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Stimulation of Lysergic Acid Alkaloid Production in Submerged Cultures of *Claviceps paspali* by Arsenate

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Abstract: Analysis of the culture filtrates of lysergic acid alkaloid-producing fermentations at intervals during growth of the cultures showed that phosphate is most rapidly depleted from the medium in fermentations that produce the highest levels of alkaloid. In view of the known interference with phosphate metabolism by arsenate, sodium arsenate was added to the culture medium with the intent of creating, in effect, a phosphate deficiency. The addition of arsenate at levels between one-fiftieth and one-twentieth the molar concentration of phosphate resulted in increases in alkaloid production up to 100 per cent. Experiments with ten-times the usual concentration of phosphate showed that the ratio of arsenate to phosphate rather than the absolute concentration of arsenate was most significant in obtaining this effect. Increasing the concentration of phosphate alone (up to twenty times the usual concentration) resulted in substantial increases in alkaloid production. The effect of arsenate on both the rate and the extent of phosphate uptake was determined. Among several inhibitors of electron transport and/or oxidative phosphorylation, only the uncoupler of oxidative phosphorylation (dinitrophenol) was effective in increasing alkaloid production.

Analysis of the culture filtrates of lysergic acid alkaloid-producing fermentations at intervals during growth of the culture showed that phosphorus is most

rapidly depleted from the medium in fermentations that produced the highest levels of alkaloids (Mary *et al.*, 1965). In a fermentation that produced 700 μg/ml total alkaloids, for example, phosphate was depleted from the medium three days prior to the attainment of peak alkaloid production and when growth was one-half its maximal value. The doubling of mycelial dry weight that occurred after phosphorus had been depleted from the medium was presumably at the expense of stored phosphate inside the cells. Low alkaloid-producing fermentations did not exhibit phosphorus depletion until one or two days prior to the attainment of peak growth and at approximately the same time as peak alkaloid production.

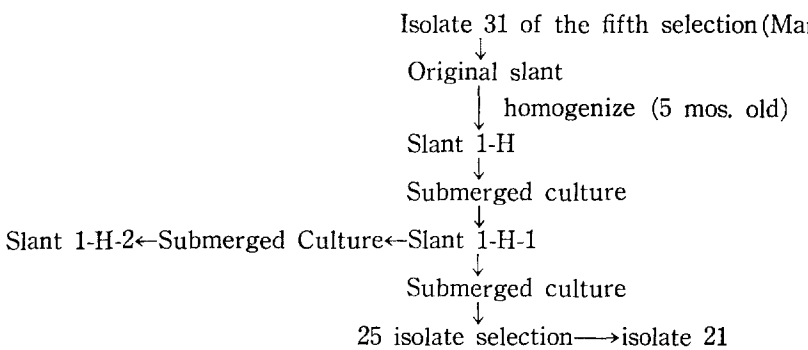
It appeared that early phosphate depletion favored alkaloid synthesis. Low-phosphate media had previously been reported by others to support the production of alkaloids (DeWaart and Taber, 1960; Taber and Vining, 1958; Taber and Vining, 1963). In studies on the inorganic nutrition of the isolate of *Claviceps paspali* used in this laboratory, however, it was shown that low phosphate levels were not essential for high alkaloid production. These studies did not measure the extent of utilization of medium phosphate.

Because of the known interference with phosphate metabolism by arsenate, the addition of sodium arsenate to the medium appeared to be a ready means of creating, in effect, a phosphate deficiency. Experiments were therefore undertaken to examine the effect of arsenate added at various times to alkaloid-producing fermentations.

Material and Methods

Fermentation Studies

The origin of the isolates used in these studies is shown in the following scheme.



The origin of isolate 31 of the fifth selection on the 8-salt medium has been described (Mary *et al.*, 1965). Slant 1-H-1 and slant 1-H-2 were used in these experiments. These were all maintained on Potato-Dextrose-Agar (PDA).

Submerged cultures were conducted in the usual manner on the 8-salt mannitol-succinate medium (Mary *et al.*, 1965). In certain cases, 1% KH_2PO_4 was used instead of 0.1% KH_2PO_4 in the medium employed for the third submerged culture.

