BIOCHEMICAL EFFECTS OF MALEIC HYDRAZIDE (1,2-DIHYDROPYRIDAZINE-3,6-DIONE) ON *RAPHANUS SATIVUS*

By

Lowell Ernest Weller

A THESIS

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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BIOCHEMICAL EFFECTS OF MALEIC HYDRAZIDE (1,2-DIHYDROPYRIDAZINE-3,6-DIONE) ON *RAPHANUS SATIVUS*

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AN ABSTRACT

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Approved C. L. Ball

Many reports have been published concerning the use of maleic hydrazide[1] to prolong the shelf life of a number of plant root crops and storage organs. It is assumed that storage life is prolonged through decreased respiration rates and/or growth inhibition.

Dewey and Wittwer (1) noted that a preharvest foliar treatment of radishes (Raphanus sativus) with maleic hydrazide (2500 ppm or 0.02 M) solution inhibits new root and shoot growth of topped radishes (Radishes clipped near the base of the petioles without removal of the vegetative growing points). The radish plant appeared to be an ideal choice of biological material to study the growth inhibition induced by maleic hydrazide since it is readily cultured under a variety of conditions and the effectiveness of the treatment can be ascertained after only a few days storage.

An investigation of the MH-treated and control fleshy radish root samples taken 48 hours after a preharvest foliar treatment with maleic hydrazide and also topped radishes held in storage for five days after the 48 hour treatment revealed that there was no difference in beta amylase, phosphorylase and phosphatase activities. Alpha amylase and pectin-methyl-esterase activities could not be detected.

Gross chemical analysis of both the fleshy root (48 hours after treatment and stored as topped radishes for 7 days) and shoot (48 hours after treatment) tissues revealed that there were no essential differences induced by treatment. Both the root and shoot tissues of control

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[1] Maleic hydrazide is 1,2-dihydropyridazine-3,6-dione and is abbreviated MH.

and treated plants were the same with respect to per cent dry weight, ether extractives, Kjeldahl nitrogen, reducing and non-reducing sugars, and polysaccharides other than starch. Starch in the treated shoot tissue was about double that of the corresponding control tissue.

A comparative quantitative examination of the DPNH[1] oxidase system of shoot tissue (48 hours after treatment) and topped radishes (48 hours after treatment, stored 7 days and separated into fleshy root and shoot stub) was conducted. The oxidative system was analyzed quantitatively for the activity of its known constitutive enzymes; that is, (a) diaphorase, (b) DPNH-cytochrome c reductase and (c) cytochrome oxidase (2).

The DPNH oxidase activity was the same in both the control and treated root tissue. The activity of the constitutive enzymes was also the same in both types of tissue.

The MH-treated shoot tissue exhibited a marked inhibition of the DPNH oxidase system. This inhibition resulted from partial failures in both DPNH-cytochrome c reductase and cytochrome oxidase systems.

The DPNH oxidase activity of the treated shoot stub tissue was somewhat lower than that of the corresponding control tissue. The inhibition resulted from a reduced activity of the diaphorase system. The activity of the cytochrome systems was the same for both the control and MH-treated tissue.

Muir and Hansch (3) have suggested that maleic hydrazide inhibits growth by reacting with thiol compounds in a manner similar to the

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[1] DPNH is reduced diphosphopyridine nucleotide.

addition of thiols to maleic acid. An investigation of the *in vitro* interaction of thiols with maleic hydrazide indicated that no addition compound resulted from the incubation of maleic hydrazide and thiols at a neutral pH, either in the presence or absence of tissue homogemate. The interaction of maleic acid and a thiol compound was noted.

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1. D. H. Dewey and S. H. Wittwer, Proc. Am. Hort. Soc., 57, In press (1956).
2. E. C. Slater, Biochem. J., 46, 484, 499 (1950).
3. R. M. Muir and C. Hansch, Plant Physiol., 28, 218 (1953).

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HISTORY OF THE DEVELOPMENT OF THE CONCEPT OF PLANT GROWTH SUBSTANCES

Our earliest records show that naturalists of the seventeenth century, in their concern for an understanding of the world about them, brought forth speculation on the nature of the factors underlying the development of plants. It was not until the latter half of the nineteenth century, when modern science of plant physiology was really born, that the German botanist Sachs started a detailed study designed towards an understanding of plant development. As a result of his studies he proposed a generalized theory involving "organ-forming substances" to explain the facts of plant growth and organ production.

The original observations which led directly to the first isolation of a plant hormone have been attributed to the British naturalist Charles Darwin. Darwin was primarily concerned with the elucidation of the mechanism of plant responses to external stimuli, for example, unilateral light and gravity. As a result of his research he proposed that the stimuli were perceived by one part of a plant and that some "influence" must be transmitted since the results of the stimuli were expressed in a growth response at some other site of the plant. Boysen-Jensen (1) demonstrated that the "influence" would pass through such a non-living substance as gelatin. Thus, the response of a coleoptile to unilateral light was unimpaired by inserting a gelatin block between a severed tip and the shoot stump. Paal (2) showed that the tip of the coleoptile could influence the growth of the stump

independent of any external stimulus. These studies as well as others demonstrated that there exists in the tip of the coleoptile a substance which passes into and down the side of the stump in contact with an asymmetrically replaced severed tip, stimulating the extension of growth of the zone below the tip and giving rise to the curvature observed.

It was not until 1926 following the now classical studies of Went, that the isolation of the chemical "messenger" was finally accomplished. The isolation and the technique which arose from it marked the beginning of the modern era of plant growth substances. Went's original contribution to the study of plant growth substances was that of a quantitative method for the measurement of the curvature produced by small agar blocks containing the hormone. Since these hormones are active at extremely low concentrations, below the sensitivity of chemical tests, the development and refinement of the biological test now set the stage for the final isolation of the hormone in pure crystalline form and its subsequent structural elucidation.

The chemists, in an attempt to isolate a sufficient quantity of the growth hormones for structural elucidation, chose a more readily available starting material than coleoptiles. Kögl and Haagen-Smit (3) discovered that human urine was a source of particularly active material. They devised a purification procedure which yielded less than a gram of crystals which was quite active in the Avena test. The chemical constitution of this compound was established and it was subsequently named "auxin A". These workers then attempted to obtain "auxin A" from a plant source, namely corn oil and barley malt. A

like series of purifications as used in the case of the urine yielded "auxin A" and a new compound of similar structure which they called "auxin B". Three years later these workers in conjunction with Erxleben (4) isolated a third active substance from urine called "heteroauxin" (now known as 3-indoleacetic acid).

Following these historic years in the development of our knowledge of plant hormones, "auxin A and B" were regarded as the natural plant growth substances and "heteroauxin" was probably a product elaborated by microorganisms. From the time of the first report of the isolation of "auxin A and B" many investigations have been directed towards obtaining these auxins from a great variety of plants. This includes efforts by the original investigators to repeat their earlier isolation. To date, no one has been able to obtain either of these auxins from any source. Efforts of the organic chemist to synthesize compounds of the structure assigned to "auxins A and B" have also been unsuccessful. Such evidences as these and others have caused several research workers to "privately express doubt as to the existence of auxins A and B". Even those who are most sympathetic toward the "auxin A and B" existence must admit that there is no rigorous evidence to which they can point.

While there is considerable doubt as to the existence of "auxins A and B", there is certainly no doubt as to the existence or importance of 3-indoleacetic acid in the auxin economy of a wide variety of plants. The investigation associated with the isolation and discovery of the unique properties of 3-indoleacetic acid is a nearly typical example of a fundamental investigation which has led to discoveries of great economic importance.

3-Indoleacetic acid was first obtained as a degradation product of protein as early as 1885 (5). It was first synthesized in Germany by Ellinger in 1905 (6). However, it remained for Kögl, Haagen-Smit and Erxleben to recognize the unique growth regulating properties of this compound. It was this compound which has served as a model for the chemists who subsequently synthesized a very wide range of compounds of similar structure for use in the control of physiological processes occurring in plants.

Much of the early work on growth hormones has been concerned chiefly with the stimulation of growth processes. It was soon recognized, however, that a single substance may both inhibit and stimulate a plant growth pattern; the type of response depending, in part, upon the concentration of growth regulating substance. Thusly, the classification of growth regulating substances on the basis of stimulators or inhibitors becomes a relative situation depending upon the conditions of its use.

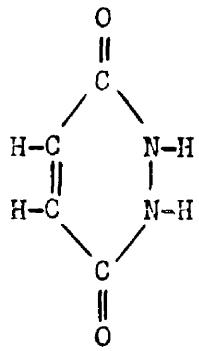
It seemed natural that the growth stimulation process would attract the most attention in the early studies. Consequently, many synthetic growth regulating substances structurally related to 3-indoleacetic acid have been recognized as a result of the research initiated by the discovery of the unique properties of this naturally occurring hormone. However, it was soon realized that under certain conditions growth inhibition could be a desirous effect. One compound which is generally regarded as a growth inhibitor is maleic hydrazide (1,2-dihydropyridazine-2,6-dione). Although this compound is not structurally related to 3-indoleacetic acid, it is one of the compounds whose

biological properties were discovered in the search for growth regulating substances. Literally hundreds of reports have been received with regard to the practical applications of this growth inhibitor. As is usually the case the more fundamental studies have followed. However, very little information is as yet available as to how this compound can bring about growth inhibition.

INTRODUCTION

INTRODUCTION

In 1895, Foersterling (7), during an investigation of the reactions of hydrazine hydrate with phthalic and maleic anhydride, isolated a compound which he called maleic acid hydrazide[1] having the following structure:



It was not until nearly fifty years later that any report concerning this compound appeared in the literature.

Arndt (8) reported the use of diazomethane in a study of the tautomeric forms of such amides as maleimide, cyclic hydrazides, malonamide, uracil and barbituric acid. He found that dihydrazides tautomerize half-way in many cases, particularly if favored by two hydrazide groups on the same carbon atom or by six-membered aromatic ring formation. In later studies Arndt and co-workers (9) report that a hydrazinedicarbonyl group $-\overset{\overset{O}{\|}}{C}-NH-NH-\overset{\overset{O}{\|}}{C}-$ lying between two atoms can

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[1]Maleic acid hydrazide is known more commonly now as simply maleic hydrazide. Both of these names are actually misnomers. From the point of systematic organic nomenclature it is 1,2-dihydropyridazine-3,6-dione. Although all three names appear in the literature today, the most common name, maleic hydrazide, will be used exclusively throughout this manuscript and will be abbreviated MH.

have only one -CONH- group tautomerize due to the adjacent positive charges of the $^{-}OC-NH-^{+}NH-CO^{-}$ mesomer which facilitates movement of a H^{+} from nitrogen to oxygen.

Maleic hydrazide is slightly acid in character and forms salts readily with alkalies. The free compound is completely water soluble at 0.2% but is not soluble at the 1.0% level. Heavy metals precipitate insoluble salts from solution. Both the soluble salts and the relatively insoluble salts function as growth regulants. The melting point is reported as in excess of 250° (7), 260° dec. (9) and 299.5-300° (10). Advantage is taken of the acid property to render maleic hydrazide more soluble and therefore more versatile biologically. Two common formulations which are available commercially are: MH-30, which is a formulation containing 58% diethanolamine salt of maleic hydrazide (equivalent to 30% maleic hydrazide) and 42% inert ingredients which include wetting agent and sticker and MH-40, which is a formulation containing 48.3% sodium salt of maleic hydrazide (equivalent to 40% maleic hydrazide) and 51.7% inert ingredients.

Schoene and Hoffman (11) first recognized the unique biological properties of maleic hydrazide in 1949. They demonstrated that maleic hydrazide, when applied as a spray, temporarily inhibited stem growth of tomato. Plants showing this response resumed growth from lateral buds about two months after treatment. They also observed that some chlorosis appeared and that root growth was also inhibited in several other species of plants. Since this first report of the unique growth regulating properties of maleic hydrazide, a great many reports have appeared in the literature describing various responses of plants to

this compound, usually related in some way to inhibition of growth. The question that still remains unsolved is how does this compound affect the metabolic systems in plants.

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

There are scores of reports in the literature concerned with many varied responses of plants to treatment with maleic hydrazide. A great many of these reports are primarily concerned with the practical application of this compound to gain a particular objective without regard as to why or how a certain result came about. There are much fewer reports of a more fundamental nature designed to gain an understanding of how maleic hydrazide alters the biochemical reactions occurring within the organism. This literature review will cover only a selected group of papers falling into the latter category. This then requires a certain amount of selection on the part of the reviewer; certainly the selection of any two reviews might conceivably vary considerably. No attempt will be made to maintain the chronology of reports with respect to the elucidation of our knowledge of the interaction of maleic hydrazide and plants.

Effect of Maleic Hydrazide on Plant Cell Division and Enlargement

Darlington and McLeish (12) reported that roots of Vicia faba which had been exposed to an aqueous solution containing 0.005 M maleic hydrazide for twenty-four hours showed no mitosis for two days. Lower concentrations did not stop mitosis, but did cause breakage of chromosomes at mitosis. Breakage was confined to heterochromatin whereas x-rays are known only to break euchromatin. These authors did not

observe this phenomenon with all plant species studied. Deysson and Deysson (13) also investigated the mitosis-inhibiting action of maleic hydrazide and other known mitotic poisons. Using mitotic poisons bearing structural similarities to uracil, namely, maleic hydrazide, barbital and antipyrine, they could not demonstrate that added uracil would reverse the mitosis-inhibiting action of the above agents. Therefore, they concluded that these mitotic poisons did not exert their effect through interference with the metabolism of uracil.

Greulach and co-workers (14) observed that maleic hydrazide at concentration of 1 to 2000 ppm inhibited mitosis and cell division in onions in proportion to the concentration of maleic hydrazide. Mitosis was resumed after transferring from maleic hydrazide solution with recovery marked at low concentration. At concentrations of 1 ppm many more roots were produced indicating a possible stimulating effect. Additionally (15) sectioned terminal buds from young beans sprayed with 0.01 M solution of maleic hydrazide showed only a few scattered mitotic figures at one week and none at two weeks following treatment. Apical cells of the treated plants were enlarged and vacuolated. Finally, Gruelach and Haesloop (16) concluded from a study of the effects of maleic hydrazide on internode elongation, cell enlargement and stem anatomy that cell enlargement was only slightly affected. Inhibition of cell division accounted for practically all of the observed growth inhibition.

Effect of Maleic Hydrazide on the Morphology of Plants

Moore (17) listed the easily visible effects of maleic hydrazide on plants as (a) a temporary suspension of stem elongation from terminal buds or death of terminal buds and adjacent tissues, (b) expansion of lateral buds some time after the terminal bud had been affected, (c) a transient intensification of green in leaves of stunted plants, (d) a localized accumulation of anthocyanins or other non-green pigments, (e) narrowing of leaves on both monocots and dicots, (f) several patterns of leaf chlorosis, (g) an interference with water adsorption, apparently caused by death of root tips, (h) suppression of nodule formation on bush beans, and (i) total, temporary or male sterility.

A number of plant species were treated with maleic hydrazide at 0.05 to 0.4% as reported by Naylor and Davis (18). They found that maleic hydrazide is remarkably uniform in plant responses from species to species with some loss of sensitivity developing with age. The effect of maleic hydrazide in every species was, (a) cessation of activity of terminal meristems, (b) cessation of elongation of internodal region and (c) increase in stem diameter. New leaves were not permanently affected since normal leaves were eventually produced.

Histological studies by Rao (19) revealed that a pre-harvest foliar spray of maleic hydrazide induced inhibition of differentiation of tissues in the bud and root primordia of potato tubers and onion bulbs. There was also a retardation of cell division.

Effect of Maleic Hydrazide on the Auxin Economy of Plants

Growth inhibitors, in general, do not cause lateral buds to develop. However, one agent that does inhibit stem elongation and that also breaks apical dominance is x-ray irradiation, which also causes the destruction of 3-indoleacetic acid or auxin. Also, it is known that auxins control apical dominance (20). Many reports have been received that maleic hydrazide also breaks apical dominance (4, 11, 17, 18, 21, 22) and it is, therefore, concluded that maleic hydrazide in some way interferes with the normal auxin metabolism.

Leopold and Klein (23) reported that maleic hydrazide did not show growth stimulation in standard pea and straight growth tests at low concentrations but does inhibit growth in the above tests at dilutions of one part in ten million. 3-Indoleacetic acid completely overcame the maleic hydrazide inhibition. Additionally, there is no reaction in vitro between maleic hydrazide and 3-indoleacetic acid as determined by diffusion studies. Other studies by these same workers (24) revealed that growth inhibition by high concentrations of auxin can be relieved by the addition of maleic hydrazide. These authors conclude that maleic hydrazide is not a growth regulator since it is not capable of promoting growth in the absence of auxin but it is an antiauxin and acts in opposition to auxin in growth. Other reports (25, 26) have been presented that show that maleic hydrazide accelerates the rate of 3-indoleacetic acid destruction. The antiauxin effect of maleic hydrazide on growth then is ascribed to the accelerated removal of endogenous auxin.

Gautheret (27) found that maleic hydrazide at low levels enhances the stimulating effect of 3-indoleacetic acid and at higher levels the two chemicals act antagonistically. A similar direction of response was obtained in root elongation studies (28). Cell proliferations which can be induced by 3-indoleacetic acid can be completely inhibited by maleic hydrazide at 10^{-4} to 10^{-6} M (29).

Effect of Maleic Hydrazide on the Carbohydrates of Plants

A common response of plants treated with maleic hydrazide is the accumulation of anthocyanin pigments (17, 18, 30, 31, 32, 33). Such a response is usually interpreted to mean that in some way carbohydrate metabolism has been impaired and that the resulting carbohydrate accumulation results in increased anthocyanin production.

Currier and collaborators (34) working with barley plants and Naylor (35) working with young corn plants both noted that maleic hydrazide treated plants exuded a fluid rich in sucrose. Naylor additionally found that quantitative sugar analysis indicated a tremendous accumulation of sucrose in shoots and roots twenty days after treatment depending upon the concentration of maleic hydrazide. No glucose accumulation occurred as a result of treatment.

Probably few other plants have been so widely used in investigations of the effect of maleic hydrazide on the carbohydrate economy of plants than the sugar beet. Of particular importance in these investigations was the effect of maleic hydrazide upon the sucrose content. Mikkelsen and collaborators (36) found that foliar sprays of maleic

hydrazide produced significantly higher sucrose percentage and did not influence yield. Wittwer and Hansen (37) used a pre-harvest foliar application of maleic hydrazide (2500 ppm) to suppress top growth, root growth and storage breakdown and preserve the sucrose content. In addition to preserving sucrose content, such treatment also increased sugar content.

Wittwer and coworkers (38, 39, 40) used a pre-harvest foliar spray of maleic hydrazide to extend the storage life of a number of roots and other storage organs. Pre-harvest application of maleic hydrazide to carrots had little effect upon the carbohydrates of organs subsequently held in storage (38). Conflicting reports have been received as to the effect of pre-harvest application of this compound to potatoes. Highlands (41) and Kennedy and Smith (42) report that maleic hydrazide had no effect upon the reducing sugar content following storage. Patterson and coworkers (39) showed that reducing sugars accumulated in chemically treated tubers. Although these results seem contradictory, different experimental conditions may account for the seemingly contradictory results.

