
0527804

354 K

D52780/84

TRAYNOR, J. D.

Double pages.
Over-lay

354

CITY OF LONDON Poly.

RESISTANCE TO PROTEIN SYNTHESIS INHIBITORS IN *COPRINUS CINEREUS*

Presented in partial fulfilment by

JOHN DAVID TRAYNOR

for the degree of

Doctor of Philosophy

to the

Council for National Academic Awards.

September, 1983.

City of London Polytechnic, Old Castle St., London E1 7NT.

DECLARATION.

I, John Traynor, declare that while registered as a full-time candidate for the degree of Doctor of Philosophy, at the City of London Polytechnic between 1977 and 1980, I was not registered for any other award. The work undertaken during that period was carried out by myself, with guidance from Dr. J. North at the City of London Polytechnic and Dr. A. Bollen at the Université Libre de Bruxelles.

Several advanced studies were undertaken in conjunction with my research programme in partial fulfilment of the degree of Doctor of Philosophy. These studies at the City of London Polytechnic included; a postgraduate course introducing analytical and photographic techniques, and a one week course on Radioactivity. I also presented several research seminars to the academic staff and was involved in practical demonstrations given to undergraduate students of which one demonstration was an application of a biochemical technique, designed by myself.

I attended several meetings of the Genetical and Biochemical Societies and presented a short communication at the former. I also attended a course sponsored by the European Molecular Biology Organisation at the Université Libre de Bruxelles which dealt with the theory and practical techniques of eukaryotic gene expression research.

J. D. Traynor September, 1983.

ABSTRACT.

Cycloheximide, a potent eukaryotic protein synthesis inhibitor (Sisler and Siegel,1967), was used in a biochemical and genetical investigation of the basidiomycete fungus, Coprinus cinereus .

An optimised polyuridylic acid dependant cell-free polyphenylalanine synthesising system was developed for Coprinus cinereus, in order to identify the cellular component conferring cycloheximide-resistance in two cycloheximide-resistant mutant strains, CY 8.2 and CY9.23. In both of these strains, resistance to cycloheximide was found to be associated with the cytoplasmic ribosome fraction. It was not possible to identify the particular cytoplasmic ribosomal subunit which conferred cycloheximide-resistance.

Analysis of cytoplasmic ribosomal proteins by two-dimensional gel electrophoresis did not reveal any difference between the small subunit proteins of CY 8 and CY 8.2. There were a considerable number of differences between the proteins extracted from the large subunit of CY 8 and CY 8.2, and CY 9 and CY9.23. There was no conclusive evidence to identify a cytoplasmic ribosomal protein associated with cycloheximide resistance although several candidates were proposed. An analysis using carboxymethyl-cellulose chromotography did not identify any cytoplasmic ribosomal proteins conferring cycloheximide resistance.

CY 8.2 was one of 174 cycloheximide-resistant mutant strains produced by the ultraviolet mutagenesis of cycloheximide-sensitive strains, according to a method modified from North (1982). Cycloheximide-resistance in each mutant strain was considered to be conferred by a single gene, which in those strains examined, was recessive in heterozygous cycloheximide-resistant dikaryons and diploids . The cycloheximide-resistance mutation in all strains examined belonged to the cy-2 complementation group (North,1982) and hence were allelic with the resistance gene carried by CY 9.23. A classification of the cycloheximide-resistant mutants was proposed on the basis of their growth responses to cycloheximide.

Dedicated to Nora and Peter Traynor.

ACKNOWLEDGEMENTS.

I would like to take this opportunity to express my gratitude to all of the people who helped me to realise my ambition. I would particularly like to thank:

Dr. J. North for her supervision, encouragement, editorial skills and above all, patience.

Dr. L. Casselton and Dr.P. Williams for their criticism.

Dr. A. Bollen and Dr. R. Herzog for their hospitality and assistance whilst at the Université Libre de Bruxelles.

The City of London Polytechnic, the Inner London Education Authority and the European Molecular Biology Organisation for their financial support.

The Libraries of the University of Manchester and Kings College London for their co-operation.

Judy and Sheila Bowker for their commendable typing.

The late Alex Campbell for his photographic expertise.

Maxine for her enthusiastic help, and
finally, to all my friends at the City of London Polytechnic, especially Pat McCarthy and the diligent workers in 006, who helped me to keep my sanity.

CONTENTS.

Section	Contents	Page
	Title page.	i
	Declaration.	ii
	Abstract.	iii
	Dedication.	iv
	Acknowledgements.	v
	Contents.	vi
	Abbreviations.	xiv
CHAPTER 1	INTRODUCTION.	1
1.1	Prologue.	2
1.2	*Coprinus cinereus.*	
	a) Identification.	3
	b) Life-history.	3
	c) The choice of *Coprinus cinereus*: nuclear interaction.	5
1.3	Cycloheximide.	
	a) Choice of cycloheximide-resistant mutants.	8
	b) Structure and biological activity.	8
	c) Mechanism of action: inhibitor of protein synthesis.	10
1.4	Cytoplasmic ribosomes.	
	a) Location.	12
	b) Physical properties.	12
	c) Cytoplasmic ribosomal genes.	15
	d) Cytoplasmic ribosomal synthesis.	15
	e) Structure and function.	16
1.5	Objectives.	18
CHAPTER 2	MATERIALS AND METHODS.	19
2.1	Materials.	20
2.2	Strains.	21
2.3	Culture of *Coprinus cinereus*.	
	a) Culture medium.	22
	b) Culture conditions.	22
	c) Stock maintenance.	23

Section	Contents	Page
2.4	Ultraviolet mutagenesis and selection of cycloheximide-resistant mutants.	24
	a) Nomenclature.	24
2.5	Genetic analysis of cycloheximide-resistant mutants.	
	a) Growth test.	25
	b) Determination of the number of genes conferring cycloheximide-resistance in a strain.	26
	c) Dominance test.	27
	d) Complementation test.	28
	Biochemical analysis.	
2.6	Buffers.	29
2.7	Preparation of Coprinus cinereus cell-extracts.	30
2.8	Preparation of cytoplasmic ribosomal subunits and analysis of cell-extracts.	34
2.9	Cell-free polyphenylalanine synthesis.	38
	a) Response of cell-extracts to cycloheximide.	40
2.10	Preparation of cytoplasmic ribosomal proteins.	41
2.11	Two-dimensional polyacrylamide gel electrophoresis.	42
2.12	Carboxymethyl-cellulose chromotography.	44
CHAPTER 3	PRODUCTION AND GENETIC ANALYSIS OF CYCLOHEXIMIDE-RESISTANT MUTANTS IN COPRINUS CINEREUS.	46
	INTRODUCTION.	
3.1	Genetics of Coprinus cinereus.	47
	a) Cycloheximide resistance.	47
	b) Nuclear interaction and gene expression.	
	i) Expression of cycloheximide resistance.	50
	ii)Expression of other genes.	50
3.2	Genetics of the cycloheximide response.	52
	a) Dominance.	54
	b) Modifier genes.	55
	c) Suppressor genes.	56
	d) Number and location of genes.	57

Section	Contents	Page
3.3	Pleiotrophic effect of cycloheximide resistant genes;	
	a) Cross-resistance with other protein synthesis inhibitors.	58
	b) Temperature sensitivity.	58
3.4	Objectives.	60
	RESULTS.	
3.5	Production of cycloheximide-resistant mutants.	
	a) Spontaneous mutation frequency.	61
	b) Ultraviolet radiation induced mutation frequency.	61
3.6	Number of loci conferring cycloheximide-resistance in each strain.	65
	Growth response of strains to cycloheximide:	
3.7	General observations.	
	a) Lag period.	67
	b) Constant growth rate.	67
	c) Morphology.	68
	d) Irreversible effect on growth.	68
	e) Typical response to cycloheximide.	68
	f) Definition of cycloheximide-resistance.	69
3.8	Growth response of monokaryotic strains to cycloheximide.	
	Cycloheximide-resistant mutant strains derived from:	
	a) CY3.	70
	b) CY6.	74
	c) CY8.	79
	d) CY9.	79
	e) Other strains.	85
3.9	Growth response of dikaryons to cycloheximide: Dominance test.	90
3.10	Growth response of diploids to cycloheximide.	94
3.11	Complementation test.	97

Section	Contents	Page
	DISCUSSION	
3.12	Induction of mutants at the cy-2 locus.	100
3.13	The effect of cycloheximide on the growth of Coprinus cinereus.	
	a) Measurement of growth.	103
	b) Characterisation of cycloheximide dose-growth response.	103
	c) Classification.	104
	d) Interpretation.	106
3.14	Nuclear interaction and expression of cycloheximide resistance.	108
3.15	Summary.	110
CHAPTER 4	DEVELOPMENT OF A COPRINUS CINEREUS CELL-FREE POLYPHENYLALANINE SYNTHESISING SYSTEM.	111
	INTRODUCTION.	
4.1	Cytoplasmic protein synthesis.	112
	a) Amino acid activation.	115
	b) Initiation.	115
	c) Elongation.	116
	i) Binding of aminoacyl-tRNA.	116
	ii) Peptide bond formation.	116
	iii) Translocation.	117
	d) Termination.	117
4.2	Translation of polyuridylic acid.	119
4.3	Objectives.	121
	RESULTS.	
4.4	Optimisation of the individual constituents of the Coprinus cinereus polyphenylalanine synthesising system.	122
4.5	Coprinus cinereus cell-extracts.	125
	a) Cytoplasmic ribosome fraction, RP-100.	125
	b) Cytoplasmic ribosome-free supernatant fraction, S-100.	131
	c) Relationship between RP-100 and S-100.	135
	d) Effect of preparation.	139
	e) Effect of storage.	140
	The effect on polyphenylalanine synthesis of:	
4.6	Polyuridylic acid, and transfer RNA.	143
4.7	Adenosine 5-triphosphate, guanosine 5-triphosphate, creatine phosphate and creatine phosphokinase.	146

Section	Contents	Page
4.8	Magnesium acetate and spermidine.	149
4.9	Monovalent cations: ammonium acetate and potassium chloride .	152
4.10	Tris-HCl and dithiothreitol .	155
4.11	L-(U-C^{14}) phenylalanine .	158
4.12	Temperature and reaction time.	162
	DISCUSSION.	
4.13	Preoptimised and optimised reaction mixtures.	165
4.14	Efficiency of polyphenylalanine synthesis in Coprinus cinereus and other species.	171
4.15	Summary.	174
CHAPTER 5.	RESPONSE OF CELL-EXTRACTS TO CYCLOHEXIMIDE.	175
	INTRODUCTION.	
5.1	Intracellular site of cycloheximide action.	176
5.2	Objectives.	180
	RESULTS.	
5.3	The effect of cycloheximide on monokaryotic cell-extracts.	
	a) Analysis of CY8 and CY8.2.	181
	i) Homologous cell-extracts.	182
	ii) Heterologous cell-extracts.	187
	b) Analysis of various other strains.	189
5.4	Localisation of the intracellular site of cycloheximide resistance.	195
5.5	The effect of RP-100 concentration on the cycloheximide dose-response.	197
5.6	The effect of cycloheximide on RP-100 fractions from dikaryons and diploids.	203
	a) Dikaryons : CY8xCY13, CY8.2xCY13, CY9xCY3, CY9.23xCY3.	203
	b) Diploids : CY9/CY14 and CY9.23/CY14.	207
	DISCUSSION.	
5.7	A screening procedure to identify cycloheximide-resistant cytoplasmic ribosomes.	209
5.8	Characterisation of the effect of cycloheximide on polyphenylalanine synthesis.	212
	a) Interpretation of the cycloheximide dose-in vitro response.	212
5.9	The effect of cycloheximide on monokaryons, dikaryons and diploids.	216
	a) Monokaryons.	216
	b) Dikaryons.	219
	c) Diploids.	221
5.10	Summary.	224

Section	Contents	Page
CHAPTER 6	ANALYSIS OFCYTOPLASMIC RIBOSOMAL PROTEINS.	225
	INTRODUCTION.	
6.1	Cytoplasmic ribosomal proteins associated with cycloheximide resistance.	226
6.2	Objectives.	230
	RESULTS.	
6.3	Characterisation of Coprinus cinereus small cytoplasmic ribosomal subunit proteins.	231
6.4	Comparison between CY8 and CY8.2 small cytoplasmic ribosomal subunit proteins.	234
6.5	Characterisation of Coprinus cinereus large cytoplasmic ribosomal subunit proteins.	236
6.6	Comparison of large cytoplasmic ribosomal subunit proteins from monokaryotic strains.	238
	a) CY8 and CY8.2.	242
	b) CY9 and CY9.23.	242
	c) CY8 and CY9 with CY8.2 and CY9.23.	244
6.7	Comparison of large cytoplasmic ribosomal subunit proteins from dikaryotic strains.	245
	a) CY8 x CY13 and CY8.2 x CY13.	245
	b) CY9 x CY3 and CY9.23x CY3.	250
	c) CY8 x CY13 and CY9 x CY3 with CY8.2x CY13 and CY9.23x CY3.	250
6.8	Analysis of cytoplasmic ribosomal proteins from the large subunit by carboxymethyl-cellulose chromatography.	252
	DISCUSSION.	
6.9	Interpretation of electropherograms and chromatograms.	255
	a) Contaminants.	255
	b) Artifacts.	256
	c) Detection.	257
6.10	Characterisation of Coprinus cinereus cytoplasmic ribosomal proteins.	259
	a) Cross-contamination.	259
	b) Losses.	260
	c) Comparison with other species.	261
6.11	Identification of cytoplasmic ribosomal proteins conferring cycloheximide resistance.	264
	a) Analysis of proteins from the small subunit.	264

Section	Contents	Page
	b) Analysis of proteins from the large subunit.	265
	i) Monokaryons.	266
	ii) Dikaryons.	269
6.12	Summary.	271
CHAPTER 7.	GENERAL DISCUSSION: MOLECULAR AND GENETIC BASIS OF CYCLOHEXIMIDE-RESISTANCE IN COPRINUS CINEREUS.	272
7.1	Conclusion from the genetic analysis of cycloheximide-resistant mutants.	273
7.2	Conclusions from the biochemical analysis of cycloheximide-resistant mutants.	274
7.3	Objectives.	275
7.4	Comparison of the effects of cycloheximide *in vivo* and *in vitro*.	276
7.5	The intracellular site of cycloheximide action.	282
7.6	Nucleo-cytoplasmic interaction and the expression of cycloheximide resistance.	286
	a) Dikaryons.	
	i) Heterozygous for the *cy-2* locus.	287
	ii) Heterozygous for *cy-2* and *modcy* loci.	290
	b) Diploids.	
	i) Heterozygous for the *cy-2* locus.	292
	ii) Heterozygous for *cy-2* and *modcy* loci.	292
7.7	Further development for the investigation.	295
	APPENDIX.	299
SECTION A	Effect of cycloheximide *in vivo*.	
	i) Determination of the growth response to cycloheximide by indirect comparison; specimen calculation.	300
	ii) Growth responses to cycloheximide of strains derived from:	
	CY3.	302
	CY6.	303
	CY8.	304
	CY9.	305
	CY9.23.	306
	Growth responses to cycloheximide of:	
	CY strains.	306
	Various other strains.	307
	Dikaryons derived from CY3 mutants.	308
	Dikaryons derived from CY8 mutants.	309
	Diploids.	309

Section	Contents	Page
SECTION B	In vitro polyphenylalanine synthesis.	
	i) A postmitochondrial supernatant - dependant polyphenylalanine synthesising system.	310
	ii) Estimation of sedimentation coefficients of Coprinus cinereus cytoplasmic ribosomal subunits: specimen calculation.	313
	iii) Fractionation of Coprinus cinereus cytoplasmic ribosomes.	316
	iv) Effect of variable RP-100 and S-100 concentrations on polyphenylalanine synthesis.	320
	v) Expression of cytoplasmic ribosome concentration.	
	a) A_{260}unit.	321
	b) mg cytoplasmic ribosomal protein.ml.$^{-1}$	321
	c) mg cytoplasmic ribosome.ml.$^{-1}$	322
	d) Molality.	323
SECTION C.	Effect of cycloheximide in vitro.	324
	Effect of cycloheximide on polyphenylalanine synthesis produced by RP-100 and S-100 fractions from CY8.2 and CY8.	325
	Effect of cycloheximide on polyphenylalanine synthesis using RP-100 fractions from various monokaryotic strains.	326
	Effect of RP-100 concentration on the response of CY9.23 to cycloheximide.	327
	Effect of cycloheximide on polyphenylalanine synthesis by RP-100 fractions from dikaryotic strains.	327
	BIBLIOGRAPHY.	328
	THE END.	339

ABBREVIATIONS.

Abbreviation	Name
ad	adenine requirement.
ATP	adenosine 5 -triphosphate.
A260	unit of absorbance at 260 nm.
A280	unit of absorbance at 280 nm.
Bisacrylamide	N,N' - methylene bisacrylamide.
Bistris	Bis (2-hydroxymethyl)-imino-tris (hydroxy-methyl) methane.
BSA	bovine serum albumin.
chol	choline requirement.
CMC	carboxymethyl cellulose.
cy^r	cycloheximide resistant.
D-(3-H^3)glucose	D-glucose, carbon 3 labelled with H^3.
D- (U-C^{14}) glucose	D-glucose,uniformlylabelled with carbon.^{14}
GTP	guanosine 5-triphosphate.
me	methionine requirement.
mod cy^+	dominance modifier of cycloheximide resistance.
nic	nicotinic acid requirement.
paba	p-aminobenzoic acid requirement.
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis.
phe	phenylalanine.
L-(U-C^{14})phe	phenylalalanine,uniformly labelled with carbon 14.
Poly (U)	polyuridylic acid.
mRNA	messenger ribonucleic acid.
rRNA	ribosomal ribonucleic acid.
$tRNA^{phe}$	transfer ribonucleic acid,phenylalanine specific.
TCA	trichloroacetic acid.
TEMED	N,N,N',N', - tetramethylene diamine.
Tris	Tris (hydroxymethyl)-amino methane.

ABBREVIATIONS.

Abbreviation	Name
ad	adenine requirement.
ATP	adenosine 5 -triphosphate.
A260	unit of absorbance at 260 nm.
A280	unit of absorbance at 280 nm.
Bisacrylamide	N,N' - methylene bisacrylamide.
Bistris	Bis (2-hydroxymethyl)-imino-tris (hydroxy-methyl) methane.
BSA	bovine serum albumin.
chol	choline requirement.
CMC	carboxymethyl cellulose.
cy^r	cycloheximide resistant.
D-(3-H^3)glucose	D-glucose, carbon 3 labelled with H^3.
D- (U-C^{14}) glucose	D-glucose,uniformlylabelled with carbon.^{14}
GTP	guanosine 5-triphosphate.
me	methionine requirement.
mod cy^+	dominance modifier of cycloheximide resistance.
nic	nicotinic acid requirement.
paba	p-aminobenzoic acid requirement.
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis.
phe	phenylalanine.
L-(U-C^{14})phe	phenylalalanine,uniformly labelled with carbon 14.
Poly (U)	polyuridylic acid.
mRNA	messenger ribonucleic acid.
rRNA	ribosomal ribonucleic acid.
$tRNA^{phe}$	transfer ribonucleic acid,phenylalanine specific.
TCA	trichloroacetic acid.
TEMED	N,N,N',N', - tetramethylene diamine.
Tris	Tris (hydroxymethyl)-amino methane.

CHAPTER 1.

INTRODUCTION.

SECTION 1.1. PROLOGUE.

This investigation was conceived as a consequence of a study undertaken by North (reported 1978). North had produced and carried out a genetic analysis on cycloheximide-resistant mutants in Coprimus cinereus. The genetic analysis had revealed that mutations in three genes conferred resistance to cycloheximide. Additionally a fourth mutation was identified, which did not itself confer cycloheximide resistance but which affected the expression of one specific gene conferring cycloheximide-resistance in dikaryons but not in the diploids.

SECTION 1.2. COPRINUS CINEREUS.

a) Identification.

The subject of this investigation was the species *Coprinus cinereus*(Schaeff ex. Fr, 1821) S.F Gray 1821, (North, 1982). *Coprinus cinereus* is the correct specific name for the dung-dwelling species which Buller incorrectly identified as *Coprimus lagopus*, (Orton, 1957). Both species have similar appearence but the habitat of *Coprimus lagopus* is woodland soils (Orton, 1957). Consequently in many early publications *Coprinus lagopus* has been referred to,when in fact the species used was *Coprimus cinereus* (eg. Casselton, 1965).

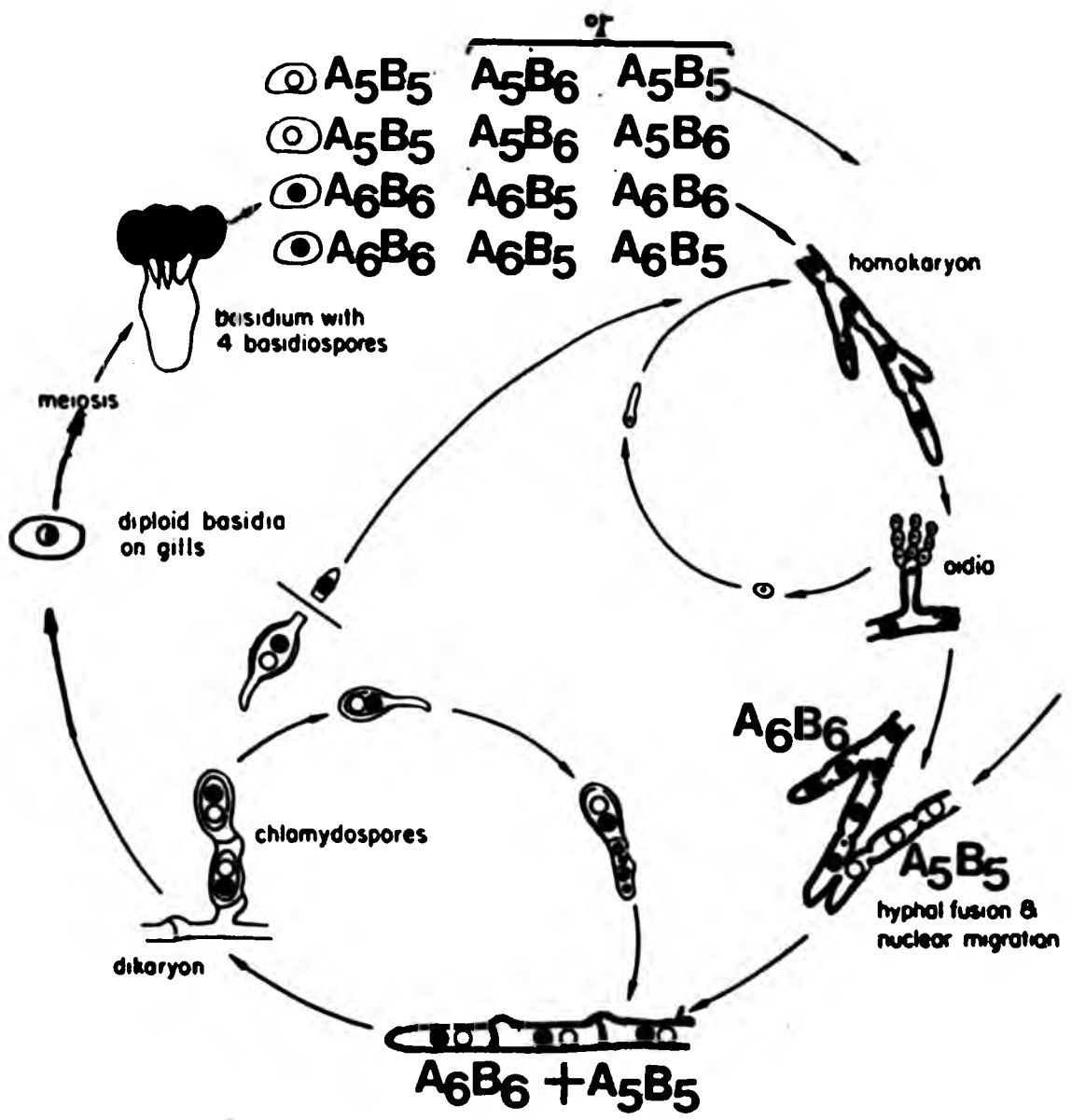
b) Life History.

The salient features of the *Coprinus cinereus* life-cycle are shown in Figure 1.1. For the majority of its life, *Coprinus cinereus* exists as a mass of white vegetative mycelium which can be distinguished as two cell-types; monokaroytic cells which possess a single haploid nucleus and dikaryotic cells which contain two haploid nuclei.

Dikaryons are produced when two monokaryons which possess compatible mating-type factors meet, fuse and transfer nuclei. The mating system in *Coprinus cinereus* is controlled by two unlinked loci, mating-type factors A and B, which control synchronous nuclear division and nuclear migration respectively. Dikaryons possess unlike alleles at the A and B mating-type loci (eg. A6 B6 and A5 B5 Figure 1.1).

If one or both alleles at the mating-type loci are identical the heterokaryons which are produced have variable nuclear contents and are unstable and infertile.

Figure 1.1. The life-cycle of Coprinus cinereus.



Based on a scheme from Fincham and Day (1971).

In advantageous environments, the monokaryons and dikaryons thrive, each capable of asexual reproduction by producing oidiospores and chlamydospores respectively (Figure 1.1).

The final phase which completes the life-cycle occurs when the individual haploid nuclei of the dikaryon fuse to produce a single diploid nucleus. The diploid cells are localised to the gills on the underside of the distinctive fruitbody which is characteristic of many basidiomycetes and of the Agaricacae in particular. Coprinus cinereus is a typical hymenomycete, possessing gills enclosed within a hymenical surface. The diploid cells undergo meiosis resulting in the production of four haploid basidiospores borne in tetrads on the basidium. When mature, the grey scaly cap dissolves by a process of autodigestion to release a black spore containing fluid. Germinated basidiospores originate monokaryotic colonies which begin the life-cycle anew.

c) The choice of Coprinus cinereus: nuclear interaction.

North's analysis (1982) of the genetic basis of cycloheximide-resistance, was investigated in Coprinus cinereus for several reasons. The species was conveniently cultured in the laboratory and was amenable to genetic analysis. It was an organism in which loci conferring cycloheximide-resistance had not previously been identified and therefore provided the opportunity for characterising the genetic map of Coprinus cinereus.

However, the primary reason for choosing Coprinus cinereus was that it was possible to investigate the effect of nuclear inter-

action on gene expression, in this particular instance, on the expression of genes conferring cycloheximide-resistance. The advantage which Coprinus cinereus afforded over other eukaryotic species was that gene complementation and dominance could be compared in the stable dikaryons and diploids, whereas in other species notably in Aspergillus nidulans (Pontecorvo 1952, 1963, and Apirion 1966) gene expression was compared between heterokaryon and diploid. In the dikaryotic cell of Coprinus cinereus, the nuclear content of the two different haploid nuclei is stable (Section 1.2 b ; Figure1.1), but in the heterokaryon the content of the different haploid nuclei is variable , often unknown, and irregularly distributed in the mycelium. In Coprinus cinereus a difference between the phenotypes of dikaryons and diploids in dominance or complementation tests may be interpreted in terms of the effect of nuclear interaction on gene expression, in the knowledge of the exact nuclear content in the dikaryon (eg. Casselton and Lewis, 1967 ; Lewis and Vakeria, 1977). However, the interpretation of the phenotypic differences between heterokaryons and diploids in terms of nuclear interaction is uncertain because of the variability of the nuclear ratios in the heterokaryons. In the heterokaryon it is probable that the prevalence of one nucleus determines which allele is expressed (Pontecorvo, 1963), rather than the interaction between transcription products in the nuclei or of the interaction of transcription and translation products in the cytoplasm, which determines the phenotype.

It was not until Casselton (1965) was able to synthesise stable diploids, that Coprinus cinereus became of value in studying the possible effects of nuclear interaction on gene expression. Prior to 1965,

naturally occurring diploids were localised in the basidia of the fruitbody and were a short-lived phase of the Coprinus cinereus life-cycle (Section 1.2 b; Figure 1.1) and as such were unsuitable for the study of gene interactions.

The first application made of the stable diploids was to compare allelic complementation of auxotrophic mutants and their suppressor genes in diploids, dikaryons and heterokaryons by Casselton and Lewis (1967). The partial expression of auxotrophic requirements in heterokaryons but not in dikaryons or diploids, was interpreted by the authors to be the result of a preponderance of auxotrophic alleles in the heterokaryon.The result therefore demonstrated the preference of dikaryons to heterokaryons in the study of nuclear interaction and gene expression.

Differences between the phenotype of dikaryons and diploids have been observed in complementation tests (eg.Day and Roberts, 1969) and dominance tests (eg.Senathirajah and Lewis, 1975; Lewis and Vakeria,1977) and have been interpreted in terms of the effect of nuclear interaction on gene expression.

Of importance to this investigation was the observation made by North (1978) in which the dominance modifier, modcy^{+}, of a specific allele conferring resistance to cycloheximide, cy-2^{r}, resulted in a partial expression of cycloheximide-resistance in dikaryons but not in diploids. One of the objectives of this investigation was to identify the molecular basis of cycloheximide resistance in Coprinus cinereus and to interpret the difference in the expression of modcy^{+} in terms of the nuclear interaction.

SECTION 1.3. CYCLOHEXIMIDE.

a) The choice of cycloheximide-resistant mutants.

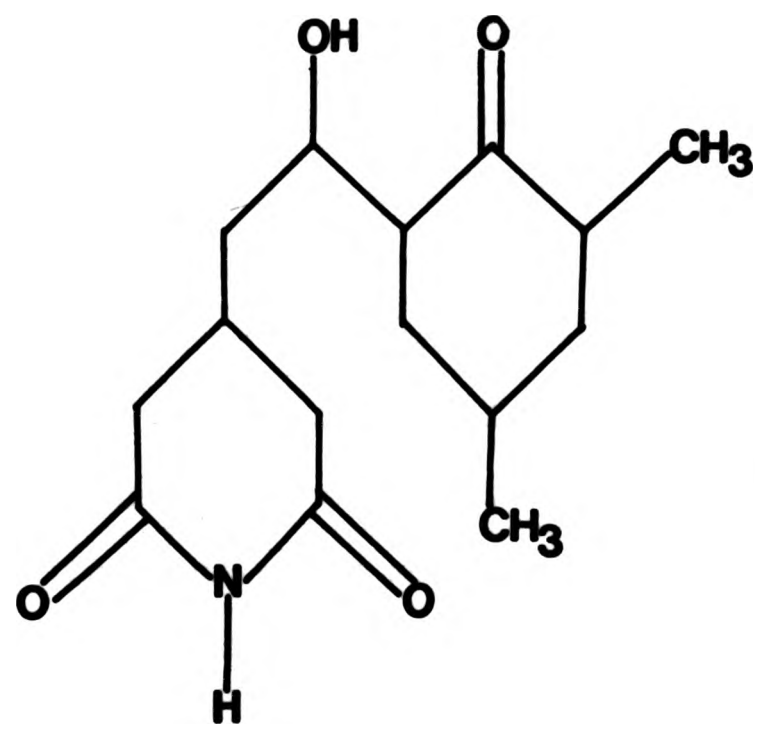
The analysis of mutants , particularly mutants exhibiting resistance to cycloheximide, has been employed as a powerful approach in the study of eukaryotic physiology , to identify gene loci , their products and their function.

Cycloheximide-resistant mutants were chosen by North (1978) and were the subject of this investigation because of the possibility of discovering the presence of genes associated with the phenotype, identifying the gene product and its function. Cycloheximide has been one of the most thoroughly investigated inhibitors of eukaryotes (Siegel, 1977). An investigation of the response of *Coprinus cinereus* strains to cycloheximide would permit a comparison of the genetical and biochemical mechanism between it and other species.

b) Structure and biological activity.

Cycloheximide belongs to the glutarimide group of inhibitors , so-called because each possesses a β -(2-hydroxymethyl) moiety, which in cycloheximide is attched to a cyclic ketone.

Figure 1.2. The structure of cycloheximide.



Originally isolated from filtrates of Streptomyces griseus (Whiffen et al, 1946), the chemical structure of cycloheximide, β - (2-(3,5, dimethyl - 2 - oxocyclohexyl) - 2 - hydroxyethyl) - glutarimide, was determined by Kornfeld et al, (1949). Of the several stereoisomeric configurations possible for the structure represented in Figure 1.2, L- cycloheximide is the steroisomeric form, which has been examined (Siegel and Sisler 1967).
L- cycloheximide is usually known as cycloheximide but has been known by the synonyms of actidione and naramycin A. Henceforth in this investigation, L- cycloheximide is referred to as cycloheximide.

When first investigated, cycloheximide was found to be toxic to a wide spectrum of eukaryotic organisms examined, but ineffective against bacteria (Whiffen, 1948). Within the diversity of the eukaryotic species examined and within different strains of the same species a differential toxicity of cycloheximide was observed (Whiffen, 1948).

Of all glutarimides and of all eukaryotic inhibitors, cycloheximide is the most toxic. The structural components essential for toxicity were the ketone-carbonyl, the hydroxyl and the imide nitrogen groups (Sisler and Siegel, 1967).

The reason why cycloheximide is one of the most thoroughly investigated antibiotics does not derive from any medical or agricultural application to control eukaryotic pathogens, but rather because of the nature of the metabolic process inhibited by the intracellular site of action of the drug.

c) Mechanism of action : inhibitor of protein synthesis.

Cycloheximide is regarded as one of the most potent inhibitors of eukaryotic protein synthesis (reviewed by Sisler and Siegel, 1967; Pestka,1977). It was first demonstrated to be an inhibitor of protein synthesis in Saccharomyces calsbergensis cells (Kerridge,1958). Subsequently inhibition of protein synthesis has been found in cells and cell-extracts from a range of eukaryotic species (Siegel and Sisler,1967). The drug inhibited protein synthesis catalysed by eukaryotic cytoplasmic ribosomes but did not inhibit prokaryotic (eg. Whiffen,1948, Ennis and Luben,1964), mitochondrial (eg. Loeb and Hubby, 1968) or chloroplastic (eg.Ellis, 1969) protein synthesis.

There have been contradictory reports concerning which specific reaction or reactions of polypeptide synthesis inhibited by cycloheximide.

There is general agreement that amino acid activation, which does not involve the cytoplasmic ribosome, is not inhibited by cycloheximide. (Siegel and Sisler, 1963; Ennis and Luben, 1964).

There is evidence that initiation (reviewed by Siegel, 1977) and termination (Godchaux et al, 1967; Rajalakshmi et al, 1971) are inhibited by cycloheximide. The majority of evidence, however, suggests that the elongation of the nascent polypeptide is reduced or prevented by cycloheximide and that the specific step of the elongation phase inhibited is the translocation of the cytoplasmic ribosomes on the mRNA (reviewed by Siegel, 1977; Vasquez, 1979).

The specificity of cycloheximide for cytoplasmic protein synthesis catalysed by the cytoplasmic ribosome, confirmed the evidence that the cytoplasmic ribosome was the intracellular site of cycloheximide action.

With the exception of studies by Trakatellis et al, (1965) and Felicetti et al, (1966) who concluded that a soluble factor in the cytoplasmic supernatant fraction of mammalian cells was inactivated by cycloheximide, all other studies, predominantly of fungal species, have implicated the cytoplasmic ribosome (reviewed by Siegel,1977; Vasquez, 1979). In particular, one mechanism by which a cell may be made more resistant to cycloheximide has been shown to be a property of the cytoplasmic ribosome (reviewed by Siegel, 1977; Vasquez, 1979).

Despite the overwhelming evidence that cycloheximide's mode of action is the inhibition of cytoplasmic protein synthesis, there is evidence which suggests that other metabolic processes are inhibited. A point of contention is whether the inhibition by cycloheximide of RNA synthesis (Horgen and Griffen, 1971; Timberlake and Griffen, 1974) and DNA synthesis (Kerridge, 1958; Bennet et al, 1964) occurs directly or as an indirect result of the primary inhibition of cytoplasmic protein synthesis.

Inhibition in vivo of RNA and DNA synthesis may be explained as a secondary effect of the drug, except for the report by Cooney and Bradley (1961) that the inhibition of DNA synthesis proceeds the inhibition of protein synthesis and the observation by Timberlake et al (1972) that the DNA-dependant RNA polymerase 1 is specifically inhibited in vitro. That the specific primary mode of action of cycloheximide on cytoplasmic protein synthesis has therefore not been proven.

SECTION 1.4. CYTOPLASMIC RIBOSOMES.

a) Location.

Eukaryotes may possess three types of ribosome, characterised by the site at which they synthesis polypeptides. Coprinus cinereus possesses cytoplasmic ribosomes located either free or bound to the endoplasmic reticulum and mitochondrial ribosomes. Eukaryotes which have chloroplasts also contain chloroplastic ribosomes.

b) Physical properties.

The physical properties of Coprinus cinereus cytoplasmic ribosomes are unknown, apart from the studies of Lovett and Haselby, (1971) and Lava-Sanchez et al, (1972). It is therefore assumed that Coprinus cinereus cytoplasmic ribosomes are similar to those examined in other fungal species. However, the most studied species are Saccharomyces cerevisiae and Neurospora crassa which are Ascomycetes wheras Coprinus cinereus is a Basidiomycete and it may be that the ribosomes from the two families do not have identical physical properties.

Fungal cytoplasmic ribosomes are composed of two subunits , the large and small cytoplasmic subunits , which together contain four molecules of RNA and between 70-80 proteins. A summary of their general physical properties is presented in Table 1.1(abridged from Russell and Wilkerson , 1980). Table 1.1 reveals variation in physical properties resulting from differences between the species but also includes variation brought about by different methods of experimentation.

Table 1.1 Summary of physical properties of fungal cytoplasmic ribosomes.

Physical properties	Monosomes	Large subunit			Small subunit
Dimension (nm)	20.5 x 25	20.2 x 21.1			18.5 x 5.2
Sedimentation coefficient (S)	80 - 82.5	56 - 61			36 - 40
Molecular weight ($x10^6$ dalton)	3.6 - 4.1	2.5 - 2.6			1.0 - 1.6
Ribonucleic acid.					
Proportion by weight %	42 - 53				
Number of molecules	4				
Sedimentation coefficient (S)		28•	5.8	5	18•
Molecular weight ($x10^4$ dalton)		130•	5.4	4.0	73•
Number of nucleotides		3300	158	120	1700
Protein.					
Proportion by weight (%)	47 - 58				
Number of molecules	70 - 80	31 - 45			21 - 34
Average molecular weight ($x10^4$dalton)		2.18 - 2.25			2.05 - 2.13

Taken from values for Saccharomyces cerevisiae and Neurospora crassa presented by Russell and Wilkerson (1980).

•=value determined in Coprinus cinereus by Lovett and Haselby (1971).

Taken from values for Saccharomyces cerevisiae and Neurospora crassa presented by Russell and Wilkerson (1980).

•=value determined in Coprimus cinereus by Lovett and Haselby (1971).

Mitochondrial and chloroplastic ribosomes, are smaller and possess fewer and smaller components than cytoplasmic ribosomes and resemble those of prokaryotic cytoplasmic ribosomes (Russell and Wilkerson, 1980). The cytoplasmic ribosomes of plants and animals possess ribosomal subunits and constituents which are on average larger than those found in fungi (Russell and Wilkerson, 1980).

The mucleotide content of the 28S and 18S rRNAs have been determined for Coprinus species (Pollard, 1964) and the high proportion of guanidine and cytosine are similar to those of other fungal species (Lava-Sanchez et al, 1972). The terminal nucleotide sequences of 28S and 18S rRNAs have been characterised in several fungi (Russell and Wilkerson, 1980) and the 5S and 5.8S rRNAs of several fungi have been completely sequenced (Russell and Wilkerson, 1980).

Each of the ribosomal proteins is presumed to be present in one copy per ribosome, with one exception in Saccharomyces cerevisiae (Zinker and Warner, 1976) and no protein is common to both cytoplasmic ribosomal subunits. No complete amino acid sequence of any cytoplasmic ribosomal protein is known, but terminal amino acid sequences of several proteins from Schizosaccharomyces pombe, Saccharomyces cerevisiae and rat liver have been recently determined by Otaka et al, (1983).

c) Cytoplasmic ribosomal genes.

The genomes of all fungal species examined contain multiple copies of cytoplasmic ribosomal RNA cistrons. The ribosomal RNA genes are arranged in repeat units which consist of 18S - 5.8S - 28S rRNA molecules separated by non-coding spacer DNA. The 5S rRNA may be closely associated with the repeat unit, but is a distinctive gene (Russell and Wilkerson, 1980). The number and location of rRNA genes is not known in Coprinus cinereus, except that they are not on linkage group 11, (Wu et al, 1983).

The identity of ribosomal protein genes is difficult to ascertain and consequantly the number of copies and locations are not known. Evidence from antibiotic resistant mutants, particularly from cycloheximide-resistant mutants has been used to identify specific cytoplasmic ribosomal protein genes from the large subunit (Coddington and Fluri, 1977: Bégueret et al, 1977; Stöcklein and Piepersberg,1980). However the identity of the majority of cytoplasmic ribosomal protein genes are not known, and none are known in Coprinus cinereus.

The genes which regulate the synthesis of rRNA and ribosomal protein, the assembly of the cytoplasmic ribosomal particles and the activity of the cytoplasmic ribosomes are also not known.

d) Cytoplasmic ribosomal synthesis.

The cytoplasmic ribosomal RNA and protein genes are transcribed in the nucleus . The rRNA precursor molecule which contains

18S, 5S and 28S rRNA is processed in the nucleolus. The mRNA molecules encoding the ribosomal proteins are translated on cytoplasmic ribosomes in the cytoplasm. The nascent ribosomal proteins associate in the nucleolus with the rRNA molecules. The ribonucleoprotein complexes develop into the large and small cytoplasmic ribosomal subunits which function in the cytoplasm. The proteins synthesised on the nascent cytoplasmic ribosomes may then partake in the transcriptional and translational processes necessary to synthesise more cytoplasmic ribosomes (Reviewed by Russell and Wilkerson, 1980).

One of the uses to which cycloheximide has been employed has been in the study of cytoplasmic ribosome synthesis . As a result of the inhibition of ribosome synthesis in the presence of cycloheximide Warner, (1974) considered that ribosome biogenesis was dependant on a continuous supply of nascent ribosomal and non-ribosomal proteins.

e) Structure and Function.

The structure of a eukaryotic cytoplasmic ribosome is envisaged as consisting of a relatively compact core in which the rRNA molecules are located. The ribosomal proteins constitute the majority of the surface of the organelle, some are located in the central core, others are less strongly bound (Wool, 1980).

The distribution of ribosomal components in the organelle is not well understood in eukaryotic species. The overall function of cytoplasmic ribosomes is to synthesise polypeptides, but few of the

individual reactions have been attributed to specific constituents of the cytoplasmic ribosome or to particular domains. The property of conferring cycloheximide-resistance has been assigned to specific ribosomal proteins (Coddington and Fluri,1977 ; Bégueret et al 1977; Stöcklein and Piepersberg,1980). There is evidence that the identified ribosmal proteins associated with the response to cycloheximide constitute the region of the organelle known as the peptidyl-transferase centre, the site at which tRNA molecules are bound and where peptide bond formation takes place (Bielka, 1978).

SECTION 1.5 OBJECTIVES.

The primary objective of this investigation was to discover the molecular basis of cycloheximide-resistance in Coprimus cinereus. However, the first objectives were to produce cycloheximide resistant mutants , to identify genes conferring cycloheximide resistance not found by North (1978) and to supplement strains made available by North for biochemical analysis.

The biochemical analysis of cycloheximide-resistance in other eukaryotic species has revealed that one way in which the mutant phenotype was brought abcut involved the modification of cytoplasmic ribosomes. The main emphasis of this investigation was to determine whether cytoplasmic ribosomes could confer cycloheximide-resistance in Coprimus cinereus.

The only previous analysis of the structure and function of Coprimus cinereus cytoplasmic ribosomes was an analysis of physical properties, (Lovett and Haselby, 1974). In order to discover the presence of cycloheximide-resistant cytoplasmic ribosomes in the cycloheximide-resistant mutant strains, it was first necessary to develop a cell-free polypeptide synthesising system in which to measure the activity of their cytoplasmic ribosomes.

A biochemical analysis of cytoplasmic ribosomal proteins from cycloheximide-resistant and cycloheximide-sensitive strains was undertaken in order to identify the specific component which conferred cycloheximide resistance.

CHAPTER 2.

MATERIALS AND METHODS.

SECTION 2.1. MATERIALS.

All chemicals were Analar grade.

The chemicals required for the culture of Coprinus cinereus were obtained from either Difco or BDH. In addition cycloheximide was supplied from Koch-Light, and Sigma supplied DNA and p-aminobenzoic acid.

The buffer constituents were purchased from BDH and Sigma. The essential chemicals for the cell-free protein synthesising system were supplied by Boehringer, with exceptions of spermidine (Aldrich) and polyuridylic acid (Calbiochem). Radiolabelled chemicals were obtained from the Radiochemical Centre, Amersham.

Chemicals required for the analysis of ribosomal proteins were obtained from Merck, except; acrylamide (Serva), glutaraldehyde (Fluka and Buchs) and pyronine yellow (G. Gurr and Sons).

SECTION 2.2. STRAINS.

Table 2.1. Strains of Coprinus cinereus used in this investigation.

Strain.	Mating-type. A	Mating-type. B	Genotype.	Origin
CY3	5	5	me^5 chol^1 cy^s	SR54 x TCA (North,1982)
CY6	5	5	nic^4 paba^2 cy^s	WMR66Ax TC4 (North,1982)
CY8	6	5	nic^4 paba^2 cy^s	WMR66Ax TC4 (North,1982)
CY9	6	6	nic^4 paba^2 cy^s	WMR66Ax TC4 (North,1982)
CY9.23	6	6	nic^4 paba^2 cy-2^r modcy$^+$	CY9 (North,1982)
CY9.23.98	6	6	me^5 cy-2^r modcy$^-$	CY9.23 x CY3 (North,1982)
CY9.23.137	5	5	cy-2^r modcy$^-$	CY9.23 x CY3 (North,1982)
CY9.23.138	5	6	me^5 cy-2^r modcy$^+$	CY9.23 x CY3 (North,1982)
CY10	6	6	nic^4 paba^2 cy^s	WMR66A x TC4 (North,1982; & unpublished)
CY11	5	6	nic^4 paba^2 cy^s	WMR66A x TC4 (North,1982; & unpublished)
CY13	5	6	me^5 chol^1 cy^s	SR54 x TC4 (North,1982)
CY14	6	5	ad^8 me^5 chol^1 cy^s	SR54 x TC4 (North,1982)
CY18	6	6	me^5 chol^1 ost cy^s	ZR336 x CY3 (North,unpub)
H1	5	5	cy-1^r	Wild strains (Day and Anderson,1961)
H2	6	5	cy-1^r	Wild strains (Day and Anderson,1961)
H5	5	6	cy-1^r	Wild strains (Day and Anderson,1961)
H9	6	6	cy-1^r	Wild strains (Day and Anderson,1961)
SR54	6	6	cy^s	Multiple crosses
TC4	5	5	cy-1^r	Multiple crosses
WMR66A	6	6	cy^s	Multiple crosses

All strains were made available by North. Abbreviations: cy^s= sensitive to 3.6 μM cycloheximide; cy^r= resistant to 3.6 μM cycloheximide; modcy$^+$ = dominance modifier of cy-2^r in dikaryons; ad = adenine requiring; chol = choline requiring; paba = para-aminobenzoic acid requiring; me = methionine requiring; nic = nicotinic acid requiring, ost = ostrich morphology.

SECTION 2.3. CULTURE OF COPRINUS CINEREUS.

a) Culture medium.

The composition of Minimal Medium and Complete Medium was as described by Lewis and North (1974). Auxotrophic supplements were added to the Minimal Medium at concentrations described by Cowan and Lewis (1961), Casselton and Lewis (1967) and Day and Roberts (1969). The concentrations of cycloheximide employed ranged from 0 to 712 μM.

All medium was sterilised for 15 minutes at a pressure of 1.06 $kg.cm^{-2}$. A stock solution of D-glucose was sterilised separately at 0.7 $kg.cm^{-2}$ for 10 minutes and added to the medium when cool. Stock solutions of heat labile chemicals, which included; choline chloride, cycloheximide, furfuraldehyde, methionine and nicotinic acid were sterilised by filtration through a 0.22 μM Millipore filter.

b) Culture conditions.

Cultures on solid medium were grown in 9 cm diameter petri-dishes from 2 mm^3 plugs of inoculum taken from within 5 mm of the margin of vigourously growing cultures. Unless stated to the contrary, all cultures were incubated at 37°C.

Cultures in liquid medium were begun as seeding-cultures. Seeding-cultures containing 50 ml of medium were inoculated with

approximately 10^8 oidiospores and were incubated for 2 days, during which time the 250 ml conical flasks were continually agitated in a Gallenkamp orbital shaker. Uncontaminated seeding-cultures were used as inocula for 1l of medium in 2l conical flasks. After 3 days incubation and continuous agitation the yield from 1l cultures was approximately 30g wet weight of mycelium.

c) Stock maintenance.

Strains in day-to-day use grown at 37°C and subcultured every 4 or 5 days. Strains which were used less frequently were kept at 4°C and subcultured monthly. Storage at 4°C was however unsuitable for dikaryotic strains and they were therefore kept at 37°C or room temperature. Long-term reserves of each strain were kept as either mycelium under paraffin oil, which were subcultured yearly , or as freeze-dried oidiospores, whose viability remained high for the duration of this investigation.

SECTION 2.4. ULTRAVIOLET MUTAGENESIS AND SELECTION OF CYCLOHEXIMIDE-RESISTANT MUTANTS.

The method used to induce and select for cycloheximide-resistant mutants was based on that of North (1982). The modifications to North's procedure were that oidiospores of the cylcoheximide-sensitive strains CY3, CY6, CY8, CY9, CY10 and CY11 were irradiated for 7.5 minutes beneath a Phillips ultraviolet light source which emitted light at 254 nm at a dose of 8.54 erg.cm^{-2}.sec^{-1}. Survival varied from 90% to 99.9% depending on the strain. The irradiated oidiospores were incubated in the dark for 24 hours on Complete Medium before they were covered with a 5mm layer of cycloheximide-agar 1% (^{W}/v) Bactoagar and 53 µM cycloheximide. Independant colonies, which emerged on the surface of the overlayered plates after 14 to 28 days, were tested on 3.6 µM cycloheximide. Each isolate was purified from an oidial suspension and was subcultured in the absence of cycloheximide. The ultra-violet induced mutation frequency was calculated from the number of isolates which were shown to be resistant to 3.6 µM cycloheximide.

a) Nomenclature.

Each of the isolates removed from the selection plates was identified according to the notation employed by North (1982) thus, CY3.16, identified this strain as being the sixteenth isolated from the cycloheximide-sensitive strain CY3.

SECTION 2.5. GENETIC ANALYSIS OF CYCLOHEXIMIDE-RESISTANT MUTANTS.

a) Growth test.

Confirmation that the strains, isolated from mutagenised oidiospores, were cycloheximide-resistant, was derived from an analysis of their growth response on solid Complete Medium containing cycloheximide.

Those strains which grew on 3.6 µM in the initial tests, were subjected to a detailed analysis of their growth response on cycloheximide concentrations up to 712 µM. Growth was measured as the average colony diameter, taken at cross-sections at right angles to each other (Casselton, 1965). Colony diameters were measured after 3 days and every 24 hours thereafter, to a maximum of 10 days. Colony diameters less than 5 mm were scored as zero. Irregularly shaped colonies were not recorded and the treatment was repeated. The growth of each strain at each cycloheximide concentration was expressed as a percentage relative to uninhibited control treatment, 0 µM cycloheximide. While the growth of the control treatment was within the confines of the petri-dish, the growth at other cycloheximide concentrations could be directly related to the control. Treatments at which growth was not recorded until after the time at which the control had outgrown the petri-dish, were indirectly related to the control by intermediate treatments of calculated relative growth (see Appendix A i for specimen calculation).

A linear regression analysis was performed over the range of cycloheximide concentrations (log.10) at which growth inhibition was recorded. The minimal and 50% inhibitory cycloheximide concentrations and the linear regression coefficients were calculated. The calculated 50% growth inhibitory cycloheximide concentrations and linear regression coefficients from different strains were compared using a Student's t-test (Bailey, 1959).

b) Determination of the number of genes conferring cycloheximide resistance in a strain.

The number of genes conferring cycloheximide resistance was inferred from the segregation ratio of cycloheximide-resistant and cycloheximide-sensitive basidiospores arising from the mating of cycloheximide-resistant mutant strains with compatible cycloheximide-sensitive test strains. The dikaryons were synthesised from monokaryotic strains which were heteroallelic at both A and B mating-type loci and which had complementary auxotrophic requirements. In many instances, repeated attempts were necessary to achieve a successful mating. The dikaryons were identified by their component monokaryotic strains, eg. CY8.2 x CY13.

Fertile dikaryons produced fruit-bodies on moistened sterile horse-dung after 2 to 3 weeks (Lewis, 1961) or on Minimal Medium in a slightly longer time. Basidiospores were germinated on Complete Medium containing 0.01% furfuraldehyde (Emerson, 1954). After 24 hours incubation, those basidiospores which had germinated were transferred to unmodified Complete Medium.

The phenotypes of the colonies arising from the basidiospores were determined from the presence or absence of growth on 3.6 μM cycloheximide.

c) Dominance test.

Dikaryons, heterozygous for cycloheximide resistance, produced in order to determine the number of genes conferring cycloheximide resistance, were tested on 1.8 μM cycloheximide; those dikaryons which grew were considered to be resistant to cycloheximide and were described as possessing dominant mutations (North, 1982). Detailed analysis of the growth response over a range of cycloheximide concentrations, similar to those described in Section 2.5 a , were undertaken to resolve fully dominant and partially dominant mutations. Irregular and unstable dikaryons were not recorded.

The dominance of cycloheximide-resistance mutations was also determined in diploids, in a similar manner to the method described for dikaryons, except that the discriminatory cycloheximide concentration used to indicate a recessive mutation was 3.6 μM (North, 1982).

Diploids were selected from the oidiospores of common A heterokaryons (Casselton, 1965) and their existence was confirmed using a dikaryon test (North, 1982). Diploids were identified by their component monokaryotic strains, eg. CY8.2/CY18.

d) Complementation test.

The number of different genes which conferred cycloheximide-resistance was determined by a complementation test (North, 1982). Only those cycloheximide-resistant mutant strains possessing single, recessive cycloheximide-resistance mutations could be tested. The necessary cycloheximide-resistant recombinant strains were derived from the progeny of the heterozygous cycloheximide-resistant dikaryons analysed in Section 2.5 b .The nomenclature of the recombinant strains was such that the cycloheximide-resistant mutant from which they were derived, and their order of isolation were identified, eg. CY 3.16.2 was derived from CY 3.16.

The dikaryons produced from the mating of cycloheximide-resistant mutants with cycloheximide-resistant recombinant strains derived from other strains, were tested for their ability to grow on 1.8 μM cycloheximide. Growth on 1.8 μM cycloheximide was considered to demonstrate that no complementation had occured, i.e. that cycloheximide resistance in the two strains was the result of mutations in the same gene.

SECTION 2.6. BUFFERS.

Constituent	Extraction Buffer Final concentration (mM).	Dissociation Buffer
Tris-HCl, pH 7.5.	50	50
Magnesium acetate.	10	1
Potassium chloride.	25	100
2-mercaptœthanol.	5	5
Sucrose.	250	0

The Extraction and Dissociation Buffers used in the preparation of Coprinus cinereus cell-free extracts were autoclaved at 0.7 $kg.cm^{-2}$. 2-mercaptoethanol was added after sterilisation. The pH. of both buffers was measured and adjusted at 4°C and the buffers were stored at 4°C for up to 2 weeks.

Constituent	CMC Start Buffer Final Concentration
Urea.	6 M
Phosphoric acid.	20 µM
2-mercaptoethanol.	7 mM

The CMC Start Buffer was adjusted to pH 6.5 with methylamine (Coppin - Raynal, 1980)

SECTION 2.6. BUFFERS.

Constituent	Extraction Buffer Final concentration (mM).	Dissociation Buffer
Tris-HCl, pH 7.5.	50	50
Magnesium acetate.	10	1
Potassium chloride.	25	100
2-mercaptoethanol.	5	5
Sucrose.	250	0

The Extraction and Dissociation Buffers used in the preparation of Coprinus cinereus cell-free extracts were autoclaved at 0.7 $kg.cm^{-2}$. 2-mercaptoethanol was added after sterilisation. The pH. of both buffers was measured and adjusted at 4°C and the buffers were stored at 4°C for up to 2 weeks.

Constituent	CMC Start Buffer Final Concentration
Urea.	6 M
Phosphoric acid.	20 µM
2-mercaptoethanol.	7 mM

The CMC Start Buffer was adjusted to pH 6.5 with methylamine (Coppin - Raynal, 1980)

SECTION 2.7. PREPARATION OF COPRINUS CINEREUS CELL-EXTRACTS.

The method used to prepare Coprinus cinereus cell-extracts was adapted from the preparation developed for Podospora anserina, (Bégueret et al, 1977).

Mycelium was collected from 3 day-old, 1 litre liquid medium cultures (Section 2.3 b) by filtration through muslin and was rinsed in distilled water and then Extraction Buffer, (Section 2.6).

All subsequent steps in the preparation and biochemical analysis were carried out at 4°C, unless stated to the contrary.

10g wet weight of freshly grown mycelium were ground by hand in a pre-chilled mortar, with an equal weight of acid-washed sand. 10 ml of Extraction Buffer were added during 3 minutes of vigourous homogenisation.

The fungal paste was subjected to the differential centrifugation steps outlined in Figure 2.1. At all stages of the preparation, the extinction coefficients of suitably diluted cell-extracts were measured at 260 and 280 nm in silica cuvettes using a Perkin-Elmer 552 Ultraviolet Spectrophotometer.

S-30 refers to the post-mitochondrial supernatant produced after centrifugation at 30,000 x gav. and S-100 and RP-100 refer to the cytoplasmic ribosome-free supernatant and resuspended cytoplasmic ribosome-rich pellet respectively, produced at 100,000 x gav.

Figure 2.1. Preparation of S-30, S-100 and RP-100 fractions.

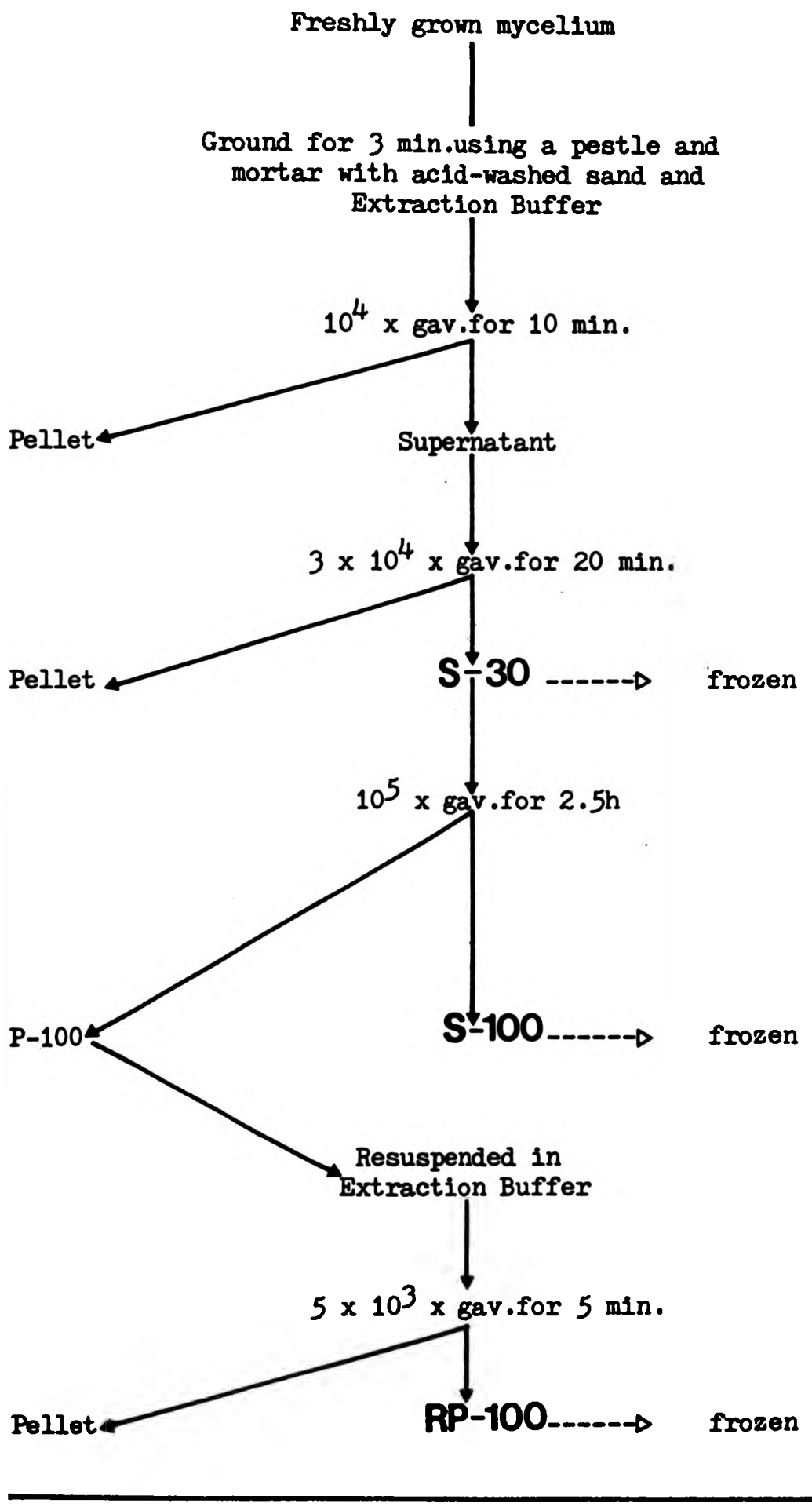
All centrifugation steps, except the clarification of the resuspended P-100 fraction, were carried out using a MSE 8 x 35 ml rotor in a MSE 65 ultracentrifuge.

The supernatant fractions were carefully removed from the pelleted material using a pasteur pipette. Resuspension of the P-100 pellet was made in the minimum volume of Extraction Buffer.

The fractions identified by bold type; **S-30**, **S-100** and **RP-100** were used in the biochemical investigation.

Cell fraction.	Typical yield (A_{260} unit) from 10g wet weight mycelium.	Typical absorbance ratio (A_{260}:A_{280}).
S-30	300	1.75 : 1
RP-100	75	1.8 : 1
S-100	150	1.95 : 1

Figure 2.1. Preparation of Coprinus cinereus cell-extracts.



S-30, S-100 and RP-100 fractions were dropped from a pasteur pipette into microcentrifuge tubes containing liquid nitrogen and were kept at -70°C (Roberts and Paterson, 1973).

The S-30 fraction was treated according to the method of Marcu and Dudock (1974), prior to its use in the cell-free polypeptide synthesising system (Appendix Bi). The S-30 was passed through a Sephadex G25 (Coarse), 20 x 1.0 cm. column. The column was eluted with Extraction Buffer at a flow rate of 3 $ml.min^{-1}$. Those 0.5 ml fractions which had an absorbance ratio ($A_{260}:A_{280}$) of 1.6:1 or greater and had a concentration of 3.0 A_{260} unit were pooled together and frozen.

Untreated S-100 and RP-100 fractions were used in the polypeptide synthesising system (Section 2.9).

SECTION 2.8. PREPARATION OF CYTOPLASMIC RIBOSOMAL SUBUNITS AND ANALYSIS OF CELL-EXTRACTS.

Sucrose density gradients were used for the small-scale preparation of large and small cytoplasmic ribosomal subunit fractions and to analyse the purity of the cell-extracts prepared, (Section 2.7). The methodology was determined by experimentation (Chapter 4, Section 4.5) and is summarised in Figure 2.2.

Linear 25 ml sucrose density gradients were prepared from 10% and 30% ($^{W}/v$) stock sucrose solutions, using a Pharmacia gradient maker. For the preparation of cytoplasmic ribosomal subunits, the sucrose density gradient was prepared in Dissociation Buffer (Section 2.6), and for the analysis of cell-extracts the gradients were prepared in Extraction Buffer minus sucrose (Section 2.6).

The samples loaded onto the top of the gradients (10% sucrose) were in a maximum volume of 500 µl. and at a maximum concentration of 40 A_{260} unit. Centrifugation was achieved in a MSE 3 x 25 swing-out rotor for 6.5 hours at 90,000 x gav. in a MSE 65 ultracentrifuge.

The distribution of cellular material after centrifugation was analysed by inserting a glass micropipette into the centrifuge tube and removing the contents, densest first, at the rate of 2 $ml.min^{-1}$. Before 0.8 ml fractions were collected, the absorption at 254 nm was monitored by a LKB Uvicord Ultraviolet Absoptiometer.

Several fractions were used to determine the profile of the sucrose density gradient, by analysis in a 0-50% sucrose refractometer

S AND

ale

it

repared,

ntation

from 10%

radient

its, the

er (Section

were prepared

% sucrose)

ntration of

swing-out

rifuge.

gation was

fuge tube

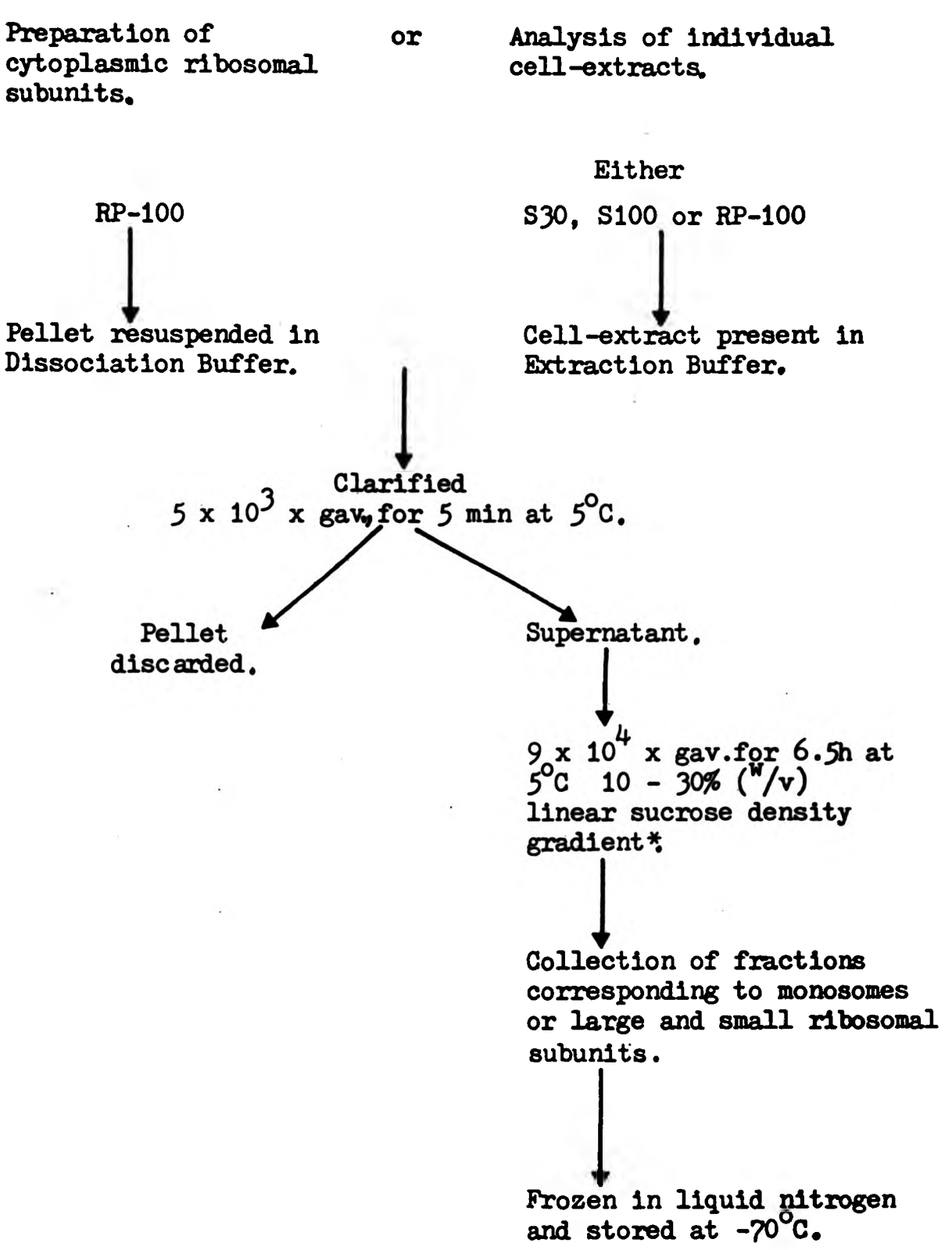
2 ml.min^{-1}.

254 nm

e of the

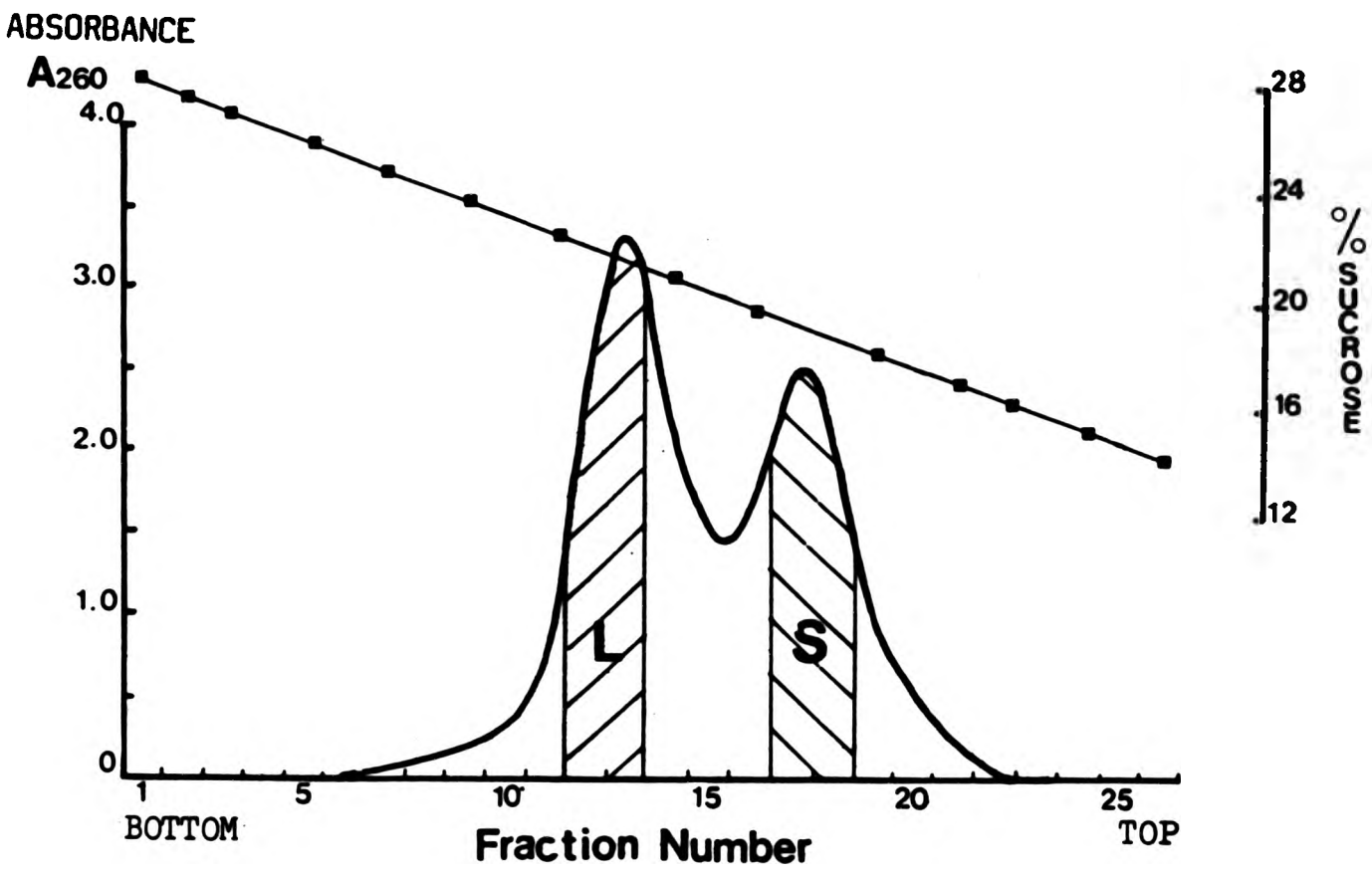
refractometer

Figure 2.2. Preparation of cytoplasmic ribosomal subunits and analysis of cell-extracts.

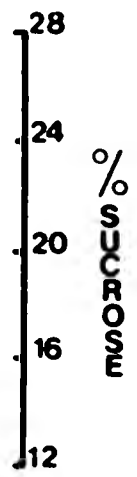


* For the preparation of cytoplasmic ribosomal subunits, the linear sucrose density gradient was prepared in Dissociation Buffer (Section 2.6) for the analysis of cell-extracts, Extraction Buffer (Section 2.6) was used instead of Dissociation Buffer.

Figure 2.3. Preparation of cytoplasmic ribosomal subunits.



Typical absorption profiles of material from the RP-100 after centrifugation (for 6.5 h at 90,000 x gav.) through a 10 - 30% sucrose density gradient in Dissociation Buffer (■——■). The shaded absorbance fractions labelled L and S were presumed to contain the large and small cytoplasmic ribosomal subunits, respectively.



OP

00 after
30% sucrose
haded
contain the
ly.

(Bellingham and Stanley Ltd). An estimate of the sedimentation coefficients of peaks of absorption were calculated according to the method of M^{c} Ewen (1967, Appendix B ii).

To minimise cross-contamination between the large and small cytoplasmic ribsomal subunits , the fractions which were pooled together were chosen from the extremes of the absorption peaks (Figure 2.3). The RP-100 fraction typically yielded approximately 40% large cytoplasmic ribosomal subunits and 30% small cytoplasmic ribosomal subunits in the pooled fractions, based on the areas of the fractions in the absorption profile. The pooled fractions of the large and small ribosomal subunits were frozen separately in liquid nitrogen and stored at - 70^{o}C.

SECTION 2.9. **CELL-FREE POLYPHENYLALANINE SYNTHESIS.**

The culmination of the experiments undertaken to develop a Coprinus cinereus cell-free polyphenylalanine synthesising system (Chapter 4) are summarised in Table 2.2.

Table 2.2. The Coprinus cinereus cell-free polyphenylalanine synthesising system; optimised for each individual component.

Constituent.	Optimised final concentration (mM, unless stated)
Adenosine - 5 - triphosphate	1.0
Guanosine - 5 - triphosphate	0.25
Creatine phosphokinase	6.0 $\mu g.ml.^{-1}$
Creatine phosphate	12.0
Tris - HCl, pH. 7.5.*	15.0
Magnesium acetate	4.5
Potassium chloride	20.0
Ammonium acetate	40.0
Spermidine	1.0
Dithiothreitol	4.0
t RNA ^{phe}	6.0 $\mu g.ml.^{-1}$
Polyuridylic acid	300.0 $\mu g.ml.^{-1}$
L - (U-C^{14}) phenylalanine(513μCi.m mole^{-1})	0.24 μM (0.125 μCi)
RP-100 cytoplasmic ribosome-rich fraction	2.0 A_{260} unit.
S100 cytoplasmic ribosome-free supernatant fraction	2.0 A_{260} unit.

