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

Nutritional Factors

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At high concentrations of heavy water, the saprophytic growth of a clavine-producing strain of *Claviceps* (strain 47A-Tyler) is highly dependent upon the carbon source. Minimal media containing only succinate as the carbon source support growth at D_2O levels up to 90 per cent, but growth is progressively inhibited by deuterium oxide, and is extremely slow at the 80–90 per cent level. The addition of glucose, however, leads to good vegetative growth not only at 90 per cent D_2O, but also at 99.6 per cent D_2O. Preliminary investigations indicate that alkaloid accumulation is also a function of both deuterium concentration and the composition of the medium.

THE SUCCESSFUL culture of living organisms under conditions in which all hydrogen is replaced by deuterium has received much attention in recent years. The expanding collection of deuteriated organisms includes bacteria, certain fungi, and a variety of algae, and is the subject of a review by Flaumenhaft *et al.* (1). The isolation (2) of fully deuteriated growth factors from algae grown in heavy water has made possible the growth of some of the more fastidious heterotrophs (3, 4). The maintenance of completely deuteriated *Euglena gracilis* under heterotrophic culture (5) is a notable example.

Isotopically substituted organisms provide a useful source of raw materials. NMR spectroscopic studies of purified photosynthetic pigments (6) obtained from deuteriated organisms have indicated the stability of the deuterium positions in chlorophyll during active photosynthesis. Deuteriated metabolic products excreted into the culture medium by an organism may be isolated, purified, and characterized. The investigation of these products may provide fundamental information concerning the pathways involved in the synthesis of these substances by the organism. Organisms which excrete pharmacologically active drugs or their congeners are especially inviting for studies of this nature.

The saprophytic culture of a strain of *Claviceps purpurea* by Abe (7) and the subsequent discovery of the water-soluble group of clavine alkaloids produced by this organism have led to a great deal of study of the culture requirements of several strains of this organism (8–10) and on the possible precursors of alkaloid synthesis (11–13). It is well established that the requirements for growth and alkaloid production vary among strains, and often slight changes in media composition affect markedly the success of the culture. With the inclusion of heavy water in the nutrient solution, the problem becomes even more complex since an optimum nutrient for growth in D_2O may not necessarily be optimum for the same strain of organism grown in an H_2O medium. With such narrow tolerance limits for nutrient changes in any single clavine-producing strain of *Claviceps*, it is of primary importance to obtain a medium which will make use of available deuteriated substrates to support effective vegetative growth as well as to induce production of alkaloids.

EXPERIMENTAL

Culture of Claviceps in H_2O.—The organism selected for these studies is a high alkaloid-yielding variety of *C. purpurea*.[1] The organism is the ergot of *Pennisetum typhoideum* and was isolated originally in French Equatorial Africa. Slants were stored at 5° on an agar medium containing Tyler's salt mixture (8) with protio-succinic acid[2] (0.5%) and protio-mannitol (10%) added as carbon sources. Aqueous shake cultures were grown on the same medium without the agar. The media were adjusted to pH 5.2 with solid potassium hydroxide and autoclaved at 15 p.s.i. for 15 min. Incubation was carried out at 25 ± 1°. Rapid vegetative accumulation was maintained by subculturing every 14 days.

Growth took place in 50 ml. of medium contained in 125-ml. cotton-plugged conical flasks on an Eberbach rotary shaker at approximately 200 r.p.m. A dense suspension of fluffy colonies was obtained which could be pipeted easily with a sterile 5-ml. wide-mouth virological pipet. All

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[1] Slant cultures of the organism, designated as strain 47A, were generously supplied by L. R. Brady and V. E. Tyler, University of Washington, Seattle.

[2] The prefix "protio-" as used throughout this paper designates the natural or hydrogen-containing compounds. The prefix "deuterio-" designates compounds containing deuterium in place of hydrogen at all positions.

inocula for experimental studies were taken from these shaken H_2O cultures at the seventh day of growth.

Adaptation Studies in D_2O.—Media used for studies of growth in various concentrations of deuterium oxide were prepared by diluting a double strength stock solution of Tyler's salt mixture (8) in deuterium oxide of greater than 99.6% isotopic purity with appropriate amounts of water and deuterium oxide. Initial adaptation studies were performed by subculturing from H_2O media to media of 50, 75, and 90% D_2O. One and two milliliters of a thick suspension of vegetative tissue were used to inoculate 25- and 50-ml. cultures, respectively. Both protio-succinic acid and deuterio-succinic acid were used in these studies. The acid was dissolved in the salt solution and the pH adjusted to 5.2 with solid potassium hydroxide. Solutions were autoclaved in 50- or 125-ml. cotton-plugged conical flasks containing 25 or 50 ml. of nutrient medium, respectively, in a Steroclave model 25X (Wisconsin Aluminum Foundry Co., Inc., Manitowoc, Wis.) which contained 99.6% deuterium oxide.

