**Threonine Biosynthesis**

**ON THE PATHWAY IN FUNGI AND BACTERIA AND THE MECHANISM OF THE ISOMERIZATION REACTION**

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(Received for publication, December 16, 1964)

The observation that an *Escherichia coli* mutant requiring exogenous succinate for aerobic growth would alternatively respond to a mixture of four amino acids (1) has now been explained in the case of three of the amino acids. Thus, N-succinyl-L-diaminopimelic acid has been shown to be an intermediate in the bacterial synthesis of lysine and diaminopimelic acid (2), and O-succinyl-L-homoserine is an intermediate in the bacterial synthesis of methionine (3–5). The requirement for threonine has not been explained. The pathway for threonine synthesis established in yeast (6), by Reactions 1 and 2, does not accommodate an intermediary role for succinate, nor does present knowledge of regulatory mechanism indicate that threonine should be required due to the presence of the other three amino acids (7).

\[ \text{L-Homoserine + ATP} \rightarrow \text{O-phospho-L-homoserine + ADP} \quad (1) \]

\[ \text{O-Phospho-L-homoserine} \xrightarrow{\text{pyridoxal-P}} \text{threonine + P}_i \quad (2) \]

The limited evidence available also indicates that the pathway in *E. coli* may be the same as that in yeast (8, 9). However, it has not previously been possible to identify Reaction 1 in extracts of *Neurospora* (10). In this paper, we wish to report a reinvestigation of these questions concerning the pathway of threonine biosynthesis (11).

Additional results bearing on the mechanism of Reaction 2 (12, 13) will also be described, including some kinetic properties of the *Neurospora* enzyme, substrate-products exchange reactions, and the unequivocal determination of the positions in threonine into which solvent hydrogen is introduced during the reaction (14).

**EXPERIMENTAL PROCEDURE**

*Microbial Culture Conditions—Escherichia coli* M-309-1 (1), obtained from Dr. B. D. Davis, was maintained on agar slants of Cold Spring Harbor Medium A supplemented with succinate, 0.3 μmole per ml. The latter medium contains (in grams per liter): K2HPO4, 10.5; KH2PO4, 4.5; sodium citrate-5H2O, 0.5; (NH4)2SO4, 1; MgSO4, 0.05; glucose, 2. Inocula for growth experiments were cultured aerobically at 37°C in liquid Medium A supplemented with succinate, 0.1 μmole per ml; near the end of the exponential growth phase the flasks were placed at 2°C, and were kept, for use, at this temperature for periods up to a week. Growth responses were measured turbidimetrically in 125-ml Bellco Nephalo flasks with a Klett-Summerson colorimeter and No. 06 filter. The flasks contained 25 ml of Medium A supplemented with various nutrients as indicated in “Results.” After inoculation with 1 ml of the above culture, they were agitated at 37°C on a shaker.

*Neurospora crassa* wild type 5997a cultures were prepared as previously described (10) except that the culture medium was that of Vogel (15). Cells were grown in carboys for the preparation of threonine synthetase, and on a rotary shaker in 2-liter Erlenmeyer flasks, containing 500 ml of medium, for studies of homoserine kinase (ATP:1-homoserine O-phosphotransferase, EC 2.7.1.39).

**Enzyme Preparations and Assays—*Neurospora* threonine synthetase was prepared and assayed as previously described (10). The fraction used in these experiments was Step 4B (10), a concentrated DEAE-cellulose eluate.

Homoserine kinase activity (Reaction 1) which previously could not be detected in *Neurospora* extracts (10) was found to be demonstrable in a protein fraction prepared as follows. Freshly harvested mycelia were added to 2 parts of cold 0.05 M potassium dimethylglutarate buffer, pH 7.3, containing 0.002 M reduced glutathione, and were briefly dispensed in a Waring Blendor to yield a thick paste. The latter was passed through a French pressure cell, and the extract was then centrifuged at 2°C. The yield of soluble protein was 40 mg per g of cells, wet weight. The extract was diluted with the above buffer to give a protein concentration of 10 mg per ml, and was then fractionated with solid ammonium sulfate at 2°C, the pH being maintained at 7.3 with NH3OH. The precipitate obtained between 30 and 50% of saturation (which contained half the original soluble protein) was collected by centrifugation, dissolved in a small volume of the above buffer, and dialyzed for 2 hours at 2°C against 0.02 M potassium dimethylglutarate, pH 7.3, containing 0.5 mM each of EDTA and β-mercaptoethanol. The dialysis bag contained several glass beads and was agitated by end over end rotation. This preparation could be stored for 1 week at −15°C.