* The pH. of Tris - HCl was measured at 20°C.

and toluene) and counted for 10 or 20 minutes in a Beckman LS7500 liquid scintillation counter (counting error = <5%, C^{14} counting efficiency = 89%).

a) Response of cell-extracts to cycloheximide.

The response of cell-extracts from various strains to cycloheximide was analysed over a range of cycloheximide concentrations to a maximum of 5.3 mM. The quantity of polyphenylalanine synthesised at each cycloheximide concentration was expressed as a percentage relative to the control treatment, which contained no cycloheximide.

A linear regression analysis was performed over the range of cycloheximide concentrations (log.10) at which polyphenylalanine synthesis was inhibited. Parameters used to describe the *in vitro* inhibition were the calculated minimum, 50%, and in some instances total, inhibitory cycloheximide concentrations, and the linear regression coefficient. The responses of two strains were compared by a Student's t-test of the 50% inhibitory cycloheximide concentration and linear regression coefficients (Bailey, 1959).

SECTION 2.10. PREPARATION OF CYTOPLASMIC RIBOSOMAL PROTEINS.

Cytoplasmic ribosomal proteins were extracted from either monosomal or subunit preparations suspended in Extraction Buffer, by the method of Hardy et al.(1969).

Cytoplasmic ribosomes were suspended in Extraction Buffer. The magnesium ion concentration of the buffer was increased to 100 mM and 2 volumes of glacial acetic acid were added. After 1 hour at $0^{o}C$, precipitated RNA was removed by 30 minutes centrifugation at 10,000 g.av. The decanted supernatant was mixed with 5 volumes of acetone and left for 2 hours at $-20^{o}C$ to precipitate ribosomal protein. Ribosomal protein was pelleted by centrifugation for 30 minutes at 10,000 g.av. The pellet was thoroughly dried in a vacuum for 15 minutes to remove excess acetone.

The ribosomal protein was resuspended in the minimum volume of CMC - Start Buffer (Section 2.6) and clarified by 5 minutes centrifugation at 5,000 x g.av. The ribosomal protein concentration was estimated from the known concentration before extraction and estimated extraction efficiency of approximately 25% (A.Bollen, personal communication).

SECTION 2.11. TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS.

The analysis of Coprinus cinereus cytoplasmic ribosomal proteins was achieved by applying a method of analysis which had been described for Escherichia coli ribosomal proteins (Cabezon and De Wilde, 1975).

First dimension analysis.

Based on the method of Knopf et al.(1975). The composition of the first dimension gel is described in Table 2.3.

Table 2.3.

Contents	Stacking gel	Separating gel
	Final concentration (M).	
Acrylamide	0.56	0.56
Bisacrylamide	0.043	0.043
Bistris	0.038	0.038
Urea	6.0	6.0
Glacial acetic acid	pH 4.5	pH 5.5

The solutions were degassed and polymerised by the addition of 1.3 mM TEMED followed by 2.15 mM, freshly made, ammonium peroxodisulphate.

Each 10 x 0.4 cm (internal diameter) perspex disc-gel tube was filled to within 2 cm of the top with Separating gel. Once polymerised, the gel was overlayered with 1 cm of Stacking gel.

A maximum of 8 gels were placed in a Bio-rad 150A gel electrophoresis cell. The upper chamber contained 10 mM bistris, adjusted to pH 3.7; the lower chamber contained 10 mM bistris at pH 7.0.

Approximately 25-50 µg of Coprinus cinereus cytoplasmic ribosomal protein in 50 µl of CMC-Start Buffer (prepared as desribed in Section 2.10) were carefully applied to the top of the Stacking gel. To facilitate the loading of the sample, the sample contained tracking dye(0.1% w/v pyronine yellow in 10% w/v glycerol).

Electrophoresis toward the cathode commenced for 15 minutes at 0.5 $mA.gel^{-1}$ and continued for 16 hours at 1.0 $mA.gel^{-1}$.

Second dimension analysis.

Based on the method of Howard and Traut,(1973). The composition of the second dimension slab gel is described in Table 2.4.

Table 2.4.

Contents	Final concentration (M).
Acrylamide	2.5
Bisacrylamide	0.32
Potassium hydroxide	0.048
Urea	6.0
Glacial acetic acid	pH 5.5

The solution was degassed and polymerised by the addition of 4.0 mM TEMED followed by 13 mM,freshly prepared, ammonium peroxodisulphate.

A disc-gel,which had been subjected to the electrophoresis conditions described for the first dimension analysis,was embedded into a slab gel (dimensions: 11.5 x 13.5 x 0.2 cm). Two slab gels were placed adjacently in the apparatus described by Howard and Traut,(1973). The buffer in the upper and lower chambers was 187 mM glycine, adjusted to pH 4.0 with glacial acetic acid. Tracking dye (pyronine yellow in glycerol) was layered across the top of the gel.

Electrophoresis was carried out towards the cathode, initially for 15 minutes at 30 mA.gel^{-1}, but increased to 60 mA. gel^{-1} for the time necessary for the tracking dye to migrate to within 1 cm of the base of the gel (approximately 7 hours). The gels were carefully removed and coded by a seriesof holes for identification.

Observation of proteins.

In the preliminary experiments the ribosomal proteins were revealed by staining with Coomassie Brilliant Blue(R-250), as described by Howard and Traut,(1973). However, a silver staining technique was found to be more suitable for staining the small quantities of ribosomal proteins which were present (Oakley et al, 1980).The resolution of the stained ribosomal proteins from the general staining of the polyacrylamide gel was dependant on the subjective decision to stop development. The gels were photographed using Kodak Panntomic-X black and white film and were kept for several weeks in 5% acetic acid.

Nomenclature of proteins.

The stained proteins on the electropherograms were identified according to the system devised by Kaltschmidt and Wittmann (1970).Each protein was assigned a prefix, either S or L, to signify their respective derivation from the small or large cytoplasmic ribosomal subunit, and was numbered according to its position. The numbering began at the origin of the electropherogram and continued in horizontal planes down to the bottom of the slab gel.

SECTION 2.12. ANALYSIS OF CYTOPLASMIC RIBOSOMAL PROTEINS BY CARBOXYMETHYL-CELLULOSE CHROMATOGRAPHY.

The analysis of cytoplasmic ribosomal proteins by carboxymethyl-cellulose (CMC) chromotography was based on the method used for Podospora anserina by Coppin-Raynal, 1980 .

Cytoplasmic ribosomal proteins were radiolabelled with either C^{14} or H^3 by modifying the source of glucose in the liquid Complete Medium (Section 2.3 a). The cycloheximide-sensitive strains CY8 and CY9 were grown in the presence of 400 µCi D -(U-C^{14}) glucose (255 mCi. $mole^{-1}$). CY8.2 and CY9.23, the cycloheximide-resistant mutants derived from CY8 and CY9 respectively, were grown in 400 µCi, of D-(3-H^3) glucose (5.6 Ci.$mmole^{-1}$).

Each strain was cultured according to the method described in Section 2.3b. The mycelium from CY8 was mixed with the mycelium from CY8.2. Similarly, CY9 and CY9.23 mycelium were mixed together. Cytoplasmic ribosomes, ribosomal subunits and ribosomal proteins were prepared following the procedures described in Sections 2.7, 2.8 and 2.10.

A 20 x 0.8 cm column containing carboxymethyl-cellulose (Whatman CM52) was equilibrated with CMC Start Buffer (Section 2.6), overnight at 4°C. The cytoplasmic ribosomal mixture of cycloheximide-resistant and cycloheximide-sensitive strains was dissolved in the CMC Start Buffer and was applied to the top of the column. The ribosomal protein samples contained approximately 2 x 10^5 dpm, (which was less than the activity suggested by Coppin-Raynal, 1980).

The cytoplasmic ribosomal proteins were eluted from the column by a linearly increasing concentration (0 - 0.3M) of LiCl in the CMC Start Buffer. Three 0.9 ml fractions were collected each hour for the duration of the LiCl gradient, which was approximately 6 days. 500 µl of most fractions were mixed with 5 ml of scintillation cocktail (Aqualuma from Lumac, Basel) and analysed in a LKB Scintillation counter. The H^3 and C^{14} radioactivity in each fraction was recorded.

In order to monitor the LiCl gradient, the conductivity of every tenth fraction was measured using a conductivity meter (Van der Heyden, Ltd). Conductivity (0-15 mho.) was equated to LiCl concentration using an experimentally determined calibration curve.

CHAPTER 3

THE PRODUCTION AND GENETIC ANALYSIS OF CYCLOHEXIMIDE-RESISTANT MUTANTS IN COPRINUS CINEREUS.

SECTION 3.1 GENETICS OF COPRINUS CINEREUS.

The genetic analysis of Coprinus cinereus has revealed 8 linkage groups (Reviewed by Lewis and North, 1974). Genes which have been identified and located on the linkage groups include those which confer resistance to amino acid analogues; ethionine (Lewis 1963), canavanine (Senathirajah and Lewis 1975), p-fluorophenyl-alanine (Barker and Lewis, 1974; Senathirajah and Lewis, 1975) and 5-fluoroindole (Veal and Casselton, 1982). In addition genes associated with resistance to the protein synthesis inhibitor, cycloheximide have also been studied, (North, 1978, 1982).

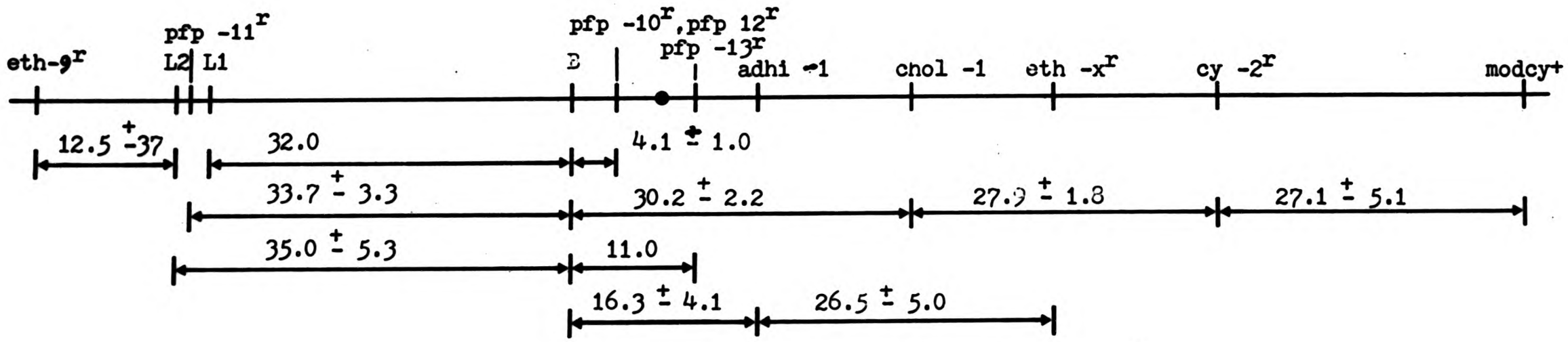
a) Cycloheximide resistance.

Day and Anderson (1961) were the first to identify a strain possessing cycloheximide-resistance in Coprinus cinereus. No details of the mutant were given other than that it was the result of ultraviolet mutagenesis in the wild strain H9.

The only other investigation of cycloheximide resistance in Coprinus cinereus was made by North (1978, 1982). Cycloheximide resistance, defined as the ability to grow on medium containing 3.6 μM cycloheximide, was found to be conferred directly by three genes and indirectly by one other.

One gene, designated by the mutant allele cy-1^{r}, was present in the strain TC4 and was believed to be common to many of the wild

Figure 3.1. Genetic map of linkage group II of Coprinus cinereus, after North (1982).



Abbreviations: eth = ethionine, pfp = p - fluorophenylalanine, B = mating-type locus B, adhi = adenine and histidine, chol = choline, cy-2^r = cycloheximide resistance, modcy$^+$ = dominance modifier of cy-2^r.

cycloheximide-resistant strains. Cy-1^r was described as dominant in both the dikaryon and the diploid and was located on the linkage group VII.

The identity of another gene was discovered when the mutant allele cy-2^r was produced by ultraviolet mutagenesis. Cy-2^r was recessive in the dikaryon and diploid and was located on linkage group II (Figure 3.1).

A third gene modcy, which was implicated in expression of cycloheximide-resistance, did not directly confer cycloheximide-resistance but did interact indirectly and specifically with cy-2^r. In heterozygous dikaryons, modcy$^+$ enabled the partial expression of cy-2^r but the heterozygous diploid was cycloheximide-sensitive. Modcy$^+$ was identified as a dominance modifier of cy-2^r and was located on linkage group II, distal to cy-2^r (Figure 3.1).

Details concerning another gene possessing a mutant allele termed cy-3^r were incomplete. It was described, by North (1982) as as a weak mutant which was not linked to either cy-1^r, cy-2^r or modcy$^+$. It was also considered to be recessive (North, unpublished).

b) Nuclear interaction and gene expression.

i) Expression of cycloheximide resistance.

North (1982) observed that the recessive cycloheximide-resistant cy-2^r in the strain CY9.23 , was partially expressed in dikaryons , but notin diploids, heterozygous for cycloheximide resistance (Table 3.1). The difference in phenotypes between the cell-types was discovered to be the result of a mutation modcy$^+$, which did not itself confer cycloheximide resistance but which modified the expression of the cy-2^r mutation in the dikaryon but not in the diploid. The effect of the dominance modifier gene modcy$^+$ was restricted to the dikaryon, in which the interaction of genetic information occurred between nuclei when the cy-$2^r$and modcy$^+$ alleles were in the cis or trans configuration, but not when their respective cycloheximide-sensitive and modcy$^-$ alleles were present in a diploid nucleus (Table 3.1).

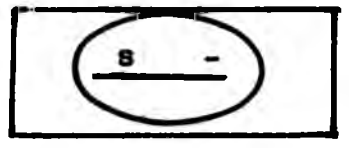
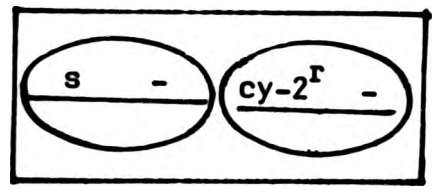
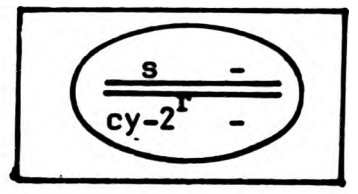
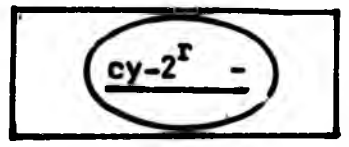
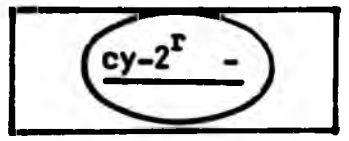
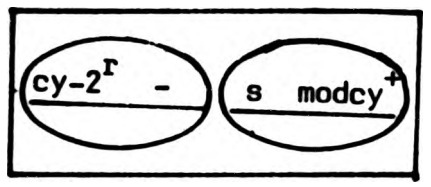
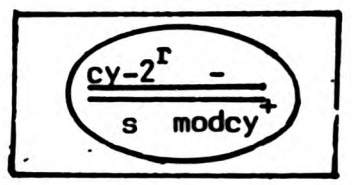
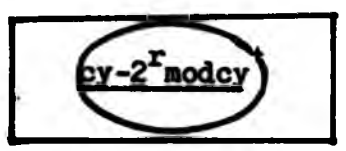
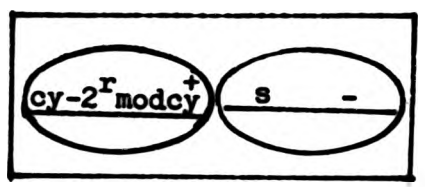
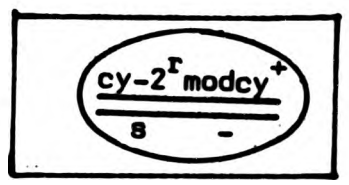
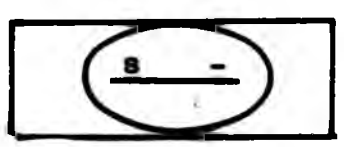
ii) Expression of other genes.

In dominance tests performed by Senathirajah and Lewis (1975), it was observed that that the canavanine resistance allele can^r was dominant in the dikaryon but was recessive in the diploid. In the same investigation, the dominance modifiers mod$^+$-10 and mod$^+$-11 were dominant in the dikaryon but recessive in the diploid in the presence of their specific genes conferring resistance to p-fluorophenylalanine, pfp-10 and pfp-11 respectively.

Complementation of several adenine requiring alleles at the ad-5 locus produced a quantitative measure of adenylosuccinase in the diploid but not in the dikaryon (Day and Roberts, 1969).

Table 3.1 Representation of the genotypes and phenotypes of strains examined by North (1982).

Table 3.1 Representation of the genotypes and phenotypes of strains examined by North (1982).

MONOKARYONS	Phenotype	DIKARYONS	Phenotype	DIPLOIDS	Phenotype
	Sensitive		Sensitive		Sensitive
	Resistant				
	Resistant		Partial Resistance		Sensitive
	Sensitive				
	Resistant		Partial Resistance		Sensitive
	Sensitive				

cy-2r and s refer to cycloheximide-resistant and cycloheximide-sensitive alleles respectively. modcy+ and - refer to the dominance modifier allele of cy-2r and its wild type allele, respectively. A resistant phenotype was expressed as able to grow on 3.6 μM cycloheximide, whereas a sensitive phenotype was unable to grow on 1.8 μM cycloheximide and partial resistance was growth observed up to 1.8 μM cycloheximide. North (1982).

SECTION 3.2. GENETICS OF THE CYCLOHEXIMIDE RESPONSE.

The first analyses of the effect of cycloheximade were made by Whiffen(1948, 1950) on a wide range of species. It was concluded that there was considerable variation in the response of eukaryotic species to cycloheximide; for example the growth of the basidiomycete Ustilago tritici was inhibited by 0.45 μM cycloheximide whereas the related Ustilago zeae was able to grow up to 36 μM(Whiffen,1950).

Except in those instances where interspecific matings were possible (eg. between Saccharomyces fragilis and Saccharomyces pastorianus, Siegel and Sisler, 1963), the amount of information which could be gleaned concerning the genetic basis for the differential cycloheximide response was limited. Whiffen (1950) had however, observed that within a species there was considerable natural variation in the response to cycloheximide; eg. in Sclerotinia fructicola the growth of one strain was inhibited by 18μM cycloheximide, whereas a more resistant strain was able to grow in up to 72μM. However, it was not until Middlekauf et al (1957) that the first evidence was presented which demonstrated a genetic basis for the variation in the cycloheximide response: it was believed that the more tolerant, cycloheximide-resistant strains in Saccharomyces cerevisiae possessed a single dominant gene. Subsequently, as many as eight genes were found to be associated with cycloheximide resistance in Saccharomyces cerevisiae (Wilkie and Lee, 1965). The genetic basis for cycloheximide-resistance in eukaryotes is described in Table 3.2.

Table 3.2. Genetic analysis of the cycloheximide response in eukaryotes.

Organism/Reference	Mutagen	No. of loci	Nomenclature of mutational loci	Min. Cycloheximide conc. for total inhibition (μg)	Dominance test	Linkage group
ASCOMYCETES						
Aspergillus nidulans						
Parr & Roper(1964)	UV.	1	*act 1*	90	semidominant	III
				(wild-type 2.5)	(in balanced heterokaryon)	
Waldron & Roberts(1974)	NNNG. spontaneous	2	*arpA*	7.2	recessive	VII
			arpB	0.5 (wild-type 7.2)	recessive (heterokaryons)	IV
Neurospora crassa						
Pratt (1967)	UV.	2	*act 1*	1000	dominant	I
			act 2	1000	dominant	V
(also Pratt & Terry,1962)				(wild-type 7.2)	(heterokaryon)	
Brockhauser *et al.* 1970)						
Tsukamoto (1972)					At least 4 genes conferring cycloheximide res. *act 1* & *act 2* and a modifier gene	
Rothschild *et al*(1975)	spontaneous 2-amino purine				Several loci conferring resistance to more than 1.4 μg and at least one modifier mutants were dominant in heterokaryons. The location of the genes was inconclusive	
Podospora anserina						
Cravat *et al* (1958)	NNNG.	3	*cyR-1*	>40	recessive	-
			cyR-2	>40	recessive	-
			cyR-3	90	recessive	-
				(wild-type 72)	(heterokaryon)	
Coppin-Raynal(1977)	NNNG,U.V EMS	1	*AS-3*	140 (wild-type 36)		-
Saccharomyces cerevisiae						
Middlekauf *et al*(1957)		1	*AC*	7.2 (wild-type <2.5)	dominant (diploid)	-
Wilkie and Lee (1965)	spontaneous UV	12	*AC 1*	3.6	semidominant	II
			AC 2	22	recessive	VII
			AC 3	3.6	semidominant	VII
			AC 4	1.8	dominant	fragment 1
			AC 5	3.6	semidominant	-
			AC 6	1.8	recessive	-
			AC 7	3.6	semidominant	-
			AC 8	72	recessive	-
			AC 9	<1.8	recessive	-
			AC 10	<1.8	recessive	-
			AC 11	<1.8	recessive	-
			AC 12	<1.8	recessive	-
				(wild-type <1.8)	(heterokaryon)	
Schizosaccharomyces pombe						
Ibrahim & Coddington (1976)	spontaneous UV, NNNG. nitrous acid	4	*cyh-1*	>712	recessive	-
			cyh-2	142	semidominant	-
			cyh-3	142	recessive	-
			cyh-4	142	recessive	-
				(wild-type <142)	(diploid)	
Ibrahim & Coddington (1978)	UV	2	*ocr-1*	> 360	-	-
			ocr-2	> 360	-	-
				(wild-type< 360)		
BASIDIOMYCETES.						
Coprinus cinereus						
Barth (1962)	UV.	3	*cy-1*	10.8	dominant	VII
			cy-2	252	recessive	II
			cy-3	-	recessive	-
			*mut cy**	0.9	dom. in dikaryon	II
				(wild-type 0.9)	(dikaryon & diploid)	
MYXOMYCETE.						
Physarum polycephalum						
Dee (1967) (also Dee & Poulter,1970)	spontaneous	1	*act 1*	90 (wild-type <14.4	recessive (diploid)	-
Hanold *et al* (1972)	UV + Caffeine NNNG.	2	*act A*	99	recessive/partial dom.	
			act B	99	recessive	-
				(wild-type <14.4)	(heterokaryon)	
Tetrahymena thermophila (*T. pyriformis* syngen 1)						
Roberts & Orias (1971) Byrne and Bruns (1974) Bleyman & Bruns (1977) Byrne (1978)	NNNG.	1	*Chx - A*	>90 (wild-type <90)	dominant (heterokaryon)	-

Abbreviations: U.V = ultraviolet radiation; NNNG = N - methyl-N-nitro-nitrosoguanidine; EMS = ethylmethane sulphonate

for comparison the effect of cycloheximide on wild-type strains is included. Also stated is the cell-type used in the dominance test.

The response of a particular individual strain (or species) to cycloheximide is generally expressed as being either cycloheximide-sensitive or cycloheximide-resistant. In the collections of strains (or species) there is usually a considerable range in the cycloheximide concentrations which are inhibitory to growth. For convenience, the strains are often classified according to their growth response at an arbitarily chosen cycloheximide concentration, which varies depending on the species (Table 3.2). In Coprinus cinereus for example, the discriminatory cycloheximide concentration employed by North (1982) was 3.6 μM.

a) Dominance.

Genes which confer cycloheximide-resistance have been described as recessive, dominant or giving rise to an intermediate form of expression, partial or semi-dominant. (Table 3.2). In some instances, eg. Tetrahymena thermophilia (Bleyman and Bruns, 1977), because of the difficulties inherent in mating, the dominance tests have yielded ambiguous results. In general there is no consistency within or between species (Table 3.2).

The variability of the results may reflect the nature of the mutagenised cell-type, the cell-type in which the dominance test was performed or the range of cycloheximide concentrations used . In many species diploid cells were mutagenised so that in order to be expressed, the cycloheximide resistant genes must, of necessity, be dominant. The effect of nuclear interaction on the expression of cycloheximide - resistant genes is demonstrated in

Coprinus cinereus (North, 1982); cy-2^{r} was partially expressed in a dikaryon possessing the dominance modifier mod cy^{+}, but not in the diploid. In the many species including Podospora anserina (Crouzet et al, 1978) and Physarum polycephalum (Haugli et al, 1972) the dominance test was performed in heterokaryons, in which conflicting results were obtained. Depending on the proportion of cycloheximide resistant and cycloheximide-sensitive alleles, the cycloheximide-resistant mutations could be described as recessive or semi-dominant. In Neurospora crassa, cy-IR was described as dominant by Hsu (1963) and recessive by Turner (1976), as a consequence of high or low proportion of cycloheximide-resistant alleles in the heterokaryon.

A classification of partial dominance depends on the range of cycloheximide concentration used. It is possible that on inter-mediate response may be misconstrued as either recessive or dominant.

b) Modifier genes.

The presence of genes which do not confer cycloheximide resistance per se, but which affect the quantitative or qualitative expression of cycloheximide-resistance genes are known as modifier genes.

Genes which modify the quantitative response are known in Saccharomyces cerevisiae (Wilkie and Lee, 1965), Neurospora crassa (Hsu, 1963; Rothschild et al, 1975) and Physarum polycephalum (Haugli and Dove, 1972). The positive interaction between the

modifier genes and specific cycloheximide-resistant genes is observed in the ability of such strains to grow at far higher concentrations of cycloheximide than is possible for strains lacking the modifier genes.

Another type of modifier gene has been described by North (1978, 1982) in which the dominance of the cycloheximide-resistance allele cy-2^{r} was modified by modcy^{+} in the heterozygous cycloheximide-resistant dikaryon but not in the comparable diploid (Section 3.1 b i).

c) Suppressor genes.

The production of cycloheximide-resistant mutants is generally considered to be the gain of the ability to grow at previously inhibitory concentrations of cycloheximide; the majority of the examples presented in Table 3.2 are of this type. However, genes affecting the response to cycloheximide have also been detected by selecting for revertants which are considered to have lost a previously acquired ability to grow on cycloheximide.

Thus suppressor genes scr-1 and scr-2, which suppressed specifically the activity of the cycloheximide-resistant gene cyh-1 were described by Ibrahim and Coddington (1976) in Schizosaccharomyces pombe. In Schizosaccharomyces pombe, two resistant alleles of cyh-1 have anti-suppressor activity (Thuriaux et al, 1975). Similarly, mutations in the AS-3 gene lead to cycloheximide resistance in Podospora anserina (Coppin-Raynal, 1977).

d) Number and location of genes.

Our present knowledge of the genetics of the response to cycloheximide varies between species. In species which have been relatively thoroughly investigated, such as Saccharomyces cerevisiae or Schizosaccharomyces pombe (Table 3.2) there is evidence that a multigenic system is involved. In other species such as Tetrahymena thermophila or Aspergillus nidulans (Table 3.2) only a single gene has been identified. It is possible that different species possess different number of genes involved in the cycloheximide response, but more probable that not all genes which confer cycloheximide-resistance have been identified in the relatively few analyses. With reference to Coprinus cinereus, the genetic analysis performed by North (1978, 1982) studied relatively few mutants and observed four genes associated with the response to cycloheximide.

The location of the genes involved in the cycloheximide response is largely unknown. From such data which is available in Coprimus cinereus (North 1982). Saccharomyces cerevisiae (Wilkie and Lee, 1965) and Neurospora crassa, (Hsu,1963) the cycloheximide-resistant genes and other genes affecting their action are dispersed throughout their respective genomes .

SECTION 3.3. PLEIOTROPHIC EFFECT OF CYCLOHEXIMIDE RESISTANT GENES.

There are several instances known of the pleiotrophic nature of genes conferring cycloheximide-resistance.

a) Cross-resistance with other protein synthesis inhibitors.

Ibrahim and Coddington (1976) demonstrated that three cycloheximide genes, cyh-2, cyh-3 and cyh-4 in Schizosaccharomyces pombe also conferred resistance to trichodermin and anisomycin Both trichodermin and anisomycin are eukaryotic protein synthesis inhibitors but have dissimilar modes of action and unrelated structures to cycloheximide (Pestka,1977), The explanation for cross-resistance proposed by Ibrahim and Coddington was that a single gene mutation rendered the cells less permeable to all three antibiotics.

b) Cycloheximide-resistance and temperature-sensitivity.

Another manifestation of the pleiotrophic nature of cycloheximide-resistant genes is their sensitivity at restrictive temperatures.

Several mutants selected for resistance to cycloheximide have been discovered to be cold-sensitive (Crouzet and Bégueret, 1978; Ibrahim and Coddington, 1976), but in one instance a mutant selected for cold-sensitivity was found to be resistant to cycloheximide (Waldron and Roberts, 1974).

In Schizosaccharomyces pombe, Ibrahim and Coddington (1976) observed that the inability to grow at 14°C was associated with two cycloheximide-resistant genes cyh-2 and cyh-3. Two suppressor genes of cycloheximide-resistance, scr-1 and scr-2, identified by the same authors (1978) in the same organism, were also found to be cold-sensitive.

Cold-sensitivity in Podospora anserina was found to be a property of the combined action of two cycloheximide-resistance genes CyR-1 and CyR-2 (Crouzet and Bégueret, 1978). Strains possessing only one mutation grew normally at both the permissive (26°C) and restrictive (13°C) temperatures. Cold-sensitivity was suppressed by spontaneous reversions in either of the two cycloheximide-resistant genes or by any of three suppressor genes which acted upon CyR-1 and CyR-2.

Cold-sensitive mutants in Aspergillus nidulans were associated with cycloheximide-resistance arpA and in one instance with ultra-sensitivity to cycloheximide, arpB (Waldron and Roberts, 1974). The response to cycloheximide and the inability to grow at 20°C were the results of single mutations which were found to possess abnormal cytoplasmic ribosomal sedimentation profiles at the non-permissive temperature.

SECTION 3.4. OBJECTIVES.

North (1978, 1982) described the production and genetic analysis of cycloheximide-resistant mutants in Coprinus cinereus.

The objectives of the experiments described in this Chapter were to produce cycloheximide-resistant strains, in a more varied genetic background than North had used.

With a larger number of cycloheximide-resistant strains available for analysis, it was probable that new genes conferring cycloheximide-resistance could be identified. There would also be an increased probability of identifying those strains possessing cycloheximide-resistant cytoplasmic ribosomes (Chapter 5), and hence modified cytoplasmic ribosomal proteins (Chapter 6), if more mutants were available.

SECTION 3.5. PRODUCTION OF CYCLOHEXIMIDE-RESISTANT MUTANTS.

a) Spontaneous mutation frequency.

No spontaneous cycloheximide-resistant mutants were obtained from any of the cycloheximide-sensitive strains examined; CY 3, CY 6, CY 8, CY 9, CY 10 and CY 11. Only a relatively small number of oidiospores were set aside for the determination of the spontaneous mutation frequency which was estimated to be less than 2×10^{-6}.

In other species values for the spontaneous mutation frequency ranged from 5×10^{-3} in Chinese hamster ovary cells (Pöche *et al*, 1979) to 9.3×10^{-9} in *Physarum polycephalum* (Haugli *et al*, 1972).

As a consequence of the inability to select spontaneously arising cycloheximide-resistant mutants a method of induced mutagenesis was employed.

b) Ultraviolet-radiation induced mutation frequency.

Irradiation of the oidiospores with ultraviolet light had been used by North (1982) to induce cycloheximide-resistant mutants in *Coprinus cinereus*. The mutagen had been commonly used for the same purpose in numerous other species (eg. Neuhäuser *et al*, 1970 in *Neurospora crassa*; Wilkie and Lee, 1965 in *Saccharomyces cerevisiae* and Haugli and Dove, 1972, in *Physarum polycephalum*) and a study by Ibrahim and Coddington

Table 3.3. Induction of cycloheximide-resistant mutants by ultraviolet radiation.

Strain	Number of experiments	% kill by UV. $\bar{x}$	Number of cycloheximide-resistant mutants	Ultraviolet radiation induced mutation frequency ($\times 10^{-4}$) $\bar{x}$	($\pm$ se)
CY 3	6	99.9	14	1.3	(0.2)
CY 6	1	98.2	11	6.6	
CY 8	2	96.7	25	4.0	(0.8)
CY 9	6	83.3	121	5.5	(1.2)
CY 10	1	93.4	2	5.7	
CY 11	1	99.1	1	1.7	
			Total 174		

Cycloheximide-resistant mutants were produced as described in (Chapter 2, Section 2.4). Mutation frequencies were calculated from the number of viable oidiospores after 7.5 minutes irradiation.

The production of cycloheximide-resistant mutants in this investigation was 100x more frequent than observed by North (1982). The higher mutation frequencies (Table 3.3) were attributed to the larger dose of ultraviolet radiation and slightly longer exposure (modified from North's technique; Chapter 2, Section 2.4). Neither analysis determined the conditions for maximum mutation frequency.

The cycloheximide-resistant mutation frequency induced by ultraviolet radiation in other species varied from 1×10^{-5} (Neurospora crassa, Neuhäuser et al, 1970) to 1.2×10^{-7}, with caffeine (Physarum polycephalum, Haugli and Dove, 1972). Ultraviolet radiation induced reversion to cycloheximide-sensitivity in Schizosaccharomyces pombe occurred at a frequency of 3×10^{-3} (Ibrahim and Coddington, 1978).

Cycloheximide-resistant mutant strains isolated and confirmed, were subjected to a physiological and genetical analysis in order to determine the nature of the mutation.

SECTION 3.6. NUMBER OF GENES CONFERRING CYCLOHEXIMIDE-RESISTANCE IN EACH STRAIN.

In each of the crosses, between cycloheximide-resistant mutant strains and compatible cycloheximide-sensitive test strains, which resulted in fertile dikaryons the ratio of cycloheximide - resistant : cycloheximide-sensitive basidiospore progeny was unity (χ^2 analysis $p>0.9$, from the segregation values presented in Table 3.4).

In accordance with Mendelian theory of segregation, a segregation ratio of 1:1 was interpreted as being the consequence of a cycloheximide resistance mutation at a single locus , or possibly , at two closely linked loci.

The response of the basidiospores to cycloheximide was determined at only one concentration. Consequently, it was not possible to infer from the analysis whether any of the cycloheximide-resistant mutants tested (Table 3.4) , possessed genes which modified the level of cycloheximide resistance : such as described by Wilkie and Lee (1965) in Saccharomyces cerevisiae. Nor was it possible to identify the presence of genes which modified the dominance of the cycloheximide genes , such as modcy$^+$ (North, 1982) from the results in Table 3.4.

NCE

istant

ains,

-

nity

able 3.4).

on,

uence

de was

ximide-

odified

ilkie

e to

the

sults

Table 3.4. Segregation of resistance mutations in basidiospore progeny

Cross	Segregation	
	Resistant	Sensitive
CY 3.1 x CY 9	46	47
CY 3.3 x CY 9	42	40
CY 3.5 X CY 9	40	43
CY 3.7 x CY 9	44	46
CY 3.8 X CY 9	47	44
CY 3.16 x CY 9	53	55
CY 6.1 x CY 18	46	48
CY 6.2 x CY 18	45	42
CY 6.3 x CY 18	43	46
CY 6.5 x CY 18	48	44
CY 6.6 x CY 18	47	48
CY 6.9 x CY 18	50	48
CY 6.11 x CY 18	52	53
CY 8.2 x CY 13	60	57
CY 8.4 x CY 13	48	44
CY 8.6 x CY 13	53	52
CY 8.7 x CY 13	51	46
CY 8.8 x CY 13	44	47
CY 8.9 x CY 13	48	49
CY 8.10 x CY 13	44	46
CY 8.12 x CY 13	42	40
CY 8.13 x CY 13	42	44
CY 8.18 x CY 13	43	46
CY 8.20 x CY 13	47	45
CY 8.23 x CY 13	48	47
CY 8.24 x CY 13	45	47
CY 8.40 x CY 13	50	52

The analysis was performed as described in Chapter 2, Section 2.5 b , Cycloheximide-resistance was identified as the growth of the haploid basidiospore colonies on 3.6 μM cycloheximide.
CY 3 x CY 9, CY 6 x CY 18, and CY 8 x CY 13 produced only cycloheximide-sensitive progeny.
The table excludes crosses which failed to dikaryotise, particularly the CY 9 mutants with CY 3, and dikayons which did not fruit.

GROWTH RESPONSE OF STRAINS TO CYCLOHEXIMIDE.

SECTION 3.7. GENERAL OBSERVATIONS.

a) Lag period.

For all strains grown on medium lacking cycloheximide there was no measureable growth within the first 24 hours incubation at 37°C. For strains grown at relatively high concentrations of cycloheximide, although the particular concentration depended upon the strain in question, there was generally no growth after 48 hours or more incubation (results not presented).

The period of incubation in which no growth occurred is a general phenomenon applying in the presence or absence of cycloheximide. In the presence of cycloheximide, the lag phase has been attributed to the period necessary to develop resistance to the antibiotic (Wilkie and Lee, 1965) for Saccharomyces cerevisiae). The higher the cycloheximide concentration employed, the more prolonged the lag phase was observed to be.

b) Constant growth rate.

Once the lag period had been completed, growth continued at a constant growth rate until the petri-dish was completely filled. For monokaryotic strains the average increase in colony diameter was approximately 0.7 $cm.day^{-1}$ in the absence of cycloheximide and 0.5 $cm.day^{-1}$ at the 50% growth inhibitory cycloheximide concentration.

The dikaryon and diploid strains also grew at constant rates. The growth rate of the dikaryons in the absence of cycloheximide was approximately 1.0$cm.day^{-1}$, almost 2 x greater than at the 50% inhibitory cycloheximide concentration. Diploids were the least vigorous cell types with a growth rate of 0.4$cm.day^{-1}$ in the absence of cycloheximide and slightly less in the presence of cycloheximide.

c) Morphology.

Observations of Coprinus cinereus strains atsub-lethal concentrations of cycloheximide did not appear to produce noticeable morphological changes to either the hyphae or the colony growth in any of the cycloheximide-resistant mutants. (also North, 1982). It is only infrequently that morphological changes have been observed, e.g. Dee and Poulter (1970) with Physarum polycephalum.

d) Irreversible effect on growth.

Inoculum which failed to grow when exposed to relatively high cycloheximide concentrations, the precise concentrations depending upon the strains examined, did not grow when transferred to medium lacking cycloheximide(ie.fungicidal).Wilkie and Lee (1965) observed a similar phenomenon with Saccharamyces cerevisiae cells but Kerridge (1958) with Saccharamyces carlsbergensis , Cooney and Bradley (1962) with Tetrahymena thermophilia and Dee (1966) with Physarum polycephalum,observed resumption of growth after transfer to cycloheximide-free medium (ie.fungistatic).

e) Typical response to cycloheximide.

The typical response to cycloheximide observed in this investigation was biphasic. At relatively low cycloheximide concentrations there was no noticeable inhibition of growth. At higher cycloheximide concentrations growth was inversly proportional to the log 10 cycloheximide concentration and, ultimately growth was totally inhibited. Inhibition of growth had a linear correlation coefficient of between -0.9 and -1.0 in all strains, when plotted as a semi-log scale against log 10 cycloheximide concentration. Two parameters used to define this phase were the 50% inhibitory cycloheximide concentration and linear regression coefficient, calculated from a linear regression analyses.

c) Morphology.

Observations of Coprinus cinereus strains atsub-lethal concentrations of cycloheximide did not appear to produce noticeable morphological changes to either the hyphae or the colony growth in any of the cycloheximide-resistant mutants. (also North, 1982). It is only infrequently that morphological changes have been observed, e.g. Dee and Poulter (1970) with Physarum polycephalum.

d) Irreversible effect on growth.

Inoculum which failed to grow when exposed to relatively high cycloheximide concentrations, the precise concentrations depending upon the strains examined, did not grow when transferred to medium lacking cycloheximide(ie. fungicidal). Wilkie and Lee (1965) observed a similar phenomenon with Saccharamyces cerevisiae cells but Kerridge (1958) with Saccharamyces carlsbergensis , Cooney and Bradley (1962) with Tetrahymena thermophilia and Dee (1966) with Physarum polycephalum, observed resumption of growth after transfer to cycloheximide-free medium (ie. fungistatic).

e) Typical response to cycloheximide.

The typical response to cycloheximide observed in this investigation was biphasic. At relatively low cycloheximide concentrations there was no noticeable inhibition of growth. At higher cycloheximide concentrations growth was inversly proportional to the log 10 cycloheximide concentration and, ultimately growth was totally inhibited. Inhibition of growth had a linear correlation coefficient of between -0.9 and -1.0 in all strains, when plotted as a semi-log scale against log 10 cycloheximide concentration. Two parameters used to define this phase were the 50% inhibitory cycloheximide concentration and linear regression coefficient, calculated from a linear regression analyses.

The cycloheximide concentrations at which these distinctive phases occurreddepended on the strain (Sections 3.9, 3.10 and 3.11).

f) Definition of cycloheximide-resistance.

The definition employed in this investigation was that used by North (1982). A cycloheximide-resistant monokaryotic or diploid strain is one which is able to grow on 3.6 μM cycloheximide after 3 days incubation at 37°C. Under the same environmental conditions,a cycloheximide-resistant dikaryon is one which grows on 1.8 μM.

SECTION 3.8. THE GROWTH RESPONSE OF MONOKARYOTIC STRAINS TO CYCLOHEXIMIDE.

a) Cycloheximide-resistant mutant strains derived from CY 3.

Of the 9 cycloheximide-resistant mutant strains derived from the cycloheximide-sensitive strain CY 3, CY 3.16 was the most resistant. CY 3.16 was approximately 2 x more resistant than any other mutant strain and 270 x more resistant than CY 3. (Figure 3.2).

CY 3.16 was significantly different from any other mutant strain based on the calculated 50% growth inhibitory cycloheximide concentration (Table 3.5; $p < 0.001$). A proposed classification of the strains based on the 50% inhibitory cycloheximide concentrations produced three groups: CY 3.16; CY 3.17, CY 3.7 and CY 3.2; and all other strains (Table 3.5; Figure 3.2). With the exception of Group 1 (Table 3.5) there was no significant difference within the group ($p > 0.5$) and a significant difference between the groups of ($p < 0.01$). It was considered that the strains in Group 1 might be subdivided into Groups 1a and 1b (Table 3.5) but that the differences were not sufficiently large to warrant classification into distinct groups.

In addition to the 50% inhibitory cycloheximide concentration, three other parameters of the growth response were recorded (Table 3.5). Classifications of the cycloheximide-resistant mutants based on the minimum and total inhibitory cycloheximide

Table 3.5. Summary of the growth response of CY 3, and strains derived from it, to cycloheximide and their classification based on calculated 50% growth inhibitory cycloheximide concentrations.

Table 3.5. Summary of the growth response of CY 3, and strains derived from it, to cycloheximide and their classification based on calculated 50% growth inhibitory cycloheximide concentrations.

Group	Strains.	Calculated 50% growth inhibitory cycloheximide concentration(μM).	Significance values within and between groups.	Calculated minimum cycloheximide concentration for inhibition (μM).	Observed total growth inhibitory cycloheximide concentration (μM).	Linear regression coefficient.
	CY 3	0.03		0.01	0.09	-101
			$p<0.001$			
1a	CY 3.5	1.8		0.4	8.9	-82
			$p>0.5$			
	CY 3.6	1.9		0.3	8.9	-67
	CY 3.9	2.5		0.2	27	-49
			$p<0.05$			
1b	CY 3.3	3.1		0.6	18	-73
			$p>0.5$			
	CY 3.8	3.2		0.4	18	-55
			$p<0.01$			
2	CY 3.17	4.1		0.6	18	-59
			$p>0.5$			
	CY 3.7	4.2		1.0	18	-80
	CY 3.2	4.3		0.8	27	-70
			$p<0.001$			
3	CY 3.16	8.1		1.5	53	-67

Original data is given in Tables A1 and A6, Appendix A.
The cycloheximide concentrations for minimum, and 50% growth inhibition were calculated from linear regression analyses over the inhibitory phase of the response to cycloheximide, (Appendix A). The linear correlation coefficients ranged from -0.92 to -0.98. Values presented for total inhibition were the minimum cycloheximide concentrations at which no growth was observed.

Figure 3.2. The effect of cycloheximide on the growth of strains derived from CY 3.

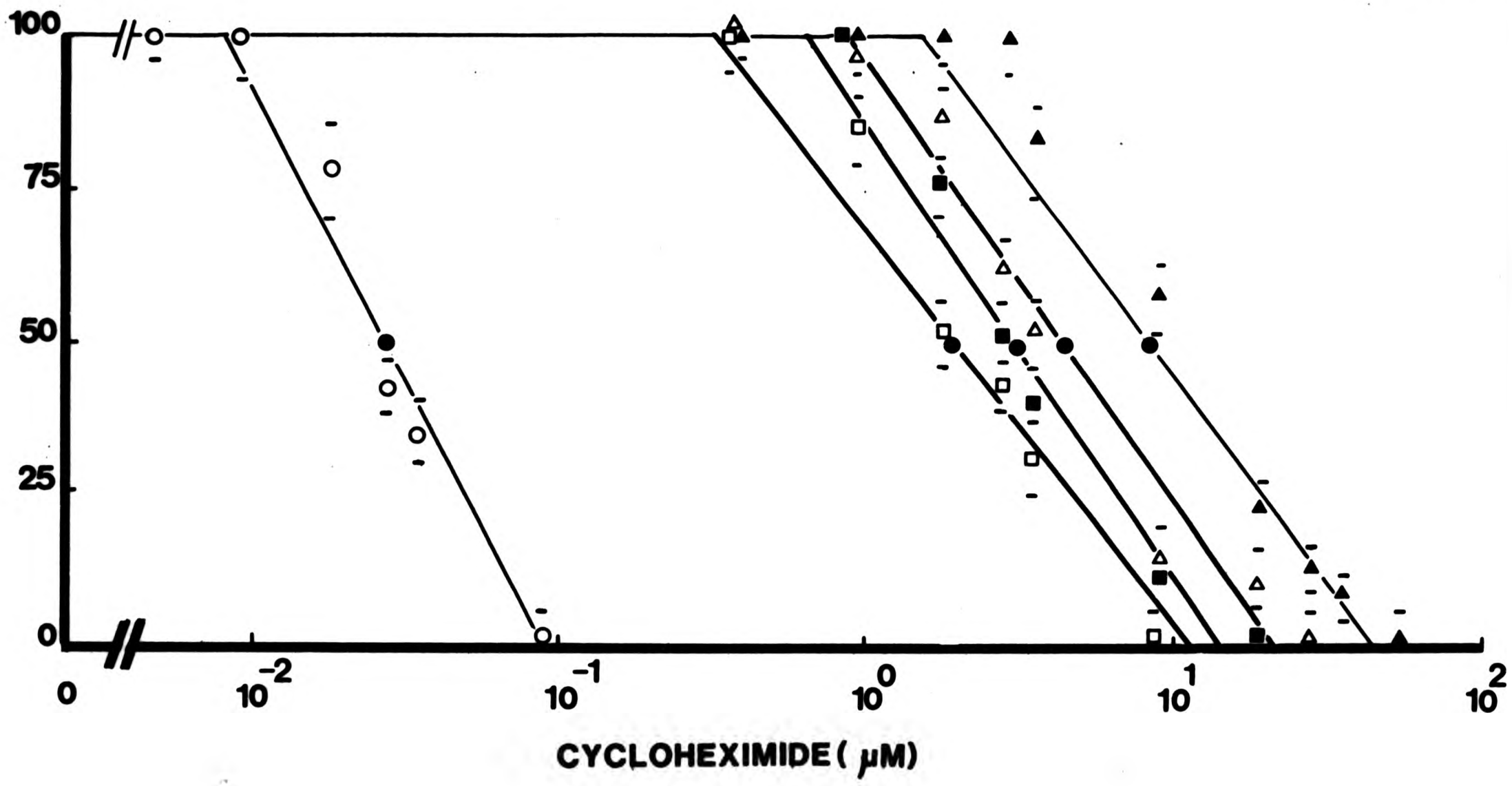
Average colony diameters from two experiments were measured for each strain over a range of cycloheximide concentrations, and the growth was expressed as a % of the uninhibited growth on 0μM. cycloheximide (Chapter 2, Section 2.5). Original data is given in Tables A1 and A6 in Appendix A.

Growth responses presented as regression lines over the cycloheximide concentrations which were inhibitory. In order to clarify the presentation, where appropriate one strain has been used to typify more than one response. Thus;

Strain	Symbol	
CY 3	○	
CY 3.6	□	The region bounded by CY 3.6 and CY 3.3 includes the responses of CY 3.5, CY 3.9 and CY 3.8.
CY 3.3	■	
CY 3.2	△	Represents CY 3.17 and CY 3.7.
CY 3.16	▲	

The 50% growth inhibitory value has been indicated (●).

FIG.3.2



concentrations were similar to the one proposed based on the 50% inhibitory cycloheximide concentration.

In contrast, an entirely different classification was possible if the linear regression coefficients were used. Such a classification grouped together CY 3.16 and CY 3.9 when CY 3.16 was 4 x more resistant than CY 3.9 and yet had CY 3.5 and CY 3.6 in different groups when they had the same 50% inhibitory cycloheximide concentration. Thus, the preferred classification was based on the 50% inhibitory cycloheximide concentration rather than on any other parameter of the cycloheximide dose-response.

b) Cycloheximide-resistant mutant strains derived from CY 6.

CY 6.1 and CY 6.2 were the most resistant of the seven strains examined and both were capable of growing at the highest cycloheximide concentration employed (712 μM). CY 6.1 and CY 6.2 were approximately 200 x more resistant to cycloheximide than the cycloheximide-sensitive CY 6, from which they were derived and approximately 10 x more resistant than any of the other strains derived from CY 6 (Figure 3.3).

Based on a statistical analysis of the calculated 50% growth inhibition cycloheximide concentration the seven cycloheximide-resistant mutant strains were classified two groups; CY 6.1 and CY 6.2 and all other strains (Table 3.6). The same grouping was also achieved if any of the other three parameters of the

concentrations were similar to the one proposed based on the 50% inhibitory cycloheximide concentration.

In contrast, an entirely different classification was possible if the linear regression coefficients were used. Such a classification grouped together CY 3.16 and CY 3.9 when CY 3.16 was 4 x more resistant than CY 3.9 and yet had CY 3.5 and CY 3.6 in different groups when they had the same 50% inhibitory cycloheximide concentration. Thus, the preferred classification was based on the 50% inhibitory cycloheximide concentration rather than on any other parameter of the cycloheximide dose-response.

b) <u>Cycloheximide-resistant mutant strains derived from CY 6</u>.

CY 6.1 and CY 6.2 were the most resistant of the seven strains examined and both were capable of growing at the highest cycloheximide concentration employed (712 μM). CY 6.1 and CY 6.2 were approximately 200 x more resistant to cycloheximide than the cycloheximide-sensitive CY 6, from which they were derived and approximately 10 x more resistant than any of the other strains derived from CY 6 (Figure 3.3).

Based on a statistical analysis of the calculated 50% growth inhibition cycloheximide concentration the seven cycloheximide-resistant mutant strains were classified two groups; CY 6.1 and CY 6.2 and all other strains (Table 3.6). The same grouping was also achieved if any of the other three parameters of the

Table 3.6. Summary of the growth response of CY 6, and strains derived from it, to cycloheximide and their classification based on calculated 50% growth inhibitory cycloheximide concentration.

Group.	Strain.	Calculated cycloheximide concentration for 50% growth inhibition (μM).	Significance values within and between groups.	Calculated minimum cycloheximide concentration for inhibition (μM).	Observed cycloheximide concentration for total growth inhibition (μM).	Linear regression coefficient.
	CY 6	0.21		0.01	0.89	-36
1	CY 6.6	3.0	$p < 0.001$	0.4	27	-56
	CY 6.11	3.4		0.5	27	-61
	CY 6.5	3.5	$p > 0.05$	0.5	27	-59
	CY 6.9	3.6		0.5	27	-61
	CY 6.3	3.7		0.6	27	-64
2	CY 6.2	39	$p < 0.001$	2.1	^{E} 750	-39
	CY 6.1	41	$p > 0.5$	2.2	^{E} 760	-40

Original data is given in Tables A2 and A6 of Appendix A.

The parameters of the response to cycloheximide were determined from a linear regression over the inhibitory phase of the response, (Appendix A). Linear correlation coefficients ranged from -0.92 to -0.99. Values presented for total inhibition were the minimum cycloheximide concentrations at which no growth was observed, except for CY 6.1^{E} and CY 6.2^{E} for which extrapolated values were used because growth occurred at the highest cycloheximide concentration examined, (712 μM).

Figure 3.3. The effect of cycloheximide on the growth of strains derived from CY 6.

The measurement and presentation of the growth responses of CY 6 strains to cycloheximide, are as described for Figure 3.2.

In order to clarify the presentation, where appropriate, one strain has been used to typify more than one response. Thus;

Strain	Symbol	
CY 6	○	
CY 6.1	□	Represents CY 6.2
CY 6.5	△	Represents CY 6.3, CY 6.6, CY 6.9 and CY 6.11

The 50% growth inhibitory value has been indicated (●).
The extrapolated response of CY 6.1 has been indicated (--------).
Original data is given in Tables A3 and A6 of Appendix A.

trains

es of

3.2.

e,

---).

FIG.3.3

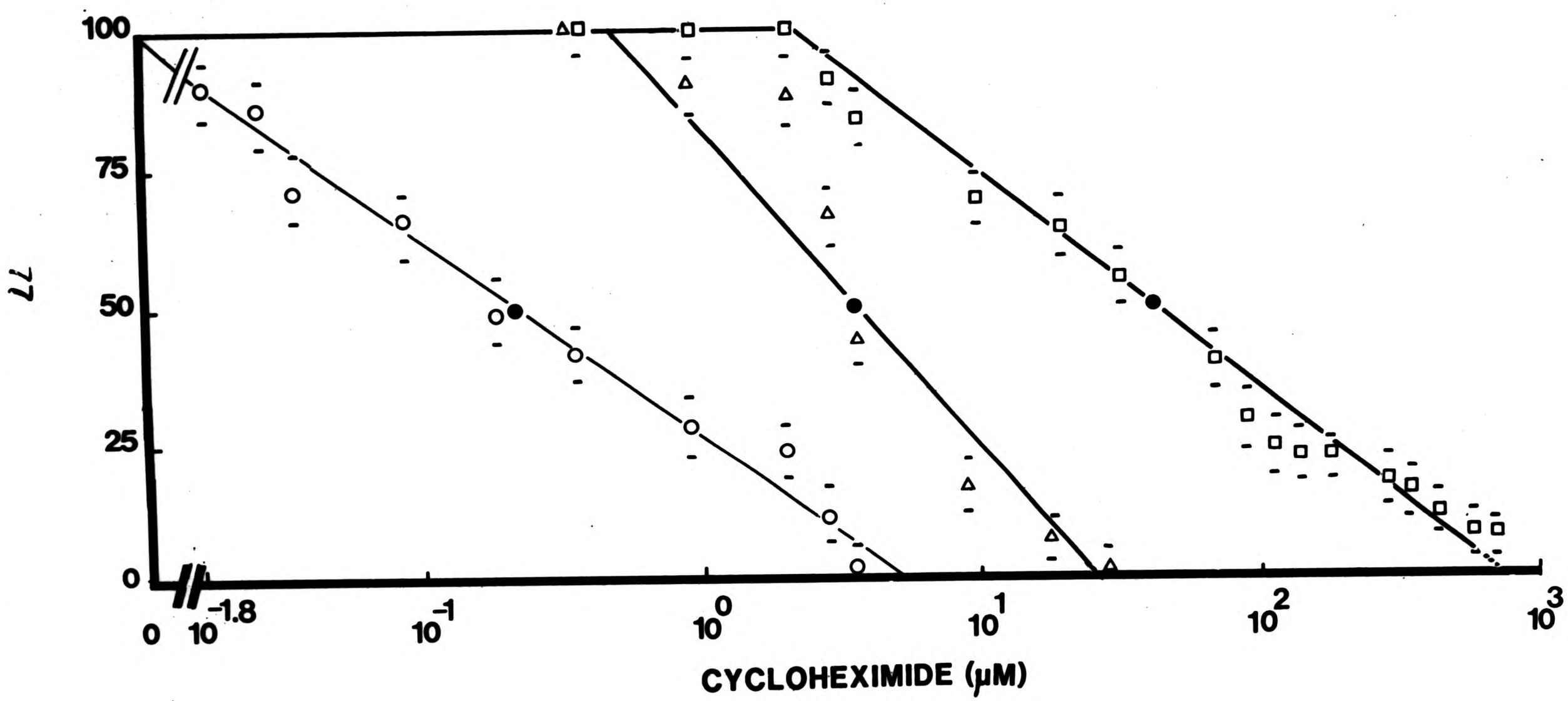


Table 3.7. Summary of the growth response of CY 8, and strains derived from it, to cycloheximide and their classification based on calculated 50% growth inhibitory cycloheximide concentrations.

Group.	Strain.	Calculated cycloheximide concentration for 50% growth inhibition (μM).	Significance values within and between groups.	Calculated minimum cycloheximide concentration for inhibition (μM).	Observed cycloheximide concentration for total growth inhibition (μM).	Linear regression coefficient.
	CY 8.	0.1	$p < 0.001$	0.01	1.8	-58
1a	CY 8.12	2.0		0.3	18	-55
	CY 8.18	2.3		0.3	18	-53
	CY 8.5	2.5		0.4	18	-62
	CY 8.8	2.5		0.4	18	-63
	CY 8.7	2.7		0.5	18	-69
	CY 8.9	2.8	$p < 0.01$	0.5	18	-64
	CY 8.10	3.0		0.6	18	-75
	CY 8.19	3.0		0.6	18	-70
	CY 8.20	3.1		0.4	18	-59
	CY 8.40	3.1		0.5	18	-61
	CY 8.23	3.2		0.7	18	-78
	CY 8.24	3.2		0.4	36	-58
	CY 8.4	3.2		0.5	18	-60
1b	CY 8.22	3.9	$p < 0.05$	0.4	36	-52
	CY 8.6	4.2	$p > 0.5$	1.4	18	-88
2	CY 8.2	37.0	$p < 0.001$	2.0	E_{750}	-40

Original data is given in Tables A3 and A6 of Appendix A.

The parameters of the responses to cycloheximide were as described for Table 3.5. Linear correlation coefficients ranged from -0.91 to -0.97 . The value for total inhibition of CY 8.2^{E} is extrapolated, because the strain grew at the highest cycloheximide concentration used.

response to cycloheximide were also used as a basis of classification (Table 3.6). The linear regression coefficients of CY 6.1 and CY 6.2 were not significantly different from that of CY 6, but were significantly lower than all other strains (Table 3.6).

c) <u>Cycloheximide-resistant strains derived from CY 8</u>.

CY 8.2 was the most resistant mutant derived from CY 8, being approximately 370 x more resistant than CY 8 and almost 10 x more resistant than any other mutant strain (Figure 3.4).

Based on the 50% growth inhibitory cycloheximide concentration the sixteen strains examined could be grouped into two major catagories; CY 8.2 and all other strains (Table 3.7). Only 2.2 μM, a 2 fold difference separated the 50% inhibitory cycloheximide concentrations of the majority of strains, from Group 1 (Table 3.7). Possible subdivisions of Group 1, Group 1a CY 8.12 to CY 8.4 inclusive (Table 3.7) and Group 1b, CY 8.22 and CY 6 (Table 3.7) are proposed by the statistical evidence for their existence is not as convincing as the differences between Groups 1 and 2 (Table 3.7).

d) <u>Cycloheximide-resistant strains derived from CY 9.</u>

The parameters of the response to cycloheximide of 31 cycloheximide-resistant mutant strains produced in this investigation from CY 9 (Chapter 2, Section 2.4) are given in Table 3.8. There was a five fold difference between the most resistant strain CY 9.77 and the least resistant strain, CY 9.29

Figure 3.4. The effect of cycloheximide on the growth of strains derived from CY 8.

The measurement and presentation of the growth responses of CY 8 strains to cycloheximide, are as described for Figure 3.2.

In order to clarify the presentation, where appropriate, one strain has been used to typify more than one response. Thus:

Strain	Symbol	
CY 8	○	
CY 8.12	□	The region bounded by CY 8.12 and CY 8.4 includes the responses of all other strains not referred to specifically.
CY 8.4	■	
CY 8.6	△	
CY 8.2	▲	

The 50% growth inhibitory value has been indicated (●).

The extrapolated response of CY 8.2 has been indicated (------)

Original data is given in Tables A4 and A6 in Appendix A.

FIG.3.4

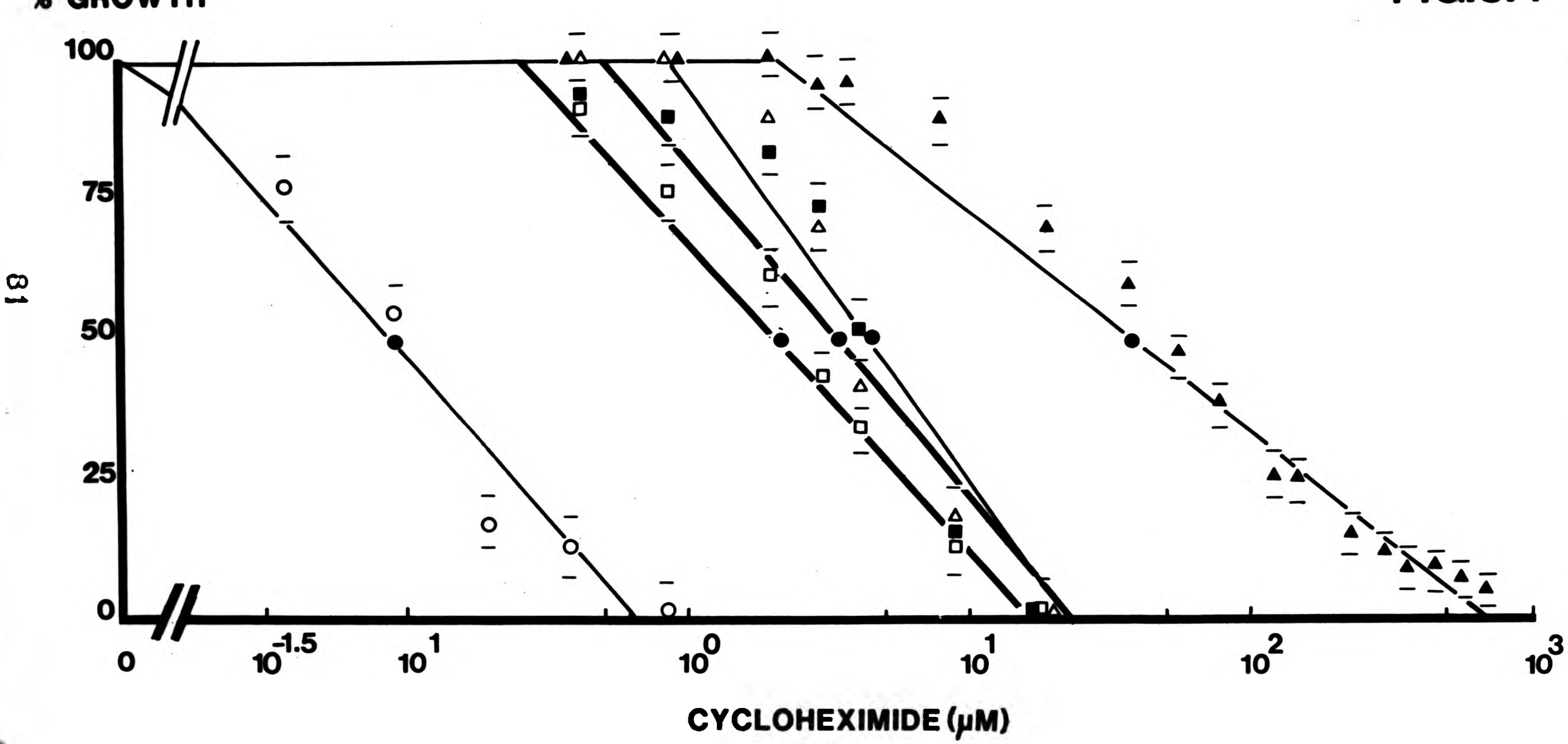

% GROWTH
100
75
50
25
0
0
$10^{-1.5}$
10^{1}
10^{0}
10^{1}
10^{2}
10^{3}
CYCLOHEXIMIDE (μM)

Figure 3.5. The effect of cycloheximide on the growth of strains derived from CY 9.

The measurement and presentation of the growth responses of CY 9 strains to cycloheximide are as described for Figure 3.2.

In order to clarify the presentation, where appropriate, one strain has been used to typify more than one response.

Thus;

Strain	Symbol	
CY 9	○	
CY 9.87	△	} The region bounded by CY 9.87 and CY 9.77 includes the responses of all other strains not referred to specifically.
CY 9.77	▲	
CY 9.29	□	
CY 9.31	■	
CY 9.23	a	
CY 9.23.98	b	
CY 9.23.137	c	
CY 9.23.138	d	

The 50% growth inhibitory value has been indicated (●). The extrapolated responses of CY 9.23, CY 9.23.98 and CY 9.23.138 have been indicated (-------). Original data is given in Tables A5 and A6 of Appendix A.

FIG.3.5

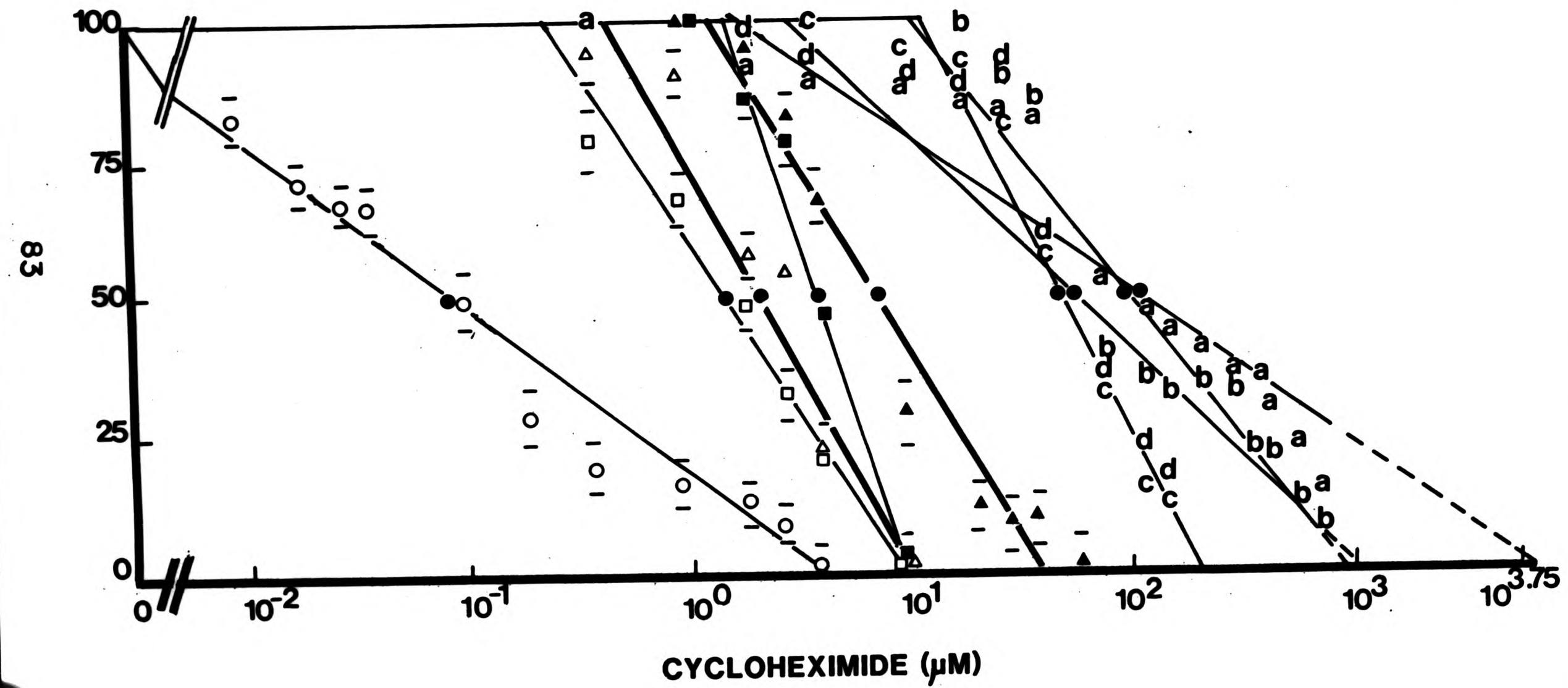

% GROWTH
100
75
50
25
0
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
$10^{3.75}$
CYCLOHEXIMIDE (μM)

Table 3.8. Summary of the growth response of CY9 and strains derived from it to cycloheximide and their classification based on calculated 50% growth inhibitory cycloheximide concentrations.

Group	Strain	Calculated 50% growth inhibitory cyclo-heximide concentration.	Observed total growth inhibitory cycloheximide concentration.	Linear regression coefficient.
	CY9	0.08	3.6	-31
1	CY9.29	1.4	8.9	-57
	CY9.89	1.4	8.9	-62
	CY9.87	2.1	8.9	-74
	CY9.10	2.6	18	-63
	CY9.35	3.0	18	-63
	CY9.30	3.4	8.9	-61
	CY9.31	3.7	8.9	-131
	CY9.48	4.0	36	-54
	CY9.66	4.1	27	-53
	CY9.75	4.1	18	-59
	CY9.71	4.2	36	-52
	CY9.103	4.2	27	-57
	CY9.88	4.3	27	-65
	CY9.151	4.5	36	-53
	CY9.85	4.8	36	-59
	CY9.117	4.8	27	-59
	CY9.116	4.9	53	-50
	CY9.70	4.9	27	-58
	CY9.37	5.0	27	-55
	CY9.61	5.1	36	-54
	CY9.150	5.2	36	-55
	CY9.73	5.3	36	-53
	CY9.118	5.4	36	-55
	CY9.129	5.5	27	-50
	CY9.69	5.5	27	-69
	CY9.86	5.6	27	-70
	CY9.16	5.7	36	-64
	CY9.127	5.9	27	-72
	CY9.64	6.3	36	-67
	CY9.77	7.0	53	-67
2	* CY9.23.137	47	200E	-80
	* CY9.23.138	54	1000E	-40
	* CY9.23.98	96	900E	-51
	* CY9.23	100	5100E	-29

Original data is given in Tables A4,A5 and A6, Appendix A
The parameters of the responses to cycloheximide were determined as described for Table 3.5. Linear correlation coefficients ranged from -0.86 to -0.99E. The values for total inhibition, for strains provided by North*, were extrapolated.

but because the 50% inhibitory cycloheximide concentrations were continuous, there was insufficient dissimilarity to propose more than one major group of strains. It was possible that the group could be subdivided but there was no clearly defined discriminatory values which could be used to define statistically significant groups. (Table 3.8). Examples of several of the responses to cycloheximide are presented in Figure 3.5.

In addition to the cycloheximide-resistant mutants derived from CY 9 in this investigation, the growth response to cycloheximide of four strains, made available by North, were also examined (Figure 3.5, Table 3.8). All of North's strains, exhibited a level of resistance which was at least 17.5 x greater than the resistance observed in strains produced by this investigation. The 50% inhibitory cycloheximide concentrations of CY 9.23 and the recombinant strain derived from it, CY 9.23.98, were significantly different from CY 9.23.137 and CY 9.23.138 ($p < 0.001$), yet all four strains possessed the same cycloheximide-resistance mutation <u>cy-2^r</u>.

e) <u>Other strains</u>

The responses to cycloheximide of the cycloheximide-sensitive CY strains and two of their ancestors SR 54 and WMR 66A were not significantly different from each other in terms of their 50% inhibitory cycloheximide concentrations ($p > 0.5$; Figure 3.6, Table 3.9). All of these strains were totally inhibited by less than 1.0 µM cycloheximide except CY 9, which grew up to 3.6 µM.

Figure 3.6. The effect of cycloheximide on the growth of various strains.

The measurement and presentation of the growth responses of various strains to cycloheximide, are as described in Figure 3.2.

In order to clarify the presentation, where appropriate, one strains has been used to typify more than one response.

Thus;

Strain	Symbol	
CY 18	○	
CY 14	△	] The region bounded by CY 14 and CY 8 includes the responses of CY 13, SR 54 and WMR 66A.
CY 8	▲	
CY 9	□	
CY 6	6	
CY 3	■	
H2	2	Represents H1 H5 H9 and TC4

The 50% growth inhibitory value has been indicated (●).

The responses of CY 3, CY 6, CY 8 and CY 9 have been presented in Figures 3.2. 3.3, 3.4 and 3.5.

nses

late, one

8
R 54

nted

FIG.3.6

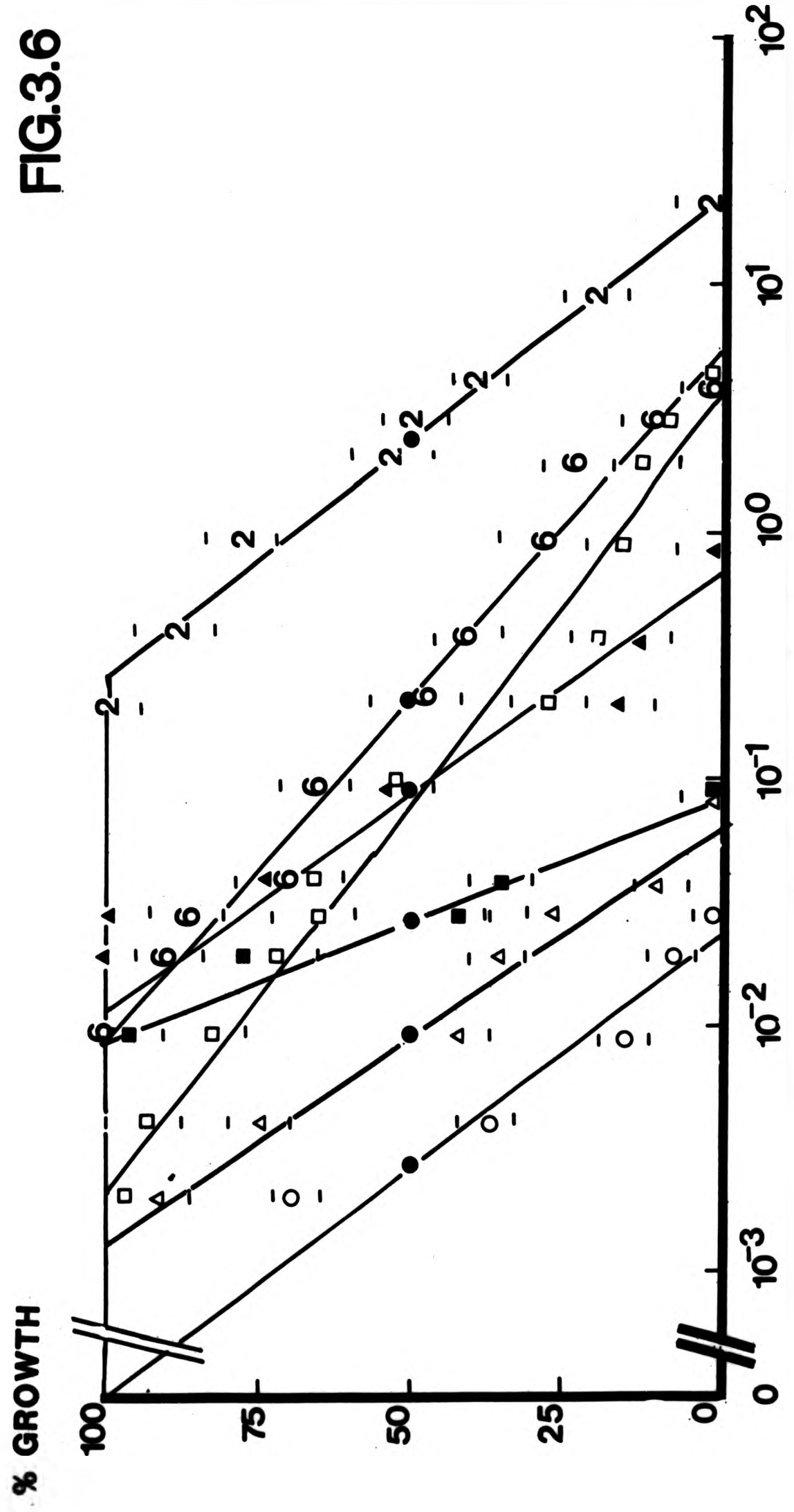


Table 3.9. Summary of the growth response to cycloheximide of cycloheximide-sensitive, wild and test strains.

