THE EFFECT OF PLANT GROWTH REGULATORS ON THE ELECTRON-TRANSPORT IN PLANTS

by

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INTRODUCTION

As the field of weed control has developed and new chemicals have been and are being discovered and synthesized for this use, the necessity of knowing the mode of action of these substances has asserted itself. It has been about ten years since the beginning of this program and various workers in the field have reported their findings.

Both Warburg respirometer and reducing dyes have been used to study the effect of these chemicals on the metabolism of the plant, and cell-free extractions as well as tissue slices have been used in preparing the plant material for study. Studies of endogenous respiration and of the responses to added substrates have given some indication of what may be happening in the plant. This study was undertaken to obtain further information on the effect of herbicides on the enzymes of plants.

I REVIEW OF LITERATURE

There is considerable evidence that plant growth regulators exert their influence on growth in close relation to respiration and energy mechanisms, but the interpretation of experimental results poses many difficulties. Smith (34, pp.78,79) has discussed some of these problems which follow:

1. Are changes in respiratory mechanisms causes or results of the treatment?
2. What basis can we use to compare treated and untreated plants, or wherein has the treatment made the least change in the plant?
3. What differences are there in the amounts and kinds of endogenous substrates in treated and untreated plants?
4. The fraction of respiration actually associated with growth may be small and therefore difficult to detect and measure.

In view of these problems, Smith (34, p.80) makes the following suggestion for future work:

"A more detailed comparison of the glycolytic mechanisms in untreated and treated tissues should be made, the products of glycolysis identified, and the rates measured. The content and utilization of the intermediary carboxylic acids, likewise should be investigated."

Smith (34, p.80) summarized his experimental results thusly:

> "Typical and consistent changes in rates of oxygen consumption, CO_2 evolution, and derived respiratory indices followed (2,4-dichlorophenoxyacetic acid, hereinafter referred to as 2,4-D) treatment. These were correlated with changes in histology and in carbohydrate and nitrogen fractions...The principal result of inhibitor studies was the difference in iodoacetate sensitivity of slices from treated and untreated plants which may indicate differences in the glycolytic phases of respiratory metabolism. Certain differences were also observed in the response of treated and untreated tissues to added intermediary metabolites."

Berger and Avery (2, pp.290-297) studied the cell-free extracts from Avena coleoptile by the Thunberg technique. Naphthaleneacetamide, and indoleacetic, naphthaleneacetic and indolebutyric acids, at low concentrations, had no accelerating affects on the activity of the malic dehydrogenase, while a high concentration of these reagents was inhibitory. Fumarase and alcohol dehydrogenase activities also were not accelerated by indoleacetic and naphthaleneacetic acids.

Berger and Avery (4, p.19) were also able to show dehydrogenase response for iso-citrate, glutamate, citrate, and cis-aconitate. No accelerated activity was observed when synthetic auxins were added to iso-citrate and glutamic dehydrogenase systems in final concentrations of 0.0025 to 1000 milligrams per liter.

At 1000 milligrams per liter, naphthaleneacetic acid inhibits both enzymes, and indoleacetic acid inhibits the glutamic dehydrogenase.

"Fluoride, malonate, sulfanilamide, ethyl carbamate, and iodoacetate, at concentrations of 0.0001 molar to 0.01 molar, do not markedly inhibit crude iso-citric dehydrogenase preparations. Iodoacetate strongly inhibits glutamic and iso-citric dehydrogenases at 0.05 molar." (4, p.18).

Miller and Burris (23, pp.548,549) studied the effect of certain synthetic growth regulators and other chemicals on crude barley juice. They found that benzoic and salicylic acids were almost as effective on ascorbic and glycolic acid oxidases as were the plant growth substances.

Wagenknecht, et al., (41, p.554) carried out a similar experiment on dicotyledons. Some thirty organic substrates were used. Bean leaf and root sap were the sources of enzymes. Oxygen uptake of bean-leaf juices was stimulated upon addition of lactic, glycolic, and ascorbic acids. Inhibition was found at the higher chemical concentrations. The growth substances had no appreciable influence on the uptake of oxygen without added substrate. This work was done in the Warburg respirometer.

Gall (15, pp.319-322) cultured thin slices of bean stem tissue in nutrient solution. The 2,4-D-treated slices showed extensive proliferation after fifteen days as compared to the control which showed very little change. Also, the "areas of starch degradation in the medium containing 2,4-D were clearer and more extensive than in the medium without 2,4-D. Reducing activity of the endodermis, phloem, cambium, and xylem parenchyma was demonstrated by the use of TZ." (15, p.323).

Teubner and Murneek (37, p.39-41) studied the effect of p-chlorophenoxyacetic acid and 3-indoleacetic acid on tomato fruit. They applied the chemicals to the flower clusters and used triphenyltetrazolium chloride (TZ) to test the enzyme activity of the fruit. They found that treated fruits gave increased malic and fumaric dehydrogenase activity and slightly reduced succinic and glutamic dehydrogenase activity.

The author has observed that succinic dehydrogenase activity is easier to demonstrate in the stem and root tissues, whereas malic dehydrogenase appears to be more active in the leaves.

It might be noted also that, in general, the stimulation of respiration by plant growth substances has been found with plant material in vivo, and inhibition of respiration is usually associated with in vitro work.

Some chemicals which will give inhibition at higher rates, will stimulate respiration at very low rates, even with the same system. (20, pp.421-425). Plants also vary in their response, peas requiring very much smaller amounts to give the same response as representative monocotyledons. (20, p.426).

Glock and Jensen (16, pp.272-277) succeeded in showing succinic dehydrogenase activity in Avena coleoptiles by blending their homogenate in vacuo. They also developed a colorimetric method using 2,2-(p-diphenylene)bis(3,5-diphenyl tetrazolium chloride), which they extracted with water-saturated n-butanol after it was reduced. The dye obeyed Beer's law from 20 to 85 per cent transmission. Glock and Jensen found a linear relationship between the enzyme concentration and the dye reduction, and also between the dehydrogenase activity and time of reaction. They used a four-hour reaction period and anaerobic conditions.

Hagen, Clagett, and Hagen (18, p.117) found oxygen uptake to be affected by 2,4-D, but no inhibition of polyphenol oxidase, alpha-hydroxy acid oxidase, or catalase was observed. Their study concerned the inhibition of castor bean lipase by 2,4-D. The sodium salt was used.

Harris (18, pp.116,117), in studying lipase, has shown that sodium 2,4-dichlorophenoxy acetate acts as a protein precipitant at relatively low concentrations.

Since the 2,4-D would be expected to combine with other proteins as well as the lipase, the effective concentration for inactivation would probably be considerably lower. From this, the conclusion might be drawn that the inactivation comes from the combination of the 2,4-D with the enzyme.

The amine and salts of 2,4-D could conceivably be converted to the free acid by simple dissociation, but this would not occur with the ester. So Hagen and coworkers (18, p.117) decided to study the castor bean lipase as an esterase on the 2,4-D ester. The inhibition of lipase by the ester of 2,4-D approached the value given by the sodium salt of 2,4-D at the end of 21 hours. It appears that the butyl ester is not effective as an inhibitor until the hydrolysis to the 2,4-D acid occurs. This might indicate the action of lipase as an esterase on 2,4-D. In this work they showed that the activity of castor bean lipase is effectively inhibited by very low concentrations of 2,4-D.

Neely, et al., (25, p.118) studied the effect of 2,4-D acid on the alpha and beta amylase activity in the stems and leaves of red kidney bean plants. They refer to work that has been reported which showed a reduction of carbohydrates and an accumulation of nitrogen in plants treated with 2,4-D. Red kidney beans were planted in 4-inch pots, two to a pot. Four replications

of 100 each were used. Application of 2,4-D was made at the rate of "one drop (0.05 milliliter) of a 1000 parts per million solution to the base of the blade of one of the primary leaves." The treatment was made when the first trifoliate leaf was expanding. The plants were harvested six days later. They were air-dried in the dark and separated, the hypocotyl, first internode, and leaf petioles being grouped together as stem tissue. Enzyme activity measurements were made separately on the dried tissue of stems and leaves.

