Analysis of Acetate Non-utilizing (*acu*) Mutants in *Aspergillus nidulans*

By S. ARMITT, W. McCULLOUGH AND C. F. ROBERTS

Department of Genetics, University of Leicester, Leicester, LE1 7RH

(Received 16 June 1975; revised 1 August 1975)

SUMMARY

Genetic analysis of 119 acetate non-utilizing (*acu*) mutants in *Aspergillus nidulans* revealed ten new loci affecting acetate metabolism in addition to the three previously recognized on the basis of resistance to fluoroacetate and acetate non-utilization. The enzyme lesions associated with mutations at seven of the *acu* loci are described. These are: *facA* (= *acuA*), acetyl-CoA synthase; *acuD*, isocitrate lyase; *acuE*, malate synthase; *acuF*, phosphoenolpyruvate carboxykinase; *acuG*, fructose 1,6-diphosphatase; *acuK* and *acuM*, malic enzyme.

The *acu* loci have been mapped and are widely distributed over the genome of *A. nidulans*. Close linkage has only been found between *acuA* and *acuD* (less than 1% recombination). There is no evidence for any pleiotropic mutation in that region affecting the expression of both these genes.

Poor induction of the enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase in mutants lacking acetyl-CoA synthase, and also in the other two classes of fluoroacetate-resistant mutants, indicates that the inducer, acetate, may be metabolized to a true metabolic inducer, perhaps acetyl-CoA, to effect formation of the enzymes. There is no evidence of any other class of pleiotropic recessive *acu* mutations affecting the expression of the *acuD* and *acuE* genes, which are therefore thought to be subject to negative rather than positive control.

INTRODUCTION

A key observation which led to the definition of the role of the anaplerotic glyoxylate cycle in the utilization of C₂ compounds for growth by micro-organisms (Kornberg, 1966*a*), was that strains devoid of isocitrate lyase activity failed to grow with acetate as sole carbon source. Mutations affecting many of the enzymes required for growth of bacteria on either glycolytic carbon sources or on gluconeogenic sources such as acetate are known (Fraenkel & Vinopal, 1973), and the genetic loci have been identified in the linkage maps especially of the enteric bacteria (Taylor & Trotter, 1972). In *Escherichia coli* the two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, constitute an operon subject to negative control by a nearby regulatory locus (Brice & Kornberg, 1968).

In eukaryotic organisms there is evidence for the association of enzymes of the glyoxylate cycle in organelles termed glyoxysomes, or sometimes termed peroxisomes (De Duve, 1969; Tolbert, 1971) and there is some evidence that similar organelles occur in fungi (Kobr, Vanderhaeghe & Combépine, 1969; O'Sullivan & Casselton, 1973). The formation of a new organelle in eukaryotes during acetate adaptation adds further interest to a genetic investigation of acetate metabolism.

In *Aspergillus nidulans*, fluoroacetate-resistant mutants map at three loci (*facA*, *facB* and *facC*) at which recessive mutations also lead to loss of ability to utilize acetate for growth (Apirion, 1965). Romano & Kornberg (1969) found that *facA* strains are defective in acetyl-

CoA synthase. Similar studies have been reported for *Coprinus lagopus* (Casselton & Casselton, 1974).

The present study in *A. nidulans* had two principal objectives. The first was to seek a better understanding of the central areas of intermediary metabolism functioning in the primary growth phase of the organism. Particular attention has been given to the regulation of isocitrate lyase and malate synthase (Armitt, Roberts & Kornberg, 1970, 1971) as key enzymes of the glyoxylate cycle – loss of either of these enzymes by mutation at either of the two unlinked structural gene loci generates acetate non-utilizing strains. The second objective was to investigate the possibility that the production of these enzymes might be genetically regulated by a system of positive control. Such systems appear common in fungi (Gross, 1969) and are characterized by the occurrence of recessive, pleiotropic mutations which are defective in the expression of several genes.

METHODS

Organisms. All the strains used were derived from the Glasgow stock of *A. nidulans* (Pontecorvo *et al.*, 1953), and the mutants were isolated in two translocation-free strains, R21 (*pabaA1 yA2*) and R153 (*wA3; pyroA4*). The 'master strain' R87 (FGSC105) *biA1; AcrA1; wA3; phenA2; pyroA4; lysB5; sB3; nicB8; coA1* was used in allocating mutant alleles to linkage groups.

Mutant alleles characterizing each of Apirion's (1965) three *fac* (fluoroacetate resistance) loci were used in strains derived as follows: locus *facA303* was used in strain R65 (master strain E, Glasgow 94) *su1adE20 yA2 adE20; wA3; galA1; pyroA4; facA303; sB3; nicB8; riboB2*. Locus *facB101* was used in strain R76 *biA1; AcrA1; (nicB8); facB101 tryC473* which derived from Glasgow strain 821 through several crosses. Locus *facC102* was used in strain R69 (Glasgow 080) *wA3; pyroA4; facC102*.

Media. The defined minimal medium (MM) was basically that of Pontecorvo *et al.* (1953), which contained (g/l distilled water): KNO₃, 6.0; KCl, 0.52; KH₂PO₄, 1.52. It was modified by the addition of a trace elements solution which supplied (mg/l medium): FeSO₄.7H₂O, 1.0; ZnSO₄.7H₂O, 8.8; CuSO₄.5H₂O, 0.4; MnSO₄.4H₂O, 0.15; Na₂B₄O₇.10H₂O, 0.10; (NH₄)₆Mo₇O₂₄.4H₂O, 0.05. The medium was brought to pH 6.5 and MgSO₄.7H₂O was added separately to 0.52 g/l.

Nutritional supplements for auxotrophic strains were added, where required, as follows (mg/l medium): adenine sulphate, 50; *p*-aminobenzoic acid, 1.0; D-biotin, 0.02; L-lysine-HCl, 200; nicotinic acid, 2; L-phenylalanine, 50; pyridoxin-HCl, 0.5; riboflavin-HCl, 2.5; L-tryptophan, 100.

Media for plates were solidified with 1.5% (w/v) agar (Oxoid No. 3).

Media for liquid culture contained the wetting agent Tween 80 diluted 10⁻⁵ (v/v), and sterile MgSO₄ solution was added after autoclaving.

The carbon sources were added aseptically to media from sterile stock solutions in distilled water. The stock solution of potassium acetate was 2 M at pH 6.5 and it was added to a final concentration of 0.1 M. Other stock solutions were 1 M and the carbon sources were supplied at the following final concentrations (M): sucrose, 0.02; glucose, 0.05; glycerol, 0.04; L-glutamate, 0.02 (sodium salt at pH 6.5); L-proline, 0.02.

Classification of the galactose non-utilizing marker *galA1* was done on MM at pH 5.5 with 0.05 M D-galactose (Roberts, 1970).

Complete medium (CM) was 2% (w/v) malt extract (Difco), 0.1% (w/v) Bacto peptone (Difco) with 2% (w/v) glucose or sucrose, and the nutritional supplements at the concentra-

tions indicated above; it was solidified with 1.5% (w/v) agar (Difco No. 3). This medium promotes rapid growth and heavy conidiation. It was routinely used for plating strains and for the preparation of slants upon which strains were maintained, with storage at 0 to 4 °C. *Aspergillus nidulans* was grown at 37 °C, unless stated otherwise.

Preparation of conidial suspensions. A suspension of conidia was spread on the surface of CM plates to give confluent growth and good conidiation after 2 days' incubation. Each plate was then flooded with about 20 ml of Tween-saline (0.08% w/v, NaCl containing Tween 80 diluted 2.5×10^{-4} , v/v) and the conidia were harvested by drawing a sterile wire across the surface of the plate. The suspensions were transferred to McCartney bottles and vigorously shaken to disrupt chains of conidia. The conidia were washed by centrifugation and resuspended in Tween-saline. The final conidial suspensions were made in neutral phosphate buffer (3 g KH_2PO_4 ; 7 g Na_2HPO_4 ; 4 g NaCl; 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, distilled water to 1 l; pH 7.2), and stored at 0 to 4 °C.

Viable conidia were counted by plating dilutions on CM. Particle numbers in suspensions were estimated from the E_{500} values, by comparison with a standard curve constructed by using a Coulter counter fitted with a 100 nm aperture tube. The viable and particle counts usually agreed to within 20%.

The isolation of acetate non-utilizing (acu) mutants. A simplified filtration procedure derived from the technique of Woodward, De Zeeuw & Srb (1954) was used to enrich cultures for acetate non-utilizing strains.

Suspensions of conidia of strains R21 or R153 were exposed to ultraviolet light (40% survival), inoculated into liquid MM-acetate to give 10^5 to 10^6 viable conidia/ml and incubated on a gyrotary shaker. They were periodically filtered through four double-layers of cotton gauze to remove growing mycelium and incubation continued until no further growth was visible. Fresh medium (0.2 vol.) was added after the second filtration. The final filtrate was stored at 4 °C and the viable count determined on MM-sucrose. Samples were pour-plated in MM-sucrose to produce about 15 colonies/dish and the colonies screened for *acu* mutants by replicating them on to MM-acetate. Presumptive mutants were cloned by single colony isolation and retested.