Strain	Cycloheximide concentration (μM) 50% inhibition (calculated)	total inhibition (observed)	Linear regression coefficient.
CY 18	0.003	0.03	-55
CY 14	0.01	0.09	-59
CY 13	0.01	0.18	-55
CY 3	0.03	0.09	-101
SR 54	0.04	0.18	-51
WMR 66A	0.08	0.89	-52
CY 9	0.08	3.6	-31
CY 8	0.09	0.89	-58
CY 6	0.21	0.89	-36
H5	2.2	17.8	-59
H1	2.2	8.9	-70
H2	2.3	17.8	-52
H9	2.4	17.8	-51
TC 4	3.3	8.9	-64

Data for CY 3, CY 6, CY 8 and CY 9 is reprinted from Tables 3.5, 3.6, 3.7 and 3.8.

Original data is given in Appendix A, Tables A5 and A6.

When initially used CY 9 had been classified as cycloheximide-sensitive because it did not grow on 3.6 μM cycloheximide, but in this analysis it was possible to detect slight growth at the concentration which was used to discriminate cycloheximide-sensitivity from cycloheximide-resistance.

Five other cycloheximide-resistant strains were also examined (Figure 3.6) H_1, H_2, H_5, H_9 and TC_4 were significantly more resistant than the cycloheximide-sensitive strains already mentioned ($p<0.001$; Table 3.8).

SECTION 3.9. GROWTH RESPONSE OF DIKARYONS TO CYCLOHEXIMIDE : DOMINANCE TEST.

The growth responses of those cycloheximide-resistant mutant strains which produced stable dikaryons are presented in Figure 3.7.

None of the responses to cycloheximide exhibited by dikaryons which were heterozygous for cycloheximide resistance were significantly different to those of the homozygous cycloheximide sensitive dikaryons, CY8 x CY13 and CY9 x CY3 ($p>0.1$, based on the 50% growth inhibitory cycloheximide concentrations, Table 3.10). With the exception of CY9.23 x CY3, none of the dikaryons grew on 1.8 μM cycloheximide (Figure 3.7, Table 3.10).

According to North (1982), dikaryons which failed to growon 1.8 uM cycloheximide were described as cycloheximide-sensitive. Therefore, all of the dikaryons examined, except CY9.23 x CY3, were considered to be cycloheximide-sensitive and to possess cycloheximide-resistance alleles which were recessive to the cycloheximide sensitive alleles. The characterisation of CY9.23 x CY3 could not be defined clearly because although it grew on 1.8 uM cycloheximide , it was indistinguishable from the other cycloheximide-sensitive dikaryons.

Table 3.10. Summary of the growth response of dikaryons to cycloheximide.

Strains	Classification of monokaryons *in vivo*.	Cycloheximide concentration (μM) for, 50% inhibition (calculated)	total inhibition (observed)	Linear regression coefficient.
CY 3 x CY 9	S x S	0.013	0.089	-65
CY 3.2 x CY 9	R x S	0.013	0.089	-78
CY 3.7 x CY 9	R x S	0.014	0.18	-57
CY 3.3 x CY 9	R x S	0.039	0.089	-138
CY 3.8 x CY 9	R x S	0.044	0.27	-49
CY 3.5 x CY 9	R x S	0.052	0.89	-50
CY 3.16x CY 9	R x S	0.057	0.89	-56
CY 9.23x CY 3	R x S	0.120	1.8	-32
CY 8 x CY 13	S x S	0.009	0.036	-57
CY 8.13x CY 13	R x S	0.009	0.027	-79
CY 8.18x CY 13	R x S	0.009	0.036	-68
CY 8.10x CY 13	R x S	0.011	0.036	-81
CY 8.23x CY 13	R x S	0.012	0.089	-61
CY 8.12x CY 13	R x S	0.013	0.036	-89
CY 8.2 x CY 13	R x S	0.014	0.089	-61
CY 8.7 x CY 13	R x S	0.020	0.089	-70
CY 8.4 x CY 13	R x S	0.022	0.089	-86
CY 8.6 x CY 13	R x S	0.023	0.089	-56
CY 8.23x CY 13	R x S	0.027	0.36	-49
CY 8.20x CY 13	R x S	0.051	0.18	-57
CY 8.9 x CY 13	R x S	0.054	0.36	-40

R = cycloheximide-resistant and S = cycloheximide-sensitive

Several cycloheximide-resistant monokaryons either failed to dikayotise or the dikaryons produced were unstable. Original data in Appendix A, Tables A8 and A9. Linear correlation coefficients ranged from -0.87 to -0.98.

FIG.3.7

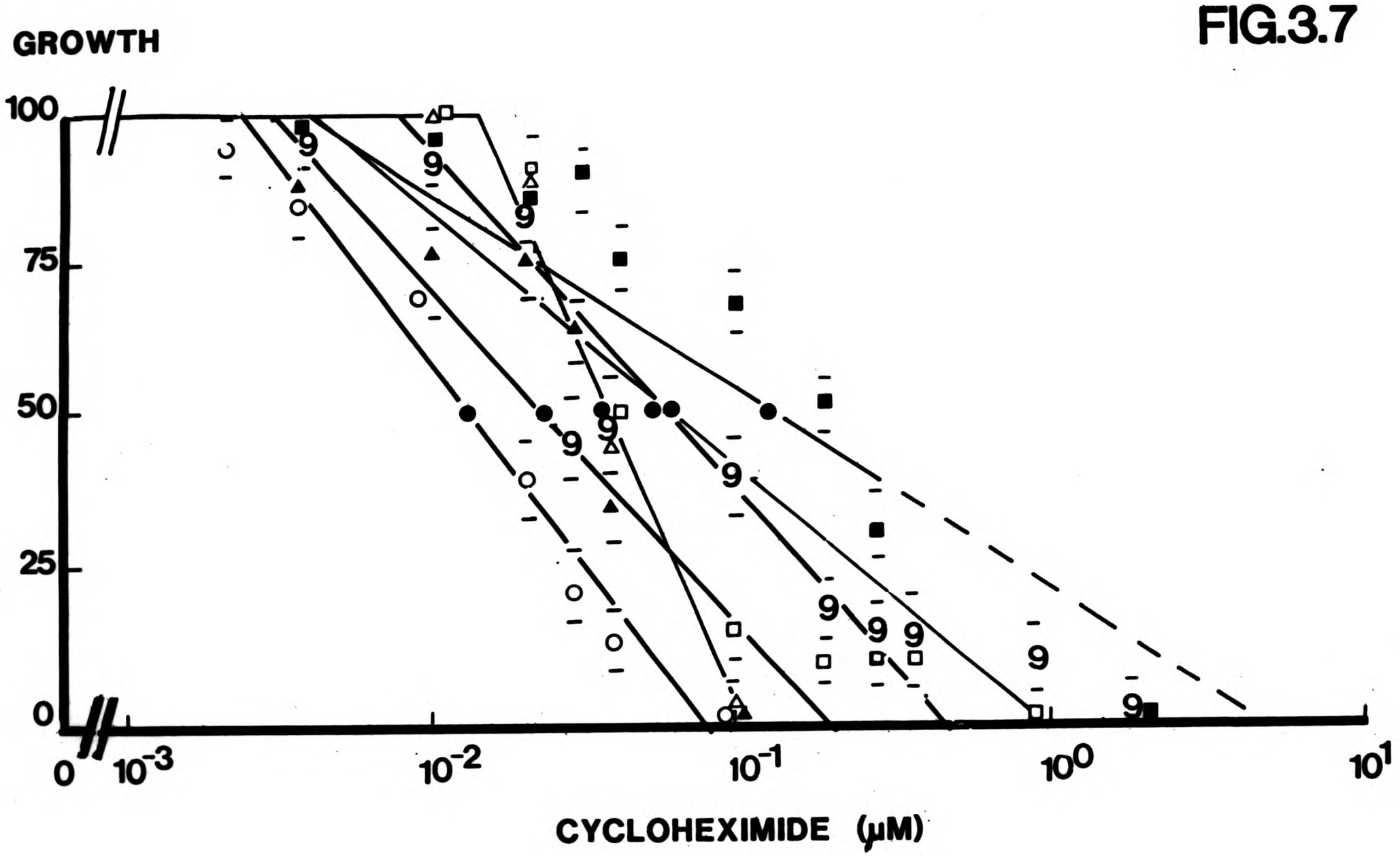


Figure 3.7. The effect of cycloheximide on the growth of dikaryons.

The measurement and presentation of the growth responses of dikaryotic strains to cycloheximide, are so described in Figure 3.2.

In order to clarify the presentation, where appropriate, one strain has been used to typify more than one response. Thus:

Strain	Symbol	
CY 3 x CY 9	O	Represents CY 3.2 x CY 9, CY 3.7 x CY 9, CY 8 x CY 13, CY 8.13 x CY 13, CY 8.18 x CY 13, CY 8.10 x CY 13, CY 8.23 x CY 13, CY 8.12 x CY 13 and CY 8.2 x CY 13
CY 3.3 x CY 9	△	
CY 8.6 x CY 13	▲	Represents CY 8.4 x CY 13, CY 8.7 x CY 13 and CY 8.23 x CY 13
CY 8.9 x CY 13	9	
CY 3.16 x CY 9	□	Represents CY 3.8 x CY 9, CY 3.5 x CY 9, CY 8.20 x CY 13 and CY 8.24 x CY 13
CY 9.23 x CY 3	■	

The 50% growth inhibitory value has been indicated (●).

Original data is given in Tables A8 and A9, Appendix A.

CYCLOHEXIMIDE (μM)

SECTION 3.10. THE GROWTH RESPONSE OF DIPLOIDS TO CYCLOHEXIMIDE.

Of the four diploid strains examined, the heterozygous cycloheximide-resistant strain CY 9.23/CY 14 was the most resistant, (Figure 3.8; Table 3.11).CY 9.23/CY 14 was approximately 7 x more resistant than the comparable homozygous cycloheximide-sensitive strain CY 9/CY 14, however the two strains were not significantly different ($p > 0.5$), based on their 50% growth inhibitory cycloheximide concentrations Table 3.11). Similarly, CY 8.2/CY 18 was more resistant than CY 8/CY 18, but the difference was not significant ($p > 0.5$). Interestingly, the response to cycloheximide exhibited by CY 8.2/CY 18 was identical to the response of CY 9/CY 14.

The only significant difference between the four strains based on the linear regression coefficients, was that the value for CY 8/CY 18 was higher than for any other strains ($p < 0.005$).

No diploid strain was able to grow on more than 1 μM cycloheximide, thus all were considered to be cycloheximide-sensitive. Therefore, the cycloheximide-resistance mutations possessed by CY 8.2 and CY 9.23 were recessive in the diploid. The dominance modifier gene <u>modcy</u>$^{+}$ present in CY 9.23 did not affect the dominance of the cycloheximide-resistance gene in the diploid, agreeing with North's observation (1982).

FIG.3.8

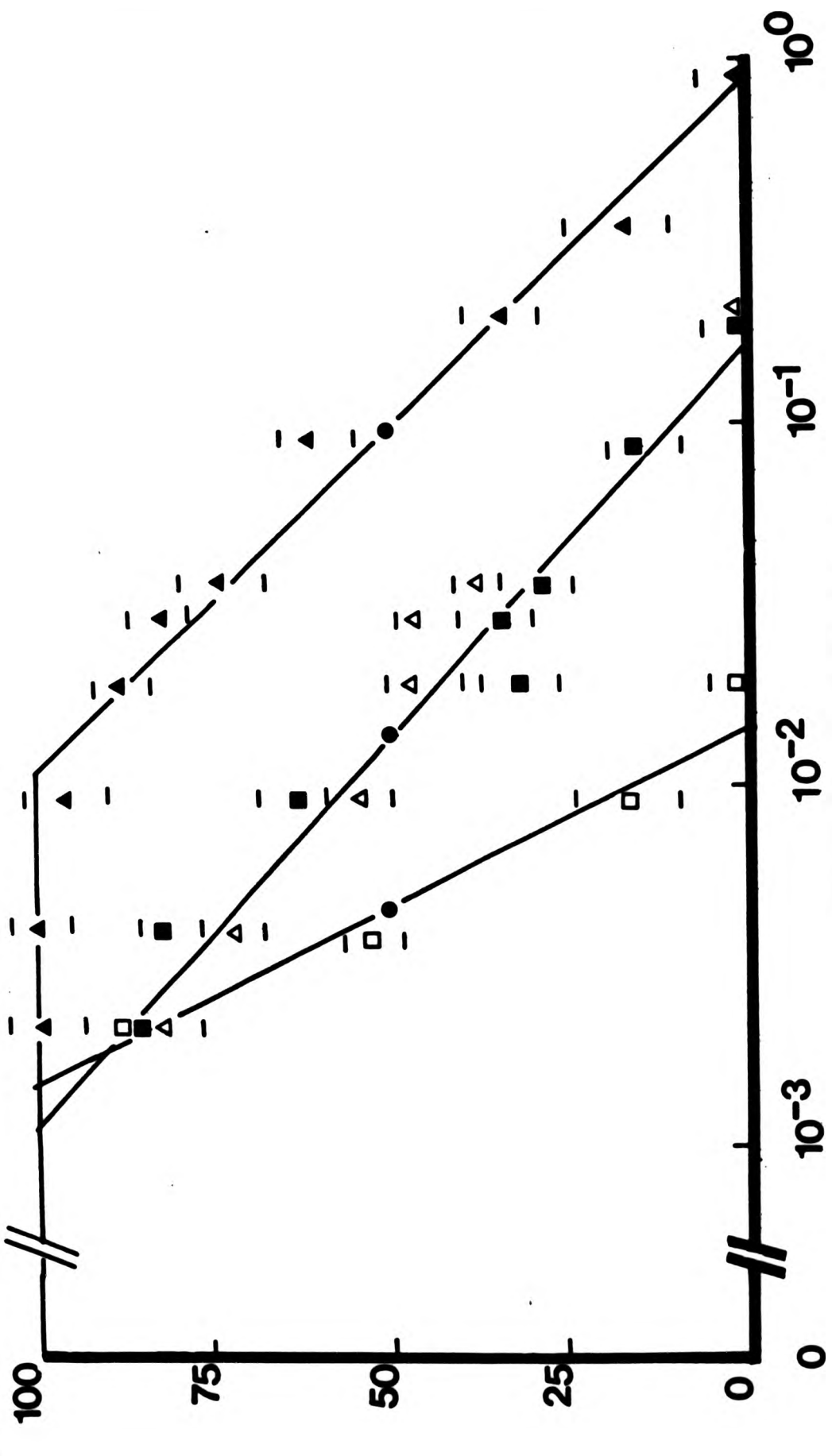


Table 3.1

Strain	
CY 8/CY 1	
CY 8.2/CY	
CY 9/CY 1	
CY 9.23/C	

Original
coefficie
R = cyclo

Figure 3

The
to cyclo
examined

The 50%

Original

Table 3.11. Summary of the growth response of diploids to cycloheximide.

Strain	Classification of monokaryotic constituents.	Cycloheximide concentration (μM) for, 50% inhibition (calculated)	total inhibition (observed)	Linear Regression coefficient.
CY 8/CY 18	S/S	0.005	0.018	-93
CY 8.2/CY 18	R/S	0.014	0.18	-47
CY 9/CY 14	S/S	0.015	0.18	-41
CY 9.23/CY 14	R/S	0.10	0.89	-52

Original data is given in Appendix A, Table A9. The linear correlation coefficients ranged from -0.98 to -0.99.

R = cycloheximide-resistant and S = cycloheximide-sensitive.

Figure 3.8. The effect of cycloheximide on the growth of diploid strains.

The measurement and presentation of the growth responses of diploids to cycloheximide, are as described in Figure 3.2. The diploid strains examined were:

Strain	Symbol
CY8/CY18	□
CY8.2/CY18	■
CY9/CY14	△
CY9.23/CY14	▲

The 50% growth inhibitory value has been indicated (●)

Original data is given in Table A10, Appendix A.

CYCLOHEXIMIDE (μM)

SECTION 3.11. COMPLEMENTATION TEST.

All cycloheximide-resistant strains were amenable to a complementation test because each had been shown to possess a single, recessive cycloheximide-resistance mutation (Sections 3.6 and 3.9).

Ideally, every cycloheximide-resistant mutant strain should have been tested with every other cycloheximide-resistant strain. However it was not possible to examine every possible combination and in order to rationalise the task, strains were selected to represent the cycloheximide-resistant mutants. The selection was based on the prepared classification of the cycloheximide-resistant mutants according to their growth responses (Section 3.8) and their ability to produce fertile dikaryons from which cycloheximide-resistant recombinant strains could be produced (Section 3.9). The strains chosen are named in Table 3.12 i .

Not all crosses produced stable dikaryons which could be tested on 1.8 μM cycloheximide, but all of those which did were deemed to be cycloheximide-resistant (Tables 3.12 i and 3.12 ii). Expression of cycloheximide-resistance in the complementation tests was interpreted as demonstrating non-complementation, ie.that all cycloheximide-resistant mutations examined were allelic and by inference that all strains represented belonged to the same complementation group.

In North's analysis (1982) 12 out of 13 mutants belonged to one complementation group, designated cy-2 . The complementation

Table 3.12i. Complementation test between cycloheximide-resistant strains selected in the same background.

Recombinant strains	Strains				
	CY 3.2	CY 3.3	CY 3.5	CY 3.8	CY 3.16
CY 3.2.16	R	R	R	R	R
CY 3.3.2	-	R	R	d	R
CY 3.5.5	-	-	R	d	R
CY 3.8.6	-	-	-	R	R
CY 3.16.1	-	-	-	-	R
	CY 6.1	CY 6.2	CY 6.3	CY 6.6	
CY 6.1.18	R	R	R	R	
CY 6.2.4	-	R	d	R	
CY 6.3.7	-	-	R	R	
CY 6.6.1	-	-	-	R	
	-	-	-	-	
	CY 8.2	CY 8.6	CY 8.10	CY 8.12	
CY 8.2.14	R	R	R	R	
CY 8.6.2	-	R	R	R	
CY 8.10.3	-	-	d	R	
CY 8.12.7	-	-	-	R	

- = result not determined.
d = failed to dikaryotise.
R = dikaryon grew on 3.6 μM cycloheximide.

No representatives from CY 9 were analysed because no dikaryons were produced (Section 3.9).

Table 3.12ii. Complementation test between cycloheximide-resistant strains selected in different backgrounds.

Recombinant Strains	Strains				
	CY 3.2	CY 3.3	CY 3.5	CY 3.8	CY 3.16
CY 6.1.18	R	R	R	R	R
CY 6.2.4	R	R	R	d	R
CY 6.3.7	R	d	R	R	d
CY 6.6.1	R	R	R	R	R
CY 8.2.18	R	R	R	R	R
CY 8.6.6	R	d	R	R	R
CY 8.10.2	R	d	R	R	R
CY 8.12.10	R	R	R	R	R
CY 9.23.98	R	R	R	R	R
	CY 6.1	CY 6.2	CY 6.3	CY 6.6	
CY 8.2.18	R	R	R	R	
CY 8.6.6	R	d	R	R	
CY 8.10.10	R	R	R	R	
CY 8.12.6	R	d	R	R	
CY 9.23.98	R	R	R	R	
	CY 8.2	CY 8.6	CY 8.10	CY 8.12	
CY 9.23.137	R	R	R	R	

d = failed to dikaryotise.
R = dikaryon grew on 3.6 μM cycloheximide.

tests using CY 9.23.98 and CY 9.23.137, which were known to possess cy-2^{r} mutations, and representatives of mutants produced in this investigation all yielded cycloheximide-resistant dikaryons. By inference, the single complementation group to which all of the cycloheximide-resistance mutant strains from CY 3, CY 6 and CY 8 belonged was cy-2 which had been shown to map on linkage group II (Figure 3.1).

	CY 8.2	CY 8.6	CY 8.10	CY 8.12
CY 9.23.137	R	R	R	R

d = failed to dikaryotise.
R = dikaryon grew on 3.6 μM cycloheximide.

No representatives from CY 9 were analysed because no dikaryons were produced (Section 3.9).

DISCUSSION

SECTION 3.12. Induction of mutants at the cy-2 locus.

All of the cycloheximide resistant mutant strains examined in the complementation test were considered to be the result of a mutation in the same complementation group, identified by North (1982) as cy-2 (Section 3.10). Unlike North's study (1982), no other genes either conferring cycloheximide resistance (ie. cy-1 or cy-3) or indirectly modifying the response in the dominance test (ie. modcy$^+$) were identified.

One of the main objectives in inducing cycloheximide-resistant mutants by ultraviolet radiation was to discover genes, other than those identified by North (1982), which affected the response of Coprinus cinereus strains to cycloheximide. In North's study (1982) mutations in the cy-2 locus accounted for the majority of cycloheximide-resistant mutants, only one mutant was found to possess a cy-3^r allele and one other to possess modcy$^+$ allele. Employing a similar method of mutagenesis with ultraviolet radiation,this investigation had produced cy-2^r mutations (Section 3.8). The mutation frequency of cy-2^r was relatively high compared with values for cy-3^r and modcy$^+$ (North, 1982). It was possible that no cy-3^r or modcy$^+$ mutants were produced in this investigation because insufficient numbers of oidiospores were irradiated. The cy-1^r mutation was identified in wild strains and was not induced by ultraviolet radiation (North, 1982).

This investigation failed to discover any new genes associated with the response to cycloheximide possibly because Coprinus cinereus did not possess any other loci. Thus Coprinus cinereus is known to have more genes conferring cycloheximide resistance than Aspergillus nidulans (Warr and Roper, 1965) but fewer than are known in Saccharomyces cerevisiae (Wilkie and Lee, 1965).

However, it was possible that mutations in genes, other than those identified by North (1982), may have been produced given different methods of mutagenesis and selection to those employed (Section 3.5 b). The method used in this investigation was similar to North's (1982) and thus it may be that the cycloheximide-resistant mutations induced by ultraviolet radiation are restricted to the cy-2 , cy-3 , modcy loci. If this was the reason, then a different experimental approach may have been gainfully employed.

Although, ultraviolet radiation has been most frequently employed to produce cycloheximide-resistant strains (Table 3.2) it is not the only one. Alternative treatments such as MNNG (Crouzet et al, 1978) or nitrous acid (Ibrahim and Coddington, 1978) have been used to produce cycloheximide resistant strains, and may have resulted in mutations in new genes had they been employed with Coprinus cinereus.

Alternatively, it may have been possible to identify new genes conferring cycloheximide-resistance by employing the strategy of Wilkie and Lee (1965) in which cycloheximide-resistant strains were exposed to ultraviolet radiation and strains were selected on increasing concentrations of cycloheximide. In this way, Wilkie and

Lee (1965) identified a multigenic system for Saccharomyces cerevisiae.

A third possibility, was the selection of revertants to cycloheximide-sensitivity induced by ultraviolet radiation in cycloheximide-resistant strains, Ibrahim and Coddington (1978) used the method in discovering two loci in Schizosaccharomyces pombe.

SECTION 3.13. THE EFFECT OF CYCLOHEXIMIDE ON THE GROWTH OF COPRINUS CINEREUS.

a) Measurement of growth.

The measurement of colony diameter on solid medium has been frequently used to measure the growth of filamentous fungi, and of Coprinus cinereus in particular (Casselton, 1965). The simplicity and convenience of the measurement made the technique attractive for use in defining the growth inhibitory effect of cycloheximide. According to Pirt (1973) a measure of the spread of a fungal colony on agar represents the actual relation between the biomass and growth limiting substrate.

North (1982) observed the effect of cycloheximide on the growth of Coprinus cinereus as either the presence or absence of growth; the result was that a value for the minimum cycloheximide concentration permitting growth was determined for each strain examined. In this investigation, the growth at each cycloheximide concentration tested was measured in relation to the growth of the uninhibited control treatment; from which it was possible to define the cycloheximide dose-growth response for each strain.

b) Characterisation of the cycloheximide dose-growth response.

The effect of cycloheximide on the growth response exhibited by all strains (Sections 3.8 to 3.10) could be differentiated into two distinct phases. At relatively low cycloheximide concentrations there was no measurable inhibition of growth. The concentration range over which the phase occurred varied between strains and was defined by calculation of the minimum growth inhibitory cycloheximide concentration

The second phase occurred over the range of cycloheximide concentrations which produced a logarithmic inhibition of growth, and ultimately prevented all growth. This phase was defined by three parameters; the 50% and total growth inhibitory cycloheximide concentrations and the linear regression coefficient.

c) Classification.

The basic classification of monokaryotic strains as either resistant to sensitive to cycloheximide, was based on their ability to grow on 3.6 μM cycloheximide (North, 1982). The detailed analysis of the response to cycloheximide made it possible to subdivide between the two general categories.

Any of the four parameters of the cycloheximide dose-growth response could have been used as the basis for classifying the strains. The calculated values of the minimum and total growth inhibitory cycloheximide concentrations were preferable to the observed values because observed values were imprecise depending on the range and interval of cycloheximide concentrations used and in the case of the total growth inhibitory cycloheximide concentration, it was not observed for all strains. A classification based on the total growth in inhibitory cycloheximide concentration was not favoured because for some strains, notably CY6.1, CY6.2, CY8.2 and CY9.23, it was necessary to use extrapolated values (Section 3.8).

Strains grouped according to their minimum or total growth inhibitory cycloheximide concentration produced a similar classification of strains to the one obtained using the 50% growth inhibitory cycloheximide concentrations (Section 3.8 to 3.10). However strains

classified according to their linear regression coefficients produced groupings which were unlike those produced when the 50% growth inhibitory cycloheximide concentration was used; for example two strains CY6 and CY6.1 had identical linear regression coefficients but their responses were those of a cycloheximide-sensitive strain and a highly resistant strain respectively.

Based on the 50% growth inhibitory cycloheximide concentrations, monokaryotic strains can be identified as either cycloheximide-sensitive or cycloheximide-resistant. The discriminatory 50% growth inhibitory cycloheximide concentration was chosen so that the same classification of strains was achieved as when North(1982) used a total inhibitory cycloheximide concentration of 3.6 µM.

Furthermore, it was possible to subdivide the cycloheximide resistant strains into two groups. There were those strains which grew at the highest cycloheximide concentration tested and which had 50% growth inhibitory cycloheximide concentrations higher than 37µM. These highly resistant strains were CY6.1, CY6.2, and CY9.23 and its recombinant strains. The second group included all other strains which were able to grow on 3.6 µM cycloheximide and which had 50% growth inhibitory cycloheximide concentrations between 1.4 and 8.1 µM. Subdivisions within each of the general catagories were difficult to define (Section 3.8).

A direct comparison of the response of strains derived from different CY cycloheximide-sensitive strains was not possible because of differences in the sensitivity of the parental strains (Section 3.8 e). In absolute terms, CY3.16 which had a 50% growth inhibitory cycloheximide concentration of 8.1 µM was less resistant

than CY6.1 which exhibited a 50% growth inhibitory cycloheximide concentration of 41 μM. However, in relation to the cycloheximide-sensitive strains from which they were derived, because CY3 was more sensitive to cycloheximide than CY6, CY3.16 was infact more resistant than CY6.1 (CY3.16 was 270 x more resistant than CY3, whereas CY6.1 was 195 x more resistant than CY6). The expression of cycloheximide resistance was determined by the nature of the mutation and the genome in which it was expressed. The CY cycloheximide-sensitive strains provided by North (Section 2.2, Chapter 2) had different origins and were not isogenic. The identity of the genes which affected the expression of the cycloheximide-resistance mutations were not known.

d) Interpretation.

The mechanism by which cycloheximide inhibited the growth of Coprinus cinereus was not known and the genetic analysis detailed in this Chapter does not provide any information regarding the identity of the mechanism. Several possible reasons could explain the biphasic growth response of cycloheximide.

In the range of cycloheximide concentration at which growth inhibition was observed, variation between the strains may have been the result of no inhibition. It was possible that the drug did not enter the cell or that concentration of the antibiotic was insufficient to produce inhibition. In cycloheximide-resistant mutant strains a change in the permeability of the cell-membrane or in the mechanism of detoxification or affinity of the intracellular site may have resulted in the high minimum inhibitory cycloheximide concentration. Alternatively, cycloheximide may have produced an inhibitory response

but the effect was masked by a mechanism which overcame the loss in function (eg. utilising a reserve capacity), but only until a threshold, the minimum growth inhibitory cycloheximide concentration, was attained.

Once the specific threshold concentration had been reached, cycloheximide was observed to inhibit growth, an inhibition which was logarithmic. There were two types of inhibitory response characterised by the linear regression coefficients. In cycloheximide-resistant mutant strains in which the linear regression coefficients were similar to those of their cycloheximide-sensitive parents, (eg CY6.1 and CY9.23 with CY6 and CY9 respectively, Section 9 a and d) the mechanism of action of cycloheximide was probably the same, only a change in the mutant strain which resulted in a higher threshold concentration distinguished the resistant and sensitive strains. In contrast, in those cycloheximide-resistant mutant strains which exhibited linear regression coefficients which were unlike their parental strains (eg CY6.3 and CY9.77 Section 3.8 a and d) there were two effects. The change which produced the change in the linear regression coefficient may have also produced the effect on the threshold concentration.

The practical value of the classification of cycloheximide-resistant mutant strains was that representatives of each of the groups of strains (the composition of which is given in Section 3.11) and not all strains need be subjected to a biochemical analysis. (Chapter 5).

SECTION 3.14. NUCLEAR INTERACTION AND EXPRESSION OF CYCLOHEXIMIDE RESISTANCE.

All but one of the dikaryons and diploids heterozygous at the cycloheximide resistance cy-2 locus were cycloheximide-sensitive indicating that the mutations at the cy-2^r were recessive. The exception was CY9.23 in which the modcy$^+$ mutation produced a partial dominance of the cy-2^r mutation which agreed with the observation made by North. No homozygous cycloheximide-resistant dikaryons and few genotypes of any diploid were examined.

The responses of monokaryons and dikaryons and diploids derived from them are summarised in Table 3.13. The response of the dikaryons tended to,or equalled the lowest values exhibited by their composite monokaryons, including in CY9.23 x CY3 in which partial resistance was expressed. The diploids produced comparable responses to the dikaryons and also tended to the reponse of the most sensitive monokaryotic constituent.

The results suggest that the product of the cy-2^r allele in the presence of the wild-type allele is not expressed, thus producing the cycloheximide-sensitive phenotype. Alternatively, the products of both alleles may exist, but that the product of the cycloheximide-sensitive allele completely prevents the functioning of the cy-2^r allele. When modcy$^+$ is present, the partial resistance to cycloheximide may be expressed by a modification of the product of the cy-2^r allele or of another, unknown allele. The disorimination between interaction between the nuclei and interaction within the cytoplasm will be discussed in Chapter 7, in the light of the biochemical evidence presented in Chapter 5.

Table

Calcu

CY8
0.1
CY8.2
37
CY9
0.08
CY9.2
100

Data

LOHEXIMIDE

ygous at the
e-sensitive
ive. The
ed a partial
bservation
dikaryons and

ploids derived
the dikaryons
eir composite
resistance
ses to the
nsitive

allele in the
producing the
products of both
lmide-sensitive
allele. When
lde may be
allele or of
leraction between
discussed in
lented in

Table 3.13. Comparison of the growth responses of monokaryons, dikaryons and diploids to cycloheximide.

Calculated 50% growth inhibitory cycloheximide concentration (μM). Strains.				
Monokaryons			Dikaryon	Diploid
CY8	CY13	CY18	CY8.x CY13	CY8/CY18
0.1	0.01	0.003	0.009	0.005
CY8.2	CY13	CY18	CY8.2 x CY13	CY8.2/CY18
37	0.01	0.003	0.014	0.014
CY9	CY3	CY14	CY9 x CY3	CY9/CY14
0.08	0.03	0.01	0.013	0.015
CY9.23	CY3	CY14	CY9.23 x CY3	CY9.23/CY14
100	0.03	0.01	0.12	0.10

Data taken from Tables 3.7, 3.8, 3.9, 3.10 and 3.11

SECTION 3.15. SUMMARY.

Cycloheximide-resistant mutants were induced as a result of ultraviolet radiation but no genes, other than those identified by North (1982) were identified; all recessive mutations examined The growth analysis confirmed North's evidence that cycloheximide resistance was partially expressed in CY9.23 x CY3. The detailed analysis of the responses of cycloheximide-resistant mutants to cycloheximide provided a method of selection of strains for biochemical analysis and would be used in Chapter 7 as a means of comparing *in vivo* effects with those observed *in vitro*.

CHAPTER 4.

DEVELOPMENT OF A COPRINUS CINEREUS CELL-FREE POLYPHENYLALANINE SYNTHESISING SYSTEM.

INTRODUCTION.

SECTION 4.1. CYTOPLASMIC PROTEIN SYNTHESIS.

Two types of protein synthesising system are found in Coprimus cinereus. Protein synthesis, which occurs in the cytoplasm and is carried out by cytoplasmic ribosomes, is the main concern of this investigation but in addition there is mitochondrial protein synthesis which takes place within the mitochondrion on mitochondrial ribosomes.

Our understanding of eukaryotic protein synthesis has been based upon the evidence of a limited number of species, including many fungal species, but excluding Coprinus cinereus. However, there are certain steps in the process which are ill-defined in eukaryotes and in order to produce an overall view of protein synthesis it has been necessary to refer to the more thoroughly investigated process in prokaryotes. Throughout this investigation, evidence from prokaryotes has been used sparingly because of the differences which are known in eukaryotic and prokaryotic protein synthesis (Cox and Godwin, 1975).

The scheme for cytoplasmic protein synthesis (represented in Figure 4.1 and summarised in Sections 4.1, a to d) is based upon a cytoplasmic ribosome model which possess two tRNA binding sites at the peptidyl-transferase centre(Watson , 1964) . The two tRNA binding sites are known as the aminoacyl-tRNA binding site (A-site) and the peptidyl-tRNA binding site (P-site).

FIG.4.1

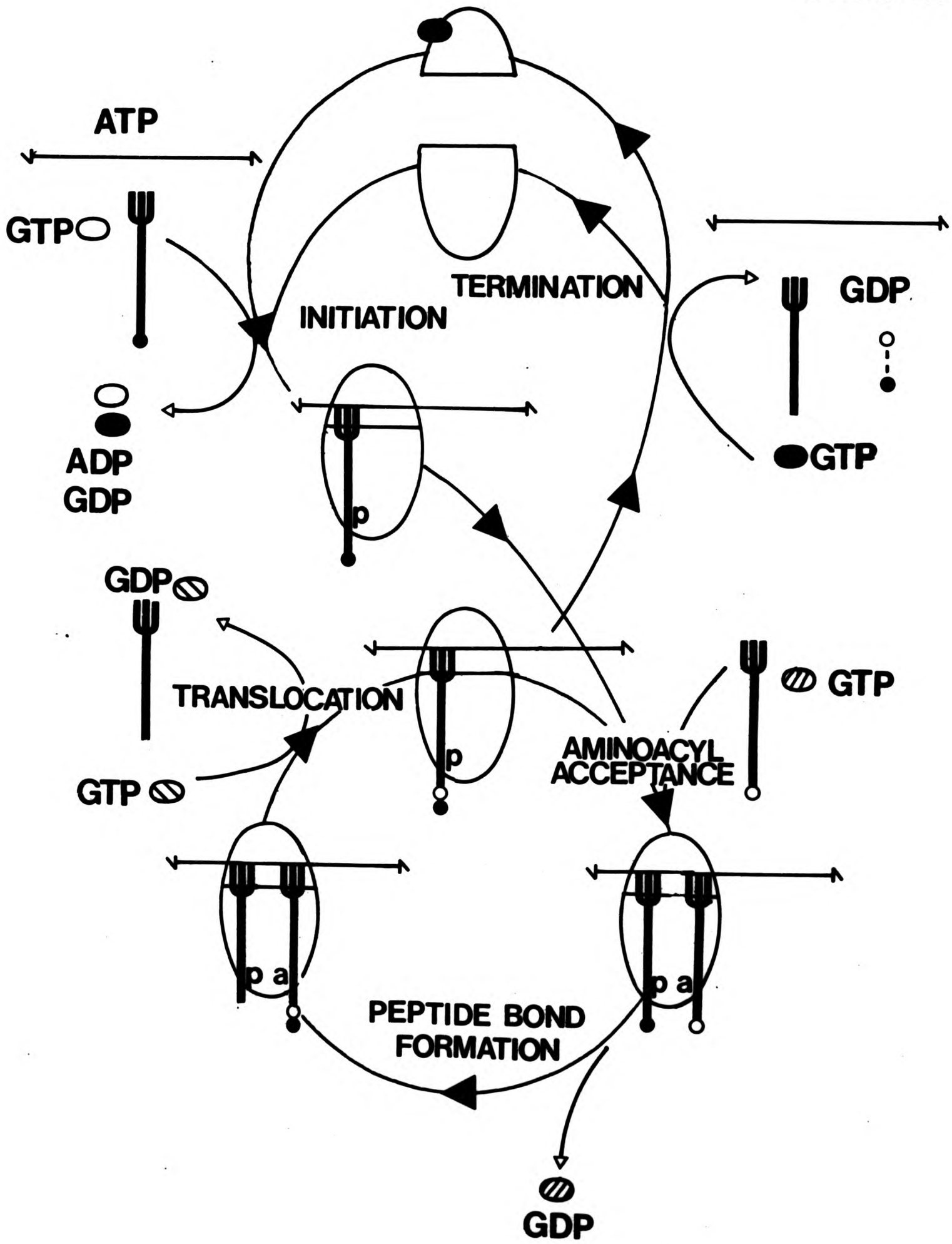

ATP
GTP
ADP
GDP
INITIATION
p
TERMINATION
GDP
GTP
GDP
GTP
TRANSLOCATION
p
GTP
AMINOACYL
ACCEPTANCE
p a
p a
PEPTIDE BOND
FORMATION
GDP

FIG.4.1

GDP

GTP

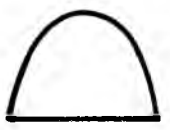
GTP

Figure 4.1. Generalised scheme for cytoplasmic protein synthesis.

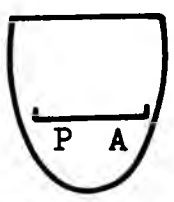
The salient features of eukaryotic protein synthesis; initiation, elongation and termination, represented in this scheme are based upon the review of Siegel (1977).

Many of the individual reactions are illdefined and for simplicity only the known substrates and final products are illustrated. The points of entry of the substrates into, and departure of the products from, the protein synthetic cycle are speculative.

The symbols used are as follows:



Small ribosomal subunit.



Large ribosomal subunit, showing peptidyl-transfease centre, P = P-site and A = A-site.

mRNA

● methionyl residue

○ aminoacyl residue

tRNA

○ soluble factor (s)

(various shadings)

a) Amino acid activation.

Prior to the involvement of the cytoplasmic ribosomes, the amino acids which are to be incorporated into polypeptides are esterified to specific tRNAs . For each different amino acid there is a specific aminoacyl - tRNA synthetase which catalyses the reaction. The process requires energy released from the pyrophosphate cleavage of ATP and the presence of magnesium ions. Aminoacyl-tRNA formation is reviewed by Ofengand (1977).

b) Initiation.

The first phase of protein synthesis catalysed by the cytoplasmic ribosome is initiation. The initiation process consists of a series of reactions during which the large and small ribosomal subunits interact with messenger RNA and with methionyl-tRNA to produce an 80s initiation complex. Initiation commences when the initiator-tRNA becomes bound to the initiation (AUG) mRNA codon at the ribosomal peptidyl-tRNA binding site (P-site).

The specific reactions involved in initiation have been reviewed by Siegel, (1977) and by Revel, (1977). The reactions require the hydrolysis of GTP, and possibly ATP as well. Siegel, (1977) refers to three soluble fungal initiation factors, but as many as eight mammalian initiation factors have been identified, Anderson et al (1977), although their function and importance has not been proven.

c) Elongation.

The addition of successive amino acids to form a polypeptide chain occurs in a stepwise manner over as many cycles as are necessary to translate the nucleotide sequence of the mRNA. Each cycle, in which a single amino acid is added, can be divided into three steps; the binding of aminoacyl-tRNA, peptide bond formation and finally, translocation.

(i) Binding of aminoacyl-tRNA.

In the first step of the elongation cycle, the appropriate aminoacyl-tRNA specified by the exposed codon at the ribosomal aminoacyl-tRNA binding site (A-site), becomes associated with the ribosomal complex. Initially the P-site is occupied by the initiator methionyl-tRNA, but as the elongation process progresses, the P-site contains a peptidyl-tRNA. The process requires a soluble elongation factor EF_1, and is dependant upon GTP hydrolysis. The reaction mechanism was reviewed by Siegel (1977) and Miller and Weissbach, (1977).

(ii) Peptide bond formation.

Elongation of the peptide chain occurs when the carboxyl ester linkage of methionyl-tRNA or of peptidyl-tRNA is broken. The released aminoacid or nascent peptide reacts with the terminal NH_2 group of the aminoacid of the aminoacyl-tRNA, and a peptide bond is formed. Consequently, the A-site is then occupied with the nascent peptidyl-tRNA, and the P-site possesses a deacylated tRNA.

The transpeptidation process is unique among the steps in protein synthesis, requiring as it does neither energy nor soluble factors. However, the process does require the ribosomal peptidyl-transferase site to be in close proximity to the reacting species for the reaction to be catalysed. The mechanism has been reviewed by Harris and Pestka, (1977).

(iii) Translocation.

The final phase of elongation is the most complex. In the process, the ribosome must move relative to the mRNA, towards the 3' end by precisely three nucleotides. During the movement, termed translocation, the deacylated tRNA at the P-site after peptide bond formation is released. The peptidyl-tRNA, previously at the A-site becomes relocated to the recently vacated P-site. The resulting unoccupied A-site exposes the next mRNA codon in readiness for the ribosomal complex to accept the next aminoacyl-tRNA.

Details of the translocation mechanism are unclear, Brot (1977), but it is known that GTP hydrolysis at a ribosome-dependant site is necessary and that a soluble elongation factor EF_2 must be present.

d) Termination.

Peptide chain termination requires the recognition of either of the three termination codons; UAA, UGA or UAG, at the A-site and the hydrolysis of the bond between the peptide

and tRNA. The result is the release of the peptide, accompanied by the dissociation of the deacylated-tRNA. mRNA. ribosome complex.

The process, reviewed by Caskey, (1977), requires a soluble release factor, Rf, and the hydrolysis of GTP. The peptidyl-transferase centre is also necessary.

SECTION 4.2. TRANSLATION OF POLYURIDYLIC ACID.

The poly (U) dependant-polyphenylalanine synthesising system was first used by Nirenberg and Matthaei (1961) with a S-30 fraction from Escherichia coli. During the development of the assay as it has been applied to eukaryotic species, the basic constituents of the reaction mixture remain the same; a suitable ionic environment ATP and a means of regeneration, an amino acid substrate and polyuridylic acid. Methods of cell fraction have improved and the reaction mixtures usually contain cell-fractions containing ribosomes and the soluble cofactors necessary for the enzymic synthesis of polypeptides.

The assay is particularly sensitive to the synthesis of small quantities of polypeptide because the amino acid substrate is radioactively labelled, at a high specific activity. Radioactivity associated with the polyphenylalanine product is differentiated from radioactivity in unreacted amino acid, or intermediate compounds because it is insoluble in trichloroacetic acid (TCA) at 90°C, (Van der Decken, 1967). Polyphenylalanine molecules with more than one peptide bond are precipitated by TCA (Bretthauer and Golichowski, 1968).

The translation of an artificial polyribonucleotide, polyuridylic acid differs from the translation of natural mRNA (Section 4.1) because the synthetic mRNA possess neither initiation or termination codons; polyuridylic acid as the name implies, is composed entirely of uridine nucleosides.

In the absence of an initiation codon it is presumed that an abnormal form of initiation occurs. Falvey and Staehelin (1970) found that a

relatively high magnesium ion concentration was necessary, although the reactions involved in the formation of an abnormal initiation complex are not known. It is not known if initiation occurs at the end of the template or along its length.

Whatever the nature of the initiation complex, polyphenylalanine is synthesised, presumably by a normal process of elongation. The cytoplasmic ribosomes would be expected to continue translating along the polyuridylic acid molecule until a constituent of the cell-free system became limiting, or until the cytoplasmic ribosome ran off the end of the template. In the absence of a termination codon, it is not known if the polypeptide chain is released from the cytoplasmic ribosome and is therefore available for another cycle of polyphenylalanine synthesis or if the nascent peptide remains attached to the ribosome rendering it unavailable.

Nevertheless, the purpose of the polyuridylic acid dependant polyphenylalanine synthesisng system in this investigation is as an assay to screen for cycloheximide-resistant cytoplasmic ribosomes, a function to which the system has been of proven value (Chapter 5).

SECTION 4.3. OBJECTIVES.

At the outset of this investigation no method existed either to prepare Coprinus cinereus cell-extracts, or to analyse their capacity to synthesise polypeptides in vitro. The objectives of the work described in this Chapter were to develop both techniques and thus achieve a satisfactory level of in vitro polypeptide synthesis. Once achieved, it would then be possible to investigate if any of the cycloheximide-resistant mutant strains (Chapter 3) possessed cycloheximide-resistant cytoplasmic ribosomes (Chapter 5).

SECTION 4.4. OPTIMISATION OF THE INDIVIDUAL CONSTITUENTS OF THE COPRINUS CINEREUS POLYPHENYLALANINE SYNTHESISING SYSTEM.

At the outset of this investigation, in the absence of a cell-free polypeptide synthesising system existing for Coprinus cinereus or any other basidiomycete species, a compromise system of several fungal species was used. The compromise,or preoptimised system,(Table 4.1) was predominantly based on the system used by Crouzet et al (1978) for Podospora anserina but took into account the common features and averaged differences of systems used by Küntzel (1969) for Neurospora crassa, Sissons (1974) for Saccharomyces cerevisiae and Berry et al (1978) for Schizosaccharomyces pombe. It was believed that there would be a greater probability of obtaining polypeptide synthesis with Coprinus cinereus cell-extracts if a compromise system were used, rather than a system based on one particular species.

Initially the activity of the preoptimised polyphenylalanine synthesising system (Table 4.1) was approximately 25 pmole polyphenylalanine synthesised. assay.$^{-1}$ hr^{-1}. (Appendix B i).

The remaining results presented in this Chapter relate to experiments aimed at improving upon the relatively low activity of the assay system, and in doing so, to economise on the resources used in the assay. Beginning with the composition of the preoptimised reaction mixture (Table 4.1), each of the

Table 4.1. The preoptimised in vitro polyphenylalanine synthesising system.

Constituent	Final concentration, (mM, unless stated otherwise)
Adenosine - 5 - triphosphate.	2.0
Guanosine - 5 - triphosphate.	0.5
Creatine phosphokinase.	4.0 $\mu g.ml.^{-1}$
Creatine phosphate.	10.0
Tris - HCl, pH 7.5.	50.0
Magnesium acetate.	10.0
Potassium chloride.	20.0
Ammonium acetate.	50.0
Spermidine.	1.0
Dithiothreitol.	2.0
$tRNA^{phe}$	10.0 $\mu g.ml.^{-1}$
Polyuridylic acid.	375.0 $\mu g.ml.^{-1}$
L - (U - C^{14}) phenylalanine.	0.98 μM (513mCi. $mmole^{-1}$).
RP-100 cytoplasmic ribosome-rich fraction.	3.0 A_{260} unit.
S-100 cytoplasmic ribosome-free supernatant fraction.	1.0 A_{260} unit.
Made to 100 μl with double distilled water.	

Additionally the reaction mixtures contained 1.0mM 2-mercaptoethanol, and 50mM sucrose derived from the cell-extracts. The preparation of the reaction mixture and determination of radioactivity insoluble in TCA at 90°C was as described in Chapter 2, Section 2.9.

constituents was individually examined in order to determine its optimum concentration for polyphenylalanine synthesis. When each of the constituents had been independantly optimised in the presence of the preoptimised concentration of all other constituents, the effect of the constituents was re-examined in the presence of the optimised concentration of all other constituents. The effects of the individual constituents in an otherwise optimised system (Chapter 2, Section 2.9, Table 2.2) are presented in the following sections, 4.5 to 4.12.

SECTION 4.5. COPRINUS CINEREUS CELL-EXTRACTS.

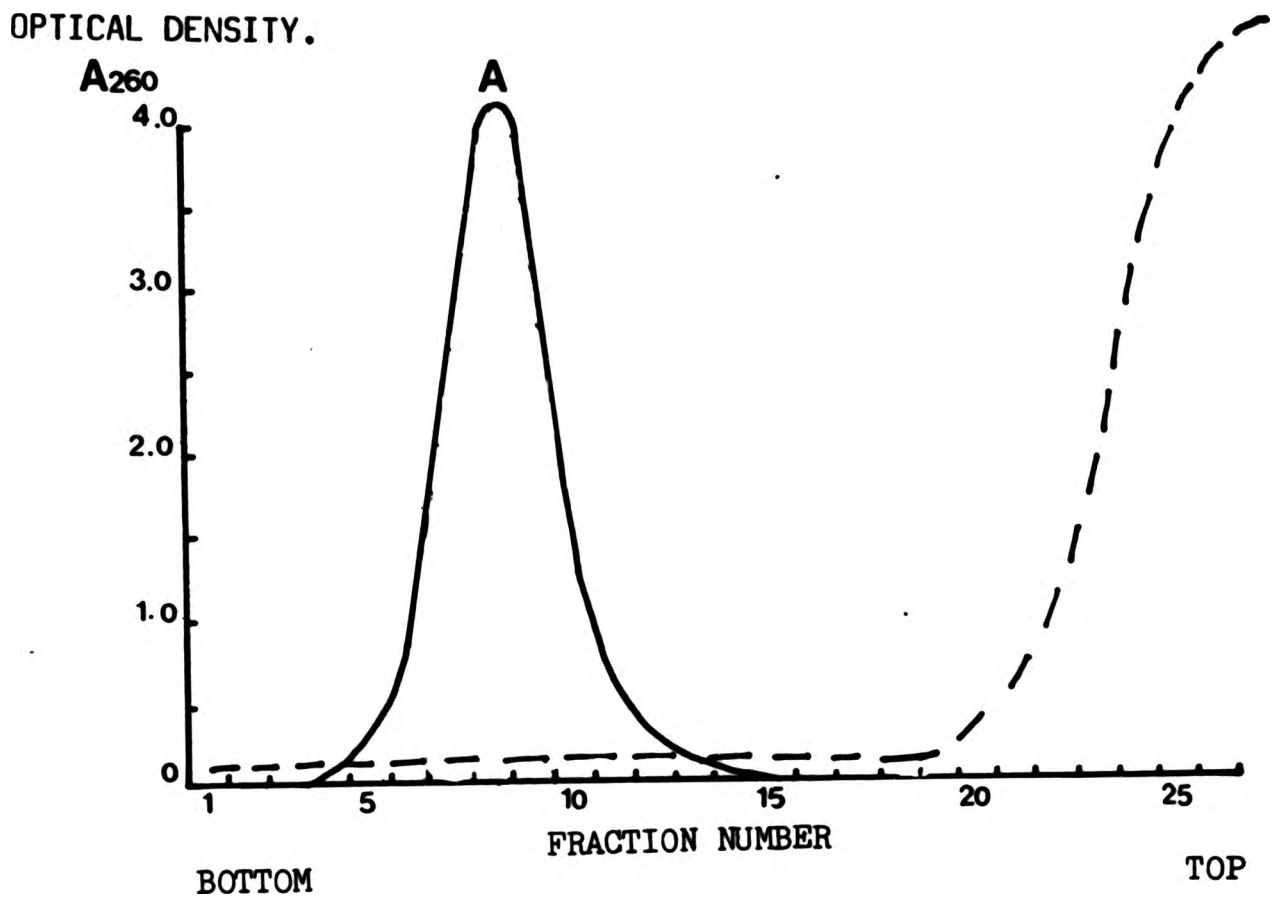
The development of the polyphenylalanine synthesising system was based on cell-extracts, derived from CY 8, and fractionated into the RP-100 and S-100 fractions (Chapter 2, Section 2.7).

a) Cytoplasmic ribosome fracticn, RP-100.

The RP-100 fraction derived from Coprimus cinereus was considered to contain the cytoplasmic ribosomes. The assumption was based on the similarity of the centrifugation conditions used to prepare the RP-100 fraction and those used to prepare fractions known to contain cytoplasmic ribosomes from other eukaryotic species (eg. Crouzet et al, 1978).

An analysis of the RP-100 fraction in sucrose density gradients added to the belief that it contained cytoplasmic ribosomes. The position of the sedimenting material derived from the RP-100 fraction was detected as a single peak of absorption at 254 nm(Figure 4.2). According to the tables published by McEwen (1967) and knowing the parameters of the sucrose density gradient, it was possible to calculate the average sedimentation coefficient of the absorbance peak. (Appendix B ii) which was found to be 74.4 S. The value was similar to that given in Chapter 1, Section 1.5 for eukaryotic cytoplasmic monosomes. There was no evidence in the sucrose density gradient of cytoplasmic ribosomal subunits, cytoplasmic polysomes or mitochondrial ribosomes. It was concluded that the RP-100 fraction was predominantly composed of cytoplasmic ribosomes.

Figure 4.2. Analysis of RP-100 and S-100 fractions in sucrose density gradients.

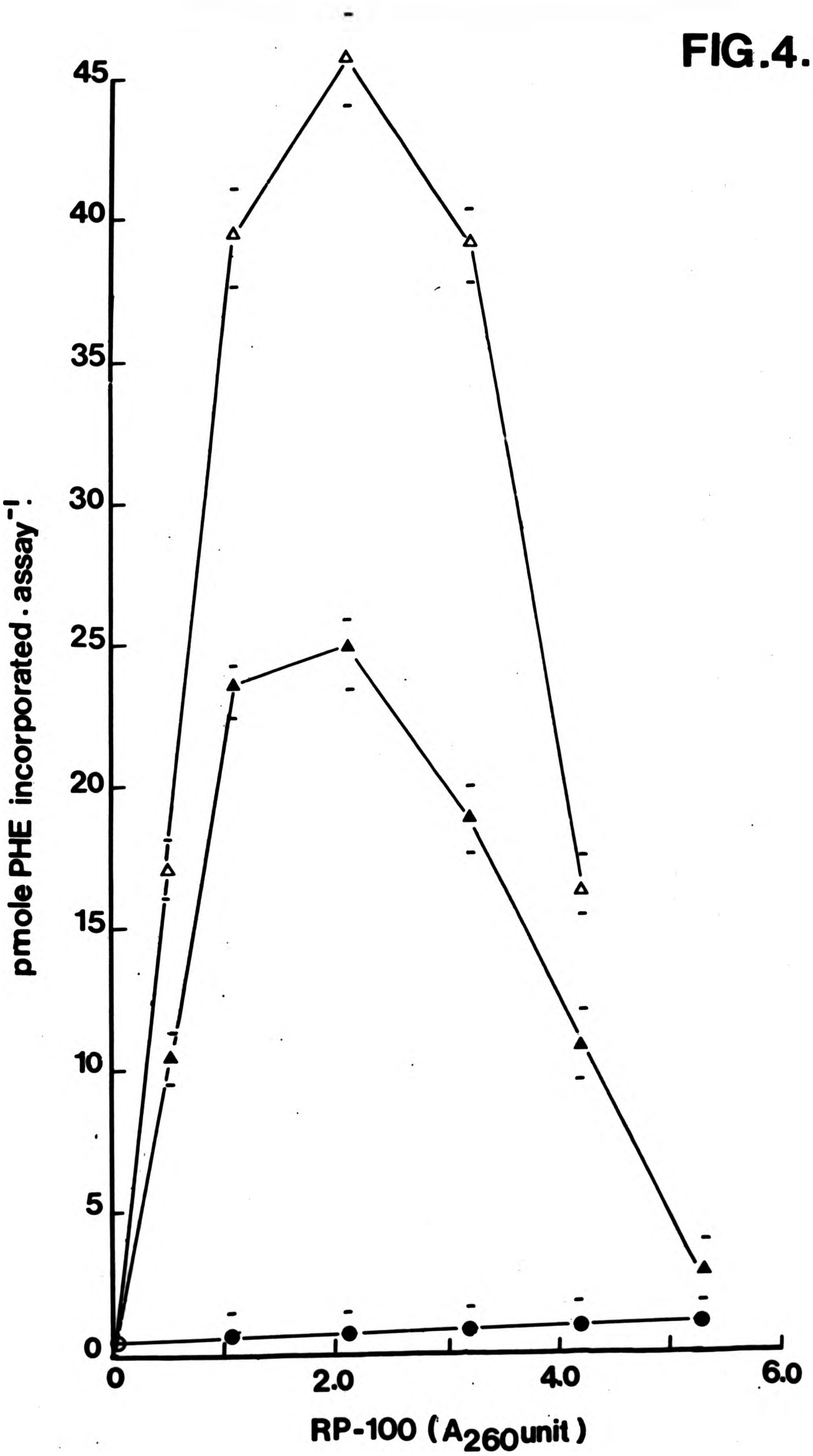


The absorbances (254nm) of a typical RP-100 fraction (solid line) and of a typical S-100 fraction (dashed line) were measured in identical linear sucrose gradients as described by the method in Chapter 2, Section 2.8.

Figure 4.3. The effect of RP-100 fraction of polyphenylalanine synthesis.

The effect of the RP-100 fraction, the cytoplasmic ribosome fraction, on polyphenylalanine synthesis (Section 2.9, Table 2.2) at three concentrations of S-100 : 0 A_{260} unit. (●) , 1.0 A_{260} unit. (▲) and 2.0 A_{260} unit. (△) , in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2). Duplicate 40 µl samples were taken from replicate experiments after 1 hour according to the method given in Chapter 2, Section 2.9. Background radioactivity, 110 cpm, was deducted from the average values (1016 cpm = 1 p mole.phe).

FIG.4.3


45
40
35
30
25
20
15
10
5
0
pmole PHE incorporated · assay⁻¹
0
2.0
4.0
6.0
RP-100 (A260unit)

The effect of the RP-100 concentration on polyphenylalanine synthesis was measured at three concentrations of S-100; no S-100, in the presence of the preoptimised S-100 concentration (1.0 A_{260} unit ; Table 4.2), and in the presence of the optimised S-100 concentration (2.0 A_{260} unit; Chapter 2, Section 2.9, Table 2.2).

Polyphenylalanine synthesis was totally dependant on the presence of the RP-100 fraction. In the absence of the RP-100 fraction, neither of the three concentrations of S-100 fraction used, produced polyphenylalanine synthesis (Figure 4.3). It was therefore concluded that the RP-100 fraction was the sole source of cytoplasmic ribosomes.

In the presence of the RP-100 fraction, polyphenylalanine synthesis was stimulated to maximum incorporation at 2.0 A_{260} unit . The maximum incorporation achieved depended on the S-100 concentration and was 1.8 x greater at the optimised S-100 concentration (2.0 A_{260} unit) than at the preoptimised concentration (1.0 A_{260} unit ; Figure 4.3).

At RP-100 concentrations above 2.0 A_{260} unit polyphenylalanine synthesis was inhibited. The degree of inhibition varied depending on the S-100 concentration, reaching 90% inhibition in the presence of 1.0 A_{260} unit S-100 and 65% inhibition in the presence of 2.0 A_{260} unit S-100 (Figure 4.3).

The RP-100 concentrations which produced 100% activity ($\pm$ 10%) were 1-3 A_{260} unit and the average value, 2.0 A_{260}unit was employed as the optimum concentration of RP-100 to be used in all subsequent optimised reaction mixtures, (Chapter 2, Section 2.9, Table 2.2).

The response observed at the three S-100 concentrations provided evidence of relationship between RP-100 and S-100 concentrations which will be discussed further in Section 4.5 c .

While the RP-100 was suboptimal, polyphenylalanine synthesis was stimulated, indicating that the RP-100 concentration was the limiting component of the cell-free system.

The decline in polyphenylalanine synthesis above the optimal RP-100 ccncentration may have been the result of several factors. It was possible that the RP-100 fraction contained a non-ribosomal constituent, possibly a nuclease or a protease which degraded the cytoplasmic ribosomes or other of the constituents. The proportion of inhibitor relative to RP-100 would be constant as the RP-100 concentration was increased. In order for the inhibition of polyphenylalanine synthesis to occur, up to the optimal concentration, the inhibitor concentration is either too low to be effective or any inhibition is made good by the system but at a particular inhibitor concentration the loss in activity is not recovered.

Alternatively, the loss of polyphenylalanine activity may be incurred as a result of increased competition for the polyurdylic

acid template by inefficient cytoplasmic ribosomes which may block or reduce the rate of translation which is manifest in a reduced quantity of polyphenylalanine. Inefficient cytoplasmic ribosomes may possess fragments of mRNA or peptidyl-tRNA or they may have been damaged by the rigours of cell-breakage.

b) Cytoplasmic ribosome-free supernatant fraction, S-100.

An analysis of S-100 fractions in sucrose density gradients (an example of which is shown in Figure 4.2, Section 4.5 a), did not reveal a detectable quantity of 254 nm. absorption in the region in which cytoplasmic ribosomal material was expected to sediment. The S-100 fraction was assumed to be devoid of cytoplasmic ribosomes and was termed the cytoplasmic ribosome-free supernatant. The S-100 fraction was assumed to contain the endogenous factors necessary for polyphenylalanine synthesis but their identity and concentration were not known.

The effect of the S-100 concentration on polyphenylalanine synthesis was measured at three RP-100 concentrations; no RP-100, in the presence of the preoptimised RP-100 concentration (3.0 A_{260}unit ; Table 4.1) and in the presence of the optimised RP-100 concentration(2.0 A_{260}unit;Section 4.5a).

When no S-100 was present in the optimised reaction mixture (Chapter 2, Section 2.9. Table 2.2) there was no polyphenylalanine synthesis, despite the presence of the RP-100 fraction (Figure 4.4) The result demonstrated that polyphenylalanine synthesis was totally dependant on the presence of the S-100 fraction. The identity of

the essential cytoplasmic constituent of the S-100 fraction was not known.

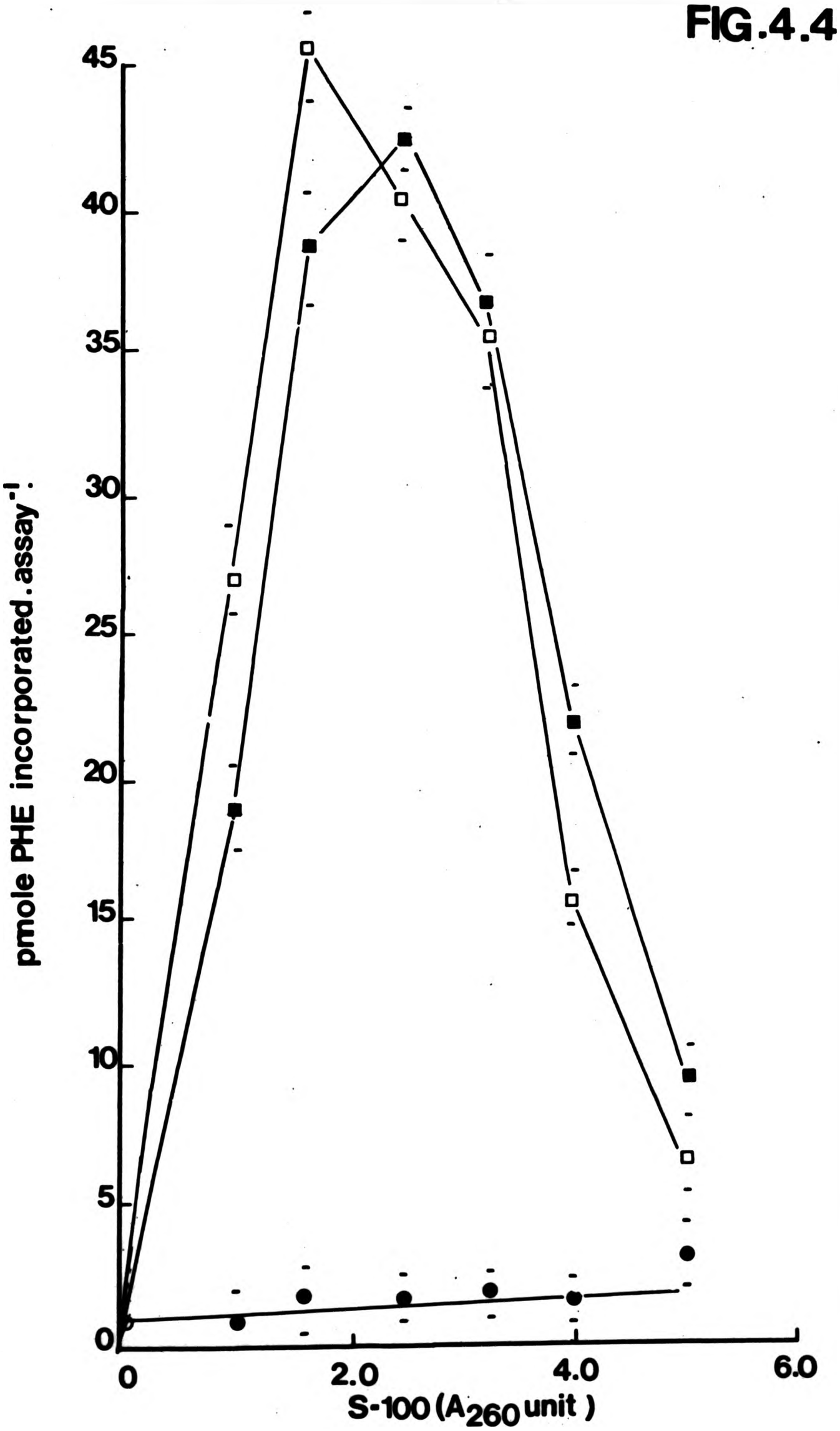
The effect of S-100 on the *in vitro* system was similar at both RP-100 concentrations(Figure 4.4). Polyphenylalanine synthesis was stimulated at relatively low S-100 concentrations,which was inferred to mean that S-100 was rate limiting. Maximum polyphenylanine synthesis was greatest and acheived at a lower S-100 concentration when 2.0 A_{260}unit RP-100 was used, rather than when 3.0 A_{260}unit was present (Figure 4.4) .At S-100 concentrations above the optimum for maximum polyphenylalanine synthesis, S-100 was inhibitory. At 5.0 A_{260}unit S-100,the maximum concentration examined, polyphenylalanine synthesis was less than 20% of the maximum activity obeerved (Figure 4.4).

The inhibitory effect of the S-100 fraction above the optimum concentration may have been a consequence of inhibitors, which acted in a similar way to those assumed in the RP-100 fraction (Section 4. 5 a). Unlike the preparation of equivalent fractions in many other species (eg. Berry *et al*,1978)or of the G-30 fraction (Appendix B i) it was not necessary to subject the S-100 fraction to for example, Sephadex chromatography, precipitation by ammonium sulphate or at pH5, or dialysis, in order to produce a satisfactory level of polyphenylalanine synthesis. It was however, possible that the removal of low molecular weight compounds from the S-100 fraction may have minimised the presence of inhibitors and endogenous assay constituents and produced greater levels of activity than those observed (Figure 4.4) and resulted in a more defined *in vitro* system.

Figure 4.4. The effect of the S-100 fraction on polyphenylalanine synthesis.

The effect of the S-100 fraction, the cytoplasmic-ribosome free supernatant, on polyphenylalanine synthesis was examined at three RP-100 concentrations : 0 A_{260} unit (●) , 2.0 A_{260}unit (□) and 3.0 A_{260}unit (■) : in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2). Duplicate 40 µl samples were taken from replicate experiments after 1 hour, according to the method given in Chapter 2, Section 2.9. Background radioactivity, 103 cpm, was deducted from the average values. (1016 cpm = 1 p mole. phe).

FIG.4.4


45
40
35
30
25
20
15
10
5
0
pmole PHE incorporated.assay⁻¹.
0
2.0
4.0
6.0
S-100 (A260 unit)

The presence of endogenous assay constituents in the S-100 fraction may have contributed toward the inhibition of polyphenylalanine synthesis. One possibility is that each preparation of the S-100 fraction may have had different concentrations of the endogenous assay constituents such that the exogenous concentration rendered the total concentration from the optimum to an inhibitory value. The presence of endogenous unlabelled L-(U-C^{12}) phenylalanine would increase as the S-100 concentration increased,thus reducing the specific activity of the radioactive amino acid and resulting in an observed decline in C^{14}-polyphenylalanine synthesis.

c) Relationship between the RP-100 and S-100 fractions.

The evidence presented in Sections 4.5a and b,revealed a relationship between the concentration of RP-100 and the concentration of S-100 on polyphenylalanine synthesis. At any given constant S-100 concentration, there was an optimum RP-100 concentration which produced maximum activity. The optimum RP-100 concentration varied deperding on the S-100 concentration.

When the concentrations of RP-100 and S-100 were expressed as a ratio and related to the % polyphenylalanine synthesis activity (Figure 4.5) the result demonstrated that the optimum concentration ratio of RP-100:S-100 for maximum activity was 1:1, but that ratios ranging from 0.5 : 1 to 2.0 : 1 produced activity which was almost as high.

When the concentration of RP-100 fraction was in excess of the S-100 concentration (ie, a low ratio) polyphenylalanine synthesis was relatively low but could be stimulated by the addition of S-100 (Figure 4.5). Conversley, when the RP-100 : S-100 concentration

Figure 4.5. The relationship between RP-100 and S-100 concentration and polyphenylalanine synthesis.

The results of several experiments are represented by the Figure 4.5. In each experiment, the effect of polyphenylalanine synthesis of various RP-100 concentrations, ranging from 0 to 4.2 A_{260}unit , were examined at a constant S-100 concentration in an otherwise optimised reaction mixture (as described in Figure 4.3) in the series of experiments, different constant S-100 concentrations were employed, ranging from 0 to 3.3 A_{260}unit.

The quantity of polyphenylalamine synthesised (pmole phe. assay^{-1},hr^{-1}) in each experiment was expressed as a percentage of the maximum activity observed (100%). The concentration ratio (A_{260} unit) of RP-100:S-100 in each experiment was calculated and related to the % activity observed for each treatment. Data used is given in Appendix B, Table B 5.

FIG.4.5

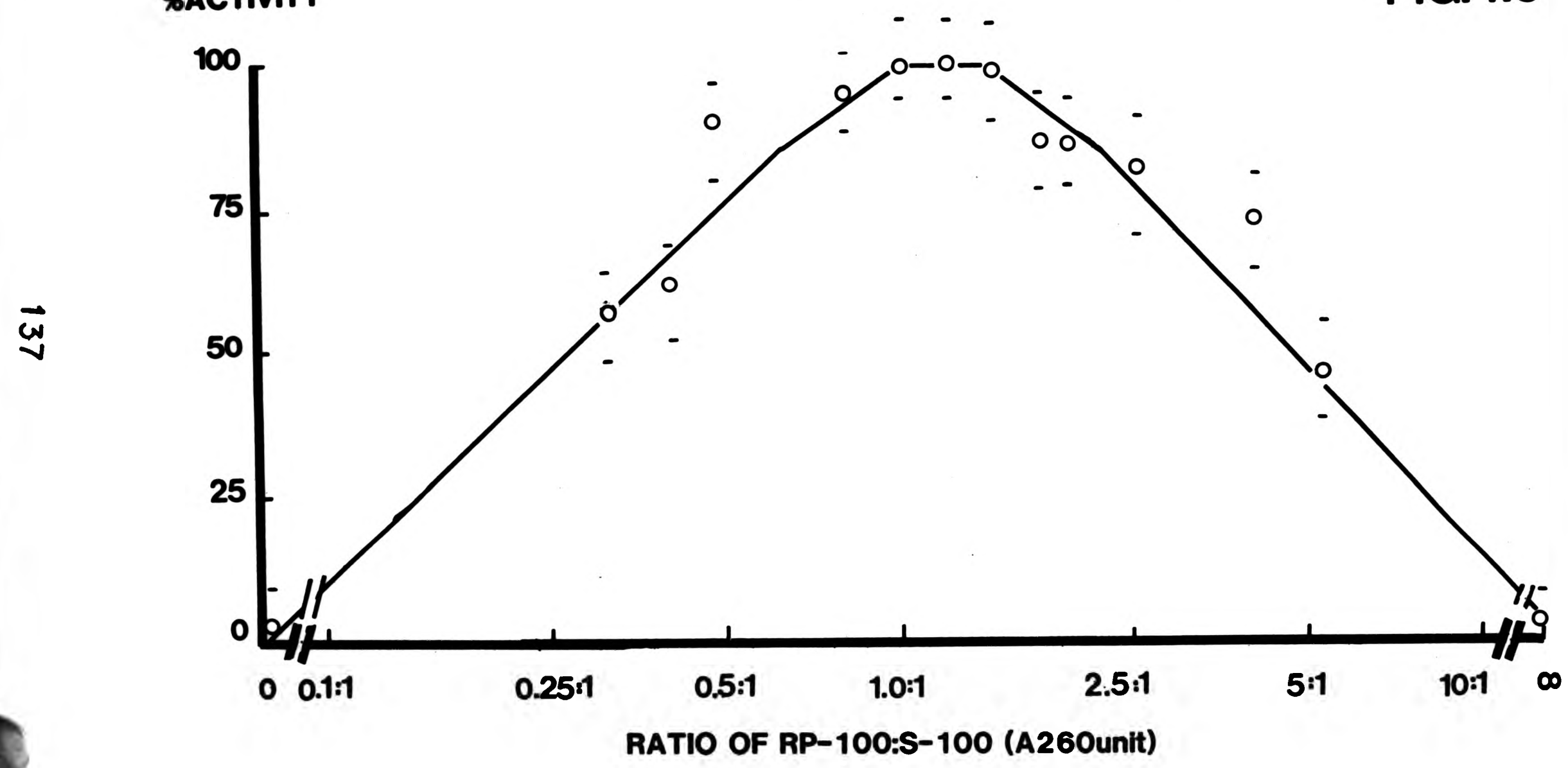

%ACTIVITY
100
75
50
25
0
0
0.1:1
0.25:1
0.5:1
1.0:1
2.5:1
5:1
10:1
∞
RATIO OF RP-100:S-100 (A260unit)

Table 4.2. The variability exhibited by preparations of CY8 cell-extracts.

