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Effects of Isonicotinic Acid Hydrazide (INH) Upon Plant Growth and Auxin (Indoleacetic Acid) Synthesis*

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Isonicotinic acid hydrazide (INH) is shown to inhibit growth of both tomato and pea seedlings as well as the conversion of exogenous tryptophan to indoleacetic acid by lyophilized tomato leaf preparations. The inhibitory effect is at least partially prevented by pyridoxine or pyridoxal phosphate. The inhibition is ascribed to formation of isonicotinyl-hydrazone between INH and pyridoxal, resulting ultimately in a depletion of pyridoxal phosphate, the coenzyme required for an essential transaminase involved in the conversion of tryptophan to indoleacetic acid. Evidence is presented which adds support to the hypothesis that the main biosynthetic pathway between tryptophan and indoleacetic acid involves indolepyruvic acid rather than tryptamine or indoleacetonitrile.

The studies described here*** were undertaken as a result of developments in two independent and unrelated fields; one in clinical medicine and pharmacology, the other in plant physiology and biochemistry. The first consisted of reports appearing soon after isonicotinic acid hydrazide (INH) was introduced for the treatment of tuberculosis, that a significant proportion of patients developed a peripheral sensory neuropathy, especially when given the drug in large dosage[1–4]. Because of the close chemical similarity between INH and nicotinamide with the resultant possibility of anti-metabolite action, it was at first suspected that the neuropathy resulted from niacin deficiency[1]. However, nicotinamide was ineffective in preventing or ameliorating the neuritis. Biehl and

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*** The first part of the work reported here was performed by one of us (G. D. L.) during 1957 in Professor Hugo Theorell's laboratory, Biochemical Department, Nobel Medical Institute, Stockholm, and a preliminary report has been made (Ludwig, G. *Federation Proc.* **17** (1958) 524, no. 2049). The work was completed in the Johnson Foundation of Biophysis (Professor Britton Chance) and Departments of Medicine (Professor Francis C. Wood) and Botany (Professor David Goddard) of the University of Pennsylvania. The authors are indebted to the above named for use of facilities and for advice rendered during the course of this work.

Vilter, recognizing the similarity of the neuritis to that produced by desoxypyridoxine[5] showed that the administration of INH led to an increased urinary excretion of pyridoxine (B_6); that the patients with the neuritis displayed other biochemical evidence of pyridoxine deficiency; and that the neuritis could be prevented by administration of pyridoxine along with the drug[6,7]. The evidence suggested that the increased urinary excretion of pyridoxine consisted of a bound form of pyridoxal, *i. e.*, the isonicotinyl hydrazone, which has been shown to occur readily *in vitro*[8].

The second set of observations that prompted this study concerned the formation of the plant growth hormone or auxin, indoleacetic acid. From the time that Thimann[9] first isolated indoleacetic acid (IAA) from a mold and suggested that it might be the main auxin of higher plants, it was suspected that it was derived from tryptophan. Abundant evidence has accumulated to support both hypotheses[10–22]. However, especially with the isolation of other indolic compounds from plants, the precise biosynthetic pathway by which tryptophan is converted to indoleacetic acid by plants has remained controversial[18–22]. The present work was based on the assumption that the conversion by plants of tryptophan to indoleacetic acid, by whatever route, would require both transamination and decarboxylation, reactions known to require pyridoxal phosphate as a coenzyme[23–27]. Moreover, pyridoxine has been shown to be required for growth of higher plants[28], but its exact role has not been further defined. Since INH is capable of binding pyridoxal phosphate, it was postulated that INH should inhibit the formation of indoleacetic acid from tryptophan, and thus, plant growth. To test this hypothesis the experiments reported here were designed.

MATERIALS AND METHODS

Tomato seeds (garden variety) were germinated in sand, and the seedlings transferred to small pots containing soil, one seedling to a pot for the initial series of experiments. All experiments were carried out in a greenhouse with controlled heat and humidity, but with natural variation of light and dark. The control plants were watered daily with de-ionized distilled water, the experimental plants with various concentrations of INH. Some plants were watered with pyridoxine, alone or in addition to the INH. In subsequent series of experiments, to exclude any effect of soil, the seedlings were transferred to 125 ml Erlenmeyer flasks containing Hoagland's nutrient solution[29], three seedlings to a flask. Various concentrations of INH varying between 10^{-6} and 10^{-2} M were added to the experimental flasks. All flasks were buffered to pH 5.1 with 0.01 M phosphate buffer. Pyridoxine (10^{-2} to 10^{-4} M) was added to some flasks with and without INH. All the flasks were continuously aerated by bubbling air through glass capillary tubes connected to the air supply through a manifold.

