THE TOXICITY, DISTRIBUTION AND MODE OF ACTION OF DICHLOBENIL (2,6 - DICHLORO - BENZONITRILE) IN PLANTS

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This is to certify that the

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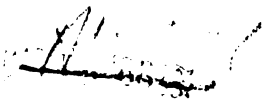

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ABSTRACT

THE TOXICITY, DISTRIBUTION AND MODE OF ACTION OF DICHLOBENIL (2,6-DICHLORO-BENZONITRILE) IN PLANTS

By

Hugh Criswell Price

Dichlobenil is used as an herbicide on perennial horticultural crops. It inhibits germinating seeds and the meristematic tissues of perennial weeds, thus is best utilized as a preemergence treatment. The objectives of this research were to determine the fate of dichlobenil in plants and to ascertain the physiological processes responsible for its phytotoxicity.

Carbon labeled dichlobenil was taken up by corn roots and translocated to all plant parts within 4 hr. Approximately 10% of the ^{14}C-dichlobenil within the shoot volatilized into the atmosphere. This volatilization was independent of light and dependent on temperature.

The ^{14}C-dichlobenil readily diffused from roots of preloaded corn plants placed in distilled water or nutrient solution. This indicates that dichlobenil is not actively held within the root cells, and readily traverses intervening membranes and cell walls. Either the lipophilic nature of the molecule or its capability of disrupting the semi-permeable nature of cellular

membranes may explain this phenomenon. The latter is substantiated by the observation that dichlobenil induces the leakage of betacyanin and reducing sugars from red beet root sections. This leakage of pigment was partially alleviated by the addition of Ca^{++}, a membrane stabilizing ion.

Respiration of red beet root sections, corn roots, and cucumber seedlings is increased by dichlobenil as evidenced by increased O_2 consumption and glucose utilization. However, mitochondria isolated from cucumber hypocotyls and cotyledons were not affected by 10^{-5}M dichlobenil. The activity of the mitochondria was assayed polarographically, manometrically and by measurement of inorganic phosphate esterified. In contrast, the monophenolic metabolite of dichlobenil (2,6-dichloro-3-hydroxybenzonitrile) effectively uncoupled oxidative phosphorylation at 10^{-5}M.

Cucumber seedlings treated 24 hr with 10^{-5}M dichlobenil showed an accumulation of polyphenolic compounds as determined by UV absorption of alkaline extracts, and phloroglucinol-HCl treatment of tissue sections.

It is postulated that a primary mechanism of action of dichlobenil is alteration of tonoplast permeability and subsequent leakage of phenolic and other ergastic substances into the cytoplasm. The phenols are thus subject to oxidation by soluble oxidase enzymes of the cytoplasm.

The oxidation products of the phenolase enzymes polymerize readily to form polyphenols, which uncouple oxidative phosphorylation and inhibit vital enzymes.

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INTRODUCTION

Although major advances have been made in understanding the practical uses for herbicides, the biochemical basis for toxicity has not been clearly elucidated for any herbicide. There is voluminous literature on physiological and morphological abnormalities induced by herbicides. From this literature it is difficult to rank plant responses in order of occurrence or to separate secondary from primary herbicidal effects. The lack of knowledge on primary sites of action for existing herbicides has necessitated the empirical screening for new herbicides. Increased knowledge on the primary sites of action should lead to the development of more specific or selective chemicals.

The objectives of this research were to study the toxicity, distribution and mode of action of dichlobenil (2,6-dichlorobenzonitrile) in plants. Mode of action studies were concentrated on events occurring over a relatively short time course with the lower physiologically active concentrations of dichlobenil. This approach was utilized to establish a logical sequence of events after plants are exposed to the herbicide.

LITERATURE REVIEW

History of Dichlobenil

The herbicidal properties of dichlobenil were first described in 1960 by Koopman and Daams (33) of Philips-Duphar. Dichlobenil was registered by the USDA in 1964 for use in cranberries (Vaccinium macrocarpon Ait.) and later registered for apples (Malus sylvestris L.), avocados (Persea americana Mill.), blueberries (Vaccinium sp. L.), brambles (Rubus sp. L.), cherries (Prunus cerasus L.), (P. avium L.), grapes (Vitis sp. L.), mangoes (Mangifera indica L.), peaches (Prunus persica (L.) Patsch), pears (Pyrus communis L.), and plums (Prunus domestica L.), (P. salicina L.) in 1966 (4). Currently, dichlobenil is being utilized for weed control in perennial horticultural crops such as orchards, vineyards, and nurseries.

Physical Properties

Dichlobenil is a white, crystalline solid with a characteristic odor (27). Its water solubility is only 18 ppm at 20 C, however, it is very soluble in most organic solvents. Dichlobenil is extremely stable thermally and is not subject to degradation by ultraviolet irradiation. The high vapor pressure of this compound, 5.5 x 10^{-4} mm Hg at 20 C, is an important physical property.

Pate and Funderburk (46) determined that 98% of the radioactivity from ^{14}C-dichlobenil was lost from an open planchet in 2 hr. Based on the rate of water loss from soil, Hartley (24) has estimated that a herbicide having about 10 times the molecular weight of water, but about one third of the diffusion rate in air, could be expected to disappear at a rate of 24 lb/A per month if its vapor pressure was approximately 10^{-4} mm Hg. Since dichlobenil has a vapor pressure 5.5 times greater than this, substantial loss could obviously occur. Vaporization may also play a significant role in the mode of action of this compound in plants (7, 11, 41).

Uses of Dichlobenil

Although dichlobenil is a powerful inhibitor of germinating seeds and actively dividing meristems (33, 41), it may be safely applied only to selected established crops. If roots of the crop come into contact with high concentrations of dichlobenil in the upper layers of soil, selectivity may be lost. Monocotyledonous and dicotyledonous weeds are controlled by both inhibition of growth of germinated seeds and young seedlings. Also, several perennial weeds, such as *Pteridium aquilinum* (L.) Kuhn (brackens), *Equisetum sp.* (horsetail), *Agropyron repens* (L.) Beauv. (quackgrass), *Artemisia sp.* (mugwort), and *Cynodon dactylon* (L.) Pers.

(bermuda grass) can be controlled if dichlobenil is applied prior to shoot emergence (27). Dichlobenil is much less effective in controlling weeds which are already established.

Fall applications of the granular formulation of dichlobenil are effective for controlling perennial weeds around fruit trees (51, 68). Applications of 6.0 lb/A provides 3-4 months of weed control in most areas. The wettable powder formulation is less effective (51), probably due to volatility. There have been a few reports of injury to tree fruits (19, 52) by dichlobenil treatment. Damage is generally manifested by marginal chlorosis of leaves and decreased terminal growth.

Early spring or late fall treatments of dichlobenil were found by Dana (15) to be effective for controlling several annual and perennial sedges, grasses and broadleaved weeds in cranberry bogs. Dichlobenil at 4.0 and 6.0 lb/A caused some reduction in terminal growth and fruit size.

The granular formulation of dichlobenil is also effective for weed control around ornamentals and in nursery plantings (1, 17). To decrease the volatility losses of dichlobenil, Ahrens (2) found that shallow incorporation after planting, provided improved weed control without injury to the nursery stock. Dichlobenil may also be incorporated into an organic mulch prior to

applying the mulch to an ornamental planting bed (17, 34). This not only reduces the loss by volatility, but facilitates application of the herbicide to irregular areas.

In an evaluation of many herbicides for activity on the submerged aquatic weeds, Potamogeton pectinatus L. (sago pondweed) and P. nodosus Poin. (American pondweed), dichlobenil was one of two compounds which performed most effectively (21, 64). However, postemergence applications did not control rooted, submerged, aquatic plants and filamentous algae (64). Dichlobenil is not acutely toxic to fish at herbicidal concentrations.

Behavior in the Soil

Massini (41), studying the adsorption of dichlobenil, found it to have a very high affinity for lignin ($k = 400-1000$) and lipids ($k = 180-300$). When a 6.0 ppm solution of dichlobenil was passed through a column containing potting soil (22% organic matter) or a sandy soil (5% organic matter), no dichlobenil could be detected in the effluent. However, sand did not adsorb dichlobenil under these circumstances. Horowitz (26) showed that organic matter had a more restrictive effect on the movement of dichlobenil than clay. The lateral spread of the herbicide near the surface was inversely proportional to the organic matter content of the soil.

Dichlobenil may also move in soil as vapor (41) but to a much greater extent in sand than in a soil high in organic matter. In moist soil, the vapor movement of dichlobenil is much less than in dry soil (26).

Incorporation of dichlobenil into the soil immediately after application increased persistence (2, 7, 55). Sheets (55) found that the initial loss of dichlobenil from plots treated with 40 lb/A and incorporated was very rapid, until the residue concentration dropped to about 10%. Subsequent loss occurred at a very slow rate, which is attributed to the slow release of the remaining herbicide from the adsorbed phase. Barnsley (7) reported similar results and concluded that persistence and rate of loss by volatilization are drastically modified by mechanical incorporation with the soil and rainfall after spraying. Experiments carried out in the tropics showed that loss by volatilization was accentuated at the higher temperatures. This is substantiated by Hein (25) who showed under laboratory conditions, that vapor losses from soil increased with increasing temperature, the greatest loss occurring from 30 to 40 C. As the moisture level is increased from air dry to field capacity, the volatility of dichlobenil increased sharply. In cranberry bogs, subjected to periodic flooding, dichlobenil was quite persistent in the 0-4 in depth with very little leaching into the 4-8 in depth (44). However, it

appeared that most of the herbicide in the 0-4 in depth was ineffective due to adsorption on the organic matter.

Uptake and Translocation

Massini (41) found that French dwarf beans (Phaseolus vulgaris L.) which had been exposed to a saturated atmosphere of dichlobenil at room temperature for 4 days absorbed the compound almost uniformly by all aerial parts. However, when applied as a foliar spray, uptake was limited (7, 46). Dichlobenil is readily taken up from an aqueous solution through the cut petiole of a bean leaf and translocated upward (41). The acropetal movement of dichlobenil is much slower than that of water which Massini attributes to the high affinity of dichlobenil for plant material. Uptake via the roots of beans from an aqueous solution gave the same type of distribution pattern as uptake via the petiole (41, 64).

There is some evidence that dichlobenil is volatilized directly from the shoots. Verloop and Nimmo (64) found that dichlobenil accumulates in bean roots about 3 fold the concentration detected in the shoots. However, the concentration in the leaves drops to about 1/4 (after 1 day) to about 1/20 (after 5 days) of the root concentrations indicating that the herbicide volatilizes from the leaves or is metabolized to CO_2.

Pate and Funderburk (46) studying the uptake of ^{14}C-dichlobenil in bean and alligator weed (Alternanthera philoxeroides (Mart.) Griseb.) found that the movement of

^{14}C-dichlobenil or its ^{14}C-labeled metabolites in a basipetal direction was limited, but acropetal movement occurred readily. Carbon labeled dichlobenil applied in a lanolin paste is absorbed and translocated, but only in an acropetal direction (41, 46).

Metabolism in Plants and Animals

A water soluble metabolite of dichlobenil, 2,6-dichlorobenzoic acid, was found in bean, alligatorweed and four genus of fungi using thin layer and gas chromatography (46). Massini (41) also found limited evidence that dichlobenil was metabolized in plants, but the metabolite was not identified. Traces of 2,6-dichloro-3-hydroxybenzonitrile and its 4-hydroxy analog were detected in apple and wheat (Triticum vulgare L.) plants 4 months after application of 2,6-dichlorothiobenzamide (chlorthiamid) to the soil (9).

Rabbits, rats and dogs (22, 59, 69) readily metabolize dichlobenil to 2,6-dichloro-3-hydroxybenzonitrile and its 4-hydroxy analog. The monophenolic metabolites account for approximately 45% of the administered dichlobenil (23). Other metabolites found are 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid, 2,6-dichloro-3-and-4-hydroxybenzoic acid and 6 other polar constituents. These metabolites are excreted primarily in the urine. The monophenolic metabolites are present both in the free state or as glucuronide conjugates.

Physiological Effects

Dichlobenil is a strong inhibitor of germination and appears to have its most pronounced effects in the meristematic regions of established plants (33). The symptoms produced by dichlobenil on established plants are similar to those induced by boron deficiency (43). Milborrow suggests that the macroscopic and microscopic appearance of dichlobenil treated and boron deficient plants are so much alike that in each case, the same basic process is affected. This hypothesis can be substantiated only when the role of boron in plant metabolism is elucidated.

Foy and Penner (20) demonstrated an uncoupling effect of dichlobenil on oxidative phosphorylation in mitochondria isolated from cucumber (Cucumis sativa L.) cotyledons. Dichlobenil consistently stimulated succinate and α-ketoglutarate utilization at concentrations of 1.45×10^{-4} and 0.73×10^{-4} M. When inorganic phosphate (Pi) esterification was measured, dichlobenil had a pronounced inhibitory effect, providing P/O ratios of 0 to 0.5. The effect of dichlobenil on mitochondria differed from that of 2,4-dinitrophenol (DNP) at 10^{-5} M, since DNP did not affect O_2 consumption but did decrease Pi esterification.

Wit and van Genderen (70) found both of the monophenolic metabolites of dichlobenil from animals to cause a significant increase in oxygen consumption of starved

yeast cells incubated with a limited quantity of glucose. Dichlobenil, 2,6-dichlorobenzoic acid and 2,6-dichlorobenzamide were inactive in this assay.

It has been proposed that in animals the monophenolic metabolites are the uncouplers of oxidative phosphorylation and also combine with glucuronic acid and sulphuric acid to give harmless conjugates (60, 71). Over a critical dose level of dichlobenil, the formation of the phenolic metabolites in the liver may be great enough to uncouple oxidative phosphorylation such that insufficient ATP will be available for further conjugation of phenols. Since the hydroxylation of dichlobenil is independent of ATP, this process may continue. Subsequently, the concentration of the free phenols in the liver may rise sharply, resulting in a complete loss of ATP and the death of the cell from a lack of energy.

Mann et al. (38) studying the effect of herbicides on protein synthesis found that dichlobenil at 2.0 and 5.0 ppm did not greatly inhibit the incorporation of ^{14}C-leucine into protein in either barley (Hordeum vulgare L.) coleoptiles or cory (Sesbania exaltata Raf.) hypocotyls. These experiments were carried out with short incubation periods (3 hr) in an attempt to detect the primary effects of the herbicides being tested. Dichlobenil does not appear to affect protein biosynthesis directly. When cory hypocotyl sections were incubated

4.5 hr in solutions containing 10 and 20 ppm dichlobenil, lipogenesis, measured by incorporation of ^{14}C from malonic acid-2-^{14}C was inhibited 30 to 40% (39). It was not determined whether dichlobenil had a direct effect on lipogenesis or whether the inhibition was due to lowered ATP levels in the treated tissue.

