ESTIMATION OF ERGOT ALKALOIDS IN CULTURES OF CLAVICEPS PURPUREA[1]

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Abstract

Methods for estimating the content of ergot alkaloids in cultures of *Claviceps purpurea* have been examined to select a simple but reliable assay procedure for evaluating the effects of cultural conditions and strain differences on yield. Modification of a solvent extraction method used for ergot sclerotia gave the most satisfactory results. Disadvantages and limitations of this and other procedures which were explored are described.

Introduction

In a study of the formation of ergot alkaloids in saprophytic culture (15), a simple, rapid method for their estimation was required. The many unsubstantiated claims of in vitro alkaloid production to be found in the literature (7) suggested that the method of assay must also be quite specific. Procedures used up to the present have been adapted from those employed for the assay of ergot sclerotia or pharmaceutical preparations, and their accuracy or usefulness for cultures has not been investigated. In the work reported below, an attempt has been made to evaluate the known methods and to compare them with some alternatives which appeared to offer certain advantages.

Materials and Methods

Solvents and Reagents

Diethyl ether was freed from peroxide by shaking with acid ferrous sulphate solution before use. Dowex-1, Dowex-50, and Dowex-21K resins were conditioned by heating on a steam bath with excess *N* sodium hydroxide, decanting, washing with distilled water, and then heating with excess *N* hydrochloric acid. The cycle was then repeated substituting 50% ethanol for water as the solvent. The resins were finally washed thoroughly with distilled water, sucked free of excess water for 10 minutes on a Buchner funnel, and stored damp in either the hydrogen or chloride form. Amberlite IRC-50 (H^+) was prepared in the same manner. It was converted to the sodium form by treatment with 4% sodium hydroxide and washed with distilled water. The sodium form was buffered to give pH values between 3.0 and 10.0 at 0.25 intervals by percolation with *N* acetate, phosphate, or borate solutions of the desired pH until the input and effluent pH's were the same. The resin was then washed thoroughly with distilled water.

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Claviceps purpurea Cultures

These were grown on 50 ml of nutrient media in 250-ml Erlenmeyer flasks at 22–25° C without shaking for periods of 35 to 50 days. Isolate PRL 1555 was grown on a mannitol – ammonium succinate (No. 1) medium and produced little alkaloid. PRL 1578 was grown on a medium containing galactose and ammonium succinate, and produced a mixture of alkaloids of which ergosine, ergosinine, ergocryptine, and ergocryptinine were the major components. Details of the cultures, conditions of cultivation, and composition of the media have been described elsewhere (15).

Standardized Culture Solids

PRL 1555.—The appropriate number of cultures were combined and macerated in a Waring blendor to a homogeneous slurry, then subdivided into 50-ml aliquots. In certain experiments, these were supplemented with known amounts of ergometrine maleate before being freeze-dried; a sufficient number of controls were left unsupplemented to establish the endogenous alkaloid content.

PRL 1578.—Cultures were combined and macerated as above. The lyophilized culture solids were then passed through a 40–60 mesh sieve, mixed thoroughly, weighed, and subdivided into equal amounts corresponding to the average weight of one culture.

Analyses were carried out in duplicate. Whenever the values obtained varied by more than 2%, additional cultures were analyzed and the results averaged.

Extraction of Cultures by Different Solvents

Extraction of Dry Lyophilized Cultures

Uniform lots of material were prepared as above except that the slurries were made alkaline with ammonium hydroxide. Each "culture" of PRL 1555 was supplemented with 127 μg of ergometrine maleate. The extractions were carried out by shaking for 1 hour with 50 ml of ether, chloroform, benzene, ethyl acetate, acetone, methanol, ethanol–water (4:1), or ethanol–water (1:1). After filtration the residue was washed twice with 10-ml portions of solvent and the filtrate evaporated carefully to dryness at 40° C *in vacuo*. One milliliter of 10% aqueous ammonia was added and after three extractions of 10 ml each with ether, the combined ethereal solution was shaken with 0.1 *N* sulphuric acid (10 ml). Three milliliters of the aqueous phase were treated with 6 ml of van Urk reagent (2). The amount of alkaloid present was measured colorimetrically and estimated by reference to a standard curve constructed by reacting known amounts of ergometrine maleate with van Urk reagent. No alkaloid was extracted with ether, chloroform, benzene, or ethyl acetate. Values for the remaining solvents are given in Table IA.

