MECHANISM OF NAPHTHALENEACETIC ACID ACCUMULATION BY SOYBEAN (GLYCINE MAX MERR.) LEAF CELLS WITH SPECIAL EMPHASIS ON TISSUE PREPARATION AND VIABILITY

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

MARK LEE BRENNER

1969

THESIS

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MECHANISM OF NAPHTHALENEACETIC ACID ACCUMULATION BY SOYBEAN (GLYCINE MAX MERR.) LEAF CELLS WITH SPECIAL EMPHASIS ON TISSUE PREPARATION AND VIABILITY

presented by

MARK LEE BRENNER

has been accepted towards fulfillment
of the requirements for

PhD degree in HORTICULTURE

Martin J. Bukovac
Major professor

Date 12/30/69

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MECHANISM OF NAPHTHALENEACETIC ACID ACCUMULATION BY SOYBEAN (GLYCINE MAX MERR.) LEAF CELLS WITH SPECIAL EMPHASIS ON TISSUE PREPARATION AND VIABILITY

By

Mark Lee Brenner

The mechanism of naphthaleneacetic acid (NAA) accumulation by leaf cells was studied with special consideration being devoted first to the improvement of methods of tissue preparation and cell isolation. A new treating medium that optimized maximum leaf tissue metabolic activity was formulated using neo-tetrazolium reduction as a quantitative index. Leaf strips, which were cut cross-sectionally 250 μm thick from a 25 mm wide piece of tissue, were used for development of a medium and as a basis of comparison to the several different preparations of isolated cells. Enzymatically isolated leaf cells exhibited approximately 50% of the viability of the leaf strip tissue, while mechanically isolated cells had minimal activity. Viability comparisons were based on oxygen exchange rates in the

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light and dark, NAA and PO_4^{3-} uptake, $^{14}CO_2$ fixation (total, and incorporation into protein), ^{14}C-acetate catabolism, tetrazolium reduction, and light and electron microscopic examinations. Polarographic analysis of light and dark oxygen exchange rates provided a rapid and reliable index of leaf tissue viability. The mechanism of NAA accumulation by the leaf strips was examined first to provide a background of NAA accumulation and a basis for comparison to isolated cells. NAA was rapidly metabolized into a compound that chromatographed identical to naphthalene-acetyl aspartate (NAAsp) and this compound appeared to play a dominant role in NAA accumulation. Once an internal concentration of about 100 pmoles of NAA/10 mg dry weight of tissue was established, the uptake rate increased rapidly. This surge was dependent upon the interaction of time and external NAA concentration. By use of metabolic inhibitors and substrates it appeared that an enzyme (likely L-aspartic acid-N-acylase) was being either activated or synthesized. Results with low temperature and metabolic inhibitors supported the concept that NAA uptake required metabolic energy. Light had a slight depressing effect on uptake. The conjugated NAA was bound tightly within the tissue. NAA uptake by enzymatically isolated leaf cells was also found related to formation of NAAsp. Unlike leaf strips, the

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MECHANISM OF NAPHTHALENEACETIC ACID ACCUMULATION BY SOYBEAN (*GLYCINE MAX* MERR.) LEAF CELLS WITH SPECIAL EMPHASIS ON TISSUE PREPARATION AND VIABILITY

By

Mark Lee Brenner

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1969

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To my wife, Ruth, I wish to express my sincere gratitude for her patience, encouragement, and support.

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INTRODUCTION

Biologically active compounds play a major role in modern agricultural practices today and their importance continues to grow. Many of these materials are applied as foliar sprays, and their effectiveness is often totally dependent upon the amount of material that penetrates into actively growing tissue. As Audus (1967) has stated that, "leaf penetration is fraught with technical difficulties", and perhaps the greatest lack of knowledge is how growth substances are accumulated in the leaf parenchyma tissue. The objective of this thesis is to study the mechanisms of foliar uptake of α-naphthaleneacetic acid (NAA) used as a model compound from the larger class of auxin type growth regulators.

Several methods have been reported for the isolation of leaf cells (Jyung et al., 1965b; Takebe et al., 1968; Price, 1967). Unfortunately none of these workers critically examined the viability of their cell preparations. The viability of these cell preparations has been questioned (Jacoby and Dagan, 1967) and a more reliable intact system

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(Smith and Epstein, 1967) has been recommended by Jacoby and Dagan (1967). Recognizing the desirability of a system using viable isolated leaf cells, a considerable effort has been directed toward the refinement of the methods of cell isolation. Special emphasis was directed to making an accurate assessment of the viability of the isolated cell preparation comparing it to an intact leaf system.

NAA uptake by stem sections has been briefly studied to establish some of the basic parameters that can be used for studying NAA accumulation by leaf tissue. Considerable detail was given to uptake of NAA by leaf strips and leaf cells.

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LITERATURE REVIEW

I. Uptake

A. Introduction

Studies on the mechanisms of molecular and ion uptake into living tissue have been voluminous. The intent of this thesis is not to review these works in a detailed manner or attempt to answer the many unsolved problems they have revealed. Rather, it is the purpose to summarize the basic aspects of uptake mechanisms, particularly in plant tissue, as a point of reference and terminology for the ensuing study of auxin uptake by leaf tissue and isolated cells. Monographs written by Stein (1967) and Troshin (1966) covered general aspects of penetration, mainly for animal cells, emphasizing problems of membrane permeability. Briggs et al. (1961), Sutcliffe (1962), Jennings (1963), and Wallace (1963), have written monographs on uptake mechanisms that are common to plant cells. Robertson (1968) in a brief monograph, emphasized charge separation in plant cells and how it relates to metabolic energetics and ion transport. Shorter reviews on uptake by plant cells have

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been presented by Arisz (1963), Brouwer (1965), and Leggett (1968).

The simplest subdivision of uptake mechanisms has been into passive and active components. Passive mechanisms imply those processes that can occur without the expenditure of metabolic energy, while active uptake involves the direct expenditure of metabolic energy to move a molecule against an electro-chemical potential (Ussing, 1949).

B. Cell Structure

The various barriers discussed in this thesis are better understood by first describing the structure of the plant cell with particular reference to its membranes. The cell is bound externally by a fibrous structure of cellulose intermixed with pectins and hemicellulose called the primary wall (Esau, 1965). The fibral net-like structure has no specific orientation and has many openings in the order of 100 Å, which means the wall will allow the passage, with little resistance, of small molecules (Briggs et al., 1961). Covering the primary wall is a layer of pectinaceous material called the middle lamella, which cements adjacent cells together (Esau, 1965). In older, woody cells, a secondary wall composed of lignin develops under the primary wall.

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Within the wall structure lies the external limiting membrane of the cell, the plasmalemma. This is the first true membranous structure a penetrating molecule must traverse. Most mature parenchymous cells contain a large vacuole bounded by a membrane called the tonoplast. Between the tonoplast and the plasmalemma is the cytoplasm, containing various organelles that carry on practically all of the cell's life sustaining functions. For a review of the structure and function of cellular organelles see Pridham, 1968. The endoplasmic reticulum appears as a continuous membrane structure within the cytoplasm. It establishes many zones or areas within the cytoplasm restricting the various organelles to limited paths during cytoplasmic streaming (Northcote, 1968).

The general structure of these membranes, all double layers, is currently (Branton, 1969) considered to be a bimolecular leaflet system with a layer of lipid bound on either side with protein (PLP), as proposed by Davson and Danielli (1943). These membranes could have specific areas of specialized structure to allow for various required functions (Branton, 1969). This hypothesis is in agreement with the sub-unit theory for membranes, but would limit their occurrence to specific areas of the cell's respective membranes. Northcote (1968) has reviewed the

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various plant cell membranes with special emphasis on the chloroplast membrane, which has a definite sub-unit matrix. Stadelmann (1969) concurs with this concept.

C. Passive Uptake

Brouwer (1965), among others, has specified that passive uptake occurs in the following two fractions for plant tissue: a) ions held in water free space, and b) ions retained in Donnan free space.

Water free space is that volume in the plant cell or tissue that may be easily penetrated by molecules from the surrounding medium. The molecules are in equilibrium and can be removed easily by washing with distilled water. The cell wall and intercellular spaces are thought to be the prime location for water free space (Briggs et al., 1961).

Donnan free space, believed to be mainly located in the wall and outer edge of cytoplasm of the plant cell (Briggs et al., 1961 and Sutcliffe, 1962), is that volume where freely diffusible ions are retained by electrostatic charges. The pectins, with their free carboxyl groups are often neutralized by calcium and magnesium. Dainty and Hope (1959) believed that these would be excellent sites for such ion exchange reactions.

The total number of molecules retained by a cell in the above two categories represents collectively the apparent

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Brouwer (1965) suggested the existence of a third fraction that is also passively accumulated. Namely, these are molecules that would accumulate within the cell plasmalemma.

Several mechanisms that could explain how passive uptake proceeds have been reviewed by Sutcliffe (1962). These include diffusion, mass flow, ion exchange, Donnan equilibrium adsorption, and chemical combination. Facilitated diffusion can also be a very important mechanism for passive uptake (Jennings, 1963, and Stein, 1967).

Diffusion as expressed by Fick's law is the movement of one component randomly within another. When speaking of a molecule passing through a membrane, the terminology changes slightly to factors of permeability instead of diffusion, thereby inferring a given barrier for the molecules moving through. This is an area that has been extensively studied. Stein (1967), in his treatise, gave an excellent review for animal tissue, while Stadelmann (1969) reviewed the same field for plant tissue. Milborrow and Williams (1968) reexamined Collander's (1954) permeability data using regression analysis and concluded that his equation for permeability of a non-electrolyte was basically correct if one includes a quadratic component for

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the partition coefficient. This means that permeability is dependent on a $\log_{10}$ function of the partition coefficient of the molecule in a quadratic expression minus a log function for the molecular weight. Most of Collander's observed data were fitted to such an equation with a 95% confidence level, providing they first rejected all molecules that were possibly metabolically transported or complexed. They agreed with Collander that olive oil: water, rather than ether: water was the best approximation for an artificial partition system with which to match the data of the plant membrane (Nitella cells). This meant that the permeability of a molecule through a plant membrane is dependent mainly on a complex function of the molecule's partition coefficient into the membrane divided by a function of the molecule's molecular weight.

Facilitated diffusion is a non-metabolic selective movement of molecules across a cell membrane, but not against a concentration gradient (Miller, 1960). Facilitated diffusion, as proposed by Danielli (1952) and reviewed by Jennings (1963), denotes that the penetrating molecule has to acquire a certain kinetic energy to pass into and then back out of the respective membrane. Thermal agitation would be one source of this energy. This results in a Q_{10} of about 2 to 3.

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The movement of one type of molecule along with the mass of a second type, is mass flow (Sutcliffe, 1962). The second type is driven by a physical force such as gravity, diffusion, or ionic charge.

Adsorption is the orientation of a molecule on the surface of a second type of material in such a manner that the surface tension at the interface is reduced (Sutcliffe, 1962).

Chemical combination occurs when the penetrating molecule is complexed by a modification of the adsorption mechanism just mentioned. This process requires usually more energy than adsorption to form the bond and therefore shows a higher Q_{10} than the simple adsorption system (Sutcliffe, 1962).

Finally, ion exchange, as mentioned earlier, is the mechanism whereby molecules of like charge exchanged (Jennings, 1963). Donnan equilibrium is a specialized ion exchange mechanism that allows for the movement of ions against a concentration gradient, but has a neutral electrochemical potential (Sutcliffe, 1962).

D. Active Uptake

Compared to the knowledge on passive mechanisms, little is known about the specific components of active uptake for plant cells. The basic premise of active uptake is that

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molecules move across an electro-chemical gradient with the energy being derived metabolically. One of the more commonly held concepts implicates various enzymatic-like carrier mechanisms (Sutcliffe, 1962).

Epstein and Rains (1965), Rains and Epstein (1965), and Epstein (1966) have described what is considered as one of the best examples of a specific carrier mechanism that is operative in plant root tissue. Without eluding to its actual nature, they have described a dual mechanism for Na^+ and K^+ uptake using enzyme kinetics. At low ion concentrations only K^+ is transported (a high affinity enzyme-mechanism I), while at ionic concentrations of 1 m$\underline{M}$ or greater, a second mechanism (low affinity-mechanism II) transports, with less specificity, either K^+ or Na^+. Torii and Laties (1966) (for a review cf. Laties, 1969), have also found the same dual mechanism in the corn root. They postulated that mechanism I lies in the plasmalemma while mechanism II probably functions in the tonoplast. Welch and Epstein (1969) have presented kinetic data that localizes both mechanisms in the plasmalemma. Pitman (1967) questioned these results, since data were obtained on salt depleted roots. Leggett (1969) also questioned the universality of Ca^{2+} in the treating medium, when it is now known that Ca^{2+} reduces passive permeability of membranes

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The above difference of opinion between Epstein's group and that of Laties is representative of the controversy on where the permeation barrier exists. Jennings (1963) offered a fine discussion on this subject, but the problem remains unresolved.

Sutcliffe (1960) reported that chloramphenicol at relatively high concentrations (1 to 2 mg/ml) retarded net ion accumulation by beet and carrot root sections. Similar results were reported for root tissue (Jacoby and Sutcliffe, 1962; Peaud-Lenoel and Gourmay-Margerie, 1962; Uhler and Russell, 1963) and several photosynthetic tissues (Parthier, 1965; Jyung et al. 1965a; Brenner and Maynard, 1966). All concluded that there was a possible direct linkage of protein synthesis with ion uptake. As Sutcliffe (1962) hypothesized, the mechanism of protein turnover (hydrolysis and resynthesis) could provide many sites for ions to be bound and subsequently released. Several of the above authors (Uhler and Russell, 1963; Peaud-Lenoel and Gourmay-Margerie, 1962) reported that chloramphenicol at high concentrations did not affect respiration. Since most of the above-mentioned studies were carried out under relatively sterile conditions, the effect of bacteria has been ruled out.

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Hanson and Hodges (1963) reported that chloramphenicol (0.8 mg/ml, 2.7 mM) uncoupled oxidative phosphorylation of corn shoot mitochondria. They found that while phosphorylation was always reduced, oxygen consumption was not affected. Thus, following respiration manometrically would not reveal the chloramphenicol effect.

MacDonald et al. (1968), studying D-threo-chloramphenicol (the only active isomer in bacterial systems) and L-threo-chloramphenicol, found that both inhibited ion uptake at low concentrations (0.03 mg/ml, 0.1 mM). Using higher concentrations (0.6 mg/ml, 2 mM), D-threo-chloramphenicol was more active than the L-threo isomer in inhibiting protein synthesis. They hypothesized that the two isomers, at low concentrations, could be acting preferentially on the ATP-ase enzyme system of the cell. At higher concentrations the D-threo-chloramphenicol would inhibit protein synthesis while the L-threo would uncouple oxidative phosphorylation.

Recently, Ellis (1969) reported that the D-threo isomer (0.15 mg/ml, 0.5 mM) specifically inhibited protein synthesis in chloroplast (suggesting that chloroplast ribosomes are similar to bacterial ribosomes) while being without effect on cytoplasmic ribosomes. The L-threo-chloramphenicol was inactive on both chloroplast and

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mitochondria preparations. These results with chloramphenicol cast considerable doubt on the assumption of a direct linkage of protein synthesis with ion uptake, especially in chloroplast-free tissue.

Skou (1964, 1965) proposed, based on work with erythrocytes, that an ATPase enzyme system functions in the cell membrane to catalyze the transfer of Na^+ out of the cell while pumping K^+ into the cell. The activity of the hydrolysis system would be regulated by the respective concentrations of Na^+ and K^+. This hypothesis has gained wide acceptance (for a review cf. Glynn, 1968) for animal tissue. The kinetics show that 3-Na^+ are transferred for every 2-K^+ with the hydrolysis of 1-ATP leaving a surplus of only 4 kcal. If the ionic balance were artifically reversed, ATP could be synthesized. Also, Glynn (1968) reported that oligomycin and the cardiac glycoside, ouabain, were both potent inhibitors of the process of pumping Na^+ for K^+.

A convincing argument is presented by Robertson (1968) that charge separation is the basic mechanism by which energy was transferred within the plant cell. Charge separation is the mechanism, proposed by Mitchell (for review cf. Mitchell, 1966), where protons are transferred, without a high energy intermediate, to form ATP or drive

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ion transport. A gradient of protons could drive a reversible ATPase enzyme to form ATP from ADP and Pi. More importantly, the reverse of this reaction could release energy to transport ions.

Robertson (1964) reviewed how such energy relations have been shown to transport ions when coupled with either oxidative or photosynthetic phosphorylation in plant cells. At present, however, only indirect evidence is available that an ATPase system is responsible for ion movements through the plasmalemma. Chang and Bandurski (1964) reported ATPase activity in cell wall preparations of corn roots. Hall (1969) also demonstrated histochemically that ATPase was associated with the cell wall, especially near the plasmalemma. Hall noted that ATPase activity was enhanced with 2 m$\underline{M}$ $Ca(NO_3)_2$. Hodges (1966) reported that oligomycin rapidly and preferentially inhibited K^+ transport into the roots of oat, rye grass, and pea without affecting respiration. These root tissues were found insensitive to ouabain. The latter substance, however, inhibited Na^+ efflux (movement out of the tissue) from carrot slices, but had no effect on K^+ influx (movement into the tissue) (Cram, 1968). Evidence is accumulating that an ATPase system, probably slightly different than in animals, might be one of the important means by which plant

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Another type of metabolic accumulation mechanism in root tissue has been described by Hiatt (1968). His findings show that organic acid synthesis, co-ordinated with transamination to form amino acids, would be a source of binding sites to accumulate cations and anions simultaneously. This means that metabolic energy would be involved only for the synthesis of the respective acceptor molecules for ionic association to create and maintain a directed chemical gradient. Actually, uptake itself would be a diffusion process. At high external ion concentrations, additional neutral salts could still enter the cell by the Donnan phenomena, even though the level of receptor sites are not increased within the cell. This system could be comparable to Epstein's carrier system (mechanism II), but the involvement of membrane carriers is not necessary. In Hiatt's concept the membrane functions only for specific molecular selectivity and retention of organic and amino acids within the cell.

II. Foliar Uptake

A. Leaf Structure as Related to Uptake

Hull (1964) gave a detailed description of leaf structure in relation to foliar penetration. Numerous

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shorter reviews are also available (van Overbeek, 1956; Currier and Dybing, 1959; Crafts, 1964; Foy, 1964; Jyung and Wittwer, 1965; Sargent, 1965; and Franke, 1967).

The above authors described the leaf's outer surface as being covered with a cuticle, a non-living membraneous structure. It is composed of a matrix of oxidized polymers of long chain fatty acids and alcohols. They impart both polar and non-polar properties to the cuticle (Foy, 1964). The cuticle is considered the most limiting barrier to penetrating molecules (Currier and Dybing, 1959; Crafts, 1964; Hull, 1964). Waxes are often found deposited on both the cuticular surface and within the matrix. On the surface they occur in the form of platelets and rodlets (van Overbeek, 1956). The hydrophobic nature of the waxes contributes to the low permeability of the cuticle (Currier and Dybing, 1959; Hull, 1964; Norris and Bukovac, 1968). A layer of pectin joins the cuticle to the subtending epidermal cells (Norris and Bukovac, 1968).

The epidermal cells are the first living cells a penetrating molecule encounters. These cells are relatively large and are free of chloroplast. Stomata are generally found within the epidermis. Guard cells (containing chloroplasts) border the stomatal opening, and regulate the aperture width (Esau, 1965). Although the stomata are

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open pores, their internal cavity is bound by cuticle (Hull, 1964). Surface tension restricts penetration of liquids into the substomatal chamber (Currier and Dybing, 1959; Hull, 1964), but some researchers have shown that the presence of a surfactant will permit stomatal penetration (Currier and Dybing, 1959; Crafts, 1964; Greene, 1969).

B. Methods of Studying Foliar Penetration

Many techniques have been employed in studying foliar uptake. One of the simplest methods is applying the chemical to the whole aerial portion of the plant (Hauser, 1955). With such a system, however, it is difficult to distinguish between uptake and transport (Audus, 1967). Day (1952), using direct leaf application of micro-droplets of auxin solutions and measuring uptake by growth response, separated translocation from uptake by removing leaves at various intervals. Pallas (1960) and Middleton and Sanderson (1965) applied radio-labeled chemicals to leaves on intact plants and found uptake was dependent upon humidity and light. Jyung and Wittwer (1964) developed a leaf immersion technique minimizing the effect of humidity.

Several excised intact leaf systems have been devised to remove the effect of translocation from leaf uptake. Luckwill and Lloyd-Jones (1962) used a micro-droplet system, Biswas et al. (1962) utilized agar blocks containing ^{14}C

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herbicides on the leaf surfaces, and Kaminura and Goodman (1964a) applied solutions in small glass cylinders attached to the leaf. Sargent and Blackman (1962, 1965) developed a glass vial technique and further reduced the translocation effect by use of leaf disks.

Since the cuticular membrane may be considered as the rate limiting barrier for foliar uptake, attempts have been made to circumvent the cuticle in uptake studies. One method for the determination of the role of the leaf cells in the uptake process was to float pieces of leaf tissue in various treatment solutions (Kylin, 1960a, 1960b, 1960c; Ariz, 1963; and Hardwick and Woolhouse, 1968). Smith and Epstein (1964) reported that leaf strip width was a limiting factor and recommended that when such a system was used, the optimum strip width should be 300 μm. This technique has been employed for many successful studies on the role of leaf cells in foliar uptake (Rains, 1967, 1968; Bowen, 1968, 1969; Osmond, 1968).

Another approach has been to separate the cuticle and cells from the leaf and study them separately (Yamada, et al., 1965; Jyung et al., 1965a, 1965b; Kannan and Wittwer, 1967; Price, 1967; Bukovac and Norris, 1966; Norris and Bukovac, 1968, 1969; Wittwer et al., 1967; Gabbott and Larman, 1968). The use of isolated leaf cells should be an ideal

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approach for studying the metabolic aspects of foliar uptake. However, this technique has been criticized (Jacoby and Dagan, 1967) for the very low viability of the cells compared to the leaf strip technique.

C. Special Aspects of Foliar Uptake

The unique role of the cuticle as a barrier to foliar penetration was not fully appreciated until isolated cuticular studies were reported. For example, temperature effects, as measured by Q_{10} values greater than 1.2, have most often been associated with metabolic activity (Sutcliffe, 1962; Sufi, 1963; Jyung and Wittwer, 1965). Sargent and Blackman (1965) reported Q_{10} coefficients as high as 4.3 for 2,4-D penetration into leaf disks, while coefficients of 5.6 and 3.6 have been determined by Norris and Bukovac (1969) and Gabbott and Larman (1968), respectively, for penetration of other organic compounds through isolated cuticle membranes. Thus, the cuticle can contribute significantly, if not totally, to high temperature coefficients of foliar penetration.

Penetration differs significantly depending upon which side of the leaf the material is applied. Kaminura and Goodman (1964), and Sargent and Blackman (1962) found greater penetration rates through the lower surface. Sargent and Blackman (1965) suggested that a higher guard

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cell to surface ratio was an important aspect. Using isolated cuticle, Norris and Bukovac (1968) found similar higher rates for penetration through the lower surface. They suggested these findings might be due to less organized wax structure (birefringent wax) in that surface.

In numerous studies, light has been reported to enhance foliar uptake (Kylin, 1960a; Arisz, 1963; Jyung and Wittwer, 1964; Sargent and Blackman, 1965, 1969;). The light effect has been attributed to the production of photosynthetic metabolites, especially when high light intensities are required (Arisz and Sol, 1956; Kaminura and Goodman, 1964b).

Rains (1967, 1968) has shown that the light effect was independent of both oxidative phosphorylation and stomatal penetration for K^+ accumulation by leaf strips. He concluded that light supplied energy for uptake either via ATP or possibly direct coupling to electron transport. This latter idea is considered one type of charge separation supported by Robertson (1968). It should be noted that even though leaf tissue is photosynthetic, light does not always enhance active uptakes; Bowen (1969) has reported light had no effect for Cu^{2+}, Zn^{2+} and Mn^{2+} uptake by leaf strips.

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III. Auxin Uptake

A. Experimental Techniques

Two basic sampling methods have been used for almost all auxin uptake studies by plant tissue. The first system measured loss of auxin from the treatment solution. This technique was mainly used before isotopes were available and was dependent on colorimetric assay analysis for auxin detection (Sutter, 1944; Reinhold, 1954). As Reinhold pointed out, this method required the use of high auxin concentration (above $1x10^{-4}$ M 2,4-dichlorophenoxyacetic acid [2,4-D]) to permit accurate detection. Sabnis and Audus (1967a) have reported a spectrofluorometric analysis procedure that is sensitive for physiological concentrations of auxin ($1x10^{-8}$ M to $5x10^{-5}$ M 3-indoleacetic acid [IAA]).

This first detection method, as originally used, was inadequate, requiring high concentrations of auxin that were far above physiological concentrations. The work of Epstein's group (1966) indicated the possibility that working at a high concentration might mean one could actually be studying a mechanism that may not normally be operative. Also, high auxin concentrations have been usually considered toxic to the tissue (Housley, 1961). The spectrofluormetric analysis, although much more sensitive than the colorimetric technique, was found to be

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The second sampling method, now the basic practice for all work in this field, is to determine net accumulation of auxin in the tissue by using radio-labeled auxins (e.g. Johnson and Bonner, 1956; Wedding and Erickson, 1957; Thimann and Wardlaw, 1963; Luckwill and Lloyd-Jones, 1962; Sargent and Blackman, 1962, 1965; Jenner et al., 1968a, 1968b; Saunders et al., 1965a, 1965b).

Two additional, less accurate assay techniques have been applied to determine auxin uptake. The first assay, which resulted in the stimulation of growth (e.g. Day, 1952), has not been very accurate since not all accumulated auxin influences growth (Andreae, 1967). The other procedure involved radioautography (e.g. Yamaguchi and Crafts, 1958) and this method gave only a qualitative determination of uptake.

Tissue size has been an important factor in facilitating optimum uptake of auxin. Reinhold (1954) and Jenner et al. (1968b) have reported that optimum accumulation occurred into their smallest prepared sections, 5 and 2 mm respectively.

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B. Factors Affecting Auxin Uptake

A time-course of accumulation has been included in most auxin uptake studies. Sutter (1944) observed that uptake in time is usually characterized by an initial rapid rate followed by a second slower phase. Similar results have been observed by others (Reinhold, 1954; Johnson and Bonner, 1956; Wedding and Erickson, 1957; Poole and Thimann, 1964; Saunders et al., 1965a, 1965b; Sabnis and Audus, 1967a, 1967b; Jenner et al., 1968a). Though the two phases of uptake have been easily recognized, their specific significance has been subjected to considerable discussion. Sutter (1944) considered both to be manifestations of passive accumulation. Reinhold (1954), as well as several others, suggested that the first phase represented a passive uptake component, while the second phase was likely an active uptake component. Saunders et al. (1965b) noted that the observed two-phase auxin uptake was followed by a third phase of net auxin loss, and that this last phase could be associated with a change in tissue properties independent of an auxin effect. From these data, Saunders et al. (1965b) categorized auxin uptake into two components: Type I uptake where auxin was accumulated and later lost from the tissue (free space) and Type II uptake where auxin was bound irreversibly within the tissue. They found that

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strong auxins were mainly associated with Type I accumulation, while weak auxins were by the Type II mechanism.

A single phase of ^{14}C-auxin uptake has been observed when the accumulating tissue was pretreated with non-labeled auxin (Zenk, 1962). A three-phase uptake of IAA has been reported by Poole and Thimann (1964). Using an auxin concentration lower ($1x10^{-8}$ M) than that used for most of the above-mentioned reports, they noted a similar two-phase uptake that was then followed by a third phase having an uptake rate faster than the second.

A linear relationship of external auxin concentration to its uptake by plant tissue has been reported by Johnson and Bonner (1956), Wedding and Erickson (1957), Sabnis and Audus (1967a, 1967b), and Jenner et al. (1968a, 1968b). Poole and Thimann (1964) have observed a similar linear relationship and established that the uptake ratio showed that the internal concentration was higher than the external concentration.

Efflux of accumulated auxin has been shown to consist only of auxin that was held in a free, non-bound form (Andreae, 1967). Jenner et al. (1968a) reported that a constant amount of auxin was released after varied periods of prelabeling. Poole and Thimann (1964) observed that almost all the auxin (IAA) accumulated in a 30-minute

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uptake period could be removed by subsequent washing. They concluded that this fraction represented accumulation into free space. Johnson and Bonner (1956) found a comparable loss for 2,4-D after a 30-minute wash and calculated this volume as the free space fraction in Avena coleoptiles as 19 to 22%. Sabnis and Audus (1967a), studying IAA accumulation by Zea mesocotyl sections, reported a free space value of 30%.

The effect of low pH has been shown by Currier and Dybing (1959), Sargent and Blackman (1962), Poole and Thimann (1964) to enhance auxin uptake. Bukovac and Norris (1966) reported that at pH values below the pK (4.2) of NAA, binding to leaf surfaces was significantly increased. Simmon and Beevers (1952) proposed that activity of weak organic acid was associated with the amount of acid in the undissociated form. Wedding and Erickson (1957) derived a model system to match their data for 2,4-D penetrability into Chlorella. They concluded that the Chlorella was 800 to 1000 times more permeable to the undissociated molecule compared to its anion. Reinhold (1954) determined that a change in the passive accumulation rate occurred one pH unit above the pK of the auxin being studied (IAA). She therefore concluded that auxin anions were accumulated at a higher rate than the uncharged molecule.

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Accumulation of auxin against a concentration gradient has been one criterion often used as evidence for active uptake. Johnson and Bonner (1956) and Poole and Thimann (1964) reported that for 2,4-D and IAA, respectively, net auxin accumulation exceeded the external concentration. Such a criterion should only be considered proof of active uptake when it has been established that the auxin is held in a free, non-bound form in excess of the external auxin concentration (Zenk, 1962).

Temperature coefficients (Q_{10}) of auxin uptake rates have been reported ranging from 1.67 for Chlorella (Wedding and Blackman, 1961) to 2.00 for coleoptiles of Avena (Johnson and Bonner, 1956) and hypocotyls for Gossypium hirsutum (Saunders et al., 1965b). Foliar penetration of auxin has been found to exhibit Q_{10} coefficients as high as 4.3 (Sargent and Blackman, 1965; Greene, 1969). As discussed earlier, these high Q_{10} values might be attributed to limiting cuticular penetration (Norris and Bukovac, 1969).

Light has been shown to stimulate the rate of auxin uptake by Chlorella (Wedding and Blackman 1961), leaf disks (Sargent and Blackman, 1965; Greene, 1969) and green pea stem sections (Thimann and Wardlaw, 1963). These reports inferred that the light effect was due to the production of photosynthetic products to thereby enhance

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The use of metabolic inhibitors has helped to resolve the magnitude of the active uptake component for auxin. Dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, has been shown to inhibit uptake of auxins by 20 to 80%, depending upon the duration of the experiment (Sabnis and Audus, 1967a; Jenner et al., 1968a; Johnson and Bonner, 1956). Potassium cyanide, a respiratory poison (Mahler and Cordes, 1966), has been shown to also inhibit auxin accumulation (Johnson and Bonner, 1956).

As Andreae (1967) summarized for pea root tissue, root metabolism of auxin can have a pronounced effect on net accumulation. For example, if decarboxylation of IAA occurred, and one were following carboxyl ^{14}C-labeled IAA, then it would appear as a net loss of auxin from the tissue. The remaining free IAA within the tissue was conjugated with L-aspartate to form indoleacetylaspartate (IAAsp) (Andreae and Good, 1955, 1957). The relationship of uptake to metabolism was described by Andreae and van Ysselstein (1960a, 1960b) and Andreae et al. (1961). Andreae (1967) reported that although free IAA is found in the roots

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in amounts above the external concentration, it was converted almost totally to IAAsp within several hours and further accumulation was only in the form of the conjugate.

Zenk (1962) found that pea epicotyl sections formed a similar aspartyl conjugate with NAA termed naphthylacetylaspartic acid (NAAsp), but that decarboxylation did not occur. Zenk's data showed a 4-hr lag before NAAsp could be found while Andreae (1967), also studying NAA uptake, reported a 6-hr lag. Both noted that after 16 hr most of the NAA was complexed. However, a small but significant amount did remain in the free form.

Andreae (1967) did not find any metabolism of 2,4-D in pea root sections, but Jenner et al. (1968a) speculated that a 2,4-D metabolite was formed by Avena mesocotyl sections.

Klambt (1961) and Zenk (1961) each reported a second conjugation product of IAA to be indoleacetyl-β-D glucose (IAGlu). Zenk (1962) also identified an NAA glucose complex (NAGlu). Zenk's (1962, 1963) data showed this glucose complex to be an earlier metabolic product that decreased with time as the aspartyl conjugate increased. Thomas et al. (1963, 1964) found that Avena stem sections metabolically hydroxylated some of the chlorinated phenoxyacetic acids which had then accumulated as their

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Formation of the aspartyl complex with NAA was the result of an inductive process, dependent upon protein synthesis (Zenk, 1962). Venis (1964) found that IAA induction of the conjugation of benzoic acid with aspartic acid could be inhibited by blocking RNA synthesis, so that benzoic acid was not conjugated. Sudi (1966) suggested that the induction of either the NAA or IAA conjugate was an example of induced enzyme formation, possibly an allosteric enzyme.

The physiological significance of the aspartyl conjugate has been described as a detoxified auxin product for most of accumulated auxin (Andreae, 1967). It is considered relatively immobile within plant tissue (Veen, 1966, 1967; Hertel and Flory, 1968; Eschrich, 1968).

Auxins, especially IAA, have been reported to occur bound to proteins (Zenk, 1963; Winter and Thimann, 1966) and nucleic acids (Kaur-Sawhney et al., 1967; Fellenberg, 1969).

IV. Isolation of Leaf Cells for Auxin Uptake Studies

A. Methods of Isolation

Racusen and Aronoff (1953) reported a mechanical separation procedure to isolate leaf cells. Their method consisted of grinding soybean leaves in a mortar with a pestle

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in 0.25 M sucrose and a phosphate buffer solution at pH 6.8. Cells were separated by filtration and centrifugation. Cells isolated in this manner had one-fifth the photosynthetic capacity of intact leaves as measured by $^{14}CO_2$ fixation. None of the carbon fixed was recovered in protein, although amino acids were labeled. Price (1967) modified this technique to obtain greater quantities of leaf cells by homogenizing soybean leaves in a high-speed blender for one minute and then recovering cells by the procedure described by Racusen and Aronoff (1953). Price tested several separation media and found that a nutrient solution containing 1.0 mM EDTA, 0.40 M sucrose and 0.05 M tricine buffer at pH 7.2 to be sufficient for the separation of cells capable of exhibiting protein synthesis, photosynthesis, and respiration. Price observed that these cells had low activity, but did not compare them to intact tissue. He assumed that his experiments were in a similar range as Racusen and Aronoff's (1953). Price emphasized that this method still permitted qualitative comparison of the cells to intact leaves.

Ball and Joshi (1965) reported that few plant species were suitable for mechanical separation of intact leaf cells. They found it was possible to tease a few cells free from peanut leaves, after which the cells were capable of

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Chayen (1952) described an enzymatic separation procedure for the isolation of root cells using a fungal pectinase from Penicillium digitatum. Zaitlin (1959), applied the enzymatic procedure to isolate tobacco leaf cells that were capable of supporting virus multiplication. Jyung et al. (1965b) modified Zaitlin's procedure and prepared tobacco leaf cell isolates for ion uptake studies. Gabbott and Larman (1968), using the technique of Jyung et al., isolated leaf cells from tobacco to follow atrazine accumulation. The latter two research groups each reported relatively low respiration rates for the cells but did not offer a comparison to intact leaf tissue. Only Jyung et al. (1965b) were able to show any metabolic involvement in their uptake studies. Takebe et al. (1968), using a different pectinase preparation called Macerozyme, isolated tobacco leaf cells that supported the growth of tobacco mosaic virus. Macerozyme (characterized by Suzuki et al., 1967a, 1967b) is mainly polygalacturonase like the pectinase mentioned above, but its optimum activity occurred at pH values of 5.0 and 6.5, compared to 4.5 and 6.0 for pectinase (Zaitlin and Coltrin, 1964).

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amounts of proteases (Zaitlin and Coltin, 1964; Suzuki et al., 1967a). Chayen (1952), aware of this, used 1% peptone to dilute the protease activity. Jyung et al. (1965b) used a lower concentration, 0.2% peptone, for the same purpose. Takebe et al. (1968) reported that high mannitol concentrations (0.6 to 0.8 M) combined with a low molecular weight dextran sulfate salt adequately protected leaf cells during isolation.

Unfortunately, none of the above reports compared the metabolic activity of the enzymatically isolated leaf cells to that of their parent tissue, so no conclusions can be drawn on how representative enzymatically isolated leaf cells were to intact tissue. Jacoby and Dagan (1967), in an effort to resolve this point, isolated bean leaf cells using the techniques of Jyung et al. (1965b) and compared them to leaf strips of the same tissue prepared according to the technique of Smith and Epstein (1964). They found that respiratory activity and Na^+ accumulation was 7.3% and 1.5%, respectively, of leaf strip tissue. They observed that only cells not completely separated, stained with neutral red. Jyung et al. (1965) noted that ion uptake by the isolated cells was only 3% of that of intact leaves.

B. Methods of Assessing Cell Viability

Thus, as ideal as isolated cells appear to be, no

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procedure is currently satisfactory for uptake studies. One reason little progress has been made may be the difficulty of applying reliable tests for cell viability.

The appearance of isolated cells under high magnification can be a very critical assay (Hongladrom, 1964; Honda et al., 1966). They described the living leaf cell as being in a dynamic state of protoplasmic streaming with organelles such as the chloroplasts having very distinct characteristics compared to dead cells.

Vital staining, particularly with neutral red, has been reported as a reliable assay for viability (Oppenheimer and Leshem, 1966), particularly when a definite distinction is to be made between living and dead tissue. Troshin (1966) has criticized such an assay, since damaged cells often stain more intensely than non-damaged ones. It should also be recognized that this technique provides a qualitative assessment of viability and not a quantitative one.

Autofluorescence, as a similar qualitative assay, has been reported as a valid criterion of life in plant tissue (Oppenheimer and Jacoby, 1961). The blue fluorescence of the vacuole, caused by ultraviolet light radiation, was used as the indicator of viability. Unfortunately, the test has seldom been successful in green tissue containing

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chloroplasts, for their red fluorescence masked the blue fluorescence of the vacuole. The red fluorescence was seldom lost in death (Oppenheimer and Jacoby, 1961).

Respiration has been one of the most often measured responses of living tissue. Hallaway (1968) indicated that bacterial contamination can often offset the true tissue respiration by a factor of 10 to 100. Thus, when measuring respiration, it is essential that the system be free of bacteria.

The ability to fix $^{14}CO_2$ has successfully been used by Racusen and Aronoff (1953) to compare metabolic activity of isolated leaf cells to intact tissue. The synthesis of protein containing fixed ^{14}C from photosynthesis may be a more critical test for viability (Davies and Cocking, 1967).

Tetrazolium reduction, an *in vivo* metabolic assay for general dehydrogenase activity (Pearse, 1960) has been used successfully to determine tissue viability on a qualitative basis (e.g. Currier and van der Zweep, 1956). Davies and Seaman (1968) reported that the triphenyl tetrazolium chloride (TTC) reaction could be colorimetrically quantified by extracting the reduced dye with ethylacetate and then measuring its absorbance at 485 nm to avoid interference of the *Elodea* pigment simultaneously extracted. The mechanism of tetrazolium reduction has been shown to be

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C. General Methods Used for Handling Plant Cells and their Organelles

Although little work has been reported on methods of preparing and handling fresh preparations of leaf cells, considerable research has been done on isolation of subcellular organelles. Some of the general techniques used for subcellular work would be expected to be applicable for intact cell systems. However, the optimum preparation for one type of organelle may be detrimental to another (Hallaway, 1965), so a suggested preparation must be evaluated again on the total system, the intact cell, or a group of them, like in leaf strips.

One of the basic principles, that has evolved from organelle work, was that the best methods were the milder ones (Hallaway, 1965, 1968). Hulme et al. (1964a) documented this point by preparing mitochondria by various grinding procedures. They reported that a roller-mill they developed yielded the most active mitochondria compared to others isolated by homogenation in either a blender or a mortar with pestle. The sensitivity of plant cells to mechanical shock was further documented by Ruesink and Thimann (1963), who enzymatically isolated protoplasts and noticed that even

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low speed centrifugation would inactivate their preparations. D'Alessio and Trim (1968) found that by using cell wall lysing enzymes, they could minimize mechanical shock and were thereby successful in isolating large numbers of leaf cell nuclei.

The importance of the medium for organelle activity was also discussed by Hallaway (1965, 1968). She reported that water, alone, is seldom used as its hypotonic stress will often rupture the membranes. For this reason, sucrose between 0.2 and 0.4 M has been used as an osmotic medium to prevent protein denaturation. Stadelmann (1966) gave a very detailed description for the determination of the optimum osmotic condition for plant cells. For Hedra leaves, he found 0.36 to 0.43 M sucrose to be equivalent to its normal osmotic potential.

The maintenance of the proper pH also has been stressed (Hallaway, 1968). Romani et al. (1969) described that their best method for isolation of pear fruit mitochondria was to carefully monitor and adjust the pH of the preparation medium during the separation procedure. They did not use a buffer because of the detrimental effects of the buffer. The use of Good's (1966) buffers might simplify such a cumbersome practice (Romani et al., 1969), and yet allow optimum activity (Good et al., 1966).

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Bovine serum albumin (BSA) has been shown to be very effective in maintaining the activity of isolated mitochondria (Jones et al., 1964; Sarkissian and Srivastava, 1968). Hongladrom (1964) and Honda et al. (1966) found that BSA at lower concentrations stabilized micro-preparations of leaf cells.

A stabilizer of phenolic compounds also has been shown to be beneficial if included in the isolating medium (Hallaway, 1968). Hulme et al. (1964b) found polyvinyl pyrrolidone (PVP) effective, while metabisulfite has been reported to be more effective (Anderson, 1968; Anderson and Rowan, 1967; Stokes et al., 1968).

Hongladrom (1964) and Honda et al. (1966) found that the incorporation of high molecular weight polymers in their treating medium stabilized microleaf strip preparations. They found that a very specific ratio of the polymers Ficoll and Dextran with sucrose and BSA gave optimum results. This medium commonly referred to as the "Honda medium", has gained wide acceptance for organelle isolation (Hallaway, 1968). Kuehl (1964) observed that other special molecules such as N-octanol and gum arabic are effective in stabilizing isolated nuclei.

Tissue culture media are another important class of treatment media (e.g. Murashige and Skoog, 1962; Lamport,

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1964). These media have been developed to permit long-term experiments on the growth and division from a small amount of tissue prepared under optimum conditions. Such a technique does not provide for the optimum recovery of all the initial material, but rather the promotion of growth and division of the healthiest cells within the initial cell population.

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EXPERIMENTAL

I. Hypocotyl Section Studies

Soybean, Glycine max Merr. var. Chippewa or Hawkeye, was chosen as the source of plant material due to the ease with which the plants could be grown and the report (Price, 1967) that cells could be mechanically isolated from the leaves.

Initial studies were conducted with soybean hypocotyl sections to establish the basic experimental parameters necessary to follow auxin uptake and to establish an optimum medium for maximum tissue activity.

A. Growing of Plants

Approximately 35 g of Chippewa soybean seeds were sown on 2 inches of vermiculite contained in 12x18x4-inch wooden flats. The seeds were covered with 1/2 inch of vermiculite and the flats were placed for 1/2 hour in a pan filled with tap water. This last step assured uniform imbibition, thus yielding a uniform population of seedlings. Subsequent daily watering was by subirrigation. The plants were grown in a growth chamber equipped with cool white and

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incandescent lamps such that the seedlings were illuminated for 12 hr daily with 1200 ft-c at the surface of the vermiculite. The day and night temperatures were maintained at 25 and 20 C, respectively. Seedlings were harvested 7 days after sowing, when their hypocotyls were approximately 9 cm long, cotyledons unfolded and the primary leaves still tightly folded.

B. Preparation of Hypocotyl Sections

Seedlings were transferred to the laboratory and their roots immersed in water for not more than 1 hr before experimental use. Prior to sectioning, the seedlings were washed for 5 min under running tap water, followed by a distilled water rinse. Two 4-mm sections were cut from each of six hypocotyls simultaneously, unless otherwise noted. The slice was made with three mounted razor blades and paraffin served as a cutting base. A total of four sections (two sets of two) were cut from each hypocotyl, the first one 8 mm below the cotyledonary node and the following three immediately proximal. After cutting, the sections were placed in filter (0.45 μm pore) sterilized treatment medium, to be accumulated with sufficient additional sections to conduct the ensuing experiment. The medium consisted of 0.35 M mannitol, 0.01 M Tris-HCl, 0.01 M sodium succinate, and adjusted to a pH of 7.4. After a 1/2-hr

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wash in the medium, the sections were collected in a Büchner funnel (No. 2A), rinsed with fresh media, and divided into groups of 12 sections each. Each group was then placed in 25 ml Erlenmeyer flasks containing the treatment medium. The flasks were stoppered with urethane foam plugs.

C. General Uptake Methods

The 25 ml Erlenmeyer flasks containing the hypocotyl sections and 7 ml of the respective treatment media were incubated in a gyrorotory water bath shaker (New Brunswick Scientific Co., Metabolyte-G77). The bath was held at 25.0 ± 0.25 C and illuminated with 500 ft-c cool-white fluorescent light. The flasks were agitated at 200 rpm within a 1/2-inch orbit.

^{14}C-Carboxyl labeled α-naphthaleneacetic acid (^{14}C-NAA) (Tracerlab, Waltham, Mass.) was chosen as the test compound, because of its stability and physiological activity. After a 1-hr pretreatment period, 1.0 ml of ^{14}C-NAA, 8x final concentration, was added to the respective flasks to give a final concentration of 5×10^{-6} M NAA, specific activity 16 μc/μmole. The ^{14}C-NAA stock solution concentration was checked using liquid scintillation counting calibrated with an internal standard of ^{14}C-toluene. The addition of the ^{14}C-NAA was staggered to allow sufficient

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time for accurate handling of the appropriate treatments. The uptake period was terminated after 3 hr by the rapid removal of the labeled treating solution using suction. The sections were quickly rinsed, followed by two 5-min washes, all with 5 ml of deionized distilled water. The wash water was rapidly administered using an automatic filling syringe.

The washed sections were placed in 1-inch planchets, oven dried at 70 C for 12 hr, and weighed to within ±0.1 mg. The weighed sections were homogenized with absolute ethanol in a 10-ml Thomas-Ten Broeck glass tissue grinder (Thomas Co., Philadelphia, Pa.). The homogenates were quantitatively transferred onto 2x5/16-inch ringed stainless steel planchets and the ethanol removed by evaporation. This procedure yielded a uniform distribution of the hypocotyl tissue within the planchet. Radioactivity was measured by using a low background proportional counter (Beckman Low Beta II). All samples were measured for at least 2000 counts (4.25% chance of error). Corrections were made for background, efficiency and sample weight. All mathematical transformations were done as a single step operation on a desktop computer (Olivetti Programa).

Statistical evaluation was achieved by analysis of variance. Comparison of means were made by the Tukey

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procedure (Steel and Torrie, 1960). Standard errors of the mean (SE) are given when the data were representative of non-homogeneous populations.

D. Establishment of Experimental Parameters

1. Determination of Self-Absorption

To facilitate the uniform labeling of a large amount of hypocotyl tissue, 10 mm sections were cut in the manner described above. Fifty of these sections were placed in a 25 ml Erlenmeyer flask. This represented material for one of four replications. The standard uptake procedure was used, except the dried labeled sections were divided into groups of varying numbers before being weighed. Thus, varying amounts (mass) of uniformly labeled hypocotyl tissue were plated onto 2-inch planchets.

As shown in Figure 1, self-absorption did not become a significant factor until more than 1.3 mg/cm^2 of tissue mass was plated in a planchet. Therefore, up to 20 mg of dried soybean tissue could be plated in a 2-inch planchet without significant self-absorption.

