Effect of Deuteration on the Growth and Antibiotic Production of a Number of Strains of *Penicillium*

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Thirteen strains of two species of *Penicillium* were surveyed for their ability to grow and produce antibiotic in a medium of high concentrations of heavy water. Mycelial dry weights, pH variation, and penicillin potency were noted over a 90-day study period. In the deuterio cultures autolysis and penicillin degradation did not occur. Phenylacetic acid was found to stimulate penicillin production in both the protio and deuterio cultures.

THE EFFECTS of heavy water on the growth rate and morphology of *Penicillium notatum* and *Aspergillus fonsecaeus* have been reported by Shaffer *et al.* (1). The production and isolation of antibiotic were not considered in that investigation. More recently Nona *et al.* (2, 3) studied the effects of D_2O on the culturing of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its properties studied. A brief review of the literature dealing with the effects of deuteration on algae, bacteria, fungi, and higher plants was included. These represent the only studies concerning the effects of D_2O on antibiotic-producing organisms.

In the present study 13 strains of two species of *Penicillium* were surveyed for their ability to grow and produce antibiotic in high concentrations of heavy water.

EXPERIMENTAL

Penicillium Strains—The penicillin-producing strains used in this investigation and the commercial sources of supply are indicated in Table I. The organisms were stored on agar slants at 5° and were reslanted every 3 months.

The agar slants used for maintenance of the molds were prepared in the following manner: Flask 1 contained peptone, 0.6 g.; tryptone, 0.4 g.; yeast extract, 0.3 g.; dextrose, 0.1 g.; beef extract, 0.2 g.; distilled water, 60 ml. Flask 2 contained potassium dihydrogen phosphate, 2.0 g.; malt extract, 2.0 g.; agar, 3.0 g. The pH of Flask 1 was adjusted to 6.7. The contents of Flask 2 were added to Flask 1 and the volume was diluted to 100 ml. with distilled water. The medium was heated to dissolve the agar, and was then dispensed into test tubes in 5-ml. aliquots. The tubes were sterilized and slanted.

Sterilization—A portable electric steroclave (Wisconsin-Aluminum) was used for sterilizing the slants at 15 p.s.i. for 20 min. Sterilization of all protio media was done under similar conditions.

Sterilization of deuterio media was effected in a pressure cooker Presto (model KE03A). Pure deuterium oxide (99.6%) was used as the sterilizing agent in place of water. Sterilization time was 15 min. in all instances.

Protio Nutrient Medium—The culture medium had the following composition in each 100 ml. of solution: sodium nitrate, 0.3 g.; magnesium sulfate, 0.025 g.; potassium dihydrogen phosphate, 0.05 g.; zinc sulfate, 0.005 g.; copper sulfate, 0.0003 g.; ferrous sulfate, 0.005 g.; dextrose, 3.0 g. The pH was adjusted to 6.5 with a 25% potassium hydroxide solution. Finally 0.5 g. of calcium carbonate was added.

For each *Penicillium* strain a series of 10 cultures was prepared in 250-ml. conical flasks. Forty milliliters of the nutrient medium was added to each flask. The flasks were plugged with nonabsorbent cotton, capped with aluminum foil, and sterilized. Protio cultures served as controls.

Deuterio Nutrient Medium—In the deuterio culture medium, 99.6% deuterium oxide was used in place of ordinary water. Otherwise, the composition of the nutrient was the same as that used in the water cultures. All exchangeable hydrogen was previously replaced with deuterium by dissolving weighed quantities of the nutrient mixture in deuterium oxide, and then lyophilizing the solution. This procedure was repeated to ensure complete exchange. The final nutrient solution contained better than 98% deuterium oxide. Ten 40-ml. cultures for each strain were prepared and sterilized as described under *Protio Nutrient Medium* and *Sterilization*, respectively.

Preparation of Seed Inoculum—A concentrated suspension of healthy spores in protio nutrient medium was prepared for each mold according to the formula shown under *Protio Nutrient Medium*, except for the dextrose content which was increased to 9.0 g. Fifty milliliters of the medium was placed in a 250-ml. conical flask and sterilized. One loopful of spores from each stock test tube slant was used for the seed inoculum. Submerged culture shake technique, as described below, was used in preparing the seed inoculum.

Culture Methods—*Shake Cultures*—The inoculum for the submerged cultures consisted of a suspension of tiny pellets, about 1 mm. in diameter obtained from the seed culture. Inoculation for the protio nutrient medium was performed using 5-ml. portions of a heavy suspension of the spores. In the case of the deuterio nutrient medium, the 5-ml.

