Effect of Deuterium Oxide on the Saprophytic Culture of *Claviceps* II

Alkaloid Production

By R. G. MRTEK*, H. L. CRESPI, M. I. BLAKE, and J. J. KATZ

The effect of growth conditions on alkaloid production by *Claviceps* in a deuterated environment was explored. Alkaloid formation was strongly inhibited in fully deuterated organisms. Consequently, a replacement culture technique was developed for the biosynthesis of highly deuterated alkaloids. Cultures grown in an ordinary hydrogen environment were transferred to a deuterated medium containing various substrates such as tryptophan, mevalonic acid, phenylalanine, succinic acid, and sugars, either as hydrogen or deuterium compounds. Multimilligram amounts of alkaloids were obtained from replacement cultures containing phenylalanine and deuterio-succinic acid as substrates. The alkaloid fraction, consisting of elymoclavine with minor amounts of agroclavine, was isolated by solvent extraction. The alkaloids were identified by thin-layer chromatography, and the incorporation of deuterated substrate into the alkaloids by the replacement culture technique was verified by NMR spectroscopy.

In the previous paper (1) of this series the nutritional factors involved in optimal growth of *Claviceps* (strain 47A-Tyler) in high concentrations of heavy water were investigated. The successful maintenance of a fully deuterated organism in saprophytic culture was achieved on a medium containing fully deuterated mixed monosaccharides (2%), deuterio-succinic acid (0.5%), and traces of vitamins in a low phosphate salt medium. However, in high concentrations of D_2O the formation of alkaloids was found to be strongly impaired. Consequently, a replacement culture technique was used to induce the elaboration of deuterated alkaloids from water-grown fungal tissue that was resuspended in a medium containing more than 98% D_2O.

EXPERIMENTAL

General Culturing Techniques—The organism used for all experiments in this investigation was a high alkaloid-yielding strain of *Claviceps purpurea*, strain 47A. Conditions for growth, culturing techniques, and adaptation studies in D_2O are described in the previous paper (1).

Replacement Culture Method—The mycelia of 1-L. sterile cultures were used as the "raw tissue" for preparative replacement cultures. This tissue was transferred aseptically from 3-L. conical growth flasks to 1-L. polypropylene bottles, which had been previously sealed with aluminum foil and then autoclaved. The bottles were then centrifuged for 15 min. at 1600 r.p.m. in an International centrifuge, size 2, model V. The supernatant nutrient was decanted and 600 ml. of sterile D_2O was added to the packed mycelia in each bottle. The contents were stirred thoroughly with a sterile glass rod and the bottles were again centrifuged. This process was repeated with 400 ml. of sterile D_2O. Then the mycelia were washed twice with 500-ml. portions of sterile 0.1 *M* phosphate buffer in D_2O, pD 7.1. Finally, 800 ml. of 0.1 *M* phosphate D_2O buffer solution plus any substrates, as indicated in the particular experiment, was added to each bottle. This constituted the replacement medium. The mycelia were dispersed in the medium and the contents were transferred to a clean sterilized 3-L. culture flask. The bottle was rinsed with 200 ml. of sterile replacement medium, and this solution was also added to the culture flask. A 5-ml. aliquot of the culture was withdrawn aseptically, centrifuged, and the supernatant solution analyzed for deuterium content. The culture flask was returned to the shaker for incubation. All manipulations of exposed cultures were performed in a bacteriological hood under sterile conditions.

In developing the optimum conditions for alkaloid production on a preparative scale and in evaluating the effect of substrates, preliminary replacement cultures were prepared using smaller volumes of medium. The tissue from 50 ml. of a dense aqueous fungal suspension was centrifuged, washed, and transferred to two replacement cultures of 50-ml. volume each in 125-ml. conical flasks, resulting in a twofold dilution of the semisolid fungal culture. All replacement culture flasks were incubated at 25° on a rotary shaker. Deuterium oxide and alkaloid analyses were performed periodically throughout the culturing period.