2. Determination of Optimum Section Length

Hypocotyl sections of varying length (2.0, 4.0, 6.0 and 8.0 mm) were prepared in the manner previously described. To maintain a constant mass of tissue per treatment, sufficient sections of the respective length sections



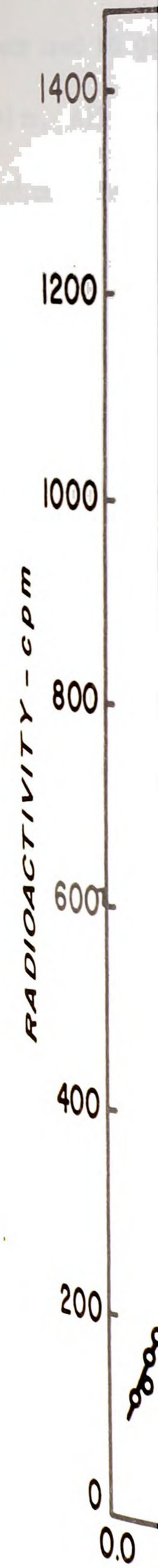
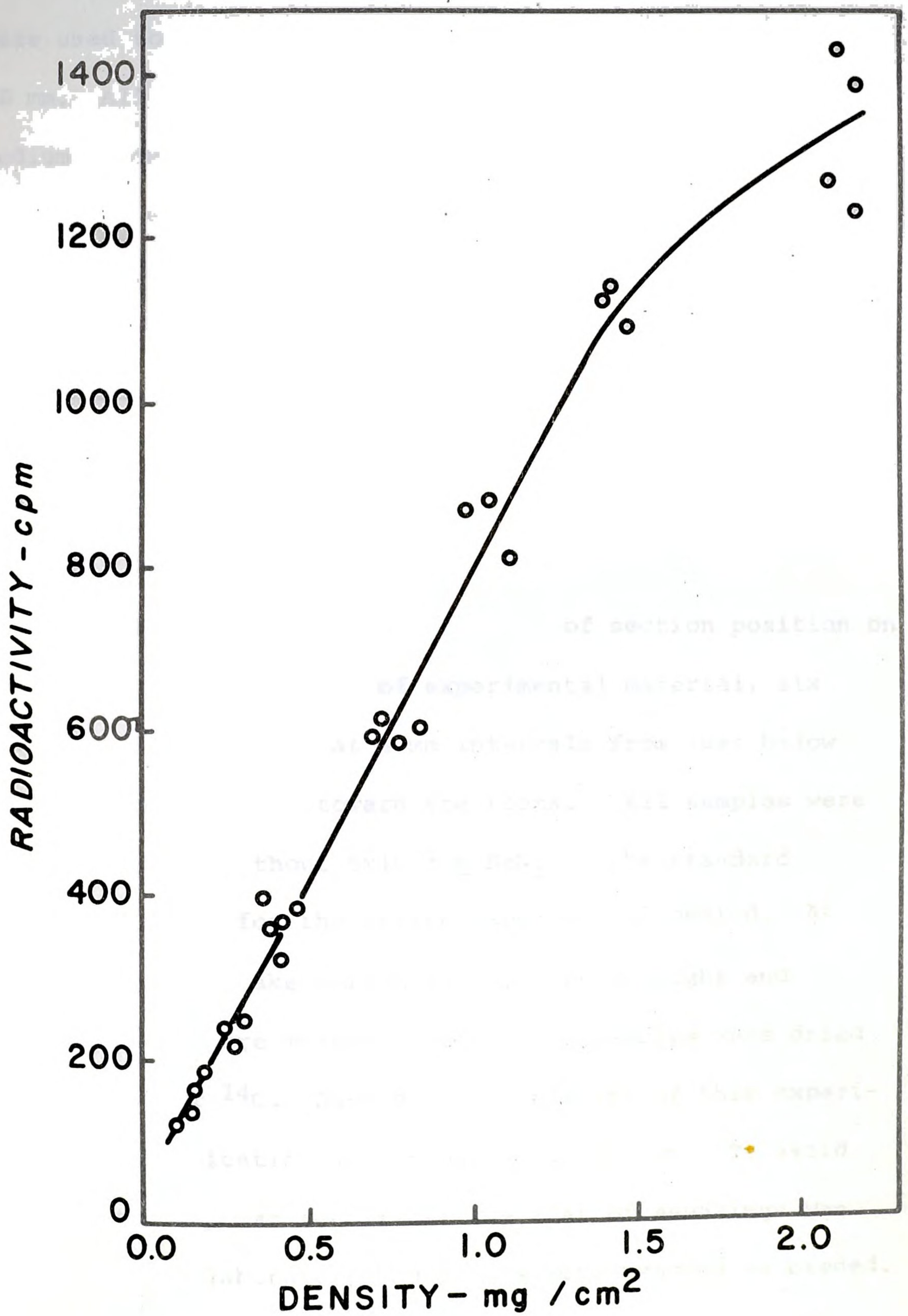


Figure 1. Self-absorption curve of ^{14}C-naphthaleneacetic acid in varying amounts of uniformly labeled homogenized hypocotyl tissue.


1400
1200
1000
800
600
400
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were used to give a total length of sections equal to 48 mm. All sizes were treated with and without $5x10^{-4}$ M sodium azide (NaN_3) in the standard treatment medium for the entire experimental period (1-hr pretreatment and 3-hr uptake period).

A section length of 4 mm was found to be optimum size for maximum NAA accumulation (Figure 2). Sodium azide inhibition of NAA uptake appeared independent of section length.

3. Effect of Section Position

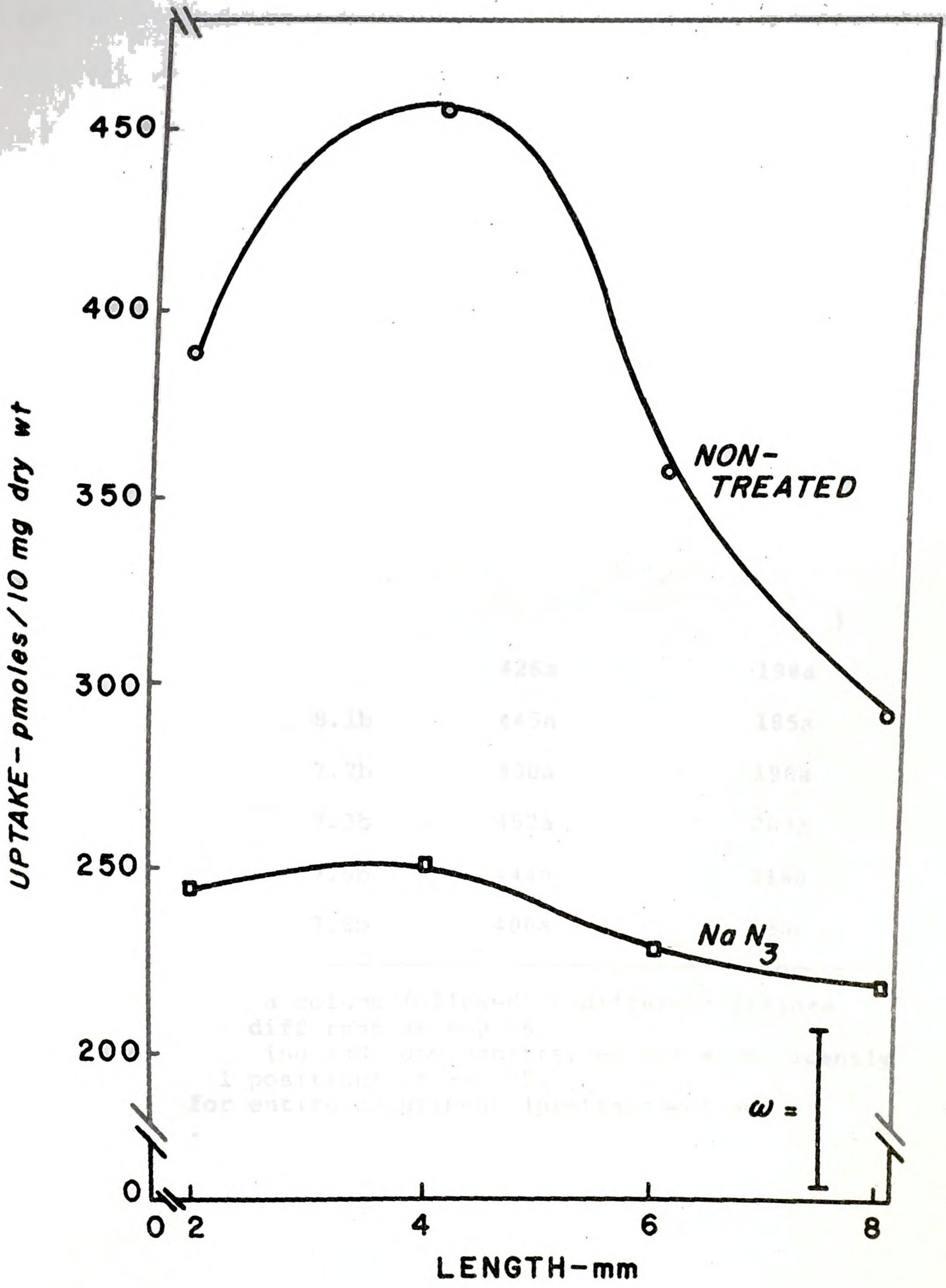
To ascertain the importance of section position on the hypocotyl as a source of experimental material, six 4-mm sections were cut at 4 mm intervals from just below the cotyledonary node toward the roots. All samples were treated with or without $5x10^{-4}$ M NaN_3 in the standard treatment medium for the entire experimental period. At the end of the uptake period (3 hr), fresh weight and section length were measured before the samples were dried and assayed for ^{14}C. Due to the complexity of this experiment, each replication was staggered by 45 min. To avoid aging of the soybean seedlings, the flat of seedlings was brought to the laboratory and plants were removed as needed.

NAA uptake (with or without NaN_3) did not vary with position on the hypocotyl as shown in Table 1. The sections



Figure 2. Effect of hypocotyl section length on naphthaleneacetic acid uptake.

Uptake was in the presence or absence of NaN_3 ($1x10^{-3}$ M).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5	113.
6	117.

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Table 1. Effect of position on the hypocotyl as a source of 4 mm sections for experimental material.

Position	Fr wt[1]	Dry wt[2]	NAA accumulated[1,2]	
			Non-treated	NaN_3[3] ($1x10^{-3}$ M)
	(mg)	(mg)	(pmoles/10 mg dry wt)	
1	108.4b	9.5a	426a	198a
2	106.4b	8.1b	445a	185a
3	110.5b	7.7b	430a	190a
4	109.5b	7.3b	452a	203a
5	113.7ab	7.5b	444a	216a
6	117.4a	7.8b	400a	230a

[1]Means within a column followed by different letters are significantly different at P=0.05.

[2]Values comparing NaN_3 and non-treated are significantly different for all positions at P=0.05.

[3]Present for entire experiment (pretreatment and uptake period).

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(at the end of the 4-hr experiment), did not elongate significantly. However, both fresh weight and dry weight differed with sampling position, especially for the apical and basal positions on the hypocotyl. The high dry weight value of position one probably reflected the zone of cell division, and the high fresh weight of position six probably reflected the area of cell enlargement and wall thickening. The sections from the four intermediate positions showed least variation and were therefore considered the optimum zone as a source of hypocotyl sections.

E. Determination of Factors Affecting Naphthaleneacetic Acid Uptake

Having determined those factors that directly affected the use of hypocotyl tissue, attention was focused on how the hypocotyl system could be used to elucidate the mechanism of NAA uptake and the development of techniques for use on leaf tissue.

1. Effect of Bacteria on the Hypocotyl System

A factorial design was used to study the effect of bacterial contamination on NAA uptake by hypocotyl tissue. Three levels of D-threo-chloramphenicol (0, 10, and 250 µg/ml) were imposed on three levels of bacterial contaminated media. NAA, oxygen uptake and bacterial levels were determined for each treatment.

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The highest level of contamination was established by inoculating the standard uptake preparation with 1 ml of media from a similar preparation contaminated with bacteria. The intermediate level was established from the standard uptake preparation. The low level was the same as the standard preparation except that the intact seedlings were washed with 1.0% (v/v) NaClO for 2 min.

After 4 hr, a 1.0 ml sample was taken from each flask, serial dilutions prepared with 0.85% NaCl, and plated on separate media for bacterial or fungal culture. The bacterial medium was prepared by autoclaving 1.0 kg of garden soil with 1.0 liter of water and 0.5 g $CaCO_3$. The resulting suspension was filtered until clear, and then to every 100 ml of solution 2.0 g agar, 1.0 g glucose, 1.5 g K_2HPO_4 was added. The pH was adjusted to 6.8. The fungal media consisted of 22 g of dehydrated potatoes (Pillsbury-Hungry Jack's) steamed 5 min in 1.0 liter of distilled water. This solution was filtered through cotton and filter paper (Whatman No. 1) and then made up to 1.0 liter. The following substances were added to the solution before it was divided into 200 ml lots for final autoclaving: 20 g glucose and 1 ml NPX (bacteriacide). The two freshly autoclaved media were stored 2 hr at 43 C before being used for respective plating in sterile disposable Petri dishes.

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Inoculation with bacteria increased markedly the bacterial population while surface sterilization lowered the bacterial level normally found (Table 2). Chloramphenicol only seemed to lower the high level of bacteria contamination. No fungal contamination was observed.

For the NAA uptake experiments all hypocotyl sections were given the standard wash, so it can be assumed that most of the bacteria were removed from the tissue before weighing and radioassay.

The presence of bacteria in the experimental system had little effect on NAA accumulation, although surface sterilization decreased NAA uptake (Table 3). Chloramphenicol at a level of 10 µg/ml had no effect, but at a concentration of 250 µg/ml significantly stimulated accumulation.

During the uptake period, two replications of all treatments were assayed for oxygen consumption using standard Warburg manometric techniques (Umbreit *et al.*, 1964). Twenty-four sections were used in 4.0 ml of treatment medium and 0.5 ml of 10% (w/v) KOH held in one of the two side arms of the flask (no centerwell). The bath temperature was 30 C and the unit was kept covered with a black cloth between the two 1-hr readings. The flasks were shaken at 120 orbits per minute.

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Table 2. Level of bacteria after 4 hr in the hypocotyl system treated with several concentrations of chloramphenicol.

Chloramphenicol concn	Bacteria level		
	Contaminated[1]	Standard[2]	Sterilized[3]
(µg/ml)		(bacteria/ml)	
0	$4x10^7$	$2x10^3$	$1x10^2$
10	$2x10^7$	$5x10^2$	$1x10^2$
250	$2x10^6$	$4x10^3$	$1x10^2$

[1]Contaminated=medium inoculated with bacteria.
[2]Standard=filter sterilized medium with water washed hypocotyl sections.
[3]Sterilized=filter sterilized medium with 1.0% (v/v) NaHClO washed hypocotyl sections.

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Table 3. Effect of bacteria on naphtheleneacetic acid uptake by hypocotyl sections treated with several concentrations of D-threo chloramphenicol.

Chloramphenicol concn	NAA accumulated[1]			
	Contaminated[2]	Standard[3]	Sterilized[4]	Mean[5]
(µg/ml)	(pmoles NAA/10 mg dry wt)			
0	687	742	683	702a
10	732	813	706	750a
250	840	876	785	833b
Mean[5]	750ab	810a	724b	

[1]At the end of the uptake period all tissue samples were washed twice to remove non-bound NAA and bacteria.

[2]Contaminated=medium inoculated with bacteria.

[3]Standard=filter sterilized medium with water washed hypocotyl sections.

[4]Sterilized=filter sterilized medium with 1% (v/v) NaHClO washed hypocotyl sections.

[5]Means followed by different letters are significantly different at P=0.05. Interaction of concentration vs. bacteria level not significant.

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Unlike the minimal effect of bacteria on NAA uptake, the presence of bacteria increased dramatically oxygen consumption of the inoculated treatments (Table 4). Chloramphenicol lowered oxygen consumption only in the inoculated treatments where the bacterial level was also decreased (Table 2). Surface sterilization further reduced hypocotyl respiration.

2. Time-Course Studies of Naphthaleneacetic Acid Uptake

Two separate experiments were conducted on different days to determine an optimum sampling time for future experiments. The standard uptake procedure was followed except for time. As seen in Figure 3A, uptake proceeded at a relatively steady rate for the entire period (5-180 min.). A second experiment, plotted in Figure 3B, revealed that the initial steady uptake rate continued for about the first 15 hr, followed by a second slower phase of uptake for the next 20 hr. The level of NAA accumulation was very different between the two experiments. However, marked variation between experiments was frequently observed. The variation within an experiment was usually quite small.

3. Methods for Killing Tissue

A prime objective of this study was to work with

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Chloramphenicol concn	
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Table 4. Effect of bacteria on oxygen uptake at 30 C by hypocotyl sections treated with several concentrations of chloramphenicol.

Chloramphenicol concn	Oxygen uptake[1]		
	Contaminated[2]	Standard[3]	Sterilized[4]
(µg/ml)	(µl/100 mg dry wt/hr)		
0	1516	581	398
10	1019	646	490
250	808	732	463

[1]Oxygen uptake (manometric) values represent measurement of the total system (tissue and bacteria) for each treatment.

[2]Contaminated=medium inoculated with bacteria.

[3]Standard=filter sterilized medium with water washed hypocotyl sections.

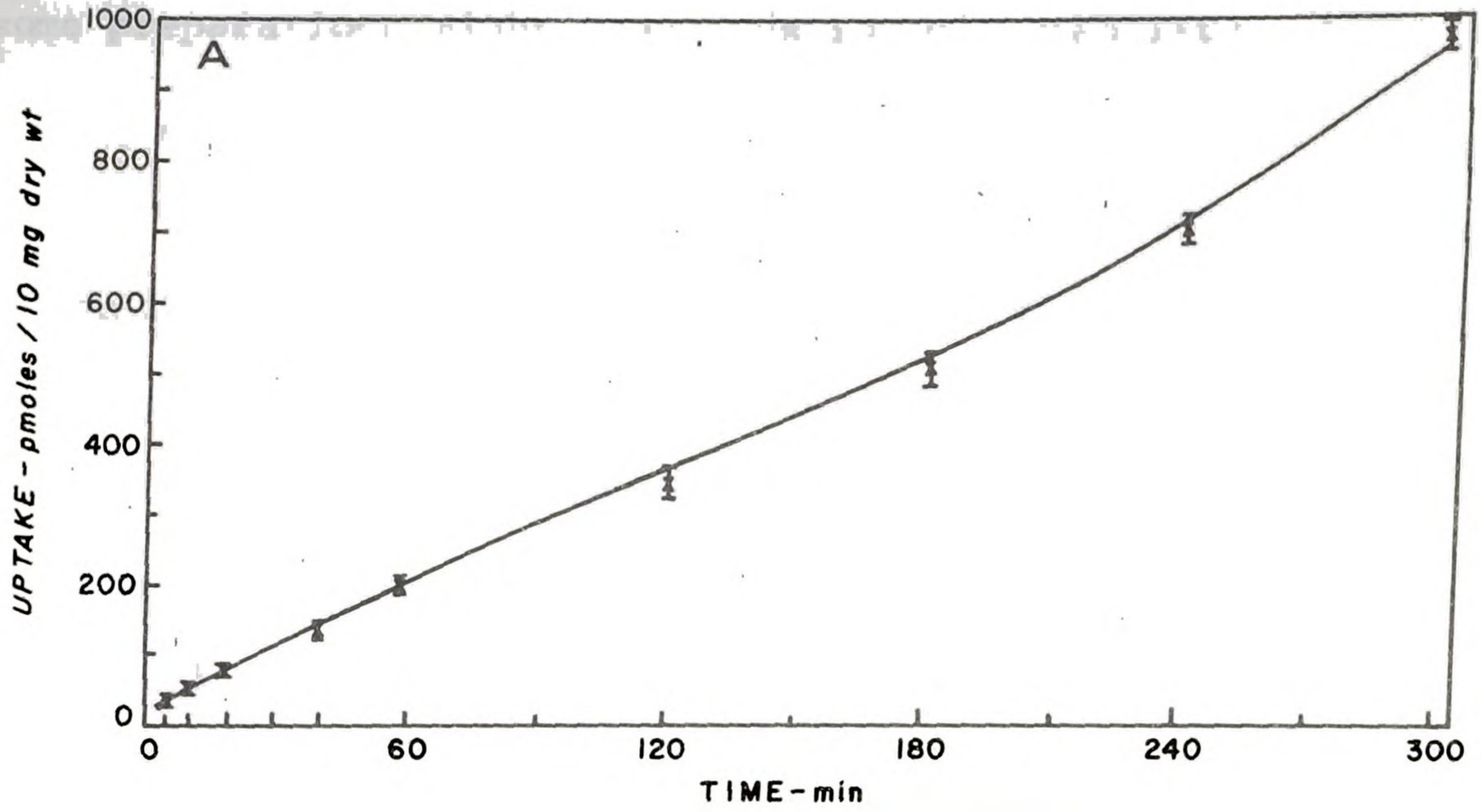
[4]Sterilized=filter sterilized medium with 1.0% (v/v) NaHClO washed hypocotyl sections.



Figure 3. Time-course of naphthaleneacetic acid uptake by hypocotyl sections.

A. Time-course of 5-300 minutes (Study A).

B. Time-course of 1-36 hours (Study B).


A
UPTAKE - pmoles / 10 mg dry wt
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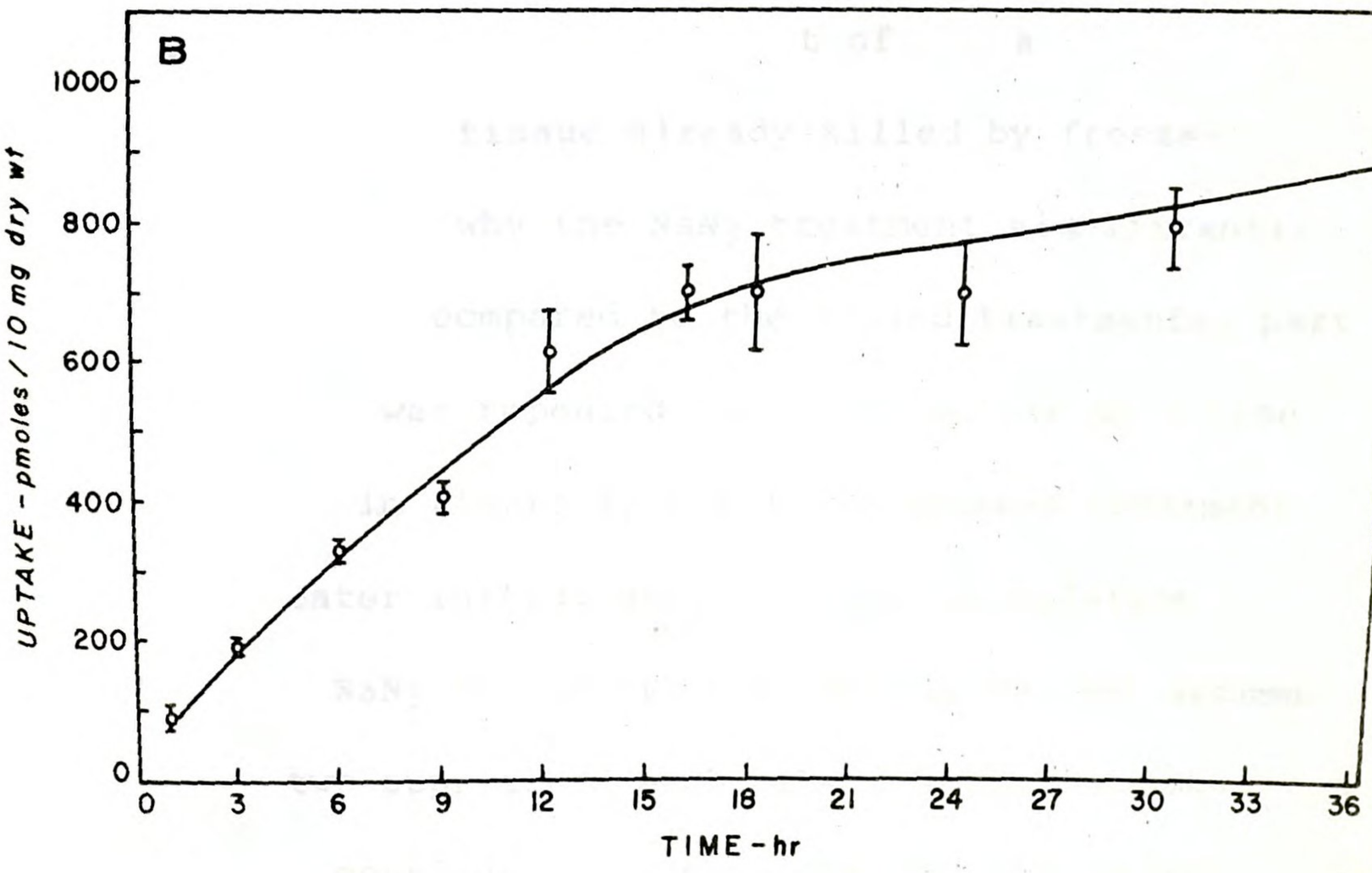
600
400
200
0
0
60
120
180
240
300
TIME - min


B
UPTAKE - pmoles / 10 mg dry wt
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leaf tissue, particularly leaf cells. Unfortunately, some preparations might have questionable viability. As a check, development of a dead control that could be applied as a base was desirable. Hypocotyl sections were killed by placing the tissue, floating in the standard medium either in a boiling water bath for 2 min or a dry-ice methanol bath until the tissue and media were frozen solid and then thawed slowly.

The data presented in Table 5 established that there was no significant difference between boiling or freezing as a method of killing the tissue. The NaN_3 treatment, which effectively reduced the amount of NAA accumulated, had little effect on tissue already killed by freeze-thawing. To examine why the NaN_3 treatment significantly reduced more uptake compared to the killed treatments, part of the experiment was repeated to follow uptake on a time basis. As shown in Figure 4, the frozen-thawed treatment permitted a greater initial surge of NAA accumulation compared to the NaN_3 treatment, but in time the net accumulation of the two approached each other, while the non-treated control continued to accumulate NAA at a steady rate.

4. Methods of Infiltrating Tissue

Preliminary experiments suggested that high levels

Table 5. Eff
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Treatment	
Non-treated	
NaN_3 ($1x10^{-3}$	
Boiled[3]	
Frozen-thawed	
Frozen-thawed	

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Table 5. Effect of several methods of killing hypocotyl sections on naphthaleneacetic acid uptake.

Treatment	NAA accumulated[1]
	(pmoles/10 mg dry wt)
Non-treated	968a
NaN_3 ($1x10^{-3}$ M)[2]	384d
Boiled[3]	587c
Frozen-thawed[3]	612bc
Frozen-thawed[3]+ NaN_3 ($1x10^{-3}$ M)[2]	647b

[1]Means followed by different letters are significantly different at P=0.05.

[2]Present for entire experiment.

[3]Pretreatment.

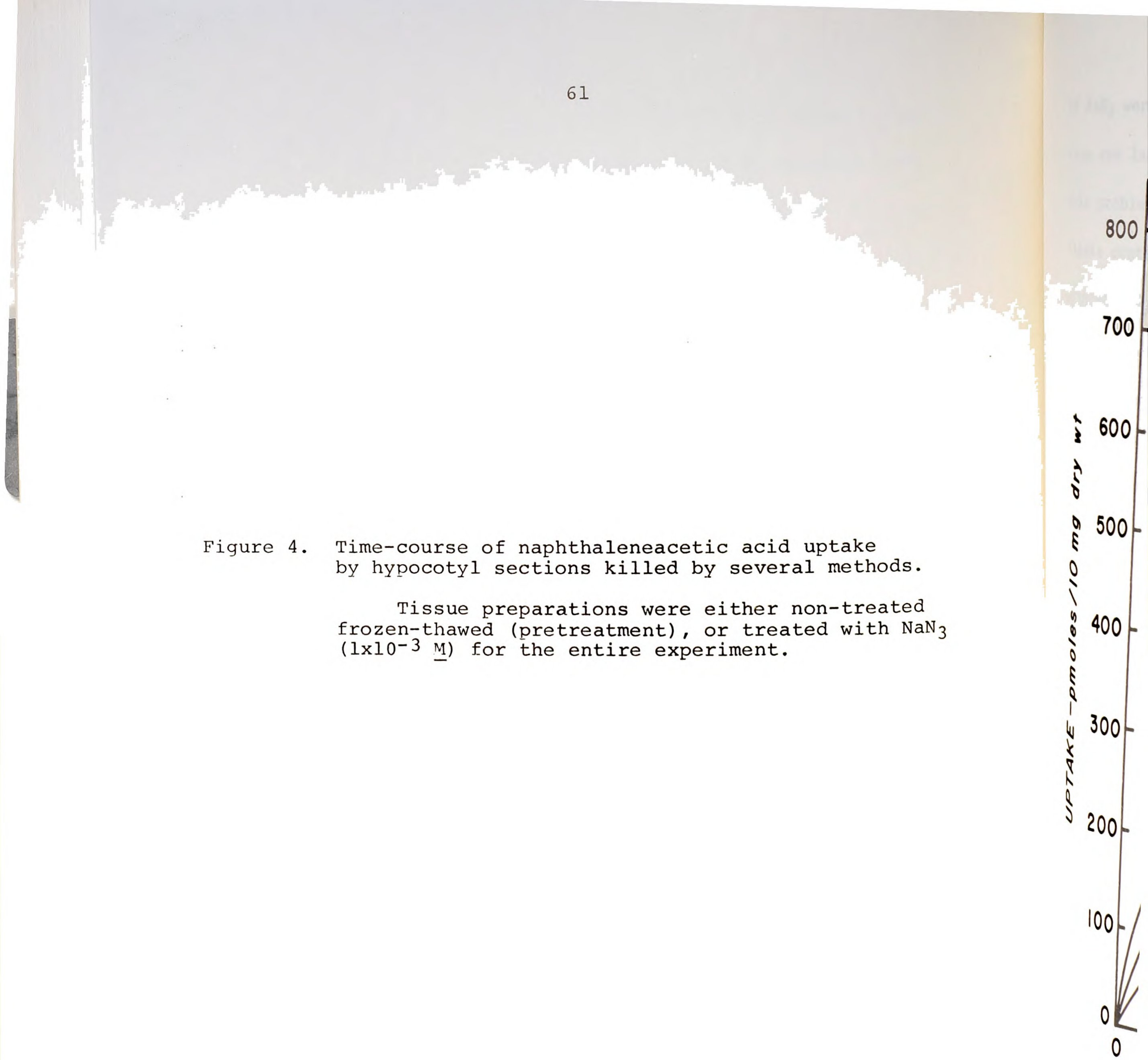
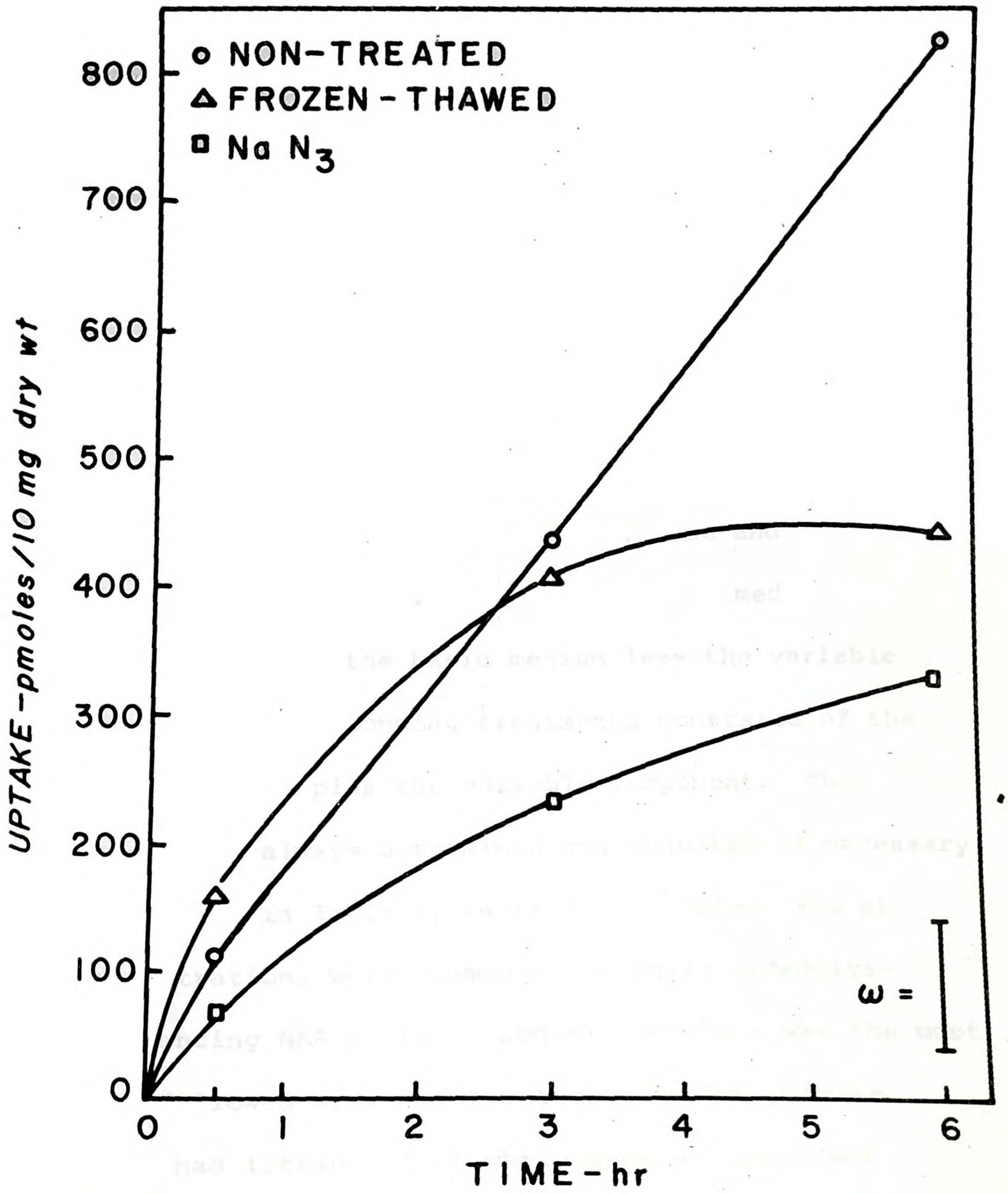


Figure 4. Time-course of naphthaleneacetic acid uptake by hypocotyl sections killed by several methods.

Tissue preparations were either non-treated frozen-thawed (pretreatment), or treated with NaN_3 ($1x10^{-3}$ M) for the entire experiment.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of NaN_3 were required to inhibit NAA uptake. One explanation was lack of penetration of the inhibitor and perhaps this problem could be overcome by infiltration. While flasks containing tissue segments and medium with or without NaN_3 were being agitated in the water bath shaker, a vacuum was drawn for 10 min and then slowly released. Infiltration had no affect on NAA accumulation (Table 6).

5. Effect of Composition of Treatment Media

When a component of the treatment medium was the variable for an experiment, the various respective treatment media were used during the pretreatment, wash and uptake portions of the experiment. The experimental media were prepared by making up the basic medium less the variable component. The corresponding treatments consisted of the experimental medium plus the variable component. The pH of the media was always determined and adjusted if necessary.

As detailed in Table 7, several substrates, all at 20 mM concentration, were examined for their effectiveness in enhancing NAA uptake. Sodium succinate was the most promotive, followed by sodium acetate. Sodium citrate and glucose had little effect while sucrose depressed uptake.

An important factor of the medium was its osmotic strength. The results of using mannitol as an osmoticum

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Frozen-thawed	

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Table 6. Effect of infiltration of the medium containing varied concentrations of sodium azide into hypocotyl sections prior to naphthaleneacetic acid uptake.

Treatment	Infiltrated	NAA accumulated[1]
		(pmoles/10 mg dry wt)
Non-treated	-	389b
Non-treated	+	388b
NaN_3 ($1x10^{-3}$ M)[2]	-	218d
NaN_3 ($1x10^{-3}$ M)[2]	+	228d
NaN_3 ($1x10^{-4}$ M)[2]	-	323c
NaN_3 ($1x10^{-4}$ M)[2]	+	315c
Frozen-thawed[3]	-	430a
Frozen-thawed[3]	+	434a

[1]Means followed by different letters are significantly different at P=0.05.
[2]Present for entire experiment.
[3]Pretreatment-no NaN_3 present.

Table 7. Eff
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Medium[1]	
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Table 7. Effect of various substrates on naphthaleneacetic acid uptake by hypocotyl sections.

Medium[1]	Substrate	NAA accumulated[2]
	(20 mM)	(pmoles/10 mg dry wt)
1	-----	234cd
2	-----	266c
2	sodium acetate	312b
2	sodium succinate	352a
2	sodium citrate	212de
2	glucose	230d
2	sucrose	190e

[1]Medium 1=0.01 M Tris-HCl adjusted to pH 7.4. Medium 2=0.01 M Tris-HCl 0.35 M mannitol and adjusted to pH 7.4.

[2]Means followed by different letters are significantly different at P=0.05.

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are shown in Table 8. A general, but inconsistent, depressive trend on NAA uptake was observed as the mannitol concentration was raised.

II. Development of a Leaf Strip Technique and an Assessment of Tissue Viability

A more direct approach to the study of NAA uptake by leaf cells was a refinement of Smith and Epstein's (1964) technique of preparing leaf strips. It was essential, however, to first develop a quantitative assessment of tissue viability and thus be able to examine uptake on tissue of known viability.

A. Growing of Plants

Soybean, cv Hawkeye, was used for the source of leaf material, as it was found to produce more vigorous plants in greenhouse culture than Chippewa. Seven to eight seeds were sown 1/2-inch deep in a sterilized mixture of soil: sand (3:1) in a 4-inch peat pot. Plants were grown in the greenhouse with a day, night temperature of 25 C and of 18 C respectively. Photoperiod was maintained at 14 hr and a minimum light intensity of 1200 ft-c at the soil surface was established during the light phase.

When the primary leaves were unfolding, all but the four most vigorous plants were removed from each pot.

Table 8.

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0.350
0.375

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Table 8. Effect of varying concentrations of mannitol on naphthaleneacetic acid uptake by hypocotyl sections.[1]

Concn[1] (M)	NAA accumulated[2] (pmoles/10 mg dry wt)
0.000	484a
0.200	434a
0.250	367bc
0.275	326d
0.300	362bcd
0.325	410bc
0.350	378bcd
0.375	342cd

[1]All treatments were present for entire experiment and contained 0.01 M Tris-HCl, 20 mM sodium succinate and adjusted to pH 7.4.

[2]Means followed by different letters are significantly different at P=0.05.

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Plants were foliar and soil fed twice a week with commercial fertilizer containing macro-elements NPK (20:20:20). Insect pests were controlled by fumigation.

B. Preparation of Leaf Strips

Fully expanded trifoliate leaves from the 2nd and 3rd node below the apex were collected and their petioles fully immersed in water for transport to the laboratory. The leaves were washed thoroughly in distilled water before use.

For initial studies, a tissue segment 25 mm wide was removed from the leaflet lamina (including the midrib) and sectioned at 125 and 225 µm (Tissue Sectioner, TC-2, I. Sorvall, Norwalk, Conn.) and 500 and 1000 µm (six razor blades with appropriate spacers). Parafilm was used as a cutting surface for the slicing procedures.

The standard cutting procedure was a modification of that described by Smith and Epstein (1964). Twelve leaflets were stacked on top of each other with their margins aligned. A 1-inch by 3-inch glass microscope slide was placed parallel to the midvein and a 1-inch wide (25 mm) strip was removed from the leaflet. These 12 strips were then cut in half and stacked together with the last freshly cut edges directly on top of each other (center edge over center edge). Thus, a block of 24 pieces of 25 mm wide strips

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was made and then placed between two pieces of fine pore compact styrofoam. The styrofoam-enclosed block of tissue was mounted on a Spencer table microtome (Thomas 7125) and sectioned with a razor blade (blue steel) held in a cyrostat razor blade holder (IEC-3351; International Equipment Co., Needham Heights, Mass.). The styrofoam-held tissue block was positioned such that it was only necessary to cut the front piece of styrofoam when cutting the block. The front piece of styrofoam was as thin as possible (2 mm) while the back piece was thicker (5 mm) to offer greater support. A small elastic band helped keep the tissue block together. The razor blade was changed frequently to allow clean cuts through the tissue. Whenever a blade was changed, a check slice was made to realign the blade to the block. The appropriate thickness was dialed before each slice.

Four consecutive slices, yielding 96 strips, 250 µm x 25 mm, represented the amount of leaf material for each treatment. Unless otherwise noted, sufficient leaf strips were cut from one tissue block to supply all the leaf strips for an entire replication. This method permitted the benefit of working with a complete block design to allow statistical removal of tissue variance.

As the strips were cut, they were immediately placed in 7 ml of filter sterilized treatment medium within a

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25 ml Erlenmeyer flask. The initial medium consisted of 0.30 M mannitol; 0.01 M Tris-HCl; 0.004 M sodium succinate; 1.0% (w/v) polvinyl pyrrolidone-k30 (PVP); 50 µg/ml bovine serum albumin, fraction V (BSA); and the pH adjusted to 7.4. When all the leaf strips for a particular experiment were cut, the media from the respective treatments containing any sloughed-off plant material was removed by suction and replaced with fresh media.

An important variation of Smith and Epstein's (1964) technique was the omission of the "tea bag" to handle the leaf strips. It was found that the strips (25 mm long) generally entwined so they could be handled en masse with forceps. The massed strips could be placed on one side of the Erlenmeyer flask where they would adhere to the wall; then the flask was tilted in the opposite direction permitting the media to be withdrawn by suction.

C. Assay of Viability by Tetrazolium Reduction

Initial studies consisted of a modification of the technique of Davies and Seaman (1968). The dye 2,3,5-triphenyl tetrazolium chloride (TTC) was used at 2.0 mg/ml unless otherwise noted. It was administered to the experimental system in a manner identical to that described for ^{14}C-NAA in the hypocotyl experiments. The treatment conditions were the same except for the previously described

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different medium (Exp. II.B.). After 3 hr, the experiment was terminated by rapid withdrawal of the medium containing the non-reduced dye by suction, followed by the addition of xylene. The tissue was stored in the dark until it was homogenized. The homogenate was quantitatively transferred into centrifuge tubes and diluted to 10 ml volume with xylene. After centrifugation (2000 x g; 10 min) the supernatant was decanted into test tubes. Trichloroacetic acid (TCA) was added (approximately 1 g crystaline) to each tube and absorption was measured (535 nm) immediately thereafter on a Beckman DU Spectrophotometer. The spectrophotometer was standardized against a xylene blank. Samples were stored in the dark to minimize photodestruction of the reduced tetrazolium.

Dry weights were determined on parallel samples of similarly treated tissue, and were measured on a macro-analytical balance (Mettler B-5).

D. Establishment of Experimental Parameters with 2,3,5-Triphenyl Tetrazolium Chloride

1. Determination of Light Absorption Curve

An absorption curve was determined on either xylene extracted leaf tissue or chemically reduced tetrazolium with a DU-spectrophotometer at 5 nm increments standardized against a xylene blank. TTC was reduced in a dilute HCl

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solution containing metallic zinc fillings. The resulting formizan compound was then partitioned into xylene. The absorption spectra of the two preparations had considerable overlap (Figure 5A). The addition of TCA resulted in a shift in the absorption maxima as well as intensification of the TTC absorption. The TCA treatment of the reduced TTC shifted the absorption maximum from 485 to 535 nm (Figure 5B).

2. Determination of Optimum 2,3,5-Triphenyl Tetrazolium Chloride Concentration

The effect of TTC concentration was examined to determine the optimum level for reduction by leaf strips. The lowest concentration, 2 mg/ml, was equally effective as 5 and 10 mg/ml, whereas 20 mg/ml slightly depressed reduction (Figure 6). Since tetrazolium salts have been shown to uncouple oxidative phosphorylation (Palmer and Kalina, 1966), 2 mg/ml of TTC was chosen for all further experiments.

3. Refinement of Leaf Strip Preparation

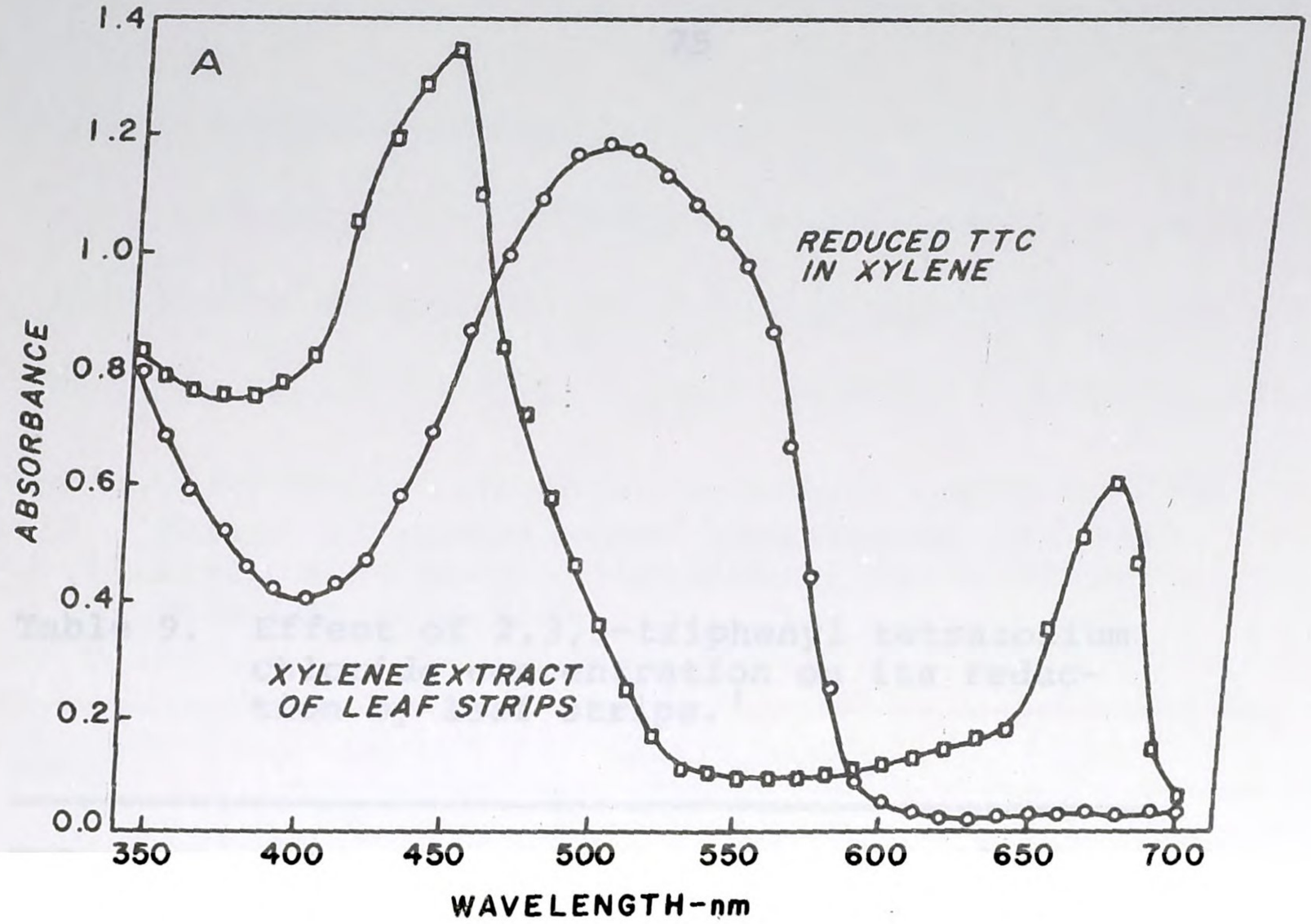
The effect of a low temperature pretreatment wash on minimizing the wound response from the cutting process, especially for thin slices, was next established. The data presented in Table 10 show that the low temperature slightly depressed reduction. The frozen-thawed pretreatment data served as a control equivalent to dead tissue (dead control). Furthermore, the thin slices (125 and 225 μm) were less

Figure 5. Light absorption curves of xylene extracts of leaf strips and reduced 2,3,5-triphenyl tetrazolium chloride (TTC) with and without trichloroacetic acid.