The irradiated conidia of strain R21 were incubated in MM-acetate, with filtration every 2 h from 12 to 24 h and thereafter at 30 and 42 h. The final filtrate contained about 40 viable conidia/ml, and the 800 colonies screened provided 62 *acu* mutants numbered *acu201* to *acu262*.

In strain R153 the suspension was filtered every 2 h from 16 to 26 h, when the viable count was 180 conidia/ml. About 1200 colonies were screened, yielding 58 mutants numbered *acu301* to *acu358*. Strain *acu345* was later discarded.

Genetic analysis. The techniques used follow those of Pontecorvo *et al.* (1953) with some minor modifications.

Heterokaryons were synthesized by mixing conidia of two strains with complementary nutritional requirements on the surface of CM and incubating overnight. Small blocks of agar (about 2 mm³) containing mycelium were transferred to the surface of thick MM-sucrose plates. Vigorously growing heterokaryon sectors were selected after 2 to 4 days incubation and maintained on MM-sucrose by transfer of mycelium from the colony boundary.

Heterokaryons between pairs of *acu* mutants in strains R21 and R153 were tested for acetate utilization on MM-acetate, using MM-sucrose as control. Each heterokaryon was tested in four replicates and generally all four controls grew normally on MM-sucrose. On MM-acetate, heterokaryons could be classified as growing like the wild type (comple-

menting mutants) or failing to grow (non-complementing mutants). When ambiguous results were obtained the growth tests were repeated and, where necessary, a new heterokaryon synthesized from its component strains.

Mutant alleles were allocated to linkage groups by mitotic haploidization (McCully & Forbes, 1965). A heterozygous diploid was synthesized between the master strain R87, which has a marker in each of the eight linkage groups, and an *acu* mutation in the R21 strain. A dilute suspension of conidia of the diploid (1 loop of conidia in 20 ml Tween-saline) was stab inoculated into CM plates containing *p*-fluorophenylalanine (0.1 g/l). After 10 to 14 days' incubation the poorly-growing colonies were replicated with filter paper discs on to CM plates where they produced good conidiation. Sectors exhibiting the segregation of recessive conidial colour genes were selected visually, and tested for the distribution of all markers.

Crosses were done on MM-glucose. Recombination analysis of crosses between the *acu* mutants was done as follows. For each cross a small sample of a suspension of ascospores from an hybrid cleistothecium was streaked on growth media with either acetate or sucrose as carbon source. The samples contained 10^3 to 10^4 ascospores and produced dense growth on the sucrose medium. If the mutants recombined freely the ascospore suspension also produced confluent growth on acetate medium, but if the mutants were closely linked few or no colonies were formed. In the latter case samples of the ascospore suspension were plated at high dilution on acetate or sucrose media and the recombination fraction calculated as twice the proportion of colonies growing on acetate.

Growth of mycelium for the preparation of cell-free extracts. Mycelium was grown in liquid culture using 2 l Erlenmeyer flasks. These had three or four vertical indentations in their walls to aid aeration and dispersion of the mycelium, and had been coated inside with a water-repellent silicone film (Siliclad; Clay Adams, Parsippany, New Jersey 07054, U.S.A.) to prevent adhesion of the mycelium. The flasks contained 500 ml medium and were inoculated with conidial suspensions to give 10^6 conidia/ml. Cultures were incubated at 37 °C on a gyrotary shaker operating at 200 to 250 rev./min.

Isocitrate lyase or malate synthase activities were measured in extracts of mycelium which had been grown in 0.02 M-sucrose plus 0.10 M-acetate for 18 h. All other enzyme activities were measured in extracts prepared from mycelium grown in 0.02 M-sucrose for 18 h, washed aseptically on the filter with C-free MM at 37 °C, then resuspended in fresh growth medium containing 0.1 M-acetate and incubated for a further 6 h.

Preparation of cell-free extracts. Mycelium was harvested by filtration under suction on Whatman No. 1 filter papers, washed with distilled water and resuspended in the buffer appropriate to each enzyme assay. The mycelium was broken by sonication (MSE 150 W ultrasonic disintegrator) for 30 s in ice-cold buffer and cell debris removed by centrifugation at 12000 g for 20 min at 0 to 4 °C. When assaying malic enzyme the cell-free extract was divided at this stage and dithiothreitol to 1 mM was added to one portion. Cell-free extracts were maintained on ice until assayed within 2 h of their preparation.

Enzyme assays. All the assays were done at 37 °C except that for malate synthase which was at 25 °C. The final incubation volume was 1 ml.

Acetyl-CoA synthase (EC. 6.2.1.1) was assayed by the method described by Jones & Lipmann (1955) using hydroxylamine as an acceptor for acetyl groups from acetyl-CoA formed in the reaction. Assay blanks lacking CoA were incubated at the same time. Each assay tube contained approximately 750 µg protein.

Malate synthase (EC. 4.1.3.1) was assayed using the polarographic method described by Weitzmann (1969). Reaction volumes of 1 ml contained 100 µmol tris-HCl buffer pH 8.0,

10 μ mol MgCl_2 , 1 μ mol glyoxylate, 0.25 μ mol acetyl-CoA, and approximately 250 μ g protein. The acetyl-CoA was prepared by acetylation of CoA as described by Stadtmann (1957).

The remaining assays were done on an SP800 recording spectrophotometer (Unicam, Cambridge).

Isocitrate lyase (EC. 4.1.3.1) was assayed by following the formation of glyoxylate phenylhydrazone at 324 nm (Kornberg, 1965). Assay mixtures of 1 ml contained 25 μ mol imidazole buffer pH 6.8; 5 μ mol MgCl_2 ; 1 μ mol EDTA; 4 μ mol phenylhydrazine hydrochloride and 2 μ mol DL-isocitrate.

Fructose 1,6-diphosphatase (EC. 3.1.3.11) was assayed in the presence of excess phosphoglucose isomerase and phosphoglucose dehydrogenase activity by following the reduction of NADP at 340 nm. Assay mixtures of 1 ml contained 100 μ mol tris buffer pH 8.5, 1 μ mol EDTA, 5 μ mol MgCl_2 , 1 μ mol FDP (fructose 1,6-diphosphate), 0.3 mg NADP, 5 units of phosphoglucose isomerase, and 1 unit of phosphoglucose dehydrogenase.

Phosphopyruvate (PEP) carboxykinase (EC. 4.1.1.32) was assayed by two methods which gave essentially similar results. Enzyme activity was measured in the presence of excess malate dehydrogenase by following the oxidation of NADH at 340 nm. The assay mixture contained 50 μ mol imidazole buffer pH 6.5, 7.5 μ mol MnCl_2 , 10 μ mol NaHCO_3 , 1 μ mol ADP, 0.1 mg NADH, 5 units malate dehydrogenase, and 1 μ mol PEP.

PEP carboxykinase activity was also assayed by measuring the bicarbonate-dependent formation of oxalacetate phenylhydrazone at 320 nm. Assay mixtures contained 66 μ mol imidazole buffer pH 6.5, 7.5 μ mol MgCl_2 , 10 μ mol NaHCO_3 , 4 μ mol phenylhydrazine hydrochloride, 1 μ mol ADP, and 1 μ mol PEP.

Malic enzyme (EC. 1.1.1.40) was assayed by following the malate-dependent reduction of NADP at 340 nm. Assay mixtures of 1 ml contained 80 μ mol tris buffer pH 7.4, 10 μ mol MgCl_2 , 2 μ mol dithiothreitol, 0.25 μ mol NADP, and 20 μ mol L-malate. Cell-free extracts for this assay contained 1 mM-dithiothreitol (McCullough & Roberts, 1974).

Protein concentration in cell-free extracts was measured with the Folin reagent. The extracts generally contained about 5 mg protein/ml.

RESULTS

Isolation of the acu mutants

The initial strains, R21 (*pabaA1 yA2*) and R153 (*wA3; pyroA4*), were chosen for their complementary conidial colour and nutritional markers, to facilitate complementation analysis through heterokaryons. The nutritional requirements are for compounds that do not provide significant amounts of carbon for growth. Only mutant strains showing normal growth on sucrose but very little or no growth on acetate were retained; we tended to discard leaky *acu* mutants. Classification of wild-type or mutant response to acetate on agar plates can be done after 24 h incubation and is perfectly clear at 36 h.

Genetic analysis of the acu mutants

Complementation analysis. Heterokaryons between *acu* mutants in strains R21 and R153 were synthesized and tested for their growth on MM-acetate. The analysis included some *acu* mutants characterized in previous reports.

Mutants representing each of the three *fac* loci identified by Apirion were included in our analysis. Strain R65 carries the allele *facA303* and was tested against all of the R21 *acu*

Table 1. *Complementation analysis of acetate non-utilizing mutants in A. nidulans*

Tester strains are in bold type. Also included in the analysis were strains carrying mutations defining *facA303* (R65), *facB101* (R76) and an isocitrate lyase mutant *acuD125*.

