APPLIED MICROBIOLOGY, Nov. 1967, p. 1270-1283
Vol. 15, No. 6

Production of Lysergic Acid Derivatives in Submerged Culture

IV. Inorganic Nutrition Studies with *Claviceps paspali*

JOHN P. ROSAZZA, WILLIAM J. KELLEHER, AND ARTHUR E. SCHWARTING

Pharmacognosy Research Laboratories, University of Connecticut, Storrs, Connecticut 06268

Received for publication 15 May 1967

The inorganic requirements for growth and alkaloid production by submerged cultures of a lysergic acid alkaloid-producing isolate of *Claviceps paspali* were determined in a defined medium. The requirement for peak growth was essentially the same as the requirement for maximal alkaloid production in the case of potassium and magnesium. With phosphorus, however, maximal growth was reached at lower levels than those required for maximal alkaloid production. Sulfur was required in smaller amounts for peak alkaloid production than for maximal growth. In the case of iron and zinc, the requirement for maximal alkaloid production was greater than for growth. Manganese and copper could be sufficiently depleted to demonstrate their essentiality for alkaloid production, but their requirements for growth could not be demonstrated.

A number of studies have described various effects of mineral salts on growth and alkaloid production by *Claviceps* species (14-16, 23, 36, 37, 43). These investigations either were conducted with complex media or failed to take special precautions to reduce the concentration of salts introduced adventitiously into the medium. Consequently, none of these studies has provided definitive results on the role of the trace elements in growth and alkaloid production.

Investigations in this laboratory with lysergic acid alkaloid-producing isolates of *C. paspali* have consistently revealed an involvement of inorganic salts with growth, and especially with alkaloid production. In early studies with the C-60 isolate, supplementation of the basal medium with a complex mixture of inorganic salts caused an increase in both growth rate and alkaloid production (27). During the course of selection and screening experiments, the composition of the tap water used to prepare the media was shown to have a very strong influence on alkaloid production (28). Efforts were, therefore, undertaken to establish a defined medium for the production of lysergic acid alkaloids. This was accomplished by obtaining a complete analysis (neutron activation) of a sample of tap water that supported high alkaloid production, creating a medium that contained the appropriate inorganic salts to simulate the medium prepared with tap water, and finally omitting each of the inorganic salts, singly, to determine which ones were essential for growth and alkaloid production (21). With this method, the requirement for certain elements was established, but the requirement for a few of the trace elements was not clarified.

On the basis of these results, two defined media were devised; one contained five salts and the other contained eights salts. A series of isolate selections was conducted with each of these media. The superiority of the eight-salt medium over the five-salt medium was demonstrated in these experiments (21).

The selection series conducted with the eight-salt medium made available several isolates capable of producing more than 1,000 μg of total alkaloids per ml in a defined medium. These isolates, together with the defined medium provided a very convenient system for a precise determination of the inorganic requirements for growth and alkaloid production. The results of such a study are presented in this report.

MATERIALS AND METHODS

Culture. An isolate of *C. paspali*, no. 31 of the fifth selection on the eight-salt medium, was used in these studies (21). Two stock slants of this isolate were employed. The first was the original slant prepared at the time the isolate was selected (21); the second was a slant, designated 1H-1, prepared from the original.

All slants were maintained on potato-dextrose-agar (PDA; 1) at 2 to 3 C in tightly closed screw-cap tubes.

TABLE 1. *Concentrations of inorganic salts in the eight-salt medium*

Salt	Salt	Salt	Cation
	moles/liter	*mg/liter*	*mg/liter*
$Ca(NO_3)_2 \cdot 4H_2O$...	1.27×10^{-3}	300	50.9
$MgSO_4 \cdot 7H_2O$.....	3.25×10^{-3}	800	79
$ZnSO_4 \cdot 7H_2O$.....	1.74×10^{-5}	5	1.14
$FeSO_4 \cdot 7H_2O$.....	1.80×10^{-4}	50	10
$CuSO_4 \cdot 5H_2O$.....	2.01×10^{-5}	5	1.27
$MnSO_4 \cdot 4H_2O$.....	4.48×10^{-5}	10	2.46
$NaNO_3$.....	1.19×10^{-3}	100	27
KH_2PO_4[a].....	7.35×10^{-3}	1,000	227.6

[a] The figure in column 4 represents milligrams of phosphorus per liter.

Media. Basal medium I contained 4% mannitol and 1% succinic acid; it was used together with appropriate salts for the first and second stage of the three-stage submerged culture procedure. Basal medium II contained 5% mannitol and 3% succinic acid; it was used together with various salts for the third stage (alkaloid-producing) of the submerged culture procedure. For all fermentations, except those in which magnesium and sulfur were studied, the salts used in both medium I and medium II were variations of the mixture shown in Table 1.

To determine the effects of magnesium and sulfur separately, modifications of the basal medium had to be employed. This was done, in the case of magnesium, by adding various amounts of magnesium as magnesium sulfate, while at the same time insuring the normal level of sulfur by adding appropriate amounts of ammonium sulfate. In the study of sulfur, this element was contributed solely by magnesium sulfate. Magnesium was maintained at its normal level in all cases by the addition of appropriate amounts of magnesium succinate. Other metal sulfates were replaced by quantities of their respective nitrates sufficient to give the same concentration of cation.

The *p*H of all media was adjusted to 5.2 with ammonium hydroxide solution.

All water used in fermentation media was prepared by redistilling laboratory-distilled water from an all-glass still. Water thus prepared was stored in a large, acid-cleaned, Pyrex container. Relative purity of glass-distilled water was checked with a Barnstead purity meter.

Mannitol was demineralized by passing a 10% solution of this polyol through a column of an anionic-exchange resin (Dowex 1-X8 hydroxyl form), and then through a column of a cationic-exchange resin (Dowex 50-X8 hydrogen form). This purified solution was stored in a large, acid-cleaned Pyrex container.

Succinic acid, potassium phosphate, and other chemicals were reagent grade. Single lot numbers of each reagent were used for all of the fermentations described in this study.

Procedure. Most of the submerged fermentations were conducted according to the three-stage procedure described below. A stock slant was removed from cold storage and used to inoculate a fresh PDA slant. This slant was incubated for 7 days at 24 C. After this period of time, the entire mycelial growth was removed from the slant, homogenized in a small volume of sterile medium I plus salts, and used as inoculum for 100 ml of this medium. This first-stage culture was incubated on the rotary shaker until heavy growth was evident (usually 7 days). The first-stage culture was homogenized, and 10 ml of the homogenate was introduced as inoculum for 100 ml of basal medium I, plus salt variations. This second-stage culture was shaken for 48 hr; 10-ml portions were then used to inoculate 100 ml of basal medium II, plus salt variations. The third stage constituted the alkaloid-producing stage of the fermentation process, and was incubated on the rotary shaker until analyses showed that growth and alkaloid production had reached their peaks.

