THE PRODUCTION OF INDOLE ACETIC ACID BY USTILAGO ZEAE, AND ITS POSSIBLE SIGNIFICANCE IN TUMOR FORMATION*

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The disease known as corn smut, caused by *Ustilago zeae* (Beckm.) Ung., is characterized by the production of large galls or tumors upon various portions of affected corn plants. Studies of the pathological histology of these galls, by De Bary[1] and Knowles,[2] indicate that both hypertrophy and hyperplasia are involved in the response of the host to fungus invasion. The first insight into the mechanism of tumor production in corn smut disease was provided by Link, Wilcox and Link,[3] and by Link, Wilcox and Eggers,[4] who reported that ether extracts of cultures of *U. zeae*, grown upon media containing tryptone, gave a positive Salkowski test for indole acetic acid. Moulton[5] found that corn smut tumor tissue contained an auxin, active in tests with the *Avena* coleoptile, in much greater amounts than normal uninfected corn tissue. Auxin was detected both in the mats and in filtrates of cultures of *U. zeae*, and it was found that considerably more auxin was produced in media containing tryptone than in a synthetic medium containing neither proteins nor amino acids. No conclusion was reached as to the chemical nature of the auxin or auxins involved.

The production of indole acetic acid by a fungus was clearly demonstrated in the case of *Rhizopus suinus* Nielsen by Thimann.[6] When grown upon media containing peptone, this organism was found to produce an auxin active in the *Avena* test. It was shown by a number of chemical tests and confirmed by isolation that the auxin formed by *R. suinus* was indole acetic acid. It was also shown that tryptophane present in the peptone was the precursor of indole acetic acid, and indole pyruvic acid was suggested as a possible intermediate in this transformation.

It is the purpose of the present paper to provide further evidence indicating that the auxin produced by *U. zeae* is indole acetic acid (IAA), to demonstrate that tryptophane is the precursor of IAA in this organism, and to present evidence regarding the conversion of tryptophane to IAA. These findings lend added weight to the hypothesis that tumor formation is a direct consequence of IAA production.

Materials and Methods.—A monosporidial culture of *U. zeae* known as 17D4, provided by Dr. E. C. Stakman, was used in all experiments except when otherwise stated. Other cultures of *U. zeae* tested for comparison were sent by Dr. R. B. Stevens, and the culture of *U. nigra* Tapke was

obtained from Dr. J. G. Dickson. These isolates were grown at room temperature in a modified Czapek's solution (containing $NaNO_3$, 3.0 g.; KH_2PO_4, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; KCl, 0.5 g.; $FeCl_3$, trace; and sucrose, 30.0 g. per liter), and in a tryptophane medium prepared by omission of sodium nitrate from the Czapek's solution, and substitution of 1.0 g. *L*-tryptophane per liter as the sole source of nitrogen. Additional details will be provided in connection with the results.

Results.—Filtrates of cultures of *U. zeae*, grown for two weeks in Czapek's solution or the tryptophane medium, were first tested for the possible presence of IAA by the Salkowski reaction. The reagent was prepared according to the directions of Berthelot, Amoreux, and Deberque.[7] Upon the addition of conc. HCl, $FeCl_3$, and boiling, a pink color is produced in the presence of IAA. The pink reaction product is soluble in *n*-butyl alcohol. When this test was applied to the filtrates of cultures grown in Czapek's medium, the results were negative. Tests with filtrates of cultures grown in the medium containing tryptophane were strongly positive for IAA.

Further tests were made using the nitrite reaction as advocated by Mitchell and Brunstetter.[8] When 0.5 ml. 5% gum arabic, 2.0 ml. 0.5% freshly prepared potassium nitrite and 0.4 ml. conc. HNO_3 are added to 50 ml. of the test solution, a cherry red color is produced by IAA or indole. Tests with filtrates of cultures of *U. zeae* on Czapek's medium were negative, while those on the tryptophane medium were again strongly positive. The *U. zeae* filtrate-nitrite reaction product was examined with a Beckman spectrophotometer, and found to have an absorption curve with a maximum near 530 mμ, indistinguishable from that given by an authentic sample of IAA upon reaction with nitrite.

Gordon and Weber[9] have pointed out a number of disadvantages of the nitrite test for IAA, including lack of specificity, low sensitivity and lack of stability of the color complex formed, and have developed a method employing ferric chloride and perchloric acid which was superior to five other methods in comparative tests. Accordingly, a reagent was prepared consisting of 1.0 ml. 0.5 *M* ferric chloride and 50 ml. 35% perchloric acid. Filtrates of cultures of *U. zeae* grown upon the tryptophane medium gave the red color characteristic of IAA when tested by this method. Further, when the filtrates of such cultures were adjusted to pH 2.0 with conc. HCl, extracted three times with an equal volume of ether, the ether removed by evaporation and the residue dissolved in water, a positive test for IAA was obtained with the resulting solution.