Measurements of stem length were made daily, and the plants were photographed at four day intervals. At the completion of each experiment, the plants in each flask were weighed after blotting and allowing to dry 8 h at room temperature. When the inhibition of growth of tomato plants by INH had been demonstrated, similar experiments were conducted using garden peas (var. "Gnesta favorit") in order to show that the inhibitory effect applied to other higher plants.

When the anticipated inhibition of plant growth by INH was observed, experiments were then performed in an attempt to show that the conversion of tryptophan to indoleacetic acid by plant tissue is inhibited by INH. Plant tissue containing the enzymes necessary for the above conversion were prepared according to the procedure described by Wildman and Muir[17] and incubated with L-tryptophan in the manner described by Henderson and Bonner[30]. Leaves from greenhouse-grown 14-day-old tomato plants were minced by sharp scissors directly into liquid nitrogen, lyophilized, pulverized, and stored in a dessicator over P_2O_5 at 0°C. Soon after preparation, aliquots of the pulverized leaf preparation (50 mg) were incubated in 100 ml Erlenmeyer flasks, with and without 50 mg L-tryptophan buffered to pH 7.0 in 0.01 M phos-

phate buffer. Various concentrations of INH between 10^{-5} and 10^{-2} M or INH plus pyridoxal phosphate were added to the experimental flasks. The flasks were incubated in the dark for 5, 10, and 15 h at 25°C. The residual plant tissue was then removed by filtration and washed with peroxide-free ether. To extract the indoleacetic acid produced, the filtrate was adjusted to pH 2.8 with 0.1 N HCl and extracted twice with 10 ml portions of peroxide-free ether, which was combined with the ether used to wash the plant particles; and the combined fractions then shaken vigourously with 2 ml of 2 % $NaHCO_3$. The bicarbonate solution was readjusted to pH 2.8 with HCl, extracted with fresh peroxide-free ether (10 ml). The ether was removed without heat (vacuum) and the residue again taken up in 2 ml of 2 % $NaHCO_3$. The IAA in the bicarbonate solutions was then assayed by two methods. A 1 ml aliquot was concentrated *in vacuo* and chromatographed in two dimensions on Whatman No. 1 filter paper using isopropanol : ammonia : water (200 : 10 : 20 v/v) for the first solvent and butanol : acetic acid : water (120 : 30 : 50 v/v) for the second solvent, according to the procedure of Jepson[31]. The papers were sprayed with Ehrlich's reagent (1 % in 1 N HCl). Indoleacetic acid was clearly identified as a red-purple spot with R_F values 0.37 and 0.91 in the two solvent systems, respectively. It is possible to detect as little as 1 μg of IAA by this method. Another 1 ml aliquot of the bicarbonate solution was treated with an equal volume of Gordon and Weber's modification[32] of Salkowski reagent. Indoleacetic acid yielded a red color, with an absorption maximum at 525 mμ. Absorbancy readings were made in a Beckman spectrophotometer at 15, 30, and 60 min, and the amount of indoleacetic acid present quantitated by comparison with a standard curve prepared by adding authentic IAA to Salkowski reagent in a manner similar to that used for the experimental solutions.

RESULTS

Tomato plants in soil; watered daily with INH: The appearance of tomato plants at the end of 19 days is shown in Fig. 1. The control plant received only distilled water, the experimental plants 10^{-5}, 10^{-4}, 10^{-2} M INH, respectively. The inhibitory effect of INH became apparent within 5–7 days, especially with 10^{-2} M INH, which completely inhibited growth; the latter concentration caused the plant to be stunted; the leaves wrinkled, dry, and shrivelled. The inhibition of growth varied in an approximately linear manner with the log of the concentration of INH, although in some experiments little difference in inhibitory effect was seen between 10^{-3} and 10^{-4} M INH.

