The Biosynthesis of Penicillin

6. A STUDY OF THE MECHANISM OF THE FORMATION OF THE THIAZOLIDINE- β -LACTAM RINGS, USING TRITIUM-LABELLED CYSTINE*

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(Received 26 February 1957)

Previous work has established that part of the penicillin molecule (I), consisting of the side-chain nitrogen atom, N₈, the β -lactam carbon atoms, C₍₅₎₋₍₇₎, and the sulphur atom, is specifically derived from L-cysteine (Arnstein & Grant, 1954*b*), whereas the carbon chain of the penicillamine moiety is derived from valine (Stevens, Vohra & DeLong, 1954; Arnstein & Grant, 1954*a*). It is not yet certain, however, whether L- or D-valine is specifically used for penicillin biosynthesis (Arnstein & Clubb, 1957).

The mechanism by which cysteine and valine give rise to the fused thiazolidine- β -lactam ring structure is still obscure. Phenylacetyl-L-cysteine (II) (Arnstein, Clubb & Grant, 1954), β -hydroxyvaline (III) (Arnstein & Clubb, 1957) and $\beta\beta$ -dimethyl-lanthionine (IV) (Stevens, Vohra, Moore & DeLong, 1954) appear to be excluded as intermediates (for a recent review, see Arnstein & Grant, 1956) and the initial reaction, therefore, may be formation of cysteinylvaline and thus of the β -lactam-peptide bond.

The present experiments deal specifically with

* Part 5: Arnstein & Clubb (1957).

the biosynthesis of the carbon-nitrogen bond common to the thiazolidine and β -lactam rings of penicillin; although this problem is, of course, connected to some extent with the nature of the initial reaction between cysteine and valine, which has not yet been fully elucidated, it has been possible to study certain of its aspects independently. In particular, two mechanisms involving possible dehydropeptide derivatives of cysteine and valine have been considered. Thus the β -lactam ring could be formed by addition of the hydrogen atom of the peptide bond to an $\alpha\beta$ -dehydrocysteine derivative (Fig. 1, A) or by addition of one of the

R = C₆H₅·CH₂· in benzylpenicillin. The broken lines indicate the metabolic origin of the thiazolidine- β -lactam ring structure from cysteine and valine.

Fig. 1. Possible mechanisms of β -lactam formation.

β -hydrogen atoms of cysteine to an imino derivative of valine (Fig. 1, *B*). These alternatives have been investigated with cystine containing tritium in the α - or β -position. Reaction *A* would involve loss of isotopic hydrogen from the α -position of cystine, whereas mechanism *B* would lead to incorporation of labelled hydrogen from the β -position of cysteine into the α -position of the penicillamine moiety of penicillin. The results of the present work show, however, that the α -hydrogen atom of cystine is stable and no migration of the β -hydrogen atoms of cysteine during penicillin biosynthesis was detected.

A preliminary communication of this work was given at the 357th meeting of the Biochemical Society (Arnstein & Crawhall, 1957).

EXPERIMENTAL

Fermentations

Penicillium chrysogenum WIS 51-20 was used in all experiments and full details of the fermentation apparatus, culture medium and estimation of penicillin by bioassay have been given in a previous paper (Arnstein & Grant 1954*b*). Details of individual experiments are summarized in Table 1.

The experiment with washed mycelium (see Table 2) was carried out essentially as described by Halliday & Arnstein (1956).

Isolation and degradation methods

Penicillin. Penicillin was extracted from the acidified culture medium and concentrated by successive extractions between buffer and ether (Smith & Hockenhull, 1952). Finally the *N*-ethylpiperidine salt was isolated and re-crystallized to constant radioactivity (see Arnstein & Grant, 1954*b*). Degradation of the *N*-ethylpiperidine salt was carried out as described by Arnstein & Clubb (1957), penicillamine being isolated as the isopropylidene derivative and penilloaldehyde as the 2:4-dinitrophenylhydrazone. The degradation of penilloaldehyde followed the procedure described earlier (Arnstein & Grant, 1954*a*), except that glycine was degraded to formaldehyde and CO₂ without prior isolation.

