Proceedings of the Iowa Academy of Science

Volume 66 | Annual Issue Article 19

1959

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Wallace H. Orgell
Iowa State University

Kunda A. Vaidya
Iowa State University

Eugene W. Hamilton
Iowa State University

Biosynthesis of Cholinesterase-inhibitory Substances from Non-inhibitory Dialkyl Thiophosphates by Plant Tissues[1]

WALLACE H. ORGELL, KUNDA A. VAIDYA and
EUGENE W. HAMILTON

Abstract. Aqueous solutions of the potassium salts of diethyl thiophosphate and diethyl dithiophosphate were applied to intact leaves of the following 10 plant species: alfalfa (*Medicago sativa* L.), carrot (*Daucus carota* L.), strawberry (*Fragaria chiloensis,* Duchesne), American elderberry (*Sambucus canadensis* L.), velvetleaf (*Abutilon theophrasti* Medic.), dandelion (*Taraxacum officinale* Weber), soybean (*Glycine max,* Merr.), lima bean (*Phaseolus limensis* Macf.), catnip (*Nepeta cataria* L.), and Virginia creeper (*Hedera quinquefolia* L.). After 24 to 72 hours, aqueous homogenates of all species, excepting carrot, inhibited the action of human plasma cholinesterase *in vitro.* Neither the plant homogenates nor the thiophosphate salts either separately or mixed brought about inhibition. It is postulated that dialkyl thio- or dithiophosphates are incorporated by intact living plant tissue into cholinesterase-inhibitory esters.

Experiments with P^{32}-labeled phosphoric acid have shown that phosphate is absorbed by both roots and foliage of higher plants and is rapidly incorporated into a variety of organic compounds including nucleotides (Loughman, 1957) and esters of choline and its derivatives (Maizel, 1956). Certain dialkyl phosphoryl esters of choline and substances related to choline are known to be potent *in vitro* inhibitors of the enzyme cholinesterase (Metcalf, 1955, p. 293). These facts suggested the possibility that dialkyl substituted phosphates and thiophosphates might likewise be absorbed by plant tissue and biosynthesized into substances which inhibit cholinesterase. To test this hypothesis, various plant tissues were treated with dialkyl thiophosphate salts and later extracted and assayed for cholinesterase-inhibitory activity.

MATERIALS AND METHODS

Plant materials consisted primarily of leaves of various species freshly excised at the base of the petiole, and supported by a plug of cotton in a vial containing either water or a solution of the thiophosphate salt. In most cases, treated plants and controls were maintained in the laboratory out of direct sunlight at about 25° C.

[1]Journal Paper No. J-3628 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1351. This investigation was supported in part by Research Grant RG-4066 from the Division of Research Grants, U. S. Public Health Service.

The compounds used as treatments were the potassium salts of diethyl thiophosphate, $(C_2H_5O)_2$ POSK, and diethyl dithiophosphate, $(C_2H_5O)_2$ PSSK, which are referred to in this paper as DTP and DDP, respectively. These substances were obtained from the American Cyanamid Company, Stamford Research Laboratories, Stamford, Connecticut. Aqueous 10,000 ppm.(w/v) stock solutions of these salts were prepared and stored at 5° C. The solutions were clear, neutral, and appeared to be stable over a period of 6 months.

Surface active agents were employed at 0.1 per cent (w/v) in the experiments involving foliar absorption. The surfactants used were Tween 20 (Atlas Powder Company, Wilmington 99, Delaware), Triton X-100 (Rohm and Haas Company, Philadelphia 5, Pa.), and Nonic 218 (Pennsylvania Salt Mfg. Company, Philadelphia 2, Pa.).

The treated plants were extracted either by grinding with sand and distilled water in a hand mortar, or by homogenizing with 10 parts of distilled water in a Waring Blendor for 3 minutes at room temperature. Aliquots of the extracts containing known amounts of tissue were added to 5 ml. of standardized human blood plasma, diluted to 50 ml. with distilled water, and allowed to incubate with mixing for 70 minutes at 37.5° C. One ml. portions of the incubated mixture were then assayed for cholinesterase activity by the electrometric method of Curry (1956). Untreated control plants were similarly extracted and assayed to allow correction for any effects of the buffer capacity of the plant extract upon the assay method.

Results

In order to determine whether the thiophosphate salts were in themselves inhibitory to cholinesterase, 1,000 μg. of each were incubated with 5 ml. of plasma by the standard procedure. No inhibition was observed in six separate experiments. Aliquots of untreated control plant extracts were also assayed against plasma cholinesterase. No inhibition was found. However, high concentrations of leaf extract (5 per cent v/v) did alter the buffer capacity of the assay mixture to the extent that a correction factor of 1 to 15 per cent, depending upon the species, was necessary.

