Biosynthesis of Deuterated Benzylpenicillins II: Isolation and Characterization of a Highly Deuterated Benzylpenicillin

BRUCE C. CARLSTEDT*†, HENRY L. CRESPI, MARTIN I. BLAKE†, and JOSEPH J. KATZ

Abstract □ By using a modified replacement culture technique in combination with deuterated substrates, highly deuterated benzylpenicillin was isolated from the broth and characterized by PMR spectroscopy. Analysis of the spectra revealed an average replacement of 89% of the protons (^1H) with deuterium (^2H) atoms. Complete replacement by ^2H is observed in the phenylacetyl group, the C-3 position of the thiazolidine ring, and the C-6 position of the β-lactam ring. Partial substitution (64%) is noted in the C-5 position of the β-lactam ring and in the methyl groups (77%) at the C-2 position of the thiazolidine ring. The methyl groups of highly deuterated benzylpenicillin are all preformed in the seed inoculum, since there appears to be no *de novo* biosynthesis of the methyl groups during fermentation.

Keyphrases □ Benzylpenicillin, deuterated—biosynthesis, isolation, PMR characterization □ Deuteration, benzylpenicillin—*P. chrysogenum* cultures, ^2H-substrates □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures, ^2H-substrates □ PMR spectroscopy—analysis, structure, deuterated benzylpenicillin

In a previous paper (1), the participation of solvent deuterium oxide in the biosynthesis of benzylpenicillin was reported. *Penicillium chrysogenum* (Wisconsin strain 53-414) was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetic acid as carbon sources and 99.8% deuterium oxide as solvent. Partially deuterated benzylpenicillin was isolated from the culture and the extent of solvent participation in the biosynthesis was determined by analysis of the PMR spectra. Complete incorporation of deuterium in the C-3 and C-6 positions and partial incorporation at the C-5 position were observed.

In the present study, a highly deuterated benzylpenicillin, produced by fermentation in the presence of ^2H-substrates, was isolated from the broth and characterized by PMR spectroscopy.

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53-414, was utilized for this study and maintained on slants as described earlier (1).

Preparation of Seed Culture—The seed culture medium was prepared according to the formula shown in Table I and involved two separate steps. The pH of Part A was adjusted to 6.9 with 25% KOH prior to the addition of $CaCO_3$. Forty milliliters of Part A was transferred to each of a series of 250-ml. conical flasks; the flasks were plugged with cotton and autoclaved. Part B of the medium containing acetate and ^2H-pyruvate was prepared separately; the solution was adjusted to pH 6.9 with concentrated HCl and added by millipore filtration to the sterile flasks containing Part A of the medium. The millipore filter was 25 mm. in diameter with a pore size of 0.22 μ.

The sodium ^2H-pyruvate used in Part B of the seed culture medium was prepared by exchanging ^1H-pyruvate with D_2O, based on a procedure of Rose (2). Sodium ^1H-pyruvate was dissolved in D_2O in a 1:8.85 ratio; the solution was filtered and heated in an autoclave at 140° for 20 min. The solvent was removed by flash evaporation at 40° under vacuum, and the residue was dissolved in sufficient D_2O to yield a 9% solution, based on the original weight of sodium ^1H-pyruvate. This solution was then heated in an autoclave for 20 min. at 140° to ensure complete exchange. This ^2H-pyruvate was used for the preparation of Part B of the seed culture medium and was added (0.4 g./100 ml. seed culture medium) after 57, 69, and 81 hr. of growth, respectively.

Table I—Composition of Seed Culture Medium

	Amount
Part A:	
Dextrose	6.00 g.
Lactose	1.00 g.
Standard salt mixture	10.00 ml.
Water to make	80.00 ml.
$CaCO_3$	1.5 g.
Part B:	
NH_4OH	0.447 g.
Acetic acid	0.273 g.
^2H-Pyruvate sodium	0.565 g.
Water to make	20.00 ml.
Standard salt mixture:	
KH_2PO_4	3.00 g.
$MgSO_4 \cdot 7H_2O$	0.25 g.
$FeSO_4 \cdot 7H_2O$	0.10 g.
$CuSO_4$	0.005 g.
$ZnSO_4 \cdot 7H_2O$	0.02 g.
$MnSO_4 \cdot H_2O$	0.02 g.
$CaCl_2$	0.05 g.
Water to make	100.0 ml.

