THE MOBILITY AND DISTRIBUTION OF 2, 4-DICHLOROPHENOXYACETIC ACID IN CANADA THISTLE

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by Woodland Hurtt

Canada thistle plants were found to be very responsive to the sodium salt of 2,4-dichlorophenoxyacetic acid (2,4-D) when it was introduced directly into the growing regions. However, when the herbicide was applied to the foliage of the plants, the majority of it remained in the leaves and the plants exhibited negligible responses. By extracting the leaves with ether and bioassaying the extracts with the Avena straight growth test, it was found that the 2,4-D was present in the tissue in a biologically active form.

To test the mobility of the 2,4-D in the extracellular liquid of the leaves, low levels of direct current were passed through the tissue in an attempt to displace the herbicide electrophoretically.

In one method the current was applied to opposite margins of the leaves such that mobility could be studied by the pattern of induced displacement of the herbicide. The 2,4-D was preferentially displaced from one-half to one centimeter toward the electropositive contact by the current levels and time intervals employed.

In the second electrophoretic technique, which was used to extract the 2,4-D from the extracellular liquid of the tissue, the current was applied to the upper and lower surfaces of the leaves such that the 2,4-D was displaced into cotton traps. Bioassays of the extracts from the cotton traps showed that 2,4-D was present in the extracellular liquid in an active form which was freely mobile under the influence of the imposed current.

It was concluded that the lack of effectiveness of 2,4-D on the thistle clones used in these experiments was caused by the lack of adequate transport to the growing regions of the plants and was not attributable to either a lack of mobility or activity of the herbicide.

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By

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INTRODUCTION

Although 2,4-dichlorophenoxyacetic acid (2,4-D) readily controls many perennial weeds, it has not proven particularly effective in the control of Canada thistle (Cirsium arvense). 2,4-D has generally been considered to be freely translocated in plants, but recent evidence indicates that it is not translocated as freely as once thought. It was, therefore, the purpose of this study to obtain information on the mobility and translocation of 2,4-D in Canada thistle in an effort to ascertain why this plant is resistant to 2,4-D.

REVIEW OF LITERATURE

Probably the most important factor in the resistance of many perennial plants to herbicides can be attributed to lack of adequate transport to the meristematic regions in the roots. Van Overbeek (34) suggested that the effectiveness of 2,4-D was due to its ability to penetrate into the protoplasm of meristems. Swanson (31) studied the histological responses of kidney beans treated with 2,4-D and reported that the meristematic tissues and those capable of reverting to a meristematic condition were the ones most readily affected by 2,4-D. Tukey et al. (33) treated bindweed and sow thistle with 2,4-D and found disorganized and greatly increased cell division in the cambial regions of the rhizomes of both plants.

According to Crafts (7) much of the available evidence indicates that the translocation of 2,4-D and similar compounds occurs in the phloem in the stream of assimilates. Movement is generally considered to be from regions of food synthesis to those of food utilization and occurs only if photosynthesis is going on or if carbohydrate reserves are being hydrolyzed and moved (9).

Crafts and Yamaquchi (10) reviewed many older papers on the translocation of 2,4-D and stated that they seemed to indicate a relationship between the movement of 2,4-D and the movement of food in the plant. They also reported that when they applied carboxyl-labeled 2,4-D and several other herbicides to the leaves of two species of plants their results suggested that the 2,4-D moved in a common stream of assimilates from the leaves to various sinks (regions of utilization).

Voevodin (35) applied C^{14}-labeled 2,4-D to field plots of Canada thistle and reported that the spraying of autumn rosettes resulted in increased uptake of 2,4-D in the roots. When the herbicide was applied to spring rosettes, the majority of it remained in the above ground parts of the thistles. These results were attributed to the direction of movement of the products of photosynthesis.

Corms (6) injected 2,4-D into perennial sow thistle plants and suggested that its transport was governed by movement of sap to the young rapidly developing parts of the plants.

The translocation of 2,4-D in plants has been considered to occur quite readily (3, 7). Rapid rates of translocation have also been reported. Day (13), for example, reported

an average speed of transport of 2,4-D in kidney bean seedlings of 50 cm per hour. The results of recent studies, however, indicate that the transport of 2,4-D does not occur as readily as previously reported (11). Crafts and Yamaquchi (10) applied labeled 2,4-D and 2,4,5-T to blocks of potato tuber tissue and found that they were accumulated and held in the living cells. On the basis of further studies in which they applied labeled 2,4-D to the leaves of Zebrina pendula, they suggested the herbicide was taken up by the living cells and any that was transported constituted a residue over and above that which was retained by the active cells (10).

Crafts (11) reviewed the previously reported work of Johnson and Bonner (20) and suggested that the low mobility of 2,4-D may be explained by the reported active accumulation of the herbicide by the living cells. These workers (20) suggested a binding action and reported that the 2,4-D was accumulated in the tissue to a higher concentration than that in the external medium.

Leonard (21) discussed the binding factors involved in the translocation of 2,4-D and postulated that some of the 2,4-D is weakly bound after entering the leaf or is precipitated as the acid of 2,4-D.

Bach and Fellig (2) studied the uptake and fate of carboxyl-labeled 2,4-D in bean stem sections and suggest the 2,4-D was bound in some way to cellular material of the tissue. They found the peak of the radioactivity was located only several millimeters from the point of application.

Canny and Katalin (5) used both carboxyl and methylene labeled 2,4-D to determine the breakdown of the herbicides in the shoots and roots of tick bean plants. They stated that the 2,4-D entered the roots of the plants in the translocation stream and was probably bound there in complexes with proteins.

Leonard (21) cited several papers which suggested that free 2,4-D as such rapidly disappears from the leaf and becomes bound with proteins.

Pallas (29) studied the effects of temperature and humidity on the absorption and translocation of labeled 2,4-D and benzoic acid in red kidney beans. He reported that movement of 2,4-D, as determined by radioautograms, followed the route of the assimilate stream out of the leaf and into the stem, bud, and roots. Further studies involving extraction and chromatography of the herbicide showed that it was translocated as free 2,4-D or its dissociable salts.

An electrophoretic method was developed by Olien (27,28) for studying the mobility of electrolytes in the extracellular liquid of wheat leaves. Anderson et al. (1) used this method to study the transport of Ca^{45} in bean plants and found a preferential transport of the calcium towards the positive electrode. It was theorized that the calcium ion was attached to a negative carrier which migrated towards the positive electrode.

MATERIALS AND METHODS

Materials

The monohydrate sodium salt of 2,4-D (Baker Chemical Company) was used in these experiments. All solutions were prepared in distilled water and stored at approximately five degrees centigrade when not in use.

Since there are several known varieties of Canada thistle (14), the plants used in these experiments were grown from one source by asexual propagation in order to eliminate possible varietal differences in response to 2,4-D. The plants were grown from one inch sections of rhizomes in four inch pots containing a one-third sand and two-thirds soil mixture. Prior to planting all sections were soaked for several minutes in a solution of Captan equivalent to two pounds per hundred gallons of water to minimize root rot. The resulting plants were watered once weekly with a complete nutrient solution and supplied with supplemental lighting when needed.

Methods

Since the lack of effectiveness of the salt form of 2,4-D on Canada thistle plants was thought to be due to its lack of transport within the plant, an electrophoretic

method was used to study its mobility.

Prior to using an electrophoretic method for displacing charged materials such as 2,4-D in the extracellular liquid, it was necessary to determine the voltage and amount of current that could be passed through leaves without damaging the tissue.

Contacts. In order to displace a substance having an electrical charge in the extracellular films of liquid, a method was needed which would extend these films into an exterior system suitable for the application of the electric current. Such a system should introduce the electric current into the tissue in a uniform manner without causing concentrated areas of stress and prevent the introduction of possible toxic substances such as copper from the anode into the tissue as a result of the current flow.

Excised leaves were used in all of the following studies because preliminary experiments indicated that the release of both 2,4-D and the dye from the xylem was similar to that obtained when 2,4-D and dye were introduced into intact plants.

Preliminary experiments in which contacts were made on the tips of two opposite leaves on the stem of each intact plant showed that electrical displacement was parallel to

the natural path of transport. For this reason the Norite contacts were made on the margins of the leaves such that the path of electrical displacement was perpendicular to the normal path of transport.

Each contact was made by burying 2.5 cm of the leaf margin about 2 mm deep in a thick charcoal paste consisting of Norite and water. The moisture content of the paste was maintained by a wick leading to a water reservoir. A graphite rod was used to complete the contact between the wire of the electric circuit and the paste. Two such contacts were made on opposite edges midway between the petiole and the tip of an excised leaf whose petiole was immersed in 3 ml of distilled water, dye, or 2,4-D (Figure 1).

Intensity limit. When a direct current of sufficiently low voltage is passed through normal tissue, the natural diffusion barriers of the protoplats are not disrupted, and in such cases the resistance of the tissue is independent of the voltage (27). At slightly higher voltages a reversible decrease in the resistance occurs which is indicative of a weakening of the diffusion barrier, but further increases in voltage results in an irreversible decrease in resistance (27). The latter indicates destruction of the diffusion barrier. For any given section of uniform tissue the voltage

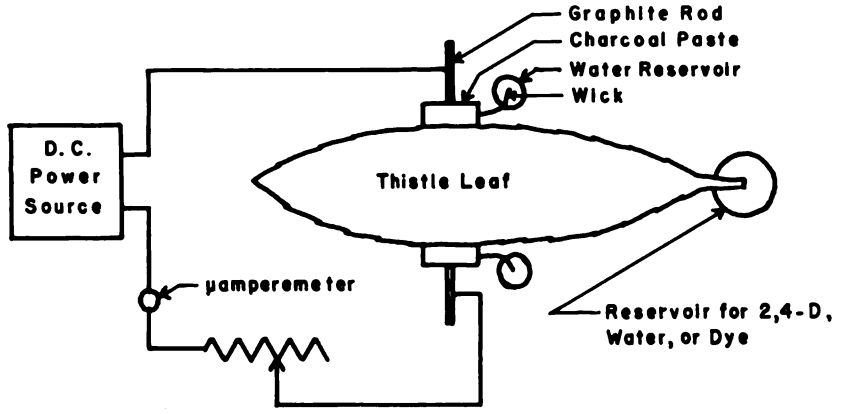

Figure 1. Diagram of method of applying an electric current to an excised Canada thistle leaf

is a function of the length of the current path. Therefore, the intensity limit is considered to be the maximum voltage per centimeter that can be applied to the tissue without affecting the diffusion barrier sufficiently to cause a decrease in resistance.

