**Saccharopine, an Intermediate of the Aminoadipic Acid Pathway of Lysine Biosynthesis**

**I. STUDIES IN NEUROSPORA CRASSA**

J. S. TRUPIN† AND HARRY P. BROQUIST

*From the Laboratory of Biochemistry, Department of Dairy Science, University of Illinois, Urbana, Illinois*

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Two pathways of lysine biosynthesis are known to exist in nature. The diaminopimelic acid pathway, studied in detail in *Escherichia coli* (1), is the route of lysine biosynthesis in bacteria, certain lower fungi, algae, and higher plants (2). In other classes of lower fungi, in higher fungi, and in Euglena, lysine is synthesized in a different manner, involving the intermediate a-aminoadipic acid (2). Recent investigations in our laboratory have been aimed at characterizing the steps in the conversion of a-aminoadipic acid to lysine. In the initial reaction(s) in yeast, a-aminoadipate is reduced to a-aminoadipic-δ-semialdehyde (3, 4). The discovery that saccharopine, e-N-(glutaryl)-2-lysine,

\[
\text{COOH} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{COOH} \\
\text{NH}_2 - \text{CH} - \text{CH}_2 - \text{COOH}
\]

is a metabolite of a-aminoadipic acid in yeast (5) suggested a unique manner of introducing the e-amino group of lysine. The pathway was visualized as shown in the following scheme.

\[
\text{a-aminoadipic acid} \\
\downarrow \\
\text{a-aminoadipic-δ-semialdehyde} \\
\downarrow \\
\text{glutamate} \\
\downarrow \\
\text{e-N-(glutaryl)-2-lysine} \\
\downarrow \\
\text{α-ketoglutarate} \\
\downarrow \\
\text{Lysine}
\]

However, the ease with which lysine can be converted to saccharopine in a yeast enzyme system (6), coupled with the fact that saccharopine is found in substantial amounts in the amino acid cell pool of yeast (7), raised the alternative possibility that saccharopine is not a precursor, but a product of subsequent lysine metabolism. In order to ascertain the role of saccharopine in lysine biosynthesis, we performed experiments with lysine-requiring mutants of *Neurospora crassa*. The results, briefly reported elsewhere (8), support the view that saccharopine is indeed a lysine precursor in this mold.

The approach used in these studies was dependent on finding a mutant blocked in the conversion of saccharopine to lysine. Such a mutant would be expected to accumulate saccharopine during growth. The saccharopine pool should become labeled when \(^{14}\text{C}-\text{aminoadipic acid}\) is added to the growth medium but not when \(^{13}\text{C}-\text{lysine}\) is administered. The reverse would be expected of mutants blocked before saccharopine. The accumulation of a compound by an auxotrophic mutant, however, is not sufficient proof per se that the compound is an obligatory intermediate on the blocked biosynthetic pathway. It is also necessary to show that the mutational event that caused auxotrophy resulted in loss of an appropriate enzyme on the postulated path (9).

**EXPERIMENTAL PROCEDURE**

**Mutant Strains and Growth Conditions**—Lysine-requiring auxotrophs of *N. crassa* were obtained from William N. Ogata, Fungal Genetics Stock Center, Dartmouth College, to whom we express our gratitude. They were maintained on agar slants according to the procedure of Doermann (10). Conidiospores or mycelial fragments from 5- to 8-day-old slants were used to inoculate the synthetic liquid medium of Horowitz and Beadle (11), supplemented with lysine as indicated. Incubation was performed in stationary culture at 28°C. L-Lysine, 2 μmoles per ml, gave maximum growth in 4 days.

**Materials**—Dl-α-Aminoadipic acid-6-\(^{14}\text{C}\) was synthesized for us by Dr. L. L. Miller, University of Rochester. Dl-α-Amino-δ-hydroxyaspartic acid was a gift from Dr. M. Bullock, Agricultural Division, American Cyanamid Company, Princeton, New Jersey. A generous sample of saccharopine, isolated from yeast, was provided by Professor S. Darling, Royal Dental College, Aarhus, Denmark. α-Aminobenzaldehyde was purchased from K and K Laboratories, Inc. All other materials were obtained from commercial sources.

**Extraction and Separation of Amino Acid Intermediates**—Mycelial pads, dried overnight in a desiccator, were extracted with a
small volume of water by immersion in a boiling water bath for 2 minutes. Enzyme reactions were stopped in the same manner. Paper electrophoresis of the mycelial extracts or enzyme reaction mixtures were performed on a Spinco apparatus with 0.05 M Veronal buffer, pH 8.2. At 330 volts, saccharopine may be separated from all common amino acids in 2 hours. Radioactivity was detected on electrophoresis strips with a Vanguard 880 auto-scanner. Separations on ion exchange columns were conducted according to the procedures of Kuo, Saunders, and Broquist (5). Aliquots of column fractions were dried on planchets and counted in a Nuclear-Chicago windowless gas flow counter.