The resulting inoculum, consisting of pellets ranging from 1 to 3 mm. in diameter, was recovered aseptically by collection on filter paper in a büchner funnel. The pad was rinsed once with sterile distilled water, rinsed twice with sterile D_2O, and stored in a sterile container.

Deuterated Culture Techniques—The modified replacement technique described earlier (1) was used for culturing *P. chrysogenum* in D_2O. The D_2O nutrient medium shown in Table II was used in this study and differed from the medium used previously (1) in that sodium pyruvate was substituted for lactic acid. The D_2O nutrient medium was adjusted to an apparent[1] pH of 6.9 (3) with concentrated HCl; 50 ml. was transferred into a series of 250-ml. conical flasks, which were plugged with cotton and autoclaved. One milliliter of vitamin mixture was added to each flask, and the culturing technique reported earlier for inoculation and fermentation was followed.

^1H-Phenylacetic acid or ^2H-phenylacetic acid in D_2O was neutralized with 2.5% KOH in D_2O. The solutions were diluted with D_2O to give a final concentration of 8.34 or 8.84 mg./ml., respectively, and were autoclaved in multiple-dose vials. Daily addition of 1.5 ml. dextrose in D_2O (8.3%) and 0.5 ml. ^1H- or ^2H-phenylacetic acid solution was accomplished with a sterile syringe and needle by injection into the culture flask through the cotton plug.

Culture Monitoring Techniques—All cultures were monitored for penicillin titer, pH values, and deuterium content by procedures already described (1).

Extraction Procedure—*Isolation and Identification of Benzylpenicillin*—Scheme I shows a flowchart for the extraction, purification, and isolation of the highly deuterated benzylpenicillin. The procedures and methods of the extraction and purification through the preparation of the *N*-ethylpiperidine salt of benzylpenicillin

[1] The apparent pH of a D_2O solution is that pH observed with a pH meter. The pD of a solution is the apparent pH plus 0.4 unit.

Table II—Composition of D_2O Nutrient Culture Medium and Vitamin Mixture

Ingredient	Amount
D_2O Nutrient Culture Medium	
Dextrose	1.00 g.
Standard salt mixture[a]	10.00 ml.
NH_4OH	0.447 g.
Acetic acid	0.273 g.
Sodium pyruvate	0.565 g.
D_2O to make	100.00 ml.
Vitamin Mixture	
Biotin	0.005 g.
Inositol	0.005 g.
Ca pantothenate	0.005 g.
Pyridoxine hydrochloride	0.005 g.
Thiamine hydrochloride	0.005 g.
D_2O to make	100.00 ml.

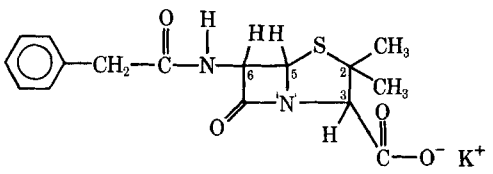
[a] Prepared as in Table I but with D_2O as the solvent.

were described, and the steps involved are indicated in the flowchart. The conversion of the *N*-ethylpiperidylbenzylpenicillin to the potassium salt of benzylpenicillin was accomplished by ion-exchange chromatography. Three grams of a resin[2] was soaked in distilled water for 1 hr. and placed in a small column (0.9-cm. i.d.) over a fiber glass plug. The resin, in the H^+ form, was converted to the potassium form by washing the column with 20 ml. of 1 *M* KOH at a flow rate of 10 drops/min. The column was then washed with water until the eluate was neutral as recorded on the pH meter. The *N*-ethylpiperidine salt was dissolved in 1 ml. distilled water and added to the column. The column was eluted with distilled water at a flow rate of 10 drops/min. The first 25 ml. was collected and lyophilized, and the residue was stored in a vacuum desiccator. A second volume of 5 ml. was collected but showed no residue on lyophilization.

Identification of Benzylpenicillin—IR and PMR spectroscopy were used for identification of benzylpenicillin and determination of deuterium uptake. The PMR spectra were obtained on a spectrometer[3] (probe temperature, 31°) after dissolving the sample in 0.01 *M* phosphate D_2O buffer (apparent pH between 6.5 and 7.5) or in deuterochloroform. The lock material was hexamethyldisiloxane, internally for deuterochloroform solutions and as an external capillary for D_2O solutions.

