Saccharopine: an Intermediate of L-Lysine Biosynthesis and Degradation in *Pyricularia oryzae*

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The aminoadipic acid pathway has previously been found to function as a biosynthetic route to lysine in higher fungi and as a catabolic pathway in mammals and higher plants. Results are now presented which suggest that in *Pyricularia oryzae* (fungi imperfecti), the aminoadipic acid pathway is important in both the biosynthesis and catabolism of lysine. *In vivo* experiments using L-[4-¹⁴C]aspartate showed that 98% of the radioactivity in protein-bound lysine and aspartate was in the latter, and with DL-amino[1-¹⁴C]adipic acid radioactivity was readily incorporated into saccharopine and free and protein-bound lysine. Endogenously supplied aminoadipic acid and saccharopine caused a rapid and substantial rise in the concentrations of free lysine in the mycelium. Cell-free extracts catalysed the conversion of aminoadipic acid to saccharopine and of saccharopine to lysine. *Pyricularia oryzae* and several other fungi were found to contain significant quantities of the intermediate saccharopine. The concentration of saccharopine increased when *P. oryzae* was supplied with lysine, and mycelia converted L-[¹⁴C]lysine into saccharopine and 2-amino-adipic acid. In addition, all of the enzyme activities necessary to break lysine down to 2-oxoadipic acid were demonstrated in extracts.

INTRODUCTION

There are two distinct pathways of L-lysine biosynthesis in Nature. One, the diamino-pimelic acid (DAP) pathway, is found in higher plants, algae, bacteria and some lower fungi (Vogel *et al.*, 1970). The second route, via 2-aminoadipic acid (AAA) and saccharopine (Fig. 1), has been found in those representatives of the remaining groups of fungi (i.e. all ascomycetes, basidiomycetes and phycomycetes with non-flagellate or posteriorly uni-flagellate zoospores) that have been tested. Evidence for the distribution of this pathway is mainly based on labelling studies in a few selected genera and enzyme studies have been largely confined to *Saccharomyces cerevisiae* and *Neurospora crassa*.

Lysine catabolism is not clearly understood and evidence for various pathways in mammals, prokaryotes and eukaryotic plants has been accumulated. Early studies have been reviewed by Meister (1965) and Broquist & Trupin (1966). Recently, Guengerich & Broquist (1976) have described a route via 6-*N*-acetyllysine in the fungi *Rhizoctonia leguminicola*, *N. crassa* and *S. cerevisiae*. A major route for lysine catabolism in mammals via the reverse half of the AAA pathway (Fig. 1) has been described (Fellows, 1973; Fellows & Lewis, 1973; Fjellstad & Robinson, 1975; Higashino *et al.*, 1971). The possibility that this pathway may also be important in fungi does not seem to have been considered, although Rothstein & Miller (1954) suggested that lysine may be degraded to aminoadipic acid and crotonyl-CoA via pipecolic acid.

This paper reports evidence which suggests that the AAA pathway is functional in both lysine synthesis and breakdown in *Pyricularia oryzae*, the causal agent of Blast Disease of rice and a member of the fungi imperfecti.

Fig. 1. Lysine metabolism in fungi and mammals according to Broquist & Trupin (1966) (—) and according to Schweet *et al.* (1955) and Rothstein & Miller (1954) (---). Several enzymes are formally called saccharopine dehydrogenase but to avoid confusion the mammalian enzymes, which appear to have different properties, have been given their trivial names used by Fellows & Lewis (1973). (1) Saccharopine dehydrogenase [lysine forming and either NAD^+ (EC 1.5.1.7) or NADP^+ (EC 1.5.1.8) forming] from yeast, *N. crassa* and other fungi. (2a) Lysine:2-oxoglutarate reductase (NADPH -dependent) and (2b) saccharopine oxidoreductase, both from mammals. (3) Saccharopine dehydrogenase [glutamate forming and either NAD^+ (EC 1.5.1.9) or NADP^+ (EC 1.5.1.10) forming] from yeast, *N. crassa* and other fungi. (4) Saccharopine dehydrogenase (NAD^+ requiring) from mammals.

