BIOCHEMICAL STUDIES ON POLLEN AND POLLEN-PISTIL INTERACTION IN *PETUNIA HYBRIDA*.

by

RAJENDER KUMAR KAMBOJ, M.Sc.

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Department of Agricultural Biochemistry,
Waite Agricultural Research Institute,
The University of Adelaide

-oOo-

March, 1986.

PREFACE

Part of the work described in this thesis has been published or submitted for publication and/or presented at scientific meetings as indicated below.

1. Uridine uptake and incorporation into RNA of germinating pollen of Petunia hybrida.
   R.K. Kamboj and J.F. Jackson.
   Proc. Aust. Biochem. Soc. 15, 77 (1983).
2. Localization of phytic acid in the floral structure of Petunia hybrida and relation to the incompatibility genes.
   J.F. Jackson, R.K. Kamboj and H.F. Linskens.
   Theor. Appl. Genet. 64, 259-262 (1983).
3. Divergent transport mechanisms for pyrimidine nucleosides in Petunia pollen.
   R.K. Kamboj and J.F. Jackson.
   Plant Physiol. 75, 499-501 (1984).
4. Energy-driven protein release from germinating pollen of Petunia hybrida.
   R.K. Kamboj, H.F. Linskens and J.F. Jackson.
   Ann. Bot. 54, 647-652 (1984).
5. Active uridine transport and non-active carrier-mediated thymidine transport in Petunia pollen.
   R.K.Kamboj and J.F. Jackson.
   Proc. Aust. Biochem. Soc. 16, 6 (1984).
6. Amino acid transport in Petunia pollen.
   R.K. Kamboj and J.F. Jackson,
   Abstracts Aust. Soc. Pl. Physiol. 25, 138 (1985).
7. Protein synthesis and release in germinating Petunia pollen.
   R.K. Kamboj and J.F. Jackson.
   Proc. Aust. Biochem. Soc. 17, 53 (1985).
8. Control of protein release from germinating pollen.
   J.F. Jackson and R.K. Kamboj.
   Biotechnology and Ecology of pollen, by Springer (1985) In Press. (369-372), 1986.
9. Control of gene expression and protein release in germinating pollen.
   R.K. Kamboj and J.F. Jackson.
   Abstracts Int. Congress Biochem, Amsterdam 13, TU101 (1985).
10. Pyrimidine nucleoside uptake by Petunia pollen: specificity and inhibitor studies on the carrier-mediated transport.
    R.K. Kamboj and J.F. Jackson.
    Plant Physiol. 79, 801-805 (1985).
11. Self-incompatibility alleles control a low molecular weight, basic protein in pistils of Petunia hybrida.
    R.K. Kamboj and J.F. Jackson.
    Theor. Appl. Genet. (1986). In Press.

ACKNOWLEDGEMENTS

I wish to express my deep and sincere thanks to Dr. J.F. Jackson for his guidance, constant encouragement and constructive criticism throughout the progress of the present investigation and the preparation of this manuscript. My sincere thanks are also due to Professor D.J.D. Nicholas for his invaluable advice and suggestions during the course of this investigation and in preparation of this manuscript.

I would also like to thank Mrs. Delrae Irving for her assistance at various stages of my studies, Mr. R. Miles for the help with electron microscopy, Mr. B.A. Palk for preparing the photographic prints, Mrs. J. Semets (GTA-Galenco) for skilled typing and to all my friends who helped me from time to time.

The award of U.R.G. scholarship by the University of Adelaide is greatfully acknowledged.

Finally, I wish to sincerely thank my parents Mr. and Mrs. K.R. Kamboj and my wife Beena for their constant encouragement.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person except when due reference is made in the text.

If accepted for the award of a Ph.D. degree, this thesis will be available for loan and photocopying.

Rajender Kumar Kamboj.

NOMENCLATURE AND ABBREVIATIONS

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by Enzyme Commission (Enzyme nomenclature 1978).

Trivial Name	E.C. Name and Number
Acid phosphatase	Orthophosphoric-monoester phosphohydrolase (acid optimum) E.C.3.1.3.2
Phytase	myo-Inositol-hexakisphosphate 6-phosphohydrolase E.C.3.1.3.26
Pyrophosphatase	Pyrophosphate phosphohydrolase E.C.3.6.1.1

The abbreviations for chemicals, symbols and units in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (Biochem. J. (1966) 101, 1-7) or the Instruction to Authors for the Biochemica et Biophysica Acta (BBA (1982) 715, 1-23).

Chemicals

ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CCCP	carbonylcyanide-m-chlorophenylhydrazone
DCCD	N,N'-dicyclohexylcarbodiimide
DIECA	diethyldithiocarbamate (sodium salt)
DNP	2,4-dinitrophenol
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (-aminoethyl ether) N,N,N'N'-tetraacetic acid
mRNA	messenger ribonucleic acid
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
NEM	N-ethylmaleimide
pCMB	p-chloro mercuribenzoate
PMSF	phenyl methyl sulphonyl fluoride
POPOP	1,4-bis (2,(4-methyl-5-phenyl oxazolyl)) benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
SDS	sodium-dodecylsulphate
TCA	trichloroacetic acid
Tris	2-amino-2-hydroxymethyl propane-1,3-diol

Symbols and Units

^{o}C	degree centigrade (Celsius)
Ci	curie
g	unit of gravitational field
h	hour(s)
kJ	kilojoule(s)
K_m	Michaelis constant
M	molar
mCi	millicurie
mg	milligram
µCi	microcurie
µg	microgram
µl	microlitre
µmol	micromole(s)
µM	micromolar
ml	millilitre
mmol	millimole(s)
mM	millimolar
min	minute(s)
nm	nanometer
nmol	nanomole(s)
%	percent
pg	picogram(s)
Pi	inorganic phosphate
pmol	picomole(s)
s	second(s)
V_{max}	rate of enzyme catalysed reaction at infinite concentration of substrate

Others

e.g.	for example
et al.	et alia (and others)
i.e.	that is
IEF	Isoelectric focussing
No.	Number
/	per
p. (plural pp.)	page
PAGE	Polyacrylamide gel electrophoresis
<	less than
v/v	volume : volume
w/v	weight : volume

TABLE OF CONTENTS

TABLE OF CONTENTS

	PREFACE	i
	ACKNOWLEDGEMENTS	ii
	DECLARATION	iii
	NOMENCLATURE AND ABBREVIATIONS	iv
	TABLE OF CONTENTS	vi
	LIST OF TABLES	xi
	LIST OF FIGURES	xiii
	SUMMARY	xvi
1.	**INTRODUCTION.**	1
1.1	**Pollen development.**	1
1.2	**Pistil (Stigma and Style).**	1
1.3	**Fertilization in flowering plants.**	3
1.3.1	Pollen germination and tube growth.	3
1.3.1.1	Uptake of nucleosides.	4
1.3.1.2	Uptake of amino acids.	5
1.3.1.3	Protein release.	6
1.3.1.4	Protein synthesis.	6
1.3.1.5	Protein phosphorylation.	7
1.3.2	Self-incompatibility.	8
1.3.2.1	Self-incompatibility genotype and phytic acid.	8
1.3.2.2	Self-incompatibility genotype associated glycoproteins.	9
1.3.2.3	Messenger RNA and gene expression.	9
1.4	**Aims of the study.**	10
2.	**MATERIALS AND METHODS.**	12
2.1	**Materials.**	12
2.1.1	Plant Material.	12
2.1.2	Chemicals.	12
2.1.3	Radioisotopes.	13
2.1.4	Solutions, buffers and solvents.	13
2.1.5	Cell-free translation extracts and marker proteins.	13
2.2	**Methods.**	13
2.2.1	Germination and pollen tube growth.	13
2.2.2	Transport assays.	14
2.2.2.1	Pyrimidine and purine nucleoside.	14
2.2.2.2	pH dependence of nucleoside transport.	14
2.2.2.3	Amino acids.	15

2.2.2.4	Identification of transported amino acids.	15
2.2.2.5	pH dependence of amino acid transport.	15
2.2.3	Efflux experiments.	16
2.2.4	Pollen intracellular space measurement.	16
2.2.5	Enzyme extraction.	16
2.2.6	Enzyme assays.	16
2.2.6.1	Phytase.	16
2.2.6.2	Acid phosphatase.	17
2.2.6.3	Acid pyrophosphatase.	17
2.2.7	Labelling and isolation of proteins.	18
2.2.7.1	Method (A).	18
2.2.7.2	Method (B).	18
2.2.7.3	Method (C).	18
2.2.8	Extraction of total RNA.	19
2.2.9	Purification of messenger RNA (mRNA).	19
2.2.10	Cell-free translation systems.	20
2.2.10.1	Rabbit reticulocyte lysate system.	20
2.2.10.2	Wheat germ system.	21
2.2.11	Preparation of protein samples.	21
2.2.11.1	For SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis).	21
2.2.11.2	For IEF (Isoelectric focussing).	22
2.2.12	Polyacrylamide gel electrophoresis (PAGE).	22
2.2.12.1	Cathodic discontinuouse - PAGE.	22
2.2.12.2	SDS-PAGE.	23
2.2.12.3	Two-dimensional gel electrophoresis.	23
2.2.13	Protein detection on gels.	23
2.2.13.1	Staining with Coomassie Blue.	23
2.2.13.2	Fluorography.	24
2.2.13.3	Autoradiography.	24
2.2.14	General techniques.	24
2.2.14.1	Electron-microscopy.	24
2.2.14.2	Liquid scintillation spectrometry.	24
2.2.15	Chemical determinations.	25
2.2.15.1	Protein.	25
2.2.15.2	ATP.	25
2.2.15.3	Phytic acid.	25
3.	**RESULTS.**	26
3.1	**Uptake of pyrimidine nucleosides.**	26
3.1.1	General characteristics.	26

3.1.2	Kinetics of Pyrimidine Nucleoside Transport.	26
3.1.3	Effect of metabolic inhibitors.	28
3.1.4	Transport of pyrimidine nucleoside analogues.	28
3.1.5	Conversion of transported nucleosides to nucleotides.	30
3.1.6	Effect of analogues on pyrimidine nucleoside transport.	33
3.1.7	Effect of thiol binding agents, chelators and other inhibitors on transport.	33
3.1.8	Effect of ions on pyrimidine nucleoside transport.	36
3.2	**Uptake of purine nucleosides.**	**36**
3.2.1	General characteristics of purine nucleosides, uptake.	36
3.2.2	Kinetic analysis of transport.	40
3.2.3	Effect of metabolic inhibitors and other agents.	40
3.2.4	Conversion of transported nucleosides to nucleotides.	42
3.2.5	Influence of metabolic inhibitors on ATP level.	42
3.2.6	Effect of analogues on purine nucleoside transport.	42
3.2.7	Effect of cations on purine nucleoside uptake.	45
3.3	**Transport of amino acids.**	**45**
3.3.1	General properties of amino acid transport.	45
3.3.2	pH dependence of transport of L-Glu, L-Met and L-Arg.	50
3.3.3	Kinetic analysis of uptake.	50
3.3.4	Effect of metabolic inhibitors and other agents.	51
3.3.4.1	Methionine and glutamic acid transport.	51
3.3.4.2	Arginine transport.	54
3.3.5	Specificity of transport of amino acids.	55
3.3.5.1	Inhibition of methionine and glutamic acid uptake by other amino acids and analogs.	55
3.3.5.2	Inhibition of arginine transport by other amino acids.	58
3.3.6	Effect of calcium, metal chelators and polyamines on L-Met and L-Glu transport.	58
3.3.7	Effect of cations and polyamines on L-Arg transport.	61
3.4	**Control of protein synthesis and protein released during germination and pollen tube growth.**	**64**
3.4.1	Control of protein release.	64

3.4.2	Effect of various inhibitors of protein release on the SDS-PAGE patterns of proteins released.	70
3.4.3	Changes in enzyme activities and enzymes released during germination and pollen tube growth.	70
3.4.4	Analysis of the SDS-PAGE patterns of pollen proteins and proteins released during germination and pollen tube growth.	72
3.4.5	Analysis of the SDS-PAGE patterns of proteins synthesized and their release during pollen germination and tube growth.	72
3.4.6	Analysis of the two-dimensional patterns of pollen proteins and proteins released during germination and pollen tube growth.	73
3.4.7	Analysis of the two-dimensional patterns of the newly synthesized pollen proteins and their release during germination and pollen tube growth.	77
3.4.8	Regulation of protein synthesis during germination and pollen tube growth of Petunia pollen.	80
3.4.9	Two-dimensional alectrophoretic analysis of regulation of protein synthesis during germination and pollen tube growth of Petunia pollen.	82
3.4.10	Two-dimensional electrophoretic analysis of the phosphorylated proteins and their release during germination and pollen tube growth.	85
3.5	**Incompatibility genotype and biochemical investigation during the interaction between pollen and pistil in Petunia hybrida.**	88
3.5.1	Distribution of phytic acid in the floral structure.	88
3.5.2	Phytase activity in the flower.	88
3.5.3	Changes in phytic acid levels after pollination and influence of the S genes.	91
3.5.4	Sparing effect of styles on pollen phytic acid breakdown.	93
3.5.5	Other factors involving stability of phytic acid in pollen.	93
3.6	**Incompatibility genotype and gene expression in pollen and pistil of Petunia hybrida.**	93
3.6.1	Self-incompatibility genotype associated proteins from the pistils.	93
3.6.2	Comparison between pistil and pollen proteins.	96
3.6.3	Isoelectric focussing and two-dimensional gel electrophoresis of pistil proteins.	99
3.6.4	Messenger RNAs in pollen and pistils of Petunia hybrida and cell-free synthesis of polypeptides.	99

4.	**DISCUSSION.**	103
4.1	**Uptake of nucleosides.**	103
4.1.1	Uptake of pyrimidine nucleosides.	103
4.1.2	Uptake of purine nucleosides.	106
4.2	**Transport of amino acids.**	108
4.2.1	L-methionine and L-glutamic acid transport.	108
4.2.2	Arginine transport.	110
4.3	**Control of protein synthesis and protein release during germination ond pollen tube growth of Petunia pollen.**	112
4.3.1	Control of protein release.	112
4.3.2	Protein synthesis during germination and pollen tube growth.	114
4.3.3	Protein phosphorylation and their release during germination and pollen tube growth.	119
4.4	**Incompatibility genotype and biochemical investigation during the interaction between pollen and pistil.**	119
4.5	**Incompatibility genotype and gene expression in Petunia pollen and pistil.**	121
4.5.1	Self-incompatibility genotype associated glycoproteins.	121
4.5.2	Messenger RNAs in pollen and pistils of Petunia hybrida and cell-free synthesis of polypeptides.	122
5.	**BIBLIOGRAPHY.**	124

LIST OF TABLES

Table 1.	Effect of metabolic inhibitors and antagonists on pyrimidine nucleoside transport into Petunia pollen.	29
Table 2.	Effect of metabolic inhibitors and other agents on deoxyuridine and 5-bromodeoxyuridine transport in Petunia pollen.	31
Table 3.	Conversion of pyrimidine nucleosides to nucleotides.	32
Table 4.	Effect of various analogues and related compounds in pyrimidine nucleoside transport in Petunia pollen.	34
Table 5.	Effect of sulphydryl and other reagents on pyrimidine nucleoside transport in Petunia pollen.	35
Table 6.	Effect of cations on pyrimidine nucleoside transport in Petunia pollen.	37
Table 7.	Effect of anions on pyrimidine nucleoside transport in Petunia pollen.	38
Table 8.	Effect of metabolic inhibitors and other agents on purine nucleoside transport and on ATP levels in Petunia pollen.	41
Table 9.	Conversion of purine nucleosides to nucleotides.	43
Table 10.	Influence of various metabolic inhibitors on the ATP level during germination and pollen tube growth.	43
Table 11.	Effect of nucleosides, analogues and polyamines on adenosine and guanosine transport into Petunia pollen.	44
Table 12.	Effect of cations on purine nucleoside transport in Petunia pollen.	46
Table 13.	Kinetic constants for L-methionine and L-glutamic acid transport by Petunia pollen.	52
Table 14.	Effect of metabolic inhibitors and thiol reagents on L-methionine and L-glutamic acid transport in Petunia pollen.	53
Table 15.	Effect of metabolic inhibitors and thiol reagents on L-arginine transport in Petunia pollen.	56
Table 16.	Effect of amino acids and analogues on L-methionine and L-glutamic acid transport in Petunia pollen.	57
Table 17.	Effect of hundred-fold concentrations of amino acids on L-methionine and L-glutamic acid transport in Petunia pollen.	59
Table 18.	Effect of amino acids on L-arginine transport in Petunia pollen.	60

Table 19.	Effect of calcium, metal chelators and polyamines on L-methionine and L-glutamic acid transport in *Petunia* pollen.	62
Table 20	Effect of cations and polyamines on L-arginine transport in *Petunia* pollen.	63
Table 21.	The effect of various compounds on protein release, germination and pollen tube length, after 5h culture of *Petunia hybrida* pollen.	65
Table 22.	The effects of inhibitors on protein release, germination and pollen tube length, after 5h culture of *Petunia hybrida* pollen.	66
Table 23.	Phytic acid distribution in the various parts of the flower and variation between clones W166K, W166H and T_2U.	89
Table 24.	Relative phytase activity in various parts of the flower in clones W166K, W166H and T_2U.	90
Table 25.	Phytic acid content of pistils 2h after pollination in various crosses between the clones.	92
Table 26.	Sparing effect of styles on phytic acid degradation during pollen germination *in vitro* for 2h.	94

LIST OF FIGURES

Fig. 1	Scanning electron micrographs of Petunia hybrida clone W166H pollen grains and pollen tubes.	2
Fig. 2	Kinetics of the transport of pyrimidine nucleosides and analogues into Petunia hybrida pollen.	27
Fig. 3a.	Effect of culture time on adenosine uptake in Petunia hybrida pollen.	39
3b.	Influence of pH on adenosine uptake.	
3c.	Time course of purine nucleoside uptake.	
3d.	Kinetic analysis of adenosine and guanosine transport.	
Fig. 4a.	Effect of culture time on transport rate of L-methionine and L-glutamic acid in Petunia pollen.	47
4b.	Influence of pH on uptake of L-methionine and L-glutamic acid.	
4c.	Double logarithmic plot of L-glutamic acid uptake as a function of external concentration.	
4d.	Double logarithmic plot of L-methionine uptake as a function of external concentration.	
Fig. 5	Lineweaver-Burk plots of L-glutamic acid (of data in Fig. 4c.) and L-methionine (of data in Fig. 4d.) uptake by Petunia pollen.	48
Fig. 6a.	Effect of cultivation time on L-arginine transport in Petunia hybrida pollen.	49
6b.	Effect of pH on L-arginine uptake.	
6c.	Double logarithmic plot of L-arginine uptake as a function of external concentration.	
6d,e,f.	Lineweaver-Burk plots of L-arginine uptake (of data shown in Fig. 6c.) by Petunia pollen.	
Fig. 7a,b.	Effect of various compounds on the time course of protein release from germinating pollen of Petunia hybrida.	67
7c.	Activities of various phosphatases during germination and pollen tube growth.	
7d.	Activities of various phosphatases released during germination and pollen tube growth.	
Fig. 8	SDS-PAGE and autoradiography of proteins released from germinating Petunia hybrida pollen by 2h.	69
Fig. 9A,B.	Coomassie Blue-stained SDS-PAGE (11% PAA) patterns of proteins released during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.	71
9C,D.	Coomassie Blue-stained patterns (C) and fluorographs (D) of SDS-PAGE (12.5% PAA) of pollen proteins and proteins released during germination and pollen tube growth in Petunia hybrida.	

Fig. 10	Coomassie Blue-stained two-dimensional gel electrophoric patterns of pollen proteins from various stages of germination and tube growth in Petunia hybrida.	74
Fig. 11	Coomassie Blue-stained two-dimensional gel electrophoretic patterns of proteins released during germination and pollen tube growth in Petunia hybrida.	76
Fig. 12	Fluorographs of two-dimensional gel electrophoretic analysis of pollen proteins synthesized during germination and pollen tube growth in Petunia hybrida.	78
Fig. 13	Fluorographs of two-dimensional gel electrophoretic analysis of newly-synthesized proteins released during germination and pollen tube growth in Petunia hybrida.	79
Fig. 14A,B.	Coomassie Blue-stained two-dimensional gel electrophoretic patterns of proteins.	81
14C,D.	Fluorographs of SDS-PAGE (12.5% PAA) of newly synthesized proteins released (C) and pollen proteins (D) synthesized during 3h culture of Petunia pollen in the presence or absence of various compounds.	
Fig. 15	Fluorographs of two-dimensional gel electrophoretic analysis of pollen proteins synthesized (A) and newly synthesized proteins released (B,C,D) during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.	83
Fig. 16A,C,D	Fluorographs of two-dimensional gel electrophoretic analysis of newly synthesized proteins released during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.	84
16B.	Coomassie Blue-stained two-dimensional gel electrophoretic pattern of proteins released in the absence of boron (i.e. 10% sucrose as culture medium).	
Fig. 17	Autoradiograms of two-dimensional gel electrophoretic analysis of phosphorylated proteins during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.	87
Fig. 18	Coomassie Blue-stained SDS-PAGE (12% PAA) pattern of pistil proteins from various clones of Petunia hybrida.	95
Fig. 19	Comparison of pistil and pollen proteins by SDS-PAGE (12% PAA).	97
Fig. 20	Schiff's stain for glycoproteins of pistil and pollen after SDS-PAGE (12% PAA).	98
Fig. 21	Coomassie Blue-stained two-dimensional gel electrophoretic patterns of pistil proteins.	100

Fig. 22 Fluorographs of SDS-PAGE (12% PAA) of proteins synthesized in the reticulocyte lysate cell-free system (A, B, C) and also in wheat germ system (D) with Petunia pollen or pistil poly(A)$^+$RNA. 101

SUMMARY

SUMMARY

1. In germinating Petunia hybrida pollen, transport of uridine, cytidine, deoxyuridine, 5-bromodeoxyuridine, adenosine and guanosine was found to show all the properties of an active, carrier-mediated process, a nucleoside transport mechanism not hitherto reported in plant cells. Thymidine transfer on the other hand behaved as if it was mediated by a non-active, carrier-mediated transport system. Thus, in Petunia hybrida pollen there is a unique situation of at least two transport systems for nucleosides having divergent properties.

2. Transport of all pyrimidine and purine nucleosides was inhibited by thiol reagents, indicating that both transport systems depend on carriers with intact sulphydryl groups.

3. The accumulation of uridine, deoxyuridine, adenosine and guanosine against a concentration gradient, in addition to evidence from the use of metabolic inhibitors (uncouplers and ATPase inhibitors) led to the conclusion that the pyrimidine and purine nucleosides with the exception of thymidine are transported by an active, carrier-mediated process.

4. ATP was very rapidly synthesized during the first 30 min of pollen germination in Petunia hybrida. All metabolic inhibitors inhibited the ATP synthesis and also decreased the level of intracellular ATP. There was no good correlation between ATP level and uptake of various substances.

5. In Petunia hybrida pollen, uptake of L-methionine and L-glutamic acid in the range of 1 μM to 2 mM could be represented by two phases. Analysis for uptake rates for L-arginine concentrations ranging from 1 μM to 2 mM showed that uptake was multiphasic with three saturable phases.

6. The accumulation of L-methionine, L-glutamic acid and L-arginine against a concentration gradient, in addition to evidence from the use of energy poisons (uncouplers and ATPase inhibitors) indicates that amino acids are transported into Petunia pollen by an active, carrier-mediated process.

7. Competition experiments using a variety of amino acids showed that L-glutamic acid was inhibited by L-aspartic acid only, thus indicating the presence of an highly specific transport carrier system for acidic amino acids. L-methionine uptake was inhibited by L-cysteine and the neutral amino acids only. Basic amino acids were without effect on both uptake systems

suggesting the presence of at least three different transport carrier systems for amino acids in Petunia hybrida pollen. Transport of all amino acids was pH-dependent, stereospecific and inhibited by thiol binding reagents.

8. In Petunia hybrida pollen, protein is gradually released into the culture medium during germination and pollen tube growth up to at least 5h, accumulation in the medium increasing with time in an hyperbolic relationship. In addition to the diffusible proteins passively released in the first hour of germination and pollen tube growth, proteins pre-existing in the pollen and newly synthesized proteins are released from the pollen by an energy-driven process during germination and pollen tube growth. The most effective inhibitors of this protein release are "energy poisons" such as CCCP, DCCD etc.

9. Several other compounds such as boron, calcium, auxins, spermine, thiol binding reagents etc. inhibited protein export from Petunia hybrida pollen. The protein synthesis inhibitors cycloheximide also inhibited bulk protein release, suggesting a role for some protein synthesis in the secretion process involving pre-existing proteins as well.

10. The two-dimensional gel electrophoretic separation showed that at least 300 different proteins are synthesized during pollen germination and tube growth, of which at least 200 are released into the culture medium. The same number and pattern of protein spots was seen whether or not α-amanitin (10 µg/ml) was included in the culture medium, indicating that mRNA present in the ungerminated pollen and the newly synthesized mRNA code for the same proteins.

11. The presence of calcium ions in the culture medium virtually stopped the release of all major storage proteins during pollen germination and tube growth, but had little inhibiting effect on the release of newly synthesized proteins. On the contrary, in the presence of calcium ions there was clear stimulation of release of a number of newly synthesized proteins. High concentration of α-amanitin (100 µg/ml) also gave similar stimulation of release of a number of newly synthesized proteins.

12. In Petunia hybrida pollen, acid phosphatase, acid pyrophosphatase and phytase activities were detected and a small fraction of the activity of all these enzymes was released during germination and pollen tube growth.

13. Ca^{2+}-dependent protein phosphorylation during pollen germination and tube growth in Petunia hybrida pollen has been demonstrated. Mg^{2+} ions also stimulated the phosphorylation of proteins but the results suggested that Mg^{2+} and Ca^{2+} ions regulate protein phosphorylation differently. However, neither Ca^{2+} nor Mg^{2+} had any effect on the release of phosphorylated proteins.

14. In three clones of Petunia hybrida with different incompatibility genes, phytic acid was detected exclusively in pollen, stigma and style. Phytase activity was detected in these tissues as well as in the ovary. The level of phytic acid and phytase activity varied between clones with different S alleles. This difference was most evident in stigma and style. The pattern of phytic acid breakdown following pollinations depended on whether pollen and pistil form a compatible or incompatible combination. Incompatible pollination resulted in an higher rate of degradation.

15. Proteins extracted from the pistils of several clones of Petunia hybrida carrying differing pairs of S alleles were examined by gel electrophoresis. The major protein of pistils, a basic glycoprotein of relatively low molecular weight, showed properties which varied in a simple manner with the S genotype. For each S allele, a specific molecular weight (ranging from 27,000 to 33,000) and isoelectric point (in the range 8.3 to 8.7) was assigned for this putative S protein. No evidence was obtained for the presence of this protein in pollen extracts.

16. Each mature ungerminated Petunia hybrida pollen grain contains approximately 100 pg of total RNA and 4 pg of poly(A)$^+$ RNA. The ability of the total RNA and poly(A)$^+$RNA to direct the synthesis of polypeptides in cell-free translation systems was a direct demonstration of the presence of presynthesized mRNA in mature Petunia pollen grains. One gram of dried mature pistils of Petunia hybrida were found to contain about 1 mg of total RNA and 25 µg of poly(A)$^+$ RNA. SDS-PAGE pattern of the in vitro translation products made with both total RNA and poly (A)$^+$ RNA from the mature pistils indicated the difficulty in locating the pistil glycoproteins that correspond to S genotype since the polypeptides synthesized in cell-free translation systems cannot have carbohydrate moities attached.

1. INTRODUCTION

1. INTRODUCTION.

1.1 Pollen development.

The pollen grain is the discrete and mobile stage of the male gametophyte of higher plants. Pollen development takes place in the microsporangia (pollen sacs) of anthers, in which cells of sporogenous tissue divide by mitosis, producing many pollen mother cells. Pollen mother cells undergo meiosis giving rise to a tetrad of microspores. After a resting period, the microspore nucleus divides in a very unequal manner, forming two cells, the vegetative and generative (Stanley and Linskens, 1974; Mascarenhas, 1975). The nucleus in the generative cell divides once more forming two sperm nuclei to give tri-nucleate pollen such as in Zea mays. The generative cell in most angiosperms pollen (e.g. Petunia hybrida) completes its division during the growth of the pollen tube (Stanley and Linskens, 1974). Pollen grains when mature have thick walls with elaborate and often beautiful surface patterns, as in Petunia hybrida (Fig. 1).

Pollen grains of Petunia are bilateral in origin (Ferguson and Collidge, 1932). When observed in dry air, they are ellipsoidal in outline when lying horizontal. There are normally three germ pores each located on a furrow extending the length of the grain (Fig. 1). The furrows are equidistant, thus giving the grains a triangular shape when viewed from the end (Sink, 1984).

Petunia hybrida pollen can be preserved for extended periods, up to several months, without great loss of viability. Thus, Petunia hybrida pollen is a suitable tool for research into the regulation of pollen germination and tube development at the biochemical and molecular biological level.

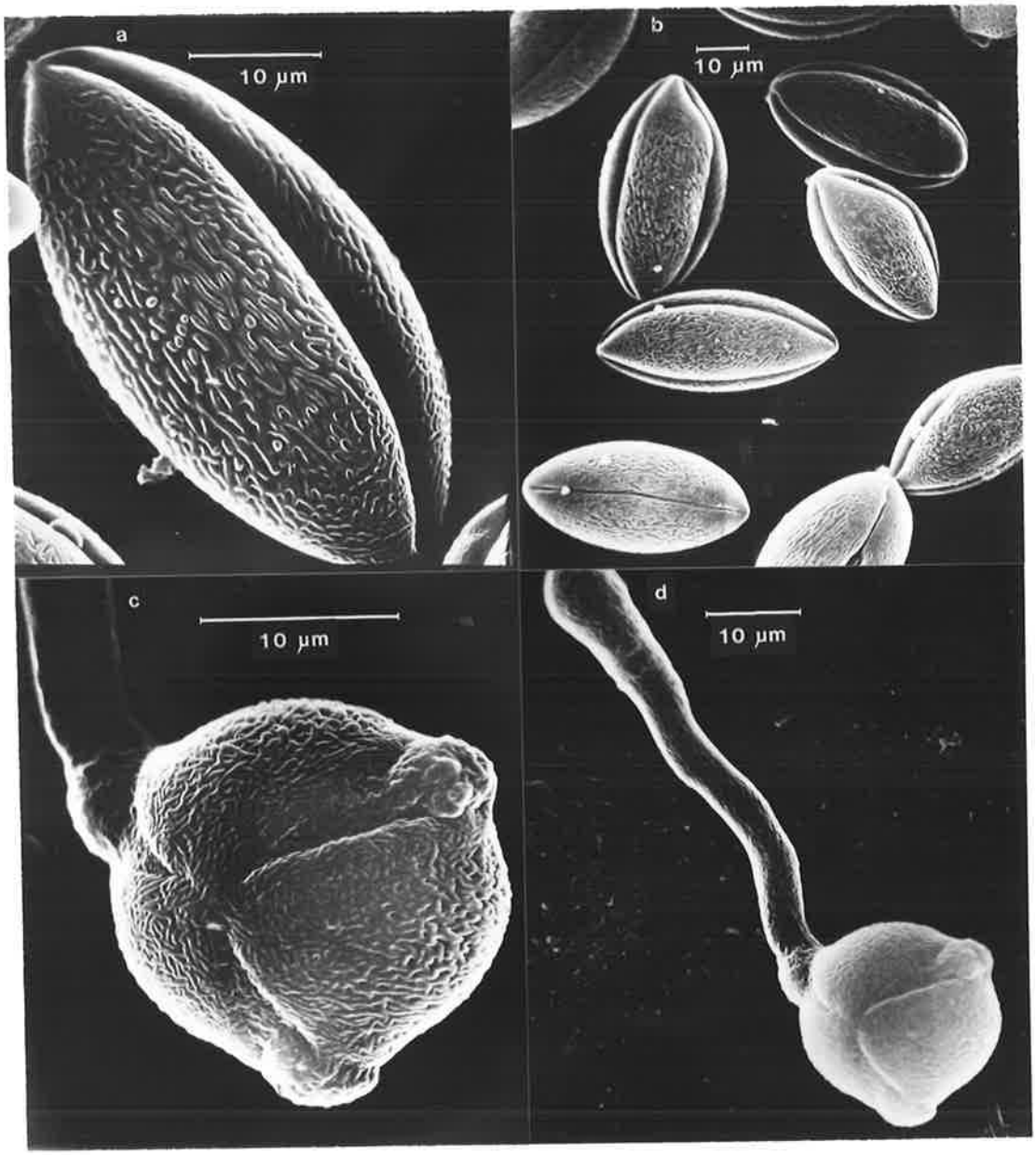
1.2 Pistil (Stigma and Style).

Petunia bears a solid pistil (stigma-style) with specialized transmitting tissue, sometimes called conductive tissue, through which pollen tubes grow to the ovary (Konar and Linskens, 1966a). Maturation of the stigma, which involved cellular degeneration

Fig. 1 **Scanning electron micrographs of Petunia hybrida clone W166H pollen grains and pollen tubes.**
Samples prepared as described in section 2.2.14.1

a,b. Dry, mature pollen grain illustrating exine wall pattern.

c,d. Germination pores and pollen tube.


a
10 µm
b
10 µm
c
10 µm
d
10 µm

and the release of stigmatic exudate, occurs at anthesis and is independent of pollination (Konar and Linskens, 1966a, b; Herraro and Dickinson, 1979). Transmitting tissue is a cylindrical mass of thick-walled cells, rich in cellular organelles and metabolites, characterized by large, matrix-filled, intercellular spaces (van der Pluijm and Linskens, 1966; Kroh, 1973; Kroh and van Bakel, 1973; Kroh and Helsper, 1974; Sassen, 1974; Herraro and Dickinson, 1979). Pollen tubes do not penetrate cells of the transmitting tissue but, rather, proceed down the style through the intercellular spaces (Schlosser, 1961; van der Pluijm and Linskens, 1966). The stigma-style of Petunia is critical in nutritive support of the male gametophyte and is the site of the self-incompatibility reaction (Ascher, 1984).

1.3 Fertilization in flowering plants.

In angiosperms or flowering plants the fertilization process can be divided into different phases: pollen germination, penetration of the pollen tubes into the stigma, growth of the pollen tubes in the style and syngamy (Linskens, 1968; Linskens and Kroh, 1970). The interaction between the pistil and pollen during the fertilization process includes several physiological and biochemical events such as nutrition of pollen tubes, incompatibility reaction, activation and growth of the ovary, translocation phenomena, accelerated senescence, wilting and abscission (Bredemeijer, 1984). All these reactions are accompanied by phase specific metabolic alterations and are little understood at the biochemical and molecular biological level.

1.3.1 Pollen germination and tube growth.

Pollen germination normally occurs in nature when pollen grains come in contact with the surface of the stigma, where they take up water and swell (Linskens and Schrauwen, 1969). On the stigma the correct conditions are present for the pollen germination and the growth of the pollen tube into the style. Germination of pollen can, to a certain degree, be imitated in vitro by placing the pollen in a suitable medium. In vitro germination experiments are, therefore, important for biochemical

and molecular biological analysis of the early activation processes.

1.3.1.1 Uptake of nucleosides.

Gene expression and RNA synthesis in pollen can be followed by incorporation of labelled uridine into RNA (Mascarenhas, 1966; Suss and Tupy, 1982), while pollen DNA repair is monitored by labelled thymidine incorporation into DNA (Jackson and Linskens, 1978, 1979, 1980, 1982). In addition it has been reported that the pool of these and other nucleic acid precursors increase in the style of Petunia hybrida following pollination (van der Donk, 1974), when there is a close interaction between style and pollen tube (Linskens and Kroh, 1970). A considerable saving in energy could be made if these precursors were taken up by the pollen, and salvaged for synthetic needs, rather than be synthesized within the pollen de novo as described earlier for other plant tissue (Ong and Jackson, 1972; Parker and Jackson, 1981). There is a need therefore to understand the uptake of these precursors by pollen since the incorporation of nucleoside precursors in other biological systems is limited by the rate of entry into the cell (Plagemann and Roth, 1969; Daentl and Epstein, 1971; Aller et al., 1980; Suss and Tupy, 1982). A knowledge of the mode of transport of these precursors into pollen may therefore be of practical importance for nucleic acid studies and useful in understanding the interaction between style and pollen tube. A similar situation exists between endosperm and embryo, where Kombrink and Beevers (1983) have shown movement of nucleosides from endosperm to embryo. These authors make the point that little is known about the mechanism of transport of nucleosides in plant cells in contrast to the wealth of information for bacterial and animal cells. In bacterial cells, nucleoside transfer takes place by an active, carrier-mediated process (Mygind and Munch-Peterson, 1975; Munch-Peterson et al., 1979; Foret and Ahlers, 1982), while animal cells carry out the transfer by a non-active, carrier-mediated mechanism (Plagemann and Richey, 1974; Wohlheuter et al., 1979; Plagemann and Wohlheuter, 1980, 1984; Young and Jarvis, 1983). In plant cells, a simple diffusion mechanism has been proposed for all pyrimidine nucleosides in Euglena (Wasternak, 1976) while Suss and Tupy

(1982) have shown that the uridine uptake system is saturable in tobacco pollen. Pollen cultures provide us with a convenient system for studying the mode of nucleoside transport in plant cells, for comparison with other cells.

1.3.1.2 Uptake of amino acids.

The study of amino acid transport and its characterization is of great importance since amino acids are essential to cell metabolism and growth. The addition of casein hydrolysate to pollen medium is reported to have a stimulatory effect on mitosis in pollen tubes of Tradescantia (Lafleur et al., 1981) and on pollen tube growth in tobacco (Tupy et al., 1983), which stresses the importance of external amino acids for pollen tube development. Protein synthesis is required for pollen tube growth (Capkova-Balatkova et al., 1980; Kamboj et al., 1984) and can be followed by incorporation of labelled amino acids into proteins (Mascarenhas et al., 1974; Zhang et al., 1982; Capkova et al., 1983; Kamboj et al., 1984). In addition it has been reported that pollen tubes of Lilium and Petunia draw nourishment (sugars and amino acids) from the stylar tissue (Vasil, 1974) and also, that compatible pollen tubes stimulate the mobilization of stylar reserves and subsequently utilize these products (Herraro and Dickinson, 1979). Since pollen tubes interact closely with stylar tissue in Petunia (Linskens and Kroh, 1970), then the style could be a good source of amino acids for protein synthesis required by the elongating pollen tube. There is a need therefore to understand the uptake of these precursors by pollen. Pollen cultures also provide us with a convenient system to characterize uptake systems in plant cells, for comparison with other cells (Kamboj and Jackson, 1984, 1985). A wide variety of amino acid uptake systems in animal and microbial cells have been identified and extensively characterized (Christensen, 1975). Although amino acid transport has been studied in several plant tissues, cultured cells and protoplasts (Reinhold and Kaplan, 1984), it has yet to be established whether one or several carriers mediate the transport of the various amino acids into higher plant cells (Reinhold and Kaplan, 1984).