They found that the alpha-amylase activity of the stems of the treated plants was about 15 per cent of the controls. There was no alpha-amylase activity in the leaves of either the treated or untreated plants. The beta-amylase activity of the stems of the treated plants was about 65 per cent of that of the controls. The beta-amylase activity of the leaves appeared not to be affected by 2,4-D.

Peroxidase activity was reported by Irma Felber (12, p.557) to be enhanced by 2,4-D. The increased activity was indicated by the greater speed and intensity of the color reaction upon introduction into the tissue of guaiacol and hydrogen peroxide.

Neely, et al., (26, pp.525-527) also studied the effect of 2,4-D on the invertase, phosporylase, and pectin methoxylase of the red kidney bean plant.

The procedure was the same as that mentioned previously. The plants were treated about 10 days after planting when the first trifoliate leaves were expanding, and were harvested six days later. The material, after being dried at 30° C, was separated into leaf, stem, and root tissue. It was then ground to pass through a 60 mesh sieve, and stored in a jar at room temperature until needed.

No invertase activity was detected. They concluded that the hydrolysis of sucrose to hexose sugars by invertase is absent in the young plant. In the stem tissue of treated plants pectin methoxylase activity was almost 50 per cent as great as the corresponding activity of the control plants, and in the leaves of the treated plants the pectin methoxylase activity was about 35 per cent of that of the control plants. The phosphorylase activity was about 65 per cent in the treated leaves and about 15 per cent in the treated stems as compared with the corresponding phosphorylase activity of the leaves and stems, respectively, of the control plants. There was no indication of the presence of a phosphatase acting on D glucose-1-phosphate. It is not surprising that some of the enzymes sought for gave no response after the way the plant material was treated. Probably the best conclusion that can be reached from these

results is that those enzymes which did show activity are quite stable enzymes.

It should be remembered that results may vary with the time elapsed after treatment, and in view of this, one of the best experiments was carried out at the University of British Columbia by Wort (44, pp.135-139). It gives a picture of the effect of 2,4-D on the enzymes over a period of time.

Wort, et al., (43, pp.175-184) carried out in vivo studies of the effects of 2,4-D on the apparent activity of a number of enzymes in the aerial portions of Marquis wheat plants. They sprayed their wheat and harvested nine hours, one day, two days, four days, and eight days after treatment. They found an increase of activity over the control plants of beta-amylase, phosphorylase, catalase and peroxidase, and a slight decrease for phosphatase. The curves all reached a maximum at one to two days, and by four days had reached normal again or were approaching it, except for amylase which was continuing to fall. The 2,4-D was applied at 5 parts per million and 500 parts per million. If the experiment had been carried beyond eight days, different relationships might have held, for there were indications of a continued fall in peroxidase activity after the fourth day and a rise in catalase activity. The

return to normal after a few days is to be expected by this light a treatment on wheat.

Starch and sugar changes in wheat have not been followed after treatment, but in most species, increases in sugar concentration above control levels in the first few days is followed by decreases to quite low levels. The increase in sugar probably results from the increased metabolism of the starch. As the starch supply is depleted, the sugar necessarily drops to a low level.

In the words of Wort, et al., (44, pp.136-138):

> "Effects of 2,4-D added to the filtrate of untreated plants in the same concentration as the concentration computed for the filtrates of treated plants, assuming that all the 2,4-D sprayed on the plants was absorbed, were determined and found to lack significance." (44, p.136).

> "An examination of the curves of values of T/C (treated over control) for phosphorylase, beta-amylase, peroxidase and catalase activity in wheat given non-lethal concentrations of 2,4-D reveals trends very similar to those for the total sugar, starch-dextrin, available carbohydrates and total nitrogen content of stems of buckwheat sprayed with lethal doses and those for total sugar and starch-dextrin in buckwheat which received non-lethal doses of the chemical. These show an increase over control levels soon after spraying, followed by a return to normal or below normal several days after treatment. Whether the influence of 2,4-D on the activity of enzymes is brought about by its direct action on the prosthetic group or the protein portion of the enzyme or indirectly on the availability of amino acids and vitamins for the elaboration of the enzymes by the plant has yet to be determined.

> "In the experiments reported above, spraying Marquis wheat plants with 5 or 500 parts per million of 2,4-D resulted in increases in the

activity of phosphorylase, beta-amylase, catalase, and peroxidase in the aerial portions of the plant. The activity of phosphatase was decreased by the treatment. The deviations from the control levels reached maxima by the first or second day after treatments." (44, p.138).

"The very considerable increase in phosphorylase activity occurring by the first day after spraying may be responsible for the early high starch content of treated plants. As the phosphorylase activity fell, beta-amylase activity rose, causing depletion of starch and the rise in sugar content usually found soon after 2,4-D treatment. The high sugar content is available for increased energy changes shown to occur in oats treated with low 2,4-D concentration. The higher activity of catalase and peroxidase may well indicate higher respiratory activity.

"Of the enzymes tested, only phosphatase showed decreased activity. This might indicate that the starch-sugar change is due primarily to the action of amylase since the decreased activity of phosphatase would block the sequence

Starch $\xrightarrow{\text{phosphorylase}}$ Glucose-1-phosphate

$\xrightarrow{\text{phosphoglucomutase}}$ Glucose-6-phosphate

$\xrightarrow{\text{phosphatase}}$ Glucose. At the same time, the condition would favor the sequence Glucose-6-phosphate $\longrightarrow$ Pyruvate and the consequent increased respiratory rate." (43, p.181).

This appears to be the most indicative work to date, showing the effect of growth substances on enzymes over a period of time which gives a much broader picture of the processes going on in the plant.

Work has been done on both susceptible and resistant plants. Wheat is resistant and little is known about the effects of 2,4-D on such species. The hydrolyzing power of wheat germ lipase is decreased by 0.009 molar 2,4-D.

Related to the study of the effect of these plant growth substances on the plant enzymes is the work of Day (10, p.151,152), who studied the absorption and translocation of 2,4-D by bean plants with respect to time. He found that 2,4-D was absorbed at approximately 30 microns per hour, but once inside the phloem, was translocated from 10 to 100 centimeters per hour--5,000 to 25,000 times faster. After application to a leaf, curvature begins in about two hours, and maximum absorption by the leaves was obtained in the first four and one-half hours after treatment under the conditions of those tests. Crafts (9, pp.51-55) has also studied the absorption and translocation of 2,4-D.

In the light of the available information it was desired to find out, if possible, some of the effects of these chemicals on the dehydrogenases of the plant. Weed killers are being widely used and a better knowledge of their mode of action might give us a clearer understanding of plant physiology and biochemistry as well as a better approach to their practical application.

The literature shows many respiratory enzymes to be present in plants. Of the available methods, triphenyl tetrazolium chloride was chosen as the electron acceptor, and thus as an indicator of respiratory activity in this present work.

2,3,5-Triphenyl tetrazolium chloride, commonly known as tetrazolium salt, TTC, or TZ, was first prepared in 1894 by von Peckman and Runge. The salt is a crystalline white to pale-yellow substance that darkens on exposure to light. It is quite water-soluble and melts with decomposition at about 245° C.

A number of substituted tetrazolium salts were synthesized in 1941 by Kuhn and Jerchel (33, p.751), using an improved method. They noticed that a dilute solution of their salts stained yeast, garden cress, and bacteria. They found, also, that the reduction of the salts to red compounds which dyed the plants was not due to the presence of glutathione, ascorbic acid, or cysteine, for the latter substances reduced the salts only above a pH of 9, whereas the observed reductions took place in neutral solutions.

Lakon (33, p.751), at about the same time, substituted TZ for the toxic compound sodium selenite in his test for the germinating ability of seeds. He was able to show that it was possible to predict the germinability of cereal grains by observation of the embryo parts in which had been deposited the insoluble red formazan. Other workers (33, pp.751,752) since then have applied this method with varying but usually satisfactory results.

Mattson, Jensen, and Dutcher (33, p.752) confirmed the work of Lakon and found that many other plant materials reduce TZ, including the fleshy parts of apples, oranges, and grapes; the gill area of mushrooms; carrot roots; white and sweet potatoes; young leaves; the stigmas and ovaries of certain pollinated flowers; bull spermatozoa; and the blastoderm of hen's eggs. This reduction is not due to reducing sugars because they cause reduction only above pH 11, whereas the above tissues were active at physiological pH's. TZ has the peculiar advantage of being one of the comparatively few organic compounds that is colored in the reduced state.

