Accumulation of Tricarboxylic Acids Related to Lysine Biosynthesis in a Yeast Mutant*

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SUMMARY
Accumulation of tricarboxylic acids related to lysine biosynthesis has been demonstrated in a yeast mutant, Ly\(_{12}\), which requires lysine as a growth factor in synthetic medium but can substitute \(\alpha\)-aminoadipic acid for the nutritional requirement of lysine.

Both radioactive and nonradioactive intermediates have been isolated from the growth medium under appropriate conditions and have been identified chromatographically, chemically, and spectroscopically as homocitric, \(\alpha\)-homoaconitic, and homoisocitric acids. Homocitric acid is in equilibrium as the free acid and lactone following treatment with formic acid.

These intermediates in the lysine pathway are not accumulated in the wild-type yeast. The mutant Ly\(_{12}\) does not accumulate \(\alpha\)-ketoacidic acid, indicating that the biochemical block in this mutant corresponds to the biosynthetic step preceding \(\alpha\)-ketoacidic acid. Accumulation of these tricarboxylic acids in the culture medium of a lysine auxotroph provides additional evidence in support of their obligate intermediary role in lysine biosynthesis.

On the basis of tracer experiments, Strassman and Weinhouse (1) postulated that the six-carbon \(\alpha\)-ketoacid representing the carbon skeleton of lysine was synthesized by way of a series of reactions analogous to those of the citric acid cycle leading to the formation of \(\alpha\)-ketoglutaric acid. Thus, homocitric acid, the homologue of citric acid, is converted successively to homoisocitric acid, homoisocitric acid, and ultimately to \(\alpha\)-ketoacidic acid and to lysine. Strassman and Ceci (2) reported in 1964 the enzymatic synthesis of homocitric acid by condensation of acetate and \(\alpha\)-ketoglutarate. Subsequently, they showed the enzymatic formation of \(\alpha\)-ketoacidic acid from homoisocitric acid (3, 4) and the reversible conversion of homoisocitric acid and \(\alpha\)-ketoacidic acid by cell-free yeast preparations (5). Independently, Weber et al. (6) also demonstrated the formation of labeled \(\alpha\)-ketoacidic acid from \(14^C\)-labeled acetate and \(\alpha\)-ketoglutarate in cell-free preparations of baker's yeast. The intermediary role of \(\alpha\)-ketoacidic acid in lysine biosynthesis is well recognized (7-10). Recently, Thomas, Kalyanpur, and Stevens (11) have shown the absolute configuration of both synthetic and yeast homocitric acid to be (\(S\))-\((+\))-2-hydroxy-1,2,4-butanetricarboxylic acid.

Further evidence for the participation of the proposed intermediates may be obtained from lysine-requiring mutants by isolation and identification of intermediates which accumulate behind the genetic blocks and overflow into the culture medium. Accumulation of homoacetocitric acid and homoconiacetic acid in a yeast mutant requiring lysine (Ly\(_{12}\)) has been reported by Maragoudakis and Strassman (12) and Maragoudakis et al. (13). In a preliminary report Hogg and Broquist (14) observed the accumulation of homocitric acid in a Neurospora mutant. The simultaneous accumulation of homocitric acid, homoacetocitric acid, and homoisocitric acid in a different lysine auxotroph (Ly\(_{12}\)) of Saccharomyces is described in this communication.

MATERIALS AND METHODS

Cultures—A wild type strain WL-1 and a lysine-requiring mutant strain 44174-2b (described hereafter as Ly\(_{12}\)) both from Lindberg's breeding stock of Saccharomyces were used. Purity of the strain was tested by plating and isolating colonies arising from single cells. Cells were grown at 30° (optimum temperature).

