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Research Articles

Effect of Deuterium Oxide on Culturing of *Penicillium janczewskii* I

Growth, Nutritional Factors, and Antibiotic Production

By D. A. NONA, M. I. BLAKE, and J. J. KATZ*

Penicillium janczewskii, **a mold which produces the antifungal antibiotic griseofulvin, was cultured in media containing 50, 75, and 99.6 per cent deuterium oxide. Protio- and deuterio-carbohydrates were used as carbon sources. Griseofulvin production decreased markedly with increase in deuterium content of the medium. There was also a marked reduction in mycelial dry weights with increase in deuterium oxide in the nutrient. The surface culture technique proved more effective than the shake method for both tissue growth and antibiotic formation.**

A VARIETY OF organisms, including bacteria, fungi, and algae, have been successfully cultured in media containing pure heavy water. The growth of the heterotroph *Euglena gracilis* in a fully deuteriated form has also been recently reported (1). Higher plants, including peppermint (2, 3), belladonna (4, 5), and duckweed (6), have been grown in nutrient solutions containing as high as 70% deuterium oxide. These studies have been summarized by Flaumenhaft (7) and by Katz (8), who have reviewed extensively the biological effects of deuterium.

Deuteriated organisms provide a practical source of metabolites containing deuterium in all

Received April 14, 1967, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612, and *the Chemistry Division, Argonne National Laboratory, Argonne, IL 60439
Accepted for publication May 25, 1967.
Presented to the Pharmacognosy and Natural Products Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by grant AI 06825-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and was performed in part under the auspices of the U. S. Atomic Energy Commission, Washington, D.C.

molecular positions where hydrogen is normally present. Many compounds of biological importance are not readily prepared by conventional synthetic methods, and for these, biosynthetic procedures are essential. Thus, Blake *et al.* (9) isolated deuteriated mannose, glucose, and several amino acids from *Scenedesmus obliquus* grown in 99% deuterium oxide, and Crespi *et al.* (10) obtained deuterio-DNA from deuteriated algae. Organisms which elaborate pharmacologically active compounds are of particular interest. A clavine alkaloid-yielding strain of *Claviceps purpurea* has been cultured (11) in pure D_2O and a highly deuteriated alkaloid has been prepared (12) by a replacement culture technique. Other molds have also attracted interest. Shaffer *et al.* (13) cultured *Penicillium notatum* and *Aspergillus fonseaceus* in heavy water some years ago, but antibiotic production and isolation were not investigated. The present study concerns the nutritional requirements for culturing the griseofulvin-producing mold, *P. janczewskii*, in a deuterium environment, and reports the effects of deuterium oxide on the production of the antibiotic griseofulvin. Griseofulvin, a systemically active antifungal agent, was originally isolated by Oxford *et al.* (14) and is produced biosynthetically by surface or submerged culture techniques (15–17) with various species of *Penicillia* (14, 18, 19). This constitutes the first report on the detection and isolation of an antibiotic obtained from a deuteriated organism.

EXPERIMENTAL

Preparation of Slant Cultures—The griseofulvin-producing strain, *P. janczewskii* (NRRL 2301), was used throughout this investigation. Slopes of malt extract agar in cylindrical glass tubes were streaked with a wire loopful of mold tissue. The tubes were plugged with cotton and incubated for 7 days at 25 ± 1°. Slants were stored in a refrigerator at 5° where they remained viable for at least 3 months.

Water Culture Techniques—A Czapek-Dox medium modified by addition of 0.5% potassium chloride (Table I) provided a well-defined synthetic medium for this investigation. The pH was adjusted, if necessary, to 4.8. The H_2O culture medium served as a control. The medium was placed in suitable culture flasks and plugged with nonabsorbent cotton. The flasks were sterilized by autoclaving for 15 min. at 15 p.s.i. in a Thermomatic-60 autoclave (Wilmot-Castle). Sugars and nutrient salt solutions were sterilized separately and combined aseptically. Carbohydrates were sterilized either by autoclaving, or by passing a solution through a Swinney adapter equipped with a Millipore filter disk of 0.22 μ porosity. Upon cooling, the medium was inoculated with 1 to 5 ml. (depending upon the medium volume) of a stock spore suspension.