Extraction of Wetted Lyophilized Cultures

Standardized culture solids of PRL 1555 (supplemented with 127 μg of ergometrine maleate) and of PRL 1578 were wetted with 1 ml of 10% aqueous

ammonia, extracted with 50 ml of ether, chloroform, benzene, or ethyl acetate and the alkaloid estimated as for dry lyophilized cultures. The results are given in Table IB.

TABLE I

Recovery of alkaloid from standardized "cultures" of PRL 1555* and PRL 1578 (as μg ergometrine maleate)

Extracting solvent	PRL 1555	PRL 1578
A. Dry cultures		
Acetone	71	49
Methanol	157	99
Ethanol–water (4:1)	162	101
Ethanol–water (1:1)	166	102
B. Wetted cultures†		
Ether	126	92
Chloroform	132	80
Benzene	87	80
Ethyl acetate	106	69

*Supplemented with 127 μg ergometrine maleate per culture. Unsupplemented control cultures extracted with ether contained 42 μg ergometrine maleate.
†Cultures were wetted with 1 ml of 10% aqueous NH_4OH.

Examination of Ether-insoluble Fractions

The fraction of the 50% ethanolic extract (from cultures PRL 1555 and PRL 1578) which was not extracted into ether in the first part of this experiment was examined by paper chromatography in *n*-butanol – acetic acid – water (4:1:5) and *n*-butanol – pyridine – water (4:1:5). Blue-fluorescent zones given by lysergic acid and its derivatives under ultraviolet irradiation were absent from the chromatograms. With 2% *p*-dimethylaminobenzaldehyde in ethanolic hydrochloric acid (3) a single blue zone appeared at the same R_f as a tryptophane standard. The amount of tryptophane in the cultures was estimated (11) to be 420 μg for PRL 1555 and 230 μg for PRL 1578.

The residue not extracted into 50% ethanol in the first part of this experiment was shaken under nitrogen with 25 ml of *N* potassium hydroxide in 50% ethanol at room temperature for 24 hours. The mixture was then filtered, the filtrate neutralized with hydrochloric acid, evaporated to dryness at 40° C *in vacuo*, and the dry solid extracted repeatedly with absolute ethanol (100 ml) containing a few drops of concentrated ammonia. The extract was concentrated almost to dryness and paper chromatographed in the solvent systems used above. A faint blue zone with the R_f of tryptophane was detected with *p*-dimethylaminobenzaldehyde spray. No blue-fluorescent zones were present. In a control in which 200 μg of ergometrine maleate was added to the culture before treatment with alkali, zones giving a blue fluorescence in ultraviolet light and a blue color with the spray reagent were detected at R_f values corresponding to those of lysergic acid, isolysergic acid, ergometrine, and ergometrinine standards. From cultures hydrolyzed with alkali at 40° C or above for 2 hours, no lysergic acid or derivatives could be detected in the controls.

Effect of Alkaloid Yield on Efficiency of Extraction

Standardized culture solids of PRL 1555 supplemented with 10, 25, 67.5, 146, 500, 1000, and 5000 μg of ergometrine maleate were wetted with 1 ml of 10% aqueous ammonia, and extracted with ether (50 ml) by shaking for 30 minutes. The mixture was filtered and the residue washed with two 10-ml portions of fresh ether. For alkaloid levels of up to 67.5 μg, the ether was evaporated to approximately 10 ml at 40° C in a stream of nitrogen, then back-extracted with 3 ml 0.1 *N* sulphuric acid. The acid layer was separated and reacted with 6 ml of van Urk reagent. For alkaloid levels of 146, 500, 1000, and 5000 μg, the ether was back-extracted directly into 10, 50, and 100 ml respectively of 0.1 *N* sulphuric acid and a 3-ml aliquot removed for the colorimetric analysis. Recoveries are given in Table II.

