system might be suitable for this separation. The results of Sparacino and Hines (1976) support this suggestion in their Figure 1 and Table II where with a silica column and heptane carrier these compounds are well separated.

With "real" samples two complications may appear. Although sample preparation schemes are intended to separate the residue from plant material, the separation is usually incomplete. In addition, the solvent the residue is in may be different from the chromatograph carrier. As an illustration, consider the determination of mesurol as mesurol sulfone phenol (Bowman and Beroza, 1969). Brussels sprouts were "spiked" with mesurol which was then converted to MSP and extracted, with the extract finally being dissolved in methanol. In carriers of low polarity the MSP was bracketed by methanol and some unidentified plant material. It was not possible to separate MSP from both simultaneously by varying the polarity of the carrier. Apparently, if a solvent other than methanol had been used for the sample, the nonpolar carrier would have provided a potentially useful separation. With polar carriers the MSP separated cleanly from both the methanol and the plant material. For example, in acetonitrile–water (1:2, v/v) the separation shown in Figure 1 was obtained.

In summary, reversed-phase liquid chromatography is a convenient analytical technique and appears to have useful potential for the analysis of residues of carbamates and substituted ureas in foodstuffs.

LITERATURE CITED

Bowman, M. C., Beroza, M., *J. Assoc. Off. Anal. Chem.* **52**, 1054 (1969).
Dorough, H. W., Thorstenson, J. H., *J. Chromatogr. Sci.* **13**, 212 (1975).
Kirkland, J. J., *J. Chromatogr. Sci.* 7, 7 (1969).
Moye, H. A., *J. Chromatogr. Sci.* **13**, 268 (1975).
Sparacino, C. M., Hines, J. W., *J. Chromatogr. Sci.* **14**, 549 (1976).
Thomson, W. T., "Agricultural Chemicals", Thomson Publications, Indianapolis, Ind., 1975.

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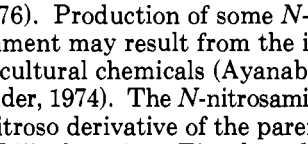
N-Nitrosamine Formation in Soil from the Herbicide Glyphosate

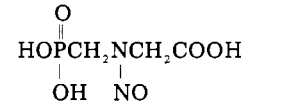
Formation of *N*-nitrosoglyphosate was observed when different soils were treated with sodium nitrite and the herbicide glyphosate at elevated levels. The highest yield was noted in soil low in organic matter and clay contents; however, nitrosation was not affected by soil pH. At low levels of glyphosate (5 ppm) and nitrite nitrogen (2 ppm) the formation of *N*-nitrosoglyphosate in soil was not observed.

Since the discovery that some *N*-nitrosamines are carcinogenic (Barnes et al., 1954), there have been many studies on the formation, action, and analysis of this class of compounds (Mirvish, 1975; Scanlan, 1975; Montesano and Bartsch, 1976). Production of some *N*-nitrosamines in a soil environment may result from the interaction of nitrite with agricultural chemicals (Ayanaba et al., 1973; Tate and Alexander, 1974). The *N*-nitrosamines that form may be the *N*-nitroso derivative of the parent compound (Elespuru and Lijinsky, 1973; Eisenbrand et al., 1975; Uchiyama et al., 1975; Wolfe et al., 1976; Egert and Greim, 1976a) or a carcinogenic *N*-nitrosamine such as *N*-nitrosodimethylamine arising from chemical modification of the pesticide (Ayanaba et al., 1973; Ayanaba and Alexander, 1974; Egert and Greim, 1976b,c). *N*-Nitroso derivatives of some insecticides are both carcinogenic and mutagenic (Elespuru et al., 1974; Siebert and Eisenbrand, 1974; Eisenbrand et al., 1975; Uchiyama et al., 1975; Lijinsky and Taylor, 1976; Seiler, 1977). *N*-Nitrosodimethylamine is stable in soil (Tate and Alexander, 1975, 1976) and can be translocated from soil into vegetable crops (Dean-Raymond and Alexander, 1976).

