AN ABSTRACT OF THE THESIS OF

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Title: The Physiological Effects and Fate of Dichlobenil (2,6-Dichlorobenzonitrile) in an Aquatic Environment

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The amounts of residual dichlobenil (2,6-dichlorobenzonitrile) in water and hydrosoil were compared periodically following application of the wettable powder and granular formulations of the herbicide to separate ponds at ten pounds of active ingredient per surface acre {0.6 ppmw (parts per million by weight)}. Dichlobenil was measured by electroncapture gas chromatography.

Maximum residual concentrations in the water after treatment with wettable powder or granules were 1.00 ppmw after four days and 0.68 ppmw after five days, respectively. After 15 days, the amounts of residue in the two ponds were similar, and decreased steadily to 0.001 ppmw or less after 126 days (last sampling date).

The maximum concentration of dichlobenil in the hydrosoil occurred 6 days after treatment with wettable powder and was 1.47 ppmv (parts per million by volume), while the granules produced a maximum concentration of 3.70 ppmv in one day. Residues in the hydrosoil of the two ponds reached similar concentrations 34 days after treatment. After 126 days the residue levels in the hydrosoil of both ponds had decreased to less than 0.04 ppmv. The persistence

of dichlobenil in pond water and hydrosoil was similar whether applied as a granular or a wettable powder formulation.

The growth rates of two aquatic vascular plants, *Potamogeton pectinatus* and *Alisma gramineum* var. *Geyeri*, and one aquatic alga, *Scenesdesmus obliquus*, were measured after exposure to various concentrations of dichlobenil in laboratory studies. *P. pectinatus* and *A. gramineum* were sensitive to 0.1 ppmw of dichlobenil, whereas *S. obliquus* was tolerant to concentrations as high as 10.0 ppmw.

Several laboratory studies were conducted in efforts to elucidate the mode of action of dichlobenil. Dichlobenil did not affect photosynthesis in either *P. pectinatus* or *S. obliquus* as determined by manometric measurements of oxygen evolution.

Utilization of oxygen by isolated potato mitochondria, measured by polarographic techniques, appeared to be stimulated slightly by 25 ppmw dichlobenil in the reaction medium during state 4 respiration. However, measurements of mitochondrial ATPase activity following additions of 10^{-5} or 10^{-4} M dichlobenil indicated that the action of dichlobenil in apparently stimulating respiration did not involve the uncoupling of oxidative phosphorylation.

The basis for the selectivity of dichlobenil was investigated by exposing *P. pectinatus* and *S. obliquus* to 10 ppmw radioactive dichlobenil for 72 hours. Two possible explanations were found for the selective action of dichlobenil. First, *S. obliquus*, the tolerant species, absorbed dichlobenil at a slower rate than the susceptible species, *P. pectinatus*. Secondly, *S. obliquus* apparently metabolized a greater percentage of the absorbed herbicide to

non-phytotoxic product(s) than did *P. pectinatus*. Proper standards were not available for the identification of the metabolite.

The Physiological Effects and Fate of Dichlobenil (2,6-Dichlorobenzonitrile) in an Aquatic Environment

by

Alex Grant Ogg, Jr.

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INTRODUCTION

The first reported synthesis of 2,6-dichlorobenzonitrile, hereafter referred to as dichlobenil, was by Norris and Klemka (1940). The herbicidal properties of dichlobenil were discovered independently by Philips-Duphar workers in Holland and by Shell Research Limited workers in Woodstock, England. Both groups found the chemical to be highly toxic to germinating seeds and buds of many species.

By 1963, dichlobenil had been shown to have herbicidal activity toward germinating seedlings of many weed species when applied to the soil, while many established perennial plants growing in the same soil appeared to be tolerant. Thus, the compound became widely recognized as a "pre-emergence herbicide". The effectiveness of pre-emergence applications of dichlobenil for the control of certain aquatic weeds was first reported in 1962 by Walker and Hiltibran.

The United States Department of Agriculture has registered dichlobenil under the trade name "Casoron" for weed control in woody ornamentals, fruit tree orchards and nurseries, shelterbelts and forest plantings, seed alfalfa, cranberries, grapes, caneberries, and impounded waters (aquatic weed control).

Early work with dichlobenil indicated effectiveness as a pre-emergence herbicide, and application was generally made before weed growth began. More recently, Van Busschbach and Elings reported it to be effective as a post-emergence herbicide in European canals.

Dichlobenil is now recognized to be effective for the control of many rooted aquatic plants and one species of attached green algae. However, in field tests, two rooted aquatic plants have been reported to be resistant to dichlobenil.

The physiological process whereby dichlobenil inhibits plant growth is relatively unknown. Physiological studies that would reveal the mode of action of dichlobenil may lead to improved methods of application and thus reduce the concentration needed for weed control. This in turn will result in lower residue levels.

The persistence of a herbicide in the environment following an application for weed control is a very important factor. It will often determine the value or commercial acceptance of a particular herbicide. Extended residual activity is usually beneficial in the control of annual weeds in perennial crops. In contrast, for aquatic weed control, it is important that the herbicide dissipate or degrade rapidly once the weeds are killed. Water from ponds and lakes is often used for irrigation, recreation, livestock water and human consumption. Herbicide residues remaining in the water can cause injury to crops and animals; thus the use of the water must be restricted until residues have decreased to safe levels.

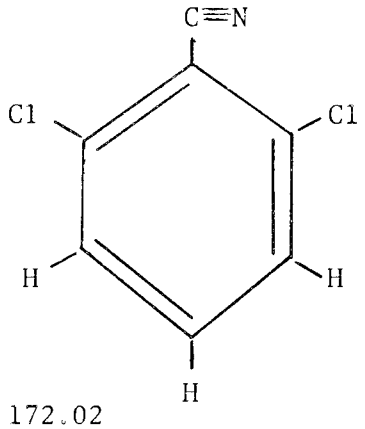
Dichlobenil is usually applied as a granular formulation either through the water or to exposed pond bottoms. It persists in the water and hydrosoil for at least 90 days, and therefore has not been registered for application in water which is to be used for crop irrigation, livestock water, or human consumption. Research is needed to find methods of reducing its residual life.

CHEMICAL AND PHYSICAL PROPERTIES OF DICHLOBENIL

In the United States, dichlobenil is manufactured by Thompson-Hayward Chemical Company. The chemical and physical properties of dichlobenil as listed in their technical information bulletin (1963) are as follows:

Chemical name: 2,6-dichlorobenzonitrile.

Structural formula:



Molecular weight: 172.02

Appearance: White crystalline solid.

Melting point: 144 degrees C..

Solubility: 25 ppm in water at 25 degrees C.. Less than 10% in most organic solvents.

Vapor pressure: 5.5×10^{-4} mm of mercury at 20 degrees C..

Stability: Relatively unaffected by heat or sunlight.

LITERATURE REVIEW

Aquatic Weed Control

The first reported uses of dichlobenil for aquatic weed control were by Walker (1962) and Hiltibran (1962). Working independently, these researchers found that dichlobenil was effective against the submersed pondweeds, *Potamogeton* spp. and *Najas flexilis* (Willd.) Rostk. and Schmidt. However, Walker noted that it did not appear to affect filamentous algae. He also reported that post-emergence applications were not effective in controlling aquatic plants.

Lawrence, Beasley and Funderburk (1963) conducted laboratory tests and found that dichlobenil applied at 2.0 ppm controlled 87% of the alligatorweed {*Alternanthera philoxeroides* (Mart.) Griseb.} after eight weeks. No new growth from dormant buds occurred within 16 weeks.

Further work by Frank, Hodgson and Comes (1963) in the laboratory and field showed that dichlobenil had outstanding activity against two troublesome aquatic plants, sago pondweed (*Potamogeton pectinatus* L. and American pondweed *Potamogeton nodosus* Poir.). All treatments were applied prior to emergence. In the laboratory tests, the shoots emerged from the hydrosoil and grew one to two inches before dying. No new leaves, branches or rhizomes were produced.

In the field trials, these authors noted that narrow leaf water plaintain (*Alisma gramineum* var. *Geyeri* Sam.) was apparently tolerant to the chemical and readily invaded the areas where the two *Potamogeton* species had been eliminated.

Field tests by Comes (1964) revealed that dichlobenil controlled the attached green alga *Chara vulgaris* L. as well as *P. pectinatus* L.. Moderate to dense infestations of *A. gramineum* var. *Geyeri* and *Zannichellia palustris* L. were observed in several plots where a high degree of *P. pectinatus* and *Chara* spp. control was obtained.

Hiltibran (1966) also reported that dichlobenil effectively prevented the growth of Chara. As indicated earlier, filamentous algae, such as *Cladophora* and *Pithophora*, were not affected.

Persistence

Massini (1961) conducted one of the first basic studies with dichlobenil. He found that its movement in soils and in plants was governed mainly by its relatively high volatility, low solubility in water, and strong adsorption on lignin, humic matter and lipid material.

In another study by Barnsley and Rosher (1961) the persistence of dichlobenil in agronomic soils was extended from a few days to several weeks when it was incorporated or watered in immediately after application. The lack of persistence of dichlobenil when applied to the soil surface and the modifying influence of soil incorporation and watering was attributed to its high vapor pressure and its relatively low solubility in water.

Hodgson (1966) investigated the effects of irrigation water contaminated with dichlobenil on alfalfa and sugar beets. He applied water containing 0.1 and 10.0 ppmw (parts per million by weight) dichlobenil to these crops by rill irrigation and determined

the effect on crop yield. Dichlobenil did not significantly reduce the yield of sugar beets at either rate, but 10 ppmw reduced yields of alfalfa.

Van Valin (1966) was the first to report on the residue levels of dichlobenil in an aquatic environment. He found only trace amounts of dichlobenil (0.001 ppmw) in the water 85 days after treatment with the wettable powder formulation at rates of 10, 20 and 40 ppmw. Concentrations of dichlobenil in hydrosoils after approximately four weeks were 0.2 ppmw or less. After 188 days, this level had decreased to 0.03 ppmw.

In another test involving the use of the granular formulation applied at 10 pounds per surface acre (0.6 ppmw in the water), the maximum concentration of dichlobenil (0.43 ppmw) occurred in the water after 20 days. At the last date of sampling, after 188 days, the dichlobenil residue was 0.001 ppm.

As would be expected, residues were highest in hydrosoils within the first few days, with the peak concentration of approximately 13 ppmw found two days after treatment. After 188 days, the residue had decreased to 0.10 ppm. The last hydrosoil sample, taken more than 14 months after treatment, contained 0.08 parts per billion by weight (ppbw).

Van Valin (1966) reported recoveries of 90% from hydrosoil, vegetation, and water samples spiked with known amounts of dichlobenil. Analysis was by electroncapture gas chromatography.

Dichlobenil residues in pond water and hydrosoil were also studied by Frank and Comes (1967). They found relatively high concentrations (8.8 to 10.7 ppmw) of dichlobenil in the upper one inch of the hydrosoil following application of 10 pounds of the granular formulation of the herbicide per surface acre. In one pond the maximum concentration of dichlobenil in the water (0.32 ppmw) occurred 36 days after treatment, while in a second pond the maximum (0.23 ppmw) occurred after eight days. On the last sampling date, 160 days after treatment, the water in these ponds contained 0.004 ppmw and 0.001 ppmw dichlobenil, respectively. Hydrosoil samples contained 1.34 and 0.23 ppmw dichlobenil, respectively, after 120 days.

Walsh and Heitmuller (1969) reported residues and compared changes in water chemistry and phytoplankton in a pond treated with 1.0 ppmw dichlobenil (wettable powder) to an untreated nearby pond. They found that the concentration of dichlobenil in the water and hydrosoil decreased steadily after treatment until negligible amounts were present after 64 days. They observed that as benthic plants died, a phytoplankton bloom and a large increase in the diel oxygen pulse occurred in the treated pond but not in the control. After treatment no distinct changes were observed in conductivity, pH, dissolved carbohydrate, alkalinity, nitrate nitrogen, and photosynthesis to respiration ratio. They concluded that four months after treatment, the pond, in general, appeared as it did before treatment.

Mode of Action of Dichlobenil

Koopman and Daams (1960) reported that dichlobenil caused the collapse of all cells in actively growing meristems, thus inhibiting the growth of young oat plants. They also stated that the germination of many weed seeds and the sprouting of stored potato tubers were inhibited by dichlobenil. Dichlobenil applied in a lanolin paste to the stems of bean plants caused a local swelling. The swelling was attributed mainly to an increase in diameter of the parenchyma cells. Experiments in which respiration or photosynthesis of tobacco leaves was measured in a Warburg apparatus revealed that neither of these processes was inhibited when the leaves were placed in a saturated solution of dichlobenil.

Daams and Barnsley (1961) found that dichlobenil did not inhibit respiration or photosynthesis in two species of green algae, *Chlorella* and *Scenedesmus*. Unfortunately, detailed descriptions of their experiments were not published.

Hull (1967) listed the symptoms resulting from a toxic level of dichlobenil in the plant as follows:

> A rapid growth inhibition, followed by a gross disruption of tissue, notably in the meristems and phloem, which may result in swelling or collapse of stem, root and petiole, and a generalized brown discoloration, frequently accompanied by the exudation of gummy material from shoots. Leaves may abscise and exhibit black discolorations over veins and a deepened blue-green color intensity interveinally.

Milborrow (1964) described symptoms of dichlobenil injury in plants and compared them with symptoms produced by boron deficiency. The effects on the macroscopic and microscopic appearance of plants

and on their ability to translocate growth regulators were so alike that Milborrow proposed that both dichlobenil and boron deficiency affected the same basic process.

The characteristic effects of boron deficiency are inhibition of shoot and root growth, followed by browning and death of the meristematic cells (Gauch and Duggar, 1954). Plants suffering from boron deficiency have been found to be less sensitive to growth regulating substances (Dyar, 1960) and are apparently less able to translocate sugar.

Milborrow (1964) applied dichlobenil in a lanolin paste to the upper portions of the stems of tomato plants. Four days later he applied 2,4-dichlorophenoxyacetic acid (2,4-D) in a lanolin paste to the lower portions of the same plants. Extreme epinasty occurred in all parts of the plants below the point of dichlobenil application, but none occurred above that point, suggesting that dichlobenil had prevented the translocation of the 2,4-D into the upper portions of the plants.

Foy and Penner (1965) studied the effect of dichlobenil on the respiration of isolated cucumber mitochondria and found that dichlobenil stimulated oxygen consumption 162 percent compared to the controls. They also reported that dichlobenil uncoupled oxidative phosphorylation. Dichlobenil at 1.45×10^{-4} M reduced the P/O ratio (micromoles of phosphate esterified per hour divided by microatoms of oxygen consumed per hour) 75 percent. The reduction in the P/O ratios was caused by both a decrease in inorganic phosphate esterified and an increase in oxygen consumed.

Wit and van Genderen (1966a) determined the metabolites of dichlobenil fed to rabbits and rats and then considered the interference of these metabolites with energy metabolism as a possible mode of action of dichlobenil (Wit and van Genderen, 1966b). They found that two of these metabolites (2,6-dichloro-3-hydroxy-benzonitrile and its 4-hydroxy analogue) added to starved yeast cells incubated with a limited quantity of glucose caused a significant rise in oxygen consumption of the cells. The same compounds induced adenosinetriphosphatase activity in isolated rat-liver mitochondria with the 4-hydroxy analogue being about three times as active as the 3-hydroxy derivative.

The experiments with the yeast-cell suspensions as well as with the isolated intact rat-liver mitochondria demonstrated the uncoupling activity of the two dichlobenil metabolites found by these authors. No activity was found with dichlobenil or 2,6-dichlorobenzoic acid.

Absorption, Translocation and Metabolism

Massini (1961) determined the adsorption coefficients (k) of dichlobenil for various substances and plant fractions. He found a k value of 400-1000 for lignin, 1 for cellulose, 17 for protein, and 180-300 for lipids. However, the values indicated for lipids represent partition coefficients between two liquid phases rather than adsorption coefficients and therefore they cannot be compared with the other values. Determination of k values for the plant fractions revealed that chloroplasts had the highest k value (230), followed by roots (80), stems (50), and seeds (10-100). Massini reasoned that the high adsorption coefficient for chloroplasts might be related to their high lipid content.