Source of RP-100 and S-100 fractions	Polyphenylalanine synthesised (pmole phe assay. hr^{-1}) n=4	(±se)
CY8.A	22.6	(2.3)
CY8.B	24.8	(3.4)
CY8.C	17.5	(2.9)
CY8.D	25.3	(3.1)

CY8A, CY8B, CY8C and CY8D identify four preparations of RP-100 and S-100 fractions derived from CY8 according to the method described in Chapter 2, Section 2.7. The capacity of each preparation to synthesise polyphenylanine was measured in a pre-optimised reaction mixture (Table 4.1) according to the method given in Chapter 2, Section 2.9.

ratio was high there was relatively low polyphenylalanine synthesis which could be stimulated by the addition of RP-100 (Figure 4.5)

To produce an optimised cell-free polyphenylalanine synthesising system for any given S-100 concentration it was possible to predict the RP-100 concentration. Similarly, given a RP-100 concentration the S-100 concentration for optimum in vitro activity could be determined.

From all of the combinations of RP-100 and S-100 concentrations examined which had a ratio of 1:1, the greatest activity was observed when both fractions were at 2.0 A_{260}unit (Appendix B,Table B5). In the optimised reaction mixture, the concentrations of the RP-100 and S-100 fractions chosen were both 2.0 A_{260} unit.

d) Effect of preparation.

In addition to the effects of RP-100 and S-100 concentrations on polyphenylalanine synthesis (Sections 4.5 a and b), there were noticable effects caused by the particular preparations of cell-extract employed.

The variability between four preparations of RP-100 and S-100 fractions derived from CY8 is revealed in Table 4.2. All were prepared according to the same method (Chapter 2, Section 2. 7) and yet the preparation identified as CY8D consistantly produced the largest quantity of polyphenylalanine. Whether the RP-100 or S-100 fractions, or both, were responsible for the variable activities of the different preparations was not examined.

A possible reason for the variability of the cell-extracts were that the vigour of manual cell-breakage had differed during their preparation and that although the yield in terms of A260 units were similar (Chapter 2, Section 2.7) the measurement did not take into account the activity of the RP-100 and S-100 fractions. Although not examined in this investigation, it is probable that minimal force applied to release the cytoplasmic contents produced more active cell-extracts (Sissons,1974; Berry et al.1978).The fact that the RP-100 fraction was composed entirely of monosomes, rather than polysomes (Figure 4.2) suggests that there was damage to the polysomes which may have adversley affected the integrity of the monosomes. Damaged monosomes may account for the slightly lower value of the sedimentation coefficient estimated for Coprinus cinereus (74.4S; Section 4.5a) relative to those of other fungal species (Chapter 1, Section 1.4,Table 1.1).

e) Effect of storage.

One of the possible sources of variation between the cell-extracts which was examined was their stability when frozen. Each preparation (Table 4.2) had been stored for different times (CY8D had been kept the least time). The method of storage of the RP-100 and S-100 fractions prior to their addition to the in vitro reaction mixture had a considerable bearing on the amount of polyphenylalanine synthesised (Table 4.3).

Freshly prepared RP-100 and S-100 fractions were the most active but it was impracticable to prepare fresh cell-extracts immediately prior to carrying out the assay. Cell-extracts frozen in liquid nitrogen and kept at -70°C produced a reasonable amount of

Table 4.3. The effect of storage of cell-extracts on their capacity to synthesise polyphenylalanine.

Treatment of freshly prepared RP-100 prior to addition to reaction mixture.	Polyphenylalanine synthesised (pmole phe. assay^{-1}. hr^{-1}.) (n=4)	(se)
On ice for 10 min	42.6	(2.3)
At 20°C for 10 min	28.3	(1.8)
S-100 on ice for 10 mins and RP-100 at 20°C for 10 mins	34.6	(3.1)
RP-100 on ice and S-100 at 20°C for 10 min.	33.4	(2.6)
Frozen at -190°C for 10 min	41.7	(3.5)
Frozen at -190°C, stored for 6 months at -70°C	28.9	(2.1)
Frozen for 10 mins at -190°C, thawed and refrozen for 10 mins	38.1	(3.3)
Frozen, thawed and refrozen for 6 months	18.3	(1.8)

Thawed samples were kept on ice. Cell-extracts were derived from CY8D (Table 4.2) and were analysed in an optimised reaction mixture according to the method described in Chapter 2, Section 2.9, Table 2.2.

polyphenylalanine (Table 4.3) and was used in the routine preparation of cell-extracts (Chapter 2, Section 2.7). In fact cell-extracts remained active, albeit a low level, for the duration of the investigation if kept frozen. However,once thawed and refrozen, activity was adversley affected. In order to reduce losses in activity due to storage, the cell-extracts were stored in small volumes to avoid the need for thawing and refreezing and its deleterious effect on activity.

Both the RP-100 and S-100 fractions produced reduced activity if left at room temperature,suggesting heat labile components existed in both fractions (Table 4.3).

SECTION 4.6. THE EFFECT OF POLYURYDILIC ACID AND TRANSFER RNA ON POLYPHENYLALANINE SYNTHESIS.

	Polyuridylic acid poly (U).	Transfer RNA, phenylalanine specific ($tRNA^{phe}$).
Role in cell-free polypeptide synthesis.	Synthetic template for polyphe. synthesis (Nirenberg & Matthaei, 1961).	Essential for the activation of phe. and incorporation of phe into polyphe.
Effect observed in vitro.	Figure 4.6.	Figure 4.7.
Activity in the absence of exogenous constituent (% of maximum activity).	5% Demonstrated that the reaction was dependant on poly (U). It was inferred that synthesis of polyphenylalanine by endogenous mRNA was insubstantial.	5% Demonstrated that the reaction was dependant on $tRNA^{phe}$. The endogenous concentration of $tRNA^{phe}$ was considered to be insignificant.
Optimum concentration for maximum activity.	20 - 30 $\mu g.ml.^{-1}$	5 - 8 $\mu g.\ ml.^{-1}$
Optimum concentration range (10% maximum activity).	17 - 38 $\mu g.ml.^{-1}$	3 - 10 $\mu g.\ ml.^{-1}$
Inhibitory effect.	At concentrations above 38$\mu g.ml.^{-1}$ poly (U) inhibited polyphenylalanine synthesis. Activity declined to 45% of the maximum at 80 $\mu g.ml^{-1}$ the highest poly (U) examined.	Inhibition above the optimum concentration was slight. Activity declined over a narrow concentration range and then activity remained constant over the concentration range examined, up to 30 $\mu g.ml.^{-1}$
Comments.	The size of the poly(U) was not known.	Competition between deacylated tRNA and phe-tRNA may have produced the observed inhibition (Kyner et al, 1973)

Unless otherwise stated, the results were obtained using an optimised reaction mixture (Chapter 2, Section 2.9).

Figure 4.6. The effect of polyuridylic acid on polyphenylalanine synthesis.

The effect of polyuridylic acid in polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Tables 2.2). Duplicate 40 µl samples were analysed from replicate experiments after 1 hour according to the method described in Chapter 2, Section 2.9. A correction was made for the background activity, 113.5 cpm (1016 c pm= 1 p mole. phenylalanine).

Figure 4.7. The effect of transfer RNA^{Phe} on polyphenylalanine synthesis.

The effect of phenylalanine specific tRNA was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described in Figure 4.6.

Figure 4.6. The effect of polyuridylic acid on polyphenylalanine synthesis.

The effect of polyuridylic acid in polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Tables 2.2). Duplicate 40 µl samples were analysed from replicate experiments after 1 hour according to the method described in Chapter 2, Section 2.9. A correction was made for the background activity, 113.5 cpm (1016 c pm= 1 p mole. phenylalanine).

Figure 4.7. The effect of transfer RNA^{Phe} on polyphenylalanine synthesis.

The effect of phenylalanine specific tRNA was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described in Figure 4.6.

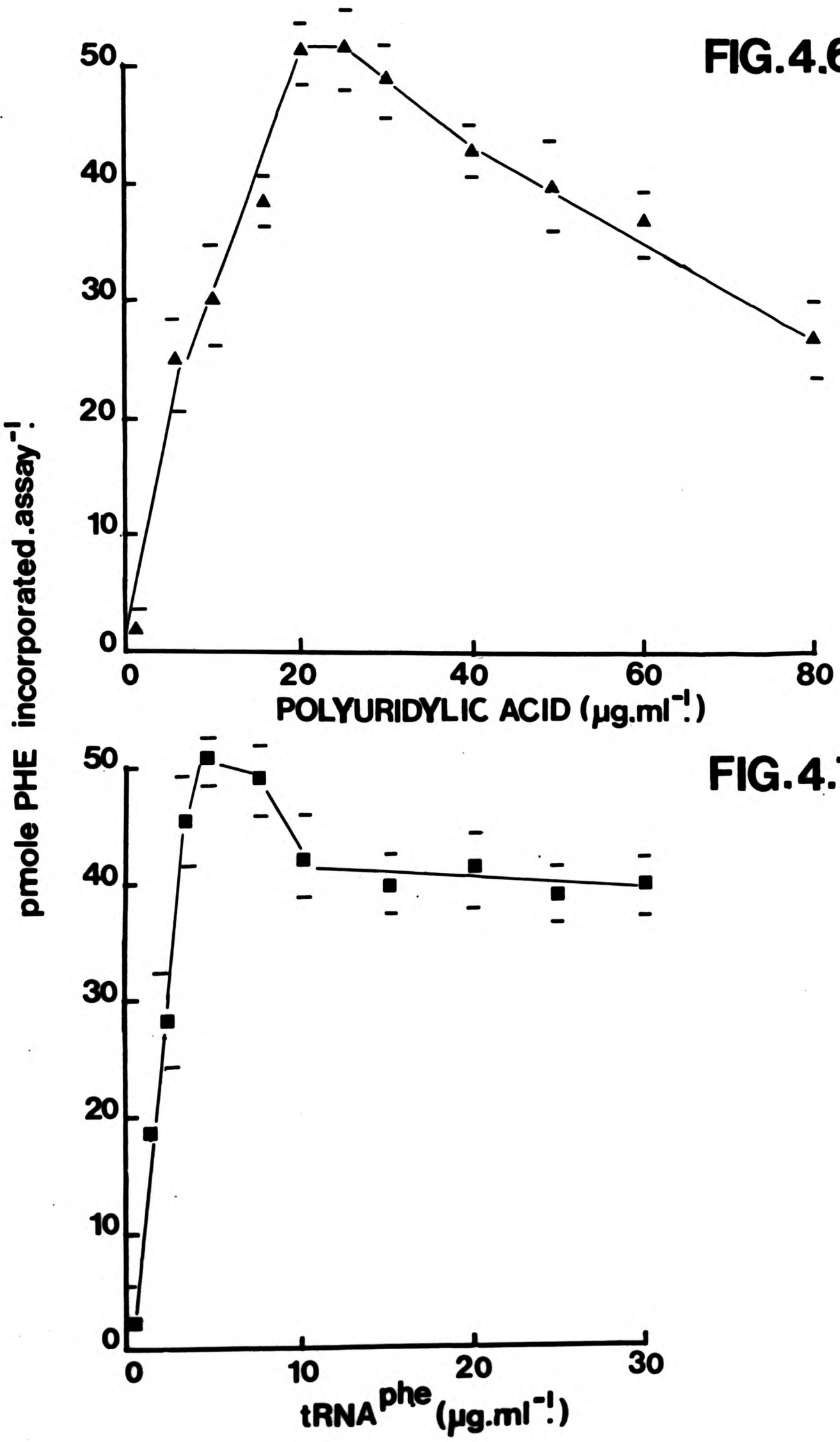

50
40
30
20
10
0
0
20
40
60
80
pmole PHE incorporated.assay⁻¹.
POLYURIDYLIC ACID (µg.ml⁻¹.)

FIG. 4.6

50
40
30
20
10
0
0
10
20
30
pmole PHE incorporated.assay⁻¹.
tRNA^phe (µg.ml⁻¹.)

FIG. 4.7

SECTION 4.7. THE EFFECT OF ADENOSINE 5-TRIPHOSPHATE, GUANOSINE 5-TRIPHOSPHATE, CREATINE PHOSPHATE AND CREATINE PHOSPHOKINASE ON POLYPHENYLALANINE SYNTHESIS.

	Adenosine 5-triphosphate (ATP)	Creatine phosphate	Creatine phosphokinase	Guanosine 5-triphosphate (GTP)
Role in cell-free polypeptide synthesis	Each polypeptide band requires the hydrolysis of at least four molecules of ATP (Swedes et al, 1979).	Creatine phosphokinase regenerates ATP from creatine phosphate and ADP thereby replenishing the pool of ATP. (Van der Decken 1967)		Specific requiremnet for the initiation of mRNA and for aminoacyl tRNA binding and translocation in elongation (Section 4.2). Figure 4.9.
Effect observed in vitro.	Figure 4.8.	Figure 4.10.	Figure 4.11.	
Activity in the absence of exogenous constituent (% of max. activity).	25% The reaction mixture was dependant on exogenous ATP. The endogenous ATP concentration, and the concentration of creatine phosphate and creatine phosphokinase and endogenous ATP produced low activity	35%	35% The result demonstrated either that there was a substantial amount of synthesis as a result of endogenous and exogenous ATP, or that the cell-extract possess an endogenous ATP regeneration couple.	60% Demonstrated a substantial endogenous concentration sufficient for most of the reactions requirement.
Optimum concentration for maximum activity	1.0 mM.	12mM	6.0 $\mu g\ ml^{-1}$	0.25 mM
Optimum concentration range (± 10% max. activity)	0.7 - 1.3 mM.	10 -17 mM	5 - 12 $\mu g\ ml^{-1}$	0.1 - 0.3 mM
Inhibitory effect	Inhibition reached 50% at the highest concentration tested 2mM.	Inhibition reached 65% at 12 mM.	Slight inhibition, 10% at 30 $\mu g ml^{-1}$.	Inhibition reached 80% at 0.75 mM.
Comments	The effect of ATP in the absence of creatine phosphate and creatine phosphokinase was not determined.	The effect of these constituents was not measured in the absence of ATP. The two constituents were used because Woodward et al (1974) reported that they were a more effective at regenerating ATF than pyruvate kinase and phosphoenol pyruvate.		With abnormal intiation the requirement for GTP is low. Translation of poly (U) required GTP and thus enzymic translation did occur (Pestka, 1974).

Unless otherwise stated, the results were obtained using an optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2).

Figure 4.8. The effect of adenosine - 5 - triphosphate on polyphenylalanine synthesis.

The effect of adenosine - 5 - triphosphate (ATP) on polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2). Duplicate 40 µl samples were taken from replicate experiments after 1 hour, according to the method given in Chapter 2, Section 2.9.

Figure 4.9. The effect of guanosine - 5 - triphosphate on polyphenylalanine synthesis.

The effect of guanosine - 5 - triphosphate (GTP) on polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described for Figure 4.8.

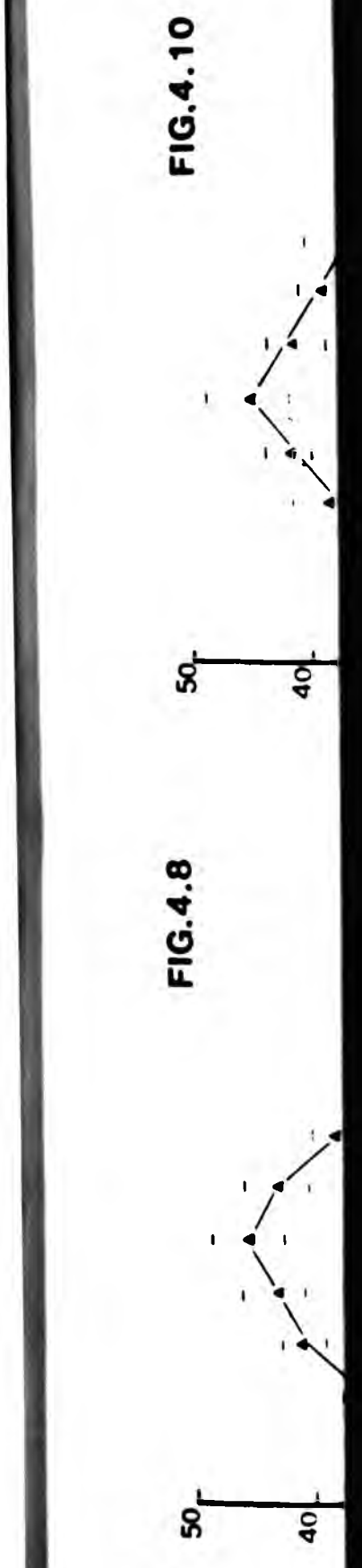
Figure 4.10. The effect of creatine phosphate on polyphenylalanine synthesis.

The effect of creatine phosphate on polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described in Figure 4.8.

Figure 4.11. The effect of creatine phosphokinase on polyphenylalanine synthesis.

The effect of creatine phosphokinase on polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2 .9, Table 2.2) as described for Figure 4.8.

In each of Figures 4.8, 4.9 and 4.11 results were corrected for a background activity of between 100 and 150 cpm (1016 cpm = 1p mole.phe).



phate on

(ATP) on poly-
ise optimised
.2). Duplicate
ts after 1 hour,
on 2.9.

phate on

(GTP) on
herwise optimised
.2) as described

olyphenylalanine

nylalanine
reaction mixture
in Figure 4.8.

on polyphenylalanine

yphenylalanine
reaction mixture
for Figure 4.8.

corrected for a

FIG.4.10

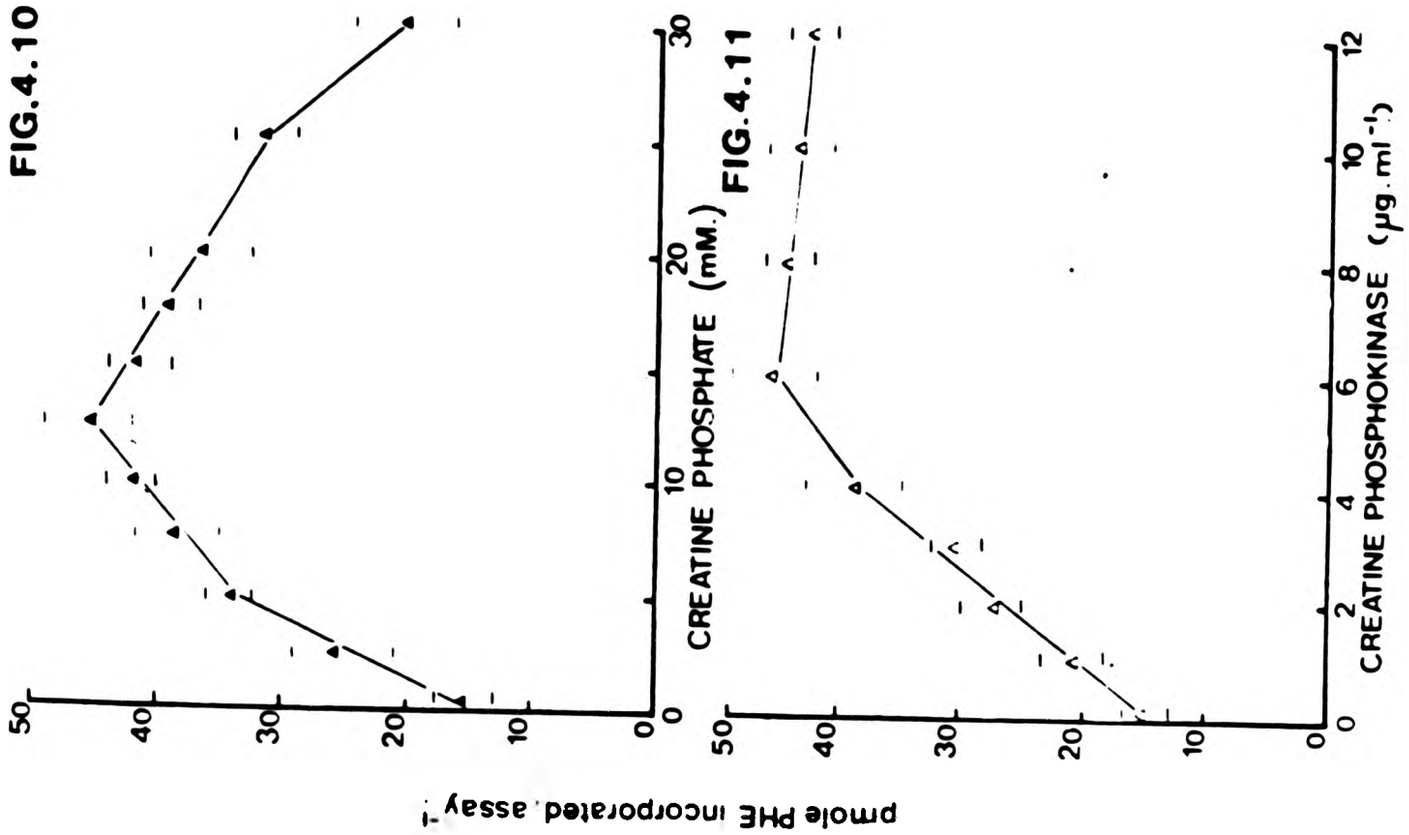


FIG.4.8

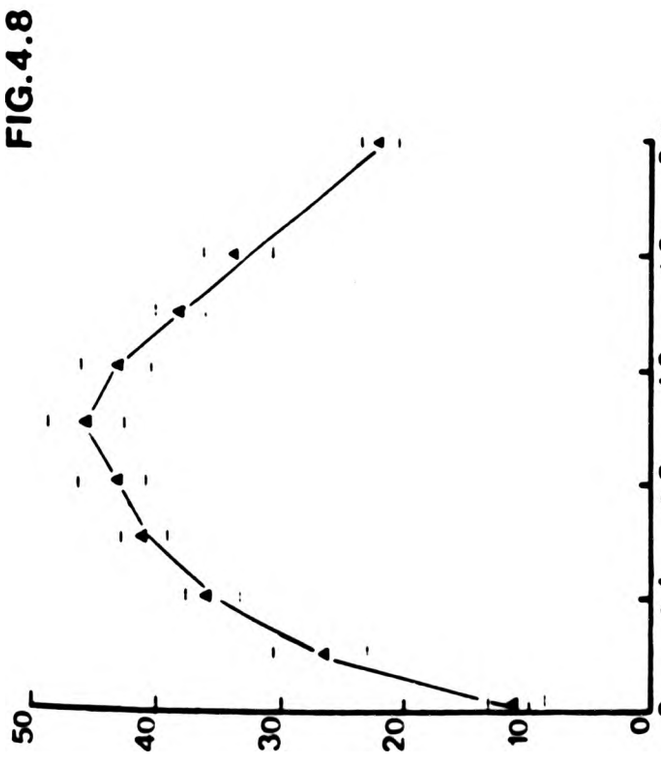
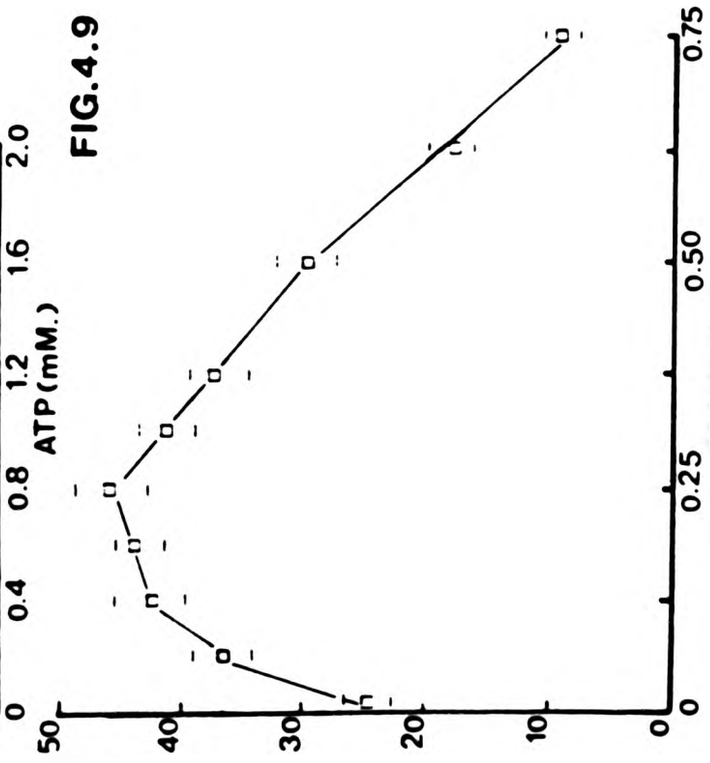


FIG.4.9



SECTION 4.8. THE EFFECT OF MAGNESIUM ACETATE AND SPERMIDINE ON POLYPHENYLALANINE SYNTHESIS.

	Magnesium acetate	Spermidine
Role in cell-free polypeptide synthesis	Magnesium acetate is a source of divalent cations which are necessary to maintain the integrity of cytoplasmic ribosomes and prevent dissociation (Petermann et al, 1958) and for initiation in a poly (U) system (Flavey and Staehelin, 1970).	Spermidine and polyamines in general are bound to ribosomes with which they are extracted (Reyes et al, 1978). Their probable function is to maintain ribosome stability, binding of ribosomes to poly (U) and tRNA and nucleic acid stability (Tabor and Tabor, 1976)
Effect observed in vitro	Figure 4.12.	Figure 4.13.
Activity in the absence of exongenous constituent (% of max. activity)	10% (2mM) Activity was not measured in the complete absence of magnesium acetate, 2mM was present in the reaction mixture derived from the cell-extract in Extraction Buffer. However, the reaction was dependant on magnesium acetate	10% The endogenous spermidine content of the assay was insufficient to produce a high activity. The reaction was dependant on the exogenous spermidine.
Optimum concentration for max. activity.	4.5 mM.	1.0 mM
Optimum concentration range (10% of max activity).	4.0 - 6.0 mM.	0.75 - 1.4 mM
Inhibitory effect.	There was a gradual inhibitory effect above the optimised concentration which reached 85% at the highest concentration tested.	Over the range of spermidine concentrations examined inhibition was negligible.
Comments.	In the absence of spermidine, activity was low and achieved only 10% of the activity observed in the presence of the optimum spermidine concentration It was inferred that there were specific requirements in the reaction mixture for magnesium acetate and spermidine Optimal monovalent cations did not fulfill the requirements for divalent and multivalent cations.	Endogenous spermidine stimulated polyphenylalanine synthesis but did not reduce the requirement for Mg^{2+}. In the absence of magnesium acetate, activity was low, only 15% of the activity observed in the presence of optimum magnesium acetate concentration.

Unless stated otherwise the results were obtained using an optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2).

The effect of magnesium acetate and spermidine on polyphenylalanine synthesis

Figure 4.12. The effect of magnesium acetate on polyphenylalanine synthesis was examined at two concentrations of exogenously supplied spermidine, 0mM (□) and 1mM (■), in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) Duplicate 40µl samples were analysed from replicate experiments after 1 hour, acccrding to the method described in Chapter 2, Section 2.9. The concentration of magnesium acetate contributed to the final concentration by the cell-extracts was 2mM (1016 cpm = 1 p mole.phe).

Figure 4.13. The effect of exogenously supplied spermidine cn polyphenylalanine synthesis was examined at two concentrations of magnesium acetate, 2mm (O) and 4.5mM (●) in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) according to the method described in Figure 4.12.

ine

FIG .4.12

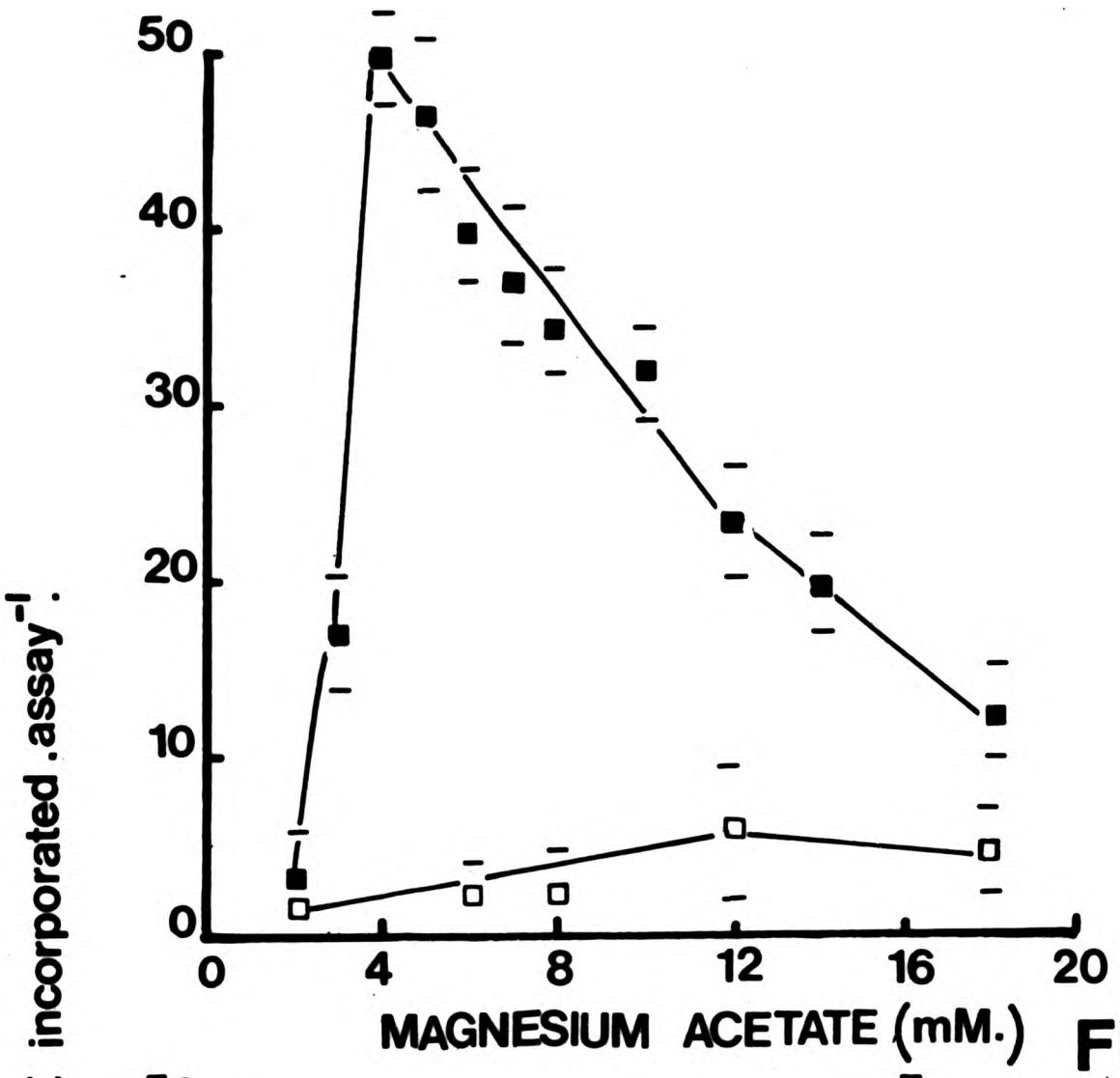
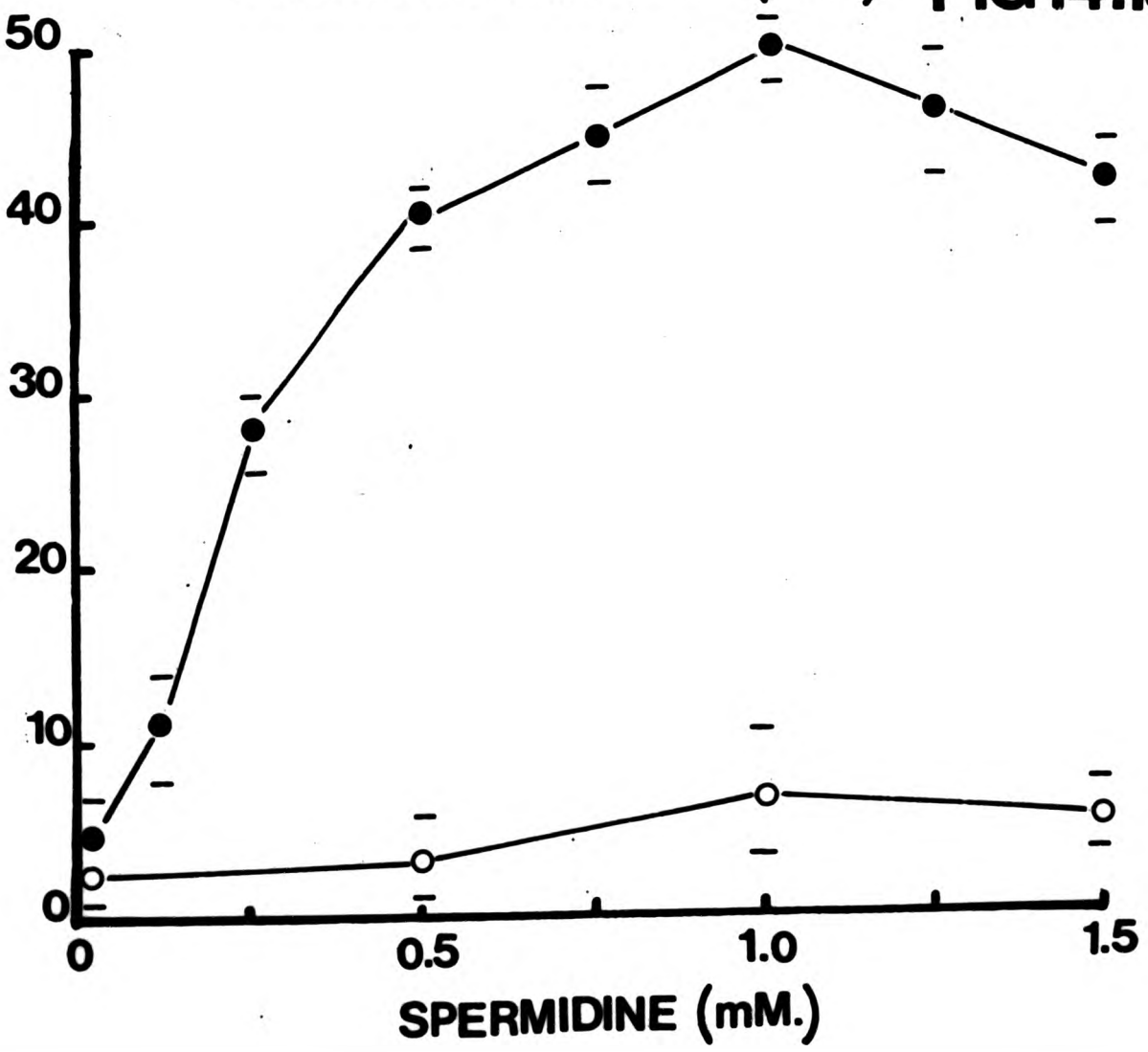


FIG .4.13



SECTION 4.9. THE EFFECT OF AMMONIUM ACETATE AND POTASSIUM CHLORIDE ON POLYPHENYLALANINE SYNTHESIS.

	Ammonium acetate.	Potassium chloride.
Role in cell-free polypeptide synthesis.	Ammonium acetate and potassium chloride provided the monovalent cations; their precise function is not known (Van der Decken, 1967).	
Effect observed in vitro.	Figure 4.14.	Figure 4.15.
Activity in the absence of exogenous constituent (% of max. activity).	50% In the presence of optimum KCl, ammonium acetate was not essential for activity. However in the presence of minimal KCL(5mM) 15% activity demonstrated that the reaction was dependant on ammonium acetate.	20% (5mM) Activity could not be measured in the absence of KCl, 5mM was present in the reaction mixture derived from the cell-extract in Extraction Buffer. In the presence or absence of ammonium acetate the reaction was dependant on KCl.
Optimum concentration for max. activity.	40 mM	20 mM (at both ammonium acetate concentrations tested).
Optimum concentration range (10% max activity).	10 - 50 mM	17 - 27 mM.
Inhibitory effect.	At concentrations greater than 40 mM, inhibition occurred, reaching 50% at 100 mM, the maximum concentration examined. Inhibition was reduced in the presence of minimum KCl concentration.	Inhibition was observed in the presence and absence of ammonium acetate and reached similar inhibitory levels, approximately 65% at the maximum KCl concentrations tested.
Comments.	The effect of ammonium acetate was similar in the presence and absence of KCl. There was a specific requirement for ammonium acetate which KCl only partially fulfilled. The optimum total monovalent cation concentration.	The effect of KCl produced similar responses in the presence and absence of ammonium acetate, but the activity was 2.5x greater when ammonium acetate was present. Inhibition may be the result of dissociation and inability of ribosomal subunits to reassociate, 100 mM KCl was used in the dissociation experiments (Appendix B iii). High chloride anions may also be inhibitory (Weber et al, 1977).

Unless stated to the contrary, the results were obtained using an optimised mixture (Chapter 2, Section 2.9, Table 2.2).

The effect of ammonium acetate and potassium chloride on polyphenylalanine synthesis.

Figure 4.14. The effect of variable ammonium acetate concentrations on polyphenylalanine synthesis was examined at two concentrations of potassium chloride, 5mM (□) and 20 mM (■) in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2). Duplicate 40 µl samples were analysed from replicate experiments after 1 hour, according to the method described in Chapter 2, Section 2.9. A correction for background activity of 127.1 cpm was made (1016 cpm = 1 p mole. phe).

Figure 4.15. The effect of potassium chloride on polyphenylalanine synthesis was determined at two concentrations of ammonium acetate, 0mM (Δ) and 40 mM (▲), in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described for Figure 4.14. The cell-extract contributed 5mM of the final potassium chloride concentration.

FIG .4.14

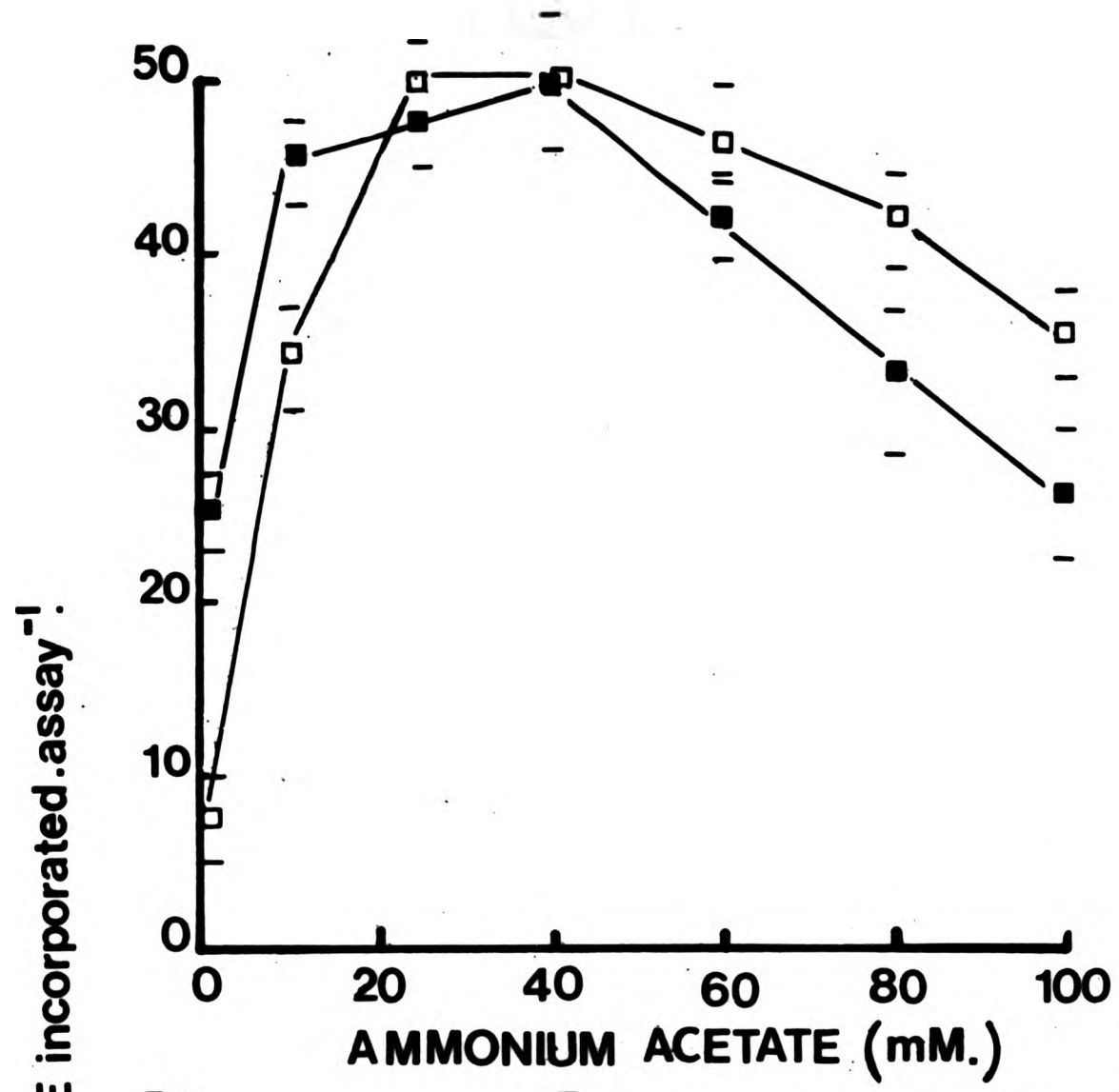
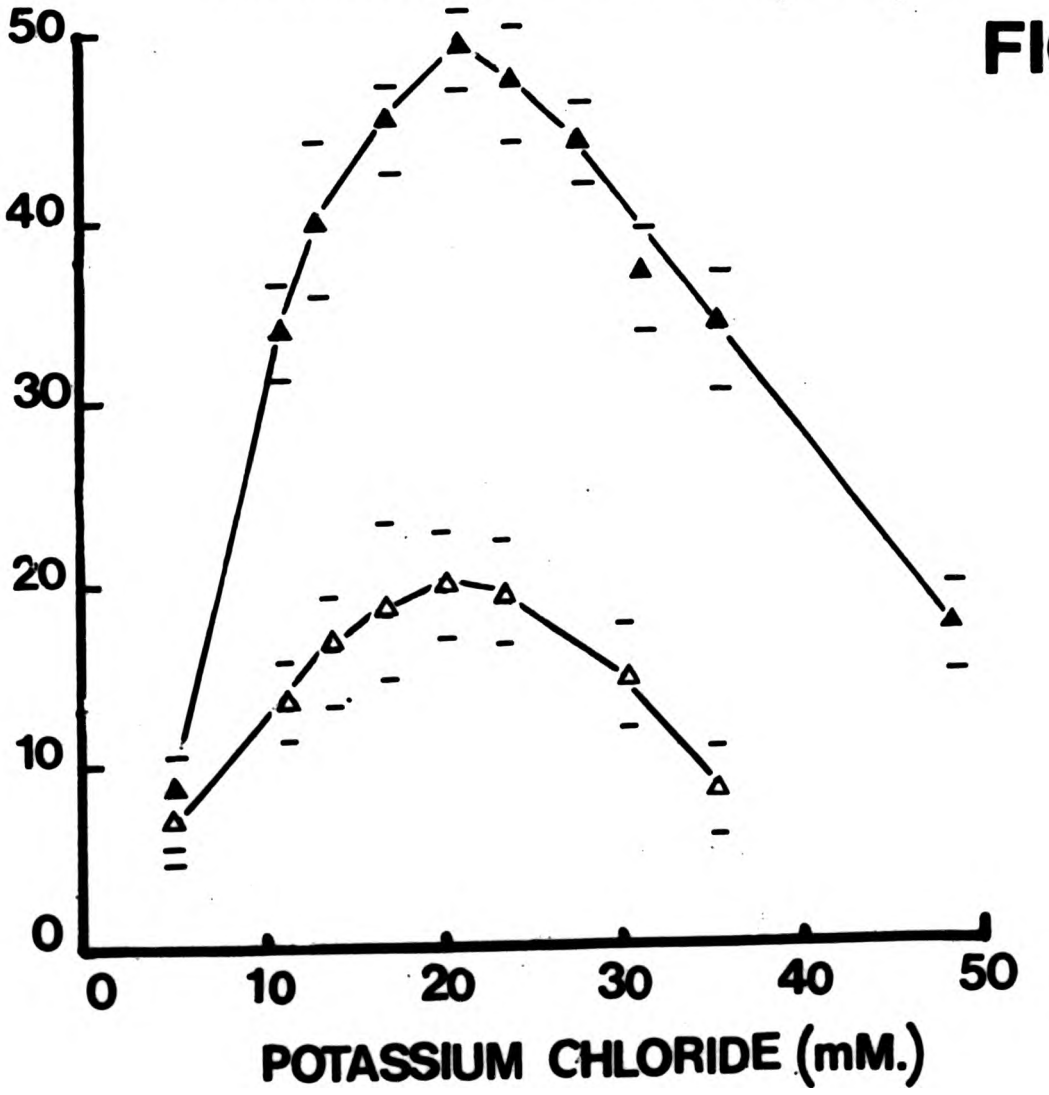


FIG. 4.15



SECTION 4.10. THE EFFECT OF TRIS-HCL AND DITHIOTHREITOL ON POLYPHENYLALANINE SYNTHESIS.

	Tris-HCl, pH7.5.	Dithiothreitol.
Role in cell-free polypeptide synthesis.	Tris-HCl was used in this reaction mixture to maintain a constant pH.	Dithiothreitol is a reducing agent which prevented inactivity resulting from oxidation, eg. of cytoplasmic proteins possessing sulphydhryl groups (Baliga et al, 1971).
Effect observed in vitro.	Figure 4.16.	Figure 4.17.
Activity in the absence of exogenous constituent (% of max. activity).	90% (10 mM) Activity was not measured in the total absence of Tris-HCl, 10 mM was present in the reaction derived from the cell-extract in Extraction Buffer. The assay was independant of additional Tris-HCl.	50% The reaction is not dependant on dithiothreitol. However, it is not known if the reaction is dependant on a reducing agent because cell-extract in Extraction Buffer contributed 2mM of 2-mercaptoethanol which is also a reducing agent.
Optimum concentration for max. activity.	15 mM.	10 mM
Optimum concentration range (10% max. activity).	15 - 25 mM	2 - 10 mM
Inhibitory effect.	Inhibition above the optimum concentration was gradual, reaching 50% at 60 mM, the highest concentration examined.	None observed.
Comments.	The effect of pH on the assay was not examined. It is possible that the effect of Tris -HCl concentration was a result of the pH rather than Tris-HCl constituent per se. In other cell-free systems, there is an optimised pH, within the range pH7 to pH8 (eg. Berry et al,1978: Crouzet et al,1978) and over this range the buffer capacity of Tris-HCl is poor. Inhibition may have been the result of deacylation of the phe tRNA (Heredia and Halvorson,1966)	

Unless stated to the contrary the results obtained using an optimised reaction mixture(Chapter 2, Section 2.9, Table 2.2).

Figure 4.16 The effect of Tris-HCl on polyphenylalanine polyphenylalanine synthesis.

The effect of Tris-HCl on polyphenylalanine synthesis was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2). Duplicate 40 µl samples were analysed from replicate experiments after 1 hour, according to the method described in Chapter 2, Section 2.9. Values were corrected for background activity of 140 cpm (1016 cpm = 1 p mole.phe).

Figure 4.17. The effect of dithiotheitol on polyphenylalanine synthesis.

The effect of dithiotheitol was examined in an otherwise optimised reaction mixture (Chapter 2, Section 2.9, Table 2.2) as described in Figure 4.17.

FIG .4.16

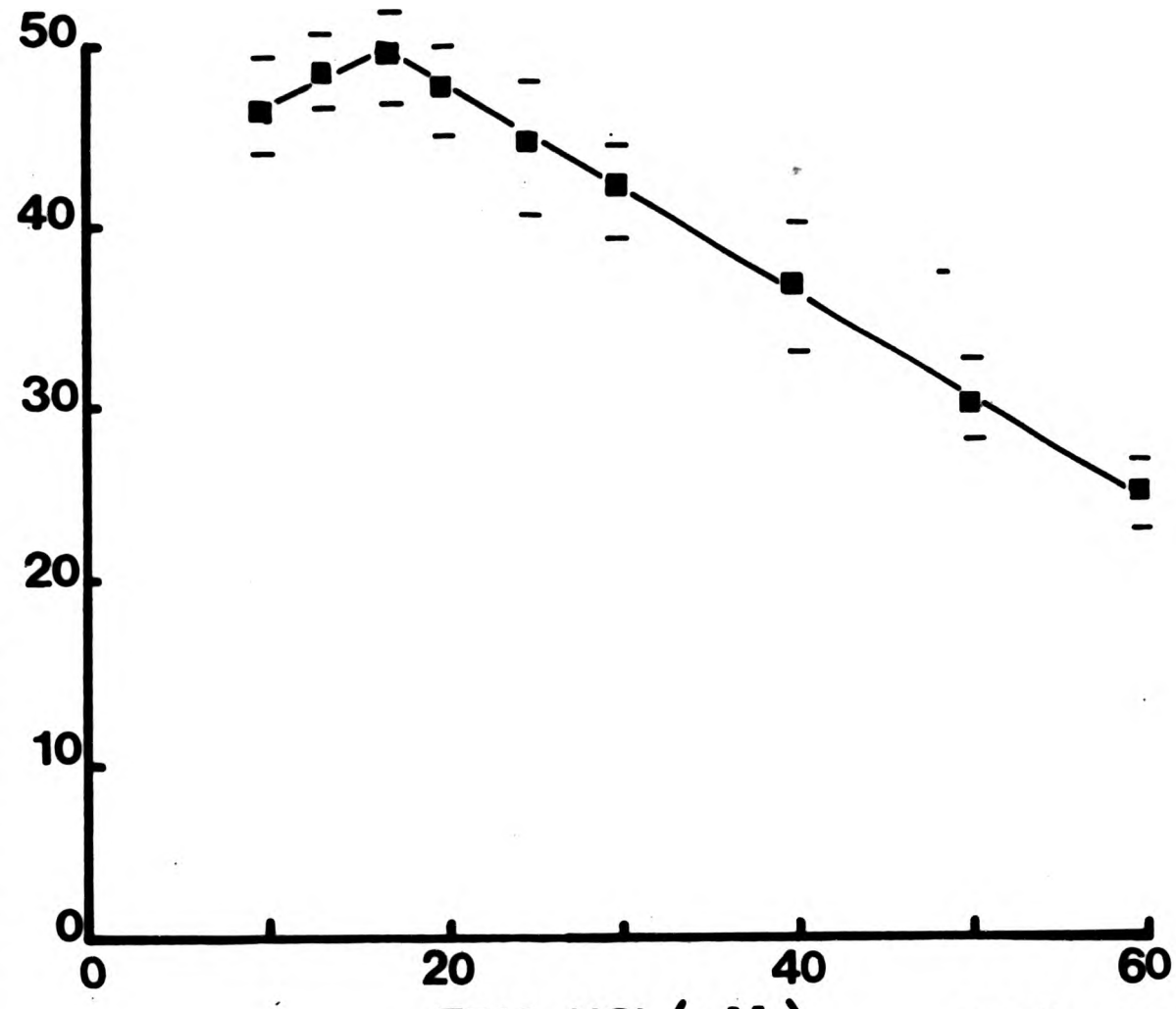
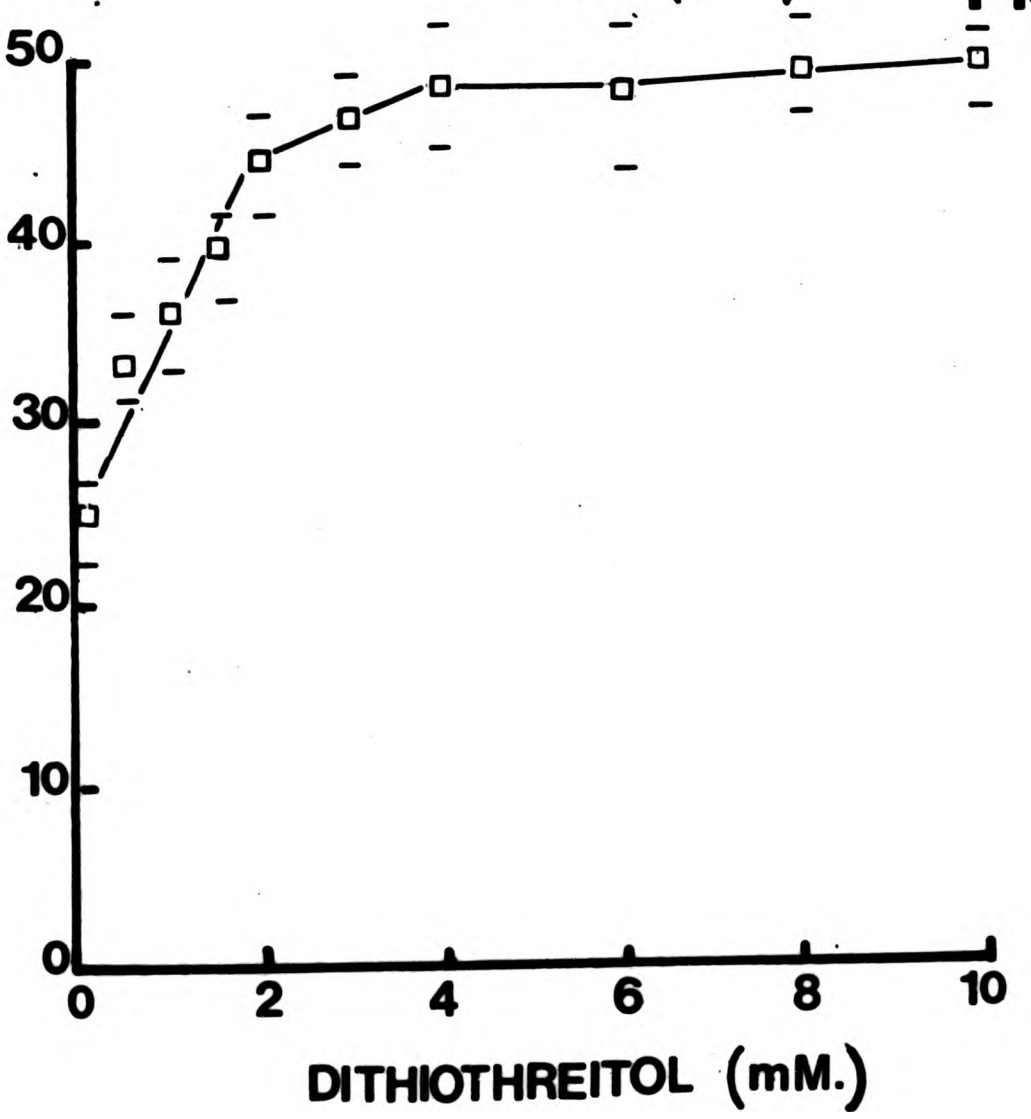


FIG .4.17



SECTION 4.11. L-(U-C^{14}) PHENYLALANINE.

The incorporation of C^{14} into material which was insoluble in TCA at 90°C, was dependant on the presence of radioactive substrate: In the absence of L - (U-C^{14}) phenylalanine, no radioactivity was detected (Figure 4.18a).

In the preoptimised cell-free system (Table 4.1, Section 4.4) maximum polyphenylalanine systhesis was achieved at the highest L - (U-C^{14}) phenylalanine concentration examined, 0.24 µM. With increasing radioactive substrate concentrations there was an approximately linear increase in product synthesised. (Figure 4.18a), but simultaneously, there was a linear decline in the efficiency of the assay system (Figure 4.18b). The L - (U-C^{14}) phenylalanine concentration used in the preoptimised reaction mixture (0.98µM) was chosen as a compromise between a reasonable level of polyphenylalanine synthesis (26.4 pmole phe.assay^{-1} hr.$^{-1}$), at an efficiency of substrate utilisation of 2.7% (Figure 4.18b) and moderated in consideration of the expense of the radiochemical.

When all other constituents in the reaction mixture had been optimised (Table 2.2, Chapter 2, Section 2.9), the effect of L - (U-C^{14}) phenylalanine was revaluated. Lower concentrations of the amino acid were required to produce the same levels of polyphenylalanine synthesis that had been observed in the preoptimised reaction mixture (Figure 4.18a). The optimised reaction mixture synthesised polyphenylalanine approximately 4 x more efficiently than the preoptimised system had achieved (Figure 4.18b).

It was therefore possible to reduce the concentration of L-(U-C^{14}) phenylalanine in the optimised reaction mixture to 0.24 µM and still maintain a satisfactory level of polyphenylalanine synthesis, which had been produced by 0.98 µM in the preoptimised assay, thereby making the assay more economic.

The cell-extracts, particularly the S-100 fraction probably contained endogenous L-(C^{12}) phenylalanine. The specific activity of L- (U-C^{14}) phenylalanine used to determine the pmole polyphenylalanine synthesised and the efficiency of translation (Figure 4.18b) was probably less than the quoted value (513 mCi. $mmole^{-1}$) but because the endogenous concentration was not known, the extent of isotopic dilution was not known. The efficiency of translation must therefore be considered to be estimated values.

Figure 4.18a. The effect of L -(U-C^{14})- phenylalanine on polyphenylalanine synthesis.

The effect L -(U-C^{14})- phenylalanine on polyphenylalanine synthesis was examined in a preoptimised (▲-▲) and an optimised (□-□) incubation mixture. (Tables 4.1 and Table 2.2, Chapter 2 Section 2.9). Duplicate 40 µl samples were analysed after one hour, from replicate experiments according to the method described in Chapter 2, Section 2.9. Values were corrected for background activity (1016 cpm = 1p mole phe).

Figure 4.18b. The efficiency of the in vitro assay.

The efficiency of the polyphenylalanine synthesis at each concentration of L -(U-C^{14})- phenylalanine was calculated from;

$$\frac{\text{pmole polyphenylalanine product}}{\text{pmole phenylalanine substrate}} \times 100\%$$

The efficiencies were obtained for the preoptimised (Δ) and optimised (■) incubation mixtures from the results presented in Figure 4.18a.

FIG.4.18a

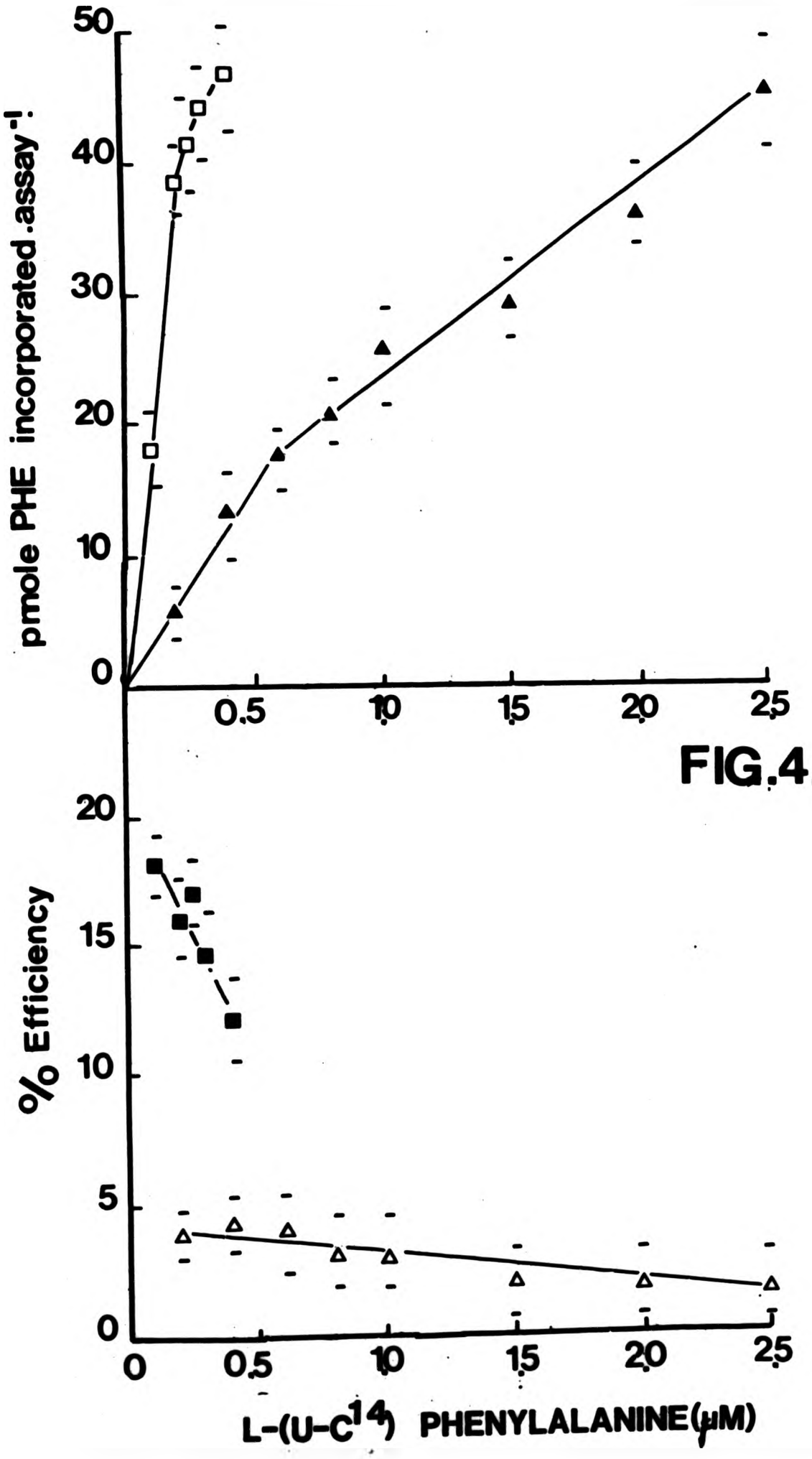


FIG.4.18b

SECTION 4.12 TEMPERATURE AND REACTION TIME.

There was no detectable polyphenylalanine synthesis at any incubation temperatures employed during the first 5 minutes incubation . Thereafter variable amounts of polyphenylalanine were synthesised at variable rates depending on the temperature. The initial period of inactivity has been observed for many other species, and has been attributed to the time required for the aminoacylation of tRNA and the formation of the initiation complex ,eg. Cannon et al.(1976).

The maximum quantity of polyphenylalanine synthesised in one hour was achieved at 30° and 34°C(legend to Figure 4.19). At 37°C, the maximum activity after one hour was less than 50% of that observed at 30° or 34°C. At 4°C,the temperature at which the reaction mixture was prepared, polyphenylalanine synthesis was negligible.

The maximum rate of polyphenylalanine synthesis at all temperatures between 27° and 37°C, was achieved between 5 and 20 minutes after the commencement of incubation. The highest rates of synthesis were obtained at 30° and 34°C, (Figure 4.18). At 37°C, synthesis stopped after 40 minutes, wheras at 30°C, polyphenylalanine synthesis contimuedat one hour.

The optimum temperature and time chosen for the incubation of the reaction mixture was 30°C and 1 hour, because it produced the largest total quantity of polyphenylalanine,at the maximum rate of synthesis.

FIG .4.19

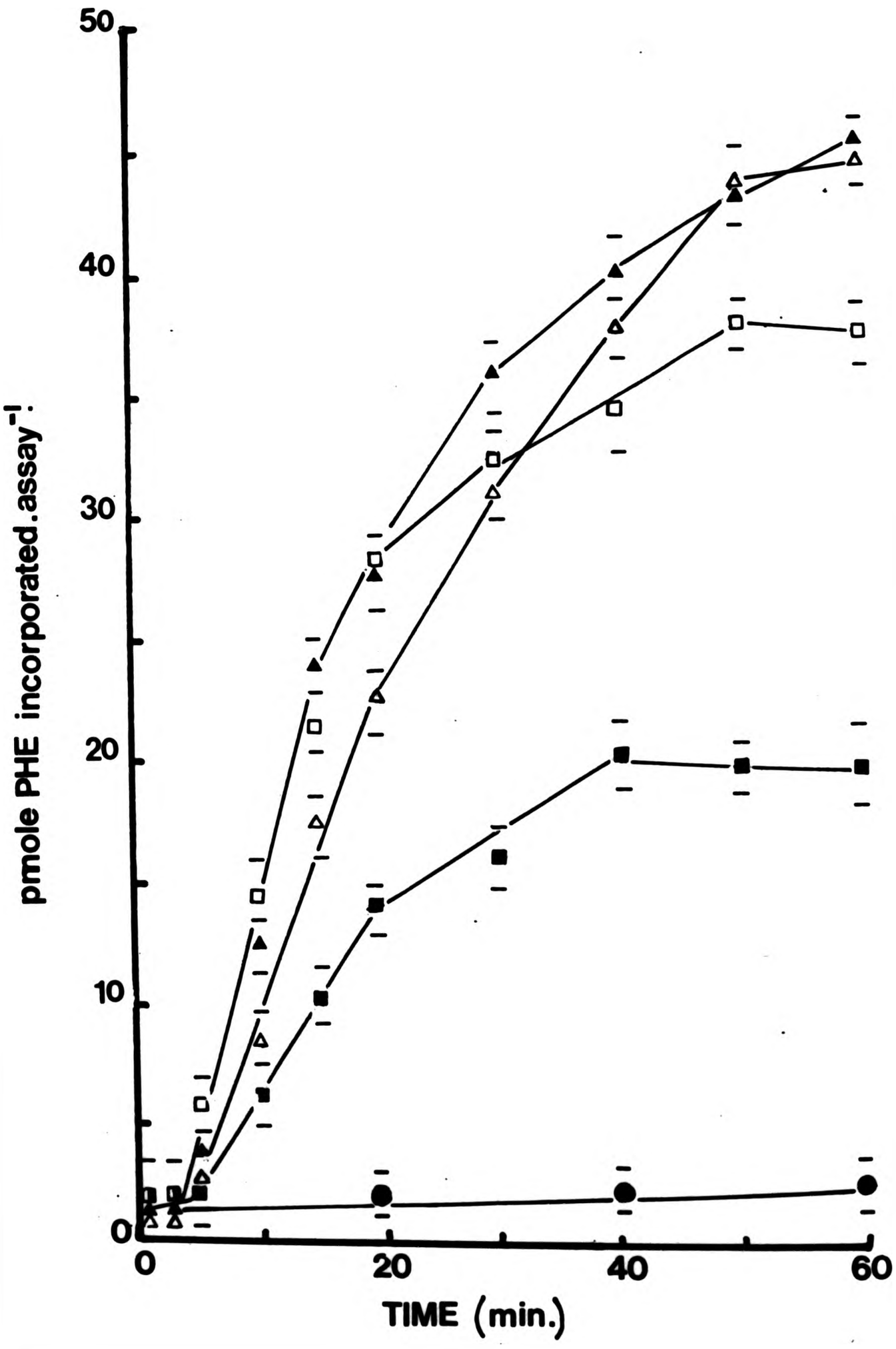


Figure 4.

Pol

samples t

Section 2

Chapter 2

.4.19

60

Figure 4.19. The effect of temperature on the kinetics of polyphenylalanine synthesis.

Polypherylalanine synthesis was determined from duplicate 40µl samples taken from 500 µl of optimised reaction mixture (Chapter2, Section 2.9, Table 2.2) according to the method described in Chapter 2, Section 2.9. The five incubation temperatures used were;

Temperature (°C)	Symbol	Maximum activity (p mole phe.100µl assay.$^{-1}$ hr.$^{-1}$)
4	●	1.8
27	△	43.1
30	▲	44.4
34	□	36.9
37	■	19.6
		(1 pmole phe = 1016 cpm)

DISCUSSION.

SECTION 4.13. THE PREOPTIMISED AND OPTIMISED REACTION MIXTURES.

In the preliminary experiments, no activity was achieved. The inability to obtain polyphenylalanine synthesis proved to be a major obstacle in the development of a Coprinus cinereus cell-free polypeptide synthesising system. Because no specific methods existed to prepare or assay Coprinus cinereus cell-extracts it was possible that techniques adapted from other species (Chapter 4.4) resulted in the preparation of inactive cell-extracts or produced unsuitable reaction mixture.

The reason for inactivity was found to be the result of using stored frozen mycelium as the source of the cell-extracts; cell-extracts prepared from freshly grown mycelium were active in the preoptimised reaction mixture (Appendix B i ; Section 4.4).

When the individual optimisation of the constituents in the preoptimised reaction mixture was undertaken, it was realised that most constituents had specific concentration ranges for maximal polyphenylalanine synthesis and that the assay system was particularly sensitive to several constituents, including; ATP (Figure 4.8), GTP (Figure 4.11), magnesium acetate and spermidine (Figures 4.12 and 4.13). It was fortuitous that most of the constituents in the preoptimised reaction mixture were within the optimal concentration range and that none were totally inhibitory (Table 4.4). Had the preoptimised system been based solely on the Podospora anserina system (Crouzet et al. 1978), there would have been no spermidine

Table 4.4. The effect of the individual optimisation of the constituents of the Coprinus cinereus cell-free system.

Constituent	Concentration (mM unless stated). Preoptimised assay.	Individually optimised assay.	% activity of preoptimised concentration relative to optimised concentration
Adenosine - 5- triphosphate.	2.0	1.0	50
Guanosine - 5 - triphosphate.	0.5	0.25	60
Creatine phosphate.	10.0	12.0	90
Creatine phosphokinase.	4.0 $\mu g.ml.^{-1}$	6.0 $\mu g.ml.^{-1}$	80
Tris-HCl, (pH 7.5).	50.0	15.0	60
Magnesium acetate.	10.0	4.5	60
Potassium acetate.	20.0	20.0	100
Ammonium chloride.	50.0	40.0	90
Spermidine.	1.0	1.0	100
Dithiothreitol.	2.0	4.0	80
$tRNA^{phe}$.	10.0 $\mu g.ml.^{-1}$	6.0 $\mu g\ ml.^{-1}$	80
Polyuridylic acid.	375.0 $\mu g.ml.^{-1}$	300.0 $\mu g.ml^{-1}$.	85
L-($U\text{-}C^{14}$) phenylalanine.	0.98 μM	0.24 μM	>100
RP-100.	3.0 A260 unit.	2.0 A260 unit.	90
S-100.	1.0 A260 unit.	2.0 A260 unit.	40
pmole phe incorporated.$assay^{-1}$. hr^{-1}.	26.4*	39.8*	
Efficiency			
pmole product/pmole substrate(%).	2.7	16.3	
pmole phe. A_{260}unit $RP\text{-}100^{-1}$.	8.8	19.9	

Polyphenlalanine synthesis was determined according to the method described in Chapter 2. Section 2.9.
* values were taken from Figure 4.18.
The activity of constituents in the preoptimised assay were expressed relative to the activity in the optimised assay by referring to the relevant results (Figures 4.3, to 4.18).

in the assay and consequently no activity (Figure 4.13, Section 4.8). By employing a preoptimised system which was based on the average of several fungal species at the outset of the investigation (Section 4.4), the probability of obtaining polyphenylalanine synthesis with Coprinus cinereus cell-extracts was greater than if a system based on one species had been used.

A comparison of the quantity of polyphenylalanine synthesised at the outset using preoptimised reaction mixture, with the quantity produced in the optimised reaction mixture demonstrated the improvement that was possible by individually optimising each assay constituent (Table 4.4). The typical activity in the preoptimised system (26.4 pmole phe. hr^{-1}.) were synthesised in an assay system which contained 3.0 A_{260} unit RP-100 fraction and 0.98 μM L- (U C^{14}) phenylalanine (Table 4.4). When the optimised system had been developed the average quantity of polyphenlalanine synthesised had been increased 1.5 x, to 40 pmole.phe assay.hr^{-1} and the concentration of RP-100 fraction and radiolabelled substrate had been reduced (Table 4.4).

The composition of the optimised reaction mixture did not substantially differ from the composition of the preoptimised reaction mixture (Table 4.4). Both reaction mixtures contained the same constituents but differed in the final concentrations of the constituents. For most constituents, the individually optimised concentrations resulting in maximum incorpororation was generally lower than in the preoptimised reaction mixture.

The objective of the experiments detailed in this Chapter was to obtain a Coprinus cinereus cell-free system able to synthesise a

The activity of the preoptimised assay mixture and constituents in the optimised assay by referring to the relevant results (Figures 4.3, to 4.18).

satisfactory amount of polyphenylalanine. The average synthesis of approximately 40 p mole polyphenylalanine.assay^{-1}.hr^{-1} (Table 4.4) was considered to be a satisfactory result. The stage in the development of the cell-free system was reached where polyphenylalanine was synthesised with a reasonable efficiency and economy of resources. It was not possible to examine the effect of all variables which may have affected in vitro polyphenylalanine synthesis. It was therefore possible that further analysis would have produced a reaction mixture capable of greater activity at highest efficeincy. For example; the pH of the reaction mixture was not optimised and pH does affect in vitro polyphenylalanine synthesis in other systems (Berry et al, 1978): the production of more active cell-extracts by ensuring that less damage was incurred during cell-breakage (Berry et al, 1978) and by maintaining their integrity by determining the most suitable extraction buffer and limiting protease and RNA ase action, instead of using minimal preparation of the cell-extracts, to produce 'washed' ribosomes (Sissons, 1974): and to remove low molecular weight molecules (endogenous assay constituents and inhibitors) from the S-100 fraction (eg.dialysis, Crouzet et al 1978; ammonium sulphate precipitation, Carter et al, 1980).

Another approach would have been to investigate the possible benefits of using alternative constituents to those used in the cell-free system, for example; an alternative buffer to Tris-HCl, possibly Hepes, because of the known inhibitory effect of Tris as a result of deacylation of phe-tRNA (Heredia and Halvorson, 1966) and its poor buffer capacity at pH 7.5. The reaction mixture may have been simplified had L-(U-C^{14}) phe-tRNA been used, obviating the requirements of ATP, creatine phosphate, creatine phosphokinase and $tRNA^{phe}$, and

possibly resulting in increased polyphenylalanine synthetic activity. One other alternative , which Weber et al, (1977) found gave improved activity, was the replacement of chloride ions by acetate ions.

The potential of Coprinus cinereus cell-free system was considered to be greater than the efficiency of translation observed in the optimised reaction mixture (Table 4.5). Without recourse to the suggested improvements which had been described, a more efficient in vitro system could have been produced by using less than 0.24 μM L- (U-C^{14}) phenylalanine (Figure 4.18a) but the yield of polyphenylalanine synthesis (Figure 4.18b) was considered to be insufficient for the purpose of the in vitro system (Figure 4.3).

The Coprinus cinereus cell-free polyphenylalanine synthesising system could not be considered as a definitive system because all possible means of improving the in vitro system were not made and because the precise composition of the RP-100 and S-100 fractions was not known. Additionally because few of the interactions between constituents were examined (eg. between magnesium acetate and spermidine Section 4.8, and ammonium acetate and potassium chloride, Section 4.9), it was possible that the individually optimised constituents may not be the most efficient and that suboptimal concentrations of some constituents may have been preferable. However, the complexity of the assay was not studied in sufficient detail for such interactions to be determined.