Enzyme systems were quite evidently the cause of this reduction because plant and animal tissues heated to 82° C or above, lost their ability to reduce TZ. Jerchel & Mohle (33, p.752) showed that the apparent redox potential of TZ was about -0.08v. This suggests dehydrogenase systems requiring coenzymes I and/or II. They found that glucose dehydrogenase with coenzyme I in the presence of its substrate reduced the salt at pH 6.6. Other experiments indicated many other enzymes with reducing ability.

There is a conflict between reports of activity in TZ-treated and radioactive-TZ-treated tissues of malignant tumors as compared with normal tissues.

This question is still being investigated. A blue dye has been synthesized which is more readily detected in animal tissue than red dyes.

Among other applications, Gall (15, p.319) used TZ to estimate the reducing activity of bean stem slices cultured in 2,4-D. Kun and Abood (21, pp.144-146) used it in a colorimetric estimation of succinic dehydrogenase, and Black and Kleiner (33, p.753) used it in the measurement of respiration of tissue slices. Many other applications have been reported and Raggio and de Raggio (27, pp.35-39) have given a review in Spanish of the uses and action of TZ salt.

Mattson and Jensen (33, p.753) reported the use of TZ in the quantitative colorimetric determination of reducing sugars at 490 millimicrons, and Trevelyan, Proctor, and Harrison (33, p.753) have used it in detecting these sugars on paper chromatograms. Such determinations are carried out under basic conditions--pH 11--in contrast to the relatively neutral conditions necessary for dehydrogenase activity.

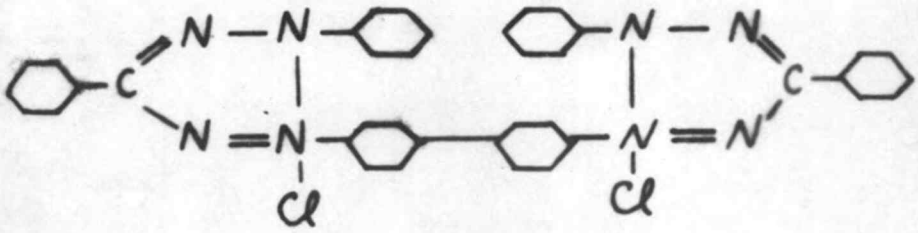
Although it is apparent that TZ is valuable to workers in many fields, it has limitations among which are its redox potential and its nonspecificity, and work has been directed towards preparation of new derivatives with similar but possibly more desirable properties.

Neotetrazolium salt, 2,2-(p-diphenylene)bis(3,5-diphenyl tetrazolium chloride) (I) is now available, its dimethoxy derivative (II) known as blue tetrazolium, and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (III). Neotetrazolium gives a deep-purple to black color on reduction in tissues and is thus more easily detected. It has been investigated and uses found for it. Blue TZ forms a deep blue pigment on reduction. It has been used in animal tissue, being well adapted because of its color on reduction, but is much more toxic to mice than TZ itself. (30, p.115).

2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyl TZ reported recently by Atkinson, Melvin, and Fox, (1, pp.385-387) with two other iododerivatives, is far less photosensitive than TZ itself and is much faster staining, with less diffusion into surrounding tissue.

Because of the rapidity of staining and the reduced photosensitivity, 2-(p-iodophenyl(-3-)p-nitrophenyl)-5-phenyl TZ (hereinafter referred to as IodoTZ) was chosen for this present work.

TZ is sensitive to heating but not to freezing. Its activity is virtually stopped at pH 6. It is reported by some (7, p.41; 13, p.169; 30, pp.113-121; 40, pp.13-15) to be more active under anaerobic conditions, presumably because of either inactivation of the components of the



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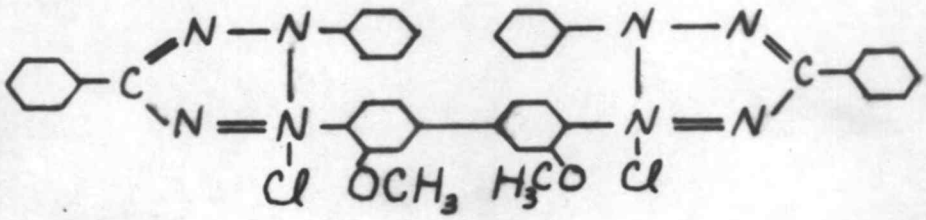

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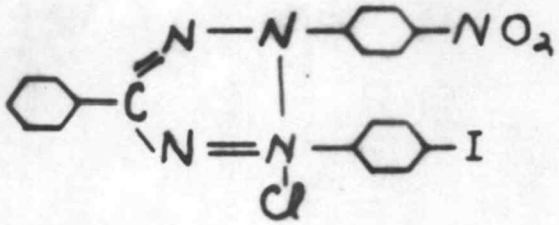
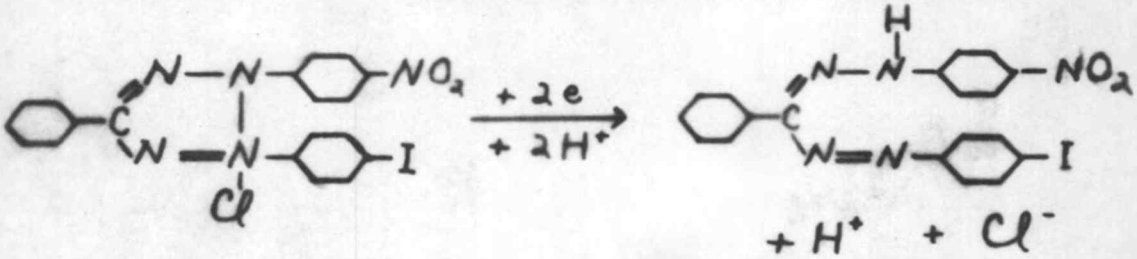


III

The reduction is thought to take place as follows:



(Colorless form) (Colored form)

in a system of the general nature:

Dehydrogenase ---> Phosphopyridine nucleotide --->

Flavoprotein ---> TZ

system by oxygen or competition by the aerobic transport system. TZ reduces to a water-insoluble dye which can be extracted by organic solvents. This lends itself very well to work with enzyme systems. Partly because of lack of specificity of known enzyme inhibitors, Roberts (28, p.693) concluded that TZ is reduced by a system of enzymes rather than by any one dehydrogenase.

II MATERIALS AND METHODS

One of the first considerations in working with enzyme systems is to prove the presence of such a system or systems. Therefore the effect of homogenate concentration was determined. Sixty grams of 17-day-old etiolated bean stem tissue were homogenized sixty seconds in a Waring blendor in thirteen milliliters of 0.25 molar phosphate buffer of pH 7.4, and the resulting brei was pressed through cheesecloth. Tubes were set up containing the enzyme preparation from zero to 1.8 milliliters in increments of two-tenths of a milliliter. Each tube contained 0.5 milliliter of 0.5 per cent IodoTZ and 0.2 milliliter of sodium succinate if substrate was used. The total volume was 2.7 milliliters and determinations were made with and without substrate. The reaction time was sixty minutes. There was a linear relationship between the amount of enzyme and the reduction of IodoTZ. There is apparently a slight dilution effect at the lower concentrations. (Fig.1).

To study the rate of enzymatic reduction of IodoTZ, four flasks of enzyme preparation were prepared with IodoTZ--two with succinate and two without. Each flask contained five milliliters of one per cent IodoTZ,

3.5 milliliters of distilled water or succinate, and 25 milliliters of enzyme preparation. The source of enzyme preparation was 190 grams of plant material which had been homogenized in 9.5 milliliters of one molar phosphate buffer of pH 7.4. Phosphate buffer of pH 7.4 was used throughout this work, because Lardy (22, p.117) gives pH 7.4 as optimum for succinic dehydrogenase.

