

IDENTIFICATION OF ETHYLENE AS A VOLATILE PRODUCT OF THE FUNGUS *PENICILLIUM DIGITATUM*¹

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(WITH THREE FIGURES)

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Introduction

This paper presents the chemical identification of ethylene as a metabolic product of the fungus *Penicillium digitatum* Sacc. It also presents a simplified method for the accumulation and identification of the small concentrations of ethylene which may be produced by plant materials.

ETHYLENE PRODUCTION BY FUNGI

Experiments reported independently by BIALE (1) and MILLER, WINSTON, and FISHER (8) revealed that the common green mold of citrus fruits, *P. digitatum*, produces a physiologically active emanation whose effects on plant tissue are the same as those caused by ethylene gas. It was shown by the latter authors and by BIALE and SHEPHERD (3) that this fungus produces this emanation whether grown on its natural host or in culture, and PRATT (10) showed that production of the emanation does not depend on major nutrient components of the culture solution but does appear to be related to active growth and respiration of the culture. Production of the active gas by fungus-infected fruit has been further studied by ROHRBAUGH (12) and BIALE (2).

Except for recent evidence of NICKERSON (9) that ethylene may be produced by *Blastomyces dermatitidis* and two other human pathogens, *P. digitatum* is the only fungus reported to have this activity. Biological testing of various other plant pathogens has given negative results (1, 14). Since *P. digitatum* is a particularly promising organism for the study of the mechanism of ethylene formation, it was of interest to verify chemically the identity of the active gas produced.

IDENTIFICATION OF ETHYLENE

The present work is based on the method of PRATT *et al.* (11), except that the accumulation of the ethylene and formation of ethylene dibromide have been greatly simplified. Instead of bromine, mercuric perchlorate has been used to absorb the ethylene from the air stream which has been passed over the plant tissue.

HOFMANN and SAND (7) found that solutions of mercuric salts take up

¹ A cooperative project of the Division of Subtropical Horticulture and the Division of Truck Crops, College of Agriculture, University of California, carried out in the laboratories of the former division.

olefins, forming complex molecules from which the olefin is released quantitatively upon addition of hydrochloric acid. HANSEN and HARTMAN (6) and DENNY (4) adapted these findings to the absorption of ethylene from an atmosphere, using mercuric nitrate solution to accumulate ethylene produced by plant tissue. They then added hydrochloric acid to the solution to free the ethylene in a small volume of air so that it could be identified by a biological test. The work of GROSS (5) suggested the use of mercuric perchlorate as the absorbent. Comparison with other mercuric salts in our laboratory showed the perchlorate to be best for the efficient absorption and subsequent quantitative release of ethylene. For the identification of ethylene produced by *Penicillium digitatum*, a solution of mercuric perchlorate was used to scrub this gas from a stream of air which was passing continuously over a series of cultures. The accumulated ethylene was subsequently released into a reaction flask by the addition of hydrochloric acid to the solution containing the ethylene mercury complex. The ethylene was brominated in the reaction flask, and suitable derivatives were produced from the ethylene dibromide.

Culture of the fungus

The strain of *Penicillium digitatum* used was collected in the field from a typically decaying grapefruit, as recommended by THOM (13, p. 244), and its purity was assured by five single-spore isolations. It was tested again on citrus fruit to verify its typical behavior. Several years' work with this fungus has shown little variability between isolates and strains with regard to gross morphology, behavior on fruit, and ability to produce ethylene (as shown by biological tests).

For this project, the fungus was grown as a surface mat on shallow layers of liquid medium, using the formula of PRATT (10) as follows (per liter of water): 25.7 gm. sucrose, 4.00 gm. NH_4NO_3 , 13.61 gm. KH_2PO_4 , 1.23 gm. $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.02 gm. $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.50 gm. Difco yeast extract, and 1.0 ml. of a micromineral supplement which provided in the final solution 0.02 p.p.m. of copper, 0.05 p.p.m. zinc, 0.01 p.p.m. molybdenum, 0.5 p.p.m. manganese, and 0.5 p.p.m. boron. The pH was adjusted to 5.0.

