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Characterization of the Phosphate-mediated Control of Ethylene Production by *Penicillium digitatum*

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AUTAR K. MATTOO[1], EDO CHALUTZ[2], JAMES D. ANDERSON, AND MORRIS LIEBERMAN
Post Harvest Plant Physiology Laboratory, Beltsville Agricultural Research Center (W), Beltsville, Maryland 20705

ABSTRACT

Characterization of the phosphate effect on ethylene production by *Penicillium digitatum* is reported. A low level of phosphate (0.001 millimolar) was about 200 to 500 times as effective as a high phosphate level (100 millimolar) in stimulating ethylene production and the stimulation was readily reversed by addition of phosphate. This phosphate effect did not operate in static cultures. The precursor of ethylene in the stimulated low phosphate system was glutamate but not α-ketoglutarate, which is a precursor in static systems. Actinomycin D and cycloheximide effectively inhibited the low phosphate/high ethylene-producing system. Alkaline phosphatase and protein kinase activities were higher in low than in high phosphate systems. We suggest that phosphate level regulates ethylene production by *P. digitatum* and that the regulation involves a phosphorylation or dephosphorylation reaction of some enzyme system associated with ethylene production. Phosphate-mediated control of ethylene production may also involve the transcriptional and translational machinery of the fungal cell. *P. digitatum* apparently can produce widely different levels of ethylene by different pathways, depending on culture conditions under which it is grown.

Ethylene production by higher plants (10, 27) and microorganisms (8, 22, 27) is now well documented. While ethylene as a plant hormone regulates many aspects of growth and development in higher plants (10), it may also be involved in plant pathogenesis (22) and in the autochemotropism of *Phycomyces blakesleeanus* (21). Methionine is now well established as a precuror of ethylene in higher plants (3, 12). However, ethylene produced by static cultures of *Penicillium digitatum*, the most extensively studied microbial ethylene producer, was shown not to derive from methionine but from α-ketoglutarate and glutamate (8). Recent studies have demonstrated that methionine not only induces ethylene production but also is a precursor of ethylene in shake cultures of *P. digitatum* (6), and perhaps in *Mucor hiemalis* (13), *Saccharomyces cerevisiae* (24), and *Escherichia coli* (18).

Although considerable information has accumulated on the biosynthesis of ethylene, regulation of ethylene production by microorganisms and higher plants remains obsecure. Chalutz *et al.* (7) demonstrated that Pi is a potent inhibitor of ethylene production by shake cultures of *P. digitatum* and that stimulation of ethylene under phosphate-limiting growth conditions was not the mechanism by which methionine induced ethylene production in similar shake cultures grown under conditions of phosphate sufficiency. The present study was carried out to characterize further the effect of phosphate on ethylene production in *P. digitatum*.

MATERIALS AND METHODS

P. digitatum (American Type Culture Collection No. 10030) was grown statically (5, 23) or as shake culture (6, 7) on modified Pratt's liquid medium with various concentrations of phosphate with or without yeast extract, as indicated at appropriate places in the text. Other inoculation and incubation procedures, culture weight determinations, and ethylene analysis were performed as previously described (23).

Particulate and supernatant fractions for enzymic assays were prepared from mycelia. The mycelia were frozen in liquid N_2, ground to powder with acid-washed sand, and extracted with buffer (100 mM Tris-HCl buffer (pH 8) and 4 mM EDTA). The homogenate was filtered with four layers of cheesecloth; and the filtrate centrifuged at 18,000*g* for 15 min. The material pelleted by centrifugation was taken up in the extraction buffer containing 0.7 M NaCl and sonicated. The sonicated extract was centrifuged at 18,000*g* for 15 min, and the supernatant preserved. This fraction is referred to as particulate fraction in the text. The first 18,000*g* supernatant was recentrifuged at 100,000*g* for 1 h in a Spinco model L ultracentrifuge. The top two-thirds of the supernatant was removed and stored at 5 C until used. This fraction is referred to as the supernatant fraction. Protein in both fractions was precipitated with 10% tricholoroacetic acid and measured by fluorescence (4).

