Effects of Some Substances on Ergot Alkaloid Production

E. O. OGUNLANA, E. RAMSTAD, and V. E. TYLER

Abstract □ Literature reports do not agree with regard to the influence of aeration on the ergot alkaloid production, but in general, the results bring out the need of adequate aeration for substantial alkaloid production. The beneficial influence of aeration and the absolute requirement of molecular oxygen in the biosynthesis of the alkaloids pointed to the possibility that oxygenases may be participating in their biosynthesis. Certain substances which are known to affect oxygenases have been tested for their influence on the ergot alkaloid production in shake cultures of *Claviceps*, strain SD/58. The effects on alkaloid production range widely: sodium L-thyroxine produces inhibition; phenobarbital, hydrocortisone, and 3,4-benzpyrene cause marked increases. The results lend support to the concept that one or more oxygenases are involved in the biosynthesis of the ergot alkaloids.

Keyphrases □ Ergot alkaloids—biosynthesis □ Oxygenases—ergot alkaloid biosynthesis □ Metal ion effect—ergot alkaloid biosynthesis

The ergoline nucleus is biosynthesized from tryptophan and mevalonic acid (1, 2). Abe's (3) first convincing demonstration of a production of ergot alkaloids by *Claviceps* species in saprophytic cultures was followed by a series of investigations on the saprophytic nutritional requirements and the influence of aeration on the alkaloid formation. The literature provides conflicting reports on aeration: Windisch and Bronn (4) reported that reduced respiration leads to an increase in alkaloid formation. Arcamone *et al.* (5) investigated the influence of aeration on the synthesis of simple lysergic acid derivatives by *Claviceps paspali* Stevens et Hall. They reported that good aeration increased the alkaloid concentration appreciably and that a reduction of the oxygen tension had a curbing effect upon the alkaloid synthesis. The diameter of the neck opening of the conical flasks and the nature of the closures of the flasks (compact or loose cotton plugs) had an appreciable influence on the alkaloid formation. Groeger and Tyler (6) confirmed these findings but the effect was less pronounced for their strain than for those studied by Arcamone *et al.* The authors have found (unpublished) that very vigorous shaking of the culture and intensive aeration invariably lead to low alkaloid concentration. It has also been observed (7) that incubation under an artificial atmosphere containing ^{18}O-enriched oxygen gas (closed vessel) resulted in a consistently low alkaloid production.

Agurell and Ramstad (8) showed the biochemical conversion of agroclavine to elymoclavine and suggested that a peroxidase or an oxygen transferase (= oxygenase) is implicated in the oxidation, and that the active hydroxyl is derived from molecular oxygen (9). Evidently the biogenesis consists in a progressive sequence of oxidative events.

Recently, Floss *et al.* showed (7) that the oxygen of the hydroxyl groups of chanoclavine-I and of elymoclavine must originate from molecular oxygen. It is evident, therefore, that the oxidation of the methyl group occurs by an initial hydroxylation.

Hayaishi (10) has used the term hydroxylases or mixed-function oxygenases to denote enzymes which catalyze the incorporation of 1 atom of atmospheric oxygen into various substrates. Oxygen is necessary as the specific oxidizing agent. Gunsalus *et al.* (11) have shown that in addition to molecular oxygen, a two-electron donor is required and water is formed from the atom of oxygen not incorporated into the substrate. The electron donor may be the reduced pyridine nucleotide, ascorbic acid, or even the substrate to be oxygenated. In most of the systems studied in detail, a metal ion plays a functional role in the oxygenation, presumably serving as the site of activation of the oxygen molecule.

Considering the influence of aeration on the alkaloid production as well as the involvement of molecular oxygen in the biosynthesis, it was thought that a study of the effects on the ergot alkaloid production by substances which are known to induce or inhibit oxygenases was appropriate. A possibly positive correlation would then provide a foundation for a subsequent program to study oxygenase involvement in detail.

METHODS

Organism—The studies were conducted with the ergot strain SD/58 originally isolated from sclerotia-parasitizing *Pennisetum typhoideum* Rich. This laboratory used the strain obtained from Dr. Heinz G. Floss.

Cultivation—Cultures were prepared by growing the mycelium on an agar slant for 10 days, and the resulting mycelium was transferred aseptically to the growth medium, 50 ml. (12) in 250-ml. conical Flasks A. The culture was shaken on a rotary shaker at 25° for 10–12 days when a copious growth had resulted. One to two milliliters of the culture was inoculated into Flasks B in order to produce the seed inoculum. Five days after inoculation of Flask B, 1 ml. of the seed culture was used for final inoculation. The organism was then grown in Flasks C for 12 days at 25° in shake culture. Triplicate runs of Flasks C were prepared for each test substance and for the control. Aseptic techniques were utilized in the addition of the substances to the medium.

Growth Correlations—At the end of the incubation period, each culture was carefully mixed by shaking, and 15 ml. of the homogeneous sample was transferred to a graduated centrifuge tube. After centrifugation at 2,000 r.p.m. for 5 min., the level of mycelium sediment in the tube was recorded immediately in order to relate the growth from each set of conditions to that of the control flasks.

