The isolation of D-valine from *Penicillium chrysogenum*

During the course of work on the identification of possible intermediates in penicillin biosynthesis, we have isolated, from the mycelium of *Penicillium chrysogenum*, several compounds containing cystine/cysteine and valine. Since these amino acids are precursors of penicillin[1], intermediates in the biosynthetic pathway must contain them in some form. The valine moiety in the final penicillin molecule has the D-configuration although L-valine is the probable ultimate precursor[2,3]. It was of interest, therefore, to examine the configuration of the valine derived from the above compounds.

Mycelium of *P. chrysogenum* strain Wis. 51–20F3 was grown in the medium of JARVIS AND JOHNSON[4], and harvested and washed as previously described[5]. The washed mycelium was resuspended in 0.01 *M* phosphate buffer, pH 7.0, containing 0.025 g/l potassium phenylacetate (100 g wet mycelial pad in 800 ml of liquid in ten 500-ml flasks), and shaken for 1 h at 24°. These conditions permit the continued synthesis of benzylpenicillin[5]. The mycelium was then washed again with three changes of water at 2°. The aim of this washing at reduced temperature was to minimize further metabolism of any accumulated intracellular intermediates.

After the final washing, the mycelium was subjected to mild acid hydrolysis (800 ml 1 *N* HCl, 56°, 2 h), filtered, and the cooled filtrate extracted with diethyl ether. Some purification of the extract was effected by extraction into aqueous buffer at pH 7 and back into ether at pH 2. The extract was then evaporated to about 0.5 ml, and portions were fractionated by paper chromatography (Whatman No. 1 paper, butanol–acetic acid–water (100:24:100, v/v/v), samples neutralized with ammonia vapor before running), and the paper sprayed with ammoniacal $AgNO_3$. The most prominent spots which appeared were colorless against the brown background and had R_F values of 0.61, 0.79 and 0.86; they gave no color reaction with ninhydrin.

Complete hydrolysis of the substances eluted from these spots yielded the same mixture of amino acids in each case, as judged by conventional paper chromatography and spraying with ninhydrin. The amino acids appeared to be valine, cystine/cysteine, glycine and leucine. Although attempts were made to demonstrate phenylacetic acid—the side-chain precursor of penicillin—in the hydrolysates, this has not yet been accomplished to our satisfaction. It is presumed that the three substances yielding amino acids on further hydrolysis are peptides.

The yield of valine from 100 g of mycelial pad (10 g dry wt.) was approximately 50 μg. Because of the small quantities of material available, the following micro methods were used to examine the configuration of the isolated valine:

1. *Paper chromatography.* Conventional chromatography, with the conditions detailed above, showed that D- and L-valine differed slightly but consistently in their R_F values.

2. *Microbiological assay.* Species of lactobacilli differ in their growth response to the optical isomers of amino acids[6]. The responses (acid production after 3-days' incubation) of *Lactobacillus arabinosus* 17–5 and *L. fermenti* ATCC 9338 to the isolated valine and to graded amounts of D- and L-valine were measured in a synthetic valine assay medium.

3. D-*Amino acid oxidase.* The isolated valine was treated with crude D-amino

acid oxidase (Sigma Chemical Co.) of known activity, in a 0.02 *M* pyrophosphate buffer, pH 8.3. The ammonia liberated during 5-min incubation was determined colorimetrically with Nessler's reagent. Known quantities of D- and L-valine were similarly treated.

4. *Ninhydrin reaction.* The total valine was determined by the quantitative ninhydrin reaction[7].

The results of these four determinations are shown in Table I.

TABLE I

RESPONSES OF L-, D- AND ISOLATED VALINE TO VARIOUS TESTS

Method:	*1*	*2*		*3*	*4*
	R_F *(Butanol–acetic acid)*	*L. arabinosus*	*L. fermenti*	*D-amino acid oxidase*	*Ninhydrin*
L-valine	0.49	+	+	—	+
D-valine	0.51	—	+	+	+
Isolated valine	0.51	—	+	+	+

Quantitatively, the positive responses of the isolated valine were similar; for example, a single sample assayed 13 (± 3) μg, 12 (± 3) μg and 12 (± 1) μg by methods 2, 3 and 4 respectively, indicating the absence of L-valine within experimental error. Thus all the valine in each of the three peptides was D-valine.

The presence of peptides of D-valine in a penicillin-producing organism might be expected, in view of the structure and origin of the penicillin molecule. However, this is the first report of the isolation of D-valine from the mycelium of *P. chrysogenum*, where it has been claimed to be absent[8]. The existence of intracellular, ether-soluble peptides containing cystine/cysteine and D-valine has important implications for theories of the mechanism of penicillin biosynthesis. These will be discussed in a more detailed account to be published elsewhere.

This work has been supported by the Australian National Health and Medical Research Council. The authors wish to thank Drs. S. A. KOSER, M. J. JOHNSON and H. C. LICHSTEIN for supplying cultures.

Department of Bacteriology, University of Queensland, Brisbane (Australia)

W. J. HALLIDAY
BETTY E. BRADFIELD
M. K. SHAW

[1] H. R. V. ARNSTEIN AND P. T. GRANT, *Biochem. J.*, 57 (1954) 353, 360.
[2] C. M. STEVENS, E. INAMINE AND C. W. DELONG, *J. Biol. Chem.*, 219 (1956) 405.
[3] H. R. V. ARNSTEIN AND H. MARGREITER, *Biochem. J.*, 68 (1958) 339.
[4] F. G. JARVIS AND M. J. JOHNSON, *J. Bacteriol.*, 59 (1950) 51.
[5] F. H. GRAU AND W. J. HALLIDAY, *Biochem. J.*, 69 (1958) 205.
[6] S. A. KOSER AND J. L. THOMAS, *J. Bacteriol.*, 73 (1957) 477.
[7] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
[8] C. M. STEVENS, P. E. HALPERN AND R. P. GIGGER, *J. Biol. Chem.*, 190 (1951) 705.

Received June 7th, 1960