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PHYSIOLOGY OF ALKALOID PRODUCTION BY CLAVICEPS PURPUREA (FR.) TUL.

CORRELATION WITH CHANGES IN MYCELIAL POLYOL, CARBOHYDRATE, LIPID, AND PHOSPHORUS-CONTAINING COMPOUNDS[1]

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Abstract

Production of ergot alkaloids by cultures of *Claviceps purpurea* was regulated by altering single experimental variables and the pattern of changes in mycelial constituents examined. Cultures which produced alkaloid ceased to accumulate polyols, carbohydrate, and lipid in the mycelium just prior to the onset of alkaloid synthesis whereas the concentration of water-extractable nitrogen and ribonucleic acid remained high or increased. Cultures which failed to produce alkaloid continued to accumulate carbohydrate, polyols, and lipid, but not nitrogenous constituents in the mycelium throughout the late growth phase. The differences between producing and non-producing cultures were consistent irrespective of method used to control the formation of alkaloid. The results suggest that production occurs only under conditions where the accumulation of certain non-nitrogenous cell components has ceased. No consistent correlation was observed between alkaloid production and changes in condensed inorganic phosphate or orthophosphate in the mycelium.

Introduction

The metabolic products of fungi have been divided by Foster (7) into two classes, cell constituents and "shunt" products. To the latter he assigned substances such as organic acids, carbohydrates, and lipids when, because of growth on rich or unbalanced media, they are accumulated in excessive amounts. He also suggested that compounds which appear to have no essential metabolic function such as antibiotics might belong in the latter category and be formed by a similar process, described as "shunt" or "overflow" metabolism. Bu'Lock (4) favors the term "secondary metabolite" to describe products of unusual chemical structure as well as normal intermediates when they are produced in large amounts. Much is now known about biosynthetic pathways involved in the formation of these secondary metabolic products (4).

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Our understanding of the factors controlling their formation is, however, still very limited. For compounds of economic importance the conditions of culture affording a maximum yield of product are usually arrived at empirically. A few attempts have been made to correlate production of penicillin (12, 23), hydroxytetracycline (24), and aureomycin (2, 8) with changes in mycelial composition, particularly of phosphorus-containing compounds, but these have not led to any conclusions of a general nature. The objective of the present study was to determine whether changes in conditions of culture which lead to an increase in secondary metabolite formation also induce a common pattern of changes in the fungus. The "secondary metabolism" examined is the formation of ergot alkaloids by the fungus *Claviceps purpurea* (Fr.) Tul. It should perhaps be noted that compounds of this type may represent a distinct subgroup of secondary metabolites, all of which contain nitrogen and may be derived, at least in part, from amino acid precursors.

Earlier work (18, 22) had detected a decrease in the economic coefficient and in the rate of mycelial dry weight accumulation during the late growth phase when alkaloid was formed. This suggested a change in mycelial composition. Attention has therefore been directed to changes in intracellular components such as polyols, carbohydrates, reducing sugars, lipids, water-extractable nitrogen, nucleotides, and inorganic phosphates during growth. Phosphorus-containing compounds were included because earlier work (8, 12, 24) in this field has been concerned mainly with such components. Experimental variables which were used to affect the production of alkaloids by the fungus were (*a*) phosphate concentration in the medium, (*b*) adaptation of the inoculum to galactose when this sugar was used as a major source of carbon, (*c*) the type of nitrogen source, and (*d*) supplementation of cultures grown on a glucose-containing medium with additional sugar at the time when the initial supply was nearly exhausted.

Materials and Methods

Cultures and Culture Conditions

The two natural isolates of *Claviceps purpurea*, PRL 1578 and 1565, have been described previously (17, 19). Medium No. 10-B, which was found to support alkaloid production by PRL 1578 in shaken culture, contained a galactose–glucose (19:1) mixture (65 g/liter), ammonium succinate (7.2 g/liter), filter-sterilized L-tryptophan (0.5 g/liter), mineral salts, and biotin. Its composition and preparation have been given in detail in an earlier publication (17). It was modified in the experiment designed to test the effect of different levels of phosphate on production by increasing the concentration of KH_2PO_4 from 250 to 750 mg/liter. To obtain alkaloid production by PRL 1565, medium No. 10-B was modified by using glucose (65 g/liter) as a carbon source and Difco yeast extract (17 g/liter) as a nitrogen source. Succinic acid was included at a rate of 3.5 g/liter. In the experiment with this strain where the nitrogen source was varied, the medium not allowing production contained ammonium succinate (8.6 g/liter) in place of yeast extract.

