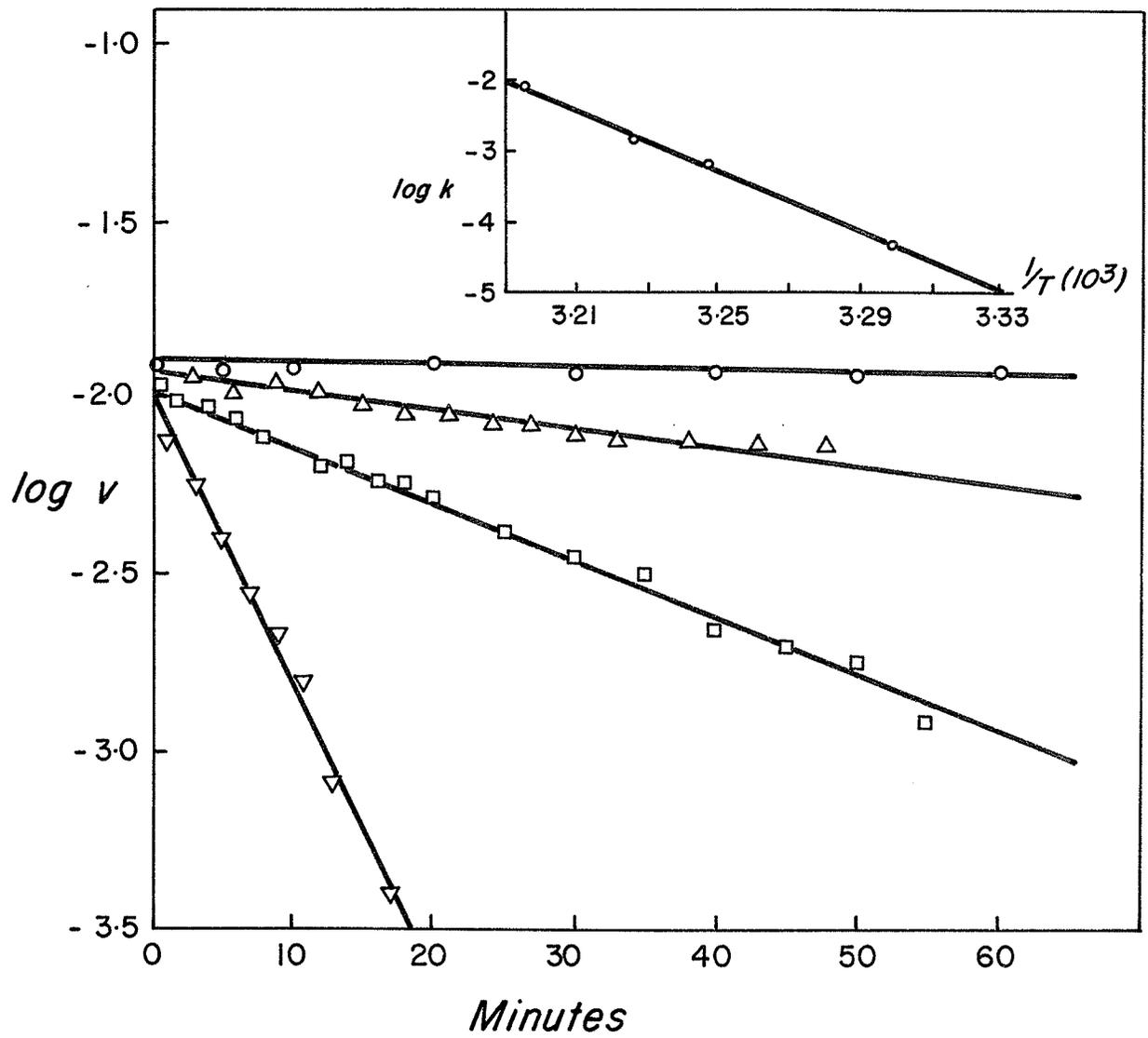


TABLE XI

Halftimes for heat denaturation of Pythium and Achlya  
NAD-specific glutamic dehydrogenases.

Temperature °K	<u>PYTHIUM</u> $t_{(0.5)}$ min	<u>ACHLYA</u> $t_{(0.5)}$ min
303	462.5	346
308	115.5	154
310	43.3	86.7
313	8.6	19.8
$\Delta H_a$	$5.75 \times 10^4$	$2.48 \times 10^4$

for the same period. Some protection by low NADH and P-enolpyruvate concentrations was observed, but the results do not indicate if these compounds act directly with the enzyme, or by influencing the rate of a degradative process occurring in the crude extract.

## DISCUSSION

NADP-type Mechanism

The initial velocity studies shown for the Pythium enzyme for glutamate oxidation and  $\text{NAD}^+$  reduction demonstrated that in the presence or absence of activators the enzymatic reaction corresponds to Equation (10)

$$1/v = 1 / V (1 + K_{ia} K_b / AB + K_a/A + K_b/B) \quad (10)$$

where A and B are the substrates of the oxidative deamination reaction,  $K_a$  and  $K_b$  are the Michaelis constants of A and B, and  $K_{ia}$  is the dissociation constant of A. From product inhibition studies,  $\text{NAD}^+$  should correspond to A. The presence of the  $K_{ia}K_b/AB$  term in Equation (10) dictates that both A and B must bind to the enzyme before any product can be released. Therefore B would be glutamate.

The products are designated P, Q, and R in the order of their release from the enzyme, during the reaction. The product inhibition patterns of Figures 11 b, 13 c and 16 a, 17 a specify that NADH will be the last substrate released. NADH therefore corresponds to R. The order of release of  $\alpha$ -ketoglutarate and ammonia was not established unequivocally from the binary-ternary kinetic mechanism

and product inhibition patterns. If Scheme 1, the NAD-type mechanism, is the correct sequence, then  $\alpha$ -ketoglutarate should be an uncompetitive inhibitor when either  $\text{NAD}^+$  or glutamate is used as varied substrate. Ammonia should act as non-competitive inhibitor of glutamate and  $\text{NAD}^+$ . The results obtained indicate that part of this prediction is not satisfied. Although ammonia is non-competitive with respect to varied glutamate, it displayed an uncompetitive pattern with  $\text{NAD}^+$ .  $\alpha$ -Ketoglutarate remained non-competitive in both cases, even in the presence of activator. The same results were obtained when activators were present singly and in multiple combinations (Table VII). This observation indicated that the activators do not participate in the kinetic mechanism. Their role may be physiological, changing the turnover number of the enzyme.

The reaction sequence of Scheme 2, the NADP-type mechanism, appears to fit the product inhibition patterns better than Scheme 1. The predictions are as discussed above, but  $\alpha$ -ketoglutarate is substituted for ammonia. However, ammonia as an inhibitor remained anomalous, since it displayed non-competitive inhibition with glutamate. This difficulty was resolved when product inhibition was conducted in the reverse reaction (reductive amination of  $\alpha$ -ketoglutarate) with glutamate as an inhibitor. This reaction, according to Cleland's nomenclature, is Ternary-Binary. The steady state algebraic equation for such a system (Cleland 1967, 1963a,b,c) in

reciprocal form is as follows:

$$\begin{aligned}
 1/v = & \frac{K_{ia}K_{ib}K_c}{V_{ABC}} + \frac{K_{ib}K_c}{V_{BC}} + \frac{K_{ia}K_b}{V_{AB}} + \frac{K_c}{V_C} + \frac{K_b}{V_B} + \frac{K_a}{V_A} \\
 & + \frac{1}{V} + \frac{K_qP}{K_{eq}ABC} + \frac{K_qP}{K_{eq}ABC} + \frac{K_qP}{BCK_{ia}K_{eq}} \quad (11) \\
 & + \frac{K_qP}{CK_{ia}K_{ib}K_{eq}} + \frac{K_qP}{K_{ia}K_{ib}K_{ic}K_{eq}}
 \end{aligned}$$

As this is the reverse reaction, the notations have been altered so that reactants are designated A, B, and C, and products as P and Q. The terms containing Q have been reduced to zero on the assumption that Scheme 2 is the correct pattern. The equation predicts that Substrate B should be uncompetitive with respect to P when A and C are saturating; and that C should be non-competitive with respect to P when A and B are saturating. Algebraic descriptions of the predictions are presented in Equation (12) and (13) respectively.

$$1/v = \frac{1}{B} \left( \frac{K_b}{V} + \frac{1}{V} \right) + \left( \frac{K_qR}{K_{ia}K_{ib}K_{ic}K_{eq}} \right) \quad (12)$$

$$1/v = \frac{1}{C} \left( \frac{K_c}{V} + \frac{K_qP}{K_{ia}K_{ib}K_{eq}} \right) + \left( \frac{1}{V} + \frac{K_qP}{K_{ia}K_{ib}K_{ic}K_{eq}} \right) \quad (13)$$

In the experiments of Figure 15 a & b, only glutamate was used as inhibitor. In initial reaction rates,  $NAD^+$  concentration would be nil. With  $\alpha$ -ketoglutarate as the varied substrate, glutamate as inhibitor, the inhibition pattern was non-competitive

whereas ammonia and glutamate interaction was uncompetitive. From the theoretical considerations, C is  $\alpha$ -ketoglutarate and B, ammonia. This fits Scheme 2, the NADP-type mechanism.

The deviation observed when ammonia was used as inhibitor in the binary-ternary mechanism can be accounted for by the fact that there seems to be an allosteric site for ammonia binding. The results shown in Figure 15 b indicate quite clearly that at high ammonia concentrations there is a change in the slope of the inhibition curve as a second ammonia site becomes filled. Presumably, in the binary-ternary inhibition studies with ammonia, only one of the two sites is filled.

#### NADP<sup>+</sup> Allosteric Regulation :

The NAD-specific glutamate dehydrogenase of these fungi have been shown to be activated by NADP<sup>+</sup> and NADPH, and to use an NADP-type kinetic mechanism. These characteristics provide an interesting system to consider in terms of the glutamate dehydrogenase found in animal sources, which is non-specific for the two coenzymes NAD(H) and NADP(H) (Frieden 1959a). The kinetic scheme used by the ox liver glutamate dehydrogenase is apparently dependent on the coenzyme used, with Scheme 1 operating for NAD(H) and scheme 2 for NADP(H) as substrate (Frieden 1959c, Fahien and Strmecki 1969).

The NAD- and NADP- specific enzymes of Thiobacillus novellus use the Scheme 1 and Scheme 2 mechanisms respectively (LéJohn et al 1968). The use of  $\text{NAD}^+$  as substrate with an NADP-type mechanism is unique in these fungi, and the presence of an allosteric site for  $\text{NADP}^+$  makes the enzyme an interesting object in terms of speculation about the evolution of the protein.

The presence of an  $\text{NADP}^+$ -binding site influences the order in which substrate sites become available, as seen in these studies, and as reported for the ox liver enzyme by Cross and Fisher (1970). In that case the NADPH-binding site includes "subsites" that are involved in binding selective groups of all substrates. The presence of the allosteric  $\text{NADP}^+$  site on the glutamate dehydrogenases of Oömycetes endowed the catalyst with a particular kinetic mechanism preserved for NADP-specific enzymes. The simplest explanation for this peculiarity is that these enzymes evolved primarily to meet the needs of the cell for NADPH metabolism, as is discussed later.

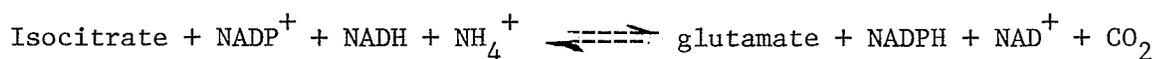
Dixon (1966) speculated on cistron duplication and evolution of a metabolic pathway, based on the formulations of Horowitz (1967). He envisaged that an enzyme could have evolved with an allosteric site by an early modification of part of duplicated active sites. A new site can no longer provide the proper steric fits required for catalysis, but interacts sufficiently to induce conformation changes. It is conceivable that the enzyme was originally of the

nonspecific type, like those of higher animals, and that one of the duplicated coenzyme active sites became modified into an allosteric site. This modification permitted  $\text{NADP}^+$  and NADPH to participate as allosteric ligands while  $\text{NAD}^+$  and NADH were retained as substrates.

Nucleotide Balance Control:

In many organisms the existence of both NAD- and NADP-linked glutamate and isocitric dehydrogenases has been considered as a possible route allowing electron transfer, thus providing reducing power for biosynthesis (NADPH) or catabolism (NADH) as required (Kaplan 1963, Atkinson 1969). In the fungi used in this study, only one glutamate dehydrogenase and one isocitrate dehydrogenase were found. The glutamate dehydrogenase appears to function in a dual capacity as a catabolic and biosynthetic enzyme. The catabolic role is defined by the repressive action of glucose metabolism on the synthesis of the enzyme. Isocitrate dehydrogenase is biosynthetic by this same argument (Smaluck 1971).

Transhydrogenase activity was not detected in these fungi, suggesting that the mechanism by which the cells maintain a balance of their pyridine nucleotides during growth may involve a cooperative interaction of the NADP-specific isocitrate dehydrogenase and the NAD-specific glutamate dehydrogenase as follows:



When glucose is being actively catabolized, isocitrate dehydrogenase would provide an ample supply of NADPH from  $\text{NADP}^+$ . A low level of glutamate dehydrogenase is maintained under these conditions for the conversion of  $\alpha$ -ketoglutarate to glutamate. Since citrate (isocitrate) inhibition is quantitatively a unidirectional feature, afflicting the oxidative deamination reaction predominately, it may not interfere with this transformation process significantly. Nevertheless, the multivalent effect of P-enolpyruvate and  $\text{NADP}^+$  can adequately prevent this inhibition by citrate.

The reason for P-enolpyruvate being involved in such control rather than ATP may involve the fact that the unidirectional control of this enzyme is to favour biosynthesis. P-Enolpyruvate is at a central point in the bridge between fermentation and aerobic metabolism. It is also a precursor of reactions for the biosynthesis of aromatic amino acids, and indirectly alanine and glutamate. The role of  $\text{NADP}^+$  as an activator seems to be prompted by a demand for NADPH in biosynthesis. All of the reactions of amino acid biosynthesis to which P-enolpyruvate is connected to utilize glutamate in an amination process. In addition, glutamate biosynthesis is a fundamental step that leads to several biosynthetic reactions. Activation of glutamate dehydrogenase by P-enolpyruvate for glutamate synthesis can therefore be considered as a reasonable evolutionary step in metabolic regulation. With the same argument the nucleotide equilibrium can be maintained if conditions are reversed so that active catabolism of

glutamate became necessary. Any excess of reducing power (NADPH) may be converted to  $\text{NADP}^+$  as  $\text{NAD}^+$  is reduced to NADH, by the same process shown in the reaction above. This situation would lead to active gluconeogenesis and P-enolpyruvate may not be acting as a precursor in amino acid metabolism under these conditions. Therefore it does not have to activate the oxidative deamination reaction at nonequilibrium conditions.

#### Multivalent Effects:

A total of five activators have been found for the NAD-specific glutamate dehydrogenases of these fungi, being  $\text{NADP}^+$ , P-enolpyruvate, short chain acyl-CoA derivatives, GTP and ATP. These activators were shown to function cumulatively to antagonize the effects of the inhibitors, AMP, citrate, and long chain acyl-CoA derivatives. The activators acted as unidirectional stimulants of the biosynthetic (reductive amination) reaction, while some of them inhibited the catabolic reaction unidirectionally. In addition, the activators modulated the inhibition of the enzyme by allosteric action of the substrates  $\alpha$ -ketoglutarate and ammonia. These various controls can be examined in terms of the mechanisms by which the enzyme effects its biosynthetic and catabolic roles in amphibolic reactions of the citric acid cycle.

As discussed above, the involvement of  $\text{NADP}^+$  and P-enolpyruvate as modulators may be in the cooperative action of NADP-specific isocitrate dehydrogenase and NAD-specific glutamate dehydrogenase in maintaining the balance of pyridine nucleotides. Under energy-rich conditions, GTP and P-enolpyruvate activation of glutamate dehydrogenase is a reasonable effect because the citric acid cycle would be operating as a biosynthetic unit, supplying its intermediates for amino acids and nucleotides.

The reason for the sensitivity of the enzymes to their substrates, ammonia and  $\alpha$ -ketoglutarate, and the reason for antagonism of this effect by activators only at high substrate concentrations are not known. Two possible explanations may be offered. First, if  $\alpha$ -ketoglutarate is liable to accumulate during its production from carbohydrates and transamination reactions of glutamate, then this metabolite may act directly as a substrate and indirectly as an end product (transaminase-glutamate dehydrogenase couple) feedback effector of glutamate dehydrogenase. One role of the activators would be to relieve the enzyme from allosteric inhibition by substrates and permit continued biosynthesis. The second and more attractive proposal is that substrate control is related to catabolism. The enzyme must have evolved as a catabolic catalyst, because it is subject to catabolite repression. During deamination reactions,  $\alpha$ -ketoglutarate and ammonia may accumulate. This may have led to the development

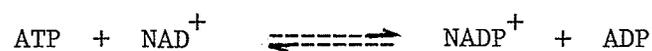
of a product inhibition control mechanism which is observed as substrate inhibition in these studies. The multivalent control by activators may have evolved subsequently to overcome this inhibition when the enzyme has to function in a biosynthetic capacity.

According to the transhydrogenase hypothesis, NADPH is produced at the expense of NADH. One use to which the NADPH might be put is the biosynthesis of fatty acids. Activation of the enzyme by short chain intermediates of fatty acid synthesis in a manner that favours NADH utilization fits this concept very well. Palmitoyl-CoA and Oleyl-CoA, which may be regarded as "end products" of fatty acid biosynthesis, feedback inhibit the enzyme. This is a common method of control of the first enzyme involved in most biosynthetic sequences studied. Although citrate is an inhibitor, its effect is qualitatively unidirectional on the catabolic reaction of the enzyme. Therefore the anomaly of citrate inhibition would not contradict acetyl-CoA activation of the enzyme. In fact it does support the concept that the enzyme has catabolic function because citrate can be looked on as an end product.

A reasonable explanation for the unidirectional stimulation of the enzyme by GTP is difficult because many interrelated reactions utilize GTP. For example, substrate-level oxidative phosphorylation of succinyl-CoA produces GTP; a GTP-linked fatty

acid activation enzyme (acyl-CoA synthetase) is present in mitochondria and closely linked to the oxidation of  $\alpha$ -ketoglutarate; GTP is utilized during synthesis of P-enolpyruvate from oxalacetate via P-enolpyruvate carboxykinase action. It is unlikely that GTP has the same function as ATP because the latter compound responds very differently in multivalent studies. The only definite conclusion which can be made is that GTP, like the other activators, activates the enzyme for biosynthesis.

There are two suggestions that satisfactorily can account for the effect of adenylates on the enzyme. First, the adenylate control may reflect the physiological response of the enzyme to the energy state of the cell. Under energy-rich conditions, there would be an ample supply of ATP that could be utilized for biosynthesis. The biosynthetic reaction of glutamate dehydrogenase would consequently be encouraged. Second, ATP may be involved in an NAD-kinase reaction of the type



which would assure a continued production of  $\text{NADP}^+$  for the transhydrogenase couple of isocitrate dehydrogenase and glutamate dehydrogenase.

The various physiological reactions that may be directly influenced by the activity of glutamate dehydrogenase are summarized in Table XII. This only includes those interrelationships pertinent to the discussion of the effects observed in these studies, and is not exhaustive.

Table XII

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

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

Enzyme Control Mechanisms and Taxonomy:

Two different mechanisms of allosteric control of NAD-specific glutamate dehydrogenase have been observed among members of the simple fungi, Phycomycetes. In the enzymes reported on here,  $\text{NADP}^+$ , P-enolpyruvate, GTP, ATP, and short chain fatty acyl CoA derivatives acted as activators, and AMP, citrate and long chain fatty acyl-CoA derivatives as inhibitors. In other groups of fungi showing multivalent regulation, the allosteric modifiers are ATP, AMP, citric acid cycle intermediates, glycolytic intermediates, and divalent cations ( $\text{Mn}^{++}$ , and  $\text{Ca}^{++}$ ) (LéJohn et al 1969). In this type of enzyme the kinetic mechanism was found to be of the NAD-type. A survey of some forty species of the lower fungi has been made (LéJohn 1971), and it was seen that the regulatory type of glutamate dehydrogenase found in these organisms may have phylogenetic significance. The correlations of enzyme regulation, lysine pathways, and cell wall structure have been discussed by LéJohn (1971).

## PART II

## CYCLIC AMP METABOLISM

## RESULTS AND DISCUSSION

Various studies were carried out in an attempt to demonstrate the occurrence of cyclic AMP or the enzymes responsible for its synthesis and degradation in Achlya. The results obtained were frequently negative or ambiguous, reflecting some of the difficulties that have accompanied the attempts to establish a role for this compound in plant regulation (Sutherland et al 1962, Kuo & Greengard 1969 , Vandepeute et al 1973, Alvarez et al 1974, Drlica et al 1974).

Cyclic AMP Uptake

<sup>3</sup>H-Cyclic AMP was supplied to Achlya germlings in G<sub>2</sub>Y medium, and retention in samples of cells after filtration and washing was measured over a period of time. Uptake proceeded apparently slowly at first, then at an increasing rate. This could reflect a breakdown prior to uptake, but thin layer chromatography of samples of

medium indicated that the major part of the radioactivity was still in the form of cyclic AMP, suggesting that any extracellular phosphodiesterase was not highly active. Low radioactivity recovered in cell extracts after 5 hours exposure to cyclic AMP resulted in the fate of the nucleotide not being clearly determined from chromatography. In other studies, uptake of cyclic AMP over a period of time was apparently blocked by AMP, caffeine, theophylline, and 2, 4-Dnp, but kinetic studies did not clarify these effects.

#### Cyclic AMP Enzymes

Attempts were made to demonstrate that enzymes capable of degrading or synthesizing cyclic AMP were present in Achlya, as one approach to demonstrating a functional role for the compound. Adenylyl cyclase activity in supernatant extracts of germlings and of cell mats was assayed, using 100  $\mu$ l of assay mixture containing 50  $\mu$ l of extract, 10  $\mu$ l  $^{14}$ C-ATP (53 mCi/mmol), and a buffer of 0.1 mM Tris-HCl, pH 8.5 and 0.1 mM  $MgSO_4$ . Following 30 minutes incubation at 30° C, the reaction was stopped by boiling, the assay tubes were centrifuged, and the supernatants were chromatographed on silica gel plates with 1 M ammonium sulfate and ethanol (30:75). A significantly high amount of radioactivity appeared to be associated with the cyclic AMP carrier in the extracts but not in the boiled control. However, later work indicated that silica gel plates developed with this solvent system could produce two ultraviolet absorbing areas from adenine, one of which was overlapping

with cyclic AMP. Possibly then, the activity in the cyclic AMP spot related to activity of an enzyme involved in ATP degradation.

Subsequent assays used the method described by Wood et al (1972), with NaF added to the assay mixture, and precipitation of non-cyclic nucleotides by addition of ZnSO<sub>4</sub> and Na<sub>2</sub>CO<sub>3</sub> after termination of the reaction. Radioactivity in aliquots of the assay supernatant correlated well with the activity recovered from the cyclic AMP carrier spot on silica gel plated developed with isopropanol: 1 M ammonium hydroxide: H<sub>2</sub>O (7:1:2). Activity correlated with increased volumes of extract used, which were in this case prepared from the particulate fraction of briefly sonicated cells. Addition of digitonin to solubilize the enzyme, after the manner of Wood et al (1972) proved ineffective. Subsequent attempts to demonstrate this activity resulted in failure, possibly due to the use of freezing and grinding as a method of breaking the cells. Assays for cyclic AMP phosphodiesterase activity seemed to suggest that this treatment resulted in loss of that activity. Phosphodiesterase activity was assayed using the system of Thompson and Appleman (1971) with Bio-Rad AG 1-X2 anion exchange resin.

#### Cyclic AMP Assays

Extracts of Achlya cells were prepared and assays for cyclic AMP levels in the tissue were carried out according to the procedure

described by Gilman (1970), using the commercially available kit from Amersham/Searle, Oakville, Ontario. These assays indicated a level of about 1 to 2 pmoles/g wet weight tissue, close to the limits of the assay system. The presence of an endogenous binding protein might interfere with accurate determinations if it removed free cyclic AMP during preparation of the extracts.

#### Possible Cyclic AMP Function

Studies on Achlya failed to provide evidence to support a role for cyclic AMP in cell regulation. However, plants in general have not proven as amenable to such studies as have animal and bacterial systems. Observed actions of cyclic AMP on some plant systems (Dufus & Dufus 1969, Galsky & Lippincott 1969, Wood & Braun (1973) and those briefly described for Achlya in Part IV and V suggest that the existence of a function for cyclic AMP cannot yet be eliminated in these cases.

## PART III

## TRANSPORT SYSTEMS FOR PURINES, PYRIMIDINES AND NUCLEOSIDES

## RESULTS

A. Characterization of Nucleic Acid Base Uptake Processes

Kinetic values and specificity: The ability of germinated sporangiospores of Achlya to take up a variety of nucleic acid bases was examined over a range of substrate concentrations, using the methods described previously. The rate-concentration plots from these studies are shown in Figure 38, with the Lineweaver-Burk double-reciprocal plots as insets. The half-saturation values,  $S_{0.5}$ , are recorded in Table XIII. An analysis of guanine uptake was not possible because it is largely insoluble at the physiological pH levels at which these uptake studies were conducted.

The saturation curves indicate that uptake of nucleic acid bases is mediated by a carrier. Support for this conclusion came from competition studies in which the uptake of one base is competitively inhibited by the others (Figure 39). Uptake of uracil was inhibited by its analogues thymine and cytosine, and by the purine adenine. Adenine, used at equimolar concentrations with the other

Figure 38: Rate-concentration plots and Lineweaver-Burk insets of the initial reaction velocities of purine and pyrimidine uptake processes. (a) uracil; (b) adenine; (c) thymine and (d) cytosine.

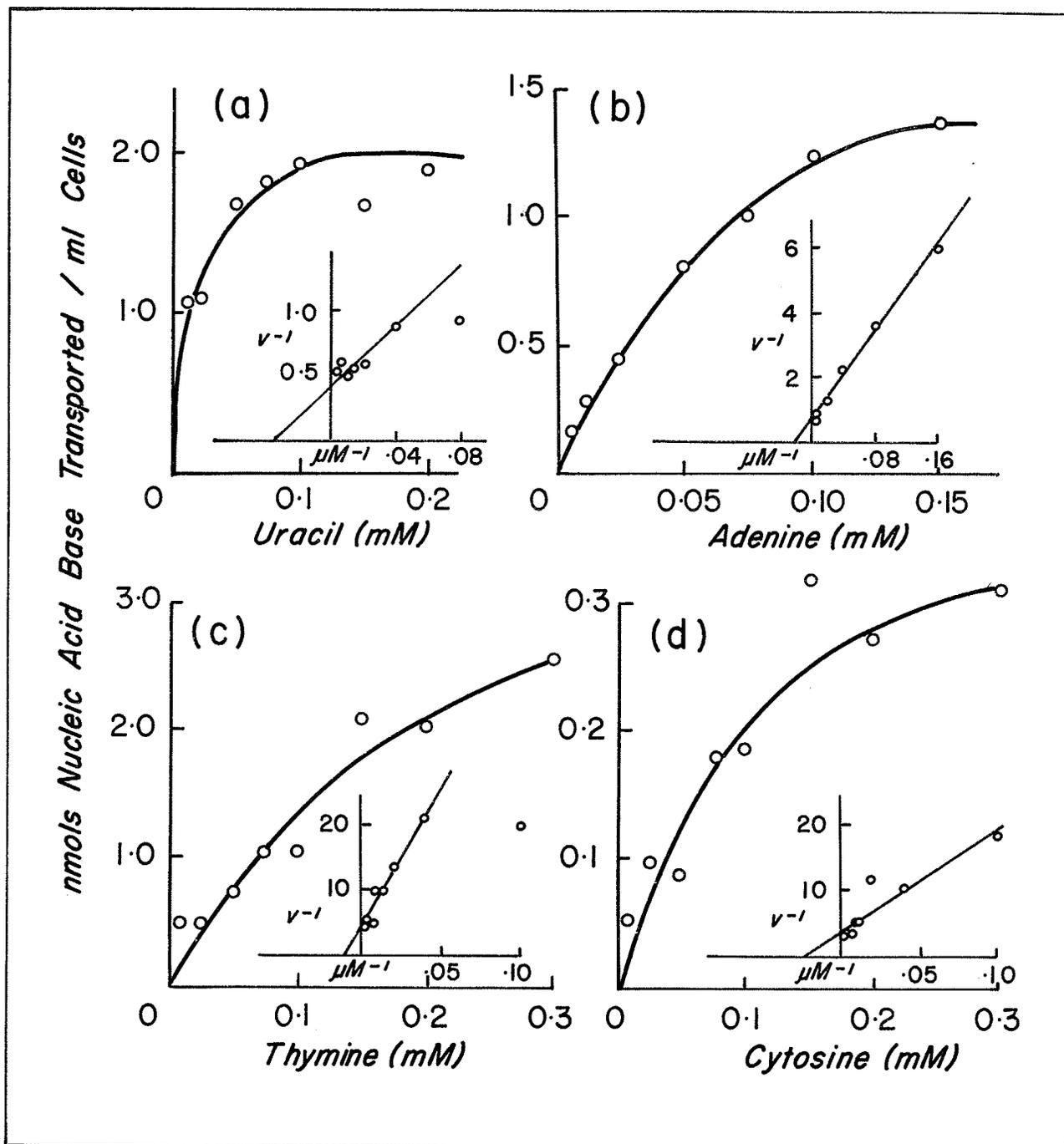


Table XIII

A summary of the  $S_{(0.5)}$  values for the transport of purines, pyrimidines and nucleosides by germinated sporangiospores of Achlya.

Metabolite	$S_{(0.5)}$ <u>M</u>
Purines and Pyrimidines	
Thymine	$1.11 \times 10^{-4}$
Adenine	$5.0 \times 10^{-5}$
Cytosine	$5.0 \times 10^{-5}$
Uracil	$2.78 \times 10^{-5}$
Nucleosides	
Thymidine*	$5.4 \times 10^{-6}$ ( $5.5 \times 10^{-5}$ )
Adenosine	$3.85 \times 10^{-6}$
Cytidine	$4.44 \times 10^{-6}$
Uridine	$4.76 \times 10^{-6}$
Guanosine	$3.8 \times 10^{-6}$

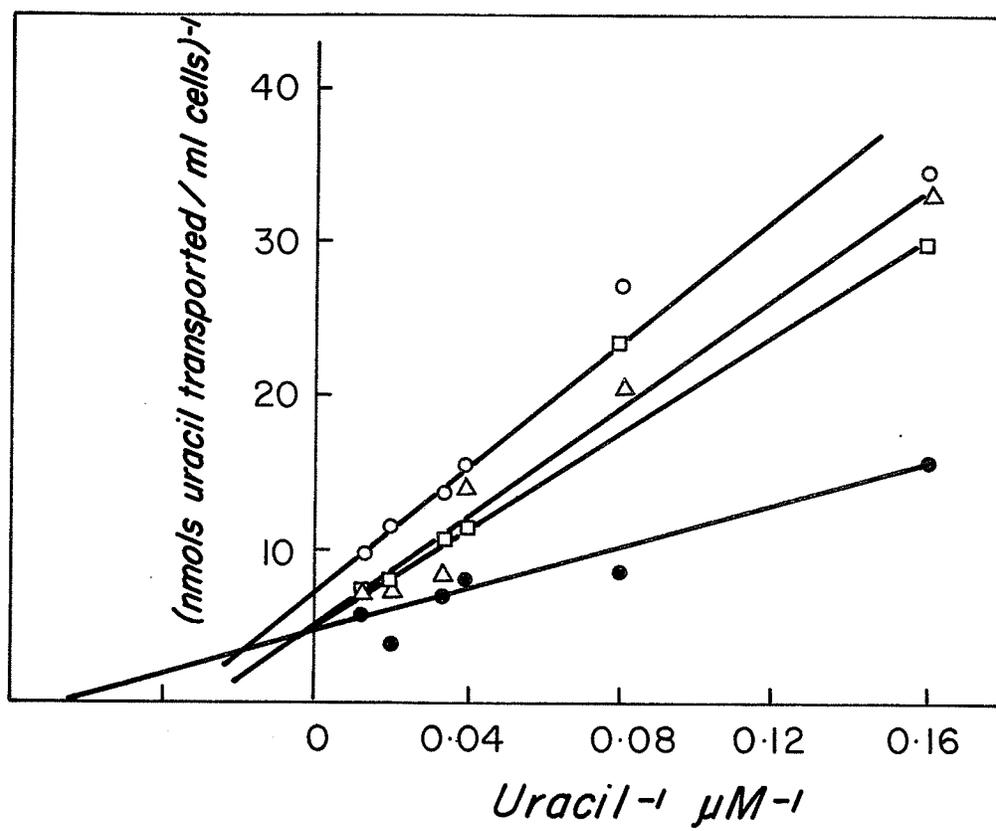
\*Two Michaelis constants determined.

Figure 39: Inhibition of uracil transport by ( $\Delta$ )  
cytosine, ( $\circ$ ) adenine, and ( $\square$ )  
thymine, all at 0.5 mM concentration.

inhibitors, was the most inhibitory, but the inhibition pattern was noncompetitive. The reason for this becomes clear when later results are considered. It is unlikely that uracil uptake would be affected in the manner that it is by the other nucleic acid bases if intake were purely by passive diffusion. Thus the results indicate that there is a common site of interaction for all the bases.

A nucleoside has little or no influence on the uptake of its corresponding nucleic acid base. The reverse situation is also true. It would therefore seem that the bases are not transported by an illicit system, but by a carrier specific for the bases, in a facilitated diffusion process.

Metabolic inhibitors: Although the purine and pyrimidine bases are transported by a mediated system, metabolic energy does not appear to be involved in the activity. Metabolic energy and electron transport inhibitors, 2,4-Dnp, CCCP, azide, and cyanide, were used at concentrations which abolish transport activity for nucleosides (see later section), amino acids, and sugars (unpublished results of LéJohn, Singh, and Goh), with no apparent effect. This indicates that if the bases are indeed taken up by a carrier-mediated process, the carriers are not linked to the metabolic energy processes of the cell.



pH and temperature effects: A study of the influence of pH and temperature was no more informative than competition studies in demonstrating different transport carriers. Except for adenine, the nucleic acid bases do not demonstrate a marked pH dependence in the rate of the reaction (Figure 40). The reaction rate is virtually unchanged from pH 5.5 to pH 9. The effects of temperature on uptake rates are shown in Figure 41. Transport of thymine, uracil, and adenine showed definite temperature effects, with optima of 30°. This is in harmony with the temperature optimum found for all the metabolites, nucleosides (later section), amino acids (unpublished results of LéJohn), and sugars (unpublished results of Goh and LéJohn).

#### B. Characterization of Nucleoside Transport Processes

Kinetic values: Rate-concentration curves showing the concentration-dependent uptake of the nucleosides are presented in Figure 42. The half-saturation values determined from the Lineweaver-Burk plots (Figure 42, insets) are listed in Table XIII. These  $S_{0.5}$  values are within one order of magnitude difference, varying from  $2.5 \times 10^{-6}$  M for guanosine to  $1.7 \times 10^{-5}$  M for thymidine.

Metabolic inhibitors: Studies on the inhibition of nucleoside transport by energy uncouplers and electron inhibitors are

Figure 40: Influence of pH on the initial reaction rates of cytosine, uracil, adenine and thymine uptake.

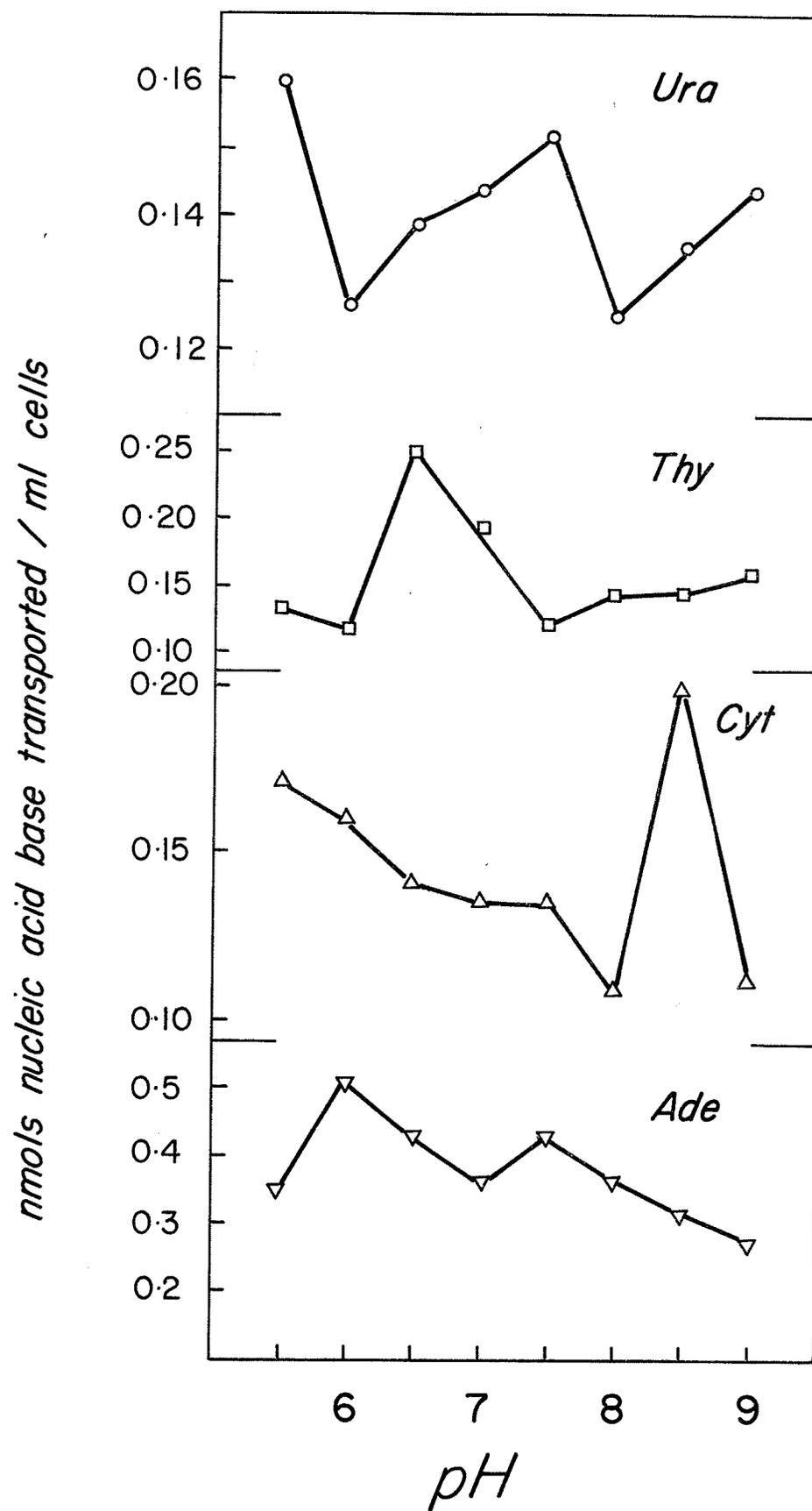


Figure 41: Influence of temperature on the initial reaction of cytosine, uracil, adenine and thymine uptake.

