GIBBERELLINS AND THE PHOTOPERIODIC CONTROL OF STEM GROWTH IN THE LONG-DAY ROSETTE PLANT SPINACH

By

James David Metzger

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

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The long-day rosette plant spinach responds to a transfer from short to long days with increased petiole growth, stem elongation, and flower formation. At least the first two responses appear to be mediated through modulation of the gibberellin status.

Analysis of highly purified extracts from spinach shoots by combined gas chromatography-mass spectrometry has demonstrated the presence of six gibberellins (GAs): GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, and GA_{29}. The two major GAs were GA_{19} and GA_{20}. These two GAs were probably responsible for most of the GA-like activity detected in spinach shoot extracts with the d-5 corn bioassay. The other four GAs found in extracts from spinach shoots occurred in much smaller amounts. Structural considerations suggest that the six GAs identified in spinach shoots are related in the following metabolic pathway: $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{17} \rightarrow GA_{20} \rightarrow GA_{29}$.

The changes in the levels of five of these GAs in relation to photoperiodic treatment were examined by combined gas

chromatography-selected ion current monitoring. Long-day treatment caused a 5-fold decline in the level of GA_{19}, while GA_{20} and GA_{29} increased almost 7-fold during the same period. In absolute terms, the level of GA_{20} increased from 0.8 µg per 100 g dry weight in short days to 5.5 µg per 100 g weight after 14 long days. The levels of GA_{17} and GA_{44} did not change significantly with long-day treatment. These results are consistent with the idea that GA_{19} is converted to GA_{20}, and that this conversion is under photoperiodic control. Since stem growth in spinach is correlated with an increase in the level of GA_{20}, one major aspect of photoperiodic control of stem growth might be the availability of GA_{20} through regulation of the conversion of GA_{19} to GA_{20}.

Analysis of spinach root extracts by either combined gas chromatography-mass spectrometry or combined gas chromatography-selected ion current monitoring showed that roots contained only four of the six GAs found in spinach shoots: GA_{53}, GA_{44}, GA_{19}, and GA_{29}. Neither GA_{17} nor GA_{20} were detected in root extracts. Both phloem and xylem exudate had patterns of GA-like activity similar to those found in shoots and roots, respectively. Since foliar application of [^{3}H]-GA_{20} resulted in transport of unmetabolized [^{3}H]-GA_{20} to the roots, part of the endogenous GA_{20} present in the phloem must have been transported to this organ. Thus, if GA_{20} is made in, or transported to the roots, it is rapidly metabolized in that organ. This is a clear indication that the regulation of GA metabolism is greatly different in

roots and shoots.

The subcellular distribution of GA_{19} and GA_{20} in relation to photoperiod was also investigated. Analysis by the d-5 corn bioassay showed that chloroplast fractions from spinach leaves contained about 15% of the total GA_{20} found in the leaf and 1% of the total leaf GA_{19}, regardless of prior photoperiodic treatment. It was concluded that photoperiod does not act through a redistribution of GAs between the chloroplasts and the cytoplasm. Comparison of two methods to extract GA-like substances from chloroplasts of spinach or wheat showed that extraction by non-ionic detergents was not more efficient than the conventional methanolic extraction technique.

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To two fine teachers, Larry B. Adams and Guy L. Steucek.

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LIST OF ABBREVIATIONS

GA	Gibberellin
MeTMS-GA	Methyl ester trimethylsilyl ether of gibberellin
C_{19}-GA	Gibberellin with 19 carbons
C_{20}-GA	Gibberellin with 20 carbons
GGPP	Geranylgeranyl pyrophosphate
CPP	Copalyl pyrophosphate
ABA	Abscisic acid
m/e	Mass to charge ratio
GLC	Gas liquid chromatography
GLC-MS	Combined gas liquid chromatography mass spectrometry
GLC-SICM	Combined gas liquid chromatography selected ion current monitoring
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
SD	Short day(s)
LD	Long day(s)
EDTA	Ethylenedinitrilotetraacetic acid

Chapter 1

General Introduction and Literature Survey

GIBBERELLINS AND THE PHOTOPERIODIC CONTROL OF STEM GROWTH IN THE LONG-DAY ROSETTE PLANT SPINACH

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1.1 INTRODUCTION

The terrestrial environment is a dynamic, multifaceted system. Plants, like all other organisms which inhabit land environments, must be able to cope with drastic changes in temperature, light parameters, and water availability. Unlike mobile animals which can migrate to survive unfavorable environmental conditions, sessile plants adjust to changing conditions by altering growth and development. In many instances, a change in some environmental factor does not act directly in altering growth and development, but instead acts through hormone mediators. There are many examples of such responses. In certain plants, the change from vegetative to reproductive growth is controlled by photoperiod. The site of perception of daylength is in the leaves, yet the site of the morphological change from the vegetative to the flowering state is in the shoot apex (Zeevaart, 1976). From physiological experiments it could be shown that an uncharacterized "floral stimulus" is transported from the leaves to the apex where it causes a drastic morphological change (Zeevaart, 1976).

When plants are placed under conditions of water stress, the guard cells in the epidermis become flaccid, and the stomates close, thereby minimizing water loss. Water stress also causes a tremendous increase in the level of the hormone abscisic acid (ABA) in the leaves. Moreover, exogenous ABA

has the same effect as water stress on stomatal aperture: rapid closure. This indicates that ABA is a hormone mediator which controls water loss by regulating the stomatal aperture (Raschke, 1975). The preceding two examples can be pictorialized in the following manner:

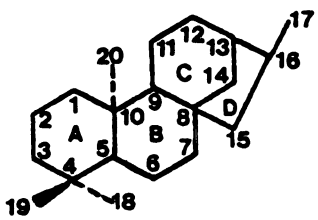
Long-day (LD) rosette plants such as Rudbeckia bicolor, spinach, and Silene armeria are plants which grow vegetatively under short day (SD) conditions and have very short stems (little or no internodes). Upon transfer to LD conditions, these plants show a bolting response, i.e. rapid stem elongation and flowering. The evidence indicates that flowering and stem elongation in rosette plants are two closely coupled, but separate developmental events (Zeevaart, 1976). Moreover, evidence presented later will show that photoperiodic control of stem growth appears to be mediated through some change in the gibberellin (GA) status. The term GA status refers to any qualitative or quantitative changes in GAs that occur, as well turnover rates, compartmentation, and tissue sensitivity to GA. Therefore, photoperiodic control of stem growth represents a useful model system in which to study how environmental factors control plant growth and development through hormone mediators.

1.2 GAs: CHEMISTRY AND ANALYTICAL TECHNIQUES

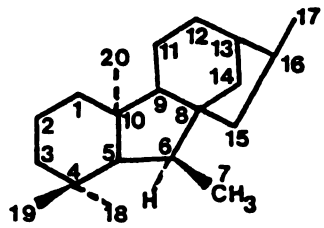
1.2.1. Structure. GAs represent a class of tetracyclic diterpene acids. At present, 56 different GAs are known. The structures of all GAs are based on ent-gibberellane (Fig. 1a). The familiar trivial nomenclature of individual GAs (e.g. GA_1, GA_{56}, etc.) refers only to naturally occurring compounds with the ent-gibberellane skeleton, and whose structure and stereochemistry have been fully elucidated (MacMillan and Takahashi, 1968). The most common structural features of GAs as a class of compounds include: i) the four rings labeled A, B, C, and D, ii) an exocyclic methylene group at C-17, and iii) a carboxyl group at C-6. GAs as a group can be broken down into two basic categories: GAs with 20 carbons (C_{20}-GAs) and GAs that contain 19 carbons (C_{19}-GAs). The latter group represents GAs in which C-20 has been removed, and in an overwhelming majority of cases, a 5-membered γ-lactone bridge between C-19 and C-10 has been formed (Fig. 1b). C-20 of C_{20}-GAs can exist in a number of oxidation states e.g., a methyl, hydroxyl, aldehyde, or carboxyl group. Free hydroxyl groups do not appear at C-20, but instead, exist as a part of a δ- or six membered lactone bridge with C-19. Both C_{19}- and C_{20}-GAs also vary in the position and number of hydroxyl groups as substituants on the four rings (Fig. 1b). Other minor modifications of the ent-gibberellane skeleton include $\Delta^{1,2}$ or $\Delta^{2,3}$ double bonds, a 2,3 epoxide group, and hydration of the $\Delta^{16,17}$ double bond. In this thesis "GA-like substances" will refer to substances of unknown structure,

Figure 1-1. (A) Numbering system of gibberellins and their immediate precursors, the kaurenoids. Note that ring B of the gibberellins contains 5 carbon atoms, whereas ring B of the kaurenoids contains 6. (B) Examples of C_{19}- and C_{20}-GAs. Note various oxidation states of C-20 of C_{20}-GAs, and different positions that can be hydroxylated.

A



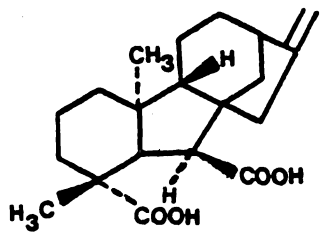
ent-Kaurane



ent-Gibberellane

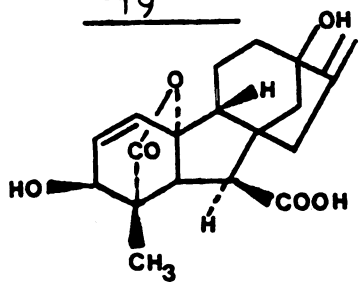
B

C_{20}-GAs

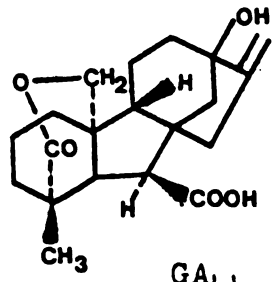


GA_{12}

C_{19}-GAs



GA_3



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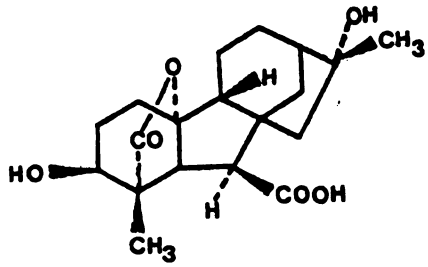
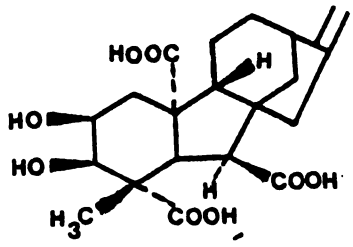
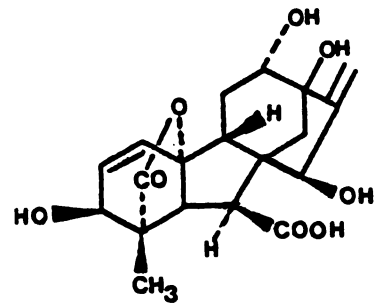


GA_2



GA_{43}



GA_{32}

but which elicit a response in a bioassay. Only chemically identified compounds will be referred to as "GAs".

1.2.2. Biological Activity. Comparative studies of the various GAs in a number of different bioassays has led to a few generalizations on structure-activity relationships (Reeve and Crozier, 1974). In general, C_{19}-GAs tend to have greater biological activity than C_{20}-GAs. However, the position and degree of hydroxylation is of paramount importance to biological activity. C-3β, C-13 dihydroxylated C_{19}-GAs have the highest biological activity in most bioassays, but C-3β or C-13 monohydroxylated GAs also have fairly high biological activity. Moreover, a $\Delta^{1,2}$ desaturation appears to somewhat enhance biological activity. On the other hand, C-2β hydroxylation destroys biological activity of the molecule, regardless which other positions are hydroxylated. The stereochemistry of the hydroxylations is also important to biological activity. If the C-3β hydroxyl group of GA_1 is epimerized to the α-configuration (epi-GA_1), biological activity is greatly diminished. GA_{51} and GA_{40} are both C-2 monohydroxylated GAs. The β-epimer, GA_{51}, is completely inactive in all bioassays tested, while GA_{40}, the α-epimer, has some biological activity in a number of bioassays (Sponsel et al., 1977).

The oxidation state of C-20 in C_{20}-GAs also affects biological activity. If C-20 is an aldehyde or part of a C-19 → C-20 δ-lactone bridge, moderate to high biological activity results. A methyl or a carboxyl group at C-20

results in low to zero biological activity (Reeve and Crozier, 1974).

1.2.3. Purification and Quantification. In most initial studies of GA physiology, an attempt is made to correlate levels of the hormone with some physiological event. However, quantitative analysis of GAs poses special difficulties. In green tissues, GAs occur in extremely minute quantities, usually in the order of a few micrograms per kilogram fresh weight of plant material. Moreover, GAs represent only a small fraction of the many different acidic compounds present in green plant tissue. Therefore, extensive cleanup and purification must be accomplished before effective quantification can take place. Another problem in measuring GA levels is that plants usually contain more than one GA. Since the GAs in a given plant are probably metabolically related, the investigator has an interest in determining the levels of the individual GAs. Consequently, methods for the separation of GAs prior to quantification are necessary. In general, the tissue is first extracted followed by one or more group purification steps (procedures which separate GAs as a class of compounds from other compounds). Solvent partitioning is the most common group separation technique used for GAs, but charcoal adsorption chromatography (Zeevaart, 1971), silicic acid adsorption chromatography (MacMillan et al., 1960), gel filtration chromatography on Sephadex (Crozier et al., 1969), anion exchange chromatography (Browning and Saunders, 1977), and chromatography on polyvinylpyrrolidone to remove

phenolics (Glenn et al., 1972) all have been used for group separation purposes as well. These techniques can also be used in combination to give a purer GA-enriched fraction.

Prior to quantification, the partially purified, GA-enriched fraction is usually subjected to a chromatography step which separates GAs and affords further purification. In early work paper chromatography was utilized (see Phinney and West, 1960), but this has been largely supplanted by thin-layer chromatography (TLC) with silica gel (MacMillan and Suter, 1961). Other chromatography techniques that separate GAs have been successfully employed: silica gel partition chromatography using either open bed columns (Powell and Tautvydas, 1967; Durley et al., 1972) or high performance liquid chromatography (HPLC) (Reeve et al., 1976; Reeve and Crozier, 1978), normal phase partition chromatography on columns of Sephadex LH-20 (MacMillan and Wels, 1973), DEAE-Sephadex chromatography (Gräbner et al., 1976), and reverse phase HPLC (Jones et al., 1980). Gas chromatography (GC) of the GA-methyl esters, the methyl ester trimethylsilyl ethers (Binks et al., 1969), or the trimethylsilyl ethers and esters (MacMillan and Pryce, 1973) gives fine resolution for mixtures of GAs, as well as serving as a necessary adjunct to certain quantitative techniques.

Quantification of GAs has been done mostly by bioassay. There are three basic types of bioassay used for GAs. The earliest used type was the dwarf seedling bioassays. Most common of these are the dwarf maize (Phinney, 1961), dwarf

pea (Brian and Hemming, 1955), and the Tan-ginbozu dwarf rice bioassay (Ogawa, 1963; Murakami, 1968). All dwarf seedling bioassays use increased growth as a measure of GA activity. The second class of GA bioassays are the ones which depend on increased elongation of seedling hypocotyls. Of these, there are two that are most often used: the lettuce hypocotyl assay (Frankland and Wareing, 1960) and the cucumber hypocotyl bioassay (Halevy and Cathey, 1960). These assays have the advantage over the dwarf seedling assays in that they are more rapid, and more easily performed. However, they are not as sensitive, and do not have as broad a spectrum of response to all the GAs. The third and last of the major types of GA bioassays are the ones which employ the response of cereal aleurone layers to GA. These cells, in response to GA, secrete hydrolytic enzymes into the endosperm, the most notable of which is α-amylase. One can make use of this fact by determining the amount of reducing sugars produced by deembryonated seeds (Nicholls and Paleg, 1963), or by measuring the increase in α-amylase activity directly (Jones and Varner, 1967).

All bioassays suffer several inherent defects. First of all, not all GAs give the same response in a given bioassay, and in fact, dose response curves for each GA might have different slopes. Secondly, there are many substances in plant extracts, phenolics and ABA to name a few, which can prove to be inhibitory in the test system. Finally, unless standards of the particular compound in question are

available, only relative amounts of GAs can be estimated (Graebe and Ropers, 1978).

Because of the disadvantages of bioassays, techniques for physico-chemical estimation of the levels of GAs have been developed. The most commonly used techniques employ some detector immediately following gas chromatography. Usually the detectors are either flame ionization detectors or mass spectrometers (e.g. Davis et al., 1968; Frydman et al., 1974). Combined gas chromatography-mass spectrometry (GLC-MS) has the added advantage of providing unequivocal identification of the compound in question (Graebe and Ropers, 1978). One disadvantage in using GLC with a flame ionization detector for quantification of GAs in plant extracts is that one needs standard compounds (which in many cases are not available) in order to know which peaks are actually GAs.

1.3 GAs AS REGULATORS OF STEM GROWTH

In this section the evidence is reviewed that GAs are natural regulators of stem growth. It is now well established that GAs are natural products from higher plants (Lang, 1970; Graebe and Ropers, 1978). Moreover, GAs have been applied to a large number of species of angiosperms and gymnosperms and a promotion of stem growth is almost always found (Goodwin, 1978; Pharis and Kuo, 1977). However, even though applied GAs cause a dramatic response in stem growth, this evidence per se does not permit the conclusion that GAs are involved in the regulation of stem growth. Indeed,

responses from exogenous substances may be due to some non-specific "pharmacological" effect (Lang, 1970). Proof of the role of GAs as endogenous regulators of plant growth is provided by several lines of evidence.

1.3.1. Correlations Between Stem Growth and Endogenous GA Content. Stem growth in many species is positively correlated with endogenous GA content. The peak of extractable GA-like activity coincides with internode elongation in the barley inflorescence (Nicholls and May, 1964; Nicholls, 1974). Similar correlations have been found in apple shoots (Robitaille and Carlson, 1976), in shoots of Picea abies (Dunberg, 1976), as well as in internodes of rice (Osada et al., 1973).

Many genetic dwarfs contain little or no extractable GA-like activity. Normal growth can often be obtained by application of GA. For example, the an_1, d_3, and d_5 mutants of Zea mays (Phinney, 1960), and dwarf mutants of Pharbitis nil (Ogawa, 1962), red clover (Stoddart, 1962), rice (Suge and Murakami, 1968), and Phaseolus vulgaris (Goto and Esashi, 1973) all are plants which lack detectable GA-like activity, but normal stem growth is restored with exogenous GAs. However, some genetic dwarfs contain the same amount of endogenous GA-like substances as their normal counterparts, and do not respond to applied GA. These "GA-insensitive" mutants include certain cultivars of dwarf rice (Suge and Murakami, 1968), Norin 10 types of wheat (Radley, 1970), and a dwarf variety of Silene armeria (Suttle and Zeevaart, 1978).

1.3.2. Growth Retardants. Several synthetic organic compounds have been discovered which retard stem elongation without causing malformations or affecting rate of development. These compounds are called growth retardants (Cathey, 1964). Use of such compounds as Amo-1618 and CCC have provided further evidence that GAs are involved in the regulation of stem growth. Both of these compounds appear to block GA biosynthesis (see section 1.4.3.). The resulting reduction in the level of extractable GA-like substances is correlated with a reduction of stem growth in a number of plants including Pharbitis nil (Zeevaart, 1966), Phaseolus vulgaris (Gerhard, 1966), and Cupressus arizonica (Kuo and Pharis, 1973). In all of these cases, exogenous GA was able to restore normal growth.

Clearly, growth retardants can be a powerful tool in investigations on GA physiology. However, as with all inhibitor studies, care must be exercised when interpreting experimental results. Not all plants are suitable for study of GA physiology by growth retardants (Lang, 1970). There are some cases in which applied GA could not completely reverse the dwarfing effects of the growth retardant, indicating that the compound acted at sites other than just GA biosynthesis (Lang, 1970; Goodwin, 1978). Indeed, both Amo-1618 and CCC also inhibit sterol biosynthesis in Nicotiana tabacum (Douglas and Paleg, 1974).

1.3.3. Stem Growth in LD Rosette Plants. LD rosette plants represent a class of plants which are physiological

dwarfs during a certain phase of their life cycle. This dwarfism is, however, environmentally imposed since exposure to LD causes the plant to assume a caulescent growth habit. Thus, these plants represent good experimental objects to study regulation of stem growth because there is an easily manipulable external factor that will cause stem growth. In many LD rosette plants exogenous GA is able to substitute for LD in inducing the stem elongation response (see Lang, 1965; Zeevaart, 1978). In a number of instances, application of growth retardants prevents LD induced stem elongation, while exogenous GA can overcome the inhibition, e.g. Silene armeria (Cleland and Zeevaart, 1970), spinach (Zeevaart, 1971), and Agrostemma githago (Jones and Zeevaart, 1980a). This implies that the photoperiod regulates stem growth in LD rosette plants through modulation of the GA status. Most investigators found that LD treatment causes both quantitative and qualitative changes in the content of GA-like substances (see Lang, 1965; Zeevaart, 1978). Moreover, in a few cases, overall GA turnover was greatly increased with LD treatment. These aspects will be dealt with in greater detail in section 1.5.3. Finally, LD also seem to increase the sensitivity of the tissue to GA (Cleland and Zeevaart, 1970; Zeevaart, 1971; Jones and Zeevaart, 1980a). Thus, photoperiod can affect many aspects of the GA status, all of which could contribute to regulation of stem growth in rosette plants.

1.3.4. Cytology of the GA-response. GA-induced stem elongation must arise from either an increase in the mean

cell length, an increase in cell number, or both. In peas (Griffith, 1957), Pharbitis nil (Okuda, 1959), and watermelon (Liu and Loy, 1976), application of GA results in a substantial increase in cell number; there is little or no increase in the mean cell length. In the case of watermelon, the greater cell division is due to a reduced S period in the cell cycle (Liu and Loy, 1976). However, in other plants, GA-promoted growth can be ascribed almost entirely to an increase in cell length. Haber and Luippold (1960) found that Triticum seedlings from seeds exposed to gamma radiation to prevent cell division, showed the normal stem elongation response to exogenous GA, indicating that stem growth was due to cell elongation. In Avena internodes, the primary locus of response to exogenous GA is the intercalary meristem. In this system, GA halts multiplication, while longitudinal cell elongation is strongly promoted (Kaufman, 1965).

In most plants, it seems that both cell division and cell elongation are integral parts of the GA response (Sachs, 1965; Jones, 1973). Application of GA_3 to the LD rosette plants Hyoscyamus niger and Samolus parviflorus in non-inductive conditions caused an increase in the frequency of transverse cell divisions within 24 hours of application of GA and an increase in the mean cell length within 72 hours (Sachs et al., 1959). Moreover, the cytological response to LD was very similar to the GA response and could be suppressed by growth retardants (Sachs and Kofranek, 1963). Stem growth in both rosette and caulescent plants was due to activity in

the subapical meristem, and this is the region which shows the response to exogenous GA (Sachs, 1965).

In summary, several lines of evidence show that GAs are natural regulators of stem growth. First of all, there is an excellent positive correlation between the presence of GAs and the ability of stems to elongate. Extractions of GAs or GA-like substances from many plants show the highest level of GA-like substances when plants have the most rapid stem elongation. Genetic dwarfs which lack detectable GA-like substances will respond to exogenous GA, restoring normal growth. LD rosette plants respond to exogenous GAs in SD with increased stem growth. LD treatment also causes quantitative and qualitative changes in GAs, increases GA turnover, and tissue sensitivity to GA. Moreover, the cytological response to exogenous GA in certain rosette plants grown under non-inductive conditions is similar to the response elicited when the plant is placed in conditions conducive to stem growth. This is compelling evidence for the role of GAs in regulation of stem growth.

1.4 BIOSYNTHESIS AND METABOLISM OF GAs

1.4.1. Biosynthesis of GAs. In the previous section, evidence was provided showing the changes in the levels of GAs or GA-like substances that were correlated with stem growth. This implies that plants have mechanisms by which they are able to regulate GA biosynthesis and metabolism, and that this regulation is of physiological and developmental

significance to the plant. However, the metabolic pathway must be known before any study on the nature of the regulation of stem growth by GAs can be made.

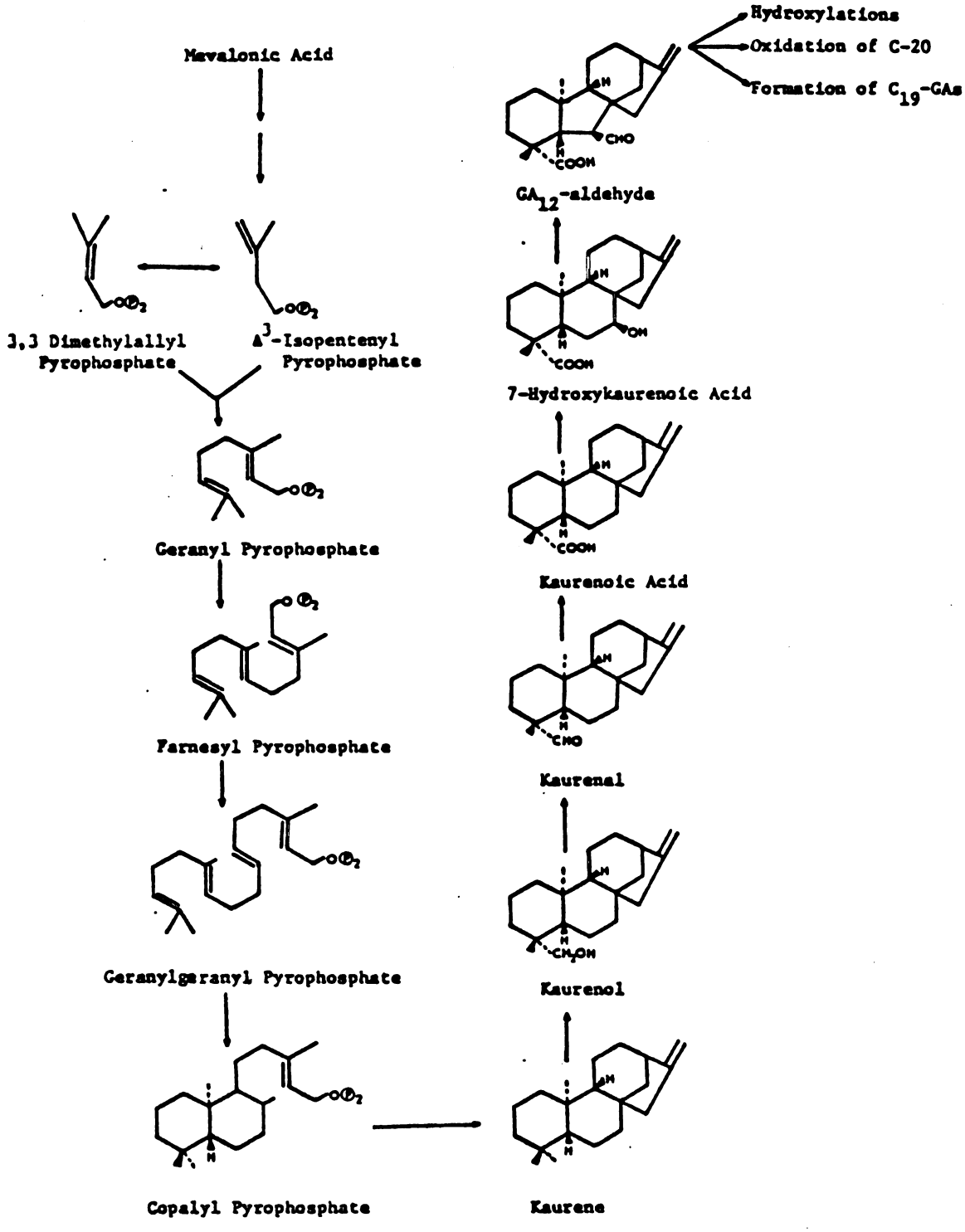
Early structural studies on GA_3 suggested that this compound might be related biogenetically to diterpenes (Cross et al., 1956). Birch et al. (1958, 1959) showed that either [2-^{14}C]-acetate or [2-^{14}C]-mevalonic acid were converted by cultures of the fungus Gibberella fujikuroi to GA_3, thus providing convincing evidence for the diterpene nature of GAs as well as common features of the biosynthetic pathway shared by isoprenoid compounds in many organisms. In subsequent years, considerable effort was expended by a number of laboratories around the world in elucidating the individual steps leading from mevalonic acid to the gibberellins. From metabolic studies on Gibberella and cell-free preparations from the endosperm of Marah macrocarpus and Cucurbita maxima, a common biosynthetic sequence emerged. Figure 2 shows this sequence. The following is a brief account of the numerous studies which delineated the biosynthetic pathway of GAs.