Analytical Methods

For determination of total alkaloid, samples were taken after the fourth or fifth day of incubation and at one or two-day intervals thereafter. Alkaloids were determined colorimetrically by the modified procedure employing p-dimethylaminobenzaldehyde (Michelon and Kelleher, 1965).

Samples for phosphate determination were removed immediately after inoculation and at one or two-day intervals thereafter. Orthophosphate was determined directly on the filtrates according to the colorimetric method of Berenblum and Chain (1938).

Mycelial dry weights were determined by filtering the samples through tared Whatman no. 1 filter paper discs, washing the mycelium with distilled water, and drying for 24 hours at 70°C.

Experimental and Results

In the first experiment, sodium arsenate was added to the production medium at levels that provide molar concentrations of arsenate equal to 1 mM, 7.35 mM and 20 mM. The basal medium contained 0.1% KH_2PO_4 or the equivalent of 7.35 mM phosphate. These fermentations were conducted in triplicate. In all cases, growth was totally inhibited, the pH remained unchanged and no alkaloids were detected up to 12.5 days after inoculation. The fermentations were discontinued after this time.

TABLE I : Effect of Delayed Arsenate Additions on Growth and Alkaloid Production. *1

Additions	Age at Peak (days)	pH	Mycelial*2 Dry Wgt. (g/100ml)	Peak Alkaloid Cencentration (μg/ml)
None	8.5	5.35	2.34 (6.5)	387
7.35 mM Na_2HASO_4 (added on 5th day) *3	8.5	4.95	0.89	168

*1 Slant 1-H-1 was used for these fermentations. The basal medium was the usual 8-salt mannitol-succinate.

*2 The numbers in parentheses are the times (days) at which peak mycelial dry weight was reached. When no number is given, the time is the same as that given for peak alkaloid production.

*3 Analyses of these cultures after 4.5 days of incubation gave the following average values: pH, 4.9; alkaloid concentration, 117 μg/ml; and mycelial dry weight, 1.45 g.

In the same experiment, sodium arsenate was added to give 7.35 mM arsenate five days after inoculation. The effect of this addition was given in Table I. The results show that arsenate caused a sharp curtailment of alkaloid production and a loss in mycelial dry weight. Analysis of these cultures at 6.5 days and comparison of the results to those obtained at 4.5 days (just prior to arsenate addition) revealed that virtually all of the loss in weight had occurred in this interval and that alkaloid production ceased entirely.

TABLE II: Effect of Low Levels of Arsenate on Growth and Alkaloid Production.*1

Additions	Age at Peak (days)	pH	Mycelial Dry Wgt.*2 (g/100ml)	Peak Alkaloid Concentration (μg/ml)
None	11	6.0	2.33	301
Na_2HAsO_4 0.0735 mM	11	5.40	2.33(9)	371
Na_2HAsO_4 0.00735 mM	9	5.40	2.25(11)	319

*1 Slant 1-H-1 was used for these fermentations.
The basal medium was the usual 8-salt mannitol-succinate.
*2 The numbers in parentheses are the times (days) at which peak mycelial dry weight was reached. When no number is given, the time is the same as that given for peak alkaloid production.

In an effort to establish the concentration at which arsenate was less than totally inhibitory, an experiment was conducted in which sodium arsenate was added to the medium at levels of 0.0735 mM and 0.00735 mM. The results of this experiment are presented in Table II.

The concentrations of arsenate used in these fermentations were essentially without effect on mycelial dry weight; the higher concentration, 0.0735 mM caused, on the average, approximately a 25 per cent increase in peak alkaloid level.

These results provided sufficient cause to examine the effect of arsenate concentrations between 0.0735 and 0.735 mM. For these experiments the 1-H-1 culture was employed and the concentrations of arsenate added to the medium were 0.0735 mM, 0.184 mM, 0.368 mM, 0.551 mM, 0.735 mM and 1.0 mM.

The cultures were assayed for total alkaloid, pH and dry weight. The results are shown in Table III.