In order to determine the voltage maximum for a Canada thistle leaf, marginal contacts were placed on a leaf and starting at one volt, succeedingly higher voltages were applied. At each voltage a reading was made of the current flow in microamperes and the resistance of the tissue calculated.

It was found that the resistance of the tissue between the contacts was essentially independent of the voltage up to 17 volts per centimeter, at which point it started to decrease and continued to do so with each increased increment of voltage. To go beyond this voltage would be unnecessary because the current started to drift and continued to do so if this voltage was held for more than a few seconds. The resistance of the tissue at this point was 1.3×10^6 ohms per centimeter.

Areas of electrical stress in which the voltage maximum had been exceeded and the diffusion barriers weakened or destroyed rapidly became necrotic. Prior to the appearance

of visible necrosis the resistance of this area of tissue decreased and as a consequence the current drifted to higher levels. As the tissue became necrotic it lost moisture and became dessicated if it was in a dry atmosphere. This in turn caused the resistance to increase and the current to bridge this area with the resulting formation of new areas of electrical stress on either side of the dessicated tissue.

Necrotic areas of tissue formed as a consequence of electrical stress were quite different in appearance from those resulting from 2,4-D treatments. The latter were always purple or black in color, whereas the former were light brown to brown in color.

In order to ensure that the maximum voltage drop of 17 volts per centimeter of tissue was not exceeded in subsequent experiments, it would have been possible to measure the voltage drop across the contacts of each leaf. This, however, would have been more difficult because the contacts themselves offer resistance to the flow of current. Since the current flow is constant in all parts of a circuit, it was more convenient to measure the level of current by means of a microamperemeter in series with the circuit. By applying contacts to the same cross-sectional area of each leaf, the voltage drop per centimeter could be maintained

below the maximum level by varying the external resistance until the desired microampere reading was obtained. This method compensated for the normal variation in the width of the leaves which affects the resistance of the tissue between the contacts.

If inadvertently the maximum voltage value was reached or exceeded, the experiment was discontinued. The stress limit was then re-evaluated and subsequent experiments were carried out within a safe range.

Capacity limit. The application of a sufficiently high voltage per centimeter of tissue caused irreversible injury to the tissue. In any given electrophoretic unit the limit of the electrical treatment at a low electrical stress is dependent upon the total amount of electricity passed through the tissue rather than the total energy (watts) applied (27). The electrical treatments were therefore measured in coulombs or ampere-seconds.

It was found that approximately 1.0 coulomb was the upper limit of electrical treatment with the type of electrophoretic unit employed in these experiments. Assuming an average leaf thickness of 0.1 cm and a contact length of 2.5 cm, the maximum limit of 1.0 coulomb was applied through a cross-sectional area of 0.25 square

centimeters. The capacity limit of the tissue was therefore considered to be 4.0 coulombs per square centimeter. The majority of current applications employed a current level which was 40-50 percent of this value. When the upper limit of current application had been exceeded and the tissue collapsed in a dry atmosphere, the current was diverted and concentrated on the sides of each spot. The result was the formation of lines or wrinkles of necrotic tissue which were perpendicular to the current path (Figure 2). However, when such an excess of current was applied in a moist atmosphere, the loss of moisture from the injured tissue was prevented and a general collapse of the entire area of tissue between the contacts occurred.

Figure 2. The effect of an excessive application of an electric current to an excised Canada thistle leaf

RESULTS

The Introduction of 2,4-D and Dye in Canada Thistle Plants

Preliminary experiments were carried out to obtain observational information on the uptake and translocation of 2,4-D and dye in Canada thistle plants. Several methods were used for applying the herbicide to the plants. One consisted of pipetting 10, 50, or 100 λ drops on the surface of one or several leaves. In some cases the 2,4-D was confined within a small area by means of a ring of stopcock grease and in others it was spread over the entire surface of the leaf. A second method consisted of immersing a leaf in a small vial containing the herbicide. In some instances the leaf tip was excised prior to immersion. An injection technique was also used in which a hypodermic needle was inserted in the stem and the 2,4-D was added through the hollow shank of the needle. A red dye, Amaranth, was used in conjunction with the 2,4-D in the experiments concerned with translocation. The dye was introduced into the plants in the same manner as was the 2,4-D.

The majority of these experiments were carried out under relatively low light intensities in the greenhouse with supplemental incandescent light or in the laboratory

under fluorescent lighting. A few experiments were conducted in the greenhouse in late spring or early summer under high light intensities. The plants used in these experiments were 12-16 cm tall and approximately two months old.

The application of 2,4-D to the surface of the leaves either resulted in no response or in very slight epinasty in the upper leaves. The only exception were treatments in which some of the herbicide ran back into the axils of the leaves but even then, death of the plants never occurred.

When Amaranth was injected into the stems of plants a characteristic pattern of staining resulted. Generally, the first and thereafter every third leaf was stained. A similar pattern of necrosis occurred when plants were treated with 2,4-D. The movement of the dye and 2,4-D was found to be upward and outward into the leaves. Neither lateral nor downward translocation of 2,4-D and dye occurred since leaves on the opposite side of the stem or below the point of injection were never stained or necrotic.

The lack of downward transport was further substantiated by repeated applications of 2×10^{-3} M 2,4-D through the shank of a needle inserted and left in the stem for 13 days. At the conclusion of this interval only the leaves above the point of injection were injured or dead. Other plants on the

same rhizome exhibited no symptoms of 2,4-D injury.

Immersing intact leaves and those with the tip excised in 2,4-D generally had no lethal effect. Such treatments usually caused epinasty of the upper portion of the plant. Neither necrosis nor epinasty was observed in leaves below the ones immersed in the 2,4-D. Immersion of leaves in the dye, with or without the tip excised, usually failed to produce any visual evidence of translocation out of the treated leaf.

The leaf immersing procedure revealed that the occurrence of epinastic responses in the plant was dependent on the development of necrosis up to the petiole of the leaf which was immersed in the 2,4-D solution. This was favored either by high concentrations of 2,4-D or the use of small plants (3-5 inches tall). The observation of crystalline particles of 2,4-D on such necrotic leaves seemed to indicate that the mechanism of uptake and transport was not one involving the vascular system but merely one resulting from the absorption and free diffusion of 2,4-D in the damaged tissue.

The above observations were substantiated by artificially injuring a leaf with a small flame. The tip of the leaf was then placed in a solution of 4×10^{-3} M 2,4-D for 34 hours.

After 24 hours the top two leaves of the plant exhibited typical epinastic effects. However, at 34 hours when the leaf was removed from the solution of 2,4-D it was approximately 80 percent necrotic and the upper stem of the plant was exhibiting severe epinasty.

Sectioning of plants in which dye had been introduced revealed that it was transported in the xylem. Because of similar behavior of 2,4-D to the dye, it was assumed to move in the xylem also.

The Electrophoretic Displacement of Dye

The uptake of dye by excised leaves. This series of experiments was conducted to characterize the uptake and movement of dye in excised Canada thistle leaves.

The dye was introduced by placing the leaf petioles in 3 ml of an aqueous solution of Amaranth (3.5 g/l).

In one-half to one hour the dye was visible in most of the trichomes of the leaves. This was the first visual indication of uptake of dye by the leaves and occurred consistently in all experiments. After one, or at most two hours, the dye was observed in the xylem vessels by placing the leaf over a light source and by observations under the microscope of cross sections of the leaves. The time interval

required for staining of the interveinal areas varied between four and six hours.

Examination of sections of the leaves under the microscope at intervals showed that the interveinal areas were stained by the gradual diffusion of the dye from the smaller network of vessels.

Displacement. The following experiments were made to determine if a low voltage direct current applied transversely through an excised leaf by marginal contacts could effect a displacement of the negatively charged dye.

The petioles of several leaves were placed in the dye solution and marginal contacts were applied. The current was then adjusted to 10 μamperes in each circuit.

After several hours the results of the imposed current were very evident in the visible gradient of dye distribution through the leaf. A definite band of concentrated dye paralleled the positive contact. The dye between this band and the midrib of the leaf appeared to be much less concentrated. On the electronegative side of the midrib the dye was fairly concentrated in the tissue up to within several millimeters of the negative electrode. Paralleling the negative electrode was a narrow strip of tissue which was free of any visible dye. This strip was comparable

in size to the area paralleling the positive contact in which the dye was concentrated.

This experiment was repeated except that a current level of 30 μamperes was used. After one hour the dye was visible in all of the larger vessels but there was no differential distribution between the electropositive and electronegative sides of the leaves. After two hours dye accumulated at the end of a vessel next to the positive contact. At approximately three hours the accumulation of dye on the positive side of the leaf was quite evident.

A current level of 10 μamperes for approximately three hours was ample to cause a lateral distribution of the dye between the electropositive and electronegative sides of excised leaves.

The Electrophoretic Extraction of Dye

Vertical displacement. The results of the previous experiments established that it was possible to displace the dye within the plant tissue. Further experiments were designed to extend this procedure to ascertain if an electric current could displace the dye in a vertical path through living tissue without injury.

A pad of Norite was constructed on a glass plate

with the negative electrode and a wick buried below the surface. Ten layers of 1 cm square filter paper were placed on top of this pad. The top five layers were cut and separated in such a manner that the midrib of an excised leaf fitted in the resulting groove. Approximately ten layers of 1 cm square filter paper were placed on top of the leaf. A Norite pad containing the positive electrode and wick was constructed on top of this layer of paper. The petiole of the leaf was placed in a container of water and the wicks were placed in small vials containing distilled water. The water level in each vial was adjusted such that a siphoning action resulted and kept the Norite contacts from drying out. The current was adjusted to 60 μamperes and passed through the tissue for eight hours after which one small spot of dye was found on the top layer of paper.

This experiment and others of a similar nature established that the dye could be transported vertically and accumulated in filter paper on the electropositive side. However, the current levels were high and generally resulted in injury to the leaves. The dye exhibited a tendency to accumulate in these injured areas. It was thought that the upper cuticle of the leaf might be a barrier to the movement of the dye from the upper side of the leaf.