A few years after the initial discovery that mevalonic acid is an early precursor of GA_3 in Gibberella cultures, a second metabolite of feeds of [2-^{14}C]-mevalonic acid was discovered. This compound turned out to be kaurene. Because of its structural similarities to GA_3, it was suggested that kaurene is an intermediate in the GA_3 biosynthetic pathway (Cross et al., 1963). Later Cross and coworkers (1964) found that refeeding ^{14}C-kaurene to fungal cultures led to the

formation of ^{14}C-GA_3, thereby establishing its role as an intermediate in the overall GA biosynthetic pathway.

The individual steps leading up to kaurene formation were first studied in cell-free preparations derived from the endosperm of Marah (Graebe et al., 1965). The biosynthesis of kaurene shares most of the common features of terpene biosynthesis in that the molecule is built with successive condensations of 5-carbon isoprene subunits. Geranylgeranyl pyrophosphate (GGPP), a 20-carbon diterpene (four isoprene units), is the last non-cyclic compound in the pathway (Oster and West, 1968). The cyclization of GGPP to kaurene is a two-step process involving the formation of a short-lived, but stable, intermediate, copalyl pyrophosphate (CPP) (Schechter and West, 1969). The enzyme kaurene synthase catalyzes both steps. The first step, the conversion of GGPP to CPP, involves the formation of rings A and B of kaurene ("A" activity of kaurene synthase), while the "B" activity converts CPP to kaurene in the second step. The "A" and "B" activities were inseparable by a number of chromatographic procedures and hence were considered part of the same enzyme or enzyme complex. However, the "A" and "B" activities had different sensitivities to a number of inhibitors and different pH optima, indicating that different sites on the enzyme were responsible for the two activities (Frost and West, 1976). Recently, it was reported that indeed the "A" and "B" activities can be partially separated (West and Duncan, 1979).

Figure 1-2. Biosynthetic pathway of GAs from mevalonic acid. This sequence has been found in both higher plants and in the fungus Gibberella fujikuroi (From Graebe and Ropers, 1978; Hedden et al., 1978).


Mevalonic Acid
3,3 Dimethylallyl Pyrophosphate
Δ3-Isopentenyl Pyrophosphate
Geranyl Pyrophosphate
Farnesyl Pyrophosphate
Geranylgeranyl Pyrophosphate
Copalyl Pyrophosphate
Kaurene
Kaurenol
Kaurenal
Kaurenoic Acid
7-Hydroxykaurenoic Acid
GA12-aldehyde
Hydroxylations
Oxidation of C-20
Formation of C19-GAs

Most of the essential features of the pathway from mevalonic acid to kaurene have been confirmed in a number of cell-free systems: immature seeds of Cucurbita maxima (Graebe, 1969) and Pisum sativum (Anderson and Moore, 1976), pea shoots (Coolbaugh et al., 1973), expanding cotyledons of Ricinus communis seedlings, and germinating tomato seeds (Yafin and Schechter, 1975). Moreover, the enzymes involved in the conversion of mevalonate to kaurene appear to be soluble, i.e. they occur in the 105,000xg supernatant of cell extracts.

The conversion of kaurene to the first compound with the ent-gibberellane skeleton, GA_{12}-aldehyde, involves the sequential oxidation of C-19 from a methyl to a carboxyl group, hydroxylation of C-7, and finally contraction of ring B from a six to a five-membered ring to form the ent-gibberellane skeleton. Again, the elucidation of this part of the GA biosynthetic pathway relied on studies using the cell-free system derived from Marah endosperm along with complementary studies in Gibberella. In Marah, the C-19 methyl group of kaurene is first oxidized to a hydroxyl group to form kaurenol (Graebe et al., 1965). The hydroxyl group is oxidized to an aldehyde (kaurenal) which in turn is converted to kaurenoic acid (Dennis and West, 1967). C-7 of kaurenoic acid is then hydroxylated to form the last compound in the GA biosynthetic pathway that lacks the ent-gibberellane skeleton, 7β-hydroxykaurenoic acid (Lew and West, 1971). Contraction of ring B occurs at this point beginning with

the abstraction of a hydrogen from the 6β position followed by the extrusion of C-7 from the ring to form GA_{12}-aldehyde (West and Fall, 1972; Graebe et al., 1975). Subsequently, the sequence of kaurene to GA_{12}-aldehyde has been shown to operate in cell-free systems derived from immature seeds of Cucurbita maxima (Graebe et al., 1972) and Pisum sativum (Ropers et al., 1978).

Whereas the enzymes responsible for the conversion of mevalonic acid to kaurene are soluble enzymes, the oxidative enzymes responsible for the conversion of kaurene to GA_{12}-aldehyde are microsomal (Dennis and West, 1967; Lew and West, 1971; West, 1973). Moreover, they require molecular O_2, NADPH, and have other properties that indicate these enzymes are mixed function oxidases which involve the participation of a species of cytochrome P-450 (Murphy and West, 1969; West, 1973).

1.4.2. Interconversion of GAs. GA_{12}-aldehyde, the first compound in the biosynthetic sequence to have the ent-gibberellane skeleton, is first converted to C_{20}-GAs, which are in turn converted to C_{19}-GAs. C_{19}-GAs are generally believed to be responsible for biological activity (Graebe and Ropers, 1978). There are two main centers of GA metabolism in the formation of the various GAs from GA_{12}-aldehyde: the C-20 methyl group can be oxidized to a variety of oxidation states to form other C_{20}-GAs, or it can be removed to form C_{19}-GAs. Both C_{20}- and C_{19}-GAs can be hydroxylated in any number of combinations, so all GAs can be derived from a

common precursor, GA_{12}-aldehyde.

One of the early events in the conversion of GA_{12}-aldehyde is the oxidation of the C-7 aldehyde to a carboxyl group. In the cell-free systems derived from immature seeds of Cucurbita and Pisum, GA_{12}-aldehyde is converted to GA_{12} (Graebe et al., 1974; Ropers and Graebe, 1978). In Gibberella, GA_{12}-aldehyde is first hydroxylated at C-3 to form GA_{14}-aldehyde just prior to the oxidation of the C-7 aldehyde group (Hedden et al., 1974).

The exact pathway leading to the removal of C-20 and the formation of the C-19→C-10 lactone bridge is unclear. Cell-free preparations from Cucurbita endosperm are able to convert GA_{12}-aldehyde to GA_4 (a C-3 monohydroxylated C_{19}-GA). This system is also able to sequentially oxidize C-20 from a methyl to a carboxyl group (Graebe et al., 1974a,b). However, the oxidation state of C-20 at which C_{20}-GAs are converted to C_{19}-GAs is still not known. In fact, no C_{20}-GA with an oxidation state higher than a methyl group has ever been shown conclusively to be converted to a C_{19}-GA (Graebe and Ropers, 1978). It is known that both oxygen atoms of the γ-lactone bridge of C_{19}-GAs are derived from the C-19 carboxyl group of C_{20}-GAs (Bearder et al., 1976). Moreover, the loss of C-20 results in the evolution of $^{14}CO_2$ when [^{14}C-20]-kaurene is fed to cultures of Gibberella (Dockerill and Hanson, 1978). Apparently, the conversion of C_{20}-GAs to C_{19}-GAs involves a nucleophilic attack by the C-19 carboxyl group on an electrophilic center at C-10 generated by the removal of C-20

(Bearder and Sponsel, 1977). While these facts eliminate many possibilities, the exact path of conversion remains unknown (Hedden et al., 1978).

As pointed out earlier in section 1.2.1., the position and degree of hydroxylation of the ent-gibberellane skeleton controls, in a large part, the degree of biological activity of the molecule. Hydroxylation reactions can occur soon after the formation of GA_{12}-aldehyde, e.g. the formation of GA_{14}-aldehyde in Gibberella (Hedden et al., 1974), GA_{53} in Pisum (Ropers et al., 1978), and GA_{36} in Cucurbita (Graebe et al., 1974a). Hydroxylations can also occur after the formation of C_{19}-GAs: the C-2β hydroxylation of GA_{20} to form GA_{29} in intact immature seeds of Pisum (Frydman and MacMillan, 1975), or the formation of GA_3 from GA_7 in Gibberella (Bearder et al., 1975).

Unlike the microsomal mixed-function oxidases which convert kaurene to GA_{12}-aldehyde, the enzymes responsible for oxidation of C-20 in the Cucurbita endosperm are soluble and require Fe^{+3} for activity (Graebe et al., 1974a,b). Likewise, a cell-free preparation from germinating bean seeds which converts GA_1 to GA_8 by a C-2β hydroxylation is also a soluble enzyme that requires Fe^{+3} and NADPH (Nadeau and Rappaport, 1972; Patterson and Rappaport, 1974). Thus, while the conversion of kaurene to GA_{12}-aldehyde shares the common property of increasing the oxidation state of the substrate with the metabolism of GA_{12}-aldehyde to other GAs, they occur in different parts of the cell. This is probably a reflection of

the solubility properties of the different substrates in question. Kaurene and the intermediates of its oxidation to GA_{12}-aldehyde are not very water soluble, and probably need to be in a hydrophobic environment if an enzymatic reaction is to occur. On the other hand, the products of GA_{12}-aldehyde metabolism become increasingly polar in nature, and the enzymes are correspondingly water soluble (Hedden et al., 1978).

Catabolism of C_{19}-GAs is not well understood. Some plant material, particularly mature seeds, contain "bound" or conjugated GAs. For the most part, such GAs are glucosyl esters or ethers (Graebe and Ropers, 1978; Hedden et al., 1978). While conjugated GAs could simply represent biologically inactive end-products of metabolism, it has been suggested that they serve as storage (Lang, 1970) or transport forms (Sembdner, 1968). C_{19}-GAs can be metabolized further in other ways. Pea seeds will catabolize GA_{29} by oxidation of the C-2 hydroxyl group and opening of the lactone ring to form the C_{19} α,β-unsaturated ketone (Sponsel and MacMillan, 1978; Durley et al., 1979). Somatic cell cultures of carrot are able to convert GA_1 to a $\Delta^{1,10}$ unsaturated, non-lactonic counterpart of GA_1 (Noma et al., 1979). Apparently, a very early catabolic process of C_{19}-GAs is the destruction of the lactone bridge and concomitant formation of the C-19 acid.

1.4.3. Mechanism of Action of Growth Retardants. Certain synthetic compounds are known to reduce growth in a number of plants, and are therefore called growth retardants. The dwarfing activity of these compounds suggests that they

act through some alteration in the GA status. Early work had shown that both CCC and Amo-1618 inhibited GA_3 production by Gibberella (Kende et al., 1963). Later it was found that these compounds also caused a reduction in extractable GA-like substances in a number of higher plants (see Graebe and Ropers, 1978). It now appears that Amo-1618 and CCC, both quaternary ammonium compounds, as well as the phosphons (phosphonium salts) prevent the cyclization of GGPP to kaurene in both Gibberella (Fall and West, 1971) and Marah (Frost and West, 1977). However, CCC was nearly ineffective in Marah unless high concentrations (10^{-2}M) were used. Moreover, the "A" activity of kaurene synthase is much more sensitive to the inhibitors than the "B" activity. Other quaternary ammonium compounds are also effective at this step, e.g. N,N,N-trimethyl-1'-methyl-(2',6',6'-trimethylcyclohex-2'-en-1'-yl) pro-2-enylammonium iodide (Hedden et al., 1977). Interestingly, some of these quaternary ammonium compounds also inhibit the cyclization of squalene to cholesterol in enzyme preparations from rat liver, indicating a similar mechanism of action of animal squalene epoxidase and plant kaurene synthase (Douglas and Paleg, 1972; Ono and Bloch, 1975).

Other growth retardants inhibit GA biosynthesis at different steps in the GA biosynthetic pathway. Ancymidol has been shown to block the oxidation of kaurene, kaurenol and kaurenal in cell-free preparations from the endosperm of Marah (Coolbaugh and Hamilton, 1976). It has been suggested that ancymidol interacts directly with the cytochrome P-450

of the mixed function oxidases that is responsible for the oxidation of the kaurenoids (Coolbaugh et al., 1978). 1-alkylimidazoles appear to inhibit GA biosynthesis at the same locus as ancymidol (Wada, 1978). It should be pointed out, however, that not all growth retardants act by blocking GA biosynthesis. Compounds such as B-995 (Alar) apparently dwarf plants through some other mechanism than reduction of endogenous GA levels (Lang, 1970).

1.4.4. Dwarfism. In section 1.3.1., several examples of genetic dwarfs which lack GA-like substances were cited. However, the molecular mechanism underlying dwarfism is not well understood. The most intensely studied plant has been the dwarf pea. Dwarfism in this case is not due to a lack of GAs, and so for the purposes of this literature review will not be considered further (see Goodwin, 1978). Perhaps the best understood of the GA-deficient dwarfs is the d-5 maize mutant. Apparently, the lesion lies at kaurene synthase. Cell-free preparations from normal maize coleoptiles are able to convert mevalonic acid to kaurene. Similar preparations from the d-5 dwarf convert mevalonic acid and GGPP to iso-kaurene (kaur-15-ene) instead of kaurene (kaur-16-ene). Iso-kaurene cannot be converted to GAs (Hedden and Phinney, 1979).

Waito-C, a dwarf variety of rice, contains the same amount of extractable GA-like substances as normal rice varieties. However, this variety responds only to C-3β hydroxylated GAs such as GA_1 and GA_3. The major GA in rice (both normal and Waito-C) appears to be GA_{19}, a C_{20}, C-13

monohydroxylated GA, which is inactive in promoting stem growth in Waito-C. Thus, it is likely that this mutant lacks the ability to convert GA_{19} to C-3β hydroxylated GAs (Murakami, 1971). These examples illustrate the potential use of mutants in elucidating metabolic pathways and mode of action of hormones.

The foregoing discussion should help to answer questions about the bewildering number of GAs known to exist. If one accepts the view that most GAs are precursors, intermediates, or deactivation products of a few biologically important ones, GA physiology is a bit easier to comprehend.

1.5 CONTROL OF GA BIOSYNTHESIS AND METABOLISM

1.5.1. Control of Enzymes. The level of a particular substance is determined by the sum of its rates of synthesis and conversion. Unfortunately, little is known about regulation of the individual enzymes and reactions that occur in GA biosynthesis and metabolism. However, one possible control point that has been studied is the conversion of GGPP to kaurene by kaurene synthase. Since metabolism of GGPP is a branch point to a number of different compounds such as the phytol chain of chlorophyll and carotenoids, the cyclization of GGPP to kaurene is seen as a likely candidate for a major regulatory step in GA biosynthesis (Simcox et al., 1975). Coolbaugh and Moore (1969) found the rate of ent-kaurene synthesis from mevalonic acid in different stages of pea seed development was correlated with the amount of

extractable GA-like substances. Also, the change in the rate of kaurene synthesis was correlated with the change in the rate of growth of pea seedlings (Eckland and Moore, 1974). However, in both of these cases, it was assumed, but not shown, that the enzyme under regulation was kaurene synthase. It is possible that other enzymes in the pathway from mevalonic acid to kaurene are under regulation as well. In the endosperm of Marah macrocarpus, kaurene synthesis from mevalonic acid is regulated by the energy charge. However, the enzyme found to be most subject to regulation was pyrophosphomevalonate decarboxylase, not kaurene synthase (Knotz et al., 1977).

A number of C-2β hydroxylated GAs are known to exist and all have little or no biological activity. Therefore, conversions from active GAs to C-2β hydroxylated GAs may serve as a mechanism by which plants can control the level of the active GA by regulating the rate of its deactivation. Presumably, this kind of deactivation is irreversible. In pea seeds, the C-2β hydroxylation reaction occurs only at a certain developmental stage, indicating a regulatory role for the conversion (Sponsel and MacMillan, 1977).

Conjugates of GAs, most common of which are the glucosyl ethers or esters, are generally thought to be biologically inactive (Graebe and Ropers, 1978; Hedden et al., 1978). Since a number of plant enzymes are able to hydrolyse GA conjugates, particularly the glucosyl esters, to form free GAs (Knöfel et al., 1974), it is possible that the relative

rates of conjugate formation and hydrolysis control the levels of the active substance. Mature bean seeds have a higher content of glucosyl esters than immature seeds. The situation is reversed for the levels of free GAs (Hiraga et al., 1972; 1974). Moreover, when the seeds germinate, the level of the conjugates declines while free GAs increase (Yamane et al., 1975). Exogenous [^{3}H]-GA_3-glucosyl ester is much more rapidly hydrolysed in germinating seeds than in 14-day-old seedlings (Liebisch, 1974). This suggests a storage role for conjugates in maturing seeds to be utilized after hydrolysis during germination. However, GA conjugates are not prevalent as natural components of green plant tissue, so it seems unlikely that conjugation as a mechanism of regulation of GA levels is of widespread importance.

1.5.2. Compartmentation. The availability of a GA to an active site could possibly be controlled by compartmentation. This means that absolute tissue levels would be irrelevant. The regulated release of sequestered molecules from an organelle or some compartment could be responsible for control of physiologically relevant levels of GA that have access to the active site. Presently, chloroplasts seem the most likely candidate to fill such a role. First of all, chloroplasts from a number of plants are known to contain GA-like substances: Brassica oleracea and Hordeum vulgare (Stoddart, 1968); Pisum sativum (Railton and Reid, 1974); Triticum aestivum (Browning and Saunders, 1976). Secondly, there is some evidence to suggest that plastids and sonicated

chloroplasts have the ability to synthesize kaurene from GGPP (Simcox et al., 1975; Coolbaugh and Moore, 1976). Moreover, chloroplast preparations from Hordeum are able to convert kaurenol to 7β-hydroxykaurenoic acid (Murphy and Briggs, 1975). Thus, chloroplasts may have the ability to synthesize GAs from mevalonic acid.

Etioplasts derived from leaves of Hordeum (Evans and Smith, 1976) or Triticum (Cooke et al., 1975) significantly increased in GA content when given short exposures to red light. Within 20 minutes, the GAs produced in etioplasts were secreted into the medium. The regulated release from etioplasts could modulate the effective levels of GAs and therefore control biological responses (Cooke et al., 1975).

1.5.3. Photoperiodic Control of GA Metabolism. In a number of rosette plants, application of GA will substitute for the inductive photoperiod in eliciting stem growth, indicating that photoperiod controls stem growth in these species through regulation of some aspect of the GA status. In the most simple case, the plants in the unfavorable photoperiod lack GAs. Indeed, there are several examples in which analysis by bioassay has demonstrated a correlative increase in the level of GA-like substances when plants are subjected to photoperiodic conditions that cause stem growth: Hyoscyamus niger (Lang, 1960); Rudbeckia bicolor (Harada and Nitsch, 1959); Nicotiana sylvestris (Grigorieva et al., 1971). However, the relationship between absolute levels of GAs and stem growth in other photoperiodically sensitive species is

not so clear cut. The LD rosette plant Agrostemma githago shows a large transient increase in the level of GAs when the plants are subjected to LD. Although photoperiodically controlled stem growth in this species is mediated through regulation of the GA status, the absolute level of GAs does not correlate with stem growth. LD treatment greatly increases overall GA turnover (Jones and Zeevaart, 1980b). In spinach there is no change in the total level of endogenous GA-like substances when plants are transferred from SD to LD conditions. However, there is a balance between a decline in one GA-like substance and a corresponding increase in another during LD treatment. Also, there is an increase in overall GA turnover under LD conditions (Zeevaart, 1971; 1974) as found in Agrostemma. Silene armeria, a LD rosette plant related to Agrostemma, also shows increased GA turnover in LD (Cleland and Zeevaart, 1970; Van den Ende and Zeevaart, 1971). Moreover, all three of these plants showed greater sensitivity to exogenous GA in LD than SD in eliciting stem growth. Thus, greater GA turnover combined with higher tissue sensitivity to GA in LD might regulate stem growth in plants rather than absolute levels of GA (Jones and Zeevaart, 1980a,b; Zeevaart, 1971; Cleland and Zeevaart, 1970).

Peas, like other monocarpic annuals, die following fruiting. Preceding death are a series of processes termed apical senescence that lead to death of the apex and ultimately to the death of the plant. There exists a genetic line of peas termed G2 in which apical senescence following

the reproductive phase is dependent on photoperiod. If fruiting G2 plants are kept in LD, apical senescence and death of the plant will ensue. However, if the same plants are maintained under SD conditions, apical growth continues unabated for some time (Proebsting et al., 1976). Moreover, G2 plants produce a graft-transmissible substance which delays apical senescence in photoperiodically insensitive lines of peas. Apparently, this graft-transmissible substance is gibberellin (Proebsting, et al., 1977). Analysis by bioassay and GLC-MS of the G2 line showed that plants under SD conditions contained higher levels of two GAs than plants in LD. Moreover, the apparent rate of GA interconversion is also higher in SD than LD (Ingram and Browning, 1979; Proebsting et al., 1978).

Light, not necessarily duration of the light period, is also known to affect the rate of GA metabolism. Short bursts of red light cause suspensions of wheat or barley etioplasts to increase in the level of extractable GA-like substances (Cooke and Saunders, 1975; Evans and Smith, 1976). Exposure to light decreases metabolism of [^{3}H]-GA_{20} to GA_1 in tobacco callus cultures. Since greater growth of the callus is obtained in the dark, it was suggested that light regulation of growth in tobacco callus was due, in part, to control of the conversion of GA_{20} to GA_1 (Lance et al., 1976). Likewise, dark-grown seedlings of Pisum sativum metabolized [^{3}H]-GA_5 faster than light-grown seedlings. Moreover, the dark-grown seedlings had a greater response to exogenous GA_5 (Musgrave

and Kende, 1970). In Phaseolus coccineus, metabolism of [^{3}H]-GA_4 was correlated with growth and the level of extractable, endogenous GA-like substances. Light caused greater metabolism of exogenous [^{3}H]-GA_4, lower levels of extractable GA-like substances, and a reduction in growth (Brown et al., 1975). Cell-free preparations from light-grown pea seedlings had a greater capacity to synthesize kaurene from mevalonic acid than did corresponding cell-free extracts from dark grown seedlings (Eckland and Moore, 1974).

While it is obvious that photoperiod or light can substantially alter GA metabolism, it is also unclear as to what mechanisms are involved in such control. As mentioned earlier, the conversion of mevalonic acid to kaurene in cell-free extracts of Marah macrocarpus endosperm is regulated by the adenylate energy charge of the system, which a higher energy charge favoring synthesis of kaurene (Knotz et al., 1977). Since variations of the energy charge in green plant cells can occur during changes between light and dark conditions (Santarius and Heber, 1965), it is possible that one aspect of light regulation of GA biosynthesis and metabolism is control by light over the level of adenylates.

Although control of the energy charge might explain some aspects of light regulation of GA metabolism, photoperiodic effects on GA metabolism are not so easily explained. Many photoperiodic effects on growth have been attributed to phytochrome control of GA metabolism (Vince-Prue, 1975), but exact mechanisms remain almost totally unknown. Leaf

unrolling in etiolated cereal leaves is controlled by the phytochrome status of the leaf. Exogenous GA_3 can substitute for red light in causing the leaves to unroll. Moreover, etiolated wheat leaves show an increase in the level of extractable GA-like substances 15 minutes following a brief exposure to red light (Beevers et al., 1970). As stated in section 1.5.2., the increases in GA-like activity are confined to the etioplast fraction where phytochrome apparently regulates the release of GAs from the etioplast (Cooke et al., 1975; Evans and Smith, 1976). Phytochrome has been detected in the envelope membranes of barley etioplasts. It has been suggested that phytochrome in the P_{fr} form acts to increase the permeability of the envelope membrane to GAs, thereby decreasing the internal GA concentration in the etioplasts. This depletion of GAs within the etioplast could activate further GA biosynthesis through some feedback mechanism (Evans and Smith, 1976; Evans, 1975). Thus, in this model, the action of phytochrome on GA metabolism is indirect; the primary effect of phytochrome in the P_{fr} form is the redistribution of GAs between compartments. Whether or not this model can be expanded to cover photoperiodic effects on GA metabolism in other species remains to be seen. In any event, much more preliminary work must be done before a unified theory on the mechanism of photoperiodic control of GA metabolism can be made. Endogenous GAs must be identified and the metabolic relationships between GAs and GA precursors should be definitively established before one can reasonably

hope to understand how various reactions in a pathway are regulated.

1.6 GAs: ROOT-SHOOT INTERRELATIONSHIPS

The growth and development of roots and shoots are separate, but highly coordinated events. Tightly coupled development of the various organs suggests that there exist means of communication between organs. Hormones transported between organs could act as integrating factors that enable one organ to signal another when to initiate or terminate a certain phase of development. Went (1938) observed that root excision or flooding roots of tomato (Went, 1943) caused an inhibition of stem growth. Because the inhibition was not due to mineral or water uptake problems, it was suggested that the roots produced a hormone-like factor which is transported from the roots to the shoot where it controls stem growth. This factor was termed "caulocaline" (Went, 1943). Because stem growth appears to be regulated by some aspect of the GA status (Jones, 1973; Goodwin, 1978), it has been suggested that at least part of caulocaline is a GA (Phillips, 1964; Reid and Crozier, 1971). If GA is identical to, or part of, caulocaline then three criteria have to be met: i) GAs should be produced in the roots, and this production should be correlated with observed effects of flooding, etc. on stem growth; ii) transport of GAs from the root to the shoot in the xylem should reflect changes in production of GAs in the roots; iii) exogenous GA should at least partly

be able to overcome the inhibition of stem growth by root excision or flooding.

Roots have been shown by indirect means to have the apparent capacity to synthesize GAs. First of all, Butcher (1963) has shown that a clone of excised tomato roots maintained in culture for five years contained GA-like substances. This implied that the GAs had been synthesized by the roots since carry-over from the mother plant after so many subcultures is unlikely. Secondly, through use of a diffusion technique, the sites of GA synthesis in Helianthus were deduced to be the root and shoot tips (Jones and Phillips, 1966). Today, it is generally believed that root and shoot tips are sites of GA synthesis in other plants as well (Lang, 1970; Graebe and Ropers, 1978). However, besides certain immature seeds, no definitive biochemical data (i.e. location of specific enzymes) yet exist that indicate where in the plant GAs are made.

