The crude shoot and root extracts were then each combined into a single solution, one for each time period (1 hr to 7 days and 8 to 14 days); the pigments were removed by the liquid–liquid partition method described by Peterson (1971); these purified extracts were evaporated to just dryness on a water bath (45–50°) under nitrogen. The residues were redissolved in a mixture of acetone and methanol (1:1). The purified solutions and 4-aminopyridine-^{14}C standards were each applied to cellulose and alumina tlc plates and processed as described for the corn samples. After development of autoradiograms of those plates containing the purified extracts, the areas on the developed plates containing ^{14}C were removed by scraping for counting by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Developed autoradiograms of both the cellulose and alumina tlc plates containing purified corn extracts revealed only single spots for the shoot and root applications, with R_f values the same as those of the 4-aminopyridine-^{14}C standards and spiked samples. Analysis of the acetone extracts and tissue residues showed that the extraction procedure removed 83.5–95.0% of the ^{14}C activity.

Autoradiograms prepared from crude sorghum extracts showed considerable tailing but suggested that 4-aminopyridine-^{14}C was present in both shoots and roots. Analysis of the crude acetone extracts and tissue residues revealed some difference with time in the amount of acetone-extractable ^{14}C present. Extraction of root tissues removed about the same amount of radioactivity from the earlier as from the later samples (87.5% for the 1-hr, 1-day, and 7-day composite *vs.* 90% for the 8- and 14-day composite) but removed more ^{14}C from the earlier sample of shoot tissues (99.5% for the 1-hr, 1-day, and 7-day composite *vs.* 80.7% for the 8- and 14-day composite). This suggests that some of the radiolabeled parent compound, or derived radioactivity of a polar character, was bound or incorporated in the tissues of those plants maintained in the nutrient solutions for the longer periods.

An autoradiogram prepared from the purified sorghum extracts (Figure 1) showed degradation of the translocated 4-aminopyridine within 2 weeks, with three similar major metabolites in both the shoot and root tissues (Figure 1, Table I); about 90–93% of the extractable radioactivity was suggested to be present as the parent chemical, or possibly as 4-aminopyridine resulting from the hydrolysis (during the cleanup procedure) of sugar and/or amino acid conjugates.

These preliminary data suggest that 4-aminopyridine absorbed and translocated by corn and sorghum plants 1–2 weeks following treatment is not degraded in appreciable quantities to acetone-extractable, nonconjugated products potentially more toxic than the parent chemical. However, this study indicates that additional work is warranted regarding possible conjugate formation of 4-aminopyridine with plant sugars and/or amino acids.

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Pathway of Nitro Reduction of Parathion by Spinach Homogenate

Takashi Suzuki* and Mitsuru Uchiyama

The nitro reduction of parathion to aminoparathion by spinach homogenate under anaerobic conditions proceeds *via* hydroxylaminoparathion as intermediate metabolite. Hydroxylaminoparathion was stable in the acidified reaction medium and therefore detectable by thin-layer chromatography. However, in the alkaline medium, it underwent autoxidation to nitrosoparathion, which was detectable by both thin-layer chromatography and gas chromatography. Nitrosoparathion itself was not demonstrated as an intermediate metabolite, but the one added to the subject reaction system was easily reduced to aminoparathion *via* hydroxylaminoparathion. It was also converted rapidly to hydroxylaminoparathion by NADPH. These results suggest the possibility that nitrosoparathion may act as one of two consecutive intermediate metabolites.

Our previous study demonstrated nitro reduction of parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) or reduction of the nitro group of the organophosphorus insecticide to the amino group, in the presence of spinach homogenate containing NADP, G-6-P, and FAD under anaerobic conditions, whereby the gas chromatogram revealed a peak suggestive of an intermediate metabolite (Suzuki and Uchiyama, 1974). The investigation reported herein was undertaken to isolate and identify the intermediate metabolite in the nitro reduction of parathion. The report also describes the findings noted for the pathway of nitro reduction of parathion by spinach homogenate.

Pharmaceutical Institute, Tohoku University, Sendai, Japan.

EXPERIMENTAL SECTION

Preparation of Spinach Homogenate and Reaction

Medium. Twenty-five grams of spinach leaves was ground together with quartz sand, approximately one-fourth the amount of the sample, and a small quantity of 1.15% KCl aqueous solution, in a mortar, and then the mixture was centrifuged at 2000 rpm for 2 min. The resulting supernatant was transferred to a 100-ml volumetric flask, an adequate amount of 1.15% KCl aqueous solution was added to the sediment, the mixture was agitated and then centrifuged in the same manner as above, and the supernatant fluid phase was collected and combined with the first supernatant in the volumetric flask. Then 1.15% KCl aqueous solution was added to volume. This was designated spinach homogenate and used as the enzyme source. Unless otherwise specified, the reaction medium and conditions employed were as follows: spinach homogenate, 1 ml; 1 *M* Tris-HCl buffer (pH 8.3), 2 ml; NADP, 1.0 μmol; G-6-P, 2.5 μmol; FAD, 0.5 μmol; parathion, 0.2 μmol (58.2 μg); and deionized water to make 5 ml.