Isolation of Radioactive Products Accumulated in Medium—Cells were grown in 50 ml of synthetic minimal medium (12) supplemented with growth-limiting concentrations of L-lysine (30 mg per liter). After 24 hours, 25 \(\mu\)C of \(1^4\)C-sodium acetate were added to the medium. At the time of addition of the tracer, the growth was essentially complete and the enzyme system was assumed to be at a derepressed state. The incubation was continued for an additional 72 hours; the cells were removed, and the medium was evaporated to dryness under reduced pressure. The residue was extracted with ether, and the ether extract was chromatographed on a column (1 \(\times\) 30 cm) of Dowex 1-formate and toluene-ethanol solvent system.

Isolation of Nonradioactive Products Accumulated in Growth Medium—A fresh slant culture of Ly\(_{12}\) was seeded into 200 ml of synthetic medium (12) which was supplemented with lysine...
(30 mg per liter), and the culture was then grown with aeration for 36 to 48 hours. This culture was used as inoculum in a fermentor containing 10 liters of the same medium. The cells were grown with vigorous aeration for 4 days, then harvested by continuous flow centrifugation. The medium was evaporated to approximately 100 ml, filtered, acidified, and extracted with ether for 7 days. The ether extract was evaporated to dryness; the residue was dissolved in 10 ml of water, neutralized with sodium hydroxide, and adsorbed on an analytical column (3 X 30 cm) of Dowex 1-formate. Then, 250 fractions of 10 ml each were eluted with a gradient of 6 N formic acid flowing into a constant volume mixing flask containing 400 ml of water. The eluent was monitored for ultraviolet light-absorbing substances and tested for KMnO₄-reacting substances (12). The same peaks of KMnO₄-reacting or ultraviolet light-absorbing material or both were pooled and evaporated to dryness.

The elution pattern of the synthetic material used as standard on a Dowex 1-formate column was determined by titration of the eluate fraction with NaOH solution.

Chemicals—Homocitric lactone, homoaconitic acid, and homoisocitric acid were synthesized as described in earlier publications (4, 5, 15). Synthetic 1,2,4-butanetricarboxylic acid was obtained through the kindness of Dr. R. H. Perry, Jr., Esso Research and Engineering Company, Linden, New Jersey.

Paper Chromatography of Radioactive Products—The solvent systems used are shown in Tables II to IV. Chromatograms were scanned for radioactivity and then sprayed with 0.04% bromphenol blue solution to determine the Rp of known organic acids used as controls.

Partition Chromatography—Homocitric acid and homoisocitric acid were separated by silicic acid column chromatography with the method of Ramsey (16). Chromatography on Dowex 1-formate (described earlier) failed to separate homocitric acid from homoisocitric acid.

RESULTS

The lysine-requiring mutant strain, Ly₁₂, was able to grow in the synthetic medium supplemented with either lysine or α-aminoadipic acid (Table I). The ability of the mutant strain to substitute α-aminoadipic acid for the requirement of lysine indicates that the mutant is blocked at a biosynthetic step prior to α-aminoadipic acid of the lysine pathway.

Column chromatograms containing several radioactive peaks were obtained from the ether extract of the acidified growth medium of Ly₁₂, while fewer radioactive peaks were obtained from the wild type strain (Fig. 1). A study of the accumulation products common to both the wild type and the mutant strain was currently in progress. Three of the radioactive peaks appearing in the profile of the mutant strain were not present in the profile of the wild type strain.

Accumulation of Homocitrate—The radioactivity in one of the three characteristic peaks from Ly₁₂ was eluted in the same fractions (52 to 62) as synthetic homocitric lactone and ran identically with synthetic homocitrate in several paper chromatographic systems. An aliquot from the combined radioactive Fractions 52 to 62 and carrier homocitric lactone (20 mg) were eluted in the same chromatographic fractions. However, following the exposure to formic acid the mutant product was resolved into two peaks of radioactivity in Fractions 36 to 43 and 52 to 62. The carrier homocitrate, determined by titration, also gave two peaks in the same fractions (Fig. 2). The earlier peak (Fractions 36 to 43) is perhaps due to the formation of free homocitric acid from the lactone.