METHODS

Chemicals. Most chemicals were bought commercially and were of the highest purity available. All radiochemicals with the exception of ^3H saccharopine were purchased from The Radiochemical Centre, Amersham. Saccharopine was isolated from a saccharopine-accumulating mutant of *N. crassa*, strain FGSC 108 (LYS IV), by a method adapted from Kuo *et al.* (1964) and Trupin & Broquist (1965). Radioactive saccharopine, N^6 -(2-L-glutaryl)-L-[U- ^3H]lysine, was prepared enzymically from [U- ^3H]lysine using crude extract-2 (see below). The incubation mixture (1.5 ml) contained 6 μmol [U- ^3H]lysine (0.25 mCi; 9.3 MBq), 2 μmol potassium 2-oxoglutarate, 3.75 μmol phosphate buffer pH 6.8, 1.3 μmol NADH (10 μl) plus 40 μl enzyme preparation (0.24 mg protein). The last two were added every 7 to 10 min. After 45 min the reaction was stopped by adding sulphosalicylic acid (50 mg in 0.5 ml water) and the denatured protein was removed by centrifugation. The saccharopine formed was purified using preparative high-voltage electrophoresis (see below) on Whatman 3MM paper run at 5 kV for 30 min. The area corresponding to authentic saccharopine was eluted with water in an airtight tank, and the eluate was rotary evaporated, taken up in water and the volume reduced under N_2 to 0.5 ml. This solution was kept at 0 °C. Its specific radioactivity was calculated as 42 mCi mmol^{-1} saccharopine (1.55 GBq mmol^{-1}) and 15.5 μCi (0.57 MBq) were obtained, corresponding to an overall yield of 6.2%.

Organisms, media and growth conditions. *Pyricularia oryzae* was provided by Shell Research Ltd, Sittingbourne, Kent and was maintained on potato dextrose agar plates (Difco, 39 g l^{-1}). Spores were produced on sterile moistened straw contained in 250 ml conical flasks and inoculated with mycelial plugs from potato dextrose agar plates. After 21 d incubation the spores were removed in 10 ml sterile water, washed by centrifugation and finally suspended in sterile distilled water. The density of the spore suspension was estimated using a haemocytometer and adjusted as required. The spores were used to inoculate three types of liquid media: (i) Czapek Dox medium (Oxoid) supplemented with 5 μg biotin l^{-1} and 1 mg thiamin $\cdot\text{HCl}$ l^{-1} ; (ii) Takahashi's medium (complex) which contained, per litre, 10 g sucrose, 5 g NaCl , 10 g Bacto-peptone

(Difco) and 5 g yeast extract powder (Oxoid); (iii) lysine-free defined medium – this was designed to ensure maximal growth of *P. oryzae* in lysine-free defined conditions (Wade, 1977) and was composed of (a) L-alanine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-histidine, L-methionine and L-serine, all at 1 mM, (b) 10 mM-NH₄NO₃, (c) 0.1 ml stock solution of trace minerals containing, per litre, 0.1 g Na₂B₄O₇ · 10 H₂O, 0.02 g (NH₄)₆Mo₇O₂₄ · 4H₂O, 0.2 g FeCl₃ · 6H₂O, 0.1 g CuSO₄ · 5H₂O, 0.02 g MnCl₂ · 4H₂O and 0.2 g ZnCl₂, and (d) vitamins (5 µg biotin l⁻¹ and 1 mg thiamin · HCl l⁻¹); all made up in Czapek Dox medium pH 6.8.

Five *Gaeumannomyces-Phialophora* complex isolates were provided by the Plant Pathology Department of this Institute: *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *Phialophora radicicola* var. *graminicola* and *P. radicicola* var. *radicicola*. The isolates were grown on 2% (w/v) malt extract agar plates. Small plugs of mycelium from the plates were used to inoculate either Czapek Dox medium supplemented with vitamins as for *P. oryzae* or 2% (w/v) malt extract broth (Difco).

Saccharomyces cerevisiae and *S. carlsbergensis* were provided by Professor M. J. Lewis, University of California, Davis, U.S.A. The yeasts were maintained on agar and grown in liquid culture in the same manner as the *Gaeumannomyces-Phialophora* isolates.

Spores of *Erysiphe graminis*, the causal agent of Powdery Mildew disease of barley, were collected from the leaves of Proctor barley plants infected with *E. graminis* using a cyclone separator.

All sterilization was by autoclaving at 103 kPa for 15 min except for medium containing malt extract when a treatment of 69 kPa for 10 min was used. Cultures in volumes of 20 ml or less were grown without shaking. Cultures in volumes greater than 20 ml were grown on a rotary shaker at 100 rev. min⁻¹. All culture growth was at 27 °C and 4000 lx.