NH_2OH-HCl was added, when required, in a final concentration of 5×10^{-3} *M* to the reaction medium. The sample was introduced into a Thunberg tube and incubated at 37° in a stream of nitrogen gas. Upon conclusion of reaction, 2 ml of reaction mixture was transferred to a test tube and 2 ml of *n*-hexane was added to it for extraction of reaction products, followed by gas chromatographic analysis.

Gas chromatography (gc) was performed under the following conditions: apparatus, Shimadzu gas chromatograph (Model GC-1C); detector, flame thermionic detector (temp at 230°); column, 5% SE-30 on Shimalite W (60–80 mesh), 1.5 m in length, 4 mm in calibre, and temperature at 190°; carrier gas, N_2 at a flow rate of 50 ml/min; hydrogen gas flow rate, 30 ml/min; air, pressure at 0.8 kg/cm²; injection port temperature, 250°. Under these conditions, retention times (minutes) of parathion, aminoparathion, and nitrosoparathion were 3.6, 2.9, and 1.9, respectively.

Thin-Layer Chromatography (Tlc). Silica gel thin layers (0.25 mm in thickness) supported on glass plates (20 × 20 cm, Merck Inc.) were used. Two solvent systems were used for the separation of reaction products: *n*-hexane–acetone–methanol (18:1:1, solvent system I) and *n*-hexane–acetone (7:3, solvent system II). When solvent system I was used, the thin layers were allowed to develop twice, 15 cm each. Organothiophosphates were visualized on the plates by spraying a $PdCl_2$ solution (0.5 g of $PdCl_2$ and 2 ml of concentrated HCl in 98 ml of water). Nitrosoparathion and hydroxylaminoparathion were also visualized by spraying a 0.5% (w/v) sodium pentacyanoammine ferroate in methanol–water (3:1).

Preparation of Aminoparathion. To a given amount of parathion, 1 ml of $TiCl_3$ solution in 1 *N* HCl was added, and the resulting mixture in a test tube was warmed in a water bath at 50° for 30 min, and then made alkaline to litmus, followed by extraction with *n*-hexane.

Reduction of Parathion with Zinc Dust–NH_4Cl System. With a view to preparation of hydroxylaminoparathion, approximately 0.1 g of zinc dust and 5 ml of 0.5% NH_4Cl in aqueous solution were added to a glass-stoppered test tube containing 100 μg of parathion. The mixture was shaken vigorously with subsequent extraction with 5 ml of ethyl ether.

Preparation of Nitrosoparathion Standard Solution. Parathion was reduced with the Zn–NH_4Cl system in the same manner as above, and then the ether extract was shaken with Tollen's reagent. Nitrosoparathion in the ether phase was isolated by tlc and its ether solution was prepared. A concentration of nitrosoparathion in the solution was determined by gc analysis of aminoparathion formed from a given volume of the solution by the treatment with 0.5% $TiCl_3$ in 1 *N* HCl solution.

Oxidation of Aminoparathion with Caro's Acid. Caro's acid, or peroxysulfuric acid, oxidizes primary amino compounds to nitroso or nitro compounds. Caro's acid was prepared by decomposition of $K_2S_2O_4$ with concentrated H_2SO_4 and neutralized. One-half milliliter of neutral Caro's acid and 0.2 ml of dioxane were added to a glass-stoppered test tube containing 100 μg of aminoparathion, and the contents were mixed thoroughly by vigorous shaking for 5 min and then subjected to extraction with ethyl ether.

