STUDIES ON CARBON METABOLISM IN WILD TYPE
AND MUTANTS OF *ASPERGILLUS NIDULANS*

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Aan mijn ouders

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Studies on carbon metabolism in wild type and mutants of *Aspergillus nidulans*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
op woensdag 3 november 1982
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

STELLINGEN

I

De bewering dat *Aspergillus nidulans* D-galacturonaat als koolstofbron niet kan gebruiken is onjuist.

Hankinson, O. (1974). J.Bacteriol. 117, 1121-1130.
McCullough, W., Payton, M.A. and Roberts, C.F. (1977) in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London pp. 97-129.
Dit proefschrift.

II

De veronderstelling van Payton dat glycerol door *A.nidulans* op dezelfde wijze wordt afgebroken als in *Escherichia coli* wordt niet bevestigd door zijn enzymassays en werkt remmend op de analyse van mogelijke alternatieven.

Payton, M.A. (1978). Ph.D. Thesis. Univ. Leicester.

III

Het gebruik van de massa als grootheid om een hoeveelheid schimmelsporen aan te geven is onpraktisch.

van Etten, J.L. and Freer, S.N. (1978). Appl.Environ.Microbiol. 35, 622-623.

IV

Om te voorkomen dat mutanten tijdens een verrijkingsprocedure ontsnappen, dient voor alles de chemische zuiverheid van het gebruikte substraat in ogenschouw te worden genomen.

V

De tot nu toe gebruikte procedures zijn niet geschikt om mutanten te isoleren, betrekking hebbend op de overige genen coderend voor enzymen van het pyruvaatdehydrogenase-complex in *A.nidulans*.

Payton, M.A., McCullough, W., Roberts, C.F. and Guest, J.R. (1977).J.Bacteriol. 129, 1222-1226.
Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981).J.Bacteriol. 148, 594-599.
Dit proefschrift.

VI

Het belang van de ligandconcentratie, gebruikt in affiniteitschromatografische procedures,wordt onderschat indien deze concentratie niet wordt vermeld.

VII

De vervanging van Latijnse benamingen op verpakkingen van chemicaliën door een veelvoud van vertalingen in moderne talen, draagt niet bij tot een beter inzicht in de inhoud ervan.

VIII

Bij de toenemende bezuinigingen op het wetenschappelijk onderzoek dreigen labmiddelen vervangen te worden door lapmiddelen.

Proefschrift van J.H.A.A. Uitzetter

Studies on carbon metabolism in wild type and mutants of *Aspergillus nidulans*

Wageningen,3 november 1982

Voorwoord

Terugblikkend op de afgelopen jaren die ik op de afdeling Erfelijkheidsleer heb doorgebracht wil ik op deze plaats graag iedereen bedanken, die op welke wijze dan ook, heeft bijgedragen aan de tot standkoming van dit proefschrift.

Allereerst wil ik mijn promotor, prof. van der Veen, dankzeggen voor de tijdens mijn onderzoek getoonde belangstelling. Vooral in de laatste fase daarvan, tijdens de correctie van het manuscript, heb ik een nuttig gebruik kunnen maken van de waardevolle suggesties die U heeft gegeven.

Mijn bijzondere dank gaat voorts uit naar Jaap Visser. Je hebt gedurende de afgelopen periode meestal met besuisde raadgevingen op enthousiasmerende wijze het onderzoek begeleid, ook buiten de diensturen, en niet alleen op het lab. Ik zal daar met genoegen aan terug denken.

Kees Bos en Marijke Slakhorst, jullie wil ik graag bedanken voor de vooral in de beginfase getrooste inspanningen om mij de nodige kennis bij te brengen over de schimmel die ik als onderzoeksobject heb gebruikt.

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Jan Maassen wil ik bedanken voor zijn technische hulp (in nood) en Mevrouw Selbach zonder wier inspanningen mijn praktische aktiviteiten aanzienlijk minder gemakkelijk zouden zijn verlopen.

Een speciaal woord van dank verdient Mevrouw Schedler-van Mierlo, die op zeer accurate wijze de tot stand koming van het typoscript heeft verzorgd.

Alle medewerkers van de vakgroep Erfelijkheidsleer wik ik bedanken voor het prettig verblijf.

Contents

ABBREVIATIONS

Gene symbols in carbon metabolism are listed on pp. 20-22.
Other gene symbols have their usual meaning (see Clutterbuck, A.J. (1981). Loci and linkage map of *Aspergillus nidulans*. Asp.News Lett. 15, 58-74).

A_{280}	absorbance at 280 nm
ADP	adenosine-5'-diphosphate
APNAD	acetylpyridine nicotinamide adenine dinucleotide
ATP	adenosine-5'-triphosphate
cAMP	cyclic adenosine 3':5'-monophosphate
CM	complete medium
CoA	coenzyme A
EDTA	ethylenediaminetetraacetate
FAD	flavine adenine dinucleotide
FDP	fructose 1,6-diphosphate
FGSC	Fungal Genetics Stock Centre
HPLC	High pressure liquid chromatography
K_i	inhibition constant
K_m	Michaelis constant
L	length
LDH	lactate dehydrogenase
M	molar
min	minute
MM	minimal medium
M_r	relative molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NTG	N-methyl-N'-nitro-N-nitroso guanidine
PC	Phabagen Collection
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
SDS	sodium dodecylsulphate
TPP	thiamine pyrophosphate
UDP	uridine diphosphate
UV	ultra violet (light)
v	volume
w	weight
WG	Wageningen Genetica

GENERAL INTRODUCTION

In this thesis several aspects of carbon metabolism in *Aspergillus nidulans* are studied.

Aspergillus nidulans has been used for several decennia as an eukaryotic model to investigate gene-protein relationships. The possibilities offered by *Aspergilli* as a research object are well illustrated by the genes known thus far in relation to carbon metabolism. These are reviewed in chapter I and a list of mutations is added. *Aspergillus nidulans* has the ability to degrade a wide variety of carbon sources. The differences observed between classes of mutants in their ability to utilize certain carbon sources provide detection criteria preceding genotypic characterization. Moreover suggestions can be given about the possible metabolic routes followed. This opens perspectives for developing specific strategies for the isolation of new mutants in carbon metabolism (chapter II). This is illustrated with mutants unable to use D-galacturonate. Their classification on the basis of the criteria developed in chapter II is described in chapter III.

When isolating glycolytic mutants the majority concerns enzymes catalyzing the synthesis and conversion of pyruvate.

I focussed my attention on two of these enzymes viz: the pyruvate dehydrogenase complex (chapter IV) and pyruvate kinase (chapter V). Affinity chromatographic techniques which are well developed in this laboratory play an important role in the purification of these enzymes.

The purified pyruvate kinase was used to raise antibodies in a rabbit. This forms the basis of chapter VI in which immunological methods are used to characterize pyruvate kinase mutants and wild type under different nutritional conditions.

I General information

I.1. Introduction

Detailed knowledge about microbial catabolic and biosynthetic pathways as well as the regulation of these processes is available, particularly with respect to prokaryotes. Mutants have proven to be very useful to study metabolism and its regulation in these relatively simply structured organisms.
The organisation of the eukaryotic cell is more complicated since compartimentalisation occurs which introduces another dimension to regulation phenomena. These cells are characterised by a membrane surrounded nucleus and possess organelles such as mitochondria and glyoxysomes with specialised metabolic functions and either or not with their own genetic information. Because of this higher complexity less is known about genome structure and organisation and about the processes involved in expression of the genetic information.
Since the early fifties the fungus *Aspergillus nidulans* has been the object used as a model to study gene expression in lower eukaryotes. Genetic aspects of this fungus have been investigated in detail.
As a result of mutation experiments many markers have been found on the eight chromosomes. Via the vegetative as well as the generative cycle recombinants can easily be obtained to study effects of the genetic background on fungal physiology.
In bacterial genomes genes functional in metabolic route are often clustered. These operons interact with other genes responsible for regulation. In eukaryotes genes are normally unlinked and scattered over the genome. Expression of these genes is still strictly regulated although control mechanisms different from those in prokaryotes are necessary. The variation in metabolic control mechanisms on the level of the gene products as known in bacteria is also expected in eukaryotes. Elucidation of the processes involved in eukaryote gene expression is an important reason for studying carbon metabolism in fungi. Besides, it is of practical importance to obtain more fundamental knowledge about metabolism of these fungi considering the fact that *Aspergilli* are used for the production of

metabolites and enzymes.
Micro-organisms used in biotechnological processes deviate from those naturally occurring with respect to their metabolic regulation. Moreover the knowledge of recombination mechanisms of these micro-organisms is generally limited and examples of improvement of the productive capacities otherwise than by mutation are still scarce.

Although *Aspergillus nidulans* itself is not used at this time in biotechnological applications, related *Aspergilli* have been used for a long time especially in food industry. Improvement of citric acid production by mutation in *Aspergillus niger* strains is a well known example.
Due to the detailed knowledge of its genetics, *Aspergillus nidulans* has proven to be a good model to study metabolic diversity of carbon metabolism and its regulation in relation to the genes involved. The key position of phosphoenolpyruvate and pyruvate in the metabolism of various carbon sources were the main reason to focus attention on the identification and characterisation of these genes and their corresponding enzymes involved in converting these metabolites in *A.nidulans*.

I.2. Aspergillus

The name *Aspergillus* was used for the first time by Micheli, a priest, who in 1729 defined the genus *Aspergillus* because of the resemblance of the spore forming structures of these fungi to a holy-water sprinkler (Lat. *aspergillium*). *Aspergilli* show a world wide propagation due to their ability to act as biodeteriorating organisms attacking organic materials and even inorganic compounds (Thomas, 1977). Attention has been focussed on some *Aspergilli* because of their medical importance (Young *et al.*, 1970; Edwards and Al-Zubaidy, 1977; Bennett, 1980) as organisms causing aspergillosis (*A.fumigatus*) and as mycotoxin producers in food (*A.flavus*). *Aspergilli* are also important in the commercial production of organic acids such as citric, gluconic and itaconic acid formed by various strains of *A.niger* and *A.oryzae* (Berry *et al.*, 1977; Jakubowska, 1977) for the production of spirituous liquors *e.g.* sakê with *A.oryzae* and for the industrial production of enzymes both extracellular ones such as amylases, amyloglucosidase, pectinase and proteases and intracellular ones such as glucose oxidase and lactase (Barbesgaard, 1977).

I.3. Aspergillus nidulans

I.3.1. Growth cycle

Aspergillus nidulans (Lat. nestling) is a member of the genus *Aspergillus*

belonging to the *Ascomycetes*, fungi which generally have both a vegetative as well as a generative growth cycle, the latter with spores formed within an ascus. These fungi can be grown in different ways: on a surface forming colonies with radially expanding hyphae which form the mycelium or under certain conditions as multicolonial aggregates in spherical pellets in liquid culture. Hyphae are threadlike structures consisting of multinucleate cells, subdivided at regular intervals by septa (cross-walls) with a pore allowing exchange of nuclei and cytoplasma. Hyphae bear conidiophores with sterigmata ending in chains of uninucleate conidiospores which are normally green. However spore colour mutants are known.

The sequence: hyphae ⟶ conidiospores ⟶ hyphae is called a vegetative growth cycle.

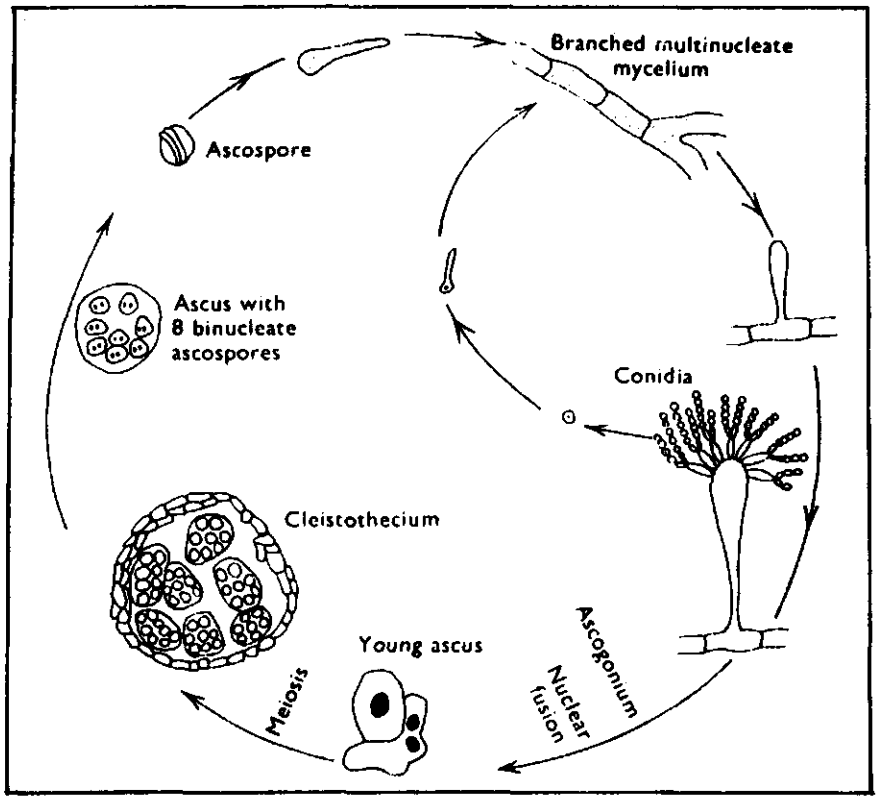

Fig. I.1. Life cycle of *Aspergillus nidulans*.

In the generative (sexual) growth cycle (see Fig. I.1.) the mycelium differentiates into ascogenous hyphae with binuclear cells which develop in an ascocarp (cleistothecium). In the top cells of the branched ascogenous hyphae the nuclei

fuse producing diploid zygotes (karyogamy). The zygote undergoes meiotic and mitotic divisions resulting in eight haploid cells. These are subject to another mitosis producing an ascus with eight binucleate ascospores. In a heterokaryon homokaryotic and heterokaryotic ascogenous hyphae are formed. When the zygotes are heterozygous recombinant ascospores may be obtained. Upon germination ascospores form a mycelium leading to haploid colonies.
Pontecorvo and his coworkers (1953; 1954) discovered processes of recombination which are different from the normal sexual cycle as "alternatives to sex" (Haldane, 1955). The sequence: anastomosis between genetically different hyphae resulting in heterokaryon formation, nuclear fusion leading to diploid formation, mitotic crossing over and haploidisation as a result of non-disjunction has been termed the parasexual cycle (Pontecorvo, 1956).

The result of these events is the recombination of genes as in the meiotic process, but the recombination frequencies are not comparable. Especially in fungi lacking a sexual cycle (*e.g. A.niger*) the parasexual cycle is a powerful tool for the generation of recombinant strains which may be useful for the improvement of industrial strains. In *A.nidulans* the parasexual cycle is experimentally used for the determination of linkage groupsand for the linear arrangement of the genes on a chromosome. Heterozygous diploids are useful to study complementation, especially in cases where this fails in heterokaryons.
A.nidulans is preferred for the use in genetic studies because of the possibility to obtain meiotic recombinants in contrast to *A.niger*.

I.3.2. Mapping

To assign a mutant gene to one of the eight linkage groups present in *A.nidulans* (Käfer, 1958) a master strain with a marker on each chromosome can be used. After diploidisation with the strain carrying the mutation haploidisation can be induced with p-fluorophenylalanine (McCully and Forbes, 1965), benlate (Hastie, 1970) or chloralhydrate (Singh and Sinha, 1976). During this process the chromosomes are reassorted. The linkage group can be assigned because the mutant gene segregates together with the wild type allele of the marker located on the same chromosome besides the possibility of recombination as result of mitotic crossing over which coincides at low frequency. The linear arrangement of the markers on the chromosome with respect to the centromere is determined by mitotic recombination in a heterozygous diploid. The exact localisation of the mutant marker is performed by analysis of meiotic recombinants from a cross between the mutant strain and a tester strain with a number of markers close to the mutant gene on that particular linkage group.

These techniques used in the analysis of mutant genes have been described by Pontecorvo *et al.*(1953) and Clutterbuck (1974). An updated version of the loci and linkage map of *A.nidulans* has recently been published (Clutterbuck, 1981a).

I.3.3. Origin of Aspergillus nidulans strains

All *A.nidulans* strains used in this study were of Glasgow origin or were obtained from these strains by mutagenic treatment. The Glasgow strains are genetically marked strains deposited in a collection built up in Glasgow (U.K.) at the Department of Genetics, University of Glasgow and in the Fungal Genetics Stock Centre, Humbold State University Foundation, Arcata California (U.S.A.). They derive from a single wild isolate FGSC4 (Barratt *et al.*, 1965) first mentioned by Yuill (1939).

I.3.4. Maintenance and preservation of strains

Viable cultures of *A.nidulans* used in this study were maintained on complete medium (CM) according to Pontecorvo *et al.* (1953) with acetate 0.1 M and sucrose 0.01 M instead of glucose for all strains except for pyruvate kinase mutants which were grown on CM with 0.1 M acetate as carbon source. Conidiospores of all strains were stored upon silicagel at 4°C according to Perkins (1962) and are held in stock at the Department of Genetics at the Agricultural University, Wageningen, the Netherlands.

I.3.5. Culturing of A.nidulans wild type

Minimal medium (MM) was used as described by Pontecorvo *et al.* (1953). It consists of 6.0 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 1.5 g KH_2PO_4, traces of $FeSO_4$, $MnCl_2$ and $ZnSO_4$ per litre demineralised water. The medium is adjusted to pH 6.0 with NaOH before autoclaving for 20' at 120°C. Filter sterilised solutions of C-sources were added at a final concentration of 0.05 M or otherwise as mentioned in the text and supplemented if necessary. Complete medium (CM) had the same basal composition as MM to which was added per litre: 2 g neopeptone (Difco); 1 g vitamin assay casaminoacids; 1 g yeast extract; 0.3 g sodium ribonucleate and 2 ml vitamin stock solution. The vitamin stock solution contained in mg per 100 ml: thiamine, 100; riboflavine, 100; p-aminobenzoic acid, 10; nicotinamid, 100; pyridoxin.HCl, 50; panthothenic acid, 10 and biotin, 0.2. Media were solidified with 1.5 % (w/v) agar (Difco).

To obtain large quantities of mycelial biomass for the preparation of cell free extracts from which enzymes were to be isolated, *A.nidulans* was batch cultured in liquid medium. The following procedure was adopted: Conidiospores

suspended in saline (0.8 % NaCl w/v) containing the wetting agent Tween-80 (0.005 %v/v) were inoculated on CM acetate 0.1 M and sucrose 0.01 M. (Sucrose or glucose alone caused more rapidly a wrinkling of the agar surface hindering an easy harvesting of spores.) After two days incubation at 37°C uniformly aged spores (Smith and Anderson, 1973) could be harvested by adding saline/Tween and spores were easily removed. The conidial suspension obtained was thoroughly shaken to break spore chains. Spore concentrations were determined in a haemocytometer. For the batch cultures the procedure was as follows: A preculture was inoculated with approx. 10^7 spores ml^{-1} in 1 litre infusion flasks with 300 ml MM 0.06 M sucrose and incubated horizontally in a home made incubator at 92 movements min^{-1} for 5-6 hours at 37°C. A preculture of 600 ml was used to inoculate 7 l of MM with the same composition in a 10 l flask.
Agitation and aeration was achieved by passing cotton wool filtered air under pressure at the bottom of the medium (approx. 7 l min^{-1}). After a growth period of 15-16 hours at 37°C mycelium was harvested by filtration over muslin, washed extensively with cold saline, squeezed to remove excess of liquid and stored at -20°C until use.

I.3.6. Culturing of A.nidulans mutants

The main difference between culturing *A.nidulans* mutants disturbed in their carbon metabolism and culturing wild type is that conditions have to be chosen that enable mutants to grow and that prevent catabolite repression or inhibition. With a sound knowledge of fungal carbon metabolism it is easy to find a proper medium composed of a digestible C-source or a mixture of C-sources. When inducible enzymes are studied in mutants and in wild type the best way for a biochemical analysis is first to grow these strains in a suitable medium to obtain biomass, and then transfer the mycelium to a medium by which the particular enzyme is induced.

Pyruvate kinase is an example of an enzyme which is only necessary for growth on glycolytic carbon sources (Payton and Roberts, 1976). The activity of this enzyme in acetate grown mycelium is very low in mutants as well as in wild type. Therefore mutants and wild type are first grown on acetate and after washing transferred to a pyruvate kinase inducing C-source *e.g.* sucrose.

I.4. Carbon metabolism in Aspergilli

I.4.1. Transport

Information about uptake systems in fungi is scarce. The problem is that their mycelial mode of growth makes it difficult to obtain uniform samples from

a suspension since within mycelium the cells tend to aggregate and they also differ in age and vary in their physiological state (Romano, 1973).
Studies on transport of carbon sources in *Aspergilli* are mainly confined to *A.nidulans*. This fungus has separate transport systems for D-glucose, D-galactose and D-fructose which are constitutive and carrier mediated (Mark and Romano, 1971). The transport system for D-fructose is highly specific since none of the sugars tested competed with its uptake (Mark and Romano, 1971), such in contrast to the glucose uptake system for which galactose has a low affinity (Romano, 1973). Another specific uptake system was demonstrated for L-sorbose (Elorza and Arst, 1971) by comparing uptake of labeled L-sorbose and D-glucose by wild type and a mutant resistant to sorbose (*sorA*), which grows almost normally on a variety of other carbon sources.
Lactose permease and β-galactosidase, the two proteins involved in lactose utilization, are induced by lactose and galactose (Paszewski *et al.*, 1970). Phosphorylation of hexoses is not required for sugar uptake as mentioned by Brown and Romano (1969), who demonstrated active transport of 2-deoxy-D-glucose against a concentration gradient. They observed high concentrations of intracellular sugar phosphates.
Romano and Kornberg (1968, 1969) showed that in *A.nidulans* the utilization of glucose, fructose, maltose, lactose, galactose, mannose, mannitol, sorbitol and glycerol, but not of sucrose was inhibited by acetate. This inhibition was also observed in a mutant lacking a component of the pyruvate dehydrogenase complex which no longer showed pyruvate formation and excretion when these carbon sources (except sucrose) were added to an acetate grown culture. This provides an indication for the presence of a specific uptake system for sucrose. Acetate itself is probably not the regulating metabolite because a mutant defective in acetyl-CoA synthetase was not inhibited by adding acetate to a glucose or glycerol grown culture. In their study on the regulation of glucose transport in *A.nidulans* Desai and Modi (1977a) showed inhibition of glucose uptake by acetate or pyruvate. Although there were significant variations in glucose uptake rate in normal and biotin deficient cultures (resulting in approx. 20% reduction of glucose uptake), they found a nearly identical intracellular acetate concentration again indicating that acetate itself was not the regulating factor in glucose uptake.
The rate of glucose transport in liquid culture declines with culture age (Kurtz and Champe, 1979). The rate of decline is the same in cultures containing carboxypolymethylene (carbopol), an additive which prevents pellet formation (Elmayergi *et al.*, 1973) although in the latter case the uptake rate is somewhat higher than in the control.

I.4.2. Main metabolic pathways in Aspergilli

The main metabolic pathways such as glycolysis, pentose phosphate pathway (Blumenthal, 1965), tricarboxylic acid cycle (Niederpruem, 1965) and glyoxylate cycle (McCullough and Roberts, 1974) have been reported for fungi. Most of the enzymes present in these metabolic pathways have been reported for *Aspergilli* (McCullough *et al.*, 1977). In Fig. I.2. these pathways are depicted including the names of the corresponding enzymes (pp12,13).

In view of the commercial importance of *Aspergilli* in acid production some aspects of the processes involved in the production of gluconic and citric acid are briefly reported in the following sections.

I.4.3. Gluconic acid production

Gluconic acid is an important product of direct monosaccharide oxidation by glucose oxidase from *A.niger* (Ward, 1967). After harvesting, the mycelium itself can be used as a source of glucose oxidase which is added as colour and flavour stabilizer in drinks and used for medical analysis (Lockwood, 1974). Gluconate is obtained from the culture filtrate as its sodium or calcium salt. Gluconates are used for medical purposes, as mild acidulants in industrial processes and as additives in food.

Gluconate can be metabolised further in *Aspergilli*. Some investigators proposed a phosphorylative degradation through the pentose phosphate pathway (Lakshminarayana *et al.*, 1969) whereas others gave some evidence for a non-phosphorylative pathway by the consecutive action of gluconate dehydrase and 2-keto-3-deoxygluconate aldolase .These reactions are supposed to lead to pyruvate and glyceraldehyde (Elzainy *et al.*, 1973a, b; 1975, 1978).

I.4.4. Citric acid production

Aspergillus niger is the organism most commonly employed in the large scale production of citric acid for use as acidulating agent in food and beverage industries. In spite of the various methods used, detailed public knowledge about the processes involved is scarce. This is not surprising considering the great commercial interests (Lockwood, 1974).

Strains with improved citric acid production can be found after elaboration of mutation-selection programmes (Gardner *et al.*,1956; Das and Nandi, 1969; Das, 1972). Thoma (1971) presented a strain improvement scheme with succesive use of different mutagens. Hannan *et al.* (1976) confirmed their results of stepwise improvement of production although they only used gamma rays. Attempts have been made to improve citric acid production by heterokaryosis and diploidy (Ilczuk,

1971; Das and Roy, 1978).

Some information is available on the analysis of factors important in the production of citric acid. It remains difficult however to come to general conclusions because different citric acid producing strains were object of the many studies.

To obtain a large citric acid production, usually a high yielding strain of *A.niger* is cultured at low pH on a minimal medium containing molasses as carbohydrate source. Two stages can be distinguished according to the physiological state of the mycelium: a phase of growth in which cells proliferate followed by a stationary acidogenic phase when citric acid is accumulating due to exhaustion of the medium (Wold and Suzuki, 1976a). Citric acid accumulation seems to be the result of a disruption of the citric acid cycle, so it is not surprising that several investigators focussed their attention on citric acid cycle enzymes in *A.niger*. Ramakrishnan (1955) found a loss of aconitase and isocitrate dehydrogenase activity, but the presence of these enzymes was clearly demonstrated by La Nauze (1966). Many investigators looked into the effects of trace elements and cofactors on citric acid production. In their studies on the transition of the growth to the acidogenic phase, Wold and Suzuki (1973; 1976a;b) found that Zn^{2+} deficiency causes this transition and that cAMP is a factor regulating physiological activity: stimulating during growth but inhibitory during citric acid accumulation. This is in agreement with the results of Al Obaidy and Berry (1980) who found high cAMP levels during the optimal physiological state for citric acid production. Kubicek and Röhr (1977) found Mn^{2+} deficiency to result in repression of enzymes of the pentose phosphate pathway and of the tricarboxylic acid cycle (except citrate synthase) during acidogenesis. In a later study the same authors (Kubicek and Röhr, 1978) noticed the absence of 2-ketoglutarate dehydrogenase activity and found high levels of oxaloacetate (inhibiting succinate dehydrogenase), fumarate and pyruvate, which suggests that the citric acid cycle was operating partly in both directions. Mn^{2+} deficiency also leads to elevated intra and extracellular amino acid pools in citric acid accumulating *A.niger* strains suggesting that protein synthesis is impaired and membrane permeability is changed (Kubicek *et al.*, 1979). The latter could be due to lower lipid levels affecting membrane structure (Orthofer *et al.*, 1979).

Phosphofructokinase has been mentioned as a key point in regulatory control of citric acid accumulation since this enzyme is inhibited by citrate. This inhibition can be antagonized by elevated levels of NH_4^+ (Habison *et al.*, 1979) emerging from intensified proteolysis or impaired protein synthesis due to

The following enzymes are indicated in Fig. 1.2.

1. hexokinase
2. phosphoglucose isomerase
3. phosphofructokinase
4. fructose-1,6-diphosphate aldolase
5. triose phosphate isomerase
6. glyceraldehyde-3-phosphate dehydrogenase
7. phosphoglycerate kinase
8. phosphoglyceromutase
9. enolase
10. pyruvate kinase
11. pyruvate dehydrogenase complex
12. pyruvate carboxylase
13. citrate synthase
14. aconitase
15. isocitrate dehydrogenase
16. oxoglutarate dehydrogenase complex
17. succinate dehydrogenase
18. fumarate dehydratase
19. malate dehydrogenase
20. fructose-1,6-diphosphatase
21. glucose-6-phosphatase
22. glucose-6-phosphate dehydrogenase
23. 6-phosphogluconate dehydrogenase
24. phosphoketopentoepimerase
25. phosphoriboisomerase
26. transketolase
27. transaldolase
28. alcohol dehydrogenase
29. aldehyde dehydrogenase
30. acetamidase
31. acetyl-CoA synthase
32. malate synthase
33. isocitrate lyase
34. NADP-glutamate dehydrogenase
35. NAD-glutamate dehydrogenase
36. malic enzyme
37. phosphoenolpyruvate carboxykinase
38. galactokinase
39. UDP-galactose pyrophosphorylase
40. UDP-glucose epimerase
41. UDP-glucose pyrophosphorylase
42. phosphoglucomutase
43. mannokinase
44. phosphomannose mutase
45. phosphomannose isomerase
46. mannose dehydrogenase
47. mannitol dehydrogenase
48. sorbitol dehydrogenase
49. mannitol kinase
50. mannitol-1-phosphate phosphatase
51. mannitol-1-phosphate dehydrogenase
52. fructokinase
53. invertase
54. glucose oxidase
55. glycerol kinase
56. glycerol-3-phosphate dehydrogenase

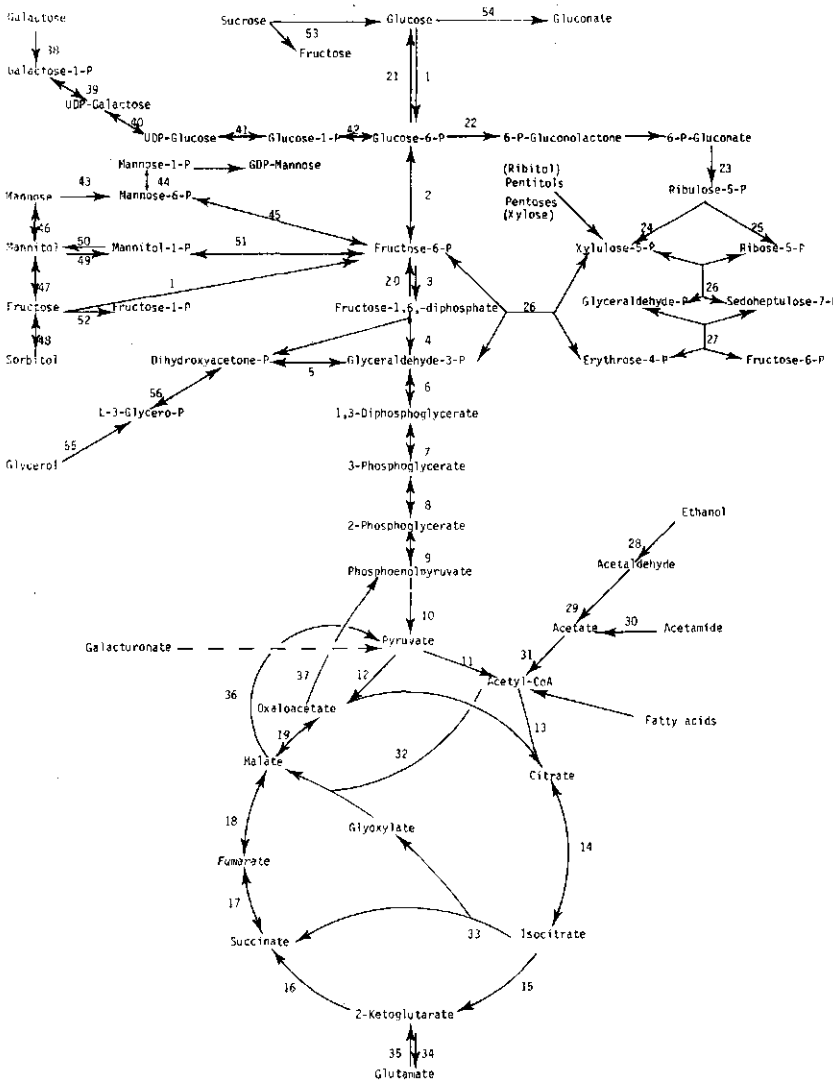

Fig. I.2. Pathways in carbon metabolism of *Aspergilli*. (partly after McCullough *et al.*, 1977) Only main metabolites are shown.

inorganic nutrient limitation (Orthofer *et al.*, 1979). Mattey (1977) mentioned three processes influencing citrate accumulation: 1) the regulation of phosphofructokinase by citrate concentration controlling citrate formation from glycolytic precursors; 2) external pH affecting the reentry of citrate into the cell; 3) the degree of isocitrate dehydrogenase inhibition dependent on the intracellular citrate concentration counteracted by the appearance of Mn^{2+} (Bowes and Mattey, 1979). These investigators found a good correlation between the amount of citric acid produced and the $NADP^+$-isocitrate dehydrogenase inhibition by citrate in different citrate accumulating *A.niger* strains (Bowes and Mattey, 1980).
Although the picture of processes involved is still far from complete the important factors mentioned in the preceding section serve as a good example of the complexity of biochemical processes involved in citric acid accumulation in *A.niger*.

I.4.5. Reserve carbohydrates

Among storage compounds in fungi, trehalose (α (1 $\longrightarrow$3)D-glucosyl-D-glucose) and mannitol are the most abundant. Glycogen as insoluble reserve carbohydrate polymer seems to play a minor role (Cochrane, 1958). Mannitol and trehalose are quickly formed by *Aspergilli* during early growth on glycolytic carbon sources such as glucose (Foster, 1949; Lee, 1967) as well as on gluconeogenic acetate (Barker *et al.*, 1958). Because mannitol is extremely soluble there is a maximum to the intracellular concentration above which this compound is excreted into the culture medium. Once the culture is devoid of sugar the intracellular mannitol level drops quickly. Evidence was given for mannitol as main endogenous carbon source during germination of*A.oryzae* where fructose is formed by NAD^+ or $NADP^+$-linked mannitol dehydrogenase (Horikoshi *et al.*, 1965). They suggested mannitol to be the more important reserve carbohydrate because it competitively inhibited trehalase activity. Two mannitol dehydrogenases were also found in *A.candidus* and even a higher activity was found in extracts of glucose grown mycelium than in the mycelium of a mannitol grown culture (Strandberg, 1969).
The pentose phosphate pathway seems to oppose the metabolism of mannitol. Hankinson and Cove (1975) found in *A.nidulans* a reduced activity of mannitol-1-phosphate dehydrogenase under conditions which stimulated the pentose phosphate pathway enzymes. Because NAD^+-dependent mannitol-1-phosphate dehydrogenase is competing with glucose-6-phosphate isomerase for fructose-6-phosphate, the levels of these enzymes influence the distribution of this compound over glycolysis and pentose phosphate pathway and thus mannitol formation.
Finally Zonneveld (1977) has presented evidence for the involvement of reserve

carbohydrates in fungal differentiation. Only if enough energy is deposited as reserve material differentiation after growth is possible. Zonneveld mentioned $\alpha(1 \longrightarrow 3)$ glucan as an important reserve carbohydrate in hyphal cell walls. This compound is essential for the formation of cleistothecia for he could demonstrate a correlation between the absence of $\alpha(1 \longrightarrow 3)$ glucan and the absence of cleistothecia. The mutants used were isolated before (Clutterbuck, 1969; Zonneveld, 1974).

I.4.6. Effects of substrates

Substrates are known to interact not only on the level of their uptake systems as proven in *A.nidulans* for *e.g.* glucose in combination wich acetate (Romano and Kornberg, 1968, 1969; Desai and Modi, 1977b) but also intracellularly on the level of catabolite repression and enzyme inactivation (Bailey and Arst, 1975; Hynes and Kelly, 1977). Arst and Bailey (1977) classified several carbon sources on the basis of their relative ability to prevent ethanol consumption by *pdhA* strains, which are deficient in pyruvate dehydrogenase complex activity and therefore unable to use glycolytic substrates as sole carbon sources. Sucrose, D-glucose and D-xylose were strongly carbon catabolite repressing; D-mannose, D-galactose, sorbitol, D-fructose and mannitol were intermediate, whilst glycerol, m-inositol, lactose and L-arabinose were strongly derepressing.

Carbon metabolism in *A.nidulans* interacts with other metabolic processes. The relation with nitrogen metabolism is most obvious. It is known that ammonium or nitrate as a source of nitrogen have a different effect on the ratio of glucose used through glycolysis and the pentose phosphate pathway. A higher activity of several enzymes of the pentose phosphate pathway and a lower activity of mannitol-1-phosphate dehydrogenase was observed when nitrate was added to a glucose grown culture with urea as nitrogen source compared to urea alone (Hankinson, 1974; Hankinson and Cove, 1974; 1975).

Evidence was presented that agar can provide a carbon source through C_2 metabolism (Payton *et al.*, 1976; Armitt *et al.*, 1976). Most mutants deficient in acetate metabolism cannot grow on agar as sole carbon source in contrast to wild type *A.nidulans* and glycolytic mutants which grow very slightly.