*Neurospora* homoserine kinase was inactivated by more prolonged dialysis but could be desalted by passage over Sephadex G-25. Activity was assayed in two ways. The first measured the rate of threonine formation (10) in the presence of added excess threonine synthetase according to Reaction 3 (sum of Reactions 1 + 2).

\[ \text{L-Homoserine + ATP} \rightarrow \text{L-threonine + ADP} + \text{P}_i \quad (3) \]

The second, based on the method of Wormser and Pardee (9), measured the rate of formation of ninhydrin-positive material which could be eluted with water from a small Dowex 50 (H+)
column. Although the latter method was more convenient, high voltage electrophoresis of these eluates indicated that occasionally there were ninhydrin-positive products other than phosphohomoserine present. To correct for this, three control incubations were run, with enzyme, ATP, and homoserine omitted. Net ninhydrin values were then corrected for these blanks. A unit of activity was defined as that amount of enzyme catalyzing the formation of 1 μmole of phosphohomoserine in 1 min.

Chemical Preparations and Materials—O-Phospho-L-homoserine was prepared with yeast homoserine kinase as previously described (10) with the following changes. For avoidance of subsequent precipitation of BaSO₄, the yeast ammonium sulfate fraction was dialyzed overnight against 0.02 M Tris-HCl, pH 7.4, containing 1 mM each of EDTA and reduced glutathione. Some further purification was obtained by descending an inactive protein precipitate which formed after freezing and thawing of the dialyzed fraction. After the O-P-homoserine was isolated from the enzymatic reaction mixture as the barium salt, it was purified by passage over Dowex 1-acetate-X10 (200 to 400 mesh). A linear acetic acid gradient from 0 to 8 N was used. The O-P-homoserine was present in the eluate from 6.2 to 7 N. The purified product was recovered as a faintly yellow, amorphous powder by lyophilization. It was homogeneous by paper chromatography and at least 95% pure by both total phosphate content and by assay with threonine synthetase. On the basis of the ATP initially added, the yield was 30% of theoretical (560 mg). A portion was crystallized from aqueous ethanol, m. p. 178°, with decomposition, in accord with previous results (16).

O-Succinyl-DL-homoserine and N-succinyl-DL-homoserine were prepared as previously described (4). D-Homoserine was a gift from Dr. M. D. Armstrong, L-homoserine was purchased from Calbiochem, nucleoside triphosphates from Pabst, and crystalline muscle lactate and yeast alcohol dehydrogenases from Boehringer.

Analytical Procedures—Oak Ridge 32P-labeled orthophosphate was purified by autochlorination overnight at 120° in 1 N HCl, followed by heating for 20 min at 100° in 1 N NaOH. The solution was then neutralized at once and kept frozen. The alkali treatment reduced the unextractable 32P (17) from 0.5 to 0.015% of the total. In the exchange experiment, 32P1 was separated from O-phosphohomoserine by extraction into isobutyl alcohol as the phosphomolybdate complex (17).

The over-all reversibility of Reaction 2 was studied by incubating labeled threonine with threonine synthetase as follows. The reaction mixture was incubated for 60 min at 30° in 1-ml final volume containing: uniformly labeled L-threonine, 3.5 μmoles (5 μC); O-P-homoserine, 8 μmoles; potassium phosphate, 4 μmoles; step 1B threonine synthetase, 0.2 unit. The pH was maintained at 7.3. Two control mixtures without O-P-homoserine contained 0 and 10 μmoles of potassium phosphate, respectively. Aliquots of deproteinized reaction mixtures were chromatographed in solvent mixtures which separate threonine, O-P-homoserine, homoserine, and α-amino butyric acid (13). Carrier α-ketobutyrate and dinitrophenylhydrazine were added to other aliquots. Carbon tetrachloride extracts of the latter were then also chromatographed in ethanol-water-ammonia (8:1:1) and 1-butanol-water-diethylamine (100:15:1). The chromatograms were examined by radioautography.

