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Biosynthesis of Deuterated Benzylpenicillins I: Solvent Deuterium Oxide Participation

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Abstract □ The 53–414 strain of *Penicillium chrysogenum* 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. The extent of solvent participation in the biosynthesis of the penicillin molecule was determined by analysis of the proton magnetic resonance spectra. Incorporation of deuterium appears almost complete at two positions: the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. A partial incorporation of deuterium at the C-5 position is also observed. The deuterium atoms in the C-5 and C-6 positions apparently arise during biosynthesis of the amino acid cysteine. The deuterium atom at the C-3 position apparently arises either in the biosynthesis of the amino acid valine or in the closing of the thiazolidine ring.

Keyphrases □ Deuterated benzylpenicillin—biosynthesis □ *Penicillium chrysogenum* cultures—benzylpenicillin deuteration □ Biosynthesis, deuterated benzylpenicillin—*P. chrysogenum* cultures □ Proton magnetic resonance—analysis, structure □ IR spectrophotometry—structure

A variety of organisms has been successfully cultured in pure deuterium oxide (D_2O) (1); in several instances, pharmacologically active principles containing deuterium have been isolated and studied. Nona *et al.* (2–4) studied the effects of D_2O on the growth of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its antifungal activity evaluated. Mrtek *et al.* (5, 6) cultured a strain of *Claviceps purpurea* in D_2O and examined the biosynthesis of deuterated clavine alkaloids isolated from the culture. Katz and Crespi (7) reviewed the literature on isotope effects in biological systems.

Shaffer *et al.* (8) observed the effects of D_2O on the growth rate and morphology of *Penicillium notatum*, but penicillin production was not investigated. Mohammed *et al.* (9, 10) reported the effects of D_2O on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *Penicillium chrysogenum*. Here again, deuterated antibiotic was not isolated. Behrens *et al.* (11) synthesized deuterophenylacetyl-^{15}N-DL-valine and found the deuterophenylacetyl moiety was incorporated into the benzylpenicillin molecule. Demain (12) used partially deuterated benzylpenicillin, which contained five deuterium atoms in the benzene ring, to study the stability of penicillin during fermentation. Laskar and Mrtek (13) synthesized and studied the activity of a benzylpenicillin which was fully deuterated in the benzyl moiety. A reduction in antibiotic activity was reported.

In the present study, *P. chrysogenum* 53-414, a mutant of the Wisconsin Q-176 strain, was cultured in a defined medium containing D_2O as the solvent. Partially deuterated benzylpenicillin was isolated, and the degree of solvent participation in its biosynthesis was determined by proton magnetic resonance (PMR) analysis of this isotope hybrid compound.[1]

EXPERIMENTAL

Preparation of Slants—*P. chrysogenum*, Wisconsin strain 53–414, was obtained from the American Type Culture Collection (number 12690) in the lyophilized form. Agar slants were prepared as described by Mohammed *et al.* (9). The lyophilized material was suspended in Difco nutrient broth, streaked on the surface of the agar

[1] "Isotope hybrid compound" refers to a compound that contains one type of atom but also more than trace amounts of one of its isotopes (14).

Table I—Composition of Protio Culture Media

Seed Culture Medium		Protio Culture Medium	
Dextrose	6.00 g.	Lactose	3.00 g.
Lactose	1.00 g.	Dextrose	0.50 g.
Standard salt mixture	10.00 ml.	Standard salt mixture	10.00 ml.
NH_4 Acetate	0.35 g.	NH_4OH	0.559 g.
NH_4 Lactate (80%, syrupy)	0.693 g.	Lactic acid	0.520 g.
		Acetic acid	0.273 g.
Water to make	100.0 ml.	Water to make	100.00 ml.
$CaCO_3$	1.50 g.		

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	100.00 ml.

slants, and allowed to grow at room temperature for 5–7 days. The agar slants were stored at 5°; fresh slants were prepared every 4–6 months from lyophilized spores.

