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Incorporation of Double-Labeled L-Cystine and DL-Valine in Penicillin

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L-[3,3'-^{3}H]cystine was incorporated into penicillin with retention of one tritium. This result can be explained by β-lactam formation through ring closure between C3 of cysteine and NH of valine. No radioactivity of DL-[2,3-^{3}H]valine was incorporated into penicillin. The loss of isotope at C2 occurs during the inversion of configuration. The loss of label at C3 is discussed in terms of possible intermediates for the formation of the thiazolidine ring of penicillin.

Studies on the biosynthesis of penicillin have shown that the β-lactam-thiazolidine rings of the antibiotic can be considered as condensation products of cysteine and valine; however, little is known about the biosynthetic pathways leading to the formation of the dual ring system.

Arnstein and Grant (3, 4) demonstrated by incorporation studies with DL-[3-^{14}C]cystine and L-[3-^{14}C]cystine-^{15}N-^{35}S that the intact cystine molecule was used for the biosynthesis of penicillin. They also noted that L-cystine was a better precursor than D-cystine. Arnstein and Crawhall (2) investigated the incorporation of DL-[2-^{3}H]cystine and found that some label was retained in penicillin. The antibiotic was also labeled from DL-[3,3'-^{3}H]cystine. These experiments, however, did not allow final conclusions to be drawn since the tritiated and the ^{14}C-labeled amino acids were not added to the same culture. For this reason we reexamined these results using double-labeled L-[3,3'-^{3}H-U-^{14}C]-cystine.

It has been shown that the carboxy group of penicillin corresponds to the carboxy group of valine (12) and, using ^{13}C-labeled chiral valine, a correlation between the methyl groups of valine and penicillin could be made (8, 10). Evidence has also been given that the intact carbon chain of valine is used for penicillin biosynthesis (1). Since no work has been done on the fate of the hydrogens in positions two and three of valine, we investigated the incorporation of DL-[2-^{3}H]valine and DL-[3-^{3}H]valine into penicillin.

MATERIALS AND METHODS

Radiochemicals. Tritiated water was obtained from I. R. E., Mol, Belgium. L-[3,3'-^{3}H]cystine and L-[U-^{14}C]cystine were products of The Radiochemical Centre, Amersham, England. L-[2,3-^{3}H]valine, L-[4,4'-^{14}C]valine, L-[1-^{14}C]valine, and DL-[1-^{14}C]valine were purchased from Schwarz/Mann, Orangeburg, N.Y.

Preparation of DL-[2-^{3}H]valine. L-Valine (1.17 g) (10 mmol) was refluxed with 20 ml of acetic anhydride and 1 ml of tritiated water (100 mCi) for 2 h. After evaporation to dryness under vacuum, the *N*-acetyl-DL-[2-^{3}H]valine was refluxed with 20 ml of 2 N HCl for 2 h (5). DL-[2-^{3}H]valine was precipitated from ethanol by addition of aniline in a yield of 400 mg (34%) and with $[\alpha]_D = 0°$ (c = 1.0, 5 N HCl). Specific activity was 188.37 μCi/mmol. Part of the DL-[2-^{3}H]valine was recrystallized from water-ethanol; specific activity was 188.30 μCi/mmol.

Preparation of DL-[3-^{3}H]valine. L-[2,3-^{3}H]valine was racemized by boiling with acetic anhydride and acetic acid. The resulting DL-[3-^{3}H]valine, after oxidation to acetone (5), contained 0.46% tritium in position four (methyl groups). Before use, the DL-[3-^{3}H]valine was mixed with DL-[4,4'-^{14}C]valine and recrystallized from water-ethanol.

Culture conditions. *Penicillium chrysogenum* Wis 49-2105 was maintained on a complete medium containing sucrose, 30 g; corn steep liquor, 10 g; Difco Casitone, 2 g; Difco yeast extract, 2 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1 g; $FeSO_4 \cdot 7H_2O$, 10 mg; DL-methionine, 50 mg; agar, 20 g; tapwater, 1,000 ml; pH 6.0. Fermentations were carried out in the medium of Jarvis and Johnson (7). Fifty milliliters of this medium in 300-ml Erlenmeyer flasks were inoculated with 3×10^7 conidia from 6-day-old cultures on complete medium and incubated at 27 C and 300 rpm on a rotary shaker. After 48 h, the suspensions were filtered with suction on sterile Whatman no. 1 filter papers and washed three times with sterile water. The mycelium from each Erlenmeyer was resuspended in 50 ml of Jarvis and Johnson medium and 1 ml of 1.5% sodium phenoxyacetate, pH 7.0; a sterile solution of the radioactive precursor was added. The cultures were further incubated for 24 h at 27 C and 300 rpm.

