A COMPARISON OF ISOLATES OF CLAVICEPS SPP. FOR THE ABILITY TO GROW AND TO PRODUCE ERGOT ALKALOIDS ON CERTAIN NUTRIENTS[1]

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Abstract

Isolates of *Claviceps purpurea* and *Claviceps* spp. obtained from various geographical areas were compared for their ability to grow and to produce ergot alkaloids in vitro on various carbon and nitrogen sources. While some differences in utilization of carbon sources for growth were found, there was no observed correlation between utilization of carbohydrates and the capacity to produce ergot alkaloids. The amount of alkaloid produced by different strains depended upon both the carbon and nitrogen sources. In general, those cultures capable of alkaloid production were able to do so on more than one carbon source, but the carbon source allowing greatest production differed from one strain to another. Both producing and non-producing strains could utilize succinic acid as a carbon source for growth, but neither could utilize L-tryptophane as a carbon or nitrogen source for growth.

Introduction

A previous survey (11) of 41 isolates of *Claviceps purpurea* (Fr.) Tul. demonstrated that strain differences existed with respect to ability to produce ergot alkaloids on a medium containing mannitol as a carbon source. A more detailed survey has now been undertaken to determine whether isolates might differ with respect to the carbon and nitrogen sources essential to production, and also to determine whether a correlation exists between the capacity to produce ergot alkaloids and the ability or inability to utilize certain nutrients in growth. Such information would be useful not only in understanding the biology of *Claviceps purpurea* and in improving yields, but also in designing a screening procedure for the selection of alkaloid producers from among either fresh isolates or induced mutants.

As carbon sources, glucose, sucrose, a mixture of galactose and glucose, mannitol, and lactose were selected to compare the ability of strains to produce alkaloids on various carbon sources. The galactose–glucose mixture, mannitol, and sucrose were selected because they have each been found suitable in various laboratories (1, 2, 6, 11) and glucose and lactose were selected because they are contaminants of the commercial galactose used in this laboratory (11), which led to the discovery that the mixture could be a superior carbon source (11). Ammonium succinate, yeast extract, and L-asparagine were used as nitrogen sources since they have been already tested previously (11) for one strain. Ammonium and asparagine nitrogen have also been used by other workers (1, 6, 14).

Glucose, mannitol, pure galactose, and maltose were selected as carbon sources to compare the nutritional limitations of the isolates. A direct comparison of three strains made in this laboratory (9) in 1956 failed to show

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differences in the utilization of sugars as carbon sources for growth. These results (9) did not agree, however, with nutritional studies reported by other workers (1, 4, 5, 15). While a direct comparison of the strains used in the different laboratories has not been made, a study of the effect of inoculum size on utilization of mannitol by one of our strains (7) suggested that the differences observed in the various laboratories might have been due to the use of inocula of different sizes and histories, rather than to the existence of nutritionally distinct strains. Through another and direct comparison of several strains, when using comparable inocula, it was hoped that sufficient evidence could be obtained to determine whether strain differences in utilization of carbon sources for growth do in fact exist in detectable frequencies. Succinic acid was also tested as a carbon source for growth since it was previously established that this nutrient, which stimulated alkaloid production by PRL 1578, was not only available for growth, but was, in fact, also utilized in preference to the mixture of galactose and glucose during the early period of growth (12).

Strain PRL 1578 cannot utilize L-tryptophane as a nitrogen source for growth, yet this amino acid stimulates production (11) and tracer studies have shown that it is incorporated into the ergot alkaloids (13). Since inability to utilize tryptophane for growth might be a requisite for production of the ergot alkaloids, and could, therefore, be used as a screening criterion, several isolates differing in capacity to produce ergot alkaloids were examined for their ability to utilize tryptophane in growth and to oxidize and decarboxylate tryptophane.

Another fungus, *Penicillium roqueforti* PRL 1463, was included in the study because it was found to produce substances giving a positive Ehrlich reaction (11). If it should possess nutritional properties in common with the alkaloid-producing strains of *Claviceps purpurea*, this fact would provide a basis for relating certain aspects of nutrition to ergot alkaloid biosynthesis.