Extraction of amino acids. (a) Free amino acids. Fungal material was placed in a boiling tube and an excess (50-fold, by weight) of 70% (v/v) ethanol was added. The contents were boiled for 3 min, and the liquor was decanted and retained. The procedure was repeated with 70% (v/v) ethanol and finally with distilled water. The extracts were pooled, evaporated to dryness *in vacuo* and the residue was taken up in a known volume of water. (b) Protein amino acids. Protein hydrolysates were prepared from washed material from which the free amino acids had been extracted. The samples were placed in screw-capped glass tubes containing a large excess of constant boiling point 6 M-HCl (100-fold, by weight). The tubes were closed with Teflon-lined caps and evacuated with ultrasonication for 30 min, flushed with N₂ and kept at 100 °C for 24 h (48 h for spore material). HCl was removed by evaporation *in vacuo* and the residue was taken up in a known volume of water.

Purification of amino acids. Samples for gas-liquid chromatography (g.l.c.) and heavily salted samples for high-voltage electrophoresis (h.v.e.) were put on an appropriately sized column of Dowex 50 (H⁺ form, 8% cross-linked, 100 to 200 mesh). The column was washed thoroughly with distilled water and eluted with 8 M-ammonia. The eluate was collected, evaporated to dryness *in vacuo* and the residue was taken up in a known volume of water.

This procedure was also used for separating the amino acids from the organic acids of *in vitro* enzyme assays prior to chromatography (see later). In this case, the column wash fraction containing the organic acids was retained, evaporated to dryness *in vacuo* and taken up in a known volume of water.

Chromatography. Glass plates (20 × 20 × 0.3 cm) for two-dimensional t.l.c. were coated with a 0.25 mm layer of cellulose-pulver Mn300 (Machery Nagel & Co., Duren, F.R.G.) and dried. After spotting the samples, the plates were run first in butan-1-ol/formic acid/water (70:12:20, by vol.) and then, after drying at room temperature and turning through 90°, in butan-1-ol/acetone/triethylamine/diethylamine/water (70:70:7:7:35, by vol.). This procedure was used for the separation of organic acids. Oxo-acids were visualized by spraying with 2,4-dinitrophenylhydrazine.

A Pye Unicam GCV chromatograph with a flame ionization detector was used for all g.l.c. analyses. The columns were packed with 3% (w/w) SE-30 on 80 to 100 mesh Gas Chrom Q (Applied Sciences Laboratories). All samples were purified on a cation exchange resin, as described earlier, before esterification and the amino acids were then chromatographed as their *N*-heptafluorobutyl *n*-propyl esters according to the method of Kirkman (1974). The amino acids were quantified using DL-meso-2,6-diaminopimelic acid as an internal standard.

Electrophoresis. Shandon high-voltage electrophoresis (h.v.e.) apparatus model L24 was used for the separation of amino acids. Whatman 3MM paper strips (11 × 63 cm) were used. Electrophoresis was at 6 kV for 25 min in 0.05 M-sodium barbital buffer pH 8.6. Amino acids were visualized by spraying with 0.1% (w/v) ninhydrin in 95% (v/v) ethanol and heating for 3 min at 105 °C.

Thin layer electrophoresis (t.l.e.) was carried out on glass plates (20 × 20 cm) coated with 0.25 mm-cellulose-pulver Mn300 using 25 mM-potassium acetate/acetic acid buffer pH 5.0. The voltage applied was usually greater than 2 kV and the current less than 100 mA. Amino acids were located and removed from the plate by the procedure of Davies & Mifflin (1978).

Free lysine determination. Samples were extracted in aqueous ethanol, as above, and lysine was determined by the colorimetric method of Hutzler *et al.* (1968), using the lysine decarboxylase assay in which lysine is converted to cadaverine. Cadaverine was determined as its 1-fluoro-2,4-dinitrobenzene derivative and estimated by measuring the absorption at 400 nm.

Liquid scintillation counting. Samples were counted using a Beckman LS-250 liquid scintillation spectrometer. Radioactive spots were located on h.v.e. papers and t.l.c. plates using a radiochromatogram spark chamber (Birchover Instruments). Radioactive spots were cut from h.v.e. papers or scraped from t.l.c. plates and added directly to 7 ml scintillation fluid [containing 8 g 2-(4'-*tert*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole and 0.5 g 2-(4-biphenyl)-6-phenylbenzoxazole per litre toluene]. Aqueous samples were either dried on paper and counted as above, or added to 7 ml of a mixture of 2 parts scintillation fluid (as above) and 1 part Triton X-100. Samples were allowed to equilibrate in the counting chamber for 4 h before counting. All counts were converted to disintegrations per minute (d.p.m.).