A. Xylene extract of TTC-treated leaf strips compared to reduced TTC.

B. Xylene extract of TTC-treated leaf strips and reduced TTC, both in the presence of 20% trichloroacetic acid (TCA).

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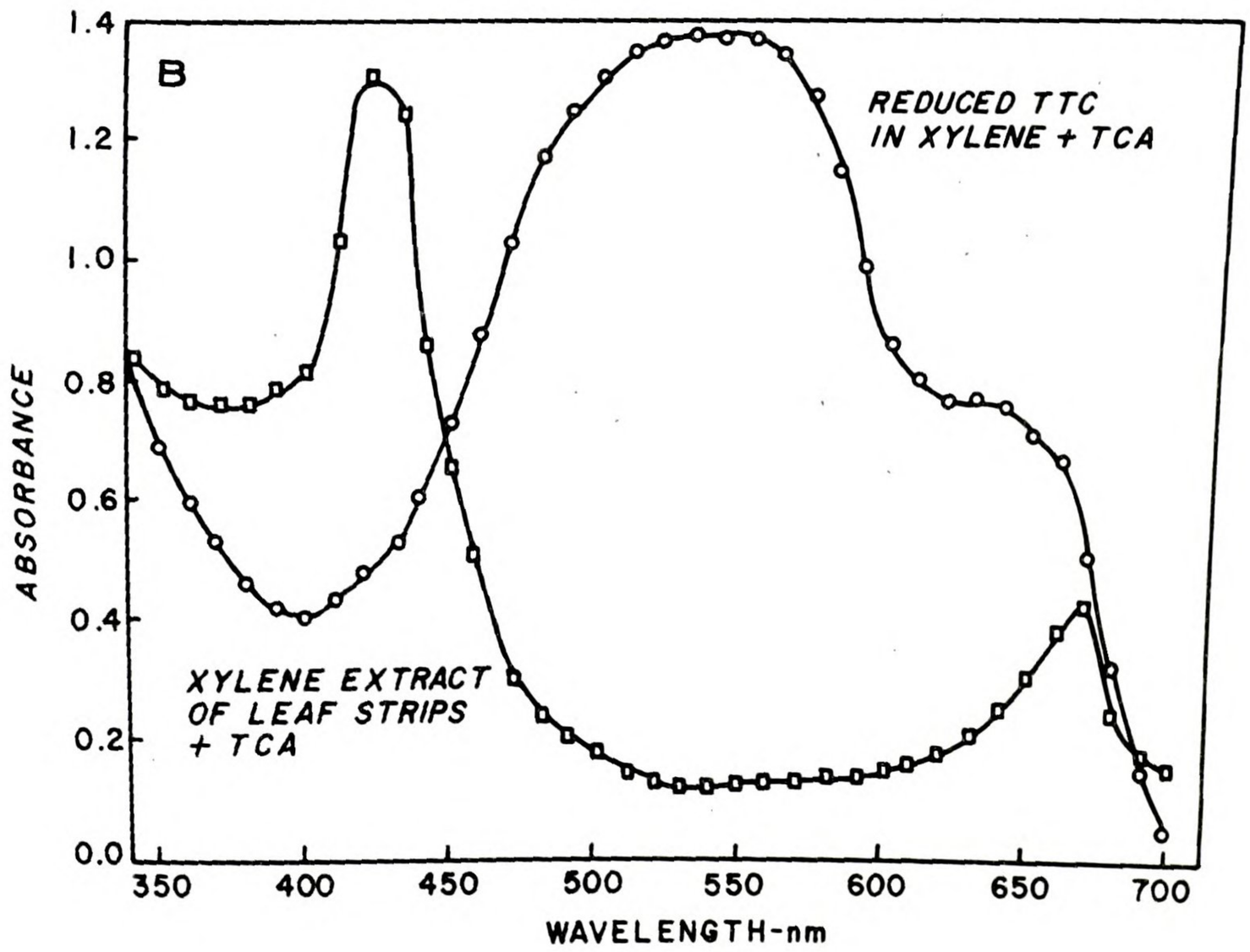
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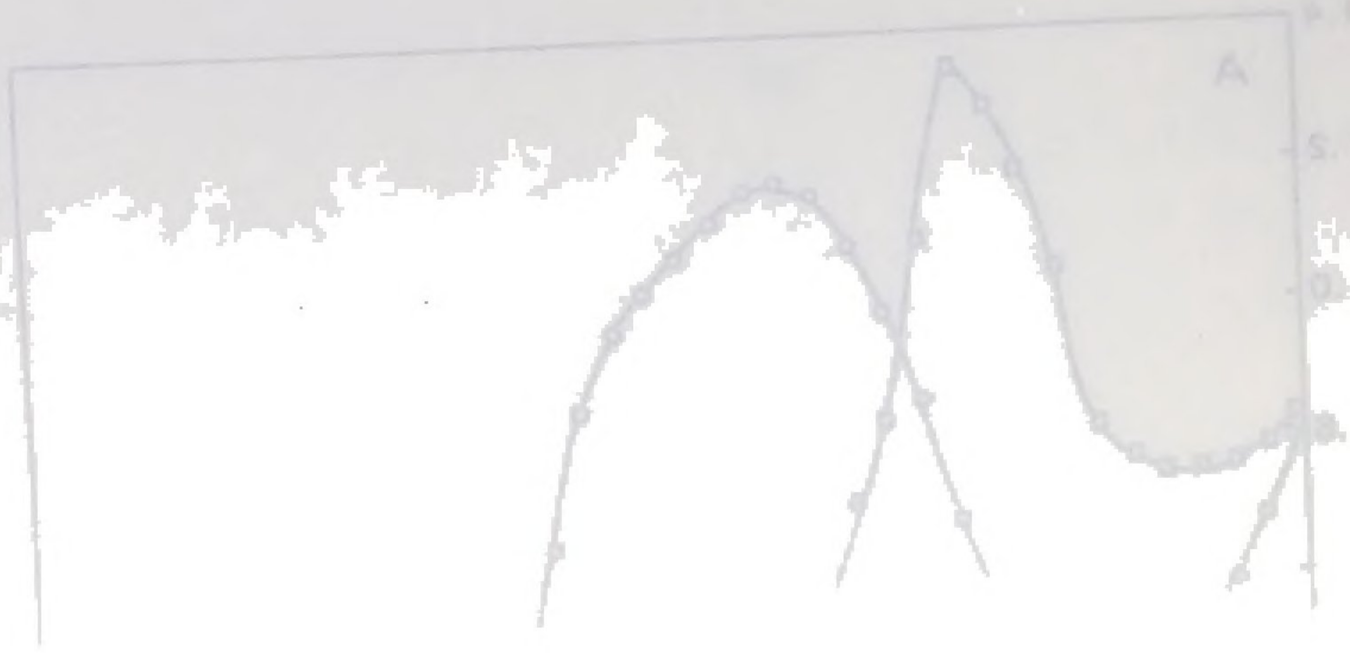

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Table 9. Effect of 2,3,5-triphenyl tetrazolium chloride concentration on its reduction by leaf strips.[1]

TTC concn (mg/ml)	TTC reduced[2] (A-535 nm)
0	112c
2	230ab
5	250a
10	236ab
20	188b

[1]Leaf strips were 1000 µm thick; each replicated treatment was cut from a separate leaf.

[2]Means followed by different letters are significantly different at P=0.05.

Table 10.

Leaf strip width (μm)	Mean
125	
225	
1000	

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Table 10. Effect of pretreatment temperature and leaf strip size on 2,3,5-triphenyl tetrazolium chloride reduction.

Leaf strip width	TTC reduced		
	Wash temperature		
	0 C	23 C	Frozen-thawed
(μm)	(A-535 nm/10 mg dry wt)		
125	0.057	0.068	0.006
225	0.099	0.120	0.023
1000	0.321	0.366	0.012
Mean	0.151[1]	0.184[1]	0.014[2]

[1]Wash temperature means are significantly different at P=0.10 by orthogonal comparison.

[2]Freeze-thawing is significantly different from wash treatments at P=0.01.

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The preliminary experiments, to this point, consisted of material from single leaves for each replicated treatment, and the resulting data showed considerable variation between replications of a given treatment. One explanation was variability in leaf age of the material on its inherent ability to reduce the TTC. When leaves of various physiological ages were sampled for the TTC reduction capacity, considerable variation was found (Table 11). A definite trend for increasing capacity to reduce TTC was apparent with age. The 2nd and 3rd leaves were chosen as the source of future experimental material because of similar activity.

4. Modification of Treatment Media and Conditions

The importance of the proper osmotic strength of the treatment medium has been considered to be one of the most important factors in developing a suitable medium (Hallaway, 1968). In a preliminary study, no significant effect of osmotic strength on TTC was observed using a mannitol concentration range from 0.20 M to 0.35 M (Appendix-Table A1). However, all levels of mannitol resulted in significantly more TTC reduction than did the 0.5 mM $CaSO_4$ base medium recommended by Smith and Epstein (1964). Therefore, a wider range of mannitol concentration was employed only during the initial wash of the cut sections. This

Table 11.

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Table 11. Effect of age of leaf used as a source of leaf strips on 2,3,5-triphenyl tetrazolium chloride reduction.

Position of leaf in relation to shoot apex	TTC reduced[1]
	(A-535 nm)
1st-unfolded	0.486d
2nd-expanding	0.625c
3rd-expanding	0.655c
4th-expanded	0.867b
5th-expanded	1.027a
7th-expanded-senescent	0.511cd
4th-expanded-picked three days earlier	0.644c
3rd-expanded-frozen-thawed	0.105e

[1]Means followed by different letters are significantly different at P=0.05.

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experiment was designed to examine Takebe's et al. (1968) hypothesis that high osmotic strength during this critical period might minimize the uptake of detrimental material released from damaged cells. Data in Table 12 show that the use of a hypertonic or hypotonic media were detrimental as compared to an isotonic solution (0.25 M) for TTC reduction. Therefore, all of the following experiments were conducted in media with 0.25 M mannitol.

Results with hypocotyl sections suggested that sodium succinate (20 mM) was an effective substrate for stimulation of ^{14}C-NAA uptake. For leaf strips a lower concentration (8 mM) was optimum for TTC reduction when exposed to light (Figure 6). Also, it was apparent that light, per se, plays an important role in TTC reduction by leaf strips.

An attempt was made to separate uptake of TTC from subsequent reduction by preloading the tissue with TTC and then following reduction under varying conditions. A time-course of TTC reduction is illustrated in Figure 7, where treatments were removed from the TTC and allowed to incubate in TTC-free medium until the end of the experiment. Consistently less net reduced TTC was found in samples removed from the TTC medium and allowed to incubate in TTC-free medium compared to when first removed from the

Table 12.

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Table 12. Effect of mannitol concentration during the initial wash of leaf strips prior to 2,3,5-triphenyl tetrazolium chloride reduction.

Mannitol concn[1,2]	TTC reduced		
	Strip width (µm)		
	500	1000	Mean[3]
(M)		(A-535 nm)	
0.10	0.397	0.492	0.444bc
0.25	0.462	0.581	0.521a
0.45	0.408	0.468	0.451b
0.60	0.387	0.459	0.423d
0.75	0.421	0.439	0.429cd

[1]Pretreatment wash medium=treating medium except variable mannitol concentration.

[2]Treatment medium during reduction=0.25 M mannitol, 0.01 M Tris-HCl, 4 mM sodium succinate, 10 mg/ml PVP 50 µg/ml BSA and adjusted to pH 7.4.

[3]Means followed by different letters are significantly different at P=0.05. Interaction of size and mannitol concentration is not significant.

Figure 6. Effect of sodium succinate concentration and light on 2,3,5-triphenyl tetrazolium chloride reduction by leaf strips.

Leaf strips were 1000 µm. The interaction of 8 mM sodium succinate with light and dark is significant at P=0.05 by orthogonal comparison.



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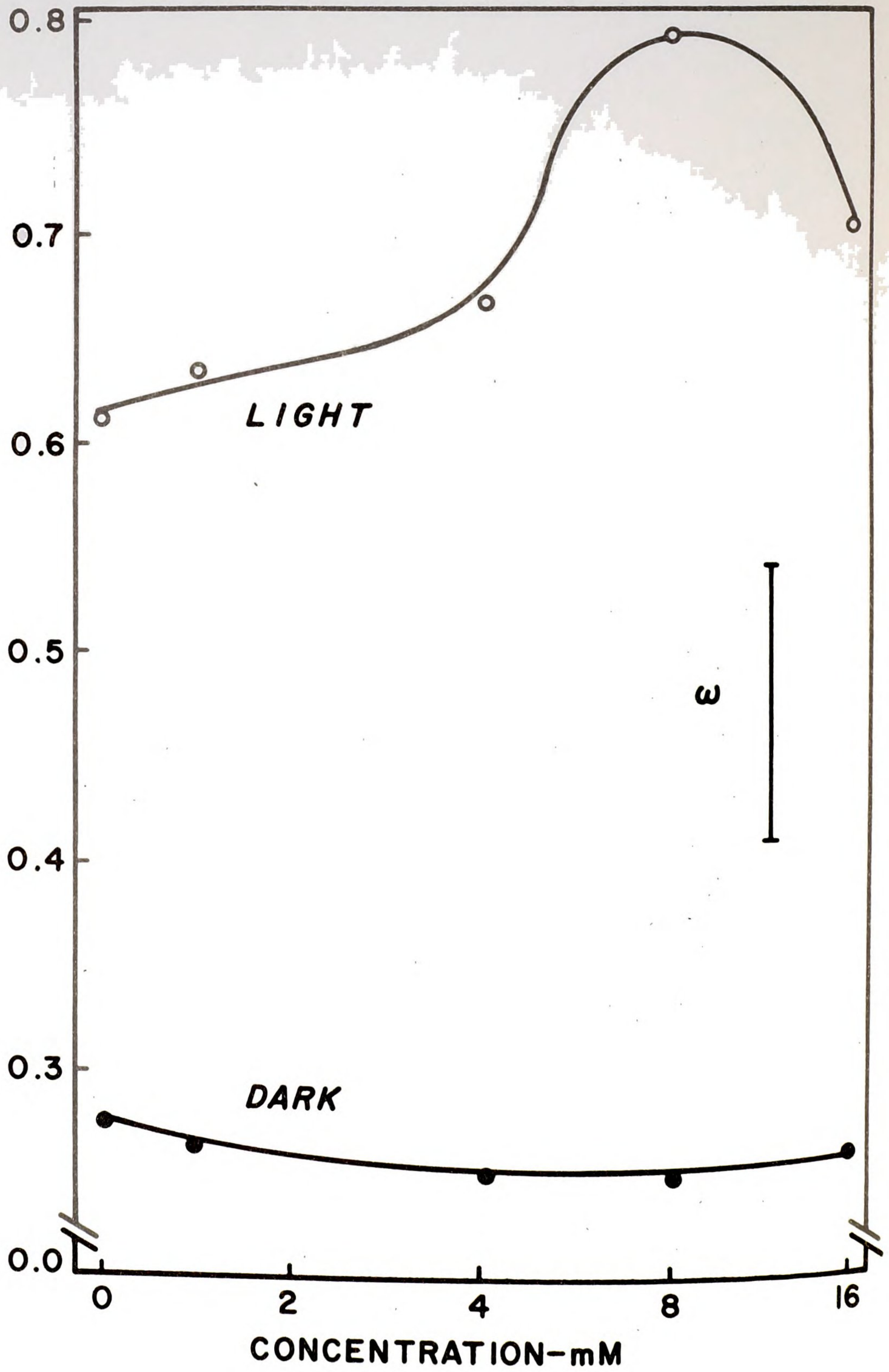
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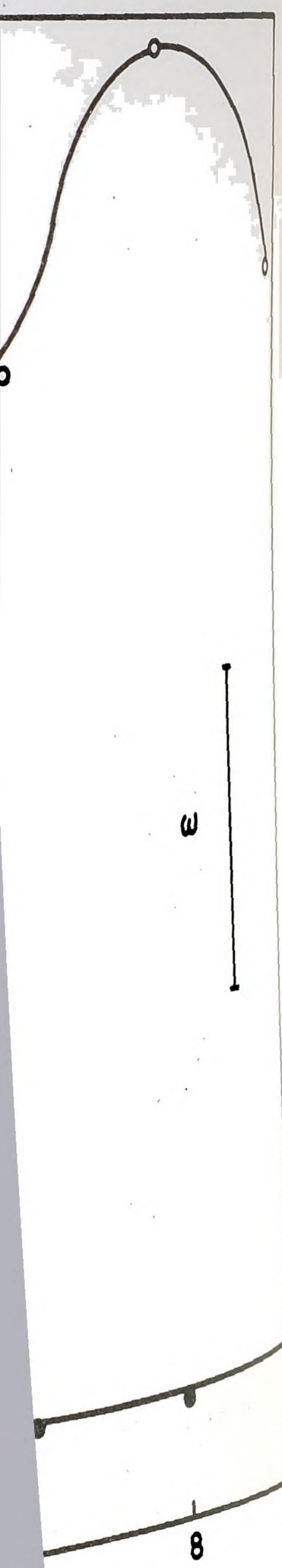
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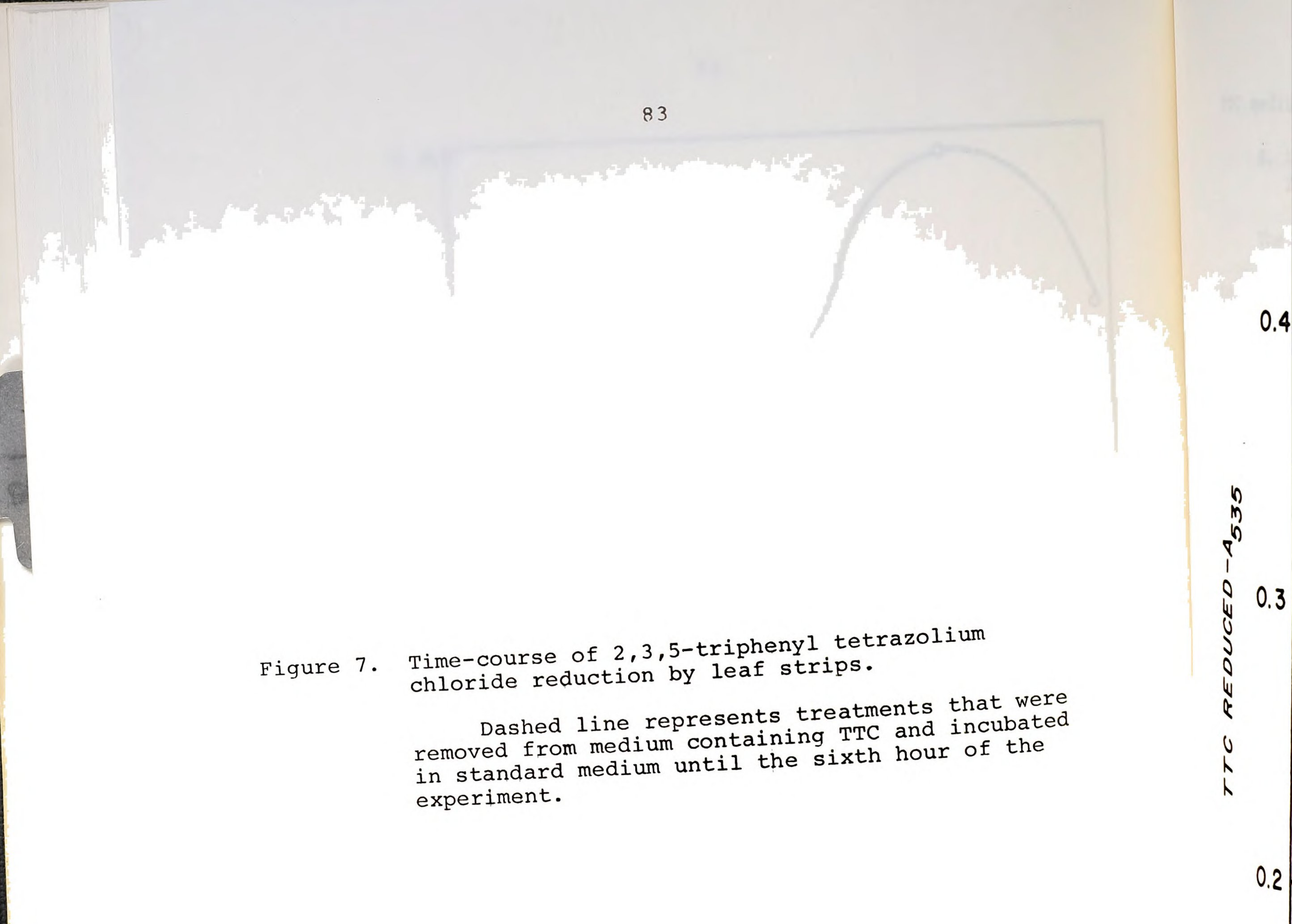
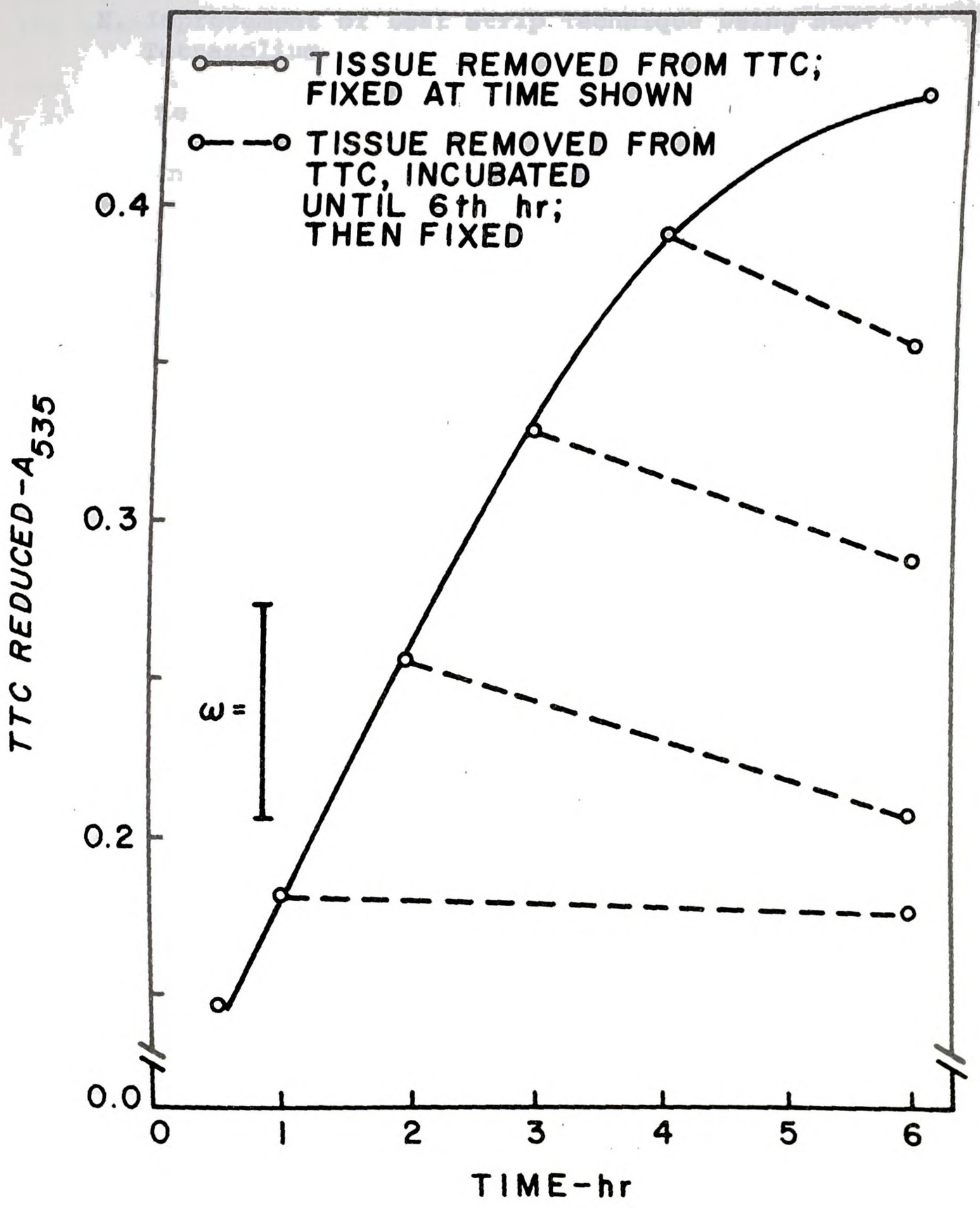


Figure 7. Time-course of 2,3,5-triphenyl tetrazolium chloride reduction by leaf strips.

Dashed line represents treatments that were removed from medium containing TTC and incubated in standard medium until the sixth hour of the experiment.



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E. Improvement of Leaf Strip Technique Using Neo-Tetrazolium Chloride

The net loss of reduced TTC within the tissue exposed to light indicated the need for a tetrazolium compound stable under these conditions. The usefulness of neo-tetrazolium chloride (NTC) (Nutrititional Biochemical Corp.) was next established. Unless otherwise noted, NTC was used at 0.25 mg/ml.

The staining procedure was identical to that used for TTC. However, because of low water solubility, NTC was first dissolved in 2 ml 50% ethanol and then the aqueous medium solution was added. The ethanol was then removed by partial flash evaporation. Finally, the resultant NTC solution was made to volume with the treatment medium. At concentrations in excess of 2.0 mg/ml, NTC precipitated out of solution.

The assay for the reduced form of NTC was the same as the method for the reduced TTC with the exception that the absorbance was measured directly at 565 nm in a spectrophotometer (Bausch-Lomb Spectronic 20) after centrifugation of the xylene homogenate.

1. Determination of Light Absorption Curve

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xylene. The light absorption curves were determined by the methods described for the reduced TTC. The use of TCA was found unnecessary, for there was a distinct separation between the absorption curves of the reduced NTC and the tissue extract (Figure 8). This wide separation permitted the use of a colorimeter to determine the absorbance of the reduced NTC at 560 nm.

2. Determination of Optimum Neo-Tetrazolium Chloride Concentration

The optimum concentration of NTC was 0.25 mg/ml NTC (Figure 9). The 0.10 mg/ml was significantly less effective while concentrations above 0.50 mg/ml also were lower. A dead control (frozen-thawed) treatment indicated the level of reductive capacity of dead tissue was nominal.

3. Determination of Optimum Strip Width

Smith and Epstein (1964) reported that the leaf strips 300 μm in width were optimum for potassium uptake studies. Because of possible light destruction of reduced TTC, the experiments with the TTC may have been misleading in identifying optimum strip width. Narrow strips would have more exposure to light and therefore may have lost more of their reduced TTC. Also, the previous experiments with NTC revealed the outer edges of the leaf strips stained more intensely. For these reasons, the leaf strip width

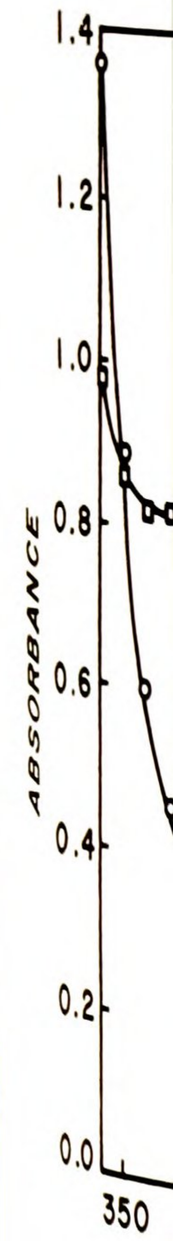

Figure 8. Light absorption curves of xylene extract of non-treated leaf strips and reduced neo-tetrazolium chloride.

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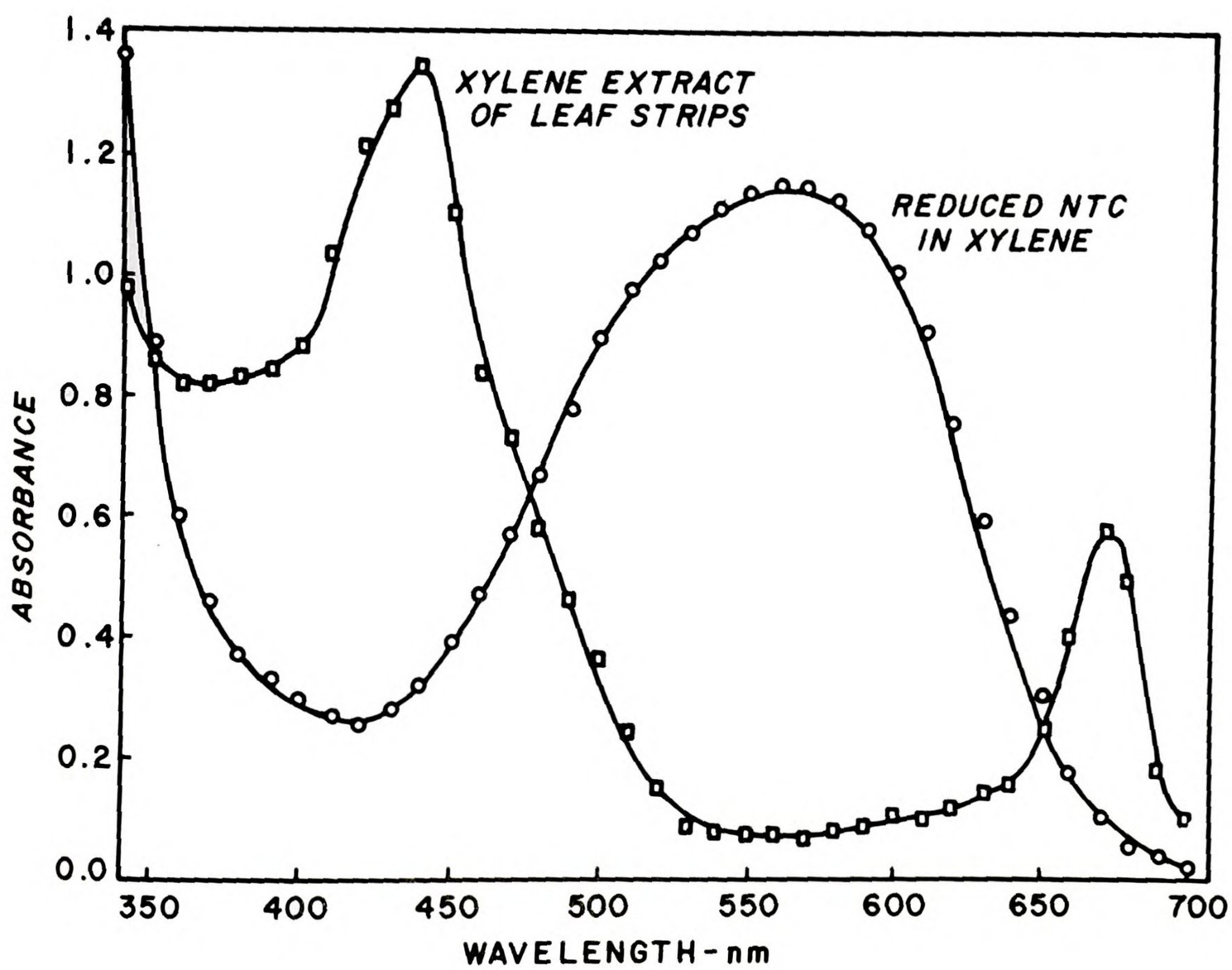

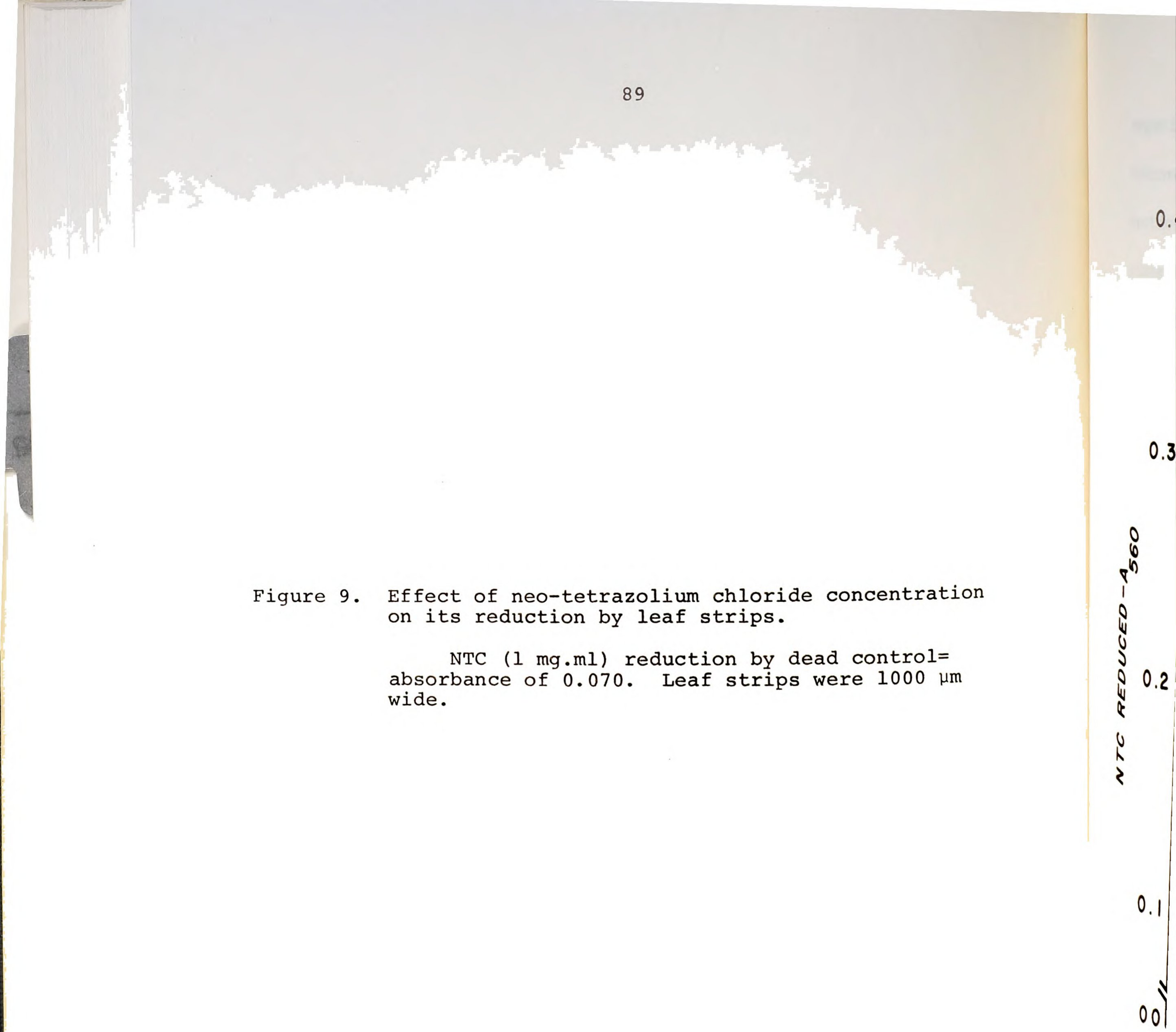

Figure 9. Effect of neo-tetrazolium chloride concentration on its reduction by leaf strips.

NTC (1 mg.ml) reduction by dead control= absorbance of 0.070. Leaf strips were 1000 µm wide.

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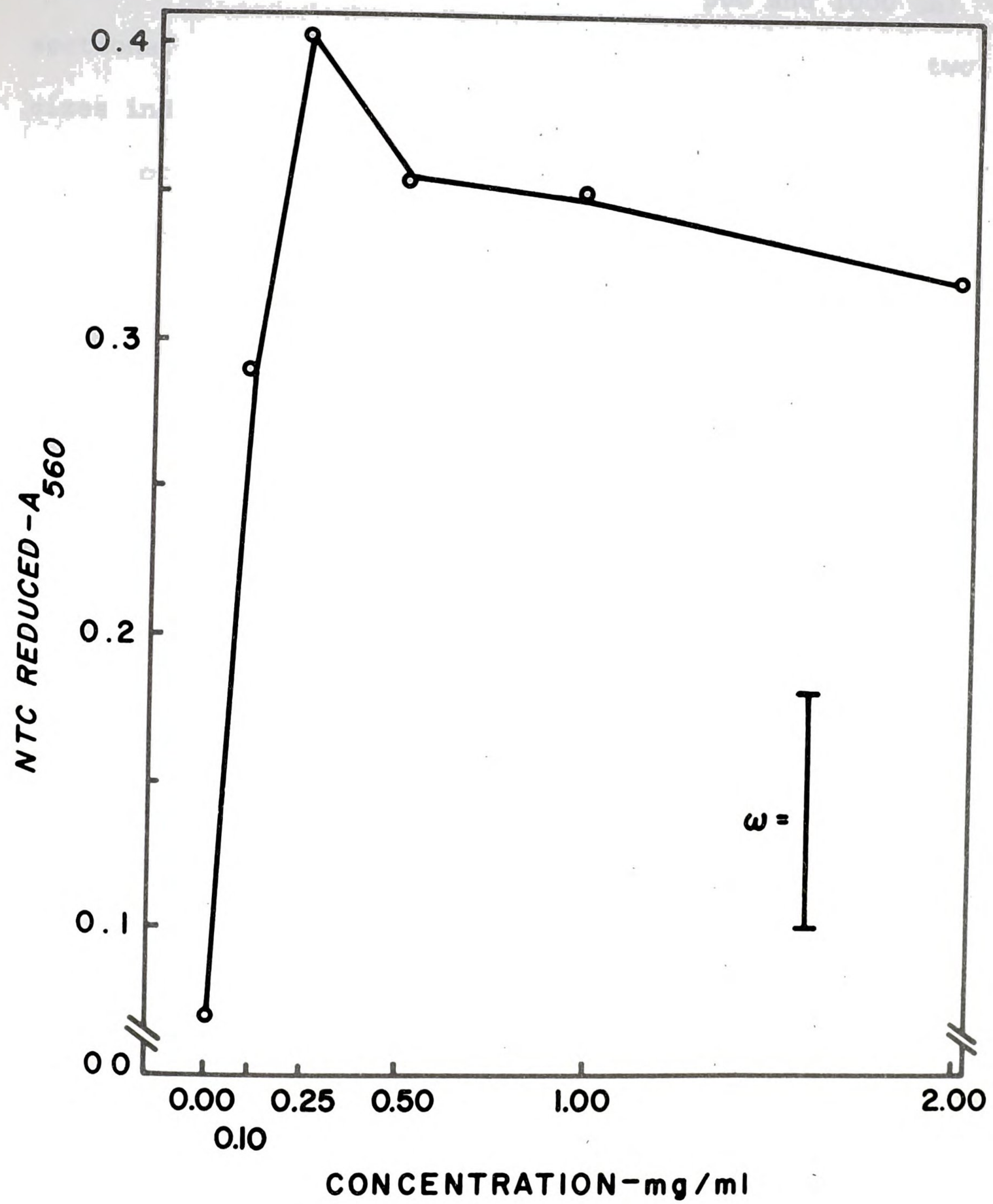

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experiment was repeated using NTC. The thin strips (250 µm) stained more intensely than the thicker (500 and 1000 µm) sections. The consistent downward trend between these two sizes indicated that the 250 µm size was probably the best width for maximum tissue exposure to the solution with minimum cutting damage (Figure 10).

To examine the possibility that the enhanced staining of the 250 µm sections was the result of a wound response, respiration rates were determined on sections cut at 250, 500 and 1000 µm. Warburg respiration manometry was employed. The respiration rates were determined hourly for 5 hr on sections given the standard pretreatement wash. As recorded in Table 13, oxygen uptake was determined on strips of different width (3 reps each) with either a constant mass of tissue per flask or a constant cut surface per flask. There was no difference between the various strip widths when the data were expressed as oxygen uptake per unit dry weight and thus wounding was not a dominant factor.

F. Refinement of Treatment Media

1. Effect of pH and Buffers

Hallaway (1968) indicated that pH was one of the major factors in a treatment medium. Leaf strips were pretreated for 2 hr in media containing 0.01 M Tris-maleate at various pH values and then allowed to reduce

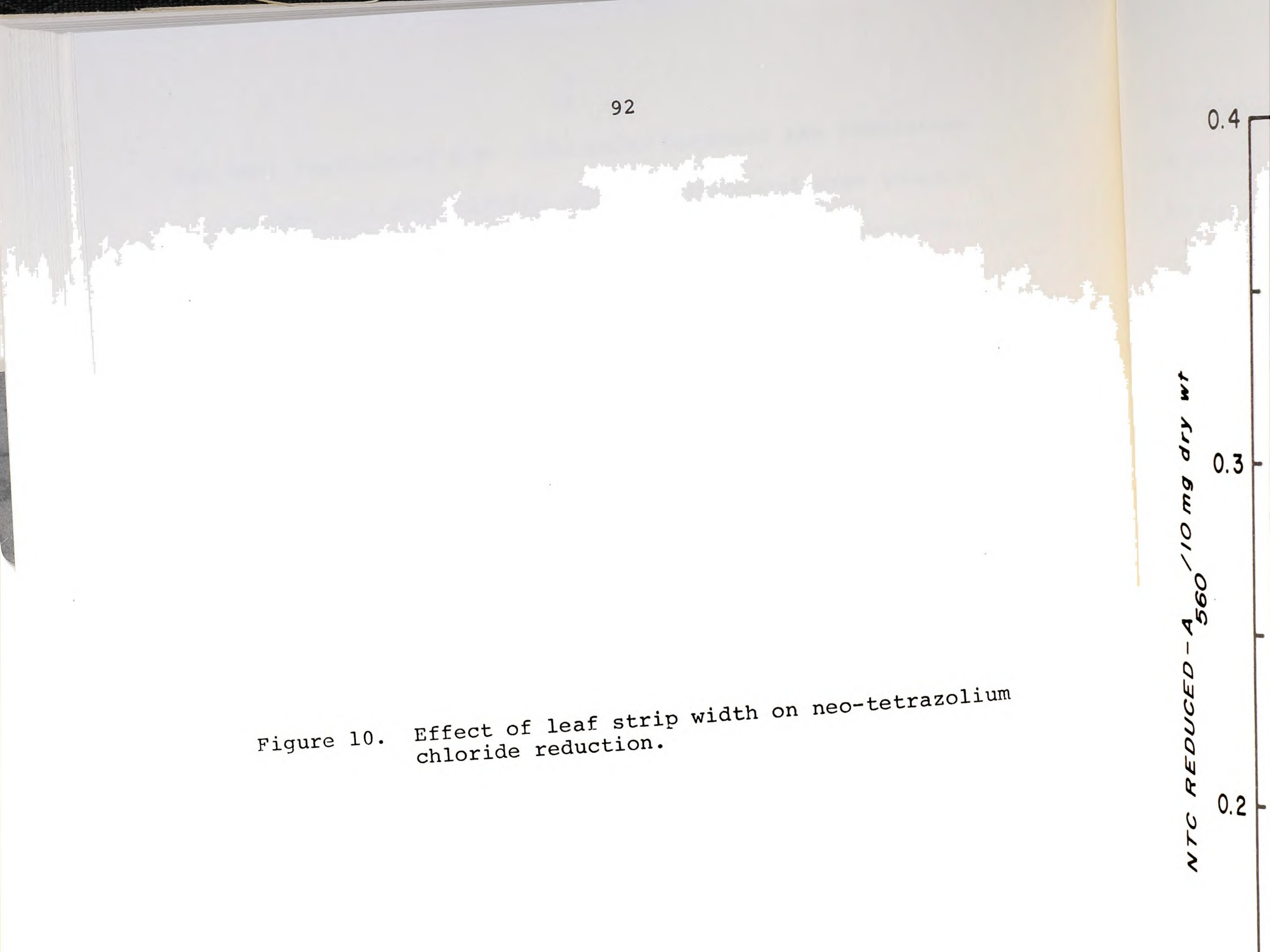

Figure 10. Effect of leaf strip width on neo-tetrazolium chloride reduction.

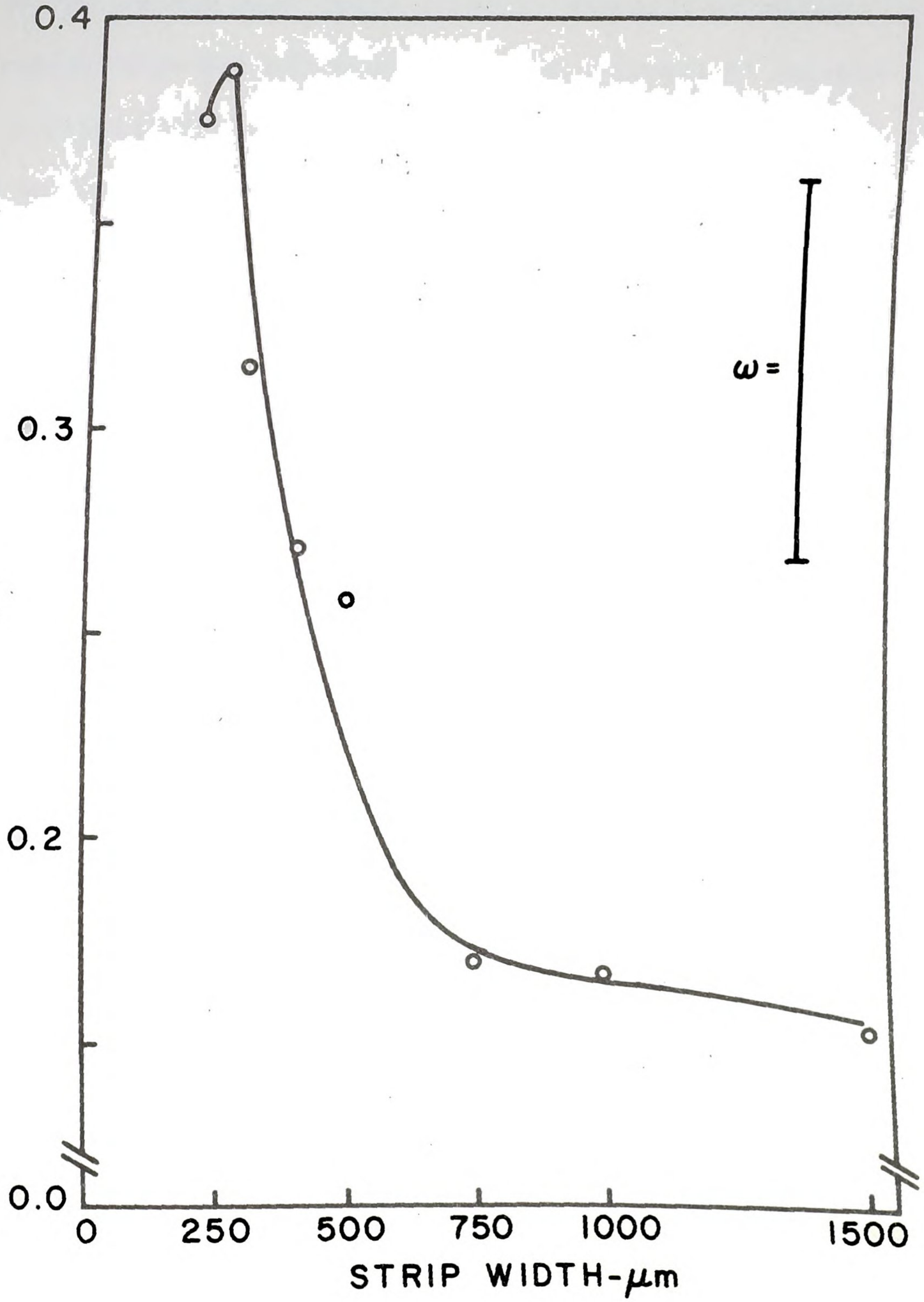

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Table 13. E

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Table 13. Effect of leaf strip width on oxygen uptake.

Number of strips	Strip width	Dry wt	Oxygen uptake[1,2]	
	(μm)	(mg)	(μl O_2 per hr)	
			(per total wt)	(per 100 mg dry wt)
20	250	2.9	10.5d	371a
20	500	5.9	23.6c	419a
20	1000	12.2	46.2b	378a
160	250	23.3	85.2a	368a
48	500	13.9	54.8b	394a

[1]Means within a column followed by different letters are significantly different at P=0.05.
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NTC either in the same media (freshly changed) or media containing Tris-HCl adjusted to pH 7.4. Figure 11 depicts that a significant trend toward pH 6.4 was the optimum hydrogen ion concentration. Because treatments at the same pH leveled stained similarly, irrespective of the pH during the NTC treatment (details in Figure legend), it was concluded that the pH effect was mainly on tissue reduction capacity and not on the uptake of the NTC. This meant that all subsequent pH experiments could be conducted in one step with NTC uptake and reduction, the various pH treatments occurring simultaneously.

The same pH range was re-examined more critically using the same buffer. This more detailed study indicated that maximum NTC reduction occurred at pH 6.6 (Figure 12).

Good et al. (1966) reported several new hydrogen buffers that would be better suited for biological systems than Tris and similar buffers. As detailed in Table 14, there was no difference between the buffers at a concentration of 0.01 M, but at 0.05 M HEPES appeared superior.

Using HEPES and MES buffers, the pH range was re-examined for optimum reduction. As illustrated in Figure 13, the two buffers complimented each other with pH 6.7 being optimum for NTC reduction. Subsequent media was made up with HEPES (0.01 M) as the buffer adjusted to pH 6.7

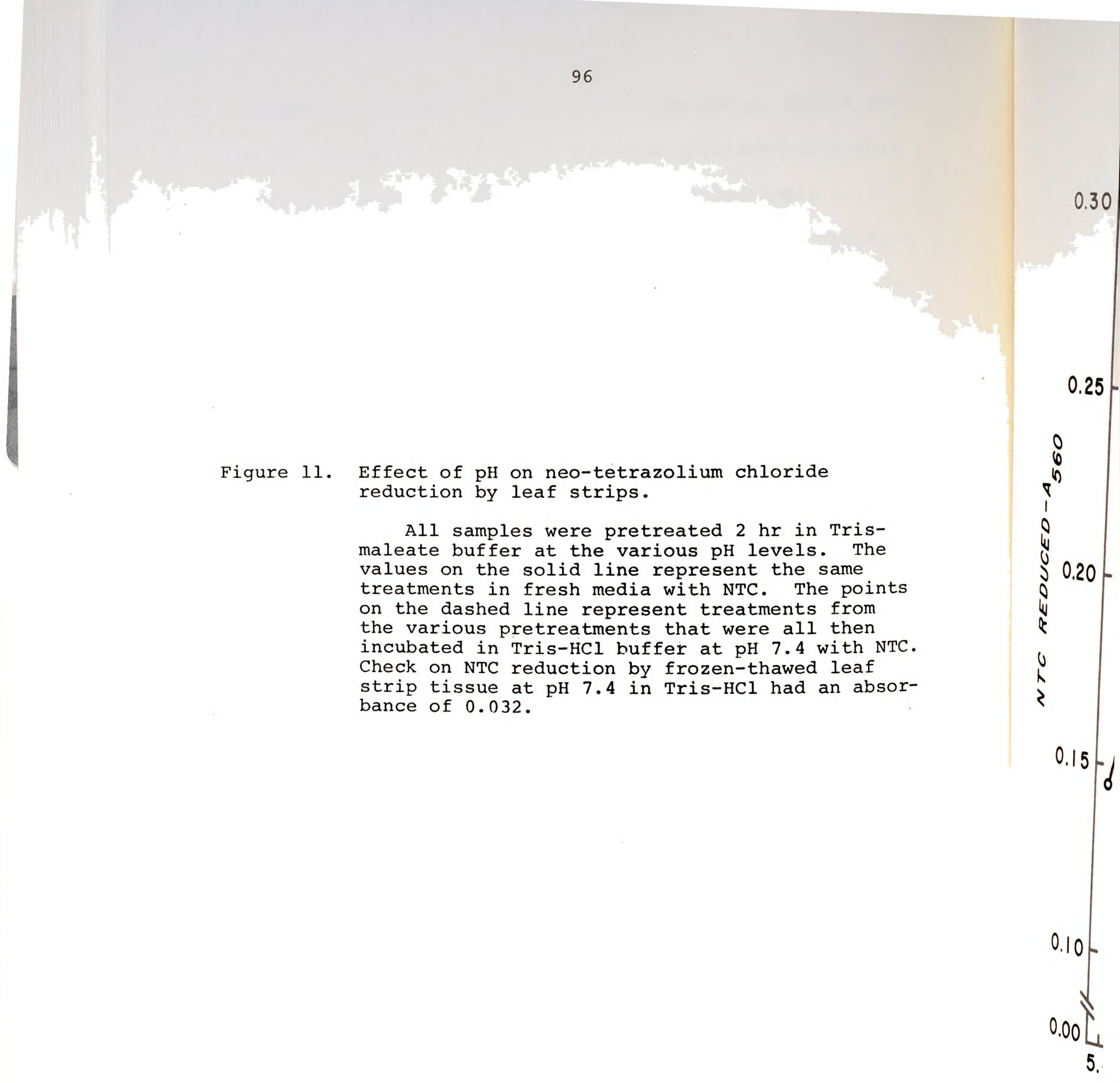

Figure 11. Effect of pH on neo-tetrazolium chloride reduction by leaf strips.