Since dichlobenil is an effective seed germination inhibitor, its effect on the activity of various enzymes during germination has been investigated. Ashton et al. (5) found that the proteolytic activity in the cotyledons of 3-day-old squash seedlings germinated in 10^{-4} M dichlobenil, was 30% of the control. However, the activity of the proteolytic enzymes isolated from control plants, was not affected by the presence of dichlobenil in the reaction mixture. In cotyledons from 2-day-old squash seedlings, the inhibition of proteolytic activity induced by 10^{-4} M dichlobenil could be partially overcome by the addition of 10^{-5} M benzyladenine to the culture solution (48). Dichlobenil may affect the synthesis or action of the hormone which controls the synthesis of proteolytic enzymes. The deficiency of ATP caused by uncoupled oxidative phosphorylation, may also affect the development of proteolytic activity.

Using intact barley seeds, Penner (49) showed that treatment with 10^{-4} M dichlobenil for two days caused amylase activity to decrease to 49% of the control. This

inhibition was not overcome by adding 10^{-6} M gibberellic acid to the incubating medium. However, with de-embryonated half seeds, a similar inhibition of amylase activity was observed but could be eliminated by adding gibberellic acid to the incubation solution. Thus, dichlobenil appears to interfere with the control of amylase synthesis and not with its activity per se.

In studies conducted by Funderburk and Carter (22), the first trifoliate leaf of bean plants treated with 20 ppm dichlobenil was exposed to $^{14}CO_2$ for 24 hr in continuous light. Subsequent extraction and identification of labeled compounds showed that dichlobenil did not affect the distribution of fixed $^{14}CO_2$.

Morphological Studies

Nodal tissue of alligator weed from plants treated with 2.0 lb/A dichlobenil was sectioned and investigated by Pate et al. (45). After 24 hr, the end walls of young sieve tubes had collapsed. Four days after treatment, damaged tissues were observed both within and above the nodes, however, inactive buds generally were not injured. Coagulation of protoplasm and collapsed cell walls in the phloem and surrounding parenchyma cells was also observed. Seven days after treatment, large segments of phloem and associated tissues appeared to be destroyed. The lack of injury to axillary buds was attributed to the fact that

vascular connection between inactive buds and the stem was not differentiated, thus no herbicide was translocated into the bud.

Devlin and Demoranville (16) made the observation that cranberry bogs treated with dichlobenil produced berries with more intense red color. Berries from plots receiving spring applied dichlobenil at 3.0 and 4.0 lb/A contained 41% and 64% more anthocyanin respectively than control berries.

Welker (69) made applications of dichlobenil at 4.0 and 8.0 lb/A each year for the control of Virginia chain fern (Woodwardia virginica L.) in cranberry bogs. The first injury symptoms on the fern were observed 4 to 6 weeks after herbicide treatment. The initial symptom was a brittleness that caused numerous broken stems. Similar symptoms were also observed on cherry trees which had received an excessive application of dichlobenil (52).

MATERIALS AND METHODS

Toxicity of Dichlobenil to Quackgrass

Rhizomes of quackgrass were collected from the field and cut into 2 cm single-node sections. After washing, the sections were planted in quartz sand in 11 x 7 cm plastic pots; 10 sections per pot. The pots were watered with Hoagland's No. 2 nutrient solution containing 0, 5.8×10^{-8}, 2.9×10^{-7}, 5.8×10^{-7}, 2.9×10^{-6} and 5.8×10^{-6}M of either dichlobenil or 2,6-dichlorobenzoic acid. Each treatment was replicated 4 or 5 times in a completely randomized design. Every third day, the pots were flushed with distilled water and fresh solutions added. After 2 weeks the number of buds which had initiated growth and the fresh weight of shoots were recorded.

To compare the effect of dichlobenil on intact quackgrass plants vs clipped plants, single node rhizome sections were grown for 3 weeks in quartz sand and watered with Hoagland's No. 2 nutrient solution. After thinning each pot to 5 uniform plants, the shoots in one half of the pots were cut off at the surface of the sand. The pots were treated with nutrient solution containing 0, 5.8×10^{-6}, 2.9×10^{-5} or 5.8×10^{-5}M dichlobenil for a

period of 2 weeks. The treatments were replicated 4 times and completely randomized on a greenhouse bench. At the termination of the experiment the shoots were removed, dried and weighed.

Uptake and Translocation Studies

Preliminary experiments with quackgrass plants indicated that after an initial uptake of ^{14}C-dichlobenil there was a subsequent loss of radioactivity from the shoots. Seedling corn plants (Zea mays L. cv. Harris Gold Cup) were selected for further studies, so that uniform plants could be obtained rapidly. Seeds were germinated in vermiculite moistened with distilled water. After the first leaf emerged from the coleoptile, the seedlings were transplanted into 11 x 7 cm plastic pots containing quartz sand. The pots were watered daily with Hoagland's No. 2 solution and grown at 27 C with 2,000 ft-C light (16 hr) and at 17 C for an 8 hr night. When the plants were approximately 15 cm high, they were removed from the sand and the seed coats were removed. After rinsing the roots, 8 plants were placed in each 250 ml beaker containing 200 ml of nutrient solution which was continuously aerated. The plants were supported by placing the shoots through perforated aluminum foil which covered the top of each beaker. Treatments

were applied after the plants were allowed to equilibrate for 24 hr.

Uptake of dichlobenil was studied by replacing the nutrient solution with fresh solutions containing 1 μc nitrile labeled ^{14}C-dichlobenil (specific activity 4.65 mc/mM) per 100 ml of solution. The concentration of dichlobenil in the solution was 2.1 x 10^{-6}M. Because of the volatility of this compound, all tracer studies were carried out in a hood under a bank of fluorescent lights. At time intervals of 4, 12, 36 and 72 hr, plants were removed from the radioactive solutions and their roots rinsed 5 times in distilled water. The roots were blotted between paper towels and dissected from the shoots. The tissue was cut into small sections, placed in glass vials and quick-frozen in acetone-dry ice. The tissue was maintained in the frozen state prior to extraction.

Preliminary experiments using the extraction procedures of Meulemans and Upton (42) and Pate and Funderburk (46) demonstrated that there were no water soluble metabolites of dichlobenil formed in corn plants within 72 hr. Therefore, a single benzene extract was ascertained to be sufficient to account for all ^{14}C-dichlobenil within corn plants at this stage of growth.

The frozen samples were weighed and homogenized thoroughly with 4.0 ml benzene in a glass hand homogenizer. The homogenate was fast-filtered through Whatman No. 1

filter paper and the residue rinsed 3 times with 1.0 ml aliquots of benzene. The filtrate was brought to a constant volume and a 1.0 ml aliquot placed in a scintillation vial containing 15 ml toluene-BBOT. After counting for 10 min, the data were corrected for quenching and the results expressed as dpm per mg fresh weight.

The radioactivity in the nutrient solution was also assayed at each sampling time. This was accomplished by first measuring the volume of the nutrient solution and then removing 1.0 ml which was placed in a scintillation vial containing 15 ml of toluene BBOT:Triton X-100 (10:4) (47).

The radioactivity in plant extracts and nutrient solution was identified by spotting 10-30 µl of the condensed samples on a prepared (Kodak) silica gel G thin layer plate. Pure ^{14}C-dichlobenil was also spotted on each plate as a reference. After developing the chromatograms in an appropriate solvent system for 10 or 15 cm, they were cut into sections 1.0 cm wide and each section placed in a separate scintillation vial for counting. Dichlobenil had an Rf of 0.40 when developed in ethanol: hexane (1:9 v/v) at 25 C and an Rf of 0.65 when developed in diethylether:petroleum ether (1:1 v/v) at 3 C.

Volatilization of Dichlobenil from Plants

Ten-day-old corn plants were placed in nutrient solutions containing 2.0 µc ^{14}C-dichlobenil for 24 hr.

At the end of the preloading period, the roots were rinsed 6 times with distilled water and 3 plants were placed in each of 3, 25 ml erlenmeyer flasks containing 20 ml of fresh nutrient solution. Duplicate plants were frozen immediately for subsequent determination of the initial ^{14}C-dichlobenil concentration. The top of each flask was sealed with a plug of cotton and polyethylene glycol 1500 which was found to be impermeable to ^{14}C-dichlobenil. The 3 flasks were placed inside the quart mason jar component of the trapping device (Figure 1). Glass tubes were inserted into the metal top to provide an air inlet and outlet. To the outlet tube 2, 200 ml gas washing bottles with glass fritted disks were connected in series. The gas washing bottles were filled with 10 ml toluene, the trapping solvent, and maintained in an ice bath. Filtered, compressed air was passed through a $CaCl_2$ moisture trap, into the jar containing the plants and then bubbled through the trapping solvent. The toluene solutions were adjusted to a constant volume, 20 ml aliquots were removed, and 80 mg BBOT was added to each aliquot prior to counting. The remainder of the trapping solvent was condensed by flash evaporation and the radioactivity identified by thin layer chromatography. Also at the termination of the experiment, the radioactivity was assayed in the roots, shoots, and nutrient solution.

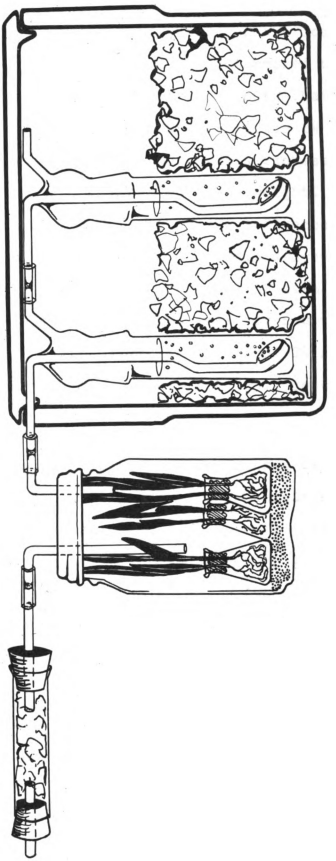


Figure 1. Apparatus for trapping ^{14}C-dichlobenil volatilized from corn leaves.

The influence of temperature on the loss of ^{14}C-dichlobenil from corn plants was determined by placing preloaded corn plants in separate growth chambers at 27 C and 10 C with continuous darkness. After 24 hr the plants were extracted and the results expressed as per cent of the initial dpm per mg fresh weight. Similar studies were conducted to study the influence of light on the loss of ^{14}C-dichlobenil. Preloaded corn plants were placed in a growth chamber at 27 C with continuous light. One-half of the plants were covered with a box which excluded all visible light. After 24 hr the plants were removed and extracted as previously described.

Mode of Action of Dichlobenil

Membrane permeability.--The leakage of betacyanin from red beet (Beta vulgaris L. cv. Detroit Dark Red) root tissue was used as an assay for the effect of dichlobenil on membrane permeability. The technique adopted was modified from Veldstra and Booij (63). Cylinders, 4.0 mm in diameter, were taken from fresh red beet roots with a cork borer and cut into sections, 5.0 mm thick, with equally spaced coupled raxor blades. The sections were rinsed with distilled water until all pigment from disrupted cells had been removed. Any sections containing large amounts of vascular tissue or those which were exceedingly colored were removed prior to initiating the

experiment. Ten root sections were placed in a 50 ml erlenmeyer flask containing 20 ml of solution of the compound to be investigated. All treatments were prepared in distilled water containing 20 ppm of streptomycin sulfate. The flasks were stoppered with a porous plug and placed in a water bath at 30 C. After 2, 4, 8, 12, 16, and 24 hr, 3.0 ml aliquots were removed from each flask and their absorbance measured at 540 mμ on a Beckman DB-G spectrophotometer using fresh treating solution as a blank. Immediately after removing the 3.0 ml aliquot for assay, 3.0 ml fresh treating solution was added to the flask.

The leakage of reducing sugars into the ambient solution was also measured as an index of permeability changes. Nelson's Test (14) was utilized on aliquots of the solution at each sampling period. All treatments were replicated 5 times and experiments were repeated at least 3 times.

Respiration of intact tissues.--The oxygen consumption of red beet root sections, described in the previous paragraph, was measured using manometric techniques (59). Two root sections were placed in a Warburg flask containing 4.0 ml of a solution of the compound being investigated plus 20 ppm of streptomycin sulfate. After allowing the flasks to equilibrate in a water bath at 30 C for

15 min, the manometers were closed and readings taken periodically over a 12 hr interval. At the end of each experiment, the tissue was removed from the flasks, dried, and weighed. Results were expressed as µl O_2 consumed per mg dry weight. Similar experiments were carried out using roots from 5-day-old corn plants which had been grown in petri dishes. The roots were excised 3.0 cm from the tip and 3 roots placed in a Warburg flask containing 3.0 ml of .05 M phosphate buffer (pH 6.5) to which 0, 5.8×10^{-6} and 5.8×10^{-5}M dichlobenil had been added.

Oxidative phosphorylation in isolated mitochondria.--Mitochondria were isolated from etiolated, 4-day-old cucumber hypocotyls or cotyledons. The seedlings were germinated on cheesecloth placed on hardware cloth suspended in a plastic tray containing tap water. The trays were covered with perforated polyethylene and placed in a growth chamber. All light was excluded from the chamber and the temperature was maintained at 30 C.

Cotyledons and hypocotyls were excised and placed in separate beakers which were packed in ice until extraction. Prior to extraction, the cucumber hypocotyls were cut into segments 2 to 3 cm long and divided into 25 g lots. Each 25 g lot was macerated for 30 sec in a prechilled mortar and pestle with 50 ml of grinding medium. The cotyledons were also divided into 25 g lots

and ground with 50 ml grinding medium, but the length of grinding was increased to 60 sec. All grinding and subsequent contrifugation operations were carried out between 0 and + 4 C.

The mitochondria were isolated using the procedure of Ikuma and Bonner (29) which was modified by including 0.01M N-2-hydroxyethyl piperazine-N-ethane sulfonic acid (HEPES) pH 7.2 in the grinding, washing and reaction mediums. The final volume of the mitochondrial suspension was 1.0 to 1.4 ml containing 15 to 25 mg protein as determined by micro-Kjeldahl.

Oxygen uptake was measured polarographically using a Clark type oxygen electrode connected to an amplifier and recorder assembly. The electrode was encased in a lucite plunger which was placed in the sample chamber containing the reaction medium. Materials were introduced into the sample chamber by means of a hypodermic syringe inserted in an access groove in the plunger. The temperature of the sample chamber was maintained at 25 C with a constant temperature circulating water bath. 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 (720 mμmoles O_2/ 3 ml) in the aerated medium as calculated by Chance and Williams (13). Oxygen consumption rates were expressed as mμmoles O_2/min per 3 ml of reaction medium.