Arsenate was effective in increasing alkaloid production by the cultures. The optimum concentration, 0.184 mM, caused approximately a doubling of alkaloid production with the 1-H-1 culture. At this concentration of arsenate, the culture did not suffer a decrease in peak mycelial dry weight.

The similarity of arsenic to phosphorus and the known effect of arsenate on

TABLE Ⅲ: Effect of Arsenate Additions on Growth and Alkaloid Production by the 1-H-1 Culture.

Additions	Age at Peak (days)	pH	Mycelial *1 Dry Wgt. (g/100ml)	Peak Alkaloid Concentration (μg/ml)
None	11	5.75	2.26	577
Na_2HAsO_4 0.0735 mM	13	5.47	2.19(11)	742
Na_2HAsO_4 0.184 mM	11	5.03	2.44	1213
Na_2HAsO_4 0.368 mM	11	5.07	2.12(13)	1098
Na_2HAsO_4 0.551 mM	13	5.05	1.34	662
Na_2HAsO_4 0.735 mM	13	5.10	0.36(11)	32
Na_2HAsO_4 1.0 mM	Did not grow			None

*1 The numbers in parentheses are the times (days) at which peak mycelial dry weight was reached. When no number is given, the time is the same as that given for peak alkaloid production.

phosphate metabolism suggested that the ratio of arsenate to phosphate, rather than its absolute amount, was critical in determining its effect. This was tested in an experiment in which the concentration of phosphate in the basal medium was increased by a factor of ten. Arsenate was added to the high-phosphate medium at the levels of 0.184 mM and 1.84 mM. The experiments were conducted with fermentations originating from slant 1-H-1. The results are given in Table Ⅳ.

TABLE Ⅳ: Effect of Arsenate on Growth and Alkaloid Production by Isolate 1-H-1 in High-Phosphate Medium.

Medium	Age at Peak (days)	pH	Mycelial *1 Dry Wgt. (g/100ml)	Peak Alkaloid Concentration (μg/ml)
Control 0.1% KH_2PO_4	9	5.60	2.49(7)	622
0.1% KH_2PO_4 and 0.184 mM Na_2HAsO_4	11	5.27	2.23(9)	1442
1.0% KH_2PO_4	11	5.73	2.70(7)	930
1.0% KH_2PO_4 and 0.184 mM Na_2HAsO_4	11	5.80	2.61(9)	1146
1.0% KH_2PO_4 and 1.84 mM Na_2HAsO_4	13	5.68	2.16(11)	1225

*1 The numbers in parentheses are the times (days) at which peak mycelial dry weight was reached. When no number is given, the time is the same as that given for peak alkaloid production.

These data clearly show that a concentration of arsenate (1.84mM) which would

severely inhibit growth and alkaloid production of these cultures on the low phosphorus medium, caused only a slight decrease in mycelial dry weight and an increase in alkaloid concentration when added to the high-phosphorus medium. Contrary to the reports of numerous other workers (DeWaart and Taber, 1960; Windisch *et al.*, 1960; Taber and Vining, 1963), low phosphate is not essential for high alkaloid production by these cultures. In fact, increasing the phosphorus concentration caused an appreciable increase in peak alkaloid production. Other work now being completed in this laboratory has shown that phosphate levels up to two per cent KH_2PO_4 are not harmful, but slightly beneficial to alkaloid production.

There seemed to be little doubt that arsenate is capable of causing an increase in alkaloid production by the isolate. The addition of arsenate was motivated by the observation of a correlation between high alkaloid production and rapid depletion of phosphate from the medium (Mary *et al.*, 1965). Because of its favorable effect on alkaloid production it was decided to determine its effect on the uptake of phosphate from the medium. These experiments were conducted with the same fermentations as those reported in Table Ⅳ. Samples were taken at zero time and at the early stages of the fermentations, and were assayed for inorganic phosphate. Analyses were conducted in triplicate, and a recovery sample was included with each set of analyses. The results are shown in Figure 1.