Massini also studied the absorption and translocation of radioactive dichlobenil in French dwarf bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), and oat (*Avena sativa*). He found similar results for all species and therefore reported only those data for the dwarf bean in detail.

Bean seedlings were exposed to a saturated atmosphere of radioactive dichlobenil at room temperature for four days, and absorbed the herbicide almost uniformly by all aerial parts. The rate of uptake was constant for the first three days, then it decreased. He calculated that in three days the bean plant had accumulated about 350 micrograms of dichlobenil per gram dry weight. Massini used autoradiograms to measure absorption of dichlobenil and therefore was not able to state whether the compound was merely adsorbed on the epidermis or if it was actually taken up by the plant. In a subsequent experiment he was able to show that at least a small part of the radioactivity was taken up and had reached the vascular tissues.

Massini reported also on the absorption and translocation of dichlobenil from an aqueous solution through the roots of whole plants and stem cuttings of bean plants. He concluded that the absorbed dichlobenil moved with the transpiration stream, but much more slowly than water because of the strong affinity between the herbicide and the plant tissue. When Massini applied radioactive dichlobenil in a lanolin paste around the epicotyl of a bean seedling, the substance was taken up very slowly and translocated, mainly upwards, with the transpiration stream. Downward translocation was very feeble.

The metabolism of dichlobenil was studied in bean seedlings which had been exposed to the vapor of radioactive dichlobenil for six days. Plants were then ground in liquid nitrogen and freeze-dried. The plant powder was extracted with 2N HCl in ethanol for 10 minutes at the boiling point. The extract was analysed by thin-layer chromatography and two spots of equal activity were detected. One spot coincided with dichlobenil; the other spot was not identified, but thin-layer chromatography revealed that it was not 2,6-dichlorobenzoic acid.

Additional studies of absorption, translocation and metabolism of radioactive dichlobenil were conducted by Pate and Funderburk (1966). Autoradiograms of bean plants and alligatorweed indicated that radioactive dichlobenil was only slightly absorbed into the leaf and little translocation occurred following foliar application. However, plants with roots submersed in nutrient solution containing labeled dichlobenil absorbed and translocated the herbicide throughout the plants.

In a second study, autoradiograms indicated limited absorption and basipetal translocation of radioactive dichlobenil following an application on the leaf surface. Acropetal movement appeared to occur readily. The rate of movement and the distribution pattern observed following acropetal translocation indicated that radioactive dichlobenil moved within the xylem tissues, but more slowly than the transpiration stream. These author's findings support Massini's (1961) data.

Two higher plants (bean and alligatorweed) and four fungi (*Fusarium* sp., *Geotrichum* sp., *Penicillium* sp. and *Trichoderma* sp.) were selected by Pate and Funderburk for metabolism studies. Bean plants and alligatorweed were placed in Hoagland and Arnon's nutrient solutions containing radioactive dichlobenil and the herbicide was added to liquid cultures containing the fungi for 12 to 120 hours. Extracts from the plants and fungi were chromatographed on thin-layer plates of silica gel. Autoradiograms of the thin-layer chromatograms of aqueous extracts revealed a radioactive compound with an R_F of 0.25 while dichlobenil had an R_F of 0.6. The former compound was subsequently identified by co-chromatography as 2,6-dichlorobenzoic acid.

The aqueous extract was also esterified and chromatographed. A ^{14}C-labeled compound having an R_F of 0.95 was observed. The esterified aqueous extracts chromatographed precisely with methyl-2,6-dichlorobenzoate. Gas chromatography of the ^{14}C-labeled compound with an R_F of 0.95 also exhibited a retention time identical to that of methyl-2,6-dichlorobenzoate. Therefore, the radioactive compound observed at R_F 0.25 was thought to be a metabolite (2,6-dichlorobenzoic acid) and not merely a complex or aggregate which might occur in a non-living system.

A larger quantity of the metabolite was found in the alligatorweed extracts than in the bean extracts. Since no data were recorded on the quantity of radioactive dichlobenil absorbed by each species, no conclusions could be drawn on the relative ability of the two species to degrade dichlobenil.

Radio-assay of aqueous extracts of the various micro-organisms indicated that *Fusarium* sp. degraded considerably more dichlobenil than did other fungi.

Wit and van Genderen (1966a) studied the metabolism of dichlobenil in rabbits and rats. Following oral administration of dichlobenil to the animals, 2,6-dichloro-3-hydroxy-benzonitrile and its 4-hydroxy analogue were identified as metabolites in the urine. A small amount of unchanged dichlobenil was recovered from the feces.

Since only insignificant amounts of the amide and benzoic acid derivatives were found, Wit and van Genderen concluded that the hydrolysis of the nitrile group of the molecule occurred to a very minor extent.

Recent studies by Verloop and Nimmo (1969) utilizing bean confirmed Wit and van Genderen's findings that 3-hydroxy-2,6-dichlorobenzonitrile and its 4-hydroxy analogue were the major metabolites of dichlobenil. They found that the ratio of these two metabolites, one to another, was four to one, with the 3-hydroxy metabolite predominating. Their results also indicated that hydrolysis of dichlobenil to 2,6-dichlorobenzoic acid was a minor metabolic route.

Dichlobenil was absorbed by the bean roots and accumulated about three-fold before being translocated throughout the plant. After translocation to the leaves two competitive processes took place. Approximately 90% of the dichlobenil evaporated and about 75% of the remaining dichlobenil was metabolized.

Verloop and Nimmo correlated phytotoxicity in bean leaves to total metabolites and found the critical concentration to be about

one microgram per gram fresh weight.

Price and Putnam (1969a) also found that dichlobenil evaporated from leaves. Working with corn seedlings, they found that 70 to 80% of the absorbed dichlobenil was emitted within 24 hours. They also found that dichlobenil could be lost directly to the atmosphere from an aerated solution. The loss was found to be proportional to the amount of aeration.

Price (1969) found that dichlobenil induced the leakage of beta-cyanin and reducing sugars from red beet root sections, indicating a disruption of the tonoplast and plasmalemma.

PURPOSE OF STUDY

Although dichlobenil has been used to control aquatic weeds in ponds and lakes, its effects and fate in the aquatic environment are relatively unknown. When applied as a granular material, dichlobenil may persist in the water and hydrosoil for at least 90 days and in some cases longer. Since this residue may injure crops and animals the use of the treated water must be restricted.

One of the purposes of this research was to compare the persistence of dichlobenil in water and in hydrosoil following treatment with equivalent amounts of the granular and wettable powder formulations. Hopefully, utilization of the wettable powder formulation might allow a more rapid breakdown or dissipation of dichlobenil. Changes in dichlobenil concentrations that occurred in the water and in the hydrosoil from the two types of treatments were monitored during a period of 126 days after application.

Elucidation of the physiological processes whereby dichlobenil inhibits plant growth would aid greatly in developing improved methods of application and would possibly lead to reduction of the residue in the environment.

Although dichlobenil has been used primarily as a pre-emergence treatment, there have been reports that it is also effective as a post-emergence herbicide. Post-emergence activity could indicate that dichlobenil was interfering with some phase of photosynthesis or respiration. Therefore, the effects of dichlobenil on photosynthesis and respiration were thoroughly investigated. Dichlobenil

has also been reported to uncouple oxidative phosphorylation. Tests were made to determine if this uncoupling activity is operating in the mitochondrial system used in this study.

Several field trials have indicated that some vascular aquatic plants are less susceptible than others to dichlobenil. Most algal forms are tolerant, but one alga (*Chara* spp.) has been reported to be susceptible. The basis for this selectivity has not been investigated. A better understanding of this selectivity could provide information about the mode of action of dichlobenil and ultimately could lead to improved application methods and less environmental contamination. Thus, the tolerance or susceptibility of several species of aquatic plants to dichlobenil was determined, and the basis for selectivity was examined.

RESIDUES OF DICHLOBENIL IN WATER AND HYDROSOIL

Introduction

Several investigators have reported that dichlobenil persists in the water and hydrosoil of treated ponds for as long as 188 days following application of the four percent granular formulation at recommended rates. Herbicide residues in the water can cause injury to crops and animals, hence use of the water must be restricted until residue levels decrease to a safe level. Since restricting the use of the water for prolonged periods is not always feasible, methods should be developed to reduce the persistence of dichlobenil without impeding its weed control potential.

As mentioned earlier, when Van Valin (1966) applied granular dichlobenil at the recommended rate of 10 pounds per surface acre (approximately 0.6 ppmw in the water if all the herbicide was dissolved at once), the concentration in the water increased to a maximum of 0.43 ppmw after three weeks and then steadily decreased. However, when dichlobenil was applied as a wettable powder formulation at 20 ppmw (approximately 33 times the recommended rate), the concentration in the water 11 days after treatment decreased to 1.60 ppmw and no residue could be detected after 85 days.

If this same relationship could be repeated with the recommended rate of dichlobenil, the wettable powder formulation might prove to be more useful than the granular formulation in situations where prolonged restriction of water use is not possible. This was the basis for conducting this residue study.

Materials and Methods

Commercial formulations of granular and wettable powder dichlobenil were obtained from Thompson-Hayward Chemical Company, Kansas City, Missouri. The granular material was formulated as a coarse clay particle and contained 4% dichlobenil. The wettable powder was formulated as a fine powder and contained 50% dichlobenil. The granular material was applied with a hand-powered granular herbicide spreader. The wettable powder was mixed with water and sprayed uniformly over the water surface.

A small man-made pond having a surface area of 0.45 acres and an average depth of five feet was selected as the test site. The pond was located adjacent to Soap Creek in the MacDonald Forest area 10 miles north of Corvallis, Oregon. Water from springs was diverted into the pond to maintain the water level. There was no overflow from the pond. One-eighth of the area was partitioned from the main portion of the pond by a heavy plastic film. The plastic extended from 12 inches above the water surface into the hydrosoil below, thus forming two separate bodies of water. The smaller area was treated on June 15, 1967, with the wettable powder at the rate of 10 pounds of dichlobenil per surface acre (0.6 ppm in the water). The remaining larger area was treated on the same date with the granular material at the same rate.

Water and hydrosoil samples were taken from each pond one day after treatment and periodically thereafter. Each sample consisted of 400 ml and was made up of five subsamples taken at random in each

of the treated areas. Water samples were collected 12 inches above the hydrosoil. Hydrosoil samples were collected with a special sampling device (Figure 1) that collected only the upper one-fourth inch of the semifluid hydrosoil layer. The last samples were taken 126 days after treatment. At that time, heavy rains had caused flooding in the plot area and further sampling was not possible. All samples were stored in a refrigerator at approximately 0° C until they were analyzed.

Samples were analyzed for dichlobenil by modifications of procedures developed by Meulemans and Upton (1966). The water was extracted by vigorously shaking the sample in a 1000-ml separatory funnel with 100 ml of redistilled benzene for three minutes. After discarding the aqueous phase, sufficient anhydrous sodium sulfate was added to dry the benzene. After decanting the dried benzene into a graduated cylinder, five to ten ml of benzene were used to wash the sodium sulfate. These washings were added to the original benzene portion and the total volume recorded. Recovery of dichlobenil from samples containing known amounts of herbicide averaged 97% by this method.

Extraction of the highly organic hydrosoil samples proved to be much more difficult. When the samples were shaken with benzene, a viscous emulsion formed and separation of the two layers in a separatory funnel was not possible. A special technique was developed to overcome this problem. Each hydrosoil sample was divided approximately into thirds by transferring the sample into three 250-ml centrifuge bottles. After adding 50 ml of redistilled benzene, each

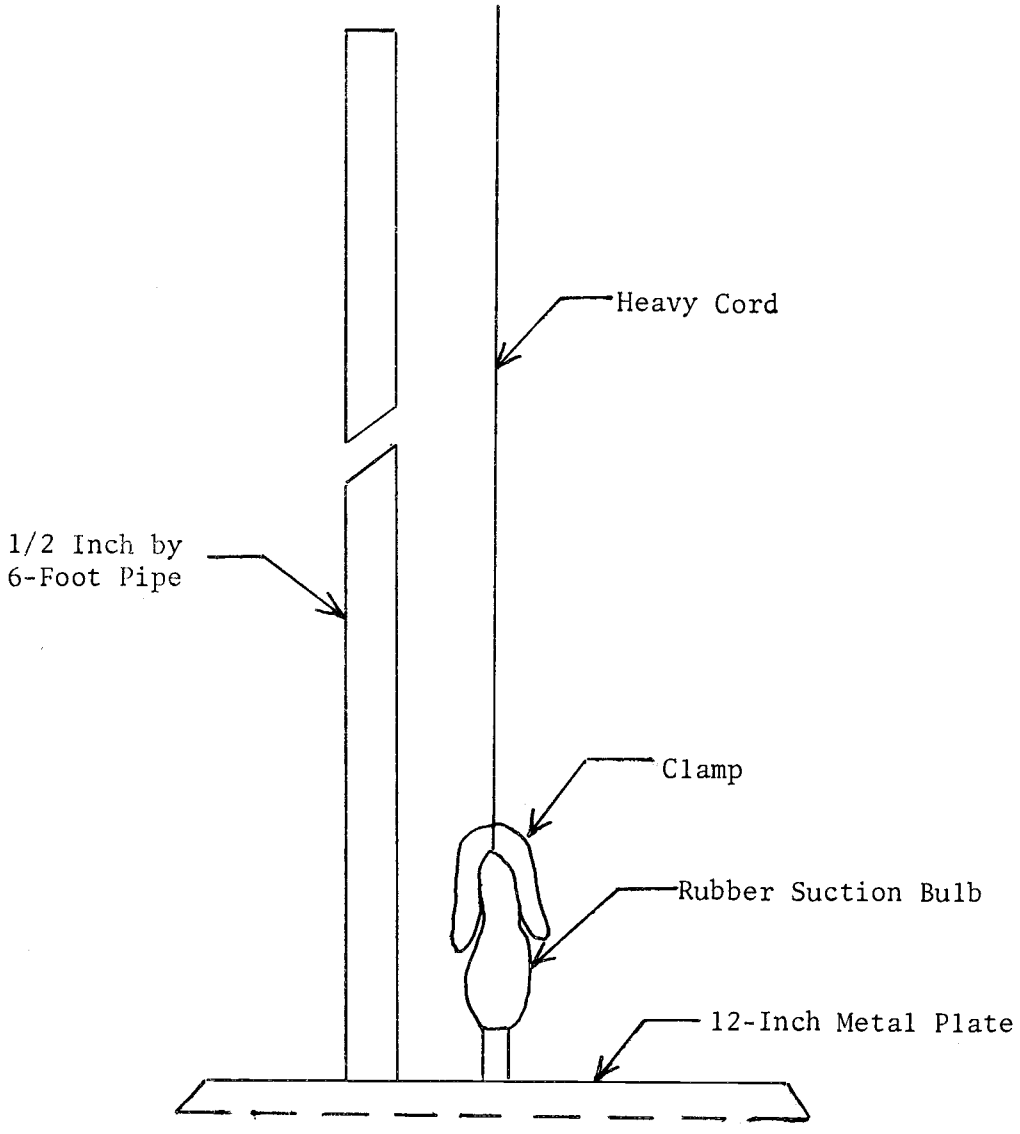


Figure 1. Hydrosoil sampler. After placing sampler on the pond bottom, the cord was pulled to remove the clamp and the bulb expanded, drawing in approximately 80 ml of the hydrosoil.

centrifuge bottle was tightly sealed and shaken vigorously for three minutes. Next, the tubes were centrifuged for 10 minutes at 5,000 x g to separate the two phases. The benzene layer was carefully removed by aspiration until only a thin film remained. Five to 10 ml of benzene were carefully added to the surface of the aqueous layer. After gentle swirling, the remaining benzene was removed by aspiration and combined with the original extract. With some samples, it was necessary to repeat the extraction procedure to remove all of the organic material. The final step was the addition of anhydrous sodium sulfate to dry the benzene. As before, the sodium sulfate was washed with benzene and the solvent portions combined and the final volume recorded. Recovery of dichlobenil from samples containing known amounts of herbicide averaged 83% by this method.

