Biochemical and Molecular Genetic Studies on Acetate Utilization in Neurospora crassa

A thesis submitted by

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for the degree of

Philosophiae Doctor

Institute of Animal Genetics

University of Edinburgh

January 1984

I declare that this thesis has been composed by myself and that the work described herein is my own except where acknowledged in the text.

ACKNOWLEDGEMENTS

It is my privilege and pleasure to thank the following people for their help in this work.

I sincerely thank Professor John R.S. Fincham for originally suggesting the project, for his excellent supervision, and for reading the entire manuscript of this thesis making many helpful suggestions, and Dr. R.L. Baxter for throwing his enthusiasm and expertise into the NMR work.

I would also like to thank Mrs. Margaret A. Keighren, Dr. Kathleen Borck and especially Dr. Jane H. Kinnaird for their help and guidance as I learnt to use various techniques described in this thesis.

I am also grateful for occasional technical assistance from Mrs. Annette J.B. Campbell, and to the staff of the photographic unit, Mr. Alan McEwan and Mr. Frank Johnston (who demonstrated astonishing perseverance with Plate 4.18), for their help in the preparation of the prints, and to the many people in the Genetics department with whom I have had many fruitful discussions.

Finally I would like to thank the Medical Research Council for their studentship which supported me during the course of this work.

SUMMARY

Acetate adaptation in Neurospora crassa was studied.

1) A series of medium transfer and starvation experiments (using sucrose, acetate and carbonless media) failed to show whether the primary regulatory mechanism for the glyoxylate cycle and gluconeogenic enzymes was mediated via induction or repression. Both isocitrate lyase and PEP carboxykinase activities showed an increase on starvation but this response was shown to depend on the growth state of the mycelium, and the two enzymes responded differently in some cases. A role for storage triacylglycerides in the adaptation process is suggested.

2) Nuclear magnetic resonance (NMR) has been used to follow the fate of [2-^{13}C]-sodium acetate in wild type and acetate non-utilizing strains transferred from sucrose to acetate medium. The results can be interpreted in terms of a three phase model for the overall sequence of the adaptation process. The first phase is characterised by the accumulation of alanine presumably as a response to an increasing pyruvate pool. This latter, it is suggested, arises from a residual glycolytic flux combined with acetyl-coA inhibition of pyruvate dehydrogenase. In the second phase the glyoxylate cycle is active in the absence of gluconeogenesis, whilst in the third the essentially fully adapted mycelium, with a large gluconeogenic flux, synthesizes the disaccharide trehalose.

3) The NMR experiments suggest a re-interpretation of the lesion in the acp strain isolated by Rao & deBusk (Biochem. Biophys. Acta. 470(1977)475-83). It is suggested that, rather than being defective in a cellular acetate-inducible permease, this mutant is impaired in some glyoxysome specific function or in the organelle's biogenesis.

4) The NMR experiments also support the tentative conclusion of Flavell & Fincham (J. Bacteriol. 95(1968)1063-8) that acu7 is defective in the enzyme 2-oxoglutarate dehydrogenase.

5) In vitro translation of mRNA isolated from sucrose grown and acetate adapted mycelium revealed several acetate-specific polypeptide bands. One of these, with an apparent molecular weight of 67,000, was shown to co-migrate with purified isocitrate lyase and another of 60,000 daltons co-migrates with purified PEP carboxykinase. The latter also has the same size as the subunit molecular weight of malate synthase (previously reported in the literature).

6) Molecular cloning experiments have resulted in the isolation of five Neurospora genomic clones by a differential screen using end-labelled mRNA isolated from sucrose grown and acetate adapted mycelium. The major transcripts corresponding to three of these are detectable only in mRNA of acetate-adapted mycelium. The fourth shows some expression in sucrose grown mycelium and is increased in acetate-adapted mycelium. The fifth shows the reverse behaviour; decreased expression on acetate medium.

7) Hybrid selection experiments using one of the three sequences transcribed only in response to acetate yeilded an mRNA which translated *in vitro* to give a 60,000 dalton polypeptide which matched in size with both the polypeptide monomer of PEP carboxykinase (purified in this study) and that of malate synthase (previously reported in the literature).

8) The *acu7* lesion, known to cause over-production of enzymes produced during adaption to acetate, was shown to cause an increase in the amount of RNA complementary to the acetate-specific clones.

ABBREVIATIONS

A_x	absorbance at x nm
ADP	adenosine-5′-diphosphate
ala	alanine
AMP	adenosine-5′-monophosphate
asp	aspartate
ATP	adenosine-5′-triphosphate
cDNA	DNA synthesized on an RNA template
cit	citrate
CoA	coenzymeA moiety
CoA-SH	free reduced coenzymeA
dATP	2-deoxyadenosine-5′-triphosphate
dCTP	2-deoxycytosine-5′-triphosphate
DEAE	diethyl amino ethyl
dGTP	2-deoxyguanosine-5′-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dTTP	2-deoxythymidine-5′-triphosphate
EDTA	ethylene diamine tetra-acetic acid
gl	glycerol
gln	glutamine
glu	glutamate
G6P	glucose-6-phosphate
kb	kilobase pairs
mal	malate
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance

PEP	2-phosphoenol pyruvic acid
P_i	inorganic orthophosphate
PP_i	pyrophosphate
ppm	parts per million
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
suc	succinate
TCA	tricarboxylic acid
Tris	tris-(hydroxymethylamino)-methane

AMN	^{13}C-acetate medium
BSA	Bovine serum albumen
dH_2O	distilled water
DTT	dithiothreitol
EB	elution buffer
EEO	electro-endo-osmotic
FID	free induction decay
HB	hybridization buffer
LB	L-broth
$mRNA^a$	poly(A)$^-$mRNA prepared from adapted mycelium
$mRNA^s$	poly(A)$^-$mRNA prepared from sucrose grown mycelium
SET	0.15M NaCl- 30mM Tris.Cl pH8- 2mM EDTA
SSC	0.15M NaCl- 0.015M sodium citrate

ENZYME COMMISSION NUMBERS

The following enzymes have been mentioned in the discussion of the work in this thesis.

a) TCA cycle

i) citrate synthase (EC 4.1.3.7)

oxaloacetate + acetyl-CoA + H_2O ---> citrate + CoA-SH

ii) aconitase (EC 4.2.1.3)

citrate <---> cis-aconitate + H_2O

iii) NAD-linked isocitrate dehydrogenase (EC 1.1.1.41)

isocitrate + NAD^+ <---> 2-oxoglutarate + NADH + H^+

iv) NADP-linked isocitrate dehydrogenase (EC 1.1.1.42)

isocitrate + $NADP^+$ <---> 2-oxoglutarate + NADPH +H^+

v) 2-oxoglutarate dehydrogenase (EC 1.2.4.2)

2-oxoglutarate + CoA-SH + NAD^+ <---> succinyl-CoA + CO_2 + NADH + H^+

vi) succinyl-CoA synthetase (EC 6.2.1.5)

succinyl-CoA + GDP + P_i <---> succinate + GTP + CoA-SH

vii) succinate dehydrogenase (EC 1.3.99.1)

succinate + FAD <---> fumarate + $FADH_2$

viii) fumarase (EC 4.2.1.2)

fumarate + H_2O <---> malate

ix) malate dehydrogenase (EC 1.1.1.37)

malate + NAD^+ <---> oxaloacetate + NADH + H^+

b) Glyoxylate cycle

i) isocitrate lyase (EC 4.1.3.1)

isocitrate <---> succinate + glyoxylate

ii) malate synthase (EC 4.1.3.2)

glyoxylate + acetyl-CoA + H_2O <---> malate + CoA-SH

c) Gluconeogenesis

i) PEP carboxykinase (ATP) (EC 4.1.1.32)

oxaloacetate + ATP <---> PEP + CO_2 + ADP

ii) fructose-1,6-diphosphatase (EC 3.1.3.11)

fructose-1,6-diphosphate + H_2O <---> fructose-6-phosphate + P_i

d) Others

i) acetyl-CoA synthetase (EC 6.2.1.1)

ATP + coenzymeA + acetate <---> AMP + acetyl-CoA + PP_i

ii) pyruvate carboxylase (EC 6.4.1.1)

pyruvate + CO_2 + ATP <---> oxaloacetate + ADP + P_i

iii) pyruvate dehydrogenase (EC 1.2.4.1)

pyruvate + NAD^+ + CoA ---> acetyl-CoA + CO_2 + NADH + H^+

iv) malic enzyme (EC 1.1.1.40)

pyruvate + CO_2 + NADPH + H^+ <---> malate + $NADP^+$

v) alanine aminotransterase (EC 2.6.1.2)

glutamate + pyruvate <---> 2-oxoglutarate + alanine

vi) NADP-linked glutamate dehydrogenase (EC 1.4.1.4)

glutamate + $NADP^+$ + H_2O <---> 2-oxoglutarate + NH_4^+ + NADPH + H^+

vii) NAD-linked glutamate dehydrogenase (EC 1.4.1.2)

glutamate + NAD^+ + H_2O <---> 2-oxoglutarate + NH_4^+ + NADH + H^+

CONTENTS

CHAPTER 1

Literature Review

INTRODUCTION

The opening sentences in the theses of my two predecessors who worked on acetate utilization in Neurospora are:-

i)"During the last twenty five years rapid advances have been made in the understanding of gene action and how it is controlled" -R.B.Flavell (1967).

ii)"During the last twenty five years the science of biology has been revolutionised by the elucidation, at least in broad terms, of many of the processes underlying the phenomenon of heredity" -R.E.Beever (1972).

Either of these would probably suffice for this thesis, but without the 'twenty'. Not long after Beever's thesis was written the beginnings of what has become known as 'recombinant DNA technology' began to evolve. This was truly a revolutionary development since it resulted in a completely new set of tools and methods with which to probe genes and their expression. After 1977, with the appearance of two rapid DNA sequencing techniques (Sanger et al, 1977; Maxam & Gilbert, 1977), the rate of progress has been nothing short of staggering.

Regulation of gene action can occur at many levels (see Darnell, 1982 for review), but it seems pertinent in this introduction to limit myself to a review of only the levels that are likely to be

accessible through the techniques used in this study. I will therefore first examine some of the better known fungal regulatory networks, investigated at the genetic and physiological level, and then briefly review some of the known regulatory sequences in and around genes that have been found after gene cloning and sequencing, before finally introducing acetate utilization, the subject of study in this thesis.

SECTION I

Genetic Investigations

In moving up the ladder of evolutionary complexity from prokaryotes to higher eukaryotes, fungi have proved extremely useful organisms for genetic study. Their organisation is sufficiently advanced to make them useful model eukaryote systems and yet they can be manipulated almost with the ease of a prokaryote. Powerful genetic techniques can be combined with molecular genetics in these organisms to great effect. This section aims to try and indicate the general strategy behind the regulatory processes in these organisms. Because of the ease of selection for auxotrophs, most of the better known regulatory networks concern general enzymatic metabolism rather than developmental pathways.

The regulatory systems can be roughly divided into two categories. Both types come under general or master controls. The first concerns the regulation of catabolic pathways, which are generally switched on in response to their substrate, and the second concerns the regulation of anabolic pathways which are normally switched off by their product. Most of the studies are on the former type and this bias is reflected in this review.

Master control systems are especially prominent amongst the catabolic systems and serve to coordinate the use of several substrates when several are present; often one is 'preferred' for biochemically rational reasons. Such systems also exist for anabolic pathways but their exact mechanism is obscure.

Two recurrent themes are apparent in the regulatory systems I will discuss. First is the predominance of positive rather than negative control systems. Metzenberg (1979) has argued that positive regulation requires 3-400 fold less regulatory protein than negative control and that this helps to decrease the osmotic pressure that has to be borne by the organism. The second theme is the frequent regulation of pathways by master genes that operate by inducer exclusion. That is, rather than repressing all the genes in a pathway directly the permease for the substrate is repressed so that induction cannot occur.

Master Controls

Nitrogen metabolism

Most of the catabolic hierarchies are organised around the assimilation of one element (C,N,S,P, etc.) from the available substrates. The best characterised of these is in Aspergillus nidulans and concerns nitrogen acquisition.

The master gene in this network is designated areA (for ammonia regulation, Arst & Cove, 1973; Arst, 1981), and mutations at this locus have a variety of effects, all of them pleiotropic. $areA^{R}$ (repressed) mutants lack the ability to utilize many nitrogen sources available to wild type mycelium (e.g. nitrate, nitrite, hypoxanthine and other purines, many amino acids, formamide, acetamide, casein) while the use of ammonium is largely unaffected. All these nitrogen sources show a common phenomenon, that of ammonium repression; although these sources will induce the enzymes for their use, they will only do so in the absence of ammonium ions.

The latter is apparently used in preference to the others (at least when both are in excess), and since the end product of these assimilatory pathways is ammonium (or glutamate to which ammonium is assimilated in one step) it seems reasonable not to waste energy producing it if it is already there.

Another, less frequent class, of mutants are designated *areA*^{D} (derepressed) which are derepressed for the enzyme activities needed for the use of the above compounds as nitrogen sources (N.B. they may still require induction). Further, both *areA*^{R} and *areA*^{D} alleles can show different pleiotropic effects depending on which particular variant of each is considered. Thus, *areA*^{R}-1 grows worse on nitrate, nitrite, proline and hypoxanthine at 37^{o}C than at 25^{o}C whilst showing no such sensitivity on other nitrogen sources; *areA*^{R}-2, however, is only temperature sensitive on proline and formamide. Similarly *areA*^{D} alleles may show different derepression spectra. In spite of this diversity of mutant phenotypes the locus behaves as a single gene.

Dominance relationships show that *areA*^{R} is recessive to wild type, whilst *areA*^{D} is dominant to a degree dependant upon the allele in question (but is never fully so). *areA*^{R} also shows some interaction with *areA*^{D}, again in an allele-dependent manner, and is epistatic to constitutive mutants in the regulatory genes of individual pathways (e.g. *nirA*^{C} in nitrate utilization- see later). The best model consistent with these data calls for a positively acting regulatory protein specified by the *areA* locus which, in the absence of ammonium, allows the induction of the various pathways by

their substrates (or metabolites thereof). It would mediate this by binding to regulatory sites on the DNA, and would be prevented from doing so by the presence of ammonium ions. The differential effects of various pleiotropic mutations would reflect changes which alter the regulatory proteins affinity for the various loci in different ways. The most frequent class of mutant would be expected to disrupt the positive action of the protein thus preventing the induction of several pathways (i.e. $\underline{areA}^{R}$). A less frequent class is also expected which has lost its affinity for the co-repressor, be it ammonium or a metabolite thereof. This mutant protein would be unable to release from the DNA and so ammonium repression would be lost. This is thought to be the explanation for the $\underline{areA}^{D}$ alleles. They should be infrequent since they must not affect DNA binding ability.

A modifier of areA action, areB, has recently been isolated but it action is uncertain (Tollervey & Arst, 1982).

areA-mediated regulation also interacts with carbon catabolite repression where substrates can act as a source of both nitrogen and carbon (Arst & Cove, 1973; Hynes, 1972, 1973; amdT is allelic to areA, Arst & Cove, 1973; Cove, 1976). This is discussed in the next section.

A gene which seems to be the Neurospora counterpart of areA is nit2 (Reinert & Marzluf, 1975; Facklam & Marzluf, 1978; also known as amr), although its specific interactions with pathway-specific regulatory pathways are less well characterised than those of the

areA locus. In nit2, as with areA mutants there is a pleiotropic loss of the enzymes concerned with ammonium production from amino acids, purines, nitrate and extracellular protein. Again a positive role in the regulation of ammonium repression is indicated.

Although the above examples have become known as ammonium regulation there is now a considerable body of evidence to suggest that the true co-repressor is in fact glutamine. In Neurospora mutants defective in the glutamine synthetase enzyme (gln-1a) ammonium can no longer exert its repressive effects (Premakumar et al, 1979; Dunn-Coleman et al, 1979), whereas glutamine can. In a strain carrying a more extreme allele, gln-1b, glutamine is less effective as a repressor (Dunn-Coleman & Garrett, 1981) and so a regulatory role for the enzyme itself has been proposed. A similar situation is apparent in Aspergillus (MacDonald, 1982).

The oligomeric state of glutamine synthetase from Neurospora varies dependent upon the nitrogen supply (Dunn-Coleman & Garrett, 1980, 1981). Under conditions of nitrogen sufficiency it is an octamer of molecular weight 400,000, whilst under conditions of nitrogen limitation it is a tetramer. The octamer was proposed to inactivate or repress the nit2 gene product but the evidence is inconclusive.

Carbon Metabolism

Just as some nitrogen sources are subject to repression in the presence of ammonium, many carbon sources (e.g. ethanol) will not be used if glucose or other readily utilizable carbon sources are

repression has been extensively studied and has proved to be very complex. The regulatory genes involved fall into two main categories (Entian & Zimmerman, 1982), those in which mutation prevents repression of carbon catabolite repressible enzymes (loci HEX1, HEX2, CAT80) and those in which mutation prevents derepression of several enzymes (loci CAT1, CAT3, CCR2, CCR3). Another gene CAT2 falls loosely into the later category since it causes accelerated derepression of the enzymes. Mutations in both the HEX genes affect hexose phosphorylation (Entian & Zimmerman, 1980). hex1 mutations decrease glucose phosphorylation (Entian et al, 1977; Entian & Zimmerman, 1980) and this locus is the structural gene for hexokinase isozyme PII (Entian, 1980). hex2 mutants, on the other hand, increase glucose phosphorylation by increasing the production of hexokinase PII (Entian & Zimmerman, 1982). Mutation at a third locus, CAT80, prevents the hexokinase PII increase in hex2 mutants and, on their own, cat80 mutations do not affect phosphorylating activity. All three mutations prevent the repression of invertase and malate dehydrogenase. Additionally hex1 and hex2 mutations also affect respiration, and the regulatory influence of HEX2 also extends to maltase and maltose uptake (Entian & Zimmerman,1982).

The other set of mutants, cat1, cat3, ccr2 and ccr3 (Ciriacy, 1977; Zimmerman et al, 1977) are all pleiotropically deficient in their ability to derepress the enzymes of the glyoxylate cycle and gluconeogenesis (op cit; Entian & Zimmerman, 1982). In addition to this ccr2 and ccr3 prevent full development of respiratory activity (Ciriacy, 1977) and cat1 and cat3 affect maltase synthesis, thus overlapping with the HEX2 action spectrum. The CAT1 locus as well

as mutating to the recessive cat1 phenotype, which prevents derepression, can also mutate to a dominant allele which, like cat2, accelerates the derepression of the repressible enzymes after glucose exhaustion (Entian & Zimmerman, 1982).

This regulatory system is evidently very complex and no coherent model has been produced yet. It will be interesting to see if the network is as complex in other organisms.

Sulphur Metabolism

A superficially similar sort of situation to that of creA is seen with a gene designated cys3 in Neurospora. Mutations at this locus lead to the loss of expression of several gene products which are produced on sulphur starvation. These include sulphate permeases, a choline-o-sulphate permease, choline sulphatase and aryl sulphatase (Metzenberg, 1979; Marzluf & Metzenberg, 1968; Burton & Metzenberg, 1972; Marzluf, 1972). cys3 appears, like areA and creA, to be a positively acting regulator but another gene appears to be involved as well. The scon locus mutates to give a constitutive phenotype for all the enzymes and permeases lost in the cys3 lesion (Burton & Metzenberg, 1972). The cys3 phenotype is epistatic to $scon^{C}$ mutations and this suggests that scon is either responsible for producing the co-repressor of the cys3 enzyme family or that its product in some way interacts with the cys3 product, or with cys3 directly preventing the expression of the above activities, possibly itself recognising the co-repressor. The situation may be more complex than this, since methionine repressibility of the above activities is lost by mutation at a separate locus eth1 (Marzluf &

Metzenberg, 1968).

The cys3-scon^{C} relationship has some parallels with phosphorus regulation (see below).

Phosphorus metabolism

Here again one finds a gene that is apparently in charge of a whole family of structural loci in Neurospora- nucl. Phosphorus starvation causes the derepression of at least eight enzymes (alkaline & acid phosphatases, three nucleases, a 5'-nucleotidase, a high affinity phosphate permease and a phosphoethanolamine permease), all of which act extracellularly or in the membrane to scavange for phosphorus and convert it to either inorganic phosphate or phosphoethanolamine, the only two forms of phosphorus to which Neurospora is known to be permeable. There are also constitutive equivalents of the above enzymes which are cytosolic (Metzenberg, 1979; Lehman et al, 1973; Metzenberg & Chia, 1979; Metzenberg & Nelson, 1977; Grove & Marzluf, 1980). The signal to which these enzymes respond may be pyrophosphate level (Stellwag et al, 1982), since this is the only phosphorus pool which consistently changes in accordance with the mycelial phosphorus status. nucl mutations prevent the derepression of the above activities.

The regulation of this system is considerably more complex than this one gene. Three other loci, nuc2, preg and pgov (op cit) are known which are involved in a so-called cascade control. Few data have been published on pgov (unpublished data were mentioned in a recent review, Metzenberg, 1979; and in Stellwag et al, 1982), but

its behaviour is apparently like that of preg, although they map at different loci. It has been suggested that they may act in parallel. Mutations at preg cause the constitutive expression of all the above enzymes, while the majority of the mutants at the nuc2 locus pleiotropically lose these activities. Other lesions apparently allelic to nuc2 and originally designated $pcon^{C}$, are constitutive for all these enzymes.

In double mutants nuc1 is epistatic to all the other regulatory loci (i.e. preg, pgov & nuc2/$pcon^{C}$), whilst preg and pgov are epistatic to nuc2. Put together this leads to an operational hierarchy in which, it is postulated, the nuc2 product responds to phosphorus starvation by inhibiting the preg product which, when active inhibits the positive action of the nuc1 product that promotes the expression of the enzymes previously mentioned.

It has been suggested that the preg-nuc1 interaction is a protein-protein one, that is the preg product does not directly affect nuc1 expression, but interacts with the protein produced by nuc1 either directly or on a cis-acting DNA site. The evidence for this comes from the one constitutive mutation known at the nuc1 locus. It turns out that the constitutivity of this mutant is dependent upon the dosage of the preg genes (and by implication its product). In a series of partial diploids it has been shown that with an increasing $preg^{+}$:$nuc1^{C}$ locus ratio, that the expression of the genes goes from constitutive to normally repressible. $nuc1^{C}$ has therefore been interpreted as an over-producer of the $nuc1^{+}$ protein which exceeds the controllable limit of one preg gene; increasing

the number of the latter titrates out the nucl product (Metzenberg & Chia, 1979; Metzenberg & Nelson, 1977).

A limited parallel can be drawn between the phosphorus and sulphur regulatory systems. It can be seen that cys3 and $scon^{C}$ possess a very similar relationship to one another as do nucl and $preg^{C}$, and it is tempting to suggest that there may be a common type of protein interaction in both systems. In yeast a similar relationship is seen in the regulation of galactose utilization (between the GAL80 product and the GAL4 positive effector, Matsumoto et al, 1978; Perlman & Hopper, 1979), and in phosphorus regulation (between the PHO80/85 product and the PHO82/4 positive effector, Toh-E et al, 1981).

The parallels between these various master control genes can only be drawn so far, however. The operation of areA in Aspergillus (& nit2 in Neurospora), in the absence of ammonium and that of creA, in the absence of a repressing carbon source also in Aspergillus, merely give 'permission' to transcribe the other genes, and this will only occur, in most cases, in the presence of the appropriate inducer. With cys3 and nucl and their associated systems, starvation for sulphur or phosphorus leads to derepression of the genes directly.

General control of amino acid biosynthesis

This last master control which I wish to discuss is an example of the integration of many biosynthetic pathways. In contrast to the

catabolic systems whose action is easily rationalised in biochemical terms the exact mechanism of operation of this network remains somewhat enigmatic. This is phenomenon is known as cross pathway regulation or general control of amino acid biosynthesis.

If in Neurospora, for example, a tryptophan auxotroph is starved for tryptophan then, as well a derepressing the tryptophan biosynthetic enzymes, the enzymes for arginine and histidine biosynthesis are also derepressed (Carsiotis & Jones, 1974). Furthermore starvation of a histidine auxotroph for histidine derepresses the same three sets of enzymes (Carsiotis et al, 1974). Starvation of the appropriate auxotrophic mutants for lysine and leucine has virtually no effect (Kemp & Flint, 1982), although it has recently been shown that some lysine auxotrophs do show the derepression response (H.J.Flint personal communication).

The signal for this effect is elusive. Whilst during starvation for histidine using the inhibitor 3-amino-1,2,4- triazole the histidine pool decreased, a similar experiment which inhibited tryptophan synthesis using indoleacrylic acid did not cause a fall in the tryptophan pool implying that amino acid pool size is not the signal (at least not the overall pool; Spurgeon & Matchett, 1977). However, both these inhibitors cause the accumulation of intermediates which are inhibitory to the amino-acyl tRNA synthetases of these two amino acids, and it was postulated that tRNA molecules themselves may be involved in the regulatory process.

A mutation which impairs this derepression (cpc1) has recently

been isolated (Barthelmess, 1982).

In Saccharomyces, a similar regulatory system is apparent (review Jones & Fink, 1982). Enzymes of histidine, methionine, cystein, tryptophan, phenylalanine and proline biosynthesis have been shown to be involved and again tRNA's have been implicated. A temperature-sensitive mutant in the isoleucyl-tRNA synthetase was shown to be derepressed and possessed decreased charged $tRNA^{ile}$ but unaffected amino acid pool levels.

Many mutations affect the regulation in yeast (op cit) and they fall into two classes; those which are constitutive for the enzymes (tra3, tra5, gen^{C}) and those which fail to derepress on starvation (aas1, aas2, aas3). Interestingly all of the constitutive mutations are epistatic to all the non-derepressible ones (even to pathway specific ones in the latter category) suggesting that in the absence of the negatively acting regulators that the positively acting ones are not required.

Regulation of individual pathways

Proline usage in Aspergillus

This system belongs to both the creA and areA repression systems (Cove, 1976), and is induced by the presence of proline in the medium. There are four genes involved in the entry and conversion of proline to glutamate, and these are found in a cluster on linkage group VIII (Arst & MacDonald, 1975; Arst et al, 1980; Arst & MacDonald, 1978). The genes specify two enzymes, proline oxidase (prnD) and pyrroline-5-carboxylate dehydrogenase (prnC), a specific

proline permease (prnB) and a gene specifying a positively acting regulatory product (prnA). The gene order is prnA prnD prnB prnC (Jones et al, 1981). A cis-acting regulatory region between prnD and prnB has been identified (prn^D), mutations in which relieve the effects of areA mediated repression (Arst et al, 1980). This areA mediated control is specifically aimed at the permease thus preventing induction by inducer exclusion. The prnA product mediates the induction by proline of prnD and prnC (and to a lesser extent prnB). Mutations at the prnA locus characteristically preclude any expression of prnD or prnC, and decrease the expression of prnB (at 37°C).

A cluster of genes in a eukaryote always provokes the question of whether they are transcribed into a polycistronic messenger and thus constitute an operon and the answer is usually no, the genes either turn out to be separately expressed or to be distinct domains of a multi-functional protein. However, in the proline cluster there does seem to be at least the vestiges of an operon like organisation in that there is some evidence for a dicistronic messenger (Arst & MacDonald, 1978). Deletions from within prnD to within prnB severely curtail the expression of prnC, something which prnD prnB double mutants do not, implying that a major transcript from prnB through into prnC may have been lost. This could be due to the loss of a promoter in the prn^D region. prnC is still expressed to some extent so there may be multiple transcripts from this region.

Nitrate utilization in Aspergillus and Neurospora

In Aspergillus two closely linked genes niaD and niiA specify

nitrate and nitrite reductase respectively, although other genes are required to produce molybdenum co-factors for these enzymes (the *cnx* loci).The genes are subject to ammonium repression mediated by *areA* and are inducible by nitrate and nitrite *via* the *nirA* gene, a positively acting regulatory element (Pateman & Cove, 1967; Cove 1969; Rand & Arst, 1977, 1978; Arst *et al*, 1979). Mutants at this locus may be of two types, $nirA^{C}$ which leads to constitutivity (these are the most common) and $nirA^{D}$ which remove ammonium repressibility (Tollervey & Arst, 1981) suggesting that the *nirA* and *areA* products may directly interact. The two genes are not thought to constitute an operon because their expression is non-coordinate and the two genes have been separated by an insertional translocation leaving their regulation unaffected, although *niiA* did then show a slight degree of constitutivity (Pateman & Cove, 1967; Arst *et al*, 1979).

It is interesting to note that although $areA^{R}$ alleles are generally epistatic to constitutive nir^{C} mutations this relationship can be reversed particularly when the $areA^{R}$ mutation is leaky (Rand & Arst, 1978). $nirA^{C/D}$ double mutants can also suppress $areA^{R}$ (Tollervey & Arst, 1981).

There is some evidence that *nirA* may be involved in regulating more than just the two reductases. Mutants at the *nirA* locus show a greatly reduced ability to use some other nitrogen sources, such as glutamate and ornithine, in the presence of nitrate (which they cannot use), whilst the use of other nitrogen sources such as uric acid remains unaffected. If *nirA* is involved in general regulation

of nitrogen metabolism it is clearly of a different range of compounds than those controlled by areA, perhaps a specific subset of them as with the case of intA (see later).

The niaD gene is also thought to provide part of the regulatory network by recognising the presence of nitrate, so this gene may be autogenously regulated. niaD mutations are of two types, $niaD^{C}$ which are constitutive for the expression of nitrite reductase and in which nitrate no longer has an inhibitory effect on the use of other nitrogen sources, and $niaD^{I}$ which lose only the nitrate reductase activity, but not its regulatory functions (Cove, 1976).

In Neurospora nitrate reductase is coded for by the nit3 locus and is induced by nitrate or nitrite, and is repressed by ammonium (Chang & Sorger, 1976; Coddington, 1976). As in Aspergillus, molybdenum co-factors have been implicated in nitrate reduction and the mutant nit1 appears to affect their formation. Two genes are known which affect the induction by nitrate, nit4/5, mutations in which lead to non-inducibility (Coddington, 1976; nit4 and nit5 are allelic, Tomsett & Garrett, 1980) and the locus nit2 which mediates ammonium repression, probably by inducer exclusion as nit2 mutants lose their permeability to nitrate (Metzenberg, 1979; Reinert & Marzluf, 1975; Coddington, 1976).

Nitrate reductase in Neurospora may also be autogenously regulated (Tomsett & Garrett, 1981) since mutants in this gene (and others affecting its co-factors) lead to the constitutive expression of nitrite reductase.

Acetamidase - multiple independent controls

The locus for acetamidase, amdS, in Aspergillus is regulated in an extremely complex manner in response to several signals (Hynes, 1972, 1977, 1978ab). This enzyme is induced by source of acetyl-CoA (acetamide, acetate, threonine), benzoate and benzamide, ω-amino acids (in particular β-alanine), and is subject to both ammonium and carbon catabolite repression (op cit, Cove, 1976).

A plethora of cis-acting regulatory mutations are known for this gene, most such being isolated as suppressors of mutations in various regulatory genes. Two, amdI18 and amdI205, have general promoter up and down effects respectively with no specificity for any regulatory signal in particular. amdI9 causes an enhanced response to induction by acetyl-CoA sources only, whilst amdI93, as well as having a general down-promoter effect, specifically affects the induction by ω-amino acids.

Two unlinked regulatory genes are known for this locus, apart from areA and creA; these are intA (= amdR) which mediates induction by w-amino acids, and amdA whose exact function is not known, but mutations in which cause a general derepression of amdS without affecting the inducibility of the locus. It has been suggested that it may mediate induction by acetyl-CoA sources but there is no firm evidence.

It should be noted that none of the so-called independent effects is truly independent and that the effects predominantly affect one

or other control function. Parallel control is a better term which has been used (Arst, 1976), based on the fact that various combinations of the intA and areA mutants have strictly additive effects on enzyme activities.

The structural gene, amdS, has recently been cloned (Hynes et al, 1983) and so the molecular basis of the cis-acting mutations should be known before long.

ω-amino acid metabolism

The genes for the γ-amino-n-butyric acid (GABA) transaminase, gatA, and permease, gabA (and acetamidase- see above), are all regulated in part by the gene intA which responds to w-amino acids. B-alanine is the best inducer but GABA is also effective (Arst, 1976; Penfold & Arst, 1977; Bailey et al, 1979, 1980). gabA is also subject to areA repression (another case of inducer exclusion) and a closely linked cis-acting site, gabI, has been found that suppresses $areA^R$ mutants.

Two types of intA mutant are known. The first $intA^-$ halves acetamidase levels (in response to ω-amino acids) and prevents the use of GABA, β-alanine and δ-amino-n-valerate, as nitrogen sources and GABA as a carbon source. The second type of mutation at this locus, designated $intA^C$, suppresses the effect of $areA^R$ alleles on the use of acetamide as a nitrogen source and, as with areA, different alleles have varying pleiotropic effects. For example, $intA^C305$ affects only gabA whilst $intA^C304$ affects both gabA and gatA.

The control of ω-amino acid and amide utilization follows much the same pattern seen before with a gene mediating induction by a specific substrate (or class of substrates), and on top of this the influence of a ?repressive master control. It is evident that amdS falls into several regulatory systems and forms a point of overlap between ω-amino acid induction (intA) and induction by acetyl-CoA sources, which derepress a different group of enzymes (isocitrate lyase and, by implication the rest of the glyoxylate cycle enzymes; Hynes, 1977).

Quinate catabolism in Neurospora

As with Aspergillus, one of the most intensively studied systems in Neurospora is a cluster of four genes. These are concerned with quinate catabolism and lie in the order qa-1,3,4,2 on linkage group VII specifying a positively acting regulatory molecule, quinate dehydrogenase, 5-dehydroshikimate dehydrolase, and catabolic dehydroquinase respectively. The three enzymes are induced by dehydroquinate (via qa-1) and are subject to carbon catabolite repression. qa-1 mutants may either cause the pleiotropic loss of all three enzymes ($qa\text{-}1^{-}$) whilst $qa\text{-}1^{C}$ alleles cause constitutivity for the same three enzymes (Valone et al, 1971; Jacobson et al, 1977). These genes have recently been cloned by complementing the aroD6 mutation in E.coli, and two other previously unknown quinate regulated transcripts, qa-Y and qa-X have been identified in the cluster (Patel et al, 1981).

SECTION II

Regulatory sequences - promoters, enhancers and terminators

Eukaryotic nuclear genes fall into three distinct classes. These are characterised by transcription by RNA polymerase I, II or III (A, B or C) which are differentially sensitive to inhibition by α-amanatin (Roeder & Rutter, 1970). In mammals the order of sensitivity is I >III >>II but in insects and yeast RNA polymerase III tends to be more resistant than RNA polymerase I (see Lewin, 1980). RNA polymerase I is confined to the nucleolus where it transcribes ribosomal RNA. RNA polymerases II and III are found in the nucleoplasm, the former transcribing the bulk of non-ribosomal genes while the latter transcribes tRNA's, 5S RNA and some other small RNA's.

In the context of this introduction the most important sequences concerning a genes' regulation are those which facilitate the initiation of transcription by the appropriate RNA polymerase (promoters) and those which halt transcription and release the messenger (terminators). Sequences which may interact with regulatory proteins, known as operators in bacterial systems, are included with the promoters since the extent of either of these is not well defined at the sequence level.

Promoters are generally located on a sequence in two stages. First the 5' flanking sequences are sequentially deleted (either from the 3' or 5' direction), and this is followed by more subtle alteration of the sequences by one of a number of directed

mutagenetic techniques. The effects of each alteration are assessed either in an *in vitro* transcription system or "*in vivo*" after transforming or injecting the modified sequences into cells.

i)RNA polymerase I transcription units

Promoter: As noted above, the primary responsibility of this enzyme is the transcription of ribosomal RNA genes in the nucleolus. The essential promoter sequences for RNA polymerase I, delimited by deletion, lie within 150base pairs of the transcription start site and extend a few bases past it (-150 to +4 or +6; Sollner-Webb *et al*, 1983; Kohorn & Rae, 1983; Learned *et al*, 1983). This promoter has been subdivided in *Drosophila* into two important regions -43 to -27 and +1 to +4 (Kohorn & Rae, 1983). Deletion of either of these decreases transcription, and deletions in both decreases transcription even further. Similarly, in *Xenopus laevis*, regions around -75 and +1 are required (Sollner-Webb *et al*, 1983).

In *Xenopus*, however, sequences further upstream of this promoter are also important. The entire -145 to +4 region is duplicated at various locations in the spacer DNA between the rDNA repeats (Moss & Birnsteil, 1979; Sollner-Webb & Reeder, 1979) and part of this region from -119 to -72 is duplicated yet more times (see Moss, 1983). Deletion of these duplicated sequences decreases transcription some five to ten fold (Busby & Reeder, 1983) and they appear to act by attracting some transcription factor(s) or by acting as RNA polymerase I 'loading sites' since these sequences allow efficient competition for transcription (Moss, 1983).

Terminator: The 3′ end of the rRNA precursor in Xenopus maps to a T cluster in an AT-rich region and is preceeded by a sequence that contains a dyad symmetry and a GC-rich region (Sollner-Webb & Reeder, 1979). This shows some resemblance to RNA polymerase III termination sites (see below).

ii) RNA polymerase II transcription units

Promoter: Genes transcribed by RNA polymerase II produce the most diverse collection of products concerned with general cell construction, division, differentiation and metabolism. Consequently, although their promoters seem to have a common ground plan, they differ in detail presumably reflecting their differing regulation.

Comparison of the 5′ flanking sequences of many genes reveals the conserved sequence TATA$\frac{A}{T}T\frac{T}{A}$ some 30bases upstream of the start site, known as the TATA box (where $\frac{T}{A}$ means T or A at that position; see for example Corden et al, 1980). This sequence is essential for transcription in vitro (Hu & Manley, 1981; Grosveld et al, 1981; Mathis & Chambon, 1981; Tsai et al, 1981; Tsujimoto et al, 1981) but its in vivo role seems to be to direct initiation to the correct location since its deletion causes the production of novel 5′ ends (Grosschedl & Birnsteil, 1980a&b; Benoist & Chambon, 1980, 1981). However, point mutations in the TATA box can cause a quantitative decrease in transcription (Grosschedl et al, 1981). The TATA box region has recently been shown to bind a transcription factor necessary for accurate in vitro transcription (Davison et al, 1983). The consensus start site for transcription directed by this sequence

is YCAYYYYY (Y = a pyrimidine; Corden et al, 1980).

Sequences upstream (5′) of the TATA box also determine the rate of transcription, but the sequences involved are more diverse than the TATA sequence. The consensus 5′-CCAAT-3′ is found around position -80 to -75 in several genes, for example, 5′ of the α- and β-globin genes (Efstratiatidis et al, 1980; Mellon et al, 1981), the histone genes (Hentschel & Birnsteil, 1981) and the herpes simplex virus (HSV) thymidine kinase gene (McKnight et al, 1981). The absence of this sequence does not affect transcription in vitro (Grosveld et al, 1981) but in vivo it is more-or-less essential (Dierks et al, 1981, 1983; Mellon et al, 1981).

Since the many different genes being studied are regulated in a multitude of different ways, presumably dependent upon the specificity of their promoters, then consensus sequences are only likely to be present where the regulation is the same or when common functions, such as binding RNA polymerase II, are involved. Evidence that 5′ sequences do specify the regulation of a gene comes from the fusion of heterologous promoters to genes. For example, the interferon-α promoter confers viral inducibility on the β-globin gene (Weidle & Weissmann, 1983), the metallothionein promoter confers cadmium inducibility on the HSV thymidine kinase gene (Brinster et al, 1982), and a heat-shock promoter will also regulate the latter (Pelham & Bienz, 1982).

The HSV thymidine kinase gene is normally not highly regulated and is produced constitutively as a 'housekeeping enzyme' and its

natural promoter has been the subject of extensive study (McKnight et al, 1981; McKnight, 1982ab; McKnight & Kingsbury, 1982). The promoter appears to consist of three regions- the proximal (-16 to -32), the first distal (-47 to -61) and the second distal (-80 to -105). Mutation of the proximal region (which contains the TATA box) and either of the distal regions individually decreases expression some ten fold; and mutation in either of the the distal sequences and the proximal sequence further decreases expression. But mutation of both the distal sequences has no worse effect than mutating one of them, so they are presumably involved in the same regulatory step (McKnight, 1982a).

Other less well characterised promoters illustrate the diversity of the sequences that are involved in regulating these genes. In yeast the HIS3 gene has a very AT-rich 5′ flanking sequence (Struhl, 1981) and, along with the HIS1, HIS4 and TRP5 genes, shares the sequence 5′-TGACTC-3′ which is believed to be implicated in general control of amino acid biosynthesis (Donahue et al, 1983). A long string of A's has been implicated in the control of the yeast alcohol dehydrogenase locus, ADC1, since mutation which enhance its expression affect this region (Russell et al, 1983). In contrast to these yeast sequences, which are very A- or AT-rich, the human preproenkephalin-A gene has essential regulatory sequences between -67 and -171 a region that is 81% GC (Terao et al, 1983).

Terminator: The termination site(s) of RNA polymerase II transcription are not very amenable to analysis since the 3′ end of the message is not a stable entity. It is cleaved off just

downstream of the sequence 5′-AAUAAA-3′ prior to polyadenylation (Proudfoot, 1982; Montell et al, 1983) and transcription probably continues past this point for several hundred bases- see, for example, studies on the β-globin gene in mice (Hofer & Darnell, 1981; Hofer et al, 1982). Histone mRNAs, however, are not polyadenylated and their 3′ ends may therefore represent the termination site. The sequences implicated here are a 12 base pair inverted repeat adjacent to the sequence ACCA (Birchmeier et al, 1982).

In yeast all messages are polyadenylated and this occurs at the termination site; the sequence 5′-AAUAAA-3′ is not common 3′ of yeast genes (see Henikoff et al, 1983), and abnormal length messages are polyadenylated at their 3′ terminii (Zaret & Sherman, 1982). Zaret & Sherman (1982) noted that termination of transcription of the yeast cyc1 gene occurred near the consensus sequence 5′-TAG--TA$\underset{G}{\overset{T}{}}$GT--TTT-3′ where the spacing between the three blocks (--) was somewhat variable. However, Henikoff et al (1983- using the Drosophila ADE8 gene) showed termination at the sequence 5′-TTTTTATA-3′ in yeast with a conserved sequence 5′ of this - 5′-CAA$\underset{G}{\overset{T}{}}$CTTTG-3′. There may thus be some heterogeneity in the termination processes.

Enhancers: Another class of sequence elements affecting the expression of these genes are the so-called 'enhancers'. First discovered in viral genomes (e.g. Benoist & Chambon, 1981) they increase the expression of almost any gene within a distance of up to several kilobases when they are in cis on either its 3′ or 5′

side (Yaniv, 1982). One hypothesis concerning their operation suggests that they are bi-directional RNA polymerase entry sites (Wasylyk et al, 1983; Khoury & Gruss, 1983), but the identification of an enhancer whose natural position is within immunoglobulin genes (Queen & Baltimore, 1983; Gillies et al, 1983; Banerji et al, 1983) must throw doubt on this. More probable is the idea that they cause a change in the local DNA or chromatin conformation. Nordheim & Rich (1983) have shown that the SV40 enhancer (72bp repeat) contains short regions capable of forming left-handed Z-DNA and that such regions also occur in other enhancers so in these cases a change in DNA conformation is a possibility, since the transition to or from a Z-DNA conformation will decrease or increase the negative superhelicity of the DNA respectively. This could affect nucleosome stability or base pairing within a chromosomal domain.

The demonstration of some tissue-specificity in enhancer action (Queen & Baltimore, 1983; Gillies et al, 1983; Banerji et al, 1983) and their response to specific transcription factors (Parker, 1983; Scheidereit et al, 1983; Chandler et al, 1983) suggests that they may be recognition sites for proteins. If this is so then the binding of the proteins may cause DNA or chromatin conformational changes that favour transcription.

iii) RNA polymerase III transcription units

Promoter: RNA polymerase III transcribes a number of small RNA species such as the 5S ribosomal RNA and the tRNA's. The promoters for these genes involve sequences within the genes themselves. However, sequences in the 5′ flanking region may still be important;

Dingerman et al (1982) showed that the transcriptional efficiency of several Drosophila tRNA genes in vitro was affected by upstream sequences.

The internal promoter, located by deletion, consists of two sequence blocks around bases 8 to 19 and 51 to 62 (Galli et al, 1981; Ciliberto et al, 1982). The importance of these sequences has been confirmed by more subtle mutagenesis (Folk & Hofstetter, 1983; Newman et al, 1983) which also indicated a role for some of the sequence not included in the two promoter blocks. Hall et al (1982) speculated that the DNA conformation may have to be folded like the tRNA molecule for efficient initiation. However, the more recent results are not consistent with this hypothesis (Newman et al, 1983; Folk & Hofstetter, 1983), although some degree of secondary structure is implicated by a pair of compensating mutations in the anti-codon loops stem (Folk & Hofstetter, 1983). Whenever a base pair could not be formed between these two bases transcription was severely impaired.

The Xenopus 5S RNA genes are probably the best defined eukaryotic transcription system in a biochemical sense since a specific protein, TFIIIA, has been found which binds to the 5S RNA (Honda & Roeder, 1980) and forms a stable initiation complex with the DNA (Sakonju et al, 1981; Gottesfeld & Bloomer, 1982; Bogenhagen et al, 1982; Woodland, 1982) protecting the internal promoter sequences.

Terminator: RNA polymerase III terminates in vitro without the addition of any factors suggesting that the enzyme itself recognises

some sequence (Cozzarelli et al, 1983). Conserved sequences to the 3′ of several 5S genes resemble those of bacterial terminators (Korn & Brown, 1978; see Glass, 1982 for bacterial references) in that there is a short GC-rich region, a region of dyad symmetry and an AT-rich stretch at the termination site. Deletion analysis suggests that a run of at least four T's is required for the actual termination event (Bogenhagen & Brown, 1981).

The acetate-specific genes with which this thesis is concerned are presumably transcribed by RNA polymerase II, and so may be expected to show some of the features described above. However, it is by no means certain that fungal genes will strictly adhere to the higher eukaryote pattern. It is already becoming clear, for example, that the TATA box occurs at a very variable location in Saccharomyces (anywhere between -37 and -180; see Sentenac & Hall, 1982 for review). In Neurospora the TATA sequence nearest to the start site of the am gene is some 40-60 base pairs away (Kinnaird & Fincham, 1983) and none is present 5′ of the qa-2 or qa-3 genes within over 100 bases of their transcription start-points (Alton et al, 1982).

SECTION III

Glyoxylate Cycle

The existence of the glyoxylate cycle was first indicated by studies of acetate utilization by various prokaryotes (see Kornberg & Elsden, 1961, for an early historical overview). Cells pre-adapted to acetate were fed with ^{14}C-acetate and within the first 5 seconds label appeared in citrate and malate. The surprise was that label was incorporated into malate prior to its appearance in succinate. This is clearly unexpected if only the tricarboxylic acid (TCA) cycle is involved. Later, however, about 10-20 seconds into the experiment, the amount of label in malate and succinate had equilibrated as expected for TCA cycle activity. It was subsequently shown that ^{14}C-acetate incorporation into cell-free extracts was greatly stimulated by addition of oxaloacetate and isocitrate (more than by 2-oxoglutarate, succinate or malate). Two new enzymes proved to be responsible for this phenomenon; isocitrate lyase, which cleaves isocitrate to produce succinate and glyoxylate and malate synthase which condenses glyoxylate with the acetate moiety of acetyl-coenzyme-A (acetyl-CoA) to produce malate. Together with the TCA cycle enzymes malate dehydrogenase, citrate synthase and aconitase, isocitrate lyase and malate synthase make up what has become known as the glyoxylate shunt or cycle. This pathway effectively bypasses the two decarboxylating steps of the TCA cycle and, incorporates an extra acetate molecule to form malate.

The overall equation is :-

$$2 \text{ acetyl-CoA} + 2H_2O + NAD^+ \text{ ----> succinate} + 2 \text{ CoA-SH} + NADH + H^+$$

The role of this cycle is as an anaplerotic (or topping up) process (Kornberg, 1966). If an organism is to grow on any carbon source it must be able to synthesize cellular components from TCA intermediates. But the cycle can only be self-maintaining if the oxaloacetate produced in one round is conserved to accept acetyl-moieties at the start of the next round. If the pool of C4 acids is depleted the cycle will run down. During glycolysis the C4 pool is maintained by a single anaplerotic enzyme carboxylating pyruvate to produce oxaloacetate; pyruvate carboxylase (Beever, 1972; Ruiz-Amil *et al*, 1965; Skinner & Armitt, 1972). In Escherichia coli (E.coli) the equivalent enzyme seems to be phosphopyruvate carboxylase (Ashworth & Kornberg, 1966) which uses phosphoenolpyruvate (PEP) instead of pyruvate as a substrate for carboxylation.

During growth on acetate as sole carbon source, acetate is activated by acetyl-CoA synthetase to acetyl-CoA and, since pyruvate dehydrogenase is effectively irreversible, pyruvate cannot be synthesized directly. Thus pyruvate carboxylase is no longer a viable anaplerotic route. The glyoxylate cycle provides a means of converting acetate to succinate, and hence oxaloacetate, without loss of carbon and is the anaplerotic route used by many organisms. In a recent review, Cioni *et al* (1981) cite an 'incomplete list' of sixty five organisms in which the glyoxylate cycle has been

demonstrated to be present.

Compartmentalization - The glyoxysome

In eukaryotes containing the glyoxylate cycle it is usually in part or wholly compartmentalized into a unique organelle called a 'glyoxysome' (deDuve, 1969; Cioni, 1981). In the castor bean glyoxysomes, for example, all the reactions of the glyoxylate cycle are present, including those in common with the TCA cycle (Breidenbach & Beevers, 1967). In Neurospora the glyoxysome is also present (Kobr et al, 1969; Wanner & Theimer, 1982) but does not contain the entire cycle (Schwitzgeubel et al, 1981). The glyoxysome was shown to be less dense than the mitochondrion and to contain isocitrate lyase and malate synthase, whereas citrate synthase, and NAD- and NADP-linked isocitrate dehydrogenase were confined to the latter. In order for the glyoxylate cycle to operate there must therefore be a close cooperation between the glyoxysome and the mitochondrion, since the pathway is split between these two organelles. On this basis I have proposed the model seen in Figure 1.1 for Neurospora where citrate or isocitrate and succinate and/or malate are exchanged between the two organelles. A similar model was proposed by Casselton (1976) for a different reason.

Biogenesis of the Glyoxysome

Glyoxysomes are believed to be derived from the endoplasmic reticulum (Bowden & Lord, 1976; Beevers, 1979; Lord & Roberts, 1983) and an earlier claim that they contained RNA and DNA, and could thus synthesize their own proteins (Ching, 1970) has been attributed to

Figure 1.1; The proposed scheme for the interactions between the glyoxylate and TCA cycles. ?- indicates uncertainty over which compound is exported to the glyoxysome.

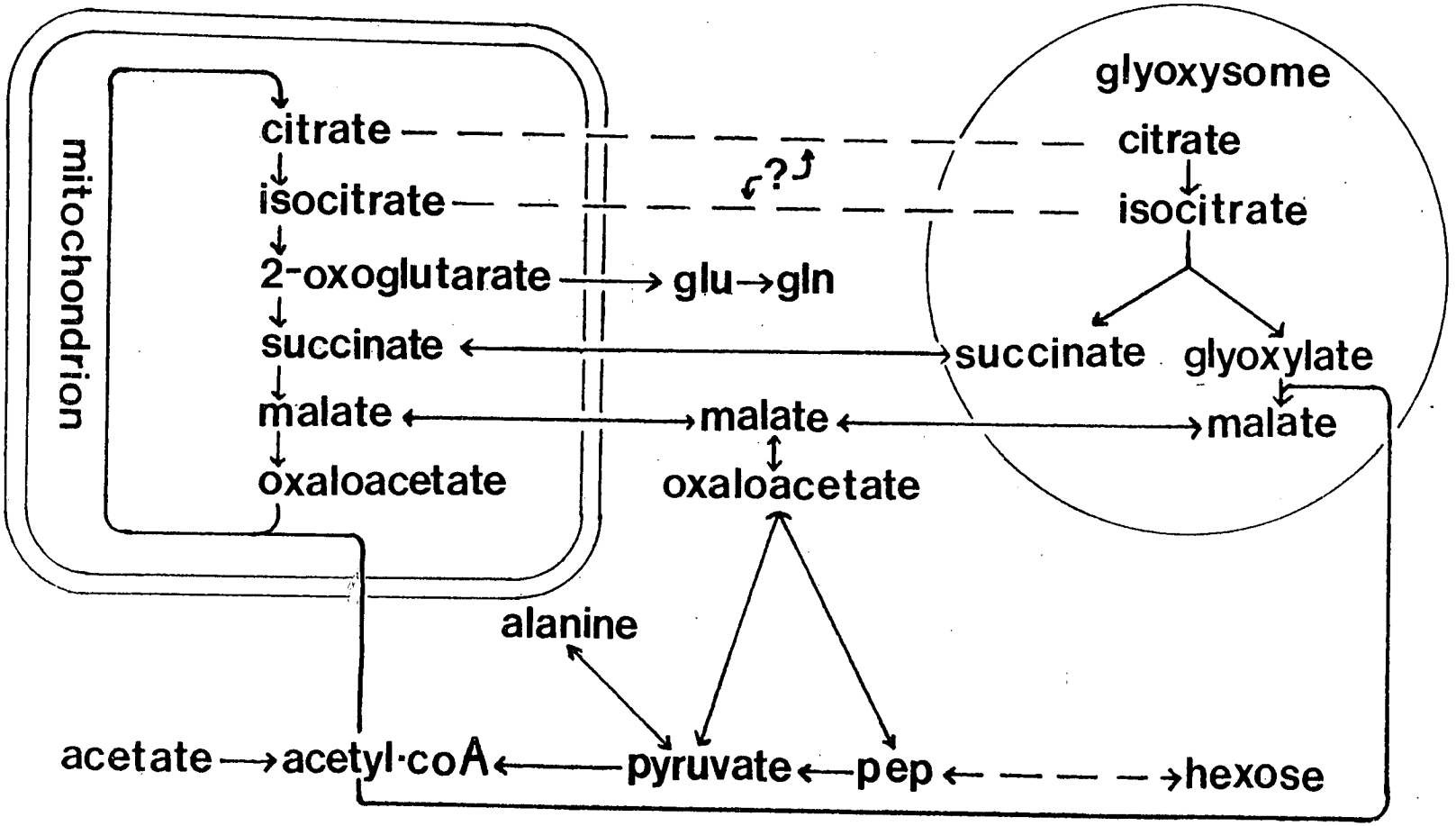

mitochondrion
citrate
isocitrate
2-oxoglutarate
glu→gln
succinate
malate
oxaloacetate
glyoxysome
citrate
isocitrate
succinate
glyoxylate
malate
malate
oxaloacetate
alanine
acetate
acetyl·coA
pyruvate
pep
hexose

Figure 1,1

contamination (Douglas *et al*, 1973). The first models (Bowden & Lord, 1976; Beevers, 1979) suggested that the glyoxysome is filled with the appropriate enzymes as it 'buds' from the rough endoplasmic reticulum, the enzymes having entered the lumen by vectorial discharge from membrane-bound polysomes. The alternative mechanism suggests that protein transport into the glyoxysome is post-translational (Lord & Roberts, 1983). The actual mechanism is somewhat enigmatic. Studies on malate synthase in plants has failed to reveal any vectorial discharge or the presence of a leader peptide normally associated with this process (Lord & Roberts, 1982), and the transient appearance of the enzyme in the cytosolic fraction (Gonzalez, 1981) implies synthesis of this enzyme on free polysomes. However, malate synthase does show a specific affinity for endoplasmic reticulum (Gonzalez, 1982). The situation with isocitrate lyase is rather more clear. Both in castor bean (Roberts & Lord, 1981) and *Neurospora* (Zimmerman & Neupert, 1980) the enzyme is synthesized on free polysomes and has the same molecular weight when immunoprecipitated, either after labelling *in vivo* or from an *in vitro* translation. The use of formyl ^{35}S-methionine (Zimmerman & Neupert, 1980) showed that no N-terminal processing of isocitrate lyase occurred *in vitro*. These authors also demonstrated the specific entry of isocitrate lyase into glyoxysomes *in vitro*, but a substantial amount of glyoxysome breakage occurred during the experiment and so enclosure during resealing cannot be ruled out (Lord & Roberts, 1983).

