Biochimica et Biophysica Acta, 661 (1981) 247–254
Elsevier/North-Holland Biomedical Press

BBA 69380

EVIDENCE THAT THE NADP-LINKED GLUTAMATE DEHYDROGENASE OF *COPRINUS CINEREUS* IS REGULATED BY ACETYL-CoA AND AMMONIUM LEVELS

DAVID MOORE

Department of Botany, The University, Manchester M13 9PL (U.K.)

(Received 1st April, 1981)

Key words: Enzyme regulation; Acetyl-CoA; Ammonium ion; NADP; Glutamate dehydrogenase; (Coprinus cinereus)

During development of the mushroom carpophore of the basidiomycete *Coprinus cinereus*, and through the operation of endogenous control mechanisms, the enzyme NADP-linked glutamate dehydrogenase (L-glutamate: $NADP^+$ oxidoreductase (deaminating), EC 1.4.1.4) increases greatly in activity in the developing cap, while remaining at a barely detectable level in the stipe and parental mycelium. This behaviour can be reproduced in vegetative mycelium which, after growth in a rich medium, is transferred to a medium lacking in nitrogen source and containing 100 mM pyruvate as sole carbon source. Such treatment immediately causes induction of activity of NADP-linked glutamate dehydrogenase. Only glucose, fructose, dihydroacetone, acetate and propan-1-ol share with pyruvate the ability to induce this enzyme activity. A mutant mycelium which is known to lack the enzyme acetyl-CoA synthetase failed to show induction of glutamate dehydrogenase activity on acetate medium, although normal induction occurred on medium containing either glucose or pyruvate. It is concluded that induction requires synthesis of acetyl-CoA and that this latter compound is the probable intracellular regulator. Inclusion of as little as 2 mM NH_4Cl in the transfer medium is sufficient to prevent enzyme induction. Some other nitrogen sources are also able to prevent induction but all seem to operate through the formation of ammonium which is excreted into the medium. Other compounds, like alanine or glutamate are unable either to promote or prevent induction. External concentrations of ammonium which are able to prevent induction do not correlate with elevated internal ammonium levels, so it is concluded that, perhaps through some membrane-reaction, the external level of ammonium determines whether induction will occur. The regulation mechanism is therefore interpreted as one in which the enzyme is induced by elevated intracellular levels of acetyl-CoA providing external levels of ammonium are low.

Introduction

During development of the mushroom carpophore of the basidiomycete *Coprinus cinereus* the enzyme NADP-linked glutamate dehydrogenase increases greatly in activity in the developing cap, while remaining at a barely detectable level in the stipe and parental mycelium [1]. Cap and stipe are intimately connected, particularly in the early developmental stages when this increase in NADP-linked glutamate dehydrogenase activity is initiated, and both tissues possess a high, and increasing, activity of NAD-linked glutamate dehydrogenase. Increase in activity of the NADP-linked enzyme is therefore seen as a morphogenetic regulatory event which enables the enzyme specifically to contribute to the metabolism of the developing cap.

Both glutamate dehydrogenase enzymes have been characterized [2], and carbohydrate metabolism and nitrogen metabolism in the developing carpophore have been investigated [3,4]. It has emerged that NADP-glutamate dehydrogenase is kinetically special-

ised for amination while NAD-glutamate dehydrogenase is able to aminate or deaminate as conditions require. During development of the cap, NADP-glutamate dehydrogenase is co-ordinately induced with glutamine synthetase and the two enzymes may form an 'ammonium scavenging' system akin to the glutamate synthase/glutamine synthetase mechanism. Induction of NADP-glutamate dehydrogenase is also correlated with induction of enzymes involved in synthesis of arginine and loss of urease activity. The functional significance seems to be that with the (demonstrated) presence of arginase an accumulation of urea occurs, and that this contributes to osmoregulation at a time when the cells of the cap are undergoing very large increases in volume.