The product of poly (U) directed polypeptide synthesis was not determined. It was assumed that the radioactivity which was absorbed onto the GF/A filter discs and precipitated by

90°C TCA was polyphenylalanine. The C^{14}-containing product was not characterised; the polypeptide may have been di- or tri- phenylalanine as reported by Bretthauer and Golichowski (1968), or it may have been a polypeptide with a large chain length as described by Sissons (1974). Benvienste et al,(1976) and Atkins et al,(1978) found that potassium ion and spermidine concentrations affect the size of the polypeptide produced in a cell-free system primed with mRNA: the effect of these constituents in a poyl(U) dependant cell-free system is not known. An important factor in determining the size of the polypeptide was the size of the polyuridylic acid molecule (Marcus et al,1963). A small poly(U) template can only translate small polypeptides , regardless of the efficiency of the cytoplasmic ribosomes. The size of the poly(U) employed in this investigation was not known.

SECTION 4.14. EFFICIENCY OF POLYPHENYLALANINE SYNTHESIS IN COPRINUS CINEREUS AND OTHER SPECIES.

The efficiency of the optimised Coprinus cinereus cell-free polypeptide synthesising system compared favourably with in vitro systems from other fungal species (Table 4.6). Efficiency has been calculated in different ways by different authors and in order to compare the results of the optimised Coprinus cinereus system with those of other species each of the expressions of efficiency have been determinded (Table 4.5).

Based on the simplest determination of efficiency, the utilisation of substrate to synthesise product, Coprinus cinereus was one of the most efficient poly (U)-dependant polyphenylalanine synthesising systems catalysed by a RP-100 (or equivalent fraction, Table 4.5). Depending on how the cytoplasmic ribosome concentration was evaluated the Coprinus cinereus cell-free system was as good as any of the other sytems for which efficiency was calculated (Table4.5, when the incubation time was taken into account).

Possibly the most meaningfull expression of efficiency is pmole phenylalanine incorporated. pmole cytoplasmic ribosome^{-1}. Taking into account the assumptions which were necessary in order to calculate the molality of the cytoplasmic ribosome concentration (Appendix B v), it was estimated that approximately 7 molecules of phenylalanine were incorporated by each ribosome present in the cell-free reaction mixture (Table 4.5). However, the estimated value is calculated on the basis of several assumptions (Appendix B v), the most important of which is that all

Table 4.5. Efficiency of cell-free polyphenylalanine synthesis.

Organism/Reference	Cell-extract	Incubation (min)	Product / substrate(%)	Efficiency: Pmole phe. A_{260} unit^{-1}	Efficiency: pmole phe. mg protein^{-1}.	Efficiency: pmole phe. mg ribosome^{-1}.	Efficiency: pmole phe. pmole ribosome^{-1}.
Coprinus cinereus.							
(Appendix Bi)	S-30	60	1.6	9	600	-	-
(Figure 4.18)	RP-100/S-100	60	16	200	3600	1800	7.1
Podospora anserina.							
Crouzet et al,(1978)	RP-140/S-140	30	1.7[c]	1.9[c]	-	-	-
Saccharomyces cerevisiae.							
Sissons (1974)	S-30	30	1.6[c]	180[c]	4300[q]	2350[c]	7.7[q]
	RP-100/S-100	30	-	-	-	-	3.5
Schizosaccharomyces pombe.							
Berry et al,(1978)	RP-100/S-100	45	8	99[c]	-	1085[q]	4.4

c= calculated from published data. q = quoted value, - =insufficient data. Values for Coprinus cinereus were calculated from the optimised reaction mixture (39.8 pmole phe. assay^{-1}. hr^{-1}), RP-100 concentration was determined as described in Appendix Bv.

cytoplasmic ribosomes were active.

The determination of efficiency assumes that all cytoplasmic ribosomes wich contribute towards the absorbance at 254 nm were functional and able to translate the poly (U) template. The absence of polysomes in the RP-100 fraction (Figure 4.2) demonstrates that no cytoplasmic ribosomes which were actively involved in polypeptide synthesis were added to the assay. The presence of only monosomes (Figure 4.2) does not differentiate between active and inactive ribosomes. The proportion of ribosomes participating in polyphenylalanine synthesis was not known, but it is probable that initiation on the poly(U) template was not possible by all ribosomes. Damage during the vigours of cell-breakage may have rendered many ribosomes inactive.

It is therefore probable that the efficiency of each active cytoplasmic ribosome is greater than the quoted estimated value (Table 4.5).

as described in Appendix Bv.

SECTION 4.15. SUMMARY.

The individual optimisation of most constituents of the *Coprinus cinereus* cell-free polyphenylalanine synthesising system produced a system, whose efficiency compared favourably with similar systems in other species and whose quantity of polyphenylanine synthesised was considered to be satisfactory. The cell-free system was deemed suitable to examine the effect of cycloheximide *in vitro* and to screen for cycloheximide-resistant cytoplasmic ribosomes.

CHAPTER 5.

THE RESPONSE OF COPRINUS CINEREUS CELL-EXTRACTS TO CYCLOHEXIMIDE IN THE IN VITRO POLYPHENLYALANINE SYNTHESISING SYSTEM.

INTRODUCTION.

SECTION 5.1. THE INTRACELLULAR SITE OF CYCLOHEXIMIDE ACTION.

The differential response to cycloheximide may be the result of several factors. Georgopoulos (1977) lists several biochemical changes which may confer the mutational resistance to fungicides including; decreased membrane permeability to the drug, detoxification of the drug and modification of the cellular component, reducing the sensitivity of the target site at which the drug interacts. The mechanism of resistance to cycloheximide has been demonstrated to occur by reduced uptake (Wescott, and Sisler, 1964) but the emphasis of the analyses undertaken have attempted to identify the intracellular target.

The concern of this investigation was the identification of the intracellular site of cycloheximide action. Early evidence (Siegel and Sisler, 1964; Ennis and Luben, 1964) suggested that cycloheximide affected a component within the cell. However, the relatively crude cell-extract could not be used to identify a specific cellular component which could be implicated in the inhibition of protein synthesis except to discount the mitochondrial ribosomes (Ennis and Luben, 1964).

Subsequently, it became possible to fractionate the cell-extract into a cytoplasmic ribosome-rich fraction and a cytoplasmic ribosome-free supernatant fraction (equivalent to RP-100 and S-100 fractions used in this investigation; Chapter 2, Section 2.7).

In one of the earliest investigations it was concluded by Traketellis et al.,(1965) and Felicetti et al.,(1966) that the cytoplasmic ribosome-free supernatant of mammalian cells was associated with cycloheximide binding. The conclusion was based on the observation that supernatant fractions, from a cycloheximide-sensitive strain, treated with cyclo-

Table 5.1. Identification of the intracellular site of cycloheximide action.

Reference	Organism	Response to cycloheximide (identity of cycloheximide -resistant mutation when known)
Whole cell-extract (S-7).		
Siegel & Sisler (1964)	Saccharomyces pastorianus.	S
Post-mitochondrial supernatant (S-30).		
Ennis & Luben (1964)	Rat Liver	S
Cytoplasmic ribosome free supernatant (S-100).		
Trakatellis et al (1965)	Mouse Liver	S
Felicetti et al (1966)	Rabbit reticulocytes	S
Cytoplasmic ribosome fraction (RP-100).		
Siegel & Sisler (1965)	Saccharomyces pastorianus.	S
	Saccharomyces fragilis.	R
Cooper et al (1967)	Saccharomyces cerevisiae.	cyh-8.
Pongratz & Klingmuller (1973)	Neurospora crassa	act 1, act 2.
Vomvoyanni (1974)		act1, act 2, act 5.
Rothschild et al (1975)		act 2.
Haugli et al (1972)	Physarum polycephalum.	act A.
Large cytoplasmic ribosomal subunit.		
Rao & Grollman (1967)	Saccharomyces fragilis.	R
Battaner & Vazguez (1971)	Saccharomyces cerevisiae.	R
Jiménez et al (1972)	" "	cyh-2
M^{c} Laughlin (1974)	" "	cyh-2
Stöcklein & Piepersberg (1980)	" "	cyh-2
Coddington & Fluri (1977)	Schizosaacharomyces pombe.	cyh-1
Berry et al (1978)	" "	cyh-1
Béguéret et al (1977)	Podospora anserina.	cyR-1
Crouzet et al (1978)	" "	cyR-1
Pöche et al (1979)	Chinese hamster ovary	R
Pöche et al (1979)	Human fibroblast.	R
Large and small cytoplasmic ribosomal subunit		
Sutton et al (1978)	Tetrahymena thermophila.	chx B

R = cycloheximide -resistant strain or species.

S = cycloheximide-sensitive species.

heximide did not synthesise polypeptide when reconstituted with untreated cytoplasmic ribosomes. The reciprocal experiment in which cycloheximide-sensitive cytoplasmic ribosomes were preincubated with cycloheximide did produce polypeptide when reconstituted with untreated supernatant. Felicetti et al.(1966) speculated that the component of the supernatant fraction to which cycloheximide bound was an elongation factor.

A similar methodology to that used by Trakatellis et al.(1965) was used by Battaner and Vazquez (1971) in Saccharomyces cerevisiae cells However, when it was possible to examine the cell-extracts from species and strains which exhibited markedly different responses to cycloheximide, (ie. cycloheximide-resitant and cycloheximide-sensitive species and mutant strains) a different methodology was employed. The overwhelming evidence produced (Table 5.1) implicated the cytoplasmic ribosomes as the intracellular site of cycloheximide action.

The first evidence that cycloheximide interacted with the cytoplasmic ribosome was made by Siegel and Sisler (1965). Cell-extracts prepared from a cycloheximide-resistant species, Saccharomyces fragilis and a cycloheximide-sensitive species, Saccharomyces pastorianus, were fractionated into two fractions, one containing cytoplasmic ribosomes, the other containing soluble supernatant fractions. The responses to cycloheximide of heterologous combinations of cytoplasmic ribosomes and supernatant fraction from each species proved that resistance to cycloheximide was a property of cytoplasmic ribosomes.

Sutton et al (1978) Tetrahymena thermophila. chx B

R = cycloheximide -resistant strain or species.
S = cycloheximide-sensitive species.

Subsequently, cell-extracts were prepared from strains which were cycloheximide-sensitive and cycloheximide-resistant and employing a similar protocol to that of Siegel and Sisler (1965). Cycloheximide-resistant cytoplasmic ribosomes have been identified from the response of the organelle to cycloheximide; in the majority of instances, using polyurdylic acid-dependant polypeptide synthesing systems (Table 5.1).

The component of the cytoplasmic ribosome which conferred cycloheximide-resistance had been localised to the particular cytoplasmic subunit by analysing the response to combinations of the large and small cytoplasmic ribosomal subunit derived from cycloheximide-resistant and cycloheximide-sensitive species (Rao and Grollman, 1967) and strains (Table 5.1). With one exception, the large cytoplasmic ribosomal subunit was identified as conferring the response to cycloheximide (Table 5.1) The exception was discovered by Sutton et al, (1978) who found that cycloheximide-resistance in mutants of Tetrahymena thermophila was conferred by both the large and small cytoplasmic ribosomal subunits.

The localisation of the component of the cytoplasmic ribosome which confers cycloheximide resistance has not been determined in a cell-free polypeptide-synthesing system. In prokaryotes it has been possible to fractionate the ribosomal subunits into their ribosomal protein and RNA constituents and by producing reconstituted ribosomes from different sources, to identify the components conferring antibiotic resistance (reviewed by Cannon and Cundliffe, 1973). A similar analysis in eukaryotic species has not been possible because not all cytoplasmic ribosomal components have been purified (Wool, 1979).

SECTION 5.2 OBJECTIVES.

Chapter 3 described the production and genetic analysis of *Coprinus cinereus* strains resistant to cycloheximide *in vivo*.

Chapter 4, detailed the development of a *Coprinus cinereus* cell-free system capable of synthesising polypeptides and provided a means of investigating the cellular basis for the *in vivo* cycloheximide resistance.

The objectives of the experiments described within this chapter were:

to analyse and classify *Coprinus cinereus* strains according to their *in vitro* responses to cycloheximide,
to identify the component of the cell-extracts which conferred resistance to cycloheximide,
to identify those strains which possessed cycloheximide-resistant cytoplasmic ribosomes,
and to compare the *in vitro* responses of monokaryons, dikaryons and diploids to cycloheximide.

RESULTS.

SECTION 5.3. EFFECT OF CYCLOHEXIMIDE ON CELL-EXTRACTS FROM MONOKARYONS IN VITRO.

a) Analysis of CY 8 and CY 8.2.

The investigation into the effect of cycloheximide on cell-free polyphenylalanine synthesis began with an analysis of the effects of the drug on cell-extracts derived from CY 8 and CY 8.2. In the analysis of the effect of cycloheximide on the growth of these strains (Chapter 3, Section 3.9 c), CY 8 was described as being sensitive to cycloheximide and the mutant strain derived from CY 8, CY 8.2 was classified as being highly resistant to cycloheximide.

The cell-extracts prepared from these two strains were the cytoplasmic ribosomal-rich fraction RP-100 and the cytoplasmic ribosome-free supernatant fraction S-100 (Chapter 2, Section 2.7). In the development of the cell-free polyphenylalanine synthesising system (Chapter 4), both the RP-100 and S-100 fractions from CY 8 had been shown to be essential for polyphenylalanine synthesis (Sections 4.6 a and b). All other constituents of the cell-free assay system had been optimised (Chapter 2, Section 2.9, Table 2.2) and were kept constant in these analyses. Therefore any differences in the effect of cycloheximide on the cell-extracts of CY 8 and CY 8.2, could be attributed to the only source of variation between the treatments, the RP-100 and S-100 fractions.

(i)Homologous cell-extracts.

Two types of cell-extracts were analysed. In the first experiments the RP-100 and S-100 fractions were derived from the same strain. Thus the two treatments were RP-100 and S-100 from CY 8, and RP-100 and S-100 from CY 8.2. The effect of cycloheximide on these homologous treatments are presented in Figure 5.1.

The cycloheximide dose-responses of CY 8 and CY 8.2 consisted of two discrete phases which were similar to the biphasic growth responses of these strains discussed in Chapter 3, Section 3.13b.. At low cycloheximide concentrations there was no noticeable inhibition of polyphenylalanine synthesis. The second phase occurred over approximately two orders of magnitude of cycloheximide concentrations when there was an exponential decline in activity and total inhibition was observed. Similar responses were observed in other analyses, presented in Figures 5.2 to 5.4.

Although the shapes of the cycloheximide response curves of CY 8 and CY 8.2 were similar (Figure 5.1), the displacement of the response of CY 8.2 towards the higher cycloheximide concentrations illustrated that the RP-100 and S-100 fractions from CY 8.2 were more resistant to cycloheximide than those of CY 8.

The growth responses of all strains to cycloheximide (Chapter 3, Sections 3.9, 3.10 and 3.11) produced biphasic

Figure 5.1. Effect of cycloheximide on polyphenylalanine synthesis by cell-extracts from CY 8 and CY 8.2

The composition of the optimised reaction mixture and method of measurement of polyphenylalanine synthesised from replicate 40 µl samples of duplicate experiments were as described in Chapter 2, Section 2.9.

Source			100% activity
RP-100	S-100	Symbol	(p mole phe incorporated.assay^{-1}.hr^{-1}.)
CY 8	CY 8	□	36.0
CY 8.2	CY 8.2	△	46.3
CY 8	CY 8.2	■	46.6
CY 8.2	CY 8	▲	43.6

The average quantity of C^{14} radioactivity insoluble in 90°C TCA was measured at each cycloheximide concentration examined and was expressed as a % relative to the maximum activity of the uninhibited control, 0µM cycloheximide. The original data was used in a linear regression analysis (Appendix C1). The 50% inhibitory value has been indicated (●) and the extrapolated responses involving CY 8.2 RP-100 fractions are indicated (-------) Regression coefficients ranged from -0.96 to -0.98.

FIG.5.1

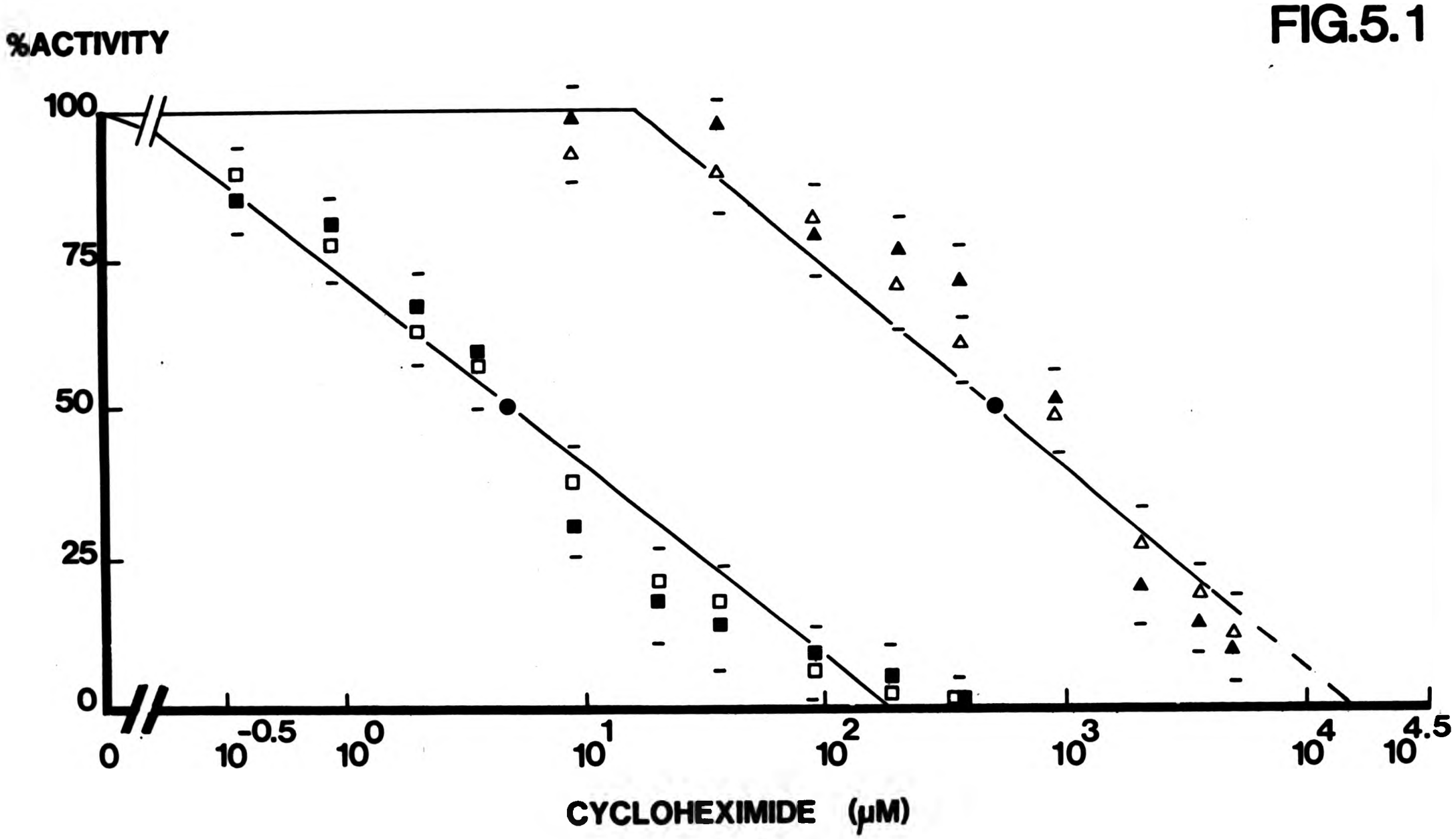

%ACTIVITY
100
75
50
25
0
0
$10^{-0.5}$
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
$10^{4.5}$
CYCLOHEXIMIDE (μM)

Table 5.2. Response of CY 8 and CY 8.2 cell-extracts to cycloheximide.

Strain		Cycloheximide concentration (μM) for		Linear
Source of RP-100	Source of S-100	50% inhibition and total inhibition (calculated)	(observed)	regression coefficient
CY 8	CY 8	5.1	360	-32
CY 8	CY 8.2	4.6	360	-33
CY 8.2	CY 8.2	490	17000[E]	-32
CY 8.2	CY 8.0	530	12000[E]	-37

The cycloheximide concentrations producing 50% inhibition and the linear regression coefficients were calculated from the original data (Appendix C1). All linear corelation coefficients in the inhibitory phase ranged from -0.96 to -0.98. [E]Total inhibition was not observed at the cycloheximide concentrations examined, (5300 μM): the values presented were extrapolated from the linear regression analysis.

to cycloheximide.

for ion	Linear regression coefficient
	-32
	-33
	-32
	-37

ibition and the original in the hibition was ed, he linear

responses similar to the response of the cell-extracts to cycloheximide (Figure 5.1). The parameters of the growth response which were used to describe the cycloheximide dose-responses *in vivo* (Chapter 3) were applied to the *in vitro* responses: the cycloheximide concentration at which total inhibition was observed, the calculated cycloheximide concentration from an analysis of linear regression which produced 50% inhibition, the linear regression coefficient and the linear correlation coefficient. The parameters describing the responses of CY 8 and CY 8.2 homologous extracts are given in Table 5.2.

In terms of the calculated 50% inhibitory cycloheximide concentration, the RP-100 and S-100 extracts from CY 8.2 were 128 x more resistant, and highly significantly different from those of CY 8 ($p < 0.001$). The total inhibitory cycloheximide concentration of CY 8.2 was estimated to be 50 x higher than that of CY 8. There was no significant difference between the linear regression coefficients of the two strains ($p > 0.1$). The linear correlation coefficients over the inhibitory phases of the cycloheximide-dose response curves, -0.98 and -0.99 for CY 8 and CY 8.2 respectively, demonstrated the inverse relationship between cycloheximide concentration and polyphenylalanine synthesis.

(ii) Heterologous cell-extracts.

The results of the homologous combination of cell-extracts established that the cell-extract of CY 8.2 was cycloheximide-resistant and that the cell-extract of CY 8 was cycloheximide-sensitive. In order to determine which of the CY 8.2 fractions, either RP-100, S-100 or possibly both, conferred *in vitro* resistance to cycloheximide, heterologous combinations of CY 8.2 and CY 8 fractions were analysed. Neither RP-100 or S-100 could be analysed independently of the other because both fractions were essential in order to synthesise polyphenylalanine (Chapter 4, Section 4.5 a and b). The cycloheximide dose-responses of two treatments were examined; the combination of RP-100 from CY 8.2 and S-100 from CY 8 and the reciprocal hybrid of RP-100 from CY 8 and S-100 from CY 8.2.

The treatment which contained RP-100 from CY 8.2 and S-100 from CY 8 was more resistant to cycloheximide than the treatment containing RP-100 from CY 8 and S-100 from CY 8.2 (Figure 5.1). The cycloheximide concentration necessary to produce 50% inhibition was 125 x higher when RP-100 was derived from CY 8.2 than when it was derived from CY 8 ($p < 0.001$; Table 5.2) but the treatments had similar linear regression coefficients ($p > 0.1$). The response to cycloheximide of CY 8.2 RP-100 fraction with CY 8 S-100 fraction was similar to that of the CY 8.2 homologous cell-extract, (Figure 5.1). Both treatments containing CY 8 RP-100 fractions were indistinguishable

from each other (Figure 5.1).

It was concluded that the RP-100 fraction from the cycloheximide-resistant strain CY 8.2 conferred resistance to cycloheximide *in vitro*. The S-100 fraction was considered to have no detectable role in conferring cycloheximide-resistance.

b) Analysis of various other strains.

It was not practicable to investigate all 174 cycloheximide-resistant mutants produced during the course of this investigation (Chapter 3, Section 3.5 b). It was considered that a more rewarding investigation would be achieved if a limited number of selected strains were thoroughly analysed, rather than a relatively superficial analysis of all strains.

On the basis of the growth response of the cycloheximide-resistant mutants to cycloheximide (Chapter 3, Section 3.8) and genetic analysis (Chapter 3, Sections 3.10 and 3.12), the mutants were rationalised into 11 groups from which one or more representative strains from each group were chosen for in vitro analysis (listed in Table 3.12i, Section 3.11, Chapter, 3).

CY 18 played an important role in all subsequent analyses because by using a CY 18 S-100 fraction as a standard component of the in vitro assay, it became possible to screen directly for cycloheximide-resistant cytoplasmic ribosomes in other strains. The S-100 fraction from CY 18 was chosen because it did not confer cycloheximide resistance in vitro (Table 5.3) and because of the high polyphenylalanine synthesising capacity it conferred with RP-100 fractions from any source. For example CY 8 or CY 8.2 RP-100 fractions in combination with the CY 18 S-100 fraction, produced a higher activity than the CY 8 or CY 8.2 homologous cell-extracts (legend to Figures 5.1 and 5.2). Additionally, by employing the CY 18 S-100 fraction, the response to

Figure 5.2. The effect of cycloheximide on polyphenylalanine synthesis by RP-100 from various monokaryotic strains.

The composition of the optimised reaction mixture and method of measurement of polyphenylalanine synthesis, from replicate 40 µl samples of duplicate experiments, were as described in Chapter 2 Section 2.9. The responses are presented as described in Figure 5.1.

In all analyses, the S-100 fraction was derived from CY 18. The source of the RP-100 fractions were as listed.

Source of RP-100	Symbol	100% activity (p mole phe incorporated.assay^{-1}. hr $^{-1}$.)
CY 8	□	47.8
CY 8.2	■	48.5
CY 9.23	△	18.6
CY 9.23.98	▲	20.2
CY 9	○	15.4
The CY 9 response also represents;		
CY 3		37.2
CY 13		30.8
CY 14		36.4
CY 18		40.1

Original data and the results of the linear regression analysis are presented in Appendices C2 and C3 i .(●) indicates the 50% inhibition of each treatment.

FIG.5.2

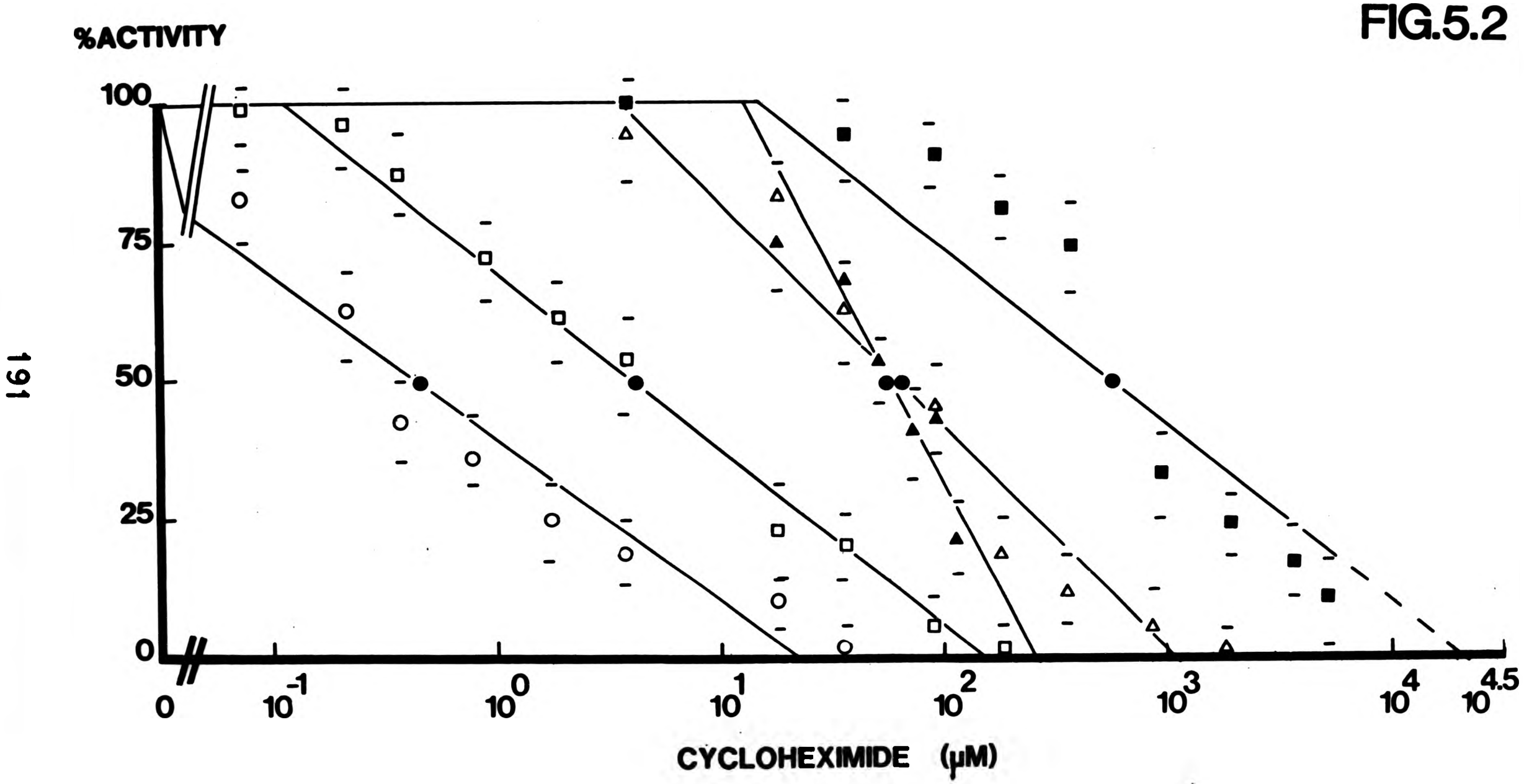

%ACTIVITY
100
75
50
25
0
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
$10^{4.5}$
CYCLOHEXIMIDE (μM)

Table 5.3. Response of RP-100 fractions from monokayotic strains, in the presence of S-100 from CY 18, to cycloheximide.

Source of RP-100	Classification *in vivo*	Cycloheximide concentration (μM) calculated for 50% inhibition of polyphenylalanine synthesis	Linear regression coefficient
CY 8	S	4.2	-25
CY 8.2	R	550.	-32
CY 9	S	0.45	-29
CY 9.23	R	64	-41
CY 9.23.98	R	58	-77
CY 3	S	0.35	-25
CY 13	S	0.22	-23
CY 14	S	0.42	-26
CY 18	S	0.47	-26

R denotes cycloheximide-resistance and S denotes cycloheximide-sensitivity *in vivo*. The calculations are based on the data given in Appendices C2 and C3.

cyclohexamide of a RP-100 fraction from any source was not noticeably changed in relation to the response of homologous cell-extracts (Figures 5.1 and Figures 5.2).

The responses to cycloheximide of RP-100 fractions from various monokaryotic sources were examined in the presence of S-100 from CY 18 (Figure 5.2).

The presence of a cycloheximide-resistant RP-100 fraction for CY 8.2 has already been reported (Section 5.3a). On the basis of the evidence for of the response to cycloheximide of a RP-100 fraction from CY 9.23, it too possessed a cycloheximide-resistant RP-100 fraction. The RP-100 fraction from CY 9.23 was 150 x more resistant than the cycloheximide-sensitive strain CY 9 from which it was derived (Table 5.3). However the level of cycloheximide resistance exhibited by the CY 8.2 RP-100 fraction was 10 x greater than that of CY 9.23. The RP-100 fraction from CY 9.23.98, the recombinant strain of CY 9.23 and CY 3, produced a response to cycloheximide which was similar to that of CY 9.23, but had a significantly higher linear regression coefficient than CY 9.23 ($p < 0.001$, Table 5.3). All three strains, CY 8.2, CY 9.23 and CY 9.23.98 (Chapter 3, Section 3.8 c and d) were resistant to cycloheximide *in vivo*.

All other strains analysed were cycloheximide-sensitive *in vivo* (Chapter 3, Section 3.8). The response of CY 8 RP-100 fraction to cycloheximide (Figure 5.2), previously reported to be cycloheximide-sensitive (Section 5.3 a), was 10 x more resistant than the RP-100 fractions from CY 3, CY 9, CY 13, CY 14 or CY 18 ($p < 0.001$; Table 5.3).

There was no significant difference between the responses of RP-100 fractions from CY 3, CY 9, CY 13, CY 14 or CY 18 in terms of their 50% inhibitory cycloheximide concentrations ($p > 0.5$) or their linear regression coefficients ($p > 0.5$).

A classification of the monokaryotic strains was proposed, based upon the response to cycloheximide of their RP-100 fractions. Four groups were recognised, each significantly different from each other ($p < 0.001$) and yet significantly similar within each group ($p > 0.5$). The groups were: CY 8.2 the most resistant; CY 9.23 and CY 9.23.98; CY 8; and CY 3, CY 9, CY 13, CY 14 and CY 18.

The description of cycloheximide-sensitive and cycloheximide-resistant RP-100 fractions was assigned to agree with the in vivo classification of the strains from which the RP-100 fractions were derived (Chapter 3, Section 3.8). Using a discriminatory cycloheximide concentration of 5 μM, the RP-100 fractions of CY 3, CY 8, CY 9, CY 13 CY 14 and CY 18 were classified as cycloheximide-sensitive and those of CY8.2, CY9.23 and CY9.23.98 were identified as cycloheximide-resistant.

The response to cycloheximide of RP-100 fractions from other strains did not progress beyond a preliminary analysis because of insufficient polyphenylalanine synthesis in the controls. (results not presented).

SECTION 5.4. LOCALISATION OF THE INTRACELLULAR SITE OF CYCLOHEXIMIDE RESISTANCE.

The evidence from the analysis of the response to cycloheximide of cell-extracts from monokaryotic strains revealed that the RP-100 fraction of the cycloheximide-resistant mutants CY 8.2, CY 9.23 and CY 9.23.98 conferred cycloheximide-resistance *in vitro* (Section 5.3).

The RP-100 fraction was considered to be the sole source of cytoplasmic ribosomes (Chapter 4, Section 4.5a). Evidence from other species had identified the equivalent cytoplasmic ribosomal containing fractions as the site of cycloheximide resistance (Section 5.2) and in several instances had specifically identified the large cytoplasmic ribosomal subunit as conferring cycloheximide-resistance (Section 5.2).

In order to identify the particular cytoplasmic ribosomal subunit which conferred cycloheximide-resistance, the cytoplasmic ribosomes in the RP-100 fractions from CY 8.2, CY 9.23 and CY 9.23.98 were dissociated. (Chapter 2, Section 2.8). The dissociation conditions were experimentally determined to produce the most satisfactory resolution of large and small cytoplasmic ribosomal subunits (Appendix B iii).

Despite numerous attempts at reconstitution of the large and small cytoplasmic ribosomal subunit fractions, in all possible combinations of sources and at variable

concentrations, no polyphenylalanine synthesis was achieved. Several reasons for inactivity were possible. The most plausible explanation was that the cytoplasmic ribosomal subunit fractions had been rendered inactive during their preparation. The separate storage of frozen large and small cytoplasmic ribosomal subunit fractions in Dissociation Buffer (Chapter 2, Section 2.8) were similar to the conditions described by Baierlein and Infante (1974) which rendered Strongylocentrotus purpuratus cytoplasmic ribosomal subunits incapable of reassociation and polypeptide synthesis. Alternatively it was possible that the cytoplasmic ribosomal subunits were active but that they had specific requirements for polyphenylalanine synthesis (eg. increased magnesium ion concentration, Berry 1976) or that the large and small cytoplasmic ribosomes were mixed in unsuitable proportions.

In the absence of any polyphenylalanine synthesis the identification of the particular cytoplasmic ribosomal subunit which conferred cycloheximide-resistance could not be made.

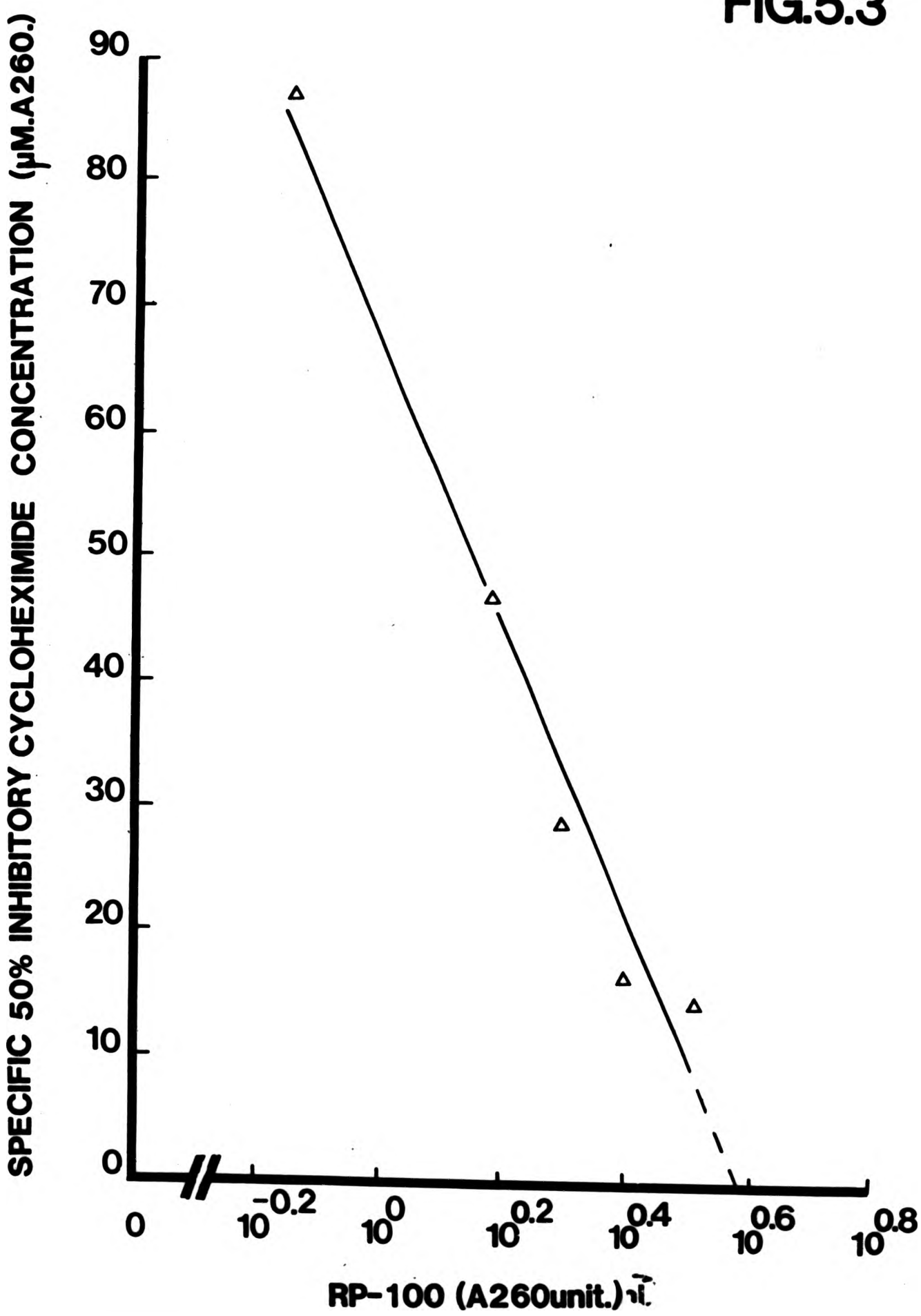
SECTION 5.5. THE EFFECT OF RP-100 CONCENTRATION ON THE CYCLOHEXIMIDE DOSE-RESPONSE.

The comparison of the effect of cycloheximide on the RP-100 fraction derived from different monokaryotic strains (Section 5.3) was made at one specific RP-100 concentration, 2.0 A_{260} unit . The concentration was used because in Chapter 4, Section 4.5 a , it produced the maximum polyphenylalanine synthesis observed for CY 8.

The experiments described in this section investigated the relationship between RP-100 concentration and response to cycloheximide, using RP-100 derived from the cycloheximide-resistant recombinant strain CY 9.23.98. In Section 5.3 b it was demonstrated that 58 μM cycloheximide was necessary to produce a 50% inhibition of the activity of the CY 9.23.98 RP-100 fraction and it was concluded that the RP-100 fraction was cycloheximide-resistant.

The response to cycloheximide at each of the five CY 9.23.98 RP-100 concentrations examined (Table 5.4) were significantly different from each other in terms of their 50% inhibitory cycloheximide concentration ($p > 0.001$) and linear regression coefficients ($p > 0.01$). The trend which the results showed was that with increasing RP-100 concentration the 50% inhibitory cycloheximide concentration decreased. Apparantly, depending on the concentration of RP-100 used, the degree of resistance of the fraction could be changed; CY 9.23.98 ribosomes were 1.7 x more resistant at the highest RP-100 concentration than at the lowest RP-100 concentration examined.

FIG.5.3


SPECIFIC 50% INHIBITORY CYCLOHEXIMIDE CONCENTRATION (μM.A260.)
90
80
70
60
50
40
30
20
10
0
0
10^-0.2
10^0
10^0.2
10^0.4
10^0.6
10^0.8
RP-100 (A260unit.)/ml.

5.3

$10^{0.8}$

Figure 5.3. The effect of RP-100 concentration on the response to cycloheximide

The composition of the optimised reaction mixture and method of measurement of polyphenylalanine synthesis from replicate 40 µl. samples of duplicate experiments, were as described in Chapter 2 Section 2.9. The RP-100 fraction was derived from CY 9.23.98 and the S-100 fraction was prepared from CY 18.

The responses of various concentrations of RP-100 to cycloheximide (Appendix C3 ii) were analysed by linear regression analysis. The values obtained for the 50% inhibitory concentration at each of the five RP-100 concentrations were related to each of the RP-100 concentrations to obtain the 'specific 50% inhibitory cycloheximide concentration' ($\mu M. A_{260} unit^{-1}$). The linear correlation coefficient of the effect of 'specific 50% inhibitory cycloheximide concentration' on the RP-100 concentrations was -0.99.

Table 5.4. The effect of CY 9.23.98 RP-100 concentrations on the inhibition by cycloheximide of polyphenylalanine synthesis.

RP-100 concentration A_{260} unit.	$\log_{10}$ A_{260} unit.	Maximum activity (p mole phe synthesised . assay^{-1} . hr^{-1} .)	Calculated cycloheximide concentration (μM) for 50% inhibition	Linear regression coefficient	Specific 50% inhibitory cycloheximide concentration (μM . A_{260} unit^{-1}.)
0.7	-0.16	9.8	61	-64	87
1.5	0.18	23.9	71	-87	47
2.0	0.30	31.5	58	-77	29
2.5	0.40	30.4	42	-62	17
3.2	0.51	26.1	48	-59	15

Original data is presented in Appendices C 3 i and C 3 ii.

The effect of RP-100 concentration on the response to cycloheximide was more clearly demonstrated when the 50% inhibitory cycloheximide concentration for each RP-100 concentration was expressed relative to the RP-100 concentrations used. The resulting'specific 50% inhibitory cycloheximide concentrations' (Table 5.4) were related to each of the RP-100 concentrations used, (Figure 5.3). There was an exponential decrease in the 'specific 50% inhibitory cycloheximide concentration' with increasing RP-100 concentration (linear corelation coefficient = -0.99 and linear regression coefficient of -115.3).

For CY 9.23.98, the choice of RP-100 concentration determined the expression of cycloheximide resistance *in vitro*. Using 5 μM cycloheximide as the 50% inhibitory concentration to differentiate between cycloheximide resistance and sensitivity *in vitro* (Section 5.3 b), CY 9.23.98 would be characterised as cycloheximide-resistant at all of the RP-100 concentrations examined. However, had the discriminatory 50% inhibitory cycloheximide concentration chosen been 50 μM, CY 9.23.98 would have been classified as cycloheximide-resistant at less than 2.0 A_{260} unit. RP-100 but sensitive at higher RP-100 concentrations.

If the results observed for the CY 9.23.98 RP-100 fraction were applicable to other strains, then it would be necessary to define more accurately cycloheximide-sensitive and -resistant responses *in vitro*. It would be necessary to stats the RP-100 concentration used.

In the analysis of monokaryotic strains (Section 5.3) a constant RP-100 concentration of 2.0 A_{260} unit was employed. The same RP-100 concentration was subsequently used for the analysis of dikaryons and diploids (Section 5.6) thereby enabling a comparison of their relative responses.

SECTION 5.6. THE EFFECT OF CYCLOHEXIMIDE ON RP-100 FRACTIONS DERIVED FROM DIKARYONS AND DIPLOIDS

The effect of cycloheximide on the growth response of dikaryons and diploids has already been presented (Chapter 3, Sections 3.9 and 3.10). The effect of cycloheximide on the responses of RP-100 from various monokaryotic strains in polyphenylalanine synthesis revealed that CY 8.2 and CY 9.23 possessed cycloheximide-resistant RP-100 fractions.

a) Dikaryons

The effect of cycloheximide on polyphenylalanine synthesis by dikaryons is presented in Figure 5.4. The responses of the homozygous cycloheximide-sensitive strains CY 8 x CY 13 and CY 9 x CY 3 were not significantly different from each other ($p > 0.5$).

Both dikaryons heterozygous for cycloheximide-resistance, CY 8.2 x CY 13 and CY 9.23 x CY 3, were more resistant to cycloheximide than were their respective homozygous cycloheximide-sensitive dikaryons, CY 8 x CY 13 and CY 9 x CY 3. (Figure 5.4, Table 5.5). The level of resistance exhibited by CY 9.23 x CY 3 cytoplasmic ribosomes was significantly higher than that of CY 8.2 x CY 13 ($p < 0.001$).

The homozygous cycloheximide-resistant dikaryons CY 8.2 x CY 9.23.138, and other dikaryotic strains, did not produce sufficient polyphenylalanine synthesis for their response to cycloheximide to be analysed.

Table 5.5. Response of RP-100 fractions from dikaryotic strains to cycloheximide.

RP-100 fraction.	Calculated cycloheximide concentration (μM) for 50% inhibition.	Linear regression coefficient.
CY 8 X CY 13	1.1	-29
CY 8.2 X CY 13	6.3	-23
CY 9 X CY 3	0.9	-31
CY 9.23 X CY 3	9.6	-22

Original data and statistical analysis given in Appendix C4.

FIG.5.4

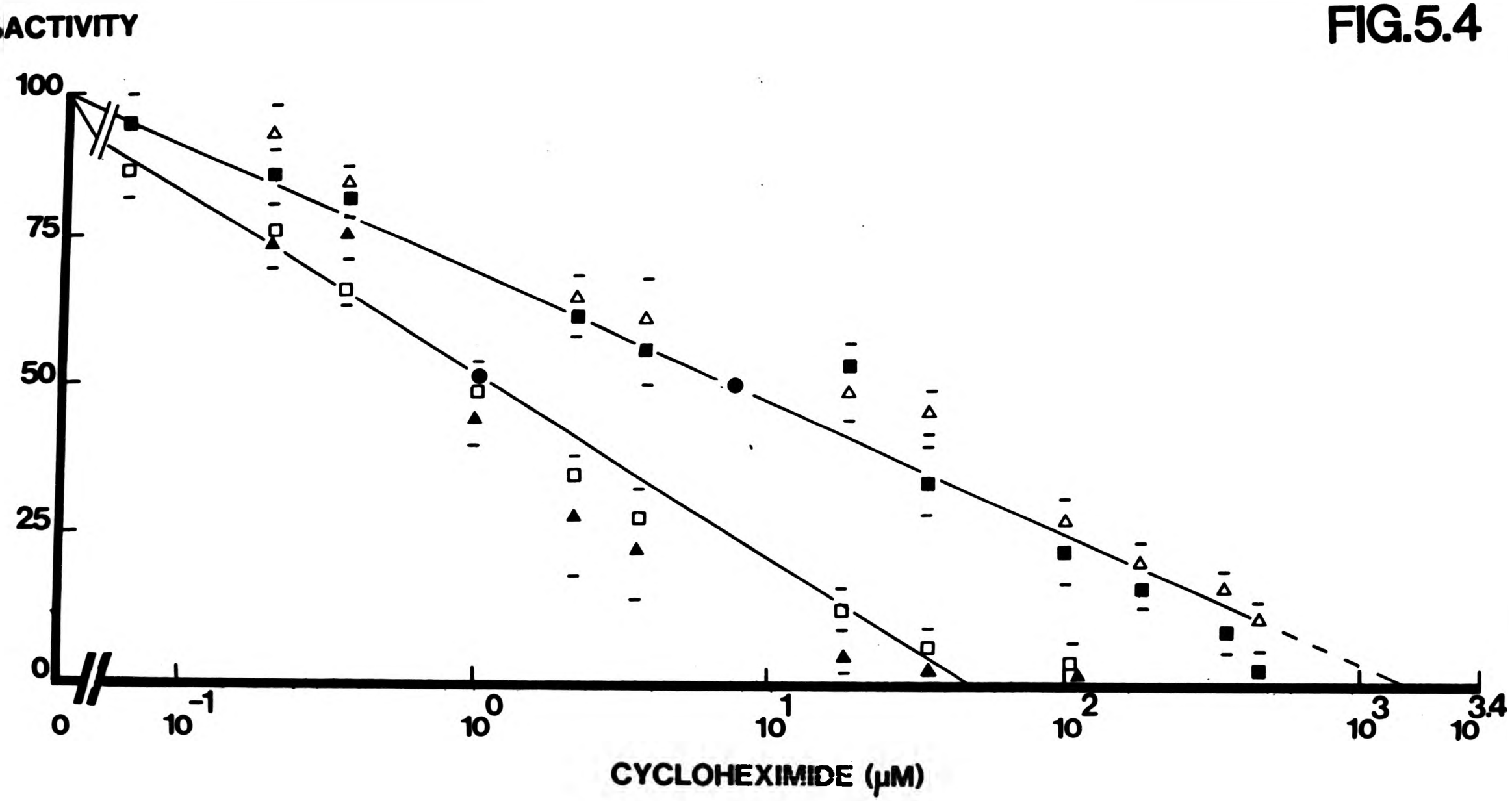

%ACTIVITY
100
75
50
25
0
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
$10^{3.4}$
CYCLOHEXIMIDE (μM)

Figure 5.4. Effect of cycloheximide on polyphenylalanine synthesis by RP-100 fractions from dikaryotic strains.

The composition of the optimised reaction mixture and the method of measurement of polyphenylalanine synthesis, from replicate 40 μl samples of duplicate experiments, were as described in Chapter 2. Section 2.9. The responses were represented as described in Figure 5.1.

In all treatments, the S-100 fraction was derived from CY 18.

Source of RP-100.	Symbol.	100% activity (p mole phe incorporated.assay^{-1}.hr^{-1}.)
CY 8 x CY 13	□	16.1
CY 8.2 x CY 13	■	20.9
CY 9 x CY 3	▲	14.9
CY 9.23 x CY 3	△	16.7

Original data and linear regression analyses are presented in Appendix C4. The 50% inhibition value of each treatment is indicated (●). The extrapolated response of CY 9.23 is indicated (------).

b) Diploids.

With the exception of RP-100 fractions from CY 9/ CY14 and CY 9.23/CY 14, no other diploid strain produced any polyphenylalanine synthesis in the *in vitro* system. Even the quantity of polyphenylalanine synthesised achieved with CY 9/CY 14 and CY 9.23/CY 14 was inadequate to permit a thorough analysis of their responses to cycloheximide (Table 5.6).

It was not possible to determine accurately the 50% inhibitory cycloheximide concentration for either strain, merely to suggest that the value was between 0 and 0.18 μM (Table 5.6). Consequently, it was not possible to differentiate between the two strains on the basis of their *in vitro* response to cycloheximide based on the results presented in Table 5.6. However, with the 50% inhibitory cycloheximide concentration less than 5 μM, both strains were regarded as sensitive to cycloheximide.

Table 5.6. Response of RP-100 fractions from diploid strains to cycloheximide.

Cycloheximide concentration. (μM)	Polyphenylalanine synthesis (% of uninhibited control) Source of RP-100 CY9/CY14	CY9.23/CY14
0	100 (1.0)	100 (1.9)
0.18	40	31
3.6	24	11

The composition of the optimised reaction mixture and the measurement of polyphenylalanine synthesis (p mole phe incorporated assay.$^{-1}$ hr.$^{-1}$) are described in Chapter 2, Section 2.9. The S-100 fractions were derived from CY 18.

DISCUSSION.

SECTION 5.7. A SCREENING PROCEDURE TO IDENTIFY CYCLOHEXIMIDE-RESISTANT CYTOPLASMIC RIBOSOMES.

One of the objectives of the experiments described in this chapter, was to identify cycloheximide-resistant mutant strains (Chapter 3, Section 3.8) which possessed cycloheximide-resistant cytoplasmic ribosomes. The cell-free polyphenylalanine synthesising system which had been developed in Chapter 4 had been designed so that the response to cycloheximide of cytoplasmic ribosomes could be analysed.

From the evidence of the responses of heterologous combinations of RP-100 and S-100 fractions to cycloheximide, it was calculated that the RP-100 fractions of CY8.2, CY9.23 and CY9.23.98 conferred cycloheximide resistance, (Section 5.3b). The experimental evidence of the sucrose density gradient and dependancy of the *in vitro* polyphenylalanine synthesising system indicated that the RP-100 fraction was rich in cytoplasmic ribosomes (Section 4.5a). However, because the RP-100 fraction was prepared with the minimum of centrifugation steps, it was possible that a non-ribosomal cellular constituent was associated with the cytoplasmic ribosome or that a soluble factor from the S-100 fraction contaminated the ribosomal pellet and that these, and not the cytoplasmic ribosomes, conferred cycloheximide resistance. However equivalent fractions to the RP-100 fraction in other species, prepared by similar techniques to those used in *Coprinus cinereus*, have been found to confer resistance and in those species the intracellular site of cycloheximide action has been stated as the cytoplasmic ribosome (Table 5.1, Section 5.1). Further evidence that cytoplasmic ribosomes conferred cycloheximide resistance in CY8.2, CY9.23 and

CY9.23.98 was not obtained because the cytoplasmic ribosomal subunits were not active *in vitro* (Section 5.4).

The responses to cycloheximide when CY8 and CY8.2 RP-100 fractions were examined with either CY8, CY8.2 or CY18 S-100 fractions were not identical (Section 5.3 a and b). The differences resulting from the source of the S-100 fraction were not large, but were consistently observed. It was possible that the S-100 fraction conferred a degree of resistance to cycloheximide; albeit small relative to that of the RP-100 fraction and not to the extent that Trakatellis *et al*,(1965) and Felicetti *et al*,(1966) observed in mammalian cell-extracts. The effect of the S-100 fractions did not prevent the identification of cycloheximide-resistant RP-100 fractions. Neither the effect of the S-100 fractions in cycloheximide resistance, nor the possibility that cytoplasmic ribosomes in the S-100 fraction were responsible for cycloheximide resistance, were investigated.

From an original cell-free system, in which RP-100 and S-100 fractions derived from the test strains were analysed in heterologous combination with cell-extracts derived from another strain, the system was standardised with a cycloheximide-sensitive CY18 S-100 fraction, so that the cell-free system could be directly identifiy those RP-100 fractions which conferred cycloheximide-resistance.

For practical reasons, cytoplasmic ribosomes from only three cycloheximide-resistant mutant strains, CY8.2, CY9.23 and CY9.23.98 were examined. Instead of analysising the cytoplasmic ribosomes from all other cycloheximide-resistant mutant strains or all strains chosen to represent the different groups of *in vivo* responses to cycloheximide (Chapter 3, Section 3.8), detailed analyses of the

responses to cycloheximide of CY8.2, CY9.23, CY9.23.98 and cycloheximide-sensitive test strains, were undertaken. All cycloheximide-resistant mutant strains analysed were shown to be allelic at the cy-2 locus (Chapter 3, Section 3.11) and because the product of the cy-2^r alleles of CY8.2 and CY9.23 were cycloheximide-resistant cytoplasmic ribosomes it was assumed that other strains would possess cycloheximide-resistant cytoplasmic ribosomes.

It was not necessary to undertake a thorough analysis in order to determine if a strain possessed cycloheximide-resistant cytoplasmic ribosomes. The basis of a simplified screening technique was that the presence or absence of detectable quantities of polyphenylalanine at one specific cycloheximide concentration could distinguish between the responses of cycloheximide-resistant and cycloheximide-sensitive cytoplasmic ribosomes. Based on the evidence in Section 5.3, a discriminatory cycloheximide concentration of 200 µl could be used to distinguish between the phenotypes of cytoplasmic ribosomes from strains which were resistant or sensitive to the drug in vivo (Chapter 3, Section 3.8). It was therefore possible that cytoplasmic ribosomes from strains which were less resistant than CY8.2, CY9.23 and CY9.23.98 in vivo might also be less resistant in vitro, in which case it would be necessary to re-evaluate the proposed discriminatory cycloheximide concentration.

SECTION 5.8. CHARACTERISATION OF THE EFFECT OF CYCLOHEXIMIDE ON POLYPHENYLALANINE SYNTHESIS.

The effect of cycloheximide on cytoplasmic ribosome-dependant polyphenylalanine synthesis could be differentiated into two distinct phases. In the first phase at relatively low cycloheximide concentrations no measurable inhibitory effect was observed. In the second phase increasing cycloheximide concentration resulted in a logarithmic inhibition of polyphenylalanine synthesis.

The biphasic response to cycloheximide *in vitro*, was similar to that observed for the effect of cycloheximide on the growth of the same strains (Chapter 3, Section 3.13b); and like the *in vivo* response, the *in vitro* response could be characterised by four parameters.

On the basis of the response of their RP-100 fractions to synthesise polyphenylalanine in the presence of cycloheximide, strains could be classified according to their minimum, 50% and total inhibitory concentrations into two groups; strains possessing cycloheximide-resistant cytoplasmic ribosomes, CY8.2, CY9.23 and CY9.23.98 ; and all cycloheximide-sensitive strains examined which were shown to have cycloheximide-sensitive cytoplasmic ribosomes, including CY8 (Section 5.3). The linear correlation coefficients of cycloheximide-resistant and cycloheximide-sensitive RP-100 fractions, with the exception of CY9.23.98 were not sufficiently dissimilar to be used as a basis for classification.

a) Interpretation of the cycloheximide dose-*in vitro* response.

The biphasic cycloheximide dose-growth responses were interpreted without knowing the basis for the effect of cycloheximide on growth

(Chapter 3, Section 3.13 d). A similar biphasic response of cycloheximide on polyphenylalanine synthesis may be interpreted in the knowledge that the cytoplasmic ribosomes are the probable site of cycloheximide action. A discussion of the contribution of the cytoplasmic ribosomes in the growth response will be discussed in Chapter 7.

It was not known whether cycloheximide reacted with cytoplasmic ribosomes which were translating poly (U) or with non-functional organelles, whether the interaction was reversible or irreversible, and whether as a result of the interaction the translation of poly (U) was totally inhibited or took place at a reduced rate.

Over the cycloheximide concentration range at which no inhibition of polyphenylalanine synthesis was detected, it was possible that the proportion of cytoplasmic ribosomes affected by cycloheximide was insignificant relative to the total number of unaffected functional organelles, and therefore, that the amount of inhibition was small, and possibly less than the sensitivity of the assay. If the amount of inhibition were small and the reaction mixture contained unaffected cytoplasmic ribosomes it was possible that the net loss in polyphenylalanine synthesis could be made good and thus there was no apparant inhibition.

In cycloheximide-resistant strains, the non-inhibitory range of cycloheximide concentrations was greater than those exhibited by cycloheximide-sensitive strains (Section 5.3 b). The difference may be explained by the mutational change in the cycloheximide-resistant strains which resulted in an alteration in the site or sites on the cytoplasmic ribosome which reacted with cycloheximide. If the modified reactive

site had less affinity for the inhibitory molecule, if the inhibitory complex were less stable or if the inhibitory effect resulting from the interaction were less pronounced, the cytoplasmic ribosomes from CY8.2, CY9.23 and CY9.23.98 would be less responsive to cycloheximide at relatively low cycloheximide concentrations than those from the cycloheximide-sensitive strains. Variation between the cycloheximide-resistant strains may be the result of difference in the different cy-2^r alleles which produced different types of reactive sites.

The identity of the cytoplasmic ribosomal reactive site, or sites, for cycloheximide interaction and the nature of the structural modifications brought about by mutations at the cy-2 locus, will be discussed in Chapter 7.

The second phase of the in vitro response to cycloheximide was the measurable logarithmic inhibition of polyphenylalanine synthesis, which ultimately resulted in total inhibition. The cycloheximide concentration at which inhibition commenced may have been the point at which the proportion of inhibited cytoplasmic ribosomes was too great for unaffected organelles to make good the losses in polyphenylalanine synthesis.

The inhibitory response of CY9.23.98 cytoplasmic ribosomes was unlike those from CY8.2 and CY9.23; CY9.23.98 possessed a similar first phase but exhibited an inhibitory phase which was achieved in approximately half the cycloheximide concentration (Section 5.3b). The response for CY9.23.98 was based on fewer observations from those of CY 8.2 and CY9.23 and may be inaccurate. However, the result of the recombinant strain from CY9.23, which therefore possessed the same mutation as CY9.23 but lacked the

modcy^{+} allele, may be a consequence of the mutation at the modcy locus, but CY8.2 which did not possess modcy^{+} responded like CY9.23 in the inhibitory phase. The difference between CY9.23 and CY9.23.98 may reflect a difference in the consequence of the formation of the inhibitory complex rather than a difference in the formation of the complex.

SECTION 5.9. THE EFFECT OF CYCLOHEXIMIDE ON MONOKARYONS, DIKARYONS AND DIPLOIDS.

In order to examine the molecular relationship that occurred between cycloheximide cytoplasmic ribosomes and to compare the results obtained in Coprinus cinereus, with those of cther species, the 50% inhibitory cycloheximide concentration and cytoplasmic ribosome concentration of each strain examined,were expressed as a molal ratio, (Table 5.7). The values for the molal ratio were based on the assumption necessary to equate cytoplasmic ribosomal concentration (A_{260} unit) with (pmole. ml^{-1}). (Appendix B.v); the values were therefore estimates.

a) Monokaryons.

Between 4 and 8 molecules of cycloheximide were necessary to produce 50% inhibition of polyphenylalanine synthesis in cycloheximide-sensitive strains, excluding CY8 (Table 5.7); the variation was insignificant. In contrast, in order to inhibit cytoplasmic ribosomes from CY8 to the same extent, it was necessary to have approximately 10 x the number of molecules of cycloheximide and in the cycloheximide-resistant strains CY9.23 and CY8.2 considerably greater numbers of cycloheximide molecules were required (Table 5.7).

The interpretation of the relationship between cycloheximide and cytoplasmic ribosomes, expressed in terms of pmole cycloheximide: pmole cytoplasmic ribosomes, was speculative in the absence of knowledge concerning the number of cycloheximide binding sites. Assuming that there was only one reactive site on the cytoplasmic ribosomes to which cycloheximide binds, the variation in the ratios obtained for the different strains indicated the relative probability

Table 5.7. The effect of cycloheximide on the in vitro response of monokaryons, dikaryons and diploids.

pmole cycloheximide: pmole cytoplasmic ribosome for 50% inhibition of polyphenylalanine synthesis.

Monokaryons			Dikaryons	Diploids
CY8	CY13	CY18	CY8 x CY13	CY8 x CY18
75:1	4:1	8:1	20:1	-
CY8.2	CY13	CY18	CY8.2 x CY13	CY8 x CY18
10000:1	4:1	8:1	110:1	-
CY9	CY3	CY14	CY9 x CY3	CY9 x CY14
8:1	6:1	8:1	16:1	< 3:1
CY9.23	CY3	CY14	CY9.23 x CY3	CY9.23 x CY14
1100:1	6:1	8:1	170:1	< 3:1

Values calculated from Tables 5.3, 5.5 and 5.6.

- = no data and values for diploids are estimates.

1 μM cycloheximide = 100 pmole. assay^{-1}; each assay contained 2 A_{260} unit. RP-100 = 5.6 pmole cytoplasmic ribosome.assay^{-1} (Appendix B v d).

of formation of a complex and the effect of the complex on inhibiting polyphenylalanine synthesis. It was improbable that a mutation at the cy-2 locus could result in a change from 4 to 1100 reactive sites in the case of CY9 and CY9,23 and a more dramatic change from 75 to 10,000 site for CY8 and CY8,2. Rather,the formation and stability of the inhibition complex between cycloheximide and cytoplasmic ribosome was approximately 135 x less probable in CY8.2 and CY9.23 than in their respective parental strains. The mutations at the cy-2 locus were considered to be the result of an alteration of the cytoplasmic ribosome which affected its affinity for cycloheximide. The consistency of the difference between CY8.2 and CY8, and between CY9.23 and CY9 suggests that the modification to the reactive site was similar in both mutants, despite the difference in the wild-type state of the reactive site. The possible identify of the structural site and the nature of the modification which brought about cycloheximide resistance will be discussed in Chapter 7.

In other species, the ratio of cycloheximide to cytoplasmic ribosomes is greater than unity. In order to produce a 50% inhibition in the inital rate of polyphenylalanine synthesis 4.8 molecules of cycloheximide are necessary for each molecule of cytoplasmic ribosomes from a cycloheximide-sensitive strain of Schizosaccharomyces pombe (Berry et al, 1978). In Saccharomyces cerevisiae the ratios for 50% inhibition were 1.4:1 and 50:1 for cycloheximide-sensitive and cycloheximide-resistant cytoplasmic ribosomes respectively (Cooper et al, 1967).

b) Dikaryons.

The response of the homozygous cycloheximide-sensitive dikaryon CY9 x CY3 was more resistant than those of the monokaryotic strains from which they were derived, (Table 5.7). The response exhibited by the other homozygous cycloheximide-sensitive dikaryon examined CY8 x CY13, was similar to that of CY9 x CY3 but perhaps because of the relatively high resistance of CY8, the response of CY8 x CY13 was less resistant than both of its parental monokaryons (Table 5.7). The probable difference between the dikaryons and monokaryons resulted from the proportion of functional cytoplasmic ribosomes in the RP-100 fractions. The concentrations of RP-100 fractions measured in absorbance units, did not distinguish between functional and nonfunctional cytoplasmic ribosomes.

If cycloheximide reacted with only those cytoplasmic ribosomes taking part in polyphenylalanine synthesis, the proportion of functional cytoplasmic ribosomes in the RP-100 fractions, determined the number of molecules of cycloheximide which were necessary to inhibit polyphenylalanine synthesis by 50%; with fewer functional cytoplasmic ribosomes fewer molecules of cycloheximide would be required. Thus the monokaryons may have fewer functional cytoplasmic ribosomes than the dikaryons.

The responses of CY8.2 x CY13 and CY9.23 x CY3 exhibited a degree of resistance to cycloheximide which was between 5 and 10 x greater than their respective cycloheximide-sensitive dikaryons CY8 x CY13 and CY9 x CY3 (Table 5.7). The responses of the dikaryons, which were heterozygous for the cy-2^r allele, were of an intermediate order of magnitude between their constituent monokaryotic strains (Table 5.7). Their level of resistance was not compared to homozygous cycloheximide-resistant dikaryons, but their degree of resistance was less than that of the cycloheximide-resistant monokaryons, CY8.2 and CY9.23 and so they were

considered to represent an expression of partial dominance.

The partial expression of cycloheximide resistance exhibited by the CY8.2 x CY13 and CY9.23 x CY3 could be interpreted by assuming that the cytoplasmic ribosomes prepared and assayed were either, identical and exhibited an intermediate level of resistance, or consisted of a mixed population of cycloheximide-resistant and cycloheximide-sensitive types.

The possibility of mixed population of two distinct types of cytoplasmic ribosome has been suggested to explain the intermediate degree of cycloheximide-resistance exhibited *in vitro*; by a *Saccharomyces cerevisiae* diploid, heterozygous for *acr 8* (Cooper *et al*, 1967) and by a heterokaryon of *Podospora anserina*, heterozygous for *cyR1* (Crouzet *et al*, 1978). The expression of various mixtures of cytoplasmic ribosomes derived from the cycloheximide-resistant and cycloheximide-sensitive parental strains in *Coprinus cinereus* were not examined in the cell-free polyphenylalanine synthesising reaction mixture and thus it was not known if mixtures of the two types of cytoplasmic ribosomes resulted in an intermediate response to cycloheximide.

The hypothesis proposed by Lederberg *et al* (1964) to explain the recessivity of streptomycin resistance in *Escherichia coli* was adapted to explain the results obtained for partial dominance of cycloheximide resistance in *Saccharomyces cerevisiae* and in *Podospora anserina* (Cooper *et al*, 1967; Crouzet *et al*, 1978). According to Lederberg *et al* (1964) the translation of mRNA by resistant ribosomes would be blocked by the presence of sensitive ribosomes engaged on the mRNA..

The greater the proportion of cycloheximide-sensitive cytoplasmic ribosomes in the total population the more effective is the blockage

and the more the level of expression tends to the response of the cycloheximide-sensitive monokaryons. This may explain the results of CY8.2 x CY13 and CY9.23 x CY3 (Table 5.7). Furthermore, the effect of the sensitive cytoplasmic ribosomes would be greater when the two types of ribosomes compete for available poly (U) template (demonstrated by Crouzet et al, 1978). In the Coprinus cinereus cell-free reaction mixture (Table 4.5), poly (U)was optimal for polyphenylalanine synthesis but the length of the molecules was not known. If the chain length of the poly (U) were long, there would be fewer initiation sites accessible and the competition for initiation between the cycloheximide-sensitive and-resistant strains would be greater than if the chain length were small and more sites were available. Crouzet et al (1978) envisaged that sensitive ribosomes would be dominant when poly (U) was limiting but semi-dominant when poly (U) was saturating the in vitro system. The semi-dominant response of Coprinus cinereus heterozygous resistant dikaryons suggested that poly(U) was saturated. The effects of the responses to cycloheximide at different concentrations of poly(U) were not determined.

The nature of the interaction between the cycloheximide resistant cy-2^r allele and the cycloheximide sensitive cy-2^s allele in CY8.2 x CY13 and CY9.23 x CY3, and additionally in CY9.23 x CY3, the interaction between the dominance modifier modcy$^+$ and the wild-type allele which would be necessary to transcribe, translate and synthesise an intermediate form of cytoplasmic ribosome, or a mixed population of the original parental forms, will be discussed in Chapter 7.

c) Diploids.

Only two diploid strains were analysed, because of practical difficulties and because of their poor synthetic capacity in vitro, their response to cycloheximide was defined imprecisely (Section 5.5b). It was possible that the poor growth of the diploid strains was a result of the

poor capacity of their cytoplasmic ribosomes to synthesise polypeptide.

With the 50% inhibition of polyphenylalanine synthesis occurring at a maximum of 3 molecules of cycloheximide for each ribosome in the reaction mixture, the diploids were more sensitive to cycloheximide than either the monokaryotic strains from which they were derived, or their comparable dikaryotic strains, CY9 x CY3 and CY9.23 x CY3 (Table 5.7).

The response of CY9.23/CY14 was indistinguishable from that of the homozygous cycloheximide-sensitive strain CY9/CY14. The cycloheximide-resistance mutation at the cy-2 locus did not confer cycloheximide resistance to the cytoplasmic ribosomes in the diploid. The recessive response of the diploid was in contrast to the partial dominance of the dikaryon. The modcy^{+} allele had no observable effect of the cytoplasmic ribosomes of the diploid.

It was possible that only functional cycloheximide-sensitive cytoplasmic ribosomes were synthesised, in which case they should respond like the cytoplasmic ribosomes of the cycloheximide-sensitive parent, CY14. The result that diploidic ribosomes were more sensitive to cycloheximide than those of CY14 (Table 5.7) may be a consequence of the smaller proportion of functional cytoplasmic ribosomes in the preparation from the diploid than in the monokaryons; if fewer functional cytoplasmic ribosomes were present in the reaction mixture, fewer molecules of cycloheximide would be needed for inhibition in the diploid than in the monokaryon. Evidence that fewer function cytoplasmic ribosomes were present was demonstrated by the low yield of polypeptide synthesis by the diploids compared with the monokaryons (Figure 5.4, Section 5.6b: Figure 5.3b, Section 5.).

Alternatively it was possible that the RP-100 fraction from CY9.23/CY14 did contain cycloheximide-resistant cytoplasmic ribosomes, but in too small a number to synthesise polyphenylalanine in the presence of competition from cycloheximide-sensitive cytoplasmic ribosomes.

SECTION 5.10. SUMMARY.

It was possible to classify strains according to the effect of cycloheximide on the response of their cytoplasmic ribosomes to synthesise polyphenylalanine. All three cycloheximide-resistant mutant strains examined CY8.2, CY9.23 and CY9,23.98. possessed cycloheximide-resistant cytoplasmic ribosomes. The identity of the particular cytoplasmic ribosomal subunit which conferred cycloheximide resistance *in vitro*, could not be determined.

The *cy-2*^{r} mutation which conferred cycloheximide resistance in CY8.2 and CY9.23 resulted in partial resistance in dikaryons which were heterozygous for cycloheximide resistance. Cy9.23 x CY3 possessed *modcy*$^{+}$, CY8.2 x CY13 did not. Cytoplasmic ribosomes from diploid strains examined were sensitive to cycloheximide.

CHAPTER 6.

ANALYSIS OF COPRINUS CINEREUS

CYTOPLASMIC RIBOSOMAL PROTEINS.

INTRODUCTION.

SECTION 6.1. CYTOPLASMIC RIBOSOMAL PROTEINS ASSOCIATED WITH CYCLOHEXIMIDE RESISTANCE.

It was concluded in Chapter 5 that the mutation at the cy-2 locus, which conferred cycloheximide resistance in CY8.2 and CY9.23, resulted in the possession of cycloheximide-resistant cytoplasmic ribosomes. In order to produce cycloheximide-resistant cytoplasmic ribosomes, the translation product of the cy-2^r allele was either a structural component of the organelle, or it was an enzyme which modified one or more of the ribosomal components. Additionally, the product of the modifier gene, mod cy^+, which affected the dominance of cy-2^r in CY9.23 (North, 1982), may or may not result in a mutation in the same cytoplasmic component as cy-2^r. The experiments described in this Chapter were based on the hypothesis that the cy-2^r and modcy^+ mutations affected cytoplasmic ribosomal proteins.

In other species (Table 6.1), the identity of the cytoplasmic ribosomal component which conferred cycloheximide resistance has centered on the role of the cytoplasmic ribosomal protein. By comparison of the cytoplasmic ribosomal proteins from cycloheximide-sensitive strain with those of the cycloheximide-resistant strains, analysed by two-dimensional polyacrylamide gel electrophoresis, it has been possible to identify differences in electrophoretic mobility in specific proteins. The proteins identified (Table 6.1) are considered to be cytoplasmic ribosomal components which confer cycloheximide-resistance.

Table 6.1. Cytoplasmic ribosomal proteins associated with cycloheximide resistance.

Organism/Reference	Cycloheximide resistance locus.	Identity of protein.	Molecular weight of protein. (Dalton)	Difference between mutant and wild-type protein.
Schizosaccharomyces pombe				
Coddington and Fluri,(1977).	cyh-1	No.66 (large subunit)	22,000	The mutant protein was approximately 3,000 dalton larger than the wild-type protein.
Podospora anserina				
Bégueret et al,(1977).	cyR-1	L21	16,500	The mutant protein has the same apparent weight but is more basic than the wild-type protein;L21.
*Crouzet and Bégueret,(1980).	cyR-1	L21	16,500	A revertant strain to cycloheximide sensitivity was more acidic than the wild-type protein, L21.
Saccharomyces cerevisiae				
Stöcklein and Piepersberg, (1980).	cyh-2	L29	-	The mutant protein was considered to be more basic and possibily to have a higher molecular weight than the wild-type form.
Stöcklein et al,(1981).	cyh-2	YL24 (=L29)	-	Identified the sequence of amino acid replacements in the mutant protein which was responsible for cycloheximide-resistance.