In certain experiments, the succession of submerged cultures was continued into a fourth and a fifth stage to achieve satisfactory depletion of certain elements in the medium.

Fermentations employed for the study of K, Ca, Mn, and Cu were conducted in duplicate; all others were in triplicate.

Cotton-plugged 500- or 1,000-ml Erlenmeyer flasks containing 100 or 200 ml of culture medium, respectively, were used in all fermentations.

All cultures were grown on a New Brunswick rotary shaker, model G-53, at 24 C, in the dark. The shaker was operated at 250 rev/min, and described a circle 5 cm in diameter.

Sterilization. Sterilization of culture medium, pipettes, and other items was accomplished by heating in an autoclave at 121 C, for 15 min.

Homogenization. Two homogenization procedures were used in the fermentation process: one to homogenize mycelial growth taken from fresh PDA slants and the other to homogenize mycelium in fully grown first submerged cultures.

Homogenization of mycelial growth taken from slants was accomplished by transferring a fragment of mycelium to an 80-ml homogenizing can along with a 20-ml sample of sterile, first-stage culture medium. The mixture was homogenized for 15 sec and then added to the remainder of the first-stage medium in the fermentation flask (total volume, 100 ml).

Fully grown first-stage cultures were homogenized by transferring the contents of the fermentation flask to a 400-ml homogenizing can. The contents of the can were homogenized for 10 sec, and 10 ml of the homogenate was used to inoculate 100 ml of basal medium I containing salt variations.

Cleaning of glassware. All equipment was first washed in hot detergent solution, rinsed in tap water, dried, and submerged in a bath of hot, concentrated nitric-sulfuric (2:1) acid for approximately 15 min. The glassware was removed from this bath and rinsed five times with laboratory-distilled water, and finally two times with glass-distilled water. All glassware used for medium preparation, storage, sampling, analyses, and the flasks in which fermentations were conducted were cleaned according to this procedure.

General analytical methods. A Bausch & Lomb

Spectronic-20 colorimeter was used in the analyses for total alkaloid, zinc, phosphate, and iron. In these analyses, absorbances were measured in 0.5-inch (1.3 cm), uniformly round test tubes.

A Cary double-beam spectrophotometer, model 15, was used in the determinations of copper and manganese. Its use in the copper analysis was dictated by the small volume of the final colored solution. Its use in the manganese determination was based on the ability of the instrument to accommodate special capillary absorption cells constructed according to the specifications of Kelleher and Johnson (17). Such cells had a light path of approximately 5 cm and required only about 0.5-ml samples for filling. This combination of long light path and small volume requirements permitted approximately a 100-fold increase in the sensitivity of the manganese determinations.

Conformance to Beer's law over the concentration ranges encountered was established for all of the analyses employed. All methods were evaluated with recovery samples added to culture filtrates. A method was not considered suitable for use with experimental samples unless recovery was 100 ± 5%.

Concentrations of elements in experimental samples were calculated from the measured absorbances; these values were related to standard curves by the inclusion of reference standards for each analysis. The reference standards were run through the entire analytical procedure (including wet digestion) in all cases.

Sampling procedure. Samples (5 or 10 ml) of third-stage cultures were taken at various intervals during the course of fermentations. Mycelium was separated from culture broth by suction filtration through tared Whatman no. 1 filter-paper discs. The mycelium was then washed with 30 ml of distilled water, dried in an oven for 24 hr at 70 C, and weighed to determine mycelial dry weights. The filtrate was analyzed for total alkaloids and for *pH*.

Samples for the determination of inorganic constituents in the medium were withdrawn immediately after inoculation. Mycelium was separated from the medium by filtration through sintered-glass funnels. The filtrates were placed in 10-ml, acid-cleaned vials and were stored in the frozen state (−20 C) until needed for analyses. One of the following analyses was performed on culture filtrates obtained in this manner: phosphate, copper, iron, manganese, or zinc.

Ashing procedure. Oxidation of organic matter present in culture filtrates was accomplished by a wet-ashing procedure with the use of a ternary acid mixture. This mixture contained concentrated nitric, 60% perchloric, and concentrated sulfuric acids in the proportion 10:3:1, respectively (39). A 5-ml amount of this mixture was found to be adequate for the complete digestion of all the organic matter contained in 10 ml of the culture filtrate.

Samples were placed in micro-Kjeldahl digestion tubes, along with 5 ml of the digestion mixture and an acid-cleaned glass bead. The tubes were placed on a rotary, electrically heated Kjeldahl digestion apparatus equipped with a glass fume hood. The tubes were heated so that boiling of the acid mixture (15-ml volume) usually occurred within 3 min from the time the digestion was initiated. Charring of organic material began after nitric acid had been distilled from the mixture. When the contents of the tubes had cleared, the digestion was continued for an additional 0.5 hr. At the end of the digestion process, approximately 0.5 ml of a mixture of sulfuric acid, perchloric acid, and inorganic salts remained in the digestion tubes.

For the manganese analysis, it was necessary to employ a different digestion procedure to avoid the interference caused by insoluble perchlorate salts. Because the level of acid present in the final analysis mixture is critical for the complete formation and stability of the permanganate color, it was necessary to control the amounts of acid which remained in the digestion tubes after organic matter had been destroyed.

To accommodate these requirements, the following wet-ash procedure was employed.

A 5-ml amount of a mixture of concentrated nitric-sulfuric acids (9:1) was added to the sample contained in micro-Kjeldahl digestion tubes. An acid-cleaned glass bead was added, and the samples were heated on the apparatus described previously. Charring of organic material commenced after all the nitric acid had been distilled from the digestion mixture. Charring was usually accompanied by the appearance of fumes of sulfuric acid. At this point, the digestion tubes were removed from the burners. Thirty per cent hydrogen peroxide was added dropwise to the charred mixture until the mixture cleared. The cleared mixtures were reheated on the burners until charring commenced again. The addition of hydrogen peroxide was repeated until the mixture in the tubes remained clear after heating for 10 min. At the end of this digestion procedure, approximately 0.5 ml remained in the digestion tubes.

Alkaloid. Total alkaloid was determined colorimetrically by the method of Michelon and Kelleher (22). Optical densities were obtained at 590 mμ. Total alkaloid was calculated from the optical density of an ergonovine maleate standard and was expressed as ergonovine maleate.

Inorganic phosphate. Inorganic phosphate was determined by the molybdate method of Berenblum and Chain (2).

Copper. This element was determined by a modification of the methods of Borchardt and Butler (5) and Diehl and Smith (9). Bathocuproine was employed as the colorimetric reagent.