Thirty-eight Fernbach culture flasks were supplied with 200 ml. of culture solution each, capped with paper, and sterilized in the autoclave at 15 pounds for 15 minutes. Assemblies of rubber stoppers with glass input and outlet tubes and attached cotton-containing guard tubes (fig. 1, E... E') were placed in paper bags and autoclaved separately. After sterilization, the flasks were inoculated with equal portions of a heavy spore suspension, and the sterilized stopper assemblies were inserted and tied down. The flasks were then assembled in series with cotton guard tubes between each flask, and air was passed through the series at approximately 350 ml. per minute. Throughout the experiment, the cultures and absorption train were maintained at 25° C. (Later work has shown that the absorber should be maintained in an ice bath for maximum efficiency.)

Accumulation and identification of ethylene

ABSORPTION TRAIN

The apparatus used for the absorption of the active emanation of *Penicillium digitatum* is diagrammed in figure 1. Air was passed through a tower of brominated activated charcoal (Columbia 4ACW, size 6/14) to remove possible active contaminants. This tower consisted of a tube 50 mm. in diameter and 80 cm. long, filled one-third full (A) of brominated carbon (24 ml. of bromine on 200 gm. of carbon), followed by twice this volume of unbrominated carbon (B). The efficiency of this method of removal of possible contamination from the air stream has been verified previously (11).

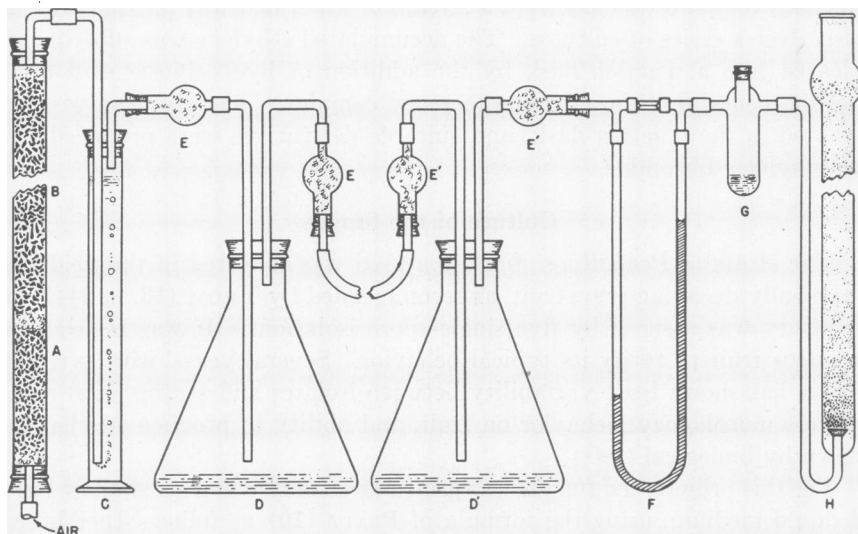


FIG. 1. Absorption train for collection of ethylene produced by fungus.

After purification, the air was humidified by a water tower (C) and passed over the fungus cultures contained in 38 Fernbach flasks (D . . . D') connected in series, through a capillary flowmeter (F), and then through a small bulb (G) containing *n*-butyl alcohol. The slow evaporation of the butyl alcohol into the air stream maintained the low concentration of alcohol in the absorber necessary to give optimum bubble size and efficiency. The absorber (H) consisted of a tube 25 mm. in diameter and 60 cm. long with a fritted Pyrex disc sealed in the inlet tube.

The absorbent solution was made up as follows: 50 gm. of mercuric oxide were dissolved in 177 ml. of 70% perchloric acid diluted to approximately 8 *N*. This solution was filtered by suction through an asbestos pad, and made to a final volume of one liter with water. The resulting solution was approximately 5% mercuric oxide in 2 *N* perchloric acid. For the fungus emanation series 100 ml. of solution were used in the absorber.