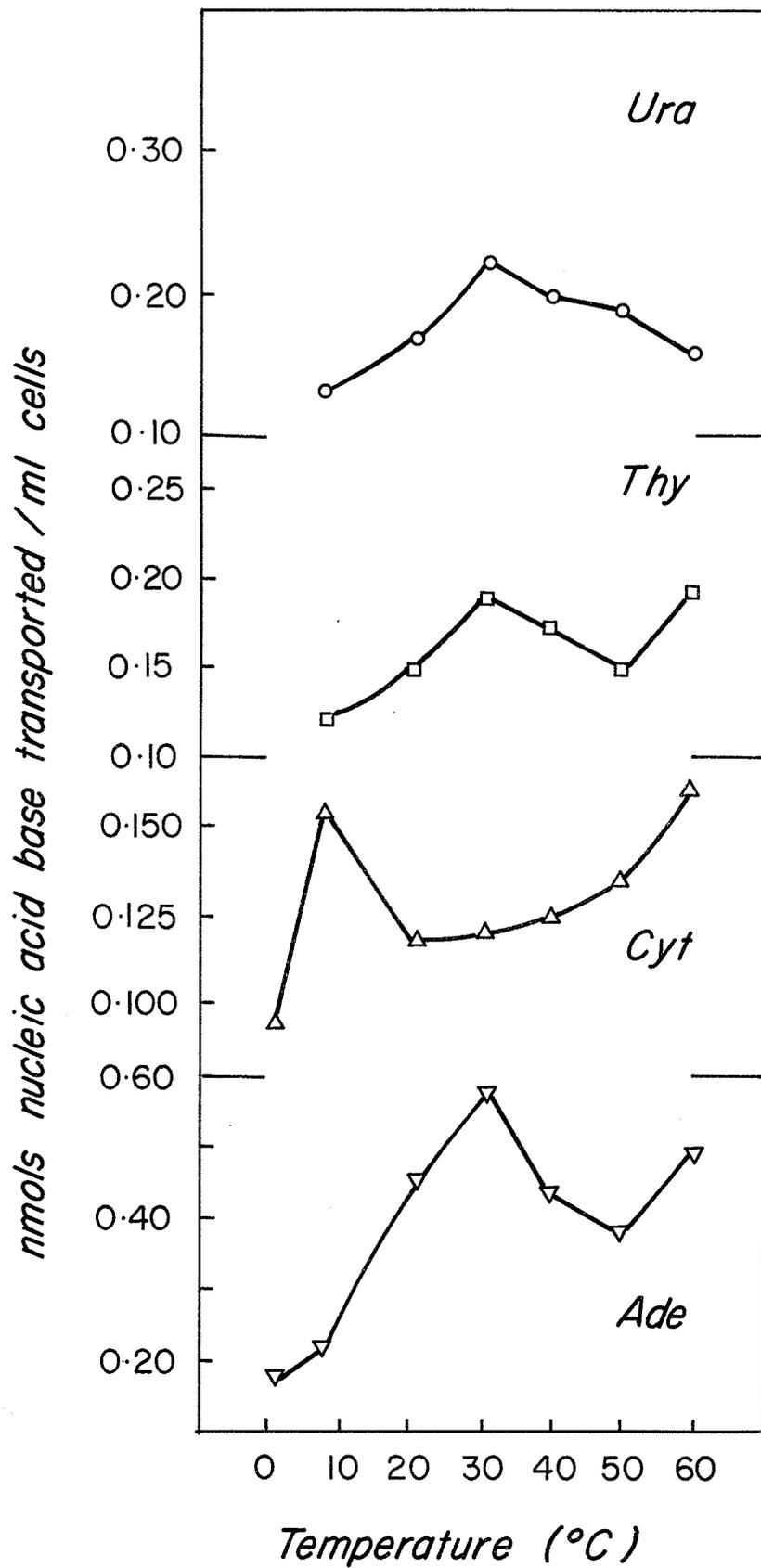
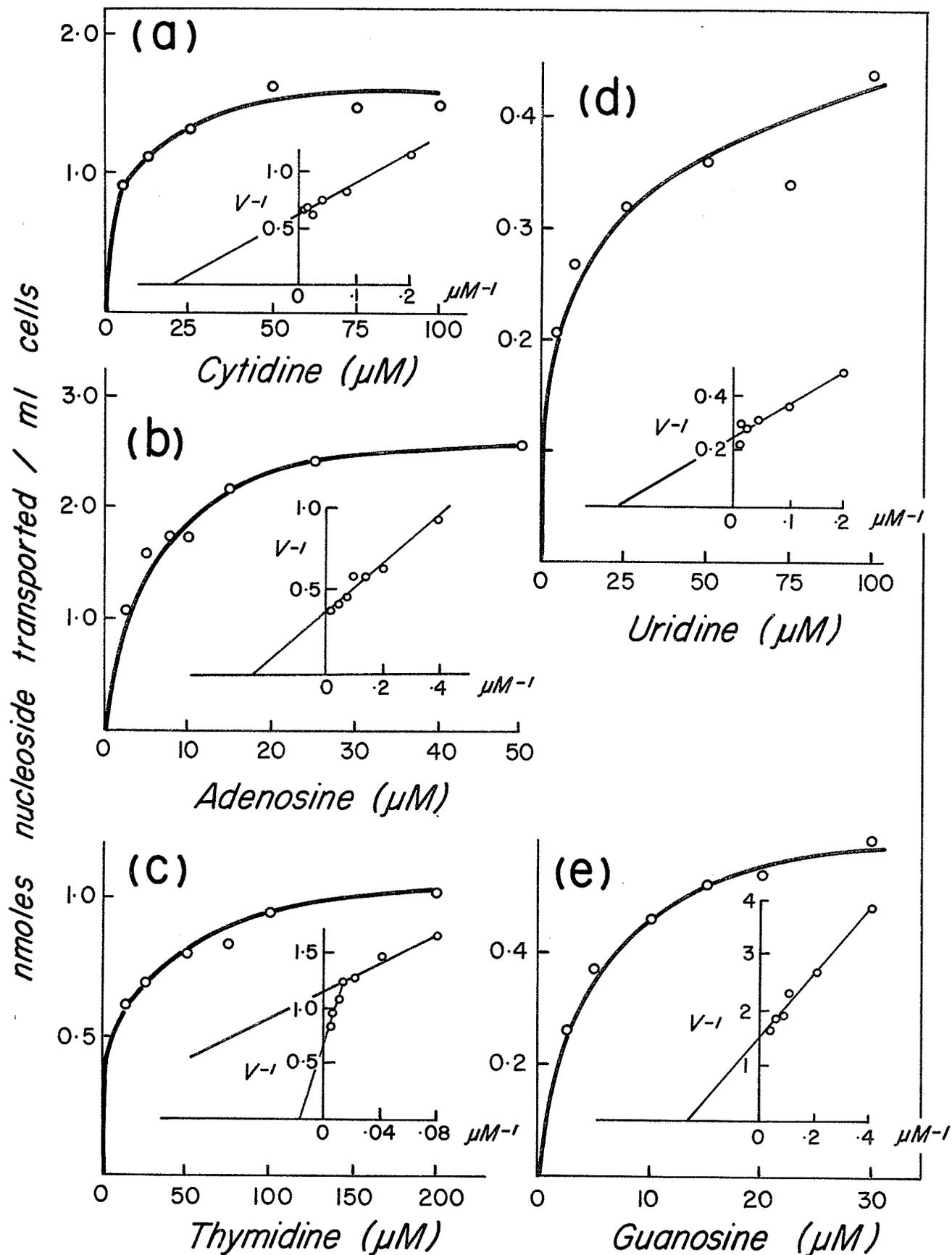


Figure 42: Rate-concentration plots and Lineweaver-Burk insets of the initial reaction velocities of nucleoside transport systems. (a) cytidine; (b) adenosine; (c) thymidine; (d) uridine and (e) guanosine.



shown in Figure 43. The most effective inhibitors were CCCP and the thiol-reacting agent  $\text{HgCl}_2$ . These two inhibitors were maximally effective at  $10 \mu\text{M}$  in most cases, when tested over the range of 10 to  $100 \mu\text{M}$ . The electron transport inhibitors, azide and cyanide, were only partially effective, not inhibiting completely at 1 mM. The uncouplers, 2,4-Dnp and CCCP were more effective. Guanosine transport appeared to be affected differently when compared to the other nucleosides.  $\text{HgCl}_2$  was not tested on this system.

To affirm that active transport was being observed for nucleosides, exit experiments were performed as described under Methods. The results obtained for two nucleosides, thymidine and adenosine, are shown in Figure 44. Thymidine and adenosine exit at the same rate, the cells losing as much as two-thirds of the accumulated material in 15 min. In the absence of  $10 \mu\text{M}$  CCCP, but with a large concentration of unlabelled adenosine present as a control culture, less than 10% of the label was released. This suggests that, when the energy system of the cell is unperturbed, the captured molecules of nucleosides are retained against a concentration gradient, or probably, recaptured by an efficient system.

Figure 43: Inhibition of the initial reaction rates of nucleoside transport by metabolic energy uncouplers, 2,4-Dnp, ( $\Delta$ ) CCCP, ( $\nabla$ ) electron inhibitors, KCN ( $\square$ ) and  $\text{NaN}_3$  ( $\circ$ ); and by  $\text{HgCl}_2$  ( $\bullet$ ). The concentrations of CCCP and  $\text{HgCl}_2$  varied from 0  $\mu\text{M}$  to 100  $\mu\text{M}$  and the other inhibitors were used between 0  $\mu\text{M}$  and 1 mM.

PERCENT CONTROL

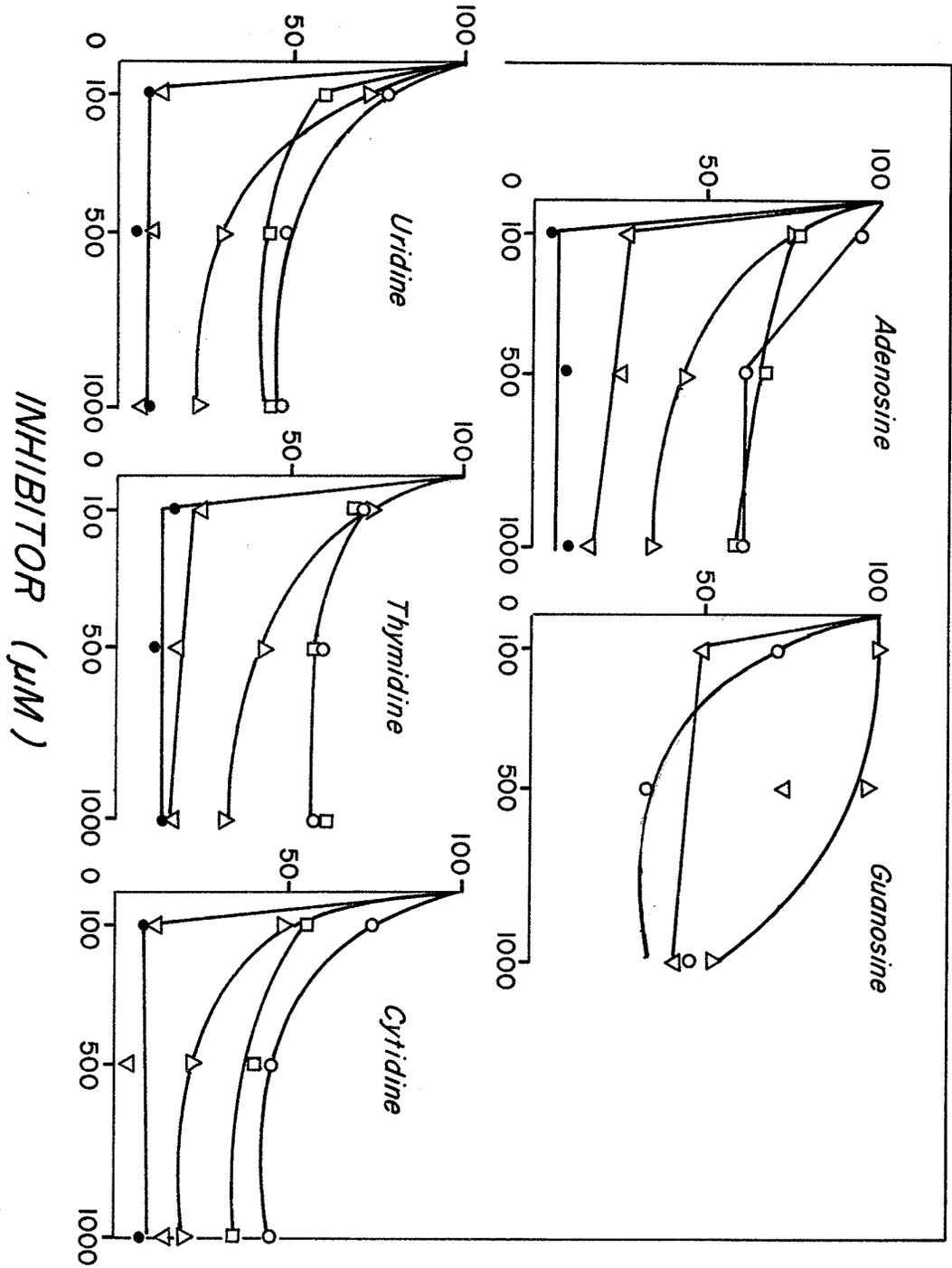
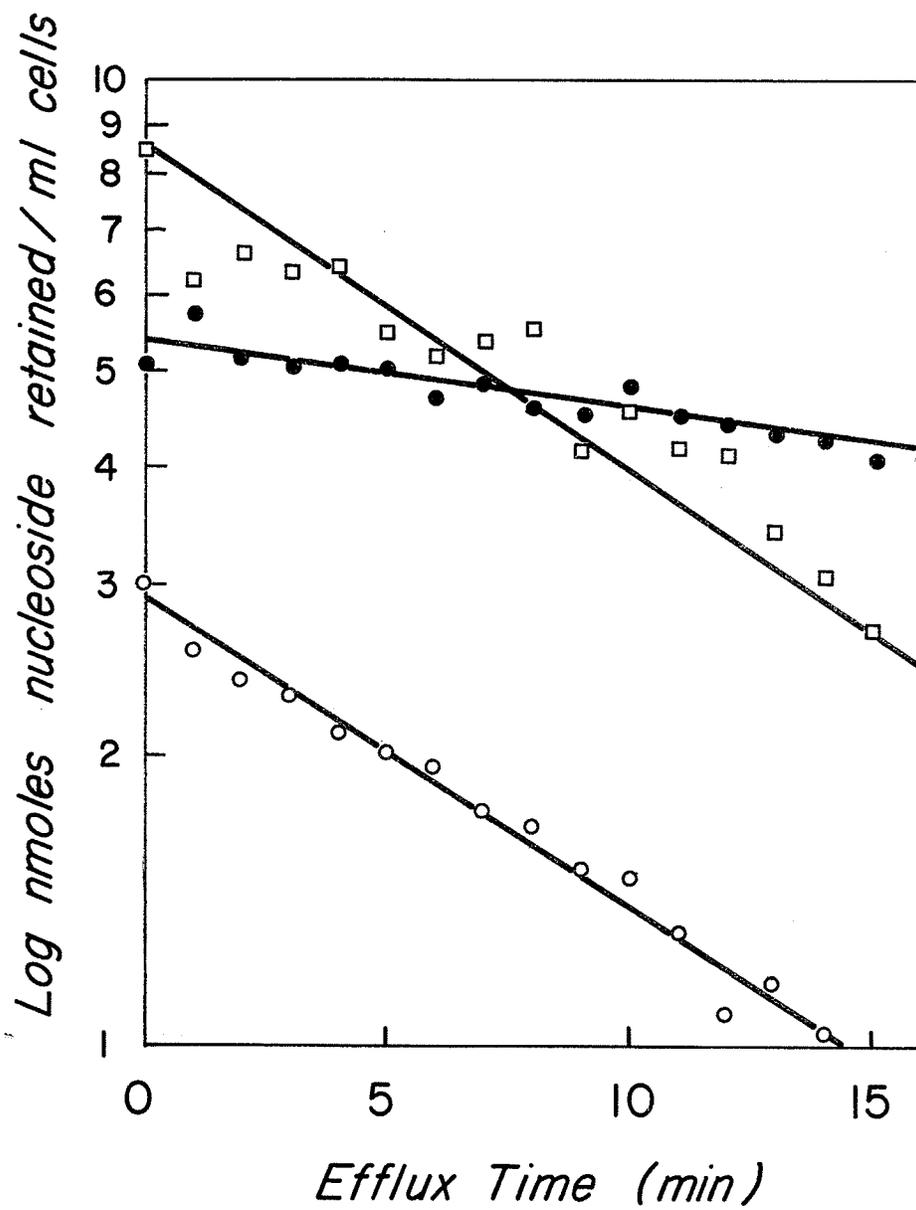


Figure 44: Semi-log plot of the time-course kinetics of efflux of adenosine and thymidine from cells pre-loaded with the specific nucleoside in the presence of 28  $\mu\text{M}$  cycloheximide. Efflux facilitated by addition of 10  $\mu\text{M}$  CCCP. In the absence of the uncoupler, but in the presence of excess unlabelled adenosine, efflux was minimal. Adenosine efflux is represented by (  $\square$  ) with CCCP, and by (  $\bullet$  ) with external adenosine. Thymidine efflux with CCCP is represented by (  $\circ$  ).



pH and temperature effects: Nucleoside transport was observed to depend on the pH of the medium, as shown in Figure 45. Uridine, cytidine, and thymidine transport systems showed rather well-defined optima, each at pH 6.5. Adenosine and guanosine transport systems were less clearly defined, with some suggestion from the pH profiles that more than one permease might be involved for each nucleoside. In particular, guanosine uptake was characterized by optima at pH 6.5 and pH 8, suggesting that this system might be distinct from that of the other nucleosides. Cytidine, uridine and thymidine appear to be taken up by a common permease, on the basis of pH studies.

Temperature studies however, revealed that the permease system of thymidine might differ from those of the other nucleosides. It alone was not affected by temperatures above 30° - the optimum for the other systems (Figure 46). A  $Q_{10}$  greater than 2 is observed for all the permeases as the temperature rose from 10° to 20°, indicating that a process requiring high activation energy is involved. For the systems other than thymidine and cytosine, the  $V_{max}$  of the transport systems dropped sharply between 30° and 40°. The process was not reversible above 40°, which suggests that a protein subject to heat denaturation may be involved.

Figure 45: Influence of pH on the initial reaction rates of nucleoside uptake.

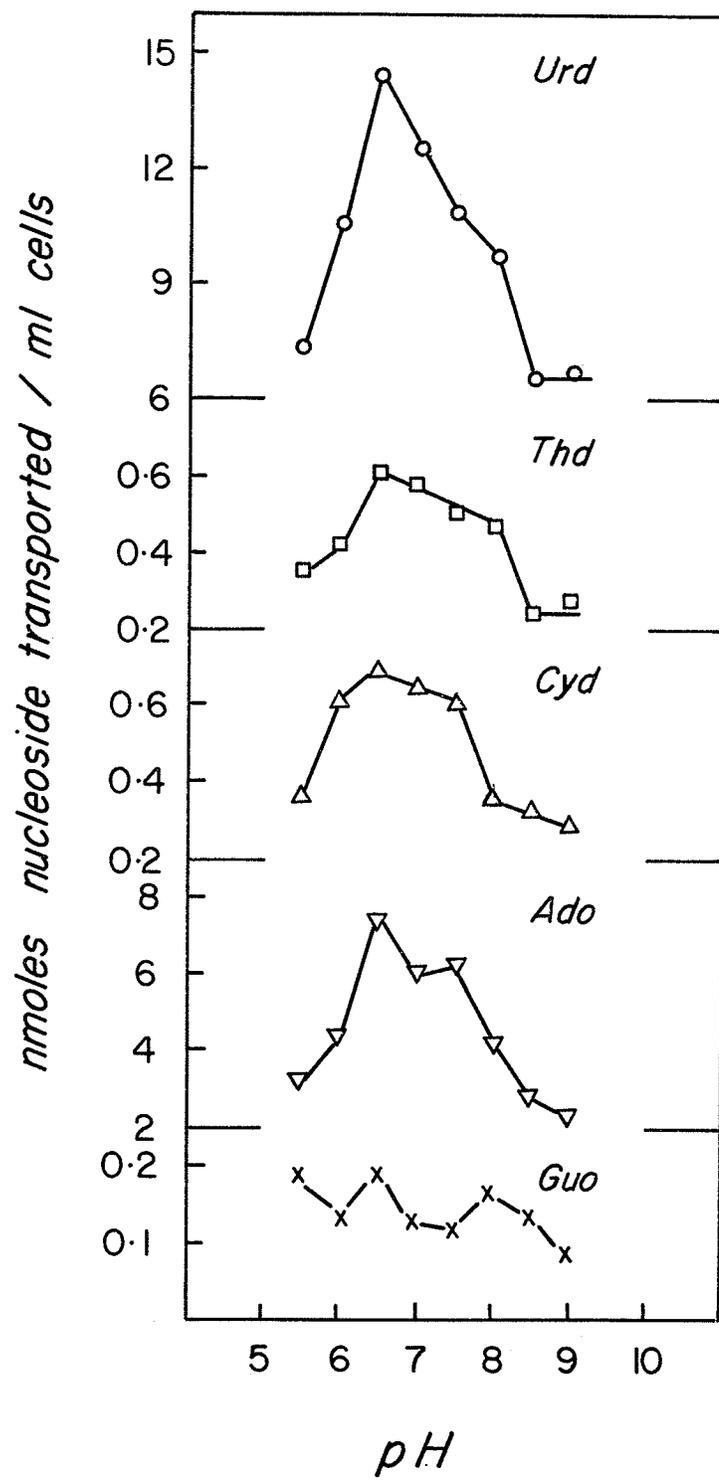
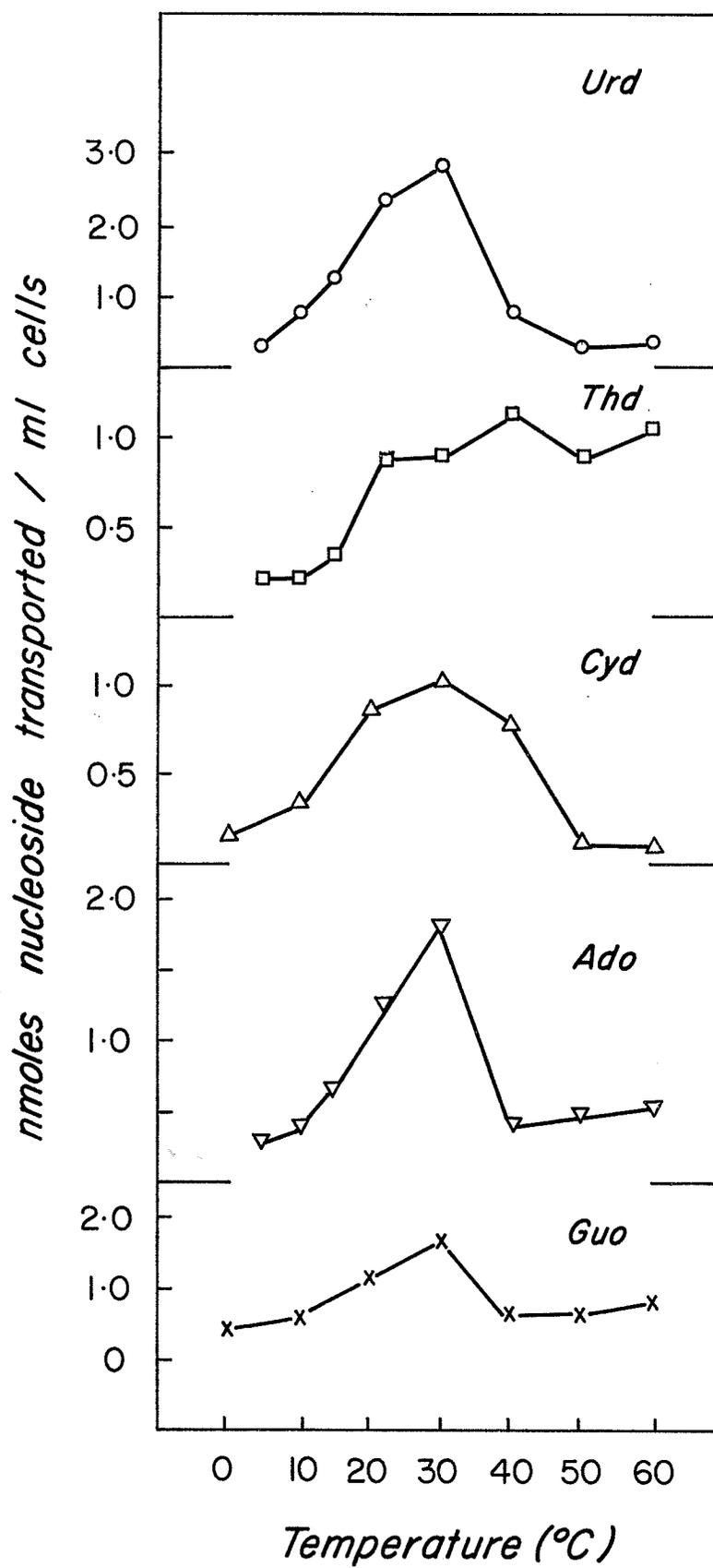


Figure 46: Influence of temperature on the initial reaction rates of nucleoside uptake.



Competition kinetics: To determine if more than one transport system exists for the nucleosides, studies of competition between the compounds were carried out. In Figure 47 a and b the saturation curves and Lineweaver-Burk forms of the competition plots for uptake of adenosine and thymidine respectively are shown. Guanosine proved to be a very poor inhibitor of adenosine transport, and did not inhibit competitively (Figure 47 a, inset). Thymidine and cytidine were strong competitive inhibitors of adenosine uptake. The corollary was also true, as cytidine and adenosine inhibit thymidine uptake competitively (Figure 47 b, inset). Values plotted in Figure 47 b also demonstrate that thymine had no effect on the uptake rate for thymidine.

When cytidine and uridine were used as the varied substrates, the inhibition patterns for thymidine and adenosine were competitive (Figure 48 a and b). Thus it seems that thymidine, adenosine, cytidine and uridine share a common transport system. Cytosine was ineffective on the transport system for cytidine (Figure 48 a), again indicating the specificity of the system for nucleosides.

In view of the pH pattern observed for guanosine (Figure 45), it was not surprising to observe that uptake of guanosine was not affected by cytidine, and only weakly inhibited by thymidine and adenosine (Figure 49). When guanosine was used as inhibitor, it had no effect on the transport of cytidine (Figure 50 a) or thymidine (Figure 50 b). The only nucleoside

Figure 47: Rate-concentration plots and Lineweaver-Burk form of the competition kinetics of nucleoside transport: (a) effect of cytidine ( ● ), thymidine ( △ ) and guanosine ( □ ) on adenosine uptake; (b) effect of cytidine ( ● ), adenosine ( △ ) and thymine ( □ ) on thymidine uptake. All inhibitors at 0.5 mM in these and following figures, unless otherwise indicated.

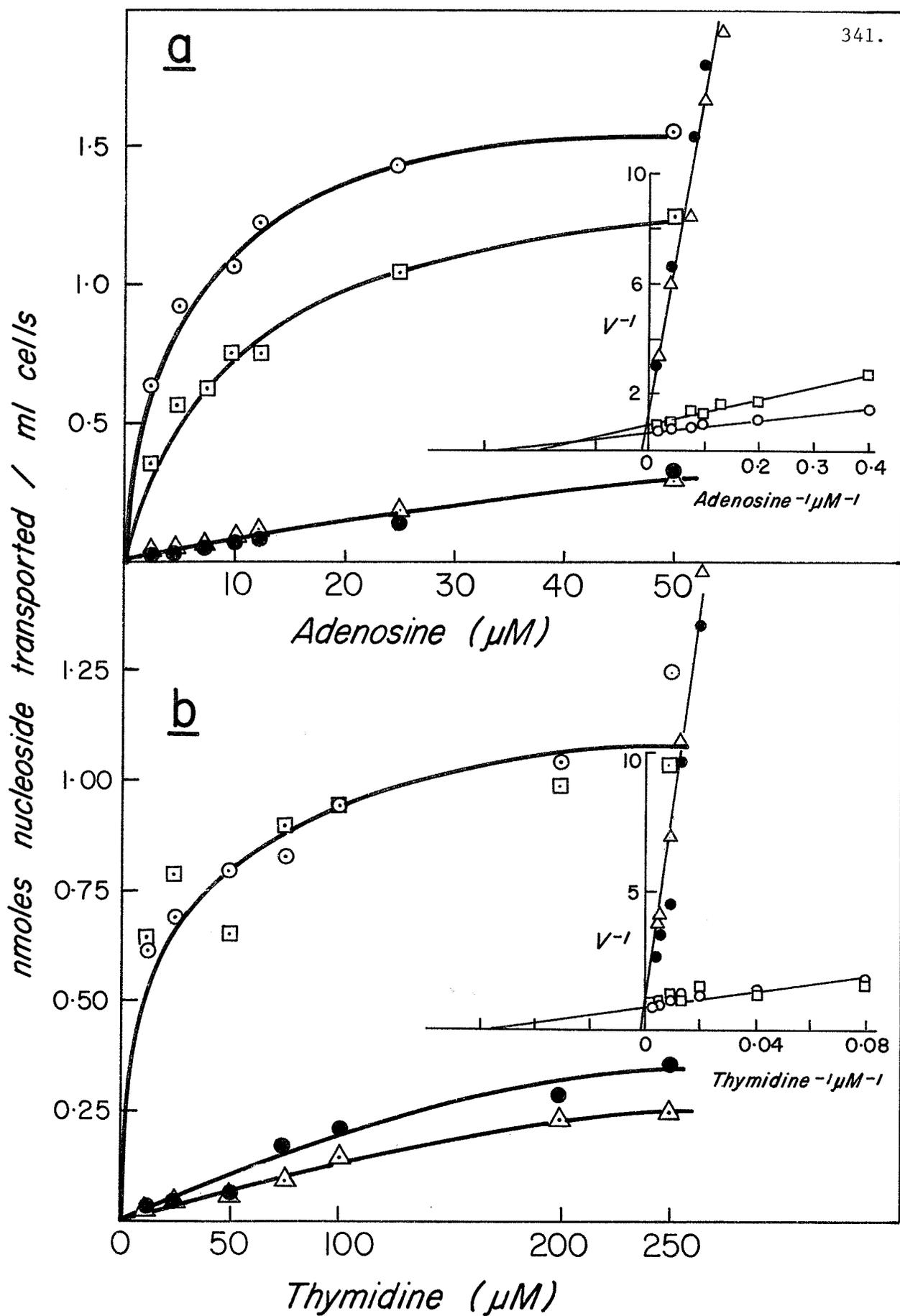


Figure 48: Rate-concentration plots and Lineweaver-Burk form of the competition kinetics of nucleoside transport: (a) effect of thymidine ( ● ), adenosine ( △ ) and cytosine ( □ ) on cytidine uptake; (b) effect of thymidine ( □ ) and adenosine ( △ ) on uridine uptake.

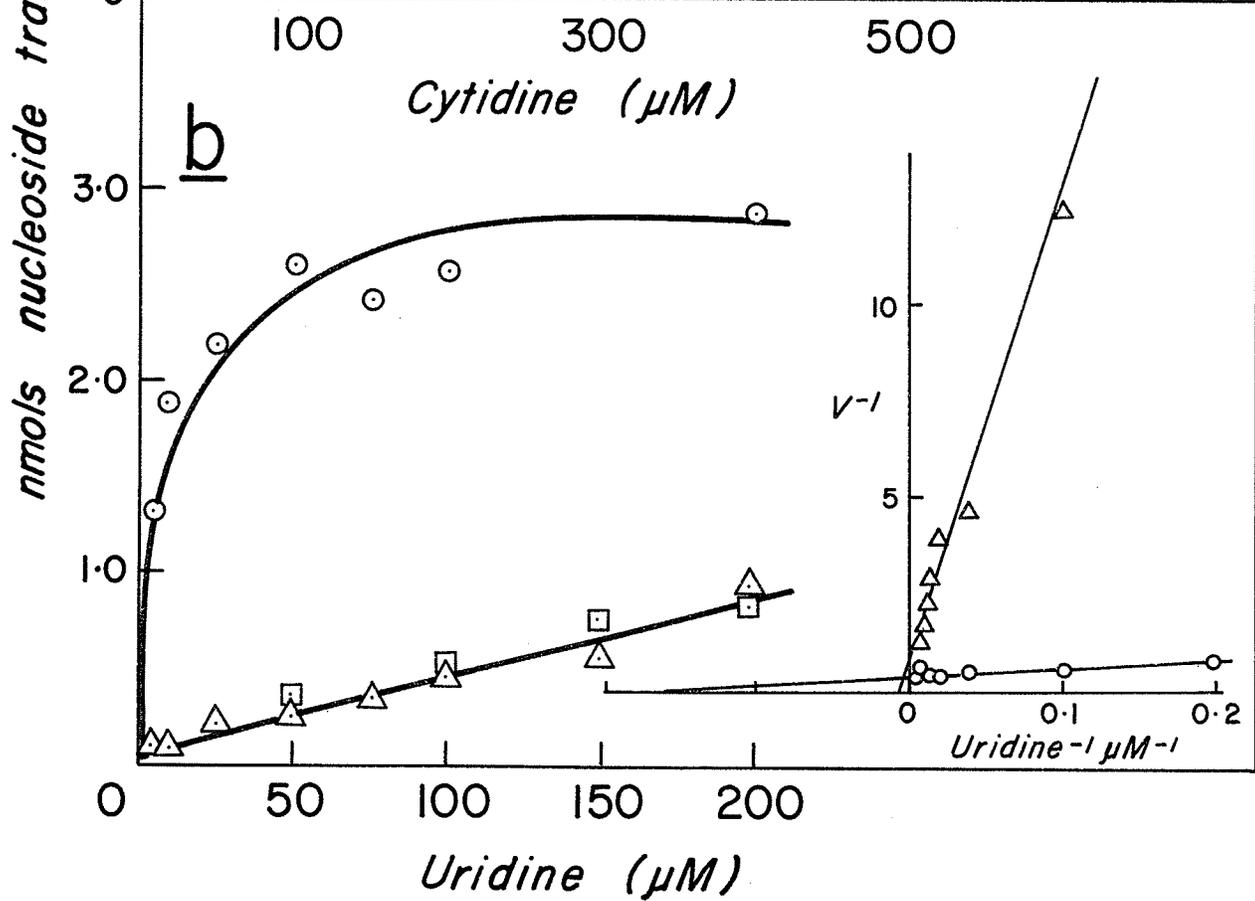
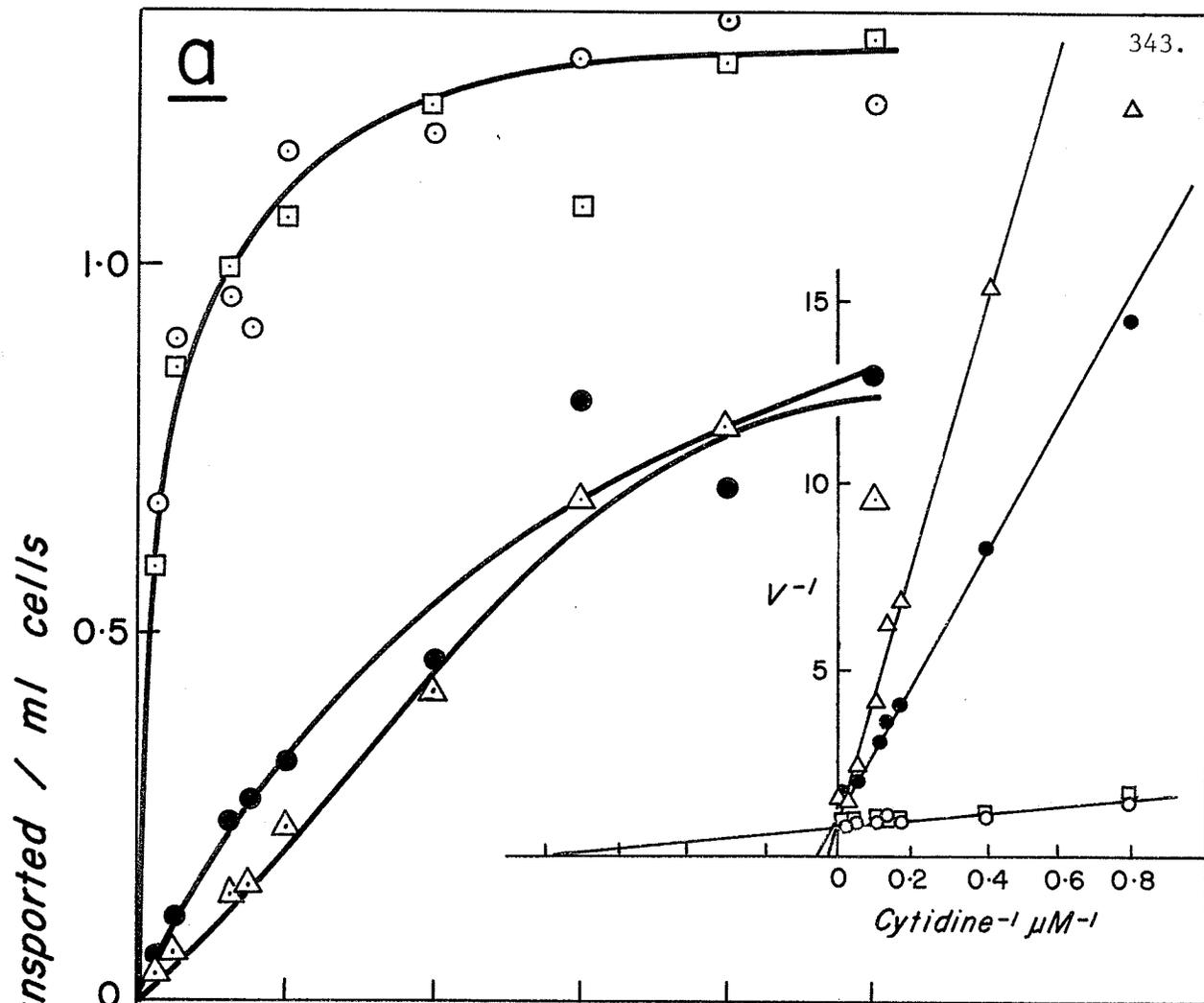


Figure 49: Rate-concentration plots and Lineweaver-Burk form of the competition kinetics of nucleoside transport: effect of thymidine (  $\square$  ) and adenosine (  $\triangle$  ) on guanosine uptake.