Regulatory events which occur endogenously in the developing cap can at least in part be reproduced in vegetative cultures subjected to particular growth conditions. Especially effective is transfer of an actively growing mycelium of the monokaryon strain BC9/6,6 from a comparatively rich medium into a synthetic medium containing 100 mM pyruvate and no nitrogen source. Such a transfer immediately induces co-ordinate increases in activity of NADP-glutamate dehydrogenase, glutamine synthetase and arginine-synthetic enzymes, and the loss of urease activity [4]. There is no suggestion that transfer to such a peculiar medium has any relevance to normal mycelial growth of the fungus. Rather, this sort of experiment is interpreted as enforcing on the mycelium similar conditions to those which prevail normally within the developing carpophore cap; so that the transfer mimics cellular conditions within the cap and elicits the appropriate regulatory response.

In the work described here the transfer procedure is used to establish the identities of metabolites which effect the regulation of NADP-linked glutamate dehydrogenase.

Materials and Methods

Organism. Except where otherwise stated, all the experiments were done with the haploid monokaryon BC9/6,6. Table III describes some comparative work done with a set of strains which were isolated from the wild in various parts of the world. All of the strains used are fully fertile when intercrossed, except for those which have mating type alleles in common.

Culture methods. Most of the experiments made use of the transfer technique [4] in which the mycelium is first grown in a medium capable of supporting good growth and is later transferred to the experimental medium. In both cases, the media [5] were based on a solution of 10 mM Na_2HPO_4/10 mM KH_2PO_4/2 mM Na_2SO_4/0.5 mM $MgSO_4$/3 μM thiamine hydrochloride (solution A). In all experiments, during the initial growth period, this solution was supplemented with 30 mM NH_4Cl, 10 mM glucose and 1% (w/v) Bacto-Casaminoacids (casein hydrolysate). Supplements to the transfer medium varied with the demands of the experiment. The standard transfer procedure involved 5 days incubation in the casaminoacids medium using 250 ml flasks containing 50 ml medium and an orbital incubator operating at 37°C and 120 strokes min^{-1}. After this incubation period mycelia were separated from the medium by centrifugation; washed once with 40 ml sterile water, centrifuged again, and then resuspended in the transfer medium (prewarmed). Incubation was then resumed for 20–22 h. Final harvesting was done by filtration through Whatman GF/A filters. Samples awaiting assay were stored at –40°C. Both glutamate dehydrogenase enzymes were found to be stable for up to 4 weeks at this temperature.

Enzyme assays. Crude extracts were prepared by grinding tissue with 100 mM sodium phosphate buffer, pH 7.5. After filtration through Whatman GF/A the extracts were used without further treatment. Glutamate dehydrogenase activities were measured using the amination assays described by Al-Gharawi and Moore [5]. Protein content was determined by the method of Lowry et al. [6].

Ammonium assay. Ammonium contents of mycelium were measured using portions of the filtered crude extract prepared for enzyme assay. The method of McCullough [7] was used to determine ammonium content. Mycelia intended for ammonium assays were washed during harvesting with 2-times 100 ml quantities of freshly deionised water. Known additions of NH_4Cl made to nine sample extracts gave an average recovery of 98% and showed the extracts to be free of interfering solutes. Considerable interference was encountered when attempts were made to measure ammonium contents of medium samples with the colorimetric assay. Samples of the medium were therefore assayed using an MSE-Orion 95-10 ammo-

nia electrode coupled to an MSE-Orion 701A Ionalyzer specific ion meter.

Terminology. The term induction is used to describe the increase in specific activity of the enzyme without implication as to the mechanism by which that increase in activity is brought about. The term ammonium is used to refer indiscriminately to NH_3 and NH_4^+.

Results and Discussion

The effect of transfer into the solution A + 100 mM pyruvate is shown in Table I. On average there was a 21-fold increase in the specific activity of NADP-linked glutamate dehydrogenase which was dependent on the presence of pyruvate in the medium and was observed whether the medium was autoclaved or filter-sterilised. Although there was considerable variation in enzyme activities of different samples, and consequently considerable overlap in the range of activities of NAD-linked glutamate dehydrogenase in mycelia before and after transfer, these two populations of results are significantly different (even at the 0.5% level) so it can be concluded that reduction in the specific activity of this enzyme is also a consequence of transfer to solution A + pyruvate.