Three milliliter aliquots were drawn out and pipetted in duplicate into six milliliters of 1,4-dioxane at fifteen minute intervals. Time was plotted against micrograms of IodoTZ reduced. Dehydrogenase activity with added succinate as substrate was linear with time. (Fig. 2). IodoTZ reduction can be used as an index of dehydrogenase activity because the quantity of IodoTZ reduced is linearly related to the concentration of the enzyme preparation.

Two milliliters of enzyme preparation and 0.5 milliliter of 0.5 per cent IodoTZ with 0.2 milliliter of 0.1 molar substrate were found to give a reading within the range of the spectrophotometer when incubated for thirty minutes. This gave a total volume of 2.7 milliliters. To stop the reaction, 7.4 milliliters of dioxane were added with a ten milliliter syringe and the reaction tubes were centrifuged at about 1000 x G

Figure 1
Amount of formazan produced by varying concentrations of enzyme preparation in 60 minutes. Total volume, 2.7 mls

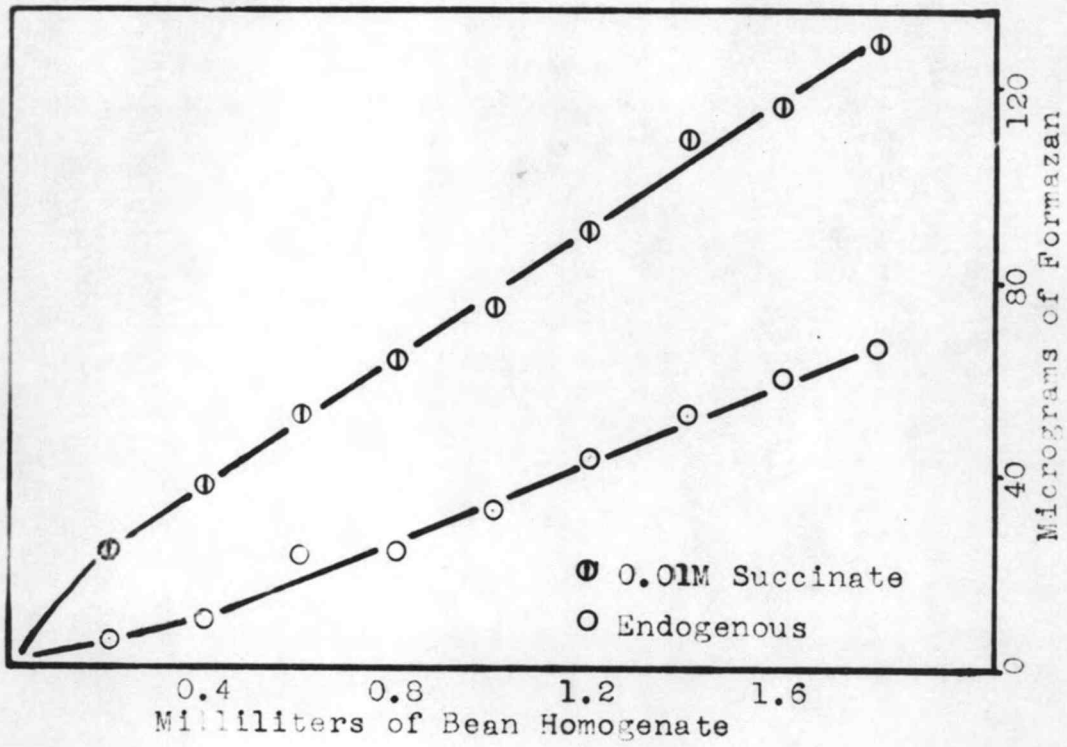
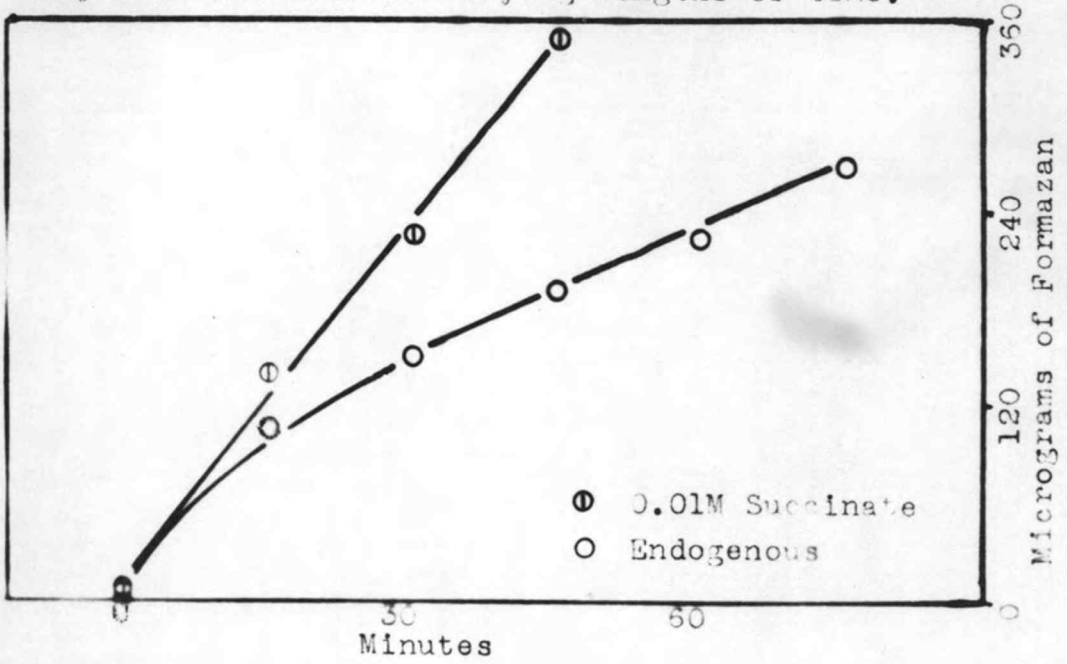


Figure 2
Amount of formazan produced by 2.5 milliliters of enzyme preparation in varying lengths of time.



to give a clear solution. This quantity gave sufficient to be read in the Coleman spectrophotometer.

To prepare a standard curve, known amounts of the dye were reduced with a few crystals of sodium hydrosulfite and serially diluted. An excess of reducing agent was avoided to prevent cloudiness of the solvent from occurring. The solutions were read at 500 millimicrons and the transmission was found to be a linear function of the quantity of formazan present in the range from twenty to ninety-five per cent transmission.

Green plants may also be used for enzyme preparations. The development of this procedure will be discussed later, but the method utilizes the characteristic which mineral oil has of differentially extracting formazan in the presence of chlorophyll.

Plastic centrifuge tubes of fifty milliliter size were chosen for reaction tubes, three milliliters of reaction mixture were added, and ten milliliters of mineral oil were pipetted in last. An automatic burette with the mineral oil under slight air pressure facilitates the addition of the solvent to the reaction tube. At the end of the reaction time fourteen milliliters of water were added, the tubes were stoppered with corks, and were shaken on a mechanical shaker for fifteen minutes. If they were not diluted, the small volume

would not mix well. The corks were then removed and the tubes were centrifuged for fifteen minutes at 1500 x G. The mineral oil was decanted and read in the spectrophotometer. Very small amounts of plant pigments, if any, were extracted by the mineral oil. Care must be taken not to have more formazan present than will be extracted by the mineral oil in a reasonable length of time.

The beans and corn used to prepare leaf homogenates were grown in the green house in mineral aggregate with NPK fertilizer. The beans were used when the trifoliates were unfolded. The treated plants received one drop each of 250 parts per million isopropyl amine salt of 2,4-D on a trifoliate leaf. They were harvested at four hours, one day, two days, four days, and eight days after treatment, the treated leaf was clipped off and discarded, and the plants were used immediately.

For the etiolated plants, Pencil Pod Black Wax beans were soaked ten to twenty minutes in a dilute Clorox solution, washed under running water, and germinated for about a day in a germinator. They were then planted in trays of vermiculite and grown in a dark cabinet at 24° to 28° Centigrade. They were watered once with a nutrient solution. For <u>in vitro</u> studies, they were

usually harvested between ten days and two weeks of age--when the seedlings were six to ten inches tall. The ClIPC and CMU treated plants received no nutrient solution.