The hydrosoil samples were a variable mixture of mineral soil, organic matter and water. Usually the solid portion comprised about 50% of the samples. Due to the inherent variability of these samples, results will be expressed on the basis of parts per million by volume (ppmv).

For these analyses, a Wilkens Model 500-D gas chromatograph equipped with an electron capture detector and a one millivolt recorder was used. Nitrogen gas flowing at 42 cc per minute carried the sample through a five-foot by one-eighth inch stainless steel column packed with 10% S. E. 30 on Gas Chromosorb Q (60/80 mesh). Injector, column, and detector temperatures were 150, 150, and 175° C, respectively. Duplicate injections of two to four microliters of each sample were made to determine the concentration of dichlobenil.

Quantification was based on comparison of recorded peak height with suitable standards. Retention time for dichlobenil was two and a half minutes and the detection limit was 0.001 ppm.

Results and Discussion

Concentrations of dichlobenil in the water and in the hydrosoil were compared after field applications of wettable powder and granules to separate ponds at 10 pounds per surface acre (0.6 ppmw in the water).

Maximum concentrations in the water after treatment with wettable powder and granules were 1.00 and 0.68 ppmw, respectively (Figure 2 and Appendix Table 1). These maxima were reached after four and five days, respectively. After 15 days, the concentrations in the water were similar, and both decreased rapidly to approximately 0.1 ppmw after 34 days. Thereafter, there was a much slower, but steady decrease in the concentration. On the last sampling date, 126 days after treatment, the concentration of herbicide in the water of both ponds was so low as to not be reliably detectable (<0.001 ppmw).

The maximum concentration measured in the water following treatment with the wettable powder formulation was 67% greater than the initial treatment concentration. Stratification of dichlobenil near the bottom of the pond would explain this result, since the water samples were taken 12 inches from the hydrosoil surface. Wilson and Bond (1969), investigating the effects of dichlobenil on pond invertebrates, observed that dichlobenil formed a concentrated layer near the bottom of their test vessels. Their results were based on

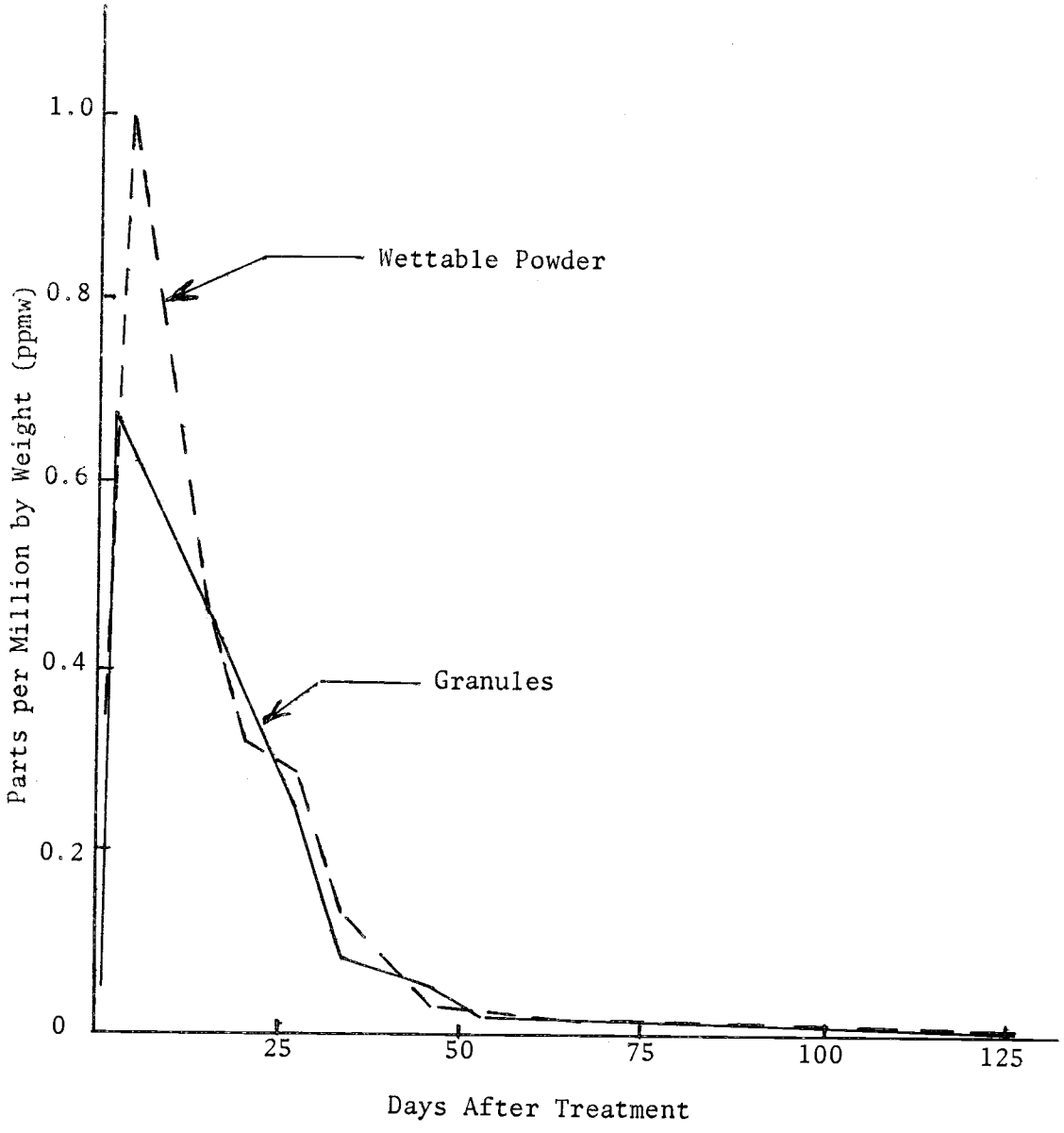


Figure 2. Residual dichlobenil in water samples.

observations of the immobilization effect of dichlobenil on the invertebrates.

The results of Frank and Comes (1967) also strengthen the stratification hypothesis. In a pond treated with granular dichlobenil, they found higher concentrations at the 10-foot depth than at the three-foot depth. The differences in concentrations were most noticeable during the period when maximum concentrations occurred.

Van Valin (1966) also found higher concentrations of dichlobenil in the water than were initially applied. He discounted stratification of dichlobenil as a probable cause since his samples were made up of subsamples taken from different depths. He theorized that some of the powder from the formulation could have been floating at the surface where it could easily have become part of the sample.

The maximum concentrations of dichlobenil in the hydrosoil after treatment with the wettable powder and granular formulation were 1.472 and 3.700 ppmv, respectively (Figure 3 and Appendix Table 2). These concentrations occurred 6 and 1 days after treatment respectively. Residues in the hydrosoil of both ponds were approximately 0.4 ppmv 34 days after treatment. One hundred and twenty-six days after treatment, the concentration in the pond treated with the wettable powder had decreased to 0.039 ppmv and to 0.025 ppmv in the pond treated with granules.

Residues in the hydrosoil of the pond treated with the wettable powder increased from 0.560 ppmv after one day to 1.470 ppmv after six days. These data also suggest that stratification of the

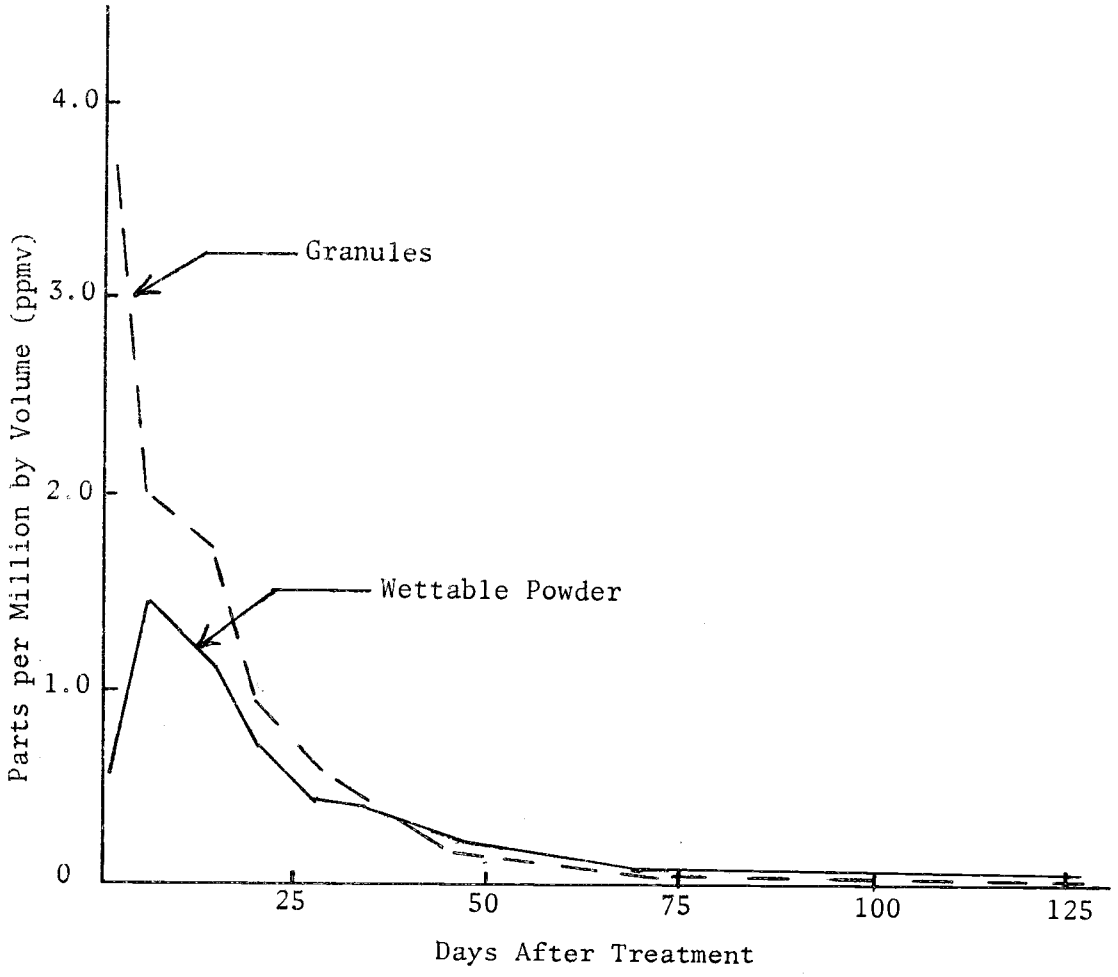


Figure 3. Residual dichlobenil in hydrosoil samples.

dichlobenil near the bottom occurred following an application of the wettable powder formulation. The persistence of dichlobenil in the water and hydrosoil of the pond studied was similar, whether applied as a granular or wettable powder formulation.

EFFECTS OF DICHLOBENIL ON THE RATE OF GROWTH IN SELECTED AQUATIC PLANTS

Introduction

Most aquatic vascular plants are controlled by field applications of dichlobenil at rates of one to two ppmw. However, *Alisma gramineum* var. *Geyeri* and *Zannichellia palustris* have been reported as being tolerant species. Except for Chara species, algal forms have also been reported as being tolerant to field applications of dichlobenil.

Growth studies were conducted in the laboratory using various rates of dichlobenil to determine the susceptibility or tolerance of two aquatic vascular plants, *Potamogeton pectinatus* and *A. gramineum*, and one species of green algae, *Scenedesmus obliquus*.

Materials and Methods

Potamogeton pectinatus plants were grown from subterranean tubers planted in washed river sand contained in one gallon glass jars. Each jar contained 500 cc (cubic centimeters) of sand and 3000 ml of one-tenth strength Hoagland's solution. Water temperature was maintained at 20° C and light intensity from cool white fluroescent lamps was 250 foot-candles at the water surface. Compressed air was bubbled through the water at the rate of 200 cc per minute.

Plants were two to four inches tall and had two to four leaves at the time of treatment. Fresh nutrient solution was added just before the treatments were applied. Granular dichlobenil was added

to randomly selected jars to give final water concentrations of 0.0, 0.1, 0.5, 1.0, 5.0 or 10.0 ppm. Each treatment was replicated five times.

Two weeks after treatment all plants were clipped at the sand surface and dry weights determined after drying for 48 hours at 65° C.

Alisma gramineum plants were grown from seed that was planted in sandy loam soil and covered with one-fourth inch of washed sand. Other culture conditions were similar to those described for *P. pectinatus*, except that the light intensity was 75 foot-candles.

When the plants were three to five inches tall and had three to four leaves they were treated with the granular form of dichlobenil. Granules were applied to randomly selected jars to give final water concentrations of 0.0, 1.0, 5.0 or 10.0 ppmw. Each treatment was replicated three times.

Visual observations of the degree of control were made two weeks after treatment.

A culture of *Scenedesmus obliquus* (Turp.) Kutz, (strain D_3), a unicellular green alga, was obtained from Dr. N. I. Bishop's laboratory at Oregon State University. Suspensions of this organism were grown in a lighted growth room maintained at 25° C. A 96:4 mixture of air and carbon dioxide was bubbled through the inorganic growth medium. The composition of the medium is given in Appendix Table 3.

Six culture tubes each containing 250 ml of sterile culture medium were inoculated with ten µl (microliters) each of *S. obliquus* cells. Analytical grade dichlobenil was dissolved in 100% ethanol

and added to randomly selected culture tubes to give final dichlobenil concentrations of 0.0, 1.0, 5.0 or 10.0 ppm. Each treatment was replicated twice. The experiment was also repeated.