TABLE I—COMPOSITION OF BASIC CULTURE MEDIUM FOR *P. janczewskii* (NRRL 2301)

Culture Medium	
Sodium nitrate	2.3
Potassium dihydrogen phosphate	1.0
Magnesium sulfate · $7H_2O$	0.5
Potassium chloride	0.5
Minor element concentrate	1.0
Dextrose	75.0
Distilled water, dilute to	1000.0
Minor Element Concentrate	
Ferrous sulfate	0.1
Copper sulfate	0.015
Zinc sulfate	0.1
Potassium molybdate	0.01
Distilled water, dilute to	100.00

Fermentation was carried out by employing one of two techniques.

Shake Method—Sixty milliliters of culture medium was placed in 250-ml. conical flasks which provided a liquid depth of approximately 2 cm. The inoculated culture flasks were agitated on an Eberbach rotary shaker at 200 r.p.m., and incubation was allowed to proceed in the dark at 25 ± 2°.

Surface Method—For small scale fermentation studies, 60 ml. of nutrient medium was placed in a 250-ml. conical flask. For preparative cultures, 500 ml. of medium was placed in 3-L. conical flasks, providing a liquid depth of about 2 cm. After inoculation, the cultures were incubated in darkness at 25 ± 2° without agitation.

Preparation of Deuteriated Media—Exchangeable hydrogen of inorganic salts and protio-sugars was replaced with deuterium by dissolution in pure deuterium oxide. The solutions then were evaporated to dryness under reduced pressure. This procedure was repeated to ensure complete exchange. A concentrate of minor elements of the culture medium was prepared with pure deuterium oxide (Table I). Partially deuteriated media composed of the basic nutrients and protio-glucose (7%) were prepared using 50, 75, and 99.6% deuterium oxide. A partially deuteriated medium was also prepared with fully deuteriated sugars (7%) and ordinary water. Fully deuteriated media were prepared using deuterio-sugars (7%) and pure deuterium oxide. The deuteriated monosaccharides were obtained from deuteriated algae according to the procedure of Blake *et al.* (9). Sugars obtained by this method contain 40% deuterio-glucose, 50% deuterio-mannose, and 10% of other hexoses and pentoses. Flasks containing deuteriated media were plugged with cotton and covered with aluminum foil to minimize isotopic dilution by exchange with atmospheric moisture.

Organic Substrates and Additives—A variety of carbon sources were tested for assimilation by the mold in media containing deuterium oxide. Glucose, glycerin, mannose, sodium acetate, and succinic acid were studied in concentrations of 3, 5, 7, and 9%, and dihydroxyacetone was tested in concentrations of 7%. All carbon sources were exchanged with D_2O prior to sterilization. For these experiments 60 ml. of the Czapek-Dox medium was prepared with pure deuterium oxide. Each of the substrates to be evaluated was sterilized separately and then added

TABLE II—COMPOSITION OF VITAMIN SUPPLEMENT ADDED TO FULLY DEUTERIATED MEDIA

Vitamin	Concn., mg./100 ml. D_2O
Biotin	0.5
Calcium pantothenate	0.5
Pyridoxine HCl	5.0
Riboflavin	0.5
Thiamine HCl	5.0

to the sterilized nutrients contained in 250-ml. conical flasks. An analogous series of controls was prepared with H_2O. Culture flasks were inoculated with 1 ml. of a stock spore suspension prepared with D_2O, whereas an H_2O spore suspension was used for the control cultures. The cultures were then incubated as static cultures at room temperature in the dark. Mycelial growth in each case was observed and recorded as a graded response during a 60-day period.

An additive of vitamin B-complex (Table II) was evaluated as a medium supplement in cultures grown in pure deuterium oxide. Concentrations of 1 to 2% were added to the culture medium. Algae extract (1%), bacto-peptone (2%), and corn steep solids (1–2%) were also studied as additives.