**Enzyme Extraction**—Mycelial pads were grown for 2 days in Fernbach flasks containing 300 ml of basal medium (11), supple-

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutant</th>
<th>Chromosome linkage group</th>
<th>Growth response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-1</td>
<td>33933</td>
<td>V</td>
<td>a-Aminoadipic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lysine</td>
</tr>
<tr>
<td>Lys-3</td>
<td>4545</td>
<td>I R</td>
<td>a-Amino-γ-hydroxyeproic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lysine</td>
</tr>
<tr>
<td>Lys-2</td>
<td>37101</td>
<td>V R</td>
<td>Lysine</td>
</tr>
<tr>
<td>Lys-4</td>
<td>15069</td>
<td>I R</td>
<td>Lysine</td>
</tr>
</tbody>
</table>

**FIG. 1.** Electrophoretic patterns of amino acids in mycelial extracts of lysine auxotrophs. Mutants were grown for 5 days in 9 ml of basal medium containing 5 μmoles of L-lysine. Mycelial pad extracts were prepared and subjected to paper electrophoresis as described in “Experimental Procedure,” and the chromatograms were visualized with ninhydrin. Abbreviation used in figures: *ASP*, aspartate; *AAA*, α-aminoacidipic acid; *SAC*, saccharopine; *HHS*, hexahomoserine (α-amino-γ-hydroxyeproic acid); *LYS*, lysine.
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RESULTS AND DISCUSSION

Saccharopine Accumulation Studies—Screening of mutants for saccharopine accumulation was performed with four different lysine auxotrophs. Pertinent nutritional and genetic data on these mutants, summarized in Table I, were gleaned from the theses of Doermann (10) and Good (12). A fifth genetically distinct mutant, STL 7 was found to grow on α-aminoacidic acid, and was therefore of little interest to this study. Since α-amino-ε-hydroxyacaproic acid is reportedly oxidized directly to α-aminoacidicic semialdehyde (13), these mutants may be assigned to a postulated lysine pathway as follows (cf. also Good, Heilbrunner, and Mitchell (14)).

![Diagram of saccharopine and lysine biosynthesis]
For examination of the amino acid pools for accumulated intermediates, the mutants were grown on a low level of lysine and the mycelial extracts were subjected to paper electrophoresis. Ninhydrin treatment of the electrophoresis strips indicated an intense saccharopine band in mutant 15069 (Fig. 1). When the mutants were grown on an excess of lysine (2 to 4 μmoles per ml, data not shown), all mutants showed intense saccharopine bands, although the band in mutant 15069 was somewhat diminished. The aminoadipic acid band, exhibited by mutant 4845 in Fig. 1, was completely abolished by growth on excess lysine.

The results of a radioactive tracer experiment described in Fig. 2 clearly established that α-aminoadipic acid rather than lysine is the precursor of saccharopine in mutant 15069 (compare Tracing 1 with Tracing 2 in Fig. 2). Moreover, the existence of a lysine-sensitive mechanism affecting the conversion of aminoadipic acid to saccharopine was suggested by a decrease in the saccharopine peak and a correspondingly larger aminoadipate peak when the mutant was grown on a higher concentration of lysine (Tracing 2, Fig. 2).

Fig. 3 depicts the results of a similar experiment in which mutants 15069 and 37101 were compared. The use of an ion exchange column was necessitated by the failure of paper electrophoresis to separate saccharopine from substances accumulated by mutant 37101. Conclusive identification of radioactive saccharopine formed from α-aminoadipic acid-6-14C in mutant 15069 was achieved by radioautography, following paper chromatography of the column effluent. Fractions eluted in the saccharopine region were pooled, concentrated, and chromatographed in tert-butyl alcohol-formic acid-water (70:15:15). No radioactive saccharopine was detectable when radioactive aminoadipate was administered to mutant 37101 (Fig. 3). On the other hand, administration of labeled lysine to this mutant yielded a radioactive saccharopine peak (data not shown).

In confirmation of the previous electrophoretic results (Fig. 2), column fractions appeared to be devoid of radioactive saccharopine when mutant 15069 was grown on radioactive lysine. However, a more sensitive radioautographic examination of the eluate showed a low level of radioactive saccharopine. In view of the large amount of unlabeled saccharopine accumulated by this mutant (Fig. 1), lysine must represent, at best, a minor source.