1.3.1.3 Protein release.

The pollen of many angiosperms release proteins during germination and pollen tube extension, which could play a role in the complex interaction between pollen and stigma (Heslop-Harrison, 1975; Pacini et al., 1981). Some of these proteins have been delineated as sporophytic or gametophytic, and appear to be released from the pollen grain wall (Heslop-Harrison et al., 1975). It is no surprise then that enzyme activity has been shown to be associated with released proteins (Poddubnaya-Arnoldi et al., 1959; Makinen and Brewbaker, 1967; Matousek and Tupy, 1983), while the pollen of at least one species contains enzymes that can break down pectins (Kroh and Loewus, 1978). Several of the proteins have antigenic properties in some species (Heslop-Harrison, et al., 1974).

The controls operating on protein release during pollen tube extension must then have important implications for interaction between pollen and style and the rate of growth of pollen tube. It was observed by Stanley and Linskens (1964, 1965) that protein was steadily given up to the medium during Petunia hybrida pollen germination, while Kirby and Vasil (1979) investigated washings of Petunia pollen which yielded proteins. It was accepted in all these cases that protein diffuses out passively from the pollen. In the present study further investigations were carried out with Petunia hybrida pollen in order to uncover the controls operating on protein release.

1.3.1.4 Protein synthesis.

Protein synthesis is initiated during the early stages of Petunia pollen germination (Linskens et al., 1970; Zhang et al., 1982) and similar results have been found with Tradescantia pollen (Mascarenhas and Bell, 1969). Studies with the protein synthesis inhibitor cycloheximide indicated that protein synthesis is required for pollen tube growth (Mascarenhas, 1971; Capkova-Balatkova et al., 1980; Kamboj et al., 1984) and also for generative cell division (Shivanna et al., 1974).

In pollen from several plant species mRNA synthesis only has been observed during pollen germination and tube growth (Linskens et al., 1971; Mascarenhas, 1971; Steffensen, 1971). However, synthesis of rRNA, tRNA and mRNA has been reported in Nicotiana tabacum pollen (Tupy, 1977; Suss and Tupy, 1979) and in Malus domestica pollen (Bagni et al., 1981). When attempts are made to block RNA synthesis by the addition of actinomycin D to pollen cultures, pollen germination and early tube growth are not inhibited in several plant species (Mascarenhas, 1966; Mascarenhas, 1975; Kamboj et al., 1984). These results suggest that most of the proteins required for pollen germination are already present in the mature pollen. Apparently protein synthesis occurs in the absence of RNA synthesis, implying the presence of sufficient rRNA, tRNA and mRNA in mature pollen grain for germination and early pollen tube growth.

There are however problems associated with the use of actinomycin D. The specificity of actinomycin D in inhibiting DNA-dependent RNA synthesis has been questioned (Honig and Rabinovitz, 1965; Laszlo et al., 1966). There is increasing evidence that actinomycin D is only partially effective at inhibiting RNA synthesis in germinating seeds (Payne, 1976) and in germinating wheat embryos (Jendrisak, 1980), perhaps due to poor penetration of actinomycin D into these tissues (Neumann, 1964). Actinomycin D, a chromopeptide which forms nonionic complexes with DNA and in so doing, inhibits the action of RNA polymerases (Harbers and Muller, 1962). Further investigation using more effective and specific mRNA inhibitors such as α-amanitin which interacts directly with the RNA polymerase molecule (Cochet-Meilhac and Chambon, 1974), is needed.

1.3.1.5 Protein Phosphorylation.

In animal cells protein phosphorylation catalyzed by cyclic nucleotide or Ca^{2+}-dependent protein kinases represents the major general mechanism by which intracellular events respond to external physiological stimuli (Cohen, 1980). Attempts have been made to explore the role of cyclic AMP as a second messenger in plants. Although convincing evidence was obtained for the existence of cyclic AMP in plants, sufficient evidence could not

be obtained for its physiological role as a second messenger (Brown and Newton, 1981). Thus, there is increasing interest in defining the possible role of calcium as a second messenger in plants (Marme, 1982).

Ca^{2+}-dependent protein kinases have been resolved from higher plants recently (Hetherington and Trewavas, 1982; Polya and Davies, 1982; Polya et al., 1983; Polya and Micucci, 1984), adding evidence for an intracellular 'second messenger' function for Ca^{2+} in plants. Recently, Ca^{2+}-promoted phosphorylation of proteins of corn coleoptiles was reported (Veluthambi and Poovaiah, 1984). Never-the-less relatively little is known about the role of calcium in the phosphorylation of proteins from plant systems in general and pollen systems in particular.

1.3.2 Self-incompatibility.

Sexual incompatibility can be defined as the inability of hermaphroditic organisms to produce certain zygotes, notwithstanding that both the male and female gametes are normal and have the capacity to produce all possible zygotes (Linskens, 1975). There are two forms of sexual incompatibility: interspecific incompatibility and intraspecific or self-incompatibility. Interspecific incompatibility represents the barrier which may create species and races. All forms of incompatibility are governed by the genome and can be controlled by 1 or 2 genes with multiple alleles.

From a developmental point of view, self-incompatibility can be divided into two types. If the genome of the pollen-producing plant determines the reaction, it is called sporophytic incompatibility, and if the pollen genome is determinent, the type is called gametophytic incompatibility (Linskens, 1975). Petunia hybrida exhibits a multi-allelic single locus gametophytic self-incompatibility system (Linskens, 1975a).

1.3.2.1 Self-incompatibility genotype and phytic acid.

Gametophytic self-incompatibility involving control of pollen tube growth in the style, as seen for example in Petunia hybrida, is genetically directed by one multi-allelic S locus

(Linskens, 1975a). When the pollen is compatible with the pistil, tube growth continues down the style to the ovary and fertilization follows. Incompatibility between pollen and pistil leads to cessation of tube growth halfway down the style so that fertilization cannot be achieved. Factors relating to control of the elongation of the pollen tube are therefore worthy of investigation in seeking a mechanism for this type of incompatibility. Recently, it has been shown that pollen from species which need to develop tubes long enough to negotiate styles greater than about 4 mm, have significant quantities of phytic acid (Jackson et al., 1982). Furthermore, pollen from species showing strong gametophytic self-incompatibility of the single multi-allelic S locus type (Petunia hybrida, Antirrhinum majus, Lilium henryi) had an extaordinary high phytic acid content. These findings, together with the realization that little is known about the distribution of phytic acid within the flower other than its presence in pollen, prompted the present investigation into the interaction of the S genes with both the localization of phytic acid and phytic acid metabolism.

1.3.2.2 Self-incompatibility genotype associated glycoproteins.

Self-incompatibility in Petunia hybrida is of the gametophytic type controlled by a single S locus (Linskens, 1975a). S allele specific antigens have been detected in pistil extracts of Petunia hybrida (Linskens, 1960), while Gilissen (1978) has shown that the S allele products are present in the style before pollination. This is in agreement with the observations of Lewis (1952) who concluded that the pistil incompatibility substance is preformed in Oenothera organensis, which also shows gametophytic self-incompatibility. Certain stylar proteins have been correlated with S genotype in Nicotiana alata (Bredemeijer and Blass, 1981) and Prunus avium (Mau et al., 1982), both species exhibiting gametophytic incompatibility.

1.3.2.3 Messenger RNA and gene expression.

The existence of pre-synthesized (stored) mRNA has been described for a number of plant systems (Dure, 1975; Payne, 1976;

Brooker et al., 1978). The concept of stored (pre-synthesized) mRNA in mature pollen has been proposed by several workers. The evidence for this has been obtained indirectly, based on the rapidity of polysome formation and protein synthesis during germination (Mascarenhas and Bell, 1969; Linskens et al., 1970; Tupy, 1977) and also, on the effects of inhibitors of RNA and protein synthesis on germination and tube growth (Mascarenhas, 1975). Recently, direct evidence for the presence of mRNA in the mature pollen of Tradescantia paludosa and Zea mays has been obtained (Frankis and Mascarenhas, 1980; Mascarenhas et al., 1984). These mRNA preparations from Tradescantia pollen code for at least 230 polypeptides (Mascarenhas and Mermelstein, 1980). The presence of stored mRNA in the mature pollen of Petunia hybrida has not been directly demonstrated hitherto.

1.4 Aims of the study.

The present investigations are concerned with the biochemical and molecular biological aspects of pollen germination, pollen tube growth and the interaction between pollen and pistil of Petunia hybrida. The following lines of enquiry were pursued:

(i) Characterization of pyrimidine and purine nucleoside uptake systems in germinating Petunia hybrida pollen. In addition, the effects of metabolic inhibitors on pollen ATP levels during germination and pollen tube growth were also investigated.

(ii) Identification and characterization of amino acid transport systems in pollen tubes of Petunia hybrida.

(iii) Investigation of controls operating on protein release during germination and pollen tube growth in Petunia hybrida.

(iv) Studies on regulation of protein synthesis and protein phosphorylation during germination and pollen tube growth in Petunia hybrida.

(v) Investigation of interaction of the self-incompatibility genes with both the localization of phytic acid and phytic acid metabolism.

(vi) Detection and partial characterization of self-incompatibility genotype associated proteins in Petunia hybrida.

(vii) Investigation to directly demonstrate the presence of pre-synthesized (stored) mRNA in mature pollen grains of Petunia hybrida.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS.

2.1 Materials.

2.1.1 Plant material.

Pollen was collected on the day of anthesis from plants of Petunia hybrida (clone W166H and 924) grown under glasshouse conditions. After harvest, the pollen was dried overnight in a desiccator with silica gel and stored at -15°C before use. The other parts of the flower were excised on the day of anthesis and snap-frozen in liquid nitrogen prior to freeze drying. The freeze dried components were stored at -15°C before analysis.

In some parts of this study, pollen and pistils used were harvested from the collection of Petunia hybrida clones maintained by Prof. Dr. H. F. Linskens at the Catholic University, Nijmegen, Netherlands. Plant material was dried over P_2O_5 before shipment by air to Adelaide, South Australia. Petunia hybrida clones used are as follows (self-incompatibility alleles in brackets): W_{43} (S_1S_1), Ka_3D (S_2S_2), T_2UD (S_3S_3), Ka_3O (S_3S_3), Ka_3 (S_4S_4), KaD_3 (S_4S_4), W166H (S_2S_3), W166K (S_1S_2), T_2U (S_3S_3).

2.1.2 Chemicals.

All pyrimidine and purine bases, nucleosides, nucleotides, amino acids, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), N,N'-dicyclohexyl carbodiimide (DCCD), Tris (hydroxymethyl)-aminomethane, p-chloromercuribenzoic acid (pCMB), 2-mercapto-ethanol, Bovine serum albumin (BSA) and Ethylene glycol-bis (-aminoethyl-ether) N,N,N'N'-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co., U.S.A. 2, 5-diphenyloxazole (PPO) and 1,4 bis (2,(4 methyl-5-phenyl oxazolyl)) benzene (POPOP) were from Packard Instrument Co., U.S.A. Phase combining system (PCS) liquid scintillation fluid was obtained from Amersham, England.

All other chemicals, the best purity available, were purchased from the following sources: B.D.H. Chemicals Ltd.

(England), Ajax Chemical Co. (Australia) and Aldrich Chemical Co. (U.S.A.).

2.1.3 Radioistopes.

$^{3}H_2O$ and {^{14}C} sucrose were purchased from New England Nuclear (NEN), U.S.A. All other radioisotopes were obtained from Amersham, England.

2.1.4 Solutions, buffers and solvents.

Unless stated otherwise, all aqueous solutions, buffers and reagents used were prepared in double distilled water. The organic solvents used were of analytical grade.

2.1.5 Cell-free translation extracts and marker proteins.

Nuclease-treated reticulocyte lysate was purchased from Amersham, England. Wheat germ extract was kindly supplied by Dr. P. Langridge of this department. Molecular weight standards were from Pharmacia Fine Chemicals, Sweden.

2.2 Methods.

2.2.1 Germination and pollen tube growth.

Before germination the Petunia hybrida pollen was placed in a chamber with 100% humidity for 2h, then quickly transferred to an Erlenmeyer flask containing a solution of 10% (w/v) sucrose in 0.01% (w/v) boric acid (pH 5.5) for germination of clone W166H and a solution of 10% (w/v) sucrose, 0.01% (w/v) boric acid in 1mM $Ca(NO_3)_2$ (pH 5.5) for germination of clone 924 at 25°C in a shaking water bath. The cultivation medium was sterilized by autoclaving. Unless otherwise stated, the concentration of pollen in the medium was 5 mg/ml.

Germination percentage and pollen tube length were determined microscopically on at least 500 randomly selected pollen grains. For germination percentage only those which had a pollen tube longer than half the diameter of the pollen grain were considered as germinated.

2.2.2 Transport assays.

2.2.2.1 Pyrimidine and purine nucleoside.

Petunia hybrida (clone W166H) pollen was cultured in aqueous solution as described in section 2.2.1. Transport was measured over the first 30 min of culture and was initiated by the addition of sterile aqueous {6-^{3}H} uridine (2μCi/ml, 24 Ci/mmol), {6-^{3}H} thymidine (2μCi/ml, 23.8 Ci/mmol), {2-^{14}C} cytidine (0.1 μCi/ml, 27.2 mCi/mmol, {2-^{14}C} doexyuridine (0.1 μCi/ml, 58 mCi/mmol), {2-^{14}C-5} bromodeoxyuridine (0.1μCi/ml, 49 mCi/mmol), {8-^{14}C} adenosine (0.1 μCi/ml, 28.4 mCi/mmol) or {U-^{14}C} guanosine (0.1 μCi/ml, 281 mCi/mmol) at different carrier nucleoside concentrations. Transport was terminated by washing pollen with ice-cold medium containing 1 mM of the appropriate unlabelled nucleoside, this treatment being repeated three times before determination of radioactivity in the pollen by liquid scintillation spectrometry. The amounts of nucleoside transported was calculated from the TCA soluble radioactivity following extraction of washed pollen with 10% (w/v) TCA. Examination of this acid-soluble fraction by paper electrophoresis (1h at 26.5 V/cm in 0.1 sodium citrate, pH 3.5) showed that all the radioactivity was present as nucleoside, nucleoside monophosphate, -diphosphate, -triphosphate, and allowed the calculation of the proportion of label in each (as described by Jackson and Linskens, 1980). Radioactivity was determined in a Packard Tricarb 460 CD liquid scintillation spectrometer with external standardization. Statistical analysis of kinetic data was carried out by the method of Cleland (1967) and energy of activation was calculated from an Arrhenius plot.

2.2.2.2 pH dependence of nucleoside transport.

pH dependence of transport rate was measured by assaying in normal, unbuffered transport medium that had been brought to the indicated pH with NaOH or HCl. Petunia hybrida (clone W166H) pollen was cultured for 30 min and then pollen was collected by centrifugation, washed with fresh medium and resuspended for 15 min in cold medium at various pH values. Transport was measured over 5 min. The pH did not change during this 5 min transport time.

2.2.2.3 Amino acids.

Petunia hybrida (924) pollen was cultured for 1h as described in section 2.2.1 and then transport was initiated by the addition of sterile aqueous L-{^{35}S} methionine (1 µCi/ml, 1390 Ci/mmol), L-{U-^{14}C} glutamic acid (0.1 µCi/ml, 270 mCi/mmol) or L-{guanido-^{14}C} arginine monohydrochloride (0.1 µCi/ml, 56 mCi/mmol) at different carrier amino acid concentrations. After 30 min transport, the germinated pollen was collected by centrifugation and washed three times with ice-cold transport medium containing 1 mM of the appropriate unlabelled amino acid. The amount of the amino acid transported was calculated from the TCA soluble radioactivity following extraction of washed pollen with 10% TCA. Radioactivity was determined by liquid scintillation counting in a Packard Tricarb 460 CD liquid scintillation system with external standardization. The method of Cleland (1967) was used for statistical analysis of kinetic data.

For L-arginine transport, Petunia hybrida (924) pollen was cultured for 1h as described in section 2.2.1 and then, the pollen was collected by centrifugation and washed with medium lacking calcium (10% (w/v) sucrose in 0.01% (w/v) boric acid). Washed pollen was resuspended in medium lacking calcium for transport experiments, because calcium was found to be a strong inhibitor of L-arginine transport.

2.2.2.4 Identification of transported amino acids.

Examination of the TCA soluble labelled material within the pollen by paper electrophoresis (15 min at 3000 V in formic acid/acetic acid, pH 1.75) indicated that at least 70 - 80% of the intracellular labelled material was identified as free amino acid (electrophoresis as described by Tate, 1968; 1981).

2.2.2.5 pH dependence of amino acid transport.

Petunia hybrida (924) pollen was cultured for 60 min and then pollen was collected by centrifugation, washed with fresh medium and resuspended in cold medium. pH dependence of transport rate was measured over 5 min by assaying in normal, unbuffered transport medium that had been brought to the indicated pH

with NaOH or HCl. The pH did not change during the 5 min transport time.

2.2.3 Efflux experiments.

Efflux was determined after culture with labelled nucleoside or amino acid by resuspension of pollen in transport medium containing the agent to be tested. After 30 min, pollen was collected by centrifugation and radioactivity in the pollen and in the supernatant determined.

2.2.4 Pollen intracellular space measurement.

Intracellular space was determined by using $^{3}H_2O$ (for total pellet water), {^{14}C} sucrose (for total pellet water-intracellular water) and {^{14}C} inulin (for extra-cellular water) as described by Rottenberg (1979). In this way the intracellular water space for Petunia hybrida (clone W166H) pollen was found to be 1.64 $\pm$ 0.12 µl/mg (dry weight).

2.2.5 Enzyme extraction.

Extracts for enzyme assays were prepared by grinding frozen plant material in a mortar precooled in liquid nitrogen and allowing 50 mM sodium acetate, pH 5.0 buffer to freeze over the powder. The frozen buffer and plant material were then powdered together, allowed to thaw while being ground, and centrifuged at 10,000 Xg for 30 min. The supernatant was poured off and adjusted to 90% saturation ammonium sulphate by the addition of solid ammonium sulphate. After mixing for 20 min with a magnetic stirrer, the precipitate was collected by centrifugation at 20,000 Xg fo 10 min. The pellet was dissolved in 50 mM acetate buffer, pH 5.0 and finally passed through a Sephadex G-25 column (equilibrated with 50 mM acetate buffer, pH 5.0) to desalt.

2.2.6 Enzyme assays.

2.2.6.1 Phytase.

Phytase activity was determined at a substrate concentration of 0.5 mM sodium phytate in 50 mM acetate buffer, pH 5.0 at 45°C.

The reaction was stopped by the addition of TCA to 5% and the inorganic phosphate released determined after separation from phytic acid substrate (Irving and Cosgrove, 1970).

2.2.6.2 Acid phosphatase.

The acid phosphatase activity was determined by measuring the release of inorganic phosphate (Pi) from β-glycerophosphate (For β-glycerophosphatase activity). The reaction mixture in a total volume of 2 ml, contained enzyme extract, 50 mM acetate buffer (pH 5.0), 1 mM $MgCl_2$ and 5 mM β-glycerophosphate (sodium salt). Incubation was at 37°C in a water bath shaker. After an appropriate incubation period, the reaction was terminated by adding 1 ml chilled 10% TCA and then centrifuging at 10,000 g for 15 min. The Pi released from β-glycerophosphate was then determined by the method of Fiske and Subbarow (1925).

The acid phosphatase activity was also determined by measuring the release of p-nitrophenol from p-nitrophenylphosphate (for p-nitrophenyl-phosphatase activity). The reaction mixture in a total volume of 2 ml contained enzyme extract, 50 mM acetate buffer (pH 5.0), 1 mM $MgCl_2$ and 2.5 mM p-nitrophenyl-phosphate. Incubation was at 37°C in a water bath shaker. After an appropriate incubation period, the reaction was terminated by adding 5.0 ml of 0.1N NaOH and absorbance was read at 410 nm (Jones, 1969).

2.2.6.3 Acid pyrophosphatase.

Acid pyrophosphatase activity was determined by measuring the release of inorganic phosphate (Pi) from sodium pyrophosphate. The reaction mixture in a total volume of 2.0 ml contained enzyme extract, 50 mM acetate buffer (pH 5.0) and 2.5 mM sodium pyrophosphate. Incubation was at 37°C in a water bath shaker. After the appropriate incubation period, the reaction was terminated by adding 1 ml chilled 10% (w/v) TCA and then, centrifuging at 10,000 Xg for 15 min. The Pi released from sodium pyrophosphate was then determined by the method of Fiske and Subbarow (1925).

2.2.7 Labelling and isolation of proteins.

2.2.7.1 Method (A).

For labelling pollen proteins, 5 μCi/ml L-{^{35}S} methionine (1390 Ci/mmol) was added in the germination medium and after 1h, 2h, 3h and 4h germination, pollen tubes were separated by centrifugation. Proteins from the supernatant medium were precipitated by adding 2 volumes of ethanol and stored overnight at -15°C. The precipitated proteins were washed twice with chilled 70% (v/v) ethanol.

The pelleted pollen tubes extracts were prepared by freezing the plant material in liquid nitrogen in a pre-cooled mortar, covering the pollen tubes with buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ and 1 mM PMSF (Phenylmethyl-sulphonyl fluoride) and after all had frozen, allowing the mixture to gradually thaw while grinding vigorously with the pestle. Proteins from the homogenates were precipitated and washed as described above.

2.2.7.2 Method (B).

For labelling pollen proteins, 2 μCi/ml {U-^{14}C} protein hydrolysate (a mixture of ^{14}C-labelled amino acids) was added in the culture medium and after 1h, 2h, 3h and 4h germination, pollen tubes were separated by centrifugation. Proteins from the supernatant medium were precipitated by adding an equal volume of ice-cold 10% (w/v) TCA, and allowing to stand at 0°C for 3h. The precipitated proteins were washed twice with chilled distilled water and finally with chilled 90% ethanol.

The pelleted pollen tubes extracts were prepared as described in section 2.2.7.1. Proteins from the homogenates were precipitated and washed as described above.

2.2.7.3 Method (C).

For labelling phosphorylated proteins in pollen, 10 μCi/ml {^{32}P} orthophosphate was added in the germination medium and

after 1h, 2h, 3h and 4h germination, pollen tubes were separated by centrifugation. Proteins from pollen tubes and supernatant medium were isolated as described in section 2.2.7.2.

2.2.8 Extraction of total RNA.

In all experiments involving RNA isolation and use, all glassware was washed with 0.5 M KOH and baked for 4h at 200°C to minimise RNAase contamination. All solutions were prepared using autoclaved, double glass-distilled water and sterilized immediately after preparation by autoclaving. Any residual ribonuclease contamination was removed by treating solutions with 0.1% (v/v) (final concentration) diethyl pyrocarbonate for 16h. This reagent was removed by heating (100°C, 15 min) before use of the solution because it can react with nucleic acids.

Pollen or pistils were weighed and ground with crushed glass in liquid nitrogen in a pre-cooled mortar. The powder was transferred to another clean mortar containing 16 volumes of the buffer containing 4 M guanadium chloride, 2% Sarkosyl, 0.1 M 2-mercaptoethanol, 0.2 M Tris-acetate (pH 8.0) and ground vigorously with pestle. The homogenate was centrifuged at 8500 g for 10 min at 10°C. 1 gram CsCl was added to each 2.5 ml of supernatant and overlaid onto 3 ml of 0.965 grams/ml CsCl in 0.1 M Tris-HCl (pH 8.0) following the method described previously (Chirgwin *et al.*, 1979). This was centrifuged at 80,000 Xg (30,000 rpm in a Beckman L265B Ultracentrifuge, fixed angle rotor type 65) for 16h at 4°C. The top part of the tube was wiped clean carefully and the supernatant discarded. The pelleted RNA was transferred to an Eppendorf tube, washed twice with chilled 80% (v/v) ethanol, dried under vacuum and dissolved in appropriate volume of sterile water. The yield and purity of RNA was determined by scanning the ultraviolet absorbtion spectrum of an appropriately diluted sample from 320 nm to 230 nm. The yield of RNA was calculated using the equation 1 OD_{260} = 45 µg/ml RNA (approximately).

2.2.9 Purification of messenger RNA (mRNA).

Poly(A)$^+$RNA (mRNA) was purified by affinity chromatography on oligo (dT)-cellulose following the method of Aviv and Leder

(1972). Poly(A)$^+$RNA was hybridized to the oligo (dT)-cellulose by mixing total RNA and oligo (dT)-cellulose together in washing buffer (0.5M NaCl in 0.5 M Tris-HCl, pH 7.5) on an orbital shaker overnight at 4^{o}C. The slurry was then packed into a small 1 ml column and the column was then washed extensively with washing buffer. RNA species lacking poly(A) (mainly rRNA and tRNA) failed to bind to oligo (dT)-cellulose and hence passed through the column. The poly(A)$^+$RNA (mRNA) was then eluted with eluting buffer (10 mM Tris-HCl, pH 7.5). The mRNA was then recovered by precipitation with 80% (v/v) ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at -20^{o}C overnight. Poly(A)$^+$RNA was collected by centrifugation (10,000 Xg, 10 min), the pellet rinsed in 70% (v/v) ethanol, dried under vacuum and dissolved in sterile water and stored at -80^{o}C.

2.2.10 Cell-free translation systems.

2.2.10.1 Rabbit reticulocyte lysate system.

Nuclease-treated reticulocyte lysate was obtained from Amersham (England) and the recommended Amersham method for translation assays was followed. For each incubation (total volume 25 µl), 5 µCi of labelled amino acid was taken in a sterile microcentrifuge tube, frozen the contents in liquid nitrogen and lyophilized. When the labelled amino acid had completely dried, the following components were added carefully on ice in the tube:

1. 17.5 µl of amino acid depleted reticulocyte lysate (thawed immediately before use)
2. 1.5 µl of 2 M potassium acetate solution
3. 1.5 µl of 2 mM amino acid mixture minus L-leucine or minus L-methionine.
4. 4.5 µl of mRNA or sterile distilled water.

The contents of tubes mixed thoroughly and incubated for 2h at 30^{o}C. 2 µl aliquots were spotted on glass fibre discs, boiled in 5% TCA for 10 min and washed five times for 5 min with 100% ethanol. The dried filters were counted using liquid scintillation.

2.2.10.2 Wheat germ system.

Wheat germ extract was kindly supplied by Dr. P. Langridge of this department. For each incubation (total volume 25 µl), the following components were mixed carefully on ice in a sterile microcentrifuge tube:

1. 10 µl of wheat germ extract (thawed immediately before use).
2. 1.5 µl of 2 M potassium acetate solution.
3. 1.5 µl of 2 mM amino acid mixture minus L-leucine or minus L-methionine.
4. 2.5 µl of "salts-energy" mixture (10 mM magnesium acetate, 12.5 mM ATP, 1.25 mM GTP, 25 mM DTT, 0.5 mM spermidine, 0.5 M Hepes, pH 7.6 with KOH).
5. 2.5 µl of creatine phosphate-creatine phosphokinase (0.16 M creatine phosphate, 7 mg/ml creatine phosphokinase).
6. 3 µl of label (5 µCi L-{^{35}S} methionine or L-{^{3}H} leucine).
7. 4 µl of mRNA or sterile distilled water.

The contents of tubes were mixed thoroughly and incubated for 2h at 30°C. 2 µl aliquots were spotted on glass fibre discs, boiled in 5% (w/v) TCA for 10 min and washed as described in section 2.2.10.1. The dried filters were counted in a liquid scintillation spectrometer.

2.2.11 Preparation of protein samples.

Proteolytic degradation was prevented by carrying out all operations as rapidly as possible and by including protease inhibitors PMSF (1 mM) in buffers used for sample preparation.

2.2.11.1 For SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Protein samples were dissolved directly in SDS buffer solution (0.0625 M Tris-HCl, pH 6.8 - 2% (w/v) SDS - 10% (v/v) glycerol - 5% (v/v) 2-mercaptoethanol - 0.01% (w/v) bromophenol

blue). The samples were heated at 100°C for 3 min and centrifuged (Eppendorf) for 5 min before loading onto the gel.

Protein samples from cell-free translation experiments and self-incompatibility genotype associated proteins experiments were mixed with an aqual amount of SDS buffer (0.125 M Tris-HCl, pH 6.8 - 4% (w/v) SDS - 20% (v/v) Glycerol - 10% (v/v) 2-mercapto ethanol - 0.01% (w/v) bromophenol blue), heated at 100°C for 3 min and centrifuged prior to loading on the gel.

2.2.11.2 For IEF (Isoelectric focussing).

Protein samples were dissolved directly in IEF buffer solution (6.7 M Urea - 2% (v/v) ampholines (LKB, pH range 3.5 to 10.0) - 2% (v/v) triton X-100, 3% (v/v) 2-mercaptoethanol). The final mixture was centrifuged (Eppendorf) for 5 min before loading onto the gel.

2.2.12 Polyacrylomide gel electrophoresis (PAGE),

2.2.12.1 Cathodic discontinuous - PAGE.

Cathodic discontinuous, nondenaturing PAGE was carried out in 7.5% (w/v) polyacrylamide tube gels (Reisfeld et al., 1962; Davis, 1964). In this system migration of proteins is towards the cathode. The stacking gel was 3% (w/v) polyacrylamide in 60 mM KOH - 62 mM acetic acid buffer, pH 6.8 and the running gel 7.5% (w/v) polyacrylamide in 60 mM KOH - 375 mM acetic acid buffer, pH 4.3. The electrode buffer contained 350 mM β-alanine - 140 mM acetic acid, pH 4.5. Electrophoresis was carried out at 2 mA per gel rod at constant current. Gels were stained for protein with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) methanol, 10% acetic acid staining solution and allowed to stain for 14h at room temperature. The gels were destained with a solution containing 25% (v/v) ethanol - 10% (v/v) acetic acid. Destaining solution was changed after 2 hours.

Phytase and acid phosphatase isoenzymes were detected in the gels, washed once in cold 50 mM acetate buffer, pH 5.0. Then for phytase, gels were incubated at 45°C for 60 min in 50 mM acetate buffer, pH 5.0 containing 1 mM sodium phytate and 3 mM

$Pb(NO_3)_2$ in a water bath shaker. For acid phosphatase, gels were incubated at 25°C for 5 min in 50 mM acetate buffer pH 5.0 containing 15 mM β-glycerophosphate (sodium salt) and 3 mM $Pb(NO_3)_2$ in a water bath shaker. After incubation the gels were washed in distilled water for 1h with repeated changes and isoenzyme bands were detected by immersing the gels in 1% (w/v) ammonium sulphide for 1 min (Allen and Gackerman, 1964). The gels were rinsed in distilled water and stored in 7% (v/v) acetic acid until photographed.

2.2.12.2 SDS-PAGE.

Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis was carried out using a modified Laemmli discontinuous buffer system (Laemmli, 1970; Laemmli and Favre, 1973). The gels were calibrated with the following marker protein standards: Phosphorylase b (94,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (20,100 daltons), α-lactalbumin (14,400 daltons).

2.2.12.3 Two-dimensional gel electrophoresis.

Two-dimensional slab gel electrophoresis was carried out using a modification of the methods of O'Farrell (1975), and of Iborra and Buchler (1976) as described by Hallenbeck et al., (1982). First dimension isoelectric focussing was carried out with slab gels containing 4.5% (w/v) acrylamide - 8.4 M urea - 2% (v/v) ampholines (LKB, pH range 3.5 to 10.0). SDS-PAGE in the second dimension was performed as described in section 2.2.12.2

2.2.13 Protein detection on gels.

2.2.13.1 Staining with Coomassie Blue.

After migration, the gels were placed directly into staining solution (0.1% (w/v) Coomassie Blue R-250 - 45% (v/v) methanol - 10% (v/v) acetic acid) and stained overnight. The gels were destained with a solution containing 45% (v/v) methanol - 10% (v/v) acetic acid.

2.2.13.2 Fluorography.

The method of Bonner (1984) was followed and the dried gels were exposed to Fuji NIF X-ray films at -80°C for 1 to 5 weeks.

2.2.13.3 Autoradiography.

The autoradiographic detection of ^{32}P-labelled proteins on gels was carried out according to the method of Desmarquets et al. (1984). After protein migration, gels were soaked in 16% (w/v) TCA and heated for 45 min at 95°C. Then, the gels were incubated for 2h at room temperature in 25% (v/v) methanol - 7.5% (v/v) acetic acid, dried under vacuum and subjected to autoradiography.

2.2.14 General techniques.

2.2.14.1 Electron-microscopy.

Dry pollen grains were brushed onto a double sided tape fixed to a brass stub and gold coated (10 nm) in a JOEL JEE 4B vacuum evaporator and the sample viewed in a JOEL electron microscope (Model JEM 100 CX) with S.E.M. (Scanning Electron Microscopy) attachment.

Pollen tubes were fixed in 2.5% (v/v) glutaraldehyde in cacodylate buffer, washed in 50 mM phosphate buffer (pH 7.0) and from the suspension a drop was spotted on the brass stub. The excess moisture was allowed to evaporate prior to examination in the JOEL electron microscope with S.E.M. attachment.

2.2.14.2 Liquid scintillation spectrometry.

Radioactivity in aqueous samples (^{32}P, ^{35}S, ^{14}C or ^{3}H) was measured by counting in 'PCS' scintillation fluid in Packard glass vials. The ratio of sample volume to scintillation fluid volume was 1:5.

Radioactivity on dried samples was measured in toluene based scintillation fluor (0.3% (w/v) PPO and 0.03% (w/v) POPOP in

toluene) in glass vials. The vials were assayed in a Packard Tricarb 460 CD liquid scintillation spectrometer with external standardization.

2.2.15 Chemical determinations.

2.2.15.1 Protein.

Protein was determined by the dye binding method of Bradford (1976), using bovine serum albumin as a standard. The absorbance at 595 nm was recorded in 1 cm quartz cuvettes.

2.2.15.2 ATP.

ATP was determined by the firefly method of Stanley and Williams (1969). ATP from pollen was extracted by boiling for 10 min in 5 mM EDTA (pH 7.0) with 100 mg of polyclar AT (acid washed) (Lundin and Thore, 1975). An appropriate aliquot from the supernatant was taken for ATP assay. The reaction mixture in a scintillation vial contained 1 ml 10 mM sodium phosphate (pH 7.5), 0.9 ml distilled water, 0.1 ml 5 mM $MgCl_2$ and 50 ul firefly extract. The vial was then placed in a liquid scintillation spectrometer (Packard Tricarb model 3375) set at maximum sensitivity with the two photomultipliers switched out of coincidence and assay continued for 6 sec. Standard solution of ATP (5-50 pmol) was also assayed. The ATP concentration was calculated from a calibration of freshly prepared ATP standard.

2.2.15.3 Phytic acid.

Phytic acid was determined as described previously (Jackson *et al.*, 1982). Plant material was extracted in Na_4EDTA and phytic acid was estimated from supernatant by paper electrophoresis (0.1 M oxalic acid, pH 1.5; 550 V for 2h; on Whatman 3 MM paper 10 x 43 cm) following the method of Jackson *et al.* (1982).

3. RESULTS

3. RESULTS

3.1 Uptake of pyrimidine nucleosides.

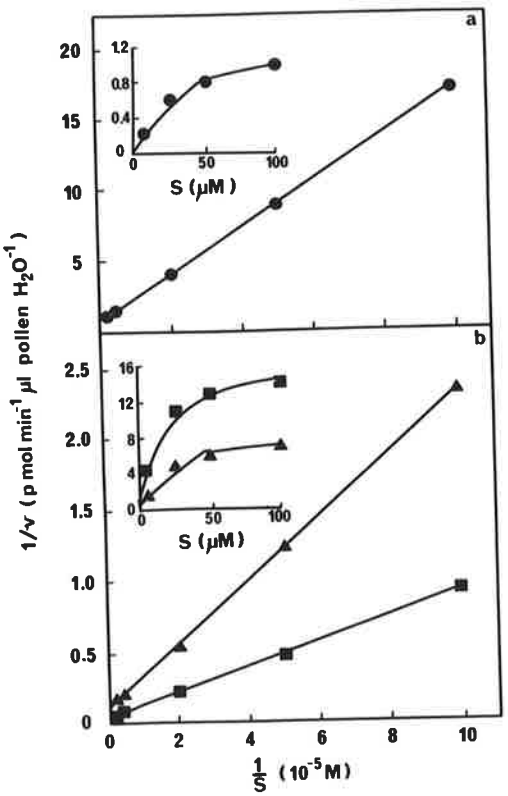
3.1.1 General characteristics.

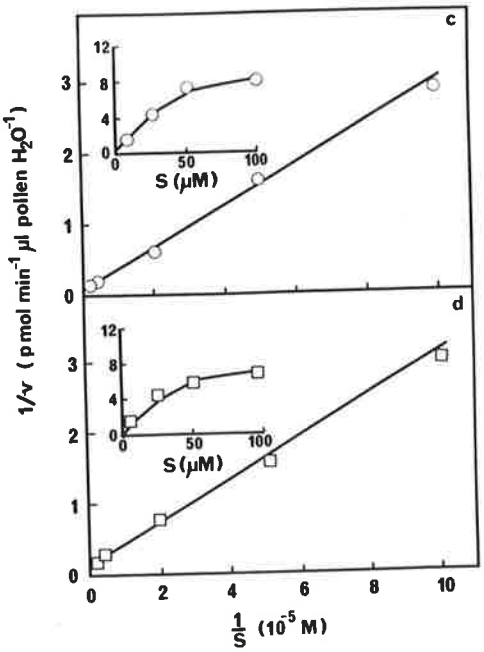
Pyrimidine nucleosides were transported into Petunia hybrida pollen grains at a linear rate up until at least 30 min of culture, the rate of uridine and cytidine transfer being substantially higher than that for thymidine. Transport was maximal in pollen from the first 30 min of culture and a gradual decline was observed in germinated pollen taken from 2h, 4h, 6h and 8h cultures. The transport of all pyrimidine nucleosides showed a broad pH optimum between 4.5 and 8.5

3.1.2 Kinetics of pyrimidine nucleoside transport.

The variation of rate with substrate concentration indicated a transport system which was saturable with substrate and therefore was carrier-mediated for all pyrimidine nucleosides (Fig. 2). Kinetic analysis of the substrate saturation curves showed no great difference in K_m between uridine, cytidine and thymidine (11.8 $\pm$ 1.1 µM, 16.3 $\pm$ 1.9 µM and 18.1 $\pm$ 0.9 µM respectively), but a large variation in V_{max} (15.86 $\pm$ 0.43, 8.27 $\pm$ 0.47 and 1.10 $\pm$ 0.02 pmol nucleoside/min/µl pollen grain H_2O, respectively). An investigation of the effect of temperature showed that uridine, cytidine and thymidine transport had an activation energy of 75.4, 67.3 and 42.0 kJ mol^{-1} $^oK^{-1}$ respectively. These results already suggest some major difference in transport between thymidine and the other pyrimidine nucleosides. This was confirmed when pre-loaded pollen with labelled thymidine (5 µM, for 30 min.), was resuspended in fresh medium containing 100 µM uridine or cytidine, and observations made on the efflux of labelled thymidine compared with controls without uridine or cytidine. There was no significant difference in thymidine efflux (found to be 30% of total transport into the pollen), indicating that thymidine might be transferred by a different carrier to that for uridine or cytidine. Similarly, thymidine had no significant effect on uridine efflux, which however is negligible (found to be 5% of total transport).