Addition of Substrates—A variety of metabolites was examined by the replacement culture technique. Phenylalanine and succinic acid were incorporated directly into the medium prior to autoclaving. Heat labile components, such as mevalonic acid and vitamin mixtures, were sterilized by filtration of a concentrate through a Millipore filter into the sterile medium.

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Mevalonic acid was obtained from a commercial source[1] as a high purity dibenzylethylenediamine salt. The free acid was prepared by passing an aqueous solution of the salt through an ion-exchange resin column,[2] 1 × 5 cm. The free acid was eluted from the column with water until the pH was neutral, and the eluate was then concentrated to a small volume with a rotary evaporator. Ten milliliters of phosphate buffer was added, and the solution was then lyophilized. The solids were dissolved in 25 ml. of D_2O and the solution was lyophilized again. Finally, the solids were dissolved in 10 ml. of D_2O and the solution was added to the culture medium by filtration through a Millipore filter.

Tryptophan, which is relatively insoluble and heat labile, was incorporated directly into the replacement medium. The solution was then filtered through a Millipore filter. The filtrate was collected in a sterile 2-L. flask to which was attached a short length of cotton-plugged sterile pressure tubing.

All replacement cultures were prepared in duplicate and were incubated on a rotary shaker. Deuterium analyses of the nutrient were performed at the start of an experiment and periodically throughout the course of the study. Periodic alkaloid determinations were performed during the incubation period.

Alkaloid Analysis—Total alkaloids were determined by a modification of the method of Michelon and Kelleher (2). A sample of medium (5 to 10 ml.) was withdrawn aseptically and clarified by centrifugation. An aliquot of the supernatant solution containing between 5 and 50 mcg. of total alkaloid was transferred to a 25-ml. Squibb funnel. The solution was made basic with a few drops of ammonium hydroxide and extracted with two 7-ml. portions of chloroform. The chloroform extracts were placed in a separator containing 2.0 ml. of water. Exactly 2.0 ml. of 0.1% *p*-dimethylaminobenzaldehyde in 50% (v/v) H_2SO_4 was pipeted into the separator which was then stoppered and shaken vigorously for 1 min. The aqueous layer was transferred quantitatively to a 15-ml. centrifuge tube and was allowed to stand until 15 min. had elapsed from the time that the acidic reagent had been added to the separator. Then 0.10 ml. of a 0.1% aqueous sodium nitrite solution was added to the centrifuge tube. A blue color developed upon gentle agitation of the tube. The tube was centrifuged for 1 min. and the clear supernatant solution was transferred to a 1-cm. cell by a dropping pipet. Absorbance measurements were made at 585 mμ with a Cary-14 spectrophotometer. Total alkaloid calculated as elymoclavine was determined from a standard curve prepared with authentic elymoclavine.

Deuterium Analysis—The deuterium oxide content of the nutrient was determined by the infrared absorption method described by Crespi and Katz (3).

Isolation of Alkaloids—The mycelia in the replacement cultures were separated from the liquid by filtration through a coarse sintered-glass funnel and the filtrate was clarified by passage through a bed of diatomaceous earth.[3] After the volume was reduced to 300 ml. on a rotary evaporator at 30°, the solution was rendered basic with 25 ml. of ammonium hydroxide and extracted with 400 ml. of chloroform in a 1-L. separator. Three extractions with 200-ml. portions of chloroform were carried out and the combined chloroform extracts were concentrated to 200 ml. in a rotatory evaporator at 30°. The concentrate was washed three times with 50-ml. portions of 1 *N* ammonium hydroxide, and the ammoniacal washings were discarded after showing a negative alkaloid test with the modified Ehrlich's reagent. The chloroform solution was then extracted with 20-ml. portions of 2% tartaric acid solution until the extracting solution gave a negative alkaloid test.