TABLE II

Per cent recovery of alkaloid from standardized cultures of PRL 1555 supplemented with various amounts of ergometrine maleate

Ergometrine maleate added, μg	Recovered, %
10	98
25	91.5
67.5	83
146	75
500	77
1,000	78
5,000	77

Procedure A

Cultures were taken from the growth chamber and frozen for 2 hours at −40° C. After lyophilization 10% aqueous ammonia (1.0 ml) and ether (50 ml) were added and the flask stoppered and shaken for 30 minutes. The contents were filtered through a coarse sintered glass funnel into a separatory funnel and the residue washed twice with 10 ml of fresh ether. Where a low yield of alkaloid was expected and it was advisable to concentrate the ether solution before back-extraction into a small volume of 0.1 *N* sulphuric acid, this was done by placing the vessel in a temperature-controlled bath at 40° C and directing a stream of nitrogen onto the surface of the liquid. The ether solution was shaken with 3 ml or more of dilute acid, according to the yield of alkaloid expected. After settling, the aqueous phase was withdrawn and 3 ml treated with 6 ml of van Urk reagent. Minimum yields of alkaloid (calculated as ergometrine) estimated by this method where a 3-ml volume of acid was used for back-extraction were 4 μg per culture (80 μg per liter of culture medium). This was determined as the level at which a distinct blue color was just discernible to the eye after the reagent was added.

Procedure B (Repeated Extraction)

Cultures were extracted with ether as in the previous experiment and the alkaloid estimated. The residual culture solids were then shaken with 50 ml of fresh ether, filtered, washed, and the alkaloid in this extract estimated in

the same manner. This process was repeated twice more. Where the method was used to obtain an accurate value for total alkaloid in a culture the extracts from four successive extractions with ether were pooled before back-extraction with an appropriate amount of dilute acid.

Continuous Ether Extraction

Standardized culture solids of PRL 1555 (supplemented with 146 μg of ergometrine maleate) and of PRL 1578 were resuspended in water (50 ml) containing sodium bicarbonate (1 g) and extracted continuously with ether in the dark. At intervals fresh ether was substituted in the receiver, the extract back-extracted with an appropriate amount of 0.2 *N* sulphuric acid, and the alkaloid content estimated colorimetrically. Replicate cultures were assayed by procedure B.

In a separate experiment additional replicate cultures were suspended in 50% aqueous ethanol containing tartaric acid (1 g) and shaken for 1 hour. They were then filtered, washed with fresh solvent, and the filtrate concentrated at 40° C *in vacuo* to approximately 25 ml and shaken with ether (50 ml). The aqueous phase was made alkaline with sodium bicarbonate and extracted continuously with ether as above.

Procedure C

Cultures were shaken for 1 hour with tartaric acid (1g) and ethanol (50 ml), filtered, washed with 50% aqueous ethanol, and the filtrate applied to a column containing 5 g (= 6 meq) of Dowex-50 × 2 (H^+). The resin was washed with 50% aqueous ethanol and eluates up to this stage discarded. Three per cent of ammonium hydroxide in 50% aqueous ethanol was used to desorb the alkaloids and the first 100 ml of eluate collected. A 5-ml aliquot was reacted with 4 ml of van Urk reagent and the color measured at 550 mμ after 10 minutes. The alkaloid content of the culture was estimated by reference to a standard curve prepared by using known amounts of ergometrine maleate in the same solvent. The smallest amount of alkaloid (as ergometrine) which could be determined in this way was 75 μg per culture (1.5 mg per liter of culture medium).

TABLE III

Alkaloid content (as μg ergometrine maleate) of identical "cultures" analyzed by procedures A, B, and C

Ergometrine maleate added, μg	Procedures		
	A	B	C
PRL 1555			
Nil	56	43	–
127	129	174	177
508	419	540	533
1,016	793	1,021	1,025
5,080	3,882	5,111	5,049
PRL 1578			
Nil	812	945	1,065
Nil	942	1,062	1,170
Nil	1,040	1,156	1,240

Comparison of Procedures A, B, and C

Standardized culture solids of PRL 1578 and of PRL 1555 supplemented with 127, 508, 1016, and 5080 μg of ergometrine maleate were assayed by procedures A and B. Replicate "cultures" were reconstituted by suspending the culture solids in 50 ml of water and the alkaloid content determined by procedure C. The results are given in Table III.