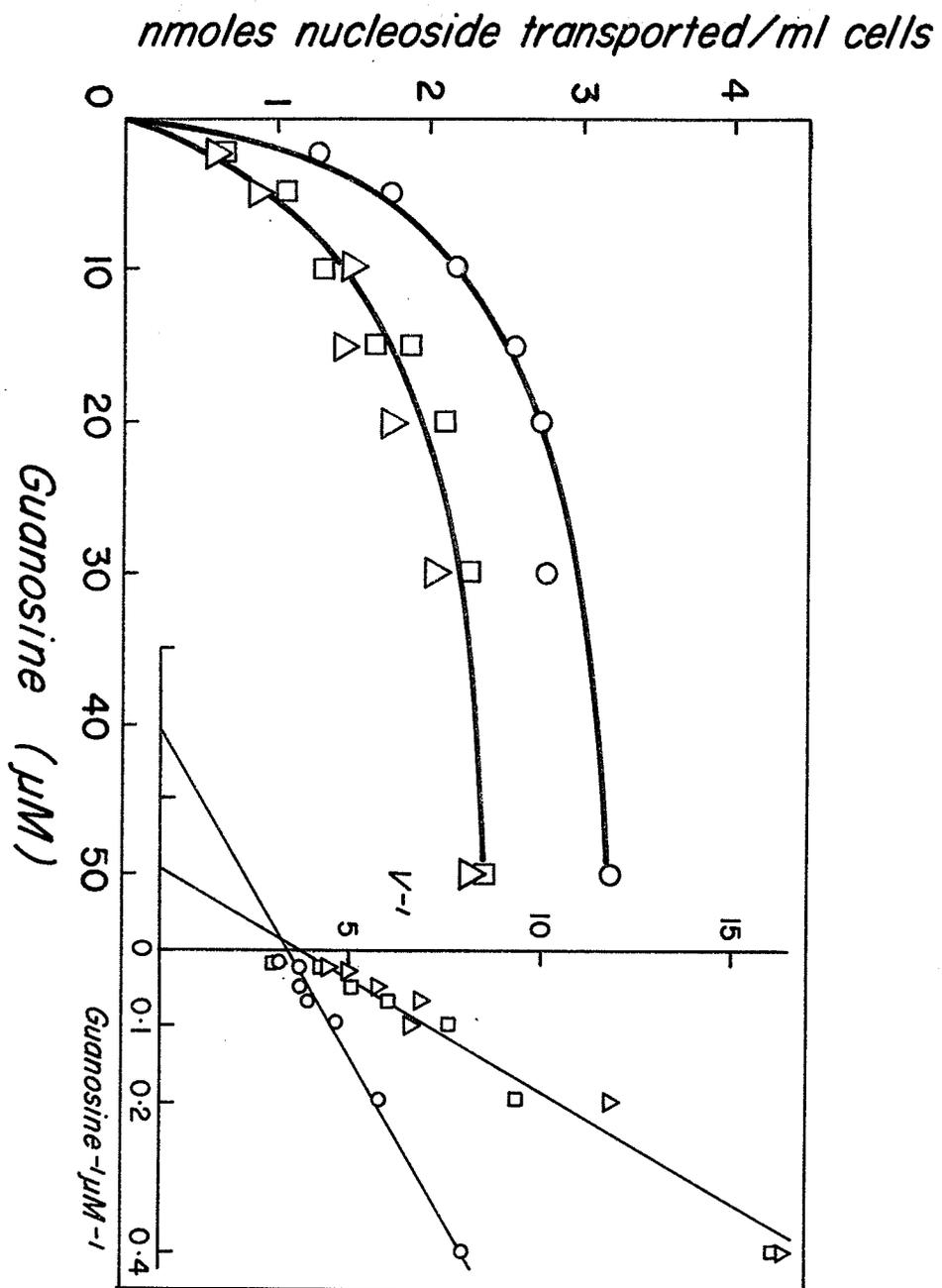
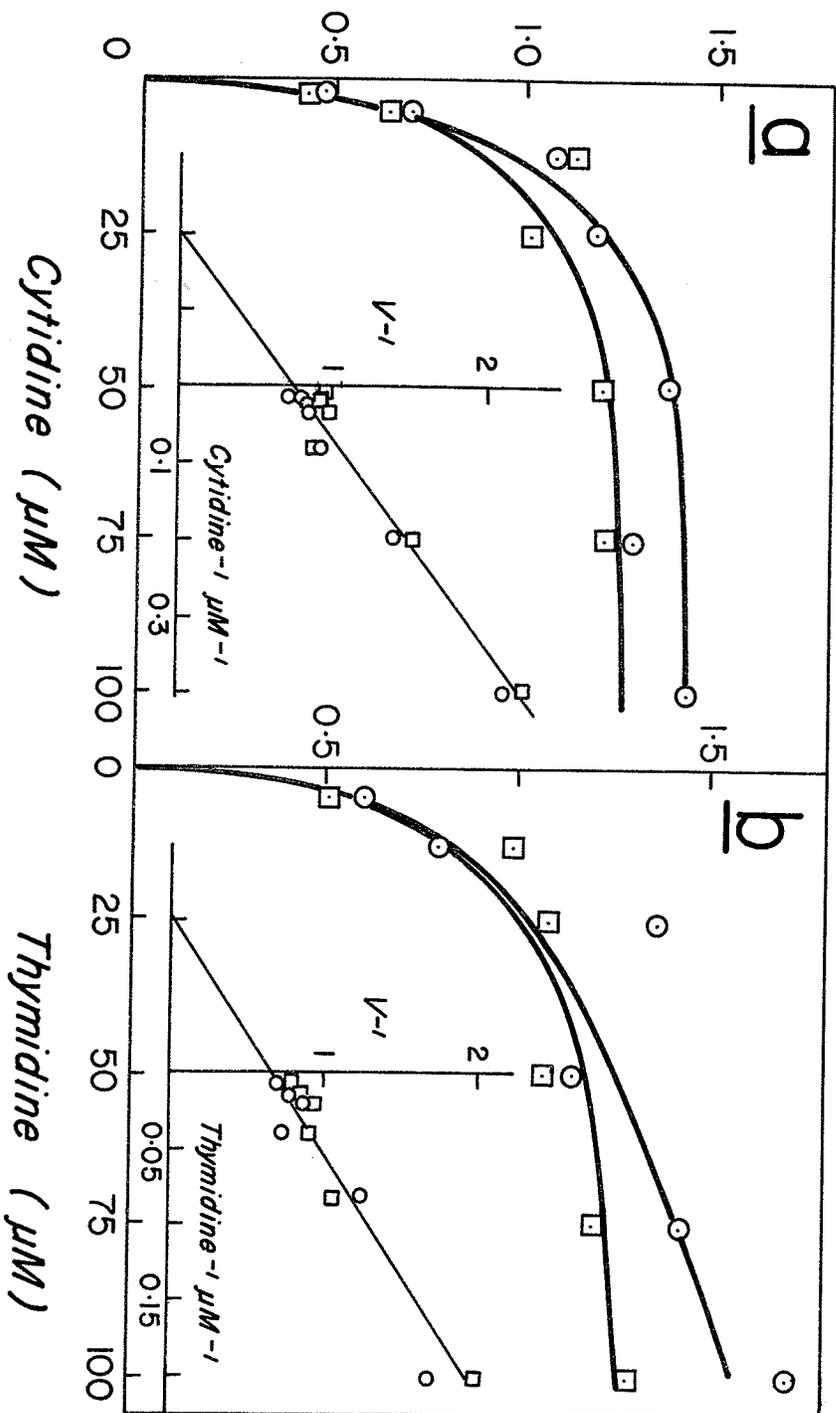


Figure 50: Rate-concentration plots and Lineweaver-Burk form of the competition kinetics of nucleoside transport: effect of guanosine on (a) cytidine (  $\square$  ); (b) thymidine (  $\square$  ) uptake.

*nmoles nucleosides transported / ml cells*

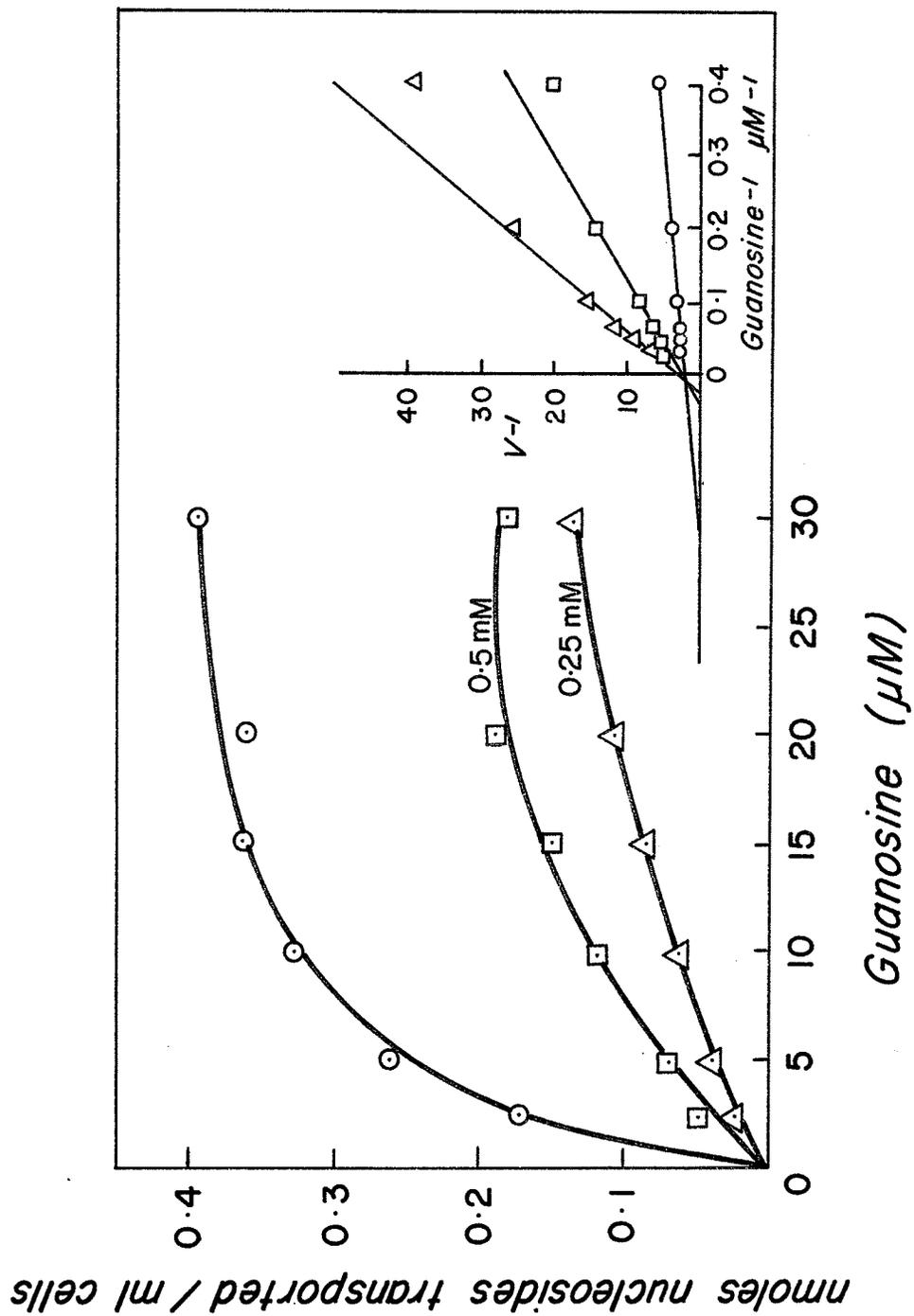


found to inhibit guanosine transport strongly was inosine, as shown in Figure 51. At 0.25 mM inosine compared to 0.5 mM adenosine, inosine was more effective an inhibitor. At 0.5 mM inosine, guanosine uptake was almost abolished. The inhibition pattern was essentially competitive for both inhibitors (Figure 51, inset). Studies of inosine as a potential inhibitor of the transport systems for cytidine, thymidine, and uridine showed no effect by inosine, synonymous with the results obtained for guanosine as inhibitor.

The most logical conclusion that can be drawn from all these observations is that there are two types of nucleoside transport systems; one specific for guanosine and its analogues, and the other with a broad specificity for purine and pyrimidine nucleosides. This then would resemble the systems of Neurospora crassa (Schiltz & Terry 1970). Nucleotide transport has not been examined closely in Achlya, but inhibition effects suggest that the nucleoside uptake systems may be involved in some manner.

Influence of citrate: The effect of citrate on the uptake of nucleosides was of interest in connection with studies of the role of calcium in the membrane transport systems of Achlya. Addition of citrate to germlings had already been found to produce immediate cessation of protein synthesis, followed by a shut-off of RNA synthesis, in a process that was relieved by addition of calcium (Cameron & LeJohn 1972 a). In connection with these studies, citrate had been found to inhibit amino acid transport.

Figure 51: Influence of inosine (  $\Delta$  ), adenosine (  $\square$  )  
on the initial rate of uptake of guanosine.

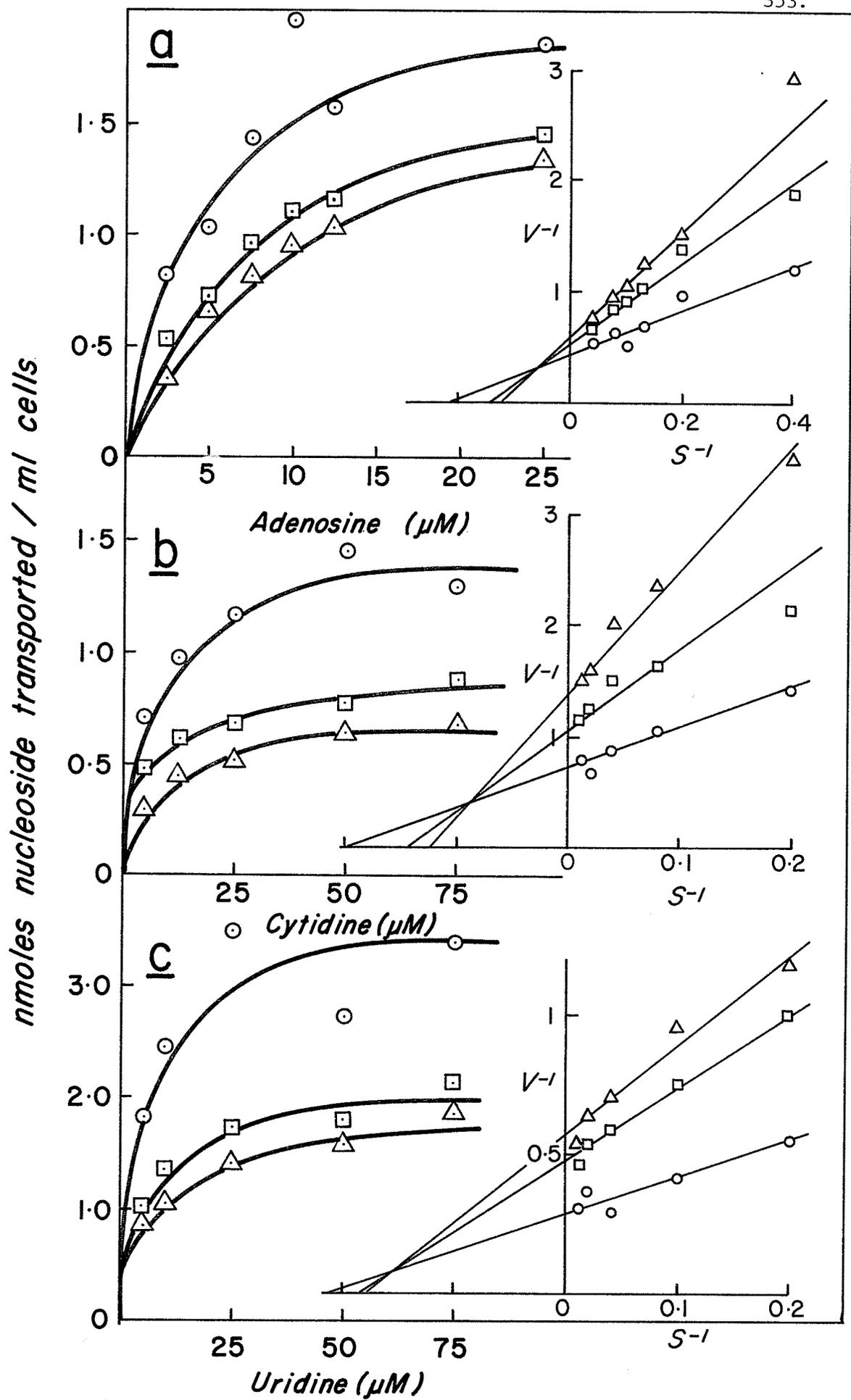


The effects of citrate on nucleoside transport was examined, and the data obtained for adenosine, cytidine, and uridine are shown in Figure 52 a, b, and c. The rate of uptake was reduced in the presence of citrate, both cytidine and uridine uptake being reduced by 50% with 10 mM citrate. Adenosine uptake was only moderately affected by an equivalent amount of citrate. The pattern of inhibition was noncompetitive (Figure 52, insets), both  $V_{max}$  and  $S_{0.5}$  being affected. In contrast, while amino acid transport was inhibited noncompetitively, the  $S_{0.5}$  values for the different permeases remained unchanged (Singh and LéJohn unpublished results). Furthermore, citrate inhibition of nucleoside transport was far less than that of amino acids for which inhibition may be as much as 80% or 90%.

A reasonable conclusion that can be drawn from these observations is that citrate inhibition of nucleoside transport is indirect. This would be consistent with citrate acting on a calcium-related function, reducing the metabolic capacity of the cell or altering some membrane feature, and affecting uptake of amino acids and nucleosides. Further investigation of this possibility was carried out using osmotically-shocked cells, devoid of a component of the  $Ca^{++}$  binding and transport system (LéJohn & Cameron 1973).

Osmotically-shocked cells: The osmotically-shocked cells, prepared as described under Methods, were tested for their ability to accumulate nucleosides. As previously determined for

Figure 52: Inhibition of nucleoside transport by citrate.  
The concentration of citrate used were 0 mM  
(  $\circ$  ); 5 mM (  $\square$  ); and 10 mM (  $\triangle$  ).



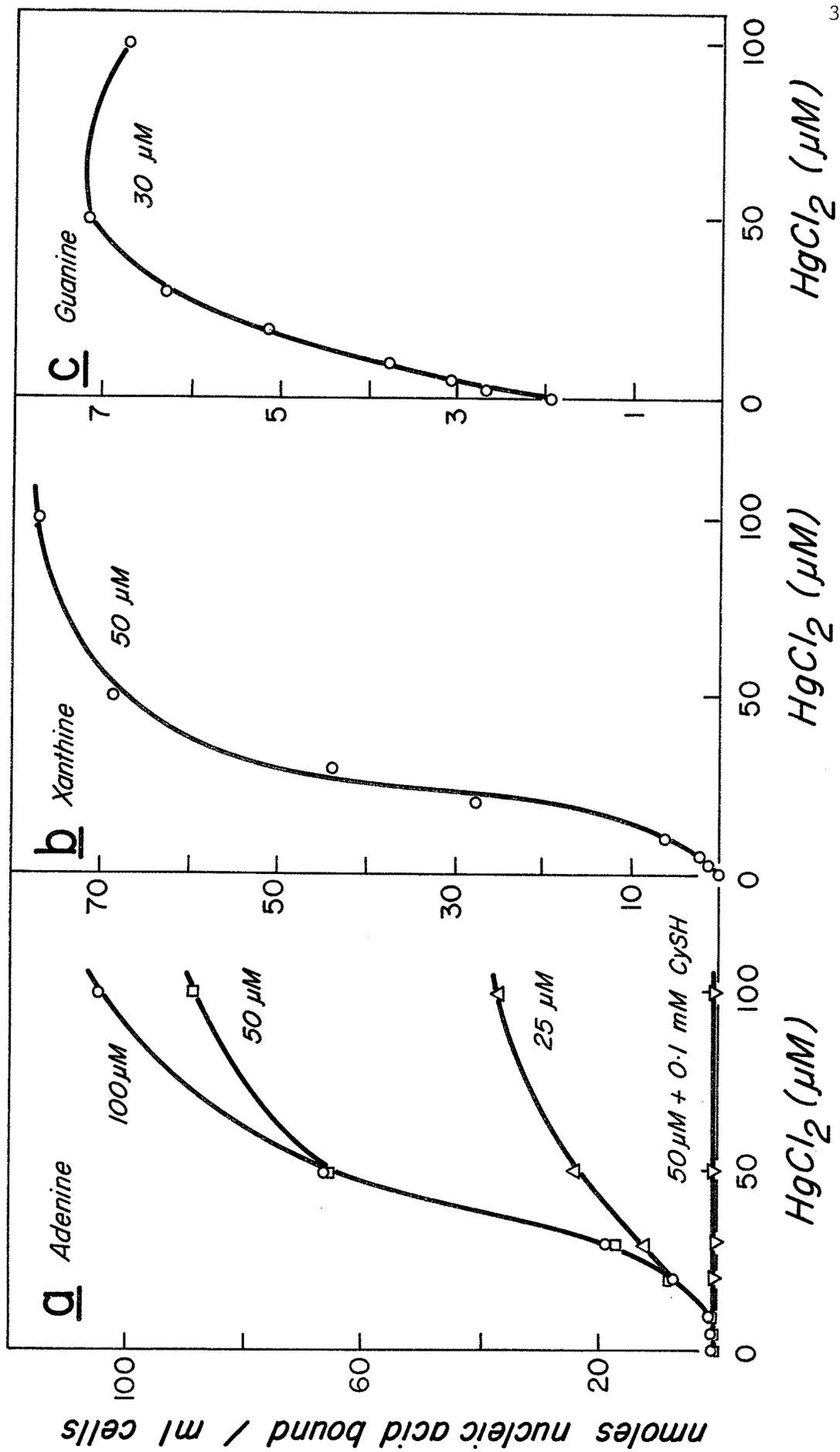
amino acids (Cameron, unpublished data) the cells were unable to transport nucleosides. Addition of calcium did not enhance transport or repair the deficiency.

### C. Binding of Purine Bases

Mercurial-enhancement effect: In studies of the effect of  $\text{HgCl}_2$  on uptake of nucleic acid bases, it was observed that, while the other bases are unaffected, adenine uptake appeared to be greatly stimulated. A more detailed study of this phenomenon was carried out, with varied  $\text{HgCl}_2$  concentration. As shown in Figure 53 a, adenine bound was increased about 100-fold by application of 0.1 mM  $\text{Hg}^{++}$ . Enhancement of adenine binding was pronounced only at concentrations above 10  $\mu\text{M}$ , and the stimulation could be reversed or prevented by the addition of cysteine (50  $\mu\text{M}$ ) to the system. This is shown in the bottom line of Figure 53 a. The same effect was obtained if 2-mercaptoethanol or dithiothreitol replaced cysteine.

Adenine was not the only purine base to be affected in this manner, and stimulation of the binding of xanthine and guanine are shown in Figure 53, b and c respectively.  $\text{HgCl}_2$  was more effective in stimulating xanthine binding than adenine binding, as 10  $\mu\text{M}$   $\text{HgCl}_2$  stimulated significantly. The half-saturation value for  $\text{HgCl}_2$  when adenine was the bound ligand was about 40  $\mu\text{M}$ , and about 25  $\mu\text{M}$  when xanthine was bound. In both cases, the maximal stimu-

Figure 53: Stimulation of the binding of purine bases to Achlya germlings by  $\text{HgCl}_2$ . (a), adenine; (b) xanthine and (c) guanine.



lation was about 100-fold. In Figure 53 c, it is seen that guanine binding is only stimulated 3 to 4-fold.

Agents other than mercurials were also found to stimulate purine binding. Figure 54 a demonstrates that  $\text{Ag}^+$  was also an excellent stimulatory agent, although the total amount of adenine bound was only one-half that bound when  $\text{Hg}^{++}$  was the activator.  $\text{I}_2$  served as a comparatively weak activator, with the amount of adenine bound being doubled maximally when the concentration was varied from 0.1 mM to 0.25 mM (Figure 54 b). This is seen with osmotically-shocked cells only.

The diverse nature of the activators of purine binding described above suggests that the process involves interaction with thiol-reactive binding groups, rather than formation of complexes between the various agents and adenine.  $\text{HgCl}_2$ ,  $\text{I}_2$  and  $\text{Ag}^+$  are all excellent oxidizing agents for thiol groups. The reversal of the mercurial enhancement by addition of cysteine has previously been mentioned; this is used again in studies of the purine binding described below.

A time course study of adenine binding was carried out, in which the cells were exposed to labelled adenine for a period of 10 min and then treated with 0.1 mM  $\text{HgCl}_2$ . The results are shown in Figure 55 a. Adenine binding was dramatically enhanced immediately upon addition of  $\text{HgCl}_2$ , but the degree of enhancement was much lower than that observed in the kinetic-type studies where the cells are exposed to adenine and  $\text{HgCl}_2$  simultaneously. It

Figure 54: Stimulation of adenine binding to osmotically-shocked cells by (a)  $\text{Ag}^+$  and (b)  $\text{I}_2$ .

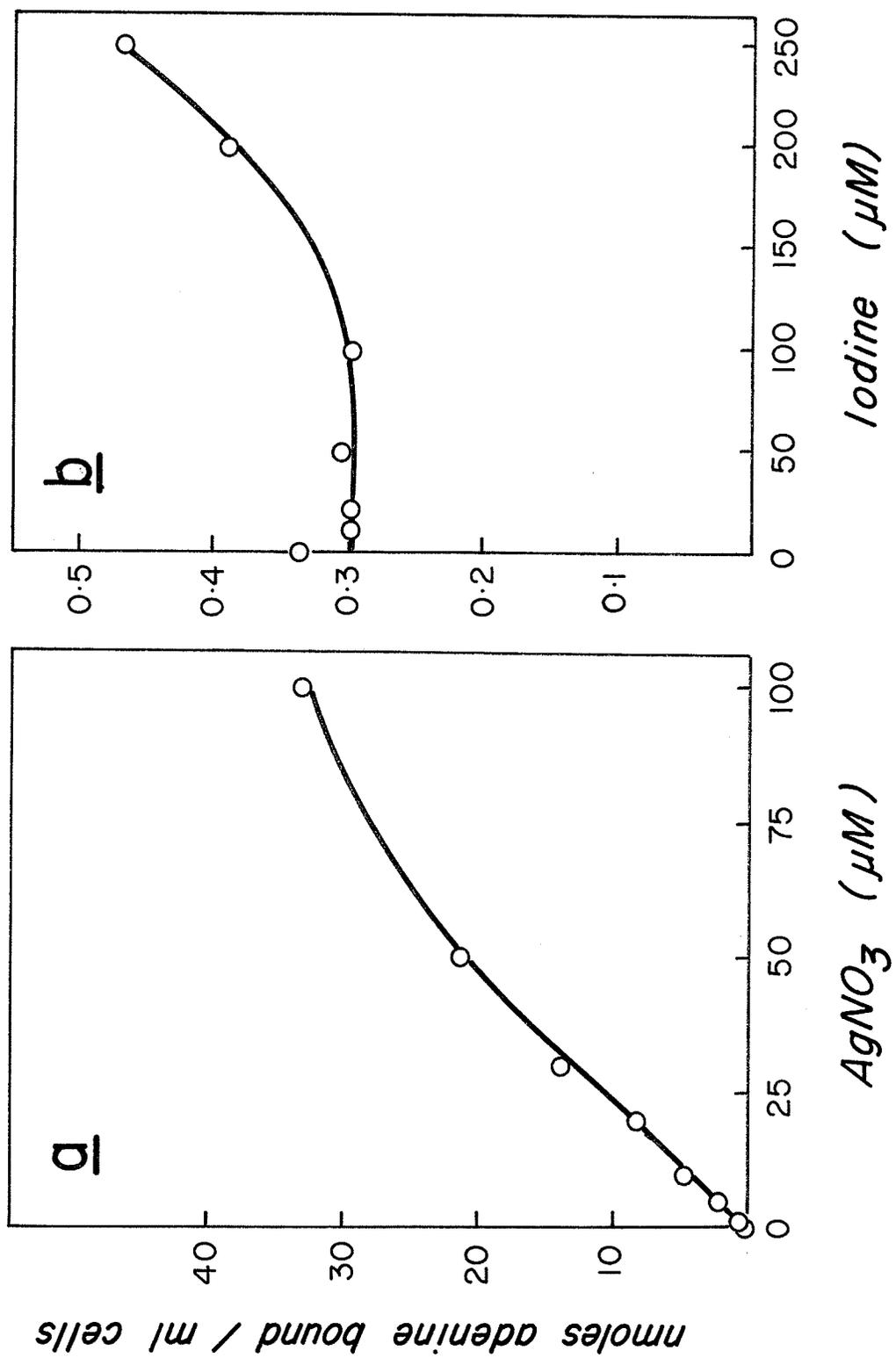


Figure 55: (i) Time-course analysis of the activating effect of  $\text{HgCl}_2$  on (a) non-shocked and (b) shocked cells pre-treated with  $50 \mu\text{M}$  adenine; and (ii) the effect of cysteine ( $0.1 \text{ mM}$ ) and excess unlabelled adenine ( $1 \text{ mM}$ ) on the release of  $^3\text{H}$ -adenine from the cell membrane surface.



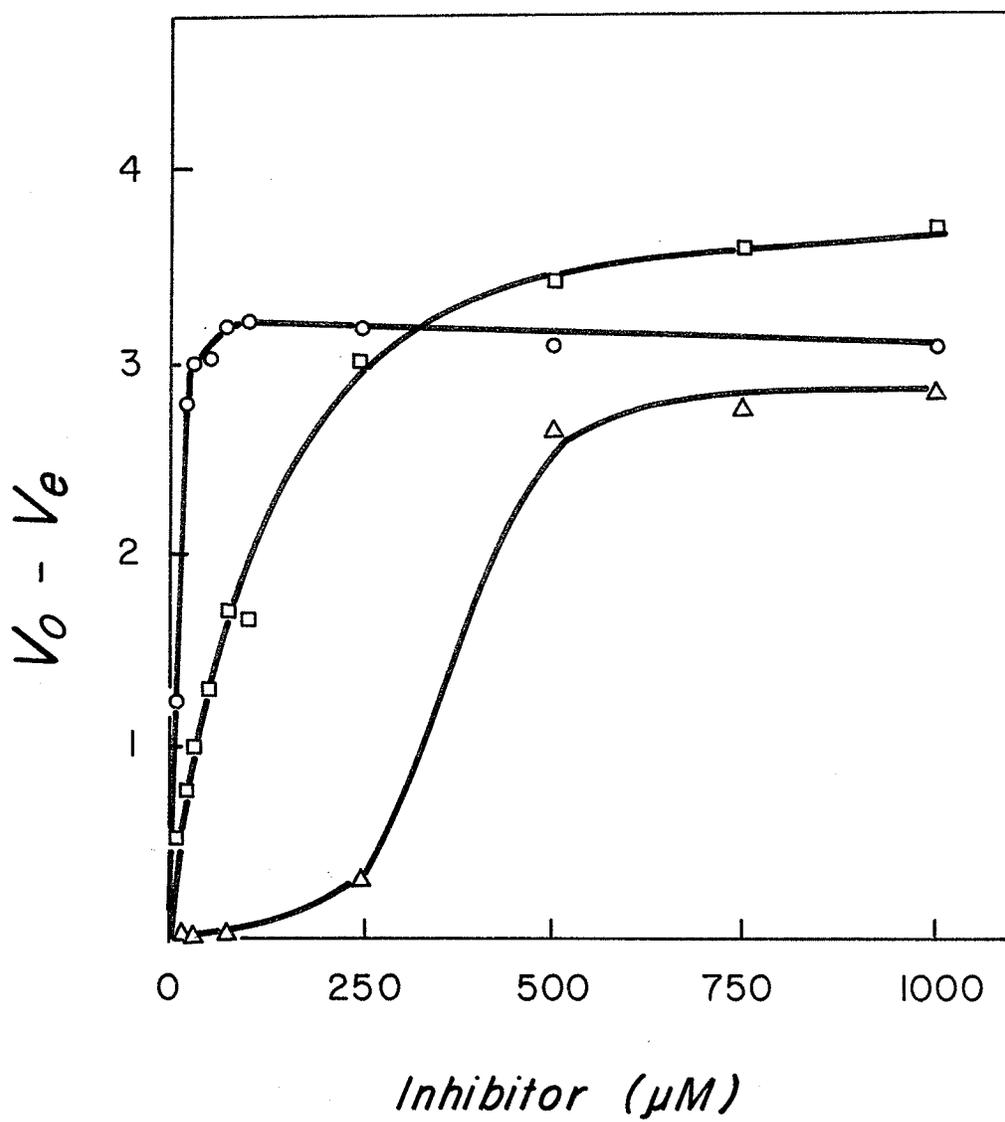
appears that the adenine binding sites are easily desensitized when the cells are exposed to large quantities of the base without the activator. The nature or function of this effect is not known. Addition of excess unlabelled adenine and 0.1 mM cysteine after 10 min of interaction with  $\text{HgCl}_2$  produced an immediate rapid release of bound adenine, as shown in the same figure. The same studies were carried out on osmotically-shocked germlings, with generally similar results as seen in Figure 55 b. While the 5-fold stimulation observed with normal cells reflects both uptake and binding, the 40-fold stimulation in the case of shocked cells represents true binding. On reversal by cysteine, the two cell types show the same rate of adenine outflow, confirming that the adenine binding occurs by the same system in both cell types.

Further tests to check the effect of order of addition of adenine and  $\text{HgCl}_2$  indicated that for the maximum stimulation to be observed, the cells had to be exposed to both substances simultaneously. Exposure of the cells to either  $\text{HgCl}_2$  or adenine prior to addition of the other resulted in the reduced level of stimulation seen in the experiment described above.

Purine binding and calcium translocation: These studies of  $\text{Hg}^{++}$  - stimulated purine binding by osmotically-shocked cells are of interest in understanding the complexities of the transport systems in this organism. Studies by LéJohn and associates

(Cameron & LéJohn 1973, LéJohn et al 1974) on the  $\text{Ca}^{++}$  transport and binding systems of this organism are pertinent in considering these results. Purines were found to release  $\text{Ca}^{++}$  that had been sequestered by a  $\text{Hg}^{++}$  - stimulated glycopeptide, as shown in Figure 56. The  $\text{Ca}^{++}$  release by purines only affects the  $\text{Ca}^{++}$  that is bound to cells under the influence of mercurials. Presumably, the rest of the  $\text{Ca}^{++}$  is taken up by the cell and is no longer available for free exchange as is the  $\text{Ca}^{++}$  sequestered on the membrane surface by the glycopeptide.

Figure 56: Stimulation of  $^{45}\text{Ca}^{++}$  release from  $\text{Hg}^{++}$ -activated Achlya cells by adenine ( $\Delta$ ), hypoxanthine ( $\square$ ), and xanthine ( $\circ$ ).  $v_0$  represents reaction rate in the absence of purine compound but fully activated by  $\text{HgCl}_2$ .  $v_e$  is the reaction rate in the presence of the specified purine compound at the concentration stated.



## DISCUSSION

Characterization of the Transport Systems

Transport of purines, pyrimidines and their nucleosides in Achlya appears to proceed by means of carrier-mediated processes, as shown by substrate saturability of the systems, the modifying influence of pH and temperature, and metabolic inhibitors in the case of the nucleosides.

Characterization of the nucleoside uptake systems indicate that they are separate and distinct from those of nucleic acid bases, and operate by an active mechanism. The competition plots for inosine, adenosine, and guanosine indicate that these compounds share a common carrier. Absence of inhibition by pyrimidine nucleosides of the guanosine transport system while adenosine transport is inhibited, implies that guanosine uses a purine-specific permease whereas adenosine enters both by this and a general purine-pyrimidine nucleoside carrier. Guanosine evidently does not use the general permease, as the transport of cytidine and thymidine proceed unhindered in its presence (Figure 50 a & b). In Figure 42 c, there is some indication that the uptake of thymidine may be a two component system, with affinities one order of magnitude different.

This is of interest as thymidine is the only nucleoside that is transported at an increased rate at temperatures above 30° (Figure 46).

Purines and pyrimidines are apparently taken up by a single common carrier system that operates by facilitated diffusion. That this system is not coupled to metabolic energy, and is distinct from the transport systems for nucleoside uptake, is of significance when the interaction between purine derivatives and a cell wall glycopeptide to which  $\text{Ca}^{++}$  also binds is considered. The binding reaction of adenine, as distinct from its uptake may explain why its interaction with other nucleic acid bases in transport is noncompetitive in nature (Figure 39). In cells not activated by mercurials or other agents, only a minor fraction of the adenine taken up is binding to the cell surface entity. This site is, however, fairly specific for adenine and closely related analogues, with guanine being ineffective as a ligand.

#### Purine Binding and Interactions with $\text{Ca}^{++}$

Activation effects: The activating influence of substances like  $\text{Ag}^+$ ,  $\text{Hg}^+$ , merthiolate, and  $\text{I}_2$  on both purine binding and  $\text{Ca}^{++}$  binding, the latter as reported by LéJohn et al (1974), signifies that thiol groups play a key role in these processes. The possibility that the stimulation of adenine binding, and the release

of  $\text{Ca}^{++}$  by the action of purines, might be due to chelation or complex formation between  $\text{Hg}^{++}$  and adenine is unlikely for several reasons. The different chemical properties of the activators make a chelation process unlikely. The fact that the effect on purine binding is relatively specific for only certain purine compounds also supports this, as nucleosides, nucleotides, and pyrimidines failed to show stimulated binding. Similarly, not all adenine analogs produced  $\text{Ca}^{++}$  release, and again nucleosides, nucleotides and pyrimidines were ineffective.

Exposure of intact cells to either  $\text{HgCl}_2$  or adenine before the second agent is added leads to a reduction in the stimulatory capacity of the activator. This indicates that simultaneous interaction of the two substances,  $\text{Hg}^{++}$  and adenine, or adenine with any of the other activators, with the cell is crucial. It may be that adenine by itself inactivates the receptor site on the cell unless the latter is interacting at the same time with an oxidizing agent. The significance of this effect is not clear, in terms of molecular interactions and cell regulation.

$\text{Ca}^{++}$  in transport: Previously Cameron and LéJohn (1972 a) suggested that  $\text{Ca}^{++}$  may act as a regulator of amino acid transport in Achlya. In those studies it was demonstrated that cycloheximide stops protein synthesis but not amino acid transport, indicating that the two processes are dissociated. Similarly, thymidine uptake was not initially interrupted by cycloheximide. A role of

$\text{Ca}^{++}$  distinct from effects on synthesis was thus indicated in this regulation. The studies presented here indicate that nucleoside transport is under similar  $\text{Ca}^{++}$  - related controls as is the transport of amino acids. Metabolic poisons are effective against both amino acid and nucleoside uptake, indicating that the systems are under metabolic control. Chelation of  $\text{Ca}^{++}$  by citrate, or the loss of a component of the  $\text{Ca}^{++}$  transport system by osmotic shock treatment, inhibits uptake of these metabolites. The role of  $\text{Ca}^{++}$  in maintaining active transport activity may involve a function in maintaining structural integrity of the membranes, an involvement in the function of the specific transport systems, or an effect through energy-linked processes of the cell, such as the activity of a  $\text{Ca}^{++}$  - stimulated  $\text{Mg}^{++}$  - ATPase.

Purine regulation of  $\text{Ca}^{++}$  transport: The results shown in Figure 56 illustrate the ability of purines to release  $\text{Ca}^{++}$  bound to  $\text{Hg}^{++}$  -activated cells. Concurrent studies in this laboratory on the  $\text{Ca}^{++}$  transport and binding system of Achlya (LéJohn et al 1974) demonstrated that the thiol-reacting binding capacity resided with a  $\text{Ca}^{++}$  - binding glycopeptide, which was released by osmotic shock treatment. The facilitated diffusion process of  $\text{Ca}^{++}$  uptake is apparently distinct from the binding of  $\text{Ca}^{++}$  as it continues in osmotically shocked cells (Cameron & LéJohn, unpublished results). It was proposed that the glycopeptide acts as a  $\text{Ca}^{++}$  sequesteror

in the cell wall complex (LéJohn et al 1974, Cameron & LéJohn, manuscript submitted for publication). If the glycopeptide does act as such a storage system, then a mechanism should exist that permits release of  $\text{Ca}^{++}$  when required for cell function. Purine compounds, particularly xanthine, were found to produce such a release. In examining the purines capable of producing this effect, a variety of cytokinins,  $\text{N}^6$ -(substituted)-adenines which are plant hormones, were tested, and a number were active (LéJohn et al 1974).

These observations suggest that the purine binding site observed in these studies reflects a cytokinin regulatory site for the  $\text{Ca}^{++}$  uptake system. The effect of mercurials and other thiol-reactive agents on both purine and  $\text{Ca}^{++}$  binding could be the result of mimicing a natural activator of the system, or of desensitizing a regulatory component. Further examination of the effects of cytokinins, particularly on transport, is presented in the next section of this thesis (Part IV).