Preparation of Seed Culture—The seed culture medium was prepared according to the formula shown in Table I. The pH of the medium was adjusted to 6.9 with 25% KOH prior to the addition of $CaCO_3$. Fifty milliliters of medium was transferred into a series of 250-ml. conical flasks, and the flasks were autoclaved. Each flask was inoculated with a loopful of the aerial mycelium from an agar slant and placed on an Eberbach rotary shaker at 185–190 r.p.m. for 96 hr. 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. For H_2O cultures, the mycelial pad was washed with distilled water and stored in a sterile container. For D_2O cultures, the mycelial pad was rinsed once with distilled water, rinsed twice with D_2O, and stored in a sterile container.

Water Culture Techniques—The protio medium was prepared according to the formula shown in Table I. The pH was adjusted to 6.85 with 25% KOH (aqueous) prior to autoclaving. Three hundred and fifty milliliters of the medium was added to a series of 2-l. conical flasks and autoclaved. Twenty-eight grams (wet weight) of the seed culture mycelium was added to each culture flask by aseptic technique. All flasks were placed on an Eberbach rotary shaker at 185–190 r.p.m. for approximately 160 hr. at which time the cultures were harvested. Phenylacetic acid (PAA), as the potassium salt, was added as a sterile solution at the rate of 29.2 mg. PAA/350 ml. culture medium/day. Protio cultures were used as controls and for penicillin isolation studies.

Deuterated Culture Methods—A modified replacement technique was used for culturing *P. chrysogenum* in D_2O. The D_2O nutrient medium (Table II) differed from that of the protio cultures in the following ways: D_2O replaced water as the solvent, a vitamin mixture (Table II) was added, and a daily addition of carbohydrate was required. The D_2O nutrient medium was adjusted to apparent[2] pH (15) 6.9 with 25% KOH in D_2O; 50 ml. was transferred into 250-ml. conical flasks and autoclaved. One milliliter of the vitamin mixture was added to each flask. Each flask was inoculated with 4 g. (wet weight) of mycelial pad which had been previously rinsed with D_2O. The flasks were allowed to ferment on an Eberbach rotary shaker at 185–190 r.p.m. for approximately 160 hr. Anhydrous D-glucose, exchanged once with D_2O, was dissolved in sufficient D_2O to give a final concentration of 0.125 g./1.5 ml., transferred to a multiple-dose vial, and autoclaved.

PAA in D_2O was neutralized with 2.5% KOH in D_2O. The solution was diluted with D_2O to give a final concentration of 4.17 mg./0.5 ml., added to a multiple-dose vial, and autoclaved.

Daily addition of the D-glucose and PAA was accomplished with a sterile syringe and needle by injection through the cotton plug.

Bioassay Procedures—A 1-ml. aliquot of the culture medium was removed each day by means of a sterile disposable pipet and was placed in a 3-ml. test tube which was stoppered to reduce dilution of the deuterium by atmospheric moisture. The aliquot was used for three analyses: pH or apparent pH, penicillin titer, and D_2O content where applicable. The USP XVII (16) bioassay procedure was employed, except that the test organism was *Sarcina lutea* (17), obtained from the American Type Culture Collection (number 9341). The organism was maintained on an agar slant of Difco Bacto Antibiotic Medium 1 and was reslanted every 7–10 days. A 10-ml. base layer of Difco Bacto Antibiotic Medium 2 and a 6-ml. seed layer of Difco Bacto Antibiotic Medium 1 were utilized. Sodium penicillin G[3] was used as the reference standard. Microliter (10–100) portions of the aliquot were diluted to 10 ml. with 1% phosphate buffer, pH 6.0. Reference standard penicillin was included with each cup plate assay. The plates were incubated at 30° for 18–24 hr. A graph of inhibition zone diameter *versus* penicillin concentration was plotted. Titer values were read from the graph, multiplied by the dilution factor, and expressed as units per milliliter of culture medium. No correction factor was applied for the deuterated penicillin. All values of penicillin are in terms of sodium penicillin G reference.