For this reason attempts were made to remove the cuticle from excised leaves without injuring the underlying tissue. One square inch of the upper surface of five leaves were scrubbed with a pad of cotton soaked in one of the following materials: soap, Tween-20, a one percent solution of Tween-20, ether, and a half and half water and ether mixture. One drop of concentrated dye was placed on each treated area. The petioles of the leaves were placed in water and moist cotton pads complete with the previously described system of wicks and vials of water were put in place.

After 11 hours the pads were removed and the leaves were examined. It was found that both ether treatments were effective in removing the cuticle and allowing the dye to penetrate into the upper tissue of the leaf. However, both of these treatments caused fairly large necrotic areas. The soap and the concentrated Tween-20 treatment allowed penetration of the dye and did not injure the leaf. One percent Tween-20 was ineffective. Although ether was the most effective in removing the cuticle, Tween-20 was selected as the most desirable treatment because it did not cause visible injury.

The initial experiments concerned with the vertical displacement of dye through tissue were repeated with the

following innovations. Rectangular cotton pads replaced the filter paper and a 2 X 3 cm area of the leaf surface was treated to remove the cuticle prior to the application of the pads. One leaf was treated with soap and another with Tween-20. The dye was added to the lower pad and a wick and carbon electrode were placed in each pad. The pad containing the positive electrode was placed over the treated area on the upper surface of each leaf. The petioles and wicks were placed in their respective containers of water and the current adjusted to 40 μamperes.

After two hours it was found that the cotton pad on the electropositive side of the leaf treated with Tween-20 was appreciably stained with the dye. Examination under the microscope of sections from this leaf showed that the dye appeared to be in the extracellular fluid around the palisade and epidermal cell walls. Since there was no dye on the upper pad on the leaf treated with soap, this experiment was continued for an eight hour period and still exhibited no sign of dye on its upper surface or in the pad.

A current level of 40 μamperes was, therefore, sufficient to displace the dye vertically through the leaf, providing the cuticle was removed under the contacts. The observations suggested that the dye had moved in the

extracellular fluid in the tissue in its passage through the leaf.

Extraction. Having established that it was possible to transport dye in a vertical path through living tissue with an electric current, attempts were made to extract the dye from leaves which had previously been treated by immersing the petioles in 3 ml of the dye solution. A Norite pad was constructed as in previous experiments. The leaf was removed from the dye solution and treated as previously with Tween-20 to remove the cuticle. The margins of the leaf opposite this area were coated with a thin film of silicone stopcock grease to prevent moisture films from creeping around the leaf. The upper surface of the leaf was divided into two equal parts by a 3 cm long ridge of stopcock grease placed over the midrib. The leaf was then centered on a Norite pad and its petiole placed in water. Two cotton pads approximately 2 cm square were prepared containing the wicks and the positive and negative electrodes respectively. Each pad was then placed on the surface of the leaf such that they were separated by the silicone ridge. The wicks were placed in their respective vials and the current was adjusted to the desired level. The arrangement of wicks and electrodes is shown in Figure 3.

The result of the above arrangement was the establishment of three regions of charge separation. The cotton

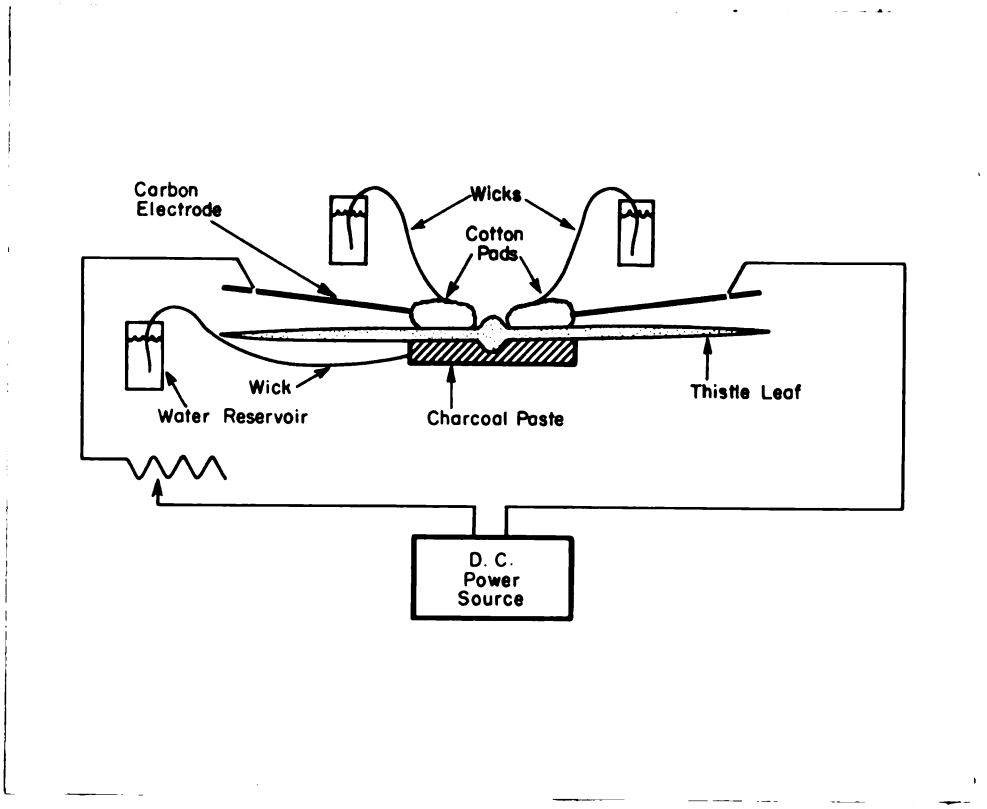

Figure 3. Diagram of method of applying an electric current to an excised Canada thistle leaf

pad containing the negative electrode was electronegative to the Norite underneath this area of the leaf. This portion of Norite was electronegative to the portion of Norite below the opposite side of the leaf which in turn was electronegative to the cotton pad containing the positive electrode directly above. It can thus be seen that a diffusible substance with a negative charge in the tissue under the negative electrode would tend to be drawn into the Norite below, whereas a similar substance in the leaf under the cotton pad containing the positive electrode would tend to be drawn up into the cotton pad.

The design of this electrophoretic unit was based principally upon observations that the diffusion of water from the cotton pads into the tissue had a slight tendency to wash the dye out of the tissue under the cotton pads if no current was applied.

Previous observations indicated the necessity of cuticle removal. The upper epidermis seemed more resistant to the injurious effects of cuticle removal than the lower, and the upper cell structure of the leaves was more dense and uniform than that of the lower.

For these reasons the electrophoretic unit was designed such that the contacts were applied to comparable

regions of tissue of the upper leaf surface. Thus, there was no greater tendency, other than that caused by the electric current, for a charged substance to diffuse into or out of one cotton pad than the other.

In the first experiment the petioles of the leaves were soaked in the dye for four hours prior to making the contacts. After five hours of current application at 40 μamperes a noticeable red color was evident on the positive pad. The current was adjusted to 20 μamperes and continued for 3.5 hours longer. At this time the distribution of red dye in the positive pad was somewhat greater and more intense in color. Examination of the leaf surface with a lens revealed that the dye appeared to be more concentrated in the area under the positive pad than in surrounding parts of the leaf or under the negative pad.

Petioles of several other leaves were soaked in the dye solution for six hours. One leaf was selected for electrophoretic extraction at 20 μamperes. After two hours a faint red color was present only on the positive pad. Examination of the leaf under the microscope revealed that the dye was concentrated in the vessels and seemingly in regions bordering the upper and lower epidermis. Sections from tissue which had been directly under the positive pad

showed that the dye appeared to be more concentrated near the upper epidermis, but the dye in a similar section taken from the tissue under the negative pad appeared equally distributed. The upper epidermis of the leaves was considered to be more of a barrier to the movement of the dye than was the lower even when the cuticle was removed from both.

Methylene blue, a positively charged dye, was selected for similar experiments to those performed with Amaranth as a check for electroosmotic displacement. A concentration of 6.5 g/l was found adequate to produce reasonably stained leaves after five hours of soaking.

The results obtained with methylene blue were the opposite of those obtained with Amaranth. The negative pad, rather than the positive, was found to be stained with the dye. Thus, electroosmosis was not considered to be a factor in respect to electrophoretic displacement and extraction.

The Electrophoretic Displacement of 2,4-D

The uptake of 2,4-D by excised leaves. The methods evolved and the results obtained were sufficient to suggest that a negatively charged diffusable substance such as 2,4-D could be displaced in the extracellular liquid of a

leaf by a noninjurious low level of current. The following experiments were designed to establish the appropriate levels of current and 2,4-D.

The method used to introduce the 2,4-D into the excised leaves was identical to that used for the dye. Leaves similar in size and shape were excised and their petioles were placed in 3 ml of solution containing the desired concentration of herbicide.

In order to determine the most desirable concentration of the herbicide, an exponential series of 2,4-D solutions were prepared ranging in concentration from 8×10^{-3} M to 8×10^{-6} M (2088 ppm - 2 ppm). A water control was also included. Five leaves were excised and placed in the solutions. Observations were made at 12 and 24 hours.

In 12 hours the 8×10^{-4} M concentration of 2,4-D was the most dilute solution that caused necrosis. The leaf in the 8×10^{-3} M solution was entirely necrotic and varied in color from purple to black. After 24 hours, the control leaf in water and the leaves in the two most dilute concentrations of the herbicide were normal in appearance. The leaf in the 8×10^{-4} M concentration of 2,4-D was severely wilted and had large black necrotic areas scattered over its surface.

Additional work was done to determine optimum periods of immersion in 2,4-D for an intermediate injurious effect. The concentration of 2,4-D selected for use was 2×10^{-3} M (522 ppm). Four to six hours were required before the first symptoms of 2,4-D injury appeared in the form of several small necrotic spots, one of which invariably was located on the periphery of the leaf. With increasing time more necrotic spots appeared, and the necrotic areas at the leaf tip and those on the margin coalesced to form a complete or almost complete rim of purple to black tissue surrounding the now mottled inner leaf area.

The effects observed when the leaves were removed from their respective solutions were as follows. The leaf which had been in the 2,4-D for the shortest time period (eight hours) had four small necrotic spots on its surface. The first necrotic spot, which was marginal in location, developed at five hours. The leaf which had been in the 2,4-D nine hours at the time of removal had approximately 20 necrotic spots on its surface plus almost complete marginal and leaf tip necrosis. The leaves subjected to the longer periods of treatment were almost completely necrotic.