The consensus opinion (extensively reviewed in Lord & Roberts, 1983) seems to be that the glyoxysome is derived from the

endoplasmic - reticulum and that the matrix proteins enter post translationally.

Control of Enzyme Synthesis

The glyoxylate cycle specific enzymes, isocitrate lyase and malate synthase, are only produced when the metabolic situation demands it (Cioni *et al*, 1981). In *N.crassa* the regulation of the glyoxylate and TCA cycle enzymes has been extensively studied (Kobr *et al*, 1965; Flavell, 1967; Flavell & Woodward, 1970a&b, 1971; Beever, 1972; Beever, 1975). Developmental regulation of isocitrate lyase has also been studied (Turian, 1961; Turian *et al*, 1962). Several of the genes required for acetate utilization in *N.crassa* have also been identified (Flavell & Fincham, 1968a&b). In two cases the mutations have been shown to lie in the structural genes for glyoxylate cycle or gluconeogenic enzymes; *acu3* is the structural gene for isocitrate lyase (Leckie & Fincham, 1971), and *acu6* is the structural locus for PEP carboxykinase (Beever & Fincham, 1973).

Flavell (1967) suggested that isocitrate lyase and malate synthase are regulated by repression dependent upon high levels of a TCA intermediate or a closely related metabolite, possibly glutamate or NADH. This suggestion was largely based upon experiments with a *suc* mutant which lacks pyruvate carboxylase activity, and which he found had elevated isocitrate lyase and malate synthase levels when grown on sucrose. This strain had been previously shown to have high levels of glycolytic intermediates (Strauss, 1957) such that pyruvate leaked into the medium. Flavell's conclusion was never

published other than a mention as 'unpublished results' (Flavell & Woodward, 1970a). In a series of three papers Flavell & Woodward (1970a&b, 1971) argue themselves away from this conclusion and around to the suggestion that regulation is actually by a glycolytic intermediate. The thread of reasoning here seems quite uncertain. They attributed the enhanced catabolite repression of the TCA cycle enzymes in the suc mutant to a high cytosolic energy charge (Flavell & Woodward, 1970a) and assumed this was also the case for the glycolytic enzymes (Flavell & Woodward, 1971) and, that this enhanced repression in suc was responsible for the somewhat elevated isocitrate lyase and malate synthase levels. The direct implication of this is that their metabolic (i.e. glycolytic) repressor is low in the suc mutant. This has been demonstrated not to be the case (Strauss, 1957); glycolytic intermediates are high in this mutant.

Beever (1975) came to a similar conclusion as Flavell (1967) regarding the nature of the repressor except that he favours a C4 acid as the repressing molecule; this being the logical 'end product' of the glyoxylate cycle (but see concluding discussion of chapter 3).

In the third paper (Flavell & Woodward, 1970b) the levels of glyoxylate and TCA cycle enzymes were shown, under certain conditions, to vary non-coordinately with one another. Flavell & Woodward's hypothesis to account for this suggests that each gene is regulated by a separate metabolic pool. Sometimes these pools are in equilibrium with one another and the enzymes change coordinately while, at other times, these pools are not in equilibrium and so the

enzyme variation is non-coordinate. In the light of recent studies on other systems one is more likely to interpret their results in terms of multiple, overlapping regulatory networks (such as with the regulation of amdS in A.nidulans; Hynes, 1978*a*; see section I). This is especially so since the variations in enzyme level are readily rationalised in relation to their various roles. As an example, on transfer to acetate those TCA cycle enzymes which are required to complete the glyoxylate cycle are elevated to a greater extent than those which still only function in the TCA cycle (Flavell & Woodward, 1970b).

It seems fairly likely that the regulation of the gluconeogenic enzyme PEP carboxykinase is different from that of isocitrate lyase. Beever (1972) found non-coordinate changes between isocitrate lyase and PEP carboxykinase on transfer to acetate, and notes the lack of even slight derepression of PEP carboxykinase in the suc mutant (Beever, 1975). My results from the following experiments also support this contention but the situation is confused.

In E.coli the enzymes isocitrate lyase and malate synthase are thought to comprise an operon (Brice & Kornberg, 1968; Malloy & Nunn, 1982; genes aceA & aceB respectively). Polar mutations indicate that transcription is from aceB into aceA (Malloy & Nunn, 1982) and there are two regulatory genes involved, mutation in either of which leads to constitutive glyoxylate cycle activity (iclR & fadR, Brice & Kornberg, 1968; Malloy & Nunn, 1982). fadR also regulates the utilization of fatty acids (Simmons et al, 1980).

There are two malate synthase isozymes in E.coli (Vanderwinkel & deVlieghere, 1968; Vanderwinkel et al,1963) which differ in their metabolic role. Malate synthase A is derepressed along with isocitrate lyase whereas malate synthase G is present during growth on glycollate.

Kornberg (1963, 1966) suggested that the control of the glyoxylate cycle is by repression, and on the basis of a plethora of mutant feeding experiments, he proposed that the likely co-repressor was PEP or a closely related metabolite. More recently the levels of PEP have been estimated in various over and under-producing strains of E.coli (Lakshmi & Helling, 1978) and the predicted correlation with enzyme level (Kornberg, 1966) does not hold. Thus the precise regulation of this operon is still unclear.

In A.nidulans a series of mutants which cannot use acetate as sole carbon source have been selected (Armitt et al, 1970, 1971, 1976). The mutations in this organism affect the same enzymatic activities as those found in N.crassa (Flavell & Fincham, 1968a&b) plus certain classes not yet identified in the latter. Armitt et al (1976) also identified lesions in malate synthase, fructose-1,6-diphosphatase and the malic enzyme. Two regulatory mutations have also been recovered which lead to constitutive isocitrate lyase synthesis (icl^{C}-A, icl^{C}-B, McCullough & Roberts, 1980) but do not affect malate synthase. Clearly these two enzymes are differently regulated in this organism. The fact that acetyl-CoA synthetase deficient mutants show poor induction of isocitrate lyase and malate

synthase (Armitt *et al*, 1976) points to acetyl-CoA or a metabolically related compound as being the regulating pool. Many compounds catabolised *via* acetyl-CoA induce another enzyme; acetamidase (Hynes, 1977) so the glyoxylate cycle enzymes may be part of a larger regulatory network in *Aspergillus*.

Several features of the acetate utilization system in *Neurospora* make it attractive for the study of genetic regulation. Firstly the regulatory change is large and is both easily and reproducibly induced (Flavell, 1967). Secondly, since the genes involved are mostly unlinked (Flavell & Fincham, 1968a), their simultaneous response to acetate suggests that they may have common 5′ regulatory sequences flanking them. The cloning and comparative analysis of several of these sequences could thus aid our understanding of their regulation.

Since the regulation is very probably transcriptional (Flavell, 1967) this provided a good opportunity to try and clone several of these genes at once, using a differential screening technique. Further biochemical and physiological characterisation of the system was also undertaken.

CHAPTER 2

Starvation Experiments

INTRODUCTION

The increase in the acetate-specific enzyme activities is very large (Flavell & Fincham, 1968b), and the production of these enzymes must represent a substantial investment of energy and materials. It is thus of interest to know whether Neurospora specifically produces these activities in response to acetate by an induction mechanism, or whether it is simply the shortage of a glycolytic carbon source that stimulates their production.

The experiments described in this chapter were originally designed to try and distinguish between induction and derepression as the primary mechanisms involved in the production of the glyoxylate cycle and gluconeogenic enzymes. The results obtained were inconclusive.

MATERIALS AND METHODS

Strains used

The following strains were used in the work described in this thesis.

Mutant Gene	Allele	Comments	FGSC No.
-	-	St.Lawrence 74A wild type (STA)	262
acu5	JI18	Acetyl-CoA synthetase deficient	1733
acu3	JI38	Isocitrate lyase deficient	1732
acu6	JI37	PEP carboxykinase deficient	--
ac-73-5*	JI31	Acetate regulatory mutant, but see text	1734
acu7	JI36	No detectable 2-oxoglutarate dehydrogenase activity	1735
acp	FS800	Inducible acetate permease deficient	3546

All strains used were of mating type A. FGSC is the Fungal Genetics Stock Centre, Humboldt State University Foundation, Arcata, California, 95521, USA.

References: acu3 ,5 ,6 7 - Flavell & Fincham (1968a&b)

: acu3 - Leckie & Fincham (1971)

: acu6 - Beever & Fincham (1973)

: ac-73-5* - Beever (1972)

: acp - Rao & DeBusk (1977)

Culture Media

Sucrose medium (SM) consisted of Vogels salts (Vogel, 1964) plus 1.5% sucrose. Acetate medium for enzyme assays (AME) used Vogels salts plus 40mM sodium acetate (1M stock solution pH5.8). [2-^{13}C]-acetate medium for use in the nuclear magnetic resonance (NMR) experiments (AMN) contained Vogels salts and 66mM sodium acetate (90% ^{13}C; supplied by the Prochem division of B.O.C. Ltd.). For *in vivo* NMR experiments the citrate was omitted from the Vogels salts to minimise the background ^{13}C NMR signals; the natural abundance of carbon-13 is 1.1%. It should be noted that the citrate acts as a chelator aiding the solubility of the various metals in Vogels salts and its omission prevents the preparation of a greater than 1x concentration stock of these salts.

Growth Conditions and Transfer from Sucrose to Acetate Medium

All growth was carried out at 25^{o}C.

For general preparation of mycelium, initial growth was from conidia inoculated to a density of $5x10^{5}$/ml in 1.2L of SM in a 2L conical flask shaken in an orbital incubator at 210rpm.

For *in vivo* NMR experiments young mycelium was required for efficient circulation in the NMR tube. The conidial inoculum was $2x10^{6}$/ml and this was germinated at 10-12^{o}C for 8-12hrs and then shifted to 25^{o}C and shaken at 250rpm until the turbidity (estimated as an optical density at 650nm) reached 0.7.

Transfer of mycelium from one medium to another was carried out by filtration on to Whatman No.1 filter paper in a sintered glass funnel, washing briefly with isothermal distilled water and resuspension in pre-warmed medium at the appropriate density.

For *in vivo* NMR studies mycelium (250mg wet weight) was resuspended in 20ml of AMN containing 10% D_2O. In all other NMR experiments mycelium was resuspended at a density of 10% wet weight/volume.

Samples were removed from cultures at intervals when required (either using a reversed 25ml pipette or by decanting), and filtered to recover the mycelium. Mycelium was pressed dry and stored at -20°C until lyophilised.

Enzyme Assays

Crude extracts for enzyme assays were produced by grinding the lyophilised mycelium and extraction with 10mM Tris.Cl buffer (pH 7.0, 50ml/g dry weight) at 0°C with intermittent vortexing, for 15-20 minutes. Extracts were clarified in a microfuge (11,600g for 5min). Assays were performed as in Flavell & Fincham (1968b) using the following protocols.

Isocitrate lyase (EC 4.1.3.1) Dixon & Kornberg (1959)

Solution	ml	µmol
1. 0.1M sodium phosphate pH 6.8	2.1	210
2. 0.15M $MgCl_3$	0.1	15
3. 0.1M phenylhydrazine HCl	0.1	10
4. 60mM 2-mercaptoethanol	0.1	6
5. 30mM EDTA	0.1	3
6. Enzyme solution	0.1	-
7. 12.5 mM DL-isocitric acid	0.4	5
Final volume	3.0	

Comments. Incubation temperature is 28°C and the formation of the phenylhydrazone is monitored at 324nm. The reaction was initiated after 3-5 minutes by the addition of solution 7 and mixing by inversion. Solutions 3,4,7 were made fresh each time. A standard curve was produced by the addition of known quantities of glyoxylic acid in place of the extract.

Malate synthase (EC 4.1.3.2) Dixon & Kornberg (1959)

Solution	ml	µmol
1. 0.2M sodium phosphate pH7.1	0.5	100
2. 15mM $MgCl_2$	0.67	10
3. 0.198mM acetyl-coenzyme-A	0.63	0.125
4. Enzyme solution	0.025	-
5. 50mM glyoxylic acid	0.1	5
Final volume	1.925	

Comments. Incubation temperature is 30°C and the disappearance of acetyl-coenzyme-A (acetyl-CoA) is monitored at 232nm. The reaction was initiated after 3-5 minutes by the addition of solution 5 and mixing by inversion. Solution 5 is made up fresh each time and solution 3 is stored as a 100x concentrate at -20°C. A standard curve was produced by measuring the absorbance of various quantities of acetyl-CoA in the reaction mixture with the extract replaced by water.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.a) Beever(1972)

Solution	ml	µmol
1. 0.1M Tris.Cl pH7.2	2.3	230
2. 0.1M $MgCl_2$	0.1	10
3. 0.25 $KHCO_3$	0.1	25
4. 30mM ADP	0.1	3
5. 0.1mg $NADH_2$	0.1	-
6. Extract	0.1	-
7. 15mM phosphoenolpyruvate	0.2	3

Final volume	3.0 ml	

Comments: Incubation temperature is 30°C and the loss of $NADH_2$ is monitored at 340nm. The reaction was initiated after 3-5 minutes by the addition of solution 7 and mixing by inversion. Solutions 3,4,5 &7 were prepared freshly each time. A standard curve was produced by measuring the absorbance of various quantities of $NADH_2$ in the mixture in the absence of extract. This is a coupled assay and malate dehydrogenase must be in excess.

Acetyl coenzyme-A synthetase (EC 6.2.1.1) Jones & Lipman (1955)

Solution	ml	µmol
1. 0.5M sodium phosphate pH 7.5	0.2	100
2. 100uM coenzyme-A	0.1	0.1
3. 0.1M ATP	0.1	10
4. 0.2M potassium acetate	0.1	20
5. 2M hydroxylamine HCl pH7.4	0.1	200
6. 0.5M potassium fluoride	0.1	50
7. 0.1M $MgCl_2$	0.1	10
8. 0.1M Glutathione pH4.5	0.1	10
9. Extract	0.1	-
10. Ferric chloride reagent:-		
10% (w/v) $FeCl_3 6H_2O$		
3.3% (w/v) trichloroacetic acid		
0.66M HCl	1.5	-

Final volume	2.5ml	

Comments: Incubation temperature is 37°C. This assay is not continuous. Solutions 1 to 8 are added to a small glass tube and brought to reaction temperature in a water bath. The assay is started by the addition of the extract and mixing. Incubation is usually for 20-40 minutes (timed), whereupon the reaction is halted and the colour developed by the addition of solution 10. This precipitates protein from the extract so the mixture must be clarified by centrifugation. The absorbance is determined at 505nm after zeroing using a dummy reaction with each extract containing

0.1ml of water instead of coenzyme-A. Solutions 3,5,8 & 10 are made fresh each time and solution 2 is stored as a 10x concentrate at -20°C. A standard curve is produced by replacing the extract with a range of acetyl phosphate concentrations.

Slope estimation

Given the ready availability of programmable calculators and computers the measurement of line gradients from a large series of continuous enzyme assays can be greatly speeded by using the following simple procedure. First the slope of the line is estimated using a protractor giving an angle (a). The slope of the line is then given by multiplying the tangent of this angle by a scaling factor which depends on the chart recorder settings thus:-

gradient = tan(a).([cm/min]/[OD units/cm])

Protein assays

Protein was estimated using the microbiuret method. 50µl of protein solution was added to 2.45ml of water and 0.5ml of biuret reagent. This was vortexed and allowed to stand at room temperature for 20 minutes. The A_{310} was read having zeroed the blank against a cell containing 50µl of the relevant buffer in place of the protein solution. A standard curve was produced using bovine serum albumen.

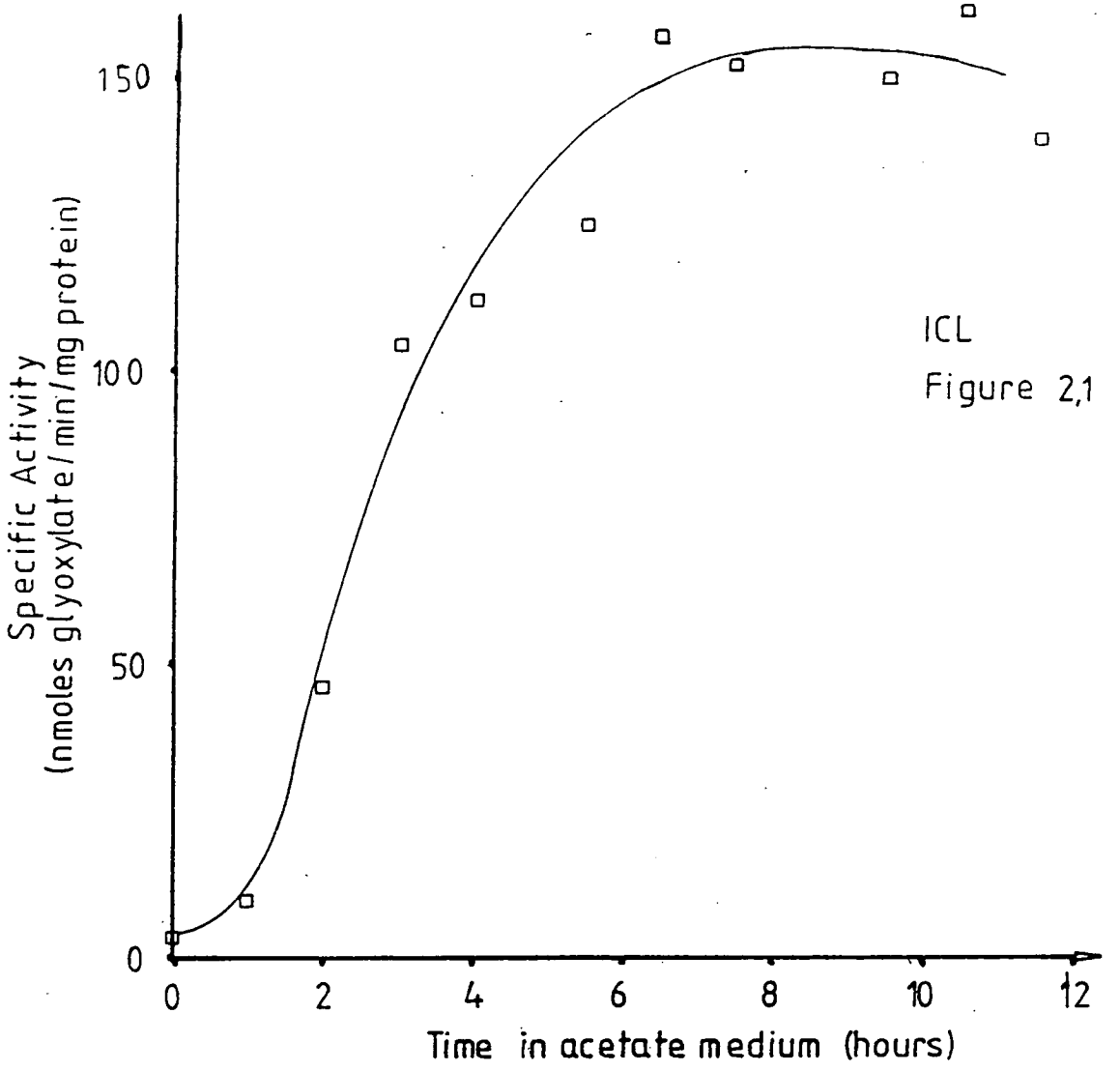
Biuret solution is made by adding 40ml of 1% copper sulphate in water to 150ml of 40% aqueous sodium hydroxide. This was stored in a plastic bottle at 4°C.

RESULTS AND DISCUSSION

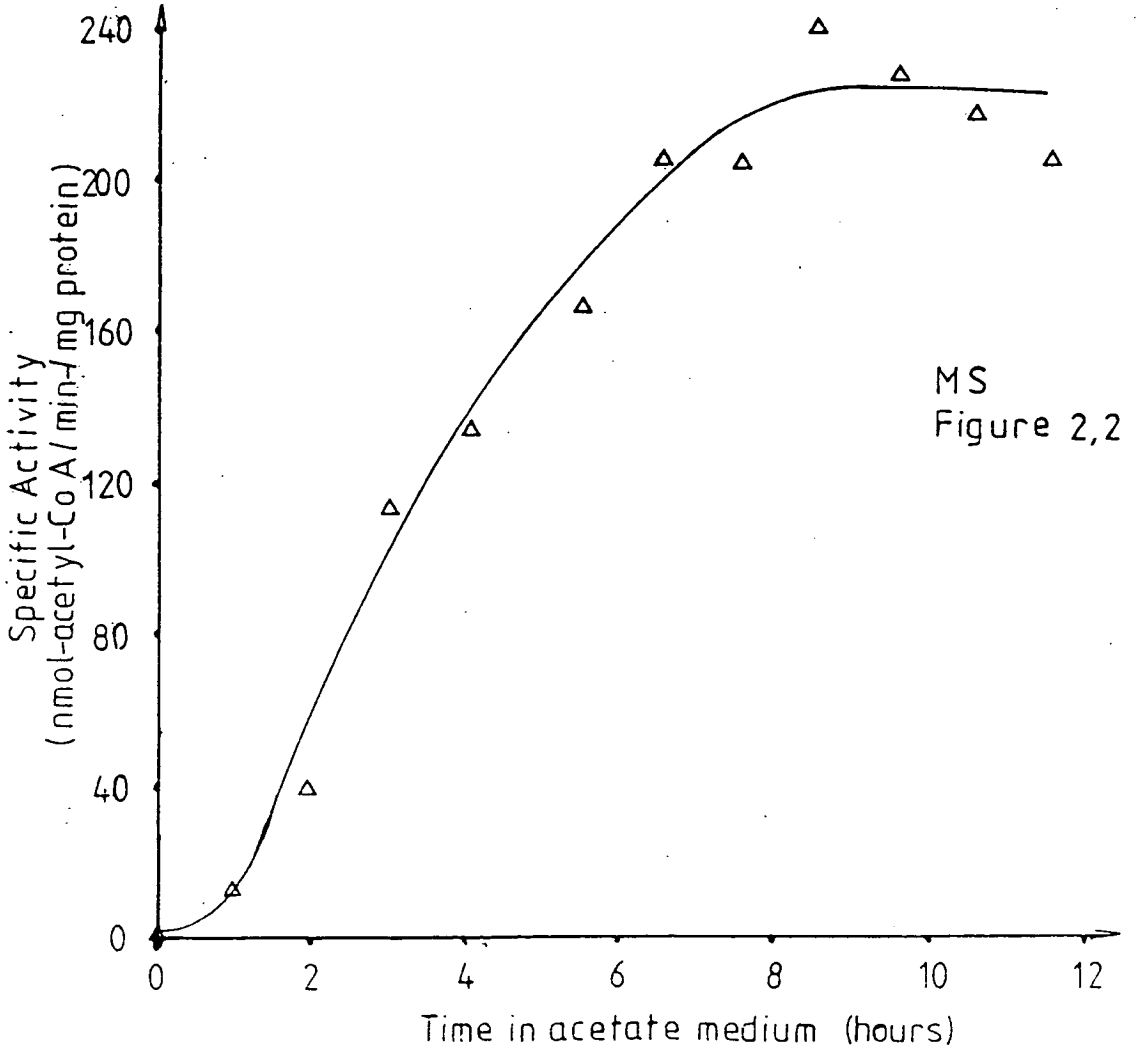
When late log phase mycelium was transferred from sucrose to acetate medium, the four assayed enzymes (isocitrate lyase, malate synthase, acetyl-CoA synthetase & PEP carboxykinase) all showed an increase in specific activity of about 50-100 fold (Figures 2.1-2.4). The induction curve shows a lag of 0.5-1 hours followed by a rise in specific activity which levels out at 6-8 hours post-transfer. Such behaviour has been well documented (Flavell, 1967). When the specific activity of isocitrate lyase was plotted against the specific activity of any of the other three enzymes a straight line resulted (Figures 2.5-2.7). This coordinacy has also been seen previously (Flavell, 1967), and suggests that all the enzymes are responding to the same signal. None of the genes for these enzymes are closely linked (Flavell & Fincham, 1968a) which implies that their non-coding flanking regions may contain a common regulatory sequence. This was one of the hypotheses to be tested after cloning the genes (see Chapter 4).

On closer examination, however, this phenomenon is far from clear cut. Flavell (1967) found coordinacy between isocitrate lyase and malate synthase, isocitrate lyase and PEP carboxykinase, but not between isocitrate lyase and acetyl-CoA synthetase as was seen in the present study (Figure 2.5). Furthermore, in a subsequent study (Flavell & Woodward, 1970b), coordinacy was not found between isocitrate lyase and malate synthase. In a third report (Beever, 1975) conditions were found where the levels of PEP carboxykinase and isocitrate lyase were apparently uncoordinated. In some ways

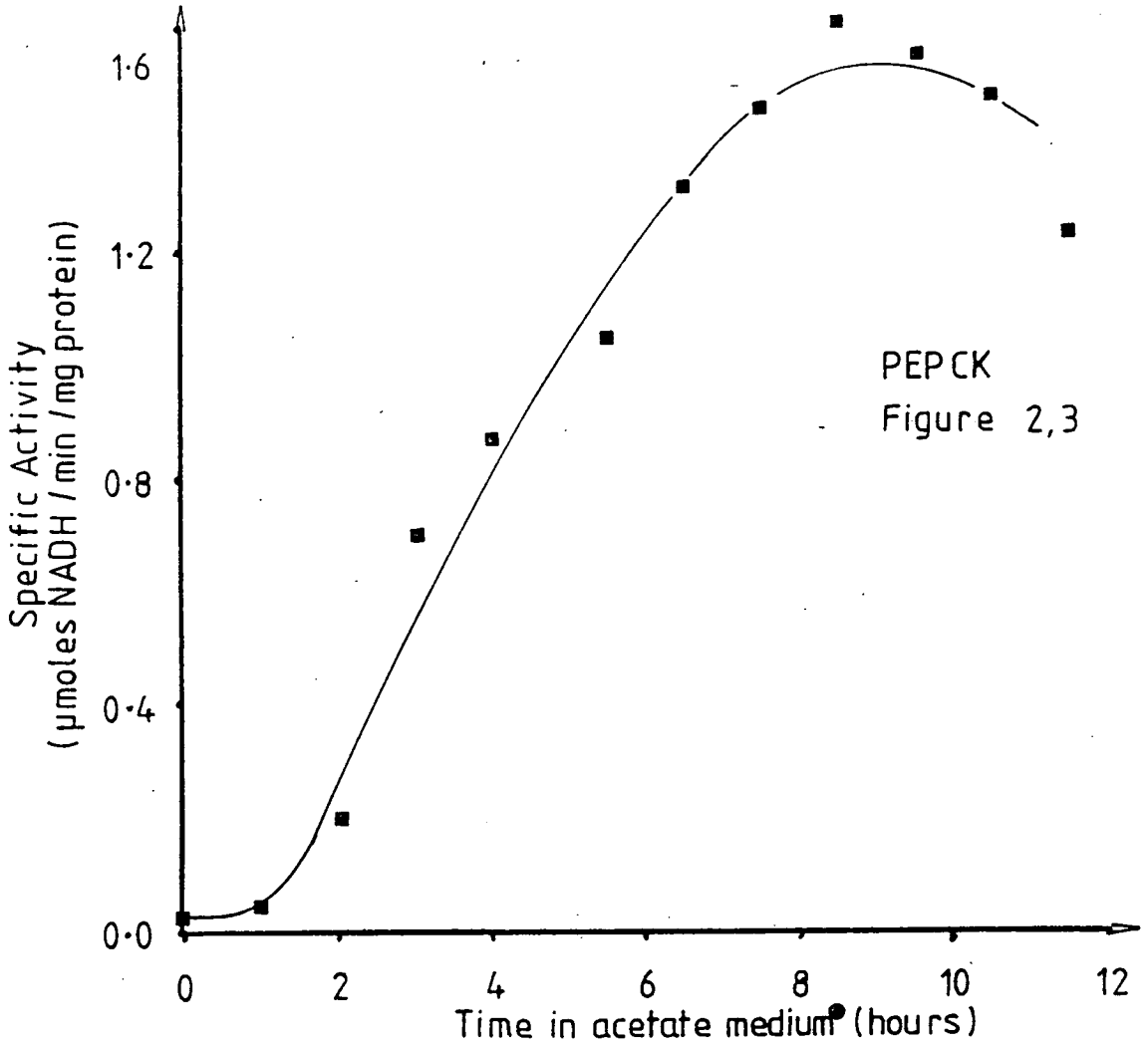
Figures 2.1-2.4; The increase in specific activity of isocitrate lyase (ICL), malate synthase (MS), PEP carboxykinase (PEPCK) and acetyl-CoA synthetase (ACOAS) when mycelium is transferred from sucrose to acetate medium at time zero. Each point is the mean of three replicates.

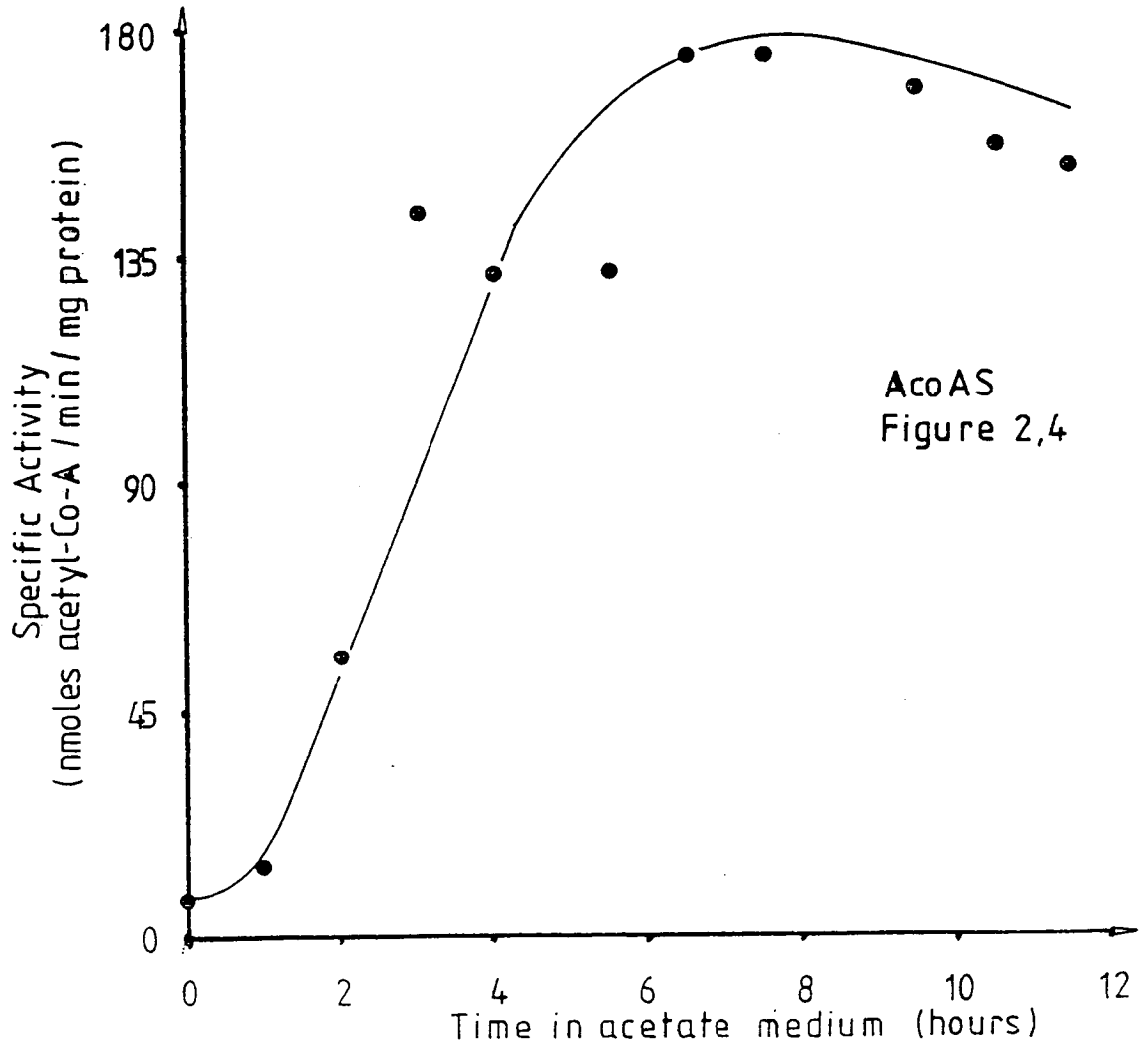


ICL
Figure 2,1



MS
Figure 2,2


Specific Activity
(μmoles NADH / min / mg protein)
1·6
1·2
0·8
0·4
0·0
0
2
4
6
8
10
12
Time in acetate medium (hours)
PEPCK
Figure 2,3


Specific Activity
(nmoles acetyl-Co-A / min / mg protein)
180
135
90
45
0
0
2
4
6
8
10
12
Time in acetate medium (hours)
AcoAS
Figure 2,4

Figures 2.5-2.7; The specific activities in Figures 2.1-2.4 replotted against one-another at corresponding times. The lines were fitted by linear regression; r is the correlation coefficient.

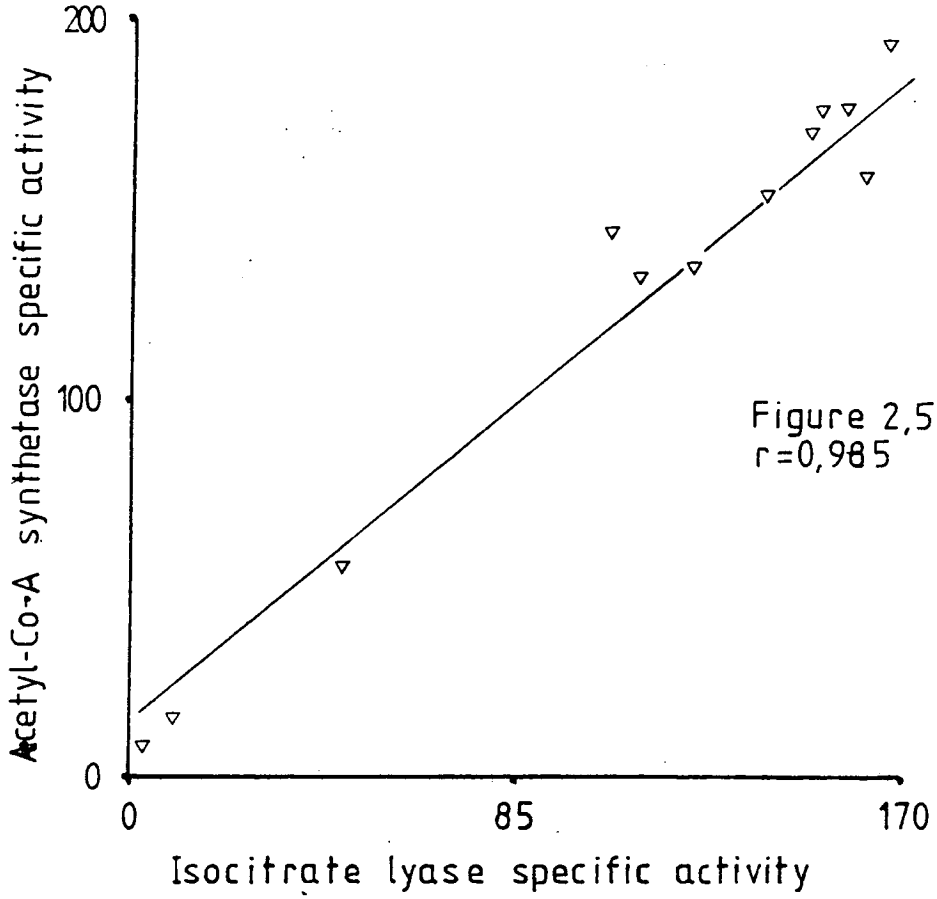


Figure 2,5
r=0,985

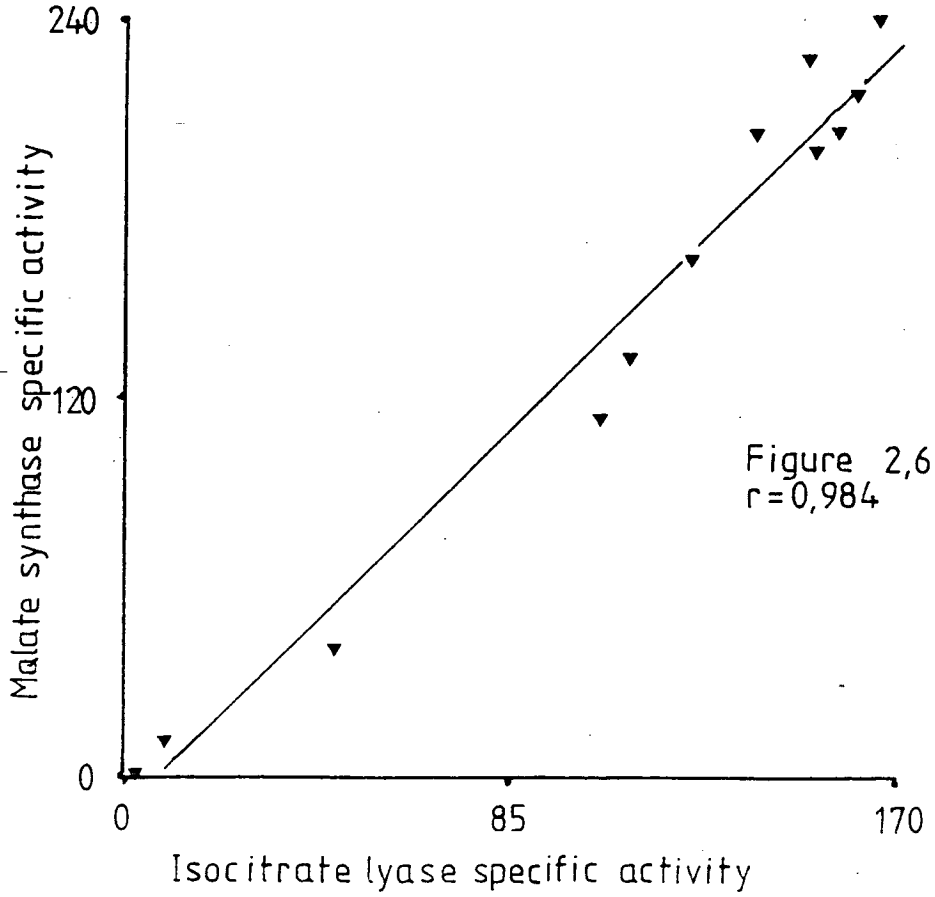
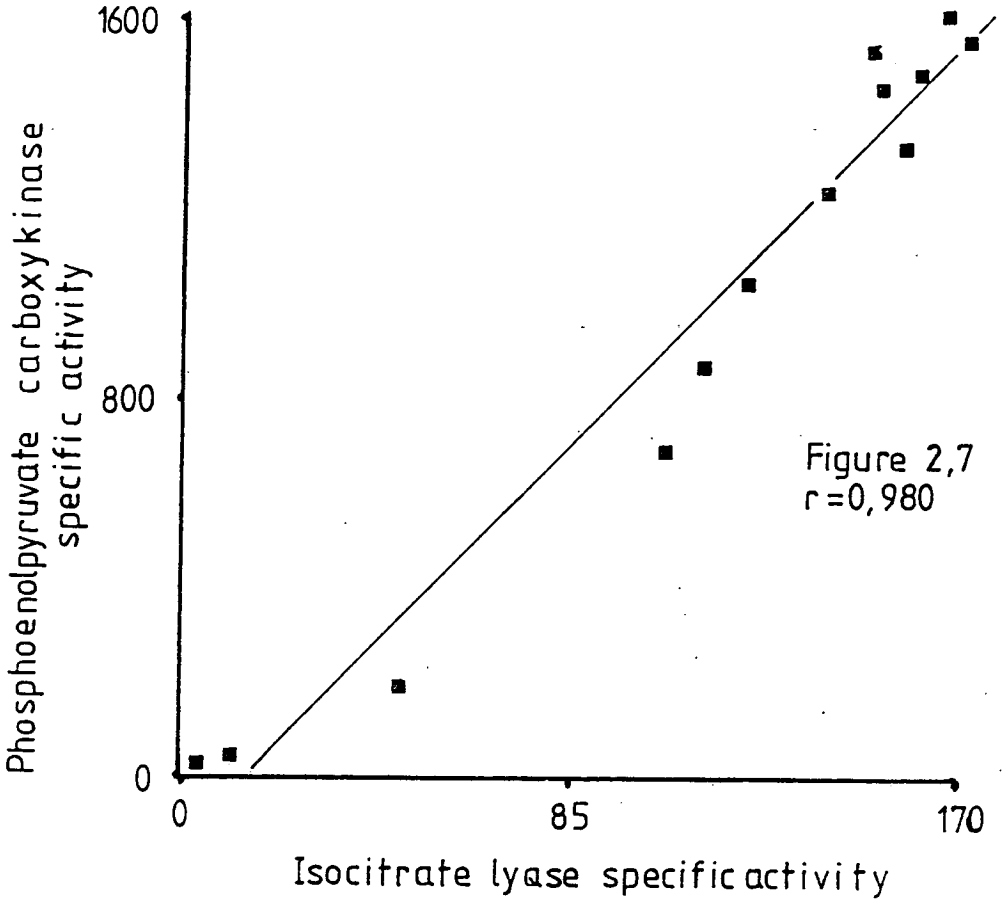


Figure 2,6
r=0,984

All scales refer to- nmoles product formed / min / mg protein



All scales refer to - nmoles product formed / min/mg protein

this variability lends some credence to the idea of concurrent regulation suggested by Flavell & Woodward (1970b), in which several different pools are involved in regulating specific enzymes or groups of enzymes. These pools are hypothesized to change coordinately producing a sort of pseudo-coordinate regulation. Under certain circumstances these pools might be induced to vary independently so that coordinacy broke down.

In this study non-coordinacy was occasionally observed between isocitrate lyase and PEP carboxykinase, however, it is usually witnessed only amongst one set of replicates in one experiment. Throughout this study the assaying of enzymes has been plagued by a greater than expected variability which makes the comparison of absolute values very difficult. The usual way round this would be to use a specific activity ratio to the basal level on sucrose medium. However, the sucrose level is often virtually zero or undetectable, and standardisation by reference to any point after treatments have diverged is meaningless. Thus, although the general pattern and timing of enzyme derepression is reproducible, activity ratios can seldom be stated very precisely. To try to keep variation to a minimum a strict protocol was followed - with a constant inoculum size and age, a constant ratio of buffer to mycelium during extraction, and the use so far as possible of the same stock solutions for successive enzyme assays.

Other studies on the regulation of these enzymes have been discussed in chapter 1, section III.

Flavell (1967) has shown that the addition of sucrose to the medium during the induction of the enzymes, causes a cessation in their increase. This phenomenon is known as carbon catabolite repression. In essence this means that the organism has a mechanism that prevents a certain carbon source from being used when a 'preferred' one is present. In this case acetate utilization is suppressed. This process, as with the nitrogen equivalent (Cove & Arst 1973), is a general system of control over-riding a number of specific controls governing the utilization of particular carbon sources. It is conceivable, however, that derepression due to relief of carbon catabolite repression might be the only regulatory mechanism in this case with induction by an acetate metabolite, for example, playing no part.

A second variation on this possibility is that some of the enzymes may be subject to different or overlapping regulation. In particular PEP carboxykinase might be thought to be regulated differently from the glyoxylate cycle enzymes. It is a gluconeogenic enzyme and may be expected to be used whenever the glycolytic flux is low or non-existent, as would be the case during growth on alanine, for example. To probe these two possibilities a, with hindsight, rather naive series of experiments was undertaken. By starving mycelium of all utilizable carbon one would expect many pools to fall to very low levels , whereas an inducing pool would have to increase if induction were a factor. Thus, mycelium was grown up in sucrose medium and transferred to either acetate medium or to medium containing no utilizable carbon for four hours and thence to acetate medium. Samples of mycelium were extracted at

various times and prepared for enzyme assays. The results are shown in Figure 2.8. The graphs showed that whilst PEP carboxykinase commenced its increase at the same time whether the mycelium starved or not, isocitrate lyase showed only a small increase in response to starvation alone and did not increase further until transfer to acetate mycelium. This implies a primarily derepressive mechanism for PEP carboxykinase and a primarily inductive one for isocitrate lyase.

To try to establish whether the inducing molecule was acetate or one of its metabolites a similar experiment was carried out with an acu5 mutant lacking detectable acetyl-CoA synthetase activity (Flavell & Fincham, 1968ab), and thus unable to activate acetate. The results shown in Figure 2.9 demonstrate that isocitrate lyase behaved as in the wild type even when the acu5 mycelium was starved for 8 hours instead of 4 hours. This suggested either that acetate itself was the co-inducing molecule or that acetate was still being utilized in some way. The latter proved to be true (see Chapter 3), leaving the matter unresolved.

An interesting anomaly is apparent between the two replicates of this experiment (Figure 2.8, A & B) which were done several weeks apart, in that the isocitrate lyase in replicate B responds substantially faster to the medium changes than A. Some uncontrolled factor seemed to be at work. The most likely possibility seemed to be the growth state of the mycelium, so the experiment was repeated with mycelium grown for 24 hours and 40 hours on sucrose medium from the standard inoculum. These times

Figure 2.8; Solid symbols show the increase in specific activity of isocitrate lyase (i) and PEP carboxykinase (ii) after transfer from sucrose to starvation medium at time zero and subsequently to acetate medium at four hours (↑). A and B are replicate experiments. Open symbols show the same enzyme activities on transfer from sucrose medium directly to acetate medium.

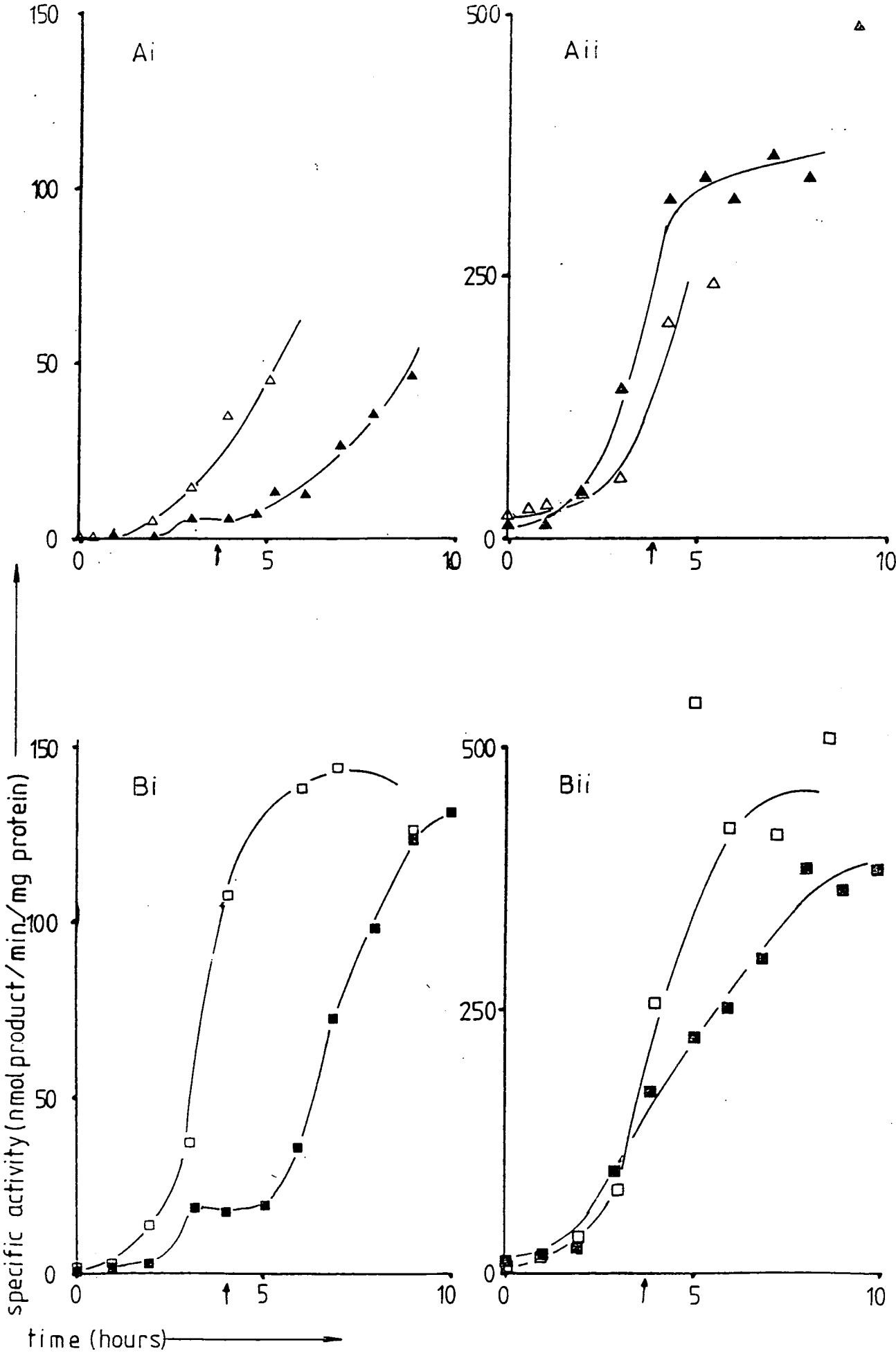

Ai
Aii
Bi
Bii
150
100
50
0
500
250
0
5
10
specific activity (nmol product / min / mg protein)
time (hours)

Figure 2.8

Figure 2.9; The increase in isocitrate lyase activity in acu5 mycelium after transfer from sucrose medium to- (■) acetate medium at time zero, (▲) starvation medium at time zero and then to acetate medium at four hours or, (□) starvation medium at time zero and then to acetate medium at eight hours.