Fig. 2 **Kinetics of the transport of pyrimidine nucleosides and analogues into Petunia hybrida pollen.**
Transport was measured as described in section 2.2.2.1
Uridine (■); Cytidine (▲); Thymidine (●); Deoxyuridine (□); 5-bromodeoxyuridine (○).


a
b
1/v (p mol min^{-1} µl pollen H_2O^{-1})
S (µM)
$\frac{1}{S}$ (10^{-5} M)


c
d
1/v (p mol min^{-1} µl pollen H_2O^{-1})
S (µM)
$\frac{1}{S}$ (10^{-5} M)

3.1.3 Effect of metabolic inhibitors.

Further differences between thymidine and the other two pyrimidine nucleosides became apparent, when the effect of the ATPase inhibitors DCCD and NBD-Cl was tested on the pyrimidine transport system. Both these reagents inhibited uridine and cytidine transport, and were without effect on thymidine transport (Table 1). Preincubation of pollen for 1h with DCCD or NBD-Cl did not affect thymidine transport, but increased inhibition of uridine transport by a further 20%. The proton translocators (uncouplers) CCCP and DNP were also effective inhibitors of uridine and cytidine transport in Petunia pollen (Table 1), and had a minimal effect on thymidine transport. These results suggest that uridine and cytidine were taken up in pollen by an active transport system, while thymidine transport was a non-active process.

The transfer of uridine was also inhibited by low concentrations of oligomycin (2µg/ml gave 29% inhibition), sodium azide (5×10^{-5}M gave 27% inhibition) and vanadate (5×10^{-5}M gave 17% inhibition). In contrast, thymidine permeation is little affected by these or other metobolic inhibitors.

These metobolic inhibitors did not increase efflux of label, as compared to controls, after 30 min resuspension in the presence of the inhibitors. Therefore, these compounds inhibited transport of uridine and cytidine by affecting the energy metabolism rather than by altering membrane integrity under the conditions of the experiments.

3.1.4 Transport of pyrimidine nucleoside analogues.

Kinetic analysis of the transport of the analogues deoxyuridine and 5-bromodeoxyuridine (Fig. 2) showed that both were transferred by a saturable system with a K_m (23.9 $\pm$ 1.1 µM and 39.1 $\pm$ 1.4 µM respectively) somewhat higher than that for the other pyrimidine nucleosides. Maximal velocities however were found to be 11.48 $\pm$ 0.16 and 8.21 $\pm$ 0.11 pmol analogue/min/µl pollen grain H_2O respectively, and thus, were more in line with transport rates obtained for uridine and cytidine, and much higher than for thymidine. This difference was supported by the

Table 1. Effect of metabolic inhibitors and antagonists on pyrimidine nucleoside transport into Petunia pollen.

Nucleosides were added to a final concentration of 5 μM. Control rates of transport for uridine, cytidine and thymidine were 4.26 ± 0.17, 1.89 ± 0.05 and 0.24 ± 0.01 pmol/min/μl pollen grain H_2O respectively.

Inhibitor or Antagonist	Concentration (μM)	Inhibition of Transport (%)		
		Uridine	Cytidine	Thymidine
DCCD	50	35	30	0
NBD-Cl	50	61	50	0
CCCP	10	83	68	18
DNP	50	65	55	15
ETHANOL	(0.5%)	0	0	0
Uridine	50	-	65	59
Cytidine	50	52	-	44
Thymidine	50	74	70	-
Adenosine	50	10	10	5
Guanosine	50	8	5	5

effect of the metabolic inhibitors on analogue transport (Table 2). The ATPase inhibitors DCCD and NBD-Cl were effective inhibitors of deoxyuridine and 5-bromodeoxyuridine transport (as for uridine and cytidine), unlike thymidine transport which was not affected by these compounds (Table 2). Similarly, the proton translocators DNP and CCCP inhibited analogue transport (Table 2) to the same extent as uridine and cytidine transport, and to a much greater extent than observed with thymidine. As shown in Table 2, uridine and thymidine inhibited analogue transport to approximately the same degree, while deoxyuridine was an effective inhibitor of 5-bromodeoxyuridine transport.

The uptake of 4-thiouridine was also briefly investigated, utilizing the high absorption of this compound at 330 nm (E_{mM} = 28.6 x 10^3; pH 5). At an external concentration of 0.2 mM, this nucleoside was taken up at an average rate of 12.4 pmol/min/µl pollen grain H_2O over a period of 30 min, a rate not greatly lower than for uridine itself.

3.1.5 Conversion of transported nucleosides to nucleotides.

As shown in table 3 transported nucleosides were readily converted to nucleoside-monophosphate, -diphosphate and -triphosphate to a varying extent. However, over the time studied, thymidine alone did not show significant conversion. As reported earlier, at longer incubation times and especially after induction of DNA repair, transported thymidine label can be shown to be converted to the nucleotide derivative (Jackson and Linskens, 1980). The results indicate that the concentration of free nucleoside accumulating inside the pollen exceeded that of nucleoside in the medium for both uridine and deoxyuridine, but was not significantly higher for the other pyrimidine nucleosides. Under the conditions of experiment, at an external concentration of 5 pmol/µl, uridine and deoxyuridine accumulated in the pollen grain to the extent of 14.05 and 17.04 pmol/30 min/µl pollen grain H_2O respectively. These estimates are conservative because they do not account for subcellular compartmentation or the initial pollen nucleoside concentration.

Table 2. Effect of metabolic inhibitors and other agents on deoxyuridine and 5-bromodeoxyuridine transport in *Petunia* pollen.

Nucleoside analogue substrate concentration was initially 5 μM. When investigating metabolic inhibitors, an ethanol control was carried out (0.5% ethanol) because these inhibitors were dissolved in ethanol. This amount of ethanol had no significant effect on analogue transport. Control rates of transport for deoxyuridine and 5-bromodeoxyuridine were 1.42 ± 0.04 and 1.28 ± 0.04 pmol/min/μl pollen grain H_2O respectively.

Inhibitors	Concentration (μM)	Inhibition of Transport (%)	
		deoxyuridine	5-bromodeoxyuridine
DCCD	50	34	35
NBD-Cl	50	69	71
DNP	50	69	70
CCCP	10	79	82
Uridine	50	74	72
Deoxyuridine	50	--	70
Thymidine	50	76	77

Table 3. Conversion of pyrimidine nucleosides to nucleotides.

The figure for nucleoside diphosphate also includes a contribution from nucleoside diphosphosugar compounds, which migrate on paper electrophoresis under the conditions of the experiment, close to the nucleoside diphosphate.

Nucleoside or Analogue	Percent of total uptake			
	Nucleoside	Nucleoside mono-phosphate	Nucleoside di-phosphate	Nucleoside tri-phosphate
Uridine	10 ± 2	47 ± 5	35 ± 4	7 ± 2
Cytidine	10 ± 2	17 ± 3	45 ± 6	26 ± 4
Thymidine	82 ± 8	5 ± 2	3 ± 1	2 ± 1
Deoxyuridine	38 ± 4	19 ± 3	30 ± 4	6 ± 2
5-Bromodeoxyuridine	13 ± 3	28 ± 4	47 ± 6	11 ± 2

3.1.6 Effect of analogues on pyrimidine nucleoside transport.

Each of the pyrimidine nucleosides were able to inhibit the transport of any other (Table 4). Unlike the animal (Young & Jarvis, 1983) and bacterial (Mygind & Munch-Petersen, 1975) pyrimidine nucleoside transport systems, transport in Petunia pollen was inhibited only slightly by the purine nucleoside adenosine (Table 4). Likewise, guanosine, which is an effective inhibitor of the animal system, showed very little inhibition in pollen. These results were supported by the further observation that inosine and the potent inhibitor of animal cell pyrimidine nucleoside transport, nitrobenzylthioinosine (Young & Jarvis, 1983), were both without significant effect on pyrimidine nucleoside permeation in pollen (Table 4). Another inhibitor of mammalian cell pyrimidine nucleoside transport, dipyridamole (Young & Jarvis, 1983), was also without effect on the Petunia pollen systems. While deoxyuridine, deoxycytidine, 5-bromodeoxyuridine, and 5-fluorodeoxyuridine all inhibited thymidine transport, these pyrimidine nucleoside analogues inhibited uridine or cytidine transport even more. The analogues 4-thiouridine and 5-azauridine inhibited uridine transport, but did not give significant inhibition of thymidine transport (Table 4). The bases thymine, uracil, cytosine and the free sugar ribose, were without significant effect on pyrimidine nucleoside transport (Table 4).

3.1.7 Effect of thiol binding agents, chelators and other inhibitors on transport.

Pyrimidine nucleoside transport in both bacterial and animal cells was severely inhibited by sulphydryl reagents (Mygind & Munch-Petersen, 1975; Young & Jarvis, 1983), and the pollen transport systems were no exception. Both N-ethylmaleimide and pCMB inhibited pyrimidine nucleoside transfer in Petunia pollen. Inhibition of uridine transport by pCMB could be largely overcome by addition of 100 μM 2-mercaptoethanol. Phenylarsine oxide, a dithiol binding agent (Walker-Smith & Payne, 1983), was found to be an extremely potent inhibitor of uridine transport (Table 5). However, highlighting the difference between uridine and thymidine transport in pollen, phenylarsine oxide did not affect

Table 4. Effect of various analogues and related compounds on pyrimidine nucleoside transport in Petunia pollen.

Nucleoside substrates were added to a final concentration of 5 μM, inhibitor nucleosides, bases and ribose to 50 μM and dipyridamole, 20 μM. Control rates of transport for uridine, cytidine and thymidine were 4.26 ± 0.17, 1.89 ± 0.05 and 0.24 ± 0.01 pmol/min/μl pollen grain H_2O respectively.

Inhibitor	Inhibition of Transport (%)		
	uridine	cytidine	thymidine
Deoxyuridine	66	--	57
Deoxycytidine	52	65	30
5-Bromodeoxyuridine	48	36	28
5-Fluorodeoxyuridine	38	30	25
6-Azauridine	15	--	<5
4-Thiouridine	30	--	<5
Inosine	<5	--	--
Nitrobenzylthioinosine	<5	--	<5
Uracil	<5	<5	<5
Cytosine	<5	<5	<5
Thymine	<5	<5	<5
Dipyridamole	<5	--	<5
D-ribose	<5	--	--

Table 5. Effect of sulphydryl and other reagents on pyrimidine nucleoside transport in Petunia pollen.

Nucleosides were added to a final concentration of 5 µM. Control rates of transport for uridine, cytidine and thymidine were 4.26 ± 0.17, 1.89 ± 0.05 and 0.24 ± 0.01 pmol/min/µl pollen grain H_2O respectively.

Inhibitor	Concentration (µM)	Inhibition of Transport (%)		
		uridine	cytidine	thymidine
N-Ethylmaleimide	50	45	35	20
pCMB	50	48	43	31
Phenylarsine oxide	200	42	--	<5
Iodoacetamide	50	18	--	--
Cycloheximide	(100 µg/ml)	<5	<5	<5
Actinomycin D	(200 µg/ml)	10	--	--
Ouabain	50	<5	--	--
DIECA	50	<5	--	--
O-Phenanthroline	50	40	--	28
EDTA	1,000	65	--	<5
EGTA	1,000	35	--	(41% (stimulation))

thymidine transport at all. The other sulphydryl reagents had a lesser effect on thymidine transport also (Table 5). Of a number of other reagents tried, only certain metal chelators gave inhibition of pyrimidine nucleoside transport. Thus, although DIECA was not effective on either uridine or thymidine transport, o-phenanthroline inhibited both, only uridine transport more than thymidine (Table 5). It was also observed that o-phenanthroline induced the efflux of pyrimidine nucleosides. A clear-cut difference between uridine and thymidine transfer became apparent when EDTA and EGTA were included in the culture medium. EDTA was found to inhibit uridine transport and was without effect on thymidine transfer, while EGTA likewise inhibited uridine transport and actually stimulated that of thymidine.

3.1.8 Effect of ions on pyrimidine nucleoside transport.

Of the cations, only the heavy metals showed inhibition of pyrimidine nucleoside transport at concentrations of 1 mM or less. Uridine transport was affected rather more than thymidine transport in this regard (Table 6). Sodium and potassium ions did give inhibition of both, but only at relatively high concentrations, while calcium and magnesium had little effect on the transport systems at 1 mM.

Several anions were found to inhibit nucleoside transport in Petunia pollen (Table 7). The polyanions citrate and phosphate were most effective , but succinate, tartarate and acetate also gave significant inhibitions at concentrations of 1 mM or greater (Table 7). Nitrate, sulphite and sulphate ions were without effect at this concentration. Both uridine and thymidine transport responded similarly to anions wherever both were tested.

3.2 Uptake of purine nucleosides.

3.2.1 General characteristics of purine nucleosides uptake.

Purine nucleosides were transported into Petunia hybrida pollen at a linear rate for at least 30 min (Fig. 3). Transport was at a maximum in germinating pollen over the first 30 min

Table 6. Effect of cations on pyrimidine nucleoside transport in Petunia pollen.

Nucleosides were added to a final concentration of 5 μM. All cations were present as chlorides. Control rates of transport for uridine and thymidine were 4.26 ± 0.17 and 0.24 ± 0.01 pmol/min/μl pollen grain H_2O respectively.

Cation	Concentration (mM)	Inhibition of Transport (%)	
		uridine	thymidine
K^+	1	<5	<5
	10	41	40
	100	76	54
Na^+	1	<5	<5
	10	10	<5
	100	70	46
Ca^{2+}	1	<5	<5
Mg^{2+}	1	<5	(20% stimulation)
Mn^{2+}	1	10	<5
Ni^{2+}	1	45	15
Co^{2+}	1	32	15
Zn^{2+}	1	92	--
Cu^{2+}	1	88	30

Table 7. Effect of anions on pyrimidine nucleoside transport in *Petunia* pollen.

Nucleosides were present at an initial concentration of 5 μM. All anions were added as sodium salt. Control rates of transport for uridine and thymidine were 4.26 ± 0.17 and 0.24 ± 0.01 pmol/min/μl pollen grain H_2O respectively.

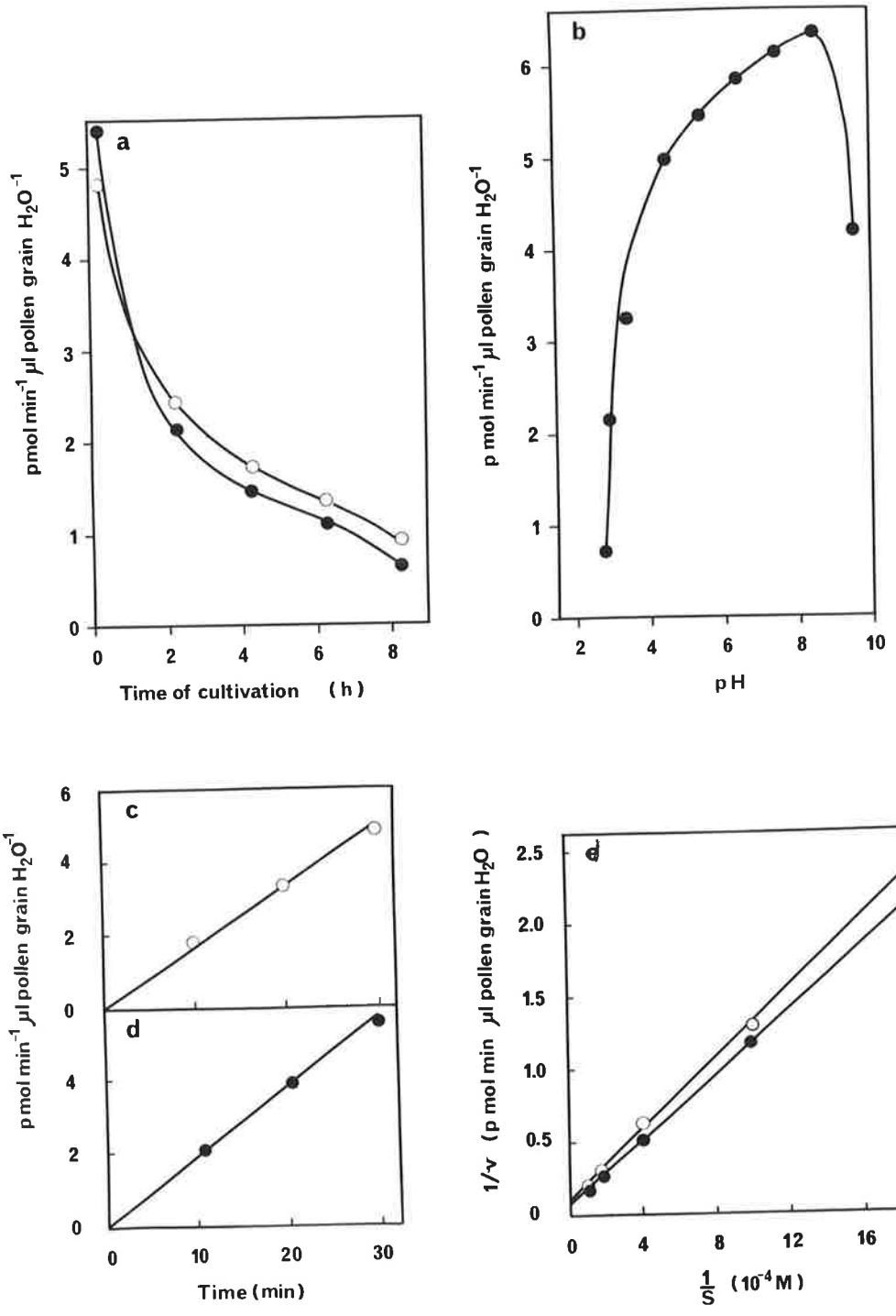
Anion	Concentration (mM)	Inhibition of Transport (%)	
		uridine	thymidine
acetate	1	10	<5
	5	38	--
citrate	1	33	35
	5	73	--
succinate	1	10	--
	5	47	--
tartrate	1	10	<5
phosphate	1	29	20
nitrite	1	10	<5
nitrate	1	<5	<5
	5	<5	<5
fluoride	1	10	<5
sulphite	1	<5	<5
sulphate	1	<5	<5
molybdate	1	10	10

Fig. 3a. **Effect of culture time on adenosine uptake in** Petunia hybrida **pollen.**
Uptake was measured in 100 µM adenosine in transport medium as described in section 2.2.2.1. 10% sucrose in 0.01% boric acid medium containing 1 mM $Ca(NO_3)_2$ (○).

b. **Influence of pH on adenosine uptake.**
Transport was measured as described in section 2.2.2.1.

c. **Time course of purine nucleoside uptake.**
Initial concentration of nucleoside was 100 µM and transport was determined as described in section 2.2.2.1.

d. **Kinetic analysis of adenosine and guanosine transport.**
Transport was measured as described in section 2.2.2.1.
Adenosine (●), Guanosine (○).


a
pmol min^{-1} µl pollen grain H_2O^{-1}
Time of cultivation (h)
b
p mol min^{-1} µl pollen grain H_2O^{-1}
pH
c
d
p mol min^{-1} µl pollen grain H_2O^{-1}
Time (min)
e
1/v (p mol min µl pollen grain H_2O)
$\frac{1}{S}$ (10^{-4} M)

of culture, and after a sharp decline thereafter up until 2h of culture time, a more gradual decline with time was observed in germinated pollen taken from 4h, 6h and 8h cultures (Fig. 3). The transport of both purine nucleosides showed a broad pH optimum between 4.5 and 8.5 (Fig. 3).

3.2.2 Kinetic analysis of transport.

The effect of nucleoside concentration on the transport of adenosine and guanosine indicated that purine nucleoside transport system in Petunia pollen was saturable and therefore carrier-mediated. Both adenosine and guanosine had a similar K_m (187.1 $\pm$ 7.9 µM and 190.8 $\pm$ 16.8 µM respectively) and V_{max} (16.25 $\pm$ 0.46 and 14.36 $\pm$ 0.89 pmol nucleoside/min/µl pollen grain H_2O, respectively) (Fig. 3).

3.2.3 Effect of metabolic inhibitors and other agents.

Purine nucleosides transport was inhibited by a variety of compounds known to affect cellular energy metabolism (Table 8). The proton translocators (Uncouplers) CCCP and DNP were the most effective inhibitors of adenosine and guanosine transport and also decreased the ATP concentration in Petunia pollen most effectively (Table 8). ATPase inhibitors DCCD and NBD-Cl also inhibited transport of purine nucleosides and lowered the ATP content (Table 8). It was observed that DCCD decreased the ATP level more strongly than the purine transport. However, NBD-Cl inhibited both equally effectively.

The sulphydryl reagent NEM was found to be a potent inhibitor of purine nucleoside transport, which is in agreement with the observations of several workers using a variety of organisms (Mygind and Munch-Petersen, 1975; Young and Jarvis, 1983). Phenylarsine oxide, a dithiol binding agent (Walker-Smith and Payne, 1983) was found to be an effective inhibitor of purine nucleoside uptake (Table 8). Of the other reagents tried, only metal chelators gave appreciable inhibition of purine nucleoside transport. Cycloheximide was without effect on either adenosine or guanosine transport (Table 8).

The metabolic inhibitors did not increase efflux of label

Table 8. Effect of metabolic inhibitors and other agents on purine nucleoside transport and on ATP levels in *Petunia* pollen.

Nucleoside substrate concentration was initially 100 µM. Control rates of transport for adenosine and guanosine were 5.66 ± 0.17 and 4.93 ± 0.29 pmol/min/µl pollen grain H_2O respectively. ATP level in the control was 1.81 ± 0.07 umol/µl pollen grain H_2O.

		Inhibition of Transport or ATP level (%)		
Inhibitors	Concentration (µM)	Adenosine	Guanosine	ATP
CCCP	10	64	58	76
DNP	50	50	50	55
DCCD	50	26	25	48
NBD-Cl	50	48	46	50
Ethanol	(0.5%)	0	0	0
NEM	100	40	37	-
PAO	100	34	36	-
EDTA	1000	36	33	-
EGTA	1000	45	36	-
Cycloheximide	(100 µg/ml)	<5	<5	-

as compared to controls, efflux measurement being carried out after 30 min resuspension in the presence of the inhibitor. Thus, it was concluded that these compounds inhibited purine uptake by affecting energy metabolism, rather than by any possible effects on efflux.

3.2.4 Conversion of transported nucleosides to nucleotides.

As shown in table 9 transported nucleosides were readily converted to nucleoside-monophosphate, -diphosphate and -triphosphate to a varying extent. Based on the determinations of the labelled free nucleoside pools, it was calculated that free adenosine and guanosine accumulation in the intracellular pool was 7.1 ± 0.3 and 10.9 ± 0.4 pmol/h/µl pollen grain H_2O respectively at 5 pmol/µl nucleoside concentration in the culture medium. These results indicated that the concentration of free nucleoside building up inside the pollen exceeded that of the purine nucleoside in the transport medium. These figures are conservative estimates because they do not consider the initial nucleoside pools of the pollen and the size of the compartment into which purine nucleosides are transported.

3.2.5 Influence of metabolic inhibitors on ATP level.

During the early stages of Petunia hybrida pollen germination, ATP was found to increase very rapidly up until at least 30 min (Table 10). The uncoupler CCCP was the most effective inhibitor in decreasing the ATP levels of Petunia pollen. DNP was also found to be an effective inhibitor in lowering the ATP levels. The decrease of ATP level of the Petunia pollen in the presence of ATPase inhibitor DCCD was very slow as compared to other inhibitors (Table 10). These results are in agreement with the observations of other workers using a variety of organism (Jeanjean, 1976; Takeuchi and Kishimoto, 1983).

3.2.6 Effect of analogues on purine nucleoside transport.

The effect of various nucleosides and analogues on the uptake of adenosine and guanosine was tested to obtain information on the specificity of adenosine and guanosine transport systems (Table 11). All nucleosides tested were able to inhibit

Table 9. Conversion of purine nucleosides to nucleotides.

The figure for nucleoside diphosphate also includes a contribution from nucleoside diphosphosugar compounds, which migrate on paper electrophoresis under the conditions of the experiment, close to the nucleoside disphosphate.

	Percent of total uptake			
Nucleoside	Nucleoside	Nucleoside mono phosphate	Nucleoside disphosphate	Nucleoside triphosphate
Adenosine	24±4	10±2	16±3	49±5
Guanosine	39±5	7±2	21±4	29±4

Table 10. Influence of various metabolic inhibitors on the ATP level during germination and pollen tube growth.

A standard deviation is indicated for each measurement.

		ATP (µmol/µl pollen grain H_2O)		
Culture Time (min)	Control	CCCP (10 µM)	DNP (50 µM)	DCCD (50 µM)
1	1.06±0.06	0.94±0.08	1.00±0.07	1.06±0.07
2	1.25±0.06	0.69±0.06	0.75±0.07	1.06±0.10
15	1.69±0.04	0.50±0.05	0.81±0.10	1.00±0.08
30	1.81±0.07	0.44±0.07	0.81±0.08	0.94±0.07

Table 11. Effect of nucleosides, analogues and polyamines on adenosine and guanosine transport into Petunia pollen.

Nucleoside substrates were added to a final concentration of 100 µM. Inhibitors were added at the concentration indicated. Control rates of transport for adenosine and guanosine were 5.66 ± 0.16 and 4.93 ± 0.29 pmol/min/µl pollen grain H_2O respectively.

Inhibitor	Concentration (µM)	Inhibition of transport (%)	
		Adenosine	Guanosine
Guanosine	500	30	-
Adenosine	500	-	33
Deoxyadenosine	500	20	-
Deoxyguanosine	500	-	18
Uridine	500	38	37
Thymidine	500	42	40
Inosine	500	17	-
Nitrobenzyl-thioinosine	20	<5	<5
Dipyridamole	20	<5	<5
Spermine	125	33	32
Spermidine	125	26	27
Putresine	125	<5	<5

the transport of adenosine and guanosine, except for the potent inhibitor of animal cell nucleoside transport, nitrobenzyl-thioinosine (Young and Jarvis, 1983) which was without significant effect on purine nucleoside permeation in Petunia pollen (Table 11). Another effective inhibitor of animal cell nucleoside transport, dipyridamole (Young and Jarvis, 1983) had no effect on the purine nucleoside transport in Petunia pollen. Polyamines (spermine and spermidine) with the exception of putrescine were found to inhibit purine nucleoside uptake in Petunia pollen (Table 11).

3.2.7 Effect of cations on purine nucleoside uptake.

Of the cations, heavy metal ions were the most potent inhibitors of purine nucleoside transport at concentrations of 1 mM or less (Table 12). Calcium or magnesium ions at 1 mM had little effect on pyrimidine nucleoside uptake in Petunia pollen (Section 3.1.8), however calcium ions at the same concentration gave about 20% inhibition of purine nucleoside uptake. Magnesium ions at 1 mM gave 15% inhibition of guanosine uptake but had little effect on adenosine transport (Table 12).

3.3 Transport of amino acids.

3.3.1 General properties of amino acid transport.

Amino acids were transported into Petunia pollen at a linear rate for at least 30 min. The rate of methionine transfer being substantially higher than that of glutamic acid or arginine. After a sharp increase up until one hour of culture, (Fig. 4) the rate of methionine transport was maximal in pollen tubes from 2h cultures and then declined gradually at 4h, 6h and 8h cultures (Fig. 4). Glutamic acid transport increased gradually with culture time whereas arginine transport was maximal in pollen for the first 30 min of culture and declined continuously thereafter (Fig. 6).

When labelled, amino acid-loaded pollen tubes were incubated for 30 min with unlabelled amino acid, less than 10% of the label was lost. These results indicate that transport and not isotopic accumulation via exchange diffusion was measured. Analysis of

Table 12. Effect of cations on purine nucleoside transport in *Petunia* pollen.

Nucleosides were added to a final concentration of 100 μM. All cations were present as chlorides. Control rates of transport for adenosine and guanosine were 5.66 ± 0.16 and 4.93 ± 0.29 pmol/min/μl pollen grain H_2O respectively.

		Inhibition of transport (%)	
Cation	Concentration (mM)	Adenosine	Guanosine
Ca^{2+}	1	18	23
Mg^{2+}	1	<5	15
Be^{2+}	1	30	25
V^{2+}	1	40	38
Fe^{3+}	1	80	50
Al^{3+}	1	80	50

Fig. 4a. **Effect of culture time on transport rate of L-methionine and L-glutamic acid in Petunia pollen.** L-methionine (●); L-glutamic acid (○).

b. **Influence of pH on uptake of L-methionine and L-glutamic acid.**

c. **Double logarithmic plot of L-glutamic acid uptake as a function of external concentration.** Two phases of uptake are indicated.

d. **Double logarithmic plot of L-methionine uptake as a function of external concentration.** Two phases of uptake are indicated.

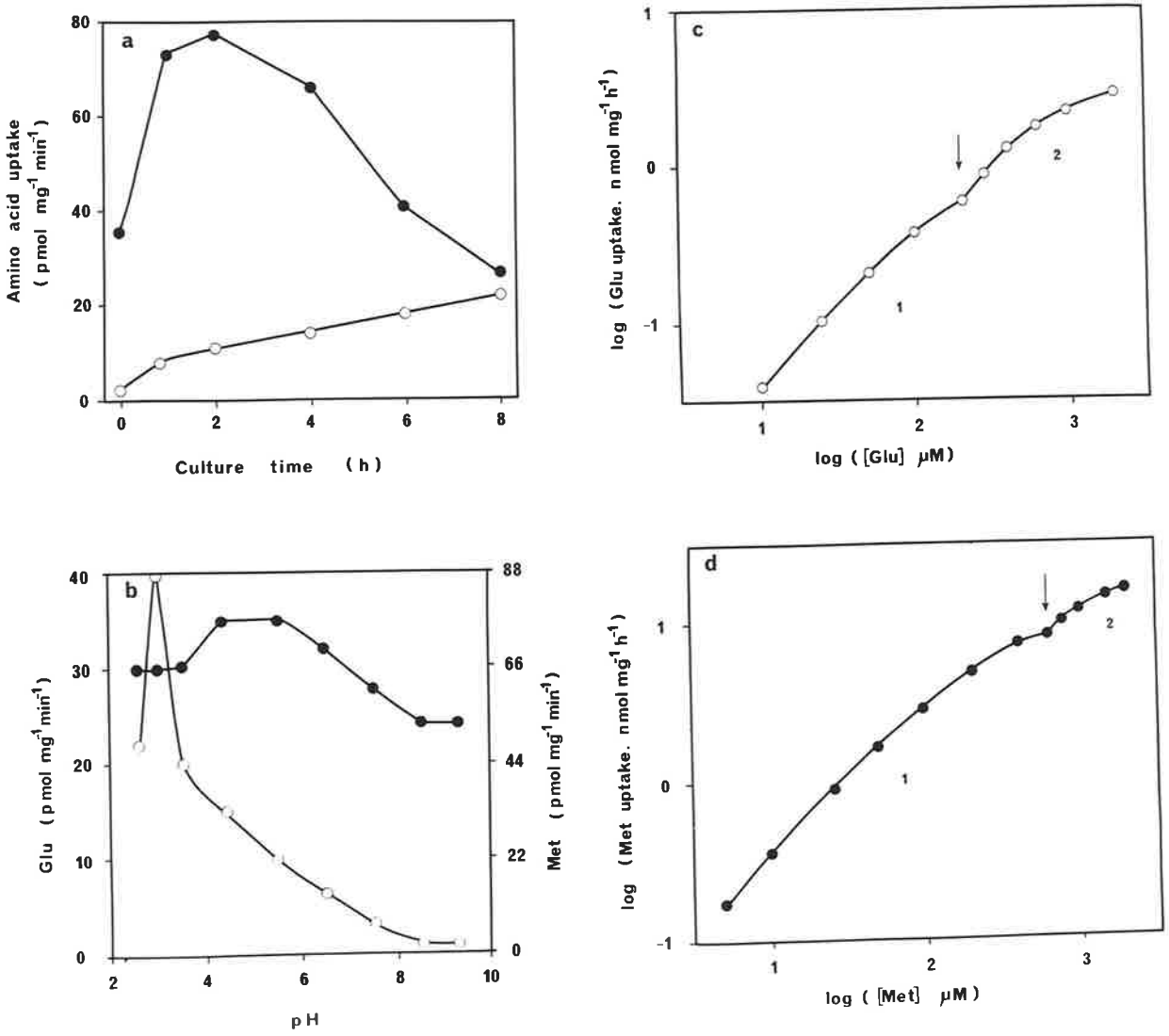

a
Amino acid uptake
(p mol mg-1 min-1)
Culture time (h)
b
Glu (p mol mg-1 min-1)
Met (p mol mg-1 min-1)
pH
c
log (Glu uptake. n mol mg-1 h-1)
log ([Glu] μM)
d
log (Met uptake. n mol mg-1 h-1)
log ([Met] μM)

Fig. 5 **Lineweaver-Burk plots of L-glutamic acid (of data in Fig. 4c.) and L-methionine (of data in Fig. 4d.) uptake by Petunia pollen.**

a. L-glutamic acid phase 1.
b. L-glutamic acid phase 2.
c. L-methionine phase 1.
d. L-methionine phase 2.

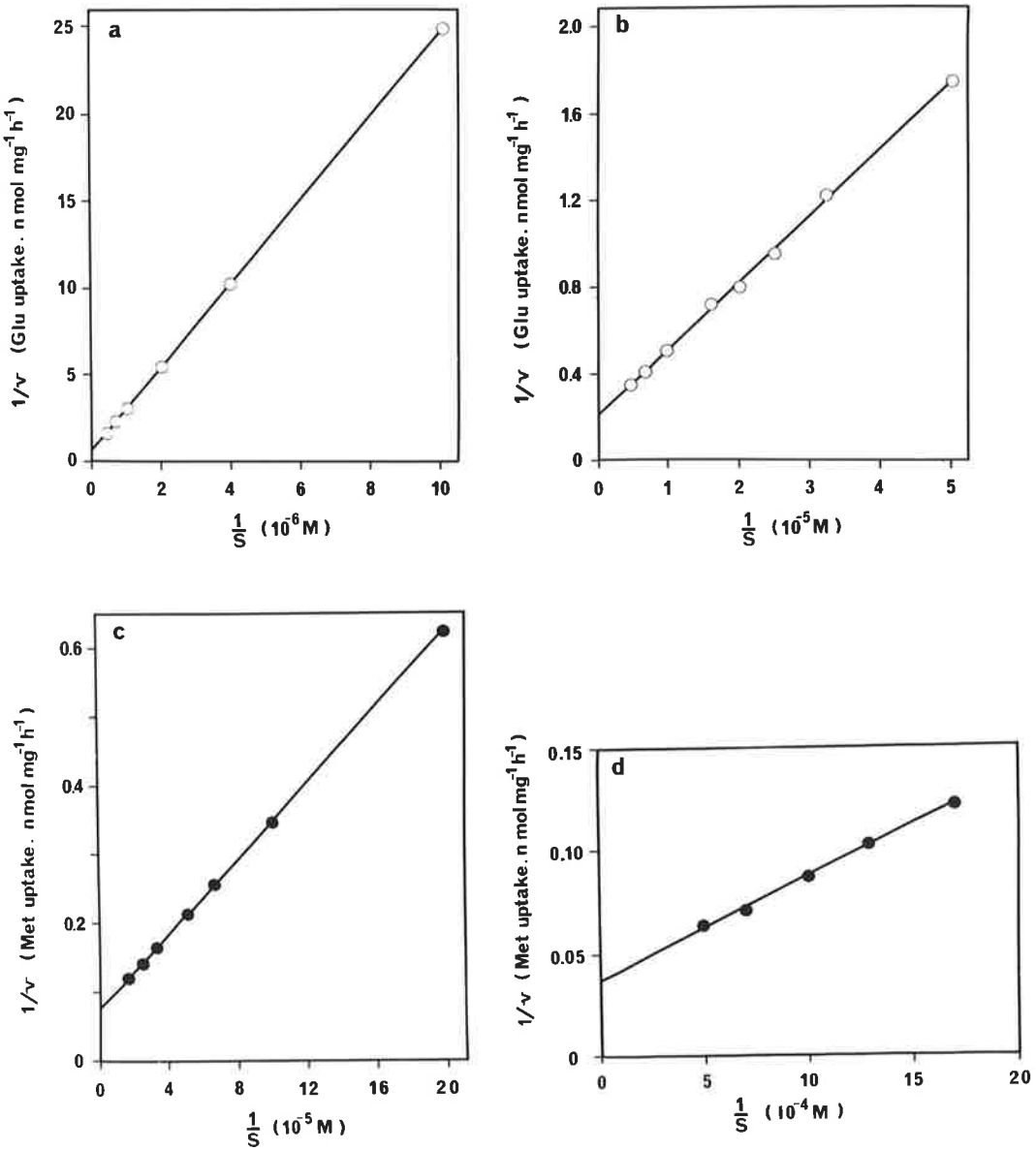
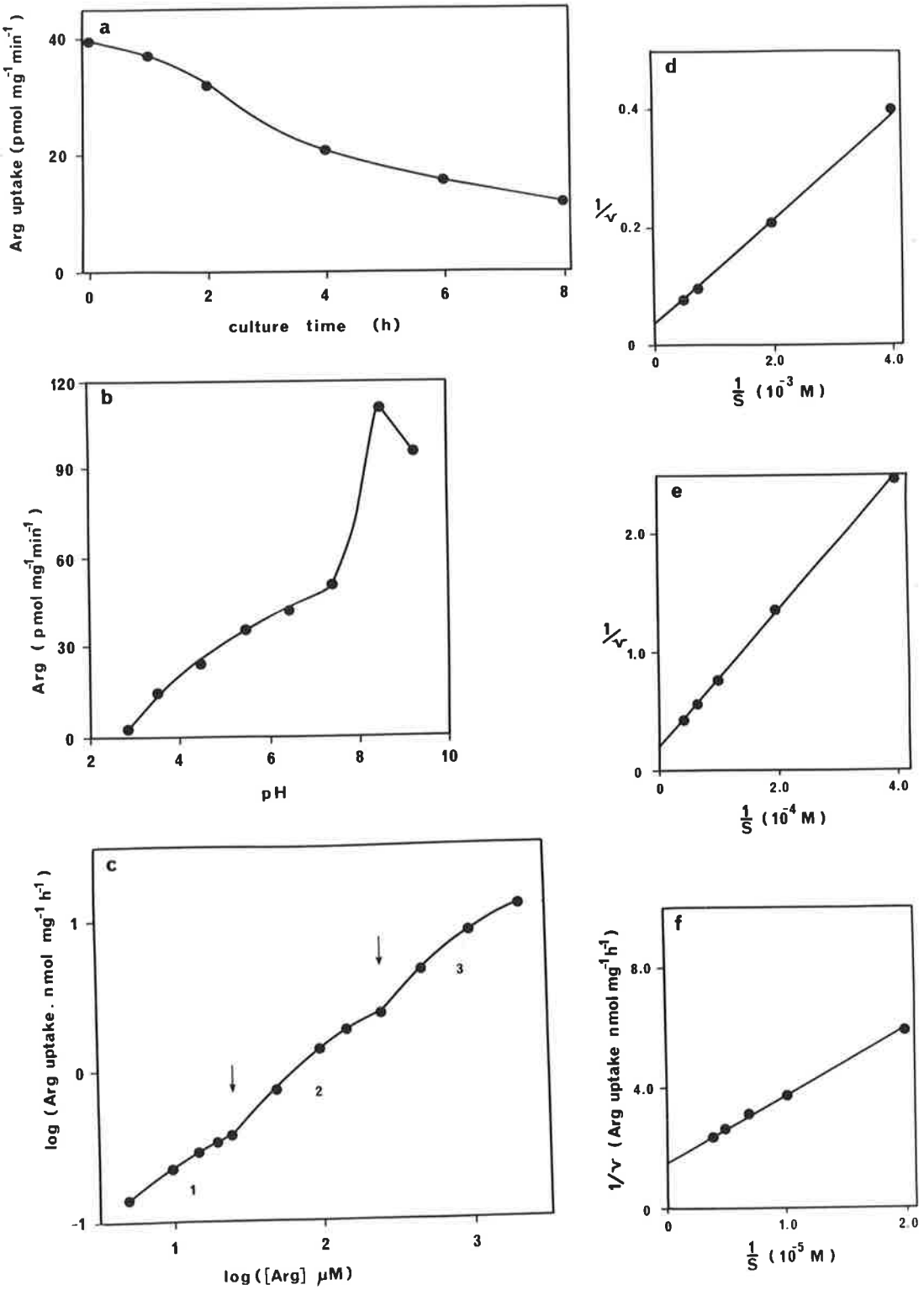

a
1/v (Glu uptake. n mol mg-1 h-1)
25
20
15
10
5
0
0
2
4
6
8
10
1/S (10-6 M)
b
1/v (Glu uptake. n mol mg-1 h-1)
2.0
1.6
1.2
0.8
0.4
0
0
1
2
3
4
5
1/S (10-5 M)
c
1/v (Met uptake. n mol mg-1 h-1)
0.6
0.4
0.2
0
0
4
8
12
16
20
1/S (10-5 M)
d
1/v (Met uptake. n mol mg-1 h-1)
0.15
0.10
0.05
0
0
5
10
15
20
1/S (10-4 M)

Fig. 6a. Effect of cultivation time on L-arginine transport in Petunia hybrida pollen.

b. Effect of pH on L-arginine uptake.

c. Double logarithmic plot of L-arginine uptake as a function of external concentration.
Three phases of uptake are indicated.

d,e,f. Lineweaver-Burk plots of L-arginine uptake (of data shown in Fig. 6c.) by Petunia pollen.


a
Arg uptake (pmol mg⁻¹ min⁻¹)
40
20
0
0
2
4
6
8
culture time (h)
b
Arg (p mol mg⁻¹ min⁻¹)
120
90
60
30
0
2
4
6
8
10
pH
c
log (Arg uptake . n mol mg⁻¹ h⁻¹)
1
0
-1
1
2
3
1
2
3
log ([Arg] μM)
d
1/v
0.4
0.2
0
0
2.0
4.0
1/S (10⁻³ M)
e
1/v
2.0
1.0
0
0
2.0
4.0
1/S (10⁻⁴ M)
f
1/v (Arg uptake nmol mg⁻¹ h⁻¹)
8.0
4.0
0
0
1.0
2.0
1/S (10⁻⁵ M)

the labelled materials within the pollen tubes indicated that when the incubation period was one hour, at least 75, 80 and 70% of the labelled material was identified as methionine, glutamic acid and arginine respectively.