The etiolated plants were treated when they were about four inches tall by placing them in a tray containing 500 milliliters of one per cent glucose and 25 parts per million of 2,4-D or 200 parts per million of ClIPC or CMU. The vermiculate of each tray, which contained approximately one hundred plants, absorbed about 460 milliliters of the solution through holes in the bottom of the tray in fifteen minutes.

The 2,4-D-treated plants were harvested at four hours, one day, two days, and four days. The ClIPC-treated plants were harvested at one day, three days, and six days. Those treated with CMU were harvested at six days. They were treated with CMU when only about an inch high.

In preparing a homogenate, the seedlings were broken off near the surface of the vermiculite, the cotyledons and leaf buds removed, and the stems broken or cut into one- to two-centimeter lengths and homogenized in a Waring blendor for sixty seconds at room temperature. For large quantities--100 to 200 grams--five per cent of their weight of one molar phosphate buffer of pH 7.4 was used. For quantities less than forty or fifty grams,

one-fifth molar buffer equivalent to one-third of the weight of the plant material was added. The homogenate was immediately squeezed through eight thicknesses of cheesecloth and used.

The tubes used were six-inch rimless test tubes and were strong enough to use in the centrifuge. Two milliliters of homogenate were used in each tube, with 0.5 milliliter of 0.5 per cent IodoTZ, and 0.2 milliliter of 0.1 molar substrate--if substrate was used. The tubes were brought to equal volume with distilled water and incubated at room temperature for thirty minutes. Unless otherwise noted, this procedure was followed hereinafter.

III RESULTS AND DISCUSSION

Green plants are the natural source of material for work with plant growth substances. But in adapting the use of TZ to work with green plants, it was necessary to find a method of extracting the reduced dye from the chlorophyll ever present in normal plant material. The work recorded in the literature using TZ has been done with material containing no chlorophyll, such as germinated seedlings. There being no chlorophyll present, only a simple solvent extraction of the formazan was necessary. In searching for a solvent which would differentially extract formazan in the presence of chlorophyll, forty colorless, water-immiscible organic solvents were tried, including those in Table 1. The evaluations were made by visual examination.

Mineral oil was the only promising solvent and it gave a relatively stable emulsion when shaken with water. This difficulty was overcome with centrifugation. Mineral oil apparently is not toxic to enzymes, so the extraction was carried out as rapidly as possible. An alternative is to lower the pH of the reaction mixture below 6 with a predetermined amount of acid. This will vary with the

TABLE I

Some of the solvents tried for extracting formazan in the presence of chlorophyll.

Solvents	Chlorophyll Extracted?
Xylene	Yes
Toluene	Yes
Cyclohexane	Yes
Iso-octane	Yes
Iso-heptane	Yes
Benzene	Yes
B,B'-Dichloroethyl ether	Yes
Chloroform	Yes
Socal Solvent No. 2	Yes
Ethylene dichloride	Yes
Orthodichlorobenzene	Yes
Tetrachloroethylene	Yes
Velsicol AR 60	Yes
Cyclohexanone	Yes
Iso-phorone	Yes
1,4-Dioxane	Yes
Petroleum ether	Some
Mineral oil	No

buffer strength and the plant material. If the solution is too strongly acidified, difficulty may be encountered with flocculence in the mineral oil fraction. Heating to stop the reaction allows some plant pigments to be extracted by the mineral oil. Plastic centrifuge tubes of fifty milliliter size were used to eliminate unnecessary transfers. Other organic solvents should not be used in plastic containers, however.

In determining the optimum buffer pH, runs were made with phosphate buffers of pH 6.0, 7.0, 7.4 and 8.0 at a

final buffer concentration of approximately one-fifth molar. (Fig. 3). Although the endogenous activity was higher at pH 8.0, the activity over the endogenous with added substrate was greater at pH 7.4. Succinate, malate, fumarate, and glutamate were used as their potassium salts. It was also found in a study of buffer concentration in the range of zero to one-tenth molar that the higher the buffer concentration, the greater the dye reduction. (Fig. 4). The general procedure has been to use five-hundredths molar buffer.

When green plants were used, the top part of the plant was separated from the lower stem and the roots and they were used separately. Of the four substrates used, malate elicited the greatest response from the dehydrogenases of the leaf homogenate. (Fig. 5). The enzymes responded somewhat to glutamate and succinate. Root homogenate dehydrogenases responded to succinate and also slightly to glutamate and malate. The dehydrogenases from neither leaves nor roots gave much response to fumarate. This appears to indicate a very limited amount of fumarase. According to the information shown in Figure 3, the enzyme preparation gave the highest response to fumarate at pH 7.4, but it still may not be the optimum for that enzyme.

Figure 3
Effect of buffer pH on dehydrogenase activity of bean leaf homogenate.

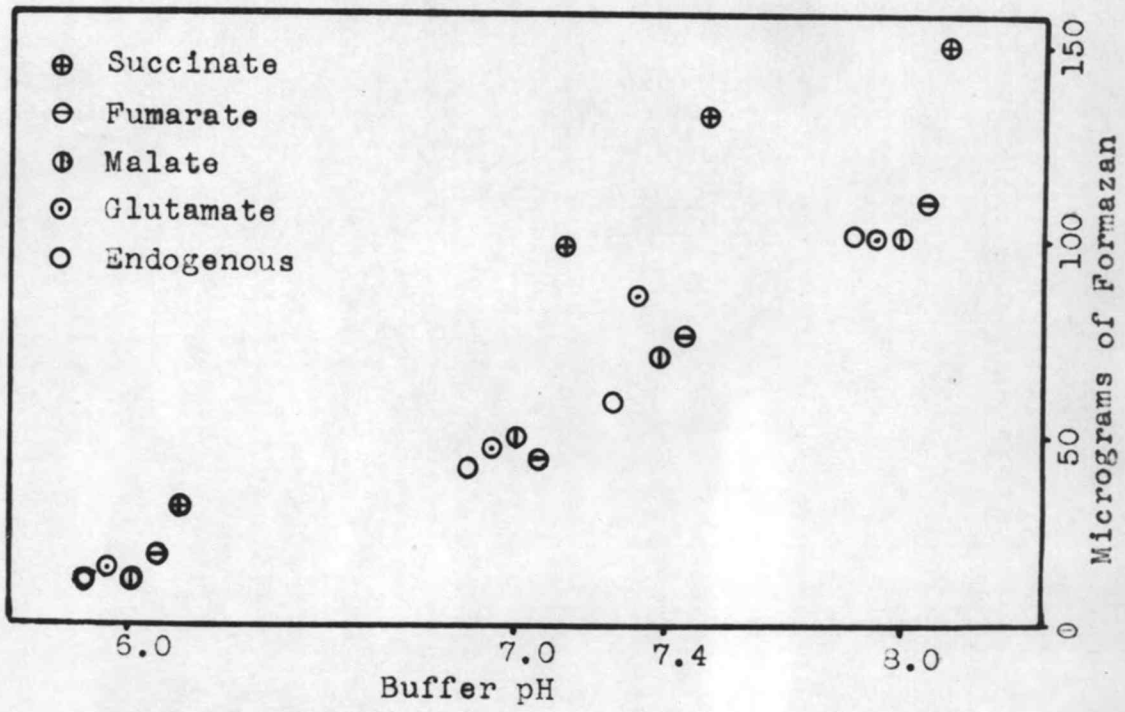


Figure 4
Effect of buffer concentration on dehydrogenase activity of bean leaf homogenate.