Materials and Methods

Cultures

Cultures obtained from various geographical regions were selected for this study (Table I). With the exception of PRL 1555, which is derived from high-alkaloid-producing strains from Spain, none of the strains had been examined for alkaloid production prior to their receipt in this laboratory.

Inoculum

Cultures to be analyzed for alkaloids were started from approximately 5 milligrams dry weight equivalent of washed mycelium which was prepared as described previously (11). Cultures grown on plates of agar media for growth estimations were started from a small quantity of washed mycelium (ca. 0.1 mg dry weight equivalent per petri plate) to avoid stimulation attributable to the presence of nutrients in larger quantities of mycelium (7).

Media

Liquid media to be used for the production of ergot alkaloids were based on basal medium No. 10-B (11).

TABLE I

History of cultures of *Claviceps* spp. used in the study

PRL No.	Source	Region	Host
1578	W. P. Campbell	Alberta	*Phleum pratense*
1564	W. P. Campbell	British Columbia	*Bromus ciliatus*
1570	W. P. Campbell	British Columbia	*Agropyron dasystachyum*
1583	W. P. Campbell	Alberta	*Calamovilfa longifolia*
1555	Centraal Bureau voor Schimmelcultures	Spain	*Secale cereale krebs*
1565	W. P. Campbell	Alberta	*Glyceria borealis*
1715*	W. P. Campbell	Alberta	Unknown
1716†	W. P. Campbell	Alberta	Wild rice
1556	Centraal Bureau voor Schimmelcultures	Portugal	*Secale cereale*
1585	W. P. Campbell	British Columbia	*Bromus ciliatus*
1596	W. P. Campbell	Saskatchewan	*Calamagrostis inexpansa*
1595	W. P. Campbell	Alberta	*Avena fatua*
1580	W. P. Campbell	Alberta	*Bromus inermis*
1557	Authors	Saskatchewan	*Secale cereale*
1574	W. P. Campbell	Ontario	*Lolium perenne*
1734	From sclerotia donated by Eli Lilly Company	Midwest state	Unknown
1735	From sclerotia donated by Eli Lilly Company	Europe	Unknown
1566	W. P. Campbell	Alberta	*Agropyron albicans*

**Claviceps maximensis.*
†*Claviceps* sp.

The carbon sources were: Fisher reagent sucrose and anhydrous dextrose (glucose), Baker and Adams mannitol, NBC beta lactose U.S.P., and Difco galactose. The latter contained glucose and lactose and it was previously found (11) that growth and alkaloid production were due to the presence of glucose but not lactose. Therefore, this carbon source is referred to in this paper as a mixture of galactose and glucose. Liquid media used to test for alkaloid production on various nitrogen sources contained approximately the same quantity of succinic acid and they were adjusted to pH 5.2 with either hydrochloric acid or potassium hydroxide.

Solid media used to test for growth were solidified with 1.5% Difco Bacto Agar. The medium containing L-tryptophane as a carbon source was prepared by mixing a solution of filter-sterilized tryptophane, adjusted to pH 3 with HCl, with No. 10-B basal medium but containing ammonium phosphate as the nitrogen source. While still molten, 20 ml of this medium was poured into petri plates, each containing 500 mg sterile, dry $CaCO_3$. The final concentration of tryptophane was 1% and the final pH was 6.4. The medium containing L-tryptophane as the nitrogen source was prepared by replacing the ammonium succinate of medium No. 10-B with filter-sterilized tryptophane. The agar media used to test for growth on various carbon sources were prepared by replacing commercial galactose with the indicated substance. The carbon sources were: Fisher reagent anhydrous dextrose, purified Difco galactose, Baker and Adams mannitol, Matheson, Coleman, and Bell maltose, Eastman L-tryptophane and analar succinic acid.