Mycelial Dry Weights—Two series of cultures were prepared, each consisting of ten 250-ml. conical flasks. Sixty milliliters of culture medium was prepared with protio-glucose and pure D_2O and placed into each flask of the first series. The same volume of an H_2O medium was placed into a second series of flasks to serve as controls. Each culture was inoculated with 1 ml. of a stock spore suspension in D_2O (H_2O for controls). At varying time intervals, up to a total of 64 days, the mycelium from a culture of each series was separated from its fermentor broth by filtration. The mycelium was dried to a constant weight in a vacuum oven at 100°. Both series were run in duplicate and the dry weights obtained were averaged and plotted as weight *versus* time in days.

Determination of Griseofulvin in Fermentor Broth—A Beckman DU spectrophotometer, model 2400 (Beckman Instruments, Inc.) equipped with matched silica cells of 10 mm. light path was employed for estimating griseofulvin in fermentor broth.

The analytical procedure employed in this study was a slight modification of the technique for the estimation of griseofulvin in fermentor broth developed by Holbrook *et al.* (20). The method is based on a process for the isomerization of griseofulvin to isogriseofulvin by acid treatment in methanol, as described by Grove *et al.* (21). Since untreated fermentor broth is unsuitable for spectrophotometric measurement, preliminary extraction of griseofulvin is necessary. Ethyl acetate was selected as the extractant because it does not absorb light between 260 mμ and 280 mμ and griseofulvin is reasonably soluble in it. The applicability of the procedure to a deuterium oxide medium was established by assaying fermentor broth both before and after additions of known quantities of standard griseofulvin.

Deuterium Analysis—At the conclusion of the growth period an aliquot of the culture medium was analyzed spectrophotometrically for D_2O content according to the method of Crespi and Katz (22). Harvested mycelium was analyzed for fixed deuterium which included both exchangeable and non-exchangeable deuterium. Five grams of dried tissue in a combustion boat was placed in a microfurnace and burned in a stream of oxygen at 700°. The water of combustion was collected in a −78° trap and transferred to an ampul which was then analyzed for deuterium content.

RESULTS AND DISCUSSION

Growth Observations—*P. janczewskii* grows well in a shake culture of protio-sugars and water. Growth is observed after 1 or 2 days of incubation. Tan pellicles are formed which reach a maximum diameter of about 70 to 75 mm. after 15 to 20 days. The medium becomes highly pigmented, imparting an amber color to the culture. When the mold is grown in a nutrient consisting of protio-sugars in pure D_2O, a lag period of about 10 days is observed before growth begins. Typically, the maximum pellicle size ranges between 10 and 20 mm. in diameter after 30 to 40 days of incubation (Fig. 1). Pigmentation of the fermentor broth is also delayed until approximately the fifteenth day, at which time the medium becomes tinged with yellow.

The mold also shows excellent growth in surface culture. In the H_2O controls, growth is observed 1 day after inoculation and by the seventh day a floating mycelial mat is formed which covers the entire surface of the culture medium. After about 10 days the mycelial mat begins to change color from white to gray-green. The fermentor broth becomes a deep amber by the eleventh day of incubation. In a culture medium containing 50% deuterium oxide, the appearance of the mycelial mat formed is much like that of the H_2O controls. The growth rate is delayed, but not drastically. A thick mycelial felt covers the entire surface by the fifteenth day of incubation as shown in Fig. 2. Pigmentation imparts a yellow color to the culture liquid early in the fermentation. As the D_2O content of the culture medium is increased to 75%, a further increase in lag period is observed. Little growth is noted before the seventh day of incubation, and by the fifteenth day the mycelial mat does not quite cover the entire surface of the culture medium. The gross morphology of the mycelium was similar to that cultured in 50% D_2O or the water controls. The mycelium differs, however, in that the mat becomes yellow by the fifteenth day. The mold can grow directly in a culture medium containing pure deuterium oxide; subculturing is not required. Only scant growth is observed before the tenth day, and isolated colonies begin to connect, forming a white lattice-type of growth after 15 days of incubation, as shown in Fig. 3. After 35 days, the surface of the culture medium becomes essentially fully covered. At the completion of the fermentation period the mycelium is not as mature as is the H_2O controls (Fig. 4). Figure 5 shows a marked reduction in the mycelial dry weights of the D_2O cultures when compared to the controls. The fermentation period necessary to reach the maximum weight was more than twice that of the water cultures. Dry weights obtained from both the water and deuterium cultures somewhat decrease, after reaching a maximum, because