Digested samples were rinsed from the micro-Kjeldahl tubes into acid-cleaned 125-ml Squibb-type separatory funnels, with several small portions of glass-distilled water (total volume, 15 ml). A small square of congo red indicator paper was placed in the solutions in the separatory funnels, and 28% ammonium hydroxide solution was added until the paper turned red. Then, 10 more drops of the 28% ammonium hydroxide solution was added to each sample. The solution was treated, in succession, with 2 ml of copper-free hydroxylamine hydrochloride

solution (10% aqueous), 1.0 ml of 0.01 M bathocuproine in hexanol-chloroform (1:9), and exactly 2.0 ml of the hexanol-chloroform mixture. The contents of the separatory funnels were then vigorously shaken and allowed to separate. The absorbance of the lower, orange layer, containing the bathocuproine-copper complex, was determined at 479 mμ.

The hexanol-chloroform (1:9) mixture was found to be superior to the hexanol or isopentanol used by others as an extractant for the bathocuproine-copper complex (5, 9). Cloudiness did not persist in the hexanol-chloroform mixture as it did with either of these alcohols alone. The alcohols also tended to form stable emulsions when shaken with the aqueous acid-digested samples.

Iron. Iron was determined by a combination of the procedures used by Diehl and Smith (10) and Rubins and Hagstrom (29). Bathophenanthroline was employed as the colorimetric reagent for these assays.

A 5-ml amount of glass-distilled water was added to each of the digested samples, and the mixtures were brought to a boil. The solutions were transferred to 125-ml Squibb-type separatory funnels, and the digestion tubes were rinsed several times with glass-distilled water. The washings were added to the solutions in the separatory funnels (total volume in each separatory funnel, 20 ml). Two ml of iron-free hydroxylamine hydrochloride (10% aqueous) and a small square of congo red indicator paper were added to the separatory funnels. Ammonium hydroxide solution was added dropwise to the mixtures in the separatory funnels until the indicator papers just turned red. Adjustment of *p*H was completed by the addition of a *p*H 3.5 potassium formate buffer (4 M). Five ml of 0.001 M bathophenanthroline solution [50%(v/v)water-ethyl alcohol] was then added, followed by 10 ml of the isoamyl alcohol-chloroform mixture (1:9). The contents of the separatory funnels were shaken, and the layers were allowed to separate. The lower organic layer was withdrawn, and its optical density was determined at 535 mμ.

Diehl and Smith (10) employed a sodium acetate-acetic acid buffer in their assay procedure for this element. In these studies, this procedure gave recoveries of 60 to 90% when applied to the analysis of broth containing standard additions of iron. Investigation of the possible causes of such low recoveries was undertaken. The presence of other metals, singly and in combination, was shown to have no significant effect on the recovery of 5 μg of iron. These metals and their concentrations in 10 ml of culture filtrate were: magnesium, 0.8 mg; copper, 0.01 mg; potassium, 2.7 mg; calcium, 0.5 mg; manganese, 0.025 mg; and zinc, 0.01 mg. The effect of *p*H was also investigated. It was found that the *p*H of the system with sodium acetate-acetic acid buffer ranged from 4.55 to 4.8 in a number of samples. According to Rubins and Hagstrom (29), recoveries of iron added to samples were best in the *p*H range of 3.2 to 4.0. It was therefore concluded that a different buffer was needed for this analysis. Formic acid, with a pK_a of 3.76 and a buffering range centered at this value was chosen as the new buffer. Recoveries of iron obtained with this buffer system averaged 101%.

Manganese. Manganese was determined by a periodate oxidation of Mn^{+2} to Mn^{+7} valency state (18, 31) and colorimetric determination of the permanganate color.

Digested samples were diluted with 3.5 ml of phosphoric acid solution (145 ml of 85% phosphoric acid per liter), and 15 mg of potassium *meta*-periodate was added to samples in the micro-Kjeldahl digestion tubes. The tubes were returned to the heating apparatus and the contents were brought to a boil. Boiling was continued until development of the purple permanganate was noted. Heating was continued to maintain the contents just below their boiling points for 30 min. Samples were removed from the burners, cooled, and diluted to 5.0 ml with periodate-treated water. The solutions were then heated in a boiling water bath for 30 min to insure complete development of the permanganate color. After the samples had cooled, optical densities were determined at 523 mμ in the capillary absorption cells described previously.

Zinc. Zinc was determined by a modification of the methods of Butts et al. (6) and Serfass et al. (34). Digested samples were rinsed into 125-ml Squibb-type separatory funnels with several small washings of glass-distilled water (total volume, about 30 ml). Two ml of sodium citrate solution (10% aqueous) that had been rendered zinc-free and 2 drops of methyl red indicator solution were added to the samples. Ammonium hydroxide solution was then added dropwise until the indicator turned yellow. This treatment was followed by the addition of 1 ml of potassium cyanide (1% aqueous). Glacial acetic acid was then used to adjust the indicator to a neutral peach color (approximately *p*H 6). The indicator was removed from the samples by shaking the contents of the separatory funnels with 10 ml of carbon tetrachloride which had been distilled in an all-glass apparatus. This carbon tetrachloride layer was then discarded. One ml of bis-2-hydroxyethyldithiocarbamate (6) and 10 ml of dithizone in carbon tetrachloride (0.01%) were added to the mixtures in the separatory funnels. The mixtures were shaken vigorously for about 30 sec. After the two immiscible layers had separated, the green to purple zinc-dithizonate layer (lower layer) was drained into a clean separatory funnel. A second extraction with a 10-ml portion of dithizone solution was performed. This organic layer was combined with that removed in the first extraction. Ten ml of sodium sulfide solution (0.04% aqueous) was then added to the combined zinc-dithizonate solutions to remove excess dithizone. This mixture was shaken for about 15 sec, the layers were allowed to separate, and the upper aqueous layer remained colorless, or only faintly yellow. The red zinc-dithizonate complex solutions were diluted to 25 ml with glass-distilled carbon tetrachloride, and the optical densities were measured at 535 mμ.