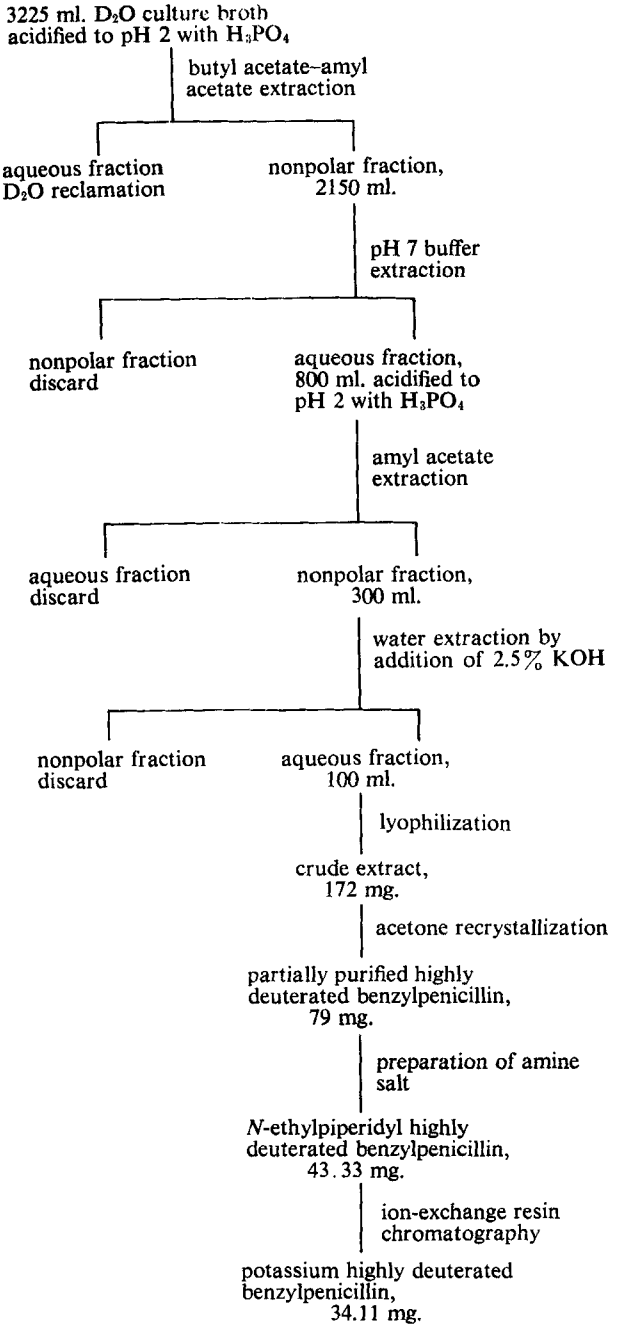
RESULTS AND DISCUSSION

A partially deuterated benzylpenicillin, III (Table III), isolated from a fermentation broth in which all the components of the culture medium except solvent D_2O were in the protio form, showed that deuterium atoms from the solvent were incorporated into benzylpenicillin during biosynthesis (1). The inclusion of ^2H-substrates in the culture medium is necessary if additional deuterium atoms are to be incorporated into this molecule. Nona *et al.* (4) and Mrtek *et al.* (5) utilized ^2H-substrates to produce deuterated griseofulvin and ergot alkaloids, respectively. The objectives of the present study were: (*a*) to examine the biosynthetic processes in both the seed inoculum and the D_2O nutrient culture medium by incorporating ^2H-pyruvate into the nutrient solutions, and (*b*) to produce, isolate, and characterize a benzylpenicillin containing a high enrichment of deuterium in nonexchangeable positions in the molecule.



potassium benzylpenicillin
I

[2] Biorad AG 50W-X8.
[3] Varian HA 100.