After five days, treatments were evaluated by measuring packed-cell volumes and total chlorophyll produced in the culture. Packed-cell volumes were determined by centrifuging 5.0 ml of each culture suspension in a graduated sedimentation tube for ten minutes at approximately 1,000 x g. Total chlorophyll was determined by the spectrophotometric method of Arnon (1949). One milliliter of alga suspension and 9.0 ml of 100% methanol were placed into a glass stoppered Pyrex centrifuge tube and heated with constant shaking for two minutes in a water bath at 65° C. Tubes were centrifuged for 10 minutes at 1,000 x g in a clinical centrifuge. Absorbance was measured at 652 mμ (millimicrons) in a Beckmann Spectronic 20 spectrophotometer and total chlorophyll calculated by the following formula:

$$\text{Total chlorophyll (mg/l)} = \frac{\text{O.D. (652 m}\mu\text{)} \times 1000}{34.5}$$

Results and Discussion

The addition of 0.1 ppm dichlobenil to the nutrient solution in which *P. pectinatus* were grown for two weeks reduced plant dry weight by 48.5% (Figure 4). Further decreases in dry weights occurred when the dichlobenil concentration was increased from 0.1 to 5 ppmw. Increasing the concentration of dichlobenil from 5 to 10 ppmw did not cause a comparable decrease in dry weight. Visual observations of the plants treated with 5 and 10 ppmw revealed that they did not

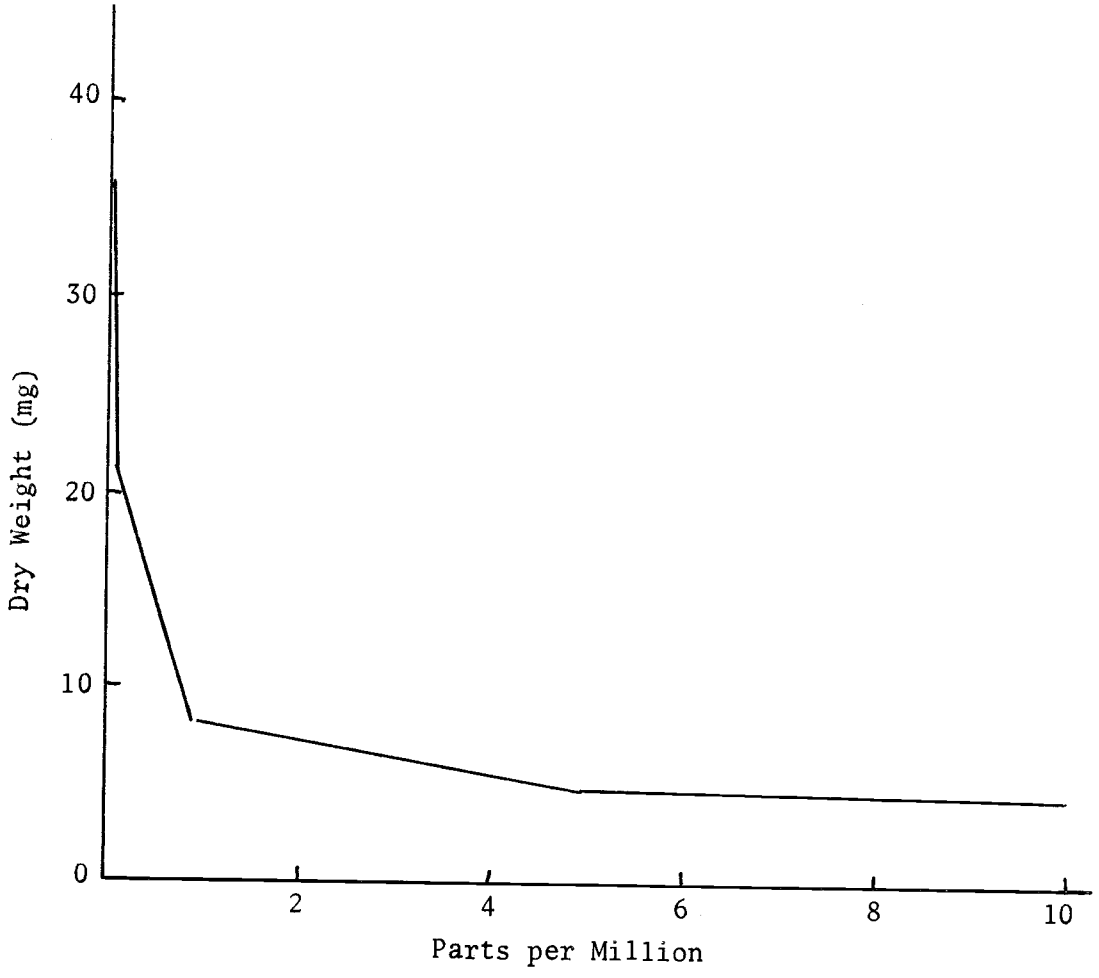


Figure 4. Average dry weights of *P. pectinatus* plants two weeks after treatment with various concentrations of dichlobenil.

make any further growth after treatment. The dry weight values for plants treated with 5 and 10 ppmw probably represent the weights present at the time of treatment. At the time of evaluation, all treated plants were brown and necrotic, except those in the 0.1 ppmw treatment, and only the basal stem portions of these were green. In all of the treated plants all of the meristematic tissue appeared dead.

Although 0.1 ppmw of dichlobenil caused a significant reduction in the dry weight of *P. pectinatus*, 0.5 ppmw was required to kill all of the tissue. These results support the published information that *P. pectinatus* can be classified as a susceptible species.

Visual observation of *A. gramineum* plants two weeks after treatment with dichlobenil at rates of 1, 5 and 10 ppmw revealed that all of the plant tissue was brown and necrotic. On the other hand, untreated plants appeared green and healthy. The tolerance of *A. gramineum* reported from studies of applications of dichlobenil in the field could not be demonstrated under laboratory conditions and this plant appeared to be as susceptible to dichlobenil as *P. pectinatus*.

Dichlobenil did not interfere with the growth (Figure 5) or chlorophyll production (Figure 6) of *S. obliquus* under the conditions of this test. Daily observations of the algal cultures revealed no distinct differences. *Scenedesmus obliquus* was capable of normal growth and chlorophyll production and appeared completely resistant to dichlobenil.

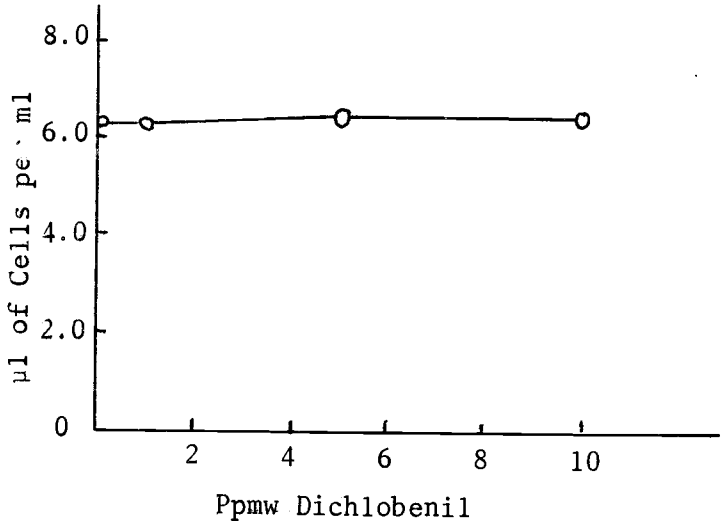


Figure 5. Averaged packed cell volume of *S. obliquus* treated with various concentrations of dichlobenil.

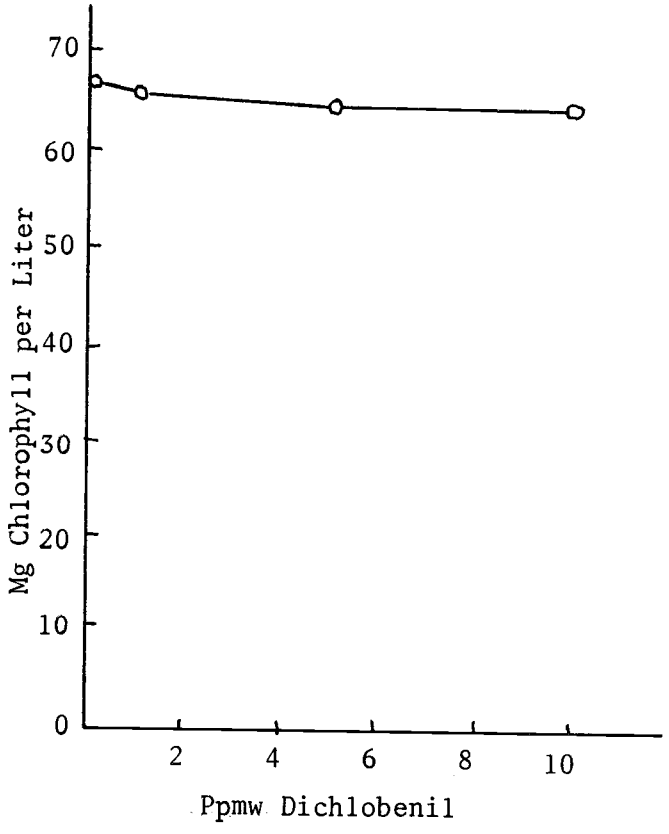


Figure 6. Concentration of total chlorophyll in *S. obliquus* treated with various concentrations of dichlobenil.

Potamogeton pectinatus was selected as the susceptible species and *S. obliquus* as the tolerant species for additional physiological studies.

EFFECT OF DICHLOBENIL ON PHOTOSYNTHESIS, RESPIRATION AND OXIDATIVE PHOSPHORYLATION.

Introduction

Reports in the literature of the effect of dichlobenil on photosynthesis, respiration, and oxidative phosphorylation appear to be contradictory. Koopman and Daams (1960) and Daams and Barnsley (1961) reported that dichlobenil did not affect photosynthesis or respiration of tobacco leaves or of whole cells of *Chlorella* and *Scenedesmus*. Wit and van Genderen (1966a) observed no effect on the respiration of yeast cells or on the activity of adenosine triphosphatase (ATPase) in rat-liver mitochondria following the addition of dichlobenil. On the other hand, Foy and Penner (1965) found a marked stimulation of respiration and a decrease in phosphate esterification in isolated cucumber mitochondria exposed to dichlobenil *in vitro*.

In this study, several physiological experiments were conducted to help elucidate the effect of dichlobenil on photosynthesis, respiration and oxidative phosphorylation.

Photosynthesis

Materials and Methods. The unicellular green alga, *S. obliquus*, and the aquatic vascular plant, *P. pectinatus*, grown as previously described, were utilized to study the effect of dichlobenil on oxygen evolution (photosynthesis). A Gilson Differential Respirometer was used to measure changes in oxygen evolution using standard manometric techniques (Umbreit, Burris, and Stauffer, 1964).

Twenty-five µl of *S. obliquus* cells from a four-day old culture were resuspended in two ml of nutrient solution and added to the main compartment of a standard Warburg flask. Five-tenths ml of a 1% carbon dioxide buffer solution was placed in one side arm of the flask. Dichlobenil dissolved in 20% methanol was added to the other side arm of each flask to give final concentrations of 5, 10, and 20 ppm in a total of 4 ml. Final methanol concentration was 5%. Distilled water and methanol controls were also included. Each treatment was replicated two times and the experiment was repeated twice.

Three 10-ml aliquots of the algal culture, each containing 93.5 µl of cells, were transferred to preweighed beakers and dry weights determined.

After allowing the flasks to equilibrate in the water bath at 25° C and 750 foot-candles of light for 10 minutes, the manometers were closed and readings taken every 10 minutes for 30 minutes. Treatments were then tipped in from the side arm and readings taken for another 30 minutes.

Leaves were harvested from two-week-old *P. pectinatus* plants and randomly separated into lots of 20 each. The upper 60 mm of the leaves in each lot were cut into 2-mm segments and placed in the main compartments of Warburg flasks each containing 3 ml of one-tenth strength Hoagland's solution. Preliminary experiments revealed that it was necessary to cut the leaves into 2-mm segments to prevent the tissue from clinging to the walls of the flasks during the shaking process. Dichlobenil, dissolved in water, was added to the flasks to

give a final concentration of 4.0 ppm in a total volume of 4.0 ml. Methanol was not used as a solvent because it greatly reduced oxygen evolution in this species. One side arm contained 0.5 ml of a 1% carbon dioxide buffer solution. Manometric conditions and procedures were similar to those used with *S. obliquus* except that the light intensity was reduced to 500 foot-candles. Dry weight of the plant material in each flask was determined at the conclusion of the measurements. Treatments were replicated three times.

Results and Discussion. Oxygen evolution in *S. obliquus* was reduced by 40.3% when methanol was added to the manometric flasks (Table 1). No further significant reduction in the rate of oxygen evolution occurred when dichlobenil was added at 5, 10, or 20 ppm. The use of ethanol in place of methanol as a solvent for dichlobenil caused an even greater adverse effect on oxygen evolution. Because of the great effect of the solvent on oxygen evolution, no definite conclusions could be made concerning the effect of dichlobenil on photosynthesis in *S. obliquus*. However, there was no evidence that dichlobenil was interfering with oxygen evolution (photosynthesis) in this species. The data supported the findings of the previous growth study.

Oxygen evolution from *P. pectinatus* leaves was not significantly reduced by the addition of 4.0 ppm of dichlobenil (Table 2). Since water was used as a solvent for the dichlobenil, higher rates in the flasks could not be attained. Although dichlobenil has been shown to drastically interfere with the growth of *P. pectinatus* at rates as low as 0.1 ppm, no effect on oxygen evolution (photosynthesis) could be demonstrated.

Table 1. The effect of dichlobenil on oxygen evolution of *S. obliquus*.

Treatment	µl of O_2 evolved/60 minutes/mg dry wt.		
	Before	After	% Reduction
Water-Control	127.4	121.7	4.5
5% MeOH-Control	134.3	80.2	40.3
5.0 ppm dichlobenil	138.3	75.5	45.4
10.0 ppm dichlobenil	139.7	80.0	42.7
20.0 ppm dichlobenil	132.0	81.6	38.2

Table 2. The effect of dichlobenil on oxygen evolution of *P. pectinatus*.

Treatment	µl of O_2 evolved/60 minutes/mg dry wt.		
	Before	After	% Reduction
Water-Control	20.5	20.3	1.0
4.0 ppm dichlobenil	22.0	21.5	2.3

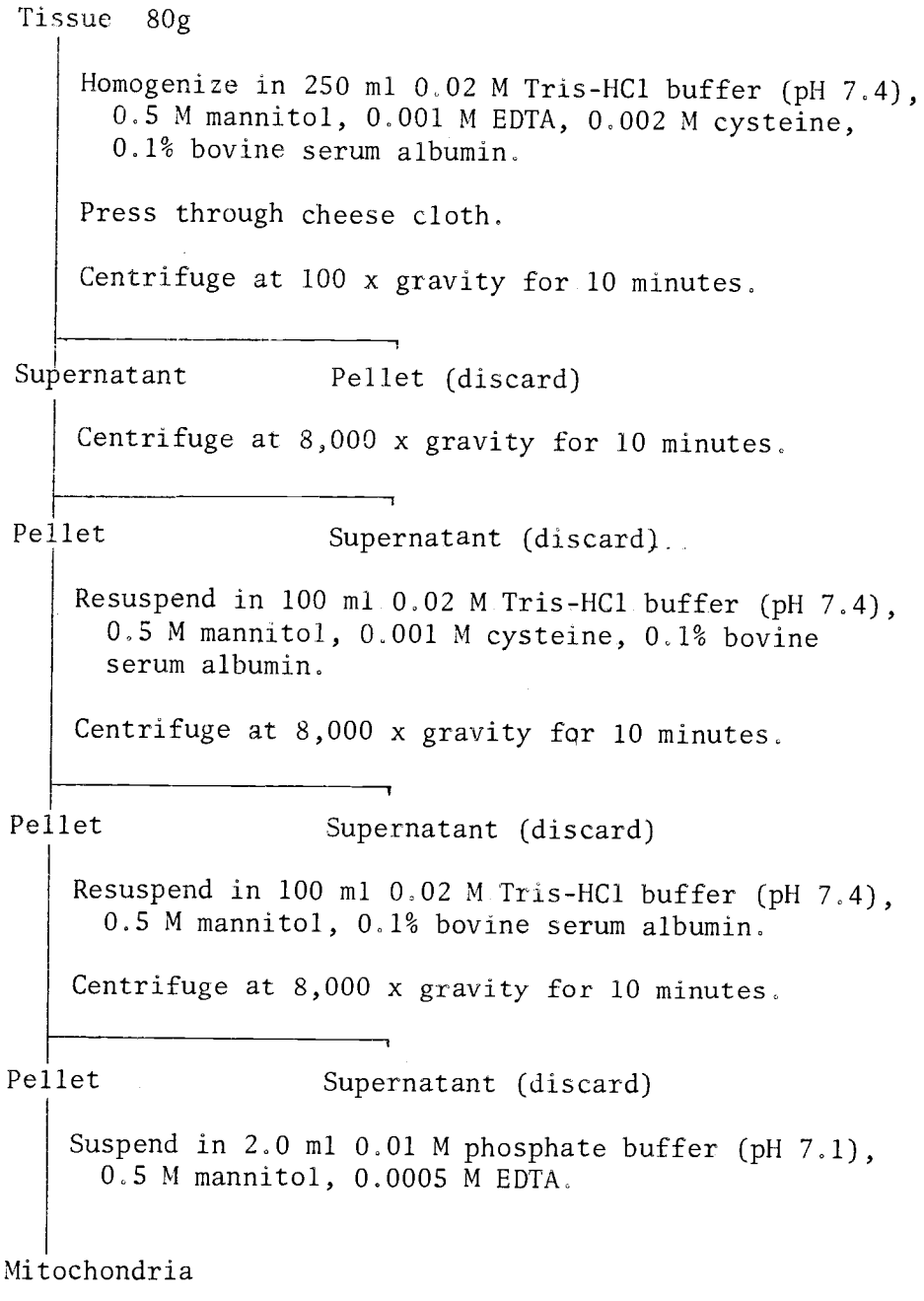
Respiration and Oxidative Phosphorylation

Materials and Methods. Isolated mitochondria were utilized in studying the effect of dichlobenil on respiration and oxidative phosphorylation. Mitochondria were isolated from white potato tubers (*Solanum tuberosum* L. variety Netted Gems) obtained from a local market. Tubers were stored in the dark at 5° C prior to use. Eighty grams of tissue from the center of potato tubers were cut into approximately one-cm cubes which were homogenized in a Servall Omni-mixer. Mitochondria were isolated from the homogenate by Method II as outlined by Verleur (1965) and later modified by Ferrari and Moreland (1969). An outline of the procedure and the reagents used is given in Figure 7. All steps in the isolation procedure were carried out between 0 and 3° C.