RESULTS

A preliminary experiment was conducted to establish the approximate requirements for the

TABLE 2. *Effect of omitting single salts on growth and alkaloid production by Claviceps paspali*

Salt omitted	3rd stage culture (1:100 dilution)			4th stage culture (1:1,000 dilution)			5th stage culture (1:10,000 dilution)		
	Age	Alkaloid	Mycelium	Age	Alkaloid	Mycelium	Age	Alkaloid	Mycelium
	days	*μg/ml*	*g/100 ml*	*days*	*μg/ml*	*g/100 ml*	*days*	*μg/ml*	*g/100 ml*
None	10	581	2.361	11	470	2.470	5	284	2.991
	10	597	2.461	8	344	2.291	5	189	2.840
$Ca(NO_3)_2 \cdot 4H_2O$	11.5	810	2.711	18.5	510	2.350[a]	13	498	2.164
	11.5	856	2.462	18.5	397	2.451[a]	12	510	2.213
	11.5	788	2.671	18.5	352	2.430[a]	11	458	2.050
$MgSO_4 \cdot 7H_2O$	7	145	0.411	7	10	0.040	Did not grow		
	7	134	0.403	7	8	0.013	Did not grow		
	6	121	0.401	7	9	0.014	Did not grow		
$ZnSO_4 \cdot 7H_2O$	—	None	0.358	—	None	0.180	Terminated		
	—	None	0.353	—	None	0.153	Terminated		
	—	None	0.310	—	None	0.230	Terminated		
$FeSO_4 \cdot 7H_2O$	10	267	1.593	7	128	0.469	6	23	0.466
	9	226	1.490	10	90	0.539	6	18	0.398
	10	216	1.317	8	94	0.546	7	17	0.578
$CuSO_4 \cdot 5H_2O$	10	165	2.890	10	223	2.895	10	89	2.970
	9	176	2.948	10	179	2.937	10	80	2.302
	9	176	2.918	11	189	2.803	11	95	2.797
$MnSO_4 \cdot 4H_2O$	9	301	2.612	8	214	2.937	9	78	2.142
	8	301	2.697	8	220	2.573	7	74	2.476
	8	301	2.697	8	245	2.921	7	80	2.307
$NaNO_3$	10	533	2.427	13	453	2.584	9.5	435	2.053
	10	458	2.384	11	526	2.562	9.5	457	2.213

[a] These numbers do not represent peak values.

eight salts listed in Table 1. This was carried out in a manner similar to that used in an earlier experiment with isolate no. 2 (21): each salt was omitted, singly, from all stages of the fermentation beyond the first. In this experiment, basal medium I plus the eight salts shown in Table 1 was used for the first stage; the same basal medium containing only KH_2PO_4 was used for the second stage. Basal medium II with all salts except one was used for the third stage. All of these fermentations were carried into a fourth and fifth stage in order to dilute adequately the salts added to the first-stage medium. The third and fourth stages were used as inocula for the next higher-numbered culture prior to their attainment of peak growth. In each case, however, these cultures were returned to the shaker and permitted to continue growing until peak growth and alkaloid production were reached (except in the case of calcium, as indicated). The results of this experiment are shown in Table 2.

The results presented in Table 2 with isolate no. 31 show that the omission of magnesium sulfate, zinc sulfate, or iron sulfate from the culture medium caused a decrease in both growth and alkaloid production. The omission of copper sulfate or manganese sulfate caused a decrease in alkaloid production but not in mycelial growth. In contrast to the results obtained in a similar previous experiment with isolate no. 2 (21), the omission of calcium nitrate in this present experiment did not cause a decrease in growth or alkaloid production. The rate of growth in calcium-deficient medium was significantly retarded, but alkaloid production was higher than that attained by the controls. The omission of sodium nitrate was without appreciable effect on growth, but resulted in a higher alkaloid production than that of the controls. In previous experiments with isolate no. 2, the omission of sodium nitrate had no effect on growth or alkaloid production. It should be noted there was a gradual decline in peak alkaloid production by successive stages of the control cultures. This had been observed many times in the past, and it is the result of transfer. This effect demonstrates the necessity for preparing fresh inoculum for each fermentation.

Aside from the amount of growth in each flask, the appearance of all of the cultures was normal except in the case of those grown under conditions of magnesium and copper deficiency. Normal cultures have a faint yellow-brown color during the first 2 to 3 days, then assume a transient pink-brown color, and finally turn to deep brown over the course of several

days. When magnesium sulfate was omitted from the medium, the cultures in the third and fourth stages turned black and elaborated a dark pigment into the medium. The last three stages of the copper-deficient cultures were virtually without pigmentation.

The alkaloid and mycelial dry weight data contained in Table 2 represent peak values. Mycelial dry weights shown in this table have been corrected for the weights of mycelium introduced into the culture flasks at the time of inoculation. The third- and fourth-stage cultures were used for inocula after 6 and 7 days of incubation, respectively.

Macro-salt requirements. To study more precisely the inorganic requirements without the disadvantage of going into a fourth and a fifth stage of the fermentation process, first-stage cultures were prepared with a concentration of a salt which was shown by preliminary studies to be just sufficient to support growth of the organism. Otherwise, the medium was as described in Materials and Methods. Variations in the concentration of only one salt were made in the medium employed for the third stage of the fermentations.

Unless otherwise stated, all alkaloid and mycelial dry weight data represent peak values. Mycelial dry weights have been corrected for the amount of mycelium introduced into the third-stage cultures as inoculum. Weights of mycelium given in tables, therefore, represent the amount of growth that took place in each culture.

The results of studies on the macro-salt requirements are expressed on the basis of the amount of a salt added to the culture medium rather than on the amount present. Generally, the concentrations of a macro-salt added to a medium are large enough to render insignificant those errors arising from the small amounts of the element that are adventitiously introduced.

Analyses were performed only for phosphate. Thus, results reported for phosphorus include phosphate that had been added to the culture medium as well as that carried over with the inoculum. It was necessary to add KH_2PO_4 to second-stage cultures to obtain normal growth.

Potassium. When potassium was omitted from first-stage media, the organism failed to grow. Therefore, an experiment was conducted in which potassium phosphate (monobasic) was added to the first-stage medium in normal concentrations and replaced by sodium phosphate (monobasic) in the second stage. Control flasks containing 0.1% KH_2PO_4 in basal medium II and others containing 0.1% NaH_2PO_4 were established for the third-stage culture. The results of this experiment showed that potassium was

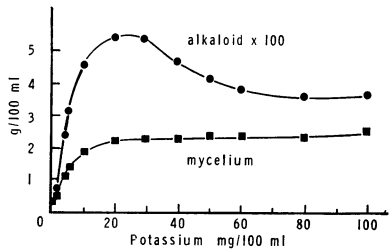

FIG. 1. *Effect of potassium on growth and alkaloid production by isolate no. 31.*

necessary for growth in the third stage. No growth was observed in those fermentations with potassium omitted from the medium. The mycelium in potassium-depleted cultures maintained its character and was like that of the controls with respect to color and form. The addition of potassium to the potassium-depleted cultures after 11 days stimulated growth, and these cultures produced nearly twice the alkaloid levels produced by the controls.