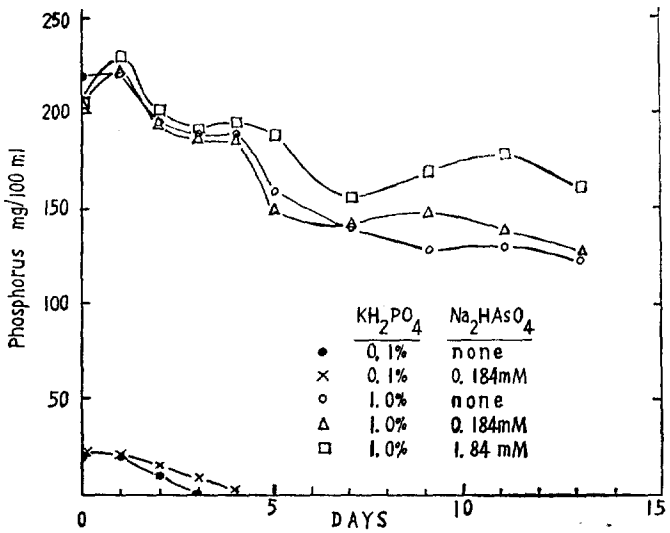


FIGURE 1: Pattern of Phosphate Utilization by Isolate 1-H-1

In the low-phosphate control, phosphate was depleted from the medium within three days after inoculation. The addition of 0.184 mM arsenate to this medium

caused a slower uptake of phosphate. In this case, depletion was not achieved until after four days of incubation.

The high-phosphate medium contained ten times more phosphate than the low-phosphate medium. In the high-phosphate control, approximately 90 mg of phosphorus was removed from 100 ml of medium. This is approximately four times more than that removed from the low phosphate medium, but it is less than 50 per cent of the total available phosphorus. The addition of 0.184 mM arsenate to this medium showed little effect on the rate or extent of phosphate uptake, but it did cause approximately a 20 per cent increase in peak alkaloid production. The addition of 1.84 mM arsenate, however, reduced the phosphorus uptake to approximately 40 mg or about one-half of that incorporated by the high-phosphorus controls; at the same time, it caused an increase of greater than 30 per cent in peak alkaloid production. The *rate* of phosphorus uptake was also somewhat retarded, especially after the fourth day of incubation.

The slight increases in the phosphate level of the high-phosphate media observed in the four-day samples was unusual. This would normally be attributed to an erroneous set of analyses, but agreement among triplicate analyses for each sample was good and a recovery sample assayed at the same time gave 99.5 per cent recovery of added phosphate. Thus, there was more than a remote possibility that these results were real.

TABLE V: Effect of Various Concentrations of Arsenate on Growth and Alkaloid Production in High-Phosphate Medium*[1]

Medium	Age at Peak (days)	pH	Mycelial*[2] Dry Wgt. (g/100ml)	Peak Alkaloid Concentration (μg/ml)
Control 0.1% KH_2PO_4	11	6.32	2.33(9)	553
0.1% KH_2PO_4 and 0.184 mM Na_2HAsO_4	11	5.20	1.76	610
1.0% KH_2PO_4	13	6.05	2.48(9)	922
1.0% KH_2PO_4 and 0.184 mM Na_2HAsO_4	15	7.05	1.89(9)	717
1.0% KH_2PO_4 and 3.68 mM Na_2HAsO_4	19	6.00	1.32(11)	1300
1.0% KH_2PO_4 and 5.51 mM Na_2HAsO_4	17.5	5.30	0.81(11)	1364

*[1] Slant 1-H-1 was used for these fermentations. The basal medium was the usual 8-salt mannitol-succinate.

*[2] The numbers in parentheses are the number of days at which peak mycelial dry weight was reached. Because dry weight determinations were not conducted beyond the 11th day, the values given for the 11th day might not represent peak values.