When the threonine synthetase reaction is carried out in tritium-labeled water, or in 3H₂O, solvent hydrogens are incorporated into threonine. Partial degradation of the threonine

<table>
<thead>
<tr>
<th>Material assayed for tritium content</th>
<th>Specific radioactivity (cpm/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Water-periodate reaction vessel</td>
<td>1100</td>
</tr>
<tr>
<td>Water-bisulfite trap after H₂O oxidation</td>
<td>730</td>
</tr>
<tr>
<td>CH₃CO₂⁻ benzyloxythiourea salt</td>
<td>540</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Water-periodate reaction vessel</td>
<td>920</td>
</tr>
<tr>
<td>Water-bisulfite trap after KMnO₄ oxidation</td>
<td>380</td>
</tr>
<tr>
<td>CH₃CO₂⁻ benzyloxythiourea salt</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table I

Exchange of γ hydrogen atoms of threonine with 3H₂O during oxidative degradations to glyoxylate and acetate.
The reaction mixtures contained, in 0.5 ml final volume: potassium dimethylglutarate, pH 7.3, 25 μmoles; MgCl₂, 7.5 μmoles; NaF, 5 μmoles; ATP, 5 μmoles; dl-homoserine, 15 μmoles; and ammonium sulfate step enzyme, 1.4 mg of protein. Incubations were for 60 min at 30°C and were terminated by placing the tubes in a 100°C bath for 5 min. After the protein was centrifuged down, the supernatants were placed onto Dowex 50 (H⁺) columns, 1 ml bed volume, and the H₂O eluates from 0.5 to 2.5 ml were assayed for amino acid with ninhydrin (18).

<table>
<thead>
<tr>
<th>Omission</th>
<th>Ninhydrin-positive material eluted from Dowex 50 (H⁺) μ mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.40</td>
</tr>
<tr>
<td>ATP</td>
<td>0.02</td>
</tr>
<tr>
<td>dl-Homoserine</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Table II**

**Substrate specificities of Neurospora homoserine kinase**

The reaction mixtures contained, in 1 ml final volume: potassium dimethylglutarate, pH 7.3. 50 μmoles; MgCl₂, 15 μmoles; NaF, 10 μmoles; pyridoxal-P, 0.2 μ mole; Step 4B threonine synthetase, 0.06 unit; 30 to 50% saturation ammonium sulfate homoserine kinase fraction, 4 mg of protein; nucleoside triphosphate, as indicated, 8 μ moles; and homoserine derivative, as indicated. Incubations were for 60 min at 30°C and were terminated by placing the tubes in a 100°C bath for 5 min. Aliquots of the supernatant, after centrifugation, were assayed for threonine as described in the text.

<table>
<thead>
<tr>
<th>Homoserine or derivative added</th>
<th>Amount added μ moles</th>
<th>Nucleoside triphosphate μ moles</th>
<th>Threonine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Homoserine</td>
<td>5</td>
<td>ATP 1.02</td>
<td></td>
</tr>
<tr>
<td>D-Homoserine</td>
<td>5</td>
<td>ATP 0.14</td>
<td></td>
</tr>
<tr>
<td>O-Succinyl-dL-homoserine</td>
<td>5</td>
<td>ATP 0.10</td>
<td></td>
</tr>
<tr>
<td>N-Succinyl-dL-homoserine</td>
<td>5</td>
<td>ATP 0.09</td>
<td></td>
</tr>
<tr>
<td>DL-Homoserine</td>
<td>25</td>
<td>UTP 0.88</td>
<td></td>
</tr>
<tr>
<td>DL-Homoserine</td>
<td>25</td>
<td>GTP 0.06</td>
<td></td>
</tr>
<tr>
<td>DL-Homoserine</td>
<td>25</td>
<td>ITP 0.37</td>
<td></td>
</tr>
<tr>
<td>DL-Homoserine</td>
<td>25</td>
<td>CTP 0.31</td>
<td></td>
</tr>
</tbody>
</table>