A final experiment was designed to establish the effect of various concentrations of potassium on growth and alkaloid production in the third stage of the fermentation. Potassium was eliminated from the second and third fermentation stages by employing sodium phosphate as the phosphate source. Potassium was added in various amounts to the third-stage medium as the sulfate salt. This salt, rather than the phosphate was used because of reports in the literature which linked alkaloid production to phosphate deficiency. The results of this experiment are presented in Fig. 1. The results indicate that growth and alkaloid production were proportional to the concentration of potassium up to approximately 20 mg/100 ml of potassium. The concentration of potassium added to the normal eight-salt medium was 28.7 mg/100 ml. The addition of higher concentrations (as the sulfate salt) caused a significant decline in alkaloid production. A separate study in which increasing amounts of sulfate (as ammonium sulfate) were added to a control medium showed that this decline could be attributed to excess sulfate.

Some growth occurred in cultures to which no potassium had been added. This was presumably a result of the presence of potassium as a contaminant of the other medium constituents or the replacement of the function of potassium to some degree by another element such as sodium, or a combination of these causes.

Potassium has been shown to be involved in the formation of tryptophan in extracts of *Bacillus subtilis* (33). It enhances the activity of the en-

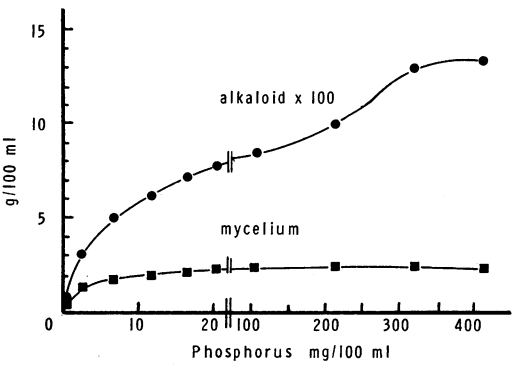

FIG. 2. *Effect of phosphorus on growth and alkaloid production by isolate no. 31.*

zyme that converts indole and serine to tryptophan. This role becomes significant when one considers that the major portion of the ergoline nucleus is derived from tryptophan (42).

Phosphate. In planning an experiment to establish the relationship between phosphate and growth and alkaloid production, cognizance had to be given to the earlier finding that potassium was required for growth. Potassium sulfate was added in sufficient amounts to insure that at least 28 mg/100 ml of the cation was present in all flasks.

The effects of phosphate on growth and alkaloid production were investigated by adding various concentrations of KH_2PO_4 to the culture medium. The first stage of the fermentation was conducted in the normal manner. Only 0.05% KH_2PO_4 was added to basal medium I for the second-stage culture. The third stage was conducted with various levels of phosphate. Samples were taken at zero-time, and the culture filtrates were analyzed for total phosphate. The results of this experiment are presented in Fig. 2.

Growth was limited when the phosphorus concentration fell below 20.5 mg/100 ml. This level of phosphorus corresponds to approximately 91 mg/100 ml of KH_2PO_4. Alkaloid production, on the other hand, continued to rise with an increase in the concentration of potassium phosphate up to the maximal level (1.8 g of KH_2PO_4 per 100 ml) employed in this study. These results stand in strong contrast to those of others who found that a phosphate deficiency is required for optimal alkaloid production (8, 38, 43).

Cultures grown on media containing less than 90 mg/100 ml of KH_2PO_4 became violet in color early in the third stage. The intensity of the violet color was greatest in cultures containing the lowest phosphate levels, and it ranged from dark purple in phosphate-deficient cultures to a faint violet tinge in the mycelium of cultures grown in medium containing 90 mg/100 ml of KH_2PO_4. As the fermentation progressed in time, the color of the medium became progressively darker. Eventually it changed from a purple to a black color. The black substance was shown to be particulate and filterable.

Appearance of the violet pigment in phosphate-deficient cultures has been repeated on numerous subsequent occasions. Pigment production resulting from a phosphate deficiency has been reported for *Penicillium chrysogenum* (13).

Calcium. In the preliminary studies with isolate no. 2, omission of $Ca(NO_3)_2 \cdot 4H_2O$ from the culture medium caused a decrease in growth and alkaloid production (21). In a similar experiment with isolate no. 31, omission of this salt from the medium had relatively little effect on growth but had a stimulatory action on alkaloid production (Table 2).

Another experiment was conducted to determine whether calcium nitrate could be omitted from all three stages of the fermentation. A slant of isolate no. 31 was used to initiate the first stage of the fermentation. Cultures grew in the absence of calcium, but the time required for maximal mycelial growth was about 12 days. This is compared to the normal duration of about 7 days in the first stage. Growth of the second stage on medium deficient in calcium appeared to be normal. Two media were employed in the third stage: basal medium II plus all salts (Table 1) as the control and basal medium II plus all salts except calcium. Samples were not taken after 13 days of growth in the calcium-depleted cultures. At this point, neither the alkaloid nor mycelial dry weights were at their peaks (Fig. 3).

The results demonstrate the decrease in growth rate brought about by the omission of calcium from all three stages of the fermentation. The control in this case contained the usual concentration of calcium in the third-stage medium, but the inoculum was derived from a common calcium-deficient fermentation. The mycelial dry weight of the calcium-deficient cultures was only on the order of one-half of that produced in controls; alkaloid production was approximately 30% higher. Alkaloid production per unit of dry weight, therefore, was approximately 2.5 times greater in calcium-deficient cultures.

These results can be rationalized in a number of ways: calcium might be inhibitory to a reaction leading to the synthesis of alkaloids; calcium might stimulate "growth metabolism" to such an extent that reactions leading to alkaloid synthesis are unable to compete favorably for certain common precursors; calcium might act

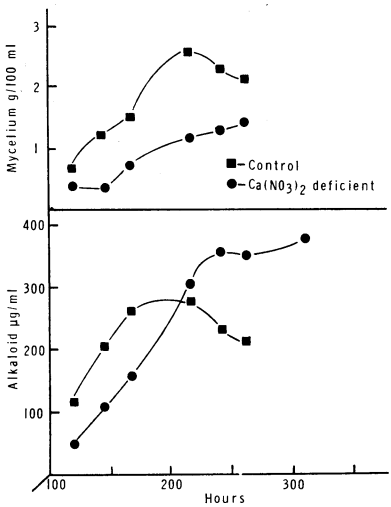

FIG. 3. *Effect of calcium nitrate on growth and alkaloid production by isolate no. 31.*

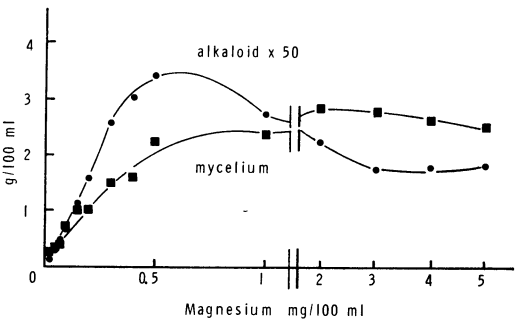

FIG. 4. *Effect of magnesium on growth and alkaloid production by isolate no. 31 (slant 1H-1).*

to displace certain elements from their complexes with natural chelators; these elements, in turn, might stimulate "growth metabolism" to a greater extent than metabolism leading to alkaloid production. Finally, these effects might be due to the nitrate anion instead of the calcium cation. Some support is given to this latter possibility by the observation in the preliminary studies that the omission of sodium nitrate resulted in higher alkaloid production than that attained by the controls. Calcium nitrate contributes about two-thirds of the nitrate contained in the eight-salt medium; sodium nitrate contributes the remainder.