Complementation group	Acetate non-utilizing mutants									
	R21 (<i>pabaA1 yA2</i>)					R153 (<i>wA3; pyroA4</i>)				
<i>acuA</i> (= <i>facA</i> *)	204 227 251	209 229 ^{ts} 260	210 244 262	212 245	225 249	312 330	318 333	319 338	321	
(<i>facB</i> *)	—					—				
<i>acuC</i> (= <i>facC</i> *)	203 231 ^{ts} 255	218 232 259	220 235	221 242	222 243	311 348	323 355	326	341	
<i>acuD</i>	202 ^e 240	207 ^{ts,e} 250 ^{ts,e}	219 ^e 254	230 ^e 261 ^e	240	306 ^e 342 ^e	313 ^{ts,e} 344 ^e	320 353 ^{ts,e}	325	
<i>acuE</i>	201 ^{ts}	208	215	224 ^{ts}		307	316	317	327	
<i>acuF</i>	205	238				305	315	328	347	
<i>acuG</i>	223	226	239	241	252	310				
<i>acuH</i>	206	236	253	258		309	322	329	357	
<i>acuJ</i>	211 228	213 256	214 257	216		302 349	335 351	343	346	
<i>acuK</i>	233	237	248			303 331	308 339	314	324	
<i>acuL</i>	217	234	246	247		304 354	334 358	337	350	
<i>acuM</i>			—			301 352	332	336	340	
<i>acuN</i>			—			356				
Total			62				57			

^{ts}, Temperature-sensitive allele; ^e, alleles showing intragenic complementation.

* Apirion (1965).

mutants. Strains R76 (*facB101*) and R69 (*facC102*) were only tested against R21 *acu* mutants characterizing groups *acuD* to *acuN* (see below).

Armitt *et al.* (1970) reported *acu* mutants lacking activity for the enzyme isocitrate lyase, and a suitable recombinant of one of these mutants (*acu125*) was tested against all the R21 *acu* mutants.

Synthesizing and testing all combinations of *acu* mutants in a grid 62 × [57 (+2)] would have been both tedious and unnecessary. The strategy adopted was to begin with a few R153 *acu* mutants together with *facA303* and *acu125* and test these against all the R21 *acu* mutants. Once a non-complementing pair was found, each mutant was taken as a 'tester' for that complementation group in the two sets of mutants. The R21 tester was then tested against all the remaining R153 *acu* mutants. Other mutants non-complementing with a tester were regarded as allocated to that complementation group and not examined further. Mutants complementing known testers were retained in the programme until a non-complementing mutant was found in the opposite set, when a new pair of testers and a new complementation group was established.

Allocation of the *acu* mutants to the first ten of the complementation groups listed in

Table 2. *Recombination analysis of acetate non-utilizing mutants in A. nidulans*

The results of crosses between *acu* mutants characterizing complementation groups *acuA* to *acuN*. +, Many colonies formed when a sample of ascospores from a hybrid cleistothecium was tested on medium containing acetate as carbon source; —, no colonies formed. The figures are the recombination fractions between mutants. Group B was represented by Apirion's (1965) *facB101* mutant either in strain R76 or recombined into the R153 genotype (*facB3-101*). Group C was represented in the R153 genotype by Apirion's *facC102* mutant (R69).

R21 strains	R153 strains												
	<i>acuA</i>	<i>acuB</i>	<i>acuC</i>	<i>acuD</i>	<i>acuE</i>	<i>acuF</i>	<i>acuG</i>	<i>acuH</i>	<i>acuJ</i>	<i>acuK</i>	<i>acuL</i>	<i>acuM</i>	<i>acuN</i>
	312	3-101	102	306	317	305	310	329	302	308	304	301	356
<i>acuA204</i>	.	+	+	< 1	.	+	22	50	+	+	+	+	.
<i>acuB101</i>	.	.	.	.	.	.	.	.	.	.	.	+	.
<i>acuC220</i>	.	+	—	.	.	+	+	+	+	+	+	+	.
<i>acuD254</i>	.	.	.	—	.	+	22	40	+	+	+	+	33
<i>acuE201</i>	.	.	.	.	—	+	+	+	+	+	+	50	.
<i>acuF205</i>	.	+	.	.	.	.	+	+	+	+	+	+	.
<i>acuG223</i>	.	+	.	.	.	.	.	50	+	+	+	+	45
<i>acuH253</i>	.	+	.	+	.	.	.	.	50	+	+	+	24
<i>acuJ211</i>	.	+	.	.	.	.	.	.	.	+	.	6	.
<i>acuK248</i>	.	+	.	.	.	.	.	.	.	.	.	50	.
<i>acuL217</i>	.	+	.	.	.	.	.	.	+	+	.	+	.
<i>acuM2-301</i>	.	50	.	.	.	.	.	.	.	.	.	.	.

Table 3. *Allocation of acu mutations to linkage groups by mitotic haploidization*

Linked markers assort into haploid strains from the heterozygous diploids only in the two parental configurations, and classes essentially of this pattern are shown in bold type. Markers on different chromosome pairs assort into four classes.

Linkage group	Strain of origin*	Known markers	No. of haploids†									
			<i>acuF</i>	<i>acuF</i> ⁺	<i>acuH</i>	<i>acuH</i> ⁺	<i>acuJ</i>	<i>acuJ</i> ⁺	<i>acuK</i>	<i>acuK</i> ⁺	<i>acuL</i>	<i>acuL</i> ⁺
I‡	R21 <i>acu</i>	<i>pabaA1 yA2</i>	19	25	37	30	36	0	17	16	28	29
	MS	<i>biA1</i>	11	8	17	12	0	11	10	15	3	9
II	R21 <i>acu</i>	—	16	20	22	12	21	4	15	5	18	23
	MS	<i>acrA1 wA3</i>	14	13	32	30	15	7	12	26	14	25
III§	R21 <i>acu</i>	—	27	30	53	41	36	11	26	31	31	48
	MS	<i>phenA2</i>	3	3	1	1	0	0	1	0	0	0
IV	R21 <i>acu</i>	—	14	24	30	21	23	5	27	2	31	0
	MS	<i>pyroA4</i>	16	9	24	21	13	6	0	29	0	48
V	R21 <i>acu</i>	—	26	24	53	9	28	6	14	24	22	41
	MS	<i>lysB5</i>	4	9	1	33	8	5	13	7	9	7
VI	R21 <i>acu</i>	—	21	16	24	16	14	8	7	14	12	13
	MS	<i>sB3</i>	9	17	30	26	22	3	20	17	19	35
VII	R21 <i>acu</i>	—	29	12	29	14	19	4	13	10	16	21
	MS	<i>nicB8</i>	1	21	25	28	17	7	14	21	15	27
VIII	R21 <i>acu</i>	—	NC	NC	48	42	32	10	22	28	24	46
	MS	<i>coA1</i>	NC	NC	6	0	4	1	5	3	7	2

NC, Not classified.

* The haploid component strains of the heterozygous diploids were *pabaA1 yA2*; *acu* (R21 *acu*) and the master strain (MS) R87, the genotype of which is given in the text. The mutant alleles tested were *acuF238*, *acuH206*, *acuJ211*, *acuK248* and *acuL217*.

† Strains recovered from the haploidization procedures were classified as haploid on the segregation of a recessive conidial colour marker and at least one unlinked nutritional marker.

‡ Haploids carrying the *wA3* allele (white conidia) were classified by the biotin marker (*biA1*).

§ The use of *p*-fluorophenylalanine to promote haploidization tended to eliminate haploid strains requiring phenylalanine (*phenA2*).

Fig. 1. Mapping of the *acu* loci in *A. nidulans*. The *acu* loci which have been mapped meiotically within linkage groups are shown in relation to previously mapped markers (Dorn, 1967; Clutterbuck & Cove, 1974). Figures are percentage recombination fractions; those in parenthesis are rough estimates. Loci shown in parenthesis have been allocated to the linkage group by mitotic haploidization, but not mapped meiotically. The *acuL* locus (linkage group IV; M. Payton, unpublished) does not map in the interval *methG* to *pyroA*, but has consistently shown weak linkage (40% recombination) with *pyroA*. The *acuH* locus (linkage group V) is excluded from the interval *acuA* to *acuN*. The mapping of two other loci of interest is also shown. The *pdhA* locus (linkage group I) affects activity of the pyruvate dehydrogenase complex (Romano & Kornberg, 1968). The *pycA* locus (linkage group III) affects pyruvate carboxylase activity (Skinner & Armit, 1972).

Table 1 required 1031 heterokaryon tests out of the 3658 necessary to complete a 62×59 grid. Each of the tester mutants characterizing a complementation group was combined with most of the mutants in the opposite set.