Scheme I —Synopsis of Extraction, Purification, and Isolation Procedure for Highly Deuterated Benzylpenicillin

Biosynthesis of Benzylpenicillin and Expected Isotopic Composition—Benzylpenicillin containing deuterium atoms in the benzyl side chain has been produced by biosynthetic and synthetic means. Behrens *et al.* (6) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule during biosynthesis. Laskar and Mrtek (7) synthesized deuterobenzyl-d_7-penicillin from 6-aminopenicillanic acid and ^2H-phenylacetic acid. Since phenylacetic acid is a precursor for benzylpencillin (8), inclusion of ^2H-phenylacetic acid in the fermentation medium should oblige the mold to biosynthesize a benzylpenicillin which is fully deuterated in the benzyl side chain.

Previous work demonstrated that in nutrient D_2O solutions (protio substrates) the incorporation of deuterium is almost complete at the C-3 position (Structure I) of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation (approximately 67%) of deuterium at the C-5 position was also

Table III—Chemical Shifts (δ, p.p.m.) of Protio and Deuterated Benzylpenicillins[a,b]

	Compound	Side-Chain Phenyl	Amide —NH—	C-5 Proton	C-6 Proton	C-3 Proton	Side-Chain $-CH_2-$	Thiazolidine $(CH_3)_2$
Solvent[c]: 0.01 *M* phosphate D_2O buffer, apparent pH 6.5–7.5								
I	K Benzylpenicillin[d]	7.38		5.53d (4 Hz.)	5.43d (4 Hz.)	4.23	3.68	1.56, 1.49
II	K pd-Benzylpenicillin	7.36		5.51	—	4.22[e]	3.67	1.56, 1.48
III	K pd-Benzylpenicillin	7.36		5.50	—	4.22[e]	3.68	1.54, 1.46
V	K pd-Benzylpenicillin	7.43		5.51	5.42[e]	4.18[e]	3.62	1.44, 1.36
VIII	K Benzylpenicillin	7.34		5.51d (4 Hz.)	5.41d (4 Hz.)	4.22	3.65	1.55, 1.48
IX	K hd-Benzylpenicillin	—		5.51	—	—	—	1.57, 1.49
Solvent[f]: $CDCl_3$								
IV	NEP[g] pd-Benzylpenicillin	7.38	6.28	5.63	—	4.36[e]	3.70	1.63, 1.53
VI	NEP hd-Benzylpenicillin	7.30[e]	6.30	5.58	—	4.29[e]	3.65[e]	1.58, 1.48
VII	Benzylpenicillinic acid[h]	7.34	6.35d (8.5 Hz.)	5.52	5.70d (4 Hz.) 5.49d (4 Hz.)	4.38	3.67	1.52, 1.45

[a] d denotes doublet; *J* values in Hz. [b] pd denotes partially deuterated; hd denotes highly deuterated. [c] Referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. [d] Assignments from (1). [e] Very small peak. [f] Referred to tetramethylsilane. [g] *N*-Ethylpiperidyl. [h] Green *et al.* (15).

observed. The methyl groups on the thiazolidine ring remained free of deuterium. It was shown that penicillin is biosynthesized from L-cysteine (9) and valine (10). In the biosynthesis of valine (11), two molecules of pyruvate are condensed to form the methyl groups of valine, which is in turn incorporated into penicillin (12) to give the methyl groups on the thiazolidine ring. If deuterated pyruvate is utilized by the mold, then the methyl groups of penicillin should be fully deuterated under our experimental conditions.

Preparation and Utilization of ^{2}H-Pyruvate by *P. chrysogenum* —Rose (2) described a technique for tritiation of pyruvate by heating sodium pyruvate in tritiated water to achieve exchange of the protons of the methyl groups with ^{3}H of the solvent, A modification of this procedure, as described under *Experimental*, yielded ^{2}H-pyruvate by heating sodium ^{1}H-pyruvate dissolved in D_2O.

Fermentation of *P. chrysogenum* 53-414 in a D_2O nutrient culture medium containing ^{2}H-pyruvate (Table II) should yield benzylpenicillin with deuterium incorporated in the methyl groups. Chemical shifts for partially deuterated benzylpenicillin II (Table III) obtained from such a fermentation showed a spectrum very similar to that of the partially deuterated benzylpenicillin III (Table III) obtained from the solvent participation study (1). These results indicate that ^{2}H-pyruvate from the fermentation medium is not utilized for the biosynthesis of benzylpenicillin in D_2O nutrient cultures. When ^{2}H-pyruvate was used in the fermentation medium, the PMR spectrum of the material purified through the acetone recrystallization step exhibited fewer and smaller peaks in the upfield region of the spectrum (due to impurities) than did corresponding samples from fermentations using lactate.