Oxidation and Decarboxylation

Preparation of substrates.—Conventional Warburg techniques (16) were used and the regime was as described previously (8). Oxidation and decarboxylation of L-tryptophane were tested on 50 μmoles of tryptophane. The solution used for oxidation determinations contained sufficient glucose to give 1 μmole of glucose per vessel. The control also contained glucose. The tryptophane solutions were prepared by suspending L-tryptophane in distilled water and then adjusting the pH to 3 with HCl. Solutions adjusted to pH 10.5 with KOH were also used but since these solutions began to color within hours and therefore were constantly changing, the results obtained from their use are not reliable and are therefore not reported. Succinic and malonic acids were dissolved in distilled water, and glutamic acid was suspended in distilled water, warmed, and adjusted to pH 7–7.1 with KOH, giving a final concentration of 50 μmoles per vessel. The glutamate solution contained sufficient glucose to give a final concentration of 1 μmole per vessel. All solutions were prepared on the day they were to be used.

Preparation of mycelium.—Mycelium was obtained from 3-day-old secondary shaken cultures (11) grown on a medium consisting of 1% glucose and 1% Difco yeast extract (YD broth). Twenty hours before beginning an experiment the mycelium was blended 3 seconds and the culture returned to the shaker. Twelve hours before use, the mycelium was washed three times with sterile distilled water and suspended in *M*/15 phosphate buffer, pH 6.9. It was returned to the shaker until just before use in the respirometer. At this time the mycelium was removed by centrifugation and washed three times with phosphate–HCl buffer (8), pH 4. Finally the mycelium was suspended in this buffer to give a final concentration equivalent to 7 to 10 mg dry weight per milliliter. One milliliter was added to each vessel.

Methods used for estimation of alkaloids and other analyses have been described in earlier publications (7, 8, 9, 10, 11, 12, and 17). Cultures were incubated at $23 \pm 2°$ C.

Results

Alkaloid Production Using Various Carbon Sources

The amounts of ergot and/or clavine (14) alkaloids produced by 14 cultures on the five sugars are given in Table II. Not only do isolates differ with respect to the carbon sources which permit optimal alkaloid synthesis, but they also differ in the number of carbon sources on which they can synthesize detectable quantities of alkaloid. Glucose served as a carbon source for synthesis for 5 isolates, sucrose for 8, the mixture of galactose and glucose for 10, lactose for 1, and mannitol for 11.

One culture produced alkaloid on each of the five carbon sources; three produced on glucose, sucrose, mannitol, and the mixture of galactose and glucose; and two on the mixture and on mannitol. Two cultures produced alkaloid on mannitol only; two on sucrose, on the mixture, and on mannitol; one on sucrose and on mannitol; one on the mixture only; and one on glucose,

sucrose, and the mixture. Strain differences certainly exist, and although mannitol was not always the superior carbon source, it permitted detection of production in 11 of 13 cultures.

TABLE II

Effect of carbon source on production of alkaloids by strains of *Claviceps purpurea*

PRL No.	Day of maximal yield*	Mg alkaloid per liter				
		Glucose	Sucrose	Galactose + glucose	Lactose	Mannitol
1578	35	0.9±0.1‡	1.8±0.2	48.1±1.7	Nil	21.2±0.4
1596	45	Nil	Nil	Nil	Nil	1.1±0.1
1566	45	0.3±0.1	0.5±0.1	0.3±0.1	Nil	Nil
1565	45	10.1±3.5	13.7±1.6	3.1±0.2	4.2±0.3	9.5±1.2
1583	45	Nil	Nil	0.3±0.1	Nil	1.0±0.1
1570	45	4.3±0.5	8.4±1.2	21.2±2.6	Nil	5.5±1.3
1564	25	Nil	Nil	Nil	Nil	0.7±0.1
1585	—	Nil	Nil	Nil	Nil	Nil
1715	25	Nil	Nil	3.8±0.1	Nil	3.0±0.5
1716	25	Nil	Nil	1.4±0.1	Nil	Nil
1555	45	Nil	0.8±0.2	Nil	Nil	0.7±0.1
1735†	35	0.3±0.1	0.4±0.1	0.4±0.1	Nil	0.3±0.1
1734	35	Nil	4.6±0.2	0.5±0.1	Nil	0.3±0.1
1574	35	Nil	0.3±0.1	1.2±0.2	Nil	1.3±0.2

*Each culture was tested at 25, 35, and 45 days, and the yield is expressed as the arithmetic mean and standard error of three replicate cultures.
†The color test was blue-green, not blue.
‡The smallest standard error listed is 0.1.