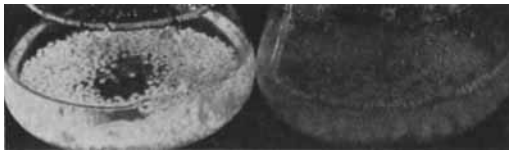

Fig. 1—Shake cultures 30 days after inoculation in 99% D_2O (left) and H_2O (right). Both cultures contain 7% protio-glucose.

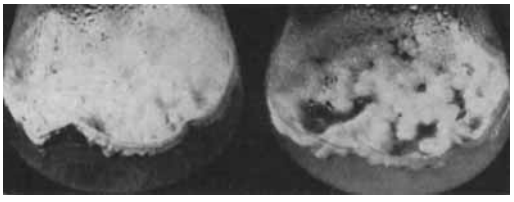

Fig. 2—Surface cultures 15 days after inoculation in 50% D_2O (left) and H_2O (right). Both cultures contain 7% protio-glucose.

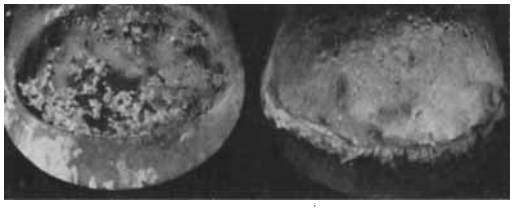

Fig. 3—Surface cultures 15 days after inoculation in 99% D_2O (left) and H_2O (right). The cultures contain 7% deuterio-glucose and 7% protio-glucose, respectively.

Fig. 4—Surface culture after 60 days' growth in medium containing 99% D_2O and 7% deuterio-glucose.

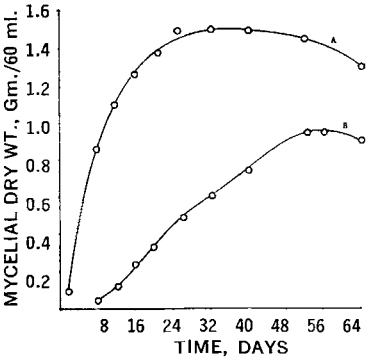

Fig. 5—Comparison of mycelial dry weights of surface cultures grown in (A) water and (B) 99% D_2O. Both cultures contained 7% protio-glucose.

of autolysis. Mature mycelium produced at the 99.6% deuterium level maintains a felt-like appearance. However, the color becomes yellowish-tan and the liquid portion of the medium shows a yellow pigmentation. At the conclusion of each fermentation period, a sample of the broth was analyzed for deuterium content. In no case was the final deuterium oxide concentration found more than 2% below the initial concentration.

Nutritional Factors—An organic substrate is essential to the growth of the mold. Protio-substrates were chosen for study which were also available as deuterio-moieties, and their ability to support growth in pure deuterium oxide was determined. Several concentrations (3, 5, 7, and 9% w/v) of each protio-substrate were prepared with 99.6% D_2O culture media. Growth was evaluated visually by assigning graded responses. The results are recorded in Table III. It is apparent that the mold can efficiently use glucose, mannose, glycerin, and, less successfully, succinic acid. Sodium acetate and dihydroxyacetone were considered as possible substrates since griseofulvin may be considered a condensation of acetic acid units, but the experimental results did not substantiate this hypothesis. Glucose or a deuterio-glucose and deuterio-mannose mixture in a concentration of 7 to 7.5% was employed in all D_2O culture media.