The tartaric acid solution was cooled in an ice bath, adjusted to pH 8.5 with ammonium hydroxide, and extracted three times with 15-ml. portions of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate, concentrated to a small volume in a rotary evaporator at 25°, and transferred quantitatively to a tared glass tube sealed at one end. The solution then was evaporated to dryness at room temperature under a stream of nitrogen gas. Last traces of solvent were removed by evacuation in a vacuum line for 30 min., and the yield of alkaloid determined by weighing.

Thin-Layer Chromatography—Chromatographic analysis of alkaloid fractions was performed on Silica Gel G plates. Plates were spread to a uniform 250-μ layer with a Desaga variable thickness spreader, dried at room temperature overnight, and stored in a desiccator until used. Immediately before use, the plates were activated by heating for 20 min. at 110°. After the plates had cooled in a desiccator, spots containing 5 to 20 μl. of sample in chloroform were applied.

The chromatogram was developed in an oversaturated chamber containing ethyl acetate–acetone–diethylamine (10:10:2). The solvent front was marked, and the chromatogram was allowed to dry thoroughly at room temperature. The dried chromatogram was sprayed with von Urk's reagent prepared by dissolving 0.5 Gm. *p*-dimethylaminobenzaldehyde in a mixture of 50 ml. of 25% HCl and 50 ml. of 95% ethanol. After the chromatogram was sprayed, it was heated at 110° for 5 min. The plate was cooled and first sprayed lightly with a fresh solution of 0.05% $NaNO_2$ in 50% ethanol and then sprayed with a mixture of equal volumes of 95% ethanol and 25% HCl. The spots containing the indole alkaloids became intensely blue. R_f values were measured from densitometric scans prepared when the color of the spots was at maximum intensity.

RESULTS AND DISCUSSION

In cultures grown under fully deuterated conditions (1, 4) alkaloids were never detected in the medium in greater than trace amounts. Inocula size, aeration, light, and varying culture volumes had little, if any, effect in stimulating the production of alkaloids, even after incubation periods as long as 3 months. Fully deuterated cultures incubated in closed cabinets containing a D_2O saturated atmosphere were observed for periods up to 6

[1] Calbiochem, Los Angeles, Calif.
[2] The column contained AG 50W-X8 in the hydrogen form, Bio-Rad Laboratories, Richmond, Calif.
[3] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

months. Tissue growth appeared to cease after about 2 months. The deuterio-culture became pigmented, as in the case of ordinary cultures, but no alkaloids formed. Thin-layer chromatograms failed to reveal the presence of even trace amounts of alkaloids in any culture medium obtained from fully deuterated *Claviceps*.

Decantation of the medium from a deuterated culture, followed by replacement with fresh complete D_2O medium, caused additional tissue growth and elaboration of pigment, but no alkaloid synthesis. Essentially the same observations were made when the deuterated cultures were supplemented with added deuterio-monosaccharides and vitamins. Addition of (D_2O-exchanged) protio-phenylalanine or protio-tryptophan also failed to stimulate alkaloid production. Different stock slants of *Claviceps* were adapted to growth in D_2O, but the resultant cultures all failed to produce alkaloids. The protio-ancestor of each deuterium experiment was carried through successive H_2O transfers to test for strain regression. Such "control" protio-cultures were still capable of producing clavine alkaloids at the conclusion of the deuterium experiments.

Thus, the prospect of obtaining alkaloids from a culture of *Claviceps* grown under fully deuterated conditions is not good. The absence of alkaloids may be the result of (*a*) an incomplete medium, or (*b*) a primary repression of one or more enzymatic mechanisms involved in alkaloid synthesis. As for the first possibility, the development of a defined medium for any biologic clone is a highly empirical task. Apart from the obvious additions of selected substrates and variation in phosphate concentration which is known to have a strong effect on alkaloid production in water, there are no obvious nutritional factor or factors that may be invoked to allow *Claviceps* to produce clavine alkaloids. To locate the specific sites of the deuterium isotope effect in the biochemical mechanisms of the fungus requires a much more thorough understanding of individual enzyme systems responsible for alkaloid production than is now available for the organism under normal conditions.