Studies using radio-labeled GAs applied to roots have shown that GAs will move from the roots to the shoots (particularly to the mature leaves) in the transpiration stream (Davies and Rappaport, 1975). Moreover, GA-like substances have been detected in xylem exudate in a number of species (e.g. Carr et al., 1964; Phillips and Jones, 1964; Sitton et al., 1967; Sweet et al., 1974). Since the sites of GA synthesis are not definitively known, the origin of GAs in the xylem is not clear. Nevertheless, there exists some evidence, albeit weak, which indicates that GAs transported

from the roots in the xylem regulate stem growth. Application of GA_3 to the base of the stem of de-rooted soybean seedlings completely overcame the inhibition of stem growth caused by the treatment (Holm and Key, 1969). Exogenous GA_3 was partially able to reverse the stunting effects caused by waterlogging tomato plants (Reid et al., 1969; Reid and Crozier, 1971). In this particular case, waterlogging was associated with a precipitous decline in the level of GA-like substances found in the xylem exudate. In addition, the level of extractable GA-like substances found in the roots and the shoots was also sensitive to waterlogging. This led the authors to conclude that root-produced GAs, sensitive to O_2 deprivation, controlled stem growth (Reid et al., 1969; Reid and Crozier, 1971). However, there are other equally viable explanations. Since the ultimate origin of GAs in the xylem is unknown, it is possible that GAs produced in the shoot are transported down to the roots via the phloem and then exported back to the shoot in the xylem. Such recirculation patterns have been observed for other substances as well (see Ziegler, 1975). Girdled Citrus sinensus trees accumulated GA-like substances in the lateral shoots and bark above the ring while the roots showed a significant decline in GA-like activity. This suggests that in reality root and xylem GAs are substantially derived from the shoot (Wallerstein et al., 1973). Likewise, the reduced stem growth in waterlogged tomato plants may not even be due to a reduction in the levels of GAs. The stems of waterlogged

plants produce copious amounts of ethylene, a plant hormone known to reduce stem growth (Bradford and Dilley, 1978). Apparently O_2 deprivation of the roots stimulates the production of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid in the roots. This substance is then transported up to the shoot in the transpiration stream where it is converted to ethylene (Bradford and Yang, 1980). Incidently, this provides a perfect example of a chemical signal produced in one organ, transported to another, where it initiates a sequence of events that modifies growth and development i.e. a hormone.

In conclusion, the evidence supporting a role for root-produced GAs in the regulation of shoot growth is equivocal at best, and points out the absolute necessity of determining the location of enzymes involved in GA biosynthesis and metabolism.

Studies using exogenous GAs indicate that GAs travel with the flow of assimilates from source leaves to sinks such as shoot tips and roots (Zweig et al., 1961; McComb, 1964). GA-like substances have been detected in the phloem sap of a number of species (Kluge et al., 1964; Hall and Baker, 1972; Hoad, 1973). It is not known whether GAs transported in the phloem regulate growth processes in the sink end of the transport system. In general, exogenous supplies of GAs to roots on intact plants have little or no effect on root growth (Goodwin, 1978). GAs have been reported to increase the elongation of isolated root tips in a number of species

including maize (Mertz, 1966), pea (Pecket, 1960), and tomato (Butcher and Street, 1960). However, in all these cases, growth proceeds well in the absence of externally supplied GAs; this is evidence that the cultured root tip is self-sufficient for GAs (Goodwin, 1978). This, coupled with the fact that root tips are purported sites of GA biosynthesis, (Jones and Phillips, 1966) indicates that shoot-produced GAs are not important in the regulation of root growth.

Considerable radial transport of assimilates from the phloem to nearby tissues can occur (Peel, 1967). GAs behave similarly and could conceivably regulate physiological processes such as cambial division and subsequent expansion, as well as maintenance metabolism of cortical cells (Bowen and Wareing, 1969). GAs traveling with the flow of assimilates also accumulate at the shoot tip, a site where GAs are known to affect stem growth. It is not known if GAs transported from the mature leaves to the shoot tip in the phloem actually regulate stem growth since the shoot tip is supposed to be a site of GA synthesis (Jones and Phillips, 1966).

In conclusion, GAs are known to be transported over long distances in the phloem and in the xylem. However, the precise physiological role of long-distance GA transport is obscure at best. Some investigators feel that GAs are produced at, or near the site of utilization (see Graebe and Ropers, 1978). The presence of GAs in the two transport streams might only be coincidental, and conceivably, have little or no physiological importance.

1.7 STATEMENT OF PURPOSE

In the beginning of this chapter, it was proposed that photoperiodic control of stem growth in LD rosette plants offers a useful model system in which to study environmental control of growth and development in plants. Early work by Zeevaart (1971; 1974) has established that photoperiodic control of stem growth in spinach is mediated by a change in the GA "status" of the plant. This work also demonstrated that photoperiod affected two related aspects of the GA status in spinach: the relative levels of the various GA-like substances and the overall GA turnover rate. This thesis is an account of further investigations on the role of photoperiodically-induced changes in GA metabolism in the regulation of stem growth in spinach. Specifically, I was interested in four areas:

1) Chemical identification of the GA-like substances present in spinach plants.
2) The quantitative relationships between the GAs as a function of photoperiod.
3) Nature and physiological significance of long-distance GA transport between roots and shoots of spinach.
4) Preliminary information on the nature of subcellular distribution of GAs in spinach leaves.

Chapter 2

The Identification of Six Endogenous Gibberellins in Spinach Shoots

2.1 INTRODUCTION

Long-day rosette plants respond to the transfer from SD to LD with increased stem elongation and subsequent flower formation. It has been demonstrated in Silene armeria (Cleland and Zeevaart, 1970), Agrostemma githago (Jones and Zeevaart, 1980a), and spinach (Zeevaart, 1971) that photoperiodic control of stem growth is mediated through regulation of the GA status (see 1.5.3.). In spinach, LD treatment of plants previously maintained under SD did not increase the total level of GA-like substances, but levels of individual GA-like substances did vary. Moreover, overall GA turnover was enhanced considerably in LD. This indicates a profound change in GA metabolism had occurred (Zeevaart, 1971; 1974). It is assumed that this change in GA metabolism is of physiological significance in the photoperiodic control of stem growth in spinach. However, before the mechanism of photoperiodic regulation of GA metabolism and its relation to stem growth can be fully investigated, the chemical identity and quantitative relationships of the endogenous GAs should be known. This chapter represents an account of the identification of six endogenous GAs in spinach shoots.

2.2 MATERIALS AND METHODS

2.2.1. Plant Material. Seeds of spinach, Spinacia oleracea L.cv. Savoy Hybrid 612 (Harris Seed Co., Rochester, New York) were sown in the field in early August, 1978. Whole shoots were harvested 1.5 months after sowing. The harvested shoots were washed with distilled water, and the yellow, senescing leaves discarded. The remaining plant material was frozen in liquid N_2, lyophilized, and stored at -15°C prior to extraction. Approximately 2,500 plants yielded 2.5 kg of dried plant material.

2.2.2. Bioassay. The presence of GA-like substances was detected with the d-5 corn bioassay. Homozygous seeds (Zeevaart, 1966) were germinated in moist vermiculite in a humid, dark incubator at 27°C. After six days the seedlings were transferred to plastic boxes (20 x 10 x 8 cm) containing half-strength Hoagland's solution. Fractions from extracts were dissolved in 0.5 ml of 0.1% Tween 20 in water. The solution was then distributed equally on each of four seedlings. The first leaf, at this stage, had not yet unrolled, and so formed a convenient cup with a capacity of about 0.15 ml. After application of the test solutions, the plants were then placed in a growth chamber at 27°C with 16 hours of light daily from fluorescent lamps (Gro-lux, Sylvania) and 40 W incandescent bulbs (total irradiance = 28 $W m^{-2}$). One week after treatment, the sum of the lengths of the first two leaf sheaths was determined and the data converted to percentages of an untreated control. A standard curve

(1ng to 1 µg) was prepared using GA_3 (Sigma).

2.2.3. Extraction and Purification Procedures. Freeze-dried spinach shoots, in 200 g lots, were homogenized with 10 liters of ice-cold 80% aqueous methanol (20 ml per gram dry weight) in a Waring Blender. The extract was filtered, and the residue was stirred overnight at room temperature in four liters of 100% methanol. After a second filtration, the two extracts were combined, and the methanol removed under reduced pressure in a rotary evaporator. An equal volume of 1 M phosphate buffer (pH 8.2) was then added to the remaining aqueous residue, and the resulting mixture was partitioned three times with petroleum ether. The aqueous phase was adjusted to pH 2.5 with 6 N HCl and purified on a charcoal-celite column as described by Zeevaart (1971), except that two grams of charcoal were used for every 10 g of dry plant material extracted. Elution of GAs was achieved with 80% aqueous acetone. The acetone was removed under reduced pressure in a rotary evaporator. The remaining aqueous residue was adjusted to pH 2.5 with 6 N HCl followed by partitioning 4 times against equal volumes of ethyl acetate.

The acidic ethyl acetate fraction obtained after charcoal chromatography was purified by silicic acid adsorption chromatography as described by Zeevaart (1971), except that 60% ethyl acetate in chloroform was used as the elution mixture. Two grams of silicic acid (Mallinkrodt, 100 mesh) were used for every gram of lyophilized plant material extracted.

The eluate from the silicic acid adsorption column was fractionated via preparative reverse phase HPLC using a Waters Model 5000A liquid chromatograph equipped with four stainless steel columns (each 60 cm x 0.65 cm i.d.) packed with Bondapak C_{18}/Porasil B (Waters Associates). GAs were eluted from the column with a linear gradient of 95% ethanol in 1% aqueous acetic acid (30-100% in 25 minutes) controlled by a Waters 660 solvent programmer. Dried samples were redissolved in 5 ml of 30% aqueous ethanol, and the solution filtered through a 0.45 μm HA Millipore filter. The filtered sample was loaded onto the column via a Waters U6K Universal Injector with a 5 ml loop. The gradient was started 1 min after injection; the solvent flow rate was 9.9 ml min^{-1}. Fractions were collected every minute from the time of injection and corresponding fractions from 12 HPLC runs were combined and dried. The remaining residues were redissolved in 2 ml of ethanol and 1.0% of each of the combined fractions was tested for the presence of GA-like substances with the d-5 corn bioassay as described in section 2.2.2. Fractions which contained biological activity, or fractions in which authentic GAs eluted (Jones et al., 1980) were further purified by silicic acid partition chromatography. In this procedure the method of Powell and Tautvydas (1967) was followed, except that the stationary phase consisted of water with the pH adjusted to 3.0 by the addition of a few drops of trifluoracetic acid. Elution of GAs was achieved with a gradient of increasing concentration in 5% increments of ethyl

acetate in hexane. Both solvents were saturated with water at pH 3.0 before mixing.

Fractions which contained biological activity, or eluted at the same step as authentic GAs, were subjected to final purification using analytical reverse phase HPLC with a μ-Bondapak C_{18} column (30 cm x 0.4 cm i.d.). Elution of GAs from this column was achieved with either a 30-100% linear gradient of methanol in 1% aqueous acetic acid (grad A), or a 10-70% linear gradient (grad B). In both cases the gradient was completed in 30 min and the flow rate was 2 ml min^{-1}. The gradient was started 2.5 minutes after injection, and fractions were collected every minute from the time of injection. Other procedures were identical to those described for preparative HPLC. Grad B was used only for eluting polyhydroxylated GAs (e.g. GA_1, GA_{29}, GA_8; see Jones et al., 1980).

2.2.4. TLC. Preparative TLC was carried out on 20 x 20 cm glass plates coated with silica gel H (EM Reagents). Partially purified acidic extracts were applied as a narrow band 12 cm long. Authentic GAs were spotted 2 cm to the side of this band. The thin layer plates were developed to 15 cm from the origin in chloroform-ethyl acetate-acetic acid (60:40:5, v/v). The resulting chromatogram was divided into 10 equal zones, and the silica gel was scraped off into test tubes. The silica gel was then eluted twice with water-saturated ethyl acetate and once with acetone. The combined eluates from each of 10 zones were assayed for the presence of GA-like substances using the d-5 corn bioassay. The side

of the plate which was spotted with the authentic GAs was left intact and sprayed with a sulfuric acid-ethanol mixture (5:95, v/v). The plate was heated at 100°C for 10 min to visualize the reference GAs.

2.2.5. Derivatization. Appropriate fractions from analytical reverse phase HPLC were methylated with ethereal diazomethane. The trimethylsilyl ethers of the methyl esters were prepared by adding 100 µl of a solution containing pyridine-hexamethyldisilazane-trimethylchlorosilazane (9:3:1, v/v) to dry methylated samples in Reacti-vials (Pierce Chemical Co.).

2.2.6. GLC. Derivatized samples were chromatographed on a Hewlett-Packard 402B gas chromatograph equipped with a U-shaped glass column (183 cm x 0.3 cm i.d.) packed with 4% SE-33 on Gaschrom Q, 80/100 mesh. All runs were isothermal at either 215 C° or 225 C°. The carrier gas was He, and the flow rate was 40 ml min^{-1}.

2.2.7. GLC-MS. Derivatized samples were chromatographed on a Hewlett-Packard 5840-A gas chromatograph with a glass column packed with 2% SP-2100 on 100/120 Supelcoport. The column temperature was programmed from 170 to 280°C at 10°C min^{-1} with a 2 min isothermal hold at the beginning of the program and a 5 min isothermal hold at the end. The flow rate of the carrier gas (He) was 25 ml min^{-1}. The GLC was connected to a Hewlett-Packard 5985 mass spectrometer by a jet separator, and mass spectra were collected every 4.5 second. The ionizing potential was 70eV.

2.3 RESULTS

2.3.1. Characterization of GA-like Substances in Spinach Shoots. A sample of field-grown spinach, 20 g dry weight, was extracted and subjected to charcoal adsorption chromatography, silicic acid adsorption chromatography, and TLC. Figure 2-1 shows the biological activity associated with 10 equal strips of the chromatogram. Two zones of biological activity are apparent. The zone at R_f 0.2, called fraction I, co-chromatographed with GA_1, while the less polar zone at R_f 0.4-0.5 (fraction II) co-chromatographed with GA_{20}. These results indicate that the pattern of GA-like activity from field-grown spinach is similar to that from plants grown in growth chambers (Zeevaart, 1971; 1974). When I and II were first separated by TLC and then subjected to preparative reverse phase HPLC, fraction I no longer behaved chromatographically like GA_1 (Fig. 2-2A), while fraction II still co-chromatographed with GA_{20} (Fig. 2-2B).

2.3.2. Identification of GAs found in Spinach Shoots. In order to identify the GAs present in fractions I and II, it was necessary to extract and purify large amounts of plant material. After charcoal and silicic acid adsorption chromatography, followed by preparative reverse phase HPLC, I and II were separated by silicic acid particion chromatography. Fractions I and II were eluted with 55% and 40% ethyl acetate in hexane, respectively. Final purification of both I and II was achieved by analytical reverse phase HPLC. Small aliquots (0.5%) of each fraction were assayed for the

Figure 2-1. GA-like activity present in an extract of lyophilized spinach shoots (20 g). The partially purified acidic extract was fractionated by TLC and the resulting chromatogram divided into ten equal strips. Each strip was eluted and the eluate was assayed for the presence of GA-like substances by the d-5 corn bioassay.

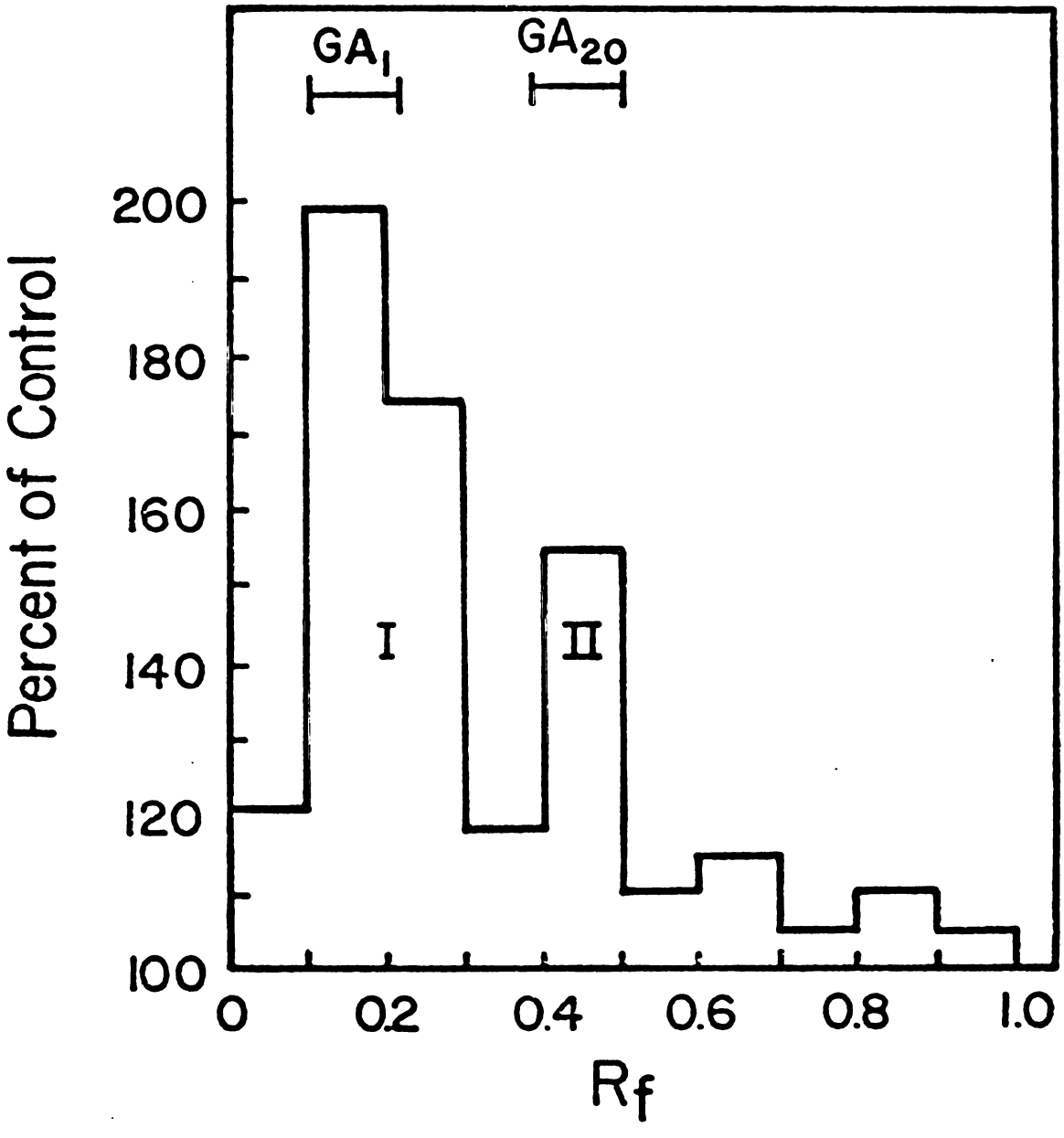
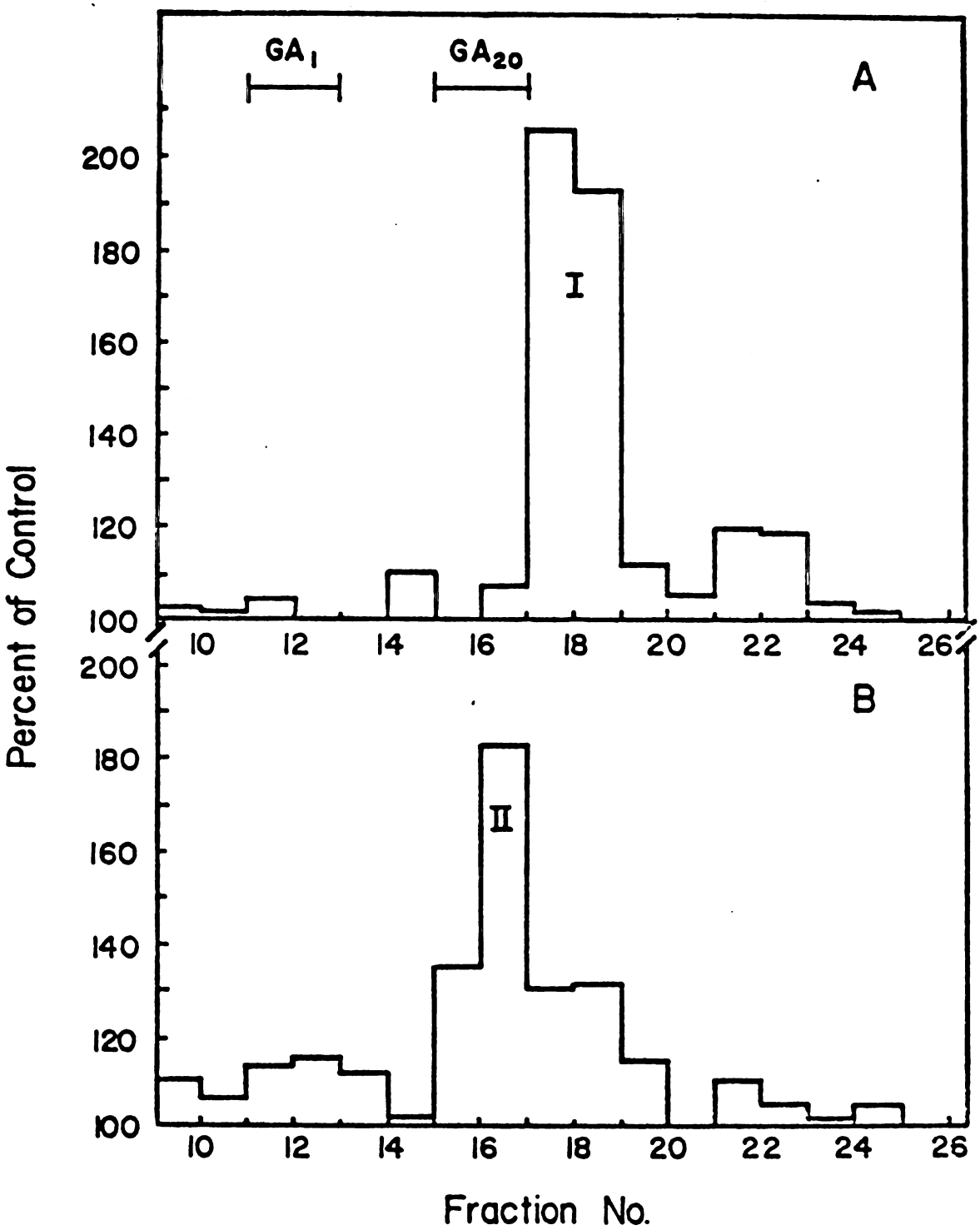

GA1
GA20
200
180
160
140
120
100
Percent of Control
I
II
0
0.2
0.4
0.6
0.8
1.0
Rf

Figure 2-2. Chromatographic behavior of fractions I(A) and II(B) when separately subjected to preparative reverse phase HPLC as detected by the d-5 corn bioassay. An acidic extract from 20 g dry weight of plant material was prepared, and I and II separated by TLC. Standard GA_1 and GA_{20} were run separately for comparison of the chromatographic behavior.


GA1
GA20
A
B
I
II
200
180
160
140
120
100
10
12
14
16
18
20
22
24
26
Percent of Control
Fraction No.

presence of GA-like substances by the d-5 corn bioassay. Both I and II were contained in single fractions. Figure 2-3 shows that II co-chromatographed exactly with GA_{20}(B), whereas I(A) co-chromatographed with none of the available GA standards (see Jones et al., 1980).

The MeTMS-derivatives of the fractions resulting from analytical HPLC that contained I and II were prepared. Gas chromatography of the derivatized fractions indicated that both I and II were contained in fractions that had only one major peak (Fig. 2-4A,B). Thus, reverse phase HPLC, used in conjunction with other chromatography techniques, is extremely useful in purifying small quantities of substances from plant material. Authentic MeTMS-GA_{20} had the same retention time as the main peak of MeTMS-II (Fig. 2-4B). GLC-MS analysis of this peak showed that its mass spectrum was identical to that of authentic MeTMS-GA_{20} (Table 2-1). None of the other minor peaks from this fraction had fragmentation patterns recognizable as any of the known GAs. When the derivatized fraction containing I was analyzed by GLC-MS, the mass spectrum of the large peak seen in GLC analysis (Fig. 2-4A) closely resembled the published mass spectrum (Binks et al., 1969) of MeTMS-GA_{19} (Table 2-1). Unfortunately, a reference sample of GA_{19} (Fig. 2-5) was not available for further confirmation. However, on the basis of the similarities between the published spectrum (Binks et al., 1969) and the mass spectrum obtained from I, it is concluded that I is comprised of GA_{19}.

Figure 2-3. GA activity as measured by the d-5 corn bioassay associated with 0.5% aliquots of individual fractions resulting from analytical reverse phase HPLC of purified fractions I(A) and II(B) using grad A.

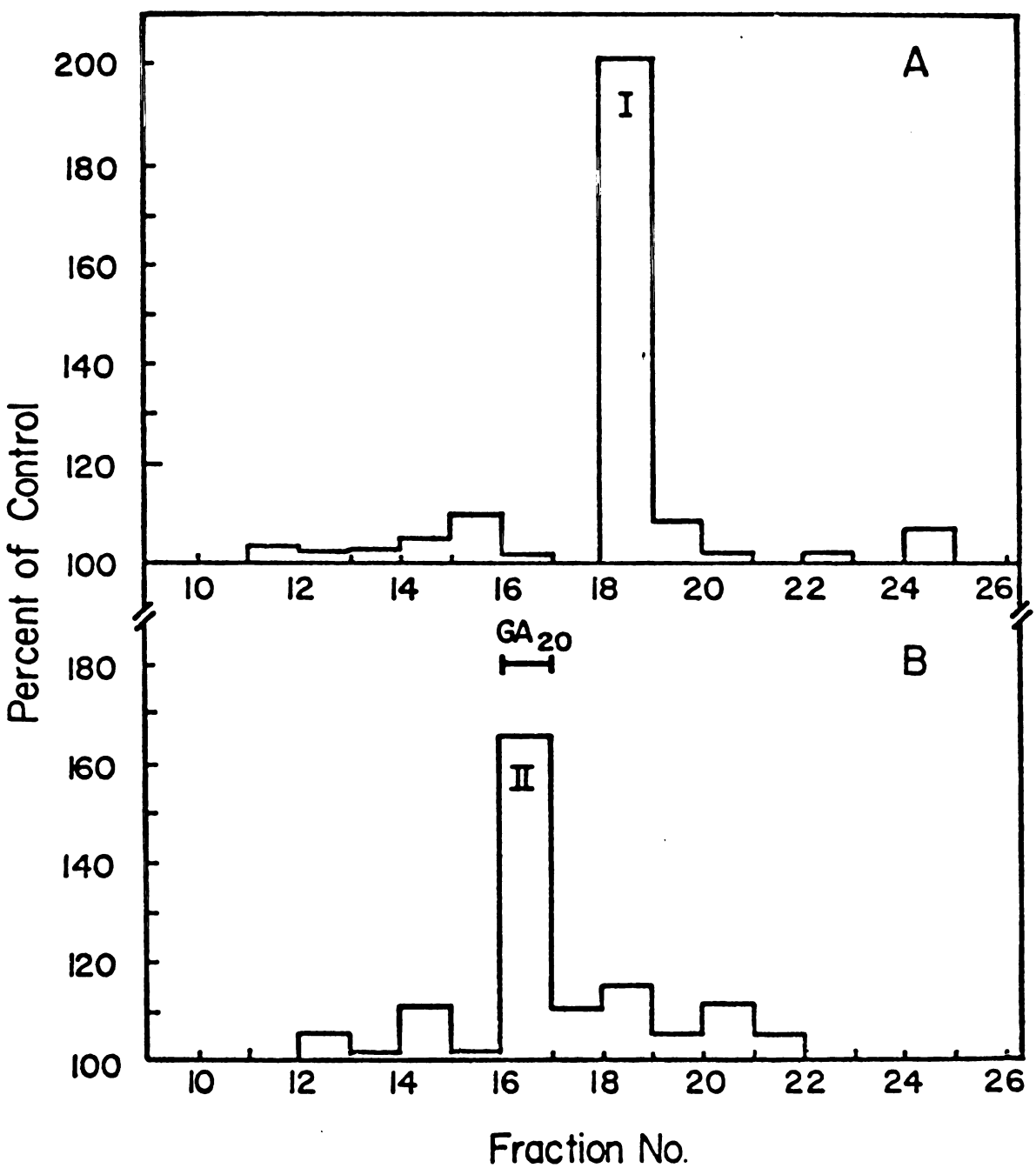

A
I
B
II
GA20
Percent of Control
Fraction No.
200
180
160
140
120
100
10
12
14
16
18
20
22
24
26

Figure 2-4A. GLC analysis of MeTMS-I. Derivatized samples were chromatographed on a U-shaped glass column (183 cm x 0.3 cm i.d.) packed with 4% SE-33 on 80/100 mesh gaschrom Q. All runs with MeTMS-I were isothermal at 225°C.