All solutions were prepared in distilled water and sterile filtered through a 5 μ Millipore filter immediately before use. The concentration of ADP was measured spectrophotometrically at 260 mμ on the basis of its mM extinction coefficient of 14.5. The concentration of other chemicals was measured gravimetrically. Stock solutions of dichlobenil, 2,6-dichloro-3-hydroxybenzonitrile and DNP were prepared in ethanol and added to the reaction media in 5 μl aliquots.

Phosphate esterification and determination of P/O ratios.--Time course phosphorylation experiments were conducted by placing 0.1 ml of the mitochondrial suspension (0.15 mg mitochondrial N) in a 50 ml erlenmeyer flask containing 2.9 ml of the reaction medium to which 0.5 mg hexokinase and 50 mM glucose was added. After removing 0.1 ml for Pi determination, the flasks were stoppered and placed in a 25 C water bath. Aliquots were removed at 15, 30, 45, and 60 min and assayed for Pi by the Taussky and Shorr (58) method as modified by Penner (50). The Pi consumed was plotted as a function of time and the slopes compared using Student's T test.

The P/O ratios were determined manometrically by placing 0.1 ml of the mitochondrial suspension (0.80 mg mitochondrial N) in Warburg flasks containing 2.7 ml of the hexokinase reaction medium. The substrate, 20 mM succinate, 820 mμmoles ADP, and the inhibitor being

investigated were placed in the side arm in 0.3 ml of the reaction medium. After removing a 0.1 ml aliquot for Pi determination, the flasks were attached to the manometers and placed in a 25 C water bath. After allowing the flasks to equilibrate for 15 min, the manometers were closed and the substrate-inhibitor mixture tipped into the flasks. Oxygen consumption readings were taken at 10 min intervals for 40 min. The flasks were removed and immediately placed on ice prior to removing a 0.1 ml aliquot for Pi determination. The quantity of Pi esterified at 0 and 40 min was determined as described above.

^{14}C-Glucose utilization.--The utilization of C_1 and C_6 labeled glucose by 4-day-old cucumber seedlings was determined using a radiorespirometer. The device was similar to that designed by Wang (66). Single cucumber seedlings treated for 24 hr with 10^{-5}M dichlobenil were placed in reaction flasks and compared to controls. Each treatment was then divided and one half of the flasks received 3.0 ml of sterilized, distilled water containing 0.5 µc of C_1 labeled glucose (specific activity 3.34 mc/mM); the other half received the same quantity of C_6 labeled glucose (specific activity 3.60 mc/mM). The flasks were attached to the CO_2 trapping system and placed in a circulating water bath at 25 C. The $^{14}CO_2$ evolved was trapped in 10 ml ethanol:ethanolamine (1:1). After adjusting the air flow rate to 60 ml per min, the

air flow was diverted through the alternate $^{14}CO_2$ trap containing fresh solution to initiate the experiment. The trapping solutions were sampled at 1 hr intervals for a period of 4 hr. At each sampling time, the ethanol: ethanolamine was removed and the chamber rinsed with 3.0 ml of ethanol. The trapping solution and washing were adjusted to 15 ml with ethanol and a 5.0 ml aliquot removed for counting by liquid scintillation. At the termination of each experiment, the tissue was removed from the reaction flask and oven dried. The sample was then combusted using the Schöniger technique (67) and the $^{14}CO_2$ liberated was collected in ethanol:ethanolamine (1:1) for counting by liquid scintillation.

Changes in free amino acids pools.--Oat seeds were planted in styrofoam cups containing quartz sand and watered with Hoagland's No. 2 solution. After 1 week, 0, 10^{-6}M or 10^{-5}M dichlobenil were applied in the nutrient solution. Throughout the growth and treatment period, the plants were maintained in a growth chamber with a 16 hr photoperiod at 24 C and an 8 hr night at 18C. After 24 hr the plants were removed and all sand washed from the roots. Shoots and roots were placed in separate aluminum containers and quick-frozen in acetone-dry ice prior to lyophylization. One hundred mg of the dried tissue was ground in a mortar and pestle with 70% ethanol containing a small number of glass grinding beads. The homogenate

was transferred with several washings to a 50 ml polyethylene centrifuge tube; the total volume of the extract was 20 ml. After gentle boiling of the extract for 15 min, it was centrifuged at 1000 x g for 10 min. The supernatant was decanted and the residue extracted 2 more times with 70% ethanol. The supernatants were combined and condensed to dryness in a flash evaporator. The residue was then carefully taken up in 10% isopropanol, 1.0 ml for root extracts and 2.0 ml for shoot extracts. The free amino acids present were determined by the ninhydrin colorimetric method described by Rosen (53). Results are expressed as μmoles per mg dry weight.

Lignin-like Polyphenol Accumulation

Spectrophotometric assay.--Three-day-old etiolated cucumber seedlings were placed on filter paper in petri dishes containing either 0, 10^{-6} or 10^{-5}M dichlobenil. After 48 hr, the plants were removed, the cotyledons discarded, and the roots dissected from the hypocotyls. The tissue was oven dried and ground in a Wiley mill with a 40 mesh screen. Thirty mg of the dried tissue was extracted with 3.0 ml of 0.5 N NaOH for 16 hr in a water bath shaker (70 C). Using the method of Stafford (56) the ultraviolet absorption spectra were determined on two aliquots. One was diluted with 0.05 N NaOH (pH 12.3) and the other with 0.05 M phosphate buffer (pH 7.0), the

difference spectrum being obtained by subtraction. Absorbance was measured using a Beckman DB-G spectrophotometer at intervals of 10 mμ from 230 to 450 mμ. Similar studies were conducted using 1-week-old oat plants which had been grown in quartz sand.

Histological test for lignin.--Three-day-old cucumber seedlings in petri dishes were treated for 48 hr with distilled water or 10^{-5}M dichlobenil. The roots were killed and fixed in FAA (40% formaldehyde-acetic acid-50% ethanol 5:5:90 v/v/v). After dehydration of the tissue through a tertiary-butyl alcohol series (32), it was infiltrated with paraffin and imbedded in Tissuemat. Sections 30 μ thick were prepared on a rotary microtome and affixed to glass slides using Haupt's adhesive. The paraffin was removed with xylene and the sections stained with phloroglucinol:HCl (54).

RESULTS AND DISCUSSION

Toxicity of Dichlobenil to Quackgrass

Sprouting of axillary buds was completely inhibited by dichlobenil at 2.91 x 10^{-6}M (Figure 2). The lethal concentration for these active buds was between 0.58 x 10^{-6} and 2.91 x 10^{-6}M. Established plants are more tolerant, however, their dry weight was reduced 50% when 5.8 x 10^{-6}M dichlobenil was added to the nutrient solution for a 2 week period. The susceptibility of established plants was increased by removing the shoots prior to application of dichlobenil to the nutrient solution (Table 1). Growth from intercalary meristems was completely inhibited. This data confirmed field results where spring applications of dichlobenil provided better quackgrass control when the tops were removed either by mechanical mowing or application of paraquat (51).

These results agree with several workers (33, 41, 46) who have found dichlobenil to be most toxic to meristematic tissue and germinating seeds. The fact that dichlobenil is less toxic to intact plants may be due to several factors; it is metabolized to a non-toxic form, it becomes complexed with plant constituents, or the concentration in the shoots is so dilute that it cannot

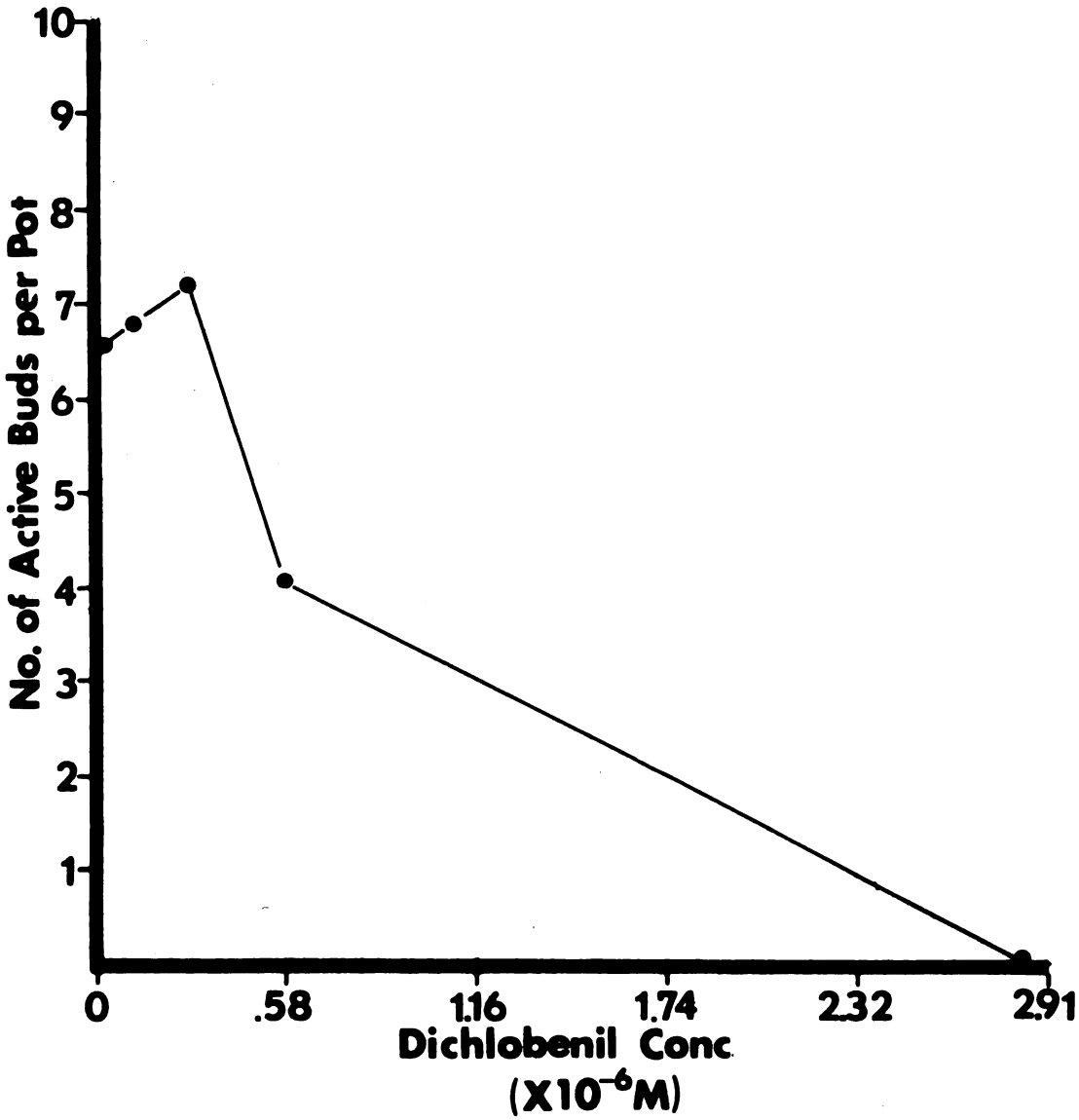


Figure 2. Effect of dichlobenil on growth of axillary buds of quackgrass.

accumulate at its site of action. To answer these questions, uptake studies were carried out using ^{14}C-dichlobenil.

TABLE 1.--The growth of intact and clipped quackgrass plants receiving dichlobenil.[1]

Dichlobenil conc (M)	Dry wt (g)	
	Clipped	Intact
0	.37	1.33
5.8×10^{-6}	.08	.63
2.9×10^{-5}	.04	.38
5.8×10^{-5}	.03	.48

[1]The F value for clipped vs intact X linear response to dichlobenil is significant at the .05 level.

Uptake and Translocation Studies

With rapid aeration (100 ml air per min) of the ^{14}C-dichlobenil solutions, 57% of the radioactivity was lost from the solution within 12 hr (Figure 3). This loss of radioactivity was not accounted for by plant uptake, which suggested volatilization directly from the nutrient solution. With this aeration system, the highest concentration of ^{14}C-dichlobenil in corn roots was attained at 4 hr and then continuously decreased until the experiment was terminated at 72 hr (Figure 4A). The decrease in