Petersen (43) suggested that a block in normal carbohydrate metabolism exists which either induces protein use as a respiratory substrate or else inhibits protein formation. This suggestion was prompted by the observation that maleic hydrazide treated tobacco plants contained larger amounts of reducing sugars, reduced soluble nitrogen, and less protein. Phouphas and Goris (44) found that <u>in vitro</u> cultures of artichoke containing maleic hydrazide showed an increased concentration of sucrose and a corresponding decrease in inulin.

Cytological examination of floral structures treated with high concentration of maleic hydrazide, 5.0 to 10%, showed an abundance of starch in the parenchyma cells which remained for a longer period of time than in flowers not treated. The preservation of starch was correlated with retarded flower maturation (45). The author suggested that retardation of starch digestion may account in part for lower respiration observed by other investigators.

Effect of Maleic Hydrazide on Enzyme Systems

Only a few reports have been received with regard to the effect of maleic hydrazide on enzyme systems. Some of the reports which have been received are rather incomplete.

Greulach (46), studying the effect of maleic hydrazide on starch synthesis and breakdown, reported that this chemical does not block the starch breakdown process. Further, he suggests that it may block either hydrolysis or phosphorolysis but it certainly does not block both processes. The observed delayed disappearance of starch from the attached treated leaves could be due to the larger quantity of starch initially present in them or to a slowing of their processes by maleic hydrazide but was more likely due to the lower rates of respiration, assimilation and to interference with translocation in the treated plants owing to maleic hydrazide injury of the sieve tubes.

Isenberg and coworkers (47) found that maleic hydrazide sprayed upon the foliage of onion affects respiration through partial inactivation or inhibition of one or more of the dehydrogenases. In a later publication (48), they report that succinic dehydrogenase and

respiration were stimulated at low maleic hydrazide concentration and inhibited at higher concentrations.

Marré (49, 50) found that maleic hydrazide and other antiauxins, in concentrations near those that reversibly inhibit growth in vivo, inhibited the activity of a preparation of dehydrogenase in vitro. Concentrations of 3-indoleacetic acid which were inactive in the absence of an inhibitor appear to be able to reverse the inhibiting effect of antiauxin upon the dehydrogenase system.

Maleic hydrazide at concentrations of 10^{-2} to 10^{-5} M stimulates the enzymatic oxidation of 3-indoleacetic acid by indoleacetic acid oxidase with an optimum pH of 5.6 (51). In contrast to this is the report of Gortner and Kent (52) that pineapple indoleacetic acid oxidase is not inhibited by maleic hydrazide at a concentration of 1.8×10^{-3} M, pH optimum 3.25.

Morel and Demetriades (53) have reported that maleic hydrazide inhibits colony growth of culture tissue, and decreases peroxidase activity. Both 3-indoleacetic acid and 2,4-dichlorophenoxyacetic acid enhanced the activity of Jerusalem artichoke tissue with respect to polyphenol oxidase but maleic hydrazide, which inhibits growth, also activates this enzyme. There is no correlation between enzymatic activity and proliferation. Maleic hydrazide also decreased catalase activity.

Effect of Maleic Hydrazide on Plant Respiration

The inhibiting effect of maleic hydrazide on the respiration of plant organs has been reported by many investigators (47, 48, 54, 55).

Some of these reports have been mentioned earlier. Naylor and Davis (54) suggested from the evidence now available from growth and respiration studies that maleic hydrazide acts either as a poison or as a biological antagonist competing for the receptor portion of an enzyme(s) concerned in respiration. A phenomenon generally assumed to be associated with growth is respiration although it is now known that the proportion of respiration energy actually utilized in growth, that is, in cell division and increase in size, is small (56, 57). Though there is no positive correlation between growth and respiration, some respiration is essential for growth. Certainly a partial or complete inhibition of respiration by lack of oxygen or respiratory poison is accompanied in higher plants by cessation of growth.

Toxicity of Maleic Hydrazide

The development of the use of maleic hydrazide on human foodstuff necessitated the study of the toxicity of this chemical. The extensive studies by Tate (58) in this regard will be summarized. Chronic oral toxicity studies with the sodium salt of maleic hydrazide have been conducted on rats and dogs. Rats have been kept exclusively on a diet containing from 0.5 to 5.0% active maleic hydrazide as sodium salt (equivalent to 0.6 to 6.0% sodium salt of MH) two years from weaning and through successive generations. Dogs similarly were fed from 0.5 to 2.0% active maleic hydrazide as sodium salt (equivalent to 0.6 to 2.4% sodium salt of MH) for one year. Rats of both sexes grew to normal adult weight on the sodium maleic hydrazide diets and the dogs responded in weight gains equal to or better than controls.

The parent generation of rats were mated, and their progeny were mated through three successive generations to produce through weaning two successive litters in each generation. Sodium maleic hydrazide had no effect on fertility of rats regardless of food level as judged by ratio of pregnancies to matings, ratio of litters born to pregnancies and by size and weight of litters at birth. No deviations from normal were noted in periodic blood and urine examinations of both rats and dogs. Autopsies were performed on rats that died during the two-year test and on those checked for this purpose, to observe any deviations from normal including occurrence and characteristics of any tumorous growth such as cysts, abscesses or neoplasms. No changes were noted which could be correlated in frequency or severity with dosages of the chemical. No evidence of tissue damage related to sodium maleic hydrazide was noted in either the rats or dogs at autopsy. Histopathological examination of liver, spleen, kidneys, and bone marrow of dogs fed daily doses of 1.0 g. of sodium salt of maleic hydrazide for one month showed no significant effect in one animal and some increase in destruction of red blood cells in a second animal but there were no changes of any significance in kidney or in bone marrow.

The acute toxicity for rats, reported as the LD50, has been found to be 2.35 g. per kg. for the diethanolamine salt of maleic hydrazide and 6.95 g. per kg. for the sodium salt of maleic hydrazide. Chronic feeding studies of maleic hydrazide as diethanolamine salt at a 1.0% active maleic hydrazide level (equivalent to 1.9% of the diethanolamine salt of MH) in the daily diet of rats showed a mortality of 21 out of 24 animals at the end of 11 weeks feeding. Further chronic

feeding studies of the diethanolamine fraction proved that the diethanolamine itself was responsible for this mortality and not the active ingredient maleic hydrazide.

Since maleic hydrazide as diethanolamine salt has desirable production advantages and response characteristics on plants, studies have been initiated to determine the residues of diethanolamine itself when this salt of maleic hydrazide is applied on foliage. A preharvest foliar spray of 3 pounds active maleic hydrazide (1 gallon MH-30 contains 2.8 pounds diethanolamine) per acre of potatoes showed 8.7 ppm maleic hydrazide residue in these tubers. No diethanolamine was present by chemical analysis using paper chromatography, indicating that diethanolamine does not translocate into potatoes.

Maleic hydrazide does not affect the growth of mice or the growth of testicular tumors in mice. Mice sprayed daily with 0.001 to 0.2% maleic hydrazide or given drinking water with the same concentration for three weeks gained as rapidly as checks and showed no toxic symptoms. Older white mice with testicular tumors were injected subcutaneously for ten days with 0.1 ml. each of nine concentrations from 0.05% to the undiluted 30% maleic hydrazide as diethanolamine salt. Growth of tumors was not inhibited nor did any concentration produce symptoms of toxicity.

Embryos of Rana pipiens were placed in 0.1 and 0.05% maleic hydrazide as diethanolamine salt for 48 hours then transferred to tap water and observed for six weeks (59). Length and body width measurements were not significantly different. No observable effect was noted at 0.05% maleic hydrazide but at 0.1% there was retardation of eye development, smaller myotomes and gills and a more sluggish response to

light and touch when compared to controls. Circulation was apparently normal.

Eggs of Bufo sp. in the early blastula stage were placed in 0.02% maleic hydrazide for 48 hours then transferred to tap water and observed. Five days later larvae were selected for tail tip amputation to study the rate and amount of regeneration. Maleic hydrazide had no observable effect on embryonic or larval development, reflexes, or amount and rate of tail tip regeneration (59).

Gastrulae of the salamander Amblyostoma punctatum were placed in 0.5, 1.0 and 2.0% maleic hydrazide for ten days, then transferred to tap water and observed. The posterior third of the tail was clipped from larvae and mitosis from the regenerated tail tip examined. The 0.5% maleic hydrazide was not particularly toxic but higher concentrations were. There were no superficial morphological differences between controls and survivors in size or size of the regenerated tail tips. The mean number of mitotic figures was lower in maleic hydrazide treated larvae but this was considered due to starvation rather than a direct effect of maleic hydrazide itself (59).

STATEMENT OF THE PROBLEM

STATEMENT OF THE PROBLEM

It has been well established that maleic hydrazide is a plant growth inhibitor under a variety of conditions. A preharvest foliar application of this chemical will prolong the storage life of a number of economically important roots and storage organs such as onions, potatoes, beets and carrots. An appropriate foliar spray of this chemical, when applied as a preharvest treatment to radish plants, will cause growth inhibition in the roots subsequently stored. Visual observations suggested that storage life was extended as a result of growth inhibition and lowered respiration rates.

The immediate problem at hand becomes that of attempting to gain some understanding of why a maleic hydrazide treated root shows inhibition of growth when compared with an untreated root.

It is realized that this study will not yield direct information as to the mechanism of the action of maleic hydrazide but should yield some information as to the effect of this chemical in inhibiting growth. It is recognized that the changes in the plant brought about by this compound may be termed a primary effect or more likely an effect many steps removed from a primary one. Data obtained in such a study as this may yield information or suggest an approach to the study of the mechanism of maleic hydrazide inhibition of growth.

EXPERIMENTAL

EXPERIMENTAL

Effect of Maleic Hydrazide on the Carbohydrase and Phosphatase Activity of Radishes

Preparation of Biological Material

The results of a preliminary investigation by Dewey and Wittwer (60) suggested that the radish would be a nearly ideal plant for a biochemical study of growth inhibition induced by maleic hydrazide. They noted that a preharvest foliar spray of a maleic hydrazide solution would inhibit new shoot growth in topped radishes which were stored for short periods of time. Topped radishes were clipped near the base of the petioles without removal of the vegetative growing points. The radish can be cultured readily either in the greenhouse or in the field and develops a fleshy root in a few weeks. Additionally, the effectiveness of the treatment can be determined only a few days following harvest.

A commercial variety of radish (Raphanus sativus), Ferry Morse's Red Comet, was chosen for this study. The plants were cultured using conventional techniques in plots in the greenhouse. When the roots had developed to a commercially feasible size (about 8-10 g.) the plants were ready for treatment. Each plot was divided into two equal portions. The foliage of one-half of each plot was treated with a 2500 ppm (0.02 molar) solution of the sodium salt of maleic hydrazide prepared by the proper dilution of MH-40. The spray was applied with a hand sprayer at

the rate of one liter per one hundred square feet. Forty-eight hours after treatment samples from the respective plots were taken for investigation. This time interval was sufficient to cause inhibition of new shoot growth on topped radishes (approximately 1/4 inch of shoot portion remaining) as determined by a previous field study (60).

Assays to determine whether treatments were effective were conducted as follows. Representative plants were harvested from both treated and control plots and washed. The shoot portion was clipped so that approximately 1/4 inch of shoot remained attached to the fleshy root. These roots were then placed in ventilated polyethylene bags and stored for five days at room temperature or for longer periods of time at lower prevailing greenhouse temperatures. After such a period of storage, the controls exhibited appreciable new shoot growth in contrast to little or no new shoot growth for the treated radishes.

Tissue samples. Tissue samples, obtained after being subjected to various experimental conditions, were prepared for investigation according to the procedures described below and referred to in this section with the following designations:

Tissue A. Root tissue was obtained forty-eight hours after treatment with a foliar spray of a 0.02 M solution of maleic hydrazide. The tissue was macerated in a Waring Blendor and the resulting macerate lyophilized. Such tissue preparations were stored at temperatures below 0° until used. Replicate samples were prepared in this manner from plants obtained from two separate plantings.

Tissue B. Root tissue was obtained forty-eight hours following the treatment described above. The topped roots were stored in

ventilated polyethylene bags for 96 hours at room temperature, at which time the effectiveness of the treatment was evident. After removal of the remaining shoot stub, the tissue was frozen and an acetone powder prepared as follows. The frozen tissue was macerated with two weight equivalents of cold acetone in a pre-cooled Waring Blendor at 0°. The slurry was filtered and the residue again macerated with one weight equivalent of acetone, re-filtered and washed with cold acetone. The residue was then placed in a vacuum desiccator over calcium chloride and stored in the cold. Following the removal of the last traces of solvent, the powders were stored at a temperature below 0°.

Tissue C. Root tissue was obtained forty-eight hours after a foliar treatment with an 0.02 M solution of maleic hydrazide. The topped radishes were stored in ventilated polyethylene bags for 96 hours at room temperature. The remainder of the shoot portion was removed after storage and the root tissue placed in polyethylene containers, immediately frozen and stored in the frozen state until used.

For comparative purposes, control tissue preparations, corresponding to the preparations Tissue A, B and C, were also prepared in the same manner from similar plants of the same planting but were not treated with maleic hydrazide.

Analytical Methods

Phosphorylase. Enzyme solutions for investigation were prepared from both control and MH-treated tissue as follows:

1 g. of Tissue A was extracted with 25 ml. of water for 1 hr. at 25°;

1 g. of Tissue A was extracted with 25 ml. of succinate buffer pH 6.5 for 1 hr. at 25°;

1 g. of Tissue A was extracted with 25 ml. of acetate buffer pH 5.0 for 1 hr. at 25°;

100 mg. of Tissue B was extracted with 20 ml. of water for 1 hr. at 38°.

50 g. Tissue C was extracted with 50 ml. of water for 1 hr. at 25°. After the extraction period had elapsed the samples were centrifuged, filtered and the enzyme activity was determined.

Preparation of reagents. Cori ester solution. A solution free from inorganic phosphate was prepared as follows. One gram of the dipotassium salt of glucose-1-phosphate was dissolved in 10 ml. of water. Approximately 25 mg. of calcium oxide was added, the solution thoroughly mixed and allowed to stand for 10 minutes. After filtering normal sulfuric acid was added to make neutral to a phenol red internal indicator. The solution was diluted to 40 ml. and 60 ml. of succinate buffer was added. This solution required refrigeration for storage.

Succinate buffer. A mixture of 59 g. of succinic acid and 81 g. of sodium carbonate was cautiously dissolved in distilled water and boiled to remove carbon dioxide. The solution was cooled, diluted to one liter and mixed. The pH should be 6.4.

Starch amylose. One hundred milliliters of succinate buffer was added to 800 ml. of water, heated to boiling and removed from heat source. Ten grams of Merck's Lintner soluble starch suspended in 100 ml. of water was added with stirring. The mixture was cooled to room temperature and centrifuged clear. The clear solution of amylose was decanted off, preserved with toluene and stored at room temperature.

Determination of phosphorylase activity (61). Two 50 ml. volumetric flasks were placed in a water bath at 25°. To each flask was added 1 ml. of tissue extract and 1 ml. of 1% starch amylose previously brought to 25°. One milliliter of Cori ester in succinate buffer, pH 6.4, was added to one tube and mixed. After 30 minutes, 5 ml. of 6.66% molybdate solution was added to both flasks. This inactivated the enzyme. Now 1 ml. of Cori ester was added to the flask to which none was added initially. This became the blank. Both flasks were diluted to about 30 ml., 5 ml. of 7.5 N sulfuric acid was added and mixed. Five milliliters of freshly prepared 4% ferrous sulfate was added and the solution diluted to the 50 ml., mixed and the optical density determined with a photoelectric colorimeter using a red filter (630 mμ) (62). The optical density readings were translated into μg. of phosphorus by reference to a previously prepared standard curve. The phosphorylase unit is expressed as μg. of phosphorus set free from Cori ester at 25° at pH 6.4 in 60 minutes by the phosphorylase enzyme in 1 ml. of tissue extract.

Phosphatase. Enzymic solutions for investigation were prepared from control and MH-treated tissues as described:

1 g. Tissue A was extracted with 50 ml. of water for 1 hr. at 38°;

200 mg. Tissue B was extracted with 40 ml. of water for 1/2 hr. at 25°;

50 g. Tissue C was extracted with 50 ml. water for 1 hr. at 25°.

The samples were then centrifuged, filtered and an aliquot of the filtrate used for the determination.

Determination of phosphatase activity (63). Five milliliters of the tissue extract was added to 10 ml. of water and equilibrated at 38°. The substrate, 10 ml. of 5% β-glycerophosphate at pH 5.8, previously warmed to the same temperature, was added. Three milliliter samples were taken at appropriate times thereafter and delivered immediately into 5 ml. of 6.66% molybdate. The phosphorus determination was conducted as outlined in the previous section under phosphorylase. A phosphatase unit is expressed as the mg. of phosphorus set free from β-glycerophosphate at 38°, pH 5.8 in 60 minutes by the phosphatase enzyme in 1 ml. of tissue extract.

Alpha amylase. An enzyme extract was prepared from control and MH-treated tissue by extracting 50 g. of Tissue C with 50 ml. of water for 1 hour at 25°.

Preparation of reagents.

1. A stock iodine solution was made up to contain 11 g. of iodine and 22 g. of potassium iodide in 500 ml. of water.
2. Iodine solution A. To 15 ml. of the stock solution, 8 g. of potassium iodide were added and diluted to 500 ml. with water.
3. Iodine solution B. To 2 ml. of the stock solution, 20 g. of potassium iodide were added and diluted to 500 ml. with water.
4. The dextrin solution contained 0.6 g. of Merck's reagent dextrin made up to 1000 ml. with water.
5. A buffer of pH 4.4-4.5 was prepared with 120 ml. of glacial acetic acid, 164 g. of anhydrous sodium acetate diluted to a volume of 1000 ml.

6. A beta amylase solution was prepared using a commercial preparation of beta amylase so that 5 ml. of solution contained 100 mg. of beta amylase.
7. A buffered alpha amylodextrin solution was prepared from a suspension of 10 g. of Merck's Lintner soluble starch, 25 ml. of acetic acid-sodium acetate buffer, 5 ml. of beta amylase solution. This solution was brought to 500 ml. with water and then saturated with toluene.
8. A color standard was prepared by pipetting 5 ml. of iodine solution A into a comparator tube and adding 1 ml. of dextrin solution. This standard should be prepared just prior to using and should not be used longer than four hours.

Determination of alpha amylase activity (64). Twenty milliliters of buffered alpha amylodextrin was placed in a 50 ml. flask containing 5 ml. of water and the mixture placed in a water bath at 25°. After temperature equilibration, 5 ml. of the extract to be tested was added. At appropriate time intervals 1 ml. of the reaction mixture was added to 5 ml. of iodine solution B in a comparator tube, then shaken and compared with the standard.

Alpha amylase units are the number of mg. of soluble starch dextrinized by the alpha amylase in 1 ml. of tissue extract in one hour at 25° and pH 4.4.

Beta amylase. Extractions of control and MH-treated tissues were conducted as follows:

1 g. of Tissue A was extracted with 50 ml. of water for 1 hr. at 25°;

500 mg. of Tissue B was extracted with 50 ml. of water for 1 hr. at 25°.

Centrifugation of the filtrate yielded a solution which was used for the determination.

Preparation of reagents.