\- not published.
* study of cycloheximide-sensitive revertants.

It is not known if the three proteins associated in cycloheximide-resistance;

Schizosaccharomyces pombe (Coddington and Fluri, 1977)

Podospora anserina (Bégueret et al, 1977)

Saccharomyces cerevisiae (Stöcklein and Piepersberg, 1980)

are the same proteins. There has been no direct comparison between the species, and each investigation has used different conditions for electrophoresis and different nomenclatures. However, on the basis of the molecular weights (Table 6.1), the proteins are apparently different.

In all three species, the proteins were located in the large cytoplasmic ribosomal subunit which agrees with the evidence from the in vitro data (Coddington and Fluri, 1977; Bégueret et al, 1977; Stöcklein and Piepersberg, 1980) that cycloheximide resistance was conferred by the large cytoplasmic ribosomal subunit.

An alternative approach to studying cycloheximide-resistant mutant strains was used by Crouzet and Bégeuret (1980). Their analysis of revertant strains to cycloheximide-sensitivity resulted in the identification of a different form of the same protein L21 which Bégueret et al (1977) had found conferred cycloheximide resistance (Table 6.1).

Many cycloheximide-resistant strains in several species have been demonstrated to possess cycloheximide-resistant cytoplasmic ribosomes (Table 5.1, Chapter 5). However, only three cycloheximide resistant cytoplasmic ribosomal proteins are known (Table 6.1). Thus, either cytoplasmic ribosomal proteins rarely confer cyclo-

which was responsible for cycloheximide-resistance.

- not published,
- * study of cycloheximide-sensitive revertants.

heximide resistance or the changes in the cycloheximide-resistant cytoplasmic ribosomal proteins are so small so as to be undetectable. In the majority of analyses, a variety of 2D -PAGE conditions have been employed. In this investigation an analysis of cytoplasmic ribosomal proteins by carboxymethyl-cellulose chromatography (CMC-chromatography) was used because it was possible that this method would resolve differences which 2D-PAGE could not (Harvey and Martinelli, 1983).

SECTION 6.2. OBJECTIVES.

Coprinus cinereus cytoplasmic ribosomal proteins were analysed for four reasons:

To compare two-dimensional polyacrylamide gel electrophoresis and carboxymethyl-cellulose chromatography as methods of analysis.

To characterise *Coprinus cinereus* cytoplasmic ribosomal proteins.

To determine which, if any, of the cytoplasmic ribosomal proteins could be implicated in the expression of cycloheximide resistance.

To compare the cytoplasmic ribosomal proteins of monokaryons, dikaryons and diploids.

SECTION 6.3. CHARACTERISATION OF COPRINUS CINEREUS SMALL CYTOPLASMIC RIBOSOMAL SUBUNIT PROTEINS.

The characterisation of small cytoplasmic ribosomal subunit proteins by two-dimensional polyacrylamide gel electrophoresis was based upon the analysis of CY8 (Plate A) and CY8.2 (Plate B). Because of technical difficulties and low yields of proteins, Plates A and B were the best examples of small cytoplasmic ribosomal subunit protein resolution obtained.

The majority of the proteins were well stained by Coomassie Brilliant Blue and were readily differentiated from the general background staining and are distinguishable on the photographic reproductions of the electropherograms (Plates A and B). It was possible to identify 24 well-stained proteins in the electropherograms of CY8.2 (Plate B), whereas the inferior resolution of CY8 proteins(PlateA) did not permit as many proteins to be identified. The proposed nomenclature of the Coprinus cinereus small cytoplasmic ribosomal subunit proteins was based on that of Kaltschmidt and Wittmann(1970) and is superimposed above the position of the proteins in Plate B. To distinguish the proteins from the small cytoplasmic ribosomal subunit from those of the larger organelle, the former group of proteins are prefixed by S when used in the text (eg. S1 to S24)

In addition the the 24 readily observed proteins, there were several which were weakly stained or poorly resolved from neighbouring proteins. Some were visible on the original polyacrylamide slab gels but were difficult to record on photographic negatives and reproduce, (Plates A and B). The existence of such proteins was less confidently identified,in contrast to the well stained proteins S1-S24 inclusive and in recognition of their speculative existence, these proteins are referred to by a subscript , together with the number

of their nearest neighbour. Six proteins were identified; S2a, S5a, S7a, S13a, S13b and S21a. Their positions are shown in Plate B.

The analysis of small cytoplasmic ribosomal subunit proteins from replicate preparations of CY8 and CY8.2, of other monokaryotic strains, and of dikaryotic and diploidic strains, did not have sufficient resolution to be of value in the characterisation, and have not been presented.

Plates A and B. Two dimensional electrophoretic analysis of Coprinus cinereus small cytoplasmic ribosomal subunit proteins.

Proteins from the small cytoplasmic ribosomal subunit of the cycloheximide-sensitive strain, CY8 (Plate A) and the cycloheximide-resistant strain CY8.2 (Plate B) were prepared and analysed by 2D-PAGE according to the methods described in Chapter 2, Sections 2,7, 2.8, 2.10 and 2.11.

Migration of the proteins was towards the cathode, from left to right in the first dimension and from top to bottom in the second dimension. The proteins were applied at the origin, represented by ▼. The proteins were revealed by staining with Coomassie Brilliant Blue (Chapter 2, Section 2.11). Photographic magnification was 1.5x. Super-imposed above the electropherogram of CY8.2. (Plate B) is the proposed nomenclature for the Coprinus cinereus small cytoplasmic ribosomal subunit proteins.


PLATE A

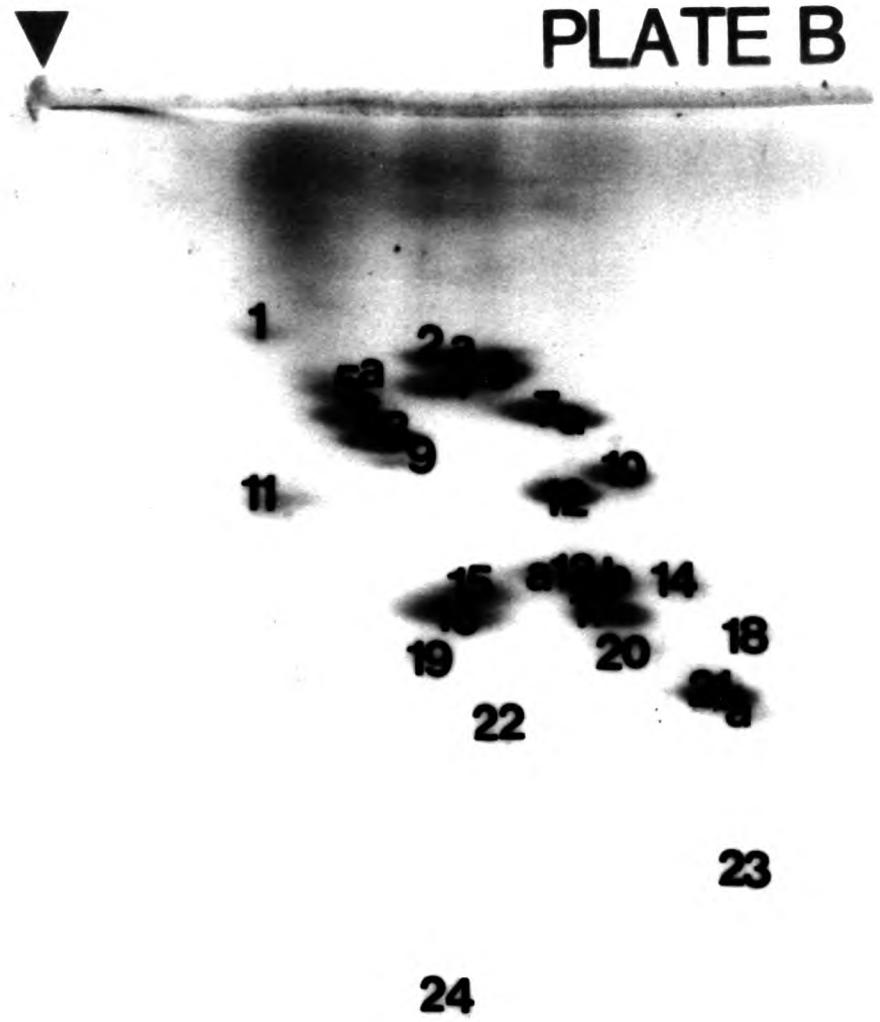
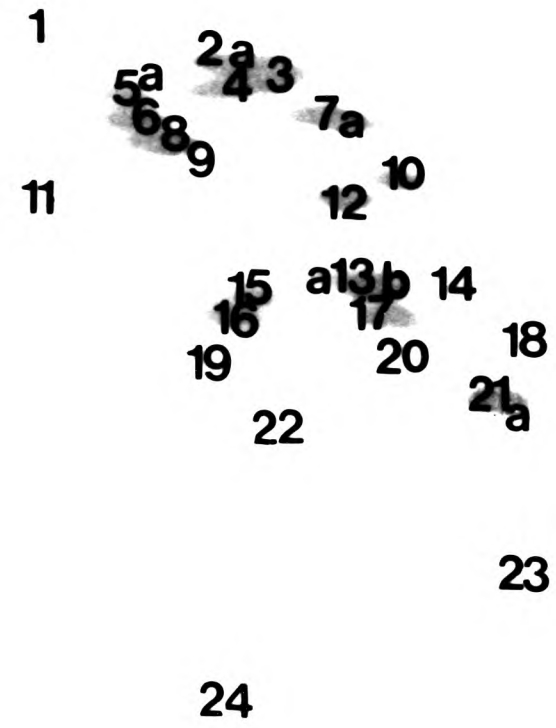


PLATE B
1
2a
2b
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

PLATE A




1
2a
4
3
5a
6
8
9
7a
10
11
12
15
a13b
14
16
17
19
20
18
21a
22
23
24

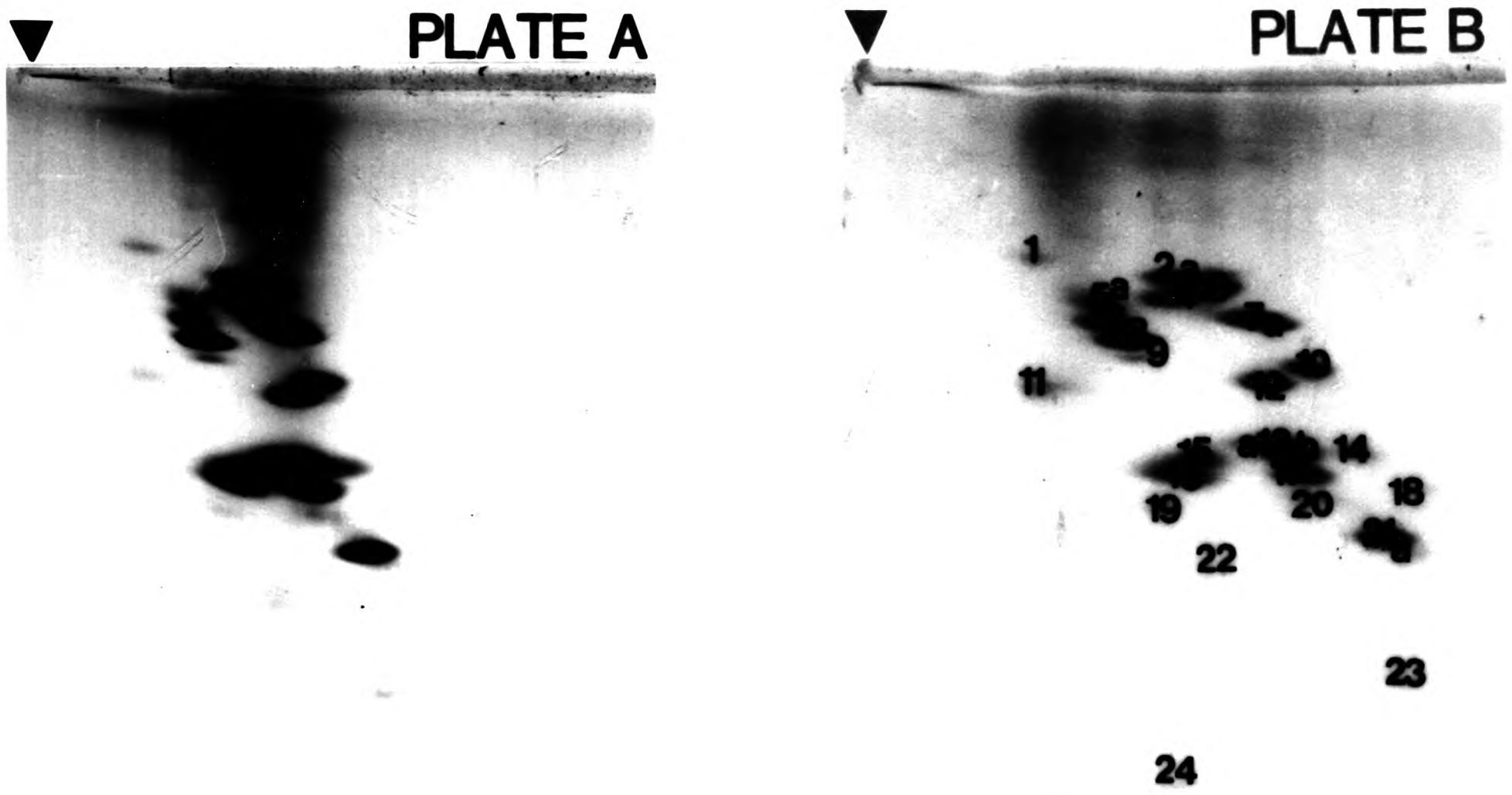
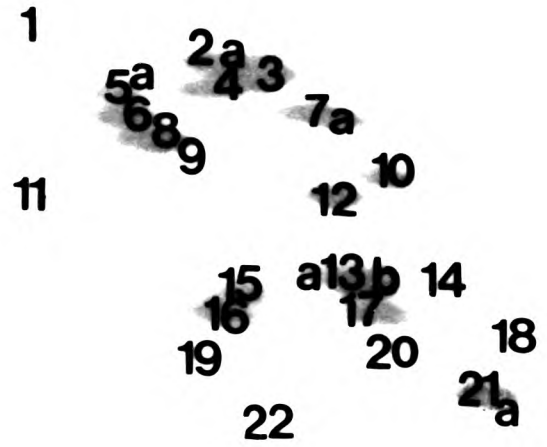


PLATE A
PLATE B
1
2
3
9
11
14
15
19
20
18
22
23
24

PLATE A



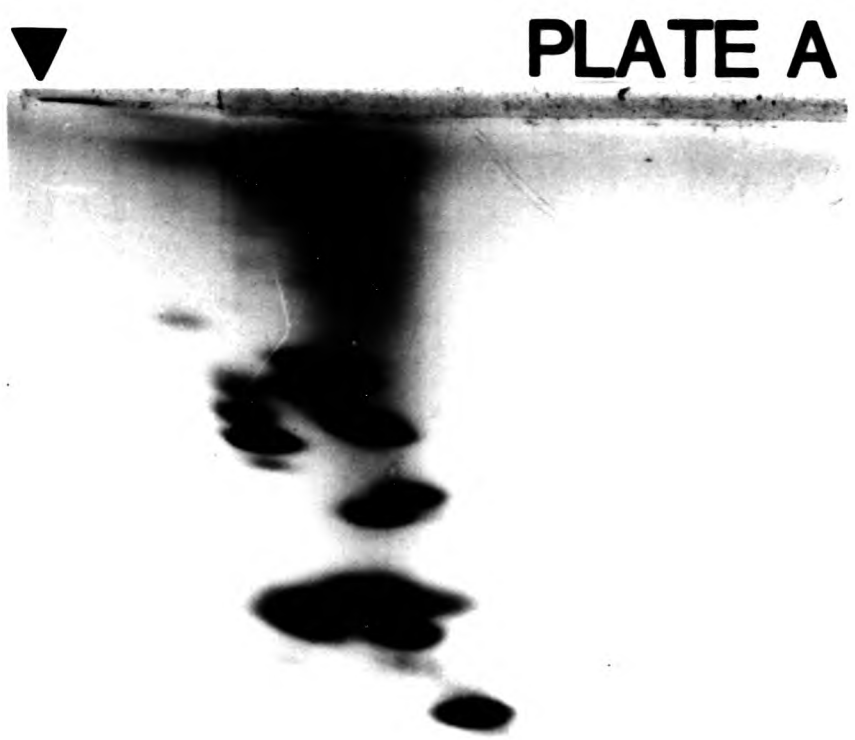


PLATE A

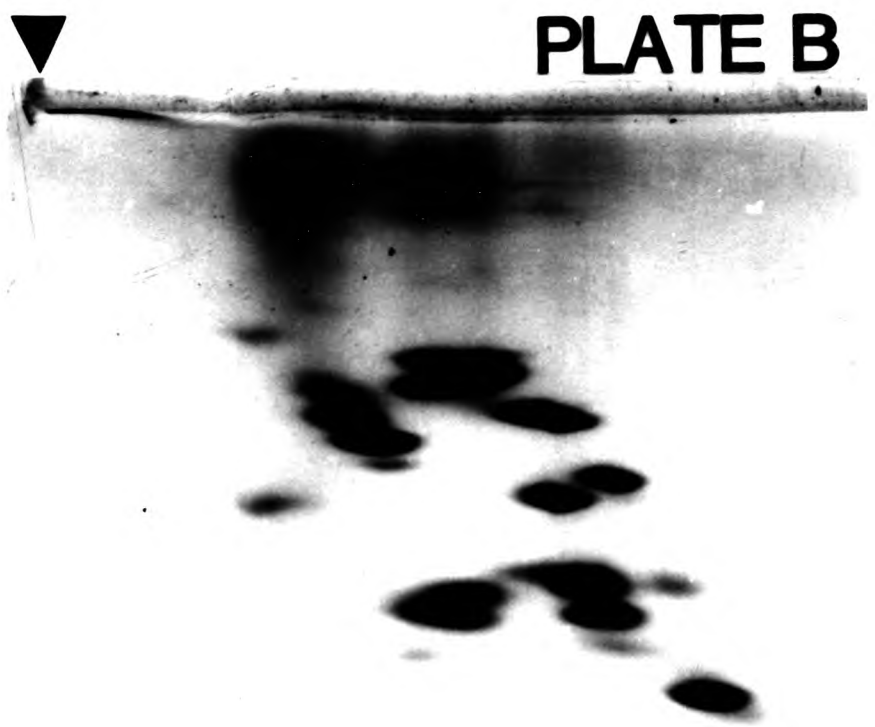
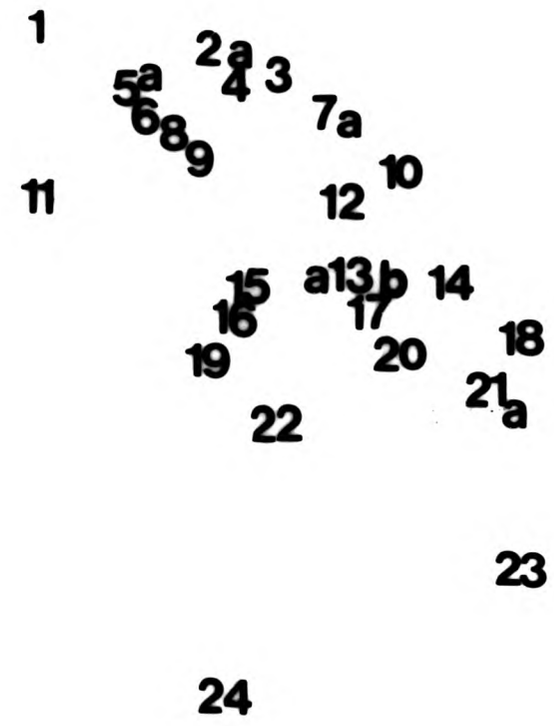


PLATE B

PLATE A

PLATE B


1
2a
4
3
5a
6
8
9
7a
10
11
12
15
a13b
14
16
17
18
19
20
21a
22
23
24


1
2a
5a
4
3
6
8
9
7a
11
10
12
15
a13b
14
16
17
19
20
18
21
a
22
23
24

SECTION 6.4. COMPARISON BETWEEN CY8 AND CY8.2 SMALL CYTOPLASMIC RIBOSOMAL SUBUNIT PROTEINS.

There was no observable difference between the small cytoplasmic ribosomal subunit proteins derived from the cycloheximide-sensitive strain CY8 (Plate A) and those of the cycloheximide-resistant strain CY8.2 (Plate B). It was concluded that there was no protein from the small cytoplasmic ribosomal subunit which was associated with cycloheximide-resistance. However, Plates A and B were the only examples of electropherograms which exhibited sufficient resolution for such a comparison to be made.

Homologous proteins from CY8 and CY8.2 were identified on the basis of their relative staining and their migration in the second dimension. However, the migration in the first dimension produced an inferior resolution of CY8 proteins to that of CY8.2 proteins and led to the impression of dissimilarity between the electropherograms, (Plates A and B). All CY8 proteins consistently migrated 70% of the distance moved by equivalent CY8.2 proteins in the first dimension. Replicate analyses of CY8 and CY8.2 small ribosomal subunit proteins (Not presented) although less resolved than Plates A and B migrated identical distances in the first dimension.

If the reduced migration of the CY8 proteins in the first dimension in Plate A was taken into account when comparing Plates A and B, then there were no discernable differences between the two strains. Homologous proteins appeared to be present in both Plates A and B but the inferior resolution of CY8 proteins in the first dimension made the

detection of closely migrating proteins in CY8 more difficult. Homologous CY8 and CY8.2 proteins migrated identical distances in the second dimension and, if a correction factor of x 1.4 were applied to the CY8 proteins, they were also identical in the first dimension. There were no apparant differences in the relative intensity of staining of the proteins from the two species.

A possible reason for the inhibition of migration of CY8 proteins in the first dimension was that an air-bubble, unnoticed during the electrophoresis, was trapped in the rod gel.

SECTION 6.5. CHARACTERISATION OF COPRINUS CINEREUS LARGE CYTOPLASMIC RIBOSOMAL SUBUNIT PROTEINS.

No one electropherogram, from monokaryotic (Plates C to F) or dikaryotic strains (Plates G to J), exhibited all the proteins which were observed. In order to illustrate the proteins from the large cytoplasmic ribosomal subunit a composite electropherogram has been drawn (Figure 6.1).

The large numbers of proteins and greater variability between electropherograms (Plates C to J inclusive) made the characterisation of the large cytoplasmic ribosomal subunit more difficult than that of the small subunit (Section 6.4).

The 36 proteins which were observed in the majority of electropherograms, identified as L1 to L36, were typically well stained and well resolved. In addition there were eighteen other proteins which were well resolved from neighbouring proteins but whose presence was less confidently assumed, because they were not observed in all analyses and because they were weakly stained. These proteins have been identified by a letter, from La to Lz. There was also a group of eight proteins whose presence was inferred from the size, shape and intensity of staining of proteins which partially obscured them and these proteins were identified with reference to the protein with which they were associated, namely L1a, L5a, L8a, L11a, L28a, L30a, L30b and L32a. In the text all proteins from the large ribosomal subunit were prefixed by L.

Of the potential 64 proteins identified, the maximum number observed in any one electropherogram was 50 (PlateI) and the minimum was 41 (Plate J).

FIG .6.1

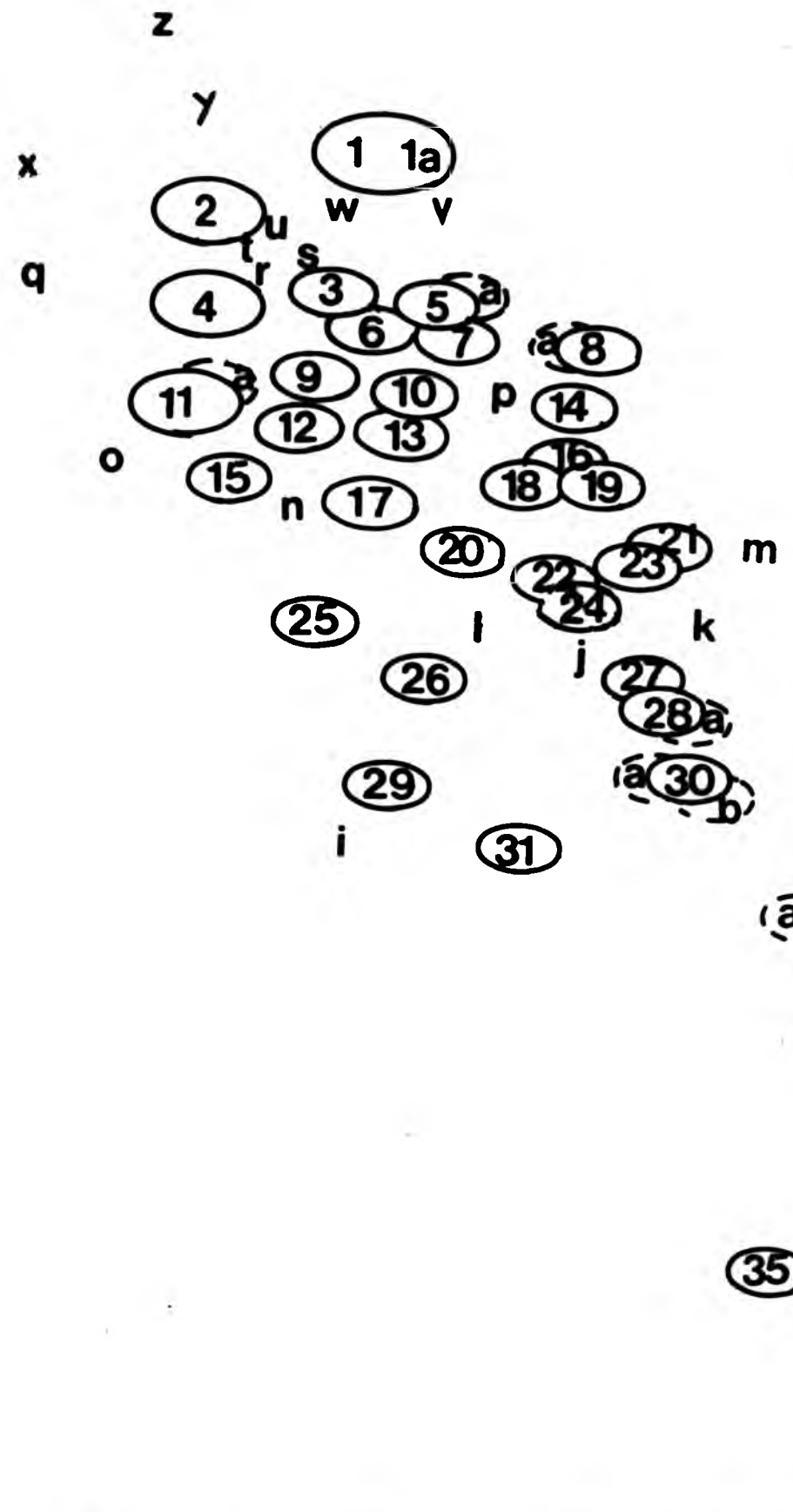


Figure 6.1. Composite illustration of an electropherogram of all observed proteins from the large cytoplasmic ribosomal subunit and their proposed nomenclature.

The illustration is based on the electropherograms of monokaryotic and dikaryotic strains present in Plate C - J inclusive. Intensly and consistenely observed proteins are bounded by solid lines and are numbered from 1 to 36. The position of less reproducable proteins are bounded by broken lines and are identified by a subscript, eg. 32a, or have been identified by a letter from i to z inclusive.

SECTION 6.6. COMPARISON OF LARGE CYTOPLASMIC RIBOSOMAL SUBUNIT PROTEINS FROM MONOKARYOTIC STRAINS.

The comparison of large cytoplasmic ribosomal subunit proteins from monokaryons was restricted to an analysis of four adequately resolved electropherograms from each of four strains, CY8, CY8.2, CY9 and CY9.23 (Plates C to F inclusive).

Two phenomena were observed in these electropherograms (Plates C to F), which were not observed in the replicates of these strains (not presented). It was believed that the double-imaging of proteins in the vicintiy of L3 in Plates C and E, and the distinctive bands in the region above L1 in Plates D and F, were artifacts of the experimental technique which occured only in these electropherograms. The cause of the double-imaging was not known, but a similar banding pattern to that of Plates D and F, was observed by Kaltschmidt and Wittmann(1970), they believed that overloading of the protein sample resulted in the production of insoluble aggregates which over the period of the electrophoresis migrated in the tracks of intially soluble proteins.

Plates C and D. Electropherograms of proteins from the large cytoplasmic ribosomal subunit of CY8 and CY8.2.

The preparation and analysis by 2D-PAGE of proteins from the large cytoplasmic ribosomal subunit of CY8 (Plate C) and CY8.2 (Plate D) was according to the methods described in Chapter 2, Sections 2.7, 2.8, 2.10 and 2.11. The proteins were stained with ammoniacal silver nitrate, Chapter 2, Section 2.11.

Migration of the proteins was towards the cathode, from the origin on the left to the right in the first dimension and from top to bottom in the second dimension. Photographic magnification was 1.8 x. Superimposed above Plate D is the proposed nomenclature of large cytoplasmic ribosomal subunit proteins (Section 6.5), and included on Plate C are the additional proteins not observed in Plate D.

Plates E and F. Electropherograms of proteins from the large cytoplasmic ribosomal subunit of CY9 and CY9.23.

The preparation analysis by 2D-PAGE of proteins from the large cytoplasmic ribosomal subunit of CY9 (Plate E) and CY9.23 (Plate F) and presentation of the electropherograms is as stated for Plates C and D. The photographic magnification 1.8x. The presence of proteins,found to be unique to either electropherogram , are identified.

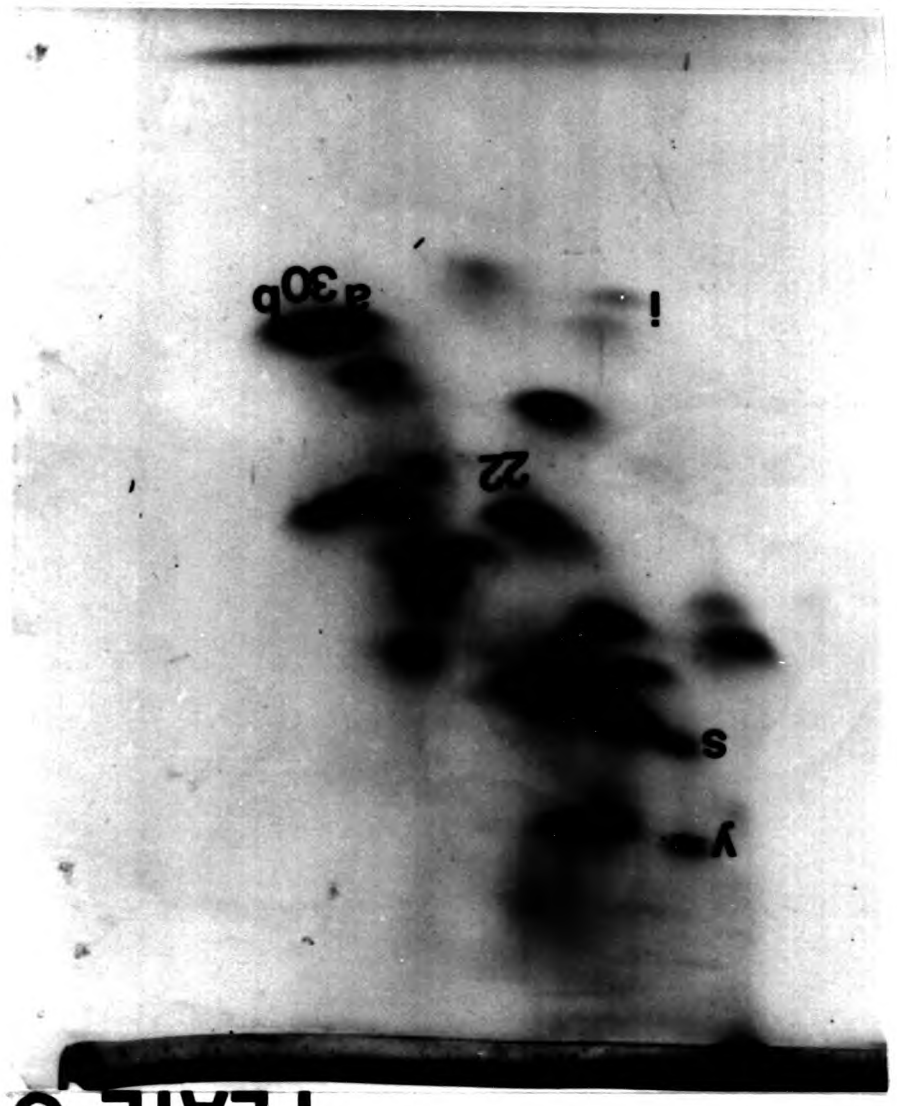


PLATE C

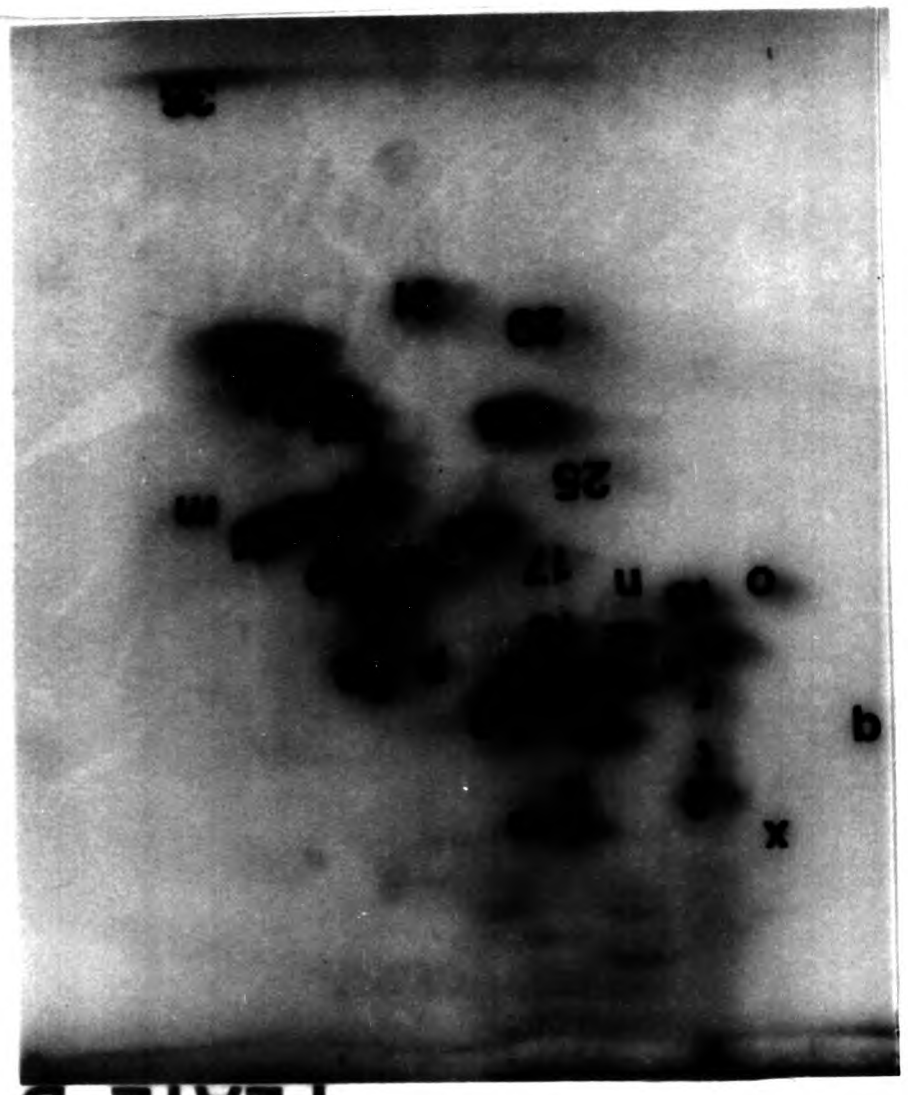


PLATE D


x
1
a
2
w
t
q
3
5
a
r
9
10
7
p
a
8
11
a
12
13
14
o
15
n
17
16
19
20
18
21
m
25
24
23
i
26
27
28
a
30
29
31
36

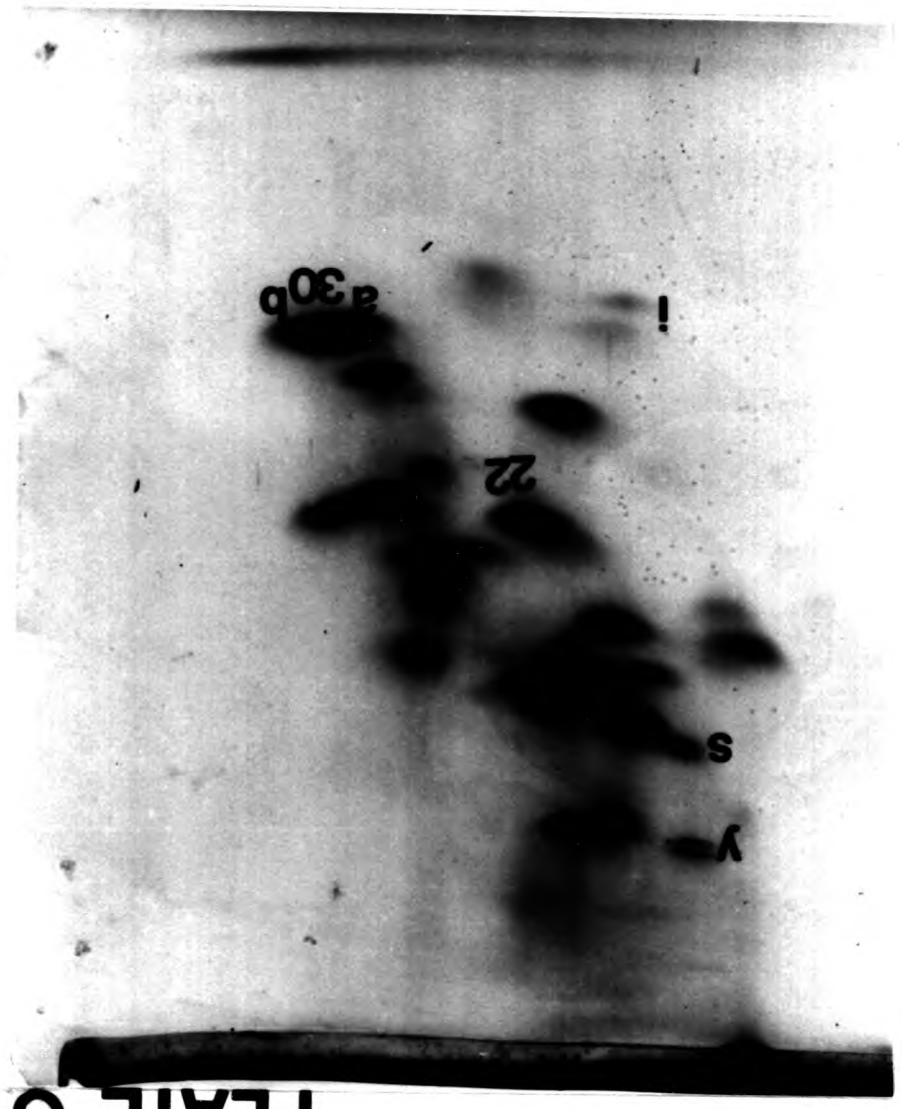


PLATE C

PLATE D

PLATE E

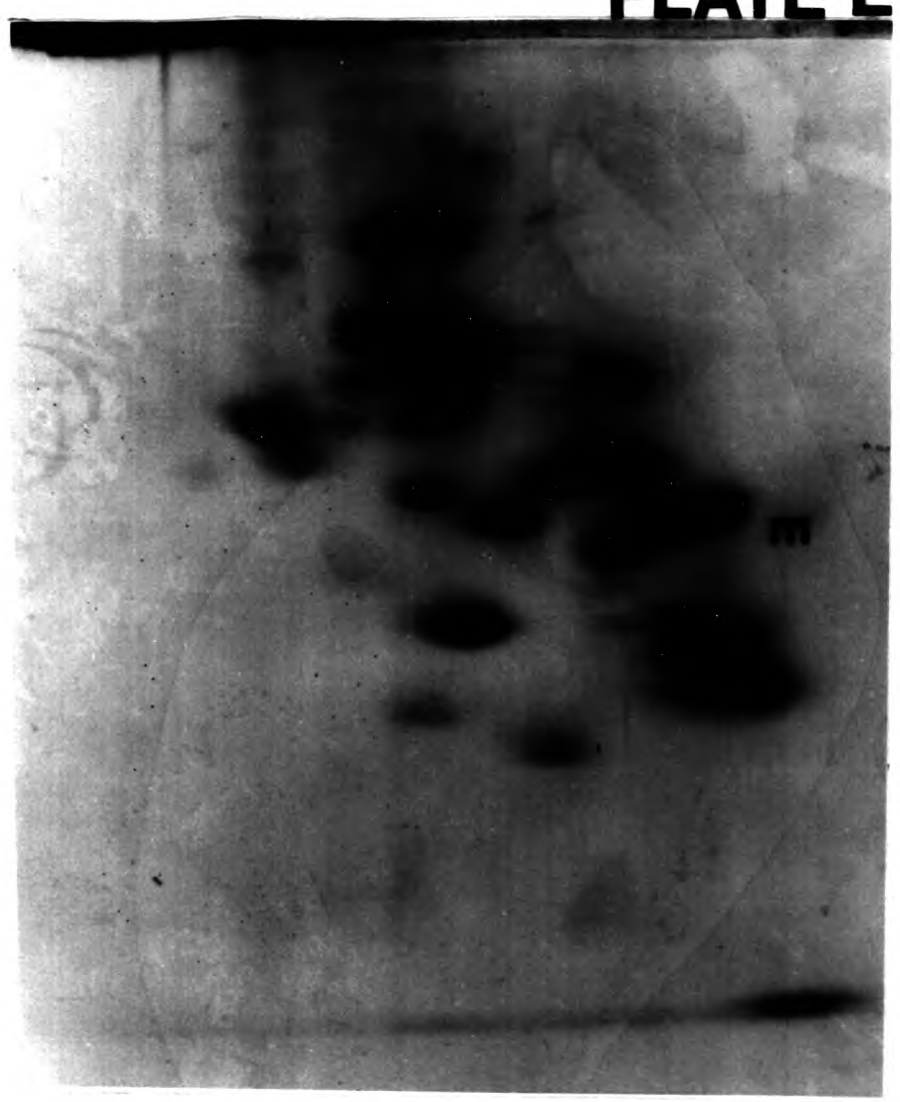
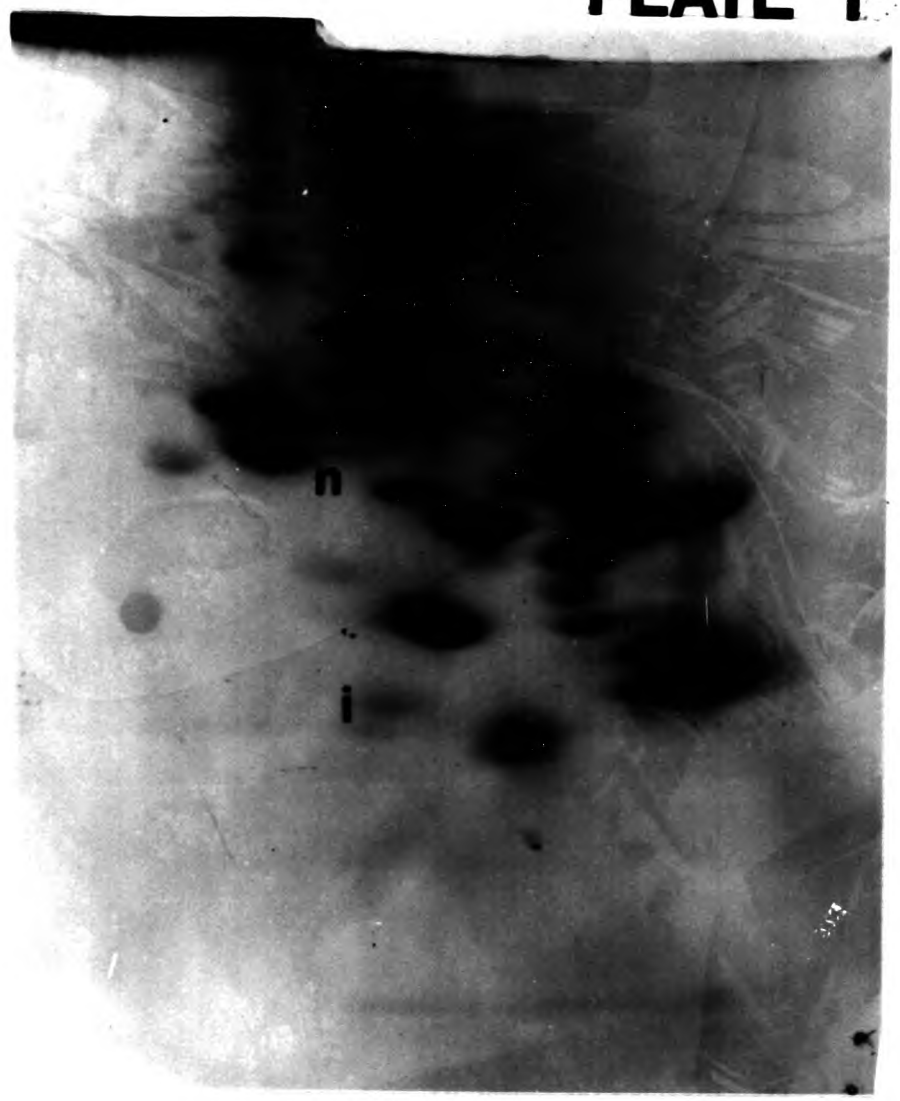


PLATE F



a) Comparison between CY8 and CY8.2.

There were a considerable number of differences between the electropherograms of the cycloheximide-sensitive strain CY8 (Plate C) and the cycloheximide-resistant mutant strain CY8.2 (Plate D) which was known to possess cycloheximide-resistant cytoplasmic ribosomes (Chapter 5, Section 5.3 a).

Discounting the differences resulting from the possibility of the artifacts (Section 6.6), the majority of the differences between the strains related to the presence of weakly stained proteins identified in only one strain. (Summarised in Table 6.2). However there were three proteins, L2, L12 and Lo which were relatively well-stained and present only in the electropherogram of CY8.2 proteins (Plate D). Differences in the relative intensity of staining between homologous proteins of CY8 and CY8.2 were not easily observed but L13 appeared to be more intensly stained in CY8 than it was in CY8.2. Differences in the relative positions of homologous proteins were not found.

b) Comparison between CY9 and CY9.23.

The electropherograms presented for CY9 (Plate E) and CY9.23 (Plate F) show double-imaging and banding at the top of the gels respectively. If these phenomena are considered to be artifacts and are discounted, then the strains show only four differences, all of which concern weakly stained proteins. L14, Ln and Li are only observed in CY9.23, and Lm is exclusive to CY9 (Table 6.2). There were no obvious differences in the relative position or intensity of any of the other proteins.

een the

8 (Plate C)

D) which

bosomes

bility

ences

ned

le 6.2).

re

ogram

ntensity

re not

d in

tions of

of the

artifacts

nces, all

re

6.2).

or

Table 6.2. Summary of the differences between the monokaryotic strains.

Protein	CY 8 (Plate C)	CY 8.2 (Plate D)	CY 9.0 (Plate E)	CY 9.23 (Plate F)
y	+	-	-	-
x	-	+	-	-
2	-	+	+	+
t	-	+	-	-
s	+	-	-	-
r	-	+	-	-
q	-	+	-	-
8a	-	+	-	-
12	-	+	+	+
14	+	+	-	+
o	-	+	+	+
n	-	+	-	+
m	+	+	+	-
22	+	-	-	-
28a	-	+	+	+
30a	+	-	+	+
30b	+	-	+	+
i	+	-	-	+

+ denotes presence and - denotes absence of stained protein.

c) Comparison between CY8 and CY9 with CY8.2 and CY9.23.

The only protein difference between the cycloheximide-sensitive and cycloheximide-resistant strains was the presence of the weakly stained protein, Ln , in the mutant strain but not in the parental strain. Ln was therefore considered to be a candidate for a ribosomal protein associated with cycloheximide resistance.

There were however a considerable number of dissimilarities between each pair of strains, ie. between CY8 and CY9, and CY8.2 and CY9.23 (Table 6.2).

SECTION 6.7. COMPARISON OF LARGE CYTOPLASMIC RIBOSOMAL SUBUNIT PROTEINS FROM DIKARYONS.

The resolution achieved for the analysis of proteins from the large cytoplasmic ribosomal subunit of the dikaryons CY8.2 x CY13 CY9 x CY3 and CY9.23 x CY3 (Plates G to J) was superior to any other analysis undertaken. However, technical problems prevented equally good resolution of replicate analyses.

a) Comparison between CY8 x CY13 and CY8.2 x CY13.

The electropherograms of the homozygous cycloheximide-sensitive strain CY8 x CY13 (Plate G) and the heterozygous cycloheximide-resistant strain CY8.2 x CY13 (Plate H) differed in several respects (summarised in Table 6.3).

CY8.2 x CY13 possessed four proteins which were not found in CY8 x CY13; Lq, L22, L30a and L30b. All were weakly stained proteins and with the exception of Lq, were difficult to observe because of neighbouring proteins. CY8 x CY13 possessed a well-stained, clearly resolved protein Lv which was not found in CY8.2 x CY13.

A less obvious difference between the dikaryotic strains was the relative intensity of staining of L2. All of the proteins in CY8.2 x CY13 were more heavily stained than in CY8 x CY13, except L2. In CY8 x CY13, L2 was a more diffuse spot and more heavily stained than in CY8.2 x CY13.

Plates G and H. Electropherograms of proteins from the large cytoplasmic ribosomal subunit of the dikaryons CY8 x CY13 and CY8.2 x CY13.

The electropherograms for CY8 x CY13 (Plate G) and CY8.2 x CY13 (Plate H) were produced according to the method for 2D-PAGE analysis (Chapter 2, Section 2.11) from cytoplasmic ribosomal proteins prepared according to Chapter 2, Sections 2.7, 2.8 and 2.10. The migration of proteins in both dimensions was as described for Plate C, Section 6.6. The photographic magnification was 2.3x. The proposed nomenclature for the large ribosomal subunit proteins (Section 6.5) is superimposed above Plate H. Proteins not found on Plate H are identified on Plate G.

Plates I and J. Electropherograms of proteins from the large cytoplasmic ribosomal subunit of the dikaryons CY9 x CY3 and CY9.23 x CY3.

The electropherograms of CY9 x CY3 (Plate I) and CY9.23 x CY3 (Plate J) were prepared and produced as described for Plates G and H. Photographic magnification was 1.6x. Those proteins not previously identified on Plate H, and differences between Plates I and J, are included on Plate I.

PLATE G

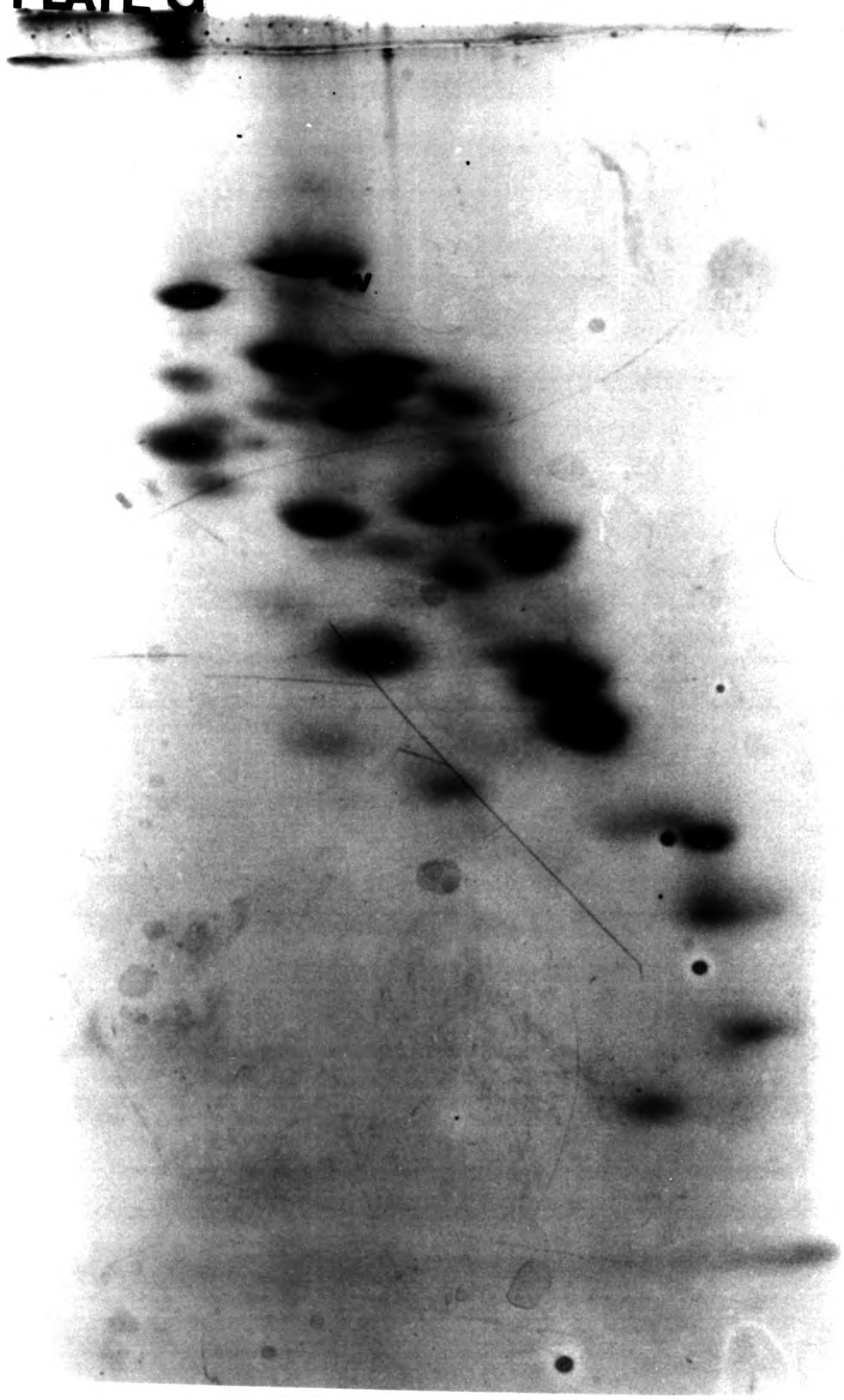
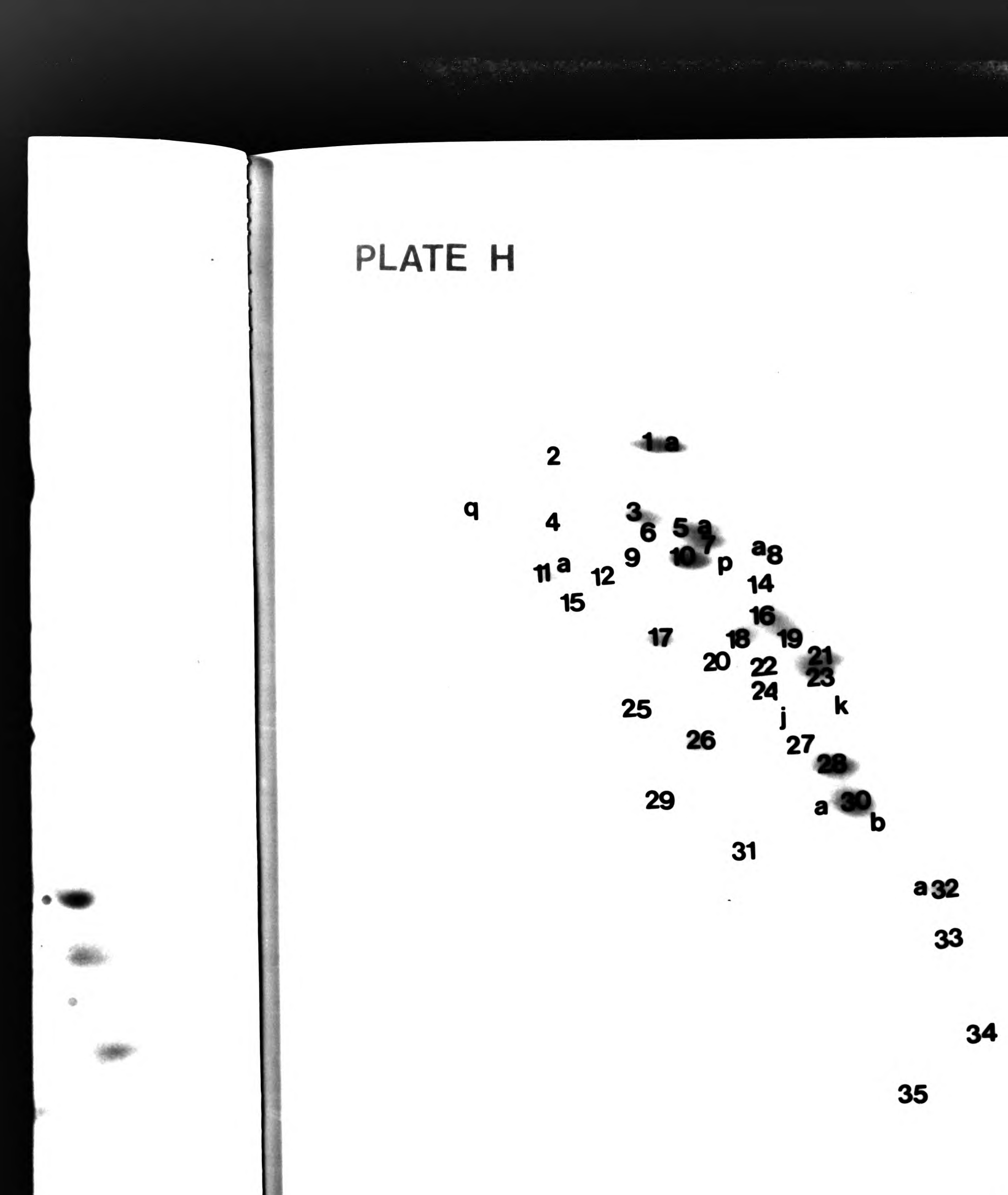
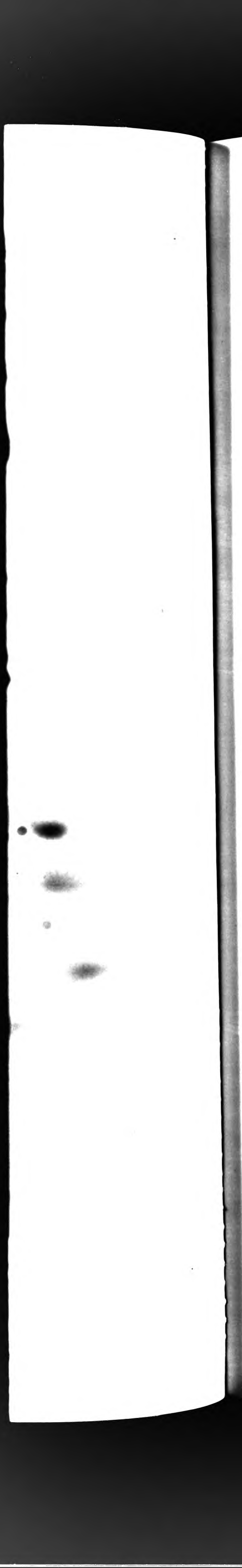


PLATE G

PLATE H

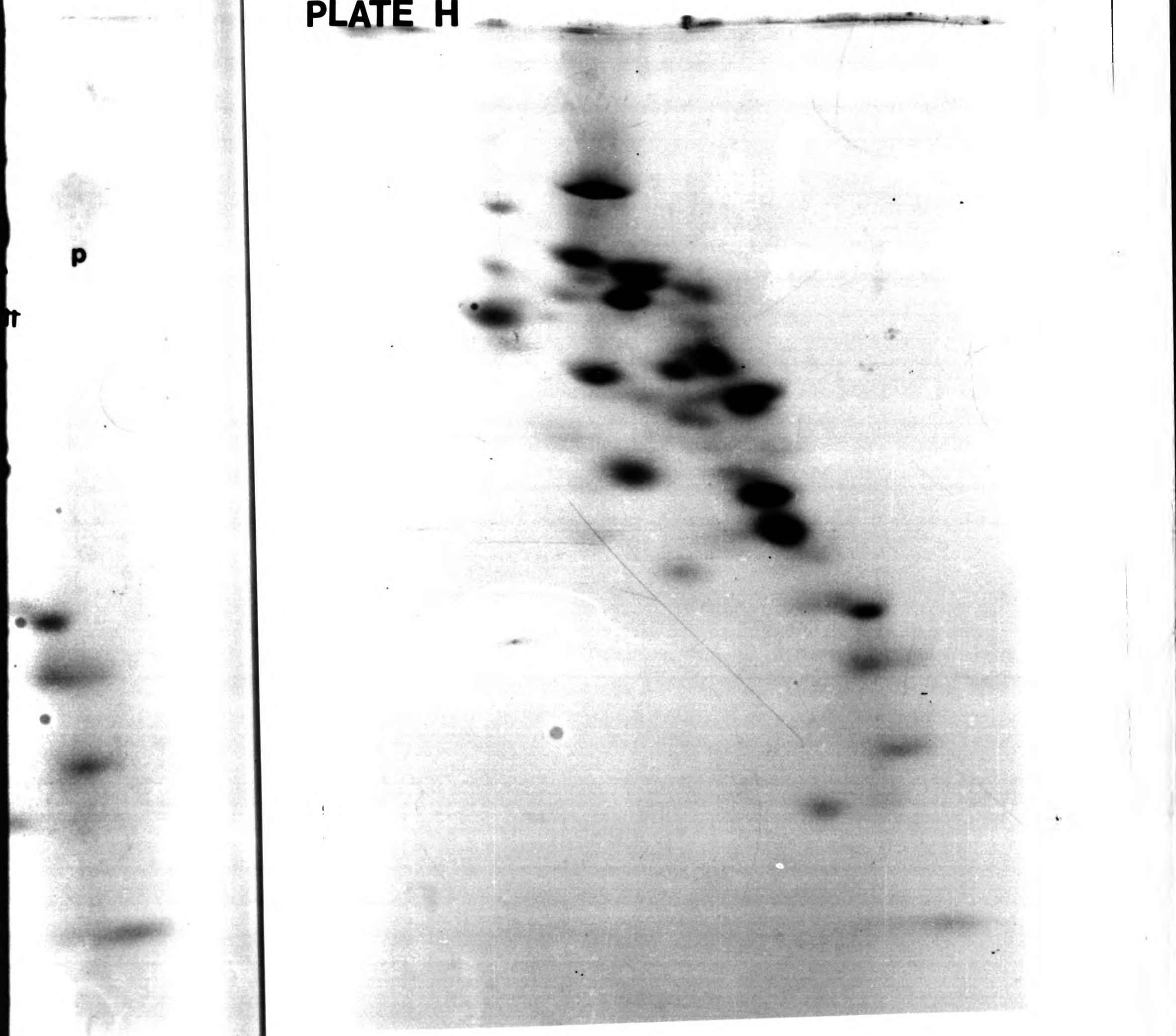

1a
2
q
3
4
6
5
a
7
a
8
9
10
p
11
a
12
14
15
16
17
18
19
21
20
22
23
24
25
j
k
26
27
28
29
a
30
b
31
a
32
33
34
35
36



1 a
2
q
3
4
6
5 a
7
9
10
p
a 8
11 a
12
14
15
16
17
18
19
21
20
22
23
24
25
j
k
26
27
28
29
a 30
b
31
a 32
33
34
35
36

PLATE H

PLATE H



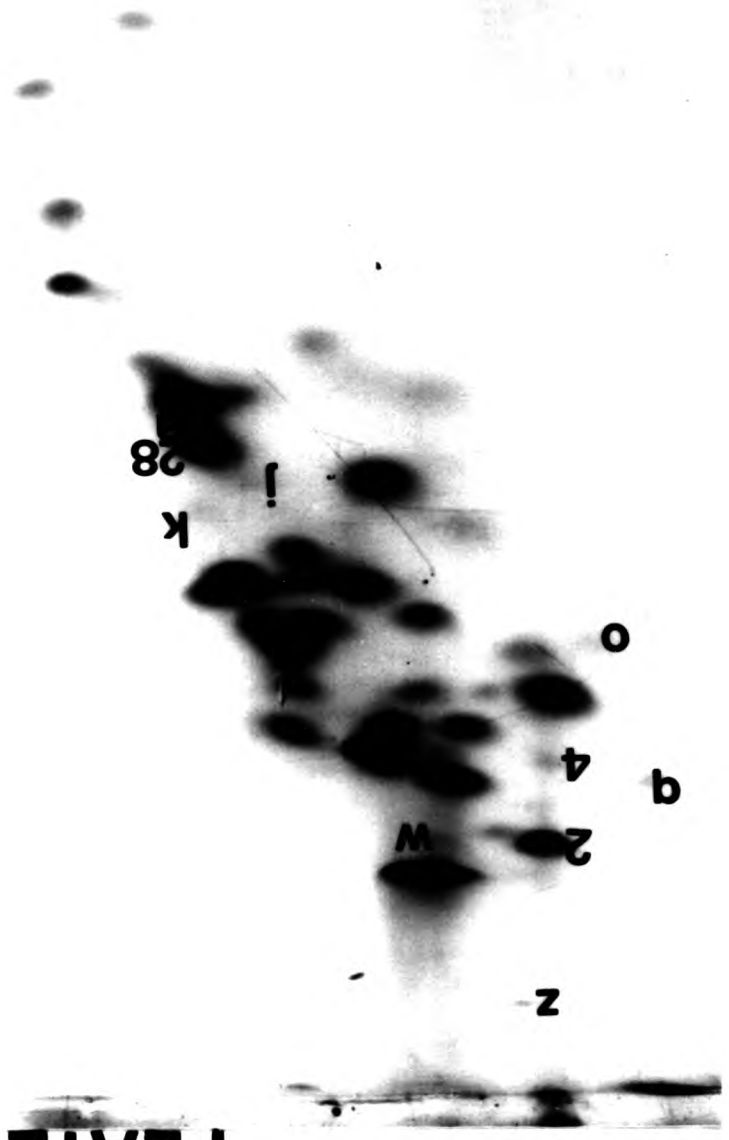


PLATE I

PLATE J

PLATE I

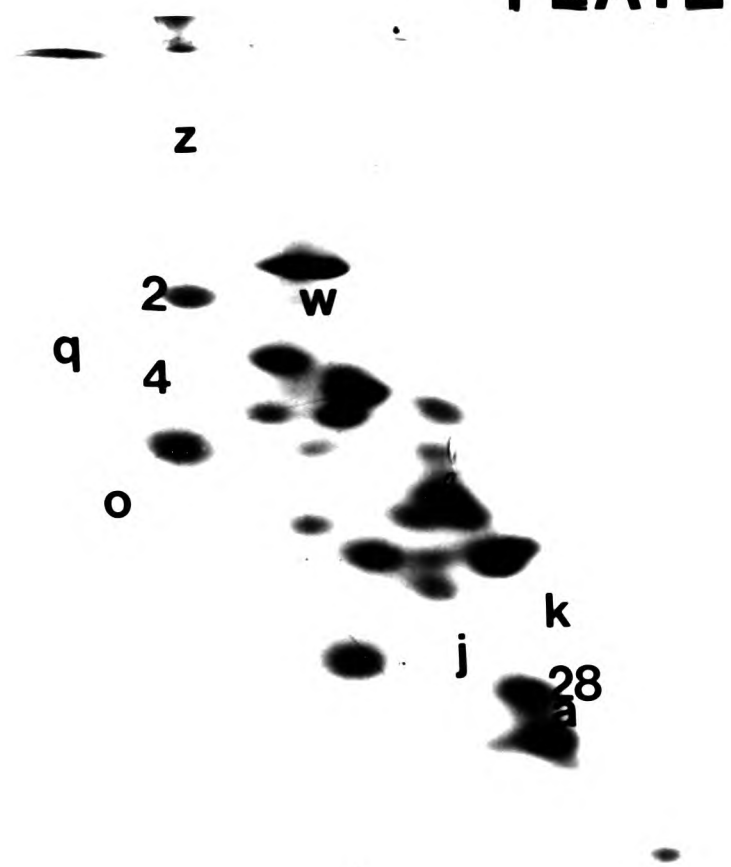


PLATE J

b) Comparison between CY9 x CY3 and CY9.23 x CY3.

The electropherogram of CY9.23 x CY3 (Plate J) revealed only 41 proteins, whereas the largest number observed in one analysis, 50, were found in CY9 x CY3 (Plate J). The majority of the difference between the two strains, was the presence of nine additional, mostly weakly stained proteins in CY9 x CY3. These are identified in Table 6.3. Included amongst these proteins was the intensly stained L2. In addition L9, L10 and L28 were relatively more intensly stained in CY9 x CY3. There was no discernable difference in the relative positions of homologous proteins between the two strains.

c) Comparison between CY8 x CY13 and CY9 x CY3 with CY8.2 x CY13 and CY9.23 x CY3.

A comparison between the two homozygous cycloheximide-sensitive dikaryons, CY8 x CY13 and CY9 x CY3 and the two heterozygous cycloheximide-resistant dikaryons CY8.2 x CY13 and CY9.23 x CY3 revealed that there was no common protein shared by both strains of a pair, which differed between the pairs, except for the relative intensity of staining of L2. L2 was more intensly stained in homozygous cycloheximide-sensitive dikaryons than in the heterozygous cycloheximide-resistant dikaryons. There were more differences within each pair, than between pairs.

Note: No results are presented for the large or small cytoplasmic ribosomal subunit proteins derived from diploid strains, or for the analysis of total cytoplasmic ribosomal protein from monokaryons, dikaryons and diploids, as a result of technical difficulties, the resolution achieved was unsuitable for meaningful observations.

Table 6

Protein
z
2
w
v
u
q
4
5a
p
11a
13
o
22
1
k
j
28a
30a
30b
+ signi

Table 6.3. Summary of the differences between the dikaryotic strains.

Protein	Strain			
	CY 8 x CY 13 (Plate G)	CY 8.2 x CY 13 (Plate H)	CY 9 x CY 3 (Plate I)	CY 9.23 x CY 3 (Plate J)
z	-	-	+	-
2	+	+	+	-
w	-	-	+	-
v	+	-	-	-
u	-	-	+	+
q	-	+	+	-
4	+	+	+	-
5a	+	+	-	-
p	+	+	-	-
11a	+	+	-	-
13	-	-	+	+
o	-	-	+	-
22	-	+	+	+
1	-	-	+	+
k	+	+	+	-
j	+	+	+	-
28a	-	-	+	-
30a	-	+	+	+
30b	-	+	+	+

+ signifies presence and - denotes absence of stained protein.

SECTION 6.8. ANALYSIS OF CYTOPLASMIC RIBOSOMAL PROTEINS FROM THE LARGE SUBUNIT BY CARBOXYMETHLY-CELLULOSE CHROMATOGRAPHY.

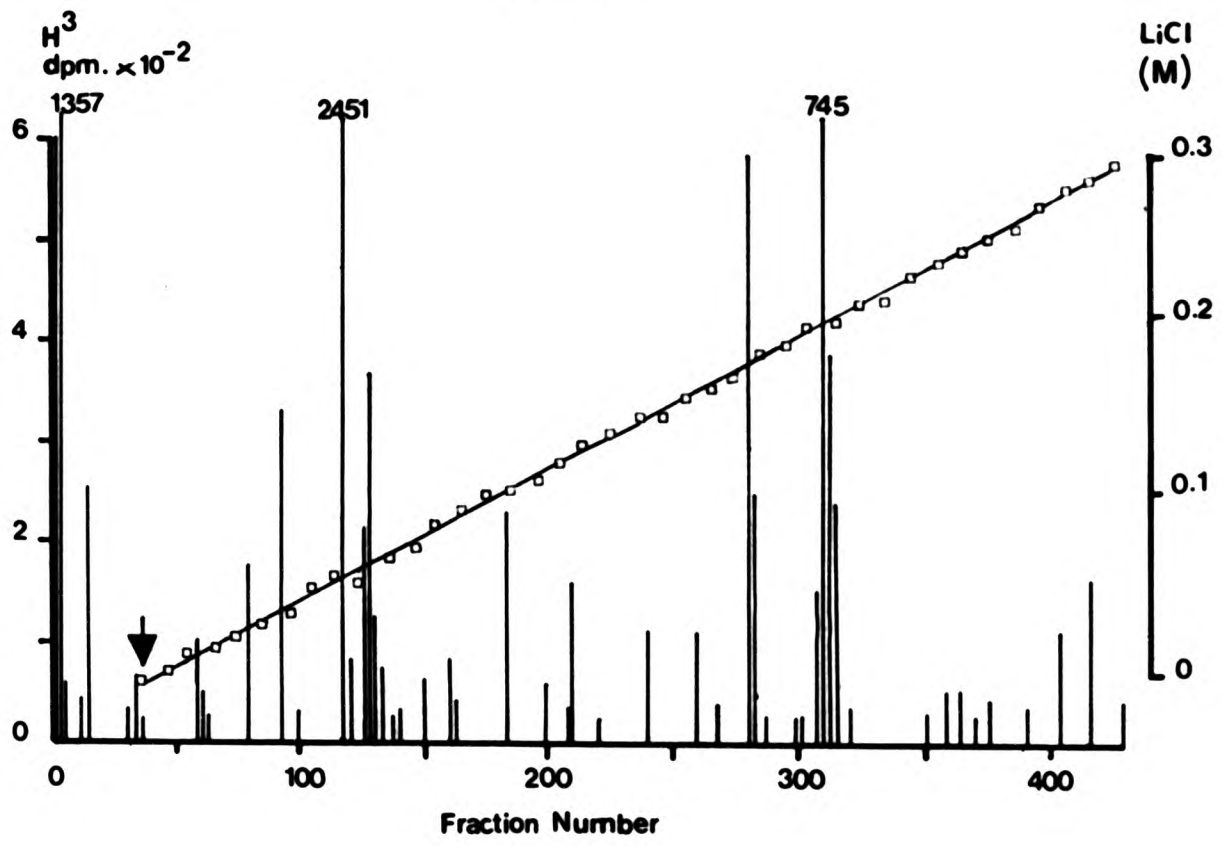
In theory, the cytoplasmic ribosomal proteins of cycloheximide-sensitive strains grown in medium containing C^{14}, could be co-extracted and co-chromatographed with those of their comparable cycloheximide-resistant mutants, grown in medium containing H^3. The cytoplasmic ribosomal proteins in the elution profile could then be directly compared in an attempt to identify cytoplasmic ribosomal protein or proteins conferring cycloheximide resistance.

In practice, both of the cycloheximide-sensitive strains CY8 and CY9 grown with D-(C^{14}) glucose became contaminated and were discarded. With no more C^{14} available, the analysis continued in a curtailed form as a comparison of CY8.2 and CY9.23 cytoplasmic ribosomal proteins. However, because both strains were grown in D -(H^3) glucose the cytoplasmic ribosomal proteins were prepared and analysed separately . The amount of cytoplasmic ribosomal protein prepared, permitted only one chromatogram for each strain and even then was 100x less than the load recommended by Coppin-Raynal (1980).