This procedure quickly revealed eight new complementation groups (*acuD* to *acuL*; Table 1), which together with groups A and C accounted for all of the mutants except six in R153. These six mutants were crossed into the R21 background and when tested amongst themselves five were non-complementing and allocated to a ninth new group (*acuM*). One mutant (*acu356*) complemented all testers and was shown to represent yet another locus (*acuN*) by recombination analysis. The most remarkable feature of the distribution of the *acu* mutants amongst the complementation groups was the absence of any that were functionally allelic to Apirion's *facB* mutant. The isolation of *acuM* and *acuN* mutants only in strain R135 might have been only a matter of chance, although it could reflect a property of these mutants causing their elimination in the longer period of filtration applied to R21.

One objection to this method of analysis is that intra-genic complementation could result in failure to allocate complementing mutant alleles to their correct group. While we have detected intra-genic complementation amongst the *acuD* mutants, this was not a problem since one of the testers (*acu254*) is a totally non-complementing allele while the other (*acu125*) only gave a single positive result. We assumed that each mutant strain contained only one *acu* mutation, and that these mutations are recessive. If either assumption was untrue for a given strain that strain would be non-complementary to more than one tester. That situation was not found.

Recombination analysis. Apirion (1965) mapped the *facA* locus in linkage group V, and

the *facB* and *facC* loci are far apart in linkage group VIII. The isocitrate lyase locus (*acuD*) is closely linked to *facA* (Armitt *et al.*, 1970). The malate synthetase locus (*acuE*) is in linkage group IR close to the *bia1* locus (Armitt *et al.*, 1971).

Close linkage between *facA* and *acuD* prompted us to test representative mutants from each complementation group for close linkage with the established *acu* loci and with one another. The close linkage between *facA* and *acuD* was confirmed and these loci were found to be loosely linked (22% recombination) to *acuG* (Table 2). Weak linkages noted between *acuD*, *acuH* and *acuN*, result from the presence of each of these loci in linkage group V (Table 3; Fig. 1). Only one further case of close linkage was found, that between *acuJ* and *acuM* (6% recombination).

Mapping the acu loci. Five of the new *acu* loci for which no clear linkage was discovered in the preliminary recombination analysis (Table 2) were allocated to linkage groups by mitotic haploidization (Table 3). The ten new *acu* loci (*acuD* to *acuN*; the serial I has not been used) are thus allocated to linkage groups and most of them have been mapped meiotically within the linkage groups (Fig. 1).

Five of the thirteen *acu* loci so far discovered fall in linkage group V; otherwise, the loci are well scattered over the genome. The only case of very close linkage between *acu* loci remains that found between *facA* (= *acuA*) and *acuD* which are less than one map unit apart in linkage group V.

Ascospore viability. When using genetic crosses, suspensions of ascospores from hybrid cleistothecia were pour-plated in CM to which sucrose (0.02 M) was added. There was no indication of poor viability of ascospores associated with any of the *acu* mutations, in contrast to the situation in *Neurospora crassa* where some *acu* mutations prevent growth of ascospores (Flavell & Fincham, 1968*a*).

Properties of mutants at the acu loci

The complementation analysis (Table 1) indicates that all the *acu* mutants result from recessive mutations. Had any strain carried a dominant *acu* mutation this strain would have yielded acetate non-utilizing (mutant) heterokaryons, and this result was not found. Moreover, diploid strains often arose from the heterokaryons as sectors producing only green (wild-type) conidia. Such diploid strains, when tested for growth upon acetate, always gave the same result as that for the corresponding heterokaryon. In addition, the heterozygous diploid strains, synthesized between a master strain and certain *acu* strains for the purpose of allocating *acu* mutations to linkage groups (Table 3), grew as does the wild type on acetate. Thus the *acu* mutations are recessive in both heterokaryons and heterozygous diploids.

The recessive nature of the *acu* mutations agrees with the expectation of their loss of enzyme functions required for acetate utilization. To discover the enzyme lesions, the *acu* strains were first tested for growth on other related carbon sources and then representative mutants examined for enzyme activities.

Growth characteristics of the acu mutants. The *acu* mutants were tested for their growth responses on agar plates to the carbon sources shown in Table 4. In the main, all mutants within a complementation group had the same characteristics. These are discussed below in relation to the known enzyme lesions associated with mutations at each locus.

Resistance to fluoroacetate. All *acu* mutants resistant to fluoroacetate fell into groups A or C (Table 4). Growth of group C mutants in the presence of the agent was characteristically less vigorous than that of group A mutants or the *facB* mutant. Further properties of mutants in these loci are discussed below.

Table 4. *Growth characteristics of acetate non-utilizing mutants in A. nidulans*

Growth responses of the *acu* mutants on solid medium with different carbon sources are shown in comparison with the growth of wild type on hexose, + + +. No growth is indicated —, and increasingly better growth as (+), +, + +. Generally all mutants within a group had the same growth response. Where mutants differed within a group this is indicated —/+. Growth tests were read after 48 h incubation. For growth on butyrate, only mutants representing each group in the R21 strain were tested. Results for the B group are based upon *facB101* crossed into strain R21. Certain *acu* mutants showed different growth responses to glutamate from those described in the Table when they were tested in liquid medium rather than on agar plates. The most striking differences were found amongst the *acuD* mutants (see text also) and the *acuL* mutants; all the mutants grew well on glutamate in liquid culture whereas some of the mutants in each group failed to grow on plates. The *acuK* and *acuM* mutants all grew poorly on glutamate in liquid culture compared to wild-type controls, whereas their responses on plates ranged from no growth to good growth. Similarly, the *acuE* mutants grew rather poorly in liquid medium. The remaining mutants gave the same growth responses to glutamate presented either in solid medium or in liquid culture. The growth responses of all *acu* mutants to hexose, glycerol, butyrate, ethanol or acetate were the same in liquid media as on solid media.

Locus	Fluoroacetate (0.51 M)	Sucrose (0.02 M)	Glycerol (0.04 M)	Glutamate (0.02 M)	Butyrate (0.1 M)	Ethanol (0.1 M)	Acetate (0.1 M)
	+ Glucose (0.05 M)	or glucose (0.04 M)					
Wild type	—	+ + +	++	++	++	++	++
<i>acuA</i> (= <i>facA</i>)	+ + +	+ + +	++	++	++	—	—
<i>facB</i>	+ + +	+ + +	++	++	++	—	(+)
<i>acuC</i> (= <i>facC</i>)	+ +	+ + +	++	++	++	—	—
<i>acuD</i>	—	+ + +	++	—/ +	—	—	—
<i>acuE</i>	—	+ + +	++	++	—	—	—
<i>acuF</i>	—	+ + +	++	—	—	—	—
<i>acuG</i>	—	+ + +	—	+	+	+	(+)
<i>acuH</i>	—	+ + +	++	++	—	—	—
<i>acuJ</i>	—	+ + +	++	++	—	—	—
<i>acuK</i>	—	+ + +	++	—/ +	—	—	—
<i>acuL</i>	—	+ + +	++	—/ +	—	—	—
<i>acuM</i>	—	+ + +	++	++	—	—	—
<i>acuN</i>	—	+ + +	++	++	—	—	—

Growth on agar plates without an added carbon source. A chance observation led us to test representative *acu* mutants for growth on defined medium containing no other source of carbon than agar (1.5%, w/v). The wild type makes appreciable growth; colonies increase in diameter at about the rate they would on glucose agar plates but the amount of mycelium and conidiation is drastically decreased. The resultant colonies are 'sparse' and poorly conidiated, with the low mycelial density which is typical of nutrient starved growth. We were surprised to find that most *acu* mutants did not grow at all after 3 days' incubation, and the two strains (*facB* and *acuG*) which grew very poorly are those which gave leaky growth on acetate plates (Table 4). The wild type and the *acu* mutants gave similar responses, although in varying degrees, to all the commercial agars we tested (Davis, Difco, Fisons and Oxoid).

Despite the conventional wisdom that carbon sources in agars are hexoses, in which case the *acu* mutants would be expected to grow like the wild type, it is evident that for *A. nidulans* these carbon sources are lipids or some other compounds metabolized by degradation to C₂ units. It seems unlikely that the organism is able to cleave the agar polymers since there is no loss of the gel property around colonies. Either extracellular enzymes are releasing C₂ residues from the polymers or there is significant contamination of agar with lipids. Sykes (1956) states that long chain fatty acids are often present in agars.

Table 5. *Enzyme activities in acetate non-utilizing mutants of A. nidulans*

All figures are enzyme specific activities expressed as $\mu\text{mol product/mg/h}$. In general, enzyme activities were assayed in different cell-free extracts. Activities of isocitrate lyase and of malate synthase were done on extracts prepared from mycelium grown on 0.02 M-sucrose + 0.1 M-acetate for 18 h. All other activities were measured on extracts prepared from mycelium grown for 18 h on 0.02 M-sucrose and then transferred aseptically to a fresh medium containing 0.1 M-acetate and incubated for a further 6 h before harvesting.