Amino acids from mycelium. In Expt. no. 1*a*, the mycelium was extracted with ethanol-ether (3:1, v/v), 5% (w/w) trichloroacetic acid, water, ethanol and ether, dried (dry wt., 5.9 g.) and hydrolysed with 6*N*-HCl (cf. Arnstein & Grant, 1954*b*). The hydrolysate was treated with Br₂ to oxidize cystine to cysteic acid, the acidic amino acids were separated by chromatography on Deacidite FF resin (Permutit Co. Ltd., London) in the acetate form and the non-acidic

fraction from this column was further chromatographed on Zeo-Karb 225 resin (Permutit Co. Ltd., London), with essentially the same procedure as described by Hirs, Moore & Stein (1954) for the analogous Dowex 1 and Dowex 50 resins.

Cysteic acid was isolated as the Ba salt by evaporating the appropriate fractions from the Deacidite FF column to dryness, and adding dilute Ba(OH)₂ soln. till the pH was 6, followed by increasing quantities of ethanol. The following fractions were thus obtained, the first being precipitated by an equal volume of ethanol: (1) 19.6 mg., 107 counts/min.; (2) 10.1 mg., 265 counts/min.; (3) 10.6 mg., 291 counts/min. Fractions nos. 2 and 3 were combined and Ba²⁺ ions removed as BaSO₄ by adding dilute H₂SO₄. The residual aqueous solution was evaporated almost to dryness and the cysteic acid was crystallized by adding ethanol. The radioactivity of this material (384 counts/min.) was in good agreement with the average radioactivity of the Ba salt of fractions no. 2 and 3, when due allowance is made for the difference in molecular wt.

Glutamic acid and aspartic acid were isolated by evaporating the relevant fractions to dryness and crystallizing the residue from aqueous ethanol.

The remaining amino acids in Table 3 were isolated from the appropriate fractions of the Zeo-Karb 225 column, the hydrochlorides being converted into the free amino acids by means of a small column of Zeo-Karb 215 resin (Arnstein & Stanković, 1956). The amino acids were crystallized from aqueous ethanol.

Radioactivity estimations

Radioactive ¹⁴C. Samples of 'infinite thickness' were mounted on 1 cm.² polythene disks (Popják, 1950) and counted with a thin end-window Geiger-Müller tube.

Tritium. Compounds described in the section on the synthesis of labelled cystine were counted at St Bartholomew's Hospital Medical College on the windowless counter described by Banks, Blow & Francis (1956), which has an efficiency of about 0.3% when 10 mg. samples are counted after dilution with an equal wt. of graphite. All other samples were counted at the National Institute for Medical Research with the windowless flow-type Geiger counter described by Garrow & Piper (1955), which was found to have a similar efficiency. Samples were mixed with an equal wt. of graphite (Banks, Crawhall & Smyth, 1956) and mounted on a 1 cm.² stainless-steel disk. The consistency of tritium assays on the latter counter was checked by comparing a sample of DL-[α -³H]cystine with a poly[¹⁴C]methyl methacrylate reference standard of specific radioactivity 1 μ C/g., obtained from the Radiochemical Centre, Amersham, Bucks. Over a period of about 4 weeks, the average of ten determinations of the [α -³H]cystine was found to be 426.2 counts/min., the range being 383.8-457.5. The maximum

Table 1. *Experimental details of fermentations with tritium-labelled cystine*

All fermentations contained 1.5 l. of medium.

Expt. no.	Precursor added	Amount of precursor added (mg.) and times of addition after inoculation (hr.) in parentheses	Yield of penicillin (i.u./ml.)
1 <i>a</i>	DL-[α - ³ H]Cystine	84 (1.5), 84 (26), 84 (50), 68 (75), 68 (102)	265 at 114 hr.
1 <i>b</i>	DL-[α - ³ H]Cystine	23 (44), 23 (61), 16.5 (68)	157 at 107 hr.
3	DL-[β - ³ H]Cystine	26.5 (46), 26.5 (70), 26.5 (94)	174 at 110 hr.

DL-[α - ^3H]Cystine. *S*-Benzyl-[α - ^3H]cysteine was dissolved in liquid NH_3 and small pellets of Na were added till there was a persistent blue colour. The colour was just discharged with NH_4Cl and the liquid NH_3 was allowed to evaporate. The residue was dissolved in water (30 ml.) and extracted with ether. The aqueous layer was acidified with HCl, and I_2 in ethanol was added until a persistent brown colour appeared. Excess of I_2 was decomposed with $\text{Na}_2\text{S}_2\text{O}_3$ and the solution was neutralized with dilute NH_3 , when DL-[α - ^3H]cystine (50.9 mg.) separated. A second crop (23.1 mg.) was obtained by adding an equal volume of ethanol to the mother liquors. The total yield was 74.0 mg. (37%).

