THE CHEMISTRY OF MOLD TISSUE

XI. ISOLATION OF LEUCINE AND ISOLEUCINE FROM ASPERGILLUS SYDOWI*

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In view of the rather high percentage of nitrogen in molds it is surprising to note the scarcity of reports concerning their amino acid content. Few amino acids have been reported, and still fewer have been isolated and characterized by analysis and by the formation and examination of suitable derivatives. Leucine, both free (1) and combined (2), has been reported in Aspergillus niger, but no report of the presence of isoleucine in molds has been found.

By continuous extraction of the dry, defatted mycelium of Aspergillus sydowi with acetone for several weeks we have obtained a mixture of amino acids from which leucine and isoleucine have been isolated in considerable quantities and identified. It would thus appear that at least these two amino acids occur free in the mycelium, or that they are very loosely combined and are split off during drying and extraction of the material.

In the separation and purification of a mixture of amino acids whose properties are so nearly alike as those of leucine and isoleucine considerable losses are inevitable. For this reason, the quantities isolated represent the minimum amounts present; the actual amounts present are undoubtedly much greater. Of the defatted mold, 0.39 per cent was isolated as leucine, and 0.87 per cent as isoleucine. However, if all the amino nitrogen of the extract is assumed to be due to leucine and isoleucine, these two amino acids were obtained to the extent of 2.3 per cent of the defatted mold.

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EXPERIMENTAL

Extraction of Mycelium—The mold was grown on glucose-inorganic salts medium in large sterilizer incubators as previously described (3). 900 gm. of dried, ground, defatted 1 mycelium were placed in a large Soxhlet type extractor, and extracted continuously for several weeks with acetone. Large amounts of white, crystalline material separated out in the boiling flask beginning about the 1st day. From time to time as the material accumulated, it was filtered off from the acetone, and the extraction continued with fresh acetone. In a pilot run, with 110 gm. of defatted mold, 11.3 gm. of material were obtained after 18 days. Further extraction for 6 days yielded only 0.4 gm. of material, but the mold still contained amino acids, as shown by the ninhydrin reaction. With the large extractor, which did not siphon as often as the smaller one, 69 gm. were obtained after 3 weeks. Further extraction yielded small quantities of material.

Fractionation of Extract—The 69 gm. of solids were warmed with water and filtered from insoluble matter amounting to 5 gm. The water solution contained 3.00 gm. of N (Kjeldahl) and 2.19 gm. of α-amino N (Van Slyke). To this solution was added the water solution of the extract from the pilot run mentioned above. The total water solution then contained 3.36 gm. of N and 2.46 gm. of α-amino N.

To remove purines and similar compounds that may have been present, the solution was concentrated under reduced pressure to 400 cc., and was then treated with a solution of mercuric chloride. A small amount of light yellow precipitate formed and was filtered off. About 6 per cent of the total nitrogen was precipitated by this treatment. In order to separate the amino acids from the large amounts of mannitol 2 and other non-nitrogenous compounds, the solution was now treated with sodium

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1 The mold was defatted by extraction with alcohol-ether (1:1) and was found to contain 12 per cent crude lipids.

2 The presence of mannitol was proved by evaporating to dryness some of the original water solution of the extract from a separate sample of the same batch of mold, decolorizing with norit, and recrystallizing twice from alcohol. Typical mannitol crystals, melting at 165°, were obtained. The acetate was prepared and was found to melt at 120°. The melting points of mannitol and its acetate are 166° and 119° respectively.
carbonate and mercuric acetate (Neuberg's reagent), and alcohol was added to a concentration of 25 per cent. The resulting precipitate was filtered off and decomposed with H₂S.

About 29 per cent (980 mg.) of the total nitrogen was not precipitated by Neuberg's reagent. None of this nitrogen reacted by the Van Slyke method. After concentration of the filtrate, a second treatment with sodium carbonate and mercuric acetate failed to precipitate any of this nitrogen. The mercury was removed from this filtrate, and to the resulting filtrate was added an equal volume of alcohol. No nitrogenous matter was precipitated by this procedure. As yet no nitrogen compounds have been isolated from the Neuberg filtrate.

Mercuric Chloride Precipitate—The precipitate obtained with mercuric chloride was decomposed with H₂S, filtered, and after removal of the excess H₂S, the filtrate was treated with ammoniacal silver nitrate. In this way the material was divided into two fractions, neither of which gave a positive ninhydrin test. No compounds have been identified in either fraction.

The filtrate from the decomposed Neuberg precipitate was evaporated to about 350 cc. under reduced pressure at 40°. This solution contained 2.30 gm. of N (Kjeldahl) and 2.20 gm. of N by the Van Slyke method.

Isolation of Leucine—The solution was boiled up with an excess of freshly precipitated copper hydroxide, and filtered while hot. The residue was entirely inorganic. The filtrate was evaporated to dryness, triturated with cold water, and washed with cold water until the washings were nearly colorless. About 1200 cc. of water were used. The light blue residue was dried in vacuo.

