Analysis of Tobacco and Smoke Condensate for Penicillic Acid

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Gas chromatographic analyses of smoke condensate from commercial, unfiltered cigarettes spiked with penicillic acid (500 or 1,000 ppm), a reported carcinogenic substance from certain fungi, indicated approximately 3% of unchanged compound was transported in the smoke. Analysis of tobacco on which either Aspergillus ochraceus or Penicillium cyclopium was grown revealed microgram quantities of the compound. Small amounts of the material were also found in moldy tobacco from commercial storage. The results of these investigations suggest that fungi may be a source of carcinogenic compounds in tobacco and tobacco smoke.

Fungi produce numerous biologically important compounds on the substrates they inhabit. Many of these substrates, such as tobacco, are utilized by human beings. Because of its implicated role in the smoking and health problem, tobacco is currently receiving considerable attention.

Aspergillus ochraceus Wilhelm and Penicillium cyclopium Westling are two of the many fungi found on tobacco during marketing and storage (11). Like numerous other microorganisms, these fungi produce substances hazardous to some test animals (4, 5, 6) and therefore are potentially hazardous to human beings.

Penicillic acid (PA) was first isolated in 1913 (1) during an investigation of the cause of pelagra. The compound, C_6H_{14}O_4 (2), exists in tautomeric equilibrium with its lactone. The toxic properties of penicillic acid have been studied by several workers (4, 5, 6); however, only recently has the carcinogenic nature of the material been reported (3).

The work reported in this paper is part of a larger study of the fungi and their metabolic relationship to tobacco.

MATERIALS AND METHODS

Moldy tobacco samples. Samples of stored, moldy (damaged) tobacco were obtained from a commercial tobacco company. After removal of midribs, a total of 3.7 kg (sample 1) of leaf lamina was macerated in chloroform in a large commercial blender. The chloroform extract was filtered through cheesecloth and Whatman no. 42 filter paper and concentrated to a thick gum.

Sample 2 (35.4 kg) of moldy commercial tobacco was air dried at 35°C to 5 to 10% moisture and shattered by hand. Enough chloroform was added to cover the tobacco which was allowed to stand overnight. The chloroform was decanted and the tobacco extracted a second time with the same volume of chloroform. The extracts were combined, filtered through Whatman no. 42 filter paper, and evaporated to a thick gum.

Cultures of A. ochraceus and P. cyclopium were isolated from tobacco in 1968, lyophilized, and stored at 3 to 4°C until used for the following experiments. To obtain monocultures of moldy tobacco, 50 g of commercial, shredded tobacco and 50 ml of distilled water were placed in each of 15 flasks. The flasks were plugged with cotton and autoclaved for 20 min at 20 psi. Five flasks were each inoculated with 5 ml of a spore suspension of either A. ochraceus (2.5 × 10⁴ spores/ml) or P. cyclopium (5.0 × 10⁴ spores/ml). The remaining five flasks each received 5 ml of sterile, distilled water and served as controls. The flasks were covered with aluminum foil to prevent desiccation and incubated at 25°C for 4 weeks.

After incubation, 300 ml of chloroform was added to each flask and they were allowed to stand overnight. The chloroform extract was filtered through cheesecloth and Whatman no. 42 filter paper. Each flask was flushed with an additional 300 ml of chloroform which was filtered and combined with the original extract. The combined extracts were then evaporated to a thick gum.

Smoke samples. PA for the following experiments was isolated from liquid cultures of A. ochraceus. After incubation, the culture medium was filtered and evaporated to 300 ml. Two hundred ml of

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xylene was added to form a liquid-liquid interface. After warming and stirring the liquids for 2 hr, the xylene was decanted and evaporated to dryness. The concentrate was dissolved in 50 ml of carbon tetrachloride and stored at –18 C. Crystals of PA began to form in 3 to 6 hr. Verification of identity was made by infrared and mass spectral analyses.

Twenty commercially manufactured 82-mm cigarettes (sample 1) were spiked with 10 ppm of PA (0.2 mg/2.0 ml of distilled water), 20 cigarettes (sample 2) were spiked with 100 ppm of PA (2.0 mg/2.0 ml of distilled water), 100 cigarettes (sample 5) were spiked with 200 ppm of PA (20 mg/10 ml of distilled water), 20 cigarettes (sample 3) were spiked with 500 ppm of PA (10 mg/2.0 ml of distilled water, and 40 cigarettes (samples 4 and 6) were spiked with 1,000 ppm of PA (40 mg/4.0 ml of distilled water). The core of each cigarette received 0.1 ml of solution from a microsyringe drawn along the length of the cigarette as the solution was expelled. Control cigarettes were spiked with distilled water.