Methionine, glutamic acid and arginine were accumulated against a concentration gradient. Based on the above determination of the labelled free amino acid pools, it was calculated that free methionine, glutamic acid and arginine in the intracellular pool was 3525, 464 and 1554 pmol/mg respectively at 200 µM (200 pmol/µl) amino acid concentration in the incubation medium. These results indicated that intracellular concentration of methionine, glutamic acid and arginine were at least 18-fold-, 2-fold- and 8-fold-greater than the external medium amino acid concentration respectively after 60 min. These figures are conservative estimates because they do not consider the initial amino acid pools of the pollen and the size of the compartment into which amino acids are transported.

3.3.2 pH dependence of transport of L-Glu, L-Met and L-Arg.

The rate of uptake as a function of pH over a pH range of 2.5 to 9.5 was measured for the three amino acids (Figs. 4,6). Uptake was found to be affected by pH, and there was a clear difference between glutamic acid (an acidic amino acid) on one hand and the basic amino acid, arginine, on the other. The following pH optima were recorded: for glutamic acid pH 3.0, for arginine pH 8.5 and for methionine a broad range of pH 4.0 - 6.5 (Figs. 4,6).

3.3.3 Kinetic analysis of uptake.

The effect of amino acid concentration on the uptake of methionine and glutamic acid indicates that amino acid transport over a 10^{-6} to 2×10^{-3} M concentration range was characterized by a biphasic uptake isotherm (Figs. 4,5). For descriptive purposes, the two phases were designated as phases 1 and 2. Phase 1 had higher affinities for methionine and glutamic acid with K_m values of 0.39 ± 0.02 and 0.39 ± 0.05 mM respectively as compared to phase 2, which had low affinities for methionine and glutamic

acid with K_m values of 1.27 ± 0.02 and 1.40 ± 0.07 mM respectively. Although these results showed no great difference in K_m values between methionine and glutamic acid in both phases, a large variation in V_{max} was found in both phases (Table 13). The uptake of glutamic acid was considerably slower than that of methionine, being only 12% and 18% of phase 1 and phase 2 respectively (Table 13). The Kinetic constants for L-Glu in Petunia pollen are similar to those found for L-Glu phase 1 and 2 in barley leaf slices by Lein and Rognes (1977). These authors found three phases for L-Glu uptake in the concentration range of 0.05 - 22 mM. Analysis of uptake rates for arginine concentrations ranging form 10^{-6} to 2 x 10^{-3} M showed that uptake was multiphasic with three saturable phases (Fig. 6). Phase 1 had very high affinity for arginine, with a K_m of 19.2 ± 4.8 µM and a V_{max} of 0.68 nmol/mg fresh weight/h. K_m of arginine for phase 1 was much lower as compared to K_m values for phase 1 of methionine and glutamic acid transport. Phase 2 had low affinity for arginine (K_m = 0.32 ± 0.02 mM and V_{max} 5.75 ± 0.26 nmol/mg fresh weight/h) whereas phase 3 had even lower affinity for arginine (K_m = 2.58 ± 0.37 mM and V_{max} = 15.14 ± 1.39 nmol/mg fresh weight/h).

At amino acid concentrations greater than 2 mM, interpretation of transport experiments became confused by the expected occurrence of diffusion of amino acids into the pollen tubes. The amount of diffusion was directly related to the amino acid concentration. Diffusion at higher amino acid concentrations have also been noted in suspension cultured tobacco cells (Blackman and McDaniel, 1980).

3.3.4 Effect of metabolic inhibitors and other agents.

3.3.4.1 Methionine and glutamic acid transport.

Methionine and glutamic acid transport was inhibited by a variety of compounds known to affect cellular energy metabolism (Table 14). The proton translocators (uncouplers) CCCP and DNP were the most effective inhibitors of methionine and glutamic acid transport in Petunia pollen (Table 14). ATPase inhibitors DCCD and NBD-Cl were also effective inhibitors of transport of both the amino acids (Table 14).

Table 13. Kinetic constants for L-methionine and L-glutamic acid transport by Petunia pollen.

Amino acids		K_m(mM)	V_{max} (nmol mg fresh weight $^{-1}h^{-1}$)
L-methionine	Phase 1	0.39±0.02	14.01±0.24
	Phase 2	1.27±0.02	26.21±0.19
L-glutamic acid	Phase 1	0.39±0.05	1.71±0.14
	Phase 2	1.40±0.07	4.72±0.13

Table 14. Effect of metabolic inhibitors and thiol reagents on L-methionine and L-glutamic acid transport in Petunia pollen.

Control rates of transport for 200 µM and 400 µM L-methionine were 4.70 ± 0.07 and 7.2 ± 0.09 nmol/mg fresh weight/h respectively. Control rates of transport for 200 µM and 400 µM L-glutamic acid were 0.59 ± 0.20 and 1.06 ± 0.05 nmol/mg fresh weight/h respectively.

Inhibitor	Concentration (µM)	Inhibition of Transport (%)			
		L-methionine concentration (µM)		L-glutamic acid concentration (µM)	
		200	400	200	400
CCCP	20	82	76	80	78
DNP	100	75	71	69	70
NBD-Cl	50	53	49	56	54
DCCD	100	41	34	60	63
NEM	200	56	51	49	47
PAO	200	39	-	0	-
Ethanol	(0.5%)	0	-	0	-
Cycloheximide	(100 µg/ml)	0	-	40	-

The sulfhydryl reagent NEM was found to be a potent inhibitor of methionine and glutamic acid transport, which is in agreement with the observations of several workers using a variety of organisms (Harrington & Henke, 1981; Nelson *et al.*, 1975). Phenylarsine oxide, a dithiol binding agent (Walker-Smith & Payne, 1983), was also found to be an effective inhibitor of transport of both the amino acids.

Cycloheximide inhibits protein synthesis in *Petunia* pollen. When the effect of cycloheximide was tested on L-Met and L-Glu uptake, it was found to be a potent inhibitor of L-Glu transport, and without effect on L-Met transport (Table 14).

Several workers have demonstrated the pitfalls involved in using several of the above inhibitors to study transport, due to the fact that these inhibitors may affect membrane integrity (Harrington & Smith, 1977; Smith, 1978). In the presence of some inhibitors, transport may proceed at a normal rate, but net transport (uptake minus efflux) may be lower due to membrane damage. The metabolic inhibitors discussed above, at concentrations inhibitory to transport, did not increase label efflux as compared to controls, after 30 min resuspension in the presence of the inhibitor. Therefore, these compounds inhibited transport of methionine and glutamic acid by affecting the energy metabolism rather than by altering membrane integrity under the conditions of the experiments described here.

3.3.4.2 Arginine transport.

The effect of metabolic inhibitors on arginine uptake was tested either when present in the transport medium or in pre-incubation experiments. In the latter, pollen tubes were pre-incubated for 15 min in culture medium containing the metabolic inhibitor and washed with culture medium three times before addition to the transport medium. For the proton translocators (uncouplers) CCCP and DNP, there were large differences in uptake rates determined by these two methods. When the uncouplers were included in the transport medium, no inhibition of arginine transport was observed; instead the uncouplers actually stimulated the uptake of arginine. DNP showed more stimulation of transport than CCCP. Both these reagents inhibited arginine

transport when these were used in preincubation experiments and removed before initiation of transport (Table 15), but the level of inhibition was much less as compared to inhibition of methionine or glutamic acid uptake.

For the other inhibitors tested, there were no significant differences in uptake rate determined by either of above methods. At both 20 and 40 μM arginine concentration, the ATPase inhibitors NBD-Cl and DCCD inhibited arginine transport (Table 15). The effects of thiol binding agent, NEM and a dithiol binding agent, phenylarsine oxide on arginine transfer were also examined. Both these reagents inhibited arginine transfer in Petunia pollen (Table 15). For all the inhibitors tested, arginine uptake at a concentration of 400 μM was generally inhibited to a greater extent than at the 20 μM arginine concentration (Table 15). The protein synthesis inhibitor, cycloheximide had no effect on arginine transport.

The inhibitors discussed above did not increase efflux of label, as compared to control, after 30 min resuspension in the presence of the inhibitor. The inhibition by these metabolic inhibitors was therefore probably due to the effects of these compounds on energy metabolism required for transport, and in the case of NEM and phenylarsine oxide, due to effects on protein structure rather than on membrane integrity.

3.3.5 Specificity of transport of amino acids.

3.3.5.1 Inhibition of methionine and glutamic acid uptake by other amino acids and analogs.

The influence of various amino acids and amino acid analogs on the uptake of L-Met and L-Glu was tested to obtain information on the specificity of L-Met and L-Glu transport systems (Table 16). In these experiments, the antagonistic compound was present at a concentration five-fold higher than that of L-Met or L-Glu. Uptake of L-Met was most sensitive to inhibition by cysteine and leucine at both substrate levels. At 400 μM L-Met, alanine, valine, proline and glycine gave greater inhibition of L-Met transport than at 200 μM L-Met. D-Met was a poor inhibitor at

Table 15. Effect of metabolic inhibitors and thiol reagents on L-arginine transport in Petunia pollen.

Control rates of transport for 20 μM and 400 μM L-arginine were 0.34 ± 0.03 and 3.80 ± 0.21 nmol/mg fresh weight/h respectively.

Inhibitor	Concentration (μM)	Inhibition of Transport (%)	
		L-arginine concentration (μM)	
		20	400
CCCP	20	32	51
DNP	100	19	32
NBD-Cl	50	38	47
DCCD	100	35	51
NEM	200	30	42
PAO	200	32	45
Ethanol	(0.5%)	0	0
Cycloheximinde	(100 μg/ml)	0	0

Table 16. Effect of amino acids and analogues on L-methionine and L-glutamic acid transport in Petunia pollen.

Amino acid concentration was five-fold higher than L-methionine or L-glutamic acid substrate concentration. Control rates of transport were as given in Table 14. legend.

Amino Acid	Inhibition of Transport (%)			
	L-methionine concentration (μM)		L-glutamic acid concentration (μM)	
	200	400	200	400
D-Met	<5	<5	-	-
D-Glu	-	-	<5	<5
L-Ala	7	31	<5	<7
L-Leu	22	43	0	0
L-Ileu	11	-	0	0
L-Glu	0	0	-	-
L-Asp	0	0	14	34
L-Cys	26	47	0	0
L-Pro	5	21	0	<5
L-Val	5	19	0	0
L-Met	7	32	0	0
Gly	-	-	0	<5

both substrate levels. L-Met transport was not inhibited by acidic and basic amino acids (Table 16). Even when a hundredfold higher concentration of antagonistic compounds was tested, the uptake of L-Met was only marginally inhibited by D-Met, acidic and basic amino acids, however cysteine, leucine, alanine, valine, and proline proved to be effective inhibitors of L-Met transport (Table 17).

At 200 and 400 µM L-Glu, aspartic acid was the only inhibitor of L-Glu transport, whereas all other amino acids and analogs including D-Glu had little or no inhibitory effect (Table 16). L-Asp was the only effective inhibitor of L-Glu transport, even when a hundred-fold higher concentration of antagonistic compounds was tested, D-Glu or alanine was the only other marginal inhibitor of L-Glu uptake (Table 17) highlighting the specificity of L-Glu transport system in Petunia pollen.

3.3.5.2 Inhibition of arginine transport by other amino acids.

The effect of various amino acid and D-Arg was tested to determine the specificity of L-Arg transport (Table 18). At a L-Arg concentration of 20 µM, the uptake was not inhibited by D-Arg, L-Lys, L-Orn, L-His, L-Met, L-Glu or L-Gln and was only marginally inhibited by L-Leu, L-Ala, L-Val and L-Pro (Table 18). When a hundredfold higher concentration of antagonistic amino acids was tested, L-Arg transport was inhibited by all the tested amino acids except that the uptake of L-Arg was only slightly inhibited by D-Arg (Table 18). At 400 µM L-Arg concentration, the uptake L-Arg was most sensitive to inhibition by L-Leu, whereas all the other amino acids had some inhibitory effect (Table 18).

3.3.6 Effect of calcium, metal chelators and polyamines on L-Met and L-Glu transport.

In the absence of calcium, transport of L-Met and L-Glu was inhibited to the extent of 35% and 56% respectively. This inhibition was reversed by the presence of 0.25 mM calcium in the

Table 17. Effect of hundred-fold concentrations of amino acids on L-methionine and L-glutamic acid transport in *Petunia* pollen.

L-methionine or L-glutamic acid substrate concentration was 20 µM. Control rates of transport for L-methionine and L-glutamic acid were 0.75 ± 0.03 and 0.09 ± 0.01 nmol/mg fresh weight/h respectively.

Amino acid	Inhibition of Transport (%)	
	L-methionine	L-glutamic acid
D-Met	14	-
D-Glu	-	11
L-Cys	61	0
L-Ala	45	10
L-Leu	57	0
L-Val	39	0
L-Pro	37	0
L-Glu	13	-
L-Asp	10	40
L-Arg	8	0
L-Met	-	0

Table 18. Effect of amino acids on L-arginine transport in *Petunia* pollen.

Amino acid concentration was ten-fold (a) and hundred-fold (b) higher when L-arginine concentration was 20 µM and five-fold higher when L-arginine concentration was 400 µM. Control rates of transport were as given in Table 15. legend.

Amino acid	Inhibition of Transport (%)		
	L-arginine concentration (µM)		
	20		400
	(a)	(b)	
D-Arg	0	15	18
L-Lys	0	24	25
L-Orn	0	33	13
L-His	0	-	29
L-Met	0	35	33
L-Glu	0	33	31
L-Glu	0	-	15
L-Ala	9	22	27
L-Val	17	33	33
L-Leu	20	30	44
L-Pro	17	30	38

culture medium. When the calcium concentration in the culture medium was raised to 0.5 or 1.0 mM, there was no further stimulation in uptake of either of the amino acids, but when calcium concentration was further raised to 5.0 mM, the transport of L-Met and L-Glu was stimulated by 9 and 47% respectively. When 25.0 mM calcium concentration was tested, L-Glu transport showed no stimulation as compared to control (Table 19).

Of the metal chelators, EDTA gave similar inhibition of transport of both the amino acids whereas EGTA gave more inhibition of L-Glu transport as compared to L-Met. These results supported the earlier observation that in the absence of calcium, L-Glu transport is inhibited more than L-Met transport. It was also observed that EDTA and EGTA induced the efflux of both the amino acids. Of the polyamines tested, spermine and spermidine showed some stimulation of the transport of both the amino acids (Table 19). Spermine stimulated L-Glu uptake more than L-Met uptake.

3.3.7 Effect of cations and polyamines on L-Arg transport.

The effect of several cations on L-Arg uptake was examined and all tested cations at 1 mM concentration inhibited L-Arg uptake. Of all the cations, Al^{3+} gave the strongest inhibition of L-Arg uptake and all divalent cations tested gave severe inhibition of L-Arg uptake (Table 20). Even monovalent cations such as Na^+, K^+ and NH_4^+ gave significant inhibitions at concentrations of 1 mM (Table 20). These results are in direct contrast to the stimulatory effect of calcium on L-Met and L-Glu transport.

This contrast was further supported with the observation that polyamines which stimulated the uptake of L-Met and L-Glu, were very potent inhibitors of L-Arg uptake (Table 20). Even putrescine which was without effect on L-Met or L-Glu transport, was found to be a very good inhibitor (Table 20). Of the three polyamines, spermine and spermidine were the most effective inhibitors of L-Arg transport.

Table 19. Effect of calcium, metal chelators and polyamines on L-methionine and L-glutamic acid transport in Petunia pollen.

Control rates of transport were as given in Table 14. legend.

Treatment	Concentration (mM)	Inhibition of Transport (%)	
		L-methionine (200 μM)	L-glutamic acid (200 μM)
$Ca(NO_3)_2$ (Control)	1.0	0	0
$Ca(NO_3)_2$	0	35	56
$Ca(NO_3)_2$	0.25	9	0
$Ca(NO_3)_2$	0.50	0	0
$Ca(NO_3)_2$	5.00	(9% stimulation)	(47% stimulation)
$Ca(NO_3)_2$	25.00	-	0
EDTA	1.00	65	69
EGTA	1.00	35	68
Spermine	0.125	(16% stimulation)	(28% stimulation)
Spermine	0.500	(19% ")	(33% ")
Spermidine	0.125	(12% ")	(11% ")
Putrescine	0.125	(<5% ")	0

Table 20. Effect of cations and polyamines on L-arginine transport in Petunia pollen.

Cation concentration was 1 mM and all cations were present as chlorides. Control rates of transport were as given in Table 15. legend.

Cations	Inhibition of Transport (%) L-arginine (400 µM)	Polyamines	Concentration mM	Inhibition of Transport (%) L-arginine (20 µM)
Na^+	27	Spermine	0.125	58
K^+	24		0.250	73
NH_4^+	50			
Ca^{2+}	74	Spermidine	0.125	55
Mg^{2+}	71		0.250	60
Mn^{2+}	82			
Co^{2+}	77	Putrescine	0.125	23
Cu^{2+}	80		0.250	27
Al^{3+}	95			

3.4 Control of protein synthesis and protein release during germination and pollen tube growth.

3.4.1 Control of protein release.

When Petunia hydrida pollen from clone W166H is cultured in 10% sucrose, no germination or tube growth is observed as long as precautions are taken to exclude boron (e.g. use of plastic Erlenmeyer flasks and pipettes). Under these conditions massive amounts of proteins are released to the medium (Table 21), the amounts rising steadily with time. By 5h, 5mg protein has been given up by 35 mg pollen. When small amounts of boric acid are added, pollen germination and tube length increases dramatically, while protein release is reduced, but much more slowly (Table 21). Calcium salts also reduce the amount of protein released, however quite high concentrations are required for significant effects, while calcium has no effect on germination and tube growth for this clone of Petunia hybrida (Table 21). Similar effects of boron and calcium on protein release from pollen of Nicotiana tabacum have been described by Capkova-Balatkova et al. (1980).

As can be seen in Fig. 7, a substantial amount of protein is gradually released to the medium from germinating pollen over 5h, protein accumulation increasing with time in an hyperbolic relationship. By 5h approximately 40 per cent of the total protein content of the pollen had appeared in the medium. Addition of the RNA synthesis inhibitor actinomycin-D does not greatly change this. However, cycloheximide, an inhibitor of protein synthesis, reduces the amount of protein exported from germinating pollen by more than 50% (Fig. 7). The uncouplers (Heytler, 1979) and ATPase inhibitors (Linnett and Beechey, 1979) are the most effective inhibitors of protein release (Table 22). The time course of inhibitors of the uncoupler DNP is shown in Fig. 7; after 1h, no further protein is released from the pollen. A similar inhibition is shown by the other proton translocator (uncoupler) CCCP, and by the other energy poisons, the ATPase inhibitors DCCD and NBD-Cl (Table 22). Unlike cycloheximide, the energy poisons have a dramatic effect in reducing pollen germination and pollen tube elongation (Table 22). The amount of protein given up to the medium in the presence of these energy inhibitors is about 25% of that released after 5h of normal in

Table 21. The effect of various compounds on protein release, germination and pollen tube length, after 5 h culture of *Petunia hybrida* pollen.

A standard deviation is indicated for each measurement. The basic culture solution contained 35 mg pollen in 5 ml of 10% sucrose.

Inhibitor	Concentration	Protein released (mg/35 mg) pollen	Germination (%)	Tube length (µM)
None	-	5.0±0.3	0	0
H_3BO_3	(0.0001%)	3.5±0.3	17±2	121±22
H_3BO_3	(0.01%)	2.0±0.1	55±3	162±58
$Ca(NO_3)_2$	1.0 mM	2.5±0.1	0	0
H_3BO_3+$Ca(NO_3)_2$	0.01%+1.0 mM	0.6±0.05	54±4	160±65
H_3BO_3+IAA	0.01%+0.5 mM	0.5±0.05	18±2	90±51
DNP	0.05 mM	0.5±0.05	0	0

Table 22. The effects of inhibitors on protein release, germinatio and pollen tube length, after 5h culture of *Petunia hybrida* pollen.

A standard deviation is indicated for each measurement. The basic culture solution contained 35 mg pollen in 5 ml of 10% sucrose in 0.01% boric acid.

Inhibitor	Concentration (mM)	Protein released (mg/35 mg) pollen	Germination (%)	Tube length (µM)
None	-	2.0 ±0.1	55±3	162±58
DNP	0.05	0.45±0.02	0	-
CCCP	0.01	0.45±0.03	0	-
DCCD	0.05	0.43±0.02	0	-
NBD-Cl	0.05	0.44±0.04	0	-
Cycloheximide	(100 µg/ml)	0.75±0.03	34±2	75±55
Actinomycin D	(200 µg/ml)	1.4 ±0.10	49±2	146±42
N-Ethylmaleimide	0.01	0.45±0.04	0	-

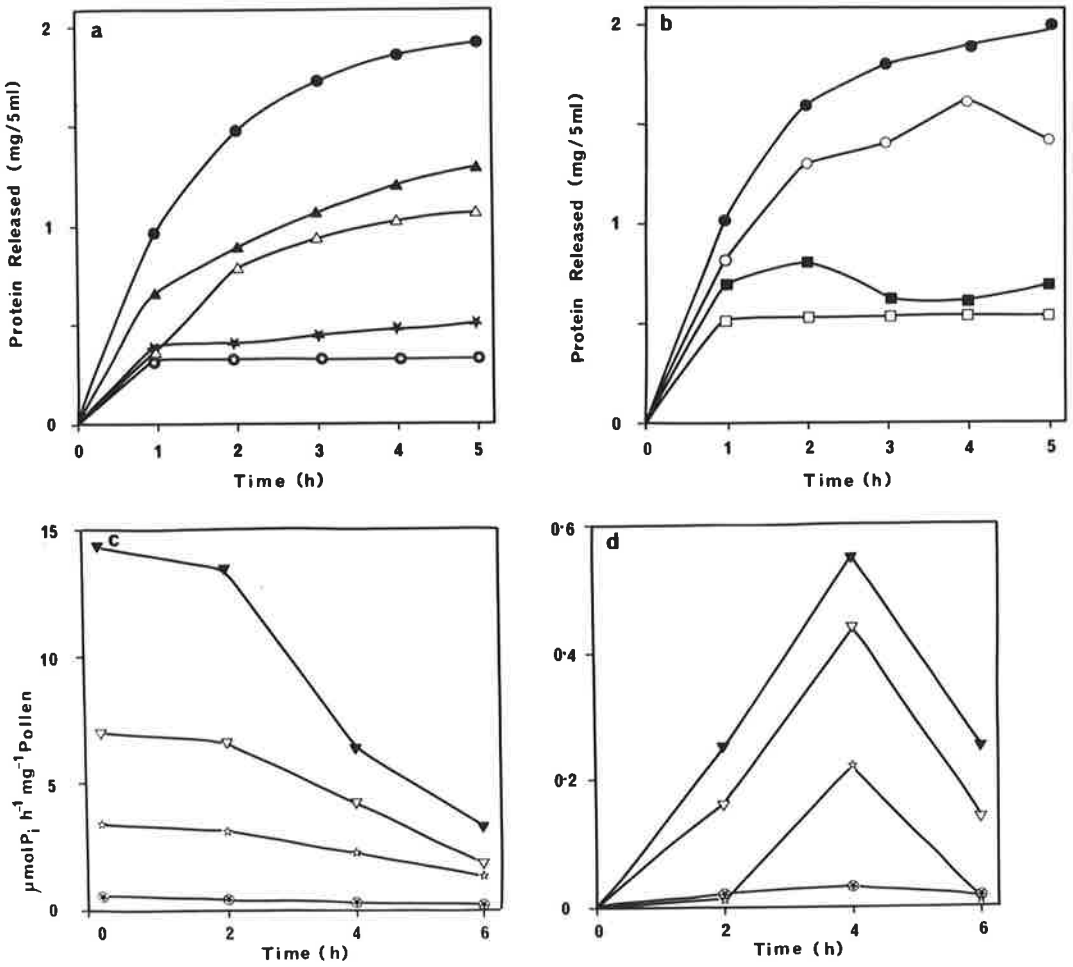
Fig. 7a,b. **Effect of various compounds on the time course of protein release from germinating pollen of Petunia hybrida.**

a. No addition to normal medium (●); + 0.25 mM Ca^{2+} (▲); + 0.1 mM spermine (△); 1.0 mM Ca^{2+} (✶); 0.5 mM spermine (✪).

b. No addition to normal medium (●); 200 µg/ml actinomycin D (○); 100 µg/ml cycloheximide (■); 0.05 mM DNP (□).

Fig. 7c. **Activities of various phosphatases during germination and pollen tube growth.**
Acid pyrophosphatase (▼); Glycerophosphatase (▽); p-nitrophenylphosphatase (✲); Phytase (✠).

d. **Activities of various phosphatases released during** germination and pollen tube growth.


a
b
c
d
Protein Released (mg/5ml)
Time (h)
µmolP$_i$ h^{-1} mg^{-1}Pollen

vitro germination, and only 10% of the total protein content of the pollen. This then may well be protein which freely diffuses out of the pollen, needing no energy or metabolic driving force for its release.

As shown in Table 21, energy poisons like DNP prevent protein release in the absence of boron also and similar experiments in the presence of calcium (and absence of boron) show that DNP is equally effective in this case. Several other compounds inhibit protein export from Petunia hydrida pollen. Auxins and polyamines exerted a considerable effect, although only at relatively high concentrations. At 0.5 mM, IAA, 2,4-D and spermine showed significant inhibition. Another type of compound found to inhibit protein release was the sulphydryl-binding reagent, such as NEM (Table 22), or phenylarsine oxide, which was equally effective. In the germinating barley embryos, active peptide transport was also severly inhibited by these thiol reagents (Walker-Smith and Payne, 1983).

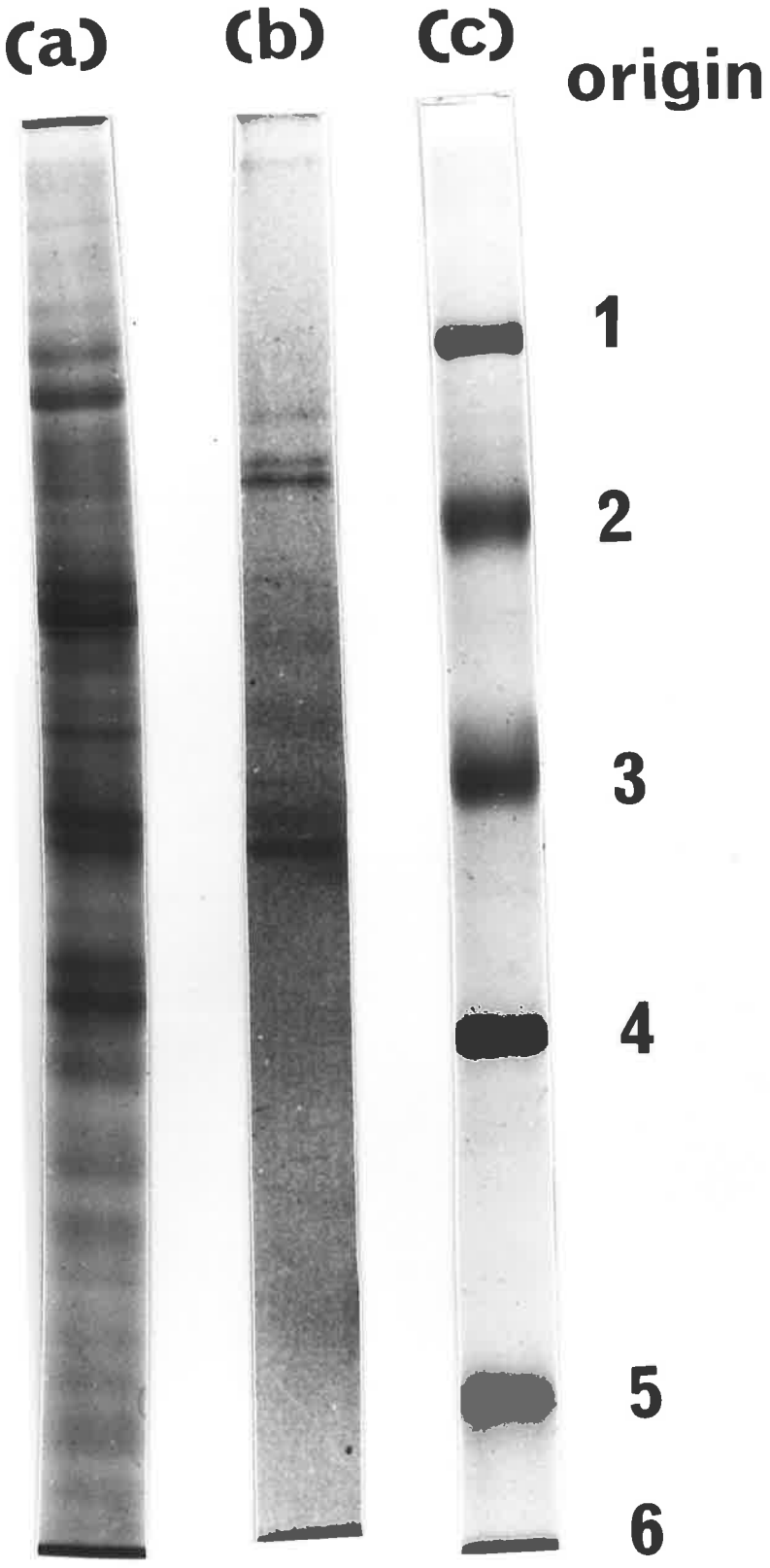
SDS-PAGE of proteins released by 2h in normal germination medium, supplemented with {^{35}S} methionine to monitor protein synthesis, showed that there were many different proteins exported from germinating Petunia pollen (Fig. 8). Autoradiography demonstrated that of the 12 major protein bands, only two had significant radioactivity. These were the two bands with a molecular weight slightly less than the ovalbumin standard protein (43,000 daltons). The other labelled bands did not appear to coincide with any of the major protein bands (Fig. 8) and must therefore be minor components of total released protein. While further work is needed to sort out the temporal relationships and patterns of protein released, it is already apparent that while some of the proteins exported during germination in the absence of uncouplers and ATPase inhibitors may be labelled and thus, represent newly synthesized protein, nevertheless the number of unlabelled protein is too large (Fig. 8) to represent solely protein diffusing from the pollen grain walls. It seems then that the proteins released in the absence of energy inhibitors (and not including the freely diffusing fraction) consist of both stored and newly synthesized proteins.

Fig. 8 **SDS-PAGE and autoradiography of proteins released from germinating Petunia hybrida pollen by 2h.**

a. Coomassie Blue-stained gel of proteins released from germinating pollen.

b. Autoradiogram of dried gel of proteins released from germinating pollen.

c. Coomassie Blue-stained gel of low molecular weight calibration kit proteins (Pharmacia). The protein standards consist of the following: 1, phosphorylase b (94,000 daltons); 4, carbonic anhydrase (30,000 daltons); 5, trypsin inhibitor (20,100 daltons); 6, α-lactalbumin (14,400 daltons).


(a)
(b)
(c)
origin
1
2
3
4
5
6

3.4.2 Effect of various inhibitors of protein release on the SDS-PAGE patterns of proteins released.

The effect of the several compounds (which inhibit protein export from Petunia hybrida pollen) on the SDS-PAGE patterns of proteins released was investigated (Fig. 9). Of the several compounds tested, only calcium at 1 mM concentration had some effect on the pattern of proteins released. Calcium at 1 mM severly inhibits the protein release from Petunia pollen and consequently, at least four times as much pollen was used to achieve a Coomassie Blue stained gel pattern of proteins released in the presence of 1 mM calcium. When the gel patterns of proteins released in the presence of 1 mM calcium was compared with the control gel pattern, it was clear that calcium stimulated the release of at least two proteins of molecular weight 28,000 and 20,000 (Fig. 9). None of the other compounds had any effect on the pattern of protein released (Fig. 9) despite the fact that all of these compounds are good inhibitors of protein release from Petunia pollen. Since the resolution of single dimension gels, is relatively poor, pollen proteins and proteins released from pollen were analysed by two-dimensional gel electrophoresis.

3.4.3 Changes in enzyme activities and enzymes released during germination and pollen tube growth.

Various phosphatase activities (pyrophosphatase, glycerophosphatase, p-nitrophenylphosphatase and phytase) were investigated during Petunia pollen germination and tube growth. In the Petunia pollen, the activities of the phosphatases decreased after dispersion in the culture medium. Subsequently, the activities continued to decrease with elongation of the pollen tubes (Fig. 7). Pyrophosphatase activity was the highest among the phosphatases investigated in this study.

The release of the various phosphatases into the culture medium from the germinating Petunia pollen was also investigated. Only a small fraction of the activity of the various phosphatases was released into the culture medium and the activities continued to increase up to 4h pollen tube growth stage. Subsequently, the activities of all the phosphatases started to decline (Fig. 7).

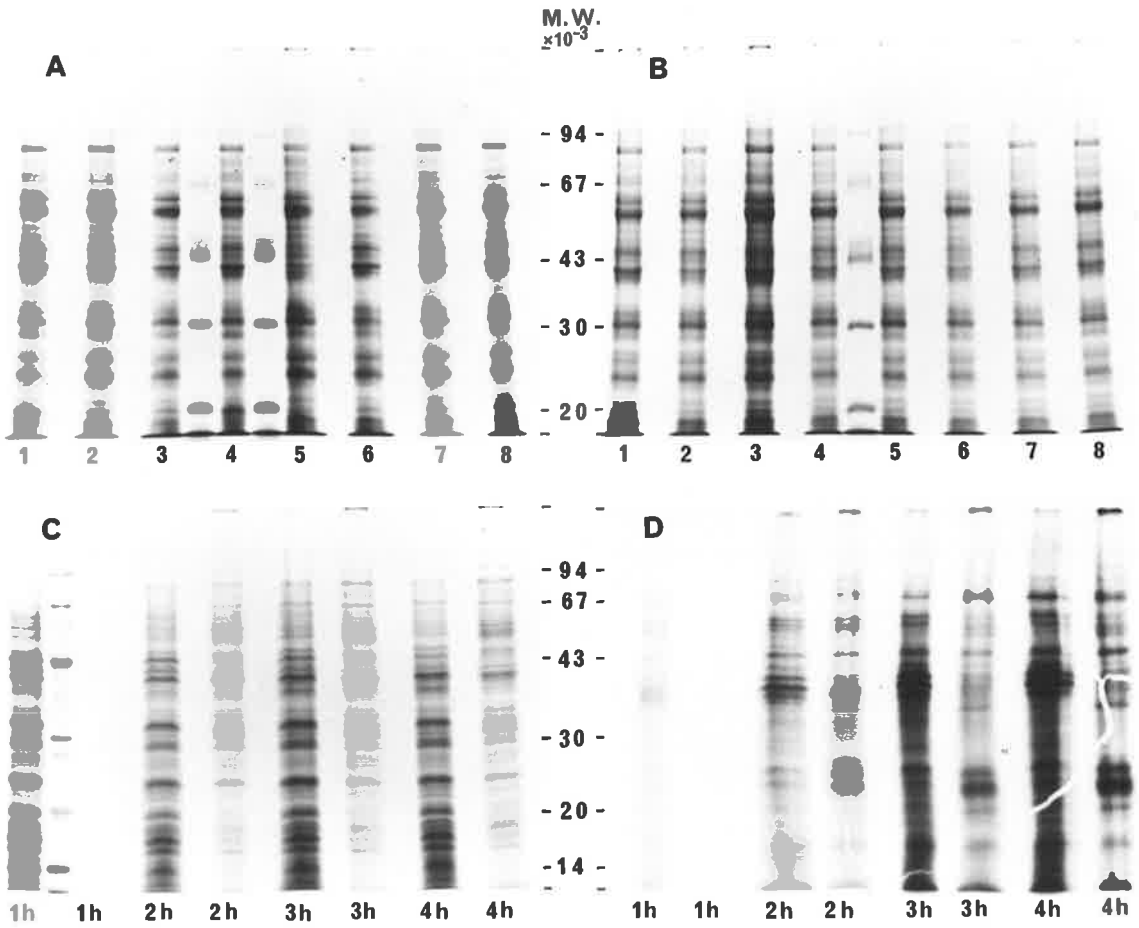
Fig. 9A,B. **Coomassie Blue-stained SDS-PAGE (11% PAA) patterns of proteins released during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.**

A. 1, minus boron and plus 1 mM Ca^{2+} (i.e. 10% sucrose in 1 mM Ca^{2+}); 2, No addition to normal medium (i.e. 10% sucrose in 0.01% boric acid); 3, plus 0.25 mM Ca^{2+}; 4, plus 1 mM Ca^{2+}; 5, plus 1 mM EGTA; 6, plus 0.1 mM PAO; 7, plus 1 mM phosphate; 8, plus 1mM citrate.