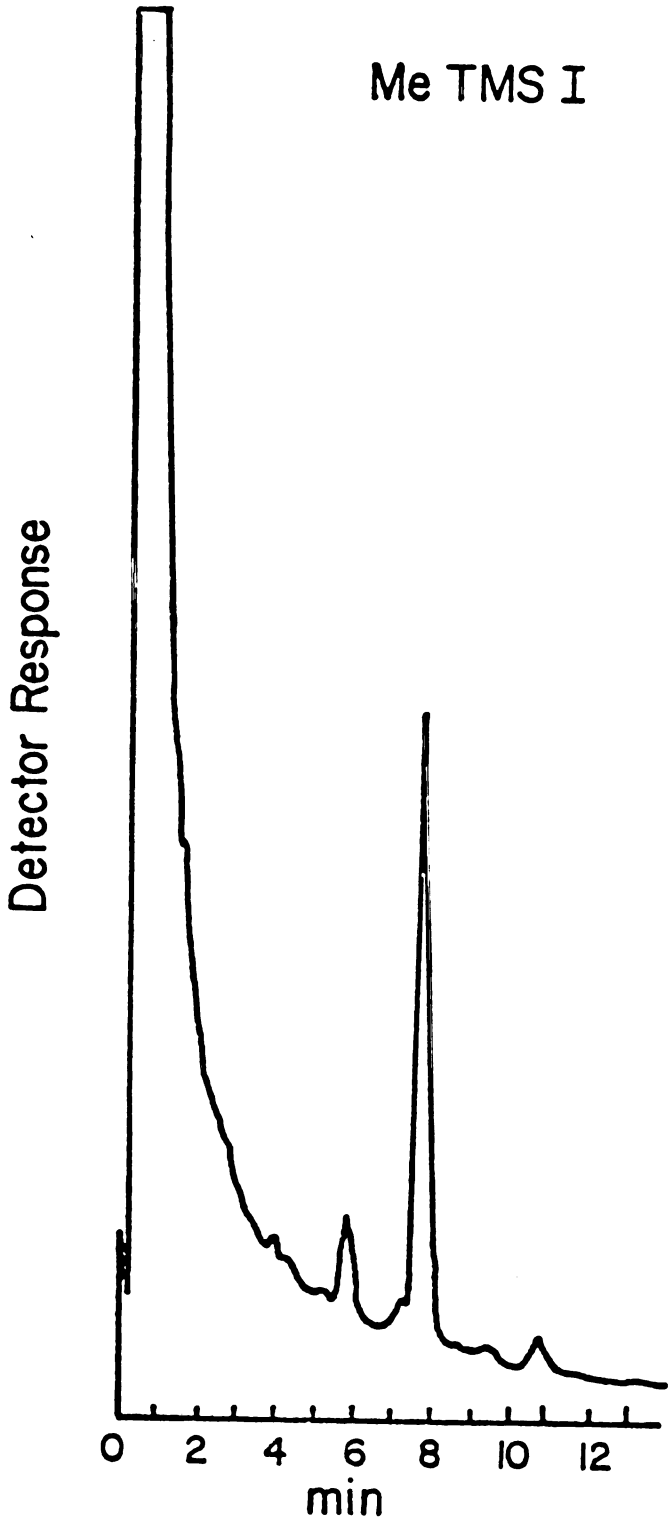

Me TMS I
Detector Response
0
2
4
6
8
10
12
min

Figure 2-4B. GLC analysis of MeTMS-II. Derivatized samples were chromatographed on a U-shaped glass column (183 x 0.3 i.d.) packed with 4% SE-33 on 80/100 mesh gas-chrom Q. All runs with MeTMS-II were isothermal at 215°C. Authentic MeTMS-GA_{20} was also run for comparison.

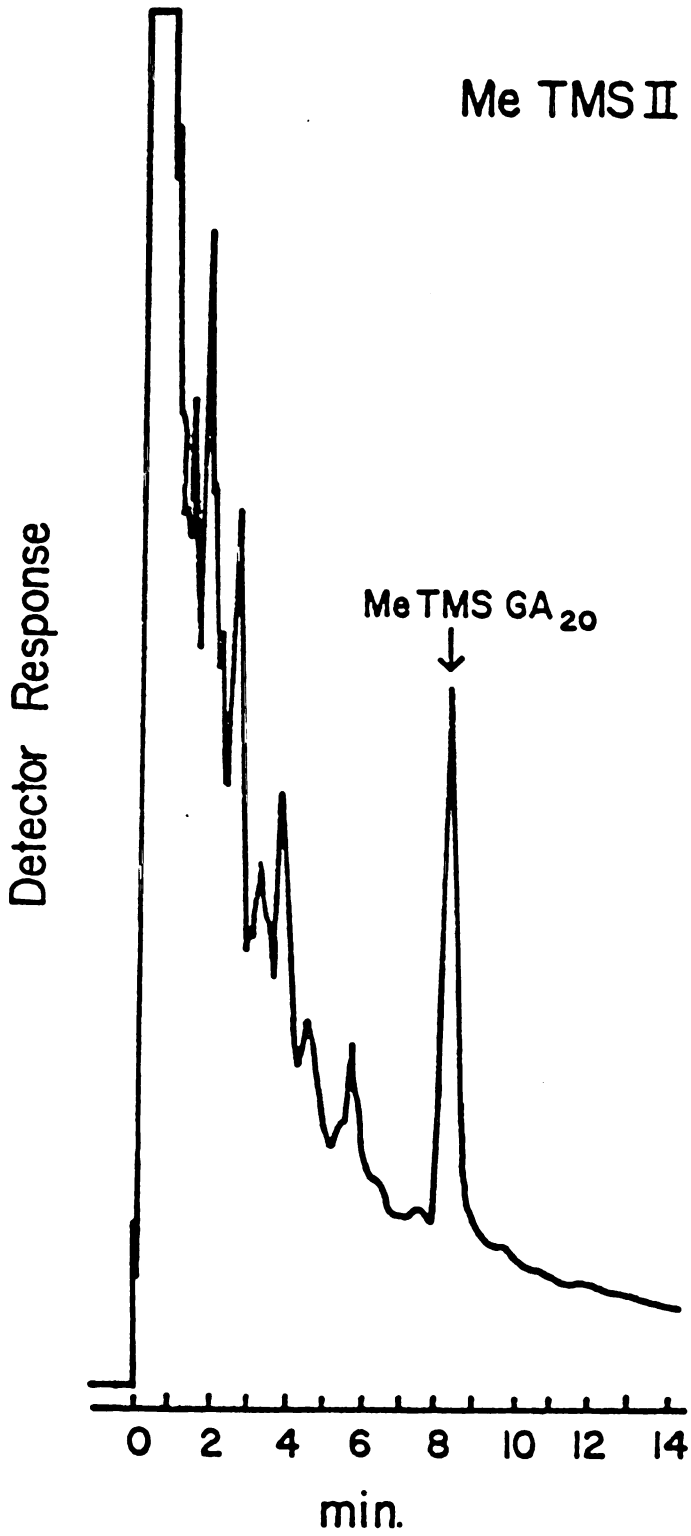
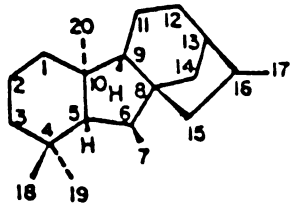

Me TMS II
Me TMS GA20
Detector Response
0
2
4
6
8
10
12
14
min.

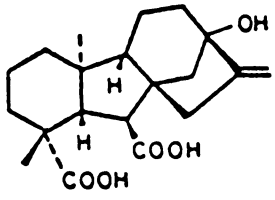
Table 2-1. GLC-MS data obtained with samples from spinach and with authentic GAs. In cases where no authentic GAs were availabe, published data from mass spectral analysis are presented: MeTMS-GA_{19} (Binks et al., 1969); MeTMS-GA_{44} (Frydman et al., 1974); MeTMS-GA_{53} (Bearder et al., 1975). Fraction numbers refer to fractions eluted from analytical HPLC system.

Sample	Time of scan (min)	Peaks in mass spectrum (m/e values) with relative abundances in parentheses.					
I	11.5	462(M^+,6)	447(4)	434(100)	402(30)	374(42)	208(39)	207(37)
MeTMS-GA_{19}		462(M^+,12)	447(7)	434(100)	402(36)	374(61)	208(72)	207(47)
II	10.8	418(M^+,100)	403(21)	375(68)	359(17)	208(10)	207(33)	
MeTMS-GA_{20}	10.8	418(M^+,100)	403(17)	375(72)	359(16)	208(10)	207(20)	
Fraction 19	11.4	492(M^+,13)	460(22)	432(13)	401(5)	373(13)	208(100)	
MeTMS-GA_{17}	11.4	492(M^+,14)	460(25)	432(21)	401(16)	373(26)	208(100)	
Fraction 14	12.6	506(M^+,100)	491(12)	477(15)	447(9)	208(25)	207(40)	
MeTMS-GA_{29}	12.6	506(M^+,100)	491(16)	477(5)	447(14)	208(38)	207(41)	
Fraction 17	13.1	432(M^+,33)	417(8)	373(17)	251(8)	238(42)	208(53)	207(100)
MeTMS-GA_{44}		432(M^+,83)	417(18)	373(21)	251(9)	238(36)	208(47)	207(100)
Fraction 21	11.2	448(M^+,31)	419(10)	416(23)	389(44)	208(80)	207(100)	
MeTMS-GA_{53}		448(M^+,34)	419(8)	416(9)	389(22)	208(98)	207(100)	

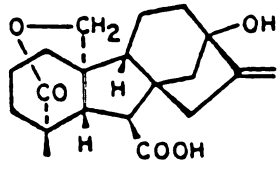
Figure 2-5. Numbering system of ent-gibberellane (A), and structures of the six GAs identified by GLC-MS in spinach shoot extracts (B).



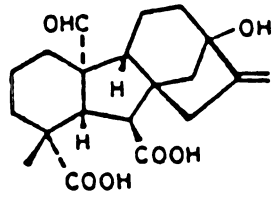
A



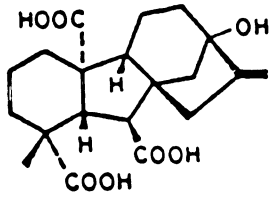
GA_{53}



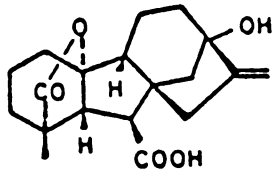
GA_{44}



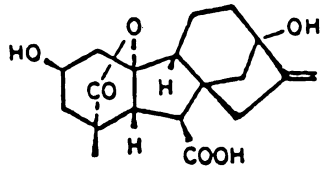
GA_{19}



GA_{17}



GA_{20}



GA_{29}

B

Since GA_{19} and GA_{20} are both C-13 hydroxylated GAs, it would seem logical that other C-13 hydroxylated GAs are present in spinach shoots. If these GAs were biologically inactive, or were present in minute quantities, they would escape detection in the bioassay. Fractions resulting from preparative reverse phase HPLC which would contain various C-13 hydroxylated GAs (see Jones et al., 1980), if present in spinach shoot extracts, were purified by silicic acid partition chromatography. Final purification was achieved by analytical reverse phase HPLC. Corresponding fractions from the spinach extract where various C-13 hydroxylated GAs are known to run in this system (Jones et al., 1980) were derivatized and analyzed by GLC-MS. Fraction 19 (grad A) and fraction 14 (grad B) contained substances that had retention times and mass spectra identical to those of authentic MeTMS-GA_{17} and MeTMS-GA_{29}, respectively (Table 2-1). Fractions 17 and 21 (grad A) contained compounds which had mass spectra similar to those published for MeTMS-GA_{44} (Frydman et al., 1974) and MeTMS-GA_{53} (Bearder et al., 1975), respectively (Table 2-1). Both GA_{44} and GA_{53} (Fig. 2-5) occurred in such low quantities that they would have escaped detection in the bioassay. No GA_1, GA_3, GA_5, or GA_8 was detected in extracts of spinach shoots. Bar graphs from the mass spectra of these compounds and available reference compounds can be found in the Appendix.

2.4 DISCUSSION

The above results demonstrate the presence of at least six GAs in spinach shoots: GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, and GA_{29} (Fig. 2-5). All six GAs have in common a C-13 that is hydroxylated. Only GA_{29} has an additional hydroxyl at the C-2β position. Four of the six GAs are C_{20}-GAs: GA_{53}, GA_{44}, GA_{19}, and GA_{17} with C-20 as a methyl, δ-lactone, aldehyde, or carboxyl group, respectively. Both GA_{20} and GA_{29} are C_{19}-GAs of which C-20 has been removed and a C-19 → C-10 lactone bridge has been formed. The same six GAs present in spinach shoots have recently been identified in immature seeds of Vicia faba (Sponsel et al., 1979), whereas GA_{53}, GA_{44}, GA_{19}, GA_{17}, and GA_{20} have been found in young tassels of Zea mays (Phinney, 1979) and shoots of Agrostemma githago (Jones and Zeevaart, 1980b). Thus, this combination of C-13 hydroxylated GAs appears to occur commonly in higher plants and suggests a biogenetic relationship between these various GAs.

If one assumes that the six GAs found in spinach shoots are related metabolically in a pathway, one can postulate a sequence based on the sequential oxidation of C-20 from a methyl to a carboxyl group, its subsequent removal with the formation of a C-19 → C-10 lactone bridge, and finally C-2β hydroxylation. This would indicate the existence of the following pathway: GA_{53} → GA_{44} (in the open lactone or hydroxy-diacid form) → GA_{19} → GA_{17} → GA_{20} → GA_{29}. However, there is little direct evidence from other plant systems that indicates the existence of this pathway in GA metabolism.

Conversion of GA_{20} to GA_{29} has been observed in a variety of higher plant systems including Phaseolus vulgaris seeds (Yamane et al., 1977), immature seeds of Pisum sativum (Frydman et al., 1974), and leaves of Bryophyllum daigremontianum (Durley et al., 1975). This type of reaction may serve as a deactivation process, since GA_{29} and other C-2β hydroxylated GAs have little or no biological activity (Hedden et al., 1978). In a cell-free system derived from Cucurbita maxima endosperm, C-20 of GA_{12} (a non-hydroxylated analog of GA_{53}) was sequentially oxidized from a methyl (GA_{12}) to an aldehyde (GA_{24}, GA_{36}), and finally to a carboxyl group (GA_{13}, GA_{25}, GA_{43}) (Graebe and Hedden, 1974). However, no C_{20}-GA with a higher oxidation state than a methyl group at C-20 has been found to act as an intermediate in the conversion of C_{20}-GAs to C_{19}-GAs. Thus, the identity of the direct precursor of C_{19}-GAs remains unknown.

Zeevaart (1971; 1974) observed that the levels of two GA-like substances in spinach shoots, here called I and II, changed in relation to photoperiod. The biological activity associated with these two GA-like substances can now be attributed to GA_{19} and GA_{20}. The other GAs found in spinach shoots are either present in quantities too low for detection by bioassay, or are biologically inactive in the d-5 corn bioassay. It appears from the earlier bioassay results (Zeevaart 1971; 1974), that the level of GA_{19} declines with LD treatment, while the level of GA_{20} increases during the same period. Moreover, the bioassay results indicated that

the total GA level remained fairly constant during LD treatment. Since GA_{19} and GA_{20} elicit similar responses in the d-5 corn bioassay (Crozier et al., 1970), the bioassay data should reflect absolute differences in the levels of both GAs. Taken together, these results could be interpreted as a precursor-product relationship between GA_{19} and GA_{20}. In the next chapter, quantitative analysis by GLC-SICM of photoperiodically-induced changes in the levels of the spinach GAs will provide further circumstantial evidence favoring this hypothesis.

Chapter 3

The Effect of Photoperiod on the Levels of Endogenous Gibberellins in Spinach Shoots

3.1 INTRODUCTION

In the previous chapter, the identification of six C-13 hydroxylated GAs (Fig. 2-5) in spinach shoots was described. Moreover, the bioassay results of Zeevaart (1971; 1974) combined with the identification of the two GAs responsible for GA-like activity in spinach shoot extracts indicate that the level of GA_{19} declines with LD treatment, whereas the GA_{20} level increases during the same period. Bioassays have, however, several limitations, most important of which is the difference in sensitivity to various GAs (see section 1.3). Thus, important quantitative information is lost because the d-5 corn bioassay is not sensitive to GA_{29} (Crozier et al., 1970) or GA_{17} (Yokota et al., 1971). This set the stage for an analysis of photoperiodically-induced changes in the levels of the GAs found in spinach shoots by GLC-SICM.

3.2 MATERIALS AND METHODS

3.2.1. Plant Material and Photoperiodic Treatment. Spinach seeds (see 2.2.1.) were sown on vermiculite. After ten days, the seedlings were transferred to 340-ml plastic cups containing a gravel-vermiculite mixture (1:2), and were watered twice daily with half-strength Hoagland's solution. The plants were maintained under SD conditions until ready

for experimentation, approximately six weeks after sowing. SD treatments consisted of an 8 hr period of light from fluorescent and incandescent lamps (total irradiance = 33 $W m^{-2}$), followed by 16 hours of darkness. LD treatment consisted of the same 8 hr illumination as in the SD treatment, followed by 16 hours of low intensity illumination from incandescent bulbs (total irradiance = 0.7 $W m^{-2}$). LD treatments were staggered in such a way that all plants were harvested at the same time. Each treatment consisted of ten plants. At the end of an experiment, the stem length of each plant was determined. The shoots were then cut off at the soil level, frozen in liquid N_2, lyophilized, and stored at -15°C prior to extraction.

3.2.2. Extraction and Purification Procedures. The extraction and purification procedures were identical to those described in 2.2.2. Methanolic extracts were reduced to a small aqueous residue and purified by charcoal adsorption chromatography and silicic acid adsorption chromatography. The eluate resulting from silicic acid adsorption chromatography was fractionated by preparative reverse phase HPLC as described earlier. Fractions known to contain GA_{44}, GA_{19}, GA_{17}, and GA_{20} (all monohydroxylated GAs) were combined and subjected to analytical reverse phase HPLC as described before (2.2.3.) for monohydroxylated GAs (grad A). The fractions containing these four GAs were then combined. The fraction from preparative HPLC that contained GA_{29} was also purified with analytical reverse phase HPLC, using the

gradient system described previously for dihydroxylated GAs (grad B, see 2.2.3.).

The resulting final two fractions were then methylated with ethereal diazomethane. The trimethylsilyl ethers of the methyl esters were prepared by the addition of 20 µl of a solution containing pyridine-hexamethyldisilazane-trimethylchlorosilazane (9:3:1, v/v) to methylated samples dried in a capillary.

3.2.3. GLC-SICM. GLC-SICM was performed using a Hewlett-Packard 5985 mass spectrometer that was interfaced by a glass jet separator with a Hewlett-Packard 5840-A gas chromatograph. The four monohydroxylated GAs were chromatographed using a glass column (183 x 0.2 cm, i.d.) packed with 2% SP-2401 on 100/120 mesh Supelcoport. Samples were injected (2 µl) onto the column at 180°C. Following a 2 min isothermal hold, the temperature was programmed 10°C min^{-1} until the column temperature was 205°C, whereupon the rate was slowed to 1°C min^{-1}. When the temperature reached 215°C, the rate was increased to 20°C min^{-1} until the column reached the maximum temperature of 255°C. GA_{29} was chromatographed on 2% SP-2100 with column conditions identical to those described in 2.2.7.

For each GA, three fragments with the following m/e values were monitored: GA_{44}-432, 373, and 207; GA_{19}-462, 434, and 374; GA_{20}-418, 419, and 375; GA_{17}-492, 460, and 208; GA_{29}-506, 507, and 207. The dwell time for each fragment monitored was 200 msec. The relative level of each GA was calculated from the SICM response of the molecular ion of

each compound, except for GA_{19}, in which case the base peak (m/e 434) was used. The ratios of the SICM response of the three fragments monitored for each GA were checked in every sample to ensure that interfering compounds did not affect the SICM response. Each sample was analyzed twice, and the average of the two readings was used in the subsequent calculations. No pair of readings ever differed by more than 5%. Other parameters of the mass spectrometer were the same as described in 2.2.7.

3.2.4. Application of GA_{20}. GA_{20} (a gift from Drs. N. Murofushi and N. Takahashi, Department of Agricultural Cheimistry, University of Tokyo, Japan) was dissolved (0.4 µg/l) in an aqueous solution of 0.1% Tween 20 and 5% ethanol. Fifty µl were applied to the shoot tips of spinach plants maintained under SD. Ten such applications, for a total of 100 µg GA_{20} were made on alternate days. The stem of each plant was measured one week following the last application. Five plants were used for both treatment and control.

3.3 RESULTS

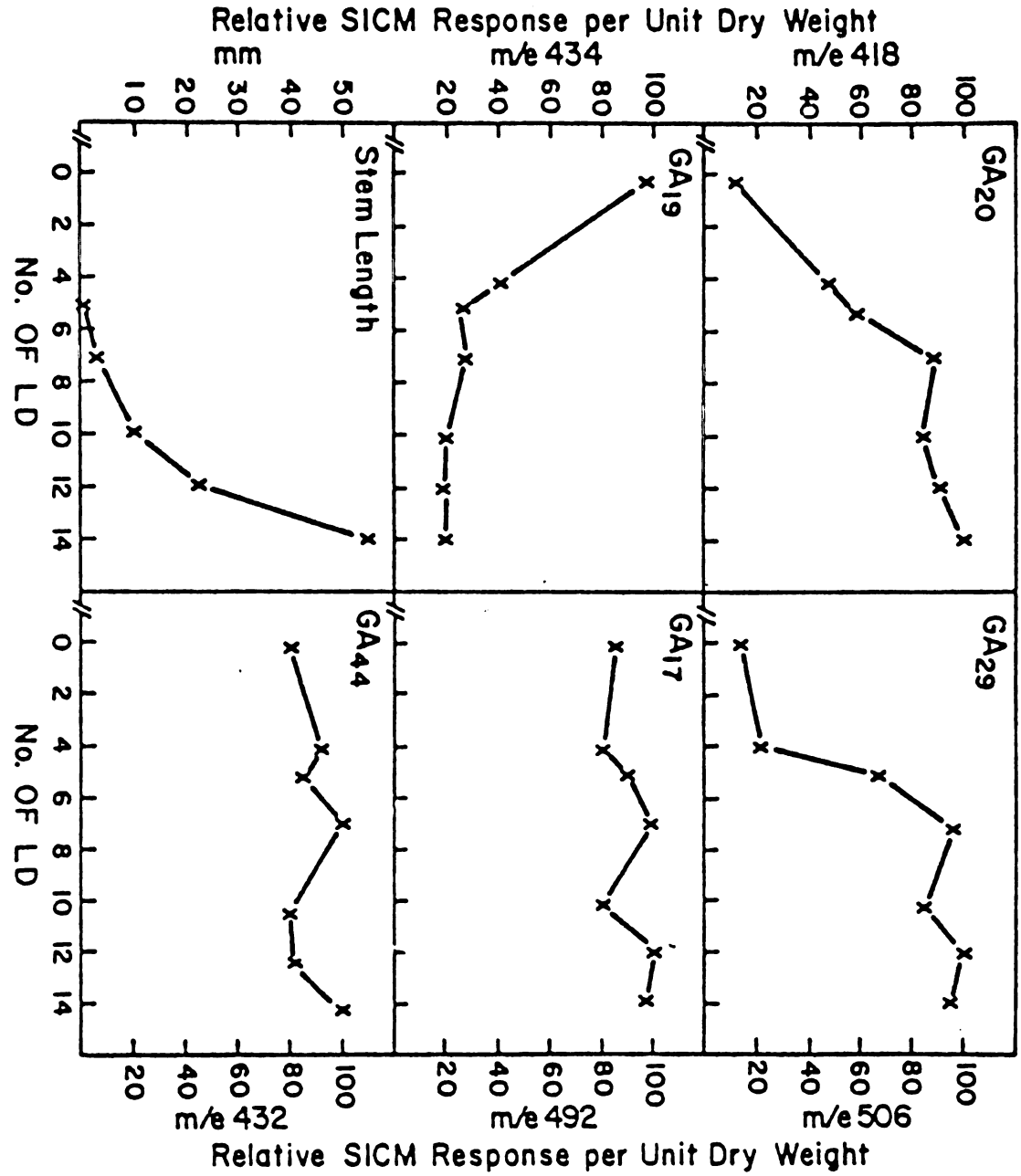
Using GLC-SICM, a standard calibration curve was constructed for GA_{20} so that the absolute amount of GA_{20} present in the plant material could be determined. This curve was linear over a range from 10 to 200 ng. The lower limit of sensitivity was 1 ng, which is about the same limit of sensitivity as the d-5 corn bioassay (Phinney and West, 1960). In contrast, at least 100 ng was necessary to get a good

spectrum using repetitive scanning GLC-MS. Because authentic samples of the other GAs present in spinach were either not available, or available in quantities too small to permit accurate weighing, standard calibration curves for these GAs could not be constructed. In these cases, only changes in the relative levels could be expressed. The largest SICM response for a given GA from the series of photoperiodic treatments was normalized to a unit dry weight basis and then arbitrarily assigned a value of 100. Normalized values from the other treatments were then expressed in proportion to the highest SICM value.

In order to provide for an estimate of losses incurred during the purification procedures, a spike of 50,000 dpm of [^{3}H]-GA_1 was added to a separate methanolic extract of spinach shoots, and the extract purified as described in section 3.2.2. Typically, 50-60% of the radioactivity was recovered after analytical reverse phase HPLC. Similar results were obtained by Jones and Zeevaart (1980b) using the same purification procedures with extracts of shoots from Agrostemma githago. These estimated losses were not used as correction factors in any of the quantitative determinations made in this chapter.