Uptake and metabolism of radioactive substrates by *P. oryzae*. Vitamin-supplemented Czapek Dox medium (2 ml) was inoculated with 10^6 spores of *P. oryzae* and incubated for 6 d. Radioactive substrates were added and the cultures were incubated on a rotary shaker at 100 rev. min⁻¹. The mycelium was harvested, and extractions and separations were carried out as described above. All treatments were carried out in duplicate.

Preparation of cell-free enzyme extracts. *Pyricularia oryzae* mycelium (50 g fresh wt) grown in Takahashi's or lysine-free defined medium was added to 40 ml 0.1 M-potassium phosphate buffer pH 6.8 containing 15% (v/v) glycerol and 1.4 mM-mercaptoethanol. The mixture was placed in a top drive 'tulip bulb' homogenizer and treated for 5 min. The homogenate was placed in a double-layered muslin bag and pressed. The filtrate was collected and centrifuged at 10000 *g* for 30 min. Protamine sulphate (75 mg in 2.5 ml water) was added dropwise to the supernatant and stirred for 30 min. The precipitate so formed was removed by centrifugation and the supernatant used as extract-1. Alternatively, the supernatant was made 85% saturated with (NH₄)₂SO₄ and stirred for 30 min, and the precipitate was collected by centrifugation at 10000 *g* for 25 min. The precipitate was resuspended in 3 ml of the original extraction buffer, desalted on a Sephadex G-10 column and used as extract-2. All steps were carried out at 0 to 4 °C and the extracts were stored at -18 °C.

Protein determination. Protein content was estimated by the Lowry method with bovine serum albumin as a standard.

Enzyme assays. (a) Saccharopine dehydrogenase (EC 1.5.1.7; NAD⁺, L-lysine forming) activity was assayed in the lysine-forming direction at 27 °C by two methods. (i) By measuring the rate of change of absorbance of the assay solution at 340 nm (1 cm light path) due to the reduction of NAD⁺. The assay solution contained 4 μmol L-saccharopine, 4 μmol NAD⁺, 15 μmol Na₂CO₃/NaHCO₃ buffer pH 9.6, enzyme solution and water in a total volume of 1.5 ml. The reaction was initiated by the addition of the 4 μmol saccharopine and incubated at 27 °C. (ii) By estimating the conversion of [³H]saccharopine to lysine. The assay mixture contained 15 μmol Na₂CO₃/NaHCO₃ pH 9.6, 12 μmol [³H]saccharopine (0.32 μCi; 11.8 kBq), 4 μmol NAD⁺ and 300 μl extract-2 with water to 750 μl. Saccharopine was added to start the reaction but NAD⁺ (4 μmol) was added every 5 min. The mixture was incubated at 25 °C for 50 min and the reaction was terminated by the addition of 50 mg sulphosalicylic acid. The protein was sedimented and the supernatant was removed and mixed with 1 mg lysine (as carrier). The precipitated protein was washed with water, the wash was added to the original supernatant and the volume was made up to 5 ml. This amino acid mixture was put on a Dowex 50 column, as described previously, and eluted with 8 M-ammonia. The eluate (25 ml) was rotary evaporated to dryness taken up in 1.5 ml 70% ethanol and blown down under N₂ to 0.2 ml; 50 μl samples were spotted on to Whatman 3MM paper and run on h.v.e. as described above. The strips were cut into 25 pieces (2 cm wide) and each of these was counted for radioactivity. Alternatively, 20 μl samples were spotted on to cellulose thin-layer plates and run on t.l.c. as described above. After running, portions of the chromatogram were removed by the method of Davies & Mifflin (1978) and counted for radioactivity.

Enzyme activity in the saccharopine-forming direction was assayed at 27 °C by measuring the rate of change of absorbance of the assay solution at 340 nm (1 cm light path) due to the oxidation of NADH. The assay solution contained 150 μmol potassium phosphate buffer pH 6.8, 8 μmol L-lysine, 0.15 μmol NADH, 4 μmol 2-oxoglutarate, enzyme and water to a total volume of 1.0 ml. The reaction was initiated by the addition of the 4 μmol 2-oxoglutarate.

(b) Saccharopine dehydrogenase (EC 1.5.1.9; NAD⁺, glutamate forming) was measured in the L-glutamate-forming direction following the saccharopine-dependent reduction of NAD⁺ at 340 nm. The assay solution was the same as for (i) above.