No response was observed to pyruvate concentrations of 10 mM and below (Fig. 1), and the optimum concentration was in the region of 50 mM. In these data, again, there was an indication of reciprocal regulation of the two glutamate dehydrogenase enzymes as increased activity of the NADP-linked enzyme seemed to correlate with a slight decline in the level of the NAD-linked glutamate dehydrogenase. The response to the transfer to pyruvate was

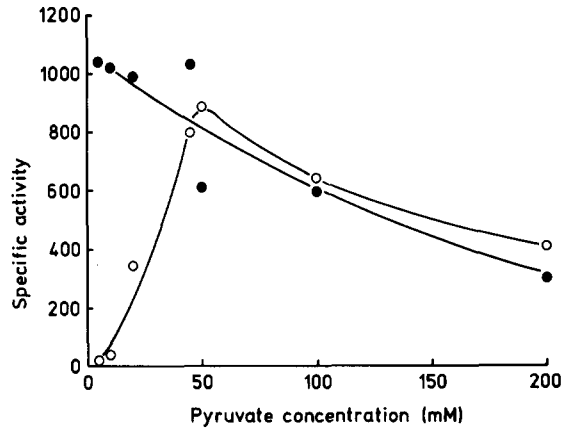


Fig. 1. Effect of varying the concentration of pyruvate in the transfer medium. Mycelia were grown and then transferred to solution A supplemented with various concentrations of pyruvate for a further period of 20 to 22 h incubation. The medium lacked any source of nitrogen. The specific activities of NAD-linked glutamate dehydrogenase (•———•) and NADP-linked glutamate dehydrogenase (○———○) are shown in units of nmol substrate used per min^{-1} (mg protein)$^{-1}$.

TABLE I

GLUTAMATE DEHYDROGENASE ACTIVITIES IN MYCELIA OF *COPRINUS CINEREUS* BEFORE AND AFTER TRANSFER TO DEFINED MEDIA

The mycelia were grown for 5 days in a medium containing 30 mM NH_4Cl/10 mM glucose/1% (w/v) bacto-casaminoacids dissolved in solution. A. They were then separated from this medium by centrifugation, washed and either assayed immediately or transferred to fresh solution A containing the supplements listed below and incubated for a further 20 to 22 h before final harvest and assay. Enzyme specific activities are shown in units of nmol substrate utilised · min^{-1} (mg protein)$^{-1}$.

Treatment	Specific activity	
	NAD-linked glutamate dehydrogenase	NADP-linked glutamate dehydrogenase
Control, untransferred	799 ± 293 (48) [a]	31 ± 28 (46) [a]
Transferred to solution A without supplements	844	43
Transferred to solution A containing 100 mM pyruvate (autoclaved)	599 ± 346 (47) [a]	644 ± 329 (47) [a]
Transferred to solution A containing 100 mM pyruvate (filter sterile)	603	318

[a] These entries show the mean and standard deviation of the number of replicates indicated in brackets. Other entries are the means of three replicate mycelia.

extremely rapid (Fig. 2). There was no indication of any lag period, but NADP-glutamate dehydrogenase activity clearly declined after reaching a maximum between 5 and 22 h after transfer. An interesting point is that the activity of NAD-linked glutamate dehydrogenase did not change during the initial period, when the NADP-linked enzyme was going through a 150-fold increase in activity. Only the samples harvested after 22 h exposure to pyruvate showed a reduced activity of NAD-linked glutamate dehydrogenase, so both enzymes must have been declining in activity between the 5- and 22-h samples. The reality of the reciprocal regulation assumed from the data of Table I and Fig. 1 is therefore put very much in doubt. Although NAD-linked glutamate dehydrogenase activities were routinely determined in all experiments, no clear regulatory pattern emerged for this enzyme and from this point on reference will be made only to the NADP-linked enzyme.