Based on the experiments described above, a new set of experimental conditions was devised to study alkaloid production in *Claviceps* at high levels of D_2O. These experiments were designed to require the organism to utilize deuterium under physiologic conditions in which alkaloid synthesis is known to occur.

Replacement culture techniques have been employed by other investigators in studies involving the saprophytic culturing of strains of *Claviceps* in aqueous media. Thus, Brady and Tyler (5) investigated the effect of certain precursors on the production of clavine alkaloids, while Groger and Erge (6) reported the effects of mannitol solutions in supporting alkaloid production by the replacement technique. Abe *et al.* (7) used a surface replacement culture technique in their studies of alkaloid production. Suitable choice of replacement media allows alkaloid production to be studied under conditions independent of growth. Thus, replacement studies permit the separate analysis of the effects of deuterium isotope substitution on vegetative growth and on alkaloid production.

In these experiments the organism was allowed to mature on an aqueous medium containing 10% mannitol and 0.5% protio-succinic acid added to a stock solution of Tyler's salt mixture (8). The organism was then introduced into a D_2O medium containing only deuterium exchanged phosphate buffer salts and selected substrates. Growth was virtually stopped by selecting a buffer of high ionic strength and a pH far removed from the growth optimum. No trace elements or other substrates normally required in a complete growth medium were added. Under such conditions the clone may either undergo autolysis or it may supply its own respiratory requirements by whatever metabolic routes are still available to it. It is recourse to the latter alternative that usually results in the elaboration of metabolites which are the end products of normally nonobligatory pathways.

In preliminary studies performed on replacement cultures a number of compounds, singly and in combination, were tested for their ability to stimulate alkaloid production. Alanine, 5-fluorotryptophan, *p*-fluorophenylalanine, and anthranilic acid were not found to induce alkaloid synthesis. The amino acid fraction isolated from fully deuterated algae (9) likewise had no demonstrable effect on alkaloid production. Although fully deuterated aspartic acid, glutamic acid, alanine, and glycine are present in large amounts in this mixture, no alkaloids are formed. In accordance with conclusions arrived at by studies in H_2O medium, only phenylalanine and tryptophan of the amino acids tested are capable of serving as substrates for clavine alkaloid synthesis in D_2O replacement cultures.

Figure 1 demonstrates the effect of various substrates on alkaloid production in a replacement culture. These include 0.1% tryptophan, 0.2% mevalonic acid, 2.0% protio-glucose, and 2.0% deuterio-sugars (glucose–mannose, 1:1), obtained from fully deuterated algae (9). In most instances a vitamin mixture supplement was added (1). In the absence of additives no alkaloid production took place (curve F). Mevalonic acid alone was a poor substrate (curve E), but tryptophan alone did serve as an adequate substrate (curve D). However, in the presence of mevalonic acid and vitamins, alkaloid production increased somewhat (curve C). Maximum alkaloid production occurred when tryptophan, mevalonic acid, sugars, and vitamins were included in the medium. Protio and deuterio-sugars produced essentially the same effect as can be seen in curves A and B.

In another series of experiments (Fig. 2) protio-phenylalanine and protio-succinic acid were utilized as substrates in replacement cultures. Phenylalanine alone at a concentration of 0.5% was the most effective in stimulating alkaloid formation (curve E). When succinic acid was added, alkaloid production diminished (curve D). Severe inhibition resulted when the succinic acid concentration was increased tenfold and the phenylalanine concentration was reduced to one-fifth (curve C). After 20 days not even a trace of alkaloid was detected in the medium. The addition of protio-glucose suppressed alkaloid synthesis (curve A). This may be a result of the utilization of the substrates for growth rather than for alkaloid production. When all substrates were omitted the organism failed to produce alkaloids in any measurable quantity.