Halliday and Arnstein (13) developed a replacement technique for the study of benzylpenicillin biosynthesis by a washed mycelium technique and observed that only oxygen and phenylacetic acid were necessary for the continued biosynthesis of benzylpenicillin under the conditions studied. They concluded that intracellular substances that are not lost in the washing procedure are readily available for penicillin biosynthesis. Since a modified replacement technique (1) was utilized for the present study, it was felt that incorporation of ^{2}H-components into the seed culture medium might prove useful. Reformulation of the seed culture medium used earlier was necessary, and the formula shown in Table I proved suitable.

Preliminary experiments indicated that not only was ^{2}H-pyruvate necessary in the seed culture medium for deuterium incorporation in the C-2 methyl groups but that greatest ^{2}H incorporation occurred when additional ^{2}H-pyruvate was added to the seed culture medium near the end of the seed inoculum growth. A fermentation was conducted in which the ^{2}H-pyruvate was added to the seed culture medium and ^{1}H-phenylacetic acid was added to the D_2O nutrient culture medium. A normal fermentation showed an antibiotic titer of approximately 73 units/ml. The partially deuterated benzylpenicillin V (Table III) was extracted from the broth, and the *N*-ethylpiperidine salt was prepared. Chemical shifts for this compound (IV) are shown in Table III, and the PMR spectrum is shown in Fig. 1.

The resonance for the amide proton (*g*) represents a single proton; thus, the area beneath this peak is the area equivalent to one proton. Deuterium incorporation at the C-5 position was found to be 64%, an amount similar to that reported earlier. The expected value for the ratio of the area for the amide proton to the area for the phenyl protons is 1:5; the experimental ratio was 1:5.1. The expected ratio for the area of phenyl protons to the area for side-chain methylene protons is 5:2, but the experimental value was 5:1.5, indicative of exchange of the methylene protons with deuterium from the solvent during the fermentation. The *N*-ethylpiperidine salt was converted back to the potassium salt by ion exchange. The chemical shifts for partially deuterated benzylpenicillin V are recorded in Table III, and the PMR spectrum is shown in Fig. 2. The ratio of the area for the phenyl protons to the area for the methyl groups on the thiazolidine ring would be expected to be 5:6, but the experimental value was 5:1.45, indicating that 76% of the protons normally expected to be present in the methyl groups had been replaced by deuterium from the ^{2}H-pyruvate in the seed inoculum.

The resonances for the partially deuterated methyl groups seen in Fig. 2 are sharp peaks and are not the usual broad peaks associated with a partially deuterium-substituted functional group. The resonances are presumably not due to a mixture of $-CH_3$, $-CH_2D$, and $-CHD_2$ but probably result from methyl groups on the thiazolidine ring that are either all $-CH_3$ or all $-CD_3$. These methyl groups arise from the methyl groups of pyruvate, which is

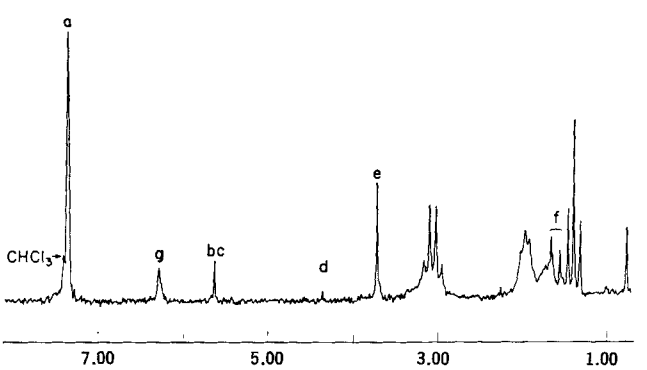


Figure 1—*PMR spectrum of* N-*ethylpiperidyl partially deuterated benzylpenicillin IV in* $CDCl_3$. *Key: a, side-chain phenyl; b, C-5 proton; c, C-6 proton; d, C-3 proton; e, side-chain methylene; f, thiazolidine dimethyl; and g, amide proton.*