RESULTS AND DISCUSSION

Tlc of Reaction Products. The previous study on nitro reduction of parathion by spinach homogenate had shown gas chromatographic evidence of increased accumulation of a substance, possibly an intermediate metabolite, in the reaction mixture with NH_2OH-HCl (10^{-3} *M*) added to it, as compared with samples not containing NH_2OH-HCl (Suzuki and Uchiyama, 1974). By taking advantage of this property of NH_2OH-HCl to inhibit intermediate metabolism of parathion, tlc analysis was carried out on a reaction system with NH_2OH-HCl added to it. Solvent system I as developing solvent and a $PdCl_2$ solution as spraying reagent were used. A thin-layer chromatogram showed three spots for unreacted parathion (R_f 0.49), aminoparathion (R_f 0.10), and a substance with an R_f value of 0.57, as reaction products from the subject system. The portion showing an R_f value of 0.57 was dissolved in acetone and analyzed by gc which revealed a peak retention time (R_t = 1.9) coinciding with that of the unidentified substance reported previously. As the spot on tlc developed a yellowish brown color by spraying with a $PdCl_2$ solution, the substance was indicated to be a P=S compound. Furthermore, the fact that a yellow color was developed following a spray with 0.5% *p*-dimethylaminobenzaldehyde in ethanol–acetic acid (1:1) after spraying with 0.5% $TiCl_3$ in 1 *N* HCl solution indicated the substance to be an oxidized form of aromatic amino compound, probably an intermediate metabolite occurring in the process of transformation from parathion to aminoparathion.

Chemically, the nitro group is reduced to the amino group through three consecutive steps as follows:

$$\underset{\text{nitro}}{\text{R-NO}_2} \xrightarrow[-H_2O]{+2H} \underset{\text{nitroso}}{\text{R-NO}} \xrightarrow{+2H} \underset{\text{hydroxylamino}}{\text{R-NHOH}} \xrightarrow[-H_2O]{+2H} \underset{\text{amino}}{\text{R-NH}_2}$$

Since it is probable that the enzymic nitro reduction *in vivo* or *in vitro* proceeds by a similar pathway wherein nitroso and hydroxylamino compounds occur as intermediate metabolites, further experiments were performed to identify the unknown compound.

Identification of the Unknown Substance by Tlc Analysis and Color Reactions. Tlc of reduction products from parathion with the Zn–NH_4Cl system showed two major spots with R_f values corresponding to parathion (R_f 0.49) and situated nearly at the point of origin (R_f 0.06). Three minor spots with R_f values of 0.10, 0.40, and 0.57 were also detected. Tlc of oxidation products from aminoparathion with Caro's acid showed three spots with R_f values corresponding to parathion, aminoparathion (R_f 0.10), and the unknown substance (R_f 0.57).

Table I shows the results of color reactions for nitroso and hydroxylamino radicals as functional groups and of gc analysis of these spots. The unknown substance with an R_f value of 0.57 proved to give positive color reactions for the nitroso group (reactions A, B, and C), and the spot with an R_f value of 0.06 was positive for the hydroxylamino group (reactions A, B, D, and E). Consequently, they were identified as nitrosoparathion and hydroxylaminoparathion, respectively. Gc analysis of the spot with an R_f value of 0.06 (hydroxylaminoparathion) disclosed peaks for nitrosoparathion and parathion alone and no other peak at all. From this result, it was suggested that hydroxylaminoparathion was presumably undemonstrable by

Table I. Qualitative Test of Reaction Products by Color Reactions and Gas-Chromatographic Analyses[a]

R_f value of spots on tlc	Color reaction						R_t value on gc anal.
	A	B	C-1	C-2	D	E	
0.06	Blueish violet	Pale violet	−	±	Red	++	1.9, 3.6
0.10	Pale green	Yellow	−	−	Pale yellow	−	2.9
0.49	Yellow	Yellow	−	−	Pale yellow	−	3.6
0.57	Violet	Reddish violet	+	++	Pale yellow	−	1.9

[a] Color reactions: A, spot test by sodium pentacyanoammine ferroate; B, test by potassium ferricyanate; C, Liebermann reaction; C-1, phenol plus concentrated H_2SO_4 (red); C-2, addition of 4 *N* NaOH to C-1 (blue); D, test by benzoyl chloride and $FeCl_3$; E, eyeglass formation (by Tollen's reagent).

Table II. Nitrosoparathion Formation from Hydroxylaminoparathion

No.	Treatment	Peak ht (mm) of nitrosoparathion on gc
1		84
2	Shaking with 1 *N* HCl	95
3	Shaking with 1 *N* NaOH	135
4	Shaking with Tollen's reagent (extraction from acidic medium)	175
5	Shaking with Tollen's reagent (extraction from alkali medium)	176

gc under the conditions employed and that it underwent oxidation in part to nitroso and nitro compounds in the stage of extraction out of tlc. In view of these results, the following were investigated.

