Metabolism of Threonine by *Penicillia*: Growth on Threonine as the Sole Carbon and Nitrogen Source

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SUMMARY

Two species of *Penicillium* were isolated from soil by their ability to grow aerobically on L-threonine as sole source of energy and cellular carbon and nitrogen. Threonine-grown fungi contained a highly active inducible L-threonine:NAD⁺ dehydrogenase and a significantly less active constitutive 'biosynthetic' threonine dehydratase, but possessed no L-threonine:acetaldehyde lyase activity. The 2-amino-3-oxobutyrate formed by initial dehydrogenation of threonine was subsequently cleaved in both fungi to acetyl-CoA and glycine in a coenzyme-A-dependent cleavage catalysed by a 2-amino-3-oxobutyrate:CoA ligase which was inducibly synthesized during growth on threonine and not during growth on acetate plus glycine.

During growth of both fungi on threonine, ¹⁴C from L-[U-¹⁴C]threonine was rapidly incorporated into glycine and malate, and thereafter into citrate, aspartate, glutamate, succinate and various other metabolites. The time-dependent distribution of ¹⁴C among metabolites in these short-term incubations with L-[U-¹⁴C]-threonine showed that acetyl-CoA produced by the NAD⁺ plus coenzyme-A-dependent cleavage of threonine was metabolized via the tricarboxylic acid cycle plus glyoxylate cycle.

Comparative enzyme induction patterns after growth of both fungi on a wide range of carbon sources showed that glycine produced by the NAD⁺ plus coenzyme-A-dependent cleavage of threonine was metabolized via the glycerate pathway.

There was no evidence from either comparative enzyme induction patterns or incorporation of ¹⁴C from L-[U-¹⁴C]threonine of aminoacetone production and further metabolism by both fungi, even though a small amount of this amino-ketone appeared in culture media during growth on threonine.

INTRODUCTION

The metabolism of L-threonine by fungi is previously unrecorded. Several alternative pathways of threonine catabolism have been recognized among different bacterial species. A pathway involving an initial L-threonine dehydratase (deaminating) and subsequent metabolism of the 2-oxobutyrate produced via propionate to succinyl-CoA and hence the tricarboxylic acid cycle has been demonstrated in several bacteria (Whiteley, 1957). The alternative metabolism of threonine by L-threonine:acetaldehyde lyase to yield acetaldehyde and glycine has been recorded in a *Bacillus* species (Willetts & Turner, 1971) and also in a *Pseudomonas* species (Morris, 1969). A third pathway, the so-called 'aminoketone pathway', involving L-threonine:NAD⁺ dehydrogenase and subsequent metabolism of the initial dehydrogenation product, 2-amino-3-oxobutyrate, via aminoacetone to pyruvate, has been recorded in a *Bacillus* species (Willetts & Turner, 1970). A fourth pathway, also involving an initial L-threonine:NAD⁺ dehydrogenase, but the alternative subsequent

metabolism of the 2-amino-3-oxobutyrate produced to acetyl-CoA and glycine by the enzyme 2-amino-3-oxobutyrate:CoA ligase, has been recorded in an *Arthrobacter* species (McGilvray & Morris, 1969) and in *Pseudomonas oxalaticus* (Blackmore & Turner, 1971). The glycine produced by the combined action of the dehydrogenase plus ligase was further metabolized via the folate-dependent serine pathway (Sagers & Gunsalus, 1961) in the *Arthrobacter* species, but by the alternative glycerate pathway (Kornberg & Elsdén, 1961) in *P. oxalaticus*.

The present paper reports the presence of L-threonine:NAD⁺ dehydrogenase and 2-amino-3-oxobutyrate:CoA ligase in two species of *Penicillium* chosen for aerobic growth on threonine as sole source of energy and cellular carbon and nitrogen, and presents evidence that these two enzymes are integrally involved in the catabolism of threonine via acetyl-CoA and glycine. Evidence is presented for the subsequent metabolism of both separate C₂ units.