Alkaloid Production Using Various Nitrogen Sources

Five cultures were compared for their ability to synthesize alkaloids on three nitrogen sources: ammonium succinate, yeast extract, and L-asparagine (Table III). Two cultures produced alkaloid on ammonium and yeast extract nitrogen and two on yeast extract nitrogen but not ammonium nitrogen. One culture did not produce alkaloid on any nitrogen source and none could utilize asparagine for synthesis.

The data given in Table III illustrate again that strain differences exist with respect to alkaloid synthesis and it is evident, when considering the data of Tables II and III, that a medium compounded for production by one strain cannot be assumed to be satisfactory for production by another strain.

TABLE III

Production on different nitrogen sources
(35 days; galactose + glucose carbon source)

PRL No.	Mg alkaloid per liter		
	Ammonium succinate	Asparagine	Yeast extract
1578	97.2±4.0	0	Trace
1570	0	0	1.4±0.3
1566	0	0	0
1565	4.8±0.6	0	49.1±2.5
1596	0	0	1.0±0.1

Comparative Growth on Carbon Sources

Sugars.—Each isolate utilized glucose and mannitol for growth but only 7 grew on galactose and 14 on maltose (Table IV). Galactose was not readily utilized by any strain. Our standard production strain, PRL 1578, which produces the highest yield on a mixture of 95% galactose and 5% glucose (11) did not grow at all on pure galactose. The data of Table IV show, however, that inability to grow on pure galactose is not associated with the ability to produce ergot alkaloids. The data given in Table IV also show that nutritionally different strains exist within *Claviceps purpurea*. Utilization of these carbon sources cannot be used to distinguish *C. purpurea* from two other species, *C. maximensis* and *Claviceps* sp. PRL 1716.

TABLE IV

Comparative utilization of various carbon sources*
(15 days)

Strain	Alkaloid produced†	Glucose	Galactose	Maltose	Mannitol	L-Tryptophane	Succinic acid
1578	+	+++	0	++	+++	0	+++
1556	−	+++	0	+	+	0	++
1564	+(−)	+++	0	++	+	0	++
1557	+	+++	+	+	++	0	+++
1570	+	+++	+	++	+	0	+
1583	+	+++	++	0	++	0	+++
1555	+	+++	0	+	++	0	+++
1565	+	+++	0	++	++	0	+++
1715‡	+	+++	++	++	+++	0	+
1716‡	+	+++	0	++	++	0	++
1585	−	+++	+	0	++	0	+
1596	+(−)	+++	+	0	++	0	+
1595	−	+++	+	+	++	0	+++
1580	−	+++	0	++	+	0	+
1574	+	+++	0	+	+	0	+++
1734	+	+++	0	+	+	0	+++
1735	+	+++	0	+	+	0	++
1463 (*P. roqueforti*)	+(?)	+++	+++	+++	+++	++	+++

NOTE: Plates of agar devoid of added carbon source were inoculated with each strain and only growth on experimental plates exceeding this quantity was considered as due to utilization of the carbon source.
*Growth estimated by visual inspection using colony diameter, thickness, and quantity of aerial hyphae as criteria of growth. Three plus signs refers to maximal growth of the indicated strain during the experiment.
†The negative sign indicates that there is no evidence for the production of alkaloid (Table II and ref. 11).
‡Different species of *Claviceps*.

Penicillium roqueforti grew on all carbon sources indicating that lack of growth by other isolates was not due to an error in the preparation of media.