Magnesium. The results of a preliminary study with magnesium sulfate indicated that the amount of magnesium employed in the control media was 20 to 30 times greater than the amount required for maximal growth and alkaloid production. If this amount was added to the first-stage culture medium and omitted from the second- and third-stage media, the carry-over alone would provide 20 to 30% of the magnesium required by the third-stage culture. To obtain a satisfactory depletion of magnesium in the experimental cultures (third stage), it was necessary, therefore, to reduce the concentration of $MgSO_4 \cdot 7H_2O$ in the first-stage basal medium I to 8 mg/100 ml (instead of 80 mg/100 ml). The second-stage medium consisted of mannitol, ammonium succinate, and KH_2PO_4, only. Third-stage culture media consisted of the basal medium II with all salts, except $MgSO_4 \cdot 7H_2O$, in their usual concentration. Magnesium sulfate was added in amounts sufficient to give 15 different levels of Mg cation per 100 ml. Sulfate was maintained at its normal level by adding sufficient ammonium sulfate to replace the sulfate lost by the reduction of the magnesium sulfate levels. Each variation was studied in triplicate fermentations. The results are shown in Fig. 4.

Alkaloid production increased with increasing magnesium concentration up to 0.5 mg/100 ml of magnesium, as shown in Fig. 4; this value is less than one-tenth of the concentration of magnesium normally employed in the eight-salt medium. Mycelial growth had reached about 80% of its peak value at this concentration. Further increases to values above 0.5 mg/100 ml brought about a gradual decline in peak alkaloid production and a very slow increase in mycelial growth.

Sulfur. In the experiments with sulfur variations, the usual eight-salt medium had to be modified to insure a proper depletion of sulfur. Cations normally added as their sulfates (Fe, Cu, Mn, and Zn) were added as their nitrates in amounts calculated to provide the same concentration of cation. To avoid a significant increase in the total nitrate concentration, sodium nitrate was replaced by an amount of sodium hydroxide sufficient to provide the same concentration of sodium ion. Sulfur was added as magnesium sulfate. A solution of magnesium succinate (prepared by dissolving 50 mg of the metal in 10 ml of a 5% solution of succinic acid) was added in sufficient quantities to maintain the level of magnesium at the value used in the usual eight-salt medium.

The first-stage culture was conducted on basal medium I with all of the sulfates except magnesium sulfate replaced by equivalent amounts of nitrates. Magnesium sulfate was added in its usual concentration. The second-stage culture was conducted on basal medium I containing only mannitol, ammonium succinate, and potassium phosphate. Third-stage cultures were

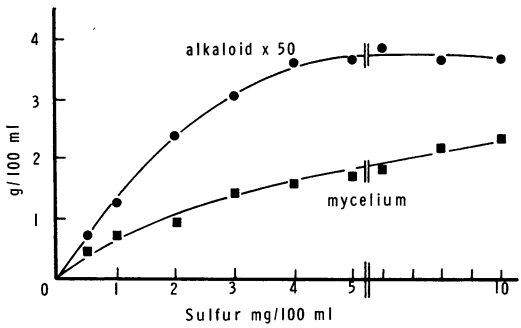

FIG. 5. *Effect of sulfur on growth and alkaloid production by isolate no. 31 (slant 1H-1).*

conducted on basal medium II with all sulfates except magnesium sulfate replaced by equivalent amounts of their respective nitrates. Magnesium sulfate was added in amounts that provided 10 different levels of sulfur. Each variation was conducted in triplicate. The results are presented in Fig. 5.

Alkaloid production was proportional to sulfur concentration up to approximately 4 mg/100 ml of sulfur. Further increases in the sulfur concentration to 10 mg/100 ml had essentially no effect on alkaloid production. The highest concentration of sulfur employed was approximately equal to the concentration in the usual eight-salt medium. Mycelial dry weight was greater with increases in sulfur concentration over the entire range examined. These increases in weight, however, were not proportional to the sulfur concentration over any portion of the range. This behavior remains unexplained, but it might be attributable to the accompanying changes in the concentrations of magnesium and succinate.

The independent variation of magnesium (as Mg^{++}) and sulfur (as SO_4^{-2}) necessitated changes in the concentrations of other ions. The question naturally arises as to the influence of these changes. This can best be answered by an examination of their magnitude. In the case of magnesium, the sulfate concentration was maintained at its normal level by the addition of ammonium sulfate. This salt was chosen because the medium already contained a relatively high concentration of ammonium ion. The highest concentration of ammonium sulfate added was 42.88 mg/100 ml (equivalent to 11.7 mg/100 ml of ammonium ion). Previous analyses of medium II (21) had shown that it contained 600 mg/100 ml of ammonia nitrogen or 770 mg/100 ml of ammonium ion. Therefore, the percentage increases in ammonium ion concentration as a result of added ammonium sulfate were small (1.5% at the most).

In the case of sulfur, the magnesium concentration was maintained at its normal level by adding appropriate amounts of a solution of magnesium succinate that contained 10 mg of succinic acid for each milligram of magnesium. The highest concentration of magnesium that had to be added in this manner was 7.90 mg/100 ml. The maximal amount of succinate introduced, therefore, was 79 mg/100 ml; this amount is small compared to the amount of succinate (3%, w/v) already present in the medium. The replacement of the sulfates of Fe, Cu, Mn, and Zn with amounts of their nitrates calculated to supply the same amount of the respective cation caused a small increase in the concentration of nitrate. This increase, however, was essentially cancelled by the substitution of sodium hydroxide for sodium nitrate as a source of sodium ion. The small net change in nitrate concentration (about 0.1 mg/100 ml) is negligible compared to the amount contributed by the 30 mg/100 ml of calcium nitrate.

It appears, therefore, that the modifications of the basal medium II used to study magnesium and sulfur separately are not of sufficient consequence to affect the fermentations significantly.

Micro-salt requirements. Experiments similar to those described under *Macro-salt requirements* were conducted to establish the micro-salt requirements for growth and alkaloid production. The elements designated as micro-medium components are manganese, copper, iron, and zinc.