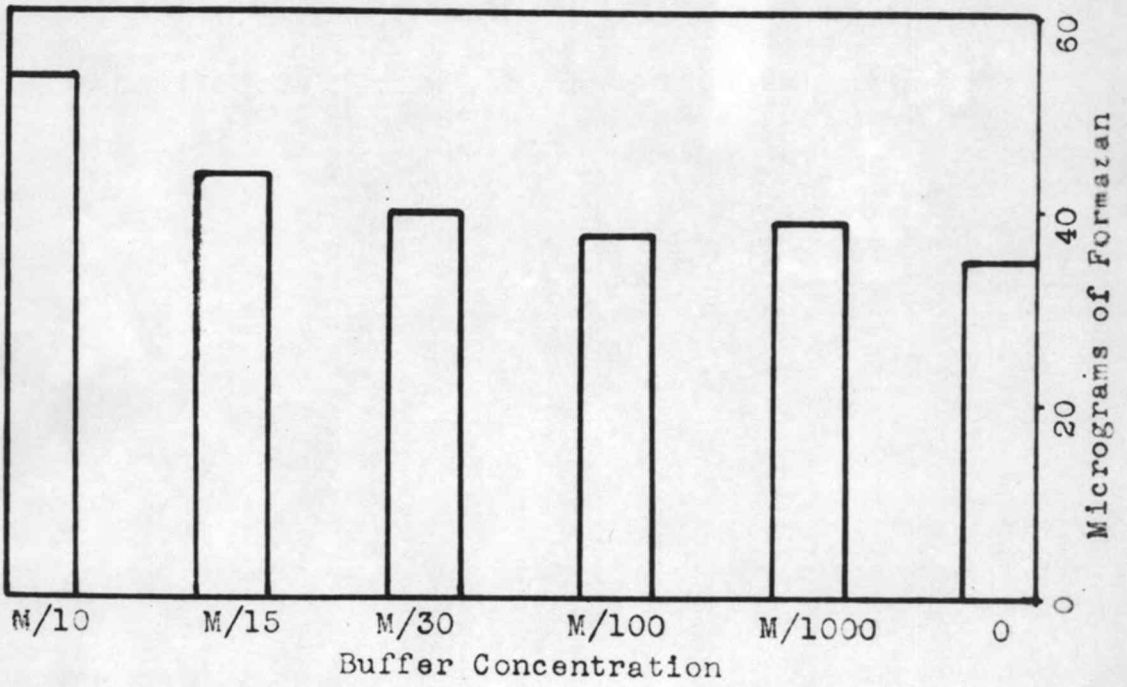


Figure 5

Response to substrates of dehydrogenases of leaves and of roots of bean in 100 minutes *in vitro*.

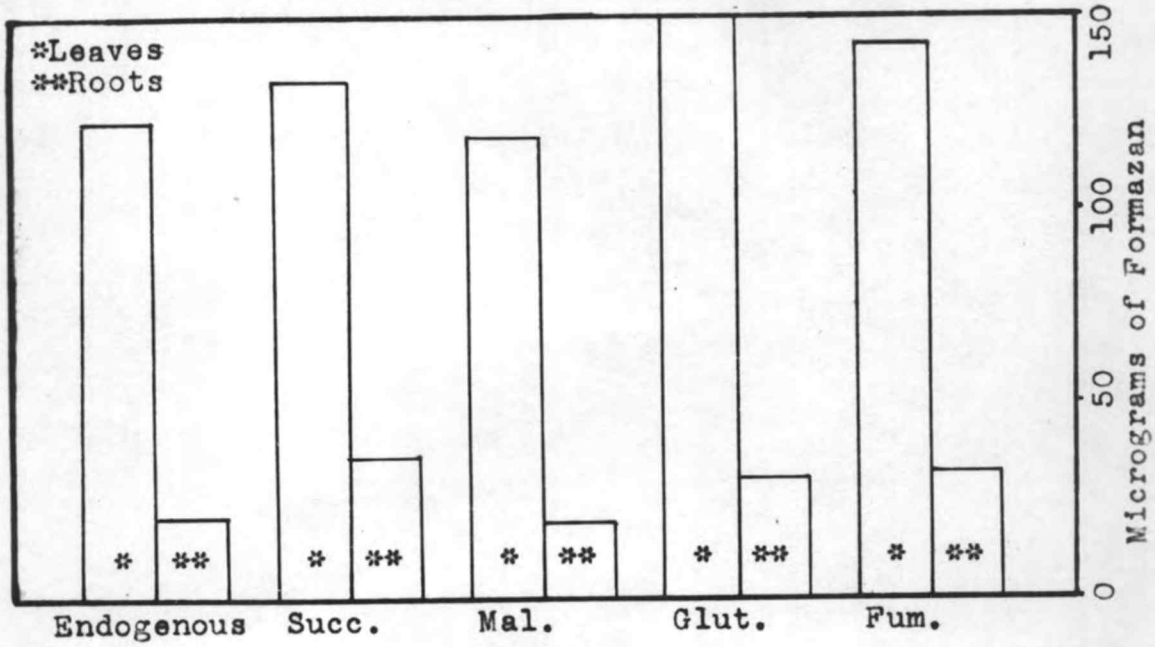
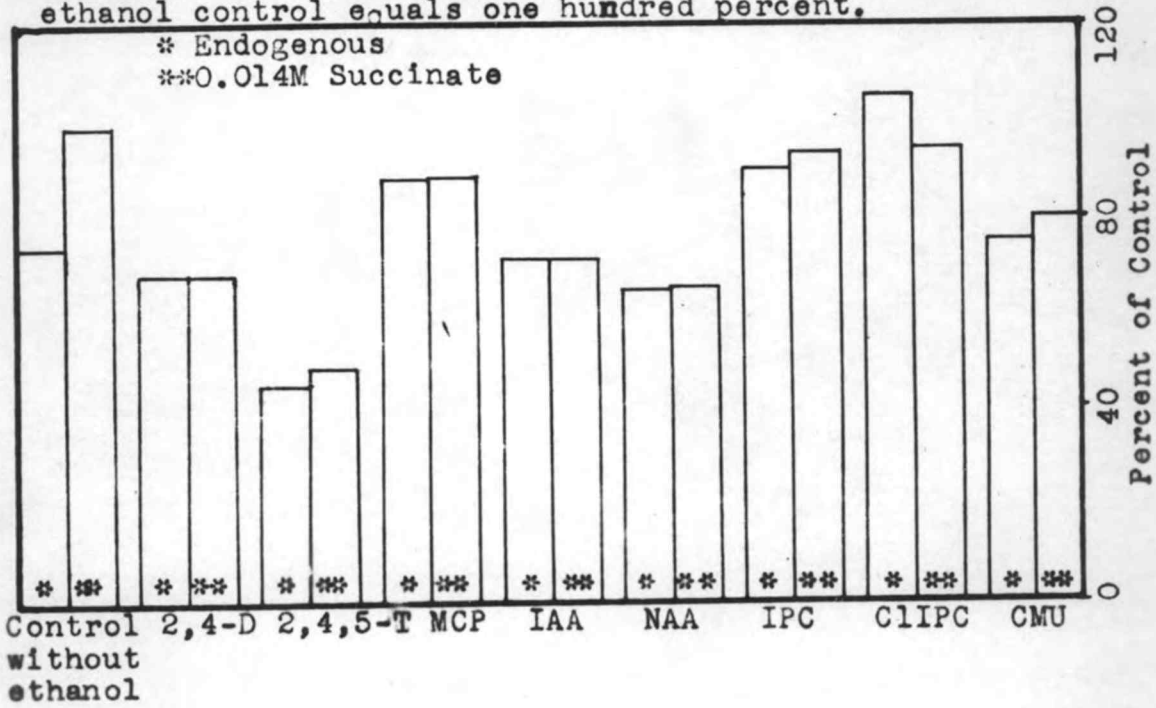


Figure 6

The relative effect of some plant growth substances and other chemicals on the dehydrogenase activity of etiolated bean tissue homogenate. Two milliliters of homogenate with one thousand parts per million of chemical and ten percent ethanol. Ten percent ethanol control equals one hundred percent.



In comparing homogenation at room temperature with homogenation at 10° C, it was found that there was only a slight reduction in activity of the latter, probably due to the short time necessary for the homogenate to come to room temperature. This would bear further investigation.

Comparison was also made between homogenation and grinding in a mortar. Ten milliliters of tissue extract from that ground in the mortar weighed 0.30 grams after being dried, and ten milliliters of homogenate weighed 0.32 grams dried. For comparison of methods of enzyme preparation, one milliliter of enzyme preparation, 0.25 milliliter of IodoTZ (1%), and 0.2 milliliter of substrate were used. The endogenous dehydrogenase activity appeared to be about the same from the two preparations, (Table 2), but the response to substrate is greater in the homogenized material for all the substrates used. This may have been due to better separation of the enzymes from the cellular material, but that does not explain why the endogenous activities were the same.