L-*Tryptophane.*—L-Tryptophane stimulates production (Fig. 1) of the ergot alkaloids and increases the quantity of clavine alkaloids (11, 13). β-C^{14}-labelled tryptophane is a precursor in the biogenesis of the ergot alkaloids in PRL 1578 (13) even though tryptophane cannot be used as a sole carbon or nitrogen source for growth. This amino acid has no pronounced influence on growth or on utilization of other carbon and nitrogen sources, but does increase the total yield of alkaloid (11) (Figs. 1 and 2) and suppresses the release of organic nitrogen. The production of alkaloids may be related to the inability to use L-tryptophane as carbon or nitrogen source for growth.

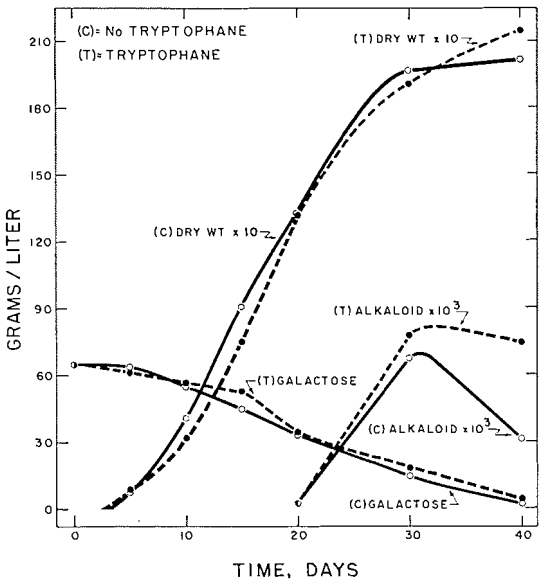

FIG. 1. Growth, utilization of the carbon source, and alkaloid production in the presence and absence of L-tryptophane by PRL 1578. Figures 1 and 2 portray data of the same experiment.

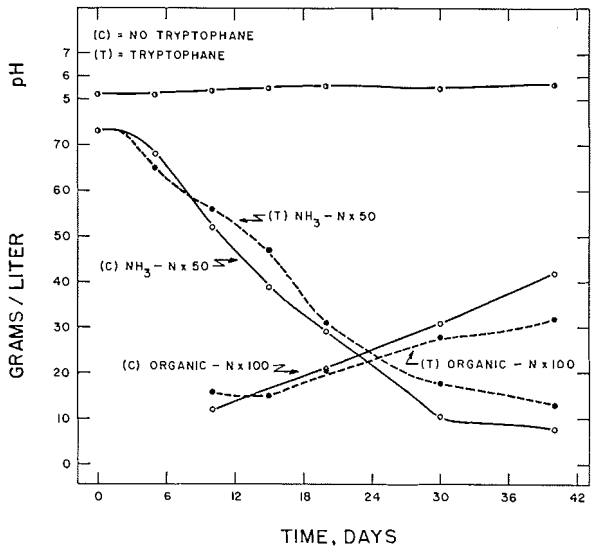

FIG. 2. Utilization of ammonium nitrogen, release of organic nitrogen, and changes in pH in the presence and absence of L-tryptophane. Figures 1 and 2 portray data of the same experiment.

However, neither producing nor non-producing isolates tested could use this amino acid as a carbon source (Table IV) and a similar survey (data not given) of these strains showed that none could use tryptophane as a nitrogen source for growth. *C. maximensis* and *Claviceps* sp. (PRL 1716) managed slight growth on a medium containing tryptophane as the sole nitrogen source. Failure to utilize tryptophane, then, is not a requisite for alkaloid synthesis. *P. roqueforti* PRL 1463 could use L-tryptophane in both capacities though to only a limited extent. Isolates PRL 1578, 1715, and 1716 were examined, in the Warburg respirometer, for ability to oxidize and decarboxylate L-tryptophane and results with PRL 1578 are given in Fig. 3. Under conditions allowing oxidation of glutamic acid, L-tryptophane was not oxidized by any of the three strains. The direct method of measurement did not reveal decarboxylation of tryptophane. Gröger *et al.* (3), however, found that tryptophane labelled with C^{14} in the carboxyl group was not incorporated into ergot alkaloids by *C. purpurea*, indicating that decarboxylation of tryptophane probably takes place during biosynthesis of the ergoline ring. Loss of the carboxyl group