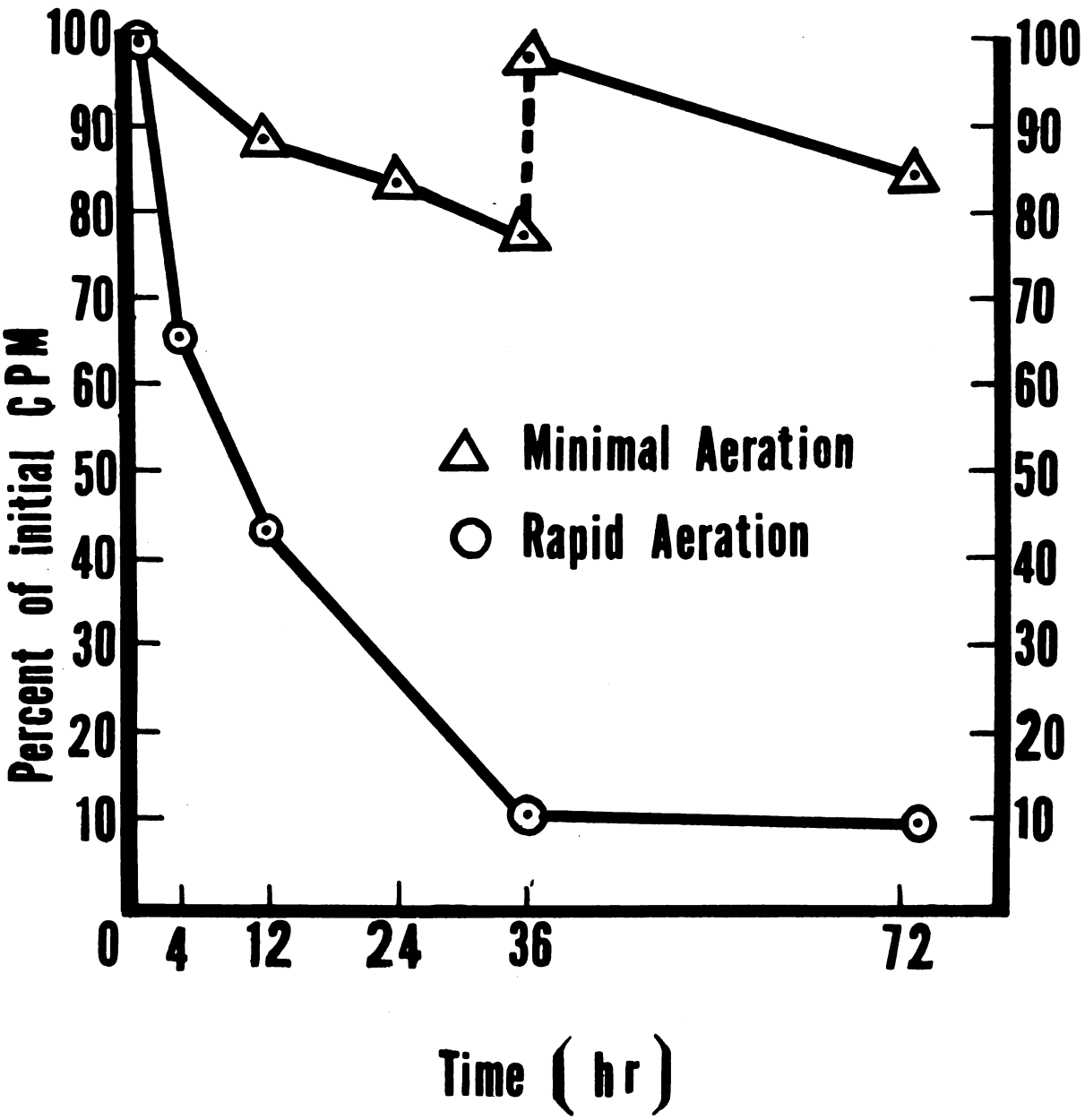
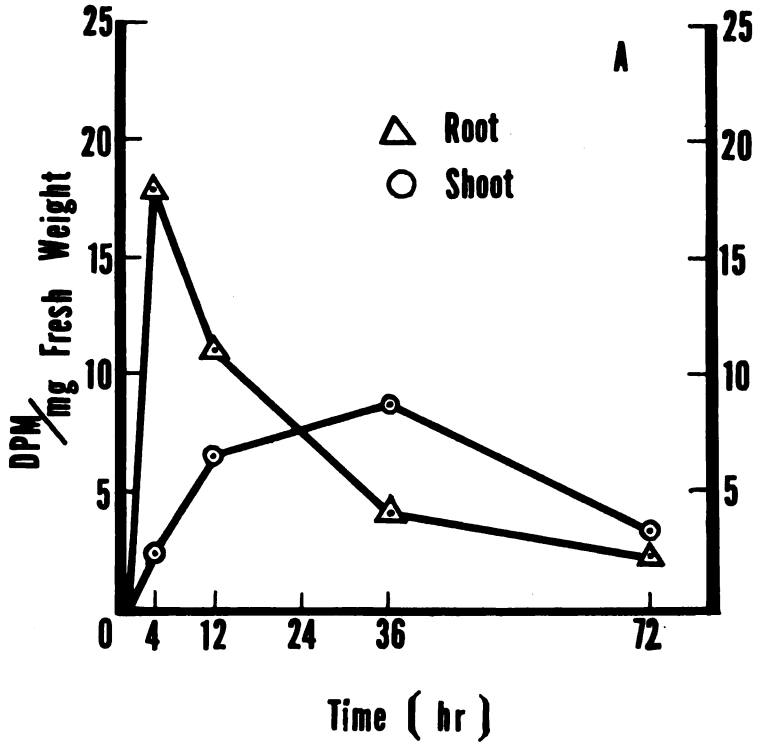


Figure 3. The per cent of the initial CPM in the nutrient solution, using minimal (15 ml/min) and rapid aeration (100 ml/min). At 36 hr, the minimal aeration scheme was brought up to the original concentration by adding sufficient ^{14}C-dichlobenil.



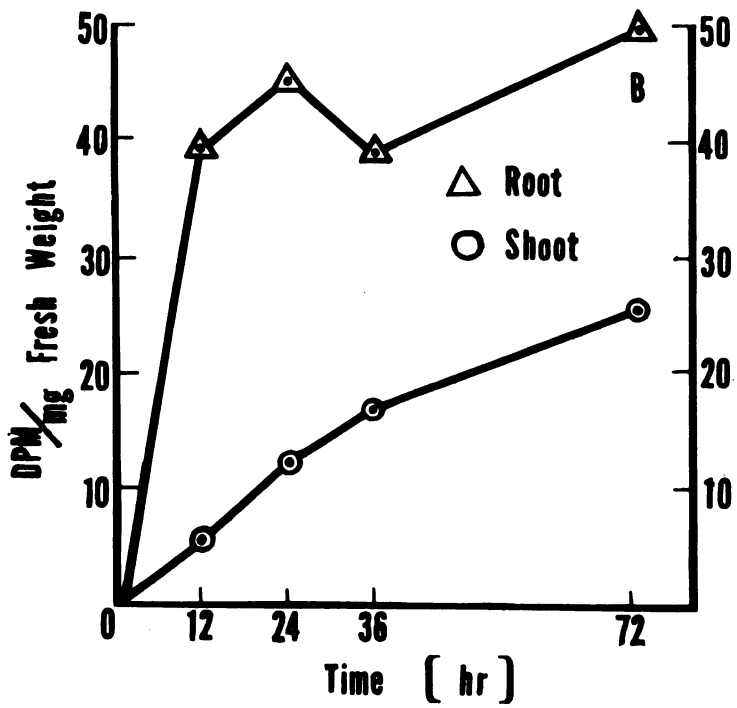


Figure 4. Radioactivity recovered from the roots and shoot of corn plants grown in nutrient solution containing ^{14}C-dichlobenil with rapid aeration (100 ml/min) (A) and mineral aeration (15 ml/min) (B).

^{14}C-dichlobenil in the roots closely paralleled the decrease of ^{14}C-dichlobenil in the nutrient solution. The concentration of ^{14}C-dichlobenil in the shoots continued to increase for 36 hr but decreased over the subsequent 36 hr (Figure 4A). The data indicates that with a limited source of ^{14}C-dichlobenil, there is a net loss of radioactivity from the shoots. The observed loss may be a result of volatilization from the leaves, a recycling and loss from the roots, or metabolism of the herbicide to $^{14}CO_2$.

A relatively constant concentration of ^{14}C-dichlobenil in the nutrient solution was attained by reducing the aeration to a minimum (15 ml/min) and adding sufficient ^{14}C-dichlobenil after 36 hr to return the nutrient solution to its original concentration (Figure 3). Utilizing this procedure, both roots and shoots contained increasing amounts of radioactivity up to 72 hr (Figure 4B). Under these conditions, any loss of ^{14}C-dichlobenil from the shoots was masked by continued uptake.

Volatilization of Dichlobenil from Plants

Corn plants extracted immediately after the 24 hr preloading period contained 64% of the total radioactivity in the roots and 36% in the shoots. However, after 24 hr in the vapor trapping device, only 12% and 7% of the

initial radioactivity was detected in the root and shoot respectively (Figure 5). Of the initial radioactivity, 10% was recovered in the toluene traps. The ^{14}C-compound had the same Rf value as dichlobenil on thin layer chromatograms utilizing two solvent systems. No radioactivity was recovered from stoppered control flasks containing nutrient solution to which 0.05 µc ^{14}C-dichlobenil had been added. These experiments show that ^{14}C-dichlobenil is transported to the shoots and may volatilize directly from the leaves of corn plants to the atmosphere. Massini (41) showed that vapors of dichlobenil can be readily taken up by all aerial plant parts, thus it seems logical that the reverse process could also occur.

The largest quantity (71%) of the radioactivity was detected in the nutrient solution (Figure 5), indicating rapid efflux of dichlobenil from the roots with the reversed concentration gradient. Since the amount lost to the nutrient solution plus that recovered from the roots was greater than the initial quantity in the roots after preloading, there appeared to be limited cycling of ^{14}C-dichlobenil from the shoot to the root. The total amount of radioactivity recovered after the 24 hr trapping period was within 5% of the initial radioactivity in the preloaded plants.

The efflux of ^{14}C-dichlobenil from the shoots and roots of preloaded corn plants over a 24 hr period was

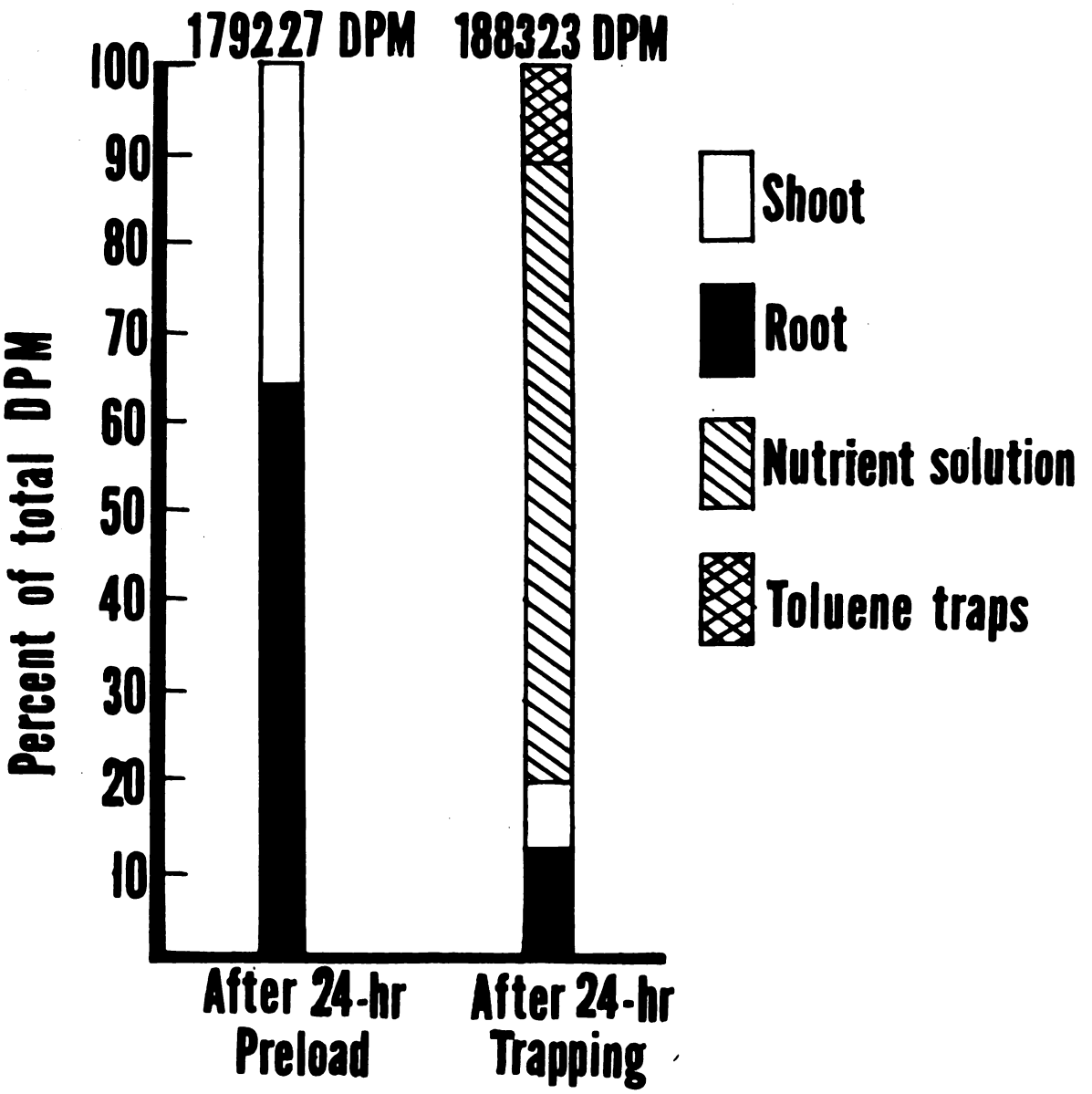


Figure 5. The distribution of radioactivity in corn plants after preloading with ^{14}C-dichlobenil for 24 hr followed by 24 hr vapor trapping period.

similar in the light and dark at 27 C (Figure 6). This supports the hypothesis that the volatilization of dichlobenil is not primarily through open stomates but occurs directly through the cuticle.

At 10 C there was only a 6% loss in radioactivity from the shoots, however, at 27 C the shoot contained only 58% of the initial radioactivity (Figure 7). Similarly, the efflux from the roots was greater at the higher temperature. At both temperatures the efflux from the roots was much greater than that from the shoots.

The rapid efflux of ^{14}C-dichlobenil from the roots of preloaded plants when placed in nutrient solution indicates that it is not actively held within the root cells, but readily passes through intervening membranes and cell walls. The lipophilic nature of the dichlobenil molecule may explain the apparent ease of movement through the plasmalemma and perhaps, other membranes. Another possibility is that the dichlobenil molecule partitions into the lipid-bilayer causing a disruption of the specific permeability properties of membranes. Hence, not only dichlobenil, but other soluble cell substances could be expected to leak from the treated root tissue.

Mode of Action of Dichlobenil

Membrane permeability.--The efflux of betacyanin from red beet root sections incubated in 10^{-6}, 10^{-5}, or

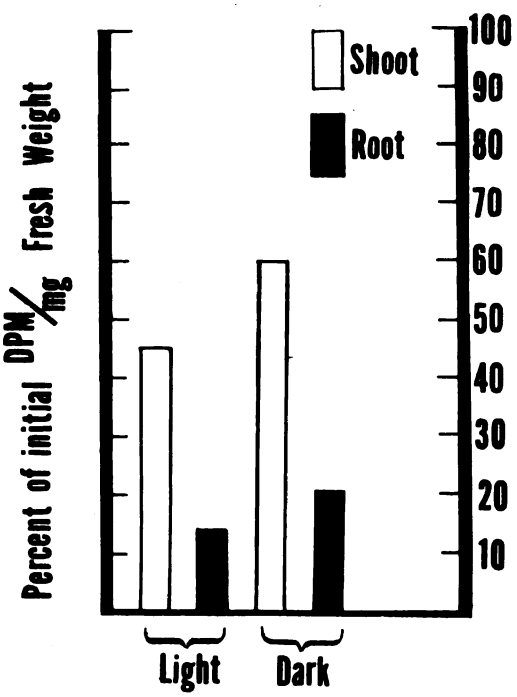


Figure 6. The per cent of initial radioactivity remaining in preloaded corn plants after 24 hr in the light or dark at 27 C.