Tomato and pea seedlings in Hoagland's nutrient solution: The results obtained with tomato plants in nutrient solution, to which were added various concentra-

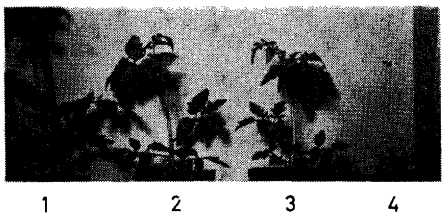

Fig. 1. Appearance of tomato plants grown in soil for 19 days; the control plant was watered with deionized distilled water, and the experimental plants with various concentrations of isonicotinic acid hydrazide (INH). 1) Control; 2) 10^{-5}, 3) 10^{-4} and 4) 10^{-2} M INH.

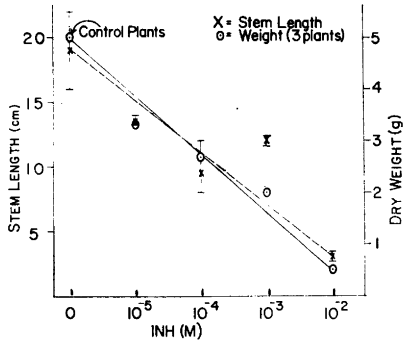

Fig. 2. Stem length and dry weight of tomato plants expressed as a function of INH concentration to which they were exposed for 21 days while growing in nutrient solution.

Fig. 3. Pea plants at the end of 10 days in nutrient solution containing various concentrations of INH. The control flasks contained no INH. 1) and 6) controls; 2) 10^{-2}, 3) 10^{-3}, 4) 10^{-4} and 5) 10^{-5} M INH.

Fig. 4. Effect of pyridoxine HCl (10^{-3} M) in preventing inhibition by INH (10^{-3} M) (compare flasks 2 and 3). High concentrations of pyridoxine HCl alone (10^{-2} M) appear to be slightly inhibitory (Flask 4). 1) Control; 2) 10^{-3} M INH; 3) 10^{-3} M INH + 10^{-3} M pyridoxine; 4) 10^{-2} M pyridoxine.

tions of INH, were similar to those obtained when the seedlings were grown in soil. The measurements of stem length and dry weight at the end of 21 days, as a function of INH concentration, are given in Fig. 2. Similar results were obtained when pea seedlings were grown under these conditions, as shown in Fig. 3. Definite evidence of at least partial protection against inhibition by INH was obtained when pyridoxine HCl ($10^{-3} - 10^{-4}$ M) was added along with INH to either tomatoes or peas. However, the reversal of inhibition was never complete as indicated by the results obtained with tomato plants shown in Fig. 4. Evidence was obtained that suggested that pyridoxine HCl alone may be slightly inhibitory to growth under these conditions, if it is applied at very high concentrations (Fig. 4, flask 4).

Conversion of tryptophan to indoleacetic acid by tomato leaf preparations: The amounts of IAA produced at 5, 10, and 15 h as a result of incubating quick-frozen, lyophilized, minced tomato leaf preparations with L-tryptophan with and without INH of various concentrations are shown graphically in Fig. 5. The highest values were obtained at 5 h incubation. The lower values at longer times may reflect destruction by an oxidase system of some of the IAA formed. It is apparent that 10^{-2} M INH almost completely inhibited IAA formation. When no tryptophan was added to the flask containing the plant material, no IAA was formed as measured by spectrophotometric assay after extraction or by paper chromatography. In this series of experiments 10^{-4} pyridoxal phosphate appeared to almost completely reverse the inhibition of color formation (*i. e.*, IAA formation) obtained with 10^{-2} M INH.

