The Biosynthesis of Penicillin

7. FURTHER EXPERIMENTS ON THE UTILIZATION OF L- AND D-VALINE AND THE EFFECT OF CYSTINE AND VALINE ANALOGUES ON PENICILLIN SYNTHESIS*

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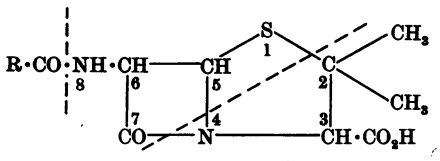
(*Received* 3 *July* 1957)

The biogenetic origin of the entire penicillin ring structure with the exception of the thiazolidine nitrogen atom is now well established. The side-chain nitrogen atom, the sulphur atom and the carbon and hydrogen atoms of the β-lactam ring of penicillin are derived from L-cystine (Arnstein & Grant, 1954*b*; Arnstein & Crawhall, 1957) whereas the carbon chain of the penicillamine moiety is synthesized from valine (Arnstein & Grant, 1954*a*; Stevens, Vohra & DeLong, 1954; Arnstein & Clubb, 1957) as shown in formula (I). It has, however, not



(I; R = $C_6H_5 \cdot CH_2 \cdot$ in benzylpenicillin)

been shown conclusively whether the immediate precursor is D- or L-valine or the keto acid (α-oxo-*iso*valeric acid) (see Arnstein & Clubb, 1957). Moreover, little is known about the structure of intermediates or the mechanism of reactions involved in penicillin biosynthesis.

In the present work, the effect of amino acids related to cystine and valine on the formation of penicillin has been studied for two reasons: first, it seemed possible, in view of the retention of the α-hydrogen atom and one of the β-hydrogen atoms of cystine during its conversion into penicillin (Arnstein & Crawhall, 1957), that α- or β-substituted cystines might give rise to penicillins substituted at $C_{(6)}$ or $C_{(5)}$ of the β-lactam ring respectively; secondly, selective inhibition of one step in penicillin biosynthesis by analogues of cystine or valine might result in accumulation of intermediates and thus facilitate a study of the biosynthetic mechanism.

The effect of amino acid analogues on penicillin biosynthesis was tested with the washed mycelium of *Penicillium chrysogenum*. Of several cystine analogues, only α-methylcystine inhibited penicillin production markedly and a similar effect was obtained with two amino acids related to valine, isoleucine and α-methylvaline. A more detailed study indicated that penicillin biosynthesis was inhibited by α-methylvaline without any apparent decrease in the synthesis of mycelial protein. Moreover, the conversion of D-valine into penicillin was decreased more than that of L-valine, indicating that D-valine is not a normal penicillin precursor.

EXPERIMENTAL

Methods

Organism and maintenance of cultures. Two strains of *Penicillium chrysogenum* were used: WIS 51/20, kindly supplied by Dr M. Lumb, Boots Pure Drug Co. Ltd., and WIS 51/20 F 3, a more stable mutant, which was obtained from Professor M. J. Johnson, University of Wisconsin. Subcultures and spore inocula were prepared as described previously (Arnstein & Grant, 1954*b*).

Details of fermentations. All fermentations were carried out in a stirred aerated jar fermenter containing 1–1·5 l. of synthetic medium (Jarvis & Johnson, 1947) under conditions essentially identical with those used earlier (Arnstein & Clubb, 1957), except that with *P. chrysogenum* WIS 51/20 F3 addition of potassium phenylacetate (0·1%/24 hr.) was begun 24 hr. instead of 40 hr. after inoculation. A vegetative inoculum was used except in the experiments described in Table 2, where the medium was inoculated with spores. The vegetative inoculum was prepared by adding 5 ml. of a suspension of washed spores from one medical flat (36 in.² area) in water (10 ml.) to 40 ml. of the synthetic medium described by Jarvis & Johnson (1947) in a 500 ml. conical flask and incubating at 24° on a rotary shaker (250 rev./min.). At the end of the incubation period (approx. 50 hr.) the entire culture was added to 1·5 l. of fermentation medium.

Washed mycelium experiments. Washed mycelium, prepared from 48–72 hr.-old fermentations as described by Halliday & Arnstein (1956), was incubated at 24° on a rotary shaker (250 rev./min.) at a concentration of 0·1 g. (wet wt.)/ml. of medium. The mycelial dry wt. was usually 10–20% of the wet wt. The composition of the incubation mixtures was as follows: medium *A*, 0·05M-glucose (0·9%), 0·15M-potassium phosphate buffer (pH 6·9), 0·005%

* Part 6: Arnstein & Crawhall (1957).

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potassium phenylacetate (pH 6·9); medium *B*, 0·1 M-potassium phosphate buffer (pH 6·9), 0·005% potassium phenylacetate (pH 6·9); medium *C*, medium *B* + 1% of glucose.

Penicillin bioassay. Penicillin was estimated by the cup-plate method with *Bacillus subtilis* (ICI strain, NCTC 8241) as test organism (Humphrey & Lightbown, 1952).

Isotope estimations. ^{14}C was estimated by counting 'infinitely thick' samples on 1 cm.^{2} polythene disks with a thin end-window Geiger–Müller tube (Popják, 1950), a 1 cm.^{2} disk of poly[^{14}C]methyl methacrylate, obtained from the Radiochemical Centre, Amersham, Bucks, being used as reference standard. The standard error of all counts is less than 5%.

For analyses of ^{15}N a 60° mass spectrometer was used as in earlier work, the accuracy being $\pm 0{\cdot}003$ atom % excess of ^{15}N (Arnstein & Grant, 1954*b*).

Isolation and degradation of penicillin. At the end of the fermentation, the mycelium was filtered off and washed with 0·1 M-potassium phosphate buffer (pH 6·9). In the washed-mycelium experiments with ^{14}C-labelled valine, non-isotopic sodium benzylpenicillin and DL-valine were added as carrier before filtration of the medium. Penicillin was extracted from the combined filtrate and washings with ether at pH 2 and isolated as the *N*-ethylpiperidine salt (cf. Arnstein & Grant, 1954*a*, *b*). The salt was purified by recrystallization from $CHCl_3$–acetone until the radioactivity remained unchanged and was degraded by acid hydrolysis as described recently by Arnstein & Clubb (1957).