Twenty-four hours after the experiment was begun, or nine hours after all the leaves had been placed in

distilled water, all of the leaves were shriveled and completely necrotic except the one which had been in the 2,4-D solution for the shortest interval (eight hours). The only difference between this leaf and the others was that the necrotic areas had not yet coalesced. Thus, the shortest time interval used for immersing the petioles of excised leaves in 2×10^{-3} M 2,4-D was found to be too long.

Subsequent work showed that two hours of petiole immersion in 2×10^{-3} M 2,4-D produced intermediate symptoms of 2,4-D injury which developed more slowly and did not cause such a rapid collapse of the tissue.

In order to ascertain if the development of necrotic symptoms on excised leaves was a sufficiently valid criterion for symptoms of 2,4-D injury, a solution containing 2×10^{-3} M acetic acid was prepared. Two leaves were excised, the petiole of one was placed in 2 ml of the acetic acid solution, and the other in a comparable molar concentration of 2,4-D.

After ten hours the leaf whose petiole had been in the acetic acid solution was perfectly normal in external appearances, and the other leaf was completely necrotic.

The results of this series of experiments established the molar concentration of 2,4-D used in all subsequent work and the boundaries used for later variations in the intervals

of immersing in both the herbicide and water. Observations of the development of necrosis in excised leaves over hourly intervals very closely paralleled the movement of dye obtained in previous work in which a similar peripheral movement was repeatedly observed.

Displacement. These experiments were based on the assumption that the herbicide taken up by the leaf was carrying a negative charge such as the dye previously used. Evidence of electrophoretic displacement was based upon the differential development of necrosis in the leaf between the positive and negative sides.

Two types of experiments were carried out. In one the petioles of the leaves were placed in 2 X 10^{-3} M 2,4-D at the same time the current application was made. In the second the petioles of the leaves were immersed in 2,4-D before the current was applied. The previous procedure was modified to conduct both experiments in a dessicator over water to maintain a high relative humidity.

In the first experiment the petiole of a leaf was placed in the 2,4-D solution at the same time 10 μamperes of current was applied. After two hours the petiole was removed from the 2,4-D solution and placed in distilled water with a continued application of current.

After six hours of current application no symptoms of 2,4-D injury were observed. At eight hours faint marginal necrosis was evident. At 19 hours, marginal necrosis was evident in addition to three small necrotic spots next to the anode. At 31 hours these spots had enlarged somewhat, but at 44 hours, after a total of 1.58 coulombs of current had been applied, these spots had enlarged to such an extent that they were 4 X 10, 3 X 10, and 6 X 7 mm in size respectively (Figure 4). There was no indication of current injury to the leaf, nor were there any necrotic areas next to the electronegative contact.

In the previous experiment the 2,4-D was presumably swept along in the path of the current. This would have allowed little time for the herbicide to react with the tissue or to be altered in some manner in the extracellular liquid. In order to allow the herbicide to diffuse out of the vascular elements the following modification was made.

The petiole of an excised leaf was placed in 2×10^{-3} M 2,4-D for 2.5 hours and then removed from the 2,4-D solution and washed. The leaf was then set up with marginal contacts and a 10 μampere current was applied.

After 20 hours of current had been applied the only necrosis present was on the electropositive side of the leaf.

Figure 4. The effect of 1.58 coulombs of current on the lateral distribution of 2,4-D in a Canada thistle leaf whose petiole had been immersed in a 2 X 10^{-3} M solution of the herbicide for the first two hours of the current treatment

The purple to black color of the necrotic area was typical of that caused by 2,4-D rather than that of an excessive current application. At 37 hours a faint trace of marginal necrosis was evident and a necrotic area approximately 3 cm wide had developed on the positive side of the midrib. The results after 44 hours at 10 μamperes may be seen in Figure 5. The necrosis at the leaf tip was slightly more severe and the necrotic area on the positive side of the leaf was very prominent.

The differential development of necrosis on the electropositive side of the leaves indicated that 2,4-D was laterally displaced in the leaves toward the positive contact. The displacement in this and other leaves varied between 0.5 and 1.0 cm.

The Extraction of 2,4-D by a Combination of Methods

In the following experiments water extractions were made of the cotton contacts following the predetermined interval of current used for electrophoretic displacement. Each pad was separately shredded and packed in the stem of a funnel. The funnel was placed in a suction flask such that the liquid drawn through it was caught in a small vial graduated to contain 5 ml. After a vacuum was applied, distilled water was slowly dripped through the cotton until

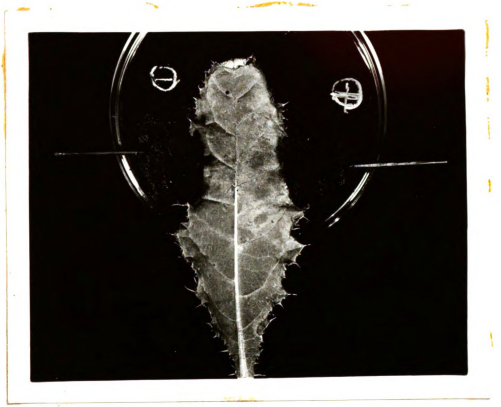

Figure 5. The effect of 1.58 coulombs of current on the lateral distribution of 2,4-D in a Canada thistle leaf whose petiole had been immersed in a 2×10^{-3} M solution of the herbicide for 2.5 hours prior to the current application

a total of 5 ml was obtained which was refrigerated and later bioassayed with the Avena straight growth test which is described in Appendix A. Prior to assaying the solution was diluted one to one with a phosphate-citrate buffer. The control consisted of an identical extraction procedure with leaves whose petioles had been immersed in distilled water instead of 2,4-D. By comparison with a standard curve, the amount of 2,4-D extracted from the cotton was determined.

Extraction with one interval of current. The petiole of an excised leaf was immersed in 3 ml of 2×10^{-3} M 2,4-D for 2.25 hours, removed from the solution, thoroughly washed in water, and placed in distilled water. A current application of 20 μamperes was applied for seven hours and water extractions were made of the positive and negative cotton pads.

The results (Table 1) show that nine times as much 2,4-D was extracted from the positive pad as from the negative pad. It was observed that the extractions from the positive and negative pads of the control leaf did not differ from each other nor did they differ from the buffer-water control.

TABLE 1. The amounts of 2,4-D recovered from cotton pads used as traps in the electrophoretic extraction of 2,4-D from an excised Canada thistle leaf, whose petiole had been immersed in 2 X 10^{-3} M 2,4-D for 2.25 hours, by an application of 20 μamperes of current for seven hours

Treatments	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (μmoles x 10^3)
Control leaf			
Positive pad	17	1.00	
Negative pad	17	1.00	
Treated leaf			
Positive pad	39	2.29	1.8
Negative pad	26	1.53	0.2
Buffer-Water control	17		

*Mean elongation expressed in grid units (64 g.u. = 1 cm), coefficient of variation for all treatments = 6.5%.

**Elongation of treated sections divided by elongation of control sections.

Extraction with varying intervals of current. The purpose of this experiment was to determine the effect of four intervals of current on the amount of 2,4-D recovered from excised leaves.

The petioles of four leaves were immersed in 3 ml of 2 X 10^{-3} M 2,4-D for 1.5 hours. The control leaf consisted of a leaf whose petiole was immersed in distilled water for 1.75 hours. A current application of 20 μamperes was applied to the leaves for two, four, six, and eight

hours respectively. An intermediate value of four hours of current was used for the control leaf. Extracts from these pads were stored under refrigeration and later bioassayed.

The results are shown in Table 2. The low value obtained for elongation of the coleoptile sections with the extract from the positive pad of the control leaf was disregarded because of a known contamination in the extract. Since the previous elongations for the positive and negative extracts of the control leaf were equal (Table 1), the value for the negative extract in this experiment was used for calculating the E_t/E_c ratios (elongation of treated sections divided by elongation of control sections) for the electrophoretic treatments.

Since the quantities of 2,4-D extracted from these leaves were rather small (Table 2), a two hour period was used for immersion of the petioles in future experiments. When the electrophoretic extraction was extended up to the longest interval of eight hours it did not result in the extraction of any material having physiological activity from the negative pad.

The recovery obtained at two hours indicated that as much 2,4-D could be recovered from the leaves with this interval as could be obtained with an eight hour interval.

TABLE 2. The amounts of 2,4-D recovered from cotton pads used as traps in the electrophoretic extraction of 2,4-D from excised Canada thistle leaves, whose petioles had been immersed in 2 X 10^{-3} M 2,4-D for 1.5 hours, by applications of 20 μamperes of current for two, four, six, and eight hours respectively

Treatments	Hrs. of current	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (μmoles x 10^3)
Control leaf				
Positive pad	4	13***		
Negative pad	4	16	1.00	
Treated leaves				
Positive pad	2	28	1.75	0.4
Negative pad	2	17	1.06	
Positive pad	4	23	1.44	0.2
Negative pad	4	17	1.06	
Positive pad	6	21	1.31	0.2
Negative pad	6	17	1.06	
Positive pad	8	27	1.69	0.4
Negative pad	8	16	1.00	
Buffer-Water control		15		

*Mean elongation expressed in grid units (64 g.u. = 1 cm), coefficient of variation for all treatments = 7.0%.

**Elongation of treated sections divided by elongation of control sections.

***This value not used in calculating E_t/E_c because of a known contaminant.

It was thought, however, that at the eight hour interval an equilibrium had been reached in which the amount of 2,4-D extracted was balanced by the amount which was being inactivated at the electropositive electrode. Since a two hour interval might not be long enough to allow for possible changes in the mobility of the herbicide, a six hour interval was selected for use in subsequent experiments.

Electrophoretic displacement and extraction by ether. Experiments with charged dyes and 2,4-D indicated that these materials were displaced horizontally in the leaves toward the oppositely charged electrodes. This experiment was designed to obtain information of a more quantitative nature on the displacement of 2,4-D. The amount or relative amounts of material displaced toward the respective electrodes should give an indication of whether the mobility or the physiological activity of the herbicide was altered in the extracellular liquid.

For the purpose of determining the amount of 2,4-D, selected areas of a leaf were excised following the electrophoretic treatment. This tissue was then ground in a Waring blendor, boiled, acidified, and extracted with ether in accordance with the technique described in Appendix B.