The major difficulty that the mold experiences in growing in a deuteriated environment appears to be in adapting to the solvent D_2O. The incorporation of deuteriated substrates into a culture medium seems to cause less of a problem for the fungus, no matter what the level of solvent deuteriation. Growth response of the organism in a nutrient solution composed of deuterio-sugars (7%) in ordinary water was not unlike that observed in culture media composed of protio-sugars in H_2O. The fixed deuterium of the dried mycelium in this experiment (obtained by combustion analysis) was found to be 18.8%. Growth characteristics observed in a fully deuteriated culture medium composed of deuterio-sugars and D_2O resembled closely those shown in media composed of protio-sugars in D_2O. A greater time period was required before initial growth, but thereafter no significant difference could be detected in the growth pattern. Combustion analysis of the dried mycelium showed the deuterium level to be slightly above 94% in the fully deuteriated mold.

Earlier studies have established that deuteriated fungi often show an increased nutritional fastidiousness. For instance, the yeasts *Torulopsis utilis* and *Saccharomyces cerevisiae* developed additional requirements for vitamins, particularly those of the B-complex group (23). Vitamins as well as algae extracts were necessary for the growth of fully deuteriated *Euglena gracilis* (1). The effect of medium fortification by vitamins was, therefore, studied. The carbohydrate concentration of the culture medium was 7%, and the response to various medium supplements was noted. The vitamin additive appeared to have little effect

TABLE III—UTILIZATION OF VARIOUS CARBON SUBSTRATES IN D_2O CULTURES

Substrate	% w/v	Incubation Period, Days[a] 6	10	12	14	16	18	20	24	28	32	38	60
Glycerin	3	0	±	±	±	+	+	+	+	+	+	+	+
	5	±	±	+	+	+	++	++	++	++	++	++	++
	7	±	±	+	+	++	++	++	+++	+++	+++	+++	+++
	9	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
Glucose	3	±	±	±	±	+	+	+	++	++	++	++	++
	5	±	±	±	+	++	++	++	++	++	+++	+++	+++
	7	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
	9	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
Mannose	3	0	0	±	±	±	±	+	+	++	++	++	++
	5	0	±	±	±	±	+	++	++	++	++	+++	+++
	7	0	±	±	+	+	+	++	+++	+++	+++	+++	+++
	9	0	±	±	+	+	+	+++	+++	+++	+++	+++	+++
Sodium acetate	3	0	0	0	0	0	±	±	±	±	±	±	±
	5	0	0	0	0	±	±	±	±	±	±	±	±
	7	0	0	0	0	±	±	±	±	+	+	+	+
	9	0	0	0	0	±	±	±	+	+	+	+	+
Succinic acid	3	0	0	0	0	±	±	±	±	±	±	±	+
	5	0	0	0	0	±	±	±	±	+	+	+	+
	7	0	0	0	±	±	±	+	+	+	++	+++	+++
	9	0	0	0	±	±	±	+	+	+	++	+++	+++

[a] 0, No growth; ±, marginal growth; +, ++, +++, graded response.

on the rate of mold growth. However, the mycelial mat formed was decidedly thicker and obviously superior to the nonvitamin control. Algae extract and bacto-peptone supplements exhibited no marked advantage. Corn steep solids showed a dramatic increase in growth in both water controls and in pure deuterium oxide media. The indefinite composition and the introduction of protio-atoms from such a supplement may interfere with deuteriation of the antibiotic biosynthesized, and was therefore not employed in further studies.

Antibiotic Production—The extent of griseofulvin production in surface cultures is noted in Fig. 6. Each point on the curve represents an average of values obtained from 5 separate batches. Antibiotic production in both surface and shake culture is observed to reach a plateau, which was followed by a gradual reduction in concentration. The effect of D_2O concentrations on peak antibiotic levels and fermentation periods are summarized in Table IV. Shake fermentation methods resulted in lower antibiotic titers than static culture conditions (Fig. 7). Griseofulvin titers in shake cultures of 99% D_2O exhibited a marked depression. Although there was good growth at this D_2O level,

TABLE IV—EFFECT OF D_2O CONCENTRATION ON PEAK GRISEOFULVIN PRODUCTION LEVELS

D_2O, %	Griseofulvin Titer, mcg./ml.	Time, Days
	Surface Culture	
0	115	17
50	90	24
75	52	40
99.6[a]	50	46
	Shake Culture	
0	55	17
99.6[a]	25	45

[a] Vitamin supplementation.