A pathway that might account for this interconversion, namely from lysine, through α-keto-ε-aminocaproic acid to α-aminoadipic-δ-semialdehyde, was suggested some time ago by Schweet, Holden, and Lowy (15). Rigorous identification of saccharopine in mutant 15069 was achieved by isolating the radioactive amino acid from an extract of mycelium grown in the presence of α-aminoadipic acid-6-14C. The mycelium was grown for 5 days in 300 ml of medium containing 170 μmoles of l-lysine. One day after inoculation, 500 μmoles of unlabeled α-aminoadipic acid and 1 μC of α-aminoadipic acid-6-14C were added. A 2-mg sample of a crystalline substance was obtained by the procedure of Kuo et al. (5). An infrared spectrum of this material, compared with a spectrum of authentic saccharopine isolated from yeast, is shown in Fig. 4.

To establish that the radioactivity was not due to associated impurities, we chromatographed the material in four solvent systems: tert-butyl alcohol-formic acid-water (70:15:15); n-butyl alcohol-acetic acid-water (4:1:1); n-butyl alcohol-pyridine-water (1:1:1); and ethanol-water (77:23). In all cases, radioactivity, detected by direct scanning, migrated at the same rate as yeast saccharopine. The remainder of the radioactive material (approximately 0.5 mg) was then mixed with 8 mg of nonlabeled yeast saccharopine and recrystallized three times from acetone-water. Successive specific activities were 667, 718, and 618 cpm per mg.

Accumulations by Mutant 37101—α-Aminoadipic-δ-semialdehyde may be identified by criteria used by Aspen and Meister (16). The only substance likely to be confused with the semialdehyde is α-keto-ε-aminocaproic acid. Under physiological conditions, both compounds cyclize spontaneously to their respective Schiff bases, Δ2-piperideine-6-carboxylic acid and Δ2-piperideine-2-carboxylic acid. Consequently, both form orange dihydroquinazolinium derivatives with o-aminobenzaldehyde, and are reduced to piperolic acid upon catalytic hydrogenation. They may be distinguished, however, by the optical properties of their reduction products. The optical configuration of the α carbon of α-aminoadipic-δ-semialdehyde will be retained on reduction to piperolic acid whereas α-keto-ε-aminocaproic acid has no asymmetrical carbon atom.

Addition of o-aminobenzaldehyde to culture filtrates of several mutants gave the results shown in Table II. No substances that react with o-aminobenzaldehyde were ever detected in growth
media of mutants 4545 or 33933. Mutants 37101 and 15069, during growth on limiting lysine, discharged o-aminobenzaldehyde-positive material into the medium, although 15069 did so to a lesser extent.

In an effort to identify this material, mutant 37101 was grown in 600 ml of medium under conditions of limiting lysine (0.5 μmole per ml). After 5 days of growth, the mycelial pad was removed; the spent medium was concentrated 10-fold in a vacuum and divided into two equal portions. One portion was subjected to catalytic hydrogenation, essentially as described by Aspen and Meister (16). Both portions were then passed through cation exchange columns to separate amino acids from residual salts and sucrose, and the column effluents were examined for pipecolic acid by paper chromatography in the solvent systems described previously. A visual comparison of the chromatograms showed that some pipecolic acid had indeed been formed during hydrogenation. However, a significant amount of the amino acid had evidently been present in the medium prior to reduction. This complicated further efforts to determine optical configuration.

Insofar as was determined, the accumulations and nutritional requirements of mutant 37101 resembled those of certain lysine-requiring mutants of *Aspergillus nidulans* studied by Aspen and Meister (16). These workers were able to identify the o-aminobenzaldehyde-positive substance as the cyclized form of α-keto-ε-aminocaproic acid. It seems likely that the two piperedine carboxylic acid isomers and pipecolic acid are not intermediates on the lysine pathway, but rather are derived from the intermediate α-aminoadipic-δ-semialdehyde. Indeed, pipecolic acid can serve as a precursor of lysine in *Euglena gracilis* (17), but may simply be converted to aminoadipic semialdehyde and thence to lysine via saccharopine, for studies now in progress have shown that both *E. gracilis* and *A. nidulans* (wild strain)

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**TABLE II**

o-Aminobenzaldehyde reactions of culture filtrates of lysine auxotrophs

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Lysine in medium</th>
<th>Mycelial dry weight</th>
<th>Absorbance at 450 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4545</td>
<td>5 μmole</td>
<td>37 mg</td>
<td>0.034</td>
</tr>
<tr>
<td>50</td>
<td>98</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>37101</td>
<td>5</td>
<td>22 mg</td>
<td>0.417</td>
</tr>
<tr>
<td>50</td>
<td>109</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>15069</td>
<td>5 μmole</td>
<td>24 mg</td>
<td>0.232</td>
</tr>
<tr>
<td>50</td>
<td>116</td>
<td>0.011</td>
<td></td>
</tr>
</tbody>
</table>