B. 1, minus boron (i.e. 10% sucrose); 2, 10% sucrose plus 0.5 mM 2, 4-D; 3, 10% sucrose plus 0.5 mM IAA; 4, normal medium (10% sucrose in 0.01% boric acid); 5, plus 0.1 mM IAA; 6, plus 0.1 mM 2, 4-D; 7, plus 0.01 mM IAA; 8, plus 0.01 mM 2, 4-D. Molecular weight markers were run in unmarked lanes.

Fig. 9C,D, **Coomassie Blue-stained patterns (C) and fluorographs (D) of SDS-PAGE (12.5% PAA) of pollen proteins and proteins released during germination and pollen tube growth in Petunia hybrida.**

1h, 2h, 3h, 4h pulse-labelled {U-^{14}c} protein hydrolysate was given at 0h to pollen culture. Proteins were separated by SDS-PAGE and detected by fluorography.


A
B
C
D
M.W.
×10⁻³
94
67
43
30
20
14
1
2
3
4
5
6
7
8
1h
2h
3h
4h

The isoenzyme patterns of phytase and glycerophosphatase were analysed by polyacrylamide disc gel electrophoresis. Five isoenzymes of phytase were detected in the Petunia pollen. The relative mobilities of various isoenzymes were 0.14, 0.19, 0.26, 0.39 and 0.46. Only one isoenzyme with relative mobility of 0.26 was detected for glycerophosphatase in Petunia pollen.

3.4.4 Analysis of the SDS-PAGE patterns of pollen proteins and proteins released during germination and pollen tube growth.

Pollen proteins during germination and pollen tube growth were investigated by SDS-polyacrylamide gel electrophesis (Fig. 9). About 50 protein bands of pollen proteins of 1h germinated pollen can be seen in Fig. 9. However, even after a careful comparison of the SDS-PAGE patterns of protein bands of pollen proteins from 1h, 2h, 3h, and 4h germinated pollen, no qualitative differences were found (Fig. 9). The proteins released from germinating pollen were also analysed by SDS-PAGE (Fig. 9). During the first hour of germination, very little protein is released and consequently very few protein bands were obtained after Coomassie Blue staining. Before 2h of germination about 50 protein bands of released proteins can be seen (Fig. 9). A careful comparison of the SDS-PAGE patterns of released proteins from 3h and 4h germinated pollen as well as pollen germinated for 2h or less shows that there are no qualitative differences in the patterns of protein bands seen at these times (Fig. 9).

3.4.5 Analysis of the SDS-PAGE patterns of proteins synthesized and their release during pollen germination and tube growth.

Single dimension SDS polyacrylamide gel electrophoresis of proteins synthesized during the 1h, 2h, 3h and 4h of pollen germination and tube growth seem to indicate that at least 35 proteins are synthesized during germination and pollen tube growth (Fig. 9). A careful comparison of the fluorographs shows that there are no qualitative differences in the patterns of protein bands seen in 2h, 3h and 4, lanes (Fig. 9). The newly synthesized proteins released from germinating pollen were also investigated by SDS-PAGE and fluorography (Fig. 9). Since during

the first hour of germination, very little protein is released, very few newly synthesized protein bands can be seen in the 1h lane (Fig. 9). About 35 bands of newly synthesized released proteins are evident in Fig. 9. A careful comparison of the fluorographs of released proteins shows that there are no qualitative differences in the patterns of protein bands seen in the 2h, 3h and 4h lanes (Fig. 9).

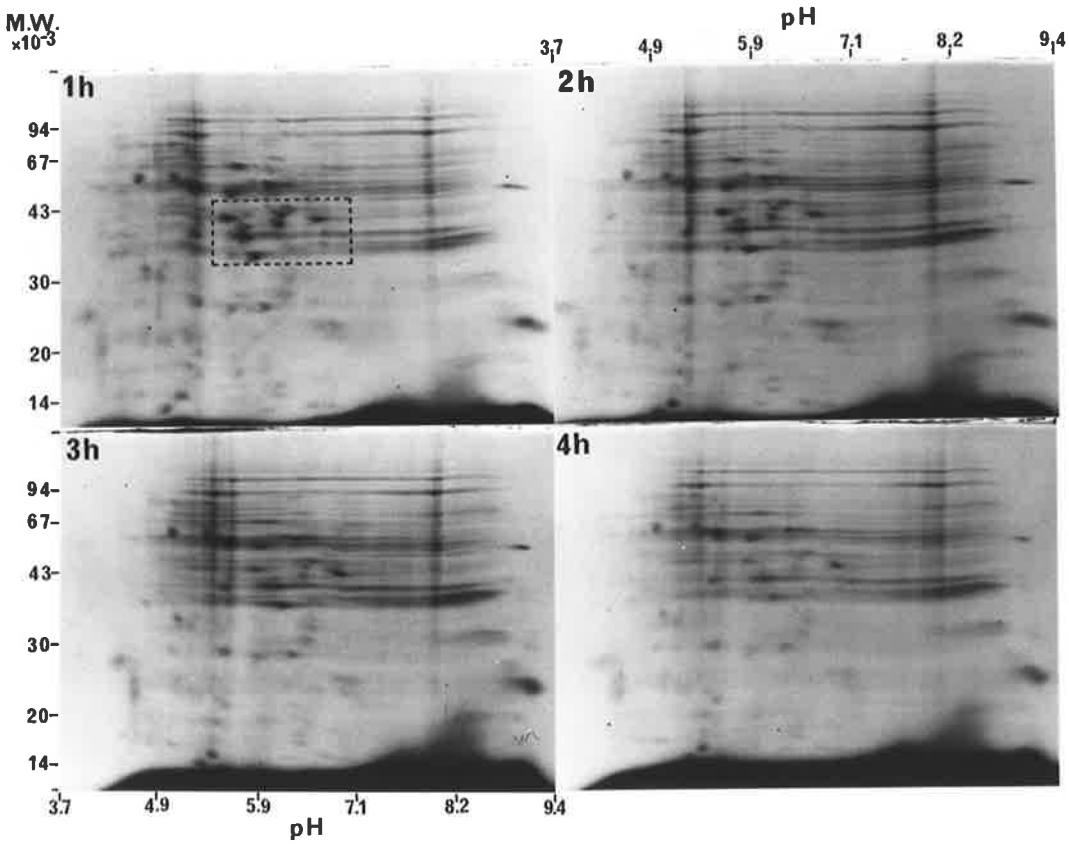
Since the resolution of the single dimension gels is relatively poor, pollen proteins and proteins released from germinating pollen were analysed by two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient in the first dimension followed by SDS-polyacrylamide gel electrophoresis.

3.4.6 Analysis of the two-dimensional patterns of pollen proteins and proteins released during germination and pollen tube growth.

Two-dimensional electrophoresis of total protein developed by O'Farral (1975) is capable of resolving hundreds or even thousands of polypeptides and of detecting allelic charge differences (Leigh-Brown and Langley, 1979). Two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient in the first dimension, was performed on pollen proteins from germinated pollen. Examples of the patterns of pollen proteins from 1h, 2h, 3h and 4h germinated pollen obtained after Coomassie Blue staining are shown in Fig. 10. Approximately 300 proteins are evident in the pattern of 1h germinated pollen proteins, mainly in the acidic half of the gel. From the patterns of 2h, 3h and 4h germinated pollen proteins, approximately the same number of proteins are evident as in 1h germinated pollen protein pattern. But the intensity of stain of most proteins goes down progressively with germination time. Even after careful comparison of the patterns, there are no major qualitative differences in the patterns of protein spots seen in the four gels.

In the acidic half of the gel the proteins occur as discrete spots while in the basic half they are streaked in the IEF dimension. This streaking is presumably due to instability in the basic end of the pH gradient, which decays with time (O'Farrel et al., 1977). This streaking could account for a deficiency of

Fig. 10 **Coomassie Blue-stained two-dimensional gel electrophoretic patterns of pollen proteins from various stages of germination and tube growth in Petunia hybrida.**
Proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA).

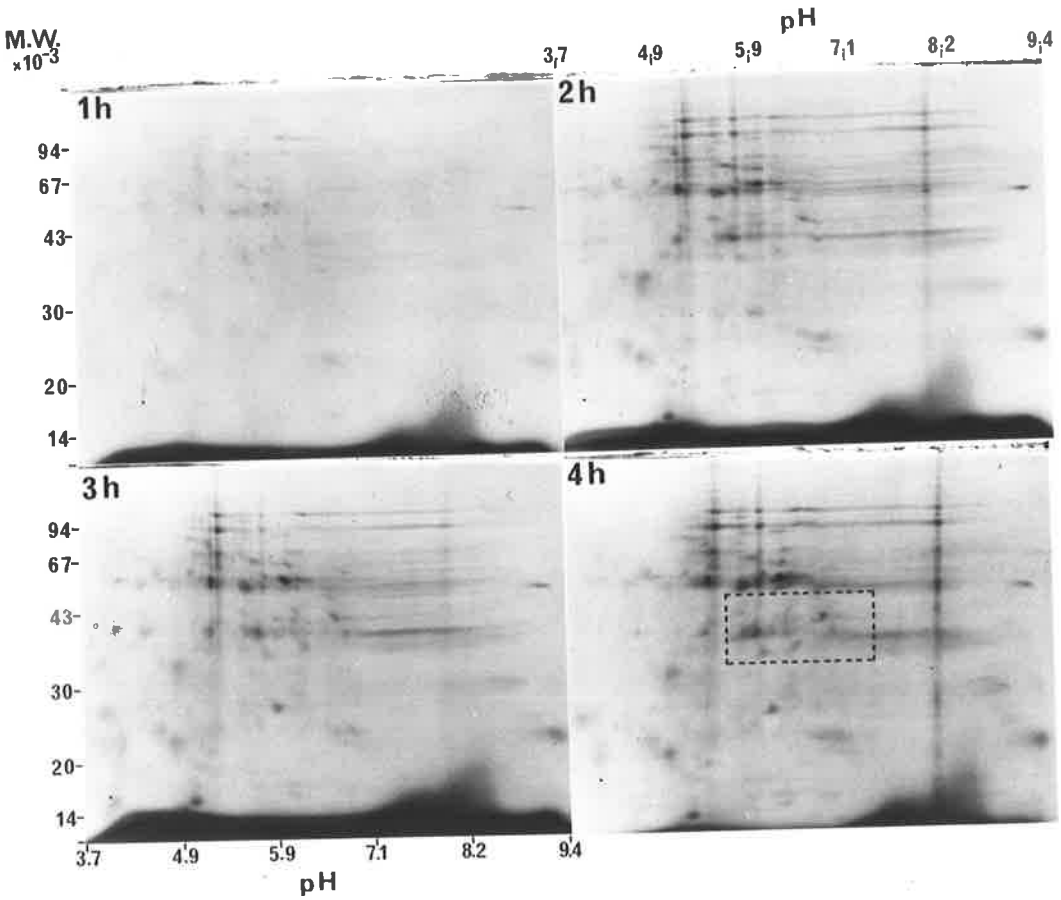

M.W.
×10⁻³
pH
3.7
4.9
5.9
7.1
8.2
9.4
1h
2h
3h
4h
94
67
43
30
20
14

proteins in the basic half of the gel, since streaking of faint spots might render them less visible. However, in general the patterns suggest that there are in fact fewer basic proteins than acidic proteins.

Two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient in the first dimension was also performed on the proteins released during germination and pollen tube growth. Examples of the patterns of released proteins from 1h, 2h, 3h and 4h germinated pollen obtained after Coomassie Blue staining are shown in Fig. 11. From the pattern of released proteins during the first hour of pollen germination only about 100 faint protein spots are evident, mostly in the acidic half of the gel. The limited number of proteins detected may be attributed to the small amount of protein released during the first hour of pollen germination. From the patterns of released proteins from up to 2h, 3h and 4h germinated pollen, approximately 250 proteins are evident, mainly in the acidic half of the gels. However, the intensity of the stain of most proteins goes up progressively with germination time. A careful comparison of the patterns of the released protein from up to 2h, 3h and 4h germinated pollen shows that there are no major qualitative differences in the patterns of protein spots seen in the gels (Fig. 11).

As expected, the general pattern obtained for released proteins from germinated pollen do not differ markedly from that obtained for germinated pollen proteins. The major differences are in the region shown in dashed rectangle (Fig. 11). It can be seen from the pattern of pollen proteins that the major proteins present in the dashed rectangle region do not appear on the pattern of proteins released from germinated pollen (Fig. 11) even though intensity of these major proteins spots in the dashed rectangle region also goes down along with the intensity of other major proteins spots seen in the germinated pollen protein gel patterns. Apart from proteins from this region (dashed rectangle Fig. 11), most of the major proteins present in the pattern of pollen proteins can be seen in the pattern of released proteins during germination and pollen tube growth.

Fig. 11 **Coomassie Blue-stained two-dimensional gel electrophoretic patterns of proteins released during germination and pollen tube growth in Petunia hybrida.** Proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA).


M.W.
×10⁻³
pH
3.7
4.9
5.9
7.1
8.2
9.4
1h
2h
3h
4h
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH

3.4.7 Analysis of the two-dimensional patterns of the newly synthesized pollen proteins and their release during germination and pollen tube growth.

Pollen proteins synthesized during germination and pollen tube growth were investigated by two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient followed by SDS-PAGE and fluorography. Examples of the patterns of proteins synthesized in 1h, 2h, 3h and 4h germinated pollen obtained after fluorography are shown in Fig. 12. About 150 radioactive protein spots are evident in the fluorograph of pollen germinated for one hour (Fig. 12). From the fluorographs of 2h, 3h and 4h germinated pollen proteins, approximately 300 radioactive protein spots are evident, mainly in the acidic half of the fluorographs. However, intensity of the most radioactive protein spots goes up progressively with germination time. A careful comparison of the fluorographs of 2h, 3h and 4h germinated pollen protein patterns shows that there are no major qualitative differences in the patterns of radioactive protein spots seen in the fluorographs.

Two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient in the first dimension followed by SDS-PAGE and fluorography was also performed on the newly synthesized proteins released during germination and pollen tube growth. Examples of the patterns of radioactive spots of released proteins from 1h, 2h, 3h and 4h germinated pollen obtained after fluorography are shown in Fig. 13. From the fluorograph of radioactive released proteins during the first hour of pollen germination, no spots are evident. This may be attributed to the very small amount of the newly synthesized proteins released during the first hour of pollen germination. From the fluorographs of the radioactive proteins released from up to 2h, 3h and 4h germinated pollen, about 150 radioactive protein spots are evident, mostly in the acidic half of the fluorograph. However, the intensity of the radioactive protein spots goes up progressively with germination time. A careful comparison of the fluorographs of the newly synthesized proteins released from up to 2h, 3h and 4h germinated pollen shows that there are no major qualitative differences in the patterns of labelled protein spots seen in the fluorographs (Fig. 13).

Fig. 12 **Fluorographs of two-dimensional gel electrophoretic analysis of pollen proteins synthesized during germination and pollen tube growth in Petunia hybrida.**

1h, 2h, 3h, 4h pulse-labelled {U-^{14}C} protein hydrolysate was given at 0h to pollen culture. Proteins were separated by isoelectric focussing (3.5 to 10.0 pH rage) in the first dimension followed by SDS-PAGE (12.5% PAA) and detected by fluorography.

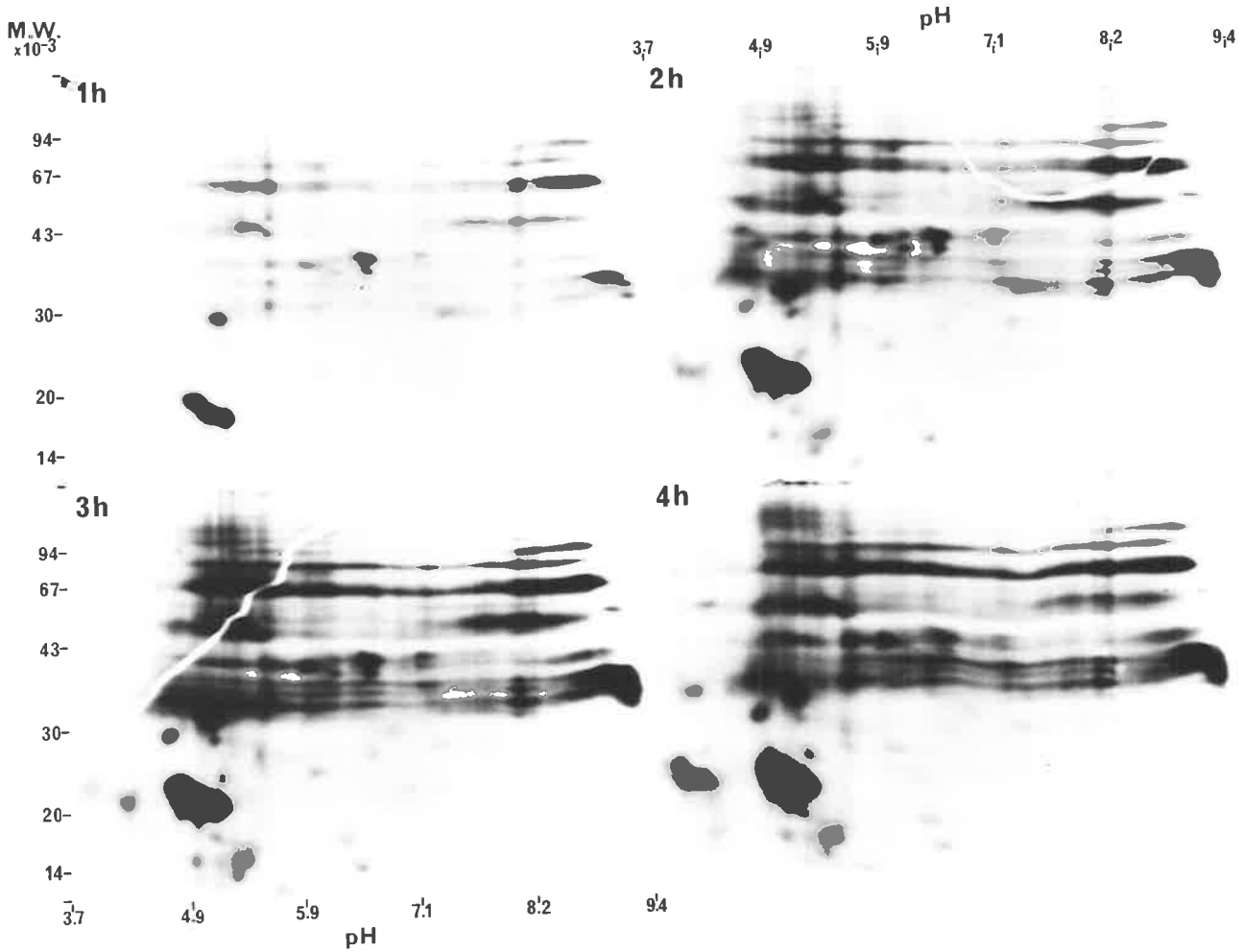
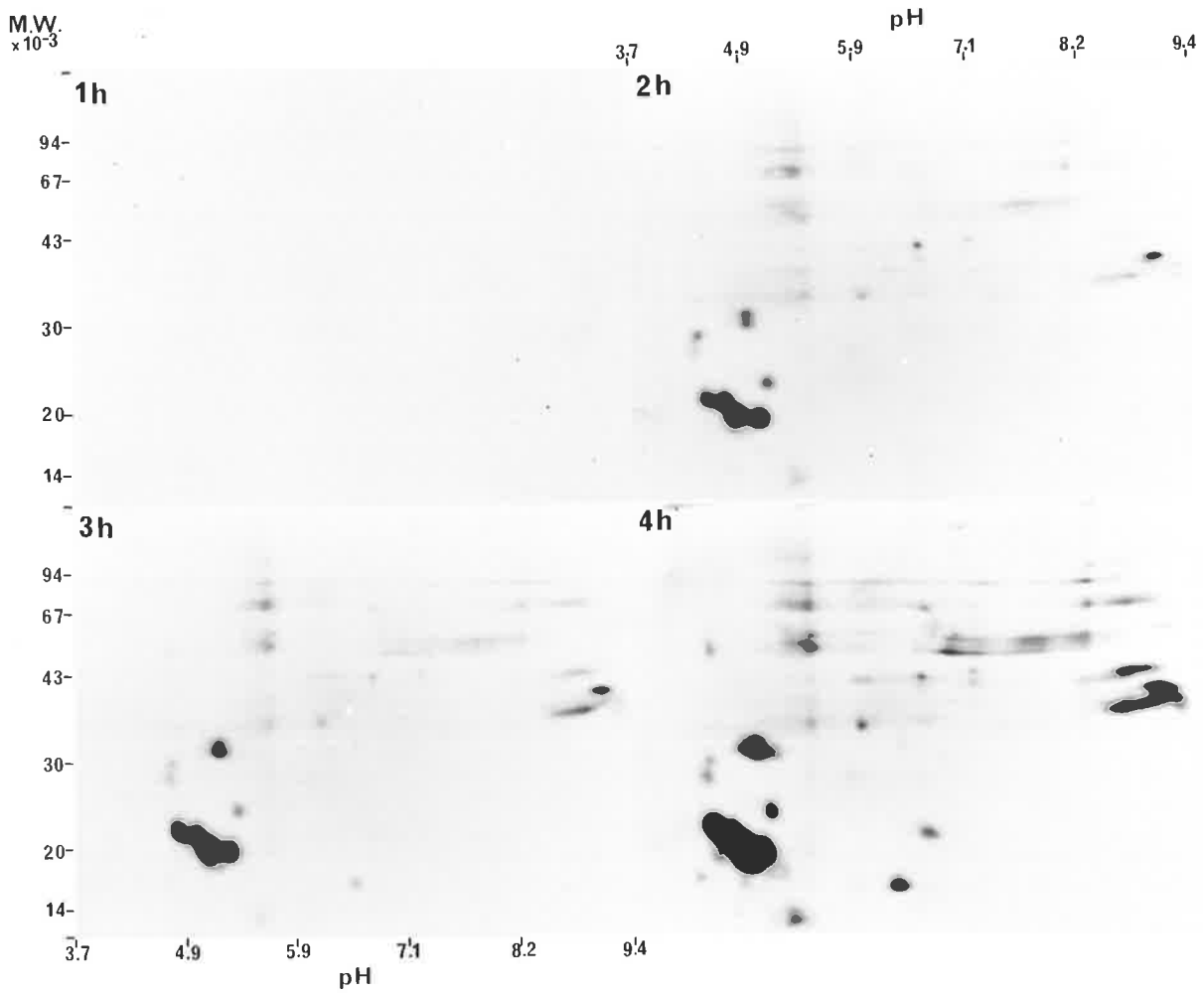

M.W.
x10-3
1h
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH
2h
3h
4h

Fig. 13 **Fluorographs of two-dimensional gel electrophoretic analysis of newly-synthesized proteins released during germination and pollen tube growth in Petunia hybrida.**

1h, 2h, 3h, 4h pulse-labelled {U-^{14}C} protein hydrolysate was given at 0h to pollen culture. Released proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA) and detected by fluorography.


M.W.
x10-3
pH
3.7
4.9
5.9
7.1
8.2
9.4
1h
2h
3h
4h
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH

3.4.8 Regulation of protein synthesis during germination and pollen tube growth of Petunia pollen.

The effect of cations (Mg^{2+}, Ca^{2+}), polyamine (spermine) and α-amanitin (inhibitor of mRNA synthesis) on protein synthesis during the first 3h of Petunia pollen germination and tube growth was investigated by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 14). A careful comparison of the different lanes of the fluorograph of the proteins synthesized in the presence or absence of various cations shows that there are no major qualitative differences in the patterns of proteins synthesized during germination and pollen tube growth. Also, a comparison of the proteins synthesized during the first 3h of pollen germination and tube growth in the presence and absence of α-amanitin seems to indicate that the presynthesized mRNA and the newly synthesized mRNA code for the same proteins (Fig. 14). The newly synthesized proteins released from germinating pollen in the presence and absence of various cations or α-amanitin was also investigated by SDS-PAGE and fluorography (Fig. 14). A comparison of the different lanes of the fluorograph of the radioactive proteins released in the presence or absence of magnesium or spermine or α-amanitin shows that there are no major qualitative differences in the patterns of the newly synthesized proteins released during the first 3h of pollen germination. The presence of 1 mM or 5 mM calcium ions in the culture medium virtually stops the release of all major storage proteins from the germinating pollen, but has little inhibitory effect on the release of newly synthesized proteins from the germinating pollen. On the contrary, as can be seen in lane 6 of Fig. 14C, which represents the pattern of the newly synthesized released proteins from germinating pollen in the presence of 5 mM calcium ions, there is clear stimulation of the release of a number of newly synthesized proteins during germination and pollen tube growth. Calcium promoted the release of newly synthesized proteins of M_r 67,000, 55,000, 43,000, 39,000, 23,000, 22,000 and 18,000 (Fig. 14). Since the resolution of the single dimension gels is relatively poor, pollen proteins and proteins released from germinating pollen in the presence or absence of various cations or α-amanitin was analysed by two dimensional electrophoresis.

Fig. 14A,B. **Coomassie Blue-stained two-dimensional gel electrophoretic patterns of proteins.**

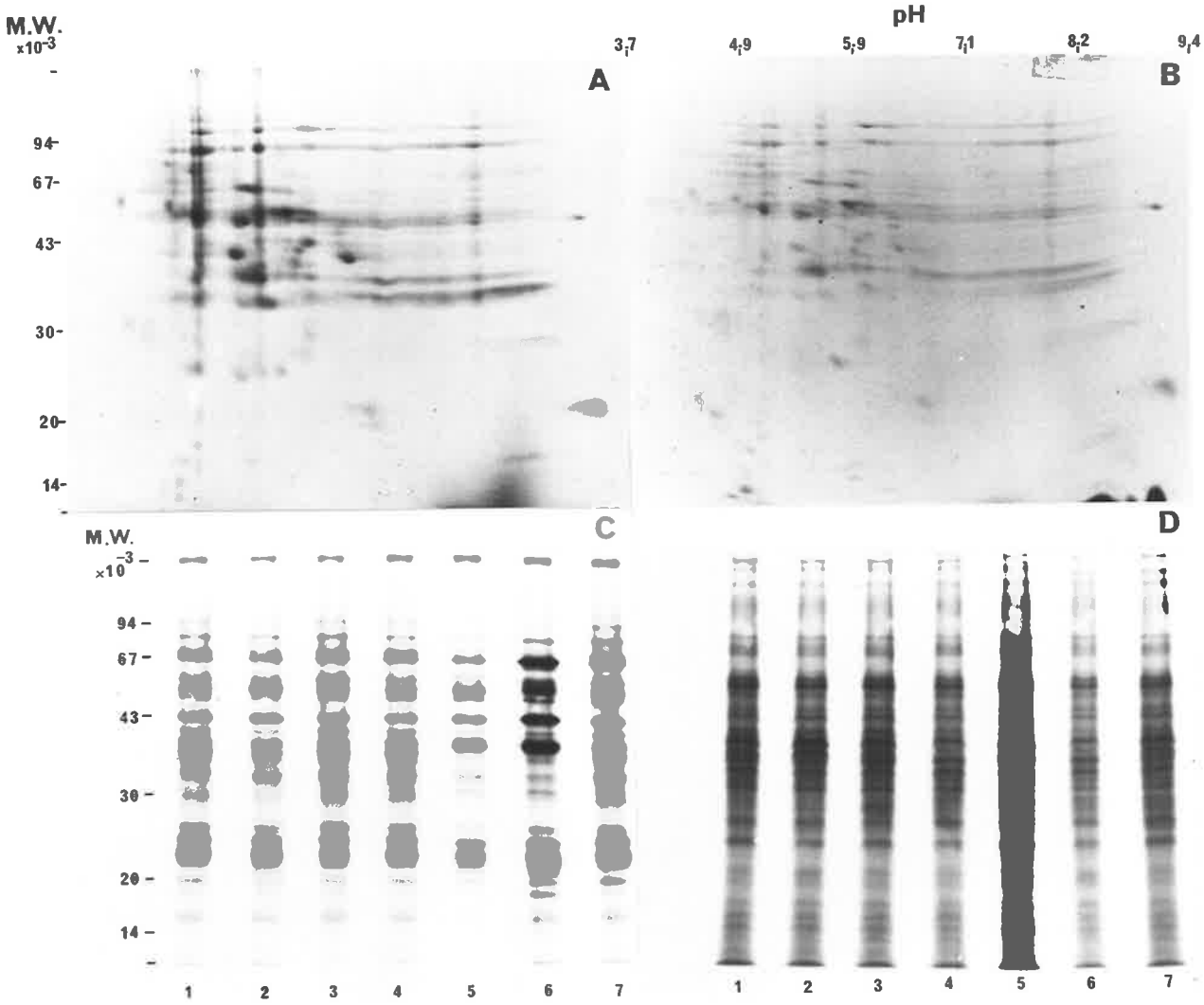
A. Pollen proteins from 3h cultured pollen.

B. Proteins released during 3h culture of pollen.

Proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA).

Fig. 14C,D. **Fluorographs of SDS-PAGE (12.5% PAA) of newly synthesized proteins released (C) and pollen proteins (D) synthesized during 3h culture of Petunia pollen in the presence or absence of various compounds.**

1, no addition to normal medium; 2, 1 mM Mg^{2+}; 3, α-amanitin (10 µg/ml); 4, α-amanitin (100 µg/ml): 5, 1 mM Ca^{2+}; 6, 5 mM Ca^{2+}; 7, 10 µM spermine.


M.W.
x10-3
94
67
43
30
20
14
pH
3,7
4,9
5,9
7,1
8,2
9,4
A
B
C
D
1
2
3
4
5
6
7

3.4.9 Two-dimensional electrophoretic analysis of regulation of protein synthesis during germination and pollen tube growth of Petunia pollen.

The effect of cations (Mg^{2+}, Ca^{2+}), and α-amanitin (inhibitor of mRNA synthesis) on protein synthesis during the first 3h of Petunia pollen germination and tube growth was investigated by two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient followed by SDS-PAGE and fluorography. Examples of the patterns of proteins synthesized in pollen germinated for 3h and of labelled proteins released from germinated pollens obtained after fluorography are shown in Fig. 15. Various cations or α-amanitin had no effect on the pattern of pollen proteins synthesized during the first 3h of germination and pollen tube growth. The newly synthesized proteins released from germinating pollen in the presence or absence of various cations or α-amanitin was also analysed by two-dimensional electrophoresis and fluorography (Fig. 15). A careful comparison of the fluorographs of newly synthesized released proteins in the presence or absence of α-amanitin (10ug/ml) shows that there are no major qualitative differences in the pattern of protein spots seen in the two fluorographs.

Two-dimensional electrophoresis of labelled proteins released during the first 3h of pollen germination and tube growth in the presence or absence of 1 mM Mg^{2+} ions seem to indicate that the presence of magnesium ions inhibits the release of several newly synthesized proteins (Fig. 15). The region most affected by the presence of magnesium ions is shown in dashed rectangles. However, the general pattern of protein spots seen in both the fluorographs is essentially similar (Fig. 15). Two-dimensional electrophoresis of radioactive proteins released during the first 3h of pollen germination and tube growth in the presence or absence of 5 mM Ca^{2+} ions shows that the presence of calcium ions inhibited the release of several newly synthesized proteins (Fig. 16), and at the same time stimulated the release of several other newly synthesized proteins. The maximum inhibition of release occurs with proteins in the region shown within the dashed rectangle. The maximum stimulation of release occurs with proteins in the acidic pI region shown in dashed rectangles (Fig. 16). At

Fig. 15 **Fluorographs of two-dimensional gel electrophoretic analysis of pollen proteins synthesized (A) and newly synthesized proteins released (B,C,D) during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.**

A. Pollen protein (no addition to normal medium).

B. Proteins released (no addition to normal medium).

C. Proteins released in the presence or α-amanitin (10 µg/ml).

D. Proteins released in the presence of 1 mM Mg^{2+}. 3h pulse-labelled {U-^{14}C} protein hydrolysate was given at 0h to pollen culture. Proteins were separated by isoelectric focussing (3.5 to 10.00 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA) and detected by fluorography.

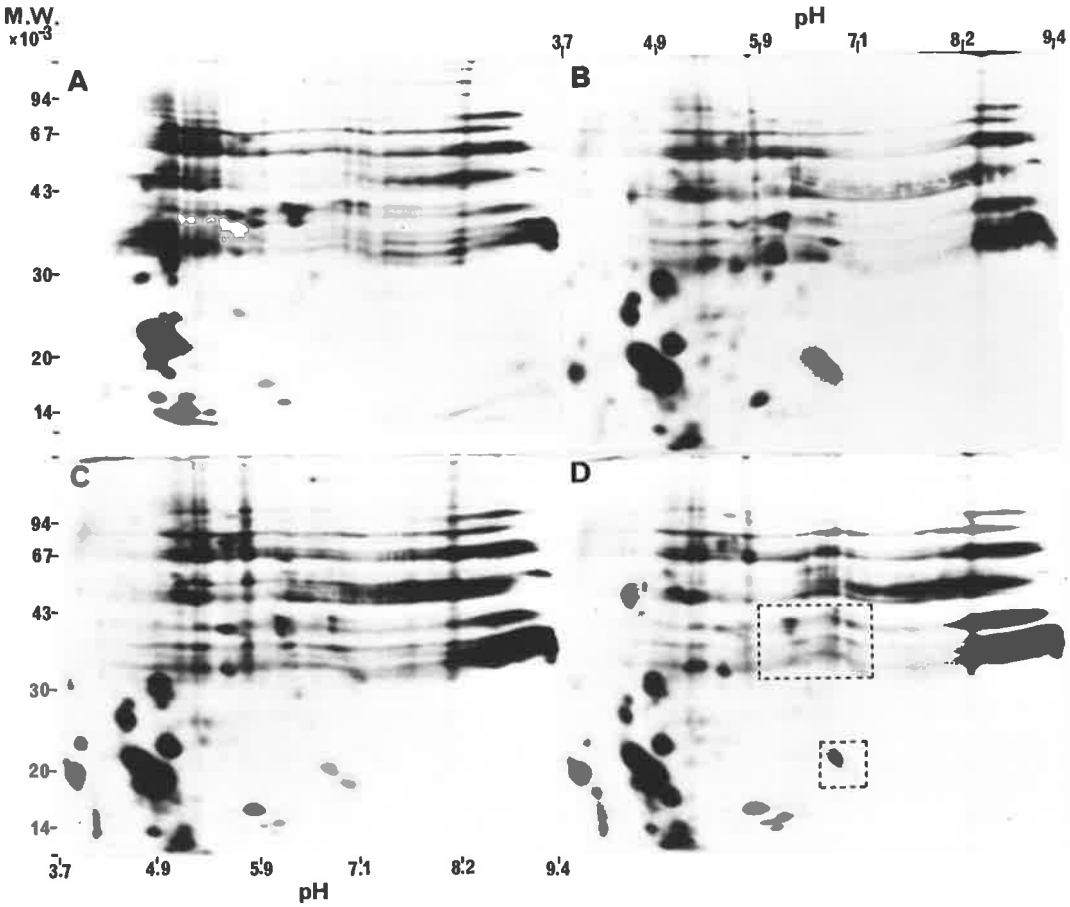

M.W.
×10⁻³
pH
3.7
4.9
5.9
7.1
8.2
9.4
A
B
C
D
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH

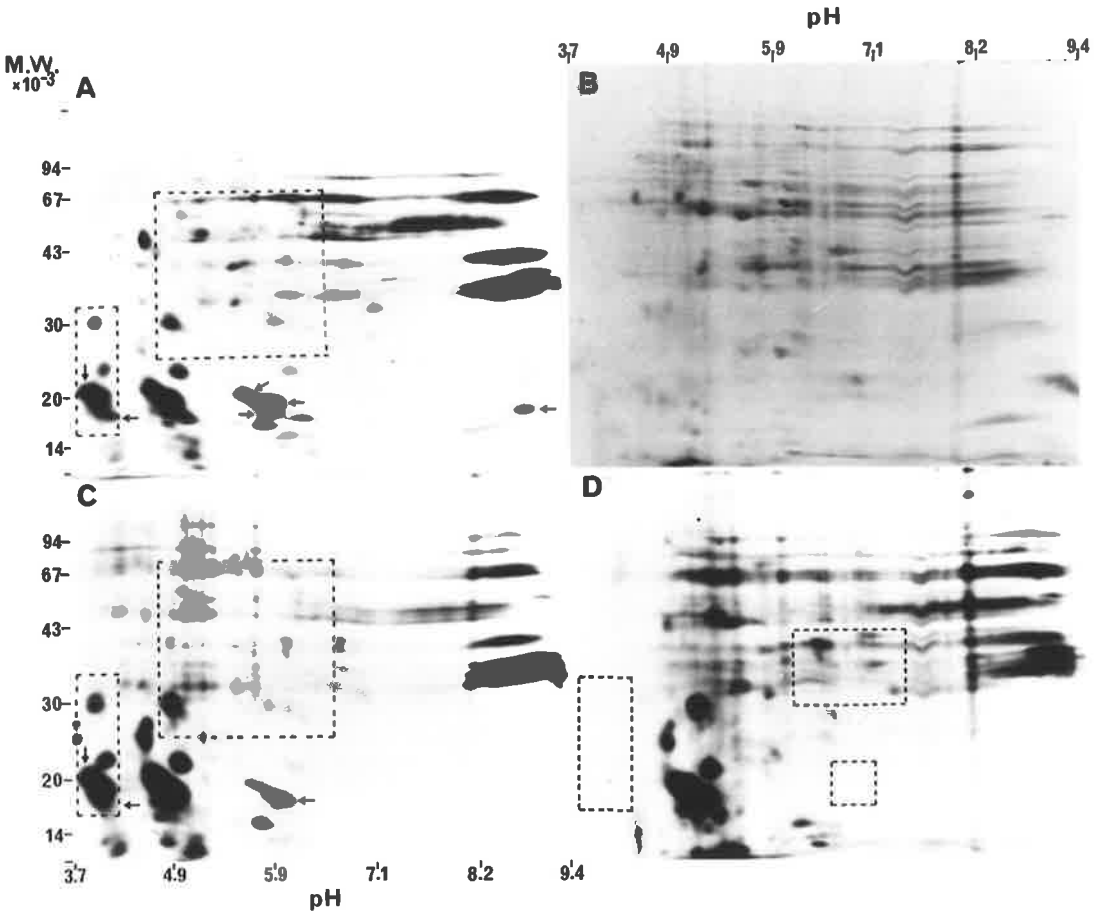
Fig. 16A,C,D Fluorographs of two-dimensional gel electrophoretic analysis of newly synthesized proteins released during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.