Strains	Treatment of mycelium					
	Transfer from sucrose (0.02 M) to acetate (0.1 M)	Grown on sucrose (0.02 M) + acetate (0.1 M) for 18 h		Grown on sucrose (0.02 M) for 18 h, then transferred to acetate (0.1 M) for 6 h		
	Acetyl-CoA synthase	Isocitrate lyase	Malate synthase	Malic enzyme	PEP carboxykinase	Fructose 1,6-diphosphatase
R21	8.5	9.6	3.06	2.00	15.0	10.9
<i>acuA204</i>	0.9	1.02	0.55	0.06	3.2	2.8
<i>facB2-101</i>	2.8	0.36	0.0	0.27	3.6	1.2
<i>acuC203</i>	4.6	1.86	0.02	0.18	4.5	2.5
<i>acuC220</i>		0.96				
<i>acuD202</i>	3.3	0.54	2.80	1.13	6.3	5.6
<i>acuD207</i>	4.2	0.0				
<i>acuD219</i>	5.6	0.6				
<i>acuD230</i>	3.1	0.0				
<i>acuD240</i>	3.9	0.0				
<i>acuD250</i>		0.0				
<i>acuD254</i>	3.4	0.0				
<i>acuD261</i>		0.0				
<i>acuE201</i>		6.18	0.0	0.54	5.6	3.1
<i>acuE224</i>		11.64				
<i>acuF205</i>	1.2	9.4				
<i>acuF238</i>		12.9	2.88	16.3	<0.3	5.2
<i>acuG223</i>	3.2	9.66	3.20	15.0	50.0	0.2
<i>acuH206</i>		7.08	4.60	0.0	10.2	2.66
<i>acuJ211</i>		8.04	5.60	0.11	4.9	4.43
<i>acuK248</i>		8.64	1.76	0.0	8.4	1.04
<i>acuL217</i>	1.5	5.82	1.89	0.57	3.3	1.50
<i>acuM2-301</i>	4.0	6.00	1.87	0.0	1.3	1.20
<i>acuN2-356</i>		6.59	3.26	2.2		

We also found, like Arst & Cove, (1973) and Polkinghorne & Hynes (1975), that agars can similarly serve as a significant source of nitrogen for growth of *A. nidulans* in the absence of an added nitrogen source but with provision of a carbon source. Growth of the wild type was similar to that described above, and in this case with glucose as carbon source the growth of all the *acu* mutants was indistinguishable from the wild type.

Enzyme lesions in the acu mutants. A series of mutants in the R21 background representing each of the *acu* loci was examined for a number of enzyme activities, the loss of any one of which might be expected to lead to inability to grow on acetate (Table 5; Fig. 2). The glyoxylate cycle enzymes, isocitrate lyase and malate synthase, were assayed in cell-free extracts of mycelium grown with a mixture of sucrose (0.02 M) and acetate (0.10 M), which supports approximately 30% of the fully induced (acetate grown) enzyme activities in the wild type (R21). The other enzyme activities, which were present at low levels in extracts of

Fig. 2. Pathways of glucose or acetate metabolism in *A. nidulans*. The diagrams have been simplified by omitting the phospho-pentose pathways (see Hankinson & Cove, 1974; Hankinson, 1974). (a) Glycolytic mutants: *pycA* (pyruvate carboxylase; Skinner & Armit, 1972); *pdhA* (pyruvate dehydrogenase; Romano & Kornberg, 1968). Mutants lacking pyruvate kinase have also been found recently (M. Payton, personal communication). The significance of a PEP carboxylase activity, reported by Bushell & Bull (1974) in addition to pyruvate carboxylase, in relation to the *pycA* mutants has not been established. (b) Acetate mutants: *acuA* (acetyl-CoA synthase); *acuD* (isocitrate lyase); *acuE* (malate synthase); *acuF* (PEP carboxykinase); *acuG* (fructose 1,6-diphosphatase); *acuK*, *acuM* (malic enzyme). It is unlikely that PEP is oxidized to acetyl-CoA during acetate metabolism since pyruvate kinase activity is very low under this condition (McCullough & Roberts, 1974). Moreover, pyruvate dehydrogenase and pyruvate carboxylase are not required for acetate metabolism since mutants lacking enzymes grow well with acetate as sole carbon source.

wild-type mycelium grown on a mixture of sucrose and acetate, were measured in extracts of mycelium which was first grown on sucrose medium and then transferred aseptically to acetate growth medium for a further 6 h.

In addition to the deficiency of acetyl-CoA synthase activity in *acuA* mutants (Romano & Kornberg, 1969), five other enzyme lesions have been identified and these are associated with six of the twelve remaining *acu* loci (Tables 5 and 6). The lesions are: isocitrate lyase (*acuD*), malate synthase (*acuE*), PEP carboxykinase (*acuF*), fructose 1,6-diphosphatase (*acuG*) and malic enzyme (*acuK* and *acuM*). Mutants lacking isocitrate lyase and malate synthase have been described previously (Table 6).

The possibility that some of the uncharacterized mutants, especially *acuH*, *acuJ* or *acuL*, affect enzymes of the tricarboxylic acid cycle was examined in extracts of mycelium grown on sucrose and also extracts of such mycelium after transfer to acetate medium. None were found to lack either succinic dehydrogenase or fumarase activities.

Table 6. *Enzyme lesions identified in acetate non-utilizing mutants of A. nidulans*

Locus	Linkage group	Enzyme lesion	Reference
<i>acuA</i> (= <i>facA</i>)	V	Acetyl-CoA synthase	Apirion, 1965; Romano & Kornberg, 1969
<i>facB</i>	VIII	Not known	Apirion, 1965
<i>acuC</i> (= <i>facC</i>)	VIII	Not known	Apirion, 1965
<i>acuD</i>	V	Isocitrate lyase	Armitt <i>et al.</i> , 1970
<i>acuE</i>	IR	Malate synthase	Armitt <i>et al.</i> , 1971
<i>acuF</i>	VII	PEP carboxykinase	
<i>acuG</i>	V	Fructose 1,6-diphosphatase	
<i>acuH</i>	V	Not known	
<i>acuJ</i>	IL	Not known	
<i>acuK</i>	IV	Malic enzyme	McCullough & Roberts, 1974
<i>acuL</i>	IV	Not known	
<i>acuM</i>	IL	Malic enzyme	McCullough & Roberts, 1974
<i>acuN</i>	V	Not known	

Glyoxylate cycle mutants

The essential role of the glyoxylate cycle during growth on acetate was confirmed by finding mutants lacking either isocitrate lyase or malate synthase among the *acu* mutants. These two groups of mutants map at two unlinked loci designated *acuD* (lacking isocitrate lyase) and *acuE* (lacking malate synthase). The loci were previously designated *icl* and *mas* respectively (Armitt *et al.*, 1970, 1971).

Temperature-sensitive mutations at the *acuD* locus have been found (Table 1) in which isocitrate lyase in crude cell-free extracts was much more sensitive to heat inactivation at 37 °C than was the enzyme from wild type. Since the only class of mutants which lack isocitrate lyase activity alone are those at the *acuD* locus, it seems likely that this is the structural gene for the enzyme.

Heat inactivation of malate synthase has not been studied, although two *acuE* temperature-sensitive alleles were found (Table 1). It seems likely that *acuE* is the structural gene for malate synthase, since this was the only group of mutants found specifically to lack that enzyme activity.

Mutants at the *acuD* locus lacking isocitrate lyase activity were still capable of forming malate synthase activity when grown on a mixture of sucrose and acetate (Table 5). This result demonstrates that malate synthase is not induced sequentially by glyoxylate, a product of the isocitrate lyase reaction. Furthermore, both *acuD* and *acuE* mutants, when grown on sucrose and transferred to acetate medium, are still capable of forming the glyconeogenic enzymes PEP carboxykinase and fructose 1,6-diphosphatase as well as malic enzyme (Table 5). Therefore, there is no evidence that a lesion in the glyoxylate cycle prevents the formation of other enzymes as might be expected if enzyme synthesis during adaptation to acetate was sequentially induced.

In the course of complementation analysis, intragenic complementation was observed among *acuD* alleles. Of sixteen alleles examined (*acuD125* was from previous studies by Armitt *et al.*, 1970), eleven complemented in heterokaryons with at least one other allele to permit growth on acetate. This contrasts with the situation in *Neurospora crassa* where no complementation was found at the analogous locus, *acu3* (Flavell & Fincham, 1968*a,b*; Leckie & Fincham, 1971). We did not find intragenic complementation at any other of our *acu* loci.