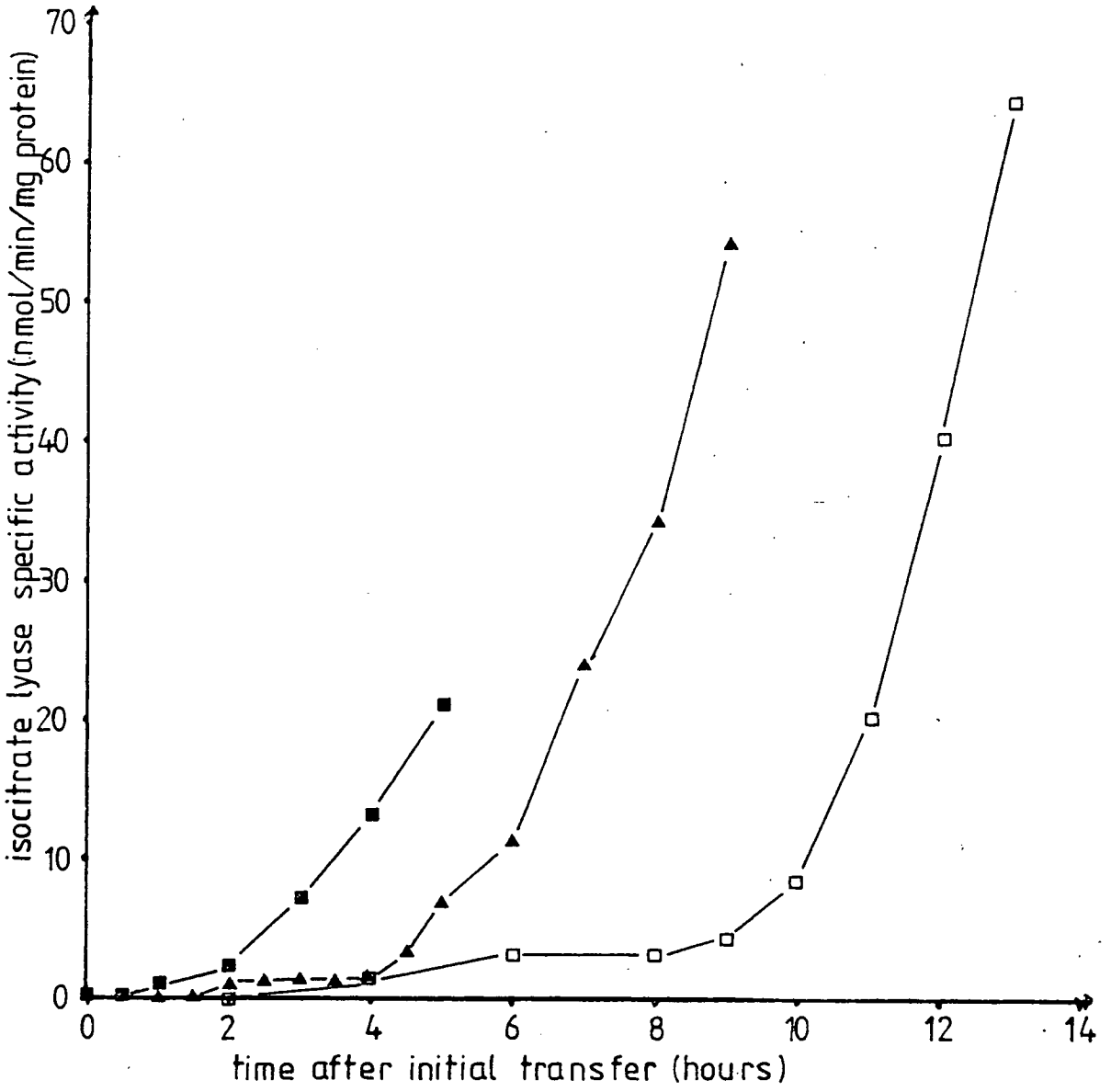

isocitrate lyase specific activity (nmol/min/mg protein)
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
time after initial transfer (hours)

Figure 2,9

corresponded to late log phase and stationary phase respectively (see growth curve - Figure 2.10). Starvation was for eight hours and the results are shown in Figure 2.11. The response of the two enzymes was essentially identical after 24 hours growth on sucrose but differed markedly after 40 hours growth. In the latter treatment isocitrate lyase behaved as in the first experiments (Figure 2.8, A) whereas PEP carboxykinase behaved somewhat differently in that it showed a similar pattern to the first experiments but did not reach the same specific activity. As before it did not respond to the presence of acetate to any great degree.

In the 24 hour mycelium, however, PEP carboxykinase did respond to acetate since the specific activity climbs again after the 8 hour transfer. Also in the 24 hour mycelium, isocitrate lyase showed a very rapid response to starvation in contrast to the previous experiments.

In order to try to define where in the growth curve this response changes, mycelium was grown for 24, 30, 36, 42, and 48 hours in sucrose medium and was then starved of carbon for eight hours followed by a shift to acetate medium. The activities of isocitrate lyase and PEP carboxykinase were monitored at 4,8, and 12 hours after the initial transfer in each treatment. The results shown in Figures 2.12A and 2.13A show that on this occasion both the enzymes show essentially the same step-like response. PEP carboxykinase thus contradicted its previous response at 24 hours. A plot of the starved 4 hour activity of each enzyme against the initial growth time (Figures 2.12B & 2.13B) shows a fairly dramatic decrease

Figures 2.10; Growth curve for wild type mycelium grown as described in the methods section. Dry weights were estimated after drying at 65°C.

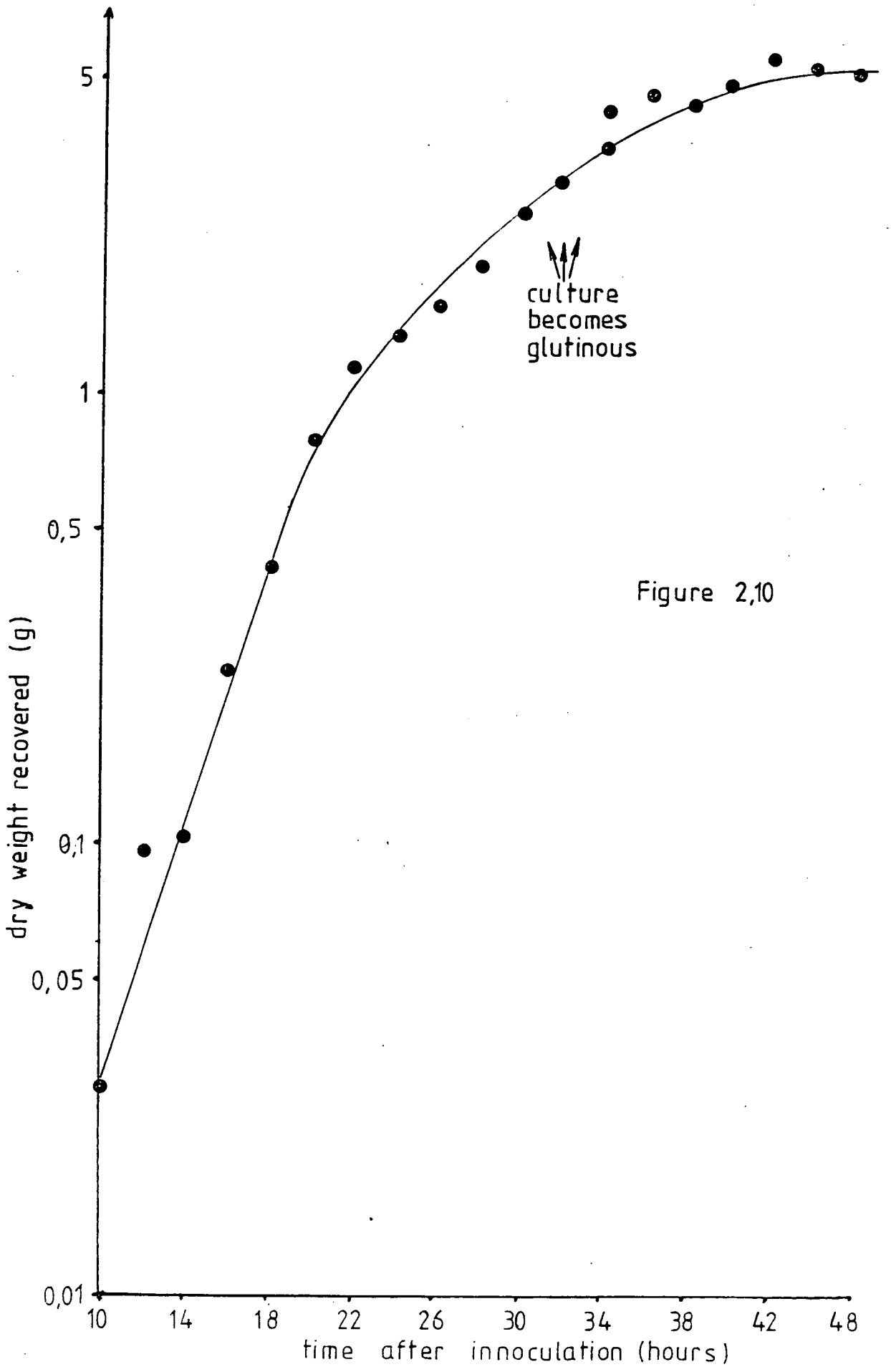

5
1
0,5
0,1
0,05
0,01
dry weight recovered (g)
culture
becomes
glutinous
10
14
18
22
26
30
34
38
42
48
time after innoculation (hours)

Figure 2,10

Figure 2.11; Changes in specific activity of- a) isocitrate lyase (ICL) and b) PEP carboxykinase (PEPCK) on transfer to starvation and acetate media as indicated. Initial growth was in sucrose medium for 24 (+) or 40 (o) hours.

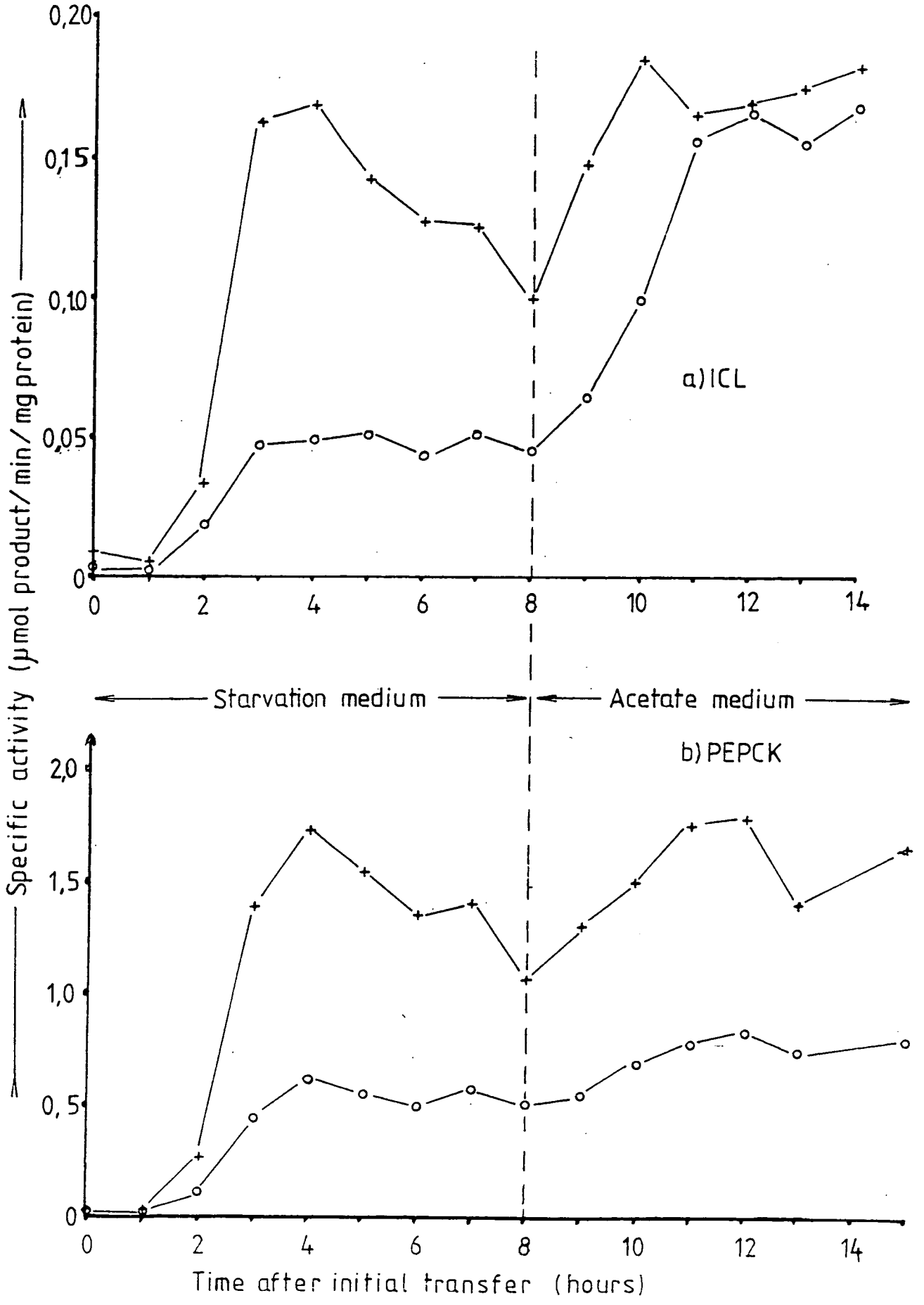

0,20
0,15
0,10
0,05
0
a) ICL
0
2
4
6
8
10
12
14
Starvation medium
Acetate medium
b) PEPCK
2,0
1,5
1,0
0,5
0
Specific activity (µmol product/min/mg protein)
Time after initial transfer (hours)

Figure 2,11

Figure 2.12; A) The specific activity of isocitrate lyase assayed at the indicated times after transfer from sucrose medium to starvation medium and acetate medium as indicated. Each curve is the result obtained from mycelium pre-grown for the indicated time on sucrose medium. B) The four hour activities from A) above replotted against the growth time on sucrose.

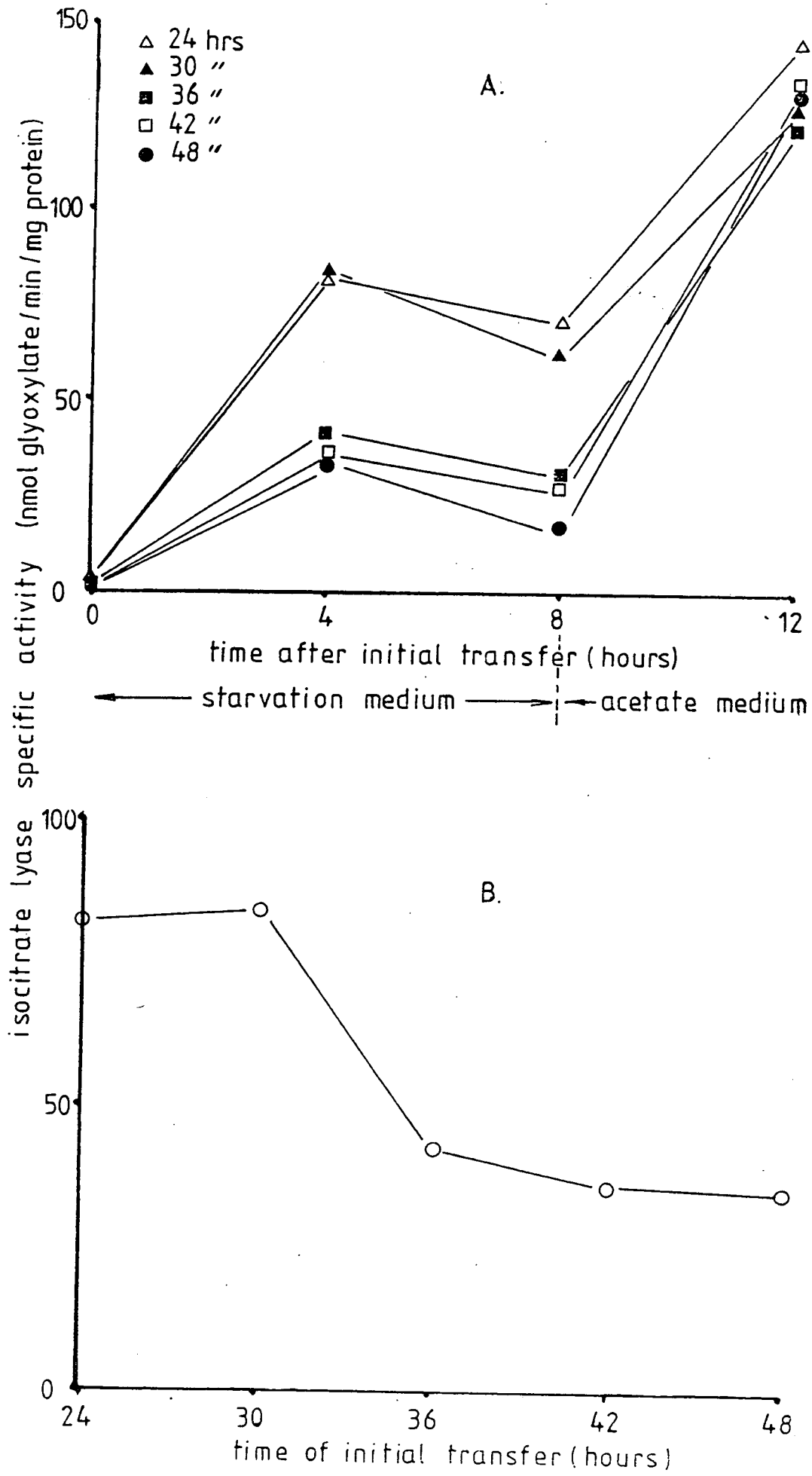

A.
24 hrs
30 "
36 "
42 "
48 "
isocitrate lyase specific activity (nmol glyoxylate/min/mg protein)
150
100
50
0
0
4
8
12
time after initial transfer (hours)
starvation medium
acetate medium
B.
100
50
0
24
30
36
42
48
time of initial transfer (hours)

Figure 2,12

Figure 2.13; These data are from the same samples as in Figure 2.12 but assaying for PEP carboxykinase activity. Plots A) and B) are explained in the legend to the previous figure.

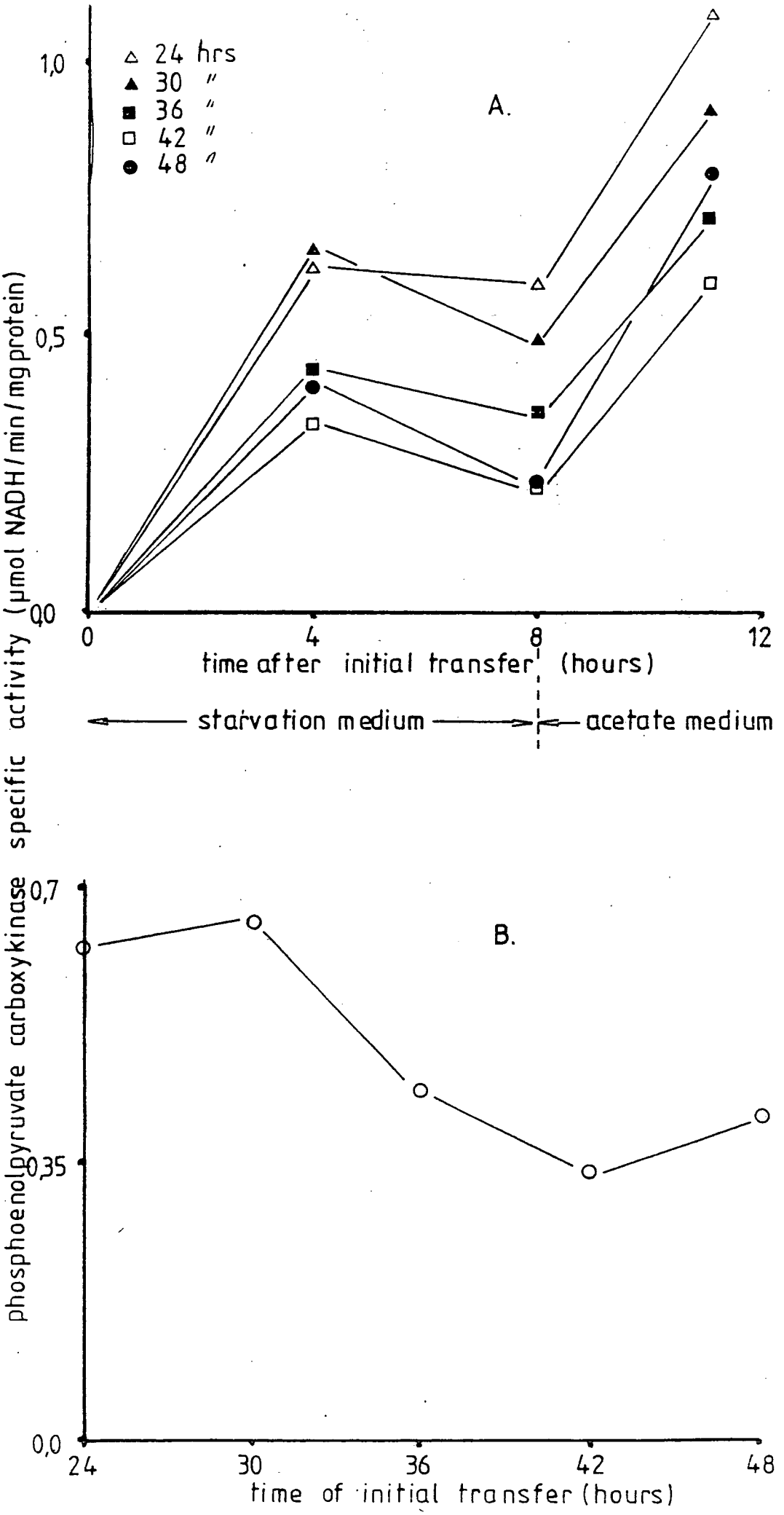

A.
24 hrs
30 "
36 "
42 "
48 "
1,0
0,5
0,0
0
4
8
12
time after initial transfer (hours)
starvation medium
acetate medium
specific activity (µmol NADH/min/mg protein)
B.
0,7
0,35
0,0
24
30
36
42
48
time of initial transfer (hours)
phosphoenolpyruvate carboxykinase

Figure 2,13

between 30 and 36 hours.

As N.crassa cultures go into stationary phase the mycelium seems to produce some sort of extracellular material, possibly polysaccharide, which makes the culture become fairly glutinous and freshly harvested mycelium slimy to the touch. This transition was seen (felt!) to occur at around 34 hours growth under these conditions and perhaps reflects the same metabolic transition seen in Figures 2.12B and 2.13B.

It is very difficult to rationalize these results since conditions evidently become largely uncontrollable in and around stationary phase. A complicating factor in the interpretation of the experiments is the possible involvement of storage compounds which may be mobilized on starvation. Mobilization of stored triacylglycerides will result in the production of acetyl-CoA by β-oxidation and may mimic any inductive effects of acetate. Thus one might make the hypothesis that in the experiment shown in Figure 2.12 the anomalous behaviour of isocitrate lyase after 24 hours growth was due to the high level of triacylglycerides at this time which resulted, on starvation, in the release of copious amounts of acetyl-CoA, leading to isocitrate lyase induction. Whereas after 40 hours growth on sucrose these compounds may be low resulting in a very small increase in the enzyme on starvation. This being the case one might expect a transient increase in the specific activity of isocitrate lyase if acetyl-CoA produced by the consumption of these stored compounds between 24 and 40 hours were utilized via the glyoxylate cycle. This proved not to be so. Samples assayed for

isocitrate lyase in two experiments between 18 and 42 hours (Figure 2.14) growth on sucrose showed no such increase and, if anything, a decrease is seen after 32 hours.

If the triacylglycerides are continually being turned over even in log phase then a cessation in their synthesis could lead to their gradual depletion using only the basal levels of isocitrate lyase or its low level constitutive isozyme (Sjorgren & Romano, 1967; Flavell & Woodward, 1971a). The existence of the latter is now questioned, however (Rougemont & Kobr, 1973). It is even possible that they could be consumed *via* the TCA cycle if glycolytically derived oxaloacetate was plentiful.

If stationary phase was entered for reasons other than carbon starvation then stored compounds may never have been mobilized. Indeed the production of copious amounts of extracellular material may be due to the continued uptake of sucrose after true mycelial growth has stopped. The extracellular material is transferred with the mycelium in medium changes and appears to be firmly attached. It is also metabolised in acetate or starvation medium since the mycelium looses the characteristic texture on reharvesting. If it were a polysaccharide its metabolism *via* glycolysis might cause some degree of catabolite repression causing the suppression of the increase in both the isocitrate lyase and PEP carboxykinase activities.

Figure 2.14; Isocitrate lyase specific activity (ICL SA) assayed in two experiments (. and x) during growth on sucrose medium for the indicated times. Each point is the mean of two replicates.

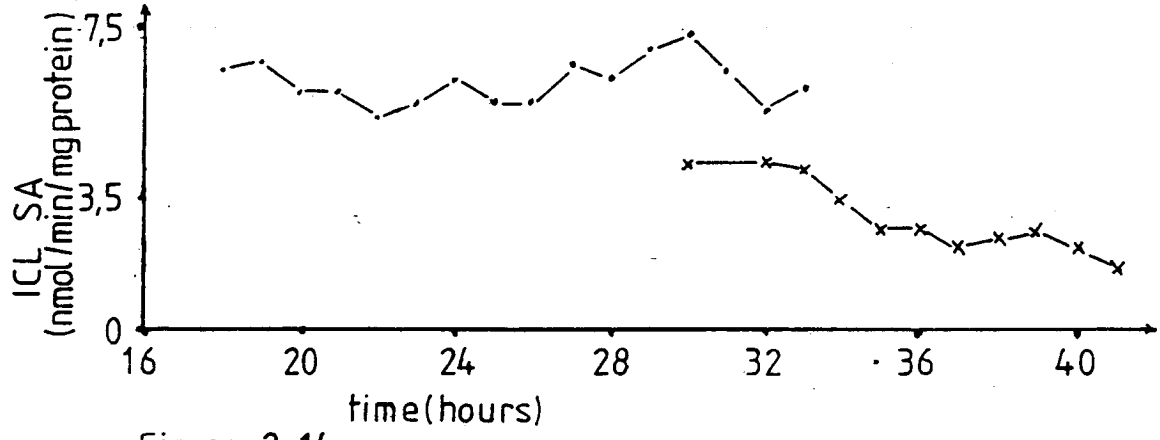


Figure 2,14

CONCLUDING DISCUSSION

The combination of (1)relief from external catabolite repression in starvation medium, (2)continued catabolite repression when extracellular polysaccharide is carried over; (3)external induction in acetate medium, and (4)internal induction by mobilization of stored triacylglycerides could together account for most of the phenomena seen in these experiments. In this model both enzymes are subject to catabolite repression and isocitrate lyase is also subject to induction. Specifically, log phase mycelium transferred to starvation medium would have high levels of triacylglycerides which, when degraded to acetyl-CoA would induce isocitrate lyase whilst the drop in the glycolytic pool would derepress PEP carboxykinase. On the other hand stationary phase mycelium would show little enzyme increase since metabolism of the putative polysaccharide would keep both enzymes repressed.

This is, however, rather speculative at present, and the results obtained are insufficiently consistent to support firm conclusions. As an example, the third series of experiments on the wild strain (Figures 2.12 & 2.13) seem to indicate a response to acetate by PEP carboxykinase that is not seen in the others. The conditions in and around stationary phase seem to be too difficult to control to get meaningful results.

CHAPTER 3

Nuclear Magnetic Resonance Experiments

INTRODUCTION

Nuclear Magnetic Resonance

This introduction aims to explain nuclear magnetic resonance (NMR) experiments without venturing into the mathematics describing the process which are complex and are described in a number of standard works (e.g. Farrer & Becker, 1971; Abraham, 1961). The work described in this chapter was largely carried out in collaboration with Dr R.L. Baxter, to whom I am grateful for accumulating the spectra and assigning the peaks. Two useful reviews on ^{13}C-NMR in biological systems are Scott & Baxter (1981) and Baxter *et al*, (1983).

Many types of atomic nuclei possess a property known as nuclear-spin which confers a magnetic moment on the nucleus and is characterised by the spin quantum number I. These nuclei can in some ways be thought of as behaving like very small bar magnets in the presence of an applied magnetic field- taking up one of a number of quantized orientations or spin-states. There are (2I+1) possible spin-states for any given nucleus. I is in units of h/2pi and increases from zero by increments of 0.5. The most common nuclei useful for NMR studies have I=0.5 and thus have two spin-states. Nuclei with this value of I include several which are biologically useful; ^{1}H, ^{13}C, ^{15}N, ^{31}P.

In a magnetic field the alternate spin-states correspond to different energy levels and transitions between them can be induced by electromagnetic radiation of the appropriate energy (radio

frequencies) much as electrons can be excited to different energy levels. The frequency at which the transition occurs is directly proportional to the field strength experienced by the nucleus and depends upon the strength of the applied magnetic field, the electronic environment of the resonant nucleus and its magnetogyric ratio (γ).

NMR is relatively insensitive because the energy difference between the spin-states is small and so the population imbalance between them is only small. The excited states are also relatively long lived so too much excitation equalizes the spin populations resulting in no net energy emission. Different nuclei also differ in their sensitivity due to differences in receptivity. The size (integral) of the signal received in an NMR experiment is directly proportional to the number of absorbing nuclei in the probe.

The average time for the restoration of the original spin-state population after excitation (the relaxation time) depends on many factors and varies for the same nucleus in different environments. An optimum acquisition time after the exciting pulse must therefore be established for any given nucleus. For example, in the spectra given later in this chapter the carboxyl carbons are not particularly intense, since their relaxation time is significantly longer than other carbons in the metabolites studied and are hence partially saturated under the acquisition conditions used.

All nuclei of a given type in the same molecule do not necessarily experience the same magnetic field. The experienced

field will differ from that applied due to screening by surrounding electrons (deshielding). Each nucleus thus requires a different frequency to promote it to an excited spin-state. The shift of frequency induced by the electronic environment is called the chemical shift and is, in most cases, unique for any given ^{13}C nucleus in a molecule. Since the extent of the screening is directly related to the strength of the applied field, the proportionate extent to which nuclei are deshielded does not vary with changes in the external field strength. The chemical shift can therefore be expressed in dimensionless units (parts per million, ppm) of the applied field and are thus instrument independent. In ^{13}C- and ^{1}H-NMR they are usually expressed relative to tetramethylsilane ($Si(CH_3)_4$) at 0ppm.

The prediction of chemical shifts is very difficult. From observations on many nuclei certain rules of thumb apply but for exact identification of a signal 'spiking' is often used. An attempt is made to boost that signal specifically by adding authentic compound to the sample.

Spin-Spin Coupling

As well as experiencing perturbations in the applied magnetic field due to electron-dependent deshielding, nuclei also experience interactions due to other nuclei in the same molecule. If these other nuclei have more than one allowed orientation relative to the applied field the interactions will be quantized and a multiple signal is seen for each of the interacting nuclei. For two ^{13}C atoms bonded together two signals are seen for each nucleus. These

are separated by a characteristic coupling constant (J) which is again independent of the applied field. These are given in Hz and in general for a ^{13}C nucleus bonded to n other ^{13}C nuclei, 2n signals will be recorded.

Coupling also occurs between different types of nuclei, for example ^{13}C and ^{1}H or ^{13}C and ^{15}N. ^{13}C-^{1}H coupling presents a special problem due to the high natural abundance of ^{1}H (>99%). Nearly all ^{13}C nuclei in biological molecules would show ^{13}C-^{1}H coupling and this would complicate the interpretation of the results. Thus, during the acquisition of a ^{13}C spectrum the sample is continuously irradiated at proton resonant frequencies. This decouples the protons and also enhances the ^{13}C signal strength (the nuclear Overhauser effect).

With nuclei whose natural abundance is low the use of enriched precursors allows spin-spin coupling to become very useful. As an example, if a specific pair of adjacent carbons are enriched for ^{13}C in a compound this will effectively label the integrity of the bond between them since any breakage and replacement with a ^{12}C atom will be revealed as a change from a doublet to a single signal. Similarly the proportion of different isotopic-isomers (isotopomers) of a compound formed (after supplying specifically enriched precursors) can be distinguished amongst a mixture. This can be important with respect to the stereochemistry of various reactions (see RESULTS section in this chapter).

In a typical NMR experiment therefore the sample is placed in an

intense magnetic field (usually in a helium-cooled superconducting magnet) and is subjected to an intense radio-frequency pulse to excite all the nuclei. Energy is subsequently re-emitted over a short period of time and is picked up by a receiver (this signal is called the free induction decay, FID) and the length of the reception period is governed by the relaxation time of the nuclei being observed. The FID is related to the frequency spectrum by Fourier transformation, carried out by a computer attached to the receiver. The pulse/receive cycle is repeated until an acceptable signal to noise ratio is achieved.

Since this process is non-destructive NMR can be used non-invasively to study metabolism in vivo. The minimum practical concentration of an enriched compound (100% ^{13}C) detectable in a reasonable time (overnight under the conditions described) would be about 30uM so only relatively large and long lived pools of intermediates will be detected.

The NMR experiments described in this chapter were designed to try and increase our understanding of the adaptation process at the biochemical level in Neurospora.

MATERIALS AND METHODS

Strains and Media

These are as described in the previous chapter.

Amino Acid Analysis

Extracts for amino acid analysis were prepared by extracting 20mg of ground lyophilised mycelium in 1ml of 10% sulphosalicylic acid, incubating at 0°C for 20-30 minutes and clarifying by centrifugation. The resulting supernatants were analysed using a Beckman IR120 amino acid analyser with 150µl of 1mM norleucine as an internal standard.

Extracts for NMR Analysis

0.5-1.5g (wet weight) of mycelium was either a) incubated with 5ml of 60% perchloric acid at 0°C for 10-20 minutes, or b) incubated with 5ml of 10% perchloric acid for 1 hour at 0°C. The mixture was then brought to pH7 with potassium hydroxide. Salt and cellular debris was removed by centrifugation. The supernatant was freeze-dried and resuspended in 20%D_2O in water containing 20µl/ml dioxan as an internal standard. The pellet was dried *in vacuo*, extracted with a 1:1 mixture of diethylether and ethanol (20ml) for 30 minutes at room temperature and the extracts clarified by filtration. Organic extracts were evaporated *in vacuo* and the residue redissolved in 400µl of deuterochloroform ($CDCl_3$) containing 25µl/ml dioxan for NMR analysis.

Isolation of alanine for NMR analysis

^{13}C-enriched alanine from cell extracts was isolated by preparative paper electrophoresis at pH2.1 (Ambler, 1963) on Whatman 3MM paper at 300v/cm. The alanine band was eluted using 0.1M ammonium hydroxide and the eluate freeze-dried.

NMR analysis

High resolution ^{13}C-NMR spectra were obtained using a Brucker WM300 wide-bore spectrometer operating at 75MHz. For *in vivo* studies freshly harvested mycelium grown and prepared as above was transferred to a 20mm NMR tube (250mg in 20ml). Immediately prior to the experiment [2-^{13}C] sodium acetate (0.5ml 2.7M) was added. The suspension was continuously oxygenated at *ca* 20ml/minute throughout the experiment. Spectra were accumulated in blocks of 1K pulses (16K time domain data points, acquisition time 0.27s, angle 30 degrees) and summed to 5K after visual inspection of the free induction decays (FID). A temperature of 25±2 degrees was maintained throughout.

The spectra of aqueous extracts were recorded in a 10mm probe (16K time domain data points, acquisition time 0.54s, angle 30 degrees, with a 1.5s time delay. FID were zero filled to 32K prior to Fourier transformation with a line broadening of 2.0Hz). High power broad band decoupling was used during the acquisition and low power during the delays. Spectra of lipid extracts were measured in a 5mm probe. ^{13}C chemical shifts are expressed relative to dioxan at 67.40ppm. The assignment of resonances in extracts was based on

enhancements of the appropriate signals on addition of known concentrations of authentic materials in spectra measured at pH7.2 and 6.0.

^{1}H-NMR spectra were measured in D_2O at 200MHz using a Brucker WP200 spectrometer. The spectra were referenced to external tetramethylsilane at 0.00ppm.

RESULTS AND DISCUSSION

Nuclear Magnetic Resonance (NMR) Studies using a Wild Strain

The experiments described in the previous chapter are very unsatisfactory and have many pitfalls, and there is always the feeling that one doesn't really know what is happening. Until recently the only way to do detailed studies of flux movements was to use a radioactive tracer which involves subsequent identification of all the labelled compounds. While this technique still has its advantages, many of its results can now be obtained much more rapidly using NMR and, since this technique is applicable to certain non-radioactive nuclides (^{1}H, ^{13}C, ^{15}N, ^{31}P), there is also no isotope containment problem. This technique seemed ideally suited therefore to the study of adaptation to acetate. It should be emphasised that a biological NMR experiment cannot really exist in isolation; one still only sees whatever is labelled. Other complementary studies, such as amino acid analyses and enzyme assays are still necessary to obtain a complete picture.

The isotope chosen for this study was carbon-13 and the enriched substrate was [2-^{13}C]-acetate. It is important to note that the enrichment at this position is only 89% and so there is a 10% possibility of a ^{12}C atom being present where there would be a ^{13}C atom with 100% enrichment. Similarly the natural abundance of this isotope is 1.1% and so some signals from compounds not labelled by added ^{13}C may be seen if they are present in high concentrations.

The first experiment took advantage of the wide bore magnet

attached to the Brucker WM300 spectrometer to look at the incorporation of isotope *in vivo*; as the mycelium was adapting inside the NMR tube. The results are shown in Figures 3.1 and 3.2. Figure 3.1 shows the time course from about one hour into the experiment until it was terminated at about 16 hours. Figure 3.2 shows in more detail the adaptation from time zero until 10 hours. Each trace represents the sum of the previous 5000 pulses (equivalent to 22.5 minutes). Label is initially seen to be incorporated into carbons 2,3 and 4 of glutamate and glutamine. After 1.5-2 hours a signal corresponding to bicarbonate (161.2ppm) appears and increases. At the same time signals corresponding to carbons in the disaccharide trehalose are evident, indicating that gluconeogenesis is taking place. Concomitant with the onset of gluconeogenesis, the consumption of acetate accelerates (see also Figure 3.3).

While this sort of study shows the overall trends in the adaptation, the resolution of the method is limited by two factors. First the low instantaneous concentrations of the enriched metabolites means that spectra must be accumulated for a relatively long time to achieve acceptable signal to noise ratios. Secondly, signals are progressively broadened due to coupling between adjacent carbon atoms. To obtain detailed data on the flux of ^{13}C during the experiment it was necessary to accumulate spectra of extracts of mycelium harvested at various times after transfer to [2-^{13}C] acetate medium.

Figures 3.1 & 3.2; ^{13}C-NMR time course of the metabolism of [2-^{13}C] sodium acetate by N.crassa. Each spectrum is the result of 5K pulses. HCO_3^- bicarbonate anion, glu and gln refer to resonances of glutamate and glutamine respectively.

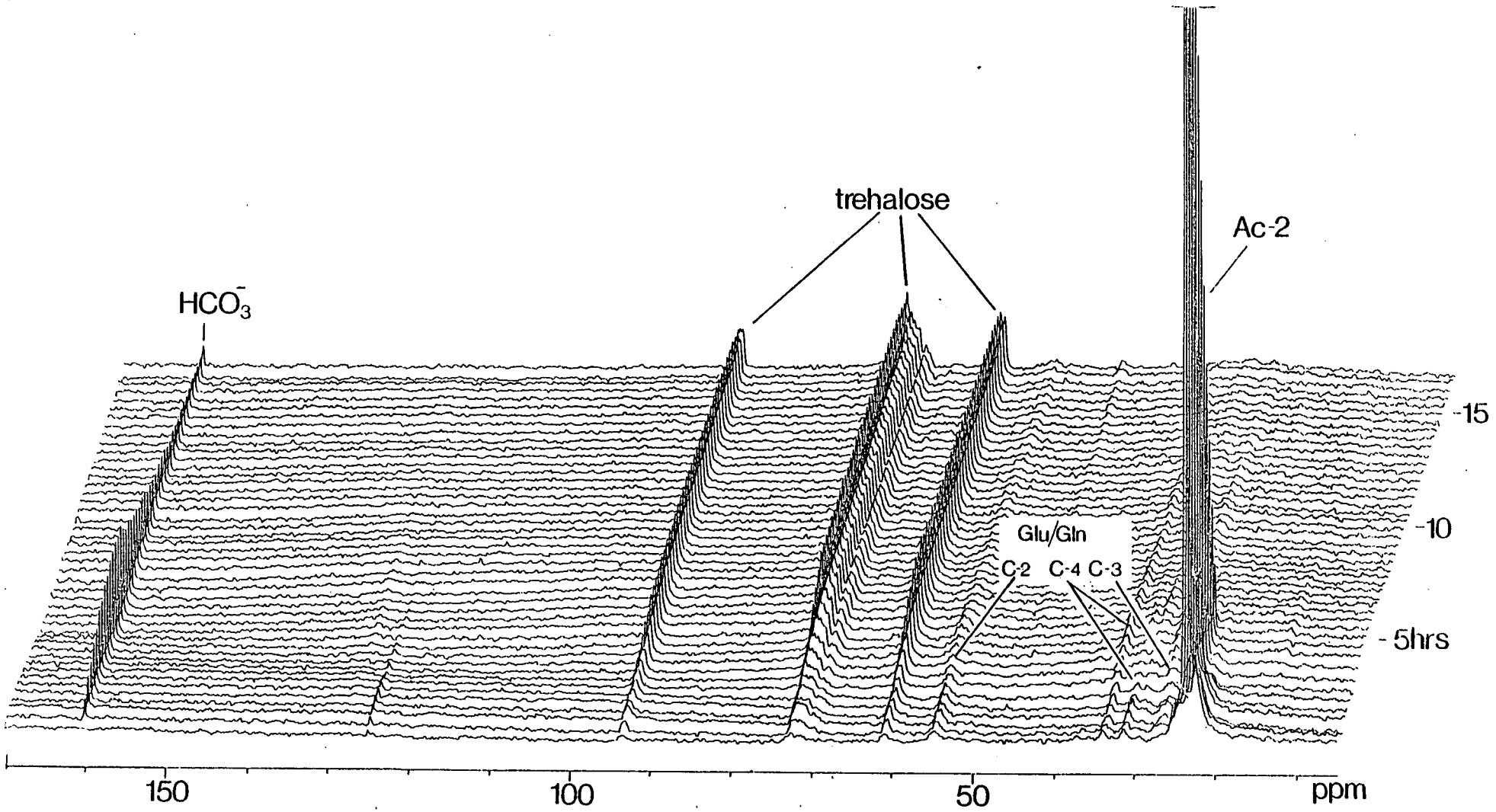


Figure 3,1

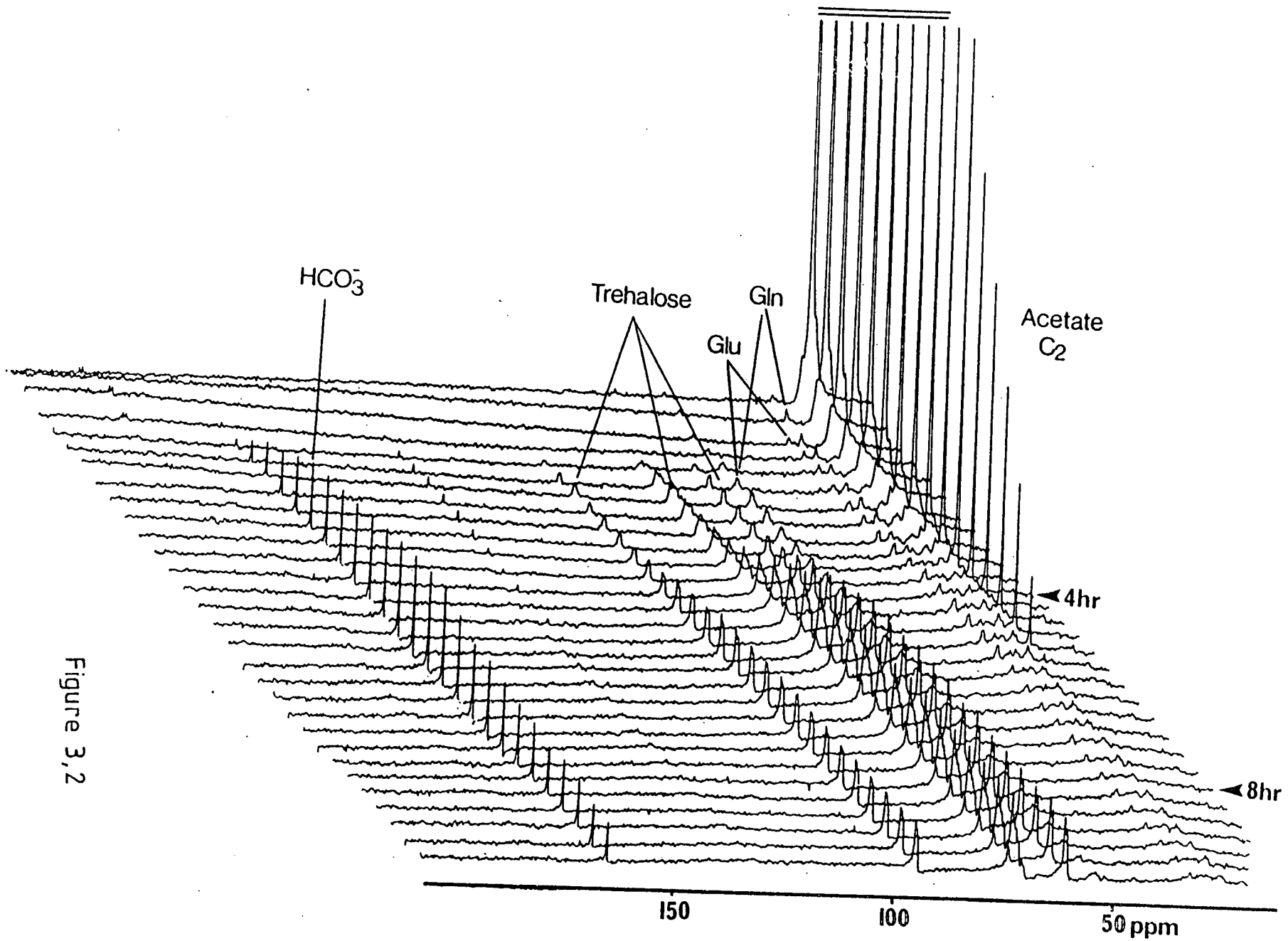


Figure 3,2

Figure 3.3; Change in the methyl peak intensities in the acetate medium as a function of time after mycelial transfer. The results for each strain are as indicated. 'wt lipid' denotes the peak intensities of lipid methyl resonances (18.4ppm) in ether-ethanol extracts from the wild type.

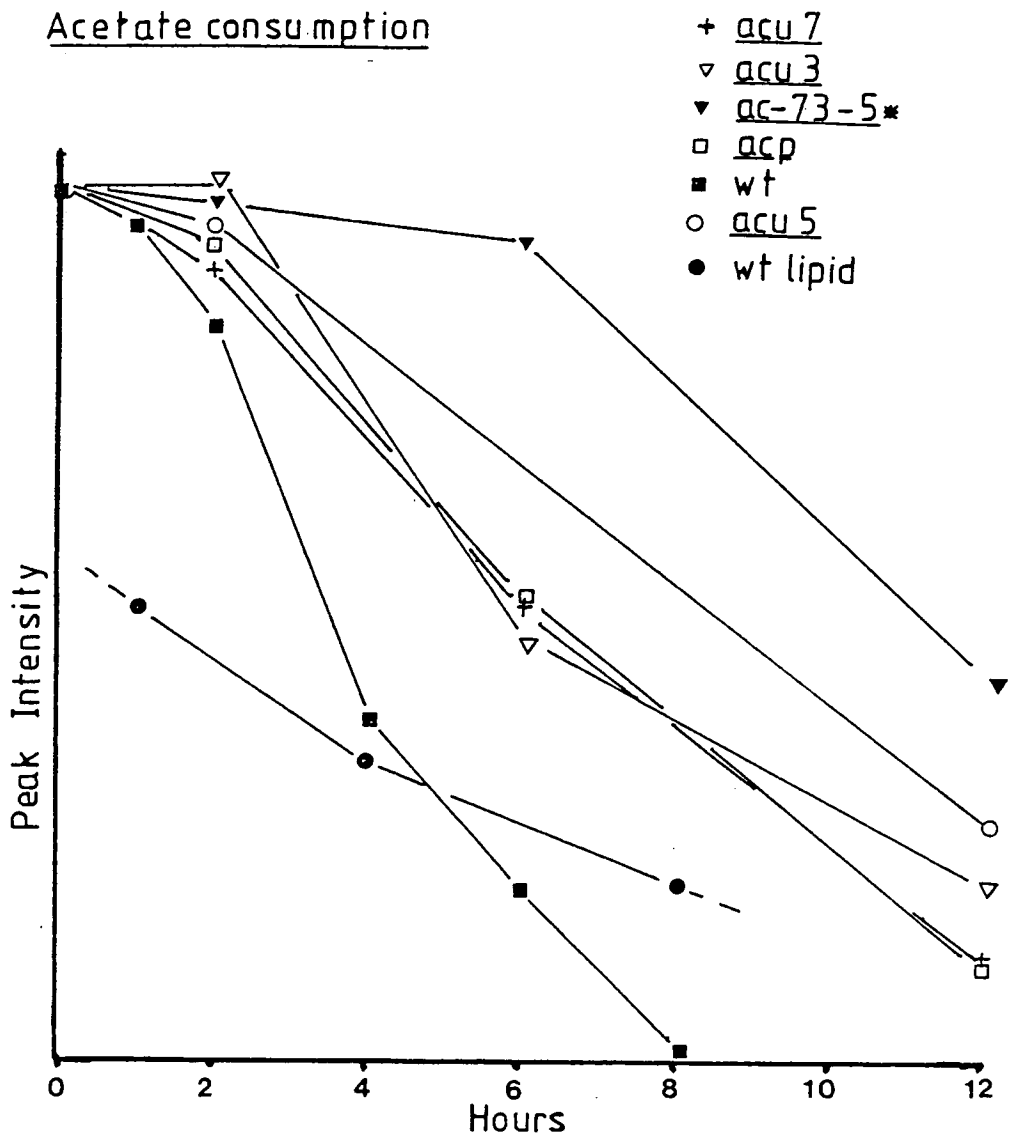

Acetate consumption
acu 7
acu 3
ac-73-5*
acp
wt
acu 5
wt lipid
Peak Intensity
0
2
4
6
8
10
12
Hours

Figure 3,3

General Considerations

Perchloric acid extraction was performed as described in the methods section on samples harvested at 1,2,4,6,7.5,12 and 16 hours after transfer. The spectra from 2 hours onwards are shown in Figure 3.4. During the first four hours after transfer label was initially seen in alanine (C2 at 51.6ppm, C-3 at 17.1ppm) and was gradually incorporated into glutamate and glutamine. At first the latter are primarily enriched at their C-4 (glutamate 34.3ppm, glutamine 31.3ppm). Subsequently label appeared in the C-3 and C-2 of these compounds (glu C2-55.8 ;C3-27.8 ;gln C2-55.2 ;C3-27.0). At four hours a signal for succinate C-2 (34.9ppm) was also evident.

Gluconeogenesis had commenced after six hours, indicated by the appearance of resonances corresponding to trehalose (C-1,1′ 94.1ppm, C-2,2′ 71.9ppm, C-3,3′ 73.4ppm, C-4,4′ 70.6ppm, C-5,5′ 73.0ppm, C-6,6′ 61.4ppm). At this time signals from enriched citrate (C-2/4 46.5ppm, C-3 76.8ppm) and malate (C-3 43.4ppm) were also apparent. In subsequent extracts the trehalose signals increase.

A replicate experiment (Figure 3.5) done in parallel with the mutants (see later) showed essentially the same data. In addition, however, the better quality of these extracts allows the resolution of some components present at lower concentrations - glycerol (C-1/3 64.0ppm, C-2 74.0ppm), glucose (C1 alpha 92.9ppm, C1 beta 96.7ppm), and some enrichment in various carboxyl groups was also evident (inset Figure 3.5 citrate 3′ 182.5ppm, citrate 1/5 179.7ppm and glutamate C-1 175.2ppm).

Figure 3.4; ^{13}C-NMR spectra of perchloric acid lysates of wild type mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The figures to the right of the spectra indicate hours in acetate medium. Intensities are scaled to a dioxan standard at 67.40ppm (this peak has been omitted for clarity in some cases). Spectra of extracts made at 7.5 and 16 hours are drawn at 0.5x the scale of the earlier spectra. T-1 to T-6 refer to α,α'-trehalose carbons C-1,1′ to C-6,6′; Cit-2,4 and Cit-3 refer to C-2, C-3 and C-4 of citrate; suc-2 to C-2 of succinate; glu-2, glu-3 and glu-4 to C-2, C-3 and C-4 of glutamate; gln-2, gln-3 and gln-4 to C-2, C-3 and C-4 of glutamine; ala-2 and ala-3 to C-2 and C-3 of alanine; mal-3 to C-3 of malate. No significant enrichment of the carboxyl carbons was observed in these samples.

Subsequent figures also show cit-1,5 which refers to citrate C-1 and C-5; glu-1 to glutamate C-1; asp-2 to aspartate C-2; G-1β and G1-α to glucose C-1 beta and alpha isomers; gl-2 and gl-1,3 to glycerol C-1, C-2 and C-3; ala-1 to alanine C-1; D to dioxan.

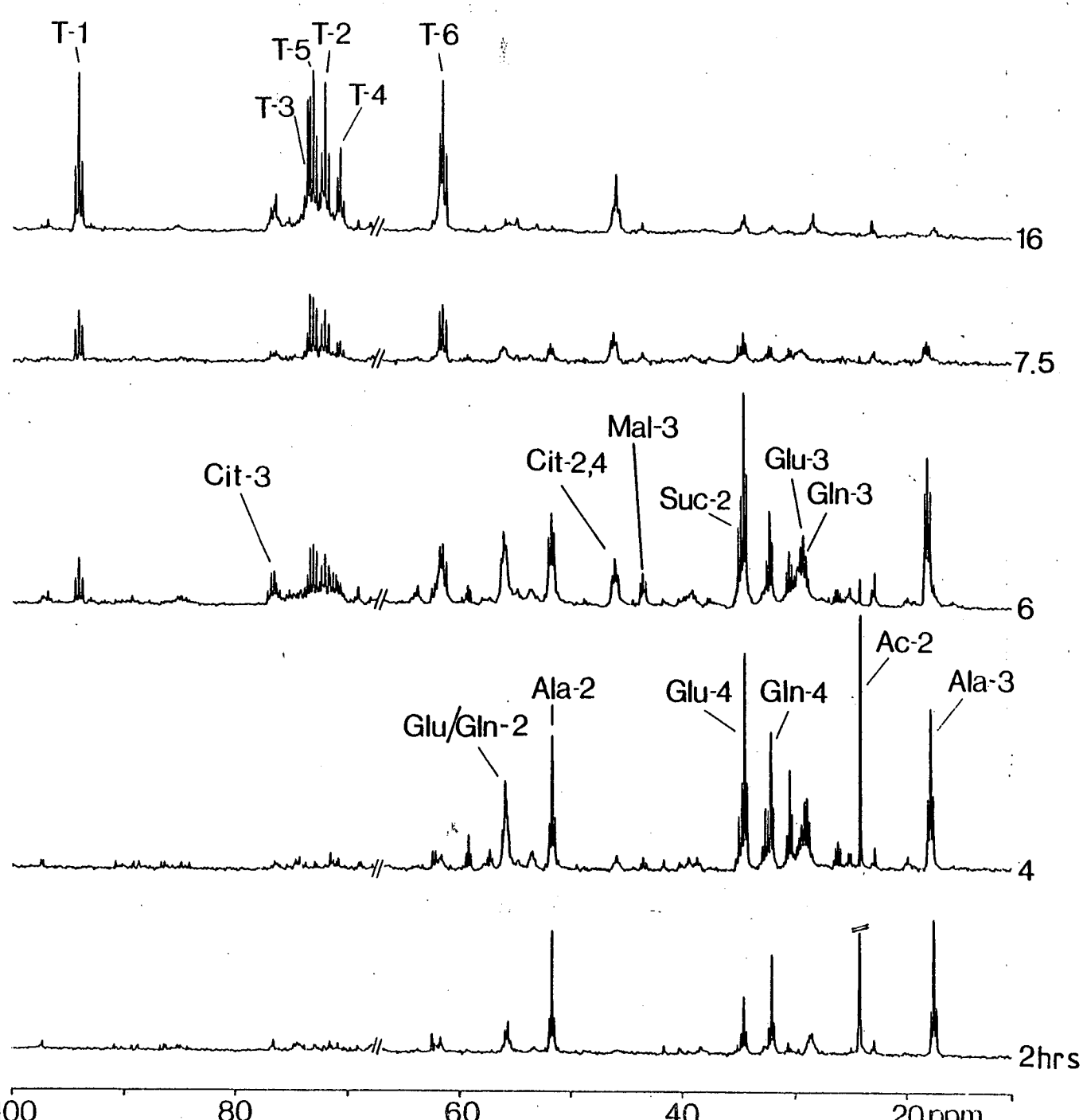

T-1
T-5
T-2
T-6
T-3
T-4
16
7.5
Mal-3
Cit-3
Cit-2,4
Suc-2
Glu-3
Gln-3
6
Ac-2
Ala-2
Glu-4
Gln-4
Ala-3
Glu/Gln-2
4
2hrs
100
80
60
40
20 ppm

Figure 3-4

Figure 3.5; Replicate of the experiment shown in Figure 3.4. The peaks are labelled as in Figure 3.4. The inset shows the carbonyl region of the 12 hour spectrum with a 5/3x expanded vertical scale.