1. A buffer of pH 4.4-4.5 was prepared using 3 ml. of glacial acetic acid, 4.1 g. of anhydrous sodium acetate and made up to 1 l. with water.
2. A N/20 alkaline ferricyanide solution was prepared using 16.5 g. of potassium ferricyanide, 22 g. of anhydrous sodium carbonate and diluting to one liter. This solution was stored in a dark container.
3. An acetic acid reagent containing 200 ml. of glacial acetic acid, 70 g. of potassium chloride, 20 g. of zinc sulfate heptahydrate made up to one liter with water.
4. A buffered starch solution consisting of 10 g. of Merck's Lintner soluble starch, 25 ml. of acetate buffer pH 4.4 was brought to 500 ml. with water and saturated with toluene.

Determination of beta amylase activity (66, 67). To 20 ml. of the buffered starch solution in a 125 ml. Erlenmeyer flask was added sufficient water so that on subsequent addition of the enzyme solution the total volume was 30 ml. When the flasks and contents had come to 25°, the enzyme solution was added and the hydrolysis allowed to proceed for fifteen minutes or longer. At the end of this time the reaction was stopped by the addition of 20 ml. of one percent sulfuric acid.

To a 2 ml. aliquot of the resulting solution was added 10 ml. of N/20 ferricyanide reagent and the tube was immersed in a boiling water bath. After 20 minutes the tube was cooled in cold running water and the contents poured immediately into a 125 ml. flask. The tube was then rinsed with 25 ml. of acetic acid reagent and the rinsings were added to the flask. One ml. of fifty percent potassium iodide solution was added and the contents of the flask were thoroughly mixed. Titration was then carried out with N/20 sodium thiosulfate to the complete disappearance of the blue color of the starch indicator.

Beta amylase unit is designated as the number of mg. of soluble starch converted to maltose by the beta amylase of 1 ml. of tissue extract in one hour at 25° and pH 4.4.

Pectin-methylesterase. One gram of Tissue A was extracted with 30 ml. of water at 38° for one hour, centrifuged, filtered and the resulting solution used for the determination below.

Substrate. A 0.5% citrus pectin solution in 0.1 M sodium chloride was prepared by adding five grams of citrus pectin slowly with stirring to approximately 600 ml. of boiling 0.1 M sodium chloride. The solution was cooled rapidly and diluted to one liter. A drop of toluene was added and the solution stored in the refrigerator until used.

Determination of pectin-methylesterase activity (65). A 250 ml. beaker equipped with a stirring device was immersed in a 25° constant temperature bath. Fifty milliliters of substrate was added and allowed to temperature equilibrate. The pH of the solution was adjusted to 7.0 (glass electrode) by the addition of 0.05 N sodium hydroxide. At zero time 5 ml. of enzyme

solution was added and the pH again adjusted to 7.0. The pH was maintained near 7 by the periodic addition of 0.05 N sodium hydroxide. The amount of alkali added over a definite period of time gives a criterion of enzymatic activity.

A unit of pectin-methylesterase is defined as the mg. of methoxyl liberated from pectin by the enzyme contained in 1 ml. of tissue extract in 60 minutes at 25°.

Results and Discussion

Beta amylase: The results of the investigation of the beta amylase activity of the respective tissue preparations are summarized in Table I.

TABLE I

EFFECT OF MALEIC HYDRAZIDE ON THE BETA AMYLASE ACTIVITY OF TISSUE PREPARATIONS

Extraction Solvent	Reaction Time (min.)	Beta Amylase Activity*	
		Control	MH-Treated
Water (3 ml. = 60 mg. Tissue A)	15	59.6	58.6
		59.6	58.0
Water (5 ml. = 100 mg. Tissue A)	20	46.5	46.5
		46.2	45.8
Water (5 ml. = 50 mg. Tissue B)	20	16.0	17.1
		17.1	18.8
		16.0	18.8
		16.0	17.4

*Results expressed as mg. soluble starch converted to maltose by the beta amylase in 1.0 ml. of tissue extract in one hour at 25° and pH 4.4.

Upon inspection of the results in Table I it becomes immediately apparent that the beta amylase activity is essentially the same in both

the control and treated tissue preparations.

Additionally, the effect of added maleic hydrazide on the enzymatic activity of a commercial preparation of beta amylase (Nutritional Biochemicals Corporation) of unknown purity was studied. In addition to yielding information as to the effect of maleic hydrazide on the activity of beta amylase in vitro, such a study also yielded a check upon the experimental procedures used in estimating the activity of the enzyme. The results of such a study are given in Table II.

TABLE II

EFFECT OF MALEIC HYDRAZIDE ON ENZYMIC ACTIVITY OF A COMMERCIAL BETA AMYLASE PREPARATION

Maleic Hydrazide (Molarity)	Beta Amylase Activity*	
0 (control)	34.1	34.1
1×10^{-5}	33.9	34.2
1×10^{-4}	33.7	33.9
1×10^{-3}	35.6	35.2

*Expressed as mg. of soluble starch converted to maltose by 1.0 mg. of beta amylase preparation in one hour at 25° and pH 4.4.

Here again, it is evident that under the conditions used in this investigation that at concentration of 10^{-3} to 10^{-5} M. maleic hydrazide had no observable effect on beta amylase activity.

Alpha amylase: The results of the study of the alpha amylase activity of the tissue preparations can be stated summarily as follows. None of

the extracts from any of tissue preparations A, B or C exhibited any detectable alpha amylase activity over periods as long as 90 minutes. Various procedural changes were made but none of the changes resulted in a measurable activity. As a further check on the validity of the experimental procedure a commercial preparation of alpha amylase (Nutritional Biochemicals Corporation) of unknown purity was assayed. Additionally, the in vitro effect of maleic hydrazide on the activity of the commercial alpha amylase was studied. Under the conditions of this investigation, concentrations of maleic hydrazide up to 10^{-3} M. were without any detectable effect on the activity of alpha amylase.

Phosphorylase: Typical data obtained in the study of the phosphorylase activity of the various tissues are given in Table III.

TABLE III

PHOSPHORYLASE ACTIVITY OF TISSUE PREPARATIONS

Extraction Solvent	Reaction Time (min.)	Phosphorylase Activity*	
		Control	MH-Treated
Water (1 ml. = 20 mg. Tissue A)	30	87 (blank 83) 89	85 (blank 83) 88
Water (1 ml. = 5 mg. Tissue B)	20	79 83 (blank 84) 80	82 83 (blank 83) 80
Water (1 ml. = 1 g. Tissue C)	20	86 (blank 83) 84	93 (blank 87) 95

* Results expressed as μg. of inorganic phosphorus liberated from Cori ester by the phosphorylase enzyme in 1 ml. of tissue extract in one hour at 25° and pH 6.4.

None of the various tissue extracts examined exhibited any appreciable amount of phosphorylase activity. The variations in μg. of phosphorus expressed in Table III fall within the limits of experimental error of the procedure which amounts to approximately ±4 μg. Various extraction procedures using acetate and succinate buffers were used in an effort to obtain a more active enzyme preparation. The Cori ester solution was increased from 1% to 3% and the starch concentration increased from 1% to 5% in the experimental procedure without any appreciable effect. Increasing the volume of tissue extract increased the "blank" phosphorus reading so that it was beyond the upper limit of the method used to determine phosphorus. However, using larger amounts of tissue extract did not give appreciably greater phosphorylase activity.

Phosphatase: Typical data obtained in the study of the phosphatase activity of the tissue preparations are given in Table IV.

TABLE IV

PHOSPHATASE ACTIVITY OF TISSUE PREPARATIONS

Extraction Solvent	Time (min.)	mg. of Phosphorus*	
		Control	MH-Treated
Water (5 ml. = 100 mg. Tissue A)	0 (blank)	0.260	0.242
	15	1.53	1.47
	30	1.19	1.27
	30	1.29	1.30
Water (5 ml. = 25 mg. Tissue B)	0 (blank)	0.365	0.380
	15	0.440	0.440
	30	0.380	0.360
Water (5 ml. = 5 g. Tissue C)	0 (blank)	0.565	0.555
	15	1.720	1.540
	30	1.170	1.24

* Results are expressed as mg. of inorganic phosphorus liberated from β-glycerophosphate by the action of the phosphatase enzyme in 1 ml. of the tissue extract in one hour at 38° and pH 5.8.

Pectin-methylesterase: This pectin enzyme was chosen for study over other possible pectin enzymes because of its simplicity in assaying. No evidence of any pectin-methylesterase activity could be obtained from the tissue preparations investigated initially. Because of the inability to demonstrate activity in the early experiments this enzyme system was not studied in other preparations. However, the validity of the experimental procedure was checked using a tissue preparation from another plant source whose pectin-methylesterase activity had been previously demonstrated.

Admittedly, the study of the carbohydrase enzymes outlined above has not been extensive. However, it should be borne in mind that throughout the investigations the main objective was an attempt to find an enzyme system(s) which may have been affected by chemical treatment and in turn could be correlated with growth inhibition. Having demonstrated such a phenomenon would then justify a more exhaustive study of the enzyme system(s) involved. However, the results of the investigation are sufficient to justify the following conclusion. Under the conditions described in this section a preharvest foliar application of maleic hydrazide was without affect on the carbohydrase and phosphatase activity of the radish roots. Additionally, maleic hydrazide did not affect an in vitro system of either alpha or beta amylase.

It should be noted that tissue samples were obtained from radishes which had been subjected to two different sets of experimental conditions. In the one instance samples were taken 48 hours after chemical treatment and immediately preserved for investigation. There was no criterion available in this case to indicate whether maleic hydrazide had affected the radishes in any way. In the second case the radishes were harvested

48 hours after treatment, topped and held in storage for a period of time. Such tissue exhibited visible evidence of some endogenous difference since the maleic hydrazide treated radishes did not show any appreciable new shoot growth at the shoot stub whereas the untreated radishes did. The changes in biochemical and physiological conditions brought about by this chemical treatment and expressed as growth inhibition may also have existed in the radish at harvest and prior to storage. In the absence of any criterion of difference this remains a moot question.

It seemed desirable at this stage of the investigation to concentrate further studies on tissue which had been previously treated with maleic hydrazide and stored for a sufficient period of time so that there was some evidence of an effect of the chemical treatment.

Effect of Maleic Hydrazide on the Composition of Radishes

Preparation and Analysis of Radish Samples

Replicate samples containing 25 radish plants averaging from 8 to 10 g. per root were collected at appropriate times using care to obtain as nearly equivalent replicates as possible with respect to total fresh weight. Shoot samples were collected 48 hours following a foliar spray of a solution containing 2500 ppm (0.02 molar) maleic hydrazide. Root samples were collected also 48 hours after treatment but were stored in ventilated polyethylene bags for 168 hours with approximately 1/4 inch of the shoot portion remaining intact during the storage period. This shoot fragment was removed prior to drying. The fresh weight of the shoot and root portions was obtained separately for each replicate and

then the samples were dried in a forced air oven at 60° for twenty-four hours. Re-weighing the samples yielded the dry weight and from the data the percent moisture was calculated. The samples were ground in an intermediate Wiley mill to pass 60 mesh and used for the analysis to be described below (68).

$$\% \text{ moisture} = \frac{\text{fresh weight (100)}}{\text{dry weight}}$$

Ether extract. Exactly 5.0000 g. of ground tissue was weighed into an extraction thimble and extracted with anhydrous ether in a Soxhlet extractor for sixteen hours. During the extraction the extractor averaged 20 cycles per hour. Following the completion of the extraction, the ether was removed on a steam bath and the residue remaining dried in a vacuum oven over phosphorus pentoxide at 60° for six hours. The flask and contents were weighed and, knowing the tare weight of the flask, the per cent ether extract was calculated.

$$\% \text{ ether extract} = \frac{\text{g. ether extract (100)}}{\text{5.0000 g. weight of sample}}$$

Alcoholic extraction for sugar analysis. The residue remaining in the thimbles after the ether extraction was freed of ether, replaced in the Soxhlet extractor and extracted for sixteen hours with 80% ethanol. During the alcoholic extraction the extractors averaged fifteen cycles per hour. This extract was used for the sugar determinations described below.

Sugars. A 100 ml. aliquot of the 80% alcoholic extract was transferred to a casserole and evaporated to about 50 ml. on a steam bath. During

the evaporation small portions of water were added and the evaporation continued until the odor of alcohol was no longer detectable. At no time did the volume of liquid fall below 50 ml. Fifty milliliters of water was added and the mass heated to 80°. The solution was transferred quantitatively to a 200 ml. volumetric and diluted nearly to volume with water. Three milliliters of saturated neutral lead acetate was added and the flask shaken vigorously and permitted to stand for fifteen minutes. The supernatant liquid was then tested for complete precipitation, diluted to volume and thoroughly mixed. The resulting solution was then filtered into a dry beaker containing one gram of dry sodium oxalate. After shaking and allowing the precipitate to settle, the solution was filtered through a dry paper into a dry flask.

Direct reducing sugars. A 50 ml. aliquot of the filtrate and 25 ml. each of Fehling's solution A (Dissolve 34.639 g. of copper sulfate pentahydrate in water and dilute to 500 ml.) and B (Dissolve 173 g. of sodium potassium tartrate and 50 g. of sodium hydroxide in water and dilute to 500 ml.) were transferred to a 400 ml. beaker. The beaker was covered with a watch glass and heated on a Precision electric heater which had been pre-adjusted so that under these conditions boiling would begin after four minutes of heating and continue to boil for an additional two minutes. The hot solution was filtered through a tared Selas crucible. The cuprous oxide remaining in the beaker was transferred quantitatively with the aid of 80° water to the crucible. The contents of the crucible were then thoroughly washed with 80° water, dried at 105° in an oven and the crucible and contents re-weighed to ascertain the amount of cuprous oxide present (aliquot 1/8).

Calculate the milligrams of glucose from Munson Walker table.

$$\% \text{ reducing sugar} = \frac{\text{g. glucose (8) (100)}}{\text{5.0000 g. dry wt. of sample}}$$

Non-reducing sugars. A 50 ml. aliquot of the above clarified aqueous sugar solution was transferred to a 400 ml. beaker. Five milliliters of 12 N. hydrochloric acid was added to the beaker and then covered with a watch glass. Hydrolysis was allowed to proceed for twelve hours with a laboratory temperature in excess of 25°. After the hydrolysis period had elapsed the solution was neutralized with about 20 ml. of 3 N. sodium carbonate to make the solution neutral to methyl orange. The contents of the beaker were then transferred to a 100 ml. volumetric flask and diluted to volume. The amount of reducing sugar present in a 50 ml. aliquot was determined as described earlier. (aliquot 1/16)

Calculate the milligrams of invert sugar from Munson Walker table.

$$\% \text{ non-reducing sugar} = \frac{(\text{g. of invert sugar after inversion} - \text{1/2 g. of invert sugar before inversion}) (0.95) (16) (100)}{\text{5.0000 g. dry wt. of sample}}$$

Starch. The dried insoluble residue remaining after the 80% alcoholic extraction was transferred to a 300 ml. flask. Sixty milliliters of water were added in 10 ml. portions and the mixture stirred to assure complete wetting of the mass as well as complete dispersion. The mixture was then heated to boiling in a water bath for one hour with occasional shaking. After cooling, 100 mg. of malt diastase was added to the flask followed by two milliliters of toluene and the mass incubated for 24 hours at 38°. In order to prevent the dispersed material from settling,

the flask was shaken from time to time and any residue on the sides of the flask was washed down with water. After incubation, the enzymes were inactivated by heating in a boiling water bath for fifteen minutes. The solution was diluted to a volume of about 90 ml. and 160 ml. of 95% ethanol were added in 25 ml. portions with thorough shaking between additions. This procedure precipitated gums, pectins and other interferring polysaccharides. After the solution had attained room temperature, it was filtered into a 500 ml. volumetric flask and washed to volume with 60% ethanol (by volume). A 100 ml. aliquot was freed of alcohol by evaporation on a steam bath according to procedure as described under the sugar section above. The volume was adjusted to approximately 75 ml. and 5 ml. of 12 N. hydrochloric acid was added and the hydrolysis carried out in a water bath at 80° for three and one-half hours. After cooling, one milliliter of a 10% phosphotungstic acid in 1% hydrochloric acid solution was added and the solution mixed and permitted to stand for fifteen minutes. The solution was then diluted to a volume of 100 ml. and filtered. Fifty milliliters of the filtrate was neutralized with about 10 ml. of 3 N. sodium carbonate to a methyl orange endpoint. The resulting solution was diluted to a volume of 100 ml. and a 50 ml. aliquot used to determine the glucose present according to the procedure described earlier. (aliquot 1/20)

Calculate the milligrams of glucose from Munson Walker table.

$$\% \text{ starch} = \frac{\text{g. glucose } (20)\ (100)\ (0.90)}{5.0000 \text{ g. dry wt. of sample}}$$

Polysaccharides. The insoluble residue from the starch determination was transferred to a 300 ml. wide mouthed flask and 100 ml. of water was added. Ten milliliters of 12 N. hydrochloric acid was added and the flask heated in a boiling water bath for two and one-half hours with intermittent shaking. After cooling, one milliliter of 10% phosphotungstic acid in 1% hydrochloric acid was added and the contents thoroughly mixed and permitted to stand for fifteen minutes. The resulting solution was then diluted to 500 ml. in a volumetric flask. (Three milliliters of water in excess of 500 ml. was added to allow for the presence of the insoluble material present). A 100 ml. aliquot was removed and neutralized to a methyl orange endpoint with about 8 ml. of 3 N. sodium carbonate. The solution was made to volume in a 200 ml. volumetric flask and the reducing sugar determined in a 50 ml. aliquot. (aliquot 1/20)

Calculate the milligrams of glucose from Munson Walker table.

$$\% \text{ polysaccharides} = \frac{\text{g. of glucose (20) (0.9) (100)}}{\text{5.0000 g. dry wt. of sample}}$$

Nitrogen. The semi-micro Kjeldahl method was used for the determination of nitrogen. Thirty to forty milligrams of sample tissue was digested in a Kjeldahl flask with 3 ml. of concentrated sulfuric acid and approximately 100 mg. of a catalyst consisting of one part potassium sulfate and three parts copper sulfate. Digestion was continued for

eight hours. The samples were distilled into excess standard acid and the excess acid determined by titration with standard base using a methyl purple indicator.

$$\% \text{ nitrogen} = \frac{(\text{ml. of } 0.0097 \text{ N. HCl})\ (0.014)\ (100)}{\text{g. weight of sample}}$$

Results and Discussion

The results of the analysis for gross nutritive constituents of radish root and shoot tissues are given summarily in Tables V and VI. These tables are self explanatory and require only a few comments.

In the instance of the root analysis (Table V), the chemical treatment has had little effect upon the plant as revealed by these analyses. The combined reducing and non-reducing sugars in the treated tissues were about one per cent higher than that in the corresponding control tissue. There was some difference in starch content, treated samples being somewhat less, but on an absolute basis this difference was small and probably not significant.

The data on the analysis of the shoot tissue (Table VI) reveal that maleic hydrazide treatment has had no large effect upon the gross constituents. It should be noted that the starch content of the treated tissue was double that of the corresponding controls.