Mutants totally lacking isocitrate lyase activity were unable to grow on L-glutamate as carbon source on agar plates (Table 4) although they could use the amino acid as a nitrogen

source when sucrose was a carbon source. Moreover there was a correlation between leaky growth of certain *acuD* mutants on glutamate and their possession of measurable amounts of isocitrate lyase activity (5 to 10% of wild type). In contrast, all malate synthase mutants grew on glutamate as a carbon source under the same conditions. Similar results were found in both cases for the utilization of L-proline as carbon source. It seems that although isocitrate lyase activity is required for the metabolism of glutamate, the presence of a complete glyoxylate cycle is not essential. This result is surprising since it implies a role for isocitrate lyase in addition to that in the anaplerotic glyoxylate cycle. A role for isocitrate lyase separate from the glyoxylate cycle has been postulated in other fungi, where synthesis of glycine from glyoxylate may be important (Galbraith & Smith, 1969; Ulane & Ogur, 1972).

However, the situation in *Aspergillus* appears more complex, since all *acuD* mutants grow as well as the wild type on L-glutamate or L-proline as sole carbon sources when tested in fully defined liquid media (M. Payton, W. McCullough and C. F. Roberts, unpublished). Similar differences have also been found in the utilization by *acu* mutants and certain glycolytic mutants of other amino acids or organic acids as sole carbon sources for growth in fully defined liquid media as compared with the same media solidified with a variety of commercial agars.

The fluoroacetate resistant mutants

Among our *acu* mutants two groups, *acuA* and *acuC*, were allelic with *facA* and *facC* mutants isolated by Apirion (1965) through their resistance to fluoroacetate in the presence of glucose (Tables 1 and 4). We did not isolate any mutants at Apirion's *facB* locus. However, most of Apirion's *fac* mutants are leaky in their growth on acetate and we may have eliminated such mutants either during filtration enrichment or in the deliberate retention of mainly non-leaky *acu* mutants. Apirion's *facB101* mutation was crossed into the same genetic backgrounds as the *acu* mutants (R21 or R153) and included in the analysis.

The *acuA* and *acuC* mutants all showed a similar degree of resistance to fluoroacetate as Apirion's original mutants. These, with *facB*, were the only fluoroacetate-resistant mutants found (Table 4).

While it is known that *acuA* mutants lack acetyl-CoA synthase (Romano & Kornberg, 1969; also Table 5), the lesions in the *facB* and *acuC* mutants have not been discovered. Strains with either *acuA*, *facB* or *acuC* mutations do not grow on acetate as sole carbon source, but all grow almost as well as wild type with butyrate (Table 4). Since butyrate is metabolized through acetyl-CoA, it may be deduced that *facB* and *acuC* mutants are unable to make acetate available to the mitochondria as acetyl-CoA. This conclusion is also apparent from the property of fluoroacetate resistance in the mutants, in which the lesion must serve to prevent the accumulation of mitochondrial fluorocitrate since this is a potent inhibitor of aconitase (Peters, 1957). The lesion is unlikely to be in citrate synthase because such mutants would be defective in the tricarboxylic acid cycle and would therefore not grow on hexose media.

Resistance to fluoroacetate and inability to utilize acetate for growth might be caused by failure of the mycelium to accumulate these compounds. However, there is some evidence against this hypothesis. (i) Fluoroacetate at a concentration (0.005%, w/v) not inhibiting growth on a glycerol medium prevents the formation of the yellow conidial pigment in strain R21, which carries the *yA2* allele. The same concentration of fluoroacetate also prevents yellow-pigment formation in *facB* or *acuC* strains carrying the *yA* marker, but not in similar *acuA* strains which form yellow pigment. This shows that *facB* and *acuC* mutants must accumulate fluoroacetate in the mycelium and also indicates that acetyl-CoA is

probably a pigment precursor. (ii) Mutants of *A. nidulans* have been reported which are resistant to the inhibitor of glycolysis, iodoacetate (Warr & Roper, 1965). If it is assumed that both fluoroacetate and iodoacetate are taken into the cell by the same transport system, then mutants defective in uptake should show cross-resistance to both inhibitors. However, all the fluoroacetate-resistant mutants were sensitive to iodoacetate. (iii) Measurement of the accumulation of radioactive isotope by mycelium incubated with [2-¹⁴C]acetate (0.1 mM) showed that *facBro1* contained 85% of the counts accumulated by the wild type after 1 h and *facCro2* 11% of the counts. Accumulation by *facA303* was negligible (< 2% of wild type). Taken together, these observations make it unlikely that *facB* or *acuC* mutants are unable to take up acetate into the mycelium.

Examination of enzyme formation in *acuA*, *facB* and *acuC* mutants helps one understand the regulation of the glyoxylate cycle in *A. nidulans*. When such mutants are grown in a mixture of sucrose and acetate, both isocitrate lyase and malate synthase activities are markedly less than in the wild type (Table 5). This suggests that an early product of acetate metabolism, perhaps acetyl-CoA, rather than acetate itself is the metabolic inducer of the glyoxylate cycle enzymes.

The structural gene for isocitrate lyase (*acuD*) and the presumed structural gene for acetyl-CoA synthase (*acuA*) are closely linked in *A. nidulans*. Such tight linkage in other fungi often indicates the presence of a gene cluster which may code for the structural components of a multi-enzyme aggregate transcribed from a single multi-cistronic mRNA (Fink, 1966; Case & Giles, 1968). In such cases nonsense polar mutations are found which affect the expression of more than one structural gene in the cluster and thus are pleiotropic with regard to complementation properties and enzyme lesions. We examined both *acuA* and *acuD* mutations for such possible pleiotropism. None of the mutants were found to be non-complementary with both *acuA* and *acuD* mutants. None of the *acuD* mutants were resistant to fluoroacetate, and those tested for acetyl-CoA synthase activity were not obviously defective (Table 5). Only a few *acuA* mutants were examined for induction of isocitrate lyase activity, but none had lost the activity completely. We have, as yet, no indication of pleiotropic mutations affecting the expression of both the *acuA* and *acuD* genes.

Gluconeogenic mutants

Organisms which grow on acetate as sole carbon source synthesize glycolytic compounds from tricarboxylic acid cycle intermediates. The enzyme responsible for the first step in this process in both bacteria and other fungi is PEP carboxykinase. That this is also the case in *Aspergillus* was verified by finding that mutants (*acuF*) lacking this enzyme activity (Table 5) were unable to grow on acetate or glutamate media, but were capable of normal growth on sucrose or glycerol media (Table 4). *Neurospora* mutants lacking this enzyme are also unable to grow on acetate (Flavell & Fincham, 1968*b*). *Neurospora* mutants exhibit intragenic complementation (Beever & Fincham, 1973), but we did not find this in five heterokaryon tests with the *Aspergillus* mutants.

Another gluconeogenic enzyme, fructose 1,6-diphosphatase, is required to bypass the irreversible reaction in glycolysis catalysed by phosphofructokinase. Mutants (*acuG*) lacking this enzyme activity showed leaky growth on ethanol, acetate, butyrate or glutamate as carbon sources, presumably due to the presence of a non-specific phosphatase activity. A similar leaky growth did not occur on glycerol medium (Table 4).

Mutants lacking fructose 1,6-diphosphatase activity yielded high (derepressed) levels of PEP carboxykinase activity when sucrose-grown mycelium was transferred to acetate medium for 6 h (Table 5). This suggests that PEP carboxykinase may be subject to repression

Table 7. *Malic enzyme activities in certain acetate non-utilizing mutants of A. nidulans*

Malic enzyme activities ($\mu\text{mol NADP reduced/mg/h}$) were measured in cell-free extracts prepared from mycelium: grown on sucrose (0.02 M) for 18 h and transferred aseptically to acetate (0.1 M) for a further 6 h incubation before harvesting; grown on glycerol (0.04 M) for 24 h; grown on glycerol (0.04 M) + glutamate (0.02 M) for 24 h.

Strains	Treatment of mycelium		
	Grown on sucrose, transferred to acetate	Grown on glycerol	Grown on glycerol + glutamate
R21	2.0	0.59	1.0
<i>acuH206</i>	0	0.45	0.35
<i>acuJ211</i>	0.11	0.6	0.3
<i>acuK248</i>	0	0	0
<i>acuM2-301</i>	0	0	0

by hexose phosphates which would not be formed from acetate in a mutant which lacks fructose 1,6-diphosphatase. PEP carboxykinase is strongly repressed during growth of *Saccharomyces* or *Tetrahymena* on glucose (Cazzulo, Claisse & Stoppani, 1968; Diesterhaft *et al.*, 1972).

Neither of the probable structural genes for the gluconeogenic enzymes was closely linked to another *acu* marker, although there was loose linkage between *acuG*, *acuA* and *acuD* in linkage group V.

Malic enzyme mutants

Malic enzyme activity was not detected in two groups of the *acu* mutants (*acuK* and *acuM*) under all conditions of growth tested (Table 7). Since no temperature-sensitive alleles were obtained at either locus it was impossible to determine whether one or both of these are structural genes for malic enzyme. Only *acuM* is closely linked to another *acu* marker (*acuJ*, 6% recombination). We have argued that the role of malic enzyme in growth on acetate media is in the supply of pyruvic acid from malate, and low activities of pyruvate kinase in extracts of acetate-grown mycelium compared with sucrose grown mycelium support this contention (McCullough & Roberts, 1974).