## PART IV

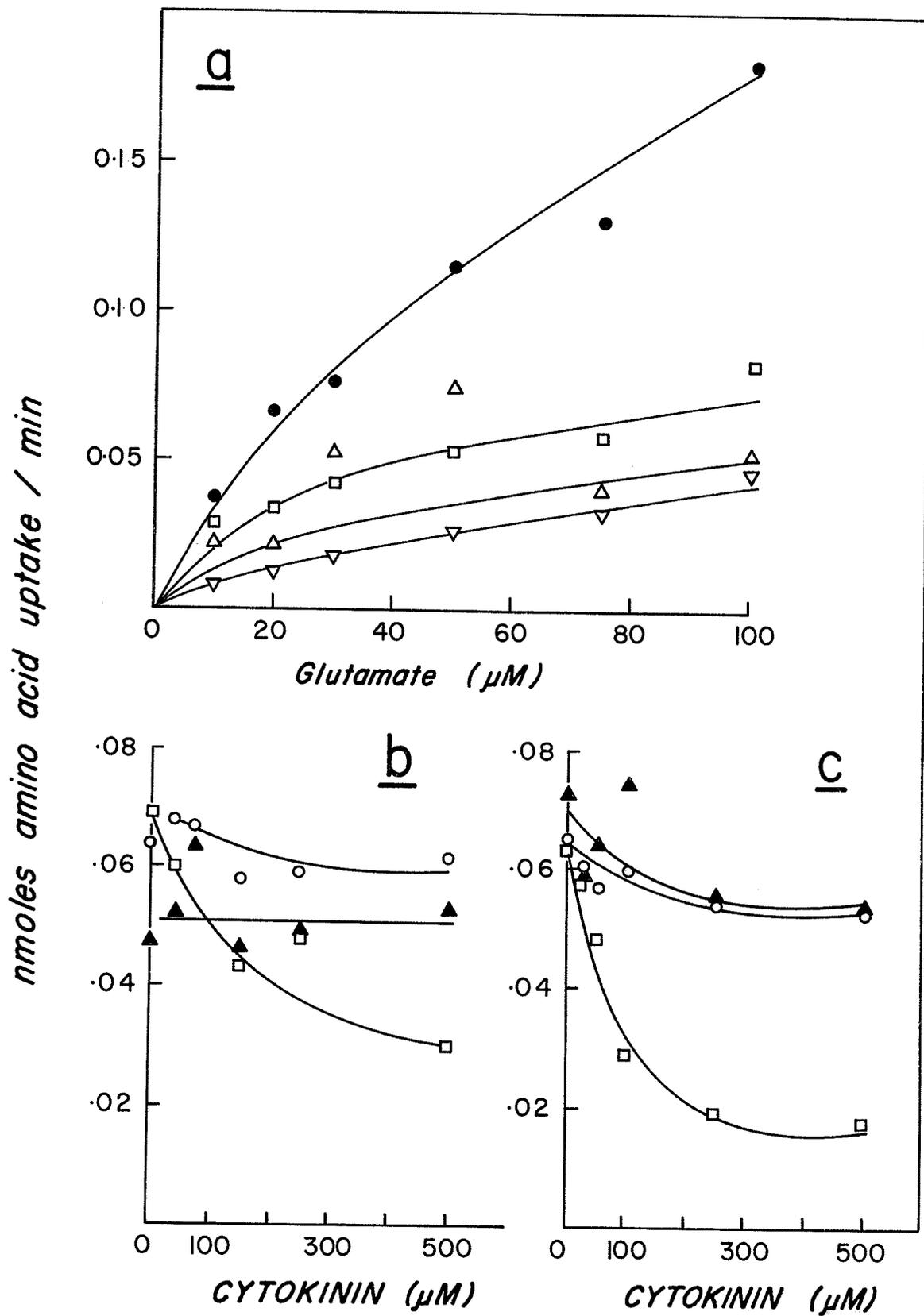
## CYTOKININ EFFECTS ON TRANSPORT

## RESULTS

A. Possible Transport Effects

The previous section mentions the effect of purines, particularly cytokinins, on the  $\text{Ca}^{++}$  transport system of Achlya. The integral part played by  $\text{Ca}^{++}$  in overall transport processes, as witnessed by the effects of citrate and osmotic shock treatment on metabolite uptake, raised the possibility that the cytokinin effect on  $\text{Ca}^{++}$  might in some way be transmitted to the level of metabolite transport. Some indication of support for the existence of such an effect came from studies on the effect of exogenous purines on glutamate uptake, in connection with induction studies on glutamate dehydrogenase. Under certain circumstances, adenine and other compounds were observed to inhibit uptake of glutamate (Figure 57 a). Subsequent examination of purine effects on uptake of glutamate and aspartate demonstrated that some compounds with cytokinin activity were particularly effective as inhibitors (Figure 57, b and c). As a result, a more detailed study of the effects of cytokinins on metabolite uptake was made.

Figure 57: Effects of purine compounds on uptake of glutamate and aspartate. (a) Glutamate uptake with adenine at (  $\nabla$  ), 1.5 mM; (  $\triangle$  ), 1.0 mM; (  $\square$  ), 0.5 mM; and (  $\bullet$  ), 0 mM. In (b) Glutamate uptake, and (c) aspartate uptake with (  $\circ$  ), xanthine; (  $\blacktriangle$  ), kinetin riboside; (  $\square$  ), isopentenyladenine varied. Amino acids fixed at 100  $\mu$ M.



### B. Effects on Nucleoside Transport

The effect of both isopentenyladenine and isopentenyladenosine on the uptake of adenosine was examined. Figure 58 a demonstrates that both compounds inhibit uptake, with the riboside form being more effective at equivalent concentrations of inhibitors. The Lineweaver-Burk replot in Figure 58 b shows that the isopentenyladenine inhibition is of a noncompetitive form. The pattern observed for isopentenyladenosine differs somewhat from that of isopentenyladenine, probably reflecting some degree of competition by the adenosine moiety for the transport site. As adenine is taken up by a system that is different from that of adenosine (previously described) no such interference from the adenine group would be expected. Adenine uptake is inhibited only slightly by isopentenyladenine, and in a competitive manner (Figure 59 a). Uptake of cytidine shows inhibition patterns with isopentenyladenine similar to those seen in adenosine uptake (Figure 59 b).

### C. Effects on Amino Acid Transport

The effects of cytokinins on amino acid uptake were studied more extensively because (a) of our interest in the induction of glutamate dehydrogenase and a related problem of "glucose effect" on its synthesis; and (b) it displayed a wider spectrum of transport effects than possible with nucleosides. Information regarding the

Figure 58: Uptake of adenosine; effects of isopentenyladenine;  
( X ), 250  $\mu\text{M}$ ; ( ■ ), 100  $\mu\text{M}$ ; ( ▲ ), 50  $\mu\text{M}$ ;  
( ● ), 0  $\mu\text{M}$ ; and of isopentenyladenosine; ( △ ),  
50  $\mu\text{M}$ ; ( □ ), 100  $\mu\text{M}$ .

Rate - concentration plot in (a), and double  
reciprocal plot in (b).

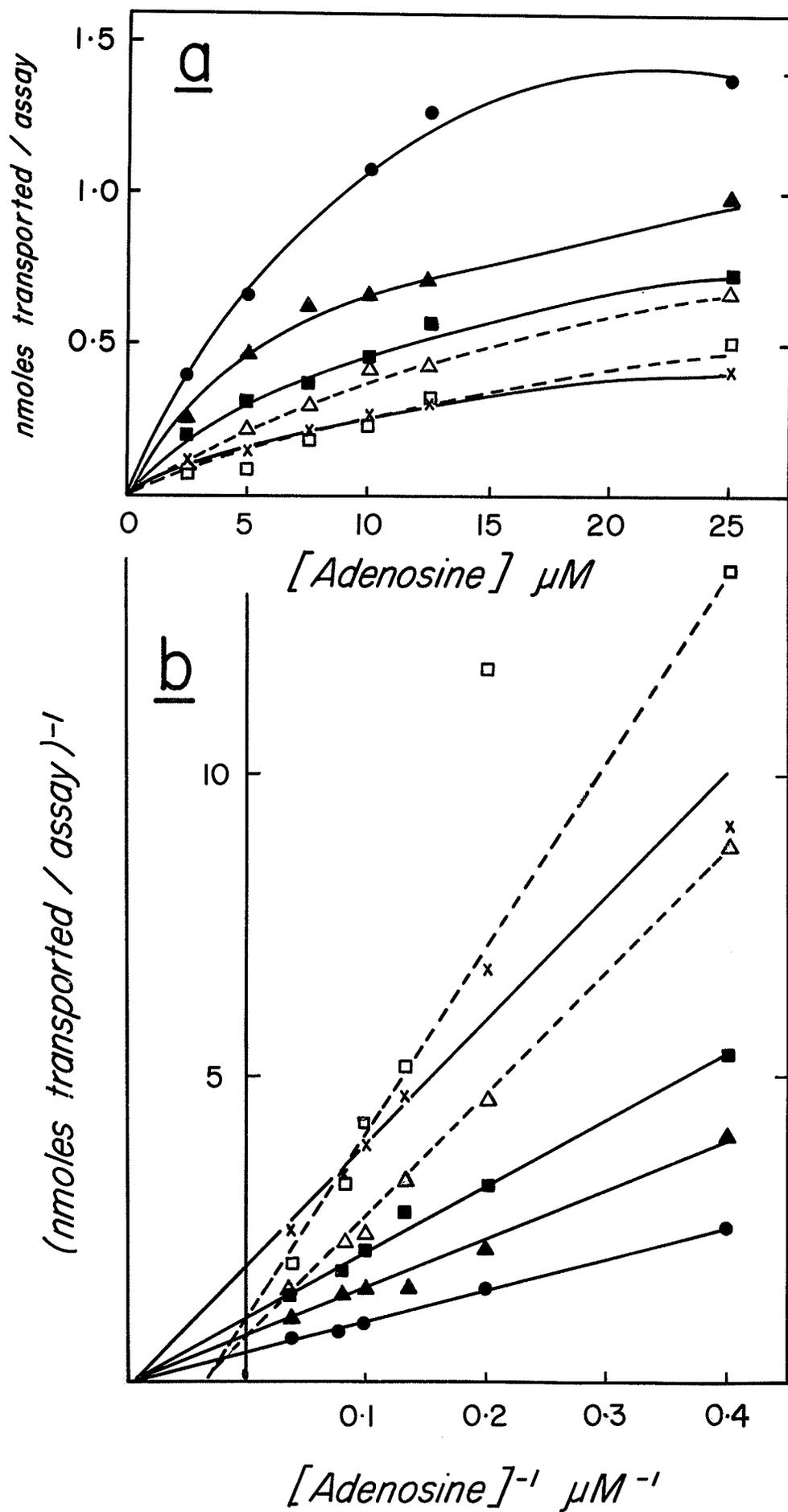
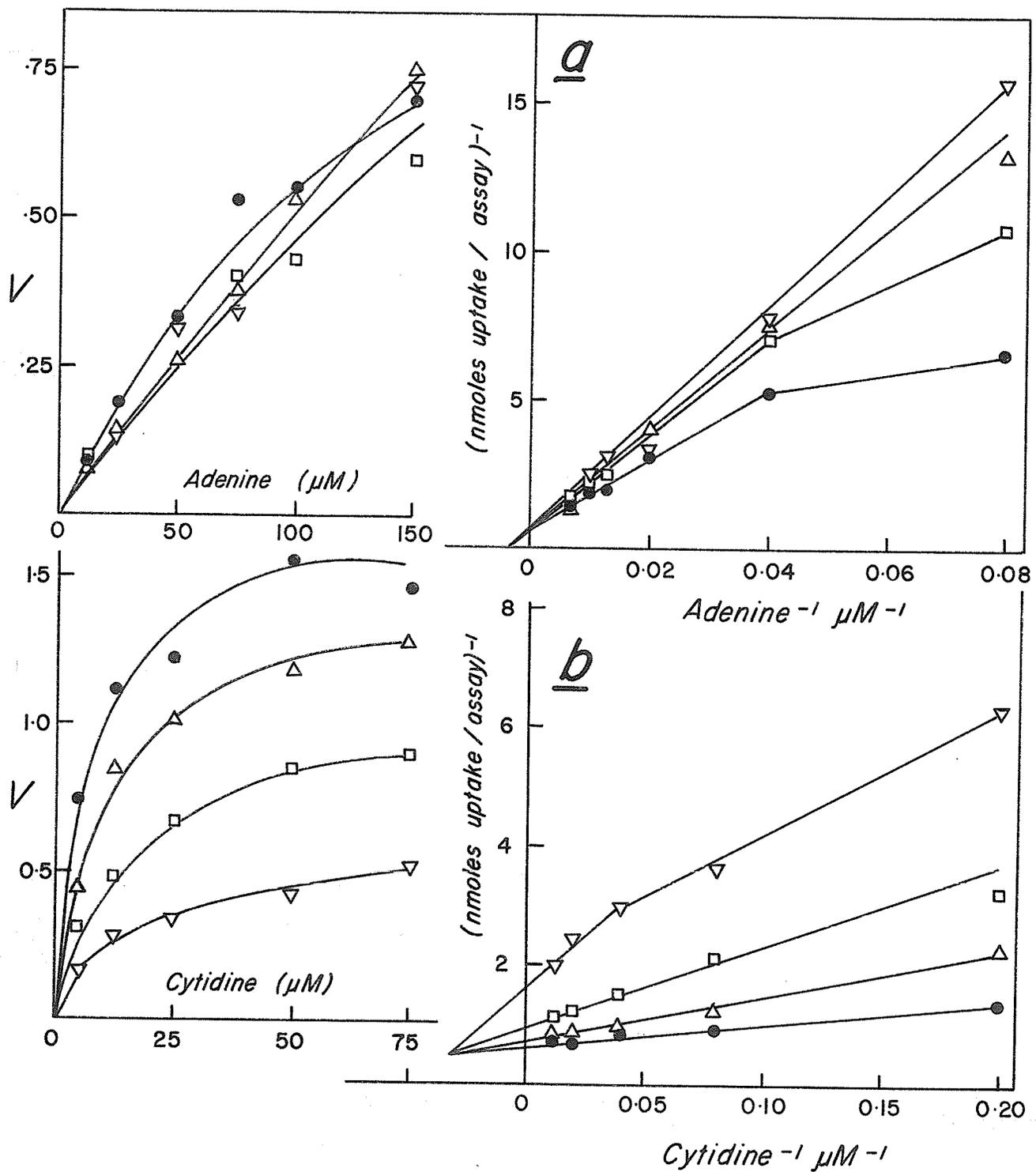


Figure 59: Effects of isopentenyladenine on uptake of adenine (a), and cytidine (b) by Achlya.  
Isopentenyladenine concentrations; (  $\nabla$  ),  
250  $\mu\text{M}$ ; (  $\square$  ), 100  $\mu\text{M}$ ; (  $\triangle$  ), 50  $\mu\text{M}$ ;  
(  $\bullet$  ), 0  $\mu\text{M}$ .



specificity and other characteristics of amino acid transport systems in Achlya is already available (Singh 1974).

1. Characterization of cytokinin effects: The active uptake of amino acids was effectively blocked by relatively low concentrations of isopentenyladenine, and the inhibition was concentration dependent. Figure 60 a shows the inhibition of methionine uptake by isopentenyladenine dissolved in DMSO. Isoleucine transport was studied with isopentenyladenine dissolved in water and in DMSO, and the effects were essentially similar (Figure 60 b). Increasing concentrations of DMSO (0 to 0.5%) alone, without cytokinin, had a slight stimulating effect on transport, probably due to increased permeability of the cell membrane. Since a solution of isopentenyladenine in DMSO produced the same effect as a water solution, and since DMSO does not seriously perturb transport, DMSO solutions could be used to add cytokinins to test systems without adding large volumes, and to study cytokinin-active compounds that are less soluble in water than isopentenyladenine.

Inhibition of uptake by isopentenyladenine and other cytokinins was immediate, occurring within 15 seconds of exposure of the cells to the cytokinin. This is shown in Figure 61, for the effect of 6-n-hexylaminopurine on histidine transport. Cells were exposed to histidine for 1 minute before adding 100  $\mu$ M 6-n-hexylaminopurine. At 15 second intervals cell samples were withdrawn, filtered, and washed. Histidine uptake was reduced by about 50 per

Figure 60: Inhibition of methionine (a) and isoleucine (b) transport by isopentenyladenine. In (b), the influence of DMSO on the transport activity is shown.

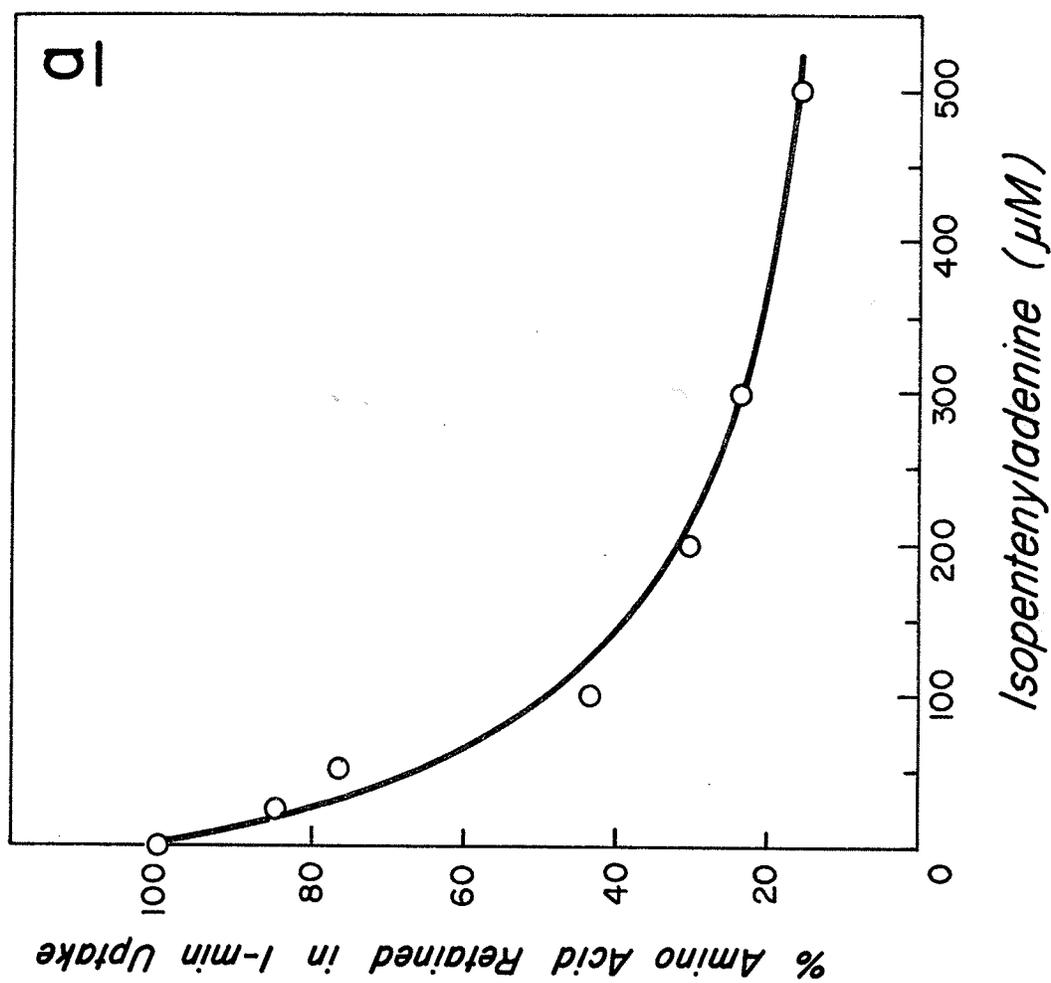
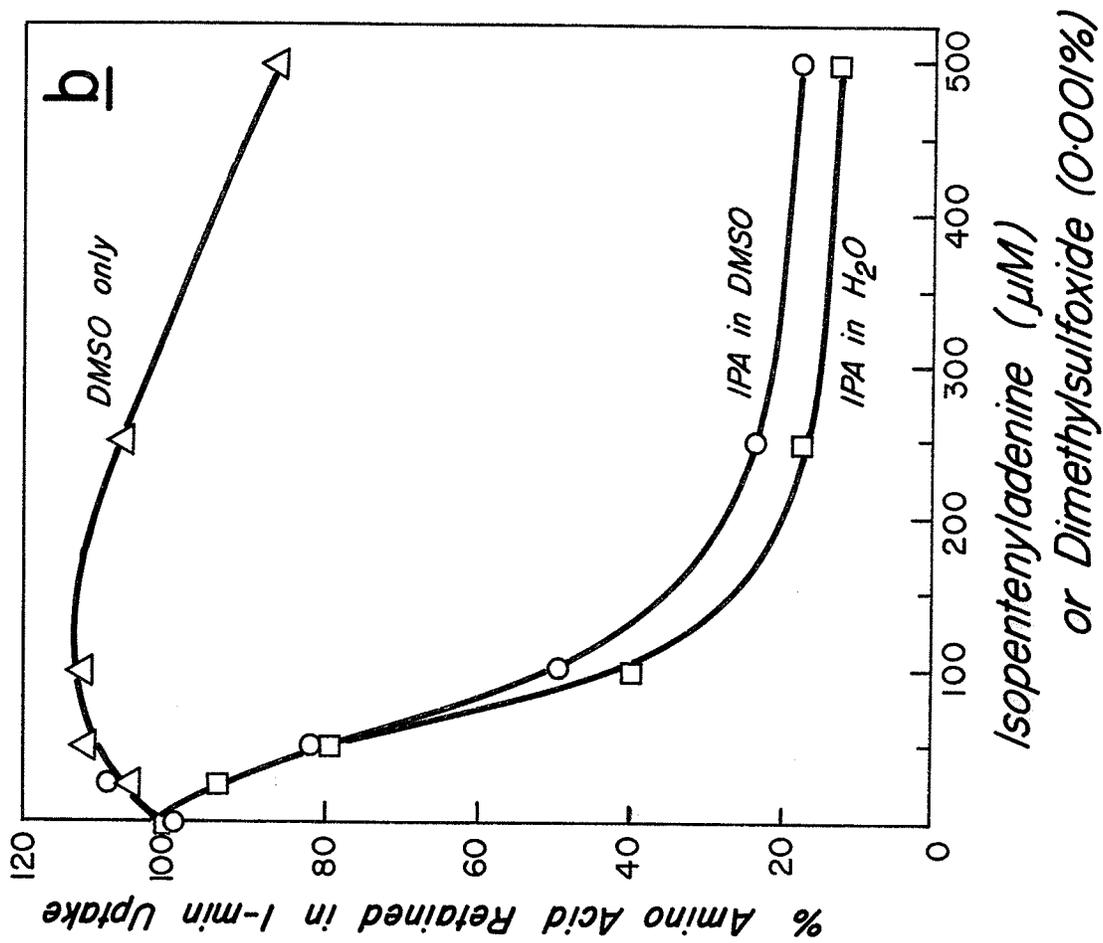
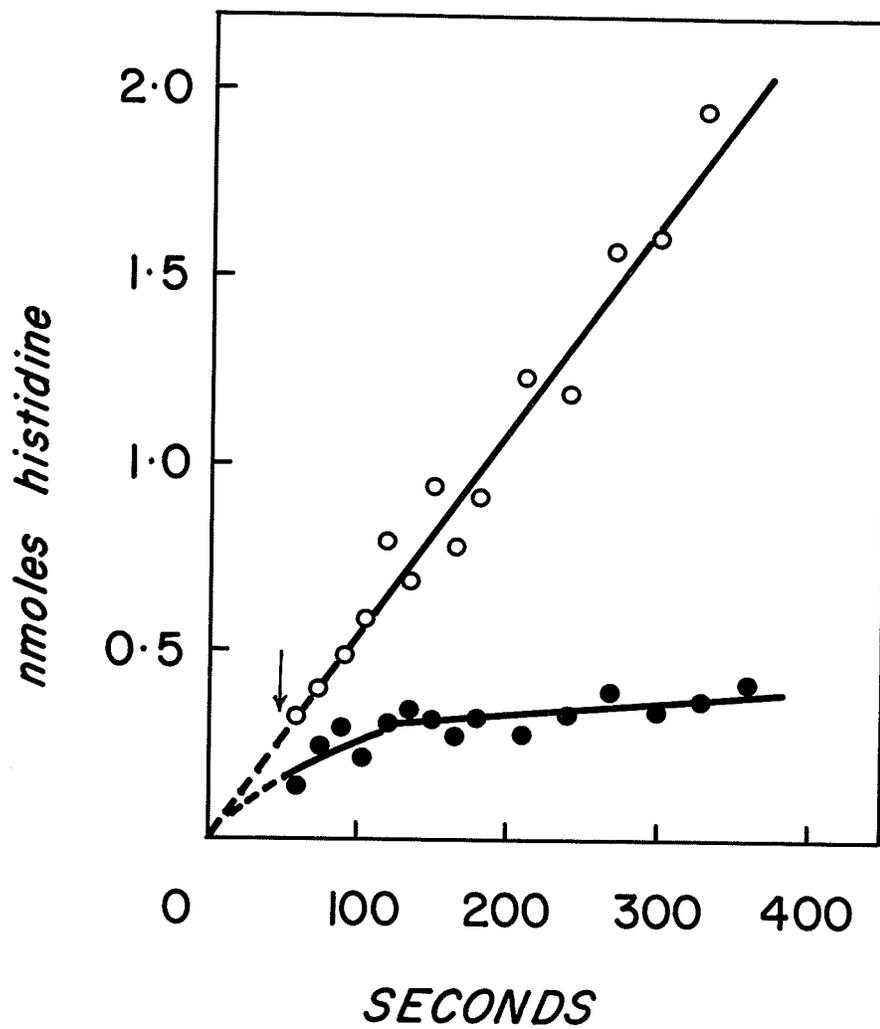


Figure 61: Effect of the cytokinin, 6-n-hexylaminopurine (n-HAP) on the rate of histidine transport by *Achlya germlings*. ( ● ), with n-HAP; ( ○ ), no n-HAP.



cent within 15 seconds in the cytokinin-treated culture. In the control culture, the transport rate increased linearly for 5 minutes without reaching saturation as cycloheximide in the transport medium effectively blocked incorporation of the precursor into proteins.

2. Kinetics of isopentenyladenine inhibition: Initial rates of uptake of methionine and isoleucine in the presence of isopentenyladenine were measured at several different concentrations of amino acids and cytokinins. The results, in Lineweaver-Burk form, are shown in Figure 62, a and b. In both cases, inhibition was noncompetitive. The effect of isopentenyladenine was to reduce the  $V_{\max}$  of amino acid transport without altering the  $K_m$ . Qualitatively similar data have been obtained in the case of other amino acids, six of which are illustrated in Lineweaver-Burk form in Figure 63. Some differences were observed in response to the cytokinin, particularly with regard to the effectiveness of a 50  $\mu\text{M}$  concentration.  $K_i$  values (inhibition constants) for isopentenyladenine estimated from intercept replots were approximately the same (70-150  $\mu\text{M}$ ).

3. Influence of pH on cytokinin inhibition: Transport of all amino acids in Achlya proceed by an active uptake system that depends on pH and temperature (Singh 1974). It was of interest to determine to what extent the inhibition by cytokinin was affected by pH changes.

Figure 62: Lineweaver-Burk plots of the inhibitory effect of isopentenyladenine on (a) methionine and (b) isoleucine transport by Achlya. The concentrations of isopentenyladenine used are specified in the figures.

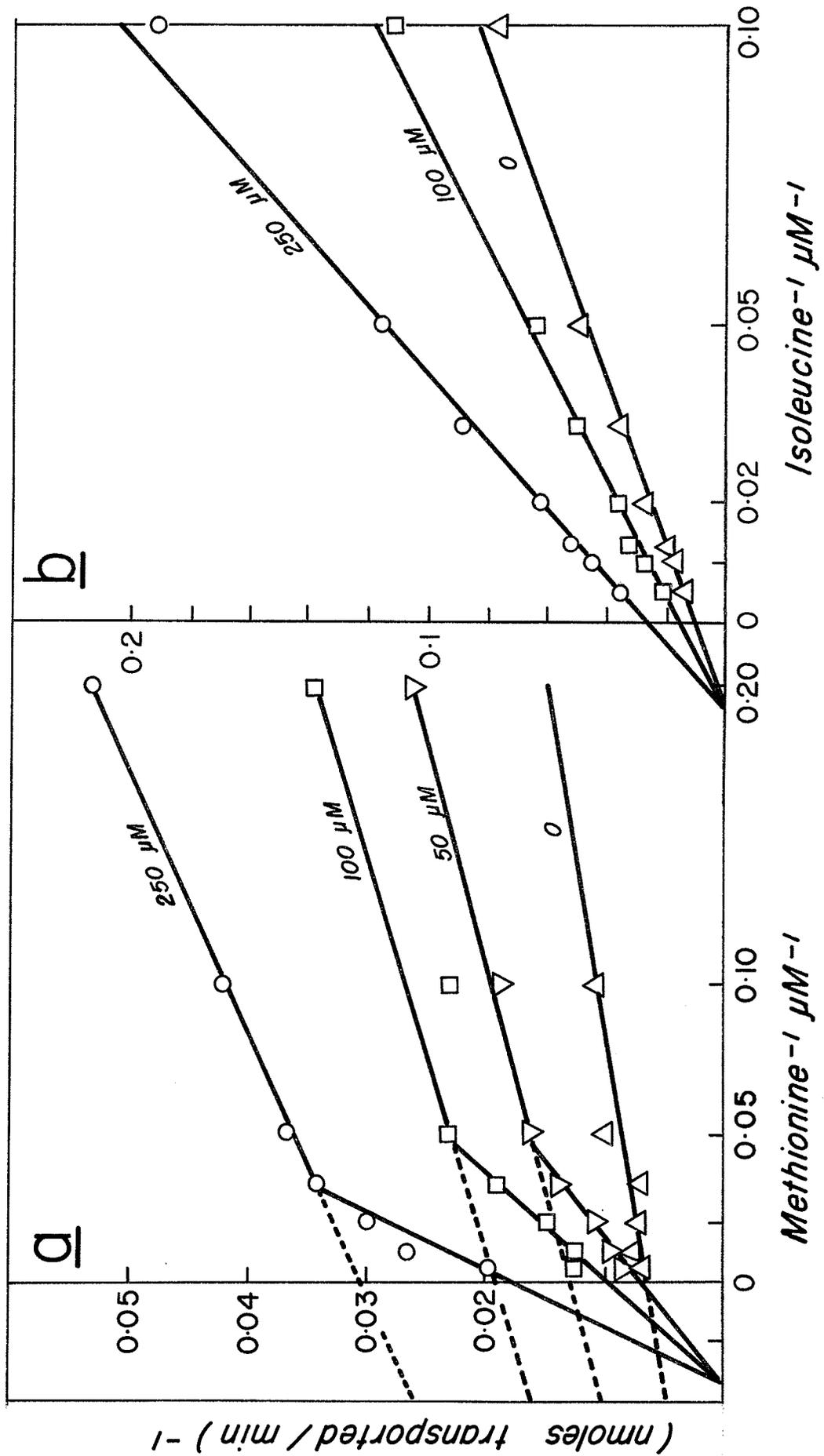
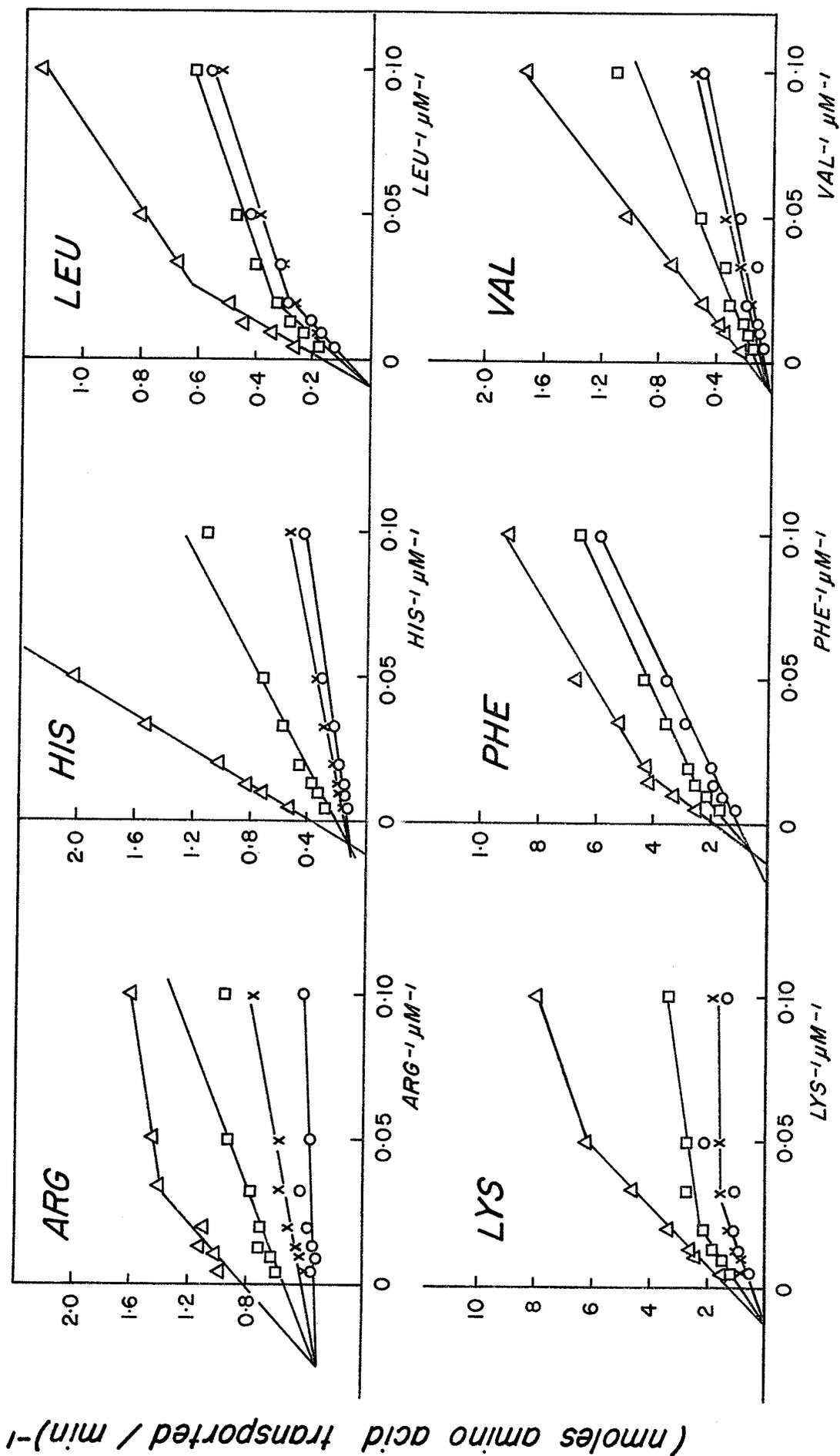


Figure 63: Lineweaver-Burk plots of isopentenyladenine effects on transport of arginine, histidine, leucine, lysine, phenylalanine, and valine. Isopentenyladenine concentrations; (  $\Delta$  ), 250  $\mu\text{M}$ ; (  $\square$  ), 100  $\mu\text{M}$ ; (  $\times$  ), 50  $\mu\text{M}$ ; (  $\circ$  ), 0  $\mu\text{M}$ .



The transport of all 20 amino acids was determined between pH 5.5 and pH 9, in the presence and absence of isopentenyladenine (200  $\mu\text{M}$ ). The results for 10 of the amino acids are shown in Figure 64. Most of the amino acids were inhibited in transport at all pH values, with pronounced inhibition between pH 6 and pH 7.5. The one exception to the inhibition of transport was tryptophan where uptake was enhanced by cytokinin. This was in agreement with earlier findings from kinetic studies, and the phenomenon will be discussed later.

#### D. Comparative Effectiveness of Cytokinins

About 30 different compounds have been examined for their effects on amino acid transport in Achlya, and it has been found that only compounds reported to display cytokinin activity in tobacco and soybean callus assays can inhibit the transport of 19 of the amino acids, and stimulate the transport of tryptophan. A survey made of the effects of 9 cytokinin compounds on uptake of 20 amino acids is shown in Table XIV. Spot checks were made using a single fixed amino acid concentration (100  $\mu\text{M}$ ) and two concentrations of the cytokinin (100 and 250  $\mu\text{M}$ ). Results were expressed as a percentage of uptake with no cytokinin. Most effective inhibition was seen with 6-n-hexylaminopurine, isopentenyladenine, and benzyladenine. 6-histaminopurine, a synthetic compound, appeared stimulatory in these studies, and the reason for this effect is not known.

Figure 64: Influence of pH on the inhibition of amino acid transport by isopentenyladenine. Control ( ○ ) and in the presence of 200  $\mu$ M isopentenyladenine ( ● ). The different amino acids used are specified in the figure.

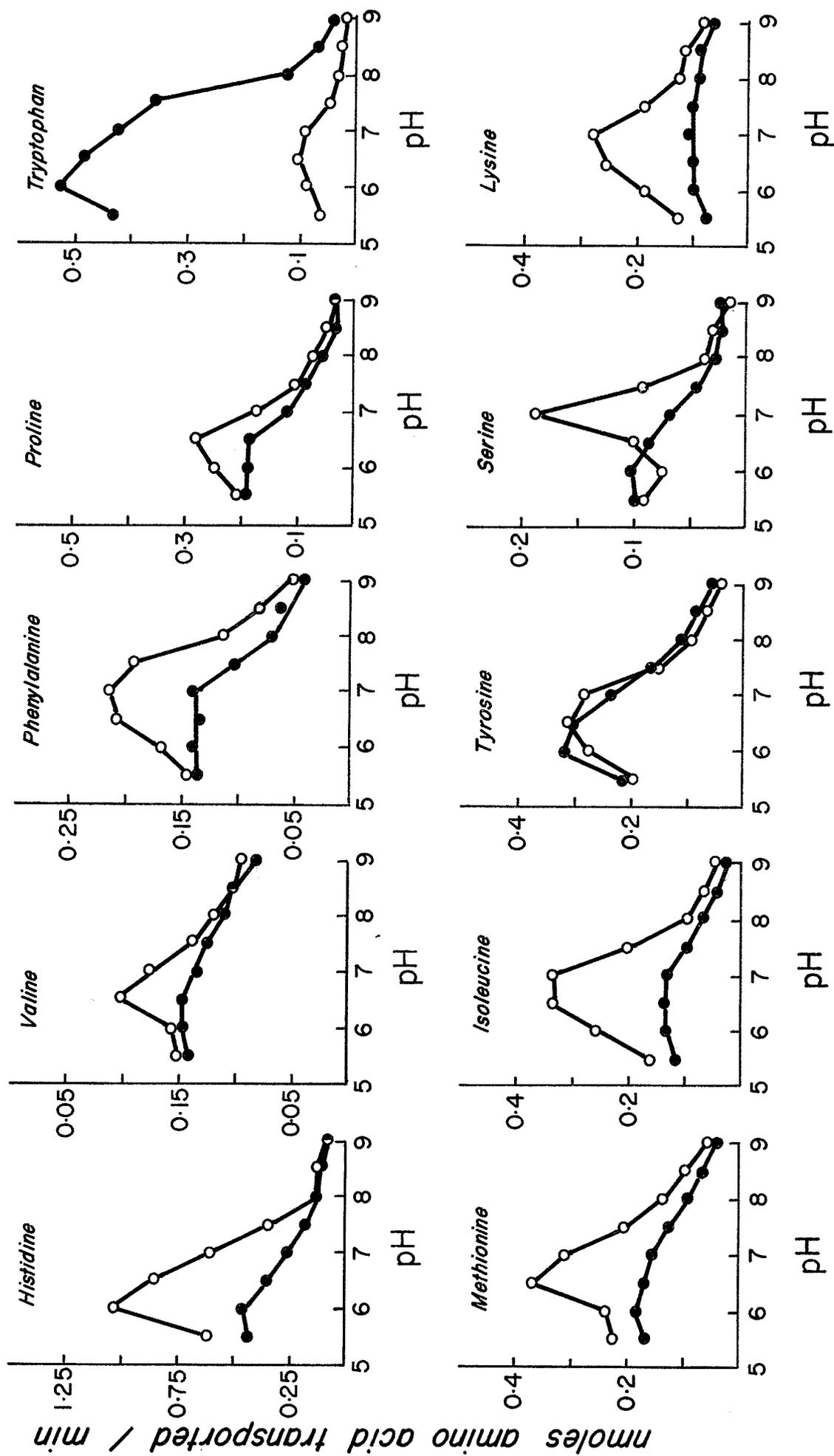


TABLE XIV

Effects of Various Cytokinin Compounds on Amino Acid Uptake

Uptake of amino acids, supplied at a fixed concentration of 100  $\mu$ M, is expressed as a percentage of uptake in a control test. The first row of figures refer to uptake at 100  $\mu$ M cytokinin concentration, the second row to uptake at 250  $\mu$ M.