On the assumption that the starting material contains three exchangeable hydrogen atoms (i.e. two carboxyl hydrogens and one hydrogen on the acetamido nitrogen atom), one of which becomes stable to further exchange with non-isotopic water after decarboxylation, the maximum radioactivity of the labelled cystine could be 3.62×10^5 counts/min. The actual radioactivity of the DL-[α - ^3H]cystine was 1.15×10^5 counts/min., or 32% of the theoretical value.

(b) *Ethyl hydrogen α -acetamido- β -benzylthiomethylmalonate* (VI). To a solution of the diester (V) (1.757 g., 5 m-moles) in ethanol (10 ml.) *N*-NaOH (5 ml.) was added. After 18 hr. at room temperature, the solution was evaporated to dryness *in vacuo* (bath temperature $<45^\circ$). The residue was dissolved in water, extracted with ether and concentrated *in vacuo*. The solution was acidified with 2*N*-HCl, when a white precipitate was obtained which was filtered off, washed with water and dried. Recrystallization from chloroform by adding light petroleum (b.p. 60 – 80°) afforded the *half-ester* (VI), m.p. 126 – 127° with evolution of CO_2 (Found: C, 56.6; H, 6.07; N, 4.63; S, 10.6. $\text{C}_{15}\text{H}_{19}\text{O}_5\text{NS}$ requires C, 55.4; H, 5.88; N, 4.31; S, 9.85%). The high figure for C is unlikely to be due to contamination with unchanged starting material, since decarboxylation of the crude product (m.p. 125°) at 130 – 150° resulted in the evolution of more than 70% of the theoretical amount of CO_2 collected as BaCO_3 .

***S*-Benzyl-DL-[α - ^3H]Cystine.** The *half-ester* (VI) (1.63 g., 5 m-moles) was dissolved in dry tetrahydrofuran, 0.09 ml. (5 m-moles, 6.67 mc) of tritiated water was added from a 0.1 ml. blood pipette and the mixture was kept at room temperature overnight. The solvent was removed by distillation *in vacuo* and the residue was decarboxylated by heating for 0.5 hr. at 130° *in vacuo*. Next day the residue had crystallized. 6*N*-HCl (20 ml.) was added and the mixture was boiled under reflux for 5 hr. The HCl was removed by distillation *in vacuo*, the residue was dissolved in warm water and the solution was neutralized with *N*-NaOH. Yield of *S*-benzylcystine, 0.88 g. (83%).

DL-[α - ^3H]cystine. A portion of the *S*-benzyl-DL-[α - ^3H]cystine (0.7 g.) was converted into cystine, as described under (a) above. Yield, 0.15 g. (38%). After addition of 100 mg. of carrier DL-cystine, 105 mg. of a second crop of DL-[α - ^3H]cystine was isolated. This material had 15% of the specific radioactivity of the first crop and the total yield of labelled cystine is therefore 41%. The radioactivity of the main crop was 3.13×10^4 counts/min., or 33% of the maximum possible activity (9.4×10^4 counts/min.).

DL-[β - ^3H]Cystine

[α - ^3H]Glycine. Ethyl aminomalonate hydrochloride (2.1 g., 10 m-moles) was dissolved in tritiated water (1 ml.,

55.5 m-moles, 86 mc) and 0.1 ml. of conc. HCl. The solution was boiled under reflux for 2 hr., when decarboxylation occurred. Next day 6*N*-HCl (20 ml.) was added and the solution was boiled for a further 4 hr. The HCl was removed by evaporation to dryness, water (2×10 ml.) was then added and the evaporation was repeated. The residue was dissolved in ethanol and diethylamine was added till no further precipitation took place. The product was washed with ethanol and acetone; yield 0.92 g. (122%; the high yield is probably due to some contamination with diethylamine hydrochloride). The glycine was assayed for radioactivity after a 1:100 dilution with carrier and the radioactivity of the undiluted material was calculated to be 1.58×10^5 counts/min. (30% of the maximum possible counting rate).

