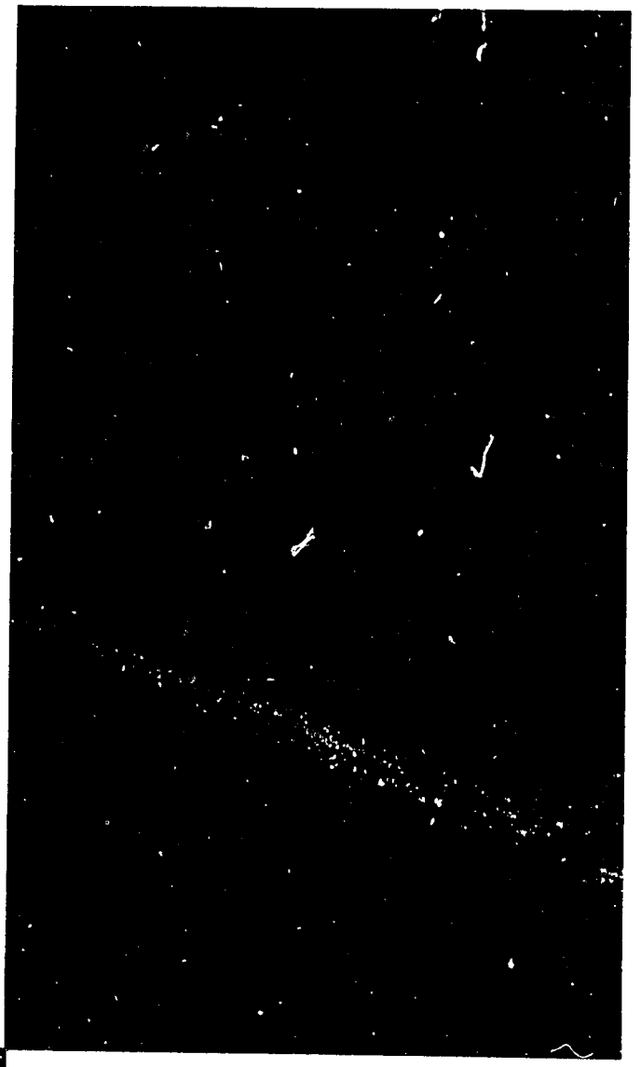

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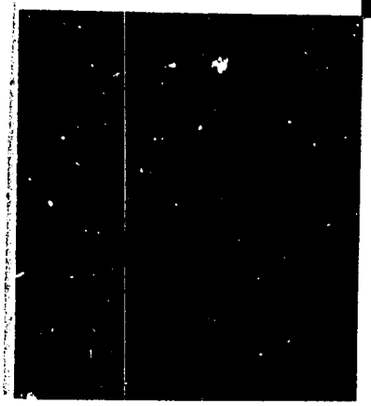

The Agricultural Experiment Station
University of Nebraska – Lincoln College of Agriculture
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## SUMMARY

Herbicides were shown to interfere in the normal process of enzymatic nitrite reduction by the plant in photosynthetic and non-photosynthetic plant tissues with little effect on nitrate reduction. This preferential inhibition caused nitrite to accumulate. The occurrence of free nitrite within the plant can help to explain the toxicity symptoms, nastic growth reactions, abnormal metabolism and rapid death due to herbicide action.

This blockage of nitrite reduction was shown with all photosynthetic inhibitor herbicides tested and with numerous other herbicides. The effect was demonstrated using an *in vivo* assay, intact green plants and germinating seedlings.

A basic *in vivo* method was presented which can provide rapid screening tests for photosynthetic inhibitor herbicides, a measure of herbicide specificity and surfactant specificity.

# A Mode of Action of Herbicides: Inhibition of the Normal Process of Nitrite Reduction

**Lowell Klepper**[1]

## INTRODUCTION

Although herbicides have been widely used for the past two decades and are an integral part of agricultural production, the mechanisms by which herbicides kill weeds have not been fully known. A large group of herbicides in use today such as the amino triazines, substituted ureas and uracils act as photosynthetic inhibitors and can readily be identified by their inhibition of the Hill reaction (52). The rapid development of such symptoms as chlorophyll degradation, leaf necrosis and the final death of the plant which are observed after application of these herbicides points to a mechanism of killing far different from starvation by photosynthetic inhibition.

The mechanisms of rapid killing action of many other herbicides such as the derivatives of the phenoxys, carbamates, anilides, and benzoic acids have eluded investigators for years, though much is known concerning the effects which these herbicides exert on certain metabolic systems within the plant (24). For example, "the mechanisms of action of 2,4-D have been studied more than those for any other herbicide. Investigators have shown that respiration, food reserves, and cell division are affected, but the primary mode of action has not been clearly established" (3).

This bulletin presents evidence that a wide variety of herbicides including photosynthetic inhibitors, 2,4-D and a variety of other chemical classes of herbicides, interfere in the normal process of nitrite reduction which can result in nitrite accumulation. Free nitrite within the plant can help to explain the initial symptoms of injury and the rapid death of the plant.

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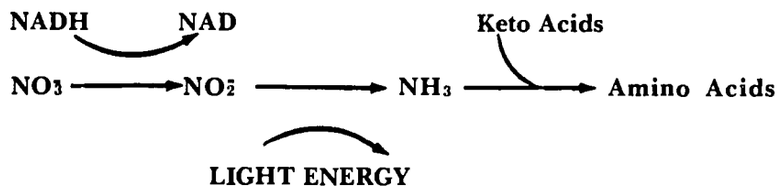
[1]Assistant Professor, Plant Physiology, Department of Agronomy, University of Nebraska – Lincoln. Cooperative investigations of the Nebraska Agricultural Experiment Station and the Agricultural Research Service, U. S. Department of Agriculture, Lincoln, Nebraska, under Contract No. AID/csd-1208 with the Agency for International Development, Department of State.

## LITERATURE REVIEW

### Nitrate Assimilation by Green Plants

Nitrate, taken up from the soil bv the plant root system and translocated throughout the plant, must be reduced to ammonia before it can combine with keto acids to form amino acids and, finally, protein. The normal pathway of nitrate assimilation by green plants is:




The first reaction of nitrate assimilation, the reduction of nitrate to nitrite, is catalyzed by nitrate reductase which utilizes NADH as its electron donor (4, 5, 64, 66). In green leaves, nitrate reductase has been localized outside the chloroplast (62), thus this reductive step does not appear to be directly linked to the photosynthetic reactions. The prime source of NADH used for nitrate reduction is believed to be generated by the glycolytic oxidation of a sugar phosphate (39). In contrast, nitrite reductase, which reduces nitrite to ammonia, has been located within the chloroplast (62) and can accept electrons directly from light-reduced ferredoxin (14, 32, 42, 57).

The enzymes responsible for nitrate and nitrite reduction in the green leaf are located in different parts of the cell, use different electron donors and have different systems for the generation of energy to perform these reactions. Nitrate and nitrite reduction are also known to occur in nonphotosynthetic plant tissues (40, 49, 50, 63), such as plant roots. However, the systems of energy generation for these reactions and their localization within the cell are not clearly understood.

### *In Vivo* Nitrate Reductase Assay

The reduction of nitrate to nitrite is believed to be a rate limiting step in protein synthesis (5, 12, 16, 39) and the level of *in vitro* nitrate reductase activity is highly correlated with grain protein content in wheat (12, 16). Techniques for the measurement of *in vitro* nitrate reductase activity have been available for more than 20 years (19). Advanced techniques for the measurement of nitrate reduction rates *in vivo* or *in situ* are now available (21, 39). An *in vivo* procedure for the measurement of nitrate reduction can offer several advantages over the *in vitro* analyses. These advantages would be: (a) a more rapid and

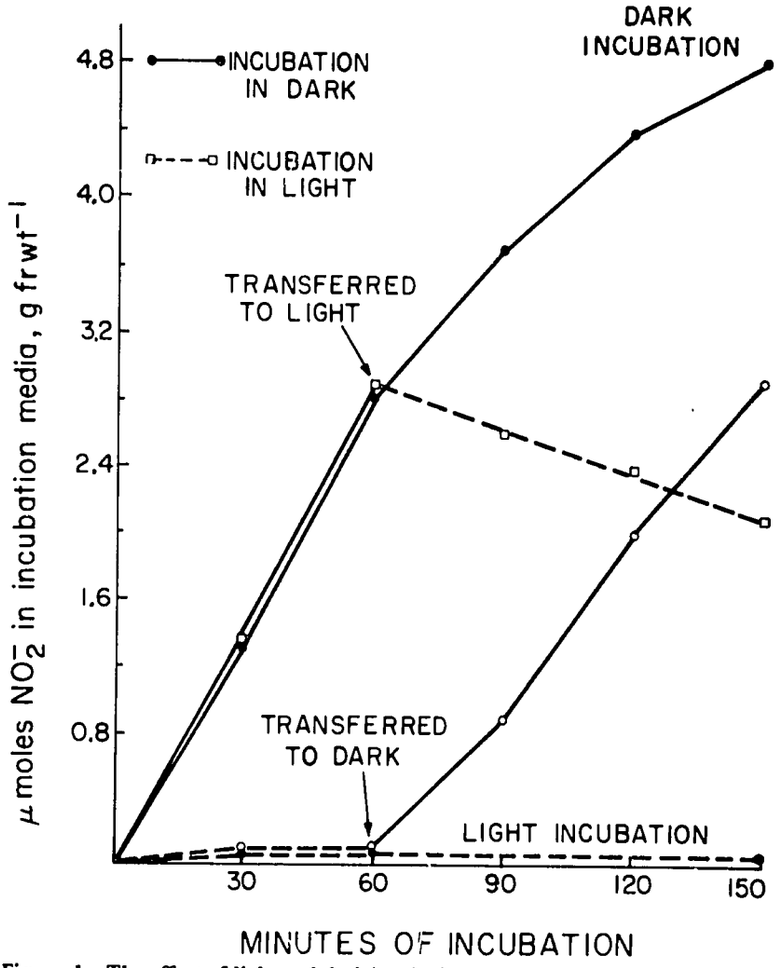



**Figure 1. The effect of light and dark incubation upon *in vivo* nitrate reduction by corn (Oh 43 x B 14) leaf sections. From Klepper *et al.* (39).**

simple analysis, (b) no aqueous extraction of enzymes is required, (c) a better measure of the actual rates of nitrate reduction which are occurring within the plant.

In the procedure for the *in vivo* nitrate reductase assay (39), it was necessary to incubate the green leaf tissue in darkness. Photosynthetic electron flow is blocked by darkness which stops the energy generation necessary for nitrite reduction. The effect of light and dark incubation upon green leaf tissue of corn is shown in Figure 1. When sections or discs of green leaf tissue were incubated in the dark under the pre-

scribed assay conditions, nitrite not only accumulated in the tissue but diffused into the incubation solution. Normally, only traces of nitrite were detectable during incubation in the light. These small amounts of nitrite are thought to be due to leakage by peripheral, ruptured cells.

Leaf discs incubated in the light did not accumulate nitrite until transferred to darkness. In contrast, leaf discs incubated in darkness rapidly accumulated nitrite until they were transferred to the light. Upon exposure to light, nitrite is rapidly reduced to ammonia (73). Although nitrite accumulation occurs in the *in vivo* nitrate reductase assay under the prescribed assay conditions, nitrite does not normally accumulate in the intact plant in light or in darkness (5). The process of nitrite reduction has been shown to occur in the green leaf in darkness (11, 39), but the mechanism of this reaction is not understood.

**Nitrite Toxicity**

Nitrite is known to be toxic to plants (7, 8, 9, 13, 15, 23, 27, 31, 41, 44, 45, 46, 47, 48, 51, 56, 58, 59, 65, 68, 70). The plant has an efficient and carefully designed enzyme system which quickly reduces nitrite to ammonia. Nitrite does not normally accumulate in plants because:

1. Nitrite reductase activity is commonly measured at 4- to 40-fold the level of nitrate reductase activity (5, 33).
2. The Km of nitrite reductase for nitrite is $10^{-6}$ M (33) as compared to a Km of $1.4 \times 10^{-4}$ M of nitrate reductase for nitrate (64).
3. Nitrate reductase is more unstable both *in vitro* and *in vivo* than nitrite reductase (5, 33, 34, 35) so that in period of plant stress such as drought or extreme heat, nitrate reductase is one of the first enzymes to decrease in activity (32).
4. Unlike nitrate, when nitrite is detectable in healthy green leaf tissue, it is thought not to occur free but as an enzyme-bound intermediate of nitrate assimilation (5, 33, 46).