Microbiologic assays. The penicillin content of the culture broth was assayed by the agar diffusion method with *Sarcina lutea* ATCC 9341 as test organism.

Purification of penicillin. After filtration of the culture, the broth was cooled to 1 to 4 C; after addition of 220 mg of penicillin V (potassium salt) and 10 g of NaCl, and adjustment of the pH to 2.2, the solution was twice extracted with 40 ml of cold *n*-butyl acetate. The combined organic layers were

washed with 10 ml of a saturated NaCl solution and extracted with 8 and 4 ml of 2.5% $NaHCO_3$. The water layer was washed with 10 ml of ether and after adjusting the pH to 6.0 to 6.2; the extracted penicillin was precipitated with 1.50 ml of *N,N'*-dibenzylethylenediamine diacetate (45.6 mg/ml). The *N,N'*-dibenzylethylenediamino penicillin V was twice recrystallied from pyridine-water and once from pyridine and petroleum ether (40 to 60 C)-benzene 2:1.

Distribution of radioactivity. The mycelium was successively extracted with 5 ml of acetone, 10 ml of 75% acetone, and 15 ml of 80% ethanol. Determinations of radioactivity were carried out on the culture filtrate and the extracted mycelium after combustion with a Packard Sample Oxidizer, model 306. For aqueous samples (0.5 to 1.0 ml), 400 to 600 mg of cellulose and 0.2 to 0.4 ml of Combustaid (Packard) was added. Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer, model 3390, with Absolute Activity Analyzer, model 544. Standardization was carried out with [^{14}C]- and [^{3}H]*n*-hexadecane.

RESULTS

Incorporation of L-[U-3,3'-^{3}H-^{14}C]cystine. Two experiments were carried out. In experiment A, 30 μmol of L-cystine containing 5.44 μCi of carbon 14 and 17.50 μCi of tritium (ratio ^{3}H/^{14}C = 3.217) were added to each of five cultures. In experiment B, 60 μmol of L-cystine containing 13.36 μCi of carbon 14 and 47.78 μCi of tritium (ratio ^{3}H/^{14}C = 3.576) were added to each of three cultures. The data in table 1 show that the ^{3}H/^{14}C ratio in penicillin was 45% of the ^{3}H/^{14}C ratio in the precursor amino acid. The loss of one tritium during the incorporation should give labeled penicillin with 50% of the starting ratio ^{3}H/^{14}C of the added L-[3,3'-^{3}H-U-^{14}C]cystine.

Incorporation of DL-[2-^{3}H]valine. In a preliminary study to check whether D-valine was taken up in the mycelium, *P. chrysogenum* was incubated with 50 μmol of L-[1-^{14}C]valine and 50 μmol of DL-[1-^{14}C]valine. The results are given in Table 2 and are the mean of three experiments. The high level of radioactivity in the mycelium and the low activity in the culture filtrate indicated good incorporation of both isomers. It should be noted that there were only minor differences in the isotopic distribution between cultures labeled with L-[1-^{14}C]valine or DL-[1-^{14}C]valine.

Two experiments (addition of 50 and 100 μmol) were carried out to study the loss or retention of label from DL-[2-^{3}H]valine after its incorporation in penicillin. The high tritium content in the culture filtrate and the low incorporation in the mycelium (Table 2) indicated an extensive racemization of the added valine, which resulted in a high level of tritiated water in the culture filtrate. Practically no tritium was incorporated into penicillin (Table 3); the very low level of radioactivity in the penicillin (0.0015%) was most likely due to trace impurities.

Incorporation of DL-[3-^{3}H-4,4'-^{14}C]valine. Four experiments were carried out; each culture was incubated with 60 μmol of DL-valine, containing 12.173 μCi of tritium and 4.044 μCi of carbon 14 (ratio ^{3}H/^{14}C = 3.0103). The ratios ^{3}H/^{14}C of the penicillin formed in the four experiments were 0.05447, 0.05292, 0.04947, and 0.05050, respectively. If only the tritium on carbon 4 of valine (=0.46%) was incorporated, the ratio ^{3}H/^{14}C in penicillin would have been 0.01385. From the very low mean isotope ratio of 0.05184 it could be calculated that the retention of tritium from carbon 3 of valine was approximately 100 times lower than the incorporation of carbon 14. From these experiments it was concluded that no significant amount of tritium from DL-[3-^{3}H]valine was retained in penicillin.