Table II records the results of a series of experiments in which mycelia were transferred for about 20 h to solution A containing supplements other than

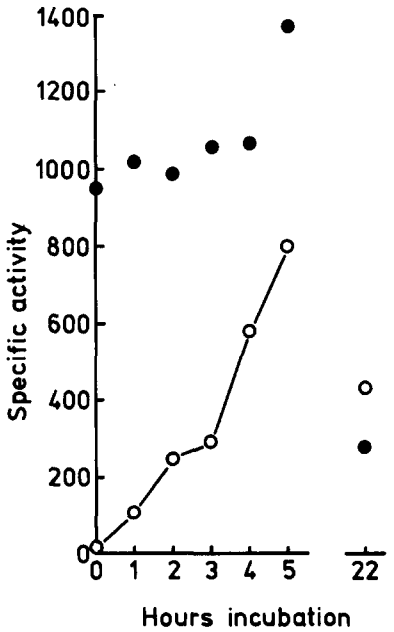


Fig. 2. Time course of induction of NADP-linked glutamate dehydrogenase (o———o) following transfer to solution A supplemented with 100 mM pyruvate. The NAD-linked glutamate dehydrogenase (•———•) was also assayed. Specific activity units are the same as for Fig. 1.

pyruvate. The only supplements able to induce NADP-linked glutamate dehydrogenase were those able to give rise to pyruvate through glycolysis (glucose, fructose and dihydroxyacetone) and the compounds acetate and propan-1-ol. It is especially interesting that Krebs cycle intermediates were ineffective, and that alanine, too, was unable to induce the enzyme. Although lack of penetration cannot be ruled out in all cases as a reason for lack of induction, it is particularly significant that both alanine and glutamate (at 100 mM concentration) support good growth when provided as sole sources of C and N. Non-inducers were mostly simply lacking in effect; they did not prevent induction when present in mixture with pyruvate (Table II). On the other hand there were some compounds which did prevent the enzyme induction by pyruvate. Ammonium was the simplest of these, and it seems likely that the effects of the others can be understood through their ability to cause formation of ammonium. A constitutive urease [4] is able to account for the formation of ammonium in medium containing urea, and direct measurement revealed ammonium levels between 1 and 3 mM, at the time of harvest, in media which contained 50 mM concentrations of either arginine, citrulline or ornithine but were initially free of ammonium. That this level of ammonium is sufficient to prevent induction of NADP-linked glutamate dehydrogenase was demonstrated by transferring mycelia to solution A + 100 mM pyruvate supplemented with various concentrations of ammonium chloride (Fig. 3).

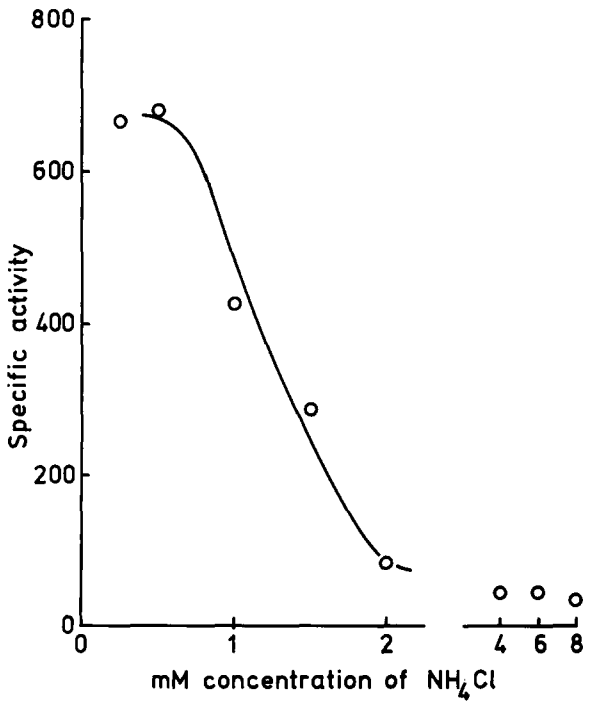
In all the work reported so far one particular haploid monokaryon (strain number BC9/6,6) has been used on the assumption that it would be representative of other haploid *C. cinereus* cultures. A survey of wild types in which enzyme activities were measured after 5 days incubation in casaminoacids medium and after a further 22 h following transfer to solution A + 100 mM pyruvate revealed that BC9/6,6 is definitely not representative (Table III). BC9/6,6 is a laboratory 'wild type' produced by nine generations of backcrossing between the strains H1 and H9 which were isolated as basidiospores from a carpophore collected in Hertfordshire [8]. Of the 17 strains listed in Table III only BC9/6,6 and its parent H9 showed induction of NADP-linked glutamate dehydrogenase on transfer to the pyruvate medium; this suggests that the ability