Because many micro-organisms produce indole, which might have been responsible for the positive findings in some of the colorimetric tests described, an experiment testing the indole production of *U. zeae* was performed by Mr. James Walton, employing the technique of Goré.[10]

Cultures of *U. zeae* were grown upon malt agar slants and in 1% Bacto-tryptone broth for two weeks. To the cotton stoppers of these tubes were applied several drops of a solution consisting of 1.0 g. *p*-dimethyl-aminobenzaldehyde, 95 ml. 95% ethyl alcohol and 20 ml. conc. HCl, as well as several drops of saturated aqueous potassium persulphate. The cotton stoppers were then pushed down into the tubes to a level about an inch above the medium, and the tubes were boiled in a water bath for 15 minutes. A red coloration on the cotton plug indicates the presence of indole. Negative results were obtained with *U. zeae*.

One-dimensional paper chromatography according to the ascending method of Williams and Kirby[11] was employed in an attempt to characterize the compound present in *U. zeae* filtrates. The solvent chosen was a 9:1 mixture of phenol and water. Filtrates of *U. zeae* cultures were extracted three times with an equal volume of ether, and the ether was evaporated to dryness. The residue was resuspended in a small quantity of water. Spots of this extract were placed near the bottom of strips of Whatman No. 1 filter paper, and the phenol-water mixture was allowed to ascend the paper strips for 12 to 15 hours. The strips were then dried and sprayed with the nitrite reagent to determine the location of the nitrite-reacting materials on the paper strip. Three spots appeared, with R_F values averaging 0.88, 0.80 and 0.70. When the paper was spotted with a sample of known IAA and treated as before, a single spot appeared at a location corresponding to R_F 0.88. When the papers were spotted simultaneously with two spots, one containing *U. zeae* filtrate alone, the other containing *U. zeae* filtrate plus IAA, it was found that the uppermost spot (R_F 0.88) was intensified in color in the latter instance. This is strong evidence that the uppermost spot in the paper chromatograms of *U. zeae* filtrates is due to IAA. No information was obtained concerning the identity of the nitrite-reacting materials responsible for the spots at R_F 0.70 and 0.80.

Since the preceding data indicate the presence of IAA in filtrates of *U. zeae* when grown in media containing tryptophane, and the absence of detectable amounts of this compound in cultures grown in Czapek's medium, experiments were performed to investigate the role of tryptophane in IAA production. When 0.5% gelatin was incorporated into media as the sole source of nitrogen for *U. zeae*, tests for the presence of IAA in the filtrate, by means of the nitrite reagent, were negative. When tryptophane was added to this medium, positive tests for IAA were obtained. Similarly, *U. zeae* does not form IAA in media in which the nitrogen is supplied as casein hydrolysate, but IAA is produced when the medium is supplemented with tryptophane.

A number of amino acids were tested for their ability to promote IAA production by *U. zeae*. Each amino acid was added to Czapek's medium

(minus $NaNO_3$) in the quantity of 100 mg. per 100 ml., and the pH was adjusted to 5.5. The amino acids tested, in addition to *L*-tryptophane, were glycine, *DL*-alanine, *DL*-serine, *DL*-threonine, *DL*-valine, *L*-aspartic acid, *L*-asparagine, *L*-glutamic acid, *L*-leucine, *DL*-isoleucine, *DL*-norleucine, *L*-cysteine·HCl, *DL*-methionine, *L*-lysine·HCl, *L*-arginine·HCl, *L*-histidine·HCl, *L*-tyrosine, *L*-proline and *DL*-phenylalanine. Results were negative for IAA production in every instance, except for tryptophane. It may therefore be concluded that tryptophane is a specific precursor in the formation of IAA by *U. zeae*.