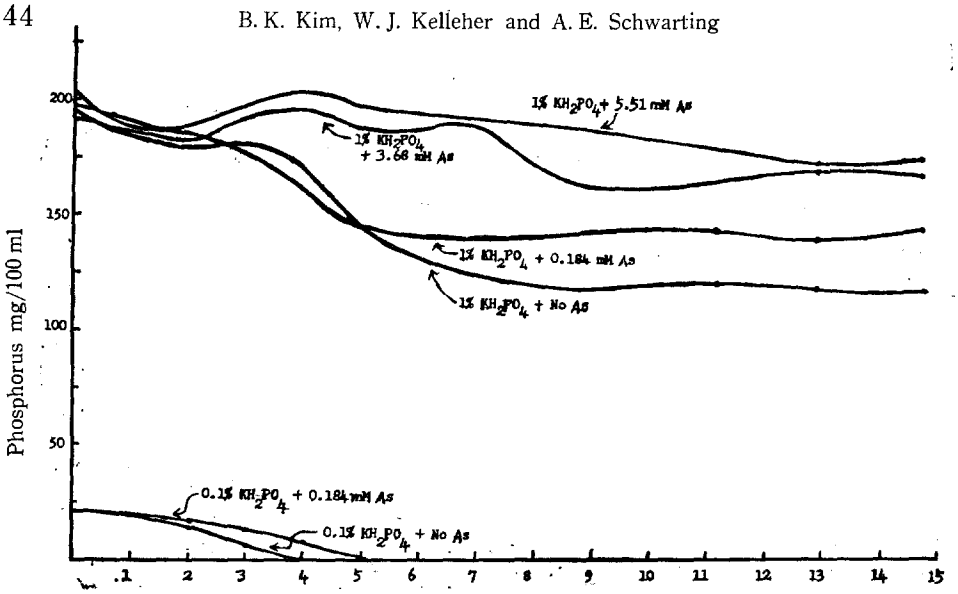


FIGURE 2: Pattern of Phosphate Utilization by Isolate 1-H-1 in the Presence of Various Concentrations of Arsenate.

Further evidence in support of this unusual pattern of phosphate uptake and release was produced by another experiment conducted with the same isolate as that used in the above experiment. In this experiment, more variations in the amount of arsenate added to the high-phosphate medium were studied. The effect of such additions on growth and alkaloid production are presented in Table V. The patterns of phosphate uptake from the medium are shown in Figure 2.

The data in Table V show that only a slight increase in alkaloid production was given by the addition of 0.184 mM arsenate to the low phosphate medium. The ten-fold increase in phosphate concentration, alone, caused an increase in peak alkaloid production of approximately 67 per cent. The addition of low levels of arsenate to the high-phosphate medium caused a slight decrease in alkaloid production. Increases were given by the higher levels, however. The highest alkaloid production, *viz.*, 1364 μg/ml (ave.) was given by the high phosphate medium with 5.51 mM sodium arsenate. The duration of this fermentation was significantly increased and the dry weight of mycelium significantly decreased.

Examination of the rate and extent of phosphate utilization as shown in Figure 2 reveals that arsenate again prolonged the time required for the disappearance of phosphate from the low-phosphate medium.

Arsenate is known to act as an uncoupler of substrate-level oxidative phosphorylation and respiratory chain-linked oxidative phosphorylation. In addition, it

might have a direct action on one or more of the reactions leading to lysergic acid alkaloid synthesis. In order to gain some insight into its mode of action in enhancing alkaloid production, several other substances, each known to uncouple oxidative phosphorylation or inhibit the respiratory chain, were added to these fermentations. These substances were: potassium cyanide, sodium azide, amytal, dicoumarol, and dinitrophenol. In a preliminary experiment to establish the proper level of addition, each of these substances was added to third submerged cultures at concentrations of 0.01 mM, 0.1 mM, and 1.0 mM. Concentrations were appropriately adjusted on the basis of the results of these preliminary experiments and the experiments were repeated. The results are presented in Table Ⅵ.