METHODS

Micro-organisms, maintenance and culture. *Penicillia* were isolated from soil samples by the enrichment culture technique using a medium which was also employed for the culture of these micro-organisms. The medium contained: L-threonine, 5 g; KH₂PO₄, 5.4 g; K₂HPO₄, 1 g and MgSO₄·7H₂O, 0.1 g, made up to 1 l with glass distilled water. When carbon compounds containing no additional organic nitrogen were used as energy sources, 1 g (NH₄)₂SO₄ was added to supplement cellular nitrogen requirements. Media were adjusted to pH 7.0 and sterilized by autoclaving. The two isolates subsequently used in this investigation were kindly identified as species of *Penicillium*, one a strain of *Penicillium chrysogenum* and the other a strain of *P. janthinellum*, by Dr D. Pitt of the Department of Biological Sciences, University of Exeter. For the sake of clarity these strain names are retained below. Both isolates have been deposited with the Culture Collection of The Commonwealth Mycological Institute, Kew, Surrey, England, as numbers CMI 166561 and CMI 166562 respectively. Both strains were maintained on slopes of threonine growth medium solidified with 1.5% of Oxoid Ionagar no. 2. Growth on threonine was optimal at 25 °C to 28 °C, and was routinely carried out at 26 °C. Liquid cultures were incubated on a rotary shaker, usually in 2 l conical flasks containing 1 l of growth medium for 24 to 30 h at 26 °C.

Preparation of washed suspensions. Cultures were harvested by straining through fine muslin, washed twice with 0.1 M-potassium phosphate buffer, pH 7.0, and resuspended in the appropriate buffer by gentle homogenizing with a Potter-type tube and pestle.

Preparation of cell extracts. Pellets of fungi after thorough washing and re-collection through muslin were frozen with the minimum quantity of liquid nitrogen and subsequently dry-ground in a mortar and pestle. Fragmented hyphae were subsequently suspended in a minimal volume of 0.1 M-potassium phosphate buffer, pH 7.0, and centrifuged at 20000 g for 10 min at 2 °C, and the supernatant solutions were used for experimental purposes.

Determination of protein. Soluble protein was measured by the biuret method described by Turner (1966).

Incorporation of ¹⁴C from ¹⁴C-labelled substrates. The procedures used to study the incorporation of ¹⁴C from L-[U-¹⁴C]threonine into constituents of the ethanol-soluble fraction of fungi growing on L-threonine were similar to those described for *Pseudomonas* metabolizing acetate (Kornberg, 1958).

Preparation of L-[U-¹⁴C]threonine. Radioactive threonine was obtained from the Radio-

chemical Centre, Amersham, Buckinghamshire, and was subsequently purified by chromatography (Large, Peel & Quayle, 1961).

Identification and assay of aminoacetone

On chromatograms. Aminoacetone was detected on chromatograms by spraying with ninhydrin reagent (0.5% in ethanol). Aminoacetone gave a characteristic yellow spot that slowly changed to purple on standing at room temperature.

In solution. Aminoacetone was determined colorimetrically by the method of Mauzerall & Granick (1956) as previously described (Turner, 1966).

Spectrophotometric procedures.

All spectrophotometric assays were performed at 30 ± 1 °C in silica cuvettes (3.5 ml volume, 1 cm light-path) using a Unicam SP 8000 recording spectrophotometer.

Enzyme assays

2-Amino-3-oxobutyrates:CoA ligase was assayed in the synthetic direction with acetyl-CoA and glycine as substrates (2-amino-3-oxobutyrates is unstable). The activity of this enzyme was measured spectrophotometrically by continuous determination of the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) by coenzyme-A released by condensation of acetyl-CoA with glycine, as expressed as the rate of increase in absorbance at 412 nm (McGilvray & Morris, 1969). Use of a suitable blank consisting of reaction mixtures minus glycine corrected for coenzyme-A formation from acetyl-CoA by any glycine-independent acylase activity.

ATP-D-glycerate phosphotransferase was assayed spectrophotometrically at 340 nm by following the formation of ADP consequent on the phosphorylation of glycerate. The ADP production was coupled to the oxidation of NADH by addition of PEP and pyruvate kinase and lactate dehydrogenase (Heptinstall & Quayle, 1970).

Citrate synthase was assayed spectrophotometrically by following oxaloacetate-dependent release of coenzyme-A from acetyl-CoA; the increase in absorbance at 412 nm was measured as 5,5'-dithiobis (2-nitrobenzoic acid) reduced by liberated coenzyme-A (McGilvray & Morris, 1969).

Erythro-β-hydroxyaspartate dehydratase was assayed spectrophotometrically by coupling with an excess of NADP-linked malate dehydrogenase and following oxidation of NADPH on addition of erythro-β-hydroxyaspartate by the decrease in absorbance at 340 nm (Kornberg & Morris, 1965).

Glycine decarboxylase (the 'glycine-cleavage enzyme') was measured by assaying the rate of formation of 5,10-methylene- H_4 PteGlu by a coupled spectrophotometric method using endogenous 5,10-methylene- H_4 PteGlu dehydrogenase (Willets & Turner, 1971).