The amounts of these elements introduced to the medium as contaminants might frequently constitute a significant portion of the total requirement. Expression of results on the basis of the amount of the element added, therefore, becomes meaningless in studies of micro-element requirements. A quantitative determination of either the amount of an element introduced adventitiously or the total amount present in a given medium is essential. The latter determination is normally more convenient because it involves greater amounts of the elements and is less demanding on the sensitivity of the analytical method.

Manganese. The results of preliminary studies had shown that the omission of manganese from the medium caused no significant decrease in growth but brought about a considerable decrease in alkaloid production (Table 2). It was apparent that the demonstration of the essentiality of this element for growth would necessitate additional efforts to reduce the level of adventitious manganese in the medium.

Attempts were made to remove manganese by two different methods from a basal medium

containing all of the macro-medium constituents: extraction with dithizone at *p*H 9.5, where 75 ml of 0.1% dithizone in carbon tetrachloride was shaken with 400 ml of the basal medium (26) and coprecipitation of manganese with copper-diethyldithiocarbamate (25).

Media treated in the above manner failed to support growth, even when manganese was later added to the cultures. This failure was attributed to the presence of a great excess of ammonium phosphate, which was introduced as phosphoric acid and ammonium hydroxide in the adjustment of *p*H to values at which the extraction and precipitation of manganese would be effective. No further attempts to reduce manganese levels by these means were made.

In a final experiment, conducted to establish the relation between manganese concentration and growth and alkaloid production, the basal medium with components purified in the usual manner was employed. In this study, the concentration of manganese sulfate added to the first stage of the fermentation was reduced to 0.25 mg/100 ml. Basal medium I plus KH_2PO_4 was used for the second stage. The third stage was used for the second stage. The third stage was conducted on basal medium II plus the usual concentrations of salts (Table 1) except manganese sulfate. Managanese was added as its sulfate in amounts ranging from 0 to 5 mg/100 ml. Results of this experiment are shown in Fig. 6.

The results presented fail to demonstrate the essentiality of manganese for growth; they do, however, provide evidence of its requirement for alkaloid formation. Manganese has been shown to be required by a number of fungi. This requirement is small: generally on the order of 0.005 to 0.01 ppm (7). With the methods employed in the present experiments, it was possible to reduce the manganese concentration in the unsupplemented medium to 0.028 ppm. Therefore, it is quite possible that manganese is required for growth of this organism but that the requirement is extremely low. The amount of growth remained essentially unchanged when the manganese concentration of the medium was varied from 2.8 to 1,400 μg/100 ml. Increasing the manganese concentration to 1,400 μg/100 ml had no effect on alkaloid production.

Manganese has been shown to be required for the formation of secondary metabolites, generally in amounts greater than those required for growth of the organisms (4, 12, 19, 40, 41).

Copper. In preliminary studies, reduction of the concentration of this element to 10^{-4} times its normal value caused a sharp reduction in the amount of alkaloid produced but had no effect on its growth (Table 2).

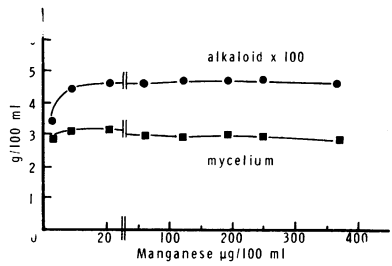

FIG. 6. *Effect of manganese on growth and alkaloid production by isolate no. 31 (slant 1H-1).*

It was later found that satisfactory growth occurred in the first stage of fermentations to which no copper was added. This suggested that copper was finding its way into the medium in amounts adequate to support growth of the organism.

An experiment was conducted to demonstrate the necessity of copper for growth. All of the macro medium components were dissolved in glass-distilled water, and the solution was treated in two different ways to remove copper: extraction with a solution of dithizone in carbon tetrachloride (26) or precipitation with hydrogen sulfide (25). Fermentations were conducted on treated medium, and on treated medium plus 0.5 mg of $CuSO_4 \cdot 5H_2O$.

Experience with similar attempts to remove traces of manganese from the basal medium showed that the substances contributed by the removal treatments could not be ignored. Therefore, a number of control fermentations, each conducted on media representing various stages of the removal processes, were established.

None of the treatments reduced the concentration of copper to levels that caused a significant decrease in growth, nor did they cause a substantial reduction in the alkaloid-producing capacity as compared with controls. In all cases, whether they received these treatments or not, the ratio of alkaloid produced by copper-supplemented media to unsupplemented media was between 6 and 7. Thus, there appeared to be no advantage in the employment of such procedures in future experiments dealing with this element.

Slant 1H-1 was used in a final experiment involving copper. Basal medium I plus all salts except copper sulfate was employed for the first stage of this fermentation. Basal medium I plus KH_2PO_4, only, was used to carry the fermentation through the second stage. Variations of copper concentration were employed in basal medium II for the third stage. Copper sulfate was added in amounts ranging from 0 to 3 mg/100 ml. Samples of medium obtained immediately after the third-stage cultures were inoculated were

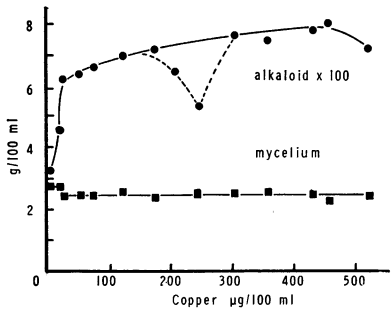

FIG. 7. *Effect of copper on growth and alkaloid production by isolate no. 31 (slant 1H-1).*

analyzed for copper; this permitted the expression of results on the basis of total copper present in third-stage culture medium.

In preliminary experiments, simple omission of copper from the culture medium did not cause a decrease in the growth of the organism. Removal of copper from the basal medium by dithizone extraction and hydrogen sulfide precipitation also failed to inhibit growth of the organism. Such treatments, however, reduced peak alkaloid production to approximately one-seventh that reached by controls. A requirement for copper would be anticipated because of the well-known role of this element in porphyrin biosynthesis. The methods employed in this preliminary experiment, however, did not permit the demonstration of such a requirement.

Results of the final experiment with copper are presented in Fig. 7. Analysis of samples obtained from freshly inoculated cultures showed that about 5 µg/100 ml of copper was present in cultures to which no copper was added. It may be assumed that a similar level of copper was present in the media used in the preliminary experiments (Table 2).

In addition to failing to reach a growth-limiting concentration of copper, these experiments also failed to establish toxic concentrations of copper, despite the fact that a level of approximately six times that used in the eight-salt medium was tested.