Analysis—Aliquots of the clear supernatants were removed and diluted suitably for total alkaloid analysis according to the method of Michelon and Kelleher (13). Alkaloid concentrations were determined from a standard curve prepared with elymoclavine, reference grade, and expressed as mg./l. of the culture medium.

RESULTS AND DISCUSSION

Except for the variations in procedure as stated above, all test substances were incorporated aseptically into the medium in Flask C prior to inoculation.

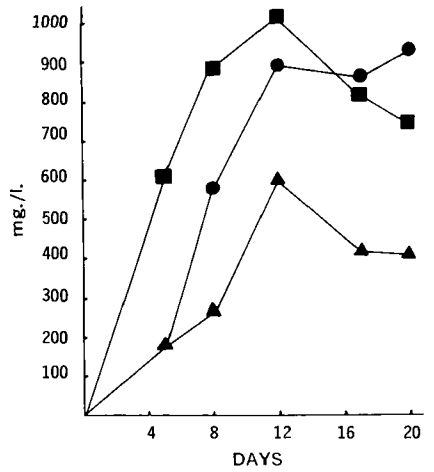

Figure 1—*Alkaolid content of medium. Key:* ■, *initial addition of* L-*tryptophan (2 m*M); ●, *addition of* L-*tryptophan after 5 days incubation;* ▲, *control culture.*

The means of each triplicate set were determined for total alkaloid content and the standard error calculated for each set at the 95% confidence level. The examination of controls showed the average coefficient of variation for replicates to be 3.5% while the coefficient of variation of all values was 6% (14).

Preliminary studies indicated (Fig. 1) that, under the conditions of the experiment, the peak of alkaloid production was reached at about the twelfth day of incubation. Therefore, subsequent total alkaloid-content determinations were carried out after 12-day incubation periods. The analyses were carried out as soon as possible on the day of the harvest, with limited exposure of the medium to light in order to prevent changes in alkaloid content.

Claviceps strain SD/58 used in these studies is known to excrete a mixture of alkaloids into the nutrient medium, principally elymoclavine. Consequently, the total alkaloid-content determinations were based on the use of elymoclavine as the standard.

The results (Table I) show that the effects of the various substances incorporated into the medium vary greatly. Only α,α'-dipyridyl caused a decrease of growth but some of the other substances led to appreciable increase in growth. It is interesting that *p*-chlorophenylalanine caused the largest increase in growth and, at the same time, provided an inhibitory effect on the total alkaloid production.

L-Ascorbic acid gave slight increase in alkaloid production; perhaps higher concentration of the substance might have caused further increase.

Catalase produced a slight decrease in alkaloid production, but the values are not large enough to be very significant. Recently, it has been observed in this laboratory that high concentrations of catalase are present in SD/58 and it is to be expected that the amount of catalase added to the cultures would have very small, if any, effect on the overall alkaloid production.

The influence of L-tryptophan on alkaloid production has been studied by previous workers, who have found an increase (5, 15). This stimulatory effect of tryptophan was confirmed with this strain. A combination of L-tryptophan and L-ascorbic acid was also tested. The values obtained with a medium containing the mixture were lower than by addition of tryptophan alone.

Studies with mammalian tissues and liver (16–18) have indicated a stimulatory effect of certain hormones on oxidative enzyme reactions. Hydrocortisone causes induction of some oxidative enzymes (17, 19) as well as *de novo* synthesis of protein. The present finding with ergot may therefore be assumed to have a similar basis as in the animal system. Phenobarbital, 2,3-benzanthracene, and 3,4-benzpyrene also caused an increase in alkaloid production and these substances have been shown to induce oxygenase in animal systems. The enzyme-inductive action of phenobarbital has been found to be nonspecific in liver microsomes but the polycyclic hydrocarbons exert considerable specificity (20), and they are more potent than phenobarbital as enzyme inducers.

Phenobarbital stimulates markedly the activity of TPNH-dependent oxidative enzymes in liver microsomes (20). The increase in alkaloid production may therefore well be an expression of an induction of TPNH-oxygen-dependent enzymes.

The tested polycyclic hydrocarbons, 2,3-benzanthracene and 3,4-benzpyrene, produced marked increases in alkaloid production, but caused practically no increase in growth when added alone or in combination with L-tryptophan. The polycyclic hydrocarbons also induce the synthesis of an enzyme system that metabolized the hydrocarbon used (20). The characteristic pink-red coloration normally developing in most of the SD/58 cultures was replaced by a slightly darker (brown) color in media containing the polycyclic hydrocarbons, but whether this phenomenon is related to alkaloid product is left open for future studies.