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TABLE I—MYCELIAL DRY WEIGHT AND ANTIBIOTIC PRODUCTION OF *Penicillium* STRAINS GROWN IN AN H_2O AND D_2O ENVIRONMENT

Strain	Culture Method	Mycelial Dry Weight, g./l. H_2O	Mycelial Dry Weight, g./l. D_2O	Antibiotic Production, Units/ml. H_2O	Antibiotic Production, Units/ml. D_2O
PN[a]-35548[c]	Surface	4.80	3.80	8.6	1.1
	Shake	4.00	3.85	7.5	1.4
PC[b]-37767[d]	Surface	4.88	4.11	1.1	1.1
	Shake	5.00	4.80	3.2	0.0
PN-15378[d]	Surface	6.50	6.11	10.0	2.8
	Shake	15.51	10.80	11.1	0.0
PC-167[e]	Surface	20.10	15.89	3.0	2.1
	Shake	30.36	22.88	1.1	3.1
PC-1428[e]	Surface	6.38	6.60	8.1	3.2
	Shake	8.13	6.30	6.1	2.1
PC-1531[e]	Surface	9.88	7.30	8.7	3.8
	Shake	18.91	12.01	8.0	0.0
PN-230[f]	Surface	35.50	18.98	6.6	0.0
	Shake	38.00	23.91	8.0	0.0
PN-284[f]	Surface	8.00	3.82	2.9	0.5
	Shake	8.75	5.72	1.8	0.1
PC-451[f]	Surface	10.01	10.34	10.1	0.0
	Shake	16.00	8.72	8.3	0.0
PN-612[f]	Surface	8.80	9.02	3.9	4.0
	Shake	23.60	16.71	3.0	2.1
PC-1177[f]	Surface	12.00	10.00	4.0	4.0
	Shake	21.66	13.81	1.9	1.2
PN-821[g]	Surface	10.25	3.00	10.0	3.1
	Shake	6.21	4.00	3.2	0.5
PN-1249B21[g]	Surface	5.00	3.75	28.1	0.0
	Shake	8.50	4.89	12.1	0.0

[a] *Penicillium notatum.* [b] *Penicillium chrysogenum.* [c] Centraalbureau Voor Schimmelcultures, Baarn, Nederland. [d] Commonwealth Mycological Institute, Kew, Surrey, England. [e] Imperial Chemical Industries Ltd., Macclesfield, Cheshire, England. [f] Laboratorie de Cryptogamie, Paris, France. [g] Northern Utilization Research & Development Div., Peoria, Ill.

portions of spores were lyophilized before inoculation. The cultures were incubated at 24–25° on an Eberbach rotary shaker at 215 r.p.m., for as long as 3 months in certain instances.

Surface Cultures—Protio and deuterio cultures were inoculated with 5-ml. aliquots of the seed inoculum. However, the water was removed by lyophilizing prior to inoculating the deuterio nutrient medium. Incubation was at 24–25°. Care was taken to ensure that the surface pellicle was not disturbed. It was noted that when the pellicle sank below the surface of the nutrient solution, growth ceased and autolysis followed immediately.

Phenylacetic Acid Additive—In several experiments 0.3% phenylacetic acid was added to the nutrient medium. The pH of the solution was adjusted to 6.5 with potassium hydroxide before sterilization.

Analysis for Penicillin Potency—All cultures were periodically assayed for antibiotic activity. The cylinder-plate method described in USP XVII (4) was employed.

Mycelial Dry Weight—At varying intervals during the experiment, the mycelium from a culture was separated from the fermented broth by vacuum filtration. The mycelium was dried to constant weight in a convection air oven at 70°.

Determination of pH and pD—At intervals throughout the study period the pH of the protio cultures and the pD of the deuterio cultures were determined. A Beckman pH meter (model 76A) was used for both series. In the case of the deuterio cultures the apparent pH was obtained and an appropriate correction (5) to the reading was made.

Deuterium Analysis—At the conclusion of each experimental period, an aliquot of the deuterio culture broth was analyzed spectrophotometrically for deuterium oxide content according to the method of Crespi and Katz (6).