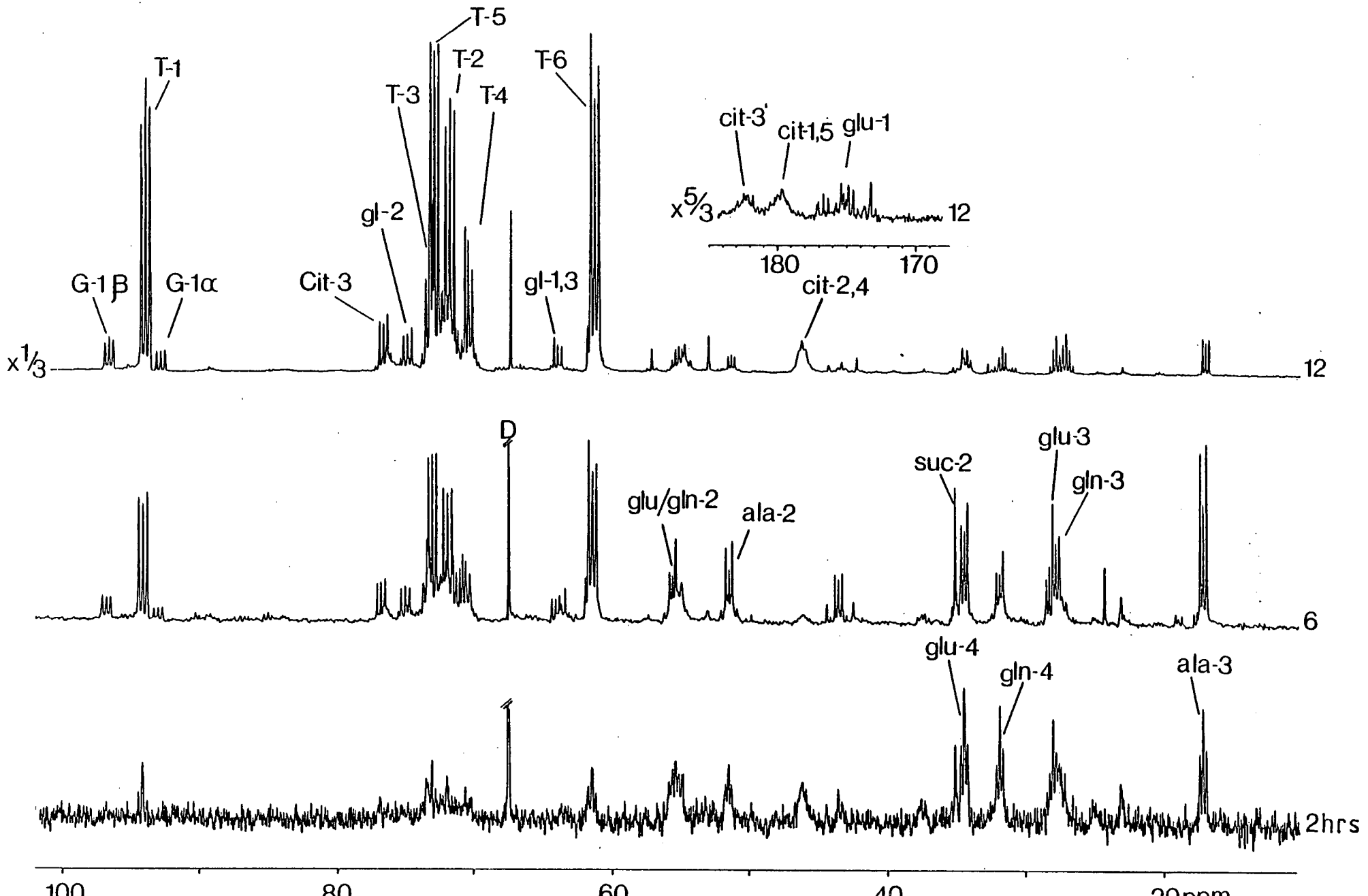

T-5
T-1
T-2
T-6
T-3
T-4
cit-3'
cit-1,5
glu-1
gl-2
x5/3
12
180
170
G-1β
G-1α
Cit-3
gl-1,3
cit-2,4
x1/3
12
D
glu-3
suc-2
gln-3
glu/gln-2
ala-2
6
glu-4
gln-4
ala-3
2hrs
100
wt
80
60
40
20ppm

Figure 3-5

These results correspond well with the in vivo experiment. In addition a parallel experiment in which the enzymes isocitrate lyase and PEP carboxykinase were monitored, showed (Figure 3.6) that their induction corresponds well with the onset of gluconeogenesis between 4 and 6 hours (Figure 3.4).

Coupling patterns

a)Glutamate and Glutamine

As mentioned in the introduction to this chapter when two ^{13}C are adjacent to one another in a molecule their spins interact and the resultant resonances are observed as doublets. This phenomenon known as spin-spin splitting can be extended for several interactive magnetic nuclei in a molecule. For a general case the signal of an atom adjacent to (or interacting with) n non-equivalent magnetic nuclei will be observed as 2n lines (Scott & Baxter, 1981). In principle therefore it should be possible (knowing the absolute ^{13}C-enrichment at each position and the integral values of each peak) to work out the proportion of each species (isotopomer) present in the mixture (London et al, 1975, Walker et al, 1982, Dickinson et al, 1983).

Some specific predictions of the isotopomer ratios and patterns can be made based on the known stereochemistry of various pathways. The entry of label from [2-^{13}C] acetate into the TCA cycle intermediates, is shown in Figure 3.7. Initially the acetate moiety of acetyl-CoA combines with oxaloacetate to produce citrate labelled at C-4. Due to the symmetry of succinate, molecules labelled thus will give rise to oxaloacetate labelled either in the C-2 or the

Figure 3.6; Increase in the specific activities of a) isocitrate lyase (ICL) and b) PEP carboxykinase (PEPC) on transfer to acetate medium under the conditions of the ^{13}C-NMR experiments.

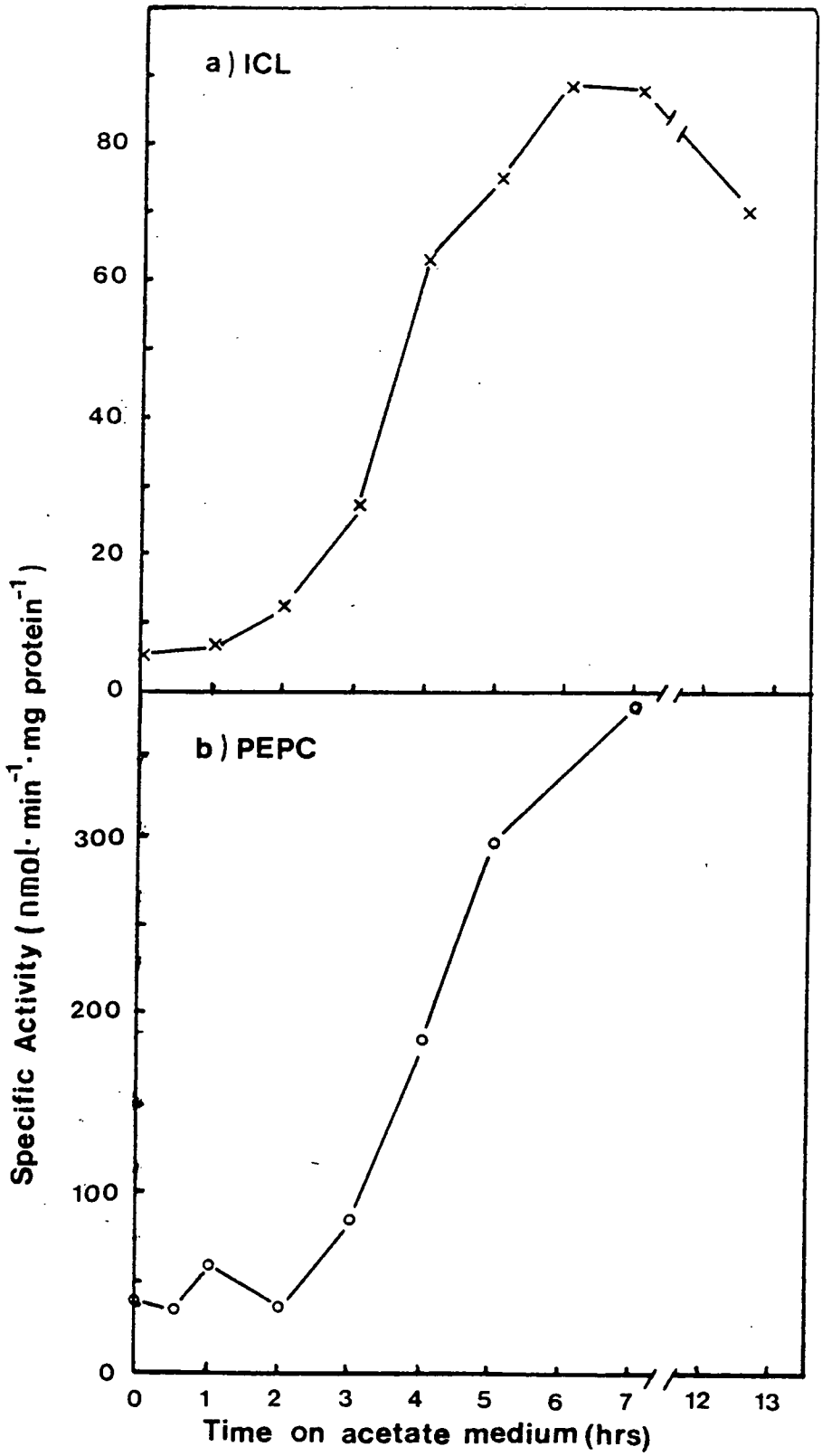

a) ICL
b) PEPC
Specific Activity (nmol·min⁻¹·mg protein⁻¹)
Time on acetate medium (hrs)
0
20
40
60
80
100
200
300
0
1
2
3
4
5
6
7
12
13

Figure 3,6

Figure 3.7; The progressive randomization of ^{13}C, supplied as [2-^{13}C] sodium acetate, via the TCA cycle. Abbreviated structures (see Key) are used for clarity.

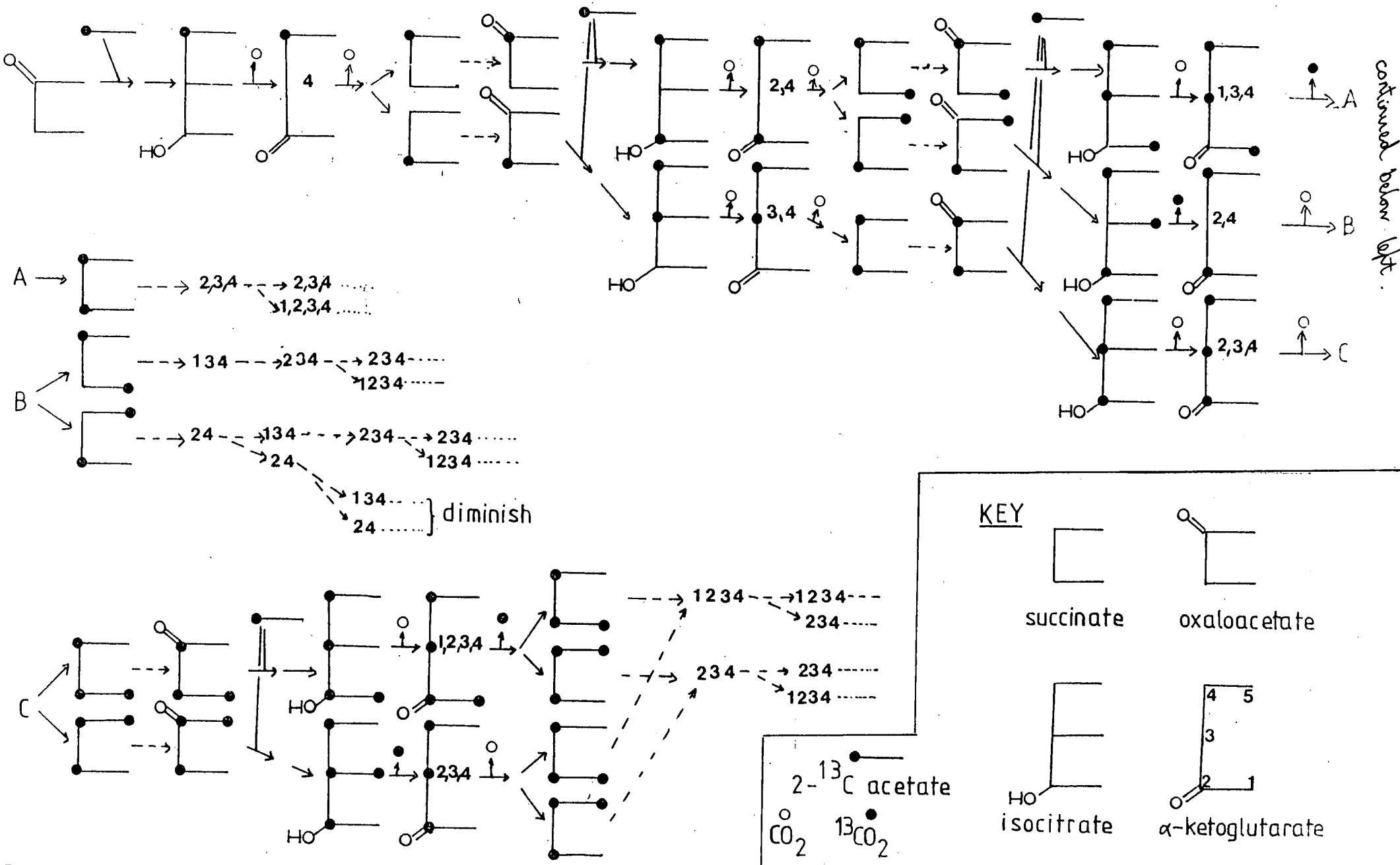


Figure 3,7 : TCA isotopomers

C-3. If these combine with another acetate moiety, [2,4-$^{13}C_2$] and [3,4-$^{13}C_2$] citrate will result in equal proportions and so on.

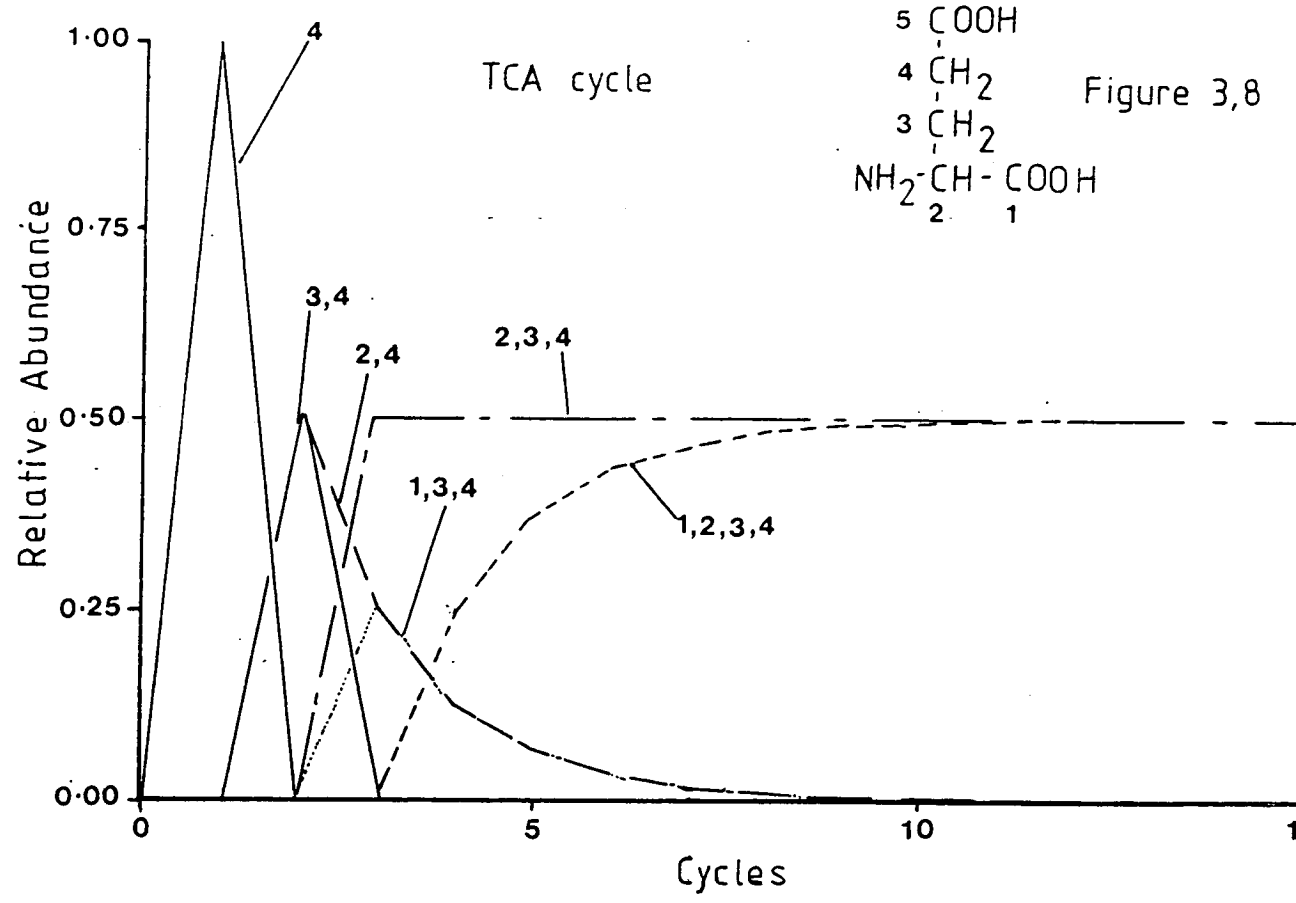
The succession of glutamate isotopomers produced in this way from 2-oxoglutarate is graphed in Figure 3.8. This essentially corresponds to a simple model where a single pool of intermediates is cycling in synchrony. After ten cycles an equilibrium is reached where the two isotopomers [1,2,3,4-$^{13}C_4$] and [2,3,4-$^{13}C_2$] are produced in equal amounts.

None of the TCA cycle intermediates has a sufficiently large ^{13}C-enriched pool size in the early extracts to be detectable, however, glutamate is synthesized directly from 2-oxoglutarate and will thus reflect the latter's labelling pattern. Whether the glutamate pool acts as an accurate monitor of TCA cycling will depend on its rate of equilibration with 2-oxoglutarate.

The same exercise can be carried out for a system where the glyoxylate cycle is operating to bypass the decarboxylating steps of the TCA cycle. The succession of isotopomers in this case is shown in Figure 3.9. An equilibrium is again reached after about ten cycles, but in contrast to the TCA cycle situation, only [2,3,4-$^{13}C_3$] 2-oxoglutarate (derived from glyoxylate cycle isocitrate) results - there can be no incorporation into the C-1. Thus any excess of [2,3,4-$^{13}C_3$] glutamate over the [1,2,3,4-$^{13}C_4$] isotopomer must be due to glyoxylate cycle activity and it should be possible to estimate the relative predominance of the two cycles (Dickinson et al, 1983).

Figure 3.8; The predicted succession of ^{13}C-isotopomers of glutamate (from 2-oxoglutarate) derived from TCA cycle activity.This simplified model uses a single synchronous pool of intermediates.

Figure 3.9; The predicted succession of ^{13}C-isotopomers of glutamate (from 2-oxoglutarate) derived from glyoxylate cycle activity. This simplified model uses a single synchronous pool of intermediates.


TCA cycle
5 COOH
4 CH2
3 CH2
NH2-CH-COOH
2 1
Figure 3,8
Relative Abundance
1·00
0·75
0·50
0·25
0·00
4
3,4
2,4
2,3,4
1,3,4
1,2,3,4
0
5
10
Cycles

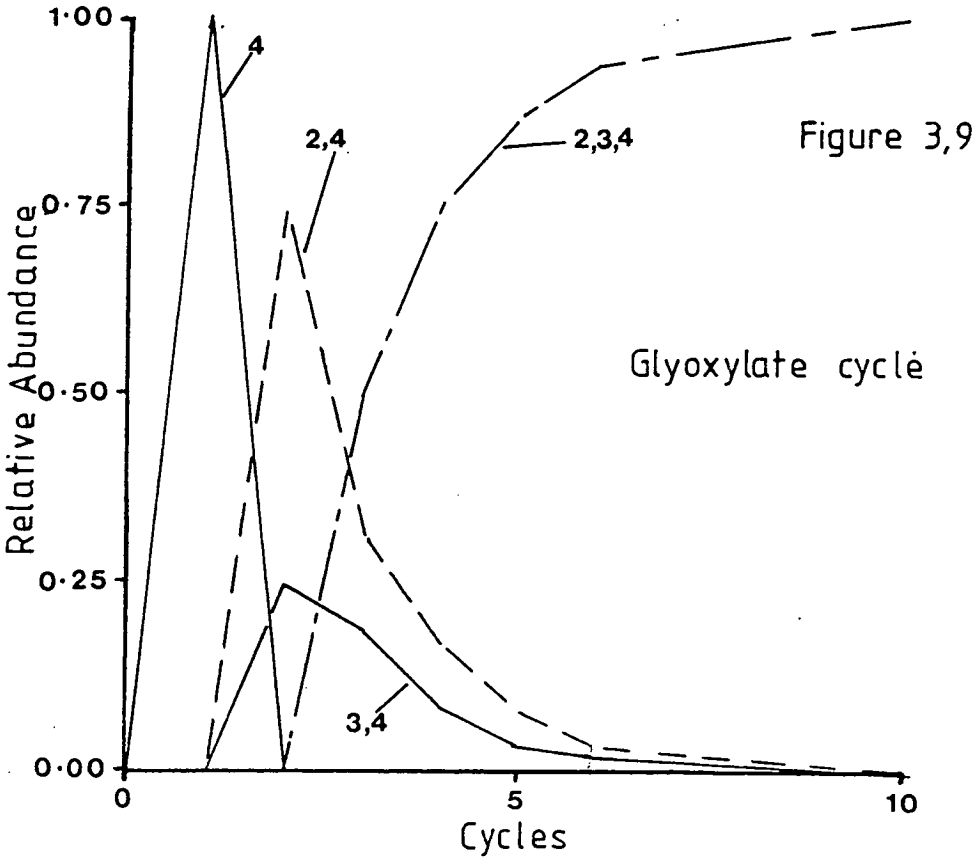


Figure 3,9
Glyoxylate cycle
Relative Abundance
1·00
0·75
0·50
0·25
0·00
4
2,4
2,3,4
3,4
0
5
10
Cycles

While it was not possible to determine the absolute ^{13}C-enrichments for glutamate or glutamine in these experiments it is clear from the spectra of perchloric acid extracts (Figure 3.4) that an increasing proportion of multiply enriched species is produced. The visualization of carboxyl groups such as the C-1 of glutamate is poor using these accumulation parameters, due to their long relaxation time. Determination of quantitative enrichments for these carbons is not possible. However, the virtual absence of signals corresponding to C-1 of glutamate or glutamine suggests that incorporation at these positions is very low. It is possible to get some measure of the enrichment at the C-1 in multiply enriched metabolites from the multiplets at the C-2. In the spectra of the perchloric acid extracts the C-2 resonances of glutamate and glutamine are observed as overlapping multiplets with central singlets at 55.2 and 55.8 ppm respectively. While both signals exhibit doublets ($^1J_{2,3}$=34.4 and 34.7 Hz respectively), doublets of doublets corresponding to C-1,C-2 coupled species ($^1J_{1,2}$=53.6 Hz) are not present at appreciable intensities indicating that only very small amounts of the [1,2,3,4-$^{13}C_4$] isotopomer is present. In contrast the presence of doublets for both C-2 and C-4 resonances and of overlapping doublets and triplets for the C-3 resonances of these amino acids indicates that [3,4-$^{13}C_2$], [2,4-$^{13}C_2$] and [2,3,4-$^{13}C_3$] isotopomers are present as significant proportions.

It can be deduced that the [2,3,4-$^{13}C_3$] over [1,2,3,4-$^{13}C_4$] ratio is large from the glutamate/glutamine coupling as follows. At the C-4 positions the singlet peak was initially dominant, but, at later times the doublets exceeded the singlets. Thus most of the 4-^{13}C

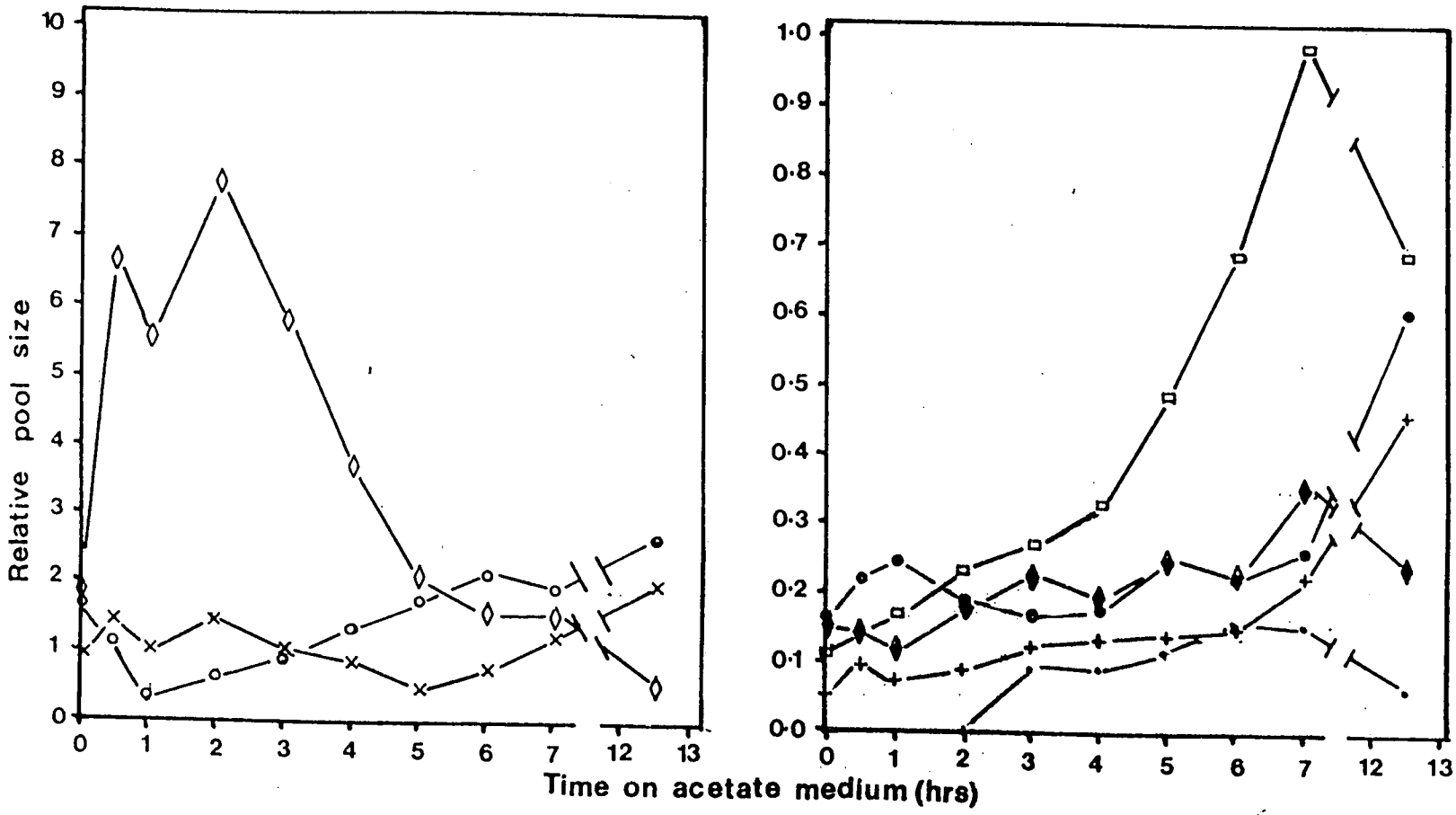
labelled species were 3,4 coupled at later times. Consideration of the C-3 position shows that the doublet signal was less than those indicating 2,3,4 coupling. Finally the C-2 signals showed only very low 1,2,3 coupling. Thus the most abundant isotopomer was apparently [2,3,4-$^{13}C_3$] with only a very little [1,2,3,4-$^{13}C_4$] present. This suggests that the glyoxylate cycle is responsible for randomizing most of the label seen in glutamate and glutamine.

The accuracy of glutamate as a monitor for the equilibration of label is supported by the amino acid analyses on extracts made at various times after transfer to acetate medium in a parallel experiment (Figure 3.10). This shows that over the first six hours of the NMR experiments there was no net increase in this pool and suggests that the glutamate pool is in continual equilibration with 2-oxoglutarate. Indeed it may be that a large amount of cycling between 2-oxoglutarate and glutamate is brought about by the transamination of pyruvate to produce alanine (Figure 3.10). The glutamate/alanine transaminase which catalyses this reaction is known to be present in large amounts (Fincham, 1951).

b)Alanine

Over the first four hours of acetate adaptation the size of the intracellular alanine pool quadruples (Figure 3.10). Analysis of the labelling intensity (Table 3.1 - described below) indicated that this is around 90% unlabelled at its maximum size so it would appear that this accumulation was derived from pre-existing precursor pools (sucrose, glycolytic and TCA intermediates). A factor in this may be an abrupt increase in the inhibition of pyruvate dehydrogenase by

Figure 3.10; Pool sizes of amino acids determined as described in the methods section relative to 150ul of 1mM norleucine as an internal standard. ◊ - alanine; o - glutamate; × - glutamine; □ - aspartate; ● - glycine; + - methionine; ♦ - valine; • - isoleucine.


Relative pool size
Time on acetate medium (hrs)

acetyl-CoA (Harding et al, 1970) derived from acetate in the medium; this would prevent oxidation of pyruvate to acetyl-CoA and any glycolytic flux could be diverted into alanine.

As an aside this effect would prevent the efficient oxidation of pyruvate via the TCA cycle and explain the observed inhibition of growth on sucrose by acetate above 50mM in concentration (Flavell, 1967; Beever, 1972). Carbon could only enter the TCA cycle through the anaplerotic pyruvate carboxylase and would be dependent on acetyl-CoA influx from acetate in the medium for continued cycling. The acetate influx would be limited to basal levels by catabolite repression of acetyl-CoA synthetase. When present together both acetate and sucrose are used simultaneously (Flavell & Woodward, 1971; Kobr et al, 1965). Some of the alanine increase may be due to a flux from oxaloacetate, as gluconeogenic precursors build up (label does enter it). The existence of such a flux would help to explain why the carbon diverted into alanine from glycolysis is not metabolised to oxaloacetate and results in its accumulation. If this route is used to a significant extent there must be some anaplerotic flux at a very early stage since carbon is leaving the TCA cycle (to accumulate as alanine). This may imply a basal or constitutive glyoxylate cycle activity. Some evidence exists for a constitutive isocitrate lyase isozyme (Sjorgren & Romano, 1967, Flavell & Woodward, 1971), but this may be artifactual (Rougemont & Kobr, 1973).

Pyruvate carboxylase from N.crassa is apparently not stimulated to produce oxaloacetate by acetyl-CoA (Beever, 1973) as it is in

other organisms such as Saccharomyces cerevisiae (S.cerevisiae) (Cazzulo & Stoppani, 1968), and it would be interesting to know whether such regulation prevents alanine from accumulating. In an NMR study on S.cerevisiae (Dickinson et al, 1983), in which [2-^{13}C] alanine was administered, there was no accumulation of labelled acetate. However, their cells were grown on a nitrogen-poor medium to induce sporulation and, since alanine levels respond to nitrogen supply as well as carbon (Kanamori et al, 1982), the comparison has limited validity.

The label seen to enter the alanine pool (Figures 3.4 & 3.5) is almost all in carbons 2 and 3 (51.6 and 17.1 ppm respectively). Enrichment at the C-1 position (176.3 ppm) is negligible and, together with the low intensity of the C-2 satellites corresponding to the [1,2,3-$^{13}C_3$] isotopomer, this indicates that the labelled alanine was derived via pyruvate, primarily from [2-^{13}C], [3-^{13}C] and [2,3-$^{13}C_2$] oxaloacetate. This is consistent with equilibration of label mainly via the glyoxylate cycle since the TCA cycle should produce significant enrichment in the C-1 position of alanine precursors at any stage when it is producing the [2,3-^{13}C] isotopomer.

The ^{13}C-enrichment at each position of alanine in four perchloric acid extracts (at 2, 4, 6 7.5 hours after transfer to acetate medium) was estimated from ^{1}H-NMR spectra of the amino acid purified by paper electrophoresis. Since ^{13}C and ^{1}H nuclei demonstrate coupling analagous to ^{13}C-^{13}C coupling described above, quantification of satellite peaks allows direct determination of

^{13}C-enrichment from these spectra.

The methyl hydrogen resonance was observed as a complex signal consisting of a central (^{12}C-H) doublet ($^{3}J_{HH}$=7.3 Hz) and a doublet of doublets ($^{3}J_{HH}$=7.3 Hz, $^{1}J_{CH}$=130 Hz). In multiply enriched species both central and satellite signals showed further coupling to the adjacent C-2 ($^{2}J_{CHH}$=4.7 Hz). Comparison of the integrals for each of these signals allowed the estimation of the ^{13}C-enrichment at C-3 and C-2 (Table 3.1). Multiplet analysis using these values and doublet/singlet ratios obtained from ^{13}C-NMR spectra enabled estimations of the relative ^{13}C-isotopomer populations for alanine in each extract (London et al, 1975; Walker et al, 1982; Dickinson et al, 1983).

The relatively high initial proportions of [2-^{13}C] and [3-^{13}C] singly enriched isotopomers (Table 3.1) reflects the significant dilution of added ^{13}C label by intermediates at natural abundance, during the early phase of adaptation. It can be seen that the C-2 and C-3 singly enriched isotopomers are present in similar amounts, suggesting that the oxaloacetate precursory to alanine is enriched to the same extent at C-2 and C-3. Initial derivation from precursors randomized via the glyoxylate cycle would be expected to be primarily enriched at the C-3 of alanine. In the early samples this result can be accounted for either by some labelled precursors coming from the TCA cycle prior to complete equilibration of the label (glutamate equilibration is not seen until 4-6 hours -Figure 3.4), or by the activity of a cytoplasmic fumarase (Cohen et al, 1981). The latter would equilibrate carbons 2 and 3 via the

Table 3,1 Proportions of ^{13}C-enriched alanine isotopomers produced during acetate adaptation.

time (h)	atom % ^{13}C at C-3 (a)	[3-^{13}C] : [2-^{13}C] : [2,3-$^{13}C_2$]		
2	12 (b)	0.8	1.0	1.2
4	24	0.8	1.0	1.6
6	50	1.0	1.0	6.4
7.5	52	0.9	1.0	7.2

(a) Enrichments at C-2 were estimated from the integrals of the C-3 satellite signals arising from 3-bond coupling of the methyl protons with ^{13}C at C-2. The values obtained were identical to those measured for C-3. No significant enrichment at C-1 could be detected by ^{13}C-NMR spectroscopy.

(b) Adjusted for the presence of unenriched alanine.

symmetry of fumarate. At later times, [2,3-$^{13}C_2$] alanine becomes predominant without the concomitant appearance of the [1,2,3-$^{13}C_3$] isotopomer which would signal TCA cycle derivation of alanine precursors, reflecting the increase in glyoxylate cycle activity. Thus there may be a transition in the source of alanine precursors from the TCA to the glyoxylate cycle as one might predict. Detectable glyoxylate cycle activity is, however, definitely present by two hours.

As gluconeogenesis starts so the alanine pool decreases again presumably indicating a flux through pyruvate carboxylase. After the peak at two hours the alanine pool must be in equilibrium with oxaloacetate since its enrichment increases (Table 3.1) as the pool size decreases (Figure 3.10). The behaviour of this pool is quite consistent with its proposed role as a pyruvate and amino nitrogen store (Kanamori et al, 1982) and suggests that its' build up in this case is caused by an increase in the pyruvate pool.

c)Trehalose

Between four and six hours after transfer to acetate medium, when the enzymes are approaching their maximal activities (Figure 3.6), signals corresponding to ^{13}C-enriched trehalose are seen in the spectra of the extracts (Figure 3.4). The disaccharide is principally enriched at C-1, C-2, C-5 and C-6 of the constituent glucose units. Signals corresponding to the C-3 and C-4 are present at low levels to begin with but form an increased proportion of the population at later times (Figure 3.4).

Labelling at the C-3 and C-4 is indicative of C-1 labelling at the triose-phosphate level. ^{13}C-enrichment at this position is indicative of either: 1)a TCA cycle contribution to the precursor pools or 2)metabolism of [2,3-$^{13}C_2$] enriched alanine to a [1,2-$^{13}C_2$] enriched acetate moiety in acetyl-CoA, if inhibition of pyruvate dehydrogenase has decreased due to acetate depletion in the medium. The latter would also give rise to glutamate (C5-C4) and succinate (C1-C2) coupling which is not observed.

All the carbons of the constituent glucose units were highly coupled as expected for well equilibrated precursors. An interesting asymmetry in the labelling is, however, apparent. This is the more singlet nature of the trehalose C-1,1′ as opposed to the C-6,6′ position (Figures 3.4 3.5), both of which are equivalent at the triose level. This distribution of label probably arose from pentose phosphate pathway activity. In its classic formulation which oxidizes one glucose unit completely, the net reaction affects the labelling as follows:-

$$6[1,2,5,6\text{-}^{13}C_4]\ \text{D-glucose-6-phosphate (G6P)} \longrightarrow$$
$$6\,^{13}CO_2 + 12\text{NADPH} + 12H^+ + 2[1,3,5,6\text{-}^{13}C_4]\ \text{G6P}$$
$$+2[1,5,6\text{-}^{13}C_3]\ \text{G6P} + 1[1,2,5,6\text{-}^{13}C_4]\ \text{G6P}$$

Thus at the expense of oxidizing 6 carbons to CO_2 four glucose units have now become uncoupled in their C-1 position whilst all are still 5-6 coupled. The difference in the doublet to singlet ratios at the trehalose C-1,1′ and C-6,6′ is thus a measure of the pentose phosphate pathway activity (I am grateful to Dr R.L. Baxter for

pointing this out to me). The C-1,1′ position is 58% singlet, whilst the C-6,6′ is 41% singlet (12hours Figure 3.5); thus there is 17% excess singlet at the C-1,1′ which represents 60% of the glucose-6-phosphate that entered the pentose phosphate pathway (20% is lost as CO_2 and 20% has unchanged coupling). Thus very roughly 28% of the gluconeogenically synthesized sugar passes through this pathway.

As the [2-^{13}C] acetate in the medium is consumed catabolism of fatty acids is occurring (Figure 3.3). This will dilute the ^{13}C-enriched acetyl-CoA pool with natural-abundance material. This dilution will become increasingly significant at later incubation times since the acetate in the medium is completely exhausted prior to the complete depletion of the fatty acids (Figure 3.3). This is reflected in a return to a less coupled pattern in trehalose between 7.5 and 8 hours (Figure 3.4). This effect was less marked in the other experiment (Figure 3.5).

d)Other Considerations - citrate, succinate and malate

The biosynthesis of trehalose is accompanied by the appearance of signals for C-2/4 and C-3 of citrate in the extract spectra (Figures 3.4 3.5). This accumulation appears after the induction of PEP carboxykinase and suggests that some step in the consumption of citrate may be inhibited after gluconeogenesis has started. Purified isocitrate lyase from N.crassa has been shown to be inhibited by PEP and fructose-1,6-diphosphate (Johanson et al, 1974), so this is a likely control step. This has also been suggested in E.coli (Ashworth & Kornberg, 1963). Another

contributing factor may be inhibition of the isocitrate dehydrogenase isozymes. In yeast, the NADPH/NADP ratio in acetate grown cells is about twice that in glucose grown cells (Satrustegui et al, 1983) and, since in this organism NADPH inhibits both NADP and NAD-linked isocitrate dehydrogenases, this will inhibit utilization of citrate via the TCA cycle. This mechanism was postulated to aid the channelling of isocitrate to the glyoxylate cycle (Satrustegui et al, 1983). If this applies in N.crassa as well then the increase in NADPH during adaptation will further inhibit citrate consumption.

Signals for malate and succinate showed peak incorporation around 6 hours (Figures 3.4 & 3.5). Both of these are glyoxysome export products in the compartmentalized model (Figure 1.1) so their accumulation may be in this organelle; however, this is only surmise. They both subsequently decrease (or become diluted) as the acetate is exhausted.

Signals are also seen corresponding to the carbons of glycerol (Figures 3.4 & 3.5) indicating some flux out of the gluconeogenic pathway at dihydroxyacetone phosphate. The physiological significance of this is not apparent.

In conclusion the wild type adaptation pattern in these experiments seems to consist of three stages :-

1) A lag phase where the enzymes of the glyoxylate cycle and gluconeogenesis are starting their increase. This is characterised by slow acetate consumption and a rapid build up of alanine with label fluxing into this pool and that of glutamate and glutamine.

2) A pre-gluconeogenic phase where glyoxylate cycle activity is clearly indicated by the labelling patterns of alanine, but no trehalose is synthesized.

3) A gluconeogenic phase characterised by the rapid consumption of acetate, the accumulation of labelled citrate, malate, succinate and trehalose, and the consumption of fatty acid reserves. This occurs as the enzymes are approaching their maximal levels.

A second part to the gluconeogenic phase where exogenous acetate has been completely consumed and dilution of label by continued metabolism of endogenous fatty acids at natural abundance is seen in the dilution of label in trehalose.

The glyoxylate cycle appears to predominate from a very early time (prior to maximal enzyme induction), as evinced by the labelling patterns.

Studies on acetate non-utilizing mutants using NMR

A number of mutants have been isolated which lack the ability to grow on acetate as sole carbon source (Flavell & Fincham, 1968a). These have been shown to lack various enzymatic activities concerned with the glyoxylate cycle, TCA cycle and gluconeogenesis (Flavell & Fincham, 1968b; Leckie & Fincham, 1971; Beever & Fincham 1973). To further complement the NMR studies on adapting wild type mycelium, several of these mutants were examined. All showed slower acetate consumption than the wild type (Figure 3.3).

a)acu3 - the structural locus for isocitrate lyase

Isocitrate lyase along with malate synthase forms the TCA cycle bypass section of the glyoxylate cycle. Without this activity the cycle cannot function and no anaplerosis should be possible on acetate as sole carbon source. The ^{13}C-NMR spectra of extracts from this strain (Figure 3.11 & 3.12) graphically illustrate this. No trehalose is synthesized at any time and only a trace of labelled glucose is seen at 12 hours. This latter observation probably indicates that in spite of the absence of a net gluconeogenic flux some degree of equilibration between enriched TCA cycle derived intermediates and the monosaccharide pool does occur.

Another very striking abnormality in this mutant is the accumulation of an appreciable quantity of ^{13}C-enriched citrate. Spectra of the growth medium showed that a significant amount of this compound had leaked out of the mycelium during the course of the incubation.

Figure 3.11; ^{13}C-NMR spectra of perchloric acid lysates of acu3 mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4. The duplicate 6 hour spectrum was from the same extract but with the pH corrected (Note the abnormal locations of the citrate C-2,4 and glutamate C-4 peaks in the lower of the two).

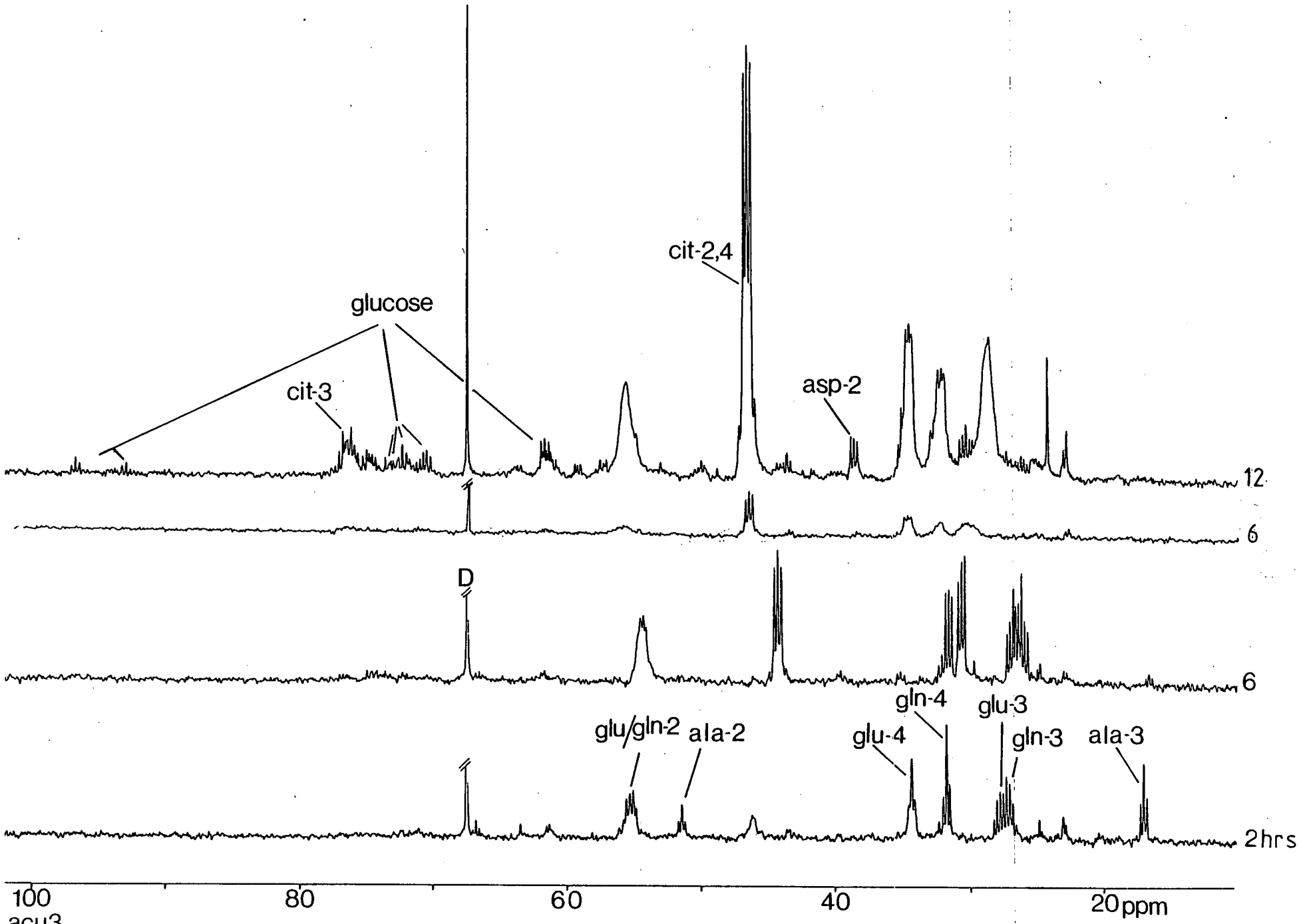
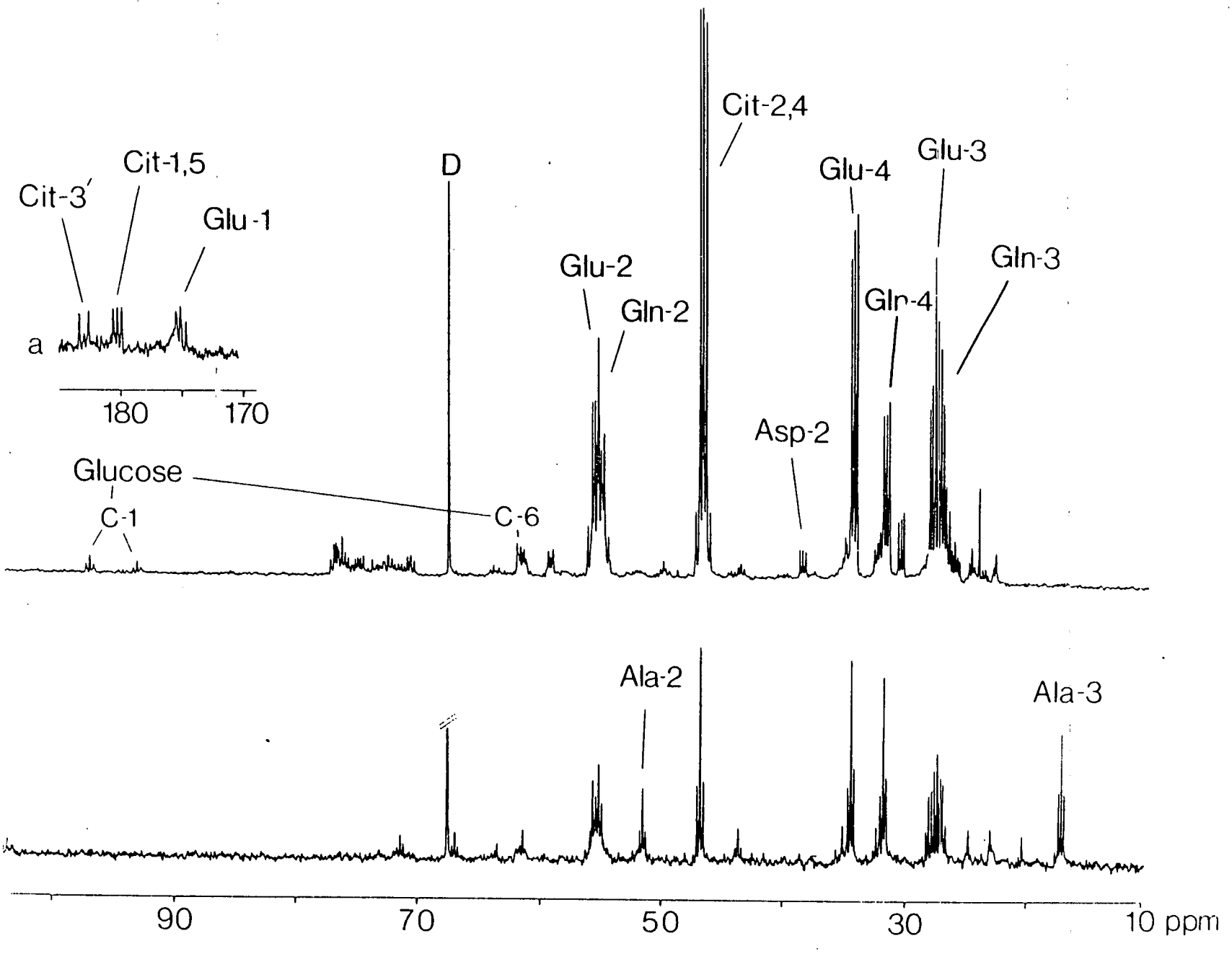

cit-2,4
glucose
cit-3
asp-2
12
6
D
6
gln-4
glu-3
glu/gln-2
ala-2
glu-4
gln-3
ala-3
2hrs
100
80
60
40
20ppm
acu3

Figure 3.11

Figure 3.12; Spectra of the 2 and 12 hour extracts shown in Figure 3.11 but in the presence of 5mM EDTA. Inset (a) shows the carbonyl region of the 12 hour spectrum with a three time expanded vertical scale.


Cit-3′
Cit-1,5
Glu-1
a
180
170
D
Cit-2,4
Glu-4
Glu-3
Glu-2
Gln-2
Gln-4
Gln-3
Asp-2
Glucose
C-1
C-6
Ala-2
Ala-3
90
70
50
30
10 ppm

The spectrum of the 12 hour extract shows significant enrichment of the carboxyl carbons of glutamate, glutamine, and citrate, together with enhancement of the outer lines of the multiplet resonances corresponding to glutamate/glutamine C-2 and citrate C-2 (and C-4). This indicates more extensive equilibration of label via the TCA cycle than is the case in the wild type.

The accumulation of so much citrate demonstrates how effectively the TCA cycle flux is partitioned and diverted to the glyoxylate cycle, since the former is evidently prevented from oxidizing this pool. This may be due to isocitrate dehydrogenase inhibition by NADPH (discussed above in the wild type section), or the export of citrate from the mitochondrion to the glyoxysome. The latter may be most likely since the lack of gluconeogenesis also implies that the pentose phosphate pathway is generating less NADPH.

