BIOSYNTHESIS OF PENICILLINS

II. UTILIZATION OF DEUTEROPHENYLACETYL-N\(^{16}\)-DL-VALINE IN PENICILLIN BIOSYNTHESIS*

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In Paper I in this series (2) the stimulation of penicillin production by certain compounds containing the phenylacetyl group was reported. It was realized that a stimulation of yield of this biological product following addition of a given substance to the medium may result from any one of several possible metabolic mechanisms. The stimulating substance may act as a vitamin or growth promoter, as a building block to be incorporated into the organism, or it may be required to satisfy any one of several other types of metabolic requirements. Thus, the early observations of increased yields were subject to varied interpretations, and there was need for proof of the direct utilization of the substances in penicillin biosynthesis.

To provide such proof the preparation and use as a precursor of deutero-phenylacetyl-N\(^{15}\)-valine was proposed (L, 13, 17).\(^1\) Considerations in the choice of this particular compound included the knowledge that considerable specificity was exhibited by both acyl and amide portions of the molecule, and the fact that the amide portion of the molecule contained a carbon skeleton similar to that in penicillamine. The deutero-phenylacetyl-N\(^{15}\)-valine was prepared in the Lilly laboratories (L, 23, 8) from deutero-phenyl-\(^{15}\)-valine.

* The work reported here in detail is briefly discussed in the monograph on penicillin (1).

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\(^1\) The references give the number and page of reports made to the Office of Scientific Research and Development.
acetic acid and N-valine (L, 22, 1). The cultural work and preliminary purification were conducted in the Abbott laboratories and final purification and crystallization of the penicillin was carried out in the Upjohn laboratories (U, 22, 8). The isotopic determinations were performed by Dr. Rittenberg. By means of the deuterium analysis, it was shown that 92.5 per cent of the penicillin obtained was derived from the precursor. In sharp contrast the N content of the isolated penicillin was only 2.69 per cent of the value expected if the phenylacetylvaline had supplied 1 nitrogen atom to the penicillin. These results demonstrated that the phenylacetyl moiety appeared in the penicillin formed, but that the valine portion of the precursor was not directly utilized in penicillin formation. The role of the amide portion of such precursors thus remains undefined.

EXPERIMENTAL

N-valine (L, 22, 1)—N-valine was prepared by the method of Knoop (3) as adapted for isotopic synthesis by Schoenheimer and Ratner (4).

Ammonium nitrate prepared by the Eastman Kodak Company, containing about 32 per cent of N in the ammonium moiety, was used. Ammonia was generated from 4.0 gm. (0.05 mole) of this ammonium nitrate and was absorbed in 35 ml. of ethanol containing 4 gm. of palladium black and cooled in a dry ice bath. After absorption of the ammonia was complete, 2.90 gm. (0.025 mole) of α-oxoisovaleric acid (5) in 35 ml. of water were added.

The mixture of isotopic ammonia and α-oxoisovaleric acid was hydrogenated under a pressure of 2 atmospheres. Almost the theoretical amount of hydrogen was absorbed. The excess N was recovered as directed by Schoenheimer and Ratner (4).

The contents of the hydrogenation vessel were treated with hot water, filtered, and evaporated to dryness in vacuo. Ethanol was added and the evaporation was repeated. The resulting white, microcrystalline powder was suspended in ethanol, collected on a filter, and washed with ether. After drying, the product weighed 1.26 gm. (43 per cent yield).

Analysis C H N O. Calculated, N 12.19; found, N 12.00

Deuterophenylacetyl-N-valine (L, 23, 8)—A mixture of 2.90 gm. (0.021 mole) of deuterophenylactic acid (in which 41 per cent of the hydrogen atoms in the benzene nucleus had been replaced with deuterium) and 5 ml. of pure thionyl chloride was allowed to stand at room temperature for 18 hours. The excess thionyl chloride, the hydrogen chloride, and the sulfur di-

2 Sample furnished by Dr. David Rittenberg, College of Physicians and Surgeons, Columbia University.

3 The calculated value is based on 32 per cent N.
oxide were evaporated at room temperature under a pressure of 5 to 10 mm. The phenylacetyl chloride was dissolved in 20 ml. of dry benzene. This benzene solution was added dropwise over a period of 10 minutes to a rapidly stirred solution of 2.60 gm. (0.022 mole) of the N\textsuperscript{15}-DL-valine in 25 ml. of 2.6 N sodium hydroxide solution, cooled in an ice-salt bath. After the addition of the phenylacetyl chloride was complete, the cooling bath was removed, and the stirring was continued for 1 hour at room temperature. The benzene layer was separated and the aqueous layer was washed with 50 ml. of ether. The combined benzene and ether solutions were washed with two 15 ml. portions of water. The total water solution was acidified with hydrochloric acid. After standing, the precipitated oil crystallized. The product was collected, washed with a little water, dried, and then washed with 50 ml. of hot petroleum ether (b.p. 60-68°). The yield of white crystalline solid was 4.14 gm. (84 per cent).