The toxicity of nitrite to plants has been documented since 1861. First experiments by Goppelsroeder (27) and Molisch (51) showed that plants could metabolize nitrite when low concentrations were used but that higher concentrations of nitrite were always toxic. A large number of investigators (7, 13, 15, 23, 45, 47, 48, 59) have reported that nitrite toxicity is critically related to acidity. Their findings have shown that at neutral or alkaline pH's, nitrite does not appear to be toxic but it becomes toxic as acidity increases. In a carefully controlled experiment to test for nitrite toxicity dependent upon the pH of nutrient solution cultures, Bingham *et al* (7) demonstrated that, at pH 4.0, 2 ppm nitrite was very toxic to Marglobe tomatoes, Atlas barley and blackeye beans. At pH 6.0 comparable injury required 20 ppm nitrite. At pH 5 to 6 the concentration which reduced growth of tomato plants by 50 percent was attributed to 0.1 – 0.2 ppm of the undissociated nitrous acid ($HNO_2$).

In other studies when plant roots were furnished nitrite as their

nitrogen source, losses of nitrogen from the roots were recorded (45, 48, 53). The loss of nitrogen was attributed to the Van Slyke reaction (deamination of an amine by nitrous acid):

$$R-NH_2 + HNO_2 \rightarrow ROH + H_2O + N_2\uparrow$$

In this reaction an amine is deaminated to form an alcohol, water and nitrogen gas. The exact products depend upon the structure of the amine (primary, secondary or tertiary). Regardless of the structure all reactions result in destruction of the amine and disappearance of nitrous acid. Amides, amino acids and proteins also undergo deamination with the same basic reaction. The rate of this reaction is dependent upon the concentration of nitrous acid which explains its relation to acidity. Normal physiological pH's within the plant can permit these reactions to occur in the presence of free nitrite.

In experiments to demonstrate the dependence of nitrite reduction upon light, Vanecko and Varner (73) vacuum infiltrated solutions of $^{15}N$-nitrite ($^{15}NO_2^-$) into green leaf discs. Approximately 82 percent of the $^{15}NO_2^-$ was recovered as amino-$^{15}N$ ($^{15}NH_2$), which demonstrated its reduction in the light with a concomitant evolution of oxygen. However, as much as 15 percent of the collected gases was identified as labeled $N_2$ ($^{15}N_2$). This loss of nitrogen in the gaseous form can be explained by the Van Slyke reaction when free nitrite is present within leaf tissue.

The Van Slyke reaction has been considered to be a protective mechanism to remove nitrite from plant tissues but has not been thought to be a physiologically important reaction since free nitrite does not normally occur in healthy plant tissues (46). If the Van Slyke reaction does occur upon nitrite accumulation, nitrite would be constantly disappearing, but at the expense of the plant's enzymes, amino acids or metabolites containing a free amino group. With sufficient nitrite accumulations, the plant's entire enzyme systems could be inactivated and destroyed, resulting in death.

Since nitrite is in an intermediate stage of reduction, it can function as either a reducing agent or as an oxidizing agent. As a reducing agent, nitrite would be oxidized back to nitrate; as an oxidizing agent, nitrite could be reduced to ammonia or converted into nitrogen gas ($N_2$) or several of the nitrogen oxide gases. In addition to reacting with amide, amino and amine nitrogen as shown in the Van Slyke reaction, nitrite has been shown to react with or destroy many other key biological compounds necessary for normal plant growth and metabolism.

Evans and McAuliffe (18) identified nitrogen ($N_2$), nitric oxide (NO) and nitrous oxide ($N_2O$) as products of the nonenzymatic reduction of free nitrite by ascorbate or reduced nicotinamide adenine dinucleotide (NADH). The rates of these reactions also increased with increased acidity. Nitrite has been shown to deaminate polyribonucleotides which explains its mutagenic action (43). The formation of

methemoglobin by the reaction of nitrite with hemoglobin has long been known. It is possible that similar hemes in the plant necessary for electron transport could be affected in the same manner. Nitrite is known to chemically destroy indoleacetic acid (71), an important growth hormone. The destruction of this growth hormone would result in undirected plant growth and could help to explain the various nastic reactions, abnormal growth and metabolism often seen in herbicide-treated plants.

The high reactivity of nitrite can also be illustrated by experiments in which nitrite was applied to sterile soils and the reaction products were measured. These products were identified as nitric oxide (NO), nitrogen ($N_2$), nitrous oxide ($N_2O$), and nitrogen dioxide ($NO_2$) (55, 67, 69). These gases were shown to be reaction products between nitrite and the organic matter, lignins and phenolic constituents of the soil.

In experiments with ammonia fertilization, damage to seedlings often has been attributed to high levels of ammonia. It was shown recently by Birch and Eagle (8) that toxicity to seedlings only occurred in the presence of nitrite and that high levels of ammonia were not toxic in the absence of nitrite. Work by Winely and San Clemente (75) demonstrated that the herbicides CIPC and EPTC prevented the oxidation of nitrite to nitrate by *Nitrobacter*. An earlier observation by Casely and Luckwill (10) demonstrated that the herbicide monuron had an inhibitory effect on soil nitrification. This confirmed previous research (25, 60) indicating that *Nitrosomas*, which converts ammonia to nitrite, was more tolerant to monuron than *Nitrobacter* which oxidizes nitrite to nitrate. This effect resulted in abnormally high concentrations of nitrite in soil treated with monuron. These data suggest that certain herbicides can interfere in the nitrogen metabolism of obligatory soil bacteria and result in an incomplete oxidation of ammonia. Toxicity symptoms often attributed to ammonia could actually be due to abnormal nitrite accumulations in the soil due to this incomplete oxidation of ammonia.

The primary symptom of nitrite toxicity in citrus is leaf-wilting (13). In tomatoes, symptoms are chlorosis of the leaves and lignification of the roots (8, 58). With increasing concentrations of nitrite application, tomato plants showed a decrease in nitrogen, potassium and phosphorus content and decreased dry matter production (7, 58). In some experiments, nitrite application had to be delayed until the plants had reached an advanced stage of growth since nitrite was extremely toxic to seedlings (56).

The level of nitrite required for toxicity appears to vary with different plants. Legumes have been reported to be more sensitive than grasses (45). In nutrient solution and soil experiments, toxicity levels for a wide range of plants varied from 2 to 100 ppm nitrite (7, 9, 13, 31, 41, 48, 56).

## MATERIALS AND METHODS

### Plant Species Tested

Tissues from the following plant species were utilized:

| *Monocot Plants* | *Genus, Species* | *Variety* |
|---|---|---|
| Barley | *Hordeum vulgare*, L. | Custer |
| Corn | *Zea mays*, L. | NB611 |
| Crabgrass | *Digitaria sanguinalis*, L. | |
| Giant foxtail | *Setaria faberii*, Herrm. | |
| Triticale | | Armadillo "S"-Beaver |
| Wheat | *Triticum aestivum*, L. | Centurk, Scout 66 |

| *Dicot Plants* | *Genus, Species* | *Variety* |
|---|---|---|
| Cocklebur | *Xanthium pennsylvanicum*, Wallr. | |
| Cucumber | *Cucurbita sativus*, L. | Burpee pickling |
| Mallow | *Malva neglecta*, L. | |
| Mustard greens | *Brassica perviridis*, Bailey | Tendergreen |
| Pennycress | *Thlaspi arvense*, L. | |
| Pigweed | *Amaranthus retroflexus*, L. | |
| Soybean | *Glycine max*, (L.) Merr. | Amsoy |
| Squash | *Cucurbita Pepo*, var. *torticollis* | Golden crookneck |
| Tomato | *Lycopersicum esculentum*, L. | Pixie |
| Velvetleaf | *Abutilon theophrasti*, Medic. | |

### Basic Technique Used with Photosynthetic Inhibitor Herbicides

Green leaf tissue of all monocot species tested was cut into 1 cm sections. Leaf tissue of all dicot species was cut into discs of 1 cm diameter. Incubation medium for all monocot species was composed of 0.05M $KNO_3$, 0.05M $KH_2PO_4$ and 0.2 percent (w/v) Neutronyx 600 (a nonionic surfactant). Technical grade herbicides (no commercial derivatives or formulations), when included in the medium, were present at 0.01 percent solution (w/v). Final pH of the solution (7.5) was adjusted with 3N KOH.

Leaf samples were cut, weighed (0.25 g) and placed in 50-ml beakers containing 5 ml of the incubation medium. Vacuum infiltration of the tissue was done in the beakers within a vacuum desiccator using a rotary vacuum oil pump. A vacuum was applied (72 cm Hg) and released twice. The beakers and their contents were incubated in shaking water baths at 33° C in darkness or in light of 300-400 $\mu$einsteins $sec^{-1}m^{-2}$ for one hour. An appropriate aliquot (normally 0.2 ml) of the incubation medium was taken and analyzed for nitrite content.

Nitrite was determined by the immediate addition of the aliquot to 2 ml of a solution (nitrite reagent) containing 0.5 percent sulfanilamide, 1.5N HCl and 0.01 percent $\alpha$-naphthylethylenediamine·diHCl and brought up to a total of 4 ml with deionized water. Optical density of the solutions were determined at 540 nm for nitrite content. Stock

solutions of 1.0 percent sulfanilamide in 3N HCl and 0.02 percent $\alpha$-naphthylethylenediamine·diHCl in water were maintained in separate containers and mixed in a 1:1 ratio immediately prior to the nitrite test.

### Technique for Measuring Photosynthetic Inhibitor Specificity

The basic technique was modified by the elimination of vacuum infiltration. The herbicide concentrations were decreased to $10^{-4}$ M and $10^{-5}$ M as indicated in Figures 4 and 5. Aliquots for nitrite determinations were taken at various intervals during the incubation period.

### Modified Technique to Test for Inhibition of Photosynthetic Nitrite Reduction by Other Herbicides

The basic technique was modified by substitution of 4 percent ethanol in place of Neutronyx 600 and by lengthening the incubation times as noted in Table 4 for wheat and soybean leaf tissue. Stainless steel screens were placed in the beakers over the leaf tissue during vacuum infiltration so that contact between tissue and incubation medium would be maintained in the absence of a surfactant. Screens were removed immediately after infiltration. Aliquots of the incubation solution were often taken at timed intervals during incubation.

### Spraying Experiments

In different experiments, young soybean and cucumber plants were removed from the growth chamber and sprayed to the point of run-off with 1 percent solutions of the herbicides 2,4-D, pebulate and 2,4-DNP. The plants were placed back into growth chambers in the light and duplicate leaf samples were removed at various intervals. The leaf samples (0.5 to 1.0 g) were cut into small pieces and 10 ml of the nitrite reagent added for nitrite determinations.

An ester of 2,4-D was used to spray soybeans and represents the only treatment in the entire study when a herbicide derivative was used. All spray solutions contained 0.01 percent Neutronyx 600 including control plants.

### Germinating Seedling Experiments

In herbicide studies with germinating mustard green seedlings, all seeds were immersed and stirred in a 5 percent solution of Clorox for 20 minutes. The seed was blotted dry and 0.25-g samples were placed in 50-ml beakers. A sufficient quantity of 0.001M $KNO_3$ was maintained in the beaker to assure adequate moisture for germination without submerging the seeds. All beakers were covered with Saran wrap with holes for adequate aeration. The beakers were placed in the dark at room temperature for germination. After three days, while germination was occuring, 1 mg of each of the various herbicides was

placed in the beaker with the germinating mustard green seedlings and mixed as thoroughly as possible to assure contact between the herbicide and seedlings. The seedlings were then re-incubated in the dark for 18 hours. At the end of the incubation period, nitrite content of the material in the beakers (seedlings and solution) was determined.

In similar studies with germinating summer squash seedlings, the same basic sterilization and germination procedures were used. After four days of incubation in the dark, the roots were removed from the plant and the remaining portions of the plants placed in covered petri dishes containing 2 ml of water and 1 mg of herbicide, and only water as a control. Again, the contents were mixed as well as possible to assure contract between plant tissue and herbicide. After 18 hours of incubation in the dark, nitrite was determined on the entire contents (seedlings and solution) of the petri dishes.

### Plant Culture

Green leaf tissue was utilized from both field-grown and growth chamber-grown plants. All plant tissue selected was healthy and in a stage of active growth. Plants from the growth chamber were grown at light energy of 400-500 $\mu$einsteins $sec^{-1}$ $m^{-2}$, in a vermiculite base, subirrigated daily with a modified Hoagland's nutrient solution (39).

Values presented for all experimental data represent the mean of duplicate analyses. All experiments have been repeated two or more times. Chemical nomenclature of herbicides used or mentioned in the text is listed in Appendix I. Surfactants used and their manufacturers are listed in Appendix II. Techniques for measurement of surfactant specificity are noted in the text.