Results and Discussion

Solvent Extraction Methods

In earlier studies of ergot alkaloid formation several authors (1, 8, 10, 14, 16) have found it convenient to separate the mycelium from the culture liquid and to analyze the two independently. The filtrate was reacted, either directly or after dilution, with van Urk reagent and the amount of blue color measured. Unless alkaloid yields are high or amounts of interfering substances present are negligible, such a procedure may be misleading. In this laboratory many of the cultures examined were found to contain pigments and other compounds (e.g., tryptophane) which interfere with the van Urk reaction. For the estimation of alkaloid in the mycelial solids, one of the procedures used for ergot sclerotia (6) has invariably been used. In these the dried and powdered product is first extracted with petrol, in which the alkaloids are insoluble. The free bases are then formed by addition of an alkali and are extracted with a suitable solvent. They may be re-extracted from an ethereal solution into dilute acid, and the alkaloid content of the aqueous solution determined colorimetrically. The partial purification resulting from this procedure, which ensures that only ether-soluble bases are estimated, has been found to add the required specificity to the test. Since it is usually unnecessary to have separate values for the amount of alkaloid present in the broth and mycelium, the whole culture may be reduced by lyophilization to a dry solid suitable for analysis by this method (12, 13). This step has the added advantage of permitting larger experiments since growth can be arrested in many cultures simultaneously by freezing. Tests have shown that maceration of the mycelial pad to give a finely powdered product is unnecessary. (Randomly sampled replicate flasks from a single experiment analyzed in both ways gave average values of 2.00 (macerated) and 2.28 (unmacerated) mg of alkaloid per culture.) The culture was successively frozen, dried, rewetted, alkalinized, and extracted with solvent in the flask in which it was grown.

For the extraction, ether was found to be the most suitable solvent. The alkaloid was recovered in high yield by a single treatment (Table IB) and in addition could be transferred to dilute acid for estimation with van Urk reagent by a direct back-extraction of the solution. Chloroform, the only other water-immiscible solvent found to give equivalent recovery, retained a large proportion of some alkaloids in the back-extraction step. Wetting the culture was necessary to release the alkaloid from adsorptive binding to the culture solids since none could be extracted with dry non-polar solvents. A sufficient volume of water was required to wet the solids adequately, but an excess

had to be avoided to prevent formation of sticky lumps. One milliliter was usually optimal, and with cultures containing a large proportion of unused sugar, the addition of Hyflo supercel before wetting was necessary. As alkalinizing agent, 10% ammonium hydroxide was preferred since it is more convenient to use and, in comparative runs, permitted at least as good extraction as did magnesium oxide or sodium bicarbonate. A shaking period of 30 minutes was sufficient for maximum extraction of the alkaloid, and protection of samples from light was unnecessary during the time required for the analysis.

The procedure based upon the results of these tests, henceforth called procedure A, was used to estimate the alkaloid content of a series of standardized samples of dried culture solid, which had been supplemented with various amounts of ergometrine. Recoveries appeared to improve as the alkaloid content decreased (Table II); at the higher levels it remained constant at about 77%. To establish that failure to recover total added alkaloid by a single extraction was due neither to degradation nor to irreversible binding to cellular material, cultures have been extracted repeatedly by this procedure. In successive extractions PRL 1555 cultures supplemented with ergometrine gave, respectively, 75, 15, 9, and less than 1% of the total alkaloid present. Cultures of PRL 1578, which contained mainly the water-insoluble alkaloids, gave successively 89, 9, 2, and less than 1% recoveries. This appreciable difference in the proportion of total alkaloid which can be recovered from the two strains by a single extraction suggests that the kind of alkaloids produced by cultures will influence the analytical result. Comparisons of alkaloid yield can be made with accuracy only between cultures producing a mixture of constant composition, or by ensuring complete removal of the alkaloid through successive extraction of the solids (procedure B). Variation encountered in the analysis of duplicate cultures in all of the work described above has been $\pm 3\%$.