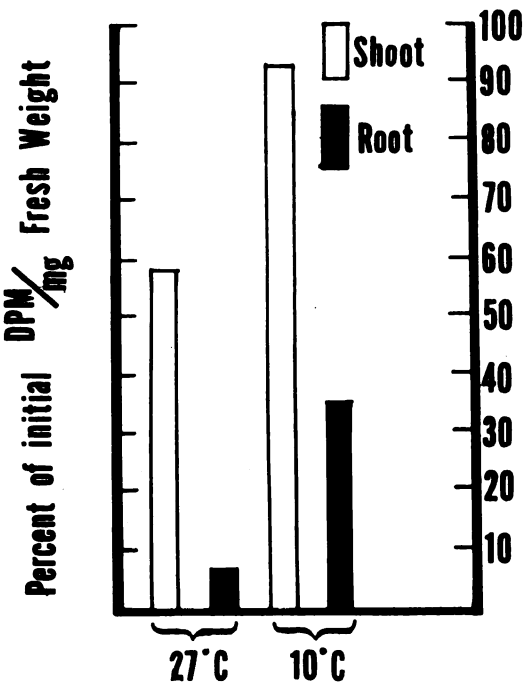


Figure 7. The per cent of initial radioactivity remaining in preloaded corn plants after exposure to 27 C and 10 C for a 24 hr loss period.

10^{-4}M solutions of dichlobenil was characterized by a lag phase for the first 8 hr (Figure 8A). However, after 8 hr of incubation, the leakage was rapid and increased linearly until 24 hr when the experiment was terminated. When equimolar concentrations of DNP were used, only the 10^{-4}M concentration caused a significant increase in betacyanin leakage into the ambient solution (Figure 8B). With 10^{-4}M DNP, rapid leakage occurred within 2 hr of incubation and subsequent efflux of betacyanin continued over the 24 hr period. Since DNP influenced permeability more rapidly than dichlobenil, the rate of uptake of the compounds into the tissue may differ or the two compounds may affect permeability in different manners. DNP is a well known uncoupler of oxidative phosphorylation, and its effect on permeability may be due to a reduction of available energy (ATP) necessary to maintain the integrity of the tonoplast and plasmalemma. With dichlobenil, a time interval is required either to accumulate a threshold concentration necessary to affect both membranes or to induce physiological changes preceding permeability changes. An alternative hypothesis is that dichlobenil itself is inactive, and must first be metabolized to a form which is active.

When Ca^{++}, which is known to stabilize membranes (37, 40, 61) was added as 1.6 x 10^{-4}M $CaCl_2$ to the incubating solution at 12 hr, it reduced the efflux of betacyanin

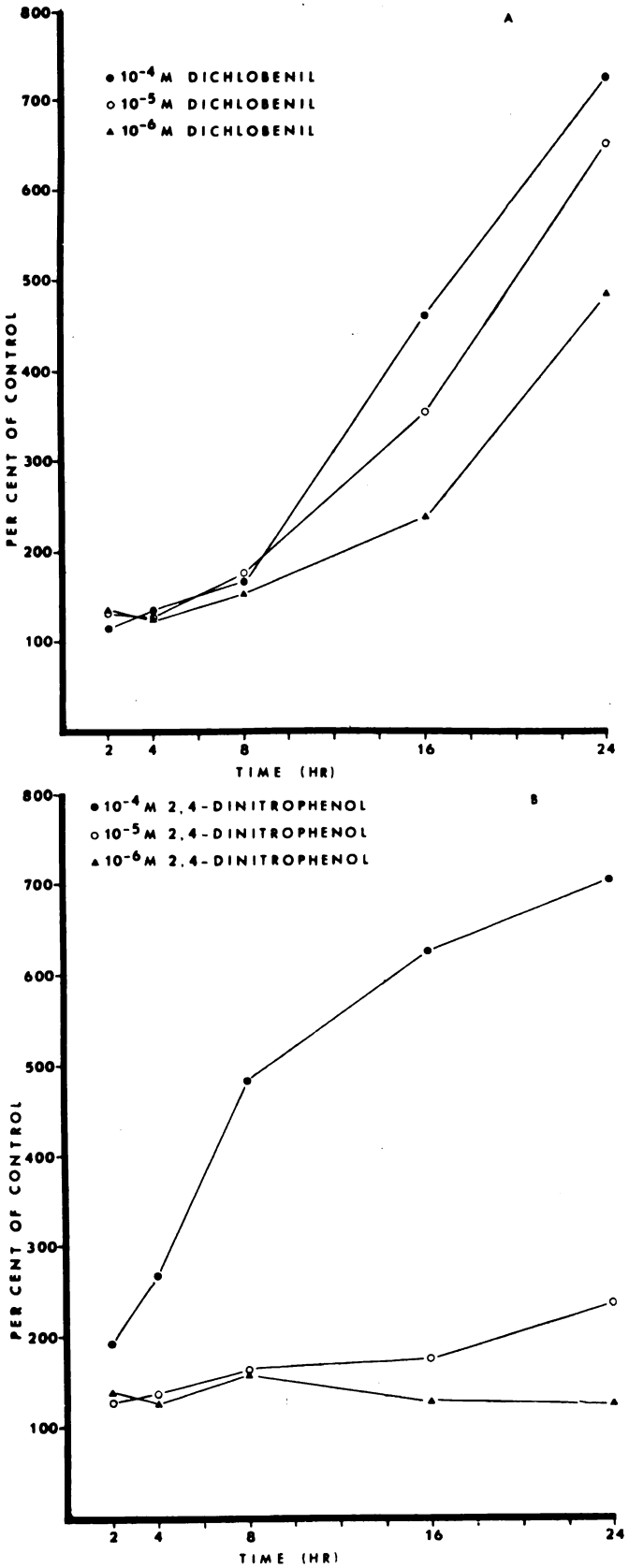


Figure 8. Leakage of betacyanin, expressed as per cent of control, from red beet root sections treated with dichlobenil (A) and 2,4-dinitrophenol (B).

induced by 10^{-4}M dichlobenil (Figure 9A). However, a similar concentration of $CaCl_2$ added to red beet root sections treated with 10^{-4}M DNP for 12 hr had no effect on subsequent leakage (Figure 9B). Again, there is an indication that the two compounds are acting differently. The membranes of the tissue treated with DNP may be irreversibly disrupted such that they cannot be stabilized with Ca^{++}. When 1.6 x 10^{-4}M $CaCl_2$ was added to the incubating solution initially, it alleviated the affect of 10^{-5}M dichlobenil and 10^{-4}M DNP similarly (Figures 10A and 10B).

The leakage of reducing sugars from red beet root sections treated with 10^{-6} and 10^{-5}M dichlobenil occurred in a similar manner as betacyanin (Figure 11). DNP at 10^{-4}M, however, induced no leakage of reducing sugars. It may be postulated that the DNP treated tissue had metabolized all free reducing sugars due to the uncoupled respiration, hence, no reducing sugars were available for leakage into the ambient solutions.

Respiration of intact tissue.--The rate of O_2 consumption by red beet root sections incubated in 10^{-6} or 10^{-5}M dichlobenil increased in the same magnitude as with 10^{-4}M DNP (Figure 12). The rate of O_2 consumption of all treatments was significantly greater than the control. Thus, the difference observed in leakage of reducing sugars from red beet root sections, treated with dichlobenil or DNP, cannot be explained by differences in respiration. The increased O_2 consumption of the

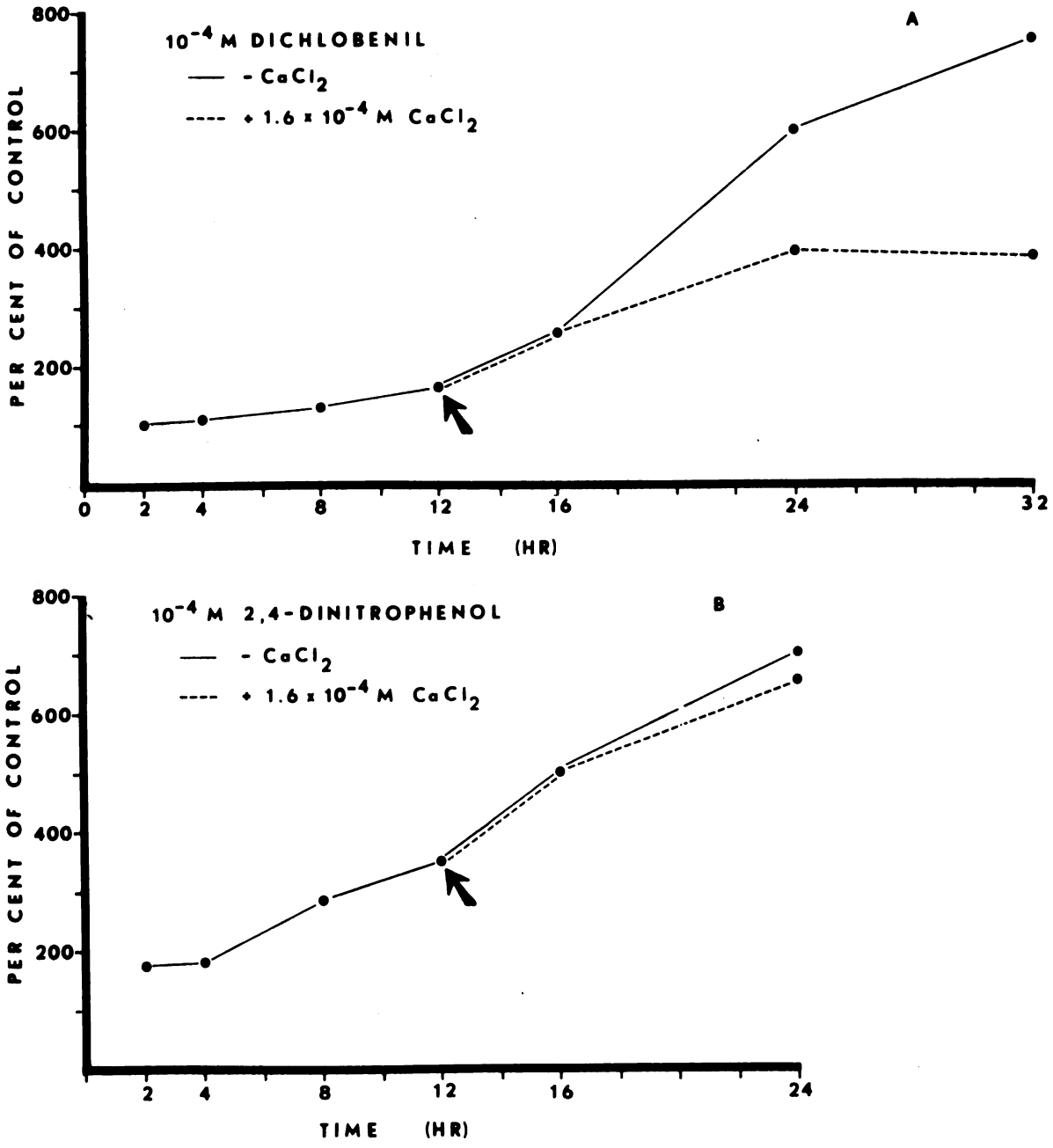


Figure 9. Effect of Ca^{++}, added at 12 hr on leakage of betacyanin induced by treatment of red beet root section with 10^{-4}M dichlobenil (A) and 10^{-4}M 2, 4-dinitrophenol (B).

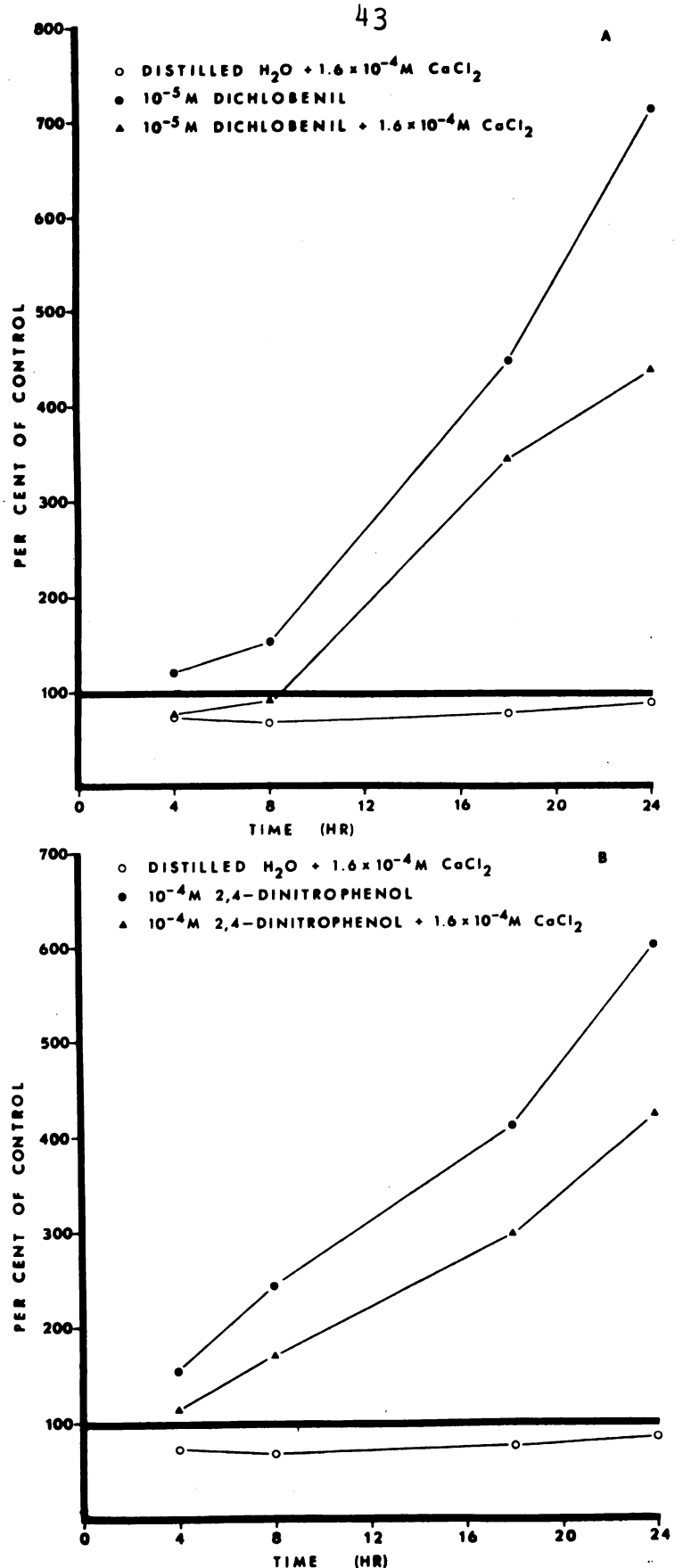


Figure 10. The influence of 1.6×10^{-4}M $CaCl_2$ on the leakage of betacyanin from red beet root sections induced by 10^{-5}M dichlobenil (A) and 10^{-4}M 2,4-dinitrophenol (B). The $CaCl_2$ was added to the incubating solution initially.