## RESULTS AND DISCUSSION

### Inhibition of Nitrite Reduction in Green Leaves by Photosynthetic Inhibitors

Earlier procedures for measurement of nitrate reduction by the *in vivo* nitrate reductase assay (39) necessitated incubation of green leaf tissue in darkness because nitrite did not accumulate in the light (Figure 1). However, it was known that certain amounts of nitrite were being lost due to "dark" nitrite reduction by the leaf tissue (11, 39). In first attempts to stop this nitrite loss so that *in vivo* nitrate reductase activity could be more accurately measured, photosynthetic electron flow was physically blocked by incubation in darkness and also chemically blocked by including *o*-phenanthroline, a well known photo-

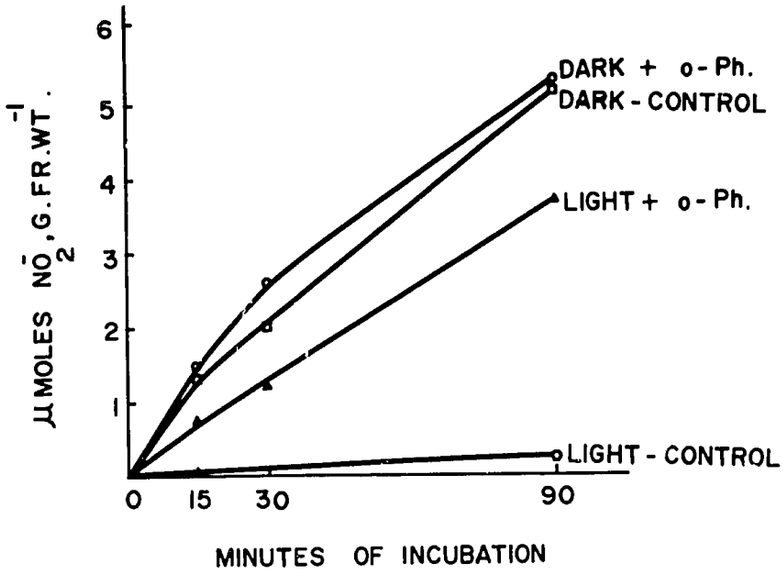



**Figure 2. The effect of *o*-phenanthroline upon nitrite accumulation by Scout 66 wheat leaf tissue with light and dark incubation using the *in vivo* assay. The *o*-phenanthroline was present at 0.1 mg/ml of reaction solution.**

synthetic inhibitor, in the reaction medium. Results of this initial experiment using Scout 66 wheat leaf tissue are shown in Figure 2.

The dark control represents the conventional method of measuring *in vivo* nitrate reduction in green leaf tissue. Both light and dark controls contained only the basic incubation solution of nitrate and phosphate buffer. Nitrite accumulated in the dark control but not in the light control. With the addition of *o*-phenanthroline to the incubation solution in the dark, slightly more nitrite accumulated than in the dark control. More importantly, nitrite accumulated under light incubation when *o*-phenanthroline was present in the reaction medium.

These data illustrate that the addition of a photosynthetic inhibitor to the incubation solution can effectively block the reduction of nitrite to ammonia. This effect would be expected since *o*-phenanthroline is known to inhibit photosynthesis, and nitrite reduction in the green leaf is considered to be directly dependent upon photosynthetic energy. In contrast, as evidenced by the dark values in Figure 2, the process of nitrate reduction was not greatly affected since it is not directly dependent upon photosynthetic energy.

It was known that a number of commercial herbicides were classified as photosynthetic inhibitors which could possibly be capable of

**Table 1. Inhibition of nitrite reduction by "photosynthetic inhibitor" herbicides.**

| Herbicide | Nitrite accumulated with light incubation[a] | |
|---|---|---|
| | Wheat leaves | Soybean leaves |
| Ametryne | 2.5 | 4.8 |
| Atrazine | 2.1 | 7.8 |
| Bromacil | 2.1 | 6.9 |
| Chloroxuron | 2.1 | 4.1 |
| Dicryl | 2.6 | 5.7 |
| Linuron | 2.4 | 4.8 |
| Monuron | 1.3 | 7.4 |
| Prometone | 1.7 | 5.1 |
| Propazine | 1.9 | 5.0 |
| Simazine | 2.0 | 5.1 |
| Solan | 1.6 | 5.3 |
| Control—no herbicide (light) | 0 | 0.1 |
| Control—no herbicide (dark) | 2.6 | 15.3 |

[a]Measured as μmoles $NO_2^-$ g fr wt$^{-1}$ hr$^{-1}$.

the same effect. Table 1 lists the photosynthetic inhibitor herbicides which were tested initially with the same basic technique as was used with *o*-phenanthroline. All of these herbicides interfered with the normal process of nitrite reduction in wheat and soybean leaf tissue. The dark control for each plant denotes the amounts of nitrite accumulated under the normal assay procedures for the *in vivo* nitrate reductase assay. The light control (no herbicide added) shows none or only traces of nitrite accumulation. Inhibition of nitrite reduction in the light by these herbicides ranged from 27 to 100 percent. In this initial screening, all herbicides were included in the reaction medium at 0.1 mg/ml and most were not completely soluble at this concentration. For this reason, these data can be used to demonstrate the common effect by all of the herbicides but cannot be used as a basis for selecting superior herbicides. Data presented later in this bulletin concerning a measure of herbicide specificity in wheat will show the effects of much lower concentrations of different herbicides and can provide a basis for the selection of a superior herbicide for a given plant species.

Two of the herbicides shown in Table 1, dicryl and solan, are rated as insoluble in water (3), yet they effectively inhibited nitrite reduction. With the aid of surfactants in the reaction solution and by the use of vacuum infiltration, sufficient amounts of these two herbicides penetrated the wheat leaf tissue to inhibit nitrite reduction.

All photosynthetic inhibitors tested, using the *in vivo* nitrate reductase assay with surfactants and vacuum infiltration, caused nitrite to accumulate in the light with leaf tissue of wheat and soybeans (Table 1). Most of these herbicides were tested on leaf tissues of mallow, velvetleaf, cocklebur, triticale, corn, pigweed, pennycress, and tomato (data not shown). The results suggest that, if a sufficient quantity of any photosynthetic inhibitor can enter the leaf tissue and the chloroplast,

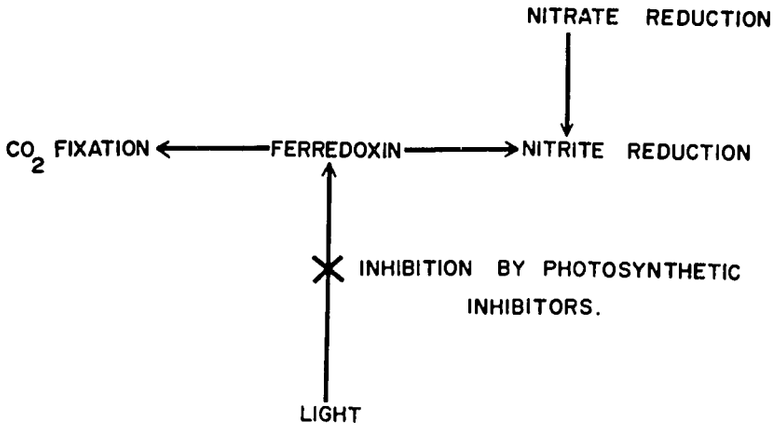



**Figure 3. Illustration of the inhibition of photosynthetic electron flow necessary for nitrite reduction in green leaves.**

photosynthetic reactions are blocked and little plant specificity is shown for any of this type of herbicide.

The green plant has a well controlled enzyme system which prevents the occurrence of free nitrite within the plant cell. Nitrite is usually detectable in plant tissue, but does not accumulate and is not known to occur except as an enzyme-bound intermediate in the intact plant in light or in the dark (5). The processes for nitrate reduction and nitrite reduction are separate. Each process has different enzymes, different electron donors, different energy generation systems, and occurs in distinctly different locations within the cell. Energy needed for nitrite reduction is blocked by photosynthetic inhibitors but nitrate reduction can continue, resulting in nitrite accumulation within the plant.

In the case of the photosynthetic inhibitors, this nitrite accumulation can be readily explained (Figure 3). Ferredoxin, a key electron acceptor and donor in the process of photosynthesis, acts as a pivotal compound capable of shunting electrons for reductive processes such as sugar synthesis and nitrite reduction in the plant leaves. Photosynthetic inhibitors are known to block the electron flow to ferredoxin and in this manner inhibit photosynthesis. Since nitrite reductase in the green leaf is directly dependent upon reduced ferredoxin as its electron donor, the process of nitrite reduction is considered as an integral component of photosynthesis, more closely linked to the light reactions than $CO_2$ fixation. Thus, the inhibition of photosynthesis can cause not only the cessation of sugar synthesis but more importantly can also block the normal process of photosynthetic nitrite reduction. This pathway for nitrite reduction in green leaves is well documented in the literature (5). While it was known that photosynthetic inhibitors stop-

ped sugar synthesis, other reactions dependent upon photosynthetic energy, such as nitrite reduction, appear to have been overlooked.

In contrast to nitrite reduction, nitrate reduction is only indirectly dependent upon photosynthesis for a source of oxidizable sugar phosphates and can effectively utilize reserve carbohydrate present within the leaf as a source of reducing power in the absence of photosynthesis (39). This is illustrated by *in vivo* reduction of nitrate by leaf tissue in the dark with no external source of energy (Figures 1 and 2).

When plants are placed in darkness and photosynthesis is physically blocked with no net sugar synthesis, death by starvation occurs very slowly. By chemical inhibition of photosynthesis, death often occurs within hours or within a single day. The symptoms of injury which develop during death and the rapidity of death cannot be explained by the mere blockage of sugar synthesis or starvation but point to a completely different mechanism. This phenomenon of rapid death and the symptoms of herbicidal injury can be explained by abnormal accumulations of nitrite within the plant.

## Specificity of the *In Vivo* Assay for the Identification of Photosynthetic Inhibitors

To further test this procedure for its capability and specificity of rapidly detecting chemicals with the ability to inhibit photosynthetic nitrite reduction, a number of herbicide analogs (triazine derivatives) were tested using wheat leaf tissue (Table 2). Treatments of simazine, atrazine and *o*-phenanthroline were capable of inhibiting nitrite reduction in the light while most of the analogs were not. Rates of nitrate reduction (data not shown) were not decreased by any of these compounds. Only the two 2-chloro-4-amino-*s*-triazine derivatives showed any interference with nitrite reduction. These two compounds are very similar to simazine, propazine, and atrazine, which are also 2-chloro-4-amino-*s*-triazine derivatives. The substitution of a hydroxyl group on the 2-position of the triazine ring appears to render the molecule inactive as a photosynthetic inhibitor. Previous research has shown that the corn plant is capable of chemically converting atrazine into a 2-hydroxy derivative (29, 30). This process appears to be an effective method of deactivation of the atrazine molecule.

Recent data obtained by personal communication from Dr. D. D. Kaufman using these same analogs in field experiments with oats at rates of 2, 10, and 50 lb/A indicates that this *in vivo* assay is quite specific for the rapid identification of promising photosynthetic inhibitor herbicides. Herbicide toxicity in the Kaufman field experiment was measured as percent decrease in fresh weight of the oats. Only one of the analogs was rated as toxic in the field experiments (76 percent decrease in fresh weight at 2 lb/A). This analog, which was 2-chloro-

**Table 2. The effect of triazine herbicides, herbicide analogs and *o*-phenanthroline on photosynthetic nitrite reduction of Centurk wheat leaves.**

| | Analog or inhibitor | $\mu$moles $NO_2^-$ accumulated with light incubation, g fr wt$^{-1}$ hr$^{-1}$ |
|---|---|---|
| Control (light, no herbicide | | 0 |
| Control (dark, no herbicide | | 4.50 |
| Simazine | | 1.00 |
| Atrazine | | 3.90 |
| *O*-phenanthroline | | 0.50 |
| Hydroxysimazine | 2-hydroxy-4,6-*bis*(ethylamino)-*s*-triazine | 0 |
| Hydroxyatrazine | 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine | 0 |
| Hydroxypropazine | 2-hydroxy-4-6-*bis*(isopropylamino)-*s*-triazine | 0 |
| | 2-hydroxy-4-methylamino-6-ethylamino-*s*-triazine | 0 |
| | 2-chloro-4-amino-6-ethylamino-*s*-triazine | 0.32 |
| | 2-chloro-4-amino-6-isopropylamino-*s*-triazine | 2.60 |
| | 2,4-*bis*(hydroxy)-6-ethylamino-*s*-triazine | 0 |
| Ammelide | 2,4-*bis*(hydroxy)-6-amino-*s*-triazine | 0 |
| Ammeline | 2-hydroxy-4,6-*bis*(amino)-*s*-triazine | 0 |
| | 2-hydroxy-4,6-*bis*(methylamino)-*s*-triazine | 0 |
| | 2,4-*bis*(hydroxy)-6-methylamino-*s*-triazine | 0 |
| | Cyanuric acid, 2,4,6-*tris*(hydroxy)-*s*-triazine | 0 |

4-amino-6-isopropylamino-*s*-triazine, is the only compound which appreciably blocked nitrite reduction (Table 2). The analog, 2-chloro-4-amino-6-ethylamino-*s*-triazine, accumulated a small amount of nitrite in the *in vivo* assay and was rated as possibly toxic. All other analogs tested were rated as not toxic or slightly toxic (0-10 percent decrease in fresh weight at 10 and 50 lb/A). Thus, the data presented in Table 2 agree completely with independently-conducted field tests with the same triazine derivatives.