Inocula were prepared as described earlier (17, 18) except in one experiment with PRL 1578 on medium No. 10-B. Here half of the flasks were inoculated

with a line adapted to galactose by serial transfer through a medium containing galactose as the carbon source until rapid growth was evident. In all experiments Erlenmeyer flasks (250-ml) containing 50 ml of medium were each inoculated with the equivalent of 5 mg dry weight of homogenized and washed mycelium and incubated at 25° C in the dark on a shaker with a half-inch radius of motion and rotating at 110 r.p.m.

Six culture flasks from an experiment were removed at each sampling time. Three were separately filtered and the mycelium washed with distilled water, collected on tared filter paper, freeze-dried, and weighed. The cells were then combined and crushed to a fine powder. The pooled filtrate was frozen and stored at −30° C whenever analyses could not be completed on the day of harvest. The remaining three cultures were analyzed separately for alkaloid as described in an earlier paper (21).

Mycelial Components

Hot water-extractable compounds were obtained by suspending the powdered mycelium (200 mg) in distilled water (10 ml) and heating for 20 minutes in a boiling-water bath. Insoluble material was separated and washed three times with distilled water. The combined extracts and washings were brought to 50 ml and clarified by high speed centrifugation at 3° C. Reducing sugar in the solutions was estimated with the Somogyi reagent (15), total carbohydrate with the anthrone reagent (15), nitrogen by the Kjeldahl method using a K_2SO_4–HgO catalyst, and polyol by the periodate oxidation procedure (15). The difference between the values obtained with the Somogyi and anthrone reagents was considered to be caused by the presence of polysaccharides. The Kjeldahl procedure used gave nitrogen contents for commercial yeast ribonucleic acid (RNA), adenine, uracil, and chitin in good agreement with the calculated values. Where necessary, the results of the polyol assay were corrected for color estimated to be due to reducing sugar in the extract by including both glucose and mannitol standards in each assay. The amount of tryptophan in the solution was found to be too low to cause error in the determination of reducing sugar. When the hot water extraction procedure was repeated no additional sugar and only a trace of additional nitrogen was extracted from the insoluble material. Statistical analysis of variance of the values obtained by determinations in triplicate made on four equal samples from the same batch of mycelium indicated that the procedure was reproducible.

Acid-extractable carbohydrate was obtained by suspending hot water extracted mycelium in 0.5 *N* HCl and heating the suspension in a boiling water bath for 20 minutes. The soluble fraction was assayed for total carbohydrate with the anthrone and reducing sugar with the Somogyi reagents. Since paper chromatography showed the main reducing sugar to be galactose, this was used as a standard in the estimations. Re-extraction of the residue with fresh acid removed an additional amount of carbohydrate, equal to about one-fifth of that obtained by the first extraction. Thirty to forty percent of the total carbohydrate in this fraction consisted of reducing sugar, and the proportion is relatively constant regardless of the age of cultures. Since the

monosaccharide component is galactose whereas the main constituents of the cell wall are glucose and glucosamine, the material extracted by this procedure is probably derived from an acid-labile polymer, solubilized by hydrolysis. The amount measured by the anthrone test after a single extraction may not represent the whole of this fraction but is probably a constant proportion of it.