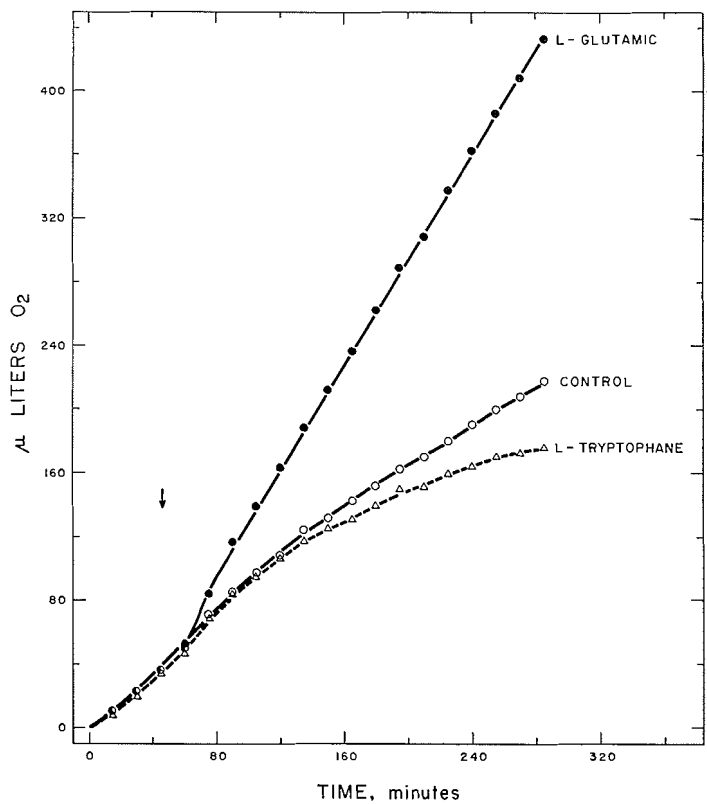

FIG. 3. Failure of PRL 1578 to oxidize L-tryptophane under conditions suitable for oxidation of glutamic acid. The side arm contained 50 μmoles amino acid + 1 μmole glucose. The control contained 1 μmole glucose. Each vessel contained the equivalent of 7.3 mg dry weight of mycelium. The pH of each vessel at the end of the experiment was 6.4.

may therefore occur only by a concurrent reaction involving condensation of a second molecule, e.g. mevalonic acid and pyrophosphate, with the activated α-carbon.

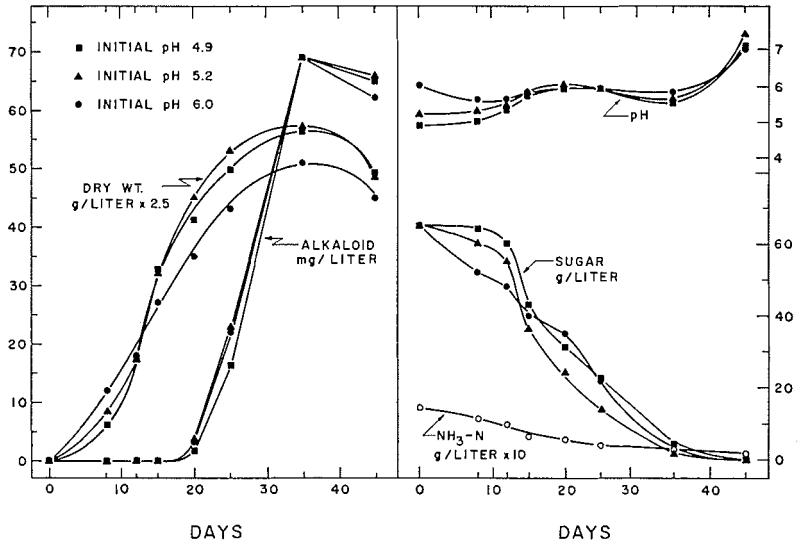

FIG. 4. Growth, utilization of sugar and ammonium nitrogen, and production of ergot alkaloids in the presence of three concentrations of succinic acid by PRL 1578. Succinic acid was added to each lot of medium to bring it to the indicated pH. The ammonium nitrogen value is the average for the three sets of cultures.