TABLE Ⅵ : Effect of Various Inhibitors on Growth and Alkaloid Production by Isolate no. 31*[1]

Inhibitor		Age at Peak (days)	pH	Mycelial*[2] Dry Wgt. (g/100ml)	Peak Alkaloid Concentration (μg/ml)
Potassium cyanide	0.5 mM	13	6.45	2.65(15)	280
	0.25 mM	13	5.65	2.64(11)	432
	0.10 mM	13	6.05	2.36(15)	776
	0.05 mM	11	5.73	2.54(9)	559
Sodium Azide	0.01 mM	13	5.85	2.44(15)	724
	0.005 mM	13	6.43	2.34(13)	718
Amytal	0.5 mM	11	5.65	2.42(11)	502
	0.1 mM	11	5.87	2.42(13)	600
Dicoumarol	0.05 mM	9	5.23	0.58(17)	44
	0.01 mM	11	5.28	2.86(13)	218
Dinitrophenol	0.025 mM	Did not grow		0	0
	0.01 mM	15	5.83	2.26(13)	986
	0.005 mM	13	5.95	2.48(13)	742
None (Control)		11	5.60	2.52(15)	660

*[1] Slant 1-H-2 was used for these fermentations. The basal medium was the usual 8-salt mannitol-succinate.

*[2] The numbers in parentheses are the times (days) at which peak mycelial dry weight was reached. When no number is given, the time is the same as that given for peak alkaloid production.

These results show that dinitrophenol was the only inhibitor that caused an appreciable (50 per cent) increase in alkaloid production. Increases of approximately 10 per cent and 15 per cent were given by certain concentrations of sodium azide and potassium cyanide, respectively.

Discussion

The results of several experiments on the addition of arsenate to alkaloid-producing fermentations showed that an increase in alkaloid production could be expected as a result of this addition. The magnitude of the increase as well as the optimum concentration of arsenate was somewhat variable, but this treatment could be relied upon to act in a beneficial manner over a considerable range of arsenate concentrations. Thus, the addition of arsenate provides an opportunity for much gain with little risk of a decrease in alkaloid production.

The optimum concentration of arsenate is strongly dependent on the phosphate concentration. It appears that the maximum increase in alkaloid production is given when the molar ratio of phosphate: arsenate is between 50:1 and 20:1. Concentrations of arsenate up to the level that gives the greatest increase in alkaloid production have little effect on mycelial dry weight; concentrations above this optimum generally inhibit growth.

Investigation of the rate and extent of phosphate utilization in a high- and low-phosphate medium showed that phosphate was depleted from the low-phosphate medium in three~four days; all of the phosphorus in the high-phosphate medium was not used but the uptake was approximately four times more than that in the low-phosphate medium. Arsenate caused a decrease in the *rate* of uptake from both media and a decrease in the *extent* of uptake from the high-phosphate medium. The levels of arsenate that were most effective in stimulating alkaloid production in the high-phosphate medium reduced the extent of phosphate utilization to approximately the value found with the low-phosphate medium.

Neither of the effects caused by arsenate, *viz.*, reduction of the rate of phosphate utilization and reduction of the extent of phosphate utilization, were consistent with conditions previously shown to favor alkaloid production. In an earlier study, rapid rate of phosphate depletion was associated with the highest alkaloid production (Mary *et al.*, 1965) In a separate study of the inorganic requirements of this culture (Rosazza *et al.*, 1967), as well as in the present study, a ten-fold increase in the phosphate content of the mediun resulted in an increase in phosphate utilization and an increase in alkaloid production.

The results of the experiments with the respiratory and phosphorylation inhibitors, although somewhat restricted, showed that only dinitrophenol caused a substantial increase in alkaloid production. This substance. like arsenate, is classified by Racker (1965) as a true uncoupler of oxidative phosphorylation. These sub-

stances limit phosphorylation and either stimulate or have no effect on respiration. The other agents tested inhibit respiration and thus, depress phosphorylation that is coupled to respiration. These results tend to rule out a direct action of arsenate on one or more of the reactions leading to alkaloid synthesis. They suggest, on the other hand, that interference with ATP production linked to respiration is the basis for its action. The effect of other inhibitors and uncouplers will be examined to test this hypothesis.

The apparent release of phosphate into the high-phosphate medium during the fourth or fifth day of incubation was also observed by Taber (1964) with a culture of *Claviceps purpurea*. This phenomenon has not yet been explained.