DISCUSSION

It is noteworthy that growth of both tomatoes and peas, as well as the conversion of exogenous L-trypthophan to indoleacetic acid by plant tissue, was practically completely inhibited by 10^{-2} M INH. This same concentration of INH has been shown to inhibit completely the growth of certain strains of *Lactobacilli* and *Saccharomyces*[33], and decarboxylation reactions[34], and tryptophan to indole conversion[35] by *E. coli*. However, the latter inhibitory effects were attributed to competitive metabolic antagonism between INH and pyridoxal phosphate for a site on the enzyme protein. It appears more likely that

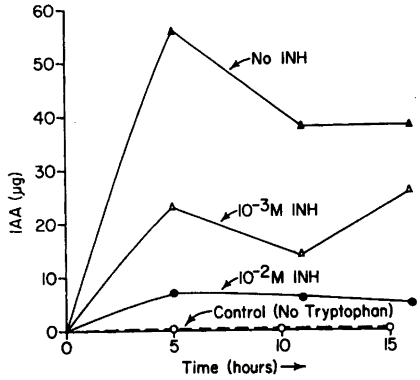

Fig. 5. Indoleacetic acid formed from L-tryptophan by quick-frozen, lyophilized tomato leaf preparations in 5, 10, and 15 h incubations at 25°C.

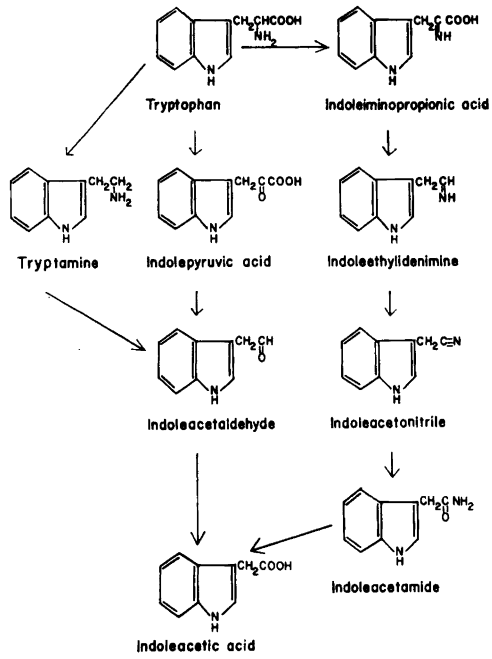

Fig. 6. Possible biosynthetic pathways for conversion by higher plants of tryptophan to indoleacetic acid that have been postulated and for which some experimental evidence exists.

the latter results as well as those reported here are due to formation of the pyridoxal-isonicotinyl hydrazone as suggested by the urinary excretion studies in the human neuropathy induced by INH[6] and by effects on certain other bacterial and mammalian enzyme systems[36,37]. In the course of these studies we have confirmed the observations of Sah[8] that the isonicotinyl-hydrazone forms easily between INH and pyridoxal phosphate, *in vitro*.

Wort[38], who tested the effect of INH on plants on an empirical basis, also has observed inhibition of growth. However, he attributed it to a decrease in catalase, to a depletion of available photosynthate for cell material synthesis and also of available energy, and in part to an inhibition of respiration.

The possible biosynthetic pathways for conversion of tryptophan to indoleacetic acid by higher plants that have been postulated and for which some experimental support exists are depicted in Fig. 6. For years, the main pathway was considered to involve either indolepyruvic acid (IPyA)[10,44] or tryptamine[18,19,39]. The demonstration of the activity and presence of indoleacetaldehyde (IAc) and its conversion to IAA in plants[22] placed it as a logical intermediate since it could arise from either IPyA or tryptamine. With the isolation of indoleacetonitrile (IAN) from plants[40,41] and the demonstration of its convertibility to IAA[10,42], it was proposed that IAN and possibly indoleacetamide (IAM) might be on the direct pathway between tryptophan and IAA[40]. However, later work showed that IAM arises from exogenous IAA added to respiring plants[43], and it has been speculated further that IAN might arise as a hydrated condensation product of IAc and hydroxylamine[21].