Protein kinase assays contained enzyme, substrate, 0.2 ml of buffer (pH 7.0) containing 50 mM 5-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 10 mM $MgCl_2$, 0.3 M EGTA,[3] and 0.5 mM [γ-^{32}P]ATP (25 μCi, 100 cpm/pmol) at 30 C for 3 and 6 min in the presence or absence of 40 μM cyclic AMP in a final volume of 0.4 ml. Protein kinase activities of particulate and supernatant fractions were determined for endogenous substrates as well as for an exogenous substrate, casein (1.8 mg), in the standard reaction mixture described above. Under the conditions of the assay, the reaction rate was linear with time and proportional to protein concentrations used in this study. Reaction was stopped by addition of 0.1 ml of 50% trichloroacetic acid and 0.5 mg of casein (as carrier). Aliquots (50 μl) were applied on Whatman[4] GF/C filters, then washed five to six times with 10% tricholoracetic acid containing 1 mM sodium pyrophosphate, twice with ethanol, and once

[1] On leave from the M.S. University of Baroda, India.

[2] On leave from the Agricultural Research Organization, The Volcani Center, Israel.

[3] Abbreviations: EGTA: ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CHI: cycloheximide; rhizobitoxine: L-2-amino-4-(2'-amino-3'-hydroxypropoxy)-*trans*-3-buteonic acid; AD: actinomycin D.

[4] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may also be available.

with ether. The dried filters were then placed in glass scintillation vials with 5 ml of liquifluor (New England Nuclear). Radioactivity was determined in a Packard liquid scintillation spectrometer. The terms "phosphorylation" or "protein kinase activities" used in this paper indicate the net amount of acid-stable ^{32}P bound to protein at the end of a period of incubation. The amount represents the sum of protein kinase (EC 2.7.1.37) and phosphoprotein phosphatase (EC 3.13.1, EC 3.13.2, and EC 3.1.3.3) reactions occurring during the incubation period.

Alkaline phosphatase activity of the supernatant fraction was determined as described in the Worthington Manual (26).

For tracer studies, 1 μCi of L-[3,4-^{14}C] methionine (5.75 mCi/mmol) or L-[3,4-^{3}H] glutamate (11.9 cpm/pmol) was added to each 25-ml Erlenmeyer flask containing 3 ml of incubation medium and about 0.5 g fungal cells from 3-day-old shake cultures. Labeled ethylene produced at different time intervals was trapped into 0.1 M mercuric acetate in methanol as described previously (5, 6). Incorporation of the amino acids into material insoluble in 10% trichloroacetic acid was also studied as described previously (1). Additional methods and conditions appear under legends of figures and tables. All experiments were repeated at least twice and representative experiments are presented. Radioactive isotopes were obtained from New England Nuclear.

RESULTS

Inhibition of Ethylene Production by Phosphate. Shake cultures of *P. digitatum*, when cultivated in a chemically defined (synthetic) medium containing 0.01 mM phosphate, a concentration 10^{-4} times that of the normal control medium (phosphate concentration 100 mM), produced about 100 times as much ethylene as control in 96 h (7). With the addition of yeast extract to such cultures ethylene production was stimulated by 176-fold at 44 h and by 528-fold at 68 h (Table I). In contrast, relatively little increase in ethylene production occurred in 4-day-old static cultures (5) and methionine-induced shake cultures (6) cultivated under conditions of phosphate limitation (Table I). Therefore, experiments reported below were carried out on shake cultures of *P. digitatum* grown on synthetic medium.