The identity of the radioactive material in Fractions 36 to 43 and 52 to 62 with synthetic homocitrate was further confirmed.

![Fig. 1. Profiles of radioactive peaks obtained from the growth media of a lysine-requiring yeast mutant, Ly₁₂, and a wild type yeast, WL-1. The strains were grown in synthetic media with the supplement of L-lysine and 1-¹⁴C-sodium acetate. The growth conditions and the isolation procedures with analytical column of Dowex 1-formate are described in the text. Fractions of 5 ml were collected, and the radioactivity contained in aliquots of different fractions was determined with a Packard scintillation spectrometer. The upper profile represents the results from the wild type yeast, and the lower profile represents the results from the mutant strain, Ly₁₂. Peaks A, B, and C are characteristic of Ly₁₂ profile. A, homocitric acid and homoisocitric acid; B, homocitric lactone; C, homoaconitic acid.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Absorbance at 510 mp</th>
<th>Minimal medium</th>
<th>L-lysine</th>
<th>D₃-α-aminoadipic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>44174-2b (Ly₁₂)</td>
<td>0.08</td>
<td>0.59</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>WL-1 (wild type)</td>
<td>1.50</td>
<td>1.45</td>
<td>1.55</td>
<td></td>
</tr>
</tbody>
</table>
Fractions 36 to 43 and 52 to 62 from tubes 52 to 62 were pooled and rechromatographed on a Dowex 1-formate column. The eluate was assayed for radioactivity and was also titrated with NaOH as described in the text. The titration peak representing the carrier homocitrate and the major part of radioactivity appeared as superimposed peaks on tubes 29 to 62. A second radioactive peak appeared in tubes 36 to 43. The radioactivity and the carrier homocitrate from tubes 52 to 62 were pooled and rechromatographed on a Dowex 1-formate column under similar conditions. Two titration peaks, A and B, for the carrier homocitrate were obtained in Fractions 36 to 43 and 52 to 62. The radioactivity also appeared in the same fractions (36 to 43 and 52 to 62).

**TABLE II**  

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Mutant product (36 to 43) and 52 to 62</th>
<th>Synthetic homocitrate</th>
<th>Mutant product (97 to 115)</th>
<th>Synthetic homoaconitic acid (28 to 43)</th>
<th>Synthetic homoaconitic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>0.31</td>
<td>0.54</td>
<td>0.54</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Ethanol-formic acid</td>
<td>0.51</td>
<td>0.51</td>
<td>0.80</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>1-Butanol-formic acid-water</td>
<td>0.73</td>
<td>0.73</td>
<td>0.84</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>2-Butanol-acetic acid (saturated with water)</td>
<td>0.50</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>3-Butanol-methyl ethyl ketone-formic acid-water (4:30:15:15)</td>
<td>0.38</td>
<td>0.80</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>3-Butanol-formic acid-water (70:15:15)</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Ethyldisuse-formic acid-water (10:2:3)</td>
<td>0.96</td>
<td>0.96</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81</td>
</tr>
<tr>
<td>2-Butanol-formic acid-water (67:11:22)</td>
<td>0.70</td>
<td>0.70</td>
<td>0.78</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>1-Butanol-formic acid-water (5:1:1)</td>
<td>0.50</td>
<td>0.60</td>
<td>0.60</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>1-Butanol-acetic acid-water (4:1:1)</td>
<td>0.62</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The radioactivity and the homocitric acid accumulated in a bulk medium gave two distinct peaks when eluted from a silicic acid column whose positions coincided with those of the formic acid-treated synthetic homocitrate. Synthetic cis-homoaconitic acid also absorbs ultraviolet light and is eluted in Fractions 97 to 115. The radioactive material from Fractions 97 to 115 was chromatographed on papers along with synthetic homocitric acid. Synthetic homoaconitic acid invariably gave two spots on chromatograms; the faster moving component is the trans form, and the slower moving component is the cis form of homoaconitate (5). The RF values of the mutant product were identical with cis-homoaconitic acid (Table II).