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

D_2O Nutrient Culture Medium		Vitamin Mixture[a]	
Dextrose	1.00 g.	Biotin	0.005 g.
Standard salt mixture[b]	10.00 ml.	Inositol	0.005 g.
		Ca pantothenate	0.005 g.
NH_4OH	0.55 g.	Pyridoxine HCl	0.005 g.
Acetic acid	0.273 g.	Thiamine HCl	0.005 g.
Lactic acid	0.52 g.	D_2O to make	100.00 ml.
D_2O to make	100.00 ml.		

[a] Filtered through Millipore filter for sterilization. [b] Prepared as in Table I but with D_2O as the solvent.

Determination of pH—The pH of the protio cultures and the apparent pH of the D_2O nutrient cultures were determined daily on the aliquot removed from the culture medium.

Deuterium Analysis—D_2O content was determined daily by the spectrophotometric method of Crespi and Katz (18). Aliquots for analysis were centrifuged to yield a clear sample prior to determination.

Extraction Procedures—Centrifugation of the broth and mycelium and vacuum filtration on a Büchner funnel through double filter paper yielded a culture broth which contained essentially all of the penicillin produced in the fermentation. The extraction procedure was modified from a general solvent transfer method described by Chain (19). The method utilized amyl acetate, buffer, chloroform, and water as the four separate extraction steps. The method of Seifter and Richardson (20) involved amyl acetate, bicarbonate buffer, ether, and silica gel column with buffer elution as the four extraction steps. In the present study, butyl and/or amyl acetate were used as the nonpolar solvents. The broth was cooled to 3–5° in an ice-salt bath, acidified to pH 2.0 with phosphoric acid, and then shaken with cold (0–5°) 20% butyl acetate in amyl acetate saturated with water. Emulsions were broken by centrifugation. The butyl acetate–amyl acetate fraction was extracted with cold (0–5°) 3% pH 7 phosphate buffer. The buffer fraction was acidified to pH 2.0 and extracted with cold (0–5°) amyl acetate saturated with water. The amyl acetate fraction was extracted into distilled water with 2.5% KOH until the pH approached 7 as recorded on the pH meter. The water fraction was lyophilized, and the crude extract was stored in a vacuum desiccator.

Isolation and Identification of Penicillin—Initial purification of the crude extract was accomplished by recrystallization. The crude extract was dissolved in 85% acetone in water and kept cold in an ice-salt bath. Small flakes of penicillin formed upon addition of cold acetone. These flakes were isolated by centrifugation, dissolved in water, lyophilized, and the residue was stored in a vacuum desiccator. This proved to be sufficient purification for the protio penicillin, but further purification was required for the partially deuterated penicillin. The *N*-ethyl piperidine salt of the partially deuterated penicillin was prepared (21) and recrystallized, first from chloroform by additions of cold acetone and then from chloroform by additions of cold amyl acetate. The *N*-ethyl piperidine salt of a reference

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

[3] Nutritional Biochemicals Corp.

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

Compound	Side-Chain Phenyl	β-Lactam CH—CH (*J*, c.p.s.)	Thiazolidine —CH—	Side-Chain —CH_2—	Thiazolidine $(CH_3)_2$
I. K benzylpenicillin[b,c,d]	7.38	5.54d(4 c.p.s.), 5.43d(4 c.p.s.)	4.26	3.68	1.58, 1.51
II. K benzylpenicillin[e,c,f]	7.38	5.53d(4 c.p.s.), 5.43d(4 c.p.s.)	4.23	3.68	1.56, 1.49
III. K benzylpenicillin[g,c,f]	7.37	5.53d(4 c.p.s.), 5.43d(4 c.p.s.)	4.22	3.68	1.56, 1.49
IV. K partially deuterated benzylpenicillin[h,c,f]	7.36	5.50	4.22[i]	3.68	1.54, 1.46

[a] d denotes doublet; *J* values in c.p.s. [b] Green *et al.* (28). [c] Referred to DSS. [d] Solvent—D_2O. [e] Purchased from Nutritional Biochemicals Corp. [f] Solvent—apparent pH 7.4 0.01 *M* phosphate buffer D_2O. [g] Protio benzylpenicillin prepared in this laboratory. [h] Partially deuterated benzylpenicillin prepared in this laboratory. [i] Very small peak.