TABLE II

ACTIVITY OF NADP-LINKED GLUTAMATE DEHYDROGENASE FOLLOWING TRANSFER TO MEDIUM CONTAINING SUPPLEMENTS OTHER THAN PYRUVATE

Transfer experiments were carried out exactly as described for Table I except that the mycelia were transferred either to solution A containing 100 mM concentrations of single supplements, or to mixtures consisting of 100 mM pyruvate + 50 mM concentrations of the listed supplements. All entries are the means of at least three separate determinations.

Single supplements at 100 mM concentration	Specific activity (nmol · min^{-1} (mg protein)$^{-1}$)	Mixed supplements 100 mM pyruvate plus 50 mM concentrations of the following	Specific activity (nmol · min^{-1} (mg protein)$^{-1}$)
Glucose	769	Oxaloacetate	479
Fructose	482	Citrate	717
Dihydroxyacetone	110	2-Oxoglutarate	1 373
Acetate	287	Succinate	1 270
Propan-1-ol	286	Malate	1 124
Propan-2-ol	85	Alanine	279
Oxaloacetate	31	Aspartate	1 205
Citrate	5	Glutamate	298
2-Oxoglutarate	36	Arginine	52
Succinate	22	Ornithine	138
Malate	19	Citrulline	97
Lactate	87	NH_4Cl	54
Alanine	15	Methylamine	444
Aspartate	16	Hydroxylamine	262
		Urea	44
		KNO_3	737



to respond in this way may be a character which segregates in crosses. Such a possibility was tested by analysing progeny from a cross between BC9/6,6 and Singapore-7. Only four progeny were tested but they all belong to the same tetrad (i.e., represent the four products of a single meiosis) and the results (Table IV) show clearly that induction of NADP-glutamate dehydrogenase by the pyruvate transfer procedure segregated as though controlled by a single major gene. It must also be significant that the two progeny which failed to show induction excreted unusually large amounts of ammonium into the medium. This was evident in the original casaminoacids medium,

Fig. 3. Effect of the inclusion of ammonium chloride in the transfer medium on activity of NADP-linked glutamate dehydrogenase. Mycelia were grown and then transferred to solution A containing 100 mM pyruvate and supplemented with various concentrations of ammonium chloride. They were harvested for assay 20 to 22 h after transfer. Specific activity units are the same as for Fig. 1.

TABLE III

ACTIVITIES OF NADP-LINKED GLUTAMATE DEHYDROGENASE IN MYCELIA OF DIFFERENT WILD TYPE MONOKARYONS OF *COPRINUS CINEREUS* FOLLOWING TRANSFER TO PYRUVATE INDUCTION MEDIUM

Cultures were grown as described in Table I and transferred for 20 to 22 h to solution A containing 100 mM pyruvate. Entries show specific activities (nmol · min^{-1} (mg protein)$^{-1}$) and are the means of at least three replicates.