[^3H]Formaldehyde. [^3H]Glycine (0.375 g., 5 m-moles; 1.58×10^5 counts/min.) was dissolved in 0.25*M*- KH_2PO_4 (20 ml.) and ninhydrin (2.2 g.) was added. The mixture was distilled almost to dryness, water (10 ml.) was added and the distillation continued till the total volume of the distillate was 25 ml. The formaldehyde content was determined gravimetrically by preparing the dimedon derivative from 0.7 ml. of the distillate. The yield of formaldehyde dimedon was 4.6 mg. (11%). The poor yield of formaldehyde may have been due largely to inadequate distillation.

DL-[β - ^3H]Cystine. Diethyl *N*-benzylthiothiocarbonylaminomalonate (VII; Crawhall & Elliott, 1951) (1.70 g., 5 m-moles) was dissolved in pyridine (40 ml.) and the remainder of the solution of [^3H]formaldehyde (24.3 ml.) was added. After 18 hr. at room temperature carrier formaldehyde (0.3 ml. of a 40% solution) was added to complete the reaction. After a further 24 hr., the solvent was removed by distillation *in vacuo* and the residue was dried thoroughly in a vacuum desiccator. The product, which crystallized on addition of light petroleum (b.p. 60 – 80°) was dissolved in dry ether (5 ml.) and freshly distilled thionyl chloride (15 ml.) was added at 0° . After 3 hr. excess of thionyl chloride was removed *in vacuo* at 20° and the residue was dried in a vacuum desiccator over NaOH. The residual oil was dissolved in ethanol (20 ml.) and sat. aqueous NaOH was added till well alkaline (pH about 11). After 45 min., the solution was adjusted to pH 7 with 3*N*-HCl and evaporated to dryness. After addition of 3*N*-HCl (40 ml.) the solution was boiled under reflux for 2 hr. and again evaporated to dryness. The residue was dissolved in water (25 ml.) and NaOH was added till the solution was strongly alkaline. After 30 min., the solution was acidified with an excess of conc. HCl, phenylmethanethiol was removed by extracting once with ether and the aqueous phase was boiled under reflux for 18 hr. Excess of HCl was removed by distillation to dryness and the residue was thoroughly extracted with ethanol (3×20 ml.). The insoluble material (NaCl) was removed by centrifuging and the ethanolic solution was treated with I_2 in ethanol till the oxidation was complete (starch being used as external indicator). Diethylamine was added to precipitate DL-[β - ^3H]cystine, which was centrifuged, washed with ethanol and acetone and dried (yield, 0.157 g., 26%). The radioactivity of this material, assayed after 100-fold dilution of a portion with non-radioactive DL-cystine, was found to be 1.25×10^4 counts/min. The molar radioactivity of the DL-[β - ^3H]cystine was thus about 13% of that of the [α - ^3H]glycine, in agreement with the approx. ninefold dilution of the [^3H]formaldehyde with non-isotopic formaldehyde.

RESULTS

Synthesis of ^3H -labelled cystine

Both [α - ^3H]cystine and [α - ^3H]glycine used for synthesizing [β - ^3H]cystine had only about 30 % of the expected specific radioactivity. This result may be due either to incomplete incorporation of tritium during the initial exchange reaction between the malonic esters and labelled water or, more probably, to loss of labelled α -hydrogen atoms during the subsequent acid hydrolysis. Cystine and glycine are known to undergo exchange of hydrogen when heated with 6N-HCl for several days (Rittenberg, Keston, Schoenheimer & Foster, 1938) and even under the less drastic conditions used in the present work (boiling with 5 or 6N-HCl for some hours) partial equilibration of the labelled α -hydrogen atoms with the unlabelled water may have occurred. It seems possible that hydrolysis with more dilute acid or for shorter periods would have reduced this exchange reaction, but this possibility was not investigated since sufficient labelled cystine was obtained.

Oxidative decarboxylation of an α -amino acid to the corresponding aldehyde is known to take place without loss of labelled hydrogen from the α -position (see Spenser, Crawhall & Smyth, 1956) and [^3H]formaldehyde was therefore prepared from [α - ^3H]glycine by this route, which involves only two stages with tritiated water as starting material.

Chemical yields were generally good, but the conversion of labelled glycine into formaldehyde was unsatisfactory. In earlier experiments yields of at least 50–60 % were obtained in this reaction, and the poor yield of labelled material may have been due to less efficient steam-distillation.