Effect of pH on the Formation of Nitrosoparathion. A reaction mixture resulting from reduction of parathion with the Zn–NH_4Cl system was extracted with ethyl ether, and the extract treated as described in Table II. Nitrosoparathion in the ether phase was analyzed by gc. As may be noted from Table II, there was little increase of nitrosoparathion in the acid-treated ether phase (no. 2), but marked increase was observed in the alkaline-treated ether phase (no. 3), as compared with that in the untreated ether phase (no. 1). These findings suggest that hydroxylaminoparathion forms a relatively stable salt in acid solution, whereas in alkaline solution it remains free and therefore unstable to be readily oxidized to nitrosoparathion.

Aromatic hydroxylamino compounds are known to be oxidized to nitroso compounds in the presence of Tollen's reagent, thereby giving a positive eyeglass reaction. In the present experimentation, the samples, when treated with Tollen's reagent (no. 4 and no. 5), were noted to yield actively nitrosoparathion and there was a thin eyeglass formation or dark-brown precipitate near the interface between the ether and water layers. No significant difference was observed between no. 4 and no. 5 in this respect since the pH was altered only after hydroxylaminoparathion had been oxidized to nitrosoparathion.

Time Course of Change from Hydroxylaminoparathion into Nitrosoparathion. Hydroxylaminoparathion was isolated from a reaction mixture of parathion-Zn/NH_4Cl system by tlc and its acidic solution was prepared. One milliliter of the solution was added into 2 ml of 1 *M* Tris-HCl buffer (pH 8.3), used in the foregoing experiments with spinach homogenate, and incubated at 37°

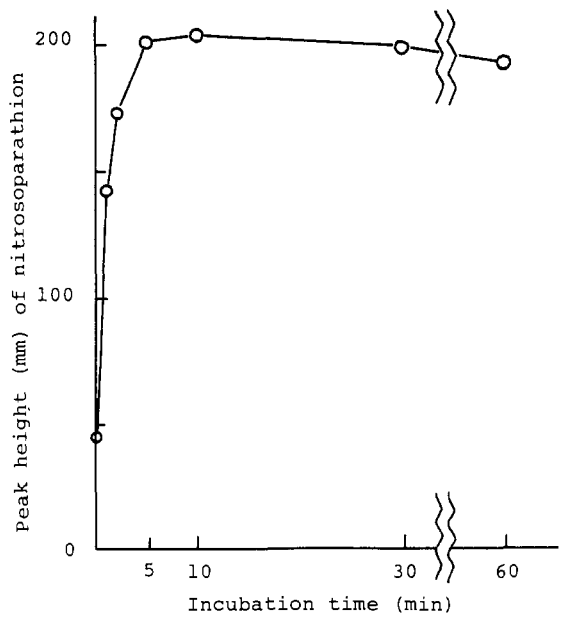

Figure 1. Time course of autoxidation of hydroxylaminoparathion in 1 *M* Tris-HCl buffer (pH 8.3).

under aerobic conditions. The time course of change from hydroxylaminoparathion to nitrosoparathion was followed. Upon conclusion of reaction, the reaction mixture was acidified by addition of 1 ml of 1 *N* HCl and then 2 ml of *n*-hexane was added to it for extraction of nitrosoparathion, followed by gc analysis. As can be seen from Figure 1, a remarkably rapid autoxidation of hydroxylaminoparathion occurred. There was no evidence of parathion formation even at 1 hr after initiation of the reaction.

Intermediate Metabolite in the Reaction Medium with Spinach Homogenate. The results previously obtained indicated that the peak at R_t = 1.9 on the gas chromatogram was nothing but nitrosoparathion and that hydroxylaminoparathion was undemonstrable at least under the gc condition employed in these experiments; however, it may be shown to be present indirectly by its oxidation to nitrosoparathion. It was yet to be determined, nevertheless, whether the intermediate metabolite which occurred in the process of nitro reduction of parathion with spinach homogenate might be a nitroso compound or, otherwise, a hydroxylamino compound or both, unless the possibility that nitrosoparathion detectable by gc analysis is derived from hydroxylaminoparathion during extraction was ruled out.

On this point the following experiments were carried out. Parathion was incubated with spinach homogenate as