Cytokinin:	HAP	IPA	BAP	MeAP	Me <sub>2</sub> AP	Zea	PMP	BxP	HisAP
ala	54.8 56.7	45.5 49.2	68.8 51.5	72.1 82.5	97.8 94.9	108.1 126.6	87.7 48.8	47.9 42.5	172.3 140.5
arg	- 26.3	- 31.8	- 31.7	- 105.9	- 62.4	- 112.6	- 36.3	- 41.6	- 97.16
asn	25.2 21.2	41.5 23.5	43.6 27.5	118.7 -	110.1 117.5	106.1 118.3	58.1 52.5	52.5 26.9	127.0 133.2
asp	30.9 35.0	45.8 30.1	45.2 27.6	105.0 92.4	93.0 95.4	104.0 112.6	63.0 23.5	61.9 25.0	111.7 114.4
cys	37.8 39.1	50.2 38.5	63.2 40.7	96.9 84.8	92.6 84.2	98.6 95.1	76.3 41.9	65.7 42.2	103.1 95.9
gly	56.9 63.7	74.9 53.2	72.2 65.7	164.8 145.3	153.4 84.2	204.1 193.5	104.4 53.5	87.8 77.2	176.0 209.3
glu	19.3 20.9	38.4 19.0	46.1 20.6	89.2 94.8	99.6 92.5	99.0 116.9	55.9 20.3	54.8 27.4	118.4 134.9
gln	18.2 12.8	39.1 18.3	42.6 16.5	96.0 108.6	108.6 104.6	88.8 -	50.0 -	45.7 -	105.4 113.6
his	16.7 13.8	29.8 6.1	34.2 15.0	95.6 -	99.4 80.8	85.0 78.9	51.8 18.8	42.0 20.0	81.0 69.2
ile	26.1 20.4	50.1 26.8	54.6 26.4	83.2 101.0	122.0 110.7	98.7 95.3	52.7 32.3	74.2 33.9	100.0 110.0
leu	39.3 39.1	56.9 46.4	69.5 52.1	88.1 110.9	75.4 117.1	130.6 116.5	77.8 53.9	78.2 49.9	89.3 124.1
lys	90.0 61.7	88.0 81.0	97.7 66.2	154.3 198.7	135.6 171.1	173.8 198.7	99.3 76.0	95.8 74.4	172.5 247.3
met	23.7 20.3	26.9 23.5	53.6 27.1	63.7 50.0	99.7 99.1	88.4 94.4	35.3 25.5	54.3 27.3	114.0 123.4

Table XIV Continued

	HAP	IPA	BAP	MeAP	Me <sub>2</sub> AP	Zea	PMP	BxP	HisAP
phe	30.9 25.8	54.4 33.0	55.5 34.9	100.4 98.1	106.0 104.4	103.8 109.8	64.4 39.0	65.6 39.2	116.0 112.7
pro	24.8 17.2	49.5 24.3	52.7 25.2	97.3 56.5	99.1 89.4	95.7 85.8	64.5 31.1	62.2 30.7	93.5 87.4
ser	60.0 60.3	68.8 50.5	83.7 53.3	152.6 166.5	115.0 176.3	190.2 182.7	110.5 55.7	92.5 50.9	188.3 205.3
thr	36.9 21.9	46.6 37.2	50.2 26.9	105.0 91.7	104.0 95.8	105.3 99.5	80.9 68.9	76.4 54.0	111.0 112.7
trp	83.8 53.4	80.0 80.1	81.9 83.3	120.3 101.1	120.7 119.1	116.6 114.6	92.5 72.7	91.9 77.2	133.8 134.3
tyr	32.5 23.7	55.7 35.6	63.4 37.8	93.6 118.3	114.5 115.7	118.6 129.1	79.4 42.1	72.1 48.1	123.8 125.5
val	20.2 13.3	37.4 22.0	42.6 20.4	88.2 92.9	94.0 94.4	90.7 112.0	55.3 25.6	63.7 22.5	104.6 103.8

Cytokinin abbreviations: HAP, hexylaminopurine; BAP, benzylaminopurine; IPA, isopentenyladenine; MeAP, methylaminopurine; Me<sub>2</sub>AP, dimethylaminopurine; Zea, zeatin; PMP, propylmercaptopyrine; BxP, butoxypurine; HisAP, histaminopurine.

In order to clarify the results obtained in the preliminary survey, a more detailed study of the effects of various cytokinins was carried out. The results of this study in the case of methionine are shown in Figure 65, for 10 of these compounds. The concentration of cytokinin-related compound that inhibited the transport of methionine by 50 per cent was taken as  $I_{50}$  for that agent. The values for all the agents used have been summarized in Table XV, although some were inert in the concentration range examined.

Both naturally-occurring and synthetic cytokinin-active compounds were effective as inhibitors of amino acid transport. Kinetin and its riboside, isopentenyladenine and isopentenyladenosine, zeatin, methylaminopurine, and dimethylaminopurine are the only naturally-occurring compounds known to have cytokinin activity that were tested. The nucleoside forms, kinetin riboside and isopentenyladenosine, were inactive. If anything, they had significant stimulatory effects at low concentrations up to 50  $\mu\text{M}$ . Cytokinin nucleosides were also inactive, and the possible requirement for hydrolysis of the sugar moiety for cytokinin activity has been discussed (LéJohn, manuscript submitted).

The structure of the various amino acids did not appear to affect the pattern of inhibition by cytokinins observed. Transport of a few amino acids, proline, cysteine, and glycine, was only weakly affected, and tyrosine uptake was unaffected, as was also seen in the pH studies of Figure 64. The ring structure of this compound may be resulting in interaction with the tryptophan uptake

Figure 65: Effect of various cytokinin-related compounds on the rate of transport of methionine by Achlya germlings. The abbreviations used in the figures are as follows: BAP, 6-benzyladenine; BMP, 6-benzylmercaptapurine; BxP, 6-n-butoxy-purine; CEMMP, 6-carbethoxymethylmercaptapurine; IPAd<sub>e</sub>, N<sup>6</sup>-( $\Delta^2$ -dimethylallyl)aminopurine; HpMP, 6-n-heptylmercaptapurine; HAP, 6-n-hexylamino-purine; HMP, 6-n-hexylmercaptapurine; Kn, kinetin; PMP, 6-n-propylmercaptapurine.

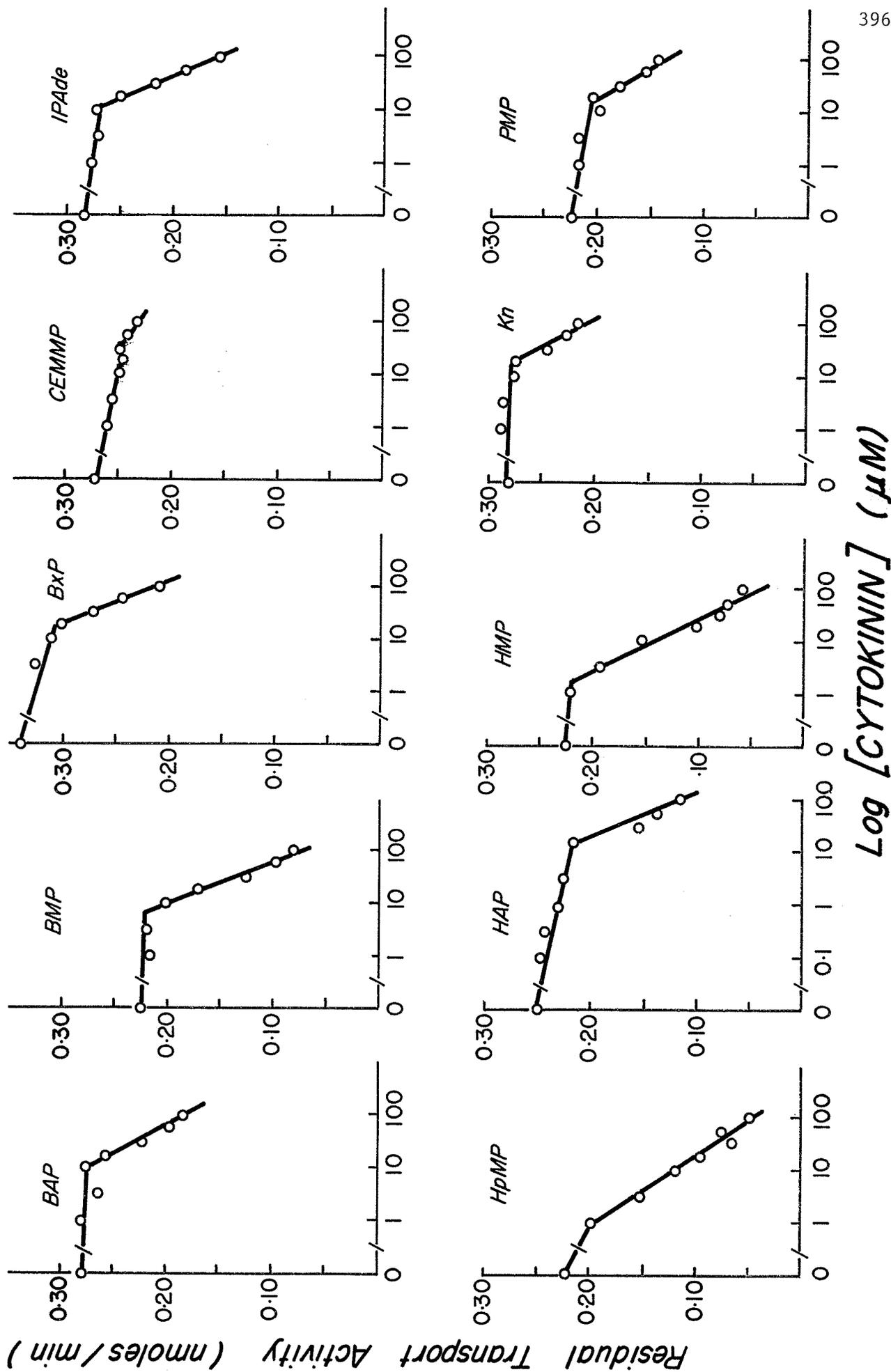


Table XV

A summary of the relative inhibitory effects of various cytokinin-related compounds on the rate of active transport of methionine by germinated sporangiospores of *Achlya*. Methionine was supplied at 50  $\mu\text{M}$  and the cytokinin-active compounds were varied from 1 nM to 100  $\mu\text{M}$ . Assay was conducted at pH 6.5. The  $I_{50}$  values were obtained from Lineweaver-Burk plots of  $1/(v_0 - v_e)$  against  $1/S$ , where  $v_0$  is the transport rate of methionine without cytokinin,  $v_e$  is the transport rate in the presence of cytokinin, and  $S$  represents cytokinin concentration. The  $I_{50}$  values were determined as  $K_i$  values from the Lineweaver-Burk plots.

Cytokinin-related compound	$I_{50}$ (M)
6-Amino-3-dimethylallylamine	- a
6-Benzylaminopurine	$3.3 \times 10^{-5}$
6-Benzylmercaptapurine	$1.3 \times 10^{-4}$
6-n-Butoxypurine	$4.0 \times 10^{-4}$
6-n-Butylmercaptapurine	$8.5 \times 10^{-5}$
6-Carboxymethylmercaptapurine	$> 10^{-3}$
6-(4-Carboxybutyl)mercaptapurine	$> 10^{-3}$
6-Carboxymethylmercaptapurine	$> 10^{-3}$
6-( $\Delta^2$ -Dimethylallyl)aminopurine	$4.1 \times 10^{-5}$
6-( $\Delta^2$ -Dimethylallyl)aminopurine riboside	- a
6-Dimethylaminopurine	$> 10^{-3}$
6-Ethoxypurine	- a
6-Ethylmercaptapurine	$2.0 \times 10^{-4}$
6-n-Heptylmercaptapurine	$1.8 \times 10^{-5}$
6-n-Hexylaminopurine	$1.5 \times 10^{-5}$

Table XV Continued

Cytokinin-related compound	I <sub>50</sub> (M)
6-n-Hexylmercaptapurine	3.5 x 10 <sup>-5</sup>
6-Histaminopurine	- a
6-bis-(β-Hydroxyethyl)aminopurine	- a
6-Isopropoxypurine	- a
Kinetin	8.0 x 10 <sup>-5</sup>
Kinetin riboside	- a
6-Methoxypurine	- a
6-Methylaminopurine	- a
6-Methylmercaptapurine	2.5 x 10 <sup>-4</sup>
6-n-Propoxypurine	> 10 <sup>-3</sup>
6-n-Propylmercaptapurine	1.3 x 10 <sup>-4</sup>
Zeatin	2.1 x 10 <sup>-4</sup>

<sup>a</sup>: signifies inactive

system, although phenylalanine, which shares a general aromatic permease (Singh 1974), is not affected. The effects of inhibition by isopentenyladenine on uptake of the amino acids are summarized in Table XVI, from values obtained from Lineweaver-Burk plots.

#### E. Tryptophan Interactions

The studies summarized in Table XVI, and shown in the pH studies of Figure 64, demonstrated that tryptophan transport alone was enhanced by cytokinins. Kinetic analysis of the interaction of tryptophan, cytokinin, and the cell is shown in Figure 66a. At low concentrations of tryptophan, cytokinin enhanced the rate of uptake, with the cytokinin effect becoming significantly reduced at higher tryptophan concentrations. This Lineweaver-Burk plot indicates that cytokinin and tryptophan were interacting with the same entity. A similar result was obtained for uptake of the tryptophan analogue, IAA (Figure 66b). The significance of this in relation to the  $\text{Ca}^{++}$ -binding glycopeptide of Achlya is considered later.

#### F. Isopentenyladenine Transport and Binding

In view of the effects of isopentenyladenine on transport, and the complexities of tryptophan and cytokinin interactions, it became of interest to examine the relationship of isopentenyladenine with the cell. Radioactively labelled isopentenyladenine was ob-

TABLE XVI

Relative inhibition of the transport of the L-isomers of the 20 common amino acids into the germinated sporangiospores of *Achlya* by the cytokinin, isopentenyladenine. Kinetic studies of amino acid transport were conducted with the concentration of amino acids varied from 1  $\mu$ M to 200  $\mu$ M, and the rates of amino acid transport determined in the presence and absence of 200  $\mu$ M isopentenyladenine. The  $V_{\max}$  values were determined from Lineweaver-Burk plots of the uptake rates under the two conditions. The expression  $V_{\max}^0$  represents the maximal reaction rate determined on the ordinate of the Lineweaver-Burk plot when cytokinin is not present, and  $V_{\max}^e$  the maximal rate of transport when cytokinin is present.

Amino Acid	$V_{\max}^0$ (nmoles/min)	$V_{\max}^e$ (nmoles/min)
Alanine	0.5	0.33
Arginine	2.2	0.87
Asparagine	0.67	0.50
Aspartic acid	0.67	0.30
Cysteine	-	-
Glycine	4.0	1.25
Glutamine	10.0	2.80
Glutamic acid	-	-
Histidine	13.33	3.33
Isoleucine	10.0	2.22
Leucine	7.5	1.33
Lysine	2.5	1.0
Methionine	20.0	3.3
Phenylalanine	10.0	3.3
Proline	6.67	1.4
Serine	4.0	1.1

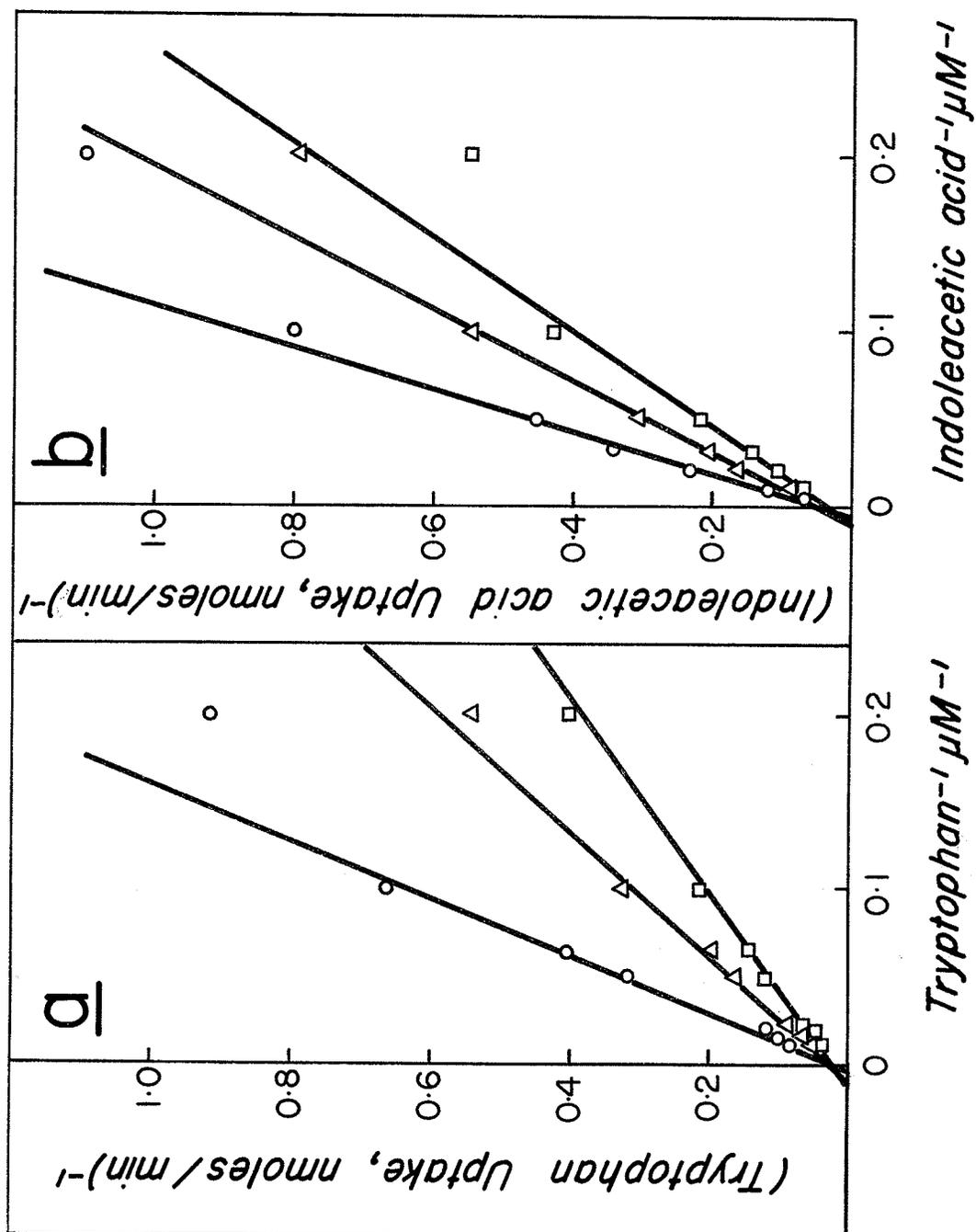
Table XVI Continued

Amino Acid	$V_{\max}^o$	(nmoles/min)	$V_{\max}^e$	(nmoles/min)
Threonine		10.0		2.86
Tryptophan		3.3		30.0 **
Tyrosine		6.6		2.0
Valine		6.6		1.43

\*\* These are not true  $V_{\max}$  values, they are relative rates at less than saturating concentration of tryptophan.

Figure 66: Enhancement of (a) tryptophan and (b) indole-acetic acid uptake by isopentenyladenine at concentrations of (  $\square$  ), 100  $\mu\text{M}$ ; (  $\triangle$  ), 50  $\mu\text{M}$ ; (  $\circ$  ), 0  $\mu\text{M}$ .

Plots are Lineweaver-Burk form.



tained and prepared as described in Materials and Methods, and uptake was examined in the same manner as for other metabolites. The uptake rate was concentration dependent, but the rate-concentration plot, shown in Figure 67, was not a typical saturation curve. In studies to determine the fate of isopentenyladenine taken up by the cells, it was found that the recovery of radioactivity in perchloric acid extracts was extremely low, even after 30 minutes exposure to the cytokinin. This suggests that the isopentenyladenine seen to be absorbed is taken into the cells very slowly if at all. Some problems were discovered with high background radioactivity resulting from binding to filters, but removal of the cells from the filters before determining radioactivity showed that cytokinin binding to the cells does in fact occur (Figure 68).

The effects of various compounds on the binding of isopentenyladenine were examined. Another cytokinin analogue, 6-n-hexylaminopurine, strongly reduced binding, but adenine was only weakly effective (Figure 69 a). Cyclic AMP was found to be an effective inhibitor, and a more formal kinetic study revealed that the inhibition was competitive (Figure 69 b). Non-cyclic adenine nucleotides, cyclic GMP, tryptophan, and IAA were ineffective. Theophylline and caffeine were not significantly inhibitory when used at the same concentrations as cyclic AMP.

Figure 67: Isopentenyladenine absorption by Achlya  
germlings.

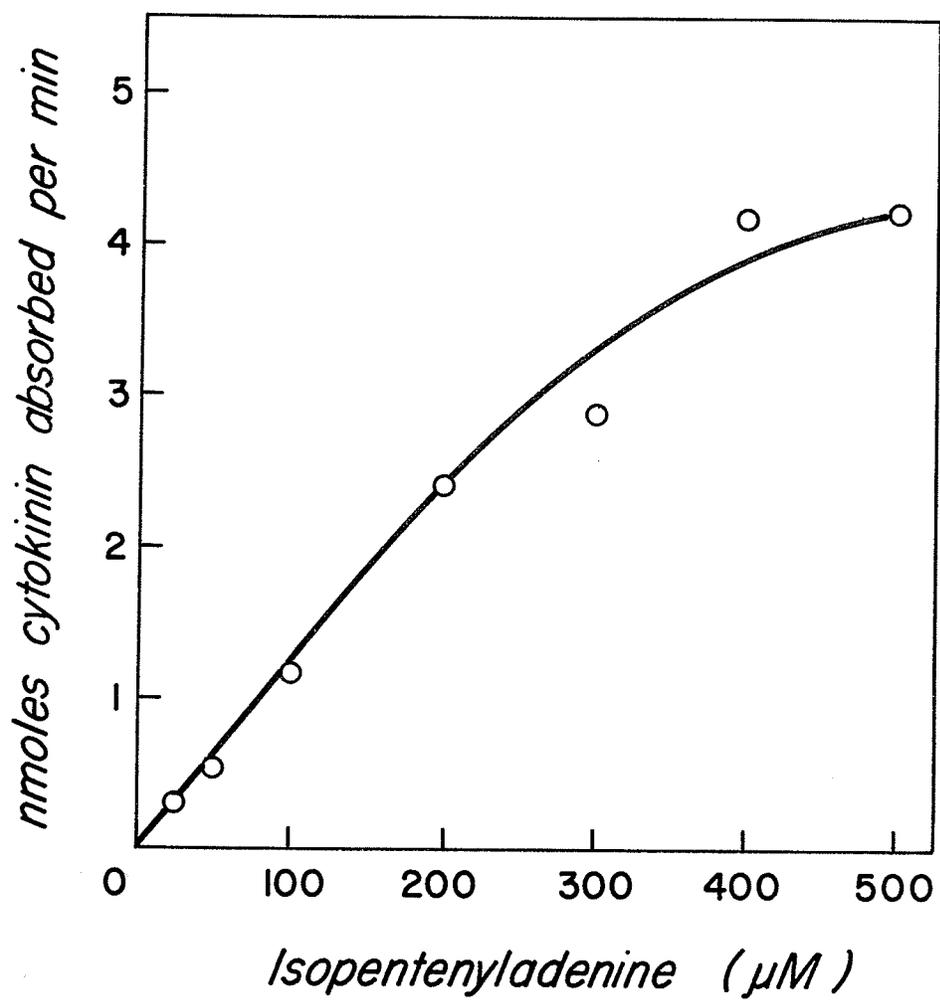


Figure 68: Isopentenyladenine binding to filters and Achlya cells. ( ■ ) represents a series cells and filters measured together, while ( ● ) represents the total radioactivity of a second series of cells ( ○ ) and scraped filters ( X ) measured separately. ( ▲ ) represents a duplicate series of scraped cells.

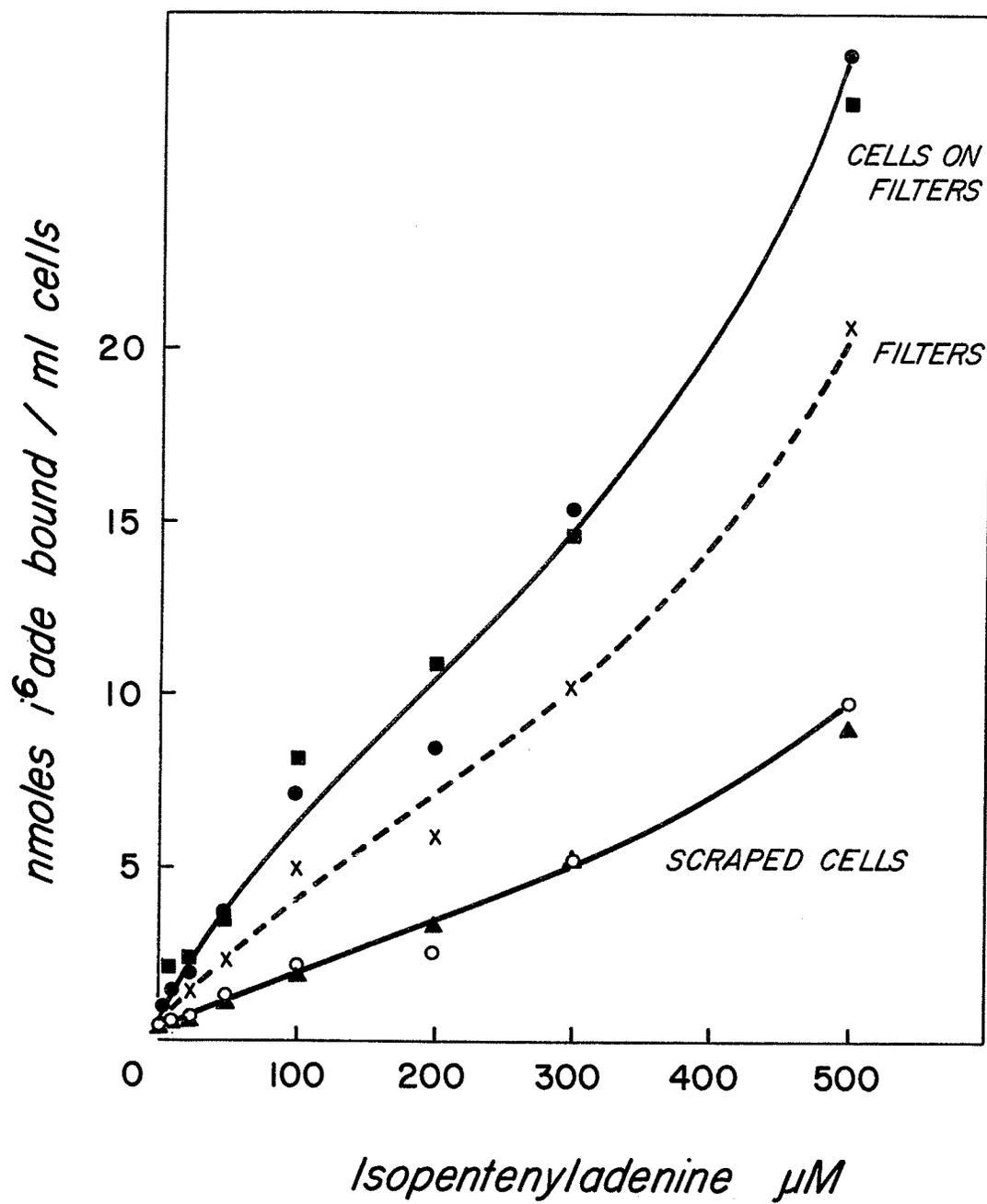
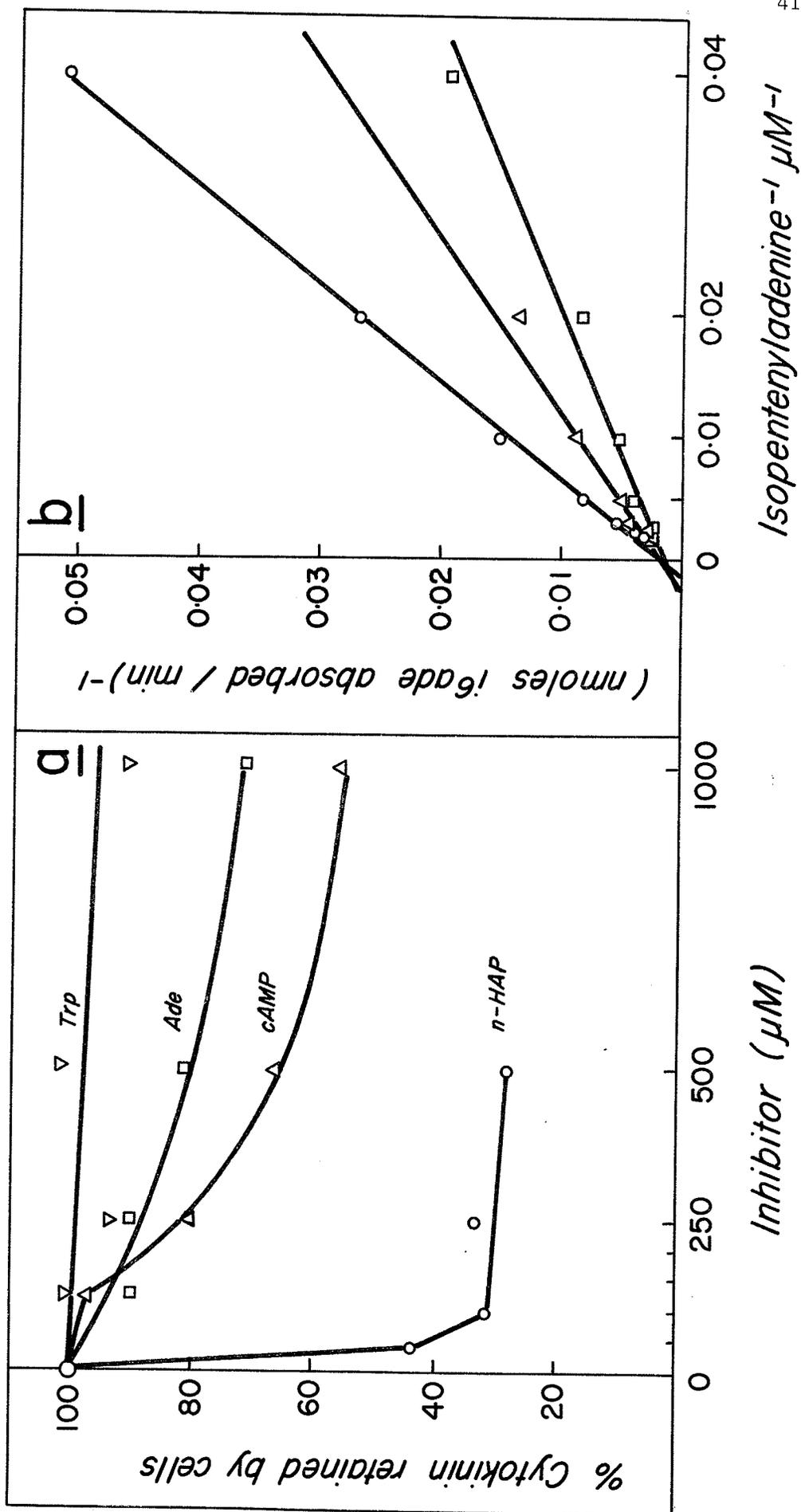


Figure 69: Isopentenyladenine absorption by Achlya germlings;  
(a) competition between isopentenyladenine and  
(  $\circ$  ) 6-n-hexylaminopurine; (  $\triangle$  ) cyclic AMP;  
(  $\square$  ) adenine; and (  $\nabla$  ) tryptophan. (b)  
Lineweaver-Burk plots of cyclic AMP inhibition  
at (  $\circ$  ), 1 mM; (  $\triangle$  ) 0.1 mM; and (  $\square$  ), 0 mM.



## DISCUSSION

### Inhibition of Transport

Purine compounds with cytokinin activity in higher plants have been found to inhibit active transport processes in Achlya. Effects on nucleoside and amino acid uptake have been reported here, and the effects on sugar transport have been examined by S.H. Goh and H.B. LéJohn (manuscript in preparation). Facilitated diffusion processes, such as for nucleic acid bases, are not affected. The inhibition of active uptake is a concentration dependent process, and the response to exposure to cytokinins is immediate. Kinetic studies show noncompetitive inhibition for both nucleosides and amino acids. The inhibition is pH-mediated, with pronounced inhibition only at pH values less than 7.5. The exception to inhibition of uptake of amino acids occurs in the case of tryptophan, where a stimulatory effect is observed.

### Relative Effectiveness of Cytokinins

A variety of cytokinin compounds were tested for their relative effectiveness as inhibitors of amino acid uptake. From these studies (Tables XIV to XVI), and other studies assessing the procedure as a bioassay (LéJohn, manuscript submitted), the relative

order of effectiveness was established as: isopentenyladenine > benzylaminopurine  $\approx$  (kinetin  $\approx$  zeatin) > hexylaminopurine > propylaminopurine. Other compounds, dimethylaminopurine, methylaminopurine, propoxyaminopurine, isopropoxyaminopurine, butoxyaminopurine, and methoxyaminopurine, were less potent. Triacanthine, the 3-isomer of isopentenyladenine, was inactive as were the riboside forms of the cytokinin bases. All effective cytokinins strongly inhibited metabolite transport between  $10^{-6}$  M and  $10^{-3}$  M, while stimulation of tryptophan uptake by the most active compounds could be detected at  $10^{-10}$  M (LéJohn, manuscript submitted).

#### Tryptophan Stimulation and Interactions

Analysis of the stimulatory effect of isopentenyladenine on tryptophan uptake demonstrated that this amino acid and the cytokinin were interacting with the same component (Figure 66 a), as was also the case for IAA (Figure 66 b). Both of these substances have been found to bind to the cell wall-membrane localized glycopeptide which also binds cytokinin (Cameron & LéJohn, manuscript submitted), and it is thought that this might be one of the sites of interaction. Both tryptophan and isopentenyladenine have been shown to reduce  $\text{Ca}^{++}$  binding by this glycopeptide (Cameron and LéJohn, and LéJohn, manuscripts submitted), perhaps by competition for binding sites. The possibility that auxin and cytokinin could act synergistically to release  $\text{Ca}^{++}$  from the glycopeptide suggests that resulting alterations in the cell wall and membrane, and increases in internal

$\text{Ca}^{++}$  levels, may be mechanisms by which plant hormones regulate cell activities. The effects of cytokinins on active transport processes in Achlya may reflect such alterations in membrane structure, or effects of  $\text{Ca}^{++}$  concentrations on cell regulatory processes.

It is not known whether the rapid interaction of isopentenyladenine with the cell and the immediate effects resulting from exposure to the cytokinin are solely the response to binding at the glycopeptide, or whether another surface binding site is also involved.

#### Implications

The effect of cytokinins in retarding amino acid uptake while stimulating specifically the transport of IAA and its amino acid analogue, tryptophan, can provide the basis for a bioassay of cytokinin concentrations, and the detailed procedures for such an assay have been described by LéJohn (manuscript submitted). Such an assay is more rapid than many previously used (see Table II), and provides for examination of both a positive and a negative response to the cytokinins tested.

The close correlation between the degree of response to various cytokinins by Achlya and by such systems as the tobacco callus bioassay (Skoog & Armstrong 1970) suggests that these plant hormones may well play a role in physiological regulation in this group of fungi. It is of interest that the cytokinin effect described here

has only been observed in members of the Oömycetes (LéJohn, unpublished data). For example, the active transport of metabolites by Blastocladiella emersonii, a taxonomically distinct aquatic mould, is not affected by either isopentenyladenine or hexylaminopurine. Consistent with the absence of a cytokinin effect in this organism is the observation that adenine and other nucleic acid bases appear to be transported by an active system, rather than the facilitated diffusion process described for Achlya (LéJohn and Stevenson, unpublished results).

Several species of Achlya as well as other members of the Saprolegniales and Peronosporales appear to be agents of root and grain rot (Webster et al 1970). It is of significance that roots and seeds are particularly rich in cytokinins (Kende 1971), as pathogens affecting such plant organs must either tolerate high levels of these substances, or have a means of detoxifying them. At the concentrations found in plant tissues, these fungi must not be adversely affected. In fact, at very low concentrations ( $10^{-10}$  M to  $10^{-7}$  M) cytokinins appear to protect Achlya germlings against rapid degeneration through starvation (LéJohn, unpublished results). It is not clear whether the observed effect of cytokinins on Achlya reflects a mechanism which in some way either allows the fungus to establish itself as a parasite, or permits the host plant to limit growth of the pathogen. Extensive knowledge, both of cytokinin-auxin relationships with cell metabolism, and of host-parasite interactions will be required before a functional role can definitely be attributed to this cytokinin action.

## PART V

## CYTOKININ EFFECTS ON MACROMOLECULAR SYNTHESIS

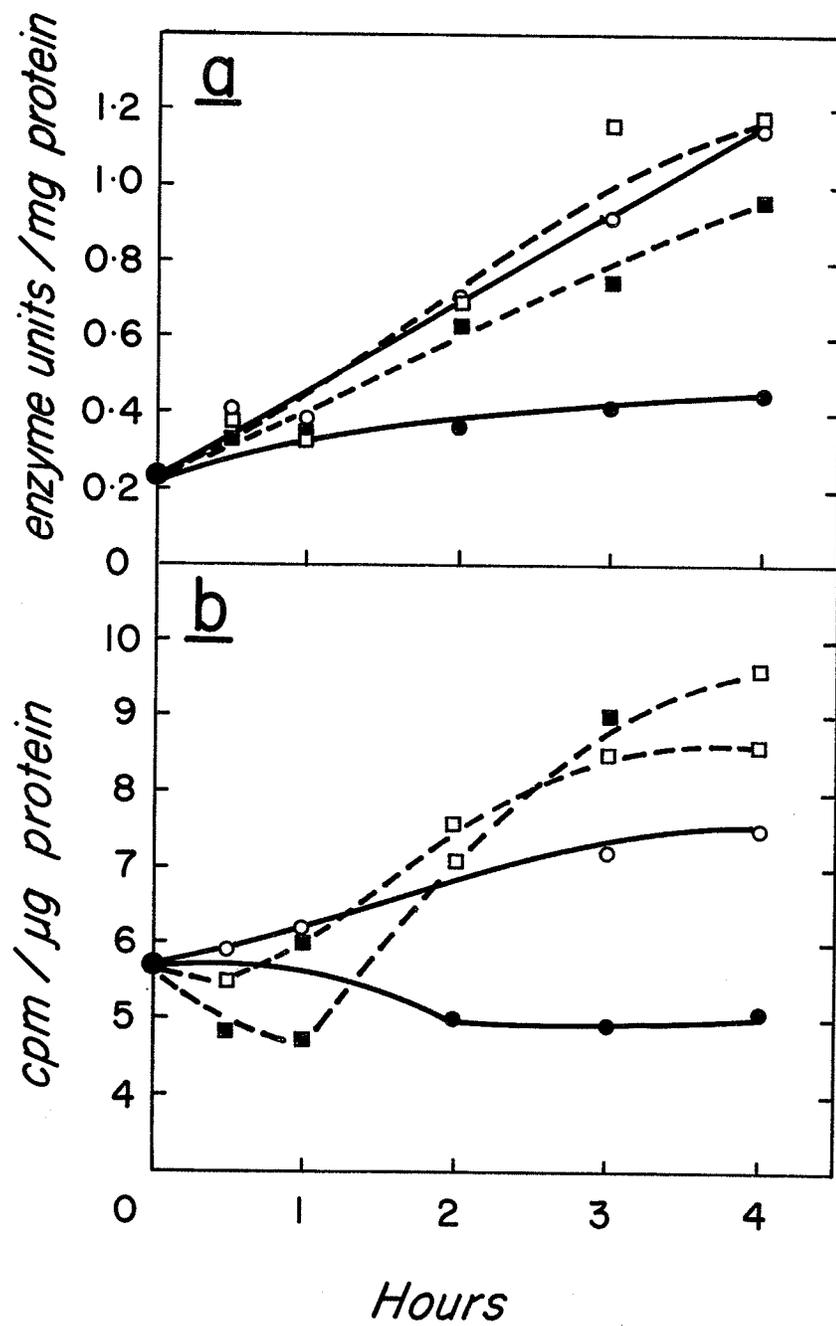
## RESULTS

A. Effects on Induction of Glutamate Dehydrogenase

Achlya germlings, when exposed to 5% glutamate, showed an increase in the specific activity of glutamate dehydrogenase of approximately six-fold over a four hour period. This contrasted with a one-fold increase in enzyme activity of control cells suspended in 5% glucose medium (Figure 70 a, solid lines). Addition of isopentenyladenine at a final concentration of 250  $\mu$ M, produced little change in the specific activity of glutamate dehydrogenase in the glutamate induced cells, but such a treatment significantly increased enzyme levels five-fold in cells growing in glucose (Figure 70 a, broken lines).