(c) For L-2-aminoadipate:2-oxoglutarate aminotransferase (EC 2.6.1.39), the reaction mixture contained 4 μmol potassium 2-oxoglutarate, 6 μmol DL-2-amino[1-¹⁴C]adipic acid (0.2 μCi; 7.4 kBq), 0.5 μmol pyridoxal phosphate, 150 μmol phosphate buffer pH 6.8, enzyme (extract-2) and water in a total volume of 1 ml. The reaction was initiated by the addition of the 4 μmol 2-oxoglutarate, incubated at 27 °C for 1 h

Fig. 2. Uptake and incorporation of DL-2-amino[1-¹⁴C]adipic acid by *P. oryzae* growing in Czapek Dox medium (a) into total protein (○), protein-bound lysine (●) and total soluble amino acid pool (▼) and (b) into 2-aminoadipic acid (Δ), saccharopine (▲), free lysine (□) and free glutamate (■). Spores of *P. oryzae* (10⁶ per replicate) were grown in 2 ml vitamin-supplemented Czapek Dox medium; DL-2-amino[1-¹⁴C]adipic acid (2.5 μCi, 80 nmol) was added to a series of 6 d cultures and incubated for the period indicated before the radioactivity in the fraction was determined.

then stopped with 0.1 ml 1 M-HCl and boiled for 10 min. After centrifugation the supernatant was evaporated to dryness *in vacuo*, taken up in 0.2 ml water and samples were spotted on t.l.c. plates for two-dimensional chromatography, as described above. The developed plates were autoradiographed and the amount of radioactivity in 2-oxoadipic acid was determined.

(d) Conversion of DL-2-amino[1-¹⁴C]adipic acid to saccharopine and lysine. Extract-1 (3.2 mg protein) was incubated for 3 h at 27 °C with 1 μmol glutathione, 4 μmol glutamic acid, 4 μmol DL-2-amino[1-¹⁴C]adipic acid (0.2 μCi; 7.4 kBq), 0.5 μmol NAD(P)H, 5 μmol ATP, 5 μmol MgCl₂, 250 μmol phosphate buffer pH 7.5 and water in a total volume of 1.3 ml. The reaction was initiated by the addition of the DL-2-aminoadipic acid and stopped by the addition of 0.1 ml 1 M-HCl and boiled for 10 min. The precipitate was removed by centrifugation and the amino acid fraction of the supernatant was separated from the organic acids by cation exchange chromatography, as above. The amino acid fraction was analysed by h.v.e. Radioactive spots were localized by autoradiography and the radioactivity in lysine and saccharopine was determined.

RESULTS

Evidence for the operation of the AAA pathway for L-lysine synthesis

Labelling studies with the intact organism. *Pyricularia oryzae* was grown in Czapek Dox medium in the presence of 5 μCi (0.6 μmol) L-[4-¹⁴C]aspartate for 24 h. At the end of the incubation period the specific activity in protein-bound aspartate and lysine was determined and values of 3.12 and 0.055 μCi mmol⁻¹, respectively, were obtained. The relative specific radioactivity in lysine expressed as a percentage of that in aspartate was therefore 1.8 which is in close agreement with values quoted by Vogel (1964) of between 0 and 5 for other fungi using the AAA pathway and greatly different from the values of 65 to 130 obtained for fungi using the DAP pathway.

When *P. oryzae* was fed DL-2-amino[1-¹⁴C]adipic acid, radioactivity was readily incorporated into protein-bound lysine. After 4 h, 50% of the total radioactivity in protein was in lysine (Fig. 2a). Analysis of the soluble fraction showed a substantial incorporation of radioactivity into saccharopine (Fig. 2b) suggesting that it may be an intermediate in the formation of lysine.

The concentration of radioactive 2-aminoadipic acid in the soluble pool rose continuously during the experiment (Fig. 2b) which may in part be due to the accumulation of the D-isomer.

Effect of non-radioactive compounds. When exogenous L-saccharopine, L-lysine and DL-2-aminoadipic acid were supplied to *P. oryzae* all three compounds caused a distinct increase

Table 1. *Effect of exogenous L-lysine, L-saccharopine and DL-aminoadipic acid on the concentrations of free lysine in the mycelium of P. oryzae*

The fungus was grown in 10 ml vitamin-supplemented Czapek Dox medium. The amino acids were added to 1 mM final concentration during the period of exponential growth. The cultures were incubated for a further 2 or 8 h and then the concentrations of free lysine in the mycelium were determined. All results are the mean of four replicates. The fresh weight of the mycelium of each culture was $43 \text{ mg} \pm \text{s.d. } 2.9 \text{ mg}$.