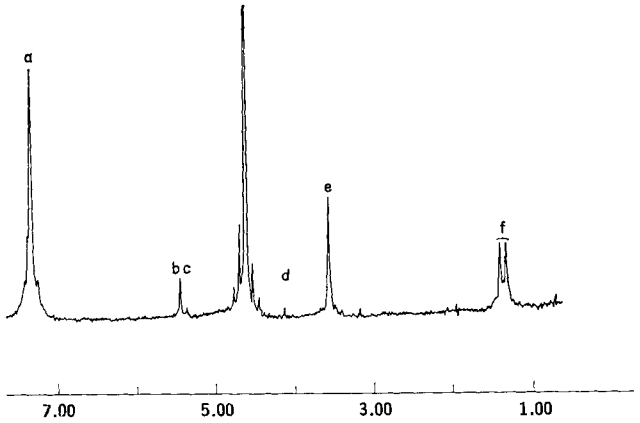


Figure 2—*PMR spectrum of potassium partially deuterated benzylpenicillin V in D_2O buffer. Key: see Fig. 1.*

present from the pyruvate added to the seed inoculum or which arises from the metabolism of lactose and dextrose in the seed inoculum. The pyruvate generated from the sugars would yield methyl groups containing predominantly protons, and even though large amounts of ^{2}H-pyruvate are added to the seed inoculum, utilization of some of the ^{1}H-pyruvate would be expected.

Other points of deuteration were observed (C-3, C-5, and C-6 positions) and were similar in isotopic composition to those observed in the earlier solvent participation study (1).

Highly Deuterated Benzylpenicillin—The information gathered from the described experiments was utilized for the production of a highly deuterated benzylpenicillin. A fermentation was conducted utilizing the ^{2}H-pyruvate additions to the seed culture medium and ^{2}H-phenylacetic acid additions to the D_2O nutrient culture medium. The fermentation proceeded normally, except that the initial drop in apparent pH was not as great as previously noted but rose to a plateau as expected. The cultures were harvested at 120 hr.; the extraction, purification, and isolation procedures shown in Scheme I were performed. An overall yield of 34.1 mg. antibiotic was attained. The IR spectrum of the isolated material exhibited the three carbonyl absorption maxima at 1763, 1662, and 1608 cm.$^{-1}$, which were in accord with expected values (14).

Chemical shifts for the *N*-ethylpiperidine salt (VI) are shown in Table III. The PMR spectrum was normal, except for the conspicuous absence of side-chain phenyl (7.30 p.p.m.) proton and methylene (4.29 p.p.m.) proton resonances because of deuteration

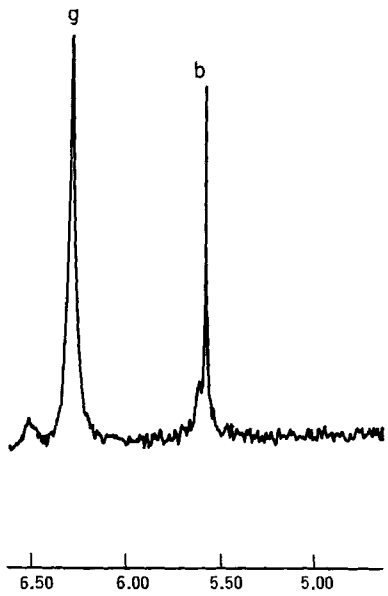


Figure 3—*PMR spectrum (5.0–6.5 p.p.m.) of N-ethylpiperidyl highly deuterated benzylpenicillin in $CDCl_3$. Key: see Fig. 1.*

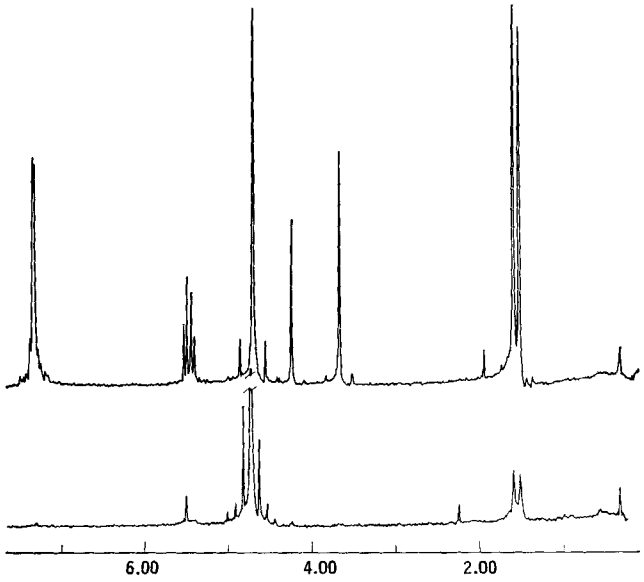


Figure 4—*PMR spectrum of equimolar solutions of potassium ^{1}H-benzylpenicillin VIII (upper) and potassium highly deuterated benzylpenicillin IX (lower) in D_2O buffer.*