The basic reaction medium for respiration studies contained the following components in a total volume of 1.6 ml: 0.5 M mannitol, 0.01 M potassium phosphate buffer (pH 7.1), 5×10^{-4} M EDTA, 8.0 µmole succinate, 0.2-ml aliquot of the final mitochondrial preparation (containing 0.21 mg nitrogen), and ADP additions as indicated in the text. Dichlobenil and oligomycin were dissolved in ethanol and added as indicated in the text.

Oxygen uptake by mitochondrial suspensions was measured polarographically with a Clark type oxygen electrode connected to an amplifier and recorder assembly. All reactions were carried out in a 1.6-ml reaction cell at 27° C. The reaction cell was designed to minimize exposure of the reaction mixture to atmospheric oxygen when

Figure 7. Procedure for isolation of mitochondria from potato tubers.



reagents were added during an experiment. The reaction medium was thoroughly aerated prior to adding mitochondria and substrates. Respiratory rates were calculated from a recorder trace on the basis of 240 μM O_2 (384 millimicromoles O_2/1.6 ml) in the aerated medium as calculated by Chance and Williams (1955). Rates of oxygen consumption were expressed as mμmoles O_2/minute/1.6 ml. The respiratory control (R.C.) ratios were calculated by dividing the maximum rate of O_2 uptake in the presence of the added ADP by the rate when all ADP had been consumed. The ADP/O ratios were represented by the quotient of the amount of ADP added (0.3 μmole) and the total oxygen uptake in μatoms O during state 3.

Chance and Williams (1955) have defined states 3 and 4 of the mitochondrial respiratory chain as ADP-stimulated and ADP-limited rates of oxygen utilization, respectively. When uncoupling activity (respiratory stimulation) was measured, a limited amount of ADP was added to the reaction medium to obtain a state 3 respiration rate. Dichlobenil and oligomycin were added at least two minutes after state 3 ended and state 4 commenced.

An aliquot of the mitochondria preparation was washed with redistilled water, recentrifuged at 20,000 x g for 10 minutes. After discarding the supernatant, nitrogen in the mitochondrial pellet was determined by the micro-Kjeldahl method (A. O. A. C., 1966).

The reaction mixture used to follow ATPase activity was maintained at 30° C and consisted of the following in a total volume of 5.0 ml: 0.02 M tris-HCl buffer (pH 7.4), 0.003 M ATP (Adenosine Triphosphate), 0.002 M $MgCl_2$, and 0.5 ml of the mitochondrial

preparation containing 0.53 mg nitrogen. Dichlobenil dissolved in 20% methanol was added at rates of 10^{-5} M (1.72 ppm) and 10^{-4} M (17.2 ppm). A 2% methanol control was also included. One milliliter of the reaction medium was removed after 0, 5, 10, and 15 minutes and added to an equal volume of ice cold, 10% trichloroacetic acid. The denatured protein was removed by centrifugation at 1000 x g for 10 minutes and the inorganic phosphate in the supernatant determined colorimetrically after the method of Fiske and Subbarrow (1925). The experiment was repeated twice.

Results and Discussion. Isolated potato mitochondria typically demonstrated the respiratory pattern shown in Figure 8. The low rate of oxygen uptake in the presence of succinate was markedly accelerated by addition of ADP, indicating a respiratory control by ADP. In the course of the oxygen electrode measurements, five states of the mitochondrial respiratory chain have been distinguished by Chance and Williams (1955): state 1, with only mitochondria added to the reaction medium; state 2, after subsequent addition of succinate; state 3, starting with the addition of ADP and terminating when the ADP has been consumed (as appears from the decreasing rate of oxygen uptake); state 4, the period after depletion of the added ADP; and state 5, the anaerobic state in which all of the oxygen has been exhausted from the reaction medium. During the study, respiratory control (R:C) ratios with freshly isolated mitochondria occasionally approached values as high as 4; however, R:C ratios were generally between 2 and 3. ADP/O ratios obtained with freshly isolated mitochondria averaged approximately 1.4.

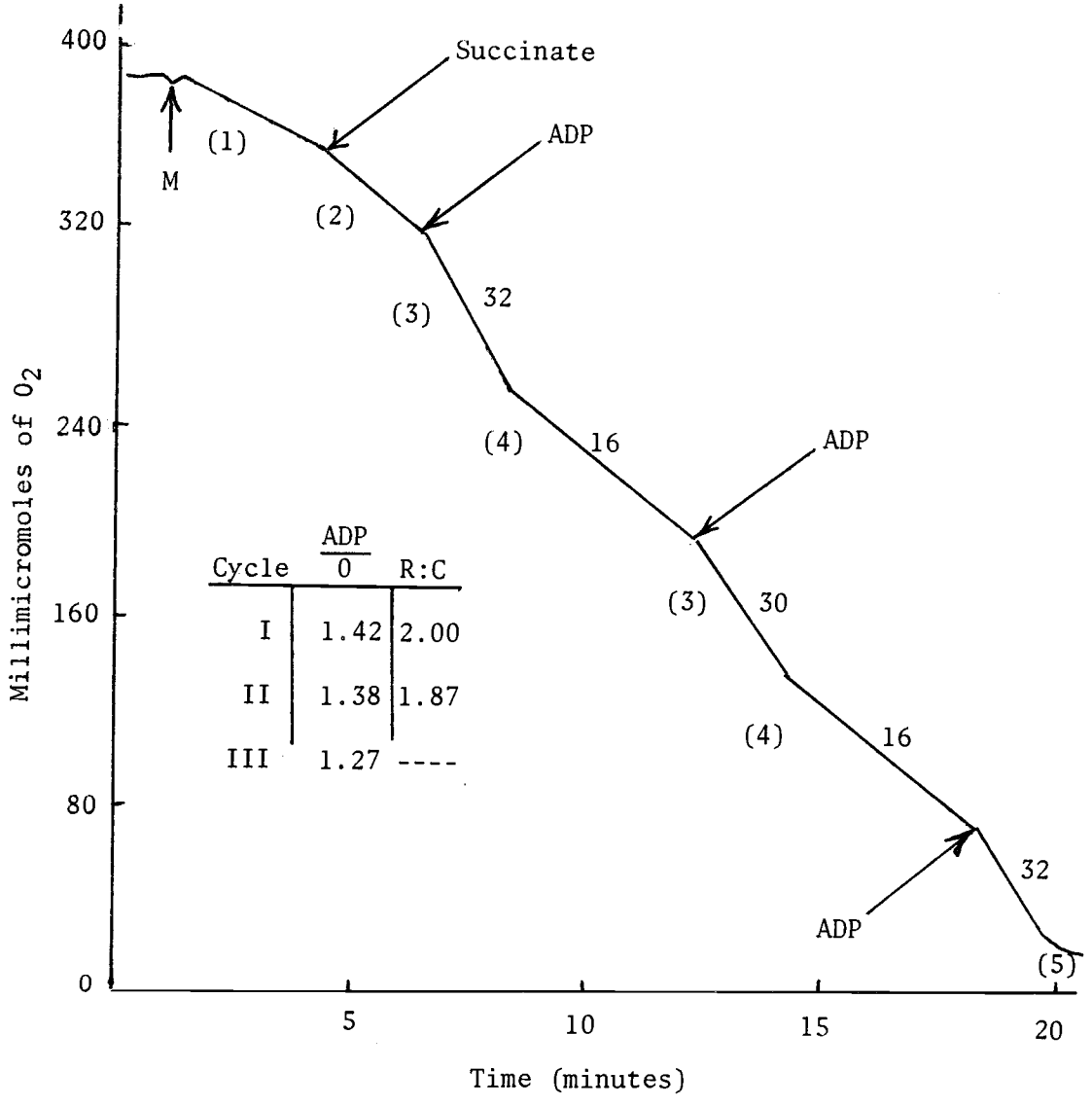


Figure 8. Representative polarographic trace illustrating oxygen utilization during succinate oxidation in potato mitochondria. Mitochondria (M) containing 0.21 mg nitrogen, succinate (8.5 µmoles), and ADP (0.3 µmoles) were added at the points indicated. Rates of oxygen utilization are presented above the trace as mµmoles of O_2/per minute/1.6 ml. States of respiration are indicated by numbers in parenthesis. ADP/O and R:C ratios are presented in tabular form.

When 1.45×10^{-4} M (25 ppm) dichlobenil was added during state 4 respiration, there appeared to be a slight stimulation of ADP-dependent respiration (Figure 9). This agreed with Foy and Penner (1965) and Moreland (1969), but conflicted with Price (1969). The stimulation of oxygen utilization indicated that dichlobenil was "uncoupling" oxidative phosphorylation. True uncouplers, such as 2,4-dinitrophenol (DNP), are also able to circumvent oligomycin-inhibited oxygen uptake. Oligomycin presumably inhibits respiration by blocking a transfer reaction involving a high-energy intermediate (Lardy, Connelly and Johnson, 1964). Attempts to demonstrate oligomycin inhibition of oxygen uptake in this system were not successful. Possibly the oligomycin had deteriorated. However, fresh solutions were prepared just before the polarographic measurements were made and should have been active. The other possible explanation was that the mitochondria were not strongly coupled. That is, respiratory activity and oxidative phosphorylation were not strongly interdependent. If this was the case, a blockage in the phosphorylation process would not necessarily be accompanied by an inhibition of respiration. Although the rate of substrate oxidation was readily controlled by ADP, R:C and ADP/O ratios were low and indicated that the mitochondria were not strongly coupled.

Moreland (1969) was able to demonstrate that dichlobenil could circumvent oligomycin inhibition, while Price (1969) reported the contrary. This point remains unresolved.

In addition to stimulation of state 4 respiration, a true uncoupler also promotes mitochondrial ATPase activity. The term

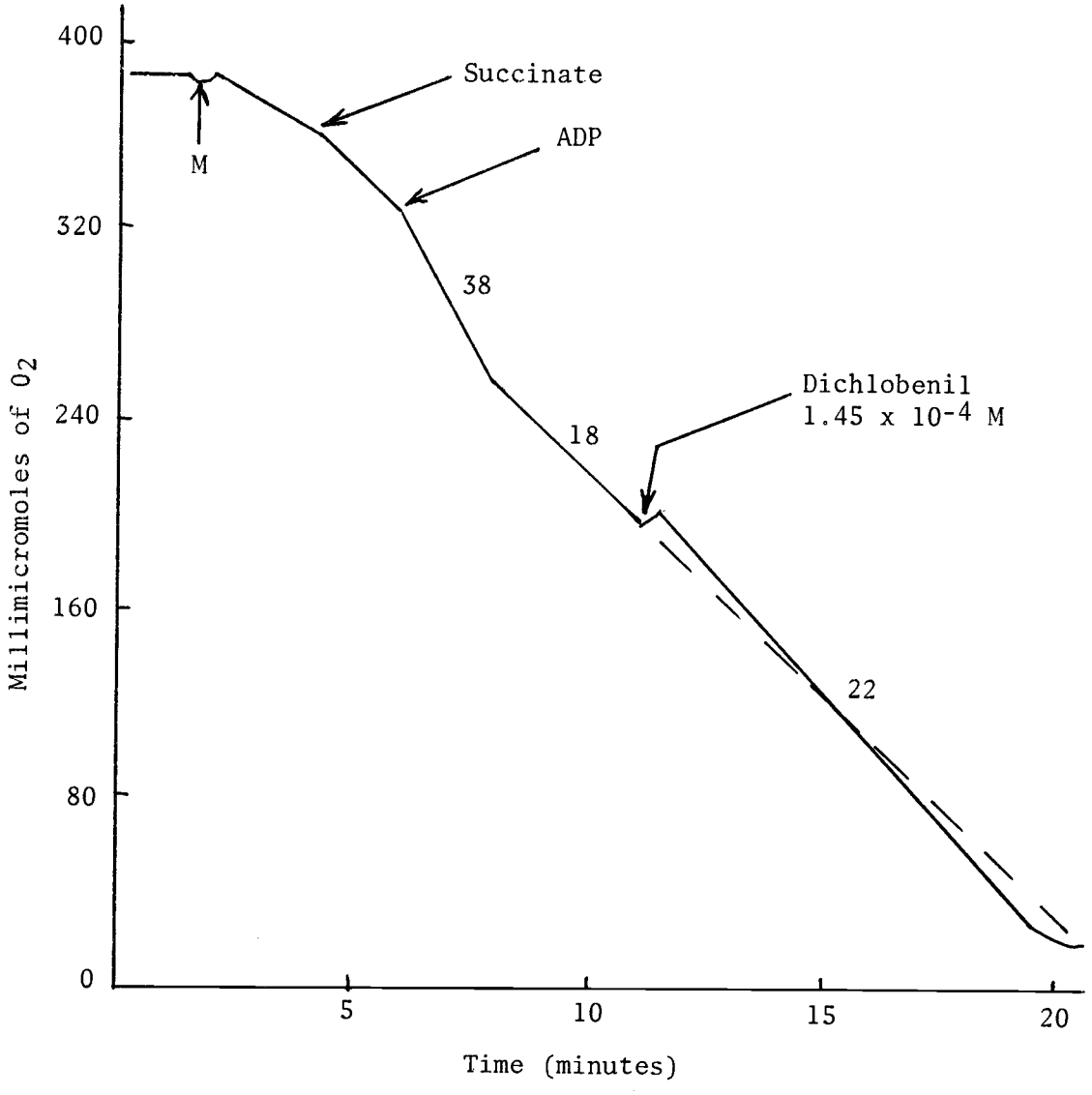


Figure 9. Apparent stimulation of state 4 respiration of potato mitochondria by dichlobenil with succinate as a substrate. Rates of oxygen utilization (mμmoles O_2/minute/1.6 ml) are indicated above the trace. Dashed line indicates projection of untreated state.

ATPase will be used to indicate the enzymatically catalyzed release of Pi (inorganic phosphate) from ATP, but its use does not necessarily imply the existence of a single or specific enzyme.

Two percent methanol did not influence the activity of the mitochondrial ATPase (Table 3). Dichlobenil did not stimulate ATPase activity at either of the concentrations tested. This indicated that the site of action of dichlobenil in stimulating respiration was not involved in the uncoupling of oxidative phosphorylation. However, if the electron transport chain of the mitochondrial system was only weakly coupled to the phosphorylating system, a change in the activity of one would not necessarily be accompanied by an equivalent change in the other.

Results of these experiments with isolated mitochondria are generally inconclusive as to the effect of dichlobenil on respiration and oxidative phosphorylation. Since reports in the literature are also contradictory, it could be assumed that differences in techniques and species used by the various investigators might be responsible for the conflicting results.

Table 3. Effect of dichlobenil on ATPase activity in isolated potato mitochondria.

Treatment	µg of Pi/ml at different time intervals			
	0 minute	5 minutes	10 minutes	15 minutes
H_2O-Control	2.0	6.5	11.6	16.6
McOH-Control	2.0	6.5	11.1	17.6
1.72 ppm	2.0	5.1	11.0	15.2
17.2 ppm	2.0	6.1	11.5	16.0

BASIS FOR THE SENSITIVITY OF CERTAIN PLANTS TO DICHLOBENIL

Introduction

Dichlobenil is phytotoxic to a wide range of plant species; however, several species are reported to be tolerant. Studies conducted as part of this research have shown the growth of *Potamogeton pectinatus* extremely sensitive to dichlobenil, while *Scenedesmus obliquus* appeared to be very tolerant. The objective of this research was to investigate the basis for this selectivity utilizing these two plant species and radioactive dichlobenil-^{14}C-nitrile.