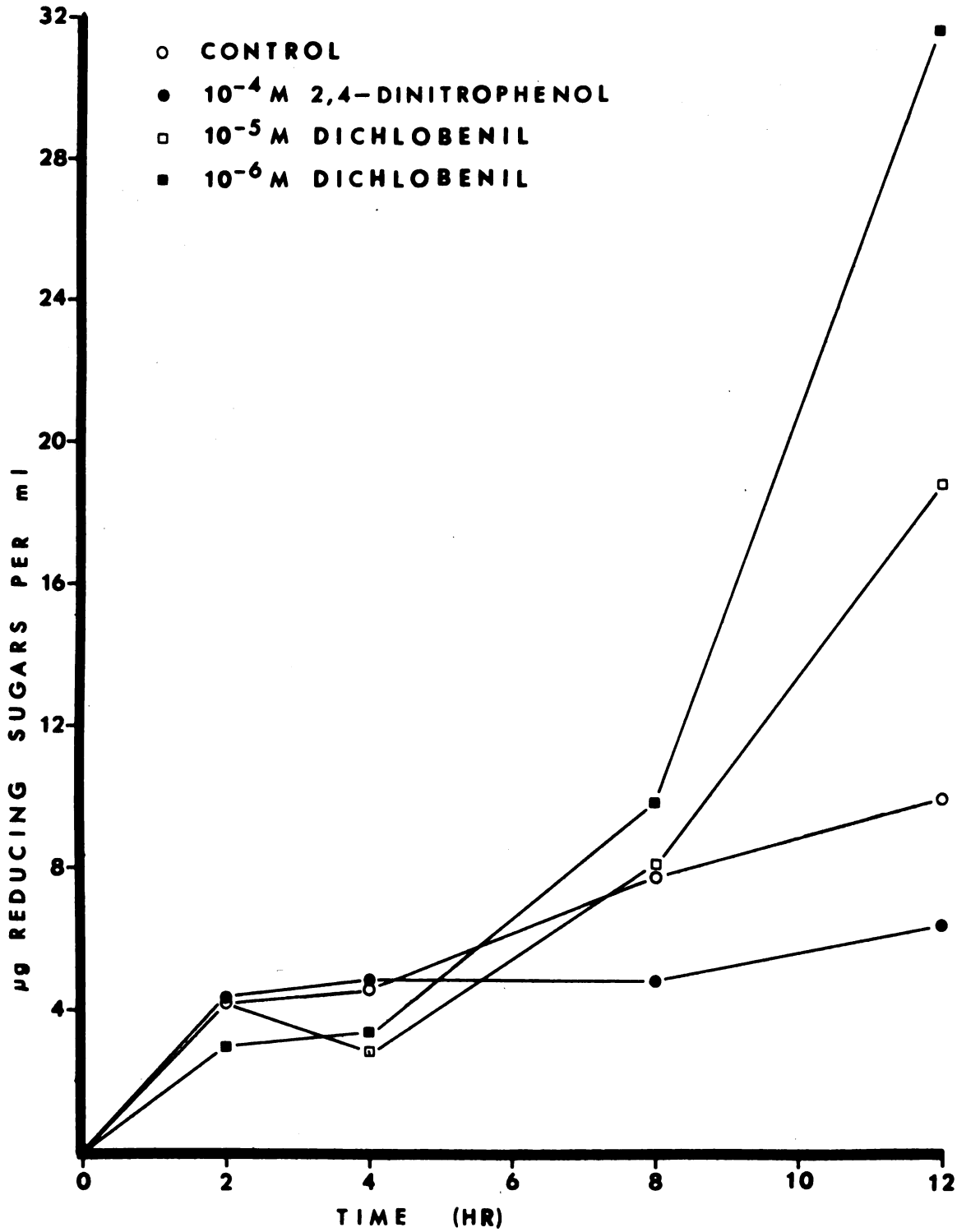


Figure 11. Time course of the leakage of reducing sugars from red beet root sections treated with dichlobenil or 2,4-dinitrophenol.

dichlobenil treated sections appears to be typical of that occurring in an uncoupled system. Similar results were obtained with corn root tips incubated in buffer containing 3 concentrations of dichlobenil (Figure 13). The experiment failed to show a significant difference between concentrations, however, the rates of O_2 consumption for all dichlobenil treatments were significantly greater than the control. Since both experiments showed no differences between concentrations of dichlobenil, it apparently affects in vivo respiration at a very low concentration. Although the increased respiration rate induced by dichlobenil is indicative of uncoupled oxidative phosphorylation, this is not conclusive and must be verified with concomitant measurement of Pi esterification in isolated mitochondria.

Oxidative phosphorylation in isolated mitochondria.-- When 10^{-5}M dichlobenil was added during state 4, there was no stimulation of the ADP dependent respiration (Figure 14A) as was induced by 8 x 10^{-5}M DNP (Figure 14B). Subsequent additions of ADP after dichlobenil treatment showed that the phosphorylation mechanism was coupled and typical respiratory control and ADP:O ratios were maintained. Similar results were obtained using rates as high as 10^{-4}M dichlobenil on mitochondria isolated form cucumber hypocotyls, potato tubers and cauliflower florets. Dichlobenil at 10^{-5}M also did not circumvent

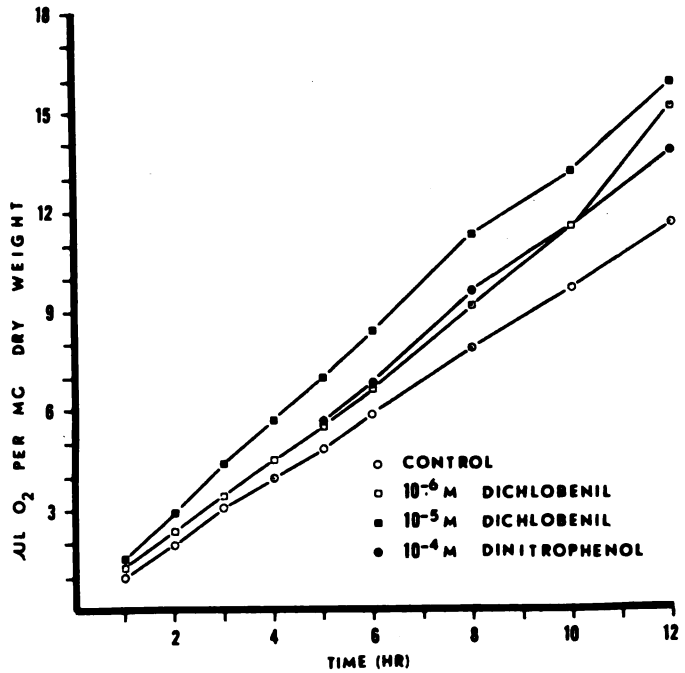


Figure 12. O_2 consumption of red beet root sections incubated in solutions of 10^{-6} or 10^{-5}M dichlobenil and 10^{-4}M dinitrophenol.

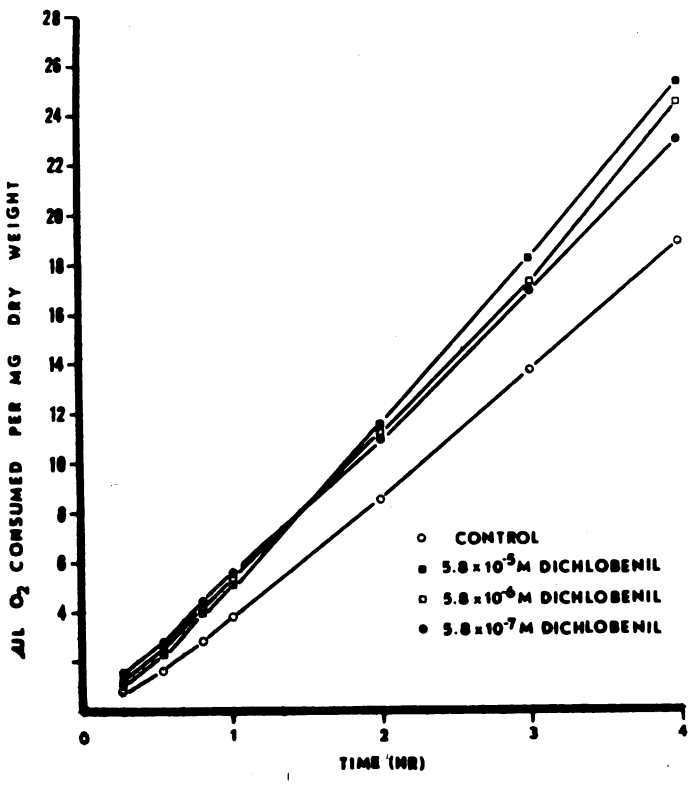


Figure 13. O_2 consumption of corn root tips incubated in .05M phosphate buffer (pH 6.5) containing various concentrations of dichlobenil.

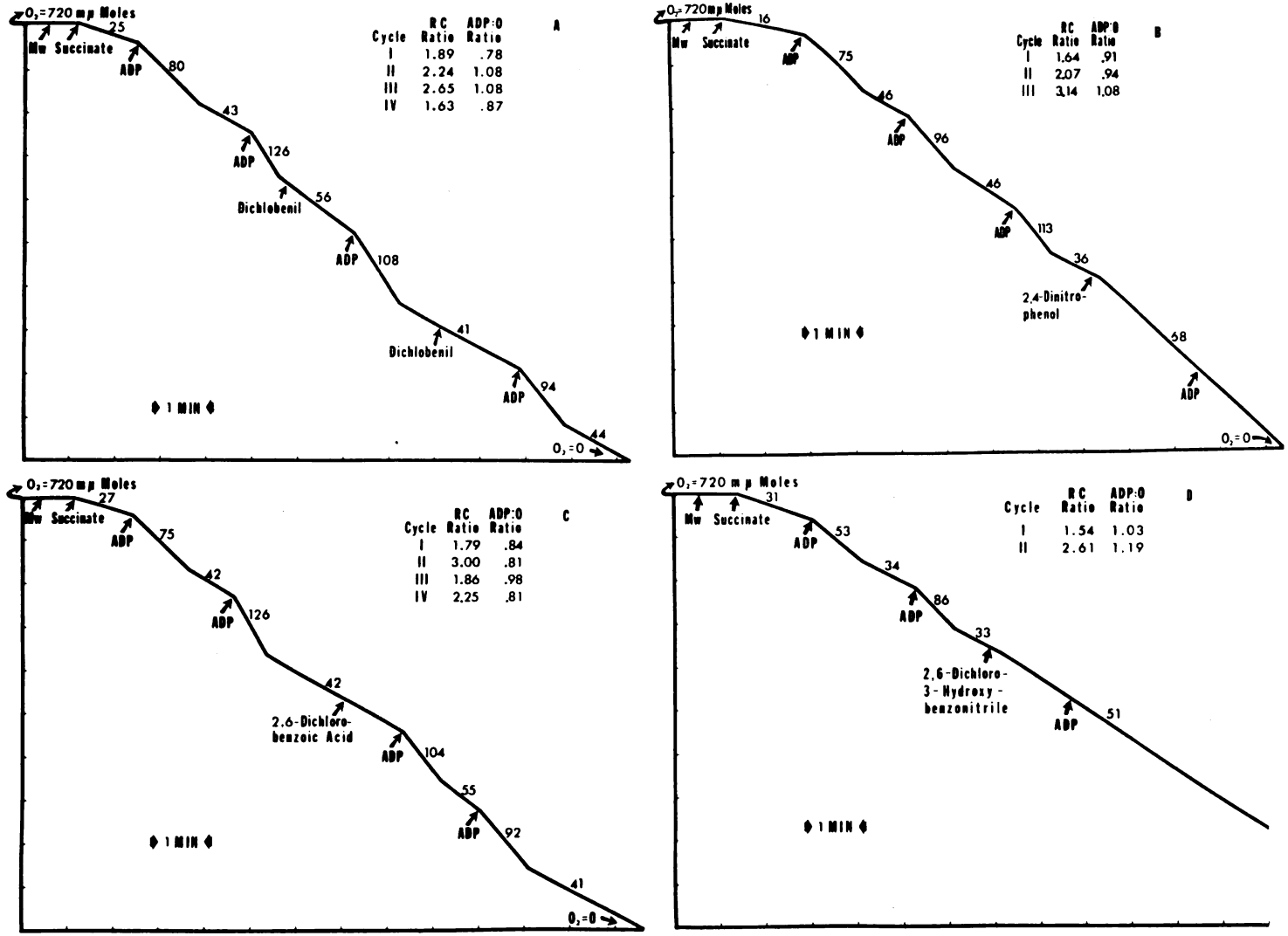


Figure 14. Polarographic traces showing the effect of 10^{-5}M dichlobenil (A), 8 x 10^{-5}M 2,4-dinitrophenol (B), 10^{-5}M 2,6-dichlorobenzoic acid (C) and 10^{-5}M 2,6-dichloro-3-hydroxybenzonitrile (D) on ADP dependent respiration in mitochondria (.20 mg N) isolated from cucumber cotyledons. The concentration of succinate is 8 mM and each addition of ADP is 55 µM. Numbers along the traces are rates of O_2 uptake in mµ moles/min per 3 ml of reaction medium.

oligomycin inhibited O_2 uptake (Figure 15A) as is typical of most uncoupling agents (30). These results conflict with those reported by Foy and Penner (20) who found dichlobenil at 0.73×10^{-4}M to be an effective uncoupler of oxidative phosphorylation as determined by manometric techniques. This discrepancy in results may be due to the concentration of dichlobenil used, however, concentrations which increased in vivo oxygen consumption did not uncouple oxidative phosphorylation in mitochondrial preparations.