The quantitative production of ethylene by the cultures was followed by

withdrawing $\frac{1}{2}$ -ml. aliquots of the absorbent solution daily. From these the ethylene was released and measured manometrically by a method to be reported elsewhere (15). As shown by figure 2, ethylene production dropped markedly on the 10th day after inoculation, and the experiment was then terminated.

A parallel series without the cultures was set up and operated as a control on the air stream and reagents during the course of the absorption. In this series, 25 ml. of solution were used in an absorber of the same type but

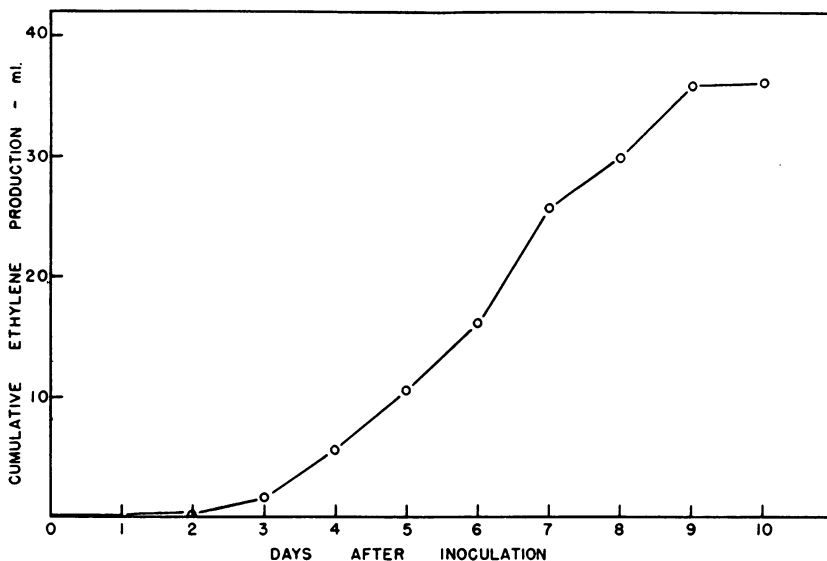


Fig. 2. Cumulative ethylene production of 38 cultures of *Penicillium digitatum*. (Final dry weight 34 gm.)

of proportionally smaller size. Absence of any absorbed gas in the control was established by use of the quantitative manometric determination mentioned above.

RELEASE AND BROMINATION OF ETHYLENE

The reaction assembly used for the release of ethylene from the mercury complex and for bromination of the ethylene is illustrated in figure 3. It consisted of two 250-ml. Erlenmeyer flasks with 24/40 standard taper joints, joined by a stopcock assembly for transferring the released gas from flask A to flask B.

The absorbent solution containing the ethylene-mercury complex was placed in flask A, and the system was evacuated to a pressure of 50 mm. of mercury through stopcock C, with E closed and F open. Stopcock C was then turned so as to close the system but leave flasks A and B connected, and a separatory funnel containing 6 N hydrochloric acid was connected to stopcock E. Approximately 100 ml. of the acid was admitted through E,

and the mixture was vigorously shaken. (The operator had to be alert to close stopcock F temporarily if too much foam developed.) The ethylene was released rapidly and passed into flask B. Additional small portions of the acid were added, with shaking, until no further evolution of the gas was observed, and then acid was allowed to run in and fill flask A to stopcock C, displacing all gas from the flask. Stopcocks C and F were then closed, and