Aeration was accomplished by passing a stream of D_2O-saturated sterile air through a bubbler-top fitted conical culture flask. Cultures were agitated on an Eberbach rotary shaker. Stationary cultures were placed into large cabinets and incubated in the dark. The medium in the standing cultures was mixed daily by gentle hand rotation of the culture flask to make certain that any surface pellicles which formed were able to derive sufficient nutrient. Cultures containing D_2O concentrations of 95% or higher were incubated in a small closed cabinet in an atmosphere saturated with deuterium oxide. Blank solutions of various isotopic concentrations were treated in the same manner as cultures and analyzed periodically (14) for isotopic dilution by atmospheric water. Aliquots of nutrient media were also analyzed by this method after clarification by centrifugation.

Modifications in Culture Medium.—It is generally accepted that organisms in the process of adaptation to growth in high concentrations of deuterium oxide may undergo a series of changes in nutrient requirements (1). For this reason several modifications were incorporated into the medium, either as individual changes or in combinations. (See Table III.) The phosphate content of the medium was adjusted to be either 0.1% KH_2PO_4 (1 Gm./L.) as in Tyler's original medium, or 0.01% KH_2PO_4 (0.1 Gm./L.).

Since fully deuteriated mannitol was unavailable, protio-glucose (reagent grade), protio-mannose (C.P.), and a mixture of fully deuteriated monosaccharides (2) were chosen to replace the hexitol nutrient component. The mixed deuteriated sugars contain approximately 40% deuterio-glucose, 50% deuterio-mannose, and 10% of other hexoses and pentoses (15). The sugars were autoclaved in deuterium oxide as a separate portion of the medium.

Succinic acid was used in varying concentrations in the medium, either in combination with a sugar mixture or alone. Succinic acid (either protio- or deuterio-) was added to media in concentrations of 0.5, 1.0, 2.0, 5.0, and 7.0% w/v. Fully deuteriated succinic acid is routinely prepared in this laboratory in gram quantities by the reduction and exchange of acetylene dicarboxylic acid in deuterium oxide. The product is recrystallized from hot deuterium oxide, yielding a compound which has deuterium in all of the molecular positions where hydrogen is normally found.

A vitamin mixture containing the following concentrations of vitamins, in mg./L., was added as a supplement: calcium pantothenate, 5.0; nicotinic acid, 5.0; thiamine hydrochloride, 50; biotin, 50; pyridoxine hydrochloride, 50; riboflavin, 5.0. The vitamin mixture (0.1–0.2 ml.) was diluted to 2 ml. with D_2O and sterilized by filtration upon addition to the sterile nutrient medium. A freeze-dried extract of whole autoclaved deuteriated *Scenedesmus obliquus* cells (algae extract) (3) was tested at a concentration of 1%. It was added to the salt solution prior to pH adjustment and autoclaved with that fraction.

Growth Response, Alkaloid Production.—Cultures in heavy water which showed growth were subcultured at 10- to 20-day intervals into nutrient media of the same deuterium concentration and into a higher deuterium concentration. Growth response and alkaloid production were evaluated on the basis of data obtained from the second subculture of each series. Growth response was visually graded in terms of degree of difference between the best growing culture and one which had not grown at all or had deteriorated. Mycelial dry weights were obtained from 25-ml. cultures. The entire mycelial content of a 25-ml. culture was centrifuged, washed twice with deuterium oxide, and lyophilized to a constant weight.

The production of alkaloids was measured periodically by analyzing the medium with the modified *p*-dimethylaminobenzaldehyde–sodium nitrite reagent described by Michelon and Kelleher (16).