DISCUSSION

The enzyme lesions identified in mutants at seven of the *acu* loci in *A. nidulans* are shown in Fig. 2. The lesions and their effects are readily interpreted by the known pathway for acetate metabolism and, together with data on enzyme formation in wild type grown in the presence of acetate (Table 5), support the conclusion that this is the pathway operating in the organism.

Mutants lacking either isocitrate lyase activity (*acuD*) or malate synthase (*acuE*) did not grow on acetate, thus confirming the essential role of the glyoxylate cycle for acetate utilization in *A. nidulans*. Mutants lacking PEP carboxykinase do not grow on acetate or any other carbon source metabolized through the tricarboxylic acid cycle (proline, glutamate, butyrate or ethanol) and therefore demonstrate the essential physiological role of this enzyme as the first step in the gluconeogenic synthesis of glycolytic intermediates. This role for PEP carboxykinase is well established in a variety of micro-organisms (Kornberg, 1966*b*; Diesterhaft *et al.*, 1972) and has also been demonstrated in other fungi (Flavell & Fincham, 1968*b*; Hartman & Keen, 1974). Similarly, mutants lacking malic enzyme grow either very

poorly or not at all on tricarboxylic acid cycle precursors, and we have argued that the role of the enzyme in *A. nidulans* in this situation is the provision of pyruvate from malate for amino acid synthesis, since pyruvate kinase which normally fulfills this role is absent during acetate metabolism (McCullough & Roberts, 1974). The *acuG* mutants lacking fructose 1,6-diphosphatase, as expected, did not utilize any gluconeogenic carbon sources (including glycerol). Moreover the high levels of PEP carboxykinase activity found in *acuG* mutants on transfer from sucrose growth medium to acetate indicates that the repressing metabolite of this enzyme is a glycolytic intermediate located before fructose 1,6-diphosphate in the glycolytic pathway.

Metabolic lesions have not been identified for the remaining *acu* loci (*facB*, *acuC*, *H*, *J*, *L* and *N*). None are defective in succinate dehydrogenase or fumarase. Moreover, it seems unlikely that they have lesions in the tricarboxylic acid cycle since these could be expected to affect growth on glucose as well as acetate. An extra-nuclear mitochondrial mutant of this type has been recovered amongst *acu* mutants in *C. lagopus* (Casselton & Condit, 1972). A consideration of which other enzymes might be important to acetate metabolism would suggest isocitrate dehydrogenase, since this enzyme acts at a branch point with isocitrate lyase (Bennett & Holms, 1975) and is also known to be present in glyoxisomes (Müller, Hogg & De Duve, 1968). It is probable that some of the *acu* mutants have lesions affecting enzymes specific to the glyoxisome or perhaps the structural proteins essential for the integrity of the organelle. Identification of such lesions must await a detailed study of the enzyme complement of this organelle in *A. nidulans*. Another possibility is that the mutants are defective in the intracellular permeation of metabolites, derived from acetate, into either mitochondria or glyoxisomes.

Mapping the *acu* mutants in *A. nidulans* has produced few surprises with regard to genetic organization. As is the general case in fungi – and indeed for certain groups of bacteria (Levinthal, 1974), particularly the pseudomonads (Holloway, Krishnapillai & Stanisich, 1971) – the structural genes for metabolically-related enzymes are not clustered but are well distributed over the fungal genome (Gross, 1969). A similar absence of close linkage has been found for *acu* mutations in *N. crassa* (Flavall & Fincham, 1968*a*) and in *C. lagopus* (Casselton & Casselton, 1974). Close linkage of the structural genes for acetyl-CoA synthase (*acuA*) and isocitrate lyase (*acuD*) in *A. nidulans* seems potentially interesting since acetyl-CoA synthase catalyses the first step in acetate metabolism and isocitrate lyase is the first enzyme of the glyoxylate cycle. However, we have yet to find any evidence for pleiotropic mutations in the region affecting the expression of both genes, and close linkage may prove fortuitous; the two analogous loci in *N. crassa*, *acu5* and *acu3*, are not linked.

If the regulation of the formation of the glyoxylate cycle enzymes in *A. nidulans* were by a genetic system of positive control (Englesberg & Wilcox, 1974; Beckwith & Rossow, 1974), one should find, among the *acu* strains, recessive pleiotropic mutations of regulatory genes which are uninducible for both isocitrate lyase and malate synthase, and perhaps other enzymes also required for acetate metabolism. Although the *fac* (fluoroacetate resistant) mutants appear such a class, we have data indicating that they are deficient in the synthesis of acetyl-CoA from acetate or in the distribution of acetyl-CoA across membranes within the mycelium; we believe that they fail to induce, due to deficiency in conversion of the inducer (acetate) to the true metabolic inducer (possibly acetyl-CoA), rather than providing evidence for a system of positive genetic regulation. We must therefore consider the hypothesis of negative genetic control, and the isolation of recessive constitutive mutants for isocitrate lyase not linked to the *acuD* locus (W. McCullough and C. F. Roberts, unpublished) supports such a model.

A recent study of fluoroacetate-resistant mutants in *C. lagopus* (Casselton & Casselton, 1974) reveals a striking parallel to the situation in *A. nidulans* (Apirion, 1965). In both fungi there are three unlinked loci at which recessive mutations confer both resistance to the analogue and also loss of acetate utilization. One locus determines acetyl-CoA synthase activity, *acuI* in *Coprinus* and *facA* (= *acuA*) in *Aspergillus*. Mutants at a second locus are only rarely recovered (*acuI2* in *Coprinus* and *facB* in *Aspergillus*), while mutants at the third locus are readily isolated *acuI1* in *Coprinus* and *facC* (= *acuC*) in *Aspergillus*. In both studies there is also a class of mutants which are fluoroacetate-resistant but utilize acetate normally. A feature of Apirion's (1965) study was the apparent failure of mutants at different *fac* loci to complement when combined in heterokaryons. Casselton & Casselton (1974) find normal complementation of their mutants combined either in dikaryons or heterozygous diploids. We have not found any difference in the normal complementation of the *Aspergillus* mutants in heterokaryons or diploids and think that Apirion may have been misled by the leaky growth of the mutants studied.

An unexpected result from this study of *acu* mutants is the demonstration that carbon sources present in agar for *A. nidulans* are utilized through C_2 metabolism and thus cannot be hexoses as is commonly believed. In addition, certain *acu* mutants gave different growth responses to L-glutamate, and also to other amino acids or organic acids, presented as carbon sources in either fully defined liquid media or the same media solidified with agar (M. Payton, W. McCullough and C. F. Roberts, unpublished). This result emphasises the care needed to evaluate the utilization of potential carbon sources both in wild-type and in mutant strains, particularly since growth tests are usually first done on agar plates and would not often be repeated in liquid culture, especially for non-growing strains. We suspect that such differences could also be found for the utilization of nitrogen sources, especially amino acids (Arst & Cove, 1973; Polkinghorne & Hynes, 1975; Kinghorn & Pateman, 1975).

We thank Professor H. L. Kornberg for his interest in this work, his enthusiasm and encouragement. We also are very grateful to M. Payton for data mapping *acuK* and *acuL* in linkage group IV, and to Mr J. Mackley and Mrs Angela Thomas for excellent technical assistance. The work has been supported by the Science Research Council (research grants B/SR/5493 and R/RG/1667-9).

REFERENCES

- APIRION, D. (1965). The two-way selection of mutants and revertants in respect of acetate utilisation and resistance to fluoroacetate in *Aspergillus nidulans*. *Genetical Research* **6**, 317-329.
- ARMITT, S., ROBERTS, C. F. & KORNBERG, H. L. (1970). The role of isocitrate lyase in *Aspergillus nidulans*. *FEBS Letters* **7**, 231-234.
- ARMITT, S., ROBERTS, C. F. & KORNBERG, H. L. (1971). Mutants of *Aspergillus nidulans* lacking malate synthase. *FEBS Letters* **12**, 276-278.
- ARST, H. N. & COVE, D. J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. *Molecular and General Genetics* **126**, 111-141.
- BECKWITH, J. & ROSSOW, P. (1974). Analysis of genetic regulatory mechanisms. *Annual Review of Genetics* **8**, 1-13.
- BEEVER, R. E. & FINCHAM, J. R. S. (1973). Acetate non-utilising mutants of *Neurospora crassa*: *acu-6*, the structural gene for PEP carboxykinase and inter-allelic complementation at the *acu-6* locus. *Molecular and General Genetics* **126**, 217-226.
- BENNETT, P. M. & HOLMS, W. H. (1975). Reversible inactivation of the isocitrate dehydrogenases of *Escherichia coli* ML308 during growth on acetate. *Journal of General Microbiology* **87**, 37-51.
- BRICE, C. B. & KORNBERG, H. L. (1968). Genetic control of isocitrate lyase activity in *Escherichia coli*. *Journal of Bacteriology* **96**, 2185-2186.