Reduction of ATP production would be expected to cause a decrease in the cellular content of condensed phosphate compounds, both organic and inorganic. Taber and his co-workers expended considerable effort in studies of the intracellular distribution of phosphorus compounds (DeWaart and Taber, 1960; Taber and Vining, 1963; Taber, 1964). They usually conducted a comparative study of the same culture grown on a low- and high-phosphate medium. Alkaloid production occurred only in the low-phosphate medium and, at best, was on the order of several milligrams per liter. The employment of uncouplers provides a means of controlling alkaloid formation and phosphate metabolism. A system is thereby available which permits a study of the relationship of alkaloid formation and phosphate metabolism in high alkaloid-producing cultures. Preliminary work in such a study is being undertaken in this laboratory.

References

Berenblum, I., and Chain, E. B. (1938), XL. An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295.

DeWaart, C., and Taber, W. A. (1960), Some aspects of phosphate metabolism of *Claviceps purpurea* (Fr.) Tul. *Can. J. Microbiol.* **6**, 675.

Mary, N. Y., Kelleher, W. J., and Schwarting, A. E. (1965), Production of lysergic acid derivatives in submerged culture. Ⅲ. Strain selection on defined medium. *Lloydia* **28**, 218.

Michelon, L. E., and Kelleher, W. J. (1963), The spectrophotometric determination of ergot alkaloids. A modified procedure employing para-dimethylaminobenzaldehyde. *Lloydia* **26**, 192.

Racker, E. (1965), *Mechanisms in Bioenergetics*, Academic Press, New York.

Rosazza, J. P., Kelleher, W. J., and Schwarting, A. E. (1967), Production of lysergic acid derivatives in submerged culture. Ⅳ. Inorganic nutrition studies with *Claviceps paspali*. *Appl. Microbiol.* **15**, 1270.

Taber, W. A., and Vining, L. C. (1958), Influence of certain factors on the *in-vitro* production of ergot alkaloids by *Claviceps purpurea*. *Can. J. Microbiol.* **4**, 611.

Taber, W. A., and Vining, L. C. (1963), Physiology of alkaloid production by *Claviceps purpurea* (Fr.) Tul. *Can. J. Microbiol.* **9**, 1.

Taber, W. A. (1964), Sequential formation and accumulation of primary and secondary shunt metabolic products in *Claviceps purpurea*. *Appl. Microbiol.* **12**, 321.

Windisch, S., Hermsdorf, B., and Bronn, W. (1960), Production of ergot alkaloids. *U.S. Patent* No. 2, 936, 266.

〔국문초록〕

*Claviceps paspali*의 액내배양에서의 라이써직산 알카로이드 생성에 대한 비산의 촉진작용

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라이써직산 알카로이드를 생성하는 발효에서 맥각균 *Claviceps paspali*의 성장과정중 경시적으로 발효액을 분석하여 본바, 가장 높은 농도의 알카로이드를 생성하는 발효액에서 인산이 가장 신속히 소모됨을 알았다. 인산대사가 비산에 의해 방해된다는 점에 착안하여 일종의 인산결핍상태를 만들 목적으로 배양액에다 비산나트륨을 첨가하였다. 인산의 0.02 및 0.05 몰 농도에 해당하는 비산을 첨가하였을때 알카로이드 생성이 100%까지 증가하는 결과를 나타내었다. 배지의 정상농도의 10배의 인산을 사용한 실험에서, 알카로이드의 생성증가에 중요한 영향을 미치는 것은 비산의 절대농도가 아니라 인산 대 비산의 비 임을 알게 되었다. 인산 농도만을 증가시켰을 때(정상 농도의 20배까지), 알카로이드 생성이 상당히 증가되는 결과를 초래하였다. 맥각균의 인산 섭취의 범위와 속도에 대한 비산의 영향을 측정하였다. 전자 수송 혹은 산화적 인산화 또는 두 가지 모두를 저지하는 수종의 억제제 중에서 다이나이트로훼놀만이 알카로이드 생성을 촉진시키는 데 유효하였다.