Following the usual procedures the petiole of an

excised leaf was placed in 2 X 10^{-3} M 2,4-D with current of 10 μamperes applied through marginal Norite contacts. After a two hour interval the leaf was removed from the 2,4-D solution and placed in distilled water. Following a six hour interval of current, the contacts were removed and 1.5 X 3 cm sections of tissue were excised from the leaf adjacent to the positive and negative contacts respectively. These sections of tissue were subjected to the ether extraction procedure.

The results indicated that considerable response was obtained in the bioassay of the ether extracts from both sections of tissue (Table 3). However, the extraction of the tissue on the electropositive side of the leaf resulted in the recovery of approximately twice as much 2,4-D than that obtained from the electronegative side of the leaf.

Electrophoretic and ether extractions. Varying time intervals were used in this experiment between the conclusion of the 2,4-D treatments and the beginning of the extraction procedures in order to obtain information on the inactivation or altering of the 2,4-D in the extracellular liquid with increasing incriments of time. Since it is conceivable that the 2,4-D could either lose its electrical charge or acquire a positive charge as a result of a chemical reaction or

TABLE 3. The amounts of 2,4-D recovered by ether extractions of sections of tissue from the electropositive and electronegative sides of an excised Canada thistle leaf following a two hour immersion of the petiole in 2 X 10^{-3} M 2,4-D with a parallel application of 10 μamperes of current followed by an additional four hours of current.

Treatments	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (μmoles X 10^3)
Positive side of leaf	35	2.06	5.0
Negative side of leaf	31	1.82	2.6
Control	17	1.00	

*Mean elongation expressed in grid unit (64 g.u. = 1 cm), coefficient of variation for all treatments = 7.2%.

**Elongation of treated sections divided by elongation of control sections.

complexing with constituents in the extracellular liquid, the results obtained from electrophoretic extractions could be expected to differ at each increment of time or perhaps no response would be obtained after a sufficient interval had elapsed. A comparison of the amount of physiologically active substance extracted by the electrical and ether extraction techniques should yield additional information on the fate of the herbicide. If inactivation of the herbicide were occurring, no response could be expected in the bioassay of the ether extractions of the tissue. This would be anticipated at the longer time intervals.

The petioles of excised leaves were uniformly immersed in 3 ml of 2 X 10^{-3} M 2,4-D for two hours, removed, thoroughly washed, and placed in distilled water. Four time intervals were employed between the conclusion of the 2,4-D treatments and the beginning of the electrophoretic extraction. These intervals were as follows: zero, three, six, and twelve hours. Appropriate controls were included. At the conclusion of each electrophoretic treatment extractions were made of the cotton pads and the terminal 3 cm portion of the leaf by water and ether respectively.

Treatments were included to obtain an approximate evaluation of the recovery of 2,4-D from the extraction apparatus, with and without cotton packed in the stems of the funnels, when known amount of 2,4-D were added. The procedure was as follows. A funnel was placed over one of the 5 ml graduated vials which was used for collecting the water extract from the pads. In this case, however, a pad was not shredded and packed in the stem of the funnel. Twenty-five microliters of 3.9 X 10^{-4} M 2,4-D was pipetted into the funnel and distilled water was added drop by drop in the same manner as when the stem of the funnel was packed with cotton. The resulting 5 ml of solution was refrigerated and then bioassayed with the other extracts.

This procedure was repeated and a second 5 ml of solution was obtained. The entire process was repeated with cotton in the stems of the funnels. In this case, however, 25 λ of distilled water was added to two funnels and 25 λ of the 2,4-D solution was added to two other funnels.

It may be seen in Table 4 that the 2,4-D solution was approximately one half as active as it theoretically should have been. Since this solution of 2,4-D was quite old, the low response was not considered as an experimental error. However, the adsorption of such a small volume of herbicide (25 λ) to the surface of the glassware was also considered as a contributing factor. Assuming that somewhat less than 5×10^{-3} μmoles of 2,4-D was obtained from the extraction apparatus for bioassaying (Table 4), the recovery of 7×10^{-4} μmoles is approximately equal to a 15 percent recovery. Thus, it must be assumed that the responses obtained from the water extraction of the cotton pads used as contacts in the electrophoretic extractions of treated leaves is only indicative of approximately 15 percent of the actual materials present.

The results of the series of extractions employing the four time intervals are presented in Table 5. From a standpoint of comparison of the amounts of 2,4-D recovered

TABLE 4. The amounts of 2,4-D recovered from the extraction apparatus used for recovering 2,4-D from cotton, with and without the cotton packing, to which 10^{-2} µmole of 2,4-D had been added

Treatments	Material added	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (µmoles X 10^3)
Extraction Apparatus				
Without cotton	2,4-D	42	2.62	5.1
Without cotton	2,4-D	41	2.56	4.5
Buffer-Water Control		16	1.00	
Extraction Apparatus				
With cotton	2,4-D	20	1.54	0.7
With cotton	water	13	1.00	
With cotton	2,4-D	22	2.00	0.8
With cotton	water	11	1.00	

*Mean elongation expressed in grid units (64 g.u. = 1 cm), coefficient of variation for all treatment = 7.3%.

**Elongation of treated sections divided by elongation of control sections.

by the ether extractions of the tissue with the amounts electrophoretically extracted, it can be seen that considerably more recovery was obtained by the ether extractions. It should be noted, however, that the area of tissue under the electrical contacts was considerably smaller than the area of tissue subjected to the ether extractions.

Because the slightly lower response obtained from the water extract of the positive pad at the 12 hour interval (Table 5) suggested the possible formation of a neutral

TABLE 5. The amounts of 2,4-D recovered from cotton pads used as traps in the electrophoretic extraction of 2,4-D from excised Canada thistle leaves and from ether extracts of a portion of each leaf following a zero, three, six and twelve hour interval during which their petioles were immersed in distilled water. The petioles of all treated leaves had previously been immersed in 2 X 10^{-3} M 2,4-D for two hours. All leaves were subjected to a current application of 20 μamperes for six hours.

Treatments	Hrs. in water	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (μmoles X 10^3)
Control leaf				
Positive pad	6	16	1.00	
Negative pad	6	17	1.00	
Tissue	6	13	1.00	
Treated leaves***				
Positive pad	0	31	1.94	1.0
Tissue	0	27	2.08	8.0
Positive pad	3	24	1.50	0.5
Tissue	3	30	2.31	13.0
Positive pad	6	28	1.75	0.7
Tissue	6	26	2.00	6.5
Positive pad	12	19	1.19	0.3
Tissue	12	31	2.38	15.0
Buffer-Water Control				
For tissue		16		
For pads		15		

*Mean elongation expressed in grid units (64 g.u = 1 cm), coefficient of variation for all treatments = 7.3%.

**Elongation of treated sections divided by elongation of control sections.

***Values for negative pads omitted because responses were not greater than control.

complex or the loss of electrical charges as a consequence of an alteration of the molecule, the previous experiment was repeated. An interval of ten hours was selected as an intermediate period between the end of the 2,4-D treatment and the beginning of the electrophoretic extraction. All procedures, solutions, and conditions were the same as in the previous experiment.

The results, as given in Table 6, show that considerably more 2,4-D was recovered from the positive pad than was recovered from the positive pads in the previous experiment (Table 5). This value, however, is not indicative of any change in the mobility of the herbicide.

The recovery obtained by the ether extraction compared favorably with those shown in Table 5.

TABLE 6. The amounts of 2,4-D recovered from cotton pads used as traps in the electrophoretic extraction of 2,4-D from an excised Canada thistle leaf and from an ether extract of a portion of the leaf following a ten hour interval during which its petiole was immersed in distilled water. The petiole had previously been immersed in 2 X 10^{-3} M 2,4-D for two hours. The leaf was subjected to a current application of 20 μamperes for six hours.

Treatments	Coleoptile* elongation	E_t/E_c **	2,4-D recovered (μmoles X 10^3)
Control leaf			
Positive pad	15	1.00	
Negative pad	17	1.00	
Tissue	12	1.00	
Treated leaf			
Positive pad	31	2.07	1.8
Negative pad	18	1.06	
Tissue	25	2.08	9.2
Buffer-Water control	16		

*Mean elongation expressed in grid units (64 g.u. = 1 cm), coefficient of variation for all treatments = 7.0%.

**Elongation of treated sections divided by elongation of control sections.

DISCUSSION

The electrophoretic method used in this study was found to be effective in extracting 2,4-D from the extracellular liquid of Canada thistle leaves. Several limitations of the method, however, had to be considered. One of these was the possibility of electrically induced flow of the extracellular liquid. The results of experiments with oppositely charged dyes indicated that electroosmosis was not a major factor in electrical displacement. The low recoveries obtained from cotton pads to which known amounts of 2,4-D had been added was a more serious limitation of the method. However, the relative differences between the recoveries obtained from the positive and negative pads furnished the desired information concerning the possible changes in the mobility of the herbicide. The method could probably be modified to make it more quantitative. One such method of achieving this end might be the utilization of a continuous flow system of liquid contacts in which the liquid was retained in a suitable trap for later assaying or chemical analysis. Such a method would eliminate the retention of the herbicide by the cotton pads.

Although the leaves used in these experiments were selected on the basis of uniform size and position on the plants, there was considerable variation in the amounts of

2,4-D and dye taken up by them. In some cases this variability was as high as sixfold, but in most instances the range of variability was between two and threefold. The petiole immersion method of introducing the 2,4-D into the excised leaves was employed in spite of this variation because it provided a means of introducing the herbicide directly into the extracellular liquid. Improved environmental control might have resulted in more uniform uptake of the dye and herbicide.

The results of the experiments utilizing the electrophoretic extraction technique were in agreement with those of observational experiments in which the 2,4-D and dye were found to be transported in the extracellular water. The 2,4-D was repeatedly recovered from the positive contacts. In only one instance was evidence obtained which suggested a change in the electrical charge of the growth regulator (Table 1). In this instance approximately nine times as much active material was recovered from the positive pad as was obtained from the negative pad. All subsequent data from extractions of cotton pads indicated that the herbicide was not altered by an interaction with substances in the extracellular liquid. The preferential displacement of an applied electrolyte to an electrode of the same charge has recently been reported by Anderson et al. (1). An oppositely charged carrier was

suggested as the explanation for this response. However, the conflicting value in Table 1 was probably the result of diffusion of the 2,4-D outside of the electrical field as a result of a poor contact.