Culture code name	Geographical origin	Glutamate dehydrogenase activity	
		Before transfer	After transfer
BC9/6,6	Laboratory	58	222
H1	Hertfordshire, U.K.	50	33
H6	Hertfordshire, U.K.	32	27
H9	Hertfordshire, U.K.	32	287
Brum-b	Birmingham, U.K.	92	14
CBS394.65	Lincolnshire, U.K.	11	21
ZBw601/40,40	Czechoslovakia	36	4
Singapore-7	Singapore	19	98
Singapore-12	Singapore	34	40
Java-a	Java, Indonesia	18	14
Java-b	Java, Indonesia	14	26
PA1	Poland	88	15
mc-1,3	Connecticut, U.S.A.	62	58
Penn-a	Pennsylvania, U.S.A.	62	16
ATCC 24926	Canada	35	27
ATCC 20120/1	Japan	8	2
TC-4	Laboratory	25	14

where ammonium levels were 50% higher in these two progeny; but more significantly, the post-transfer medium (initially lacking in ammonium) showed levels almost 60-times greater than in the cultures of the other progeny. Indeed, the ammonium concentration, at around 2 mM, is in the range which prevents induction even in BC9/6,6 (Fig. 3). Paradoxically, though, these high external concentrations of ammonium do not seem to be correlated with elevated mycelial ammonium contents. The lack of such a correlation could indicate that ammonium regulation depends upon a reaction which takes place on the membrane, as suggested for *Aspergillus* [8]. There is clearly a very profound difference between the nitrogen metabolism of inducible and non-inducible progeny. Presumably, some component of the casamino-acids medium is processed to ammonium in the non-inducers. It should be possible to establish the identity of this component and the enzymology of the metabolic difference between the genotypes. BC9/6,6 must be defective in the reaction which, in other strains, is responsible for excretion into the medium of sufficient ammonium to obscure the induction of glutamate dehydrogenase activity by pyruvate.

Since neither ammonium analogues (methylamine and hydroxylamine) nor amino acids available for transamination (alanine and glutamate) were able to prevent induction of NADP-linked glutamate dehydrogenase by pyruvate, the implication is that the ammonium molecule itself is involved in a reaction

TABLE IV

ACTIVITIES OF NADP-LINKED GLUTAMATE DRHYDROGENASE IN MYCELIA OF A TETRAD OF PROGENY FROM THE CROSS BC9/6,6 × SINGAPORE-7 BEFORE AND AFTER TRANSFER TO THE PYRUVATE MEDIUM

Progeny	Medium pH	Medium ammonium (mM)	Mycelial ammonium (μmol · mg protein^{-1})	Glutamate dehydrogenase activity (nmol · min^{-1} (mg protein)$^{-1}$)
a. Before transfer				
T1a	7.25	76.5	7.19	21
T1b	6.82	51.7	8.99	54
T1c	7.37	77.9	6.12	10
T1d	6.92	51.2	5.61	39
b. After transfer				
T1a	6.77	1.54	0.002	45
T1b	7.10	0.034	0.026	479
T1c	6.75	2.32	0.053	73
T1d	7.32	0.034	0.012	488

which prevents such induction. The effect of ammonium in this case could be similar to the effects of exogenously supplied ammonium on metabolic regulation in other organisms [9], but in no other fungus is there any reason to believe that the regulatory phenomena observed have significance with regard to anything other than the vegetative hyphal organization. In *Coprinus,* it is clearly the case that ammonium and ammonium-assimilating enzymes are involved in control processes in the cap, a part of the carpophore which is so well removed from the substrate that regulatory changes observed must be responses to endogenous conditions in the cells and tissues of the fruit body. Fundamental differences between *Coprinus* and, say, *Aspergillus* and *Neurospora* are a real possibility. It is already clear that glutamate fails to prevent induction of NADP-linked glutamate dehydrogenase in *Coprinus* (Table II), whereas *Neurospora, Aspergillus* and *Escherichia coli* grown on glutamate showed low glutamate dehydrogenase activities [10]. In the same study [10] it was shown that glutamate dehydrogenase and glutamine synthetase activities were negatively correlated, whereas in *Coprinus* these two enzyme activities show a very strong positive correlation both in native carpophore tissues and in manipulated mycelia [4].