After air drying overnight, cigarettes at each PA level were mechanically smoked on a Phipps and Bird smoking machine (capacity of 20 vacuum ports). Each port was programmed to draw a 35 ± 2 cm³ puff for 10 sec of 60-sec intervals. Smoke was collected in Pyrex condensors, each containing 2 g of Sokaflow (Brown Company, Berlin, N.H.), and partially submerged in a dry ice-acetone bath. Contents of the condensors were combined and twice flushed with reagent grade acetone. After filtering through Whatman no. 42 filter paper, the combined sample was evaporated to dryness.

Analysis of samples. Gas chromatographic analyses for PA were carried out by using a Varian Aerograph model 2100 gas chromatograph with a flame ionization detector and an accessory model 480 Varian-Aerograph digital integrator with printout. Derivatization and programming were carried out according to the method reported by Pero et al. (8).

RESULTS

The results of gas chromatographic analyses for PA in extracts of moldy tobacco are summarized as follows. The smaller sample of commercial moldy tobacco (sample 1) contained approximately 23 µg of PA per 100 g of tobacco. Sample 2 contained approximately 11 µg of PA per 100 g of tobacco, about half as much PA as sample 1. Sample 2, however, was visibly less moldy than sample 1, which might account for some of the difference. Also, sample 1 was macerated in a blender, whereas sample 2 was shattered into pieces no larger than about ¼ inch in diameter.

More PA was found in samples of tobacco on which A. ochraceus (1,800 µg/100 g) was grown than in samples on which P. cyclopium (320 µg/100 g) was grown. This suggests that A. ochraceus may produce more compound under storage conditions than does P. cyclopium. No gas chromatographic peaks with the same retention time as PA were found in extracts of nonmoldy tobacco.

Table 1 summarizes the results of gas chromatographic analyses of smoke condensates. A trace of PA was detected in condensate from cigarettes spiked with either 10 or 100 ppm (samples 1 and 2), whereas samples 3 and 4 contained 2.4 and 3.9%, respectively, of the amount originally added. For samples 5 and 6, a finer-mesh Sokaflow (SW-60) was used to trap the smoke, and no PA was detected in the condensate. Lack of recovery of PA in these samples may have been due to decreased air flow through the finer-mesh filter.

DISCUSSION

Several workers (7, 9, 12) have detected aflatoxin and aflatoxin-like compounds in tobacco. Aflatoxin, like PA, is a carcinogenic compound produced by a fungus, Aspergillus flavus, cultured from flue-cured tobacco.

The presence in tobacco of a carcinogenic compound produced by fungi growing on tobacco and its transport in cigarette smoke, although in small amounts, emphasizes the importance of evaluating the possible contribution of microbial contaminants to tobacco in general and to the solids and volatile components of cigarette smoke. A. ochraceus and P. cyclopium are only two of numerous fungi which have been isolated from moldy, commercial tobacco (10). When one considers the myriads of compounds produced by these organisms, the chances of one or more being harmful to man are considerable. If mold-

| Sample | Amt spiked (mg/ppm) | No. of cigarettes | Sokaflow used | Amt detected (µg) | Per cent transported |
|--------|---------------------|-------------------|--------------|-------------------|---------------------|
| 1      | 0.2/10              | 20                | SW-40        | Trace             | Not calculated      |
| 2      | 2.0/100             | 20                | SW-40        | Trace             | Not calculated      |
| 3      | 10/500              | 20                | SW-40        | 240               | 2.4                 |
| 4      | 20/1,000            | 20                | SW-40        | 775               | 3.9                 |
| 5      | 20/200              | 100               | SW-60        | 0                 | 0                   |
| 6      | 20/1,000            | 20                | SW-60        | 0                 | 0                   |
damaged tobacco is blended with undamaged tobacco, PA or other fungal toxins could find their way into commercial products. At very low concentrations, these compounds, acting alone or in combination with carcinogens or co-carcinogens of fungal or other origin, may after many years of exposure produce effects similar to those obtained when the compounds are tested on a short-term high-dose basis. Further studies are needed to determine the effects of long-term exposure to these materials alone and in combinations with other tobacco and smoke components.

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