When ether extractions were made of portions of a leaf from the positive and negative sides following an electrophoretic displacement, it was found that a response was obtained from the tissue on the negative side (Table 3). This response is readily explained when it is recalled that a similar displacement of dye indicated that the midrib was a barrier to transverse movement. Since the 1.5 X 3.0 cm portion of excised tissue from each side of the leaf included areas near the midrib, it was probable that part of this tissue on the negative side of the leaf included an area in which the herbicide was concentrated as a result of the barrier imposed by the midrib.

The responses obtained from ether extractions of the terminal 3 cm portion of leaves over varying intervals of time (Table 5) suggested that inactivation of the herbicide over periods up to 12 hours did not occur. When this experiment was repeated with a ten hour interval the recovery obtained (Table 6) was in good agreement with the data in Table 5. The data from ether extractions in Table 5 and 6 indicate gradual increases in the amounts of extractable material over increasing time intervals. This substantiated previous

observations (obtained from parallel experiments with dye and 2,4-D) that the 2,4-D was transported toward peripheral areas.

It should be noted that in these experiments it was considered essential to work only with tissue which was normal in appearance. Any tissue which had become necrotic was discarded because of the possible release and subsequent extraction of inhibitory substances.

The recoveries obtained from the positive cotton pads up to and including the six hour interval were essentially constant (Table 5). This was interpreted as evidence that the herbicide was not readily taken up by the living cells and was not involved in the formation of an immobile complex in the extracellular liquid. However, the somewhat lower value obtained for the 12 hour interval did not negate the latter possibility. This lower response could not be validly interpreted as inactivation since the response from the tissue extract for this interval was quite high. When the experiment was repeated with an intermediate interval of ten hours (Table 6), the recovery obtained was considerably higher than those given in Table 5 but was in exact agreement with that given in Table 1 for the positive pad. On the basis of these results, it was considered unlikely that the 2,4-D formed a neutral complex. The higher recoveries obtained in the last experiment were probably caused by a greater uptake of the herbicide by the leaf.

The possibility of the 2,4-D molecule forming a complex in the extracellular liquid such that it had an effective positive charge was disproven since no response was obtained in any of the extracts from the negative pads at any of the time intervals.

The transport of 2,4-D which occurred in these experiments was principally, if not entirely, confined to the xylem vessels. That the vessels were capable of functioning in transport was evidenced by the observations of dye transport in the vessels and by the observation of lack of protoplasm and the presence of secondary walls in the form of annular and spiral rings.

The peripheral movement of the herbicide to the margins of the leaves to which it had been applied was clearly indicative of xylem transport. A similar peripheral movement of a herbicide from the point of application on Canada thistle leaves has previously been reported (19). The upward and outward movement of the herbicide which occurred regardless of the method of introduction was quite typical of transport of a material in the transpirational stream. The parallel movement of the dye and its presence in the xylem, as found by examination of sections under the microscope, were considered as additional evidence of xylem transport. Corms (6) studied translocation of 2,4-D in perennial sow thistle and suggested a similar

xylem pattern of movement within the plant but also reported extensive downward movement.

The same pattern of response was obtained when leaves whose tips had been excised were immersed in 2,4-D. Movement in the former was attributed to a reversal of the transpiration stream. Since essentially the same responses were obtained by both methods, the same avenue of transport was considered to be operative in both cases. A similar movement of 2,4-D in the transpiration stream as a result of leaf tip excising has been reported (37).

The enhanced transport of 2,4-D that was obtained when the leaf and petiole were injured or when a portion of the applied 2,4-D ran back down the petiole of the leaf into the axil was considered to be evidence of placement of the herbicide nearer the vigorous upward transpirational stream and nearer to larger vascular bundles. There is evidence in the literature that the position of placement of a herbicide on a plant leaf has considerable bearing on the effectiveness of such an application (7, 21, 36). More extensive growth inhibition was obtained when 2,4-D was applied at the base of the leaves than when it was applied nearer the apex. These results, however, may be interpreted as either enhanced xylem or phloem transport.

The explanation for the lack of phloem transport in these plants could be attributed to a number of causes. One

of which concerns the position of the treated leaf on the stem. Crafts (11, 12) reported that applications of 2,4-D to the lower leaves of plants resulted in the downward translocation of 2,4-D whereas applications to upper leaves resulted in transport to the upper regions of the plants. It was further reported that applications of 2,4-D to the middle leaves of morning glory plants resulted in either downward or both upward and downward transport (8). Since the applications of 2,4-D to the Canada thistle plants used in these experiments were always made on leaves intermediate in their position on the stems and since the roots of these plants were actively growing, some transport into the roots was anticipated. Such a downward path of transport, however, was never observed.

Further explanations for the lack of adequate transport of 2,4-D could involve a binding mechanism or active accumulation with the tissue (2, 11, 20, 21). Since the evidence obtained with the electrophoretic technique used in these experiments indicated that the 2,4-D was freely mobile in the extracellular liquid, it can only be assumed that if such a mechanism occurred it must have resulted in the formation of strong bonds between a small portion of the 2,4-D and the cellular materials or else such a mechanism occurred within the protoplasm out of the path of the electric

current.

The most probable explanation for the lack of transport and hence lack of effectiveness of 2,4-D on these Canada thistle plants is that it was not taken up by the functioning sieve elements. This lack of uptake is attributed either to the polar nature of the sodium salt and the apolar nature of the lipoidal properties of the protoplasm or to the lack of the proper activated metabolic processes necessary for the accumulation of 2,4-D in the phloem and its transport once in the phloem, or possibly to both. Evidence for the necessity of metabolic energy in the transport of 2,4-D has been presented in several publications (16, 18, 23).

The results obtained in these experiments also indicated that there was no appreciable metabolism or inactivation of the 2,4-D up to the 12 hour interval used. If the 2,4-D was metabolized to any extent, it must be assumed that it evoked the formation of material of similar physiological activity and mobility.

SUMMARY AND CONCLUSIONS

An electrophoretic method was used to study the mobility of the sodium salt of 2,4-D in the extracellular liquid of Canada thistle leaves since the ineffectiveness of the herbicide might be attributed to the formation of an inactive or immobile complex. Extraction of the 2,4-D was accomplished electrophoretically and by an ether extraction procedure. The concentration of the herbicide in the extract was assayed by the Avena straight growth test.

These studies provided the following conclusions:

1. The transport of 2,4-D was found to occur principally in the extracellular liquid of the tissue. Because of the identical peripheral movement of the 2,4-D and a water soluble dye (Amaranth) and the visual presence of the dye in the xylem vessels, it was concluded that the transport of the 2,4-D was confined to the xylem.
2. Transport in the phloem was considered to be negligible.
3. On the basis of electrophoretic studies there was no evidence of a change of or a lack of mobility of the 2,4-D in the extracellular liquid. The herbicide

was consistently extractable at the electropositive contact.

4. There was no loss of biological activity of the herbicide up to and including a 12 hour interval as a result of metabolism or other detoxification processes.

5. The limited uptake of the herbicide by the living protoplasm was thought to be the principal factor in the lack of transport.

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APPENDIX A

THE BIOASSAY PROCEDURE

The Avena straight growth test was used to determine the amounts of 2,4-D present in extracts obtained from treated Canada thistle leaves by ether extractions of the tissue (Appendix B) and by water extractions of cotton pads which had been used as traps in the electrophoretic extraction procedure.

The oats, Avena sativa var. Brighton (Bio-Lab Equipment Company) were grown in a constant temperature cabinet at 25 degrees centigrade and approximately 90 percent relative humidity. The cabinet was equipped with a small fan to provide a uniform circulation of air and a source of red light 30 cm above the plant bed. The light source consisted of two General Electric Ruby red bulbs. During the interval in which the lights were on the temperature rose approximately three degress and remained at this level until the light period ended. The normal range in temperature variation that occurred between the heating and cooling cycles was approximately one-half of a degree centigrade.

Brighton oats were selected for use rather than the more commonly used Victory variety because it had been

established that they were a suitable substitute and eliminated the dehulling procedure (25). The method, as first employed, was essentially the same as that outlined by Leopold (22). Literature citations are given to major deviations from the outlined method.

The seed were placed in a suction flask, soaked in water under a vacuum for two hours, removed and placed on glass strips covered with paper towels such that the grooved side was down and the embryo end projected over the side. The strips were placed in a glass tray with sufficient distilled water to cover the bottom to a depth of 5 mm and placed in the dark constant temperature cabinet.

Twenty-four hours later the seed were exposed to two hours of red light and 72 hours later, or when the coleoptiles were 2.0 to 3.0 cm long, they were removed from the cabinet in the dark. All subsequent manipulations were performed under a low intensity Ruby red light. One 5 mm section was cut from each coleoptile 3 mm from the apical end and placed in distilled water. The primary leaf was pushed out and the hollow sections were threaded on sealed glass capillaries and placed in small glass boats after the method of Luckwill (24). The boats contained 1.0 ml of the solution to be tested and 1.0 ml of a pH 5.0 buffer to

avoid variations in growth due to pH effects. The composition of the buffer as given by Nitsch and Nitsch (26) was as follows: citric acid monohydrate, 2.038 g/l; K_2HPO_4, 3.588 g/l; sucrose, 40 g/l. The boats, containing two capillaries with five sections each, were placed in Petri dishes in the constant temperature cabinet.

Twenty-four hours later the sections were removed and the elongation measured with a binocular microscope equipped with an eyepiece micrometer (4). One centimeter was equal to 64 grid divisions.

The results of the initial experiments indicated that insufficient red light had been used to suppress the elongation of the first internode. As a result of subsequent experiments it was established that four hours of red light from two 25 watt bulbs provided the optimum intensity and duration of light needed.

Growth in the Avena straight growth test, as originally suggested by Bonner (4), is approximately proportional to the log of the concentration of the applied auxin. In order to determine a range of concentrations of 2,4-D that would give a linear response a serial dilution series of 2,4-D was prepared ranging in concentration from 2×10^{-3} µmoles/l to 2.0×10^{2} µmoles/l and a bioassay was run on these solutions.