From a nutritional aspect it is of interest to note here that root and shoot samples were also analyzed which were obtained 216 hours

OUTLINE OF THE METHOD OF CARBOHYDRATE ANALYSIS

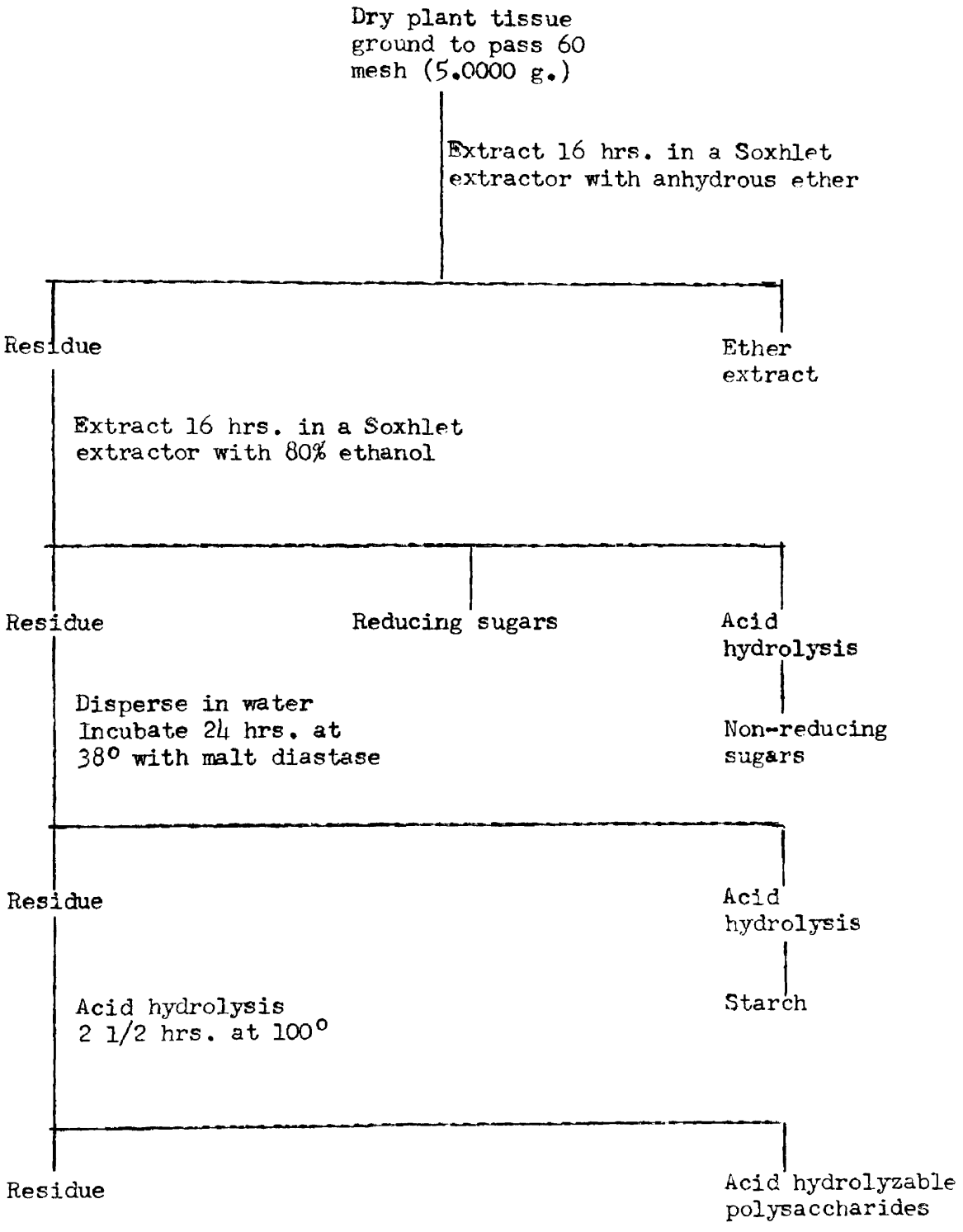

TABLE V

THE EFFECT OF A PREHARVEST FOLIAR SPRAY OF MALEIC HYDRAZIDE ON THE COMPOSITION OF RADISHES

(Expressed as per cent dry weight)

Plant Constituent	Root							
	Control				Maleic Hydrazide Treated			
	Replicate				Replicate			
	1	2	3	Average	1	2	3	Average
	%	%	%	%	%	%	%	%
Dry weight	4.39	4.51	4.46	4.45	4.66	4.51	4.55	4.57
Ether extract	1.77	1.92	1.77	1.82	2.34	2.25	1.96	2.15
Kjeldahl nitrogen	3.26	3.32	3.38	3.32	3.30	3.20	3.25	3.25
Reducing sugars	19.74	19.40	19.09	19.41	19.62	19.98	20.19	19.93
Non-reducing sugars	1.47	1.98	1.95	1.80	2.25	2.25	2.58	2.36
Starch	0.58	0.59	0.69	0.62	0.30	0.40	0.43	0.38
Other polysaccharides	1.73	1.51	1.91	1.72	1.37	1.55	1.69	1.54
Total carbohydrates determined	23.52	23.48	23.64	23.55	23.54	24.18	24.89	24.21

TABLE VI

THE EFFECT OF A PREHARVEST FOLIAR SPRAY OF MALEIC HYDRAZIDE ON THE COMPOSITION OF RADISHES

(Expressed as per cent dry weight)

Plant Constituent	Shoot							
	Control				Maleic Hydrazide Treated			
	Replicate				Replicate			
	1	2	3	Average	1	2	3	Average
	%	%	%	%	%	%	%	%
Dry weight	6.58	6.48	6.55	6.54	6.68	6.68	6.69	6.68
Ether extract	3.98	3.95	4.12	4.02	3.94	3.80	3.80	3.85
Kjeldahl nitrogen	5.86	5.86	5.74	5.82	5.57	5.52	5.68	5.59
Reducing sugars	0.54	0.83	0.62	0.67	0.42	0.45	0.47	0.44
Non-reducing sugars	3.10	3.10	3.01	3.07	3.22	3.59	3.16	3.33
Starch	0.66	0.89	0.71	0.76	1.42	1.40	1.51	1.45
Other polysaccharides	2.56	2.84	2.56	2.67	2.56	2.59	2.52	2.56
Total carbohydrates determined	6.86	7.66	6.90	7.17	7.62	8.03	7.66	7.78

following treatment. This period corresponds to the period of 48 hours after treatment plus the 168 hours of storage used previously for the samples analyzed. These treated root tissues had less reducing sugar, more non-reducing sugar and slightly more than one per cent more total reducing and non-reducing sugar than the controls. The ether extractives of the treated samples were also about one per cent less than the corresponding control. Per cent dry weight of the treated samples was approximately one per cent higher. Analysis of the shoot tissue (216 hours after treatment) showed that the treated tissue had a higher dry weight and lower nitrogen content than the controls. The reducing sugars of the treated samples were double that of the untreated samples. The control tissue gave no reducing sugar but the treated tissues did contain less than one per cent.

Additionally, mineral analyses were obtained on samples equivalent to those used for gross constituent analysis. These results, obtained spectrographically, did not reveal any marked difference in mineral content as a result of maleic hydrazide treatment.

Effect of Maleic Hydrazide on the DPNH Oxidase Activity of Radishes

Introduction

The almost complete inhibition of cellular respiration by agents which interfere with the normal functioning of cytochrome oxidase or of other cytochrome components of the respiratory chain indicated the obligatory role of these enzymes in the production of biosynthetically useful energy. Although this is only one of a number of systems involved

in the transfer of electrons to molecular oxygen it is generally believed to attribute substantially to the sum of the total electrons transferred in biological systems.

It has long been known for many years that DPN is necessary for the oxidation of a number of intermediary metabolites by tissue. The DPN[1] is reduced by the metabolite, activated by a specific dehydrogenase, to DPNH.[1] There are two well known ways whereby the DPNH may be oxidized to reform DPN, (a) by the anaerobic reaction with a substrate (S):

$$S + DPNH \longrightarrow DPN + SH_2$$

which requires a single enzyme, SH_2 dehydrogenase and (b) by the aerobic reaction:

$$H^+ + DPNH + 1/2\ O_2 \longrightarrow DPN + H_2O$$

which requires a complex of enzymes which may be called DPNH oxidase system. A less well known system involved in the possible oxidation of DPNH is:

$$DPNH + \text{fumarate} \longrightarrow DPN + \text{succinate}.$$

This reaction was demonstrated indirectly by Dewan and Green (69) who found an oxido-reduction system linking β-hydroxybutyrate, which is a typical DPN-requiring dehydrogenase and succinic dehydrogenase, which does not require DPN. It has been shown by Slater (70) that this is a slow reaction and it is doubtful whether it is of great quantitative importance in vivo.

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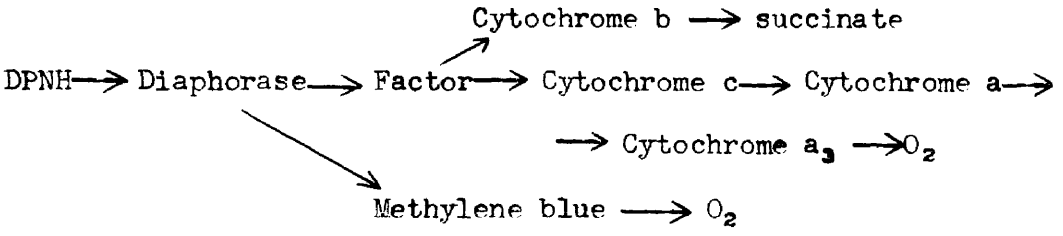
[1] DPN and DPNH are abbreviations used for the oxidized and reduced forms respectively of diphosphopyridine nucleotide.

Another system which has been described recently which may also be involved in the oxidation of DPNH is a system functioning with the aid of ascorbic acid, or more likely an oxidation product of ascorbic acid. Such a system was first observed in green peas by Mathews (71) and more recently found in cucumbers by Beevers (72). As yet very little is known of this system and its importance in the oxidation of DPNH remains to be evaluated.

Slater, (70, 73, 74) on the basis of his experiments, suggested that the DPNH oxidase system consisted of the following components, the arrows denoting the pathway taken by the hydrogen atoms or electrons on their way from substrate to oxygen.



The addition of methylene blue enables the reaction to by-pass all the components except diaphorase. The factor is the BAL[1]-sensitive (Slater factor or antimycin sensitive factor) component of the system and is probably the same as the factor previously found necessary for the oxidation of succinate. This proposed scheme does not, of course, exclude the possibility that further components, not yet identified, may also be required.

It is known that the reactions between succinate and cytochrome b is easily reversible. If the reaction between cytochrome b and the factor is also reversible to a certain extent, the slow reduction of

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[1]BAL is a common abbreviation for British Anti-Lewsite (2,3-dimercaptopropanol).

cytochrome b by DPNH and the anaerobic oxidation of DPNH by fumarate are understandable since in the absence of oxygen and the presence of fumarate the reaction proceeds as follows:

DPNH ⟶ Diaphorase ⟶ Factor ⟶ Cytochrome b ⟶ fumarate.

The system designated as the DPNH oxidase system then can be resolved into the following enzyme components:

a) diaphorase, a flavoprotein catalyzing the direct oxidation of DPNH.

b) DPNH-cytochrome c reductase, which mediates the oxidation between DPNH and cytochrome.

c) cytochrome c oxidase, which facilitates the oxidation of reduced cytochrome c.

Although there is some question as to what entities constitute DPNH-cytochrome c reductase, these entities affect the enzymatic removal of hydrogen from DPNH with the transfer of electrons to cytochrome c. Thus, in keeping with the general system as defined by Slater and outlined above, DPNH-cytochrome c reductase will be considered in this thesis to include that activity of diaphorase together with the essential elements which accomplish the oxidation of DPNH and the reduction of cytochrome c.

Preparation of DPNH Oxidase System

The radish plants, which were used as a source of biological material in this investigation, were treated with a 2500 ppm solution of maleic hydrazide (0.02 M) applied as foliar spray. Samples were taken 48 hours after the treatment described above. Shoot samples were

immediately frozen and stored until used. Topped radish roots were stored for 168 hours at greenhouse temperature. Following storage, the fleshy roots and shoot stubs were separated and preserved by freezing. Samples of both treated and untreated radishes were prepared in a similar manner.

Tissue extracts for the study of the respiratory enzymes were prepared by a method similar to that used by Keilin and Hartree (75) for the extraction of cytochrome c oxidase. Essentially this procedure had been used by Slater and others for the preparation of DPNH oxidase system in animal tissues. The procedure consisted of extraction with phosphate buffer and precipitation of the enzymes by acidification with acetic acid.

Schneider and Hogeboom (76) have observed that the majority of cytochrome oxidase activity in animal tissue was found in the mitochondrial cell fraction. These investigators (77, 78) have also shown that the presence of DPNH-cytochrome c reductase is not restricted to any definite cell structure. The greatest amount of this enzyme was found in the mitochondrial and submicroscopic particles; some being present in the nucleus. Since the individual enzyme components of the DPNH oxidase system were found in various sedimentable and non-sedimentable fractions, only a general tissue preparation was studied and no attempt was made to analyze cell fractions which might be obtained by differential centrifugation methods.

It was realized that using an extraction procedure originally designed for animal tissue might involve some problems when plant tissue was used. Of particular concern here would be such problems

as buffer concentration, type of buffer and hydrogen ion concentration. Whatever influence these and other factors might have, the modified procedure of Keilin and Hartree did, indeed, yield an active DPNH oxidase system when applied to plant tissue. Whether the conditions used yielded the most active preparation possible is not known. If absolute enzyme activities were the prime purpose of the investigation this problem would be of upmost importance. However, when enzymatic activity of the preparations obtained under like conditions are compared, then this problem is minimized.

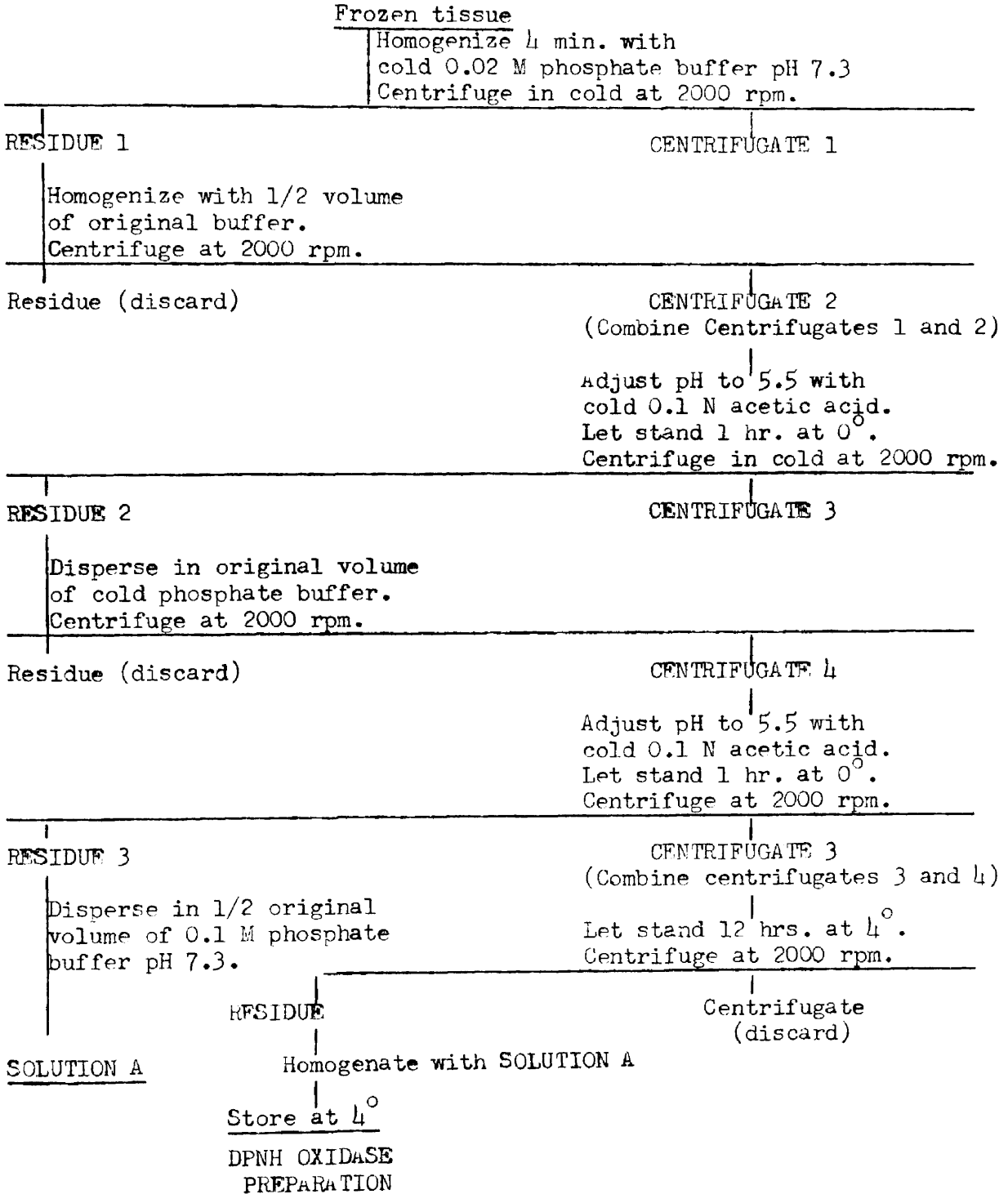
The extraction procedure varied depending upon the kind of tissue used. The particular conditions associated with special plant organs were arrived at generally by the trial and error methods.

Extraction of root tissue: Thirty grams of frozen tissue was macerated with 90 ml. of cold 0.02 M phosphate buffer pH 7.3 for four minutes in a pre-cooled blendor. The resulting slurry was strained through four thicknesses of cheese cloth and the solution centrifuged in the cold at 2000 rpm for five minutes. The centrifugate was collected and the residue re-extracted with 45 ml. of cold 0.02 M phosphate buffer. Centrifugation was repeated and the second centrifugate added to the original centrifugate and refrigerated if necessary to bring the temperature to 0-4°. The pH was then adjusted to 5.5 by the addition of cold 0.1 N acetic acid and the solution permitted to stand for one hour at about 0°. Centrifugation for five minutes at 2000 rpm yielded a residue and a centrifugate. The centrifugate was held at 4° in the refrigerator while the residue was dispersed in 90 ml. of cold 0.02 M

phosphate buffer and the acid precipitation repeated. The resulting residue was homogenized with 45 ml. of cold 0.1 M phosphate buffer pH 7.3. This solution was stored at 4° (Solution A). The combined centrifugates from the acid precipitations were held at 4° for 12 hours and centrifuged again. The resulting centrifugate was discarded and the residue homogenized with Solution A. This solution was stored, when necessary, at 4°. All enzymatic studies using this and all other preparations were conducted within 48 hours of preparation. During the preparation of the solution of enzymes great care was exercised to keep the extraction temperature below 10° at all times. (1 ml. of extract was equivalent to 2/3 g. fresh tissue).

Extraction of shoot tissue: The enzymatic preparation was obtained from the leaf tissue in essentially the same way as described for the root tissue. However, in the case of the leaves, the presence of large amounts of chlorophyll was a disturbing factor. This necessitated some minor changes in the extraction procedure. Since the procedure was nearly the same as outlined above only the changes will be described in this section. Fifteen grams of frozen leaf tissue was macerated with 90 ml. of phosphate buffer. This solution was then permitted to stand for one hour at 4° prior to centrifugation. All centrifugations were conducted for ten minute intervals at a speed of 2000 rpm. The final preparation was not completely free of chlorophyll but since blank solutions of the enzyme preparation were used consistently the presence of chlorophyll was not a prohibitive factor. (1 ml. of extract was equivalent to 1/3 g. fresh tissue).