Another feature of the acu3 spectra is the absence of any accumulated succinate or malate as seen in the wild type (Figures 3.4 & 3.5) which peak around 6 hours. This lends credence to the idea that these pools are of glyoxysomal origin as postulated above.

Label still appears in alanine in acu3 (Figure 3.11 & 3.12) but is remetabolised much more quickly than in the wild type. Since, as noted above, there was no net gluconeogenic flux, this means that the movement of carbon into the alanine pool in the wild type (Figure 3.10) probably arose mainly from a pre-existing pool of intermediates. To be absolutely safe in this conclusion amino acid analyses would need to be done on samples of the mutant to see

whether an equivalent increase in the alanine pool size is seen in this mutant.

The metabolism of the alanine to oxaloacetate *via* pyruvate may be the source of the carbon for the citrate build-up. Alternatively storage carbohydrate (glycogen, Zalokar, 1965) could have been mobilized to provide oxaloacetate for continued TCA cycle function.

The appearance of the signal corresponding to aspartate (C-2, 37.4ppm) in the 12 hour extract probably reflects the general accumulation of TCA cycle intermediates behind citrate.

b)acu6 - the structural locus for PEP carboxykinase

PEP carboxykinase is the first step in gluconeogenesis catalysing the ATP-dependent (in *N.crassa*) decarboxylation of oxaloacetate to generate PEP. This mutant has a complete glyoxylate cycle and its primary lesion should allow the accumulation of TCA cycle intermediates and, in particular, those that are gluconeogenic precursors (i.e. oxaloacetate), but not the accumulation of trehalose.

The ^{13}C-NMR spectra of extracts from this mutant are shown in Figure 3.13. Clearly the situation was not as simple as predicted, although obvious differences from the wild type are visible.

Firstly trehalose *is* synthesized, though not in as large a quantity as in the wild type (Figure 3.5). This particular allele, therefore, may be "leaky", although it showed no detectable activity

Figure 3.13; ^{13}C-NMR spectra of perchloric acid lysates of acu6 mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4.

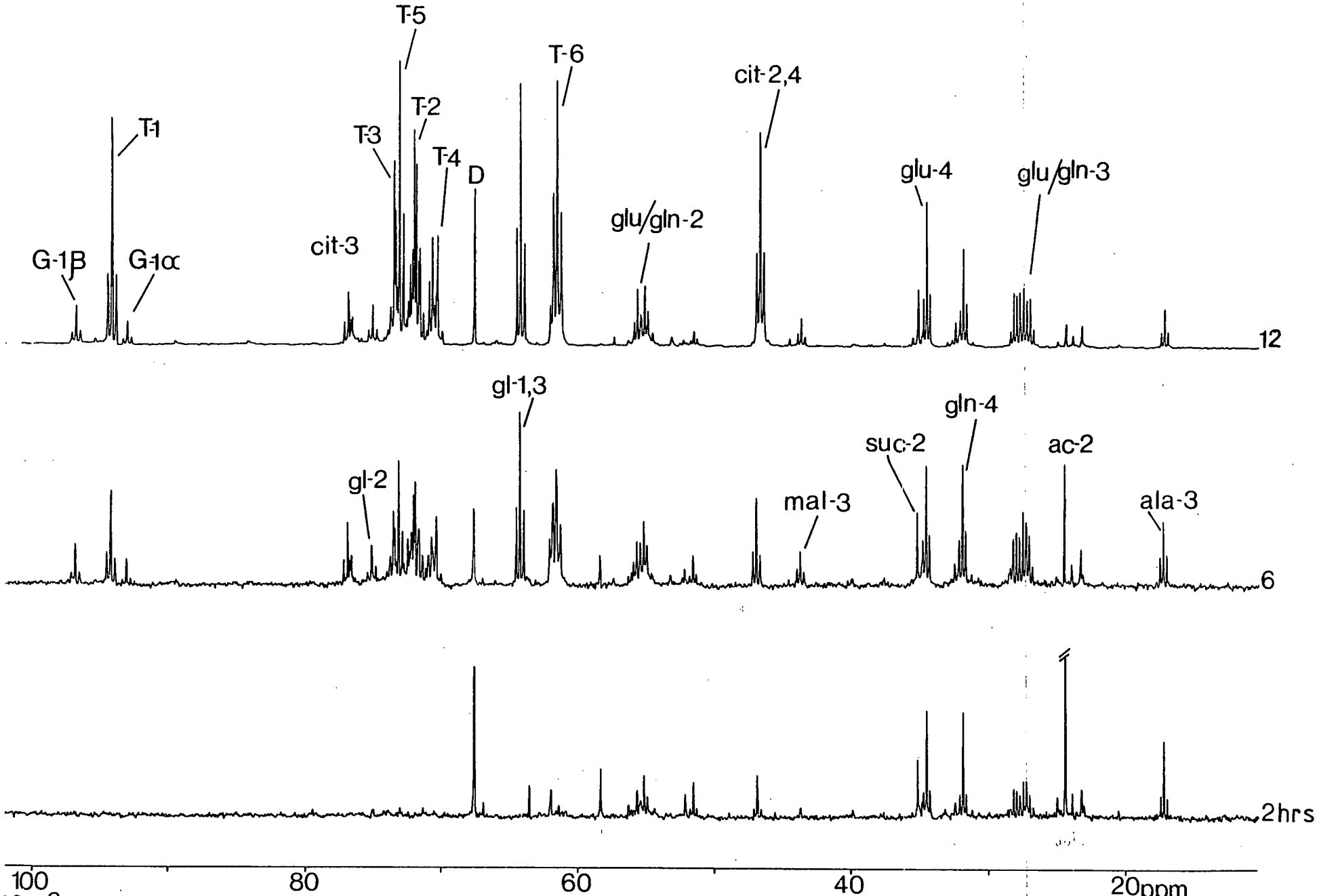

T-5
T-6
cit-2,4
T-2
T-1
T-3
T-4
D
glu-4
glu/gln-3
glu/gln-2
cit-3
G-1β
G-1α
12
gl-1,3
gln-4
suc-2
ac-2
gl-2
mal-3
ala-3
6
2hrs
100
80
60
40
20ppm

Figure 3-13

of PEP carboxykinase *in vitro* (Flavell, 1967). This assay is, however, insensitive at very low activities (Flavell, 1967) so it may have gone undetected. An accumulation of precursors may still push a measurable flux past a partially defective enzyme. That this may be what is occurring is suggested by the maintenance of larger ^{13}C-enriched alanine, glutamate, glutamine, citrate, succinate, and malate pools than in the wild type up to 12 hours.

Elevated levels of TCA cycle intermediates would tend to inhibit TCA cycling activity (e.g. oxaloacetate inhibition of succinate dehydrogenase and succinyl-CoA inhibition of citrate synthase- Lehninger, 1975), and since some of the steps are shared with the glyoxylate cycle this would be inhibited as well. Such a situation seems to be reflected in the much reduced randomization of label seen in these extracts (Figure 3.13 vs 3.5 -coupling is lowered).

Somewhat unexpected was the appearance of quite a substantial signal corresponding to glycerol in this mutant. The reason for this is still unclear.

c)*ac-73-5** - a regulatory mutant with delayed acetate adaptation

This strain is a triple mutant (Beever, 1972). As well as carrying a regulatory mutation, *ac-73-5*, which slows markedly the induction of enzymes on transfer to acetate, it carries a morphological mutation *coi11*, which causes spiral growth of the mycelium, and a mutation in the PEP carboxykinase gene (*acu6*). All three mutations are separable (Beever, 1972).

The ^{13}C-NMR spectra of extracts from this strain show a very long lag before [2-^{13}C] acetate becomes incorporated into any metabolites (Figure 3.14), whereupon the spectra look not dissimilar to that of the 2 hour acu6 (Figure 3.13) or wild type (Figure 3.5) spectra. There is a corresponding delay in the acetate consumption curve for the ac-73-5* strain (Figure 3.3) even compared to the other mutants.

acu6 mutants induce/derepress the other enzymes (isocitrate lyase, malate synthase, acetyl-CoA synthetase) in the normal way on transfer to acetate medium (Flavell & Fincham, 1968b), so unless there is some ancillary effect of the coill1 mutation the observed delay in label uptake is due to the delay in enzyme increase caused by the regulatory mutation.

This has important implications. Combined with the starvation results (Chapter 2) which eliminate inducer exclusion as a reason, it implies that some inducible function prior to acetyl-CoA is essential for the synthesis of this intermediate; there is no constitutive system. There are two possible candidates for this vital function, 1) acetyl-CoA synthetase, or 2) a permease function. Mutational defects in both were investigated by NMR.

d)acp - a lesion in an inducible acetate permease (?)

Two acetate permease functions have been described in N.crassa (Rao & DeBusk, 1977). One of these is constitutive, while the other increases on acetate. Mutants in the inducible function have been isolated (Rao & DeBusk, 1977) and are designated acp. ^{13}C-NMR spectra of extracts prepared from acp after transfer to acetate

Figure 3.14; ^{13}C-NMR spectra of perchloric acid lysates of ac-73-5* mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4. Inset shows the carbonyl region of the 12 hour spectrum at the same scale.

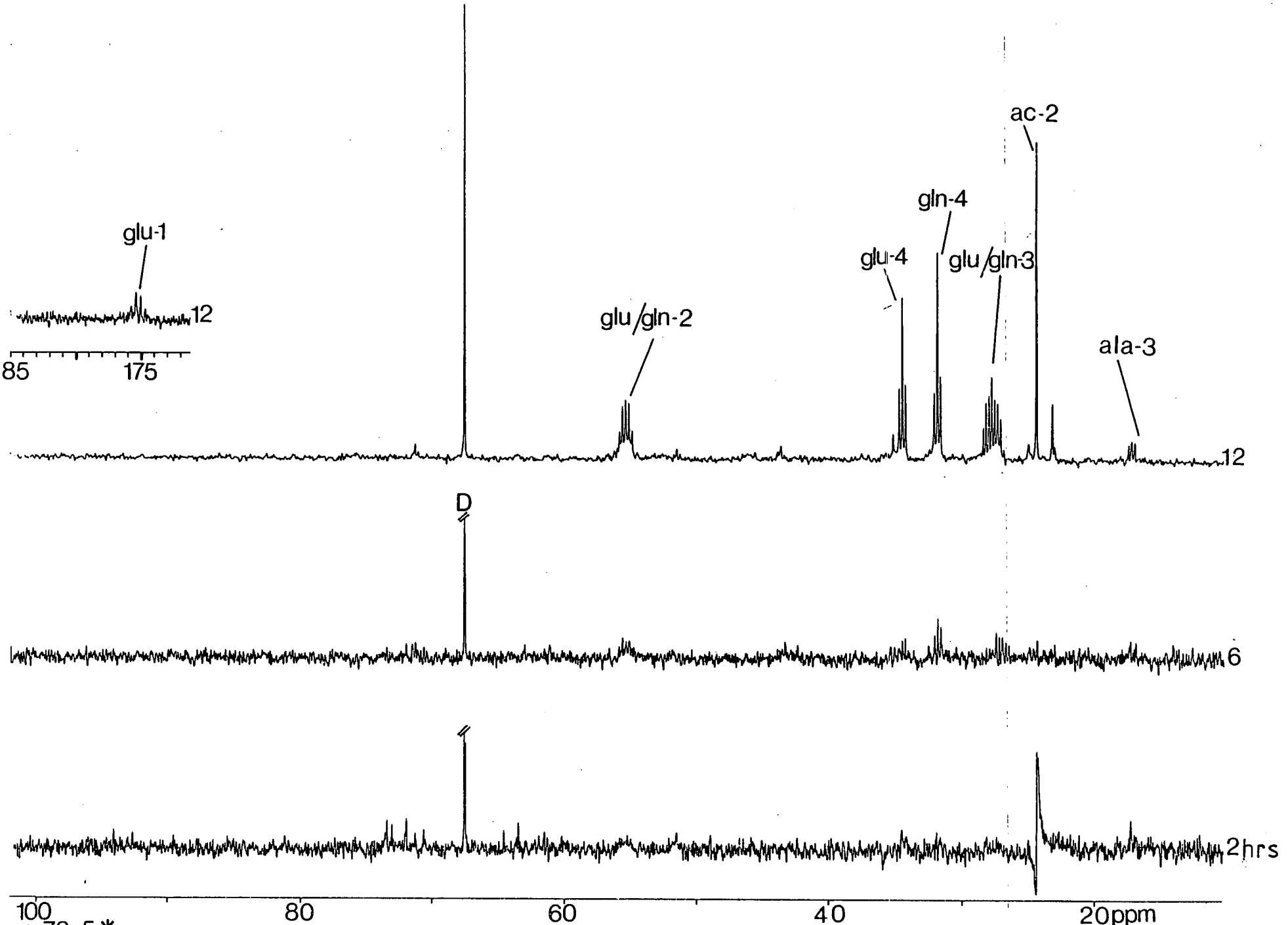

glu-1
12
185
175
glu/gln-2
glu-4
gln-4
glu/gln-3
ac-2
ala-3
12
D
6
2hrs
100
80
60
40
20ppm

Figure 3.14

medium are shown in Figure 3.15.

Two conclusions can be drawn from these spectra. first the constitutive permease is quite adequate for substantial uptake and incorporation of ^{13}C, throwing responsibility for the lack of incorporation in ac-73-5* on to lack of acetyl-CoA synthetase. Secondly the effects of the mutation are more complex than simple loss of a cellular permease function. All the enzymes of the glyoxylate cycle and gluconeogenesis were still present (Table 3.2), but, the spectra revealed no detectable glyoxylate cycle activity whatsoever. The labelling pattern was solely TCA cycle-derived as shown by the relatively large signal in the glutamate C-1 and the large outer satellites on the glutamate/glutamine C-2.

Thus acp seems to affect some fundamental function of the glyoxysome. Perhaps it is an organelle specific permease or is involved in its biogenesis. The induction of the acp function seen by Rao and DeBusk (1977) may just reflect the induction of glyoxylate cycle activity which is clearly prevented from operating in this mutant. The unexpected properties of this mutant mean that some uncertainty must remain over the conclusion that the constitutive permease is adequate, since there may still be a genuine inducible cellular permease.

There is little enrichment of alanine in this mutant, presumably because the carbon which enters this pool in the wild type is very rapidly used in the mutant to provide oxaloacetate for the extensive TCA cycle activity seen.

Figure 3.15; ^{13}C-NMR spectra of perchloric acid lysates of *acp* mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4. Inset shows the carbonyl region of the 12 hour spectrum at the same scale.

Table 3.2; Specific enzyme activities in wild type and *acp* mycelium at variuos time after transfer from sucrose to acetate medium. ICL - isocitrate lyase; MS - malate synthase; PEPCK - PEP carboxykinase; ND - ND not determined

Strain	Time	Enzyme		
		ICL	MS	PEPCK
wt	0	0.001	0.003	0.002
	6	0.084	0.097	0.921
	12	0.070	0.108	0.988
acp	0	ND	ND	ND
	6	0.202	0.179	0.889
	12	0.252	0.241	1.252

All activities are given as μmol product/min/mg protein.

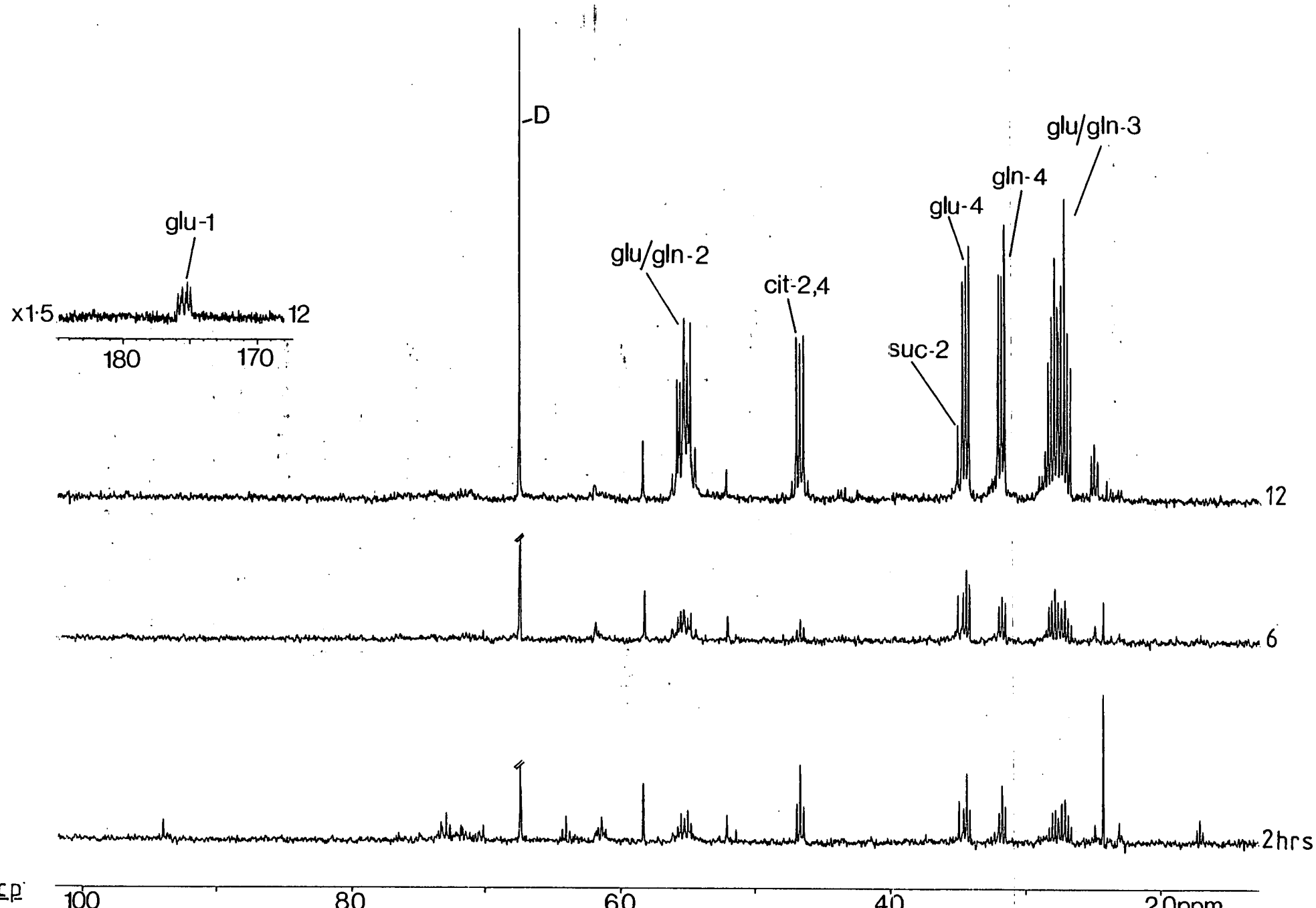

glu-1
x1·5
12
180
170
D
glu/gln-2
cit-2,4
suc-2
glu-4
gln-4
glu/gln-3
12
6
2hrs
100
80
60
40
20ppm

Figure 3·15

e) acu5 - lacking acetyl-CoA synthetase activity

This locus has not been proven to be the structural locus for acetyl-CoA synthetase, but, both mutant alleles at this locus showed an identical loss of function of this enzyme (Flavell, 1967). The function of the enzyme is to initially activate the acetate to acetyl-CoA so that it may enter cellular metabolism. A possibility raised by the previous two mutants described, acp and ac-73-5*, was that this enzyme was essential for any ^{13}C incorporation into intermediates beyond acetyl-CoA. From the ^{13}C-NMR spectra of extracts from acu5 (Figure 3.16) it can readily be seen that quite extensive incorporation of [2-^{13}C] acetate did occur. There are two possible reasons for this. Either the mutation has some residual enzyme activity in vivo, despite showing no activity in vitro, or in ac-73-5* there is some other effect of the ac-73-5 or coil1 mutations, that prevents the constitutive permease or an isozyme of acetyl-CoA synthetase from being expressed. The former possibility may be the most likely explanation since conidia from the mutant allele used (JI18) will germinate and exhibit visible growth in acetate medium after several weeks of incubation. Testing the other acu5 allele, JI32, might resolve this (see also below).

The spectra (Figure 3.16) show that trehalose is synthesized. However, there is a very marked decrease in the amount of cycling is seen in all the intermediates, as all the peaks remain more singlet in nature. An increase in the degree of equilibration via the TCA cycle is apparent from the appearance of a very prominent signal corresponding to the C-1 of alanine at 2 hours and citrate C-1 (and

Figure 3.16; ^{13}C-NMR spectra of perchloric acid lysates of acu5 mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4. Insets show the carbonyl region of both spectra, as indicated, at the same scale.

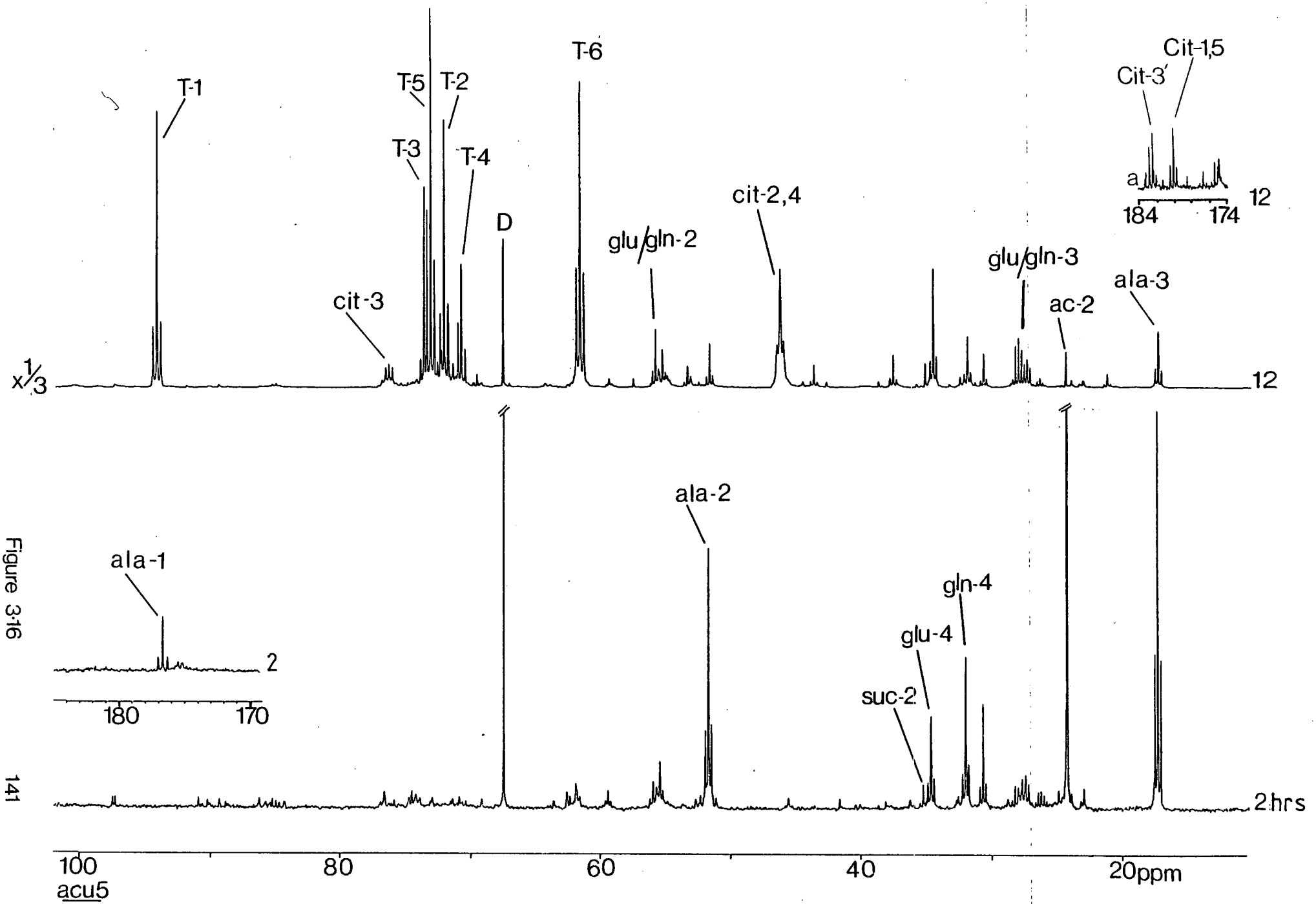

T-1
T-5
T-2
T-6
T-3
T-4
D
cit-3
glu/gln-2
cit-2,4
glu/gln-3
ac-2
ala-3
Cit-1,5
Cit-3
a
184
174
12
x 1/3
12
ala-2
ala-1
gln-4
glu-4
suc-2
2
180
170
2 hrs
100
80
60
40
20ppm
acu5

Figure 3-16

C-5) at 12 hours (insets Figure 3.16). This suggests that the lesion may be primarily associated with the glyoxysome, acting by slowing down the glyoxylate cycle flux at the malate synthase step by limitation of acetyl-CoA. Some glyoxylate cycle activity is indicated by the appearance of label in trehalose; the acu3 mutant clearly showed that lack of such a flux prevents gluconeogenesis. The existence of this flux supports the 'leaky' idea. The predominance of the TCA cycle, however, may also mean that there is a mitochondrial acetyl-CoA synthetase isozyme unaffected by this mutation. If this enzyme was also induced/derepressed on acetate, but did not function in the assay used, then this might explain the paradox.

The amount of ^{13}C-enriched alanine synthesized seemed much greater than previously seen at this time, certainly with respect to the relatively consistent signal seen for glutamate and glutamine. However, for reasons that are unlikely ever to become apparent the weights of the mycelial samples extracted for these spectra were not recorded so it is quite difficult to compare them, other than on a qualitative basis, with the others.

f) acu7 - a possible 2-oxoglutarate dehydrogenase deficiency

Not all of the induced mutations isolated as acetate non-utilizers could be assigned to lesions in the glyoxylate cycle or gluconeogenesis. Two loci (acu1 & acu7) showed no detectable activity for the TCA cycle enzyme 2-oxoglutarate dehydrogenase (Flavell & Fincham, 1968b). This enzyme unambiguously belongs to the TCA cycle since it lies in the section of it which is bypassed

by the glyoxylate cycle. The fact that its deficiency prevented utilization of acetate as sole carbon source reveals the intimate relationship between the two cycles:- the TCA cycle produces energy to drive acetate fixation and to drive gluconeogenesis, whilst the glyoxylate cycle provides gluconeogenic precursors and acetate acceptors for energy production.

acu7 will germinate and grow quite readily on sucrose so this lesion was expected to be "leaky". The ^{13}C-NMR spectra of the perchloric acid extracts (Figure 3.17) showed that this is indeed the case. There is a substantial incorporation of the ^{13}C into intermediates and continued TCA cycle activity is evident from the appearance of signals corresponding to glutamate C-1 and outer satellites on the citrate C-2 (and C-4). The lesion in 2-oxoglutarate dehydrogenase is reflected in channelling of carbon out of the TCA cycle into glutamate and glutamine:- glutamine shows a very much greater enrichment than glutamate in marked contrast to the wild type (Figure 3.17 vs 3.4 & 3.5).

Significant quantities of trehalose do not appear until later than in the wild type in this mutant, and the glutamate, glutamine, and citrate signals all have elevated levels in the 12 hour sample. Malate and succinate enrichments also remain high after 6 hours instead of disappearing.

This strain has an intact glyoxylate cycle so the most likely rationalisation of these results is that there was an energy shortage for driving acetate fixation and gluconeogenesis, due to

Figure 3.17; ^{13}C-NMR spectra of perchloric acid lysates of *acu7* mycelium sampled during adaptation to growth on [2-^{13}C] sodium acetate. The symbols are described in the legend to Figure 3.4. Inset shows the carbonyl region of the 6&12 hour spectrum at the same scale.

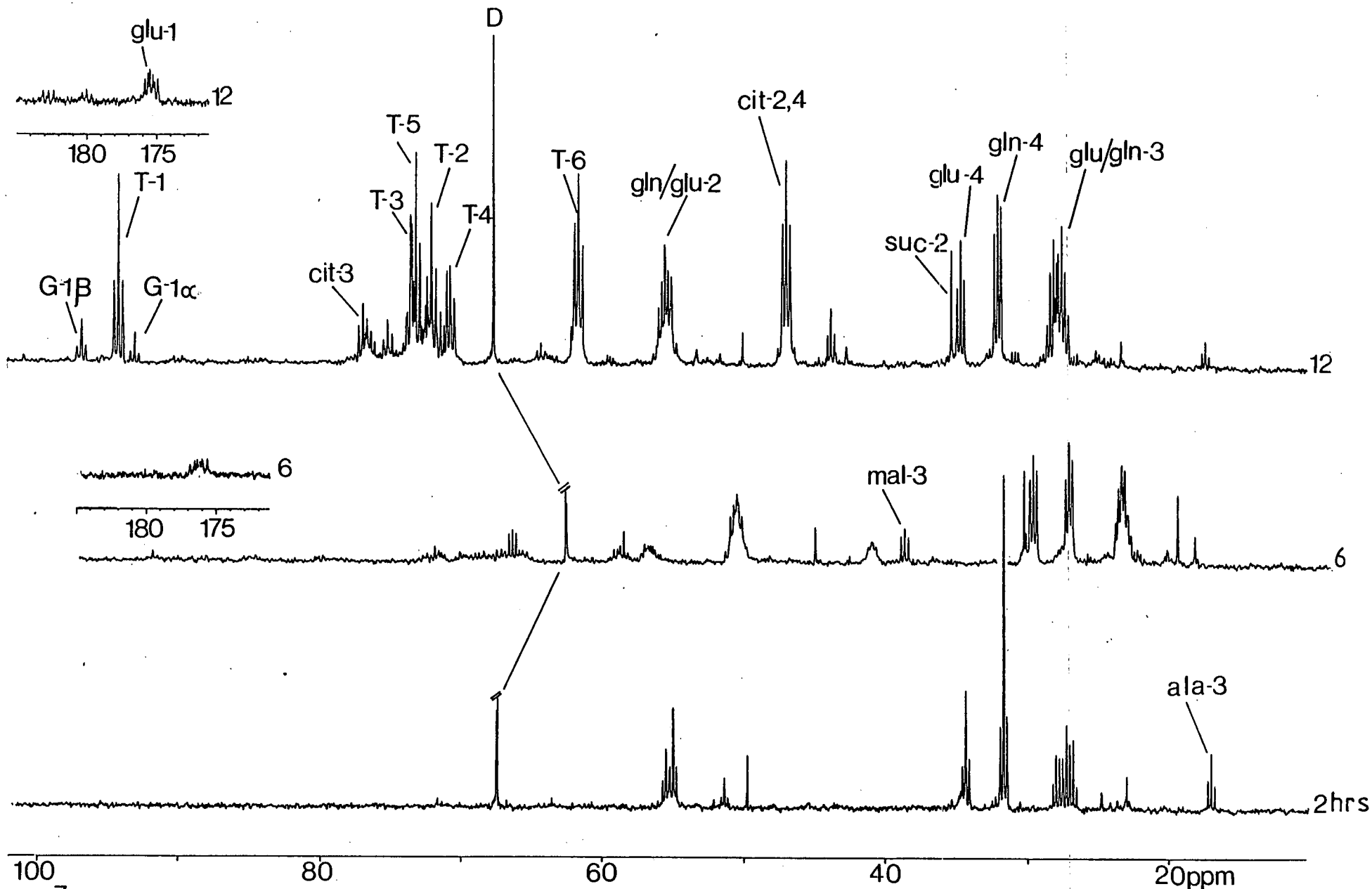

glu-1
12
180
175
T-1
G-1β
G-1α
cit-3
T-3
T-5
T-2
T-4
D
T-6
gln/glu-2
cit-2,4
suc-2
glu-4
gln-4
glu/gln-3
12
6
180
175
mal-3
6
ala-3
2hrs
100
80
60
40
20ppm

Figure 3-17

the impaired TCA cycle.

This mutation has some interesting regulatory consequences which will be discussed in the next section.

CONCLUDING DISCUSSION

a) Adaptation

The NMR studies support the following conclusions.

i) acu3 demonstrates the the expected dependence of gluconeogenesis on glyoxylate cycle activity. The large accumulation of citrate in this mutant on acetate medium is compatible with its deficiency for isocitrate lyase (Flavell & Fincham, 1968b). It also suggests that there may be some active partitioning of the glyoxylate and TCA cycle fluxes since the latter is not able to consume all the citrate.

The malate and succinate pools which reach a maximum in the wild type (in the 6 hour extract) are not seen in this mutant suggesting that they may be glyoxysomal pools.

Alanine is still labelled in acu3 at an early stage but it is metabolised fairly rapidly. This suggests that the alanine increase in the wild type does originate from endogenous pools and that its maintenance in the wild type could be explained by gluconeogenic production of oxaloacetate which does not occur in acu3 (see earlier in this chapter).

ii) ac-73-5* showed that the whole adaptation process is probably dependent on some acetate specific function to produce acetyl-CoA. Mutants of both the likely steps were examined; acp -supposedly a mutation in an inducible acetate permease (Rao & deBusk, 1977), and

acu5 an acetyl-CoA synthetase deficiency (Flavell & Fincham, 1968b).

iii) The acp mutation did not prevent the uptake of label from the medium. However, this strain showed no glyoxylate cycle activity whatever, in spite of producing the glyoxylate cycle enzymes. Thus I suggest that the actual lesion in this strain is either affecting glyoxysome biogenesis, structure or function. One specific possibility would be that it has a defective glyoxysome specific acetate permease. Since this mutation is not what it was originally thought to be (Rao & deBusk, 1977) it is still not certain that uptake is not limiting in ac-73-5*.

iv) An acu5 mutation defective in acetyl-CoA synthetase was examined, but found to be 'leaky' although the rate of acetate consumption was significantly impaired.

v) A TCA cycle mutant tentatively identified as a 2-oxoglutarate dehydrogenase deficiency (Flavell & Fincham, 1968b) was also 'leaky' but showed a significant diversion of the TCA cycle flux into glutamate which is compatible with the postulated lesion. The impairment in the TCA cycle markedly slows gluconeogenesis re-emphasising the necessity of this cycle to produce energy for the process.

The overall model for the adaptation process that I propose is as follows. Most of the details have been considered in the preceding section.

Initially the transfer of the mycelium from sucrose to acetate medium causes a carbon shortage since the enzymes for acetate utilization are not present. The response in terms of enzyme synthesis must be quite rapid since influx of label and glyoxylate cycle activity are detectable two hours after transfer (Table 3.1), and some basal acetyl-CoA synthetase activity may be present (although the ac-73-5* result makes this rather unlikely). Thus sometime during the first 60 minutes in acetate medium it is probably reasonable to assume that acetyl-CoA is synthesized from acetate. I suggest that this may be sufficient to inhibit pyruvate dehydrogenase and that the alanine pool increases enormously as a response to an accumulation of pyruvate. Most of the alanine is unlabelled, so this carbon pre-existed in the mycelium. A residual glycolytic flux may be the source of this carbon; however a degree of equilibration with oxaloacetate is apparent since label entered the alanine pool by two hours.

After two hours a second phase can be recognised in which acetate-specific activities have increased, glyoxylate cycle perturbation of the labelling patterns is clearly present, but no trehalose has yet been synthesized. There are two obvious possible explanations for the lack of gluconeogenesis. First the PEP carboxykinase activity may still be limiting, and second the energy charge may still be insufficient to drive gluconeogenesis. In the NMR experiments the PEP carboxykinase increase did lag slightly behind that of isocitrate lyase, but this was not generally the case. However, such induction curves may be misleading and whether a given specific activity measured in vitro is limiting or not will

depend on the enzyme's particular characteristics. In the acu7 mutant, where energy generation is expected to be decreased, gluconeogenesis appeared significantly impaired.

By six hours after transfer significant synthesis of trehalose has occurred and this is the main characteristic of the third phase in the adaptation process. Maximal labelling of the succinate and malate pools are also seen in this extract. The absence of such pools in the acu3 strain suggests that these may be glyoxysomal. The citrate pool also increases at this time and there are two probable scenarios to account for this. Firstly an inhibition of the TCA cycle at isocitrate dehydrogenase by NADPH as suggested for yeast (Satrustegui et al, 1983), or secondly the proposed active export of citrate from the mitochondrion to the glyoxysome combined with feedback inhibition of isocitrate lyase by PEP. Of these two possibilities the latter is more likely. acu3 which has no net gluconeogenic flux will be low in NADPH, since the pentose phosphate pathway may not be as active as in the wild type, and yet it still accumulates citrate implying that it is isolated from the TCA cycle; presumably by export.

Two further pieces of evidence are relevent to this. Firstly if mutational loss of isocitrate lyase in acu3 is likened to extreme inhibition compared to the wild type; a much greater citrate build up is seen than in the wild type (i.e. inhibition at this point will cause citrate accumulation). Secondly, during the first phase of adaptation to acetate in the wild type when the glyoxylate cycle but not gluconeogenesis is operative the citrate pool is low. Thus when

gluconeogenesis is operating and citrate does build up it is presumably due to inhibition by a gluconeogenic intermediate. PEP and fructose- 1,6-diphosphate are known to inhibit isocitrate lyase from N.crassa (Johanson et al, 1974).

b)Regulation

Previous studies in Neurospora on the regulation of acetate specific enzyme activities (Flavell, 1967; Beever, 1972; Flavell & Woodward, 1970a&b, 1971; Beever, 1975) had to rely on inference about flux movements and intermediate pool sizes. Whilst not a totally invalid approach this depends on a lot of assumptions (see section III, Chapter 1). Having carried out the NMR study a more detailed insight has been gained into the adaptation process and I have constructed the plausible model outlined above.

A continual problem even so is compartmentalization. If regulation is transcriptional and of the classical kind there is presumably a repressing or inducing protein of some kind that responds to a co-repressing or -inducing molecule. Evidence for such proteins in eukaryotes does exist (Grove & Marzluf, 1981; Phillipides & Scazzocchio, 1981) and extensive measurement of steady state pool sizes can reveal pools which relate to enzyme levels (Barthelmess et al, 1974) in the predicted manner for allosteric repression (Burns & Kascer, 1977). However, is there an expected compartment for the regulating molecule? Presumably it needs access to the nucleus unless it acts merely as a 'key' for the regulatory protein to enter the nucleus. In the studies cited above (Grove & Marzluf, 1981; Phillipides & Scazzocchio, 1981) the protein was

specifically eluted from a DNA affinity column under conditions which favour the idea of a cytosolic co-repressor with access to the nucleus. For a compartmentalized pool to regulate transcription it would be necessary to postulate some sort of cascade-like regulation. Such systems, involving several genes interacting successively, do exist in fungi but their relationship to compartmentalization is not clear- see for example phosphorus regulation in Neurospora (reviewed in Metzenberg, 1979), and the induction of the galactose utilizing enzymes in Saccharomyces (Douglas & Hawthorn, 1966; Perlman & Hopper, 1979; Matsumoto et al, 1978).

How would a regulating pool be expected to behave during adaptation? In a negatively-regulated repressing system the pool must decrease and stay somewhat below its previous level; conversely in a negatively-regulated inducing system the pool must increase and remain somewhat elevated. In positive systems the pool response for induction and repression is reversed.

An important mutant in previous studies (Flavell, 1967; Beever, 1975) was suc which has a defect in the anaplerotic enzyme, pyruvate carboxylase. These mutants accumulate large quantities of glycolytic intermediates and leak pyruvate into the medium (Strauss, 1957). They thus require a TCA cycle intermediate such as succinate as a supplement. Growth will occur without a supplement after a long lag, presumably due to other enzyme activities (e.g. the malic enzymes). The level of isocitrate lyase in this mutant when grown on sucrose medium is slightly elevated while that of PEP

carboxykinase is not (Beever, 1975). Isocitrate lyase was thus interpreted as being repressed by a TCA cycle intermediate and PEP carboxykinase by a glycolytic intermediate (or closely related metabolites thereof).

Based on some ancillary evidence and a lot of reasoning Flavell (1967) concluded that glutamate or NADH was the likely co-repressor of isocitrate lyase. Beever (1972) favoured a C4 acid as the repressor, being the more logical end product for feedback regulation. I am not sure that the arguments for either of these is particularly convincing. While I agree with the general conclusions from the suc mutant I feel that an equally strong case can be made for other TCA cycle intermediates and I fail to see why C4 acids should be singled out as the likely regulator. Since the glyoxylate cycle is 'topping up' the TCA cycle, assessment of the level of any pool in the latter should suffice for end product repression. Also, given the interrelated nature of the two cycles, I would argue that it is more logical to use an intermediate that purely represents the TCA cycle. There are two possibilities for this; 2-oxoglutarate and succinyl-CoA. If either of these two fell it would indicate that the TCA cycle is being starved of carbon. Turian (1963) has shown that the 2-oxoglutarate pool size during growth on acetate is some twelve times greater than on sucrose so this seems an unlikely regulator pool unless the system is an inducing one. It seems possible therefore that the key regulatory signal could be succinyl-CoA but, although it has some attractive features it is by no means compelling. However, this is not the end of the story since PEP carboxykinase is clearly regulated differently (see

Chapter 2; and Beever, 1972, 1975), and I shall not consider how the succinyl-CoA pool communicates with the nucleus.

On transfer from sucrose to acetate medium the mycelium may be temporarily starved of TCA intermediates. If the TCA cycle pools are reduced the low succinyl-CoA pool would allow enzyme derepression to start. As acetate consumption increases acetyl-CoA synthetase may deplete the CoA-SH pool and limit the formation of succinyl-CoA and TCA cycle activity. The accumulation of 2-oxoglutarate on acetate medium (Turian, 1963) may be indicative of this. The steady state levels of succinyl-CoA on acetate medium would have to be slightly lowered so that the enzyme activities will be maintained during growth, but the enzyme increases level-off so the size of this pool must be sufficient for some repression.

The acu7 mutant which was tentatively thought to be mutated in the 2-oxoglutarate dehydrogenase enzyme (Flavell & Fincham, 1968b) and had the characteristics of such a mutation in the NMR experiments is expected to be defective in the synthesis of succinyl-CoA from 2-oxoglutarate. The behaviour of this mutant is compatible with the model since it over-produces the glyoxylate cycle enzymes (Beever, 1972; see also Chapter 4). Similarly the suc mutant on limiting succinate would have a limited capacity to form succinyl-CoA along with the other TCA cycle intermediates producing the slight derepression seen (Flavell, 1967; Beever, 1975).

Any readily used compound that would boost the TCA cycle intermediate levels or remove a drain from the cycle would be

expected to repress the enzymes at least partially. Glutamate has this effect (Flavell, 1967; Beever, 1975).

Citrate can probably be eliminated as a regulatory molecule since its presence in large quantities in acu3 (see earlier) has no effect on enzyme production (Flavell & Fincham, 1968b).

The first step which must be necessary to understand the regulation of the acetate specific enzyme activities, is an accurate description of the adaptation process. The above experiments have produced a testable model for adaptation to acetate in Neurospora. It is to be hoped that further experiments will refine (or refute) this model and eventually produce enough details about adaptation to allow reasonable hypotheses to be constructed and tested on its regulation.

CHAPTER 4

Cloning Experiments

INTRODUCTION

There are now many methods for molecular cloning. The actual procedure used for any given gene depends largely upon the properties of that gene and its expression. This is analagous to the choice of selection procedure in a mutagenesis experiment. Where a gene or set of genes shows strong differential expression, which has a transcriptional basis, and a lambda genomic library is available there are two useful approaches. Firstly one may enrich for the sequences of interest in the probe, for example, by a cascade hybridization procedure (Zimmerman et al, 1980), or secondly by a differential (+/-) screening procedure (St.John & Davis, 1979).

When the transcriptional difference between the 'on' and 'off' situation is expected to be large the latter technique is simpler technically. Since the differences in enzyme activity between sucrose grown and acetate 'induced' mycelium are large (Flavell, 1967; Beever, 1972; this thesis) and the regulation is probably transcriptional (Flavell, 1967), the differential screening technique was used in the present study. First, however, further evidence for transcriptional regulation was sought.

MATERIALS AND METHODS

Strains and Media Used

a)Escherichia coli

ED8654 (NM259) - host for lambda vector Charon 4A

- Genotype: *metB1*, r_{K12}^-, m_{K12}^-, *supE*, *supF*, *trpR*
- Murray *et al* (1977)

HB101 - general host for plasmid vectors

- Genotype: r_B^-, m_B^-, *recA*, *pro*, str^r, *gal*
- Boyer & Roulland-Dussoix (1969)

Bacterial Media

LB medium - Oxoid Tryptone 10g/l
- Difco Yeast Extract 5g/l
- NaCl 10g/l

BBL medium - Baltimore Biological Laboratories Trypticase 10g/l
- NaCl 5g/l

Solid media also contained - 15g/l agar (Bottom layers)
- or 7g/l agar or agarose (Top layers)

Growth was at 37°C; Liquid media was shaken at 250rpm in an orbital incubator.

Antibiotic Supplements

Antibiotic	Final concentration	Stock solution
Na Ampicillin	50µg/ml	25mg/ml aqueous
Tetracyclin HCl	15µg/ml	12.5mg/ml 50% ethanol
Chloramphenicol	170µg/ml	34mg/ml absolute ethanol

b)Bacteriophage lambda

Strain used

Charon 4A - general cloning vector for 7-20kb EcoRI fragments

- Genotype: Aam32, Bam1, lac5, bio256, VKH54, vNIN5, ø80QSR
- Blattner et al, 1977; Williams & Blattner, 1979; deWet, 1980

Media

Phage Buffer

(Storage and dilution buffer)- KH_2PO_4 3g/l
- Na_2HPO_4 7g/l
- $MgSO_4$ 10ml of 0.1M stock/l
- $CaCl_2$ 10ml of a 10mM stock/l
- gelatin 1ml of a 1% stock/l

All media used for bacteriophage infection were brought to 10mM $MgSO_4$

Electrophoresis

a)Polyacrylamide gel electrophoresis (Laemmli, 1970)

Separating gels contained 10% acrylamide, 0.27% bis-acrylamide (Sigma), 0.375M Tris.Cl pH8.8, 0.03% w/v ammonium persulphate, 0.1% sodium dodecyl sulphate (SDS), 0.06% v/v N,N,N′,N′-tetramethyl-ethylenediamine (TEMED, BDH analar).

Stacking gels contained 5% acrylamide, 0.13% bis-acrylamide, 0.125M Tris.Cl pH6.8, 0.05% w/v ammonium persulphate, 0.1% SDS, 0.05% TEMED

Running buffer contained 25mM Tris.Cl pH8.3, 192mM glycine, 0.1% SDS and sample buffer (2x) contained- 125mM Tris.Cl pH6.8, 4% SDS, 40% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol

Protein samples were mixed with an equal volume of 2x sample buffer, heated at 95-100°C for five minutes and then chilled on ice. Reticulocyte lysates treated thus require 3 minutes centrifugation in a microfuge.

Electrophoresis in gels 13cm wide x14cm tall x1.5mm thick was at a constant 100v through the stacking gel and a constant 200v through the separating gel.

Staining with coomassie blue

1)Gels were immersed in 500ml of staining solution (45% ethanol, 10% acetic acid, both v/v in dH_2O, plus 0.2% coomassie brilliant blue ,GURR or Sigma - pre-heated to 65°C) and were left for 30 minutes.

2)Destaining was started in 500ml of staining solution minus the coomassie blue at 65°C for 20-30 minutes and was completed by overnight incubation in 10%acetic acid at room temperature with gentle agitation.

Fluorography of acrylamide gels

This method is described in Bonner & Laskey (1974) and Laskey & Mills (1975).

After staining gels were soaked for 1 hour in 500ml dimethyl sulphoxide (DMSO) with one change, for 1 hour in 100ml 25% 2,5-diphenyloxazole (PPO) in DMSO at 37°C with gentle agitation and finally for 1 hour in dH_2O with one change. Gels were dried *in vacuo* prior to autoradiography.

b)Agarose gel electrophoresis of DNA

Two buffer systems were used: Tris-acetate (10x stock= 0.4M Tris.Cl pH8.2, 0.2M sodium acetate, 20mM EDTA, 0.18M NaCl), and Tris-borate (10x stock= 0.9M boric acid, 0.9M Tris base, 25mM EDTA final pH8.3). Gels were prepared by heating the appropriate quantity of agarose (Sigma low EEO) in gel buffer in a boiling water bath until fully dissolved. The gel was allowed to cool to around 50°C and was cast into the appropriate mould. Electrophoresis was generally at $\leq$5V/cm. 0.1 volumes of sample buffer (100mM EDTA pH7.5, 30% Ficoll, 0.05% Bromophenol blue) was added to the DNA digest and the mixture was heated at 65°C for 5-10 minutes and quenched on ice prior to loading.

c)Agarose gel electrophoresis of RNA with formaldehyde

The method of Lehrach *et al* (1977) was used as described in Maniatis *et al* (1982).

1.2% Agarose gels were cast containing running buffer (40mM morpholinopropanesulphonic acid pH7.0, 10mM sodium acetate, 1mM EDTA) and 2.2M formaldehyde. RNA samples were prepared by heating them for 15 minutes at 55°C in running buffer containing 2.2M formaldehyde and 50% formamide. To this was added a tenth volume of

loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol blue). Identically treated DNA fragments were used as size markers and were localised by hybridization after transfer to nitrocellulose.

Recovery of DNA from agarose gels

DNA was recovered from pieces of agarose gels by electroelution, either from 1ml pipette tips into dialysis bags in a tube gel apparatus or using the method described in Maniatis et al (1982) in which the agarose block is placed inside a dialysis bag in a submerged electrophoresis apparatus.

Ethidium bromide was removed by extraction 3-4 times with butan-2-ol. Passage through a Sephadex G50 spun column (Maniatis et al, 1982) was found to be useful for removing salt and contaminating agarose prior to ethanol precipitation.

Transfer of DNA & RNA from agarose gels to nitrocellulose

Transfer to nitrocellulose filters (Schleicher & Schull BA85) was accomplished by the method of Southern (1975).

DNA agarose gels were pre-treated by immersion in 2x500ml of 0.5M NaOH- 1.5M NaCl for 1 hour and 2x500ml of 0.3M Tris.Cl pH7.0- 3M NaCl for 1 hour.

RNA formaldehyde agarose gels were pre-treated with a 5 minute wash in dH_2O, soaking in 500ml of 50mM NaOH- 10mM NaCl for 30 minutes, soaking in 500ml of 0.1M Tris.Cl pH7.5 for 30 minutes and

in 20xSSC (3M NaCl, 0.3M Na citrate) for 45 minutes.

When transfer was complete the filters were washed briefly in 2xSSC, blotted dry and baked at 80°C *in vacuo*.

Plaque Transfers

DNA from plaques was transferred to nitrocellulose by the method of Benton & Davis (1977).

Filters lifted off plates were placed (plaques topside) on to a pad of Whatman 3MM paper soaked in 0.5M NaOH for 5 minutes. They were then passed successively through one wash in 0.1M NaOH- 1.5M NaCl, two washes in 0.5M Tris.Cl pH7.5- 1.5M NaCl and finally one wash in 2xSSC. Each wash lasted 20 seconds. Filters were then blotted dry and baked at 80°C *in vacuo* between Whatman 3MM filter paper.

RNA preparation

This procedure is based on that of Holland *et al* (1977), and Lucas *et al* (1977), and has since been published (Kinnaird *et al*, 1982).

Everything was kept as sterile as possible. 2-3g of lyophilised mycelium was finely powdered and resuspended with vigorous stirring in 40ml of a 1:1 mixture of Buffer A (0.15M sodium acetate pH5.0, 4% SDS, 20mM sodium iodoacetate, 100µg/ml heparin) and a phenol (redistilled, water saturated) -chloroform -isoamyl alcohol mixture (49:49:2).

The phases were separated by centrifugation at 20,000g for 20 minutes.

The aqueous phase was re-extracted three times with fresh phenol/ chloroform/ isoamyl alcohol mix.

The first two interphases were retained and back extracted with 5ml of Buffer A. The aqueous phase from this back extraction was pooled with the main aqueous phase for the fourth phenol extraction.

The RNA in the aqueous phase was precipitated with two volumes of absolute ethanol at -20°C and the precipitate was recovered by brief (2-5 minute) centrifugation at 20,000g.

The pellet was resuspended in 1mM EDTA and diluted to an absorbance (260nm) of <50. The solution was then brought to 0.1M Tris.Cl pH9- 0.5% SDS- 1mM EDTA and was again extracted with the phenol/ chloroform/ isoamyl alcohol mixture until no interphase was seen (usually twice).

The aqueous phase was then brought to 0.2M sodium acetate, aliquoted and precipitated with 2 volumes of absolute ethanol at -20°C.

Isolation of poly(A)$^+$-mRNA

Poly(A) containing RNA was isolated on an oligo(dT) cellulose column (Bethesda Research Laboratories). The column and tubing was sterilised prior to the addition of the cellulose using 25ml of a

0.1% solution of diethyl pyrocarbonate, and was rinsed with 100ml of sterilised dH_2O.

The cellulose (4ml) was added to the column and washed with 25ml of 0.1M NaOH, 50ml of sterile dH_2O and 25ml of binding buffer (0.5M NaCl, 10mM Tris.Cl pH7.5, 1mM EDTA, 0.5% SDS).

Total RNA, prepared as above, was resuspended in binding buffer and was heated to 65°C for 10-15 minutes to dissociate any remaining polysomes and secondary structure. After cooling on ice the RNA was passed through the column four times.

The cellulose was washed extensively with binding buffer to remove all unbound RNA and residual protein. Poly(A)$^+$ RNA was eluted with 10mM Tris.Cl pH7.0- 1mM EDTA and collected in 40 drop fractions. Those fractions including the A_{260} peak were pooled and brought to 0.2M sodium acetate and precipitated with 2.5 volumes of absolute ethanol at -20°C.

Oligo(dT) fractionation was repeated on the eluate if the RNA was to be end-labelled.

For in vitro translation the RNA was fractionated once on the column, reprecipitated a further two times from 0.2M sodium acetate with 2.5 volumes of absolute ethanol and was stored at -70°C in aqueous solution.