Phosphorus-containing compounds were extracted from mycelium and fractionated by a procedure similar to that of Krishnan *et al.* (11). Briefly, powdered mycelium was extracted with ice-cold 5% trichloroacetic acid (TCA) and ice water to give an "acid-soluble" fraction. This extract was immediately treated with ether to remove TCA and then assayed for orthophosphate by the method of Berenblum and Chain (1). Tests for orthophosphate in commercial pyrophosphate or "hexametaphosphate" by this procedure were negative indicating the specificity of the method. Nucleotides were estimated by their absorption at 260 mμ using yeast RNA as a standard. Total "acid-soluble" phosphorus was estimated after digestion of an aliquot by the Kjeldahl procedure. The difference between total phosphorus and orthophosphate was considered to be "acid-soluble condensed phosphate". After the residual mycelium had been extracted with solvent to remove lipid (see below), it was re-extracted with 5% TCA at 90° C to give the "acid-insoluble" fraction. This was assayed for RNA by the ultraviolet absorption method after a detailed comparison had shown the results obtained to be comparable with those given by the orcinol procedure of Markham (14). The hot TCA extract did not contain sufficient deoxyribonucleic acid to be detectable by the diphenylamine method of Markham (14). Total "acid-insoluble" phosphorus was estimated as above. "Acid-insoluble" polyphosphate was estimated as the difference between total phosphorus and RNA-phosphorus (assuming the phosphorus content of RNA to be 10%) and also, in one experiment (Table I), as phosphate labile to hot 1 *N* HCl after nucleotide had been removed with Norit A (6). The two methods were comparable. The former may be in slight error if the percentage of phosphorus in the ultraviolet-absorbing material changes with time (5) or if other material absorbing at 260 mμ is present. The presence of "acid-insoluble" polyphosphate in the extract was also demonstrated by the paper chromatographic method of Kolloff (10). Pyrophosphate was estimated by the method of Lehninger and Smith (13).

Lipid, the residue left after cold TCA treatment, was extracted with ethanol and then with a mixture of ethanol–ether (1:3) (11). The extracts were combined, evaporated, and weighed. A representative sample contained 0.6% phosphorus and 6% nitrogen.

"Cell wall" material was separated in a crude form by steeping the hot water extracted residue overnight in ethanol, then subjecting the insoluble material to two successive extractions with 4.5% aqueous KOH under reflux for 15 minutes. The residue was washed successively with acetic acid, ethanol, and water, then freeze-dried, weighed, and assayed for nitrogen. The nitrogen content varied between 1.2 and 1.6% and the ash averaged 0.2%.

TABLE I

Effect of phosphate concentration in the medium on growth, alkaloid production, and changes in the mycelial constituents of PRL 1578*

		Age of cultures, days								
		3	7	9	12	16	18	20	25	30
Alkaloid	H	0	0	0	0	0	0	0	0	0
	L	0	0	0	0	0	0	0	5	5
Dry weight	H	1,200	6,000	6,800	6,400	8,100	10,800	14,700	16,800	15,900
	L	700	4,800	5,500	9,000	11,300	12,500	13,700	14,800	13,500
Total N	H	84	330	380	390	486	540	706	696	684
	L	46	288	336	495	622	538	685	755	689
Polyol	H	140	930	980	850	1,100	1,610	2,400	2,830	2,620
	L	70	780	720	1,310	1,560	1,650	1,930	1,970	1,480
Carbohydrate	H	93	830	1,108	781	689	940	1,103	1,210	932
	L	68	586	732	891	904	1,063	932	681	675
Reducing sugar	H	91	798	1,088	698	559	637	617	974	890
	L	63	576	715	684	825	800	589	524	527
Lipid	H		578	789	920	1,102	1,350	1,882	2,285	2,099
	L		531	688	1,386	1,751	1,825	2,000	1,954	1,959
H_2O-extractable N	H	32	114	146	109	300	270	441	336	349
	L	5	91	110	243	192	263	219	311	149
RNA	H		198	177	192	203	302	309	302	254
	L		182	176	288	237	275	315	370	351
"Acid-insoluble" polyphosphate	H		17	5	5	20	32	63	48	14
	L		4	0	0	0	0	0	0	0
Orthophosphate	H		17	22	15	15	21	22	32	27
	L		7	7	3	3	2	0	3	3

*H (high) and L (low) indicate the initial phosphate concentration in the medium on which the cultures were grown. The figures represent the amount of substance found as mg/liter of culture. Orthophosphate and "acid-insoluble" polyphosphate are reported as elemental P.