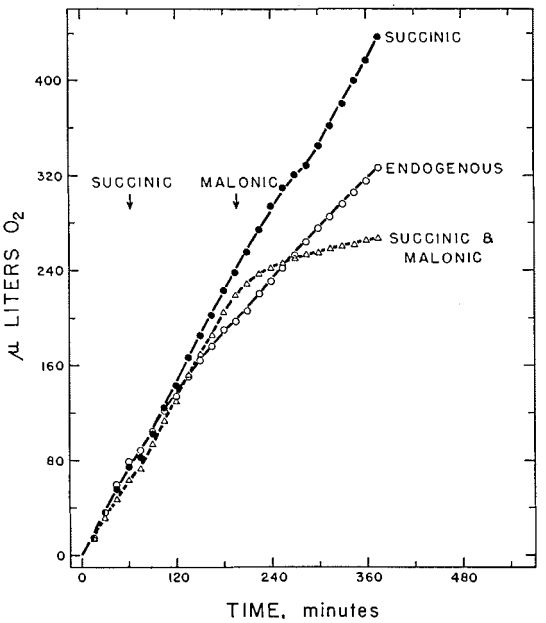

FIG. 5. Oxidation of succinic acid by PRL 1578 and inhibition by 1×10^{-2} M malonic acid. Each vessel contained the equivalent of 11 mg dry weight mycelium.

Succinic acid.—PRL 1578 produced maximum yields of alkaloid when succinic acid was present in the medium (11). It has now been shown that the specific quantity originally used is not required (Fig. 4). The role of succinic acid has not been determined but it is known that it can be used as a carbon source for growth (Fig. 4) (12) and that it spares the carbon source during the early phase of growth. Either the high concentration of succinic acid, or the low pH caused by it, however, retards this early growth (Fig. 4). Succinic acid is slowly oxidized and oxidation, including endogenous, is inhibited by 1×10^{-2} *M* malonic acid (Fig. 5). All of these data illustrate the fact that succinic acid is metabolized by a producing culture and the possibility was considered that the ability to produce ergot alkaloids was associated with the ability to metabolize succinic acid. However, when producing and non-producing strains were tested for ability to use succinic acid as a carbon source (Table IV), all were found capable of using it.

Discussion

This study has demonstrated that strain differences exist with respect to utilization of carbon and nitrogen sources for the production of ergot alkaloids. While some differences in utilization of carbon sources for growth exist, there is no correlation between utilization of any of them for growth and the capacity to synthesize ergot alkaloids. The comparative utilization of two substances involved in the biogenesis of ergot alkaloids, succinic acid and tryptophane, was examined. Both producing and non-producing strains could use succinic acid as a carbon source for growth and none could use L-tryptophane as a carbon or nitrogen source for growth.

The ability to synthesize ergot or clavine alkaloids is not limited to *C. purpurea*. Two other species, *C. maximensis* and *Claviceps* sp. PRL 1716* synthesized alkaloids.

Mannitol would be the best single carbon source of those tested to use in a screening procedure since most strains produced detectable quantities of alkaloid on it. The fact that strains often produced alkaloid on more than one carbon source and that they differed as to the "optimal" carbon source for production indicates, however, that a medium which allows maximum production for one strain may not be satisfactory for production by another strain. The nitrogen source is also an important variable, and, although ammonium nitrogen appears to be widely used for production, a second nitrogen source, yeast extract, included in a screen might result in the detection of additional isolates capable of production.

The fungus *Penicillium roqueforti* PRL 1463, which produces substances giving a positive reaction with *p*-dimethylaminobenzaldehyde, bears no strict nutritional similarities to alkaloid-producing strains of *Claviceps purpurea*.

*These species were obtained from and identified by Dr. W. P. Campbell, Plant Pathology Laboratory, Canada Department of Agriculture Research Branch, Edmonton, Alberta.

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