Isolation and subsequent analysis of mutants altered in carbon catabolite regulation provide a tool to study regulatory processes within carbon metabolism (Arst and Cove, 1973; Bailey and Arst, 1975; Hynes and Kelly, 1977). Because it is beyond the scope of this study to treat nitrogen metabolism in detail the reader is referred to a recent review of nitrate assimilation and its effects on carbon metabolism in *A.nidulans* (Cove, 1979).

I.5. Isolation of mutants

I.5.1. Induction

Although in *A.nidulans* spontaneous mutations occur at a frequency of 10^{-6} - 10^{-7}, both chemical and physical means have been used to enhance the mutation frequency. Especially nitrogen mustards, diethylsulphate, nitrous acid, diepoxybutane and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) have been used as chemicals to induce mutations (Burnett, 1975; Payton, 1978). Among the physical methods UV radiation (mercury-resonance lamps as a source of UV light with 95% of their output confined to the resonance line at 254 nm) (Pontecorvo *et al.*, 1953; Alderson and Hartley, 1969) and X-ray treatment are used (Burnett, 1975; this study). In nearly all cases suspensions of conidiospores are object of mutagenic treatment. It is not preferable to use conditions causing low survival levels because damage in the genetic background can cause considerable difficulties in the genetic analysis of the desired mutants. In this study only X-rays were used to induce mutations.

I.5.2. Selection

Various selection methods can be applied which depend on the particular nature of mutants desired.

Selection techniques often used in the isolation of mutants in *Aspergillus* carbon metabolism are:

1. Resistance of mutants to substrate analogues toxic for non-mutants *e.g.* fluoroacetate resistant mutants unable to utilize acetate as sole carbon source (Apirion, 1965; Armitt *et al.*, 1970, 1976; McCullough and Roberts, 1974).
2. Filtration enrichment. In this technique a suspension of conidiospores is incubated in an "enrichment medium" after a mutagenic treatment. It is essential to use a shake culture, for aeration and to stimulate quick germination and growth of non-mutants but also to prevent heterokaryon formation. After 8-12 hours incubation at 37°C when some growth has become visible, mycelium is filtrated over cotton-wool and a filtrate containing non-germinated spores is obtained. Refreshing the medium in this stage is useful to prevent growth of mutants on metabolites excreted in the medium by growing non-mutants. The spores are concentrated on a membrane filter, resuspended in one tenth of the volume of the medium and incubated generally for another 12-16 hours. When no further growth is observed the culture is again subjected to the filtration procedure. A spore suspension finally obtained this way is plated on CM with a suitable carbon source and replicated upon MM supplemented with carbon sources to discriminate mutants.

The disadvantages of this method are:

- Loss of "leaky" mutants which grow but with lower effectiveness.
- Death of proper mutants due to prolongued starvation conditions. Once a strong indication for a certain mutation has been found in grow tests, the analysis can be taken further by enzymatic assays and complementation in heterokaryons or diploids with similar strains obtained before.

I.5.3. Aspergillus nidulans mutants in carbon metabolism

A complete list including generally accepted gene symbols of *Aspergillus nidulans* mutants thus far found in carbon metabolism is given in Table I.1. Although it is not my intention to discuss these mutants individually it is useful to comment on some general aspects regarding the isolation of such mutants. It is obvious that the mutant types are merely determined by the way they are selected. It is possible to classify these mutants according to some general characteristics they fulfil.

A. Glycolytic mutants were commonly selected after enrichment on a hexose and rescue on a gluconeogenic substrate. Although at first sight the expectation would be a variety of mutants in different steps of glycolysis, the majority however has a deficiency in pyruvate kinase, pyruvate carboxylase or in the pyruvate dehydrogenase complex, enzymes catalyzing nearly irreversible reactions. Why these mutants are found in abundancy, whereas others such as hexokinase and phosphofructokinase mutants are not found is not quite clear.

There are several possible explanations for this:

- Escape of mutants by use of substrate for enrichment contaminated with readily digestible carbon sources.
- The presence of sugar phosphorylating systems of different specificity allowing growth of mutants. Gancedo *et al.* (1977) for instance, showed ability of hexokinase mutants of *Saccharomyces cerevisiae* to grow on glucose due to the presence of different hexokinases.
- The generation of toxic metabolite levels preventing growth of mutants (as explained later in this paragraph).
- Pathways operating at the same time (*e.g.* the pentose phosphate pathway during growth on glucose together with the glycolytic pathway.
- Phosphofructokinase, as a key regulatory enzyme in eukaryotic systems, acting on glycolysis as well as the citric acid cycle. In *E.coli* only phosphofructokinase mutants have been isolated with a residual low activity of this enzyme (Fraenkel and Vinopal, 1973). Maybe futile cycling of fructose-6-phosphate plays a role in carbon metabolism (Katz and Rognstad, 1976).

In contrast to the principle generally used in the isolation of glycolytic mutants in *A.nidulans*, there are two alternatives applied especially for prokaryotic mutants.

a. To use a carbon source for rescue which enters not far behind the metabolic step of interest. If glucose is used for enrichment, mannitol could serve as a permissive carbon source for phosphoglucoisomerase mutants and fructose for phosphofructokinase mutants etc. (Fraenkel and Vinopal, 1973; Bachmann *et al.*, 1976).

b. To use a substrate mixture in the rescue medium containing two carbon sources each one supplying metabolites at a different side of the metabolic block. Such an approach was successful for the isolation of *E.coli* mutants defective in glyceraldehyde-3-phosphate dehydrogenase, glycerate-3-phosphate kinase and enolase. They were maintained on a mixture of glycerol and succinate (Irani and Maitra, 1974).

In the case of *A.nidulans* the latter approach gives rise to problems:

1. Tricarboxylic acid cycle intermediates are bad substrates to allow growth;
2. Substrate mixtures may cause inhibition of uptake of one component or may lead to catabolite repression or inhibition (I.4.6.).

B. Gluconeogenic mutants were mainly obtained by use of the inverse rationale applied to glycolytic mutant isolations or by screening for resistance to a toxic substrate (fluoroacetate). As a result several classes of mutants were discovered in the early steps of acetate assimilation (*facA*), glyoxylate cycle (*acuD,E*), anaplerotic routes (*acuF,K,M*) as well as in a much later step of gluconeogenesis (*acuG*). (For gene symbols and references see Table I.1 pp. 20-22).

C. Mutants in side pathways which are essential for the degradation or certain specific carbon sources. Mutants requiring galactose (*pgmA*) or mannose (*manA, mnrA*) were used to show the essential role of these sugars in the biosynthesis of cell wall components. Mutants unable to utilise galactose (*galA,D*) and comparative studies with wild type *A.nidulans* not only proved the existence of the Leloir pathway for galactose metabolism but also gave evidence for the existence of a second pathway (still not elucidated) operating at alkaline pH.

D. Mutants defective in the citric acid cycle are unknown for *A.nidulans*. Such mutants could be very useful for the elucidation of mechanisms involved in metabolite accumulation.

Only mutants with a reduced inhibition of succinate dehydrogenase activity were found by selecting for carboxin resistance (*cbx*).

E. Mutants with altered regulation of carbon metabolism were isolated by selecting in an *areA* strain, altered in ammonium repression (Hynes, 1975), for the ability to grow on acetamide in the presence of glucose or sucrose. The *areA* mutation

itself caused an inability to grow on a range of compounds as sole nitrogen sources, or to grow on those serving both as C as well as N source in the presence of compounds such as glucose or sucrose causing catabolite repression. Three regulatory genes (*creA,B,C*) were found to be involved in carbon catabolite repression. Some of these mutations have pleiotropic effects such as a hypersensitivity to fluoroacetate, a diminished catabolite repression of acetamidase and alcohol dehydrogenase and elevated activities of extracellular protease and of β-glucosidase.

F. Mutants altered in the way they use compounds which can serve both as C and as N source (e.g. glutamate or acetamide) are useful to study the interference of C and N metabolism. Mutants selected for their sensitivity to high ammonia concentrations appeared to be defective in $NADP^+$-glutamate dehydrogenase (*gdhA*) which has a biosynthetic function. Mutants defective in NAD^+-glutamate dehydrogenase (*gdhB*) are unable to use L-glutamate as sole carbon source. The isolation of a third type of mutant (*gdhC*), which grew better on glutamate than wild type did, made it plausible that NAD^+-glutamate dehydrogenase activity was at least partly regulated by glucose repression because wild type *A.nidulans* has an undetectable level of NAD^+-glutamate dehydrogenase activity when utilising glucose, this in contrast to the *gdhC* mutant.

G. Mutants obtained with the aid of a mutant background.

In bacteria mutants blocked at certain steps in carbon metabolism have been used to isolate mutants with other metabolic lesions especially in such cases where an easy escape could be the cause of unsuccessful experiments. Examples are glucose-6-phosphate dehydrogenase mutants of *E.coli* selected as phosphoglucose isomerase mutants unable to grow on glucose and gluconate-6-phosphate dehydrase mutants (which are gluconate positive) in a 2-keto-3-deoxy-6-phosphogluconate aldolase mutant (a strain which is, in spite of an operative pentose phosphate pathway, gluconate negative, probably due to a toxic KDPG level). Both types of mutants are still uronic acid negative (Fradkin and Fraenkel, 1971; Pouysségur and Stoeber, 1971).

In *A.nidulans pycA* mutants (sucrose negative) were used to select isocitrate lyase constitutive (*iclA,B*) mutants as sucrose positive strains. (McCullough and Roberts, 1980). Experiments in this laboratory to obtain glucose negative mutants in a *pppA* background were unsuccessful, due to extreme cold lability of the conidial suspension. In contrast pyruvate kinase mutants with altered sucrose tolerance were easily isolated as colonies with wild type morphology (conidial color and density) on a mixture of sucrose and acetate (unpublished results).

Table I.1. Mutations in carbon metabolism of *Aspergillus nidulans*

Gene symbol	Metabolic defect	Used mutagen	Selection	Rescue	References
acuD	Isocitrate lyase	UV	Acetate enr.	Sucrose	Armitt *et al.*,1970; 1976
acuE	Malate synthase	UV	Acetate enr.	Sucrose	Armitt *et al.*,1971; 1976
acuF	PEP-carboxykinase	UV	Acetate enr.	Sucrose	Armitt *et al.*,1976
acuG	Fructose 1,6 diphosphatase	UV	Acetate enr.	Sucrose	Armitt *et al.*,1976
acuH,I	Acetate ut.	UV	Acetate enr.	Sucrose	Armitt *et al.*,1976
acuK	Malic enzyme	UV	Acetate enr.	Sucrose	McCullough and Roberts,1974; Armitt *et al.*,1976
acuL	Acetate ut.	UV	Acetate enr.	Sucrose	Armitt *et al.*,1976
acuM	Malic enzyme	UV	Acetate enr.	Sucrose	McCullough and Roberts,1974; Armitt *et al.*,1976
acuN	Acetate ut.	UV	Acetate enr.	Sucrose	Armitt *et al.*,1976
alcA	Alcohol dehydrogenase	a)	a)	a)	Page and Cove,1972
aldA	Aldehyde dehydrogenase	a)	a)	a)	Page and Cove,1972
araA	L-Arabinose ut.	a)	a)	a)	Clutterbuck,1981b
bgaA,B,C	Galactose ut. β-galactosidase	a)	a)	a)	Fantes and Roberts,1973
bguA	Benzoate ut.	a)	a)	a)	Hynes and Kelly,1981
cbxA,B,C	Succinate oxidation	NTG	Carboxin res.		Van Tuyl,1975; Gunatilleke *et al.*, 1976
creA	Carbon catabolite repression	NTG	See I.5.3. E		Arst and Cove,1973
creA	Carbon catabolite repression	NTG	*pdhA* Ethanol+sucrose pos.		Bailey and Arst, 1975
creB,C	Carbon catabolite repression	NTG	See I.5.3. E		Hynes and Kelly,1977
facA	Acetyl-CoA synthethase	None	Fluoroacetate res.		Apirion,1965; Armitt *et al.*, 1976
facB,C	Acetate ut.	None	Fluoroacetate res.		Apirion,1965; Armitt *et al* , 1976
fanA,B,C *fanD,E*	Effect not specified	None	Fluoroacetate res.		Apirion,1965

Table I.1. Continued

frA	Fructokinase	UV	None	Glucose	Roberts,1963a; Mark and Romano, 1971
gaaA	Galacturonate ut.	X	Galacturonate enr.	Acetate	Uitzetter, 1982
galA	Galactokinase and galactose-1-P uridyl transferase reg.	UV	None	Glucose	Roberts,1963b
galB	Galactose and arabinose ut.	UV	None	Glucose	Roberts,1963b
galC	Galactose ut.	UV	None	Glucose	Roberts,1963b
galD	Galactose-1-P uridyl transferase	UV	None	Glucose	Roberts,1963b
gamA,B,C	Galactose ut.	None	Molybdate res.		Arst and Cove,1970
gdhA	$NADP^+$-glutamate dehydrogenase	NTG	NH_4 sens.		Kinghorn and Pateman,1973
gdhB	NAD^+-glutamate dehydrogenase	NTG	Glutamate neg.		Kinghorn and Pateman,1973
gdhC	NAD^+-glutamate dehydrogenase reg.	NTG	See I.5.3. F		Kinghorn and Pateman,1974
glcA,B	Glycerol ut.	UV or NTG	Glycerol enr.	Acetate	Payton,1978; McCullough *et al.*, 1977
glcB	Glycerol ut.	X	Galacturonate enr.	Acetate	Uitzetter, 1982
glrA,B,C,D glrE,F,G	D-Glucuronate ut.	a)	a)	a)	Hynes and Kelly,1981
icLA,B	Isocitrate lyase reg.	NTG	See I.5.3. G		McCullough and Roberts, 1980
lacA,B	Lactose ut.	UV	None	Glucose	Roberts,1963a
lacC,D	β-Galactosidase	a)	a)	a)	Gajewski *et al.*,1972; Fantes and Roberts, 1973
lacE,F	Lactose ut.	a)	a)	a)	Gajewski *et al.*,1972; Fantes and Roberts, 1973
lacG,H,I	β-Galactosidase	a)	a)	a)	Gajewski *et al.*,1972; Fantes and Roberts, 1973

a) Not mentioned in literature wt = wild type enr.= enrichment
ut.= utilisation pos.= positive neg.= negative res.= resistance reg.= regulation sens.= sensitivity

Table I.1. *Continued*

Gene symbol	Metabolic defect	Used mutagen	Selection	Rescue	References
malA	Maltose ut.	UV	None	Glucose	Roberts,1963a
manA	Phosphomannose isomerase	a)	a)	a)	Valentine and Bainbridge,1978
mmrA	Phosphomannose mutase	a)	a)	a)	Valentine and Bainbridge,1978
pdhA,B	Pyruvate dehydrogenase complex	NTG	Sucrose enr.	Acetate	Payton *et al.*,1977.
pdhA,B,C	Pyruvate dehydrogenase complex	UV	Glucose enr.	Acetate	Bos *et al.*,1981
pdhA,B,C	Pyruvate dehydrogenase complex	X	Galacturonate enr.	Acetate	Uitzetter, 1982
pgmA	Phosphoglucomutase	UV	Glucose enr.	Galacto-se + Acetate	Boschloo and Roberts,1979
pkiA	Pyruvate kinase	NTG or UV	Hexose enr.	Acetate	Payton and Roberts,1976; Payton, 1978
pkiA	Pyruvate kinase	UV	Glucose enr.	Acetate	Bos *et al.*,1981
pppA	Pentose phosphate pathway, transaldolase	NTG	None	NH_4^+ Glucose	Hankinson,1974
pppB	Pentose phosphate pathway	NTG	None	NH_4^+ Glucose	Hankinson ,1974
pycA	Pyruvate carboxylase	NTG	None	Acetate	Skinner and Armitt,1972
pycA,B	Pyruvate carboxylase	UV	Glucose enr.	Acetate	Bos *et al.*,1981
pycA,B	Pyruvate carboxylase	X	Galacturonate enr.	Acetate	Uitzetter, 1982
sbA	Sorbitol ut.	UV	None	Glucose	Roberts,1963a
sorA	Sorbose uptake	None	Sorbose res.	-	Elorza and Arst,1971
sorB	Phosphoglucomutase	None	Sorbose res.	-	Elorza and Arst,1971

a) Not mentioned in literature
enr.= enrichment
ut.= utilization pos.= positive neg.= negative res.= resistance reg.= regulation sens.= sensitivity

REFERENCES

Alderson, T. and Hartley, M.J. (1969). Specificity for spontaneous and induced forward mutations at several gene loci in *Aspergillus nidulans*. Mutation Res. 8, 255-264.

Al Obaidy, Z.S. and Berry, D.R. (1980).cAMP concentration, morphological differentiation and citric acid production in *Aspergillus niger*. Biotechnol. Lett. 2, 5-10.

Apirion, D. (1965). The two-way selection of mutants and revertants in respect of acetate utilization and resistance to fluoro-acetate in *Aspergillus nidulans*. Genet.Res.Camb. 6, 317-329.

Armitt, S., McCullough, W. and Roberts, C.F. (1976). Analysis of acetate non-utilizing (*acu*)mutants in *Aspergillus nidulans*. J.Gen.Microbiol. 92, 263-282.

Armitt, S., Roberts, C.F. and Kornberg, H.L. (1970). The role of isocritrate lyase in *Aspergillus nidulans*. FEBS Lett. 7, 231-234.

Armitt, S., Roberts, C.F. and Kornberg, H.L. (1971). Mutants of *Aspergillus nidulans* lacking malate synthase. FEBS Lett. 12, 276-278.

Arst, H.N. and Bailey, C.R. (1977). The regulation of carbon metabolism in *Aspergillus nidulans*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 131-146.

Arst, H.N. and Cove, D.J. (1970). Molybdate metabolism in *Aspergillus nidulans* II. Mutations affecting phosphates activity or galactose utilization. Molec.Gen. Genet.108, 146-153.

Arst, H.N. and Cove, D.J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. Molec.Gen.Genet. 126, 111-141.

Bachmann, B.J., Low, K.B. and Taylor, A.L. (1976). Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40, 116-167.

Bailey, C. and Arst H.N. (1975). Carbon catabolite repression in *Aspergillus nidulans*. Eur.J.Biochem. 51, 573-577.

Barbesgaard, P. (1977). Industrial enzymes produced by members of the genus *Aspergillus*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*.Academic Press, London, pp. 391-404.

Barker, S.A., Gómez-Sanchez, A. and Stacey, M. (1958). Studies of *Aspergillus niger*. X. Polyol and disaccharide production from acetate. J.Chem.Soc. 1958, 2583-2586.

Barratt, R.W., Johnson, G.B. and Ogata, W.N. (1965). Wild-type and mutant stocks of *Aspergillus nidulans*. Genetics 52, 233-246.

Bennett, J.E. (1980). Aspergillosis. in: Thorn, G.W. *et al.* Harrisons Principles of

internal medicine. 9th edition. McGraw Hill, New York, pp. 1210-1214.
Berry, D.R., Chmiel, A. and Al Obaidy, Z. (1977). Citric acid production by *Aspergillus niger*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 405-426.
Blumenthal, H.J. (1965). Glycolysis. in: Ainsworth, G.C. and Sussman, A.S. (eds.) The Fungi. Vol. I. Academic Press, London, pp. 229-268.
Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.
Boschloo, J.G. and Roberts, C.F. (1979). D-galactose requiring mutants in *Aspergillus nidulans* lacking phosphoglucomutase. FEBS Lett. 104, 17-20.
Bowes, I. and Mattey, M. (1979). The effect of manganese and magnesium ions on mitochondrial $NADP^+$-dependent isocitrate dehydrogenase from *Aspergillus niger*. FEMS Microbiol. Lett. 6, 219-222.
Bowes, I. and Mattey, M. (1980). A study of mitochondrial $NADP^+$-specific isocitrate dehydrogenase from selected strains of *Aspergillus niger*. FEMS Microbiol. Lett. 7, 323-325.
Brown, C.E. and Romano, A.H. (1969). Evidence against necessary phosphorylation during hexose transport in *Aspergillus nidulans*. J.Bacteriol. 100, 1198-1203.
Burnett, J.H. (ed.) (1975). Mycogenetics. An introduction to the general genetics of fungi. John Wiley and Sons, London, New York.
Clutterbuck, A.J. (1969). A mutational analysis of conidia development in *Aspergillus nidulans*. Genetics 63, 317-327.
Clutterbuck, A.J. (1974). *Aspergillus nidulans*. in: King, R.C. (ed.) Handbook of Genetics. Vol. I. Plenum Press, New York, pp. 447-510.
Clutterbuck, A.J. (1981a). Loci and linkage map of *Aspergillus nidulans*. Asp. News Lett. 15, 58-72.
Clutterbuck, A.J. (1981b). An arabinose non-utilizing mutant *araA1*. Asp. News. Lett. 15, 21.
Cochrane, V.W. (ed.) (1958). Physiology of fungi. John Wiley, New York.
Cove, D.J. (1979). Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol.Rev. 54, 291-327.
Das, A. (1972). Strain selection in citric acid fermentation. A review. Current Science 41, 593-596.
Das, A. and Nandi, P. (1969). Improved production of citric acid from *Aspergillus niger* by mutagenic treatment. Ind.J.Exp.Biol. 7, 275-276.
Das, A. and Roy, P. (1978). Improved production of citric acid by a diploid strain of *Aspergillus niger*. Can.J.Microbiol. 24, 622-625.

Desai, J.D. and Modi, V.V. (1977a). Growth, glucose metabolism and melanine formation in biotine deficient *Aspergillus nidulans*. Folia Microbiol. 22, 55-60.

Desai, J.D. and Modi, V.V. (1977b). Regulation of glucose transport in *Aspergillus nidulans*. Experientia 33, 726-727.

Edwards, J.H. and Al-Zubaidy, T.S. (1977). Medical aspects of *Aspergillus*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, Londen, pp. 525-538.

Elmayergi, H.,Scharer, J.M. and Moo-Young, M. (1973). Effects of polymer additives on fermentation parameters in a culture of *Aspergillus niger*. Biotechnol.Bioeng. 15, 845-859.

Elorza, M.V. and Arst, H.N. (1971). Sorbose resistant mutants of *Aspergillus nidulans*. Molec.Gen.Genet. 111, 185-193.

Elzainy, T.A., Hassan, M.M. and Allam, A.M. (1973a). Occurence of the non-phosphorylative pathway for gluconate degradation in different fungi. Biochem. Systematics 1, 127-128.

Elzainy, T.A., Hassan, M.M. and Allam, A.M. (1973b). New pathway for non phosphorylated degradation of gluconate by *Aspergillus niger*. J.Bacteriol.114, 457-459.

Elzainy, T.A. Hassan, M.M. and Allam, A.M. (1975). Fate of pyruvate formed non phosphorolytically from gluconate by extracts of *Aspergillus niger*. Zentralbl. Bakteriol.Parasit.Infektionskr.Hyg. Zweite Naturw.Abt. 130, 201-205.

Fantes, P.A. and Roberts, C.F. (1973). β-Galactosidase activity and lactose utilization in *Aspergillus nidulans*. J.Gen.Microbiol. 77, 471-486.

Foster, J.W. (ed.) (1949). Chemical activities of fungi. Academic Press, New York.

Fradkin, J.E. and Fraenkel, D.G. (1971). 2-Keto-3-deoxygluconate 6-phosphate aldolase mutants of *Escherichia coli*. J.Bacteriol. 108, 1277-1283.

Fraenkel, D.G. and Vinopal, R.T. (1973). Carbohydrate metabolism in bacteria. Ann. Rev.Microbiol. 27, 69-100.

Gajewski, W. Litwinska, J., Paszewski, A. and Chojnacki, T. (1972). Isolation and characterization of lactose non-utilizing mutants in *Aspergillus nidulans*. Molec.Gen.Genet. 116, 99-106.

Gancedo, J.M. Clifton, D. and Fraenkel, D.G. (1977). Yeast hexokinase mutants. J.Biol.Chem. 252, 4443-4444.

Gardner, J.F., James, L.V. and Rubbo, S.D. (1956). Production of citric acid by mutants of *Aspergillus niger*. J.Gen.Microbiol. 14, 228-237.

Gunatilleke, I.A.V.N., Arst Jr., H.N. and Scazzocchio, C. (1976). Three genes

determine the carboxin sensitivity of mitochondrial succinate oxidation in *Aspergillus nidulans*.Genet.Res.Camb. 26, 297-305.

Habison, A. Kubicek, C.P. and Röhr, M. (1979). Phosphofructokinase as a regulatory enzyme in citric acid producing *Aspergillus niger*. FEMS Microbiol.Lett. 5, 39-42.

Haldane, J.B.S. (1955). Some alternatives to sex. New Biol. 19, 7-26.

Hankinson, O. (1974). Mutants of the pentose phosphate pathway in *Aspergillus nidulans*. J.Bacteriol. 117, 1121-1130.

Hankinson, O. and Cove, D.J. (1974). Regulation of the pentose phosphate pathway in the fungus *Aspergillus nidulans*. J.Biol.Chem. 249, 2344-2353.

Hankinson, O. and Cove, D.J. (1975). Regulation of mannitol-1-phosphate dehydrogenase in *Aspergillus nidulans*. Can.J.Microbiol. 21, 99-101.

Hannan, M.A., Sarwar, M.G., Baten, A. and Chaudhury, N. (1976). Stepwise mutational improvement of *Aspergillus niger* for citric acid productivity in cane molasses. Folia Microbiol. 21, 409-412.

Hastie, A.C. (1970). Benlate-induced instability of *Aspergillus* diploids. Nature 226, 771.

Horikoshi, K., Iida, S. and Ikeda, Y. (1965). Mannitol and mannitol dehydrogenase in conidia of *Aspergillus oryzae*. J.Bacteriol. 80, 326-330.

Hynes, M.J. (1975). Studies on the role of the *areA* gene in the regulation of nitrogen catabolism in *Aspergillus nidulans*. Austr.J.Biol.Sci. 28, 301-313.

Hynes, M.J. and Kelly, J.M. (1977). Pleiotropic mutants of *Aspergillus nidulans* altered in carbon metabolism. Molec.Gen.Genet. 150, 193-204.

Hynes, M.J. and Kelly, J.M. (1981). Threonine, quinate and glucuronic acid utilization mutants of *Aspergillus nidulans*. Asp.News Lett. 15, 18-21.

Ilczuk, Z. (1971). Genetik der Citronensäure erzeugende Stämme *Aspergillus niger* 4. Mitt. Citronensäuresynthese durch heterozygotische Diploide von *A.niger*. Die Nahrung 15, 381-388.

Irani, M. and Maitra, P.K. (1974). Isolation and characterization of *Escherichia coli* mutants defective in enzymes of glycolysis. Biochem.Biophys. Res.Comm. 56, 127-133.

Jakubowska, J. (1977). Itaconic and itatartaric acid biosynthesis. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 427-451.

Käfer, E. (1958). An 8-chromosome map of *Aspergillus nidulans*. Adv.Genet. 9, 105-145.

Katz, J. and Rognstad, R. (1976). Futile cycles in the metabolism of glucose. in: Current topics in cellular regulation. Vol. 10, pp. 237-289.

Kinghorn, J.R. and Pateman, J.A. (1973). NAD and NADP L-glutamate dehydrogenase activity and ammonium regulation in *Aspergillus nidulans*. J.Gen.Microbiol. 78, 39-46.

Kinghorn, J.R. and Pateman, J.A. (1974). The regulation of NAD L-glutamate dehydrogenase in *Aspergillus nidulans*.Genet.Res.Camb. 23, 119-124.

Kubicek, C.P. and Röhr, M. (1977). Influence of manganese on enzyme synthesis and citric acid accumulation in *Aspergillus niger*. Eur.J.Appl.Microbiol. Biotechnol. 4, 167-175.

Kubicek, C.P. and Röhr, M. (1978). The role of the tricarboxylic acid cycle in citric acid accumulation by *Aspergillus niger*. Eur.J.Appl.Microbiol.Biotechnol. 5, 263-271.

Kubicek, Z.P., Zehentgruber, O. and Röhr, M. (1979). An indirect method for studying the fine control of citric acid formation by *Aspergillus niger*. Biotechnol.Lett. 1, 47-52.

Kurtz, M.B. and Champe, S.P. (1979). Genetic control of transport loss during development of *Aspergillus nidulans*. Developm.Biol. 70, 82-88.

Lakshminayarana, K., Modi, V.V. and Shah, V.K. (1969). Studies on gluconate metabolism in *Aspergillus niger*. II. Comparative studies on the enzyme make-up of the adapted and parent strains of *Aspergillus niger*. Archiv f.Mikrobiol. 66, 396-405.

La Nauze, I.M. (1966). Aconitase and isocitric dehydrogenase of *Aspergillus niger* in relation to citric acid production. J.Gen.Microbiol. 44, 73-81.

Lee, W.H. (1967). Carbon balance of a mannitol fermentation and the biosynthetic pathway. Appl.Microbiol. 15, 1206-1210.

Lockwood, L.B. (1974). Organic acid production. in: Smith, J.E. and Berry, D.E. The filamentous fungi Vol. I. Edward Arnold, London, pp. 140-157.

Mark, C.G. and Romano, A.H. (1971). Properties of the hexose transport systems of *Aspergillus nidulans*. Biochim.Biophys.Acta 249, 216-226.

Mattey, M. (1977). Citrate regulation of citric acid production in *Aspergillus niger*. FEMS Microbiol. Lett. 2, 71-74.

McCullough, W. Payton, M.A. and Roberts, C.F. (1977). Carbon metabolism in *Aspergillus nidulans*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 97-129.

McCullough, W. and Roberts, C.F. (1974). The role of malic enzyme in *Aspergillus nidulans*. FEBS Lett. 41, 238-242.

McCullough, W. and Roberts, C.F. (1980). Genetic regulation of isocitrate lyase activity in *Aspergillus nidulans*. J.Gen.Microbiol. 120, 67-84.

McCully, K. and Forbes, E. (1965). The use of p-fluorophenylalanine with

'master strains' of *Aspergillus nidulans* for assigning genes to linkage groups. Gen.Res.Camb. 6, 352-359.

Micheli, P.A. (1729). Nova plantarum genera iuxta Tournefortii methodium disposita Illus Florence.

Niederpruem, D.J. (1965). Tricarboxylic acid cycle. in: Ainsworth, G.C. and Sussman, A.S. (eds.) The Fungi Vol. I. Academic Press, New York, pp. 269-300.

Orthofer, R., Kubicek, C.P. and Röhr, M. (1979). Lipid levels and manganese deficiency in citric acid producing strains of *Aspergillus niger*. FEMS Microbiol. Lett. 5, 403-406.

Page, M.M. and Cove, D.J. (1972). Alcohol and amine catabolism in the fungus *Aspergillus nidulans*. Biochem.J. 127, 17 p.

Paszewski, A., Chojnacki, T, Litwinska, J. and Gajewski, W. (1970). Regulation of lactose utilization in *Aspergillus nidulans*. Acta Biochim.Pol. 17, 385-391.

Payton, M.A. (1978). A genetic study of sugar metabolism and transport in *Aspergillus nidulans*. Ph.D.Thesis Univ.Leicester.

Payton, M.A., McCullough, W. and Roberts, C.F. (1976). Agar as a carbon source and its effect on the utilization of other carbon sources by acetate non-utilizing *(acu)* mutants of *Aspergillus nidulans*. J.Gen.Microbiol. 94, 228-233.

Payton, M.A., McCullough, W., Roberts, C.F. and Guest, J.R. (1977). Two unlinked genes for the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 129, 1222-1226.

Payton, M. and Roberts, C.F. (1976). Mutants of *Aspergillus nidulans* lacking pyruvate kinase. FEBS Lett. 66, 73-76.

Perkins, D.D. (1962). Preservation of *Neurospora* stock cultures with dehydrous silica gel. Can.J.Microbiol. 8, 591-594.

Pontecorvo, G. (1954). Mitotic recombination in the genetic systems of filamentous fungi. Caryologia (suppl.) 6, 192-200.

Pontecorvo, G. (1956). The parasexual cycle in fungi. Ann.Rev.Microbiol. 10, 393-400.

Pontecorvo, G., Roper, J.A., Hemmons, L.J., MacDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans*. Adv.Genet. 5, 141-238.

Pouysségur, J.M. and Stoeber, F.R. (1971). Etude de rameau dégradatif commun des hexuronates chez *E.coli* K 12. Purification, proprietés et individualité de la 2-ceto-3-désoxy-6-phosphogluconate aldolase. Eur.J.Biochem. 21, 363-373.

Ramakrishnan, C.V., Steel, R. and Lentz, C.P. (1955). Mechanism of citric acid formation and accumulation in *Aspergillus niger*. Arch.Biochem.Biophys. 55, 270-273.

Roberts, C.F. (1963a). The genetic analysis of carbohydrate utilization in

Aspergillus nidulans. J.Gen.Microbiol. 31, 45-58.

Roberts, C.F. (1963b). The adaptive metabolism of D-galactose in *Aspergillus nidulans*. J.Gen.Microbiol. 31, 285-295.

Romano, A.H. (1973). Properties of sugar transport systems in *A.nidulans* and their regulation. in: Vanek, Z, Hostalek, Z. and Cudling, J. (eds.) Genetics of industrial micro organisms. Vol. II. Elsevier, Amsterdam, pp. 195-212.

Romano, A.H. and Kornberg, H.L. (1968). Regulation of sugar utilisation by *Aspergillus nidulans*. Biochim.Biophys.Acta 158, 491-493.

Romano, A.H. and Kornberg, H.L. (1969). Regulation of sugar uptake by *Aspergillus nidulans*. Proc.Roy.Soc.B. 173, 475-490.

Singh, M. and Sinha, U. (1976). Chloral hydrate induced haploidization in *Aspergillus nidulans*. Experientia 32, 1144-1145.

Skinner, V.M. and Armitt, S. (1972). Mutants of *Aspergillus nidulans* lacking pyruvate carboxylase. FEBS Lett. 20, 16-18.

Smith, J.E. and Anderson, J.G. (1973). Differentiation in the *Aspergilli*. in: Ashworth, J.M. and Smith, J.E. (eds.) Microbial differentiation. 23th Symposium of the Society for General Micriobiology. Cambridge University Press, pp. 295-337.

Strandberg, G.W. (1969). D-mannitol metabolism by *Aspergillus candidus*. J.Bacteriol. 97, 1305-1309.

Thoma, R.W. (1971). Use of mutagens in the improvement of production strains of micro-organisms. Folia Microbiol. 16, 197.

Thomas, A.R. (1977). The genus *Aspergillus* in biodeterioration. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 453-479.

Uitzetter, J.H.A.A. (1982). This thesis, Chapter III.

Valentine, B.P. and Bainbridge, B.W. (1978). The relevance of a study of a temperature-sensitive ballooning mutant of *Aspergillus nidulans* defective in mannose metabolism to our understanding of mannose as a wall component and carbon/energy source. J.Gen.Microbiol. 109, 155-168.

Van Tuyl, J.M. (1975). Acquired resistance to carboxin in *Aspergillus nidulans*. Neth.J.Pl.Pathol. 81, 122-123.

Ward, G.E. (1967). Production of gluconic acid, glucose oxidase, fructose and sorbose. in: Peppler, H.J. (ed.) Microbial Technology. Reinhold Publishing Company, London, pp. 200-221.

Wold, W.S.M. and Suzuki, I. (1973). Cyclic AMP and citric acid accumulation by *Aspergillus niger*. Biochem.Biophys.Res.Comm. 50, 237-244.

Wold, W.S.M. and Suzuki, I. (1976a). The citric acid fermentation by *Aspergillus*

niger: regulation by zinc of growth and acidogenesis. Can.J.Microbiol. 22, 1083-1092.

Wold, W.S.M. and Suzuki, I. (1976b). Regulation by zinc and adenosine 3',5'-cyclic monophosphate of growth and citric acid accumulation in *Aspergillus niger*. Can.J.Microbiol. 22, 1093-1101.

Young, R.C., Bennet, J.E., Vogel, C.L., Carbone, P.P. and de Vita, V.T. (1970). Aspergillosis, the spectrum of the disease in 98 patients. Medicine (Baltimore) 49, 147-173.

Yuill, E. (1939). Two new *Aspergillus* mutants. J.Bot. 77, 174-175.

Zonneveld, B.J.M. (1974). α-1,3. glucan synthesis correlated with α-1,3 glucanase synthesis, conidiation and fructification in morphogenetic mutants of *Aspergillus nidulans*. J.Gen.Microbiol. 81, 445-451.

Zonneveld, B.J.M. (1977). Biochemistry and ultrastructure of sexual development in *Aspergillus*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and physiology of *Aspergillus*. Academic Press, London, pp. 59-80.

II Growth characteristics of *Aspergillus nidulans* mutants defective in carbohydrate metabolism [1)]

SUMMARY

Several mutants of *Aspergillus nidulans* defective in carbohydrate metabolism were tested for growth on different carbon sources. D-Galacturonate was found to be a substrate, useful to discriminate between mutants in pyruvate kinase, pyruvate dehydrogenase complex or pyruvate carboxylase. The results of these tests indicate how particular classes of mutants can be obtained and which substrates can be used preferentially for a rapid phenotypical screening of unknown mutants.