Ethylene production by 4-day-old shake cultures growing under phosphate limitation was effectively inhibited by adjustment of the phosphate concentration to 0.01 or 1 mM Pi or PPi (Fig. 1). The degree of inhibition depended on the concentration of phosphate. Initially, PPi was more effective than Pi, probably because it hydrolyzed and thus increased the phosphate concentration. Ethylene production by cultures cultivated under phosphate-limitation could be completely inhibited at any stage of growth by the exogenous addition of phosphate to a final concentration of (0.15 M). When 3-day-old mycelia from phosphate-limiting growth medium (phosphate level 0.05 mM) were aseptically transferred to fresh medium containing high phosphate (100 mM) in the absence or presence of glucose, ethylene production decreased faster than in the above experiments with the 4-day-old cultures and glucose promoted this decrease (Fig. 1, inset). Earlier reports (6, 24) showed that glucose elevates the methionine-induced ethylene production in microorganisms. Our results indicated that in whatever way the cultures were exposed to phosphate, the net result was a strong inhibition of ethylene production and mycelial weight remained constant or increased slightly. It appeared that phosphate depletion triggered the onset of ethylene production and that phosphate addition suppressed ethylene production.

Effects of Inhibitors of Transcription and Translation. In order to ascertain whether ethylene production under phosphate-limiting conditions is regulated by *de novo* synthesis of the ethylene-synthesizing system, we carried out experiments in the presence of AD and CHI. We found that either ethylene was not produced or its rate of production rapidly decreased when AD or CHI (15 μg/ml or 14 μg/ml final concentration, respectively) was added at hour 12, 24, 48, 72, and 96 of fungal cultivation. (Fig. 2, a–e). Also, the inhibition of ethylene production caused by the addition of Pi to phosphate-limiting cultures was increased by the presence of AD or CHI.

Influence of Substrates for Ethylene. The effects of putative ethylene precursors on the production of ethylene by shake cultures grown in low and high phosphate media are shown in Table II. While glutamate stimulated ethylene production as much as two times in low phosphate cultures without affecting their growth, it inhibited by 50% the ethylene production by the high phosphate cultures. On the other hand, whereas methionine has a slight stimulatory or inhibitory effect on ethylene production by low phosphate cultures, it markedly induced ethylene production in the fungus growing in high phosphate medium (Table II) and caused about a 50% decrease in the mycelial weight of the culture. Increased ethylene production in methionine shake cultures in high phosphate may be due to nonenzymic extracellular systems in the medium (6). Methionine added to the cultures after 48 h

Table I. Effect of phosphate level in the cultivation medium on ethylene production by shake and static cultures of P. digitatum

Cultural condition	Incubation time, hr	Ethylene Production nl/hr/g		Fold increase in C_2H_4 Production over 100 mM phosphate
		phosphate addition		
		100 mM	0 mM	
Shake culture[1]	44	5.1	900	(+) 176
	68	2.9	1530	(+) 528
Shake culture[1,2] (methionine-induced)	24	42.0	137	(+) 3.5
Static culture[3]	24	740.0	831	(+) 1.1

[1] Cultivated in the presence of yeast extract (5, 27).

[2] 4-day-old shake cultures, induced by 7 mM methionine (6), were collected aseptically and transferred to fresh medium containing either 100 or 0 mM phosphate, and assayed for ethylene at the indicated incubation times.

[3] The medium underneath the mycelial mat of 4-day-old cultures was removed aseptically as described earlier (5) and replaced with fresh medium (23) containing either 100 or 0 mM phosphate. At the indicated incubation times ethylene from the gaseous phase was determined.