Utilization of tritium-labelled cystine for penicillin biosynthesis

DL-[α - ^3H]Cystine was readily utilized for penicillin formation both in complete fermentations and by washed mycelium, although the dilution of isotope was significantly greater than with ^{14}C -labelled cystine (Table 2, Expt. nos. 1a, 2, 5 and 6), probably owing to an isotope effect, as discussed below. DL-[β - ^3H]Cystine also gave rise to labelled penicillin (Table 2, Expt. no. 3). An accurate comparison of the utilization of α - and β - ^3H -labelled cystine is not possible since the experimental details, particularly the amount of labelled compound added and the times of addition, were different (see Table 1). It has, however, been previously found that the specific radioactivity of penicillin is approximately proportional to the amount of labelled L-cystine added, at least in the range 35–135 mg./l. (Arnstein & Grant, 1954b), and the higher isotope dilution in the experiment with DL-[β - ^3H]cystine (Expt. no. 3) is therefore not unexpected.

As in previous experiments with cystine labelled with ^{14}C , ^{35}S or ^{15}N (Arnstein & Grant, 1954b) the molar radioactivity of penicillin derived from [α - ^3H]cystine was somewhat greater than that of the cysteine in the mycelial protein (Table 3). The radioactivity of the other eight amino acids isolated from the mycelium was insignificant compared with that of cysteine. It is clear, therefore, that reutilization of isotopic hydrogen, arising from catabolism of the labelled cystine, is accompanied by large dilution of specific radioactivity.

Degradation of the penicillin biosynthesized from tritium-labelled cystine (Table 4) showed that the penicillamine moiety contained less than 10 % of the total radioactivity.

Table 2. Comparison of penicillin biosynthesis from ^{14}C - and ^3H -labelled cystine

The radioactivity is expressed as μC of ^{14}C /m-mole of substance or, for tritium, as counts/min. $\times$ mol.wt. $\times 10^{-6}$. Details of Expts. nos. 1a and 3 are given in the Experimental section and in Table 1. In the washed-mycelium expt. 500 ml. conical flasks, each containing 5 g. (wet wt.) of mycelium, 50 ml. of 0.005 % potassium phenylacetate, pH 6.9, and 0.25 mg. of labelled cystine, were incubated on a rotary shaker for 4.5 hr. (see Halliday & Arnstein, 1956), two flasks being used for each expt. At the end of the incubation the appropriate duplicate flasks were combined, penicillin was estimated by bioassay and isolated in the usual way after addition of carrier sodium benzylpenicillin (50 mg.).

Expt. no.	Precursor	Amount added (mg./l.)	Molar radioactivity		
			Cystine added* (A)	Penicillin (B)	Dilution (A/B)
1a	DL-[α - ^3H]Cystine	258.6	0.86	0.119	7.2
2†	DL-[β - ^{14}C]Cystine	267.5	1.91	0.47	4.1
3	DL-[β - ^3H]Cystine	52.9	1.51	0.076	19.9
4†	L-[β - ^{14}C]Cystine	34.6	2.01	0.145	14.3
Washed-mycelium expt.					
5	DL-[α - ^3H]Cystine	5.0	13.7	0.68†	20.2
6	DL-[β - ^{14}C]Cystine	5.0	61.4	6.75†	9.1

* Calc. as cysteine.

† From Arnstein & Grant (1954b).

‡ Corrected for dilution by carrier sodium benzylpenicillin.

When DL-[α - ^3H]cystine was used as a precursor, most of the radioactivity was found in the formaldehyde which arises from $\text{C}_{(6)}$ of penicillin (Table 4). The quantitative measurement of the radioactivity of the formaldehyde dimedon was, however, not entirely satisfactory. In degradation no. III a persistently high counting rate was obtained, and the observed radioactivity of the dimedon in degradation no. I was, if anything, lower than that expected. The reason for this variation is unknown, but no change was observed on repeated recrystallization of these samples. The figures for the radioactivity of formaldehyde dimedon must therefore be considered less reliable than those for the other compounds, although the interpretation of these experiments is not affected by the difficulty in assaying this compound.