A. Proteins released in the presence of 5 mM Ca^{2+}.

C. Proteins released in the presence of α-amanitin (100 µg/ml).

D. Proteins released in the absence of boron (i.e. 10% sucrose as culture medium).

Fig. 16B. Coomassie Blue-stained two-dimensional gel electrophoretic pattern of proteins released in the absence of boron (i.e. 10% sucrose as culture medium). 3h pulse-labelled {U-^{14}C} protein hydrolysate was given at 0h to pollen culture. Proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA).


M.W.
×10⁻³
A
B
C
D
pH
3,7
4,9
5,9
7,1
8,2
9,4
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH

least 6 new major protein spots (shown by arrows) can be seen in the pattern of newly synthesized released proteins in the presence of calcium. Two-dimensional electrophoresis of the newly synthesized protein released in the presence of α-amanitin (100 µg/ml) was also investigated. Unexpectedly, the pattern of the released protein spots is very similar to the pattern that is obtained in the presence of 5 mM Ca^{2+} ions (Fig. 16). The inhibition of the release of the newly synthesized proteins (again shown in dashed rectangle) is less severe in this case as compared to that shown in pattern obtained in the presence of 5 mM Ca^{2+} ions (Fig. 16). The maximum stimulation of release of newly synthesized proteins (in the pH 4.0 region shown in dashed rectangle) is also very similar to that obtained in the presence of 5 mM Ca^{2+} ions (Fig. 16). In addition at least 3 new major protein spots (shown by arrows) can be seen in the pattern of newly synthesized proteins released in the presence of α-amanitin (100 µg/ml) as compared to the control pattern (Figs. 15,16).

Two-dimensional electrophoresis of proteins released (storage and newly synthesized) from Petunia pollen when cultured in 10% sucrose only (i.e. boron is excluded) was also investigated. Example of the pattern obtained after Coomassie Blue staining and also, obtained after fluorography are shown in Fig. 16. A careful comparison of the Coomassie Blue patterns of released proteins in the presence or absence of boron reveals that there are no major qualitative differences in the patterns of protein spots seen in the gels (Figs. 14,16). As can be seen from the fluorographs (Figs. 15B,16D), there is inhibition of the release of several newly synthesized proteins in the absence of boron. The areas of maximum inhibition of release of newly synthesized proteins are shown within the dashed rectangles (Fig. 16). When compared to the control pattern it can be seen that the release of several major newly synthesized proteins is completely stopped by the exclusion of the boron from the culture medium (Figs. 15B,16D).

3.4.10 Two-dimensional electrophoretic analysis of the phosphosylated proteins and their release during germination and pollen tube growth.

Protein phosphorylation during germination and pollen tube

growth was investigated by two-dimensional electrophoresis and autoradiography. Example of the pattern of phosphorylated proteins in the first 3h of pollen germination obtained after autoradiography is shown in Fig. 17. Approximately 50 phosphorylated protein spots are evident in the autoradiogram of 3h germinated pollen phosphorylated proteins (Fig. 17). A careful comparison of the patterns of the autoradiograms of 1h, 2h, 3h and 4h germinated pollen phosphorylated proteins revealed that there were no major qualitative differences in the patterns of phosphorylated protein spots seen in the autoradiograms.

Two-dimensional electrophoresis of the phosphorylated proteins from 3h germinated pollen in the presence of 1 mM Ca^{2+} ions indicates that calcium stimulated the phosphorylation of the protein during germination of Petunia pollen (Fig. 17). Approximately 70 phosphorylated protein spots are evident in the autoradiogram of 3h germinated pollen in the presence of 1 mM Ca^{2+} ions. When compared with the control pattern (Fig. 17), it can be seen that calcium stimulates the phosphorylation of all the phosphorylated proteins seen in the absence of calcium. In addition several new phosphorylated protein spots can also be seen in the autoradiogram of phosphorylated proteins in the presence of calcium (Fig. 17). The major new phosphorylated protein spots are indicated by arrows (Fig. 17).

The effect of 1 mM Mg^{2+} ions on phosphorylation of protein during germination and tube growth of Petunia pollen was also investigated by two-dimensional electrophoresis and autoradiography. As can be seen from the pattern (Fig. 17), magnesium stimulated the phosphorylation of all the existing phosphorylated proteins. However, no new major phosphorylated protein spots can be seen.

The effect of α-amanitin (10 µg/ml) on phosphorylation of proteins during germination and pollen tube growth of Petunia pollen was also investigated, but the presence of α-amanitin in culture medium had no effect on the pattern of phosphorylated proteins as compared to the control pattern of the phosphorylated proteins.

The release of phosporylated proteins during germination and

Fig. 17 **Autoradiograms of two-dimensional gel electrophoretic analysis of phosphorylated proteins during 3h culture of Petunia hybrida pollen in the presence or absence of various compounds.**

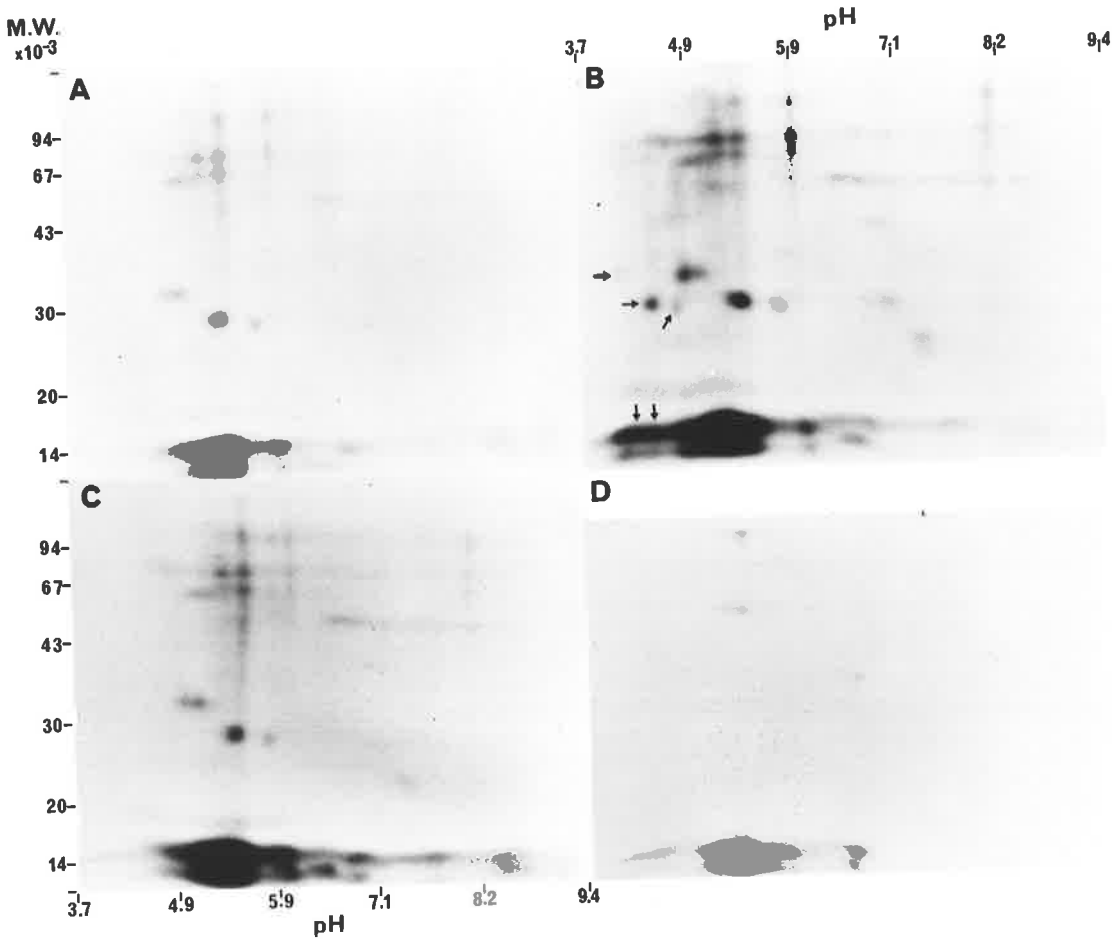
A. Phosphorylated pollen proteins (no addition to normal medium).

B. Phosphorylated pollen proteins in the presence of 1 mM Ca^{2+}.

C. Phosphorylated pollen proteins in the presence of 1 mM Mg^{2+}.

D. Phosphorylated proteins released (no addition to normal medium).

3h pulse-labelled {^{32}P} orthophosphate was given at 0h to pollen culture. Proteins were separated by isoelectric focussing (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12.5% PAA) and detected by autoradiography.


M.W.
x10-3
A
B
C
D
94-
67-
43-
30-
20-
14-
3.7
4.9
5.9
7.1
8.2
9.4
pH

pollen tube growth was also investigated by two-dimensional gel electrophoresis and autoradiography. Approximately, 15 phosphorylated protiein spots are evident in the autoradiogram of phosphorylated protein released during the first 3h of pollen germination and tube growth (Fig. 17). The majority of the phosphorylated protein spots are seen in the low molecular weight region, mainly in the acidic half of the autoradiogram. No phosphorylated proteins were evident in the 1h released protein pattern obtained after autoradiography. A comparison of the patterns of the autoradiograms of released proteins during 2h, 3h and 4h of pollen germination showed that there were no major qualitative differences in the patterns of autoradiograms of phosphorylated protein spots. The presence of 1 mM Ca^{2+} or 1 mM Mg^{2+} or α-amanitin (10 µg/ml) in the culture medium had no effect on the release of phosphorylated proteins from germinating pollen.

3.5 Incompatibility genotype and biochemical investigation during the interaction between pollen and pistil in Petunia hybrida.

3.5.1 Distribution of phytic acid in the floral structure.

Phytic acid was found to be localized in the pollen (anthers) and in the stigmas in all three clones tested, with pollen always having the highest concentration (Table 23). The filaments however contained no significant amounts of phytic acid. While in two of the three clones the style showed some phytic acid, the concentration was always lower than the stigma and increased towards the stigma end. No stylar phytic acid was detected in clone W166H (S_2S_3). Although there was some correlation between phytic acid and total phosphate in the styles, this was not true of the pollen from the three clones (Table 23).

3.5.2 Phytase activity in the flower.

By far the highest concentration of phytase activity to be found in the floral structure resides in mature pollen (Table 24). Unlike the striking localizaion of substrate phytic acid, all parts of the flower showed some phytase activity. Some difficulty lies in the interpretation of these results since the specifi-

Table 23. Phytic acid distribution in the various parts of the flower and variation between clones W166K, W166H and T_2U. The corresponding incompatibility genes are shown in brackets.

	Stigma	Upper style	Lower style	Ovary	Pollen	Filaments	Petals & other
W166K S_1S_2)							
Phytic Acid (% by weight)	0.3	0.02	0	0	2.0	0	0
P (µmol per mg)	0.17	0.14	0.14	-	0.52	-	-
Av. wt. (mg)	0.85	0.53	0.49	-	-	-	-
W166H (S_2S_3)							
Phytic acid (% by weight)	0.3	0	0	0	1.8	0	0
P (µmol per mg)	0.18	0.14	0.15	-	0.3	-	-
Av. wt. (mg)	0.92	0.50	0.46	-	-	-	-
T_2U (S_3S_3)							
Phytic acid (% by weight)	0.6	0.06	0.01	0	1.0	0	0
P (µmol per mg)	0.22	0.16	0.17	-	0.42	-	-
Av. wt. (mg)	0.70	0.41	0.30	-	-	-	-

Table 24. Relative phytase activity in various parts of the flower in clones W166K, W166H and T_2U.

	W166K (S_1S_2)	W166H (S_2S_3)	T_2U (S_2S_2)
Stigma	0.1	0.01	0.03
Upper style	0.1	0.04	0.03
Lower style	0.05	0.03	0.03
Ovary	0.30	0.33	N.D.
Pollen	1.0	1.0	1.0

city of phytase in Petunia hybrida is not known. At least three acid phosphatases of unknown but probably quite wide specificity are present in some plant tissue (Tanksley et al., 1981), any of which may hydrolyse phytic acid. However, regardless of the specificity of these acid phosphatases the fact remains that these or other enzyme(s) that can hydrolyse phytic acid are present in all parts of the flower tested. Thus no simple explanation relative to phytic acid disappearance via a phytase after pollination suggests itself from these results. However, it is apparent that the S_3 allele could be associated with low phytase in the pistil and the S_1 allele with high pistil phytase levels (Table 24).

3.5.3 Changes in phytic acid levels after pollination and influence of the S genes.

It has recently been established that phytic acid in pollen is rapidly degraded during germination in vitro (Jackson and Linskens 1982a). When pollen from clones W166H and W166K is used to pollinate W166H styles, phytic acid is similarly reduced to lower levels over a period of several hours. However the pattern of its disappearance varies depending on whether the pollination is compatible (W166K x W166H) or self-incompatible (W166H x W166H). The latter shows a steady reduction, while the compatible combination has a significantly higher phytic acid level at 2h than the incompatible one, even though both are reduced to lower levels over a longer period. Other combinations with W166K, W166H and T_2U styles showed a similar comparison at 2h; the compatible combination had higher phytic acid than the incompatible (Table 25). It may well be that some phytic acid is synthesized over the first two hours in the pollen-stigma-style combination in the compatible situation, or perhaps synthesis occurs in both compatible and incompatible combinations and phytic acid is utilized more vigorously in the incompatible one. At present the latter hypothesis has more support as it seems to tie in well with the observed patterns of translocation of organic substances within the flower after pollination. Thus in the first few hours after pollination there is a stronger influx into selfed (incompatible) styles than into crossed (compatible) styles (Linskens 1975, 1975a).

Table 25. Phytic acid content of pistils 2h after pollination in various crosses between the clones. Two different repetitions.

Style	Pollen	Phytic acid (% by wt)
W166K (S_1S_2)	W166K (S_1,S_2)	0.20; 0.15
W166K (S_1S_2)	T_2U (S_3,S_3)	0.40; 0.40
T_2U (S_3S_3)	T_2U (S_3,S_3)	0.40; 0.35
T_2U (S_3S_3)	W166H (S_2,S_3)	0.50; 0.55
W166H (S_2S_3)	W166H (S_2,S_3)	0.50; 0.45
W166H (S_2S_3)	T_2U (S_3,S_3)	0.55; 0.55

3.5.4 Sparing effect of styles on pollen phytic acid breakdown.

As referred to above, Petunia hybrida pollen when germinated in vitro quickly loses contained phytic acid (Jackson and Linskens 1982a). When W166K styles are added to germinating pollen in vitro, the loss of phytic acid in the pollen after two hours is reduced, particularly with W166H (or compatible) pollen (Table 26). After the two hours shaking, styles reisolated from the culture solution had little or no phytic acid.

3.5.5 Other factors involving stability of phytic acid in pollen.

Germination of Petunia hybrida pollen in vitro is greatly improved by prior hydration of the pollen at high humidity (Gilissen 1977). There was no breakdown in pollen phytic acid as a result of this treatment (up to 48 hours at 100% relative humidity, 20°). Breakdown of phytic acid subsequently takes place in pollen from all three clones when shaken (or merely stood) in 10% sucrose solutions with or without added boron. When a wheat germ phytase preparation (2 mg per 35 mg pollen) was added to germinating pollen in vitro, there was no acceleration of phytic acid degradation after one hour, nor was there any subsequent effect on the germination rate or average tube length achieved after 14 hours for any of the three clones tested.

3.6 Incompatibility genotype and gene expression in pollen and pistil of Petunia hydrida.

3.6.1 Self-incompatibility genotype associated proteins from the pistils.

The proteins of pistil extracts were examined for molecular weight differences among the dissociated, denatured components by SDS-PAGE. Nine clones of Petunia hybrida with varying pairs of S alleles when analysed in this way gave the protein patterns shown in Fig. 18. It was apparent from this pattern that there was a simple relationship between the S alleles carried by the pistil and the molecular weight of the major protein in the extract which migrated in the region of the 30,000 dalton marker protein.

Table 26. Sparing effect of styles on phytic acid degradation during pollen germination *in vitro* for 2h.

Pollen	Style[a,b]	Phytic acid (% by wt)
W166H (S_2S_3)	-	0.15; 0.15
W166H (S_2S_3)	W166K (S_1S_2)	0.35; 0.30
W166K (S_1S_2)	-	0.05; 0.07
W166K (S_1S_2)	W166K (S_1S_2)	0.05; 0.10

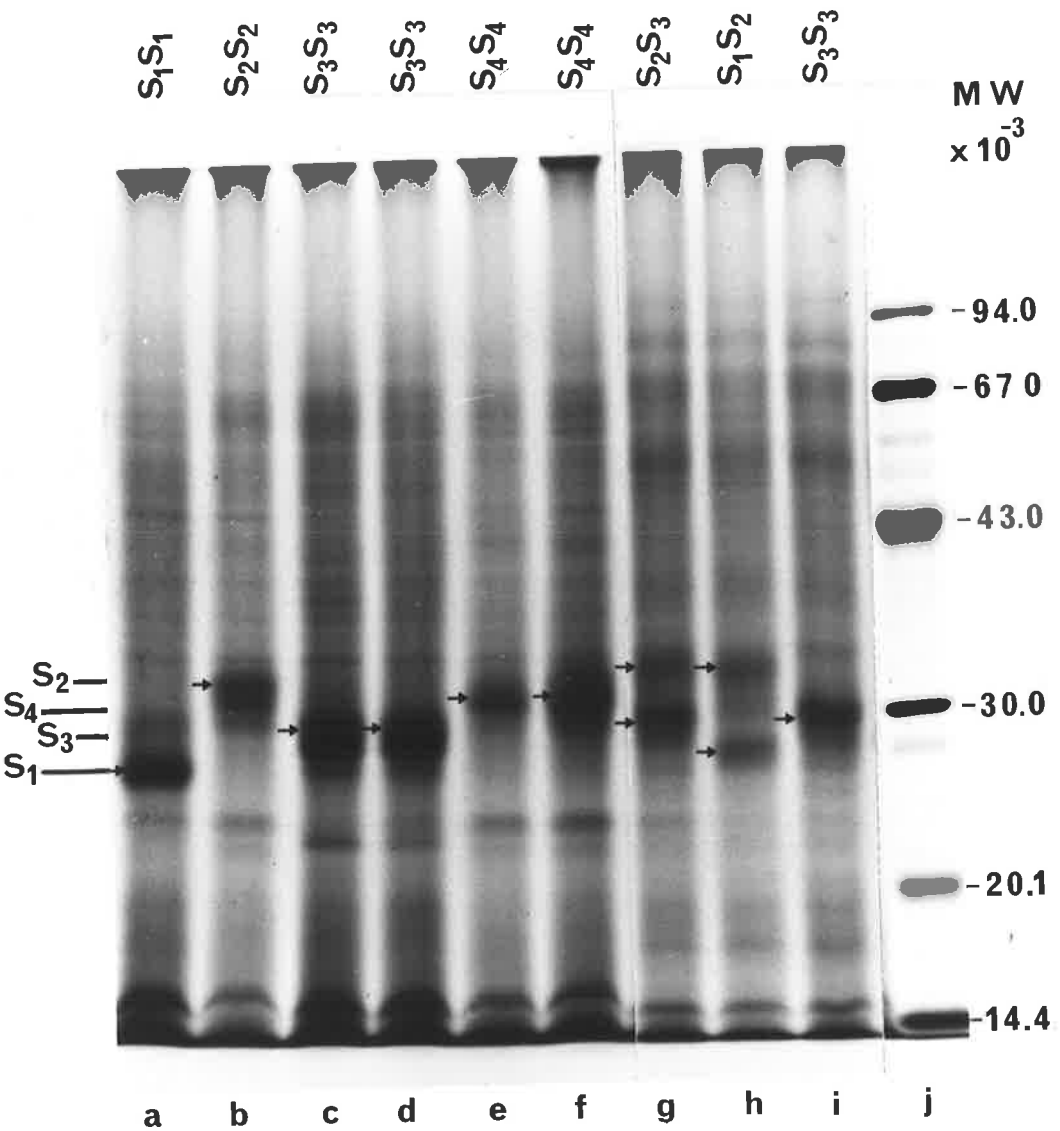
a Styles had 0.02% phytic acid after the incubation

b 3 Styles oer 35 mg pollen in 5 ml medium

Fig. 18 **Coomassie Blue-stained SDS-PAGE (12% PAA) pattern of pistil proteins from various clones of Petunia hybrida.**

Clones used and lettering adopted are as follows (S alleles in brackets): a - W43 (S_1S_1), b - Ka3D (S_2S_2); c - T_2UD (S_3S_3); d - KA30 (S_3S_3); e - KA3 (S_4S_4); f - KAD_3 (S_4S_4); g - W166H (S_2S_3); h - W166K (S_1S_2); i - T_2U (S_3S_3).

The position of major proteins assigned to each S allele is indicated by arrows.


S1S1
S2S2
S3S3
S3S3
S4S4
S4S4
S2S3
S1S2
S3S3
MW
x10-3
-94.0
-67.0
-43.0
-30.0
-20.1
-14.4
S2
S4
S3
S1
a
b
c
d
e
f
g
h
i
j

By comparison with molecular weight markers it was possible to assign a specific molecular weight for each putative "S protein", varying from 27,000 daltons for S_1, up to 33,000 daltons for S_2, the values for S_3 and S_4 lying in between (30,000 daltons for S_3 and 31,000 daltons for S_4). Where the pistil was homozygous for an S allele, only one major protein band was seen, while two major bands appeared for extracts from pistils heterozygous at the S locus, as expected. As can be seen in Fig. 18, there was some variation in the intensity of several minor bands between the clones, but none of these showed the clearcut difference or consistent relationship with the S genotype that was evident for the major protein in the 30,000 dalton region.

3.6.2 Comparison between pistil and pollen proteins.

Proteins in extracts of pistils and pollen from two heterozygous clones (bearing S_2S_3 and S_1S_2 alleles) and one homozygous (S_3S_3) clone were compared by SDS-PAGE. The results, after staining the gels with Coomassie Blue, are shown in Fig. 19. It can be seen that while the major protein of pistils varied according to the S genotype as noted above, no such simple relationship is apparent with the protein patterns of pollen extracts. The major proteins of pollen have a much higher molecular weight than the pistil counterpart and do not vary markedly according to the S alleles carried. Due to the multiplicity of protein bands obtained with pollen extracts, it cannot be said whether or not the "S protein" seen in pistil extracts is present in pollen. However, one thing is clear that it is not a major protein in pollen, and that no other correlations with S genotype is evident from the SDS-PAGE pollen protein patterns.

A similar gel to that shown in Fig. 19 was stained with periodic acid - Schiffs reagent instead of Coomassie Blue, in order to reveal glycoproteins. As shown in Fig. 20, the major "S protein" of pistils stains as a glycoprotein; there is little evidence for any other glycoproteins in pistils extracts. In contrast, pollen extracts show many glycoprotein bands, but none however in the "S protein" 30,000 dalton region, and none showing any type of correlation with the S genotype.

Fig. 19 **Comparison of pistil and pollen proteins by SDS-PAGE (12% PAA).**
Molecular weight markers were run in the unmarked lanes. Proteins were detected by Coomassie Blue staining.

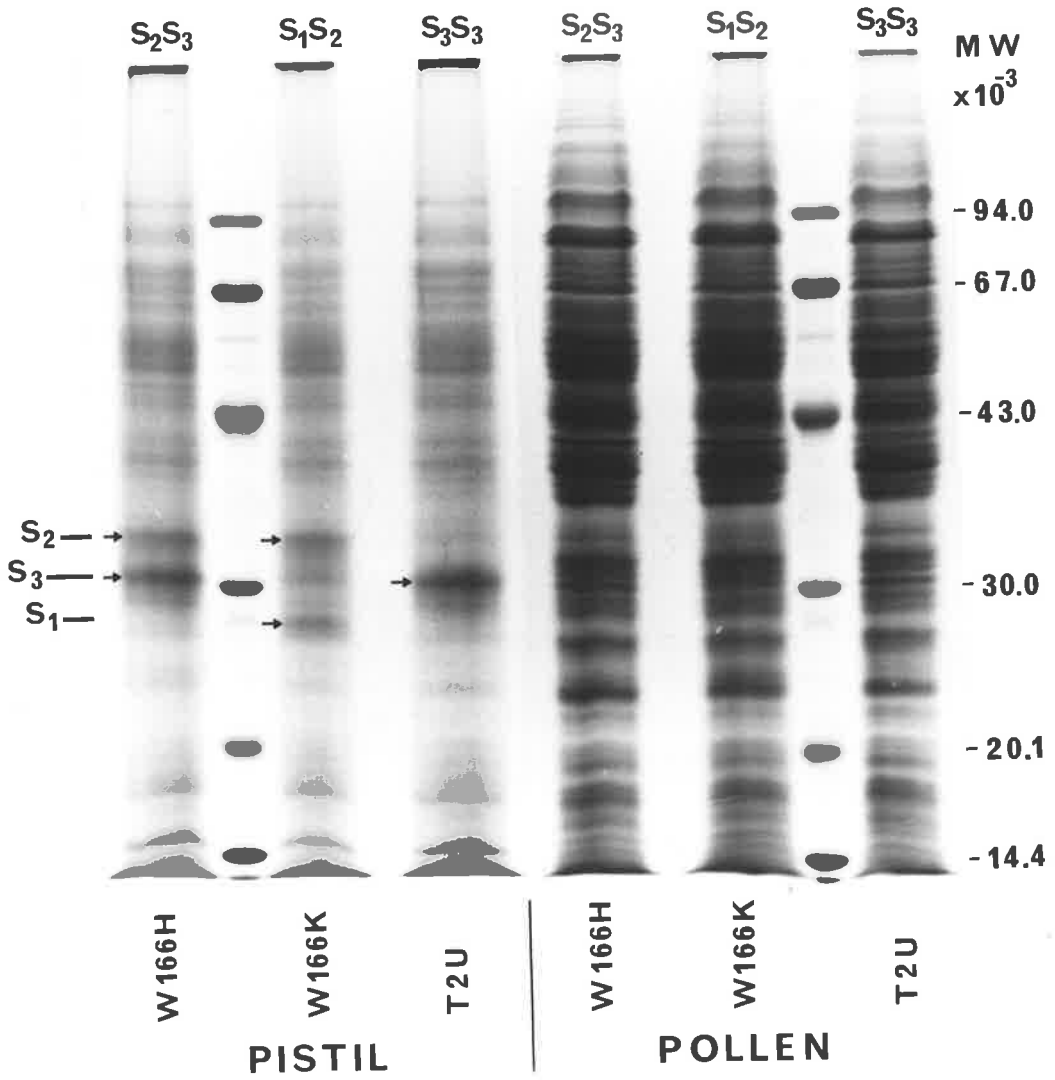
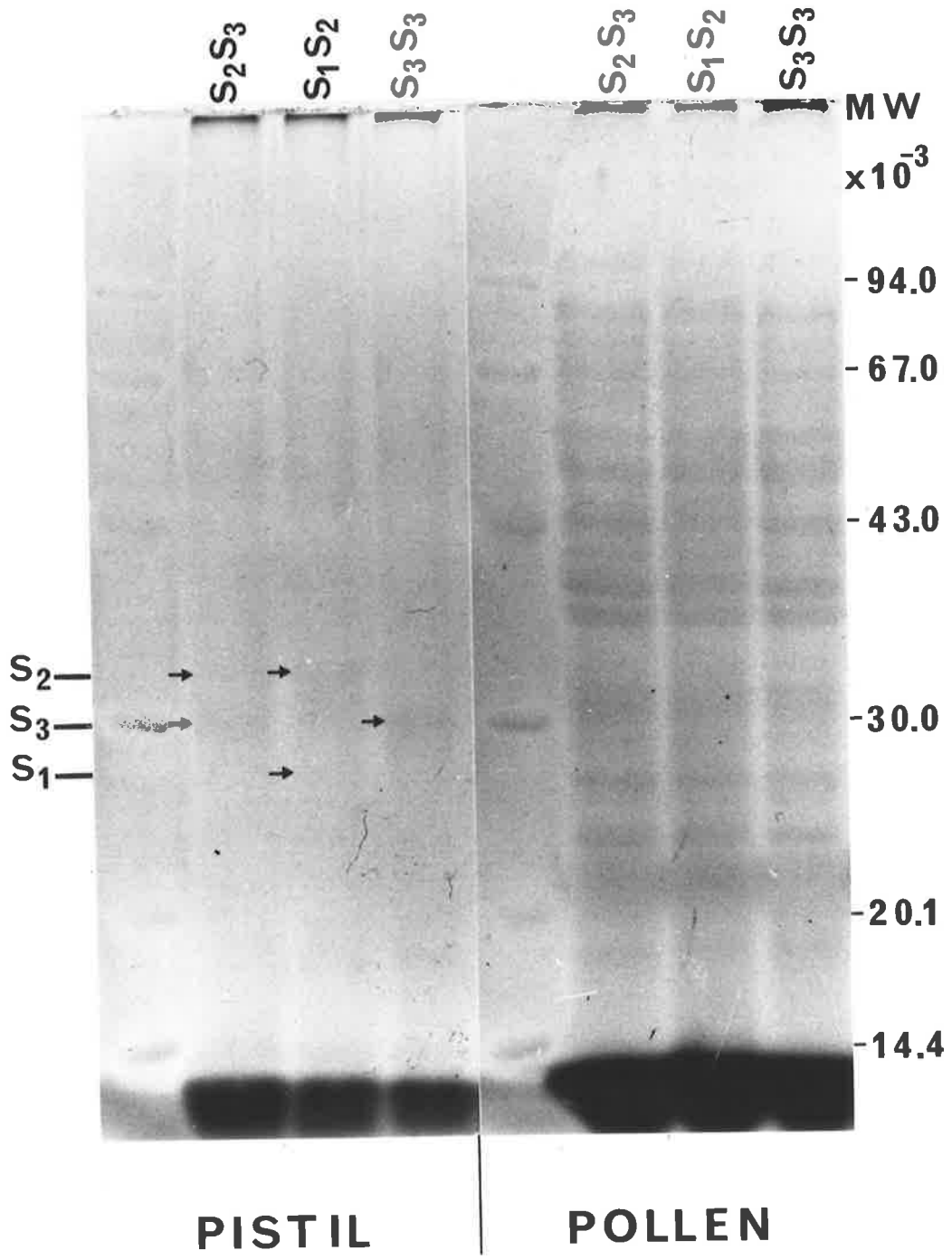

S2S3
S1S2
S3S3
S2S3
S1S2
S3S3
MW
x10-3
-94.0
-67.0
-43.0
-30.0
-20.1
-14.4
S2
S3
S1
W166H
W166K
T2U
W166H
W166K
T2U
PISTIL
POLLEN

Fig. 20 **Schiff's stain for glycoproteins of pistil and pollen after SDS-PAGE (12% PAA).**
Molecular weight markers were run in the unmarked lanes.


S2S3
S1S2
S3S3
S2S3
S1S2
S3S3
MW
x10-3
-94.0
-67.0
-43.0
-30.0
-20.1
-14.4
S2
S3
S1
PISTIL
POLLEN

3.6.3 Isoelectric focussing and two-dimensional gel electrophoresis of pistil proteins.

Pistil extracts (four homozygous and two heterozygous at the S locus) were first subjected to isoelectric focussing in one direction, and then to separation by molecular weight in another, to achieve a two-dimensional separation of proteins. The spreading of minor proteins in the pistil extracts by this means results in the "S protein", by virtue of its abundance, being the only protein visible on the gel after staining with Coomassie Blue (Fig. 21). A simple correlation with S genotype is again evident, and specific isoelectric points can be assigned to each "S protein", all around the region of 8.5. Specific isoelectric points calculated from Fig. 21 and from a one-dimensional isoelectric focussing gel are ranging from a pI of 8.3 for S_1 protein to 8.7 for S_3 protein, the values for S_2 and S_4 lying in between (8.5 for S_2 and 8.6 for S_4).

3.6.4 Messenger RNAs in pollen and pistils of Petunia hybrida and cell-free sythesis of polypeptides.

Total RNA was isolated from ungerminated pollen grains and from this analysis 100 mg of pollen was found to contain approximately 1 mg of total RNA. Using 100,000 pollen grains per mg of Petunia hybrida pollen, the total amount of RNA per Petunia pollen grain is 100 pg. Poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose and on an average each Petunia pollen grain contains approximately 4 pg of poly(A)$^+$ RNA. Total RNA and poly(A)$^+$ RNA were also isolated from pistils of Petunia and from this analysis one gram of dried mature pistils were found to contain about 1 mg of total RNA and 25 µg of poly(A)$^+$ RNA.

Total RNA and poly(A)$^+$ RNA isolated from pistils and ungerminated pollen grains were used as templates for protein synthesis in both the wheat germ and reticulocyte lysate cell-free translation systems. Total RNA and poly(A)$^+$ RNA stimulated the protein synthesis several fold over that of the endogenous activities of the wheat germ and reticulocyte lysate systems. The polypeptides synthesized in vitro were analysed by single dimension SDS-PAGE and fluorography (Fig. 22). A large number of polypeptides were

Fig. 21 **Coomassie Blue-stained two-dimensional gel electrophoretic patterns of pistil proteins from clones W43 (S_1S_1), W166K (S_1S_2), Ka3D (S_2S_2), W166H (S_2S_3), T_2U (S_3S_3) and KaD3 (S_4S_4).** Proteins were separated by IEF (3.5 to 10.0 pH range) in the first dimension followed by SDS-PAGE (12% PAA).

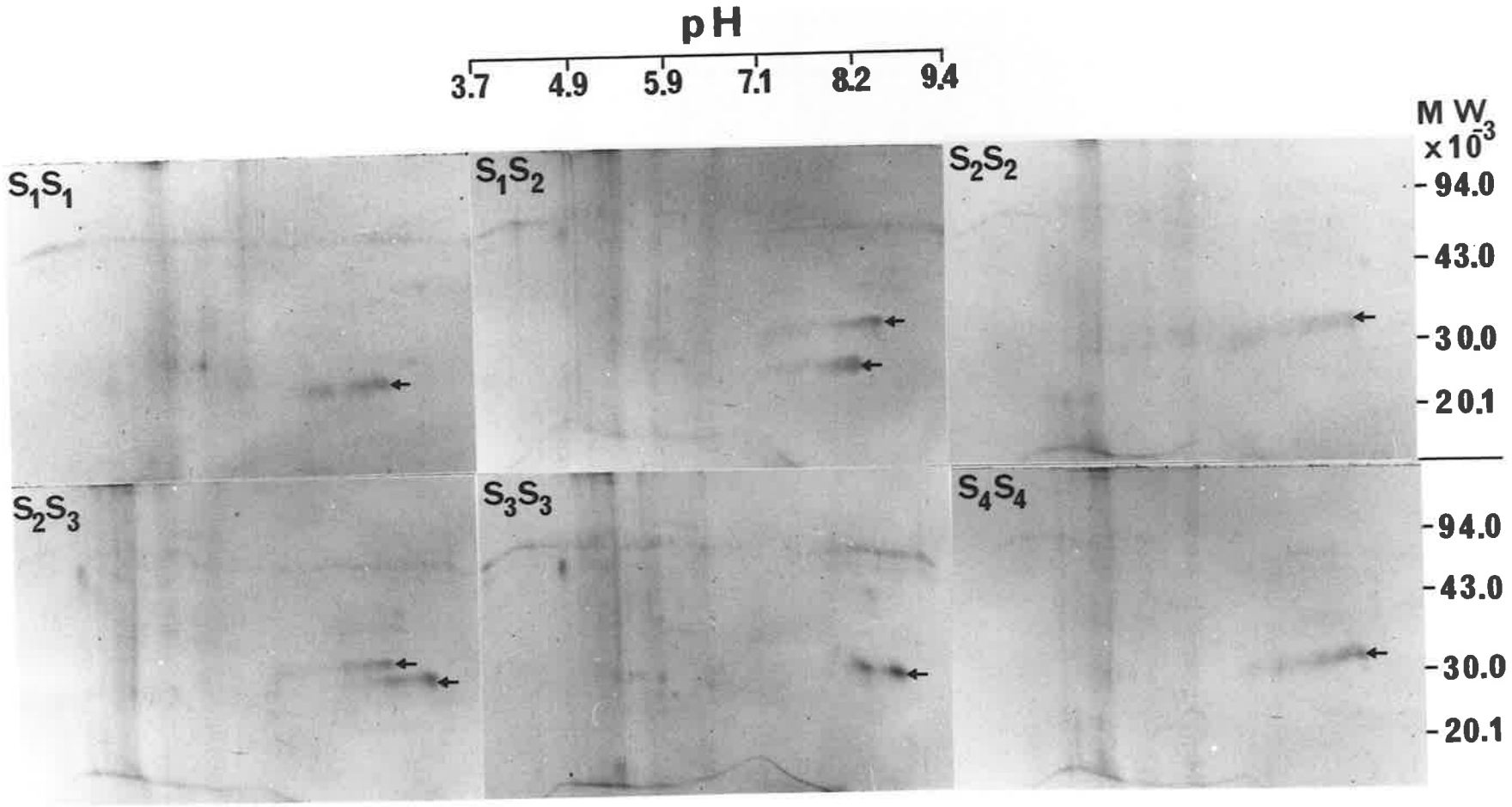
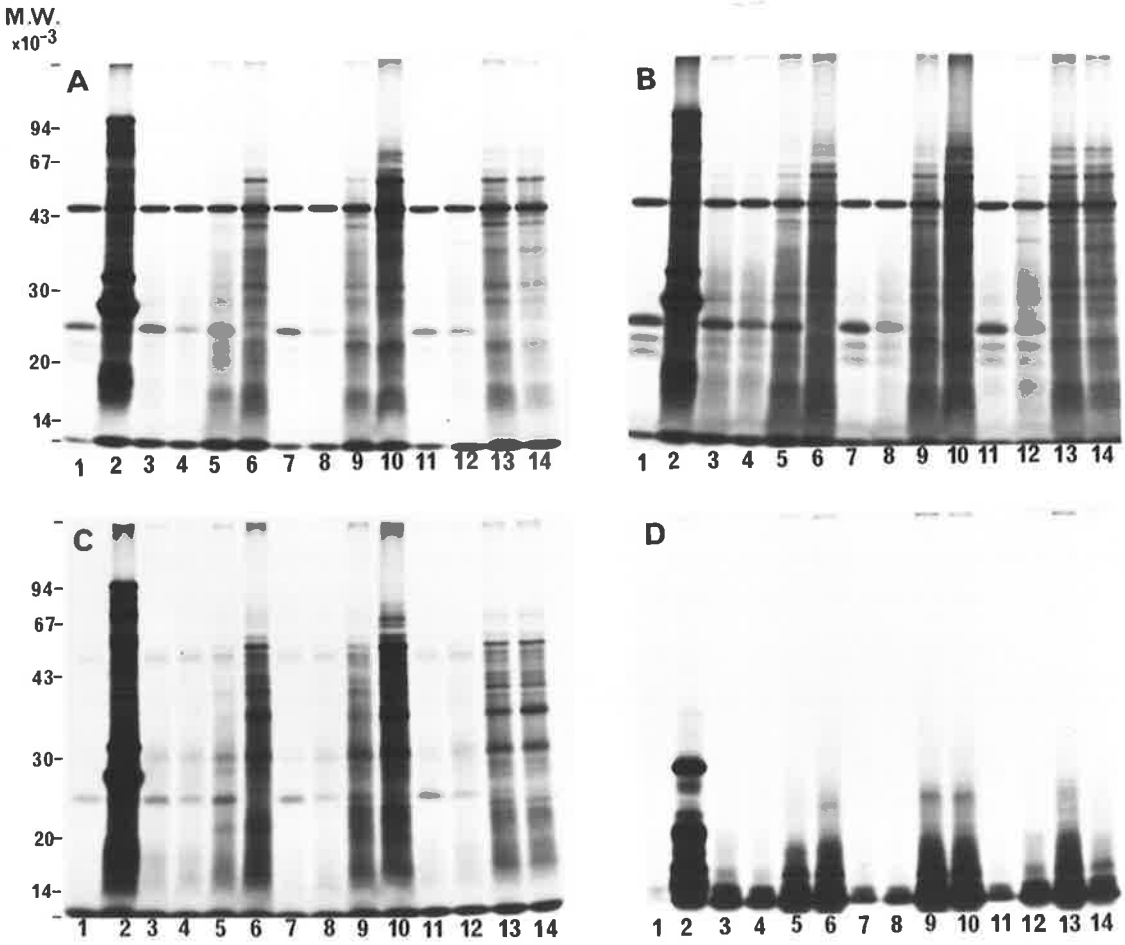

pH
3.7
4.9
5.9
7.1
8.2
9.4
MW
x10^3
94.0
43.0
30.0
20.1
S1S1
S1S2
S2S2
S2S3
S3S3
S4S4

Fig. 22 **Fluorographs of SDS-PAGE (12% PAA) of proteins synthesized in the reticulocyte lysate cell-free system (A, B, C) and also in wheat germ system (D) with Petunia pollen or pistil poly(A)$^+$RNA.**

Proteins were labelled with L-{^{35}S} methionine in A, B, D and with L-{^{3}H} leucine in C as described in section 2.2.10.