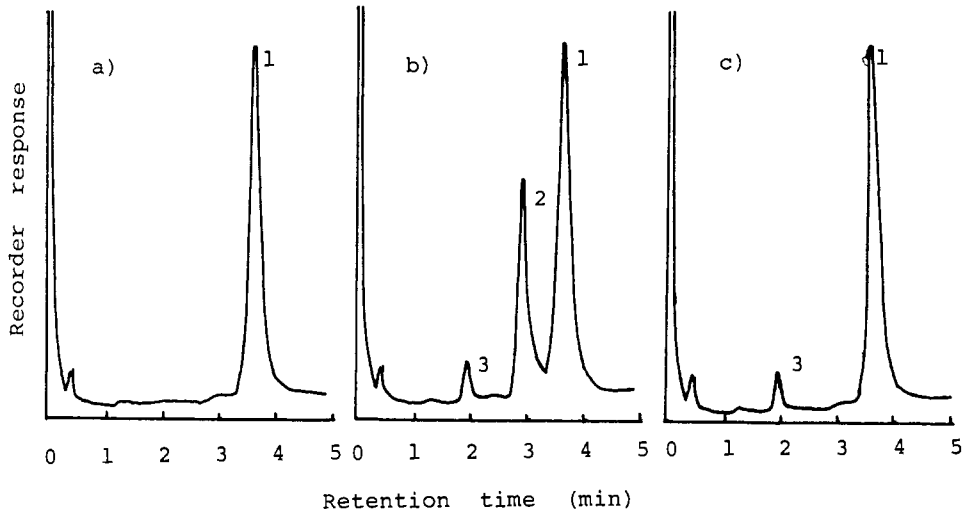

Figure 2. Gas chromatogram of nitro-reduction products of parathion by spinach homogenate: peak 1, parathion; peak 2, aminoparathion; peak 3, nitrosoparathion.

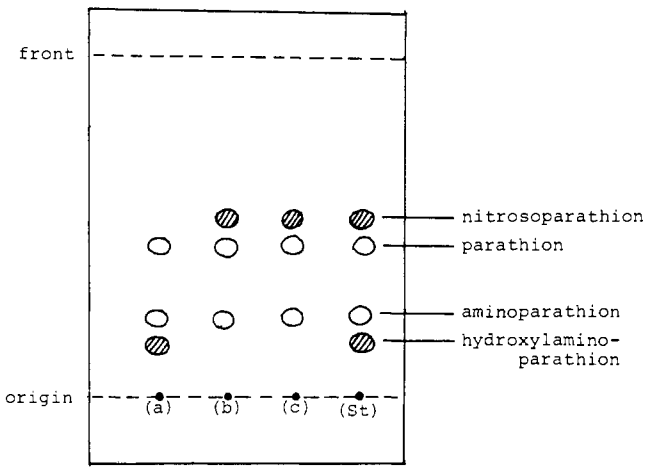

Figure 3. Thin-layer chromatogram of reaction products: solid circles indicate the spots detected by spraying of a $PdCl_2$ solution; hatched circles indicate the spots detected by spraying of a sodium pentacyanoammine ferroate solution as well as a $PdCl_2$ solution; St, standard chromatogram of authentic compounds.

mentioned in the Experimental Section and the reaction products were extracted as follows for gc analysis: (a) to 2 ml of reaction medium, 1 ml of 1 *N* HCl and 2 ml of *n*-hexane were added and shaken; (b) to 2 ml of reaction medium, 2 ml of *n*-hexane was added and shaken; (c) after the analysis of b, 1 ml of 1 *N* HCl was added and shaken. For tlc analysis, 500 μg of parathion was added to the incubation medium and the products were extracted by 1 ml of ethyl ether instead of 2 ml of *n*-hexane.

Figure 2 shows the gas chromatogram obtained in this experiment. Nitrosoparathion was undemonstrable in the extract from acidic medium a while it was found to be present in the extract b. The peak characteristic of nitrosoparathion noted in b did not disappear (c) even after acidification of b. As evident from Table II, it was the rate of autoxidation of hydroxylamino compound to nitroso compound that was affected by the pH of the aqueous phase, and not the rate of extraction of nitroso compound into *n*-hexane. That nitrosoparathion, undemonstrable in a, emerged in b and c indicates the possibility of occurrence as hydroxylaminoparathion of the intermediate metabolite in the subject reaction system and also that, in alkaline medium, it may undergo autoxidation to nitrosoparathion in the extraction stage.

In order to further investigate this possibility, the reaction products extracted with ethyl ether mentioned above were analyzed by tlc. The thin layer was allowed to develop with solvent system II and sprayed with a $PdCl_2$ solution. As can be seen from Figure 3, the chromatogram of extract a showed three spots with R_f values of 0.44, 0.23, and 0.16. These values correspond to parathion, aminoparathion, and hydroxylaminoparathion, respectively. On the other hand, when the extraction procedures of b and c were used, hydroxylaminoparathion with an R_f value of 0.16 was not detected, but nitrosoparathion with an R_f value of 0.49 was detected. When 0.5% (w/v) sodium pentacyanoammine ferroate in methanol-water (3:1) was used as a color developing reagent, the spots other than hydroxylaminoparathion were not visualized as a violet spot on the chromatogram of extract a, while the spots other than nitrosoparathion were not visualized as a violet spot on the chromatograms of extracts b and c. Similar results were obtained by use of the solvent system I.