In searching for a method of treating etiolated seedlings, soaking the seeds in a dilute solution of 2,4-D was tried. Beans were soaked for twenty-four hours in solutions of 2,4-D from one part per million, the concentration doubled each time up to 256 parts per

million. The roots were measured at nine days old and it was found that the concentration of 2,4-D was linearly related to the reciprocal of the root length. (Table 3).

Some of these roots were tested andfound to have a higher dehydrogenase activity than their controls. (Table 2). This difference might be attributed partly to the smaller plant size and therefore possibly more concentrated enzyme systems. The treating of seeds at time of germination was abandoned because of the difficulty of getting material of sufficient size and age with which to work. This method of treatment may also contribute to the greater differences between treated and control plants.

When using young plant material, the cotyledons should be removed because they contain sufficient substrate to mask a desired substrate response.

The dry weight of treated and control homogenates was practically the same. Two and one-half milliliters of control homogenate weighed 75 milligrams after drying in a forced draft oven for 13 hours at 90° C, whereas the same amount of homogenate from bean seedlings treated with 2,4-D four days before weighed 74 milligrams.

One of the common methods used when working with TZ is to extract the formazan from the water-acetone or water-dioxane mixture with xylene. It was found that

TABLE II

Micrograms of IodoTZ reduced by one milliliter of bean homogenate in twenty minutes.

Substrate	*Method of Enz. Prep.		**2,4-D-soaked Beans	
	Mortar	Blendor	Treated	Control
Endogenous	72	69	95	58
Succinate	104	144	180	110
Malate	74	118	93	57
Glutamate	102	121	125	68
Citrate	82	134	127	70

*Thirty grams of etiolated bean stem tissue ground or homogenized 60 seconds in 18 milliliters of one-fifth molar buffer.

**Fifteen grams of nine-day-old roots from beans which had been soaked in 2,4-D before germination were homogenized 15 seconds in 35 milliliters of one-fifth molar buffer.

TABLE III

Root length in inches of nine-day-old beans soaked twenty-four hours in 2,4-D before germination. 2,4-D concentration in parts per million. Average of twenty roots per sample.

Inches	2,4-D
0.010	256
0.020	128
0.055	64
0.088	32
0.141	16
0.218	8
0.281	4
0.425	2
0.625	1
0.950	0

extraction was not necessary, but that the solution could be cleared up with concommitant precipitation of the denatured plant material by centrifugation. The problem then faced was the increase in color of the formazan in the dioxane with time. Tertiary butanol, butyl cellosolve, propylene glycol, triethylene glycol, and glycerol were tried. They reacted similarly and with poorer results. The cause of the variation was not determined. Whether the formazan was broken down in the solvents, or whether electrons were donated by the enol form of these solvents is only speculation. To minimize this difficulty, the time of manipulation should be kept at a minimum, ten to fifteen minutes being sufficient time to elapse between addition of solvent and reading of color. It may be necessary to go back to extraction with a water-immiscible solvent to eliminate this problem completely.

Triethanolamine reduces TZ very rapidly. Triethanolamine may donate electrons to the TZ reagent and hydrogen ions may be picked up from the solution, hydroxyl ions being attracted and more or less loosely held by the nitrogen of the triethanolamine in place of its lost electrons. This is only a possibility. For <u>in vitro</u> work, therefore, the plant growth regulators and other chemicals used were made up in ethanol. Indole-3-acetic acid also reduces TZ slowly in the presence of succinate.

Work with fresh beef liver homogenates and 2,4-D and 2-methyl-4-chlorophenoxyacetic acid (hereinafter referred to as MCP) at 0.1 per cent did not give very striking results with the high level of plant growth regulator present. The beef liver homogenate was incubated at 12° C with the chemicals. Aliquots were drawn out at 1½, 3½, and 6 hours. The 1½-hour aliquot indicated a possible slight stimulation of endogenous respiration. The 3-hour aliquot suggested possibly a slight inhibition, especially with 2,4-D. At six hours there was definite inhibition of endogenous respiration. Other work with beef liver was inconclusive.

In vitro studies were carried out with 2,4-D, 2,4,5-trichlorophenoxyacetic acid(2,4,5-T), MCP, indole-3-acetic acid(IAA), naphthaleneacetic acid(NAA), isopropyl-N-phenyl carbamate(IPC), 3-chloro isopropyl-N-phenyl carbamate(ClIPC), and p-chlorophenyl-N,N'-dimethyl urea (CMU) on bean homogenate. Figure 6 shows their relative effects on the system, as compared to a control with the same amount of ethanol. There is also a control without ethanol to show the effect of ethanol itself on the system. The same experiment was carried out on homogenates from two different kinds of beans and the results are similar. (Fig. 6).

It is interesting to note that 2,4,5-T is the most inhibitory at this level of 0.1 per cent, whereas ClIPC

may possibly have a slight stimulatory effect. These are not the results that might be expected from such active substances as were used, however, at these concentrations. Perhaps the response of dehydrogenases to these chemicals is a secondary effect. On the other hand, this observed effect may be sufficient after a period of time to unbalance the equilibrium of the plant to the extent of causing death, even at lower rates than those used here. Another possibility is that the enzyme which is affected may not have been studied yet. Or, the response of the plant to the chemical may be the cause of its death.

There is an inverse relationship between dehydrogenase activity and concentration of 2,4-D from zero to one thousand parts per million. (Table 4).

TABLE IV

Micrograms of formazan produced in 85 minutes by one milliliter of bean homogenate with varying amounts of 2,4-D. 2,4-D in parts per million.

2,4-D	Formazan
2000	80
1000	112
200	136
100	160
0	160

In the work from here, citrate has been substituted for fumarate, which was not very effective in eliciting a response from the enzyme systems being studied.

In view of the relatively small response to the plant growth regulators *in vitro*, it was decided to try some time studies on treated plants. The etiolated seedlings were treated through the roots with the chemical, and glucose at one per cent. 2,4-D was used at 25 parts per million and ClIPC and CMU at 200 parts per million. The 2,4-D-treated plants showed very slight, if any, visual symptoms after nine days. Thirty grams of plant material were harvested and the enzymatic activity measured. The calculations were made by dividing the amount of formazan produced by the homogenates from the treated plants by the amount of formazan produced by the homogenates from the control plants. (Fig. 7). It is interesting to note the relatively large response of the dehydrogenase systems to citrate as compared with succinate, malate, and glutamate which elicited somewhat the same response among themselves. (Table 5). Perhaps the glucose which was given to the plants at the time of treatment may have contributed to this difference.

When everything is compared to the control, there is little difference between substrates or between substrate and endogenous. This probably indicates a general effect on the dehydrogenase activity, rather than any specific response. On the other hand, a small but definite difference may be masked by using an indicator

Figure 7
Dehydrogenase activities of treated plants compared to controls, harvested varying periods after treatment-2,4-D.