All samples were pretreated 2 hr in Tris-maleate buffer at the various pH levels. The values on the solid line represent the same treatments in fresh media with NTC. The points on the dashed line represent treatments from the various pretreatments that were all then incubated in Tris-HCl buffer at pH 7.4 with NTC. Check on NTC reduction by frozen-thawed leaf strip tissue at pH 7.4 in Tris-HCl had an absorbance of 0.032.

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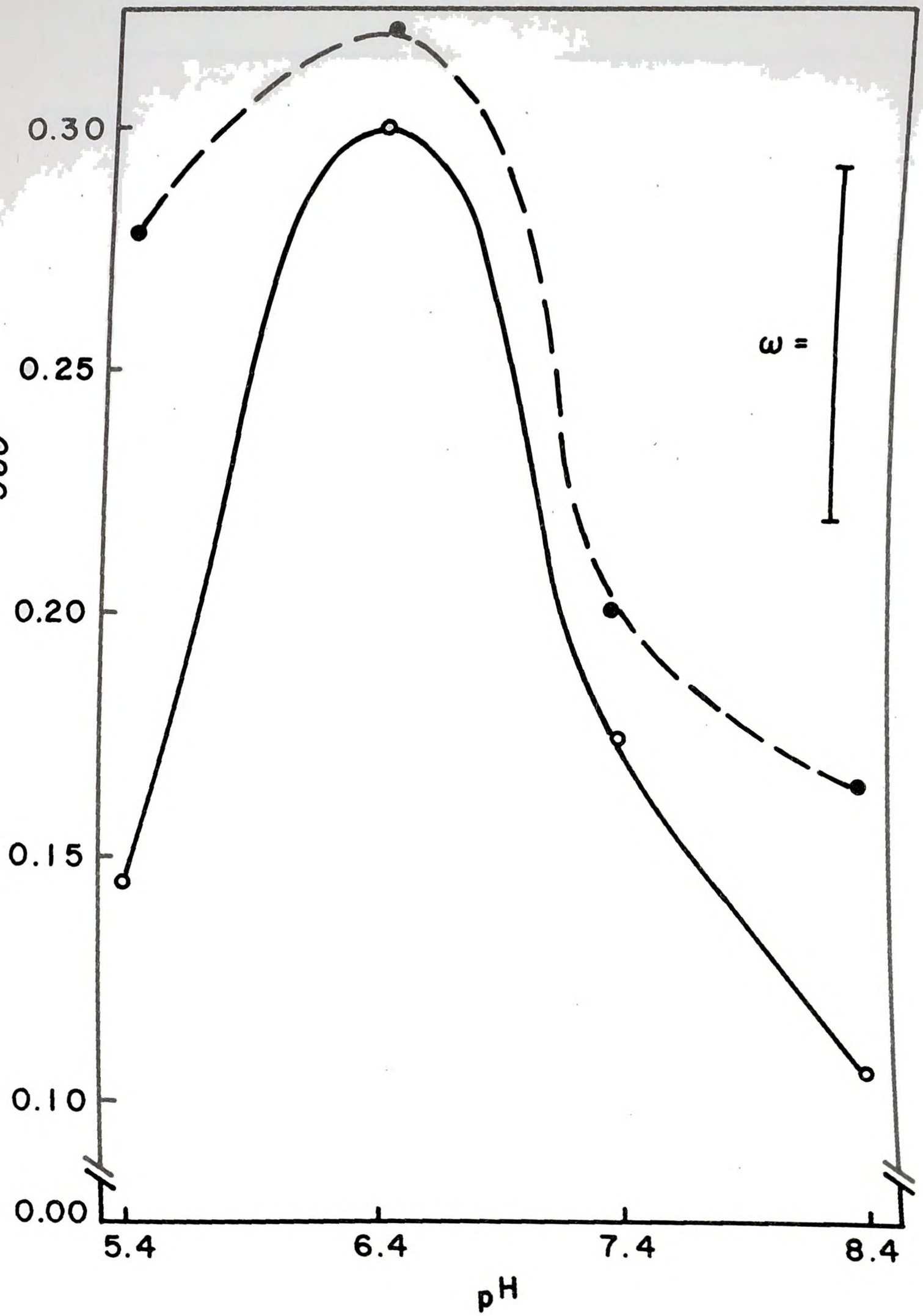

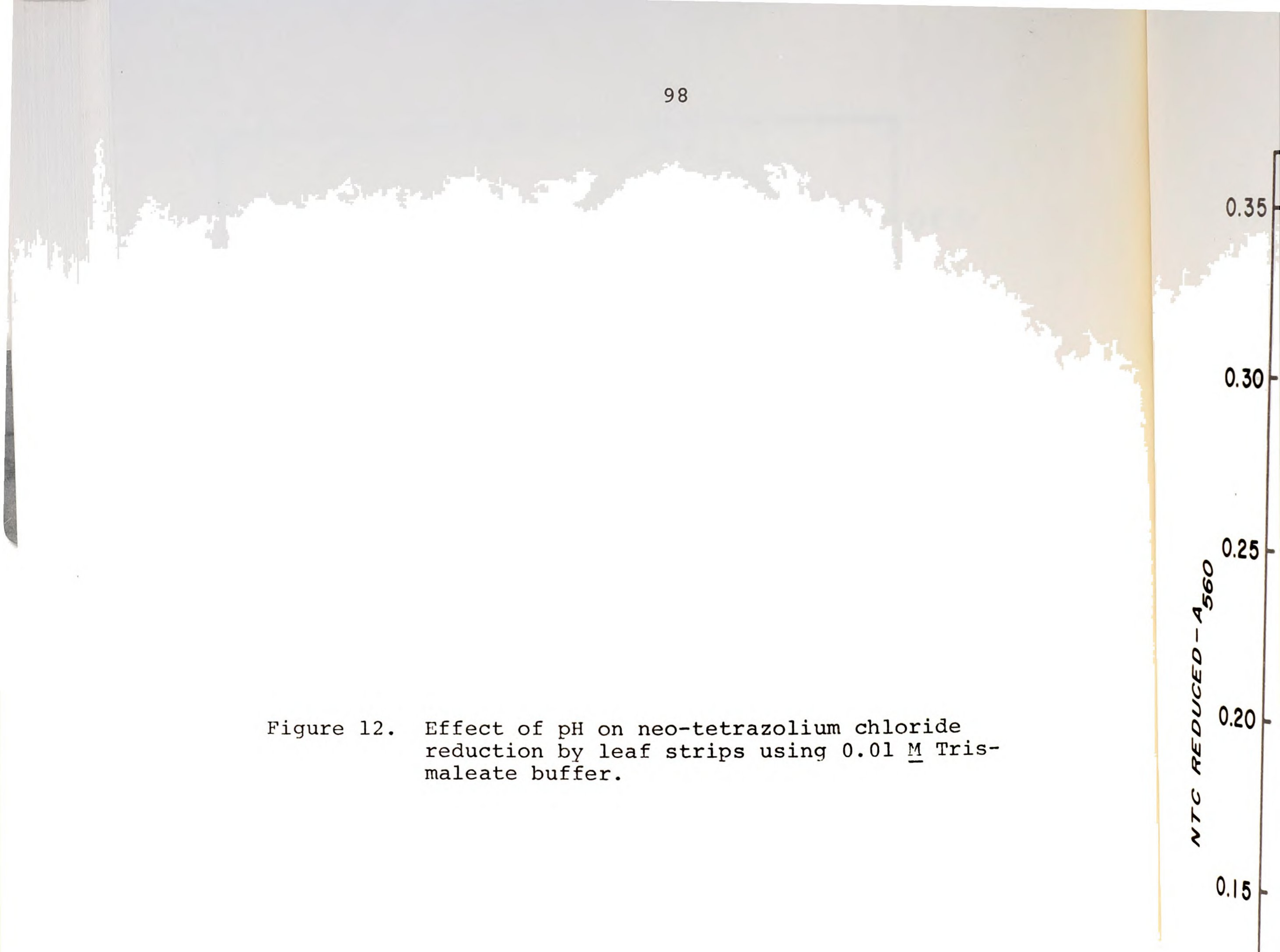

Figure 12. Effect of pH on neo-tetrazolium chloride reduction by leaf strips using 0.01 M Tris-maleate buffer.

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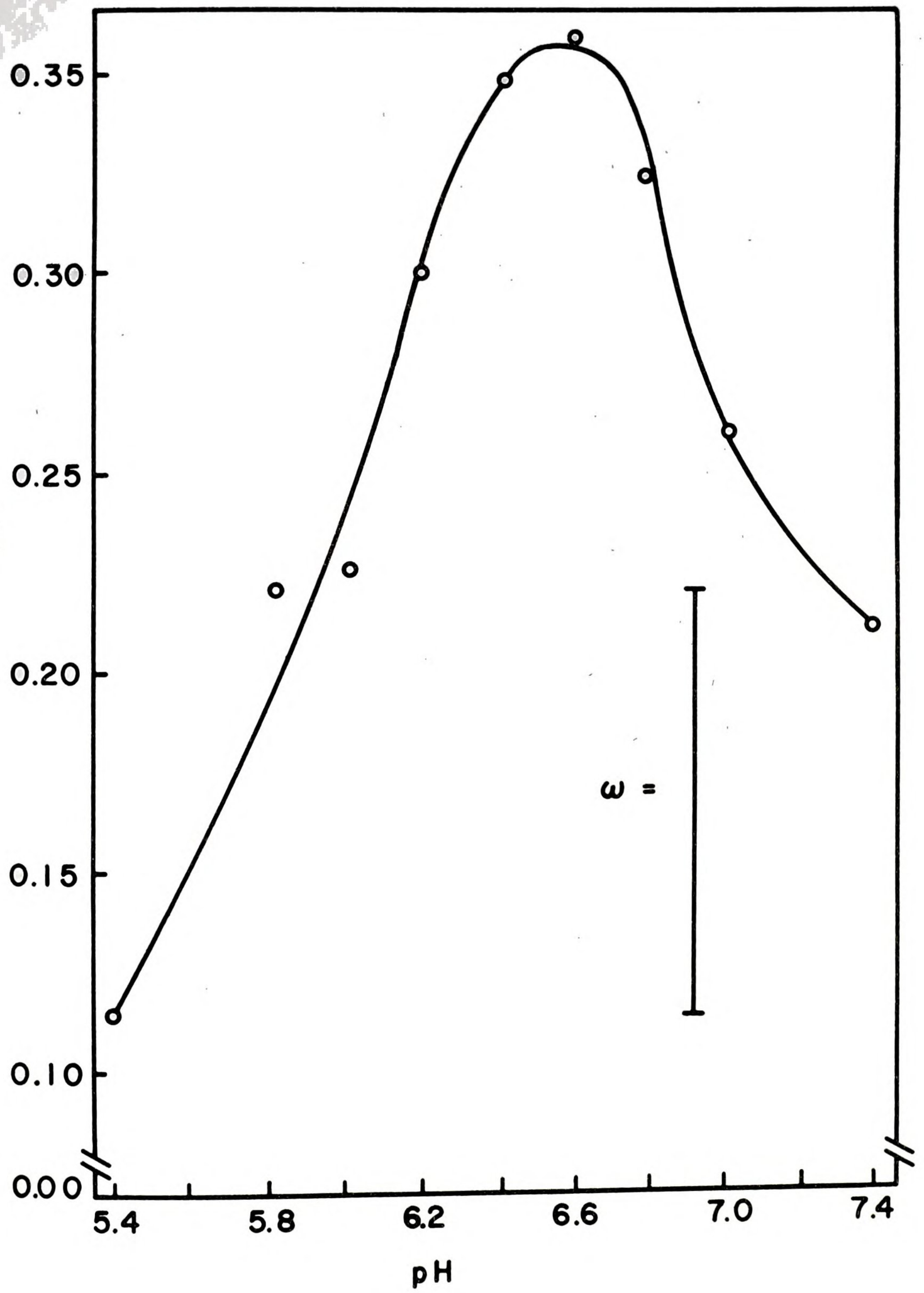


Table 14.

Buffer	
Tris-maleat	
KH_2PO_4-K_2HP	
MOPS[4]	
MES[5]	
HEPES[6]	

[1]Means
are signifi
[2]Tris-
pK_a 6.15.
[3]pK_a 6
[4]Morph
[5]2-(N-
20 C.
[6]N-2-h
pK_a 7.55 at

Table 14. Effect of several buffers at pH 6.6 on neotetrazolium chloride reduction by leaf strips.

Buffer	Buffer concn:	NTC reduced[1] 0.01 M	0.05M	Mean
			(A-560 nm)	
Tris-maleate[2]		0.207ab	0.114c	0.161c
KH_2PO_4-K_2HPO_4[3]		0.225a	0.178b	0.202abc
MOPS[4]		0.236a	0.197abc	0.217ab
MES[5]		0.218ab	0.154bc	0.186bc
HEPES[6]		0.243a	0.242a	0.242a

[1]Means within a column followed by different letters are significantly different at P=0.05.

[2]Tris-(hydroxymethyl aminomethane), pK_a 8.3; maleate-pK_a 6.15.

[3]pK_a 6.8 at 20 C.

[4]Morpholinopropane sulfonic acid, pK_a 7.2 at 20 C.

[5]2-(N-morpholino) ethanesulfonic acid, pK_a 6.15 at 20 C.

[6]N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pK_a 7.55 at 20 C.



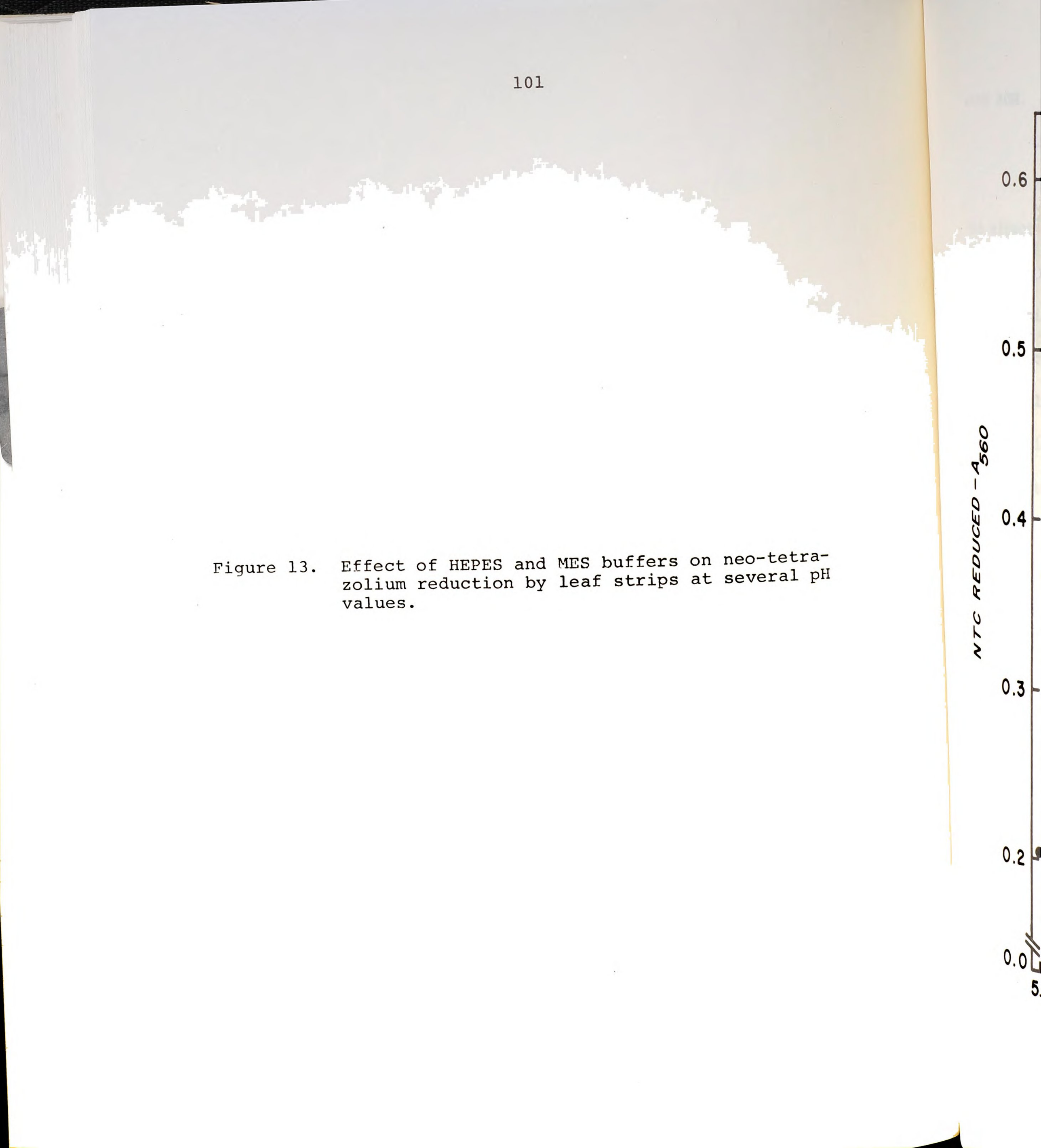
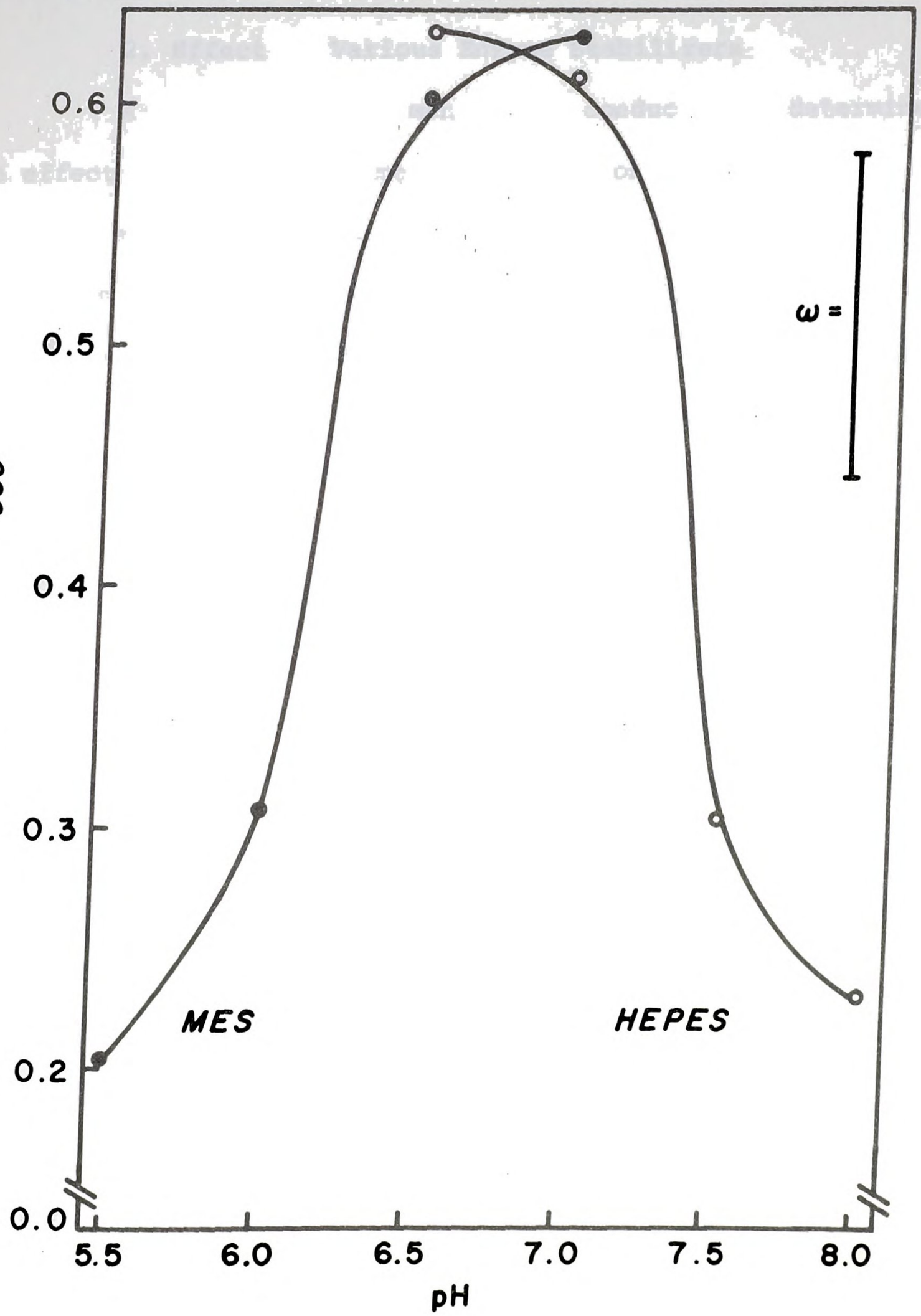


Figure 13. Effect of HEPES and MES buffers on neo-tetrazolium reduction by leaf strips at several pH values.



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2. Effect of Various Enzyme Stabilizers

A series of experiments were conducted to determine the effect of several enzyme stabilizers on preserving metabolic activity of intact leaf strips.

The high molecular weight polymers Ficoll and Dextran-40 (Pharmacia, Uppsala, Sweden) were first evaluated using a factorial design. Ficoll had a very negative effect on NTC reduction while Dextran-40 had no effect (Table 15). The inhibition by Ficoll was partially attributed to a dialyzable inhibitor (Appendix, Table A-2).

Both polyvinyl pyrrolidone-k30 (PVP) and potassium metabisulfite ($K_2S_2O_5$) significantly enhanced the leaf tissue reduction of NTC when used at up to 5.0 mg/ml and up to 0.4 m$\underline{M}$, respectively (Table 16). Because higher concentrations of $K_2S_2O_5$ were more detrimental than those of PVP, 1.0 mg/ml (0.1% w/v) PVP was chosen for subsequent experiments.

The factorial effect of bovine serum albumin (BSA) and PVP is recorded in Table 17. BSA (50 µg/ml) was beneficial, while PVP had no effect in this experiment. The 50 µg/ml BSA was confirmed as an optimum level for subsequent leaf strip work.

Table 15. F
2

Ficoll concn	Dex (g/
(g/100 ml)	
0	
2.5	
5.0	
Mean[2]	

[1]Basic
KOH, 8 mM soc
[2]Means
different at
was not sign

Table 15. Effect of Ficoll and Dextran-40 on neo-tetrazolium chloride reduction by leaf strips.[1]

Ficoll concn	NTC reduced			
Dextran concn- (g/100 ml):	0	1.0	2.5	Mean[2]
(g/100 ml)	(A-560 nm)			
0	0.558	0.458	0.565	0.536a
2.5	0.420	0.405	0.387	0.404b
5.0	0.341	0.356	0.316	0.336c
Mean[2]	0.440b	0.415b	0.423b	

[1]Basic treatment medium=0.25 M mannitol, 0.01 M HEPES-KOH, 8 mM sodium succinate and adjusted to pH 6.7.

[2]Means followed by different letters are significantly different at P=0.05. Interaction of Ficoll and Dextran-40 was not significant.

Table 16.

PVP	K
(mg/ml)	([illegible]
-----	-
1.0	-
5.0	-
10.0	-
50.0	-
-----	0
-----	0
-----	1
-----	4
-----	8

[1]Treat
8 mM sodium
[2]Means
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Table 16. Effect of polyvinyl pyrrolidone (PVP) and potassium metabisulfite ($K_2S_2O_5$) on neotetrazolium chloride reduction by leaf strips.[1]

PVP	$K_2S_2O_4$	NTC reduced[2]
(mg/ml)	(m$\underline{M}$)	(A-560 nm)
-----	-----	0.435b
1.0	-----	0.528a
5.0	-----	0.534a
10.0	-----	0.345b
50.0	-----	0.189c
-----	0.1	0.515a
-----	0.4	0.590a
-----	1.0	0.258c
-----	4.0	0.182c
-----	8.0	0.125c

[1]Treatment medium=0.25 $\underline{M}$ mannitol, 0.01 $\underline{M}$ HEPES-KOH, 8 m$\underline{M}$ sodium succinate and adjusted to pH 6.7.

[2]Means followed by different letters are significantly different at P=0.05.

Table 17.

BSA concn (µg/ml)	
0	
50	
100	
500	
1000	
Mean[1]	

[1]Means
different a
significant

Table 17. Effect of bovine serum albumin (BSA) and polyviny1 pyrrolidone (PVP) on neo-tetrazolium chloride reduction by leaf strips.

BSA concn	NTC reduced		
PVP concn (mg/ml):	0	2.5	Mean[1]
(μg/ml)		(A-560 nm)	
0	0.534	0.531	0.533b
50	0.638	0.620	0.629a
100	0.510	0.533	0.522b
500	0.548	0.539	0.544b
1000	0.419	0.473	0.465c
Mean[1]	0.543b	0.539b	

[1]Means followed by different letters are significantly different at P=0.05. Interaction of BSA and PVP was not significant.

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3. Effect of Mannitol

Having significantly refined the original medium, the effect of mannitol concentration as an osmoticum was reinvestigated. The optimum NTC reduction occurred within the range of 0.20-0.40 M, with 0.25 M mannitol being optimal (Table 18). The $CaSO_4$-based medium was inferior to the refined medium for NTC reduction (Table 18). Having established 0.25 M mannitol as a suitable concentration for future experiments, the final modified medium had the composition listed in Table 19.

G. Time-Course of Neo-Tetrazolium Chloride Reduction

A time-course of NTC reduction was followed in both light and dark to establish the role of light in the reduction process. As detailed in Figure 14, light was required only for the high level of reduction while sufficient NTC accumulation occurred in either the light or dark. In contrast to the TTC experiment, sufficient NTC accumulated during the first hour to supply the tissue with enough dye for additional reduction for the following 2 hr.

H. Further Assessment of the Leaf Tissue Viability

1. Measurement of Protein Synthesis

Davies and Cocking (1967) reported that green tissue would incorporate ^{14}C into protein following fixation of $^{14}CO_2$ via photosynthesis. Testing the leaf strip

Table

Mannit (M)	
0.00	
0.10	
0.15	
0.20	
0.25	
0.30	
0.35	
0.40	
0.25	
0.01 M PVP,	

signi

8 mM

Table 18. Effect of mannitol concentration on neo-tetrazolium chloride reduction by leaf strips in the refined medium.

Mannitol concn[1] (M)	NTC reduced[2] (A-560 nm)
0.00	0.459c
0.10	0.504bc
0.15	0.490bc
0.20	0.518abc
0.25	0.593a
0.30	0.519abc
0.35	0.565ab
0.40	0.535abc
0.25 in the dark	0.108e
----- 0.5 mM $CaSO_4$[3]	0.198d

[1]Treatment medium=variable concn mannitol, 0.01 M HEPES-KOH 8 mM sodium succinate, 1 mg/ml PVP, 50 µg/ml BSA and adjusted to pH 6.7.

[2]Means followed by different letters are significantly different at P=0.05.

[3]In place of treating medium, but including 8 mM sodium succinate.

Table

Compon
Mannit
HEPES-
Sodium
PVP-k3
BSA

Table 19. Modified treatment medium.[1]

Component	Concn
Mannitol	0.25 M
HEPES-KOH	0.01 M
Sodium succinate	0.008 M
PVP-k30	1.0 mg/ml
BSA	50.0 µg/ml

[1]Final solution adjusted to pH 6.7.

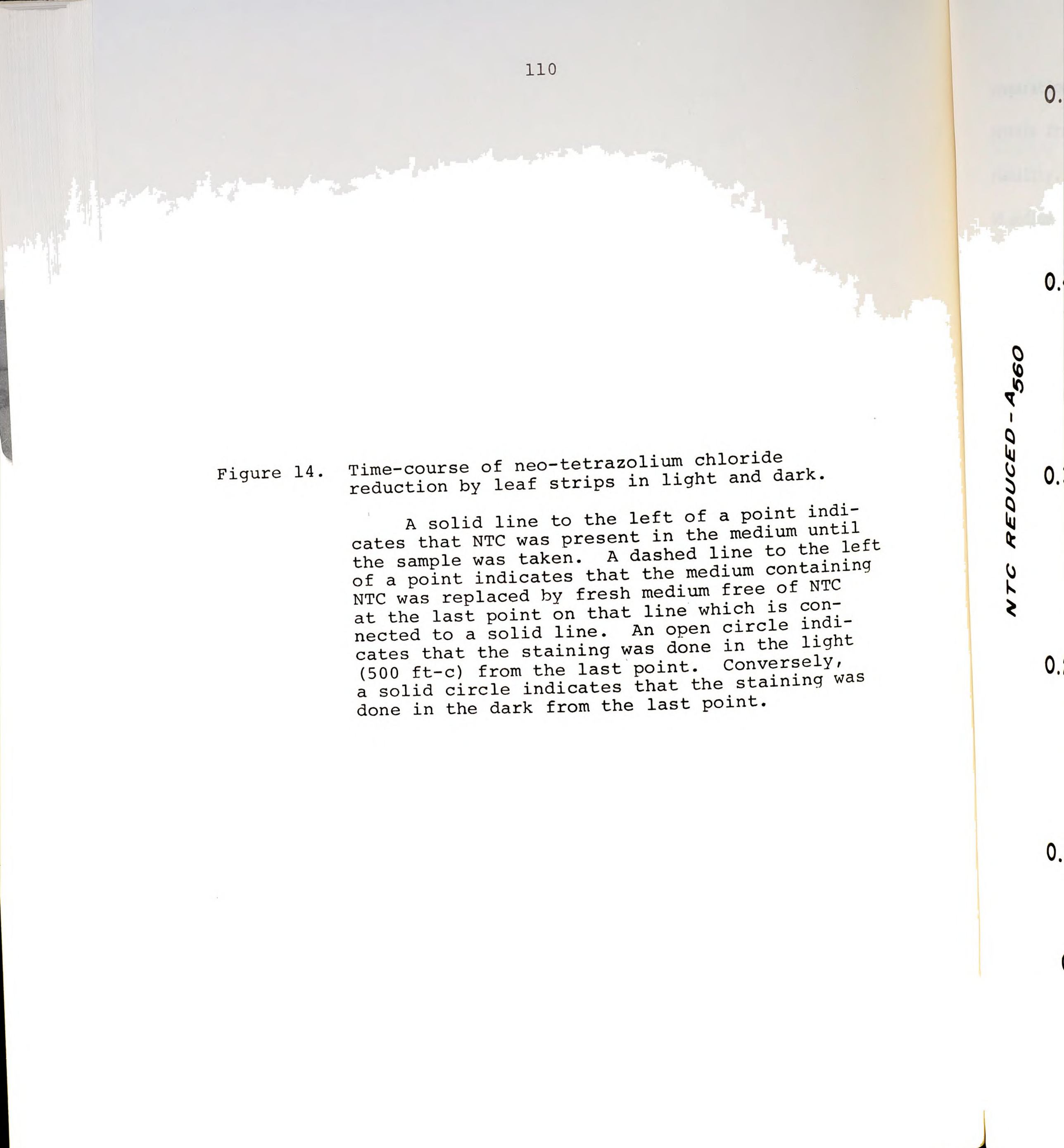

Figure 14. Time-course of neo-tetrazolium chloride reduction by leaf strips in light and dark.

A solid line to the left of a point indicates that NTC was present in the medium until the sample was taken. A dashed line to the left of a point indicates that the medium containing NTC was replaced by fresh medium free of NTC at the last point on that line which is connected to a solid line. An open circle indicates that the staining was done in the light (500 ft-c) from the last point. Conversely, a solid circle indicates that the staining was done in the dark from the last point.

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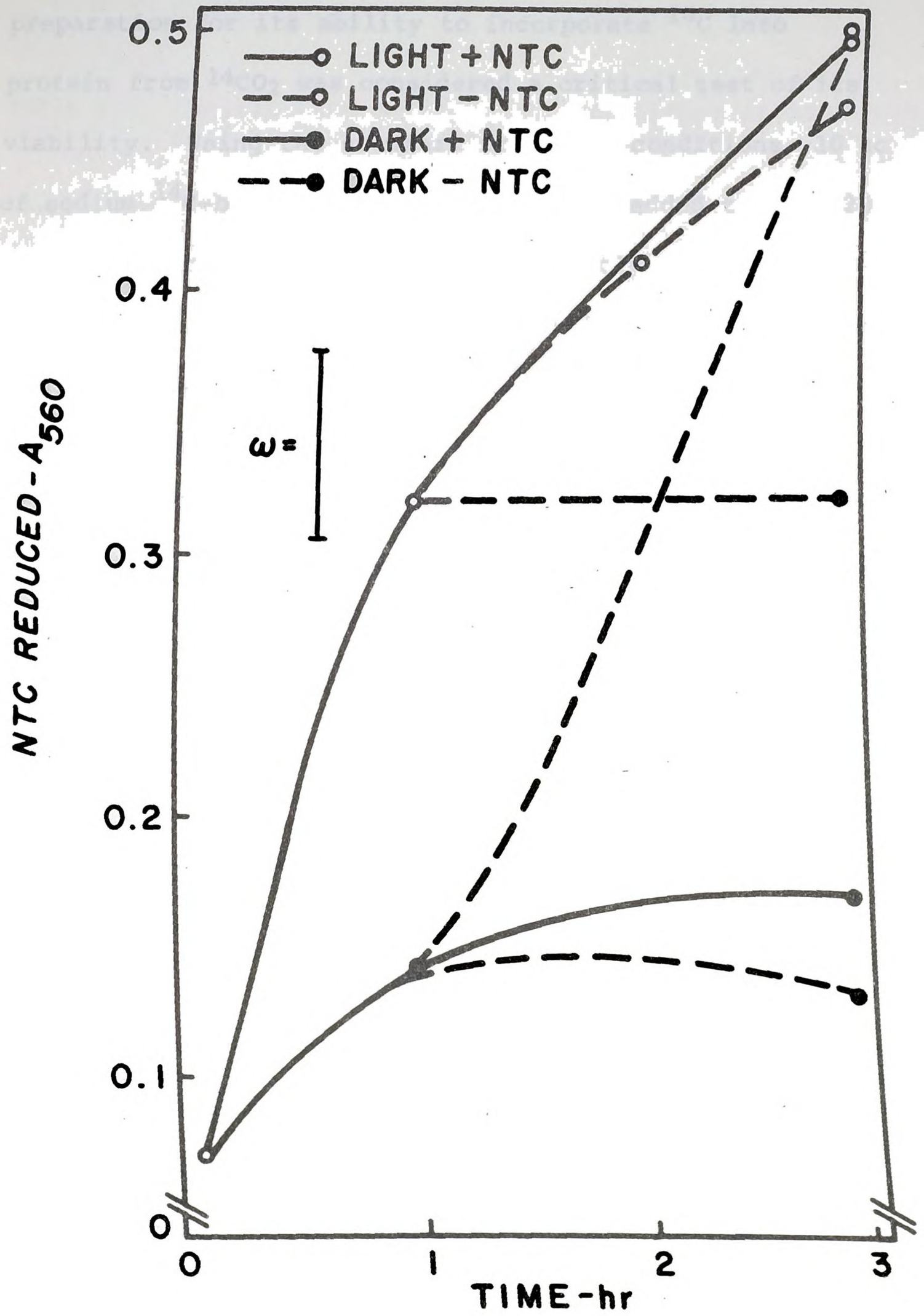

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preparation for its ability to incorporate ^{14}C into protein from $^{14}CO_2$ was considered a critical test of its viability. Using the standard treatment conditions, 10 µc of sodium ^{14}C-bicarbonate ($NaH^{14}CO_3$) was added to each 25 ml Erlenmeyer flask which was then tightly stoppered. After 30 min in either light (500 ft-c) or dark the treatment medium was withdrawn, the leaf strips were washed twice and resuspended, all with 5 ml distilled water. Sufficient HCl was added to adjust the pH to 2. One set of samples was homogenized in 80% (v/v) ethanol and total ^{14}C fixed was determined. A second set of samples was homogenized in 80% (v/v) ethanol and fractionated using Davies and Cocking's (1967) method. High speed centrifugation (48,000 x g) for 30 min was necessary to separate fractions 8-13 (Table 20).

Considerable quantities of ^{14}C were fixed in the light (Table 20). Fractions 1 and 2, representing sugars and amino acids, were most highly labeled. The next largest labeled fraction was the perchloric acid solubilized material representing starch. Fractions 14 and 15, containing protein, were also heavily labeled. The low activity recovered from ethanol washes between major fractions showed the various fractions were distinct entities.

Table 20.

Fraction number	Total
1	
2	
3	
4	
5	
6	
7-9	
10	
11	
12-13	
14	
15	
16	

Table 20. Distribution of $^{14}CO_2$ in leaf strips after 30 min of fixation in either light or dark.

Fraction number	Solvent	$^{14}CO_2$ fixed			
		Light (500 ft-c)		Dark	
		(cpm)	(%)	(cpm)	(%)
Total		36,000	100.0	1,000	100.0
1	80% Ethanol	9,984	27.7	486	48.6
2	80% Ethanol	1,599	4.4	70	7.0
3	20% Trichloroacetic acid	960	2.7	19	1.9
4	20% Trichloroacetic acid	230	0.6	3	0.3
5	95% Ethanol	25	0.1	3	0.3
6	100% Ethanol	22	0.1	2	0.2
7-9	Chloroform, Chloroform-Ether (1:1), Ether	610	1.7	11	1.1
10	1.0 N Perchloric acid	7,428	20.0	165	16.5
11	100% Ethanol	461	1.3	5	0.5
12-13	Ethanol-Ether (1:1), Ether	97	0.3	2	0.2
14	0.25 N Sodium Hydroxide 60 C	3,976	11.4	143	14.3
15	1.0 N NaOH	978	2.7	47	4.7
16	Residue	882	2.4	10	1.0
	Unaccounted	9,000	25.0	34	3.4

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2. Measurement of Oxygen Exchange Rates

Oxygen exchange rates in both light and dark were determined with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, Model 53) and stirrer assembly connected to a Temp-unit (Oxford Lab) water bath at 25 ± 0.1 C. The electrode was attached to a custom-built Johnson polarographic amplifier (George J. Johnson, Jr., Baltimore, Maryland). The output was recorded on a Sargent SR linear 1-100 mv strip chart recorder.

The electrode was standardized in air-saturated distilled water and then bucked down, such that 10x amplification of the remaining signal could be used. This provided sufficient sensitivity so that a change of greater than 0.05 µl O_2/min could be detected in 6.0 ml of solution. For measurement of net oxygen production in the light (the Hill reaction coupled to photosynthesis minus respiration), the leaf strips were illuminated (approx. 1000 ft-c) with an American Optical microscope lamp (A.O. 653) at a 7.5 v setting.

Data are reported in Table 21 from an initial experiment where an oxygen exchange assay was used to measure the effect of infiltration of the medium. Infiltration significantly reduced oxygen production, but had little effect on dark respiration.

Table 21.

Pressure d infiltrati	
(mm)	
760 (contr	
500	
350	
100	

[1]Mean
different
[2]Nega
[3]Posi

Table 21. Effect of infiltration of the treatment medium into leaf strips on oxygen exchange rates in dark and light.

Pressure during infiltration	Net oxygen exchange[1]	
	Dark uptake[2]	Light production[3]
(mm)	(µl/100 mg dry wt per hr)	
760 (control)	-240a	234a
500	-240a	147b
350	-251a	106b
100	-243a	113b

[1]Means followed by different letters are significantly different at P=0.05.
[2]Negative numbers indicate oxygen consumption.
[3]Positive numbers indicate oxygen production.

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I. Interaction of Neo-Tetrazolium and Naphthaleneacetic Acid

Experiments were designed to establish the feasibility of using NTC to monitor leaf strip viability simultaneously during NAA uptake experiments.

The NAA uptake was studied by using the procedures outlined for hypocotyl studies (Exp. I.C). The uptake studies were terminated by rapidly removing the labeled medium with suction and washing the tissue twice for 5 min each with 5 ml of 0.01 M phosphate buffer (K_2HPO_4-KH_2PO_4) at pH 6.7. The tissue was stored at 4 C (not more than 12 hr), homogenized in xylene, and centrifuged for 10 min at 2000 x g. The supernatant was removed and the reduced NTC measured. The respective xylene solutions were then transferred to 2-inch planchets and dried. The ^{14}C in the xylene supernatant, as well as that in the xylene insoluble material, was determined by the methods described for the hypocotyl sections (Exp. I.C.). Data transformations and statistical analysis were also identical.

As recorded in Table 22, $5x10^{-6}$ M NAA had no effect on the amount of NTC reduced. However, NTC markedly reduced NAA accumulation (Table 23). Furthermore, most of the NAA accumulated in the NTC-free treatment was in the xylene insoluble fraction and suggested that it was tightly bound

Table 22.

Treatment		
NAA[2]	NTC[3]	
+	-	
-	+	
+	+	

[1]Mean
different
[2]NAA
[3]NTC

Table 22. Time-course of the effect of naphthaleneacetic acid on neo-tetrazolium reduction by leaf strips.

Treatment			NTC reduced[1]		
NAA[2]	NTC[3]	Time:	1 hr	3 hr	6 hr
			(A-560 nm)		
+	-		0.168d	0.171d	0.151d
-	+		0.226c	0.349b	0.478a
+	+		0.284c	0.393b	0.494a

[1]Means followed by different letters are significantly different at P=0.05.
[2]NAA concn=5×10^{-6} M
[3]NTC concn=0.1 mg/ml

Table 23.

Fraction	
Xylene sol	
-NTC	
+NTC	
% inhi	
Xylene ins	
-NTC	
+NTC	
% inh	
Total upta	
-NTC	
+NTC	
% inh	

[1]Mea
the total
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Table 23. Time-course of naphthaleneacetic acid ($1x10^{-6}$ M) uptake by leaf strips as affected by neo-tetrazolium chloride.

Fraction	NAA accumulated[1]		
Time:	1 hr	3 hr	6 hr
	(pmoles/10 mg dry wt)		
Xylene soluble			
-NTC	265de	358d	611a
+NTC	257de	276de	300de
% inhibition	3%	22%	51%
Xylene insoluble			
-NTC	481cde	1569b	3086a
+NTC	510cde	502cde	737c
% inhibition	-6%	68%	76%
Total uptake			
-NTC	746c	1927b	3697a
+NTC	767c	778c	1037c
% inhibition	-3%	60%	72%

[1]Means within or between the two fractions, and within the totals followed by different letters are significantly different at P=0.05.

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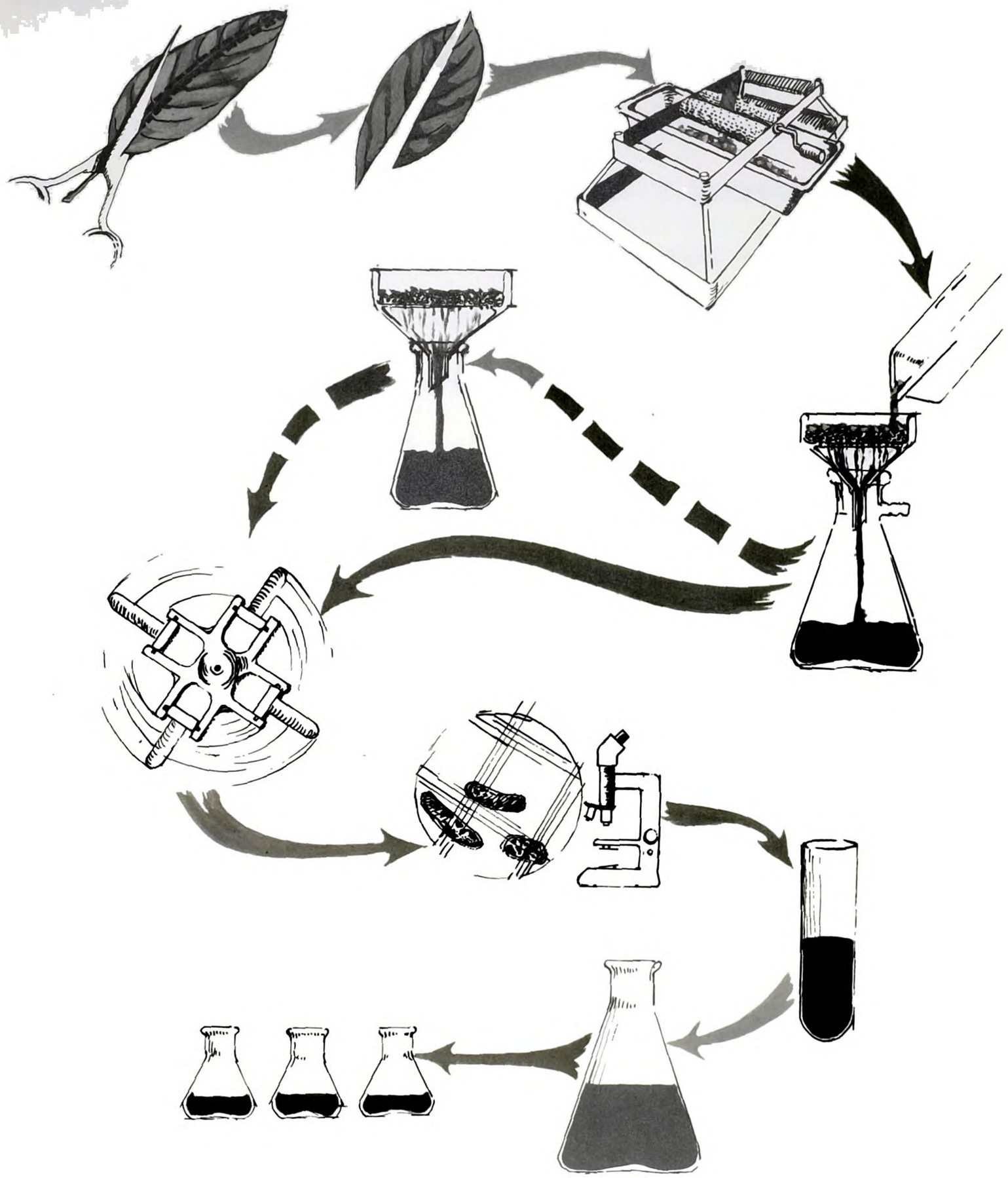
III. Development of a Cell Isolation Technique and Viability Comparisons to Leaf Strips

After refining the medium for leaf tissue, its usefulness in the preparation of viable leaf cells was then established. Jyung et al. (1965b) and Takebe et al. (1968) reported on enzymatic separation methods for leaf cells. Using both techniques in preliminary experiments, leaf cells of low metabolic activity were recovered. Similar results were obtained using the mechanical isolation procedure described by Price (1967). The following modifications of the above two approaches, enzymatic and mechanical separations, were made in developing an isolated cell preparation. Simultaneously, cell viability was monitored.

A. Methods of Mechanical Isolation of Leaf Cells

Soybean plants were grown and the freshly picked leaves washed using procedures described for leaf strips (Exp. II.A). Cell separation was done at 4 C. As diagrammatically represented in Figure 15, 25 g of the leaflets, petioles removed, were homogenized by passing through a roller mill in groups of two or three. The rollers were flushed with 5-10 ml of medium with each group of

Figure 15. Schematic diagram of mechanical isolation technique for leaf cells.



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The leaf homogenate collected in a plastic container was poured into a Büchner funnel (No. 2A), filled 3-4 cm deep with glass beads (3 mm diameter). Almost all tissue and cell clumps larger than one or two cells were usually trapped in the glass beads. An extra filtration step was possible by passing the suspension through a commercial milk filter (15 cm diameter) supported in a Büchner funnel (Figure 15). This step permitted only single cells and small debris (fragmented cells) to pass, but was usually omitted for it was too time-consuming and many viable cells were lost. The cell suspension, usually slightly less than 600 ml in volume and containing broken cell debris, was then divided into four 150 ml polycarbonate centrifuge tubes. After 5 min at 75 x g on a horizontal rotor centrifuge (Sorvall, Norwalk, Conn. GLC-I), the debris was carefully decanted and the sedimented cells massed into one 150 ml centrifuge tube. The cells were resuspended in 100 ml of medium, and washed a minimum of four times using the

---

[1]The roller mill was reported by Hulme et al. (1964) to be the best means of homogenizing tissue to yield mitochondria with high respiratory control values. The roller mill's design was described in a personal communication by J. D. Jones to D. R. Dilley of the Michigan State University, Department of Horticulture.