The hydroxytriazines also are very water-insoluble, but on the basis of the work already presented in which the water-insoluble herbicides, dicryl and solan were used, it was assumed that these derivatives also entered the leaf tissue. Compounds related to herbicides such as ethylurea, urea, ethylcarbamate, biguanides, amines, biuret, and indoleacetic acid also were tested with no apparent effect on nitrite reduction (Table 3).

The use of surfactants and vacuum infiltration assures the rapid penetration of a potential herbicide molecule into the appropriate cell site for inhibition. This basic technique can be used to determine

**Table 3. Effect of herbicide-related compounds on nitrite reduction by Centurk wheat leaves.**

| Chemical tested | μmoles $NO_2$ accumulated with light incubation, g fr wt$^{-1}$ hr$^{-1}$ |
|---|---|
| $N^1$, $N^5$-Diisopropylbiguanide | 0 |
| $N^1$, $N^5$-Diethylbiguanide | 0 |
| N-Ethylurea | 0 |
| Urea | 0 |
| Ethylcarbamate (Urethane) | 0 |
| Methylamine | 0 |
| Diethylamine | 0 |
| Indoleacetic acid | 0 |
| Biuret | 0 |
| Simazine | 1.00 |
| Atrazine | 3.90 |
| *O*-Phenanthroline | 0.50 |
| Control (light) | 0 |
| Control (dark) | 4.50 |

whether a given molecular structure is capable of inhibiting photosynthetic nitrite reduction in green leaf tissue. This portion of the test does not measure important factors of herbicide specificity such as foliar penetration, root uptake, translocation within the plant or possible deactivation processes, but modifications of the basic method can aid in idemifying factors of herbicide specificity for a given plant species.

## Photosynthetic Inhibitor Specificity of Wheat

Bromoxynil, a photosynthetic and respiratory inhibitor (3), when used in combination with a proper surfactant and with vacuum infiltration, was found to effectively inhibit nitrite reduction in wheat leaf tissue. Since this herbicide is registered for weed control in wheat as a foliar application, it was chosen to further investigate factors of specificity using wheat leaf tissue.

Wheat leaf sections were floated (no vacuum infiltration) on solutions containing surfactant and four different chemical classes of photosynthetic inhibitor herbicides. All herbicides were included at concentrations of $10^{-4}$ M and incubation was conducted in the light and in the dark. Nitrate reduction rates were not decreased in any of the dark treatments with herbicides (data not shown). Figure 4 shows that the dark control (no herbicide) maintained highest rates of nitrite accumulation while the light control accumulated no nitrite. In the light, bromacil and atrazine treatments accumulated the largest amount of nitrite while monuron was intermediate and bromoxynil was lowest. The bromoxynil treatment also showed no nitrite accumulation for the first 60 minutes of incubation while all others rapidly and effectively blocked nitrite reduction.

Further comparisons of the effect of atrazine and bromoxynil at

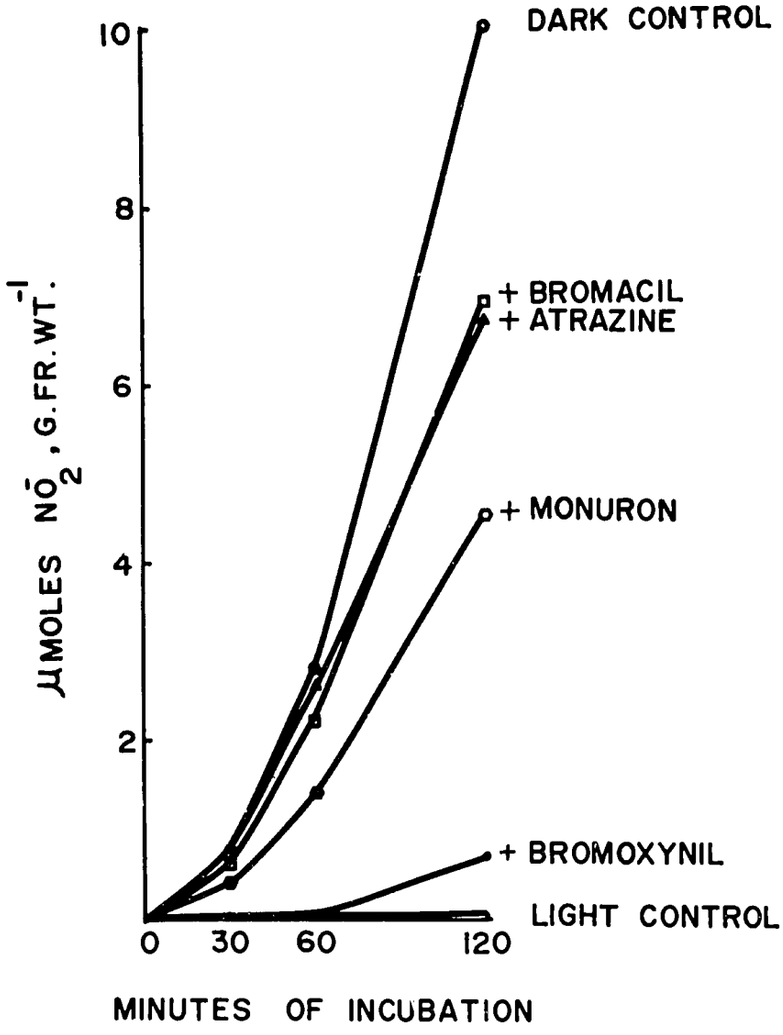



**Figure 4. The blockage of nitrite reduction in the light with wheat leaf tissue (Scout 66) by several classes of photosynthetic inhibitor herbicides. $10^{-4}$ M concentration of all herbicides were used. Leaf tissue sections were not vacuum infiltrated.**

$10^{-4}$ and $10^{-5}$ M concentrations are shown in Figure 5. Both concentrations of atrazine were effective in partially blocking nitrite reduction while $10^{-4}$ bromoxynil also was partially effective. At $10^{-5}$ M bromoxynil, nitrite accumulation in the light was barely detectable. It is not

known whether atrazine penetrates the wheat leaf tissue more effectively than bromoxynil or whether atrazine is a more effective inhibitor of photosynthesis in wheat. These experiments provide information as to why bromoxynil can be used as a foliar spray on wheat for weed control and a herbicide such as atrazine cannot, although both are known photosynthetic inhibitors. The surfactant (Neutronyx 600)

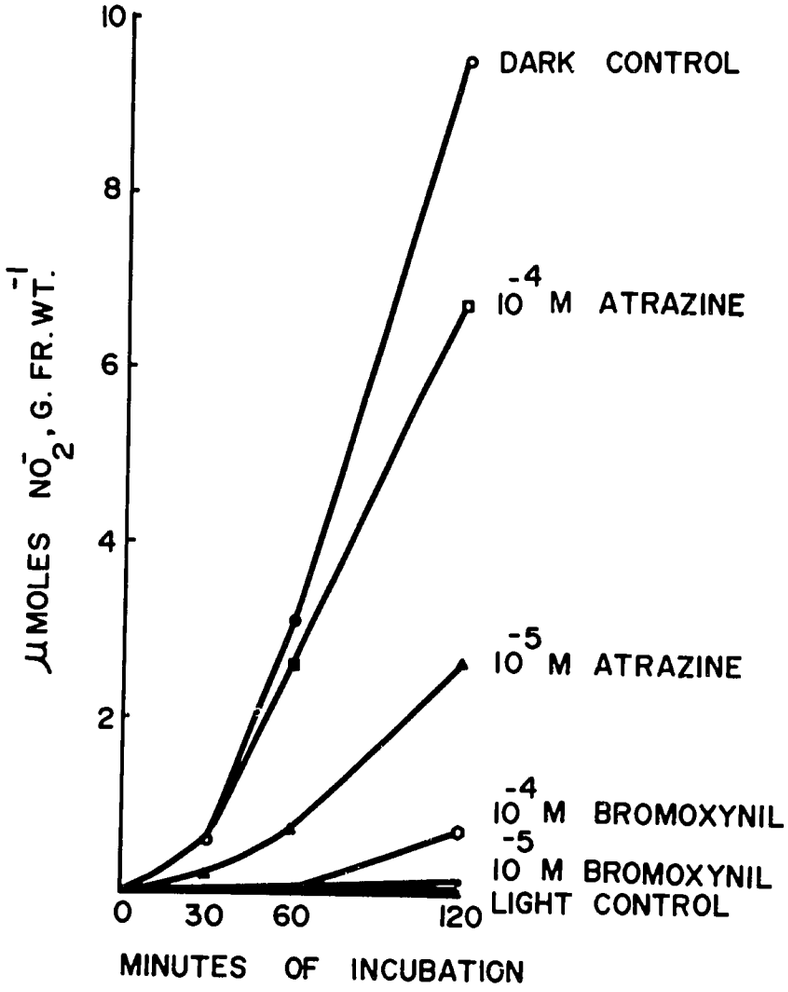



**Figure 5.** The blockage of nitrite reduction in the light by atrazine and bromoxynil ($10^{-4}$ M and $10^{-5}$ M). No vacuum infiltration of the tissue was used in the *in vivo* assay.

used in this test was highly effective in permitting the entry of herbicides into wheat leaf tissue. Bromoxynil, used in combination with a surfactant not as effective in penetrating wheat leaf tissue, could provide an even safer treatment for wheat.

### Effects of Other Classes of Herbicides on Photosynthetic Nitrite Reduction

There are many other chemical classes of herbicides which are not normally classified as inhibitors of photosynthesis. These include the phenoxy-, aniline-, benzoic acid-, carbamate-derivatives and others. Although there is much known of the effects that these herbicides exert on the many metabolic reactions and processes that occur within the plant, there has been no satisfactory explanation as to their exact mechanism for rapidly killing plants. In contrast to the action of most photosynthetic inhibitors which kill the plant after emergence from the soil and greening have taken place, many of these other classes of herbicides kill the germinating weed seedlings before emergence from the soil. Others, such as 2,4-D, are used primarily as contact herbicides. Yet, death with these herbicides normally is rapid and often symptoms of injury are similar to those caused by the photosynthetic inhibitors.

The effects of this wide range of chemical classes of herbicides upon the *in vivo* nitrate assay system were tested with green leaf tissue to determine if some of these herbicides also were capable of interfering with photosynthetic nitrite reduction. The assay system was modified by substitution of 4 percent ethanol in place of surfactant and incubation time was lengthened. Levels of surfactant used for optimal reduction of nitrate in the dark and for optimal penetration of photosynthetic inhibitor herbicides in the light appeared to interfere with the accumulation of nitrite in the light using these other herbicides. This effect is not understood but some of its aspects will be discussed.

Initial data obtained from this slightly modified assay system with a diverse group of herbicides on wheat and soybean leaf tissue are shown in Table 4. With wheat leaf tissue, only 2,4-D and propham treatments showed any appreciable accumulation of nitrite in the light. Other treatments were ineffective or inconclusive even though the test was conducted for a 4-hour period. The treatments of pebulate, dinoben and alachlor appeared to reduce nitrate reduction rates at the concentration of herbicides used (0.1 mg/ml). Percent inhibition is calculated as the percent nitrite accumulated in the light as compared to the dark control. Both wheat and soybean leaf tissue light control values illustrate that some leakage of ruptured peripheral cells does occur with prolonged incubation. All herbicides tested with soybean leaf tissue showed a positive response after 90 minutes of incubation. The herbicides 2,4,5-T, pebulate and propham inhibited nitrite reduction from 57 to 79 percent. Many of the herbicides listed (Table 4) are not commonly used as foliar sprays but appear capable of blocking nitrite

**Table 4. Inhibition of photosynthetic nitrite reduction by a variety of herbicides.**

| | μmoles $NO_2$ accumulated/g fr wt leaf in light | | | |
|---|---|---|---|---|
| Herbicide | Wheat (240 min.) | % Inhibition | Soybeans (90 min.) | % Inhibition |
| Alachlor | 1.40 | 0 | 3.50 | 13 |
| Amitrole | 2.45 | 9 | 2.75 | 11 |
| Dinoben | 1.40 | 0 | 6.00 | 23 |
| Paraquat | 2.65 | 9 | 6.03 | 23 |
| Pebulate | 0.78 | 0 | 20.50 | 79 |
| Propham | 4.75 | 17 | 14.90 | 57 |
| 2,4-D | 4.23 | 15 | 2.80 | 10 |
| 2,4,5-T | 2.93 | 10 | 19.40 | 74 |
| Control (no herbicide-light | 1.53 | 5 | 1.70 | 7 |
| Control (no herbicide-dark) | 28.30 | | 26.10 | |

reduction upon penetration into the soybean leaf. This suggests that they are capable of inhibiting normal photosynthetic nitrite reduction.