Dried gels were exposed for 40h in A and 11 days in B, C, D.

1, endogenous activity of the reticulocyte lysate system (A, B, C) or wheat germ system (D) without added mRNA; 2 to 14, *in vitro* translation products of poly(A)$^+$RNA from as follows: 2, cucumber mosaic cirus (CMV) as standard mRNA; 3,4, W166H pistils; 5,6, W166H pollen; 7,8, 924 pistils; 9,10, 924 pollen; 11,12, *Petunia nana* pistils; 13,14, *Petunia nana* pollen.


M.W.
×10⁻³
A
94
67
43
30
20
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
94
67
43
30
20
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14

synthesized in both the reticulocyte lysate and wheat germ systems. In the wheat germ system very few larger polypeptides (molecular weight in excess of 60,000 daltons) are synthesized. The wheat germ system is known to have a tendency to produce incomplete products due to premature termination and the release of peptidyl RNA (Clemens, 1984). In the reticulocyte lysate system larger polypeptides are also synthesized (Fig. 22).

The cell-free translation systems operate on the basis of the incorporation of labelled amino acids during polypeptide synthesis. Protein patterns obtained after the use of only one labelled amino acid can be misleading because of variation in amino acid composition of various proteins. As shown in Fig. 22, the polypeptide pattern obtained after using ^{3}H-leucine differs from the polypeptide patterns obtained after using ^{35}S-methionine.

SDS-PAGE of the *in vitro* translation products made with both total RNA and poly(A)$^{+}$ RNA from the mature ungerminated pollen shows that some of the *in vitro* synthesized polypeptides (Fig. 22) have similar relative mobilities as the proteins synthesized *in vivo* during pollen germination (Fig. 9). The large number of polypeptides synthesized in cell-free translation systems give direct evidence of a diverse population of the pre-synthesized (stored) mRNA in the mature ungerminated pollen of *Petunia*.

Total RNA and poly(A)$^{+}$ RNA isolated from ungerminated *Petunia* pollen were more actively translated as compared to total RNA and poly(A)$^{+}$ RNA isolated from the mature pistils (Fig. 22). Consequently, fewer protein bands can be seen in the lanes of total RNA and poly(A)$^{+}$ RNA from the mature pistils. From the SDS-PAGE pattern of the *in vitro* translation products, it is difficult to locate the pistil glycoproteins that correspond to S genotype (Fig. 22).

4. DISCUSSION

4. DISCUSSION.

4.1 Uptake of nucleosides.

4.1.1 Uptake of pyrimidine nucleosides.

The mechanism of pyrimidine nucleoside transport varies according to the organism, having been shown to be active, carrier-mediated in certain bacterial cells (Mygind and Munch-Petersen, 1975; Munch-Petersen and Phil, 1980; Foret and Ahlers, 1982) and non-active, carrier-mediated in animal cells (Plagemann and Richey, 1974; Wohlheuter et al., 1979; Plagemann and Wohlheuter, 1980; Young and Jarvis, 1983; Plagemann and Wohlheuter, 1984). However, in these cases it has either been shown or inferred that all the nucleosides are transported by a similar mechanism in any particular cell. Little is known about nucleoside transport in plant cells; a simple diffusion mechanism has been proposed for all pyrimidine nucleosides in Euglena (Wasternack, 1976) while Suss and Tupy (1982) have shown that the uridine uptake system is saturable in tobacco pollen. The results presented in section 3.1 support the hypothesis that transport of pyrimidine nucleosides into germinating Petunia hybrida pollen is carrier-mediated, and, except for thymidine, is inhibited by a variety of metabolic inhibitors.

The carrier-mediated transport characteristics shown for uridine and cytidine has been demonstrated to apply also to the pyrimidine nucleoside analogues 5-bromodeoxyuridine and deoxyuridine. Despite the fact that these two compounds are usually considered to be thymidine analogues and as such are incorporated into DNA in a wide range of organisms (Kornberg, 1980), the pollen grain transport of these analogues is more like that for uridine and cytidine than for thymidine. The maximal velocity of 5-bromodeoxyuridine and deoxyuridine is considerably higher than that for thymidine, and there may be therefore considerable practical advantage in using these analogues for DNA labelling studies with germinating pollen where advantage can be taken to increase analogue concentration to overcome the disadvantage of a higher K_m. These considerations may be especially useful in DNA repair studies of the type reported earlier (Jackson and Linskens, 1978, 1979, 1980, 1982).

It seems in Petunia pollen, thymidine transport differs from that for uridine and cytidine in several ways. That thymidine transport is achieved by a different carrier is suggested from the lack of effect of uridine on thymidine efflux. In addition to the fact that the rate of transfer is significantly lower than that for the other pyrimidine mucleosides (including the analogues), thymidine entry is not so prone to inhibition by chelating agents, and is less sensitive to both sulphydryl binding reagents and heavy metal ions. The accumulation of uridine and deoxyuridine against a concentration gradient, in addition to evidence from the use of energy inhibitors, shows that the pyrimidine nucleosides, with the exception of thymidine, are transported by an active (energy-coupled) process. Active (energy-coupled) transport of all pyrimidine nucleosides occurs in E. coli (Mygind and Munch-Petersen, 1975; Munch-Petersen et al., 1979) and yeast cells (Losson et al., 1978), this interpretation of transport experiments being supported by observations with certain E. coli mutants (Munch-Petersen, et al., 1979). However, until plant mutants unable to phosphorylate pyrimidine nucleosides become available, it will be difficult to prove conclusively that active (energy-coupled) nucleoside transport does occur in plant cells. The results put forward here could possibly be interpreted as phosphorylation (during or immediately after transport) being the step which is affected by energy poisons, although accumulation of uridine and deoxyuridine against a concentration gradient is a strong counter-argument in favour of energy-coupled transport of these nucleosides.

All pyrimidine nucleosides enter cultured animal cells (e.g. erythrocytes) by an non-active process, involving a carrier of wide specificity, which is a protein of similar size and properties to one involved with sugar transport (Wu et al., 1983; Young and Jarvis, 1983). The recent advances with animal cell transport have been made possible by the highly specific and potent inhibitor nitrobenzylthioinosine, which can be used as a probe for the pyrimidine nucleoside transporter (Young and Jarvis, 1983). Such a probe is not available for further studies in Petunia pollen (or, presumably, for the E. coli or yeast systems either), since nitrobenzylthioinosine is without effect on pyrimidine nucleoside transport in pollen. This observation

in itself is of interest, as, among the eukaryotes, it sets the plant cells apart from the animal cells in so far as the mode of pyrimidine nucleoside transport is concerned, in addition to the difference in possible energy-linked characteristics observed with pollen. Tapeworm is the only other eukaryote believed to show the characteristics of active transport of pyrimidine nucleosides (Page and Macinnis, 1975). Sugar transport in many plant cells proceeds by an energy-linked proton symport (Baker, 1978), a mechanism which has recently been shown to occur for sugar uptake in Lilium longiflorum pollen (Deshusses et al., 1981). This would also seem to set pollen apart from cultured erythrocytes and other cultured animal cells where a non-active, carrier-mediated process prevails for sugar uptake (Plagemann and Wohlheuter, 1980; Young and Jarvis, 1983). While the maximal rates of transport of pyrimidine nucleosides in E. coli (Mygind and Munch-Petersen, 1975) are of the same order as found, for example in Navikoff rat hepatoma cells (Plagemann and Richey, 1974; Wohlheuter et al., 1979) (approximately 50 pmol/sec/µl cell H_2O), these rates are substantially higher than observed here for uridine transport in Petunia hybrida pollen (0.26 pmol/sec/µl pollen grain H_2O). There does not therefore seem to be any obvious advantage in the expenditure of energy on uridine uptake, unless it is that a higher rate of entry of uridine into the cell per molecule of transporter protein utilized can be achieved this way. There is no means of determining this in pollen at present. However, pollen does have a thick and presumably impenetrable outer wall (Stanley and Linskens, 1974) which may considerably restrict the area of membrane available for transport processes, perhaps even to the three pores through which a pollen tube eventually emerges. These areas are quite small, and so perhaps there is sufficient reason for the use of energy if it can indeed increase the throughput of the uridine or cytidine transported in that small area for the needs of the RNA (Mascarenhas and Bell, 1970; Tupy, 1977), polysaccharide and membrane lipid biosynthesis that takes place during pollen germination. Although DNA replication does not normally take place during Petunia hybrida pollen germination (Jackson and Linskens, 1978), and one could attribute the different and low rate of thymidine transport to a lack of demand for this precursor, there is however considerable unscheduled DNA synthesis in response to ultraviolet irradiation (Jackson and Linskens,

1978) or the presence of mutagens (Jackson and Linskens, 1979, 1980, 1982) and this may require immediate and substantial input of precursor for a short time. There is considerable evidence for a close interaction between pollen and pistil, including uptake of small molecular weight compounds from pistil to pollen for synthesis of high molecular weight materials essential for pollen tube development and other needs (Linskens and Kroh, 1970). The findings reported herein are consistent with process occurring during pollination and show that pollen has the transport systems necessary to take up pyrimidine nucleosides from Petunia styles, where it is known that the concentration of nucleic acid precursors increase after pollination (van der Donk, 1974).

4.1.2 Uptake of purine nucleosides.

The general characteristics of adenosine and guanosine transport have been determined for Petunia hybrida pollen. In most respects, the transport of purine nucleosides is identical to that of pyrimidine nucleosides with the exception of thymidine (Section 3.1; Kamboj and Jackson, 1984, 1985). The results presented in section 3.2 support the hypothesis that transport of purine nucleosides into germinating Petunia hybrida pollen is carrier-mediated and is restricted by a variety of metabolic inhibitors. Similar uptake systems have recently been described for leucine uptake in tobacco pollen (Capkova et al., 1983), and for sugar uptake in Lilium longiflorum pollen (Deshusses et al., 1981).

The accumulation of adenosine and guanosine against a concentration gradient, in addition to the evidence from the use of metabolic inhibitors suggests that purine nucleosides are transported in germinating Petunia hybrida pollen by an active (energy-coupled) process. The results put forward here could possibly be interpreted as phosphorylation (during or immediately after transport) being the step which is affected by metabolic inhibitors, although accumulation against a concentration gradient is a strong counter-argument in favour of energy-coupled transport of purine nucleosides. This is in general agreement with the active (energy-coupled) transport characteristics of nucleosides for a variety of organisms such as bacterial cells

(Mygind and Munch-Petersen, 1975; Munch-Petersen et al., 1979), yeast cells (Losson et al., 1978) and pollen (Kamboj and Jackson, 1984, 1985). However, in animal cells all nucleosides enter by a non-active process, involving a carrier of wide specificity. The nucleoside carrier protein in animal cells is of similar size and properties to one involved with sugar transport (Wu et al., 1983; Young and Jarvis, 1983). As discussed in previous sub-section, the recent advances with animal cell transport have been made possible by the highly specific and potent inhibitor nitrobenzylthioinosine, which can be used as a probe for the nucleoside transporter (Young and Jarvis, 1983). Since nitrobenzylthioinosine is without effect on purine nucleoside transport in Petunia pollen, such a probe is not available for further studies in Petunia pollen (or presumably, for the bacterial or yeast systems either).

Pollen is an highly differentiated tissue, its primary function being to deliver the gametes to the ovule through a rapidly synthesized pollen tube which has to interact with and make its way through stigma and style (Linskens and Kroh, 1970). Adenosine and guanosine nucleosides are in immediate and urgent demand in the early stages of pollen germination for protein synthesis, RNA synthesis, polysaccharide and membrane lipid biosynthesis (Mascarenhas, 1975), DNA repair (Jackson and Linskens, 1978, 1979, 1980, 1982), and uptake of metabolic substrates (Deshusses et al., 1981; Capkova et al., 1983; Kamboj and Jackson, 1984, 1985). Results from this study also showed that adenosine triphosphate was rapidly synthesized during the early stages of Petunia pollen germination. So there is sufficient reason for the use of energy if it can indeed increase the throughput of the adenosine or guanosine transported in the small membrane area available for uptake (as discussed in previous sub-section) for the needs of the various metabolic activities during early stages of pollen germination. This study suggests that purine nucleosides could be salvaged from the stylar tissue, where pools of nucleic acid precursors increase after pollination (van der Donk, 1974), when there is a close interaction between pistil and pollen (Linskens and Kroh, 1970).

4.2 Transport of amino acids.

4.2.1 L-methionine and L-glutamic acid transport.

The results presented in section 3.3 support the hypothesis that Petunia pollen has active, carrier-mediated transport systems for uptake of metabolic substrates. Similar systems have recently been described for leucine uptake in tobacco pollen (Capkova et al., 1983), for sugar uptake in Lilium longiflorum pollen (Deshusses et al., 1981) and also, for pyrimidine nucleosides uptake, with the exception of thymidine, in Petunia pollen (Kamboj and Jackson, 1984, 1985). While the pyrimidine nucleoside transport rates decreased soon after pollen germination, a steady increase of L-Glu uptake into Petunia pollen is shown here for at least 8h of culture. On the other hand, L-Met uptake rate reaches its maximum in 2h of culture and decreases thereafter. Capkova et al., (1983) have related the increase of amino acid uptake to the initial increase of pollen tube surface during their growth, but in Petunia pollen, the increase in amino acid uptake cannot be related to the increase of pollen tube surface during culture.

The accumulation of L-Met and L-Glu against a concentration gradient, in addition to evidence from the use of metabolic inhibitors suggests that amino acids are transported in Petunia pollen by an active (energy-coupled) process. This is in general agreement with the active (energy-coupled) transport characteristics of amino acids for a wide variety of plant tissues, cells, and protoplasts (Reinhold and Kaplan, 1984). The sulphydryl reagent NEM and dithiol binding agent phenylarsine oxide were good inhibitors of transport of both the amino acids. Since both these inhibitors did not increase the label efflux as compared to controls, the inhibition by these inhibitors is probably due to effects on protein structure of the carriers rather than on membrane integrity.

Kinetic analysis of L-Met and L-Glu uptake in Petunia pollen indicates two saturable phases and one unsaturable phase. Multiphasic uptake of amino acids has been observed previously in several plant tissues (Shtarkshall and Reinhold, 1974; Lein and Rognes, 1977; Soldal and Nissen, 1978) and in cultured cells

(King, 1976; Harrington and Smith, 1977; Blackmann and McDaniel, 1980; Harrington and Henke, 1981). Nissen (1974) put forward the hypothesis that a single membrane-bound component of the transport system undergoes all-or-none transitions at certain critical external solute concentrations. Data which fit this multiphasic hypothesis has been obtained for amino acid uptake by several workers (Soldal and Nissen, 1978; Blackmann and McDaniel, 1980; Despeghel and Delrot, 1983). Alternatively multiphasic uptake may be due to the presence of several carriers as shown by Pall (1969, 1970) in Neurospora crassa, where several permeases have overlapping specificity for various amino acids. In the present study, the meaning and significance of the multiphasic pattern observed for L-Met and L-Glu is not clear and requires further study. However, the discontinuities observed in double logarithmic plots demonstrate that there are sudden shifts over small substrate concentration changes in the apparent kinetic parameters of the systems involved in the transport of L-Met and L-Glu and these shifts may be consistent with multiphasic uptake hypothesis (Nissen, 1974).

A number of separate systems of defined specificity for the transport of various amino acids have been distinguished in bacterial and animal cells (Christensen, 1979). However in higher plant cells, it has yet to be established whether one or several carriers mediate transport of the various amino acids (Reinhold and Kaplan, 1984). The data so far collected for higher plants suggests a single system shared by the various amino acids for a wide variety of tissues, cells, and protoplasts (Reinhold and Kaplan, 1984). By contrast, in Petunia pollen the L-Glu transport was inhibited by L-Asp only, indicating the presence of a highly specific transport carrier system for acidic amino acids. A similar conclusion can be drawn for a neutral amino acid transport carrier system for neutral amino acids because L-Met uptake was inhibited by cysteine and the neutral amino acids only. Since basic amino acids were without effect on both uptake systems, the data from this study indicates that at least three uptake carrier systems for various amino acids exist in Petunia pollen. This is in general agreement with the reports suggesting the multiple uptake carrier systems for various amino acids e.g. in bacterial and animal cells (Christensen, 1970); in cultured soybean root cells (King and Hirji, 1975)

and also in barley roots (Soldal and Nissen, 1978).

The effects of cycloheximide either when present in the transport medium or in preincubation experiments on transport of the two amino acids, in being inhibitory for L-Glu and without any effect on L-Met transport, provide further evidence for separate uptake carriers of these amino acids in Petunia pollen. Some workers have highlighted the problems of using cycloheximide as an inhibitor of protein synthesis (Ellis and MacDonald, 1970; McMohan, 1975; Harrington et al., 1981). However, in Petunia pollen, cycloheximide does not reduce ATP levels during pollen germination (data not shown) and is without effect on L-Met transport. The simplest interpretation of these results is that protein synthesis is required for the L-Glu transport carrier system whereas no protein synthesis is required for the protein associated with the L-Met transport carrier.

Separate uptake carrier systems for L-Met and L-Glu may also be distinguished on the basis of their differential stimulation by Ca^{2+} as well as their differential sensitivities to EGTA.

The results presented in this study support the hypothesis that L-Met and L-Glu are transported by separate carriers into Petunia pollen via energy-dependent, pH-dependent, stereospecific, multiphasic, saturable systems. Competition experiments using a variety of amino acids indicate the presence of at least three uptake carrier systems for various amino acids in Petunia pollen.

4.2.2 Arginine transport.

The active, carrier-mediated transport characteristics shown for L-Met and L-Glu in germinating Petunia pollen (section 3.3), has been demonstrated to apply also to the L-Arg uptake. The uptake rate for L-Arg in Petunia pollen decreased soon after pollen germination and a gradual decline in uptake rate was observed in pollen tubes from 2h, 4h, 6h and 8h cultures, which is in contrast to the steady increase of L-Glu uptake rate observed in Petunia pollen at least up to 8h of culture. A steady increase in leucine uptake rate has also been reported for tobacco pollen (Capkova et al., 1983). A similar decrease in

transport rates of pyrimidine nucleosides was observed in Petunia pollen (Kamboj and Jackson, 1985).

The accumulation of L-Arg against a concentration gradient suggests that L-Arg is transported in Petunia pollen by an active (energy-coupled) process. This is in general agreement with the active (energy-coupled) transport characteristics of amino acids for a wide variety of plant tissues, cells, protoplasts (Reinhold and Kaplan, 1984) and pollen (Capkova et al., 1983).

Kinetic analysis of L-Arg transport in Petunia pollen indicates three saturable phases and one unsaturable phase. Multiphasic uptake of amino acids has been observed in Petunia pollen (section 3.3) and also in several plant tissues, cultured cells and protoplasts (Reinhold and Kaplan, 1984). The discontinuities observed in double logarithmic plot demonstrate that there are sudden shifts over small substrate concentration changes in the apparent kinetic parameter of the system involved in the transport of L-Arg and these shifts may be consistent with a multiphasic uptake hypothesis (Nissen, 1974). The kinetic constants for phase 2 and phase 3 for L-Arg uptake in Petunia pollen are similar to those found for L-Arg phase 2 and phase 3 in barley leaf slices by Lein and Rognes (1977). These authors did not assign any K_m and V_{max} values for phase 1 of L-Arg uptake by barley leaf slices. Soldal and Nissen (1978) reported three phases for arginine uptake in barley root system. The kinetic constants for phase 1 and 3 for arginine uptake are similar to those found for phase 1 and 2 in the present study.

It is tempting to postulate that the basic amino acids are taken up by a general amino acid carrier, but several lines of evidence go against this hypothesis. That L-Arg transport is achieved by a different carrier is suggested from the wide difference in pH-optima for L-Arg transport observed in this study and low pH-optima for L-Glu and also a broad pH-optima for L-Met transport (section 3.3). In addition to the fact that the basic amino acids were without effect on L-Met and L-Glu uptake systems (section 3.3), L-Arg entry responded uniquely to inhibition by metabolic inhibitors, and is not sensitive to cycloheximide. Also the observation that L-Arg uptake rate started declining soon after pollen germination is quite different to a

steady increase of L-Glu uptake and a jump in L-Met uptake soon after germination (section 3.3).

Christensen and Handlogten (1969) proposed that cations weakly bound to the side chain of certain neutral amino acids could well mimic a cationic amino acid and hence, be recognised as such. This could explain the inhibition of L-Arg uptake by most of the amino acids tested.

The severe inhibition of L-Arg transport by calcium and polyamines also suggested that L-Arg uptake is achieved by a different carrier than that for acidic amino acids and neutral amino acids, because calcium and polyamines caused no inhibition of L-Glu and L-Met uptake, and on the contrary had a stimulatory effect on their transport (section 3.3) Berry et al. (1981) examined the uptake of arginine into cultured tobacco cells. In contrast to the present study, the uptake of arginine was stimulated by calcium in a time dependent fashion and in the absence of calcium, transport declined to relatively low rates. They also reported that arginine transport is favoured by lower pH values up to pH 5, which is again different to the high pH-optima observed for L-Arg uptake in Petunia pollen. In contrast to the present study, McDaniel et al. (1982) using suspension cultured tobacco cells reported a pH optima of 3.0 for L-Arg uptake. Therefore it seems that L-Arg uptake system in Petunia pollen is quite different to those reported for other plant cells.

The results presented in this study support the hypothesis that L-Arg is transported into Petunia pollen via energy-dependent, pH-dependent, stereospecific, multiphasic, saturable system.

4.3 Control of protein synthesis and protein release during germination and pollen tube growth of Petunia pollen.

4.3.1 Control of protein release.

The results presented in section 3.4.1 support the hypothesis that a large proportion of protein export occurring during germination represents an energy-driven release, and that a

number of these proteins are synthesized during germination. It is apparent that while some of the proteins exported during germination in the absence of uncouplers and ATPase inhibitors may be labelled and thus represent newly synthesized protein, nevertheless the number of unlabelled proteins is too large to represent solely protein diffusing from the pollen grain walls. It seems then that the proteins released in the absence of energy inhibitors (and not including the freely diffusing fraction) consist of both stored and newly synthesized proteins.

The freely diffusing protein, comprising about half of that normally appearing in the first hour of germination, may correspond with that described by Kirby and Vasil (1979), who obtained protein by cold elution of Petunia pollen. The remainder of the protein appearing in the first hour, together with all protein that is progressively released into the medium upto at least 5h, represents an energy-driven protein export. Data on energy requirements from protein export or release from cells is scanty. In animal cells it is known that certain steps in protein secretion are energy dependent (Kern et al., 1979), while in bacteria it is known, for example, the coliphage protein procoat transfer across the membrane bilayer requires, at least indirectly, an electrochemical gradient inhibited by the energy poisons (Date et al., 1980). For subcellular organelles the situation is clearer; uptake of cytoplasmic polypeptides by chloroplasts (Grossman et al., 1980) is known to be energy dependent, as is the proteolytic processing of cytoplasmically made precursors to mitochondrial proteins (Nelson and Schatz, 1979). In the present experiments with pollen, the metabolic requirements of protein synthesis can explain part of the energy dependence of this protein export. It is possible that the remainder represents transmembrane protein transport not coupled to translation, with either electrochemical gradient or proteolytic-processing energy requirements. Like the active peptide transport system in germinating barley embryo (Walker-smith and Payne, 1983), energy-dependent protein release in Petunia pollen is inhibited by the thiol reagent, N-ethylmaleimide, a finding which tends to support the hypothesis that the release is an active protein transport. Stanley and Linskens (1965) observed a higher rate of protein release with Petunia hybrida at 25°C than at 4°C (which has been verified

in the present study, data not shown), and suggested that this showed that the protein 'diffusate' was a product of 'metabolic activities'. So, it is proposed that the protein described by Stanley and Linskens (1965) as being exported at 4°C is indeed a diffusate, and that the extra released at 25°C represents an energy-driven protein release (possibly transmembrane transport) including with it a small portion of newly synthesized protein.

4.3.2 Protein synthesis during germination and pollen tube growth.

In Petunia pollen, a large number of proteins are synthesized during the early stages of pollen tube growth. There appears to be no major qualitative differences between the Coomassie Blue stained protein bands in SDS-PAGE patterns obtained from pollen cultured for varying times up to 4h. Similar results have been found for proteins synthesized during germination and pollen tube growth. Since the resolution of the single dimension gels is relatively poor, pollen proteins from various stages of germination and pollen tube growth were analysed by two-dimensional electrophoresis with IEF on a pH 3.5 - 10.0 gradient in the first dimension followed by SDS-PAGE. The two-dimensional electrophoretic separations of Coomassie Blue stained pollen proteins at various stages of germination and pollen tube growth shows that at least 300 different proteins are present at various stages of pollen germination and tube growth. This is likely to be an underestimate of the number of proteins. Since there are no detectable differences in proteins present at various stages of pollen tube growth, the previous results with SDS-PAGE are confirmed. Using two-dimensional electrophoretic separations, similar results have been found for proteins synthesized during germination and pollen tube growth of Petunia pollen. By means of single dimension SDS-PAGE, Mascarenhas et al. (1974) observed similar results for Coomassie Blue stained protein patterns and also for proteins synthesized during germination and pollen tube growth of Tradescantia. However, these authors did not investigate the patterns of proteins released during germination and pollen tube growth, as is described here.

As discussed in section 4.3.1, in Petunia hybrida pollen, in addition to the diffusible proteins passively released in the

first hour of germination and pollen tube growth, proteins pre-existing in the pollen as well as newly synthesized proteins are released by an energy-driven process during germination and pollen tube growth. Although there are some differences in the patterns of released proteins and pollen proteins, there appear to be no major qualitative differences in the SDS-PAGE and two-dimensional electrophoretic patterns of the released proteins (both proteins pre-existing and newly synthesized) at various stages during germination and pollen tube growth. This implies that the proteins made on newly synthesized mRNA are not different from the proteins translated from the pre-existing mRNA in the pollen.

More evidence for the presence of stable or pre-existing mRNA in the ungerminated Petunia pollen grain was obtained, based on the effects of inhibitors of RNA and protein synthesis on germination and pollen tube growth, and also on protein synthesis during germination and pollen tube growth in Petunia. There appears to be no major qualitative differences between the newly synthesized protein bands in single dimension (SDS-PAGE) gels obtained from pollen tubes cultured in the presence or absence of α-amanitin (10 μg/ml). The two-dimensional electrophoretic separations of newly synthesized proteins showed that at least 300 different proteins are synthesized during pollen germination and tube growth. The same number and pattern of protein spots is seen whether or not α-amanitin (10 μg/ml) is included in the culture medium, indicating that mRNA present in the ungerminated pollen, and those newly synthesized, code for the same proteins. Similar results have been found with Tradescantia pollen using actinomycin-D as RNA synthesis inhibitor (Mascarenhas et al., 1974; Mascarenhas and Mermelstein, 1981). Again, these authors did not report on the patterns of proteins which may have been released in the presence or absence of RNA synthesis inhibitors during germination and pollen tube growth, so it is possible some differences have been missed.

In Petunia a reasonably large number of proteins (pre-existing and newly synthesized) are released during germination and pollen tube growth. From the results in sections 3.4.8 and 3.4.9, it can be seen that there are some minor differences in the pattern of proteins synthesized in pollen and in the pattern

of newly synthesized proteins released during germination and pollen tube growth. However, there appear to be no major qualitative differences, between the released protein bands (both pre-existing and newly synthesized) in single dimension (SDS-PAGE) gels obtained from pollen cultured with and without α-amanitin (10 μg/ml). The two-dimensional electrophoretic separations of released proteins (both pre-existing and newly synthesized) in the presence or absence of α-amanitin (10 μg/ml) during germination and pollen tube growth confirms this. It is concluded that the pre-existing mRNA present in the pollen grain and the mRNA newly synthesized during germination and pollen tube growth, code for the same proteins. In other words, the genes active during the latter part of pollen maturation prior to anthesis and those active during germination and pollen tube growth appear to be the same.

The patterns (single dimension SDS-PAGE and two-dimensional electrophoretic separations) of proteins synthesized in the presence or absence of cations (Mg^{2+} or Ca^{2+}), polyamine (spermine) or α-amanitin, during germination and pollen tube growth, reveal that there are no major qualitative differences between the proteins synthesized as a result of these treatments. However, the presence of 1 mM or 5 mM Ca^{2+} ions in the culture medium virtually stops the release of all the major pre-existing proteins during germination and pollen tube growth, but has little inhibitory effect on the release of newly synthesized proteins. On the contrary, in the presence of Ca^{2+} ions there is clear stimulation of the release of a number of newly synthesized proteins during germination and pollen tube growth. Two-dimensional electrophoretic separation of newly synthesized released proteins in the presence of 5 mM Ca^{2+} ions during germination and pollen tube growth shows that at least 6 new major protein spots can be seen in the pattern. 5 mM Ca^{2+} also inhibited the release of several newly synthesized proteins. Although α-amanitin (10 μg/ml) has no effect on the pattern of newly synthesized proteins released, the two-dimensional electrophoretic pattern of newly synthesized released proteins in the presence of higher α-amanitin concentration (100 μg/ml) during germination and pollen tube growth indicates that the pattern of released protein spots is very similar to the pattern that is

obtained in the presence of 5 mM Ca^{2+} ions. The region of maximum stimulation of release of newly synthesized proteins is similar to that obtained in the presence of 5 mM Ca^{2+} and in addition at least 3 new major protein spots can be seen as compared to the control pattern. Stimulation of synthesis of some proteins by high concentration of α-amanitin (100 µg/ml) has been observed in maize (P. Langridge, unpublished observations).

Heat shock or other environmental stresses have been shown to elicit the enhanced synthesis of a small set of heat shock proteins in eukaryotes (Ashburner and Bonner, 1979; Schlesinger et al., 1982; Tanquay, 1983). Altschuler and Mascarenhas (1982) reported that heat shock proteins are not synthesized in Tradescantia pollen tubes. Similarly in this study, the exposure of Petunia pollen tubes to heat shock and abscisic acid did not enhance the synthesis of heat shock proteins. In addition, there was no specific effect on the release of newly synthesized proteins.

It is proposed that protein release from Petunia hybrida pollen is not merely a passive diffusion, but rather goes by way of Golgi-derived secretory vesticles which make their way to the plasma membrane and there give up proteins to the outside after vesicle fusion and exocytosis. To complete the cycle of membrane flow, fragments of membrane released by an endocytosis process, migrate back to the Golgi area. These processes are thought to be involved in pollen tube extension (Picton and Steer, 1983). As known from work with animal cells, several steps in such process could well be energy-dependent, including packaging of proteins into secretory granules and exocytosis itself. Many of these proteins exported may be destined for the cell wall that is built up during pollen tube extension, it is suggested that boron is needed for this process, so that in the absence of boron, proteins normally inserted into the wall are released to the culture medium. However, protein export cannot be completely stopped, and so it is suggested that much of the protein released during exocytosis as the pollen tube is extended, escapes outside the tube and interacts with the tissue of pistil, helping to make way for further pollen tube elongation.

The above hypothesis may well apply to the bulk of the protein stored in the pollen grain at anthesis. During pollen germination, a small amount of protein is newly synthesized. This newly synthesized protein is also exported; however it cannot be controlled to the same extent as stored protein, so that the amount secreted is largely independent of boron and calcium added to the culture medium. It is however stopped as expected by the energy poisons, and by cycloheximide. Cycloheximide also inhibits bulk (stored) protein release, suggesting a role for some protein synthesis in the secretion process involving stored proteins as well.

For the situation where, as for Petunia hybrida, the pollen tube makes its way through a solid style, active release of proteins (presumably including enzymes needed to break down stylar tissue and other protein interacting with the style) is perhaps important. It is of interest to add that in early stages of this work pollen from Lilium longiflorum was also investigated and the results showed that it releases very little protein during germination (at most 10 per cent of that exported by Petunia hybrida pollen and only 3 per cent of its contained total protein). Perhaps this reflects the fact that in this case the pollen tube does not make its way through a solid style, but grows in a stylar canal. In addition, other evidence suggests that mobile proteins are not involved in the incompatibility response in Lilium (Fett et al., 1976).

Enzyme activities have been shown to be associated with the released proteins during germination and pollen tube growth (Poddubnaya-Arnoldi et al., 1959; Makinen and Brewbaker, 1967; Matousek and Tupy, 1983). Acid phosphatase is active as an antigen (Howlett et al., 1975) and so could be involved in pollen stigma interactions. In Petunia pollen, various phosphatase activities (pyrophosphatase, glycerophosphase, p-nitrophenyl-phosphatase and phytase) were detected. A small fraction of the activity of all the phosphatases was released during germination and pollen tube growth. Five isoenzymes of phytase and one isoenzyme of glycerophosphatase were detected by polyacrylamide disc gel electrophoresis. Attempts to locate the activities of these enzymes after detergent removal on SDS-PAGE and two-dimensional gels after detergent removal were not successful.

4.3.3 Protein phosphorylation and their release during germination and pollen tube growth.

Ca^{2+}-dependent protein phosphorylation is now recognized to be a major general mechanism by which intracellular events in mammalian tissues respond to external physiological stimuli (Cohen, 1982). Ca^{2+}-promoted protein phosphorylation in corn coleoptiles has been reported recently (Veluthambi and Poovaiah, 1984) and Ca^{2+}-dependent protein kinases have been recently resolved from plants (Polya et al., 1983; Polya and Micucci, 1984). In this study Ca^{2+}-dependent protein phosphorylation during germination and pollen tube growth in Petunia pollen has been demonstrated. These observations suggest that there could be similarities in Ca^{2+}-mediated signal transduction in animal cells and in Petunia pollen tubes.

Mg^{2+} is an important cofactor for kinases and its effect on protein phosphorylation was investigated. Mg^{2+} also stimulated the phosphorylation of proteins during germination and pollen tube growth. However, no new major phosphorylated protein spots can be seen as compared to the control. Whereas, in the presence of Ca^{2+} several new major phosphorylated proteins spots can be seen. These results indicated that Mg^{2+} and Ca^{2+} regulated protein phosphorylated differently. Even though Ca^{2+} or Mg^{2+} stimulated the phosphorylation of several proteins during germination and pollen tube growth, neither Ca^{2+} nor Mg^{2+} had any effect on the release of phosphorylated proteins. There is thus no evidence for involvement of protein phosphorylation in the mechanism of protein release.

4.4 Incompatibility genotype and biochemical investigation during the interaction between pollen and pistil.

Earlier investigations into the physiology of phytic acid dealt mainly with the development of the seed after fertilization and the concentration of phytic acid in the aleurone layer of the monocotyledenous seed (Cosgrove 1980; Jennings and Morton 1963; Tanaka et al. 1973; Tanaka et al. 1974). Recently Jackson and co-workers have looked for phytic acid in reproductive tissue before fertilization and investigated as wide a range of species

as possible. They found that phytic acid occurs in pollen (Jackson et al., 1982; Jackson & Linskens 1982b), particularly in dicotyledenous species with longer styles, and that in Petunia hybrida at least, phytic acid is degraded during in vitro pollen germination (Jackson & Linskens 1982a).