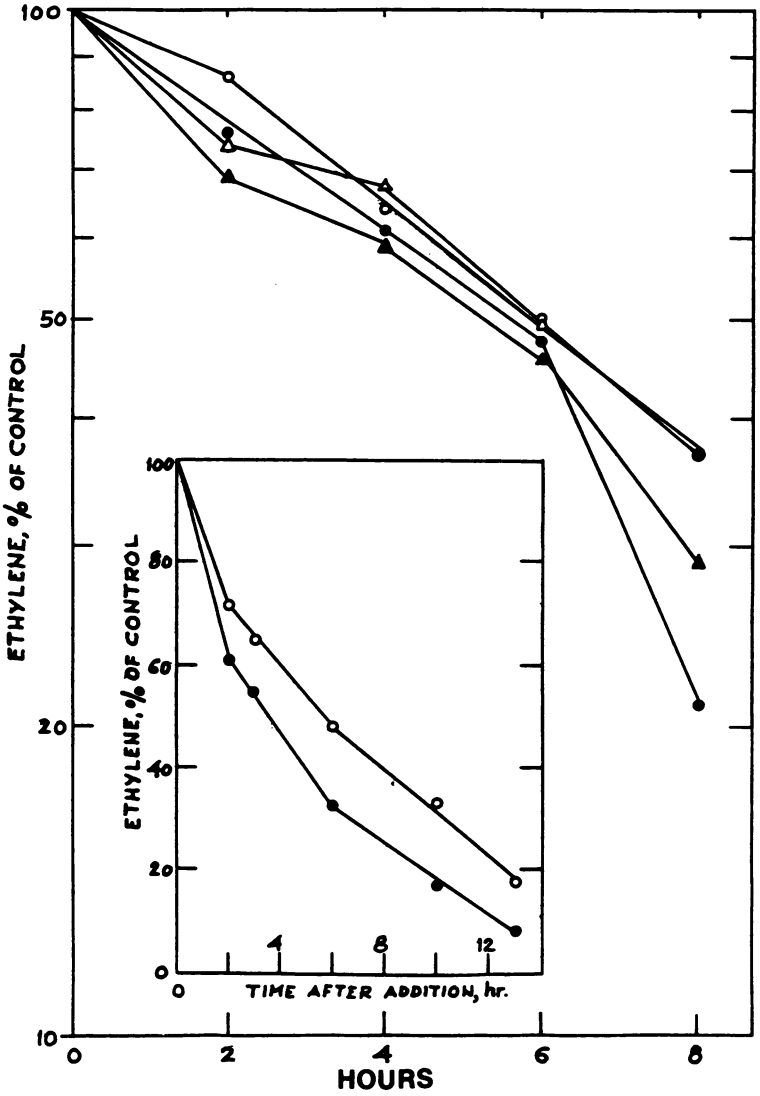

FIG. 1. Effect of exogenously added Pi and PPi on ethylene production by shake cultures of *P. digitatum* grown under conditions of phosphate limitation. Cultures were grown for 4 days on modified Pratt's medium without yeast extract but containing 0.001 mM phosphate in 50-ml Erlenmeyer flasks; then 1 ml of Pi (○, ●) or PPi (△, ▲) solution was added to a final concentration of 0.01 mM (○, △) or 1 mM (●, ▲). Ethylene was measured at indicated time intervals after phosphate addition. Inset shows ethylene production of 3-day-old cultures after transfer from a 0.05 mM phosphate medium to fresh solutions of 100 mM phosphate with (●) and without (○) glucose (7 mM). Absolute value of ethylene produced by control cultures at time 0 was 750 nl/h·g fresh mycelial weight.

