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, Ly4, which requires lysine as a growth factor in synthetic medium but can substitute a-ketoacid 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, cis-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 Ly4 does not accumulate a-ketoacid, indicating that the biochemical block in this mutant corresponds to the biosynthetic step preceding a-ketoacid. 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.
(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 effluent 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 Product—The solvent systems used are shown in Tables II to IV. Chromatograms were scanned for radioactivity and then sprayed with 0.04% bromophenol 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 homoisocitric 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 is 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 homocitrate lactone and run identically with synthetic homocitrate in several paper chromatographic systems. An aliquot from the combined radioactive fractions 52 to 62 and carrier homocitrate 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.
in the same fractions (36 to 43 and 52 to 62). The radioactive peak appearing in tubes 36 to 43. The radioactivity and the carrier homocitrate acid from a Dowex 1-formate column. The eluate was assayed for mutant product is identical with the cis form.

Fractions 36 to 43 and 52 to 62. The radioactivity also appeared as superimposed peaks in tubes 52 to 62. A second radioactive peak appeared in the growth medium of Lyiz. The radioactive peak appearing in tubes 52 to 62 (Fig. 1) was pooled, and an aliquot was mixed with 20 mg of synthetic homocitric lactone and eluted with 6 N formic acid from a silicic acid column whose positions coincided with those of the formic acid-treated synthetic homocitrate.

Formic acid-treated homocitrate, synthetic or mutant product, was resolved into two components in most solvent systems; the faster moving component is the lactone (compare first five solvent systems) and the slower moving component is the free acid. The synthetic homoaconitate contains both cis and trans forms; the mutant product is identical with the cis form.

Fig. 2. Identification of labeled homocitrate accumulated in the growth medium of Lyiz. The radioactive peak appearing in tubes 52 to 62 (Fig. 1) was pooled, and an aliquot was mixed with 20 mg of synthetic homocitric lactone and eluted with 6 N formic acid from 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 in tubes 52 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 homoaconitate</th>
<th>Mutant product (28 to 43)</th>
<th>Synthetic homocitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether-benzene-formic acid-water (21:9:7:2)</td>
<td>0.31</td>
<td>0.54</td>
<td>0.54</td>
<td>0.19</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>1-Butanol-formic acid-water (7:3:12)</td>
<td>0.50</td>
<td>0.64</td>
<td>0.64</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>2-Butanol-acetic acid-water (saturated with water) (85:15)</td>
<td>0.73</td>
<td>0.56</td>
<td>0.56</td>
<td>0.84</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>3-Butanol-methyl ethyl ketone-formic acid-water (40:30:15:15)</td>
<td>0.63</td>
<td>0.81</td>
<td>0.80</td>
<td>0.54</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>3-Butanol-formic acid-water (70:15:15)</td>
<td>0.85</td>
<td>0.75</td>
<td>0.75</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Ethylacetate-formic acid-water (10:2:3)</td>
<td>0.91</td>
<td>0.70</td>
<td>0.69</td>
<td>0.81</td>
<td>0.81</td>
<td>0.81</td>
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<tr>
<td>2-Butanol-formic acid-water (67:11:22)</td>
<td>0.70</td>
<td>0.78</td>
<td>0.78</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>1-Butanol-formic acid-water (8:1:1)</td>
<td>0.50</td>
<td>0.60</td>
<td>0.60</td>
<td>0.61</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>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
</tbody>
</table>

The radioactive material was then eluted from an analytical column of Dowex 1-formate along with 20 mg of carrier cis-homoaconitic acid. The synthetic cis-homoaconitic acid showed ultraviolet absorption in the same fractions in which the radioactive peak from the mutant material was obtained.

A total of 174 mg of homoaconitic acid was isolated from 10 liters of Lyiz culture supernatant. The infrared spectrum of the biosynthetic homoaconitic acid was identical with the spectrum of synthetic cis-homoaconitic acid (Fig. 3).

The authenticity of the mutant-accumulated homoaconitic acid was further verified by reducing the mutant product to 1,2,4-butanetriolcarboxylic acid by catalytic hydrogenation (5). The reduction product was identified by its absence of ultraviolet light-absorbing capacity and by the similarity of its chromatographic property with that of the synthetic material in nine different solvent systems (Table III). The melting point of the mutant product also compared with that of the synthetic cis-homoaconitic acid (5).