The rates of nitrite accumulation by soybean leaf tissue after treatment with 2,4,5-T in the light and in the dark are shown in Figure 6. The light control shows some nitrite accumulation. Again, this is believed to be escape of nitrite by injured peripheral cells. With monocot leaf tissue, such as corn or wheat, this effect is rarely seen except with prolonged incubation. The dark control had linear rates of nitrite accumulation for a 90-minute period.

The addition of 2,4,5-T and incubation in the dark increased the rate of nitrite accumulation. It is not known whether this apparent stimulation is due to increased rates of nitrate reduction or due to a more complete blockage of nitrite reduction in the dark or a combination of the two possiblilities. Apparent stimulation of nitrate reduction rates has been noted repeatedly with many herbicide treatments and plant tissues during the course of this study. The accumulation of nitrite in the light when 2,4,5-T was added to the medium was over 50 percent of that in the dark without 2,4,5-T. This accumulation of nitrite in the light caused by 2,4,5-T illustrates that the phenoxy herbicides which were not commonly known to inhibit photosynthesis can interfere with the normal process of nitrite reduction in the green leaf.

The effects of apparent stimulation in rates of *in vivo* nitrate reduction in the dark by 2,4,5-T also were investigated with wheat leaf tissue using three concentrations of 2,4,5-T (20, 100 and 200 μg/ml of reaction mixture) as shown in Figures 7 and 8. All concentrations of 2,4,5-T gave immediate increases in nitrite accumulation in the dark over that of the control for the first hour of incubation (Figure 7). With 200 μg/ml, nitrate reduction ceased after 90 minutes of incubation. With 100 μg/ml, nitrate reduction remained above that of the control for 2 hours but showed decreasing rates after an hour of incubation. With 20

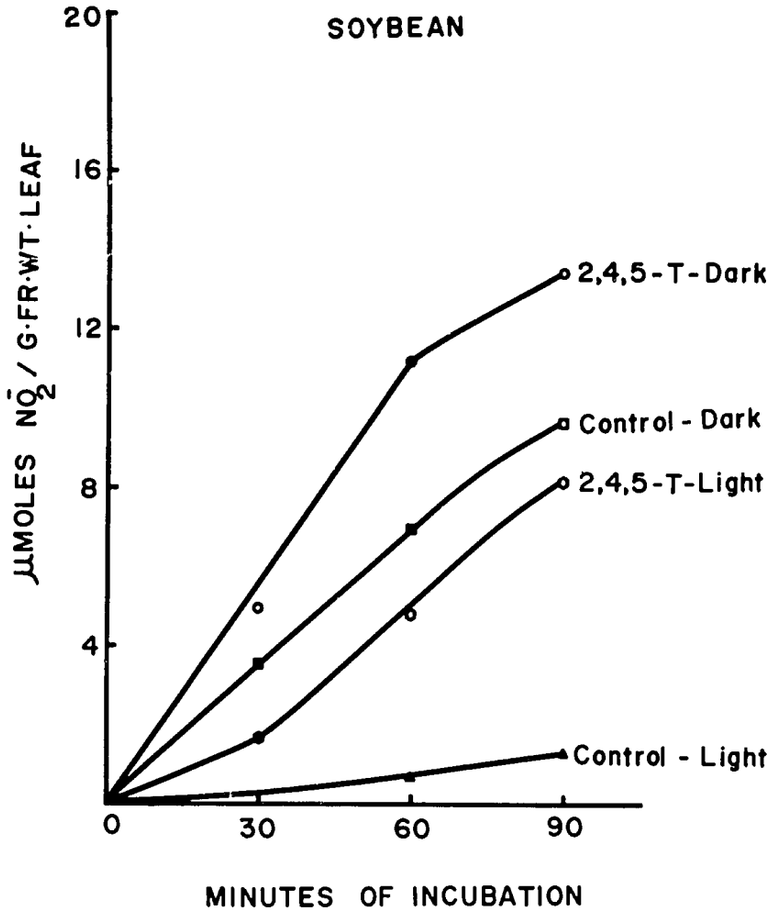



**Figure 6. The rates of nitrite accumulation in the light and in the dark with the *in vivo* assay of soybean leaves (Amsoy) as affected by 2,4,5-T. 2,4,5-T was included at 0.1 mg/ml of reaction solution.**

µg/ml an increased rate of nitrate reduction continued for the entire incubation period.

In a companion experiment, the same concentrations of 2,4,5-T were used with light incubation to test their effects upon nitrite reduction (Figure 8). Without 2,4,5-T, nitrite did not accumulate in the light. Nitrite accumulated in the light with all concentrations of 2,4,5-T but to greater extents and at different times during the incubation. Every treatment showed at least a 30-minute lag before nitrite accumulated in the light whereas an immediate effect was seen on the rates of nitrite

accumulation with dark incubation (Figure 7). The treatment of 2,4,5-T, 100 μg/ml, was most effective in the inhibition of nitrite reduction. With 200 μg/ml, 2,4,5-T inhibition began at 30 minutes but decreased at 2 hours similar to the decrease in nitrate reduction rates in the dark (Figure 8). Percent inhibition of nitrite reduction (light treat-

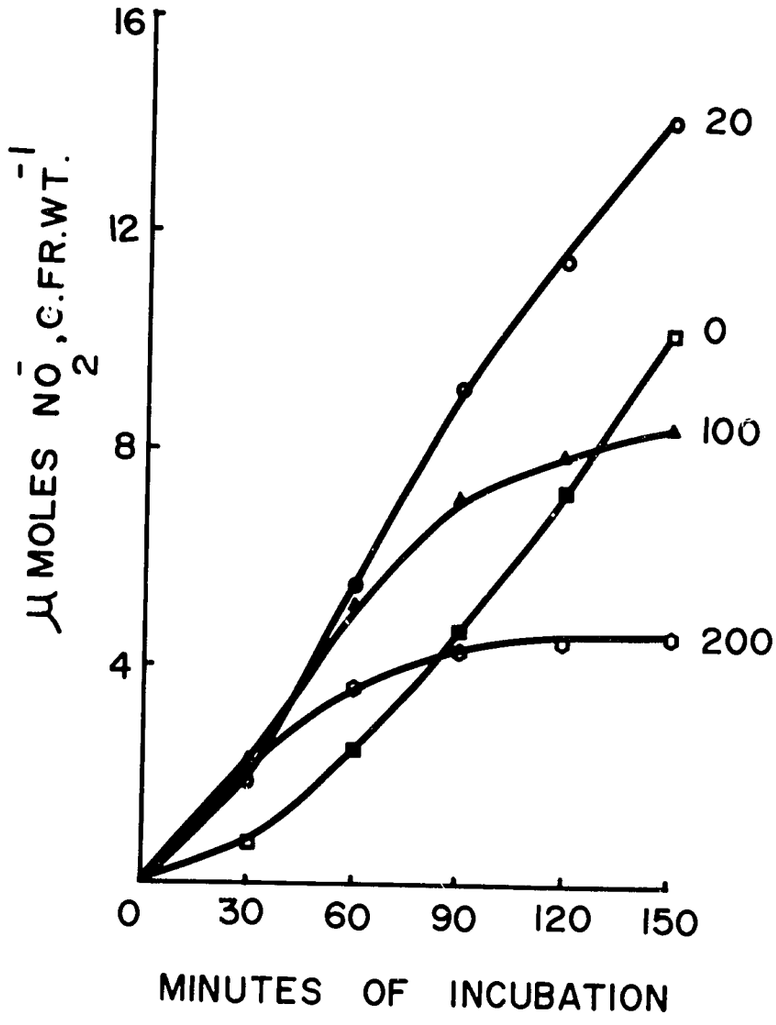



**Figure 7.** The effect of varying concentrations of 2,4,5-T (20 μg/ml, 100 μg/ml and 200 μg/ml of reaction solution) on *in vivo* nitrate reduction rates by Scout 66 wheat leaf tissue. Only dark incubation is shown.

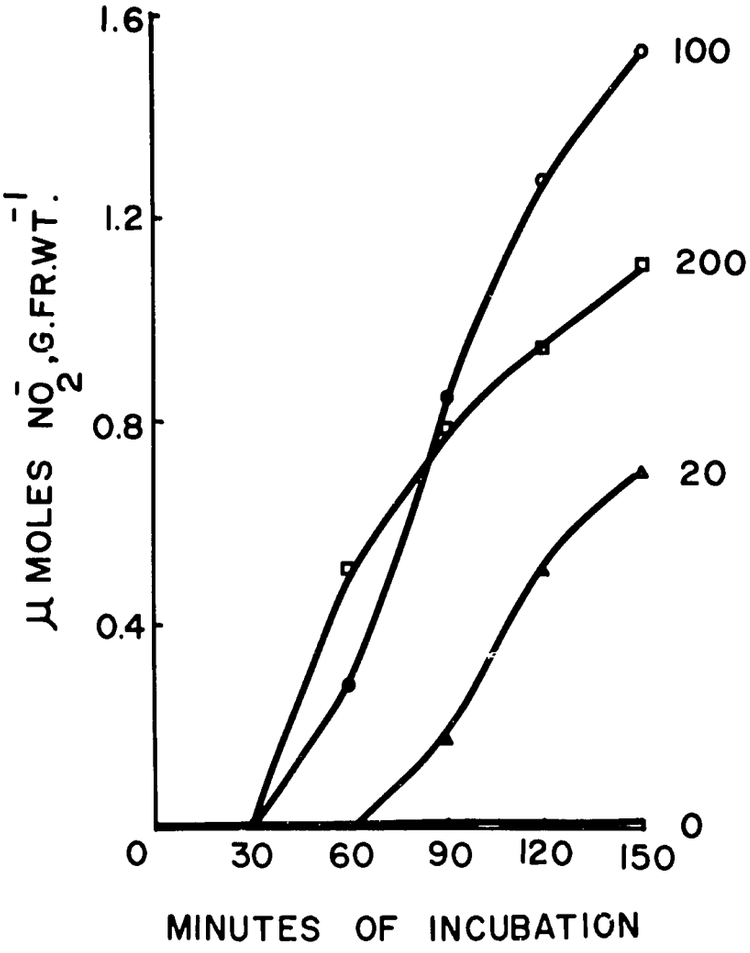



**Figure 8. The effect of varying concetrations of 2,4,5-T (20 μg/ml, 100 μg/ml, 200 μg/ml of reaction solution) on nitrite reduction by Scout 66 wheat leaf tissue. Only light incubation is shown.**

ments with 2,4,5-T in Figure 8 as compared to dark treatments with 2,4,5-T in Figure 7 increased with time. With 100 μg 2,4,5-T, during the 90- to 150-minute period of incubation, nitrite reduction in the light was inhibited over 50 percent, although rates of nitrate reduction were greatly decreased after prolonged incubation.

This decrease in nitrate reduction rates at 100 μg/ml and 200 μg/ml can possibly explain inconclusive data of the treatment of 2,4,5-T on wheat as presented earlier in Table 4. For nitrite to accumulate, nitrite

reduction must be inhibited while nitrate reduction continues. At the higher concentrations of 2,4,5-T it is evident that nitrate reduction rates were decreased with longer periods of incubation (Figure 7).

### Accumulation and Disappearance of Nitrite in Intact Plants After Foliar Applications of Herbicides

Although nitrite was shown to accumulate in the light in green leaf tissue only when herbicides were included in the medium using the *in vivo* nitrate reductase assay, it was necessary to test whether nitrite also accumulated in intact, growing plants sprayed with herbicides. Soybean plants with expanding, first trifoliolate leaves were sprayed with a 1 percent solution of a 2,4-D ester to the point of run-off and replaced in the light. The trifoliolate leaves were sampled at various timed intervals and analyzed for the presence of nitrite (Figure 9). Within one hour after spraying, abnormally high concentrations of nitrite were present within the leaves. This nitrite concentration increased for 4 hours. Visual symptoms of 2,4-D toxicity such as leaf necrosis and epinasty appeared during this 4-hour period. Although nitrite did not accumulate to such high levels as in the *in vivo* assay, it was present in much larger quantities than found in normal plant tissue. The nitrite concentration remained constant for approximately 8 hours. At the end of 24 hours, the plants were dead and nitrite was no longer detectable.