Treatment	L-Lysine concn	
	$\mu\text{g in sample}$	% of control
Control	5.1	100
1 mM-L-lysine, 2 h	15.2	298
1 mM-L-lysine, 8 h	16.8	329
1 mM-L-saccharopine, 2 h	8.8	172
1 mM-L-saccharopine, 8 h	12.8	252
1 mM-DL-2-aminoadipate, 2 h	7.3	143
1 mM-DL-2-aminoadipate, 8 h	11.1	218

in the concentration of free lysine in the mycelium of *P. oryzae* (Table 1). Lysine caused a threefold increase over 2 h and a further slight increase after 8 h, and DL-2-aminoadipic acid and saccharopine had raised the concentration of free lysine more than twofold after 8 h. These results suggest that 2-aminoadipic acid and saccharopine are lysine precursors in *P. oryzae* and are indicative of the AAA pathway.

Studies with cell-free extracts. Using extract-2 from mycelium grown on Takahashi's medium and the assay system (ii) with [^3H]saccharopine as substrate, radioactive lysine was formed. The identity of lysine was checked by co-migration with authentic lysine using h.v.e., t.l.c. and t.l.e. The specific enzyme activity of the most active preparation was $0.33 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ based on the conversion measured over 1 h. The radioactivity in the 'no enzyme' and 'zero time' blanks was less than 10% of that in the complete reaction mixture.

Attempts to demonstrate the conversion of DL-2-amino[1- ^{14}C]adipic acid to saccharopine and lysine in extract-1 from the same mycelium were not particularly successful. Approximately 1% of the radioactivity supplied was recovered in saccharopine but none was found in lysine. The majority of the radioactivity metabolized was found in oxo-acids. Attempts to increase the conversion to saccharopine and lysine by using NADPH instead of NADH were unsuccessful.

Evidence for the operation of the AAA pathway for lysine degradation

Studies with the intact organism. When L-[U- ^{14}C]lysine was supplied to *P. oryzae* a large proportion (> 70%) of radioactivity taken up over 4 h was incorporated into protein as lysine (Fig. 3a). However, examination of the soluble pool after short periods of incubation showed that in the first 7.5 min [^{14}C]lysine had been taken up and converted to saccharopine (Fig. 3b). The amount of radioactivity in the saccharopine fraction dropped after 15 min with a concomitant rise in the radioactivity in the 2-aminoadipic acid fraction. This suggests that at least the lysine to 2-aminoadipic acid section of the AAA pathway is freely reversible and may be used for lysine degradation.

Studies with cell-free extracts. The rapid oxidation of NADH in the presence of 2-oxoglutarate and lysine was readily demonstrated using cell-free extracts of mycelium grown in either lysine-free or Takahashi's (complex) media. The specific activities for extracts-1 and -2 averaged 0.46 and $1.36 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively, based on the

Fig. 3. Uptake and incorporation of L-[U-¹⁴C]lysine by *P. oryzae* growing in Czapek Dox medium (a) into total protein (○), protein-bound lysine (●) and total soluble amino acid pool (▼) and (b) into 2-aminoadipic acid (△), saccharopine (▲), free lysine (□) and free glutamate (■). The conditions were as described in Fig. 2 except that L-[U-¹⁴C]lysine (2 μ Ci, 0.2 μ mol) was added to each culture instead of the DL-2-amino[1-¹⁴C]adipic acid.

initial rate of NADH oxidation. The reaction was dependent on the presence of 2-oxoglutarate, lysine and NADH and the pH optimum was 6.8. The activity of glutamate dehydrogenase in the extract was too low to explain NADH oxidation in terms of deamination of lysine. The reaction product was confirmed as saccharopine by analysis of the reaction mixture by h.v.e. and g.l.c. The enzyme was strongly specific for NADH compared with NADPH and was not stimulated by the addition of FAD or FMN.