Valine was isolated from mycelial protein as in previous work (Arnstein & Clubb, 1957).

Estimation of the uptake of valine by mycelium. The aqueous solution remaining after extraction of penicillin from the filtered-culture fluid was passed through a column of Zeo-Karb 215 ion-exchange resin (Permutit Co. Ltd., London) in the H^+ form, sufficient resin being used to retain all the amino acids (approx. 5 g. of resin/m-mole of valine + isoleucine or α-methylvaline). After washing with water, the resin was eluted with dil. aq. NH_3 soln. (usually 0·02 or 0·1 M) and the eluate containing the amino acids was evaporated to dryness. The residue was weighed accurately and its specific radioactivity was then determined by counting an infinitely thick sample in the usual way. The total radioactivity in the eluted amino acid fraction, which was assumed to be due to valine, was then calculated. In Expts. 1 and 5 (Table 4), valine isolated by the above method was recrystallized from aq. ethanol and the total radioactivity was obtained by multiplying its specific radioactivity by the weight of carrier valine added (40·1 mg.). In both, the amount of valine taken up by the mycelium is defined as the difference between the total radioactivity added initially as [*carboxy*-^{14}C]valine and that remaining in the valine or in the amino acid fraction isolated from the medium at the end of the incubation period.

MATERIALS

Amino acids related to cystine. The synthesis of ^{14}C-labelled and unlabelled β-methyl-DL-cystine (thiothreonine disulphide) and α-methyl-DL-cystine has been described in the preceding paper (Arnstein, 1958). The β-methyl*threo*-DL-[U-^{14}C]cystine (U indicates uniformly labelled) used in Expt. 2 (Table 2) was prepared from a mixture of L[U-^{14}C]-threonine (obtained from the Radiochemical Centre, Amersham) and unlabelled DL-threonine. Since inversion of the α-carbon atom probably occurs in this synthesis (Arnstein, 1958), the isotopic carbon would be expected to be present only in the D-enantiomorph of the product.

S-Methyl-L-cysteine was prepared by methylation of the sodium derivative of L-cysteine with methyl iodide in liquid NH_3 (du Vigneaud, Loring & Craft, 1934). *S*-Benzyl-L-cysteine was prepared by Dr D. Morris by the method of Wood & du Vigneaud (1939).

N-Methyl-L-cystine was synthesized by reducing 4-carboxythiazolidine, prepared from L-cysteine and formaldehyde (Ratner & Clarke, 1937), with Na in liquid NH_3 (Cook & Heilbron, 1949), followed by oxidation of the *N*-methyl-L-cysteine to the disulphide with air in the presence of a trace of $FeCl_3$ (Bloch & Clarke, 1938).

DL-Cysteic acid was prepared by Dr P. T. Grant (cf. Arnstein & Grant, 1954*b*).

Samples of L- and D-penicillamine were kindly supplied by Dr J. Wilkinson, Wellcome Research Laboratories. The thiol compounds were oxidized to the disulphides by aeration of solutions at pH 8 in the presence of a trace of $FeCl_3$ until no purple colour was obtained with nitroprusside.

L-Methionine was a gift from Dr T. S. Work.

Amino acids related to valine. *N*-Methylvaline has been synthesized from α-bromo*iso*valeric acid and methylamine (Friedmann, 1908), but in the present synthesis methylation of *p*-toluenesulphonyl-DL-valine was used, followed by hydrolysis of the *p*-toluenesulphonyl group. A solution of toluene-*p*-sulphonyl chloride (19 g.) in ether (50 ml.) was added to DL-valine (5·85 g.) in 2 N-NaOH (27·5 ml.) and the mixture was shaken at room temp., 25 ml. portions of 2 N-NaOH being added after 1 and 2 hr. After a further 1 hr., the aqueous layer was separated and acidified and the crude product was filtered off. After recrystallization from dil. ethanol, p-*toluenesulphonyl*-DL-*valine*, m.p. 168–169° (10·6 g., 79%), was obtained (Found: N, 4·9. $C_{12}H_{17}O_4NS$ requires N, 5·2%). The *p*-toluenesulphonyl-DL-valine (5·43 g.) was dissolved in 2 N-NaOH (40 ml.) and heated with methyl iodide (5·68 g.) in a sealed tube at 65–68° for 2 hr. The cooled mixture was acidified with HCl, when a yellow oil separated. The oil was dissolved in $NaHCO_3$ and reprecipitated with HCl. After some hours, 4·72 g. (83%) of crude *N*-methyl-*p*-toluenesulphonyl-DL-valine crystallized. The crude material (4·4 g.) was heated with conc. HCl (20 ml.) in a sealed tube at 105° for 24 hr. After cooling to 0°, the *p*-toluenesulphonic acid was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in ethanol and pyridine was added, when a white ppt. separated. The ppt. was recryst. from 95% ethanol (150 ml.), giving *N*-methyl-DL-valine (0·364 g., 17%), m.p. 240–250° (Found: N, 10·3. Calc. for $C_6H_{13}O_2N$: N, 10·7%).

α-Methyl-DL-valine has been synthesized by Kurono (1922) by hydrolysis of the amino nitrile obtained from methyl *iso*propyl ketone, but we were unable to prepare the amino acid in satisfactory yield and purity by this route. Excellent yields were, however, obtained by preparing the hydantoin instead of the amino nitrile; this method has been used recently for the synthesis of several other α-methylamino acids (Pfister *et al.* 1955).