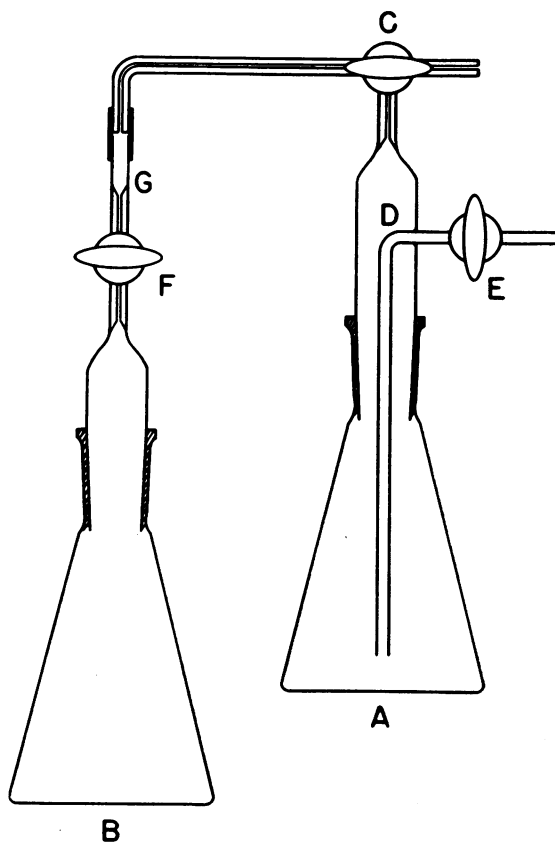


FIG. 3. Apparatus for release and bromination of ethylene.

the space between them was swept with several small portions of air by alternately opening C with F closed and then opening F with C closed. (It was important to retain a considerable vacuum in flask B to draw in subsequent reagents.)

With stopcock F closed, flask assembly B was disconnected, and a few drops of bromine were placed in the chamber at G and cautiously drawn in by opening the stopcock. Most of the bromine added evaporated, and the reaction took place rapidly, the dibromide condensing out on the vessel walls. The reaction flask was warmed gently over a steam bath to increase the reaction rate and encourage the product to flow down the walls of the

flask and collect at the bottom. After 30 minutes, small portions of sodium bisulphite solution were drawn into the flask until all free bromine disappeared. The reaction mixture was then extracted with ether, the first portion being drawn into the flask through the stopcock by the residual vacuum. After three more extractions, the ether solution was transferred to a 12-inch test tube. The ether was carefully evaporated over steam, leaving a small amount of yellow oil, presumably ethylene dibromide.

PREPARATION OF DERIVATIVES

For final identification derivatives were prepared as previously reported (11). An excess of aniline was added, and the mixture was heated on the steam bath for three and one-half hours. The unreacted aniline was then removed by steam distillation, and the resulting aqueous mixture was extracted with chloroform. After separation the chloroform was evaporated off, leaving a dark brown residue, presumably *N,N'*-diphenylethylenediamine. This was recrystallized three times from 50% ethanol, and the resulting crystals melted at 66.5-67.0° C, as did a known sample (Eastman White Label) and a mixture of these. The melting points were uncorrected and were taken simultaneously on the aluminum block of a Fisher-Johns melting-point apparatus.

The above derivatives were converted to *N,N'*-diphenyl-*N,N'*-dianilinothioformylethylenediamine by reaction with phenylisothiocyanate, and the products were washed three times with Skellysolve B and three times with 95% ethanol, filtered with suction, and dried under vacuum for 36 hours over P_2O_5 . Melting point data (taken as above): from *Penicillium digitatum* derivative 188.5-189.0° C; from *N,N'*-diphenylethylenediamine (Eastman White Label), 189.0-189.5° C; mixture of these, 189.0-189.5° C.

Summary

A greatly simplified method has been devised for the collection and identification of small concentrations of ethylene from gas streams, the principal feature being the concentration of the ethylene in a mercuric perchlorate solution with subsequent release and bromination under easily controlled conditions. The ethylene dibromide obtained was identified as *N,N'*-diphenylethylenediamine and *N,N'*-diphenyl-*N,N'*-dianilinothioformylethylenediamine. Using this technique, ethylene was identified as a volatile product of the common green mold of citrus fruits, *Penicillium digitatum* Sacc.

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