The mutant material from either peak invariably gave two radioactive spots on the paper; the faster moving spot had the same RF value as the synthetic homocitric lactone. In another experiment, 700 mg of homocitrate were isolated from 10 liters of the Lyz culture medium. The melting point of the homocitric lactone synthesized from quinic acid was reported as 155-157° (11) and that of the mutant product (lactone) was 154-155°.

Both the radioactive material and the homocitric acid accumulated in a bulk medium gave two distinct peaks when eluted from a silicic acid column whose positions coincided with those of the formic acid-treated synthetic homocitrate. The biosynthetic homoaconitic acid was identical with the spectrum of synthetic cis-homoaconitic acid (Fig. 3).

The radioactive material was then eluted from an analytical column of Dowex 1-formate along with 20 mg of carrier cis-homoaconitic acid. The spectrum of the mutant material obtained was identical with the spectrum of synthetic cis-homoaconitic acid (Fig. 3).

The radioactivity and the homocitric acid accumulated in a bulk medium gave two distinct peaks when eluted from a silicic acid column whose positions coincided with those of the formic acid-treated synthetic homocitrate. The biosynthetic homoaconitic acid was identical with the spectrum of synthetic cis-homoaconitic acid (Fig. 3).

**Accumulation of Homoconitrate**—Homocitrate and homoisocitric acids were not separated on Dowex 1-formate chromatography. However, they separated well on silicic acid columns and could be
Further distinguished by the characteristic double zonation, described earlier for homocitric acid, following treatment with formic acid. Homoisocitric acid moved in all systems as a single entity.

Synthetic homoisocitric acid appeared in Fractions 38 to 43 when eluted from a Dowex 1-formate column. The bulk of the homocitric acid in Fractions 35 to 40 (Fig. 1) was removed as lactone by repeated equilibration and separation on Dowex 1-formate columns. The remaining radioactive material in Fractions 36 to 43 was mixed with 20 mg of carrier homoisocitric acid and eluted from a Dowex 1-formate column. Both the radioactivity and the titratable homoisocitrate appeared in identical fractions. They also ran together in several paper chromatographic systems (Table II). This radioactivity and the carrier homoisocitrate remained together and appeared in Fractions 127 to 136 on silicic acid column chromatography (Fig. 4). On the other hand, formic acid-treated homoisocitrate was eluted in Fractions 80 to 85 and 118 to 123 in the same system.

Malic acid also accumulates to a considerable extent in yeast culture medium, and this accumulation will be described more fully in another communication. Whereas on silicic acid column chromatography homoisocitrate could not be completely separated from malate, separation could be effected on Dowex 1-formate. Homaioisocitrate was eluted in Fractions 38 to 43 and malate in Fractions 26 to 30 on Dowex 1-formate column.

Homoisocitrate isolated from the culture medium of the mutant strain was purified, and the authenticity of the mutant product was determined by column, paper, and partition chromatography. This homoisocitrate was tested as a substrate of homoaconitase (5) and was found to be acted upon by purified homoaconitase. Both synthetic and biological homoisocitrate were not acted upon by homoaconitase for reasons yet unknown (the enzymatic work will be elaborated in a future publication). The same enzyme fraction failed to act on citrate and isocitrate, indicating the specificity of homoaconitase, and suggesting that the material accumulated by Lyn is indeed homoisocitrate. The homoisocitrate was also distinguishable from citrate and isocitrate on both paper and column chromatography.

The homoisocitrate isolated from the growth medium of Lyn was further characterized by converting it to L-ketoadipic acid 2,4-dinitrophenylhydrazone (17). The RF values of the hydrazone prepared from the mutant product and authentic L-ketoadipic acid hydrazone were identical. In two other experiments, radioactive homoisocitrate from Lyn was mixed with carrier homoisocitrate and converted to L-ketoadipic acid hydrazone with the radioactivity appearing in the derivative (Table IV).