In vitro translation

The *in vitro* translation of poly(A)$^+$-mRNA was performed in a rabbit reticulocyte lysate (Pelham & Jackson, 1976). A commercial amino acid supplemented lysate (N90) was obtained from Amersham U.K. and 8µl of this was incubated for 1 hour at 30°C with 2µl (10uCi) of ^{35}S-methionine (Amersham) and 1ul of RNA solution (see text for concentration).

Incorporation of label was estimated by adding 5µl of lysate to 15µl of 100vol hydrogen peroxide, followed by 0.25ml of 1M sodium hydroxide. This was incubated on ice for 15-30 minutes and protein was precipitated by the addition of 0.5ml of 25% trichloroacetic acid and was recovered by filtration through Whatman GFC filters. The filters were washed through with 6x 5ml of 10% TCA and dried *in vacuo*. Scintillation counting was done with the filters in in toluene containing 0.3% PPO and 0.03% 1,4-di-(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP).

DNA preparations

a)Plasmids

i)Bulk preparations were made as described in Maniatis *et al* (1982). Bacteria were grown in 500ml of LB medium in a 2l conical flask at 37°C shaken at 250rpm in an orbital incubator. The appropriate antibiotic supplement was added to prevent plasmid loss. At a turbidity (measured as an optical density (OD) at 600nm) of 0.4, chloramphenicol was added to 170µg/ml to amplify the plasmid. Incubation was continued for a further 12-16 hours.

The bacteria were harvested by centrifugation at 4000g for 10 minutes at 4°C. The cells were then washed by resuspending them in 45mls of ice cold STE (10mM Tris.Cl pH7.8, 0.1M NaCl, 1mM EDTA) and recentrifuging as above.

The bacteria were lysed in alkali by a method (Maniatis et al, 1982) based on that of Birnboim & Doly (1979). This procedure is most easily carried out in the 250ml bottles used to harvest the bacteria initially.

The washed pellet was resuspended in 10ml of solution 1 (50mM glucose, 25mM Tris.Cl pH8.0, 10mM EDTA, 5mg/ml lysozyme) and incubated at room temperature for 5 minutes.

20ml of solution 2 (0.2M NaOH, 1% SDS -freshly made) and 15ml of 5M potassium acetate were successively added with thorough mixing, the mixture being left on ice for 10 minutes after each addition. After centrifuging at 9,000rpm for 1 hour in a Sorvall GSA rotor at 4°C, DNA was precipitated from the supernatant by the addition of 0.6 volumes of iso-propanol and pelleted by centrifugation (20,000g for 30 minutes at 20°C) after standing at room temperature for 15 minutes.

The pellet was washed with 70% ethanol at room temperature and the ethanol was removed in vacuo. The DNA was redissolved in 8ml of 10mM Tris.Cl pH8- 1mM EDTA.

To the 8ml of DNA solution was added 7.6g of CsCl and 0.8ml of a

10mg/ml ethidium bromide solution. The remaining space in the tube was filled with light paraffin oil. After centrifugation at 45,000rpm for 36-40 hours at 20°C in a Beckman type 65 rotor the plasmid band (visible in ordinary light) was collected through the side of the tube with a 21 gauge hypodermic needle and syringe. Ethidium bromide was removed by extraction 4 times with an equal volume of water saturated butan-2-ol and a further 2 times with an equal volume of unsaturated butan-2-ol. CsCl was then removed by dialysis against 10-15l of 10mM Tris.Cl pH8- 1mM EDTA (2changes).

ii)Rapid plasmid preparation for screening transformants was carried out by the method of Birnboim & Doly (1979). A single transformed colony was inoculated into 2ml of LB medium containing the appropriate antibiotic and was incubated overnight at 37°C with agitation. Bacteria from 1.5ml of this culture were harvested by centrifugation at 11,600g for 2 minutes in a microcentrifuge (microfuge). The supernatant was drained off and the pellet was resuspended in the medium retained (by surface tension) by vortexing. 100µl of solution 1 (50mM glucose, 25mM Tris.Cl pH8, 10mM EDTA, 2mg/ml lysozyme) was added and mixed briefly by vortexing. After incubation on ice for 30 minutes, 200µl of solution 2 (0.2M NaOH, 1% SDS) was added and mixed in gently and left on ice for 5 minutes. 150µl of 5M potassium acetate was added, mixed in gently and left on ice for 1 hour. The precipitate was removed by centrifugation for 5 minutes in a microfuge. The supernatant was transferred to a fresh tube, and the nucleic acid precipitated with 1ml of absolute ethanol at -70°C for 30 minutes.

After recovery by centrifugation for 5 minutes in a microfuge, the pellet was dried *in vacuo* and redissolved in 100µl of 0.1M sodium acetate- 50mM Tris.Cl pH8. The nucleic acid was again precipitated with 2 volumes of of ethanol as above.

For restriction analysis this precipitation was repeated a further two times and the DNA was finally resuspended in 40µl of dH_2O. 1ug of RNase (1µl of a 1:10 dilution of a stock solution of 10mg/ml, boiled for 10 minutes to destroy DNase) was added to restriction digests of these preparations.

b)Preparation of bacteriophage lambda DNA

This procedure is based on that of Yamamoto *et al* (1970).

An overnight culture of ED8654 in LB medium was used to inoculate 100ml of fresh LB in a 1l conical flask to an OD_{650} of 0.1. This was incubated at 37°C with vigorous shaking until the OD_{650} was between 0.45 and 0.6. 1ml of 1M $MgSO_4$ and $2.4x10^{10}$ phage were then added (multiplicity of infection approximately one), and incubation was continued at 37°C. The OD_{650} typically increased to around 1.8 and then dropped rapidly to around 0.4, 2-4 hours after infection.

At this point the remaining bacteria were killed by the addition of 200µl of chloroform and a further 10 minutes incubation. Debris was removed by centrifugation at 7000rpm for 20 minutes in a Sorvall GSA rotor.

RNase and DNaseI (Sigma) were added to the supernatant each to a

final concentration of 10µg/ml and the mixture was incubated at 37°C for 30 minutes.

The solution was then brought to 0.7M NaCl and, after this had dissolved, to 10% polyethylene glycol (average molecular weight 6,000). This was allowed to stand overnight at 4°C. The precipitate was then recovered by centrifugation at 9,000rpm for 20 minutes at 4°C in a Sorvall GSA rotor.

The precipitate was resuspended in 7ml of phage buffer with gentle agitation. This was extracted with an equal volume of chloroform and layered on to a tripartite CsCl step gradient (2ml 1.3g/ml, 2ml 1.5g/ml, 1.5ml 1.7g/ml -all made up in phage buffer and clarified by filtration). This was spun at 38,000rpm for 2 hours at 20°C in a Beckman SW40 rotor. The phage band was collected using a 21 gauge hypodermic needle and a syringe.

The phage from the step gradient were brought to 8ml with 41.5% w/w CsCl in phage buffer and were spun at 35,000rpm at 4°C for 18-24 hours in a Beckman type 65 rotor. The phage band was collected as above.

CsCl was removed by dialysis against two changes (4l each) of 10mM Tris.Cl pH8- 1mM EDTA for 3 hours.

Coat protein was removed by three gentle phenol extractions. Phenol was redistilled ,water saturated, stored frozen and equilibrated with an equal volume of 0.5M Tris.Cl pH8 prior to use.

Residual phenol was removed from the aqueous phase by two extractions with chloroform. The DNA was then dialysed extensively against 10mM Tris.Cl pH7.5- 1mM EDTA.

Radioactive labelling of Nucleic Acids

a)End-labelling RNA with Polynucleotide kinase

RNA at a concentration of 1µg/4µl was subjected to mild alkaline hydrolysis in 50mM $NaCO_3$ in degassed dH_2O at 55°C for 1 hour. This reaction was stopped by the addition of 0.1 volumes of 2M sodium acetate pH5 followed by precipitation with 2.5 volumes of ethanol. This produces 5′ hydroxyl groups which are available as a substrate for polynucleotide kinase (Glynn & Chappell, 1964).

In the labelling reaction, 3µg of hydrolysed RNA was resuspended in 17µl of kinase buffer 1 (10mM Tris.Cl pH7.5, 1mM Spermidine, 1mM EDTA), heated at 60°C for 3 minutes and chilled on ice. To this was added 2.5µl of 10xkinase buffer 2 (500mM Tris.Cl pH9, 100mM $MgCl_2$, 50mM dithiothreitol, DTT), 1µl of 0.1mM ATP, 2.5µl dH_2O, 1µl (10µCi) γ-^{32}P-dATP (Amersham) and 1ul (7 units) of polynucleotide kinase (Boehringer). After incubation at 37°C for 45 minutes to 1 hour the reaction was stopped by the addition of 1µl of 0.4M EDTA pH7.5.

Unincorporated nucleotide was removed by fractionation on a sterile Sephadex G50 (Pharmacia) column equilibrated with 0.1xSSC. This was done in a disposable 10ml pipette.

b)Nick Translation of DNA

Two procedures have been used, both based on the original method of Rigby et al (1977).

Procedure 1:Nicking prior to labelling.

DNaseI was stored as a 1mg/ml solution in 10mM HCl in 50µl aliquots at -20°C. The enzyme was activated by adding 450µl of 10mM Tris.Cl pH7.5- 5mM $MgCl_2$- 1mg/ml bovine serum albumen (BSA) and incubating on ice for 2 hours.

2µg of DNA was nicked in 30µl of 66mM Tris.Cl pH7.5- 6mM $MgCl_2$- and 1µg of DNaseI (10ul of the activated solution) for 7 minutes at room temperature. The enzyme was inactivated by heating at 70°C for 10 minutes.

10µl of nicked DNA (0.67µg) was incubated for 3 hours at 14°C with 1µl each of 1mM dATP, dGTP, and dTTP, 10µCi of α-^{32}P-dCTP (Amersham) and 1µl (5 units) of DNA polymeraseI (Boehringer) in a final volume of 30µl of 66mM Tris.Cl pH7.5- 6mM $MgCl_2$.

Procedure2:Simultaneous nicking and labelling.

This procedure is more rapid than the above but gives comparable incorporations.

1µg DNA is labelled in 50µl containing the following; 5µl of 10x

NT buffer (0.5M Tris.Cl pH7.2, 0.1M $MgSO_4$, 1mM DTT, 500µg/ml BSA), 1µl each of 1mM dATP, dGTP, dTTP, 2µl of 50µM dCTP, 1µl α-^{32}P-dCTP (10µCi), 1µl DNA polymerase (5 units) an 1µl of a 10^{-4} dilution of a 1mg/ml DNaseI stock solution (stock 1mg/ml DNaseI in 0.15M NaCl- 50% glycerol; diluted using 1xNT buffer- 50% glycerol). The mixture was incubated for 1 hour at 16°C and the reaction was stopped by the addition of 5µl of 0.4M EDTA pH7.5.

In both procedures unincorporated label was removed by the spun column procedure (Maniatis et al, 1982) using a 1ml Sephadex G50 column in a 1ml syringe. The standard spin was for 5 minutes (including acceleration) at mark 4 on an MSE bench centrifuge. Separating ability under these conditions was checked using a mixture of orange G and dextran blue.

c)cDNA synthesis

0.5µg of poly(A) RNA was annealed at 40°C for 15 minutes with 50ng of oligo$(dT)_{12-18}$ in 20µl of 50mM Tris.Cl pH8.3- 6mM $MgCl_2$- 50mM KCl- 40µM DTT. The mixture was brought to 1mM dTTP, dGTP and dATP- 100µM dCTP- 100µg/ml actinomycin D (to prevent second strand synthesis), 10µCi α-^{32}P-dCTP was added. The reaction was started by the addition of 5 units of reverse transcriptase. After incubation at 37°C for 1 hour the RNA template was destroyed by bringing the mixture to 1mg/ml denatured salmon sperm DNA- 0.1% SDS- 0.2M NaOH and heating at 65°C for 10 minutes.

Unincorporated label was removed using a Sephadex G50 column as described for the end-labelled RNA.

All DNA probes were heated in a boiling water bath for 5 minutes prior to their addition to hybridization mixtures.

Estimation of nucleotide incorporation

Two lul aliquots of the mixture to be estimated were removed. One was spotted directly on to a Whatman GFC filter, the other was added to 100µl of 500µg/ml denatured salmon sperm DNA- 20mM EDTA in a small glass test tube. 5ml of 10% trichloroacetic acid was added to this and the mixture was left on ice for 15 minutes. This was then filtered through a Whatman GFC filter which was washed six times with 5ml of TCA and once with 5ml of absolute ethanol.

Both filters were dried in vacuo. Scintillation counting was as described before.

Hybridization conditions

a)DNA or RNA to filter bound DNA or plaques

The method used is based on that of Maniatis et al (1978). Filters were wetted in dH_2O at room temperature for at least 20 minutes and were rinsed in 4xSET (1xSET is 0.15M NaCl, 30mM Tris.Cl pH8, 2mM EDTA). This was followed by a three hour wash at 65°C in 4xSET 10x Denhardt's solution 0.1% SDS with constant agitation. 10x Denhardt's solution is 0.2% BSA, 0.2% polyvinylpyrolidone 360, 0.2% Ficoll 400 (Denhardt 1966).

Prehybridization was carried out in a heat sealed polythene bag for at least 1 hour at 65°C in 4xSET- 10xDenhardt's solution- 50µg/ml denatured salmon sperm DNA- 10µg/ml poly(A)- 0.1% SDS- 0.1%

sodium pyrophosphate.

Filters were hybridized for 16-18 hours at 65°C with gentle agitation using a new bag containing fresh prehybridization mixture plus the ^{32}P-labelled probe.

After hybridization, the filters were washed once with agitation in 4xSET- 10xDenhardt's solution- 0.1% SDS- 0.1% sodium pyrophosphate for 1 hour at 65°C; twice in 3xSET- 0.1% SDS- 0.1% sodium pyrophosphate at 65°C ; twice in 1xSET- 0.1% SDS- 0.1% sodium pyrophosphate at 65°C ; and once in 0.5xSET at room temperature. Each wash was for 20 minutes.

The filters were blotted with filter paper and dried *in vacuo* between two sheets of Whatman 3MM paper.

b)DNA to filter bound RNA

Filters were wetted in dH_2O for at least 20 minutes and were then rinsed in 4xSSC. Prehybridization was done in heat sealed polythene bags with 50% formamide- 4xSSC- 6xDenhardt's solution- 10µg/ml denatured salmon sperm DNA- 0.1% SDS at 42°C for 4-6 hours. The filters were then transferred to another bag and hybridized for 48 hours at 42°C in prehybridization mixture plus ^{32}P-labelled probe and twice the concentration of salmon sperm DNA (20µg/ml). The filters were washed for 5 minutes in a large excess of 2xSSC- 0.5% SDS and for 15 minutes in a similar quantity of 2xSSC- 0.1% SDS both at room temperature. This was followed by two successive washes (120 and 30 minutes respectively) at 65°C in 0.1xSSC- 0.1% SDS for 2

hours with gentle agitation. -

The filter was blotted and dried *in vacuo* between two layers of Whatman 3MM paper.

Autoradiography

All autoradiography used Kodak Ortho-G X-ray film and was carried out at -70°C. Intensification of the ^{32}P-signal was obtained using Kodak Lanex Regular screens.

Restriction Endonuclease Cleavage

Restriction digests were performed using the buffers suggested by Maniatis *et al* (1982). They are:-

1) Low salt buffer-10mM Tris.Cl pH 7.5
   -10mM $MgCl_2$
   -1mM DTT

2) Medium salt buffer-50mM NaCl
   -10mM Tris.Cl pH 7.5
   -10mM $MgCl_2$
   -1mM DTT

3)High salt buffer-100mM NaCl
   -50mM Tris.Cl pH 7.5
   -10mM $MgCl_2$
   -1mM DTT

Reactions were carried out at 37°C for the appropriate time, generally in 20µl.

Digestion of some DNA preparations was slow and was assisted by the addition of spermidine, typically to 4mM, although the optimum varied from preparation to preparation.

Subcloning DNA fragments into pBR322

20ng of plasmid, cut with the desired enzyme(s), was co-precipitated with a three-fold molar excess of the fragment to be cloned from 0.2M NaCl with 2 volumes of absolute ethanol at -70°C for 1 hour. The DNA was recovered by centrifugation for 5 minutes in a microfuge and was washed with 70% ethanol.

The pellet was redissolved in 6µl of 10mM Tris.Cl pH 7.5- 1mM EDTA and the solution was brought to 10mM DTT, 1mM ATP and 1xligation buffer (10xstock is 500mM Tris.Cl pH7.5, 100mM $MgCl_2$, 2mM spermidine) in 9µl. 1µl (2 units) of T4 DNA ligase (Boehringer) was then added and the mixture was incubated at 12-16°C for at least 8 hours. The DNA was used to transform E.coli.

Transformation of E.coli strain HB101

This procedure was based on that of Mandel and Higa (1970) as described in Maniatis et al (1982).

40ml of LB medium in a 250ml conical flask was inoculated with HB101 and grown with vigorous shaking at 37°C to an OD_{550} of 0.5. The culture was chilled on ice for 10 minutes and the sedimented by centrifugation at 4,000g for 5 minutes at 4°C.

The cells were resuspended in half the original volume of ice

cold 50mM $CaCl_2$- 10mM Tris.Cl pH8 and were incubate on ice for 15 minutes. The bacteria were resedimented as before, resuspended in one fifteenth of the original volume of the same solution and was left at 4°C for 12-24 hours.

200µl of the cell suspension was then incubated with the ligated DNA on ice for 30 minutes, heated to 42°C for 2 minutes and then placed at 37°C. 1ml of pre-warmed LB medium was added to the tube and incubation continued for 1 hour to allow expression of the antibiotic resistances.

100-200µl aliquots were spread on plates of selective medium and incubated at 37°C. Transformation frequencies achieved were generally around 10^5 transformants/µg DNA. Insertional inactivation was checked by replica plating.

Hybridization-selection of RNA for translation

a)Selection on filters

DNA was bound to small filters (e.g. 3-4mm square) as described by Maniatis et al (1982). 10µg of clone DNA was heated to 100°C in 10mM Tris.Cl pH7.5- 1mM EDTA for 10 minutes and cooled rapidly on ice. An equal volume of 1M NaOH was added and the mixture was left at room temperature for 20 minutes. Then 0.5 volumes of 1M NaCl- 0.3M sodium citrate- 0.5M Tris.Cl pH8- 1M HCl was added followed, after mixing, by an equal volume of 6xSSC bringing the final volume to 120µl.

The mixture was applied to a damp nitrocellulose filter on a

stack of filter paper using a disposable 1ml pipette. By pressing the tip of the pipette on to the surface of the filter the DNA is confined to a small area 2mm in diameter. The filter was dried with a hair drier and washed twice with 50ml of 6xSSC at room temperature. After blotting dry the filters were baked at 80°C *in vacuo*.

To remove loosely bound DNA the filters were heated in dH_2O in a boiling water bath for 5 minutes.

If the filters had been used before the were recycled by treating them with 1ml of 2xSSC- 0.1M NaOH at room temperature for 30 minutes, followed by five 1ml washes with 2xSSC and a further wash with 1ml of dH_2O.

The hybridization mixture (50% formamide, 4xSSC, 0.1% SDS and 50ug of poly(A)$^+$-mRNA) was heated at 70°C for 2 minutes and then incubated with the filter at 42°C overnight.

The hybridization mixture was removed by aspiration and the filters were washed with 1ml of 2xSSC at room temperature. This was followed by ten 1ml washes in 1xSSC- 0.1% SDS at 60°C and a final wash in 2mM EDTA at 60°C.

RNA was eluted from the filter by boiling in 150µl of distilled water for 3 minutes and then freezing in a dry-ice/ ethanol bath. After thawing the solution was removed to a fresh tube, brought to 0.2M sodium acetate and precipitated with 2.5 volumes of ethanol at

-20°C.

The RNA was recovered for translation by centrifugation in a microfuge for 40-45 minutes and was resuspended in 5ul of sterile dH_2O.

b)Selection using DNA immobilized on a cellulose column

70µg of an approximately 900 base pair fragment (see text) was coupled to epoxy-activated cellulose (Bethesda Research Laboratories) by the method of Moss et al (1981), as follows.

The DNA was denatured by heating in a water bath at 100°C for 5 minutes in 180µl of dH_2O, followed by the addition of 20µl of 1M NaOH.

50mg of the activated cellulose was washed eight times with 1ml of 0.1M NaOH. As much of the liquid as possible was removed from the cellulose and the DNA solution was added. The cellulose/ DNA mixture was vortexed and scraped on to a sterile microscope slide. (An even distribution on the slide is most easily achieved by gently pressing it on to a whirlimixer.) This was left in a dessicator with a water tray for 8 hours, and then it was removed and air dried for a further two hours.

The cellulose was scraped into a fresh tube and washed twice with 1ml of dH_2O. Estimation of the DNA present in the wash gives the binding efficiency. (Note. The binding efficiency proved quite low -around 30%- so the above procedure was repeated three times in

order to bind the DNA)

Hybridization was carried out in a small column as shown in Figure 4.1. The column (300ul in volume) was pre-washed with 25ml of 0.1M NaOH and then 50ml of dH_2O (the pH was checked for neutrality). This was followed by 10ml of elution buffer (EB is 99% formamide, 10mM Tris.Cl pH7.8) at 65°C and 10ml of hybridization buffer (HB is 50% formamide, 4xSSC, 0.1% SDS) at 42°C.

The hybridization mixture was 100ul of HB containing 200ug of poly(A)$^+$ RNA. This was heated at 70°C for 2 minutes and then run into the column and left overnight at 42°C.

The column was then washed with 10ml HB and another 10ml of HB minus the SDS (washing should probably have been more extensive- see text).

Bound RNA was eluted with EB at 65°C. 200ul of EB (pre-warmed to 65°C) was run into the column and the water bath allowed to come to 65°C. The column was given 15 minutes to equilibrate. EB was then pumped through the column until 1ml (5x200ul fractions) had been collected.

The column was again allowed 10 minutes to re-equilibrate and another 1ml was collected. Fractions were diluted to 50% formamide and brought to 0.2M sodium acetate. RNA was precipitated by the addition of 2.5 volumes of absolute ethanol and incubation in a dry-ice/ ethanol bath for 20 minutes and recovered by spinning in a

Figure 4.1; Cross section of the apparatus used for selection of complementary RNA by hybridization to DNA-cellulose. Arrows A and B indicate the flow of water from the bath and of the column buffer respectively.

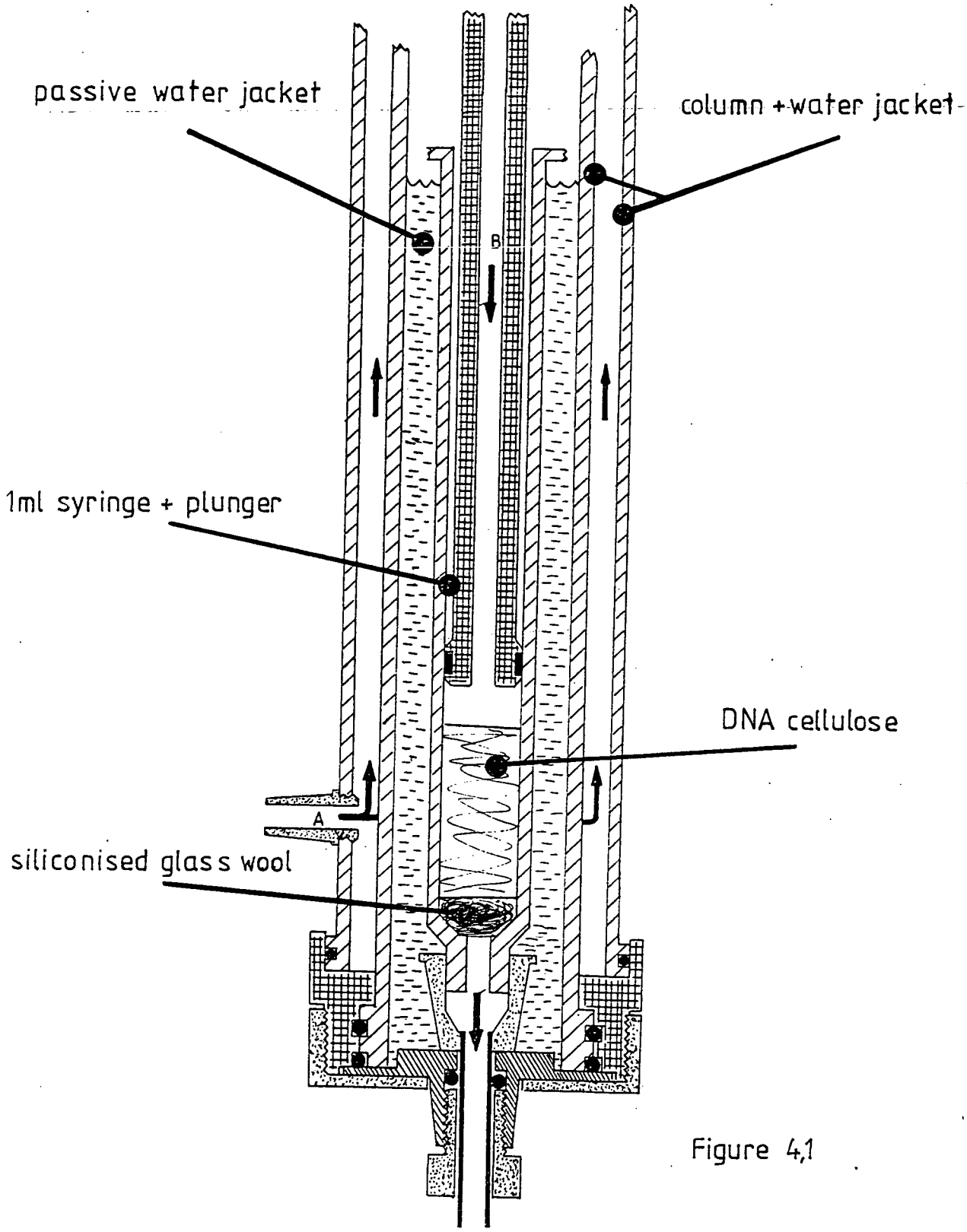

passive water jacket
column + water jacket
B
1ml syringe + plunger
DNA cellulose
A
siliconised glass wool

Figure 4,1

microfuge for 30 minutes. Fractions were resuspended, pooled and reprecipitated as described in the text.

RESULTS AND DISCUSSION

a)In vitro Translation of messenger RNA

As de novo protein synthesis has been shown to be necessary for the production of the various acetate induced/derepressed enzyme activities (Flavell, 1967; Wanner & Theimer, 1982) it was decided to see if this was reflected in the appearance of new mRNA species. mRNA was prepared as described in the methods section. The optimum RNA concentration for the translation reaction was determined by using a series of dilutions of mRNA from acetate adapted mycelium ($mRNA^a$; mRNA from sucrose-grown mycelium is abbreviated $mRNA^s$). 1ul of each dilution was translated in 4ul of lysate plus 1ul (5uCi) of ^{35}S-methionine. The trichloroacetic acid (TCA)- precipitable radioactivity was determined after an hour's incubation; the results are shown in Figure 4.2.

The optimum for 4µl of lysate lies around 0.25µg. Inhibition with greater quantities of $mRNA^a$ was presumably due to increasing quantities of contaminating material in the preparation. 8µl of lysate was routinely used for polyacrylamide gel electrophoresis so 0.5µg mRNA was used in these reactions.

Incorporation curves for $mRNA^{a\&s}$ are given in Figure 4.3. Typically around $2x10^5$ TCA precipitable counts were loaded per track in a polyacrylamide gel. This allowed visualization of bands after 2 days fluorography, although the best results were sometimes obtained after a few more days exposure.

Figure 4.2; Determination of the optimum concentration of mRNA for *in vitro* translation. See text for method.

Figure 4.3; ^{35}S-incorporation time courses using- 0.5µg of $mRNA^{s}$ (o); 0.5µg of $mRNA^{a}$ (x); no RNA added (+) in 8µl of lysate.

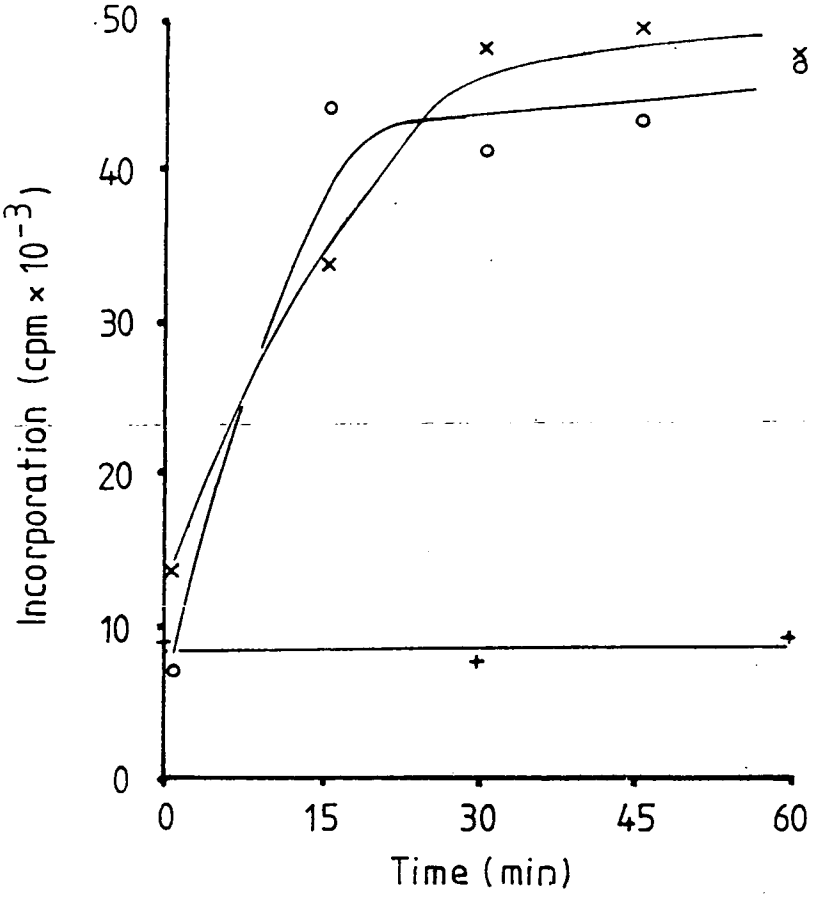
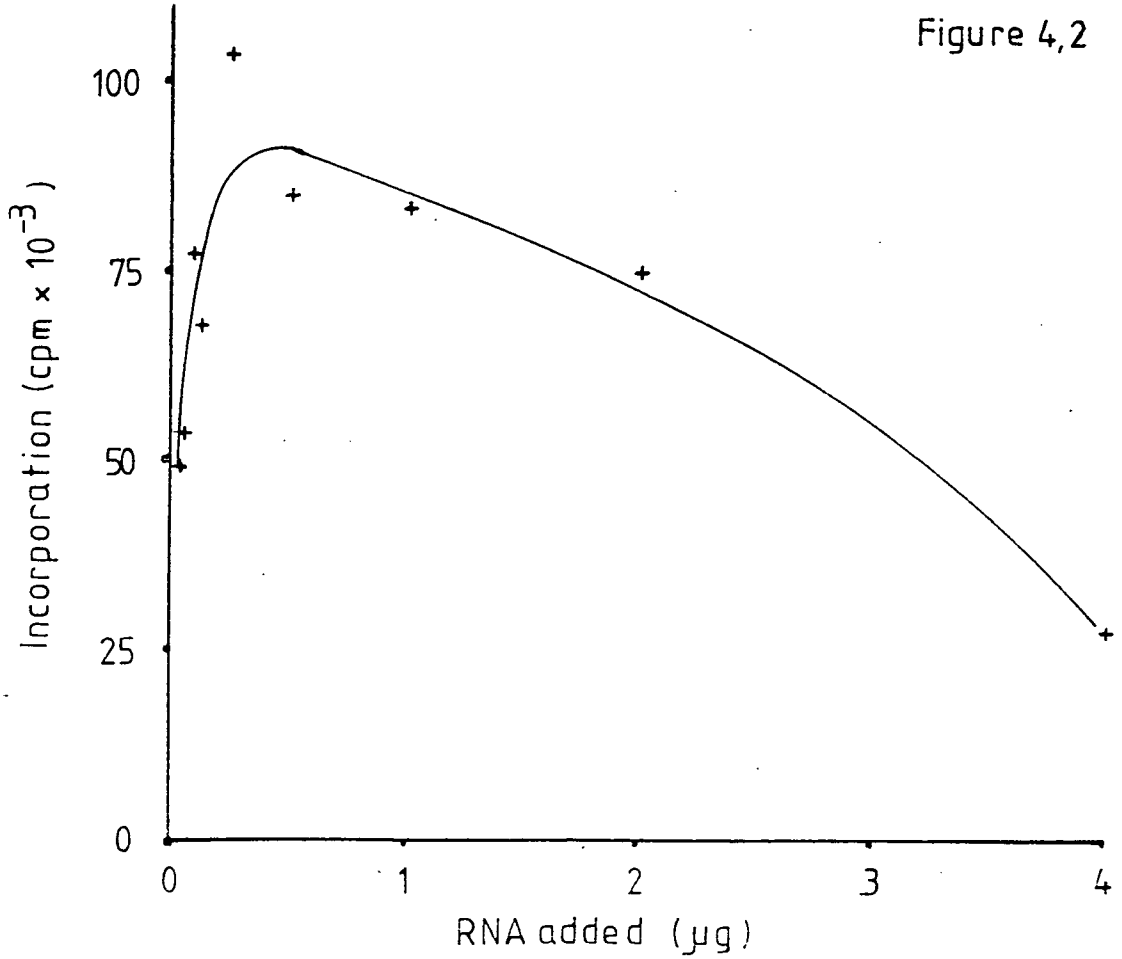


Figure 4,3

Figure 4,2



Samples of poly(A)$^+$-mRNA were made from wild type mycelium at various times during adaptation and were translated *in vitro*. Fluorographs of polyacrylamide gels run on these samples are shown in Plate 4.1. The most prominent changes seen are certain translation products that decrease in intensity with an increasing time in acetate medium.

A similar experiment was carried out on an acetate non-utilizing strain, *acu7* (allele JI36), which has been reported to lack detectable 2-oxoglutarate dehydrogenase activity *in vitro* (Flavell & Fincham, 1968b), and which over-produces the acetate-specific enzymes (Beever, 1972). In this experiment coding capacity for three very prominent polypeptide bands (apparent molecular weights 78, 67 & 60x10^3 daltons) appeared after transfer to acetate reaching a maximum after about four hours in acetate medium (Plate 4.2). Using this fluorograph for reference it is possible to pick out these same bands in the fluorograph from the wild type experiment. This is fairly strong evidence that this *acu7* strain (JI36) is over-producing at the transcriptional level.

The 67kd acetate- specific protein is the same size as purified *N.crassa* isocitrate lyase (Desel *et al*, 1982) and co-migrates with the purified enzyme (Plate 4.3, see appendix for the isocitrate lyase purification procedure).

Immunoprecipitation experiments using anti-isocitrate lyase antibodies have also been shown to react with a polypeptide of this size among *N.crassa in vitro* translation products (Desel *et al*,

Plate 4.1; Fluorograph of *in vitro* translation of polyA$^+$ selected mRNA prepared from wild type mycelium at various time after transfer from sucrose to acetate medium. Numbers under tracks refer to the number of hours in acetate medium prior to RNA preparation; Bl- no added RNA; M- protein size markers visualized by coomassie staining prior to fluorography. Sizes are given in kilodaltons. The markers are- rabbit muscle myosin (205); *E.coli* B-galactosidase (116); rabbit muscle phosphorylase-B (97.4); bovine albumen (69); egg albumen (45); bovine erythrocyte carbonic anhydrase (29); B-lactoglobulin (18.4).

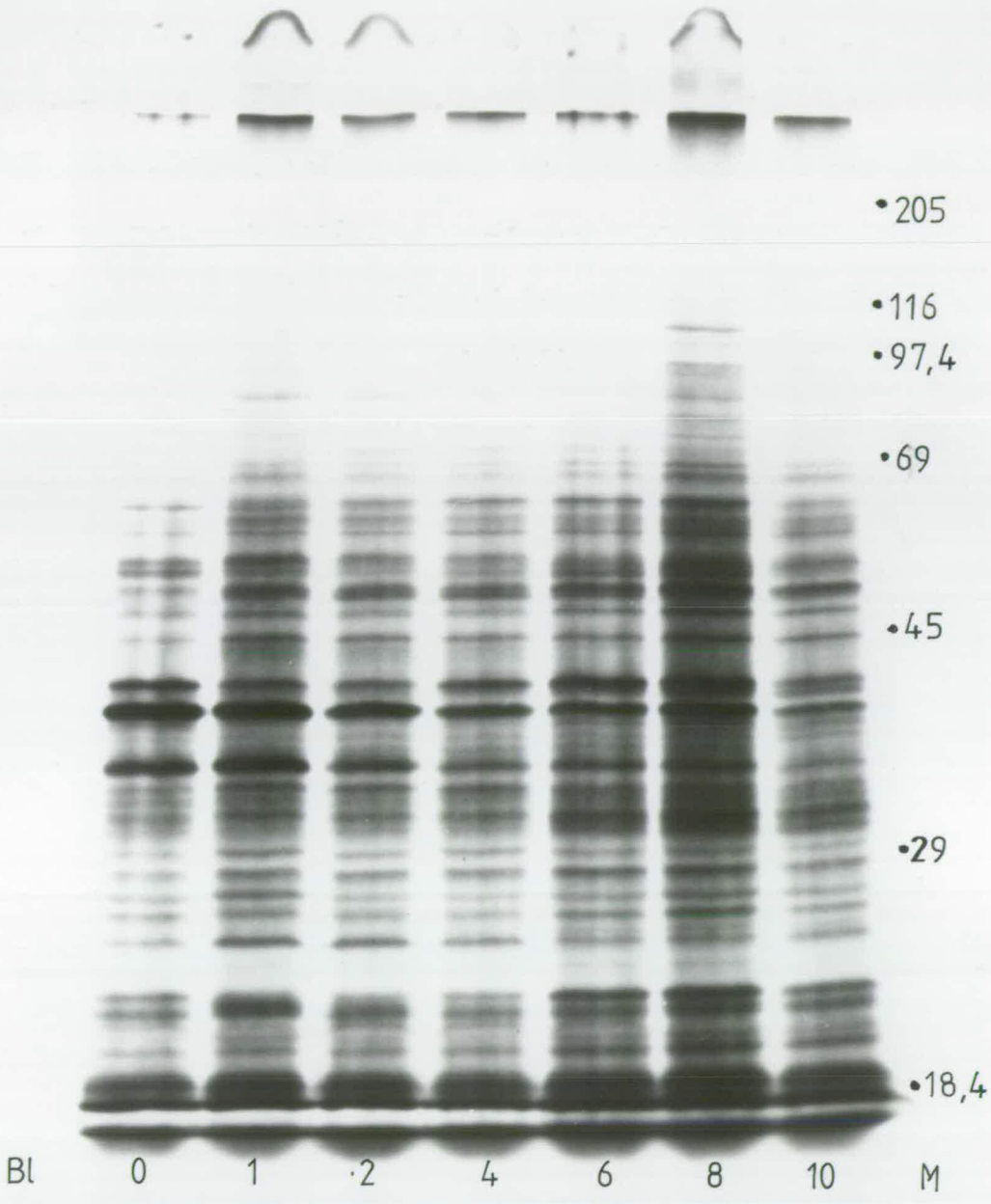

205
116
97,4
69
45
29
18,4
Bl
0
1
2
4
6
8
10
M

Plate 4.2; Fluorograph of *in vitro* translations of polyA$^+$ selected mRNA prepared from *acu7* (JI36) mycelium at various time after transfer from sucrose to acetate medium. Symbols are as described in the legend to Plate 4.1.

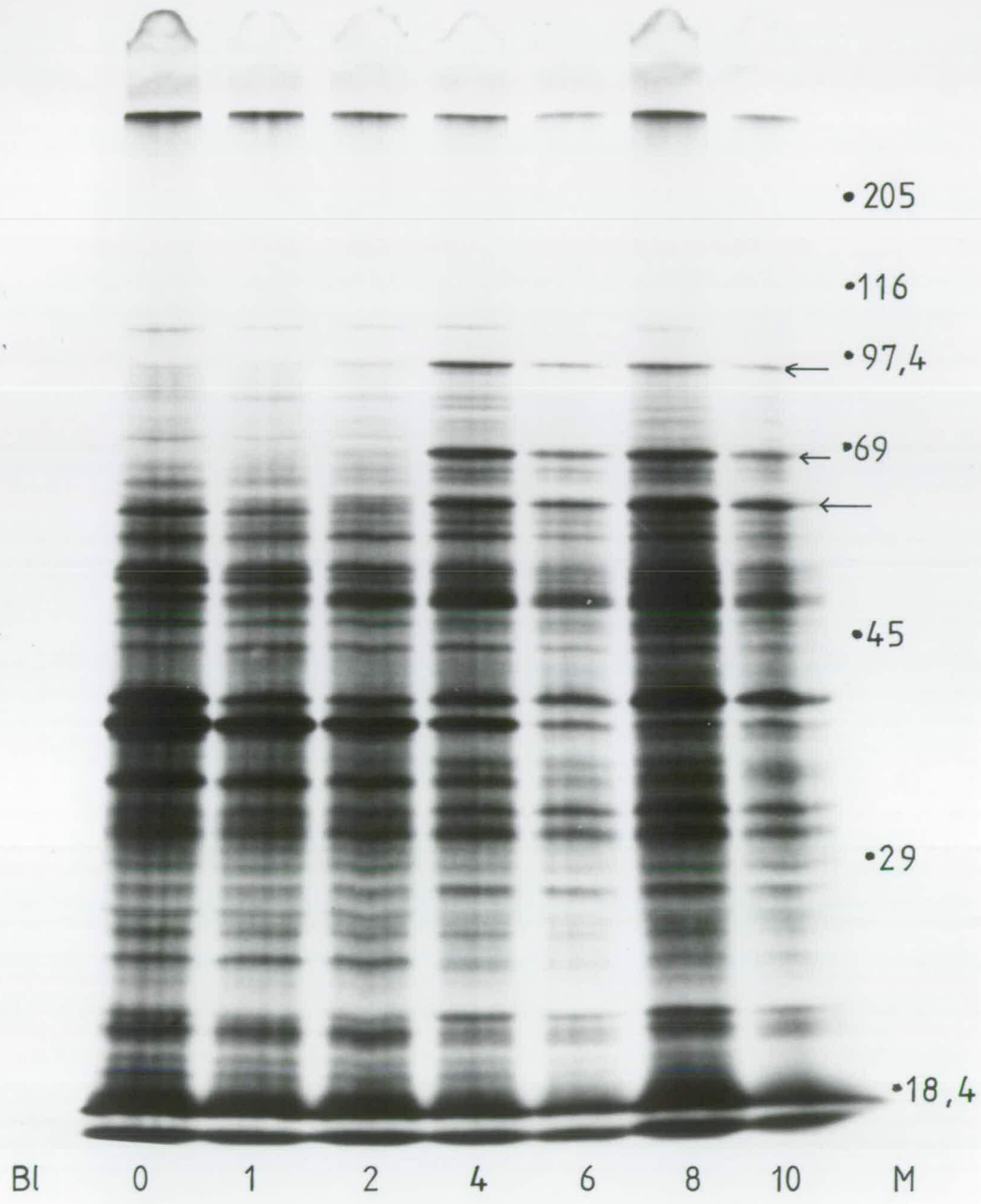
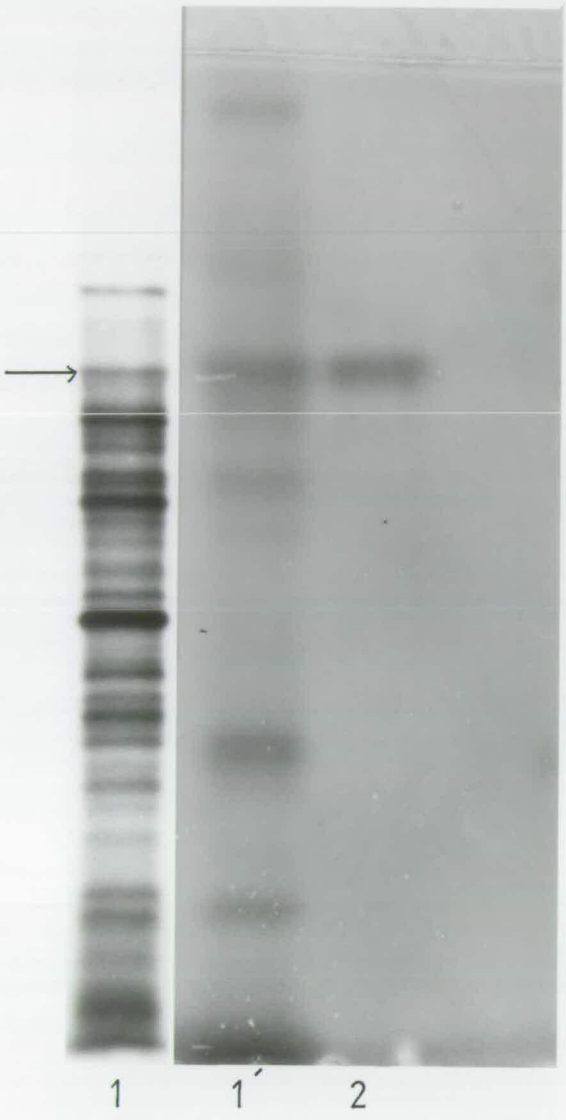

• 205
• 116
• 97,4
• 69
• 45
• 29
• 18,4
Bl
0
1
2
4
6
8
10
M

Plate 4.3; Correspondence of the molecular weight of purified isocitrate lyase and the 67kd acetate-specific band in the *in vitro* translation of *acu7* mRNA[a] (arrow). 1- autoradiographic exposure of track 1′; 1′ and 2 are the coomassie stained gel after treatment for fluorography. 1′- translation mixture+ purified isocitrate lyase; 2- purified isocitrate lyase.


1
1´
2

1982), but only when acetate induced mRNA was used. The 67kd acetate specific band is thus probably isocitrate lyase.

The enzyme PEP carboxykinase from N.crassa has a molecular weight apparently identical to the smallest prominent acetate specific band (60kd) and co-migrates with it (Plate 4.18 lanes 5 vs 7, see appendix for the purification procedure for PEP carboxykinase and later in the text). However, the N.crassa malate synthase polypeptide is very similar in size (59kd, Desel et al, 1982), so the identity of this band is less certain.

The identity of the 78kd band is unknown but two likely candidates are acetyl-CoA synthetase and fructose-1,6-diphosphatase.

The size range of the polypeptides synthesized (up to 100kd) matches that found by Lucas et al (1977) in a wheat germ translation system with N.crassa mRNA, but the patterns could not be compared since they did not publish a fluorograph.

It is clear from these results that the populations of translatable mRNA's are different between sucrose- grown and acetate- adapted mycelium, presumably reflecting differences in transcription. This difference was exploited for the differential screening of a N.crassa genomic library (see below and the introduction for a discussion of the method).

b)Synthesis of cDNA and end-labelled mRNA

The synthesis of cDNA from mRNA for differential screening is the method of choice for three reasons. Firstly, high specific radioactivities can be achieved with cDNA since several radioactive atoms are incorporated per molecule copied. Secondly one may selectively eliminate ribosomal RNA (rRNA) contamination by using an oligo(dT) primer and, finally DNA is somewhat less subject to contamination with damaging nucleases than is RNA, so there is a stability advantage. Unfortunately attempts at cDNA synthesis were largely unsuccessful, however. Incorporation time courses from four typical attempts are shown in Figure 4.4. Even the best of them barely achieved 2% incorporation. With hindsight the reason for this lack of success was probably the poor quality of the mRNA preparations; on *in vitro* translation they were, at that time, showing poor incorporation of amino acids and the synthesis of smaller peptides only. It was decided that a fresh approach was needed, and so the mRNA itself was used as a probe after end-labelling with polynucleotide kinase.

As mentioned above end-labelled RNA has a much lower specific radioactivity than cDNA since one can only add one ^{32}P atom per molecule, and consequently make a less sensitive probe. In order to generate the greatest possible number of 5'-hydroxyl groups for the end-labelling reaction a limited alkaline hydrolysis is carried out in 50mM $NaCO_3$ at $55^{o}C$ for 1hour. This had the effect of generating RNA fragments with with a slightly lower mobility than the tRNA marker (Plate 4.4, note that the gel was not denaturing).

Figure 4,4

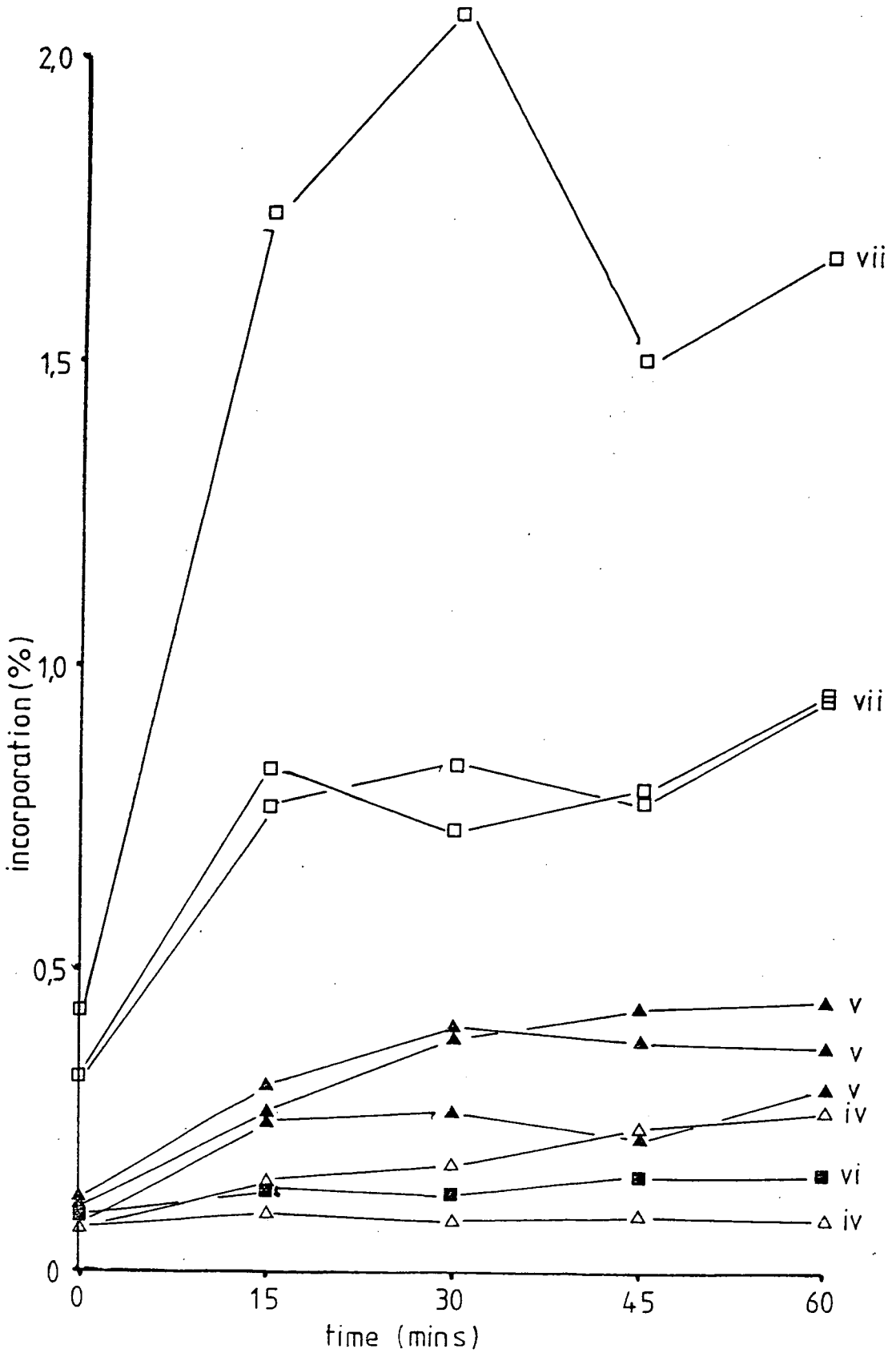
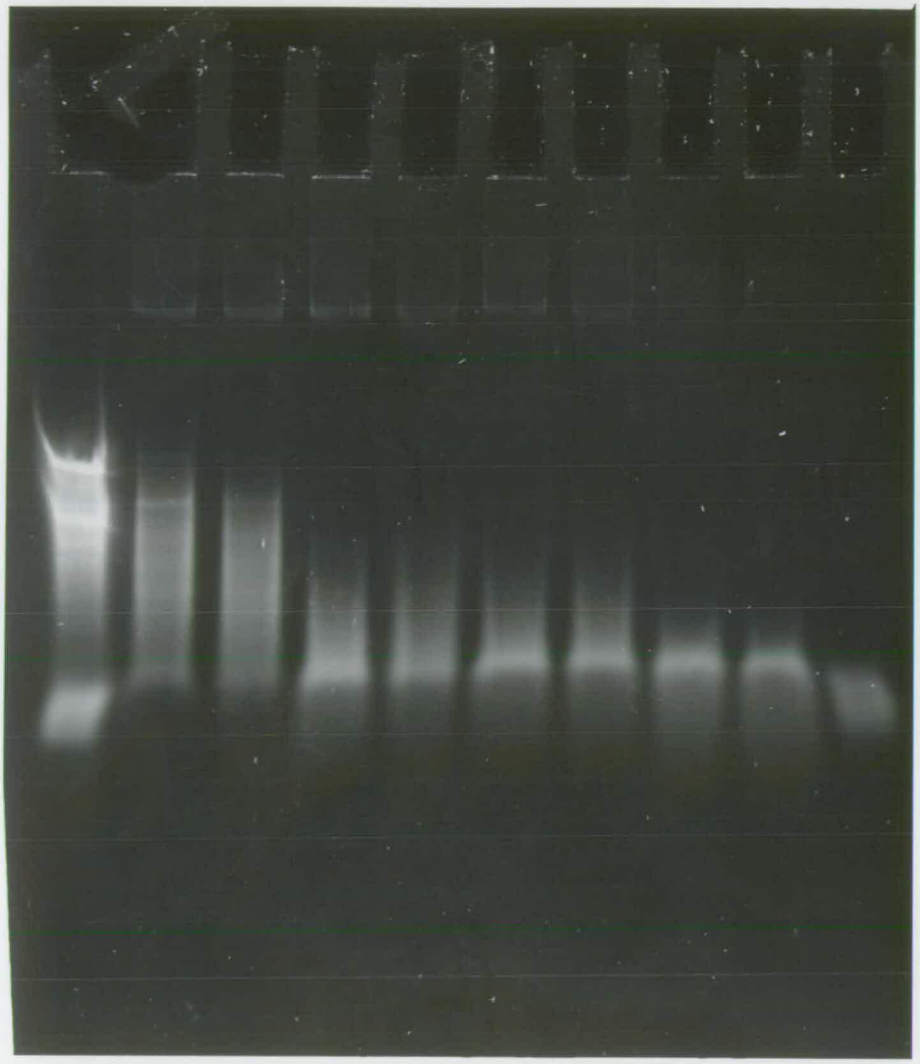

2,0
1,5
1,0
0,5
0
incorporation (%)
0
15
30
45
60
time (mins)
vii
vii
v
v
v
iv
vi
iv

Plate 4.4; Determination of the degradation conditions for mRNA for end-labelling. R- *E.coli* ribosomal RNA markers (a gift from K.Borck); T- yeast tRNA marker; S- $mRNA^S$; A- $mRNA^A$; 1- untreated; 2- treated at 50°C for 60 minutes; 3- treated at 55°C for 60 minutes; 4- treated at 60°C for 60 minutes.