Figure 3-1 shows the changes in the relative levels of 5 GAs as well as stem height as a function of LD treatment. GA_{53} occurred in quantities too small to be measured. It is clear from this figure that the relative level of GA_{19} declined 5-fold with LD treatment, whereas the levels of both

Figure 3-1. Changes in the relative levels of five GAs in spinach as measured by GLC-SICM, and stem length as affected by different durations of LD treatment. The highest concentration (SICM response/unit dry weight) of each GA was arbitrarily assigned a value of 100, and the other concentrations expressed in proportion to this value. The SICM response of the molecular ion was used in all calculations except for GA_{19}, in which case the base peak was used. Ten plants were used in each treatment.


Relative SICM Response per Unit Dry Weight
mm
m/e 434
m/e 418
Stem Length
GA19
GA20
No. OF LD
GA44
GA17
GA29
m/e 432
m/e 492
m/e 506
Relative SICM Response per Unit Dry Weight

GA_{20} amd GA_{29} increased dramatically during the same period. The levels of GA_{17} and GA_{44} remained fairly constant throughout photoperiodic treatment. In absolute terms, the level of GA_{20} increased from 0.8 μg/100 g dry weight (30 ng/plant) to 5.5 μg/100 g dry weight (200 ng/plant), a nearly 7-fold increase. The realtive changes in the levels of GA_{19} and GA_{20} with LD treatment as measured by GLC-SICM confirm earlier work on spinach by Zeevaart (1971; 1974), using the d-5 corn bioassay for quantitating the levels of GA-like substances. However, GA_{29}, which reportedly has very little biological activity in the d-5 corn bioassay (Yokota et al., 1971), would have been overlooked in the earlier work. In Chapter 2, it was shown that the biological activity associated with spinach shoot extracts is due almost entirely to the combined effected of GA_{19} and GA_{20} which give similar responses in the d-5 corn bioassay (Crozier et al., 1970). This indicates that in absolute terms, GA_{19} occurs in a similar, but inverse, range of quantities as GA_{20}.

GA_{20}, when applied to plants maintained under SD, was able to substitute (at least partially) for LD in eliciting stem growth (Table 3-1). Zeevaart (1974) found that exogenous GA_{20} could completely replace LD treatment in causing increased petiole elongation and changing leaf orientation (position). Moreover, GA_{20} was more active than GA_3 (Zeevaart, 1974).

Table 3-1. Comparison of the effect of exogenous GA_{20} and LD on stem growth of spinach plants. GA_{20} was applied to the tips of plants, on alternate days, 20 µg per plant, for a total of 100 µg of GA_{20} per plant. Stems of the plants were measured 7 days following the last GA_{20} application. Other plants given 17 LD. Five plants per treatment.

Treatment	Stem Length (mm)
SD	0
SD + GA_{20}	75 ± 6[1)]
17 LD	123 ± 15

[1)] Standard error of the mean.

3.4 DISCUSSION

In Chapter 2 the following metabolic pathway, based on structural considerations, was proposed to occur in spinach: $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{17} \rightarrow GA_{20} \rightarrow GA_{29}$ (Fig. 2-5). The decline in the level of GA_{19} with the concomitant rise in the level of GA_{20} with LD treatment is indicative of a precursor-product relationship between the two GAs. Moreover, the co-occurrence of GA_{19} and GA_{20} in a number of disparate species, including Agrostemma githago (Jones and Zeevaart, 1980b), Pharbitis nil (Jones et al., 1980), Phaseolus coccineus (Graebe and Ropers, 1978), Pisum sativum (Ingram and Browning, 1979), and Zea mays (Phinney, 1979), is also circumstantial evidence supporting the notion that GA_{19}, a C_{20}-GA, is eventually converted to GA_{20}, a C_{19}-GA. However, as yet no definitive biochemical evidence is available that proves such a conversion takes place (Graebe and Ropers, 1978; Hedden et al., 1978).

The initiation in the rise of the level of GA_{29} lagged slightly behind the increase in the amount of GA_{20} (Fig. 3-1), and was also observed in a second experiment (results not shown). This is consistent with the idea that GA_{20} is converted to GA_{29}. Indeed, this conversion has been demonstrated in a number of systems, including Pisum sativum (Frydman et al., 1974), Bryophyllum daigremontianum (Durley et al., 1975), and Phaseolus vulgaris (Yamane et al., 1977). Thus, it is probable that such a conversion is also a natural process in spinach. Since C-2 β hydroxylated GAs, such as GA_{29}, are

usually inactive in eliciting GA responses, conversions of the type proposed above are generally thought to be inactivation steps (see 1.3.1.).

Stem growth is preceded by a precipitous rise in the level of GA_{20}. Since exogenous GA_{20} is able to cause stem growth in spinach plants maintained under SD, a major factor in the control of stem growth in spinach could be the availability of endogenous GA_{20}. The level of GA_{20} is controlled through a balance of production and metabolism. If GA_{19} is a precursor of GA_{20}, the data presented in Fig. 3-1 indicate that the step(s) $GA_{19} \longrightarrow GA_{20}$ is under photoperiodic regulation. This suggests that one major aspect of photoperiodic control of stem growth in spinach is effected through regulation of this conversion. The fact that GA_{19} is at its highest level during SD could mean that it is itself inactive in promoting stem growth, and must be converted to biologically active GA_{20}. Consequently, GA_{19} could serve in spinach as a "pool" gibberellin, a role postulated for GA_{19} in rice (Kuroguchi *et al.*, 1979). Obviously, the exact metabolic relationship between the endogenous GAs must be ascertained before a clear picture of the mechanism of photoperiodic control of stem growth can be made.

Chapter 4

Comparison of the Levels of Endogenous Gibberellins in Roots and Shoots of Spinach in Relation to Photoperiod

4.1 INTRODUCTION

In Chapter 3 it was suggested that photoperiod controls stem growth in spinach by regulating the level of GA_{20}. However, the mechanism of regulation of this particular aspect of GA metabolism is not known. A priori, the levels of individual GAs in a particular organ could be regulated in at least two ways. Most obvious would be a direct control of the organ's enzymes responsible for GA biosynthesis and metabolism. It is also possible that transport of GAs or GA precursors and intermediates could play a significant role in regulating developmental events (see section 1.5). In fact, GAs are known to be present in the phloem and in the xylem, and appear to be transported in tissues over long distances (King, 1976; Graebe and Ropers, 1978). Went (1943) originally proposed that substances produced in the root exert a hormonal control over shoot growth. Later, it was suggested by other investigators that these substances are, at least in part, GAs (Reid et al., 1969; Reid and Crozier, 1971). However, the conclusions reached by these authors are disputed by others (see 1.5; Graebe and Ropers, 1978). Thus, although GAs appear to be transported in the plant over long distances, the physiological importance of such movement remains unclear.

This chapter is a preliminary study on the possible role of root-shoot interactions in GA-mediated growth responses in spinach. First of all, the GA content of roots and shoots in relation to photoperiod was analyzed, with special attention to the distribution between roots and shoots of the six GAs previously identified in spinach shoots (Chapter 2). Secondly, as an indication of the movement of GAs between roots and shoots, the GA content of both phloem and xylem exudate was examined. Finally, in an effort to ascertain the sites of GA production, the GA content of excised root tips grown in culture was determined.

4.2 MATERIALS AND METHODS

4.2.1. Plant Culture and Photoperiodic Treatments. Spinach seeds (2.2.1.) were sown on vermiculite. After 10 days the seedlings were transferred to 37 x 30 x 22 cm trays outfitted with a cover to hold 20 seedlings. The trays were filled with half-strength Hoagland's solution, which was continuously aerated. During the course of an experiment, the medium was changed once a week. The plants were maintained under SD (3.2.1.) until ready for experimentation, approximately six weeks after sowing. At the end of an experiment, whole plants were harvested and divided into roots and shoots. Both parts were frozen in liquid N_2, lyophilized, and stored at -15^{o}C prior to extraction.

4.2.2. Effect of Photoperiod on Levels of Extractable GA-like Substances from Roots and Shoots. The details of the

extraction and purification procedures have been described in 2.2.2. Methanolic extracts of roots or shoots were purified by charcoal adsorption chromatography and silicic acid adsorption chromatography. The eluate resulting from silicic acid adsorption chromatography was fractionated by preparative TLC. The chromatogram was divided into 10 equal zones and each zone analyzed for the presence of GA-like substances using the d-5 corn bioassay (2.2.4.).

4.2.3. Identification of GAs in Root Extracts. Methanolic extracts of the roots from 500 plants (ca 100 g dry weight) were purified as described before and then fractionated via preparative reverse phase HPLC (2.2.2.). Fractions known to contain spinach shoot GAs in this system were purified further by analytical reverse phase HPLC (2.2.2.). Appropriate fractions resulting from analytical HPLC were methylated with ethereal diazomethane. The trimethylsilyl ethers of the methyl esters were prepared by adding 50 µl of a solution containing pyridine-hexamethyldisilazane-trimethylchlorosilazane (9:3:1, v/v) to dry samples in Reactivials (Pierce Chemical Co.). The derivatized samples were then subjected to GLC-MS under the same conditions described before (2.2.7.).

4.2.4. GA Content of Phloem Exudate. Phloem exudate was collected from detached spinach leaves using the method of King and Zeevaart (1974). Leaves from 100 plants were detached and placed into beakers containing a 20 mM solution of EDTA (pH 7.0). The beakers contained enough solution so

that only the cut surface and a few mm of the petioles were exposed to the EDTA solution. After 2 hr the treated ends of the petioles were rinsed with distilled H_2O, and phloem exudate was then collected in double-distilled H_2O over the next 10-12 hr. The phloem exudate was frozen, lyophilized, and the dry weight of the residue determined. The residue was taken up in 50 ml of 0.1 M phosphate buffer that was previously adjusted to pH 2.5 with 6 N HCl and partitioned 4 times with equal volumes of ethyl acetate. The acidic ethyl acetate fraction was concentrated, subjected to preparative TLC, and the GA content determined as described earlier.

4.2.5. GA Content of Xylem Exudate. Spinach plants were individually grown hydroponically in 4 liter bottles that were wrapped in aluminum foil to keep the root system in darkness. Plants were maintained under SD for 4 weeks and then given 7 LD. The plants were decapitated and latex tubes placed over the cut ends. Xylem exudate was collected via the latex tubes in flasks packed in ice. Collection of the xylem exudate continued for 12 hr. The xylem exudate from 100 plants was pooled and the pH of the exudate adjusted to 2.5 with 6 N HCl. The acidified xylem exudate was partitioned 4 times with equal volumes of ethyl acetate. The GA content of the acidic ethyl acetate fraction was determined after fractionation by TLC as described before.

4.2.6. GA Content of Spinach Roots Cultured in Vitro. Spinach seeds were surface-sterilized by treatment with water saturated with bromine for 7 min. The seeds were then rinsed

thoroughly 4 times with sterile distilled water. Following the last rinse, the seeds were sown on sterile 1% agar in Petri dishes, and then placed in the dark at 27°C. After 7 days 10 root tips, 0.5 cm long, were excised and placed in a 125 ml Erlenmeyer flask containing 50 ml of modified White's medium (White, 1943). The only modification of the original formulation was replacement of the $Fe_2(SO_4)_3$ with 500 mg of Sequestrene (Geigy Industrial Chemicals) in the stock solution. The root tips were allowed to grow for 2 weeks in the dark at 27°C. During that period, the roots grew to a length of 15-20 cm.

A total of 2200 cultured roots were lyophilized (dry weight = 4.1 g), extracted, and analyzed for the presence of GA-like substances as described in section 4.2.2. The medium in which the roots were cultured was also analyzed for the presence of GA-like substances. Approximately 12 liters of culture medium in 500 ml batches were acidified to pH 2.5 with H_3PO_4, and then pumped through a column (1 x 25 cm) packed with Bondapak C_{18}/Porasil B (Waters Associates). GAs were eluted with 250 ml of 95% ethanol. Fifteen ml of phosphate buffer (pH 8.2) were added to the eluate and the ethanol removed under reduced pressure. The pH of the aqueous residue was reduced to 2.5, and the solution was partitioned 4 times with equal volumes of ethyl acetate. The acidic ethyl acetate fractions from 24 similar runs were combined, reduced to a small volume, and fractionated by TLC as described earlier. The resulting chromatogram was then analyzed for

the presence of GA-like substances as described earlier (4.2.2.).

4.2.7. [^{14}C]-Labeling of Assimilates. Blades of intact spinach leaves were enclosed in a 17 x 31 x 2 cm clear Plexiglas chamber. $^{14}CO_2$, generated by reacting 2 mg of Ba $^{14}CO_3$ (New England Nuclear, 59.7 mCi/mM) and 3 mg of non-radioactive $BaCO_3$ with a few ml of 20% lactic acid, was circulated over the enclosed blades for 8 min. During that time the blades were irradiated by light from two 250 W flood lamps filtered through 5 cm of water.

4.2.8. Transport of [^{3}H]-GA_{20} and [^{14}C]-Labeled Assimilates. Two plants were grown hydroponically under SD for 4 weeks as described in 4.2.5., and then given 7 LD. Each plant received a foliar application of 4.0 x 10^5 dpm of [2,3-^{3}H]-GA_{20} (a gift from Dr. R. P. Pharis, University of Calgary, Alberta, Canada) dissolved in an aqueous solution of 0.05% Tween 20 and 10% ethanol. The [2,3-^{3}H]-GA_{20} was diluted with cold GA_{20} from an original specific activity of 3.3 Ci/mmol to 25 mCi/mmol. After 24 hr the roots were harvested, frozen in liquid N_2, and lyophilized. The freeze-dried root systems were extracted, purified and fractionated by TLC as described in 4.2.2. The resulting chromatogram was divided into 10 zones, and each zone eluted. The resulting eluates were dried on cellulose powder in Packard Combustocones. Each of these was combusted in a Packard model 306 Tri-Carb sample oxidizer for 45 sec, and then counted for 5 min in vials containing 15 ml of Packard Monophase-40 and 2 ml of Permafluor V using

a Packard model 3255 Tri-Carb liquid scintillation spectrometer. The counting efficiency was determined, and the data converted to dpm.

In other experiments simultaneous transport of exogenous [^{14}C]-sucrose and [^{3}H]-GA_{20} out of spinach leaves was followed. A small circle, 1 cm in diameter, on a leaf from a plant subjected to 10 LD, was lightly abraded with silicon carbide powder (400 grit, Sargent-Welch). The abraded area was then bounded with a wall of softened lanolin. Fifty µl of a 5% ethanol and 0.5% Tween solution in water containing 0.5 µCi of [^{14}C]-sucrose (New England Nuclear, 658 mCi/mM) and 0.5 µCi of [^{3}H]-GA_{20} (3.3 Ci/mM) were applied in the lanolin well. To prevent evaporation, the well was covered with a small square of polyethylene plastic film. Two leaves of the same size (blade ca 7 cm long) from a plant were similarly treated, and placed under high intensity light (33 $W m^{-2}$) in a growth chamber. After 1 hr the 2 treated leaves were detached, and the cut end of the petiole from one of the leaves was treated with a 20 mM EDTA solution (see 4.2.4.) and the other treated with double-distilled H_2O (pH 7.0). The leaves in the treatment solutions were placed in a dark, humid incubator at 27^{o} for 1 hr. The cut end of the petiole from each leaf was rinsed with double-distilled H_2O, and then placed into a scintillation vial containing 2 mls of H_2O, and allowed to exude in the dark at 27^{o}C for 16 hr. The H_2O in the scintillation vial was evaporated with a stream of N_2, and 10 ml of Formula I scintillation cocktail (King and Zeevaart, 1974)

added. ^{14}C and ^{3}H were counted simultaneously using a Packard model 3255 Tri-Carb liquid scintillation spectrometer.

In a similar experiment, 2 leaves from plants subjected to 10 LD were allowed to photosynthesize in the presence of $^{14}CO_2$ as described in section 4.2.7. Following labeling, the leaves were excised and treated with an EDTA solution or double-distilled H_2O as described above. After pre-treatment, the leaves were allowed to exude into a scintillation vial containing 2 ml of H_2O for 8 hr. The H_2O was evaporated, and the residue counted as described before.

4.2.9. Carbohydrate and Protein Analysis of Phloem Exudate. Total carbohydrate was determined by a phenol-sulfuric acid test (Aminoff et al., 1970). Fifty µg of dried phloem exudate were dissolved in 2 ml of double-distilled water. Fifty µl of 80% phenol were then added, followed by 5 ml of H_2SO_4. After the solution had cooled, absorption at 488 nm was measured. Quantitative determinations were made after interpolation of the reading on a sucrose standard curve.

The characterization of individual component sugars in the phloem exudate was performed by comparison of the R_f in TLC of various authentic sugars and compounds in the phloem exudate. Ten µg of the phloem exudate residue in 10 µl of water were spotted on a 20 x 20 glass plate coated with a 0.25 mm thick layer of Silica Gel 60 (EM Reagents), along with 10 µg of 5 reference sugars: glucose, fructose, sucrose, raffinose, and stachyose. The plate was then developed 4

times to 15 cm from the origin in ethyl acetate:acetic acid: water (60:30:12, v/v). The compounds were visualized by spraying the chromatogram with a mixture of acetic acid: sulfuric acid:p-anisaldehyde (50:1:0.5, v/v) and heating for a few minutes at 100°C.

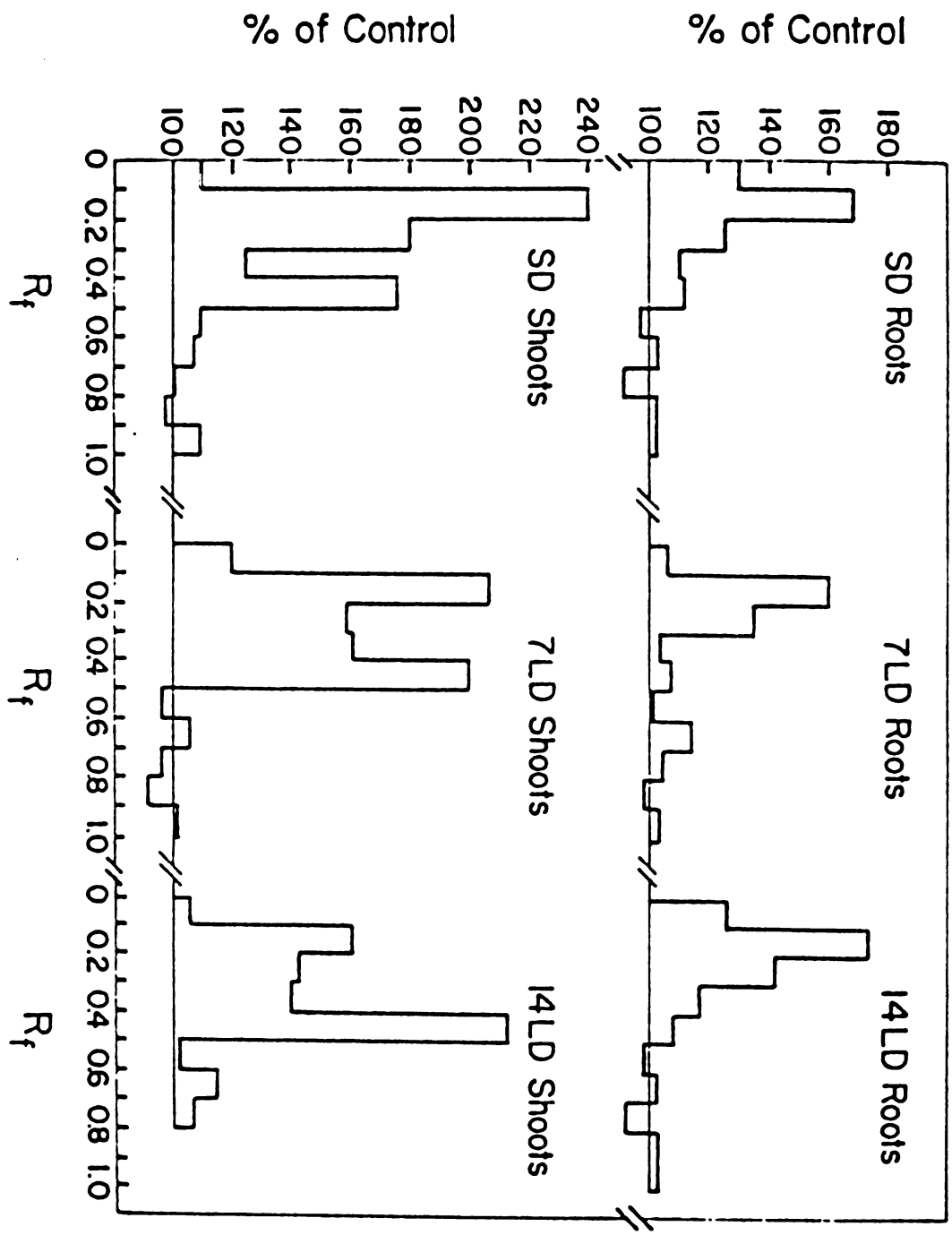
Protein determinations were made by the method described by Bradford (1976) employing Coomassie Brilliant Blue G-250 (Sigma).

4.2.10. Autoradiography. Three plants were grown hydroponically in SD as described in 4.2.5. Following 10 LD, 2 leaves from each plant were labeled with $^{14}CO_2$ (4.2.7.) and placed back into the growth chamber. After 2, 4, or 6 hr, a root system was detached, pressed between 2 pieces of cardboard, and frozen in liquid N_2. The root systems were then lyophilized. Kodak Industrial M-54 X-ray film was exposed to the freeze-dried root systems for 7 days. Development of the film was by standard procedures.

4.3 RESULTS

4.3.1. Effect of Photoperiod on Levels of GAs in Shoots and Roots. The approximate dry weight ratio of shoot material to root material from hydroponically grown plants was 5:1. Therefore, it was necessary to extract the roots from 80 plants as compared to shoots from 12 plants in order to have comparable amounts of dry plant material. Figure 4-1 shows the GA content of extracts from roots and shoots as affected by different durations of LD treatment. In

Figure 4-1. The effect of photoperiod on GA-like activity in roots and shoots. Partially purified acidic extracts were fractionated by TLC, and the resulting chromatograms divided into 10 equal zones. Each zone was assayed for the presence of GA-like substances using the d-5 corn bioassay. Roots from 80 plants (20 g dry weight), shoots from 12 plants (15 g dry weight).


% of Control
% of Control
SD Roots
7LD Roots
14LD Roots
SD Shoots
7LD Shoots
14LD Shoots
100
120
140
160
180
200
220
240
0
0.2
0.4
0.6
0.8
1.0
R_f

agreement with previous work by Zeevaart (1971), extracts of shoots showed the presence of two GA-like substances which change in level with LD treatment. The level of a polar GA (R_f 0.1-0.3), which has been identified as GA_{19}, declined with LD treatment, whereas the level of GA_{20} (R_f 0.5) increased during the same period (Chapter 2, 3). Roots, on the other hand, contained only one zone with GA-like activity with chromatographic properties identical to that of the shoot GA_{19}. The level of this substance(s) remained constant with different durations of the LD treatment. The root extracts showed no GA-like activity in the zone where GA_{20} chromatographs regardless of photoperiodic treatment. It should also be noted that the shoots had a higher GA content than roots whether expressed on a per unit weight basis (3 times) or per plant basis (20 times).

4.3.2. Identification of Root GAs. In view of the above results, it was of interest to see which of the six GAs previously identified in spinach shoots (Chapter 2) were present in root extracts. Therefore, it was necessary to extract and purify roots from ca 500 plants. Various fractions were derivatized and then analyzed by GLC-MS. Similar preparations from shoots of the same plants were used for analysis by GLC-MS. Full repetitive scans of various root fractions indicated the presence of three substances which had identical retention times and similar mass spectra to three GAs previously found in shoots (Chapter 2): GA_{44}, GA_{19}, and GA_{29} (Table 4-1). Neither GA_{53}, GA_{17}, nor GA_{20} (Fig. 2-5),

all of which were found in the shoot extracts, was detected in root extracts by repetitive scanning mass spectrometry. However, the lower limit of detection by this technique was 100 ng of GA_{20} per injection. Presumably, this figure would be similar for other GAs as well. It is possible that GA_{53}, GA_{17}, and GA_{20} were present in minute quantities and therefore escaped detection by GLC-MS with repetitive scanning. A more sensitive, albeit less definitive, detection technique was GLC-SICM which increased sensitivity by 100 times to 1 ng GA_{20} per injection. Using this technique, a compound was detected with the same retention time and with percentages for 4 m/e values similar to those of MeTMS-GA_{53} (Table 4-2). From these data it can be concluded that GA_{53} is present in trace amounts in the roots. No evidence was obtained by GLC-SICM for the presence of either GA_{20} or GA_{17} in root extracts.

4.3.3. GA Content of Phloem Exudate. Figure 4-2 shows the GA content of phloem exudate from plants subjected to either SD or 10 LD. It is clear that the pattern of GAs detected by bioassay was similar to that in extracts from leaves. The concentration of GA-like substances can be calculated, assuming that sugar constitutes about 15% (w/v) of the phloem contents, and that sugar comprises almost all of the dry material (Pate, 1976; Ziegler, 1975). The volume of phloem exudate can then be calculated from the dry weight of the exudate (Table 4-3). The total GA-like substances found in the phloem exudate can be determined by interpolation

Table 4-1. Comparison of GLC-MS data from purified extracts of spinach roots and shoots.

Sample	Time of scan	Peaks in mass spectrum (m/e values) with relative abundances in parenthesis.						
				MeTMS-GA_{19}				
Root	11.6	462(M^+,7)	447(4)	434(100)	402(30)	374(50)	208(39)	207(36)
Shoot	11.6	462(M^+,8)	447(4)	434(100)	402(27)	374(50)	208(47)	207(40)
				MeTMS-GA_{44}				
Root	13.2	432(M^+,24)	417(20)	373(12)	251(6)	238(38)	208(42)	207(100)
Shoot	13.2	432(M^+,30)	417(34)	373(17)	251(6)	238(42)	208(53)	207(100)
				MeTMS-GA_{29}				
Root	12.6	506(M^+,100)	491(18)	477(5)	447(15)	389(20)	208(20)	207(40)
Shoot	12.6	506(M^+,100)	491(12)	477(5)	447(9)	389(10)	208(12)	207(41)

Table 4-2. Identification of Gibberellin A_{53} in spinach roots by GLC-SICM. Comparison of relative abundances of 4 ions obtained by GLC-MS of MeTMS-GA_{53} from shoot extracts with the same 4 ions obtained by GLC-SICM of a MeTMS derivatized extract from spinach roots. Retention time of all ions was 11.2 min.

Ion(m/e)	Shoot	Root
207 (base peak)	100	100
448 (M^+)	31	25
416 (M^+-32)	23	16
389 (M^+-59)	44	31

Figure 4-2. The effect of photoperiod on the GA-like activity in phloem exudate and leaves. Acidic extracts were fractionated by TLC and the resulting chromatogramsanalyzed for the presence of GA-like substances using the d-5 corn bioassay. Phloem exudate derived from the leaves of 100 plants, leaf extracts from 10 plants.