The demonstration that INH inhibits both plant growth and the conversion

of tryptophan to IAA, especially when considered in conjunction with certain previously reported findings of others, permits some conclusions to be drawn regarding the principle biosynthetic pathway involved in higher plants. Gordon[20] has reported that isopropyl-isonicotinic acid hydrazide (Iproniazid, Marsilid), a potent monoamine oxidase inhibitor, completely blocked conversion of tryptamine to IAA by plant tissue, but had no inhibitory effect upon the conversion of tryptophan to IAA or upon plant growth. This result, by itself, suggests that the pathway is not through tryptamine, leaving as the most likely alternative, the tryptophan → indolepyruvic acid → indoleacetaldehyde → indoleacetic acid pathway, as Thimann's work[10,44] had previously suggested. The results obtained with INH contained in the present report add further support to this hypothesis, since INH, which has little or no effect on monoamine oxidase[45,46], is a potent inhibitor of both transamination[47] and decarboxylation reactions[36,37] by virtue of its ability to form the isonicotinyl-hydrazone, depriving the above enzymes of their requisite coenzymes. Conversely, the isopropyl analogue is a potent monoamine oxidase inhibitor[45,46]; but cannot bind with pyridoxal to form a hydrazone and consequently does not inhibit transaminases or decarboxylases[36].

Therefore, we conclude that INH probably inhibits plant growth by blocking the conversion of tryptophan to indoleacetic acid. The probable mechanism is inhibition of an essential transaminase involved in converting tryptophan to indolepyruvic acid due to formation of the isonicotinyl-hydrazone with pyridoxal, ultimately depriving the enzyme of its requisite co-factor. McCormick and Snell[48] have shown that pyridoxal-isonicotinyl hydrazone is a potent inhibitor of pyridoxal-phosphate kinase of brain tissue. They suggest that this mechanism is at least partly responsible for the depletion of pyridoxal phosphate in a tissue, and thus for the physiological effects of INH.

The next step in the sequence, the decarboxylation of IPyA, would be expected to resemble pyruvate oxidation which requires co-factors other than pyridoxal phosphate and, therefore, would not be likely to be inhibited by INH. The decarboxylation of tryptophan to tryptamine could conceivably be inhibited by INH, but as mentioned above, the previously reported results obtained with iproniazid[20] provide evidence against tryptamine being on the main biosynthetic pathway. The pathway proposed by which IAA would be formed from tryptophan through intermediate participation of indoleacetonitrile[40] would require an oxidation followed by decarboxylation, or an oxidative decarboxylation followed by subsequent oxidation and hydrolysis (Fig. 6). None of these reactions would be expected to require pyridoxal phosphate as a co-factor, hence, inhibition by INH cannot be ascribed to this pathway.

Although these results point toward the tryptophan → IPyA → IAc → IAA pathway being the major one under normal conditions, they do not exclude the possibility of tryptamine or indoleacetonitrile being intermediates in the alternate routes that become active under certain circumstances[18–20]. Indeed, the conversion by plant tissue of radioactively labelled tryptophan to both tryptamine and indoleacetonitrile has been demonstrated[21]; but even in those experiments the main pathway appeared to be through IPyA and IAc.

It is also suggested by these studies that at least one of the specific functions of pyridoxine, which has been shown to be a requirement for growth of tomatoes

and other higher plants[28], is to provide coenzyme (pyridoxal phosphate) needed for a transaminase which is, in turn, required for the synthesis of the main plant growth hormone, indoleacetic acid.