R
S A
1
S A
2
S A
3
S A
4
T

This substrate was labelled to a level corresponding to 35% incorporation of label from γ-^{32}P-dATP, and specific radioactivities in excess of 10^6cpm/μg RNA. A typical incorporation curve is shown in Figure 4.5, and the separation of unincorporated label is shown in Figure 4.6.

A further disadvantage of using end-labelled RNA as a probe is that contaminating rRNA is labelled as well. To minimise this problem mRNA preparations for end-labelling were subjected to two cycles of adsorbtion and elution from an oligo(dT) column. Hybridization of the end-labelled RNA preparations to Southern transfers of HindIII digests of genomic N.crassa DNA showed 5 distinct bands of 3.6, 4.45, 5.35, 7.5 and 8.4kb in size that stood out clearly on a background smear (Plate 4.5). The smear is presumably due to the hybridization of the many species of mRNA. The bands at 3.6 and 5.35kb correspond quite well with the published sizes of 3.4 and 5.2kb for fragments of the rDNA repeat units generated with HindIII (Cox & Peden, 1979). The 8.4kb fragment observed may have been a partial digest (i.e. the sum of the 3.6 and 5.35kb bands). The presence of the 4.45 and 7.5kb bands could result from a second family of rDNA repeat units which had an approximately 1kb deletion from their 5.35kb HindIII fragment. As a further check on the identity of these bands genomic DNA was also digested with EcoRI, XhoI and BamHI. These were run out on an agarose gel, transferred to nitrocellulose and hybridized to another batch of end-labelled RNA probe. The sizes of the prominent bands (Plate 4.6) generally correspond well with the sizes expected from the digestion of the rDNA repeat units with these enzymes (Cox &

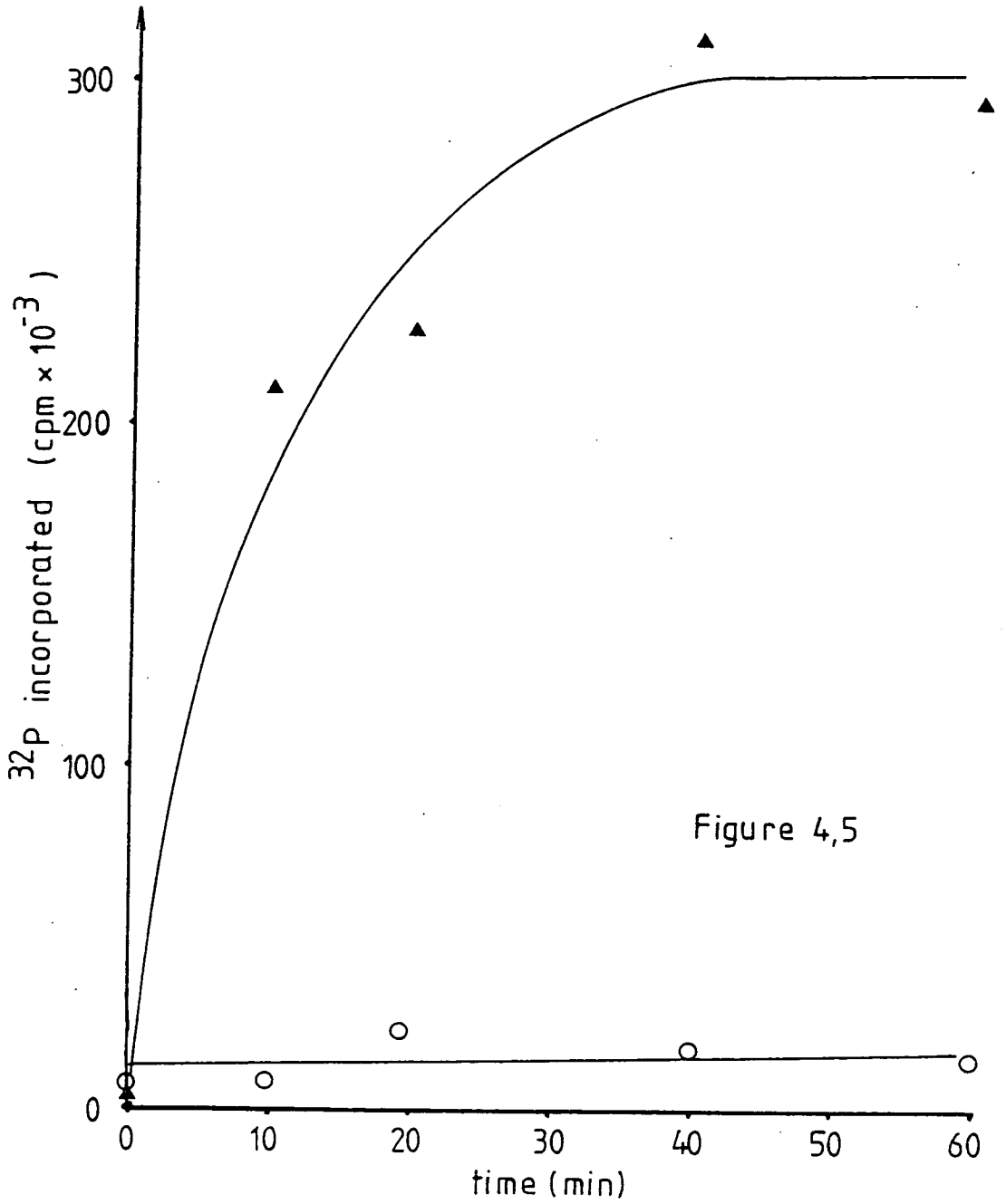

300
200
100
0
^{32}P incorporated (cpm x 10^{-3})
0
10
20
30
40
50
60
time (min)

Figure 4,5

Figure 4.6; The separation of unincorporated ^{32}P-dATP from labelled mRNA[a] after end-labelling. The fractions indicated were pooled for hybridization.

Figure 4,6

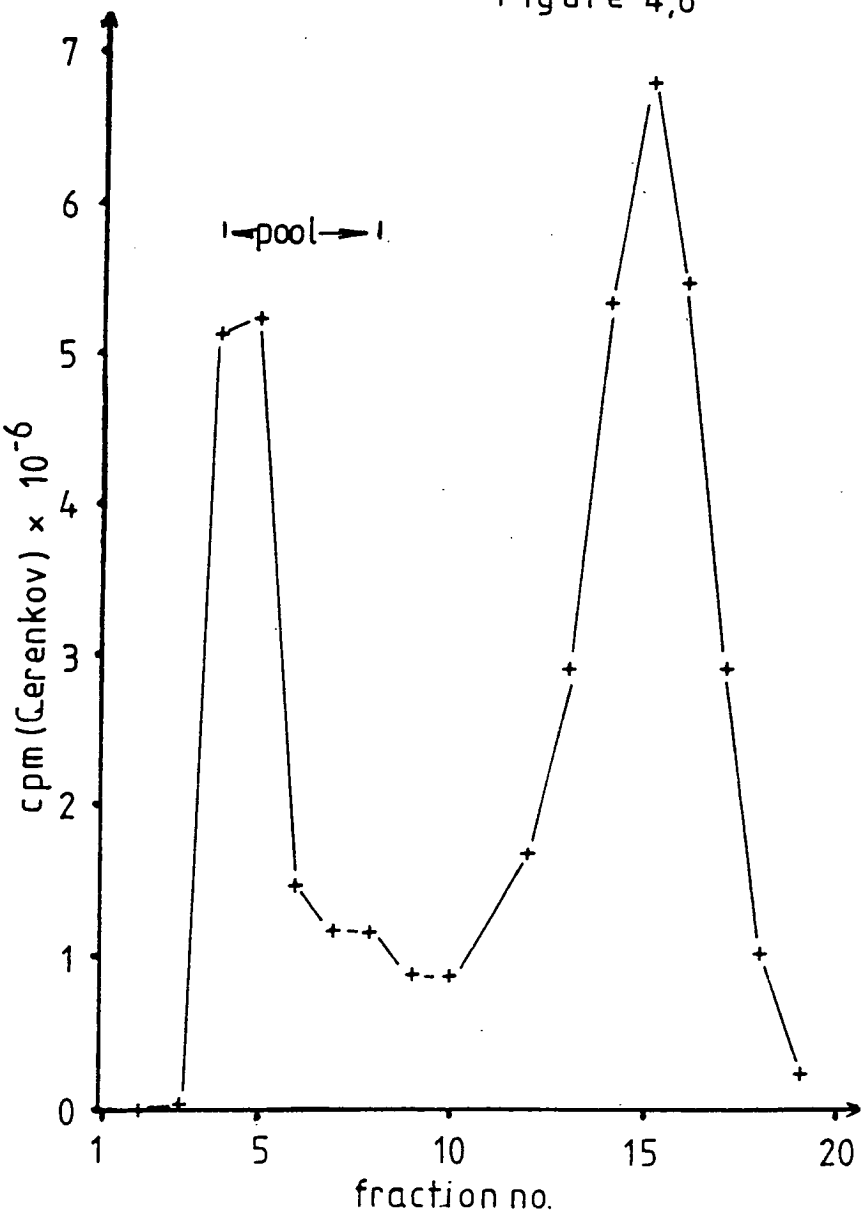


Plate 4.5; Autoradiograph of hybridization of end-labelled $mRNA^s$ (S) and $mRNA^a$ (A) populations to blots of a HindIII Neurospora genomic digest. Sizes were calculated from ethidium bromide stained markers run on the same gel and are given in kilobases.

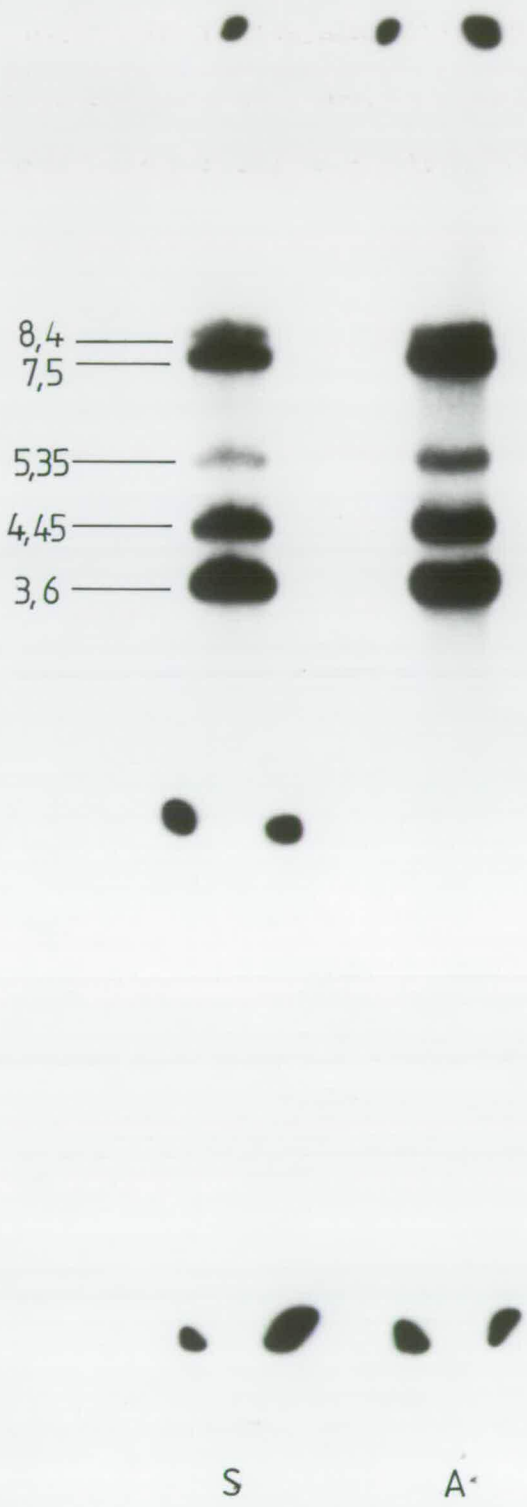
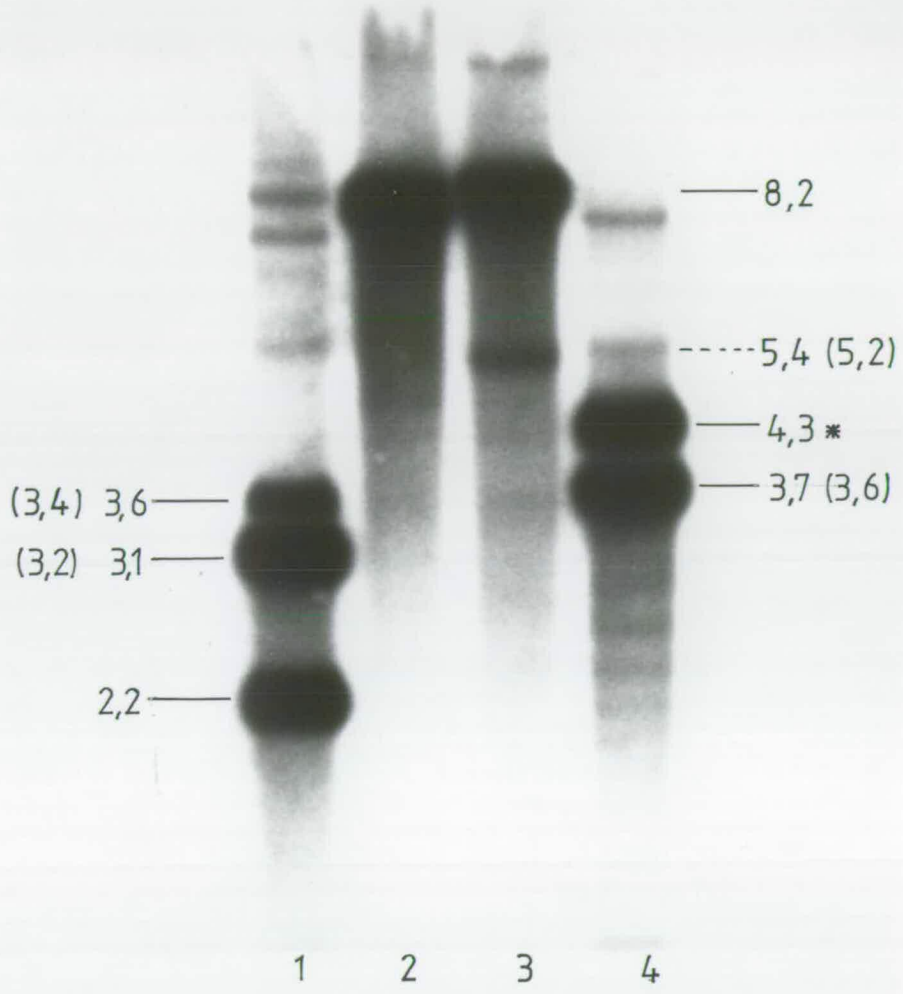

8,4
7,5
5,35
4,45
3,6
S
A

Plate 4.6; Autoradiograph of hybridization of end-labelled mRNA[a] to a blot of; 1- EcoRI; 2- BamHI; 3- XhoI; 4- HindIII digests of genomic DNA. Sizes were determined as in Plate 4.5 and are given in kb. Sizes in bracket are from Cox & Peden (1979) where their estimated size varies from that given here. *- indicates a band not seen by Cox Peden.


8,2
5,4 (5,2)
4,3 *
3,7 (3,6)
(3,4) 3,6
(3,2) 3,1
2,2
1
2
3
4

Peden, 1979). However, the HindIII digest again shows a lower molecular weight band, this time much more intense than the 5.4kb band. Since none of the other digests show any size perturbations, the simplest explanation for this observation is the presence of another HindIII site in the spacer DNA which produces a small (~1kb) fragment which does not hybridize to the rRNA. The presence of the two extra bands in the previous experiment (7.5 & 8.4kb, Plate 4.5) is explained as a partial digestion. This notion is supported by the virtual disappearance of the intensely hybridizing bands around 8kb on the second blot.

It is fairly obvious from these experiments that despite two rounds of selection on an oligo(dT)-cellulose column, the most abundant species of RNA is still rRNA. However, the presence of this RNA provided a useful bonus in allowing accurate alignment of one autoradiograph with another (see below).

c)Differential screening of a N.crassa Genomic Library

The library used had been previously constructed by Dr J.O.Bishop, in the Charon 4A bacteriophage vector (Blattner et al, 1977). Genomic DNA was methylated in vitro and cut using EcoRI* activity (Kemp et al 1979). Fragments of 12-16kb were selected and ligated into the EcoRI sites of Charon 4A after removal of the internal "stuffer" fragments (J.O.Bishop personal communication).

In order to screen the library using the technique of differential hybridization (St.John & Davis, 1979) it was necessary to ensure that multiple transfers (four per plate) could be made

without appreciable variation in the resultant signals from the plaques. Three methods of taking replicate transfers were tested using plates of a pure Charon 4A clone (JB1.1- a gift from J.Kinnaird). The methods were i)stacking four dry filters on the plate for 30- 40 minutes; ii)sequential transfer to four dry filters laid down for 0.5, 2, 5, and 10 minutes respectively and iii)sequential transfer to four filters pre-wetted in dH_2O, equilibrated in 6.6xSSC and blotted dry with filter paper. These were laid down individually for 3, 3, 5 and 5 minutes respectively. All the filters were treated as described in the methods section and were hybridized to nick-translated DNA from the same clone. Plate 4.7 shows the autoradiograph from this experiment.

Only the sequential methods gave a similar signal on successive filters. The filters towards the top of the stack had a reduced signal. Of the two sequential methods, the 'dry' protocol seemed to give less streaking due to running of loosely adsorbed DNA so this method was used for subsequent screening.

Phage from the library were plated out on E.coli (ED8654) at a fairly low density (800-900/ 9cm plate, 2500-3000/ 14cm plate) to facilitate easy recovery of putative positive clones and to minimise the masking of signals by ribosomal clones (the latter give very large spots -Plate 4.8). The first and third transfers were hybridized to end-labelled $mRNA^s$ and the second and fourth to end-labelled $mRNA^a$. It was hoped in this way to allow for any downward trend in the amount of DNA on successive transfers, and also by hybridizing the $mRNA^a$ to later transfers, to bias the

Plate 4.7; Autoradiograph of hybridizations of nick-translated charon 4A clone JB1.1 to plaque transfers of the same. i), ii) and iii) are described in the text.

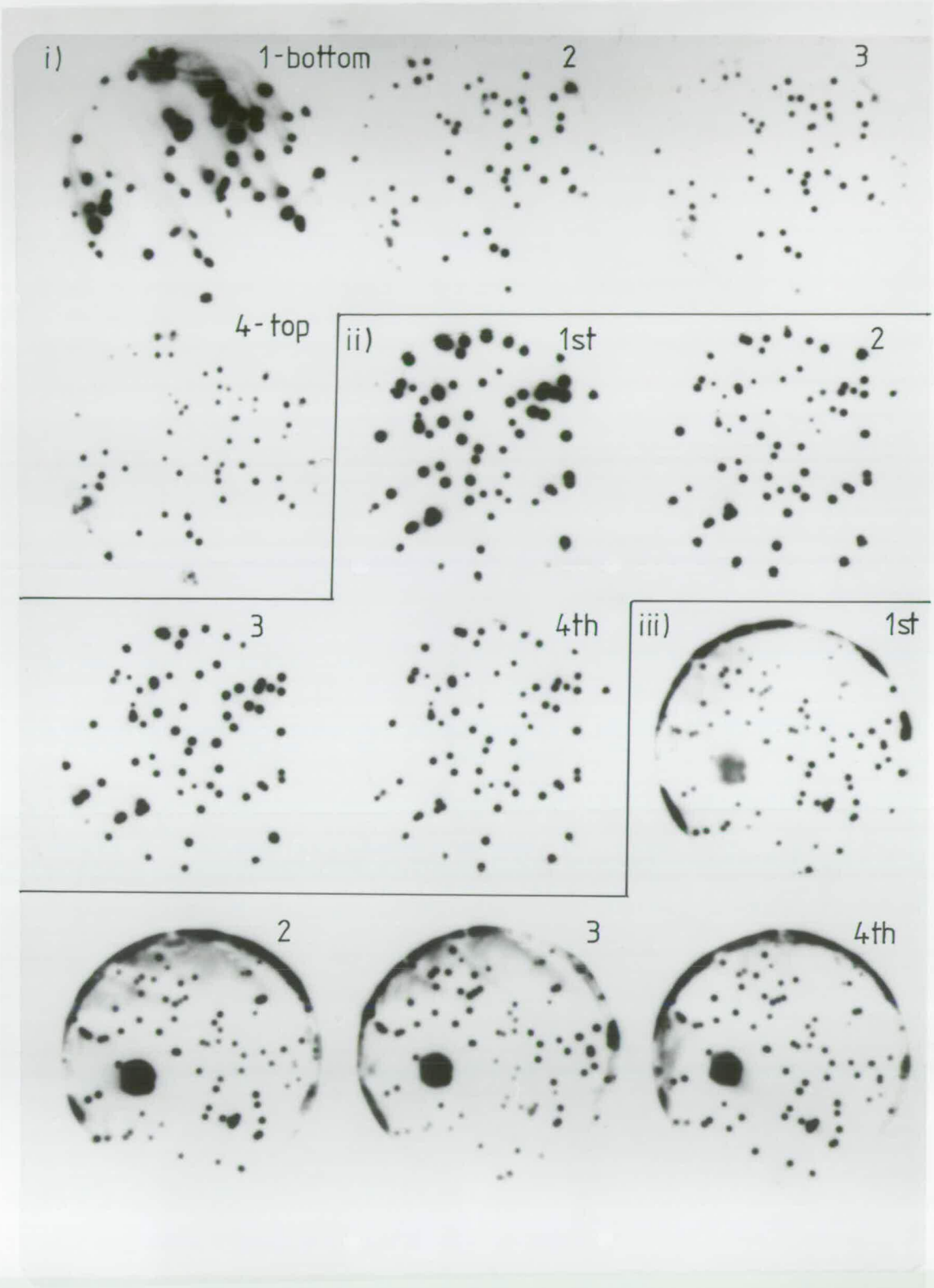
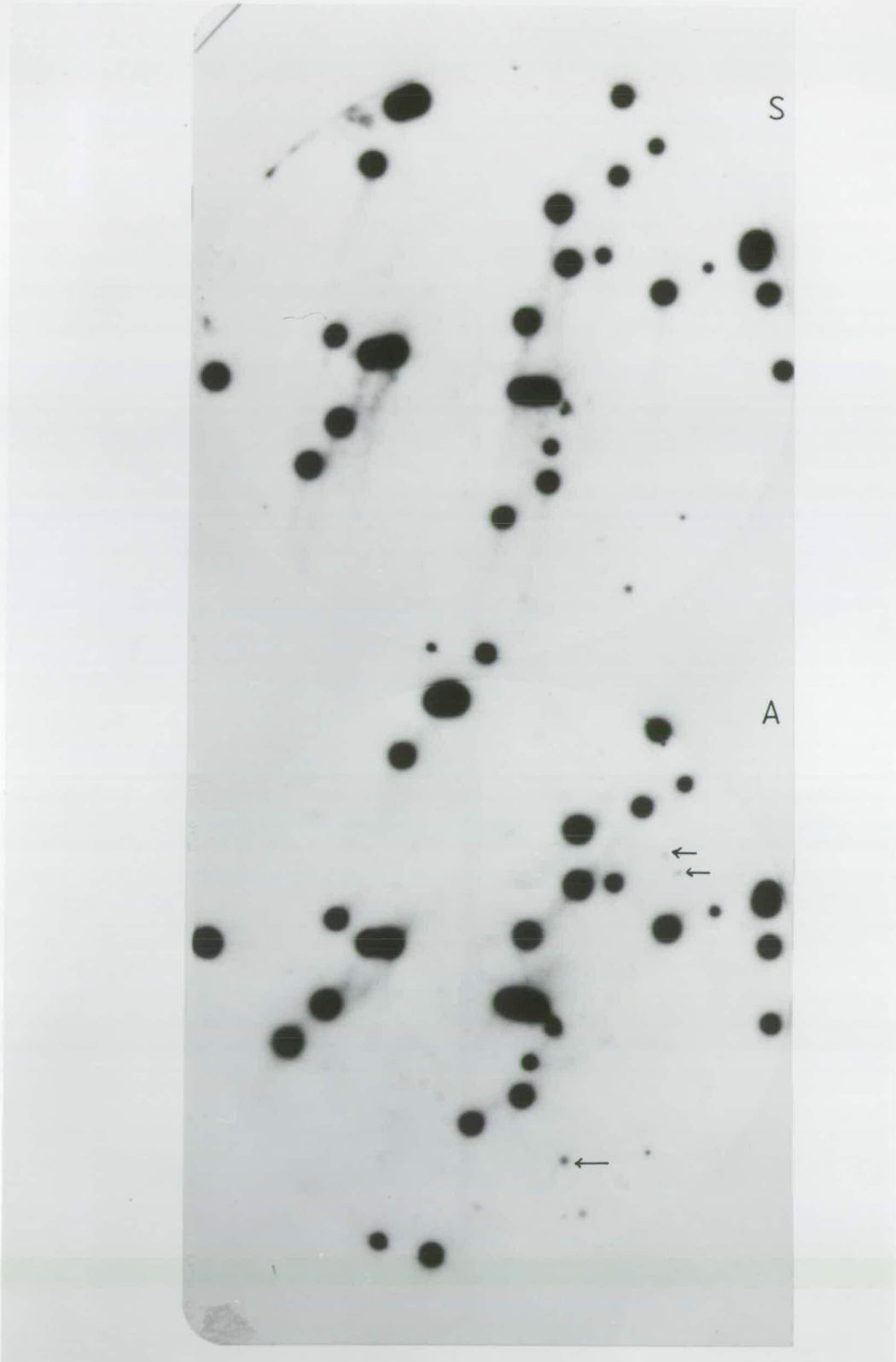

i)
1-bottom
2
3
4-top
ii)
1st
2
3
4th
iii)
1st
2
3
4th

Plate 4.8; Autoradiograph of two replicate transfers from a 14cm plate of bacteriophage from the Neurospora genomic library. Hybridization was to end-labelled $mRNA^{s}$ (S) and $mRNA^{a}$ (A). Arrows indicate example plaques showing differential hybridization.


S
A

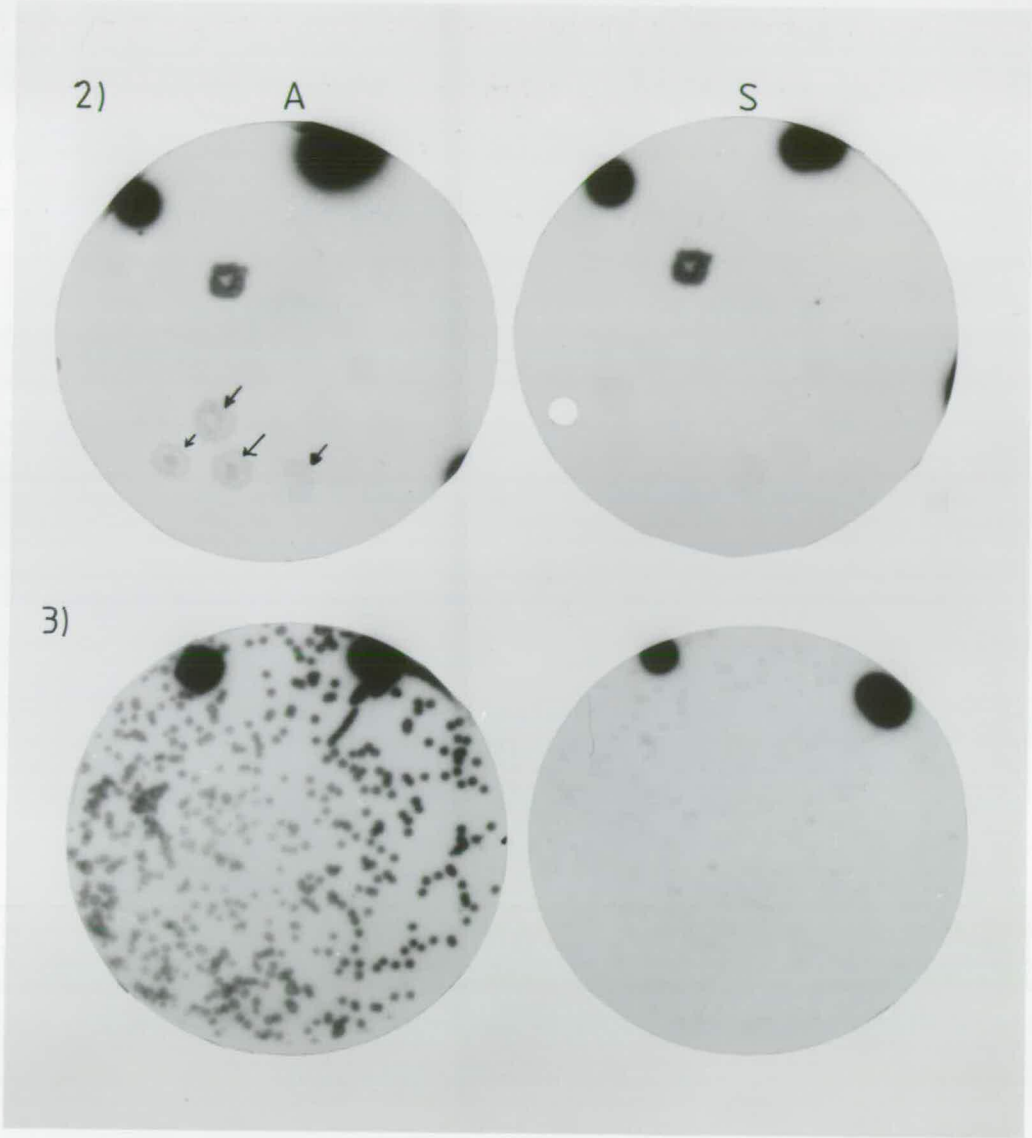
experiment against artifactual acetate- positive clones.

Positive plaques were picked with a sterile cocktail stick and was applied to an incipient lawn of ED8654 as a small circle of dots. This produces circular patches of lysis (Plate 4.9/2), and sequential transfers from these plates were again hybridized to both $mRNA^a$ and $mRNA^s$ as before. Usually several plaques were patched from around the positive spot in case the positive clone was missed due to shrinkage of the nitrocellulose or misalignment of the autoradiograph relative to the plate.

The top layer of agar containing a positive patch was lifted into 1ml of phage buffer and briefly vortexed to resuspend the phage. An appropriate dilution of this was plated out at low density and was again screened by the differential hybridization technique (Plate 4.9/3). A well isolated positive plaque was then picked in order to produce purified DNA for subsequent analysis.

Minimum expected frequency of positive clones: N.crassa has a genome size of $2x10^7$ base pairs (bp, Krumlauf & Marzluf, 1980). If the average insert size in the library is 15kb then any particular sequence should be present on average once in each 1300-1400 clones if the library is complete. The acu mutant loci are all either unlinked or only very loosely linked (Flavell & Fincham, 1968a) and so it is unlikely that more than one of the acu loci would be present in one insert. The number of known acetate- specific functions is 6 (isocitrate lyase, malate synthase, PEP carboxykinase, acetyl-CoA synthetase, the acp function, and probably

Plate 4.9; Autoradiographs of two replicate transfers from each of screens 2 and 3 hybridized to end-labelled $mRNA^s$ (S) and $mRNA^a$ (A). 2)- screen 2; phage were spotted out in a ring with cocktail sticks to produce patches of lysis. Arrows indicate positive clones. 3)- screen 3; phage from half a positive plaque were resuspended in phage buffer and plated out at an appropriate density.


2)
A
S
3)

fructose- 1,6-diphosphatase). Various TCA cycle enzymes are also modulated, but omitting these will give a minimum frequency to be expected. Thus a minimum of 0.43% (6/1400) clones would be expected to show the differential hybridization pattern.

A total of 50-60,000 plaques were screened and 160 putative positives were patched out for a second screen. Twelve of these were confirmed as positive clones. In the third screen one of the twelve proved to be constitutively expressed, leaving eleven from which DNA was prepared. A positive clone of the opposite sense (switched off on acetate) was spotted by chance and survived to a third screen; DNA was also purified from this phage.

The actual frequency of positive clones recovered was thus only 0.018-0.022% and about one twentieth of the minimum expected.

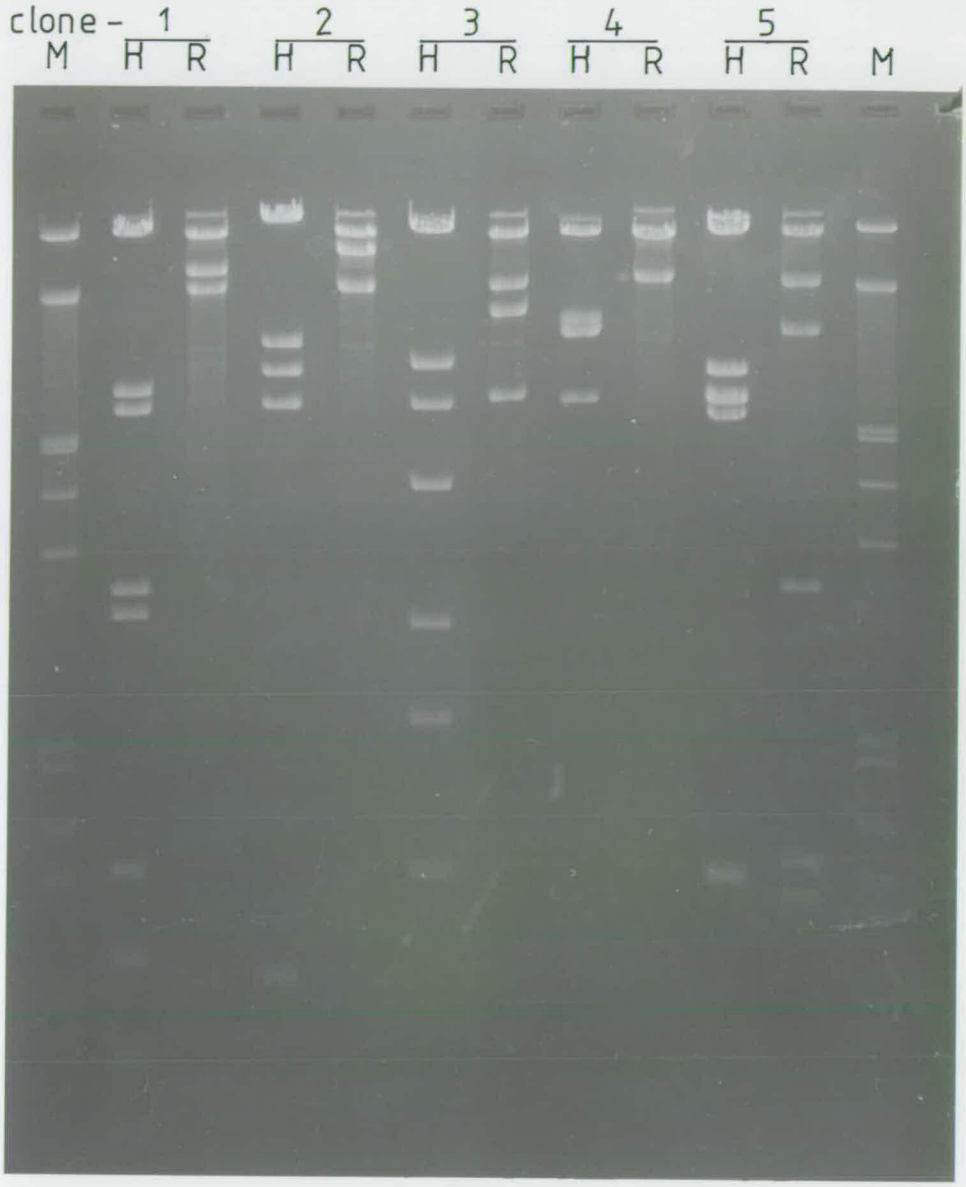
d)Characterisation of the positive clones

i)Restriction mapping

Restriction analysis of the 11 positive clones showed that 8 of them were independent isolates of the same insert, whilst the other three were all unique. Plate 4.10 illustrates the different classes. Clone 4 has only one EcoRI site, the site at the other end of the insert is presumably a hybrid EcoRI/EcoRI* site that is not recognised under these conditions. One in four of the insert/vector boundaries would be expected to show this behaviour.

The isolation of so many with the same insertion suggests that the library may not evenly represent the entire genome. The library

Plate 4.10; Restriction digests of the five classes of clone recovered (0.5ug/ track). Clones 1-4 are acetate 'induced'; clone 5 is acetate 'repressed'. H- HindIII digestion; R- EcoRI digestion; M- size markers (EcoRI/HindIII digested lambda DNA).


clone - 1 2 3 4 5
M H R H R H R H R H R M

was initially amplified some 10^7 fold and so differential amplification may account for this and possibly also for the reduced frequencies of the positives isolated, although the poor signal strength of the probes may also have contributed to the latter. Problems have also been found in the method used to make this type of library since its construction. EcoRI* activity is no longer thought to cut randomly at all the 5'-NAATTN-3' sites (where N is any base), but to show significant bias depending what base N is (Woodbury et al, 1980; reviewed in Anson, 1983). This can lead to a non-random library.

Tentative restriction maps have been constructed for some of these clones based on size measurements of fragments from complete (single double) an partial digests run on 0.5, 0.7 and 1.2% agarose gels (Figure 4.7, Plate 4.11). Further analysis using subcloned probes and end-labelled partial digests (as described in Clark et al, 1982) is needed to confirm these. The transcription units were located on them by hybridizing end-labelled poly(A)$^+$-mRNA to southern transfers of digests of the DNA (Plate 4.12).

ii)RNA Blots

The inducible nature of the four clones was confirmed by hybridizing nick translated ^{32}P-labelled DNA from each clone to RNA blots of mRNA^s and mRNA^a. The results are shown in Plate 4.13.

The most abundant transcript which hybridize to the DNA of clones 1,2 and 3 (4.1, 4.1, 4.5kb respectively) are strongly induced and not detectable in mRNA^s. The main band in clone 4 (3.9kb) is present at a reduced level in mRNA^s.

Figure 4.7; Tentative restriction maps for clones 1, 2 5. Sites; R- EcoRI; S- SalI; X- XhoI; Xb- XbaI. *- indicates an inferred fragment not visualized on a gel. Hatching indicates hybridization to end-labelled mRNA[a] Solid shading indicates the strongest hybridization in clone 1- this is the region thought to contain the acetate-specific transcript. T- direction of transcription in that fragment (see text).

224

Figure 4,7

Plate 4.11; Restriction digests of clone 1 DNA (0.5µg/track). Digestion was with; 1-EcoRI; 2-EcoRI/HindIII; 3-digestion failed; 4-HindIII/BamHI; 5-BamHI; 6-BamHI/EcoRI; 7-EcoRI/HindIII/BamHI; M-size markers (EcoRI/HindIII digested lambda DNA).

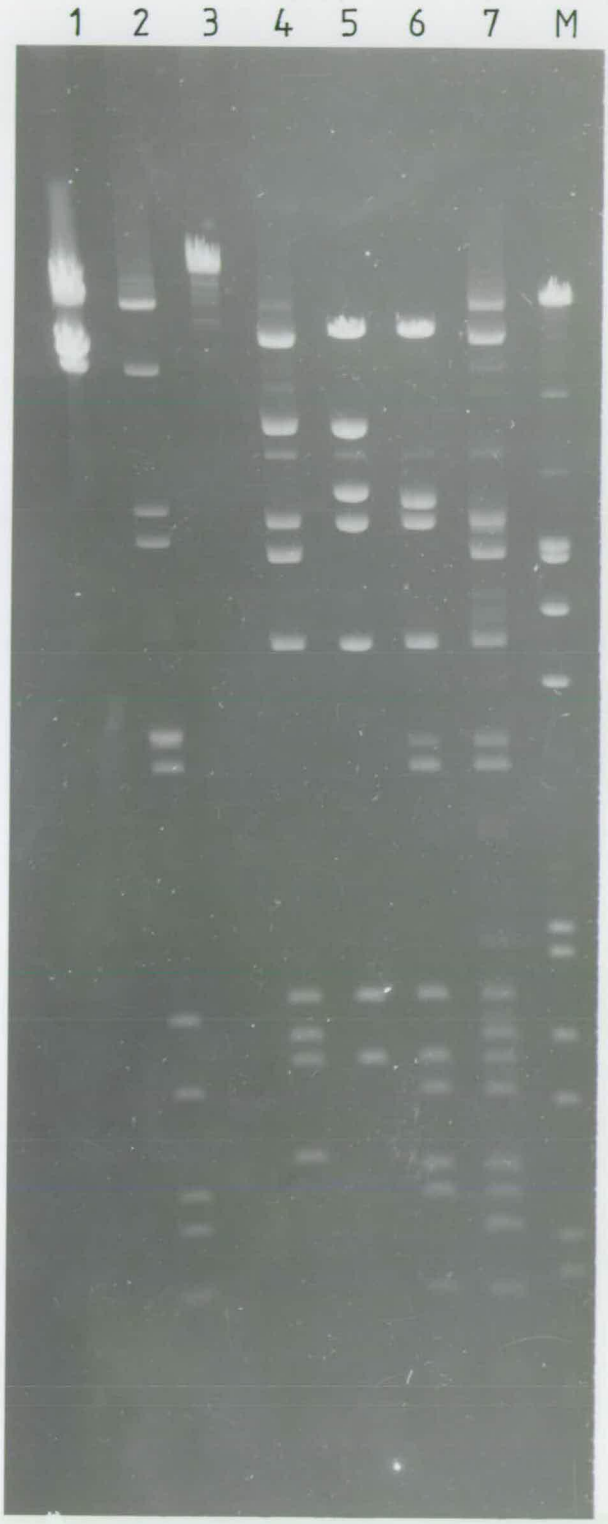

1
2
3
4
5
6
7
M

Plate 4.12; Autoradiograph of the blot from the gel shown in Plate 4.11, hybridized to end-labelled mRNA^{a}. 1-7 and M are described in the legend to Plate 4.11. Note that there is no hybridization to the lambda DNA in M.

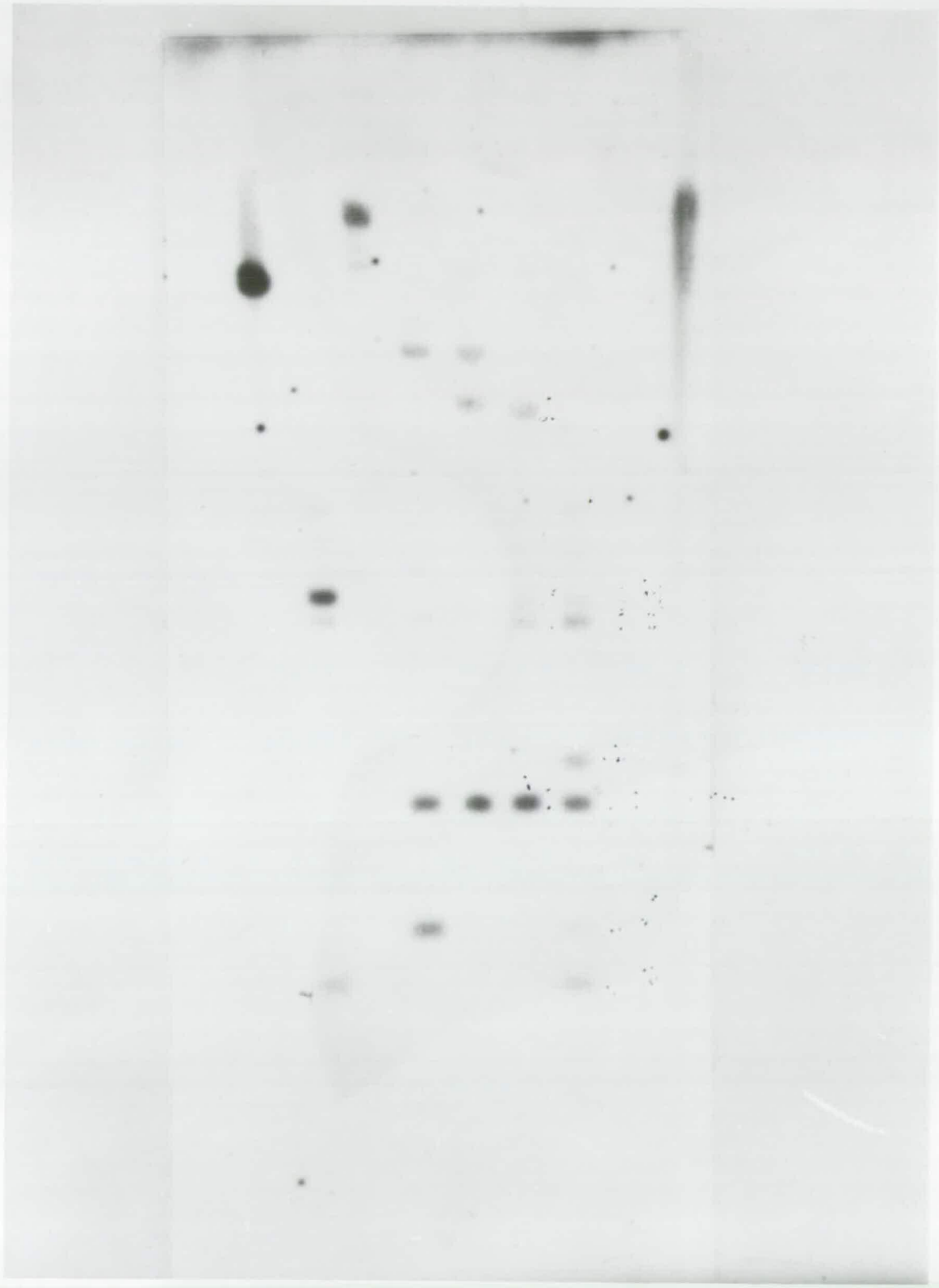
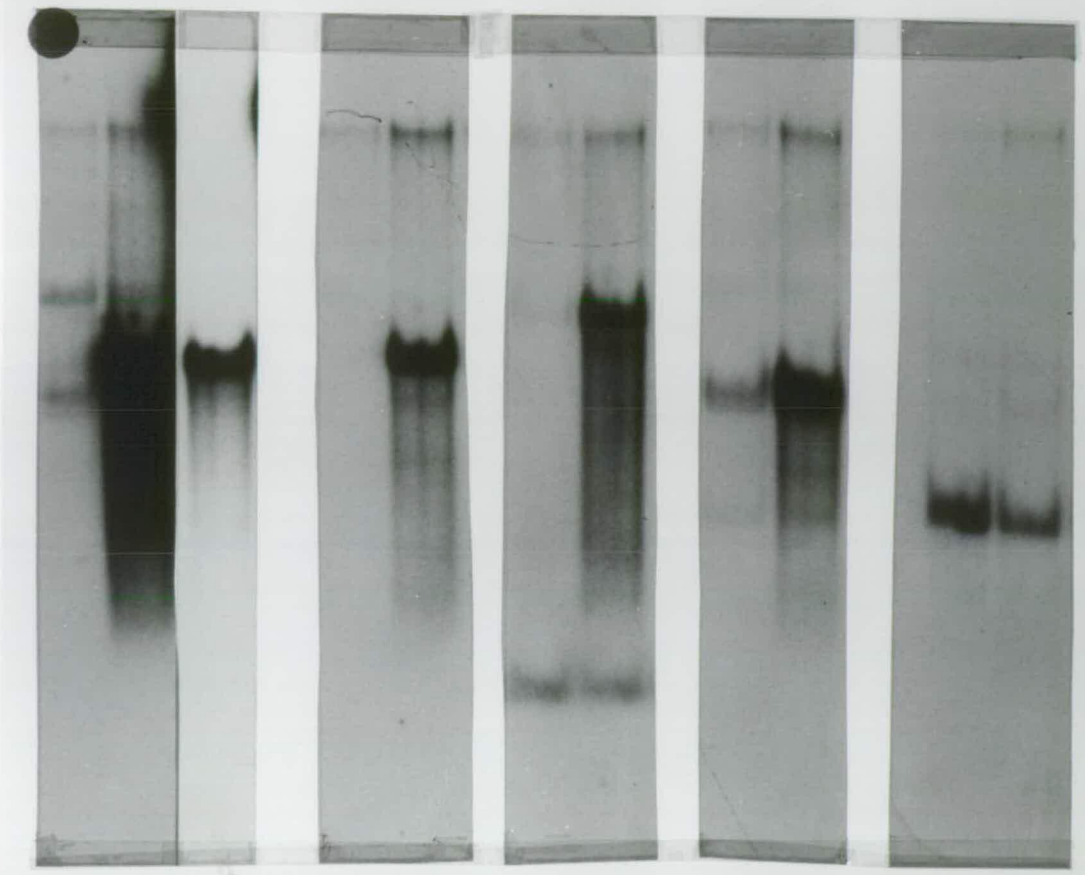

1 2 3 4 5 6 7 M

Plate 4.13; Autoradiograph of hybridization of nick-translated DNA from clones 1-5 to RNA blots of $mRNA^s$ (S) and $mRNA^a$ (A) (10µg/track) separated in a formaldehyde-agarose gel as indicated. A′ is a shorter exposure of clone 1 track A.


S A A´ S A S A S A S A
clone 1 2 3 4 5

In addition to its major transcripts clone 1 also hybridizes to two other sequences (4.5 & 3.8kb) both of which are also present in $mRNA^S$ making it unlikely that they are either precursors or in any other way related to the major clone 1 transcript. Clone 3 also hybridizes to an additional small band which is constitutively expressed on both sucrose and acetate.

The clones have also been hybridized to a time course series of total RNA blots prepared at appropriate times after transfer to acetate medium from both the wild type and acu7 (JI36) strain. The latter was shown to over-produce the acetate specific enzymes (Beever, 1972) and to produce a greater quantity of the acetate specific bands in in vitro translations than the wild type (this chapter). This experiment suggests that the over-production is transcriptionally based (Plates 4.14-16), although an alteration in messenger stability has not been ruled out.

The bands from the clone 1 hybridization to acu7 RNA have been quantified using a Joyce Lobel scanning densitometer. The integrals are plotted in Figure 4.8, and are compared with the enzyme levels in this strain found after similar times on acetate in a parallel experiment. The enzyme and mRNA levels increased in parallel over much of the time course suggesting that the rate of protein synthesis may be proportional to the amount of RNA.

iii)Attempted identification of Gene products

Having confirmed the control of transcription of the cloned sequences the next step is the identification of the proteins for

Plate 4.14; Autoradiograph of hybridization of nick-translated clone 1 DNA to a RNA blot from a formaldehyde-agarose gel. RNA was prepared from wild type (wt) and acu7 (JI36) at various times after transfer from sucrose to acetate medium (times in acetate are given in hours under the appropriate track). M- DNA markers (lambda digested with EcoRI) which hybridize to sequences in the arms of Charon 4A. T1 indicates the major transcript hybridizing to the clone. Note that this gel was run on total RNA (14µg/track).

Plate 4.15; Autoradiograph of hybridization of nick-translated clone 2 and 4 DNA to a RNA blot from a formaldehyde-agarose gel. Symbols are as described in the legend to Plate 4.14. T2 and T4 refer to transcripts complementary to clones 2 and 4 respectively. Note i) total RNA was used (14µg/track); ii) equal radioactivity from each clone was not added so the between-clone intensities are not comparable.