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same centrifugation procedure until the supernatant was clear. The washed cells were resuspended in medium to yield a final volume of 100 ml.

A 0.5 ml sample of the cell suspension was serially diluted 100-fold and the cell density determined using a Spencer bright-line phase-microscopy hemocytometer (Improved Neubauer type). Based on preliminary experiments that established a relationship of 100,000 cells to yield about 1 mg dry weight, the concentrated cell suspension was appropriately diluted to provide a final cell density of 1.0 mg dry weight of cells per ml unless otherwise noted. For all experiments, four to six 5-ml samples of the final suspension were collected on tared filter disks (2.1 cm No. 554), washed three times with 5 ml of distilled water, dried at 70 C for 24 hr and weighed to ±0.05 mg on the macro-analytical balance.

A further modification of the cell separation technique revealed that a given cell population could be partially fractionated by sedimentation at 200 x g in a linear density gradient of either Ficoll (Figure 16A) or Dextran-40 (Figure 16B). The fractionation on the Ficoll gradient demonstrated that the population of a small number of the recovered lighter weight mesophyll cells were composed of a high proportion of spongy cells, while the heavier cells

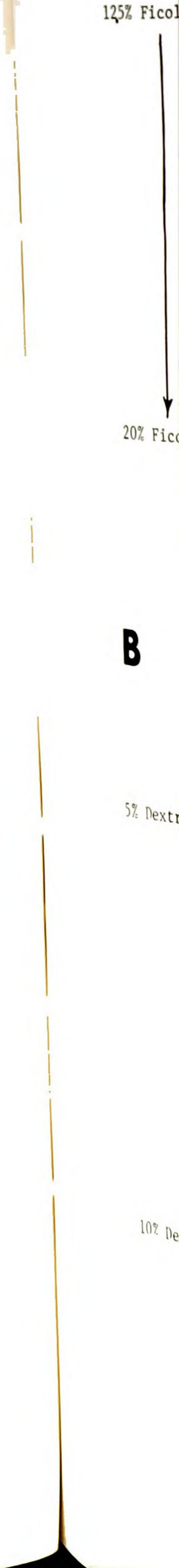

Figure 16. Density-gradient separation of leaf cells.

A. Separation of leaf cells on a Ficoll gradient.

B. Separation of leaf cells on a Dextran-40 gradient.

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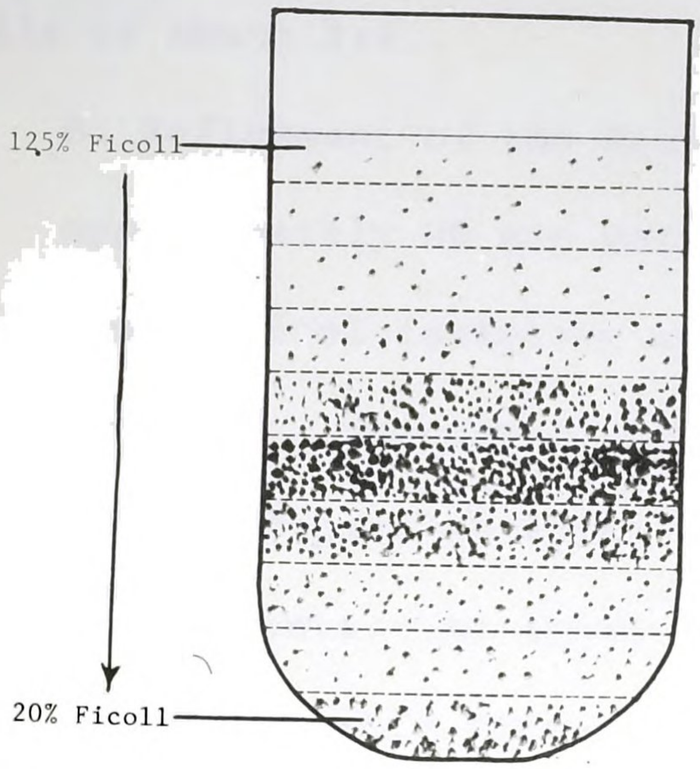

Total Cells	Clumps	Palisade	Spongy
20	0	34%	66%
29	0	34%	66%
36	0	39%	61%
89	1	49%	51%
283	24	72%	28%
690	68	66%	34%
430	39	79%	21%
241	36	73%	27%
68	18	75%	25%
158	37	75%	25%
2024	222(11%)	70%	30%

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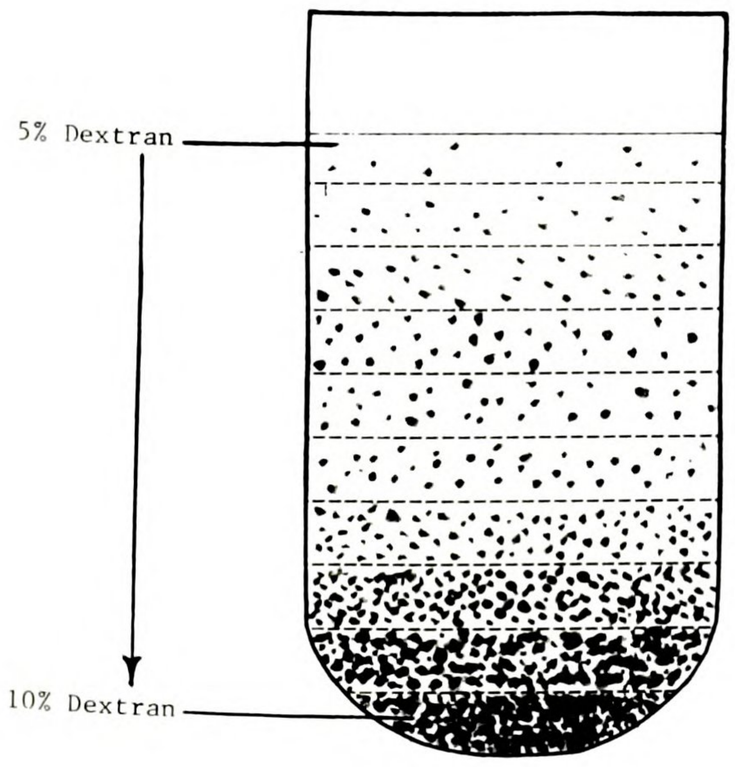

Total Cells	Clumps	Palisade	Spongy
56	1	50%	50%
80	1	52%	48%
110	3	65%	35%
98	4	59%	41%
118	6	70%	30%
125	5	66%	34%
194	9	66%	34%
193	11	66%	34%
189	18	72%	28%
3970	650	79%	21%
5183	708(14%)	75%	25%

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were mainly palisade cells. Tabulation of the total population of cells showed the ratio of palisade to spongy cells of about 3:1.

B. Refinement of the Mechanical Isolation Technique

Approximately 80 min were required to prepare cells by the mechanical isolation method for one replication of leaf material. Therefore, approximately 4 hr of work were required to prepare material for four replications for each day's experiments. As shown in Table 24, when four such leaf isolations were consecutively prepared, they were quite similar as indexed by oxygen exchange. Unless otherwise noted, all oxygen determinations were made by polarography, as described for leaf strips (Exp. II.G.2.). Samples for the time-course measurements (for the oxygen exchange measurements) were withdrawn from the respective treatments incubating in the illuminated shaking water bath (Exp. I.C.). The results presented in Table 24 were interpreted to mean that one large cell preparation would be statistically valid as a source of cells to be used in replicated experiments. Accordingly, one common isolation was made for all cells used to establish the usual replications (four) for each treatment.

Due to the success with polarographic analysis of oxygen exchange rates, two additional experiments were

Table 24.

Preparation number	
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Table 24. Oxygen exchange rates in dark and light of four separate mechanically isolated leaf cell preparations in time.

Preparation number	Net oxygen exchange[1]					
	1 hr		4 hr		8 hr	
	Dark	Light	Dark	Light	Dark	Light
	(µl/100 mg dry wt per hr)					
1	-137bc	314a	-107c	221a	-200ab	301a
2	-141bc	273a	-150b	184a	-220a	255a
3	-138bc	171a	-137c	188a	-173ab	198a
4	-130c	127a	-121c	251a	-162abc	164a

[1]Light means or dark means among all time samples followed by different letters are significantly different at P=0.05.

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conducted to determine if the treatment medium was optimum for the isolated cells. Oxygen exchange rates are presented in Table 25 for equal masses of cells resuspended in several different media to examine if certain additions to the media, such as components of tissue culture media, might increase or stabilize the metabolic activity of the cells. The cell preparation incubated in the standard medium (Table 19) in the dark showed that its photosynthetic ability did increase with incubation time (Table 25). Incubation in the light further enhanced the photosynthetic capability but the addition of nutrients severely reduced photosynthesis and dark respiration. The addition of the high molecular weight polymers Dextran-40 and Ficoll had little effect on the system; thus they were not used in further studies.

The effect of pH was re-examined. The most dramatic effect of pH occurred after the first 1 hr where cells incubated at pH 6.6 had the highest photosynthetic activity (Table 26). Proceeding from the 3-hr to the 8-hr sample, the activity of all treatments increased with the greatest activity exhibited by the samples held at pH 6.6 and 7.6. At the end of the 8-hr incubation, the pH values of the media were checked. A distinct shift toward pH 6.6 was noted for all treatments. After the pH levels of the

Table 25

| Medium[1] | | |

Table 25. Comparison of the effect of several media on oxygen exchange rates of mechanically isolated leaf cells in dark and light.

Medium[1]		Net oxygen exchange[2]			
Sample time:[1]		3 hr	5 hr	7 hr	9 hr
		(µl/100 mg dry wt per hr)			
1[3] in dark	dark	-184±13	-184±6	-178±25	-213±28
	light	391±22	458±12	550±20	630±108
1[3] in light	dark	-194±23	-217±31	-255±8	-294±16
	light	551±8	626±10	776±109	830±54
2[4] in light	dark	-200±1	-188±20	-150±19	-158±4
	light	269±53	285±50	511±27	627±15
3[5] in light	dark	-164±6	-151±8	-93±8	-122±6
	light	135±14	234±21	283±1	69±0
4[6] in light	dark	-211±8	-250±16	-225±20	-312±12
	light	520±4	620±0	772±40	595±15

[1]Cells in respective media stored in water bath shaker at 25 C in 250 rpm. A sample of the suspension was tested at the indicated time.

[2]SE given.

[3]Medium 1=0.25 M mannitol, 0.01 M HEPES-KOH, 8 mM sodium succinate, 1 mg/ml PVP, 50 µg/ml BSA and adjsuted to pH 6.7.

[4]Medium 2=Medium 1 + inorganic nutrients (Murashige + Skoog, 1962).

[5]Medium 3=Medium 1 and organic nutrients and vitamins (not hormones) (Murashige and Skoog, 1962).

[6]Medium 4=Medium 1 and 5 g/100 ml Ficoll + 2.5 g/100 ml Dextran-40.

Table 2

Sample time (hr)	
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Table 26. Effect of pH (0.01 M HEPES and 0.01 M MES buffers) on oxygen exchange rates of mechanically isolated leaf cells in dark and light.

		Net oxygen exchange[1]				
Sample time	Buffer: pH:	MES 5.6	MES 6.6	HEPES 6.6	HEPES 7.6	HEPES 8.6
(hr)		(µl/100 mg dry wt per hr)				
1	dark	-378±111	-351±1	-348±50	-442±105	-455±144
	light	-292±38	479±16	325±17	216±37	172±39
3	dark	-351±32	-351±21	-398±66	-322±34	-322±34
	light	244±54	661±83	624±28	460±6	460±6
6	dark	-398±79	-470±37	-464±23	-373±27	-360±50
	light	308±32	563±57	481±39	768±31	353±24
8	dark	-425±43	-568±31	-614±94	-971±82	-500±122
	light	289±135	702±124	892±50	926±0	599±22
		(5.8)[2]	(6.9)[2]	(6.6)[2]	(7.2)[2]	(7.9)[2]
20[3]	dark	-998±75	-1075±41	-1172±111	-1074±21	-821±110
	light	-393±32	-403±11	-354±111	-642±116	-576±67
		[0.90][4]	[1.40][4]	[1.50][4]	[1.08][4]	[0.140][4]

[1]SE given.
[2]Final pH of medium had shifted to indicated pH.
[3]Cell suspensions were washed once in fresh media containing the respective buffers at the appropriate pH values.
[4]Absorbance of 560 nm of 0.10 mg/ml NTC reduced (1 hr) by the leaf cells in the respective treatments.

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respective treatments were readjusted, the cells were incubated an additional 12 hr. Only the sample in HEPES buffer at pH 6.6 maintained a high photosynthetic activity at the 20-hr sampling. The high oxygen consumption in the dark at the 20-hr sampling was likely due to bacterial contamination.

An important secondary experiment on the 8-hr sample showed a definite similarity between oxygen production and the ability of the cells to reduce NTC. The high values of reduced NTC reflect the ease of penetration of NTC into the cells.

C. Comparisons of Mechanically Isolated Cells to Leaf Strips

A simultaneous multiple comparison of a single preparation of the mechanically isolated leaf cells with leaf strips (4 replicates) was conducted, by starting with identical leaf material. All treatments were made in 8 ml (final volume) in 25 ml Erlenmeyer flasks, with the exception for the O_2 uptake determinations. The latter measurements were followed manometrically as described for the hypocotyl sections (Exp. I.E.1). NTC (0.1 mg/ml), based on the results in Table 23, was used as an inhibitor in the uptake studies. The techniques and conditions for NAA uptake were similar to those used for the hypocotyl sections (Exp. I.C.), except

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the cell suspensions were separated from the labeled medium by centrifugation at 500 x g for 5 min. After the medium was decanted, the cells were washed twice in 5 ml of phosphate buffer (KH_2PO_4-K_2HPO_4, 0.01 M) at pH 6.7 using the same centrifugation system. The washed cells were transferred to 2-inch planchets and radioactivity determined.

Phosphorus uptake was followed in the same manner as for ^{14}C-NAA. Carrier-free $H_3{}^{32}PO_4$ (Tracerlab, Waltham, Mass.) was added to K_2HPO_4 to give a final concentration of $5x10^{-5}$ M and approximately 0.1 µc ^{32}P per treatment. Exact calibration of the specific activity was determined simultaneously with the rest of the uptake portion of the experiment.

$^{14}CO_2$ fixation was carried out as previously described for the leaf strips (Exp. II.4.1.), except 1 µc/flask of $NaH^{14}CO_3$ (S.A. 2.0 µc/µmole) was added. Total $^{14}CO_2$ fixed was measured.

NTC reduction was determined on the NTC treatment used to inhibit the NAA uptake. After the cells were washed, the absorbance of the supernatant of the xylene homogenate from the treated tissue was measured at 560 nm. The supernatant was then added to the respective planchets already containing the residue of the xylene extraction.

As detailed in Table 27, the leaf strips accumulated

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	+NTC[5]	
NAA[3] up		
	-NTC	
	+NTC[5]	
NTC[5] re		
	+NTC[5]	
PO_4[6] up		
	-NTC	
	+NTC[5]	
$^{14}CO_2$[7]		
	-NTC	

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Table 27. Comparison of leaf strips to mechanically isolated leaf cells by oxygen uptake, naphthaleneacetic acid uptake, neo-tetrazolium reduction, phosphorus uptake and $^{14}CO_2$ fixation.

Treatment	Leaf strips[1]	Mechanically[1] isolated cells
O_2 uptake[2]	$\left(\frac{\mu l}{10 \text{ mg dry wt/hr}}\right)$	
-NTC	352a	167c
+NTC[5]	297b	91d
NAA[3] uptake[4]	$\left(\frac{\text{pmoles}}{10 \text{ mg dry wt}}\right)$	
-NTC	2432a	1408b
+NTC[5]	640c	451c
NTC[5] reduced[4]	$\left(\frac{\text{A-560 nm}}{10 \text{ mg dry wt}}\right)$	
+NTC[5]	0.415b	1.097a
PO_4[6] uptake[4]	$\left(\frac{\text{nmoles}}{10 \text{ mg dry wt}}\right)$	
-NTC	72.8a	17.5b
+NTC[5]	6.6c	4.2c
$^{14}CO_2$[7] fixed	$\left(\frac{\text{cpm}}{10 \text{ mg dry wt}}\right)$	
-NTC	7106b	8594a

[1] Treatment means within a determination followed by different letters are significantly different at P=0.05.

[2] Mean of five 1-hr readings at 30 C measured manometrically.

[3] NAA concn=1×10^{-6} M.

[4] 3-hr treatment under 500 ft-c light.

[5] NTC concn=0.1 mg/ml.

[6] PO_4 concn=5×10^{-5} M.

[7] 1/2-hr treatment under 500 ft-c light, 1 µc/flask $^{14}CO_2$.

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significantly more NAA and PO_4^{3-}, especially when using the NTC inhibition as a base line for the system. The strips also had a higher respiration rate though the NTC was not as inhibitory to this determination. Interestingly, the cells fixed significantly more $^{14}CO_2$ and reduced more NTC than did a comparable weight of leaf strips.

D. Method of Enzymatic Cell Isolation

The last sequence of experiments demonstrated that mechanical leaf cell isolation might be practical, but did not exhibit metabolic activity comparable to intact tissue. Enzymatic separation was then evaluated as an alternate approach.

Growing and plant preparation procedures were similar to those described for leaf strips (Exp. II.A.). Approximately 15 leaflets at a time were stacked, rolled on their longitudinal axis and thinly sliced (3-4 mm) with a hand-held razor blade. The stack of leaf slices was then cut in the opposite direction and yielded tissue pieces about 4 mm x 4 mm in size. After a sufficient amount (60 to 80 g) of leaves were sliced, the tissue pieces were thoroughly washed with treating medium (Table 19) until all signs of broken cells were removed. The tissue pieces were then placed in ten times their fresh weight (600 to 800 ml) of fresh medium containing 1.0% (w/v) Macerozyme (a crude

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preparation of endo-polygalacturonase donated by Kanematsa-Gosha Ltd., Tokyo, Japan) unless otherwise noted and 0.5% peptone. This suspension was stirred at 300 rpm with a rotor knife assembly driven by an Omni-mixer motor (I. Sorvall, Norwalk, Conn.) with its power controlled through a voltage regulator (33 v used). The knife blades were covered with latex tubing to minimize cutting of the tissue. The container was partially submerged in a water bath (25 ± 0.5 C) illuminated with 1000 ft-c of incandescent light (photo-flood 300W lamps cooled in a plexiglass walled water jacket mounted over the water bath). After 5 hr, the cells were recovered from the tissue suspension by filtration and centrifugation as previously described (Exp. III.A.).

Suspension density was established by using a hemocytometer and also by weighing aliquots on tared filter disks. The weighing method proved to be a more reliable method to estimate final weights of cell suspensions isolated enzymatically, since variable amounts of cuticle pieces were present, that affected the final weight but were not accounted for in the counting procedure. The initial weights determined after oven drying for 1 hr at 70 C were used to calculate the appropriate cell density dilutions to yield 1 mg dry weight/ml. Final cell

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density determinations were made on aliquots dried for 24 hr at 70 C from the diluted cell suspensions.

Optimum cell to medium ratio was determined using the oxygen electrode. A uniform batch of enzymatically (Macerozyme) isolated cells was diluted sequentially to yield several different population densities. Dark respiration was independent of cell density (Table 28), whereas light driven oxygen production was optimum at a cell density of 1.0 mg dry weight/ml or less. Light was the limiting factor for the optimum oxygen production.

Two, apparently different, pectinase preparations were reported to have been successfully used to isolate tobacco leaf cells. Jyung et al. (1965b) used pectinase from Nutritional Biochemical Corp. This enzyme had a pH optimum of 4.5 and was contaminated with several other hydrolytic enzymes not fully characterized (personal communication from Nutritional Biochemical Corp., Cleveland, Ohio). Takebe et al. (1968) used Macerozyme, a fungal endo-polygalacturonase preparation with pH optima of 5.0 and 6.5. Macerozyme was also contaminated with several hydrolytic enzymes, which have been characterized by Suzuki et al. (1967a, 1967b).

While separate lots of leaflets were being treated with the two respective enzymes, a third group of leaflets

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Table 28. Effect of cell density of enzymatically isolated leaf cells on oxygen exchange rates in dark and light.

Cell density[1]	Net oxygen exchange[2]	
	Dark	Light
(mg dry wt/ml)	(µl/100 mg dry wt/hr)	
0.5	-209a	868a
1.0	-212a	830a
1.8	-207a	740a
2.7	-205a	569b
5.3	-229a	307c

[1]1.0 mg dry wt contained approximately 40,000 cells.
[2]Means within a column followed by different letters are significantly different at P=0.05.

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was used for the mechanical isolation of cells. The isolated cells were washed and appropriate cell populations established by the methods previously described.

There was a dramatic difference between the mechanically isolated cells and those isolated by either enzymatic method for their ability to produce oxygen in the light (Table 29). This measurement also established that Macerozyme was slightly better than pectinase. Dark respiration measurements showed little difference among the isolation methods. Because Macerozyme provided slightly more active cells, it was selected over pectinase for continued evaluation.

E. Comparisons Among Mechanically and Enzymatically Isolated Leaf Cells and Leaf Strips-Metabolic Studies.

Since the enzymatically isolated cells seemed to be photosynthetically more active than those isolated by the mechanical procedure, a more complete comparison including leaf strips was performed. To cover a broad spectrum of assays, the comparisons were done over a period of 3 days, each being indicated by an experiment number. For the purpose of continuity, polarographic analyses of oxygen exchange rates were measured on all three days (Experiments 1 to 3). At the start of each experiment a common source of leaf material was utilized, and the experiment was

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Table 29. Time-course of oxygen exchange rates of several different types of leaf cell preparations in dark and light.

Preparation	Time[1]	Net oxygen exchange[2]	
		Dark	Light
	(hr)	(µl/100 mg dry wt/hr)	
Mechanically isolated	1	267±30	267±10
	8	309±51	77±19
	10	325±36	-238±33
	24[3]	216±16	-103±20
Enzymatically isolated (Macerozyme)	1	444±1	730±13
	8	396±30	960±28
	10	497±19	830±56
	20	479±110	567±120
	24[3]	396±64	-18±0
Enzymatically isolated (pectinase)	1	433±21	1001±72
	8	578±100	784±227
	10	361±11	598±42
	20	227±21	258±41
	24[3]	366±25	64±39

[1]Samples incubated in water bath shaker 25 C, 250 rpm, 500 ft-c light.
[2]SE given.
[3]Sample stored at 5 C in dark, not stirred.

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Mechanically isolated cells had lower dark respiration and photosynthetic rates than leaf strips (Table 30). Dark respiration rates of enzymatically isolated cells were intermediate, while their photosynthetic capacity was highest. Cells from all three could be uncoupled with m-chloro-carbonyl cyanide phenylhydrazone (Cl-CCP). The effect of Cl-CCP could be observed almost instantaneously.

$^{14}CO_2$ fixation was studied using the techniques previously described (Exp. II.H.1.). The experiment was repeated twice, once 2 hr after each tissue preparation and again after 24 hr. Carbon fixation by leaf strips and enzymatically isolated cells was not significantly different at either sampling time, though both lost efficiency with time (Table 31). The mechanically isolated cells had about half the capacity of the strips for the 2-hr sample and then dramatically lost their capacity during the storage period.

A parallel set of samples was labeled similarly with $^{14}CO_2$ at the 2-hr measurement. The experiment was terminated by the addition of dilute HCl and the tissue was immediately frozen. The tissue was fractionated by placing leaf strips (homogenized in 80% [v/v] ethanol) and the isolated cells on respective teflon membrane filters (5 µm pore size,

Table 3

Tissue preparation	Leaf strip (Table 10), dark respiration rates	Mechanically isolated cells, dark respiration rates	Enzymatically isolated cells, dark respiration rates

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Table 30. Comparison by oxygen exchange rates in dark and light of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 1).

Tissue preparation	Sample time	Net oxygen exchange[1]			
		Dark	Light	Dark+ Cl-CCP[2]	Light+ Cl-CCP[2]
	(hr)	(µl/100 mg dry wt/hr)			
Leaf strips	1	-315±50	206±16	-524	-111
	2	-333±31	182±24	-560	-56
	4	-325±8	175±1	-452	-79
	6	-397±32	127±1	-532	-79
	24[3]	-320±1	71±8	----	----
Mechanically isolated cells	1	-110±2	183±2	-207	-174
	2	-111±6	107±10	-174	-152
	4	-104±4	84±2	-118	-136
	6	-105±10	84±6	-174	-119
	24[3]	-112±21	27±1	-146	-108
Enzymatically isolated cells	1	-194±14	117±17	-296	-191
	2	-175±13	479±49	-296	-181
	4	-224±22	253±8.5	-318	-212
	6	-235±15	357±2	-365	-232
	24[3]	-209±2	273±10	-222	-148

[1]SE given.
[2]1×10^{-6} M m-Cl-CCP added 1 min before measurement.
[3]Only sample stored at 5 C in dark; both cell preparations were stirred.

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Table 31. Comparison by $^{14}CO_2$ fixation of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 1).

Tissue preparation	$^{14}CO_2$ fixed[1,2]		
Time after preparation:	2 hr	24 hr	Change in time
	(cpm/10 mg dry wt)		
Leaf strips	5809a	3683bc	-35%
Mechanically isolated cells	2920d	407e	-86%
Enzymatically isolated cells	5460a	4610b	-15%

[1]Means followed by different letters are significantly different at P=0.05.

[2]30-min fixation period, 1.0 µc/flask, SA 2.0 µc/µmole.

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45 mm diam. Millipore Co.) and stepwise extracted with the solvents listed in Table 20 (10 ml each), except for the NaOH solutions. The ^{14}C in the residue was determined by proportional planchet counting. Net recovered counts are given in Table 32 based on initial weight of the respective tissue preparations. The leaf strips were most efficient followed by the enzymatically isolated cells in incorporating $^{14}CO_2$ into protein. The mechanically isolated cells were less than half as efficient as the enzymatically isolated cells.

Using the same experimental techniques described for the comparison of mechanically isolated cells to leaf strips (Exp. III.C.), NAA uptake and NTC reduction were followed for the three cell preparations. NTC significantly inhibited NAA uptake by leaf strips, but dramatically enhanced accumulation by either isolated cell preparation, particularly the mechanically isolated cells (Table 33).

Extraction of reduced NTC from isolated cells was found to be more difficult than from leaf strips. By first extracting the tissue with 100% ethanol to remove plant pigments, acetone effectively extracted the reduced NTC. Unlike acetone's reductive effect on TTC, NTC is not reduced by this solvent, though the reduced NTC becomes unstable in light. Therefore, the acetone extracts were

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Table 32. Comparison by $^{14}CO_2$ incorporation into protein of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 1).[1]

Tissue preparation	^{14}C in protein fraction[2,3]
	(cpm/10 mg dry wt)
Leaf strips	1550a
Mechanically isolated cells	465c
Enzymatically isolated cells	1075b

[1]Parallel sample of 2 hr $^{14}CO_2$ fixation study-Table 31.

[2]Means followed by different letters are significantly different at P=0.05.

[3]Protein fraction represents material left on filter after separate washing with solvents listed in Table 20.

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Table 33. Comparison by naphthaleneacetic acid uptake and neo-tetrazolium chloride reduction of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 1).

Tissue preparation	NAA accumulated[1,2] (pmoles/10 mg dry wt)		NTC reduced[1,3] (A-560 nm / 10 mg dry wt)
Leaf strips			
-NTC	899c		0.085d
+NTC[3]	387d		0.155b
		net change	0.070
Mechanically isolated cells			
-NTC	303d		0.112c
+NTC[3]	1940a		0.279a
		net change	0.167
Enzymatically isolated cells			
-NTC	328d		0.070d
+NTC[3]	1262b		0.158b
		net change	0.088

[1]Means within a column followed by different letters are significantly different at P=0.05.
[2]NAA concn=5×10^{-6} M.
[3]NTC concn=0.01 mg/ml.

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stored in the dark until the NTC absorbance was measured at 560 nm. As found with the NAA uptake (Table 33), the two cell preparations, particularly the mechanically isolated cells, reduced more NTC than an equal quantity of leaf tissue.

Chlorophyll content was determined by the method of Arnon (1949). Protein was measured by micro-Kjeldahl digestion of acetone-extracted residue followed by steam distillation into boric acid and subsequent titration (Hawk, 1965). Analysis of chlorophyll and protein contents (Table 34) indicated that, on a weight basis, decreasing quantities of chlorophyll and protein were found in the mechanically isolated cells followed by enzymatically isolated cells and then the leaf strips, respectively. By contrast, chlorophyll content was less in isolated cells than in leaf strips when expressed in terms of protein present (Table 34).

Polarographic analysis in Experiment 2 (Table 35) indicated the results in Experiment 2 were similar to those of Experiment 1 (Table 29), except that the level of activity for the mechanically isolated cells was lower even though it was measured at the beginning of the experiment.

Using the same techniques of the experiment described for data in Table 27, $^{32}PO_4^{3-}$ uptake was examined. Both NTC

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Table 34. Comparison by measurement of chlorophyll and protein content of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 1).

Tissue preparation	Chlorophyll[1]	Protein[1]	Chlorophyll[1,2] / protein
	(µg/10 mg dry wt)	(mg/10 mg dry wt)	(µg/mg)
Leaf strips	47.7c	1.61c	29.8a
Mechanically isolated cells	73.8a	2.96a	24.9b
Enzymatically isolated cells	55.5b	2.15b	25.6b

[1]Means within a column followed by different letters are significantly different at P=0.05.
[2]Chlorophyll per unit of protein.

Table 3

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Table 35. Comparison by oxygen exchange rates in dark and light of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 2).

Tissue preparation	Net oxygen exchange[1,2]	
	Dark	Light
	(µl/100 mg dry wt/hr)	
Leaf strips	-315±22	172±20
Mechanically isolated cells	-93±18	-44±14
Enzymatically isolated cells	-170±25	287±22

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[2]Tissue analyzed 2 hr after preparation.

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and Cl-CCP were used as inhibitors in conjunction with the non-treated control. The inhibitors reduced uptake significantly for all tissue preparations (Table 36). The uptake in the non-treated control for the leaf strips was about twice that of enzymatically isolated cells and approximately 20 times that of the mechanically isolated cells.

The measurement of catabolism of a key metabolite was used as another criterion for tissue viability. Metabolism of acetate-^{14}C was followed at 25 C in the dark in a radiorespirometer as described by Wang (1962). Millipore filtered (0.45 µm pore size) air was pumped through each flask at 60 cc/min to transfer the liberated $^{14}CO_2$ into the trapping solution of ethanol-ethanolamine (1:1 v/v). Acetate-2-^{14}C was added to the tissue preparations after a 1/2-hr pretreatment. Hourly samples were collected after the first 15-min sample was discarded. ^{14}C was determined on the collected samples, properly transferred and diluted, with a liquid scintillation counter (4g BBOT/l toluene). Counting efficiency was determined by internal standardization with ^{14}C-toluene. The $^{14}CO_2$ evolution data (Table 37) showed that leaf strips and enzymatically isolated cells had similar capacities to metabolize acetate while mechanically isolated cells were significantly less active. The rate of

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Table 36. Comparison by phosphorus uptake of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 2).

Tissue preparation	PO_4^{3-} accumulated[1,2]		
	Non-treated	NTC[3]	Cl-CCP[4]
	(nmoles/10 mg dry wt)		
Leaf strips	51.43a	1.98c	17.15b
Mechanically isolated cells	1.84a	0.80c	1.31b
Enzymatically isolated cells	21.9a	1.59b	1.59b

[1]Means within a row followed by different letters are significantly different at P=0.05.
[2]PO_4^{3-} concn=5×10^{-5} M.
[3]NTC concn=0.1 mg/ml.
[4]Cl-CCP concn=1×10^{-6} M.

Table 37.

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Table 37. Comparison by acetate-2-^{14}C catabolism of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 2).

Tissue preparation	$^{14}CO_2$ liberated[1,2]		
Sample time:	1st hr	2nd hr	3rd hr
	(cpm/10 mg dry wt/hr)		
Leaf strips	3799c	6649b	9706a
Mechanically isolated cells	749d	2185cd	2758c
Enzymatically isolated cells	3500c	5720b	9460a

[1]Means followed by different letters are significantly different at P=0.05.

[2]Acetate concn=0.1 m$\underline{M}$, specific activity 1.0 µc/100 µmoles.

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catabolism increased with time for all preparations.

Because the mechanically isolated cells had very low activity (Experiment 2) compared to earlier experiments (Tables 24, 25), a further comparison on a third set of tissue preparations was carried out. The mechanically isolated cells again had (Table 38, Table 39) very low activity.

F. Comparisons Among Mechanically and Enzymatically Isolated Leaf Cells and Leaf Strips-Microscopic Studies.

1. Light Microscope Studies

A Wild M20 research microscope, equipped with both bright field and phase contrast objective lens, was used. Photomicrographs (35 mm) were taken with the aid of a Wild photoautomatic exposure control unit.

A leaf strip (50 μm) is shown in cross-section in Figure 17. Two rows of tightly packed palisade cells stand over the more loosely arranged spongy mesophyll cells. The highly vacuolated epidermal cells are banded on the leaf exterior by a cuticle. Though not shown in this photograph, the leaf strips, used for experimental purposes, contained several vascular bundles. The surface of the leaf was characterized by a large number of trichomes.

Typical examples of mechanically isolated leaf cells are shown in oil phase contrast photomicrographs of Figure 18.

Table 38.

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Table 39.

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Enzymatical cells	

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[3]Cl-C

Table 38. Comparison by oxygen exchange rates of leaf strips and cells isolated by either mechanical or enzymatic procedures (Experiment 3).

Tissue preparation	Net oxygen exchange[1]	
	Dark	Light
	(µl/100 mg dry wt/hr)	
Leaf strips	-362±72	175±27
Mechanically isolated cells	-125±18	80±37
Enzymatically isolated cells	-309±34	610±70

[1]SE given.

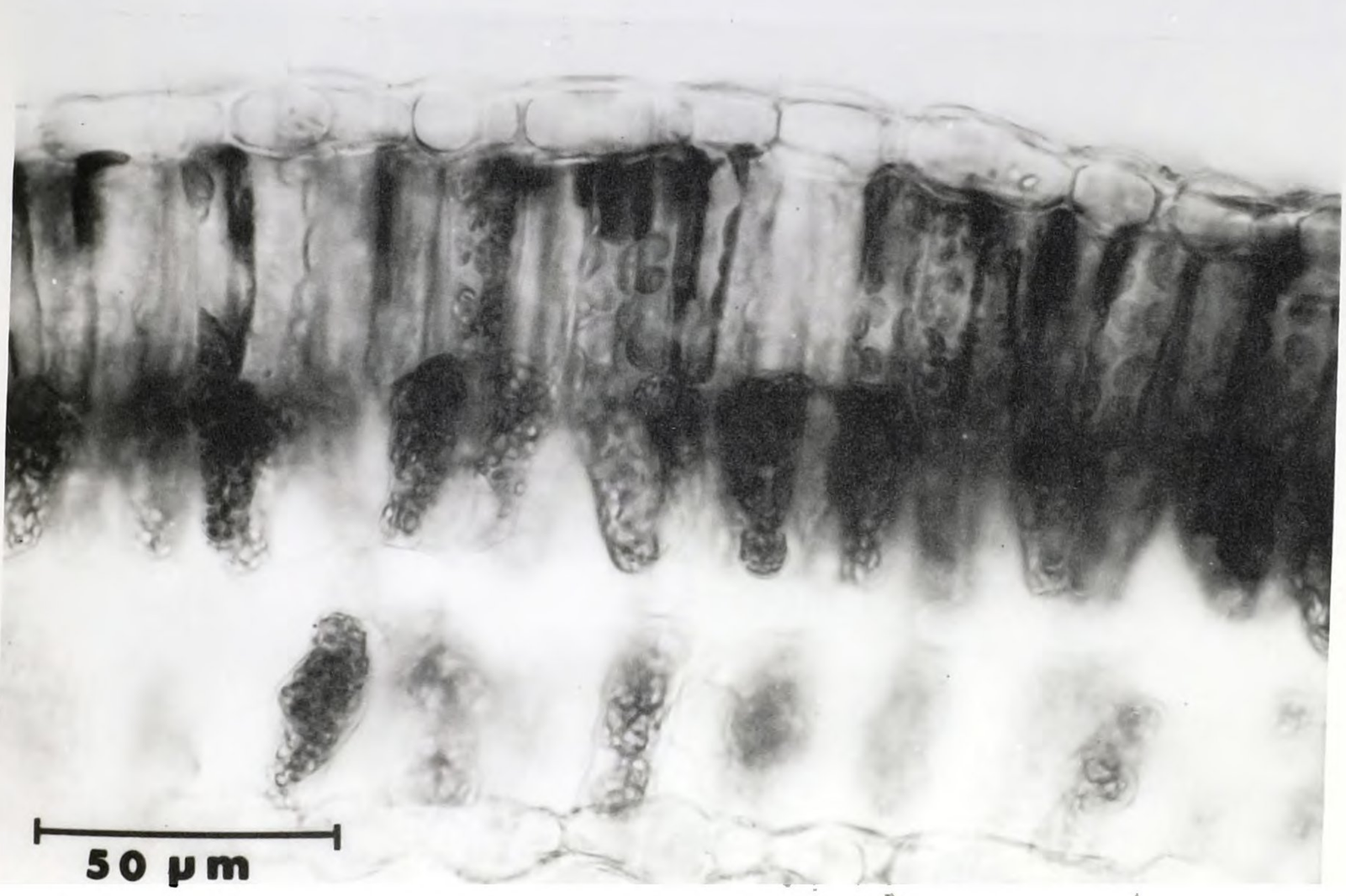
Table 39. Comparison by phosphorus uptake of leaf strips and cells isolated either by mechanical or enzymatic procedures (Experiment 3).

Tissue preparation	Phosphorus accumulated[1]		
	Non-treated	NTC[2]	Cl-CCP[3]
	(nmole/10 mg dry wt)		
Leaf strips	27.72a	1.48b	2.99b
Mechanically isolated cells	3.47a	0.80b	0.53b
Enzymatically isolated cells	8.71a	2.35b	2.77b

[1]Means within a row followed by different letters are significantly different at P=0.05.
[2]NTC concn=0.1 mg/ml.
[3]Cl-CCP concn=1×10^{-6} M.



Figure 17. Photomicrograph of a cross-section of a leaf strip (50 µm thick) viewed under bright light.



section of a leaf
under bright light.



Figure 18. Mechanically isolated cells.

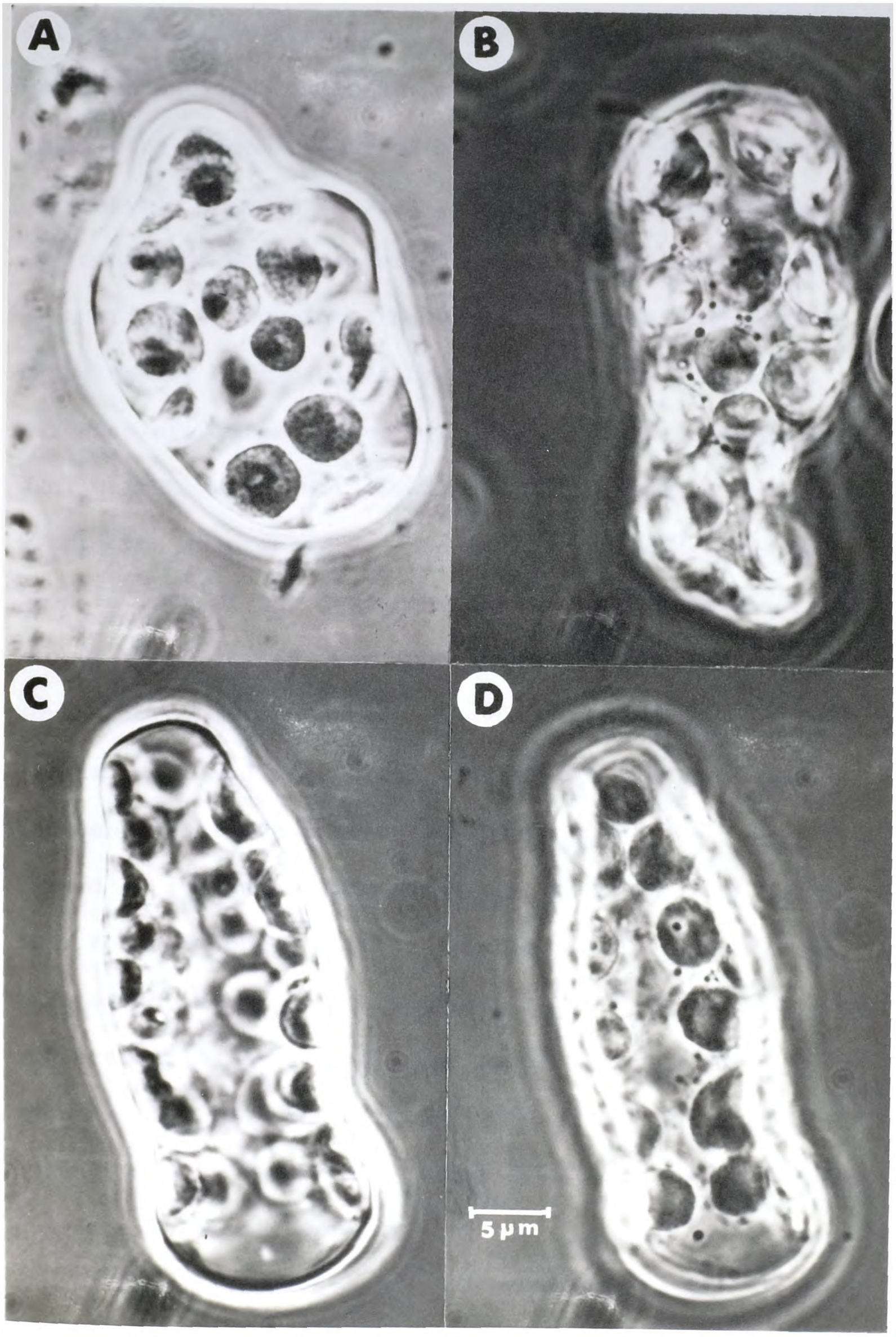
All photomicrographs taken with oil phase contrast.

A. Spongy mesophyll cell. Note distinct grana within chloroplasts.

B. Palisade mesophyll cell. Note microbodies and mitochondria.

C. Palisade mesophyll cell-focused on vacuole.

D. Palisade mesophyll cell of C focused on plane between chloroplasts and plasma membrane.



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Cell size varied from about 20x20 μm for spongy mesophyll cells to as large as 20x50 μm for palisade cells. Epidermal cells were seldom recovered by this technique. All cells were highly vacuolated with saucer-shaped chloroplasts (4 to 5 μm in diameter) oriented along the cell membrane. With critical focusing, the grana within the chloroplast occasionally became distinct (Figure 18A). When cells were isolated from leaves harvested after 6 hr of natural sun, starch gains (not shown) were also readily apparent within the chloroplasts. Distinct small bodies can be seen in most of the isolated cells. They could be microbodies (Fredrick and Newcomb, 1969), spherosomes, peroxisomes and/or mitochondria. They appeared dark or opaque (Figure 18B) and most often were observed in cytoplasm between the cell membrane and the vacuole. This is illustrated in the photomicrographs (Figures 18C,D) of the same cell focused on the central vacuole side of the chloroplasts and on the plasma membrane side of the chloroplasts, respectively. It should be stressed that not all mechanically isolated cells were as intact as those illustrated in Figure 18. About 45% of the cells showed some degree of disruption.

A representative sample of enzymatically isolated cells is shown in Figure 19A. The general description



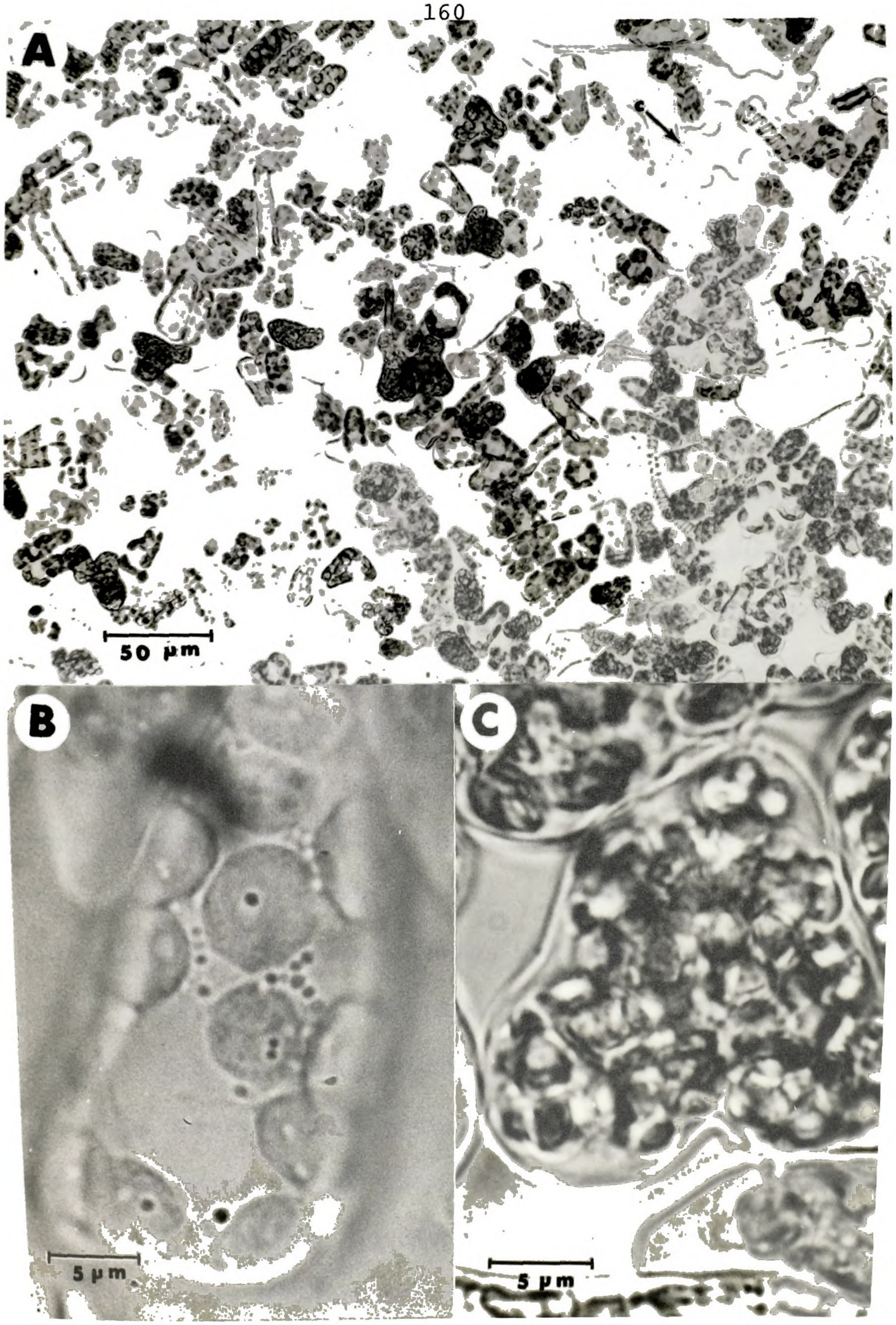
Figure 19. Enzymatically isolated cells.

All photomicrographs taken from bright field.

A. Low magnification of typical cell population. Note pieces of cuticle (C).

B. High magnification of viable palisade mesophyll cell.

C. High magnification image of damaged spongy mesophyll cell.



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of the cells is very similar to that of the mechanically isolated cells. The only difference between preparations appeared to be a larger number of cuticular fragments present in the enzymatically isolated preparation. Cells that appear darker were considered dead since their protoplasm was coagulated (Figure 19C). A typical living enzymatically isolated cell is illustrated in Figure 19B.

Cytoplasmic streaming was observed in many of the highly vacuolated cells, that had distinct chloroplasts, isolated by either enzymatic or mechanical procedures. Only Brownian movement could be seen in cells where the protoplasm was coagulated. Protoplasmic streaming is portrayed by the sequence of photomicrographs in Figure 20. The organelles often move in a slow short zig-zag pattern, but they definitely follow distinct paths that can be followed during a period of several minutes. Their movement appeared to be restricted to areas between the chloroplasts and the plasma membrane. These microbodies were not seen in the vacuole. Only by photographic comparisons can the chloroplasts be observed to be moving. None of the organelles were found going through pleomorphic changes in structure.

2. Electron Microscope Studies

Cells obtained by various tissue preparations were further examined using a Hitachi HU-11 electron microscope



Figure 20. Cytoplasmic streaming in enzymatically isolated cells as shown by time-sequence photomicrographs.

Bright field photomicrographs were taken approximately every 3 min except for F which was taken 30 min after E.

Note arrow in A and follow its point to see one example of from where microbodies and possibly mitochondria are moving. The organelles seem to follow a discreet path as shown in B, C, D, E. In time even the chloroplasts show some movement. Note chloroplast in F.