From these results, it was proved that the intermediate metabolite in the subject reaction system occurred as hydroxylaminoparathion, which underwent autoxidation to nitrosoparathion during the extraction from alkaline medium.

Reduction of Nitrosoparathion of Spinach Homogenate. Further experiments were performed to investigate the formation of hydroxylaminoparathion and aminoparathion from nitrosoparathion. Instead of parathion, 30 μg of nitrosoparathion was added into the reaction medium. Figure 4 illustrates the time course of the reaction. Nitrosoparathion extractable from acidified medium into *n*-hexane (A) was expressed as unreduced nitrosoparathion. Nitrosoparathion extractable from the medium treated with Tollen's reagent into *n*-hexane (B) represents the sum of hydroxylaminoparathion and unreduced nitrosoparathion. Therefore, the difference between A and B was expressed as nitrosoparathion reduced to hydroxylaminoparathion. As can be seen from Figure 4, more than 90% of nitrosoparathion was reduced to hydroxylaminoparathion within 1 min. Then, subsequent decrease of hydroxylaminoparathion was observed in proportion to the increase of aminoparathion.

Nonenzymatic Reduction of Nitrosoparathion to Hydroxylaminoparathion by NADPH. Figure 5 shows the nonenzymatic reduction of nitrosoparathion by NADPH. When 28 μg (0.1 μmol) of nitrosoparathion was incubated with 1.0 μmol of NADPH in 1 *M* Tris-HCl buffer (pH 8.3), the ratio of nitrosoparathion recovered from acidified medium was less than 10%, while that recovered from the medium treated with Tollen's reagent was more than 80%,

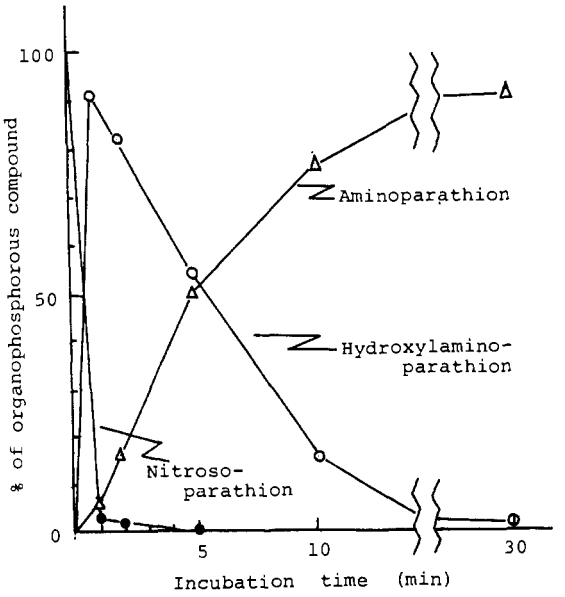

Figure 4. Time course of reduction of nitrosoparathion by spinach homogenate.

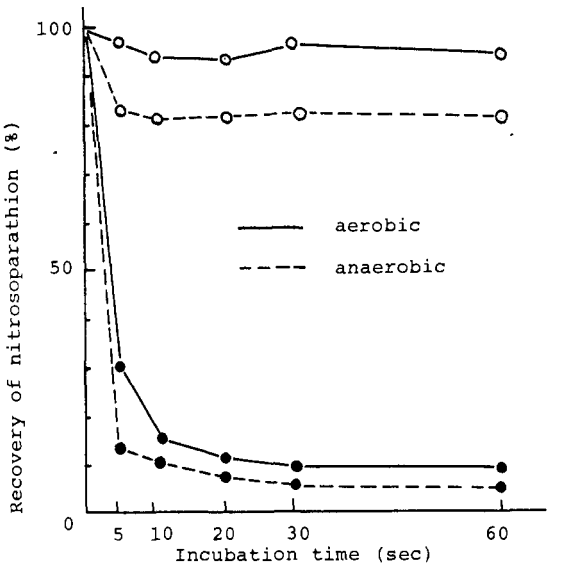

Figure 5. Nonenzymatic reduction of nitrosoparathion to hydroxylaminoparathion by NADPH: (●) extraction from the acidified medium; (○) extraction from the medium treated with Tollen's reagent.

under either aerobic or anaerobic conditions. This result suggests the possibility that, if nitrosoparathion is formed from parathion with spinach homogenate, it may be readily and rapidly reduced nonenzymatically to hydroxylaminoparathion by NADPH and/or other physiological hydrogen donors.