Recent studies with higher plants, notably those of Wildman, Ferri and Bonner[12] with spinach leaves, Wildman and Bonner[13] and Larsen[14] with the *Avena* coleoptile, Galston[15] with etiolated pea seedlings and Gordon and Sanchez-Nieva[16] with pineapple, have shown the existence of two routes in the enzymatic conversion of tryptophane to IAA. One of these involves tryptamine and indole acetaldehyde as intermediates, the other involving indole pyruvic acid and indole acetaldehyde. Experiments were planned to determine which, if either, of these mechanisms was operative in *U. zeae*. It was found that tryptamine·HCl cannot serve as a sole source of nitrogen for growth of *U. zeae*. It was therefore necessary to provide nitrogen for growth in the form of nitrate, which, as previously shown, does not lead to IAA formation. Consequently, tryptamine·HCl was added, in concentrations of 50 or 100 mg. per 100 ml., to Czapek's medium, and inoculated with *U. zeae*. Although good growth resulted, tests for IAA in the filtrate by both the nitrite method of Mitchell and Brunstetter[8] and the perchloric acid method of Gordon and Weber[9] were negative. Further, when the filtrates were acidified and extracted with ether, the ether extract taken to dryness, and the residue dissolved in water, tests on the resulting solution by the perchloric acid method were negative. It is therefore apparent that tryptamine may be excluded from consideration as a possible intermediate in the transformation of tryptophane to IAA by *U. zeae*.

Attempts to demonstrate the participation of an aldehyde in the transformation of tryptophane to IAA by trapping with dimedon failed, because of reaction of the dimedon with ingredients of the Salkowski and nitrite reagents.

Any attempt to explain tumor formation as due to IAA production must take into account the possible toxicity of IAA to the inciting fungus. Richards[17] reported the inhibition of growth of *Phycomyces blakesleeanus*, *Aspergillus candidus*, *Schizophyllum commune* and *Neurospora tetrasperma* by IAA in concentrations of 10^{-2} to 10^{-3} M. Itzerott[18] found that the tolerance of *U. zeae* to IAA is very high, growth in the presence of 0.1% IAA being 98% of that in controls. Experiments with *U. zeae*, strain 17D4, upon malt agar containing 0.01% or 0.1% IAA showed that growth

in the presence of 0.01% IAA was comparable to that of untreated controls. Considerable inhibition occurred at the 0.1% concentration, but a few colonies managed to develop. The tolerance of *U. zeae* to IAA is therefore probably high enough to allow for the possibility of tumor formation without inhibition of growth of the smut.

Four monosporidial cultures of *U. zeae*, representing all the meiotic products of a single chlamydospore, were all found to produce IAA.

There are rather few smuts which induce tumor formation upon their hosts. Demonstration that smuts which fail to produce tumors do not form IAA would constitute additional evidence favoring the thesis that IAA production by *U. zeae* is related to tumor formation in corn smut disease. *U. nigra*, which causes a loose smut of barley, does not induce gall formation. When *U. nigra* was grown upon a tryptophane medium and the filtrate was tested for IAA by the nitrite and perchloric acid reagents, negative results were obtained in several trials.

Discussion.—It is known that abnormal tissue proliferations result in many species of higher plants upon application of IAA. Friedman and Francis[19] reported cellular hypertrophy, hyperplasia and disarrangement of tissue organization in tomato roots treated with IAA. In corn, Britten[20, 21] found that the application of α-naphthalene acetic acid to unpollinated ovaries resulted in parthenocarpy and abnormal differentiation of embryonic tissues.

It is believed that the production of IAA by *U. zeae* is an important factor in the development of corn smut galls. Whether the increased auxin content of corn smut galls with respect to normal corn tissue is due solely to IAA production by the parasite has not been determined. Haagen-Smit, Leech, and Bergren,[22] Berger and Avery[23] and Haagen-Smit, Dandliker, Wittwer and Murneek[24] have demonstrated by isolation the presence of IAA in corn. An increase in IAA content of corn smut tumor tissue owing to the formation of this substance by *U. zeae* may conceivably be responsible for the pathological changes observed.

In the paper chromatography of *U. zeae* filtrates, three spots were observed when the papers were sprayed with nitrite reagent, one of which was identified as IAA. The two remaining spots could not have been due to tryptophane, which gives no color with the nitrite reagent,[8] nor to tryptamine·HCl, which produces a faint yellow color. Although proof is lacking, it would appear probable that the intermediates in the formation of IAA from tryptophane by *U. zeae* are indole pyruvic acid and indole acetaldehyde.

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Summary.—Indole acetic acid has been identified as a metabolic product of *Ustilago zeae*. The identification of this compound is based upon three independent color tests, and upon identity of R_F values obtained by paper chromatography. Tests with gelatin, casein hydrolysate and a number of individual amino acids indicate that tryptophane is the precursor of IAA in *U. zeae*. The mechanism of conversion of tryptophane to IAA is not known with certainty, but it is shown that tryptamine cannot be an intermediate.

U. nigra, a smut which does not form tumors upon its host, failed to produce from tryptophane a quantity of IAA detectable by colorimetric tests. The present findings add weight to the theory that IAA production by *U. zeae* is important in the development of the tumors characteristic of corn smut disease.

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