Glycine-pyruvate aminotransferase was assayed spectrophotometrically by assaying the rate of oxidation of NADH by a coupled spectrophotometric method using exogenous lactate dehydrogenase (Rowell, Snell, Carnie & Al-Tai, 1969).

Glyoxylate carboligase was assayed manometrically by measuring the rate of anaerobic release of CO₂ from glyoxylate (Blackmore & Quayle, 1968).

Isocitrate lyase was assayed spectrophotometrically by following the increase in absorbance at 324 nm due to the formation of glyoxylate phenylhydrazone from DL-isocitrate as described by Dixon & Kornberg (1959).

Malate synthase was assayed spectrophotometrically by using 5,5'-dithiobis (2-nitrobenzoic acid) and following the increase in absorbance at 412 nm consequent on the glyoxylate-dependent release of coenzyme-A from acetyl-CoA.

'Malate enzyme' was assayed spectrophotometrically by following the decrease in absorbance at 340 nm consequent on the CO₂-dependent oxidation of NADH in the presence of pyruvate. The activity was determined in the presence and absence of sodium bicarbonate, so that an allowance for any endogenous lactate dehydrogenase activity could be made (Watson & Dworkin, 1968).

PEP carboxykinase was determined by assaying the rate of formation of oxaloacetate by a coupled spectrophotometric method using authentic exogenous ADP and malate dehydrogenase (Large, Peel & Quayle, 1962).

PEP carboxylase was determined by assaying the rate of formation of oxaloacetate by a coupled spectrophotometric method using authentic exogenous malate dehydrogenase without ADP addition (Large *et al.* 1962).

Pyruvate carboxylase was determined by assaying the rate of formation of oxaloacetate by a coupled spectrophotometric method using authentic exogenous ATP and malate dehydrogenase (Watson & Dworkin, 1968).

Pyruvate dehydrogenase complex was measured by assaying the rate of formation of ferrocyanide ions by a coupled spectrophotometric method using exogenous Prussian blue (Hayakawa *et al.* 1966).

L-Serine dehydratase was assayed by following the rate of production of pyruvate from L-serine using a procedure based on the Friedemann & Haugen (1943) assay for keto acids as described previously (Willettts & Turner, 1971).

L-Serine hydroxymethyltransferase was assayed in the direction of 5,10-methylene-H₄PteGlu formation from H₄PteGlu and L-serine using a coupled spectrophotometric assay system dependent on the presence of endogenous 5,10-methylene-H₄PteGlu dehydrogenase (Willettts & Turner, 1971).

L-Threonine:acetaldehyde lyase was assayed by measuring acetaldehyde production consequent on the aldol cleavage of threonine, by linking acetaldehyde production in a spectrophotometric assay with a decrease in absorbance at 340 nm due to the further NADH-dependent metabolism of acetaldehyde by exogenously added alcohol dehydrogenase (Willettts & Turner, 1971).

L-Threonine dehydratase (deaminating) was assayed colorimetrically by assaying production of 2-oxobutyrate from threonine (Datta, 1966).

L-Threonine:NAD⁺ dehydrogenase was assayed spectrophotometrically by following the increase in absorbance at 340 nm consequent on the NAD⁺-dependent oxidation of threonine to 2-amino-3-oxobutyrate (Willettts & Turner, 1970).

Chemicals

Thiol-CoA esters were prepared as described by Stadtman (1957). Acyl-CoA esters were estimated by the method of Cánovas & Kornberg (1966). Alcohol dehydrogenase, L-lactate dehydrogenase, malate dehydrogenase and pyruvate kinase were purchased from C. F. Boehringer und Soehne, GmbH, Mannheim, Germany. All other reagents were of AR grade or the highest grade commercially available.

RESULTS

Growth studies

Both *Penicillium chrysogenum* and *P. janthinellum* grew well at 26 °C on basal medium supplemented with L-threonine as the sole carbon and nitrogen source. Good growth also occurred on glycine alone, and on acetate plus ammonium sulphate. Growth on an equi-

Table 1. *Enzyme activities in extracts of Penicillium chrysogenum grown on various sources of carbon and nitrogen*

The micro-organism was grown as described in the text, an extract prepared and enzymes assayed as described in the Methods. Enzyme activities were measured at 30 °C unless otherwise stated. Units of enzyme are defined as those amounts which catalyse the formation of 1 nmol of product (or transformation of substrate)/min under the conditions described.