penicillin G was also prepared for comparative purposes. The IR spectrum of isolated material was obtained with a Beckman IR 7 spectrophotometer using a Nujol mull on Irtran II plates. The PMR spectra were obtained on a Varian HA 100 spectrometer (probe temperature at 31°) after dissolving the sample in 0.01 *M* phosphate D_2O buffer (apparent pH 7.4) or in deuteriochloroform. The lock material was hexamethyldisiloxane (HMS), internally for deuteriochloroform solutions and as an external capillary for D_2O solutions. The chemical shifts for D_2O solutions (Table III) were referred to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS). The chemical shifts for the $CDCl_3$ solutions (Table IV) were referred to tetramethylsilane (TMS). Bioassays were performed on the partially deuterated penicillin as described under *Bioassay Procedures.*

RESULTS AND DISCUSSION

It has been demonstrated on numerous occasions that organisms cultured in media containing high concentrations of heavy water experience a severe inhibition in the biosynthesis of metabolic products. Experiences of the authors with ergot alkaloids (6), belladonna alkaloids (22), penicillin (9, 10), and griseofulvin (2) have been reported. Thus, poor yields of penicillin obtained in this study (Fig. 1) were not unexpected, nor does this represent a serious deterrent in studying the biosynthesis of this antibiotic.

Isolation of penicillin on a laboratory scale has been described (11) but does present certain difficulties, principally because of the extreme instability of penicillin at pH values below and above neutral. This is compounded in the present studies by low titer values imposed by the inhibitory effects of heavy water and by the necessity for developing a defined medium for biosynthetic studies.

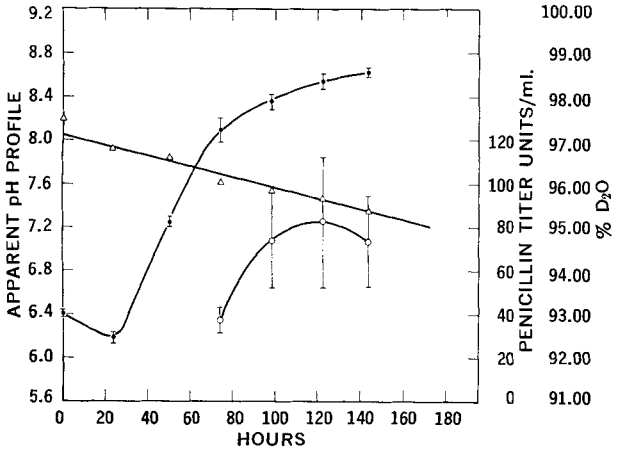


Figure 1—*Typical growth curves for* P. chrysogenum *53-414 in D_2O nutrient medium. Key:* ●, *apparent pH profile;* ○, *penicillin titer; and* △, *D_2O dilution.*

Knowledge of the composition of the medium and the availability of nutrient components and precursors in deuterated form is essential if the biosynthetic pathways are to be charted. Although optimal growth and penicillin production are achieved when corn-steep liquor is incorporated into the nutrient medium, its use here was precluded because of its unspecified composition. Further, the introduction of hydrogen atoms in this way would interfere with biosynthetic studies involving fully deuterated nutrient media needed for the production of fully deuterated penicillin.

The objectives of this study were: (*a*) to select a suitable strain of *Penicillium*, which would produce adequate amounts of penicillin under the constraints of a deuterated environment; (*b*) to develop an appropriate, defined, nutrient medium yielding maximum penicillin titers; (*c*) to isolate the antibiotic in a sufficiently pure state to permit identification, characterization, and analysis of deuterium distribution within the penicillin molecule; and (*d*) to determine the extent of participation of solvent D_2O in the biosynthesis of penicillin.