RESULTS AND DISCUSSION

Partially Deuteriated Claviceps.—*Claviceps* strain 47A can be maintained well and will produce concomitantly high yields of alkaloids in H_2O on a medium containing a mixture of 5–15% protio-mannitol and 0.5% protio-succinic acid as the carbon–hydrogen substrates (8). Cultures which were allowed to metabolize for a period of 35 days or longer showed typical rose-colored pigmentation, and analysis indicated that the organism was producing alkaloids. Because of the nonavailability

TABLE I.—EFFECT OF D_2O CONCENTRATION ON ALKALOID PRODUCTION[a]

D_2O, %,	Extent of Growth[b]	Total Alkaloids, mcg./ml. 30 Days	60 Days
Phosphate Concentration, 1 Gm./L.			
0	+++	45	115
50	+	8	544
75	±	1.5	0.75
90	0	0	0
Phosphate Concentration, 0.1 Gm./L.			
0	++	50	160
50	+	10	552
75	±	2.0	3.0
90	0	0	0

[a] Media contained 7% protio-succinic acid. [b] 0, no growth; ±, marginal growth; +, ++, +++, graded response.

TABLE II.—EFFECT OF SUCCINIC ACID CONCENTRATION ON ALKALOID PRODUCTION BY *Claviceps* IN H_2O CULTURE[a]

Concentration, Protio-succinic Acid, %	Growth	Alkaloid, mcg./ml. 60 Days
0.7	+	32
2	++	48
3.5	++	68
7	+++	115

[a] KH_2PO_4 concentrations, 1 Gm./L. [b] +, ++, +++, graded response in a particular series.

of deuterio-hexitol, the utility of succinic acid as the sole carbon source was studied in cultures at relatively low levels of D_2O (0–90%). Growth and alkaloid accumulation were achieved in submerged and mat cultures at concentrations of deuterium oxide from 0–75%, with protio-succinic acid (7%) as the only carbon source. Growth of tissue in this nutrient medium is slower than in the hexitol-containing medium and appears to cease after 30 days. Analyses performed on aliquots of the medium from the tenth to eightieth day indicated that maximum alkaloid accumulation in H_2O and D_2O cultures occurs approximately 60 days after inoculation. The quantity of total alkaloids decreases precipitously at the highest deuterium concentration (see Table I). A reduction in the phosphate content of the nutrient medium stimulated the production of alkaloids in H_2O cultures. Only slight stimulation was found in cultures containing deuterium oxide, however.

In concentrations of D_2O above 75%, growth was not reproducible, and the morphology of the organism in mat culture changed from white continuous surface mycelial pellicles to scant, dark green isolated colonies. Large or small tissue inocula showed practically no growth. Traces of alkaloids in cultures from large inocula were detected only after 70 days.

Tyler (9) has shown that high levels of succinate inhibit the growth of some strains of *Claviceps*. Consequently, the authors examined the effect of various levels of succinate upon growth in stationary culture in H_2O and 75% D_2O. In H_2O, lower protio-succinate concentrations led to reduced growth and alkaloid production, while at 75% D_2O a uniformly poor response was obtained at 0.5 to 7.0% succinate (Table II). The substitution of deuterio-succinic acid in cultures at 75% D_2O or higher failed to elicit even slight growth of the original inoculum. Aeration at 75% D_2O had no effect.

Fully Deuteriated Ergot.—The importance of supplementation with various D_2O extracts of whole autoclaved algae was realized in the successful adaptation of several bacterial strains (3) to growth in 99.7% D_2O. The growth and deuteriation of several varieties of *Saccharomyces cerevisiae* (17) and of *Euglena gracilis* (5) were achieved by the addition of both algae extracts and traces of vitamins. Preliminary studies indicated that fully deuteriated *Claviceps* could be obtained if algae extract (1%) and small amounts of vitamins were added. However, in biosynthesis studies it is of critical importance to have a defined minimal medium which supports growth under conditions of full deuteriation. The presence of crude extracts not only interferes with alkaloidal assays, but the indefinite composition of these extracts makes the observation of the effects of additives on growth or alkaloid production extremely difficult.

At very high levels of D_2O, good growth in the absence of algae extract was obtained upon the addition of protio-glucose (2%) to media containing protio-succinic acid in low phosphate salt medium. Cultures containing only 1% protio-succinic acid as a carbon source in 96% D_2O showed no growth 30 days after inoculation, but new tissue appeared within 5 days after supplementing the medium with protio-glucose (2%). Fourteen days after the addition of the sugar, a well-defined surface pellicle had formed, the central portions of which showed signs of pigmentation.