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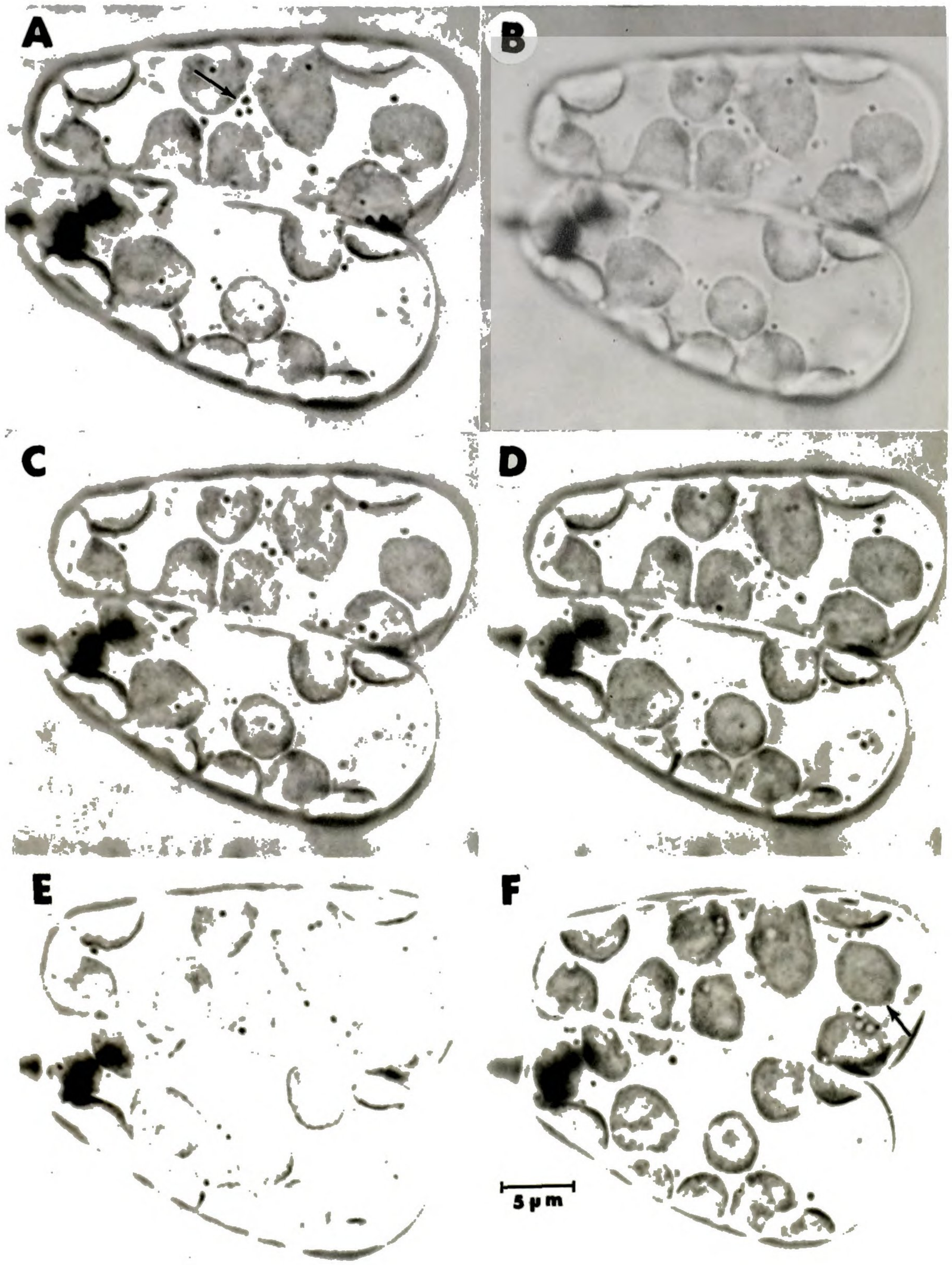

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operated at an electron accelerating voltage of 75 kV. Tissue samples were fixed and imbedded in Epon 812 as described by Valdovinos et al. (1968) with the slight modification of uranyl staining during the ethanol dehydration sequence. Sections were cut on a glass knife (L K B ultramicrotome). Lead citrate was used as a final stain (5 min). Through an unfortunate error, the samples of mechanically isolated cells were irrevocably damaged. It can only be assumed that the similarities noted in the light microscope work extend to the fine structure level.

Electron-photomicrographs (taken on Kodak EIP Plates) of representative enzymatically isolated cells and leaf strips cells revealed a great deal of similarity. The electron microscope study revealed a high number of starch grains present in cells from both preparations. Furthermore, the organelles observed streaming in the cells with the light microscope were likely both microbodies (spheresomes, lysosomes or peroxisomes) (Frederick and Newcomb, 1969) and mitochondria. The 1 μm diameter of the mitochondria noted in Figure 21A means it was just at the limit of resolution of the light microscope. The mitochondria found in the leaf strips (Figure 21B) had several forms.

Higher magnification of other viable enzymatically isolated cells (Figure 22) showed the ultrastructure of the

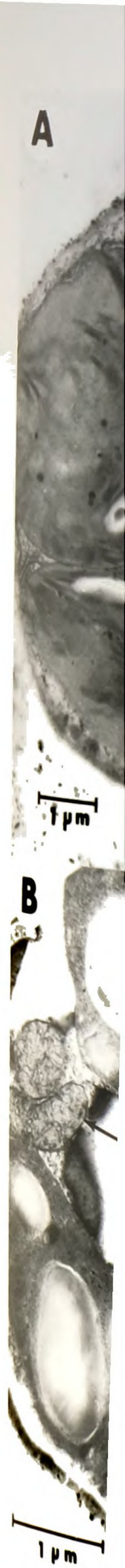
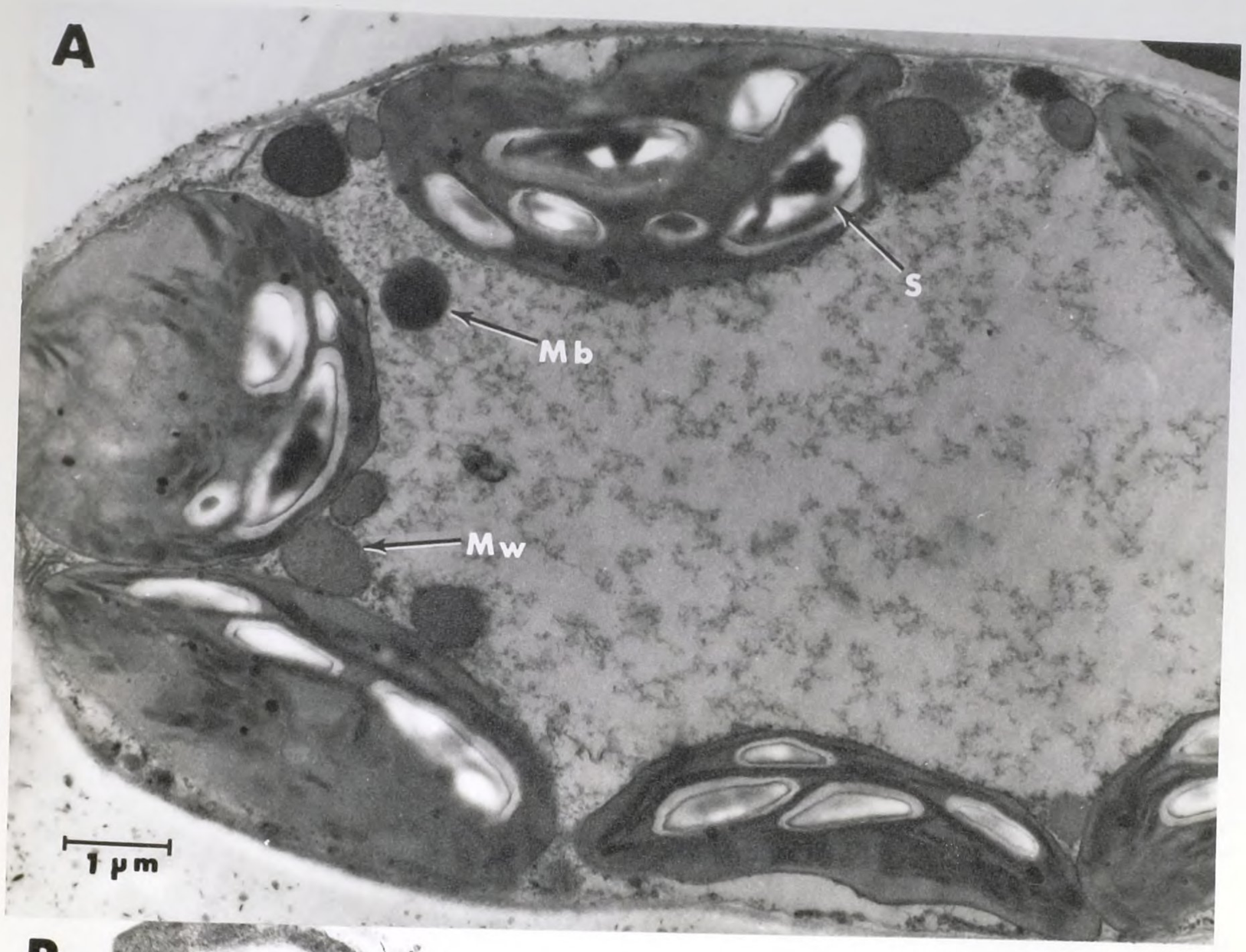


Figure 21. Electron micrograph of an enzymatically isolated cell compared to a cell in a leaf strip.

A. Enzymatically isolated cell. Examples of a starch grain (S) within a chloroplast, mitochondria (Mw) and microbody (Mb) are indicated by arrows.

B. Leaf strip cell. Examples of a starch grain (S) within a chloroplast and mitochondria are shown with arrows.



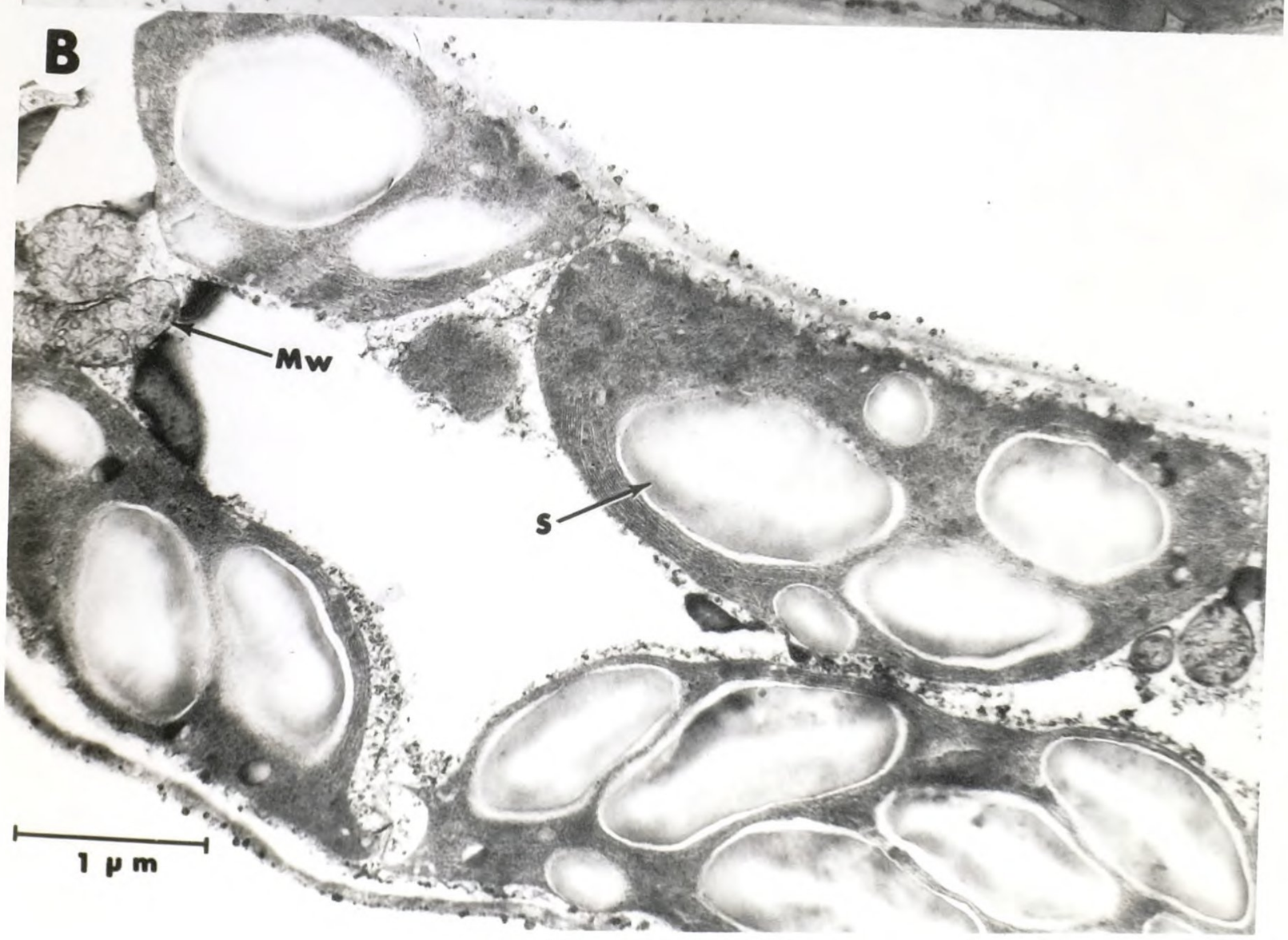


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Figure 22. Ultrastructure of viable enzymatically isolated cells.

A. Enzymatically isolated cell. Note distinct grana within chloroplasts, mitochondria (Mw) and microbody (Mb). The partially hydrolyzed cell wall (Cw) is also shown.

B. Enzymatically isolated cell. Cell wall (CW) is indicated.

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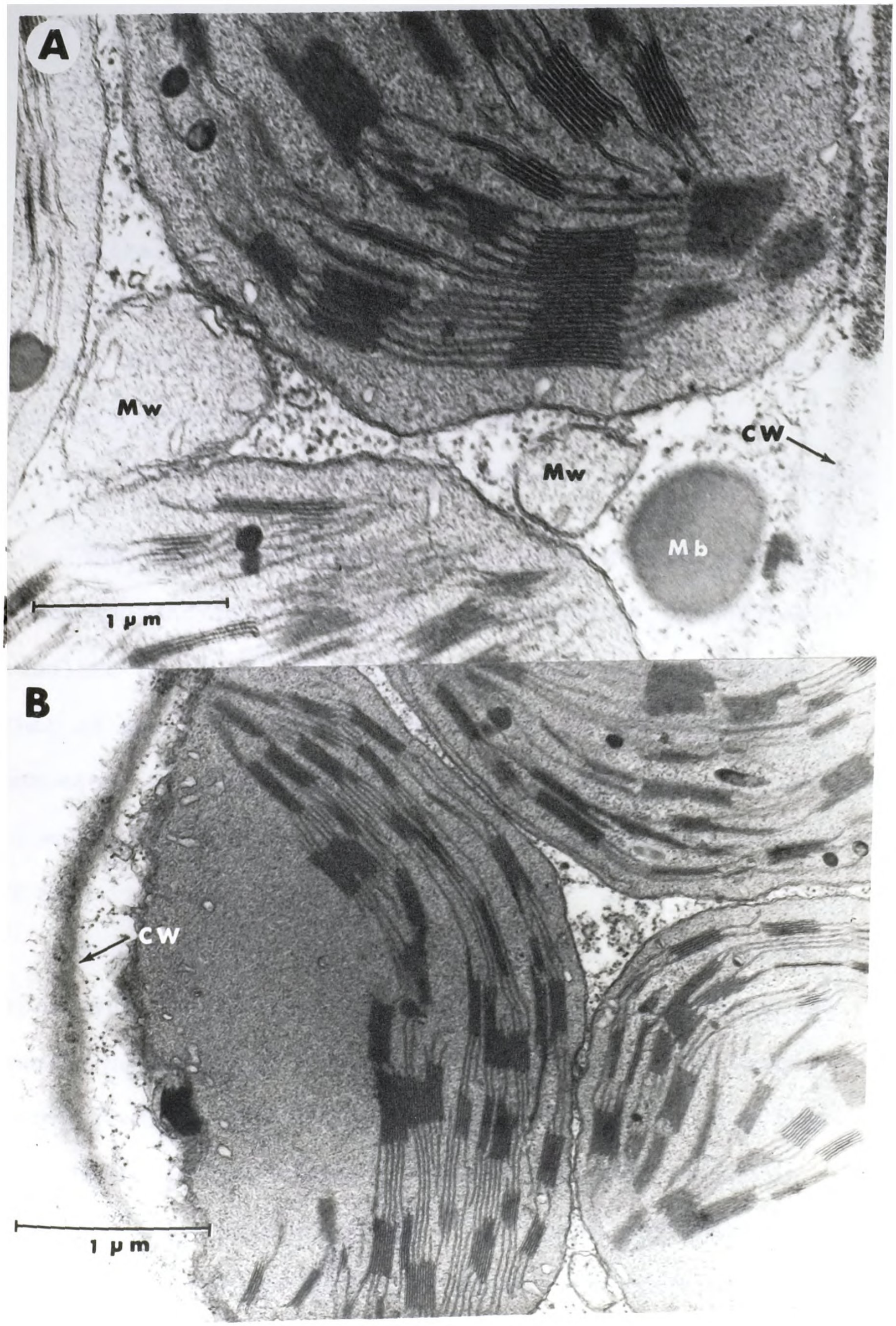

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various organelles to have distinct membranes. The cell wall of some of the cells appeared to be partially hydrolyzed (Figure 22A). As found in the light microscopic examinations, damaged or disrupted cells were also apparent at the ultrastructure level. Typically damaged enzymatically isolated cells (Figure 23) were characterized by chloroplasts with ruptured membranes. Starch grains were found randomly distributed within the cells.

G. Final Modification of Enzymatic Isolation Procedure for Leaf Cells

The several multiple comparisons of tissue preparations all indicated that both cell isolation procedures resulted in tissue of considerably lower viability than that of the leaf strips. However, cells isolated by the enzymatic technique were of sufficient activity and stability to warrant a further refinement of the method and evaluation of their usefulness for uptake studies.

Because of the time required for cell isolation, an experiment was designed to determine if cells isolated the evening before could be used the following day for experimental studies. The isolated cells had good activity that remained relatively constant for the 6-hr time period in which they were studied (Table 40). Because of this success, all the following enzymatic isolations were performed the



Figure 23. Ultrastructure of damaged enzymatically isolated cells.

A. Damaged enzymatically isolated cell.

B. Another example of damaged enzymatically isolated cell.

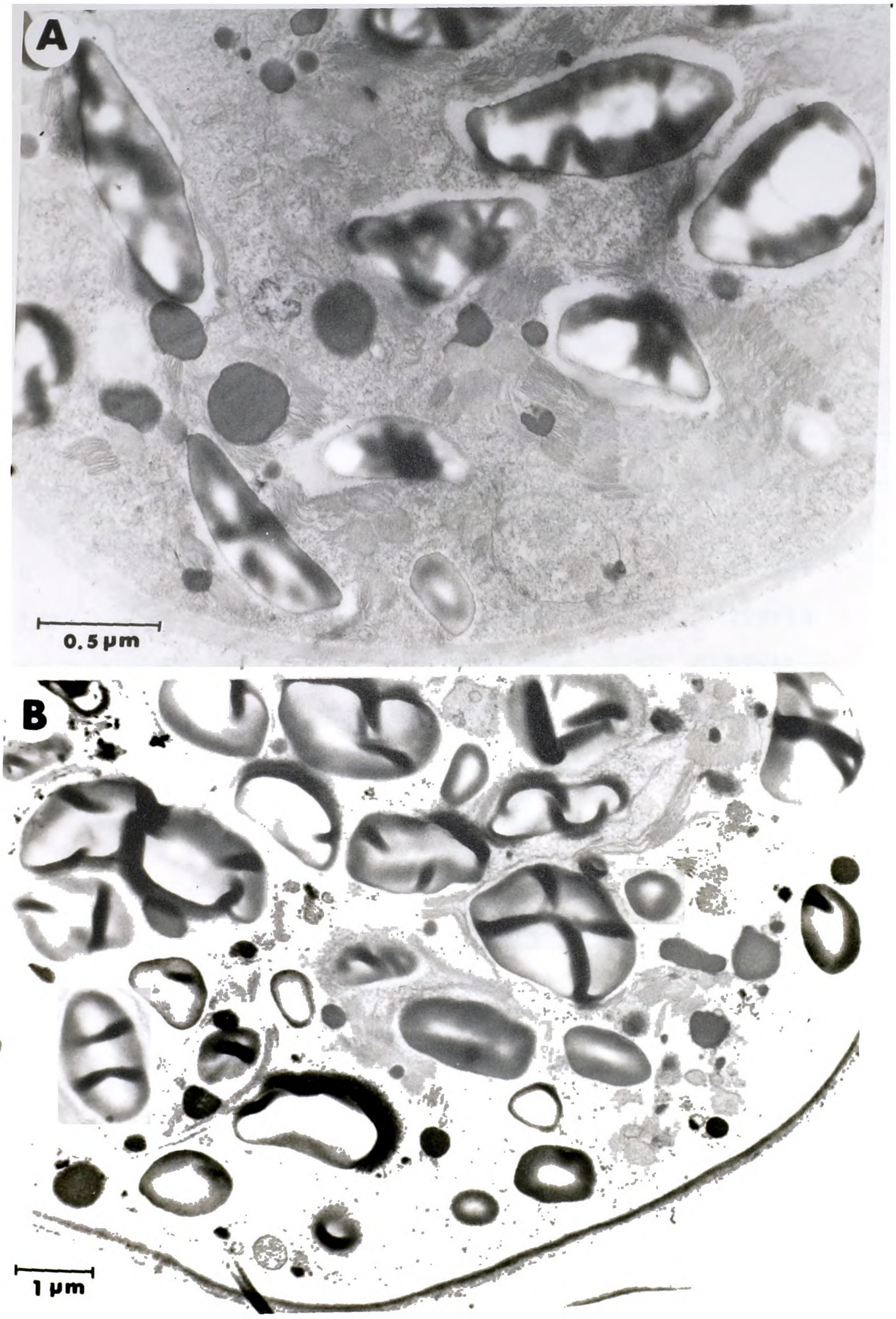

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Table 40. Analysis of stability of oxygen exchange rates of enzymatically isolated cells in dark and light.

Time at 25 C[1]	Net oxygen exchange[2]	
	Dark	Light
(hr)	(µl/100 mg dry wt/hr)	
0	-258±35	275±25
1/2	-267±9	250±43
1	-335±35	275±1
2	-327±18	241±35
3	-196±11	241±1
4	-215±9	181±9
5	-284±26	207±17
6	-293±17	250±9

[1]Cells isolated 12 hr earlier, stored fully diluted on stirrer in dark at 5 C. At the end of the storage period the cells were washed twice in filter sterilized medium and then transferred at 0 hr to 25 C at 200 ft-c light on a stirrer. Samples measured for O_2 exchanged at indicated times.

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IV. Uptake and Metabolism of Naphthaleneacetic Acid by Leaf Strips

Leaf strips (250 µm) were prepared in the same manner as previously described (Exp. II.B) and 48 such strips were used for each replicated treatment. The treatment medium listed in Table 19 was made up to a final volume of 5 ml per 25 ml Erlenmeyer flask. Uptake conditions were the same as those used for the hypocotyl sections (Exp. I.C.) except the NAA concentration was $1x10^{-6}$ M, specific activity 16 µc/µmole and the uptake period was extended to 4 hr unless otherwise noted. The experiments were terminated and the necessary procedures were used to produce the finalized data as described for the viability studies with leaf strips (Exp. II.I). The only exception was that 100% ethanol was used in place of xylene and the homogenate was quantitatively transferred to 2-inch planchets.

A. General Parameters of Naphthaleneacetic Acid Uptake

1. Time-Course

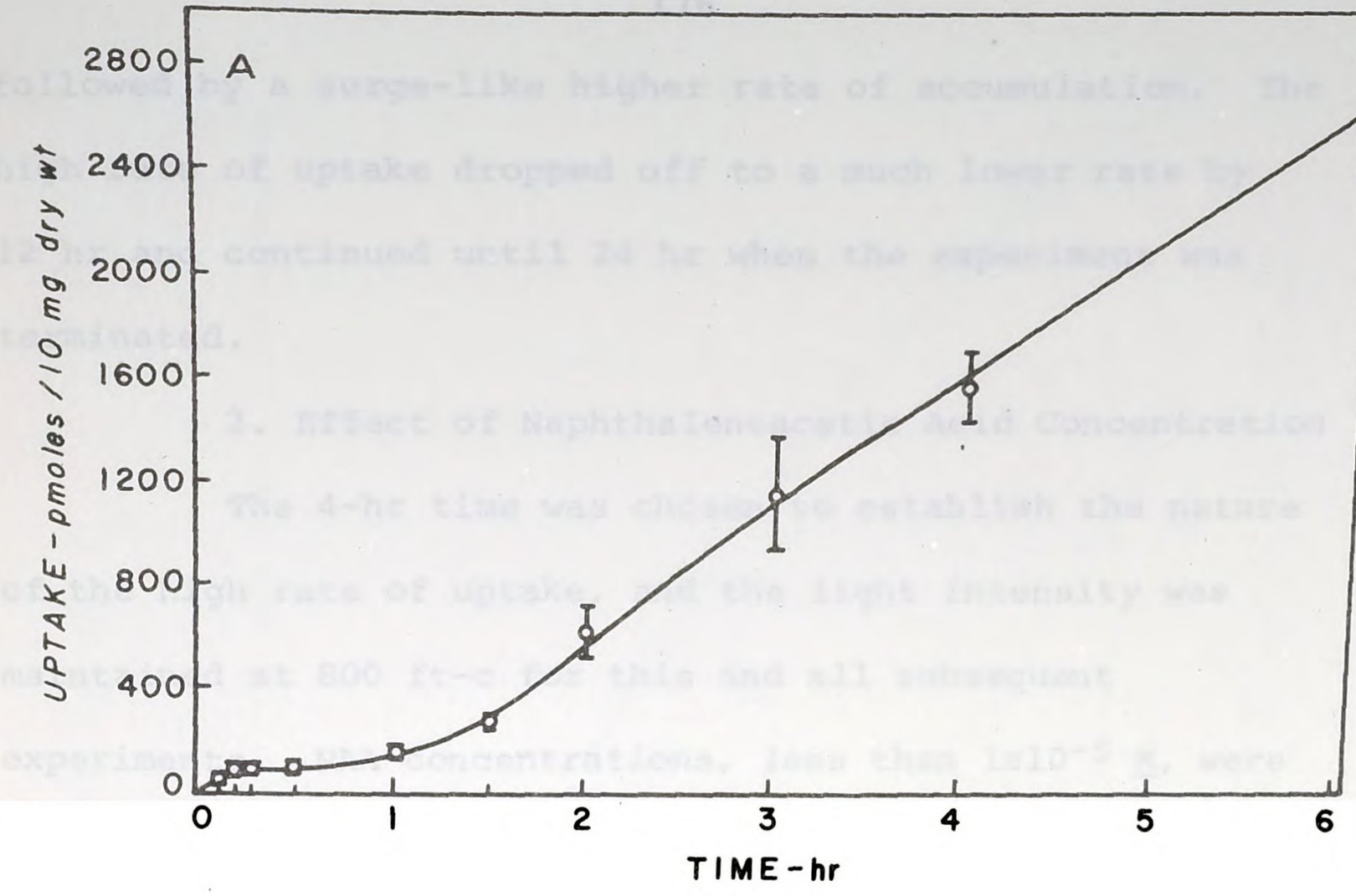
An extended time-course of NAA ($1x10^{-6}$ M) uptake by leaf strips is reported in Figure 24. Frequent samplings during the early portion of the time study (Figure 24A) revealed an initial 1.5 hr low rate of uptake that was then



Figure 24. Time-course of naphthaleneacetic acid uptake by leaf strips in the light.

A. First 6.0 hr of uptake.

B. Complete 24-hr time-course. Dead-control (frozen-thawed) check at 12 hr accumulated 51 pmoles NAA/10 mg dry wt.



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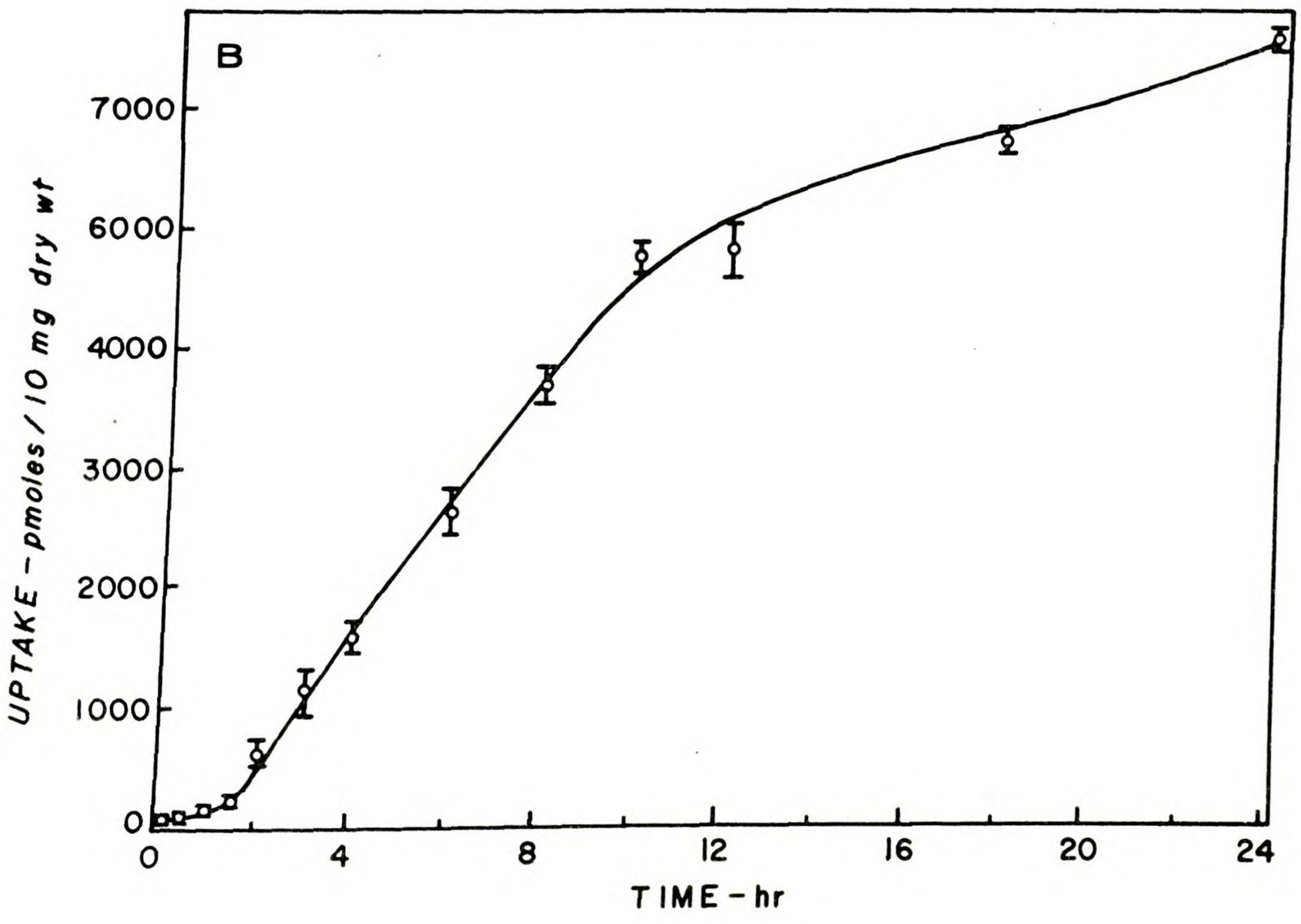

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followed by a surge-like higher rate of accumulation. The high rate of uptake dropped off to a much lower rate by 12 hr and continued until 24 hr when the experiment was terminated.

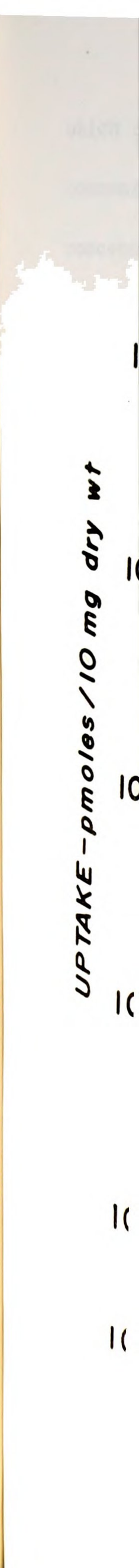
2. Effect of Naphthaleneacetic Acid Concentration

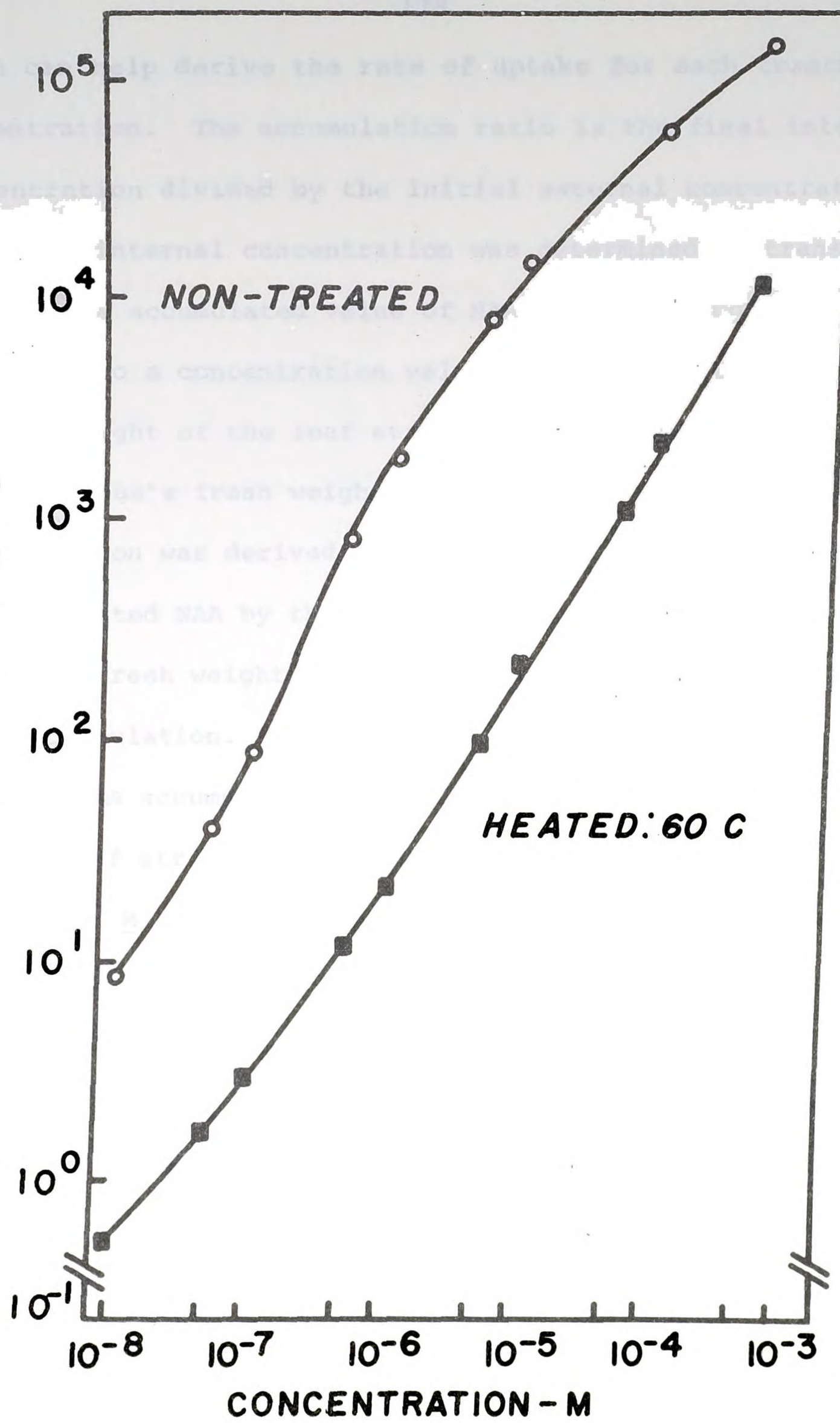
The 4-hr time was chosen to establish the nature of the high rate of uptake, and the light intensity was maintained at 800 ft-c for this and all subsequent experiments. NAA concentrations, less than $1x10^{-5}$ M, were prepared by dilution of the ^{14}C-NAA stock solution. For concentration of $1x10^{-5}$ M to $5x10^{-4}$ M, ^{14}C-NAA was mixed with non-labeled NAA to yield solutions of known specific activity and thereby maintaining a relatively constant level of ^{14}C-NAA for this concentration range. Comparable tissue pretreated for 30 min at 60 C served as a dead control. ^{14}C content of dead tissue was linear with increasing NAA concentration (Figure 25), while in the non-treated tissue, there was a slight jump in rate of accumulation at about $5x10^{-7}$ M and then dropped off as the concentration was further increased. It is important to note that the level of NAA in the non-treated tissue is larger, by an order of magnitude, than that of the heated tissue.

The results illustrated in Figure 25 can be transformed mathematically into values here termed accumulation ratios

Figure 25. Effect of naphthaleneacetic acid concentration on its own uptake by leaf strips.

Leaf strips were either non-treated or heated to 60 C for 30 min prior to the experiment.





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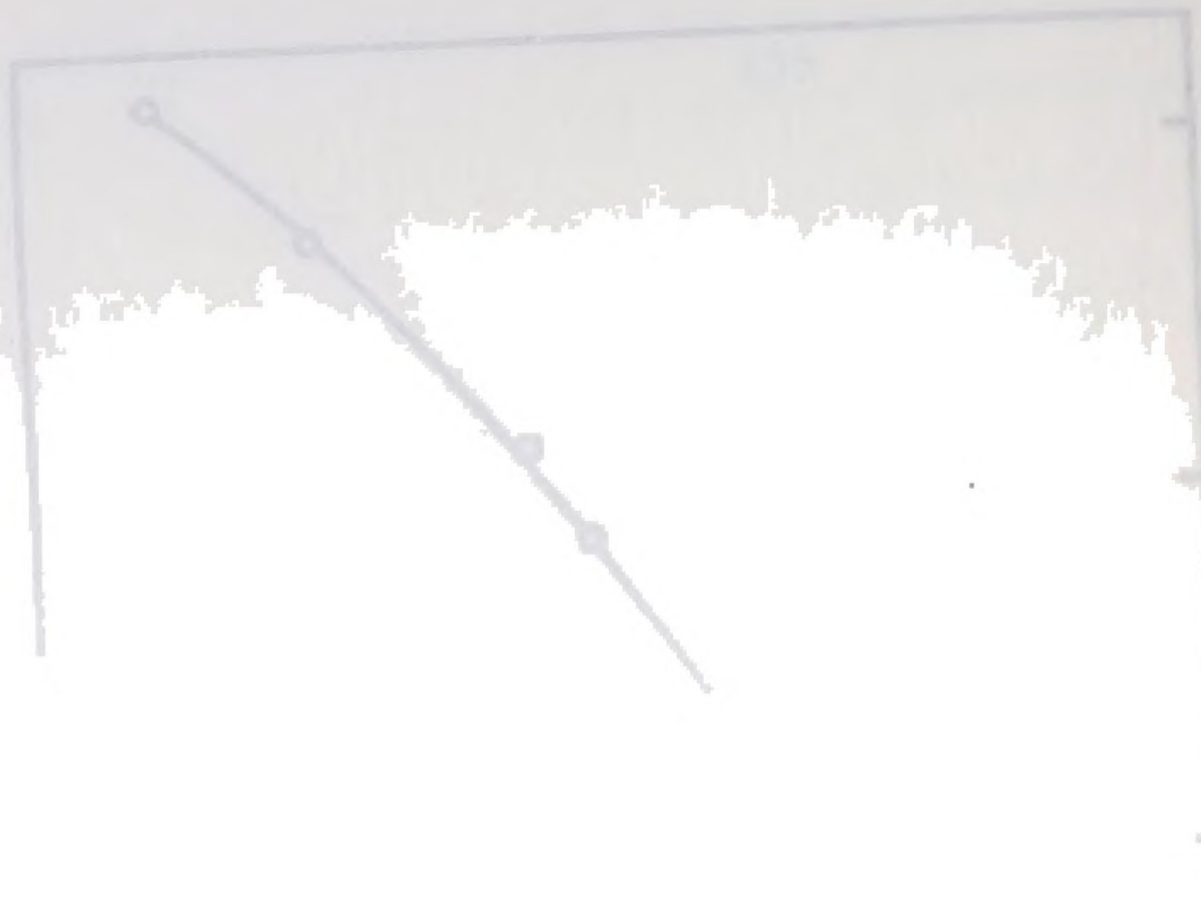

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which can help derive the rate of uptake for each treatment concentration. The accumulation ratio is the final internal concentration divided by the initial external concentration. The final internal concentration was determined by transforming the accumulated value of NAA (pmoles/10 mg dry weight) into a concentration value based on the fact the dry weight of the leaf strip represented 20.1% (w/w) of the tissue's fresh weight. Therefore, the internal NAA concentration was derived by dividing the number of moles of accumulated NAA by the volume of the tissue water content (80% fresh weight). See Figure A-1 (Appendix) for a sample calculation.

The NAA accumulation ratios (Table 41) by the non-treated leaf strips showed almost a doubling in rate, going from $1x10^{-7}$ M to $5x10^{-7}$ M external NAA concentration. As the external concentration was raised to $5x10^{-5}$ M, the rate of uptake dramatically dropped, but it did not exhibit saturation even at $5x10^{-4}$ M NAA. The accumulation ratios of the heated tissue revealed a lower rate of sorption that was relatively independent of concentration.

3. Loss of Accumulated Naphthaleneacetic Acid from Tissue and Determination of Free Space

The amount of accumulated NAA freely lost to fresh media is reported in Table 42. Rapid successive washes of

Table 41

Treating	
$1x10^{-8}$	M
$5x10^{-8}$	M
$1x10^{-7}$	M
$5x10^{-7}$	M
$1x10^{-6}$	M
$5x10^{-6}$	M
$1x10^{-5}$	M
$5x10^{-5}$	M
$1x10^{-4}$	M
$5x10^{-4}$	M

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Table 41. Effect of naphthaleneacetic acid concentration on its own uptake by leaf strips.

Treating concn	Accumulation ratio[1,2]	
	Non-treated	Heated to 60 C[3]
$1x10^{-8}$ M	22.5±4.2	1.25±0.02
$5x10^{-8}$ M	20.0±2.9	0.88±0.25
$1x10^{-7}$ M	23.0±2.7	0.75±0.08
$5x10^{-7}$ M	40.0±2.8	0.60±0.02
$1x10^{-6}$ M	46.7±4.2	0.53±0.02
$5x10^{-6}$ M	37.0±3.3	0.50±0.02
$1x10^{-5}$ M	35.0±4.5	0.55±0.02
$5x10^{-5}$ M	15.8±0.6	0.54±0.02
$1x10^{-4}$ M	12.7±1.2	0.55±0.03
$5x10^{-4}$ M	10.8±0.7	0.72±0.05

[1]SE given.
[2]Calculated final internal NAA concn/initial external concn. Actual per cent of NAA removed from medium=80% of accumulation ratio values.
[3]30-min pretreatment.

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Table 42. Effect of successive washings (2 min) on the amount of naphthaleneacetic acid retained by leaf strips after naphthaleneacetic acid[1] uptake.

Wash number[2,3]	NAA retained[4]
	(pmoles/10 mg dry wt)
0	976a
1	910ab
2	738c
3	788bc
4	698c
5	737c

[1]NAA concn=$1x10^{-6}$ M.
[2]Wash medium=0.01 M phosphate buffer (KH_2PO_4-K_2HPO_4) pH 6.6.
[3]Time for each wash=2 min.
[4]Means followed by different letters are significantly different at P=0.05.

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tissue preloaded with NAA indicated that a minimum of two 5-ml washes was required to remove that fraction of NAA not firmly bound by the tissue. Two washes were used for all leaf strip experiments. The fraction removed was equal to 236 pmoles/10 mg dry weight.

The determination of the initial rate of uptake of a compound into tissue at low temperatures is another way of assessing the amount of free space (Briggs et al., 1961). Results of a study are reported in Figure 26 where the NAA treating concentration was raised to $5x10^{-6}$ M to facilitate labeling, during the very short sampling time, for accurate measurements. One set of samples was collected but not washed, while a second set was collected and washed twice before analysis for NAA. Extrapolation back to zero time for the non-washed samples gave a value of about 590 pmoles/10 mg dry weight, while the washed had 100 pmoles/10 mg dry weight.

NAA efflux from the leaf strips is detailed in Table 43 where leaf strips were labeled in the dark with NAA. Because of the large size of the experiment, two water baths were required to label sufficient material for use in the efflux study. Unfortunately the temperature of one of the baths had shifted during the labeling portion of the experiment, but was corrected in time for the efflux study. As

Figure 26. Short-time course of naphthaleneacetic acid (5×10^{-6} M) uptake by leaf strips at 5 C in the dark.

Following uptake, one set of treatments was assayed without washing while the other set was washed twice with 0.01 M phosphate buffer (KH_2PO_4-K_2HPO_4) at pH 6.7.

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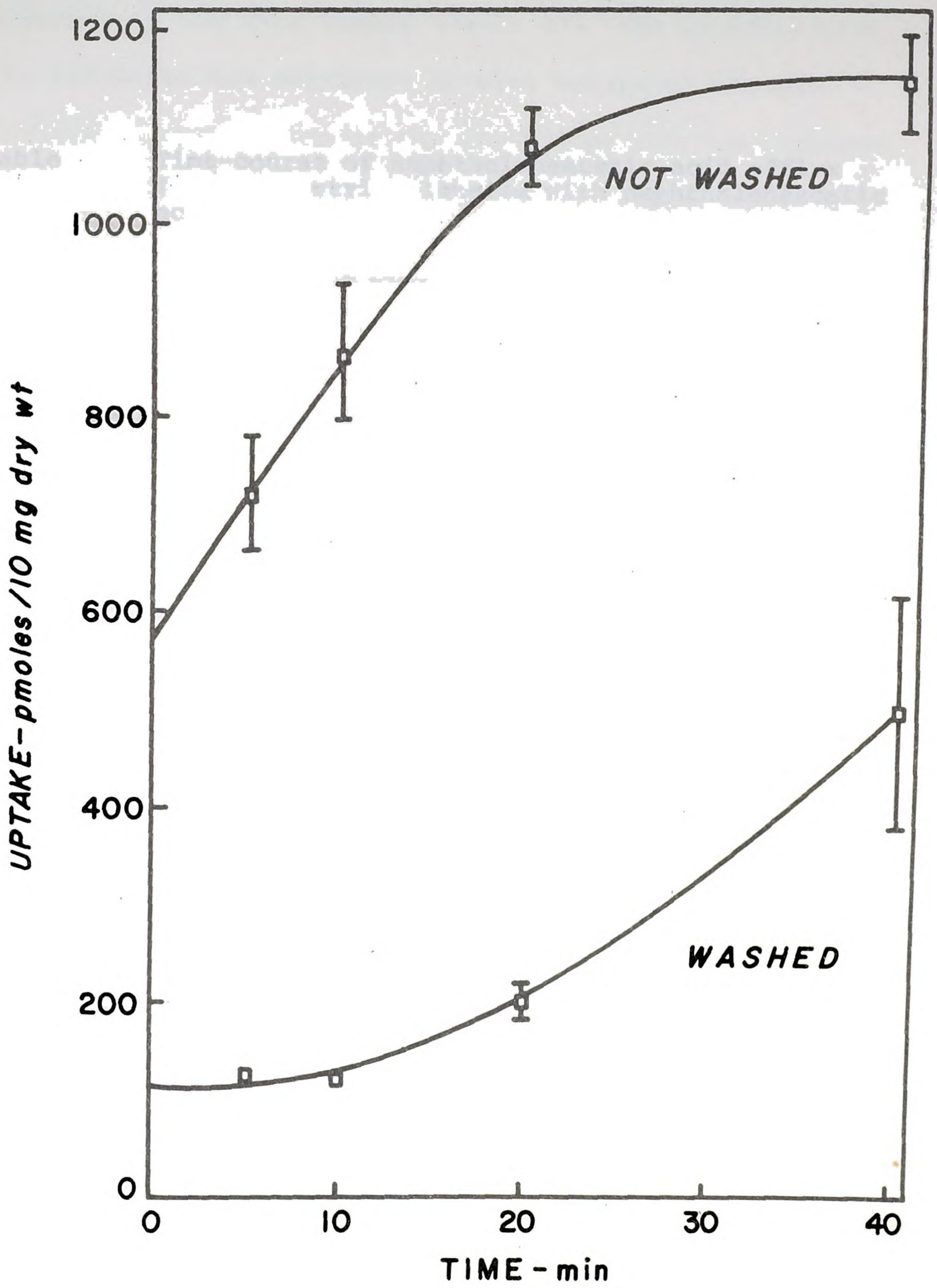

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Table 43. Time-course of naphthaleneacetic acid efflux from leaf strips labeled with naphthaleneacetic acid.[1,2]

Time	NAA retained[3]			
	Group I[4]		Group II[4]	
Wash treatment:	Medium only	NAA[5]	Cl-CCP[6]	Cl-CCP[6] + NAA[5]
(hr)	(pmoles/10 mg dry wt)			
0.0	1184a	1184a	913a	913a
0.5	1121	933	872	875
1.0	1013	990	867	890
2.0	994	919	841	881
4.0	1052	1094	881	819
Mean[7]	1022b	978b	860a	894a

[1]Labeling NAA concn-$1x10^{-6}$ M, 4 hr in dark.