The amount of radioactive protein hydrolysate incorporated into the cold trichloroacetic acid-precipitable fraction remained fairly constant in the cells in glucose medium, but increased about 50 per cent in the induced cells (Figure 70 b, solid lines). With the addition of isopentenyladenine, the increase in labelled protein in both sets of cells was similar, reaching about 80 per cent after 4 hours (Figure 70 b, broken lines). This result could be

Figure 70: Effects of isopentenyladenine (250  $\mu$ M final concentration) on levels of (a) glutamic dehydrogenase specific activity, and (b) amino acid incorporation, in cell extracts of Achlya germlings. Isopentenyladenine was added to prelabelled cells at zero time, when glutamate or fresh glucose was added. Glucose-treated cells, ( ● ) no isopentenyladenine; ( ○ ) with isopentenyladenine. Glutamate-treated cells, ( ■ ) no isopentenyladenine; ( □ ) with isopentenyladenine.



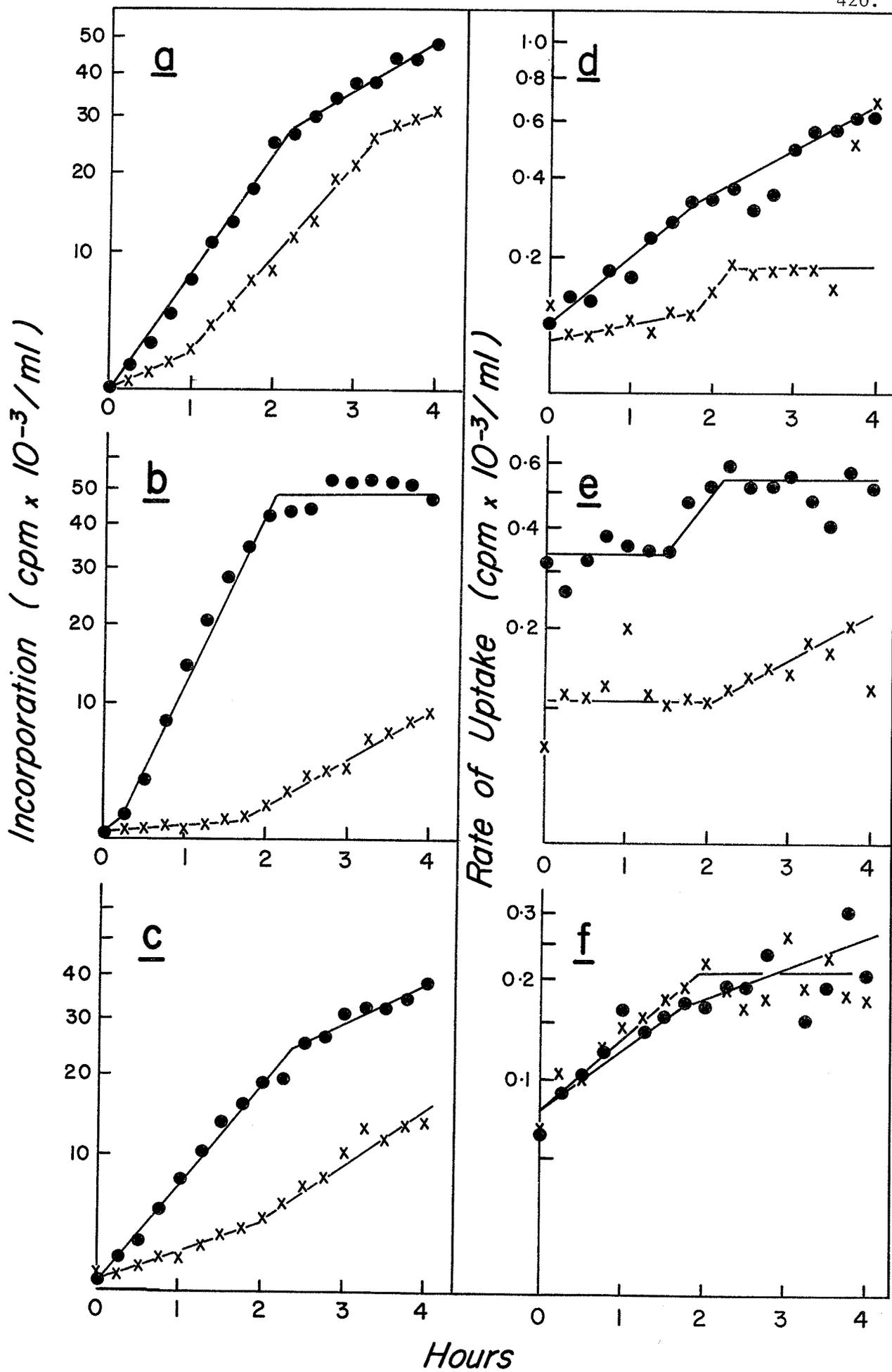
interpreted to mean that either isopentenyladenine was stimulating protein synthesis, or it was inhibiting protein degradation and turnover.

#### B. Protein and RNA Synthesis

Isopentenyladenine enhancement of glutamate dehydrogenase activity and accompanying protein synthesis raised the question whether the effect was a general stimulation of protein synthesis by the cytokinin. To examine the effects of isopentenyladenine on overall macromolecular synthesis, the incorporation of  $^{14}\text{C}$ -protein hydrolysate,  $^3\text{H}$ -uridine, and  $^3\text{H}$ -uracil into cold trichloroacetic acid-precipitable material was measured, with the cells being grown briefly in defined medium. Incorporation of the three labelled substrates into macromolecules is shown in Figure 71, a, b, and c respectively.

Isopentenyladenine at 250  $\mu\text{M}$  reduced the quantity of amino acids and uracil incorporated by about 30 per cent, and almost totally inhibited uridine incorporation for two hours. A factor that would explain such a reduction in the incorporation of labelled substances, is inhibition of uptake of the compound in the presence of isopentenyladenine. To determine the relative extent of this inhibitory effect, the ability of cells to take up the substrate after different times of exposure to the cytokinin was assayed concurrently. Both amino acid and uridine uptake were strongly inhibited by isopentenyladenine (Figure 71 d and e), which could

Figure 71: Effects of isopentenyladenine on incorporation of (a)  $^{14}\text{C}$ -protein hydrolysate, (b)  $^3\text{H}$ -uridine, and (c)  $^3\text{H}$ -uracil, and on the corresponding rate of uptake ability after exposure to isopentenyladenine, for (d)  $^{14}\text{C}$ -protein hydrolysate, (e)  $^3\text{H}$ -uridine, and (f)  $^3\text{H}$ -uracil. No isopentenyladenine ( ● ); 250  $\mu\text{M}$  isopentenyladenine ( × ).

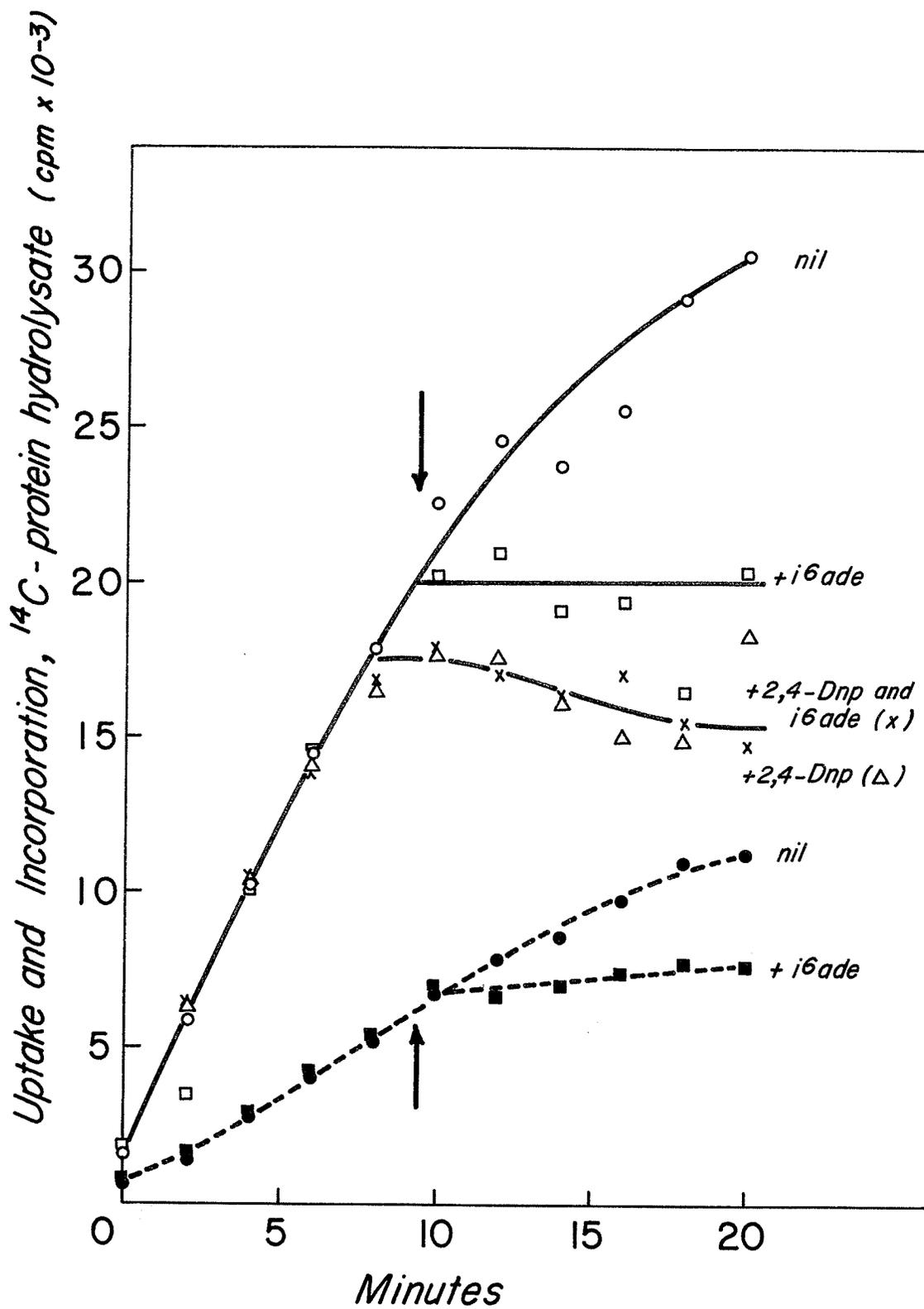


explain part of the incorporation inhibition. Uracil uptake, however, was not seriously affected (Figure 71 f) although incorporation into RNA was retarded. These transport effects are consistent with the earlier transport studies, which showed nucleic acid bases to be transported by a facilitated diffusion system, while cytokinins inhibit active transport processes, such as those of amino acids and nucleosides. It is interesting that incorporation of uracil into RNA was reduced in the presence of isopentenyladenine, despite a normal rate of uptake. This suggested that the cytokinin may also act on synthetic processes of the cell at a level other than that of metabolite transport.

It can be noted in Figure 71, that there are changes in the rate of incorporation and uptake by both the cytokinin treated and the control cells after approximately two hours in the fresh medium. This is thought to reflect a general shift in the physiology of the cells, possibly related to nuclear division events which occur at that time.

Isopentenyladenine effects on uptake and incorporation were again examined in the experiment shown in Figure 72. Addition of the cytokinin after the cells had been exposed to labelled protein hydrolysate in defined medium for ten minutes produced an immediate alteration in the rate of both transport and incorporation. The addition of 2,4-Dnp also inhibited transport, but in the presence of 2,4-Dnp, isopentenyladenine produced no increase in efflux of labelled material from the cells (Figure 72). Uracil transport was demonstrated not to be affected by cytokinin, but incorporation was

Figure 72: Effects of isopentenyladenine (240  $\mu\text{M}$ ) and 2,4-Dnp (100  $\mu\text{M}$ ) on uptake, (solid lines); and of isopentenyladenine on incorporation (broken lines), of  $^{14}\text{C}$ -protein hydrolysate. Arrow indicates the time of additions, and the specific additions are indicated in the figure.

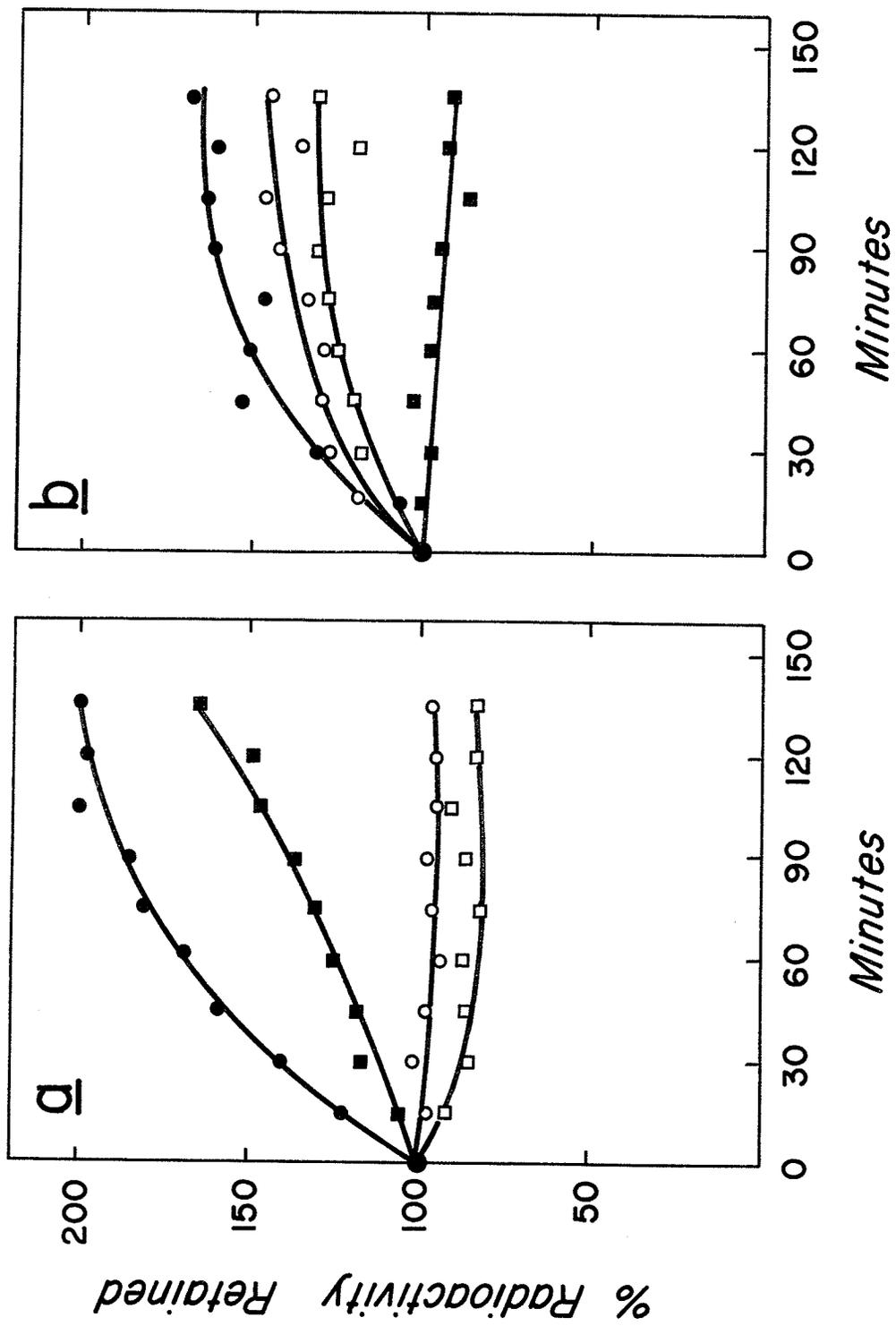


found to be immediately inhibited by addition of cytokinin in this case also. The immediate effect of cytokinins on uptake has been noted before (see Figure 61), but in this case the effect is on macromolecular synthesis as well.

### C. Protein and RNA Degradation and Synthesis

It therefore appeared that isopentenyladenine might have an additional effect on protein and RNA macromolecules aside from its effect on precursor transport. Such an effect might occur by action on the synthetic processes themselves, or by an influence on the rate of product degradation, as has been shown by Shibaoka and Thimann (1970). In order to examine these possibilities, cells were preloaded for 30 minutes with either  $^{14}\text{C}$ -protein hydrolysate or  $^3\text{H}$ -uracil in Barksdale's defined medium, collected on mesh and washed, then resuspended in Transport Buffer. Following prelabelling, radioactive material in the cells should be present both in macromolecules of protein or RNA, and in intracellular pools of precursors. After removal of the external label, it would be expected that the amount of trichloroacetic acid-precipitable material in the cells would continue to increase as the pool material was incorporated into protein and RNA. This expected rise was seen in the control cells for both protein hydrolysate (Figure 73 a) and uracil (Figure 73 b). Isopentenyladenine at 250  $\mu\text{M}$  strongly inhibited the continued incorporation of amino acids, and completely blocked further uracil incorporation. As the cells were preloaded with the

Figure 73: Effects of isopentenyladenine on the continued incorporation and retention of radioactive material in cells prelabelled with (a)  $^{14}\text{C}$ -protein hydrolysate, and (b)  $^3\text{H}$ -uracil, and washed prior to cytokinin addition. Control cells, (●) no isopentenyladenine; (■) with 250  $\mu\text{M}$  isopentenyladenine; Cycloheximide-treated cells: (○) no isopentenyladenine; (□) with 250  $\mu\text{M}$  isopentenyladenine.



radioactive material, and then resuspended in buffer lacking exogenous label or nutrients, it was unlikely that this inhibition resulted from isopentenyladenine effects on the rate of metabolite uptake. Most likely, cytokinin acted directly or through a cytokinin-directed process involving either macromolecular synthesis or degradation.

To distinguish between these possibilities, cycloheximide was used as a diagnostic agent. This antibiotic has been shown to inhibit both protein and RNA synthesis in *Achlya* (Cameron & LéJohn 1972a). If cycloheximide were to be added to interrupt further incorporation of labelled precursors from the preloaded pools, then a cytokinin-enhanced degradation of proteins or RNA should appear as a progressively increasing loss in radioactivity from the trichloroacetic acid-precipitable fraction over time in the cytokinin-treated cells. The results of this approach are also shown in Figure 73. Cycloheximide blocked further protein synthesis, and the level of labelled material remained constant for two hours (Figure 73 a). Addition of isopentenyladenine in these cycloheximide-treated cells initiated a small but significant reduction in the content of the labelled cellular protein. About 15 to 20 per cent of the labelled protein was destroyed within 30 minutes, and the level remained constant thereafter.

In contrast to the protein studies, cycloheximide seemingly reversed the inhibitory effect of cytokinin on RNA synthesis (Figure 73 b). The bulk of the RNA synthesized by these cells in the presence of cycloheximide has been characterized as non-ribosomal,

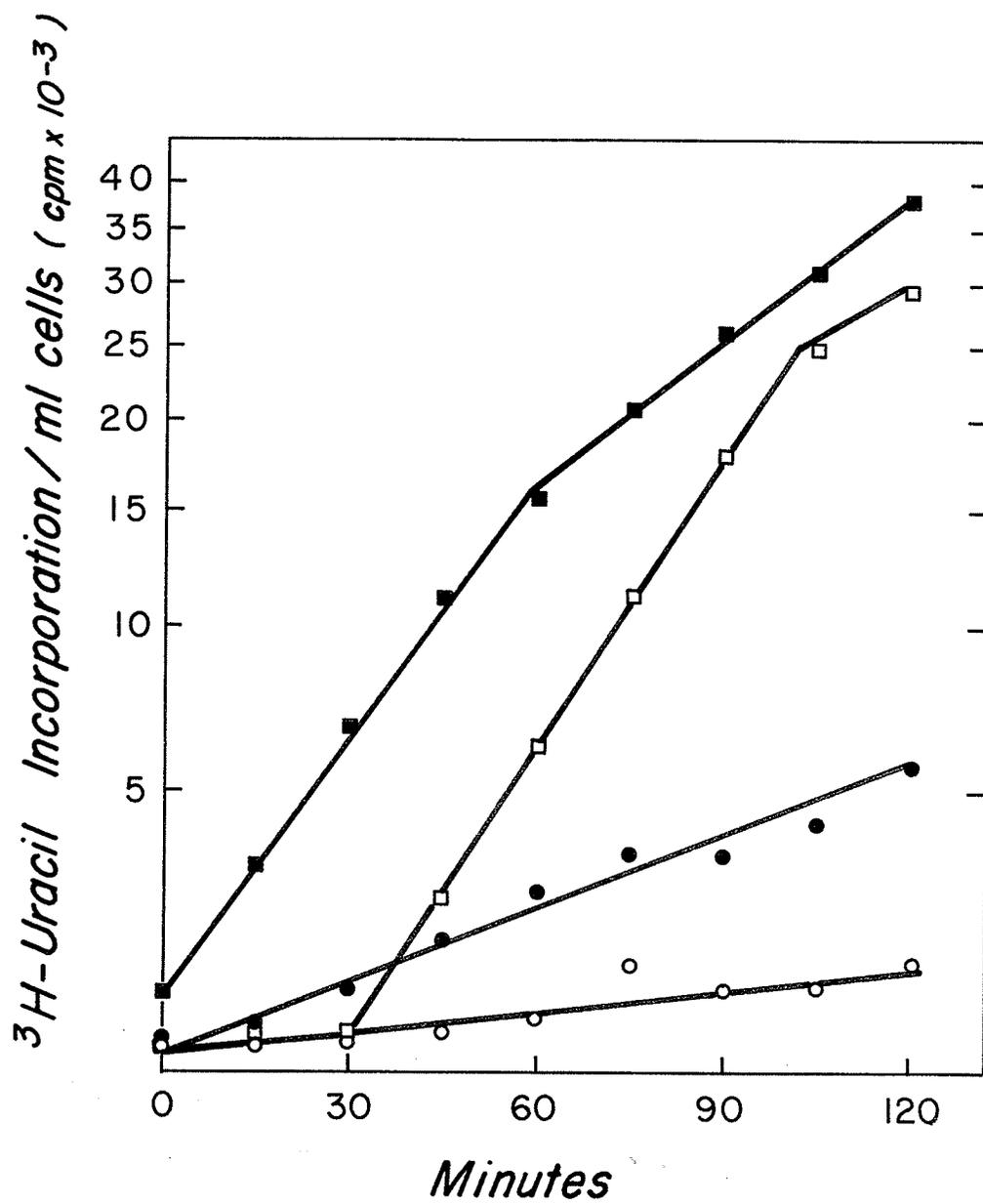
sedimenting between 4s and 12s in sucrose density gradients (unpublished data of Cameron and LeJohn). The presence of cytokinin in cycloheximide-treated cells reduced RNA synthesis slightly (about 20%). The fact that cycloheximide reversed cytokinin inhibition of RNA synthesis at a time when protein synthesis was completely blocked, suggested that continued protein synthesis was an important factor in maintenance of the level of RNA in the cells. If cytokinin either induced the synthesis of RNase, or enhanced its activity, then blocking the synthesis of more RNase molecules by cycloheximide would have repercussions in that it would either reduce or prevent its degradative action on RNA. The apparent concentration of cellular RNA would increase as observed. This is similar to the conclusions reached by Shibaoka and Thimann (1970) for cytokinin effects on RNase activity in oat leaves.

#### D. Possible Cyclic AMP Interaction

The inhibition of isopentenyladenine binding by cyclic AMP, and the role played by this compound in hormone responses in animal cells suggested an inquiry into possible interactions with the cytokinin effects on synthesis. Figure 74 shows the results of one such experiment, in which cyclic AMP not only overcame the isopentenyladenine inhibition of uracil incorporation, but also greatly stimulated incorporation with respect to the control levels. Other studies demonstrated that cyclic AMP alone greatly stimulated uracil incorporation but not protein synthesis. However, the

Figure 74: Effect of cyclic AMP (1 mM) on isopentenyladenine (240  $\mu$ M) inhibition of uracil incorporation.

No isopentenyladenine ( ● ); isopentenyladenine, alone ( ○ ); isopentenyladenine with cyclic AMP ( ■ ); isopentenyladenine with cyclic AMP added at 30 minutes ( □ ).



effects of cyclic AMP on synthesis have been found to be somewhat inconsistent, and the nature of this observed stimulation is being pursued by others in the laboratory. The reversal of cytokinin inhibition could either be a specific effect, perhaps caused by competition for a regulatory site on the cell surface, or a non-specific response, in which the stimulatory effect of cyclic AMP overwhelms the inhibitory effect of isopentenyladenine.

## DISCUSSION

Enzyme Induction and Catabolite Repression

The effect of isopentenyladenine on glutamate dehydrogenase activity in glucose-grown Achlya germlings was to reverse the 'glucose effect' on the enzyme by increasing the specific activity. Treating glutamate-induced cells with isopentenyladenine at the same time did not produce a greater increase in enzyme activity, suggesting that the mechanism by which the increase is produced may be the same in both cases. Addition of cytokinin produced an increase in the amount of radioactive amino acids incorporated into the soluble or supernatant fraction of the cell extract - a greater increase than was seen due to induction by glutamate. This might reflect changes in other enzymes such as by activation of a general induction mechanism by the cytokinin, or preservation from degradation at the normal rates by cytokinin effects on degradative enzymes. These studies do not distinguish between the various possible means by which observed enzyme activity could increase, including increased enzyme synthesis or stability, decreased degradation of messenger RNA for an enzyme, or activation of pre-existing enzyme molecules.

The increases in enzyme activity of glutamate dehydrogenase, and the increases in the radioactivity of the supernatant did not correlate with the effects of cytokinins on protein synthesis in whole cell measurements. In this way, induction of glutamate dehydrogenase was similar to the increase in activity of nitrate reductase in 6-benzyladenine-treated Agrostemma githago embryos, as described by Kende and Shen (1972). In that study there was no overall cytokinin effect on protein synthesis or degradation. In Achlya the effect of isopentenyladenine on general protein and RNA levels was inhibitory with alternate levels of action being metabolite uptake, synthesis, and RNase activity.

#### Effects on Synthesis

The effects of isopentenyladenine on synthesis and degradation processes have been discussed in connection with the results presented in Figures 71 to 73. The mechanism by which such effects on synthesis or on degradation are mediated is not obvious from these studies. Kende et al (1971) noted that the response to cytokinins in the nitrate reductase induction process described above was very rapid. The effect of isopentenyladenine on glutamate dehydrogenase activity and on protein and RNA levels was also very rapid, occurring immediately

after exposure of the cells to the cytokinin. The studies of radioactively labelled isopentenyladenine, presented in Part V, indicated rapid binding to the cell, but slow penetration of the cytokinin or its derivatives. These observations suggest a hormone-type effect occurring with cytokinin binding at the cell surface. Cyclic AMP has been demonstrated to be a major intracellular mediator of hormone responses in animal cells. Some effects of cyclic AMP in connection with cytokinin action on Achlya suggest that this molecule could be involved in the cytokinin response (Figures 69 b and 74), but the evidence is not sufficient to indicate the nature of the involvement.

C O N C L U S I O N S

## CONCLUSIONS

Studies reported in this thesis have demonstrated that the NAD-specific glutamate dehydrogenase found in the Oömycetes, Achlya and Pythium is subject to allosteric regulation by a wide variety of effectors. In Table XII an attempt was made to evaluate the observed in vitro responses of the purified enzyme with regard to the physiological activities of the cell. Allosteric modifications of an individual enzyme may be viewed as reflecting evolutionary development of a gene product that is responding to the cellular conditions under which it acts in vivo.

The occurrence of different patterns of responses to effectors by the glutamate dehydrogenases of different groups of fungi, and the implications in examining phylogeny and evolutionary relationships has been discussed by LéJohn (1971). The glutamate dehydrogenase catalyst of the Oömycetes is of particular interest as it is specific for the NAD(H) coenzyme, and no second, NADP-specific enzyme form occurs as in some other fungal species. In this connection, the proposed transhydrogenase function of NAD-specific glutamate dehydrogenase in cooperation with NADP-specific isocitrate dehydrogenase in regulating pyrimidine nucleotide balances is of interest. An NADP<sup>+</sup> allosteric site on the Oömycete NAD-specific glutamate dehydrogenase is of significance when considering evolutionary development of the Oömycetes at the molecular level.

Different types of glutamate dehydrogenase isolated from animal sources are capable of reacting with either NAD(H) or NADP(H) as coenzyme. Kinetic studies of the mechanisms of enzyme action of these nonspecific-coenzyme forms, demonstrating some variations in response to different coenzymes, suggest that further studies of the Oömycete enzyme may prove useful in elucidating protein-coenzyme interactions at the molecular level. The order of substrate interaction and product release in the NADP-activated NAD-specific enzyme is another facet of the behaviour of the fungal enzymes with potential value in examining these interactions. The NADP-type reaction order observed in this NAD-specific catalyst of the Oömycetes may reflect a sequence in evolution of the enzyme, or it may be a consequence of requirements imposed because of the NADP-binding site on the protein. Further studies at the level of physical structure, and on ligand-enzyme interactions of this particular protein might prove informative with regard to dehydrogenase reaction mechanisms in general.

As described, regulation of the activity of this key enzyme in metabolism by a diversity of effectors reflects control by the overall physiological requirements of the cell. Further examination of glutamate dehydrogenase regulation, particularly with regard to induction and repression, has provided new leads to other systems of control in overall cellular metabolism. One such is the regulation of metabolite transport at the level of the plasma membrane.

A significant regulation process at the level of metabolite transport that involves the action of cytokinin-active compounds has

been described here. This phenomenon is of interest in several different ways. First, the existence of cytokinin control in these particular fungi, but not in some related species, suggests it may have some specific function in these organisms. The parasitic mode of life of certain members of the Oömycetes raises the possibility that control exerted by a plant growth substance may reflect some basic physiological features of the host-parasite interactions in higher plants. The relationship of cytokinin controls to the growth and survival of the phytopathogen remains unclear however, requiring further information on responses at specific cytokinin concentrations, and in concert with other plant growth regulators.

A second interest in this regulatory effect is in its possibilities as a model system of molecular level mediation of plant growth hormone effects. The rapidity with which the cytokinin acts on transport makes possible an examination of the kinetics of the response. The importance of such studies of rapid responses has recently been discussed by Evans (1974), with particular reference to the effects of auxins. He considers rapid responses to be those detectable within one hour of hormone application, which would include not only cytokinin effects on transport in Achlya, but also the described effects on macromolecular synthesis and degradation. Of significance in considering this organism as a model for cytokinin action is the observed binding of IAA and tryptophan to a cell wall  $\text{Ca}^{++}$ -binding glycopeptide (LéJohn & Cameron 1973, LéJohn et al 1974), suggesting that the system

might also prove valuable in examining interactions of different groups of plant growth substances. The demonstrated effect of cytokinins on  $\text{Ca}^{++}$  binding and transport also provides an approach to understanding possible mechanisms of cytokinin action in this organism. The importance of  $\text{Ca}^{++}$  in membrane structure, in transport processes, and in cellular synthesis suggests that cytokinin action may be mediated through  $\text{Ca}^{++}$ .

The ability of cytokinins to quantitatively inhibit the active transport of metabolites and enhance the binding of tryptophan (auxin) within a short period makes its potential as a bioassay a third area of interest. The bioassays reviewed in Table II demonstrate that procedures vary in rapidity, sensitivity, specificity, and simplicity. An assay based on cytokinin effects on metabolite transport in Achlya germlings may prove a useful tool in assessing cytokinin levels and the relative effectiveness of various compounds, particularly if the basis of the response can be further elucidated.

In view of the wide variety of effects attributed to cytokinins in different systems (see Table III), the possible influence of cytokinins in Achlya on functions other than transport is also an area of interest. The results obtained from studies of macromolecular synthesis and degradation processes suggest this could be one such level of response to cytokinin action. For various plant systems, it has been proposed that different responses to cytokinin compounds may reflect different molecular mechanisms, different active forms, or the action of different groups of compounds. The Achlya system, with its variety of observed responses, provides the

opportunity of conveniently assessing such concepts. One such concept concerns the involvement of cyclic AMP in the mediation of plant hormone actions, a proposal that has been made for several systems without conclusive evidence regarding its action. The apparent interaction of cyclic AMP with the cytokinin responses of Achlya suggests that this problem could be further studied using this system.

R E F E R E N C E S

REFERENCES

- Alexopoulos, C.J. (1962) Introductory Mycology, 2nd Edn., John Wiley and Sons Inc., New York, 613 pp.
- Alvarez, R. (1970) Hormonal control of the synthesis and secretion of a soluble adenyl cyclase from barley aleurone layers. Ph.D Thesis, University of California, Davis. (Dissertation Abs. 32: 770-B)
- Alvarez, R., T.C. Moore & J. Vandepute (1974) Formation of adenosine 5'-phosphofluoridate and the assay of adenyl cyclase in barley seeds. Plant Physiol. 53: 144-148
- Anderson, M.B. & J.H. Cherry (1969) Differences in leucyl-transfer RNAs and synthetases in soybean seedlings. Proc. Nat. Acad. Sci. U.S. 62: 202-209
- Armstrong, D.J., F. Skoog, L.H. Kirkegaard, A.E. Hampel, R.M. Bock, I. Gillam & G.M. Tener (1969) Cytokinins: Distribution in species of yeast tRNA. Proc. Nat. Acad. Sci. U.S. 63: 504-511
- Atkin, R.K. & B.I.S. Srivastava (1969) The changes in soluble protein of excised barley leaves during senescence and kinetin treatment. Physiol. Plant. 22: 742-750
- Atkinson, D.E. (1968) The energy charge of the adenylate pool as a regulatory parameter: Interaction with feedback modifiers. Biochemistry 7: 4030-4034
- Atkinson, D.E. (1969) Regulation of enzyme function. Ann. Rev. Microbiol. 23: 47-68
- Atkinson, D.E., J.A. Hathaway & E.C. Smith (1965) Kinetics of regulatory enzymes: Kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. J. Biol. Chem. 240: 2682-2690
- Azhar, S. & C.R. Krishna Murti (1971) Effect of indole-3-acetic acid on the synthesis of cyclic 3'-5' adenosine phosphate by Bengal gram seeds. Biochem. Biophys. Res. Commun. 43: 58-64
- Babula, M. & A.G. Galsky (1974) The effects of cyclic AMP on the initiation of crown gall tumors on primary pinto bean leaves. Plant Physiol. 53 (Suppl.) p. 72 (Paper 409)

- Bachofen, R. (1973) Distribution of cyclic AMP in maize seedlings. Plant Science Lettr. 1: 447-450
- Balinsky, J.B., G.E.Shambaugh & P.P.Cohen (1970) Glutamate dehydrogenase biosynthesis in amphibian liver preparations. J. Biol. Chem. 245: 128-137
- Balz, H.P. (1966) Intrazelluläre Lokalisation und Funktion von hydrolytischen enzyemen bei Tabak. Planta 70: 207-236
- Barksdale, A.W. (1963) The uptake of exogenous Hormone A by certain strains of Achlya. Mycologia 55: 164-171
- Barksdale, A.W. (1969) Sexual hormones of Achlya and other fungi. Science 166: 831-837
- Barratt, R.W. (1963) Effect of environmental conditions on the NADP-specific glutamic acid dehydrogenase in Neurospora crassa. J.Gen.Microbiol. 33: 33-42
- Basile, D.V., H.N.Wood & A.C.Braun (1973) Programming of cells for death under defined experimental conditions: Relevance to the tumour problem. Proc.Nat.Acad.Sci. U.S. 70: 3055-3059
- Bauer, L. (1966) Isolierung und Testung einer kinetinartigen Substanz aus Kalluszellen von Laubmoosporophyten. Z. Pflanzenphysiol. 54: 241-253
- Beevers, L. & R.H.Hageman (1969) Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. 20: 495-522
- Benedetto, A. & A.Cassone (1974) Inhibition of uridine transport in cultured mammalian cells by theophylline. Biochim.Biophys. Acta 349: 53-60
- Benko, P.V., T.C.Wood & I.H.Segal (1967) Specificity and regulation of methionine transport in filamentous fungi. Arch.Biochem. Biophys. 122: 783-804
- Berlin, R.D. & E.R.Stadtman (1966) A possible role of purine nucleotide phosphorylases in the regulation of purine uptake by Bacillus subtilis. J.Biol.Chem. 241: 2679-2688
- Bernhardt, W., K.Panten & H.Holzer (1964) Damped oscillation of the enzyme synthesis in yeast. Angew.Chemie.,Internat. Edn. 3: 803-804
- Berridge, M.V., R.K.Ralph & D.S.Letham (1970) The binding of kinetin to plant ribosomes. Biochem.J. 119: 75-84

- Beutelmann, P. (1973) Untersuchungen zur Biosynthese eines Cytokinins in Calluszellen von Laubmoosporophyten. Planta 112: 181-190
- Bezemer-Sybrandy, S.M. & H.Veldstra (1971) Investigations on cytokinins: IV. The metabolism of 6-benzylaminopurine in Lemna minor. Physiol.Plant.25; 1-7
- Birnbaumer, L. (1973) Hormone-sensitive adenylyl cyclases: Useful models for studying hormone receptor functions in cell-free systems. Biochim.Biophys.Acta 300: 129-158
- Biswas, B.B. & S.P.Sen (1959) Relationship between auxins and nucleic acid synthesis in coleoptile tissues. Nature 183: 1824-1825
- Bitensky, M.W., K.L.Yielding & G.M.Tomkins (1965) Reciprocal changes in alanine and glutamate dehydrogenase activities after exposure of crystalline bovine L-glutamate dehydrogenase to organic mercury. J.Biol.Chem.240: 663-667
- Bonner, J.T. (1970) Induction of stalk cell differentiation by cyclic AMP in the cellular slime mold Dictyostelium discoideum. Proc. Nat.Acad.Sci. U.S. 65: 110-113
- Bonner, J.T. (1971) Aggregation and differentiation in the cellular slime molds. Ann.Rev.Microbiol.25: 75-92
- Bopp, M. (1968) Control of differentiation in fern-allies and bryophytes. Ann.Rev.Plant Physiol.19: 361-380
- Bopp, M. & W.Diekmann (1967) Versuche zur Analyse von Wachstum und Differenzierung der Moosprotonemen V. Die Kinetinwirkung bei Caulonemaregeneration. Planta 74: 86-96
- Borriss, H. (1967) Untersuchungen über die Steuerung der Enzymaktivität in pflanzlichen Embryonem durch Cytokinine. Wiss.Z.Univ. Rostock Math.Naturwiss. Reihe 16: 629-639
- Bradham, L.S. (1972) Comparison of the effects of  $Ca^{++}$  and  $Mg^{++}$  on the adenylyl cyclase of beef brain. Biochim.Biophys.Acta 276: 431-443
- Brandes, H. & H.Kende (1968) Studies on cytokinin-controlled bud formation in moss protonemata. Plant Physiol.43: 827-837
- Bray, G. (1960) A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279-285
- Brewin, N.J. & D.H.Northcote (1973 a) Variations in the amounts of 3',5'-cyclic AMP in plant tissues. J.Exptl.Bot.24: 881-888