Tate and Alexander (1974) were unable to detect any *N*-nitrosamines in soil treated with sodium nitrite and the herbicide glyphosate (I) at elevated levels. However, their method would have detected only volatile *N*-nitrosamines. We have recently developed a method of analysis for glyphosate (Young et al., 1977) that involves formation of *N*-nitrosoglyphosate (II). We carried out a study similar



I



II

to that of Tate and Alexander (1974) using our method of detection (Young et al., 1977) and now report the formation of II from I in nitrite-treated soils.

EXPERIMENTAL SECTION

To 10-g portions of air-dried and ground soils (Table I) was added a solution of 1 mg of sodium nitrite (20 ppm nitrite nitrogen) and 10 mg of glyphosate isopropylamine salt (740 ppm acid equivalent) in sufficient distilled water to bring the soils to field capacity. The samples were thoroughly mixed and incubated in the dark at 25 °C. At regular intervals the soils were extracted with distilled water (2 × 50 mL), centrifuged, the combined supernatants concentrated under reduced pressure to ca. 10 mL, and centrifuged. The supernatant was washed with methylene chloride (5 × 10 mL), concentrated to 1.0 mL under reduced pressure, and diluted with 4.0 mL of acetonitrile. This solution was transferred to a column (1 × 5 cm) of Florisil (60–100 mesh, PR grade, moisture content 0.8%) and eluted with 20% water in acetonitrile (50 mL) and

Table I. Selected Properties of Soils

Location	Soil series	Texture	pH[a]	Organic matter, %	Clay, %	Field capacity, % water
Ontario	Fox	Sandy loam	5.8	1.1	5.1	10.1
	Grenville	Sandy loam	6.1	7.5	20.0	19.0
	Brookston	Clay loam	4.3	4.4	35.0	25.4
Quebec	De l'Anse	Clay	3.8	18.0	47.9	55.5
Alberta	Grandin	Loam	4.4	1.1	15.0	18.9

[a] Soil-water 1:1 (w/v).

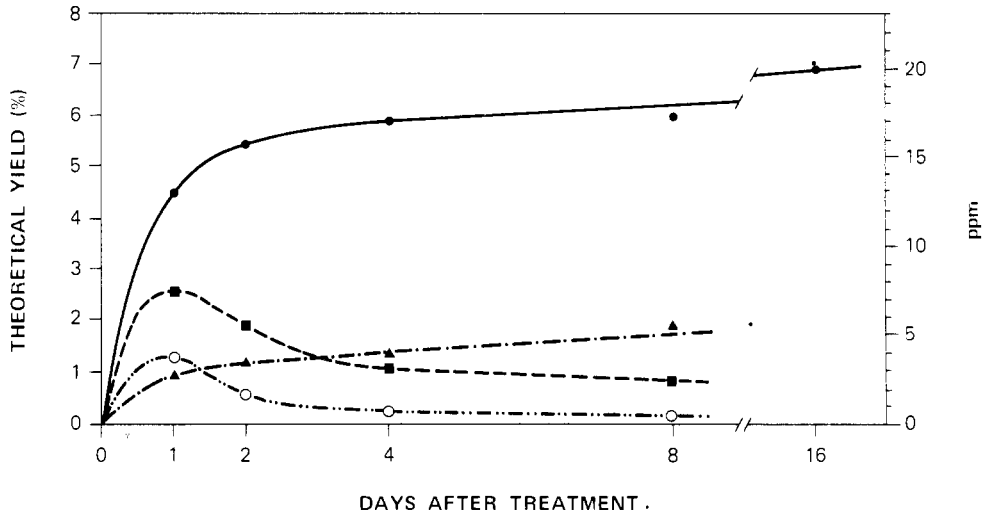


Figure 1. Formation of *N*-nitrosoglyphosate in soils incubated at 25 °C with 20 ppm of nitrite nitrogen as sodium nitrite and 740 ppm glyphosate (free acid equivalent). Values are average of duplicate samples. Soil symbols: (●) Fox, (▲) Grandin, (■), Brookston, and (○) Granville.

60% water in acetonitrile (15 mL). The latter eluate was concentrated to 10.0 mL and aliquots analyzed by thin-layer chromatography (TLC) (Young et al., 1977). As a check, blank soil and soil treated with either 1 mg of sodium nitrite or 10 mg of glyphosate isopropylamine salt were similarly analyzed.