Methyl *iso*propyl ketone (15 g.), KCN (17·2 g.) and $(NH_4)_2CO_3$ (79·2 g.) were dissolved in 50% aq. ethanol (520 ml.) and heated at 56° for 18 hr., then at 100° for

4 hr. The solution was acidified to pH 2, evaporated to 80 ml. and cooled, when *5-methyl-5-isopropylhydantoin* separated. After recrystallization from hot water, 18·05 g. (66·5%), m.p. 176–177°, was obtained (Found: N, 18·1. $C_7H_{12}O_2N_2$ requires N, 17·95%).

The hydantoin (18 g.) was boiled under reflux with $Ba(OH)_2,8H_2O$ (130 g.) in water (100 ml.) for approx. 72 hr., when no more NH_3 was evolved. The solution was filtered to remove $BaCO_3$, which was washed with hot water. The filtrate and washings were freed from remaining Ba^{2+} with CO_2 gas and $BaCO_3$ was again filtered off. The filtrate was evaporated to dryness and the residue was dissolved in hot dimethylformamide (150 ml.)–water (80 ml.). On cooling, α-methyl-DL-valine (8 g.) separated as needles, m.p. 240–260°, and by evaporation of the mother liquors to approx. 150 ml. a second crop (2·6 g., m.p. 240–260°) was obtained; the total yield was thus 10·6 g. (70%) (Found: N, 10·35. Calc. for $C_6H_{13}O_2N$: N, 10·7%).

Labelled valine. Uniformly ^{14}C-labelled L-valine was obtained from the Radiochemical Centre, Amersham. The synthesis of D- and L-[*carboxy*-^{14}C]valine and DL-[^{15}N]valine has been described elsewhere (Arnstein & Clubb, 1957).

RESULTS

Effect of cystine and its analogues on penicillin biosynthesis by washed mycelium

Of the methyl-substituted cysteine derivatives, 2·5 or 25 mM-α-methyl-DL-cystine consistently inhibited penicillin production by washed mycelium of *P. chrysogenum* WIS 51/20F3 (Table 1). This strain seems to be more sensitive than the closely related *P. chrysogenum* WIS 51/20, which was not inhibited by 7·5 mM-α-methyl-DL-cystine, but higher concentrations were not tested. Both D- and L-penicillamine disulphide (ββ-dimethylcystine)

Table 1. *Effect of cystine and cystine analogues on penicillin biosynthesis by washed mycelium of* Penicillium chrysogenum

Washed mycelium of *P. chrysogenum* WIS 51/20 F3 (0·5 g.; 0·07 g. dry wt.), harvested 58 hr. after inoculation of fermenter, was incubated for 2·5 hr. with 5 ml. of medium *A*, which also contained cystine or cystine analogues as stated.

Expt.	Compound added	Concentration (mM)	Penicillin production (i.u./ml.)	Penicillin production (% of average of control)
1	None	—	9·6, 8·5	—
	N-Methyl-L-cystine	25	9·1, 8·9	99
		2·5	8·5, 9·6	100
	S-Methyl-L-cysteine	50	8·5, 8·7	90
2	None	—	9·3, 8·1	—
	S-Methyl-L-cysteine	5	8·5, 7·4	92
	β-Methyl*threo*-DL-cystine	25	8·1, 9·3	100
		2·5	8·5, 7·4	92
	β-Methyl*erythro*-DL-cystine	25	9·1, 8·7	102
		2·5	11·75, 9·55	120
3	None	—	9·6, 9·6	—
	None (phenylacetate omitted)	—	1·2, 1·5	14
	α-Methyl-DL-cystine	25	6·6, 5·9	65
		2·5	7·1, 6·0	68
	S-Benzyl-L-cysteine	50	9·1, 8·9	94
	DL-Cystine	25	11·5, 12·0	123
4	None	—	7·8, 7·6	—
	DL-Cystine	2·5	12·8, 10·5	151
	L-Cystine	25	11·5, 10·7	144
		2·5	10·9, 10·1	136
	L-Methionine	50	7·6, 6·9	94
		5	6·9, 7·6	94
5	None	—	8·7, 8·8	—
	ββ-Dimethyl-L-cystine	25	7·4, 6·9	82
		2·5	8·1, 7·6	90
	ββ-Dimethyl-D-cystine	25	7·2, 8·1	87
		2·5	8·8, 8·9	101
6	None	—	8·1, 6·6	—
	DL-Cysteic acid	50	10·7, 9·3	135
		5	8·3, 8·3	113

also inhibited penicillin biosynthesis, but less effectively than α-methylcystine.

Stimulation of penicillin production was observed with DL-cysteic acid and with DL- or L-cystine, cystine being somewhat more potent than cysteic acid. In similar experiments with strain 51/20, DL- and L-cystine were again found to stimulate penicillin production.

No effect on penicillin biosynthesis was observed with L-methionine, *S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, *N*-methyl-L-cystine, or either isomer of β-methyl-DL-cystine (thiothreonine disulphide).

Penicillin biosynthesis in the presence of ^{14}C-labelled β-methylcystine

The benzylpenicillin *N*-ethylpiperidine salt isolated from fermentations to which ^{14}C-labelled β-methylcystine had been added was only weakly radioactive (Table 2). Since it was possible that a benzylpenicillin substituted at $C_{(5)}$ might not give an insoluble *N*-ethylpiperidine salt, the amount of ^{14}C in the supernatant solution from the precipitation of the *N*-ethylpiperidine salt and in the mother liquors from the recrystallization of the salt was also measured. From this radioactivity and the specific radioactivity of the added β-methylcystine, which is, of course, not diluted by endogenous synthesis, the maximum possible amount of penicillin derived from the ^{14}C-labelled β-methyl-DL-cystine was calculated to be less than 0·8 % of the total penicillin produced by the fermentation. It is likely, however, that these traces of radioactivity are due to an indirect utilization of isotope, for example by fixation of $^{14}CO_2$ produced by oxidation of the labelled β-methylcystine, rather than to the formation of 5-methylpenicillin.