As previously shown with the *in vivo* assay (Table 4), pebulate, classified as a pre-emergence herbicide, caused very high nitrite accumulations when incubated with soybean leaf tissue. It was also used as a foliar spray on soybean leaf tissue. Nitrite concentrations (Figure 10) rapidly increased within 30 minutes along with the appearance of severe leaf necrosis. After 60 minutes the nitrite concentration rapidly declined and the plant leaves were desiccated and presumably dead within 2 hours after the pebulate treatment. In repeated experiments with pebulate sprayed on soybean plants (data not shown) desiccation and death of the leaves always occurred within 90 to 120 minutes. Nitrite exhibited an identical pattern of accumulation and always decreased to lower concentrations within 2 hours after treatment.

Cucumber plants were sprayed with 2,4-DNP (not registered as a herbicide but a well-known uncoupler of oxidative phosphorylation) and nitrite concentrations were easily detectable in the leaf tissue. Analyses of nitrite within the plant showed that the young leaves, which were the first to die, contained 175 m$\mu$ moles of nitrite per gram of fresh weight while the older leaves contained only 28 m$\mu$ moles per gram fresh weight. Nitrite concentration in stem tissue was at the lower limit of detection.

Although nitrite did not accumulate in such large amounts in these spraying experiments as occurred in the *in vivo* assay, highly abnormal concentrations of nitrite were present within the intact plants. This

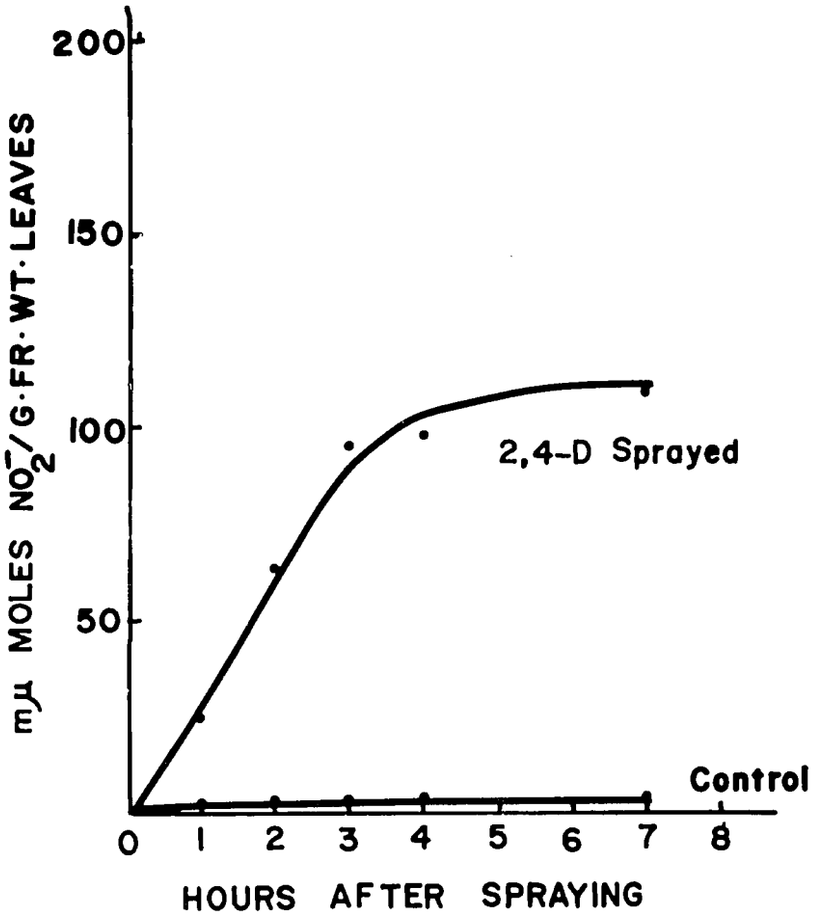



**Figure 9. The accumulation of nitrite in intact soybean leaves after spraying with a one percent 2,4-D ester. Leaf samples were taken from the plants at 1, 2, 3, 4 and 7 hours for nitrite determinations.**

provides evidence that nitrite reduction was blocked while nitrate reduction continued as in the *in vivo* assay.

All soybean plants sprayed with the herbicides 2,4-D and pebulate accumulated highly abnormal quantities of nitrite and died. Cucumber plants sprayed with 2,4-DNP also accumulated nitrite and died. The rapidity of death and desiccation of the leaves corresponded with the levels of nitrite found in the plant tissues. When 2,4-D and 2,4-DNP were used, death of the above-ground portion of the plants occurred within a 24-hour period after application of the herbicides. Much lower levels of nitrite were detected in these treatments than in the pebulate-treated soybeans which were desiccated and dead within 2

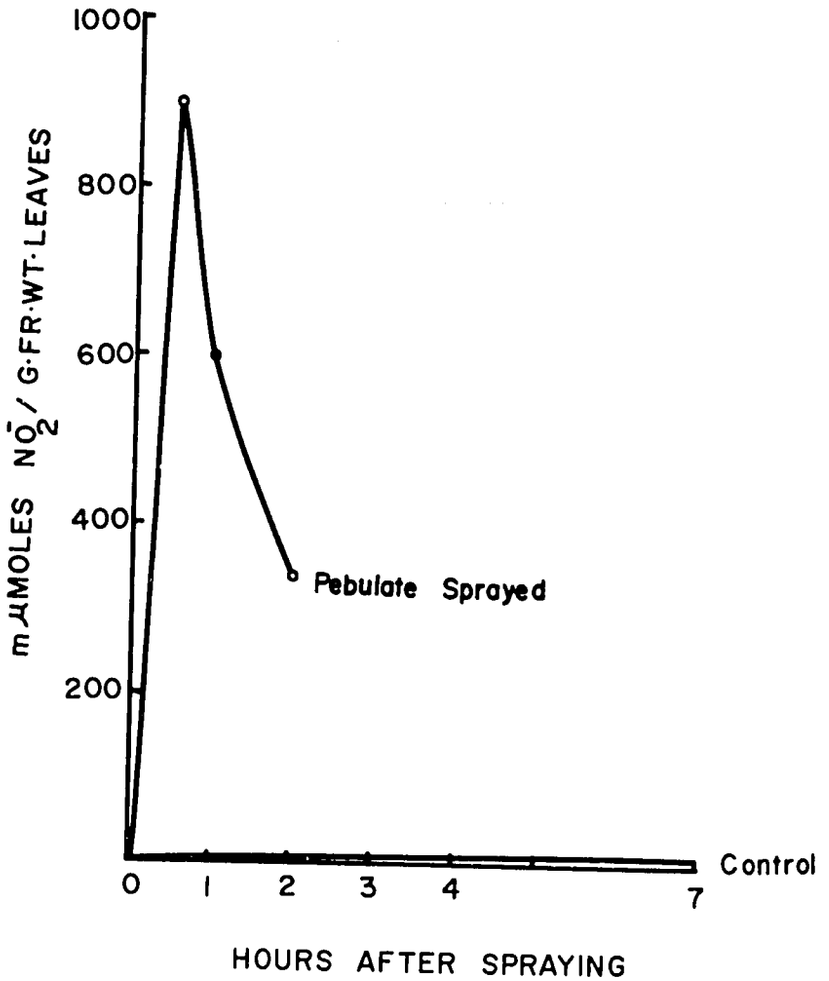



**Figure 10.** **The accumulation of nitrite in intact soybean leaves after spraying with a one percent pebulate solution. Samples were taken from the plants at 30, 60 and 120 minutes for nitrite determination.**

hours after application. Further, the youngest leaves of cucumber plants, which were the first to show symptoms of herbicidal action and the first to die, contained higher levels of nitrite than the other parts of the plant.

In all sprayed plants, nitrite accumulated while the plants were dying but was not detectable in the tissue after death. If these herbicides blocked the enzymatic reduction of nitrite, the disappearance of nitrite must be due to non-enzymatic reductions or conversions to other forms of nitrogen. Nitrite has the known capability of reacting

with a wide range of plant metabolites including amines, amides, amino acids, proteins, enzymes, hemes, hormones, RNA, DNA, ascorbate, NADH, and phenols. In the reactions of nitrite with these metabolites, they are altered or destroyed and nitrite is converted to a number of other forms of nitrogen; hence, its disappearance. A continuing reaction between these compounds and nitrite within intact plants can help to explain the nastic reactions, necrosis and rapid death without extremely high concentrations of nitrite present at any given time. If these degradative reactions causing nitrite disappearance are occurring during the death of the plant, large amounts of nitrite would not be expected to accumulate.

The lower concentrations of nitrite found in the intact leaf tissues after spraying as compared to the *in vivo* assay can be clarified by three factors present in the assay which are not available to the intact plant. First, the *in vivo* assay system has been optimized for the escape of nitrite into the reaction solution by the addition of surfactants or ethanol. After nitrite reduction is blocked in the intact plant, the nitrite formed within the plant cannot escape by this method. Disappearance of nitrite would probably be due to the degradative reactions with plant metabolites. Second, the *in vivo* assay system ordinarily utilizes 0.25 gram of plant tissue in 5 to 10 ml of reaction solution, which allows a 20- to 40-fold dilution of the nitrite as it escapes from the tissue into the reaction solution. This dilution effect is not possible within the intact plant. Third, the assay system is used with an alkaline reaction solution at a pH (7.5) at which nitrite is, supposedly, non-toxic. The physiological pH within the plant cell can provide for the occurrence of the highly reactive nitrous acid ($HNO_2$).

In all experiments discussed in the Literature Review which demonstrated toxicity of nitrite to plants, whether nutrient solution, soil culture or green leaf tissue was utilized, the plant tissue possessed an efficient, operable system for reducing nitrite. Toxicity of nitrite would not have been measurable until concentrations of nitrite were applied which overloaded the capacity of the reducing system. In plants which have their enzymatic nitrite reducing system blocked, much lower concentrations of nitrite should be required for symptoms of toxicity. In all experiments reported in literature which measured nitrite toxicity to intact plants, nitrite was supplied externally in the growth media. Entry of the nitrite into the roots and translocation to the shoot could have been limiting factors. Nitrite formed and accumulated within a plant cell rather than applied externally should require much lower amounts for toxicity.

### Blockage of Nitrite Reduction by Herbicides in Germinating Seedlings

Plant roots are known to have the capability of reducing nitrate to ammonia. It has previously been shown that active *in vitro* preparations

of both nitrate reductase and nitrite reductase can be extracted from root tissue (40, 49, 63). The exact mechanisms for energy generation for these two systems are not clearly understood. Energy generation necessary for nitrate reduction can be explained in the root by the same process as in the leaf (NADH generated by glycolytic oxidation). However, since the energy generation necessary for nitrite reduction in the leaf is directly dependent upon light energy, the process of dark nitrite reduction by nonphotosynthetic tissue is not understood.

Many of the herbicides used as pre-emergence treatments kill the weed seedlings during germination before their emergence from the soil. Experiments were designed to determine whether some of these herbicides were capable of blocking nitrite reduction in non-green germinating seedlings.

Germinating mustard green seedlings were treated with different herbicides (Table 5) as presented in Materials and Methods. After incubation in darkness for 18 hours, nitrite content was determined for the entire contents of the beaker (seedlings and solution). The control (no herbicide) contained no nitrite. All herbicide treatments contained nitrite although in widely varying amounts. Low levels of nitrite were found in treatments with trifluralin, DCPA and chloramben. Treatments with propachlor, picloram and 2,4-DNP contained intermediate levels of nitrite (6-9 $\mu$moles). The most effective treatments were alachlor and 2,4,5-T which contained 22.0 and 41.2 $\mu$moles nitrite, respectively.

In a similar experiment (Table 6), the hypocotyl and cotyledons of germinating etiolated squash seedlings were treated with different herbicides and nitrite content measured after an 18-hour incubation. The control (no herbicide) contained 0.4 $\mu$moles nitrite. It is not known whether this nitrite escaped by leakage from the tissue since the roots had been removed or was due to bacterial contamination. The most effective treatments were pebulate, 2,4-D, butylate, bromoxynil and cycloate and were much higher than the control or other treatments.

While these techniques with germinating seedlings are crude and unrefined, they demonstrate that a variety of chemical classes of herbicides (Tables 5 and 6) are capable of inhibiting nitrite reduction in

**Table 5. Nitrite accumulation by germinating mustard green seedlings.**

| | $\mu$moles $NO_2^-$ accumulated[a] |
|---|---|
| Control | 0 |
| Trifluralin | 0.1 |
| DCPA | 0.1 |
| Chloramben | 0.3 |
| Picloram | 6.0 |
| Propachlor | 9.1 |
| 2,4-DNP | 6.9 |
| Alachlor | 22.0 |
| 2,4,5-T | 41.2 |

[a] Total $NO_2^-$ accumulated after an 18-hour incubation period.