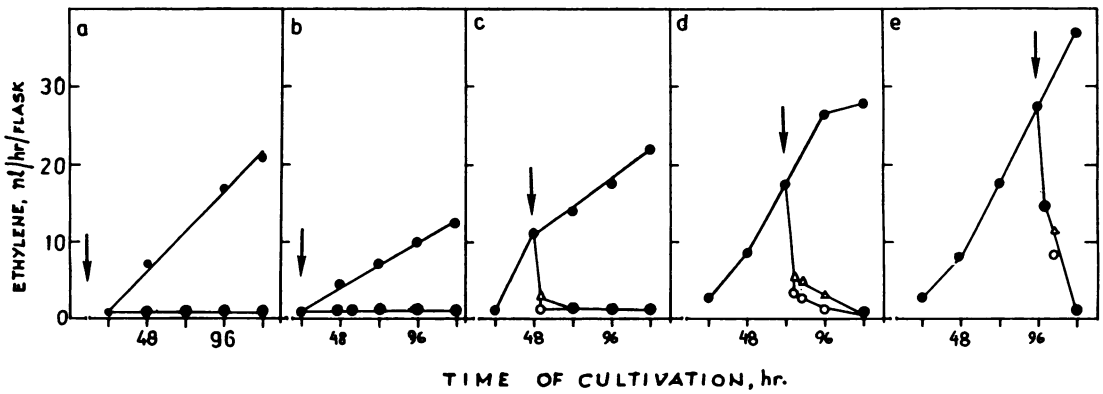

FIG. 2. Relation between time of addition of cycloheximide or actinomycin D to production of ethylene by shake cultures of *P. digitatum*. Fungus was cultivated on 0.01 mM phosphate. At 12 h (a), 24 h (b), 48 h (c), 72 h (d), or 96 h (e) of cultivation, cycloheximide (○) or actinomycin D (△) in methanol was added to the flasks such that the final concentration was 14 μg/ml or 15 μg/ml, respectively. Ethylene produced was assayed immediately after and at indicated time intervals. An equivalent volume of methanol was added to controls (●).

actually inhibited ethylene production in the low phosphate medium. Surprisingly, however, α-ketoglutartate, which along with glutamate is also a precursor of ethylene in static cultures (8, 27), strongly inhibited ethylene production in shake cultures growing under conditions of phosphate-limitation (data not shown).

In order to test which of these substrates is incorporated into ethylene by the fungus, we studied the incorporation into ethylene of [^{14}C]methionine and [^{3}H]glutamate under phosphate-limiting (0.01 mM) and phosphate-sufficient (10–100 mM) growth conditions. After 2 h, four times more [^{3}H]ethylene was produced from low phosphate than from high phosphate cultures (Table III). Incorporation of labeled methionine into [^{14}C]ethylene was approximately 35 to 50% lower in low phosphate than in high phosphate cultures. These data suggest that glutamate was converted to ethylene under phosphate-limiting growth conditions and that excess phosphate inhibited this conversion. In the methionine system, phosphate does not stimulate ethylene production and high phosphate appears to increase ethylene production somewhat. The level of phosphate in the medium apparently affected total protein synthesis, as determined by incorporation of the labeled amino acids into trichloroacetic acid-insoluble material, and dry mycelial weight (Table II). As compared with the high phosphate cultures the low phosphate cultures incorporated less [^{3}H]glutamate and [^{14}C]methionine into total protein by about 85 and 20%, respectively. These differences might have been caused in part by different glutamate and methionine pool sizes.

Ethylene Inhibitors and Increased Ethylene Production in Low Phosphate. Propyl gallate (1 mM) and the ethoxy analog of rhizobitoxine (0.1 mM), the inhibitors of ethylene production in higher plants (2, 11, 16), did not inhibit ethylene production under conditions of phosphate limitation (0.001 mM); instead, 1 mM propyl gallate stimulated it by about 50%. The methoxy analog of

Table II. Effects of glutamate and methionine on growth of and ethylene production by shake cultures of P. digitatum in phosphate-limiting and phosphate-excess media.

Chemical tested	Time of substrate addition	Time of assay	Ethylene production nl/hr/g fresh wt.		Dry mycelial wt. g	
	HR		Initial phosphate conc., mM			
			1	100	1	100
None(control)[1]	-	60	870	13	0.3	1.1
L-Glutamate	24	60	1884	6	0.4	0.9
L-Glutamate	48	60	1395	7	0.3	0.9
L-Methionine	24	60	940	706	0.3	0.5
L-Methionine	48	60	430	815	0.3	0.4

[1] Details are given in the text. The concentration of L-glutamate and methionine was 7 mM

Table III. Effect of phosphate concentration of the culture medium on incorporation of (^{3}H) glutamate and (^{14}C) methionine into labeled ethylene and into total protein by shake culture of P. digitatum

Radioactive[1] compound	Phosphate status of culture,mM	Labeled ethylene produced at hr 1	2	Incorporation of label into proteins
		pmoles		cpm/2 hr
(^{3}H) Glutamate	100	14.7	12.2	9899
(^{3}H) Glutamate	0.01	31.9	51.5	1212
(^{14}C) Methionine	100	14.0	23.4	25713
(^{14}C) Methionine	0.01	7.0	15.3	20328

[1] The fungus was grown for 3 days in 50-ml Erlenmeyer flasks containing modified Pratt's medium without yeast extract at pH 4.5. Aseptically, the cultures were harvested, pelleted by centrifugation and then redistributed into 3 ml of water containing 3 µl (3,4-^{3}H) glutamate (sp. activity 11.9 cpm/pmole) or 13.8 µl of (3,4-^{14}C) methionine (sp. activity 7 cpm/pmole). At 0.5 hr the flasks were sealed. After 1 and 2 hr, the gaseous, labeled ethylene was collected and assayed as previously described (5,6). Also after 2 hr, incorporation of label into total mycelial protein (material insoluble in hot 10% trichloroacetic acid) was determined.