Five strains of *P. chrysogenum*, Q-176, 49-133, 51-20, 51-20 F3-64, and 53-414, were examined in preliminary studies for their ability to grow and produce antibiotic on a defined medium containing heavy water as solvent. The last two strains listed demonstrated the best performance in a series of growth and production studies. After further experimentation, the 53-414 strain was selected for the present work on the basis of higher penicillin titers (about 80 units per ml.).

For the protio cultures, the nutrient medium described by Singh and Johnson (23) was utilized. Cultures of the test organisms in this medium were used as controls, as a reference for establishing the appropriate medium and culture conditions for the D_2O nutrient cultures, and to provide a source material for the development of suitable extraction, isolation, and purification procedures. The composition of the protio culture media is shown in Table I. Since only one form of penicillin, benzylpenicillin, was desired in the fermentation broth, phenylacetic acid was added as a precursor to the nutrient.

In the development of an appropriate medium for the deuterio cultures, an attempt was made to include only those components that were also available in fully deuterated form. This was considered at this time since in subsequent studies the production of a fully deuterated penicillin was planned. The basic D_2O nutrient culture medium is listed in Table II and is an adaptation of the protio medium of Singh and Johnson (23). Lactose is a component of the protio medium, since it is slowly metabolized and represents a continuous source of glucose. Soltero and Johnson (24) demonstrated that increased penicillin titers resulted when cultures were fed glucose or sucrose on a continuous basis rather than only initially as a single addition of lactose which presumably has a similar effect as continuous or repeated additions of glucose. However, since deuterated lactose is not available at this time, the authors resorted to daily addition of glucose. Deuterioglucose is readily available from algae grown in heavy water.

The culturing procedure employed in this study is a modification of the replacement medium technique suggested by Halliday and Arnstein (25) for studying the metabolites in penicillin biosynthesis.

Table IV—Chemical Shifts (δ, p.p.m.) of Protio and Partially Deuterated *N*-Ethyl Piperidyl Benzylpenicillins in $CDCl_3$[a]

Compound	Side-Chain Phenyl	Amide —NH—	β-Lactam CH—CH (*J*, c.p.s.)	Thiazolidine —CH—	Side-Chain $—CH_2—$	Thiazolidine $(CH_3)_2$
V. Benzylpenicillinic acid[b,c]	7.34	6.35d(8.5 c.p.s.)	5.52, 5.70d(4 c.p.s.) 5.49d(4 c.p.s.)	4.38	3.67	1.52, 1.45
VI. NEP-Benzylpenicillin[c,d]	7.37	6.22d(7.5 c.p.s.)	5.62, 5.69d(4 c.p.s.), 5.63d(4 c.p.s.)	4.35	3.70	1.63, 1.51
VII. NEP-Partially deuterated benzylpenicillin[c,e]	7.37	6.22	5.62	—	3.70	1.62, 1.52

[a] d denotes doublet; *J* values in c.p.s. [b] Green *et al.* (28). [c] Referred to TMS. [d] Prepared as in *Experimental* section from reference benzylpenicillin. [e] Prepared from IV in Table III.

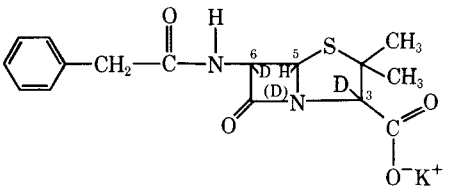
Nona *et al.* (3) and Mrtek *et al.* (6) used a replacement technique in culturing *P. janczewskii* and *C. purpurea*, respectively, in deuterated culture media. In their experiments the organism was allowed to mature in a protio medium and was then introduced into a D_2O medium containing only deuterated substrates. Hopefully the protio organism would synthesize deuterio metabolic products from the deuterated substrates. The technique was applied successfully for the production of deuterated griseofulvin and certain deuterated ergot alkaloids. It has not yet been successful for deuterated belladonna alkaloids when the method (26) is applied to a higher plant. The modification of replacement technique as applied in the present study involved the preparation of the seed culture in a protio medium. The mycelial pad from the protio seed culture was rinsed with D_2O and used as the inoculum for the deuterated culture.