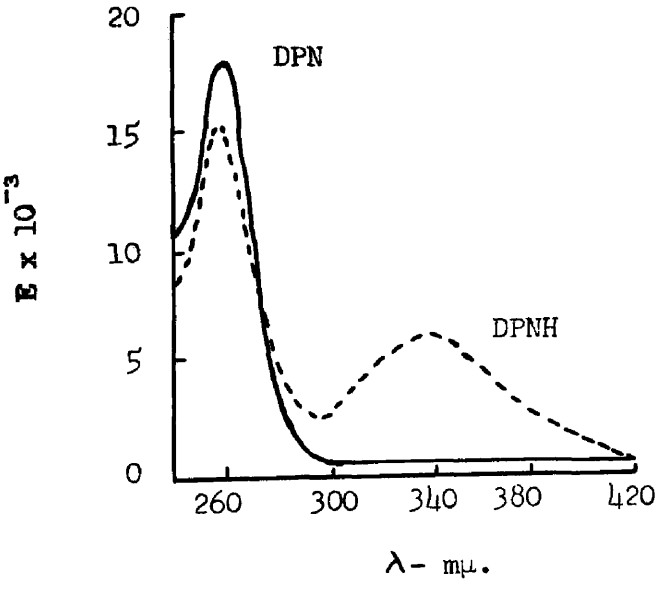
OUTLINE OF PROCEDURE FOR PREPARING DPNH-OXIDASE SYSTEM



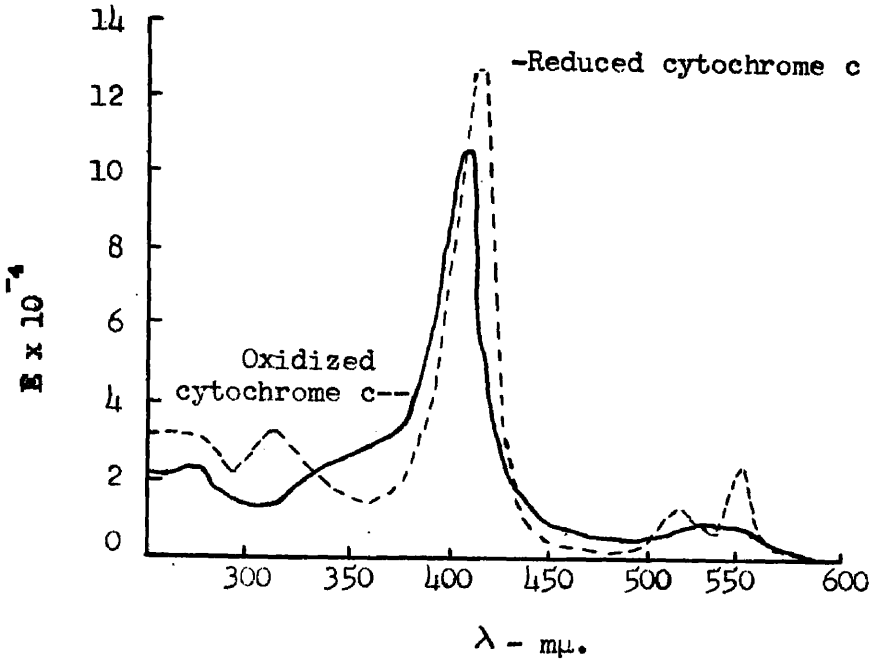
Extraction of "shoot stub" tissue: Again, the extraction procedure was essentially the same as used for the root tissue extraction. Only variations from the outlined procedure will be mentioned. Seven and one-half grams of frozen "shoot stub" was macerated with 45 ml. of cold 0.02 M phosphate buffer pH 7.3. From this point on the procedure was exactly as described above except that the volumes used were one-half of that described in the section on root extraction. The final preparation was contaminated only slightly with chlorophyll. (1 ml. of extract was equivalent to 1/3 g. fresh tissue).

Analytical Methods

The evaluation of the activity of the total DPNH oxidase system and of its component parts were conducted spectrophotometrically. The procedures used were dependent upon the spectral characteristics of both oxidized and reduced diphosphopyridine nucleotide and oxidized and reduced cytochrome c. The spectra of these compounds are as follows (79):



These spectra are typical of pyridine nucleotides in the oxidized and reduced form. Thus the oxidation of DPNH can be readily followed by noting the decrease in optical density at 340 mμ.



Reduced cytochrome c exhibits an absorption maximum at 550 mμ. which is not true of oxidized cytochrome c. Thusly the oxidation or reduction of cytochrome c can be followed by observing increases or decreases in optical density at 550 mμ.

Preparation of special reagents. Reduced diphosphopyridine nucleotide: Reduced DPN was prepared by a modification of a procedure described previously by LePage (80). To a 16 x 150 mm. test tube was added 5 mg. of DPN (Sigma Cozymase - "90" from yeast) dissolved in 0.6 ml. of water and 1.0 ml. of freshly prepared 1.0% sodium bicarbonate. Two milliliters of a freshly prepared 3.0% solution of sodium hydrosulfite in 1.0%

sodium bicarbonate were prepared in a small test tube with cautious shaking to avoid aeration (60 mg. of sodium hydrosulfite dissolved in 1.0% sodium bicarbonate and made to a 2.0 ml. volume). An 0.40 ml. aliquot of sodium hydrosulfite solution was added to the tube containing the DPN sample, mixed gently and allowed to stand twenty minutes at room temperature without further agitation. To the sample was added 18.0 ml. of a 1.0% sodium bicarbonate - 1.0% sodium carbonate (v/v). The mixture was oxygenated for five minutes to remove the excess hydrosulfite. The DPNH solution was neutralized to pH 7.3 by the dropwise addition of an 0.5 M potassium dihydrogen phosphate before use in the enzymatic studies. The nucleotide solution could be stored under refrigeration before neutralization without any appreciable oxidation; however, at pH's near neutrality, the solution oxidizes slowly even at 4°. Such a solution assayed approximately 10^{-4} molar using the molar extinction coefficient of DPNH at 340 mμ. of 6.22×10^{6} sq. cm. per mole (81).

Oxidized cytochrome c: A 10^{-4} molar solution of cytochrome c was prepared by dissolving 16.5 mg. of cytochrome c (Sigma cytochrome c, based on a molecular weight of 16,500) in 0.005 N hydrochloric acid and diluting to a volume of 10 ml. Such solutions were prepared just prior to using.

Reduced cytochrome c: A 10^{-4} molar solution of reduced cytochrome c was prepared by dissolving 16.5 mg. of cytochrome c (Sigma cytochrome c, based on a molecular weight of 16,500) in 0.10 M phosphate buffer pH 7.3 and diluted to nearly 10 ml. with additional buffer. Reduction was accomplished by adding an amount of sodium hydrosulfite sufficient to give a concentration that is 0.001 M. The preparation

was then aerated by shaking for 3 to 5 minutes to oxidize the excess reducing agent. Auto-oxidation was minimized by keeping the phosphate buffered cytochrome c solution at 4° (82).

Determination of total DPNH oxidase activity. The total system contained the following components added to the absorption cell in the order listed: 0.5 ml. of enzyme solution, 1.5 ml. of glass distilled water, 1.0 ml. of DPNH solution. The blank contained 1.0 ml. of glass distilled water in place of the DPNH solution. Upon addition of the DPNH solution the contents of the cell were mixed and the decrease in optical density at 340 mμ was obtained.

Determination of diaphorase activity (83). The components of the system employed for the measurement of diaphorase activity consisted of 0.5 ml. enzyme solution, 0.3 ml. of 2×10^{-3} M potassium cyanide, 0.1 ml. of 5.3×10^{-4} methylene blue, 1.1 ml. of glass distilled water and 1.0 ml. of DPNH solution. The blank contained the same components except for water in place of DPNH solution. The oxidation of DPNH was followed by observing the optical density decrease at 340 mμ.

Determination of DPNH - cytochrome c reductase (77). The reaction mixture (volume 3.00 ml.) contained 0.5 ml. enzyme solution, 0.3ml. of 2×10^{-3} M potassium cyanide, 0.8 ml. distilled water, 1.0 ml. of DPNH solution, and 0.4 ml. of oxidized cytochrome c. The blank contained all components except DPNH. The reaction was followed by recording the rate of increase in optical density at 550 mμ.

Determination of cytochrome c oxidase. Cytochrome oxidase was determined spectrophotometrically at 25° by the general procedure of Hogeboom and Schneider (84). The reaction mixture (volume 3.00 ml.) contained 0.5 ml. of enzyme solution, 1.0 ml. of 0.1 M phosphate buffer pH 7.3, 0.8 ml. distilled water, 0.3 ml. of 4×10^{-3} M aluminum chloride and added last 0.4 ml. of 10^{-4} M reduced cytochrome c. The blank solution contained all components except cytochrome solution. The reaction was followed for six minutes at one minute intervals by noting the decline in optical density at 550 mμ.

Measurement of enzyme activities. All spectrophotometric studies were carried out using a Beckman DU spectrophotometer equipped with a temperature control device so that the cells and contents could be maintained at 25°, the temperature at which all determinations were made. Matched cells of one centimeter light path were used exclusively throughout. All solutions used were temperature equilibrated prior to use. All zero time optical density readings were obtained within 15 seconds following the addition of the last component of the system and thorough mixing. Optical density readings, obtained at one minute intervals for six minutes, are given on the immediately succeeding pages. Duplicate readings were obtained on enzymatic preparations from two replicate tissue samples. These optical density readings are summarized graphically in Figures 1 through 7.

DPNH-OXIDASE ACTIVITY

	Time	Extinction x 10^3 at 340 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	612	638	562	560	590	595	518	525
	1	603	630	553	550	577	589	508	516
	2	593	619	541	539	565	578	497	503
	3	580	604	530	530	555	567	487	494
	4	569	592	517	515	544	552	475	483
	5	556	579	505	507	534	538	468	477
	6	542	568	494	495	524	526	458	463
Shoot:	0	538	505	708	640	447	542	587	585
	1	528	495	702	633	443	538	585	583
	2	522	490	698	629	440	536	583	581
	3	518	486	692	621	437	533	581	578
	4	512	481	688	615	433	531	577	574
	5	508	476	682	609	429	527	575	570
	6	502	472	678	603	426	525	573	568
Shoot Stub:	0	500	510	499	500	480	483	478	468
	1	495	505	493	494	476	481	475	467
	2	491	502	487	490	473	480	472	465
	3	487	498	483	486	472	478	471	463
	4	484	494	479	482	470	477	468	461
	5	481	491	476	479	469	475	467	460
	6	478	488	473	475	468	473	465	458

DIAPHORASE ACTIVITY

	Time	Extinction x 10^3 at 340 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	557	568	563	520	561	560	415	491
	1	539	545	543	498	539	536	388	471
	2	512	528	511	485	510	512	359	446
	3	491	510	493	472	497	490	339	432
	4	471	493	479	465	479	482	329	418
	5	457	480	473	449	470	472	325	402
	6	447	467	462	425	462	460	310	387
Shoot:	0	620	615	661	645	612	621	642	638
	1	571	565	616	604	570	576	584	594
	2	529	524	577	560	530	537	557	550
	3	496	490	546	528	498	508	528	520
	4	474	481	527	507	478	489	506	505
	5	459	464	510	491	460	472	498	493
	6	448	457	502	482	451	463	492	487
Shoot Stub:	0	573	576	580	593	579	574	560	564
	1	562	569	574	583	576	571	557	560
	2	552	558	562	577	570	566	552	556
	3	546	551	555	572	565	559	546	552
	4	544	549	551	564	562	558	543	547
	5	539	543	544	557	555	552	539	542
	6	535	535	537	549	548	544	534	536

CYTOCHROME C OXIDASE ACTIVITY

	Time	Extinction x 10^3 at 550 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	260	266	215	257	271	269	284	265
	1	248	256	203	244	261	258	274	251
	2	236	244	191	234	247	244	258	238
	3	226	232	175	222	233	231	245	226
	4	213	218	162	210	220	220	233	216
	5	203	206	153	202	208	210	221	206
	6	194	196	144	190	198	201	211	194
Shoot:	0	245	249	301	303	247	257	253	265
	1	235	240	292	294	243	251	246	260
	2	227	232	283	286	236	246	241	253
	3	222	227	279	281	231	242	236	250
	4	216	221	274	274	226	238	232	246
	5	211	216	266	270	222	235	226	240
	6	206	212	260	263	218	232	222	237
Shoot Stub:	0	288	280	288	270	271	268	248	274
	1	285	277	285	267	268	265	246	272
	2	281	274	282	264	265	261	245	269
	3	278	270	280	260	263	258	243	266
	4	276	268	277	258	261	256	242	264
	5	274	265	275	256	257	254	239	262
	6	271	262	273	253	256	253	236	260

EFFECT OF ADDED CYTOCHROME C ON ABSORPTION AT 550 mμ.

	Time	Extinction x 10^3 at 550 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	4	7	0	3	5	3	2	0
	1	16	21	14	14	15	12	11	10
	2	28	30	28	29	24	33	21	19
	3	39	42	40	44	36	43	33	32
	4	52	55	51	56	49	53	44	47
	5	66	70	63	69	59	65	52	57
	6	77	84	72	81	68	74	63	65
Shoot:	0	4	3	43	30	2	3	1	0
	1	15	13	54	40	9	10	9	8
	2	26	23	67	52	16	16	18	15
	3	36	33	77	61	22	22	26	22
	4	44	42	87	68	27	26	34	28
	5	51	50	95	76	31	32	40	33
	6	58	57	100	83	35	38	47	38
Shoot Stub:	0	0	2	-9	0	-2	1	-8	4
	1	4	6	-5	4	2	5	-4	8
	2	8	10	-2	7	5	9	0	11
	3	12	13	0	10	8	12	3	15
	4	14	16	4	13	12	15	5	18
	5	17	20	7	16	15	18	8	20
	6	20	22	10	18	17	20	11	22

EFFECT OF ADDED CYTOCHROME C ON DPNH OXIDASE ACTIVITY

	Time	Extinction x 10^3 at 340 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	571	566	508	512	591	570	502	510
	1	562	556	497	502	584	562	494	503
	2	549	545	485	488	571	552	485	494
	3	538	537	474	476	557	538	477	483
	4	530	524	463	464	549	530	471	477
	5	521	517	454	456	540	521	465	464
	6	508	505	443	443	531	512	447	452
Shoot:	0	394	394	634	640	410	414	598	528
	1	383	384	625	630	405	408	589	520
	2	375	376	617	621	400	403	584	514
	3	368	370	610	615	395	398	579	510
	4	361	366	602	610	390	394	574	506
	5	356	360	597	605	387	390	568	501
	6	353	358	590	598	385	386	564	498
Shoot Stub:	0	530	528	532	524	510	508	475	470
	1	525	522	528	520	506	504	471	466
	2	518	515	522	517	502	499	468	463
	3	512	511	520	510	499	496	465	461
	4	508	508	517	508	497	493	462	459
	5	503	502	513	505	493	490	460	457
	6	499	498	510	501	491	488	458	455

EFFECT OF ADDED METHYLENE BLUE ON DPNH OXIDASE ACTIVITY

	Time	Extinction x 10^3 at 340 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	563	560	511	510	544	552	518	520
	1	556	540	492	490	519	525	490	496
	2	526	518	471	471	494	500	469	473
	3	508	501	454	451	476	488	452	453
	4	491	484	437	435	459	471	434	440
	5	473	470	422	426	446	462	415	420
	6	458	457	411	509	440	453	409	410
Shoot:	0	479	475	626	640	545	495	594	612
	1	459	450	611	623	533	482	588	603
	2	439	429	594	606	521	474	580	594
	3	420	401	575	582	512	465	574	584
	4	406	393	559	564	502	457	565	573
	5	392	381	540	448	492	451	558	567
	6	381	369	526	530	483	446	543	563
Shoot Stub:	0	463	457	455	447	442	450	450	442
	1	453	448	448	439	438	446	445	438
	2	448	441	441	432	432	438	441	432
	3	440	435	437	428	428	433	437	429
	4	433	429	430	422	423	428	431	425
	5	428	422	424	417	419	422	428	420
	6	420	418	419	410	412	418	422	415

DPNH-CYTOCHROME C REDUCTASE ACTIVITY

	Time	Extinction x 10^3 at 550 mμ.							
		Control				MH-Treated			
	min.	Replicate 1		Replicate 2		Replicate 1		Replicate 2	
Root:	0	5	7	175	200	2	4	167	152
	1	17	18	182	209	12	17	180	162
	2	26	27	197	220	24	24	191	171
	3	36	39	205	233	34	34	198	182
	4	45	47	214	242	41	44	204	192
	5	53	56	222	249	52	51	215	200
	6	60	65	227	256	60	62	223	207
Shoot:	0	0	2	0	3	1	1	-1	0
	1	10	12	9	13	8	10	7	7
	2	18	18	16	22	14	14	13	14
	3	28	26	24	29	18	20	19	20
	4	36	32	29	36	24	24	25	24
	5	40	35	35	42	28	30	30	30
	6	50	44	41	48	32	32	34	34
Shoot Stub:	0	5	4	-1	1	-1	3	-4	2
	1	7	6	2	3	1	5	-1	4
	2	10	9	4	6	3	8	2	7
	3	12	12	6	8	5	10	4	10
	4	14	15	8	10	7	12	6	12
	5	15	17	10	12	8	13	7	13
	6	17	20	12	15	10	15	9	15

Results and Discussion

Cells are capable of oxidizing, with considerable ease, a great many types of metabolites many of which are quite stable in air. The enzymes that participate in these energy-yielding reactions are generally called "respiratory" enzymes. The sequence of reaction going from substrate to oxygen may be illustrated diagramatically as follows, the arrows indicating the direction of transfer of hydrogen or electrons (79).

Substrate → Pyridine nucleotides → Flavoproteins → Cytochromes → → Oxygen

Not only are cells capable of producing energy through the "respiratory" enzymes, but they also possess highly organized systems which operate to trap the energy that is released and subsequently make the energy available to the cells for energy requiring responses. The energy is trapped by the action of multienzyme systems.

A multienzyme system may be defined as an organization of enzymes which catalyzes an orderly sequence of reactions to bring about the conversion of a substrate to the desired product. Multienzyme systems must be approached with some skepticism, although they have proven of some value in revealing details as to how certain metabolic processes occur. When intact tissue is homogenized, a number of factors influence the resulting enzymatic activity observed and hence the nature of the reaction sequence in such preparations. Some of these more obvious factors are: (a) dilution effects resulting from the suspending medium used during homogenization, (b) inhibiting effects related to the liberation of sub-

stances through cell fragmentation, and (c) destructive effects such as autolytic processes which may modify the structure of the enzyme proteins and alter their enzymatic activities. Additionally, the instability of the enzymes, substrates and co-factors, the actual concentration of each of these components, the mutual affinities of substrates and co-factors for the enzymes, pH, temperature and salt effect, the introduction of spatial separation of enzyme from substrate and co-factors and the possible influence of hormonal control on enzyme systems must be considered.

The only well established effective means presently known for the coupling of energy production with energy utilization by a multienzyme system is the esterification of inorganic phosphate into the adenylate system to form adenosine triphosphate. Adenosine triphosphate may be readily transferred to energy-requiring systems.