Table IV. Effect of phosphate status of the medium on the levels of protein kinase and alkaline phosphatase in shake cultures of P. digitatum. Shake cultures of the fungus were centrifuged at hr 66 of incubation. The mycelia were harvested, immediately frozen and prepared into cell-free extracts. Other details are given under Materials and Methods.

Phosphate status mM	Substrate	Protein kinase activity cpm(10^{-4})/min/mg protein			Alkaline phosphatase activity in S
		Particulate (P)	Supt.(S)	P/S	milliunits/min/mg
1	Endogenous	2.1	1.3	1.6	-
1	Exogenous	24.3	2.2	11.0	23.3
100	Endogenous	1.7	0.7	2.3	-
100	Exogenous	1.7	1.1	1.6	5.1

Supt = supernatant

rhizobitoxine, which inhibited the incorporation of [^{3}H]glutamate into ethylene by static cultures of *P. digitatum* (5), inhibited (14% in 1 h and 30% in 2 h) the incorporation of [^{3}H]glutamate into ethylene by shake cultures of the fungus under conditions of phosphate limitation. Propyl gallate turned bluish on addition to the medium containing low phosphate. This has not been reported previously and may relate to the ineffectiveness of the antioxidant as an inhibitor of ethylene production.

Alkaline Phosphatase and Protein Kinases. Earlier we (7) showed phosphate-mediated inhibition of ethylene production is accompanied by a rapid increase in ATP level of fungal cells. We do not know whether this increase in ATP level leads to inhibition of ethylene production. We did suggest (7) that ATP level may control the phosphorylation/dephosphorylation of proteins, such as the enzyme(s) in the ethylene-synthesizing system. To test this possibility we determined the levels of alkaline phosphatase and protein kinase in phosphate-limiting and sufficient cultures and in those phosphate-limiting cultures to which more phosphate was added exogenously. The alkaline phosphatase activity in the supernatant fraction prepared from high phosphate cultures was less than one-fourth the prepared from low phosphate cultures (Table IV). Also, alkaline phosphatase activity decreased markedly with time in ethylene-producing, low phosphate-grown (0.001 mM) cultures to which phosphate (100 mM) was added (data not shown).

Table IV shows that protein kinase activities with endogenous substrates were higher in both particulate and supernatant fractions of the low phosphate system compared with the high phosphate system. The ratio of particulate to supernatant enzyme activities in these assays was lower for the low phosphate than for the high phosphate system with endogenous substrates. However, protein kinase activity of the particulate fraction prepared from low phosphate cultures was considerably higher when assayed with than without exogenously supplied substrate (casein). The enzyme activity of the supernatant fraction increased by about two times under similar assay conditions. Exogenous substrate (casein) did not significantly increase protein kinase activity in the particulate fraction from the high phosphate system. In the assay with casein, protein kinase was increased about 40% in the supernatant fraction of highest phosphate samples compared to 70% in the low phosphate supernatant. In short term experiments, the rapid decrease in ethylene production by low phosphate cultures on addition of exogenous phosphate was associated with a rapid decrease in the phosphorylating activities of protein kinase on endogenous and exogenous substrates.

These data show that under phosphate-limiting conditions, which induce high production of ethylene, the cells of *P. digitatum* contain high levels of protein kinase and alkaline phosphatase, but that addition of phosphate inhibits ethylene production and decreases protein kinase and alkaline phosphatase activities.