**Table 6. Nitrite accumulation by germinating squash seedlings.**

| | $\mu$moles $NO_2^-$ accumulated[a] |
|---|---|
| Control | 0.4 |
| DCPA | 2.5 |
| Alachlor | 0.3 |
| Trifluralin | 0.5 |
| Benefin | 0.4 |
| Pebulate | 12.9 |
| 2,4-D | 14.1 |
| Dalapon | 0.2 |
| Butylate | 7.5 |
| Bromoxynil | 12.4 |
| Cycloate | 16.9 |
| Bensulide | 1.0 |

[a] Total nitrite accumulated per 3 squash seedlings after an 18-hour incubation.

nonphotosynthetic tissue. Also, some specificity was shown by certain herbicides failing to block nitrite reduction. It is not known whether this specificity was due to failure of the herbicide to enter the plant tissue or deactivation of the herbicides by the plant tissue.

## Relationship to Previous Herbicide Research

The inhibition of nitrite reduction by herbicides and 2,4-DNP has been previously reported by other investigators in both photosynthetic and nonphotosynthetic plant tissues. Kessler reported that 2,4-DNP inhibited nitrite reduction in *Ankistrodesmus* (36) and *Chlorella* (37) and did not appear to largely affect nitrate reduction. Ahmad and Morris (1) also found that nitrite reduction was completely inhibited by 2,4-DNP in *Ankistrodesmus*.

In a review article on nitrate assimilation by plants, Kessler (36) noted that the same effect has been shown for *Azotobacter*, *Euglena* and *Anabaena* by other researchers. Ferrari and Varner (22) have shown that 2,4-DNP, and the herbicides pentachlorophenol and ioxynil, inhibit nitrite reduction in barley aleurone layers (nonphotosynthetic tissue) with little effect on the process of nitrate reduction. 2,4-DNP is known to be a potent uncoupler of oxidative phosphorylation and to interfere with mitochondrial electron transfer.

In an excellent review of herbicide research, Ashton and Crafts (2) report that the herbicides 2,4-D, 2,4,5-T, 4-CPA, 2-CPA, MCPA, DMTT, naptalam, 2,3,6-TBA, monuron, CDAA, pyriclor, amitrole, dicamba, propham, 2,4-DB, MCPB, dalapon, simazine, chloramben, diuron, EPTC, dinosam, dinoseb, PCP, bromoxynil, ioxynil, propanil, chloropropham, trifluralin, and nitralin are capable to various degrees of inhibiting $O_2$ uptake and oxidative phosphorylation.

On the basis of these data gathered from a large number of researchers and the data presented in Tables 5 and 6, it is proposed that herbicidal inhibition or interference in mitochondrial electron transfer can block the energy necessary for nitrite reduction in nonphoto-

synthetic tissues. These data also implicate the mitochondrion in nonphotosynthetic nitrite reduction as previously suggested by other researchers (49, 50, 74).

Further, it has been reported (2) that the herbicides DNBP, 3,4-DCIPC, CIPC, 2,4,5-T and 3,4,5-TBA are capable of inhibiting the Hill reaction. This interference in photosynthetic electron flow by some of the same herbicides and related herbicides resulted in nitrite accumulation in leaf tissue as presented in Table 4.

Kessler (36) has reported that arsenate can also completely inhibit nitrite reduction in *Ankistrodesmus* while not affecting nitrate reduction. This suggests that the arsenical herbicides may also cause nitrite accumulation in higher plants.

Many studies have shown that herbicide application, especially to regions of cell elongation and differentiation, results in multinucleated cells, inhibition of cell division, and the absence of spindle fiber formation (2, 26, 38). All of these actions are similar to the action shown by the alkaloid, colchicine. Colchicine is known to be toxic to plants but is useful for inhibition of cell division and interference with spindle fiber formation for the purpose of chromosome doubling. It is interesting, though perhaps coincidental, that colchicine has also been reported to inhibit nitrite reduction (54). Nitrous oxide ($N_2O$), another chemical in popular use today for doubling chromosome numbers, is a decomposition product of nitrite reacting with metabolites of the plant and organic components of soils (18, 55, 69).

On the basis of the data presented and the review of literature concerning nitrite toxicity it is postulated that herbicides such as 2,4-D and others have no direct auxin-like or hormonal properites. It is believed that the nastic reactions and abnormal metablolism often following herbicide applications and preceding death are the result of a sequence of events:

1. Blockage of electron flow (photosynthetic or nonphotosynthetic) needed for nitrite reduction.
2. Accumulation of nitrite.
3. Chemical destruction of indoleacetic acid and chemical modifications of DNA, RNA and key metabolites by nitrous acid.
4. Undirected or misdirected growth of the cell due to hormonal imbalance and a chemically altered metabolism.

It can be postulated that nitrite accumulation is an artifact which occurs after the plant has died. This theory would suggest that although "death" has occurred, vital metabolic processes are still operating within the plant. Some of the processes still functioning are glycolysis, NAD reduction and the enzymatic reduction of nitrate to nitrite. In *in vivo* tests, although nitrite reduction has been blocked by herbicides, these same vital processes have been shown to function at normal rates for hours.

Nitrate reduction rates do not appear to be greatly reduced by most

herbicide treatments. In experiments with a large number of different herbicides, an apparent stimulation of nitrate reduction has been repeatedly noted similar to those shown in Figures 6 and 7. If this apparent stimulation is real, it could help to explain the many reports of increased protein synthesis and increased nitrate reductase activity with sub-toxic applications of herbicides (4, 20, 61, 72).

The hypothesis and interpretation of data as presented point out that nitrogen metabolism and herbicidal action are inseparably coupled. By the inhibition of nitrite reduction with little or no regulative control over the preceding step, nitrate reduction, herbicides can upset the delicately balanced metabolism of plants which can result in the accumulation of nitrite.

As stated by Ashton and Crafts (2) in their recent text, "The mechanism of action of the triazine herbicides in higher plants is a blockage of photosynthesis. More specifically, their site of action is within photosytem II at the photolysis of water step. However, their herbicidal activity involves more than a mere blocking of photosynthesis. The plants do not simply die from starvation due to lack of photosynthate. The phytotoxic symptoms of the triazine herbicides are not typical of starvation and they occur more rapidly than could be accounted for by lack of photosynthate. It appears that a reaction which is probably coupled to the photolysis of water results in the formation of a secondary phytotoxic agent which is primarily responsible for their herbicidal properties."

Evidence is presented that identifies this "secondary phytotoxic agent, primarily responsible for herbicidal properties" as nitrite. Photosynthetic inhibition does not appear to be the only process involved, but a general blockage of electron flow necessary for nitrite reduction in a wide variety of plant tissues. It is believed that the majority of herbicides cause injury and death to plants by this interference in electron transfer which results in nitrite accumulation.

### Surfactant Action and Specificity

Much of this research was aided by the use of certain nonionic surfactants included in the reaction medium. These surfactants greatly increase the penetration of herbicides into leaf tissue and facilitate the escape of nitrite out of the leaf into the reaction medium. The effect of Neutronyx 600, a nonylphenyl ethoxylate, on the *in vivo* nitrate reductase assay is shown in Figure 11. Like the effects of ethanol or propanol which are commonly used in this assay, the rates of nitrate reduction are not thought to be stimulated by the surfactants, but the escape of nitrite from the tissue into the solution is increased. With light incubation, nitrite does not normally escape reduction by the green leaf even with the use of surfactants. Results similar to those shown in Figure 11 have been obtained with numerous other other surfactants tested (data not shown).

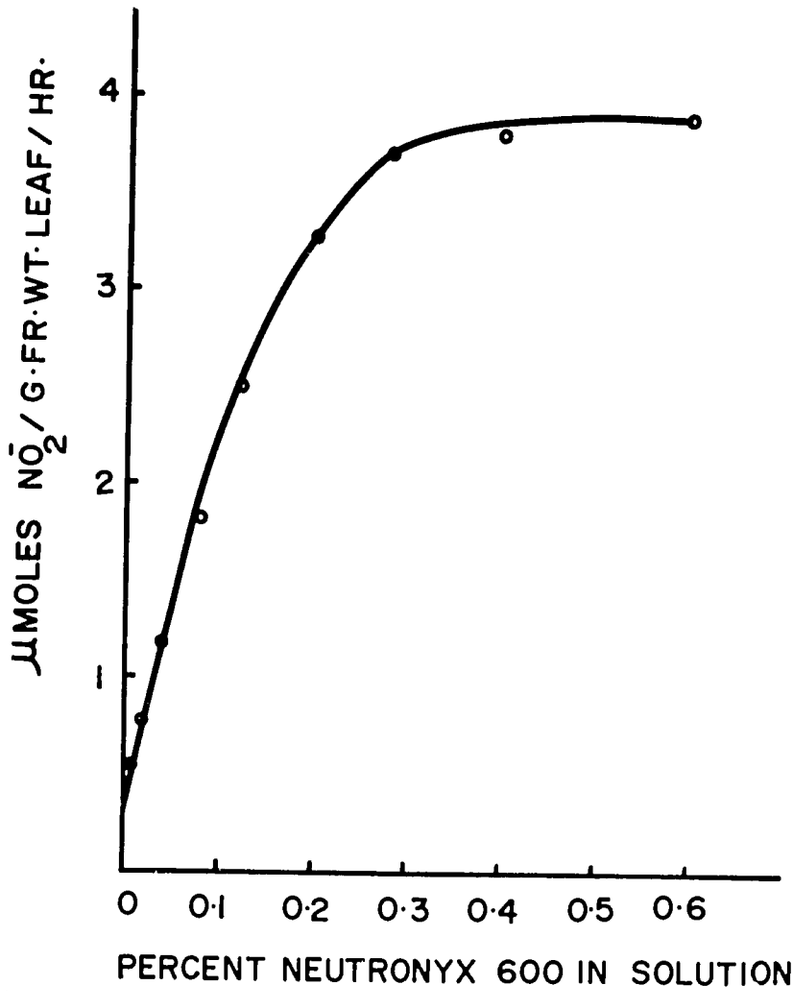



**Figure 11. The effect of Neutronyx 600 (a nonionic surfactant) concentration on rates of *in vivo* nitrate reduction by Centurk wheat leaf tissue. Only dark incubation is illustrated.**

The use of certain surfactants greatly increased the accuracy of the *in vivo* nitrate reductase assay. In comparisons of the basic reaction solution, the reaction solution containing 4 percent ethanol and the reaction solution containing 0.3 percent Neutronyx 600, the coefficient of variation for young Centurk wheat leaves with 10 replications of each treatment was 39.1, 39.3 and 12.4 for each treatment, respectively. Mean increases in measurable nitrate reductase activity were 8-fold using 4 percent ethanol and 58-fold for Neutronyx 600 over that of the reaction solution alone.

The leaf sections used in the *in vivo* assay had severed edges and vacuum infiltration was used to increase penetration of the tissue. To test whether surfactants aided in penetration of herbicides primarily through the severed edges or whether the penetration occurrred through the outer surface of the leaf, wheat leaf tissure was incubated in the light with only the terminal apices of the leaves in contact with the reaction medium. The reaction media contained $10^{-5}$ M atrazine with and without Neutronyx 600. No contact between solution and injured or cut tissue was made. The vacuum infiltration process was eliminated. Treatments containing both surfactant and atrazine began accumulating nitrite in the light within 30 minutes. Without surfactant, no nitrite accumulation was noted after 2 hours of incubation. This provides evidence that certain surfactants can aid in the penetration of leaf cuticular and epidermal layers to permit entry of relatively large foreign molecules and aid in the exit of metabolites such as nitrite which are normally contained within the cell. In another experiment in which leaf sections with their cut edges sealed by paraffin were utilized, similar results were obtained. Use of surfactants and vacuum infiltration of leaf sections allows for maximum penetration of herbicides. However, penetration occurs in intact leaf tissue without vacuum infiltration when the proper surfactants are used.

Large differences in surfactant specificity have been detected. Some nonionic surfactants permit rapid penetration of relatively large molecules into a given plant tissue while others have little effect upon the same plant tissue. Tables 7 and 8 show the effect of different surfactants upon *in vivo* nitrate reduction in the dark with leaf tissue of different plant species. In these tests, 0.2 percent of the different surfactants were included in the reaction medium and vacuum infiltration was not performed. In Table 7 different surfactants were tested for their ability to "open up" the monocots, foxtail, crabgrass and corn. For foxtail the most effective surfactant was Neutronyx 600, for crabgrass, Pronon 455 and for corn, Neutronyx 611. In Table 8 similar data for several dicot species are shown. The most effective surfactant for wild mustard was Zonyl A, for velvetleaf, Hyonic PE-70 and for soybean, either Pronon 210-T or Tergitol NP-14.