The esterification of inorganic phosphate can take place at two levels: (a) the substrate level and (b) the electron-oxygen transfer level. The substrate level phorphorylation has been studied in some detail and is characterized by the formation of adenosine triphosphate in absence of oxygen by reactions catalyzed by water soluble enzymes. The electron-oxygen transfer system involves the passage of electrons from the substrate through several electron carrier systems to the final acceptor, oxygen. Relatively large amounts of utilizable energy become available and are trapped also in "high energy" phosphate bonds.[1]

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[1]The term "high energy" has been applied to such bonds to indicate that they release a relatively large amount of energy when they are broken and not that there is a strong bonding energy between the phosphate group and the group to which it is attached.

Ball (85) depicts the sequence for the transfer of electrons to oxygen as:

Substrate · 2H → Pyridine nucleotides ($2e+H^+$) → Flavin system ($2e+H^+$) → Cytochrome system (e) → oxygen.

At each level of the electron-oxygen transport system significant quantities of utilizable energy are theoretically available. In the oxidation of DPNH a theoretical value of four "high energy" phosphates is possible. Lehninger (86) has determined experimentally that in the oxidation of DPNH by a mitochondrial preparation, three "high energy" phosphates are formed for the transfer of the two electrons from DPNH to oxygen. These results would indicate a 75 per cent trapping efficiency. The electron-oxygen transport system is characterized by its instability, oxygen uptake and existence in the mitochondria.

Respiration, that is the oxidation of metabolites with the subsequent production of biologically useful energy, is affected by multienzyme systems residing within the organism. Respiration may occur either aerobically or anaerobically. Although both types of respiration do occur, certainly in higher plants anaerobic respiration can not take place at the exclusion of aerobic respiration, at least for very long periods of time. Aerobic respiration is associated with the electron-oxygen transport system mentioned earlier and is a vital process for higher plants. The cytochrome system is generally believed to play a predominant role in aerobic respiration. However, it is true that the extent of the participation of the cytochrome system is not known. A large body of evidence has been accumulated which indicates that the cytochrome system is associated with a large part of the aerobic respiration.

One of the phenomena generally assumed to be associated with growth is respiration. However, it is now known that the proportion of respiratory energy actually utilized in growth, that is, in cell division and increase in size, is small (56, 57). Although there is no direct correlation between growth and respiration, some respiration is unquestionably essential for growth. Surely a partial or complete inhibition of respiration through lack of oxygen or respiratory poisons is accompanied in plants by the cessation of growth. Conversely, if a substance such as maleic hydrazide can bring about cessation or inhibition of growth, it seems reasonable then to suspect that the respiratory system(s) may have been affected by chemical treatment.

As mentioned earlier, the cytochrome system functions in the electron-oxygen transport system which in turn functions to produce biosynthetically useful energy. It seems logical then to expect that inhibition of growth may be reflected in a lowered respiration rate which in turn means less biologically useful energy available. Conversely, a lowered production of biologically useful energy through inhibition of the enzymes in the electron-oxygen transport system could be reflected in cessation of growth or growth inhibition. Even the inhibition of an enzyme system functioning to produce substrate(s) for the electron-oxygen transport system may be detected as a lowered activity of the enzyme systems involved directly in the electron-oxygen transport system.

A study of the electron-oxygen transport system might be approached in at least two ways. A quantitative measurement of the gaseous exchange of respiring tissues would reveal the net result of the action of the "respiratory" enzymes. A second, and a better approach to such a study

would be an investigation of the DPNH oxidase system. Such a study would yield not only a measure of the oxygen uptake through the cytochrome system but also an index of the individual activities of the other enzymes which form the component parts of the DPNH oxidase complex.

DPNH oxidase activity. Preliminary results were obtained from a study of tissue homogenates by a colorimetric procedure devised originally to measure dehydrogenase activity. In this assay tissue homogenates were incubated for several hours (up to 20 hours) in a buffered system in the presence of a substrate and a hydrogen acceptor. The method of assay depends upon the ability of DPNH to reduce the colorless 2,3,5-triphenyl-tetrazolium-chloride to its colored reduction product (87). This reaction is nonspecific and the results obtained therefrom were erratic and inconclusive. Hence, a more definitive procedure was adopted for the assay of DPNH oxidase activity of maleic hydrazide treated radishes and comparative controls. In this assay, the enzyme preparation was obtained from an acid-treated phosphate buffer extract of the respective tissues. The method of following the oxidation of DPNH was the spectrophotometric method of Warburg and collaborators based upon the change in spectral characteristics of oxidized and reduced DPN. The results of the measurement of DPNH oxidase activity are summarized in Figure 1, showing the activity of the total system unfortified by additional amounts of cytochrome c or any other co-factors. It should be noted from Figure 1 that in the root tissue the rate of DPNH oxidation is the same for both types of tissue. However, in both the shoot and shoot stub the treated tissues exhibit an activity of about one-half of the respective controls.

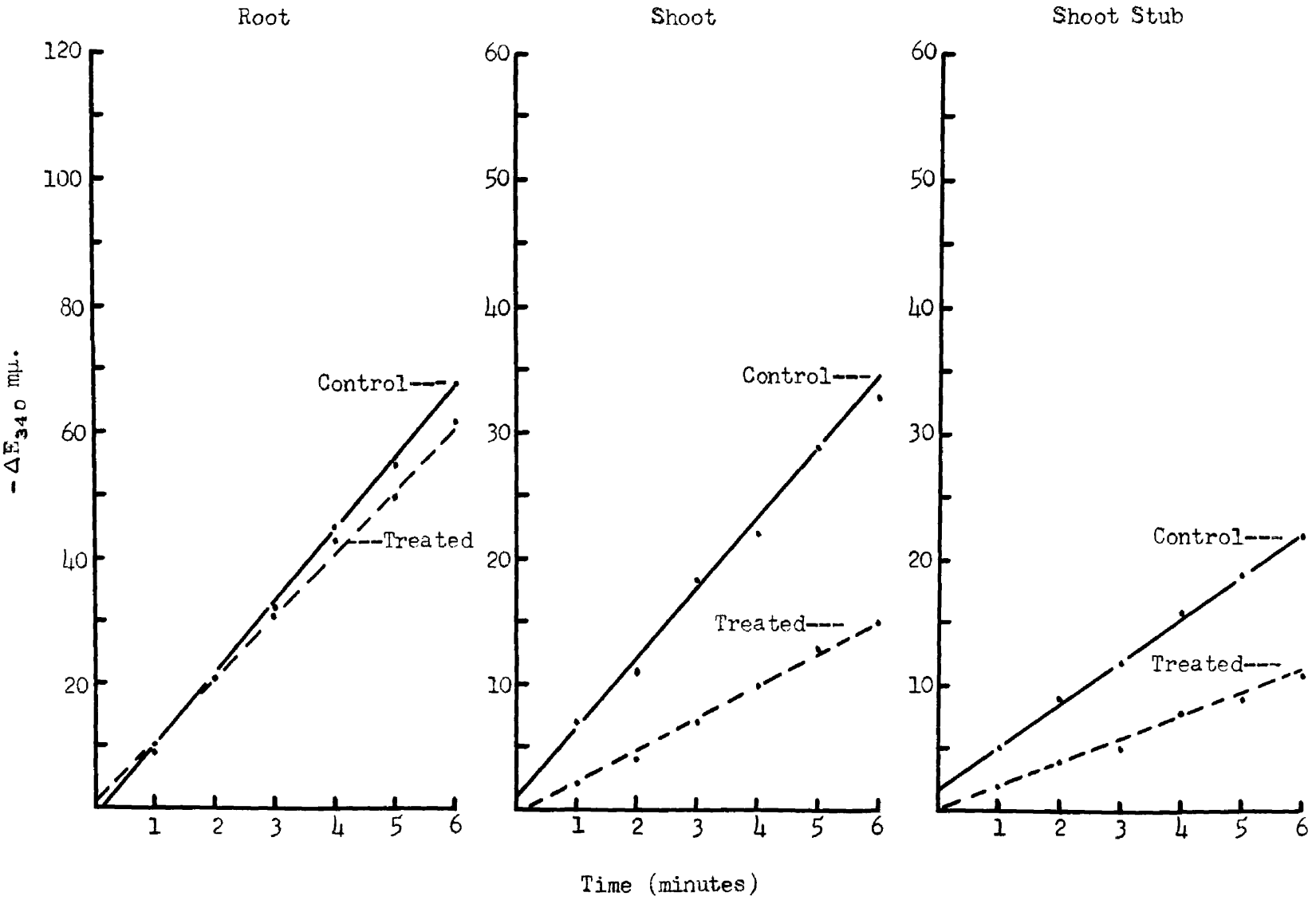


Figure 1. The DPNH oxidase activity of control and maleic hydrazide treated radish tissues. $-\Delta E_{340}$ mμ. values represent the decrease in optical density x 1000.

Before investigating the individual components of the DPNH oxidase system it seemed desirable to establish the enzymatic nature of the oxidation of DPNH. Singer and Kearney (88) have reported that cytochrome c may be reduced non-enzymatically by pyridine nucleotides, a reaction catalyzed by various flavins. Since heated enzyme preparations (90 - 100° for 5 minutes) failed to change the optical density at 340 mμ. over periods in excess of six minutes, it was assumed that the oxidation observed was indeed enzymatic.

It should be mentioned that the DPNH oxidase activity of all of the tissue preparations was considerably lower than might be anticipated when compared with similar preparations obtained from animal tissue. However, in the studies with animal tissue vital organs have usually been used. Such tissue might be expected to exhibit higher orders of enzyme activity in general. A comparison of plant and animal tissue on such a basis may be unjustified.

A lower oxidative response could conceivably be peculiar to a system containing sodium dithionite reduced substrate. Slater (70) observed that DPNH prepared by the nonenzymatic modified method of Ohlmeyer (89) was not satisfactory for certain phases of his oxidative study. Presumably this was due to interference of the products of oxidation of the reducing agent. However, an equally plausible suggestion might be that the lower oxidative level was due to impurities in the DPN used or the result of interaction of reducing agent and such impurities as might have occurred in the DPN. In this study chemically reduced DPN (cozymase - 90) was used exclusively which when assayed spectrophotometrically was determined to be more than 90 per cent pure.

Diaphorase activity. In investigating the DPNH oxidase system for factors essentially associated with the success or failure of the system in the various tissues, quantitative determinations of diaphorase activity were performed. The experimental system designed to measure this activity contained cyanide to inhibit the cytochrome oxidase portion of the respiratory chain and methylene blue to act as a hydrogen acceptor to by-pass the cytochrome system. It has been established that methylene blue is capable of oxidizing reduced diaphorase. Cyanide was used at a final concentration of 2×10^{-4} M, which concentration has been shown by Lockhart and Potter (90) to be sufficient to block completely the activity of cytochrome oxidase. These workers also showed that at concentrations of 10^{-2} M cyanide the reduction of cytochrome c itself was inhibited and concluded that even 10^{-3} M cyanide has some depressant action on the reduction of cytochrome c. That 10^{-2} M cyanide does not inhibit diaphorase was reported by Adler et al. (91) and later observed also by Lockhart and Potter (90). Meyerhof and co-workers have reported that DPN reacts with cyanide (92). Colowick and associates (93) not only confirmed the conclusion that DPN is capable of forming a complex with cyanide but also presented evidence that DPNH is unaffected by cyanide. Therefore, the site of action of cyanide in the DPNH oxidase system seems to be the terminal part of the oxidative sequence rather than the interaction with the components of the initial diaphorase system.

In the diaphorase determination, it was noted that in the systems containing both methylene blue and cyanide, the inherent rate of autoxidation of the substrate was accelerated. This effect was also noted by Slater (70) who stated that the reason for this reaction was not known. In the

data reported here the diaphorase activities are corrected for this autoxidation by use of a "blank" determination. It should be noted in Figure 2 that the diaphorase activity of the treated tissue preparations is essentially the same as the respective controls except for the treated shoot stub tissue. These data then suggest that the lowered DPNH oxidase response of the treated shoot is not associated primarily with limited diaphorase activity but rather point to a failure in the cytochrome components. Lower diaphorase activity of the treated shoot stub suggests that lowered DPNH oxidase activity may be associated with some inhibition of the diaphorase system.

Cytochrome oxidase. Direct measurement of the cytochrome oxidase by the spectrophotometric method of Hogeboom and Schneider (84) confirmed the suggestion of a deficiency in the cytochrome system in the case of the shoot (Figure 3). However, the level of cytochrome oxidase activity remained the same for both the control and treated root, as might have been expected since the DPNH oxidase was the same. The cytochrome oxidase activity of the control and treated shoot stub tissues was essentially the same.

That the metabolism of DPNH normally requires cytochrome oxidase is now generally accepted. Slater (70) has demonstrated that 5×10^{-4} M potassium cyanide produced a 96.9 per cent inhibition of the DPNH oxidase system. The fact that the oxidation of cytochrome c was indeed enzymatic was confirmed by the fact that no change in absorption at 550 mμ. occurred in the presence of 2×10^{-4} M cyanide. Cytochrome c was used at a final concentration of 1.3×10^{-5} M. This concentration of cytochrome c

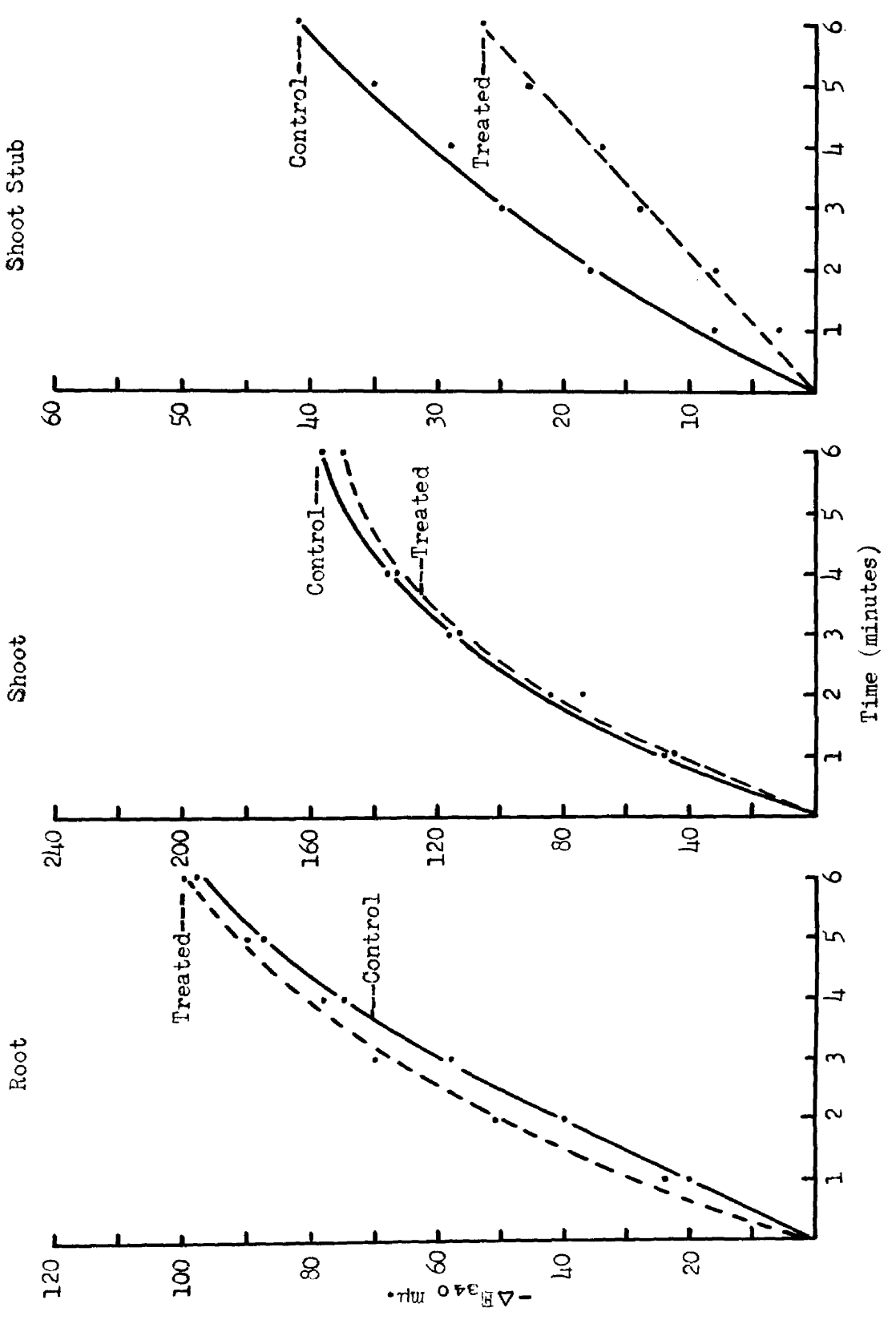


Figure 2. The diaphorase activity of control and maleic hydrazide treated radish tissues. The reaction mixture contained 0.3 ml. of 2×10^{-3} M potassium cyanide and 0.1 ml. of 5.3×10^{-4} M methylene blue. $-\Delta E_{340}$ mμ. values represent the decrease in optical density x 1000.

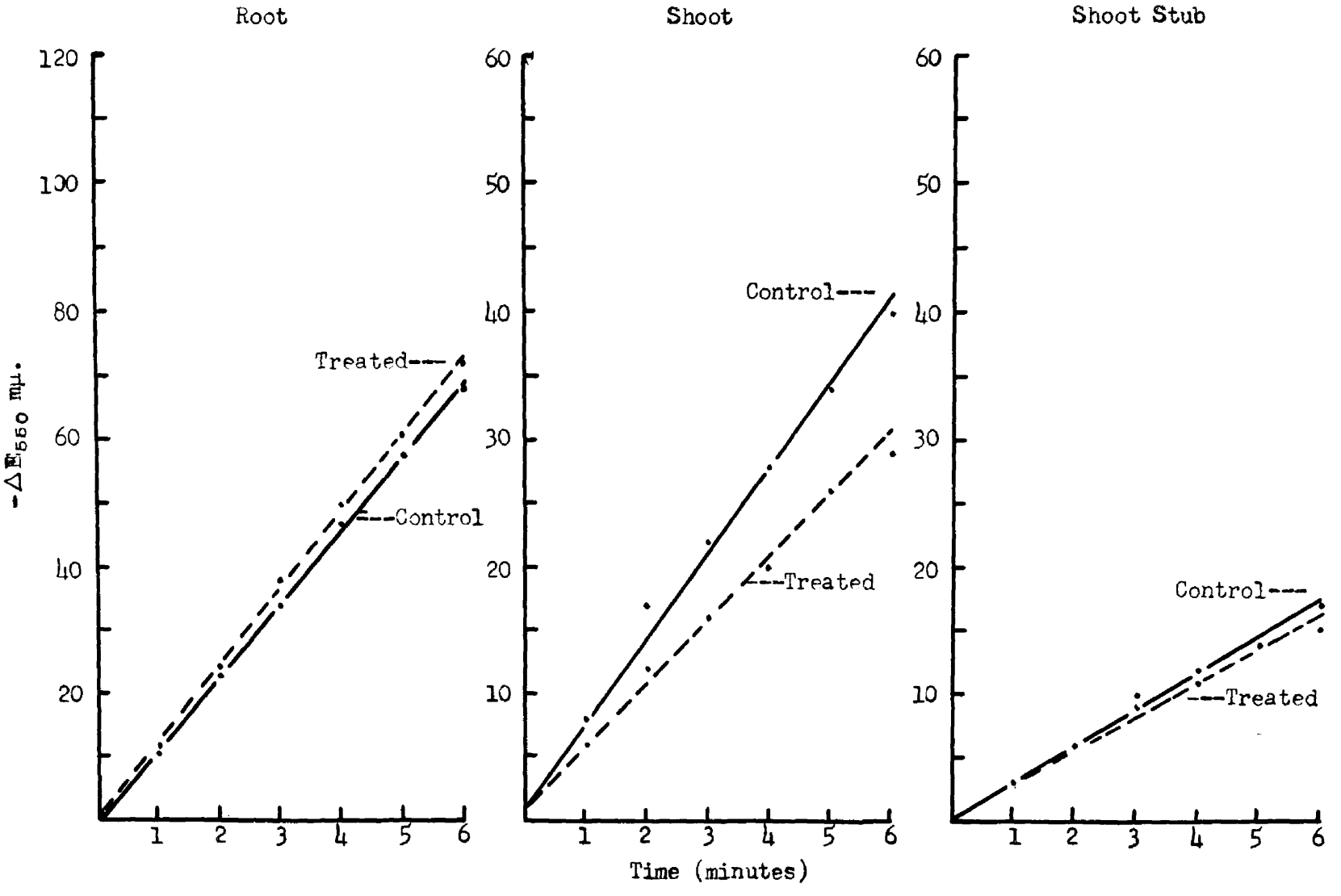


Figure 3. The cytochrome oxidase activity of control and maleic hydrazide treated radish tissues. The reaction mixture contained 0.4 ml. of 10^{-4} M reduced cytochrome c. $-\Delta E_{550}$ mμ. values represent the decrease in optical density x 1000.

was used since higher concentrations did not reflect any greater rate of oxidation of cytochrome c.