DISCUSSION

Comparison of the utilization of DL-[β - ^{14}C]- and DL-[α - ^3H]-cystine shows that the latter is utilized with approximately twice the dilution of molar

Table 3. Comparison of radioactivity of mycelial amino acids and penicillin isolated from a fermentation with DL-[α - ^3H]cystine

The amino acids were isolated from the mycelium obtained in Expt. no. 1a (Tables 1 and 2) as described in the Experimental section.

Compound	Radioactivity	
	Counts/min.	10^{-3} (Counts/min. $\times$ mol.wt.)
Leucine	1.9	<1.0
Glutamic acid	3.8	<1.0
Threonine	5.7	<1.0
Valine	5.8	<1.0
Alanine	6.0	<1.0
Aspartic acid	8.4	1.1
Serine	9.7	1.0
Glycine	13.0	1.0
Cysteic acid	384.0	65.0
Penicillin	266.7	119.2

radioactivity as the former. It is unlikely, however, that this difference is due to loss of the isotopic hydrogen by a chemical reaction, such as dehydrogenation to an $\alpha\beta$ -unsaturated amino acid derivative. Such a mechanism would result in complete removal of the label; although some indirect incorporation of isotopic hydrogen might occur during subsequent steps in penicillin formation, the radioactivity of the penilloaldehyde moiety [$\text{C}_{(5)} + \text{C}_{(6)}$ of penicillin] would then be expected to be only of the same order of magnitude as that found in penicillamine or in the mycelial amino acids other than cystine. Moreover, the retention of label during the conversion of [α - ^3H]cystine into penicillin is also demonstrated by the finding that the specific radioactivity of the penilloaldehyde moiety, which is known to be derived from L-cystine (Arnstein & Grant, 1954b), was greater than that of the mycelial cysteine. A similar relationship between the radioactivity of penicillin and that of mycelial cysteine has been observed previously in experiments with ^{14}C -labelled cystine (Arnstein & Grant, 1954b).

The less efficient utilization of tritium- compared with carbon-labelled cystine for penicillin biosynthesis is probably due to a quantitatively important 'isotope effect'. It is well known that in chemical or biochemical reactions the carbon-tritium bond is less reactive than the carbon-deuterium or carbon-hydrogen bond, which may cause considerable differences in rates of reactions involving rupture of such bonds. On the other hand, the corresponding differences between the isotopes of carbon are quantitatively much less serious, and important fractionation of isotopes thus occurs mainly with the isotopes of hydrogen (for a recent review see Arnstein & Grant, 1957). Since the α -hydrogen atom of cystine apparently does not undergo a chemical reaction during penicillin biosynthesis, the observed isotope effect must be ascribed to a physical interaction. It seems possible that during synthesis of peptide bonds such a physical interaction between the α -hydrogen atom of an amino

Table 4. Degradation of penicillin biosynthesized from DL-[α - ^3H]- or DL-[β - ^3H]-cystine

Degradations nos. I and II were carried out with mixed samples of the *N*-ethylpiperidine salt of benzylpenicillin from experiments no. 1a and 5 (Table 2), to which carrier had been added. Degradations nos. III and IV were done with the *N*-ethylpiperidine salt from Expts. nos. 1b and 3 (Tables 1 and 2) respectively, also after dilution with carrier.

Labelled precursor added to fermentation				DL-[α - ^3H]Cystine			DL-[β - ^3H]-Cystine
Degradation no.	...	...	...	I	II	III	IV
				Position(s) of H atom(s) in penicillin*			
Compounds used for tritium assay				$10^3 \times$ Molar radioactivity (counts/min. $\times$ mol.wt.)			
<i>N</i> -Ethylpiperidine salt of benzylpenicillin				All	20.7	20.9	21.5
<i>iso</i> Propylidenepenicillamine				3, 10, 11	—	—	0.9
Penilloaldehyde 2:4-dinitrophenylhydrazone				5, 6	22.2	25.1	20.0
Formaldehyde dimedon				6	16.9	—	27.6

* See formula (I).

— Signifies compound not isolated.

acid and the enzyme might result in the preferential utilization of molecules which do not contain tritium. Alternatively, uptake of the labelled cystine into the mycelial cells by a mechanism involving interaction with the α -hydrogen atom might similarly lead to discrimination against the labelled molecules. It is noteworthy that the isotope effect observed in the present work on penicillin biosynthesis appears to be quantitatively similar to that which has been found in the biological oxidation of tritium-labelled methanol to formate (Rachele, Kuchinskas, Knoll & Eidinoff, 1954), but further work would be necessary to establish this point conclusively.