The results in Table I show that the acetate obtained by this procedure (Experiment 1) had not undergone any significant exchange with solvent hydrogen, in contrast to the extensive exchange (Experiment 1) in the peroxide procedure (13). Various samples of enzymatically formed threonine-3H and -2H (13) were then degraded in unlabeled solvent in the same manner described above. In the case of threonine formed in -2H₂O, 30 mg of the crystalline sample obtained previously from Dowex 50 (13) were mixed with 150 μ moles of additional carrier. S-Benzylisothiouronium acetate, 30 mg, was isolated, m.p. 128-136°C; after one recrystallization, 18 mg were isolated, m.p. 129-134°C. In this experiment, glyoxylate was isolated again for deuterium analysis.

**RESULTS**

As previously reported (1), E. coli M-309-1 was found to grow aerobically in the presence of succinate, or alternatively, in a mixture of methionine, lysine, diaminopimelic acid, and threonine (Fig. 1). Maximal growth required a higher concentration of succinate than of the amino acids. However, contrary to previous reports, the single omission of threonine from the amino acid mixture had no effect on the rate (doubling time, 40 min) or extent of growth. No growth was observed after the single omission of either methionine or lysine (Fig. 1). Single omissions of diaminopimelic acid resulted in a small increase in turbidity (3 to 5 Klett units) followed by cell lysis and clarification of the medium.

Similar results were obtained with solid growth media, except that in this case diaminopimelic was not apparently required and the mutant responded to lysine plus methionine. In neither case was growth affected by the addition of threonine or homoserine.

Table II shows the evidence for the presence of a homoserine kinase in wild type Neurospora. Formation of O-phosphohomoserine was dependent on the addition of ATP, dl-homoserine, and the 30 to 50% ammonium sulfate fraction. The reaction product was identified as O-phosphohomoserine in the following ways. First, it migrated identically with authentic O-P-homoserine in high voltage electrophoresis, with pyridine-acetate buffer, pH 3.5, and like O-P-homoserine, gave positive ninhydrin and phosphate spot tests. When dl-homoserine-2,4C was used, the only new radioactive spot corresponded to O-P-homoserine. When excess purified threonine synthetase and pyridoxal phosphate were added to the above incubation mixture, threonine was formed. With dl-homoserine 2,4C as substrate, label appeared in the threonine. Threonine formation, too, was dependent on ATP, homoserine, and the ammonium sulfate fraction protein.

**Table IV**

**Stoichiometry of threonine synthetase reaction**

The reaction mixture contained, in 6 ml final volume: glycylglycine, pH 7.3, 300 μ moles; O-P-homoserine eluted from Dowex 1-acetate, 11.3 μ moles; Step 4B threonine synthetase, 0.4 unit. The mixture was incubated at 30°C, and aliquots were deproteinized for assays at successive short intervals until the reaction came to a stop.

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>Total phosphate</th>
<th>Inorganic phosphate</th>
<th>Threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>11.9</td>
<td>10.6</td>
<td>10.6</td>
</tr>
</tbody>
</table>

The mixture was incubated at 30°C, and aliquots were deproteinized for assays at successive short intervals until the reaction came to a stop.
The reaction rate with the ninhydrin assay was proportional to protein concentration from 1 to 7 mg per ml. The specific activity of the 30 to 50% ammonium sulfate fraction was 0.004 with the ninhydrin assay and 0.005 with the coupled assay. Although this latter assay was not strictly proportional to protein concentration, it was more specific for homoserine kinase since O-P-homoserine is the only known substrate for threonine synthetase.

Inability to detect the kinase in crude extracts (10) was not caused by exceptionally low activity but rather by destruction of substrates and products through side reactions. Equal amounts of crude extract protein decomposed threonine as much as four times faster than the rate at which it was formed from homoserine by the ammonium sulfate fraction. Similarly, if the reaction mixture used in Table II was incubated longer than 1 hour, there was a decline in measureable O-3-homoserine and after 2 hours, none was detectable.