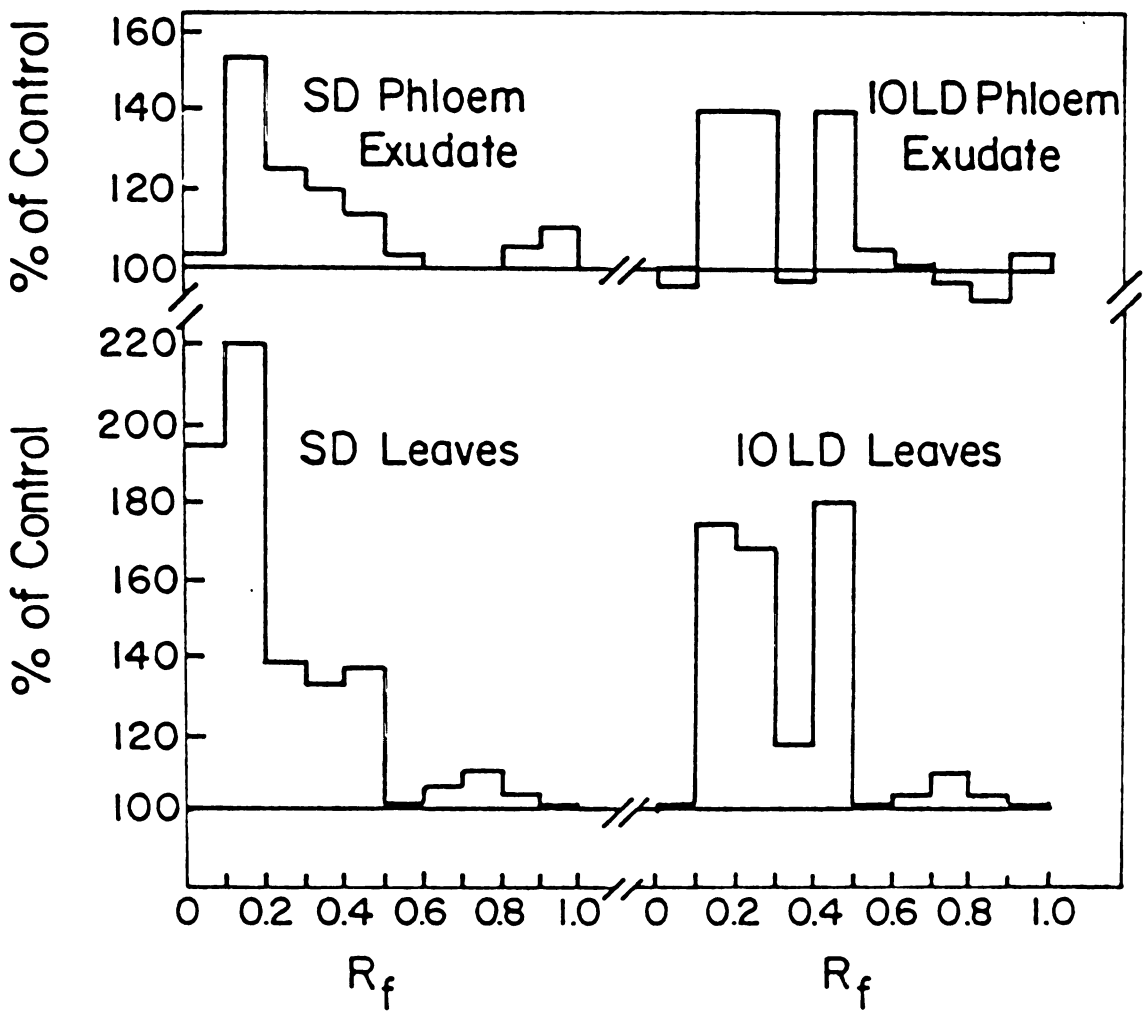

% of Control
160
140
120
100
SD Phloem Exudate
10LD Phloem Exudate
% of Control
220
200
180
160
140
120
100
SD Leaves
10 LD Leaves
0 0.2 0.4 0.6 0.8 1.0
0 0.2 0.4 0.6 0.8 1.0
R_f
R_f

Table 4-3. Comparison of the concentrations of GA-like substances in phloem and xylem exudates from 100 spinach plants under SD or LD conditions. GA-like activity was determined by the d-5 corn bioassay.

Photoperiodic treatment	Exudate	Dry weight	Volume	GA Content	Concentration
		mg	ml	µg GA_3-equivalents	µg GA_3-equivalents ml^{-1}
SD	Phloem	552	3.7[1)]	3.2×10^{-2}	86×10^{-4}
10 LD	Phloem	882	5.9[1)]	4.8×10^{-2}	81×10^{-4}
7 LD	Xylem	-	1000	10.0×10^{-2}	10^{-4}

[1)] Calculated on the assumption that phloem sap contains 15% (w/v) solute.

of bioassay data from a GA_3 standard curve. The total concentration of GA-like substances in phloem exudate from plants after SD or 10 LD was quite similar: 86×10^{-4} µg ml^{-1} and 81×10^{-4} µg ml^{-1} for SD and 10 LD phloem exudate, respectively (Table 4-3). These results were very similar to those found in 2 other experiments. The concentrations of GA-like substances found in spinach phloem exudate is in the same range as found in other species (Table 4-4). Unfortunately, the logistics of obtaining sufficient amounts of exudate for analysis by GLC-MS precluded identification of the GAs present. However, the close similarity of the GA patterns found in phloem exudate and in leaf extracts indicates that GA_{19} and GA_{20} are the major GAs present in the exudate.

4.3.4. Does EDTA Treatment Enhance Phloem Exudation or Increase General Cell Leakage? EDTA treatment was necessary in order to obtain any GA-like activity or significant dry matter in the exudate (data not shown). It is possible that EDTA damages the plasmamembrane of cells at or near the cut surface, thereby causing loss of cellular components. Therefore, several experiments were performed to ascertain the origin of substances in EDTA-enhanced exudates.

Chemical analysis of the composition of the exudate revealed that soluble carbohydrates represented about 85% of the dry weight, whereas only 1.0-1.5% of the dry weight was protein. It is not known what other substances were present in the exudate. TLC of the dry material showed that most of the carbohydrate was sucrose, but a faint spot corresponding

Table 4-4. Comparison of concentration of GA-like substances in phloem exudate from various species.

Species	Concentration (μg GA_3-equivalents ml^{-1})	Reference
Spinach[1)]	81.0×10^{-4}	This thesis
Robinia pseudoacacia L.	6.0×10^{-4}	Kluge et al., 1964
Tilia cordata	45.0×10^{-4}	Kluge et al., 1964
Quercus robur L.	17.0×10^{-4}	Kluge et al., 1964
Fagus sylvatica L.	11.0×10^{-4}	Kluge et al., 1964
Ricinus communis L.	230.0×10^{-4}	Hall and Baker, 1972

[1)] After exposure to 10 LD.

to glucose/fructose could be seen (Fig. 4-3). Sucrose is the major transport sugar in a variety of angiosperm families, including Chenopodiaceae, of which spinach is a member (Crafts and Crisp, 1971). Furthermore, the pH of the exudate collection solution was high: 7.8. Thus, these chemical and physical properties of the EDTA-enhanced exudate in spinach are similar to those of phloem exudates collected by different techniques from other plants (Ziegler, 1975).

EDTA pretreatment was also necessary for the continued movement of newly-synthesized assimilates out of the detached leaf. When leaves were allowed to photosynthesize in the presence of $^{14}CO_2$, only leaves with the cut surface of the petiole treated with EDTA showed significant radioactivity in the exudate collection solution (Table 4-5). Since chemical analysis has shown that most of the dry residue in EDTA-enhanced exudation is sucrose (the major transport sugar in spinach), it is likely that much of the radioactivity in the exudate is in the form of sucrose. Such selectivity in the enhancement of the exudation of transport sugars suggests that EDTA acts by maintaining the phloem in an "unblocked" state (King and Zeevaart, 1974). Thus, EDTA treatment causes enhancement of phloem exudation rather than non-specific leakage from damaged cells near the cut surface of the petiole. It appears, then, that EDTA treatment is a valid experimental technique, useful in obtaining relatively pure phloem exudate in sufficient quantities for the study of hormone transport.

Figure 4-3. Comparison of chromatographic behavior of substances in phloem exudate and 5 authentic sugars. Ten μg each of lyophilized phloem exudate, glucose, fructose, sucrose, raffinose, and stachyose were chromatographed 4 times on silica coated glass plates. The compounds were visualized by spraying the chromatogram with a mixture of acetic acid: sulfuric acid and p-anisaldehyde (50:1:0.5, v/v) and heating a few min at 100°C. The colored spots were outlined with a no. 2 pencil, and a photocopy made of the chromatogram.

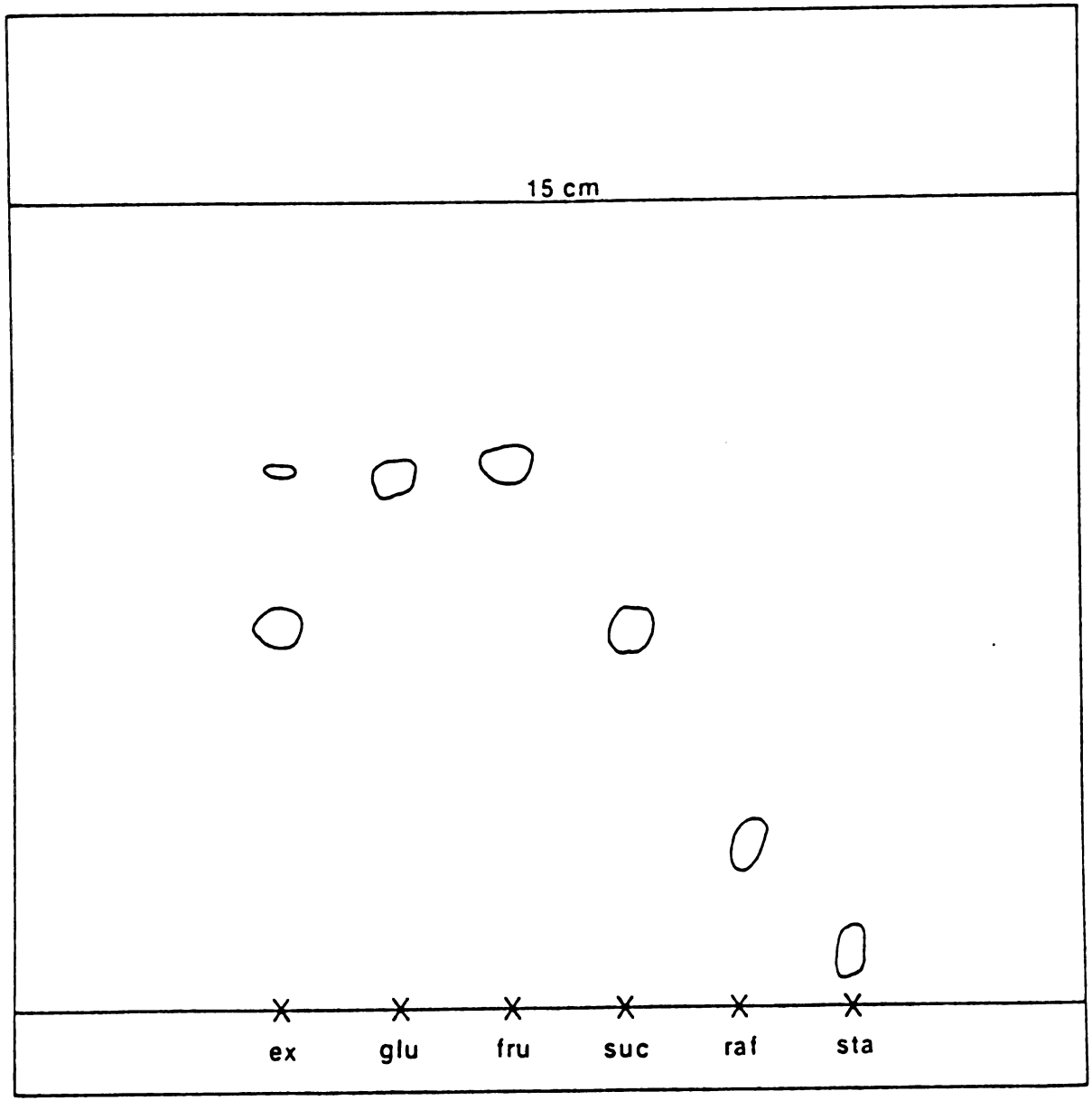

15 cm
ex
glu
fru
suc
raf
sta

Table 4-5. The effect of EDTA pretreatment on radioactivity in the phloem exudate collection solution. Two leaves from each plant were allowed to photosynthesize in the presence of $^{14}CO_2$. Immediately following labeling, both leaves were excised, and the cut end of the petiole of one was treated with 20 mM EDTA, and the other treated with water for 1 hr. Phloem exudate was collected in 2 ml H_2O over the next 10 hr. Four replicates.

Plant	EDTA	cpm
1	+	2,041
1	-	0
2	+	16,615
2	-	5
3	+	31,237
3	-	20
4	+	55,429
4	-	213

4.3.5. GA Content of Xylem Exudate. Figure 4-4 shows the GA content of 1 liter of xylem exudate collected from 100 plants treated with 7 LD. It also shows for comparison the GA content of a shoot extract derived from the plants used for obtaining xylem exudate. The pattern of GA-like substances was similar for both the xylem exudate and root extracts (compare Fig. 4-1 and Fig. 4-4) in that both lack a zone of biological activity associated with the region where GA_{20} chromatographs in the TLC system used in this study. Not enough xylem exudate was available to identify the GA-like substances by GLC-MS. It is likely, however, that at least part of the biological activity in spinach xylem exudate is due to the presence of GA_{19}, since GA_{19} is present in the roots, and the chromatographic behavior of the GA-like substance(s) found in the xylem exudate was very similar to that of GA_{19}. The total GA content in the xylem exudate was calculated to be 0.1 µg GA_3-equivalents in 1 liter of xylem exudate. Consequently, the concentration of GA-like substances was 10^{-4} µg GA_3-equivalents ml^{-1}. This is in the same range as found in other species (Table 4-6). However, the concentration of GA-like substances is much higher in phloem exudate than in xylem exudate (Table 4-3).

4.3.6. Transport of [^{3}H]-GA_{20} and [^{14}C]-Assimilates from the Shoot to the Roots. Figure 4-5 (bottom) shows autoradiograms of root systems harvested 2, 4, or 6 hr after the leaves were allowed to photosynthesize in the presence of $^{14}CO_2$. Clearly, [^{14}C]-labeled photosynthate moved from the

Figure 4-4. GA-like activity in xylem exudate and shoots from plants that were used for obtaining the xylem exudate. Xylem exudate from 100 plants, shoots from 10. All plants were exposed to 7 LD prior to use. Extracts were fractionated by TLC, and analyzed by the d-5 corn bioassay.

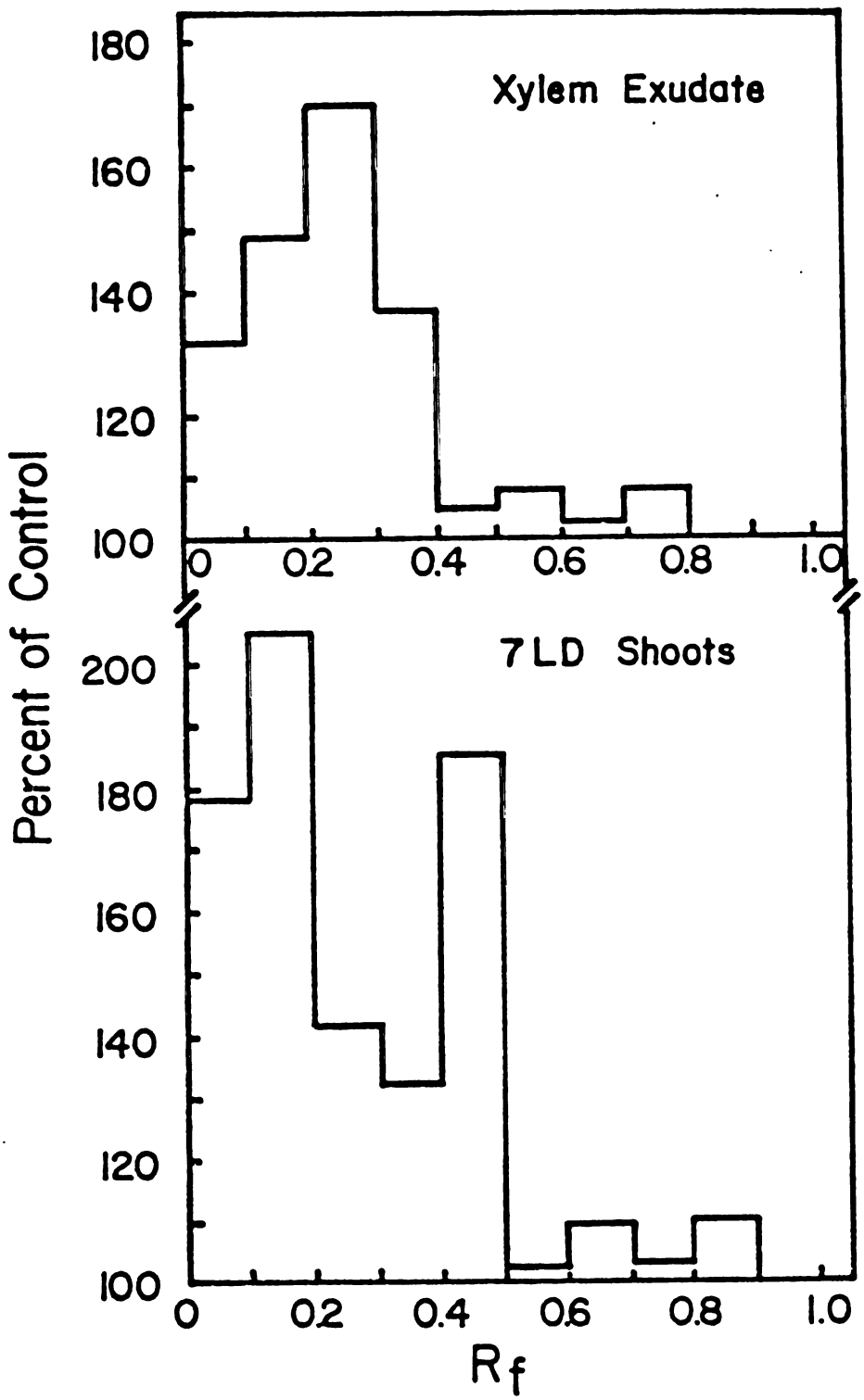
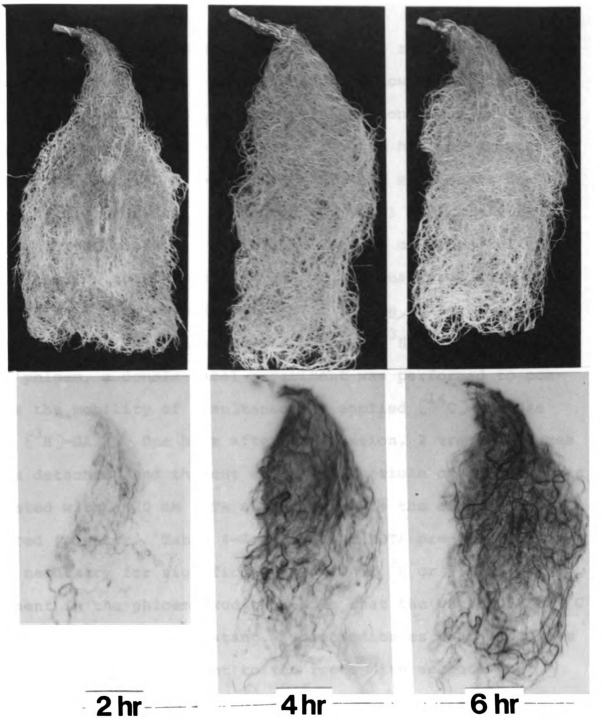

Xylem Exudate
7 LD Shoots
Percent of Control
180
160
140
120
100
200
180
160
140
120
100
0
0.2
0.4
0.6
0.8
1.0
Rf

Table 4-6. Comparison of concentration of GA-like substances in xylem exudate from various species.

Species	Concentration (μg GA_3-equivalents ml^{-1})	Reference
Spinach	1.0×10^{-4}	This thesis
Vitis vinifera	$0.7\text{-}3.0 \times 10^{-4}$	Skene, 1967
Pyrus malus	0.2×10^{-4}	Luckwill and Whyte, 1968
Impatiens glandulifera	230.0×10^{-4}	Carr *et al.*, 1964

Figure 4-5. Root systems (top) and their autoradiograms (bottom) from plants allowed to photosynthesize in the presence of $^{14}CO_2$. Roots were harvested 2, 4, or 6 hr after labeling, frozen, and lyophilized. X-ray film was exposed to the freeze-dried root systems for 7 days before development. Plants subjected to 10 LD prior to use.


2 hr
4 hr
6 hr

shoot to the roots. This movement was fairly rapid, since radioactivity was detected in the root systems detached 2 hr after labeling.

Figure 4-6 shows the distribution of radioactivity on a chromatogram of a root extract 24 hr following a foliar application of [^{3}H]-GA_{20}. Two distinct zones are apparent: a polar zone at Rf 0.1-0.2, and one which has the same Rf as authentic GA_{20} (Rf 0.4-0.5), and probably represents unmetabolized [^{3}H]-GA_{20}. Although the total radioactivity found in the roots was a small percentage of the total [^{3}H]-GA_{20} applied to the plant (0.35%), it illustrates that exogenous GA_{20} does move from the root to the shoot.

In order to test whether exogenous [^{3}H]-GA_{20} moves in the phloem, a double-label experiment was performed to compare the mobility of simultaneously applied [^{14}C]-sucrose and [^{3}H]-GA_{20}. One hour after application, 2 treated leaves were detached, and the cut end of the petiole of one leaf was treated with a 20 mM EDTA solution, while the other was placed in water. Table 4-7 shows that EDTA pre-treatment was necessary for significant amounts of ^{3}H or ^{14}C to be present in the phloem exudate. Note that the ratio of ^{3}H:^{14}C remained relatively constant. This indicates that exogenous GA_{20} moved from the shoot to the roots with sucrose and other assimilates in the phloem. Thus, it is likely that at least part of the endogenous GA_{20} present in the phloem moved to the root system.

Figure 4-6. Distribution of radioactivity on a chromatogram of a root extract from 2 plants 24 hr following a foliar application of 4 x 10^5 dpm of [2,3-^{3}H]-GA_{20} to each plant.

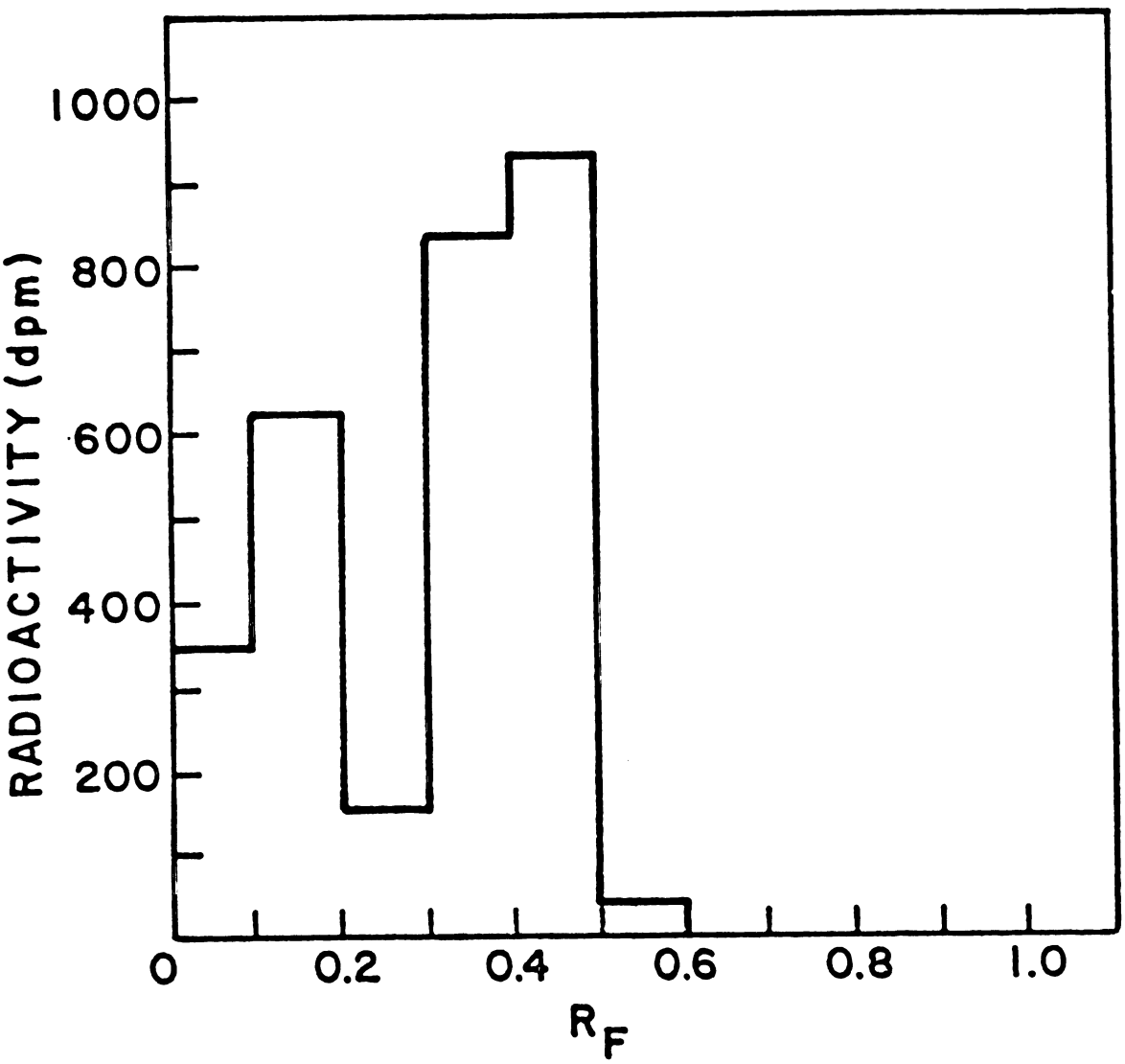

1000
800
600
400
200
RADIOACTIVITY (dpm)
0
0.2
0.4
0.6
0.8
1.0
R_F

Figure 4-7. The effect of EDTA pretreatment on the amount of radioactivity (^{3}H and ^{14}C) in the phloem exudate collection solution. Two leaves from each plant were given 0.5 µCi of [^{14}C]-sucrose and 0.5 µCi of [^{3}H]-GA_{20}. After 1 hr in the light, the leaves were excised, and one leaf was treated with a 20 mM EDTA solution and the other with H_2O for 1 hr. Phloem exudate was then collected in 2 ml of H_2O over the next 16 hr. Three replicates.