Extracts-1 and -2 also catalysed a saccharopine-dependent NAD⁺ reduction at rates of 0.083 and 0.145 μ mol min⁻¹ (mg protein)⁻¹, respectively. The products of this reaction could either be glutamate plus 2-aminoadipic semialdehyde or lysine plus 2-oxoglutarate (Fig. 1). Using non-labelled substrates and colorimetric spray reagents, only glutamate and 2-aminoadipic semialdehyde [trapped as its *o*-aminobenzaldehyde addition product (Larson *et al.*, 1963)] could be easily demonstrated as reaction products. The production of glutamate from saccharopine was confirmed by co-migration with authentic standards using h.v.e. and g.l.c. However, as shown above, [³H]saccharopine can be converted to [³H]lysine by extract-2. Subsequent work has shown that the enzyme catalysing the conversion of saccharopine to lysine can be separated from that converting saccharopine to glutamate and aminoadipic semialdehyde (D. M. Thomson, unpublished results).

The presence of aminoadipate:oxoglutarate aminotransferase in extracts-1 and -2 from mycelium grown in Takahashi's medium was readily demonstrated by their ability to convert DL-2-amino[1-¹⁴C]adipic acid and 2-oxoglutarate to 2-oxoadipic acid and glutamate. Analysis by h.v.e. and t.l.c. of the products of the reaction mixture using extract-1 showed significant incorporation of radioactivity into two other unidentified oxo-acids and saccharopine. When extract-2 and conditions specific for aminoadipate:oxoglutarate aminotransferase (see Methods) were used, 2-oxoadipic acid was the only significant radioactive product.

Concentrations of lysine and saccharopine in mycelial extracts

To see if the occurrence of relatively large pools of saccharopine is a general phenomenon and to study the relationship between lysine and saccharopine, the concentrations of these two compounds were assayed by g.l.c. in a range of fungi grown on defined (lysine-free) or Takahashi's (complex) media (Table 2). In all cases saccharopine was detected in significant amounts with the greatest concentration being in *G. graminis* var. *tritici* grown

Table 2. *Concentrations of lysine and saccharopine in the soluble amino acid fraction of extracts from a range of fungi*

The fungal material (spores of *E. graminis*, mycelia of the filamentous fungi and cells of the yeasts) was analysed by g.l.c. Concentrations of lysine and saccharopine are expressed as $\mu\text{mol (g tissue fresh wt)}^{-1}$.

Organism	Growth medium	Lysine concn	Saccharopine concn
<i>Pyricularia oryzae</i>	Czapek Dox	0.82	0.54
	Takahashi	3.28	0.54
<i>Gaeumannomyces graminis</i> var. <i>avenae</i>	Czapek Dox	0.42	0.75
	Takahashi	0.74	1.30
<i>Gaeumannomyces graminis</i> var. <i>graminis</i>	Czapek Dox	0.21	1.88
	Takahashi	1.23	1.81
<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	Czapek Dox	0.07	0.29
	Takahashi	5.54	2.27
<i>Phialophora radicicola</i> var. <i>radicicola</i>	Czapek Dox	trace	0.04
	Takahashi	4.32	0.60
<i>Phialophora radicicola</i> var. <i>graminicola</i>	Czapek Dox	1.15	0.16
	Takahashi	10.64	1.40
<i>Erysiphe graminis</i>	(on host)	1.48	0.37
<i>Saccharomyces cerevisiae</i>	Takahashi	3.21	0.76
<i>Saccharomyces carlsbergensis</i>	Takahashi	2.65	0.10
<i>Agaricus bisporus</i>	(not known)	0.82	0.13

on complex medium. In general, the amount of saccharopine rose when the fungi were grown on complex medium although not as much as that of lysine; conversely, although free lysine was at very low concentrations in the *G. graminis* series grown on lysine-free medium, substantial amounts of saccharopine remained. It is likely that the saccharopine formed in complex medium arose from lysine in the medium.

DISCUSSION

The results obtained are consistent with the operation in *P. oryzae* of the AAA pathway for lysine synthesis via saccharopine: (1) the feeding of L-[4- ^{14}C]aspartate resulted in 98% of the radioactivity incorporated into protein-bound aspartate and lysine being in aspartate; (2) DL-2-amino[1- ^{14}C]adipic acid was readily incorporated into saccharopine and free and protein-bound lysine; (3) a rapid and substantial increase in the concentrations of free lysine in *P. oryzae* was brought about by exogenous DL-2-aminoadipic acid and L-saccharopine; (4) extracts catalysed the conversion of 2-aminoadipic acid to saccharopine and of saccharopine to lysine.