Ultraviolet irradiation of II on the silica gel plate following development in ethanol–benzene–water (4:1:1) yields aminomethylphosphonic acid, which in turn affords a fluorophore when treated with fluorescamine (Young et al., 1977). Quantitation was achieved by fluorescence spectrophotometry (Young, 1976). Identity of II was confirmed by comparison of the TLC properties with those of authentic compound, the photolysis product aminomethylphosphonic acid, and the fluorescamine derivative and by comparison of the UV spectra with that of II.

Under the experimental conditions used the method gave a limit of detection of 5 ng. Recoveries of II from fortified soil samples (10 g) at 1 and 5 ppm levels were nearly quantitative.

Many *N*-nitrosamines are potent carcinogens. Although the carcinogenic properties of *N*-nitrosoglyphosate are unknown at this time, safety precautions such as those outlined in the National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens to prevent skin contact and inhalation must be exercised at all times.

RESULTS AND DISCUSSION

The formation of *N*-nitrosoglyphosate (II) was not observed in soil samples that were incubated without any treatment, or treated with either sodium nitrite or glyphosate salt alone. However, II was generated in most soil samples when both the herbicide and sodium nitrite were present. Formation of II was very rapid near the first day of incubation (Figure 1). The levels of II in Fox and Grandin soils rose slowly with increase in incubation period, whereas a decline was observed after the first day for Brookston and Grenville soils. Analyses of De l'Anse soil made throughout the incubation period did not reveal the presence of II.

Although an optimum pH of 2.8–3.0 was found for the formation of II in solution (Young et al., 1977), pH dependence of the nitrosation of I in soils of pH range 3.8–6.1 (Table I) was not observed. Mills and Alexander (1976) also reported that the amount of dimethylnitrosamine formation in soil was not affected by pH. Greater nitrosation was observed in soils with low organic matter and clay contents. Thus in Fox soil about 17 ppm of II (5.9% theoretical yield) was detected at 8 days. In another experiment, incubation of Fox soil with 74 ppm of I and 2.0 ppm of nitrite nitrogen for 8 days resulted in a similar yield of II. The absence of II in De l'Anse soil by contrast probably reflects the effect of organic matter and clay contents on nitrosation of I.

Although this study demonstrates that *N*-nitrosoglyphosate is generated in some soils from a widely used broad-spectrum herbicide glyphosate, it should be recognized that the high levels of the latter employed under the experimental conditions described are not likely to be encountered in practical agriculture. The average recommended rates of application of the herbicide are about 2 kg/ha, and the herbicide is relatively nonpersistent in soil (Herbicide Handbook, 1974). At these levels of application we cannot envisage the formation of II in soil under normal field conditions. In further experiments we were unable to detect II when Fox soil was incubated over an 8-day period with 5 ppm of I and 2 ppm of nitrite nitrogen.

The formation of *N*-nitroso compounds in soil by interaction of nitrites with certain pesticides has been the

subject of several recent investigations (Ayanaba et al., 1973; Tate and Alexander, 1974). Nitrite may be formed in soil via the nitrogen cycle or from ammonium or nitrate fertilizers. In certain soils under fairly high ammonia and pH conditions resulting from heavy fertilization with urea, concentrations of 90 ppm nitrite nitrogen have been reported to persist for several months (Chapman and Liebig, 1952). Thus in instances when nitrite accumulates temporarily in high concentration, particularly in soils low in organic matter and clay content, and exceptionally high level of the herbicide glyphosate in soil due to accidental spillage or over application may therefore represent a potential starting material for the synthesis of *N*-nitrosoglyphosate. The latter is persistent in soil as it was observed that Fox soil treated at the highest levels contained about 7 ppm of II even after 140 days. It was also observed that a standard solution of II was stable for at least 6 months. At the present time we are not aware of any reported study concerning the carcinogenic properties of compound II, although it is known to be weakly mutagenic (Seiler, 1977). It may be noted that *N*-nitrososarcosine, an *N*-nitrosoamino acid structurally related to *N*-nitrosoglyphosate, has been shown to be carcinogenic (Druckrey et al., 1963).