at these sites. Figure 3 shows the resonances for the amide proton (6.30 p.p.m.) and protons at the C-5 position (5.58 p.p.m.). Chemical shifts for benzylpenicillinic acid (VII) dissolved in $CDCl_3$ are given in Table III. Green *et al.* (15) assigned the resonance at 5.52 p.p.m. to the C-5 proton, the two doublets centered at 5.70 and 5.49 p.p.m. to the C-6 proton (which is coupled to the C-5 proton and the amide proton), and the doublet at 6.35 p.p.m. to the amide proton. However, these workers (16) later revised the assignments. The single resonance assigned to the C-5 proton, which would normally be expected to be a doublet because of coupling to the C-6 proton, had been called an anomalous result. By use of a spectrometer with greater resolution, a different compound (*para*-dimethylaminophenyl ester of benzylpenicillin), and other solvents, it was shown that the C-6 proton does yield a normal double doublet and the C-5 proton does yield a doublet as expected. These workers attributed the anomalous pattern in $CDCl_3$ to chance equivalence of the chemical shifts for the β-lactam protons. The lack of splitting observed in Fig. 3 for the amide resonance again is evidence that the protons responsible for the 5.58 p.p.m. peak reside at the C-5 position and not the C-6 position (1). The ratio of the areas beneath the amide and C-5 resonances was 1:0.36 and indicated that deuteration at the C-5 position was 64%.

Biosynthesis of Deuterated Benzylpenicillins Based on PMR Spectra—Figure 4 shows the PMR spectra of equimolar concentrations of protio potassium benzylpenicillin (upper) and highly deuterated potassium benzylpenicillin (lower); the chemical shifts for these compounds (VIII and IX, respectively) are given in Table III. The ratio of the areas beneath the methyl resonances for the upper and lower spectra is 1:0.227, respectively, and indicates that deuterium replacement in the methyl groups is of the order of 77%. This result is compatible with the 76% deuterium incorporation observed with previous compounds. Deuterium replacement in the highly deuterated benzylpenicillin was estimated to be, on

Table IV—Deuterium Incorporation in Highly Deuterated Benzylpencillin

Group	Number of Protons	Percent Incorporation
Side-chain phenyl	5	99
C-5 Proton	1	64
C-6 Proton	1	90–95
C-3 Proton	1	90–95
Side-chain methylene	2	99
Thiazolidine methyls	6	77
Amide proton	1	99

the average, approximately 89% (Table IV) based on the deuterium replacement observed in preliminary work and on a comparison of the spectra in Fig. 4.

These results can be used to study the site of penicillin biosynthetic processes in D_2O nutrient culture fermentations. It is probable that parts of the biosynthesis occur in both the seed inoculum and the fermentation, because the seed culture ^2H-pyruvate was shown to be essential for deuterium incorporation in the methyl groups of the penicillin, and antibiotic titers were only observed in the fermentation medium. Deuterium from the methyl groups of ^2H-pyruvate in the seed culture medium was incorporated into the methyl groups of valine and, hence, into the methyl groups of penicillin. Washing the mycelium by the modified replacement technique apparently does not remove the components involved in the valine biosynthesis, which are probably intracellular substances.

Preliminary experiments showed that ^2H-pyruvate in the fermentation medium was not utilized for penicillin biosynthesis. It is apparent that 23% of the methyl groups arise from valine derived from ^1H-sugars in the H_2O seed culture medium and that 77% of the methyl groups are deuterated and are derived from ^2H-pyruvate added to the seed culture medium, with no back exchange with H_2O. The α-proton of valine is incorporated into the C-3 position of the benzylpenicillin molecule, but replacement of the C-3 proton by a solvent proton (or deuterium atom) occurs during ring closure of penicillin biosynthesis (12). The biosynthesis of the amino acid cysteine, which is utilized for the β-lactam portion of the molecule, probably takes place almost wholly in the D_2O nutrient culture medium, as evidenced by the sites of deuteration in the benzylpenicillin molecule. Arnstein and Crawhill (12) concluded that the α-proton and one of the β-protons of cysteine are retained during penicillin biosynthesis.