DISCUSSION

Ethylene production by statically grown *P. digitatum* was previously (8) shown to derive from glutamate of α-ketoglutarate, but not from methionine, the precursor in higher plants (11, 27). Chalutz *et al.* (6) demonstrated that methionine is also a precursor (and an inducer) of ethylene production by the fungus in shake cultures. Similarly, shake cultures of *M. hiemalis* (13) and *S. cerevisiae* (24) have been shown to produce ethylene from methionine, but possibly by extracellular, artifactual systems. The data presented in this report indicate the existence of yet another, hitherto unknown, pathway of ethylene biosynthesis in shake cultures of *P. digitatum*. This pathway appears to utilize glutamate as a precursor, is greatly influenced by phosphate concentration, and differs from the one induced by methionine (6). Apparently, *P. digitatum* can be made to produce ethylene by different pathways, depending upon the cultural conditions.

Our data indicate that phosphate concentration regulates ethylene produced by shake cultures of *P. digitatum*. Ethylene production by the fungus grown under conditions of phosphate limitation could be inhibited completely by exogenous phosphate. This suggests that phosphate depletion triggers the onset of ethylene production. The high production of ethylene under phosphate-limiting conditions may involve derepression of an ethylene-synthesizing enzyme system in the fungal cells. This view is supported by the effectiveness of CHI and AD as inhibitors, the negligible production of ethylene under conditions of high phosphate, the existence of a lag period (7), and the rapid and pronounced inhibition of ethylene upon addition of exogenous phosphate.

Ethylene production by low phosphate cultures was stimulated by glutamate, and the cultures incorporated labeled glutamate into labeled ethylene. These findings might suggest that the pathway of ethylene production in shake cultures is similar to the one in static cultures (8). However, this does not seem to be so, since α-ketoglutarate, which like glutamate is a precursor of ethylene produced by static cultures of *P. digitatum*, strongly inhibited ethylene production by the shake cultures under phosphate-limiting conditions. Moreover, the ethoxy rhizobitoxine analog inhibited ethylene production by static cultures (5), but not by shake cultures. As in static cultures (5), the methoxy analog of rhizobitoxine inhibited the incorporation of [^{3}H]glutamate into ethylene by *P. digitatum* in shake cultures.

Phosphate may regulate ethylene production, just as it has been shown to regulate the formation of secondary fungal metabolites (25). The mechanisms(s) underlying regulatory effects of Pi in microorganisms may have some common features. A common observation has been the presence of higher amounts of phosphatase in cultures grown under phosphate-limiting than in phos-

phate-sufficient conditions (17, 25). Martin and Demain (14) showed that addition of phosphate concentrations which inhibited candicidin synthesis had no effect on protein synthesis as determined by incorporation of leucine. Later they reported (15) that the phosphate-mediated effect may have been associated with the level of intracellular ATP, which rapidly increased after phosphate addition. We have also reported (7) that a sharp rise in the intracellular ATP level accompanies inhibition of ethylene, and that the level of alkaline phosphate decreases sharply (Table IV). However, our short term experiments showed that [^{3}H]glutamate and [^{14}C]methionine were incorporated differentially into protein under conditions of phosphate limitation. This observation, along with other data presented above, suggest that phosphate mediation control of ethylene production also involves the transcriptional and translational machinery of the cells.

It is possible that the intracellular ATP level which dramatically increases after phosphate addition (7) has a regulatory effect. Among other reactions, ATP may control the phosphorylation/dephosphorylation of proteins, such as the ethylene-synthesizing enzyme, in the low phosphate system, as it does in other systems (9, 20). The affinity of cyclic AMP for protein kinase has been shown to decrease markedly with increasing concentrations of ATP (19, 20). ATP is also known to inactivate adenylate cyclase (19). Our data on the relation between phosphate concentrations and changes in protein kinase activities in particulate and supernatant fractions provide some circumstantial evidence for the association of protein kinase activities with the control of ethylene synthesis. Further work is needed to establish whether the levels of intracellular cyclic AMP, ATP, and protein kinases are related to the ethylene synthesizing capacity of *P. digitatum*.

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