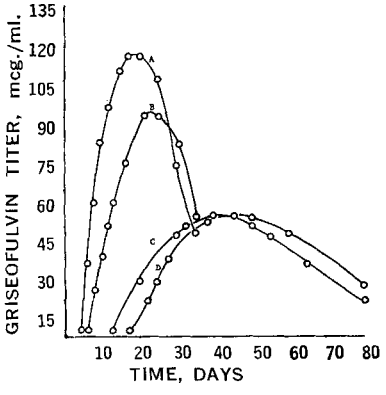

Fig. 6—Effect of D_2O content of the medium on griseofulvin production in surface culture. Key: A, *0% D_2O;* B, *50% D_2O;* C, *75% D_2O;* D, *99% D_2O plus vitamin supplement. All cultures contained 7% protio-glucose.*

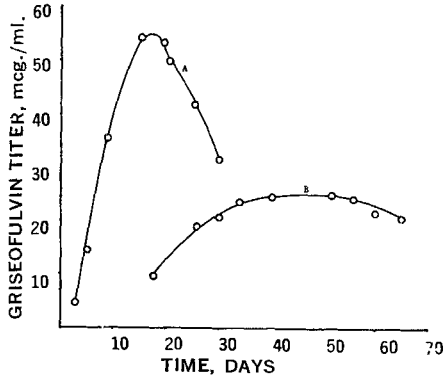

Fig. 7—Production of griseofulvin in shake cultures containing 7% protio-glucose and grown in (A) H_2O *and* (B) *99% D_2O plus vitamin supplement.*

it became apparent during the course of this study that good growth is not synonymous with good griseofulvin production. The fermentation method of choice proved to be surface culture, and was therefore used exclusively throughout the investigation after early fermentation trials.

In surface cultures the culture media, consisting of protio-sugars in 50% D_2O, exhibited about a 20% reduction in griseofulvin titer when compared with H_2O controls. When the concentration of deuterium oxide was increased to 75%, the griseofulvin titer of the fermentor broth was decreased by approximately one-half. At a D_2O concentration of 99.6%, griseofulvin production was severely impaired, and only small amounts of griseofulvin were produced. By supplementing the 99.6% D_2O culture medium with vitamin B-complex, a griseofulvin titer could be attained which approached that observed in the 75% D_2O cultures.

CONCLUSIONS

The deuterium isotope effect on the growth of the mold *P. janczewskii* is clearly a repressive one. Growth, griseofulvin production, and pigmentation of the mold are all inhibited with increasing concentrations of deuterium oxide. Corn steep solids and vitamin B-complex additives have demonstrated an enhancement of both mycelial growth and griseofulvin production in highly deuteriated media. It is tempting to infer from these experimental observations that the isotope effect in the mold is due to an incomplete medium. However, the deuterium effect in biological systems is extremely complex and no doubt many factors are involved. A normally endogenous metabolite may become a required exogenous factor. A maximum enhancement level is reached with such additives; a 5% concentration of vitamin supplement offers no advantage over a 2% concentration. Similar trends were noted with corn steep solids. High deuterium concentrations result in the utilization of available metabolites to maintain the nutrition of the isotopically altered mold. The fungus apparently becomes frugal, meeting only those needs required for survival. Griseofulvin production and elaboration of pigment can be considered to be nonessential for mold survival, and can therefore be depressed without damage to the organism. Deuterium seems to interfere with the basic integrity of the mold enzymes which are necessary to antibiotic and pigment production. The morphological changes in the deuteriated fungi appear to be due to a general disruption of the cell enzyme physiology.

It is the objective of subsequent studies to isolate and characterize the partially and fully deuteriated griseofulvin produced by the organism grown under the conditions described here. Such compounds may prove useful in studying the biogenesis of antibiotics and may contribute to the elucidation of the mechanism by which these compounds function in the body. The effect of deuterium on the antibiotic activity must also be examined. It is expected that since carbon-to-deuterium bonds are more stable than carbon-to-hydrogen bonds, and if detoxification requires the rupture of such bonds, then the deuterio form of the drug should possess a more prolonged antifungal activity. Preliminary results appear to show that this is the case.

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