RESULTS AND DISCUSSION

Growth Observations—Growth was evident after 2 days of incubation in all surface and shake protio nutrient cultures. In the surface cultures rapid growth was evidenced by the enlargement of the surface pellicle. In the shake cultures this was demonstrated by the production of numerous globular pellets ranging in size from 2 to about 10 mm. in diameter. This was followed by a progressive thickening of the nutrient medium. In water all organisms sporulated in 5 to 7 days. The cultures appeared tan, light brown, or faint yellow in color.

A somewhat different pattern was noted in the deuterio nutrient cultures. There was no sign of growth after 2 days of incubation for any of the cultures, surface or shake. However, within 10 days after incubation the surface cultures did show definite signs of growth. Clusters of colonies, varying in size from 10 to 25 mm. in diameter, were apparent over the surface of the culture. One strain, PC-37767, produced very tiny brown colonies. The others ranged in color from white to gray to light tan and generally were lighter than the corresponding protio cultures. In general the shake cultures showed better growth than the surface cultures. In 6 days the shake cultures developed a sizable number of pellets though smaller in diameter (1.5 to 6.0 mm.) when compared with those formed in the protio nutrient

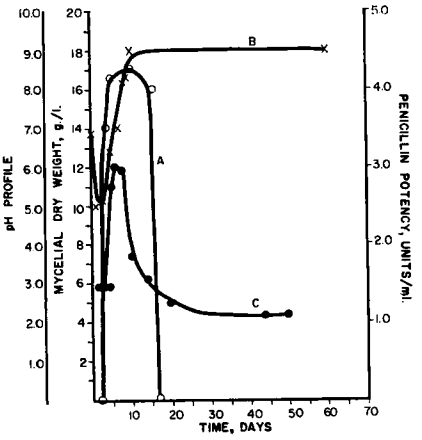

Fig. 1—The penicillin potency, pH profile, and mycelial dry weight of PC-1177 grown in protio medium by surface culture method. Key: A, penicillin potency; B, pH profile; C, mycelial dry weight.

medium. Growth was considerably slower for the 13 strains of *Penicillium* grown in the deuterio nutrient, requiring roughly twice the time to produce the same amount of growth in the deuterio cultures as in the corresponding protio cultures.

Mycelial Dry Weights—Dry weights of the mycelial tissue from the strains of *Penicillium* studied are listed in Table I. The values represent the maximum weights obtained by plotting dry weight *versus* time as typified in Figs. 1 and 2 for one of the strains in the study group. Considerable variation is apparent among the strains in both H_2O and D_2O and there appears to be little correlation between good growth and antibiotic production. Preliminary studies indicated that optimal growth was obtained when corn steep liquor was included in the nutrient medium. However, due to its indefinite composition and since the introduction of hydrogen atoms into a deuterio nutrient medium would preclude the biosynthesis of a fully deuterated antibiotic, this additive was not employed in subsequent studies.

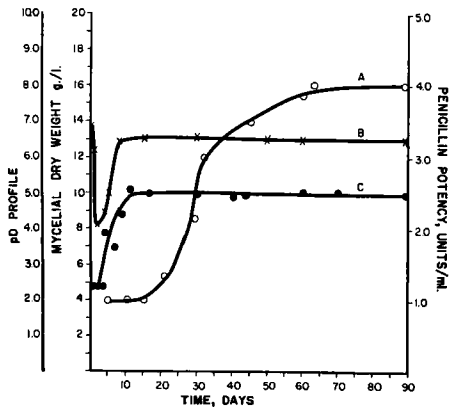

Fig. 2—The penicillin potency, pD profile, and mycelial dry weight of PC-1177 grown in D_2O medium by surface culture method. Key: A, penicillin potency; B, pD profile; C, mycelial dry weight.

Figure 1 shows the change in mycelial dry weight with time for the PC-1177 strain grown in a protio nutrient. There is a rapid increase in tissue formation for about 7 to 10 days followed by a rapid breakdown over the next 6 weeks. A different situation is apparent for the deuterio culture as shown in Fig. 2. There is a rather rapid formation of tissue over a 10-day period with little indication of autolysis in the following 80 days of the growth study period. This may be attributed to the fact that the enzymes responsible for autolysis are inhibited or inactivated in the presence of high concentrations of D_2O. Another contributing factor may be that the more stable carbon-to-deuterium bonds comprising the deuterated tissue may tend to resist autolysis