Effect of valine and related compounds on penicillin production and valine metabolism

Penicillin biosynthesis by washed mycelium of strain WIS 51/20F3 was inhibited by α-methyl-DL-valine and, to a lesser extent, by DL-valine, whereas *N*-methyl-DL-valine had no effect (Table 3). In a similar experiment with strain WIS 51/20, DL-isoleucine was found to be a powerful inhibitor of penicillin biosynthesis, a concentration of 1 mg./ml. (approx. 7 mM) decreasing penicillin biosynthesis by approx. 50 % (Table 4).

Effect of isoleucine. A comparison of the utilization of D- and L-[*carboxy*-^{14}C]valine for penicillin biosynthesis in the presence and absence of DL-isoleucine is shown in Table 4. The addition of isoleucine resulted in a striking decrease in the uptake of D-valine by the mycelial cells, but had little or no effect on that of L-valine (Table 4, columns 4 and 5). The amount of ^{14}C present in the mycelium at the end of the experiment with D-[*carboxy*-^{14}C]valine was markedly decreased by the addition of isoleucine, whereas in the experiment with labelled L-valine the radioactivity in the mycelium was increased by about 50 % when isoleucine was added (Table 4, column 6). Although the decrease in the total amount of penicillin formed in the presence of isoleucine was similar in

Table 2. *Penicillin biosynthesis in the presence of ^{14}C-labelled β-methylcystine*

Solutions of ^{14}C-labelled β-methylcystine (see text) in dil. HCl (approx. 10 ml. of 0·2 N) were sterilized by Seitz filtration and added to the fermentation medium (1·5 l. in Expts. 1 and 3, 1 l. in Expt. 2) at various times after inoculation with spores of *P. chrysogenum* WIS 51/20, as follows: Expt. 1. 98·5, 49, 49, 55 and 59 mg. of β-methyl*threo*-DL-[*carboxy*-^{14}C]-cystine (thiothreonine *B* disulphide) were added at 45, 67, 91, 110 and 115 hr. respectively. Expt. 2. 40·1 mg. portions of β-methyl*threo*-DL-[U-^{14}C]cystine (thiothreonine *B* disulphide) were added at 41, 63, 87 and 109 hr.; Expt. 3. 100, 87, and 87 mg. of β-methyl*erythro*-DL-[*carboxy*-^{14}C]cystine (thiothreonine *A* disulphide) were added at 46, 70 and 44 hr. respectively.

		Expt. 1	Expt. 2	Expt. 3
Experimental details				
Duration of fermentation (hr.)		139	130	96
Yield of penicillin (10^{-3} i.u.)		240	249	252
Labelled amino acid added	(mg.)	310·5	165	274
	(μc)	1·14	1·02	0·81
Amount of benzylpenicillin isolated as *N*-ethylpiperidine salt	(mg.)	40	85	90
	(% of penicillin produced)	22	50	47
Comparison of specific radioactivities				
Labelled amino acid added (μc/mole*)		572	830	402
Benzylpenicillin (μc/mole)		0·56	1·62	0·18
Recovery of ^{14}C				
In penicillin produced by fermentation	(mμc)	0·23	0·69	0·08
	(%)	0·02	0·07	0·01
In mother liquor from crystallization of benzylpenicillin *N*-ethylpiperidine salt	(mμc)	0·84	0·68	<1·2
	(%)	0·07	0·07	<0·15

* Calculated for sulphydryl compound.

all flasks, the incorporation of ^{14}C from labelled D-valine into penicillin was reduced to about 20% of that taking place in the absence of isoleucine, but the conversion of labelled L-valine into penicillin was reduced comparatively little (Table 4, column 9). The effect of isoleucine on the specific radioactivity of the penicillin is, in general, similar to that on the labelling of the mycelium; i.e. a marked decrease with labelled D-valine, but an increase with labelled L-valine.

Effect of α-methylvaline. The effect of α-methyl-DL-valine on the utilization of D- and L-valine was tested with washed mycelium of *P. chrysogenum* WIS 51/20F3 (Table 5). The uptake of D-valine by

Table 3. *Penicillin production by washed mycelium in the presence of valine and analogues of valine*

In Expt. 1, washed mycelium (1 g.; 0·17 g. dry wt.) of *P. chrysogenum* WIS 51/20 F3, which had been stored at 4° for 24 hr., was suspended in 10 ml. of medium *B*, containing valine or valine analogues where stated. In Expt. 2 the experimental conditions were the same as in the experiments with cystine analogues (Table 1). In Expts. 3 and 4 medium *C* was used.

Expt.	Compound added	Final concentration (mM)	Penicillin production (i.u./ml./4 hr.)	Penicillin production (% of average of control)
1	None	—	14·1, 17·2	—
	N-Methyl-DL-valine	90	17·6, 15·1	108
		9	14·1, 11·9	86
	DL-Valine	90	11·0, 11·8	75
		9	14·8, 15·1 (i.u./ml./2·5 hr.)	99
2	None	—	9·6, 8·5	—
	N-Methyl-DL-valine	50	9·1, 8·9	99
		5	8·5, 9·6 (i.u./ml./4 hr.)	100
3	None	—	17·6, 17·6	—
	α-Methyl-DL-valine	50	13·0, 13·0	74
		5	14·0, 14·5 (i.u./ml./4 hr.)	81
4	None	—	12·9, 11·8	—
	α-Methyl-DL-valine	100	7·4, 6·8 (i.u./ml./7 hr)	58
	None	—	21·2, 21·0	—
	α-Methyl-DL-valine	100	12·2, 11·5	56

Table 4. *Effect of isoleucine on penicillin biosynthesis from* D- *and* L-*valine*

Each flask contained 4 g. of mycelium (0·6 g. dry wt.) from a 70 hr. culture of *P. chrysogenum* WIS 51/20. After a preliminary incubation for 2 hr. with 20 ml. of 3% lactose solution containing DL-isoleucine (1 mg./ml.) where indicated a further amount of 3% lactose solution (21·6 ml.) containing DL-isoleucine (21 mg., in flasks nos. 3, 4, 7 and 8 only), potassium phenylacetate (42 mg.) and either D- or L-[*carboxy*-^{14}C]valine (2 mg., 56 mμC) was added; the final concentration of DL-isoleucine was therefore approx. 7 mM, on the assumption that there was no significant catabolism of isoleucine during the pre-incubation period. After incubation for 4 hr. the penicillin content of each flask was determined by bioassay, carrier sodium benzylpenicillin (41·4 mg./flask) and DL-valine (40·1 mg./flask) were added, and penicillin and valine were isolated and assayed for ^{14}C as described in the text.