REFERENCES

1. Jones, W. A. and Jones, G. P. *Lancet* **1** (1953) 1073.
2. Gammon, G. D., Burge, F. W. and King, G. *Arch. Neurol. Psychiat.* **70** (1953) 64.
3. Biehl, J. P. and Skavlem, J. H. *Am. Rev. Tuberc.* **68** (1953) 296.
4. Loring, H. N. *Am. Rev. Tuberc.* **68** (1953) 458.
5. Vilter, R. W., Mueller, J. F., Glazer, H. S., Jarrold, T., Abraham, J. P., Thompson, C. and Hawkins, V. R. *J. Lab. Clin. Med.* **42** (1953) 335.
6. Biehl, J. P. and Vilter, R. W. *Proc. Soc. Exptl. Biol. Med.* **85** (1954) 389.
7. Biehl, J. P. and Vilter, R. W. *J. Am. Med. Assoc.* **156** (1954) 1549.
8. Sah, P. P. T. *J. Am. Chem. Soc.* **76** (1954) 300.
9. Thimann, K. V. *J. Biol. Chem.* **109** (1935) 279.
10. Stowe, B. and Thimann, K. V. *Arch. Biochem. Biophys.* **51** (1954) 499.
11. Berger, J. and Avery, G. S. *Am. J. Botany.* **31** (1944) 199.
12. Haagen-Smit, A., Leech, W. D. and Bergren, W. R. *Am. J. Botany.* **29** (1942) 500.
13. Haagen-Smit, A., Dandliker, W., Wittwer, S. and Murneck, A. *Am. J. Botany.* **33** (1946) 118.
14. Wildman, S. G., Ferri, M. G. and Bonner, J. *Arch. Biochem.* **13** (1947) 131.
15. Wildman, S. G. and Bonner, J. *Am. J. Botany.* **35** (1948) 740.
16. Tsui, C. *Am. J. Botany.* **35** (1948) 172.
17. Wildman, S. G. and Muir, R. M. *Plant Physiol.* **24** (1949) 84.
18. Gordon, S. A. and Nieva, F. S. *Arch. Biochem.* **20** (1949) 356.
19. Gordon, S. A. and Nieva, F. S. *Arch. Biochem.* **20** (1949) 367.
20. Gordon, S. A. In Wain, R. L. and Wightman, F. (Ed) *The Chemistry and Mode of Action of Plant Growth Substances,* Academic Press, New York, 1952, p. 65.
21. Dannenberg, W. N. and Liverman, J. L. *Plant Physiol.* **32** (1957) 263.
22. Larsen, P. *Ann. Rev. Plant Physiol.* **2** (1951) 169.
23. Snell, E. E. *J. Am. Chem. Soc.* **67** (1945) 194.
24. Schlenk, F. and Snell, E. E. *J. Biol. Chem.* **157** (1945) 425.
25. Meister, A. *Science* **120** (1954) 43.
26. Umbreit, W. W. and Gunsalus, I. C. *J. Biol. Chem.* **159** (1945) 333.
27. Lichstein, H. C., Gunsalus, I. C. and Umbreit, W. W. *J. Biol. Chem.* **161** (1945) 311.
28. Bonner, J. and Bonner, J. *Vitamins Hormones* **6** (1948) 225.
29. Hoagland, M. B. *Arch. Biochem. Biophys.* **35** (1952) 249.
30. Henderson, J. H. M. and Bonner, J. *Am. J. Botany.* **39** (1952) 444.
31. Jepson, J. B. *Lancet* **2** (1955) 1009.
32. Gordon, S. A. and Weber, R. P. *Plant Physiol.* **26** (1951) 192.
33. Boone, I. U. and Woodward, K. T. *Proc. Soc. Exptl. Biol. Med.* **84** (1953) 292.
34. Yoneda, M., Kato, N. and Okajima, M. *Nature* **170** (1952) 803.
35. Yoneda, M. and Asano, N. *Science* **117** (1953) 277.
36. Davison, A. N. *Biochim. Biophys. Acta* **19** (1956) 131.
37. Hoare, D. S. *Biochim. Biophys. Acta* **19** (1956) 141.
38. Wort, D. J. *Science* **120** (1954) 72.
39. Skoog, F. *J. Gen. Physiol.* **20** (1937) 311.
40. Jones, E. R. H., Henbest, H. B., Smith, G. F. and Bentley, J. A. *Nature* **169** (1952) 485.
41. Henbest, H. B., Jones, E. R. H. and Smith, G. F. *J. Chem. Soc.* **1953** 3796.
42. Thimann, K. V. *Arch. Biochem. Biophys.* **44** (1953) 242.
43. Good, N. E., Andreae, W. A. and Van Ysselstein, M. W. H. *Plant Physiol.* **31** (1956) 231.
44. Stowe, B. B. and Thimann, K. V. *Nature* **172** (1953) 764.
45. Zeller, E. A. and Barsky, J. *Proc. Soc. Exptl. Biol. Med.* **81** (1952) 459.
46. Zeller, E. A., Barsky, J., Fonts, J. R., Kirchheimer, W. F. and Van Orden, L. S. *Experientia* **8** (1952) 349.
47. Meister, A. and Downey, P. F. *Proc. Soc. Exptl. Biol. Med.* **91** (1956) 49.
48. McCormick, D. B. and Snell, E. E. *Proc. Natl. Acad. Sci. U. S.* **45** (1959) 1371.

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