The beneficial effect of a vitamin mixture addition on the culturing of organisms in a deuterated medium has been demonstrated by Mohan *et al.* (27) with *Torulopsis utilis*. Nona *et al.* (2) and Mrtek *et al.* (5) also reported the effects of adding selected vitamins to the culture medium. After preliminary experimentation the vitamin combination shown in Table II was found to enhance penicillin production.

Figure 1 shows the change in apparent pH, the dilution of deuterium by exchange with atmospheric moisture, and penicillin titer values for a typical D_2O nutrient culture over a 160-hr. study period. The apparent pH profile shows an expected immediate drop to an apparent pH 6.2 followed by a rise to a plateau value of apparent pH 8.6. If the fermentation was permitted to continue beyond that indicated in Fig. 1, the apparent pH would eventually drop. The data for the curves in Fig. 1 are based on the observations from thirty 50-ml. cultures. Maximum penicillin titers (70–80 units per ml.) are obtained after about 120 hr. of fermentation, at which point the apparent pH begins to plateau. At this apparent pH, autolysis begins and penicillin breakdown is evident. Over the course of study the D_2O content of the medium diminished at the rate of about 0.26% per day.

Because of the extreme lability of the penicillin molecule in solutions of low pH, caution was exercised during the processing of the fermentation broth. The primary problem in the extraction procedure was the formation of emulsions in the first step, which was overcome by centrifugation. After recovery of the crude extract in lyophilized form, the penicillin was recrystallized from 85% acetone in water by additions of cold acetone. The partially deuterated benzylpenicillin was found to be 60% pure by bioassay. The IR spectrum showed characteristic carbonyl absorptions at 1770, 1665, and 1608 $cm.^{-1}$, which confirmed the presence of penicillin but also showed a broad shoulder between 1550 and 1575 $cm.^{-1}$ that indicated the possible presence of carbonyl groups other than those of the penicillin. The PMR spectrum showed additional peaks in the upfield region (0.70–2.30 p.p.m.) which were not due to the benzylpenicillin.

Chemical shifts for ordinary and partially deuterated benzylpenicillins are given in Tables III and IV. PMR spectrum for potassium benzylpenicillin in D_2O (II) shows a resonance at 7.38 p.p.m. for the five protons of the phenyl ring and two doublets at 5.53 and 5.43 p.p.m. due to the C-5 and C-6 protons (Structure I) of the β-lactam ring, respectively, in the downfield region. The amide proton is exchanged with the deuterium of the solvent and is not observed.



partially deuterated benzylpenicillin

I

In the upfield region of the spectrum, the C-3 proton of the thiazolidine ring is observed at 4.23 p.p.m. and the methylene protons of the benzyl side chain at 3.68 p.p.m. The two methyl groups on the thiazolidine ring appear as singlets at 1.58 and 1.51 p.p.m. The PMR spectrum for the isotope hybrid compound isolated in this study (IV) shows the same resonances for the phenyl, side-chain methylene, and thiazolidine methyl groups. The resonances at 4.22 and 5.43 p.p.m. are almost completely absent and a singlet is observed at 5.50 p.p.m. Interpretation of these spectra indicates the possibility of two points of full deuteration, which are surmised to be the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring. Also, the C-5 position of the β-lactam ring appears to be partially deuterated.

C-3 Position—In accord with the assignments made by Green *et al.* (28), the resonance at 4.22 p.p.m. (Table III) is almost completely absent (5–10% normal value) and indicates extensive deuteration at the C-3 position.[4] It has been shown that penicillin is biosynthesized from L-cysteine (29) and valine (30). The C-3 position of the thiazolidine ring arises from the α-carbon of valine, and an examination of the pathway for biosynthesis of valine (31) reveals that a deuterium atom from the solvent may be incorporated at the α-carbon by transamination. Arnstein and Crawhill (32) suggested a mechanism for the formation of the thiazolidine-β-lactam ring. In the final step for the thiazolidine ring closure, the suggested pathway allows for incorporation of a proton, or a deuterium atom, at the C-3 position, so that the deuterium atom evidenced here may be due either to previous incorporation into the valine or this last ring closure step.