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LITERATURE CITED

Ayanaba, A., Alexander, M., *J. Environ. Qual.* **3**, 83 (1974).
Ayanaba, A., Verstraete, W., Alexander, M., *Soil Sci. Soc. Am., Proc.* **37**, 565 (1973).
Barnes, J., Magee, P. N., *Br. J. Ind. Med.* 11, 167 (1954).
Chapman, H. D., Liebig, G. F., *Soil Sci. Soc. Am., Proc.* **16**, 276 (1952).
Dean-Raymond, D., Alexander, M., *Nature (London)* **262**, 394 (1976).
Druckrey, H., Preussmann, R., Blum, G., Ivankovic, S., Afkham, J., *Naturwissenschaften* **50**, 100 (1963).
Egert, G., Greim, H., *Naunyn-Schmiedeberg's Arch. Pharmacol.* **293**, 66 (1976a).
Egert, G., Greim, H., *Mutat. Res.* **38**, 136 (1976b).
Egert, G., Greim, H., *Food Cosmet. Toxicol.* **14**, 193 (1976c).
Eisenbrand, G., Ungerer, U., Preussmann, R., *Food Cosmet. Toxicol.* **13**, 365 (1975).
Elespuru, R. K., Lijinsky, W., *Food Cosmet. Toxicol.* **11**, 807 (1973).
Elespuru, R., Lijinsky, W., Setlow, J. K., *Nature (London)* **247**, 386 (1974)
Herbicide Handbook, Weed Science Society of America, Champaign, Ill., 1974, p 201.
Lijinsky, W., Taylor, H. W., *Cancer Lett.* **1**, 275 (1976).
Mills, A L., Alexander, M., *J. Environ. Qual.* **5**, 437 (1976).
Mirvish, S. S., *Toxicol. Appl. Pharm.* **31**, 325 (1975).
Montesano, R., Bartsch, H., *Mutat. Res.* **32**, 179 (1976).
Scanlan, R. A., *Crit. Rev. Food Technol.* **5**, 357 (1975).
Seiler, J. P., *Mutat. Res.* **48**, 225 (1977).
Siebert, D., Eisenbrand, G., *Mutat. Res.* **22**, 121 (1974).
Tate, R. L., Alexander, M., *Soil Sci.* **118**, 317 (1974).
Tate, R. L., Alexander, M., *J. Natl. Cancer Inst.* **54**, 327 (1975).
Tate, R. L., Alexander, M., *J. Environ. Qual.* **5**, 131 (1976).
Uchiyama, M., Takeda, M., Suzuki, T., Yoshikawa, K., *Bull. Environ. Contam. Toxicol.* **14**, 389 (1975).
Wolfe, N. L., Zepp, R. G., Gordon, J. A., Fincher, R. C., *Bull. Environ. Contam. Toxicol.* **15**, 342 (1976).
Young, J. C., *J. Chromatogr.* **124**, 17 (1976).
Young, J. C., Khan, S. U., Marriage, P. B., *J. Agric. Food Chem.* **25**, 918 (1977).

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Electrophoretic Detection of Protein in Highly Pigmented Tobacco

Measurement of protein content of tobacco leaves during and after curing, using the Lowry method, gives falsely high values due to the reaction of the reagent with pigmented components that develop during the curing. The presence of protein in the curing and cured leaf can be shown by the presence of enzymatic activity. Peroxidase can be detected in leaf extracts by disc electrophoresis, but it is impossible to use this technique for protein in general, due to the interference of the highly pigmented components with the protein stain. A preparative electrophoretic technique, using Sephadex as a support medium, has been developed for the removal of these interfering pigments from the protein. The protein fraction is then resolved by standard disc electrophoresis, and individual proteins are visualized by staining with amido black.

Recent interest in tobacco protein as a by-product of certain processing techniques and its possible utilization as a food supplement have stimulated a reexamination of methods of detection and analysis of protein that are applicable to tobacco. The Lowry (1951) method of protein analysis, which involves the use of the Folin-Phenol reagent, can usually be applied without difficulty to green tobacco leaves but not to cured tobacco. Aqueous or buffer extracts of cured tobacco contain small amounts of soluble protein. Such extracts also contain appreciable amounts of high molecular weight, brown pigmented complexes composed, at least in part, of polyphenolic, protein, and carbohydrate moieties (Bailey and Schepartz, 1974). These pigments react with the Folin reagent to give falsely high protein values (Bailey et al., 1970). Protein content is known to decrease during curing (Frankenburg, 1946; Vickery and Meiss, 1953). We have observed this decrease during the first half of the curing period, followed by an