Observations made in this study indicate that full deuteration occurs at the C-6 position and approximately two-thirds deuterium replacement occurs at the C-5 position, *i.e.*, the α-position and β-position of cysteine, respectively. If the cysteine was biosynthesized in the seed culture medium and the α-proton was retained during the biosynthesis of benzylpenicillin, then no deuterium would be expected at the C-6 position. Full deuteration of this position is observed, however. Also, if one of the β-protons of the cysteine is retained during penicillin biosynthesis, then no deuterium would be anticipated at the C-5 position of penicillin. However, a peak is observed for the C-5 position, indicating the presence of protons, but the area represents only about one-third proton when compared to an amide proton in PMR spectra (Fig. 3). Exchange of β-protons with deuterium from the solvent must occur at some point of the cysteine biosynthesis since two-thirds replacement of the protons of the β-position is observed. Thus, the major portion of cysteine biosynthesis would probably take place in the D_2O fermentation medium, although the precursors (*e.g.*, 3-phosphoglycerate) may be retained from the seed inoculum. The components for the valine biosynthesis, *i.e.*, pyruvate, apparently originate almost totally in the seed inoculum, but formation into the valine may take place in either the seed culture or the fermentation.

REFERENCES

(1) B. C. Carlstedt, H. L. Crespi, M. I. Blake, and J. J. Katz, *J. Pharm. Sci.*, **59,** 1456(1970).

(2) I. A. Rose, *J. Biol. Chem.*, **235,** 1170(1960).

(3) A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, **40,** 700(1968).

(4) D. A. Nona, M. I. Blake, H. L. Crespi, and J. J. Katz, *J. Pharm. Sci.*, **57,** 975(1968).

(5) R. G. Mrtek, H. L. Crespi, M. I. Blake, and J. J. Katz, *ibid.*, **56,** 1234(1967).

(6) O. K. Behrens, J. Corse, R. G. Jones, E. C. Kleiderer, Q. F. Soper, F. R. VanAbeele, L. M. Larson, J. C. Sylvester, W. J. Haines, and H. E. Carter, *J. Biol. Chem.*, **175,** 765(1948).

(7) P. A. Laskar and R. G. Mrtek, *J. Pharm. Sci.*, **59,** 1727 (1970).

(8) K. Singh and M. J. Johnson, *J. Bacteriol.*, **56,** 339(1948).

(9) H. R. V. Arnstein and P. T. Grant, *Biochem. J.*, **57,** 360 (1954).

(10) *Ibid.*, **57,** 353(1954).

(11) A. White, P. Handler, and E. Smith, "Principles of Biochemistry," 3rd ed., McGraw-Hill, New York, N. Y., 1964, p. 520.

(12) H. R. V. Arnstein and J. C. Crawhill, *Biochem. J.*, **67,** 180 (1957).

(13) W. J. Halliday and H. R. V. Arnstein, *ibid.*, **64,** 380(1956).

(14) H. T. Clarke, J. R. Johnson, and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949.

(15) G. F. H. Green, J. E. Page, and S. E. Stanforth, *J. Chem. Soc.*, **1965,** 1595.

(16) G. F. H. Green, J. E. Page, and S. E. Stanforth, *Chem. Commun.*, **1966,** 597.

ACKNOWLEDGMENTS AND ADDRESSES

Received April 26, 1971, from the *Chemistry Division, Argonne National Laboratory, Argonne, IL 60439*

Accepted for publication July 7, 1971.

Performed under the auspices of the U.S. Atomic Energy Commission, Washington, D. C.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, San Francisco meeting, March 1971.

Abstracted in part from a dissertation presented by Bruce C. Carlstedt to the Graduate College, University of Illinois at the Medical Center, Chicago, Ill., in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors acknowledge the assistance of Mrs. G. N. McDonald in obtaining the PMR spectra.

* Argonne Universities Association–Argonne National Laboratory Fellowship Appointee.

† Present address: Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612