C-5 and C-6 Positions—The assignments made by Green *et al.* (28) for potassium benzylpenicillin in D_2O (I) are in agreement with this work, except for the β-lactam ring protons at C-5 and C-6. They assigned the resonance at 5.43 p.p.m. to the C-5 proton and the resonance at 5.54 p.p.m. to the C-6 proton (Table III). In the partially deuterated benzylpenicillin isolated in this study, the doublet at 5.43 p.p.m. is essentially absent and the doublet at 5.53 p.p.m. is replaced by a singlet at 5.50 p.p.m. (Table III). The C-5 and C-6 positions of the β-lactam ring arise from the β- and α-carbon atoms, respectively, of cysteine (29). From an examination of the biosynthetic pathways for formation of cysteine (33), it is observed that a deuterium atom from the solvent may be incorporated at the α-carbon of cysteine (*i.e.*, the C-6 position of penicillin) by trans-

[4] The presence of protons at these points might be due to the 3–5% water in the medium or a preferential isotope incorporation of the protons over the deuterium atoms during biosynthesis.

amination. This position should then be essentially fully deuterated. Thus, the observation of deuteration at the position which normally resonates at 5.43 p.p.m. indicates that this resonance represents the C-6 proton rather than the C-5 proton. The authors conclude, therefore, that the assignments by Green *et al.* (28) for the β-lactam protons are inverted. This observation is further confirmed by the data from Table IV in which chemical shifts for penicillin in organic solvents are listed.

The assignments of Green *et al.* (28) (V) are for benzylpenicillinic acid in $CDCl_3$. The *N*-ethyl piperidyl salts of protio (VI) and partially deuterated (VII) benzylpenicillins were used in this study. Green *et al.* (28) 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. Resonances observed for *N*-ethyl piperidyl protiobenzylpenicillin (VI) differ slightly but are in good agreement with these assignments. The spectrum observed for the *N*-ethyl piperidyl partially deuterated benzylpenicillin (VII) shows singlets at 6.22 and 5.62 p.p.m. The singlet observed at 5.62 p.p.m. is the same resonance as that assigned to the C-5 proton and indicates its presence in the partially deuterated benzylpenicillin. Agreement with the conclusion that the resonance observed in D_2O solutions at 5.50 p.p.m. (Table III) is due to the C-5 proton is thus retained. Also, if the protons had been present at the C-6 position, splitting of the amide resonance would have been observed. The change in assignments to 5.54 p.p.m. for the C-5 proton and 5.43 p.p.m. for the C-6 proton indicated by this study would involve only those assignments for potassium benzylpenicillin in D_2O solution.

The assignments made by Green *et al.* (28) for other penicillins or benzylpenicillins in other solvents or under different conditions than those examined in this study are assumed correct, since factors such as solvent or conformational effects could be influencing the chemical shifts. Incorporation of deuterium at the C-6 position is probably greater than 90%,[4] since there is no observable splitting of the singlet peak at 5.50 p.p.m. (Table III). Partial deuteration at the C-5 position is estimated to be 50–75%. The amide proton in the $CDCl_3$ spectrum for VII (Table IV) was used as an internal reference. Saur *et al.* (34) observed isotopic exchange of deuterium in the β-carbon protons of phosphoenolpyruvate when examining deuterium isotope effects on fermentation of hexoses. Thus, deuterium may have been incorporated at the β-carbon of 3-phosphohydroxypyruvate during the biosynthesis of the cysteine (33). Arnstein and Crawhill (32) suggested that one of the β-protons of cysteine is retained during ring closure of penicillin biosynthesis and thus deuteration at the C-5 position would be observed.

Further studies will attempt to biosynthesize a fully deuterated benzylpenicillin, to examine its potency in relation to protiopenicillin, and to examine the fate of d_3-acetate in the fermentation.

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