Enzyme specific activities (units/mg protein)	Growth substrate			
	Threonine	Glycine	Acetate plus (NH ₄) ₂ SO ₄	Glucose plus (NH ₄) ₂ SO ₄
2-Amino-3-oxobutylate:CoA ligase	14.9	0	0.1	0
ATP-D-glycerate phosphotransferase	26.0	35.2	0	0
Citrate synthase	23.6	18.0	29.6	26.5
Erythro-β-hydroxyaspartate dehydratase	0.1	0	0	0.2
Glycine decarboxylase	0	0.2	0.3	0
Glycine-pyruvate aminotransferase	36.2	22.3	0	0.6
Glyoxylate carboligase	57.0	71.0	0.1	0.1
Isocitrate lyase	35.8	0.1	49.6	0
'Malate enzyme'	0.1	0	7.9	0.3
Malate synthase	14.0	0	26.2	0.2
PEP carboxykinase	0	0	9.6	0.2
PEP carboxylase	2.9	0.8	3.1	1.9
Pyruvate carboxylase	46.0	50.2	26.9	60.0
Pyruvate dehydrogenase	0	19.6	0	40.2
L-Serine dehydratase	0.3	0.1	0	0.4
L-Serine hydroxymethyltransferase	0	0	0	0
L-Threonine:acetaldehyde lyase	0.2	0.3	0	0
L-Threonine dehydratase	3.6	4.9	4.6	2.8
L-Threonine:NAD ⁺ dehydrogenase	20.6	0	0.2	0

molar mixture of acetate plus glycine exactly mirrored growth on an equivalent concentration of threonine as a function of both time (as assessed by continuous nephelometry) and growth potential (as assessed by dry weight yield/mole of oxidizable growth substrate).

The optimum growth temperature of both penicillia was 26 °C. The lower temperature limit of growth was approximately 12 °C, and the upper temperature limit was at 34 °C. Neither *Penicillium* species grew in any medium under strictly anaerobic conditions, and only poor growth was observed in static aerobic cultures. Best growth was obtained in shaken liquid cultures.

Enzymes utilizing L-threonine in extracts

The specific activities of key threonine, acetate, and glycine-utilizing enzymes were assayed in crude extracts of *Penicillium chrysogenum* (Table 1) and *P. janthinellum* (Table 2) grown on (i) threonine, (ii) glycine, (iii) acetate plus ammonium sulphate or (iv) glucose plus ammonium sulphate as sole carbon and nitrogen sources. No L-threonine:acetaldehyde lyase activity could be detected in any extract. The low and significantly constant L-threonine dehydratase activity present in all extracts was markedly (90 to 100%) inhibited by low concentrations of L-isoleucine, which was consistent with the hypothesis that the assayed activity was due solely to a 'biosynthetic' threonine dehydratase in both organisms that is concerned with the synthesis of L-isoleucine (Umbarger & Davis, 1962) during growth on minimal media.

In direct contrast L-threonine:NAD⁺ dehydrogenase was significantly induced in extracts

Table 2. *Enzyme activities of Penicillium janthinellum grown on various sources of carbon and nitrogen*

The micro-organism was grown as described in the text, an extract prepared and enzymes assayed as described in the Methods. Enzyme activities were measured at 30 °C unless otherwise stated. Units of enzyme are defined as those amounts which catalyse the formation of 1 nmole of product (or transformation of substrate)/min under the conditions described.

Enzyme specific activities (units/mg protein)	Growth substrate			
	Threonine	Glycine	Acetate plus (NH ₄) ₂ SO ₄	Glucose plus (NH ₄) ₂ SO ₄
2-Amino-3-oxobutyrates:CoA ligase	21.6	0	0	0
ATP-D-glycerate phosphotransferase	19.6	38.4	0.1	0.1
Citrate synthase	19.2	23.9	28.0	30.4
Erythro-β-hydroxyaspartate dehydratase	0	0	0	0
Glycine decarboxylase	0	0	0	0
Glycine-pyruvate aminotransferase	29.4	28.4	0.2	0
Glyoxylate carboligase	49.0	62.4	0	0
Isocitrate lyase	46.2	0.4	50.6	0.5
'Malate enzyme'	0	0	12.6	0
Malate synthase	23.2	0.1	31.6	0.3
PEP carboxykinase	0	0	8.2	0
PEP carboxylase	4.1	3.4	2.0	2.8
Pyruvate carboxylase	49.6	70.2	43.0	56.6
Pyruvate dehydrogenase	0	22.2	0.3	36.2
L-Serine dehydratase	0	0	0	0
L-Serine hydroxymethyltransferase	0	0	0	0
L-Threonine:acetaldehyde lyase	0.1	0	0.4	0.2
L-Threonine dehydratase	2.4	4.1	4.6	4.5
L-Threonine:NAD ⁺ dehydrogenase	27.6	0.1	0	0

of both penicillia after growth on threonine. The induction of significant levels of L-threonine:NAD⁺ dehydrogenase in both threonine-grown penicillia suggested that the catabolism of threonine occurred through 2-amino-3-oxobutyrates in both species. The relative abilities of both penicillia to metabolize further this transitory intermediate either via decarboxylation to aminoacetone or CoASH-dependent cleavage to acetyl-CoA and glycine were subsequently investigated.