Comparison of the Rate of Nitrosoparathion Reduction with That of Parathion Reduction. The rate of aminoparathion formation from nitrosoparathion was compared with that from parathion under the same conditions as described in the Experimental Section. As substrate, 58.2 μg of parathion or 27.5 μg of nitrosoparathion was added into the reaction medium. Figure 6 illustrates the time course of both reactions. Evidence has been obtained that nitrosoparathion is reduced to aminoparathion at a rate far greater than that of parathion reduction to aminoparathion. Although precise chemokinetic comparison is impracticable as the amounts of substrate in the reaction media differed, it follows from the results shown in Figures 4 and 6 that parathion reduction to aminoparathion occurs at a rate of 0.9 nmol/min, that nitrosoparathion reduction to hydroxylaminoparathion occurs at a rate of 100 nmol/min, and that hydroxylaminoparathion reduction to aminoparathion occurs at a rate of 9 nmol/min.

The studies on nitro reducing systems in mammalian tissues and bacterial cells that have been published to date suggest hypotheses which may be divided grossly into three: one, that the nitro reduction is effected by way of the above-described two consecutive intermediate metabolites (Parke, 1961; Neuberg and Werde, 1914); two, that the nitroso compound functions as a direct precursor of amino compound (Yamashina *et al.*, 1954; Yamashina, 1954); and three, that the process involves formation of the hydroxylamino compound as the only intermediate metabolite (Kato *et al.*, 1969; Yoshida *et al.*, 1968; Yoshida and Kumaoka, 1969).

The present investigation suggests that the nitro reduction of parathion to aminoparathion by spinach homogenate under anerobic conditions proceeds *via* hydroxylaminoparathion as the intermediate metabolite. Accordingly, the reaction in spinach homogenate may belong to the third category in the three metabolic pathways mentioned above. However, as shown in Figures 4, 5, and 6, we have obtained the results that nitrosoparathion is easily reduced to aminoparathion *via* hydroxylaminoparathion by spinach homogenate and also rapidly converted to hydroxylaminoparathion by nonenzymatic reaction. These results suggest that, even if nitrosoparathion occurs as the first intermediate metabolite, it may be further reduced at a fast enough rate to be nondetectable by tlc and gc. On the basis of this point, it may be possible that nitrosoparathion acts as one of the two consecutive intermediate metabolites.

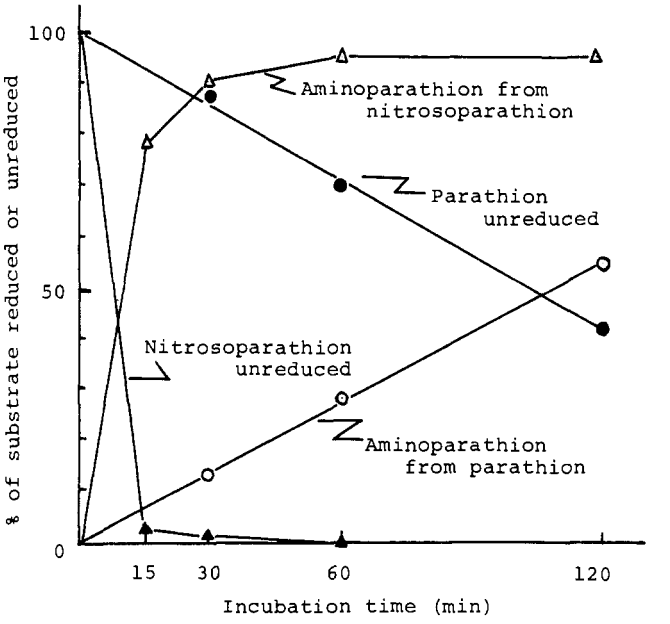

Figure 6. Time course of aminoparathion formation from parathion and nitrosoparathion by spinach homogenate.

In the previous paper, we have described that the properties of nitro-reducing systems in spinach homogenate were very similar to those in mammalian liver microsomes, *e.g.*, requirement of NADPH for a preferential hydrogen donor, stimulation by flavines, and emergence of activity only under anaerobic conditions (Suzuki and Uchiyama, 1974). Gillette *et al.* (1968) speculated that the reason why reduction of nitro compounds by liver enzyme systems did not occur under aerobic conditions might be due in part to autoxidation of hydroxylamino compounds to nitroso compounds. We could not demonstrate the autoxidation of hydroxylaminoparathion to nitrosoparathion

in the reaction medium incubated under aerobic conditions. Nitrosoparathion was rather reduced to aminoparathion *via* hydroxylaminoparathion in the presence of spinach homogenate even under aerobic conditions. This finding indicates that the requirement for anaerobic conditions is not for the entire sequence of reactions, but for the initial stage of reactions, *i.e.*, the reduction of parathion to nitrosoparathion or hydroxylaminoparathion.