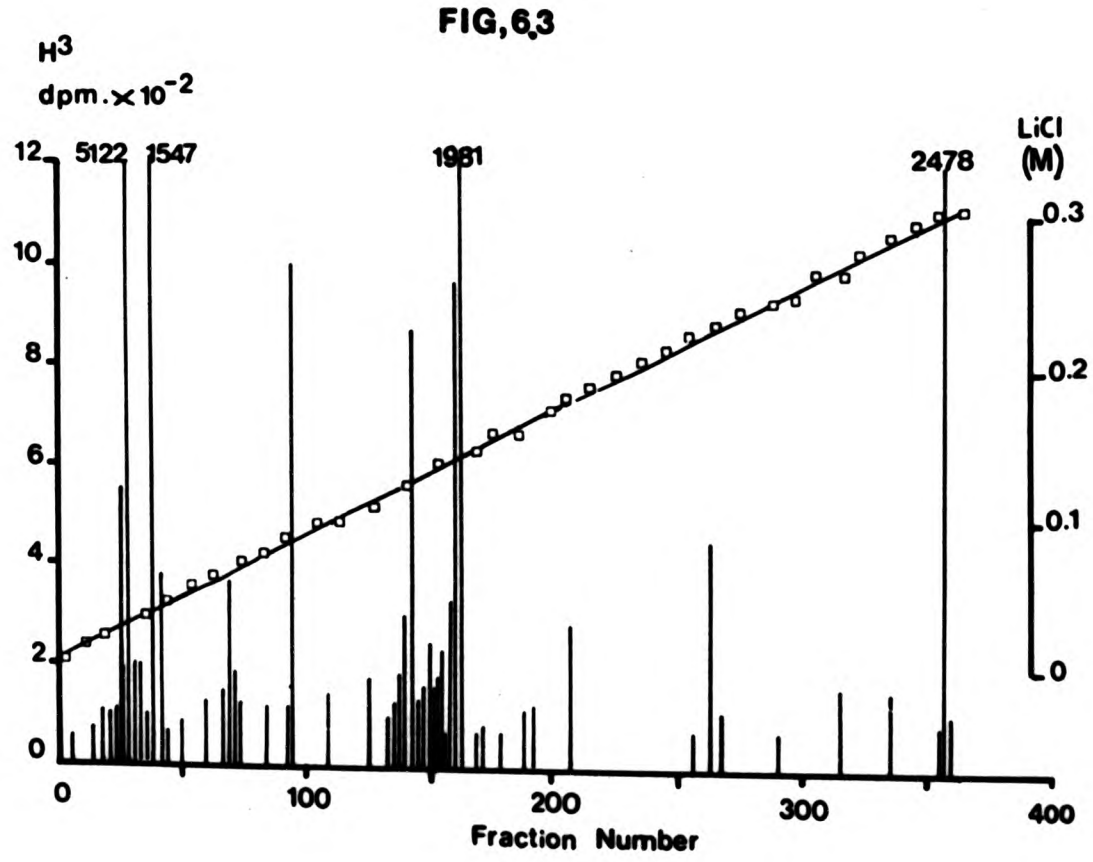
The analysis of large cytoplasmic ribosomal subunit proteins from CY8.2 and CY9.23 produced protein elution profiles in which H^3 radioactivity was usually confined to single fractions (Figures 6.2 and 6.3), contrary to all other published results (eg. Coppin-Raynal, 1980; Tsurugi *et al*,(1976),for rat liver). Consequently any interpretation of the results was speculative in the absence of further data.

Figures 6.2 and 6.3. Chromatograms of large cytoplasmic ribosomal subunit proteins from CY8.2 and CY9.23.

FIG,6.2



FIG,6.3



mic

Because the proteins of CY8.2 and CY9.23 were labelled with H^3, they could not be directly compared by co-chromatography. Additionally, because the linear LiCl concentration gradient was not applied at the same time to both treatments,the results could not be directly compared.

The elution profile of CY8.2 proteins (Figure 6.2) could be divided into two regions. The first half of the elution profile (0-0.15 M LiCl, fractions 0 to 180) contained nearly 70% of the H^3 which was recovered whereas, for CY9.23 (Figure 6.3)the distribution of radioactivity was more uniform. In both treatments the total recovery was approximately 30%. In both treatemnts, 50 fractions possessed measurable amounts of radioactivity, with the maximum activity in any one fraction being 5122 dpm for CY8.2 and 245 dpm for CY9.23.

Figures 6.2 and 6.3. Chromatograms of large cytoplasmic ribosomal subunit proteins from CY8.2 and CY9.23.

Ribosomal proteins from the large cytoplasmic ribosomal subunit of CY8.2 and CY9.23 were labelled with H^3, prepared and subjected to separate analyses by carboxymethyl-cellulose chromatography, according to the method described in Chapter 2, Section 2.12.

6×10^4 dpm of CY8.2 protein was immediately subjected to a linear LiCl concentration gradient (0-0.3M) in CMC buffer (Chapter 2, Section 2.6). Figure 6.2. 3×10^4 dpm of CY9.23 protein were equilibrated for 8 hours in CMC buffer (OM LiCL) before a linear LiCl concentration gradient (0-0.3M) was applied at fraction 40 (▼), Figure 6.3.

The distribution of H^3 radioactivity in the elution profiles of CY8.2 (Figures 6.2) and CY9.23 (Figure 6.3) were determined using a LKB scintillation counter (solid vertical lines). Radioactivity (H^3 dpm) off the scale of the figures is indicated. Figures 6.2 and 6.3 also include the LiCl concentration (□—□).

SECTION 6.9. INTERPRETATION OF ELECTROPHEROGRAMS AND CHROMATOGRAMS.

The characterisation of Coprinus cinereus cytoplasmic ribosomal proteins and the identification of cytoplasmic ribosomal proteins conferring cycloheximide resistance was based on a limited number of adequately resolved electropherograms and chromatograms. Consequently, considerably more importance was accorded to those results which were adequately resolved than would have been given had replicate analyses and analyses of other strains been of sufficient resolution. Furthermore, because the analysis was based on a limited number of results, it was not known whether the variation between electropherograms (Plates A to J) and chromatograms (Figures 6.1 and 6.2) were the consequence of experimental error or inherent differences between the strains.

The analysis of cytoplasmic ribosomal proteins was based on two assumptions. Firstly, that each stained spot detected on the electropherograms, and each peak of radioactivity on the chromatograms, consisted of a homogeneous cytoplasmic ribosomal protein; moreover, that the proteins were derived from the cytoplasmic ribosomal subunit they were purported to be from. Secondly, that each of the proteins produced only one stained spot or peak of radioactivity.

a) Contaminants.

There was no conclusive evidence that the proteins analysed by either two-dimensional polyacrylamide gel electrophoresis or carboxy-methyl-cellulose chromatography were from cytoplasmic ribosomes. Neither Coomassie Brilliant Blue, nor the ammoniacal silver nitrate stain were specific for cytoplasmic ribosomal proteins (Oakley et al, 1980).

Similarly, the C^{14}- radiolabel was not specifically incorporated into cytoplasmic ribosomal proteins.

It was possible that the samples analysed contained proteins which were not derived from, and had different physical properties to, cytoplasmic ribosomes and were therefore revealed as distinct entities. The RP-100 may have contained mitochondrial ribosomes which, if Coprinus cinereus were like other species, eg. Neurospora crassa, Küntzel (1969), have different proteins to those of the cytoplasmic ribosomes. There were insufficient mitochondrial ribosomes to be detected in the analysis on sucrose density gradients (Chapter 4, Section 4.5a) and to allow polyphenylalanine synthesis in the presence of cycloheximide (mitochondrial ribosomal resistance to cycloheximide, eg. Ennis and Luben, 1964). Additionally, there may also have been contamination by non-cytoplasmic ribosomal proteins which remained associated during the preparation of the organelle, including during the dissociation into subunits in high potassium ion concentration (eg translational factors).

A second type of contamination, the presence of proteins from the large cytoplasmic ribosomal subunit in the preparation of proteins from the small cytoplasmic ribosomal subunit, and vice versa, will be discussed in Section 6.10a.

b) Artifacts.

It was assumed that each stained spot or peak of radioactivity contained one protein. However, it was possible that the spots and peaks detected were an association of different proteins which

co-migrated or co-eleuted either because of aggregation or because they possessed similar physical properties. The large diffuse stained region on most electropherograms represented as L30, L30a and L30b or the eluted fractions with very high radioactivity,(eg. Fractions 5 and 120 of Figure 6.2) may represent heterogeneous proteins. Further fractionation of the stained proteins or eluted fractions was not undertaken, nor were conditions used to minimise aggregation and improve resolution.

It was possible that one or more of the cytoplasmic ribosomal proteins may have produced more than one distinct stained spot in the electropherogram. One or more derivatives of a protein, each exhibiting different physical properties may have been produced during the preparation or analysis, perhaps by partial degradation,aggregation, changes in redox state or incomplete unfolding by urea. The derivative or satellite, proteins might not necessarily have migrated or eluted in the proximity of the unaffected proteins. In the absence of replicate analyses, it was not possible to identify any satellite proteins except two types which were described in Plates C and E, and Plates D and F, Section 6.6.

c) Detection.

The presence of artifacts and contaminants was suspected from the considerable variability that was observed, particularly in the analysis of proteins from the large cytoplasmic ribosomal subunit. Variability between preparations from the same strain should indicate the presence of experimental variation but because replicate analyses produced poor resolution, their identity could not be proven. A comparison of the results from different strains therefore included

variation resulting from inherent differences between the strains and experimental technique; the difficulty lay in differentiating between the two sources of variation.

The artifacts and contaminants were presumed to be present in small amounts relative to the native proteins. Intensity of staining and amount of radioactivity did not necessarily indicate quantity of homogenous protein because of the possible differences in the specific reactivity and activity, respectively, of the proteins and because of co-migration and co-elution of different proteins. The qualitative nature of the stains was observed by the range of colours with which the proteins were stained; the majority stained with Coomassie Brilliant Blue were blue, but some individual spots were violet (not shown on Plates A and B) and proteins stained by ammonical silver nitrate were brown, grey and silver (not shown on Plates C to J).

Although weakly-stained proteins probably indicated low quantity and therefore possible contamination, such proteins might have been relatively unreactive. Thus equal importance was given to both weakly-stained and the well-stained proteins in the comparison of proteins from different strains.

SECTION 6.10. CHARACTERISATION OF COPRINUS CINEREUS CYTOPLASMIC RIBOSOMAL PROTEINS.

The number of Coprinus cinereus cytoplasmic ribosomal proteins identified by 2D-PAGE analysis was estimated to be between 24 - 30 for the small subunit and between 36 - 64 for the large subunit. The estimates were based on the total number of proteins observed. In the case of the proteins from the small subunit, both strains examined exhibited all 30 proteins (Section 6.3). By contrast, the maximum number of proteins observed for the large cytoplasmic ribosomal subunit was 64, but the maximum number observed on any one electropherogram was 50 (Section 6.5).

The chromatograms of proteins from the large cytoplasmic ribosomal subunit were interpreted with caution (Section 6.8) but 50 peaks of radioactivity was slightly greater than the number of stained proteins observed in the same strains (Plates D and F, Section 6.6).

The characterisation of proteins from the large and small cytoplasmic ribosomal subunit was made in the light of doubts concerning the authenticity of the stained proteins, in particular with the possibility of there being cross-contamination between the subunit particles (Section 6.9).

a) Cross-contamination.

In other eukaryotic species there is evidence that only one protein is common to both cytoplasmic ribosomal particles (Russell and Wilkerson, 1980). The electropherograms of proteins from the

large cytoplasmic ribosomal subunit (Plates C to J) were superimposed above the results of the analysis of proteins from the small cytoplasmic subunit (Plates A and B) in order to determine if the position of any proteins were identical, and thereby to demonstrate if cross-contamination of proteins from the two subunits had occurred. However, the analysis of proteins from the two subunits were not undertaken under identical conditions and in the absence of an internal marker or well-resolved electropherogram from monosomes, the proteins from the large and small subunits could not be directly compared. Indirect evidence suggested; that the well-stained consistently observed proteins in both subunits were unique, but that the weakly-stained, infrequently observed proteins of the large subunit may be evidence of contaminating proteins from the small subunit. Contamination of the large subunit proteins by proteins from the small subunit was more probable that the reciprocal contamination because in the preparation of the large subunits, it was necessary to aspirate them through the layer of small subunits which remained in the sucrose density gradient after the bulk of small subunits had been removed (Chapter 2, Section 2.8).

b) <u>Losses.</u>

In the characterisation of the large and small cytoplasmic ribosomal subunit proteins,it was assumed that all proteins were present and detected. It was possible that proteins were lost during the dissociation of the cytoplasmic ribosomes which may account for the inability to prepare functional subunits (Section 5.4, Chapter 5). It was not possible to compare the proteins from monosomes with those of the subunits to determine if losses did occur, but in other species there are instances of such differences (Sherton and Wool, 1974).

It was possible that not all proteins were prepared with equal efficiency and that some were not present in the samples for analysis, although the method of preparation was one which produced the best efficiency of extraction in mammalian cells (Sherton and Wool, 1974) and in Schizosaccharomyces pombe (Coddington and Fluri, 1977). The variation in efficiency of extraction may be the cause of the variable intensites of staining with both Coomassie Brilliant Blue and ammonical silver nitrate. However, proteins reacted differently to both stains, producing a variation in colours (Section 6.9 c), and it was therefore possible that some proteins did not react with the stains and were thus not characterised.

Only those, cytoplasmic ribosomal proteins which had a net basic charge at pH 8.7, migrated toward the cathode in the first dimension and were therefore resolved by electrophoresis. The number of acidic proteins was not determined.

The proposed characterisation and nomenclature of Coprinus cinereus cytoplasmic ribosomal proteins, despite the reservations expressed (Section 6.9), served a practical role in the comparison of different strains and of comparison with different species.

c) Comparison with other species.

The number of proteins identified from the small cytoplasmic ribosomal subunit of Coprinus cinereus, which possessed a net positive charge at pH 8.5 (Section 6.3), was similar to the numbers reported in other fungal species, in particular, and eukaryotic species in general (Table 6.4), However, the estimated number of proteins observed from the large cytoplasmic ribosomal subunit of Coprinus cinereus (Section 6.5), was considerably higher than the numbers observed in other species.

(Table 6.4). Coprinus cinereus was considered to be a typical fungal species and thus, approximately 10 - 20 of the proteins described in Figure 6.1 (Section 6.5) were not native proteins from the large subunit; the difficulty was in determining which were native proteins and which were not.

M^{c}Conkey et al (1979) demonstrated that the variation in electropherograms of cytoplasmic ribosomal proteins from different species, and strains, presented by various authors was a consequence of the methods of preparation and analysis. In the absence of a standardised analysis of eukaryotic cytoplasmic ribosomal proteins,it was possible to detect 67 or 71 proteins in Saccharomyces cerevisiae (Table 6.4) but it was not possible to use a system of nomenclature for Coprinus cinereus cytoplasmic ribosomal proteins. One consequence was that the electropherograms produced for Coprinus cinereus could not be compared directly with the results from other eukaryotic species because different methods of analysis. The only direct comparison which could be made was with the proteins of Escherichia coli from which the method of analysis had been taken (Chapter 2, Section 2.11). There were no similarities between the two species either in number or relative positions which was to be expected between the dissimilar ribosomes of a eukaryotic and prokaryotic species (Russell and Wilkerson, 1980; Table 6.4). However, the fact that cytoplasmic ribosomal proteins could be resolved from a method which was used for a prokaryotic species demonstrated that the proteins were not too dissimilar, but it was possible that the resolution of Coprinus cinereus cytoplasmic ribosomal proteins could be improved.

Table 6.4. Numbers of proteins from eukaryotic cytoplasmic ribosomes.

Organism/Reference	Total for monosomes	Large subunit Basic	(Acidic)	Small subunit Basic	(Acidic)
Podospora anserina. (Bégueret et al.(1977).	79	39	(+2)	25	(+3)
Saccharomyces cerevisiae. Zinker and Warner,(1976).	67	33	(+4)	24	(+6)
Ishiguro(1976).	68-71	40-41		28-30	
Schizosaccharomyces pombe. Coddington and Fluri,(1977).	93	31 including 36 unaccounted	(+7)	19	
Rat liver. Sherton and Wool (1974)	69-71	39	(+2)	28	(+2)
Chlamydomonas reinhardii Hanson et al.(1974). cytoplasm	65	39		26	
chloroplast	48	26		22	
Escherichia coli Kaltschmidt and Wittmann	55	27	(+7)	17	(+4)

Basic proteins which migrated to the cathode at pH 8.5. Numbers of proteins which were acidic at pH8.5 are presented, where known, in brackets.

The chromatographic analysis of eukaryotic species has been undertaken on relatively few species (including Coppin-Raynal,1980 for Podospora anserina, Küntzel,1969 for Neurospora crassa). The chromatograms produced for Coprinus cinereus (Figures 6.1 and 6.2) were unlike any other published results. The detection of 50 peaks of radioactivity in the analysis of proteins from the large cytoplasmic ribosomal subunit for CY8.2 and CY9.23 was slightly higher than the 44 and 45 proteins identified by electrophoresis (Plates D and F), but the correspondence between the two methods was not known and was not examined for practical reasons.

SECTION 6.11. CYCLOHEXIMIDE RESISTANT CYTOPLASMIC RIBOSOMAL PROTEINS.

At the outset, the intention of the investigation was to place equal emphasis on the analysis of cytoplasmic ribosomal proteins from the large and small subunits, in an attempt to determine which subunit of CY8.2 and CY9.23 conferred cycloheximide resistance. Evidence from the cell-free polypeptide synthesising system did not establish which subunit conferred cycloheximide resistance (Section 5.4, Chapter 5). In practice, the emphasis of the investigation concentrated on the proteins from the large cytoplasmic ribosomal subunit, because the yield of protein from the small subunit was small in comparison to that of the large and consequently fewer analyses were possible, almost all of which resulted in poor resolutions.

a) Analysis of proteins from the small subunit.

It was concluded from the limited evidence available, that there were no detectable differences between the proteins of the small cytoplasmic ribosomal subunit of CY8 and CY8.2, except for the difference in the migration of all proteins in the second dimension, a difference which was assumed to be an experimental artifact (Section 6.2). No analysis of the small cytoplasmic ribosomal subunit by carboxymethyl-cellulose chromatography was undertaken because the yield of radioactive proteins was insufficient. Failure to detect a difference did not prove that a protein or proteins from the small cytoplasmic ribosomal subunit of CY8.2 and CY9.23 was responsible for cycloheximide resistance. The general consensus of opinion is that cycloheximide resistance is exclusively conferred by the large cytoplasmic ribosomal subunit (Table 5.1, Section 5.1, Chapter 5). There

has been only one exception, reported by Sutton et al,(1978) in Tetrahymena thermorphilia, in which the small subunit also conferred cycloheximide resistance but no altered protein has been associated with the response.

b) Analysis of proteins from the large subunit.

Many cycloheximide-resistant mutants, in a large variety of species, are known to possess cycloheximide-resistant large cytoplasmic ribosomal subunits (Table 5.1, Section 5.1, Chapter 5), but there are few reports which have correlated the resistant phenotype with an altered protein in the large subunit. (Table 6.1, Section 6.1). In the few instances in which alterations in a protein have been discovered, the difference between the mutant and wild-type protein has required very careful observation and deliberation and necessitated a consistency of replicate analyses.

In contrast to the electrophoretic analysis of proteins from the small cytoplasmic ribosomal subunit (Section 6.4), the proteins from the large subunit revealed considerable variation between strains (Section 6.6). The difficulty in the absence of replicate analyses of suitable resolution, was in differentiating between variation resulting from experimental technique (Section 6.9) and variation resulting from inherent differences either due to cycloheximide-resistance or other phenotypic differences.

In Coprinus cinereus it was not certain that the large cytoplasmic ribosomal subunit conferred cycloheximide-resistance in CY8.2 and CY9.23. The comparison of proteins from the large subunit of the two mutant strains with their cycloheximide-sensitive parental strains did not clarify the situation.

In an attempt to identify a protein or proteins which may confer cycloheximide resistance, the results of all strains, monokaryotic and dikaryotic, were taken into account in determining common differences in the electropherograms.

i) Monokaryons.

If the product of the cy-2 was a structural cytoplasmic ribosomal protein, and if, as a result of the cy-2^r mutation, there was a detectable charge and/or size difference between the wild-type and mutant protein then there would be two differences in the electrophoretic pattern of proteins from CY8.2 and CY8, and CY9.23 and CY9; the cycloheximide-sensitive strains should possess the wild-type protein and the mutant strains should not, and the mutant strains should possess the mutant form which the parental strain did not.

In the analysis it was not possible to identify the two forms of the same protein, thus the differences between cycloheximide-sensitive and cycloheximide-resistant strains were compared with the premise that the two forms of the protein or proteins were present, amidst differences which were the result of experimental variation.

Present in CY8. absent from CY8.2.
Ly, Ls, L22, L30a and L30b.
Present in CY8.2 absent from CY8.
Lx, L2, Lt, Lr, Lq, L8a, L12a, Lo, Ln and L28a
Present in CY9, absent from CY9.23.
Ln
Present in CY9.23. absent from CY9.
L14 and Li
(Taken from Section 6.6a and b).

There was no common factor between the two pairs of strains. The only protein to feature as differences between both pairs was Ln, but its presence conferred cycloheximide-resistance in CY8.2, while its absence conferred cycloheximide-resistance in CY9.23.

It was possible that the cy-2 gene product were a modifier of the cytoplasmic ribosome proteins. It is possible that more than one mutant protein may be produced, (ie. by phosphorylation or methylation, Russell and Wilkerson, 1980). This may explain the number of differences observed, but does not explain the differences between the pairs.

Although CY8.2 and CY9.23 possessed mutations in the same cy-2 complementation group, cycloheximide-resistance may have been conferred by different alleles in each strain and consequently, it was possible that two different forms of the mutant may have been present. The presence of any of 10 proteins found in CY8.2 but not CY8, may have been homogenous with any of the 3 proteins found in CY9.23 but not CY9.

It was also possible that the difference between proteins from CY9.23 and CY9, and CY8.2 and CY8 were not the same because of the effect of the modcy^{+} allele in CY9.23 but not CY8.2. The modcy^{+} allele interacts with cy-2^{r} in the dikaryons in vivo (Chapter 3, Section 3.9) and in vitro (Chapter 5, Section 5.6a), and may affect either the same or different protein or proteins as cy-2. A comparison between CY9 and CY9.23.98 or CY9.23 137, two recombinant strains of CY9.23 which lacked the modcy^{+} allele (Chapter 2, Section 2.2), was not possible because of the poor resolution of their proteins. The effect of the allele of cy-2^{r} present in CY9.23 on the protein content was

therefore not examined in the absence of the modcy^{+} allele.

It was possible that the CY8 and CY9 strains were inherently different, because although both strains originated from the same parents, they were not isogenic (Section 2.2, Chapter 2). Variability in the cytoplasmic ribosomal proteins of Saccharomyces cerevisiae is known (Adoutte-Panvier et al, 1980) and in Coprinus cinereus variability in the cytoplasmic proteins has been demonstrated (Smythe and Anderson, 1971). However, there was no consistency in the shared differences of CY8 and CY9, and CY8.2 and CY9.23, except Ln (Section 6.6a), which suggested that the most probable reason for the differences between CY8.2 and CY8 and CY9.23 and CY9 was that variation introduced during the preparation of the cytoplasmic ribosomes cytoplasmic ribosomal protein and the analysis of the proteins (Section 6.9).

The phenomenon was not clarified by an analysis of the cytoplasmic ribosomal proteins by carboxymethyl-cellulose chromatography. The proposed experiment which would have compared CY8 with CY8.2, and CY9 with CY9.23 cytoplasmic ribosomal proteins could not be undertaken and the results which were obtained for CY8.2 and CY9.23 ribosomal proteins from the large subunit (Figures 6.1 and 6.2) were not comparable for practical reasons. The direct comparison of proteins from cycloheximide-sensitive and cycloheximide-resistant strains may have revealed differences that an indirect comparison by 2D-PAGE may not (eg Harvey and Martinelli, 1983) but did not in this investigation.

It is however, possible that differences in the cytoplasmic ribosomal proteins from the large subunit were the result of ultra-

violet radiation induced or spontaneous mutations in the genes other than those associated with cycloheximide-resistance, which produced mutant phenotypes which were not sought or detected; for example, cold-sensitivity or ribosomal ambiguity mutants (Section 3.3, Chapter 3).

ii) Dikaryons.

The comparison of the electropherograms of proteins from dikaryotic strains did not clarify the interpretation of the protein differences from the monokaryotic strains. The presence of no one protein could be correlated with the partial expression of cycloheximide-resistance in the dikaryons examined.

Present in CY8 x CY13, absent in CY8.2 x CY13.
Lv
Present in CY8.2 x CY13, absent in CY8 x CY13.
Lq, L22, L30a and L30b
Present in CY9 x CY3, absent in CY9.23 x CY 3.
Lz, L2, Lw, Lq, L4, Lo, Lk, Lj and L28a
Present in CY9.23 x CY3, absent in CY9 x CY 3.
none

Taken from Section 6.7.

The only protein difference common to both pairs of dikaryons (CY8 x CY13 and CY8,2 x CY13, and CY9 x CY3 and CY9,23 x CY3) was Lq, but its presence or absence could not be correlated with the response to cycloheximide. However, Lq was present in the monokaryotic strains and was a possible candidate for further study; Ln however which had been considered a candidate based on the result of the monokarytoic strains did not differ in the dikaryotic strains. Differences in the proteins were observed in monokaryotic and dikaryotic strains, but only

in one pair, and could not be correlated with cycloheximide resistance, namely L22, L30a and L30b, L2, Lo and L28a.

The interpretation of the proteins from the dikaryons was limited by the absence of data for CY3 and CY13. A hypothesis proposed to explain the expression of a degree of resistance to cycloheximide in CY8.2 x CY13 and CY9.23 x CY3 *in vitro* was that two populations of cytoplasmic ribosome were present (Section 5.9, Chapter 5). The presence of wild-type and mutant proteins in the heterozygous cycloheximide-resistant dikaryons could not be determined from the results. It was possible that there was no total loss of the wild-type protein but a loss of quantity, however, detection of differences in relative intensity of staining was not obvious in the electropherograms produced.

SECTION 6.12. SUMMARY.

It was probable that the inconsistencies between the strains examined were the result of experimental differences in the preparation and analysis of the cytoplasmic ribosomal proteins, rather than inherent differences between the strains. In the absence of proof that the differences were not the result of experimental technique, any conclusions regarding the results must be considered speculative.

The analysis of Coprinus cinereus cytoplasmic ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis resolved between 24 - 30 proteins from the small ribosomal subunit and between 36 - 64 proteins from the large subunit. The variability in the estimated was the result of technical difficulties which restricted the analysis to a limited number of strains and two few replicates of sufficient resolution.

There was no proof that the mutations in either the cy-2 or the modcy loci produced alterations in one or more cytoplasmic ribosomal proteins which could be associated with the response to cycloheximide; there were however several proteins from the large cytoplasmic ribosomes which were tentatively proposed as candidates. There were differences in the cytoplasmic ribosomal proteins from the large subunit of dikaryotic strains but they were not consistent with differences in comparable monokaryotic strains.

The analysis by carboxymethyl-cellulose chromatography was not of sufficient consistency to either characterise Coprinus cinereus cytoplasmic ribosomal proteins or to identify proteins associated with an altered response to cycloheximide.

CHAPTER 7.

GENERAL DISCUSSION: MOLECULAR AND GENETIC BASIS OF CYCLOHEXIMIDE-RESISTANCE IN COPRINUS CINEREUS.

SECTION 7.1. CONCLUSIONS FROM THE GENETIC ANALYSIS OF CYCLOHEXIMIDE-RESISTANT MUTANTS.

As a result of the genetic analysis of cycloheximide-resistant mutants (Chapter 3) it was concluded that, CY8.2 and CY9.23 possessed a single recessive mutation which conferred cycloheximide resistance. North (1982) had identified the allele of CY9.23 as cy-2^r. The mutations of CY8.2 and of all other cycloheximide-resistant mutant strains examined, were shown to be alleles at the cy-2 locus. In addition CY9.23 possessed modcy$^+$ which modified the dominance of cy-2^r in the dikaryon, rendering it partially dominant, but had no effect in the diploid (North, 1982). None of the cycloheximide-resistant strains produced in this investigation were shown to possess a dominance modifier mutation.

The effect of cycloheximide on the growth response of cycloheximide resistant strains was used as a means of classification, based on the 50% growth inhibitory cycloheximide concentration, and of selection for biochemical analysis.

SECTION 7.2. CONCLUSIONS FROM THE BIOCHEMICAL ANALYSIS OF CYCLOHEXIMIDE-RESISTANT MUTANTS.

CY8.2 and CY9.23 were shown to possess cycloheximide-resistant cytoplasmic ribosomes, but the specific cytoplasmic ribosomal subunit which conferred cycloheximide resistance could not be identified. The same relative degree of resistance was exhibited by CY8.2 and CY9.23 *in vitro*, in comparison with their cycloheximide-sensitive strains CY8 and CY9. The cycloheximide-resistant recombinant strain CY9.23.98 which did not possess *modcy*$^{+}$ was as resistant *in vitro* as CY9.23, which did possess *modcy*$^{+}$. The dikaryons heterozygous for cycloheximide resistance, CY9.23 x CY3 and CY8.2 x CY13 exhibited similar responses to cycloheximide *in vitro*, which were described as partial resistance. Available data showed that the diploid CY9.23/CY14 was sensitive to cycloheximide (Chapter 5).

Analysis of cytoplasmic ribosomal proteins from CY8, CY8.2, CY9 and CY9.23, by two-dimensional polyacrylamide gel electrophoresis and carboxymethyl-cellulose chromatography, did not conclusively identify altered proteins which could be associated with mutations at the *cy-2* and *modcy* loci, but did identify several possible candidates.

SECTION 7.3. OBJECTIVES.

A reappraisal of the genetic analysis of cycloheximide-resistance, both in this study (Chapter 3) and by North (1982), in the light of the biochemical investigation of cycloheximide-resistant mutant strains *in vitro* (Chapter 5) and an analysis of their cytoplasmic ribosomal proteins (Chapter 6).

SECTION 7.4. COMPARISON OF THE EFFECTS OF CYCLOHEXIMIDE IN VIVO and IN VITRO.

The detailed analysis of the effect of cycloheximide on the growth (Chapter 3) and polyphenylalanine synthesis (Chapter 5), on monokaryotic and diploid strains, made it possible to classify strains according to their response to cycloheximide, and to consider the relationship between the inhibitory effect of cycloheximide *in vivo* and *in vitro*.

The similar biphasic responses to cycloheximide *in vivo* and *in vitro* were described by the same parameters. The values for the 50% inhibitory cycloheximide concentration and linear regression coefficeint for those strains examined *in vivo* and *in vitro* are presented in Table 7.1.

In the majority of monokaryotic strains and in all dikaryons examined, polyphenylalanine synthesis was more resistant to cycloheximide than was growth (Table 7.1). All of the cycloheximide-sensitive monokaryotic strains possessed cytoplasmic ribosomes which were able to synthesis polyphenylalanine at cycloheximide concentrations which were totally inhibitory to growth; in fact the cytoplasmic ribosomes were at least 6 x more resistant than the whole cells (Table 7.1). The relatively high sensitivity of CY18 cells, in comparison to the other strains, was not observed *in vitro*. possibly reflecting the effect of the abnormal ostrich morphology of CY18 cells on cycloheximide. It should also be noted that the response of CY8 cells and cytoplasmic ribosomes were approximately 10 x more resistant than any other of the cycloheximide-sensitive strains, which led to reservations concerning the classification of the strain.

Table 7.1. Comparison of the effect of cycloheximide *in vivo* and *in vitro*.

Table 7.1. Comparison of the effect of cycloheximide in vivo and in vitro.

Strain.	Genotype.	in vivo 50% inhibitory cycloheximide concentration (μM).	in vivo Linear regression coefficient.	in vitro 50% inhibitory cycloheximide concentration (μM).	in vitro Linear regression coefficient.
MONOKARYONS					
CY8	*cy*^{s}	0.1	-60	4.	-30
CY8.2	*cy-2*^{r} *modcy*$^{-}$	40	-40	600	-30
CY9	*cy*^{s}	0.08	-60	0.5	-30
CY9.23	*cy-2*^{r} *modcy*$^{+}$	100	-30	60	-40
CY9.23.98	*cy-2*^{r} *modcy*$^{-}$	100	-50	60	-80
CY3	*cy*^{s}	0.03	-100	0.4	-30
CY13	*cy*^{s}	0.01	-60	0.2	-20
CY14	*cy*^{s}	0.01	-60	0.4	-30
CY18	*cy*^{s}	0.003	-60	0.5	-30
DIKARYONS					
CY8 x CY13	*cy*^{s} x *cy*^{s}	0.01	-60	1.0	-30
CY8.2 x CY13	*cy-2*^{r}, *modcy*$^{-}$ x *cy*^{s}	0.01	-60	6.0	-20
CY9 x xCY3	*cy*^{s} x *cy*^{s}	0.01	-70	1.0	-30
CY9.23 x CY3	*cy-2*^{r}, *modcy*$^{+}$ x *cy*^{s}	0.1	-30	10.	-20

From data presented in Chapter 3 , Section 3.8 (c,d and e), 3.9 and 3.10 and from Chapter 5, Sections 5.3 (b) and 5.6 (a and b).

The cycloheximide-resistant strain CY8.2 was, like the cycloheximide-sensitive strains examined, more resistant to cycloheximide *in vitro* than *in vivo*; CY9.23 and CY9.23.98 were the only strains examined in which cytoplasmic ribosomes were more sensitive to cycloheximide than was their growth (Table 7.1).

The greater degree of resistance, 100 x or more of the dikaryotic strains *in vitro* compared with *in vivo*, was more pronounced than for most of the monokaryotic strains (Table 7.1).

A comparison of strains based on the linear regression coefficients revealed higher values *in vivo* than *in vitro* for all monokaryotic and dikaryotic strains, except for CY9.23 and CY9.23.98 (Table 7.1). Thus CY9.23 and CY9.23.98 were exceptional based on both parameters used to describe the cycloheximide dose-responses.

The relationship between the cycloheximide-sensitive strains and their cycloheximide-resistant mutant strains (ie.between CY8 and CY8.2 and CY9 and CY9.23) was more pronounced *in vivo* than it was *in vitro*. CY8.2 was almost 3 x more resistant to cycloheximide than CY8 *in vivo* compared with *in vitro*, and similarly for CY9.23, the mutant strain was 9 x more resistant than CY9 *in vivo* compared with *in vitro* (Table 7.1).

Conversley, for the dikaryotic strains, CY8.2 x CY13 was 6 x more resistant than CY8 x CY13 *in vitro* but as sensitive *in vivo* (Table 7.1). In the comparison of CY9.23 x CY3 and CY9 x CY3, the degree of resistance was the same *in vivo* and *in vitro*.

The comparison of the effect of cycloheximide on growth and polyphenylalanine synthesis was restricted to a small number of strains because of the lack of data from the in vitro studies of many strains. For practical reasons, responses to cycloheximide of cytoplasmic ribosomes from all cycloheximide resistant mutant strains (or representatives of the strains classified according to their in vivo responses), homozygous cycloheximide resistant dikaryons and diploids derived from CY8.2 and CY9.23 were not examined.

Inhibition of growth by cycloheximide did not differentiate between the primary mode of action of the drug, which was presumed to be the inhibition of the elongation phase of translation mediated by the cytoplasmic ribosome, and the secondary inhibitory effects. Other possible mechanisms of cycloheximide action, included the effect on DNA and RNA synthesis andon initiation and termination of translation. and possible mechanisms of resistance to cycloheximide including the effect of the plasma membrane and detoxification of the drug, (Section 1.3, Chapter 1).

The polyuridylic acid template for cell-free polypeptide synthesis has been used in many species to identify cycloheximide resistant cytoplasmic ribosomes (Table 5.1, Section 5.1, Chapter 5) and it was employed for that purpose in this investigation. The poly-uridylic acid dependant polyphenylalanine synthesising system fulfilled its function as a suitable assay for identifying cytoplasmic ribosomes in CY8.2 , CY9.23 and CY9.23.98. However, polyuridylic acid was not an ideal template on which to study the mechanism of action of cyclo-heximide and its inhibitory action on cytoplasmic ribosomes because it possessed noinitiation and no termination codons. The role of

cycloheximide as an inhibitor of initiation and termination is controversial (eg Siegel, 1977). A cell-free system dependant on poly (U) was only suitable for analysing the specific effect of cycloheximide on cytoplasmic ribosomes which have undergone abnormal initiation and are in the elongation phase of polyphenylalanine synthesis. The effect of cycloheximide *in vitro* therefore represents only one possible aspect of the effect of the drug, whereas the *in vivo* effect was a measure of all aspects of the inhibitory response.

The responses *in vitro* may have been different to those observed (Table 7.1) had the cytoplasmic concentration used not been 2.0 A_{260} unit. For cytoplasmic ribosomes from CY9.23.98, the lower their concentration, the more cycloheximide was required to produce 50% inhibition (Section 5.5, Chapter 5). Thus for CY9.23.98, and possibly for the other strains examined, it was possible that the cytoplasmic ribosome concentration may have been varied so that the 50% inhibitory value *in vitro* may have equalled the value *in vivo*. The physiological concentration of functional cytoplasmic ribosomes was not known, nor was proportion of active cytoplasmic ribosomes in the RP-100 fractions from the different strains.

Despite the reservations expressed concerning the use of the poly (U) assay to measure the effect of cycloheximide *in vitro*, the value of the detailed analyses of the effect of cycloheximide on growth and on polyphenylalanine synthesis was used to indicate the importance of the role of cytoplasmic ribosomes in the expression of the response to cycloheximide. In the majority of strains the absolute values obtained for the response of the cytoplasmic ribosomes were greater than those of the growth responses,possibly indicating

that other metabolic processes or components were more sensitive to cycloheximide than the elongation phase of translation mediated by the cytoplasmic ribosomes. However, the relative differences between the strains *in vivo* was found in the response of the cytoplasmic ribosomes to cycloheximide (Table 7.1) indicating the role of the cytoplasmic ribosomes. The fact that cycloheximide-resistance was conferred by the cytoplasmic ribosomes in CY8.2, CY9.23 and CY9.23.98 (Section 5.3, Chapter 5) demonstrated that the organelle was the intracellular site of cycloheximide action.

SECTION 7.5. THE INTRACELLULAR SITE OF CYCLOHEXIMIDE ACTION.

The investigation demonstrated that CY8,2 and CY9.23 possessed cytoplasmic ribosomes which conferred cycloheximide-resistance (Section 5.3b , Chapter 5). Although several cytoplasmic ribosomal proteins were proposed as candidates for conferring cycloheximide resistance (Section 6.11, Chapter 6), the analysis did not identify the cytoplasmic ribosomal protein or proteins involved in binding cycloheximide nor the structural change produced as a result of the mutation of the *cy-2* locus.

The inability to prove the identity of one or more cytoplasmic ribosomal proteins which conferred cycloheximide resistance in *Coprinus cinereus*, was similar to the results obtained in several other species, because although many cycloheximide-resistant cytoplasmic ribosomes are known (Table 5.1, Section 5.1, Chapter 5), in only four have the modified cytoplasmic ribosomal proteins been discovered, (Coddington and Fluri, 1977; Bégueret *et al*, 1977 and Crouzet and Bégueret, 1980; Stöcklein and Piepersberg, 1980).

The probable reason why so few cytoplasmic ribosomal proteins conferring cycloheximide resistance have been identified is that the structural difference between wild-type and mutant protein is small and at the limits of sensitivity of the analytical methods.

Whilst conferring resistance to cycloheximide, the mutation of the *cy-2* locus must also allow sufficient normal function for the cell to be viable. The extent of the difference between wild-type and mutant proteins depends on its importance in cytoplasmic protein synthesis. Each of the cytoplasmic ribosomal proteins is presumed to

have a specific role in the normal functioning of the organelle, although what that role is, is not known in eukaryotic species. The wild-type cytoplasmic ribosomal proteins may be considered to be of two general types, those essential and those not essential for function. Any modification in an essential cytoplasmic ribosomal protein, for example, those proteins involved in binding mRNA or tRNA, would at least be deleterious to polypeptide synthesis, but would probably be lethal. The observation that there was only a negligible difference in polyphenylalanine synthesis between CY8.2 and CY8 and between CY9.23 and CY9 (Section 5.3b, Chapter 5), suggested that the cy-2^r mutation affected a cytoplasmic ribosomal protein which was not essential. Cytoplasmic ribosomal proteins which were not considered vital for function, if they existed, might be considered to have no specific role but which were necessary to maintain a stable functional configuration could tolerate mutations without being disadvantagous to polypeptide synthesis.

Differences in charge but not size between the mutant and wild-type proteins were assumed to be the result of amino acid substitutions in Podospora anserina (Crouzet and Bégueret, 1980) and in Saccharomyces cerevisiae (Stöcklein and Piepersberg, 1981), which in the latter was demonstrated by amino acid sequencing to be the result of a substitution of glutamine for either glutamic acid or lysine. The difference observed in Schizosaccharomyces pombe by Coddington and Fluri (1977) was in size, representing the addition of approximately 20 additional amino acids.

The particular conditions of two-dimensional polyacrylamide gel electrophoresis used by Crouzet and Bégueret (1980), Stöcklein and Piepersberg (1981) and Coddington and Fluri (1977) were capable of

detecting small differences but required consistency of replicate analysis and good resolution in order to detect the relatively small differences between the mutant protein relative to the wild-type protein. It is probable that with consistency of analysis and improved resolution using two-dimensional polyacrylamide gel electrophoresis that the cytoplasmic ribosomal protein or proteins associated with cycloheximide resistance would be found in Coprinus cinereus and in other species.

Carboxymethyl-cellulose chromatography has the advantage over two-dimensional polyacrylamide gel electrophoresis because mutant and wild-type strains may be directly compared and has revealed differences which 2D-PAGE did not detect but in phenotypes other than cycloheximide resistance (eg. Harvey and Martinelli, 1983).

It is not known whether the cytoplasmic ribosomal proteins which are known to confer cycloheximide-resistance in Podospora anserina Saccharomyces cerevisiae and Schizosaccharomyces pombe are identical and are part of an equivalent cycloheximide binding site on the cytoplasmic ribosome because of the different analytical methods used and inability to relate between species (Section 6.10c,Chapter 6). Had it been possible to relate them to the cytoplasmic proteins of Coprinus cinereus it would have been possible to concentrate the analysis on particular proteins and to improve the resolution so that proteins with similar physical properties to them were well separated in order to make the observation easier.

The site of cycloheximide binding, possibly to an individual cytoplasmic ribosomal component or to a reactive domain, should have a predictable structure to accommodate the size and configuration of the

cycloheximide molecule and possess groups which are attractive to the ketone-carboxyl, the hydroxyl and the imide-nitrogen groups which give cycloheximide its high toxicity. Siegel and Sisler (1966) postulated that the reactive site on the cytoplasmic ribosome possessed a three-point attachment for cycloheximide and the observation that the effect of cycloheximide in vivo was irreversible (Section 3.7d , Chapter 3), indicated that the drug was firmly bound to the organelle. However, no theoretical model exists for the site of cycloheximide binding and no studies have been undertaken to identify the site.

SECTION 7.6. NUCLEO-CYTOPLASMIC INTERACTION AND THE EXPRESSION OF CYCLOHEXIMIDE RESISTANCE.

As a result of a mutation at the cy-2 locus, an alteration of the cytoplasmic ribosome resulted in cycloheximide resistance. Two alternatives are possible for the product of the cy-2 locus; either it was a structural component of the organelle or it was a modifying protein which indirectly affected one or more of the structural components.

The cytoplasmic ribosomal component which conferred cycloheximide resistance in Coprinus cinereus was believed to be a protein rather than a ribonucleic acid. Although no cytoplasmic ribosomal protein was proven to be associated with cycloheximide resistance in CY8.2 and CY9.23, several candidates were proposed (Section 6.11, Chapter 6). Additionally the cytoplasmic ribosomal proteins are known to confer cycloheximide-resistance (Coddington and Fluri, 1977; Bégueret et al,1977; Stöcklein and Piepersberg, 1980) and also because no rRNA gene was known in the vicinity of the cy-2 and modcy loci (Wu et al, 1983).

The synthesis of components of cycloheximide-resistant cytoplasmic ribosomes occurred at two intracellular sites; the nucleus and the cytoplasm. The transcription of the $cy\text{-}2^{r}$ allele and the assembly of the cycloheximide-resistant component into the cytoplasmic ribosome took place in the nucleus. The translation of mRNA into cytoplasmic ribosomal protein occurred in the cytoplasm. In order for functional cytoplasmic ribosomes to be synthesised, material is exchanged in both directions between the nucleus and cytoplasm across the double nuclear membrane.

The responses to cycloheximide of different cell-types *in vivo* and *in vitro* (Chapters 3 and 5) , may be explained by a hypothesis proposed to take into account the role of cytoplasmic ribosomes and the effect of nucleo-cytoplasmic interaction on cytoplasmic ribosome synthesis and function. North (1982) had discussed *in vivo* responses in terms of a nucleo-cytoplasmic interaction but had no evidence for the role of cytoplasmic ribosomes.

a) Dikaryons.

i) Heterozygous for the cy-2 locus.

In vivo, dikaryons heterozygous for the cy-2 locus, ($cy\text{-}2^{r}$)($cy\text{-}2^{s}$) were sensitive to cycloheximide (Section 3.9, Chapter 3). The hypotheses proposed to explain why the cycloheximide-resistant $cy\text{-}2^{r}$ allele was recessive to the wild-type cycloheximide-sensitive $cy\text{-}2^{s}$ allele, are based on the phenotype of the cytoplasmic ribosomes.

It was possible that the dikaryons possessed both cycloheximide-sensitive and cycloheximide-resistant cytoplasmic ribosomes either in equal proportions or with cycloheximide-sensitive predominating ,to account for the cycloheximide-sensitive phenotype. Alternatively, it was possible that the dikaryons possessed only functional cycloheximide-sensitive cytoplasmic ribosomes.

In order to explain the possibility that cycloheximide-sensitive cytoplasmic ribosomes were preferentially or exclusively synthesised it was necessary for the product of the cy-2^{s} allele to directly or indirectly inhibit the synthesis of the cycloheximide-resistant cytoplasmic ribosomal component. The inhibitive interaction may have occurred either in the nucleus preventing the transcription of the cy-2^{r} mutation or the assembly of the cycloheximide-resistant component into the cytoplasmic ribosome, or in the cytoplasm where cycloheximide-resistant cytoplasmic ribosomal proteins were translated and where the cytoplasmic ribosome was functional.

In vitro, CY8.2 x CY13 (cy-2^{r},modcy^{-} x cy-2^{s},modcy^{-}) exhibited a response to cycloheximide which was described as partial dominance (Section 5.6, Chapter 5). The result of CY8.2 x CY13 could not have resulted if only cycloheximide-sensitive cytoplasmic ribosomes were present but responses of other dikaryons with similar genotype were not examined and may have results dissimilar to those for CY8.2 x CY13. There were two possible ways in which partial resistance to cycloheximide may have arisen in CY8.2 x CY13, depending upon the product of the cy-2 locus. If the product of the cy-2 locus was a single polypeptide which was either a structural component of the cytoplasmic ribosome, or modified one or more compoents of the ribosome, the alternative phenotypes of the organelle would be cycloheximide-sensitive and cycloheximide-resistant; CY8.2 x CY13 may have possessed equal or unequal numbers of each type. Alternatively, cytoplasmic ribosomes may have exhibited a range of responses to cycloheximide. If the component of the cytoplasmic ribosome which conferred cycloheximide-resistance were oligomeric, as North (1982) suggested based on the evidence of interallelic complementation. The proportion of cycloheximide-resistant and cycloheximide-sensitive subunits would determine the phenotype of the component and thus the organelle.

With an equal proportion of cycloheximide-sensitive and cycloheximide resistant protein subunits the cytoplasmic ribosome would probably have an intermediate response to cycloheximide. The response of CY8.2 x CY13 *in vitro* was similar to the response of the cycloheximide sensitive CY13 which suggests that in the dikaryon the proportion of cycloheximide-sensitive subunits synthesised was greater than the quantity of cycloheximide-resistant subunits. Similarly if the product of the *cy-2* gene was a monomeric component of the cytoplasmic ribosome the proportion of cycloheximide-sensitive and cycloheximide-resistant cytoplasmic ribosomes would determine the phenotype of the total organelle population *in vitro* and *in vivo*.

The interpretation on the possible effect of the products of the $cy\text{-}2^{s}$ allele on the $cy\text{-}2^{r}$ allele may be the result of unequal synthesis as a result of inhibition at any one of the stages in cycloheximide-resistant cytoplasmic ribosomal synthesis in the nucleus or in cytoplasm. However, the simplest interpretation is that biosynthesis was not affected, rather that there were an equal proportion of cycloheximide resistant and cycloheximide-sensitive cytoplasmic ribosomes present and that the effect of the cycloheximide-sensitive organelle in translocation which determined the response to cycloheximide.

Assumptions concerning the proportion of the different types of cytoplasmic ribosome are made without knowing what effect cycloheximide-sensitive organelles have on polypeptide synthesis at inhibitive concentrations of the drug. It is possible that one cycloheximide-sensitive cytoplasmic ribosome on a mRNA or poly(U) template prevents that template being translated by cycloheximide-resistant ribosomes.

A cycloheximide-sensitive phenotype would therefore be produced when sufficient cycloheximide-sensitive cytoplasmic ribosomes block translation on most mRNA or poly (U) templates so that cycloheximide-resistant cytoplasmic ribosomes were unable to synthesise sufficient polypeptide to sustain growth. If the proportion of cycloheximide-resistant cytoplasmic ribosomes is greater than that of the cycloheximide-sensitive cytoplasmic ribosomes, the probability of the translation inhibition would be reduced.

Cytoplasmic ribosomes from CY8.2 x CY13 were partially resistant *in vitro* but the growth of the dikaryon was sensitive. One possibility was that poly (U) was not limiting *in vitro*. and a detectable quantity of polyphenylalanine was synthesised by cycloheximide-resistant cytoplasmic ribosomes whose activity was not inhibited by cycloheximide sensitive organelles. *In vivo*, mRNA may have been limited and there was insufficient protein synthesised to sustain growth. Alternatively, the difference in the response to cycloheximide *in vivo* and *in vitro* may have depended on the quality of the polypeptide synthesised. A polypeptide inhibited before it has been completed *in vivo* is of no benefit to the cell and therefore resulted in no growth. However, *in vitro* all polypeptide, irrespective of size, was measured by TCA precipitation.

ii) *Heterozygous for* cy-2 *and* modcy *loci*.

The cytoplasmic ribosomes from CY9.23 x CY3 (*cy-2*^{r}, *modcy*$^{+}$ x *cy-2*^{s}, *modcy*$^{-}$) exhibited partial resistance to cycloheximide *in vitro* (Section 5.6, Chapter 5). Unlike the response of CY8.2 x CY13 which was homozygous for *modcy*$^{-}$, CY9.23 x CY3 also exhibited partial resistance to cycloheximide *in vivo* (Section 3.9, Chapter 3). Dikaryons, in which *cy-2*^{r} and *modcy*$^{+}$ alleles were organised in different nuclei, were also

A cycloheximide-sensitive phenotype would therefore be produced when sufficient cycloheximide-sensitive cytoplasmic ribosomes block translation on most mRNA or poly (U) templates so that cycloheximide-resistant cytoplasmic ribosomes were unable to synthesise sufficient polypeptide to sustain growth. If the proportion of cycloheximide-resistant cytoplasmic ribosomes is greater than that of the cycloheximide-sensitive cytoplasmic ribosomes, the probability of the translation inhibition would be reduced.

Cytoplasmic ribosomes from CY8.2 x CY13 were partially resistant *in vitro* but the growth of the dikaryon was sensitive. One possibility was that poly (U) was not limiting *in vitro*, and a detectable quantity of polyphenylalanine was synthesised by cycloheximide-resistant cytoplasmic ribosomes whose activity was not inhibited by cycloheximide sensitive organelles. *In vivo*, mRNA may have been limited and there was insufficient protein synthesised to sustain growth. Alternatively, the difference in the response to cycloheximide *in vivo* and *in vitro* may have depended on the quality of the polypeptide synthesised. A polypeptide inhibited before it has been completed *in vivo* is of no benefit to the cell and therefore resulted in no growth. However, *in vitro* all polypeptide, irrespective of size, was measured by TCA precipitation.

ii) *Heterozygous for* cy-2 *and* modcy *loci*.

The cytoplasmic ribosomes from CY9.23 x CY3 (*cy-2*^{r}, *modcy*$^{+}$ x *cy-2*^{s}, *modcy*$^{-}$) exhibited partial resistance to cycloheximide *in vitro* (Section 5.6, Chapter 5). Unlike the response of CY8.2 x CY13 which was homozygous for *modcy*$^{-}$, CY9.23 x CY3 also exhibited partial resistance to cycloheximide *in vivo* (Section 3.9, Chapter 3). Dikaryons, in which *cy-2*^{r} and *modcy*$^{+}$ alleles were organised in different nuclei, were also

partially resistant *in vivo* (CY9.23.38 × CY9.23.137; *cy-2*^{r} *modcy*$^{-}$ *cy-2*^{s}, *modcy*$^{+}$, North, 1982).

There were two possibilities for the effect of the *modcy*$^{+}$ allele: either it stimulated the production of cycloheximide-resistant cytoplasmic ribosomes or it inhibited the production of cycloheximide-sensitive cytoplasmic ribosomes. The net result, which was believed to account for the partial expression of cycloheximide-resistance of the cell and *in vitro*, was that there was an increased proportion of cycloheximide resistant cytoplasmic ribosomes.

The effect on *cy-2*^{r} in either the *cis* or *trans* configuration demonstrated that the effect of *modcy*$^{+}$ was not restricted to the nucleus, but it was not known whether the product of the *modcy*$^{+}$ allele affected the biosynthesis of the cytoplasmic ribosomes in the nucleus or in the cytoplasm or if its effect was on the functional organelle.

A physical change to the cytoplasmic ribosome as a result of the *modcy*$^{+}$ allele was not detected. It was possible that the *modcy*$^{+}$ allele affected the same component or components of the cytoplasmic ribosomes as the *cy-2*^{r} allele, either directly or indirectly. Alternatively, it may have affected the metabolic processes which enabled a greater proportion of cycloheximide-resistant cytoplasmic ribosomes to translate , perhaps the permeability of the nuclear membrane was affected resulting in increased efficiency of biosynthesis of cycloheximide-resistant organelles. In the haploid cells of CY9.23 there was no increase in resistance to cycloheximide, in comparison with CY9.23.98 (*cy-2*^{r}, *modcy*$^{-}$) (Table 7.1, Section 7.4) which demonstrated that the effect of the *modcy*$^{+}$ was only applicable to the circumstances

partially resistant *in vivo* (CY9.23.38 × CY9.23.137; *cy-2*^{r} *modcy*$^{-}$ *cy-2*^{s}, *modcy*$^{+}$, North, 1982).

There were two possibilities for the effect of the *modcy*$^{+}$ allele: either it stimulated the production of cycloheximide-resistant cytoplasmic ribosomes or it inhibited the production of cycloheximide-sensitive cytoplasmic ribosomes. The net result, which was believed to account for the partial expression of cycloheximide-resistance of the cell and *in vitro*, was that there was an increased proportion of cycloheximide resistant cytoplasmic ribosomes.

The effect on *cy-2*^{r} in either the *cis* or *trans* configuration demonstrated that the effect of *modcy*$^{+}$ was not restricted to the nucleus, but it was not known whether the product of the *modcy*$^{+}$ allele affected the biosynthesis of the cytoplasmic ribosomes in the nucleus or in the cytoplasm or if its effect was on the functional organelle.

A physical change to the cytoplasmic ribosome as a result of the *modcy*$^{+}$ allele was not detected. It was possible that the *modcy*$^{+}$ allele affected the same component or components of the cytoplasmic ribosomes as the *cy-2*^{r} allele, either directly or indirectly. Alternatively, it may have affected the metabolic processes which enabled a greater proportion of cycloheximide-resistant cytoplasmic ribosomes to translate, perhaps the permeability of the nuclear membrane was affected resulting in increased efficiency of biosynthesis of cycloheximide-resistant organelles. In the haploid cells of CY9.23 there was no increase in resistance to cycloheximide, in comparison with CY9.23.98 (*cy-2*^{r}, *modcy*$^{-}$) (Table 7.1, Section 7.4) which demonstrated that the effect of the *modcy*$^{+}$ was only applicable to the circumstances

found in the dikaryons, ie. the competition between the cycloheximide-resistant and cycloheximide-sensitive cytoplasmic ribosomes.

b) Diploids.

i) Heterozygous for the cy-2 locus.

In vivo, diploids heterozygous for cy-2^r were sensitive to cycloheximide (eg CY8.2/CY18; cy-2^r modcy$^-$/cy-2^s modcy$^-$), (Section 3.10, Chapter 3 ; CY9/CY9.23.20, North 1982). No data was obtained for the response to cycloheximide of cytoplasmic ribosomes from CY8.2/CY18.

The dominance of the cy-2^s allele in the diploid strain may be the consequence of some population of cytoplasmic ribosomes discussed for CY8.2 x CY13 (Section 7.6 a(i)); namely equal or greater numbers of cycloheximide-sensitive ribosomes relative to cycloheximide-resistant organelles or cytoplasmic ribosomes with a range of responses to cycloheximide. In the diploid, in contrast to the dikaryon, it is more probable that any interaction between cy-2^s and cy-2^r alleles occurred within the confines of the nuclear membrane, at the level of transcription of the mutant allele or the assembly of cycloheximide resistant cytoplasmic ribosomes. In vivo, CY8.2 / CY18 was more sensitive to cycloheximide than CY8.2 x CY13 which may be a consequence of interaction with the nucleus rather than between nuclei.

ii) Heterozygous for cy-2^r and modcy$^+$ alleles.

In vivo and in vitro CY9.23/CY14 (cy-2^r modcy$^+$/cy-2^s modcy$^-$) was

sensitive to cycloheximide (Section 3.10, Chapter 3, Section 5.9, Chapter 5). However, the dikaryotic strain CY9.23 x CY3 which possessed the same alleles as CY9.23/CY14 but divided into two haploid nuclei rather than one diploid nucleus, exhibited a low level of resistance to cycloheximide *in vivo* and *in vitro*. It was concluded, *in vivo* (North, 1982; Chapter 3) and *in vitro* (Chapter 5), that the difference in the expression of cycloheximide resistance was the result of the packaging of the genetic information and its effect on the cytoplasmic ribosomes possessed by each cell-type.

The inability of the diploid to exhibit cycloheximide resistance was because either, the *cy-2*^{r} and *cy-2*^{s} alleles or the *modcy*$^{+}$ and *modcy*$^{-}$ alleles, were together in the same nucleus. The second possibility could be discounted because a diploid homozygous for *modcy*$^{+}$, CY9.23.52/CY9.23.102 (*cy-2*^{r} *modcy*$^{+}$/ *cy-2*^{s} *modcy*$^{+}$; North, 1982) was sensitive to cycloheximide.

The hypothesis proposed to explain the response of CY9.23/CY14, is that the product of the *cy-2*^{s} inhibits the synthesis of cycloheximide-resistant cytoplasmic ribosomes. In the dikaryon, the product of the *modcy*$^{+}$ allele may affect the inhibitory action of the *cy-2*^{s} allele or protect or stimulate the synthesis of cycloheximide-resistant cytoplasmic ribosome because the wild-type and mutant alleles of the *cy-2* locus are separated into two nuclei. The same response whether *modcy*$^{+}$ is in the same or a different nucleus to the *cy-2*^{r} allele may be explained if the effect of *modcy*$^{+}$ on the synthesis or function of cycloheximide-resistant cytoplasmic ribosomes occurs in the cytoplasm whereas the effect of *cy-2*^{s} on *cy-2*^{r} occurs in the nucleus. Thus, in the diploid *cy-2*^{s} affects the *cy-2*^{r} product before the product of the *modcy*$^{+}$ allele exerts its influence.

The recessiveness of cy-2^{r} and the effect of modcy^{+} in the dikaryon but not the diploid, has been discussed in terms of the interaction between nuclei on the biosynthesis and function of cytoplasmic ribosomes. The speculation has been based on results of only a few strains and future investigations would be necessary to substantiate the *in vitro* results in this investigation and to test the hypothesis proposed, particularly by the analysis of dikaryons and diploids with various combinations of alleles associated with cycloheximide resistance and by determining what type of cytoplasmic ribosomes are present in each strain.

SECTION 7.7. FUTURE DEVELOPMENT OF THE INVESTIGATION.

The aim of this investigation was to identify cytoplasmic ribosomal genes in Coprinus cinereus. The approach employed was to study strains which possessed cycloheximide-resistant cytoplasmic ribosomes. The investigation could have developed into a more thorough investigation of the cy-2^{r} and modcy^{+} alleles and the effect of nuclear interaction on their expression. Additionally, the mechanisms by which other genes, cy-1, cy-3 and possibly undiscovered genes, conferred cycloheximide-resistance were possibilities for further analysis.

Alternatively, it may have been possible to identify cytoplasmic ribosomal mutants by studying their types of mutants; resistance to other antibiotics (eg, trichodermin, Fried and Warner, 1981), temperature-sensitivity and abnormal cytoplasmic ribosomal assembly (Waldron and Roberts, 1974) and translational ambiguity mutants (Coppin-Raynal, 1977). The methodology would have been similar to that used in this investigation; mutatagenesis, selection, genetic analysis and biochemical characterisation. Employing recombinant DNA technology similar to that used by Fried et al (1981) in Saccharomyces cerevisiae it may have been possible to identify directly cytoplasmic ribosomal genes.

Specific improvements which could be made to the experimental techniques employed in this investigation were;

Chapter 3.

To use alternative mutagens to ultraviolet radiation and different selection conditions to produce cycloheximide-resistant mutants, or to induce cycloheximide-sensitive revertants, in an attempt to identify mutations other than those found in the cy-2 complementation group.

To produce cycloheximide-resistant mutants with the pleiotrophic characters known in other eukaryotes (Section 3.3, Chapter 3) to be associated with the cytoplasmic ribosomes, thereby increasing the probability of identifying mutant cytoplasmic ribosomes.

Chapter 4.

To complete the optimisation of all assay constituents (eg. the effect of pH), investigation the use of alternative constituents in order to maximise polypeptide synthesis and minimise costs (eg using Hepes buffer, replacing chloride ions by acetate ions) minimising the presence of inhibitors (eg using ribonuclease and protease inhibitors and removal of endogenous constituents which may be inhibitory, eg. L-C^{12} phenylalanine).

To develop a mRNA dependant polypeptide synthesisng system in order to study the mode of action of cycloheximide, rather than using a system translating an artifical poly (U) template.

To determine the proportion of functional cytoplasmic ribosomes in the RP-100 fraction from different strains and different preparations in an attempt to improve efficiency of translation.

To determine the size of the polyphenylalanine product synthesised and the size of the poly (U) template.

Chapter 5.

To analyse more cycloheximide-resistant mutant strains, particularly those strains identified as representing the groups of growth responses (Chapter 3, Section 3.13c) and strains possessing mutations at the cy-1 and cy-3 loci.

To obtain functional cytoplasmic ribosomal subunits so that hybrid reassociation experiments are possible in order to determine which cytoplasmic ribosome confers cycloheximide-resistance.

To analyse dikaryotic and, particularly, diploidic strains, and to examine the response of various proportions of cycloheximide-sensitive and cycloheximide-resistant cytoplasmic ribosomes in order to test the hypothesis that mixed populations of cytoplasmic ribosomes may exist in heterozygous cycloheximide-resistant strains to account for the observed intermediate responses to cycloheximide (Section 5.6).

To analyse the response of varying the RP-100 concentration in strains to determine if the results obtained with CY9.23.98 (Section 5.5) are found in other strains.

Chapter 6.

To produce consistent results for replicate analysis by 2D-PAGE and CMC-chromatography, thereby identifying and minimising contamination and the presence of artifacts and allowing the study of inherent differences between the strains.

To improve the resolution of the electropherograms and chromatograms adapting the techniques to suit the specific requirements of Coprinus cinereus, so that each stained spot and peak of radioactivity is a homogenous protein, distinguished from its neighbours, possibly to analyse separately specific groups of proteins in condition which maximise their resolution making it easier to resolve differences in wild-type and mutant forms, in order to discover if cytoplasmic ribosomal protein(s) confer cycloheximide-resistance.

To identify and correlate the cytoplasmic proteins of the electropherograms with the results of the CMC analysis, so that both techniques may be used together to analyse Coprinus cinereus.

To study cytoplasmic ribosomes from other strains, particularly from CY9.23.98 (cy-2^{r}, modcy^{-}) so that the effect of modcy^{+} found in CY9.23 may be determined and in other strains which possess mutations in the cy-2 complementation group to determine if there are allelic differences in the mutant proteins.

To analyse cytoplasmic ribosomal proteins from homozygous cycloheximide-resistant dikaryons and from diploids produced from CY8.2 and CY9.23 in order to understand the nature of nuclear interaction on gene expression.

APPENDIX.

SECTION A EFFECT OF CYCLOHEXIMIDE IN VIVO.

i) Determination of the growth response to cycloheximide by indirect comparison: Specimen calculation.

A specimen calculation of the type applied to treatments exhibiting slow growth, is taken from the growth of the monokaryon CY6.2 (Table A i). After 3 and 4 days incubation, the respective growth at 36 µM and 142 µM cycloheximide averaged 60% and 20% respectively, relative to the 0µM cycloheximide control. At 4 days incubation, growth at 285 µM was measurable but liable to a relatively high experimental error and at 427 µM there was no observed growth. After 5 days, the colony on the control treatment filled the petri-dish and growth on other treatments could not be directly related to it. If the growth on 36 µM was assumed to be 60%, as it had been at 3 and 4 days incubation, the relative growth calculated at 142 and 285 µM cycloheximide were similar to those observed at 3 or 4 days. When growth was observed at 427 µM cycloheximide at 6 or 10 days, it was calculated to be 9% relative to the control, if it was related to the growth at 142 µM cycloheximide which was assumed to be 22%. The results demonstrate that the growth relationships between the different treatments was constant over the period of incubation.

Notes to tables A1 to A10.

Less than 5 mm colony diameter after 6 days was recorded as zero growth. Δ indicates growth measured at 10 days.* indicates the minimum cycloheximide concentration used in determining the linear regression analysis over the inhibitory range of cycloheximide concentrations.

Table A 1. Determination of the effect of cycloheximide on the growth response of CY 6.2.

Table A 1. Determination of the effect of cycloheximide on the growth response of CY 6.2.

Period of incubation (days)	Cycloheximide concentration (μM). 0		36		142		285		427	
	Diameter (mm)	% growth	Diameter (mm)	% growth	Diameter (mm)	% growth	Diameter (mm)	% growth	Diameter (mm)	% growth
3	42	100$^{\Delta}$	25	60$^{\Delta}$	10	24$^{\Delta}$	-	-	-	-
4	58	100$^{\Delta}$	35	60$^{\Delta}$	12	21$^{\Delta}$	8	14$^{\Delta}$	-	-
5	+		45	60$^{\blacktriangle}$	15	20$^{\blacktriangle}$	10	13$^{\blacktriangle}$	-	-
6	+		+		18	22$^{\blacktriangle}$	12	15$^{\blacktriangle}$	7	9$^{\blacktriangle}$
10	+		+		63	22$^{\blacktriangle}$	37	13$^{\blacktriangle}$	25	9$^{\blacktriangle}$
% growth over 10 days	100		60		22		14		9	

Abbreviations: - = colony diameter less than 5 mm, not recorded, + = diameter in excess of 90 mm, not recorded. Δ = % growth related directly to growth on 0μM cycloheximide, $\blacktriangle$ = % growth related to an intermediate cycloheximide concentration.

Data abridged from Appendix Table A3.

Table A1. Effect of cycloheximide on the growth of strains derived from CY3.

Cycloheximide concentration (μM)	Cycloheximide concentration (log 10 μM)	Growth as % of 0 μM cycloheximide treatment. CY3.2	CY3.3	CY3.5	CY3.6	CY3.7	CY3.8	CY3.9	CY3.16	CY3.17
0		100	100	100	100	100	100	100	100	100
0.36	-0.45	100	100	100	89*	100	86*	83*	100	96*
0.89	-0.05	96*	100	85*	82	97*	88	72	100	89
1.8	0.26	88	76*	40	51	85	69	69	100	87
2.7	0.43	62	50	31	43	76	65	49	100	78
3.6	0.56	52	40	23	30	49	56	42	83*	56
8.9	0.95	13	11	0	0	21	19	12	58	28
18	1.26	10	0			0	0	12	23	0
27	1.43	0						0	13	
36	1.55								9$^{\Delta}$	
53	1.73								0	
Linear regression coefficient		-70.1	-73.2	-81.5	-66.8	-79.6	-54.9	-68.6	-66.8	-58.8
Linear correlation coefficient		- 0.98	-0.97	-0.96	-0.98	-0.98	-0.95	-0.97	-0.92	-0.93
Calculated 50% growth inhibitory cycloheximide concentration (μM)		4.3	3.1	1.8	1.9	4.2	3.2	2.5	8.1	4.1

Table A2. **Effect of cycloheximide on the growth of strains derived from CY6.**

Cycloheximide concentration (μM)	(log 10 μM)	Growth as % of 0 μM cycloheximide treatment. Strains CY6.1	CY6.2	CY6.3	CY6.5	CY6.6	CY6.9	CY6.11
0		100	100	100	100	100	100	100
0.36	-0.45	100	100	100	95*	93*	93*	95*
0.89	-0.05	94	100	91*	90	91	88	95
1.8	0.26	100	93*	73	88	81	88	85
2.7	0.43	100	93	54	68	63	76	73
3.6	0.55	90*	96	53	44	39	56	59
8.9	0.94	81	85	17	15	14	14	14
18	1.26	69	74	7$^{\Delta}$	7$^{\Delta}$	7$^{\Delta}$	7$^{\Delta}$	7$^{\Delta}$
27	1.43	64	65	0	0	0	0	0
36	1.56	55	60					
53	1.72	40	39					
71	1.85	29	34					
110	2.04	24	29					
140	2.15	22	22					
210	2.32	23	18					
290	2.46	18	14					
360	2.56	16	14					
430	2.63	12	9$^{\Delta}$					
570	2.76	8$^{\Delta}$	6$^{\Delta}$					
710	2.85	7$^{\Delta}$	6$^{\Delta}$					
Linear regression coefficient		-39.7	-39.1	-63.5	-58.6	-56.3	-61.0	-61.0
Linear correlation coefficient		- 0.98	- 0.99	- 0.99	- 0.95	- 0.98	- 0.92	- 0.95
Calculated 50% growth inhibitory cycloheximide concentration (μM)		40.9	39.3	3.7	3.5	3.0	3.6	3.4

Table A3. The effect of cycloheximide on the growth of strains derived from CY8.

Cycloheximide concentration.		Growth as a % of 0μM cycloheximide treatment Strains																
(μM)	(log_{10}μM)	CY8.2	CY8.4	CY8.5	CY8.6	CY8.7	CY8.8	CY8.9	CY8.10	CY8.12	CY8.13	CY8.18	CY8.19	CY8.20	CY8.22	CY8.23	CY8.23	CY8.40
0		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.36	-0.45	100	94*	93*	100	96*	96*	92*	100	94*	83*	92*	100	93*	87*	100	97*	100
0.89	-0.05	100	90	83	100	93	85	92	92*	76	88	84	96*	87	87	100	93	89*
1.8	0.26	100	84	79	90*	78	77	77	73	61	71	55	84	77	81	75*	80	67
2.7	0.43	98*	74	66	70	67	65	77	69	44	75	52	73	77	71	69	77	67
3.6	0.56	98	52	43	41	59	42	52	57	35	59	48	61	52	60	61	33	57
8.9	0.94	89	18	22	18	10	21	13	13	14	16	18	24	10	27	14	18	18
18	1.26	70	0	0	0	0	0	0	0	0	0	0	0	0	12	0	7	0
36	1.56	62													0		0	
53	1.72	49																
71	1.85	38																
110	2.04	26																
140	2.15	25																
210	2.32	16																
290	2.46	13																
360	2.56	10																
430	2.63	10																
570	2.76	8																
710	2.85	6																
Linear regression coefficient.		-39.7	-59.5	-62.0	-87.7	-68.7	-63.8	-64.0	-74.9	-55.0	-54.3	-52.9	-69.5	-58.8	-52.1	-78.2	-57.5	-61.0
Linear correlation coefficient.		-0.98	-0.96	-0.95	-0.97	-0.95	-0.95	-0.91	-0.93	-0.99	-0.91	-0.96	-0.93	-0.94	-0.93	-0.91	-0.94	-0.95
Calculated 50% growth inhibitory cycloheximide concentration (μM).		36.9	3.2	2.5	4.2	2.7	2.5	2.8	3.0	2.0	3.4	2.3	3.0	3.1	3.9	3.2	3.2	3.1

Table A3. The effect of cycloheximide on the growth of strains derived from CY8.

Cycloheximide concentration. (μM)	(log_{10}μM)	Growth as a % of 0μM cycloheximide treatment. Strains																
		CY8.2	CY8.4	CY8.5	CY8.6	CY8.7	CY8.8	CY8.9	CY8.10	CY8.12	CY8.13	CY8.18	CY8.19	CY8.20	CY8.22	CY8.23	CY8.23	CY8.40
0		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.36	-0.45	100	94*	93*	100	96*	96*	92*	100	94*	83*	92*	100	93*	87*	100	97*	100
0.89	-0.05	100	90	83	100	93	85	92	92*	76	88	84	96*	87	87	100	93	89*
1.8	0.26	100	84	79	90*	78	77	77	73	61	71	55	84	77	81	75*	80	67
2.7	0.43	98*	74	66	70	67	65	77	69	44	75	52	73	77	71	69	77	67
3.6	0.56	98	52	43	41	59	42	52	57	35	59	48	61	52	60	61	33	57
8.9	0.94	89	18	22	18	10	21	13	13	14	16	18	24	10	27	14	18	18
18	1.26	70	0	0	0	0	0	0	0	0	0	0	0	0	12	0	7	0
36	1.56	62													0		0	
53	1.72	49																
71	1.85	38																
110	2.04	26																
140	2.15	25																
210	2.32	16																
290	2.46	13																
360	2.56	10																
430	2.63	10																
570	2.76	8																
710	2.85	6																
Linear regression coefficient.		-39.7	-59.5	-62.0	-87.7	-68.7	-63.8	-64.0	-74.9	-55.0	-54.3	-52.9	-69.5	-58.8	-52.1	-78.2	-57.5	-61.0
Linear correlation coefficient.		-0.98	-0.96	-0.95	-0.97	-0.95	-0.95	-0.91	-0.93	-0.99	-0.91	-0.96	-0.93	-0.94	-0.93	-0.91	-0.94	-0.95
Calculated 50% growth inhibitory cycloheximide concentration (μM).		36.9	3.2	2.5	4.2	2.7	2.5	2.8	3.0	2.0	3.4	2.3	3.0	3.1	3.9	3.2	3.2	3.1

Table A4. The effect of cycloheximide on the growth of strains derived from CY9.