INTRODUCTION

As compared with bacteria, little is known about carbohydrate metabolism and its regulation in fungi and only a few genetic lesions concerning this metabolism are known. Various aspects of sugar transport, the main metabolic pathways, as well as the reserve carbohydrates in fungi have been reviewed by Smith and Berry (1975) and Burnett (1976). *Aspergillus nidulans* is an attractive organism for a biochemical genetic study since genetic techniques are well developed for this fungus. The first "sugar mutants" in *A.nidulans* were described by Roberts (1959). The same author later used galactose non-utilizing mutants to analyse galactose metabolism and confirmed the presence of the Leloir pathway (Roberts, 1970). Mutants unable to use carbohydrates by glycolysis can be rescued on gluconeogenic substrates. The majority of such mutants lack enzyme activity either of pyruvate kinase (Payton and Roberts, 1976; Payton, 1978), pyruvate carboxylase (Skinner and Armitt, 1972) or pyruvate dehydrogenase complex (Payton *et al.*, 1977; Bos *et al.*, 1981). Among mutants unable to use gluconeogenic substrates such as ethanol or acetate are those defective in glyoxylate cycle enzymes (Armitt *et al.*, 1976). In the pentose phosphate pathway only one defined lesion in transaldolase activity has been described (Hankinson, 1974). Glycerol non-utilizing mutants have also

1) Uitzetter *et al.*, 1982.

been isolated, but have not been conclusively assigned (McCullough *et al.*, 1977; Payton, 1978).

Aspergilli are able to use a diversity of organic compounds as carbon sources (Cochrane, 1958; McCullough *et al.*, 1977); even agar can provide a compound which is likely metabolized through C_2 metabolism (Payton *et al.*, 1976). Some investigators studied the effects of carbon sources or mixtures of them on growth of mutants blocked at different steps (Roberts, 1963; Skinner and Armitt, 1972; Hankinson, 1974; Payton and Roberts, 1976). The results of such growth tests can be used in different ways:
(i) to elucidate unknown metabolic pathways; (ii) to predict classes of mutants found after mutation and enrichment and (iii) to obtain a specific class of mutants by choice of an appropriate rescue medium.

We directed our attention to the isolation of new classes of mutants to study the implications of mutations for the metabolism in *A. nidulans*. We therefore decided to investigate the growth properties of known mutants on a variety of carbon sources. Relevant categories of easily available carbon compounds were tested separately or in combination *e.g.* hexoses, pentoses, polyols, amino acids and a few sugar acids.

MATERIALS AND METHODS

Strains

The *A. nidulans* strains used in this study were derived from the original Glasgow strain FGSC 4 (Barratt *et al.*, 1965). They are listed in Table II.1 with their genotypes main metabolic lesion and references.

Chemicals

D-Galacturonic acid was obtained from Fluka or Aldrich. All other carbon sources were obtained from Merck or BDH. Bacto agar was obtained from Difco.

Media and growth tests

A.nidulans was grown on complete medium (CM) or supplemented minimal medium (MM) according to Pontecorvo *et al.* (1953), solidified with 1.5% (w/v) agar and autoclaved. Instead of glucose 0.1 M sodium acetate was used for CM. A master plate containing the wild type and the mutants was obtained by inoculating spores from the different strains on CM. Growth tests were performed by replica plating on MM containing the appropriate supplements and instead of

Table II.1. Mutant strains of *A.nidulans* used for growth tests

Strain number	Genotype	Main lesion in carbohydrate metabolism	References or source
WG096	*yA2, pabaA1*	none	Barratt *et al.* (1965) FGSC 187
WG121	*pkiA1*	pyruvate kinase (EC 2.7.1.40)	Payton and Roberts (1976)
WG230	*yA2, pabaA1;pkiA4*		a)
WG231	*yA2, pabaA1;pkiA5*		a)
WG193	*yA2, pabaA1;pkiA9*		a)
WG097	*yA2, pabaA1,pdhA1*	pyruvate dehydrogenase complex	Payton *et al.* (1977)
WG105	*wA3; pyroA4;pdhA1*		idem, see also Bos *et al.* (1981)
WG100	*yA2, pabaA1;pdhB1*		idem
WG106	*wA3; pyroA4;pdhB1*		idem
WG099	*yA2, pabaA1;pdhB2*		Bos *et al.* (1981)
WG192	*yA2, pabaA1;pdhB3*		a)
WG228	*yA2, pabaA1;pdhB4*		a)
WG107	*yA2, pabaA1; pdhC1*		Bos *et al.* (1981)
WG108	*wA3; pyroA4;pdhC1*		idem
WG084	*yA2, pabaA1;pycA2*	pyruvate carboxylase (EC 6.4.1.1.)	Skinner and Armitt (1972)
WG103	*wA3; pyroA4;pycA3*		idem
WG135	*yA2, pabaA1;pycB4*		a)
WG077	*yA2; pyroA4;lacA1; pppA1*	transaldolase (EC 2.2.1.2)	Hankinson (1974)
WG196	*yA2, pabaA1;glcA1*	glycerol metabolism	Payton (1978)
WG197	*yA2, pabaA1,glcB33*		idem

a) Isolated after UV-treatment of WG096 (Bos *et al.*, unpublished results).

glucose other carbon sources were separately added as filter sterilized solutions. Carbon sources were used at a final concentration of 0.05 M pH 6.0 except for 0.1 M acetate; 0.1 M acetate + 0.01 M sucrose; 0.02 M sucrose; 0.05 M fructose + 0.01 M glutamate; 0.01 M glycerol + 0.05 M glucose or 0.05 M fructose. The amount of growth attained by the wild type and mutant strains was scored after two days incubation at 37°C.

RESULTS AND DISCUSSION

Over fourty different carbon sources were used to test the growth behaviour of the various mutants listed in Table II.1. Except for the two glycerol non-utilizing strains the enzymatic defects of the mutants have been established. The observations with some of these substrates are summarized in Table II.2. The wild type *A nidulans* used in these tests is able to assimilate all substrates listed as sole carbon and energy source. This makes this organism very suitable for studying carbon metabolism in a lower eukaryote.

A. nidulans cannot grow on L-sorbose (not shown), because this compound is only phosphorylated and not metabolized further (Elorza and Arst, 1971). The growth of this fungus on D-galacturonate drew our attention because this is in contrast with a previous observation by Hankinson (1974). *Aspergillus spp.* are known to degrade polygalacturonate and pectin (Barbesgaard, 1977) to oligomers and also to the monomer D-galacturonate. As early as 1931 Hofmann reported growth of *A. niger* on uronic acids but he did not investigate how these compounds were subsequently degraded. We demonstrated growth of *A.nidulans* on polygalacturonate minimal medium. Therefore the ability to grow on the monomer is not surprising.

Phenotype of pyruvate kinase (pki), pyruvate dehydrogenase complex (pdh) and pyruvate carboxylase (pyc) mutants.

Mutants with a defect in one of these structural genes share common features on many carbon sources, because they are unable to use compounds which are metabolized through the glycolytic pathway *e.g.* sucrose, glucose, fructose, mannose, sorbitol or through the pentose phosphate pathway *e.g.* xylose, ribitol. They all are able to use acetate or glutamate since gluconeogenesis is not blocked as pointed out by Payton *et al.* (1977). By using mixtures of a gluconeogenic and a glycolytic carbon source the three defects can be discriminated, although some *pki* mutants tested are able to grow on some of these mixtures, erroneously suggesting a *pyc* or *pdh* genotype. *Pki* mutants grow on L-alanine

Table II.2. Growth of *A.nidulans* mutants on various carbon sources [1)]

Carbon sources	wild type	mutant					
		pkiA	*pdhA,B,C*	*pycA,B*	*pppA*	*glcA*	*glcB*
Acetate	++	++	++	++	++	++	++
L-Alanine	+++	+	.	.	+	++	++
D-Fructose	+++	-	-	-	+++	++	++
D-Galactose	+++	-	-/.	+[2)]	+++	++	+
D-Galacturonate	+++	++	-	+[2)]	.	.	-
D-Glucarate	++	-	.	++	.	+	+
D-Gluconate	+++	-	.	+[3)]	+	+	+
D-Glucose	+++	-	-	-	+++	+++	+++
L-Glutamate	+++	+	+/++	+++	+++	+	+
Glycerol	++	-	+[2)]	+[2)]	++	.	-
D-Mannitol	+++	-	-	.	+++	++	++
D-Mannose	+++	-	-	-	+++	+++	+++
Ribitol	++	-	+	+	.	+	+
Sorbitol	+++	-	-/.	.	++	++	++
D-Xylose	+++	-	-	-	-	+++	+++
No added C-source	.	.	.	.	.	.	.
Mixed carbon sources							
Acetate + sucrose	++	-[4)]	++	++	+	++	++
L-Glutamate + D-fructose	+++	-	-	++	+++	++	++
Glycerol + acetate	++	+	+/++	++	++	+	-
Glycerol + D-fructose	+++	-	-	-	++	++	-
Glycerol + D-glucose	+++	-	-	-	++	+++	.

1) +++ Good, ++ moderate, + poor, . very poor, and - no growth

2) No growth in liquid culture

3) Growth is observed in liquid culture but not with all *pyc* mutants tested

4) Growth is observed with some non leaky *pki* mutants

Payton and Roberts, 1976) and it is assumed that alanine provides pyruvate by transamination. Because even *pdh* and *pyc* mutants show a slight growth on a solid medium with alanine, this substrate is not suitable for classification (Bos *et al.*, 1981). D-Galacturonate allows growth of *pki* mutants in contrast to *pdh* mutants (no growth) and *pyc* mutants (poor growth). This indicates that degradation of this uronic acid leads in any case to pyruvate. Ashwell *et al.*(1958) reported a pathway for the degradation of D-galacturonate in *E.coli* resulting in pyruvate as well as glyceraldehyde-3 phosphate.

Roberts (1970) suggested two pathways for galactose breakdown in *A.nidulans*, the Leloir pathway being the major one whereas direct oxidation to galacturonate was suggested for the minor pathway. The latter possibility seems unlikely because *pki* mutants are unable to grow on galactose. Observations of growth on D-glucarate are instructive too. *Pyc* mutants show growth on this hexaric acid indicating that the catabolic pathway may lead directly to a tricarboxylic acid cycle intermediate. The formation of 2-keto-glutarate from D-glucarate is well established in *Pseudomonas spp.* (Trudgill and Widdus, 1966). If this is also the case in *A.nidulans*, acetylCoA has still to be provided by glycolysis which would explain why *pki* and *pdh* mutants do not grow on D-glucarate. An alternative degradation to glycerate and pyruvate has been found in *Enterobacteriaceae* (Chang and Feingold, 1970). The presence of such a pathway in *A.nidulans* however, is not consistent with the growth behaviour of *pyc* mutants.

One expects that *pki*, *pyc* and *pdh* mutants cannot grow on glycerol. Agar has been mentioned as a possible source of C_2 (Armitt *et al.*, 1976; Payton *et al.*, 1976) permitting glycolytic mutants to grow on agar plates with the assumption that glycerol itself is strongly derepressing (Arst and Cove, 1973; Bailey and Arst, 1975). For this reason *pki*, *pyc* and *pdh* mutants were also tested on liquid medium where they all failed to grow on glycerol.

The induction of the glyoxylate cycle by C_2 compounds has been established in *A.nidulans*. (Armitt *et al.*, 1976). A mixture of acetate and sucrose supports growth in *pdh* and *pyc* mutants by supplementing both acetylCoA and TCA-cycle intermediates. In this case the combination of acetate and sucrose is preferred above that of acetate with a hexose as glucose to avoid the interaction of acetate or its products with the hexose-uptake systems. Romano and Kornberg (1969) demonstrated this by the inhibition of pyruvate formation and excretion when glucose (but not sucrose) was added to a culture of a *pdh* mutant grown on acetate. A possible explanation for the inability of *pki* mutants to grow on a mixture of acetate and sucrose is the accumulation of a phosphorylated intermediate

(*e.g.* phosphoenolpyruvate) which can have an inhibitory effect on the enzymes of the glyoxylate cycle (Kornberg, 1966; Fraenkel and Vinopal, 1973; Payton and Roberts, 1976). The basis for an enlarged sucrose tolerance which we found to occur in some *pki* mutants is unknown yet.

The combination of L-glutamate and D-fructose discriminates *pyc* mutants from *pki* and *pdh* mutants. This medium does not enable *pki* and *pdh* mutants to grow because acetylCoA is not generated; *pyc* mutants, however, grow because in this case glutamate is a supplementing source of oxaloacetate.

Glycerol non-utilizing mutants (glc)

The *glcA* and *glcB* mutants were isolated by Payton (1978) after mutation and enrichment in glycerol. The two genes are unlinked. The exact nature of the enzymatic defects have not yet been elucidated, although McCullough *et al.* (1977) suggested that *glcB* mutants lack glycerol-3-phosphate dehydrogenase. In contrast to the *glcA* mutant the *glcB* mutant is strongly inhibited by glycerol in combination with glucose or fructose (Payton, 1978) presumably due to the accumulation of glycerol-3-phosphate and subsequent inhibition of glycolysis. A similar inhibition has been demonstrated in mutants of *E.coli* defective in glycerol-3-phosphate dehydrogenase but not in mutants lacking glycerol kinase (Cozzarelli *et al.*, 1965).

Glycerol non-utilizing mutants are also known in *Neurospora* (Nilheden *et al.*, 1973; Holm *et al.*, 1976; Viswanath-Reddy *et al.*, 1977). In this organism dissimilation of glycerol has been reported to occur by a phosphorylative and an oxidative pathway. One of these mutants was found to lack an inducible glycerol kinase activity but still possessed a normal residual activity of a basal non-inducible glycerol kinase (Holm *et al.*, 1976). A flavin linked glycerol-3-phosphate dehydrogenase mutant has also been found (Denor and Courtright, 1978).

Another mutant of the oxidative pathway lacked glyceraldehyde kinase (Viswanath-Reddy *et al.*, 1977). In contrast to the results reported for *N.crassa*, the *glcA* and *glcB* mutants of *A.nidulans* metabolize other polyols readily.

Another characteristic of the *glc* mutants in *A.nidulans* is the inability to use D-galacturonate. As the D-galacturonate pathway is likely to lead to C_3 compounds, this substrate opens new perspectives to study the complicated interactions between glycerol metabolism and glycolysis.

Pentose phosphate pathway mutant (pppA)

D-Xylose and also ribitol cannot support growth of a *pppA* mutant since the pentose phosphate pathway is the main route for degradation of these compounds.

The *pppA* mutant lacking transaldolase activity (Hankinson, 1974) can grow however, on glucose because this is metabolized to a large extent through glycolysis. Other hexoses and polyols can be metabolized for the same reason. Some growth is observed with D-gluconate which is not expected if this compound would only become phosphorylated and metabolized through the pentose phosphate pathway.

In *A.niger* strains Allam *et al.* (1975) claimed a second pathway in which gluconate is directly oxidized to 2-keto-3-deoxygluconate and subsequently split into pyruvate and glyceraldehyde by an aldolase reaction. The same pathway is reported for *A.fumigatus* (Elzainy and Hassan, 1978). The behaviour of both *pki* and *pdh* mutants on gluconate together with the slight growth observed in tests with *pyc* mutants using this substrate is also somewhat puzzling. There is hardly any growth of the *pppA* mutant on D-galacturonate or glucarate whereas there is on gluconeogenic substrates.

CONCLUSIONS

The growth characteristics of *pki* mutants and *pdh* mutants from *A.nidulans* with D-galacturonate as carbon source predict that pyruvate is a metabolite formed during degradation. Whether a pathway exists analoguous to the one present in *E.coli*, remains to be proven.

One can also predict that mutation followed by enrichment in D-galacturonate will lead among others to *pdh* and *glc* mutants. The actual experiment has been performed in the meantime. A majority of the galacturonate non-utilizers are *pdh* mutants, whereas others are either *glc* or *pyc* or disturbed in the galacturonate degradation itself (Chapter III). This investigation indicates that the best strategy to obtain *pki* mutants, would be an enrichment on D-glucose with subsequent rescue on D-galacturonate instead of on acetate.

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REFERENCES

Allam, A.M., Hassan, M.M. and Elzainy, T.A. (1975). Formation and cleavage of 2-keto-3-deoxygluconate by 2-keto-3-deoxygluconate aldolase of *Aspergillus niger*. J.Bacteriol. 124, 1128-1131.

Armitt, S., McCullough, W. and Roberts, C.F. (1976). Analysis of acetate non-utilizing *(acu)*mutants in *Aspergillus nidulans*. J.Gen.Microbiol. 92, 263-282.

Arst, H.N. and Cove, D.J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. Molec.Gen.Genet. 126, 111-141.

Ashwell, G., Wahba, A.J. and Hickman, J. (1958). A new pathway of uronic acid metabolism. Biochim.Biophys.Acta 30, 186-187.

Bailey, C. and Arst, H.N. (1975). Carbon catabolite repression in *Aspergillus nidulans*. Eur.J.Biochem. 51, 573-577.

Barbesgaard, P. (1977). Industrial enzymes produced by members of the genus *Aspergillus*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 391-404.

Barratt, R.W., Johnson, G.B. and Ogata, W.N. (1965). Wild type and mutants stocks of *Aspergillus nidulans*. Genetics 52, 233-246.

Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.

Burnett, J.H. (ed.) (1976). Fundamentals of mycology. 2nd edition. Edward Arnold, London.

Chang, Y.F. and Feingold, D.S. (1970). D-glucaric acid and galactaric acid catabolism by *Agrobacterium tumefaciens*. J.Bacteriol. 102, 85-96.

Cochrane, V.W. (ed.) (1958). Physiology of fungi. Wiley, New York.

Cozzarelli, N.R., Koch, J.P., Hayashi, S. and Lin E.C.C. (1965). Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J.Bacteriol. 90, 1325-1329.

Denor, P.F. and Courtright, J.B. (1978). Isolation and characterization of glycerol-3-phosphate dehydrogenase-defective mutants of *Neurospora crassa*. J.Bacteriol. 136, 960-968.

Elorza, M.V. and Arst, H.N. (1971). Sorbose resistant mutants of *Aspergillus nidulans*. Molec.Gen.Genet. 111, 185-193.

Elzainy, T.A. and Hassan, M.M. (1978). 2-keto-3-deoxygluconate aldolase in different fungi. Egypt.J.Bot. 21, 151-153.

Fraenkel, D.G. and Vinopal, R.T. (1973). Carbohydrate metabolism in bacteria. Ann.Rev.Microbiol. 27, 69-100.

Hankinson, O. (1974). Mutants of the pentose phosphate pathway in *Aspergillus nidulans*. J.Bacteriol. 117, 1121-1130.

Hofmann, E. (1931). Ueber die Bildung von Oxalsäure aus Uronsäuren durch *Aspergillus niger*. Biochem.Z. 243, 423-428.

Holm, K., Nilheden, E. and Kølmark, H.G. (1976). Genetic and enzymatic analysis of a glycerol kinase deficient mutant in *Neurospora crassa*. Molec.Gen.Genet. 144, 11-15.

Kornberg, H.L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem.J. 99, 1-11.

McCullough, W. Payton, M.A. and Roberts, C.F. (1977). Carbon metabolism in *Aspergillus nidulans*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and Physiology of *Aspergillus*. Academic Press, London, pp. 97-129.

Nilheden, E., Holm, K. and Kølmark, H.G. (1973). Glycerol non-utilizing mutants in *Neurospora crassa*. Genetics 74, S 196.

Payton, M.A. (1978). A genetic study of sugar metabolism and transport in *Aspergillus nidulans*. Ph.D.Thesis. University of Leicester.

Payton, M.A., McCullough, W., and Roberts, C.F. (1976). Agar as a carbon source and its effect on the utilization of other carbon sources by acetate non-utilizing *(acu)*mutants of *Aspergillus nidulans*. J.Gen.Microbiol. 94, 228-233.

Payton, M.A., McCullough, W, Roberts, C.F. and Guest, J.R. (1977). Two unlinked genes for the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 129, 1222-1226.

Payton, M.A. and Roberts, C.F. (1976). Mutants of *Aspergillus nidulans* lacking pyruvate kinase. FEBS Lett. 66, 73-76.

Pontecorvo, G., Roper, J.A., Hemmons, L.J. MacDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans*. Adv.Genet. 5, 141-238.

Roberts, C.F. (1959). A replica plating technique for the isolation of nutritionally exacting mutants of a filamentous fungus *(Aspergillus nidulans)*. J.Gen.Microbiol. 20, 540-548.

Roberts, C.F. (1963). The genetic analysis of carbohydrate utilization in *Aspergillus nidulans*. J.Gen.Microbiol. 31, 45-58.

Roberts, C.F. (1970). Enzyme lesions in galactose non-utilizing mutants of *Aspergillus nidulans*. Biochim.Biophys.Acta 201, 267-283.

Romano, A.H. and Kornberg, H.L. (1969). Regulation of sugar uptake by *Aspergillus nidulans*. Proc.Roy.Soc.B. 173, 475-490.

Skinner, V.M. and Armitt, S. (1972). Mutants of *Aspergillus nidulans* lacking pyruvate carboxylase. FEBS Lett. 20, 16-18.

Smith, J.E. and Berry, D.R. (eds.) (1975). The filamentous fungi. Vol. 2.

Biosynthesis and metabolism. Edward Arnold, London.

Trudgill, P.W. and Widdus, R. (1966). D-glucarate catabolism by *Pseudomonaceae* and *Enterobacteriaceae*. Nature, 211, 1097-1099.

Uitzetter, J.H.A.A., Bos, C.J. and Visser, J. (1982). Growth characteristics of *Aspergillus nidulans* mutants defective in carbohydrate metabolism. Antonie van Leeuwenhoek 48, 219-227.

Viswanath-Reddy, M., Bennet, S.N. and Howe, H.B. (1977). Characterization of glycerol non-utilizing and protoperithecial mutants of *Neurospora*. Molec.Gen. Genet. 153, 29-38.

III Isolation and characterization of *Aspergillus nidulans* mutants in carbon metabolism after D-galacturonate enrichment

SUMMARY

A method quite selective for the isolation of mutants defective in the pyruvate dehydrogenase complex in *Aspergillus nidulans* was found.
The essential steps in the procedure were a mutagenic treatment of conidiospores with X-rays yielding about 50% survival, followed by filtration enrichment in minimal medium with D-galacturonate as sole carbon source and rescue on complete medium with acetate. The mutants thus isolated were phenotypically characterized on the basis of growth tests and different genotypes were assigned on the basis of complementation tests. The majority of the galacturonate non-utilizing mutants was defective in one of the components of the pyruvate dehydrogenase complex. In addition mutants defective in pyruvate carboxylase, in glycerol catabolism and some novel mutants only unable to use D-galacturonate as sole carbon source were found. At least two genes are concerned in D-galacturonate metabolism.

INTRODUCTION

Aspergillus nidulans mutants defective in carbon metabolism have been found after mutagenic treatment with a low percentage of survival without further enrichment procedures (Skinner and Armitt, 1972). Carbon metabolism mutants are commonly selected by a filtration enrichment technique which causes selective elimination of prototrophic mycelium grown in a well defined medium. This method was used to isolate both gluconeogenic mutants (Armitt *et al.*, 1976) and glycolytic ones. For the isolation of the latter mutants a disaccharide like sucrose (Payton *et al.*, 1977) or a readily digestible monosaccharide like glucose (Payton *et al.*, 1977; Bos *et al.*, 1981) was used.
In chapter II growth of *A.nidulans* on D-galacturonate as sole carbon and energy source was reported. Tests with mutants defective in pyruvate kinase *(pki)* and

pyruvate dehydrogenase complex *(pdh)* showed good growth of the former but no growth of the latter genotype on this substrate. This indicates a metabolic pathway for D-galacturonate leading to pyruvate as one of the degradation products. Such a pathway exists in *Escherichia coli* (Ashwell *et al.*, 1958). Payton *et al.*(1977) isolated 8 *pdh* mutants among some 200 mutants selected for their inability to grow on sucrose. They used N-methyl-N'-nitro-N-nitroso guanidine as a mutagen at a 0.5% survival level.
Genetic analysis of these *pdh* mutants revealed them to belong to two different genes; *pdhA* and *pdhB* belonging to linkage group I and V respectively. Bos *et al.* (1981) reported the isolation of a number of other pyruvate dehydrogenase complex mutants which were obtained by UV-treatment at a 75-80% survival level followed by enrichment in glucose minimal medium. Of the 160 strains isolated, 54% were disturbed in their pyruvate dehydrogenase complex activity. Among these mutants a third distinct gene, *pdhC*, was found and assigned to linkage group VIII. In order to obtain other mutants defective in pyruvate metabolism, D-galacturonate was chosen as medium for enrichment. This chapter describes the results of an X-ray mutation experiment with *A.nidulans* followed by D-galacturonate enrichment and the characterization of mutant classes found.

MATERIALS AND METHODS

Organisms

The *Aspergillus nidulans* strain used for mutation was WG145 (*wA3;pyroA4*). This strain has wild-type characteristics for carbon source utilization. For complementation studies the following strains were used: WG097 (*pabaA1,yA2,pdhA1);* WG100 (*pabaA1,yA2;pdhB1*); WG107 (*pabaA1,yA2;pdhC1*); WG102 (*pabaA1,yA2;pycA2*); WG135 (*pabaA1,yA2;pycB4*); WG196 (*pabaA1,yA2;glcA1*) and WG197 (*pabaA1,yA2,glcB33*). These strains were derived from the original Glasgow strains of *Aspergillus nidulans* (Pontecorvo *et al.*, 1953). All symbols have their usual meaning (Clutterbuck, 1973; Payton, 1978): *glc* glycerol metabolism, *pdh* pyruvate dehydrogenase complex, *pyc* pyruvate carboxylase. For mutants concerned with D-galacturonate metabolism the gene symbol *gaa* is used.

Media and growth tests

A.nidulans WG145 was grown on complete medium (CM) according to Pontecorvo *et al.* (1953) modified as described in I.3.4. to obtain conidiospores. Growth tests were performed on minimal medium (MM) supplemented with pyridoxine HCl

(1 mg l^{-1}). The medium was solidified with 1.5% (w/v) agar. Unless stated otherwise glucose was omitted and other carbon sources were added instead. Carbon sources (pH6) were added separately as membrane filter sterilized solutions.

The final concentrations of the carbon sources were: D-galacturonate 0.05 M; D-gluconate 0.05 M; D-glucose 0.05 M; glycerol 0.05 M; D-xylose 0.05 M; acetate 0.1 M; acetate 0.1 M with sucrose 0.01 M; L-glutamate 0.05 M with D-fructose 0.01 M; glycerol 0.05 M with acetate 0.1 M. All acids were used as their sodium salt. The amount of growth attained by the wild-type and mutant strains was scored after two days incubation at 37°C.

Induction and isolation of mutants

Mutations were induced by X-ray treatment during 6.5 minutes at a dose yielding 51% survival using a Philips Röntgen apparatus equipped with a MCN 161 tube (160 kV, 19 mA, 2 mm Al-filter) placed at a distance of 36 cm from the bottom of a petri dish which contained 15 ml of a conidial suspension of strain WG145 (approx. 5.10^7 conidia ml^{-1}). In this case the total dose was 2.5 krad. The survival was determined before and after irradiation by plating a diluted suspension of conidiospores on CM acetate. Irradiated conidia were transferred to 300 ml minimal liquid medium in a 1 l infusion bottle with D-galacturonate 0.05 M as carbon source and supplemented with pyridoxine . HCl. The flask was incubated in a reciprocal shaker bath (160 strokes min^{-1}) at 37°C during 12 hours. Then non-germinated conidia were separated from hyphae by filtration over cotton wool, the spores were collected on a membrane filter and incubated again in 100 ml fresh medium for a further 11 hours. Finally non-germinated conidia which passed a second filtration procedure were resuspended in 0.5 ml 0.8% (w/v) NaCl. After appropriate dilution the suspension was plated at a density of approx. 60 colonies per plate in CM with acetate and incubated at 37°C for two days. From the colonies thus obtained masterplates were made on CM with acetate and replicas were made on MM with D-galacturonate and acetate respectively as carbon source in order to identify mutants in carbon metabolism. Mutants which showed leaky or no growth on D-galacturonate medium were then purified by plating conidia from a single mutant colony. Monospore cultures were made and conidia were preserved on dehydrated silicagel according to Perkins (1962).

Phenotypical classification

To characterize the mutants by their phenotype, growth tests were performed as described under Media and growth tests. Criteria to assign phenotypes are

mentioned in Table III.1. Growth behaviour on the various substrates was already discussed in chapter II.

Complementation tests

To assign mutant genes, *pdhA,B,C* and *pycA,B* tester strains with different color and vitamin markers were used for complementation. Heterokaryotic mycelium was obtained according to Pontecorvo *et al.* (1953). Twin pieces of heterokaryotic mycelium were transferred to MM with sucrose and MM acetate with sucrose respectively. The latter medium gives much better growth than acetate alone. Complementation with *glc* mutants was performed with diploids constructed with the *glcA1* and *glcB33* strain respectively. Growth tests with diploids were performed on MM with glycerol and galacturonate respectively, using MM sucrose as a control.

Analysis of uronic acid metabolism

Organisms, culture conditions and cell free extracts

Escherichia coli K12 (PC1088) was cultured on D-galacturonate. Cell free extracts were made according to Ashwell (1962).

Aspergillus nidulans WG145 was cultured for 24 hours at 37°C in minimal medium with D-galacturonate 0.05 M pH=6 as sole carbon source. After grinding 1 g of mycelium in liquid nitrogen and evaporation, 4 ml extraction buffer was added with the same composition as used to prepare *E.coli* extracts. The cell free *A.nidulans* extract was used after centrifuging for 10 minutes at 40.000 x *g* to remove cell debris.

Enzyme assays

Uronic acid isomerase and D-altronic acid dehydrogenase were assayed according to Ashwell (1962). D-altronic acid dehydrase was also assayed according to Ashwell (1962) except that 2-keto-3-deoxy-gluconic acid was detected by HPLC.

Metabolite assays

After removal of proteins by passing the assay mixture through a nitrocellulose filter, analyses of metabolites of the uronic acid pathway were carried out with 10 µl samples in a Spectraphysics SP 8000 liquid chromatograph equipped with a precolumn Chrompack Standard Column Vydac 201 SC L = 10 cm, I.D. = 2.1 mm, O.D. = ¼" catalogue number 27723 and a Chrompack Economy Column Lichrosorb 10 NH_2 L = 25 cm, I.D. = 4.6 mm, O.D. = ¼" catalogue number 27805. The eluent consisted of 80 mM boric acid, 80 mM primary sodiumphosphate

adjusted to pH 4.15 with phosphoric acid. During the elution at 20°C a constant flow of 1.5 ml min^{-1} was maintained. Detection of metabolites was achieved at 216 nm with a Pye Unicam LC-UV detector.

Chemicals

Tagaturonic acid was synthesized according to Ehrlich and Guttmann (1934), altronic acid was synthesized according to Pratt and Richtmeyer (1955). These acids were a gift of the Dept. of Organic Chemistry. 2-Keto-3-deoxy-D-gluconic acid was a gift of Dr. W.A. Wood (Michigan State Univ. East Lansing, Michigan U.S.A.). D-galacturonic acid was obtained from Fluka or Aldrich.

RESULTS AND DISCUSSION

The survival of the irradiated conidiospores was 51%. In order to prevent background damage a low dose rate was used in this mutation experiment. The mutagenesis and enrichment procedure resulted in 2232 colonies. Of these colonies 840 were tested further for their ability to grow on D-galacturonate. From these 209 (25%) showed little or no growth on this carbon source and were therefore of the desired phenotype, 145 mutants (17%) appeared to have other deficiencies. These are left out of consideration. In Table III.1. the criteria are summarized which were used to assign different phenotypes to the D-galacturonate non-utilizing or badly utilizing mutants.
The *pdh*, *pyc* and *glc* phenotypes classified by these growth tests were then verified by complementation to correspond with genotypes already known. Their frequencies are also shown in Table III.1. Mutants in glycerol metabolism (*glc*) and D-galacturonate metabolism (*gaa*) showed normal sporulation like the parental strain, such in contrast to *pdh* and *pyc* mutants.

The amount of mutants recovered is very high. This could be due to a relatively quick growth of the mycelium which made the enrichment period sufficiently short to avoid death of ungerminated conidia. Another possibility is that less metabolites were excreted. Only 9 mutants could not be assigned by a phenotypical characterization.

Table III.1. Growth criteria used to assign phenotypes to isolated *A.nidulans* mutants.[1)]

	Strains				
Carbon source	wild type	*gaa*	*glc*	*pyc*	*pdh*
D-Galacturonate	+++	-/.	-	-	-
Glycerol	++	++	-	+	-/.
Glycerol+acetate	++	++	-	++	++
D-Glucose	+++	+++	+++	-	-
D-Xylose	+++	+++	+++	-	-
D-Gluconate	+++	++	++	.	-
L-Glutamate+D-fructose	+++	+++	+++	++	-
Acetate+sucrose	++	++	++	++	++
Acetate	++	++	++	++	++
Totals[2)]		15	36	17	132
Frequency (%)		7	17	8	63

1) +++ good, ++ moderate, + poor, . very poor and - no growth

2) 9 strains could not be assigned with these criteria.

Pyruvate dehydrogenase complex (pdh) mutants

From the mutants isolated the majority (63%) turned out to have a defective pyruvate dehydrogenase complex. Complementation of these mutants was performed with the three marker genes known for this complex. *PdhA* is the structural gene for lipoate acetyltransferase (E_2) , and *pdhC* and *pdhB* are the structural genes for the α- and β-subunits respectively,of pyruvate decarboxylase (E_1) (Visser *et al.*, 1982). The distribution of the *pdh* mutants over the three genes was: 37% *pdhA*, 28% *pdhB* and 35% *pdhC*.It is obvious that other selection techniques are required to find mutants with defects in structural genes coding for the other complex components *viz.* lipoamide dehydrogenase and the two regulating enzymes pyruvate dehydrogenase kinase and -phosphatase. A possible reason for missing the E_3 mutants of this complex is the occurrence of more than one gene coding for E_3. For instance in mammalian tissues several E_3 isoenzymes are found which are functionally exchangeable between the keto-acid multienzyme complexes (Kenney *et al.*, 1972; Sakurai *et al.*, 1970). Of course *pdh* kinase mutants will not be found. In the case of *pdh* fosfatase mutants a partially active complex may still be present since phosphorylation is a post-translational event. This will result in escape of such mutants in an early stage of the enrichment. Also it is unknown whether non-specific phosphatase activities present act to some extent on the phosphorylated pyruvate dehydrogenase complex.

Mutants in glycerol metabolism (glc)

All *glc* mutants isolated belonged to one single class as shown by complementation in diploids and were identified as *glcB*. It was necessary to construct diploids for complementation analysis because heterokaryons showed very poor growth if any at all on glycerol. Such a failure to complement in heterokaryons was observed earlier with other mutants in carbon metabolism *e.g.* those unable to grow on sorbitol (Roberts, 1964) and with mutants resistant to fluoro-acetate (Apirion, 1966). *Glc* mutants were first isolated by Payton (1978) after mutagenic treatment followed by enrichment in glycerol medium. He assigned two genes: *glcA* (linkage group V) and *glcB* (linkage group I). The *glcA* mutant showed very poor growth on glycerol. The *glcB* mutant was absolute, and its growth on other carbon sources in the presence of glycerol was strongly inhibited. The exact nature of the *glcA* mutant is still unresolved. The *glcB* mutant was suspected to be deficient in glycerol-3-phosphate dehydrogenase because of the interference of glycerol with growth on other carbon sources which is also observed in *Escherichia coli* (Cozzarelli *et al.*, 1965) and *Salmonella typhimurium* (Aceves-

Pina *et al.*, 1974).
Since in this study only *glcB* mutants were found this is an indication that only part of the pathway used for glycerol metabolism seems to coincide with that of galacturonate catabolism.
Glc diploids show the same growth (if any) on glycerol or galacturonate although in the latter case the color of conidia is better developed.

Pyruvate carboxylase (pyc) mutants

Skinner and Armitt (1972) were the first to describe *pyc* mutants after mutagenic treatment of *A.nidulans* conidiospores at 1% survival level. They tested 8000 colonies of which 3 among 55 sucrose-negative survivors had *pyc* genotype. A large number of *pyc* mutants have since been isolated by Payton *et al.* (1977) and Bos *et al.* (1981). Complementation data suggest intragenic complementation to occur although the existence of two closely linked genes is not completely excluded (Bos, unpublished results).
In this experiment 17 mutants showed a *pyc* genotype (Table III.1.). These mutants isolated by the present procedure were completely unable to use D-galacturonate for growth, such in contrast to the *pyc* mutants (chapter II) isolated earlier. The *pyc* genotype was confirmed by complementation with two strains which mutually complement. Of the new *pyc* strains 16 failed to complement with both tester strains; *i.e.* mimicked allelism to both *pycA* and *pycB*; only one strain showed slight growth in a heterokaryon with the *pycB* strain. Obviously the selection method used yielded *pyc* mutants which were damaged in such a way that in nearly all cases complementation with these two tester strains was impossible.

Mutants in D-galacturonate metabolism (gaa)

In the mutation experiment described 15 mutants were isolated which were exclusively unable to use D-galacturonate as sole carbon and energy source. These mutants showed normal growth on the other carbon sources listed in Table III.1., indicating that D-galacturonate was not catabolized through one of the main pathways for carbohydrate degradation. Apart from the carbon sources already mentioned the following compounds were tested for growth: D-gluconate, D-glucarate, D-glucuronate, D-altronate, D-galactarate and D-tagaturonate. All compounds except the latter two were able to support growth both in wild-type and *gaa* mutants.