In the first series of experiments, foliar absorption of the thiophosphate salts along with their subsequent biosynthesis into inhibitory substances was studied using leaves of alfalfa (*Medicago sativa* L.). A series of 25 x 60 mm. glass shell vials was prepared, each containing 2 trifoliolate leaves supported by cotton, and with the freshly severed basal portions of the petioles immersed in tap water. One-hundredth ml. of an aqueous solution of the thiophosphate salt containing 0.1 per cent (w/v) surfactant was applied to each of the 6 leaflets per vial, thus making up one treatment. The

applied solutions contained concentrations of thiophosphate salt ranging from 5,000 to 16,666 ppm.; hence individual treatments of 6 leaflets received total dosages of thiophosphate ranging from 300 to 1,000 μg. The treated leaves were generally allowed to remain in the laboratory out of direct sunlight at 25° C for at least 2 hours prior to extraction and assay. All treatments were made in duplicate.

Biosynthesis of cholinesterase-inhibitory material was observed in each of seven separate experiments carried out as described. One thousand micrograms of DTP resulted in 70 to 83 per cent inhibition within 24 to 72 hours after treatment. DDP was biosynthesized into inhibitory material more slowly, the same dosage resulting in 46 per cent inhibition 72 hours following treatment. Dosage levels of DTP of 300 and 900 μg resulted in 41 and 77 per cent inhibition, respectively, at 72 hours. Three surfactants, Tween 20, Triton X-100, and Nonic 218, formulated at 0.1 per cent (w/v) with 1,000 μg DTP, gave 0, 11, and 34 per cent inhibition, respectively, at 4 hours, and 77, 79, and 73 per cent inhibition at 24 hours. The relative inhibition values obtained at 4 hours reflected the observed ability of these adjuvants to aid in wetting the alfalfa leaf surface. The differences in rates of absorption of the thiophosphate were apparently leveled at the end of 24 hours.

At thiophosphate dosages of 1,000 μg or higher, pronounced phytotoxicity was usually evident within 8 hours after treatment. The margins of the leaflets yellowed and were often dead at 72 hours. Marginal burn appeared sooner and was more extensive in treatments held at 25° C as compared with treatments held at 5° C.

In a second series of experiments, leaves of various plant species were allowed to take up from 300 to 500 μg of an aqueous solution of DTP through their cut petioles. Surfactants were not added to the DTP solutions. Extracts of 2.5 grams of leaves of carrot (*Daucus carota* L.), velvetleaf (*Abutilon theophrasti* Medic.), strawberry (*Fragaria chiloensis,* Duchesne), and American elderberry (*Sambucus canadensis* L.) resulted in 0, 8, 9, and 13 per cent inhibition, respectively.

Better results were achieved in a third series of experiments in which various leaves were dipped in an aqueous solution of DTP containing 0.1 per cent Nonic 218. The wetted leaves were allowed to drip free of excess liquid and were maintained with their cut petioles in tap water in the laboratory for 17 to 20 hours prior to extraction and assay. Inhibition values obtained with 2.5 grams of leaves of various species were as follows: dandelion (*Taraxacum officinale* Weber) 7 per cent, velvetleaf 7-20 per cent, catnip (*Nepeta cataria* L.) 21 per cent, soybean (*Glycine max* Merr.) 29 per cent,

lima bean (*Phaseolus limensis* Macf.) 37-75 per cent, and Virginia creeper (*Hedera quinquefolia* L.) 79 per cent.

The addition of 100 μg of DTP to 10 ml. of aqueous homogenates of various parts of 18 different untreated plant species failed to show any biosynthesis of cholinesterase inhibitor *in vitro* within a time interval of 24 hours. Preliminary observations indicated that alfalfa foliage treated with 2 per cent (w/v) DTP was not detrimental to either grasshoppers or spotted alfalfa aphids.

Since it is suspected that organic phosphate insecticides are degraded by hydrolysis within plant tissues to their non-toxic acid and alcohol or thiol moieties (Metcalf, 1955, p. 305), it is possible that the released dialkyl phosphoric or thiophosphoric acid portion may be recombined biosynthetically into substances which are still cholinesterase-inhibitory, but are not toxic to mammals or insects. This possibility introduces a note of caution into the interpretation of insecticide residue data obtained by methods of analysis based upon cholinesterase inhibition, particularly in the case of the low and lingering residue values often observed over a week after treatment of a crop with a systematic organic phosphate insecticide.

Discussion

These experiments have shown that treatment of the intact foliage of various higher plants with either diethyl thiophosphate or diethyl dithiophosphate results in the production of cholinesterase-inhibitory substances within the foliage. Foliar application of an aqueous solution of the thiophosphate plus a surfactant has been the most effective method of treatment so far examined.

Neither diethyl thiophosphate nor diethyl dithiophosphate are in themselves cholinesterase-inhibitory, although they are present as a part of the molecule in many synthetic esters which are powerful inhibitors of cholinesterase. This, along with the known capability of plant tissue to form various esters with externally supplied phosphate, suggests that the dialkyl substituted thio- and dithiophosphates can be incorporated or biosynthesized by the plant into cholinesterase-inhibitory esters.

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Department of Zoology and Entomology
Iowa State University
Ames, Iowa