Penicillin Production—The *Penicillium* strains in this study varied markedly in their ability to produce antibiotic in a protio nutrient medium ranging from a low of 1.10 units/ml. to a high of 28.11 units/ml. of fermented broth. Preliminary studies indicated that much higher production was possible when corn steep liquor was incorporated in the nutrient medium. This was not used in the present study for reasons already noted. In the deuterio nutrient medium penicillin production was severely depressed and in most cases was completely inhibited. The repressive effects of D_2O on the production of alkaloids in belladonna have been reported by Uphaus *et al.* (7). Repression in griseofulvin production by the fungus *P. janczewskii* in the presence of high concentrations of D_2O was observed by Nona *et al.* (2). Nevertheless, in the present investigation two of the strains surveyed, PN-612 and PC-1177, appeared to be less adversely affected by D_2O than were the remaining strains. The penicillin production curves for the PC-1177 strain by the surface culture method in H_2O and D_2O nutrient media are shown in Figs. 1 and 2.

Maximum production in protio medium is found to occur in about 10 days. Peak production is followed by a rapid loss in activity and within a

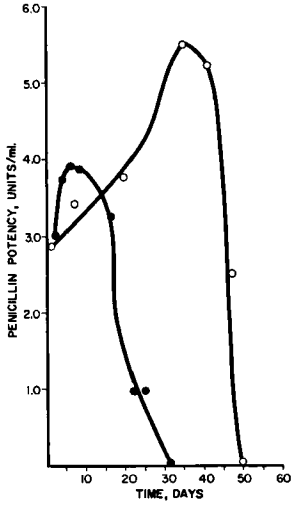

Fig. 3—Effect of adding phenylacetic acid on penicillin production by PN-612 in protio medium. Key: ●, protio medium; ○, 0.3% phenylacetic acid added to protio medium.

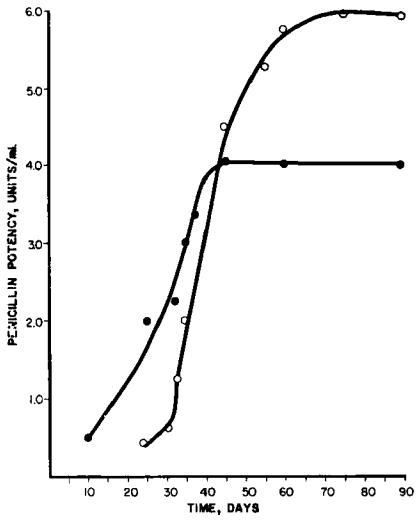

Fig. 4—Effect of adding phenylacetic acid on penicillin production by PN-612 in deuterio medium. Key: ●, deuterio medium; ○, 0.3% phenylacetic acid added to deuterio medium.

few days the penicillin titer reaches zero. In the deuterio cultures, however, the situation is quite different. There is noted a gradual increase in penicillin titer which reaches a maximum in about 60 days. There is no indication of antibiotic degradation over the 90-day study period. In the protio culture penicillin destruction took place after the pH of the medium reached its maximum value of 9.0 (see Fig. 1). Since penicillin is most stable in solutions having a pH range of 6.4 to 7.0 the observed breakdown and disappearance of penicillin is expected. In alkaline conditions the β-lactam ring of the penicillin molecule is broken producing a biologically inactive product.

In the deuterio culture, Fig. 2, the pD values remained below 7 throughout the study period. This fact apparently explains why the penicillin was not destroyed.

Effect of Phenylacetic Acid Additive—The effect of adding phenylacetic acid to the extent of 0.3% in the protio and deuterio nutrient media is illustrated in Figs. 3 and 4. Phenylacetic acid has been shown (8) to be a precursor in the biosynthesis of benzyl penicillin and to be useful in stimulating antibiotic production by fungi. This effect is clearly demonstrated in the protio culture (Fig. 3) and in the deuterio culture (Fig. 4) where the response to the phenylacetic acid additive is compared with the corresponding controls. It should also be noted that in the deuterio cultures degradation of the antibiotic did not occur after a peak in production as in the case of the protio culture.

At the conclusion of each fermentation period an aliquot of the culture medium was analyzed for D_2O content. In the series reported here all of the cultures showed a D_2O concentration above 87% and most were over 90%. Dilution of the deuterium content is accounted for by the 3% protio-dextrose in the nutrient and by exchange over the 3-month study period with atmospheric moisture. The opening of the culture flasks was plugged with cotton which may have permitted moisture exchange to a small extent.

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Keyphrases

Penicillium species—deuterium effect
Deuterium effect—*Penicillium* growth, antibiotic production
Protio nutrient media—*Penicillium* cultures
Phenylacetic acid effect—*Penicillium* cultures