Flask no.	Labelled valine added	DL-Isoleucine present (+) or absent (−)	Uptake of valine by mycelium (mμC)	Uptake of valine by mycelium (% of added valine)	Radioactivity in mycelium (mμC)	Penicillin biosynthesis (i.u./flask)	Penicillin biosynthesis Specific radioactivity (μC/mole)	Penicillin biosynthesis Total ^{14}C in penicillin (mμC)
1	D-[*carboxy*-^{14}C]-Valine	−	24·0	42	9·5	474	524	0·422
2		−	34·0	61	12·8	443	783	0·347
3		+	16·5	29	1·8	174	54	0·016
4		+	12·4	22	5·0	193	349	0·115
5	L-[*carboxy*-^{14}C]-Valine	−	55·5	99	18·2	443	913	0·688
6		−	55·4	99	14·3	461	831	0·651
7		+	54·0	97	29·1	224	1590	0·604
8		+	54·6	98	24·3	174	1780	0·527

Table 5. *Effect of α-methylvaline on utilization of* D- *and* L-*valine for penicillin biosynthesis*

Washed mycelium of *P. chrysogenum* WIS 51/20 F3 from a 72 hr. culture was frozen slowly to −8° and stored at −70° for 57 days. Before use, it was thawed and re-washed with 0·1 M-potassium phosphate buffer (pH 6·9). Portions of mycelium (5 g.; 1·4 g. dry wt.) were suspended in 250 ml. conical flasks containing 50 ml. of medium *B* + 1% of glucose, D- or L-[*carboxy*-^{14}C]valine (5 mg., 140 mμC) and α-methyl-DL-valine (final concentration 0·1M) where indicated. After incubation for 7 hr. at 24°, the mycelium was filtered off, washed with 0·1M-potassium phosphate buffer (pH 6·9) and water and dried at 100°. The amount of penicillin in the combined filtrate and washings was determined by bioassay, carrier sodium benzylpenicillin (50 mg./flask) and DL-valine (50 mg./flask) were added, and penicillin and valine were isolated and assayed for ^{14}C as described in the text.

Flask no.	Labelled valine added	α-Methyl-DL-valine present (+) or absent (−)	Uptake of valine by mycelium (mμC)	Uptake of valine by mycelium (% of added valine)	Radio-activity in mycelium (mμC)	Penicillin biosynthesis (i.u./flask)	Penicillin biosynthesis Specific radio-activity (μC/mole)	Penicillin biosynthesis Total ^{14}C in penicillin (mμC)
1	D-[*carboxy*-^{14}C]-Valine	−	56·8	40·6	8·0	800	78·4	0·107
2		−	99·4	71·1	13·8	904	139	0·214
3		+	49·6	35·6	6·7	516	42·8	0·038
4		+	58·2	41·6	7·1	511	46·3	0·040
5	L-[*carboxy*-^{14}C]-Valine	−	104·6	74·9	73·0	726	955	1·18
6		−	105·7	75·6	75·1	794	991	1·34
7		+	89·6	64·4	63·5	466	969	0·768
8		+	100·6	72·0	76·5	560	991	0·943

this strain was somewhat more efficient than that by strain 51/20, whereas L-valine was taken up more efficiently by mycelium of 51/20 than by 51/20F3. The result of these changes was thus a smaller difference between the uptake of L- and D-valine in the present experiment than in earlier work with strain 51/20 (Arnstein & Clubb, 1957).

The uptake of labelled L-valine by mycelium was affected only slightly by addition of α-methyl-DL-valine; that of D-valine was decreased somewhat more (Table 5, columns 4 and 5), but the effect was much less than when isoleucine was added to strain 51/20 (Table 4). The radioactivity present in the mycelium at the end of the experiments with labelled D-valine (flasks nos. 1–4) was much less than that with labelled L-valine (flasks nos. 5–8). The effect of α-methylvaline on the radioactivity of the mycelium was approximately proportional to that on the uptake of valine by the mycelial cells (Table 5, columns 4–6).

The incorporation of ^{14}C into penicillin from labelled L-valine was much greater than that from labelled D-valine in corresponding experiments (Table 5, columns 8 and 9), even when allowance is made for the less efficient uptake of labelled D-valine by the mycelium.

As in the experiments with isoleucine (Table 4), the incorporation of ^{14}C from labelled D-valine into penicillin was decreased by α-methylvaline to a much greater degree than that from labelled L-valine (Table 5, column 9). The specific radioactivity of penicillin biosynthesized from labelled L-valine was, however, not affected by addition of α-methylvaline, although this valine analogue caused a marked decrease in the radioactivity of penicillin when labelled D-valine was used as precursor (Table 5, column 8). It is noteworthy that this decrease was greater than the change in the uptake of labelled D-valine by the mycelial cells due to α-methylvaline (Table 5, columns 4 and 5).

Fig. 1 shows the effect of α-methylvaline on penicillin production by the same mycelium and under the same conditions as in the above experiment. After an initial lag period of about 2 hr., penicillin biosynthesis was inhibited uniformly throughout the remaining 5 hr. incubation. In another experiment with a different batch of mycelium essentially similar results were obtained, inhibition of penicillin production being 42% after incubation for 4 hr. and 44% after incubation for 7 hr. (Table 3).