(C₅H₁₁O₂N)₂Cu. Calculated, N 8.66; found, N 8.66

The copper salt weighed 4.84 gm., which corresponds to 3.88 gm. of leucine. This represents 0.39 per cent of the defatted mold. The copper salt was decomposed with H₂S and the filtrate concentrated at 40° under reduced pressure until crystallization began. Alcohol was added, and the crystals were filtered off and dried; 2.6 gm. were obtained. In water solution [α]D = -10.3° (calculated on the basis of the weight of the solution taken, not its volume).

C₄H₇NO₂. Calculated, N 10.7; found, N 10.6
The molecular weight by Linderstrøm-Lang titration with HCl (4) was 133, and by alcoholic NaOH titration to the thymolphthalein end-point was 130. Leucine has a molecular weight of 131. The copper salt was prepared and dried in vacuo.

\[(C_4H_{11}O_2N)_2Cu. \text{ Calculated, Cu 19.64; found, Cu 19.61}\]

0.1486 gm. was oxidized with 1 mole of chloramine T, and the aldehyde so formed was distilled into a solution of \(p\)-nitrophenylhydrazine in dilute acetic acid. The resulting hydrazone was recrystallized twice from dilute alcohol. Melting point 108°. The \(p\)-nitrophenylhydrazone of isovaleraldehyde melts at 109° (5).

\textit{Isolation of Isoleucine—}The dark blue filtrate from leucine copper salt was evaporated to dryness and thoroughly dried in vacuo. The salts were finely pulverized, triturated with absolute methyl alcohol, and filtered by suction. This treatment was repeated until the filtrate was only pale blue. About 2 liters of methyl alcohol were used. The residue weighed 3.7 gm.

The methyl alcohol was distilled, the residue was dissolved in water, decomposed with H₂S, filtered, concentrated at 40° under reduced pressure until crystallization began, treated with alcohol, and placed in the ice box until crystallization was complete. The crystals weighed 3.3 gm. By concentrating the filtrate and adding acetone 4.0 gm. more were obtained. In water solution \([\alpha]_D = +10^\circ\). Values in the literature range from +9.6° to 11.3° (6). Abderhalden and Zeisset (7) have recently reported 10.8°.

\[\text{C}_4\text{H}_{13}\text{O}_2\text{N}. \text{ Calculated, N 10.7; found, N 10.6}\]

The molecular weight by Linderstrøm-Lang titration was 128 (theoretical, 131). 0.1414 gm. was oxidized with chloramine T, and the \(p\)-nitrophenylhydrazone of the aldehyde was prepared. After recrystallization from dilute alcohol this derivative melted at 112–113°. Neuberg and Peterson (8) have reported 112–113° for active methylethylacetaldehyde-\(p\)-nitrophenylhydrazone. The hydrazone separated first as an oil, but crystallized after standing in the ice box for some time.

The phenyl isocyanate derivative was prepared in the usual way from 0.149 gm. of the amino acid by the action of phenyl isocyanate and NaOH at 0°. After crystallization from water it
melted at 120°. Abderhalden and Zcissect (7) found that the d-isoleucine phenyl isocyanate derivative melted at 121°.

The p-toluenesulfonate of 0.26 gm. was prepared according to the procedure used by Fischer and Lipschitz (9). It was recrystallized from water. Beautiful needles were obtained which melted at 122.5° (124° corrected). The melting point was not changed by further recrystallization.

\[ \text{C}_{12}\text{H}_{18}\text{NO}_{3}\text{S}. \] Calculated, N 4.91; found, N 4.83

We have not found any reference to this compound in the literature.

**Methyl Alcohol-Insoluble Copper Salt**—When treated with water, the methyl alcohol-insoluble residue (3.7 gm.) did not all dissolve. A green, amorphous precipitate (probably copper hydroxide or carbonate) weighing 0.66 gm. was filtered off and discarded. The copper was removed from the filtrate with \( \text{H}_2\text{S} \), the filtrate was concentrated under reduced pressure, and the amino acid precipitated with acetone; 0.8 gm. was obtained. This material was recrystallized from alcohol.

\[ \text{C}_{6}\text{H}_{14}\text{O}_{2}\text{N}. \] Calculated, N 10.7; found, N 10.6

The p-toluenesulfonate melted at 120–122° and contained 4.7 per cent nitrogen. The \( p \)-nitrophenylhydrazone formed from the aldehyde produced by oxidizing the amino acid with chloramine T melted at 109–110° and showed the same crystalline form and behavior as the \( p \)-nitrophenylhydrazone obtained from isoleucine. A mixture of the two hydrazones melted at 110°. These facts indicate that the methyl alcohol-insoluble copper salts consisted of the copper salt of isoleucine occluded by copper hydroxide or carbonate. Thus, a total of 8.7 gm. of isoleucine was isolated from the water-soluble copper salts. This corresponds to 0.87 per cent of the defatted mold.

**SUMMARY**

Leucine and isoleucine have been isolated in considerable quantities by extracting the dried, defatted mycelium of *Aspergillus sydowi* with acetone. From this method of extraction it was concluded that these amino acids must be present either free in the mycelium, or held in rather loose combination. The amino
acids were characterized by analysis and by the preparation and examination of two or more derivatives. The p-toluenesulfonate of isoleucine has been prepared apparently for the first time.

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