DISCUSSION

Arnstein and Crawhall (2) observed a significant incorporation of label in penicillin after feeding DL-[2-^{3}H]cystine, thereby excluding a 2,3-didehydrocysteine intermediate in the biosynthesis of the antibiotic. They also noted that tritium was retained in penicillin after addition

TABLE 1. *Isotope ratios in penicillin after addition of L-[3,3'-^{3}H-U-^{14}C]cystine to cultures of P. chrysogenum Wis. 49-2105*

Expt	^{3}H/^{14}C starting ratio in L-cystine	Flask no.	^{3}H/^{14}C ratio in penicillin	% of starting ratio
A	3.217	1	1.390	43.21
		2	1.371	42.62
		3	1.428	44.39
		4	1.416	44.02
		5	1.485	46.16
Avg			1.418	44.1
B	3.576	1	1.681	47.00
		2	1.683	47.06
		3	1.642	45.92
Avg			1.669	46.7
Avg A + B				45

TABLE 2. *Isotope distribution in cultures of P. chrysogenum Wis. 49-2105 after incubation with labeled valines*

Added	% Radioactivity in:	
	Culture filtrate	Extracted mycelium
L-[1-^{14}C]valine (50 μmol)	9.7	33.5
DL-[1-^{14}C]valine (50 μmol)	11.2	29.4
DL-[2-^{3}H]valine (50 μmol)	58.4	9.5
DL-[2-^{3}H]valine (100 μmol)	59.5	7.5

TABLE 3. *Incorporation of* DL-[2-^{3}H]*valine in penicillin*

Determination	Expt	
	A	B
DL-Valine (sp act, 181.1 μCi/mmol; added per Erlenmeyer flask, in μmol)	50	100
Penicillin; mg formed	3.34	2.83
Sp act of penicillin, in μCi/mmol	0.037	0.067
% of DL-[2-^{3}H]valine incorporated in penicillin	0.0017	0.0013

of DL-[3,3'-^{3}H]cystine, but they could not estimate the amount incorporated because the tritiated amino acid and DL-[^{14}C]cystine were added to different cultures. The present investigations, using L-[3,3'-^{3}H-U-^{14}C]cystine, showed that 45% of the tritium was incorporated in penicillin. This result is in agreement with a β-lactam-formation by ring closure between carbon 3 of cysteine and the nitrogen of valine, which would cause a 50% loss of isotope from L-(3,3'-^{3}H]cystine. We have no explanation for the difference of 5% between the observed and expected values of retention of label. These data also indicate that the leaving tritium atom is not transferred to another part of the penicillin molecule.

The experiments with L- and DL-[^{14}C]valine show that both products are well taken up in the mycelium. The amount of radioactivity in the mycelium after addition of DL-[2-^{3}H]valine is much lower, and practically no isotope is found in penicillin. During the biosynthesis of penicillin from L-valine, the configuration of the asymmetric carbon is changed from L to D. This epimerization is accompanied by loss of hydrogen at the chiral center. Since it has been shown that the amino group of valine is retained in penicillin (11), 2-oxoisovaleric acid cannot be an intermediate. The possibility of direct incorporation of D-valine into penicillin is ruled out, because in the present experiments no label was incorporated into penicillin after incubation with DL-[2-^{3}H]valine. Because the experiments with L- and DL-[1-^{14}C]valine did not show a great difference in uptake of isotope, and because the radioactivity of the mycelium was much lower with tritium-labeled valine than with [^{14}C]valine, the incorporation of D-valine in penicillin probably occurs after epimerization to L-valine.

Since penicillin has no hydrogen at carbon 2, the loss of tritium from DL-[3-^{3}H]valine might be expected. The absence of label in penicillin also indicates that no tritium migration occurs, e.g., to carbon 3 of penicillin. It has been proposed (6) that in the biosynthesis of penicillin, δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine is transformed to isopenicillin N via an α, β-didehydrovaline intermediate. This would explain the loss of isotope on C2 and C3 of valine and the change of configuration at C3 of penicillin. The stereotopic labeling of either the α or β methyl group of penicillin after feeding ^{13}C-labeled chiral valines (8, 10) can be an indication of such a mechanism. However, since it was discovered that the tripeptide in *Cephalosporium* (9) and also in *Penicillium* (Adriaens et al., unpublished data) contains D-valine, formation of an α, β-didehydrovaline intermediate remains possible but is no longer necessary to explain the D-configuration of the C3 of penicillin.

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