Cycloheximide concentration (μM)	($\log_{10}$μM)	Growth as a % of 0 μM cycloheximide — Strain															
		CY9.16	CY9.29	CY9.30	CY9.31	CY9.35	CY9.37	CY9.48	CY9.61	CY9.86	CY9.87	CY9.88	CY9.89	CY9.101	CY9.103	CY9.105
0		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.36	-0.44	100	78*	89	100	96*	93*	96*	96*	100	93*	100	83*	97*	90*	96*
0.89	-0.05	93*	67	89	100	92	90	96	96	93*	90	93*	63	83	97	96
1.8	0.26	86	48	84	86*	76	83	81	86	86	59	75	53	76	79	85
2.7	0.43	79	33	79	79	50	83	73	75	86	55	71	40	55	76	78
3.6	0.56	68	20	49	47	49	69	65	61	69	22	54	14	18	62	67
8.9	0.95	27	0	0	0	13	38	17	23	31	0	17	0	1[illegible]	14	34
18	1.26	15				0	10	9	23	13		14		0	14	14
27	1.43	9					0	9	11	0		0			0	11
36	1.56	0						0	0							8
53	1.72															0
Linear regression coefficient		-64.0	-59.3	-82.5	-30.6	-62.9	-54.5	-54.2	-53.8	-69.5	-72.0	-65.2	-61.9	-63.0	-56.7	-52.4
Linear correlation coefficient		-0.98	-0.97	-0.34	-0.98	-0.97	-0.94	-0.97	-0.97	-0.98	-0.95	-0.98	-0.97	-0.95	-0.94	-0.98
Calculated 50% grth inhib cyclohex conc.(μM)		5.7	1.4	3.4	3.7	3.0	5.0	4.0	5.1	5.6	2.1	4.3	1.4	2.6	4.2	5.6

(μM)	(logμM)	CY9.64	CY9.66	CY9.69	CY9.70	CY9.71	CY9.73	CY9.75	CY9.77	CY9.85	CY9116	CY9117	CY9118	CY9127	CY9.129	CY9.150	CY9.151
0		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.36	-0.44	100	86*	100	96*	93*	93*	92*	96	100	93*	97*	96*	100	91*	97*	97*
0.89	-0.05	100	86	100	93	89	93	92	100	90*	93	93	93	92*	91	90	83
1.8	0.26	85*	83	78*	85	75	83	80	96*	72	82	83	89	92	79	86	83
2.7	0.43	81	70	78	81	71	83	72	84	72	75	80	79	88	73	79	80
3.6	0.56	70	69	61	70	61	70	76	68	64	57	63	68	76	76	69	60
8.9	0.94	24	18	39	28	20	28	26	29	24	30	20	36	29	39	34	21
18	1.26	22	14	15	11	13	18	0	11	11	11	8	15	12	27	14	14
27	1.43	12	0	0	0	11	11		9	11	11	0	9	0	0	8	10
36	1.56	0				0	0		10	0	10		0			0	0
53	1.72								0		0						
Linear regression coefficient		-67.3	-52.6	-68.8	-57.5	-52.0	-53.2	-58.8	-67.0	-58.7	-50.2	-59.4	-54.7	-72.0	-48.9	-54.6	-53.4
Linear correlation coefficient		-0.98	-0.93	-0.99	-0.95	-0.97	-0.96	-0.91	-0.97	-0.98	-0.97	-0.96	-0.97	-0.96	-0.94	-0.97	-0.96
Cal.50% grth.inhib. cyclohex.conc. (μM)		6.3	4.1	5.5	4.9	4.2	5.3	4.1	7.0	4.8	4.9	4.4	5.4	5.9	5.5	5.2	4.5

Table A5. The effect of cycloheximide on the growth of CY9.23 and recombinant strains produced by North(1982).

Cycloheximide concentration		Growth as a % of 0μM cycloheximide treatment Strains			
(μM)	(log 10μM)	CY 9.23	CY 9.23.98	CY 9.23.137	CY 9.23.138
0		100	100	100	100
0.36	-0.44	100	100	100	100
1.8	0.26	91*	100	100	98*
3.6	0.56	88	90	100	94
8.9	0.95	85	100	95*	89
18	1.26	88	100	92	91
27	1.43	84	90*	82	89
36	1.56	83	85	57	60
71	1.85	52	39	34	37
110.	2.04	48	34	15	23
140.	2.15	43	32	11	18
210.	2.32	40	35	□	□
280	2.46	36	35		
360.	2.56	36	22		
430.	2.63	31	22		
570.	2.76	23	13		
710.	2.85	16	9		
Linear regression coefficient		-29.4	-51.1	-80.1	-39.5
Linear correlation coeffient		- 0.94	- 0.92	- 0.97	- 0.86
Calculated 50% growth inhibitory cycloheximide concentration (μM)		102.1	95.8	46.6	53.6

□=response not examined at this or higher concentrations.

Table A6. The effect of cycloheximide on the growth of parental and test strains.

Cycloheximide concentration		Growth as a % of 0μM cycloheximide treatment Strains						
(μM)	(log 10μM)	CY3	CY6	CY8	CY9	CY13	CY14	CY18
0		100	100	100	100	100	100	100
0.002	-2.70	100	100		96*	97*	92*	63*
0.004	-2.40	100	98		93	92	75	38
0.009	-2.05	96*	100		83	62	42	15
0.018	-1.74	78	90*		71	27	36	7Δ
0.027	-1.57	42	86	100	67	20	17	0
0.036	-1.44	34	71	78*	67	13	8Δ	
0.089	-1.05	0	66	55	54	12	0	
0.18	-0.74		48	16	28	0		
0.36	-0.44		41	13	19			
0.89	-0.05		29	0	16			
1.8	0.26		24		13			
2.7	0.43		12		9Δ			
3.6	0.56		0		0			
Linear regression coefficient		-101.0	-35.7	-58.1	-31.0	-55.0	-59.2	-54.6
Linear correlation coefficient		- 0.98	- 0.99	- 0.95	- 0.99	- 0.95	- 0.98	- 0.98
Calculated 50% inhibitory cycloheximide concentration (μM.)		0.03	0.21	0.09	0.08	0.012	0.009	0.003

Table A7. Effect of cycloheximide on various other strains.

Cycloheximide concentration		Growth as a % of 0μM. cycloheximide treatment. Strain.						
(μM)	(log 10 μM)	H_1	H_2	H_5	H_9	TC4	SR54	WMR66A
0		100	100	100	100	100	100	100
0.002	-2.74						96*	100
0.004	-2.44						98	100
0.009	-2.05						90	88*
0.018	-1.74						82	82
0.027	-1.57						74	85
0.036	-1.44						72	83
0.089	-1.05						16	40
0.18	-0.74	100	100	100	100	100	0	27
0.36	-0.44	91*	87*	90*	86*	92*		12
0.89	-0.05	89	78	88	70	90		0
1.8	0.26	69	53	45	59	84		
2.7	0.43	47	51	47	53	73		
3.6	0.56	32	42	37	50	49		
8.9	0.95	0	20	7	15	0		
18.	1.26		0	0	0			
Linear regression coefficient		-69.5	-52.4	-59.2	-51.3	-63.5	-51.0	-51.5
Linear correlation coefficient		- 0.95	- 0.99	- 0.97	- 0.98	- 0.87	- 0.90	- 0.97
Calculated 50% growth inhibitory cycloheximide concentration(μM)		2.2	2.3	2.2	2.4	3.3	0.04	0.0

Table A8. The effect of cycloheximide on the growth of dikaryons.

Cycloheximide concentration (μM)	(log 10μM)	Growth as a % of 0μM cycloheximide control: Strain CY3 x CY9	CY3.2 x CY9	CY3.3 x CY9	CY3.5 x CY9	CY3.7 x CY9	CY3.8 x CY9	CY3.16 x CY9	CY9.23 x CY3
0		100	100	100	100	100	100	100	100
0.002	-2.70	96*	100	100	100	100	100	100	93*
0.004	-2.44	85	100	100	100	89*	100	100	94
0.009	-2.05	75	83*	100	100	79	100	100	88
0.018	-1.74	40	21	89*	84*	29	66*	89*	77
0.027	-1.57	21	19	87	66	25	62	89	80
0.036	-1.44	12	8	45	55	10	54	50	75
0.089	-1.05	0	0	0	29	10	36	14	68
0.18	-0.74				12	0	33	8	51
0.27	-0.57				11		0	10	30
0.36	-0.44				10			8	-
0.89	-0.05				0			0	-
1.8	0.25								0
Linear regression coefficient		-65.4	-77.5	-137.6	-49.8	-57.4	-49.0	-56.3	-32.1
Linear correlation coefficient		- 0.98	- 0.87	- 0.96	- 0.96	- 0.92	- 0.95	- 0.90	- 0.95
Calculated 50% growth inhibitory cycloheximide concentration (μM)		0.013	0.013	0.039	0.052	0.014	0.044	0.059	0.120

- = value not determined

Table A9. The effect of cycloheximide on the growth of dikaryons.

Cycloheximide concentration (μM)	(log_{10} μM)	Growth as a % of 0μM cycloheximide treatment. Strain CY8 x CY13	CY8.2 x CY13	CY8.4 x CY13	CY8.6 x CY13	CY8.7 x CY13	CY8.9 x CY13	CY8.10 x CY13
0		100	100	100	100	100	100	100
0.002	-2.70	80*	100	100	95*	100	100	98*
0.004	-2.44	65	75*	100	90	93*	98*	94
0.009	-2.05	70	63	80*	78	75	94	66
0.018	-1.74	40	65	70	78	65	86	23
0.027	-1.57	25	38	37	65	44	43	23
0.036	-1.44	0	10	27	25	31	48	0
0.089	-1.05		0	0	0	0	40	
0.18	-0.74						17	
0.27	-0.57						14	
0.36	-0.44						12	
0.89	-0.05						10	
1.8	0.26						0	
Linear regression coefficient		-56.8	-60.5	-86.0	-55.6	-69.5	-40.4	-81.2
Linear correlation coefficient		-0.91	-0.92	-0.97	-0.89	-0.98	-0.95	-0.97
Calculated 50% growth inhibitory cyclohex conc.(μM)		0.009	0.014	0.022	0.023	0.020	0.054	0.011

(μM)	(log_{10} μM)	CY8.12 x CY13	CY8.13 x CY13	CY8.18 x CY13	CY8.20 x CY13	CY8.23 x CY13	CY8.24 x CY13
0		100	100	100	100	100	100
0.002	-2.70	100	91*	100	100	100	100
0.004	-2.44	88*	77	72*	98*	86*	96*
0.009	-2.05	76	74	48	94	48	86
0.018	-1.74	35	23	32	86	45	77
0.027	-1.57	31	0	24	76	28	28
0.036	-1.44	0		0	62	21	19
0.089	-1.05				45	0	15
0.18	-0.74				0		13
0.27	-0.57						13
0.36	-0.44						0
Linear regression coefficient		-89.2	-78.7	-68.2	-57.2	-60.8	-49.4
Linear correlation coefficient		-0.96	-0.93	-0.98	-0.94	-0.98	-0.91
Calculated 50% growth inhibitory cyclohex conc.(μM)		0.013	0.009	0.009	0.051	0.012	0.027

Table A10. The effect of cycloheximide on the growth of dipoids.

Cycloheximide concentration (μM)	(log 10 μM)	Growth as a %, 0μM cycloheximide treatment. Strains CY8/CY18	CY8.2/CY18	CY9/CY14	CY9.23/CY14
0		100	100	100	100
0.002	-2.70	88*	86*	86*	98
0.004	-2.44	51	83	71	100
0.009	-2.05	16	63	54	96*
0.018	-1.74	0	31	47	89
0.027	-1.57		34	47	83
0.036	-1.44		29	37	74
0.089	-1.05		16	18	61
0.18	-0.74		0	0	34
0.36	-0.44				17
0.89	-0.05				0
Linear regression coefficient		-93.1	-46.5	-41.2	-51.5
Linear correlation coefficient		- 0.99	-0.98	-0.99	-0.99
Calculated 50% growth inhibitory cycloheximide concentration (μM)		0.005	0.014	0.015	0.099

SECTION B. IN VITRO POLYPHENYLALANINE SYNTHESIS.

1) A postmitochondrial supernatant polyphenylalanine synthesising system.

In the early experiments with the polyphenylalanine synthesising system, described in Chapter 4, Table 4.2, no synthesis was achieved. In order to determine the cause of inactivity, a less complex *in vitro* assay was used (Table B.1) and the post-mitochondrial supernatant S-30 used, required fewer preparative steps than the RP-100 and S-100 fractions (Chapter 2, Section 2.7).

Originally *Coprinus cinereus* mycelium was frozen and kept at -70°C before the cell-extract was prepared and *in vitro* such cell-extracts produced no polypeptide. The cell-extracts prepared from freshly grown mycelium produced polyphenylalanine synthesis and were subsequently adopted for the preparation of functional cell-extracts (Chapter 2. Section 2.7).

The assay was shown to be dependant on the S-30 fraction, but the activity of the system was low. However, when the S-30 fraction was passed through a Sephadex column (Chapter2, Section2.7) it was 6 x more active that the untreated S-30 fraction (Table B.2); improved activity of the G-30 was considered to result from the removal of low moelcular weight inhibtiors from the S-30. The cell-free system was dependant on polyuridylic acid, indicating that the endogenous concentration of mRNA was low.

Table B.1 The Composition of the S-30 polypeptide synthesising system.

Constituent.	Final concentration (mM, unless stated).
Adenosine - 5 - triphosphate.	1.0
Guanosine - 5 - triphosphate.	0.1
Creatine phosphokinase.	4.0 $\mu g.ml^{-1}$.
Creatine phosphate.	10.0
Tris-HCl, pH7.5.	50.0
Magnesium acetate.	8.0
Potassium chloride.	30.0
Spermidine.	0.25
Dithiothreitol.	2.0
Polyuridylic acid.	400.0 $\mu g.ml^{-1}$.
L-(U-C^{14})-phenylalanine (513 m Ci.$mmole^{-1}$).	0.98 μ
G-30, postmitochondrial supernatant fraction.	2.0 A_{260} unit.
Made to 100 μl with double distilled water.	

The G-30 fraction was prepared from the postmitochondrial supernatant fraction, S-30, by passage through Sephadex (Chapter 2, Section 2.7).

The reaction mixture was based on Saccharomyces cerevisiae (Sissons, 1974) and wheat germ (Marcu and Dudock, 1974; Roberts and Paterson, 1973).

Table B.2. The

on p

Cell-extract.
G-30
S-30
No extract

The composition

The S-30 concen

polyphenylalani

Chapter 2, Sect

The high

the TCA precipi

substrate utili

the efficiency

presented by Si

cinereus syste

The rela

upon had the as

not developed

cycloheximide-

extract, G-30,

therefore went

synthesising sy

Table B.2. The effect of variable reaction mixture composition on polyphenylalanine synthesis.

Cell-extract.	Composition of reaction mixture.	Polyphenylalanine synthesised (pmolephe. assay.$^{-1}$ hr.$^{-1}$)
G-30	complete	18.1
	complete minus poly(U)	5.9
S-30	complete	3.4
	complete minus poly(U)	1.1
No extract	complete	0.4

The composition of complete medium is described in Table B.1 The S-30 concentration was 2.0 A_{260} unit. The quantity of polyphenylalanine synthesised was determined as described in Chapter 2, Section 2.9.

The highest activity observed, 18.1 p.mole phe incorporated into the TCA precipitate.assay^{-1} hr^{-1} (Table B2) had an efficiency of substrate utilisation of 1.9% (Efficiency = $\frac{\text{pmole phe product x100\%}}{\text{pmole phe substrate}}$) the efficiency was similar to the efficiency calculated from data presented by Sissons (1974), the system on which the Coprinus cinereus system was based (Table 4.1, Chapter 4).

The relatively low efficiency achieved may have been improved upon had the assay been developed. The post-mitochondrial system was not developed because one of the objectives was to screen for cycloheximide-resistant cytoplasmic ribosomes and the crude cell-extract, G-30, was unsuitable. The emphasis of the investigation therefore went on developing a RP-100/S-100 in vitro polyphenylalanine synthesising system (Section 4.4, Chapter 4).

ii) Estimation of the sedimentation coefficients of Coprinus cinereus cytoplasmic ribosomal subunits.

Sedimentation coefficients were estimated from the position of the 260 nm absorption peaks in sucrose density gradients according to the calculation and tables of McEwen (1967). In order to employ McEwen's method it was assumed that the ribosomal particles were inert, non-diffusing spherical particles and that the linear sucrose density gradient was stable. It was also assumed that Coprinus cinereus cytoplasmic ribosomes, like other eukaryotes (McCarty et al, 1968) had a bouyant density of 1.4 $g.cm^{-3}$.

Specimen calculation

From data presented in Figure B1 (Section Biii) carried out as described in Chapter 2, Section 2.8.

Centrifugation conditions: $\omega^2 t$.

t = time(sec) from start of acceleration to start of deceleration

= 6.5h = 2.34×10^4 sec

ω = angular velocity ($rad.sec^{-1}$) = 2.95×10^4 rpm

= 3.01×10^3 $rad.sec.^{-2}$

ω^2 = 9.54×10^6 $rad.sec.^{-2}$

Thus $\omega^2 t = 2.23 \times 10^{11}$ $rad.sec.^{-1}$

Sucrose density gradient.

Initially, the sucrose density gradient was made as a linear 10-30%(W/v) sucrose, however after centrifugation;

(z1) the sucrose concentration at the top of the gradient = 13%

(z2) the sucrose concentration at the bottom of the gradient = 27.5%

(zx) the sucrose concentration at the absorbance peak (Peak A, Figure B1) = 24%

The radial distances in the MSE 3 x 25 rotor were;

(r_1) radial distance to the top of the gradient = 7.3 cm.

(r_2) radial distance to the bottom of the gradient = 12.9 cm.

Thus, the sucrose concentration (zo) extrapolated at zero radius (ro) equalled $\frac{z_1 r_2 - z_2 r_1}{r_2 - r_1} = -5.9$.

The calculation of the integrals of the sucrose concentrations (z_x) and (z_1) was achieved by reference to Table B3 and specified by the centrifugation temperature and particle density. The column chosen is the one closest to the calculated value of (z0), namely -5.0.

Thus,

	Integral
zx = 24%	4.143
z_1 = 13%	2.417

(The value of (z_1) used was intermediate between 14% and 12%).

Table B.3 Values of time integral for sucrose gradient centrifugation.

TEMPERATURE 5.0 DEG C PARTICLE DENSITY 1.40

% SUCROSE	Z0= 5	Z0= 0	Z0= -5	Z0= -10	Z0= -15	Z0= -20	Z0= -25	Z0= -30	Z0= -40	Z0= -60	Z0=-100
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.5262	0.2854	0.1960	0.1493	0.1206	0.1011	0.0765	0.0514	0.0311
4	0.0	1.1703	0.9510	0.5461	0.3762	0.2865	0.2315	0.1937	0.1552	0.1052	0.0639
6	0.0	1.5134	1.3192	0.7913	0.5600	0.4435	0.3660	0.3087	0.2369	0.1617	0.0989
8	2.2059	2.4893	1.6534	1.0269	0.7500	0.5919	0.4892	0.4170	0.3221	0.2215	0.1364
10	3.3222	2.9769	1.9648	1.2478	0.9129	0.7241	0.6181	0.5294	0.4116	0.2850	0.1764
12	4.1319	3.4161	2.2685	1.4836	1.1181	0.8987	0.7521	0.6471	0.5062	0.3530	0.2201
14	4.8911	3.8269	2.5650	1.7196	1.3099	1.0604	0.8926	0.7712	0.6068	0.4261	0.2673
16	5.3953	4.2225	2.8617	1.9569	1.5066	1.2299	1.0409	0.9039	0.7147	0.5057	0.3189
18	5.9685	4.6129	3.1632	2.2025	1.7139	1.4091	1.1988	1.0461	0.8311	0.5913	0.3756
20	6.4817	5.0035	3.4740	2.4598	1.9383	1.6004	1.3683	1.1963	0.9575	0.6857	0.4383
22	7.0037	5.4077	3.7998	2.7322	2.1829	1.8064	1.5519	1.3619	1.0960	0.7909	0.5091
24	7.5565	5.8262	4.1426	3.0259	2.4512	2.0303	1.7524	1.5436	1.2489	0.9060	0.5904
26	8.0970	6.2651	4.5139	3.3394	2.6974	2.2758	1.9734	1.7445	1.4188	1.0360	0.6768
28	8.6777	6.7414	4.9102	3.6569	3.0017	2.5477	2.2192	1.9687	1.6095	1.1829	0.7754
30	9.2937	7.2552	5.3485	4.0079	3.3404	2.8518	2.4952	2.2213	1.8266	1.3504	0.8910
32	9.9738	7.8209	5.8355	4.4041	3.7215	3.1956	2.8083	2.5037	2.0729	1.5431	1.0248
34	10.7166	8.4518	6.3835	4.8799	4.1594	3.5886	3.1574	2.8303	2.3529	1.7671	1.1815
36	11.5506	9.1666	7.0099	5.4359	4.6659	4.0434	3.5845	3.2246	2.6915	2.0305	1.3669
38	12.5011	9.9887	7.7433	6.1023	5.2609	4.5723	4.0752	3.6785	3.0867	2.3462	1.5890
40	13.6062	10.9503	8.5856	6.9677	5.9355	5.2139	4.6613	4.2221	3.5615	2.7232	1.8589
42	14.9092	12.0764	9.6073	7.9692	6.7748	5.9836	5.3714	4.8841	4.1418	3.1986	2.1924
44	16.4358	13.4891	10.8549	9.2092	7.8034	6.9364	6.2543	5.7047	4.8634	3.7703	2.6114
46	18.2342	15.2129	12.4055	10.6038	9.1495	8.1280	7.3628	6.7383	5.7765	4.5004	3.1466
48	20.2620	17.3914	14.3758	12.2965	10.7524	9.6567	8.7853	8.0695	6.9539	5.4668	3.8436
50	23.5993	20.2090	16.9326	14.5467	12.9099	11.6579	10.6515	9.8174	8.5057	6.7340	4.7707
52	28.1417	23.9457	20.1568	17.6212	15.7979	14.3627	13.1597	12.1711	10.6007	8.4516	6.0335
54	33.7658	29.0396	24.9010	21.9572	19.7600	18.0619	16.6220	15.4249	13.5068	10.8420	7.7992
56	41.6535	36.2112	31.5655	28.0266	25.4950	23.3025	21.5569	20.0670	17.6556	14.2738	10.3457
58	53.1235	46.6758	41.1871	36.9306	33.6914	31.0513	28.8306	26.9258	23.8107	19.3748	14.1476
60	70.5283	62.6074	55.8751	50.5556	45.3966	42.0531	40.0246	37.4914	33.3099	27.2785	20.0639

Reprinted from McEwen, (1967).

$$S20.w = \frac{\text{Integral } (z_x) - \text{Integral } (z_1)}{\omega^2 t}$$

$$= \frac{4.1426 - 2.4168}{2.233 \times 10^{11}}$$

$$= 0.773 \times 10^{-11}\text{sec}$$

$$= 77.3 \times 10^{-13}\text{sec}$$

Thus $\underline{S20\ w = 77.3\ S}$ (Svedberg units).

Under dissociating conditions, Figure B4, the sedimentation coefficients at maximum absorbance at peak B (21%) and C (18.5%), were 54.5S and 36.9S respectively.

i ii) Fractionation of Coprinus cinereus cytoplasmic ribosomes.

The conditions which produced a suitable resolution of Coprimus cinereus cytoplasmic ribosomal subunits were experimentally determined by varying the cation concentration of the sucrose density gradient and the conditions of centrifugation.

Centrifugation in Extraction Buffer produced a single absorbance peak (Peak A in Figures B1 and B5), calculated by the method described in Section B(ii)to have an approximate sedimentation coefficient of 77.5 S (Table B.4) . It was assumed that this peak revealed the presence of cytoplasmic monosomes: no polysomes or cytoplasic ribosomal subunits were present.

Two absorbance peaks resolved when the potassium chloride concentration was raised to 100mM or more, and when the magnesium acetate concentration was reduced to 1.0mM or less, were assumed to be produced by cytoplasmic ribosomal subunits. The large and small cytoplasmic ribosomal subunits, identified as peak B and C respectively in Figures B2, B3, B4, B6 and B7, had estimated sedimentation coefficients of 53.1 - 57.8 S and 35.1 - 40.6 S respectively. The best resolution of cytoplasmic ribosomal subunits was achieved in 1.0 mM magnesium acetate and 100 mM potassium chloride (Figure B4) and this buffer was termed

Figures B1 - B7. Fractionation of cytoplasmic ribosomes.

The sucrose density gradients were prepared and analysed as described in Chapter 2, Section 2.8. The variable concentrations of magnesium acetate and potassium chloride (in 50mM Tris-HCl pH 7.5 and 5mM 2 - mercaptoethanol) together with the variable centriguation conditions were examined. The RP-100 fraction from CY 8 was used in all treatments. Typical absorbance profiles are shown.

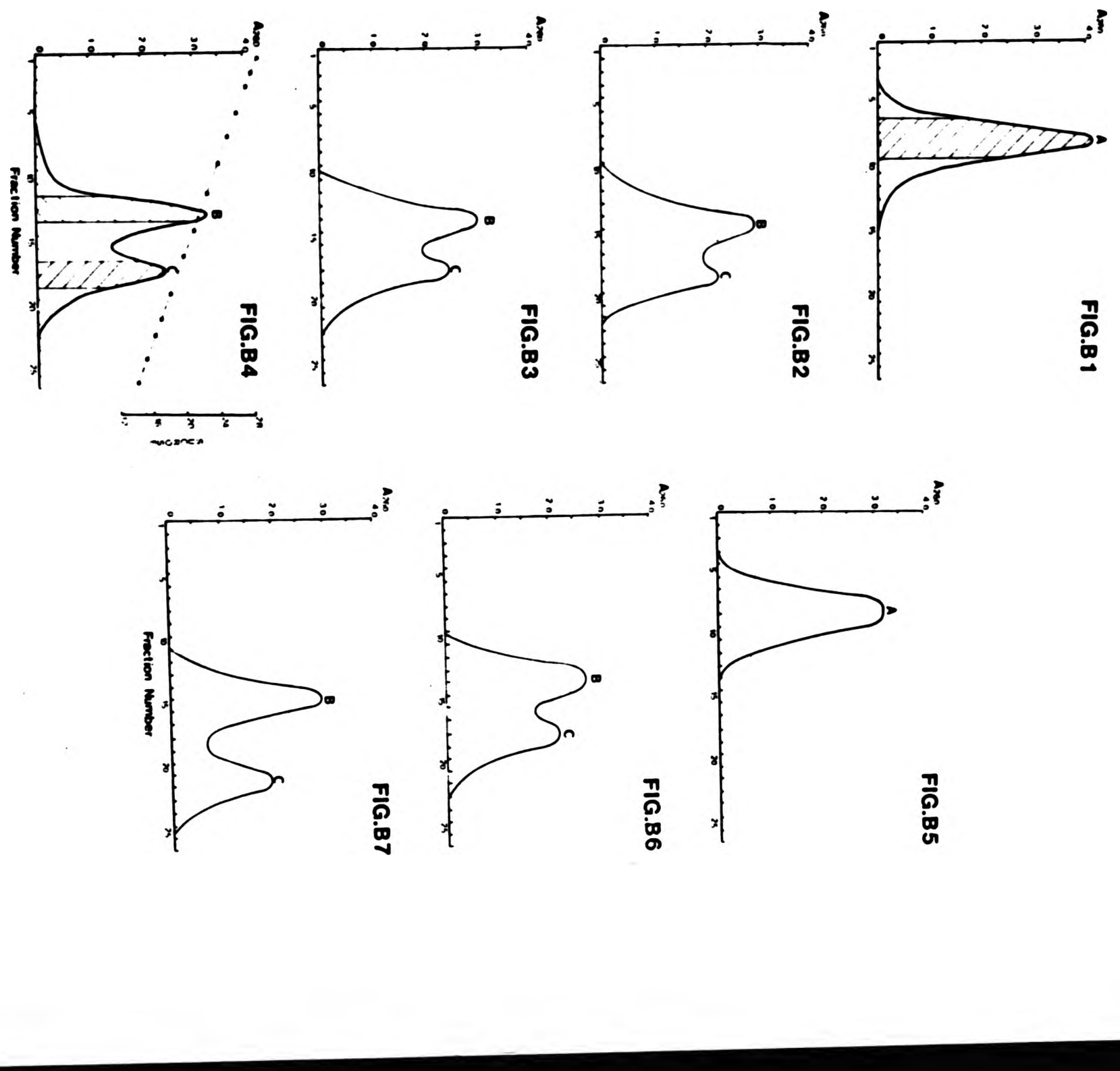
Table B.4

Figure	Magnesium acetate (mM)	Potassium chloride (mM)	Centrifugation conditions Time (h)	Centrifugal force (x gav.)	Calculated sedimentation coefficient (s) Monosome	Large subunit	Small subunit
					Average values. (± se.)		
B1	10	25	6.5	90.000	74.4 (1.2)		
B2	0	100	6.5	90,000		53.1 (0.8)	35.5 (1.7)
B3	0.1	100	6.5	90,000		55.8 (1.2)	40.6 (2.9)
B4	1.0	100	6.5	90,000		57.8 (2.8)	39.5 (1.3)
B5	10	25	16	37,000	74.8 (0.7)		
B6	1.0	100	16	37,000		53.8 (0.2)	35.1 (2.1)
B7	10	500	6.5	90,000		nd	nd

Absorbance at 254nm was continually monitored (solid line) during the fractionation of the gradients, while every 1/10th fraction was used to determine % sucrose (■——■) . All gradients were identical to that of Figure B4. Direction of sedimentation was from Fractions 1 to 27. Absorbance peaks A, B and C represent cytoplasmic monosomes, large and small cytoplasmic ribosomal subunits. Sedimentation coefficients were calculated as described in Section B(ii) (nd = not determined).

Absorbance at 254nm was continually monitored (solid line) during the fractionation of the gradients, while every $^1/_{10}$th fraction was used to determine % sucrose (■——■) . All gradients were identical to that of Figure B4. Direction of sedimentation was from Fractions 1 to 27. Absorbance peaks A, B and C represent cytoplasmic monosomes, large and small cytoplasmic ribosomal subunits. Sedimentation coefficients were calculated as described in Section B(ii) (nd = not determined).

Figures B1-B7. Fractionation of cytoplasmic ribosomes.



the Dissociation Buffer (Chapter 2, Section 2.6). In Dissociation Buffer there was no evidence of cytoplasmic monosomes. The best resolution using Dissociation Buffer was achieved in a fast, short centrifugation rather than an equivalent overnight centrifugation (Figures B4 and B6; $rpm^2 \times hr = constant$, Baierlein and Infante, 1976). The overnight centrifugation resulted in peak broadening.

Variation in the estimated sedimentation coefficients at different conditions (Table B4) may be explained by the effects of diffusion of the particles and instability and loss of ribosomal material (Baierlein and Infante, 1976).

iv) Table B5. Effect of variable RP-100 and S-100 concentrations on polyphenylalanine synthesis.

RP-100 Concentration (A260 unit)	S-100 concentration (A_{260} unit)							
	0.8		1.7		2.5		3.3	
	Conc. Ratio	Activity (%)	Conc. ratio	Activity (%)	Conc. ratio	Activity (%)	Con. ratio	Activity (%)
1.1	1.4 : 1	89	1.6 : 1	84	0.4 : 1	61	0.3 : 1	58
2.1	2.6 : 1	100	1.2 : 1	100	0.8 : 1	95	0.6 : 1	96
3.2	4.0 : 1	75	1.9 : 1	83	1.3 : 1	100	1.0 : 1	100
4.2	5.3 : 1	47	2.5 : 1	75	1.7 : 1	85	1.3 : 1	86
100% values pmole phe. assay.$^{-1}$ hr.$^{-1}$	24.7		46.9		42.2		36.6	

No RP-100 with any concentration of S-100 and no S-100 with any RP-100 concentration produced infinite ratios and activity which was less than 0.5 pmole phe. assay.$^{-1}$ hr^{-1}, these results are not included.

v) Expression of cytoplasmic ribosomal concentration.

a) A_{260} unit.

Throughout this investigation, the concentration of cell-extracts has been expressed in terms of the A_{260} unit; 1.0 A_{260} unit was defined as the quantity of material which, when dissolved in 1ml, had an absorbance of 1.0 at 260nm, in a 1cm path length.

Alternatively, the concentration of the RP-100 fraction may be expressed in terms of the protein content, (mg protein. ml^{-1}) and cytoplasmic ribosomal content, (either mg cytoplasmic ribosome.ml^{-1} or p mole cytoplasmic ribosome.ml^{-1}). However, in expressing the RP-100 concentration in terms other than A_{260} unit , several assumptions were necessary, which introduced a degree of uncertainty into the calculations. (Section B.v. (b-d).).

b) mg cytoplasmic ribosomal protein .$ml.^{-1}$

The protein concentration of the RP-100 and S-100 fractions were determined by the method of Lowry et al (1951) and employing bovine serum albumin (BSA) as a standard protein. The protein concentration was then equated with the known absorbance at 260 nm. It should be noted that the estimate of protein content by the method of Lowry et al (1951) is subject to interference from constituents in the Extraction Buffer and that different proteins used as standards react differently in the assay (Peterson, 1979).

Table B.6. The relationship between A_{260} unit and mg BSA protein. $ml.^{-1}$

Coprinus cinereus cell-extract	A_{260} unit equivalent to 1mg BSA protein. ml^{-1}.	*Optimised in vitro concentration.	
		A_{260} unit.	µg BSA. 100µl assay $^{-1}$
G-30	6.7	2.0	30
S-100	6.7	2.0	30
RP-100	18.2	2.0	11

The optimised in vitro concentrations are given in Chapter 2, Section 2.9, Table 2.2.

c) mg cytoplasmic ribosome.$ml.^{-1}$

The concentration of cytoplasmic RNA was not determined. It was assumed that Coprinus cinereus cytoplasmic ribosomes contained an equal proportion of protein and RNA (as stated for eukaryotes in Table 1.1, Chapter 1). Given that in mammalian cytoplasmic monosomes RNA absorbs at 260 nm to the same extent as ribosomal protein (Nieuwenhuysen et al, 1978; Wool, 1979) and assuming that all protein was cytoplasmic ribosomal protein then;

18.2 A_{260}unit = 1 mg ribosomal protein.ml^{-1} + 1 mg rRNA.ml^{-1}.

Therefore

9.1 A_{260} unit = 1 mg cytoplasmic ribosome.$ml.^{-1}$

In each 100 µl assay, 2.0 A_{260} unit of RP-100 is equivalent to 22 µg cytoplasmic ribosomes of which 11 µg is cytoplasmic ribosomal protein.

Alternatively, estimates for the absorbance at 260 nm of 1 mg cytoplasmic ribosomes.ml^{-1} include; 13 A_{260} unit for Saccharomyces

cerevisiae (Sissons, 1974), 11 A_{260}unit for Schizosaccharomyces pombe (Berry et al, 1978) and 9.3 A_{260}unit for Saccharomyces fragilis, (Rao and Grollman, 1967)

d) Molality of cytoplasmic ribosomes.

In order to determine the p.mole cytoplasmic ribosomes present in the in vitro assay, it was necessary to assume a molecular weight for Coprinus cinereus cytoplasmic ribosomes because no value has been measured for this particular species. Assuming that cytoplasmic ribosomes of Coprinus cinereus are similar to other fungal species, then; the molecular weight of Coprinus cinereus cytoplasmic ribosomes is approximately 3.9×10^6 dalton (Table 1.1, Chapter 1). Each 100 µl assay contains 22 µg cytoplasmic ribosomes (Section B (v.b). Therefore each assay was estimated to contain 5.6 p.mole cytoplasmic ribosomes.

Alternative molecular weights which have been used include; 3.4×10^6 daltons for Saccharomyces cerevisiae (Sissons, 1974) and Schizosaccharomyces pombe (Berry et al, 1978).

SECTION C. EFFECT OF CYCLOHEXIMIDE IN VITRO.

Note to Tables C1 to C4.

* indicates the minimum cycloheximide concentration used in determining the linear regression analysis over the inhibitory range of cycloheximide concentrations.

Table C1. Effect of cycloheximide on polyphenylalanine synthesis produced by RP-100 and S-100 fractions from CY8 and CY8.2.

Cycloheximide concentration (μM)	(log 10μM)	Polyphenylalanine synthesised (% of uninhibited control) CY 8 RP-100 CY 8 S-100	CY 8 RP-100 CY8.2 S-100	CY 8.2 RP-100 CY 8.2 S-100	CY 8.2 RP-100 CY 8	CY 8.2 RP-100 S-100
0		100	100	100		100
0.36	-0.44	90*	87	-		-
0.89	-0.05	79	81	-		-
1.8	0.26	64	68	-		-
3.6	0.56	58	59	100		100
8.9	0.95	39	30	93*		98*
18	1.26	21	18	-		-
36	1.56	18	15	90		99
89	1.95	5	8	83		81
180	2.25	3	4	71		77
360	2.56	0	0	62		73
890	2.95			39		42
1800	3.26			27		20
3600	3.56			20		14
5300	3.72			13		10
Linear correlation coefficient		-0.98	-0.97	-0.97		-0.96
Linear regression coefficient		-32.4	-33.4	-32.3		-36.7
Calculated 50% inhibitory cycloheximide concentration (μM)		5.1	4.6	490		530

- = not determined

Table C2. Effect of cycloheximide on polyphenylalanine synthesis using RP-100 fractions from various monokaryotic strains.

Cycloheximide concentration		Polyphenylalanine synthesised (% of uninhibited control) S-100 from CY18 in all tests. Source of RP-100							
(μM)	($\log_{10}\mu$M)	CY3	CY8	CY9	CY13	CY14	CY18	CY8.2	CY9.23
0		100	100	100	100	100	100	100	100
0.07	-1.15	87*	99*	84*	79*	82*	80	-	-
0.21	-0.68	58	92	64	52	61	68	-	-
0.36	-0.44	40	88	43	39	54	53	-	-
0.89	-0.05	33	79	37	27	34	33	-	-
1.8	0.26	24	62	25	20	22	22	100	100
3.6	0.56	12	55	19	15	14	18	96*	96*
18	1.26	5	23	11	8	6	12	-	84
36	1.56	4	20	0	5	4	4	95	64
89	1.95	0	6		0	0	0	91	46
180	2.26		0					81	18
360	2.56							75	11
890	2.95							33	5
1800	3.26							24	0
3600	3.56							17	
5300	3.72							12	
Linear correlation coefficient		-0.93	-0.99	-0.96	-0.93	-0.95	-0.95	-0.91	-0.97
Linear regression coefficient		-25.4	-31.9	-28.6	-22.5	-26.1	-25.7	-31.9	-40.5
Calculated 50% inhibitory cycloheximide concentration (μM)		0.35	4.2	0.45	0.22	0.42	0.47	550	64

- not determined

Table C3i. The effect of RP-100 concentration on the response to cycloheximide.

Cycloheximide concentration (μM)	(log_{10}μM)	Polyphenylalanine synthesised (% activity of uninhibited control) S-100 from CY 18, RP-100 from CY 9.23.%6. RP-100 concentration (A_{260} unit)				
		0.7	1.5	2.0	2.5	3.2
0		100	100	100	100	100
18	1.26	85*	92*	86*	73*	76*
36	1.56	61	88	69	55	53
53	1.72	54	70	54	42	51
71	1.85	50	42	41	37	41
89	1.95	41	43	44	26	31
110	2.04	30	27	21	27	30
Linear correlation coefficient.		-0.99	-0.94	-0.97	-0.99	-0.98
Linear regression coefficient.		-64.4	-87.4	-76.7	-62.0	-58.7
Calculated 50% inhibitory cycloheximide concentration (μM).		60.8	70.5	57.8	41.9	47.7

Table C 3(ii).

RP-100 concentration A_{260}unit	(log A_{260} unit)	Specific 50% inhibitory cycloheximide concentration (μM. A_{260} $unit^{-1}$)
0.7	-0.15	87
1.5	0.18	47
2.0	0.30	29
2.5	0.40	17
3.2	0.51	15
Linear correlation coefficient.		- 0.99
Linear regression coefficient.		-115.6

Table C4. The effect of RP-100 from dikaryons on the response to cycloheximide.

Cycloheximide concentration (μM)	(log 10 μM)	Polyphenylalanine synthesised (% activity of uninhibited control) S-100 from CY 18. Source of RP-100			
		CY8 x CY13	CY8.2 x CY13	CY9 x CY3	CY9.23 x CY3
0		100	100	100	100
0.07	-1.15	88*	96*	-	-
0.21	-0.68	75	86	74*	94*
0.36	-0.44	66	80	76	82
1.1	0.04	49	56	44	53
2.1	0.32	36	61	28	63
3.6	0.56	28	56	23	61
18.	1.26	12	54	4	48
36.	1.56	6	34	1	46
110.	2.04	0	22	0	28
180.	2.26		15		21
360.	2.56		8		16
450.	2.65		0		10
Linear correlation coefficient.		-0.99	-0.98	-0.95	-0.97
Linear regression coefficient.		-29.1	-23.4	-30.5	-21.6
Calculated 50% inhibitory cycloheximide concentration(μM).		1.1	6.3	0.92	9.6

- not determined

BIBLIOGRAPHY.

Adoutte-Panvier, A., Davies, J.E., Gritz, L.R. and Littlewood, B.S. (1980).
Molecular and General Genetics, 179:273-282.
Studies of ribosomal proteins of yeast species and their hybrids. Gel electrophoresis and immunological cross-reactions.

Apirion, D. (1966).
Genetics, 53:935-941.
Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons.

Anderson, W.F., Bosch, L., Cohn, W.E., Lodish, H.,

Merrick, W.C., Weissbach, H., Wittmann, H.G. and Wool, I.G. (1977).
Federation of European Biochemical Societies Letters, 76:1-10.
International Symposium on protein synthesis. Summary of Fogarty Centre -NIH workshop held in Bethesda, Maryland on 18-20 October, 1976.

Atkins, J.F., Lewis, J.B., Andersen, C.W. and Gesteland, R.F. (1975).
Journal of Biological Chemistry, 250:5688-5695.
Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines.

Baierlein, R. & Infante, A.A. (1974).
Methods in Enzymology, 30:328-345.
Pressure-induced dissociation of ribosomes.

Bailey, N.T.J. (1959).
In Statistical Methods in Biology, Chapter 6 and 10.
Ed. Bullough. W.S: London, English University Press.

Baliga, B.S., Pronczuk, A.W., and Munro, H.N. (1969).
Journal of Biological Chemistry, 244:4480-4489.
Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver.

Barker, C. & Lewis, D. (1974).
Journal of General Microbiology, 82:337-343.
Impaired regulation of aromatic amino acid synthesis in a mutant resistant to p-fluoro phenylalanine.

Battaner, E. and Vazquez, D. (1971).
Biochimica et Biophysica Acta, 254:316-330.
Inhibitors of protein synthesis by ribosomes of the 80 S type.

Bégueret, J., Perrot, M. & Crouzet, M. (1977).
Molecular and General Genetics, 156: 141-144.
Ribosomal proteins in the fungus Podospora anserina: evidence for an electrophoretically altered 60S protein in a cycloheximide resistant mutant.

Bennett, L.L., Smithers, D. & Ward, C.T. (1964).
Biochemica et Biophysica Acta, 87:60-69.
Inhibition of DNA synthesis in mammalian cells by actidione.

Berry, C.H.J., Ibrahim, M.A.K. & Coddington, A. (1978).
Molecular and General Genetics, 167:217-225.
Characterisation of ribosomes from drug resistant strains of Schizosaccharomyces pombe in a poly (U) directed cell-free protein synthesising system.

Bielka, H. (1978).
Transactions in Biochemical Sciences, 3:156-158.
The eukaryotic ribosome.

Adoutte-Panvier, A., Davies, J.E., Gritz, L.R. and Littlewood, B.S. (1980).
Molecular and General Genetics, 179:273-282.
Studies of ribosomal proteins of yeast species and their hybrids. Gel electrophoresis and immunological cross-reactions.

Apirion, D. (1966).
Genetics, 53:935-941.
Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons.

Anderson, W.F., Bosch, L., Cohn, W.E., Lodish, H.,

Merrick, W.C., Weissbach, H., Wittmann, H.G. and Wool, I.G. (1977).
Federation of European Biochemical Societies Letters, 76:1-10.
International Symposium on protein synthesis. Summary of Fogarty Centre -NIH workshop held in Bethesda, Maryland on 18-20 October, 1976.

Atkins, J.F., Lewis, J.B., Andersen, C.W. and Gesteland, R.F. (1975).
Journal of Biological Chemistry, 250:5688-5695.
Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines.

Baierlein, R. & Infante, A.A. (1974).
Methods in Enzymology, 30:328-345.
Pressure-induced dissociation of ribosomes.

Bailey, N.T.J. (1959).
In Statistical Methods in Biology, Chapter 6 and 10.
Ed. Bullough. W.S: London, English University Press.

Baliga, B.S., Pronczuk, A.W., and Munro, H.N. (1969).
Journal of Biological Chemistry, 244:4480-4489.
Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver.

Barker, C. & Lewis, D. (1974).
Journal of General Microbiology, 82:337-343.
Impaired regulation of aromatic amino acid synthesis in a mutant resistant to p-fluoro phenylalanine.

Battaner, E. and Vazquez, D. (1971).
Biochimica et Biophysica Acta, 254:316-330.
Inhibitors of protein synthesis by ribosomes of the 80 S type.

Bégueret, J., Perrot, M. & Crouzet, M. (1977).
Molecular and General Genetics, 156: 141-144.
Ribosomal proteins in the fungus Podospora anserina: evidence for an electrophoretically altered 60S protein in a cycloheximide resistant mutant.

Bennett, L.L., Smithers, D. & Ward, C.T. (1964).
Biochemica et Biophysica Acta, 87:60-69.
Inhibition of DNA synthesis in mammalian cells by actidione.

Berry, C.H.J., Ibrahim, M.A.K. & Coddington, A. (1978).
Molecular and General Genetics, 167:217-225.
Characterisation of ribosomes from drug resistant strains of Schizosaccharomyces pombe in a poly (U) directed cell-free protein synthesising system.

Bielka, H. (1978).
Transactions in Biochemical Sciences, 3:156-158.
The eukaryotic ribosome.

Bienveniste, K., Wilczek, J., Ruggieri, A. and Stern, R. (1976).
Biochemistry, 15:830-835.
Translation of collagen messenger RNA in a cell-free system derived from wheat germ.

Bleyman, L.K. & Bruns, P.J. (1977).
Genetics, 87:275-284.
Genetics of cycloheximide resistance in Tetrahymena.

Bretthauer, R.K. and Golichowski, A.M. (1967).
Biochimica et Biophysica Acta, 155:549-557.
Adsorption chromatography of phenylalanine peptides on sephadex.

Brot, T. (1977).
In Molecular mechanisms of protein biosynthesis, 375-412.
(Edited by Weissbach, H. & Pestka, S.)
Translocation.

Bryne, B.C. & Bruns, J.P. (1974).
Genetics, 77:57-58.
Selection of somatic and germ-line drug resistance mutations in Tetrahymena.

Byrne, B.C. (1978).
Genetics, 89:703-710.
Induced resistance to 6-methylpurine and cycloheximide in Tetrahymena. II Variation within vegetative cultures of micronucleate T. thermophila and amicronucleate T. pyriformis.

Cabezón T. & De Wilde, M. (1975).
In study of bacterial ribosomes, Biochemical methods, 14-42.
University of Brussels.

Cannon, M. and Cundliffe, E. (1973).
In Techniques in protein biosynthesis (Edited by Campbell, P.N. and Sargent, J.R.), 3:1-34.
The use of antibiotics and other inhibitors in studies of bacterial protein synthesis.

Cannon, M., Smith, K.E. and Carter, C.J. (1976).
Biochemistry Journal, 156:289-294.
Preventation by ribosomal-bound nascent polyphenylalanine chains, of the functional interaction of T-2 toxin with its receptor site.

Carter, J., Cannon, M. and Jimenez, A. (1980).
European Journal of Biology , 107:173-183.
A trichodermin resistant mutant of Saccharomyces cerevisiae with an abnormal distribution of native ribosomal subunits.

Casselton, L.A. (1965).
Genetical Research, 6:109-208.
The production and behaviour of diploids of Coprinus lagopus .

Casselton, L.A. & Lewis, D. (1967).
Genetical Research, 9:63-71.
Dilution of gene products in the cytoplasm of of heterokaryons in Coprinus lagopus .

Caskey, C.T. (1977).
In Molecular mechasisms of protein biosynthesis, 443-467.
(Edited by Weissbach, H. & Pestka, S.)
Peptide chain termination.

Coddington, A. & Fluri, R. (1977).
Molecular and General Genetics, 158:93-100.
Characterisation of ribosomal proteins from Schizosaccharomyces pombe by two-dimensional polyacrylamide gel electrophoresis: Demonstration that a cycloheximide resistant strain, CYH1, has an altered 60S ribosomal protein.

Cooney, W.J. and Bradley, S.G. (1961).
In Antimicrobial Agents and Chemotherapy, 237-234.
(Edited Finland, M. and Savage, G.M.)
Action of cycloheximide on animal cells.

Cooper, D., Banthorpe, D.V. and Wilkie, D. (1967).
Journal of Molecular Biology, 26: 347-350.
Modified ribosomes conferring resistance to cycloheximide in mutants of Saccharomyces cerevisiae.

Coppin-Raynal, E. (1977).
Journal of Bacteriology, 131:876-883.
Ribosomal suppressors and antisuppressors in Podospora anserina: Resistance to cycloheximide.

Coppin-Raynal, E. (1980).
Analytical Biochemistry, 109:395-398.
Analytical chromatography of ribosomal proteins in the fungus Podospora anserina.

Cowan, J.W. & Lewis, D. (1966).
Genetical Research, 7:235-244.
Somatic recombination in the dikaryon of Coprinus lagopus.

Cox, R.A. & Godwin, E. (1975).
In MTP. International Review of Science, Biocheminstry series 1, 7:181-253. (Editor, Arnstein, H.R.V.).
Ribosome structure and function.

Crouzet, M., Perrot, M., Nogueira, M. & Bégueret, J. (1978).
Biochemical Genetics, 16:271-286.
Genetic and biochemical analysis of cycloheximide resistance in the fungus Podospora anserina.

Crouzet, M. and Bégueret, J. (1980).
Molecular and General Genetics, 180:177-183.
A new mutant form of the ribosomal protein L21 in the fungus Podospora anserina: Identification of the structural gene for this protein.

Day, P.R. & Anderson, G.E. (1961).
Genetical Research, 2:414-423.
Two linkage groups in Coprinus lagopus .

Day, P.R. & Roberts, C.F. (1969).
Genetics, 62:265-270.
Complementation in dikaryons and diploids of Coprinus lagopus.

Dee, J. & Poulter, R.T.M. (1970).
Genetic Research, 15:35-41.
A gene conferring actidione resistance and abnormal morphology on Physarum polycephalum plasmodia.

Dee, J. (1966)
Genetic Research, 8:101-110.
Genetic analysis of actidione-resistant mutants in the myxomycete Physarum polycephalum, Schw.

Ellis, R.J. (1969).
Science, 163:477-478.
Chloroplast ribosomes. Stereospecifity of inhibition by chloramphenicol.

Emerson, M.R. (1954).
Plant Physiology, 29:418-428.
Some physiological characteristics of ascospore activation in Neurospora crassa.

Ennis, H.L. & Luben, M. (1964).
Science, 146:1474-1476.
Cycloheximide: Aspects of inhibition of protein synthesis in mammalian cells.

Falvey, A.K. and Staehelin, T. (1970).
Journal of Molecular Biology, 53:21-34.
Structure and function of mammalian ribosomes.II Exchange of ribosomal subunits at various stages of in vitro polypeptide synthesis.

Felicetti, L., Colombo, B. & Baglioni, C. (1966).
Biochemica et Biophysica Acta, 119:120-129.
Inhibition of protein synthesis in reticulocytes by antibiotics.
II. The site of action of cycloheximide, streptovitacin A and pactamycin.

Fincham, J.R.S. & Day, P.R. (1971).
In Fungal Genetics, Botanical Monographs, 4: Chapter 2. 3rd Edition.
Blackwell Scientific Publications; Oxford and Edinburgh.

Fried, H.M., Pearson, N.J., Kim, C.H. and Warner, J.R. (1981).
The Journal of Biological Chemistry, 256:10176-10183.
The genes for fifteen ribosomal proteins of Saccharomyces cerevisiae.

Fried, H.M. and Warner, J.R. (1981).
Proceedings of the National Acadamy of Sciences (USA), 78:238-242.
Cloning of a yeast gene for trichodermin resistance and ribosomal protein L3.

Georgopoulos, S.G. (1977).
In Antifungal Compounds, 2:439-495.
Edited by Siegel, M.R. and Sisler, H.D.
Development of fungal resistance to fungicides.

Godchaux, W., Adamson, S.D. & Herbert, E. (1967).
Journal of Molecular Biology, 27:57-72.
Effects of cycloheximide on polyribosome function in reticulocytes.

Grover, R.K. & Moore, J.D. (1961).
Phytopathology, 51:399-401.
Adaption of Sclerotinia fructicola and Sclerotinia laxa to higher concentrations of fungicides.

Hanson, M.R., Davidson, J.N., Mets, L.J. and Bogorad, L. (1974).
Molecular and General Genetics, 132:105-118.
Characterisation of chloroplast and cytoplasmic ribosomal proteins of Chlamydomonas reinhardii by two-dimensional polyacrylamide gel electrophoreris.

Hardy, S.J.S., Kurland, C.G., Voynow, P. & Mora, G. (1969).
Biochemistry, 8:2897-2905.
The ribosomal proteins of Escherichia coli. 1. Purification of 30S proteins.

Harris, R.J. & Pestka, S. (1977).
In Molecular mechanisms in protein biosynthesis, 413-442.
(Edited by Weissbach, H. & Pestka, S.)
Peptide bond formation.

Harvey, C. and Martinelli, S.D. (1983).
Heredity, 50:210
An informational suppressor in Aspergillus nidulans has an altered ribosomal protein.

Haugli, F.B., Dove, W.F., Jimenez, A. (1972).
Molecular and General Genetics, 118:97-107.
Genetics and biochemistry of cycloheximide resistance in Physarum polycephalum.

Haugli, F.B. & Dove, W.F. (1972).
Molecular and General Genetics 118:109-124.
Mutagenesis and mutant selection in Physarum polycephalum.

Heredia, C.F. and Halvorson, H.O. (1966).
Biochemistry, 5:946-952.
Transfer of amino acids from aminoacyl soluble ribonucleic acids to protein by cell-free extracts from yeast.

Horgen, P.A. & Griffen, D.H. (1971).
Proceedings of the National Academy of Sciences (USA) 68:338-341.
Specific inhibitors of the three RNA polymerases from the aquatic fungus Blastocladiella emersonii.

Howard, G.A. & Traut, R.R. (1973).
Federation of European Biochemical Societies Letters, 29:177-180.
Separation and radioautography of microgram quantities of ribosomal proteins by 2D polyacrylamide gel electrophoresis.

Howe, H.B. & Terry, C.E. (1962).
Canadian Journal of Genetical Cytology, 4:447-452.
Genetic studies of resistance to chemical agents in Neurospora crassa.

Hsu, K.S. (1963).
Journal of General Microbiology, 32:341-347.
The genetic basis of actidione resistance in Neurospora.

Ibrahim, M.A.K. & Coddington, A. (1976).
Heredity, 37:179-191.
Genetic studies on cycloheximide-resistant strains of Schizosaccharomyces pombe.

Ibrahim, M.A.K. & Coddington, A. (1978).
Molecular and General Genetics, 162:213-219.
Genetic studies on revertants to sensitivity from a cycloheximide resistant strain of Schizosaccharomyces pombe.

Ishiguro, J. (1976).
Molecular and General Genetics, 145:73-79.
Study on proteins from yeast cytoplasmic ribosomes by two dimensional gel electrophoresis.

Jimenez, A., Littlewood, B, and Davies, J. (1972).
In Molecular mechanisms of antibiotic action on protein synthesis and membranes, 292-306 (Edited by Monoz, E, Garcia-Fernandez, F. and Vazuez, D.).
Inhibition of protein synthesis in yeast.

Kaltschmidt, E. and Witmann, H.G. (1970).
Proceedings of the National Academy of Sciences, 67:1276-1282.
Ribosomal proteins, XII. Number of proteins in small and large ribosomal subunits of Escherichia coli as determined by two-dimensional gel electrophoresis.

Kerridge, D. (1958).
Journal of General Microbiology, 19:497-506.
The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis.

Knopf, U.C., Sommer, A., Kenny, J. & Traut, R.R. (1975).
Molecular Biology Reports, 2:35-40.
A new 2D gel electrophoresis system for the analysis of complex protein mixtures: application to the ribosome of Escherichia coli.

Haugli, F.B., Dove, W.F., Jimenez, A. (1972).
Molecular and General Genetics, 118:97-107.
Genetics and biochemistry of cycloheximide resistance in Physarum polycephalum.

Haugli, F.B. & Dove, W.F. (1972).
Molecular and General Genetics 118:109-124.
Mutagenesis and mutant selection in Physarum polycephalum.

Heredia, C.F. and Halvorson, H.O. (1966).
Biochemistry, 5:946-952.
Transfer of amino acids from aminoacyl soluble ribonucleic acids to protein by cell-free extracts from yeast.

Horgen, P.A. & Griffen, D.H. (1971).
Proceedings of the National Academy of Sciences (USA) 68:338-341.
Specific inhibitors of the three RNA polymerases from the aquatic fungus Blastocladiella emersonii.

Howard, G.A. & Traut, R.R. (1973).
Federation of European Biochemical Societies Letters, 29:177-180.
Separation and radioautography of microgram quantities of ribosomal proteins by 2D polyacrylamide gel electrophoresis.

Howe, H.B. & Terry, C.E. (1962).
Canadian Journal of Genetical Cytology, 4:447-452.
Genetic studies of resistance to chemical agents in Neurospora crassa.

Hsu, K.S. (1963).
Journal of General Microbiology, 32:341-347.
The genetic basis of actidione resistance in Neurospora.

Ibrahim, M.A.K. & Coddington, A. (1976).
Heredity, 37:179-191.
Genetic studies on cycloheximide-resistant strains of Schizosaccharomyces pombe.

Ibrahim, M.A.K. & Coddington, A. (1978).
Molecular and General Genetics, 162:213-219.
Genetic studies on revertants to sensitivity from a cycloheximide resistant strain of Schizosaccharomyces pombe.

Ishiguro, J. (1976).
Molecular and General Genetics, 145:73-79.
Study on proteins from yeast cytoplasmic ribosomes by two dimensional gel electrophoresis.

Jimenez, A., Littlewood, B, and Davies, J. (1972).
In Molecular mechanisms of antibiotic action on protein synthesis and membranes, 292-306 (Edited by Monoz, E, Garcia-Fernandez, F. and Vazuez, D.).
Inhibition of protein synthesis in yeast.

Kaltschmidt, E. and Witmann, H.G. (1970).
Proceedings of the National Academy of Sciences, 67:1276-1282.
Ribosomal proteins, XII. Number of proteins in small and large ribosomal subunits of Escherichia coli as determined by two-dimensional gel electrophoresis.

Kerridge, D. (1958).
Journal of General Microbiology, 19:497-506.
The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis.

Knopf, U.C., Sommer, A., Kenny, J. & Traut, R.R. (1975).
Molecular Biology Reports, 2:35-40.
A new 2D gel electrophoresis system for the analysis of complex protein mixtures: application to the ribosome of Escherichia coli.

Kornfeld, E.C., Jones, R.G. and Parke, T.V. (1949).
Journal of the American Chemical Society, 71:150-159.
The structure and chemistry of actidione, an antibiotic of Streptomyces griseus.

Küntzel, H. (1969).
Nature, 222:142-146.
Proteins of mitochondrial and cytoplasmic ribosomes from Neurospora crassa.

Kyner, D., Zabos, P. and Levin, D.H. (1973).
Biochemica et Biophysica Acta, 321:386-396.
Inhibition of protein chain initiation in eukaryotes by deacylated transfer of RNA and its reversibility by spermine.

Lava-Sanchez, P.A., Analdi, F. La Posta, A.(1972).
Journal of Molecular Evolution, 2:44-55.
Base composition of ribosomal RNA and evolution.

Lewis, D. (1961).
Genetical Research, 2:141-155.
Genetical analysis of methionine suppressors in Coprinus lagopus.

Lewis, D. (1963).
Nature, 200:151-154.
Structural gene for the methionine-activating enzyme and its mutation as a cause of resistance to ethionine.

Lewis, D., & North, J. (1974).
In Handbook of Microbiology, 4:691-699.
Edited by Laskin, A.I. & Lechevalier, H. Cleveland.Ohio: CRC Press
Linkage maps of Coprinus lagopus.

Lewis, D., & Vakeria, D. (1977).
Genetical Research, 30:31-43.
Resistance to p-fluorophenylalanine in diploid/haploid dikaryons: dominance modifier gene explained as a controller of hybrid multimer formation.

Lederberg, E.M., Cavalli-Sfora, L. and Lederberg, J. (1964).
Proceedings of the National Acadamy of Sciences (USA), 51:678- 681.
Interaction of streptomycin and a suppressor galactose fermentation in Escherichia coli K-12.

Loeb, J.N. & Hubby, B.G. (1968).
Biochimica et Biophysica Acta, 166:745-748.
Amino acid incorporation by isolated mitochondria in the presence of cycloheximide.

Lovett, J.S. and Haselby, J.A. (1971).
Archiv für Mikrobiologie, 80:191-204.
Molecular weights of the ribosomal nucleic acid of fungi.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951).
Journal of Biological Chemistry, 193:265-275.
Protein measurment with the folin phenol reagent.

Marcus, L., Bretthauer, R.K., Boch, R.M. and Halvorsen, H.O. (1963).
Proceedings of the National Academy of Sciences (USA), 50:782-789.
The effect of poly (U) size on the incorporation of phenylalanine in the cell-free yeast system.

Marcu, K. & Dudock, B. (1974).
Nucleic Acid Research, 1:1385-1397.
Characterisation of a highly efficient protein synthsising system derived from commercial wheat germ.

M^{c}Carty, K.S., Stafford, D. & Brown, O. (1968).
Analytical Biochemistry, 24:319-329.
Resolution and fractionation of macromolecules by isokenetic sucrose density gradient sedimentation.

M^cConkey, E.H., Bielka, H., Gordon, J., Lastick, S.M., Lin A., Ogata, K., Rebound, J-P, Traugh, J.A., Traut, R.R., Warner, J.R., Welfle, H. and Wool, I.G. (1979).
Molecular and General Genetics, 169:1-6.
Proposed uniform nomenclature for mammalian ribosomal proteins.

M^cEwen, C.R. (1967).
Analytical Biochemistry, 20:114:149.
Tables for estimating sedimentation through linear concentration gradients of sucrose solution.

M^cLaughlin, C.J. (1974).
In Ribosomes (Edited by Nomura, M., Tissieres, A. and Lengyel, P.). 815-827. New York:Cold Spring Harbour Laboratory.
Yeast ribosomes: Genetics.

Middlekauf, J.E., Hino, S., Young, S.P., Lindergren, C.C. & Lindergren,G. (1957).
Genetics, 42:66-71.
Gene control of resistance vs. sensitivity to actidione in Saccharomyces.

Neuhäuser, A., Klingmüller, W. & Kandewitz, F. (1970).
Molecular and General Genetics, 106:180-194.
Selektion actidion-resistenter mutanten bei Neurospora crassa sowie ihre genetische und biochemische analyse.

Nieuwenhuysen, P., De Herdt, E. & Clauwaert, J. (1978),
Analytical Biochemistry, 88:532-538
Ribonucleic acid and protein content of eukaryotic ribosomes isolated from Artemia salina.

Nirenberg, M.W. & Matthaei, J.H. (1961).
Proceedings of the National Academy of Science (USA), 47:1588-1602.
The dependance of cell-free protein synthesis in Escherichia coli upon naturally occurring or synthetic polyribonucleotides.

North, J. (1978).
Heredity, 40:331.
Modification of dominance of a cycloheximide resistance mutation in Coprinus cinereus.

North, J. (1982).
Journal of General Microbiology, 128:2747-2753.
A dominance modifier for cycloheximide resistance in Coprinus cinereus.

Oakley, B.R. , Kirsch, D.R. & Morris, N.R. (1980).
Annals of Biochemistry, 105: 361-363.
A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels.

Ofengand, J. (1977).
In Molecular mechanisms of protein biosynthesis, 8-80.
(Edited by Weissbach,H. & Pestka, S.)
tRNA and aminoacyl-tRNA synthetases.

Orton, P.D. (1957).
Transactions of the British Mycological Society, 40:263-276.
Notes on British Agarics 1-5.(Observations on the genes Coprinus).

Pestka, S. (1974).
Methods in Enzymology, 30:462-470.
Assay for nomenzymatic and enzymatic translocation with Escherichia coli ribosomes.

Pestka, S. (1977).
In Molecular mechanisms of protein biosynthesis, 468-555.
(Edited by Weissbach, H., and Pestka, S.)
Inhibitors of protein biosynthesis.

Peterson, G.L. (1979).
Analytical Biochemistry, 100:201-220.
Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall,

Petermann, M.L. (1964)
The physical and chemical properties of ribosomes.
(Elserier:Amsterdam and London)

Pirt, S.J. (1973).
Journal of General Microbiology, 75:245-247.
Estimation of substrate affinities (KS values) of filamentous fungi from colony growth rates.

Pöche, H., Zakrewski, S. & Nierhaus, K.H. (1979).
Molecular and General Genetics, 175:181-185.
Resistance against cycloheximide in cell lines from Chinese Hamster and Human cells is conferred by the large subunit of cytoplasmic ribosomes.

Pollard, C.J. (1964).
Biochemical and Biophysical Research Communications, 17:171-176.
The specificity of ribosomal ribnucleic acids of plants.

Pongratz, M. and Klingmuller, W. (1973).
Molecular and General Genetics 124:359-363.
Role of ribosomes in cycloheximide resistance.

Pontecorvo, G. (1952).
Symposium of the Society of Experimental Biology, 6:218-229.
Genetic analysis of cell organisation.

Pontecorvo, G. (1963).
Proceedings of the Royal Society of Biology, 158:1-23.
Microbial genetics; retrospect and prospect.

Rajalashmi, S., Liang, H., Sarma, D.S.R., Kisilevsky, R. & Farber, E.(1971).
Biochemical and Biophysical Communications,42:259-265.
Cycloheximide, an inhibitor of peptide chain termination or release in liver in vivo and in vitro.

Rao, S.S. and Grollman, A.P. (1967).
Biochemical and Biophysical Communications, 29:696-704.
Cycloheximide resistance in yeast: A property of the 60S ribosomal subunit.

Revel, M. (1977).
In Molecular mechanisms of protein biosynthesis, 246-323.
(Edited by Weissbach, H. and Pestka, S.)
Initiation of messenger RNA translation into protein and some aspects of its regulation.

Reyes, R., Vazquez, D. & Ballesta, J.P.G. (1978).
Biochimicia et Biophysica Acta, 521:229-234.
Structure of the yeast ribosomes. Proteins associated with the rRNA.

Roberts, B.E. & Paterson, B.M. (1973).
Proceedings of the National Acadamy of Sciences (USA), 70:2330-2334.
Efficient translation of Tobacco Mosaic Virus RNA and Rabbit Globin 9S RNA in a cell-free system from commercial wheat germ.

Roberts, C.T. and Orias, E. (1973).
Experimental Cell Research, 81:312-316.
A cycloheximide resistant mutant in Tetrahymena pyriformis.

Rothschild, H., Germershausen, J. & Suskind, S.R. (1975).
Biochemical Genetics, 13:283-300.
Biochemical genetic studies of cycloheximide resistance in Neurospora crassa.

Russell, P.J. & Wilkerson, W.M. (1980).
Experimental Mycology, 4:231-337.
The structure and biosynthesis of fungal cytoplasmic ribosomes.

Senathirajah, S. & Lewis, D. (1975).
Genetical Research, 25:95-107.
Resistance to amino-acid analogues in Coprinus dominance modifiers and dominance reversal in diploids and dikaryons.

Shafritz, D.A. & Anderson, W.F. (1970).
The Journal of Biological Chemistry, 245:5553-5559.
Isolation and partial characterisation of reticulocyte factors M_1 and M_2.

Sherton, C.C. and Wool, I.G. (1974).
Molecular and General Genetics, 135:97-112.
The extraction of proteins from eukaryotic ribosomes and ribosomal subunits.

Siegel, M.R. & Sisler, H.D. (1963).
Nature, 200:675-676.
Inhibition of protein synthesis in vitro by cycloheximide.

Siegel, M.R. and Sisler, H.D. (1964).
Biochimicia et Biophysica Acta, 87:83-89.
Site of action of cycloheximide in cells of Saccharomyces pastorianius.
II. The nature of inhibition of protein synthesis in a cell-free system.

Siegel, M.R. and Sisler, H.D. (1965).
Biochemica et Biophysica Acta, 103:558-567.
The site of action of cycloheximide in cells of Saccharomyces pastorianius.
III. Further studies on the mechanism of action and the mechanism of resistance in Saccharomyces species.

Siegel, M.R. (1977).
In Antifungal compounds 2:399-438.
(Edited by Siegel, M.R. & Sisler H.D.)
Effect of fungicides on protein synthesis.

Sisler, H.D. & Siegel, M.R. (1967).
In Antibiotics; Mechanism of Action, 1:283-307.
(Edited by Gottlieb, D. & Shaw, P.; New York: Springer-Verlag).
Cycloheximide and other glutarimide antibiotics.

Sissons, C.H. (1974).
Biochemical Journal, 144:131-140.
Yeast protein synthesis. Preparation and analysis of a highly active cell-free system.

Smythe, R. and Anderson, G.E. (1971).
Journal of General Microbiology, 66:251-253.
Electrophoretic protein spectra of wild-type and isogenic monokaryons of Coprinus lagopus.

Stöcklein, W. & Piepersberg, W. (1980).
Current Genetics, 1, 177-183.
Altered ribosomal protein L29 in a cycloheximide-resistant strain of Saccharomyces cerevisiae.

Stöcklein, W. & Piepersberg, W. (1981).
Federation of European Biochemical Societies Letters, 136; 265-268.
Amino acid replacements in ribosomal protein YL24 of Saccharomyces cerevisiae causing resistance to cycloheximide.

Sutton, C.A., Ares, M. and Hallberg, R. (1978).
Proceedings of the National Academy of Sciences, 75:3158-3162.
Cycloheximide resistance can be mediated through either ribosomal subunit.

Swedes, J.S., Dial, M.E. & M^{c}Lauglin, C.S. (1979).
Journal of Bacteriology, 138:162-170.
Regulation of protein synthesis during energy limitation of Saccharomyces cerevisiae.

Tabor, C.W. and Tabor, H, (1976).
Annual Review of Biochemistry, 45:285-306.
1,4-Diaminobutane (putrescine), spermidine and spermine.

Thuriaux, P., Minet, M., Hofer, F. and Leupold, U. (1975).
Molecular and General Genetics, 142:251-261.
Genetic analysis of anti-suppressor mutants in the fission yeast Schizosaccharomyces pombe.

Timberlake, W.E., M^{c}Dowell, L. & Griffen, D.H. (1972).
Biochemical & Biophysical Communications, 46:942-947.
Cycloheximide inhibition of the DNA-dependant RNA-polymerase I of Achlya bisexualis.

Timberlake, W.E. & Griffen, D.H. (1974).
Biochimica et Biophysica Acta, 349:39-46.
Differential effects of analogs of cycloheximide on protein and RNA synthesis in Achlya.

Trakatellis, A.C., Montjar, M. & Axelrod, A.E. (1965).
Biochemistry, 4:2065-2071.
Effect of cycloheximide on polysomes and protein synthesis in the mouse liver.

Tsurugi,K., Collatz, E., Wool, I.G. and Lin, A. (1976).
Journal of Biological Chemistry, 254:7940-7946.
Isolation of eukaryotic ribosomal proteins. Purification and characterisation of the 60S ribosomal subunit proteins.

Van der Decken, A. (1967).
In Techniques in Protein Biosynthesis, 1:65-131.
(Edited by Campbell, P.N. & Sargent, J.R.)
Methodological aspects of protein synthesis in mammalian species.

Vazquez, D. (1979).
Molecular Biology, Biochemistry and Biophysics, 30:155-158.
(Edited by Kleinzeller, A., Springer, G.F. and Wittmann, H.G.)
Inhibitors of protein biosynthesis.

Veal, D. & Casselton, L.A. (1982).
Heredity, 48:324.
Resistance to 5-fluoroindole in Coprinus.

Vomvoyanni, V. (1972).
PhD.Thesis, University of Leeds.
Genetical and biochemical studies on mutant strains of Neurospora crassa resistant to cycloheximide.

Vomvoyanni, V. (1974).
Nature, 248:508-510.
Multigenic control of ribosomal properties associated with cycloheximide sensitivity in Neurospora crassa.

Waldron , C. and Roberts, C.F (1974).
Molecular and General Genetics, 134:115-132.
Cold-sensitive mutants in Aspergillus nidulans. II Mutations affecting ribosome production.

Warner, J.R. (1974).
In Ribosome, 461-488.
(edited by Nomura, M., Tissieres, A. & Lengyel, P.; Cold Spring Harbour Laboratory, New York).
The assembly of ribosomes in eukaryotes.

Warr, J.R. & Roper, J.A. (1965).
Journal of General Microbiology, 40:273-281.
Resistance to various inhibitors in Aspergillus nidulans.

Watson, J.D. (1964).
Bulletin of the Society of Chemical Biology, 46:1399-1425.
Synthesis of proteins upon ribosomes.

Weber, L.A., Hickey, E.D. Maroney, P.A. and Baglioni, C. (1977).
Journal of Biological Chemistry, 252:4007-4010.
Inhibition of protein synthesis by Cl.

Wescott, E.W. and Sisler, H.D. (1964).
Phytopathology, 54:1261-1264.
Uptake of cycloheximide by a sensitive and resistant yeast,

Whiffen, A.J., Bohonos, N. & Emerson, R.L. (1946).
Journal of Bacteriology, 52:610-611.
The production of an antifungal antibiotic by Streptomyces griseus.

Whiffen, A.J. (1948).
Journal of Bacteriology, 56:283-291.
The production, assay and antibiotic activity of actidione, an antibiotic from Streptomyces griseus.

Whiffen, A.J. (1950).
Mycologia, 42:253-258.
The activity in vitro of cycloheximide (actidione) against fungi pathogenic to plants.

Wilkie, D. & Lee, B.K. (1965).
Genetical Research, 6:130-138.
Genetic analysis of actidione resistance in Saccharomyces cerevisiae.

Woodward, W.R., Ivey, J.L. and Herbert, E. (1974).
Methods in Enzymology, 30:724-731.
Protein synthesis with rabbit reticulocyte preparations.

Wool, I.G. (1979).
Annual Review of Biochemistry, 48:719-754.
The structure and function of eukaryotic ribosomes.

Wu,M.M.J., Rhodes-Cassidy,J. & Pukkila,P.J. (1983).
Current Genetics, 7 385-392.
Polymorphisms in DNA of Coprinus cinereus.

Zinker, S. & Warner, J.R. (1976).
Journal of Biological Chemistry, 251:1799-1807
The ribosomal proteins of Saccharomyces cerevisiae: phosphorylated and exchangeable proteins.

D527804

END