Multimilligram amounts of deuterated alkaloids

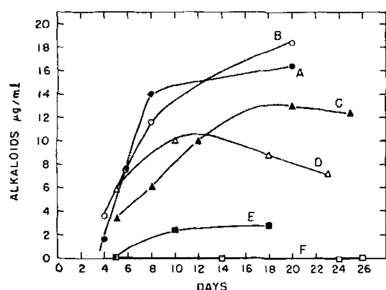

Fig. 1—Effect of substrates on alkaloid production in D_2O replacement cultures. Key: A, *0.1% tryptophan, 0.2% mevalonic acid, 2.0% protio-glucose, and vitamins in trace amounts;* B, *tryptophan, mevalonic acid, deuterio-sugars, and vitamins;* C, *tryptophan, mevalonic acid, and vitamins;* D, *tryptophan;* E, *mevalonic acid;* F, *control.*

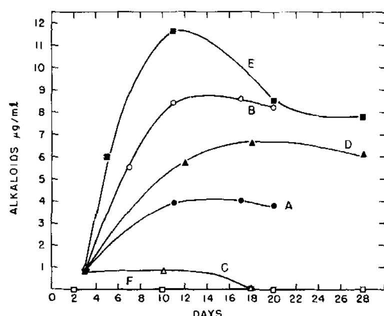

Fig. 2—Alkaloid production in D_2O replacement cultures in the presence of certain substrates. Key: A, *0.5% phenylalanine, 2.5% succinic acid, 2.0% protio-glucose;* B, *0.5% phenylalanine, 2.5% succinic acid;* C, *0.1% phenylalanine, 2.5% succinic acid;* D, *0.5% phenylalanine, 0.2% succinic acid;* E, *0.5% phenylalanine;* F, *control.*

were obtained from large-scale preparative replacement cultures in D_2O. For this purpose the replacement medium contained 0.5% protio-phenylalanine and 0.5% protio-succinic acid dissolved in 99% D_2O. The phenylalanine and succinic acid were exchanged[4] with D_2O prior to incorporation into the medium. In a second experiment protio-phenylalanine and succinic acid-d_6, both of which were exchanged with D_2O, were used as substrates in a D_2O medium. In the first culture, the only source of deuterium accessible to the organism was the solvent D_2O used in the medium. In the second culture the organism was required to utilize deuterium from both the solvent and the nonexchangeable positions of the deuterio-succinic acid. Table I gives the yield of deuterated alkaloids obtained from these cultures. A total of 24.8 mg. was obtained in the first experiment and 9.0 mg. in the second.

Thin-layer chromatography of the purified deuterated alkaloid fraction indicated that only elymoclavine was formed in the highly deuterated cultures. No agroclavine could be detected in the alkaloid fraction. With the chromatographic solvent system employed in this study protio-elymoclavine and protio-agroclavine had R_f values of 0.29 and 0.59, respectively. Deuterated elymoclavine had a distinctly lower R_f value of 0.27, as observed previously for other deuterated compounds (9).

[4] Deuterium atoms exchange only for the hydrogen of the carboxyl and amino groups.

Table I—Yield of Deuterated Alkaloids by Replacement Culture Method

Substrate	D_2O Content,[a] %	Incubation Period, Days	Vol. at Harvest, ml.	Total Alkaloids, mg.
Protio-phenylalanine (0.5%) Protio-succinic acid (0.5%)	99.0	26	1900	24.8
Protio-phenylalanine (0.5%) Deuterio-succinate (0.5%)	98.8	24	1920	9.0

[a] Determined at harvest.

A complete nuclear magnetic resonance (NMR) analysis was carried out on authentic samples of protio-clavine alkaloids and on deuterated elymoclavine. Spectra were obtained with a Varian HA-100 spectrometer using alkaloid solutions which were 0.1 *M* in pyridine-d_5. From these data it was possible to deduce the H/D ratio at the various hydrogen positions in the molecule. The elymoclavine obtained from replacement cultures in 99% D_2O with added protio-succinate and protio-phenylalanine was highly deuterated at all positions. The hydrogens at positions 10 and 17 were completely replaced by deuterium and the hydrogens at positions 4, 5, 6 (CH_3—N), 7, and 9 contained from 68 to 86% deuterium. These data indicate extensive labilization of carbon-bound hydrogen during the biosynthesis of the alkaloid. In a similar replacement culture containing deuterio-succinate in place of protio-succinate (Table I) the over-all level of deuteration was not significantly different. The complete details and analysis of these experiments are to be published in a subsequent paper.