Table III indicates that *Neurospora* homoserine kinase was specific for L-homoserine. Other nucleoside triphosphates were less effective than ATP in the order ATP > UTP > GTP > ITP = CTP.

The rest of the results are concerned with some functional properties of purified *Neurospora* threonine synthetase and the mechanism of Reaction 2. Table IV shows the stoichiometric formation of phosphate and threonine from O-P-homoserine which had been eluted from Dowex 1-acetate. A plot of threonine synthetase $V_{max}$ against pH (Fig. 2) shows an asymmetrical curve with a pH optimum of 7.3. The $K_m$ for O-P-homoserine was found to be $1.3 \times 10^{-4} \text{M}$, by use of the integrated form of the Michaelis-Menten equation (Fig. 3). The standard Lineweaver-Burk plot was unsatisfactory because it was difficult to measure small amounts of threonine accurately. The purified threonine synthetase does not, in the usual range of enzyme concentration, form threonine from the succinic ester or amide of homoserine, nor from any of several related substrates tested (10). The free energy change for transformation of a primary alcohol phosphate ester into a secondary alcohol + phosphate is not known. A sensitive test for any measurable reversibility of the threonine synthetase reaction gave negative results. When enzyme was incubated with threonine-14C and phosphate with or without a pool of O-P-homoserine, no radioactivity could be detected in the latter. Threonine also did not yield any radioactive homoserine or a-ketobutyrate.

This result did not, however, rule out the possibility of enzymatic exchange of phosphate into O-P-homoserine. Enzyme was, therefore, incubated with O-P-homoserine and various concentrations of phosphate, with a constant high amount of 32P, for brief periods so that most of the substrate remained unreacted. No labeling of O-P-homoserine was observed, under conditions in which incorporation of about 1/2000 of the added phosphate could have been detected (17).

Previous results had shown that during the enzymatic synthesis of threonine in $^3$H$_2$O, 2 solvent hydrogen atoms were incorporated into it (13). Partial degradation showed that 1 deuterium residue was in the $\alpha$, and one in the $\beta$ + the 3$\gamma$ hydrogen fractions. A complete degradation (Table V) has now established that the second deuterium is in the $\gamma$ position, since it was retained in the acetate formed by oxidation of acet-aldehyde. The results of deuterium analysis for glyoxylate were identical with those obtained previously (13), and the sum
of the deuterium present in glyoxylate and acetate exactly equaled that of the original threonine. The small excess of deuterium in acetaldehyde over that in acetate is within analytical error, and it can be concluded that no solvent hydrogen is incorporated into the beta position of threonine.

DISCUSSION

These results have removed the remaining objections to the view that threonine biosynthesis takes place by Reactions 1 and 2 in Neurospora and E. coli, as well as in yeast. The possibility that a succinylated intermediate might be involved in the former pathways had been suggested by a report that an E. coli mutant blocked in aerobic succinate formation would alternately respond either to succinate, or to a mixture of methionine, lysine, diaminopimelate, and threonine. However, we have found that the mutant responds to the first three amino acids alone, and does not require threonine. Moreover, the succinic ester and amide derivatives of homoserine cannot replace L-homoserine for Reaction 1, or O-phospho-L-homoserine for Reaction 2 in Neurospora. Finally, the existence of Reaction 1 has been definitely established in Neurospora. Thus, the bacteria and higher fungi share a common path for threonine biosynthesis, although they have evolved different synthetic routes for lysine and methionine.

A pyridoxal phosphate enzyme purified from Neurospora, which catalyzes formation of threonine and inorganic orthophosphate from O-phosphohomoserine (gamma, beta isomerization), has been shown to have a pH optimum of 7.3 and a K_m of 1.3 x 10^{-4}. The reaction stoichiometry has been established with a crystalline preparation of 0-phosphohomoserine. Reversibility could not be shown with threonine-14C, and the enzyme does not catalyze gamma replacement (exchange of orthophosphate into O-phosphohomoserine). The positions in threonine into which labeled solvent hydrogens are incorporated during the reaction has been determined.

Acknowledgment—We are indebted to Dr. B. D. Davis for the culture of E. coli M 309-1 and for helpful discussions of its nutritional characteristics.

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