The utilization of cystine labelled with tritium in the α -position excludes the participation of an $\alpha\beta$ -dehydrocysteine derivative in penicillin biosynthesis. Although the detailed mechanism of β -lactam ring formation is not clear, it seems likely that an oxidative condensation is involved. The observation that one of the β -hydrogen atoms of cystine is incorporated into the penilloaldehyde moiety, presumably at C₅, of penicillin, places certain restrictions on possible mechanisms. Thus oxidation of the β -methylene group of cysteine to a carbonyl group is excluded, since this would involve complete loss of isotopic hydrogen from [β - ^3H]cystine. On the assumption that cysteinylvaline (VIII) is an intermediate in penicillin formation, the present results are compatible with two possible alternative mechanisms, namely the

removal of two hydrogen atoms by dehydrogenation (Fig. 3, A), or oxidation of cysteine to the thioaldehyde (X) followed by formation of the C-N bond with loss of a proton from the peptide nitrogen atom (Fig. 3, B). At present it is not possible to differentiate between these two mechanisms, but the initial step of pathway B is analogous to the conversion of alcohols into aldehydes by alcohol dehydrogenase, which also involves removal of only one of the α -hydrogen atoms of the alcohol (Vennesland & Westheimer, 1954).

Conversion of the intermediate β -thiol- β -lactam (IX) into the thiazolidine- β -lactam ring structure of penicillin may be envisaged as involving $\alpha\beta$ -dehydrogenation of the valine moiety to give (XI), followed by addition of the thiol group, as shown in Fig. 3. The final step in penicillin biosynthesis would then consist of the acylation of compound (XII), which has been claimed to be formed in the absence of side-chain precursor (phenylacetic acid) in cultures of *Penicillium chrysogenum* Q 176 (Kato, 1953a, b).

SUMMARY

1. DL- $[\alpha$ - ^3H]Cystine and DL- $[\beta$ - ^3H]cystine have been synthesized from tritiated water and used to investigate possible mechanisms of penicillin biosynthesis by *Penicillium chrysogenum*.

2. Both α - and β - ^3H -labelled cystine gave rise to labelled penicillin.

3. Chemical degradation of the penicillin showed that nearly all the isotope was present in the penillo-

Fig. 3. Suggested pathways of penicillin biosynthesis.

aldehyde moiety, which corresponds to positions 5 and 6 of the molecule. Further degradation indicated that most of the radioactivity of penicillin derived from [α - ^3H]cystine was located at position 6, as expected from intact incorporation of the labelled cystine.

4. In a fermentation experiment with [α - ^3H]cystine the molar radioactivity of penicillin was greater than that of the cysteine in the mycelial protein.

5. These results show that the α -hydrogen atom and one of the β -hydrogen atoms of cystine are retained during penicillin biosynthesis.

6. The significance of these findings is discussed with particular reference to possible mechanisms of β -lactam-ring formation.

We wish to thank Dr J. W. Cornforth, F.R.S., for most helpful discussions concerning the mechanism suggested in this paper for the biosynthesis of the β -lactam ring of penicillin.

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Some Spontaneous Reactions of *OO*-Dimethyl *S*-Ethylthioethyl Phosphorothiolate and Related Compounds in Water and on Storage, and their Effects on the Toxicological Properties of the Compounds

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(Received 18 December 1956)

Shortly after beginning to study the biochemistry of Metasytox (a mixture of *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and its thionate isomer) and some related compounds, we discovered that all our samples, tested biochemically, were impure, and that most of them increased in toxicity on storage. We therefore carried out a few preliminary experiments on the whole group of compounds:

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OO-dimethyl *S*-ethylthioethyl phosphorothiolate, Isosystox (the *OO*-diethyl homologue), the thionate isomer (dimethyl ethylthioethyl phosphorothionate) and the sulphoxide and sulphone of the thiolate (*S*-ethylsulphinylethyl- and *S*-ethylsulphonylethyl-*OO*-dimethyl phosphorothiolates). Contrary to expectation, much of the toxicity of all five compounds, as determined by intravenous injection into rats, remained in the water layers after chloroform extraction of their aqueous solutions.