- BUSHELL, M. E. & BULL, A. T. (1974). Anaplerotic carbon dioxide fixation in steady state and non-steady state fungal cultures. *Proceedings of the Society for General Microbiology* **1**, 69.
- CASE, M. E. & GILES, N. H. (1968). Evidence for nonsense mutations in the *arom* gene cluster of *Neurospora crassa*. *Genetics* **60**, 49–58.
- CASSELTON, L. A. & CASSELTON, P. J. (1974). Functional aspects of fluoroacetate resistance in *Coprinus* with special reference to acetyl-CoA synthetase deficiency. *Molecular and General Genetics* **132**, 255–264.
- CASSELTON, L. A. & CONDIT, A. (1972). A mitochondrial mutant of *Coprinus lagopus*. *Journal of General Microbiology* **72**, 521–527.
- CAZZULO, J. J., CLAISSE, L. M. & STOPPANI, A. O. M. (1968). Carboxylase levels and carbon dioxide fixation in bakers' yeast. *Journal of Bacteriology* **96**, 623–628.
- CLUTTERBUCK, A. J. & COVE, D. J. (1974). Linkage map of *Aspergillus nidulans*. In *Handbook of Microbiology*, vol. 5, *Microbial Metabolism, Genetics and Immunology*, pp. 665–676. Edited by A. I. Laskin and H. A. Lechevalier. Ohio: C.R.C.
- DE DUVE, C. (1969). Evolution of the peroxisome. *Annals of the New York Academy of Sciences* **168**, 369–381.
- DIESTERHAFT, M. D., HSIANG-CHUAN, H., ELSON, C., SALLACH, H. J. & SCHRAGO, E. (1972). Enzymatic regulation of the metabolism of phosphoenolpyruvate in *Tetrahymena pyriformis*. *Journal of Biological Chemistry* **247**, 2755–2762.
- DORN, G. L. (1967). A revised map of the eight linkage groups of *Aspergillus nidulans*. *Genetics* **56**, 619–631.
- ENGLESBERG, E. & WILCOX, G. (1974). Regulation: positive control. *Annual Review of Genetics* **8**, 219–242.
- FINK, G. R. (1966). A cluster of genes controlling three enzymes in histidine biosynthesis in *Saccharomyces cerevisiae*. *Genetics* **53**, 445–459.
- FLAVELL, R. B. & FINCHAM, J. R. S. (1968*a*). Acetate-nonutilising mutants of *Neurospora crassa*. I. Mutant isolation, complementation studies and linkage relationships. *Journal of Bacteriology* **95**, 1056–1062.
- FLAVELL, R. B. & FINCHAM, J. R. S. (1968*b*). Acetate-nonutilising mutants of *Neurospora crassa*. II. Biochemical deficiencies and the role of certain enzymes. *Journal of Bacteriology* **95**, 1063–1068.
- FRAENKEL, D. G. & VINOPAL, R. T. (1973). Carbohydrate metabolism in bacteria. *Annual Review of Microbiology* **27**, 69–100.
- GALBRAITH, J. C. & SMITH, J. E. (1969). Changes in activity of certain enzymes of the tricarboxylic acid cycle and the glyoxylate cycle during the initiation of conidiation of *Aspergillus niger*. *Canadian Journal of Microbiology* **15**, 1207–1212.
- GROSS, S. R. (1969). Genetic regulatory mechanisms in the fungi. *Annual Review of Genetics* **3**, 395–424.
- HANKINSON, O. (1974). Mutants of the pentose phosphate pathway in *Aspergillus nidulans*. *Journal of Bacteriology* **117**, 1121–1130.
- HANKINSON, O. & COVE, D. J. (1974). Regulation of the pentose phosphate pathway in the fungus *Aspergillus nidulans*. *Journal of Biological Chemistry* **249**, 2344–2353.
- HARTMAN, R. E. & KEEN, N. T. (1974). The phosphoenolpyruvate carboxykinase of *Verticillium albo-atrum*. *Journal of General Microbiology* **81**, 21–26.
- HOLLOWAY, B. W., KRISHNAPILLAI, V. & STANISICH, V. (1971). *Pseudomonas* genetics. *Annual Review of Genetics* **5**, 425–446.
- JONES, M. E. & LIPMANN, F. (1955). Aceto-CoA-kinase. In *Methods in Enzymology* **1**, 585–591.
- KINGHORN, J. R. & PATEMAN, J. A. (1975). Mutations which affect amino acid transport in *Aspergillus nidulans*. *Journal of General Microbiology* **86**, 174–184.
- KOBR, M. J., VANDERHAEGHE, F. & COMBÉPINE, G. (1969). Particulate enzymes of the glyoxylate cycle in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **37**, 640–645.
- KORNBERG, H. L. (1965). Control of biosynthesis from C₂ compounds. In *Regulation chez les Micro-organisms*, pp. 183–208. Paris: Centre Nationale de la Recherche Scientifique.
- KORNBERG, H. L. (1966*a*). The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochemical Journal* **99**, 1–11.
- KORNBERG, H. L. (1966*b*). Anaplerotic sequences and their role in metabolism. *Essays in Biochemistry* **2**, 1–31.
- LECKIE, B. J. & FINCHAM, J. R. S. (1971). A structural gene for *Neurospora crassa* isocitrate lyase. *Journal of General Microbiology* **65**, 35–43.
- LEVINTHAL, M. (1974). Bacterial genetics excluding *E. coli*. *Annual Review of Microbiology* **28**, 219–230.
- MCCULLOUGH, W. & ROBERTS, C. F. (1974). The role of malic enzyme in *Aspergillus nidulans*. *FEBS Letters* **41**, 238–242.
- MCCULLY, K. S. & FORBES, E. (1965). The use of *p*-fluorophenylalanine with 'master strains' of *Aspergillus nidulans* for assigning genes to linkage groups. *Genetical Research* **6**, 352–359.
- MÜLLER, M., HOGG, J. F. & DE DUVE, C. (1968). Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes in *Tetrahymena pyriformis*. *Journal of Biological Chemistry* **243**, 5385–5395.
- O'SULLIVAN, J. & CASSELTON, P. J. (1973). The subcellular localization of glyoxylate cycle enzymes in *Coprinus lagopus* (sensu Buller). *Journal of General Microbiology* **75**, 333–337.
- PETERS, R. A. (1957). Mechanism of the toxicity of the active constituent of *Dichapetalum cymosum* and related compounds. *Advances in Enzymology* **18**, 113–159.

- POLKINGHORNE, M. & HYNES, M. J. (1975). Effect of L-histidine on the catabolism of nitrogenous compounds in *Aspergillus nidulans*. *Journal of General Microbiology* **87**, 185-187.
- PONTECORVO, G., ROPER, J. A., HEMMONS, L. J., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advances in Genetics* **5**, 141-238.
- ROBERTS, C. F. (1970). Enzyme lesions in galactose non-utilising mutants of *Aspergillus nidulans*. *Biochimica et biophysica acta* **201**, 267-283.
- ROMANO, A. H. & KORNBERG, H. L. (1968). Regulation of sugar utilisation by *Aspergillus nidulans*. *Biochimica et biophysica acta* **158**, 491-493.
- ROMANO, A. H. & KORNBERG, H. L. (1969). Regulation of sugar uptake by *Aspergillus nidulans*. *Proceedings of the Royal Society B* **173**, 475-490.
- SKINNER, V. M. & ARMITT, S. (1972). Mutants of *Aspergillus nidulans* lacking pyruvate carboxylase. *FEBS Letters* **20**, 16-18.
- STADTMANN, E. R. (1957). Preparation and assay of acyl coenzyme A and other thiol esters: use of hydroxylamine. *Methods in Enzymology* **3**, 931-941.
- SYKES, G. (1956). *Constituents of Bacteriological Culture Media*. Special report for the Society for General Microbiology. Cambridge: Cambridge University Press.
- TAYLOR, A. L. & TROTTER, C. D. (1972). Linkage maps of *Escherichia coli* strain K-12. *Bacteriological Reviews* **36**, 504-524.
- TOLBERT, N. E. (1971). Microbodies - peroxisomes and glyoxysomes. *Annual Review of Plant Physiology* **22**, 45-74.
- ULANE, R. & OGUR, M. (1972). Genetic and physiological control of serine and glycine biosynthesis in *Saccharomyces*. *Journal of Bacteriology* **109**, 34-43.
- WARR, J. R. & ROPER, J. A. (1965). Resistance to various inhibitors in *Aspergillus nidulans*. *Journal of General Microbiology* **40**, 273-281.
- WEITZMANN, P. D. J. (1969). Polarographic assay for malate synthase and citrate synthase. *Methods in Enzymology* **13**, 365-368.
- WOODWARD, V. W., DE ZEEUW, J. R. & SRB, A. M. (1954). The separation and isolation of particular biochemical mutants of *Neurospora* by differential germination of conidia followed by filtration and selective plating. *Proceedings of the National Academy of Sciences of the United States of America* **40**, 192-200.