The results as shown in Figure 6 in which the response of the sections is expressed by dividing the elongation of the treated sections by the elongation of the control sections. The control sections in this and all subsequent experiments were grown in equal proportions of distilled water and the phosphate-citrate buffer. On the basis of these responses other bioassays were performed using the concentrations of 2,4-D which corresponded to the linear range shown in Figure 6. As a result of these experiments it was established that growth was approximately proportional to the log of concentration between 5.0×10^{-2} μmoles/l and 1.95 μmoles/l.

The standard deviations of the various treatments in these bioassays were quite large. In fact, in many cases the coefficient of variation obtained for some treatments approached values as high as 15 percent. It was theorized that microbial contamination of the seed might be a factor and that the procedure of soaking them under a vacuum might be a contributing factor in infecting the seed with whatever contamination that might have been on the surface. To test this hypothesis the following three treatments were employed; one group of seed was subjected to the usual soaking under vacuum, a second group was washed continuously

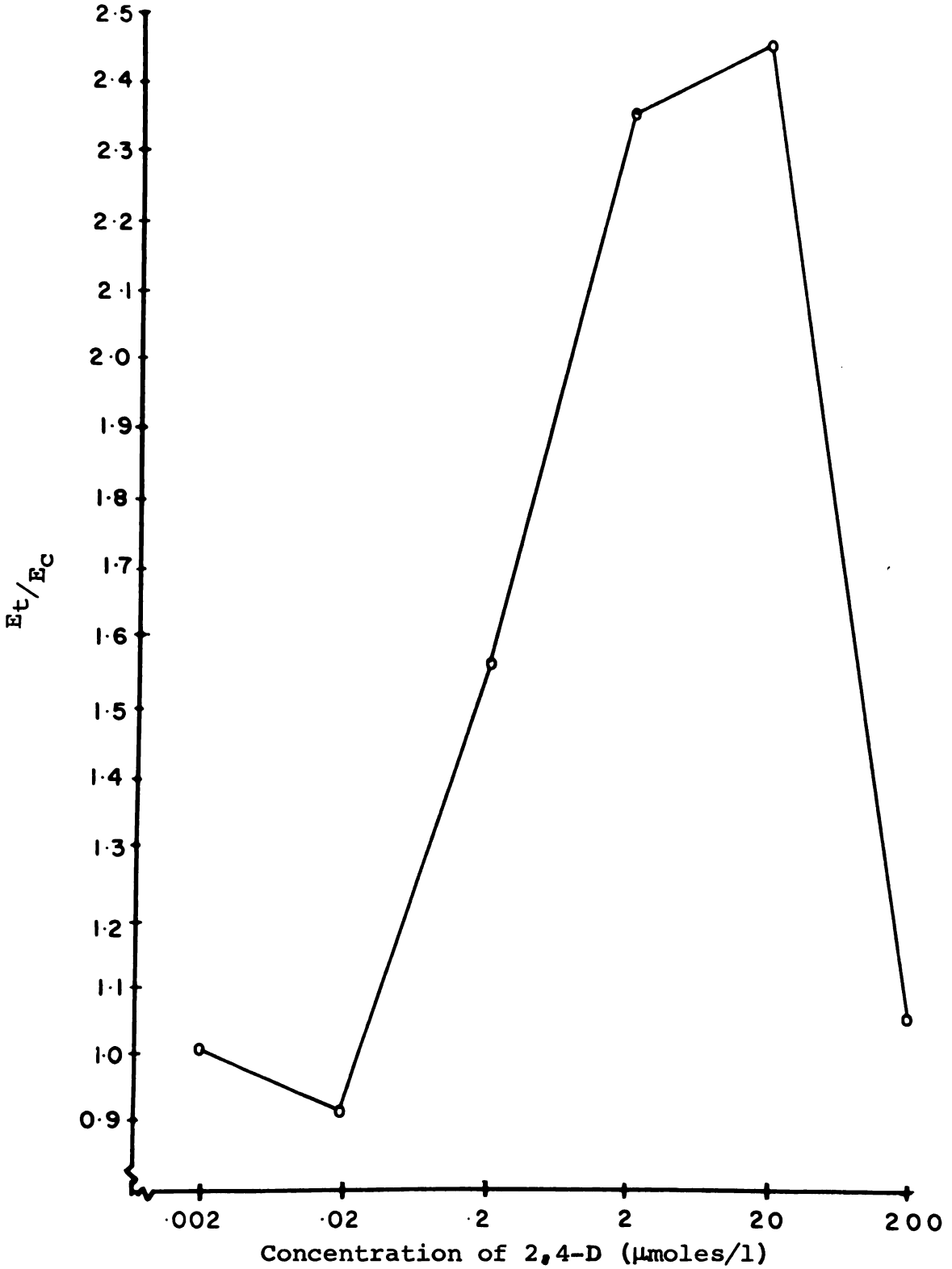

Figure 6. The response (elongation of treated sections divided by elongation of control sections) of Avena coleoptile sections to 2,4-D

in running tap water for two hours, and the third group was surface sterilized for one minute in one percent NaOCl. Each group of seed was then plated on a potato dextrose agar.

After 24 hours it was found that the seed treated with NaOCl were free of microbial growth, but the seed from the other treatments were covered with microbial growth. Of the latter two, the vacuum treatment was decidedly the most undesirable. As a result of these findings the vacuum treatment was abandoned and in all subsequent bioassays the seed were washed in running water and surface sterilized with one percent NaOCl.

In previous bioassays the buffer solution was autoclaved after using, and the unused portion was refrigerated and used in later bioassays. Since the possibility of microbial contamination in the buffer as well as on the glassware could affect the growth of the sections, the following modifications were made in the procedure. The seed were prepared for planting as described using the NaOCl. A new buffer solution was prepared and autoclaved along with a container of water and all of the glassware to be used. During the preparation of the sections all equipment which came in contact with the sections was rinsed in sterile water

following frequent surface sterilizations with NaOCl. After the cutting operation, the sections were floated on sterile water until this operation was complete. One-half of the sections were grown in a one to one dilution of sterile water and sterile buffer, and the other half were grown in a similar dilution of nonsterilized water and buffer. After 24 hours the sections were measured.

The results showed that there was little difference between the mean lengths of the two groups of sections, but the standard deviation of the sections grown in the non-sterilized buffer was 50 percent larger than that of the sterilized buffer treatment. A histogram showing the distribution of the individual section lengths around the mean is shown in Figure 7.

Freshly prepared and autoclaved buffer solutions, as well as sterilized glassware, were used in all of the following bioassays.

Although these modifications aided in reducing the variability of the growth of the Avena sections, there were inherent problems associated with growing the sections on capillary rods in glass boats which often caused erratic responses. The major problems were the curvature of the sections, the injury of the sections which occurred when the

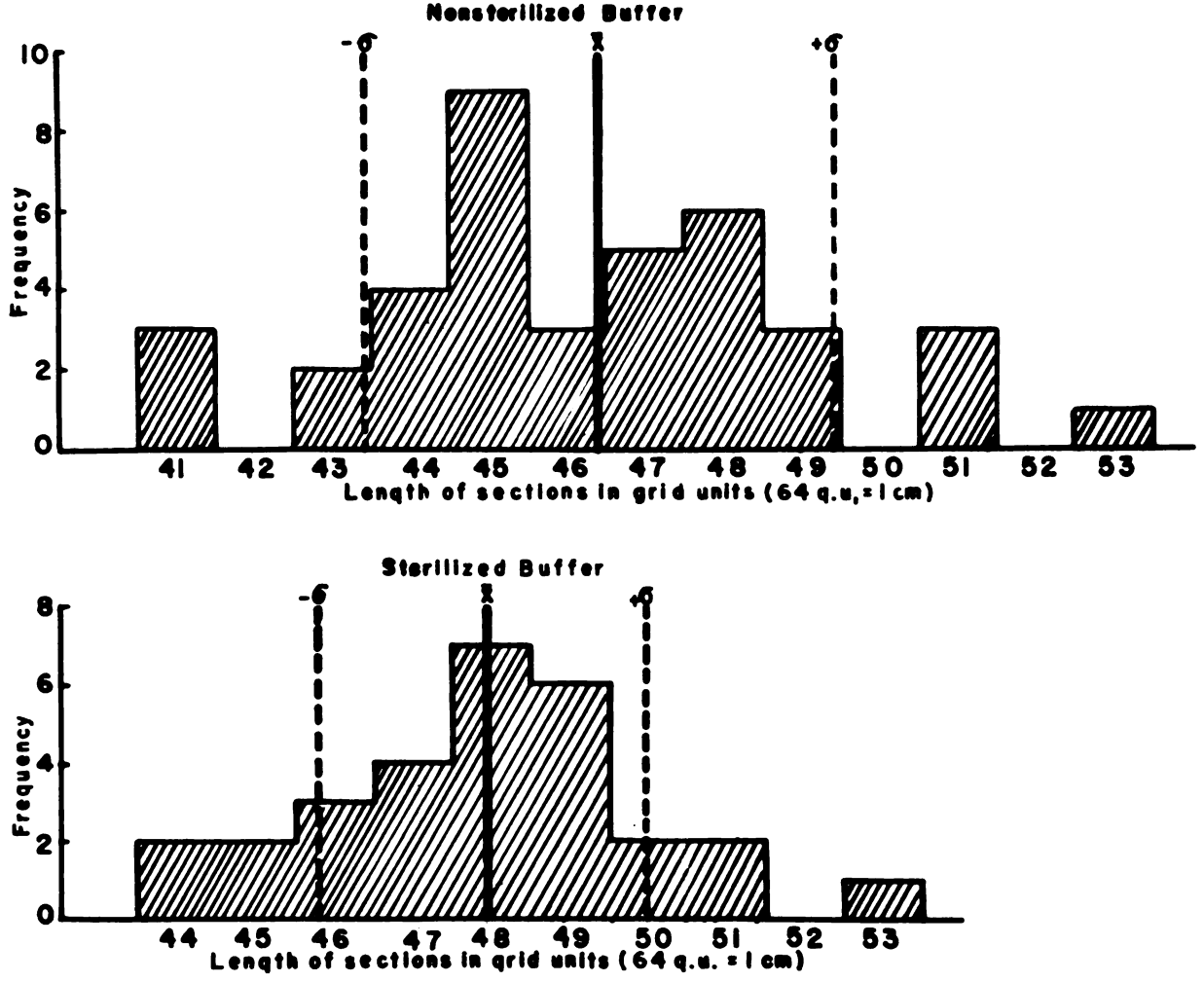

Figure 7. The frequency distribution of Avena coleoptile sections grown in a nonsterilized buffer and in a sterilized buffer without 2,4-D

primary leaf was pushed out, and the imperfect contact between the sections and the solution as a result of the capillaries becoming closely orientated or crossed when the boats were placed in the constant temperature cabinet.