While surfactants are commonly used in herbicide formulations, they have been considered primarily as "spreaders" or "stickers." These research data indicate that there is specificity in that a surfactant can "open up" leaf tissue of a given plant species while not greatly affecting another. The *in vivo* nitrate reductase assay conducted in this manner can provide a rapid screening method for surfactant specificity. Such specificity should lead to better control of monocot weeds in monocot crops and dicot weeds in dicot crops by the choice of the most effective surfactants. This selection technique for surfactants should have its greatest benefit in foliar application of herbicides. Furthermore, the same herbicide possibly could be used for weed control in

**Table 7. The effect of different surfactants on *in vivo* nitrate reductase assays of different monocot species (dark incubation).**

| Surfactant[a] | μmoles $NO_2^-$ g fr wt$^{-1}$ hr$^{-1}$ Foxtail | Crabgrass | Corn |
|---|---|---|---|
| None | 0.3 | 0 | 1.0 |
| Hyonic PE-70 | 1.4 | 3.0 | 2.6 |
| Tergitol 4 | 0.6 | 0 | 1.2 |
| Zonyl A | 1.1 | 1.3 | .... |
| F-38 | .... | 2.7 | .... |
| F-183 | 0.4 | 0.4 | 1.6 |
| Surfynol 465 | 0.3 | 0 | 1.4 |
| Tergitol NPX | 0.1 | 2.3 | 1.8 |
| Tergitol TP-9 | 1.0 | 2.4 | 1.8 |
| Surfynol 450B | 0.8 | 0.6 | 1.7 |
| Tergitol NP-27 | 1.1 | 1.9 | .... |
| Pronon 455 | 3.0 | 5.7 | 2.1 |
| Tergitol NP-35 | 0.8 | 1.5 | 1.7 |
| Neutronyx 611 | 0.5 | 1.4 | 2.9 |
| Tergitol NP-14 | 0.5 | 0.9 | .... |
| Tergitol 7 | 0.4 | 1.1 | 1.9 |
| Tergitol XD | 0.6 | 0.4 | 1.4 |
| Tergitol TMN | 2.1 | 3.7 | 2.3 |
| Tergitol XH | 0.5 | 0.3 | 1.2 |
| Tergitol NP-33 | 1.3 | 3.0 | 1.4 |
| Tergitol 7X | 0.5 | 0 | 1.1 |
| Neutronyx 600 | 3.4 | 1.6 | 1.4 |
| Pronon 210-1 | 2.4 | 1.6 | 1.2 |
| Tergitol NP-40 | 1.3 | 0.5 | 1.0 |
| Surfynol 450 | 1.9 | 1.1 | 1.1 |
| Surfynol 475 | 0.9 | 0.1 | 0.9 |
| PE 4343 | 1.1 | 0.3 | 0.8 |

[a] All surfactants included in the reaction solution at 0.2 percent (w/v). Vacuum infiltration was eliminated.

**Table 8. The effect of different surfactants on *in vivo* nitrate reductase assays of different dicot species.**

| Surfactant[a] | μmoles $NO_2^-$ g fr wt$^{-1}$ hr$^{-1}$ Penny cress | Velvetleaf | Soybean |
|---|---|---|---|
| None | 0 | 0.3 | 5.8 |
| Tergitol NPX | 4.7 | 1.5 | 10.5 |
| Tergitol TP-9 | 3.9 | 1.2 | .... |
| Pronon 210-1 | 4.3 | 1.2 | 16.0 |
| Hyonic PE-70 | 3.5 | 1.8 | 10.0 |
| Surfynol 450 | 2.4 | 1.2 | 7.8 |
| Tergitol NP-14 | 2.8 | 0.5 | 15.9 |
| Zonyl A | 6.0 | 1.0 | 9.6 |
| Tergitol 4 | 2.1 | 0.6 | .... |
| Neutronyx 600 | 3.3 | 1.4 | 8.6 |

[a] All surfactants included in the reaction solution at 0.2 percent (w/v). Vacuum infiltration was eliminated.

different crops merely by the substitution of a different surfactant in the herbicide formulation.

Surfactants can aid not only in the penetration of herbicide molecules but could be used as well in connection with basic metabolism studies involving intact tissue, male gametocide applications in hybridization programs, and aid in the entry of insecticides, fungicides, plant hormones, and growth regulators into plant tissues.

The use of surfactants has identified some differences in the action of photosynthetic inhibitor herbicides as compared to the herbicides which are not classified as photosynthetic inhibitors. When effective surfactants and vacuum infiltration are used in combination with photosynthetic inhibitors, instant inhibition of nitrite reduction occurs. Even without the vacuum infiltration process, inhibition of nitrite reduction by phototynthetic inhibitors is rapid when effective surfactants are present. When other classes of herbicides are used, surfactants tend to interfere with their inhibition of nitrite reduction. Best results were obtained when 4 percent ethanol was substituted for surfactant in the reaction solution. Also noted during the course of this research was the immediate inhibition of nitrite reduction by the photosynthetic inhibitors while other herbicides commonly lagged for a 30-to-90 minute incubation period before inhibition began.

There are several possible theories which could help explain the differences noted in herbicidal action.

First, herbicides are known to be metabolized by the plant or to react with metabolites within the plant (2, 24). These reactions are largely degradation or deactivation processes of the herbicide molecule by the plant (2, 24). It is entirely possible that certain metabolic reactions upon the herbicide molecule could be an activation process which convert a relatively harmless compound into a phytotoxin. Surfactants used in this study could have interfered with this conversion.

A second hypothesis is that the nonionic surfactants are effective in penetrating the chloroplasts where photosynthetic inhibitors act and were ineffective in penetrating other organelles, possibly the mitochondria, where many of the other herbicides are believed to act.

A third hypothesis is that these surfactants interfere with a specific binding process necessary for these herbicides to act which is different from the photosynthetic inhibitors. These hypotheses are only suggested by the unexplainable lag period in the inhibition of nitrite reduction and the apparent interference of surfactants upon the action of certain herbicides as compared to the action of the known photosynthetic inhibitors.

## CONCLUSIONS

Evidence has been presented which implicates nitrite as a secondary phytotoxic agent responsible for initial injury, abnormal metabolism and the final death of plants after herbicide treatment. While little is known concerning the exact steps of the electron transfer pathways that are being inhibited, it is clear that energy necessary for nitrite reduction is being blocked by herbicides with a common result: nitrite accumulation. Interference in electron transfer in both photosynthetic and nonphotosynthetic tissue by herbicides has been well documented but nitrite has never before been implicated.

The techniques presented using the photosynthetic inhibitor herbicides are sufficiently refined for rapid and accurate screening of new chemicals for their potential as future herbicides. While the methods used with other divergent groups of herbicides were sufficient to demonstrate their gross effect upon nitrogen metabolism, they are relatively crude and are not yet ready for use as valid, rapid screening tests.

The implications of the identification and utilization of surfactant specificity are many. The use of this specificity in combination with herbicides for better weed control is but a single aspect. It is expected that future research in the areas involving applications of such chemicals as fungicides, insecticides, gametocides, hormones and growth regulators will be affected by these new techniques.

## APPENDIX I

List of common names and chemical nomenclature for all compounds tested or mentioned in the text.

| *Common Name* | *Chemical Nomenclature* |
|---|---|
| alachlor | 2-chloro-2′, 6′-diethyl-N-(methoxymethyl) acetanilide |
| ametryne | 2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine |
| amitrole | 3-amino-*s*-triazole |
| atrazine | 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine |
| benefin | N-butyl-N-ethyl-$\alpha,\alpha,\alpha$-trifluoro-2,6-dinitro-*p*-toluidine |
| bensulide | 0,0-diisopropyl phosphorodithioate S-ester with N-(2-mercaptoethyl) benzenesulfonamide |
| bromacil | 5-bromo-3-*sec*-butyl-6-methyluracil |
| bromoxynil | 3,5-dibromo-4-hydroxybenzonitrile |
| butylate | S-ethyl diisobutylthiocarbamate |
| CCP | carbonyl cyanide *m*-chlorophenyl hydrazone |

| | |
|---|---|
| CDAA | N,N-diallyl-2-chloroacetamide |
| CDEC | 2-chloroallyl diethyldithiocarbamate |
| chloramben | 3-amino-2,5-dichlorobenzoic acid |
| chloroxuron | 3-[*p*-(*p*-chlorophenoxy)phenyl]-1,1-dimethylurea |
| chlorpropham (CIPC) | isopropyl *m*-chlorocarbanilate |
| cycloate | S-ethyl N-ethylthiocyclohexanecarbamate |
| dalapon | 2,2-dichloropropionic acid |
| dazomet | tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione |
| DCPA | dimethyl tetrachloroterephthalate |
| dicamba | 3,6-dichloro-*o*-anisic acid |
| dichlobenil | 2,6-dichlorobenzonitrile |
| dicryl | 3′, 4′-dichloro-2-methyl acrylanilide |
| dinoben | 2,4-dichloro-3-nitro-benzoic acid |
| dinosam | 2-(1-methylbutyl)-4,6-dinitrophenol |
| dinoseb | 2-*sec*-butyl-4,6-dinitrophenol |
| diuron | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| EPTC | S-ethyl dipropylthiocarbamate |
| ioxynil | 4-hydroxy-3,5-diiodobenzonitrile |
| linuron | 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea |
| MCPA | [(4-chloro-*o*-tolyl)oxy] acetic acid |
| monuron | 3-(*p*-chlorophenyl)-1-1-dimethylurea |
| naptalam | N-1-naphthylphthalamic acid |
| nitralin | 4-(methylsulfonyl)2,6-dinitro-N,N-dipropylaniline |
| paraquat | 1,1′-dimethyl-4,4′-bipyridinium ion |
| PCP | pentachlorophenol |
| pebulate | S-propyl butylethylthiocarbamate |
| picloram | 4-amino-3,5,6-trichloropicolinic acid |
| prometone | 2,4-*bis*(isopropylamino)-6-methoxy-*s*-triazine |
| propachlor | 2-chloro-N-isopropylacetanilide |
| propanil | 3′, 4′-dichloropropionanilide |
| propazine | 2-chloro-4,6-*bis*(isopropylamino)-*s*-triazine |
| propham (IPC) | isopropyl carbanilate |
| pyriclor | 2,3,5-trichloro-4-pyridinol |
| simazine | 2-chloro-4,6-*bis*(ethylamino)-*s*-triazine |
| solan | 3′-chloro-2-methyl-*p*-valerotoluidide |
| trifluralin | $\alpha,\alpha,\alpha$,-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine |
| 3,4-DCIPC | 3,4-dichloro-isopropyl carbamate |
| 2,3,6-TBA | 2,3,6-trichlorobenzoic acid |
| 3,4,5-TBA | 3,4,5-trichlorobenzoic acid |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |

| | |
|---|---|
| 2,4-DNP | 2,4-dinitrophenol |
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |

## APPENDIX II

Manufacturers of surfactants used in this research.

| *Surfactant Used* | *Manufacturer* |
|---|---|
| Hyonic PE-70 | NOPCO Chemical Co.<br>60 Park Place<br>Newark, NJ 07111 |
| Zonyl 'A' | DuPont Chemical Co.<br>Dyes & Chemical Division<br>Room 1526<br>7 S. Dearborn St.<br>Chicago, IL 60603 |
| Tergitol 4<br>Tergitol NPX<br>Tergitol TP-9<br>Tergitol NP-27<br>Tergitol NP-35<br>Tergitol NP-14<br>Tergitol 7<br>Tergitol XD<br>Tergitol TMN<br>Tergitol NP-33<br>Tergitol 7X<br>Tergitol NP-40 | Union Carbide Chemical Co.<br>30-20 Thomson Ave.<br>Long Island City, NY 11101 |
| F-38<br>F-183 | Swift & Co.<br>Chemicals for Industry Dept.<br>115 W. Jackson Blvd.<br>Chicago, IL 60604 |
| Surfynol 465<br>Surfynol 450B<br>Surfynol 450<br>Surfynol 475 | Air Products & Chemicals<br>Chemicals Group<br>Five Executive Mall<br>Swedesforo Road<br>Wayne, PA 19087 |
| Pronon 210T<br>Pronon 455<br>Prosol E-4343 | Process Chemicals Co.<br>8733 S. Dice Rd.<br>Santa Fe Springs, CA 90670 |
| Neutronyx 600<br>Neutronyx 611 | Onyx Chemical Corp.<br>190 Warren St.<br>Jersey City, NJ 07302 |

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