As already mentioned in chapter II a pathway similar to the one existing in *Escherichia coli* was considered. This pathway is shown in Fig. III.1. Such a pathway exists also in *Aeromonas* (Farmer and Eagon, 1969).

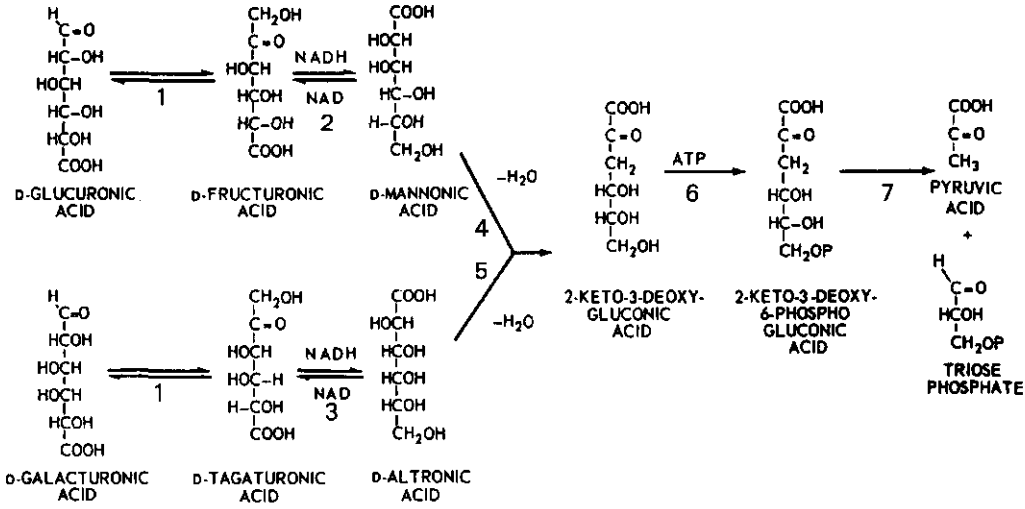

Fig. III.1. Uronic acid pathway in *Escherichia coli* (after Ashwell,1962).

The enzymes indicated are:

1. Uronic acid isomerase
2. D-mannonic acid dehydrogenase
3. D-altronic acid dehydrogenase
4. D-mannonic acid dehydrase
5. D-altronic acid dehydrase
6. Ketodeoxygluconic acid kinase
7. Ketodeoxyphosphogluconic acid aldolase

Analysis of D-galacturonate metabolism in wild-type A. nidulans

Preliminary experiments to identify the presence of a D-galacturonate metabolic pathway in *A.nidulans* similar to the one present in bacteria, were carried out with cell free extracts and cell debris of D-galacturonate grown wild-type *A.nidulans* and with cell free extracts of *E.coli* grown under similar conditions. Using the enzymatic assays described in Materials and Methods, uronic acid isomerase and D-altronic acid dehydrogenase could be demonstrated in *E.coli*. In *A.nidulans* however, no detectable amount of enzymatic activity of any of these enzymes was observed.

In *E.coli* the enzymes of the uronic acid pathway need different assay conditions to show maximal activity (Ashwell, 1962). They also differ in activity with respect to the presence of thiol reagents and metal ions:

- Uronic acid isomerase has a pH optimum range of 8.0 to 8.5 and is completely inhibited by cysteine, 2-mercaptoethanol, EDTA (10^{-3}M) and Zn^{2+} or Cu^{2+}(10^{-5}M).
- D-Altronic acid dehydrogenase has a maximum activity at pH 6.0. It is inactive with NADPH as cofactor.
- D-Altronic acid dehydrase is only stable in the presence of cysteine or 2-mercaptoethanol in the pH range 7.0 to 8.0 and it has an absolute Fe^{2+} requirement, whereas Mn^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} have no influence on the activity.

Because no detectable amount of enzymatic activity of uronic acid isomerase and D-altronic acid dehydrogenase was observed in *A.nidulans* extracts, the conditions for extraction and enzyme assays were varied. The pH of the sodium phosphate buffer (0.1 M) used for extraction was varied in the range pH 4.0 to pH 8.0 whereas sodiumphospate buffer 0.1M pH 7.0 and borate buffer 0.1 M pH 8.2 were used respectively in the uronic acid isomerase assay.
Neither in cell free extracts nor in the cell debris any activity could be demonstrated.
The same variation in extraction conditions was used in the D-altronic acid dehydrogenase assay. In this case no NAD(P)H disappearance was observed even in the presence of 0.2 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} or Co^{2+} in sodium phosphate assay buffer 0.1 M with a pH varying in the range 4.0 to 8.0. The presence of uronic acid isomerase, D-altronic acid dehydrogenase, and D-altronic acid dehydrase was also studied by the use of HPLC. An HPLC system for the separation of D-galacturonic acid, D-tagaturonic acid and D-altronic acid was developed with boric acid as component of the eluent. Boric acid has been used as an additive to improve the separation of polyols as their borate complexes on anion exchange resins (Zill *et al.*, 1953; Larsson and Samuelson, 1976) and in the separation of carbohydrates on silica gel (Jones, 1978; Ghebregzabher *et al.*, 1979).
In Table III.2. the capacity factor (*i.e.* relative retention time) is given of the various intermediates. The elution conditions were as described under Materials and Methods.

Table III.2. Capacity factors of uronic acid pathway intermediates in *E.coli*.

Compound	k'
D-galacturonic acid	1.35
D-tagaturonic acid	1.61
D-altronic acid	2.16
2-keto-3-deoxy-gluconic acid	1.69

Although uronic acid isomerase and D-altronic acid dehydrase could be demonstrated in *E.coli*, again there was no evidence for the presence of these enzymes in *A.nidulans*.
There are several possibilities to account for the results obtained: A pathway as present in *E.coli* exists but the enzyme levels are too low to allow their

detection, or the extraction and assay conditions are not optimal. The first possibility is unlikely since fungal growth on D-galacturonate still results in approx. 35% biomass as compared to growth on sucrose.
In several microorganisms other pathways are followed in the degradation of D-galacturonate.

In *Pseudomonas syringae* (Kilgore and Starr, 1959) and *Agrobacterium tumefaciens* (Chang and Feingold, 1969) D-galacturonate is oxidized to D-galactarate. In the latter organism a hexarate dehydratase gave a mixture of 2-keto-3-deoxy-glucaric acid and 4-dexy-5-keto-D-glucaric acid which finally lead to α-keto-glutaric acid (Chang and Feingold, 1970).

In a polygalacturonate adapted *Pseudomonas*, Preiss and Ashwell (1963a) mentioned the enzymatic formation of 4-deoxy-L-threo-5-hexoseulose uronic acid (4-deoxy-5-keto-uronic acid) and D-galacturonic acid as the monosaccharide endproducts of polygalacturonate degradation (see Fig. III.2.). Evidence was presented that the D-galacturonic acid formed, was metabolized via the *E.coli* pathway, whereas the 4-deoxy-5-keto-uronic acid yielded a new intermediate 3-deoxy-D-glycero-2,5-hexodiulosonic acid. This acid was first reduced to 2-keto-3-deoxy-D-gluconic acid, then phosphorylated and finally split into pyruvate and glyceraldehyde-3-phosphate (Preiss and Ashwell,1963b). Some further remarks on the various possibilities can be made. If a pathway similar to the one present in *E.coli* is operating in *A.nidulans* then it is unlikely to find mutants in the first step since *E.coli* has an uronate isomerase attacking both D-glucuronate and D-galacturonate; this is inconsistent with the phenotype of the

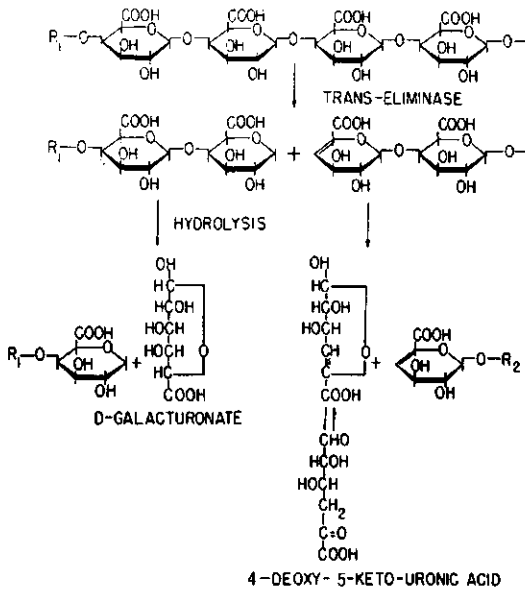

Fig. III.2. Enzymatic degradation of polygalacturonate after Preiss and Ashwell (1963a).

gaa mutants found. They all grow on D-glucuronate.
A direct oxidation of D-galacturonate to D-galactarate is also unlikely since D-galactarate supports no growth in wild type. However this might be due to the absence of a transport system for this compound. Preliminary experiments indicated that in cell free extracts of *A.nidulans* no oxygen consumption was measured in the presence of D-galacturonate. Because of the limited time available no further enzymatic analyses were made to investigate the possibility of the direct formation of an unsaturated compound similar to the one found in *Pseudomonas*.

The existence of different gaa genes and allocation of two of them

In order to allocate *gaaA* a heterozygous diploid was constructed with master strain WG203 and haploid segregants were induced with p-fluorophenylalanine according to McCully and Forbes (1965).
From the results shown in Table III.3. it can be concluded that the *gaaA* gene belongs to linkage group VI since genes on this chromosome remain together during mitotic haploidization. Because all *gaa* strains isolated were in the *wA3;pyroA4* background one of the haploid segregants was used for complementation experiments with the other *gaa* mutants isolated. Heterokaryons were tested on MM sucrose and MM galacturonate respectively. The *gaaA1* strain was able to complement with all other *gaa* mutants, but not with the parental strain from which it was derived. Unless intragenic complementation occurred,possibly at least two genes are involved in D-galacturonate metabolism.

One of the other *gaa* mutants (*gaaB2*) was also analyzed in a heterozygous diploid with the same master strain (Table III.3.). Because in this case a conclusive assignment of *gaaB* was impossible,a second heterozygous diploid was constructed with WG001 in order to examine the segregation of markers on chromosome I, III and V (see Table III.4.).

Table III.3. Allocation of *gaaA* and *gaaB* by mitotic haploidization of diploids *gaaA*//WG203 and *gaaB*//WG203

Linkage group	Markers in:		No. of haploids			
	Mutant strain	Tester strain	*gaaA1*	*gaaA*$^+$	*gaaB2*	*gaaB*$^+$
I	*yA*$^+$		0	0	4	1
		yA2	20	20	0	39
II	*wA3*		12	29	25	90
		wA$^+$	20	20	4	39
III	*meaA*$^+$		0	3	a)	a)
		meaA3	32	46	a)	a)
IV	*pyroA4*		15	14	13	57
		pyroA$^+$	17	35	16	72
V	*pA*$^+$		a)	a)	13	58
		pA1	a)	a)	16	71
VI	*sB*$^+$		32	1	16	53
		sB3	0	48	13	76
VII	*nicB*$^+$		17	25	9	77
		nicB8	15	24	20	52
VIII	*riboB*$^+$		20	20	13	56
		riboB2	12	29	16	73

a) *meaA3* and *pA1* were difficult to classify

Table III.4. Allocation of *gaaB* by mitotic haploidization of diploid *gaaB*//WG001

Linkage group	Markers in:		No. of haploids	
	Mutant strain	Tester strain	*gaaB*	*gaaB*$^+$
I	*yA*$^+$		8	0
		yA2	0	42
II	*wA3*		42	63
		wA$^+$	8	42
III	*phenA*$^+$		50	105
		phenA2	0	0
V	*lysB*$^+$		40	78
		lysB5	10	27

From Table III.4. it can be concluded that *gaaB* is located on linkage group I which confirms that at least two genes exist coding for enzymes in D-galacturonate metabolism.

CONCLUSION

D-galacturonate is a very suitable substrate for enrichment of mutants defective in galacturonate, glycerol and pyruvate metabolism. These mutants will undoubtedly give a tool to resolve the subsequent steps within galacturonate and glycerol metabolism. Because no pyruvate kinase mutants were found (in fact these do grow on galacturonate) this proves the hypothesis that D-galacturonate is degraded to a metabolite beyond phosphoenolpyruvate. At least two genes are concerned in galacturonate metabolism. No evidence has been found for the existence of a pathway for galacturonate degradation identical to the one existing in *E.coli*.

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REFERENCES

Aceves-Pina, E., Ortega, M.V. and Artis, M. (1974). Linkage of the *Salmonella typhimurium* chromosomal loci encoding for the cytochrome-linked L- α -glycerophosphate dehydrogenase and amylomaltase activities. Arch.Microbiol. 101, 59-70.

Apirion, D. (1966). Recessive mutants at unlinked loci which complement in diploids but not in heterokaryons of *Aspergillus nidulans*. Genetics 53, 935-941.

Armitt, S., McCullough, W. and Roberts, C.F. (1976). Analysis of acetate non-utilizing (*acu*) mutants in *Aspergillus nidulans*. J.Gen.Microbiol. 92, 263-282.

Ashwell, G. (1962). Enzymes of glucuronic and galacturonic acid metabolism in bacteria. in: Colowick, S.P. and Kaplan, N.O. (eds.) Methods in Enzymology. Academic Press, New York, London, pp. 190-208.

Ashwell, G., Wahba, A.J. and Hickman, J. (1958). A new pathway of uronic acid metabolism. Biochim.Biophys.Acta 30, 186-187.

Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.

Chang, Y.F. and Feingold, D.S. (1969). Hexuronic acid dehydrogenase of *Agrobacterium tumefaciens*. J.Bacteriol. 99, 667-673.

Chang, Y.F. and Feingold, D.S. (1970). D-glucaric acid and galactaric acid catabolism by *Agrobacterium tumefaciens*. J.Bacteriol. 102, 85-96.

Clutterbuck, A.J. (1973). Gene symbols in *Aspergillus nidulans*. Genet. Res. Camb. 21, 291-296.

Cozzarelli, N.R., Koch, J.P., Hayashi, S. and Lin, E.C.C. (1965). Growth stasis by accumulated L- α -glycerophosphate in *Escherichia coli*. J.Bacteriol. 90, 1325-1329.

Ehrlich, F. and Guttmann, R. (1934). Zur Kenntnis der 5-Keto-l-galactonsäure (d-Tagaturonsäure). Ber.d.Deutsche Chem.Gesellsch. 67, 1345-1347.

Farmer, J.J. and Eagon, R.G. (1969). Aldohexuronic acid catabolism by a soil *Aeromonas*. J.Bacteriol. 97, 97-106.

Ghebregzabher, M., Rufini, S., Sapia, G.M. and Lato, M. (1979). Improved thin-layer chromatographic method for sugar separations. J.Chromatogr. 180, 1-16.

Jones, S.C. (1978). Quick analytical separation of glucose and fructose with impregnated woven glass fiber. J.Chromatogr. 166, 587.

Kenney, W.C., Zakim, D., Hogue, P.K. and Singer, T.P. (1972). Multiplicity and origin of isoenzymes of lipoyl dehydrogenase. Eur.J.Biochem. 28, 253-260.

Kilgore, W.W. and Starr, M.P. (1959). Uronate oxidation by phytopathogenic *Pseudomonads*. Nature 183, 1412-1413.

Larsson, K. and Samuelson, O. (1976). Anion-exchange chromatography of alditols in borate medium. Carbohyd.Res. 50, 1-8.

McCully, K. and Forbes, E. (1965). The use of p-fluorophenylalanine with 'master strains' of *Aspergillus nidulans* for assigning genes to linkage groups. Genet.Res.Camb. 6, 352-359.

Payton, M.A. (1978). A genetic study of sugar metabolism and transport in *Aspergillus nidulans*. Ph.D.Thesis Univ. Leicester.

Payton, M.A., McCullough, W., Roberts, C.F. and Guest, J.R. (1977). Two unlinked genes for the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 129, 1222-1226.

Perkins, D.D. (1962). Preservation of *Neurospora* stock cultures with dehydrous silica gel. Can.J.Microbiol. 8, 591-594.

Pontecorvo, G., Roper, J.A., Hemmons, L.J., MacDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans*. Adv.Genet.5, 141-238.

Pratt, J.W. and Richtmeyer,N.K. (1955). Transformation of D-allose to 1.6-anhydro-β-D-allopyranose in acid solution. J.Amer.Chem.Soc. 77, 1906-1908.

Preiss, J. and Ashwell, G. (1963a). Polygalacturonic acid metabolism in bacteria. I. Enzymatic formation of 4-deoxy-L-threo-5-hexoseulose uronic acid. J.Biol.Chem. 238, 1571-1576.

Preiss, J. and Ashwell, G. (1963b). Polygalacturonic acid metabolism in bacteria. II. Formation and metabolism of 3-deoxy-D-glycero-2.5-hexodiulosonic acid. J.Biol.Chem. 238, 1577-1583.

Roberts, C.F. (1964). Complementation in balanced heterokaryons and heterozygous diploids of *Aspergillus nidulans*. Genet.Res.Camb. 5, 211-229.

Sakurai, Y., Fukuyoski, Y., Hamada, M., Hayakwa, T. and Koike, M. (1970). Mammalian α-ketoacid dehydrogenase complexes. VI. Nature of the multiple forms of pig heart lipoamide dehydrogenase. J.Biol.Chem. 245, 4453-4462.

Skinner, V.M. and Armitt, S. (1972). Mutants of *Aspergillus nidulans* lacking pyruvate carboxylase. FEBS Lett. 20, 16-18.

Visser, J., Kester, H.C.M, Derksen, A.C.G. and Uitzetter, J.H.A.A. (1982). Affinity chromatography and affinity electrophoresis: Tools to investigate protein interactions and enzyme mutants. in: Gribnau, T.C.J., Visser, J. and Nivard, R.J.F. (eds.) Affinity chromatography and related techniques. Elsevier, Amsterdam, pp. 425-435.

Zill, L.P., Khym, J.X. and Cheniae, G.M. (1953). The separation of the borate complexes of sugars and related compounds by ion-exchange chromatography. J.Amer.Chem.Soc. 75, 1339-1342.

IV The pyruvate dehydrogenase complex of *Aspergillus nidulans*: purification and characterization

SUMMARY

The pyruvate dehydrogenase complex of *Aspergillus nidulans* was purified to homogeneity by a multistep procedure including extraction and ultracentrifugation, polyethylene glycol precipitation, affinity chromatography on ethanol-Sepharose 2B, ultracentrifugation and finally sucrose density gradient centrifugation. The overall yield was 16% with a specific activity of 7.8 U/mg. Four major protein bands were identified upon SDS polyacrylamide gel electrophoresis of the complex and the molecular weights were found to correspond to the α and β subunits of the pyruvate decarboxylase, the lipoate acetyltransferase and the lipoamide dehydrogenase of other eukaryotes. In addition some evidence is presented that the complex is regulated by phosphorylation and dephosphorylation. The kinase involved is copurified.

INTRODUCTION

The pyruvate dehydrogenase complex is a multienzyme complex catalyzing an essential universal step in aerobic metabolism, the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA, carbon dioxide and NADH.
The overall reaction is:

$$\text{pyruvate} + NAD^+ + CoA \longrightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

The complex is composed of three different enzymatic activities:
pyruvate decarboxylase (E_1: EC 1.2.4.1)
lipoate acetyltransferase (E_2: EC 2.3.1.12)
lipoamide dehydrogenase (E_3: EC 1.6.4.3.)
These enzymes participate in the following multistep reaction
see *e.g.* Hucho (1975):

$CH_3COCOOH+TPP^{*)}-E_1 \longrightarrow CO_2+hydroxyethyl-TPP-E_1$ (1)

$hydroxyethyl-TPP-E_1+lipoyl-E_2 \longrightarrow S-acetyldihydrolipoyl-E_2+TPP-E_1$ (2)

$S-acetyldihydrolipoyl-E_2+CoA \longrightarrow acetyl-CoA+dihydrolipoyl-E_2$ (3)

$dihydrolipoyl-E_2+FAD-E_3 \longrightarrow lipoyl-E_2+reduced\ FAD-E_3$ (4)

$reduced\ FAD-E_3+NAD^+ \longrightarrow NADH+H^+FAD-E_3$ (5)

Much information about the pyruvate dehydrogenase complex has been obtained from studies in prokaryotes especially *Escherichia coli* (Reed, 1974), *Azotobacter vinelandii* (Bresters *et al.*, 1975), *Pseudomonas aeruginosa* (Jeyaseelan *et al.*, 1980) and *Bacillus spec.* (Henderson and Perham, 1980, Visser *et al.*, 1980). Although the general reaction scheme is the same for all organisms there are considerable differences with respect to enzyme regulation, complex composition and structure.

In *Escherichia coli* the pyruvate dehydrogenase complex consists of a core formed by the E_2 component, composed of 24 identical subunits arranged with octahedral symmetry as found by electron microscopy (Reed, 1974; De Rosier *et al.*, 1971). To this core multiple copies of E_1 and E_3 are bound independently (Reed and Oliver, 1968; Reed, 1974). There is a disagreement about the polypeptide chain stoichiometry of the complex as mentioned by various authors. A chain ratio (E_1:E_2:E_3) of 2:2:1 has been reported by Eley *et al.*(1972), Reed (1974), Speckhard *et al.* (1977), Fuller *et al.* (1979) and Angelides *et al.* (1979), whereas a chain ratio of 1.3-1.8 : 1.0 : 0.8-1.0 based on radioamidination experiments has been reported by Danson *et al.* (1979). The molecular weights of the components are: E_1: 96,000-100,000; E_2: 65,000-89,000 and E_3: 54,000-56,000 (Reed, 1974; Vogel, 1977; Gebhardt *et al.*, 1978). A recent estimation of the total complex molecular weight is 6.1×10^6 (Danson *et al.*, 1979).

In *Azotobacter vinelandii* (Bresters *et al.*, 1975) a much smaller pyruvate dehydrogenase complex has been found with a molecular weight of M_r $0.8\text{-}1.2 \times 10^6$ containing four polypeptide chains: E_1 (M_r 89,000), two different E_2-chains (M_r 82,000 and 59,000) one of which can be removed selectively by Blue Dextran Sepharose chromatography (de Abreu *et al.*, 1977) and E_3 (M_r 56,000). Although the sedimentation coefficient of the *A.vinelandii* complex is 17-20 S and that of the *E.coli* complex 53-63 S there must be a resemblance of these complexes because low concentrations of polyethylene glycol 6000 stimulate the association of the *A.vinelandii* complex and then a sedimentation coefficient is observed similar to the *E.coli* complex (Bosma *et al.*, 1980).

*) TPP = Thiamine pyrophosphate.

In *Pseudomonas aeruginosa* (Jeyaseelan *et al.*, 1980) a pyruvate dehydrogenase complex has been found containing three major components and one minor polypeptide: E_1 (M_r 92,500), two E_2 (major component M_r 76,000; minor component M_r 77,800) and E_3 (M_r 58,000). It is unknown thus far whether the transacetylase is particularly susceptible to proteolysis like in *E.coli* or whether these different molecular weights reflect a functional, posttranslational event.

In *Bacillus stearothermophilus* (Henderson *et al.*, 1979; Henderson and Perham, 1980), *Bacillus subtilis* (Visser *et al.*, 1980) and other *Bacillus* species (Visser *et al.*, 1982b),all gram-positive bacteria, there are substantial differences in the structure of the pyruvate dehydrogenase complex as compared to the complex of the prokaryotes mentioned before which are all gram-negative. The bacillar complex resembles the eukaryote counterpart with respect to its high molecular weight (9.6×10^6) and the size of the individual components including two non identical E_1 subunits: E_1 (M_r 42,000) , E_1 (M_r 36,000), E_2 (M_r 57,000) and E_3 (M_r 54,000).

Similar values have been reported for the bovine kidney and heart pyruvate dehydrogenase complexes (Barrera *et al.*, 1972). The two different E_1 polypeptide chains appear as dimers ($\alpha_2\beta_2$) in the native form. Furthermore the E_2 component of the *Bacillus* pyruvate dehydrogenase complex and of mammalian sources show a different symmetry as compared to the *E.coli* complex. In the case of *Bacillus* the E_2 core appears as a regular pentagonal dodecahedron like the mammalian complex (Reed, 1974; Koike and Koike, 1976) whereas the E_2 component of *E.coli* has octahedral symmetry (Danson *et al.*, 1979).

The large difference between the quaternary enzyme structures obtained from gram-negative bacteria at one hand and from gram-positive bacteria and mitochondria of eukaryotes at the other hand, is considered by some investigators to support the serial endosymbiosis theory for the evolution of the mitochondrion from a gram-positive ancestor (Henderson *et al.*, 1979; Kresze and Ronft, 1981b).

For the pyruvate dehydrogenase complex in eukaryotes three types of regulation have been described as reviewed for instance by Hucho (1975) and Randle (1978): The amount of enzyme is regulated by induction and repression of enzyme synthesis. Regulation occurs by metabolite effectors noticed as end product inhibition by acetyl-CoA and NADH for instance,and the complex is further regulated by two specific enzymes: pyruvate dehydrogenase kinase (firmly bound to the E_2 component), inactivating the complex by phosphorylating αE_1 subunits,and pyruvate dehydrogenase phosphatase (loosely associated) activating by dephosphorylation (Linn *et al.*, 1969; Wieland and Siess, 1970; Hucho *et al.*, 1972; Denton *et al.*, 1975). The activity of the regulating enzymes is also controlled by various metabolite

levels as reflected by different levels of complex activity. In mammalian systems the kinase reaction is modulated by the ratio's of ATP:ADP, acetyl-CoA:CoA, NADH:NAD and inhibited by pyruvate, Mg^{2+}, Ca^{2+}, and TPP whereas the phosphatase is activated by Ca^{2+} and Mg^{2+} (Denton *et al.*, 1975; Randle, 1978).
Similar control mechanisms have been reported to occur in lower eukaryotes such as *Neurospora crassa* (Wieland *et al.*, 1972), although in *Saccharomyces cerevisiae* (Kresze and Ronft, 1981a) and *S.carlsbergensis* (Wais *et al.*, 1973) regulation by interconversion (phosphorylation-dephosphorylation) is lacking.
Investigations of the pyruvate dehydrogenase complex in relation to its genetics have been reported for some prokaryotes. Mutants in all three components of the complex are known in *E.coli* (Henning *et al.*, 1966; Guest and Creaghan, 1973, 1974). The *aceE* (E_1) and *aceF* (E_2) mutant required acetate for aerobic growth on glucose or succinate but were unimpaired in their growth on unsupplemented acetate medium, whereas the *lpd* (E_3) mutant required a combination of acetate and succinate because the *lpd* gene product provides the E_3 component for both the pyruvate and the α-ketoglutarate dehydrogenase complexes (Guest, 1978). A gene-protein relationship for the pyruvate dehydrogenase complex similar to that of *E.coli* has been reported for *Salmonella typhimurium* (Langley and Guest, 1974).
In *Pseudomonas aeruginosa* four *ace* mutants were isolated lacking overall pyruvate dehydrogenase complex activity (Jeyaseelan and Guest, 1980). They resembled the *ace* mutants of *E.coli* and *S.typhimurium* in requiring acetate as an essential supplement for growth on glucose. Three strains (*aceA*) lacked activity of the E_1 component whereas one strain (*aceB*) lacked activity of the E_2 component. Genetic studies (Jeyaseelan and Guest, 1980) showed a close linkage of these genes consistent with the existence of an operon as found in *E.coli*.
In eukaryotes the relationships between the pyruvate dehydrogenase complex and the genes coding for the constituent enzymes were studied especially in fungi. By isolating acetate requiring mutants both in *Neurospora crassa* (Okumura and Kuwana, 1979; Kuwana and Okumura, 1979) and in *Aspergillus nidulans* (Payton *et al.*, 1977; Bos *et al.*, 1981; this thesis chapter III) pyruvate dehydrogenase complex mutants were obtained. In both fungi the genes found thus far which code for components of the pyruvate dehydrogenase complex are located on different chromosomes. There is no tendency to form an operon as in prokaryotes and other control mechanisms will be necessary to balance the ratio in which the enzymes are biosynthesized.
Mutants can be analyzed in several ways: by direct enzyme assay methods, complementation *in vivo* with known mutant strains, reconstitution of enzyme activity *in vitro* with extracts of mutants strains, or by immunological methods. Such

studies were undertaken with pyruvate dehydrogenase complex mutants of *E.coli* (Alwine *et al.*, 1973; Guest and Creaghan, 1974; Jeyaseelan, 1980) and *P.aeruginosa* (Jeyaseelan *et al.*, 1980).

The advantage in using of immunological methods lies in the large number of mutants which can be screened simultaneously,the minute amounts of cell free extract needed and in the information about immunological relationships which can be observed between mutants and wild type. The first step in the use of immunological methods is the purification of the protein of interest for the preparation of antibodies.

The pyruvate dehydrogenase complex has been purified from a number of organisms including bacteria such as *E.coli* (Koike *et al.*, 1960), *A.vinelandii* (Bresters *et al.*, 1975), *B.stearothermophilus* (Henderson and Perham, 1980), *B.subtilis* (Visser *et al.*, 1980) and *P.aeruginosa* (Jeyaseelan *et al.*, 1980). Among the pyruvate dehydrogenase complexes purified from eukaryotic sources are those from plants (Reid *et al.*, 1977; Randall *et al.*, 1977), birds (Jagannathan and Schweet, 1952; Furuta *et al.*, 1977), and mammals (Hayakawa *et al.*, 1966; Ishikawa *et al.*, 1966; Linn *et al.*, 1972; Roche and Cate, 1977).

Among the lower eukaryotes the yeasts *Hansenula miso* (Hirabayashi, and Harada, 1972), *Saccharomyces carlsbergensis* (Wais *et al.*, 1973) and *Saccharomyces cerevisiae* (Kresze and Ronft, 1981a, b) have to be mentioned.

A very general method for the purification of the pyruvate dehydrogenase complex of *E.coli* was described by Reed and Willms (1965) including a protamine sulphate treatment, ultracentrifugation and isoelectric precipitation. This method was further improved by introducing several precipitation steps between pH 5.7 and pH 4.9 and by applying calcium phosphate gel-cellulose chromatography (Speckhard and Frey, 1975). A more rapid method based on a constitutively producing mutant strain used Bio Gel A-50 and calcium phosphate gel-cellulose chromatography successively (Vogel *et al.*, 1972).

In eukaryotes the purification of the pyruvate dehydrogenase complex includes generally the isolation of mitochondria and preparation of a mitochondrial extract, successive fractionations with polyethylene glycol to separate the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex,and ultracentrifugation to remove low molecular weight contaminants and to concentrate the complex (Randall *et al.*, 1977; Roche and Cate, 1977).

A different approach to the purification of the pyruvate dehydrogenase complex from prokaryotes was used by the introduction of affinity chromatography. Ethanol-Sepharose 2B was used as an affinity matrix for the purification of the pyruvate dehydrogenase complex of *E.coli* (Visser *et al.*,1978; Visser and Strating, 1982).

This method was also useful in the purification of the complex from other prokaryotes such as *B.subtilis* (Visser *et al.*, 1980) and *P.aeruginosa* (Jeyaseelan *et al.*, 1980).

For the purification of the pyruvate dehydrogenase complex of lower eukaryotes the procedures described for the yeasts *Hansenula miso* (Hirabayashi and Harada, 1972), *Neurospora crassa* (Harding *et al.*, 1970; Wieland *et al.*, 1972), *Saccharomyces carlsbergensis* (Wais, *et al.*, 1973) and *S.cerevisiae* (Kresze and Ronft, 1981a, b) may serve as examples to come to a general approach.

The principles used in these purifications are based mainly on the ease to precipitate the complex with various reagents such as polyethylene glycol, ammonium sulphate or protamine sulphate combined with the property of this complex to have a high molecular weight that allows concentration by high speed centrifugation. In this chapter a combination of some of these steps (polyethylene glycol precipitation and high speed centrifugation) together with affinity chromatography on ethanolamine-Sepharose 2B is used to purify the pyruvate dehydrogenase complex of *Aspergillus nidulans*.

MATERIALS AND METHODS

Chemicals

Sepharose 2B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and ethanolamine from Fluka (Buchs, Switzerland). Coenzyme A, nicotinamide adenine dinucleotide (NAD^+) and acetylpyridine adenine dinucleotide (free acid) ($APNAD^+$) were supplied by Boehringer (Mannheim F.R.G.).D,L dithiothreitol (DTT) and thiamine pyrophosphate chloride (TPP) were purchased from Sigma (St. Louis MO, USA). Polyethylene glycol 6000 was obtained from BDH (Poole, U.K.). All other chemicals were from Merck (Darmstadt, F.R.G.).

Strains

Aspergillus nidulans WG096 (*yA2,pabaA1*), WG145 (*wA3;pyroA4*) and a green diploid strain WG096//WG145 were used to determine the levels of pyruvate dehydrogenase complex activity. The diploid strain was used to obtain fungal biomass for enzyme purification purposes.

Culture conditions

Aspergillus nidulans was maintained on CM 0.1 M acetate with 0.01 M sucrose for the preparation of mycelium with a high density of conidiospores. *A.nidulans*

was cultured in 7 l batches of minimal liquid medium with sucrose as a C-source as described in chapter I section 3.5, or for small scale experiments in 300 ml batches in a Gallenkamp orbital shaker with 200 rev. min^{-1} at 37°C.

Preparation of cell free extract

For small scale experiments up to 20 g frozen mycelium was ground manually in a mortar with liquid nitrogen and for large scale preparations up to 250 g mycelium was homogenized in a stainless steel Waring Blendor with liquid nitrogen for 10-15 minutes at 90-100 Volt. After evaporation of the nitrogen,buffer was added (approx. 2 ml g^{-1} of mycelium) consisting of potassium phosphate 50 mM pH 7.0, magnesium chloride 1 mM, EDTA 1 mM, 2-mercaptoethanol 5 mM, TPP 0.2 mM and glycerol 5% (v/v) (buffer A). After thawing the mixture was allowed to stand for 1 hour at 4°C and was occasionally stirred. The solution obtained after filtration through muslin was centrifuged for 15 minutes at 40,000 x *g* and the supernatant was used as a crude extract for further experiments. In later steps of the purification buffer B was used. This buffer had the same composition as buffer A except that TPP was omitted.

Protein determination

Protein was measured according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Sucrose density gradients

Samples were layered on linear sucrose density gradients of 10-30% (w/v) sucrose in buffer A prepared with a gradient mixer. Centrifugation was performed during 3.5 hours in a 6 x 14 MSE swing-out titanium rotor at 150,000 x *g*. Fractions of approx. 1 ml were collected by elution of the gradient from bottom to top.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Samples were dialyzed against 0.05 M sodium phosphate buffer pH 7.5 and if necessary concentrated by freeze drying.
SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970) in 10% (w/v) acrylamide slab gels pH 8.8 (length 6 cm, width 7.4 cm, thickness 0.3 cm) with 3% (w/v) acrylamide spacer gels pH 6.8. Samples in 10% (v/v) glycerol were applied on top of the gels and electrophoresis was carried out in 0.025 M Tris. - 0.19 M glycine buffer pH 8.8. A constant current of 15 mA/gel was applied during 15 minutes to allow the samples to migrate into the gel. Then

the electrophoresis buffer was circulated continuously and the current was raised to 25 mA/gel. As internal marker Bromophenol Blue was used.
After the run proteins in the gel were fixed with a mixture of methanol, water and acetic acid (454:454:92 by volume) and stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in the same solution. Destaining was achieved in a mixture of methanol, water and acetic acid (100:75:825 by volume).

Enzyme assays

Pyruvate dehydrogenase complex activity was measured spectrophotometrically by a modified assay according to Schwartz and Reed (1970), following continuously the keto-acid dependent reduction at 25°C of either 3-acetylpyridine NAD^+ ($APNAD^+$) or NAD^+ at 366 nm (APNADH, $E_{366-480} = 9.1 \times 10^6\ cm^2\ mole^{-1}$) or 340 nm (NADH, $E_{340-380} = 5.33 \times 10^6 cm^2\ mole^{-1}$) with an Aminco change DW2 spectrophotometer in the dual wavelength mode with reference at 480 nm ($APNAD^+$assay) or 380 nm (NAD^+assay).
In crude extracts $APNAD^+$ is preferred in view of the interference of NADH oxidase in the NAD^+assay. In all other steps of the purification the NAD^+assay was used to measure enzyme activity. One unit (U) is defined as the amount of enzyme required to produce 1 µmole of NADH per minute under the conditions specified.
For the NAD^+assay the reaction conditions were: DTT 2.5 mM, CoA 0.13 mM, NAD^+ 0.75 mM, TPP 0.4 mM, $MgCl_2$ 2 mM, sodium pyruvate 5 mM in potassium phosphate buffer 120 mM pH 7.0.
For the $APNAD^+$assay the reaction conditions were: DTT 2.5 mM, CoA 0.07 mM, $APNAD^+$ 0.36 mM, TPP 0.24 mM, $MgCl_2$ 1.2 mM, sodium pyruvate 6 mM in Tris.HCl buffer 61 mM pH 8.0.

α-Ketoglutarate dehydrogenase complex activity was assayed under identical conditions as the pyruvate dehydrogenase complex activity except that sodium ketoglutarate was used instead of sodium pyruvate.