In order to avoid these difficulties certain modifications were made. To prevent injury to the sections and to simplify the procedure the primary leaf was left inside the coleoptile (15). A tumbler was used to rotate the sections to prevent their orientation in the liquid surface and subsequent curvature. Since it had been reported that aeration of the sections was necessary (30, 32), the bottles in which the sections were grown were equipped with glass tubes which allowed a free exchange of air. The ends of the tubes were covered with a thin layer of silicone stopcock grease to prevent them from becoming filled with liquid. The tumbler with the bottles in the constant temperature cabinet is shown in Figure 8.

The bioassays which were performed using these modifications were greatly improved over the previous ones. Curvature of the sections was essentially eliminated and the standard deviations were reduced. However, occasionally erratic responses were obtained which resulted in nonlinearity. The procedure was then altered such that the sections were

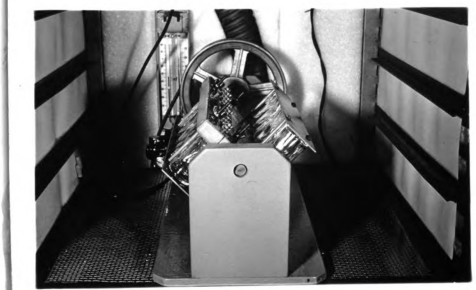

Figure 8. The tumbler and bottles used for rotating Avena coleoptile sections in the constant temperature cabinet

floated on glass distilled water containing 1.5 ppm $MnSo_4.H_2O$ for two hours prior to using (26).

As a result of the last modification, the response of the sections to 2,4-D was increased and much of the variability in growth was reduced. The coefficients of variation of the treatments were greatly reduced and seldom exceeded five percent. As a result of the increased response, a new dilution series of 2,4-D was used in which the most concentrated solution was 1.0 μmoles/l rather than 1.95 μmoles/l as previously used.

The results from the first bioassay using all of the above modifications are shown in Figure 9. The response was essentially linear over the entire range of concentrations of 2,4-D used. The coefficient of variation of the most concentrated solution of 2,4-D was 4.5 percent.

As a result of skill acquired in performing the intricate manipulations of the procedure and the establishment of a strict time schedule, the sensitivity and reliability of the procedure were further increased. Because of the greater sensitivity an additional dilution was added to the series of 2,4-D solutions. The response of the sections was approximately proportional to the log of the concentration of the 2,4-D solutions between 2.6×10^{-2} μmoles/l and 1.0 μmoles/l.

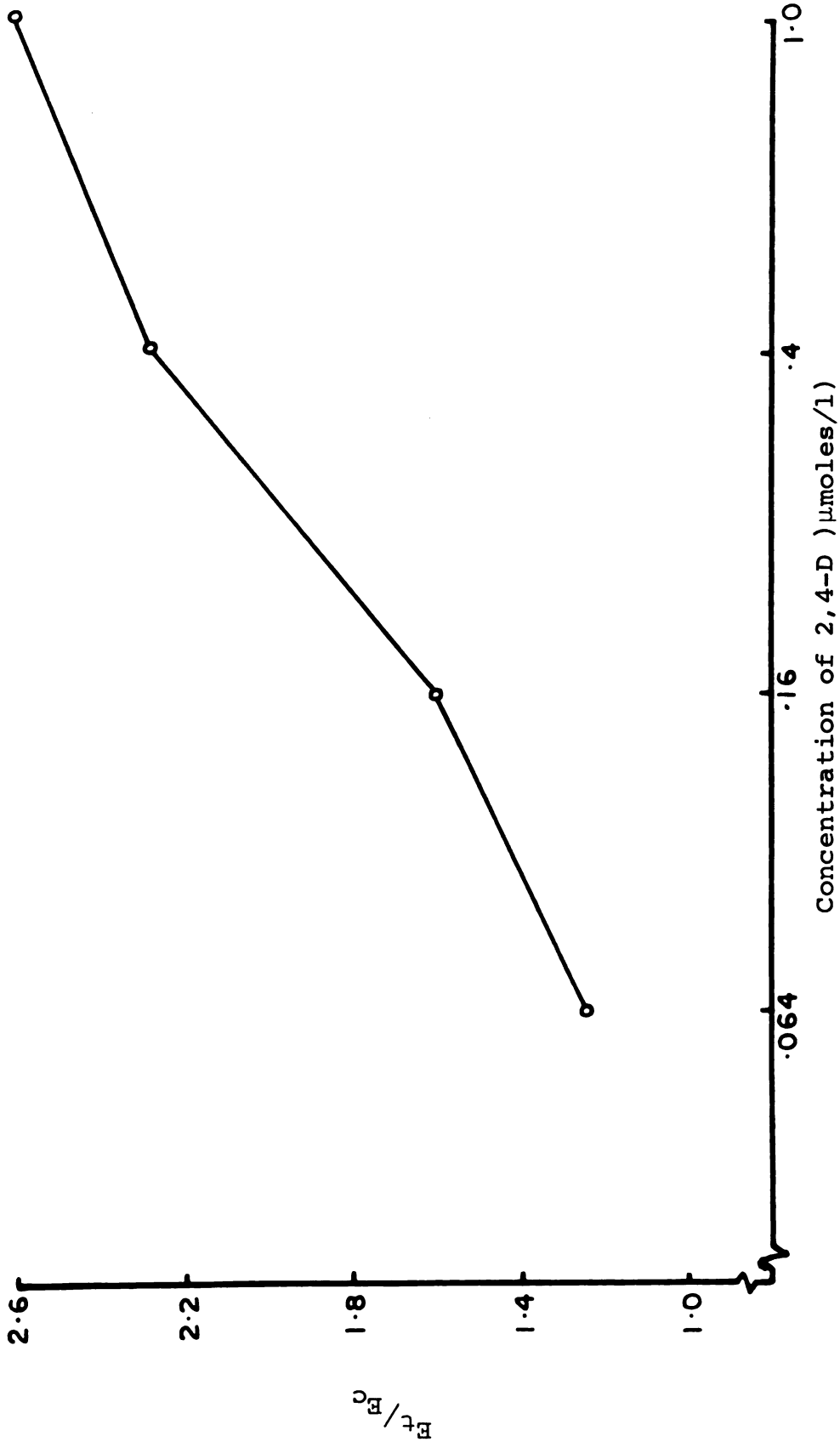

Figure 9. The response (elongation of treated sections divided by elongation of control sections) of Avena coleoptile sections to 2,4-D

In order to allow an adequate margin of error, E_t/E_c values lower than 1.3 were usually not considered when bioassays were performed on extracts of unknown concentration. A value of 1.3 normally corresponded to a concentration of 3.0×10^{-2} μmoles/l (8 μg/l). When an extract having a volume of 5 ml was diluted one to one with the buffer and assayed, a response of 1.3 was equivalent to 3.0×10^{-4} μmoles (0.1 μg) of 2,4-D.

Briefly, the procedure in its final form was as follows: the oats were screened to obtain a uniform size fraction, washed in running water for one hour, surface sterilized in one percent NaOCl for one minute, and placed on paper covered glass strips with the grooved side down and the embryo end projecting over the side. The glass strips were placed in a large glass tray with added distilled water and placed in the dark constant temperature cabinet at 25 degrees centigrade and 90 percent relative humidity. Twenty-four hours later the seed were exposed to four hours of light from two 25 watt Ruby red bulbs which were 30 cm above the germinating tray.

After 80 hours, or when the coleoptiles were 2.0-2.5 cm long, the coleoptiles were removed under a low intensity red light. All subsequent operations were performed with sterilized equipment. One 5 mm section was cut from each

coleoptile 3 mm from the apical end and placed in distilled water. When all sections were cut they were transferred to a dish containing glass distilled water and 1.5 ppm of $MnSO_4 \cdot 4H_2O$. The sections were presoaked in the solution for one hour in total darkness and then for an additional hour in a fresh solution under red light.

At the end of this period the sections were placed in 30 ml bottles containing 5 ml of the solution to be bioassayed and 5 ml of the pH 5.0 phosphate-citrate buffer containing four percent sucrose. The bottles were fitted with ground glass vent tubes and placed on the tumbler which rotated then around the horizontal axis at 20 revolutions per minute. The tumbler was placed in the constant temperature cabinet at the same temperature and humidity as used for growing the coleoptiles.

After 24 hours of rotation, the sections were measured under a binocular microscope equipped with an eyepiece micrometer in which one centimeter equaled 64 grid divisions.

APPENDIX B

THE ETHER EXTRACTION PROCEDURE

The method used for extraction of 2,4-D from Canada thistle leaves was derived from the method given by Hay and Thimann (17). All attempts to use the method exactly as outlined were unsatisfactory because of the low recoveries obtained when known amounts of 2,4-D were added to leaf tissue and extracted. The major difficulty was thought to be adsorption of the 2,4-D on the filter paper and residue in the filter paper.

The method evolved for extraction is as follows: The tissue sample (0.5-1.0 g) was ground for two minutes in a Waring blendor with 15 ml of distilled water, washed into a small beaker with a minimum of water, boiled for one minute, allowed to cool for ten minutes, acidified to pH 1.0 with concentrated sulfuric acid, and transferred to a small centrifuge tube fitted with a rubber stopper covered with aluminum foil and a stopcock to release the pressure when it became excessive. The liquid was extracted with two 10 ml portions of diethyl ether and centrifuged at a low speed for ten minutes.

The ether phase was then pipetted off, leaving the

aqueous portion and tissue residue behind, and transferred to a 60 ml separatory funnel and extracted with two 10 ml aliquots of 0.2 M $NaHCo_3$. The aqueous portion of this separation was acidified to pH 1.0 and re-extracted with two 10 ml aliquots of ether. The ether fraction was evaporated in a hood. Twenty millileters of distilled water were added to the residue and the solution adjusted to pH 5.0 by adding 0.1 M NaOH. The solution was diluted to 25 ml and stored at 5 degrees centigrade until it was bioassayed. When necessary, serial solutions were made of the extract.