Results

Phosphate as Variable

Alkaloid was produced by PRL 1578 only when the phosphate concentration in the medium was low. A comparison during the late growth phase showed that mycelia from cultures grown on the high phosphate medium had a greater dry weight but no greater total nitrogen content than mycelia from cultures grown on low phosphate. The difference can be attributed to the greater content of polyol, carbohydrate, reducing sugar, and lipid in the former (Table I). Accumulation of these substances in the mycelium of cultures grown on low phosphate ceased relatively early and before the appearance of alkaloid. On the other hand, accumulation of RNA and water-extractable nitrogen was maintained during the period of alkaloid synthesis. Phosphorus may have become a limiting factor in their metabolism since there was considerably less orthophosphate, "acid-soluble" condensed phosphate, and "acid-insoluble" polyphosphate in the cells (Table I). Nevertheless accumulation of RNA continued even after phosphate in the medium had been exhausted (Table II) and seems to have occurred at the expense of condensed inorganic phosphate. The RNA content of these cultures eventually reached a higher value than that of the cultures provided with the larger initial supply of phosphate in the medium. Presumably the fact that the latter ceased to accumulate RNA relatively early has an important bearing on this situation.

The lag in growth between the 7th and 18th day of cultures grown in a high phosphate medium is attributed to a diauxie pause after glucose in the medium had been exhausted and before utilization of galactose had begun. With an inoculum grown on glucose, galactose was evidently used only after the organism had adapted to it. The diauxie is reflected in changes in both mycelial and filtrate constituents (Tables I and II). Absence of the effect in cultures grown on low phosphate suggests that the reduced phosphate concentration in the medium limited the rate of growth and sugar utilization, even in the early period when excess phosphate was still present. The lower overall rate of growth, in addition to evidence from earlier work (17, 18, 19, 20, 22), seemed to indicate that a restriction of growth is necessary for the formation of alkaloids by *C. purpurea*.

Galactose Adaptation as Variable

It was of interest to learn whether preadaptation of the inoculum to galactose would result in more rapid growth and suppress alkaloid production even with the lower concentration of phosphate in the medium. This proved to be so (Table III). Moreover, the adapted, non-producing cultures accumulated carbohydrate and polyols but not water-extractable nitrogen or RNA during the late growth phase. The producing cultures showed a decrease in rate of accumulation of polyol and lipid and an increase in RNA and water-extractable nitrogen (Table III). No explanation is available for the continued accumulation of reducing sugar. This apparent anomaly may be the result of breakdown of a cellular polymer. The large amounts of material stored in the adapted cells is reflected in the relatively more rapid increase in dry weight than in "cell wall" during the latter stages of growth. In unadapted, alkaloid-producing cultures, "cell wall" and dry weight accumulation were more

TABLE II

Changes in filtrates of cultures of PRL 1578 grown on high and low phosphate*

		Age of cultures, days									
		0	3	7	9	12	16	18	20	25	30
pH	H	5.2	5.1	6.0	5.9	5.9	5.6	5.5	5.6	6.0	6.0
	L	5.2	5.1	6.0	5.8	5.9	5.6	5.7	5.5	5.4	5.4
NH_3 N	H	1,600	1,460	1,120	1,010	930	740	630	480	320	280
	L	1,600	1,460	1,280	1,160	740	520	440	340	360	240
Organic N	H	0	15	46	170	200	180	240	260	460	390
	L	0	60	40	80	270	290	350	500	400	430
Orthophosphate	H	171	94	51	34	50	40	0	0	0	4
	L	57	40	3	2	0	0	0	0	0	1
Reducing sugar (as galactose)	H	66,000	66,000	60,000	52,000	45,000	39,000	32,000	21,000	12,000	9,000
	L	66,000	66,000	58,000	55,000	43,000	32,000	31,000	22,000	13,000	13,000

*H (high) and L (low) indicate the initial phosphate concentration in the medium on which the cultures were grown. The figures represent the amount of substance found as mg/liter of culture. Orthophosphate and "acid-insoluble" polyphosphate are reported as elemental P.

TABLE III

Effect of adapting the inoculum to galactose on growth, alkaloid production, and changes in mycelial constituents of PRL 1578*

		Age of cultures, days					
		5	8	12	15	20	25
Alkaloid	Ad.	0	0	0	0	0	0
	Unad.	0	0	0	0	2	4
Dry weight	Ad.	4,200	9,700	11,100	11,500	14,700	
	Unad.	2,100	3,900	6,200	8,900	12,900	
Cell wall	Ad.		960		1,875	2,337	
	Unad.		331		1,380	2,141	
Polyol	Ad.		1,032		756	1,029	
	Unad.		273		961	970	
Reducing sugar	Ad.		1,151		895	2,538	
	Unad.		702		437	1,541	
Lipid	Ad.		1,251		1,622	1,970	
	Unad.		437		1,184	1,509	
RNA	Ad.		260		240	260	
	Unad.		110		270	300	
H_2O-extractable N	Ad.		126		265	235	
	Unad.		43		169	206	
Orthophosphate	Ad.		3		6	4	
	Unad.		3		6	4	

*The medium contained a mixture of galactose and glucose in the ratio of 19:1 as carbon source. Cultures grown on adapted and unadapted inocula are represented by "Ad." and "Unad.", respectively. Values are reported as described in footnote of Table I.