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1 S. T. Vaughan and H. P. Broquist, work in progress.
FIG. 5. Conversion of radioactive lysine to saccharopine by enzyme extracts of N. crassa 37101 and 15069. Reaction mixtures consisted of 0.75 μmole of L-lysine containing DL-lysine-l-14C, 1.5 μmoles of α-ketoglutarate, 3 μmoles of NADH, 2.5 μmoles of K2HPO4 buffer (pH 6.0), and enzyme extract as indicated in a previous section. The reaction was stopped by heating in a boiling water bath. Aliquots (40 μl) were analyzed by paper electrophoresis and scanned for radioactivity.

The enzyme was assayed by conversion of radioactive lysine to saccharopine as measured chromatographically and by following the oxidation of NADH spectrophotometrically. A cell-free extract, containing 0.2 mg of protein, catalyzed an essentially complete conversion of L-lysine to saccharopine (Fig. 5), but after an identical incubation with a similar extract of mutant 15069, no saccharopine could be detected even when 12 times as much protein was used. As shown in Table III, this latter extract, in contrast to 37101 extract, failed to cause any substrate-dependent NADH oxidation. The results obtained satisfy the requirements of Umbarger and Davis (9) for proof of a biosynthetic intermediate with one exception. No evidence is presented concerning the number of enzymes involved in the conversion of saccharopine to lysine. That saccharopine is not itself a lysine precursor, but is derived.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Enzyme extract</th>
<th>Protein</th>
<th>Decrease in absorbance at 340 μm</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>37101</td>
<td>0.1</td>
<td>1.0</td>
<td>0.020</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.9</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4.8</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>15069</td>
<td>0.5</td>
<td>6.2</td>
<td>-0.005</td>
<td>0</td>
</tr>
</tbody>
</table>

* One unit of specific activity is defined as the amount of enzyme catalyzing a change of 0.001 absorbance unit per min per mg of protein.
from an intermediate, is, however, an unlikely possibility in view of
the nature of the reactions involved. The accompanying paper (20)
describes a series of Saccharomyces cerevisiae lysine auxotrophs.

An unusual mechanism of transamination, with the term used in
its broadest sense, apparently operates in the biogenesis of the
\( \varepsilon \)-amino group of lysine in yeast and Neurospora. An
extension of these findings to organisms other than Ascomycetes
would permit the generalization that saccharopine is an obligatory in-
termediate in the conversion of \( \varepsilon \)-amino adipic acid to lysine.

**SUMMARY**

Lysine-requiring mutants of Neurospora crassa were examined to
determine whether saccharopine, \( \varepsilon \)-N-(L-glutaryl-2)-L-lysine,
was an intermediate in lysine biosynthesis. Mutant 15069, in-
capable of growth on either \( \varepsilon \)-amino adipic acid or \( \varepsilon \)-amino-
hydroxycaproic acid, was the only mutant found to accumulate
saccharopine when grown on low levels of lysine. Administration
of \( \varepsilon \)-amino adipic acid-6-\( ^{14} \)C during growth of this mutant
resulted in the labeling of the saccharopine pool, whereas lysine-
6-\( ^{14} \)C contributed negligible radioactivity to saccharopine. In
contrast, in mutant 37101, which is nutritionally similar to 15069,
lysine, but not \( \varepsilon \)-amino adipic acid was the source of the sac-
charopine pool.

Mutants 37101 and 15069 grown on minimal lysine excreted a
substance into the medium, presumably \( \varepsilon \)-amino adipic-\( \delta \)-semi-
aldehyde, which formed a characteristic orange color with
\( \varepsilon \)-aminobenzaldehyde. The substance predominated in mutant
37101 cultures and was repressed when the mutant was grown with
excess lysine.

An enzyme capable of converting lysine plus \( \alpha \) ketoglutarate to
saccharopine was found in mutant 37101, but was absent in
mutant 15069. These findings were all interpreted as evidence
that mutant 37101 lacks an enzyme involved in the conversion of
\( \varepsilon \)-amino adipic-\( \delta \)-semialdehyde to saccharopine, and that
mutant 15069 is blocked in the conversion of saccharopine to
lysine. The establishment of saccharopine as an intermediate in
lysine biosynthesis affords a novel mechanism of transamina-
tion of a carbonyl function.

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