Table 6 shows the effect of 0·1 M-α-methylvaline on the utilization of labelled valine, consisting of a mixture of L-[U-^{14}C]- and DL-[^{15}N]-valine, for the biosynthesis of penicillin and mycelial protein during incubation for 48 hr. Penicillin formation was reduced to about 40% of that in the control flasks, but the amount of ^{14}C in the crude mycelial protein and the specific radioactivity of the mycelial valine and of the penicillin was significantly increased by addition of α-methylvaline. The incorporation of isotopic nitrogen into penicillin and mycelial valine was also increased, but the dilution of ^{15}N relative to ^{14}C was not much changed by α-methylvaline: the ^{14}C:^{15}N ratios were $1·5 \pm 0·15$ and $1·7 \pm 0·2$ in penicillin, and $1·0 \pm 0·06$ and $1·2 \pm 0·03$ in the mycelial valine in the absence and presence of α-methylvaline respectively.

Because of the small amount of penicillin obtained in this experiment it was not possible to carry out a complete degradation, but it is clear from earlier work (cf. Arnstein & Clubb, 1957) that most of the ^{14}C must have been present in the penicillamine moiety. Whereas the ^{15}N content of penicillin given in Table 6 refers only to the average of both nitrogen atoms (N_4+N_8), determination of the ^{15}N content of penicillamine, isolated as the Hg mercaptide, and of penilloaldehyde 2:4-dinitrophenylhydrazone indicated that the distribution of ^{15}N between N_4 and N_8 was approx. 2:1 and was apparently not affected by addition of α-methylvaline. Accurate figures for the ^{15}N content of these degradation products could, however, not be obtained. The low ^{15}N content of the penilloaldehyde 2:4-dinitrophenylhydrazone samples (0·006–0·01 atom % excess of ^{15}N) resulted in a large percentage error in their ^{15}N analysis, and the amount of penicillamine Hg mercaptide was insufficient for further purification by converting it into *iso*propylidene penicillamine, as was done in earlier work (Arnstein & Clubb, 1957).

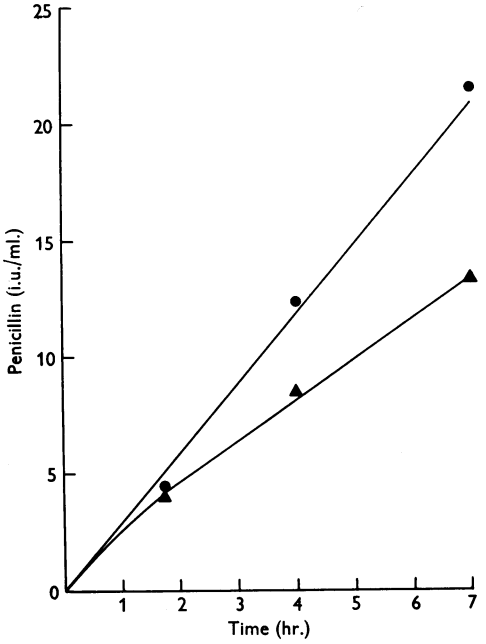

Fig. 1. Inhibition of penicillin production by α-methylvaline. Washed mycelium (2 g. portions; 0·46 g. dry wt.) of *P. chrysogenum* WIS 51/20 F3, from the same batch as used for the experiment in Table 5, was incubated with medium *C* (20 ml.) in 100 ml. conical flasks at 24°. Each point represents the mean of penicillin production in duplicate flasks in the absence (●) and in the presence (▲) of 0·1 M-α-methyl-DL-valine.

DISCUSSION

Effect of cystine and cystine analogues on penicillin biosynthesis by washed mycelium

The marked stimulation of penicillin production by washed mycelium which resulted from the addition of cystine is noteworthy because previously no consistent effect of cystine could be demonstrated (Stone & Farrell, 1946). Since the earlier work was done with complete fermentations, the biosynthesis of cystine may be rate-limiting in washed mycelium but not in a complete fermentation. Apart from cystine, only cysteic acid increased penicillin biosynthesis consistently in the present experiments. In isotope-competition experiments with $Na_2{}^{35}SO_4$ it has been shown that cysteic acid is used for penicillin synthesis in preference to sulphate, although it was a less efficient precursor than cystine (Stevens, Vohra, Moore & DeLong, 1954).

Table 6. *Utilization of valine labelled with* ^{14}C *and* ^{15}N *for penicillin biosynthesis in the presence of* α*-methylvaline*

Portions of fermentation medium + mycelium (50 ml.) from a 52 hr. fermentation of *P. chrysogenum* WIS 51/20 F3 were transferred aseptically into 12 sterile 250 ml. conical flasks (0·4 g. dry wt. of mycelium/flask). Where stated, 0·1 M-α-methyl-DL-valine (0·655 g./flask) was present. The flasks were incubated for 48 hr. on a rotary shaker (250 rev./min.) at 24°. At the beginning of the incubation and after 24 hr., 1 ml. of a serile solution containing L-[U-^{14}C]valine (approx. 0·15 mg.) and DL-[^{15}N]valine (0·1874 g.; radioactivity of mixture = 2·95 μc/m-mole; ^{15}N content = 15·52 atom % excess) was added to each flask. At 24 hr., 20% potassium phenylacetate (0·17 ml./flask) was also added. At the end of the incubation the mycelium was filtered off, defatted with boiling ethanol–ether (3:1, v/v), and extracted with 5% trichloroacetic acid. The specific radioactivity of the crude mycelial protein was 0·210 and 0·273 μc/g. in the absence and presence of α-methylvaline respectively. The filtrates from the appropriate flasks were combined, assayed for penicillin and carrier sodium benzylpenicillin (45·8 mg.) was added. Penicillin was isolated as the *N*-ethylpiperidine salt and assayed for ^{14}C and ^{15}N. The results are corrected for dilution by penicillin present at the beginning of the incubation and by carrier. The ^{15}N results are corrected for non-isotopic nitrogen in the *N*-ethylpiperidine salt.