TABLE IV

Effect of nitrogen source on growth, alkaloid production, and changes in mycelial constituents of PRL 1565*

		Age of cultures, days							
		2	3	4	5	7	9	12	15
Alkaloid	N	0	0	0	0	0	0	0	0
	Y	0	0	0	0	2	7	8	0
Dry weight	N	4,600	7,800	11,500	13,400	15,100	16,400	16,600	16,700
	Y	12,100	16,300	21,900	23,500	24,700	22,300	17,300	16,100
Total N	N	377	585	794	804	831	886	780	835
	Y	908	994	1,095	1,151	1,210	1,204	952	757
Polyol	N	320	230	140	160	240	310	420	630
	Y	650	1,660	2,540	2,350	2,070	1,050	210	190
Carbohydrate	N	235	835	1,633	1,876	1,993	1,967	1,926	1,353
	Y	1,065	1,090	1,510	987	865	558	350	241
Lipid	N	726	975	1,725	1,956	2,295	2,706	3,000	2,960
	Y	1,089	1,695	2,321	2,279	2,347	1,873	1,660	1,460
H_2O-extractable N	N	70	140	190	260	530	300	270	260
	Y	190	260	330	370	370	350	200	190
RNA	N	201	296	261	214	166	164	144	150
	Y	504	424	460	541	568	423	237	161
"Acid-insoluble" polyphosphate	N	0	0	0	0	0	0	0	0
	Y	14	20	39	26	28	40	24	8
Orthophosphate	N	2	4	6	7	2	3	3	5
	Y	36	25	28	31	62	83	111	93
"Acid-soluble" condensed P	N	6	5	5	4	6	7	13	1
	Y	41	42	55	63	43	30	0	0

*"N" refers to cultures grown on ammonium succinate and "Y" to those grown on yeast extract as the nitrogen source. Values are reported as described in footnote of Table I.

nearly parallel. The orthophosphate and "acid-soluble" condensed phosphate content of cultures grown from the two types of inocula did not differ significantly and, except for the changes in RNA content, there appeared to be no relationship between production of alkaloid and the fate of phosphorus-containing substances in the mycelium.

Nitrogen Source as Variable

PRL 1565 produced alkaloid on a modified No. 10-B medium containing glucose and yeast extract as carbon and nitrogen sources. When ammonium succinate was used in place of yeast extract, growth was slower but alkaloid was not formed. However, the relationship observed in the two preceding experiments between accumulation of mycelial components and production of alkaloid was maintained (Table IV). In this strain, reducing sugar did not accumulate in the mycelium in detectable amounts. The rapid decrease in polyol and carbohydrate in mycelium grown on yeast extract began on the fourth day, coincident with the disappearance of reducing sugar from the