Aminoacetone formation from threonine

Very low concentrations (0.026 mM and 0.020 mM) of aminoacetone were present in spent media of *Penicillium chrysogenum* and *P. janthinellum* respectively, grown on L-threonine (5 g/l) and harvested in late exponential phase. Extracts of both fungal species were unable to utilize aminoacetone in the simultaneous presence of 2-oxoglutarate and NAD⁺ under the assay conditions previously reported to ensure aminoacetone disappearance in *Pseudomonas* and *Bacillus* species (Higgins, Turner & Willetts, 1967). A wide range of variable assay parameters failed to produce any evidence of aminoacetone utilization by threonine-grown extracts of either penicillium.

Acetyl-CoA plus glycine formation from threonine

The alternative CoASH-dependent catabolism of 2-amino-3-oxobutyrates to acetyl-CoA and glycine, catalysed by the enzyme 2-amino-3-oxobutyrates:CoA ligase has been recorded in an *Arthrobacter* species (McGilvray & Morris, 1969). This enzyme, originally considered to be 'aminoacetone synthetase' (Elliott, 1958), was substantially induced in both penicillia by growth on threonine, but significantly, was not induced by growth on glycine plus

acetate, acetate plus ammonium sulphate or glucose plus ammonium sulphate. As previously observed in the *Arthrobacter* species (McGilvray & Morris, 1969), the activity was substantially inhibited (76%) by low concentrations (0.5 mM) of free coenzyme-A. Similar inhibition patterns were recorded with other thiol-containing compounds, e.g. glutathione (90% inhibition at 0.5 mM) and L-cysteine (66% inhibition at 0.5 mM).

Further metabolism of acetyl-CoA and glycine by Penicillium chrysogenum and P. janthinellum

Washed mycelial pellets of both penicillia, after pregrowth on threonine, continued growth uninterrupted by any apparent lag phase on re-inoculation into fresh media containing either acetate plus ammonium sulphate or an equimolar mixture of glycine and acetate as the sources of carbon and nitrogen. When threonine-grown penicillia were resuspended in glycine growth medium, however, a small but significant lag phase of approximately 4 to 6 h was repeatedly recorded.

Enzymatic evidence obtained from extracts of both penicillia suggested that glycine was initially metabolized to glyoxylate, and subsequently via the glycerate pathway (Kornberg & Gotto, 1961) in a metabolic sequence essentially similar to that recorded in threonine-grown *Pseudomonas oxalaticus* (Blackmore & Turner, 1971). The substantial induction of glycine-pyruvate aminotransferase, glyoxylate carboligase and ATP-glycerate phosphotransferase in extracts of both penicillia by growth on either threonine or glycine medium in contrast to either acetate plus ammonium sulphate or glucose plus ammonium sulphate medium (Tables 1, 2) was direct evidence favouring the operation of this particular metabolic sequence in both penicillia. The repeated failure to detect glycine decarboxylase, L-serine dehydratase, L-serine hydroxymethyltransferase, or erythro- β -hydroxyaspartate dehydratase in extracts of either penicillium grown on threonine or glycine was direct evidence against the alternative metabolism of glycine via either the β -hydroxyaspartate pathway (Kornberg & Morris, 1965) or the glycine-serine pathway (Sagers & Gunsalus, 1961).

Both threonine and glycine-grown *Penicillium janthinellum* and *P. chrysogenum* induced pyruvate carboxylase in preference to PEP carboxylase to ensure net C_4 production from the intermediary C_3 products of the glycerate pathway. The small and consistent levels of both the 'malate enzyme' and PEP carboxykinase in both penicillia after growth on acetate but not threonine, glycine or glucose as available carbon source, was consistent with a biosynthetic role of these two enzymes in the penicillia during growth on C_2 compounds. The induction of PEP carboxykinase in addition to the 'malate enzyme' in such acetate-grown penicillia was indirect evidence for the absence of PEP synthetase (Cooper & Kornberg, 1967) in these fungi.

One major difference between the enzymatic complement of threonine-grown and glycine-grown penicillia was the substantial induction of pyruvate dehydrogenase by growth on glycine but not threonine. This enzyme activity was also induced by growth on glucose plus ammonium sulphate but not acetate plus ammonium sulphate.