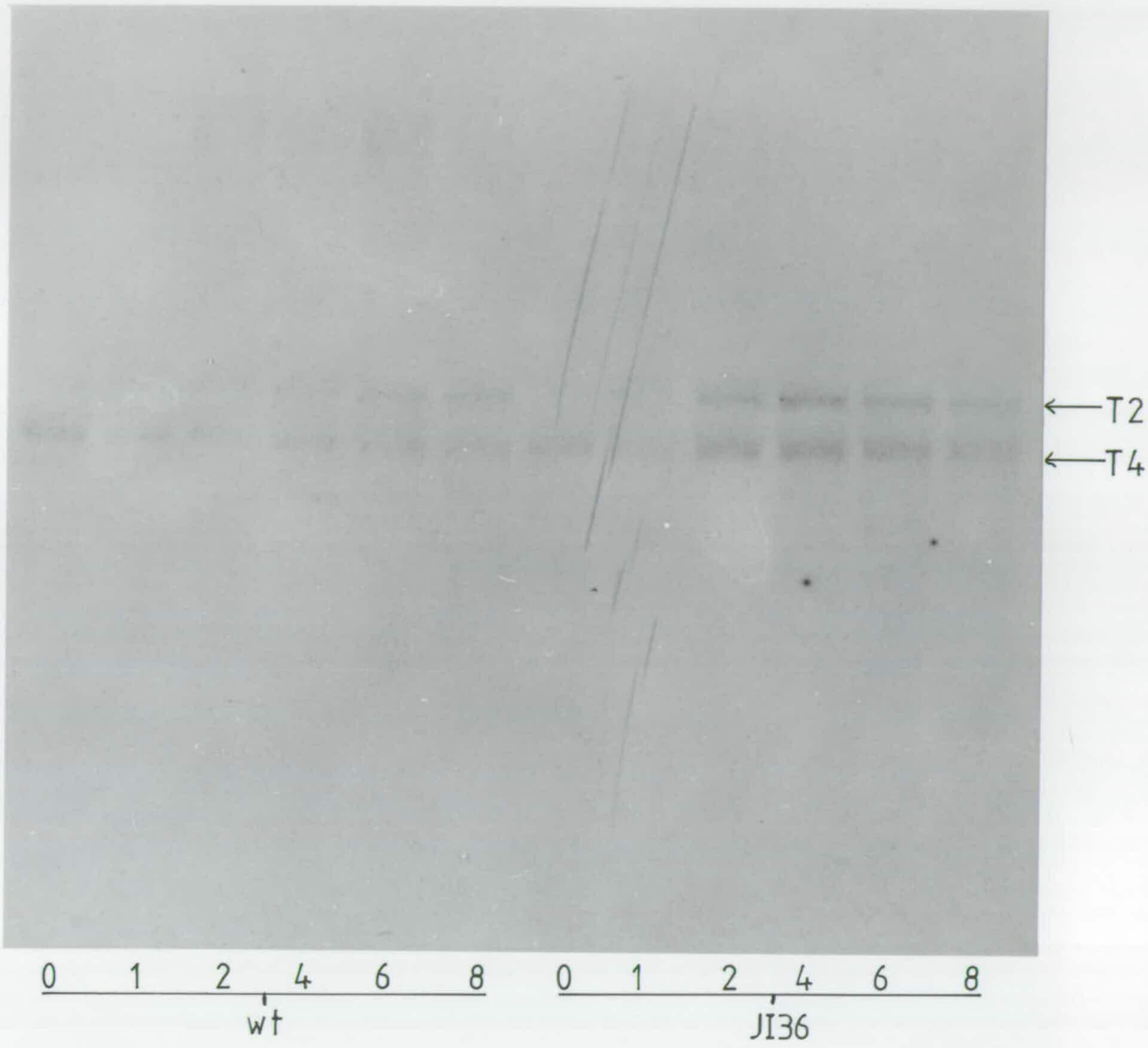

←T2
←T4
0 1 2 4 6 8
wt
0 1 2 4 6 8
JI36

Plate 4.16; Autoradiograph of hybridization of nick-translated clone 3 and 5 DNA to a RNA blot from a formaldehyde-agarose gel. Symbols are as described in the legend to Plate 4.14. T3 and T5 refer to transcripts complementary to clones 3 and 5 respectively. Note i) total RNA was used (14µg/track); ii) equal radioactivity from each clone was not added so the between-clone intensities are not comparable.

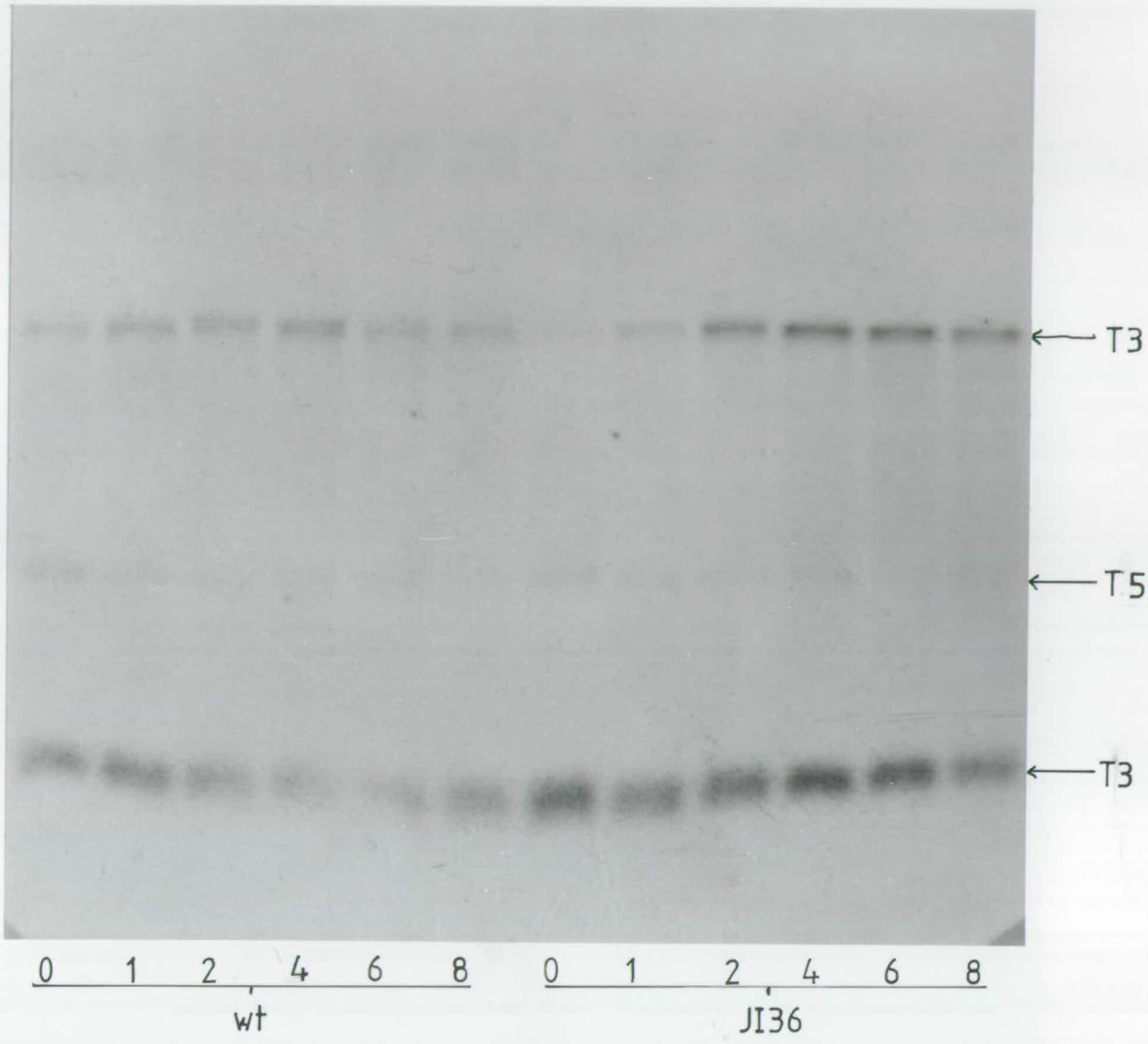
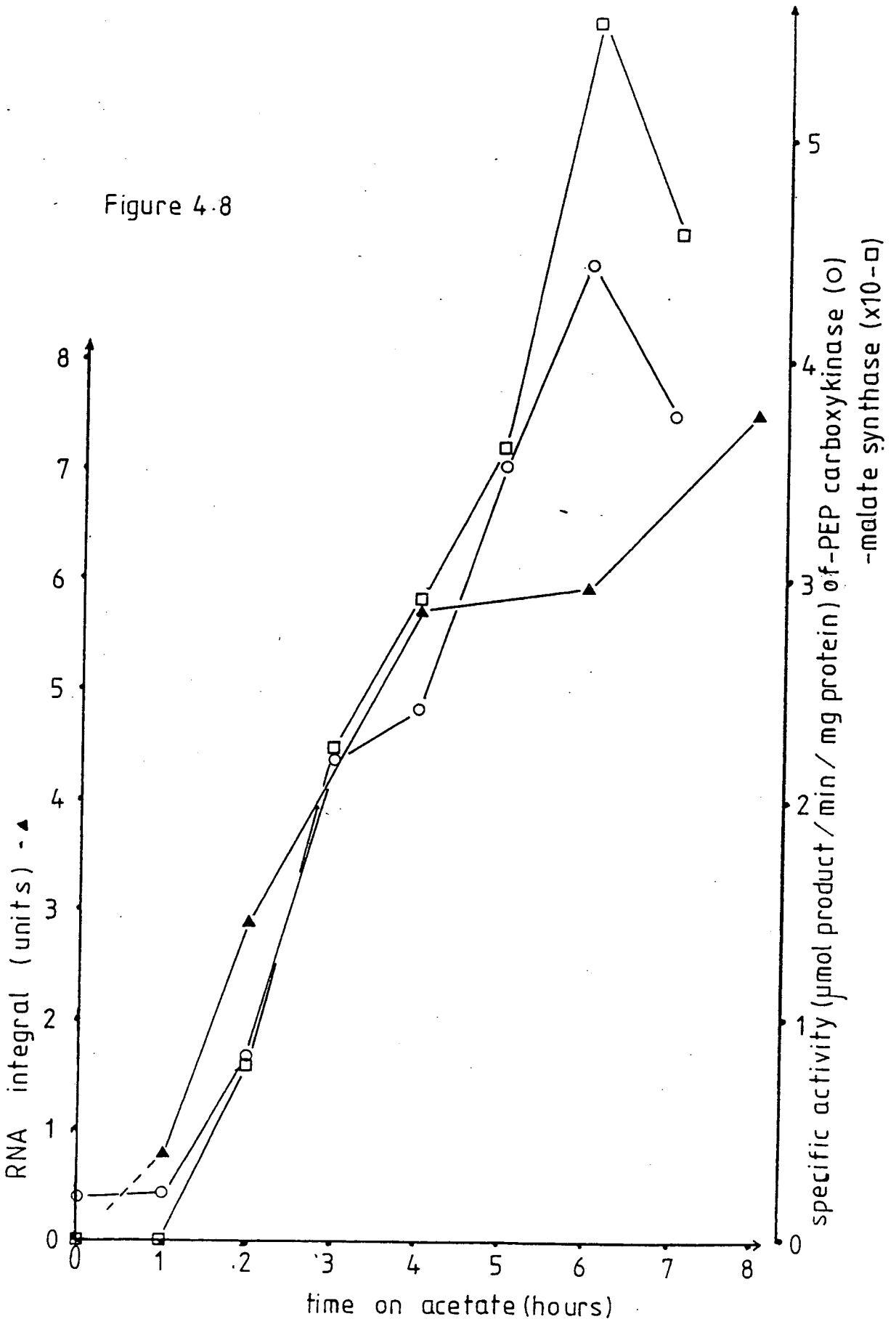

T3
T5
T3
0 1 2 4 6 8
wt
0 1 2 4 6 8
JI36

Figure 4.8; The increase in the amount of RNA complementary to clone 1(▲) Nick-translated DNA was hybridized to a blot of total RNA prepared at various times, from acu7 mycelium, after transfer from sucrose to acetate medium and separated in a formaldehyde-agarose gel. The corresponding increases in PEP carboxykinase (o) and malate synthase (□) specific activities in this strain after transfer to acetate are shown since clone 1 may code for one of these.

Figure 4.8


RNA integral (units) - ▲
specific activity (μmol product / min / mg protein) of-PEP carboxykinase (o)
-malate synthase (x10-□)
time on acetate (hours)
0
1
2
3
4
5
6
7
8

which they code. The method of choice is hybrid selection (Ricciardi et al, 1979). The principle of this procedure is very straightforward. Cloned DNA is denatured and immobilized on a filter of either nitrocellulose or diazotised paper. This is then hybridized to mRNA in solution and any complementary RNA sequences will become bound to the filter. Unbound RNA is washed away and the complementary RNA is recovered by melting it off the DNA. mRNA thus selected can be translated in vitro and the product(s) visualized on a polyacrylamide gel by fluorography.

This technique is rather fickle, mainly because of RNA degradation. Although as little as 10ng of mRNA of a single species can be detected in an in vitro translation system (Maniatis et al, 1982) this must be completely intact.

Out of 13 attempts to select mRNA with the acetate- specific clones only one (the ninth) worked and then only for clone 1. The reason for this poor success rate is unknown, but degradation of the RNA during the hybridization step seems the likely cause. Attempts to translate the non-selected mRNA were largely unsuccessful and at best resulted in only a few low molecular weight proteins being synthesized. This was despite the use of RNase inhibitors such as vanadyl ribonucleoside complexes (Berger et al, 1980; from BRL) or placental RNase inhibitor (Blackburn et al, 1977; from PS Biochemicals) in the hybridization mixture. DEP pre-treatment of the filters was also tried to no avail. It is worth noting that 50% formamide (though not SDS) was present in these hybridization mixtures, and it is not clear whether this could have reduced the

effectiveness of the RNase inhibitors.

The successful hybrid selection experiment showed a single faint band after three weeks exposure to the X-ray film (Plate 4.17). Its' apparent molecular weight of 60,000 daltons corresponds exactly to one of the prominent induced bands translated from the $mRNA^a$ preparation.

In order to increase the chances of recovering enough intact mRNA for translation, a larger scale technique was used. The strongest transcription unit contained in clone 1 is located at least in part in the right-hand-most EcoRI-HindIII fragment of the original clone. This, along with the neighbouring HindIII fragment, was subconed into pBR322 and prepared on a large scale. 0.75mg of this plasmid (designated pGT14) was digested with EcoRI and HindIII and the resulting fragments were separated on several 1% agarose gels. The 0.9kb EcoRI-HindIII fragment was eluted from the gels using the bag method (see methods) and 89µg was recovered of which 75% was successfully bound to epoxy-activated cellulose.

RNA was selected and eluted as described in the methods section and ten 200ul fractions were collected. After concentration by precipitation fractions 1, 2 and pools of 3-5 and 6-10 were reprecipitated. In vitro translation again yielded a 60,000 dalton polypeptide (Plate 4.18, in the 3-5 pool) detected this time after only 2.5 days fluorography. A longer exposure revealed a few more lower molecular weight bands, which seem to correspond to some of the more prominent translation products seen when total mRNA is

Plate 4.17; Autoradiograph of *in vitro* translation. 1- no added RNA; 2- 0.5µg of $mRNA^s$; 3- 0.5µg of $mRNA^a$; 4- protein size markers visualized by coomassie staining and described in the legend to Plate 4.1; 5- clone 1 hybrid-selected RNA; 6 to 9- clone 2 to 5 hybrid selected RNAs respectively. Tracks 2 and 3 have been held back during printing to prevent over-exposure.

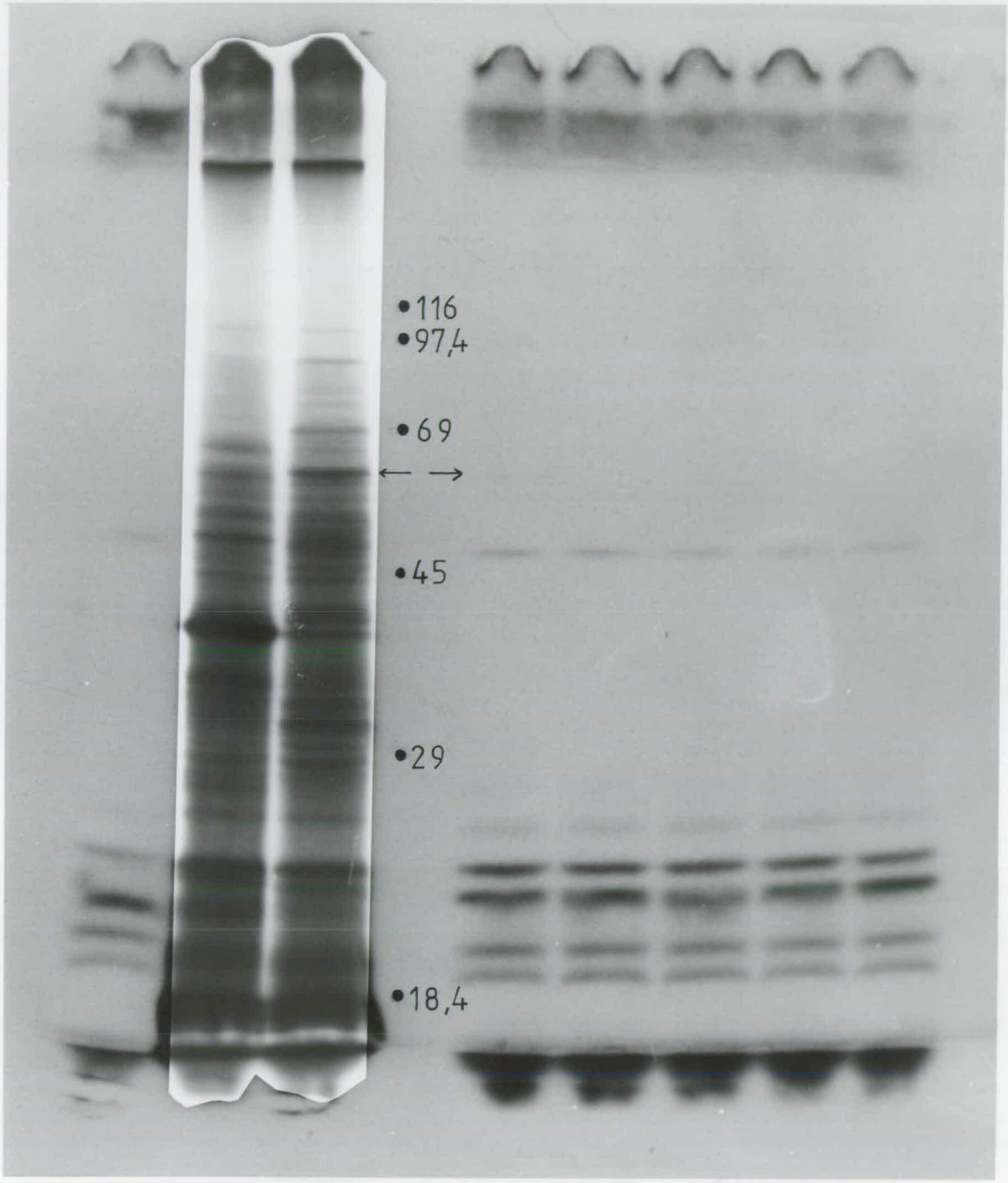
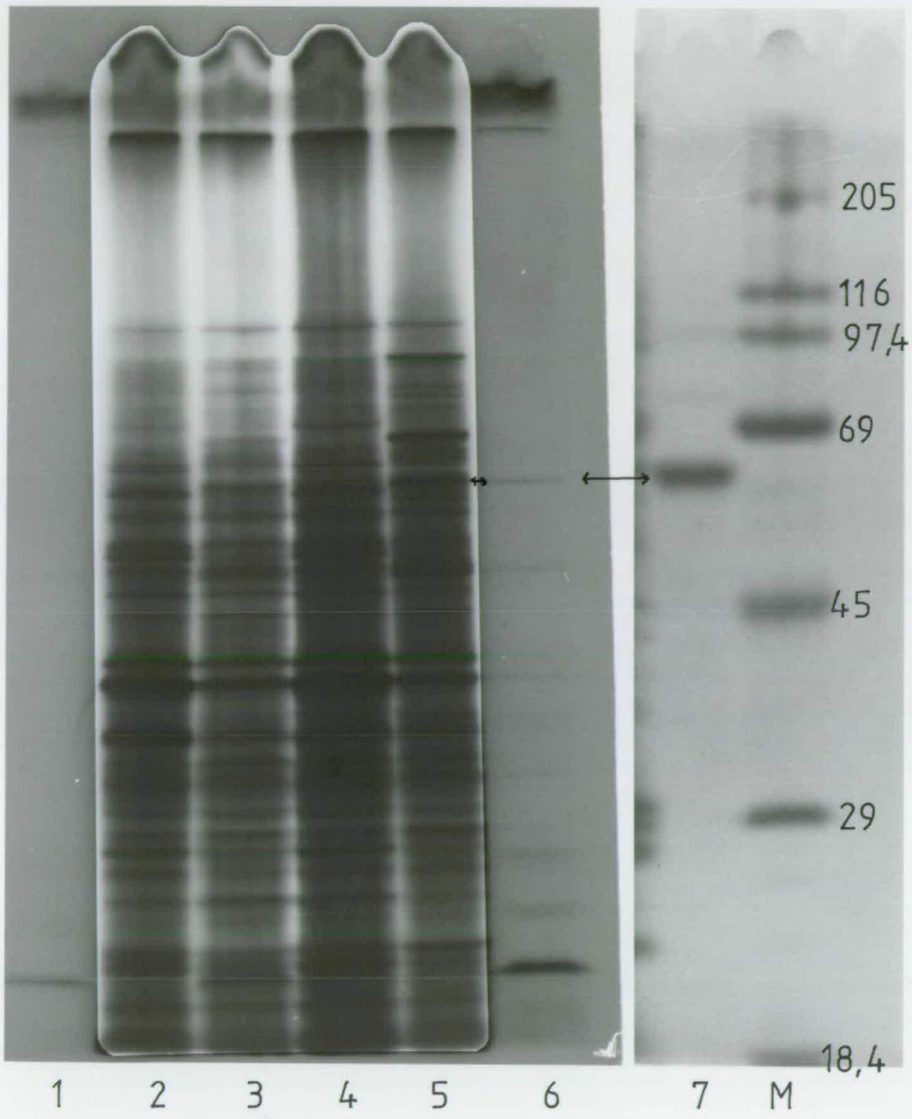

•116
•97,4
•69
•45
•29
•18,4
1
2
3
4
5
6
7
8
9

Plate 4.18; 1 to 6- Autoradiograph of *in vitro* translations. 1- no added RNA; 2- 0.5µg of $mRNA^s$ from wild type mycelium; 3- 0.5µg of $mRNA^a$ from wild type mycelium; 4- $mRNA^s$; 5- $mRNA^a$; 6- RNA selected by the DNA-cellulose column. 7 and M are from the same gel but are stained with coomassie blue. 7- purified PEP carboxykinase; M- protein molecular weight markers as described in the legend to Plate 4.1. Tracks 2-5 have been held back during printing to prevent over-exposure.


205
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97,4
69
45
29
18,4
1
2
3
4
5
6
7
M

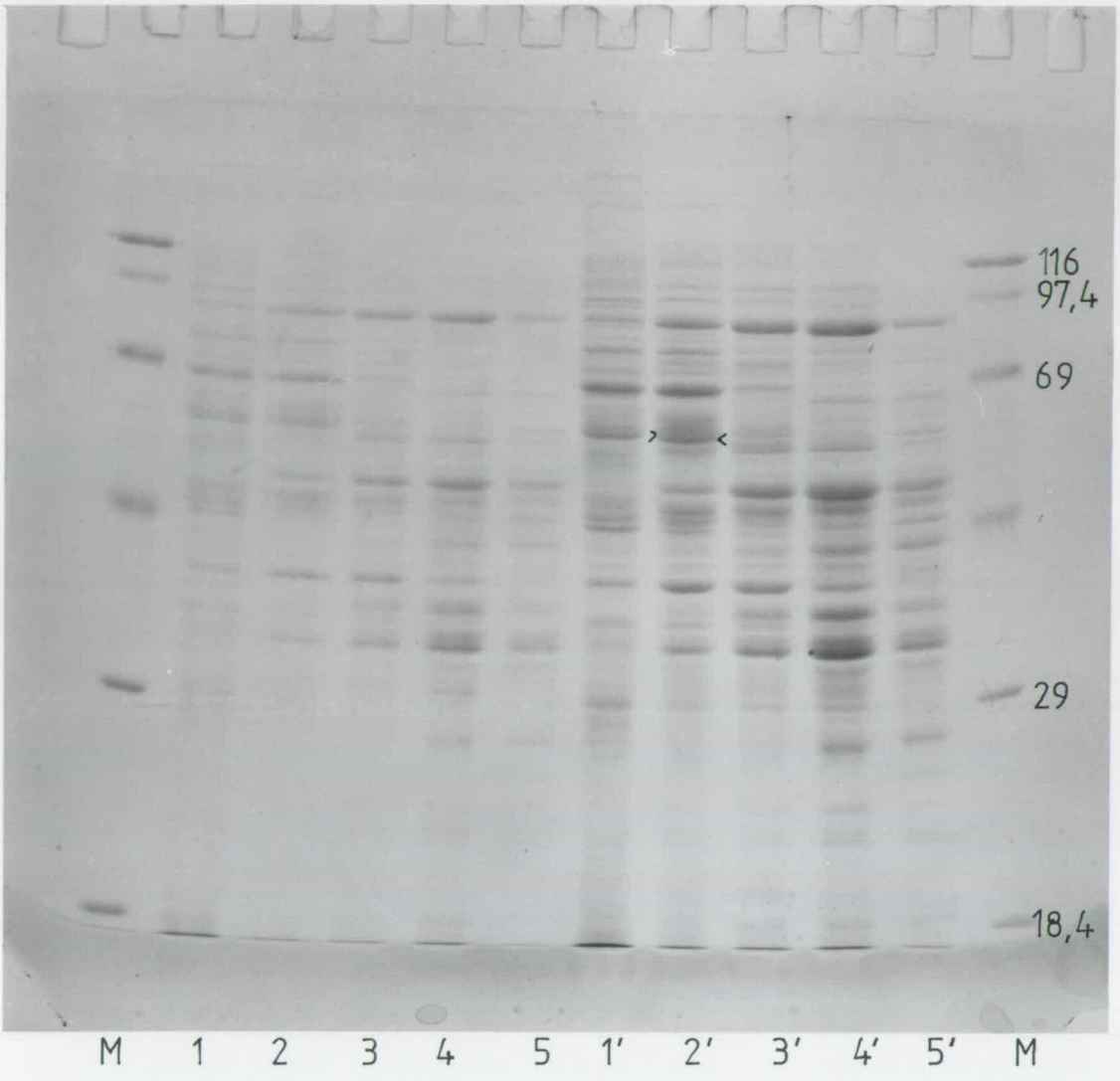
translated. This suggests that some non-specifically bound mRNA was still attached to the column at the time of elution. Clearly the column washing procedure needs to be more thorough and/or stringent. However, the enrichment of the 60,000 dalton band is very clear. Since the filter selection procedure also selected a messenger coding for a polypeptide of this size it is presumed to be the acetate-specific product whose coding sequence falls, at least partly, within clone 1.

The clone 1 product is clearly not isocitrate lyase since this enzyme has an apparent monomer molecular weight of 67,000 daltons (see earlier). This left five possible candidates (malate synthase, acetyl-CoA synthetase, PEP carboxykinase, the acp function and probably fructose- 1,6-diphosphatase) of which the first three have convenient assays. A crude extract was prepared from acetate adapted mycelium and was fractionated on a Sephadex G200 column. Every third fraction was assayed for each of malate synthase, PEP carboxykinase, and acetyl-CoA synthetase. No malate synthase activity was detectable, but acetyl-CoA synthetase and PEP carboxykinase both showed distinct peaks of activity.

Pools of the various fractions were concentrated by precipitation with 65% ammonium sulphate and, after resuspension and dialysis, were run on an SDS polyacrylamide gel to see if either active pool contained a prominent 60,000 dalton band. The PEP carboxykinase pool showed such a band (Plate 4.19), so the purification of this enzyme was undertaken (see appendix). The most highly purified fraction, contained one prominent protein of monomer molecular

Plate 4.19; SDS polyacrylamide gel stained with coomassie blue. Samples are concentrated pools from a crude extract of acetate 'induced' mycelium fractionated on a Sephadex G200 column. M- protein molecular weight markers as described in the legend to Plate 4.1; 1- pool of all the 3 preceding fractions prior to the PEP carboxykinase pool; 2- pool of 5 fractions showing PEP carboxykinase activity; 3- pool of all the 3 fractions between the pools of PEP carboxykinase and acetyl-CoA synthetase activities; 4- pool of 5 fractions showing acetyl-CoA synthetase activity; 5- pool of the 3 subsequent fractions after the acetyl-CoA synthetase pool. 1' to 5' are heavier loadings of the same samples in tracks 1 to 5 respectively.

Column conditions- 75ml of a concentrated acetate 'induced' crude extract in 50mM Tris.Cl pH7.4- 1mM EDTA- 10mM B-mercaptoethanol was brought to 60% ammonium sulphate on ice and spun for 20minutes at 20,000g. The resultant precipitate was resuspended in 15ml of the extraction buffer and clarified by centrifugation as above. 12ml of this was loaded on to a Sephadex G200 column and 20 minute (~12-13ml) fractions were collected and assayed as described in the text. Pools were concentrated by precipitation with 65% ammonium sulphate and centrifugation. The precipitates were resuspended in 10mM Tris.Cl pH7.5 and dialysed versus 5l of the same overnight prior to electrophoresis.


116
97,4
69
29
18,4
M 1 2 3 4 5 1' 2' 3' 4' 5' M

weight of 60,000 and three other very minor components (Plate A1). The main band migrated in parallel with both the prominent acetate-specific polypeptide in the mRNA^{a} translation and the 60,000 dalton polypeptide selected by the column (Plate 4.18).

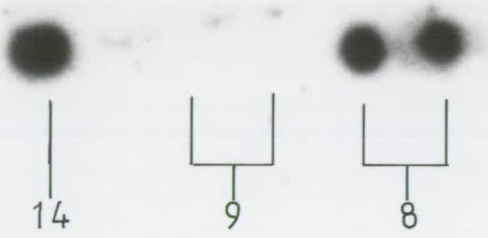
It has also been shown that the molecular weight of malate synthase from N.crassa is 59,000 (Desel et al, 1982) and thus clone 1 may code, in part, for either of the enzymes. I intend to sequence a few residues of the purified PEP carboxykinase to compare with the sequence of the subcloned 0.9kb fragment from clone 1. This fragment probably contains the 5′ end of the gene for the following reasons:-

i)The 0.9kb EcoRI-HindIII fragment was also subcloned into the replicative form of the M13 derivatives mp8 and mp9 which have their cloning sites in opposite orientations (Messing & Vieira, 1982). The mp8- and mp9-secreted DNA should therefore carry complementary 0.9kb single strands and this was demonstrated by their ability to anneal together.

DNA from each M13 clone and pGT14 were spotted on to a nitrocellulose filter and hybridized to end labelled mRNA^{a}. Only the pBR322 and the mp8 derivative hybridized to the mRNA (Plate 4.20) and so transcription must proceed rightwards (Figure 4.7) out of the clone.

ii)The neighbouring clone 1 HindIII fragment does not hybridize to the end-labelled message (Figure 4.7). This suggests that the

Plate 4.20; Autoradiograph of hybridization of end-labelled mRNA[a] to: 14- 0.5µg of pGT14 DNA; 9- single stranded DNA from the 0.9kb EcoRI/HindIII fragment described in the text, cloned into M13 derivative mp9; 8- single stranded DNA of the same fragment this time cloned into M13 derivative mp8. M13 DNA was prepared as described in Messing et al (1981).


14
9
8

0.9kb fragment contains the transcription start- point and, unless there is a very long leader sequence or the start- point is far to the right, it should contain the amino- terminal codons.

In all probability the whole gene is not present in this fragment. A 60kd polypeptide of around 600 amino acids needs around 1800bp of coding sequence which cannot all be in 0.9kb.

CONCLUDING DISCUSSION

The genes isolated are unlikely to be the complete set of acetate specific activities. Given the rather biased recovery of clones it would be a good idea to screen another Neurospora genomic library using cDNA probes if possible, to complete the set. The size of the message to which clones 1 & 2 hybridize, is the same (at this level of resolution) and clone one evidently has an EcoRI site inside the gene. These two clones may therefore represent different parts of the same gene. However with PEP carboxykinase and malate synthase being so similar in size it is possible that their messages may be of a similar size. At least part of the transcribed sequence in clone 2 lies in an EcoRI- HindIII fragment. A 'walk' along the genome by screening another library, made with a different enzyme, would help answer this question.

It should be possible to confirm or deny one identity for clone 1 by sequencing the EcoRI-HindIII fragment containing its 5' end (this is already cloned in M13) and comparing it with a partial sequence of the purified PEP carboxykinase enzyme.

It is interesting to note that clone 4 shows some expression during growth on sucrose. Several enzymes would fit this pattern of expression. Many of the TCA cycle enzymes also used by the glyoxylate cycle increase in activity some three to six fold on transfer to acetate (Flavell, 1967). The development of transformation in N.crassa (Case et al, 1979) and the construction of plasmids that can replicate in this organism (Stohl & Lambowitz,

1983) could be useful in the identification of these sequences by complementation of various existing mutations.

The regulation of the clone 1 gene is very strong which makes it ideal for a number of experiments investigating its regulation. For example, *in vitro* mutagenesis of its 5′ sequences coupled with the use of a *Neurospora* vector (Stohl & Lambowitz, 1983) for transformation would allow the regulatory and promoter sequences to be identified. Changes in the surrounding chromatin structure on expression may be expected (see Cartwright *et al*, 1982) and this is something else that can now be investigated.

The demonstration that the *acu7* mutation has a genuine regulatory effect must encourage a more detailed biochemical analysis of this mutant may well help to elucidate the control mechanism of these enzymes which remains somewhat of an enigma.

FINAL COMMENTS

Future approaches to the investigation of the control of the acetate-specific enzymes in Neurospora should logically proceed along two lines. Firstly, more genetic information is needed; in Aspergillus McCullough and Roberts (1980) have successfully isolated mutations which cause constitutive expression of isocitrate lyase activity from a strain deficient in the enzyme pyruvate carboxylase. Such an approach would also be possible in Neurospora by looking for mutations which suppress the effects of the suc mutation (which affect the same enzyme activity) on sucrose medium. These mutants lack their normal anaplerotic route for growth on sucrose (Beever, 1972). Suppression of this mutation should fall into two classes those which are revertants at the suc locus and those which provide an alternative anaplerotic route. Some or all of the latter would be expected to be regulatory mutations which cause constitutivity for the glyoxylate cycle enzymes. There may be many genes which can mutate to cause this phenotype, as is the case in yeast (see Chapter 1) where the genetic regulation of central carbon metabolism has proved very complex. This is presumably due to the amphibolic nature of the pathways which must respond to multiple an diverse 'demands' on its products.

The second approach takes advantage of the

availability of the clones described in this thesis containing sequences differentially transcribed in response to acetate. As discussed in the chapter 1, the specificity of regulation of a gene transcribed by RNA polymerase II usually lies in sequences 5′ to its transcription start-point, and this is presumably also true for these genes. This could be confirmed by, for example, fusing an acetate-specific 5′-region (e.g. that of clone 1) to another cloned Neurospora gene, such as am coding for glutamate dehydrogenase, introducing it by transformation into a suitable strain like the am deletion mutant am-132 (Kinnaird et al, 1982) and looking for a change in its regulation. In this instance on might predict acetate-specific suppression of the am phenotype.

The availability of the clones could also help identify the co-repressor (if there is a unique one). Grove & Marzluf (1981) and Phillipides & Scazzocchio (1981) have shown that DNA affinity columns can be used to demonstrate the existence of DNA-binding proteins, that are probably regulatory effectors. These proteins are eluted from DNA columns by the presumed co-repressor molecules (glutamine for the nit2 product and uric acid for the uaY gene product). A similar sort of experiment using a DNA-affinity column with bound acetate-specific 5′-sequences could be used to look for proteins that were bound or eluted specifically in the presence of

various small molecules that could be involved in glyoxylate cycle repression. Protein blots could also be used in the reverse experiments to bind labelled cloned DNA (Bowen et al, 1980).

Another approach is to try to clone the regulatory genes themselves, and through a host-vector system designed for high expression, attain their products for direct study. Cloning of an unlinked regulatory gene is probably most easily accomplished by complementation of the appropriate regulatory mutation using an homologous cloning vector such as that of Stohl & Lambowitz (1983). If a regulatory mutation were isolated that conferred constitutivity of acetate-gene expression on sucrose, the cloned gene that restored repression might be detectable as conferring resistance to fluoroacetate on sucrose medium (see Armitt et al, 1976).

I hope that the availability of the acetate-specific clones will allow someone in the not-too-distant future to try these or other experiments.

APPENDIX

Enzyme purifications

a)Isocitrate Lyase

The method used was a slightly modified version of Leckie (1969). The change was the replacement of a dialysis step for desalting with passage over a Sephadex G50 column.

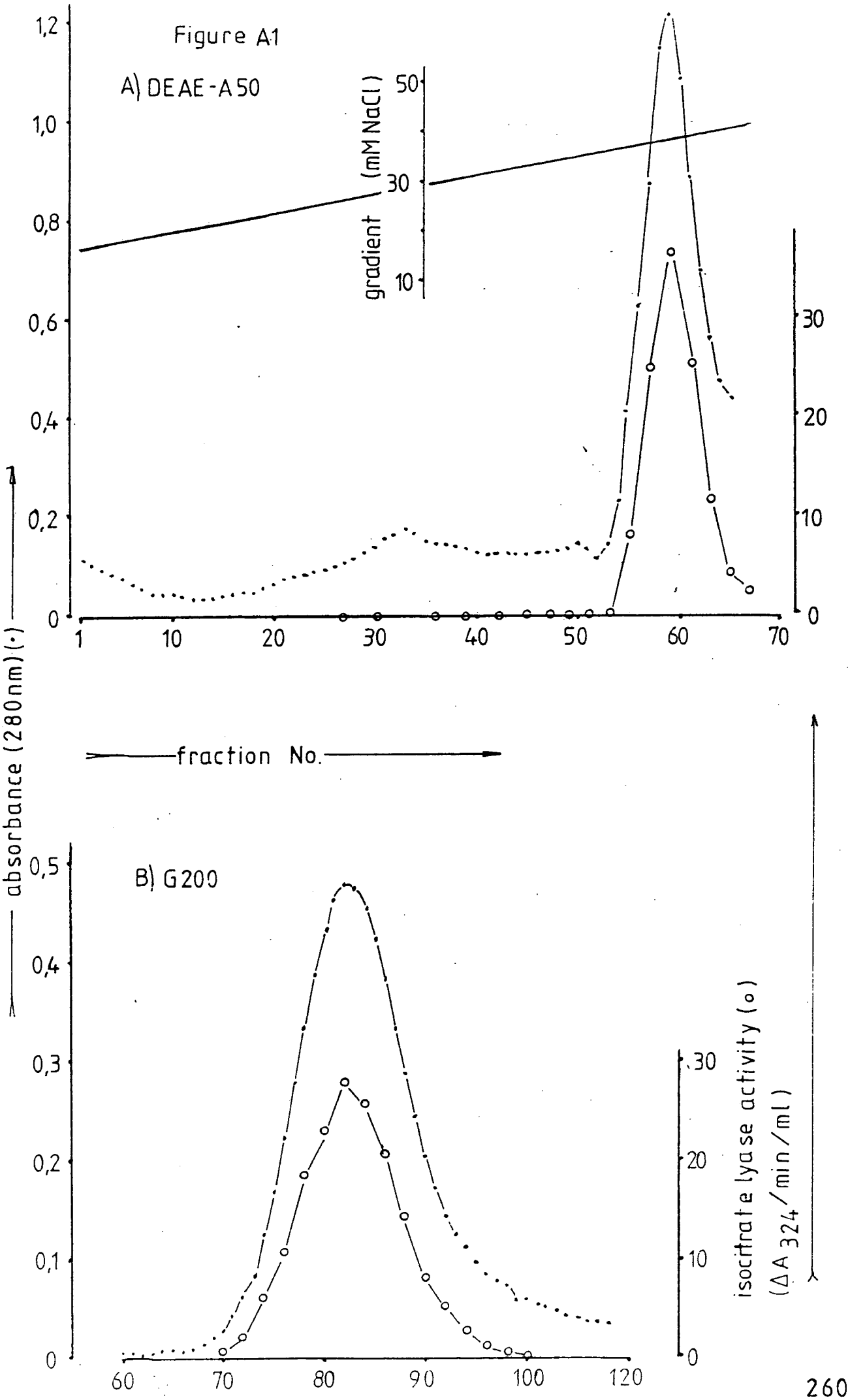
All operations were carried out at 0-4°C. 13.2g of lyophilised mycelium was powdered in an MSE Ato-Mix homogeniser and vortexed twice after the addition of 250ml of Buffer 1 (5mM Tris.Cl pH 6.8- 1mM $MgCl_2$- 1mM EDTA- 0.2mM B-mercaptoethanol). This mixture was centrifuged at 20,000g for 45 minutes after extracting for 15 minutes with stirring. The supernatant was slowly brought to 42% (of saturation) ammonium sulphate, left for 15 minutes with stirring and the precipitate removed by centrifugation as above. Further ammonium sulphate was added to the supernatant to bring it to 62% (of saturation) and the precipitate was collected by centrifugation.

The pellet was resuspended in 25ml of Buffer 1, passed through a Sephadex G50 column to desalt it, and applied to a DEAE Sephadex A50 column. Isocitrate lyase activity was recovered by elution using a linear 1-50mM $MgCl_2$ gradient in Buffer 1 (Figure A.1A), and the pool

Figure A1; A) Elution profile of the Sephadex DEAE-A50 column used in the isocitrate lyase purification. A_{280} of each fraction (.) and isocitrate lyase activity (o) are shown.

B) Elution profile of the Sephadex G200 column used in the isocitrate lyase purification. Symbols are as described in A).

Figure A1


A) DEAE-A50
gradient (mM NaCl)
absorbance (280nm)(•)
fraction No.
B) G200
isocitrate lyase activity (o)
(ΔA 324/min/ml)

precipitated by bringing it to 65% ammonium sulphate. After centrifugation the pellet was resuspended in about 5ml of Buffer 1 and carefully layered on to a Sephadex G200 column.

5.7mg of protein was recovered from the active pool of this final step (Figure A.1B). Plate A.1 shows that the final pool consists of one predominant polypeptide with an apparent molecular weight of 67,000.

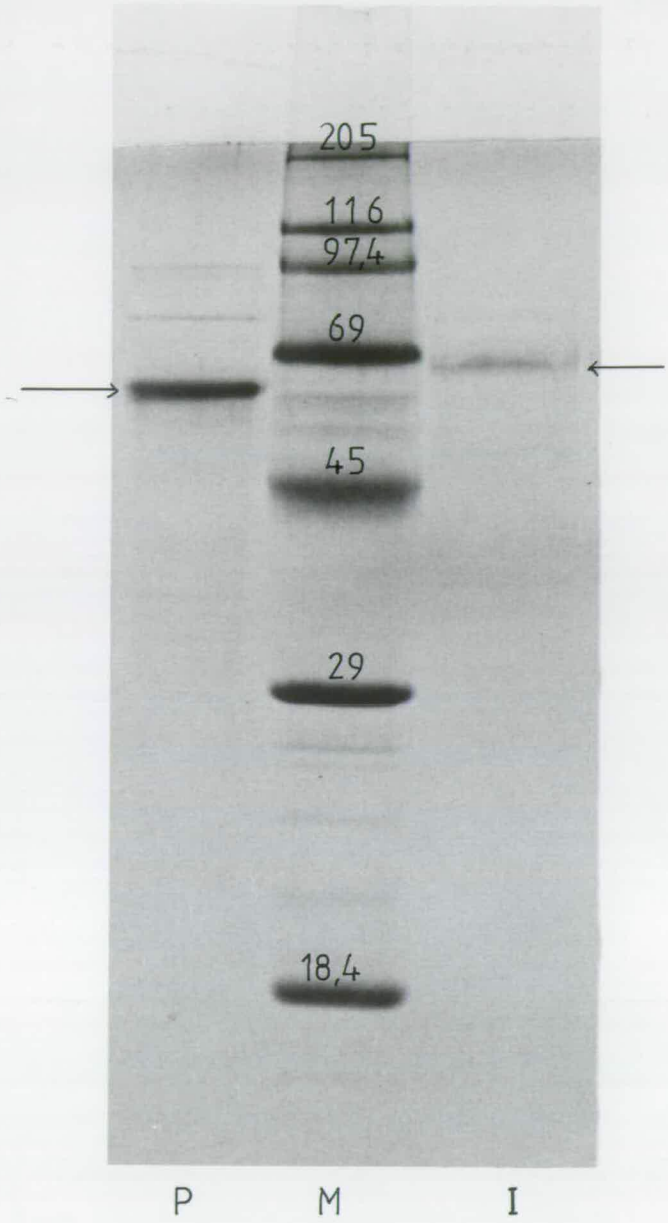
Another purification protocol for this enzyme has been published (Johanson et al, 1974) and this estimate of the molecular weight corresponds with that of Desel et al (1982).

b) PEP Carboxykinase

This method is based on that of Beever (1972) which achieved a partial purification, but is modified by the removal of both the Sephadex G25 column and a DEAE Sephadex A50 step.

A crude extract was prepared by extracting 8.5g of lyophilised induced mycelium in 80ml of Buffer 2 (50mM Tris.Cl pH 7.4- 20mM NaCl- 1mM EDTA- 10mM B-mercaptoethanol) as for the isocitrate lyase procedure. The 50-60% (of saturation) ammonium sulphate fraction was collected again as in the preceding

Plate A1; SDS polyacrylamide gel stained with coomassie blue. P- purified PEP carboxykinase; M- markers as described in the legend to Plate 4.1; I- purified isocitrate lyase.


205
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29
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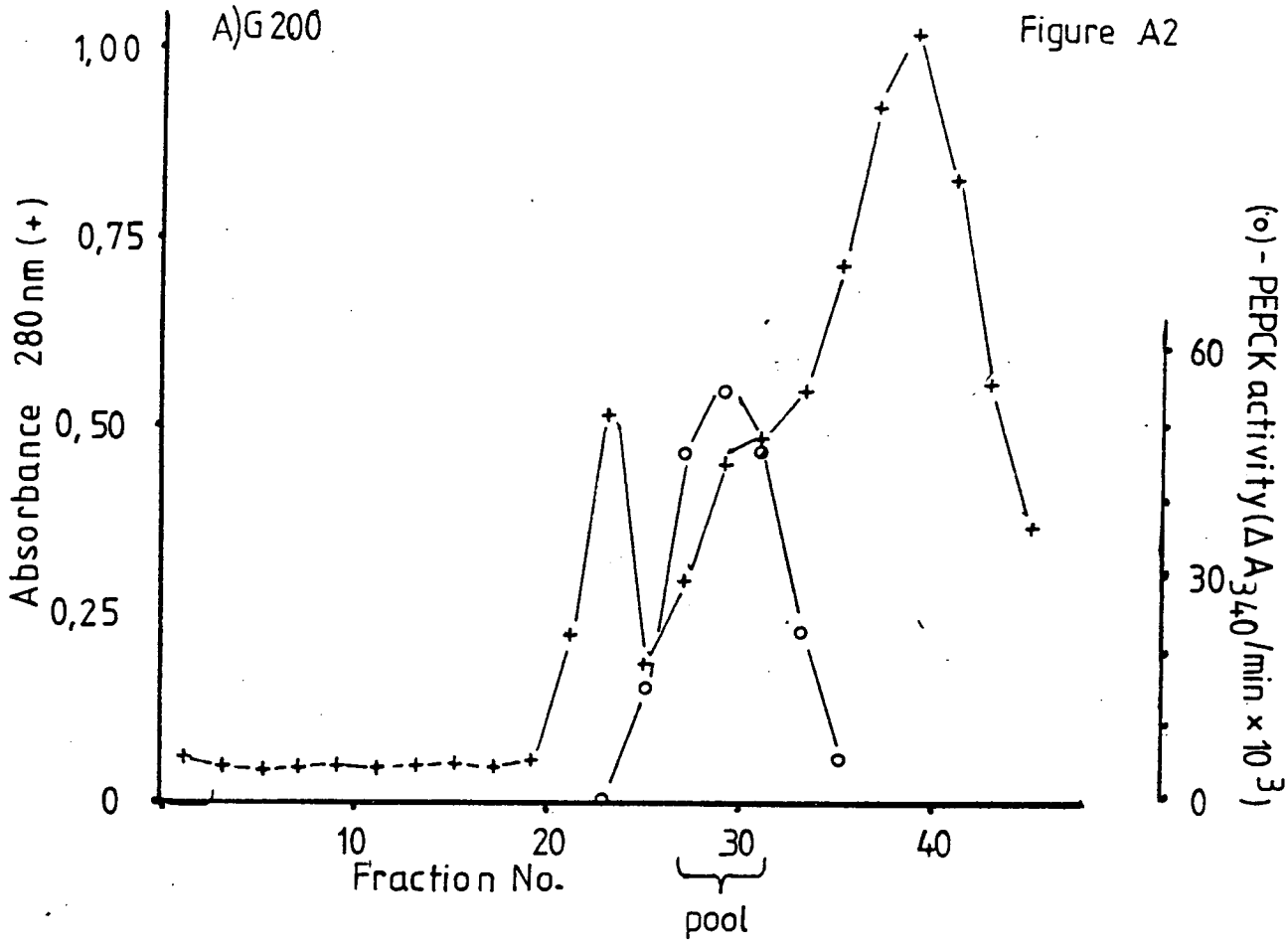
P
M
I

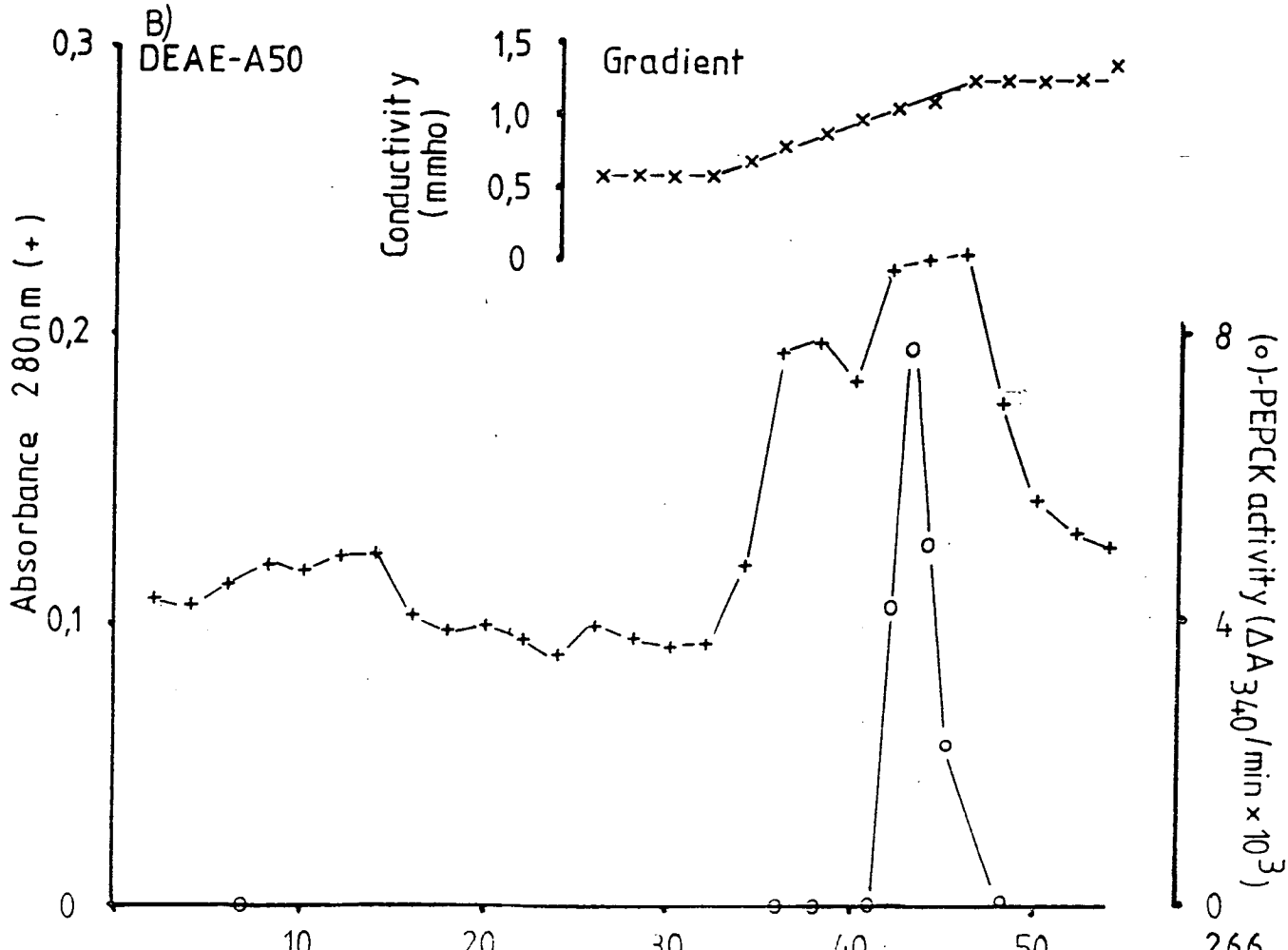
protocol. It was resuspended in about 5ml of Buffer 2 and applied to the top of a Sephadex G200 column equilibrated in the same buffer. The pooled active fractions from this column (Figure A.2A) were applied to a DEAE Sephadex G50 column again pre-equilibrated in Buffer 2 and thoroughly washed. PEP carboxykinase activity was eluted using a linear 20-250mM NaCl gradient (Figure A.2B). Approximately 0.8mg of protein was recovered from the peak fractions. It should be noted that the PEP carboxykinase is measured by a coupled assay and requires excess malate dehydrogenase to be present. During the purification procedure this activity was supplied by a crude extract of sucrose grown mycelium (prepared as described in chapter 2 METHODS) which contained no detectable PEP carboxykinase activity.

Plate A.1 shows that the major product in the final pool is a polypeptide with an apparent molecular weight of 60,000.

Figure A2; A) Elution profile of the Sephadex G200 column used in the PEP carboxykinase purification. A_{280} of each fraction (+) and PEP carboxykinase activity (o) are shown.

B) Elution profile of the Sephadex DEAE-A50 column used in the PEP carboxykinase purification. Symbols are as described in A).


A)G 200
Figure A2
1,00
0,75
0,50
0,25
0
Absorbance 280 nm (+)
60
30
0
(o) - PEPCK activity (ΔA340/min × 10-3)
10
20
30
40
Fraction No.
pool


B)
DEAE-A50
0,3
0,2
0,1
0
Absorbance 280nm (+)
Conductivity (mmho)
1,5
1,0
0,5
0
Gradient
8
4
0
(o)-PEPCK activity (ΔA340/min × 10-3)
10
20
30
40
50
266

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