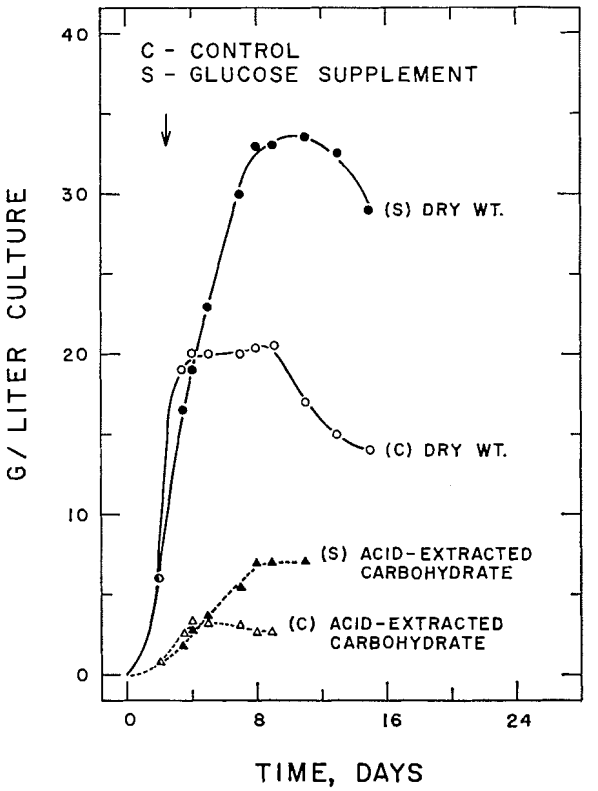

FIG. 1. Changes in mycelial dry weight and acid-extractable carbohydrate in cultures of PRL 1565 grown in a glucose – yeast extract medium. Lines marked (S) are those of cultures which received supplementary glucose at 2½ days, while (C) designates the unsupplemented controls.

medium. In the same interval, cultures grown on ammonium succinate steadily accumulated polyol in both the mycelium and filtrate. They also continued to accumulate lipid in the cells during the late growth phase whereas the producing culture ceased accumulating lipid after the fifth day.

The RNA content of cultures grown on yeast extract was at a maximum when alkaloid formation began on the seventh day and decreased shortly thereafter (Table IV). The rapid fall may be due to general dissolution of the culture after 9 days since dry weight and total nitrogen also decreased and the amount of nucleotide (collected on Dowex-1 × 8 (Cl^{-1}) (16)) in the filtrate increased. There was an inverse relationship between the amount of RNA and "acid-insoluble" polyphosphate in the mycelium until the general loss of cell constituents on the ninth day. Paper chromatography showed that the "acid-insoluble" polyphosphate was of approximately the same molecular size as the less polymerized fraction of commercial "hexametaphosphate". However, some of the polyphosphate had been hydrolyzed to smaller polymers or to orthophosphate during the hot TCA extraction since up to 22% of the

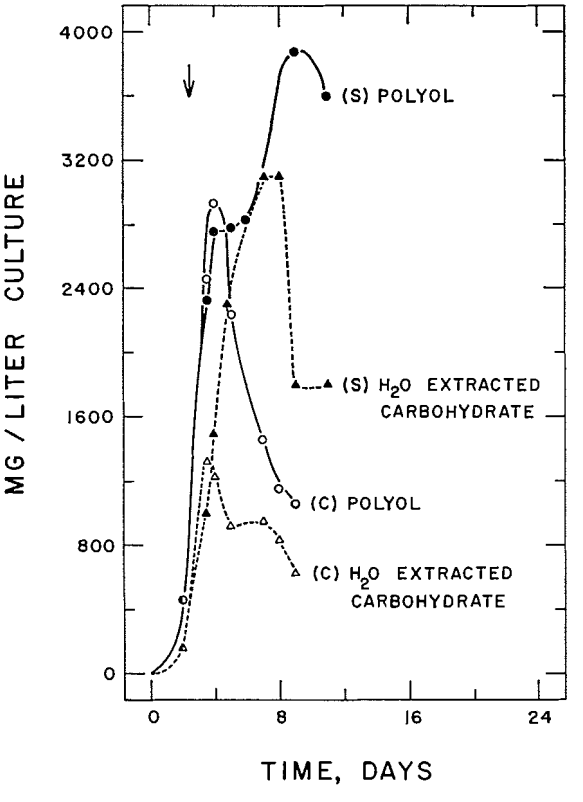

FIG. 2. Changes in mycelial polyol and water-extractable carbohydrate of cultures described in Fig. 1.

phosphorus was present as orthophosphate. This could be derived only from condensed phosphate because a preliminary cold TCA extraction had removed orthophosphate from the mycelium.

The amount of phosphate initially present in the ammonium succinate medium was lower than in the yeast extract medium. As in the experiment where cultures of PRL 1578 were grown in media containing different concentrations of phosphate, mycelium produced on a restricted supply of phosphate never contained more than a trace of "acid-insoluble" polyphosphate. Other workers (8, 12) have similarly shown that the polyphosphate content of cells is variable and depends on the growth medium. "Acid-soluble" condensed phosphate did accumulate in cells grown on the low phosphate medium, however (Table IV). There is evidence (9) that no distinction between "acid-soluble" and "acid-insoluble" polyphosphate should be made, but the "acid-soluble" condensed phosphate measured here would have included any nucleotides present.