Pyruvate kinase activity was measured as will be described in chapter V.

Preparation of ethanol-Sepharose 2B

The procedure for the preparation of the affinity adsorbent is based on the method described by Sundberg and Porath (1974). Suction-dried Sepharose 2B (50 g) was washed with water on a Büchner funnel and then mixed with 50 ml of 1.4-butanediol-diglycidyl ether and 50 ml of 0.6 M NaOH containing 2 mg of $NaBH_4$ per millilitre. The reaction mixture is gently rotated overnight (a Büchi Rotavapor is used) at 25°C. The activated Sepharose is carefully washed on a Büchner funnel with water (25-50 l). The activated matrix is then suspended in

200 ml of 0.01 M NAOH containing 0.2 M ethanolamine and allowed to react at 40°C for 2 hours. This modified coupling resulted in a constant degree of substitution (Visser *et al.*, unpublished results). The matrix finally obtained was washed extensively with deionized water, 2 M NaCl, again water and then stored at 4°C in potassium phosphate buffer pH 7.0. It contained 0.65 mmoles of ligand g^{-1} dry Sepharose 2B.

RESULTS

Enzyme levels in crude extracts of Aspergillus nidulans strains

In Table IV.1. the activities are shown of the pyruvate dehydrogenase complex and of pyruvate kinase as measured in freshly prepared crude extracts.

Table IV.1. Enzyme levels in crude extracts of *Aspergillus nidulans*[1)]

Strain	Specific Activity (U mg^{-1})	
	pyruvate dehydrogenase complex ($APNAD^+$ assay)	pyruvate kinase
WG096	0.009	0.37
WG145	0.007	0.38
Diploid	0.012	0.52

[1)]Based on at least two cultures.

The levels of enzyme activities were measured immediately after culturing 300 ml batches minimal medium with 0.06 M sucrose for 20 hours at 37°C in a Gallenkamp orbital shaker at 200 rpm. Although the pyruvate dehydrogenase complex activity was somewhat higher in the diploid strain under these conditions, there is no difference in enzyme activities of these strains in more vigorously aerated cultures. For the purification of the pyruvate dehydrogenase complex the diploid strain was used. There was another important reason to use this strain to obtain fungal biomass: The yellow conidiated WG096 strain used as a wild type strain with respect to carbon metabolism initially gave rise to several inconveniences. To obtain conidiospores this strain was cultured first on CM with sucrose 0.02 M. As mentioned in chapter I section 3.5 this medium caused wrinkling of the agar surface hindering the harvesting of spores. Moreover often bad conidiation was observed with concomittant growth of non sporulating mycelium, maybe due to the

spontaneous generation of undifferentiated variants as was observed earlier by Dorn (1970). This problem was not encountered with the diploid strain. In later experiments a mixture of sucrose and acetate was used to obtain conidiopores and sporulation of the WG096 and WG145 strain was as good as with the diploid strain.

Purification of the pyruvate dehydrogenase complex

The whole procedure was performed within four days. This was necessary because considerable loss of the enzymatic activity occurred especially if a prolonged time was used to carry out the first steps. Ultimately a scheme was developed with exclusion of a dialysis step in the early stage of the purification.
A five-step purification scheme was necessary which included: 1) the preparation of a crude extract and ultracentrifugation to concentrate the complex, 2) polyethylene glycol (PEG) precipitation, 3) affinity chromatography on ethanol-Sepharose 2B, 4) concentration by ultracentrifugation and 5) sucrose density gradient centrifugation. It was important to avoid dialysis in the early stage of the purification because even a very short dialysis time resulted in considerable loss of enzyme activity. Therefore protamine sulphate or ammonium sulphate precipitation was avoided. Unless stated otherwise all steps of the purification scheme (see Table IV.2) were carried out at $4^{o}C$.

Table IV.2. Purification of the *A.nidulans* pyruvate dehydrogenase complex

Step	Volume (ml)	Protein ($mg.ml^{-1}$)	Specific activity[1] ($U.mg^{-1}$)	Overall yield (%)
Crude extract	425			
Dissolved pellet after ultracentrifugation	50	21	0.170	100
Dissolved PEG precipitate	17	31	0.188	56
Pool ethanol-Sepharose 2B	64	0.54	2.1	41
Pellet after ultracentrifugation	1.5	7.0	6.4	38
Pool sucrose density gradient	3.9	0.94	7.8	16

1) NAD^{+}assay

Step 1. Extraction and ultracentrifugation

A crude extract of 425 ml was obtained after homogenization of 238 g frozen *Aspergillus nidulans* diploid mycelium as described in Materials and Methods. This was centrifuged during 4.5 hours at 150,000 x g. The supernatant was decanted and to the yellow pellets 2 ml of buffer B was added. The rotor tubes were placed in such a way that the pellets were submerged. The pellets were left overnight to allow the pyruvate dehydrogenase complex to dissolve.

Step 2. Polyethylene glycol precipitation

The dissolved pellets were pooled and the light yellow solution obtained was further diluted with buffer B to give an absorbance at 280 nm of ≒ 40 corresponding with approx. 10 $mg.ml^{-1}$ protein. Then $MgCl_2$ and NaCl were added up to 10 and 50 mM respectively. Precipitation with polyethylene glycol 6000 was carried out. at room temperature by dropwise addition from a stock solution of polyethylene glycol 6000 (50% w/v) to a final concentration of 2.5% (v/v) and 5% (v/v). Fifteen minutes after the successive concentrations were reached, the precipitates formed were removed by centrifugation for 15 minutes at 40,000 x g. The pyruvate dehydrogenase complex activity remained in the supernatant. Then the polyethylene glycol concentration was raised to 8% (v/v) and the precipitate obtained,containing the pyruvate dehydrogenase complex activity was also collected by centrifugation during 15 minutes at 40,000 x g. The pellets obtained were dissolved in 17 ml of buffer B. This low ionic strength buffer is necessary to achieve binding of the complex to the affinity matrix in the next step. A dialysis step to remove traces of polyethylene glycol was omitted because this caused partial inactivitation (up to 20% within 2 hours) of the complex.

Step 3. Ethanol-Sepharose 2B affinity chromatrography

The clear solution containing the pyruvate dehydrogenase complex activity was loaded on a preequilibrated ethanol-Sepharose 2B column (Ø 2.5 cm; height 12 cm). This column was eluted overnight with the same buffer to remove non binding proteins. When the A_{280} was low a linear gradient (340 ml) was applied by mixing equal volumes (170 ml) of buffer B and 125 mM potassium phosphate buffer which otherwise had the same composition. Fractions of 3 ml were collected. The pyruvate dehydrogenase complex activity was recovered between 70-90 mM potassium phosphate (see Fig. IV.1.).

Step 4. Concentration by ultracentrifugation

The active fractions obtained after ethanol-Sepharose 2B elution were pooled

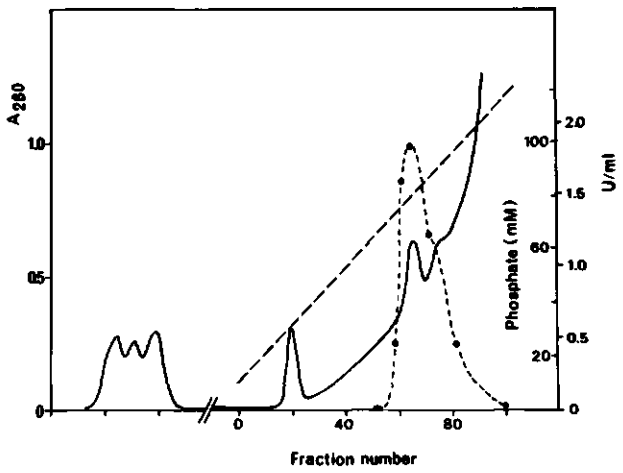

Fig. IV.1. Pyruvate dehydrogenase complex elution profile on ethanol-Sepharose 2B column. 17 ml of the dissolved 8% (v/v) polyethylene glycol precipitate were loaded on an ethanol-Sepharose 2B column (Ø 2.5 cm; height 12 cm) and washed with 300 ml buffer B. A linear gradient (340 ml) of 10-125 mM potassium phosphate was applied. Otherwise the buffer composition remained as in buffer B. Fractions of 3 ml were collected. The pyruvate dehydrogenase complex was recovered between 70-90 mM potassium phosphate.

——— A_{280} — — — potassium phosphate gradient -●--●- pyruvate dehydrogenase complex activity

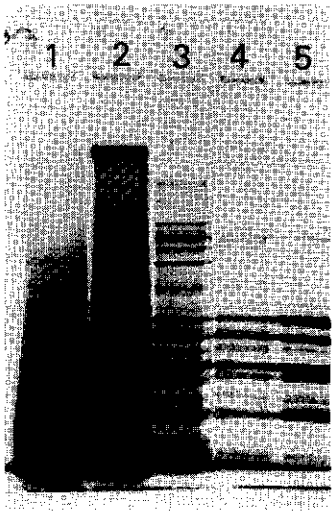

Fig. IV.2. SDS polyacrylamide gel electrophoresis of the pyruvate dehydrogenase complex at different stages of the purification. From the left to the right: 1. crude extract; 2. preparation after first ultracentrifugation; 3. after PEG precipitation; 4. pool ethanol-Sepharose 2B column; 5. after 2nd ultracentrifugation.

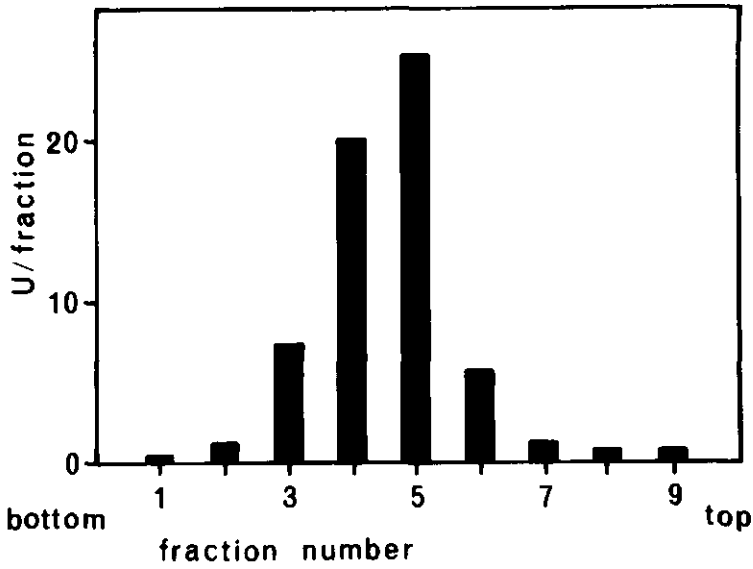

Fig. IV.3a. Sucrose density gradient centrifugation of the pyruvate dehydrogenase complex of *A.nidulans*. The dissolved pyruvate dehydrogenase complex (1.5 ml) was applied on a linear sucrose (10-30% w/v) density gradient and centrifuged for 3.5 hours at 150,000 x *g* in a 6 x 14 MSE Titanium Swing-out rotor. The gradient was eluted from bottom to top and fractions of 1.3 ml were collected and screened for activity.

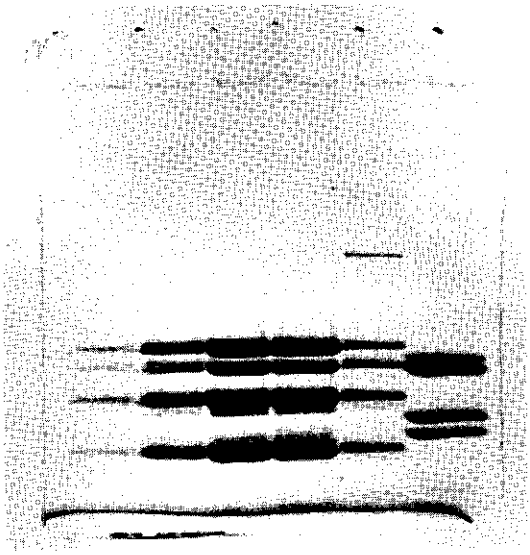

Fig. IV.3b. SDS-polyacrylamide gel electrophoresis of fractions obtained after sucrose density gradient centrifugation. From the left to the right: Fractions 2, 3, 4, 5, 6 and the purified pyruvate dehydrogenase complex of *Bacillus stearothermophilus*. (c.f. Table IV.3.)

and concentrated by ultracentrifugation during 4.5 hours at 150,000 x g. After decantation of the supernatant, which was devoid of enzyme activity the pellets were dissolved overnight in a small volume of buffer A. In Fig. IV.2. samples are shown of the pyruvate dehydrogenase complex at different stages of the purification after SDS-polyacrylamide gel electrophoresis.

Step 5. Sucrose density gradient centrifugation

The solution obtained in the previous step was clarified if necessary by centrifugation, then loaded on a linear sucrose (10-30% w/v) density gradient and centrifuged during 3.5 hours at 150,000 x g.
The gradient was eluted and fractions of 1.3 ml were collected. (see Fig. IV.3a.) These were screened for pyruvate dehydrogenase complex activity. The active fractions appearing at 17-26 % (w/v) sucrose were analyzed further by SDS-polyacrylamide gel electrophoresis (see Fig. IV.3b.). The final preparation contained no α-ketoglutarate dehydrogenase complex activity.

Characterization of the pyruvate dehydrogenase complex

Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed that the purified pyruvate dehydrogenase complex of *A.nidulans* contained several components. The molecular weights (M_r) of these components were estimated from the migration rate relative to reference proteins. The identification of the subunits was made by analogy to other pyruvate dehydrogenase complexes and by comparing SDS-polyacrylamide gel electrophoresis patterns obtained with partially purified pyruvate dehydrogenase complexes of *A.nidulans* mutants lacking E_1 or E_2 component enzyme activity respectively (Visser *et al.*, unpublished results).
In Fig.IV.4. the components of the purified pyruvate dehydrogenase complex together with their molecular weights are indicated.
In Table IV.3. the molecular weights of the components of the pyruvate dehydrogenase complex of several organisms are compared. From these data it can be concluded that the *A.nidulans* complex resembles well the complexes of eukaryotes and *Bacilli* as for subunit composition and their respective molecular weights. As is also observed in the purification of the pyruvate dehydrogenase complex of several other eukaryotes, *e.g. Neurospora crassa* (Wieland *et al.*, 1972) and bovine kidney and heart (Linn *et al.*, 1969, 1972), the pyruvate dehydrogenase kinase of *A.nidulans* is copurified during the procedure described. After the sucrose density gradient step the complex is still easily inactivated in the presence of ATP. Therefore the kinase must be tightly bound to the complex.
In Fig. IV.5 the time dependent inactivation of the complex is shown. The inacti-

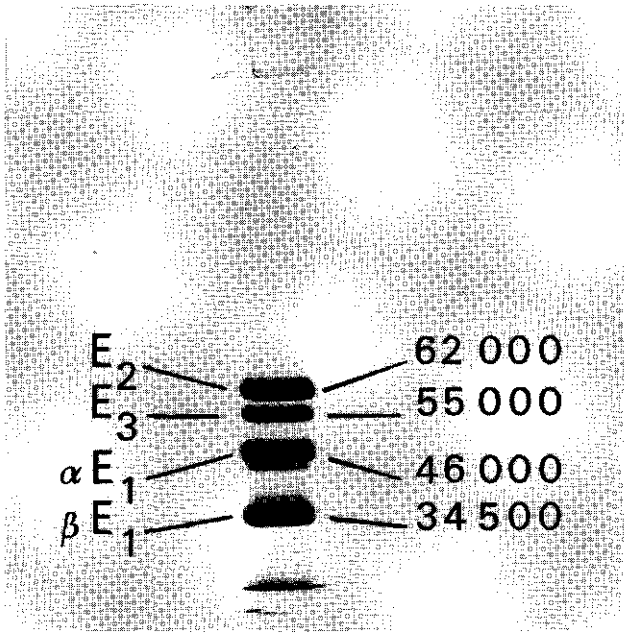

Fig. IV.4. SDS-polyacrylamide gel electrophoresis pattern of the purified pyruvate dehydrogenase complex of *A.nidulans*. The subunits and their respective molecular weights are indicated.

Fig. IV.5. Time dependent inactivation of the purified dehydrogenase complex of *A.nidulans*. 0.9 U of purified pyruvate dehydrogenase complex was dialyzed against 30 mM imidazol buffer pH 7.0. The conditions used for inactivation were: 30 mM imidazol pH 7.0, 1.5 mM $MgCl_2$, 1 mM ATP at 30°C. The residual activity (A) of the complex was measured with the NAD^+ assay and compared to a control sample incubated under the same conditions without ATP.

Insert: Pseudo first order inactivation of the pyruvate dehydrogenase complex. Data obtained above were used to plot In (A) against time.

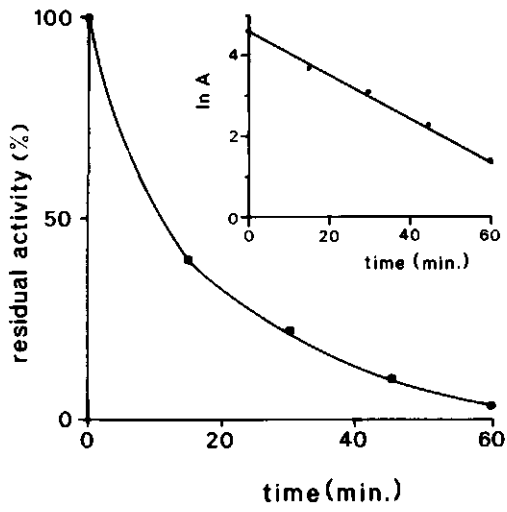

Table IV.3. Molecular weights of the pyruvate dehydrogenase complex components of several organisms.

Organism	Molecular weight E_1	E_2	E_3	References
Escherichia coli	96-100,000	65-89,000	54-56,000	Reed, 1974, Vogel, 1977 Gebhardt *et al.*, 1978
Azotobacter vinelandii	89,000	82,000 59,000	56,000	Bresters *et al.*, 1975
Pseudomonas aeruginosa	92,500	76-78,000	58,000	Jeyaseelan *et al.*, 1980
Bacillus stearothermophilus	42,000 36,000	57,000	54,000	Henderson *et al.*, 1979
Bacillus subtilis	40-41,000 37,000	58-59,000	53,000	Visser *et al.*, 1980
Saccharomyces cerevisiae	α 45,000 β 35,000	58,000	56,000	Kresze and Ronft, 1981b
Aspergillus nidulans	α 46,000 β 34,500	62,000	55,000	present author
Bovine kidney, heart	α 42,000 β 37,000	74,000	53,000	Barrera *et al.*, 1972

Table IV.4. Kinetic constants of the pyruvate dehydrogenase complex from *A.nidulans* compared with those from other eukaryotes

Source	K_m sodium pyruvate	K_m CoA	K_{m_+} NAD^+	K_i NADH	Reference
Aspergillus nidulans	0.16[a)]	1.1×10^{-3}	7.7×10^{-2}	9×10^{-3}	present author
Neurospora crassa	0.26	1.1×10^{-2}	15×10^{-2}	3×10^{-3}	Harding *et al.*, 1970
Saccharomyces cerevisiae	0.65	1.4×10^{-2}	7.2×10^{-2}	2×10^{-3}	Kresze and Ronft, 1981a
Potato tuber	0.18	0.7×10^{-3}	4.5×10^{-2}	6×10^{-3}	Crompton and Laties, 1971
Heart/kidney mammals	0.04	3×10^{-3}	2.5×10^{-2}	2×10^{-3}	see Hucho, 1975

[a)] all values are in mM

In the case of *A.nidulans*, activities were determined in 50 mM Tris buffer pH 7.5, 2.5 mM dithiothreitol, 5 mM magnesium chloride, 0.5 mM thiamine pyrophosphate at 25°C when varying one substrate concentration, the other two were held constant; namely 5 mM sodium pyruvate, 0.13 mM CoA and 0.75 mM NAD^+. In the CoA regenerating system 0.2 mM oxaloacetate and 2.5 units citrate synthase were added.

vation of the pyruvate dehydrogenase complex of *A.nidulans* obeys pseudo first order kinetics with k = 0.058 min^{-1}. (see insert Fig. IV.5.)
From Lineweaver-Burk plots kinetic constants of the pyruvate dehydrogenase complex of *A.nidulans* were obtained. Their values are shown in Table IV.4. together with data obtained from other eukaryotes. NADH is a competitive inhibitor with respect to NAD^+ as is usually observed.

DISCUSSION

The following comments can be given on the subsequent steps in the isolation and purification of the pyruvate dehydrogenase complex of *A.nidulans*. The initial step in the purification of the pyruvate dehydrogenase complex from higher eukaryotes involves generally the isolation of intact mitochondria. The mitochondria of *A.nidulans* are fragile however and are easily disrupted. (Turner and Rowlands, 1977). Therefore a procedure of total extraction of the mycelium was developed using liquid nitrogen to open the cells. Earlier attempts to use buffer A directly as homogenisation medium led to lower recoveries as compared to disruption in liquid nitrogen. The high molecular weight of the complex is advantageous in the ultracentrifugation step both to purify and to concentrate the enzyme. The limitation of this step is the amount of crude extract that can be handled. Thus far ethanol-Sepharose 2B has been used exclusively as an initial step in the purification of the pyruvate dehydrogenase complex from prokaryotes (Visser *et al.*, 1978; 1980; Visser and Strating, 1982). This study demonstrates that a multienzyme complex of eukaryotic origin is also bound. Earlier investigations of Visser *et al.* (1978, 1982a) showed the marked affinity of the pyruvate dehydrogenase complex of prokaryotes such as *E.coli* , *P.aeruginosa* and several *Bacillus species* to hydrophobic matrices with small alkyl (C_3 - C_5) and ω-amino alkyl residues as ligands. Problems however were encountered to achieve a recovery of the overall complex activity from these matrices. Accidently the strong reversible interaction of the pyruvate dehydrogenase complex with ethanolamine coupled to Sepharose was discovered. Similar results were obtained with C_3-C_5 analogues of ethanolamine coupled to Sepharose. When however glycerol or 2-mercaptoethanol were coupled no affinity was observed. The combination of an alkanolic residu with a nitrogen bond seemed therefore essential to achieve a reversible interaction of the complex. The observed affinity opens possibilities to investigate the interaction of mutant complexes with ethanol-Sepharose. From studies with *E.coli* mutants defective in one of the components of the pyruvate dehydro-

genase complex it became clear that the core of the complex, the E_2 component, was responsible for binding since mutants defective in this enzyme showed frontal elution of the other complex components (Visser *et al.*, 1982a). In the case of *A.nidulans* a similar behaviour of mutants defective in the pyruvate dehydrogenase complex was observed. As a result a *pdhC* and a *pdhB* mutant, deficient in the $E_1\alpha$ and $E_1\beta$ component respectively, showed an elution pattern similar to the wild type complex eluted under the same conditions. The pyruvate dehydrogenase complex of the *pdhA* mutant with a defective E_2 component hardly showed any affinity to the ethanol-Sepharose matrix. From these results it was concluded that the E_2 component was involved in the binding to the affinity matrix. Because the ethanol-Sepharose 2B matrix is rather aspecific in its binding behaviour of proteins, and the elution conditions used are aspecific too, it was of considerable interest to diminish the amount of contaminating proteins in the preparation of the pyruvate dehydrogenase complex prior to the use of this column. If a crude extract was directly applied to this column only a 2.5 fold purification could be achieved whereas a 12 fold purification was possible when ultracentrifugation and PEG - 6000 precipitation prior to the use of this column were introduced. Above 5% (v/v) polyethylene glycol the complex is precipitated completely. For an optimal recovery of enzyme activity it was essential to dissolve the complex under mild conditions. Dialysis to remove ethylene glycol prior to ethanol-Sepharose 2B affinity chromatography resulted in a partial loss (up to 20% within 2 hours) of enzyme activity and turned out not to be necessary for binding the complex to the matrix. Because the complex still showed some minor high molecular weight contaminations as was observed after SDS-polyacrylamide gel electrophoresis, a sucrose density gradient was used to obtain final purification. The choice of a 10-30% (w/v) sucrose density gradient is essential under the centrifugation conditions used. In the preparation finally obtained there are two minor bands visible in the gels obtained after electrophoresis, with molecular weights of M_r = 43,000 and 36,000 respectively. One of these or both could be due to the pyruvate dehydrogenase kinase. The molecular weight of the bovine heart pyruvate dehydrogenase kinase subunits are 48,000 and 45,000 respectively (Reed and Petitt, 1981) and one of the copurified proteins in this case has a similar molecular weight.

The activity of the *A.nidulans* pyruvate dehydrogenase complex is regulated by interconversion (phosphorylation-dephosphorylation) as are all eukaryotic pyruvate dehydrogenase complexes studied (see Hucho, 1975) except the complex isolated from *S.cerevisiae* (Kresze and Ronft, 1981a). In that particular case it has not been investigated whether inactivation of the pyruvate dehydrogenase kinase could have been the result of the addition of several protease inhibitors

to the buffers used. In the presence of phenylmethanesulfonylfluoride (PMSF) the *A.nidulans* complex is inactivated. Phosphorylation sites in bovine kidney are serine residues as shown by sequence analysis of the fully phosphorylated complex (Davis *et al.*, 1977).
In view of the apparent insensitivity of the *S.cerevisiae* complex towards PMSF the existence of an interconversion mechanism is not likely in this yeast, although in the final preparation obtained by Kresze and Ronft (1981a) still a minor impurity is found ($M_r \approx 53{,}000$) which is not discussed however. The inactivation by ATP seems rather slow, possibly caused by partial loss of the pyruvate dehydrogenase kinase during the purification. Reactivation of the inactivated complex was only observed in crude extracts indicating that the phosphatase is loosely bound (in agreement with observations made with other eukaryotic sources (Linn *et al.*, 1969; 1972). In *N.crassa* reactivation of the complex was also found with mammalian pyruvate dehydrogenase phosphatase (Wieland *et al.*, 1972).

The kinetic parameters of the *A.nidulans* pyruvate dehydrogenase complex show similarity with other eukaryotic complexes. The value of K_m pyruvate is much higher than reported for the mammalian heart and kidney complexes but similar to the value reported for potato tuber and *N.crassa*. The K_m CoA value resembles the mammalian complex being lower than those reported for *N.crassa* and *S.cerevisiae*. The value of K_m NAD^+ was determined in a CoA regenerating system and is similar to those of the *S.cerevisiae* and the *N.crassa* complex. Inhibition by NADH was competitive with respect to NAD^+ as is generally observed. The major difference between the kinetic properties of the complex of *A.nidulans* and of other microorganisms at one hand and those of the mammalian complexes at the other lies in the rather high affinity of the latter complexes for pyruvate.
A difficulty that remains in making such comparisons between kinetic data is the non identity of the reaction conditions used in the various studies.

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REFERENCES

Alwine, J.C., Russell, F.M. and Murray, K.N. (1973). Characterization of an *E.coli* mutant deficient in dihydrolipoyl dehydrogenase activity. J.Bacteriol. 115, 1-8.

Angelides, K.J., Akiayama, S.K. and Hammes, G.G. (1979). Subunit stoichiometry and molecular weight of the pyruvate dehydrogenase complex from *Escherichia coli*. Proc.Nat.Acad.Sci. U.S.A. 76, 3279-3283.

Barrera, C.R., Namihira, G., Hamilton, L., Munk, P., Eley, M.H., Linn, T. and Reed, L.J. (1972). α-Keto acid dehydrogenase complexes. XVI. Studies on the subunit structure of the pyruvate dehydrogenase complexes from bovine kidney and heart. Arch.Biochem.Biophys. 148, 343-358.

Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.

Bosma, H.J., Voordouw, G., de Kok, A. and Veeger, C. (1980). Self-association of the pyruvate dehydrogenase complex from *Azotobacter vinelandii* in the presence of polyethylene glycol. FEBS.Lett. 120, 179-182.

Bresters, T.W., de Abreu, R.A., de Kok, A., Visser, J. and Veeger, C. (1975). The pyruvate dehydrogenase complex from *Azotobacter vinelandii*. I. Purification and properties. Eur.J.Biochem. 59, 335-345.

Crompton, M. and Laties, G.C. (1971). The regulatory function of potato pyruvate dehydrogenase. Arch.Biochem.Biophys. 143, 143-150.

Danson, M.J., Hale, G., Johnson, P., Perham, R.N., Smith, J. and Spragg, P. (1979). Molecular weight and symmetry of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. J.Mol.Biol. 129, 603-617.

Davis, P.F., Pettit, F.H. (1977). Peptides derived from pyruvate dehydrogenase as substrates for pyruvate dehydrogenase kinase and phosphatase. Biochem. Biophys.Res.Commun. 75, 541-549.

De Abreu, R.A., de Kok, A. and Veeger, C. (1977). Transformation of the 4-component pyruvate dehydrogenase complex from *Azotobacter vinelandii* into a 3-component complex. FEBS.Lett. 82, 82-92.

Denton, R.M., Randle, P.J., Bridges, B.J., Cooper, R.H., Kerbey, A.L., Pask, H.T., Severson, D.L., Stansbie, D. and Whitehouse, S. (1975). Regulation of mammalian pyruvate dehydrogenase. Molec.Cell.Biochem. 9, 27-53.

De Rosier, D.J., Oliver, R.M. and Reed, L.J. (1971). Crystallization and preliminary structural analysis of dihydrolipoyl transsuccinylase, the core of the 2-oxoglutarate dehydrogenase complex. Proc.Nat.Acad.Sci. U.S.A. 68, 1135-1137.

Dorn, G.L. (1970). Genetic and morphological properties of undifferentiated and invasive variants of *Aspergillus nidulans*. Genetics 66, 267-269.

Eley, M.H., Namihira, G., Hamilton, L, Munk, P. and Reed, L.J. (1972). α-Keto acid dehydrogenase complexes. XVIII. Subunit composition of the *Escherichia coli* pyruvate dehydrogenase complex. Arch.Biochem.Biophys. 152, 665-669.

Fuller, C.C., Reed, L.J., Oliver, R.M. and Hackert, M.L. (1979). Crystallization of a dihydrolipoyl transacetylase-dihydrolipoyl dehydrogenase subcomplex and its implications regarding the subunit structure of the pyruvate dehydrogenase complex from *Escherichia coli*. Biochem.Biophys.Res.Comm. 90, 431-438.

Furuta, S., Shindo, Y. and Hashimoto, T. (1977). Purification and properties of pigeon breast muscle α-keto acid dehydrogenase complexes. J.Biochem. (Tokyo) 81, 1839-1847.

Gebhardt, T., Mecke, D. and Bisswanger, H. (1978). Dihydrolipoamide transacetylase from *Escherichia coli*: evidence for internal gene duplication. Biochem. Biophys.Res.Comm. 84, 508-514.

Guest, J.R. (1978). Aspects of the molecular biology of the lipoamide dehydrogenase. Adv.Neurology 21, 219-244.

Guest, J.R. and Creaghan, I.T. (1973). Gene-protein relationships of the α-keto acid dehydrogenase complexes of *Escherichia coli* K12: isolation and characterization of lipoamide dehydrogenase mutants. J.Gen.Microbiol. 75, 197-210.

Guest, J.R. and Creaghan, I.T. (1974). Further studies with lipoamide dehydrogenase mutants of *Escherichia coli* K12. J.Gen.Microbiol. 81, 237-245.

Harding, R.W., Caroline, D.F. and Wagner, R.P. (1970). The pyruvate dehydrogenase complex from the mitochondrial fraction of *Neurospora crassa*. Arch. Biochem.Biophys. 138, 653-661.

Hayakawa, T., Hirashima, M. Ide, S., Hamada, M., Okabe, K. and Koike, M. (1966). Mammalian α-keto acid dehydrogenase complexes. I. Isolation, purification and properties of pyruvate dehydrogenase complex of pig heart muscle. J.Biol. Chem. 241, 4694-4699.

Henderson, C.E. and Perham, R.N. (1980). Purification of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus* and resolution of its four component polypeptides. Biochem.J. 189, 161-172.

Henderson, C.E., Perham, R.N. and Finche, J.T. (1979). Structure and symmetry of *Bacillus stearothermophilus* pyruvate dehydrogenase multienzyme complex and implications for eucaryote evolution. Cell 17, 85-93.

Henning, U., Dennert, G., Hertel, R. and Shipp, W.S. (1966). Translation of the structural gene of the *E.coli* pyruvate dehydrogenase complex. Cold Spring Harb.Symp.Quant.Biol. 31, 227-234.

Hirabayashi, T. and Harada, T. (1972). Isolation and properties of a pyruvate dehydrogenase complex from the yeast *Hansenula miso*. J.Biochem. (Tokyo) 71, 797-804.

Hucho, F. (1975). The pyruvate dehydrogenase multienzyme complex. Angew.Chem. 14, 591-601.

Hucho, F., Randall, D.D., Roche, T.E., Burgett, M.W., Pelley, J.W. and Reed, L.J. (1972). α-Keto acid dehydrogenase complexes. XVII. Kinetic and regulatory properties of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase from bovine kidney and heart. Arch.Biochem.Biophys. 151, 328-340.

Ishikawa, E., Oliver, R.M. and Reed, L.J. (1966). α-Keto acid dehydrogenase complexes. V. Macromolecular organization of pyruvate and α-ketoglutarate dehydrogenase complexes isolated from beef kidney mitochondria. Proc.Nat. Acad. Sci. U.S.A. 56, 534-541.

Jagannathan, V. and Schweet, R.S. (1952). Pyruvic oxidase of pigeon breast muscle. I. Purification and properties of the enzyme. J.Biol.Chem. 196, 551-562.

Jeyaseelan, K. (1980). Biochemical and genetic studies of bacterial pyruvate dehydrogenase complexes. Ph.D.Thesis. Univ.of Sheffield (U.K.).

Jeyaseelan, K. and Guest, J.R. (1980). Isolation and properties of pyruvate dehydrogenase complex mutants of *Pseudomonas aeruginosa* PAO. J.Gen.Microbiol. 120, 385-392.

Jeyaseelan, K., Guest, J.R. and Visser, J. (1980). The pyruvate dehydrogenase complex of *Pseudomonas aeruginosa* PAO. Purification, properties and characterization of mutants. J.Gen.Microbiol. 120, 393-402.

Koike, M. and Koike, K. (1976). Structure, assembly and function of mammalian α-keto acid dehydrogenase complexes. Adv.Biophys. 9, 187-227.

Koike, M, Reed, L.J. and Carroll, W.R. (1960). α-Keto acid dehydrogenation complexes. I. Purification and properties of pyruvate and α-ketoglutarate dehydrogenation complexesof*Escherichia coli*. J.Biol.Chem. 235, 1924-1930.

Kresze, G.B. and Ronft, H. (1981a). Pyruvate dehydrogenase complex from bakers yeast. 1. Purification and some kinetic and regulatory properties. Eur.J. Biochem. 119, 573-579.

Kresze, G.B. and Ronft, H. (1981b). Pyruvate dehydrogenase complex from bakers yeast. 2. Molecular structure, dissociation, and implications for the origin of mitochondria. Eur.J.Biochem. 119, 581-587.

Kuwana, H. and Okumura, R. (1979). Genetics and some characteristics of acetate requiring strains in *Neurospora crassa*. Japan.J.Genet.54, 235-244.

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

Langley, D. and Guest, J.R. (1974). Biochemical and genetic characteristics of deletion and other mutant strains of *Salmonella typhimurium* LT2 lacking α-keto acid dehydrogenase complex activities. J.Gen.Microbiol. 82, 319-335.

Linn, T.C., Pettit, F.H., Hucho, F. and Reed, L.J. (1969). α-Keto acid dehydrogenase complexes. XI. Comparative studies of regulatory properties of the pyruvate dehydrogenase complexes from kidney, heart and liver mitochondria. Proc.Nat.Acad.Sci. U.S.A. 64. 227-234.

Linn, T.C., Pelley, J.W., Pettit, F.H., Hucho, F., Randall, D.D. and Reed, L.J. (1972). α-Keto acid dehydrogenase complexes. XV. Purifications and properties of the component enzymes of the pyruvate dehydrogenase complexes from bovine kidney and heart. Arch.Biochem.Biophys. 148, 327-342.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein determination with the Folin phenol reagent. J.Biol.Chem. 193, 265-275.

Okumura, R. and Kuwana, H. (1979). Gene-protein relationships in acetate-requiring mutants of *Neurospora crassa* deficient in activity of pyruvate dehydrogenase complex. Japan.J.Genet. 54, 245-257.

Payton, M.A., McCullough, W., Roberts, C.F. and Guest, J.R. (1977). Two unlinked genes for the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 129, 1222-1226.

Randall, D.D., Rubin, P.M. and Fenko, M. (1977). Plant pyruvate dehydrogenase complex, purification characterization and regulation by metabolites and phosphorylation. Biochim.Biophys.Acta 485, 336-349.

Randle, P.J. (1978). Pyruvate dehydrogenase complex - meticulous regulator of glucose disposal in animals. Trends in Biochem.Sci. 10, 217-219.

Reed, L.J. (1974). Multienzyme complexes. Acc.Chem.Res. 7, 40-46.

Reed, L.J. and Oliver, R.M. (1968). The multienzyme α-keto acid dehydrogenase complexes. Brookhaven Symp.Biol. 21, 397-411.