**Table III**

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Radioactive</th>
<th>Authentic</th>
<th>Reduced product</th>
<th>Butyrate-carboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Butanol-formic acid-water (67:11:22)</td>
<td>0.82</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate-formic acid-water (10:2:3)</td>
<td>0.73</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Butanol-acetic acid-water (3:1:1)</td>
<td>0.70</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Butanol-formic acid-water (5:1:1)</td>
<td>0.68</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Butanol-methyl ethyl ketone-formic acid-water (40:18:15:16)</td>
<td>0.71</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>77% ethanol</td>
<td>0.74</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ether-benzene-formic acid-water (21:29:7:2)</td>
<td>0.55</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Butanol-formic acid-water (7:3:12)</td>
<td>0.71</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Homoisocitrate accumulated in the yeast is probably in the form of free acid, whereas chemical synthesis produces the lactone. The free acid and the lactone appear in two distinct fractions on paper and column chromatography. In the presence of formic acid, an equilibrium is established between the free acid and the lactone. The lactone-forming property of this seven-carbon lysine precursor is characteristic of -hydroxy-carboxylic acids.
Homoisocitrate is not found to be accumulated in Lyτ cells. The isomers of homoisocitrate, and homoaconitate accumulation is greater than in its growth medium not found either in the wild type or in Lyr also accumulates large amounts of an unidentified material which indicate that Lyr, lacks homoisocitric dehydrogenase activity and Ly, lacks homoaconitase activity (19). Besides having genetic differences, Ly4 and Ly, differ from each other in that the pathway leading to lysine. Consistent with these observations is the natural form of homoaconitic acid accumulated by the mutant is the cis isomer. Synthetic homoisocitrate consists of a mixture of the four possible stereoisomers; which of these isomers is the natural form is not yet definitely known.

Another yeast mutant, Ly4, accumulates homoisocitrate (12) and homoaconitate (13). A Neurospora mutant also shows accumulation of homocitric acid (14), indicating a similarity in lysine biosynthesis in these two ascomycetous organisms. However, homoisocitrate is not found to be accumulated in Ly, cells. The genetic data (15) show that the mutant Ly3 is complementary to Ly, indicating that these two mutants belong to two different genetic loci. The ability of Ly12 culture to substitute a-aminoadipic acid for the requirement of lysine during the growth in synthetic medium suggests that the biochemical block in this mutant precedes the intermediate a-aminoadipic acid. It has been established that a-ketoglutaric acid, the precursor of a-aminoadipic acid, is not accumulated in Ly27 culture. However, a-ketoglutaric acid is accumulated in Ly5 which is blocked after a-aminoadipic acid and hence fails to substitute a-aminoadipic acid for the requirement of lysine in synthetic growth medium (10). The results described in this paper show that Ly27 has a lesion immediately following homoisocitrate of the biosynthetic pathway leading to lysine. Consistent with these observations are the results from enzymatic studies (currently in progress) which indicate that Ly27 lacks homoisocitric dehydrogenase activity and Ly4 lacks homoaconitase activity (19). Besides having genetic differences, Ly4 and Ly27 differ from each other in that Ly4 also accumulates large amounts of an unidentified material in its growth medium not found either in the wild type or in Ly12. The relationship of this product to homoisocitrate and to lysine biosynthesis is not yet known. Thus, both the genetic data and the accumulation studies indicate that the mutant Ly12 represents a different genetic locus and biochemical block than that of Ly4.

Isolation of these three natural intermediates as accumulation products in the culture medium of a lysine auxotroph and not in wild type is evidence that they are obligatory intermediates of the biosynthetic pathway leading to lysine. Demonstration of the accumulation of lysine precursors in the mutant strain of yeast along with other evidence (4, 5) strongly supports the original scheme of Strassman and Weinhouse (1) in which it was postulated that the biosynthesis of the carbon chain for lysine precursors involves a series of reactions analogous with those in the formation of α-ketoglutaric acid in the citric acid cycle.

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