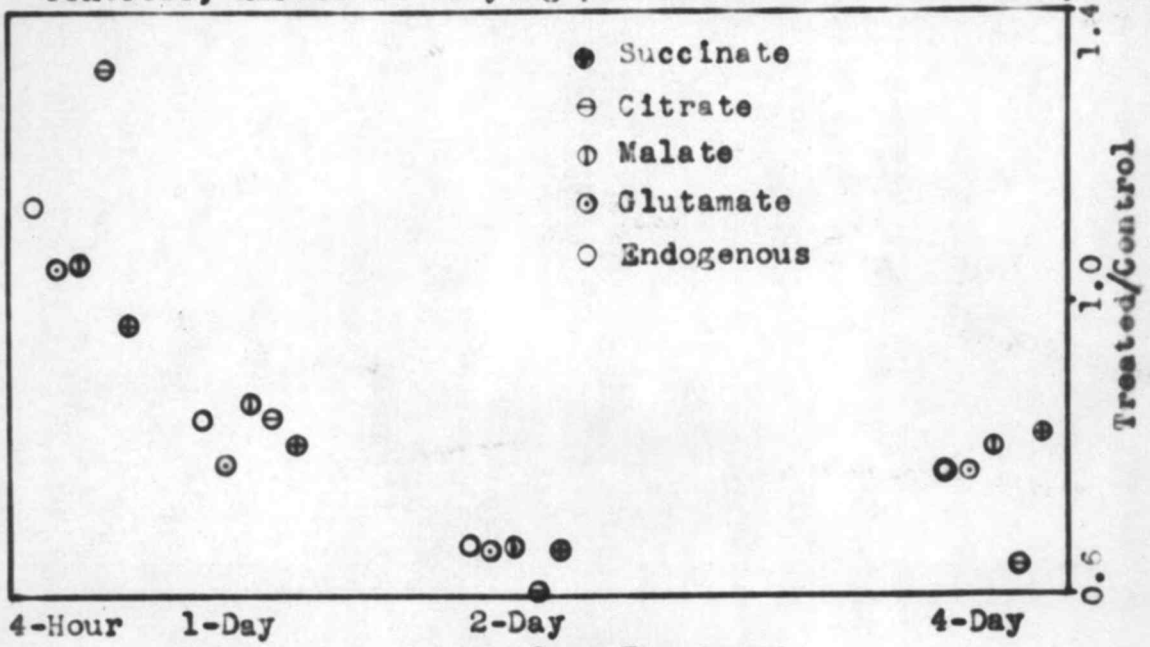
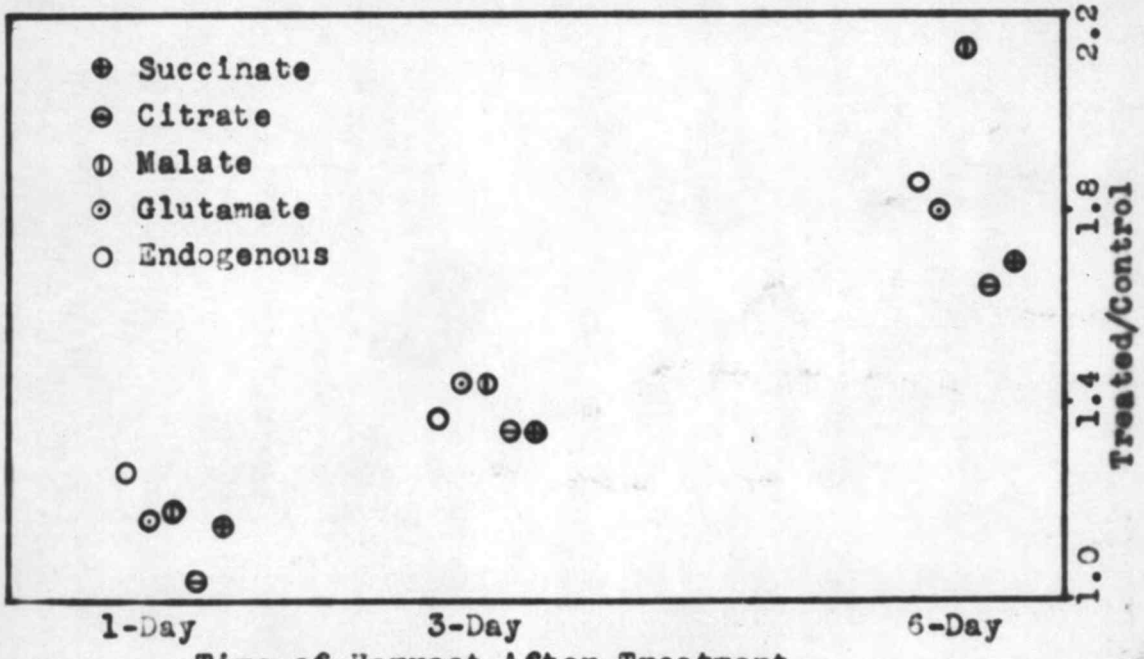


Figure 8
Dehydrogenase activities of treated plants compared to controls, harvested varying periods after ClIPC treatment



which measures the activity of a group of enzymes. It would appear that this would show up in the substrate response, but it might not. Also only four of the possible substrates were used.

3ClIPC (Fig. 8) seemed to have a more striking effect on the dehydrogenase system of beans than 2,4-D, although beans are not particularly susceptible to ClIPC. The rate of chemical application was eight times as high for ClIPC as for 2,4-D, however.

2,4-D appeared first to have given a slight stimulation to the dehydrogenases of the treated plant, then inhibition, and subsequently the enzymes recovered. (Fig. 7). This was probably a sublethal dose. The response of the dehydrogenases to citrate was greater when they were stimulated and less when they were inhibited. This may only have been a reflection of the greater response of the enzymes to citrate as a substrate, or it may indicate the presence of some other enzyme or enzymes sensitive to 2,4-D.

The slight stimulation of the dehydrogenases of 2,4-D-treated plants and subsequent inhibition bears out what is known about depletion of starch reserves of 2,4-D-treated plants. The respiration rate appears to be stimulated initially and then it tapers off as the food reserves of the plant are depleted.

Apparently ClIPC stimulates the metabolism of the plant so that the dehydrogenases become progressively more active as time elapses. (Fig. 8). It would be expected that a concurrent depletion of food reserves would occur. How the chemical exerts its effect on plants is still unknown.

Six days after treatment, the CMU-treated plants showed reduced dehydrogenase activity as compared with the controls. (Table 5). This was probably a secondary effect of the altered metabolism caused by CMU.

TABLE Va

Micrograms of IodoTZ reduced by homogenates of plants treated with chemical and harvested varying days after treatment, with their controls. Also, values calculated from ratio of treated/control. Data for figure 7. Plants were treated with 2,4-D.

Substrate	Endog.	Succ.	Mal.	Glut.	Cit.
4-Hour					
T	86	128	134	137	214
C	76	132	127	130	162
T/C	1.13	0.97	1.06	1.05	1.32
1-Day					
T	72	116	118	114	156
C	86	143	137	146	186
T/C	0.84	0.81	0.86	0.78	0.84
2-Day					
T	57	88	90	88	114
C	85	134	135	133	186
T/C	0.67	0.66	0.67	0.66	0.61
4-Day					
T	82	126	136	119	182
C	107	154	168	154	285
T/C	0.77	0.82	0.81	0.77	0.64

TABLE Vb

Micrograms of IodoTZ reduced by homogenates of plants treated with chemical and harvested varying days after treatment, with their controls. Also, values calculated from ratio of treated/control. Data for figure 8. Plants were treated with ClIPC and CMU.

Substrate	Endog.	Succ.	Mal.	Glut.	Cit.
ClIPC Treatment					
1-Day					
T	178	255	238	241	423
C	141	222	201	208	410
T/C	1.26	1.15	1.18	1.16	1.03
3-Day					
T	213	272	331	300	472
C	155	205	230	208	355
T/C	1.37	1.33	1.44	1.44	1.33
6-Day					
T	223	298	345	290	529
C	120	176	162	161	323
T/C	1.86	1.69	2.13	1.80	1.64
CMU Treatment					
6-Day					
T	108	184	152	146	264
C	136	246	194	166	367
T/C	0.79	0.75	0.78	0.88	0.72

IV SUMMARY AND CONCLUSION

Methods have been modified to detect and measure dehydrogenase activity in both etiolated and green plant material by the use of IodoTZ. The presence of an enzyme system or systems has been verified and response to substrates has been shown. The effect of some plant growth regulators and other chemicals on this system *in vitro* has been studied. A study has also been made of the dehydrogenases of plants treated with some of these chemicals.

Indications of some *in vitro* inhibition of dehydrogenases were also obtained from work with beef liver.

It must ever be borne in mind that life is a dynamic condition and that perhaps small shifts in enzymatic equilibrium may be indications of disturbed balances which may result in final death to the organism. The time factor, which is always involved, also must not be overlooked. It has been suggested that herbicides cause death by either inhibiting respiratory enzymes directly or by stimulating respiration so that the plant burns itself out. The slight stimulation by 2,4-D observed four hours after treatment suggests the latter but does not entirely

eliminate possibility of the former. And if the respiration is stimulated, it does not account for the fact that by the end of the first twenty-four hours after treatment the dehydrogenases are inhibited and are still below normal at the end of the fourth day in plants which were treated with sublethal doses, unless the starch possibly has been moved out and depleted in that short a time. It may be, however, that at higher rates the stimulation would be of a greater degree and of a longer duration. The observed inhibition may be a reaction of the plant in adjusting its metabolism as it comes back to normal.

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