With regard to alkaloid production, a very distinct effect of copper was seen. At the lowest level of copper, alkaloid production was reduced to less than half of that attained by copper-sufficient cultures. There was a very sharp rise in alkaloid production with increasing copper concentrations up to approximately 26 µg/100 ml. At this point, alkaloid production was approximately 75% of the maximal value obtained. With increasing concentrations of copper up to about 600 µg/100 ml, there was a very gradual increase in alkaloid production. In the middle of this portion of the curve, a sharp dip was observed. This dip is largely the result of a single point, and might, therefore, be anomalous.

Copper-deficient cultures were white or light tan in color; control cultures normally ranged from medium to dark brown in color. The intensity of the pigmentation was directly related to the amount of copper present. In these experiments, and in most others in the past, high alkaloid production was associated with dark pigmentation in the mycelia. The appearance of pigmentation as a function of copper concentration has been reported with other microorganisms (7, 35). Copper has been shown to be essential for the action of certain oxidases, many of which give rise to colored reaction products.

Several authors have implied or suggested that copper was important in ergot alkaloid biosynthesis (15, 16, 28, 43). None of these, however, has presented data that establish a relationship between copper concentration and alkaloid production. Other investigators have shown that copper is required (in amounts greater than those needed for growth) for the production of secondary metabolites (13, 20).

Iron. Results of the preliminary experiment showed that the depletion of iron to 10^{-4} times its usual concentration in first-stage cultures caused a decrease in growth and alkaloid production (Table 2). An experiment was undertaken to determine the effects of various concentrations of iron on growth and alkaloid production.

When iron was omitted from first-stage media, the organism failed to grow. Therefore, a first-stage culture was conducted on basal medium I plus all salts and $FeSO_4 \cdot 7H_2O$, 0.1 mg/100 ml. Iron was omitted from the second stage and was added to the third-stage medium in varying concentrations. Zero-time samples were withdrawn from inoculated media and were assayed for iron content. Difficulty in filtering zero-time samples through sintered-glass funnels was encountered with the iron assay. An iron-containing precipitate in the zero-time samples would not pass through the filters. This precipitate formed when ammonium hydroxide was used to adjust the *p*H of the completed medium to 5.2. The result of this difficulty was that the clear filtrate contained only a fraction of the total amount of iron originally added to the medium. Therefore, analyses for iron were run on samples of mycelium and culture filtrate together. The total broth samples were mixed by vigorous shaking and were accurately pipetted (with serological pipettes) into digestion tubes. Total iron added to the first-stage of the fermentation was 20 µg/100 ml. After transferring the culture through two more stages, only 0.2 µg of iron should have been carried over into the third stage of the fermentation. Results of this experiment are shown in Fig. 8.

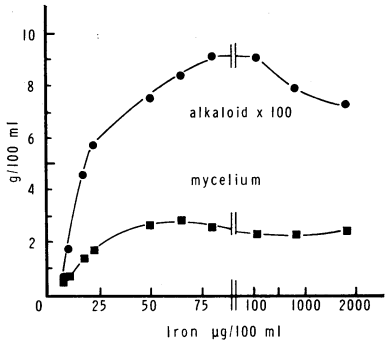

FIG. 8. *Effect of iron on growth and alkaloid production by isolate no. 31 (slant 1H-1).*

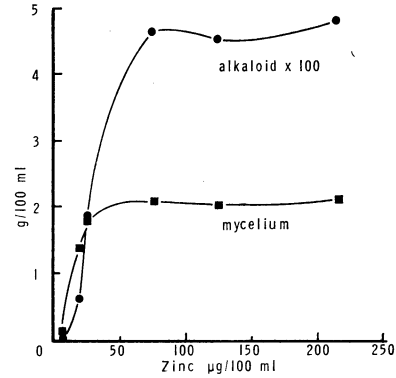

FIG. 9. *Effect of zinc on growth and alkaloid production by isolate no 31 (slant 1H-1).*

Maximal growth was given by 50 µg/100 ml of iron; maximal alkaloid production required a slightly higher level, viz., 80 µg/100 ml. This behavior pattern is similar to that observed for manganese and for copper except that the percentage difference between the concentration required for maximal alkaloid production was smaller in the case of iron.

The iron requirement for the production of secondary metabolites is generally greater than the iron requirement for growth (7). Iron has been implicated in alkaloid production by *Claviceps* species by other investigators (21, 30, 32, 36). These reports are, however, usually lacking in experimental data.

Zinc. Preliminary results on the omission of zinc from second- and third-stage cultures indicated that growth had stopped and that alkaloid production was completely inhibited (Table 2).

An experiment was conducted to determine the effects on growth and alkaloid production of various concentrations of zinc. The first stage of the fermentation was conducted on basal medium I plus all salts listed in Table 1. Basal medium I plus KH_2PO_4 was used for the second stage. The third stage of this fermentation was conducted on basal medium II plus all salts, with variations in the concentration of added zinc. Total zinc in these media was determined by analysis of samples taken immediately after the third stage was inoculated. The results of this experiment are shown in Fig. 9.

Approximately 90% of maximal growth was given by the medium that contained 25 µg/100 ml of zinc. This concentration of zinc, however, supported the formation of only 40% of the maximal alkaloid levels attainable. Alkaloid production reached a maximum when the zinc content of the medium was 75 µg/100 ml. This element, therefore, falls into the same category as manganese, copper, and iron, with respect to its requirement in greater amounts for alkaloid production than for growth.

Numerous studies have indicated that zinc is essential for alkaloid production by various species of *Claviceps* (11, 14–16, 21, 30, 36). None of these investigations showed whether the requirement was directly linked to alkaloid production.

Zinc has, however, been shown to be necessary for the catalytic action of many enzymes. In particular, this element has functioned in the activation of tryptophan synthetase in *Neurospora crassa* (24) and *Aspergillus niger* (3). This may be important because of the precursor role of tryptophan in ergot alkaloid biosynthesis.

Cobalt, molybdenum and cadmium. Addition of these elements as the following salts to normal third-stage culture medium resulted in no increase in growth: 3 $CdSO_4 \cdot 8H_2O$, 0.26 mg/100 ml; $Co(C_2H_3O_2)_2 \cdot 4H_2O$, 1.49 mg/100 ml; and $Na_2MoO_4 \cdot 2H_2O$, 0.252 mg/100 ml. There was about 5% more alkaloid produced by controls than by fermentations containing these salts. No attempts were made to deplete the medium of these elements other than by the use of the usual precautions for the purification of the medium constituents. It is, therefore, not possible to reach any conclusions regarding the essentiality of these elements.

ACKNOWLEDGMENTS

We thank Frima Botnick for her valuable technical assistance. One of the authors, J. P. R., acknowledges support as a trainee under National Institutes of Health Training Grant GM01139. This investigation was supported by Public Health Service grant GM14530.

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