When deuterated tissue was used in a replacement culture experiment, no alkaloids were elaborated. In addition, when deuterated tissue was introduced into an H_2O replacement medium, no alkaloid synthesis occurred. The phenomenon of alkaloid production is probably nonessential and organisms which elaborate this class of compounds do so only under specific and often nonobligatory conditions.

It appears that a primary effect of deuterium in biological systems is the suppression of nonessential metabolism. The stress imposed on *Claviceps* by the introduction of large amounts of deuterium results in the utilization of available metabolites to maintain the nutrition of the isotopically altered fungus at the expense of alkaloid synthesis.

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Establishment of Sink Conditions in Dissolution Rate Determinations

Theoretical Considerations and Application to Nondisintegrating Dosage Forms

By MILO GIBALDI and STUART FELDMAN

The importance of approximating perfect sink conditions for the determination of dissolution rates which may be correlated with *in vivo* results is pointed out. A theoretical treatment of zero and first-order dissolution under perfect sink and nonsink conditions is presented. It is shown that unless sink conditions are maintained, *in vitro* results will bear little relationship to *in vivo* observations. The use of an organic solvent phase which can function as a reservoir for dissolved drug and thereby maintain sink conditions in the aqueous dissolution fluid is demonstrated for a model nondisintegrating tablet exhibiting zero-order drug release. The use of an organic solvent reservoir for the determination of first-order dissolution rates is also discussed.

One of the most important as well as most difficult approaches in the biopharmaceutic evaluation of a drug is the quantitative correlation of absorption and *in vitro* dissolution kinetics. Levy *et al.* (1) have recently reported the development of a single *in vitro* dissolution rate test which correlates quantitatively with the gastrointestinal absorption, in man, of aspirin from three markedly different types of dosage forms. The basic method, however, is limited to the study of drugs which are relatively water soluble.

Dissolution rate-limited absorption implies that there is no build-up of drug concentration in the gastrointestinal fluids, *i.e.*, the fluids function as a perfect sink. Unless this condition is embodied in the design of the *in vitro* test (*i.e.*, drug concentration in the dissolution fluids does not exceed 10 to 20% of solubility), *in vitro* results will bear little relationship to *in vivo* observations (2).

As noted recently by Levy (2), those drugs which represent the greatest dissolution problem are also those which are least soluble and give the greatest difficulty with respect to maintenance of perfect sink conditions. Frequently, it is necessary to use exceedingly large volumes of solvent for this purpose and to adopt very sensitive analytical procedures. At times, it may be very difficult to follow the dissolution of more than a small fraction of the drug contained in the dosage form. Under these conditions one must assume a uniform pattern of release rate. Wood (3), however, has pointed out that in practice there are many cases in which one point in time or a rate constant does not characterize the dissolution process and a full dissolution curve (based on the total drug content of the dosage form) is required for *in vitro* to *in vivo* correlation.

A definite need exists for the development of methodology to maintain sink conditions during dissolution rate determinations of poorly soluble drugs. Levy (2) has suggested two possibilities: the use of an upper organic phase which can act as a reservoir for the dissolved drug or the addition of adsorbents to the aqueous medium. The ability of adsorbents to maintain "sink" conditions had been demonstrated previously by Wurster and Polli (4). Both approaches involve the same principle, *i.e.*, removal of dissolved drug from the dissolution medium and prevention of accumulation. The removal of drug from the dissolution fluid is analogous to removal of drug from the gastrointestinal fluids by the absorption process in dissolution rate-limited absorption.

The present report concerns the theory and application of the use of an upper organic solvent

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