Substantial activities for citrate synthase were recorded in both penicillia after growth on threonine, glycine, acetate plus ammonium sulphate or glucose plus ammonium sulphate. The enzyme was considered to be constitutive in both fungal species as a result of repeated enzymatic assay after growth on the above, plus numerous other different minimal media. Significant levels of both isocitrate lyase and malate synthase were induced in both penicillia only by growth on threonine or acetate plus ammonium sulphate: no evidence was obtained for the induction of either enzyme in both strains of *Penicillium* after growth on glucose plus ammonium sulphate, or more significantly, glycine.

Table 3. *Variation with time of the percentage distribution of ^{14}C incorporated from L-[U- ^{14}C]threonine by *Penicillium chrysogenum* growing on L-threonine as sole source of carbon and nitrogen atoms*

L-[U- ^{14}C]threonine (5.0 μmol ; 10 $\mu\text{Ci}/\mu\text{mol}$) was added to 10 ml of a culture of *Penicillium chrysogenum* (15 mg dry wt/ml) growing aerobically on L-threonine (100 μmol). Samples (1.0 ml) of culture were withdrawn at intervals into 3 ml of hot ethanol and the distribution of ^{14}C among the ethanol-soluble components was analysed by two-dimensional paper chromatography and autoradiography; radioactivity was directly assayed with a Geiger-Müller tube. The total radioactivity (minus background and residual L-[U- ^{14}C]threonine) fixed on the chromatograms after 5, 10, 15, 30, 60, 90, 120, 150 and 180 s of incubation was 196, 382, 669, 1503, 2646, 4041, 4646, 5314 and 5996 c.p.m. respectively.

	Sample time (s)								
Radioactivity in metabolite (% of total incorporation)	5	10	15	30	60	90	120	150	180
Alanine	0	0	0	0	5	5	6	4	6
Aspartate	6	9	10	13	10	8	8	11	9
Citrate	8	7	7	6	9	11	8	8	7
Cysteine	0	0	0	0	0	4	3	2	3
Glutamate	0	3	14	22	12	10	13	13	11
Glycine	40	25	18	12	9	5	5	5	5
Leucine	0	0	0	0	4	5	5	3	6
Malate	46	42	35	30	29	32	27	29	26
Methionine	0	0	0	0	0	3	4	2	5
Phosphorylated compounds	0	7	9	10	12	9	11	11	9
Succinate	0	8	9	7	10	8	10	10	8
Tyrosine	0	0	0	0	0	0	0	2	5

Incorporation of ^{14}C from L-[U- ^{14}C]threonine during growth

Washed suspensions of both *Penicillium janthinellum* and *P. chrysogenum* grown aerobically on threonine rapidly incorporated ^{14}C from L-[U- ^{14}C]threonine into both the ethanol-soluble and protein fractions. Although incorporation of ^{14}C into the protein fraction rapidly (30 s) attained a constant absolute value which remained consistent for several minutes, the incorporation of ^{14}C into the ethanol-soluble fraction increased linearly for the duration (0 to 180 s) of the experiments.

*Relative distribution of ^{14}C from L-[U- ^{14}C]threonine in washed suspensions of *Penicillium chrysogenum**

The relative distribution of ^{14}C among the various labelled metabolite components of the ethanol-soluble fractions was analysed by four techniques, i.e. two-dimensional chromatography, autoradiography, co-chromatography with authentic unlabelled samples, and direct monitoring of radioactive zones of chromatograms with a Geiger-Müller tube and scaler.

Label from L-[U- ^{14}C]threonine rapidly appeared in glycine, citrate, malate, aspartate, succinate and glutamate – but significantly not in serine. The relative distribution of label between identified metabolites as a function of time from the earliest sample taken (5 s) to completion of the experiment (180 s) is summarized in Table 3. In the earliest sample the largest percentage of label was located in either malate (46%) or glycine (40%), the remainder of the activity being approximately equally distributed between aspartate (6%) and citrate (8%). Only a minimal amount of label was found in serine. This absence of incorporation of ^{14}C from L-[U- ^{14}C]threonine into serine in washed suspensions of *Penicillium chrysogenum* was significantly different from the recorded distribution of label in an

Table 4. Variation with time of the percentage distribution of ^{14}C incorporated from L-[U- ^{14}C]threonine by *Penicillium janthinellum* growing on L-threonine as sole source of carbon and nitrogen atoms