Materials and Methods

A sample of ^{14}C-dichlobenil having a specific activity of 4.63 μc (microcuries) per μmole, was obtained from Thompson-Hayward Chemical Company, Kansas City, Missouri. The entire contents (9.3 mg) of the sealed ampule were quantitatively transferred to a 100-ml volumetric flask and brought up to volume using a 20% methanol solution.

Duplicate samples of *P. pectinatus* leaves, weighing 4.70 gm (0.2886 gm dry wt.) each, were taken from three-week-old plants grown in large aquaria in the greenhouse. The leaves were transferred to 1000-ml flasks containing 50.03 μc of ^{14}C-dichlobenil in 186 ml of nutrient solution. Concentration of labeled plus unlabeled dichlobenil was 10.0 ppmw. After stoppering the flasks with a loose cotton plug, they were placed in an oscillating shaker and allowed to incubate at room temperature (22-25^{o}) for three days.

Continuous light, approximately 200 foot-candles, was supplied by an incandescent lamp.

Duplicate 166-ml aliquots of a *S. obliquus* suspension, which had grown for four days in a carbon dioxide-enriched medium (Appendix Table 3), were transferred to sterile flasks containing 20 ml (50.03 µc) of ^{14}C-dichlobenil. The final concentration of dichlobenil was 10 ppmw. Packed-cell volume of the original *S. obliquus* suspension was 1.5 µl of cells per milliliter. Carbon dioxide-enriched air was bubbled through the medium at the rate of 60 cc per minute. Normal aeration rates of 200 cc per minute could result in excessive volatilization of dichlobenil from the solution (Price and Putnam, 1969a). Flasks were placed in an oscillating shaker to insure adequate mixing of the suspension. Light and temperature conditions were similar to those used with *P. pectinatus*.

Duplicate 1.0-ml aliquots of each of the nutrient solutions were removed before and after incubation of plant material, and diluted 1:49 with water for determination of radioactivity. Half-milliliter aliquots of each of the diluted solutions were added to 15 ml of scintillation solution in liquid scintillation vials. The scintillation solution consisted of 7 volumes of ethylene glycol monoethyl ether (Cellosolve) mixed with 10 volumes of 0.6 percent 2,5-diphenyloxazole (PPO) in toluene. Radioactivity in each vial was counted in a Packard Tricarb Liquid Scintillation Spectrometer, Model 3375. Liquid scintillation data are expressed as disintegrations per minute (dpm).

After three days (72 hours) cultured material of both species was removed from the incubating solutions, rinsed three times with distilled water, and transferred to reflux boiling flasks. The plant material was exhaustively extracted with 2N HCl in ethanol for one hour. After filtering each extract through a fritted disc Buchner funnel, they were condensed using nitrogen gas Final volumes of the *P. pectinatus* and *S. obliquus* extracts were 37.2 ml and 18.9 ml, respectively.

Radioactivity in the extracts was determined by liquid scintillation counting. One milliliter of each extract was neutralized with sodium hydroxide and 0.05 ml added to scintillation vials containing 20 ml of Bray's solution. This solution consisted of 60 g of naphthalene, 200 mg of 1,4-bis-2'-(5'-phenyloxazolyl)-benzene, 4 g of 2,5-diphenyloxazole, 100 ml of methanol, and 20 ml of ethylene glycol, made up to one liter with *para*-dioxane. All other samples were counted in the Cellosolve-0.6% PPO-toluene solution.

Five-tenths milliliter of *P. pectinatus* extract and 0.3 ml of *S. obliquus* extract were applied separately on 5 x 20 cm glass plates coated with 250 μ of silica gel G. Plates were allowed to dry before being developed in closed glass cylinders (6 x 23 cm). Gel was removed at a point 15 cm above the origin, so that the solvent front could not advance off of the plate. Plates were developed twice in benzene-methanol (95:5, v/v) with the solvent front being allowed to advance the full 15 cm each time. A duplicate set of plates was developed under similar conditions in chloroform-ethanol-acetic acid (89:10:1, v/v/v).

A stock solution of ^{14}C-dichlobenil (0.125 μc/ml) was prepared and 0.3 ml spotted on thin-layer plates for developing in both solvent systems.

Following development, the chormatograms were scanned on a Packard Radiochromatogram Scanner, Model 7201, to locate the positions of radioactive substances. Operating conditions were as follows: 300 cc of helium-butane (98.7% to 1.3%) per minute; scan speed, 0.5 cm per minute; linear range, 0-100 counts per minute (cpm) fullscale; time constant, 100 seconds; slit width, two mm. Data obtained from this scanner were of a qualitative nature; therefore, the gel from each plate that was developed in the benzene-methanol solvent system was carefully removed in 1.0-cm bands and placed in scintillation vials containing 15 ml of the scintillation solution. Radioactivity in each vial was measured by liquid scintillation counting.

Results and Discussion

Thin-layer chromatography of authentic ^{14}C-dichlobenil in benzene-methanol and subsequent scanning of the plate with the Radiochormatogram Scanner revealed that this compound moved with the solvent front to the top of the plate (Figure 10). A similar tracing was obtained when chloroform-ethanol-acetic acid was used as the solvent system (Appendix Figure 1). Analysis of the distribution of radioactivity on the plates by elution and liquid scintillation counting verified the qualitative results obtained with the scanner (Figure 11 and Appendix Table 4). Over 91% of the

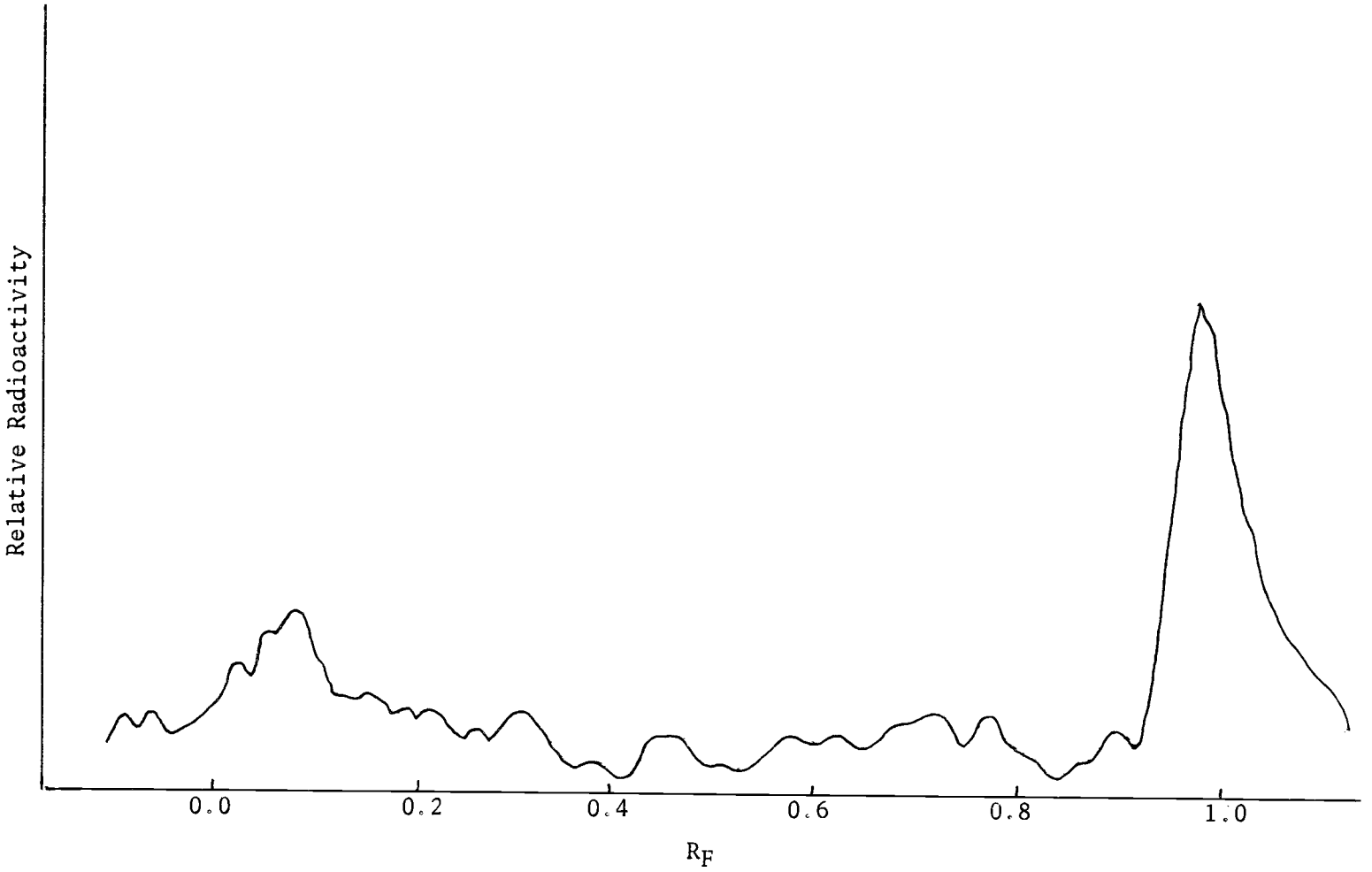


Figure 10. Tracing of a strip chart scan of a chromatogram of authentic ^{14}C-dichlobenil chromatographed on silica gel G in benzene-methanol (95:5, v/v).

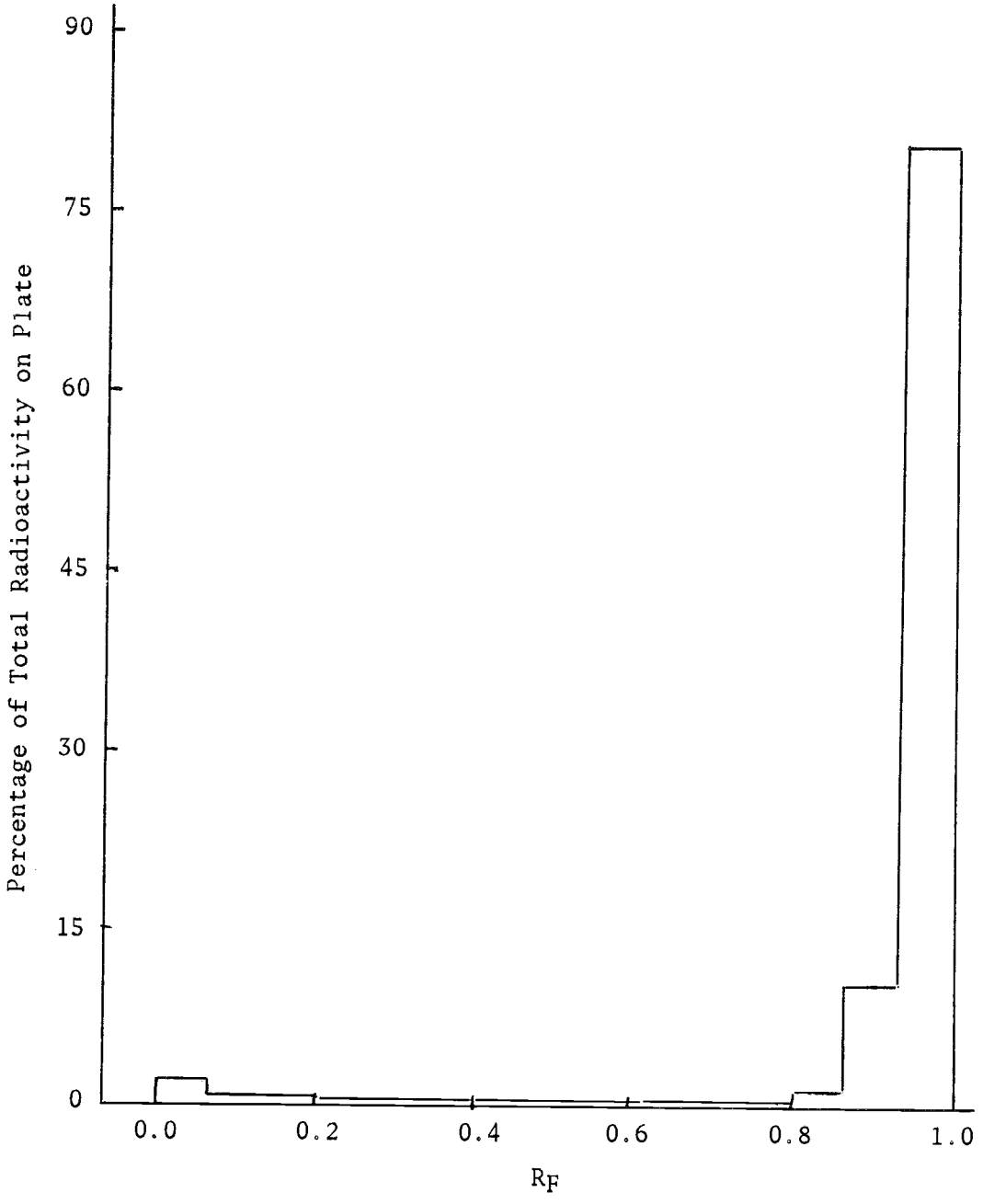


Figure 11. Distribution of radioactivity on a chromatogram of authentic ^{14}C-dichlobenil chromatographed on silica gel G in benzene-methanol (95:5, v/v).

total radioactivity was found in the last two centimeters on the plate and represents ^{14}C-dichlobenil. A small amount of radioactivity (2-2.5%) remained at the origin. It is not known if this was a contaminant or the product of a degradation process. Since dichlobenil is reported to be heat and light stable, it could be hypothesized that the small amount of radioactivity at the origin was a contaminant.

Seventy-two percent of the applied radioactivity was recovered from the *P. pectinatus* nutrient solution after 72 hours (Table 4). Only 23% of the original radioactivity of the *S. obliquus* nutrient solution was recovered after 72 hours. The low recovery of radioactivity in the *S. obliquus* nutrient solution was apparently due to volatilization of ^{14}C-dichlobenil during the aeration process.

Over eight times as much total radioactivity was recovered from the *P. pectinatus* extract as there was from the *S. obliquus* extract. A factor which must be taken into account, however, is that much of the dichlobenil-^{14}C was lost from the *S. obliquus* cultures during the experimental period. Therefore the concentration of herbicide available for absorption was constantly less in the *S. obliquus* than in the *P. pectinatus* cultures. Computations reveal that the amount of radioactivity recovered in the *P. pectinatus* extract was approximately 24% of the amount that remained in the nutrient solution, whereas for *S. obliquus* the figure was approximately 9%. When the radioactivity data for the extracts were converted to dpm per mg dry weight, there was seven times as much radioactivity in the *P. pectinatus* tissue (110,337 dpm per mg dry weight) as there was

Table 4. Radioactivity measured by liquid scintillation counting in different nutrient solutions and acidic extracts.

Solution or Extract	Radioactivity (dpm/ml)
1. Original nutrient solution	500,300
2. *P. pectinatus* nutrient solution after 72 hours	361,000
3. *S. obliquus* nutrient solution after 72 hours	115,728
4. *P. pectinatus* extract (37.2 ml)	85,600
5. *S. obliquus* extract (18.9 ml)	20,904

in the *S. obliquus* tissue (15,935 dpm per mg dry weight). Experiments conducted by Verloop and Nimmo (1969) indicated that none of the radioactivity from treated bean plants was lost as $^{14}CO_2$. Assuming that none of the absorbed radioactivity was lost as $^{14}CO_2$ and acknowledging the complication caused by volatilization of herbicide from algal cultures, the tolerance of *S. obliquus* to dichlobenil apparently can be at least partially attributed to a lower rate of absorption.