Reed, L.J. and Pettit, F.H. (1981). Phosphorylation and dephosphorylation of pyruvate dehydrogenase. Cold Spring Harbor Conferences on cell proliferation. Vol. 8: Protein phophorylation, pp. 701-711.

Reed, L.J. and Willms, C.R. (1965). Purification and resolution of the pyruvate dehydrogenase complex (*Escherichia coli*). in: Colowick, S.P. and Kaplan, N.O. (eds.). Methods in Enzymology. Vol. IX. Academic Press, New York, pp. 247-265.

Reid, E.E., Thompson, P., Lyttle, C.R. and Dennis, D.T. (1977). Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. Plant Physiol. 59, 842-848.

Roche, T.E. and Cate, R.L. (1977). Purification of porcine liver pyruvate dehydrogenase complex and characterization of its catalytic and regulatory proper-

ties. Arch.Biochem.Biophys. 183, 664-677.

Schwartz, E.R. and Reed, L.J. (1970). Regulation of the activity of the pyruvate dehydrogenase complex of *Escherichia coli*. Biochemistry 9, 1434-1439.

Speckhard, D.C. and Frey, P.A. (1975). *Escherichia coli* pyruvate dehydrogenase complex: improved purification and the flavin content. Biochem.Biophys. Res. Commun. 62, 614-620.

Speckhard, D.C., Ikeda, B.H., Wong, S.S. and Frey, P.A. (1977). Acetylation stoichiometry of *Escherichia coli* pyruvate dehydrogenase complex. Biochem. Biophys.Res.Comm. 77, 708-713.

Sundberg, L. and Porath, J. (1974). Preparation of adsorbents for biospecific affinity chromatography. I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. J.Chromatogr. 90, 87-98.

Turner, G. and Rowlands, R.T. (1977). Mitochondrial genetics of *Aspergillus nidulans*. in: Smith, J.E. and Pateman, J.A. (eds.) Genetics and physiology of fungi. Academic Press, London, New York, San Francisco. pp. 319-337.

Visser, J., Kester, H.C.M., Derksen, A.C.G. and Uitzetter, J.H.A.A. (1982a). Affinity chromatography and affinity electrophoresis: tools to investigate protein interactions and enzyme mutants. in: Gribnau, T.C.J., Visser, J. and Nivard, R.J.F. (eds.) Affinity chromatography and related techniques. Elsevier, Amsterdam, pp. 425-435.

Visser, J., Kester, H. and Huygen, A. (1980). Purification and some properties of the pyruvate dehydrogenase complex of *B.subtilis*. FEMS Microbiol. Lett. 9, 227-232.

Visser, J., Kester, H., Jeyaseelan, K. and Topp, R. (1982b). Pyruvate dehydrogenase complex from *Bacillus*. in: Colowick, S.P. and Kaplan, N.O. (eds.) Methods in Enzymology. Vol. 89. Academic Press, New York, pp. 399-407.

Visser, J. and Strating, M. (1982). Pyruvate dehydrogenase complex from *Escherichia coli*. in: Colowick, S.P. and Kaplan, N.O. (eds.) Methods in Enzymology. Vol. 89. Academic Press, New York, pp. 391-399.

Visser, J., van Dongen, W. and Strating, M. (1978). Ethanolamine, a useful ligand for large scale purification of bacterial pyruvate dehydrogenase complex by affinity chromatography. FEBS Lett. 85, 81-85.

Vogel, O. (1977). Redetermination of the molecular weights of the components of the pyruvate dehydrogenase complex from *E.coli* K12. Biochem.Biophys.Res.Commun. 74, 1235-1241.

Vogel, O., Hoehn, B. and Henning, U. (1972). Molecular structure of the pyruvate dehydrogenase complex from *Escherichia coli* K12. Proc.Nat.Acad.Sci. U.S.A. 69, 1615-1619.

Wais, U., Gillmann, U. and Ullrich, J. (1973). Isolation and characterization of pyruvate dehydrogenase complex from brewers yeast. Hoppe-Seyler's Z.Physiol. Chem. 354, 1378-1388.

Wieland, O.H., Hartmann, U. and Siess, E.A. (1972). *Neurospora crassa* pyruvate dehydrogenase: Interconversion by phosphorylation and dephosphorylation. FEBS Lett. 27, 240-244.

Wieland, O. and Siess, E. (1970). Interconversion of phospho- and dephospho-forms of pig heart pyruvate dehydrogenase. Proc.Nat.Acad.Sci. U.S.A. 65, 947-954.

V Purification of pyruvate kinase of *Aspergillus nidulans*[1)]

SUMMARY

Reactive triazine dyes coupled to a solid support have been used in the screening of a suitable affinity ligand for pyruvate kinase of *Aspergillus nidulans*. Mikacion Brilliant Yellow 6GS and Blue Dextran 2000 were used in the purification of pyruvate kinase. The addition of ethylene glycol was necessary to warrant optimal enzyme recovery. A homogeneous enzyme preparation with a specific activity of 67 U/mg could be obtained by a final gel filtration on Sephacryl S-300 to remove minor contaminations. Due to instability of the enzyme the overall yield is low (approx. 4%).

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40), one of the key regulatory enzymes of glycolysis, catalyzes the conversion of phosphoenol-pyruvate (PEP) generating adenosine-5'-triphosphate (ATP):

$$ADP + PEP \xrightarrow{K^+/Mg^{2+}} ATP + pyruvate$$

Fungal pyruvate kinases have been studied in *Saccharomyces spec.* and *Neurospora crassa*. In *A.nidulans* a number of pyruvate kinase mutants were isolated (Payton and Roberts, 1976; Payton, 1978; Bos *et al.*, 1981) and therefore interest had arisen in the development of a mild purification method which might also be useful for isolating mutant proteins.

Pyruvate kinase of *Neurospora crassa* was purified by Kapoor and Tronsgaard (1972), using a heat treatment, ammonium sulphate precipitation, elution from DEAE-Sephadex, ammonium sulphate fractionation and finally gel filtration on Sephadex G-200. The results of various investigations indicate that this enzyme

1) Parts of this chapter were published elsewhere (Visser *et al.*, 1982).

normally exists as a tetramer (molecular weight ≈ 240.000 D) composed of similar subunits (Kapoor, 1975; 1976).

Pyruvate kinase of *Saccharomyces spec.* has been studied by several investigators (Hunsley and Suelter, 1969; Fell *et al.*, 1974). The various steps normally applied in the purification scheme of this enzyme include cytolysis by toluene, ammonium sulphate precipitation,chromatography on DEAE-cellulose and on cellulose phosphate to yield a more or less homogeneous preparation. Haeckel *et al.* (1968) reported the purification of yeast pyruvate kinase using cytolysis by toluene, alkaline extraction, ammonium sulphate fractionation and gel filtration on Sephadex G-200 at a low ionic strength in the presence of Blue Dextran and a second gel filtration step at high ionic strength to separate pyruvate kinase from Blue Dextran. All investigators experienced the instability of pyruvate kinase at various steps during the purification. Fell *et al.* (1974) described various precautions to be taken in order to prevent degradation of the enzyme including a pH > 6 to keep some yeast proteases inactive and the addition of α-toluene-sulphonylfluoride as protease inhibitor. Especially in dilute solutions the activity of pyruvate kinase is lost quickly. Hunsley and Suelter (1969) reported the stabilization of pyruvate kinase by the addition of glycerol (50% v/v).
In the purification of pyruvate kinase from human erythrocytes Staal *et al.*(1971) applied DEAE-Sephadex chromatography, ammonium sulphate precipitation, heat treatment, ultrafiltration and two Sephadex G-200 gelfiltration steps with Blue Dextran similar as described for the yeast pyruvate kinase purification. Staal *et al.* mentioned the addition of glycerol and ε-aminocaproic acid to stabilize the enzyme.
In various other purification procedures for pyruvate kinase using sources such as human erythrocytes (Marie *et al.*, 1977),human kidney (Harkins *et al.*, 1977), rabbit muscle (Easterday and Easterday, 1974) and *Streptococcus mutans* (Abbe and Yamada, 1982) Reactive Blue 2 (the chromophore of Cibacron Blue 3G-A) has been used as an affinity ligand. This dye can be bound to the matrix by direct coupling or by coupling a dextran conjugate of this dye (Haff and Easterday, 1978; Dean and Watson, 1979).
Blue dextran Sepharose was used by Stellwagen *et al.*, (1975) as an affinity adsorbent specific for proteins whose nucleoside phosphate binding sites are composed of the dinucleotide fold (Schultz and Schirmer, 1974), a supersecondary structure found by X-ray crystallography in a number of enzymes. The chromophore can be oriented to mimic the aromatic rings and anionic groups characteristic of NAD^+ in binding to the dinucleotide fold of dehydrogenases, for instance (Thompson *et al.*, 1975). Also from binding studies,the relation between the

presence of an intact dinucleotide fold in enzymes and the binding to Cibacron Blue was clearly demonstrated (Thompson and Stellwagen, 1976). However, in other experiments unexpectedly proteins were found which showed binding to Blue Dextran Sepharose but which contained no dinucleotide fold. Such proteins, for instance *Staphylococcus* ribonuclease and ferrocytochrome c, can still bind nucleotides (Thompson *et al.*, 1975). In these cases elution by salt (so called "aspecific elution") required approximately the same ionic strength as necessary for "biospecific elution" using nucleotides. However for the elution of proteins containing the dinucleotide fold usually salt concentrations are required of at least an order of magnitude larger than free ligand concentrations required for biospecific elution.

Wilson (1976) demonstrated that enzymes possessing the dinucleotide fold strongly interacted with Cibacron Blue and its conjugate Blue Dextran, whereas yeast hexokinase, an enzyme without the dinucleotide fold, (Anderson *et al.*, 1974) was relatively weakly adsorbed to Cibacron Blue Sepharose and hardly at all to Blue Dextran Sepharose. The author did not mention the concentration of the chromophore in both cases, but assuming these were in the same order of magnitude, the binding to Blue Dextran Sepharose was more selective. In fact there must be other factors involved in protein binding to Cibacron Blue than a specific interaction of the dinucleotide fold because human serum albumin a protein which neither possesses a dinucleotide fold nor has a dinucleotide cofactor requirement also binds to Cibacron Blue (Leatherbarrow and Dean, 1980).

Since the discovery that a number of proteins interact with triazine dyes such as Cibacron Blue 3G-A and Procion Red HE-3B also other reactive dyes, which are produced in bulk quantities by various manufacturers as textile dyes, received attention. The application of reactive dyes in affinity chromatography for the purification of various enzymes has been reviewed on several occasions (Dean and Watson, 1979; Lowe *et al.*, 1981). It is often a very selective and rapid method as compared to other methods applied. A procedure for the purification of pyruvate kinase from *Aspergillus nidulans* based on affinity chromatography with reactive dyes as affinity ligands will be described in this chapter. The advantage in the use of substituted triazine dyes lies in their availability, the broad variation in chromophore structures and simple coupling procedure as compared to nucleotide affinity adsorbents. Moreover and of crucial importance the bond formed is quite stable. In this study mainly Procion MX-dyes were used in the first screening for affinity to pyruvate kinase. These dyes share a common dihalogenated triazine moiety, a reactive aromatic nucleus, which allows a direct coupling to a polysaccharide support under mild conditions.

A purification scheme for pyruvate kinase was developed mainly based on affinity chromatography leading to a homogeneous preparation to be used for the preparation of rabbit antibodies.

MATERIALS AND METHODS

Chemicals

Adenosine-5'-diphosphate monopotassium salt (ADP), adenosine-5'-triphosphate disodium salt (ATP), lactate dehydrogenase (LDH), fructose-1,6-diphosphate trisodium salt (FDP) and phosphoenolpyruvate tricyclohexylammonium salt (PEP) were obtained from Boehringer (Mannheim, F.R.G.).
Sepharose 4B, Sepharose Cl-4B, Blue Dextran 2000 and Sephacryl S-300 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).
Procion dyes were produced by Imperial Chemical Industries Ltd Organics Division, Blackley (Manchester, U.K.).
Mikacion Brilliant Yellow 6 GS was supplied by Nippon Kayaku Co Ltd (Tokyo, Japan).
Cibacron Blue 3G-A was obtained from Ciba Geigy (Arnhem, The Netherlands).
All other chemicals were supplied by Merck (Darmstadt, F.R.G.).

Organism and culture conditions

The *A.nidulans* strain and culture conditions used to obtain fungal biomass were as described in chapter IV.

Preparation of cell free extract

Frozen mycelium was first ground in a mortar and then homogenized in a Warring Blendor with liquid nitrogen during 10 minutes at 90-100Volt.After evaporation of the nitrogen, buffer H or buffer A was added to the powder obtained.
Buffer H consisted of potassium phosphate 50 mM pH 7.5; EDTA 0,5 mM; magnesium chloride 5 mM; 2-mercaptoethanol 5 mM. Buffer A had the same composition as buffer H except that ethylene glycol up to 5% (v/v) was added. After thawing the cell debris was removed by filtration through muslin. The filtrate was centrifuged for 10 minutes at 40.000 x g and the supernatant was used as a crude extract for further purification.

Preparation of affinity matrices

Coupling of reactive dyes to Sepharose Cl-4B

The method used was a modification of a procedure described earlier by Dean

and Watson (1979). For small scale dye screening the following procedure was adopted. Sepharose Cl-4B was washed extensively with deionized water to remove antimicrobial agents and filtrated with suction to remove excess of water. The packed beads (3 g suction dry weight) were resuspended in water (10 ml) and the reactive dye (15-60 mg) was added. The suspension was gently shaken until the dye was completely dissolved. Then 3 M NaCl (2 ml) was added. The mixture was allowed to stand at room temperature for 15 minutes. The coupling was initiated by elevating the pH to 10-11 by adding 1 M Na_2CO_3. The reaction mixture was incubated for 1 hour at 55°C. The suspension was then filtrated, washed extensively with water, 2 M NaCl, water, 4 M urea and water successively The matrix thus obtained was stored at 4°C in potassium phosphate buffer 50 mM pH 7.5, with 0.02% (w/v) sodium azide. The buffer was changed once or twice to remove traces of unreacted dye.

Coupling of Blue Dextran 2000 to Sepharose 4B

Cyanogen bromide activation of agarose was performed according to March *et al.* (1974a, b). Sepharose 4B beads (80 g suction dry weight) were washed with water and then suspended in 160 ml 1M Na_2CO_3. To this suspension a solution of 16 g CNBr in 16 ml acetonitrile (precooled at 4°C) was added within 2 minutes and precautions were taken to prevent a rise of temperature above 15°C. After stirring for 8 minutes the activation was stopped by washing the gel with approximately 4 l 0.2 M $NaHCO_3$-Na_2CO_3 buffer pH 9.8. The activated Sepharose 4B was then added to a solution of 4 g Blue Dextran 2000 conjugate in 160 ml of the same $NaHCO_3$-Na_2CO_3 buffer. The reaction was continued for 10 minutes at 4°C followed by 2 hours at room temperature. Subsequently the reaction was stopped by washing the matrix extensively with water, 2M NaCl and again water. The freshly prepared matrix was stored at 4°C in 50 mM phosphate buffer pH 7.5 with 0.02% (w/v) sodium azide. The buffer was changed one or twice to remove traces of unreacted dye.

Determination of ligand concentrations

The amount of Cibacron Blue 3G-A bound was determined by liberation of the bound ligand from the matrix by acid hydrolysis followed by spectrophotometric determination according to Chambers (1977) using a molar extinction coefficient of 5.61 $mM^{-1}cm^{-1}$ at 515 nm. The concentration of Mikacion Brilliant Yellow 6GS was determined by elemental analysis of the lyophilized matrix with Sepharose Cl-4B as a control. Elemental analyses were carried out by the Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht, the Netherlands under supervision of W.J. Buis.

Enzyme assay

Pyruvate kinase activity was determined by measuring the disappearance of NADH at 340 nm in a Shimadzu UV-210A Double Beam Spectrophotometer using a lactate dehydrogenase coupled assay according to Bergmeyer (1974).
The reaction conditions were: triethanolamine HCl buffer pH 7.6 87.3 mM; PEP 0.53 mM; $MgSO_4$ 2.5mM; KCl 10 mM; ADP 4.7 mM; NADH 0.2 mM; LDH approx. 7 U/ml. All assays were performed at 25°C. 1 Unit of pyruvate kinase activity is defined as the amount of protein which catalyzed the formation of 1 µmole of pyruvate per minute.

Determination of protein

Protein was measured according to Lowry *et al.*(1951) using bovine serum albumin as standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis according to Laemmli (1970) was performed in 10% acrylamide gels as described in chapter IV.

RESULTS

Isolation of pyruvate kinase

Unless otherwise stated all steps were carried out at 4°C. The first step in the isolation of pyruvate kinase was the preparation of a cell free extract by homogenization of the fungal cells. A cell free extract of sucrose grown *A.nidulans* prepared as described in Materials and Methods has a specific activity of approximately 0.3-0.5 U/mg pyruvate kinase activity.
In order to obtain a maximum of pyruvate kinase activity in a small volume, the amount of buffer to be used has to be limited. The low specific activity of a crude extract from *A.nidulans* as compared to extracts obtained from other sources urges the use of at least 100 g of mycelium to perform several purification steps and hence the initial volume of an extract is relatively large. As mentioned in the Introduction a heat treatment or an ammonium sulphate precipitation are often used as a first step in the purification of pyruvate kinase of fungal origin. The large initial volume makes the use of a heat treatment rather teadious because of the difficulty to attain quickly the temperature required in the extract in a reproducible way. Moreover, the application of a heat treatment in the purification of mutant proteins is not recommended because these are often more suscep-

tible to various denaturing conditions. An ammonium sulphate precipitation as first step resulted in a loss of 50% of the initial activity and therefore this method was also rejected. Since it was necessary to use a first purification step which would result in purification and also concentration of the enzyme with a good recovery, the present investigation was undertaken in which reactive dyes were used to fulfil these requirements.

Screening of dyes for pyruvate kinase affinity

Samples of cell free extract (0.5-1.0 U) obtained in buffer H as described in Materials and Methods were applied to Pasteur pipettes (0.5 x 5 cm) filled with several dye-Sepharose conjugates and eluted with the same buffer. The following dyes were tested: Procion Red P 3BN, Procion Yellow M-4G, Procion Yellow MX-6G, Procion Yellow H-5G, Procion Turquoise MX-G, Cibacron Blue 3G-A, Blue Dextran 2000, Procion Navy MX-RB, Procion Brown MX-GRN, Procion Orange MX-G and Procion Yellow MX-4G. Only the last two affinity matrices and Sepharose Cl-4B itself showed no binding of pyruvate kinase under these conditions. Surprisingly the addition of 0.5 M NaCl in the eluent resulted in only very low (< 10%) recovery of pyruvate kinase activity indicating inactivation during interaction with the dye or a very strong binding. Better results with the recovery of pyruvate kinase were achieved by the addition of ethylene glycol (10% v/v) to the crude extract and the eluents used. In most cases however the activity of pyruvate kinase appeared in the void volume of the various columns except for Procion Red P3BN, Blue Dextran Sepharose 4B, Procion Yellow MX-6G and Procion Yellow H-5G. These affinity matrices were also tested for their aptness to allow biospecific elution with 10 mM PEP. This was not achieved with Procion Red P3BN whereas in the case of the other three dyes it was partly possible.

To obtain an impression of scaling-up effects, the two yellow matrices were used in a chromatographic experiment in which larger columns (Ø = 1 cm, h = 10 cm) were used, and biospecific elution of pyruvate kinase was achieved with 10 mM PEP (10 ml). The recovery of enzyme activity was 62% in the case of Procion Yellow H-5G and 80% in the case of Procion Yellow MX-6G. During the course of this experiment the high viscosity of the solutions was recognized as a serious disadvantage. Therefore the optimum ligand concentration of the latter chromophore (Procion Yellow MX-6G, Colour Index No. 18971, Reactive Yellow 1) was finally determined in buffers containing 5% (v/v) ethylene glycol (Buffer A).

Availability of Reactive Yellow 1

At the time of these experiments Procion Yellow MX-6G was no longer available from the manufacturer (ICI, Manchester, U.K.). This dye is also produced as Ostazin Brilliant Yellow S-6G (Chemapol, Prague, Czechoslovakia); Xirion Brilliant Yellow 3G-HD (Chemiekombinat Bitterfeld, D.D.R.) and Mikacion Brilliant Yellow 6GS (Nippon, Kayaku Co Ltd, Tokyo, Japan). The purity of the latter dye served my purposes best and therefore this compound was used for further experiments.

Effect of the ligand concentration of Reactive Yellow 1 on the binding of pyruvate kinase in crude extracts

Various dye concentrations of Mikacion Yellow 6GS were coupled to Sepharose Cl-4B to look into the effect of dye concentration on elution behaviour of pyruvate kinase from a crude extract. Amounts of 5, 10, 15 and 20 mg of dye were used per g of moist Sepharose Cl-4B to prepare affinity columns A, B, C and D respectively.

The reaction conditions are described in Materials and Methods.

Columns A and B showed leakage of pyruvate kinase activity upon loading and also upon elution of the column with buffer A. Columns C and D however showed no leakage. The recovery of enzyme activity by aspecific elution with 0,2 M NaCl was 74% with column C and 57% with column D. Therefore column C was chosen for further experiments.

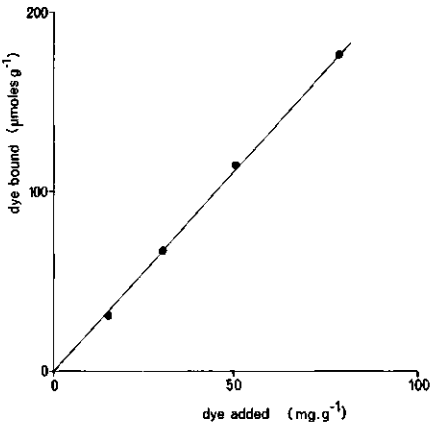
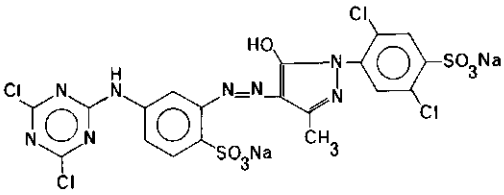


Fig. V.1. Relationship between the amount of crude Mikacion Yellow 6GS (mg/g suction dried Sepharose Cl-4B) used during coupling and actual amount of dye bound to the matrix (μmoles g^{-1} dry Sepharose Cl-4B based on N content).

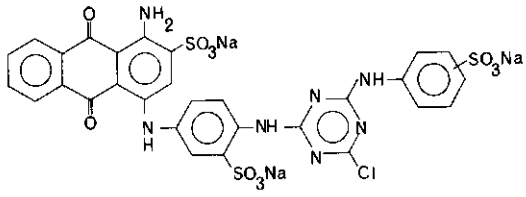
Fig.V.1. shows the relation between the amount of crude Mikacion Brilliant Yellow 6GS used during coupling to Sepharose Cl-4B and the amount of dye actually bound. Calculations were based on N content determined by elemental analysis. Even when using 80 mg of dye per g of Sepharose Cl-4B,still a linear relationship is observed between the amount of dye bound and the amount of dye added in the coupling reaction. This makes it easy to prepare a defined matrix.

Biospecific elution of pyruvate kinase from dye-Sepharose matrices

Mikacion Brilliant Yellow 6GS and Cibacron Blue are structurally quite different compounds as is seen in Fig. V.2.



Mikacion Brilliant Yellow 6GS



Cibacron Blue 3G-A

Fig. V.2. Structures of dyes used as ligand in affinity chromatography of pyruvate kinase.

For optimalization of the elution step it is relevant to know whether these two dyes bind to the same or to different binding sites of the enzyme. Therefore the effect of substrate and possible effectors on the elution behaviour of pyruvate kinase was studied in both cases. Crude extract (5U pyruvate kinase) was loaded on 1 ml columns. Elution occurred with buffer A which contained already 0.1 M and 0.15 M NaCl respectively to weaken the existing interactions. The results are shown in Table V.1.

Table V.1. Biospecific elution of pyruvate kinase from affinity matrices.

Compound added	% Recovery of enzyme activity	
	Mikacion Brilliant Yellow 6GS Sepharose Cl-4B (30 μmoles/g dry weight)	Blue Dextran Sepharose 4B (14 μmoles/g dry weight)
None	0	0
ATP	50	10
FDP	15	53
PEP	41	108

With Blue Dextran both PEP and FDP interfere with the binding whereas ATP is not effective. With the Mikacion Brilliant Yellow matrix however, ATP and PEP are effective and now FDP hardly has any influence. Increasing the salt concentrations leads to higher recoveries.
Because in the majority of these tests no quantitative elution was observed aspecific elution was used in the procedure finally developed for the purification of pyruvate kinase.

Purification of pyruvate kinase

Because both Mikacion Brilliant Yellow 6GS and Blue Dextran emerged from the first dye screening experiments as suitable affinants, these were used in the development of the purification scheme shown in Table V.2.
116 g mycelium of *A.nidulans* was used to prepare a cell free extract of 192 ml in buffer A as described in Materials and Methods. This extract was loaded on a column (Ø 3 cm, height 15 cm) with Mikacion Yellow 6GS Sepharose Cl-4B (30µmoles of dye /g of dry Sepharose) and fractions of 10 ml were collected.
Non binding proteins were eluted with buffer A until the absorption of the eluate at 280 nm was less than 0.2. The elution profile is shown in Fig. V.3. Pyruvate kinase was recovered by elution with buffer A containing 0.5 M NaCl. (arrow in Fig. V.3.). Active fractions were collected and dialyzed overnight against 2 x 2 l buffer A. After dialysis the pyruvate kinase sample was bound to a Blue Dextran Sepharose 4B column (Ø 2 cm, height 9.5 cm; 14 µmoles of Cibacron Blue 3G-A/g of dry weight Sepharose) and washed with buffer A containing 0.1 M NaCl and 50 mM L-alanine (arrow 1 in the elution profile Fig. V.4.). In earlier experiments L-alanine was tested to elute pyruvate kinase from Blue Dextran

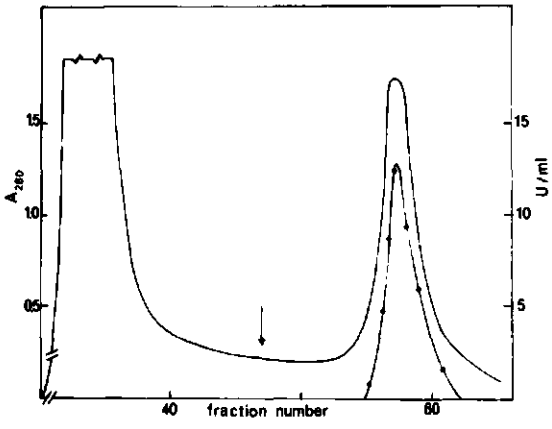


Fig. V.3. Elution profile of Mikacion Brilliant Yellow 6GS Sepharose Cl-4B. 192 ml cell free extract were loaded on a column (Ø 3 cm, height 15 cm) and fractions of 10 ml were collected. Elution of nonbinding proteins occurred with buffer A. Pyruvate kinase was eluted with buffer A containing 0.5 M NaCl (arrow). -●- Pyruvate kinase activity, — A280.

Table V.2. Purification of *A.nidulans* pyruvate kinase

Step	Volume (ml)	Activity (U)	Protein (mg/ml)	Specific activity (U/mg)	Yield (%)
Crude extract	192	484	8.75	0.29	100
Mikacion Brilliant Yellow 6GS Sepharose Cl-4B	59	398	0.84	8.1	82
Blue Dextran Sepharose 4B	30	249	0.36	23.3	53
Sephacryl S-300	27	54	n.d.	n.d.	11
Final preparation after concentration	4.4	17	0.06	67	3.5

n.d. = not determined

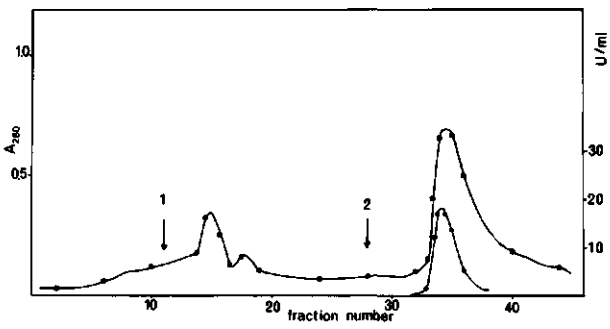


Fig. V.4. Elution profile of Blue Dextran Sepharose 4B. Pyruvate kinase was bound to a column (Ø 2 cm; height 9.5 cm) and washed with buffer A containing 0.1 M NaCl, 50 mM l-Alanine (arrow 1). Pyruvate kinase was eluted with buffer A containing 0.3 M NaCl (arrow 2). Fractions of 5.5 ml were collected. •—• A280; ○—○ Pyruvate kinase activity.

Sepharose. Although it was ineffective, it could be used to remove contaminating proteins which were also bound to this matrix. Pyruvate kinase was recovered by elution with buffer A containing 0.3 M NaCl (arrow 2 in Fig. V.4.) and pooled active fractions were dialyzed against 2 x 1000 ml buffer A. In order to concentrate the pyruvate kinase activity the dialysate was applied to a column filled with Mikacion Brilliant Yellow 6GS Sepharose Cl-4B (Ø = 1 cm, height 2.5 cm; 112 µmoles of dye/g of dry Sepharose Cl-4B), washed with 5 ml buffer A and eluted with the same buffer containing 1 M NaCl. The high concentration of NaCl is used to prevent unnecessary dilution of the pyruvate kinase. Active fractions were pooled (8.6 ml) and after dialysis

against 0.5 M NaCl applied to a column with Sephacryl S-300 (Ø = 1.7, height 86 cm). This column was eluted with buffer A and 0.5 M NaCl. Active fractions were pooled and after dialysis against buffer A to which 0.1 M NaCl was added, the enzyme was concentrated on a Mikacion Brilliant Yellow 6GS Sepharose Cl-4B matrix (Ø = 1.2 cm, height 1 cm; 66 µmoles of dye/g of dry Sepharose Cl-4B). Pyruvate kinase was eluted with buffer A with 1 M NaCl as described above. The purification was further analyzed by SDS polyacrylamide gel electrophoresis. The results are shown in Fig. V.5. From this is was concluded that a homogeneous enzyme preparation was obtained. The molecular weight of the subunit was 60,000 D as estimated by SDS polyacrylamide gel electrophoresis with reference proteins.

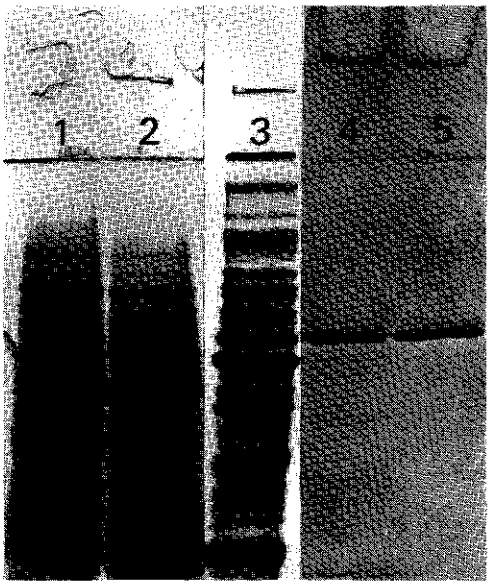


Fig.V.5. SDS-polyacrylamidegel electrophoresis of pyruvate kinase samples at different stages of the purification.

1) Crude extract
2) Eluate of Mikacion Brilliant Yellow 6GS Sepharose Cl-4B column
3) Pyruvate kinase after affinity chromatography on Mikacion Brilliant Yellow 6GS Sepharose Cl-4B column
4) Pyruvate kinase after affinity chromatography on Blue Dextran Sepharose 4B
5) Final preparation after Sephacryl S-300 and concentration on Mikacion Brilliant Yellow 6GS Sepharose Cl-4B.

DISCUSSION

Some general remarks on the use of reactive dyes for affinity chromatography purposes can be made. An essential step in the development of an enzyme purification method based on affinity chromatography is the search for a suitable ligand. Different approaches can be used to select such a ligand. The analysis of dye interactions with an enzyme by binding studies or by inactivation and competition experiments can provide information for a proper selection of the ligand (Lowe *et al.*., 1981) especially when there are large differences in the binding constants or when only a few proteins show an interaction with the ligand. The direct use of a dye immobilized on a solid support in small scale affinity chromatographic experiments as presented in this study, has certain advantages. If in such experiments a crude enzyme preparation is used information is obtained both about the interaction of the enzyme and of the contaminants with the affinity matrix and about the elution conditions which can be applied. Once an interaction of the enzyme is observed, its selectivity, recovery and specific activity can be further improved by varying the ligand concentration or altering the conditions for binding and/or elution (pH, ionic strength, ethylene glycol addition).

From a first screening scheme of dye-Sepharose matrices it was clear that many dyes showed a strong, rather aselective interaction with pyruvate kinase of *A.nidulans*. The recovery of the enzyme activity however was in most cases very low. The addition of ethylene glycol during this screening was useful to warrant the recovery of enzyme activity but it also became clear at this stage that only a few of the ligands tested showed a relatively strong, reversible interaction with pyruvate kinase. Mikacion Brilliant Yellow 6GS and Blue Dextran. Although in early experiments the possibility of a biospecific elution with some ligands was studied, ultimately an aspecific elution was used by enhancing the ionic strength. This was done not only because recoveries were higher but also because it resulted in a sharper elution profile of pyruvate kinase. From the small scale experiments in which Cibacron Blue Dextran and Mikacion Yellow 6GS were tested for biospecific elution it became clear that these dyes interact in a different way with pyruvate kinase. In both cases however binding of pyruvate kinase is very strong because partial elution only occurred in the presence of salt.

The best strategy for pyruvate kinase elution from these dye affinity columns therefore is to find a compromise between elution at high ionic strength (sharp elution profile with many contaminating proteins) and elution at the critical ionic strength, the aim being less overlap with contaminating protein. A dis-

advantage is broadening of the elution profile which enhances the rate of inactivation due to dilution.
The capacity of the dye affinity matrices used for pyruvate kinase is strongly dependent upon the degree of purity of the enzyme applied. Hence one is forced to make an analysis of the column dimensions and elution conditions to be used for each step in the purification in which the dye is used so as to optimize enzyme recovery.

Although attempts were made to use additives to improve the stability of pyruvate kinase this could not be achieved by the addition of glycerol or ε-aminocaproic acid to the various preparations. Since the enzyme was used to prepare antibodies, bovine serum albumin could not be used as stabilizer. The use of Sephacryl S-300 was ultimately necessary to remove some minor contaminations. However in this step pyruvate kinase becomes considerably diluted and tends to loose activity. No alternatives were found for a molecular sieve step. The amount of protein finally obtained in this manner was sufficient for the preparation of antibodies.

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REFERENCES

Abbe, K. and Yamada, T. (1982). Purification and properties of pyruvate kinase from *Streptococcus mutans*. J.Bacteriol. 149, 299-305.

Anderson, W.F., Fletterick, R.J. and Steitz, T.A. (1974). Structure of yeast hexokinase III. Low resolution structure of a second crystal form showing a different quaternary structure heterologous interaction of subunits and substrate binding. J.Mol.Biol. 86, 261-269.

Bergmeyer, H.U. (ed.) (1974). Pyruvat kinase.in: Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim Bnd. 1, pp. 544-545.

Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.

Chambers, G.K. (1977). Determination of Cibacron Blue F3GA substitution in Blue-Sephadex and Blue Dextran-Sepharose. Anal. Biochem. 83, 551-556.

Dean, P.D.G. and Watson, D.H. (1979). Protein purification using immobilised triazine dyes. J.Chromatogr. 165, 301-319.

Easterday, R.L. and Easterday I.M. (1974). Affinity chromatography of kinases and dehydrogenases on Sephadex and Sepharose dye derivatives. in: Dunlap R.B. (ed.) Immobilized biochemicals and affinity chromatography. Advances in experimental medicine and biology Vol. 42. Plenum Press, New York, Londen, pp. 123-133.

Fell, D.A., Liddle, P.F. and Peacocke, A.R. (1974). The preparation and properties of pyruvate kinase from yeast. Biochem.J. 139, 665-675.

Haeckel, R., Hess, B., Lauterborn, W. and Wuster, K. (1968). Purification and allosteric properties of yeast pyruvate kinase. Hoppe-Seyler's Z.Physiol.Chem. 349, 699-714.

Haff, L.A. and Easterday, R.L. (1978). Cibacron Blue-Sepharose: A tool for general ligand affinity chromatography. in: Sundaram, P.V. and Eckstein, F. (eds.) Affinity techniques. Pergamon, Elmsford, N.Y., Oxford, Paris, pp. 23-44.

Harkins, R.N., Black, J.A. and Rittenberg, M.B. (1977). M_2 Isozyme of pyruvate kinase from human kidney as the product of a separate gene: Its purification and characterization. Biochem. 16, 3831-3837.

Hunsley J.R. and Suelter, C.H. (1969). Yeast pyruvate kinase I. Purification and some chemical properties. J.Biol.Chem. 244, 4815-4818.

Kapoor, M. (1975). Subunit structure and some properties of pyruvate kinase of *Neurospora*. Can.J.Biochem. 53, 109-119.