Additional studies on the cytochrome system in the preparations were conducted in which the DPNH oxidase system was fortified with added cytochrome c. Figure 4 shows the increase in optical density at 550 mμ. in a system where cytochrome oxidase is not inhibited. Cytochrome c was used at a final concentration of 1.3×10^{-5} M. Both types of root preparation show a parallel reduction of cytochrome c with the oxidation of DPNH (Figure 4). In the case of the shoot preparations, where the DPNH oxidase of the treated preparation was about one-half that of the corresponding control, a lower rate of reduction of cytochrome c in the treated preparation suggests a lowered cytochrome c reductase activity. In the instance of the shoot stub, also, the treated preparation exhibited a lower DPNH oxidase activity but the rate of reduction of cytochrome c is essentially the same, indicating that the cytochrome c reductase system in both preparations are at about equal levels. For all tissue preparations, evidence of cytochrome oxidase was not apparent over the 6 minute assay period.

Cytochrome c. A relatively adequate amount of endogenous cytochrome c must be assumed in the root tissues since added cytochrome c did not change the rate of oxidation of DPNH appreciably (Figure 5). However, a high cytochrome c concentration in the tissue extracts, prepared in the manner described in the experimental section, was not expected. The fractionation procedure was designed to remove most soluble tissue metabolites. The addition of cytochrome c, 1.3×10^{-5} M, did accelerate

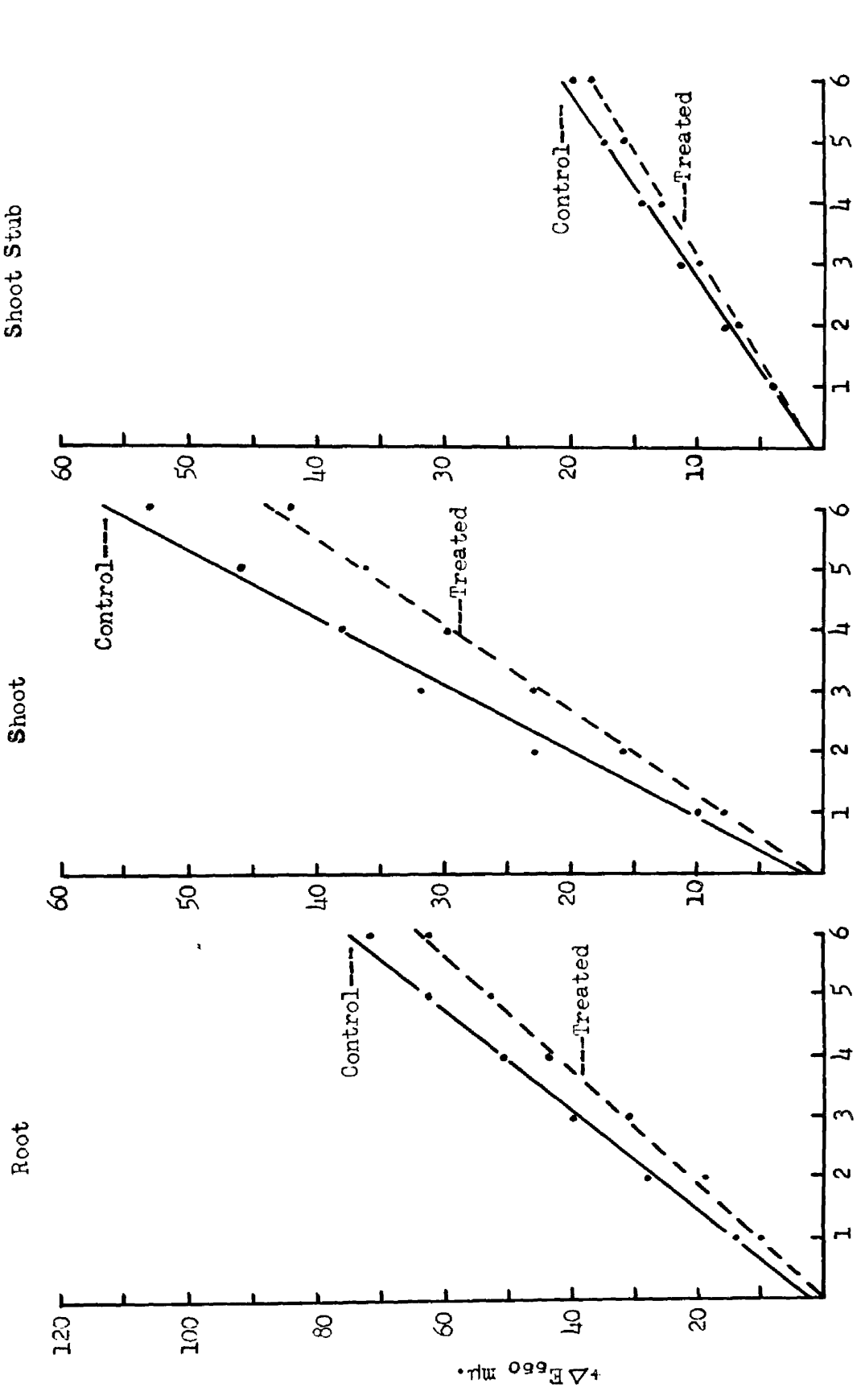


Figure 4. The effect on the absorption at 550 mμ. produced by the addition of 0.4 ml. of 10^{-4} M cytochrome c to the DPNH oxidase system of control and maleic hydrazide treated radish tissues. $+\Delta E_{550}$ mμ. represent the increase in optical density x 1000.

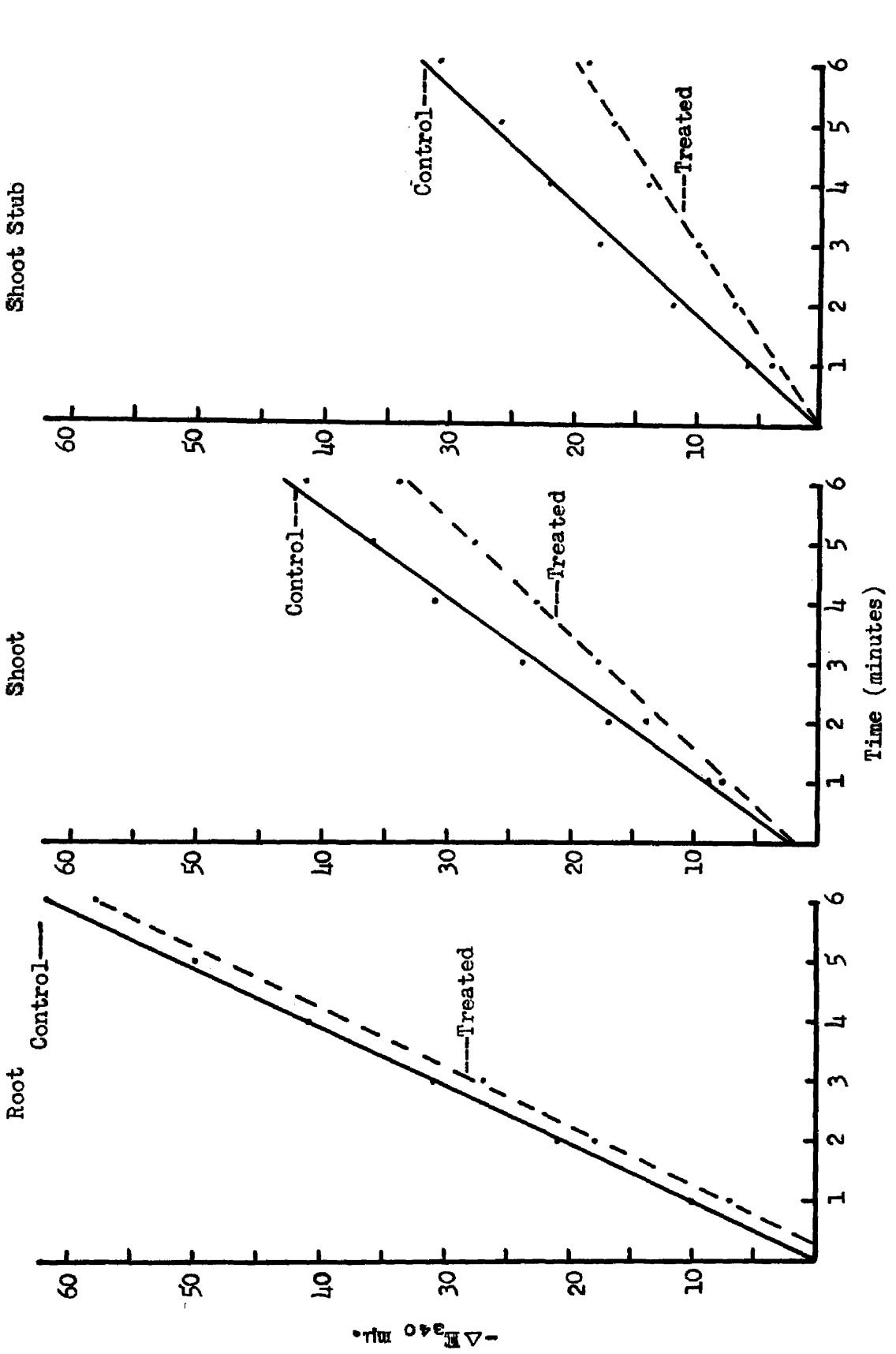


Figure 5. The effect on the absorption at 340 mμ. produced by the addition of 0.4 ml. of 10^{-4} M cytochrome c to the DPNH oxidase system of control and maleic hydrazide treated radish tissues. $-\Delta E_{340}$ mμ. values represent the decrease in optical density x 1000.

the oxidation of DPNH somewhat in both the shoot and shoot stub tissue preparations. However, added cytochrome c did not bring the level of oxidation of DPNH in the treated preparations up to the level of the corresponding control. The saturation concentration of cytochrome c for optimum activity of the DPNH oxidase system is not known. Since cytochrome c concentration greater than 1.3×10^{-5} M did not increase cytochrome oxidase activity, it is assumed that at this concentration of cytochrome the DPNH oxidase system was saturated with respect to this component. Lehninger (95) has indicated that 1.5×10^{-5} M cytochrome c is a saturating concentration for optimum activity of the succinoxidase system studied.

It should be noted that any acceleration observed in systems supplemented with cytochrome c is subject to interpretation since an absolute correlation between the amount of acceleration noted and the original cytochrome c content cannot be made with certainty since a number of factors directly influence the activity of the whole respiratory system. The catalytic effect of added cytochrome c on the DPNH oxidase was reported by Slater (70) who obtained an appreciable increase in activity when cytochrome c was added to enzyme preparations. A similar stimulation has been noted by other investigators (94, 95).

More information on specific activities of the components of the DPNH oxidase system was obtained by studying systems containing 1.3×10^{-5} M methylene blue (without cyanide) to facilitate the oxidation of reduced diaphorase. The stimulation observed (Figure 6) is markedly more than that observed by the addition of a like molar concentration of cytochrome c (Figure 5). It should be noted in the root preparations that

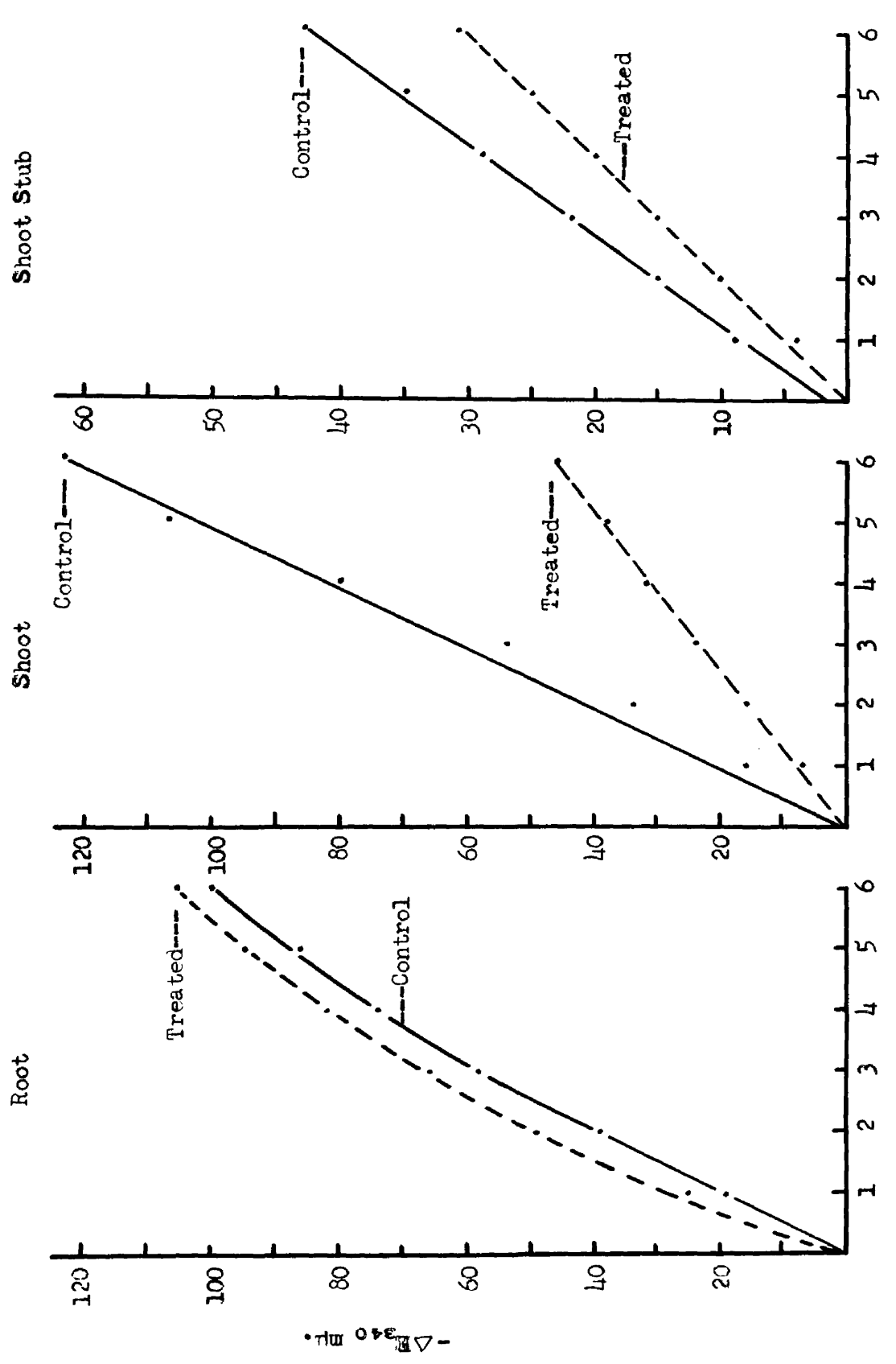


Figure 6. The effect on the absorption at 340 mμ. produced by the addition of 0.4 ml. of 10^{-4} M methylene blue to the DPNH oxidase system of control and maleic hydrazide treated radish tissues. $-\Delta E_{340}$ mμ. values represents the decrease in optical density x 1000.

an equivalent stimulation was noted for each type of preparation. This would suggest equivalent diaphorase activity for these preparations (compare Figure 2). However, in the case of the shoot stub preparations, the treated tissue activities fall below that of the control and suggest a somewhat lower diaphorase activity (compare Figure 2). The results in the case of the shoot preparations suggest a marked difference in diaphorase activity of the treated system. However, it will be noted from Figure 2 that the diaphorase activity of the two preparations is nearly the same. This seemingly divergent result may be explained since, in the case of the diaphorase determination, the cytochrome system was inhibited by the addition of cyanide with methylene blue functioning to oxidize the reduced diaphorase. In the case of the results in Figure 6, both the cytochrome system (which was inhibited in the treated shoot tissue, see Figures 3 and 7) and methylene blue are functioning to oxidize the reduced diaphorase.

DPNH-cytochrome c reductase. Cytochrome c reductase determinations showed that only in the case of the shoot tissue preparations was there any difference between activities of the treated preparations and their respective controls (Figure 6). This difference is small. Since the diaphorase activity of the treated preparations (shoot stub) was lower, it is surprising that the DPNH-cytochrome c reductase is not lower also. (This divergence may be explained as a catalytic effect of added cytochrome c in cytochrome c reductase which was not true in the diaphorase assay. However, this was not true of the other preparations studied.)