Analysis—C\textsubscript{18}H\textsubscript{17}NO\textsubscript{3}. Calculated,\textsuperscript{4} N 6.00; found, N 5.86

Benzy1penicillin from Deuterophenylacetyl-N\textsuperscript{15}-DL-valine—The isotopic precursor was sent to the Abbott laboratories where it was added at a level of 188 mg. per liter to a corn steep medium inoculated with Penicillium notatum, strain NRRL 1976.

Approximately 5 liters of filtered broth assaying 95 units per ml. were chilled to 5°, acidified to pH 2.0, and extracted with amyl acetate. The amyl acetate solution was extracted with 3 per cent phosphate buffer solution at pH 7.0. The phosphate buffer solution was chilled to 5°, acidified to pH 2.0, and extracted with chloroform. The penicillin in the chloroform solution was converted to sodium salt by stirring with water and adding sodium hydroxide dropwise until the pH reached 7.0. The aqueous solution was dried from the frozen state.

This crude sodium salt was sent to the Upjohn laboratories for the isolation of the crystalline penicillin (U, 22, 8). The sodium penicillin (352,000 units, assaying 234 units per mg.) was further purified by use of an ether-silica-phosphate buffer (pH 6.2) chromatographic column (2). The four bands of activity that were noted accounted for 99 per cent of the units applied to the column. The benzylpenicillin band containing 191,000 units was converted to the sodium salt, yielding 175,000 units of material which assayed 790 units per mg. It seemed probable that the yield of crystalline product from this material would be insufficient for adequate isotopic analysis. Hence, 100 mg. (an equal amount in terms of units of antibiotic activity) of analytically pure sodium benzylpenicillin were added. The combined material (321 mg., assaying 1150 units per mg.) was further puri-

\textsuperscript{4} The content of D and N\textsuperscript{15} being taken into account.
fied over a chloroform-silica-phosphate buffer (pH 6.2) column. All the recovered activity, representing 91 per cent of the applied penicillin, was in a single band. This material was converted to the sodium salt and was dried from the frozen state, yielding 230,000 units assaying 1590 units per mg. The product (145 mg.) was treated with dry acetone, in which it first dissolved, then reprecipitated, yielding 137 mg. of powder. The powder was dissolved in 0.65 ml. of 90 per cent acetone. Addition of 1.1 ml. of dry acetone to the solution yielded a first crop of crystals weighing 70 mg. This material, which consisted of typical benzylpenicillin platelets, was used for isotopic analysis.

Analysis—C₁₅H₁₇N₂O₁₅Na. Calculated. C 53.92, H 4.81, N 7.83
Found. " 54.02, " 5.10, " 8.02

An additional 38.4 mg. of crystalline material were obtained from the mother liquors of the first crystallization. Thus a total of 108.4 mg. of crystallized material was recovered from the acetone-insoluble residue of 137 mg.

Deuterium and N¹⁵ determinations on the penicillin and on a sample of the precursor were performed by Dr. David Rittenberg. The original phenylacetic acid used in the preparation of the precursor contained 26.7 atom per cent excess deuterium (L 28, 3). The phenylacetylvaline contained 32.7 atom per cent excess N¹⁵. The crystalline sodium benzylpenicillin isolated (which had been diluted during processing with an equal amount of non-isotopic benzylpenicillin) contained 5.81 atom per cent excess deuterium and 0.220 atom per cent excess N¹⁵.

Calculation shows that 92.5 per cent of the benzylpenicillin isolated was derived from the precursor as indicated by the deuterium analysis.

\[
\frac{0.0581 \times 17 \times 2}{0.267 \times 8} = 0.925 \text{ atoms of D in the isolated penicillin}
\]
\[
\frac{1.975}{2.136} = 0.925
\]

In sharp contrast, only 2.69 per cent of the penicillin isolated was derived from the part of the precursor containing N¹⁵.

\[
\frac{0.0022 \times 2 \times 2}{0.327 \times 1} = 0.0269 \text{ atom of N}^{15} \text{ per molecule of isolated penicillin}
\]
\[
\frac{0.0088}{0.327} = 0.0269
\]

**SUMMARY**

The preparation of deuterophenylacetyl-N¹⁵-DL-valine is described. Benzylpenicillin was isolated following use of this as a precursor. Deuterium analyses demonstrated that the phenylacetyl portion of the precursor
was incorporated directly into the penicillin. In contrast, very little $N^{15}$
was found in the penicillin product. Therefore, the rôle of the amide por-
tion is still unknown.

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