Full deuteriation of the organism and successful maintenance of the culture has been achieved on a medium containing fully deuteriated mixed monosaccharides (2%), deuterio-succinic acid (0.5%), and traces of vitamins in a low phosphate salt medium. The fungus is subcultured at 24-day intervals and metabolizes well, forming off-white surface pellicles. Deuterio-glucose or fully deuteriated mixed monosaccharides supported growth to the same extent as protio-glucose. When deuterio-sugars were used in combination with 0.5–7.0%

TABLE III.—EFFECT OF NUTRIENT MODIFICATION ON GROWTH OF *Claviceps* IN D_2O

	Modification[a]	D_2O Content, %	Age of Culture, Days	Dried Mycelia, mg.[b]
A	7% Protio-succinic acid	98.0	27	10.5
B	7% Deuterio-succinic acid	99.1	27	5.8
C	2% Mixed protio-sugars[c]	99.6	16	18.0
D	Complete media containing[d]:			
	0.5% protio-succinic acid	98.7	17	108.1
	2% protio-succinic acid	98.1	17	40.3
	5% protio-succinic acid	97.0	17	46.9
E	Complete media containing[d]:			
	0.5% deuterio-succinic acid	98.7	17	101.2
	1.0% deuterio-succinic acid	98.6	23	68.1
	2.0% deuterio-succinic acid	98.6	17	66.7
	5.0% deuterio-succinic acid	98.4	17	52.4
F	Complete media (without algae extract) containing 1% deuterio-succinic acid	99.1	23	114.9
G	1% Deuterio-succinic acid in minimal medium[e]	99.6	23	68.4

[a] The salts were Tyler's low phosphate salt mixture. [b] Based on a standardized inoculum of 1.5 ± 0.5 mg. of tissue. [c] Mannose–glucose (1:1). [d] Mixed deuterio-monosaccharides, algae extract, vitamins, low phosphate salt mixture. [e] Mixed deuterio-monosaccharides, low phosphate salt mixture.

protio- or deuterio-succinic acid and vitamins in media above 98% D_2O, growth was obtained at all succinic acid concentrations, but growth is much better at lower concentrations of succinic acid (Table III). Withdrawal of the vitamin mixture leads to reduced growth and a reduction in the size and abundance of floating mycelia. The effect of changes in phosphate concentration, vitamin withdrawal, and various additives on the production of deuteriated alkaloids is under investigation.

SUMMARY

C. purpurea, strain 47A, does not require a carbohydrate carbon source in order to metabolize and produce alkaloids in ordinary water or 50% D_2O in the presence of succinic acid. The total amount of alkaloid produced increases with increasing succinic acid concentration. Above 75% D_2O, a carbohydrate carbon source becomes indispensable, and fully deuteriated *Claviceps* can be successfully cultured.

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Release of a Drug from a Dosage Form

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The complete interpretation and use of absorption, distribution, and excretion data depend upon an understanding of the mechanism of release of the drug from the dosage form and then on the ability to utilize this information to predict its effect *in vivo*. An analog computer program suitable for using blood *versus* time data as an input and producing the *in vivo* dosage form availability *versus* time pattern as its output has been developed, tested on synthetic problems, and used to analyze published blood data for a sustained-release form and a multiple-dosage regimen of sulfaethylthiadiazole (SETD) and solution, capsule, and tablet forms of acetylsalicylic acid. Similar programming ideas have produced a program able to predict, for known systems, the effect of changes in the *in vivo* release patterns on the absorption, distribution, and excretion picture.

SINCE the advent of sustained-action, sustained-release, and delayed-availability dosage forms, considerable effort has gone into the collection and correlation of absorption, distribution, and excretion data for many drug substances (1). Proper correlation and use of these data have depended mainly upon the use of exponential (first-order) approximations for the obviously very complex natural processes that occur within the body. A tacit but simplifying assumption in most of these approximations is that the drug is either all available immediately (administration of solution to the gut or by injection), or that it becomes available in a manner described by some ordinary linear differential equation (2). In the case of I.V. solutions, while the slug of drug injected is not *truly* a well-defined concentration, uniform at time 0, as is usually assumed, the other rates (absorption and excretion) involving the drug are so much slower than the rate of injection and mixing that the error in this assumption may be safely neglected. A solution in the gut, however, and even more so for other less rapidly available dosage forms as well as for more exotic administration sites, is not and does not become a uniform concentration; nor is it reasonable to assume that the availability of a drug from it follows any linear ordinary differential equation (save perhaps a series approximation)—at least not one that can be theoretically formulated without some actual release or availability data being obtained directly in the GI tract. The approximations currently in use for calculating

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