The present investigations with Petunia hybrida reproductive tissue made before fertilization builds on the previous observation that phytic acid occurs in highest concentration in the pollen of species showing gametophytic incompatibility and demonstrates that it is found only in those parts of the plant taking part in the incompatibility reactions. Thus it was found that phytic acid is localized before pollination in the pollen, stigma and style in amounts which vary according to the S alleles present, and that the same alleles influence the time course of breakdown of phytic acid that occurs after pollination. Incompatible alleles lead to a more rapid disappearance of phytic acid than with compatible alleles, which could in turn imply greater utilization by the incompatible combination. In germinating lily pollen myoinositol is taken up readily for polysaccharide biosynthesis (Loewus et al., 1978) where the myoinositol oxidation pathway is operational for pectin synthesis (Loewus and Loewus, 1980). Assuming a similar pathway in Petunia hybrida, then the products of phytic acid degradation, myoinositol or a phosphorylated derivative (Cosgrove 1980), could well be utilized for pectin or other polysaccharide biosynthesis. Kroh and Knuiman (1982) suggest that much of the secondary thickening in walls and "callose" plugs of the pollen tube is pectin in nature, while Linskens (1975a) points out that in the incompatible pollen tube there is increased thickening and more "callose" plugs. It is tempting to suggest that the more rapid disappearance of phytic acid seen here for the incompatible pollination is in fact due to increased utilization of phytic acid for inositol which in turn is in greater demand for pectin biosynthesis in the early stages of incompatible pollen germination and tube growth. This fits well with the differences in pattern of flow of organic substances described by Linskens (1975, 1975a) for incompatible and compatible pollination. At later times one would expect the difference in phytic acid levels between incompatible and compatible combinations to decrease owing to steady uptake for pectin synthesis in the longer tubes

developing as a result of compatible pollination after incompatible tube growth has ceased (see also Linskens 1975, 1975a).

This study has shown several points of interaction of phytic acid localization and metabolism with the S alleles. Further biochemical investigation will be needed to verify the interpretation in terms of pectin biosynthesis and its control by the self-incompatibility alleles.

4.5 Incompatibility genotype and gene expression in Petunia pollen and pistil.

4.5.1 Self-incompatibility genotype associated glycoproteins.

There has been an indication from the few gametophytic incompatibility systems so far studied, that the S genotype correlates with a protein of relatively high isoelectric point. Thus the S_3S_4 genotype of Prunus avium styles has yielded an S antigen with a pI of 8.8 (Mau et al., 1982), and Nicotiana alata styles with S_2 alleles showed an "S-specific protein" with the same pI (Bredemeijer & Blaas, 1981). Moreover the S antigen of Prunus avium was shown to have a molecular weight of 37,000-39,000 (Mau et al., 1982). These parameters are remarkably close to those found here for Petunia hybrida. In addition there is an indication that the "S protein", like the S antigen of Prunus avium, is a glycoprotein. Although the S antigen of Prunus avium is not the major protein of pistils, for Nicotiana alata S_2 genotypes the "S-specific protein" is indeed the major protein of stylar extracts, as is shown here for all "S proteins" of Petunia hybrida.

A feature of the Petunia hybrida system described herein is that each of the proteins assigned to the S_1, S_2, S_3 and S_4 alleles, while being clearly separable on a molecular weight and pI basis, nevertheless, appear to belong to one particular class of proteins with a low molecular weight and high pI. This is perhaps not unexpected, if they are indeed gene products of four different alleles at the one S locus. In this respect, the Petunia hybrida system appears to differ from that described for Nicotiana alata (Bredemeijer and Blass, 1981), where only one of

the "S specific proteins" (corresponding to S_2) was the major protein and exhibited an high pI. Other genotypes in Nicotiana alata were characterized by minor protein bands.

There is no evidence so far that the pistil "S proteins" so clearly shown for each of the four alleles in Petunia hybrida, occur in pollen as well. Bredemeijer and Blaas (1981) were also unable to comment on this from their investigations with Nicotiana alata, for similar reasons. However, Linskens (1960), working with the same clones used here, was able to demonstrate serological interactions with pollen consistent with the presence of pistil S antigen in pollen extracts. If the S antigen of Linskens (1960) corresponds with the "S protein", then the concentration of the latter in pollen must be considerably lower than in pistils.

There is no conclusive proof as yet that the "S proteins" are in fact S gene products and not merely the products of genetic backgrounds. However, when taken together with the indications from other gametophytic systems so far studied, it does seem that there is a strong possibility that they are, and that it may soon be possible to test the molecular models put forward for example by Linskens (1975), Lewis (1965), Heslop-Harrison (1983) or by Dumas et al. (1984), to explain the mechanism of self-incompatibility and how it relates to other biochemical events taking place during the interaction between style and pollen (Jackson et al., 1983).

4.5.2 Messenger RNAs in pollen and pistils of Petunia hybrida and cell-free synthesis of polypeptides.

Each mature ungerminated Petunia hybrida pollen grain contains approximately 100 pg of total RNA and 4 pg of poly(A)$^+$ RNA. In comparison, each Tradescantia paludosa pollen grain contains 195 pg of total RNA and 5.1 pg of poly(A)$^+$ RNA (Mascarenhas and Mermelstein, 1981) and each Nicotiana tabacum pollen contains 230 pg of total RNA and 6.2 pg of poly(A)$^+$ RNA (Tupy, 1982). Corn pollen grain has been reported to contain between 352 - 705 pg of total RNA and 8.9 - 17.8 pg of poly(A)$^+$ RNA (Mascarenhas et al., 1984). The higher amount of RNA in corn pollen grain may be due to its large size.

The ability of the total RNA and poly(A)$^+$RNA from mature ungerminated Petunia pollen to direct the synthesis of polypeptides in both the wheat germ and reticulocyte lysate cell-free systems is a direct demonstration of the presence of presynthesized mRNA in mature Petunia pollen grains. SDS-PAGE of the in vitro translation products shows that some of the in vitro synthesized polypeptide bands comigrate with polypeptides synthesized in vivo during pollen germination while others did not. This lack of a perfect correlation was expected because proteins synthesized in vivo may undergo post-translation modifications which cannot take place in the cell-free translation systems. (Clemens, 1984). Similar results has been observed with mRNA from other systems (Cuming and Lane, 1978; Frankis and Mascarenhas, 1980; Mascarenhas et al., 1984). SDS-PAGE patterns of the in vitro translation products obtained after fluorography also highlight the significance of the use of two different radioactively labelled amino acids (^{3}H-leucine and ^{35}S-methionine) and some of the differences between the two cell-free translation systems. Similar comparisons of the two cell-free translation systems have been reported by Clemens (1984). Although the two cell-free translation systems and the two radioactively labelled amino acids used in this study produced different patterns, they all indicate the presence of a diverse population of pre-synthesized mRNA in ungerminated pollen of Petunia. Other workers have shown the stored mRNAs of mature pollen to be of similar diversity (Tupy, 1982; Willing and Mascarenhas, 1984).

SDS-PAGE pattern of the in vitro translation products made with both total RNA and poly(A)$^+$RNA from the mature pistils indicates the difficulty in locating the pistil glycoproteins that correspond to S genotype. One of the reasons for the lack of correlation could be that the polypeptides synthesized in cell-free translation systems cannot have carbohydrate moieties attached. The lack of correlation of the polypeptides synthesized in the cell-free systems and those synthesized in vivo has been observed with mRNA from other systems (Cuming and Lane, 1978; Frankin and Mascarenhas, 1980; Mascarenhas et al., 1984).

5. BIBLIOGRAPHY

5. BIBLIOGRAPHY

Allen, J. and Gackerman, J. (1964) Electrophoretic separation of multiple forms of particle associated acid phosphatase. Ann. N.Y. Acad. Sci. 121, 616-633.

Aller, P.; Leonato, R. and Diez, J.L. (1980) Modification of the rate of uridine uptake by metabolic inhibitors on salivary glands of Chironomus thummi. Insect Biochem 10, 529-533.

Altschuler, M. and Mascarenhas, J.P. (1982) The synthesis of heat-shock and normal proteins at high temperatures in plants and their possible roles in survival under heat stress. In: Heat shock, from bacteria to man, eds. Schlesinger, M.J.; Ashburner, M. and Tissieres, A. (Cold Spring Harbour, New York) pp, 321-323.

Ascher, P.D. (1984) Self-incompatibility. In: Petunia, ed. Sink, K.C. (Springer-Verlag) pp., 92-109.

Ashburner, M. and Bonner, J.J. (1979) The induction of gene activity in Drosophila by heat shock. Cell 17, 241-254.

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Nat. Acad. Sci. (U.S.A.) 69, 1408-1412.

Bagni, N.; Adamo, P; Serafini-Fracassini, D. and Villaneuva, V. (1981) RNA, proteins and polyamines during tube growth in germinating Apple pollen. Plant Physiol. 68, 727-730.

Baker, D.A. (1978) Proton cotransport of organic solutes by plant cells. New Phytol 81, 485-497.

Berry, S.L.; Harrington, H.M.; Bernstein, R.L. and Henke, R.R. (1981) Amino acid transport into cultured tobacco cells. Planta 153, 511-518.

Blackman, M.S. and McDaniel, C.N. (1980) Amino acid transport in suspension-cultured plant cells II. Characterization of L-Leucine uptake. Plant Physio 66, 261-266.

Bonner, W.M. (1984) Fluorography for the detection of radio-activity in gels. Methods Enzymol. 104, 461-465.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quanties of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.

Bredemeijer, G.M.M. (1984) The role of peroxides in pistil-pollen interactions. Theor. Appl. Genet. 68, 192-206.

Bredemeijer, G.M.M. and Blaas, J. (1981) S-specific proteins in styles of self-incompatible Nicotiana alata. Theor Appl Genet 59, 185-190.

Brooker, J.D.; Tomaszewski, M. and Marcus, A. (1978) Preformed and newly synthesized mRNA in germinating wheat embryos. Planta 144, 491-496.

Brown, E.G. and Newton, R.P. (1981) Cyclic AMP and higher plants. Phytochem. 20, 2453-2463.

Capkova, V.; Hrabetova, E.; Tupy, J. and Rihova, L. (1983) Amino acid uptake and protein synthesis in cultured tobacco pollen. Biochem Physiol Pflanzen 178, 511-520.

Capkova-Balatkova, V.; Hrabetova, E. and Tupy, J. (1980) Effects of cycloheximide on pollen of Nicotiana tabacum in culture. Biochem. Physiol. Pflanzen. 175, 412-420.

Capkova-Balatkova, V.; Hrabetova, E. and Tupy, J. (1980) Effect of some mineral ions on pollen tube growth and release of proteins in culture. Biol Plant 22, 294-302.

Chirgwin, J.M.; Przybyla, A.E.; MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. 18, 5294-5299.

Christensen, H.N. (1979) Exploiting amino acid structure to learn about membrane transport. Adv. Enzymol. 49, 41-101.

Christensen, H.N. and Handlogten, M.E. (1969) Reactions of neutral amino acids plus Na^+ with a cationic amino acid transport system. FEBS Lett. 3, 14-17.

Cleland, W.A. (1967) The statistical analysis of enzyme kinetics data. Adv Enzymol 29, 1-32.

Clemens, M.J. (1984) Translation of eukaryotic mRNA in cell-free extracts. In: Translation and transcription - a practical approach, eds. Hames, B.D. and Higgins, S.J. (IRL Press) pp, 231-245.

Cochet-Meilhac, M. and Chambon, P. (1974) Animal DNA-dependent RNA polymerases II. Mechanism of the inhibition of RNA polymerase B by Amatoxins. Biochim. Biophys. Acta 353, 160-184.

Cohen, P. (1980) In: Molecular aspects of cellular Regulation. Vol. 1, ed. P. Cohen (Elsevier, Amsterdam), pp, 255-268.

Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296, 613-620.

Cosgrove, D.J. (1980) Inositol phosphates. Their chemistry, biochemistry and physiology. Amsterdam: Elsevier Scientific Publ. Co. Stud. Org. Chem. Ser. 4, 1-191.

Cuming, A.C. and Lane, B.G. (1978) Wheat embryo ribonucleates, XI. Conserved mRNA in dry wheat embryos and its relation to protein synthesis during early inhibition. Can. J. Biochem. 56, 365-369.

Daentl, D.L. and Epstein, C.J. (1971) Developmental interrelationships of uridine uptake, nucleotide formation and incorporation into RNA by early mammalian embryos. Dev. Biol. 24, 428-442.

Date, T.; Zwizinski, C.; Ludmerer, S. and Wickner, W. (1980) Mechanisms of membrane assembly: Effects of energy poisons on the conversion of soluble M13 coliphage procoat to membrane bound coat protein. Proc. Nat. Acad. Sci. (U.S.A.) 77, 827-831.

Davis, B.J. (1964) Disc Electrophoresis II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121, 404-427.

Deshuesses, J.; Gumber, S.C. and Loewus, F. (1981) Sugar uptake in lily pollen. Plant Physiol. 67, 793-796.

Desmarquets, G.; Cortay, J.C. and Cozzone, A.J. (1984) Two-dimensional analysis of proteins phosphorylated in E. Coli cells. FEBS Lett. 173, 337-341.

Despeghel, J.P. and Delrot, S. (1983) Energetics of amino acid uptake by Vicia faba leaf tissue. Plant Physiol. 71, 1-6.

Dumas, C.; Knox, R.B. and Gaude, T. (1984) Pollen-pistil recognition: new concepts from electron microscopy and cytochemistry. Int Rev Cytol. 90, 239-272.

Dure, L.S. (1975) Seed formation. Ann. Rev. Plant Physiol. 26, 259-278.

Ellis, R.J. and MacDonald, I.R. (1970) Specificity of cycloheximide in higher plant systems. Plant Physiol. 46, 227-232.

Fergusan, M.C. and Collidge, E.B. (1932) A cytological and a genetical study of Petunia IV. Pollen grains and the method of studying them. Am. J. Bot. 19, 644-659.

Fett, W.F.; Paxton, J.D. and Dickonson, D.B. (1976) Studies on the self-incompatible response in Lilium longiflorum. Am. J. Bot. 63, 1104-1108.

Fiske, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Foret, M. and Ahlers, J. (1982) Interconversion and uptake of nucleotides, nucleosides, and purine bases by the marine bacterium MB 22. J. Bacteriol. 150, 471-482.

Frankis, R. and Mascarenhas, J.P. (1980) Messenger RNA in the ungerminated pollen grain: A direct demonstration of its presence. Ann. Bot. 45, 595-599.

Gilissen, L.J.W. (1977) The influence of relative humidity on the swelling of pollen grains in vitro. Planta 137, 299-301.

Gilissen L.J.W. (1978) Effects of X-rays on seed setting and pollen tube growth in self-incompatible Petunia. Incompat Newsletter 10, 15-19.

Grossman, A.; Bartlett, S. and Chua, N. (1980) Energy dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. Nature 285, 625-628.

Hallenback, P.C.; Meyer, C.M. and Vignais, P.M. (1982) Regulation of nitrogenase in the photosynthetic bacterium Rhodopseudomonas capsulata as studied by two-dimensional gel electrophoresis. J Bacteriol 151, 1612-1616.

Harber, E. and Muller, W. (1962) On the inhibition of RNA synthesis by actinomycin. Biochem. Biophys. Res. Commun. 7, 107-110.

Harrington, H.M.; Berry, S.L. and Henke, R.R. (1981) Amino acid transport into cultured tobacco cells. II. Effect of calcium. Plant Physiol. 67, 379-384.

Harrington, H.M. and Henke, R.R. (1981) Amino acid transport into cultured tobacco cells. I. Lysine transport. Plant Physiol. 67, 373-381.

Harrington, H.M. and Smith, I.K. (1977) Cysteine transport into cultured tobacco cells. Plant Physiol. 60, 807-811.

Herrero, M. and Dickinson, H.G. (1979) Pollen-pistil incompatibility in Petunia hybrida: changes in the pistil following compatible and incompatible intraspecific crosses. J. Cell. Sci. 36, 1-18.

Heslop-Harrison, J. (1975) The physiology of the pollen grain surface. Proc. Roy. Soc. London B. 190, 275-299.

Heslop-Harrison, J. (1983) Self-incompatibility: phenomenology and physiology. Proc Roy Soc London B 218, 371-395.

Heslop-Harrison, J; Knox, R.B. and Heslop-Harrison, Y. (1974) Pollen wall proteins: Exine-held fractions associated with the incompatibility response in the Cruciferae. Theor. Appl. Genet. 44, 133-137.

Heslop-Harrison J.; Knox, R.B.; Heslop-Harrison, Y. and Mattson, O. (1975) Pollen-wall proteins: emission and role in incompatibility responses. In: The biology of male gamete, eds. Duckett, S.G. and Racey, P.A. (Academic Press), pp., 188-202.

Hetherington, A. and Trewavas, A. (1982) Calcium-dependent protein kinase in pea shoot membranes. FEBS Lett. 145, 67-71.

Heytler, P.G. (1979) Uncouplers of oxidative phosphorylation. Methods Enzymol. 55, 462-472.

Honig, G.R. and Rabinovitz, M. (1965) Actinomycin D: Inhibition of protein synthesis unrelated to effect of template RNA synthesis. Science 149, 1504-1505.

Howlett, B.J.; Knox, R.B.; Paxton, J.D. and Heslop-Harrison, J. (1975) Pollen wall proteins: physicochemical characterization and role in self-incompatibility in Cosmos bipinnatus. Proc. Roy. Soc. London B 188, 167-182.

Iborra, F. and Buchler, J.M. (1976) Protein subunit mapping. A sensitive high resolution method. Anal Biochem 74, 503-511.

Irving, G.C.J. and Cosgrove, D.J. (1970) Interference by myoinositol hexaphosphate in inorganic orthophosphate determinations. Anal. Biochem. 36, 381-388.

Jackson, J.F. and Linskens, H.F. (1978) Evidence for DNA repair after ultra-violet irradiation of Petunia hybrida pollen. Mol. Gen. Genet. 161, 117-120.

Jackson, J.F. and Linskens, H.F. (1979) Pollen DNA repair after treatment with the mutagens 4-nitroquinoline-1-oxide, ultraviolet and near-ultraviolet irradiation and boron dependence of repair. Mol. Gen. Genet. 176, 11-16.

Jackson, J.F. and Linskens, H.F. (1980) DNA repair in pollen: range of mutagens inducing repair, effect of replication inhibitors and changes in thymidine nucleotide metabolism during repair. Mol. Gen. Genet. 180, 517-522.

Jackson, J.F. and Linskens. H.F. (1982) Metal ion induced unscheduled DNA synthesis in Petunia pollen. Mol. Gen. Genet. 187, 112-115.

Jackson, J.F. and Linskens, H.F. (1982a) Phytic acid in Petunia hybrida pollen is hydrolysed during germination by a phytase. Acta Bot. Neerl. 31, 441-447.

Jackson, J.F. and Linskens, H.F. (1982b) Conifer pollen contains phytate and could be a major source of phytate phosphorous in forest soils. Aust. For. Res. 12, 11-18.

Jackson, J.F.; Jones, G. and Linskens, H.F. (1982) Phytic acid in pollen. Phytochem. 21, 1255-1258.

Jackson, J.F.; Kamboj, R.K. and Linskens, H.F. (1983) Localization of phytic acid in the floral structure of Petunia hybrida and relation to the incompatibility genes. Theor Appl Genet 64, 259-262.

Jeanjean, R. (1976) The effect of metabolic poisons on ATP level and on active phosphate uptake in Chlorella pyrenoidosa. Physiol. Plant. 37, 107-110.

Jendrisak, J. (1980) The use of α-amanitin to inhibit in vivo RNA synthesis and germination in wheat embryos. J. Biol. Chem. 255, 8529-8533.

Jennings, A.C. and Morton, R.H. (1963) Changes in nucleic acids and other phosphorous-containing compounds of developing wheat grains. Aust. J. Biol. Sci. 16, 332-341.

Jones, K.C. (1969) Similarities between gibberellins and related compounds in inducing acid phosphatase and reducing sugar release from barley endosperm. Plant Physiol. 44, 1695-1700.

Kamboj, R.K. and Jackson, J.F. (1984) Divergent transport mechanisms for pyrimidone nucleosides in Petunia pollen. Plant Physiol. 75, 499-501.

Kamboj, R.K.; Linskens, H.F. and Jackson, J.F. (1984) Energy-driven protein release from germinating pollen of Petunia hybrida. Ann. Bot. 54, 647-652.

Kamboj, R.K. and Jackson, J.F. (1985) Pyrimidine nucleoside uptake by Petunia pollen. Specificity and inhibitor studies on the carrier-mediated transport. Plant Physiol. 79, 801-805.

Kern, H.F.' Bieger, W.; Vokl, A.; Rohr, G. and Adler, G. (1979) Regulation of intracellular transport of exportable proteins in the rat exocrine pancreas. Symposia Soc. Exptl. Bot. 33, 79-99.

King, J. (1976) Uptake by soybean root cells of {^{14}C} alanine over a wide concentration range. Can. J. Bot. 54, 1316-1321.

King, J. and Hirji, R. (1975) Amino acid transport systems of cultured soybean root cells. Can. J. Bot. 53, 2088-2091.

Kirby, E.G. and Vasil, I.K. (1979) Effect of pollen protein diffusates on germination of eluted pollen samples of Petunia hybrida in vitro. Ann. Bot. 44, 361-367.

Kombrink, E. and Beevers, H. (1983) Transport of purine and pyrimidine bases and nucleosides from endosperm to cotyledons in germinating castor bean seedlings. Plant Physiol. 73 370-376.

Konar, R.N. and Linskens, H.F. (1966a) The morphology and anatomy of the stigma of Petunia hybrida. Planta 71, 356-371.

Konar, R.N. and Linskens, H.F. (1966b) Physiology and biochemistry of the stigmatic fluid of Petunia hybrida. Planta 71, 372-387.

Kornberg, A. (1980) DNA Replication, W.H.Freeman & Co. San Francisco.

Kroh, M. (1973) Nature of the intercellular substances of stylar transmitting tissue. In: Biogenesis of plant cell wall polysaccharides, ed. Leowus, F. (Academic Press, New York), pp., 195-205.

Kroh, M. and Bakel, C.H.J. van (1973) Incorporation of label into the intercellular substance of stylar transmitting tissue from Petunia pistil labelled with tritiated myo-inositol. An electromicroscopic autoradiographic study. Acta Bot. Neerl. 22, 106-111.

Kroh, M. and Helsper, J.P.F.G. (1974) Transmitting tissue and pollen tube growth. In: Fertilization in higher plants ed. Linskens, H.F. (North-Holland, Amsterdam), pp., 167-179.

Kroh, M and Knuiman, B. (1982) Ultrastructure of cell wall and plugs of tobacco pollen tubes after chemical extraction of polysaccharides. Plant 154, 241-250.

Kroh, M. and Loewus, M.W. (1978) Pectolytic enzyme activity from Nicotiana tabacum pollen. Phytochem. 17, 979-799.

Laemmli, W.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laemmli, W.K. and Favre, M. (1973) Maturation of the head of bacteriophage T4. J. Mol. Biol. 80, 575-599.

Lafleur, G.J.; Gross, A.E. and Mascarenhas, J.P. (1981) Optimisation of culture conditions for the formation of sperm cells in pollen tubes of Tradescantia. Gamete Res. 4, 35-40.

Laszlo, J.; Miller, D.S.; McCarty, K.S. and Hochstein,P. (1966) Actinomycin D: Inhibition of respiration and glycolysis. Science 151, 1007-1009.

Leigh-Brown, A.J. and Langley, C.H. (1979) Reevaluation of level of genic heterozygosity in natural population of Drosophila melanogaster by two dimensional electrophoresis. Proc. Natl. Acad. Sci. (U.S.A.) 76, 2381-2384.

Lein, R. and Rognes, S.E. (1977) Uptake of amino acids by barley leaf slices: Kinetics, specificity and Energetics. Physiol. Plant. 41, 175-183.

Lewis, D. (1952) Serological reactions of pollen incompatibility substances. Proc Roy Soc Lond B 140, 127-135.

Lewis, D. (1965) A protein dimer hypothesis on incompatibility. Genet Today 3, 657-663.

Linnett, P.E. and Beechey, R.G. (1979) Inhibitors of the ATP synthetase system. Methods Enzymol. 55, 472-518.

Linskens, H.F. (1960) Zur frage der entstehung der Abwehr-korper bei der inkompatilitatsreaktion von Petunia III. Mitteilungser-ologische teste mit leitgewebs - und pollen-extrakten. Zeit. fur Bot. 48, 126-135.

Linskens, H.F. (1968) Egg-sperm interaction in higher plants. Acad. Naz. Dei. Lincei Guaderno 104, 47-56.

Linskens, H.F. (1974) Translocation phenomena in the Petunia flower after cross- and self-pollination. In: Fertilization in higher plants. ed. Linskens, H.F., (North-Holland Publ. Co. Amsterdam) pp., 285-292.

Linskens, H.F. (1975) The physiological basis of incompatibility in angiosperms. In: The biology of male gamete, eds. Duckett, J.G. and Racey, P.A. (Academic Press), pp., 143-152.

Linskens, H.F. (1975a) Incompatibility in Petunia. Proc. Roy. Soc. London B 188, 299-311.

Linskens, H.F. and Kroh, M. (1970) Regulation of pollen tube growth. Curr. Top. Dev. Biol. 5, 89-113.

Linskens, H.F. and Schrauwen, J (1969) The release of free amino acids from germinating pollen. Acta Bot. Neerl. 18, 605-614.

Linskens, H.F.; Schrauwen, J.A.M. and Konings, R.N.H. (1970) Cell-free protein synthesis with polysomes from germinating Petunia pollen grains. Planta 90, 153-162.

Linskens, H.F.; van der Donk, J.A.M. and Schrauwen, J. (1971) RNA synthesis during pollen germination. Planta 97, 290-298.

Loewus, F.A.; Loewus, M.W.; Maite, I.B. and Rosenfield, C. (1978) Aspects of myo-inositol metabolism and biosynthesis in higher plants. In: Cyclitols and phosphoinositides, eds. Wells, W.W.; Eisenberg, F., (Academic Press). pp. 249-267.

Loewus, M.W. and Loewus, F.A. (1980) The C-5 hydrogen isotope-effect in myo-inositol 1-phosphate synthase as evidence for the myo-inositol oxidation pathway. Carbohydr. Res. 82, 333-342.

Losson, R.; Jund, R. and Chevallier, M.R. (1978) Properties of three distinct pyrimidine transport systems in Yeast. Biochem Biophys Acta 513, 296-300.

Lundin, A. and Thore, A. (1975) Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. 30, 713-721.

Makinen, Y. and Brewbaker, J.L. (1967) Isoenzyme polymorphism in flowering plants. I. Diffusion of enzymes out of intact pollen grains. Physiol. Plant. 20, 477-482.

Marme, D. (1982) In: Plant Growth Substances, ed. P.F.Wareing (Academic Press, New York), pp, 419-426.

Mascarenhas, J.P. (1966) Pollen tube growth and ribonucleic acid synthesis by vegetative and generative nuclei of Tradescantia. Am. J. Bot. 53, 563-569.

Mascarenhas, J.P. (1971) RNA and protein synthesis during pollen development and tube growth. In: Pollen Development and Physiology, ed. J. Heslop-Harrison (Butterworths, London), pp, 201-222.

Mascarenhas, J.P. (1975) The biochemistry of angiosperm pollen development. Bot Rev 41, 259-314.

Mascarenhas, J.P. and Bell, E. (1969) Protein synthesis during germiation of pollen: Studies on polyribosome formation. Biochim. Biophys. Acta 179, 199-203.

Mascarenhas J.P. and Bell, E. (1970) RNA synthesis during development of the male gametophyte of Tradescantia. Dev Biol 21, 475-490.

Mascarenhas, J.P. and Mermelstein, J. (1981) Messenger RNAs: their utilization and degradation during pollen germination and tube growth. Acta Soc. Bot. Pol. 50, 13-20.

Mascarenhas, J.P.; Terenna, B.; Mascarenhas, A.F. and Rueckert, L. (1974) Protein synthesis during germination and pollen tube growth in Tradescantia. In: Fertilization in higher plants, ed. Linskens, H.F. (North-Holland, Amsterdam) pp, 137-143.

Mascarenhas, N.T.; Bashe, D.; Eisenberg, A.; Willing, R.P.; Xiao, C.M. and Mascarenhas, J.P. (1984) Messenger RNA in corn pollen and protein synthesis during germination and pollen tube growth. Theor. Appl. Genet. 68, 323-326.

Matousek, J. and Tupy, J. (1983) The release of nucleases from tobacco pollen. Plant Sci. Lett. 30, 83-89.

Mau, S.L.; Raff, J and Clarke, A.E. (1982) Isolation and partial characterization of components of Prunus avium L styles, including an antigenic glycoprotein associated with a self-incompatibility genotype. Planta 156, 506-516.

McDaniel, C.N.; Holterman, R.K.; Bone, R.F. and Woznaik, P.M. (1982) Amino acid transport in suspension-cultured plant cells III. Common carrier system for the uptake of L-Arg, L-Asp, L-His, L-Leu and L-Phe. Plant Physiol. 69, 246-249.

McMohan, D. (1975) Cycloheximide is not a specific inhibitor of protein syntehsis in vitro. Plant Physiol. 55, 815-821.

Munch-Petersen, A.; Mygind, B.; Nicolaisen, A. and Phil, N.J. (1979) Nucleoside transport in cells and membrane vesicles from Escherichia coli. K 12. J Biol Chem 254, 3730-3737.

Munch-Petersen, A. and Phil, N.J. (1980) Nucleoside transport in cells and membrane vesicles from E. Coli K 12. Proc. Natl. Acad. Sci. (U.S.A.) 77, 2519-2523.

Mygind B. and Munch-Petersen, A. (1975) Transport of pyrimidine nucleosides in cells of E. Coli. K 12. Eur. J. Biochem. 59, 365-372.

Nelson, N. and Schatz, G. (1970) Energy dependent processing of cytoplasmically made precursors to mitochondrial proteins. Proc. Nat. Acad. Sci. (U.S.A.) 76, 4365-4369.

Nelson, S.O.; Glover, G.I. and Magill, C.W. (1975) The essentiality of sulfhydryl groups to transport in Neurospora crassa. Arch. Biochem. Biophys. 168, 483-489.

Neumann, J. (1964)The effect of actinomycin D on lettuce seedlings and its differential uptake by roots and shoots. Physiol. Plant. 17, 363-370.

Nissen, P. (1974) Uptake mechanisms: Inorganic and organic. Ann. Rev. Plant Physiol. 25, 53-79.

O'Farrell, P.H. (1975) High resolution two dimensional electrophroesis of proteins. J Biol Chem 250, 4007-4021.

O'Farrel, P.Z.; Goodman, H.M. and O'Farrel, P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12, 1133-1142.

Ong, B.L. and Jackson J.F. (1972) Pyrimidine nucleotide biosynthesis in Phaseolus aureus. Enzymic aspects of control of carbamoyl phosphate synthesis and utilization. Biochem J 129, 583-593.

Pacini, E.; Franchi, G.G. and Sarfatti, G. (1981) On the widespread occurrence of polar saprophytic proteins in pollen of dicotyledons. Ann. Bot. 47, 405-408.

Paddubnaya-Arnoldi, V.A.; Tsinger, N.V.; Petrovskaya, T.P. and Polunina, N.N. (1959) Histochemical study of the pollen grains and the pollen tubes in the angiosperms. Recent Adv. Bot. 1, 682-685.

Page, C.R. and Macinnis, A.J. (1975) Characterization of nucleoside transport in Hymenolepidid cestodes. Parasitology 61, 281-290.

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.L. (1970) Amino acid transport in Neurospora crassa. II. Properties of two amino acid transport systems. Biochim. Biophys. Acta 203, 139-149.

Parker, N.F. and Jackson, J.F. (1981) Control of pyrimidine biosynthesis in synchronously dividing cells of Helianthus tuberosus. Plant Physiol 67, 363-366.

Payne, P.I. (1976) The long-lived messenger RNA of flowering plant seeds. Biol. Rev. 51, 329-363.

Picton, J.M. and Steer, M.W. (1983) Membrane recycling and the control of secretory activity in pollen tubes. J. Cell. Sci. 63, 303-310.

Plagemann, P.G.W. and Richey, D.P. (1974) Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture. Biochim Biophys Acta 344, 263-305.

Plageman, P.G.W. and Roth, H.F. (1969) Permeation as a rate-limiting step in phosphorylation of uridine and choline and their incorporation into macromolecules by Novikoff hepatoma cells. Competitive inhibition of phenetylalcohol, persantin and adenosine. Biochemistry 8, 4789-4789.

Plagemann, P.G.W. and Wohlheuter, R.M. (1980) Permeation of nucleosides, nucleic acid bases, and nucleotides in animal cells. Curr. Top Membr. Transp. 14, 225-330.

Plagemann, P.G.W. and Wohlheuter, R.M. (1984) Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzythioinosine or hypoxanthine. Biochim Biophys Acta 773, 39-52.

Polya, G.M. and Davies, J.R. (1982) Resolution of Ca^{2+} calmodulin activated protein kinase from wheat germ. FEBS Lett. 150, 167-171.

Polya, G.M.; Davies, J.R. and Micucci, V. (1983) Properties of a calmodulin-activated Ca^{2+}-dependent protein kinase from wheat germ. Biochim. Biophys. Acta 761, 1-12.

Polya, G.M. and Micucci, V. (1984) Partial purification and characterization of a second calmodulin-activated Ca^{2+} -dependent protein kinase from wheat germ. Biochim. Biophys. Acta 785, 68-74.

Reinhold, L. and Kaplan, A. (1984) Membrane transport of sugars and amino acids. Ann. Rev. Plant Physiol. 35, 45-83.

Reisfeld, R.A.; Lewis, W.J. and Williams, D.E. (1962) Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195, 281-283.

Rottenberg, H (1979) The measurement of membrane potential and Δ pH in cells, organelles and vesicles. Methods Enzymol 55, 547-568.

Sassen, M.M.A. (1974) The stylar transmitting tissue. Acta Bot. Neerl. 23, 99-108.

Schlesinger, M.J.; Ashburner, M. and Tissieres, A. (1982) Heat shock, from bacteria to man. Cold Spring Harbor Laboratory, New York.

Schlosser, K. (1961) Cytolegische und cytochemische untersuchunger uber das. Pollenschlauchwachstum selbststeriler Petunia. Z. Bot. 49, 266-288.

Segrest, J.P. and Jackson, R.L. (1972) Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Methods Enzymol 28, 54-63.

Shivanna, K.R.; Jaiswal, V.S. and Mohan Ram, H.V. (1974) Inhibition of gamete formation by cycloheximide in pollen tubes of Impatiens balsamina. Planta 117, 173-177.

Shtarkshall, R.A. and Reinhold, L. (1974) Multiphasic amino acid transport in leaf cells. In Membrane Transport in Plants, eds. U. Zimmerman, J. Dainty, (Springer-Verlag.) pp. 338-342.

Sink, K.C. (1984) Anatomy and morphology. In: Petunia, ed. Sink, K.C. (Springer-Verlag) pp., 10-19.

Smith, I.K. (1978) Role of calcium in serine transport into tobacco cells. Plant Physiol. 63, 941-948.

Soldal, T. and Nissen, P. (1978) Multiphasic uptake of amino acids by barley roots. Physiol. Plant. 43, 181-188.

Stanley, P.E. and Williams, S.G. (1969) Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29, 381-392.

Stanley, R.G. and Linskens, H.F. (1964) Enzyme activation in germinating Petunia pollen. Nature 203, 542-544.

Stanley, R.G. and Linskens, H.F. (1965) Protein diffusing from germinating pollen. Physiol. Plant 18, 47-53.

Stanley R.G. and Linskens, H.F. (1974) Pollen-Biology, Biochemistry, Management. Springer-Verlag, Berlin.

Steffensen, D.M. (1971) Ribosome synthesis compared during pollen and pollen tube development. In: Pollen Development and Physiology, ed. J. Heslop-Harrison (Butterworths, London), pp, 223-229.

Suss, J. and Tupy, J. (1979) Poly(A) RNA synthesis in germinating pollen of Nicotiana tabacium L. Biol. Plant. 21, 365-371.

Suss, J. and Tupy, J. (1982) Kinetics of uridine uptake and incorporation into RNA in tobacco pollen culture. Biol. Plant 24, 72-79.

Takeuchi, Y. and Kishimoto, U. (1983) Changes of adenine nucleotide levels in Chara internodes during netabolic inhibition. Plant and Cell Physiol. 15, 103-110.

Tanaka, K.; Yoshida, T.; Asada, K. and Kasai, Z. (1973) Subcellular particles isolated from aleurone layer of rice seeds. Arch. Biochem. Biophys. 155, 136-143.

Tanaka, K.; Yoshida, T. and Kasai, Z. (1974) Radioautographic demonstration of the accumulation site of phytic acid in rice and wheat grains. Plant Cell Physiol. 15, 147-151.

Tanguay, R.M. (1983) Genetic regulation during heat shock and function of heat shock proteins: a review. Can. J. Biochem. Cell Biol. 61, 387-394.

Tanksley, S.D.; Zamir, D. and Rick, C.M. (1981) Evidence for extensive overlap of sporophytic and gametophytic gene expression in Lycopersicon esculentum. Science 213, 453-455.

Tate, M.E. (1968) Separation of myoinositol pentaphosphates by moving paper electrophoresis. Anal. Biochem. 23, 141-149.

Tate, M.E. (1981) Determination of ionization constants by paper electrophoresis. Biochem. J. 195, 419-426.

Tupy, J. (1977) RNA synthesis and polysome formation in pollen tubes. Biol. Plant. 19, 300-308.

Tupy, J. (1982) Alterations in polyadenylated RNA during pollen maturation and germination. Biol. Plant. 24, 331-340.

Tupy, J.; Hrabetova, E. and Capkova, E. (1983) Amino acids and bivalent cations in the growth of tobacco pollen in mass culture. Plant Sci. Lett. 30, 91-98.

Van der Donk, J.A.W.M. (1974) Synthesis of RNA and protein as a function of time and type of pollen tube-style interaction in Petunia hybrida. Mol. Gen. Genet. 134, 93-98.

Van der Pluijm, J. and Linskens, H.F. (1966) Feinstruktur der Pellenschlauche in Griffel von Petunia. Zuechter 36, 220-224.

Vasil, I.K. (1974) The histology and physiology of pollen germination and pollen tube growth on the stigma and in the style. In: Fertilization in higher plants, ed. Linskens, H.F. (North-Holland), pp. 105-118.

Veluthambi, K. and Poovaiah, B.W. (1984) Calcium-promoted protein phosphorylation in plants. Science 223, 167-169.

Walker-Smith, D.J. and Payne, J.W. (1983) Peptide uptake in germinating barley embryos involves a dithiol dependent transport protein. FEBS Lett. 160, 25-30.

Wasternack, C.H. (1976) Uptake and incorporation of pyrimidines in Euglena gracilis. Arch. Microbiol. 109, 167-169.

Willing, R.P. and Mascarenhas, J.P. (1984) Analysis of the complexity and diversity of mRNAs from pollen and shoots of Tradescantia. Plant Physiol. 75, 865-868.

Wohlheuter, R.M.; Marz, R. and Plagemann, P.G.W. (1979) Thymidine transport in cultured mammalian cells. Biochim Biophys Acta. 553, 262-283.

Wu, J-S.R.; Kurong, F.Y.P.; Jarvis, S.M. and Young, J.D. (1983) Identification of the erythrocyte nucleoside transporter as a band 4.5 polypeptide. J Biol Chem 258, 13745-13751.

Young, J.D. and Jarvis, S.M. (1983) Nucleoside transport in animal cells. Biosci Rep 3, 309-322.

Zhang, H.G.; Croes, A.F. and Linskens, H.F. (1982) Protein synthesis in germinating pollen of Petunia: role of proline. Planta 154, 199-203.