Flask no.	α-Methylvaline	Penicillin production (mg. of sodium benzylpenicillin)	Isotope content of penicillin		Isotope content of mycelial valine	
			^{14}C (μc/m-mole)	^{15}N (atom % excess)	^{14}C (μc/m-mole)	^{15}N (atom % excess)
1–6	Absent	15·4	0·93±0·05	0·62±0·03	0·372±0·015	0·369±0·003
7–12	Present	6·3	1·34±0·07	0·80±0·05	0·524±0·026	0·429±0·003

The stimulation of penicillin production by cysteic acid may thus be explained by its conversion into cystine.

Penicillin biosynthesis by washed mycelium was reduced markedly by 2·5 mM or higher concentrations of α-methyl-DL-cystine, but lower concentrations were not tested. The inhibitory effect of α-methylcystine and α-methylvaline is of interest in view of the relatively limited number of examples of biologically active α-methyl amino acids (Umbreit, 1955). Inhibition of penicillin production was also obtained with ββ-dimethyl-L- and D-cystine (L- and D-penicillamine disulphide), but this cystine analogue was much less active than α-methylcystine. The inhibition of penicillin biosynthesis by penicillamine and the stimulation by cystine provide further support for the conclusion that cystine (Arnstein & Grant, 1954*a*, *b*; Stevens, Vohra, Inamine & Roholt, 1953) rather than penicillamine is the sulphur-containing precursor of penicillin.

Neither stereoisomer of β-methyl-DL-cystine had a significant effect on penicillin biosynthesis. The absence of inhibition of penicillin formation by *S*-methyl- or *S*-benzyl-L-cysteine in concentrations up to 50 mM is noteworthy because the closely related *S*-ethyl-DL-cysteine has been reported to inhibit penicillin synthesis by 23% at mM-concentration (Demain, 1956). It is possible, however, that the activity of the latter compound was due to the D-stereoisomer.

The possible conversion of methyl-substituted cystine derivatives into penicillin

In recent experiments with tritium-labelled cystine it was found that the α-hydrogen atom and one of the β-hydrogen atoms of this amino acid were incorporated into penicillin (Arnstein & Crawhall, 1957). It seemed worth while therefore to investigate whether α- or β-methylcystine could give rise to a penicillin substituted by a methyl group at $C_{(6)}$ or $C_{(5)}$ respectively. The present experiments with both isomers of ^{14}C-labelled β-methyl-DL-cystine, as well as the earlier work with α-methyl-DL-[*carboxy*-^{14}C]cystine (Arnstein, Clubb & Grant, 1954), show, however, that such substituted penicillins are not formed to any significant extent. It is clear that at least one of the enzymes involved in the utilization of L-cystine for penicillin biosynthesis is not able to metabolize α- or β-methyl-substituted cystine analogues.

Effect of valine and related amino acids and the configuration of valine used for penicillin biosynthesis

The inhibition of penicillin biosynthesis by DL-valine and α-methyl-DL-valine confirms earlier observations by Demain (1956), who showed moreover that the effect of DL-valine was due to the D-enantiomorph. In the present work somewhat higher concentrations of these amino acids were required to produce quantitatively similar effects, but this may be ascribed to possible differences in the strains of *P. chrysogenum*, particularly since even the closely related strains WIS 51/20 and WIS 51/20F3 apparently differed with respect to inhibition by α-methyl-DL-cystine. In addition, Demain (1956) used starved mycelium which may be more susceptible to inhibition because of depletion of endogenous valine or its precursors. We have found that DL-isoleucine is also a potent inhibitor of penicillin biosynthesis, but the present results are of greater interest in connexion with the configuration of the valine used for penicillin biosynthesis.

In an earlier paper (Arnstein & Clubb, 1957) it was shown that the uptake of D-valine by the mycelium of *P. chrysogenum* WIS 51/20 was much less rapid than that of the L-enantiomorph. Moreover, this difference accounted quantitatively for the better conversion of L-valine into penicillin compared with that of D-valine, and it was suggested that the lag period in the utilization of D-valine for penicillin synthesis which was observed by Stevens, Inamine & DeLong (1956) might also be due to the inefficient uptake of this enantiomorph. With mycelium of strain WIS 51/20F3, which was used in most of the present experiments, there was much less difference between the uptake of L- and D-valine than in the previous experiments with strain WIS 51/20. Despite this more favourable uptake of D-valine, its utilization for penicillin biosynthesis relative to that of L-valine was actually less efficient. This result suggests that L-valine is used for penicillin biosynthesis in preference to D-valine, and this interpretation is supported by the inhibition experiments with both isoleucine and α-methylvaline, which were carried out with strains WIS 51/20 and WIS 51/20F3 respectively. In these experiments neither isoleucine nor α-methylvaline inhibited the uptake of L-valine by washed mycelium to any significant extent, but the uptake of D-valine was decreased by both amino acids. However, whereas the utilization of L-valine for penicillin biosynthesis, as judged by the specific radioactivity of the penicillin, was not appreciably reduced (Table 5) or even increased (Table 4) by addition of the valine analogues, the conversion of D-valine into penicillin was inhibited markedly and to a greater extent than can be accounted for by the decreased uptake of D-valine. Since the uptake of D-valine by the mycelial cells is known to be less rapid than that of the L-enantiomorph (Arnstein & Clubb, 1957), it was considered possible that the relatively

greater reduction in the conversion of D-valine into penicillin might have been due to a considerable delay in the inhibition of penicillin biosynthesis after addition of the inhibitors. If this occurred, penicillin formation would take place mainly during the early part of the incubation period, when little or no ^{14}C-labelled D-valine would have entered the cells. In the experiment with α-methylvaline this possibility has, however, been excluded by the results in Fig. 1, which show that after a relatively short lag period inhibition of penicillin is approximately constant during the remaining 70% of the incubation time. In the experiment with isoleucine, the mycelium was pre-incubated with the inhibitor, which would presumably serve to reduce the effect of any lag period, but no check was carried out on the rate of penicillin production at various times after addition of this amino acid.