Metal ions participate in the oxygenation reactions. Oxygenases are generally heme-containing enzymes. They are activated by ferrous ions. Incorporation of the Fe^{2+}-chelating α,α'-dipyridyl into the medium showed a marked inhibition of alkaloid formation. Introduction of α,α'-dipyridyl into the medium before inoculation led to greatly stunted growth of the organism. When the chelating agent was added on the fifth day of cultivation after growth had been established, alkaloid content of medium was still greatly reduced on the 12th day. The relative reduction in growth amounted to about 33%, while the relative decrease in total alkaloid content was about 87% and it appears that α,α'-dipyridyl may be necessary in the alkaloid production itself.

The influence of L-thyroxine on hydroxylation reactions and on the entire pattern of cellular constituents has been extensively studied. Caravaca and May (21) found evidence that the hormone is of significance in the oxidation of DPNH and TPNH by peroxidase and H_2O_2, or molecular oxygen, in the presence of low levels

Table I—Total Alkaloid Content Determinations

Concentration of Substances Added to the Medium	Total Alkaloid Concn. of Medium, mg./l.	Relative Change in Alkaloid Content, %	Relative Change in Growth, %
Control	543 ± 22	—	—
L-Ascorbic acid, 0.1 m*M*	566 ± 19	+4	+4
Catalase, 0.0001 m*M* (4,562 units)	463 ± 40	−14	+4
Catalase, 0.0001 m*M* (4,562 units)[a]	500 ± 30	−8	+4
L-Tryptophan, 2 m*M*	984 ± 22	+81	+7
L-Tryptophan, 2 m*M* and L-Ascorbic acid, 0.1 m*M*	800 ± 30	+53	+7
Hydrocortisone, 0.05 m*M*	719 ± 26	+32	+55
2,3-Benzanthracene, 1 m*M*	1,290 ± 30	+137	+25
3,4-Benzpyrene, 2 m*M*	1,330 ± 67	+145	+0
Phenobarbital, 1.7 m*M*	880 ± 60	+62	+5
L-Tryptophan, 2 m*M* and 3,4-Benzpyrene, 2 m*M*[b]	1,471 ± 11	+170	+7
Sodium L-thyroxine, 0.5 m*M*	200 ± 12	−63	+35
α,α'-Dipyridyl, 1 m*M*	14 ± 3	−97	−85
α,α'-Dipyridyl, 1 m*M*[c]	73 ± 9	−87	−33
p-Chlorophenylalanine, 0.15 m*M*	262 ± 12	−52	+86

[a] Catalase was added prior to inoculation and at 4-day intervals during cultivation. [b] Tryptophan was added prior to inoculation and 3,4-benzpyrene added on the fifth day of cultivation. [c] α,α'-Dipyridyl was added on the fifth day of cultivation.

of Mn^{2+}. When sodium L-thyroxine was incorporated into the ergot culture medium, an appreciable decrease in alkaloid production resulted but an increase in growth was obtained. Thyroxine is known to inhibit cholesterol synthesis (22), a process which may be related to the effect of L-thyroxine on oxygen consumption. Evans (23), in discussing the mechanism of microbial hydroxylations, points to the involvement of oxygenases in these phenomena. One of the hydroxylase inhibitors is *p*-chlorophenylalanine. When it was incorporated into the medium, a marked decrease (52%) in alkaloid production resulted.

The results show, on one hand, that the incorporation of some oxygenase inducers into the ergot culture produces considerable increase in alkaloid production, while, on the other hand, reduction in alkaloid production is obtained from substances which have inhibitory effects on oxygenases. The findings, therefore, lend strong support to the concept that oxygenases are involved in the biogenesis of the ergot alkaloid.

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Gas Chromatographic Determination of Mebutamate, Carisoprodol, and Tybamate in Plasma and Urine

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Abstract □ A gas chromatographic method for the determination of mebutamate, carisoprodol, and tybamate in plasma and urine is described. The procedure permits the measurement of these drugs individually or in combination with meprobamate.

Keyphrases □ Carbamates—determination, biological fluids □ Plasma—mebutamate, carisoprodol, tybamate determination □ Urine—mebutamate, carisoprodol, tybamate determination □ GLC—analysis

A previous paper (1) described the gas chromatographic determination of meprobamate in plasma and urine. These observations have been extended to the analysis in biological fluids of three related carbamate compounds—mebutamate, carisoprodol, and tybamate.

EXPERIMENTAL

Equipment and Reagents—A gas chromatograph, equipped with a flame-ionization detector,[1] and a recorder[2] were employed. The chromatographic columns used were 121.9-cm. (4-ft.) glass tubes packed with 3.8% UC-W98 methyl silicone on 80–100-mesh Diataport S (Hewlett Packard). The instrument settings were as follows:

Temperature—Column, 180°; injection port, 275°; detector block, 225°.

Gas Flow Rates—Hydrogen, 20 ml./min.; helium (carrier gas), 65 ml./min.; oxygen, 50 ml./min.

Sensitivity settings were range 10 with an attenuation of 2×. Redistilled chloroform and dibutyl phthalate[3] were used.

[1] F and M model 402 dual-column.
[2] 1-mv. Minneapolis-Honeywell.
[3] Supplied by Eastman Chemical Products, New York, N. Y.