- Brewin, N.J. & D.H.Northcote (1973 b) Partial purification of a cyclic AMP phosphodiesterase from soybean callus: Isolation of a non-dialysable inhibitor. Biochim.Biophys.Acta 320: 104-122
- Brooker, G. (1972) High-pressure anion exchange chromatography and enzymatic isotope displacement assays for cyclic AMP and cyclic GMP. in Advances in Cyclic Nucleotide Research 2: 111-129, Raven Press, New York
- Brown, B.L., J.D.M.Albano, R.P.Ekins, A.M.Sgherzi & W.Tampion (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5' cyclic monophosphate. Biochem.J. 121: 561-562
- Brown, E.G. & R.P.Newton (1973) Occurrence of adenosine 3':5'-cyclic monophosphate in plant tissues. Phytochem. 12: 2683-2685
- Burrows, W.J., D.J.Armstrong, F.Skoog, S.M.Hecht, J.T.A.Boyle, N.J.Leonard & J.Occolowitz (1968) Cytokinin from soluble RNA of Escherichia coli: 6-(3-methyl-2-butenylamino)-methylthio-9-D-ribofuranosyl purine. Science 161: 691-693
- Burrows, W.J., F.Skoog & N.J.Leonard (1971) Isolation and identification of cytokinins located in the transfer ribonucleic acid of tobacco callus grown in the presence of 6-benzylaminopurine. Biochem. 10: 2189-2194
- Cameron, L.E. & H.B.LeJohn (1972 a) On the involvement of calcium in amino acid transport and growth of the fungus Achlya. J.Biol.Chem. 247: 4729-4739
- Cameron, L.E. & H.B.LeJohn (1972 b)  $Ca^{++}$  is a specific regulator of amino acid transport and protein synthesis in the water-mould Achlya. Biophys.Biochem.Res.Commun. 48: 181-189
- Carritt, B. & J.M.Eisenstadt (1973) Synthesis in vitro of high molecular weight RNA by isolated Euglena chloroplasts. Eur. J. Biochem. 36: 482-488
- Cass, C. & A.R.P.Patterson (1972) Mediated transport of nucleosides in human erythrocytes. Accelerative exchange diffusion of uridine and thymidine and specificity toward pyrimidine nucleosides as permeants. J.Biol.Chem. 247: 3314-3320
- Casselton, P.J. (1969) Concurrent regulation of two enzymes in fungi. Sci.Prog.(Oxford) 57: 207-227
- Cassman, M. & H.K.Schachman (1971) Sedimentation equilibrium studies on glutamic dehydrogenase. Biochem. 10; 1015-1024
- Chance, B. & B.Schoener (1964) Control of oscillations of the DPNH level in a cell-free extract of Saccharomyces carlsbergensis by 3'5'-cyclic AMP. Biophys.Biochem.Res.Commun. 17; 416-423

- Chance, B., B.Schoener & S.Elsaesser (1965) Metabolic control phenomena involved in damped sinusoidal oscillations of reduced diphosphopyridine nucleotide in a cell-free extract of Saccharomyces carlsbergensis. J.Biol.Chem. 240: 3170-3181
- Chang, Y.Y. (1968) cAMP Phosphodiesterase produced by the slime mold Dictyostelium discoideum. Science 161: 57
- Chen, C. & R.Hall (1969) Biosynthesis of N<sup>6</sup>( $\Delta^2$ -isopentenyl)adenosine in the transfer ribonucleic acid of cultured tobacco pith tissue. Phytochem. 8: 1687-1695
- Cherry, J.H. & M.B.Anderson (1972) Cytokinin-induced changes in transfer RNA species. in Plant Growth Substances 1970 (D.J.Carr, ed.) Springer-Verlag (1972) pp. 181-189
- Cheung, W.Y. (1966) Adenosine 3'5'-phosphate and oscillations of DPNH in a cell-free extract of Saccharomyces carlsbergensis. Biochim. Biophys.Acta 115: 235-239
- Cheung, W.Y. (1970) Cyclic nucleotide phosphodiesterase. in The Role of Cyclic AMP in Cell Function (P.Greengard & E.Costa, eds.) Raven Press, New York (1970) pp. 51-65
- Cheung, W.Y. (1971) Cyclic 3',5'-nucleotide phosphodiesterase. Evidence for and properties of a protein activator. J.Biol.Chem. 246: 2859-2869
- Chi, Y-Y. & D.Francis (1971) Cyclic AMP and calcium exchange in a cellular slime mold. J.Cell Physiol. 77: 169-174
- Cleland, W.W. (1963 a) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim.Biophys.Acta 67: 104-137
- Cleland, W.W. (1963 b) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim.Biophys.Acta 67: 173-187
- Cleland, W.W. (1963 c) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. Biochim.Biophys. Acta 67: 188-196
- Cleland, W.W. (1967) Enzyme kinetics. Ann.Rev.Biochem. 36: 77-112
- Cline, M.G., M.Edgerton & M.M.Rehn (1974) Effects of cyclic AMP on elongation in Avena coleoptile segments. Plant Physiol. 53 (Suppl.) p.58 (Paper 328)
- Constantopoulos, A. & V.A.Najjar (1973) The activation of adenylate cyclase. II. The postulated presence of (A) adenylate cyclase in a phospho(inhibited) form (B) a dephospho(activated) form with a cyclic adenylate stimulated protein kinase. Biophys. Biochem.Res.Commun. 53: 794-799

- Conway, A. & D.E.Koshland, Jr. (1968) Negative cooperativity in enzyme action. The binding of diphosphopyridine nucleotide to glyceraldehyde 3-phosphate dehydrogenase. Biochem. 7: 4011-4022
- Corbin, J.D. & C.O.Brostrom (1971) Subunit analysis of skeletal muscle cyclic AMP-dependent protein kinases. Fed.Proc. 30: 1089 (Abs.217)
- Crabeel, M. & M.Grenson (1970) Regulation of histidine uptake by specific feedback inhibition of two histidine permeases in Saccharomyces cerevisiae. Eur.J.Biochem. 14: 197-204
- Crick, F.H.C. (1966) Codon-anticodon pairing: The Wobble Hypothesis. J.Mol.Biol. 19: 548-555
- Cross, D.G. & H.F.Fisher (1970) The mechanism of glutamate dehydrogenase reaction. III. The binding of ligands at multiple subsites and resulting kinetic effects. J.Biol.Chem. 245: 2612-2621
- Cummins, J.E. & J.M.Mitchison (1967) Adenine uptake and pool formation in the fission yeast Schizosaccharomyces pombe. Biochim. Biophys.Acta 136: 108-120
- Czok, R. & L.Eckert (1963) D-3-Phosphoglycerate, D-2-phosphoglycerate, phosphoenolpyruvate. in Methods of Enzymatic Analysis, (H-U. Bergmeyer, ed.), Academic Press, New York (1963) pp. 224-233
- Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann.N.Y.Acad.Sci. 121: 404-427 (Canalco pre-print, 1961)
- Davoren, P.R. & E.W.Sutherland (1963) The cellular location of adenylyl cyclase in the pigeon erythrocyte. J.Biol.Chem. 238: 3016-3023
- DeCastro, I.N., M.Ugarte, A.Cano & F.Mayor (1970) Effect of glucose, galactose, and different nitrogen sources on the activity of yeast glutamate dehydrogenase (NAD and NADP-linked) from normal strain and impaired respiration mutant. Eur.J.Biochem. 16: 567-570
- de Crombroghe, B., B.Chen, W.Anderson, P.Nissley, M.Gottesman, I.Pastan & R.Perlman (1971) Lac DNA, RNA polymerase and cyclic AMP receptor protein, cyclic AMP, lac repressor and inducer are the essential elements for controlled lac transcription. Nature New Biol. 231: 139-142
- Dekhuijzen, H.M. & J.C.Overeem (1972) Cytokinin activity of N<sup>6</sup>,O<sup>2'</sup>-dibutyryl cyclic AMP and N<sup>6</sup>-butyryladenine. Phytochem. 11: 1669-1672

- Deleuze, G., J.D. McChesney & J.E. Fox (1972) Identification of a stable cytokinin metabolite. Biochem. Biophys. Res. Commun. 48: 1426-1432
- Dixon, G.H. (1966) Protein evolution. in Essays in Biochemistry 2 (P.N. Campbell & G.D. Greville, eds.), Academic Press (1966) pp. 147-204
- Doree, M. (1973) Metabolism of exogenous adenine by Acer pseudoplatanus cells. Phytochem. 12: 2101-2108
- Doree, M. & J. Guern (1967) Sur un mode de degradation possible de quelques cytokinins. Comptu. Rendu. Acad. Sci. (Paris) 265: 29-32
- Doree, M. & J. Guern (1973) Short-time metabolism of some exogenous cytokinins in Acer pseudoplatanus cells. Biochim. Biophys. Acta 304: 611-622
- Doree, M., J.J. Leguay, J. Guern & H. Heslot (1972 a) Metabolisme de la kinetine chez le Schizosaccharomyces pombe. Comptu. Rendu. Acad. Sci. (Paris) 275: 59-62
- Doree, M. & C. Terrine (1972) Action del'AMP desaminase sur les nucleosides-5'-monophosphates d'adenines-N<sup>0</sup>-substituees. Comptu. Rendu. Acad. Sci. (Paris) 275: 1503-1506
- Doree, M., C. Terrine & J. Guern (1972 b) Plant cell permeability to kinetin. in Hormonal Regulation in Plant Growth and Development (Proc. Adv. Study Institute, NATO, Izmir 1971), (H. Kaldewey & Y. Vardar, eds.), Verlag-Chemie, Weinheim (1972), pp. 221-231
- Dove, L.D. (1972) Environmental and chemical control of RNA breakdown in leaves. Symp. Biol. Hung. 13: 299-307
- Drlica, K.A., J.A. Gardner, C.I. Kado, I.K. Vijay & F.A. Troy (1974) cyclic adenosine 3':5'-monophosphate levels in normal and transformed cells of higher plants. Biochem. Biophys. Res. Commun. 56: 753-759
- Dubois, E., M. Grenson & J. Wiame (1973) Release of the "ammonia effect" on three catabolic enzymes by NADP-specific GDH-less mutayions in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 50: 967-972
- Duffus, C. & J. Duffus (1969) A possible role of cyclic AMP in gibberellic acid triggered release of  $\alpha$ -amylase in barley endosperm slices. Experientia 25: 581
- Dyson, W.H., J.E. Fox & J.D. McChesney (1972) Short term metabolism of urea and purine cytokinins. Plant Physiol. 49: 506-513
- Dyson, W.H. & R.H. Hall (1972) N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine: its occurrence as a free nucleoside in an autonomous strain of tobacco tissue. Plant Physiol. 50: 616-621

- Eisenkraft, B. & C. Veeger (1970) Glutamate dehydrogenase - a study on its inactivation. in Pyridine Nucleotide-Dependent Dehydrogenases (H. Sund, ed.), Springer-Verlag, Berlin, Heidelberg, New York 1970, pp. 271-278
- Elas, L.E. & L.E. Rosenberg (1967) Inhibition of amino acid transport in rat kidney cortex by puromycin. Proc. Nat. Acad. Sci. U.S. 57: 371-378
- Elliott, D.C. & A.W. Murray (1972) A quantitative limit for cytokinin incorporation into transfer ribonucleic acid by soya-bean callus tissue. Biochem. J. 130: 1157-1160
- Emmer, M., B. de Crombrughe, I. Pastan & R. Perlman (1970) Cyclic AMP receptor protein in E. coli; its role in the synthesis of inducible enzymes. Proc. Nat. Acad. Sci. U.S. 66: 480-487
- Engel, P.C. & K. Dalziel (1970) Kinetic studies of glutamate dehydrogenase. The reductive amination of 2-Oxoglutarate. Biochem. J. 118: 409-419
- Engelbrecht, L. (1971) Cytokinin activity in larval infected leaves. Biochem. Physiol. Pflanzen. 162: 9-27
- Evans, M.L. (1974) Rapid responses to plant hormones. Ann. Rev. Plant Physiol. 25: 195-223
- Fahien, L.A. & M. Strmecki (1969) Studies of gluconeogenic mitochondrial enzymes. III. The conversion of  $\alpha$ -ketoglutarate to glutamate by bovine liver mitochondrial glutamate dehydrogenase and glutamate-oxalacetate transaminase. Arch. Biochem. Biophys. 130: 468-477
- Fahien, L.A., B.O. Wiggert & P.P. Cohen (1965) Crystallization and kinetic properties of glutamate dehydrogenase from frog liver. J. Biol. Chem. 240: 1083-1090
- Fang, M. & R.A. Butow (1970) Nucleotide reversal of mitochondrial repression in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 41: 1579-1583
- Farrell, C.A. & F.J. De Toma (1973) Increased capacity for RNA synthesis in Dictyostelium discoideum nuclei induced by exposure to cyclic AMP or 5'-AMP. Biochem. Biophys. Res. Commun. 54: 1504-1510
- Faulkner, R.D. & M. Uziel (1971) Iodine modification of E. coli tRNA<sup>phe</sup> reversible modification of 2-methylthio-N<sup>6</sup>-isopentenyl-adenosine and lack of disulfide formation. Biochim. Biophys. Acta 238: 464-474

- Fawole, M. & P.J.Casselton (1972) Observations on the regulation of glutamate dehydrogenase activity in Coprinus lagopus. J.Exptl. Bot. 23: 530-551
- Ferguson, A.R. & A.P.Sims (1971) In vivo inactivation of glutamine synthetase and NAD-specific glutamate dehydrogenase: Its role in the regulation of glutamine synthesis in yeasts. J. Gen.Microbiol. 69: 423-427
- Fincham, J.R.S. (1957) A modified glutamic acid dehydrogenase as a result of gene mutation in Neurospora crassa. Biochem.J. 65: 721-728
- Fisher, H.F. (1970) The structure of the glutamic dehydrogenase molecule and its subunits. in The Mechanism of Action of Dehydrogenases (G.W.Schwert & A.D.Winer, eds.). Univ.Press of Kentucky, Lexington (1970), pp.223-254
- Fisher, H.F. (1973) Glutamate dehydrogenase-ligand complexes and their relationship to the mechanism of the reaction. in Advances in Enzymology and Related Areas of Molecular Biology 39:369-417 (A.Meister,ed.), Interscience
- Fisher, H.F.,D.G.Cross & L.L.McGregor (1962) Catalytic activity of sub-units of glutamic dehydrogenase. Nature 196: 895-896
- Fisher, H.F., R.E.Gates & D.G.Cross (1970) A ligand exclusion theory of allosteric effects. Nature 228:247-249
- Fittler, F. & R.H.Hall (1966) Selective modification of yeast seryl-t-RNA and its effect on the acceptance and binding functions. Biochem.Biophys.Res.Commun. 25:441-446
- Fittler, F., L.K.Kline & R.H.Hall (1968) Biosynthesis of N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine. The precursor relationship of acetate and mevalonate to the  $\Delta^2$ -isopentenyl group of the transfer ribonucleic acid of microorganisms. Biochem. 7: 940-944
- Flawia, M.M. & H.N.Torres (1972 a) Adenylate cyclase activity in Neurospora crassa. I. General Properties. J.Biol.Chem. 247:6873-6879
- Flawia, M.M. & H.N.Torres (1972 b) Adenylate cyclase activity in Neurospora crassa. II. Kinetics. J.Biol.Chem. 247:6880-6883
- Flawia, M.M. & H.N.Torres (1972 c) Activation of membrane-bound adenylate cyclase by glucagon in Neurospora crassa. Proc. Nat.Acad.Sci. U.S. 69:2870-2873
- Flawia, M.M. & H.N.Torres (1973) Adenylate cyclase activity in Neurospora crassa. III. Modification by glucagon and insulin. J.Biol.Chem. 248: 4517-4520

- Fox, J.E. (1966) Incorporation of a kinin, N<sup>6</sup>-benzyladenine into soluble RNA. Plant Physiol. 41:75-82
- Fox, J.E. (1969) The cytokinins. in The Physiology of Plant Growth and Development, (M.B.Wilkins, ed.), McGraw-Hill, London (1969), pp.85-123
- Fox, J.E. & C.Chen (1967) Characterization of labelled ribonucleic acid from tissue grown on <sup>14</sup>C-containing cytokinins. J.Biol. Chem. 242: 4490-4494
- Fox, J.E. & C.M.Chen (1968) Cytokinin incorporation into RNA and its possible role in plant growth. in Biochemistry and Physiology of Plant Growth Substances, ( F.Wightman & G.Setterfield, eds.), The Runge Press Ltd., Ottawa (1968) pp. 777-789
- Fox, J.E., J.Cornette, G.Deleuze, W.Dyson, C.Giersak, P.Niu, J.Zapata & J.McChesney (1973) The formation, isolation and biological activity of a cytokinin 7-glucoside. Plant Physiol. 52: 627-632
- Fox, J.E., W.D.Dyson, C.Sood & J.McChesney (1972) Active forms of the cytokinins. in Plant Growth Substances 1970 (D.J.Carr, ed.), Springer-Verlag, (1972), pp. 449-458
- Fox, J.E., C.Sood, B.Buckwalter & J.McChesney (1971) The metabolism and biological activity of a 9-substituted cytokinin. Plant Physiol. 47: 275-281
- Francis, D. (1969) Time sequences for differentiation in cellular slime molds. Quart.Rev.Biol. 44: 277-290
- Frieden, C. (1959 a) Glutamic dehydrogenase: I. Effect of coenzyme on the sedimentation velocity and kinetic behaviour. J.Biol. Chem. 234: 809-814
- Frieden, C. (1959 b) Glutamate dehydrogenase: II. Effect of various nucleotides on the association-dissociation and kinetic properties. J.Biol.Chem. 234: 815-820
- Frieden, C. (1959 c) Glutamic dehydrogenase: III. The order of substrate addition in the enzymatic reaction. J.Biol.Chem. 234: 2891-2896
- Frieden, C. (1962) The molecular weight of chicken liver glutamate dehydrogenase. Biochim.Biophys.Acta 62: 421-423
- Frieden, C. (1963 a) Glutamate dehydrogenase: IV. Studies on enzyme inactivation and coenzyme binding. J.Biol.Chem. 238: 146-154
- Frieden, C. (1963 b) Glutamate dehydrogenase: V. The relation of enzyme structure to the catalytic function. J.Biol.Chem. 238: 3286-3299
- Frieden, C. (1965) Glutamate dehydrogenase: VI. Survey of purine nucleotide and other effects on the enzyme from various

- sources. J.Biol.Chem. 240: 2028 - 2035
- Frieden, C. (1970) Molecular and kinetic properties of glutamate dehydrogenase. in The Mechanism of Action of Dehydrogenases, (G.W.Schwert & A.D.Winer, eds.), The Univ. Press of Kentucky, Lexington, (1970), pp.197-222
- Fries, N. (1960) The effect of adenine and kinetin on growth and differentiation in Lupinus. Physiol.Plant. 13: 468-481
- Fuller, W. & A. Hodgson (1967) Conformation of the anticodon loop in tRNA. Nature 215: 817-821
- Gallo, R.C. & J. Whang-Peng (1971) Observations on the regulatory effects of the transfer RNA minor base, N<sup>6</sup>-( $\Delta^2$ -isopentenyl)-adenosine, on human lymphocytes. in Biological Effects of Polynucleotides (R.F.Beers, Jr. & W.Braun, eds.), Springer-Verlag (1971), pp. 303-334
- Galsky, A.G. & J.A.Lippincott (1969) Promotion and inhibition of  $\alpha$ -amylase production in barley endosperm by cyclic 3',5'-adenosine monophosphate and adenosine diphosphate. Plant Cell Physiol. 10: 607-620
- Galsky, A.G., H.L.Monoson, F.J.Pikul & J.S.Thompson (1972) Promotion of perithecial initial formation in the imperfect fungus Monoacrosporium doedyciodes by 6-methylpurine and its reversal by cyclic AMP. Amer.J.Bot. 59: 669 (Abst.)
- Garren, L.D., G.N.Gill & G.M.Walton (1971) The isolation of a receptor for adenosine 3',5'-cyclic monophosphate (cAMP) from the adrenal cortex: The role of the receptor in the mechanism of action of cyclic AMP. Annal.N.Y.Acad.Sci. 185: 210-226
- Gefter, M.L. & R.L.Russell (1969) Role of modifications in tyrosine transfer RNA: A modified base affecting ribosome binding. J. Mol.Biol. 39: 145-157
- Ghosh, K. & H.P.Ghosh (1970) Role of modified nucleoside adjacent to 3' end of anticodon in codon-anticodon interaction. Biochem.Biophys.Res.Commun. 40: 135-143
- Giannattasio, M. & V.Macchia (1973) Adenylate cyclase and cyclic 3'5' AMP-diesterase in Jerusalem artichoke tubers. Plant Science Lettr. 1: 259-264
- Giannattasio, M., E.Mandato & V.Macchia (1974) Content of 3'5'-cyclic AMP phosphodiesterase in dormant and activated tissues of Jerusalem artichoke tubers. Biochem.Biophys.Res.Commun. 57; 365-371

- Gilbert, M.L. & A.G.Galsky (1972) The action of cyclic AMP on  $GA_3$  controlled responses. III. Characteristics of barley endosperm phosphatase induction by gibberellic acid and cyclic 3'5' adenosine mono-phosphate. Plant & Cell Physiol. 13: 867-873
- Gill, G.N. & L.D.Garren (1971) Role of the receptor in the mechanism of action of adenosine 3':5' cyclic monophosphate. Proc.Nat.Acad.Sci. U.S. 68: 786-790
- Gilman, A.G. (1970) A protein binding assay for adenosine 3'5' cyclic monophosphate. Proc.Nat.Acad.Sci. U.S. 67: 305-312
- Gits, J. & M.Grenson (1967) Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. III. Evidence for a specific methionine-transporting system. Biochim.Biophys.Acta 135: 507-516
- Goldberg, N.D., A.G.O'Toole & M.K.Haddox (1972) Analysis of cyclic AMP and cyclic GMP by enzymatic cycling procedures. in Advan. in Cyclic Nucleotide Res. 2: 63-80 (Raven Press, New York)
- Goldin, B. & C.Frieden (1971) L-Glutamate dehydrogenases. in Advan. Cell Regulation 4: 77- 117, (Academic Press)
- Goldthwaite, J.J. (1974) Activity of cyclic and non-cyclic nucleotides as senescence inhibitors in Rumex obtusifolius leaf tissue. Plant Physiol. 53 (Suppl.) p.58 (Paper 328)
- Gorton, B.S., C.G.Skinner & R.E.Eakin (1957) Activity of some 6-(substituted)-purines on the development of the moss Tortella caespitosa. Arch.Biochem.Biophys. 66: 493-496
- Greengard, P. & J.F.Kuo (1970) On the mechanism of action of cyclic AMP. in The Role of Cyclic AMP in Cell Function, (P. Greengard & E.Costa, eds.), Raven Press, New York (1970) pp. 287-306
- Grenson, M. (1966) Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. II. Evidence for a specific lysine-transporting system. Biochim.Biophys.Acta 127: 339-346
- Grenson, M. (1969) The utilization of exogenous pyrimidine and the recycling of uridine 5'-phosphate derivatives in pyrimidine uptake and metabolism. Eur.J.Biochem. 11: 249-260
- Grenson, M. (1973) Specificity and regulation of the uptake and retention of amino acids and pyrimidines in yeast. in Genetics of Industrial Microorganisms vol.II (Z.Vanek, Z. Hostalek & J.Cudlin, eds.), Elsevier Pub. Co., Amsterdam (1973), pp.179-193

- Grenson, M., M. Crabeel, J. Wiame & J. Bechet (1968) Inhibition of protein synthesis and stimulation of permease turnover in yeast. Biochem. Biophys. Res. Commun. 30: 414-419
- Grenson, M. & C. Hou (1972) Ammonia inhibition of the general amino acid permease and its suppression in NADPH-specific glutamate dehydrogenaseless mutants of Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 48: 749-756
- Grenson, M., C. Hou & M. Crabeel (1970) Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. IV. Evidence for a general amino acid permease. J. Bacteriol. 103: 770-777
- Grenson, M., M. Mousset, J. M. Wiame & J. Bechet (1966) Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. I. Evidence for a specific arginine-transporting system. Biochim. Biophys. Acta 127: 325-338
- Gross, W. & K. Ring (1969) Effect of chloramphenicol on active amino acid transport. FEBS Lett. 4: 319
- Guern, J., M. Doree, J. J. Leguay & H. Heslot (1972) Sur la degradation enzymatique de la kinetine et de quelques adenines N<sup>6</sup>-substituees, chez le Schizosaccharomyces pombe. Comptu. Rendu. Acad. Sci. (Paris) 275: 377-381
- Guilfoyle, T. J. & J. B. Hanson (1974) Greater length of ribonucleic acid synthesized by chromatin-bound polymerase from auxin-treated soybean hypocotyls. Plant Physiol. 53: 110-113
- Hackette, S. L., G. E. Skye, C. Burton & I. H. Segal (1970) Characterization of an ammonium transport system in filamentous fungi with methyl-ammonium-<sup>14</sup>C as the substrate. J. Biol. Chem. 245: 4241-4250
- Hadden, J. W., E. M. Haden, M. Haddox & N. D. Goldberg (1972) Guanosine 3',5'-cyclic monophosphate: A possible intracellular mediator of mitogenic influences in lymphocytes. Proc. Nat. Acad. Sci. U.S. 69: 3024-3027
- Hall, R. H. (1970) N<sup>6</sup>-( $\Delta^2$ -Isopentenyl)adenosine: Chemical reactions, biosynthesis, metabolism, and significance to the structure and function of tRNA. in Progress in Nucleic Acid Research and Molecular Biology, v.10 (J. N. Davidson & W. E. Cohn, eds.), Academic Press (1970), pp. 57-86
- Hall, R. H. (1971) The Modified Nucleosides in Nucleic Acids. Columbia University Press, New York & London, (1971), 451 pp.

- Hall, R.H. (1973) Cytokinins as a probe of developmental processes. Ann.Rev.Plant Physiol. 24: 415-444
- Hall, R.H., S.N.Alam, B.D.McLennan, C.Terrine & J.Guern (1971) N<sup>6</sup>-( $\Delta^2$ -Isopentenyl)adenosine: Its conversion to inosine, catalyzed by adenosine aminohydrolases from chicken bone marrow and calf intestinal mucosa. Can.J.Biochem. 49: 623-630
- Hall, R.H., W.H.Dyson, C.D.Whitty, C.B.Chheda, S.P.Dutta & C.I.Hong (1972) Modified components of tRNA: Their possible role in the process of differentiation. in Hormonal Regulation in Plant Growth and Development. (Proc.Adv.Study Inst., NATO, Izmir 1971), (H.Kaldewey & Y.Vardar, eds.), Verlag Chemie, Weinheim (1972), pp. 233-244
- Hall, R.H. & G.Mintsioulis (1973) Enzymatic activity that catalyzes degradation of N<sup>6</sup>-( $\Delta^2$ -isopentenyl) adenosine. J.Biochem. 73: 739-748
- Halpern, Y.S. & H.E.Umbarger (1960) Conversion of ammonia to amino groups in Escherichia coli. J.Bact. 80: 285-288
- Hardman, J.G. (1971) Other cyclic nucleotides. in Cyclic AMP, (G.A.Robison, R.W.Butcher & E.W.Sutherland, eds.), Academic Press, New York & London, (1971), pp. 400-421
- Hartung, W. (1973) The importance of cAMP and different sugars for the effect of phytohormones on elongation growth of Avena coleoptile cylinders. (Ger.) Zeit.für Pflanzenphysiol. 68: 329-335
- Hauschka, P.V., L.P.Everhart & R.W.Rubin (1972) Alteration of nucleoside transport of Chinese hamster cells by dibutyryl adenosine 3',5'-cyclic monophosphate. Proc.Nat.Acad.Sci. U.S. 69: 3542-3546
- Hecht, S.M., R.M.Bock, R.Y.Schmitz, F.Skoog & N.J.Leonard (1971a) Cytokinins: development of a potent antagonist. Proc.Nat.Acad.Sci. U.S. 68: 2608-2610
- Hecht, S.M., R.M.Bock, R.Y.Schmitz, F.Skoog, N.J.Leonard & J.L. Occolowitz (1971 b) Question of the ribosyl moiety in the promotion of callus growth by exogenously added cytokinins. Biochem. 10: 4224-4228
- Heinz, E. (1972) Transport of amino acids by animal cells. in Metabolic Pathways v. VI, (D.Greenberg, ed.), Academic Press, (1972), pp. 455-501
- Helgeson, J.P. (1968) The cytokinins. Science 161: 974-981
- Helgeson, J.P. & N.J.Leonard (1966) Cytokinins: identification of compounds isolated from Corynebacterium fascians. Proc. Nat.Acad.Sci. U.S. 56: 60-63

- Heppel, L.A. (1971) The concept of periplasmic enzymes. in Structure and Function of Biological Membranes, (L.I.Rothfield, ed.), Academic Press, New York, (1971), pp. 223-247
- Hierholzer, G. & H.Holzer (1963) Repression der Synthese von DPN-abhängiger Glutaminsäure-dehydrogenase in Saccharomyces cerevisiae durch Ammoniumionen. Biochem.Z. 339: 175-185
- Hill, T.A. (1973) Endogenous Plant Growth Substances. (Inst. of Biol., Studies in Biol. 40), Edw.Arnold, London, 68 pp.
- Hirschberg, K.,G.Hübner & H.Borriss (1972) Cytokinin-induzierte de novo - Synthese der Nitratreductase Embryonen von Agrostemma githago. Planta 108: 333-337
- Hochstadt-Ozer, J. (1972) The regulation of purine utilization in bacteria. IV. Membrane and pericytoplasmic enzymes. J.Biol.Chem. 247: 2419-2426
- Hochstadt-Ozer, J. & M.Cashel (1972) The regulation of purine utilization in bacteria. V. Inhibition of purine phosphoribosyltransferase activities and purine uptake in isolated membrane vesicles by ppGpp. J.Biol.Chem. 247: 7067-7072
- Hochstadt-Ozer, J. & E.R.Stadtman (1971a) The regulation of purine utilization in bacteria. I. Purification of adenine phosphoribosyltransferase from Escherichia coli K<sub>12</sub> and control of activity by nucleotides. J.Biol.Chem. 246: 5294-5303
- Hochstadt-Ozer, J. & E.R.Stadtman (1971 b) The regulation of purine utilization in bacteria. II. Adenine phosphoribosyltransferase in isolated membrane preparations and its role in transport of adenine across the membrane. J.Biol.Chem. 246: 5304-5311
- Hochstadt-Ozer, J. & E.R.Stadtman (1971c) The regulation of purine utilization in bacteria. III. The involvement of purine phosphoribosyltransferases in the uptake of adenine and other nucleic acid precursors by intact resting cells. J.Biol.Chem. 246: 5312-5320
- Hoffmeyer, J. & J.Neuhard (1971) Metabolism of exogenous purine bases and nucleosides by Salmonella typhimurium. J.Bact. 106: 14-24
- Holden, J.T. & N.M.Utech (1967) Actinomycin D inhibition of amino acid transport in Streptococcus faecalis. Biochim.Biophys. Acta 135: 351-354
- Hollenberg, C.P.,W.F.Riks & P.Borst (1970) The glutamate dehydrogenases of yeast: extra-mitochondrial enzymes. Biochim.Biophys. Acta 201: 13-19

- Holley, R.W., J. Apgar, G.A. Everett, J.T. Madison, M. Marquisse, S.H. Merrill, J.R. Penswick & A. Zamir (1965) Structure of a ribonucleic acid. Science 147: 1462-1465
- Holm, R.E. & M.E. Miller (1972) Hormonal control of weed seed germination. Weed Sci. 20: 209-212
- Holzer, H. (1966) The control of enzyme patterns in yeast. Biochem. J. 98: 37 P (Abst.)
- Holzer, H. & S. Schneider (1957) Anreicherung und Trennung einer DPN-spezifischen und einer TPN-spezifischen Glutaminsäuredehydrogenase aus Hefe. Biochem.Z. 329: 361-369
- Hong, J-S., G.R. Smith & B.N. Ames (1971) Adenosine 3',5'-cyclic monophosphate concentration in a bacterial host regulates the viral decision between lysogeny and lysis. Proc. Nat. Acad. Sci. U.S. 68: 2258-2262
- Hooper, A.B., J. Hansen & R. Bell (1967) Characterization of glutamate dehydrogenase from the ammonia-oxidizing chemoautotroph Nitrosomonas europaea. J. Biol. Chem. 242: 288-296
- Horowitz, N.H. (1967) The evolution of biochemical syntheses -retrospect and prospect. in Evolving Genes and Proteins, (V. Bryson & H.J. Vogel, eds.), Academic Press, New York (1967), pp. 15-23
- Hsu, R.Y., G. Wasson & J.W. Porter (1965) The purification and properties of the fatty acid synthetase of pigeon liver. J. Biol. Chem. 240: 3736-3746
- Hunter, D.R. & I.H. Segel (1971) Acidic and basic amino acid transport systems in Penicillium chrysogenum. Arch. Biochem. Biophys. 144: 168-183
- Hunter, D.R. & I.H. Segel (1973 a) Control of the general amino acid permease of Penicillium chrysogenum by transinhibition and turnover. Arch. Biochem. Biophys. 154: 387-399
- Hunter, D.R. & I.H. Segel (1973 b) Effect of weak acids on amino acid transport by Penicillium chrysogenum: evidence for a proton or charge gradient as the driving force. J. Bact. 113: 1184-1192
- Hunter, D.R., C.L. Norberg & I.H. Segel (1973) Effect of cycloheximide on L-leucine transport by Penicillium chrysogenum: involvement of calcium. J. Bact. 114: 956-960
- Hüttermann, A., S.M. Elsevier & W. Eschrich (1971) Evidence for the de novo synthesis of glutamate dehydrogenase during the spherulation of Physarum polycephalum. Archiv. für Mikrobiologie 77: 74-85

- Hynes, M.J. (1974) The effects of the carbon source on glutamate dehydrogenase activities in Aspergillus nidulans. J.Gen. Microbiol. 81: 165-170
- Ishikura, H., Y. Yamada, K. Murao, M. Saneyoshi & S. Nishimura (1969) The presence of N-[9-( $\beta$ -D-ribofuranosyl)purin-6-yl carbamoyl] threonine in serine, methionone and lysine transfer RNAs from Escherichia coli. Biochem. Biophys. Res. Commun. 37: 990-995
- Jablonski, J.R. & F. Skoog (1954) Cell enlargement and cell division in excised tobacco pith tissue. Physiol. Plant. 7: 16-24
- Jacquez, J.A. (1962) Transport and enzymatic splitting of pyrimidine nucleosides in Ehrlich cells. Biochim. Biophys. Acta 61: 265-277
- Janistyn, B. (1972) IAA enhanced adenyl cyclase activity in homogenates of maize coleoptile. (Ger.) Z. Naturforsch. 27 B 872
- Johnson, R.A. (1972) The luminescence assay of cyclic AMP. in Advan. in Cyclic Nucleotide Res. 2: 81-87, Raven Press, New York
- Johnson, T.B., C. Ross & R. Baker (1970) Similarity of cytokinin contents and electrophoretic banding patterns of crown gall tumor and stem RNAs. Biochim. Biophys. Acta 199: 521-524
- Johri, M.M. & S. Desai (1973) Auxins-regulation of caulonema formation in moss protonema. Nature New Biol. 245: 223-224
- Johri, J.V. & J.E. Varner (1968) Enhancement of RNA synthesis in isolated pea nuclei by gibberellic acid. Proc. Nat. Acad. Sci. U.S. 59: 269-276
- Joiris, C.R. & M. Grenson (1969) Specificity and regulation of a dicarboxylic amino acid permease in Saccharomyces cerevisiae. (Fr.) Arch. Int. Physiol. 77: 154
- Jones, R.W. & D.G. Wild (1973) Regulation of uptake of purines, pyrimidines, and amino acids by Candida utilis. Biochem. J. 134: 617-627
- Jost, J-P. & H.V. Rickenberg (1971) Cyclic AMP. Ann. Rev. Biochem. 40: 741-774
- Kaback, H.R. (1970) The transport of sugars across isolated bacterial membranes. in Current Topics in Membranes and Transport v.1: 39-99, (F. Bronner & A. Kleinzeller, eds.), Academic Press, New York (1970)

- Kamisaka, S. (1972) Auxin-induced growth of tuber tissue of Jerusalem artichoke. VII. Effect of cyclic 3',5'-adenosine monophosphate on the auxin-induced cell expansion growth. in Plant Growth Substances 1970 (D.J.Carr, ed.), Springer-Verlag, Berlin, Heidelberg, New York (1972), pp. 654-660
- Kamisaka, S. & Y. Masuda (1971) Stimulation of gibberellin-induced germination in lettuce seeds by cyclic 3',5'-adenosine monophosphate. Plant & Cell Physiol. 12: 1003-1005
- Kamisaka, S., N. Sakurai & Y. Masuda (1973) Auxin-induced growth of tuber tissue of Jerusalem artichoke. VIII. Role of cyclic AMP in the action of auxin, cytokinin and gibberellic acid. Plant & Cell Physiol. 14: 183-193
- Kamisaka, S., H. Sano, M. Katsumi & Y. Masuda (1972) Effect of cyclic AMP and gibberellic acid on lettuce hypocotyl elongation and mechanical properties of its cell wall. Plant & Cell Physiol. 13: 167-174
- Kaplan, N.O. (1963) Symposium on multiple forms of enzymes and control mechanisms: I. Multiple forms of enzymes. Bacteriol. Rev. 27: 155-169
- Kapoor, M. & A.K. Grover (1970) Catabolite-controlled regulation of glutamate dehydrogenases of Neurospora crassa. Can. J. Microbiol. 16: 33-40
- Keates, R.A.B. (1973) Evidence that cyclic AMP does not mediate the action of gibberellic acid. Nature 224: 355-356
- Kebabian, J.W., J.F. Kuo & P. Greengard (1972) Determination of relative levels of cyclic AMP in tissues or cells prelabelled with radioactive adenine. in Advan. Cyclic Nucleotide Res. 2: 131-137, Raven Press, New York
- Keirns, J.J., B. Carritt, J. Freeman, J.M. Eisenstadt & M.W. Bitensky (1973) Adenosine 3',5'-cyclic monophosphate in Euglena gracilis. Life Sci. 13: 287-302
- Kende, H. (1971) The cytokinins. Internat. Rev. Cytol. 31: 301-338
- Kende, H., H. Hahn & S.E. Kays (1971) Enhancement of nitrate reductase activity by benzyladenine in Agrostemma githago. Plant Physiol. 48: 702-706
- Kende, H. & T.C. Shen (1972) Nitrate reductase in Agrostemma githago: comparison of the inductive effects of nitrate and cytokinin. Biochim. Biophys. Acta 286: 118-125
- Kende, H. & J.E. Tavares (1968) On the significance of cytokinin incorporation into RNA. Plant Physiol. 43: 1244-1248