Tracings of strip chart scans from thin-layer chromatograms of the *P. pectinatus* extract (Figure 12) and the *S. obliquus* extract (Figure 13) indicated an additional basis for dichlobenil selectivity. A larger amount of radioactivity remained at the origin on the chromatogram of the *S. obliquus* extract compared with that of the *P. pectinatus* extract. A duplicate set of plates was developed in a different solvent system and a similar set of tracings was obtained (Figures 14 and 15).

Liquid scintillation counting data of the radioactivity in each centimeter on the plates are tabulated in Appendix Table 5. The greatest amount of radioactivity was found at the solvent front (R_F 1.0) on both chromatograms, and this material co-chromatographed at the same R_F zone with authentic ^{14}C-dichlobenil. A smaller peak of radioactivity was located at the origin on each plate. However, the counting data indicated that only 11% of the radioactivity remained at the origin of the *S. obliquus* chromatogram, whereas the strip chart scan of the chromatogram indicated 50 to 60% of the radioactivity remained at the origin. All samples were recounted, spiked

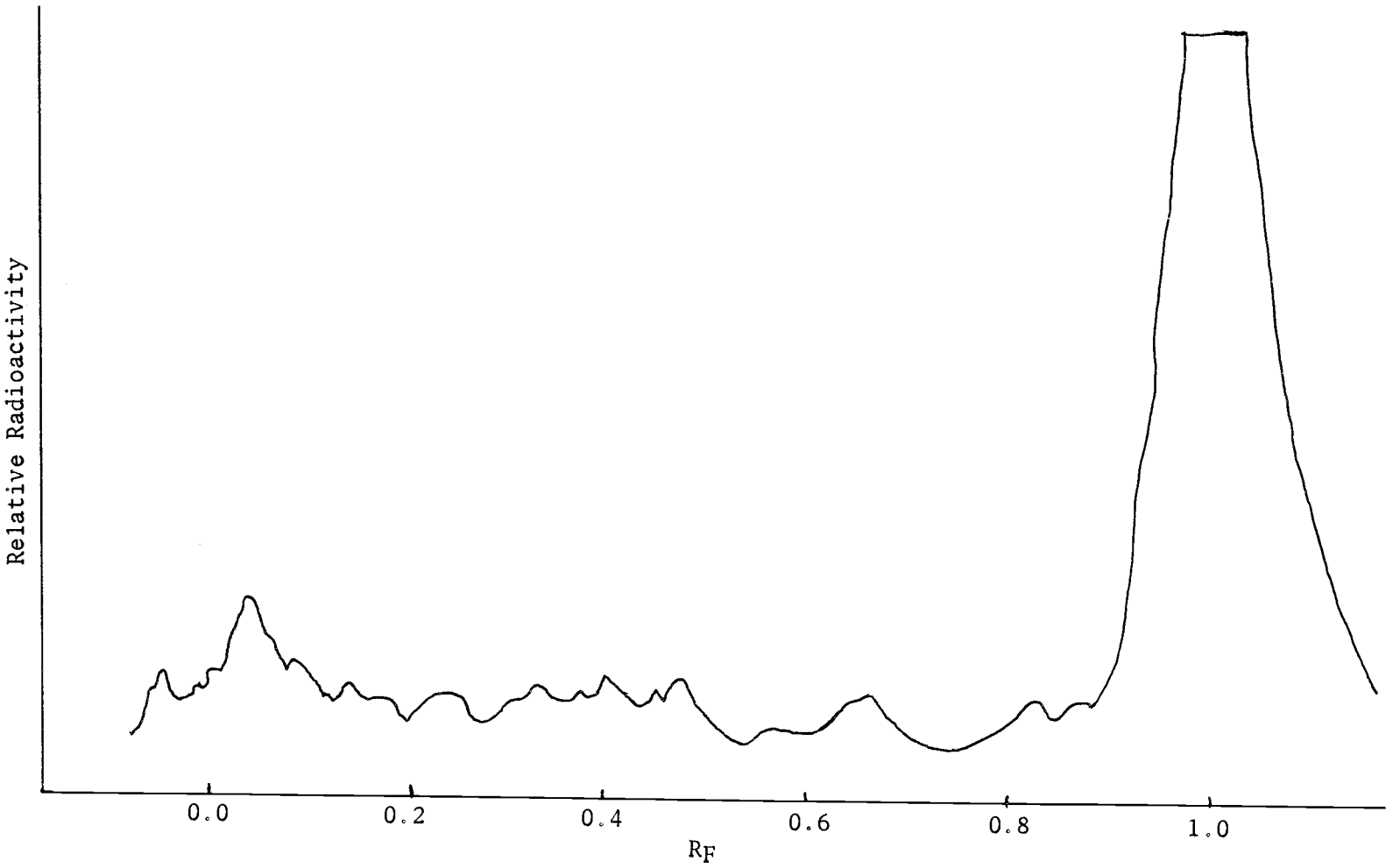


Figure 12. Tracing of a strip chart scan of a chromatogram of *P. pectinatus* extract made 3 days after treatment with ^{14}C-dichlobenil. Extract was chromatographed on silica gel G in benzene-methanol (95:5, v/v).

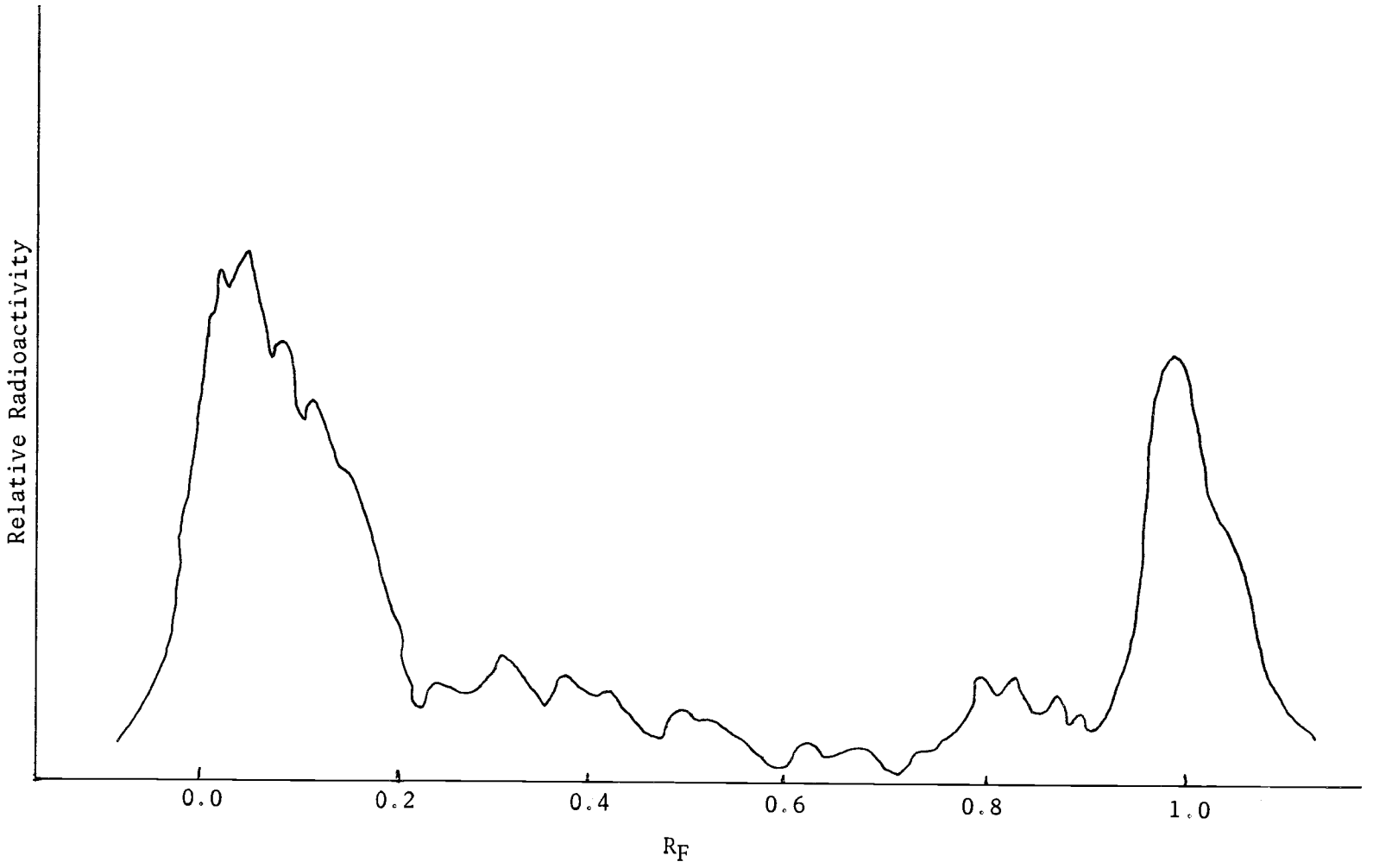


Figure 13. Tracing of a strip chart scan of a chromatogram of *S. obliquus* extract made 3 days after treatment with ^{14}C-dichlobenil. Extract was chromatographed on silica gel G in benzene-methanol (95:5, v/v).

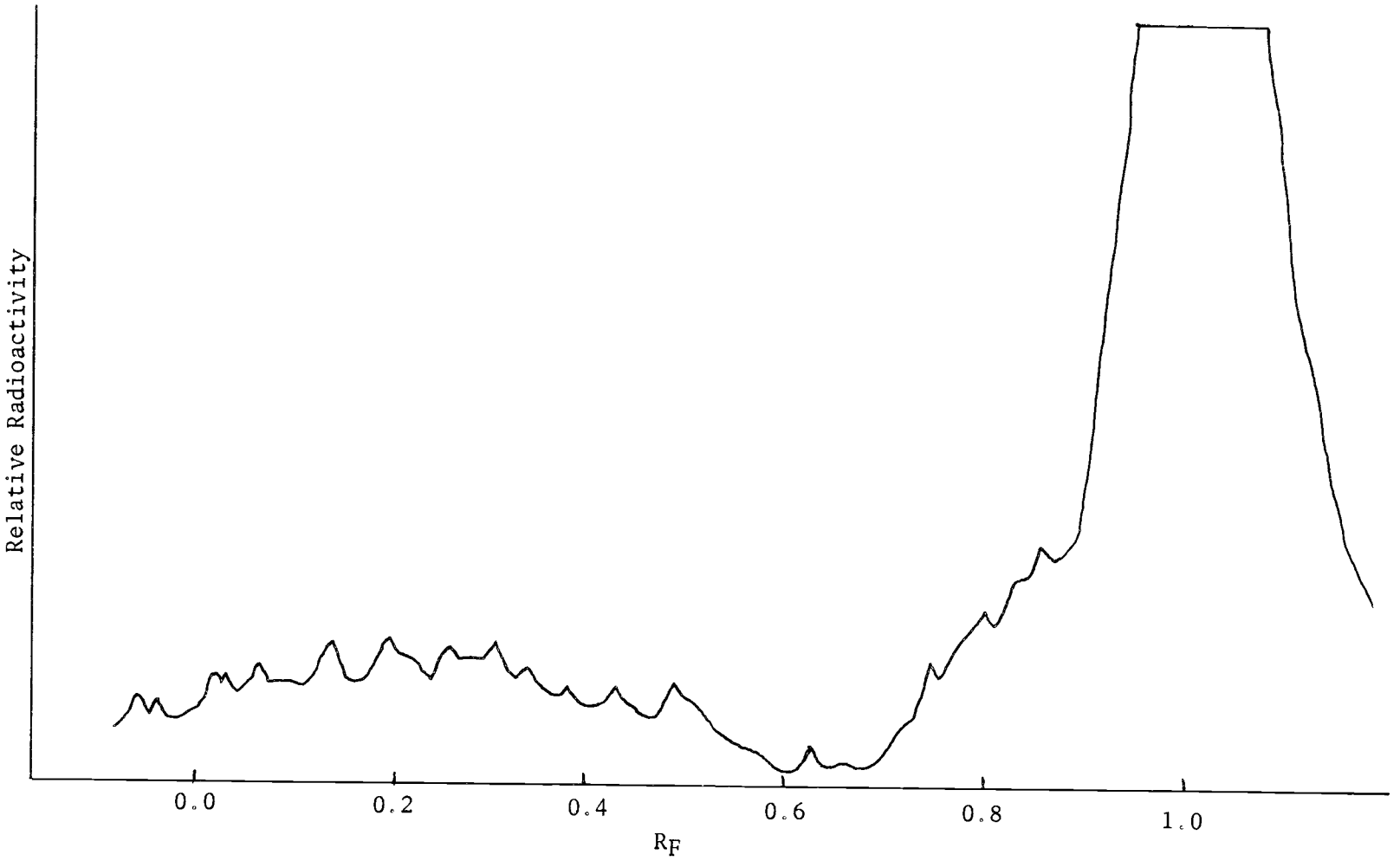


Figure 14. Tracing of a strip chart scan of a chromatogram of a *P. pectinatus* extract made 3 days after treatment with ^{14}C-dichlobenil. Extract was chromatographed on silica gel G in chloroform-ethanol-acetic acid (89:10:1, v/v/v).

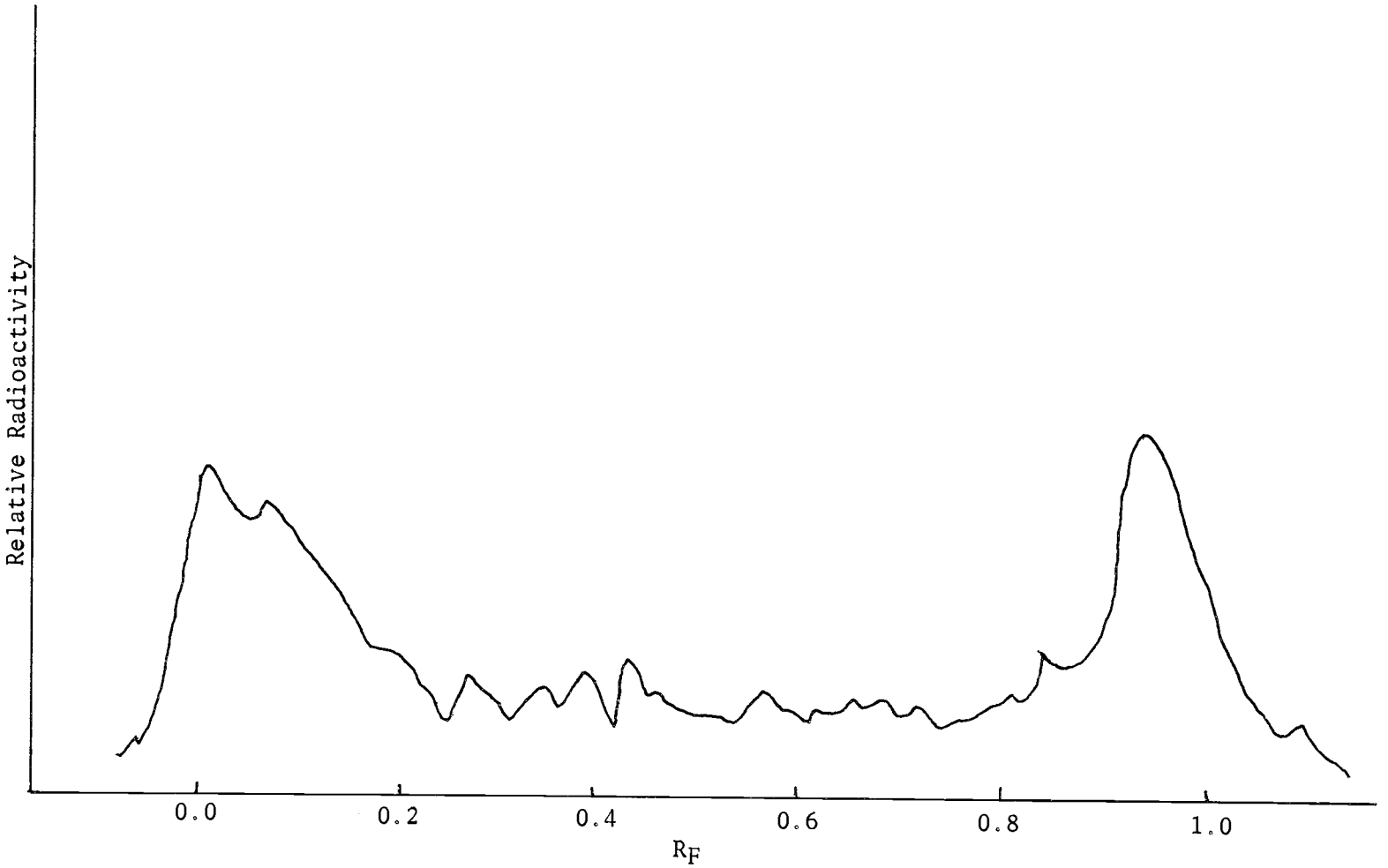


Figure 15. Tracing of a strip chart scan of a chromatogram of a *S. obliquus* extract made 3 days after treatment with ^{14}C-dichlobenil. Extract was chromatographed on silica gel G in chloroform-ethanol-acetic acid (89:10:1, v/v/v).

with toluene-^{14}C, counted again, and the dpm recalculated. Values similar to the original data were obtained for all of the samples. Apparently considerable quantities of radioactivity were lost from the plates between the time of scanning and when they were scraped into the scintillation vials. The radioactivity at the origin is believed to represent a metabolite of dichlobenil. If this was the case, *S. obliquus* was metabolizing dichlobenil at a much faster rate than *P. pectinatus*. Samples of known metabolites of dichlobenil (3-hydroxy- and 4-hydroxy-2,6-dichlorobenzonitrile) were requested, but were not available. Verloop and Nimmo (1969) using the same solvent system and acidic extracts from beans treated with ^{14}C-dichlobenil, identified these two metabolites, but listed their R_F's as 0.35 and 0.22. They observed a minor spot at the origin, but did not determine its identity. Pate and Funderburk (1966) have reported 2,6-dichlorobenzoic acid as a metabolite of dichlobenil, but its R_F in solvent systems similar to those used in these experiments is listed as 0.40.