Since it is unlikely that anaerobic conditions prevail in live higher plants or mammals, with the exception of ruminants, the biological significance of reductive metabolisms described above may be minor. On the other hand, it has been clarified that the microsomal electron-transport system contributes to the microsomal nitro-reducing system (Gillette *et al.*, 1968; Sazame and Gillette, 1969; Yoshida and Kumaoka, 1969). Therefore, nitro-reducing activity is one of the indexes of microsomal drug-metabolizing enzymes. For a routine assay of nitro-reducing activity, the method of Fouts and Brodie (1957) has been so far used by which the amino analog alone is measured colorimetrically as a reaction product. Although hydroxylamino compounds are usually determined by the complex formation with sodium pentacyanoammine ferroate, nitroso compounds also form a similar complex with it. Therefore, hydroxylamino compounds are indistinguishable from nitroso compounds by such methods. In contrast with them, amino, hydroxylamino, and nitroso analogs of parathion were separately determined by the method described here. The present analytical method will be useful as a sensitive and selective assay method of nitro-reducing systems in various organisms. Gas–liquid chromatography of oxidative metabolites of parathion was also available as a sensitive and precise index of induction of liver microsomal drug-metabolizing enzymes (Davis *et al.*, 1973).

Experiments are now in progress to study the relationship between the nitro-reducing and the photosynthetic electron-transport systems in spinach chloroplasts.

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Metabolism of Siduron in Kentucky Bluegrass (*Poa pratensis* L.)

Lowell S. Jordan,* Ahmed A. Zurqiyah, Antonio R. De Mur,[1] and Wilhelmus A. Clerx

Kentucky bluegrass "Merion" (*Poa pratensis* L.) growing in nutrient solution was treated with 1-(2-methylcyclohexyl)-3-phenylurea (siduron) and 1-(2-methylcyclohexyl)-3-phenyl-2-^{14}C-urea. Siduron and its metabolites were extracted, partitioned between chloroform and water, purified by column and thin-layer chromatography (tlc), separated by tlc, and characterized by infrared and mass spectrometry. Conjugates were hydrolyzed by enzymes. Four metabolites were isolated by tlc, three of which were characterized as 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea, 1-(2-hydroxymethylcyclohexyl)-3-phenylurea, and 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. The fourth metabolite was not identified.

The herbicide 1-(2-methylcyclohexyl)-3-phenylurea (siduron) may be used for preemergence control of certain grass weeds in newly seeded or established plantings of some cool-season turf grasses such as Kentucky bluegrass. De Mur (1971) reported on the absorption and translocation of siduron and inhibition of the Hill reaction in resistant Kentucky bluegrass "Merion" (*Poa pratensis* L.) and susceptible bermuda grass "Santa Ana" (*Cynodon dactylon* L.). He proposed that the differential rate of metabolism is the basis of selectivity of siduron. Splittstosser and Hopen (1968) reported that siduron was not metabolized in barley (*Hordeum vulgare* L.). The metabolism of siduron has been studied in animals and soils by Belasco and Reiser (1969) and Belasco and Langsdorf (1969). They also found and identified three metabolites, which were conjugates of hydroxylated siduron. The objectives of the research reported here were to study the metabolism of siduron in Merion Kentucky bluegrass and to identify the major metabolites.

Department of Plant Sciences, University of California, Riverside, California 92502.
[1] Present address: Standard Fruit & Steamship Co., Honduras Division, New Orleans, La.

MATERIALS AND METHODS

Chemicals. The herbicide 1-(2-methylcyclohexyl)-3-phenyl-2-^{14}C-urea and potential metabolite reference compounds were supplied by E. I. DuPont, Wilmington, Del. The purity of the ^{14}C-labeled compound was greater than 99%. This was determined by spotting 2 μl of a 20 μCi/ml solution of ^{14}C-labeled herbicide on silica gel G thin-layer plates. The plates were then developed in two dimensions with chloroform–ethyl acetate (8:9, v/v) and chloroform–methanol (9:1, v/v). A Berthold thin-layer radioscanner was used to determine the position of radioactive spots. The labeled siduron compound migrated as a single radioactive spot. The enzymes α-glucosidase, β-glucosidase, and hesperidinase were purchased from Sigma Chemical Co.

Treatment of Plants. Merion Kentucky bluegrass seeds were germinated in sand culture in a greenhouse. After germination, seedlings were watered daily with half-strength Hoagland solution. When the plants were approximately 5 weeks old, they were transferred in groups