Kapoor, M. (1976). Minireview pyruvate kinase: a model allosteric enzyme for

demonstration of structure-function relationships. Int.J.Biochem. 7, 439-443.
Kapoor, M. and Tronsgaard, T.M. (1972). Pyruvate kinase of *Neurospora crassa:* purification and some properties. Can.J.Microbiol. 18, 805-818.
Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.
Leatherbarrow, R.J. and Dean, P.D.G. (1980). Studies on the mechanism of binding of serum albumines to immobilized Cibacron Blue F3GA. Biochem.J. 189, 27-34.
Lowe, C.R., Small, D.A.P. and Atkinson, A. (1981). Minireview some preparative and analytical applications of triazine dyes. Int.J.Biochem. 13, 33-40.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein determination with the Folin phenol reagent. J.Biol.Chem. 193, 265-275.
March, S.C., Parikh, I. and Cuatrecasas, P. (1974a). Affinity chromatography, old problems and new approaches. Adv.Exp.Med.Biol. 42, 3-14.
March, S.C., Parikh, I. and Cuatrecasas, P. (1974b). A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal.Biochem. 60, 149-152.
Marie, J., Kahn, A. and Boivin, P. (1977). Human erythrocyte pyruvate kinase. Total purification and evidence for its antigenic identity with L-type enzyme. Biochim. Biophys. Acta 481, 96-104.
Payton, M.A. (1978). A genetic study of sugar metabolism and transport in *Aspergillus nidulans*. Ph.D.Thesis. Univ.Leicester.
Payton, M. and Roberts, C.F. (1976). Mutants of *Aspergillus nidulans* lacking pyruvate kinase. FEBS Lett. 66, 73-76.
Schultz, G.E. and Schirmer, R.H. (1974). Topological comparison of adenyl kinase with other proteins. Nature 250, 142-144.
Staal, G.E.J., Koster, J.F., Kamp, H., van Milligen-Boersma, L. and Veeger, C. (1971). Human erythrocyte pyruvate kinase. Its purification and some properties. Biochim.Biophys. Acta 227, 86-96.
Stellwagen, E., Cass, R., Thompson, S.T. and Woody, M. (1975). Predicted distribution of NAD domain among glycolytic enzymes. Nature 257, 716-718.
Thompson, S.T., Cass, K.H. and Stellwagen, E. (1975). Blue dextran-Sepharose: An affinity column for the dinucleotide fold in proteins. Proc.Nat.Acad. Sci. USA 72, 669-672.
Thompson, S.T. and Stellwagen, E. (1976). Binding of Cibacron Blue F3GA to proteins containing the dinucleotide fold. Proc.Nat. Acad. Sci. USA 73, 361-365.
Visser, J., Kester, H.C.M, Derksen, A.C.G. and Uitzetter, J.H.A.A. (1982). Affinity chromatography and affinity electrophoresis: Tools to investigate

protein interactions and enzyme mutants. in: Gribnau, T.C.J., Visser, J. and Nivard, R.J.F. (eds.) Affinity chromatography and related techniques. Elsevier, Amsterdam, pp. 425-435.

Wilson, J.E. (1976). Applications of Blue dextran and Cibacron Blue F3GA in purification and structural studies of nucleotide-requiring enzymes. Biochem. Biophys.Res.Comm. 72, 816-823.

VI Pyruvate kinase in wild type and pyruvate kinase mutants of *Aspergillus nidulans*

SUMMARY

Pyruvate kinase was measured in crude extracts of a wild type strain of *A.nidulans*, grown under different nutritional conditions. The specific activity of pyruvate kinase was high in mycelium grown on sucrose and low in mycelium grown on acetate or polygalacturonate. Antibodies raised against purified pyruvate kinase were used for immunological detection of pyruvate kinase. In the Ouchterlony double diffusion only wild type pyruvate kinase could be detected after induction by a suitable glycolytic substrate like sucrose. To optimize the detection of mutant pyruvate kinase, Western blotting was used.

Among 5 pyruvate kinase mutants, at the *pkiA* locus only one was found which totally lacked immunological and enzymatic activity. The absence of a pyruvate kinase gene product is not a prerequisite for sucrose tolerance of some pyruvate kinase mutants.

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) has been isolated from various organisms, both prokaryotes and eukaryotes. It has been recognized as a key regulatory enzyme in glycolysis. The balance between glycolysis and gluconeogenesis is controlled by allosteric regulation by substrate and product and by other allosteric effectors.

In prokaryotes in general one molecular form of pyruvate kinase has been demonstrated except for *Escherichia coli* in which two different non interconvertible pyruvate kinases coexist under different nutritional conditions (Malcovati and Kornberg, 1969; Kornberg and Malcovati, 1973; Kotlarz *et al.*, 1975): a constitutive AMP-activated enzyme called pyruvate kinase A in the genetic nomenclature of Demerec *et al.* (1966) (in other literature pyruvate kinase II) which is important under glyconeogenic growth conditions, and an inducible fructose 1,6-diphosphate activated pyruvate kinase B (pyruvate kinase I), important under glycolytic

growth conditions. Both pyruvate kinases are under independent genetic control (Pertierra and Cooper, 1977) and are different proteins, each being a tetramer of identical subunits with a molecular weight of 56,000 D (type I) and 51,000 D (type II) respectively (Valentini *et al.*, 1979). Information about pyruvate kinases in lower eukaryotes has been obtained mainly for *Saccharomyces spec.* (Hunsley and Suelter, 1969, a, b; Fell *et al.*, 1974) and *Neurospora crassa* (Kapoor and Tronsgaard, 1972; Tsao and Madley, 1975; Kapoor, 1975, 1976). In these cases the enzyme is also supposed to be a tetramer of similar or identical subunits with a molecular weight of 60,000 D. In *Mucor rouxii* however three pyruvate kinase isoenzymes have been identified (Friedenthal *et al.*, 1973): pyruvate kinase type I and III which are favoured under aerobic glycolytic and gluconeogenic conditions respectively whereas pyruvate kinase II is suggested to be a hybrid form composed of subunits of pyruvate kinase I and III. None of the forms seemed to be constitutive and their relative proportions varied according to growth conditions and also according to the alternative yeastlike or filamentous morphology of this fungus. Yeastlike cells which had a fermentative mode of hexose utilization showed almost exclusively the presence of pyruvate kinase I.
In agreement with the importance of pyruvate kinase in glycolysis Barwell and Hess (1971) demonstrated only a low specific activity of this enzyme in *Saccharomyces cerevisiae* when grown on ethanol in contrast to cultures grown on glycolytic carbon sources. A similar difference in the levels of pyruvate kinase activity in relation to the carbon source offered has been observed in *Aspergillus nidulans* where pyruvate kinase is suggested not to play a role in gluconeogenesis since the activity of acetate grown cells is low compared with cells grown on a glycolytic source such as sucrose (McCullough and Roberts, 1974; Payton and Roberts, 1976).

Additional insight in the role of pyruvate kinase in glycolysis and gluconeogenesis has been obtained from mutants defective in pyruvate kinase. Among the prokaryotes only pyruvate kinase mutants in *Escherichia coli* are known. The first pyruvate kinase mutant was isolated accidentally by Pertierra and Cooper using a rationale described by Irani and Maitra (1974). The latter authors isolated mutants defective in enzymes catalyzing triose interconversion reactions by selecting for those strains which required for their growth a combination of substrates entering metabolism before and after the metabolic block. They used a combination of glycerol and succinate as rescue medium for mutants unable to grow on either of these compounds alone. When Pertierra and Cooper (1977) used a similar procedure they found a mutant unable to grow on glycerol, gluconate, lactate, malate or acetate, but still able to grow on succinate. This mutant was devoid of both pyruvate kinase A and α-ketoglutarate dehydrogenase activity.

Revertants of this strain were able to grow on all the carbon sources mentioned and were only defective in pyruvate kinase A activity and one of these revertant strains was used for mutation to isolate double mutants defective in both pyruvate kinase A en B. These double mutants show the importance of the hexose phosphotransferase system which catalyzes the alternative formation of pyruvate as a result of monosaccharide uptake by the following reaction: glucose + PEP → glucose-6-phosphate + pyruvate. In this case pyruvate kinase is not required. The pyruvate kinase double mutants were able to grow on glucose, sorbitol, mannose and fructose but grew very poorly on glycerol, galactose, glucose-6-phosphate, maltose and lactose which compounds are not transported by the PEP-sugar phosphotransferase system. Moreover these authors confirmed earlier observations (Malcovati *et al.*, 1973; Waygood and Sanwall, 1974) of the presence of high activities of pyruvate kinase B in the pyruvate kinase A mutant (thus defective in the pyruvate kinase form important under gluconeogenic conditions), when this mutant was grown on glucose or glycerol in contrast to the low activities measured in lactate or succinate grown cultures.

In eukaryotes several pyruvate kinase mutants were obtained in *Saccharomyces cerevisiae* by mutation and selection for mutants unable to use sugars such as glucose, or which are inhibited by the addition of glucose to an otherwise permissive gluconeogenic carbon source. The mutants isolated were still able to grow on ethanol, pyruvate or lactate (Sprague jr., 1977; Maitra and Lobo, 1977; Lam and Marmur, 1977, Clifton *et al.*, 1978). A considerable amount of constitutive pyruvate kinase activity was found in alcohol grown cultures and there is evidence that this constitutive activity and the hexose induced activity result from a single pyruvate kinase gene because both activities had the same half lives. Secondly loss or reappearance of pyruvate kinase activities always occurs simultaneously in this yeast. Moreover all the mutants tested failed to complement each other in diploids showing that a single gene was responsible for pyruvate kinase (Maitra and Lobo, 1977).

The inability of pyruvate kinase mutants to grow on a glycolytic carbon source stresses the importance of this enzyme in glycolysis and shows that no other route exists which bypasses the pyruvate kinase catalyzed reaction. This is in contrast to the situation found in *E.coli* were different pyruvate kinases are present and where other pyruvate generating reactions exist.

The growth of pyruvate kinase mutants on acetate or ethanol in lower eukaryotes occurs by virtue of induction of the glyoxylate cycle and PEP carboxykinase. The marked inhibition of growth when for instance glucose or galactose is added to C_2 grown cultures of pyruvate kinase mutants suggests the following possibi-

lities:

- inhibition of uptake of these compounds;
- catabolite repression of enzymes necessary for growth on C_2 compounds (Lam and Marmur, 1977);
- accumulation of high (toxic) concentrations of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate under these conditions (Maitra and Lobo, 1977).

Pyruvate kinase mutants do not differ in the latter respect from other mutants in which phosphorylated metabolites that cannot be metabolized cause growth stasis (Cozzarelli *et al.*, 1965; Fraenkel, 1968; Maitra, 1971).

Gancedo and Gancedo (1979) reported the inactivation of the gluconeogenic enzymes fructose 1,6-diphosphatase, PEP carboxykinase and of malate dehydrogenase after addition of glucose to crude extracts of wild type and of pyruvate kinase and phosphofructokinase mutants of *S.cerevisiae*. In one peculiar strain, a pyruvate kinase mutant which was able to grow on mixtures of glucose and a gluconeogenic carbon source the malate dehydrogenase activity was not found to be inactivated.

In *A.nidulans* pyruvate kinase mutants were isolated by Payton and Roberts (1976), Payton (1978) and Bos *et al.*(1981). In all cases a rationale was used similar to the one followed for the isolation of the *S.cerevisiae* pyruvate kinase mutants mentioned before: mutation and enrichment took place on a glycolytic carbon source (glucose or sucrose), and rescue took place on acetate as sole carbon source. Pyruvate or lactate are not used for rescue since these compounds are bad carbon sources. Using this strategy also mutants were isolated defective in other enzymes of the glycolytic sequence as discussed in chapter I. Criteria were developed to recognize the phenotype of pyruvate kinase mutants. Most pyruvate kinase (*pki*) mutants do not grow on acetate in combination with sucrose (Payton and Roberts, 1976) or in combination with glycerol (Bos *et al.*, 1981), such in contrast to mutants defective in the pyruvate dehydrogenase complex or in pyruvate carboxylase. Pyruvate kinase mutants grow well however on D-galacturonate, this in contrast to *pdh* and *pyc* mutants (chapter II).

Payton and Roberts (1976) observed differences in the levels of pyruvate kinase activity in wild type *A.nidulans* when the fungus was grown on different carbon sources. Pyruvate kinase activity was high in sucrose grown mycelium, intermediate in mycelium grown on a mixture of sucrose and acetate and extremely low if grown on acetate as sole carbon source. Transfer to sucrose following growth on acetate quickly raised the level of pyruvate kinase activity to 50% of the sucrose grown culture. The pyruvate kinase mutants isolated by Payton and Roberts (1976) were identified to belong to one single gene *pkiA* based on non complementarity of mutant strains in heterokaryons, and the *pkiA* gene was located in linkage group V.

In this chapter several *pki* mutants (both sucrose tolerant and sucrose sensitive ones) have been analyzed. These mutants were screened for the presence or absence of the pyruvate kinase gene product under pyruvate kinase inducing conditions to see whether or not a relation exists between the presence or absence of the pyruvate kinase gene product and sucrose tolerance observed in some pyruvate kinase mutant strains. A regulatory function of an enzyme protein on the expression of genes is known for instance in *Klebsiella aerogenes*, where glutamine synthethase is able to regulate the synthesis of enzymes responsible for glutamate formation (Magasanik *et al.*, 1974; Tyler, 1978). Pyruvate kinase purified as described in chapter V was used to prepare a rabbit antiserum. This antiserum was used for the detection of a pyruvate kinase gene product, by Ouchterlony double diffusion, tandem crossed immunoelectrophoresis and by Western blotting.
The technique of blotting was first introduced by Southern (1975) for the transfer of DNA fragments from agarose gels to a sheet of nitrocellulose and was later used by Alwine (1979) for the transfer to diazobenzyloxymethyl (DBM) cellulose (Northern blot). A similar technique was used by Renart *et al.* (1979) to transfer proteins from polyacrylamide gels to DBM cellulose. In this study proteins were transferred to nitrocellulose by diffusion from SDS-polyacrylamide gels.

MATERIAL AND METHODS

Strains, media and culture conditions

The following *A.nidulans* strains were used: WG096 (*yA2, pabaA1*) as wild type strain with respect to carbon metabolism, WG121 (*pkiA1*) and WG224 (*wA3; pyroA4; pkiA3, nicA2*) for complementation tests with pyruvate kinase (*pki*) mutants. WG123 (*yA2, pabaA1; pkiA2 (Ts)*) was used as a temperature sensitive pyruvate kinase mutant strain. The other *pki* mutant strains used were: WG083 (*pkiA3*), WG230 (*pkiA4*), WG231 (*pkiA5*) and WG232 (*pkiA6*) isolated by Bos *et al.* (1981) in the WG096 background, all at the *pkiA* locus. Strains were maintained on complete medium (see I.3.5.) according to Pontecorvo *et al.* (1953) with acetate 0.1 M, sucrose 0.01 M as carbon source (wild type) or acetate 0.1 M (pyruvate kinase mutants).
A.nidulans WG096 (wild type) was grown in shake culture to obtain fungal biomass. Growth occurred in 1 l infusion bottles containing 300 ml of liquid minimal medium and placed in horizontal position at 37^{o}C in a home made incubator (at 92 strokes min.$^{-1}$). Approx. 10^{6} conidia ml^{-1} were inoculated. Carbon sources used were acetate 0.1 M, sucrose 0.02 M, acetate 0.1 M with sucrose 0.01 M and

polygalacturonate 0.5% (w/v).
A.nidulans pyruvate kinase mutants were grown in liquid minimal medium with acetate 0.1 M. For inoculation approx. 10^5 conidia ml^{-1} were used. If enough biomass was formed (usually after 40-48 hours), the mycelium was collected by filtration and washed with sterile saline (0.8% w/v NaCl) to remove traces of the original carbon source. The mycelium was then transferred to 0.02 M sucrose medium for approx. 5-10 hours to induce pyruvate kinase.

Complementation tests were performed using techniques described by Pontecorvo *et al.* (1953). Heterokaryons of WG224 or WG121 with other strains were made on minimal medium with polygalacturonate sodium salt 0.5% (w/v) or acetate 0.1 M and twin pieces were transferred to sucrose 0.02 M and to polygalacturonate or acetate as a control.

Chemicals

Polygalacturonic acid was obtained from U.S. Biochemical Corporation (Cleveland Ohio,U.S.A.), Freunds Adjuvant Complete was purchased from Difco (Detroit, U.S.A.), Nitrocellulose membrane filter BA85 0.45 μm was obtained from Schleicher and Schüll GmbH (Dassel, F.R.G.).
Antirabbit IgG (whole molecule) peroxidase conjugate and o-dianisidine HCl were supplied by Sigma (St.Louis, MO, U.S.A.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.).

Methods

SDS-polyacrylamide gel electrophoresis was performed as described in chapter IV. The pyruvate kinase assay was described in chapter V.
Crude extracts of small samples of mycelium were obtained by grinding the frozen hyphae with liquid nitrogen in a mortar and by adding subsequently buffer A (see chapter V) to the powder obtained (approx. 2-3 ml g^{-1}). After centrifugation for 15 minutes at 40,000 x *g* the clear supernatant was used for further analyses. Partial purification of pyruvate kinase in small samples of crude extracts was performed by affinity chromatography with Mikacion Brilliant Yellow 6GS Sepharose Cl-4B (30 μmoles of dye g^{-1} of dry Sepharose) as described in chapter V.

Preparation of antibodies

Purified *A.nidulans* pyruvate kinase (chapter V) was dialyzed against phosphate buffered saline (0.01 M sodium phosphate pH 7.1; 0.85% w/v sodium chloride). To a 1 ml solution of pyruvate kinase corresponding to 140 μg of protein, an equal

volume of Freund's Adjuvant Complete was added. With a Vortex mixer an emulsion was made which was subcutaneously distributed over several sites in a male New Zealand white rabbit at day 1. This treatment was repeated at day 11. At day 17 an antigen injection was given intraveneously. Blood was obtained from a vein of the right ear and collected at day 23, 38 and 66. Fresh blood was incubated for one hour at room temperature and allowed to stand overnight at $4^{o}C$. After decantation of the serum this was centrifuged for 15 min. at 1000 x *g*. The supernatant was frozen at $-20^{o}C$.

Immunological detection of pyruvate kinase

Crude extracts and partially purified pyruvate kinase obtained from wild type and mutants were analyzed for immunologically active material by the Ouchterlony double diffusion method, tandem crossed immunoelectrophoresis and by Western blotting.

Ouchterlony double diffusion (Ouchterlony and Nilsson, 1978) was performed using 0.8% (w/v) ion-agar (Difco) in phosphate buffered saline on glass plates precoated with agarose (1% w/v in water). The central well was filled with 15 µl antiserum whereas a serial dilution of antigen was applied to the peripheral wells. After incubating for 24 hours at room temperature the gels were washed in saline for another 24 hours. After drying the gels were stained for 10 min. with 0.1% (w/v) Coomassie Brilliant Blue R250 in ethanol/acetic acid/water = 450/100/450 (by volume) and destained in the same solvent without this dye.

Tandem crossed immunoelectrophoresis was carried out as described by Krøll (1973), using 14 ml of 0.8% (w/v) agarose in barbital buffer (pH 8.6 I = 0.02) on overhead sheets, 5 µl samples were applied. After diffusion of the samples the application holes were filled with a drop of agarose. The run in the first dimension was performed at 10 V cm^{-1} at $4^{o}C$ for one hour. Electrophoresis in the second dimension was performed at 10 V cm^{-1} at $4^{o}C$ for 2.5 hours, using 0.8% (w/v) agarose containing 0.25% (v/v) antiserum in the same barbital buffer.

Western blotting

For protein transfer the method of Bowen *et al.* (1980) was used. SDS-polyacrylamide gel electrophoresis of the various protein samples was performed in duplicate. One gel was stained with Coomassie Brilliant Blue R250 as described in chapter IV. The other gel was washed for 3 hours to remove SDS in buffer containing NaCl 50 mM, EDTA 2 mM, urea 4 M, DTT 0.1 mM and Tris HCl 10 mM

pH 7.0 (buffer C). After washing, this gel was put between two sheets of nitrocellulose and care was taken to remove air bubbles. These were then put between several layers of Whatmann 3 MM filter paper and at both sides covered with a sponge, before being mounted between alumina grids. Proteins were transferred from the gel to both nitrocellulose sheets by diffusion during 48 hours in buffer C. After 24 hours the buffer was refreshed.

Upon transfer of the protein samples, the nitrocellulose sheets were further saturated with aspecific protein by incubation with 1% (w/v) bovine serum albumin during 5 hours in Tris HCl buffer 10 mM pH 7.5 containing 350 mM NaCl. Excess protein was removed by washing several times in the same buffer without bovine serum albumin. The nitrocellulose blots were then incubated overnight in 50 ml phosphate buffered saline in the presence of 1% (w/v) of bovine serum albumin and of 0.2 % (v/v) pyruvate kinase antiserum. Excess of antiserum was removed by washing in phosphate buffered saline. Finally the blots were incubated for 2 hours in a small volume of phosphate buffered saline containing 1% (w/v) bovine serum albumin and 0.1% (v/v) goat anti-rabbit γ-globuline peroxidase conjugate. After washing for 2 hours in phosphate buffered saline the detection of pyruvate kinase was achieved by measuring peroxidase activity.

A piece of Whatmann 3 MM filter paper impregnated with 50 mM Na_2HPO_4, 25 mM citric acid containing per liter 10 mg o-dianisidine (dissolved in 2 ml of methanol) and 0.16% (v/v) H_2O_2 was carefully placed on the blot. When bands were visible, the reaction was stopped by washing the blot in a 200 ml solution of 50 mM Na_2HPO_4 and 25 mM citric acid. Itwas essential to make photographs immediately.

RESULTS AND DISCUSSION

Pyruvate kinase activities in wild type A.nidulans

The level of the pyruvate kinase activity present in mycelium varies with the carbon source offered during growth. Therefore enzyme levels were measured under different nutritional conditions *e.g.* growth on a glycolytic carbon source (sucrose), a gluconeogenic carbon source (acetate), a mixture of both and on polygalacturonate.

The latter compound was found before to be a good substrate for both wild type and *pki* mutant strains. Pyruvate kinase activities were also measured after aseptical transfer of mycelium from a gluconeogenic to a glycolytic substrate and vice versa. The mycelium was first grown for 20 hours at 37°C. The transfer period lasted 5 hours. The results are shown in Table VI.1. It is obvious that among the

Table VI.1. Pyruvate kinase activity in crude extracts of wild type *A.nidulans* grown under different nutritional conditions.

C-source	Transfer [1]	Yield[2] ($g\ l^{-1}$)	Specific activity[3] ($U\ mg^{-1}$)
Sucrose 0.02 M	-	4.3	0.34
	Acetate 0.1 M	4.5	0.14
Acetate 0.1 M	-	0.8	0.02
	Sucrose 0.02 M	2.7	0.10
Acetate 0.1 M + Sucrose 0.01 M	-	4.3	0.12
Polygalacturonate 0.5% (w/v)	-	0.8	0
	Sucrose 0.02 M	1.7	0.18

1) There is hardly any change of activity during this period if no transfer is performed and the mycelium is kept on sucrose at 37°C.

2) Wet weight based on at least 3 cultures.

3) Values corrected for NADH-oxidase activity.

carbon sources sucrose and sucrose in combination with acetate lead to the highest amount of biomass whereas almost a threefold difference in pyruvate kinase activity is found between these growth conditions. The value of the specific activity obtained for the sucrose grown mycelium is lower than the one mentioned by Payton and Roberts (1976) (1.17 U mg^{-1}), who used other assay conditions however. From Table VI.1. it can also be concluded that in wild type *A.nidulans* pyruvate kinase levels respond readily to changes in the carbon source as shown by all transfer experiments.

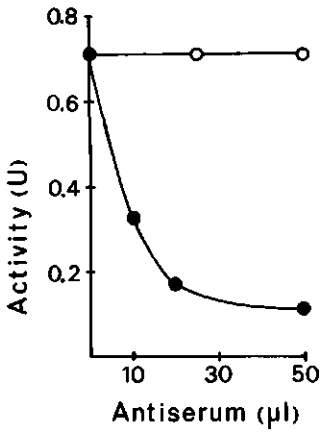
Since pyruvate kinase mutants do not grow on sucrose and the pyruvate kinase level in wild type is low on acetate, pyruvate kinase mutants were screened for the presence of the pyruvate kinase gene product by growing mycelium on acetate or polygalacturonate, followed by transfer to sucrose to induce pyruvate kinase activity. The results with pyruvate kinase mutants will be described later in this chapter.

Immunological observations with wild type A.nidulans grown under different nutritional conditions

An antiserum with a high titre was prepared against native pyruvate kinase. Both the first and the second batch of serum had a titre of approx. 3400 μg ml^{-1} based on single radial immunodiffusion with partially purified pyruvate kinase. The antibodies had an inhibitory effect on the enzymatic activity as shown in Fig. VI.1. In a crude extract 0.72 U of enzyme was inhibited for 75% upon incubation with 20 μl antiserum at 0°C. Thus the inhibition is far from complete.

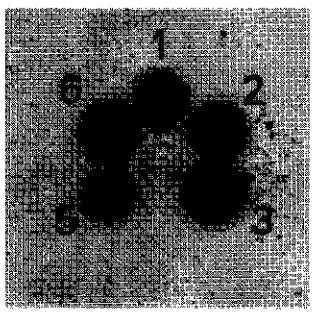
Fig. VI.1. Effect of antibodies raised against *A.nidulans* pyruvate kinase. Titration was carried out incubating samples of crude extract (0.72 U) at 0°C with different amounts of antiserum (final volume 250 μl). After 5 minutes the pyruvate kinase activity was measured at 25oC

—●— residual activity in the presence of antiserum
—○— residual activity in the presence of control serum



The Ouchterlony double diffusion method was used to investigate the presence of pyruvate kinase in crude extracts obtained from mycelium after growth for 20 hrs at 37°C on acetate, polygalacturonate and sucrose. The same was done after transfer for 5 hours from either polygalacturonate or acetate medium to sucrose and from sucrose to acetate. The results are shown in Fig. VI.2.

Fig. VI.2. Ouchterlony double diffusion of 15 μl (fourfold dilution) of anti-pyruvate kinase serum (central well) and 15μl samples of crude extracts (peripheral wells) of *A.nidulans* (wild type) obtained after growth on several carbon sources.
1) sucrose grown; 2) acetate grown;
3) polygalacturonate grown; 5) sucrose grown after transfer to acetate; 6) acetate grown after transfer to sucrose.
(Crude extracts containing approx. 5 mg protein ml^{-1})



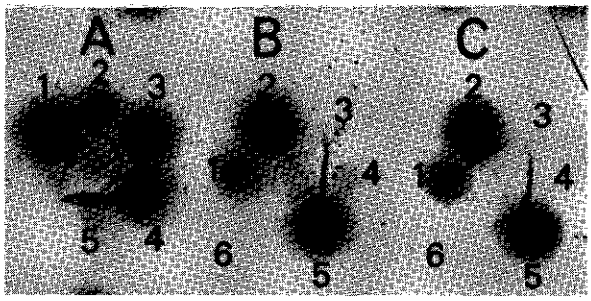


Fig. VI.3. Ouchterlony double diffusion of crude extract of WG096 (wild type) and of fractions obtained during partial purification of pyruvate kinase on Mikacion Brilliant Yellow 6GS Sepharose Cl-4B. All wells were filled with 15 µl of sample.

A: 1) crude extract; 2), 3), 4) pyruvate kinase inactive eluate of affinity column; 5) 0.5 M NaCl pulsecontaining partially purified pyruvate kinase. central well: pyruvate kinase antiserum (4-fold dilution).

B: 1), 3), 6) pyruvate kinase inactive eluate of affinity column; 2), 5) crude extract; 4) partially purified pyruvate kinase. central well: pyruvate kinase antiserum (16-fold dilution).

C: see B, central well: pyruvate kinase antiserum (32-fold dilution).

Surprisingly in the case of acetate (well 2) and polygalacturonate (3) grown mycelium a single precipitation line was observed whereas two such lines were found upon transfer to sucrose (6) and in cultures grown on sucrose (1, 5). Purified enzyme caused a precipitation line which fused with the outer precipitation line in the pattern of the sucrose grown cultures (1, 5, 6). Therefore this must be the pyruvate kinase specific precipitate. The nature of the inner precipitate observed in all cases is not clear since there is hardly any induction of pyruvate kinase activity in the acetate and polygalacturonate media. The origin of the inner precipitation line in the Ouchterlony double diffusion pattern has been investigated somewhat further. A crude extract of wild type *A.nidulans* grown on sucrose, was applied to a small size affinity column of Mikacion Brilliant Yellow 6GS Sepharose Cl-4B and eluted as described in chapter V. Fractions of the eluate and fractions obtained by washing the column with buffer A (see chapter V) did not contain pyruvate kinase activity, whereas the pyruvate kinase activity was recovered as usual by elution with buffer A containing 0.5 M NaCl. With the fractions containing non bound proteins a single precipitation line was observed corresponding with the inner precipitate in the crude extracts. The pyruvate kinase pool caused the outer precipitation line in the Ouchterlony pattern (see Fig. VI.3A.). This experiment demonstrated a simple way to separate the two immunological activities. Various dilutions of the antiserum were made in

order to optimize the antigen-antibody ratio's required for precipitation. With crude extracts the outer precipitation line only appeared and sharpened at higher dilutions of the antiserum (see Fig. VI.3A, B, C). In the same figure one notices that the two reaction lines do not really cross. This suggests at least some partial identity of the antigens. Since in the purification of pyruvate kinase Mikacion Brilliant Yellow 6GS Sepharose Cl-4B is used twice, the possibility is excluded that with purification of the antigen this second protein has been co-purified. The inner precipitate in Fig. VI.2. might reflect however the reaction of an inactive form or of subunits of pyruvate kinase with the antibody. It might also correspond to proteolytic degradation products of the enzyme or to the reaction of a different but immunologically related protein.

Western blotting of wild type pyruvate kinase

The advantage of protein blotting in combination with an immunochemical detection method for specific proteins is the higher specificity and sensitivity of this method. Since the antigen is fixed on a nitrocellulose carrier it can in principle be detected in a 1:1 labelled antibody-antigen complex. Crude extracts of mycelium (6-10 g), grown on sucrose 0.02 M and on acetate 0.1 M with sucrose 0.01 M for 20 hours at 37^{o}C were prepared as usually. A partial purification of pyruvate kinase was achieved by applying Mikacion Brilliant Yellow 6GS Sepharose Cl-4B as affinity adsorbent and eluting the enzyme in buffer A by adding 0.5 M NaCl and 1 mM PEP. Both crude extracts and active fractions obtained after partial purification were further analyzed as described in Materials and Methods. The results are shown in Fig. VI.4. In the case of both sucrose and sucrose + acetate grown mycelium several bands appeared after staining with peroxidase-labelled goat anti-rabbit IgG. With the partially purified pyruvate kinase samples a single band was observed in position with the subunit molecular weight of the enzyme. In the crude extracts two lower molecular weight bands were also present, presumably representing degradation products of the enzyme. It has to be mentioned that protein transfer is not quantitative, since proteins can still be detected on the polyacrylamide gel by staining after transfer to the nitrocellulose filter (Fig. VI.5.).

Pyruvate kinase has been found to loose easily its activity. The protein turned out to be susceptible to proteolysis as is shown by comparing the blots of a freshly prepared, partially purified enzyme and of the same preparation after storage at -18^{o}C for 5 weeks (Fig. VI.6A, B).

Degradation products were also observed in the case of a pure enzyme preparation stored at -18^{o}C for 6 months. After further storage at 4^{o}C for 8 days only small

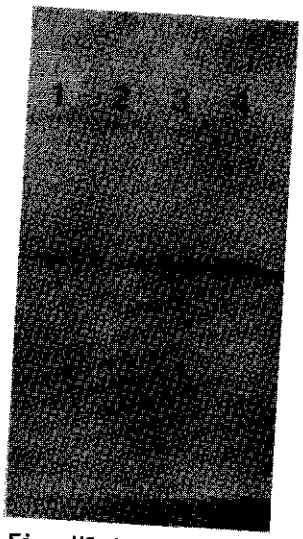


Fig. VI.4. Western blot of samples of pyruvate kinase

No.	Sample	Culture	Amount of protein applied to SDS-gel µg)	U
1	partially purified	sucrose + acetate	24	0.12
2	crude extract	sucrose + acetate	217	0.27
3	crude extract	sucrose	52	0.28
4	partially purified	sucrose	< 25	0.19

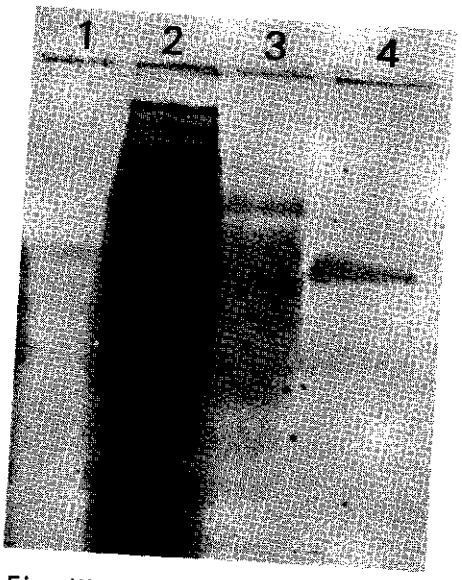


Fig. VI.5. SDS-polyacrylamide gel after Western blotting stained with Coomassie Brilliant Blue R-250.
(see also Fig. VI.4.)

Fig. VI.6. Western blot of partially purified pyruvate kinase (< 25 µg)

A) freshly applied
B) same preparation after storage at -18°C for 5 weeks.

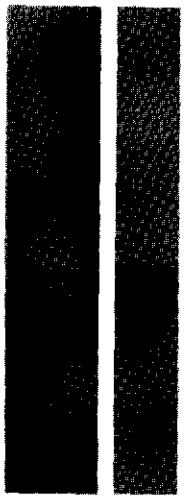
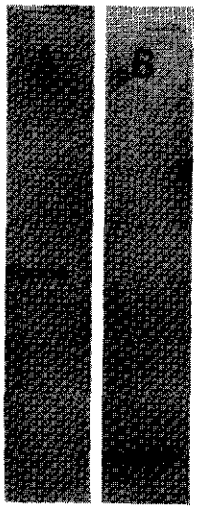


Fig. VI.7. Western blot of purified pyruvate kinase (<20 µg) after storage of the enzyme at -18°C for 6 months (A) and further incubation at 4°C for 8 days (B).



fragments were left which migrate in the front upon SDS-polyacrylamide gel electrophoresis (Fig. VI.7A, B).

Detection and immunological analysis of pyruvate kinase in pyruvate kinase mutants

Bos *et al.*(1981) isolated several mutants unable to use glucose as sole carbon source. Among the mutants some of them were found to show a leaky growth on acetate 0.1 M with sucrose 0.01 M thereby mimicking the phenotype of the pyruvate dehydrogenase complex mutants. However they were not defective in the pyruvate dehydrogenase complex since they were able to complement with *pdhA,B* and *pdhC* tester strains in a heterokaryon grown on glucose. Further tests indicated that

these mutants were D-galacturonate positive and this led to the conclusion that they had a pyruvate kinase mutant phenotype. This was confirmed by the inability to complement with WG121 and WG224, strains bearing the mutant gene *pkiA*. Since the *pki* mutants available behaved phenotypically different with respect to their sucrose tolerance when plated on a mixed substrate of acetate and sucrose, a further analysis of these strains was carried out.

For wild type mycelium it was shown that transfer from an acetate to a sucrose medium for 5 hours was sufficient to induce pyruvate kinase activity which resulted in an immunological detectable precipitation line in the Ouchterlony double diffusion pattern of crude extracts. Therefore, similar induction conditions were chosen for the mutant strains which were grown for a prolonged period (40-48 hrs) on acetate 0.1 M and then transferred to sucrose 0.02 M for 8-10 hrs. Crude extracts and partially purified fractions obtained after affinity chromatography on Mikacion Brilliant Yellow 6GS Sepharose Cl-4B were used to analyze whether partially active or inactive protein was made. By the Ouchterlony double diffusion method no pyruvate kinase specific precipitation lines were found (see Fig. VI.8).

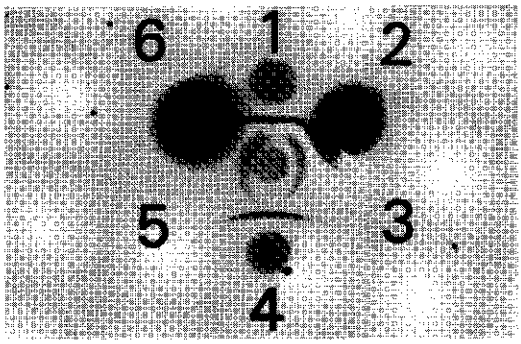


Fig. VI.8. Ouchterlony double diffusion of wild type and pyruvate kinase mutants of *A.nidulans*. Central well: pyruvate kinase antiserum 4-fold dilution (15 µl). Peripheral wells (15 µl samples): 1) and 4) pyruvate kinase, partially purified fraction obtained after affinity chromatography on Mikacion Brilliant Yellow 6GS Sepharose Cl-4B; 2) crude extract of WG096 (*pkiA*$^+$), sucrose grown; 3) crude extract of WG232 (*pkiA6*); 5) crude extract of WG121 (*pkiA1*); 6) crude extract of WG123 (*pkiA2(Ts)*) grown on sucrose at 30°C.