One metabolite of dichlobenil, 2,6-dichlorobenzoic acid, which has been found in plants (46), also had no effect on ADP-limited respiration (Figure 14C). However, 2,6-dichloro-3-hydroxybenzonitrile was found to stimulate state 4 respiration and eliminate subsequent respiratory control with ADP at 10^{-5}M (Figure 14D). It also circumvented oligomycin inhibited O_2 uptake (Figure 15B) at the same concentration. This compound at 3.4×10^{-5}M was found by Wit and van Genderen (71) to cause a significant rise in O_2 consumption of starved yeast cells incubated with a small amount of glucose. It also induced ATPase activity in isolated, intact rat liver mitochondria. Dichlobenil had no effect in either of these assays indicating that the monophenolic metabolite is the toxic form in animals. Currently this metabolite has been reported in plants only after extended periods of treatment with a

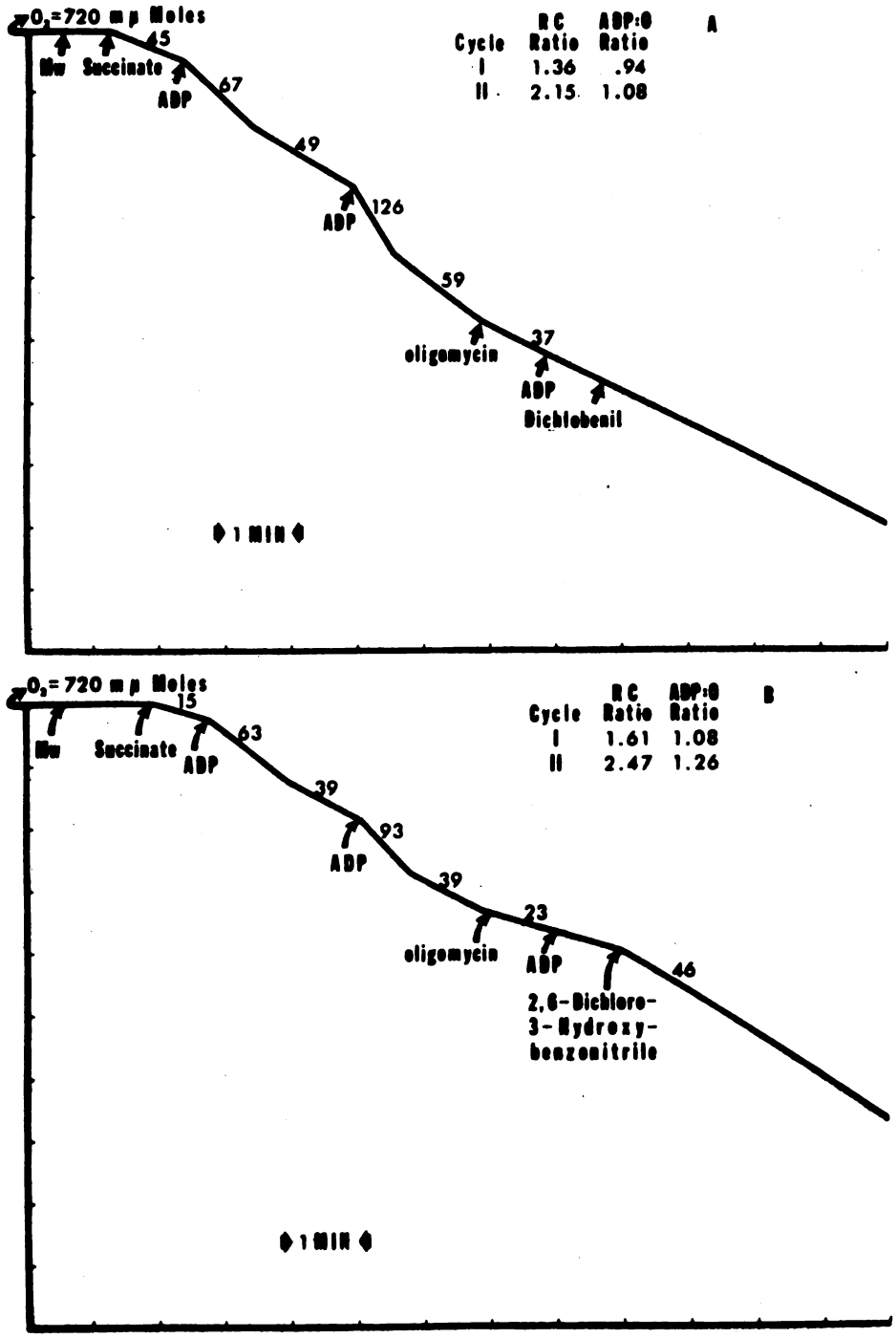


Figure 15. Polarographic traces showing the effect of 10^{-5}M dichlobenil (A) and 10^{-5}M 2,6-dichloro-3-hydroxybenzonitrile (B) on oligomycin (150 mμg) inhibited respiration in mitochondria (.20 mg N) isolated from cucumber cotylendons. The concentration of succinate is 8 mM and each addition of ADP is 55 μM. Numbers along the traces are rates of O_2 uptake in mμ moles/min per 3 ml of reaction medium.

related herbicide, 2,6-dichlorothiobenzamide (chlorthiomid) (9). Since plants do contain many hydroxylase and mixed-function oxidase enzymes, the formation of the monophenolic metabolite from dichlobenil seems feasible.

P/O ratios determined by manometric techniques.--The quantity of Pi esterfied and O_2 consumed was not affected by 2 x 10^{-5}M dichlobenil or 2 x 10^{-6}M, 2,6-dichloro-3-hydroxybenzonitrile, therefore, their P/O ratios are similar (Table 2). The monophenolic metabolite at 2 x 10^{-5}M reduced the O_2 consumption slightly and reduced the Pi esterified by nearly 70%, consequently lowering the P/O ratio to 0.42. Although DNP did not influence the O_2 consumption, Pi esterification was reduced but not as greatly as with the monophenolic metabolite. The P/O ratios substantiate the polarographic determination that dichlobenil at these concentrations does not uncouple oxidative phosphorylation. The suppression of O_2 consumption by 2 x 10^{-5}M 2,6-dichloro-3-hydroxybenzonitrile was evident only after the first 10 min of the experiment, consequently it would not have been detected in the polarographic determinations where the O_2 utilization was measured for only 2 to 3 min after adding the compound.

Pi esterification.--The rate of Pi esterified by cucumber hypocotyl mitochondria incubated in a reaction medium containing 10^{-5}M dichlobenil was not significantly

TABLE 2.--Effect of dichlobenil, 2,6-dichloro-3-hydroxybenzonitrile and DNP on oxidative phosphorylation by cucumber cotyledon mitochondria.[1]

Inhibitor	Conc (Mx2)	Pi esterified mole/40 min	O Consumed µatom/40 min	P/O ratio
None		16.5 a[2]	47.7[3]	1.03 a[2]
Dichlobenil	10^{-5}	19.1 a	50.4	1.16 a
2,6-dichloro-3-hydroxy-benzonitrile	10^{-6}	15.6 a	49.5	0.96 ab
2,6-dichloro-3-hydroxy-benzonitrile	10^{-5}	5.1 c	36.0	0.42 c
DNP	10^{-5}	9.8 b	44.9	0.66 bc

[1]20 mM succinate utilized as substrate.

[2]Within columns, means followed by the same letters are not significantly different at the .01 probability level as determined by Duncan's multiple range test.

[3]F value for comparison of control with 2 x 10^{-5}M 2,6-dichloro-3-hydroxybenzonitrile significantly different at the .05 probability level.

different than the control (Figure 16). An equimolar concentration of the monophenolic metabolite, however reduced the rate of Pi esterification compared to both the control and dichlobenil treatments.

^{14}C-Glucose utilization.--The utilization of both C_1 and C_6 labeled glucose by cucumber seedlings was doubled by treatment with 10^{-5}M dichlobenil for 24 hr (Table 3). The ratio of C_6 to C_1 glucose utilized was not significantly altered by the dichlobenil treatment. The smaller amount of radioactivity remaining in the dichlobenil treated tissue at the termination of the experiments is also indicative of increased glucose utilization. Increased glucose utilization is expected, based on previous observations of increased O_2 consumption by intact tissue. Increased catabolism may be due to uncoupling of oxidative phosphorylation induced by dichlobenil or alleviation of a limiting factor in the respiration mechanism. Since there was no change in the C_6/C_1 ratio, the same pathways of glucose metabolism appear to be operative in the treated tissue, albeit at a faster rate.

Amino acid accumulation.--Within 24 hr after treating oat seedlings with 10^{-6} or 10^{-5}M dichlobenil there was an increase in the total free amino acid content of both shoots and roots (Table 4). This accumulation may be due to reduced incorporation of the amino acids into protein

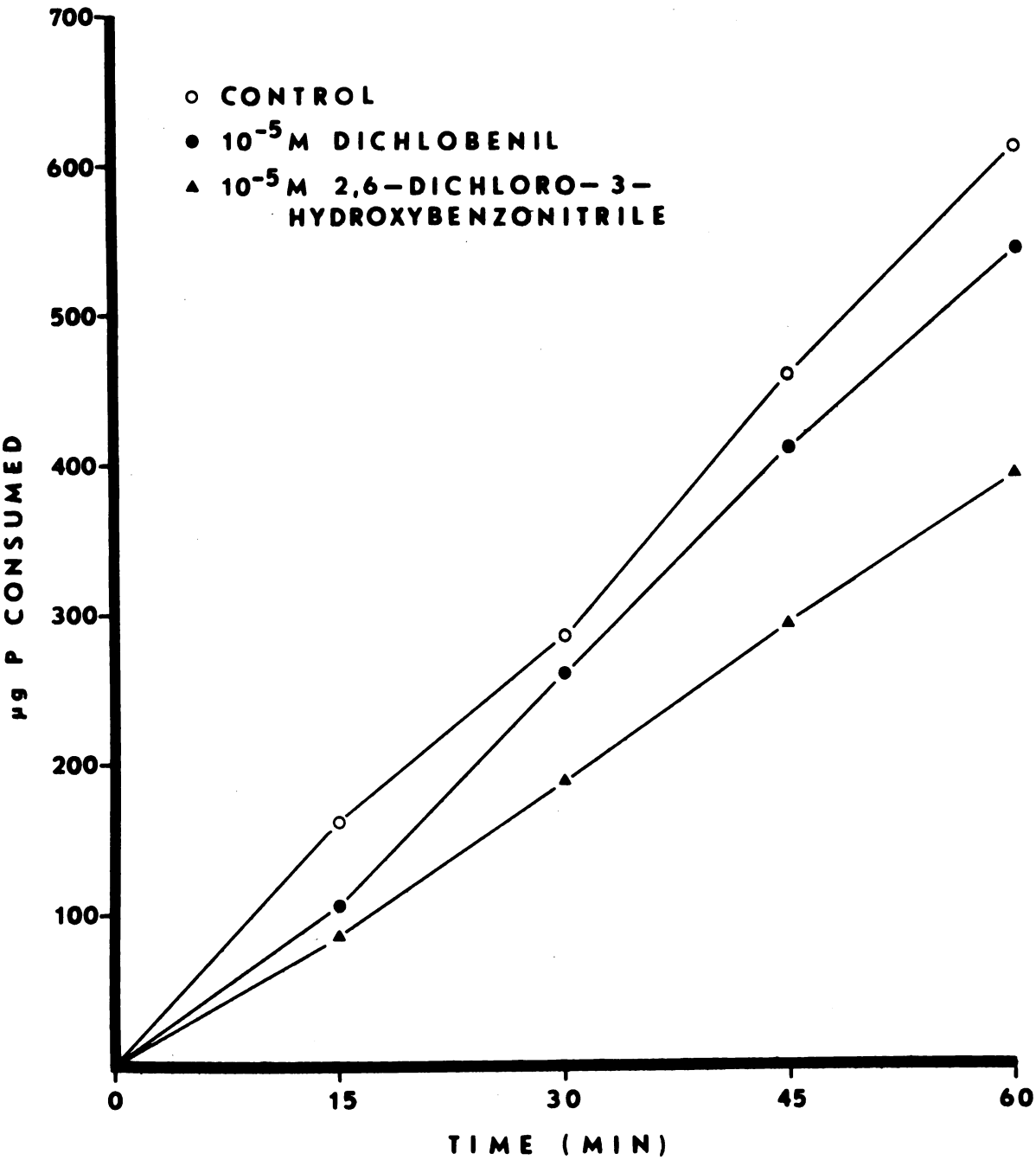


Figure 16. Time course of Pi uptake by mitochondria (.16 mg N) isolated from cucumber hypocotyls.

TABLE 3.--Effect of 10^{-5} dichlobenil on the utilization of $^{14}C_1$ and $^{14}C_6$ labeled glucose by 4-day-old cucumber seedlings.[1]

Dichlobenil Conc (M)	C_6/C_1 Ratio	$^{14}CO_2$ Evolved from fresh tissue (cpm/mg)		^{14}C-Remaining in dry tissue (cpm/mg)	
		C_1	C_6	C_1	C_6
0	.62	158 a	79 a	6394 a	7811 a
10^{-5}	.40	373 b	140 b	2813 b	3615 b

[1]Within columns, means followed by the same letters are not significantly different at the .01 probability level as determined by analysis of variance. Data are means of 16 observations.

because oxidative phosphorylation is uncoupled and the necessary ATP is unavailable. Also, as noted earlier, respiration in the treated tissue is increased, hence, amino acids may be produced at a faster rate without concomitant incorporation into protein, or more protein may be hydrolyzed.

TABLE 4.--The free amino acid content of oat plants treated with dichlobenil.

Dichlobenil conc (M)	µMoles/mg dry wt	
	Shoot	Root
0	.173 a	.089 a
10^{-6}	.214 b	.084 a
10^{-5}	.227 c	.107 b

[1]Within columns, means followed by the same letters are not significantly different at the .01 probability level as determined by Duncan's multiple range test.