- Kinghorn, J.R. & J.A.Pateman (1973) The regulation of nicotinamide-adenine-dinucleotide glutamate dehydrogenase in Aspergillus nidulans. Biochem.Soc. Transactions 1: 675-676
- Király, Z., M. El Hammady & B.I.Pozsár (1967) Increased cytokinin activity of rust infected bean and broad bean leaves. Phytopath. 57: 93-94
- Kirschner, K. (1971) Kinetic analysis of allosteric enzymes. in Advan. in Cell. Regulation 4: 167-210, Academic Press
- Kirtley, M.E. & D.E.Koshland, Jr. (1967) Models for cooperative effects in proteins containing subunits: Effects of two interacting ligands. J.Biol.Chem. 242: 4192-4205
- Klämbt, D., G.Thies & F.Skoog (1966) Isolation of cytokinins from Corynebacterium fascians. Proc.Nat.Acad.Sci. U.S. 56: 52-59
- Klein, B. (1967) Versuche zur Analyse der Protonemaentwicklung der Laubmoose. IV. Der endogene Faktor H und seine Rolle bei der Morphogenese von Funaria hygrometrica. Planta 73: 12-27
- Kline, L.K., F.Fittler & R.H.Hall (1969) N<sup>6</sup>-( $\Delta^2$ -Isopentenyl)adenosine: Biosynthesis in transfer ribonucleic acid in vitro. Biochem. 8: 4361-4371
- Knypl, J.S. (1973) Synergistic induction of nitrate reductase activity by nitrate and benzylaminopurine in detached cucumber cotyledons. Z.Pflanzenphysiol. 70: 1
- Konijn, Th.M. (1970) Microbiological assay of cyclic 3',5'-AMP. Experientia 26: 367-369
- Konijn, Th.M. (1972) Cyclic AMP as a first messenger. in Advan. Cyclic Nucleotide Res. 1: 17-31, Raven Press, New York
- Konijn, Th.M., J.G.C. van de Meene, J.T.Bonner & D.S.Barkley (1967) The acrasin activity of 3',5'-cyclic phosphate. Proc.Nat.Acad.Sci. 58: 1152-1154
- Koshland, D.E., Jr., G.Nemethy & D.Filmer (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochem. 5: 365-385
- Kram, R. & G.M.Tomkins (1973) Pleiotypic control by cyclic AMP: Interaction with cyclic GMP and possible role of microtubules. Proc.Nat.Acad.Sci. 70: 1659-1663
- Krishna, G. (1968) Estimation of cyclic 3',5'-adenosine monophosphate (c-AMP) in subnanomolar quantities by gas-liquid chromatography (GLC). Fed.Proc. 27: 649
- Kuehn, G.D. (1971) An adenosine 3',5'-monophosphate inhibited protein kinase from Physarum polycephalum. J.Biol.Chem. 246: 6366-6369

- Kuehn, G.D. (1972) Cell cycle variation in cyclic adenosine 3',5'-monophosphate dependent inhibition of a protein kinase from Physarum polycephalum. Biochem.Biophys.Res.Comm. 49: 414-419
- Kuo, J.F. & P.Greenard (1969) Cyclic nucleotide-dependent protein kinases. IV. Widespread occurrence of adenosine 3',5'-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. Proc.Nat.Acad.Sci. U.S. 64: 1349-1355
- Kuo, J.F. & P.Greenard (1970 a) Cyclic nucleotide-dependent protein kinases. VI. Isolation and partial purification of a protein kinase activated by guanosine 3',5'-monophosphate. J.Biol.Chem. 245: 2493-2498
- Kuo, J.F. & P.Greenard (1970 b) Cyclic nucleotide protein kinases. VIII. An assay method for the measurement of adenosine 3',5'-monophosphate in various tissues and a study of agents influencing its levels in adipose cells. J.Biol.Chem. 245: 4067-4073
- Kuraishi, S. (1968) The effect of kinetin on protein level of Brassica leaf discs. Physiol.Plant. 21: 78-83
- Larsen, A.D. & P.S.Sypherd (1974) Cyclic adenosine 3',5'-monophosphate and morphogenesis in Mucor racemosus. J.Bact. 117: 432-438
- Lehninger, A.L. (1970) Enzymes: mechanism, structure and regulation. in Biochemistry: The Molecular Basis of Cell Structure and Function, Worth Publishers Inc., New York (1970), pp. 169-187
- LéJohn, H.B. (1968 a) Unidirectional Inhibition of glutamate dehydrogenase by metabolites. A possible regulatory mechanism. J.Biol.Chem. 243: 5126-5131
- LéJohn, H.B. (1968 b) On the involvement of  $Ca^{++}$  and  $Mn^{++}$  in the regulation of mitochondrial glutamic dehydrogenase from Blastocladiella. Biochem.Biophys.Res.Comm. 32: 278-283
- LéJohn, H.B. (1971) Enzyme regulation, lysine pathways and cell wall structures as indicators of major lines of evolution in fungi. Nature 231: 164-168
- LéJohn, H.B. & L.E.Cameron (1973) Cytokinins regulate calcium binding to a glycoprotein from fungal cells. Biochem.Biophys.Res.Comm. 54: 1053-1060
- LéJohn, H.B., L.E.Cameron, R.M.Stevenson & R.U.Meuser (1974) Influence of cytokinins and sulfhydryl group-reacting agents on calcium transport in fungi. J.Biol.Chem. 249: 4016-4020

- LéJohn, H.B. & S.Jackson (1968 a) Allosteric interactions of a regulatory nicotinamide adenine dinucleotide-specific glutamate dehydrogenase from Blastocladiella. J.Biol.Chem. 243: 3447-3457
- LéJohn, H.B. & S.Jackson (1968 b) Selective desensitization of the allosteric glutamic dehydrogenase from Blastocladiella by  $Hg^{++}$ . Biochem.Biophys.Res.Comm. 33: 613-619
- LéJohn, H.B., S.G.Jackson, G.R.Klassen & R.V.Sawula (1969) Regulation of mitochondrial glutamic dehydrogenase by divalent metals, nucleotides and  $\alpha$ -ketoglutarate. J.Biol.Chem. 244: 5346-5356
- LéJohn, H.B. & B.E.McCrae (1968) Evidence for two species of glutamate dehydrogenases in Thiobacillus novellus. J.Bact. 95: 87-94
- LéJohn, H.B., I.Suzuki & J.A.Wright (1968) Glutamate dehydrogenases of Thiobacillus novellus. Kinetic properties and possible control mechanism. J.Biol.Chem. 243: 118-128
- Leonard, N.J., H.Iwamura & J.Eisinger (1969) Synthetic spectroscopic models relating to coenzymes and base pairs. IV. Stacking interactions an tRNA; the anticodon-adjacent base. Proc. Nat. Acad.Sci. U.S. 64: 352-359
- Letham, D.S. (1967) Regulators of cell division in plant tissues. V. A comparison of the activities of zeatin and other cytokinins in five bioassays. Planta 74: 228-242
- Letham, D.S. (1969) Cytokinins and their relation to other phytohormones. Bioscience 19: 309-316
- Letham, D.S. (1973) Transfer RNA and cytokinins. in The Ribonucleic Acids, (P.R.Stewart & D.S.Letham, eds.), Springer-Verlag, New York, Heidelberg, Berlin (1973), pp.18-106
- Lin, E. C-C. (1971) The molecular basis of membrane transport systems. in Structure and Function of Biological Membranes. (L.I.Rothfield, ed.), Academic Press, New York & London (1971), pp.285-341
- Lin, P.P-C. & J.E.Varner (1972) Cyclic nucleotide phosphodiesterase in pea seedlings. Biochim.Biophys.Acta 276: 454-474
- Lips, S.H. & N.Roth-Bejerano (1969) Light and hormones: Interchangeability in the induction of nitrate reductase. Science 166: 109-110
- Londesborough, J.C. & T.Nurminem (1972) A manganese-dependent adenyl cyclase in baker's yeast, Saccharomyces cerevisiae. Acta Chem.Scand. 26: 3396-3398

- Lowry, O.H., N.J.Rosebrough, A.L.Farr & R.J.Randall (1951) Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193: 265-275
- Lundeen, C.V., H.N.Wood & A.C.Braun (1973) Intracellular levels of cyclic nucleotides during cell enlargement and cell division in excised tobacco pith tissues. Differentiation 1: 255-260
- MacCalla, D.R., D.J.Moore & D.J.Osborne (1962) The metabolism of a kinin, benzyladenine. Biochim.Biophys.Acta 55: 522-528
- Magasanik, B. (1961) Catabolite repression. Cold Spring Harbour Sympos.Quantit.Biol. XXVI: 249-260
- Makman, R.S. & E.W.Sutherland (1965) Adenosine 3':5' phosphate in Escherichia coli. J.Biol.Chem. 240: 1309-1314
- Maltzhan, K.E. (1959) Interaction between kinetin and indoleacetic acid in the control of bud reactivation in Splachnum ampullaceum (L.) Hedw. Nature 183: 60-61
- Martin, C. & K.V.Thimann (1972 a) The role of protein synthesis in the senescence of leaves: I. The formation of protease. Plant Physiol. 49: 64-71
- Martin, C. & K.V.Thimann (1972 b) The role of protein synthesis in the senescence of leaves: II. The influence of amino acids on senescence. Plant Physiol. 50: 432-437
- Martin, R.G. & B.N.Ames (1961) A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J.Biol.Chem. 236: 1372-1379
- Mason, J.W., H.Rasmussen & F.DiBella (1971) 3',5'-AMP and Ca<sup>++</sup> in slime mold aggregation. Exptl.Cell Res. 67: 156-160
- Matthysse, A.G. (1970) Organ specificity of hormone-receptor-chromatin interactions. Biochim.Biophys.Acta 199: 519-521
- Matthysse, A.G. & M.Abrams (1970) A factor mediating interaction of kinins with the genetic material. Biochim.Biophys.Acta. 199: 511-518
- Matthysse, A.G. & C.Phillips (1969) A protein intermediary in the interaction of a hormone with the genome. Proc.Nat.Acad. Sci. U.S. 63: 897-903
- McDonald, J.J., N.J.Leonard, R.Y.Schmitz & F.Skoog (1971) Cytokinins: synthesis and biological activity of ureidopurines. Phytochem. 10: 1429-1439

- McLennan, B.D., D.M.Logan & R.H.Hall (1968) Enzymatic degradation of N<sup>6</sup>-( $\Delta^2$ -isopentenyl) adenosine. Proc.Amer.Assoc.Canc.Res. 9: 47
- McLennan, B.D. & A.Pater (1973 a) Evidence for the oxidation of N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine and N<sup>6</sup>-(3-hydroxy-3-methylbutyl)adenine by xanthine oxidase. Can.J.Biochem. 51: 1123-1126
- McLennan, B.D. & A.Pater (1973 b) The enzymatic phosphorylation of N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine in chicken liver homogenates. Can.J.Biochem. 51: 1341-1346
- Menon, M.K.C. & M.Lal (1974) Morphogenetic role of kinetin and abscisic acid in the moss Physcomitrium. Planta 115: 319-328
- Miller, C.O. (1961) Kinetin and related compounds in plant growth. Ann.Rev.Plant Physiol. 12: 395-408
- Miller, C.O. (1963) Kinetin and kinetin-like compounds. in Modern Methods of Plant Analysis v. VI; 194-202, ( H.F.Linskens, M.V.Tracey & B.D.Sanwal, eds.), Springer-Verlag; Berlin, Heidelberg, Göttingen.
- Miller, C.O. (1967) Naturally-occurring cytokinins. in Biochemistry and Physiology of Plant Growth Substances, (F.Wightman & G.Setterfield, eds.), The Runge Press Ltd., Ottawa (1968) pp.33-45
- Miller, C.O., F.Skoog, F.S.Okumura, M.H.von Saltza & F.M.Strong (1956) Isolation, structure and synthesis of kinetin, a substance promoting cell division. J.Amer.Chem.Soc. 78: 1375-1380
- Miller, C.O., F.Skoog, M.H.von Saltza & F.M.Strong (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J.Amer.Chem.Soc. 77: 1392
- Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photo-synthetic phosphorylation. Biol.Rev. 41: 445-502
- Mizel, S.B. & L.Wilson (1972) Nucleoside transport in mammalian cells. Inhibition by colchicine. Biochem. 11: 2573-2578
- Mondal, H., R.K.Mandal & B.B.Biswas (1972) RNA stimulated by indole-acetic acid. Nature New Biol. 240: 111-113
- Monod, J., J-P.Changeux & F.Jacob (1963) Allosteric protein and cellular control systems. J.Mol.Biol. 6: 306-329
- Monod, J., J.Wyman & J-P.Changeux (1965) On the nature of allosteric transitions: A plausible model. J.Mol.Biol. 12: 88-118
- Montencourt, B.S., S-C.Kuo & J.O.Lampen (1973) Saccharomyces mutants with invertase formation resistant to repression by hexoses. J.Bact. 114: 233-238

- Mothes, K. & L. Engelbrecht (1961) Kinetin-induced directed transport of substances in excised leaves in the dark. Phytochem. 1: 58-62
- Murashige, T. & F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497
- Murray, A.W., M. Spizman & D.E. Atkinson (1971) Adenosine 3',5'-monophosphate phosphodiesterase in the growth medium of Physarum polycephalum. Science 171: 496-498
- Narayanan, A., J. Vermeesch & A. Pradet (1970) Dosage enzymatique de l'acide adénosine 3',5'-monophosphate cyclique dans les semences de Laituse, variété 'Reine de mai'. Compt. Rend. Acad. Sci. (Paris) 271: 2406-2407
- Nestle, M. & M. Sussman (1972) Effect of cyclic AMP on morphogenesis and enzyme accumulation in Dictyostelium discoideum. Develop. Biol. 28: 545-554
- O'Dea, R.F., M.K. Haddox & N.D. Goldberg (1971) Interaction with phosphodiesterase of free and kinase complexed cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 246: 6183-6190
- Oliver, J.M. & A.R.P. Patterson (1971) Nucleoside transport: I. A mediated process in human erythrocytes. Can. J. Biochem. 49: 262-270
- Olson, J.A. & C.B. Anfinsen (1953) Kinetic and equilibrium studies on crystalline L-glutamic acid dehydrogenase. J. Biol. Chem. 202: 841-856
- Osborne, D.J. (1962) Effect of kinetin on protein and nucleic acid metabolism in Xanthium leaves during senescence. Plant Physiol. 37: 595-602
- Osborne, D.J. (1965) Interactions of hormonal substances in the growth and development of plants. J. Sci. Food Agric. 16: 1-13
- Oxender, D.L. (1972) Amino acid transport in microorganisms. in Metabolic Pathways v. VI, 3rd Edn., (L.E. Hokin, ed.), Academic Press, New York & London (1972), pp. 133-185
- Pačes, V., E. Werstiuk & R.H. Hall (1971) Conversion of N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol. 48: 775-778

- Pall, M.L. (1969) Amino acid transport in Neurospora crassa. I. Properties of two amino acid transport systems. Biochim. Biophys. Acta 173: 113-127
- Pall, M. (1971) Amino acid transport in Neurospora crassa. IV. Properties and regulation of a methionine transport system. Biochim. Biophys. Acta 233: 201-214
- Pardee, A.B. (1968) Membrane transport proteins. Science 162: 632-637
- Parker, C.W., D. Letham, D. Cowley & J. Macleod (1972) Raphanatin, an unusual purine derivative and a metabolite of zeatin. Biochem. Biophys. Res. Commun. 49: 460-466
- Pastan, I. & R. Perlman (1970) Cyclic adenosine monophosphate in bacteria. Science 169: 339-344
- Pastan, I. & R. Perlman (1971) Cyclic AMP in metabolism. Nature New Biol. 229: 5-7
- Pateman, J.A. (1969) Regulation of the synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. Biochem. J. 115: 769-775
- Pateman, J.A., J.R. Kinghorn, E. Dunn & E. Forbes (1973) Ammonium regulation in Aspergillus nidulans. J. Bact. 114: 943-950
- Perlman, R.L., B. de Crombroghe & I. Pastan (1969) Cyclic AMP regulates catabolite and transient repression in Escherichia coli. Nature 233: 810-812
- Perlman, R.L. & I. Pastan (1968) Regulation of  $\beta$ -galactosidase synthesis in Escherichia coli by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 243: 5420-5427
- Perlman, R. & I. Pastan (1969) Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of Escherichia coli. Biochem. Biophys. Res. Commun. 37: 151-157
- Peterofsky, A. (1968) The incorporation of mevalonic acid into the N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenosine of transfer ribonucleic acid in Lactobacillus acidophilus. Biochem. J. 7: 472-482
- Pickard, M.A., L. Phillippe & J.N. Campbell (1974) Metabolism and transport of purine nucleosides by membrane preparations of Micrococcus sodonensis. Can. J. Biochem. 52: 83-89
- Pickering, W.R. & R.A. Woods (1972) The uptake and incorporation of purines by wild-type Saccharomyces cerevisiae and a mutant resistant to 4-aminopyrazolo-(3,4-d)pyrimidine. Biochim. Biophys. Acta 264: 45-58

- Plowman, K.M. (1972) Enzyme Kinetics, McGraw Hill Book Co., New York, 171 pp.
- Polak, A. & M.Grenson (1973) Evidence for a common transport system for cytosine, adenine and hypoxanthine in Saccharomyces cerevisiae and Candida albicans. Eur.J.Biochem. 32: 276-282
- Polakis, E.S. & W.Bartley (1965) Changes in the enzyme activities of Saccharomyces cerevisiae during aerobic growth on different carbon sources. Biochem.J. 97: 284-297
- Pollard, C.J. (1970) Influence of gibberellic acid on the incorporation of 8-<sup>14</sup>C-adenine into adenosine 3'-5'-cyclic phosphate in barley aleurone layers. Biochim.Biophys.Acta 201: 511-512
- Pollard, C.J. & R.J.Venere (1970) Gibberellin action and 3',5'-cyclic AMP metabolism in aleurone layers. Fed.Proc. 29: A 670 (Abst. 2386)
- Posternak, Th. (1971) Chemistry of cyclic nucleoside phosphates and synthesis of analogs. in Cyclic AMP, (G.A.Robison, R.W. Butcher & E.W.Sutherland ), Academic Press, New York & London, (1971), pp.48-71
- Pourquié, J. (1970) Antagonism by adenine in the nutrition of Schizosaccharomyces pombe mutants. Inhibition at the level of guanine uptake. Biochim.Biophys.Acta 209: 269-277
- Pourquié, J. & H.Heslot (1971) Utilization and interconversions of purine derivatives in the fission yeast Schizosaccharomyces pombe. Genet.Res. 18: 33-44
- Pradet, A., P.Raymond & A.Narayanan (1972) Confirmation de la présence de l'AMP cyclique dans les semences de Laituse, var. 'Reine de mai'. Compt.Rendu.Acad.Sci. (Paris) 275 D: 1987-1988
- Rasmussen, H. (1970) Cell communication, calcium ion, and cyclic adenosine monophosphate. Science 170: 404-412
- Rast, D., R.Skřivanová & R.Bachofen (1973) Replacement of light by dibutyryl-cAMP and cAMP in betacyanin synthesis. Phytochem. 12: 2669-2672
- Rathbone, M.P. & R.H.Hall (1972) Concerning the presence of the cytokinin, N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine, in cultures of Corynebacterium fascians. Planta 108: 93-102
- Raymond, P., A.Narayanan & A.Pradet (1973) Evidence for the presence of 3',5'-cAMP in plant tissues. Biochem.Biophys.Res.Commun. 53: 1115-1121

- Reiman, E.M., D.A. Walsh & E. Krebs (1971) Purification and properties of rabbit skeletal-muscle adenosine 3',5'-monophosphate-dependent protein kinases. J.Biol.Chem. 246: 1986-1995
- Richmond, A., A. Back & B. Sachs (1970) A study of the hypothetical role of cytokinins in completion of tRNA. Planta 90: 57-65
- Richmond, A. & A. Lang (1957) Effect of kinetin on protein content and survival of detached Xanthium leaves. Science 125: 650-651
- Riedel, V. & G. Gerisch (1971) Regulation of extracellular cyclic AMP-phosphodiesterase activity during development of Dictyostelium discoideum. Biochem.Biophys.Res.Commun. 42: 119-124
- Riedel, V., G. Gerisch, E. Mueller & H. Beug (1973) Defective cyclic adenosine-3':5'-phosphate-phosphodiesterase (cAMP) regulation in morphogenetic mutants of Dictyostelium discoideum. J.Mol.Biol. 74: 573-585
- Rijven, A.H.G.C. & V. Parkash (1971) Action of kinetin on cotyledons of fenugreek. Plant Physiol. 47: 59-64
- Ring, K. (1970) Some aspects of the active transport of amino acids. Angew.Chem.Internat.Edit. 9: 345-356
- Robison, G.A., R.W. Butcher & E.W. Sutherland (1971) Cyclic AMP, Academic Press, New York & London, 531 pp.
- Robison, G.A., M.T. Schmidt & E.W. Sutherland (1970) On the development and properties of the brain adenylyl cyclase system. in Role of cAMP in Cell Function, (P. Greengard & E. Costa, eds.), Raven Press, New York (1970), pp. 11-30
- Rothstein, A. (1970) Sulfhydryl groups in membrane structure and function. in Current Topics in Membranes and Transport, (F. Bronner & A. Kleinzeller, eds.), Academic Press, New York & London (1970), pp. 135-176
- Rubin, M.M. & J-P. Changeux (1966) On the nature of allosteric transitions: Implications of non-exclusive ligand binding. J.Mol.Biol. 21: 265-274
- Sadorge, P., M. Dorée, C. Terrine & J. Guern (1970) Utilisation d'adénine exogène par les tissus végétaux. I. Technique de mesure de l'activité de l'adénine pyrophosphoribosyltransférase. Physiol. Vég. 8: 499-514

- Sadorge, P., Y. Signor & J. Guern (1972) Sur la synthèse des nucléosides 5'-monophosphates de cytokinines par les cellules d'Acer pseudoplatanus. Compt. Rend. Acad. Sci. (Paris) 275: 2493-2496
- Sahulka, J. (1972) The effect of exogenous IAA and kinetin on nitrate reductase, nitrite reductase, and glutamate dehydrogenase activities in excised pea roots. Biol. Plant. (Praha) 14: 330-336
- Salomon, D. & J.P. Mascarenhas (1971) Auxin-induced synthesis of cyclic 3',5'-adenosine monophosphate in Avena coleoptiles. Life Sci. 10 (II): 879-885
- Salomon, D. & J.P. Mascarenhas (1972) Auxin and cyclic 3',5' adenosine monophosphate during the isolation of chromatin from Avena coleoptiles: effects on cell-free RNA synthesis. Biochem. Biophys. Res. Commun. 47: 134-141
- Sanwal, B.D. (1970) Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. 34: 20-39
- Sanwal, B.D. & M. Lata (1961) The occurrence of two different glutamic dehydrogenases in Neurospora. Can. J. Microbiol. 7: 319-328
- Sanwal, B.D. & M. Lata (1962) Concurrent regulation of glutamic acid dehydrogenases of Neurospora. Arch. Biochem. Biophys. 97: 582-588
- Scarborough, G. (1973) Transport in Neurospora. in Internat. Rev. Cytol. 34: 103-122, Academic Press
- Schiltz, J.R. & K.D. Terry (1970) Nucleoside uptake during the germination of Neurospora crassa conidia. Biochim. Biophys. Acta 209: 278-288
- Schimke, R.T. & D. Doyle (1970) Control of enzyme levels in animal tissues. Ann. Rev. Biochem. 39: 929-976
- Schneider, M.J., J.C.J. Lin & F. Skoog (1969) Nucleic acid metabolism during cytokinin induced cellular differentiation. Plant Physiol. 44: 1207-1210
- Schwalb, M.N. (1974) Effect of adenosine 3',5'-cyclic monophosphate on the morphogenesis of fruit bodies of Schizophyllum commune. Arch. Microbiol. 96: 17-20
- Scott, W.A. & B. Solomon (1973) Cyclic 3',5'-AMP phosphodiesterase of Neurospora crassa. Biochem. Biophys. Res. Commun. 53: 1024-1030
- Shaw, M. & D.J. Samborski (1956) The physiology of host-parasite relations. I. The accumulation of radioactive substances at infections of facultative and obligate parasites including tobacco mosaic virus. Can. J. Bot. 34: 389-405

- Shibaoka, H. & K.V.Thimann (1970) Antagonisms between kinetin and amino acids: experiments on the mode of action of cytokinins. Plant Physiol. 46: 212-220
- Shimoyama, M., M.Kawai, Y.Hoshi & I.Ueda (1972 a) Nicotinamide inhibition of 3',5'-cyclic AMP phosphodiesterase in vitro. Biochem.Biophys.Res.Comm. 49: 1137-1141
- Shimoyama, M., M.Kawai, Y.Tanigawa, I.Ueda, M.Sakamoto, K.Hagiwara, Y.Yamashita & E.Sakakibara (1972 b) Evidence for and some properties of a 3',5'-cyclic AMP phosphodiesterase inhibitor in potato. Biochem.Biophys.Res.Comm. 47: 59-65
- Silverstein, E. & G.Sulebele (1973) Equilibrium kinetic study of the catalytic mechanism of bovine liver glutamate dehydrogenase. Biochem. 12: 2164-2172
- Singh, D. (1974) Characterization of the Amino Acid Transport Systems of Achlya: Regulatory Aspects of Cytokinins and Calcium. M.Sc. Thesis, University of Manitoba
- Skoog, F. & D.J.Armstrong (1970) Cytokinins. Ann.Rev.Plant Physiol. 21: 359-384
- Skoog, F., D.J.Armstrong, J.D.Charayil, A.C.Hampel & R.M.Bock (1966) Cytokinin activity: localization in transfer RNA preparations. Science 154: 1354-1356
- Skoog, F. & N.J.Leonard (1967) Sources and structure: activity relationships of cytokinins. in Biochemistry and Physiology of Plant Growth Substances, (F.Wightman & G.Setterfield, eds.), The Runge Press Ltd., Ottawa (1968), pp. 1-18
- Skoog, F. & C.O.Miller (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Sympos. Soc.Exptl.Biol. 11: 118-131
- Skoog, F., R.Y.Schmitz, R.M.Bock & S.M.Hecht (1973) Cytokinin antagonists: synthesis and physiological effects of 7-substituted 3-methylpyrazolo [4,3-d] pyrimidines. Phytochem. 12: 25-37
- Skoog, F., F.M.Strong & C.O.Miller (1965) Cytokinins. Science 148: 532-533
- Skye, G.E. & I.H.Segel (1970) Independent regulation of cysteine and cystine transport in Penicillium chrysogenum. Arch. Biochem.Biophys. 138: 306-318
- Smaluck, L.M. (1971) Transcriptional and Translational Control of Glutamic Dehydrogenases in Fungi. M.Sc. Thesis, University of Manitoba.

- Snell, W.H. & E.A.Dick (1971) A Glossary of Mycology, Harvard University Press, Cambridge, Mass. (1971), 181 pp.
- Sood, S. & R.N.Chopra (1973) A record preponement of bud-induction in the moss Entodon myurus. Z.Pflanzenphysiol. 69: 390-393
- Speziali, G.A.G. & R.van Wijk (1971) Cyclic 3',5'-AMP phosphodiesterase of Saccharomyces carlsbergensis. Inhibition by adenosine 5'-triphosphate, inorganic pyrophosphate, and inorganic polyphosphate. Biochim.Biophys.Acta 235: 466-472
- Stachow, C.S. (1965) Glutamate Dehydrogenase: Kinetic Analysis and Enzyme Regulation. Ph.D Thesis, University of Manitoba
- Stachow, C.S. & B.D.Sanwal (1967) Regulation, purification and some properties of the NAD-specific glutamate dehydrogenase of Neurospora. Biochim.Biophys.Acta 139: 294-307
- Stadtman, E.R. (1966) Allosteric regulation of enzyme activity. Advan.Enzymol. & Rel.Areas Mol.Biol. 28: 41-154
- Stein, W.D. (1967) The Movement of Molecules across Cell Membranes. Academic Press, New York & London (1967), 369 pp.
- Steiner, A.L., D.M.Kipnis, R.Utiger & C.W.Parker (1969) Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proc.Nat.Acad.Sci. U.S. 64: 367-373
- Strickland, W.N. (1971) Regulation of glutamate dehydrogenases in Neurospora crassa as a response to carbohydrates and amino acids in the media. Aust.J.Biol.Sci. 24: 905-915
- Śrivastava, B.I.S. (1967) Cytokinins in plants. in Internatl. Rev.Cytol. 22: 349-387, Academic Press
- Sueoka, N. & T. Kano Sueoka (1970) Transfer RNA and cell differentiation. Prog.Nucleic Acid Res. Mol.Biol. 10: 23-55
- Surdin, Y., W.Sly, J.Sire, A.M.Bordes & H.deRobichon-Szulmajster (1965) Propriétés et contrôle génétique du système d'accumulation des acides aminés chez Saccharomyces cerevisiae. Biochim. Biophys.Acta 107: 546-566
- Sutherland, E.W. & T.W.Rall (1958) Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J.Biol.Chem. 232: 1077-1091
- Sutherland, E.W., T.W.Rall & T.Menon (1962) Adenyl cyclase. I. Distribution, preparation and properties. J.Biol.Chem. 237: 1220-1227

- Sy, J. & D.Richter (1972 a) Separation of a cyclic 3',5'-adenosine monophosphate binding protein from yeast. Biochem. 11: 2784-2787
- Sy, J. & D.Richter (1972 b) Content of cyclic 3',5'-AMP and adenylyl cyclase in yeast at various growth conditions. Biochem. 11: 2788-2791
- Szweykowska, A. (1962) The effects of kinetin and IAA on shoot development in Funaria hygrometrica and Ceratodon purpureus. Acta Soc.Botan.Polon. 31: 553-557
- Takai, Y., H.Yamamura & Y.Nishizuka (1974) Adenosine 3':5'-monophosphate-dependent protein kinase from yeast. J.Biol.Chem. 249: 530-535
- Taketa, K. & B.M.Pogell (1966) The effect of palmityl coenzyme A on glucose-6-phosphate dehydrogenase and other enzymes. J.Biol.Chem. 241: 720-726
- Talal, N. & G.M.Tomkins (1964) Allosteric properties of glutamate dehydrogenases from different sources. Science 146: 1309-1311
- Talley, D.J., L.H.White & R.R.Schmidt (1972) Evidence for NADH- and NADPH-specific isoenzymes of GDH and the continuous inducibility of the NADPH-specific isoenzyme throughout the cell cycle of the eukaryote Chlorella. J.Biol.Chem. 247: 2927-2935
- Tao, M., M.L.Salas & F.Lipmann (1970) Mechanism of activation by adenosine 3',5'-cyclic monophosphate of a protein phosphokinase from rabbit reticulocytes. Proc.Nat.Acad.Sci. U.S. 67: 408-414
- Tavares, J. & H.Kende (1970) The effect of 6-benzylaminopurine on protein metabolism in senescing corn leaves. Phytochem. 9: 1763-1770
- Tellez-Inon, M.T. & H.N.Torres (1970) Interconvertible forms of glycogen phosphorylase in Neurospora crassa. Proc.Nat.Acad.Sci. U.S. 66: 459-463
- Terrine, C., M.Dorée, J.Guern & R.H.Hall (1972) Uptake of cytokinins by Acer pseudoplatanus cells: enzymes of the adenosine deaminase type as possible regulators of the cytokinin level inside the cell. in Plant Growth Substances 1970 (D.J.Carr, ed.), Springer-Verlag, (1972), pp. 467-475
- Terrine, C., P.Sadorge, M.Gawer & J.Guern (1969) Étude de la dégradation par voie enzymatique des analogues N<sup>6</sup>-substitués de l'adénosine. I. Action de l'adénosine aminohydrolase in vitro. Physiol. Vég. 7: 425-435

- Thimann, K.V. & T.Sachs (1966) The role of cytokinins in the "fasciation" disease caused by Corynebacterium fascians. Amer.J.Bot. 53: 731-739
- Thimann, K.V., H.Shibaoka & C.Martin (1972) On the nature of senescence in oat leaves. in Plant Growth Substances 1970, (D.J.Carr, ed.), Springer-Verlag (1972), pp. 561-570
- Thompson, W.J. & M.M.Appleman (1971) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochem. 10: 311-316
- Thwaites, W.M. & L.Pendyala (1969) Regulation of amino acid assimilation in a strain of Neurospora crassa lacking basic amino transport activity. Biochim.Biophys.Acta 192: 455-461
- Tomkins, G.M., T.D.Gelehrter, D.Granner, D.Martin, Jr., H.H.Samuels & E.B.Thompson (1969) Control of specific gene expression in higher organisms. Science 166: 1474-1480
- Tomkins, G.M., K.L.Yielding & J.Curran (1961) Steroid hormone activation of L-alanine oxidation catalyzed by a subunit of crystalline glutamate dehydrogenase. Proc.Nat.Acad.Sci. U.S. 47: 270-278
- Tomkins, G.M., K.L.Yielding, N.Talal & J.Curran (1963) Protein structure and biological regulation. Cold Spring Harbour Sympos. Quantit.Biol. 28:461-471
- Tomkins, G.M., K.L.Yielding, J.F.Curran, M.R.Summers & M.W. Bitensky (1965) The dependence of the substrate specificity on the conformation of crystalline glutamate dehydrogenase. J.Biol.Chem. 240: 3793-3798
- Torrey, J.G. (1962) Auxin and purine interactions in lateral root initiation in isolated pea root segments. Physiol. Plant. 15: 177-185
- Trewavas, A. (1972) Control of the protein turnover rates in Lemna minor. Plant Physiol. 49: 47-51
- Tsuboi, M., S.Kamisaka & N.Yanagishima (1972) Effect of cyclic 3',5'-adenosine monophosphate on the sporulation of Saccharomyces cerevisiae. Plant Cell Physiol. 13: 585-588
- Tsuboi, M. & N.Yanagishima (1973) Effect of cyclic AMP, theophylline, and caffeine on the glucose repression of sporulation in Saccharomyces cerevisiae. Archiv. für Mikrobiol. 93: 1-12
- Tuveson, R.W., D.J.West & R.W.Barratt (1967) Glutamic acid dehydrogenases in quiescent and germinating conidia of Neurospora crassa. J.Gen.Microbiol. 48: 235-248

- Uno, I. & T.Ishikawa (1973 a) Purification and identification of the fruiting-inducing substances in Coprinus macrorhizus. J.Bacteriol. 113: 1240-1248
- Uno, I. & T.Ishikawa (1973 b) Metabolism of adenosine 3':5'-cyclic monophosphate and induction of fruiting bodies in Coprinus macrorhizus. J.Bacteriol. 113:1249-1255
- Uno, I. & T.Ishikawa (1974) Presence of multiple protein kinase activities in Coprinus macrorhizus. Biochim.Biophys.Acta 334: 354-360
- Valadon, L.R.G. & R.S.Mummery (1971) Quantitative relationship between various growth substances and bud production in Funaria hygrometrica. A bioassay for abscisic acid. Physiol. Plant. 24: 232-234
- Vandepeute, J., R.C.Huffaker & R.Alvarez (1973) Cyclic nucleotide phosphodiesterase activity in barley seeds. Plant Physiol. 52: 278-282
- van der Plaats, J.B. (1974) Cyclic 3';5'-adenosine monophosphate stimulates trehalose degradation in baker's yeast. Biochem. Biophys.Res.Comm. 56: 580-587
- van Eyk, J. & H.Veldstra (1966) A comparative investigation of kinetin (6-furfurylaminopurine) and some similarly substituted purines and pyrimidines with Lemna minor. Phytochem. 5: 457- 462
- van Wijk, R. & Th.M.Konijn (1971) Cyclic 3',5'-AMP in Saccharomyces carlsbergensis under various conditions of catabolite repression. FEBS Lettr. 13: 184-186
- Walsh, D.A., E.G.Krebs, E.M.Reimann, M.A.Brostrom, J.D.Corbin, J.P. Hickenbottom, T.R.Soderling & J.P.Perkins (1970) The receptor protein for cyclic AMP in the control of glycogenolysis. in Role of cAMP in Cell Function, ( P.Greengard & E.Costa, eds.), Raven Press, New York (1970), pp.265-285
- Walton, G.M. & L.D.Garren (1970) An assay for adenosine 3',5'-cyclic monophosphate based on the association of the nucleotide with a partially purified binding protein. Biochem. 9: 4223-4229
- Warburg, O. & W.Christian (1942) Isolierung und Kristallization des Gärungsferments Enolase. Biochem.Z. 310: 384-421
- Webb, J.L. (1966) Mercurials. in Enzyme and Metabolic Inhibitors v.II, J.L.Webb, Academic Press, New York & London (1966), pp.729-1070

- Webster, R.K., D.H.Hall, J.Heeres, C.M.Wick & D.M.Brandon (1970) Achlya klebsiana and Pythium species as primary causes of seed rot and seedling disease of rice in California. Phytopath. 60: 964-968
- Wellburn, A.R., J.P.Ashby & F.A.M.Wellburn (1973) Occurrence and biosynthesis of adenosine 3',5'-cyclic monophosphate in isolated Avena etioplasts. Biochim.Biophys.Acta 320: 363-371
- Werner, D. & D.Gogolin (1970) Kennzeichnung der Bildung und Alterung von Wurzeln in Callus- und Organ-Kulturen von Daucus carot durch die Aktivität der Glutamatdehydrogenase. Planta 91: 155-164
- West, D.J., R.W.Tuveson, R.W.Barratt & J.R.S.Fincham (1967) Allosteric effects on nicotinamide adenine dinucleotide phosphate-specific glutamic dehydrogenase from Neurospora. J.Biol.Chem. 242: 2134-2138
- Westphal, H. & H.Holzer (1964) Synthese von NAD-abhängiger glutamatdehydrogenase in protoplasten von Saccharomyces carlsbergensis. Biochim.Biophys.Acta 89: 42-46
- Whitty, C.D. & R.H.Hall (1974) A cytokinin oxidase in Zea mays. Proc.Can.Fed.Biol.Soc. 17: 163 (Abst. 649)
- Wickson, M. & K.V.Thimann (1958) The antagonism of auxin and kinetin in apical dominance. Physiol.Plant. 11: 62-74
- Wold, W.S.M. (1974) The Citric Acid Fermentation by Aspergillus niger: Regulation of Growth, Cell Adhesion, and Citric Acid Accumulation by Zinc and Adenosine 3',5'-cyclic Monophosphate. Ph.D Thesis, University of Manitoba
- Wold, W.S.M. & I.Suzuki (1973 a) Cyclic AMP and citric acid accumulation by Aspergillus niger. Biochem.Biophys.Res.Comm. 50: 237-244
- Wold, W.S.M. & I.Suzuki (1973 b) Promotion of conidia aggregation in Aspergillus niger by cyclic AMP and 5'-GMP. Biochem.Biophys.Res.Comm. 55: 824-830
- Wolff, J. (1962) The effect of thyroxine on isolated dehydrogenases. II. Sedimentation changes in glutamic dehydrogenases. and III. The site of action of thyroxin on glutamic dehydrogenase, the function of adenine and guanine nucleotides, and the relation of kinetic to sedimentation changes. J.Biol.Chem. 237: 230-235, and 236-242
- Wood, H.N. (1970) Revised identification of the chromophore of a cell division factor from crown gall tumor cells of Vinca rosea L. Proc.Nat.Acad.Sci. U.S. 67: 1283-1287

- Wood, H.N. & A.C.Braun (1967) The role of kinetin (6-furfuryl-aminopurine) in promoting division in cells of Vinca rosea L. Ann.N.Y.Acad.Sci. 144: 244-250
- Wood, H.N. & A.C.Braun (1973) 8-Bromoadenosine 3',5'- cyclic monophosphate as a promoter of cell division in excised tobacco pith parenchyma tissue. Proc.Nat.Acad.Sci. U.S. 70: 447-450
- Wood, H.N., A.C.Braun, H.Brandes & H.Kende (1969) Studies on the distribution and properties of a new class of cell division-promoting substances from higher plant species. Proc.Nat. Acad.Sci. U.S. 62: 349-356
- Wood, H.N., M.C.Lin & A.C.Braun (1972) The inhibition of plant and animal adenosine 3',5'-cyclic monophosphate phosphodiesterase by a cell-division-promoting substance from tissues of higher plant species. Proc.Nat.Acad.Sci. U.S. 69: 403-406
- Wright, B.E. (1966) Multiple causes and controls in differentiation. Science 153: 830-836
- Zachau, H.G., D.Dütting, H.Feldman, F.Melchers & W.Karau (1966) Serine specific transfer ribonucleic acids. XIV. Comparison of nucleotide sequences and secondary structure models. Cold Spring Harbour Sympos.Quantit.Biol. XXXI : 417-424
- Zielke, R.H. & P.Filner (1971) Synthesis and turnover of nitrate reductase induced by nitrate in cultured tobacco cells. J.Biol.Chem. 246: 1772-1779
- Zubay, G., D.Schwartz & J.Beckwith (1970) Mechanism of activation of catabolite-sensitive genes: A positive control system. Proc.Nat.Acad.Sci. U.S. 66: 104-110