Plant	EDTA	cpm ^{3}H	cpm ^{14}C	cpm ratio $^{3}H/^{14}C$
1	+	532	804	.66
1	-	59	89	.66
2	+	3,896	5,708	.68
2	-	93	124	.75
3	+	10,849	14,577	.74
3	-	108	150	.72

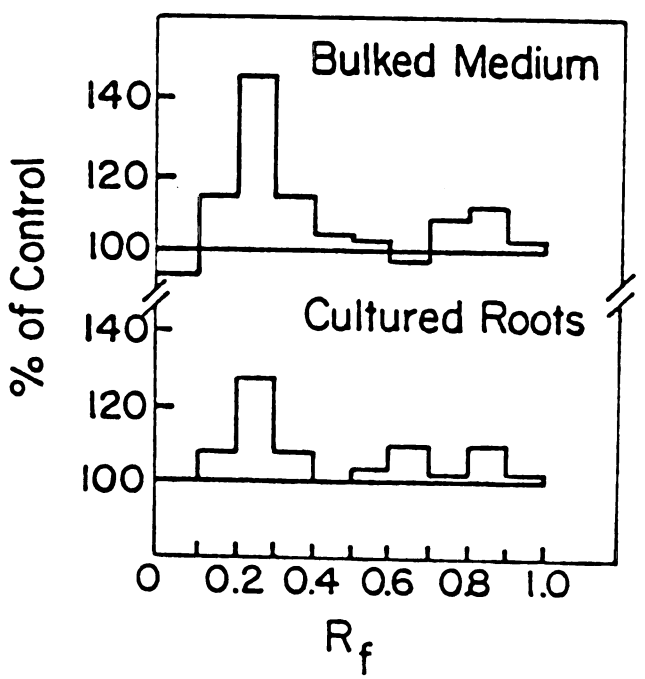
4.3.7. GA Content of Roots Cultured In Vitro. Figure 4-7 shows the distribution of GA-like substances on a thin-layer chromatogram from an acidic ethyl acetate fraction of 220 spinach roots cultured in vitro (bottom), and the culture medium in which they were grown (top). Only 1 zone of biological activity was apparent in either the cultured roots or the medium. This GA-like substance(s) had chromatographic properties similar to those of GA_{19}.

4.4. DISCUSSION

4.4.1. Distribution of GAs Between Roots and Shoots. Spinach shoots were previously shown to contain GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, and GA_{29} (Chapter 2). In this investigation, only four of the six GAs found in spinach shoots were detected in extracts of roots by GLC-MS or GLC-SICM: GA_{53}, GA_{44}, GA_{19}, and GA_{29}. Neither GA_{17} nor GA_{20} was detected in root extracts. The physiological significance of the striking qualitiative difference in the spectrum of GAs found in shoots and roots is unclear. However, since GA_{20} is present in the phloem exudate and $[^3H]$-GA_{20} applied to the leaves moves to the roots, it would appear that GA_{20} is continuously translocated from the shoot to the roots. This suggests that if GA_{20} is made in, or transported to the roots, it is rapidly metabolized in that organ. Thus, it seems that regulation of GA metabolism is very different in roots and shoots.

The very presence of $[^3H]$-GA_{20} in root extracts (Fig. 4-6) would seem to contradict the idea of rapid GA_{20} metabolism

Figure 4-7. GA-like activity in 2200 spinach roots cultured *in vitro* (dry weight = 4.1 gm) and the medium in which they were grown (12 liters). Extracts were fractionated by TLC, and analyzed by the d-5 corn bioassay.


Bulked Medium
140
120
100
% of Control
Cultured Roots
140
120
100
0
0.2
0.4
0.6
0.8
1.0
R_f

in the roots. This paradox can be explained in the following manner. The total radioactivity associated with the region of the chromatogram where GA_{20} ran was 1872 dpm. Since the specific activity of the applied [^{3}H]-GA_{20} was 25 mCi/mmol, the radioactivity present as [^{3}H]-GA_{20} in the root extracts was equivalent to 11.3 ng GA_{20}, or 5.65 ng GA_{20} per plant. However, there was only ca 0.15 ng GA_3-equivalents per plant associated with the GA_{20} region of the chromatogram from the LD phloem exudate (Fig. 4-2). Thus, the applied [^{3}H]-GA_{20} contributed 35 times more substrate to the roots than is normally transported via the phloem. This could mean that the enzymes in the roots responsible for metabolizing [^{3}H]-GA_{20} were saturated.

4.4.2. Possible Sites of GA Synthesis. The pattern of GA-like substances detected by bioassay in xylem and phloem exudate was remarkably similar to that found in roots and shotts, respectively (Fig. 4-4 and 4-2). In general, such data cannot normally be used to ascertain the sites of GA synthesis (Graebe and Ropers, 1978). But since GA_{20} was not detected in either xylem exudate or root extracts, the GA_{20} detected in shoot extracts and phloem exudate must have been synthesized in the shoots. In Chapter 3, it was suggested that in the shoots GA_{19} is converted to GA_{20}, and this conversion is under photoperiodic control.

GA_{19}, on the other hand, was detected in both root and shoot extracts, and is probably present in xylem and phloem exudate as well. It is possible that either roots or shoots

(or both) have the capacity to synthesize GA_{19}. Since LD treatment causes a decline in the level of GA_{19} in the phloem exudate, one would expect a concomitant decrease in the level of GA_{19} in extracts of roots, if the shoots are the major source of GA_{19} found in the roots. However, no such decline in levels of GA_{19} in root extracts was observed (Fig. 4-1). The maintenance of a constant level of GA_{19} can be interpreted to mean that the root system is a major source of GA_{19}, with contributions from the shoot via the phloem as a minor addition to the total GA_{19} content in the roots. However, other interpretations are also possible since the collection technique used for phloem exudate may not give an accurate picture of the flux of substances moving in the phloem from the shoot to the roots (King, 1976; Pate, 1976). Perhaps there is a compensatory increase in the rate of movement of substances in the phloem when plants are transferred from SD to LD that accounts for the steady levels of GA_{19} found in root extracts. Indeed, different light intensities are known to affect the translocation rate of assimilates in the phloem (Hatrick and Bowling, 1973).

Butcher (1962) has shown that a clone of excised tomato roots maintained in culture for five years contained GA-like substances, indicating that roots are able to synthesize GAs. Excised spinach roots cultured for two weeks, as well as the medium in which they were grown, contained a GA-like substance with chromatographic properties identical to those of GA_{19} (Fig. 4-7). This is consistent with the notion that

GA_{19} is synthesized in the roots. However, the possibility cannot be ruled out that carry-over from the germinating seed was responsible for GAs present in the cultured root tips.

4.4.3. Physiological Significance of GA Transport in the Phloem. Knott (1934) as well as Withrow et al. (1953), found that stem elongation in spinach occurred when the leaves were exposed to LD while the bud remained under SD. On the other hand, when only the bud was subjected to LD and the leaves exposed to SD conditions, stem elongation did not occur. This led Knott (1934) to hypothesize that a substance was produced in the leaves and transported to the growing point, causing stem growth. Since stem growth is correlated with an increase in the level of GA_{20} in shoot extracts (Chapter 3) and phloem exudate (Fig. 4-2), it is suggested that GA_{20} is synthesized in the leaves and transported via the phloem to the tip where it exerts control over stem growth.

4.4.4. The Physiological Significance of GA Transport in the Xylem. Skene (1967) has calculated that there was enough GA in the xylem exudate of grapevines to account for all of the GA content in the leaves of the plant simply by transport in the transpiration stream from the roots to the leaves during a 24 h period. Since GA_{19} is apparently present in the xylem exudate of spinach, it is tempting to suggest that the roots supply the shoot with GA_{19}. Assuming a transpiration rate of about 100 ml day^{-1}, approximately 0.01 μg GA_3-equivalents will pass from the roots to the shoot each day (Table 4-3). The SD shoots in Fig. 4-1 contained about 0.04 μg

GA_3-equivalents per plant in the GA_{19} region of the chromatogram. It appears, therefore, that transport of GAs from the roots to the shoot can contribute a significant portion of the GA present in the shoot. Since the site(s) of GA_{19} synthesis is not definitively known, the possibility also exists that GA_{19} is synthesized in the shoot, transported to the roots via the phloem, and then recycled back to the shoot in the xylem.

It is generally assumed that both root and shoot tips are sites of GA synthesis, and many workers feel that GAs are produced near the sites of GA utilization or action (Graebe and Ropers, 1978). However, it has also been suggested that the roots and the shoot are responsible for different steps of the total GA biosynthetic and metabolic pathway (Crozier and Reid, 1971; Kamienska and Reid, 1978). Although the data presented in this chapter are consistent with the idea of integration of GA biosynthesis and metabolism in the plant as a whole, they do not provide proof. Therefore, in order to provide for a more definitive resolution of these questions, in vitro systems must be developed to study the regulation and location of the enzymes responsible for GA biosynthesis and conversion.

Chapter 5

The Subcellular Distribution of GAs in Spinach Leaves

5.1 INTRODUCTION

In Chapter 1 it was suggested that the effective or available level of GAs might be controlled by the regulated release from some cellular compartment. At present chloroplasts seem to be the most likely candidate to fill such a role. First of all, chloroplasts from a number of species contain GA-like substances: barley and kale (Stoddart, 1968); peas (Railton and Reid, 1974); wheat (Browning and Saunders, 1977). Secondly, several enzymes in the GA biosynthetic pathway are reportedly associated with chloroplast or plastid fractions. Green et al. (1975) have shown that isopentenyl isomerase and geranylgeranyl pyrophosphate synthase, two crucial enzymes in isoprenoid biosynthesis, are associated with proplastid preparations from Ricinus communis endosperm. Kaurene synthase activity has also been demonstrated to occur in proplastids from Marah macrocarpus endosperm (Simcox et al., 1975). Finally, suspensions of etioplasts of wheat and barley have been reported to produce GA-like substances upon irradiation with red light. Etioplasts are also purportedly able to regulate the release of GAs into the medium through control of permeability of the envelope membrane by phytochrome (Cooke et al., 1975; Evans and Smith, 1976). Obviously, such a role of chloroplasts in the distribution of GAs in leaf

cells has very important implications in the control of the effective levels of biologically active GAs, as well as in the regulation of both GA biosynthesis and catabolism.

In view of these findings by other workers, it was considered possible that in spinach, LD acts to cause a redistribution of GAs which would lead to the observed physiological responses to LD treatment. This possibility was examined by analyzing spinach chloroplast fractions from plants kept under SD or LD for GA content and comparing these figures with those from whole leaf extracts.

Browning and Saunders (1977) reported that they were able to increase the yield of extractable GAs from wheat chloroplasts 1000-fold by using solutions of non-ionic detergents instead of the conventional methanolic extraction techniques. If this observation would prove true in other plant species, then levels of GAs would have been seriously underestimated. This prompted a similar investigation in spinach.

5.2 MATERIALS AND METHODS

5.2.1. Plant Material. Spinach plants were grown in 340 ml plastic cups as described in Chapter 3. The plants were maintained in controlled environment chambers under SD conditions until they were ready for experimentation, 6 weeks after sowing. Photoperiodic treatments were identical to those described in 3.2.1.

In other experiments, seeds of Triticum aestivum L. (cv. Kolibri) were sown in vermiculite. Plants were raised under

LD in growth chambers at 23°C. The light regime consisted of combined light from fluorescent and incandescent lamps (total irradiance = 12.5 W m^{-2}) for 12 hr, followed by 8 hr of light from incandescent lamps only (3 W m^{-2}). The plants were ready for experimentation 10 days following sowing.

5.2.2. Isolation of Chloroplasts. Spinach chloroplasts were isolated by the method described by Railton and Reid (1974). Approximately 100 g fresh weight of diced leaf material from 10 plantswere ground (four, 2 sec bursts at full speed) in a Waring blender containing 250 ml of ice-cold grinding medium. The grinding medium (pH 7.8) consisted of the following: 0.4 M sucrose, 0.04 M phosphate buffer, 0.25 M EDTA, 0.01 M $MgCl_2$, 0.01 M cysteine, and 0.01 M sodium metabisulfite. The resulting brei was filtered through 2 layers of cheesecloth and 1 layer of Miracloth (Chicopee Mills, Inc., Milltown, N.J.), and finally through Nytex nylon mesh (48 μm). The filtered brei was then centrifuged once at 300 x g for 2 min. The resulting supernatant was recentrifuged at 1200 x g for 20 min at 2°C to provide a chloroplast enriched pellet. The pellet was carefully resuspended in fresh grinding medium (4°C) and centrifuged once more at 1200 x g for 20 min. The supernatant was discarded, and the pellet was suspended in distilled H_2O, stirred vigorously, and frozen. The water was removed by lyophilization to provide a dried chloroplast fraction that was stored at -15°C until ready for extraction.

Procedures for the isolation of wheat chloroplasts were the same as those described by Browning and Saunders (1977). Ten gram lots of leaves from 10-day old plants were ground in a pre-chilled mortar and pestle lined with 48 μm nylon mesh cloth for 2 min in 100 ml of ice-cold Honda medium (Honda, 1974). This medium contained the following ingredients: 0.5 M sucrose, 24 mM Tricine, 8 mM 2-mercaptoethanol, 5 mM magnesium acetate, 50 mg/ml Dextran-15 (Sigma), 24 mg/ml Ficoll (Sigma), 1 mg/ml bovine serum albumin (Sigma), at pH 7.2. The liquid expressed through the nylon mesh was centrifuged for 2 min at 300 x g; the resulting supernatant was then centrifuged at 1000 x g for 10 min. The supernatant was discarded, and the chloroplast pellet was extracted immediately.

5.2.3. Extraction and Purification Procedures. Spinach and wheat chloroplast preparations were extracted by two different methods. In the first method, each chloroplast preparation was resuspended in 100 ml of 80% aqueous methanol. The extract was filtered, and the residue extracted overnight with 100 ml of 100% methanol. The 2 extracts were combined, and the methanol removed under reduced pressure. The aqueous residue was purified and fractionated by TLC as described earlier (2.2.4.) for whole leaf extracts.

In the other extraction technique, the procedures described by Browning and Saunders (1977) were closely followed. Chloroplast pellets from 1 preparation were resuspended in 50 ml of 2% Triton X-100 (Research Products

International) in water at pH 8.0, and stirred for 2 hr at room temperature. This extract was then passed through a 2 x 10 cm column of Dowex 1-X2 (100 mesh) anion exchange resin in the formate form (Bio-Rad), followed by a wash with 200 ml of H_2O at pH 8.0. GAs were eluted from the resin with 400 ml of ethanol: 1 M formic acid (4:1, v/v). The eluate was reduced to a small aqueous volume on a rotary evaporator. The residue was frozen and lyophilized. The dried residue was redissolved in a small volume of ethyl acetate, and fractionated by TLC as described before.

Lyophilized spinach leaves were extracted, and the extracts purified and fractionated as described in 4.2.1. Chlorophyll for all extracts was determined using the method described by Kirk (1968) employing a nomogram.

5.2.4. Bioassays. The d-5 corn bioassay was performed as described in 2.2.2. In other cases, GA-like substances were detected by the lettuce hypocotyl assay (Frankland and Wareing, 1960). The eluates resulting from TLC (2.2.4.) were dried on a piece of Whatman #1 filter paper lining the bottom of a 50 ml beaker. Ten germinating lettuce seeds (cv. Artic King) with the radicle protruding 1-2 mm from the testa, were placed in each of the beakers. The filter paper was then moistened with 2 ml of distilled H_2O. The beakers were covered with a polyethylene film, and placed in growth chambers ($27^{o}C$) with continuous light ($28\ W\ m^{-2}$). After 3 days, the length of the hypocotyl was measured. A standard curve of authentic GA_3 (Sigma) was also prepared using the

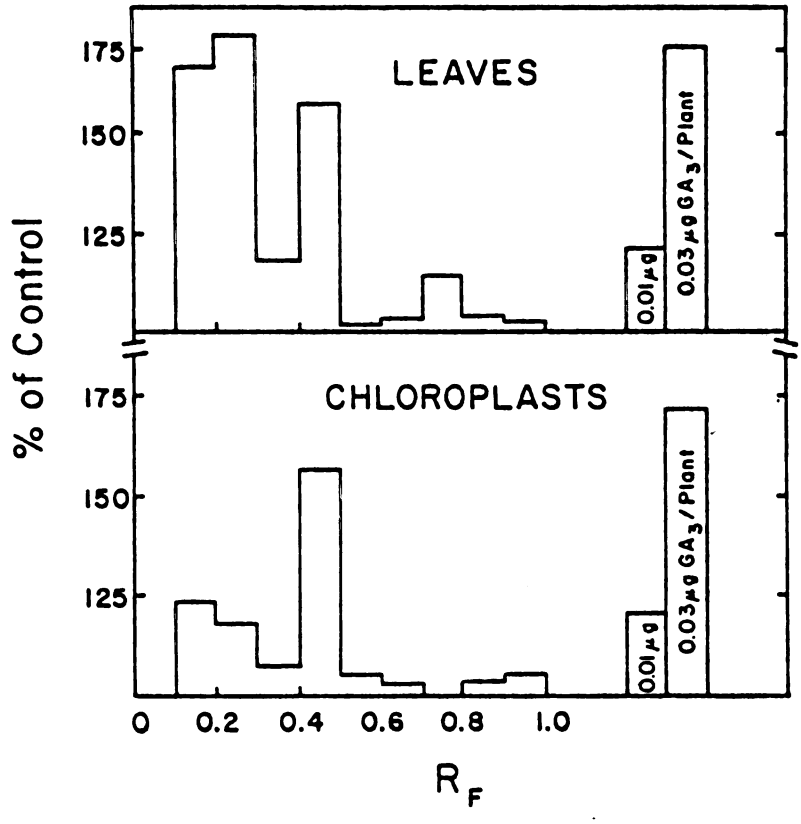
same procedures.

5.3 RESULTS

5.3.1. Subcellular Distribution of Spinach GAs. Figure 5-1 compares the pattern of GA-like substances in chloroplasts from leaves of 100 plants (10 chloroplast preparations) and leaves from 10 plants. There is an apparent enrichment of GA_{20} (R_f 0.5) in the chloroplasts over GA_{19} (R_f 0.1-0.3). When expressed on a per unit weight of chlorophyll, some 10-15% of the total leaf GA_{20} was associated with the chloroplast fraction, while only about 1% of the total leaf GA_{19} was found in the chloroplasts. Chloroplasts from SD leaves also showed a similar distribution of both GA_{19} and GA_{20}. These results were consistent in 3 separate experiments for both LD and SD chloroplasts.

5.3.2. Comparison Between Efficiency of Extraction of GAs from Spinach Chloroplasts by Methanol and Solutions of Non-Ionic Detergents. The report by Browning and Saunders (1976) that extraction of chloroplasts from wheat by Triton X-100 led to a 1000-fold increase in yield of GAs over conventional methanolic extracts prompted a similar investigation in spinach. Chloroplasts were prepared from the leaves of 100 spinach plants as described earlier. Following extraction of the chloroplasts with a solution of Triton X-100, the extracts were purified by anion exchange chromatography and then fractionated by TLC as described in 5.2.3. In 3 separate experiments, no zones of the chromatogram showed

Figure 5-1. Comparison of GA-like substances in spinach leaves and chloroplasts. Methanolic extracts were purified, and the acidic fractions fractionated by TLC. The resulting chromatogram was divided into 10 equal zones, and each zone assayed for the presence of GA-like substances with the d-5 corn bioassay. Leaves from 10 plants, chloroplasts from 100. All plants subjected to 7 LD prior to use.


LEAVES
CHLOROPLASTS
% of Control
175
150
125
0.01 µg
0.03 µg GA3/Plant
0
0.2
0.4
0.6
0.8
1.0
RF

the presence of any GA-like activity. It is possible that the negative results were due to losses in the procedure or destruction of biologically active GAs. These possibilities were tested by two additional experiments. In one experiment, a chloroplast preparation was spiked with 100,000 dpm of [^{3}H]-GA_1 (specific activity = 30 Ci/mM, New England Nuclear), extracted with a 2% Triton X-100 solution, and purified by anion exchange chromatography as described before. The eluate resulting from anion exchange chomatography was dried in a scintillation vial and counted as described in 4.2.8. The recovery of radioactivity after these procedures was 92%, indicating that GAs were not lost. In another experiment, 0.5 µg of unlabeled GA_3 (Sigma) was dissolved in 50 ml of a solution of 2% Triton X-100 at pH 8.0. The solution was subjected to anion exchange chromatography as described before. The eluate was dried, and assayed by the lettuce hypocotyl assay (5.2.4.). The response was then compared to the bioassay response of 0.5 µg of GA_3 applied directly to the test seedlings. No significant difference between the treatment and control was detected. Thus, the purification techniques apparently did not destroy GA molecules.

5.3.3. An Attempt to Repeat the Findings of Browning and Saunders with Wheat Chloroplasts. Because of the failure of Triton X-100 to increase the yield of extractable GAs from spinach chloroplasts, it was decided than an effort should be made to reproduce the findings of Browning and Saunders (1976) with the same plant material as used by these workers.

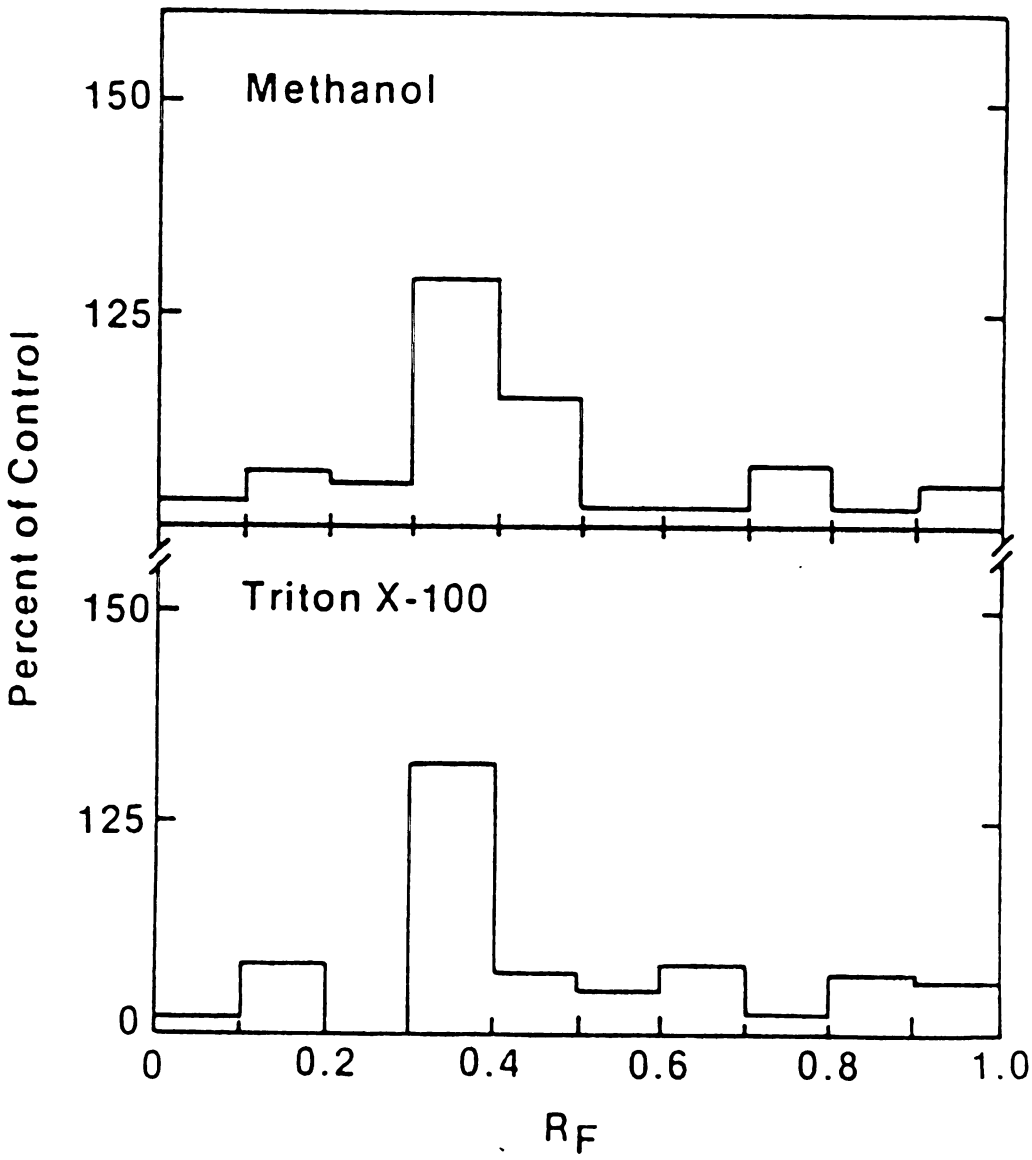
Chloroplasts were isolated from wheat (cv. Kolibri, the same variety used by Browning and Saunders) as described in 5.2.2. Every effort was made to reproduce in an identical manner the experimental procedures as described by Browning and Saunders (1976) (see 5.2.3. for details). Following fractionation by TLC, the eluates from 10 equal zones of the chromatogram were assayed for the presence of GA-like substances by the lettuce hypocotyl assay (5.2.4.). In three separate experiments, only trace amounts of GA-like activity in either methanolic or Triton X-100 extracts of wheat chloroplasts were detected (Fig. 5-2, note R_f 0.4). In no experiment was Triton X-100 any more effective than methanol in extracting GA-like substances from Kolibri wheat chloroplasts.

5.4 DISCUSSION

5.4.1. The Physiological Significance of the Distribution of GAs Between Chloroplasts and the Rest of the Leaf Cell.

In section 5.3.1., data were presented which showed that chloroplasts from spinach leaves were comparatively enriched in GA_{20} over GA_{19}. Moreover, the proportion of total leaf GA_{19} and GA_{20} found in the chloroplast fraction was the same in both LD and SD plants. This implies that photoperiod does not act through an alteration of the distribution of GAs between the chloroplast and the cytoplasm. This conclusion is correct only if the following two conditions are fulfilled:
i) no leakage of GAs occurs during chloroplast isolation;
ii) the chloroplast preparations are essentially free of

Figure 5-2. Comparison of yields of GA-like substances from chloroplasts of Kolibri wheat using a 2% solution of Triton X-100 (v/v) in water or 80% aqueous methanol as the extractant. Extracts were purified, and chromatographed on silica gel coated glass plates. The resulting chromatogram was divided into 10 equal zones, and each zone eluted. The eluates were tested for the presence of GA-like substances using the lettuce hypocotyl assay.


Methanol
150
125
Triton X-100
150
125
0
Percent of Control
0
0.2
0.4
0.6
0.8
1.0
R_F

other organelles and cytoplasmic contaminants. However, the degree to which these criteria were met was not ascertained.

In any event, the uneven distribution of GA_{19} and GA_{20} remains unexplained. Roughly 15% of the GA_{20} present in the leaf was localized in the chloroplast fraction. Stoddart (1968) found that 16% of the total GA-like substances in kale and barley leaves could be accounted for in the chloroplast fraction. Curiously, in most leaf mesophyll cells, the chloroplasts occupy between 15-20% of the total cell volume (Wilson and Loomis, 1967). Thus, if GAs are freely mobile across the chloroplast envelope membrane, the chloropast fraction would be expected to contain about the same proportion of the total cell GA as the proportion of the total cell volume that chloroplasts occupy.

The distribution of GA_{19} is not so easily explained. Only about 1% of the total leaf GA_{19} was found to be associated with the chloroplast fraction. It could be that GA_{19}, with its 2 carboxyl groups, diffuses much more slowly than GA_{20}, which has only 1 carboxyl group, across the envelope membrane. Another possible explanation is that the chloroplast could be a subcellular site of GA_{19} metabolism. If this is true, then perhaps GA_{19} is rapidly metabolized (to GA_{20}?) and never accumulates within the chloroplast. These hypotheses remain to be tested.