Supply of Glucose as Variable

It was noted in the previous experiment that the polyol and carbohydrate content of mycelia decreased when the carbon source of the medium had been exhausted. This can be prevented by adding more glucose to the culture before the initial supply is completely exhausted. For the relationship noted

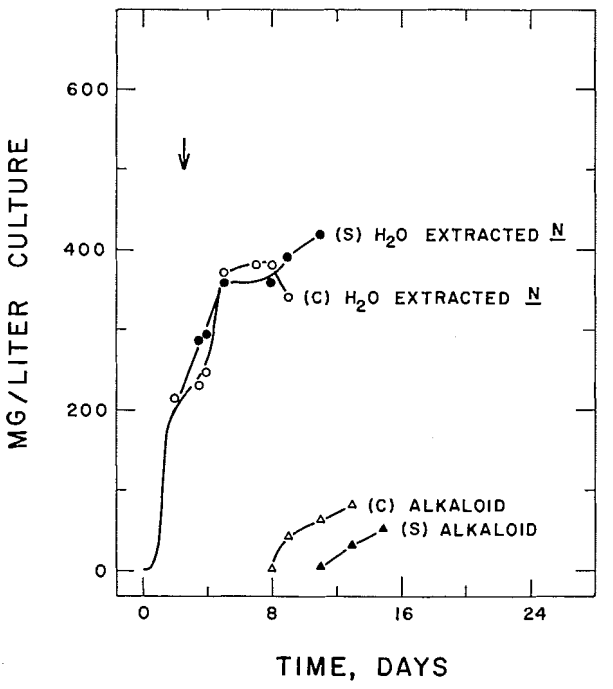

FIG. 3. Production of ergot alkaloids and changes in hot water-extractable nitrogenous constituents in the mycelium of cultures described in Fig. 1.

in all previous experiments between changes in cell constituents and alkaloid production to be valid synthesis of alkaloid should then be delayed. Figures 1, 2, and 3 show that this expectation was realized. It is noteworthy that cultures supplemented with glucose began to lose hot water extractable nitrogen from the mycelium at the time when alkaloid formation would otherwise have occurred. Eventually, however, this nitrogenous fraction increased again, polyol and carbohydrate content declined, and alkaloid was formed.

Changes in the amount of acid-extractable carbohydrate in the mycelium generally paralleled those in mycelial dry weight. Since no increases occurred at times when polyol and hot water extractable carbohydrate were decreasing, it does not appear to represent an alternate form of these substances. More likely it is structural in character, rather than a reserve or byproduct.

Discussion

Rate of growth cannot be the common factor which controls alkaloid formation but seems to be merely one way of effecting a suitable pattern of intracellular metabolism. Two of the characteristics of this pattern which were common to all conditions examined where alkaloid was formed and absent from unproductive cultures were (*a*) cessation of the increase in intracellular polyol, carbohydrate, and lipid before the end of growth, and (*b*) maintenance of a high level of hot water extractable nitrogen and RNA in the mycelium. The level of RNA and water-extractable nitrogen remains high if the culture remains active and decreases if leaching of the mycelium begins. No meaningful and consistent correlation between alkaloid formation and changes in content of either orthophosphate or condensed inorganic phosphate was detected. Other experimenters (8, 12, 22, 24) who examined the changes in phosphorus-containing substances in mycelia during secondary metabolite formation made phosphate one of the experimental variables. The results which they obtained appear to have been related more directly to the effect of phosphate concentration on the growth of the organisms than on its ability to influence "secondary metabolism".

When growth is viewed as consisting of the various phases proposed by Borrow and co-workers (3), it would appear that ergot alkaloids accumulate during a prolonged *transition phase* of a type which is characterized by a continued accumulation of nitrogenous but not carbohydrate-like reserves. Non-producing cultures pass rapidly into the *storage phase* which normally takes place (3) when nitrogen is exhausted in the presence of a carbon source. From the data presented here and elsewhere (17, 19, 22), failure to enter the *storage phase* may be due to (*a*) genetic constitution, (*b*) growth limitation due to partial starvation of phosphate or carbon source, or (*c*) exhaustion of readily available carbon source while a poor carbon source such as galactose, mannitol, or succinic acid and a nitrogen source are still available.

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