L-[U- ^{14}C]threonine (5.0 μmol ; 10 $\mu\text{Ci}/\mu\text{mol}$) was added to 10 ml of a culture of *Penicillium janthinellum* (15 mg dry wt/ml) growing aerobically on L-threonine (100 μmol). Samples (1.0 ml) of culture were withdrawn at intervals into 3 ml of hot ethanol and the distribution of ^{14}C among the ethanol-soluble components was analysed by two-dimensional paper chromatography and autoradiography; radioactivity was directly assayed with a Geiger-Müller tube. The total radioactivity (minus background and residual L-[U- ^{14}C]threonine) fixed on the chromatograms after 5, 10, 15, 30, 60, 90, 120, 150 and 180 s of incubation was 179, 372, 696, 1546, 2726, 4304, 4844, 5502 and 6116 c.p.m. respectively.

Radioactivity in metabolite (% of total incorporation)	Sample-time (s)								
	5	10	15	30	60	90	120	150	180
Alanine	0	0	0	0	0	4	6	6	6
Aspartate	10	7	7	7	13	9	11	10	8
Citrate	4	9	7	11	8	8	8	11	10
Cysteine	0	0	0	0	1	3	4	2	2
Glutamate	0	6	12	19	14	14	11	12	14
Glycine	44	21	20	12	14	6	4	5	8
Leucine	0	0	0	0	0	3	6	6	4
Malate	42	40	36	30	30	32	23	17	21
Methionine	0	0	0	1	1	4	6	3	3
Phosphorylated compounds	0	11	9	9	9	7	12	13	10
Succinate	0	6	9	11	10	10	8	12	12
Tyrosine	0	0	0	0	0	0	1	3	2

equivalent experiment using threonine-grown *Arthrobacter* species (McGilvray & Morris, 1969).

The time-course of incorporation of ^{14}C into malate showed a rapid equilibration to a value of approximately 30% after 30 s. The proportion of label in glycine fell dramatically from 40% at 5 s to a consistently low value of approximately 5% after 90 s. The time-courses of incorporation of ^{14}C into both succinate and citrate remained remarkably consistent at about 8% throughout the course of the experiment. Similarly, the incorporation of label into a pool of metabolites tentatively identified only as 'phosphorylated compounds' remained consistent at about 10% in all time-course samples analysed. In contrast, incorporation of ^{14}C into both aspartate and glutamate showed an initial rapid rise to 13% and 22% respectively after 30 s, followed by an equally rapid decline to respectively 10% and 12% after 60 s, the percentages then remaining essentially constant thereafter. The distribution of label among metabolites in the last two time samples (150 s and 180 s) indicated a general pattern which was consistent with the establishment of intracellular 'metabolic pools' of aspartate, glutamate, succinate and citrate and the subsequent distribution of ^{14}C label from these pools into secondary products of biosynthesis, such as alanine, leucine, methionine, cysteine and tyrosine.

No evidence for the significant incorporation of label into aminoacetone, propionate or serine was obtained at any time during the course of the incubation.

*Relative distribution of ^{14}C from L-[U- ^{14}C]threonine in washed suspensions of *Penicillium janthinellum**

The relative distribution of ^{14}C among the various labelled metabolite components of the ethanol-soluble fractions of washed suspension of threonine-grown *Penicillium janthinellum* incubated with L-[U- ^{14}C]threonine was essentially similar to that recorded with *P. chryso-*

genum. Although the absolute counts incorporated into individual metabolites did show some variation between the two species, the relative percentage distribution of the label was almost identical (Table 4).

DISCUSSION

The combined results obtained from growth, enzyme induction and radiotracer studies all suggest that during the growth of both *Penicillium chrysogenum* and *P. janthinellum* on C₄ threonine as the sole source of carbon and nitrogen, the amino acid undergoes an initial catabolic oxidation to yield equimolar quantities of C₂ acetyl-CoA and C₂ glycine. The net C₄ biosynthesis essential for growth and multiplication of the fungi is attained, in both cases, by a combination of the subsequent metabolism of acetyl-CoA via the tricarboxylic plus glyoxylate cycles and the subsequent biosynthetic assimilation of glycine via the glycerate pathway plus pyruvate carboxylase. This pathway of threonine assimilation, as recognized in both *P. chrysogenum* and *P. janthinellum*, is summarized in Fig. 1. The internal rearrangements of the threonine carbon skeleton which characterize this pathway can be summarized as follows:

This is the first reported occurrence of threonine metabolism in fungi. However, the pathway recorded in these two penicillia is essentially similar to that tentatively recorded in the bacterium *Pseudomonas oxalaticus* (Blackmore & Turner, 1971), and differs from that previously recorded in another bacterial species, an *Arthrobacter* (McGilvray & Morris, 1969), only in the further metabolism of the glycine moiety produced by the initial cleavage of threonine. No evidence for the operation of the 'aminoketone pathway' of threonine metabolism (Willettts & Turner, 1970), previously recorded in a bacterial *Bacillus* species, has been obtained with these two fungal species. The consistently low threonine dehydratase activity recorded in both *Penicillium* species after growth on a wide range of carbon sources is almost certainly involved in the threonine-initiated biosynthesis of isoleucine. The almost 100% inhibition of this activity by isoleucine plus the failure to detect ¹⁴C-propionate among the metabolites of L-[U-¹⁴C]threonine are both consistent with the involvement of this threonine dehydratase in a biosynthetic rather than a catabolic pathway. The absence of L-threonine:acetaldehyde lyase eliminates any catabolic pathway equivalent to the lyase-initiated pathway recorded in certain bacterial species (Morris, 1969; Willettts & Turner, 1971).

The proposed operation of a 2-amino-3-oxobutyrate:CoA ligase activity catalysing the cleavage of the initial threonine dehydrogenation product to acetyl-CoA and glycine in both penicillia is confirmed by two observations:

(i) 2-Amino-3-oxobutyrate:CoA ligase is induced only during growth on L-threonine. The enzyme is not induced by growth on an equimolar mixture of glycine and acetate, indicating that the enzyme, acting as an 'aminoacetone synthetase', is unlikely to be involved in the biosynthetic role proposed by Elliott (1960).

(ii) ¹⁴C-Glycine is one of the earliest products of L-[U-¹⁴C]threonine metabolism by threonine-grown washed cell suspension of both fungal species. The repeated failure to detect any L-threonine:acetaldehyde lyase activity in such threonine-grown fungi eliminates the possible involvement of this alternative mechanism of rapid glycine production from threonine. No other characterized enzyme activity could feasibly explain this extremely rapid labelling of C₂ glycine from C₄ threonine.

Evidence from both comparative induction patterns after growth on various carbon

Fig. 1. The route of threonine catabolism and subsequent assimilation in *Penicillium chrysogenum* and *P. janthinellum* during growth of both fungi on the amino acid as the sole source of carbon and nitrogen. The results to support this pathway originate from the growth, enzyme induction and pulse radiotracer studies described in Methods.

sources and the selective incorporation of radioactivity into certain specific metabolites after incubation with L-[U-¹⁴C]threonine indicates that the further metabolism of glycine by both *Penicillium* species occurs via the glycerate pathway (Kornberg & Elsdén, 1961).

The induction of high levels of isocitrate lyase and malate synthase in both *Penicillium*

chrysogenum and *P. janthinellum* by growth on threonine, and the equally important failure of growth on glycine or glucose to effect a similar induction pattern, is an indication of the production of significant levels of 'acetate units' from L-threonine. The direct demonstration of the production of ^{14}C -succinate as one of the earliest detectable radio-labelled metabolites of L-[U- ^{14}C]threonine, is additional confirmation that the glyoxylate cycle operates in both penicillia during growth on L-threonine.

The apparent failure of L-threonine as the available carbon source to induce either 'malate enzyme' or PEP carboxykinase, i.e. the classical enzymes associated with the production of C_3 carbon skeletons from C_4 precursors, is probably an expression of the induction of the glycerate pathway in penicillia grown under these conditions. That this failure of threonine to induce either enzyme does not represent an inherent genetic defect in these fungi is indicated by the corresponding induction of significant levels of both enzymes during growth on acetate as the sole carbon source. The significance of C_3 production from C_4 threonine via glycine and the glycerate pathway as opposed to via acetyl-CoA, the glyoxylate cycle plus one of the classical C_4 decarboxylation enzymes, remains unknown.

One major difference in induced enzyme complement between threonine and glycine-grown *Penicillium chrysogenum* and *P. janthinellum* is the induction of pyruvate dehydrogenase after growth on glycine but not threonine. The significance of this selective induction is that glycine-grown penicillia require this enzyme for the generation of essential acetyl-CoA, whereas this need is satisfied by a combination of L-threonine:NAD $^+$ dehydrogenase and 2-amino-3-oxobutyrates:CoA ligase in threonine-grown fungi. The necessary induction of pyruvate dehydrogenase to ensure growth on glycine after prior growth on threonine provides an explanation of the observed lag phase of 4 to 6 h repeatedly observed on transferring either *Penicillium* species from threonine to glycine medium. This interpretation is confirmed by the selective induction of pyruvate dehydrogenase by growth on glucose but not acetate as sole carbon source.

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