Fawole and Casselton [11] concluded that ammonium did not directly regulate glutamate dehydrogenase activity in *Coprinus cinereus* (=*lagopus*) because internal ammonium levels did not correlate with enzyme activity. This is not inconsistent with the view expressed above that it is the extracellular level of ammonium which is effective. These authors also concluded that a product of glucose metabolism (which they identified as 2-oxoglutarate) was responsible for inducing NADP-linked glutamate dehydrogenase activity. In the work reported here, pyruvate rather than 2-oxoglutarate is firmly implicated. Differences in interpretation could well be due to differences in technique. Fawole and Casselton [11] used long periods of incubation in most of their experiments, whereas the transfer technique employed here gives a much more immediate view of response to the medium. However, it is important to note that transfer to acetate was as effective as was transfer to pyruvate in inducing the glutamate dehydrogenase. It is possible that the intracellular regulator is acetyl-CoA since this is the only metabolite, prior to the Krebs cycle, which is common to glucose/pyruvate and acetate metabolism. Tests with a mutant which is known to be defective in acetyl-CoA synthetase [12] showed that induction of NADP-linked glutamate dehydrogenase was, indeed, dependent on synthesis of acetyl-CoA (Table V). The mutant mycelium failed to show any increase in NADP-glutamate dehydrogenase activity when transferred to a medium containing acetate, but behaved like the wild type when transferred either to glucose or pyruvate media, on which it is presumed that an intact pyruvate dehydrogenase complex was able to form acetyl-CoA in mutant and wild type alike.

It is thus concluded that regulation of NADP-linked glutamate dehydrogenase in *C. cinereus* depends on the intracellular level of acetyl-CoA and

TABLE V

ACTIVITIES OF NADP-LINKED GLUTAMATE DEHYDROGENASE BEFORE AND AFTER TRANSFER IN MYCELIA OF A WILD TYPE STRAIN AND OF A MUTANT WHICH LACKS ACTIVITY OF ACETYL-CoA SYNTHETASE

Entries are the means of at least three replicates.

Treatment	Glutamate dehydrogenase specific activity (nmol · min^{-1} (mg protein)$^{-1}$)	
	Wild type mycelium (strain H9)	Acetyl-CoA synthetase deficient mycelium (strain CC 20)
Control, untransferred	29	46
Transferred to		
Solution A + 100 mM pyruvate	707	557
Solution A + 100 mM glucose	647	640
Solution A + 100 mM acetate	290	14
Solution A + 100 mM propan-1-ol	491	23

the extracellular level of ammonium such that a high concentration of the former is able to induce the enzyme providing the concentration of the latter is low.

Acknowledgements

It is a pleasure to acknowledge the consistently excellent technical assistance of Mr. S.M. Crowe. Mr. F.N. Jabor is thanked for data used in Tables III and IV.

References

1 Stewart, G.R. and Moore, D. (1974) J. Gen. Microbiol. 83, 73–81
2 Al-Gharawi, A. and Moore, D. (1977) Biochim. Biophys. Acta 496, 95–102
3 Moore, D. and Ewaze, J.O. (1976) J. Gen. Microbiol. 97, 313–322
4 Ewaze, J.O., Moore, D. and Stewart, G.R. (1978) J. Gen. Microbiol. 107, 343–357
5 Al-Gharawi, A. and Moore, D. (1974) J. Gen. Microbiol. 85, 274–282
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
7 McCullough, H. (1967) Clin. Chim. Acta 17, 297–304
8 Day, P.R. and Anderson, G.E. (1961) Genet. Res. 2, 414–423
9 Pateman, J.A. and Kinghorn, J.R. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), Vol. II, pp. 159–237, Edward Arnold, London
10 Pateman, J.A. (1969) Biochem. J. 115, 769–775
11 Fawole, M.O. and Casselton, P.J. (1972) J. Exp. Bot. 23, 530–551
12 Casselton, L.A. and Casselton, P.J. (1974) Mol. Gen. Genet. 132, 255–264