It is important to emphasize that the previous enzymic evidence for the conversion of saccharopine to lysine in fungi was based on very limited data (Trupin & Broquist, 1965). Results purporting to measure this enzyme based on a saccharopine-dependent NAD⁺ reduction without proof of product formation are not valid because this assay also measures the conversion of saccharopine to 2-aminoadipic semialdehyde. Other workers in the field have relied on the back-reaction of lysine to saccharopine for evidence of the forward reaction. However, the discovery of an enzyme catalysing the irreversible conversion of lysine plus 2-oxoglutarate to saccharopine in mammals (Fellows & Lewis, 1973; Fig. 1, reaction 2a) casts doubts on the infallibility of this assumption.

These results are in agreement with the studies of Broquist and co-workers who established the AAA pathway as the major route for lysine synthesis in baker's yeast and *N. crassa* and showed that saccharopine was an essential intermediate in this process. Evidence suggesting that lysine synthesis may proceed from 2-aminoadipic acid to lysine via pipercolic

acid instead of via saccharopine in *N. crassa* has been reported by Schweet *et al.* (1955). The proposal was based on evidence of the *in vivo* conversion of Δ^1 -piperidine-2-carboxylic acid to lysine. This would involve a transamination of 6-amino-2-oxocaproic acid (see Fig. 1) but there is no evidence for the existence of this reaction in fungi. It is more likely that the conversion of Δ^1 -piperidine-2-carboxylic acid to lysine proceeds via pipercolic acid to 2-amino-adipic-5-semialdehyde and saccharopine, as has been shown to be the case in *Rhodotorula glutinis* (Kurtz & Bhattacharjee, 1975). Furthermore, the genetic evidence presented by Trupin & Broquist (1965) clearly demonstrates the role of saccharopine as a direct lysine precursor in *N. crassa*. The role of pipercolic acid in *P. oryzae* has not been investigated.

Evidence that saccharopine and the AAA pathway also play an important role in the catabolism of lysine is the demonstration in extracts of the conversion of lysine to saccharopine, saccharopine to 2-amino-adipic semialdehyde and 2-amino-adipic acid to 2-oxo-adipic acid. Enzyme activities are generally much greater in the catabolic than in the synthetic direction. The relative amounts of lysine and saccharopine in a range of fungi grown on lysine-free medium also suggest that the equilibrium of the interconversion of lysine and saccharopine favours the catabolic reaction. Consistent with the operation of this pathway of lysine breakdown *in vivo* is the rapid conversion of lysine to saccharopine and 2-amino-adipic acid (Figs 2 and 3) and the ability of *P. oryzae* to grow on lysine as a sole nitrogen source (D. M. Thomson, unpublished results). A similar role for saccharopine in lysine breakdown has been proposed in mammals (Hutzler & Dancis, 1968; Fellows, 1973; Fellows & Lewis, 1973) and higher plants (Moller, 1974, 1976*a, b*).

If our conclusions regarding the role of saccharopine in the degradation of lysine in fungi are correct then it would appear that the saccharopine pathway is a catabolic pathway common to fungi, higher plants and mammals. It may be that its role in lysine synthesis in fungi is a secondary one which may only have assumed importance after some evolutionary event [such as the loss of plastids which contain several of the enzymes of the DAP pathway (Wallsgrave *et al.*, 1979)] had led to the loss of the DAP pathway.

In the mammalian system, lysine breakdown occurs via a different enzyme (lysine:2-oxoglutarate reductase; Fig. 1, reaction 2a) present in a different compartment of the cell from the enzyme responsible for lysine synthesis (saccharopine oxidoreductase; Fig. 1, reaction 2b); neither of the enzymes is reversible (Fellows & Lewis, 1973). So far it is not known if there are two enzymes present in fungi with different roles or what is the subcellular location of the activities described. The mutant evidence of Trupin & Broquist (1965) would suggest that the same enzyme is responsible for both lysine synthesis and breakdown in *N. crassa*.

In summary, the results presented here emphasize that saccharopine is important not only in lysine synthesis but also in its degradation. The presence of significant quantities of saccharopine in fungi could also indicate that it has other possible functions since in biochemical terms a stable, free and accumulating intermediate of a transamination reaction is extremely rare, especially when the end-product of the pathway is limiting; evidence that it is not obligatory is that a transaminase capable of catalysing the direct interconversion of lysine plus 2-oxoglutarate to glutamate plus 2-amino-adipic semialdehyde has been isolated from *Flavobacterium fuscum* (Soda *et al.*, 1968; Soda & Misono, 1968). However, as yet there is no other plausible role for saccharopine.

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