The decreased utilization of D-valine for penicillin biosynthesis in the presence of α-methylvaline may be due to inhibition of D-amino acid oxidase. Inhibition of this enzyme by α-methylvaline has been reported recently (Sourkes, Heneaghe & Umbreit, unpublished experiments quoted by Umbreit, 1955). It seems possible that the effect of DL-isoleucine is due to competition of D-isoleucine with D-valine for the same D-amino acid oxidase.

These results leave little doubt that the D-configuration of the penicillamine moiety ($C_{(3)}$ of penicillin) is not derived from D-valine or from an intermediate related biosynthetically to D-valine. It is, however, not possible to decided unequivocally whether L-valine or the keto acid (α-oxo*iso*-valeric acid) is the more immediate penicillin precursor, since attempts to inhibit the reversible deamination of valine were not successful, as shown by the experiment with ^{14}C,^{15}N-labelled valine, in which addition of α-methylvaline did not significantly change the relative utilization of ^{14}C and ^{15}N. The similarity in the ^{14}C:^{15}N isotope ratios of penicillamine and mycelial valine, which has been found in earlier experiments with ^{14}C,^{15}N-labelled valine (Arnstein & Clubb, 1957), suggests, however, that transamination of L-valine may be only incidental and not essential for penicillin biosynthesis.

The introduction of the D-configuration into $C_{(3)}$ of penicillin by a mechanism not involving D-valine has some resemblance to the biosynthesis of poly-D-glutamic acid by *Bacillus subtilis*. This polypeptide is apparently synthesized from L-glutamic acid without the direct participation of D-glutamic acid (Bovarnick, 1942). Although α-iminoglutaric acid has been suggested as an intermediate (Bovarnick, 1942), inversion of configuration or racemization of L-glutamic acid residues in an intermediate peptide seems equally plausible. Regardless of the detailed mechanism, the experiments on poly-D-glutamic acid, as well as the present results with penicillin, suggest that the D-configuration of amino acid residues in polypeptides and related compounds probably arises biogenetically after formation of peptide bonds rather than by utilization of free D-amino acids.

SUMMARY

1. The effect of amino acids related to cystine and valine on penicillin biosynthesis has been studied with washed mycelium of two strains of *Penicillium chrysogenum*.

2. Penicillin formation was markedly stimulated by L- or DL-cystine and by DL-cysteic acid.

3. L-Methionine, *S*-benzyl-L-cysteine, *S*-methyl-L-cysteine, *N*-methyl-L-cystine and the *erythro*- and *threo*-isomers of β-methyl-DL-cystine (thiothreonine *A* and *B* disulphides) had no effect on penicillin biosynthesis by washed mycelium. When ^{14}C-labelled β-methylcystine was added to fermentations producing penicillin, no conversion of this cystine analogue into a $C_{(3)}$-substituted penicillin could be detected.

4. Inhibition of penicillin biosynthesis was observed with α-methyl-DL-cystine and ββ-dimethyl-L- and D-cystine (L- and D-penicillamine disulphide).

5. DL-Isoleucine, α-methyl-DL-valine and DL-valine inhibited penicillin biosynthesis, but *N*-methyl-DL-valine had no effect.

6. Both isoleucine and α-methylvaline inhibited the conversion of ^{14}C-labelled D-valine into penicillin to a greater extent than that of L-valine.

7. A different strain of *Penicillium chrysogenum* (WIS 51/10F3) from that used in previous work utilized L-valine for penicillin biosynthesis much better than D-valine.

8. It is concluded from the better conversion of L-valine into penicillin by strain WIS 51/20F3 and from the preferential inhibition of the utilization of D-valine by α-methylvaline or isoleucine that D-valine is not a penicillin precursor, but that either L-valine or, less likely, the keto acid, α-oxo*iso*-valeric acid, is the precursor of the penicillamine moiety of penicillin.

We wish to thank all those mentioned in the text for gifts of amino acids and strains of *P. chrysogenum* used in this work.

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The Amino Acid Compositions of Three Fractions from Oxidized Wool

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(*Received* 5 *July* 1957)

The complex histology of the wool fibre and its insolubility in all solvents are the main obstacles to the elucidation of its chemical structure. On histological grounds alone it is apparent that wool is not a homogeneous protein, and therefore the logical approach to the problem would be to isolate the different structural components and study them separately. Unfortunately, in our opinion, it is not possible to do this at present since it is by no means clear how many components there are in the wool fibre or how they can be separated in a pure state. Most of the chemical methods used in their isolation result in considerable degradation of material, and in some cases several components are inextricably mixed. Moreover, histologists differ about the exact sites in the fibre structure which the isolated components originally occupied, and there is some confusion in the naming of them. A good review of the histological findings has been given by Mercer (1953), and further reference should be made to the *Proceedings of the International Wool Textile Conference*.

In view of the foregoing therefore most of the past and present research into the chemical structure of wool has been, and is being, directed towards the dissolution of the whole wool fibre and the isolation of large, homogeneous peptides from the resultant solutions. Clearly such procedures are only second best, as the assignment of any given peptide to a histological component of the fibre cannot be free from doubt. Two main methods of dissolution have been used, and in both the essential preliminary degradative reaction has been the fission of the disulphide linkages. Gillespie & Lennox (1953) used alkaline thioglycollate solutions to reduce the disulphide linkages and to dissolve the wool, and Alexander & Hudson (1954) oxidized these bonds with peracetic acid and dissolved the oxidized wool in dilute ammonia solution. The latter method is analogous to that used by Sanger (1949) for the separation of the *A* and *B* chains of insulin, and has been adopted in the present work. Alexander & Hudson (1954) obtained three main fractions from oxidized wool which they named α-, β- and γ-keratoses. In our opinion it is doubtful whether the fractions are sufficiently well characterized to warrant the term 'keratoses', but we have retained this nomenclature because other workers have already made reports on their constitutions (Earland & Knight, 1955, 1956), their physical properties (Alexander, 1951; O'Donnell & Woods, 1955) and on the amounts of their *N*-terminal amino acids (Alexander & Smith, 1955). This paper describes the