Accumulation of Homoaconitate—Homoaconitic and homoisocitric acids were not separated on Dowex 1-formate chromatography. However, they separated well on silicic acid columns and could be by paper chromatography (Table II). The mutant material from either peak invariably gave two radioactive spots on the paper; the faster moving spot had the same Rp value as the synthetic homoaconitic lactone. In another experiment, 700 mg of homoaconitic acid was isolated from 10 liters of Lyiz growth medium. The melting point of the homoaconitic 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 homoaconitic 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 homoaconitic acid.

Homoaconitic Acid Accumulation—Fractions 97 to 115, in which a radioactive peak appeared in the original profile from Lyiz (Fig. 1), contained a material which absorbed ultraviolet light. 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 homoaconitic 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 Rp values of the mutant product were identical with cis-homoaconitate (Table II).
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. Homoisocitrate 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 homoisocitrate isolated from the growth medium of Lyn was purified, and the authenticity of the mutant product was determined by repeated equilibration and separation on Dowex 1-formate columns. The remaining radioactive material in Fractions 38 to 43 was mixed with 20 mg of carrier homoisocitric acid and eluted from a Dowex 1-formate column. Both the radioactivity and hydrazone appeared in identical fractions.

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 α-ketoadipic acid 2,4-dinitrophenylhydrazone (17). The \( R_F \) values of the hydrazone prepared from the mutant product and authentic α-ketoadipic acid hydrazone were identical. In two other experiments, radioactive homoisocitrate from Lyn was mixed with carrier homoisocitrate and converted to α-ketoadipic acid hydrazone with the radioactivity appearing in the derivative (Table IV).

![Graph](http://www.jbc.org/)

**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( R_F ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Authentic α-ketoadipate 2,4-dinitrophenylhydrazone</td>
<td>0.59</td>
</tr>
<tr>
<td>Hydrazone from the mutant product</td>
<td>0.59</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>0.59</td>
</tr>
<tr>
<td>2. Authentic α-ketoadipate 2,4-dinitrophenylhydrazone</td>
<td>0.56</td>
</tr>
<tr>
<td>Hydrazone from the carrier homoisocitrate</td>
<td>0.56</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>0.56</td>
</tr>
<tr>
<td>3. Authentic α-ketoadipate 2,4-dinitrophenylhydrazone</td>
<td>0.51</td>
</tr>
<tr>
<td>Hydrazone from the carrier homoisocitrate</td>
<td>0.51</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>0.51</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Homocitrate accumulated in the mutant 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 \( \gamma \)-hydroxy carboxylic acids.
In both radioactive and nonradioactive experiments, more homoisocitrate is accumulated in the culture medium of $L_{y1}$ than homoaconitate and homoaconitate accumulation is greater than homoisocitrate which indicates that $L_{y1}$ lacks homoisocitrate dehydrogenase activity and $L_{y4}$ lacks homoaconitase activity (19). Besides having genetic differences, $L_{y4}$ and $L_{y1}$ differ from each other in that the pathway leading to lysine. Consistent with these observations is the fact that $L_{y4}$ accumulates homocitrate (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 $L_{y4}$ cells. The genetic data (18) show that the mutant $L_{y4}$ is complementary to $L_{y1}$, indicating that these two mutants belong to two different genetic loci. The ability of $L_{y1}$ culture to substitute $\alpha$-aminoadipic acid for the requirement of lysine during the growth in synthetic medium suggests that the biochemical block in this mutant precedes the intermediate $\alpha$-aminoadipic acid. It has been established that $\alpha$-ketoisocitreric acid, the precursor of $\alpha$-aminoadipic acid, is not accumulated in $L_{y4}$ culture. However, $\alpha$-ketoisocitreric acid is accumulated in $L_{y1}$ which is blocked after $\alpha$-aminoadipic acid and hence fails to substitute $\alpha$-aminoadipic acid for the requirement of lysine in synthetic growth medium (10). The results described in this paper show that $L_{y4}$ 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 $L_{y4}$ lacks homoisocitrate dehydrogenase activity and $L_{y4}$ lacks homoaconitase activity (19). Besides having genetic differences, $L_{y4}$ and $L_{y1}$ differ from each other in that $L_{y4}$ also accumulates large amounts of an unidentified material in its growth medium not found either in the wild type or in $L_{y1}$. The relationship of this product to homoaconitate and to lysine biosynthesis is not yet known. Thus, both the genetic data and the accumulation studies indicate that the mutant $L_{y4}$ represents a different genetic locus and biochemical block than that of $L_{y1}$.

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 $\alpha$-ketoglutaric acid in the citric acid cycle.

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