Strain WG123 (well 6) which is a temperature sensitive mutant (specific activity of pyruvate kinase 0.03 U mg^{-1}) although grown for 20 hours at 30°C also failed to give a precipitation line. Therefore the detection level given by the double diffusion method is insufficient to decide with certainty whether the protein is synthesized or not.

In Fig. VI.9A and B the results are shown of a tandem crossed immunoelectro-

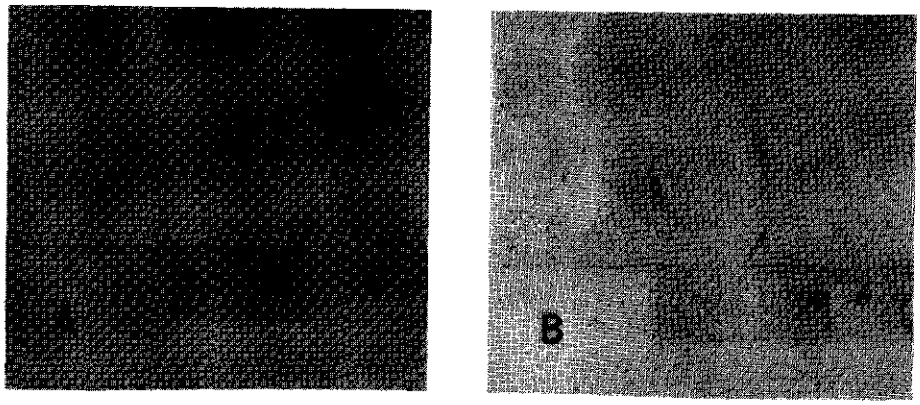


Fig. VI.9. Tandem crossed immunoelectrophoresis of pyruvate kinase.

A. From the left to the right: crude extract of WG232 (*pkiA6*) of WG096 (*pkiA*$^+$) and of partially purified pyruvate kinase of the diploid strain (see chapter V).

B. From the left to the right: crude extract of WG096 (*pkiA*$^+$), WG232 (*pkiA6*) and of partially purified pyruvate kinase.

phoresis with a crude extract of WG096 (*pkiA*$^+$), WG232 (*pkiA6*) and of a sample of partially purified pyruvate kinase of the diploid strain used in chapter V. It is clear that the pyruvate kinase of the haploid and of the diploid strain are homologous since the precipitation lines fuse (Fig. VI.9A). When the central hole was filled with an extract of the mutant, the surrounding precipitates of the wild types did not return to the base line at the neighbouring edges. The mutant extract therefore contained antigen although its concentration was too low to give rise to a visible precipitate. Therefore Western blotting was finally resorted to in order to realize a positive identification of possible gene products.

Western blotting of pyruvate kinase mutants

In Table VI.2. the strains used for Western blotting of pyruvate kinase are listed, together with the density of the inoculum, and the amount of cultures used. The sucrose tolerance was judged by growing mutant strains on acetate 0.1 M minimal medium with sucrose 0.01 M.

As is obvious from Table VI.2. the mutant strains differ largely in the yield of mycelial biomass finally obtained and even the wild type strain has a very low yield. Some caution is necessary however, since the different strains, although cultured under similar conditions, may vary considerably in their physiological age.

Table VI.2. Strains used for analysis of pyruvate kinase gene product by Western blotting

Strain	Sucrose tolerance	Inoculation ($Conidia\ ml^{-1}$)	MMA[a]	MMS[a]	Yield wet weight (g)
WG096(*pkiA*$^+$)	+	1.3×10^6	5	2	1.2
WG083(*pkiA3*)[b]	+	2.3×10^5	10	1	2.4
WG230(*pkiA4*)	-	1.3×10^5	10	2	8.2
WG231(*pkiA5*)	-	1.3×10^5	10	2	5.1
WG232(*pkiA6*)	+	1.0×10^5	6	1	2.0

a) In the table the number of 300 ml cultures (40 hours) on acetate (MMA) are listed and the number of 300 ml cultures used for subsequent transfer (10 hours) of the mycelium to sucrose (MMS).

b) To obtain sufficient conidia of the mutant strains, the yield of conidia of approx. 40 petri dishes (Ø 9 cm) was necessary.

Table VI.3. Enzymatic activities of pyruvate kinase in various strains

Strain	Crude extract protein($mg\ ml^{-1}$)	Pyruvate kinase Specific activity ($U\ mg^{-1}$)	Partially purified pyruvate kinase. Specific activity ($U\ mg^{-1}$)
WG096(*pkiA*$^+$)	4.54	0.07[a]	38.0
WG083(*pkiA3*)	3.59	0.05[b]	12.6
WG230(*pkiA4*)	1.97	0.05	16.3
WG231(*pkiA5*)	3.44	0	0
WG232(*pkiA6*)	1.11	0.05	0.33

a) Specific activity of pyruvate kinase in wild type is low probably due to ageing of the culture. The mutant cultures were stored only for a short period at -20°C.

b) In the case of WG083 colonies were found in the inoculate able to grow on minimal sucrose medium, this value is therefore not only due to mutant pyruvate kinase.

Crude extracts of the various strains were made and pyruvate kinase specific activities were determined. Also some extract was applied on small columns of Mikacion Brilliant Yellow 6GS Sepharose Cl-4B (Ø 0.5 x 5 cm) both for mutant strains (3-5 ml) and wild type (1 ml). The columns were washed with buffer A (chapter V) and then the 0.5 M NaCl eluate was collected. In Table VI.3. some data are given about the various strains used. The values of the specific activities of partially purified pyruvate kinase of the mutants differ. This could be due to differences in the stability of the mutant pyruvate kinases.
In Fig. VI.10 the results are shown of SDS-polyacrylamide gel electrophoresis of crude extracts of several strains (A) together with the results of Western blotting (B).

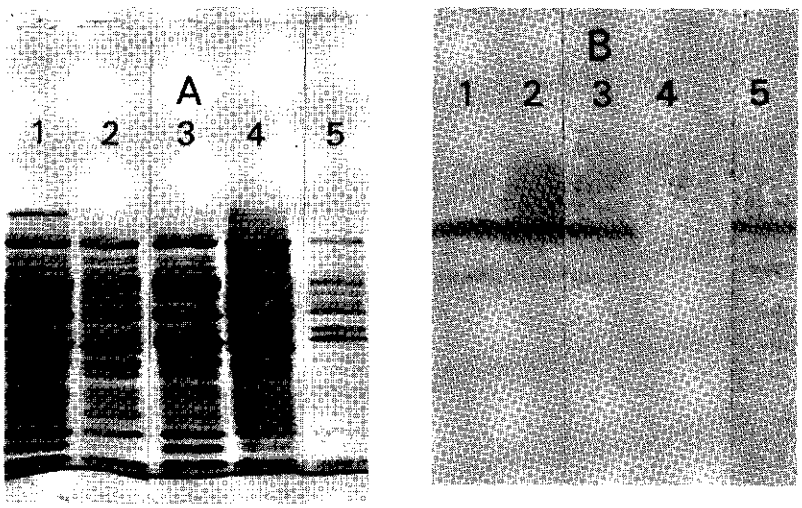


Fig. VI.10. A: SDS-polyacrylamide gels after electrophoresis and staining
Crude extracts of the following strains were used:
1) WG096 (*pkiA*$^+$), 170 µg; 2) WG232 (*pkiA6*), 42 µg
3) WG083 (*pkiA3*), 135 µg; 4) WG231 (*pkiA5*), 129 µg
5) WG230 (*pkiA4*), 74 µg.
B: Western blots after staining, similarly to A.

In Fig. VI.11. similar results are shown with the corresponding partially purified extracts. From these pictures one observes that these strains except WG231 (*pkiA5*) show immunological activity both in a crude extract and in the partially purified state. WG231 (*pkiA5*) is the only strain totally devoid of pyruvate kinase activity (Table VI.3.) and therefore this strain could be a strain incapable to synthesize the pyruvate kinase gene product.
Since WG083 (*pkiA3*) and WG232 (*pkiA6*) which have a sucrose tolerance (Table VI.2)

still synthesize a pyruvate kinase gene product, there is no direct relation between this tolerance and the absence of the pyruvate kinase protein. The Ouchterlony double diffusion does not allow detection of pyruvate kinase in acetate grown mycelium with antiserum raised against pyruvate kinase induced under glycolytic conditions. Therefore it will be interesting to screen immunological activity of pyruvate kinase in mycelium grown on that carbon source, since pyruvate kinase of this origin is inhibited by the pyruvate kinase antiserum and does have affinity for Mikacion Brilliant Yellow 6GS Sepharose Cl-4B. Unfortunately, the limited time available did not allow further experiments to optimize the detection of pyruvate kinase in acetate grown mycelium to verify whether or not a second pyruvate kinase gene exists in this fungus.

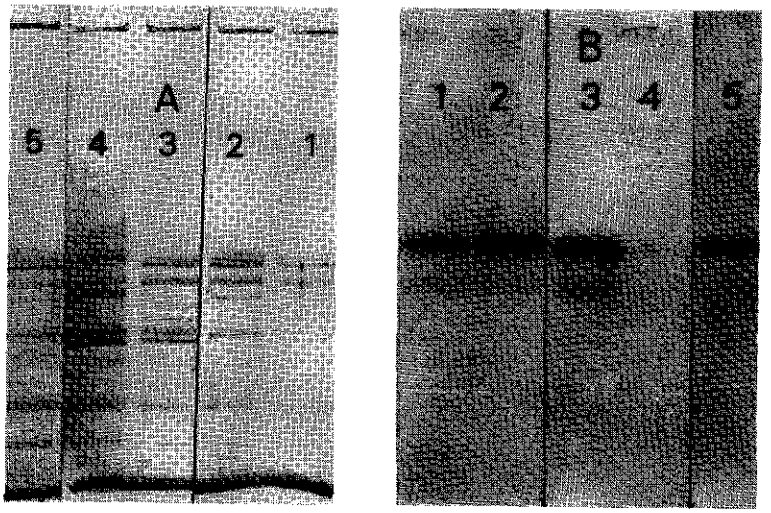


Fig. VI.11. A: SDS-polyacrylamide gels after electrophoresis and staining
Partially purified samples of the following strains were used:
1) WG096 (*pkiA*$^{+}$), 5 µg; 2) WG232 (*pkiA6*), <5 µg
3) WG083 (*pkiA3*), 5 µg; 4) WG231 (*pkiA5*), 11 µg
5) WG230 (*pkiA4*) <5 µ g.
B: Western blots after staining, similarly to A.

CONCLUSIONS

The pyruvate kinase in wild type *A.nidulans* is regulated by a mechanism that differentially responds to the carbon source offered. The levels of pyruvate kinase in polygalacturonate or acetate grown cultures are low and are raised quickly when a glycolytic carbon source like sucrose is offered. The change in

carbon source is reflected by the immunological activity of pyruvate kinase. An acetate grown culture of wild type *A.nidulans* shows a single precipitation line in the Ouchterlony double diffusion. This precipitate involves an unidentified protein in the crude extract of both sucrose or acetate grown *A.nidulans* that has no affinity for the matrix used in the partial purification of pyruvate kinase and that has no pyruvate kinase activity. After transfer to sucrose of an acetate grown culture a second precipitation line is observed in the Ouchterlony double diffusion, which can be assigned to pyruvate kinase. Detection of pyruvate kinase is also possible in a Western blot. The pyruvate kinase mutants tested all belong to a single gene *pkiA* as judged from non-complementation. Only one of the mutants (*pkiA5*) is totally devoid of pyruvate kinase activity and even in a Western blot pyruvate kinase protein is not detected. The absence of low molecular weight bands in the Western blot of this mutant supports the notion that these bands which are present in the Western blot of pyruvate kinase in crude extracts of wild type are degradation products of pyruvate kinase. Since the pyruvate kinase gene product is present in both a sucrose tolerant and a sucrose sensitive pyruvate kinase mutant strain there is no direct relationship between the absence of a pyruvate kinase gene product and sucrose tolerance.
Further research is required to establish whether or not a second *pki* gene occurs in *A.nidulans*.

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REFERENCES

Alwine, J.C., Kemp, D.J., Parker, B.A., Reiser, J., Renart, J., Stark, G.R. and Wahl, G.M. (1979). Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethylpaper. in: Colowick, S.P. and Kaplan, N.O. (eds.) Methods in Enzymology 68. Academic Press, New York, London, pp. 220-242.

Barwell, C.J. and Hess, B. (1971). Regulation of pyruvate kinase during gluconeogenesis in *Saccharomyces cerevisiae*. FEBS Lett. 19, 1-4.

Bos, C.J., Slakhorst, S.M., Visser, J. and Roberts, C.F. (1981). A third unlinked gene controlling the pyruvate dehydrogenase complex in *Aspergillus nidulans*. J.Bacteriol. 148, 594-599.

Bowen, B., Steinberg, J., Laemmli, U.K. and Weintraub, H. (1980). The detection of DNA-binding proteins by protein blotting. Nucl. Acids.Res. 8, 1-20.

Clifton, D., Weinstock, S.B. and Fraenkel, D.G. (1978). Glycolysis mutants in *Saccharomyces cerevisiae*.Genetics 88, 1-11.

Cozzarelli, N.R., Koch, J.P., Hayashi, S. and Linn, E.C.C. (1965). Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J.Bacteriol. 90, 1325-1329.

Demerec, M., Adelberg, E.A., Clark, A.J. and Hartman, P.E. (1966). A proposal for a uniform nomenclature in bacterial genetics. Genetics 54, 61-76.

Fell, D.A., Liddle, P.F., Peacocke, A.R. and Dwek, R.A. (1974) The preparation and properties of pyruvate kinase from yeast. Biochem.J. 139, 665-675.

Fraenkel, D.G. (1968). The accumulation of glucose-6-phosphate from glucose and its effect in an *Escherichia coli* mutant lacking phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. J.Biol.Chem. 243, 6451-6457.

Friedenthal, M., Roselino, E. and Passeron (1973). Multiple molecular forms of pyruvate kinase from *Mucor rouxii*. Immunological relationships among the three isoenzymes and nutritional factors affecting the enzymatic pattern. Eur.J. Biochem. 35, 148-158.

Gancedo, J.M. and Gancedo, C. (1979). Inactivation of gluconeogenic enzymes in glycolytic mutants of *Saccharomyces cerevisiae*. Eur.J.Biochem. 101, 455-460.

Hunsley, J.R. and Suelter, C.H. (1969a). Yeast pyruvate kinase I. Purification and some chemical properties. J.Biol.Chem. 244, 4815-4818.

Hunsley, J.R. and Suelter, C.H. (1969b). Yeast pyruvate kinase II. Kinetic properties. J.Biol.Chem. 244, 4819-4822.

Irani, M. and Maitra, P.K. (1974). Isolation and characterization of *Escherichia coli* mutants defective in enzymes of glycolysis. Biochem.Biophys.Res.Comm. 56,

127-133.

Kapoor, M. (1975). Subunit structure and some properties of pyruvate kinase of *Neurospora*. Can.J.Biochem. 53, 109-119.

Kapoor, M. (1976). Minireview: pyruvate kinase: a model allosteric enzyme for demonstration of structure-function relationships. Int.J.Biochem. 7, 439-443.

Kapoor, M. and Tronsgaard, T.M. (1972). Pyruvate kinase of *Neurospora crassa*: Purification and some properties. Can.J.Microbiol. 18, 805-815.

Kornberg, H.L. and Malcovati, M. (1973). Control *in situ* of the pyruvate kinase activity of *Escherichia coli*. FEBS Lett. 32, 257-259.

Kotlarz, D., Garreau, H. and Buc, H. (1975). Regulation of the amount and the activity of phosphofructokinases and pyruvate kinase in *Escherichia coli*. Biochim.Biophys.Acta 381, 257-268.

Krøll, J. (1973). Tandem-crossed immuno-electrophoresis. in: Axelsen, N.H., Krøll, J. and Weeke, B. (eds.) A manual of quantitative immuno-electrophoresis. Methods and applications. Universitetsforlaget, Oslo, Bergen, Tromsø, pp. 57-59.

Lam, K. and Marmur, J. (1977). Isolation and characterization of *Saccharomyces cerevisiae* glycolytic pathway mutants. J.Bacteriol. 130, 746-749.

Magasanik, B., Prival, M.J., Brenchley, J.E., Tyler, B.M. De Leo, A.B., Streicher, S.L., Bender, R.A. and Paris, C.G. (1974). Glutamine synthethase as a regulator of enzyme synthesis. in: Horecker, B.L. and Stadtman, E.R. (eds.) Current topics in cellular regulation. Vol. 8. Academic Press, New York, London, pp. 119-138.

Maitra, P.K. (1971). Glucose and fructose metabolism in a phosphoclucose isomeraseless mutant of *Saccharomyces cerevisiae*. J.Bacteriol. 107, 759-769.

Maitra, P.K. and Lobo, Z. (1977). Pyruvate kinase mutants of *Saccharomyces cerevisiae*. Biochemical and genetic characterisation. Molec.Gen.Genet. 152, 193-200.

Malcovati, M. and Kornberg, H.L. (1969). Two types of pyruvate kinase in *Escherichia coli* K12. Biochim.Biophys.Acta 178, 420-423.

Malcovati, M., Valentini, G. and Kornberg, H.L. (1973). Two forms of pyruvate kinase in *E.coli*: their properties and regulation. Acta Vitaminol.Enzymol. 27, 96-111.

McCullough, W. and Roberts, C.F. (1974). The role of malic enzyme in *Aspergillus nidulans*. FEBS Lett. 41, 238-242.

Ouchterlony, O. and Nilsson, L.A. (1978). Immunodiffusion and immuno-electrophoresis. in: Weir, D.M. (ed.) Handbook of experimental immunology. third edition. Blackwell Scientific Publications, Oxford, pp. 19.1-19.44.

Payton, M.A. (1978). A genetic study of sugar metabolism and transport in

Aspergillus nidulans. Ph.D.Thesis. Univ.Leicester.

Payton, M.A. and Roberts, C. (1976). Mutants of *Aspergillus nidulans* lacking pyruvate kinase. FEBS Lett. 66, 73-76.

Pertierra, A.G. and Cooper, R.A. (1977). Pyruvate formation during the catabolism of simple hexose sugars by *Escherichia coli:* studies with pyruvate kinase negative mutants. J.Bacteriol. 129, 1208-1214.

Pontecorvo, G., Roper, J.A., Hemmons, L.J., MacDonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus nidulans*. Adv.Genet. 5, 141-238.

Renart, J., Reiser, J. and Stark, G.R. (1979). Transfer of proteins from gels to diazobenzyloxymethylpaper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc.Nat.Acad.Sci. U.S.A. 76, 3116-3120.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Sprague jr., G.F. (1977). Isolation and characterization of a *Saccharomyces cerevisiae* mutant deficient in pyruvate kinase activity. J.Bacteriol. 130, 232-241.

Towbin, H., Staehlin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc.Nat.Acad.Sci. U.S.A. 76, 4350-4354.

Tsao, M.U. and Madley, T.I. (1975). Regulation of glycolysis in *Neurospora crassa:* kinetic properties of pyruvate kinase. Microbios 12, 125-142.

Tyler, B.M. (1978). Nitrogen assimilation. Ann.Rev.Biochem. 47, 1127-1162.

Valentini, G., Iadarola, P., Somani, B.L. and Malcovati, M. (1979). Two forms of pyruvate kinase in *Escherichia coli*. Biochim.Biophys.Acta 570, 248-258.

Waygood, E.B. and Sanwall, B.D. (1974). The control of pyruvate kinases in *Escherichia coli*. I. Physicochemical and regulatory properties of the enzyme activated by fructose 1,6-diphosphate. J.Biol.Chem. 249, 265-274.

Summary

This thesis deals with carbon metabolism in the lower eukaryote *Aspergillus nidulans*. This fungus is an attractive organism as a model to study genetics in relation to metabolism in lower eukaryotes.

In chapter I the present state of affairs in this field is summarized for *A. nidulans* and some of the advantages of this fungus in particular for physiological and biochemical genetic studies are indicated. The picture of the metabolic abilities of *Aspergilli* is still far from complete notwithstanding the many studies in *A. nidulans* and *A.niger*. The latter is a related fungus used for the biosynthesis of industrial enzymes and for the large scale production of metabolites of commercial and economic interest.
Ever since the early investigations into the physiology of *A.nidulans*, which started some 40 years ago, the use of mutants to study the effects of mutant genes on metabolism has become increasingly important. Therefore a brief review is given of the various ways in which mutants regarding to carbon metabolism in *A. nidulans* have been selected and isolated. An up to date list is added of mutations in carbon metabolism and, as far as these are known, of the metabolic defects involved.

In chapter II a study was made on the metabolic features of mutants in carbon metabolism, since mutations may have effects on the level of a particular enzyme directly as well as on the regulation of other steps in metabolism (*e.g.* inhibition, repression). The mutants used for this purpose were defective in pyruvate kinase, pyruvate dehydrogenase complex, pyruvate carboxylase, transaldolase and in two non-specified lesions in glycerol metabolism.
Growth tests with a variety of substrates and combinations of them were performed with the mutants and on the basis of the results obtained criteria were developed to distinguish the mutant phenotypes. Such growth tests can be used as a first step in the elucidation of unknown metabolic pathways and also to find a strategy to isolate particular mutants. From the carbon sources tested for growth, D-galacturonate emerged as a substrate useful to discriminate pyruvate kinase mutants, which showed good growth on this carbon source from mutants defective in the

pyruvate dehydrogenase complex or pyruvate carboxylase, which grow badly or not at all. The differences observed between these mutants in their ability to use D-galacturonate as sole carbon source indicated that pyruvate is a metabolite formed during the degradation of D-galacturonate. Moreover, it was predicted that selection for a mutant phenotype unable to use this carbon source would lead, among others, to mutants unable to metabolize pyruvate, for instance pyruvate dehydrogenase complex mutants.

In chapter III the results are described of an X-ray mutation experiment followed by enrichment on D-galacturonate. This led to a large number of mutants unable to grow on this carbon source. The criteria developed in chapter II were used to assign phenotypes to the mutants isolated. The majority of these were found to be defective in the pyruvate dehydrogenase complex. In addition mutants were isolated defective in pyruvate carboxylase and in glycerol metabolism, whereas a novel class of mutants was found blocked earlier in D-galacturonate metabolism.
The mutants isolated were further analyzed by complementation tests with defined tester strains. It was found that all pyruvate dehydrogenase complex mutants studied belonged to one of the three genes already known. The pyruvate carboxylase mutants were novel with respect to their inability to complement in a heterokaryon with two pyruvate carboxylase tester strains which mutually complement. All mutants found in glycerol metabolism belonged to the same gene.
Since mutants were obtained which were only defective in D-galacturonate metabolism, a preliminary study was started to analyze the metabolic route which this compound follows in wild type *A.nidulans*. A pathway for the degradation of D-galacturonate which leads to pyruvate as one of the products is known to occur in *Escherichia coli*. Although the presence of this pathway was easily demonstrated in the latter microorganism, no positive evidence has been found for a similar pathway operating in *A.nidulans*.

In chapter IV the purification and some properties of the pyruvate dehydrogenase complex of *A.nidulans* are described. A purification scheme was developed using ultracentrifugation of the complex in a crude extract, polyethylene glycol precipitation of the concentrated enzyme complex and affinity chromatography on ethanol-Sepharose 2B. After a final sucrose density gradient centrifugation step, a pure multienzyme complex was obtained with a specific activity of 7.8 U mg^{-1} with a recovery of 16%. The preparation finally obtained still contained pyruvate dehydrogenase kinase, which enzyme was copurified. SDS-polyacrylamide gel electrophoresis showed that the purified pyruvate dehydrogenase complex is composed of 4 proteins which were assigned to the α and β subunit of pyruvate decarboxylase,

lipoate acetyltransferase and of lipoamide dehydrogenase. The molecular weights of the complex enzymes resemble those of the complex of other eukaryotes and of *Bacilli*. The kinetic constants of the complex were listed and they were compared to those of the complexes of other sources.

In chapter V a purification scheme for pyruvate kinase is described based mainly on the use of affinity chromatography with reactive dyes. Screening several of these dyes coupled to Sepharose resulted in two dyes which were used in sequence to purify pyruvate kinase: Mikacion Brilliant Yellow 6GS, and Cibacron Blue 3G-A as a dextran conjugate. The addition of ethylene glycol during these purification steps was necessary to warrant an optimal recovery of the enzyme activity. Although some pyruvate kinase activity could be recovered by biospecific elution, in the final purification scheme aspecific elution of the enzyme was preferred by enhancing the ionic strength of the elution buffer. A homogeneous enzyme preparation was obtained with specific activity of 67 U mg^{-1} after a final gel filtration on Sephacryl S-300. The overall yield was low due to instability of the enzyme. The purified pyruvate kinase was used to raise antibodies in a rabbit.

In chapter VI the levels of pyruvate kinase specific activity were studied in wild type *A.nidulans* grown under different nutritional conditions. The specific activity of pyruvate kinase was high in mycelium grown on sucrose and low in mycelium grown on polygalacturonate or acetate. The latter two carbon sources allow growth of pyruvate kinase mutants. With antibodies against purified pyruvate kinase raised in a rabbit several pyruvate kinase mutants and wild type grown under pyruvate kinase inducing conditions were analyzed for the presence of a pyruvate kinase gene product in sucrose tolerant and sucrose sensitive pyruvate kinase mutant strains, by the use of immunological methods. Although pyruvate kinase could be detected easily in crude extracts of wild type by Ouchterlony double diffusion and tandem crossed immunoelectrophoresis, these methods failed when applied to extracts of pyruvate kinase mutants. Therefore Western blotting was introduced to screen for a pyruvate kinase gene product. Among 5 mutants, all at the same locus as found by non-complementation, only one was found, totally lacking immunological and enzymatic activity. Since a pyruvate kinase gene product was present in both a sucrose tolerant and a sucrose sensitive strain, the absence of such a gene product is not a prerequisite for sucrose tolerance observed with some pyruvate kinase mutants.

Samenvatting

Dit proefschrift handelt over het koolstofmetabolisme in de lagere eukaryoot *Aspergillus nidulans*. Deze schimmel is een aantrekkelijk modelorganisme voor de bestudering van de genetica met betrekking tot het metabolisme in lagere eukaryoten.

In hoofdstuk I is de huidige stand van zaken hierover voor *A.nidulans* samengevat en zijn enige voordelen voor het gebruik van deze schimmel in het bijzonder voor onderzoek van de fysiologie en de biochemische genetica aangegeven.
Het beeld van de stofwisselingsmogelijkheden van *Aspergilli* is nog verre van compleet niettegenstaande de vele studies gewijd aan *A.nidulans* en *A.niger*.
De laatstgenoemde is een verwante schimmel die gebruikt wordt voor de biosynthese van enzymen op industriële schaal, en tevens voor de productie van metabolieten die commercieel en economisch van belang zijn.
Sinds het eerste onderzoek naar de fysiologie van *Aspergillus nidulans* dat ongeveer 40 jaar geleden een aanvang nam, is het gebruik van mutanten om de effecten van mutante genen op het metabolisme te bestuderen steeds belangrijker geworden. Daarom is er een kort overzicht gegeven van de verschillende manieren waarop mutanten met betrekking tot het koolstofmetabolisme in *A.nidulans* geselecteerd en geïsoleerd werden. Een bijgewerkte lijst van mutaties in het koolstofmetabolisme met voor zover bekend de erop betrekking hebbende stofwisselingsstoornissen is toegevoegd.

In hoofdstuk II is het onderzoek beschreven naar de stofwisselingskenmerken van mutanten in het koolstofmetabolisme, aangezien mutaties niet alleen rechtstreeks invloed kunnen hebben op het niveau van een bepaald enzym maar ook op de regulatie van andere stappen in de stofwisseling (bijv. remming, repressie). De voor dit doel gebruikte mutanten waren gestoord in pyruvaatkinase, pyruvaatdehydrogenase-complex, pyruvaatcarboxylase, transaldolase en in twee niet nader bepaalde gebreken in de glycerolstofwisseling.
Groeitesten met een verscheidenheid van substraten en combinaties daarvan werden met deze mutanten uitgevoerd en op grond van de verkregen resultaten werden er

criteria ontwikkeld om de mutante fenotypen van elkaar te kunnen onderscheiden. Zulke groeitesten kunnen gebruikt worden als een eerste stap in de opheldering van onbekende stofwisselingswegen en ook om een strategie te vinden voor de isolatie van bepaalde mutanten.
Van de koolstofbronnen die gebruikt werden voor de groeiproeven bleek D-galacturonaat een nuttig substraat om enerzijds pyruvaatkinase mutanten, die hierop goed groeien, te onderscheiden van mutanten gestoord in het pyruvaatdehydrogenase-complex of pyruvaatcarboxylase, die er niet of nauwelijks op groeien. De verschillen waargenomen tussen deze mutanten in hun vermogen om D-galacturonaat als enige koolstofbron te gebruiken gaven aan dat pyruvaat een metaboliet is die gevormd wordt tijdens de afbraak van D-galacturonaat. Bovendien werd voorspeld dat selectie voor een mutant fenotype, niet in staat om deze koolstofbron te gebruiken, o.a. zou leiden tot mutanten gestoord in de pyruvaatverwerking, bijvoorbeeld pyruvaatdehydrogenase-complex mutanten.

In hoofdstuk III zijn de resultaten beschreven van een experiment waarbij Röntgenstraling gebruikt werd om mutaties te induceren. Aansluitend verrijking van mutanten in D-galacturonzuur als koolstofbron leidde tot een groot aantal mutanten die niet op deze verbinding kunnen groeien. De criteria die in hoofdstuk II werden ontwikkeld werden gebruikt om de geïsoleerde mutanten fenotypisch te karakteriseren. Het merendeel bleek gestoord in het pyruvaatdehydrogenase-complex. Daarnaast werden er mutanten geïsoleerd gestoord in pyruvaat carboxylase en gestoord in het glycerol metabolisme, terwijl een nieuw type mutanten werden aangetroffen op een eerder niveau geblokkeerd in het D-galacturonaat metabolisme zelf. De geïsoleerde mutanten werden verder geanalyseerd door middel van complementatie met gedefiniëerde tester stammen. Alle geïsoleerde pyruvaatdehydrogenase-complex mutanten hadden betrekking op een van de drie reeds bekende genen. De pyruvaatcarboxylase mutanten waren nieuw wat betreft hun onvermogen in een heterokaryon te complementeren met pyruvaatcarboxylase mutanten die dat onderling wèl kunnen. Alle mutanten in het glycerolmetabolisme hadden betrekking op hetzelfde gen. Aangezien mutanten waren verkregen die uitsluitend gestoord waren in het D-galacturonaat metabolisme, werd een inleidend onderzoek uitgevoerd om de stofwisselingsweg te analyseren die door deze verbinding wordt gevolgd in *A.nidulans*. Een afbraakweg voor D-galacturonzuur die naar pyruvaat leidt als een van de produkten is bekend voor *Escherichia coli*. Hoewel de aanwezigheid van deze weg in laatstgenoemd organisme gemakkelijk aangetoond kon worden, werd er geen positief bewijs gevonden voor de aanwezigheid van een soortgelijke weg in *A.nidulans*.

In hoofdstuk IV worden de zuivering en enige eigenschappen van het pyruvaatdehy-

drogenase-complex van *A.nidulans* beschreven. Een zuiveringsschema werd ontwikkeld, gebruik makend van ultracentrifugatie van het complex in een ruw extract, precipitatie van het geconcentreerde enzymcomplex met polyethyleenglycol en affiniteitschromatografie met behulp van ethanol-Sepharose 2B. Na ultracentrifugatie in een sucrose-dichtheidsgradient werd een zuiver multienzymcomplex verkregen met een specifieke activiteit van 7.8 U mg^{-1} in een opbrengst van 16%. Het uiteindelijk verkregen preparaat bevatte nog steeds pyruvaatdehydrogenase kinase, dat werd meegezuiverd. SDS-polyacrylamide gel electroforese toonde aan dat het gezuiverde pyruvaatdehydrogenase-complex bestaat uit 4 eiwitten toegewezen aan de α- en β-component van het pyruvaatdecarboxylase, lipoaat acetyltransferase en van het lipoamide dehydrogenase. De molecuulgewichten van de tot het enzymcomplex behorende enzymen lijken op die van het complex bij andere eukaryoten en *Bacilli*. De kinetische constanten van het complex werden vergeleken met die van het complex uit andere organismen.

In hoofdstuk V is een zuiveringsschema beschreven voor pyruvaatkinase, voornamelijk gebaseerd op het gebruik van affiniteitschromatografie met reactieve kleurstoffen. Een proefopzet waarin verscheidene van deze kleurstoffen gekoppeld aan Sepharose werden vergeleken, resulteerde in twee kleurstoffen die na elkaar gebruikt werden voor de zuivering van pyruvaatkinase: Mikacion Brilliant Yellow 6GS en Cibacron Blue 3G-A als dextraanconjugaat. De toevoeging van ethyleenglycol gedurende deze zuiveringsstappen was noodzakelijk voor een optimale terugwinning van de enzymactiviteit. Hoewel pyruvaatkinase ook met behulp van biospecifieke elutie teruggewonnen kon worden, werd uiteindelijk gekozen voor een aspecifieke elutie door verhoging van de ionsterkte van de elutiebuffer. Een homogeen enzympreparaat met een specifieke activiteit van 67 U mg^{-1} werd verkregen na een gelfiltratie met Sephacryl S-300. De opbrengst was laag tengevolge van instabiliteit van het enzym. Het gezuiverde pyruvaatkinase werd gebruikt voor het opwekken van antilichamen in een konijn.

In hoofdstuk VI werden de niveaus van de pyruvaatkinase specifieke activiteit bestudeerd in wild type *A.nidulans* gekweekt onder verschillende voedingsomstandigheden. De specifieke activiteit van het pyruvaatkinase was hoog in mycelium verkregen na groei op sucrose en laag in mycelium verkregen na groei op polygalacturonaat of acetaat. De laatstgenoemde koolstofbronnen stellen pyruvaatkinase mutanten in staat om te groeien. Met antilichamen tegen het gezuiverde pyruvaatkinase, opgewekt in een konijn, werden verscheidene pyruvaatkinase mutanten en het wild type na groei onder pyruvaatkinase inducerende omstandigheden, geanalyseerd op de aanwezigheid van een pyruvaatkinase genprodukt in sucrose tolerante

en sucrose gevoelige pyruvaatkinase mutanten, gebruik makend van immunologische methoden. Pyruvaatkinase kon gemakkelijk aangetoond worden in ruwe extracten van het wild type met de Ouchterlony dubbel diffusie en "tandem crossed immuno-electroforese". Deze methoden faalden echter wanneer extracten van pyruvaatkinase mutanten werden gebruikt. Daarom werd de "Western blotting" ingevoerd om te kijken naar de aanwezigheid van een pyruvaatkinase genprodukt. Onder 5 mutanten, alle op dezelfde locus, (zoals gevonden uit het onvermogen om te complementeren) werd slechts één mutant gevonden, die zowel immunologische als enzymatische activiteit volledig miste. Aangezien een pyruvaatkinase genprodukt zowel aanwezig was in een sucrose tolerante als in een sucrose gevoelige stam, is de afwezigheid van zo'n genprodukt geen vereiste voor de sucrosetolerantie waargenomen bij enkele pyruvaatkinase mutanten.

Curriculum vitae

De auteur van dit proefschrift werd op 20 juni 1952 te Almelo geboren. Na het doorlopen van de St.Alphonsusschool te Mariaparochie, Tubbergen (O.) van 1958 tot 1964, bezocht hij de Rijksscholengemeenschap "Erasmus" te Almelo waar hij in 1971 het diploma gymnasium-β behaalde. In datzelfde jaar begon hij de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Op 1 juli 1974 werd het kandidaatsexamen (S_2) afgelegd. Voor de doctoraalstudie werden twee hoofdvakstages gevolgd: organische chemie (Prof.dr. R.J.F. Nivard en Dr. G.I. Tesser) en chemische mikrobiologie (Prof.dr.ir. G.D. Vogels en Dr. G.P.A. Bongaerts). Daarnaast behaalde hij op 25 januari 1977 het diploma deskundigheid stralingshygiëne voor een C-laboratorium. Tijdens de doctoraalstudie verleende hij bij diverse praktika assistentie. Op 26 juni 1978 werd het doctoraalexamen afgelegd.

Op 1 januari 1979 werd hij als wetenschappelijk assistent aangesteld bij de secties microbiële- en biochemische genetica van de vakgroep Erfelijkheidsleer (Prof. J.H. van der Veen) aan de Landbouwhogeschool te Wageningen. Daar verrichtte hij o.l.v. Ir. C.J. Bos en Dr.ir. J. Visser een 3-jarig onderzoek waarin diverse aspecten van het koolstofmetabolisme bij *Aspergillus nidulans* werden bestudeerd, zoals in dit proefschrift is beschreven. Gedurende deze tijd was hij tevens free-lance recensent bij het Nederlands Bibliotheek en Lektuur Centrum.

Op 1 maart 1982 trad hij in tijdelijke dienst bij bovengenoemde vakgroep Erfelijkheidsleer voor de uitvoering van een eenjarig project.

Op 1 september 1982 trad hij in dienst als medewerker bij de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek te 's-Gravenhage.