Effect of Dichlobenil on the Formation of Lignin-like Polyphenols

Spectrophotometric determination.--The difference spectra for cucumber hypocotyl and root alkaline extracts are shown in Figures 17A and 17B, respectively. Aulin-Erdtman (6) has shown that the peak at 280-300 mµ represents the non-conjugated phenols, while phenols with large conjugated side chains such as hydroxycinnamic acid derivatives will account for peaks at wave lengths greater

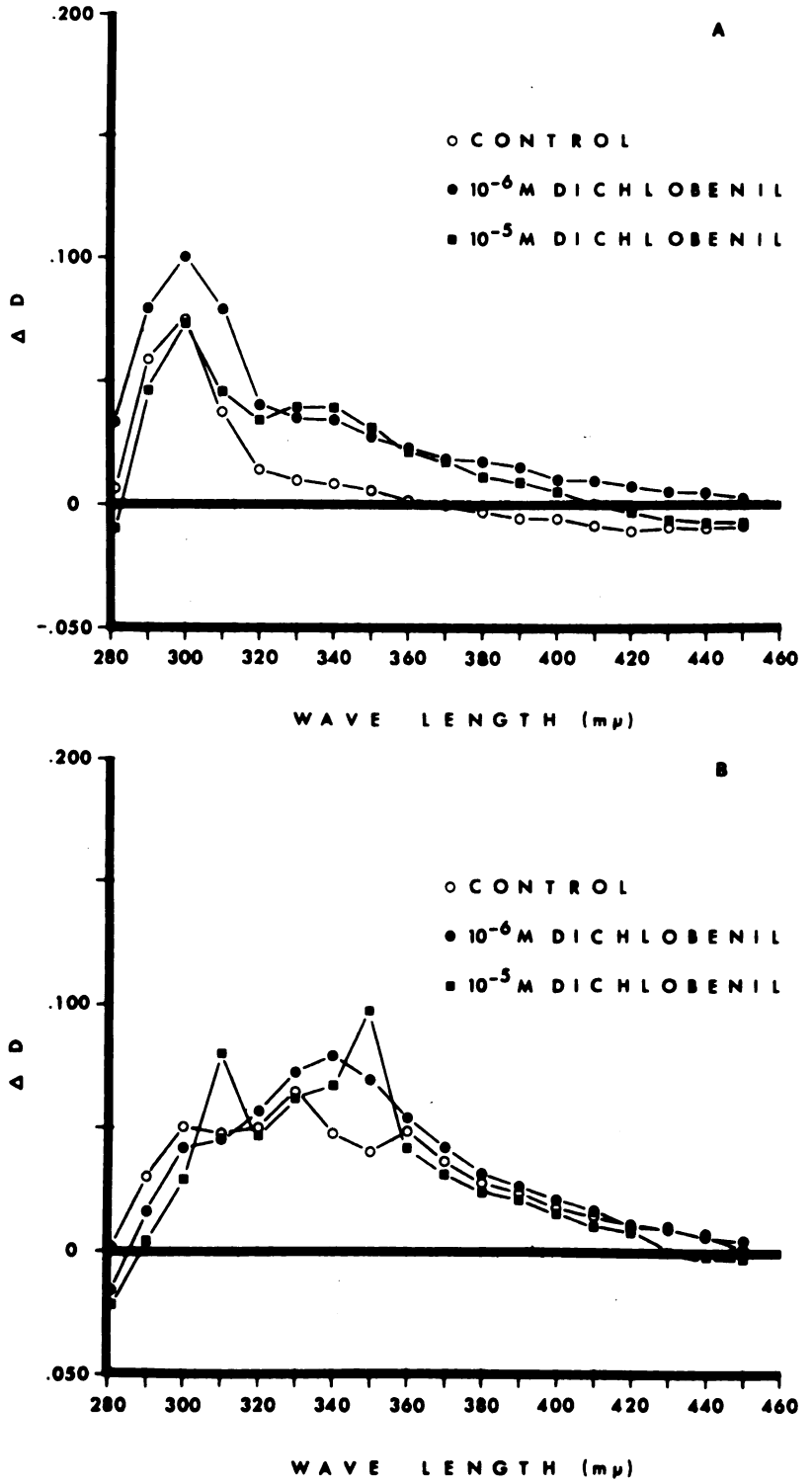


Figure 17. Difference spectra of lignin extracts of cucumber hypocotyl (A) and root tissue (B). Each ml of solution in the curvette contains an aliquot of a lignin extract equivalent to 1.5 mg dry wt root tissue, diluted either with 0.05 M phosphate buffer at pH 7.0 or with 0.05 M NaOH at pH 12.3.

than 300 mμ. Based on these difference spectra, extracts from cucumber hypocotyls treated with 10^{-6} and 10^{-5}M dichlobenil have a significantly higher peak at 350 mμ than that of the control. However, the ΔD (350 mμ) of the extract from treated root tissue was not different from the control. Only the 10^{-5}M dichlobenil concentration significantly increased the ΔD (350 mμ) of extracts from oat shoots (Figure 18A), however, both concentrations of dichlobenil significantly increased the ΔD at this wave length in the root extracts (Figure 18B). Dichlobenil causes an increase of lignin-like substances in both cucumbers and oats within 48 hr after treatment.

Reaction of dichlobenil treated tissue with phloroglucinol-HCl.--Mounted sections from cucumber roots treated with 10^{-5}M dichlobenil for 48 hr showed extensive red coloration of the epidermal and outer cortical cells when stained with phloroglucinol-HCl (Figure 19). This constitutes a positive test for lignin, however, Jensen (31) points out that tannins and other polyphenolic compounds related to lignin will react similarly with phloroglucinol-HCl. The lignin-like substances detected in these sections generally appeared as amorphous masses both within the cells and in the intercellular spaces. The stained area was localized in epidermal cells and parenchyma cells of the outer region of the cortex. Sections from

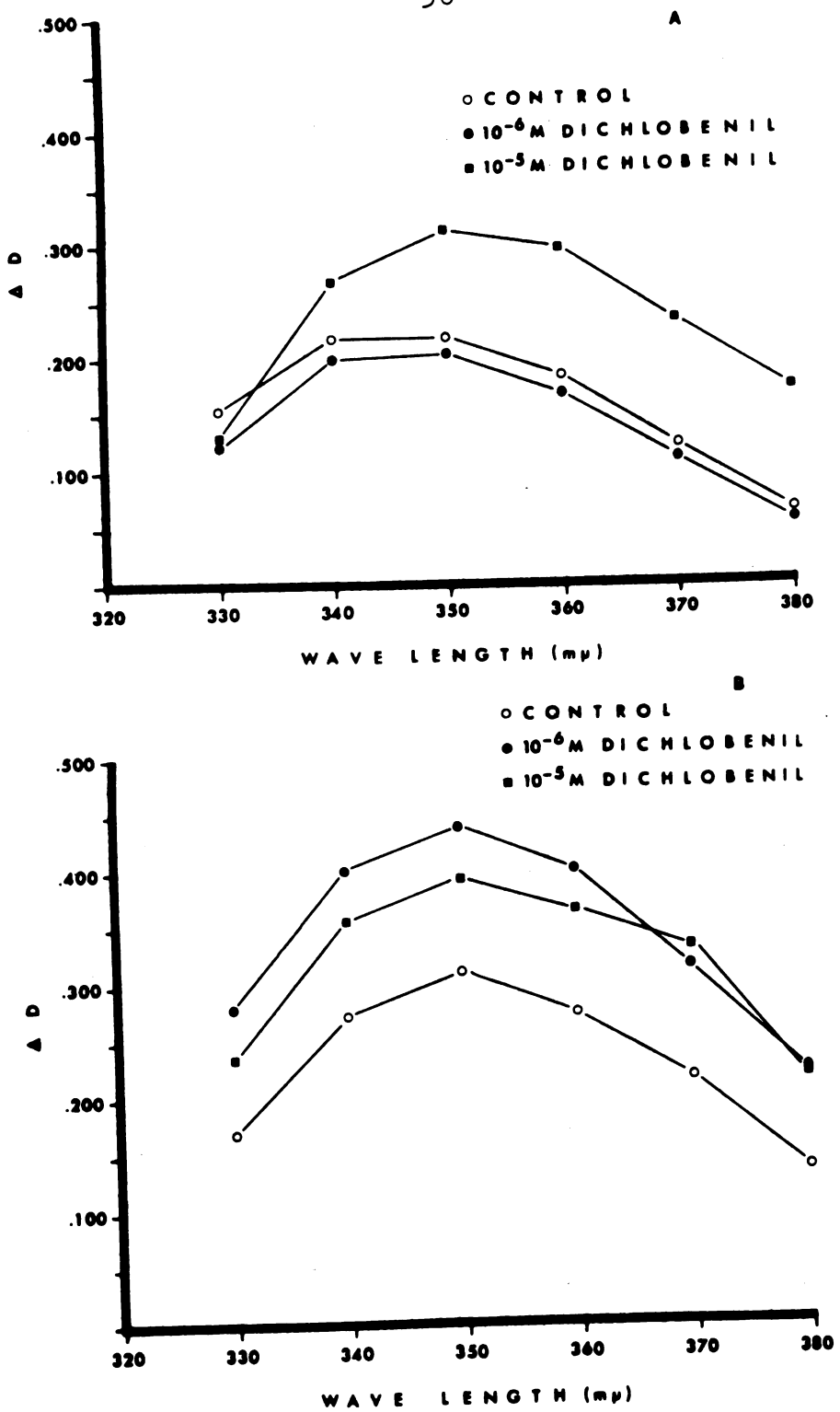


Figure 18. Difference spectra of lignin extracts of oat shoots (A) and roots (B). Each ml of solution in the cuvette contains an aliquot of the lignin extract equivalent to 1.0 mg dry wt shoot tissue or .5 mg dry wt root tissue diluted either with 0.05 M phosphate buffer at pH 7.0 or with 0.05 M NaOH at pH 12.3.

untreated roots showed only the normal lignification associated with the secondary thickening of the xylem vessels.

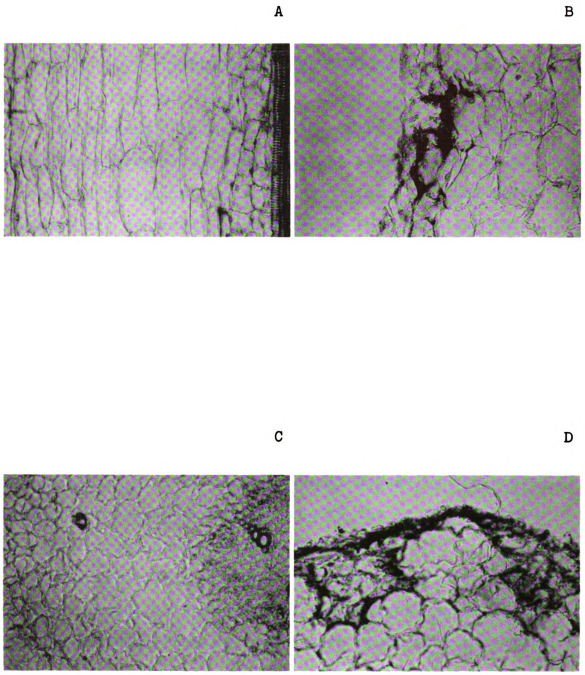


Figure 19. Comparison of the accumulation of polyphenolic substances after 48 hr in roots of control (A, longitudinal section) (C, cross section) and 10^{-5}M dichlobenil (B, longitudinal section)(D, cross section) treated cucumber roots (125 x). Sections are 30 μ thick and stained with phloroglucinol-HCl.

SUMMARY AND CONCLUSIONS

Dichlobenil is rapidly taken up by the roots of corn plants from an aerated nutrient solution and translocated to all plant parts via the xylem. The unaltered compound may be volatilized directly from the leaf into the atmosphere. Volatilization from the leaf is temperature dependent and light independent. Due to the lipophilic nature of dichlobenil, it has the capability of rapidly traversing membranes, and cuticle layers. In the leaf, which is subject to relatively high temperatures, a portion of the dichlobenil may be present in the vapor phase and thus be subject to volatility loss. Under circumstances of limited dichlobenil uptake by the roots, it does not accumulate in the shoots due to the concomitant volatility loss from the leaves.

The relatively unrestricted movement of dichlobenil in plant tissue is also evident in the root, where it readily diffuses from preloaded corn roots when placed in fresh nutrient solution. Dichlobenil also induces the leakage of betacyanin and reducing sugars from red beet root sections, indicating a disruption of the tonoplast and plasmalemma. It may be postulated that dichlobenil

partitions into the lipid bilayer of the membranes and causes them to be more permeable to cell constituents. In the presence of Ca^{++}, however, the membranes are stabilized and either the dichlobenil within the membrane has a reduced effect on permeability or inhibits dichlobenil from penetrating the membrane. DNP induces the leakage of betacyanin more rapidly than dichlobenil, but does not influence the leakage of reducing sugars. Since DNP is a known uncoupler of oxidative phosphorylation, its effect on permeability may be due to a lack of ATP necessary to maintain membrane structure. Differences in the rate and reversibility of DNP and dichlobenil induced permeability changes indicate that their initial site of action may be different.

Red beet root sections and corn roots when incubated in 10^{-6} to 10^{-5}M solutions of dichlobenil exhibit increased O_2 consumption which is similar to that produced by 10^{-4}M DNP. Cucumber seedlings treated with 10^{-5}M dichlobenil for 24 hr also show increased respiration as evidenced by the 2-fold increase in the amount of C_1 and C_6 labeled glucose utilized. Although this indicates uncoupled respiration, isolated mitochondria were unaffected by 10^{-5} to 10^{-4}M dichlobenil when assayed using the oxygen electrode, manometric techniques, or Pi consumption assays. The monophenolic metabolite of dichlobenil (2,6-dichloro-3-hydroxybenzonitrile), however, did

act as an uncoupler of oxidative phosphorylation in all 3 assay techniques.

The free amino acid content of roots and shoots of oat plants increased within 24 hr after treating with 10^{-5}M dichlobenil. This accumulation may be due to reduced incorporation of amino acids into protein because oxidative phosphorylation is uncoupled and the necessary ATP is unavailable. Also, amino acids may be formed at a faster rate because of the increased glucose utilization.

The oxidation products of endogenous phenolic compounds have been shown to be uncouplers of oxidative phosphorylation in mitochondria isolated from potato tubers (57) and sweet potato roots (35). These phenolic compounds, which are found in nearly all higher plants (10), are generally located within the vacuole (18) and thus spacially separated from the phenol oxidizing enzymes. Dichlobenil may destroy this compartmentation by altering the permeability of the tonoplast. Subsequently, the phenols would become available for oxidation by phenoloxidase, peroxidase or other oxidizing enzymes. The quinones and polyphenols formed by non-enzymatic polymerization reactions could inhibit oxidative phosphorylation.

Spectrophotometric assay of alkaline extracts of cucumber and oat seedlings indicated that 10^{-5}M dichlobenil induced the formation of lignin-like polyphenols

within 48 and 72 hr respectively. This would lend evidence that the oxidation products of phenols released from the vacuole are being conjugated into polyphenolic compounds. Histological evidence for this was also obtained. Cucumber root sections upon staining with phloroglucinol-HCl, manifested red amorphous masses around the periphery of the root. The polyphenolic compounds are specifically indicated by this procedure. The phenol oxidizing enzymes generally utilize molecular oxygen as an electron acceptor, but the concentration of oxygen required for half maximal activity is very high compared to that of respiration (3). Thus, it is only around the outside of the root that the partial pressure of oxygen is high enough to support phenolase activity.

A similar mechanism might also be postulated to explain the coagulation of cytoplasm in the phloem and surrounding parenchma of the nodal tissue of dichlobenil treated alligator weed noted by Pate and Funderburk (45). Polyphenols react rapidly to form covalent bonds with protein, thus causing denaturation (36). It is by this mechanism that polyphenols are thought to inhibit enzymes and subcellular organelles (3, 28).

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