No definite conclusions could be made as to the identity of the radioactive material located at the origin. However, the presence of a greater percentage of this radioactive substance(s) at the origin of the *S. obliquus* plate did indicate that dichlobenil was being metabolized to a greater extent in this species than in *P. pectinatus*. Since *S. obliquus* was not adversely affected by administered dichlobenil, the metabolite(s) apparently is non-toxic to the alga. Thus, the possibility of metabolic detoxification being correlated with the alga's tolerance to the herbicide is clearly indicated.

SUMMARY AND CONCLUSIONS

Dichlobenil residue levels in water and hydrosoil were compared after application of wettable powder and granule formulations of dichlobenil to separate ponds at 10 pounds of active ingredient per surface acre (0.6 ppm in the water). Water and hydrosoil samples were taken from each pond one day after treatment and periodically thereafter. A special technique that involved vigorous shaking with benzene, rapid centrifugation and vacuum separation was developed to extract dichlobenil from the highly organic hydrosoil. Approximately 83% of the dichlobenil could be recovered by this method. Dichlobenil was measured by electron-capture gas chromatography. A five-foot by one-eighth-inch stainless steel column packed with 10% SE-30 on 60/80 Gas Chromosorb Q and maintained at 150° C was utilized. Retention time for dichlobenil was 2.5 minutes and the detection limit was 0.001 ppm.

Maximum residual concentrations in the water after treatment with wettable powder or granules were 1.00 and 0.68 ppmw, respectively. These concentrations were reached four and five days after treatment, respectively. After 15 days, the residual concentrations were approximately the same, and both decreased steadily to a level at or below the least amount detectable by the analytical procedure (0.001 ppmw) after 126 days (last sampling date). The maximum concentrations of dichlobenil in the hydrosoil after treatment with wettable powder were 1.472 ppmv in six days and with granules 3.700 ppmv in one day. Residues in the hydrosoil of the two ponds did not reach similar concentrations until 34 days after treatment. One

hundred twenty-six days after treatment the residual concentrations from the wettable powder treatment had decreased to 0.039 ppmv and from the granular treatment to 0.025 ppmv.

The maximum concentration of residue in the water following treatment with the wettable powder was 67% greater than the initial treatment concentration. However, in these investigations all water samples were taken 12 inches above the hydrosoil. Apparently, stratification of dichlobenil had occurred near the bottom of the pond. Additional research in which water samples were collected from at least three different depths would substantiate this hypothesis.

The results of this experiment indicated that the persistence of dichlobenil in pond water and hydrosoil was similar whether applied as a granular or a wettable powder formulation.

The growth of two aquatic vascular plants, *Potamogeton pectinatus* and *Alisma gramineum*, and one aquatic alga, *Scenedesmus obliquus*, was measured after exposure to various concentrations of dichlobenil in laboratory studies. *P. pectinatus* was found to be extremely sensitive to dichlobenil, with concentrations as low as 0.1 ppmw causing a 48.5% decrease in the amount of dry weight produced after two weeks. Growth inhibition was rapid and complete when the concentration was increased to 5 or 10 ppmw.

Similar results were obtained with *A. gramineum*, a species reported as being tolerant to field applications of this herbicide. Apparently, some physical characteristic involved in a field application of dichlobenil either reduces the concentration or period of exposure and enables this species to survive.

Rates of dichlobenil as high as 10 ppmw did not interfere with growth of cells or chlorophyll formation in *S. obliquus*. This species either did not absorb dichlobenil in injurious concentrations, or else it was capable of metabolizing dichlobenil to a non-phytotoxic product(s) rapidly enough to prevent injury.

Several laboratory studies were conducted in an effort to elucidate the mode of action of dichlobenil in killing plants. Manometric measurements of oxygen evolution indicated that dichlobenil was not affecting photosynthesis in either *P. pectinatus* or *S. obliquus*.

Oxygen utilization by isolated potato mitochondria appeared to be slightly stimulated by the presence of 25 ppmw dichlobenil in the reaction medium during state 4 respiration. The stimulation of oxygen utilization indicated that dichlobenil was uncoupling oxidative phosphorylation and should be capable of circumventing oligomycin-inhibited oxygen uptake. Attempts to demonstrate oligomycin inhibition of oxygen uptake were not successful however; thus, the uncoupling activity of dichlobenil could not be demonstrated by this method.

A true uncoupler of oxidative phosphorylation also promotes mitochondrial ATPase activity. Dichlobenil did not stimulate ATPase activity at 10^{-5} or 10^{-4} M concentrations. Apparently, the site of action of dichlobenil in stimulating respiration was not involved in the uncoupling of oxidative phosphorylation. However, a similar result would be expected if the electron transport chain of the mitochondrial system was only weakly coupled to the phosphorylating system.

Although dichlobenil appeared to stimulate slightly the respiration of isolated mitochondria, such stimulation would not cause the death of a plant. Considerably more research needs to be conducted before the true mode of action of dichlobenil is discovered.

The basis for the selectivity of dichlobenil was investigated by exposing *P. pectinatus* and *S. obliquus* to radioactive dichlobenil at 10 ppmw for 72 hours. Measurement of the radioactivity in the extracts by liquid scintillation counting indicated that *P. pectinatus* absorbed seven times more ^{14}C-dichlobenil per mg dry weight than *S. obliquus*.

Information obtained from strip chart scans of the radioactivity on the plates and liquid scintillation counting of the radioactivity in each centimeter of gel indicated two major concentrations of radioactivity. The larger was at the solvent front and co-chromatographed with authentic ^{14}C-dichlobenil. The other spot remained at or very near the origin. Unfortunately, suitable standards were not available for identification of this compound. The data also revealed that *S. obliquus* metabolized a greater percentage of the absorbed ^{14}C-dichlobenil than did *P. pectinatus*.

The slower rate of absorption and the higher rate of metabolism to non-phytotoxic product(s) could account for the tolerance of *S. obliquus* to dichlobenil, as compared with *P. pectinatus*.

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APPENDIX

Appendix Table 1. Residues in water samples from ponds treated with two different formulations of dichlobenil.

Days after treatment	PPMW of dichlobenil	
	Granules	Wettable powder
1	.052	.353
2	.394	---
3	.558	.911
4	.596	1.000
5	.680	.937
6	.631	.898
8	.608	.657
9	.605	.614
10	.549	.571
11	.628	.545
12	.500	.560
13	.388	.462
14	.489	.459
15	.462	.466
18	.388	.394
20	.369	.322
22	.319	.263
27	.249	.292
34	.083	.133
41	.078	.059
46	.049	.029
55	.012	.022
70	.007	.010
99	.002	.002
126	.001	.001

Appendix Table 2. Residues in hydrosoil samples from ponds treated with two different formulations of dichlobenil.

Days after treatment	PPMV of dichlobenil	
	Granules	Wettable powder
1	3.700	.563
6	2.044	1.470
14	1.761	1.142
20	.979	.750
27	.640	.467
34	.453	.424
46	.160	.208
70	.054	.070
99	.036	.035
126	.025	.039

Appendix Table 3. Inorganic Nutrient Media For *Scenedesmus*.

The following spectro-grade reagents were added to 700 ml of deionized water in the following order:

I. Major Elements:

A. 20.0 ml of 1 Molar KNO_3

B. 10.0 ml of 1 Molar NaCl

C. 7.5 ml of 1 Molar KH_2PO_4

D. 5.0 ml of 1 Molar K_2HPO_4

E. 1.0 ml of 1 Molar $MgSO_4$

F. 0.5 ml of 1 Molar $CaCl_2$

II. Minor Elements:

A. 0.1 ml of 0.1% $ZnCl_2$

B. 0.1 ml of 0.1% $CoCl_2$

C. 0.1 ml of 0.1% $(NH4)_2MoO_4$

D. 0.1 ml of 0.1% $MnCl_2$

E. 0.1 ml of 0.1% $CuCl_2$

F. 0.2 ml of 10.0% $FeCl_3$

Appendix Table 4. Distribution of radioactivity on a chromatogram of authentic ^{14}C-dichlobenil chromatographed on silica gel G in two solvent systems.

Location on plate	Disintegrations per minute 0.3 ml of authentic ^{14}C-dichlobenil	
	B-M[a]	C-E-A[b]
1/2 cm below origin	78	24
1st cm above origin	99	52
2nd cm above origin	10	14
3rd cm above origin	17	17
4th cm above origin	8	9
5th cm above origin	8	8
6th cm above origin	7	10
7th cm above origin	9	9
8th cm above origin	7	7
9th cm above origin	8	7
10th cm above origin	13	3
11th cm above origin	12	27
12th cm above origin	6	13
13th cm above origin	34	29
14th cm above origin	401	781
15th cm above origin	2963	2364
Total	3680	3374

[a] Benzene-methanol (95:5, v/v)

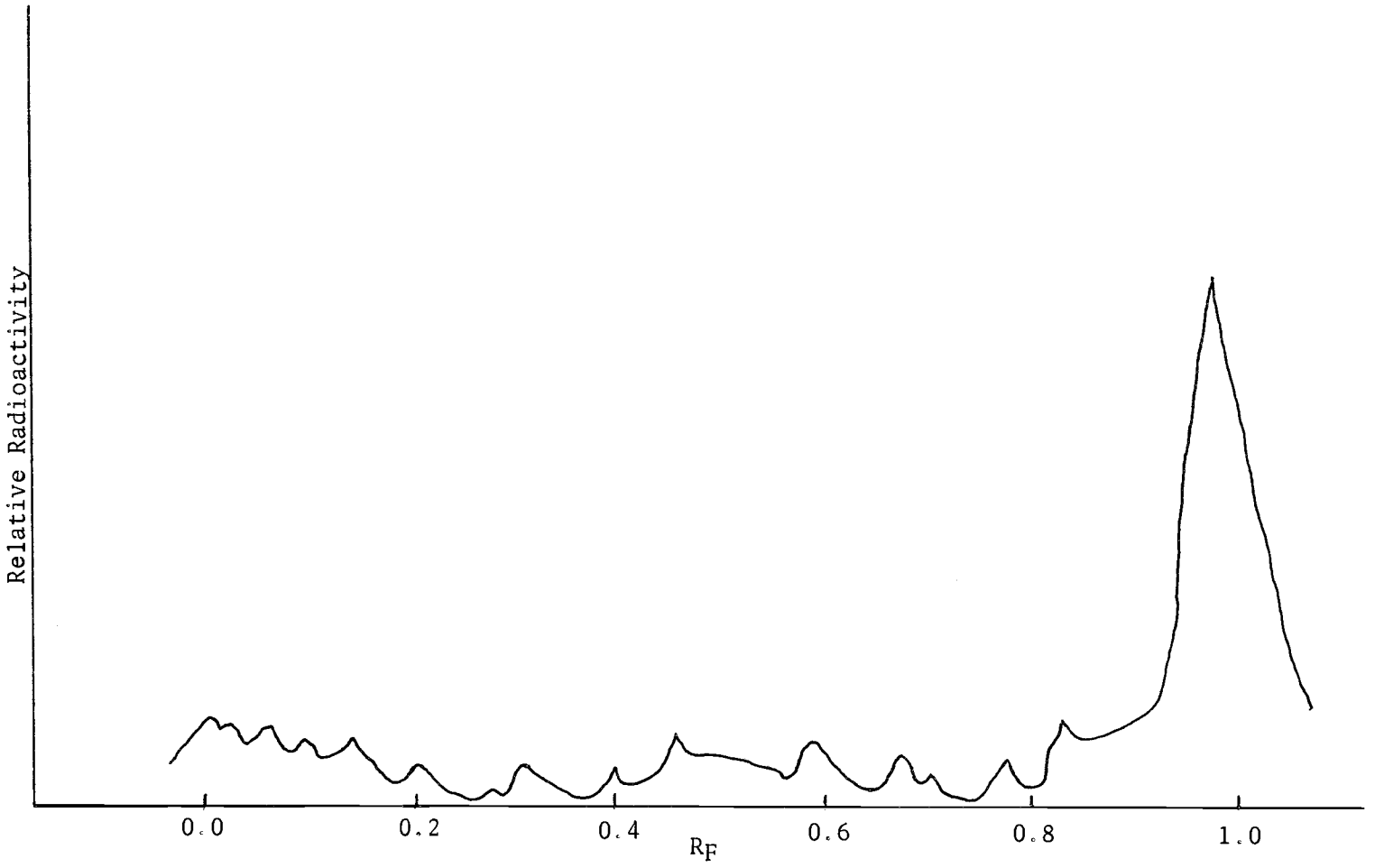
[b] Chloroform-ethanol-acetic acid (89:10:1, v/v/v)

Appendix Table 5. Distribution of radioactivity on chromatograms of *P. pectinatus* and *S. obliquus* chromatographed on silica gel G in two solvent systems.

	Disintegrations per minute			
	P. pectinatus		*S. obliquus*	
Location on plate	B-M[a]	C-E-A[b]	B-M	C-E-A
1/2 cm below origin	164	79	268	164
1st cm above origin	259	164	343	201
2nd cm above origin	107	107	162	174
3rd cm above origin	78	68	62	98
4th cm above origin	63	58	54	30
5th cm above origin	180	54	79	25
6th cm above origin	50	47	46	85
7th cm above origin	52	56	26	64
8th cm above origin	59	174	23	46
9th cm above origin	55	63	30	40
10th cm above origin	100	60	19	41
11th cm above origin	66	260	22	44
12th cm above origin	104	210	84	99
13th cm above origin	209	374	45	117
14th cm above origin	3468	5300	200	1381
15th cm above origin	10,494	11,388	1635	466
Total	15,508	19,462	3116	3075

[a] Benzene-methanol (95:5, v/v)

[b] Chloroform-ethanol-acetic acid (89:10:1, v/v/v)



Appendix Figure 1. Tracing of a strip chart scan of a chromatogram of authentic ^{14}C-dichlobenil chromatographed on silica gel G in chloroform-ethanol-acetic acid (89:10:1, v/v/v).