Modified Solvent Extraction Methods

Analysis of cultures by repeated extraction has been found too time consuming for routine use, and transferring the dry powdered solids to a column for percolation with chloroform or ether containing aqueous ammoniacal ethanol (4) also proved cumbersome. Exhaustive continuous extraction of homogenized, alkalinized cultures with ether gave poor recoveries of alkaloid, probably owing to decomposition during the prolonged treatment found necessary. (Although most of the alkaloid was transferred to the ether after 4 hours, small amounts continued to be extracted up to 24 hours.) An attempt to reduce the extraction time by first separating the alkaloid from the culture solids with aqueous ethanol was unsuccessful. The slow rate of extraction must therefore be due to an unfavorable partition coefficient for certain of the alkaloids.

Photometric and Fluorometric Methods

The complete extraction of alkaloid from culture solids into aqueous ethanol (Table I) offered the simplest method of assay if a means of estimating

the amount present in such solutions could be found. The absorption maximum of lysergic acid derivatives at approximately 312 mμ could not be used since it was obscured by interfering substances in extracts of PRL 1578 containing up to 10 mg alkaloid per culture. The alkaloids did not fluoresce in alkaline solution but in neutral or acid solution those containing a 9,10-ergolene ring gave intense activation and fluorescence maxima at 320–325 and 425–440 mμ, respectively (Table IV). Elymoclavine and agroclavine, with a double bond in the 8,9-position, had corresponding maxima at 280 and 340 mμ (cf. tryptophane maxima at 280 and 360 mμ). The intensity of absorption was higher for alkaloids derived from lysergic acid than for those derived from isolysergic acid, and, in addition, the size of the side chain substituent had an effect (see also (9)). Although these differences were less marked in ethanolic than in aqueous solution, they were sufficient to cause error when cultures producing different alkaloids were compared. PRL 1578 cultures showed strong activation and fluorescence maxima at 320 and 445 mμ, respectively, but estimations of their alkaloid content relative to an ergometrine standard gave, as expected, appreciably lower values than obtained by procedure B. However, the method is useful for the estimation of single alkaloids at low concentration.

TABLE IV

Fluorescence characteristics of ergot alkaloids

	H_2O*			EtOH*		
	λ activation, mμ	λ fluorescence, mμ	Relative intensity†	λ activation, mμ	λ fluorescence, mμ	Relative intensity†
Ergometrine	320	435	1.00	325	415	0.66
Ergotamine	320	425	3.23	325	415	0.72
Ergosine	320	440	3.24	325	420	0.62
Ergocornine	320	435	3.48	330	420	0.69
Ergocristine	320	435	3.20	325	415	0.65
Ergocryptine	320	435	3.39	325	420	0.64
Lysergic acid	320	435	2.16	325	420	0.82
Penniclavine	325	440	1.85	325	425	0.85
Ergometrinine	320	445	1.33	325	420	0.90
Ergotaminine	320	440	6.64	325	420	1.19
Ergosinine	320	440	6.51	325	420	1.22
Ergocorninine	320	440	6.34	320	420	1.19
Ergocristinine	320	445	6.40	325	420	1.13
Ergocryptinine	320	445	6.61	325	415	1.24
Agroclavine	280	350	14.7	285	345	4.81
Elymoclavine	270	350	53.8	280	340	21.8

*Solutions contain 1% (w/v) tartaric acid.
†Calculated as μM of alkaloid per liter required to give the same intensity of fluorescence as a 1% (w/v) aqueous tartaric acid solution containing 1 μM ergometrine per liter. The lowest concentration at which ergometrine fluorescence could be measured accurately in this solvent was 0.07 μM per liter. The response to increasing concentration was linear up to 6.80 μM per liter.

Ion Exchange Method

The amount of alkaloid in aqueous ethanolic culture extracts could be measured without interference from tryptophane by reacting the solution directly with van Urk reagent under modified conditions. An optical density of 0.10 which was given by 3.4 μg/ml of ergometrine in the test solution required 650 μg/ml of tryptophane. However, interference from the pigments produced by many strains of *Claviceps purpurea* rendered the method

unsuitable for general use. With PRL 1578, which is a strong pigment producer, the minimum yield which could be determined with $\pm 5\%$ error by this method was 50 mg per culture.