[2]All tissue washed twice before efflux experiment commenced.

[3]Means within Group I or II followed by different letters are significantly different at P=0.05. Interaction of wash vs time was not significant.

[4]All tissue within a group were labeled uniformly but Group I had been labeled at a higher temperature (25 C) than Group II (23 C), and consequently the two groups must be considered independently of each other.

[5]NAA wash concn=$1x10^{-5}$ M.

[6]Cl-CCP wash concn=$1x10^{-6}$ M.

[7]Mean of 0.5, 1.0, 2.0 and 4.0-hr washes.

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apparent in the 0-hr sample (Table 43), the material from the two baths had different initial levels of NAA and, therefore, the results of the two groups were not compared statistically. Within the first group, efflux was compared from tissue in medium alone to that in medium with 10x the labeling NAA concentration. No difference between these two treatments was noted, although a small, but significant, release of NAA was observed. The released NAA was totally accounted for in the wash medium. Efflux from the second group of tissue was examined in the presence of the inhibitor Cl-CCP with and without the 10x NAA. In the presence of the inhibitor no significant loss of NAA was detected irrespective of the addition of NAA in the wash medium.

B. Metabolic Aspects of Naphthaleneacetic Acid Uptake

1. Effect of Temperature

NAA uptake by leaf strip tissue held at 5, 15 and 25 C was examined on a time basis. Tissue was equilibrated at each temperature for 40 min at pH 6.7 in the standard medium. Tissue heated to 60 C followed by placement in fresh medium at pH 6.7 was used as a dead control at each temperature. A second set of dead controls was placed in fresh medium at pH 3.7 (no buffer).

As shown in Table 44, a significant temperature effect

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Table 44. Effect of temperature on naphthaleneacetic acid uptake by leaf strips in the dark.

Treatment	NAA accumulated[1]					
	5 C		15 C		25 C	
Time-hr:	2.0	4.0	2.0	4.0	2.0	4.0
	(pmoles/10 mg dry wt)					
Non-treated pH 6.7[3]	73ef	99de	105de	206c	272b	715a
Dead control[2] pH 6.7[3]	18f	22f	23f	26f	29f	25f
Dead control[2] pH 3.7[3]	123de	107de	100de	109de	152cd	100de

[1]Means followed by different letters are significantly different at P=0.05.
[2]Pretreatment 40 min by heating to 60 C.
[3]Uptake medium pH.

Table 45. Q_{10} values for naphthaleneacetic acid uptake by leaf strips.

Treatment	Q_{10} uptake					
	5 C to 15 C			15 C to 25 C		
	2-hr	4-hr	Δ time	2-hr	4-hr	Δ time
Non-treated pH 6.7[2]	1.4	2.1	----[3]	2.6	3.5	4.4
Dead control[1] pH 6.7[2]	1.3	1.2	----[3]	1.2	1.1	----[3]
Dead control[1] pH 3.7[2]	0.8	1.0	----[3]	1.5	0.9	----[3]

[1]Pretreatment 40 min by heating to 60 C.
[2]Uptake medium pH.
[3]No statistical change in time.

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was apparent only in non-treated tissue. The dead control at pH 6.7 contained less than 30 pmoles/10 mg after 4 hr. However, a similar dead control at pH 3.7, which is below the pK_a of 4.2, accumulated over five-fold more than the dead control held at pH 6.7. Calculated Q_{10} values of 1.4 to 3.5 were obtained for living tissue and 0.8 to 1.5 for dead tissue irrespective of pH (Table 45). If the change in uptake from 2.0 to 4.0 hr is considered a rate value, then the non-treated tissue exhibited a Q_{10} value as high as 4.4 in the 15 to 25 C temperature range. None of the other treatments could be analyzed in this manner since uptake did not change in time during the temperature treatments.

2. Effect of Light on Naphthaleneacetic Acid Uptake

To study the effect of light on NAA uptake, a photo-flood lamp bank (Exp. III.D) was used to provide a light intensity of 2000 ft-c. The temperature of the water bath was carefully regulated at 25.0 C. A sufficient number of leaves was harvested initially for two consecutive experiments. The first light study was on freshly harvested leaves, and the second on leaves aged for 2 days by storing in low light (200 ft-c) with their petioles immersed in distilled water. Statistical analysis of the data (only

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valid on the interaction of leaf age to light and light versus dark) showed that the interaction of light and tissue age affecting NAA uptake was not significant. However, the light significantly reduced the amount of NAA accumulated (Figure 27).

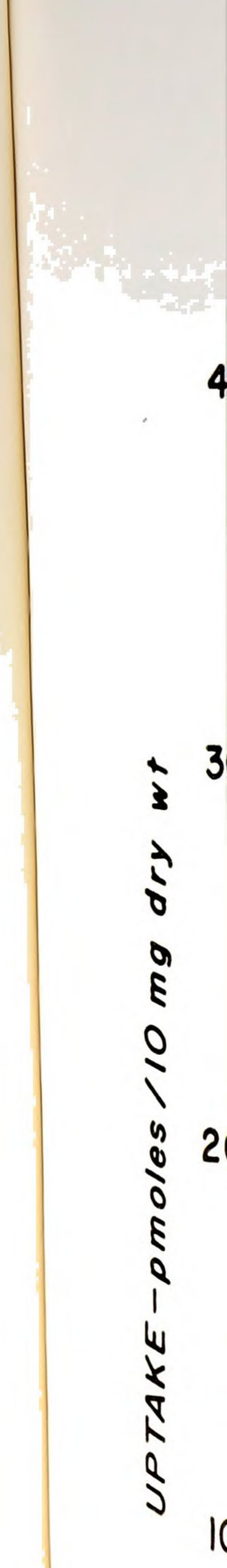
Pretreatment with NAA (10^{-6} M to 10^{-5} M) had no effect on dark O_2 respiration (Table 46), while in the light, it significantly reduced O_2 production.

3. The Effect of Metabolic Inhibitors on Naphthaleneacetic Acid Uptake

As detailed in Table 47, the action of several metabolic inhibitors, each at 3 different concentrations, was examined to determine their effect on NAA accumulation. At the lower light intensity (800 ft-c), uptake was not depressed as compared to a dark control. NaN_3 partially inhibited accumulation at concentrations of $5x10^{-5}$ M or higher, while Cl-CCP, at $1x10^{-6}$ M, almost totally inhibited NAA uptake. The photosynthetic inhibitor 3-(4-chlorophenyl)-1,1-dimethylurea (monuron, CMU), which inhibited oxygen production (Table 48) had no significant effect on NAA uptake by leaf strips in the light (Table 47). Cycloheximide, an inhibitor of plant protein synthesis (Morris, 1966, 1967; Ellis, 1969), at concentrations of $1x10^{-5}$ M to $1x10^{-4}$ M inhibited NAA uptake by more than 50%, but had no

Figure 27. Time-course of naphthaleneacetic acid uptake in light and dark by leaf strips.

Light intensity=2000 ft-c. The points represent values from two experiments; one set from freshly harvested leaves and the other from the same group of leaves aged under low light intensity for two days. The interaction of leaf age and light on NAA uptake was not significant, while the effect of light significantly reduced uptake with time (P=0.05).



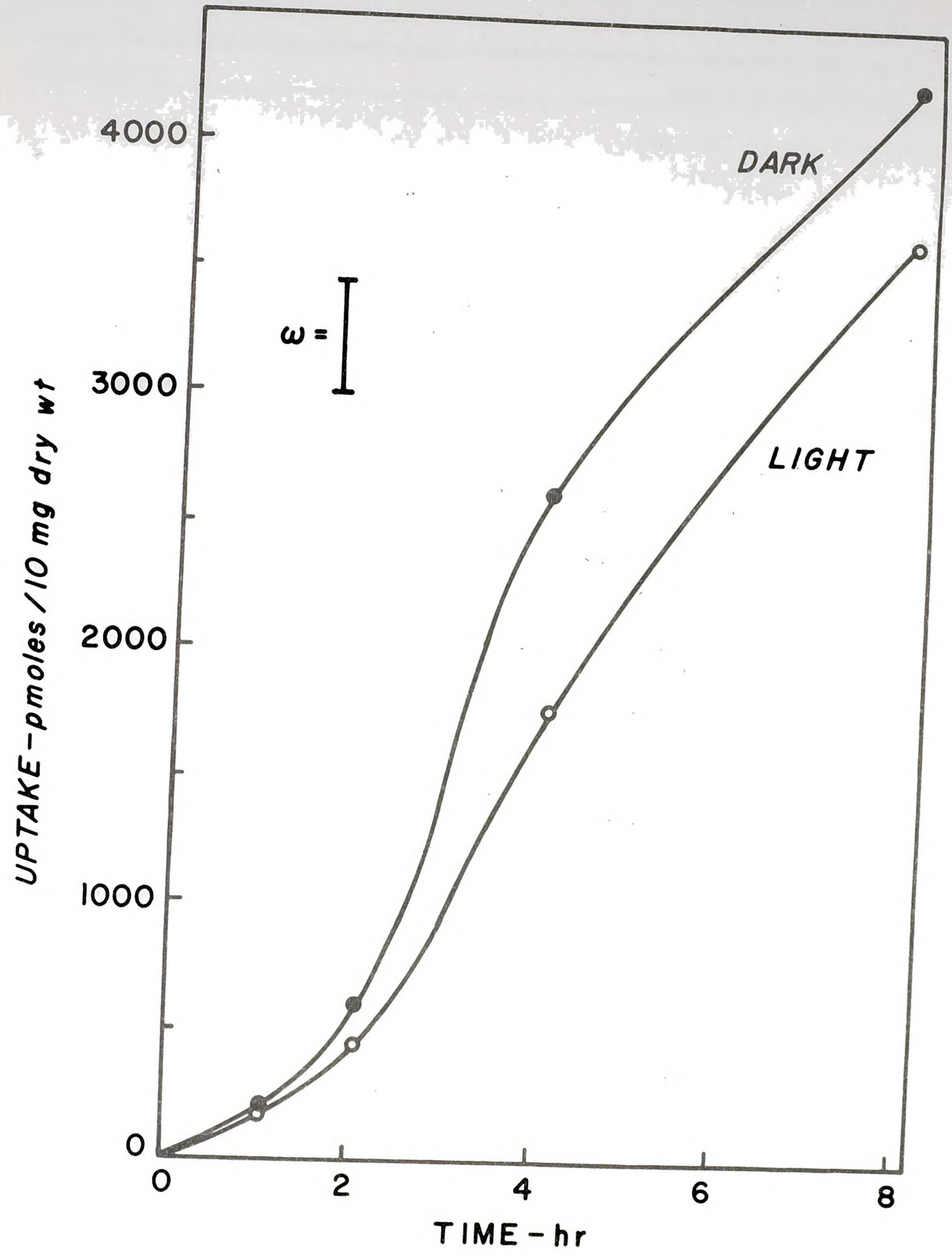


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Table 46. Effect of naphthaleneacetic acid pretreatment on oxygen exchange rates of leaf strips in dark and light.

Treatment	Net oxygen exchange[1]	
	Dark	Light
	(µl/100 mg dry wt/hr)	
Control	-297a	276a
NAA ($1x10^{-6}$ M)[2]	-307a	181b
NAA ($1x10^{-5}$ M)[2]	-317a	191b

[1]Means within a column followed by different letters are significantly different at P=0.05.
[2]2-hr pretreatment.

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Table 47. Effect of several metabolic inhibitors on naphthaleneacetic acid uptake by leaf strips.[1,2]

Treatment	NAA accumulated (pmoles/10 mg dry wt)
Non-treated-light	1037
Non-treated-dark	949
NaN_3 ($1x10^{-5}$ M)	997
NaN_3 ($5x10^{-5}$ M)	751[3]
NaN_3 ($1x10^{-4}$ M)	600[3]
Cl-CCP ($5x10^{-7}$ M)	39[3]
Cl-CCP ($1x10^{-6}$ M)	30[3]
Cl-CCP ($5x10^{-6}$ M)	22[3]
CMU ($1x10^{-6}$ M)	1054
CMU ($5x10^{-6}$ M)	1094
CMU ($1x10^{-5}$ M)	1065
Cycloheximide ($1x10^{-5}$ M)	484[3]
Cycloheximide ($5x10^{-5}$ M)	415[3]
Cycloheximide ($1x10^{-4}$ M)	389[3]
Chloramphenicol ($1x10^{-4}$ M)	1149
Chloramphenicol ($5x10^{-4}$ M)	1317[3]
Chloramphenicol ($1x10^{-3}$ M)	1434[3]
Malonic acid ($5x10^{-4}$ M)	1138
Malonic acid ($1x10^{-3}$ M)	1347[3,4]
Malonic acid ($5x10^{-3}$ M)	1797[3,4]

[1]All time treatments were present during the entire experimental time period including a 1-hr pretreatment.

[2]All treatments were conducted in light (800 ft-c) unless otherwise noted.

[3]These treatment means are significantly different from the light control at P=0.05.

[4]These treatment means are significantly different from each other at P=0.05.

Table

Table 48. Effect of 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) on oxygen exchange rates of leaf strips in dark and light.

Treatment	Net oxygen exchange[1]	
	Dark	Light
	(µl/100 mg dry wt/hr)	
Non-treated	-263±29	263±29
CMU ($1x10^{-6}$ M)	-315±46	-123±12
CMU ($5x10^{-6}$ M)	-310±22	-185±33
CMU ($1x10^{-5}$ M)	-375±29	-189±11

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effect on respiration (Table 49). Greater uptake was observed in the presence of chloramphenicol at $5x10^{-4}$ M and higher (Table 47). Malonic acid also stimulated NAA uptake with its effect becoming more significant with higher concentrations ($1x10^{-3}$ M to $5x10^{-3}$ M) (Table 47). Malonate ($5x10^{-3}$ M) had no effect on respiration (Table 49).

C. Metabolism of Naphthaleneacetic Acid by Leaf Strips

The results of the malonic acid and cycloheximide studies inferred that the metabolism of NAA may be related to uptake. This hypothesis was investigated by more critically assessing the effect of malonate and cycloheximide both independently and in combination on NAA uptake. Sufficient tissue was included in each treatment so as to determine NAA uptake and the form in which NAA was present.

These data (Table 50) confirmed the stimulatory effect of malonate and the inhibitory effect of cycloheximide on NAA uptake. The addition of malonate to the cycloheximide treatment partially overcame the effect of cycloheximide but uptake was still significantly less than for the non-treated control (Table 50).

For analysis of the form of the accumulated NAA, the leaf strip tissue was homogenized (see Exp. II.C.) in 10 ml of 80% (v/v) ethanol and quantitatively transferred

Table

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Table 49. Effect of malonic acid and cycloheximide on oxygen exchange rates of leaf strips in dark and light.

Treatment	Net oxygen exchange[1]	
	Dark	Light
	(µl/100 mg dry wt/hr)	
Non-treated	-292	147
Malonic acid ($5x10^{-3}$ M)	-315	144
Cycloheximide ($5x10^{-5}$ M)	-322	133

[1]No significant difference between means within a column at P=0.05.

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Table 50. Effect of malonic acid and cycloheximide individually and in combination, on naphthaleneacetic acid uptake by leaf strips.[1]

Treatment	NAA accumulated[2]
	(pmoles/10 mg dry wt)
Non-treated	828b
Malonic acid ($5x10^{-3}$ M)	1273a
Cycloheximide ($5x10^{-5}$ M)	357d
Malonic acid ($5x10^{-3}$ M) + Cycloheximide ($5x10^{-5}$ M)	536c

[1]All treatments were present for the entire experimental time including a pretreatment (1 hr).

[2]Means followed by different letters are significantly different at P=0.05.

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to 15 ml centrifuge tubes. After standing for 24 hr, the sample was sedimented at 2000 x g for 10 min. The supernatant, decanted into a 150 x 16 mm test tube, was condensed to 0.1 ml on a flash evaporator (Buchler Instruments, New York). The tissue residue was washed twice with 5 ml of 80% (v/v) ethanol, processed as just described and combined with the first extract.

The concentrated ethanol extract was streaked in a 3-cm band on a 5 cm x 20 cm thin-layer plate coated with silica gel G 250 μm thick. The chromatograms were developed for 15 cm (ascending) in isopropanol : ammonia : water; 8:1:1 (Solvent 1) unless otherwise noted. The thin-layer plates were scanned for radioactivity using a chromatographic scanner (Actigraph I, Nuclear, Chicago) that consisted of a 2π gas flow detector connected to a chart recorder through a linear ratemeter (Nuclear Chicago). The thin-layer plates were advanced under the detector at a rate of 12 inches per hr. The micromil window of the detector exposed to the plates was limited by a collimeter set at 3 mm.

Trace of a radiochromatogram of the extracted NAA from the leaf strip tissue is shown in Figure 28A. A comparison of authentic (Figure 28C) NAA (R_f 0.6) and that extracted from leaf tissue (R_f 0.2) were not identical (Figure 28).

Figure 28. Radiochromatogram traces of authentic and extracted naphthaleneacetic acid.

A. Extract of accumulated NAA chromatographed in Solvent 1 (isopropanol: ammonia: water; 8:1:1).

B. Extract of accumulated NAA chromatographed in Solvent 2 (n-butanol: glacial acetic acid: water; 5:1:2.2).

C. Authentic NAA chromatographed in Solvent 1.

D. Authentic NAA chromatographed in Solvent 2.

E. Major peak from A (Solvent 1) purified in Solvent 2 system, then hydrolyzed (1 N HCl, 60 C, 1 hr) and rechromatographed in Solvent 1.

F. Major peak from E hydrolyzed (2 N HCl, 100 c, 4hr) and rechromatographed in Solvent 1.

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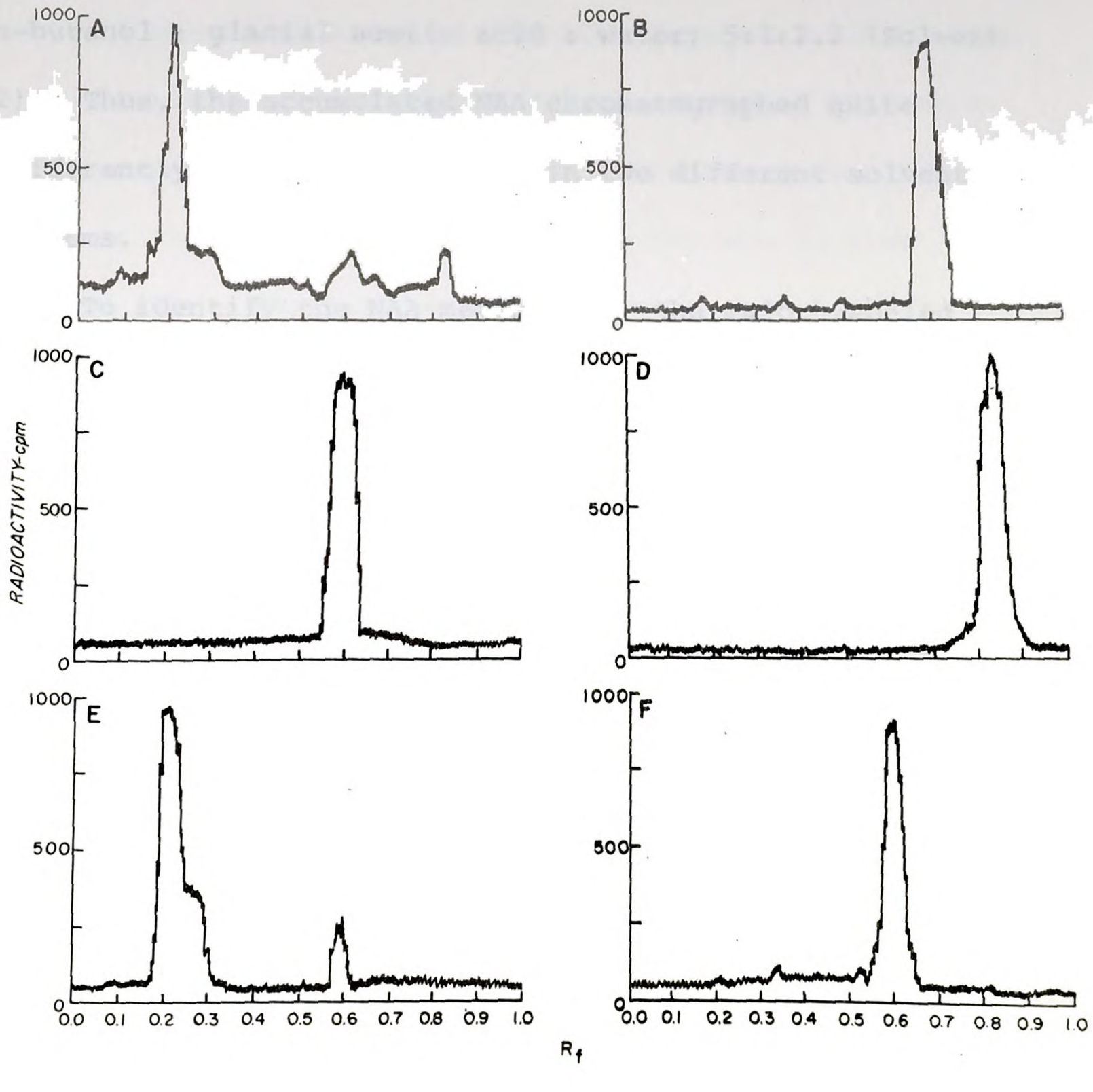

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This fact is further verified by a comparison of chromatograms of tissue extract and authentic NAA run in n-butanol : glacial acetic acid : water; 5:1:2.2 (Solvent 2). Thus, the accumulated NAA chromatographed quite differently from authentic NAA in two different solvent systems.

To identify the NAA metabolite, the major labeled peak (Figure 28A) was eluted in 80% ethanol and rechromatographed in Solvent 2. The major peak in Solvent 2 (R_f 0.7) was identical to that shown in Figure 28B. This peak was eluted again in 80% ethanol and concentrated to 1.0 ml and hydrolyzed for 1 hr at 60 C in 1 N HCl. The HCl-ethanol solution was evaporated until all traces of acid fumes were removed. The residue was resuspended in 80% ethanol and chromatographed in Solvent 1. Figure 28E shows the presence of a small peak comparable to NAA; however, it appeared that only a portion of the labeled compound was hydrolyzed. Therefore, the major peak at R_f 0.2 was again eluted, concentrated, and hydrolyzed in 2 N HCl at 100 C for 4 hr. Upon chromatography in Solvent 1, a strong peak occurred at R_f 0.6 and the original peak at R_f 0.2 was missing (Figure 28F). The new peak appeared at the same R_f as authentic NAA. Spraying the chromatogram with ninhydrin yielded a positive spot at R_f 0.05 which

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corresponded to authentic aspartic acid in a separate series of experiments. For this reason, it was believed that NAA was metabolized to NAA acyl-aspartic acid (NAAsp).

Chromatographic analysis of the remaining treatments of the experiment detailed in Table 50 showed that the malonate stimulated the formation of the NAA complex (Figure 29B). The labeled material extracted from the cycloheximide treatment was of insufficient quantity to obtain any significant chromatographic peaks so it was not illustrated in Figure 29. However, significant activity was detected in the malonate plus cycloheximide treatment and this activity corresponded in R_f to authentic NAA (Figure 29C).

Chromatographic analysis implicated the involvement of aspartic acid during NAA accumulation. A preliminary experiment revealed that pretreatment with aspartic acid ($1x10^{-3}$ M) enhanced NAA uptake. The non-treated tissue accumulated 851 pmoles NAA/10 mg dry weight while the aspartic acid-treated tissue accumulated 1161 pmoles NAA (significantly different at P=0.05).

To further examine the metabolic aspects of NAA accumulation, a detailed time-course experiment was conducted to determine if the surge in uptake noted in the earlier time-course study (Figure 24) was related to the

Figure 29. Radiochromatograph traces used to determine the form of accumulated naphthaleneacetic acid from treatments listed in Table 50.[1]

A. Extract of non-treated tissue chromatographed in solvent 1.

B. Extract of malonate ($5x10^{-3}$ M) treated tissue chromatographed in solvent 1.

C. Extract of malonate ($5x10^{-3}$ M) plus cycloheximide ($5x10^{-5}$ M) treatment chromatographed in solvent 1.

[1]Note-extract of cycloheximide treatment had no significant peaks, so trace is not illustrated.

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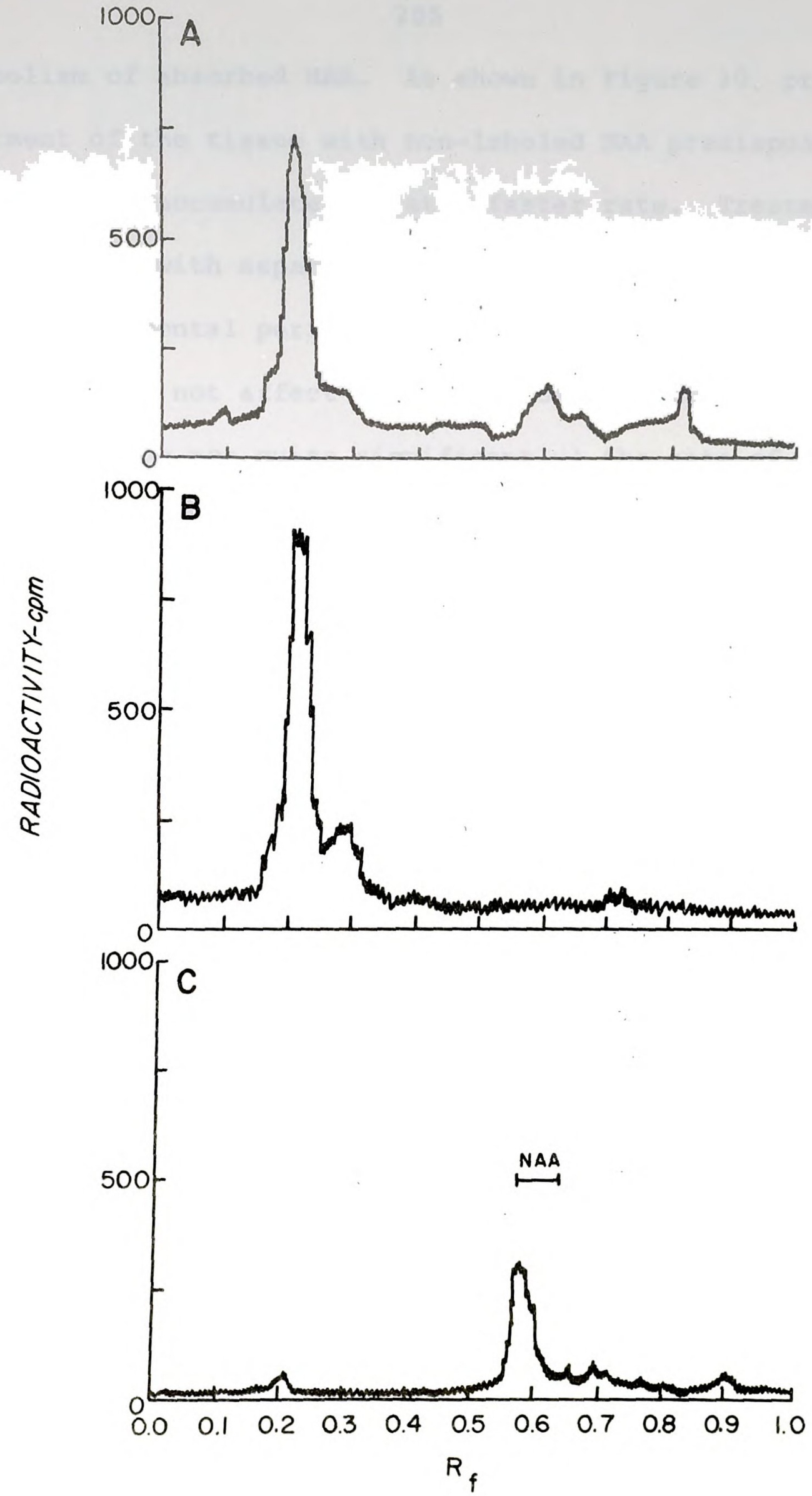


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metabolism of absorbed NAA. As shown in Figure 30, pre-treatment of the tissue with non-labeled NAA predisposed the tissue to accumulate NAA at a faster rate. Treatment of the tissue with aspartic acid ($1x10^{-3}$ M), during the entire experimental period, which included a 1-hr pre-treatment, did not affect the timing of the surge but did enhance (though not quite significantly) the rate of the uptake. Cycloheximide ($5x10^{-5}$ M) combined with or without aspartic acid ($1x10^{-3}$ M) eliminated the surge of NAA uptake.

The form of accumulated NAA after 2 hr and 6 hr was determined by eluting the NAA from planchets and analyzing by radiochromatographic procedures previously described. Pretreatment with non-labeled NAA (Figure 31B) enhanced the amount of the NAA complex running at R_f 0.2 compared to the non-pretreated tissue (Figure 31A). Cycloheximide, irrespective of the presence (Figure 31C) or absence (Figure 31E) of aspartic acid, resulted in about equal activity at both R_f 0.2 (where the complex occurred) and 0.6 (where authentic NAA occurred) for the 2-hr sample. By the 6-hr sample all the activity, though still very limited, was found at R_f 0.2. The 2-hr aspartic acid treatment had a small, but significant, amount of activity that corresponded to free NAA along with a major peak at R_f 0.2 (Figure 31D). In the 6-hr sample almost all of the activity

Figure 30. Time-course of ^{14}C-naphthaleneacetic acid ($1x10^{-6}$ M) uptake by leaf strips as affected by several treatments.

The experiment included a 1-hr pretreatment with medium only, non-labeled NAA ($1x10^{-6}$ M), aspartic acid ($1x10^{-3}$ M), cycloheximide ($5x10^{-5}$ M), and aspartic acid ($1x10^{-3}$ M) with cycloheximide ($5x10^{-5}$ M). At the end of the pretreatment time the ^{14}C-NAA was added to all flasks such that all pretreatments became the treatments for NAA uptake. The one exception was that the non-labeled NAA treatment medium was removed and replaced by ^{14}C-NAA in fresh medium. Points represent means of three experiments with two replications for each experiment. Points lying between or on two adjacent dashed lines are not significantly different at P=0.05 as determined by statistical analysis on the $\log_{10}$ transformations of all observations. The antilog values are plotted in this figure.

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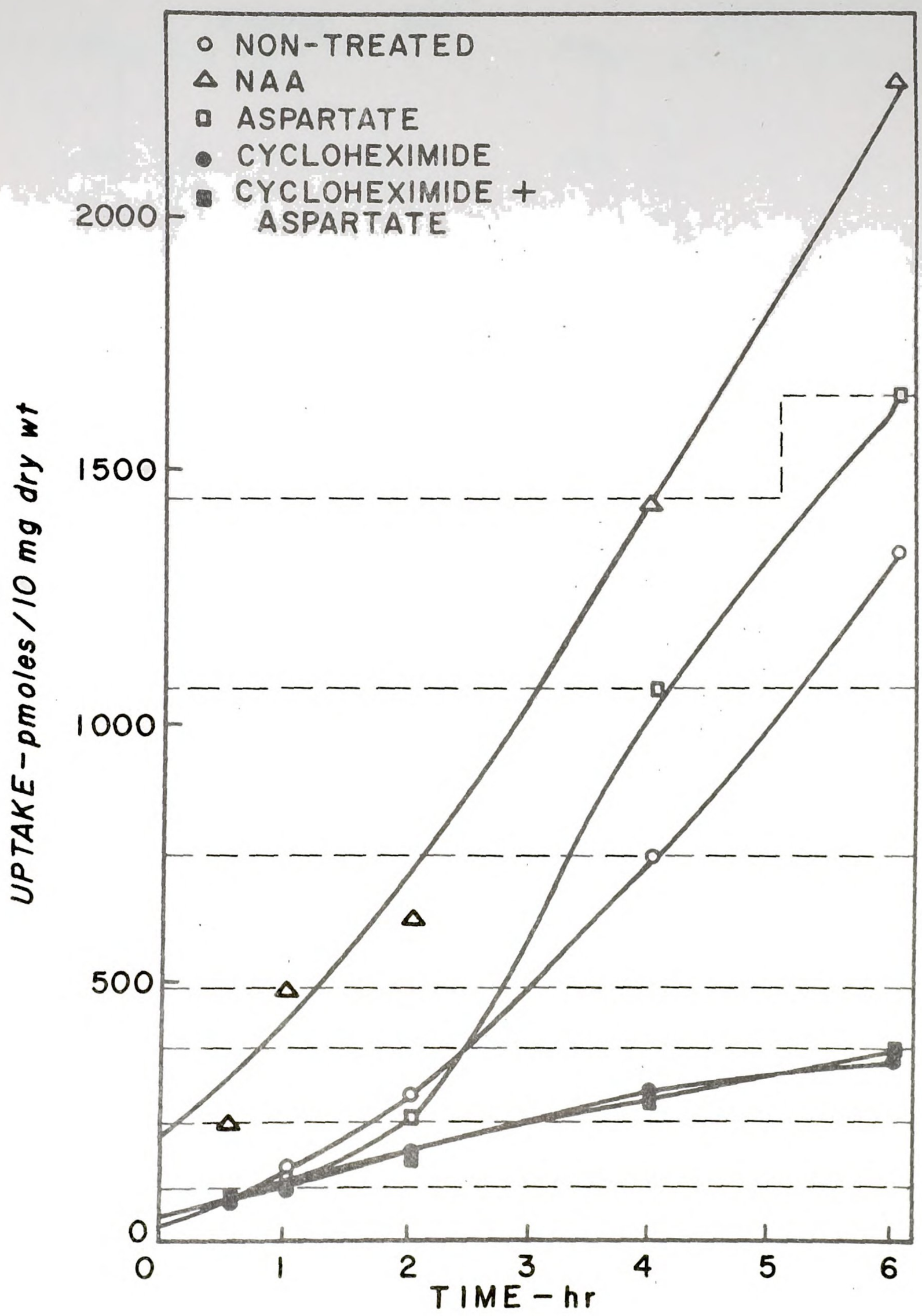

Figure 31. Radiochromatograph traces used to determine the form of accumulated naphthaleneacetic acid from treatments shown in Figure 30.

Traces represent samples from 2-hr and 6-hr measurements of all treatments chromatographed in Solvent 1.

A. Non-treated tissue.

B. Non-labeled NAA pretreated tissue.

C. Cycloheximide ($5x10^{-5}$ M) for entire experimental period.

D. Aspartic acid ($1x10^{-3}$ M) for entire experimental period.

E. Cycloheximide plus aspartic acid for entire experimental period.

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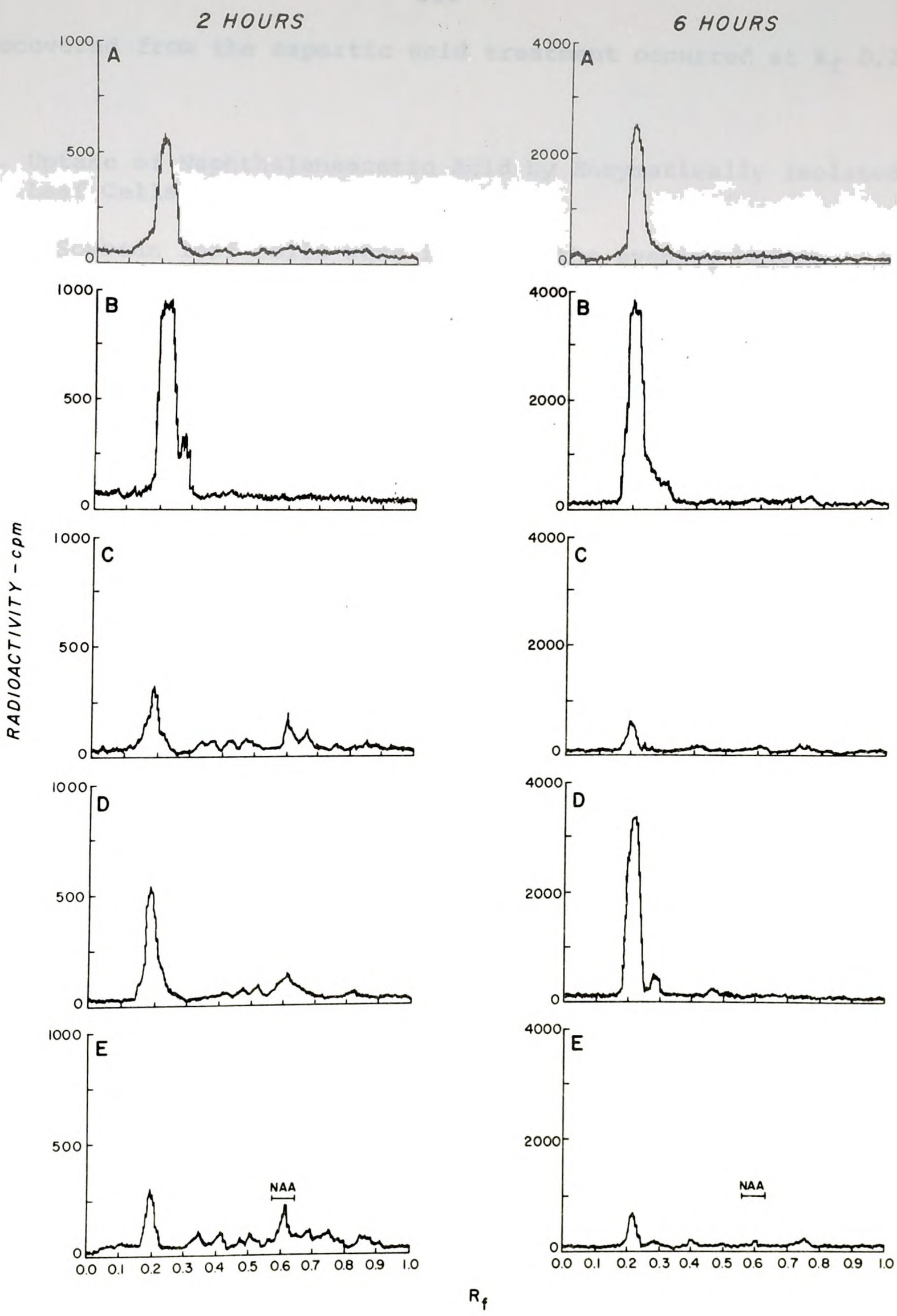

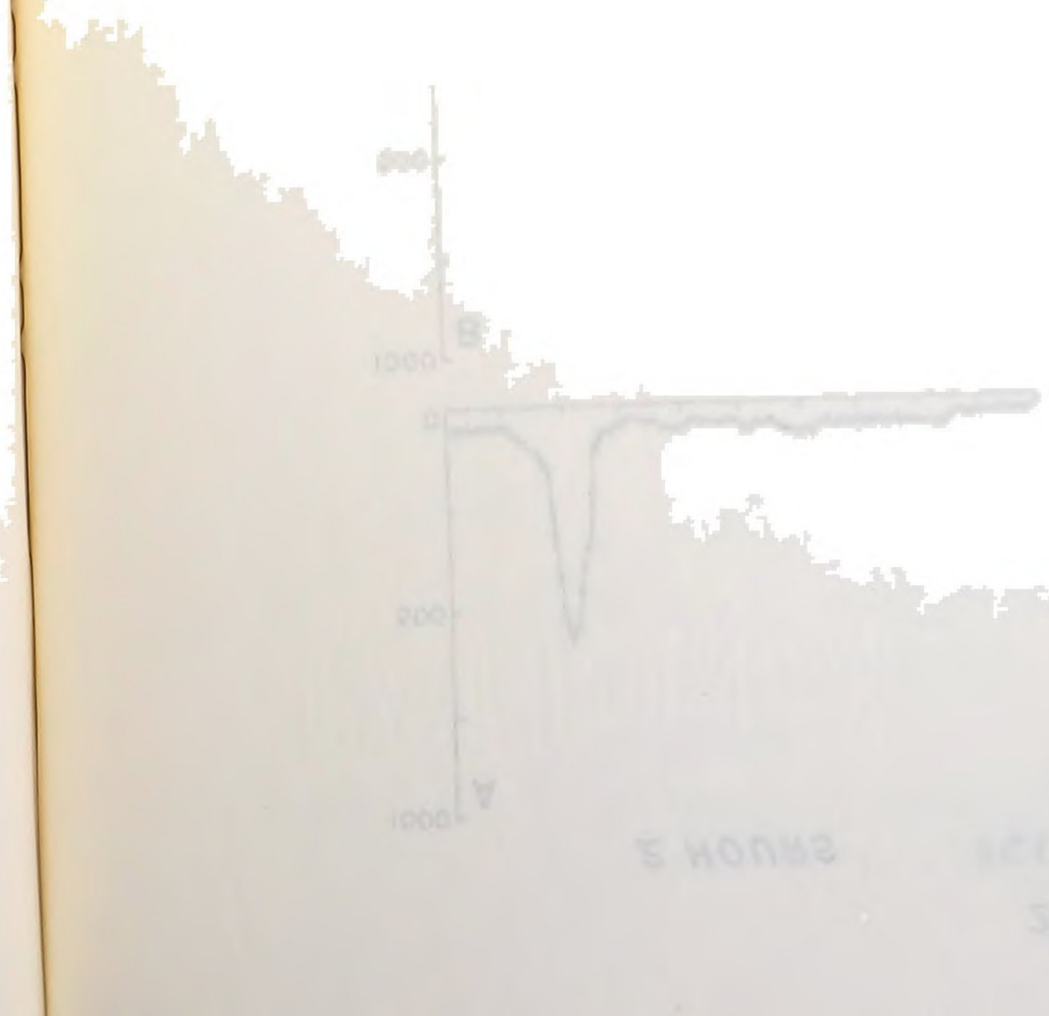
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recovered from the aspartic acid treatment occurred at R_f 0.2.

V. Uptake of Naphthaleneacetic Acid by Enzymatically Isolated Leaf Cells

Soybean leaf cells were isolated the evening before each experiment by following the procedures previously developed (Exp. III.D., III.G.). ^{14}C-NAA concentration was $1x10^{-6}$ M (S.A. 16µc/µmole) unless otherwise noted. Analysis of accumulated NAA was as described for comparison studies (Exp. III.C.).

A. General Aspects of Naphthaleneacetic Acid Uptake

1. Time-Course and Concentration Study

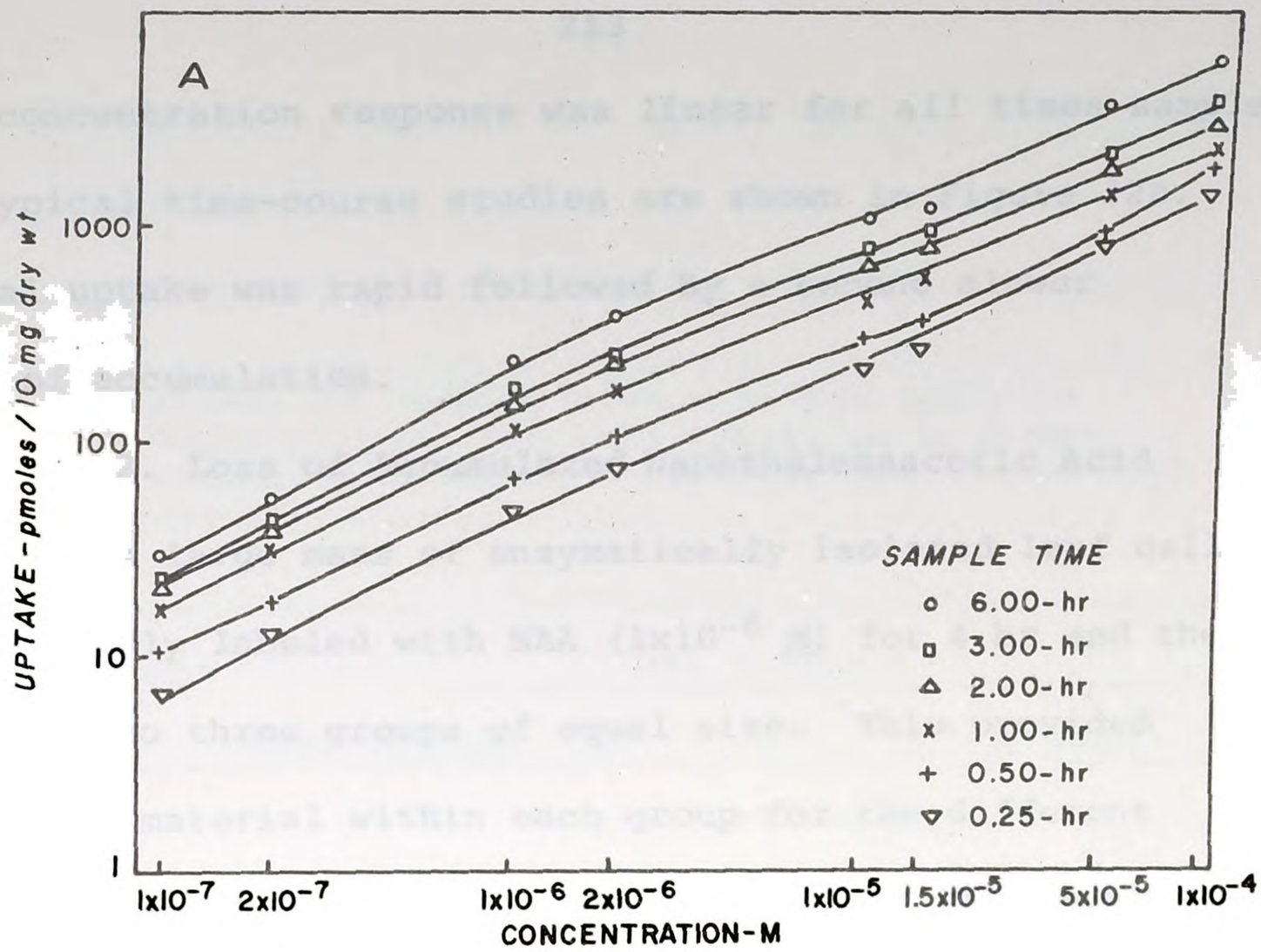
A detailed time-course and concentration study of NAA uptake was simultaneously conducted on one large preparation of enzymatically isolated cells. The standard experimental technique was modified slightly such that a final volume of 17 ml of cell suspension was treated in a 25 ml Erlenmeyer flask. This provided sufficient material to permit the removal of three 5-ml time sequence samples from each flask. As was done earlier, the experiment was staggered in sets of replications.

Examination of the uptake data (Figure 32A) showed that when expressed on a log versus log basis, there was a linear relationship between concentration and uptake.

Figure 32. The uptake of naphthaleneacetic acid by enzymatically isolated leaf cells as affected by concentration and time.

A. The uptake of NAA as affected by its own concentration. Each line represents a different time sample. The standard errors of the means are no larger than the plotted points for the respective observations. (Values and SE given in Appendix Table A-4).

B. Time-course of uptake of NAA at two NAA concentrations. Standard error of the means were plotted when larger than plotted points.



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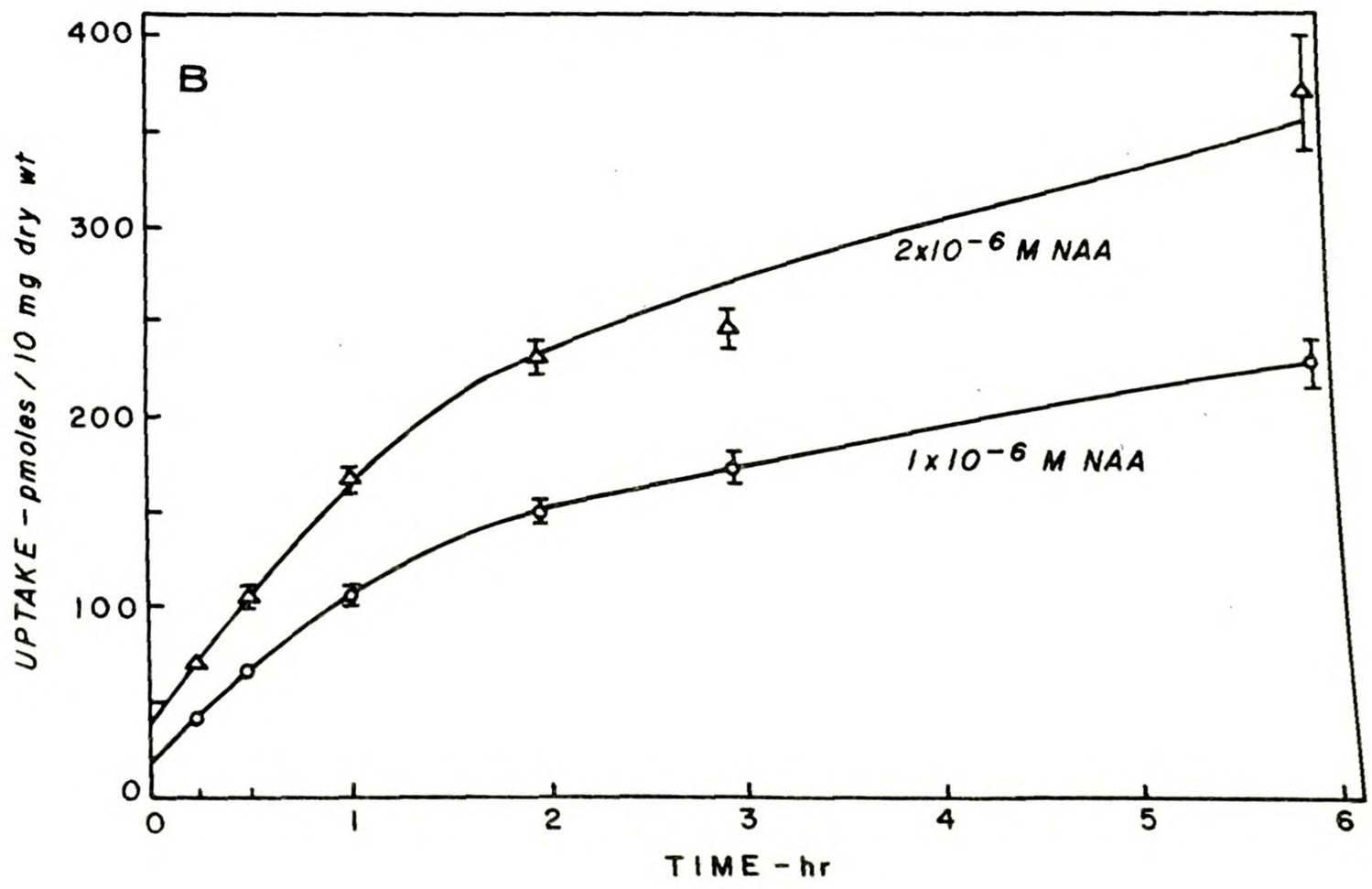



This concentration response was linear for all times sampled. Two typical time-course studies are shown in Figure 32B. Initial uptake was rapid followed by a second slower phase of accumulation.