It should be noted from Figure 3 that the rate of reduction of cytochrome c (in the absence of cyanide) is lower only in the treated

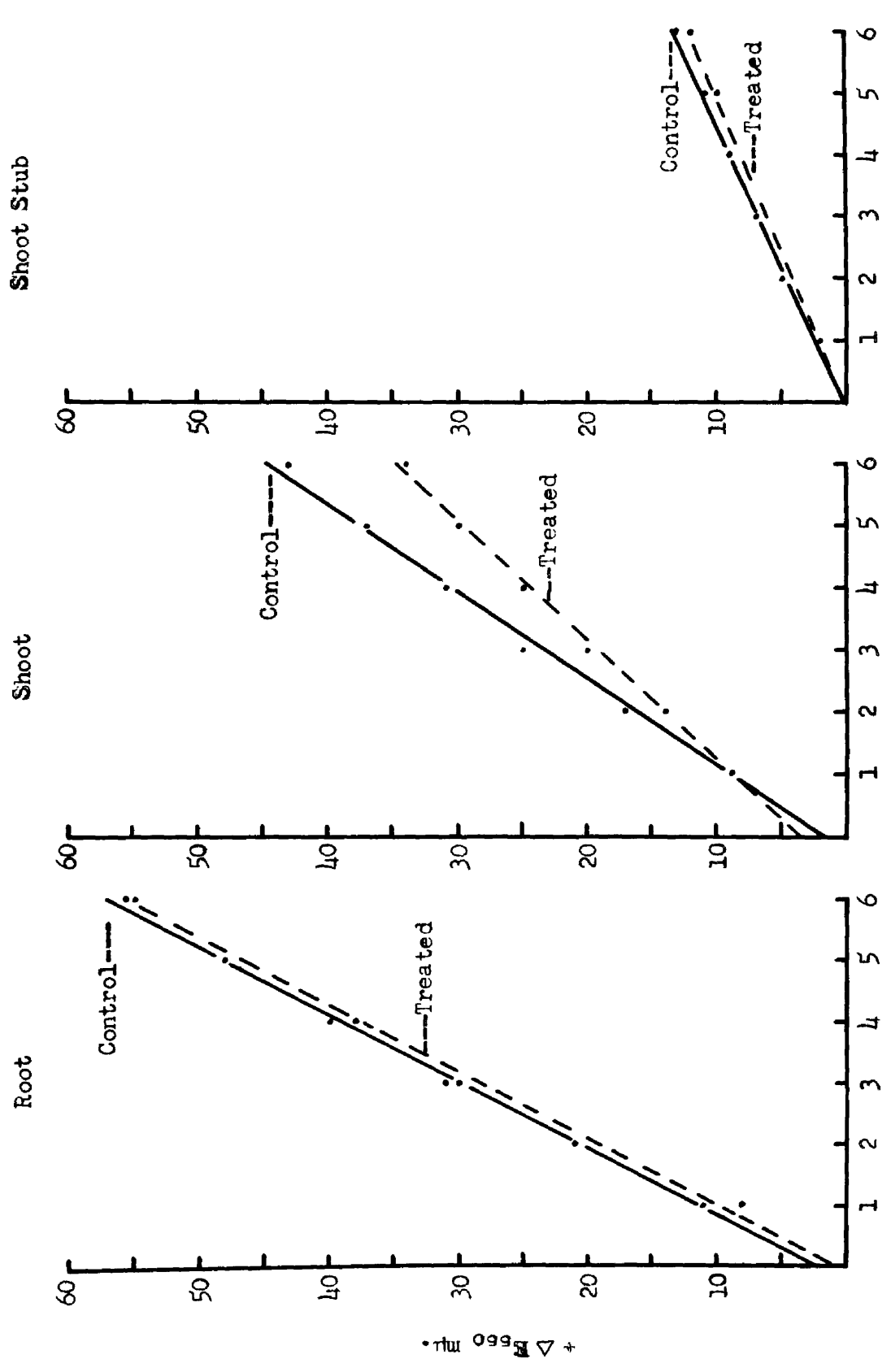


Figure 7. The DPNH-cytochrome c reductase activity of control and maleic hydrazide treated radish tissues. The reaction mixture contained 0.4 ml. of 10^{-4} M cytochrome c and 0.3 ml. of 2 x 10^{-3} M potassium cyanide. $+\Delta E_{650}$ mμ. values represent the increase in optical density x 1000.

shoot preparations. Direct measurement of DPNH cytochrome c reductase corroborates this observation. Although a lower diaphorase activity in the case of the treated shoot stub preparation was noted, a failure of any of the components constituting the factor(s) which mediates the system between diaphorase and cytochrome c was not striking even in the case of the treated shoot. The nature and mechanism of action of the intermediates involved in this transformation is not well elucidated. Some studies have implicated cytochrome b and the BAL-sensitive factor (antimycin sensitive factor), which operates between cytochromes b and c in the succinoxidase system also functions in the DPNH oxidase system between diaphorase and cytochrome c. In support of this, Slater (70) has shown that 0.01 M BAL inactivates the DPNH oxidase system to the extent of 94.6 per cent.

It should be noted that in the DPNH-cytochrome c reductase determination cyanide was added to inhibit the action of cytochrome oxidase. Potter (96) reported that cytochrome c combines with cyanide in such a way that it can not be reduced enzymatically. The formation of a cytochrome-cyanide complex has been further described by Horecker and Kornberg (97). The effect of cyanide on the system being investigated may be questionable.

TABLE VII

RELATIVE ACTIVITIES OF THE DPNH OXIDASE SYSTEM AND CONSTITUTIVE ENZYMES

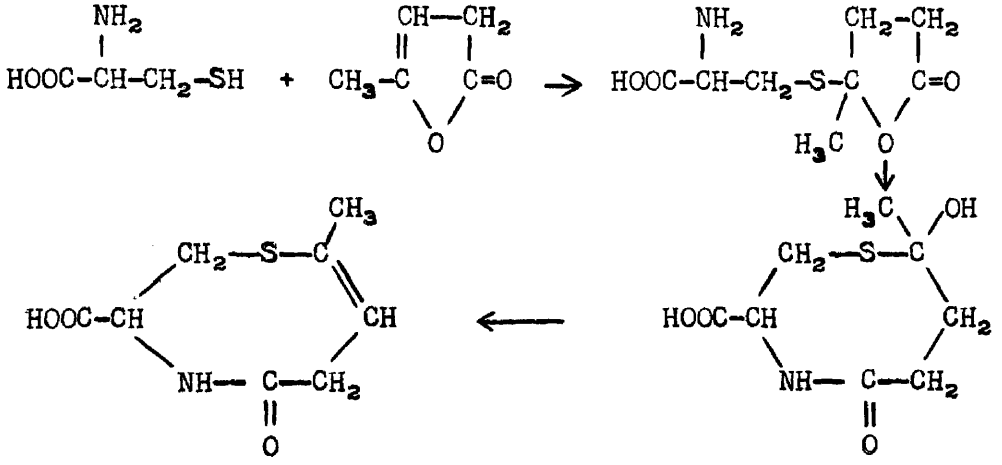
Tissue	DPNH[1] oxidase	Dia-phorase	DPNH-cytochrome c reductase	Cytochrome oxidase
Root (Control)	++++	+++++	+++	+++
Root (MH-treated)	++++	+++++	+++	+++
Shoot (Control)	++++	+++++	++++	++++
Shoot (MH-treated)	++	+++++	+++	+++
Shoot stub (Control)	++	++++	+	++
Shoot stub (MH-treated)	+	+++	+	++

[1]The number of + signs indicates only the relative magnitude of the respective activities. Actually only the activities between control and treated of one type of tissue should be compared. The relative value of the + sign in terms of ΔE is approximately the same throughout, except for the diaphorase activity.

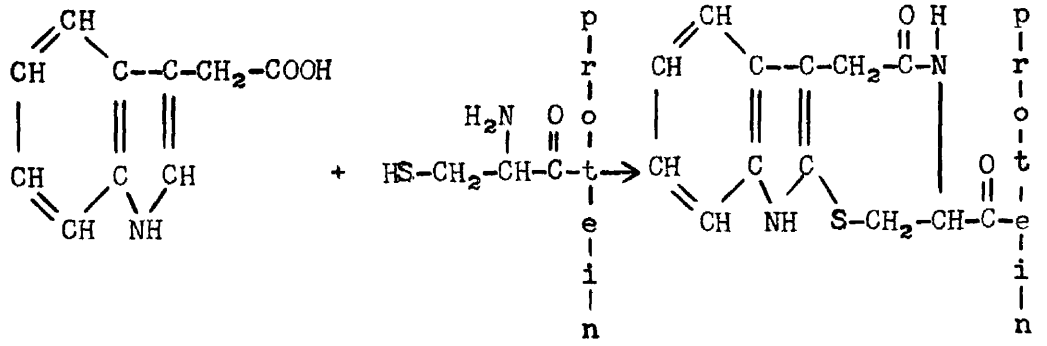
Maleic Hydrazide and Thiol Compounds

Introduction

A variety of unsaturated lactones of the general structure  are known to inhibit growth of plants (98, 99). Cavallito and Haskell (100) have shown that these lactones react with cysteine according to the following mechanism:

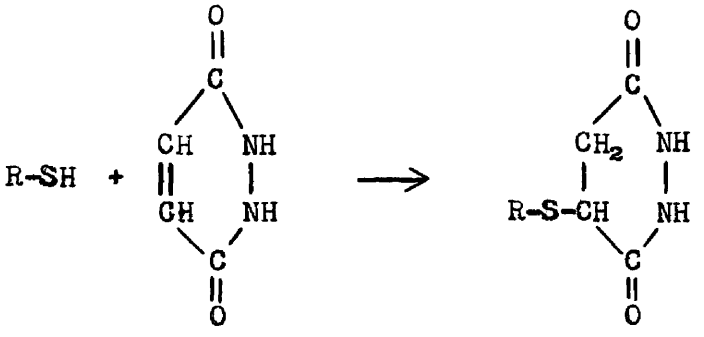


Thus, the inhibiting effect of the lactones on growth could be due to their reaction with the cysteinyl unit of a protein which prevents the growth regulator from reacting at that point. If the growth regulators activate an enzyme system by reaction with a cysteinyl part of a protein molecule as follows:

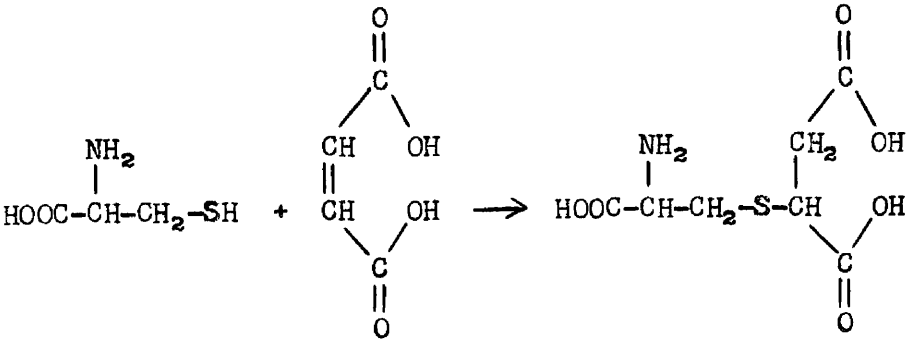


Then an unsaturated lactone, iodoacetate, arsenite or any substance which effectively compete with the growth regulator for the cysteinyl enzyme unit would inhibit growth provided the cyclic reaction product would not operate in the next step of the growth reaction.

Muir and Hansch (101) have suggested that it seems likely that the growth inhibitor, maleic hydrazide, reacts in the same fashion as the lactones by reaction with a sulfhydryl group as follows:



There are many instances of reaction between sulfhydryl groups and activated double bonds. Morgan and Friedmann (102) have shown that maleic acid will react with a variety of thiol compounds and that cysteine reacts in 93% yields as follows:



Later Hopkins and collaborators (103, 104) showed that maleic acid also reacts with the sulfhydryl groups of proteins and could thus inhibit

the action of succinic dehydrogenase. Although dehydrogenases in general contain sulfhydryl groups (79) other dehydrogenases investigated were not inhibited by the disappearance of free sulfhydryl groups. This was interpreted to mean that although a free sulfhydryl group was essential to succinic dehydrogenase, this is not necessarily true for other dehydrogenases.

Muir and Hansch (101) further suggested that undoubtedly a study of other stable organic compounds with double bonds activated by groups such as amides, esters, ketones and nitriles would lead to the discovery of many compounds quite toxic to plant cells.

Naylor and Davis (54) reported that maleic hydrazide had a deleterious effect on root tip respiration and suggested that it would seem entirely possible that it exerts its influence on growth by inhibiting respiration. Just what portion of the oxidative metabolism of the plant is affected was not indicated but they speculated that a dehydrogenase was in some way prevented from functioning normally. The speculative nature of this suggestion should be emphasized.

Isenberg, Odland, Popp and Jensen (47) reported that a number of dehydrogenases were inhibited in tissue obtained from maleic hydrazide treated plants. However, it should be pointed out that these workers used the 2,3,5-triphenyltetrazolium chloride as a means of measuring dehydrogenase activity. It has been shown by Brodie and Gots (105) that the transfer of hydrogen from enzymatically reduced DPN to triphenyltetrazolium chloride is mediated by diaphorase; thusly, inhibition of only diaphorase could appear as dehydrogenase inhibition. The long incubation period used by Isenberg and coworkers also leads to some uncertainties as to the results.

In a later publication, Isenberg and coworkers (48) presented more convincing evidence that maleic hydrazide treatment did suppress respiration and depress succinic dehydrogenase activity. In fact, these investigators reported that low concentration enhances both respiration and succinic dehydrogenase activity while just the reverse is true at higher maleic hydrazide concentrations.

Marré (50) reported that maleic hydrazide inhibits dehydrogenase activity in the presence of various substrates although the results were erratic. In another report (49) this same author indicated that maleic hydrazide in concentrations near those that reversibly inhibit growth *in vivo* have inhibited the activity of a preparation of dehydrogenase *in vitro*. Indoleacetic acid appeared to be able to reverse the inhibiting effect of maleic hydrazide upon the dehydrogenase system.

It is true that the suggestion of Muir and Hansch (101) that maleic hydrazide reacts in the same manner as lactones and maleic acid in adding sulfhydryl compounds at the double bond appears inviting. However, the similarity in structures may be misleading with respect to the ease of addition at the double bond. In view of the published reports reviewed above concerning the possible inhibition of dehydrogenase activity by maleic hydrazide, it seemed desirable to ascertain if compounds carrying a free sulfhydryl would indeed add to the double bond of maleic hydrazide. The absence of any data in regard to this problem made this area of investigation seem necessary.

Analytical Methods

A technique similar to that of Morgan and Friedmann (102) was employed for the study of the interaction of maleic hydrazide with thiol compounds. A stock solution of maleic hydrazide was prepared by dissolving 14.01 g. of maleic hydrazide in 300 ml. of water, adjusting the pH to 7 and finally making to a volume of 500 ml. (40 ml. is equivalent to 0.01 mole of maleic hydrazide). One-hundreth mole of the sulfhydryl containing compound was added to 40 ml. of stock solution. The pH was again adjusted to 7 and the resulting solution diluted to 50 ml. A tissue homogenate was prepared by macerating radish shoot tissue with two weight equivalents of water. The boiled tissue homogenate was heated in a boiling water bath for 10 minutes. In the studies involving the use of the tissue homogenates, 0.01 moles of both mercaptoacetic acid and maleic hydrazide and 10 g. of tissue homogenate were contained in a volume of 50 ml. and incubated at a pH of 7. A 1.0 ml. aliquot was titrated at zero time and at various time intervals after incubation at 37°. Titrations were carried out by adding a 1.0 ml. aliquot to 5.0 ml. of 0.01 N sulfuric acid. Twenty milliliters of 0.02 N iodine was added and the excess iodine back titrated with a standard sodium thiosulfate solution using starch as the indicator. Blank solutions were prepared which contained only maleic hydrazide, thiol compound or homogenate in amounts equivalent to that used when in combination. Maleic acid was used in place of maleic hydrazide under like conditions as a check on the experimental procedure.

Results and Discussion

The data in Table VIII clearly demonstrate that thiols do not add readily to the double bond of maleic hydrazide under the experimental conditions used. In the case of mercaptoacetic acid there is a small decrease in titratable thiol groups following incubation, however, this decrease can be accounted for in the blank. This probably arises from the slow oxidation of the thiol compound during long incubation. In marked contrast are the results with maleic acid which is known to add thiols at the double bond (102). In this instance after only four hours of incubation approximately 60 per cent of the thiol groups can no longer be titrated with iodine. The longer periods of incubation show a slower but continuous decrease in titratable groups indicating a disappearance of the thiol groups through interaction with maleic acid. The data of Table VIII also show that such other thiols as cysteine and glutathione do not add readily to the double bond of maleic hydrazide. Additionally, the studies using tissue homogenates indicated that under the experimental conditions prevailing, no biological system in the tissue was capable of catalyzing the addition of thiol with maleic hydrazide or converting the maleic hydrazide into a compound that would add thiol.

Morgan and Friedmann (102) in a study of the interaction of thiols with unsaturated compounds found that while maleic acid readily reacted with thiol compounds, the following unsaturated acids did not: citraconic, mesconic, cis and trans cinnamic.

TABLE VIII

INTERACTION OF MALEIC HYDRAZIDE AND THIOL COMPOUNDS

(Results expressed as milliequivalents x 10^3 of iodine oxidized by the thiol compound in a 1.0 ml. aliquot of solution)

System components	Time (hours)			
	0	4	7	24
Maleic hydrazide (blank)	0	3	3	5
Mercaptoacetic acid (blank)	192	192	193	191
Maleic acid (blank)	2	3	-	3
Maleic hydrazide and Mercaptoacetic acid	192	191	193	189
Maleic hydrazide and Glutathione (G-SH)	189	190	190	186
Maleic hydrazide and Cysteine	188	187	189	186
Maleic acid and Mercaptoacetic acid	191	67	52	31
	Time (days)			
	0	1	2	4
Mercaptoacetic acid (blank)	185	184	-	182
Maleic hydrazide and Mercaptoacetic acid	190	189	-	187
Shoot tissue homogenate	-	-	4	1
Boiled homogenate	-	-	3	-
Homogenate and Mercaptoacetic acid	183	183	185	182
Boiled homogenate and Mercaptoacetic acid	185	183	187	185
Homogenate, Mercaptoacetic acid and Maleic hydrazide	186	186	187	185
Boiled homogenate, Mercaptoacetic acid and Maleic hydrazide	184	184	185	187

These results indicate that the suggestion of Muir and Hansch (101), that maleic hydrazide inhibits growth by reacting with sulfhydryl groups, is not a very probable one. This assumes, of course, that maleic hydrazide remains as such in the biological system. These authors' suggestion was based primarily on an analogy between the interaction of thiol compounds and maleic acid and the probable interaction of thiols and maleic hydrazide. This analogy is not a correct one.

SUMMARY

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A preharvest foliar application of a 0.02 M solution of maleic hydrazide does inhibit new shoot and root growth of topped radishes (Raphanus sativus) harvested 48 hours after treatment and subsequently stored.

A study of the carbohydrase enzyme components in the radish root indicated that maleic hydrazide has had no observable effect on the beta amylase, phosphorylase or phosphatase activity in these tissues. Alpha amylase and pectin-methyl-esterase activity was not detected.

Chemical analysis of the gross nutritional components of both the shoot and root tissues of control and maleic hydrazide treated plants revealed that dry weight, ether extract, Kjeldahl nitrogen, reducing and non-reducing sugars and polysaccharides other than starch had not been changed significantly by chemical treatment. The starch content of the shoot tissue was nearly doubled in the treated samples as compared to the non-treated samples.

A comparative quantitative study of the ability of an acid treated extract of various control and maleic hydrazide treated radish tissues to oxidize DPNH is presented. The oxidative system was analyzed quantitatively for the activity of its known constitutive enzymes; that is (a) diaphorase, (b) DPNH-cytochrome c reductase and (c) cytochrome oxidase.

The DPNH oxidase activity was the same in both the control and treated root tissue. The activity of the constitutive enzymes was also the same in both types of tissue.

The maleic hydrazide treated shoot tissue exhibited a marked inhibition of the DPNH oxidase system. This inhibition resulted from partial failures in both DPNH-cytochrome c reductase and cytochrome oxidase systems.

The DPNH oxidase activity of the treated shoot stub tissue was somewhat lower than that of the corresponding control tissue. This inhibition resulted from a reduced activity of the diaphorase system. The activity of the cytochrome systems was the same for both the control and maleic hydrazide treated tissue.

An investigation of the in vitro interaction of thiols and maleic hydrazide indicated that no addition compound resulted from the incubation of maleic hydrazide and thiols at a pH of 7 even though the addition of thiol and maleic acid was noted. Tissue homogenates had no effect on the system investigated for the possible interaction of thiol and maleic hydrazide.

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