To increase the accuracy, a means of fractionating the extract to remove interfering substances was sought. Foote and co-workers (5) have reported, without details, a method of purifying ergot alkaloids using active charcoal. In the present study it was found that ergometrine was readily adsorbed but could not be eluted without destruction. Sulphonic acid type cation exchange resins in the hydrogen form were found to adsorb the alkaloids quantitatively and quantitative desorption was possible from resins of low cross-linkage when the alkaline eluting solvent contained approximately the same proportions of water and ethanol as that from which the alkaloid was adsorbed. Similar observations have recently been reported for morphine and other large molecules (17). Extracts prepared by shaking acidified whole cultures with an equal volume of ethanol were passed through a short column containing excess Dowex-50 $\times$ 2 (H^+). The alkaloid was adsorbed and a red acidic pigment present in PRL 1578 cultures appeared in the effluent. A brown water-soluble pigment remained on the resin and was eluted with the alkaloid when the column was treated with 50% ethanol containing 3% ammonia. Desorption of the alkaloid was complete after passage of 50 ml of elutriant but normally the first 100 ml was collected.

Using this method (procedure C), ergometrine added to a culture of PRL 1555 could be recovered within $\pm 5\%$. With cultures of PRL 1578, however, the brown pigment eluted with the alkaloid caused some error (Table III). Direct spectrophotometric estimation of the alkaloids in column eluates again failed because of interference from other substances.

The weak cation exchange resin, Amberlite IRC-50, prepared in the hydrogen form or buffered up to a pH of 4.75, adsorbed the ergot alkaloids quantitatively from slightly alkaline aqueous ethanol. The brown pigment was also adsorbed and neither alkaloid nor pigment was eluted by washing the resin with dilute aqueous acid. With 50% aqueous ethanol containing either 1% tartaric acid or 3% ammonia, the alkaloid was recovered quantitatively, but no separation from the pigment was achieved.

The pigment was found to be ampholytic and could be adsorbed on Dowex-1 (OH^-). Ergometrine in ammoniacal 50% ethanol is not adsorbed on this resin provided a porous form is used, but ergotamine, and presumably also other alkaloids containing a large peptide side chain, is non-ionically trapped on a resin of even 1% cross-linkage. On less porous resins, ergometrine is partially retained. Non-ionic adsorption of ergotamine also occurred on Dowex-3 (OH^-) and Dowex-21K (OH^-).

Conclusions

In practice it was found that the most satisfactory procedure for routine assay of cultures was a modification of the ether extraction method used extensively for estimating the alkaloid content of ergot sclerotia (procedure A).

Since it was usually not practicable to carry out more than a single extraction of each dried culture, the values obtained were low and ranged between 75 and 90% of the true value. This order of accuracy was sufficient for most survey work, but where necessary it could be improved by combining successive ether extractions of the culture solids (procedure B). The method was not subject to error caused by differences in tryptophane content or pigmentation of different *Claviceps* strains, or of the same strain grown under different conditions, whereas such variation can lead to serious error in other methods of analysis. The substances in the final test solution giving a color with the van Urk reagent were examined paper chromatographically, and, except for the presence of minor amounts of tryptamine in a few instances, were found to consist entirely of ergot alkaloids.

Of the methods which do not require initial lyophilization of cultures, the most promising was the direct fluorometric estimation of alkaloids in an aqueous ethanolic extract. The disadvantages of this method are: (*a*) that changes in relative composition of an alkaloid mixture produced by the culture could give an apparent change in yield owing to differences in the intensity of fluorescence by different alkaloids, and (*b*) those alkaloids which do not contain a 9,10-ergolene ring must be measured separately at wave lengths where common indole metabolites interfere. However, it is sensitive and selective, whereas other methods involving direct estimations on the culture filtrate are useful only when alkaloid yields are high. Fractionation of the culture filtrate by means of a cation exchange resin afforded some improvement in accuracy but again did not give reliable results at very low alkaloid concentrations.

No evidence was found that alkaloid is present in a "bound" state which cannot be extracted with wet ether or aqueous ethanol. Mild alkaline hydrolysis of culture solids which had been thoroughly extracted by these methods and subsequent paper chromatography of the hydrolyzate failed to reveal any trace of lysergic acid.

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