2. Loss of Accumulated Naphthaleneacetic Acid

A large mass of enzymatically isolated leaf cells was uniformly labeled with NAA ($1x10^{-6}$ M) for 4 hr and then divided into three groups of equal size. This provided sufficient material within each group for the different wash treatments, each replicated four times. The phosphate buffer (KH_2PO_4-K_2HPO_4, 0.1 M, pH 6.7) based wash media consisted of three levels of non-labeled NAA (none, $1x10^{-5}$ M and $1x10^{-4}$ M). Three successive washes with any of these three media were required to remove a significant amount of the accumulated NAA (Table 51). Because there was no significant interaction between the wash media and the number of washes, only the overall means were compared statistically. All three wash media were effective in reducing the retained level of NAA compared to the non-washed sample by about 16%. However, only the NAA, 100x of the labeling concentration, was effective in further lowering the level of retained NAA.

Table 51. Effect of washing with selected media on naphthaleneacetic acid retention by enzymatically isolated leaf cells prelabeled with naphthaleneacetic acid.[1]

Wash treatment[2]	NAA retained				
Wash medium:		A[3]	B[4]	D[5]	mean[6]
		(pmoles/10 mg dry wt)			
No wash	448	---	---	---	448a
One 5-min wash		423	415	425	421ab
Two 5-min washes		408	427	421	419a
Three 5-min washes		395	378	341	371bc
Three 5-min washes + one 15-min wash		387	373	336	366bc
Three 5-min washes + one 30-min wash		378	363	331	357c
Three 5-min washes + one 60-min wash		369	357	318	347c
Mean[6]	448a	394b	386b	352c	

[1]NAA concn=1×10^{-6} M; for 4-hr uptake period.
[2]All washings were done at 5 C.
[3]Wash medium A=0.01 M phosphate buffer (KH_2PO_4-K_2HPO_4) pH 6.7.
[4]Wash medium B=wash medium A+1×10^{-5} M non-labeled NAA.
[5]Wash medium C=wash medium A+1×10^{-4} M non-labeled NAA.
[6]Means within a row or column followed by different letters are significantly different at P=0.05. Interaction of wash time and wash media was not significant.

B. Metabolic Aspects of Naphthaleneacetic Acid Uptake

1. Effect of Light

The effect of light on NAA uptake was established using the same experimental conditions as used for the leaf strip study (Exp. IV.B.). Several intermediate light intensities were also examined along with the effect of light on tissue held at low temperatures. Light at all intensities studied at 25 C significantly reduced NAA uptake (Figure 33). At the low temperature, light had no effect, though uptake in either light or dark was dramatically reduced.

2. Effect of Temperature

An identical experimental design as used for studying the effect of temperature on NAA uptake by leaf strips (Exp. IV. B.1.) was utilized for enzymatically isolated cells. Temperature affected NAA accumulation only by viable cells. This temperature effect can be expressed as a Q_{10} value of 2.4 to 2.5 (Table 53). Lowering the pH of the medium to 3.6 for the dead control tissue enhanced sorption over three-fold, compared to dead cells at pH 6.7 (Table 52). The Q_{10} values for the sorption into the dead cells ranged from 1.1 to 1.4 (Table 53).

3. Metabolism of Naphthaleneacetic Acid

Analysis of the involvement of NAA metabolism as part of the accumulation process was evaluated using the same techniques applied to leaf strips (Exp. III.C.).

Figure 33. Time-course of naphthaleneacetic acid uptake by enzymatically isolated leaf cells as affected by light, dark, and temperature.

The effect of light compared to dark significantly (P=0.05) reduced uptake at 25 C but not at 5 C.

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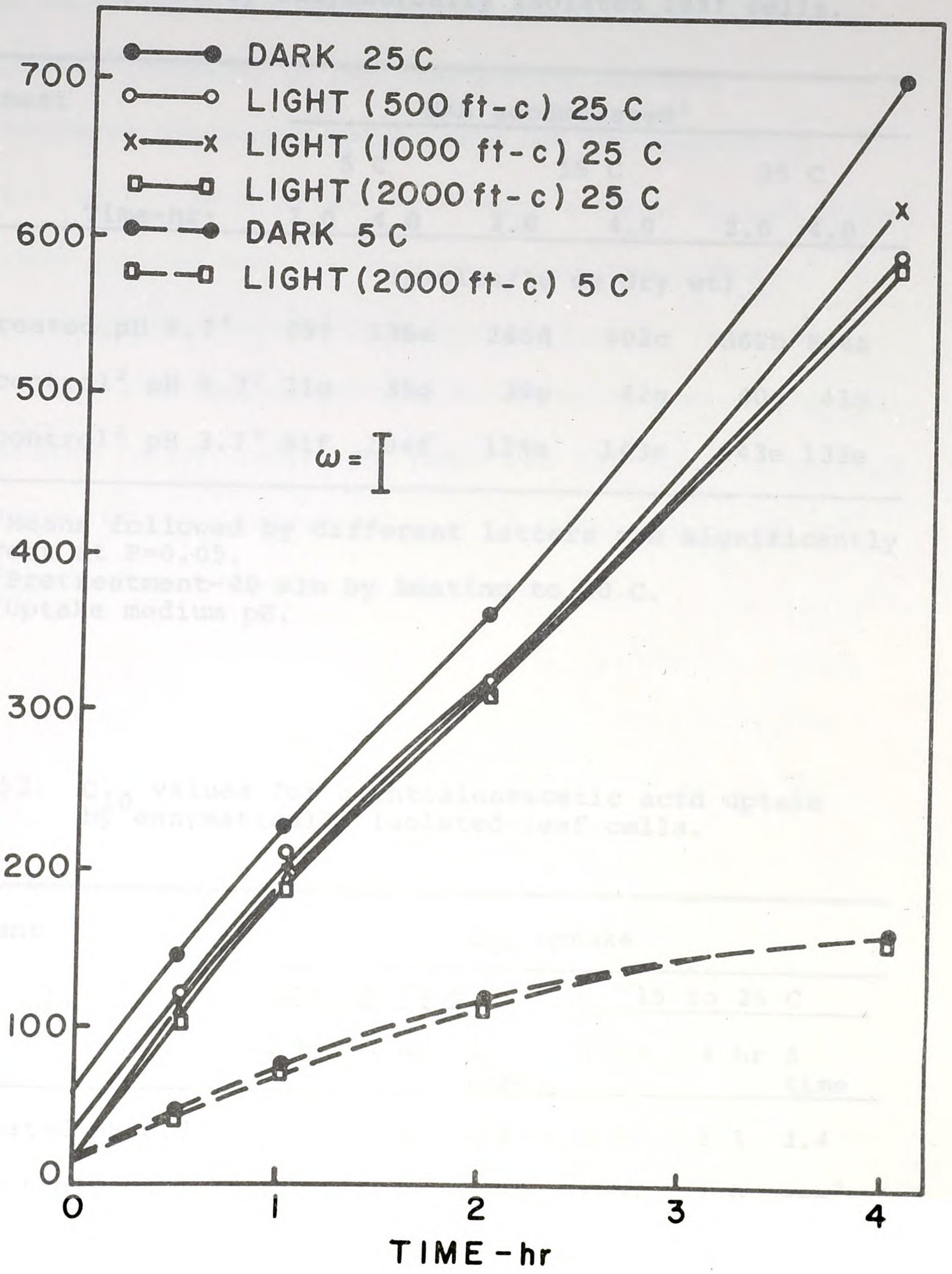



Table 52. Effect of temperature on naphthaleneacetic acid uptake by enzymatically isolated leaf cells.

Treatment	NAA accumulated[1]					
	5 C		15 C		25 C	
Time-hr:	2.0	4.0	2.0	4.0	2.0	4.0
	(pmoles/10 mg dry wt)					
Non-treated pH 6.7[3]	89f	136e	286d	402c	562b	844a
Dead-control[2] pH 6.7[3]	31g	35g	39g	42g	40g	41g
Dead-control[2] pH 3.7[3]	91f	104f	129e	143e	143e	132e

[1]Means followed by different letters are significantly different at P=0.05.
[2]Pretreatment-40 min by heating to 60 C.
[3]Uptake medium pH.

Table 53. Q_{10} values for naphthaleneacetic acid uptake by enzymatically isolated leaf cells.

Treatment	Q_{10} uptake					
	5 C to 15 C			15 to 25 C		
	2 hr	4 hr	Δ time	2 hr	4 hr	Δ time
Non-treated pH 6.7[2]	3.2	3.0	2.5	2.0	2.1	2.4
Dead-control[1] pH 6.7[2]	1.2	1.2	---[3]	1.0	1.0	---[3]
Dead-control[1] pH 3.7[2]	1.4	1.3	---[3]	1.1	1.0	---[3]

[1]Pretreatment-40 min by heating to 60 C.
[2]Uptake medium pH.
[3]No statistical change in time.

Pretreatment with non-labeled NAA had no significant effect (Table 54). Aspartic acid ($1x10^{-3}$ M) slightly increased NAA uptake, compared to the non-treated control. Cycloheximide depressed uptake only in the absence of aspartic acid. None of the treatment effects varied during the times sampled and no surge in uptake was found. Chromatographic analysis of extracts of cells from treatments after 2 hr indicated that essentially all of the NAA accumulated by the cells was complexed (R_f 0.2). A typical radiochromatograph trace of the 2-hr sample (pool of two replications) is illustrated in Figure 34.

Table 54. Time-course of naphthaleneacetic acid uptake by enzymatically isolated leaf cells as affected by several treatments.

Treatment	NAA accumulated						
Time-hr:	0.5	1.0	2.0	3.0	4.0	6.0	Mean[1]
	(pmoles/10 mg dry wt)						
Non-treated[2]	187	369	483	689	879	905	585bc
NAA[3]	188	370	491	750	905	929	605bc
Cycloheximide[4]	182	356	417	732	872	849	568c
Aspartic acid[5]	209	420	461	843	980	1005	653a
Aspartic acid[5]+ cycloheximide[4]	184	411	462	749	953	926	614ab

[1]Means followed by different letters are significantly different at P=0.05. Interaction of time and treatment was not significant.

[2]Medium only.

[3]Non-labeled NAA ($1x10^{-6}$ M) 1-hr pretreatment, removed before ^{14}C-NAA was added in fresh medium.

[4]Cycloheximide ($5x10^{-5}$ M) present for entire experimental period (included 1-hr pretreatment).

[5]Aspartic acid ($1x10^{-3}$ M) present for entire experimental period (included 1-hr pretreatment).


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Figure 34. Trace of radiochromatogram of accumulated naphthaleneacetic acid from isolated leaf cells after 2 hr of uptake.

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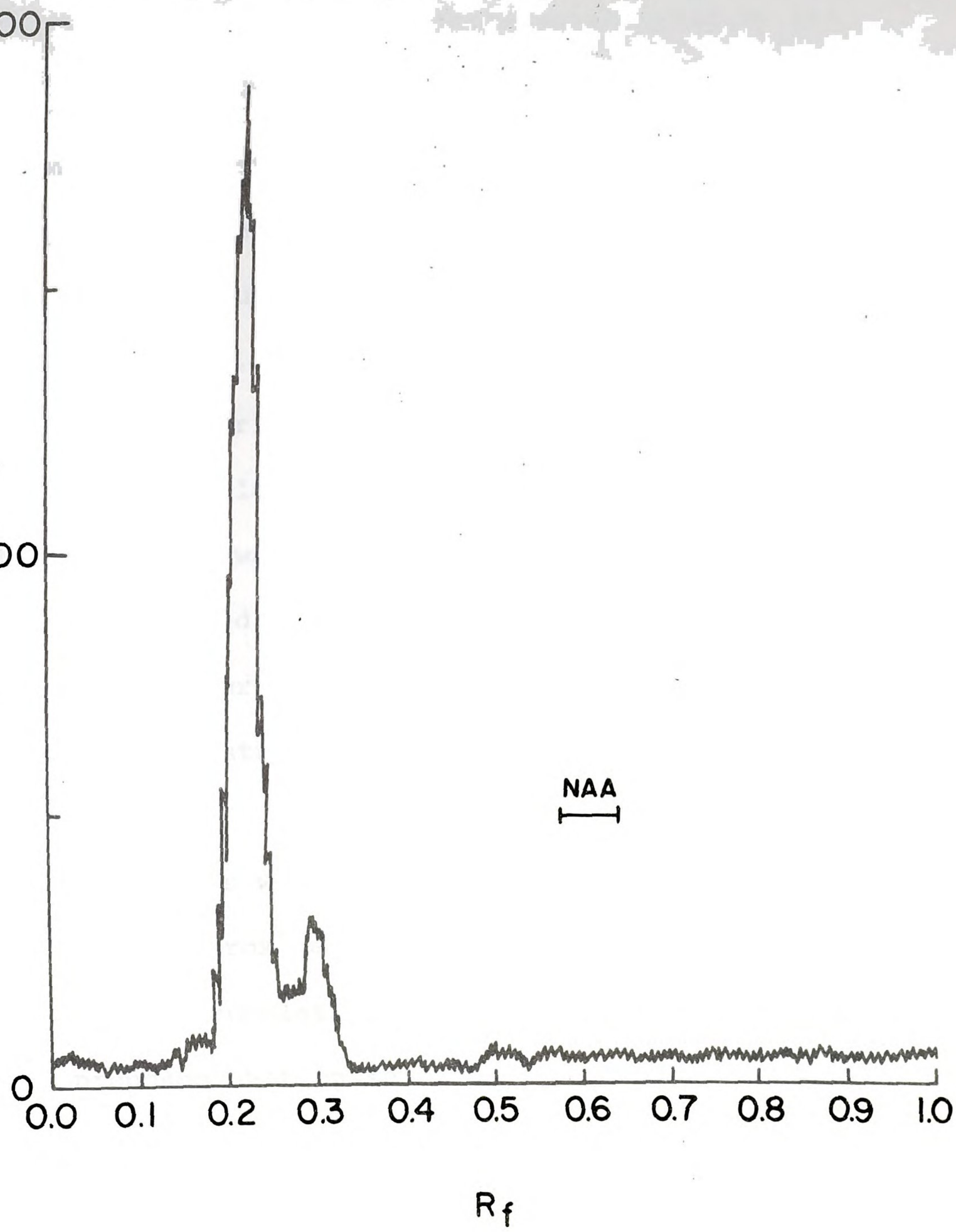

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DISCUSSION

I. Assessment of Tissue Viability

The primary objective of this thesis was to study the mechanism of NAA accumulation by leaf cells. Since previous studies (Jyung et al., 1965b; Takebe et al., 1968; Kannan and Wittwer, 1967; Price, 1967) did not consider the viability of the isolated cells in comparison to intact tissue, this aspect was first examined. Several criteria had to be established. Once acceptable criteria were established, they were used in developing techniques to obtain leaf tissue strips and isolated leaf cells of maximum viability.

The experiments with hypocotyl tissue were designed to give a first approximation of the general factors involved in NAA accumulation by soybean tissue. It was initially proposed that those factors that would maximize NAA accumulation would also be optimum for tissue viability. The results of the tissue comparison experiments did prove that high levels of NAA accumulation occurred only in viable tissue (Table 27,33). However, as this was not known when the hypocotyl experiments were conducted, only a

limited number of tests were conducted to avoid developing a system based on a possible artifact.

One of the more important facts established by the hypocotyl work was that bacterial contamination could inflate oxygen consumption values (Table 4). This meant that if several tissue preparations were compared with different levels of bacterial contamination, the interpretation of the respiration data would be very difficult. Hallaway (1968) previously reported that such bacterial contamination could easily offset true respiration rates.

The addition of chloramphenicol (D-threo-isomer) to hypocotyl segments contaminated with bacteria was done to establish if the antibiotic would reduce the effect of the bacteria on the experimental system (as suggested by Wilson, 1966; Leaver and Edelman, 1965). Chloramphenicol only at a high level (250 µg/ml) reduced the number and effect of bacteria (Table 2), but oxygen consumption was still considerably above the standard preparation (Table 4). Thus, measurements of viability based on oxygen consumption were easily influenced by bacteria and therefore were not suitable as an index in developing an experimental technique for handling isolated leaf cells. Chloramphenicol (not a bactericide [Brock, 1961]) only reduced the level of bacteria population but did not eliminate them. Since

chloramphenicol did not provide adequate control of bacterial contamination, it was not an adequate tool. The high level of chloramphenicol also enhanced NAA accumulation (Table 3), a response apparently independent of bacterial contamination. This enhancement was also observed with leaf strips (Table 47).

Bacteria removed from the system before metabolic measurements minimized the effect of contamination (Table 3). The presence of bacteria, even though not contained in the plant material being analyzed, might have had a profound effect on the system. Bacteria releasing a permease is an example (Nissen, 1968). The best practice was to use conditions as sterile as possible (Wilson, 1966) and minimize the duration of the experiments. Surface sterilization with NaHClO was detrimental to the tissue.

The establishment of a dead control to serve as a base line for metabolic comparisons proved to be an important aid for measurements of viability. The action of azide was as a metabolic inhibitor since it did not alter the high NAA binding capacity of dead hypocotyl tissue, while it markedly reduced the NAA accumulation in living tissue (Table 5). The minimal reductive capacity of the dead leaf strip tissue for tetrazolium salts demonstrated the sensitivity of the assay for living tissue.

The development of a quantitative tetrazolium assay for tissue viability played a major role in identifying the optimum treatment conditions for the leaf strips. The use of tetrazolium salts to measure reductive capacity has been described as an *in vivo* assay except for the presence of the actual tetrazolium (Pearse, 1960). The fact that most of the NTC did not penetrate deeply into the wide leaf strip tissue made its initial application ideal, since NTC was concentrated in those parts of the tissue with maximum exposure to the medium and therefore probably most affected by the treating medium. This meant that the NTC would measure mainly the responses of those cells affected by the medium, rather than assessing the entire leaf strip. The simplicity of the assay gave additional merit to the technique since numerous treatments could be conducted simultaneously to permit multiple comparisons of the treatment conditions.

The tetrazolium assay also had several deficiences that must be recognized before this procedure can be considered valid. The light sensitivity of the reduced TTC limits its use to experiments where light (Table 7) or tissue size (Table 10) (which would impose different degrees of shading) is not a variable. The poor penetration of NTC implies that factors affecting tissue permeability, such as the

different methods of tissue preparation (Tables 27 and 33), may easily lead to false interpretations of tissue viability. Future NTC viability assays, when used in conjunction with other viability factors, might best be approached by first preloading the tissue with NTC and then exposing the NTC-treated tissue to the various treatments. The toxic effect of the tetrazolium salts, due to their potent uncoupling activity (Palmer and Kalina, 1966, 1968; Clark et al., 1965) can be detrimental if viability studies are prolonged. Extended experiments would likely result in an underestimation of cell viability. This effect was apparent in the concentration experiments (Table 9, Figure 9).

One of the more sensitive assays for viability, adapted in this study, was the polarographic electrode analysis of oxygen exchange rates. The ability to rapidly measure light driven net oxygen evolution and respiratory oxygen consumption, almost simultaneously on a single small sample of tissue, was advantageous. The photo-synthetic dependent oxygen evolution appeared to be the most reliable indicator of overall tissue viability. As demonstrated in the multiple comparison experiments on the several leaf tissue preparations, those tissue preparations that exhibited high oxygen evolution rates were most effecient in metabolizing acetate (Table 37), accumulating PO_4^{3-}

(Tables 36, 39) NAA (Table 33), and fixing $^{14}CO_2$ (Tables 31, 32). These last several assays were used to establish a broad range of metabolic functions. (The initial lag observed in the acetate catabolism was typical of such experiments [Doyle and Wang, 1960]).

II. Development of Techniques to Handle Soybean Leaf Tissue

Preliminary experiments established that cells isolated following several suggested procedures (Jyung et al., 1965b; Takebe et al., 1968; Price, 1967) were of low viability. It was impossible to determine what aspects of the procedure led to a loss in viability because of the complexity of the isolation technique. An unsuitable treatment medium, severe mechanical damage to the cells during isolation or cellular disconnection from the simplast system of the leaf may be critical factors.

As Hallaway (1965, 1968) reviewed, the correct type of treatment medium was critical in order to sustain active metabolic activity of isolated organelles. Hongladrom (1964) and Honda et al. (1966) noted that a very special medium was necessary to retain cytoplasmic streaming when handling even intact micro-leaf strips.

The pH and choice of a buffer system had the most dramatic effect on tissue viability as indexed with NTC

(Figures 11, 12, 13; Table 14). The polarographic oxygen respiration analysis revealed the same effect on mechanically isolated leaf cells (Table 26). These data substantiate the findings of Romani et al. (1969) that pH must be regulated within a very narrow range. The pH optimum of 6.7 for soybean tissue was the same as for pear fruit mitochondria (Romani et al., 1969). Kim and Bidwell (1968) reported a similar optimum for incorporation of ^{14}C-glucose into root tissue.

The various buffers studied (Table 14) did not exhibit toxic effects below 0.05 M. The use of this higher level was done to simulate a long term exposure at a lower level. Under these conditions the Tris-maleate buffer was most toxic. Such results were consistent with the recent report that showed Tris buffer to inhibit the Hill reaction (Yamashita and Butler, 1969). Another disadvantage of the Tris buffer was that the pK shifted 2-3 times faster with changes in temperature than did most of the other buffers (Good et al., 1966). Thus, if a treatment medium was adjusted to pH 7.4 at room temperature (25 C) and then was used to isolate cells at 4 C, the pH would have shifted to pH 8.0. This higher pH would have been less desirable and was probably one reason why cells isolated in the

preliminary studies were of low viability. The cold wash treatment of leaf strips (Table 10) documented this detrimental effect of low temperature where the pH had likely shifted to pH 8.0.

HEPES buffer was particularly non-toxic and is in agreement with the work of Good et al. (1966). The MES buffer was equally effective (Figure 13, Table 26).

Contrary to several reports (for reviews cf. Hallaway, 1965, 1968), osmotic pressure only slightly affected leaf strip viability. The results of experiments on NAA uptake by hypocotyl sections (Table 8) demonstrated a negative relationship between mannitol concentration and NAA accumulation. This might be explained by the fact that it is more difficult for tissue under hypertonic stress to accumulate material. The effect of mannitol concentration on NTC reduction (Table 18) showed only a general trend toward a 0.25 M optimum, but a broad range of 0.20 to 0.40 M was found to be acceptable for almost the same response.

The hypothesis of the benefit of handling the tissue under hypertonic conditions, when the leaf tissue was being exposed to detrimental materials (Takebe et al., 1968), was found to be unsuitable in that the high mannitol concentrations required (0.45 M and above) damaged the leaf strip tissue (Table 12). This was one of the better experiments

with tetrazolium because the staining was carried out independent of the treatment conditions. These data also revealed that incubating the tissue under hypotonic conditions resulted in tissue injury.

To avoid starving the excised tissue preparations, the value of added substrate was established. Sodium succinate was an effective substrate for hypocotyl sections (Table 7), while sucrose, on the other hand, actually depressed the accumulation of NAA. It is possible that the use of sucrose in the preliminary studies contributed to the observed low viability. The optimum succinate concentration (0.008 M, Figure 6) for leaf strip tissue was found to be much lower than that used for hypocotyl tissue (0.02 M). Interestingly the succinate was only stimulatory in light (Figure 6), and a similar response with intact leaf tissue was observed by Dyar (1953). Sucrose has been reported to enhance leaf disk growth only in the light (Dale, 1966).

The high molecular weight polymers Ficoll and Dextran-40 along with BSA as stabilizers have been found useful in some excised tissue media (Hongladrom, 1964; Honda et al., 1966). The NTC reduction assay (Table 17) revealed that only the BSA (50 µg/ml) had any beneficial effect on viability of soybean tissue while Dextran-40 (Table 15) was

without effect and the Ficoll depressed tissue activity. There was no effect by either Ficoll or Dextran on oxygen exchange rates (Table 25) and thus they were not utilized. If Ficoll is used, one should first dialyze it to remove a toxic component. These polymers might be utilized as a practical aid in cell separations (Figure 16). The beneficial effect of BSA was consistent with results of Jones et al. (1964) and Sarkissian and Srivastava (1968).

The addition of stabilizers of phenolic compounds also proved helpful. Little difference was noted between PVP and metabisulfite (Table 16). The inhibitory effect of metabisulfite above 1.0 mM was surprising, since Stokes et al. (1968) reported that this concentration was the minimum necessary to recover active potato tuber mitochondria. The optimum PVP concentration was also about 5 to 10 fold less than that used by Hulme et al. (1964a, 1964b) for apple fruit and rose flower mitochondria.

The use of Ca^{2+} in the treatment medium was intentionally avoided, as the role that Ca^{2+} plays in membrane permeability is as yet uncertain (Leggett, 1968; Handler et al., 1965; van Stevenick, 1965). The addition of plant hormones was also intentionally avoided so as not to confound ensuing auxin uptake studies.

The development of a treatment medium appropriate for

soybean leaf tissue suggested that further failure to isolate viable leaf cells could then be attributed to either the effect of mechanical shock during isolation or to the loss of the symplastic system (Arisz, 1963). Cells isolated by the enzymatic procedure were consistently more active than those isolated mechanically (Table 29 to 39). Apparently the roller mill was too harsh on the leaf tissue. However, the roller mill has been shown as the gentlest method for tissue homogenization (Hulme et al., 1964a). Jagendorf and Wildman (1954) noted that damaged tobacco leaf nuclei resulted from harsh mechanical separation.

The enzymatic cell separation, though slower and contaminated with damaging enzymes, was sufficiently mild to permit the release of relatively active leaf cells. Pectinase enzymes have aided in isolation of plant cell nuclei by making the isolation procedure milder (D'Alessio and Trim, 1968). Even though the enzymatically isolated cells were far superior to the mechanically isolated cells, they were still much less active than a comparable cell population in leaf strips. A visual analysis of the cells from a typical isolation revealed that the protoplasm was coagulated in almost half of them. Thus, approximately half of the enzymatically isolated cells were living and

the total cell population exhibited about one-half the metabolic activity of the leaf strips. It is tempting to conclude that viability of isolated leaf cells was dependent upon mild handling of the cells in an appropriate treatment medium and that the symplastic system was unnecessary for short term metabolic studies.

Though the isolated cells were not as active as intact leaf strip tissue, they were capable of many metabolic functions associated with living cells and absent in preparations previously reported. The transformation of photosynthetically fixed $^{14}CO_2$ into protein (Table 31) indicated a considerable improvement over the data of Racusen and Aronoff (1953) where there was no incorporation into protein.

The relatively high respiration rates approached those of intact leaf tissue compared to the very low rates of oxygen consumption reported by Jyung et al. (1965b), Gabbott and Larman (1968), and Jacoby and Dagan (1967). The consistently higher photosynthetic oxygen production rates of the enzymatically isolated leaf cells, compared to leaf strips, were probably due to a more even distribution of the tissue mass in the treatment medium. This would have permitted the isolated cells to be exposed to more light. The addition of Cl-CCP, an uncoupler of both

photosynthetic and oxidative phosphorylation (Heytler, 1963), eliminated the light effect on oxygen production and enhanced the dark respiration rate. Therefore one can conclude that respiration was coupled in the leaf strips and isolated cells.

III. Naphthaleneacetic Acid Uptake

A. Hypocotyl Tissue

Uptake studies using hypocotyl sections were considered preliminary to the subsequent leaf strip and isolated cell experiments. Results obtained with hypocotyl tissue indicated that tissue size was a critical factor. Unlike the reports of Reinhold (1954) and Jenner et al. (1968b), maximum uptake occurred with the 4-mm rather than 2-mm sections, the smallest prepared. It is likely that the difference between the 4-mm and the 2-mm sections was due to a wound response in the 2-mm sections since uptake in the presence of NaN_3 was uniform for all tissue sizes.

Another important point was the relatively minor effect of bacteria on NAA uptake, providing bacteria were removed from the tissue before radioassay. MacDonald (1967) found that bacterial contamination reduced ion uptake into beet tissue. Possibly, the bacteria had only a limited capacity to accumulate NAA and therefore their effect was

minimal. Andreae and van Ysselstein (1960b) reported that bacteria did not reduce IAA uptake in pea tissue, but did reduce the level of IAA in the medium.

The frozen-thawed treatments (Table 5, Figure 4) revealed that the tissue membranes were rate limiting for NAA uptake into tissue treated with NaN_3. The freeze-thawing process likely destroyed membrane integrity permitting rapid initial entry of NAA, but the total sorptive capacity was not changed.

The addition of either succinate or acetate significantly enhanced NAA uptake. These data, along with inhibition with NaN_3 and frozen-thawed tissue, all indicated that tissue metabolism was at least partially involved in the accumulation of NAA.

B. Leaf Strip Tissue

Since the isolated cell preparations demonstrated considerably less metabolic activity than leaf strip tissue, the uptake data for the two preparations will be discussed separately.

The last several experiments described for the leaf strip tissue revealed the metabolic formation of an NAA conjugate. The synthesis of a complex is likely the major factor controlling the accumulation of exogenous NAA. Thin-layer chromatographic separations of the extracted compound

established a major peak at R_f's (0.2 Solvent 1, 0.6 Solvent 2) corresponding to NAAsp (Zenk, 1962; Veen, 1966, 1967; Sudi, 1964). Venis and Stoessl (1969) have recently reported on the recharacterization of a compound that, chromatographed like benzoylaspartic acid, was actually benzoylmalic acid. They did not find any such new compound for NAA. In this thesis study, the enhancement of the uptake rate by feeding aspartic acid to the tissue (Figure 30) along with the results of the hydrolysis of the complex strongly suggest that the complex was NAAsp. The slight shoulder occurring at R_f 0.28 might be a second compound, but no attempt was made to identify it.

There are many similarities between NAAsp and IAAsp in their formation and characterization (Zenk, 1962; Andreae, 1967; Sudi, 1964, 1966; Veen, 1966, 1967). The metabolic conjugation involving other auxins is not as well defined. 2,4-D has been shown to induce the formation of NAAsp and IAAsp (Zenk, 1962; Sudi, 1966), but the actual aspartic acid conjugate of 2,4-D has not been isolated. Several of the phenoxyacetic acid auxins, including 2,4-D, have been isolated (Thomas et al., 1964) as a complex with glucose (e.g. 1-O-2,4-dichlorophenoxyacetyl glucose). A glucose conjugate has also been recovered for NAA and IAA (Klambt, 1961; Zenk, 1962, 1963; Veen, 1966, 1967). The

amount of the auxins recovered conjugated with glucose was usually small compared to that of acyl-aspartate. For 2,4-D metabolism, the amount recovered bound is very small (Andreae, 1967). This implies that not all data from 2,4-D uptake should be directly compared to NAA and IAA data.

Luckwill and Lloyd-Jones (1962) showed that NAA was metabolized by apple leaf tissue into a water soluble neutral compound and an acidic one. This thesis reports for the first time the significance of a NAAsp-like compound as a major metabolite of NAA in leaves. Zenk (1963) found IAAsp to be formed in leaf tissue, but most of the reports on the formation of the auxin conjugates are based on stem and root tissue (Zenk, 1962; Sudi, 1964, 1966; Andreae, 1967; Andreae and Good, 1955, 1957; Andreae and van Ysselstein, 1960a, 1960b; Veen, 1966, 1967; Veen and Jacobs, 1969).

The surge in uptake noted in the time-course study (Figure 24) and later confirmed in the inhibitor study (Figures 30, 31) can be explained by the induction or activation of the enzyme forming the aspartic acid-NAA conjugate. Zenk (1962) observed a similar surge with pea shoot tissue in uptake after a 4-hr lag and attributed the lag to induction of an enzyme he termed L-aspartic

acid-N-acylase. Venis (1964) found that actinomycin-D or puromycin treatments nullified the formation of a similar conjugate, benzoyl aspartic acid. He concluded that the auxin induced a true de novo synthesis of the enzyme system (N-acylaspartate synthetase) involved in the formation of the complex.

In this study the cycloheximide inhibition of the surge (Figure 30) suggests that protein synthesis may be involved in formation of the complex. Since addition of aspartic acid to the cycloheximide treatment did not release the inhibition, while aspartic acid added alone was stimulatory, it can be concluded that protein synthesis was required for conjugation of the two components (NAA and aspartic acid) and not for synthesis of a special pool of aspartic acid. Pretreatment with non-labeled NAA showed that it was possible to precondition the tissue to respond to the labeled NAA sooner and thus eliminate the surge (Zenk, 1962, 1964; Sudi, 1964). Since some conjugated NAA was recovered from the cycloheximide treated tissue, it is impossible to conclude if de novo synthesis or activation of the enzyme occurred.

The surge in uptake might also be explained as a direct effect of auxin on membrane permeability. IAA has been found to induce beet root tissue to lose K^+ (van Stevenick,

1965), while 2,4-D enhanced penetrability of sucrose into Chlorella (Wedding et al., 1959). The presence of non-labeled NAA on the receiver-side of an isolated cuticle membrane has been shown to enhance the movement of labeled NAA through the cuticle into the receiver solution (Norris and Bukovac, 1969). Although such a phenomenon is possible, it is unlikely to be a major factor in the experiments with leaf tissue since cycloheximide treatments that reduced conjugation also removed the surge. If the auxin acted directly on the membrane, then the surge should have been independent of the cycloheximide inhibition.

The increased rate of NAA accumulation at $5x10^{-7}$ M compared to lower concentrations (Figure 25, Table 41) indicated a possible concentration dependency for the induction of conjugate formation. Examination of the data (Figures 24, 25, 27, 30) revealed that the surge usually occurred after about 100 pmoles/10 mg dry weight was accumulated. Sudi (1966) and Veen (1967) each reported a distinct optimum concentration for formation of the complex. As found in this study, Veen (1967) observed a time and concentration interaction that showed high (10^{-4} M) NAA concentrations induce the formation of the complex sooner than did the lower (10^{-5} M) concentrations. It is likely that a critical internal concentration must first be attained

before induction takes place.

Light has been shown to enhance auxin uptake in several studies (Greene, 1969; Thimann and Wardlaw, 1963; Sargent and Blackman, 1965, 1969). In contrast to reports (Sargent and Blackman, 1965, 1969) of light dependency for the surge in auxin uptake (2,4-D), light actually depressed NAA uptake by leaf strips (Figure 27). CMU, a photosynthesis inhibitor (Good, 1961), effectively blocked oxygen production in the light (Table 48), but did not affect NAA uptake in the light (Figure 27). Since substrate (succinate) was present during the experiment, the lack of an additive light effect indicated that direct coupling of photosynthetic energy (as Rains [1968] reported for K^+ accumulation) was not involved in NAA uptake. Assuming that aspartic acid is one of the key metabolites necessary for high levels of NAA uptake, its independence on light for synthesis (Ashton et al., 1961; Zweig and Ashton, 1962; Walker, 1962) might be another reason for the lack of a positive light effect. Fang et al. (1959) have shown that the formation of IAAsp is independent of light.

The negative effect of light on uptake might also be related to light enhancement of RNase (Bagi and Farkas, 1967) which may be activated by the cutting of leaf strips. They reported that high light intensities, like those used

(Figure 27), would affect the RNase system much more than lower intensity light (800 ft-c) used in other experiments where light did not have a depressive effect (Table 47).

An uncoupler of oxidative and photosynthetic electron transport, m-Cl-CCP, inhibited NAA accumulation (Table 47) equal to the level recovered in the dead controls (Table 44). The level (35 pmoles/10 mg dry weight) found in the dead controls would be slightly less than the external NAA concentration (see Appendix Figure A1 for sample calculations). Thus, there was a distinct dependency on metabolic energy to drive the accumulation mechanism. High levels of NaN_3 (10^{-4} M) only partially inhibited NAA uptake. The 33% inhibition was similar to the report of Sargent and Blackman (1969).

From these studies, it was impossible to determine if free NAA was accumulated against a concentration gradient. The chromatographic separations in all (Figures 29a, 29b, 31) but one case (Figure 29c) showed that no free NAA was extracted at levels above 40 pmoles/10 mg dry weight, which would be equal to the external concentration. In the other treatments, metabolized NAA was recovered in substantial levels even from the 2-hr sample. Thus, the question as to whether free NAA accumulated against a concentration gradient will have to be left unanswered. However, if it did accumulate against a concentration gradient, it

would have been only by a very small amount. Zenk (1962) showed that free NAA did accumulate to twice the treatment medium concentration, while Andreae and van Ysselstein (1960) found IAA to exceed the concentration gradient by 10-fold.

Malonic acid, a possible competitive inhibitor of succinic dehydrogenase (Mahler and Cordes, 1966), actually stimulated NAA uptake. This might be explained in several ways. First, since malonate did not depress respiration (Table 49), it possibly acted as a substrate (Young and Shannon, 1959) or it may have enhanced succinate dehydrogenase activity (Ojha et al., 1969). Swets and Wedding (1964) suggested that inhibition of the Krebs cycle enhanced 2,4-D uptake by Chlorella.

The stimulatory effect of high levels of chloramphenicol (above 5×10^{-4} M) observed for both the hypocotyl and leaf strip tissue is similar to the data of Sargent and Blackman (1969). One explanation might be that the chloramphenicol, which selectively acts on protein synthesis in chloroplast (Ellis, 1969), inhibited the synthesis of RNase that was activated during the cutting of the leaf strips (Bagi and Farkas, 1967).

Since Q_{10} values for NAA uptake of above 2.0 were only possible for living tissue (Table 45), it should be

assumed that the temperature dependency is another expression of the involvement of metabolic energy in the uptake process. Similar temperature effects have been reported for auxin uptake by leaf tissue (Luckwill and Lloyd-Jones, 1962; Sargent and Blackman, 1962; Greene, 1969), but they did not resolve the difference between metabolic and cuticular components. The cuticular component, likely the most limiting barrier in their system, could be contributing significantly to high Q_{10} uptake values (Norris and Bukovac, 1969).

The very limited amount of NAA lost by efflux from the tissue was likely due to most of the accumulated NAA being immobilized as NAAsp. Andreae (1967) reported that after long treatments (24 hr) with NAA, the complexed NAA could not leak out of the tissue. It has been noted generally that complexed auxins like IAAsp and NAAsp are not translocated out of the tissue in which they are metabolized (Veen, 1966; Veen and Jacobs, 1969; Hertel and Flory, 1968; Goldsmith, 1968; Eschrich, 1968).

The formation of the NAA conjugate seemed to be the major metabolic aspect of the NAA accumulation process in the leaf strip tissue. This type of mechanism might be similar to the electrostatic association uptake process that Hiatt (1968) has proposed for ion accumulation by roots.

This would mean that the NAA would enter the tissue by a passive mechanism, like diffusion, and would be irreversibly bound as the conjugate, establishing a gradient. Recalling the pronounced sensitivity of NAA uptake to m-Cl-CCP, metabolic energy was likely involved in either the formation of the conjugate or the "pumping" of the NAA to the site of electrostatic association. An ATPase system would be a possible mechanism for an active NAA pump. Low temperature had its greatest effect by negating the surge in uptake, not uptake above the NAA concentration gradient (Table 44). Thus it is likely that the conjugate forming system has a metabolic energy requirement. Further studies are needed to determine the energy requirement for enzyme synthesis or activation.

Since the pH of the medium dramatically affected tissue viability, the effect of pH on NAA uptake was not examined on living tissue. This was necessary to avoid the interaction of the two variables. Andreae (1967) reported that the conjugate formation of NAA by pea roots was reduced greatly by lowering the pH of the medium from 5.3 to 4.6. In this study the effect of pH was examined on sorption by the dead control tissue where it was found that lowering the pH to 3.7 caused a 5-fold increase in sorption compared to pH 6.7. This demonstrated the greater sorption of NAA

by the dead leaf tissue when the auxin is in an undissociated form. Poole and Thimann (1964) noted a 5-fold increase in IAA uptake going from pH 7.5 to 4.5.

The almost total recovery of the NAA in the efflux experiment confirms the many reports that NAA is not decarboxylated by plant tissue (Luckwill and Lloyd-Jones, 1962; Zenk, 1962; Veen, 1966; Andreae, 1967).

The difference in amount of NAA retained between the washed and non-washed tissue from the short-time course study was about 500 pmoles/10 mg dry weight of leaf strip tissue. The calculated volume, that a $5x10^{-6}$ M NAA solution would occupy to give an equimolar concentration (see appendix for sample of calculation, Figure A-1), was about 100 µl which was about 2.5x the water content (or volume) of the tissue. A 250% value for free space is impossible, so some special factor must be involved that did not affect similar measurements for stem sections (Johnson and Bonner, 1956; Sabnis and Audus, 1967a) where it was found that free space was equal to between 21% and 30%. The tremendous surface area of the finely cut leaf strips might be one factor. Another might be weak binding (like hydrogen bonding) that would allow the NAA to concentrate on the surface of the tissue.

C. Isolated Cells

The chromatographic analysis of the accumulated NAA from the cycloheximide treated isolated cells (Table 54) demonstrated that the conjugate-forming enzyme system was likely operating from the beginning of the experiment. The minimal response to the several treatments made this point apparent. The lack of a surge in uptake during the time-course and concentration experiment (Table 32) can also be explained by the fact that the conjugate-forming enzyme was functioning before the experiment commenced.

The results of the effect of light, temperature and pH were consistent with those of the leaf strip experiments and, therefore, need not be discussed further.

The fact that the NAA conjugating enzyme appeared to be operating at the time the NAA uptake studies had commenced does not mean that one should not consider their use. It is quite possible that the very nature of the cells being totally isolated, which facilitated a very rapid initial uptake, induced the enzyme system before the surge could be detected or effected by other treatments. It is also possible that the mechanism for auxin uptake by isolated leaf cells might be slightly different from intact tissue. It should be recognized that further improvements on the cell isolation might be required before data from the two systems will correspond. Certainly the modified technique for leaf cell isolation reported in this thesis has been a significant step toward this goal but further refinement would most likely be beneficial.

SUMMARY

Several techniques were developed to assess tissue and cell viability. Quantitative measurement of tetrazolium reduction permitted the formulation of a treating medium suitable for maximum soybean leaf tissue metabolic activity. Polarographic measurement of oxygen exchange rates in the light and dark proved to be a reliable method for assessing tissue viability.

Using these monitoring methods, an improved technique was developed to enzymatically isolate viable leaf cells and it is described. A multiple comparison, using several viability assays, revealed that the enzymatically isolated leaf cells were about 50% less active than intact leaf strips while cells mechanically isolated exhibited minimal metabolic activity.

NAA uptake by soybean hypocotyl tissue was studied to establish some of the basic experimental factors. Bacteria had no effect, while succinate enhanced NAA uptake.

NAA uptake by leaf strip tissue was characterized by the following:

1. A surge in uptake dependent upon the interaction of time and external concentration.

2. A minimum of 100 pmoles NAA/10 mg dry weight of tissue was required before the surge occurred.

3. Chromatographic separation of the compound showed that it corresponded to NAAsp.

4. Cycloheximide inhibited uptake and formation of the conjugate while aspartic acid enhanced both.

5. Light had a slight negative effect on NAA uptake and an inhibitor of photosynthesis had no effect on uptake in the light.

6. The uncoupler m-Cl-CCP blocked NAA uptake above the concentration gradient, revealing a dependency on metabolic energy for uptake.

7. Temperature coefficients above 2.0 (only for living tissue) further substantiated the involvement of a metabolic factor for NAA uptake.

8. Only limited amounts of NAA were lost by efflux from the tissue.

Extensive retention of NAA (that fraction not removed by washing with distilled water) made estimation of free space impossible. Sorption of undissociated NAA was 5-fold greater than NAA in its dissociated form.

NAA accumulation by the isolated leaf cells was also related to the formation of an NAA conjugate except that the enzyme was probably functioning at the beginning of the uptake experiments so that no metabolic control could be demonstrated.

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APPENDIX

Table A1. Effect of mannitol concentration on 2,3,5-triphenyl tetrazolium chloride reduction by leaf strips.[1]

	TTC reduced		
	Strip width (μm)		
Mannitol concn	500	1000	Mean[2]
(M)		(A-535 nm)	
0.20[3]	0.315	0.392	0.393a
0.25	0.293	0.401	0.347a
0.30	0.308	0.341	0.325a
0.35	0.292	0.354	0.343a
0.30 (frozen-thawed)[4]	----	0.095	0.095c
----0.5 m<u>M</u> $CaSO_4$[5]	0.231	----	0.231b

[1]The various media were used for entire experiment.

[2]Means followed by different letters are significantly different at P=0.05.

[3]Treatment medium=mannitol, 0.01 <u>M</u> Tris-HCl, 4 m<u>M</u> socium succinate, 10 mg/ml PVP, 50 μg/ml BSA and adjusted to pH 7.4.

[4]Pretreatment.

[5]In place of treatment medium but including 4 m<u>M</u> sodium succinate.

Table A2. Effect of dialyzed Ficoll on neo-tetrazolium reduction by leaf strips.

Treatment	NTC reduced[1]
	(A-560 nm)
Non-treated	0.305a
Ficoll (5 g/100 ml)	0.205b
Dialyzed Ficoll (5 g/100 ml)	0.276ab
Dialyzate from Ficoll (5 g/100 ml)	0.247ab
NaCl ($1x10^{-3}$ M)	0.326a
NaCl ($1x10^{-3}$ M) + dialyzed Ficoll	0.213b

[1]Means followed by different letters are significantly different at P=0.05.

Table A3. Liquid scintillation cocktail used.

Chemical	g/l
PPO[1]	4.0
POPOP[2]	0.2
Naphthalene	60.0
Absolute methanol	100 ml
Ethylene glycol	100 ml
1,4-Dioxane	made to 1000 ml

[1] 2,3-diphenyloxazole
[2] 1,4-bis-2-(5-phenyloxozolyl)-benzene

Table A4. The uptake of naphthaleneacetic acid by enzymatically isolated leaf cells as affected by concentration and time.

NAA concn	Accumulated NAA[1]					
	(min)					
Sample time:	15	30	60	120	180	360
(M)	(pmoles/10 mg dry wt)					
$1x10^{-7}$	6.5±0.3	10±0.7	16±0.5	22±0.4	24±0.9	29±1.0
$2x10^{-7}$	12±0.7	18±1.3	30±2.4	38±1.8	43±2.7	54±1.5
$1x10^{-6}$	44±2.0	66±2.0	108±4.1	148±4.9	171±8.9	229±13.0
$2x10^{-6}$	71±3.2	106±5.1	168±7.8	233±11.5	246±9.6	373±28.9
$1x10^{-5}$	200±12	295±6	428±12	605±11	733±13	1067±30
$1.5x10^{-5}$	259±13	346±16	547±18	726±26	906±38	1368±59
$5x10^{-5}$	806±72	888±37	1310±69	1746±127	2065±116	3453±284
$1x10^{-4}$	1369±54	1855±81	2212±116	2961±269	3600±201	5545±460

[1]SE given.

Figure A1. Sample calculation for final naphthaleneacetic acid internal concentration.

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1. Start with the value=100 pmoles/10 mg dry wt.

2. Convert dry wt to aqueous volume

   10 mg dry wt=50 mg fr wt

   Therefore 10 mg dry wt=40 mg H_2O

   40 mg H_2O=40 µl[1]

3. Substitute aqueous volume for dry wt

   100 pmoles/10 mg dry wt=100 pmoles/40 l

4. Simplify 100 pmoles/40 µl to 2.5 pmoles/µl

5. Finally, 2.5 pmoles/µl=2.5µ moles/l=2.5×10^{-6} M

[1]Calculating the volume occupied by the tissue
96 strips x140µ x 250µ x 25000µ
gives a calculated final volume of 42.0µl



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