It is interesting to note that a similar pattern exists in peas. Chloroplasts from pea leaves were found to contain a high proportion of a GA_{20}-like substance while being

notably deficient in a GA_1-like substance (Railton and Reid, 1974). These two substances were later identified as GA_{20} and GA_{19}, respectively (Ingram and Browning, 1979). However, the physiological significance of these observations remains obscure.

5.4.2. Comparison of Efficiency of Extraction of GAs from Chloroplasts by Methanol and Solutions of Triton X-100. Contrary to the previously published report (Browning and Saunders, 1977), no increase in the efficiency of extraction of GAs from wheat or spinach chloroplasts by Triton X-100 over conventional methanol extraction techniques was observed. These results cannot be attributed to losses or destruction of GAs during the work-up procedures. It should be noted that the results of Browning and Saunders (1976) could not be repeated in other laboratories as well (Railton and Rechov, 1977; Sponsel and MacMillan, personal communication). However, the discrepancies between the present work and the results of Browning and Saunders remain unexplained.

Chapter 6

General Discussion and Conclusions

The original purpose of this thesis as stated in Chapter 1 was to study photoperiodic control of stem growth in spinach. More specifically, the goal was to identify which aspects of the GA status are relevant in the regulation of stem growth, and to determine how photoperiod acts on the GA status. The following is a model of how photoperiod controls stem growth in spinach through regulation of the GA status, based on the results of experiments described in this thesis.

In Chapter 3, it was shown that an excellent correlation exists between LD-induced stem growth and an increase in the level of GA_{20} in shoot extracts (Fig. 3-1). Since GA_{20} was able to substitute for LD in causing increased stem growth (Table 3-1), it was proposed that photoperiod controls stem growth in spinach by regulating the level of GA_{20}.

GA_{19}, on the other hand, declined during the same period when the level of GA_{20} was increasing (Fig. 3-1). Bioassay results suggested that GA_{19} and GA_{20} occurred in a similar, but inverse, range of quantities. The quantitative relationships between GA_{19} and GA_{20}, as well as structural considerations suggest that GA_{19} is a precursor of GA_{20} (Chapter 2 and 3). If this is true, it would appear that photoperiod acts to control the level of GA_{20} by regulating the conversion of GA_{19} to GA_{20}. Perhaps the phytochrome status influences the activity of enzymes responsible for this conversion.

In Chapter 4, it was shown that the LD-induced increase in the level of GA_{20} in spinach shoots was also seen in the phloem exudate. This, coupled with the observation that the

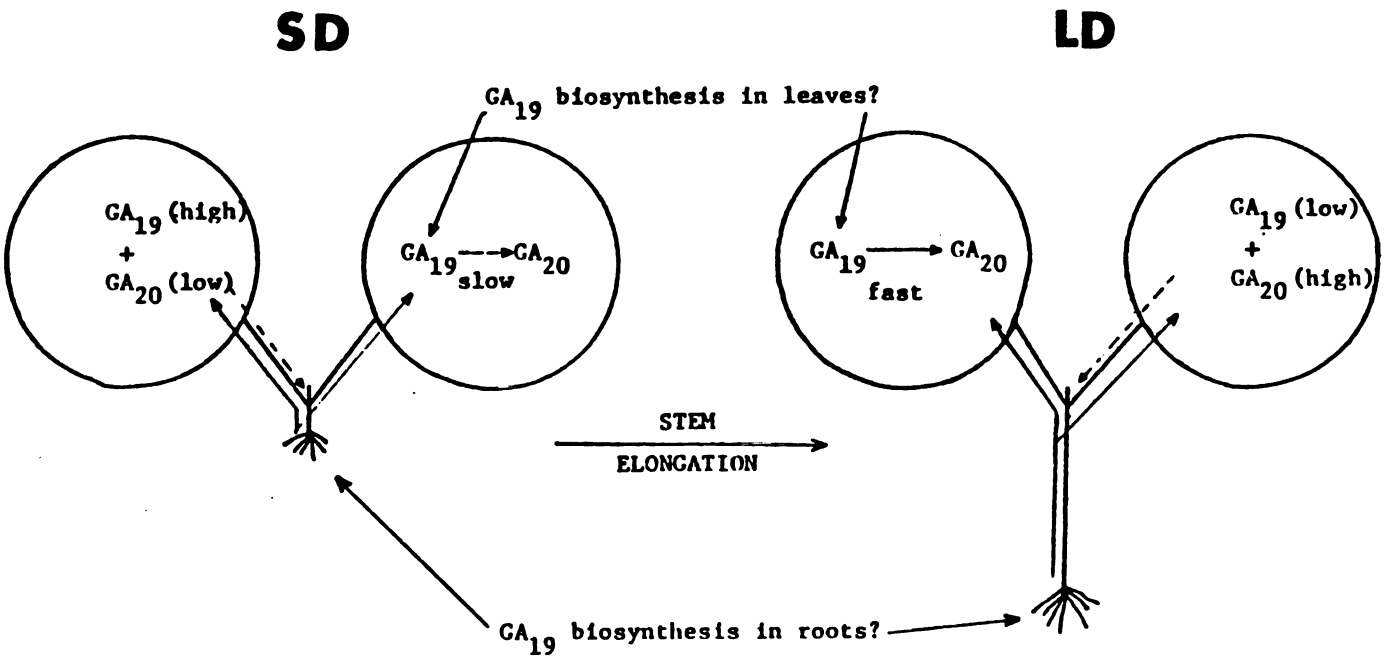
leaf is the site of perception of the photoperiodic stimulus (Knott, 1934), suggests that GA_{20} is produced in the leaf and transported via the phloem to the shoot tip, where it causes increased stem elongation.

GA_{20}, while being present in the shoot and the phloem exudate, was not detected in root extracts or in the xylem exudate (Chapter 4). GA_{19}, on the other hand, was detected in both roots and in the xylem exudate. In section 5.4.4., approximately 25% of the total GA_{19} found in the shoots of SD plants could be accounted for by movement of GA_{19} in the transpiration stream during a single 24-hr period. This suggests that the roots can contribute a significant portion of the GA_{19} present in the shoot. If this is correct, GA metabolism in roots and shoots of spinach is a highly integrated process.

Models, like the one just presented, can only provide a framework to guide further investigations. In order to evaluate these hypotheses, it is of utmost importance to establish unequivocally the metabolic relationships between the endogenous GAs in spinach. Furthermore, *in vitro* systems which support such conversions must be developed so that the sites of GA synthesis and metabolism can be firmly established. In addition, further evaluation of the assumption that the absolute level of GA controls the physiological response should be made. Since spinach is less sensitive to exogenous GA under SD than LD (Zeevaart, 1971), it is obvious that photoperiod controls stem growth in this species by more than

just regulating the absolute levels of GA_{20}. In the LD rosette plant Agrostemma githago, LD treatment causes a large, transient increase in the level of GAs which precedes stem growth. Further LD treatment restuls in a decline in the level of GAs when most stem growth occurs. While stem growth in Agrostemma is apparently regulated by the GA status, absolute levels of any particular GA do not appear to be important in the control of stem growth (Jones and Zeevaart, 1980a,b). However, overall GA turnover is greatly increased by LD treatment in several LD rosette plants, including spinach (Zeevaart, 1971), Silene armeria (Cleland and Zeevaart, 1970), and Agrostemma githago (Jones and Zeevaart, 1980b). GA turnover, combined with tissue sensitivity, are therefore important aspects of the GA status that must be considered in addition to absolute GA levels. In closing, a scheme which summarizes the main differences in the GA status of spinach plants under SD and LD conditions is presented in Fig. 6-1.

Figure 6-1. A scheme summarizing the differences in three aspects of the GA status of spinach plants grown under SD or LD conditions.



GA LEVELS

High level of GA_{19} in leaves and shoot apex, low GA_{20}; conversion of GA_{19} to GA_{20} slow.	Low level of GA_{19} in leaves and shoot apex, high GA_{20}; conversion of GA_{19} to GA_{20} fast.

GA TURNOVER RATE

Low GA turnover rate.	High GA turnover rate. (Zeevaart, 1971)

TISSUE SENSITIVITY TO GAs

Low tissue sensitivity to GAs.	High tissue sensitivity to GAs. (Zeevaart, 1971)

APPENDIX

APPENDIX

This Appendix contains the bar graphs of the mass spectra with background subtraction of the GAs found in extracts of spinach shoots, along with the similar graphs of appropriate reference compounds (if available).

Figure A-1

Spinach MeTMS-GA_{19}

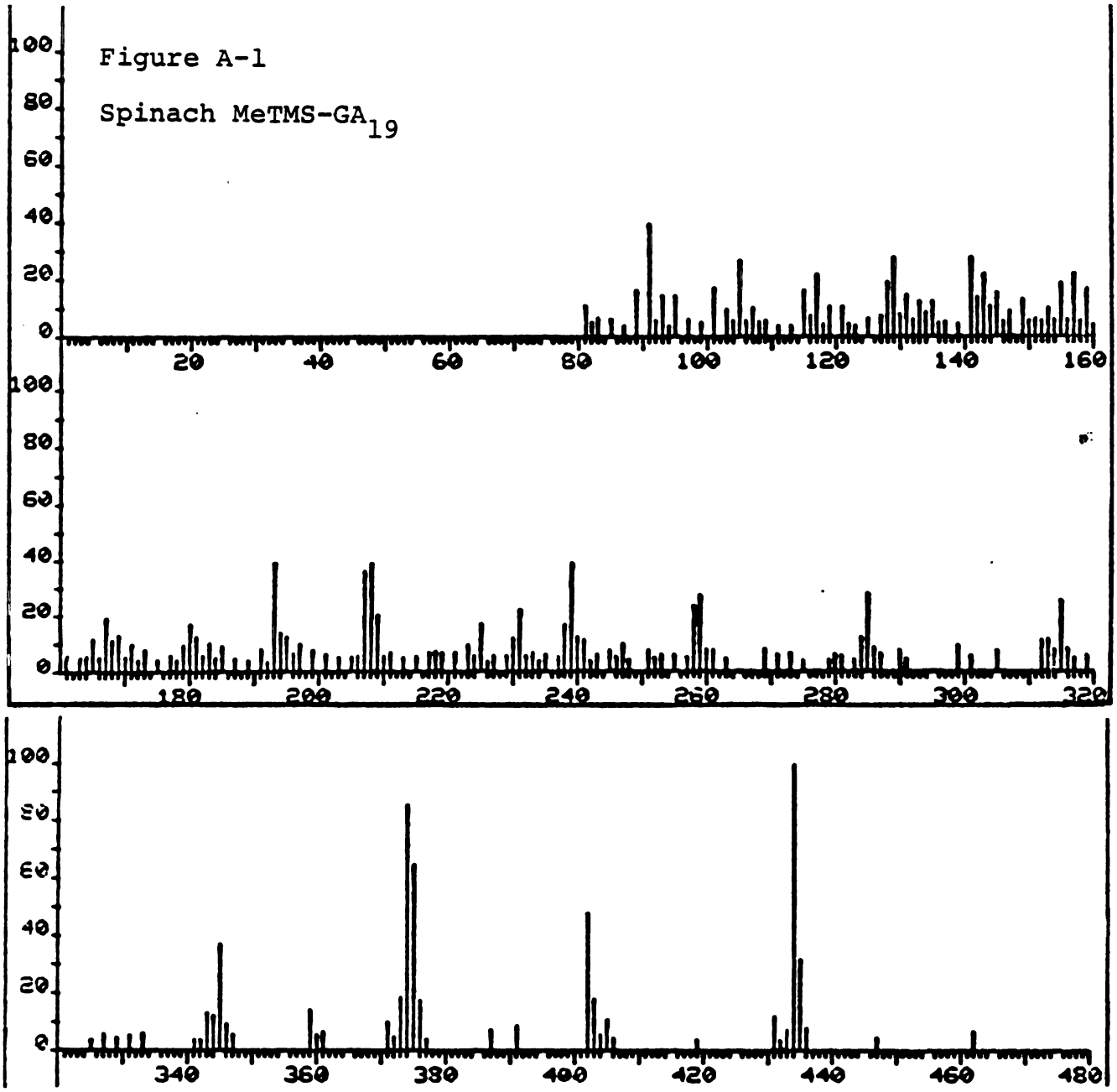


Figure A-2

Spinach MeTMS-GA_{20}

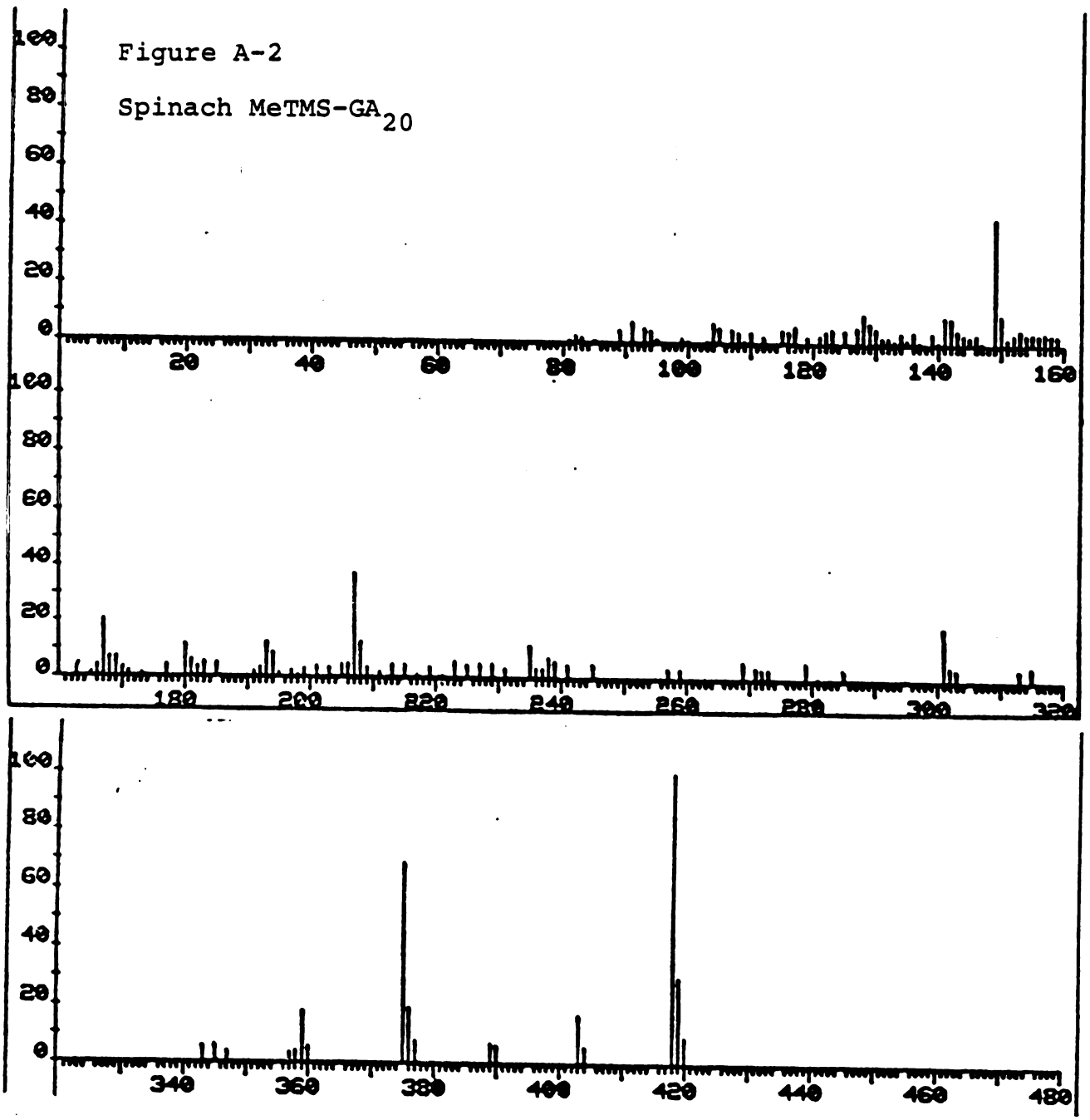


Figure A-3

Standard MeTMS-GA_{20}

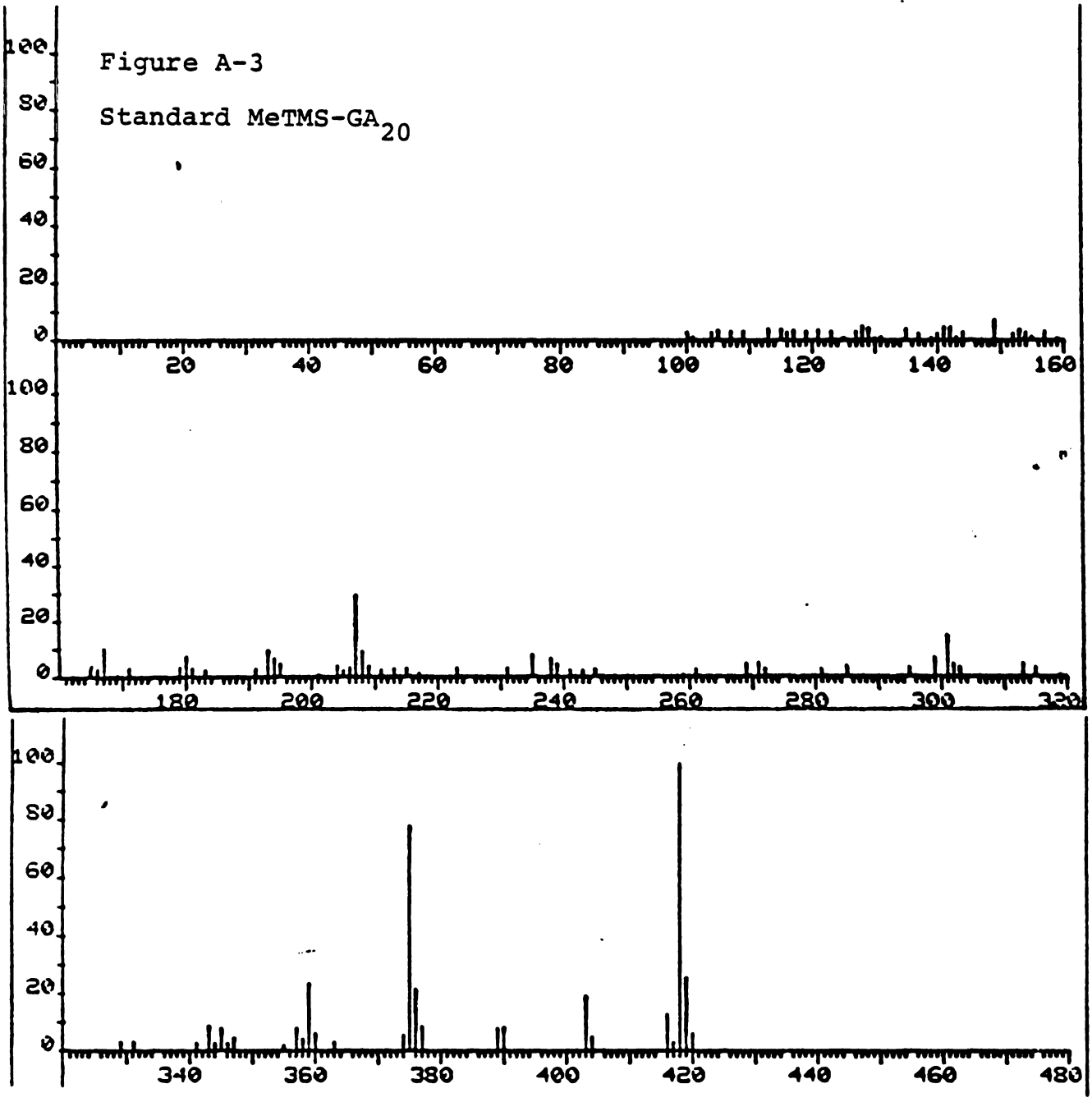
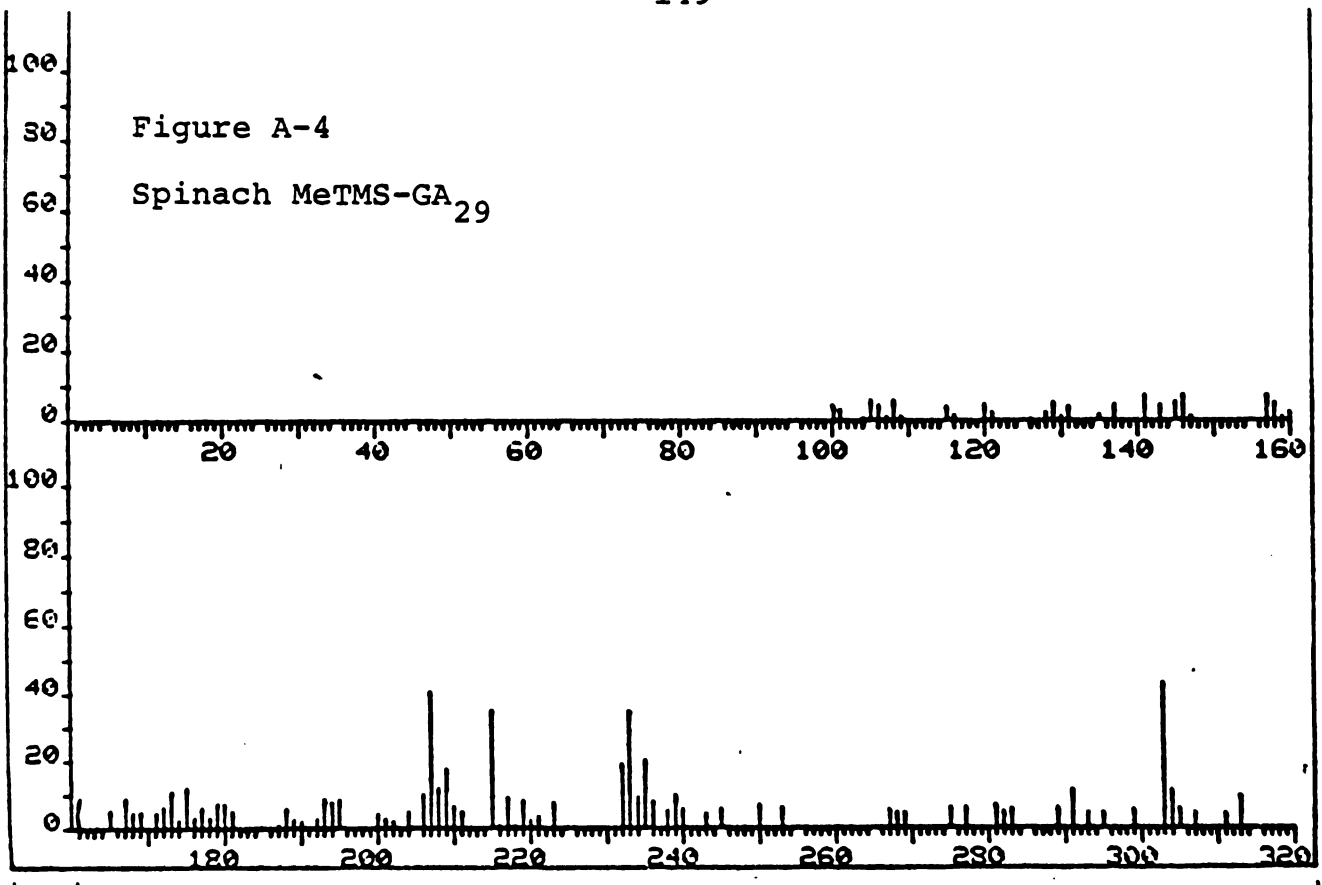


Figure A-4

Spinach MeTMS-GA_{29}



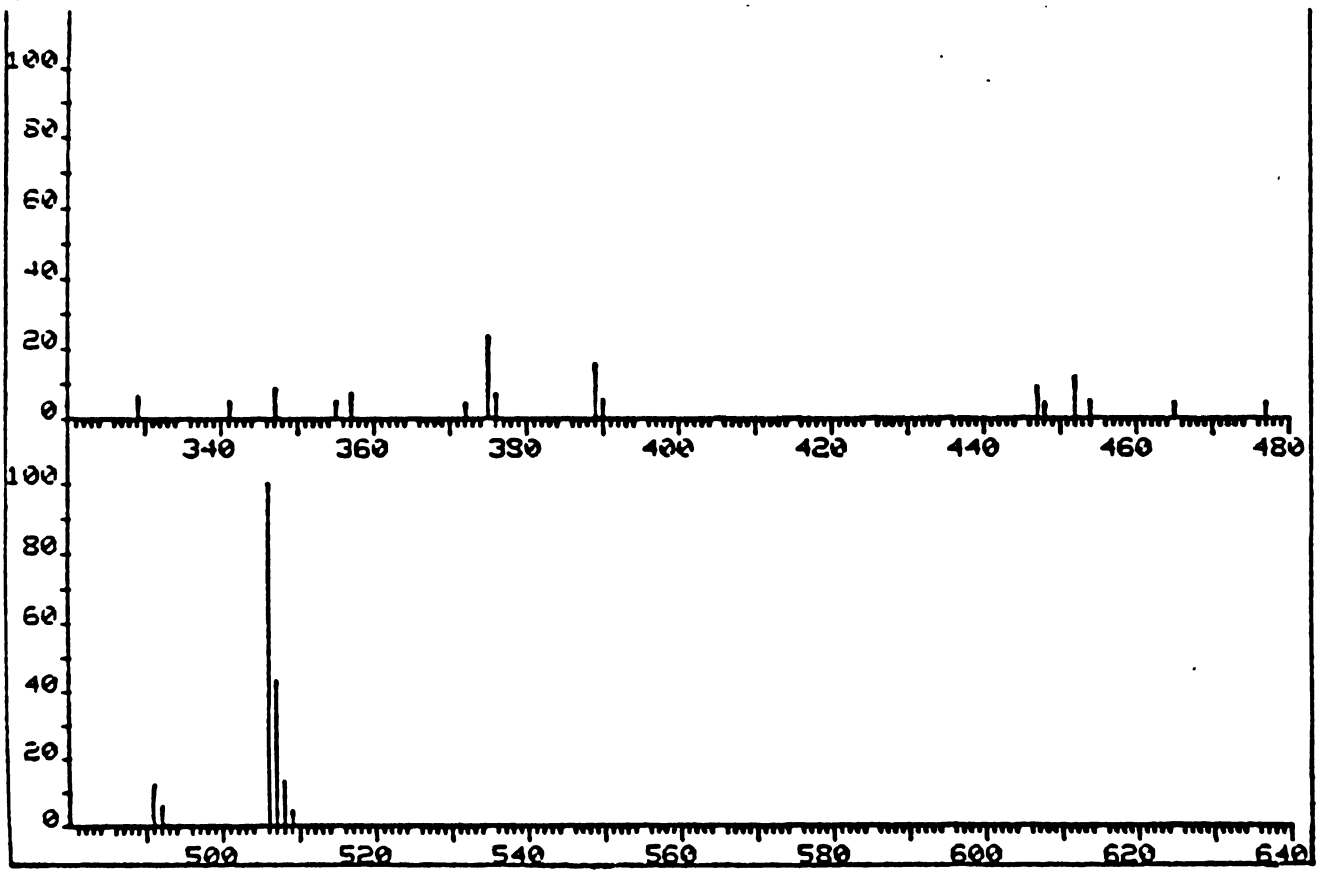


Figure A-5

Standard MeTMS-GA_{29}

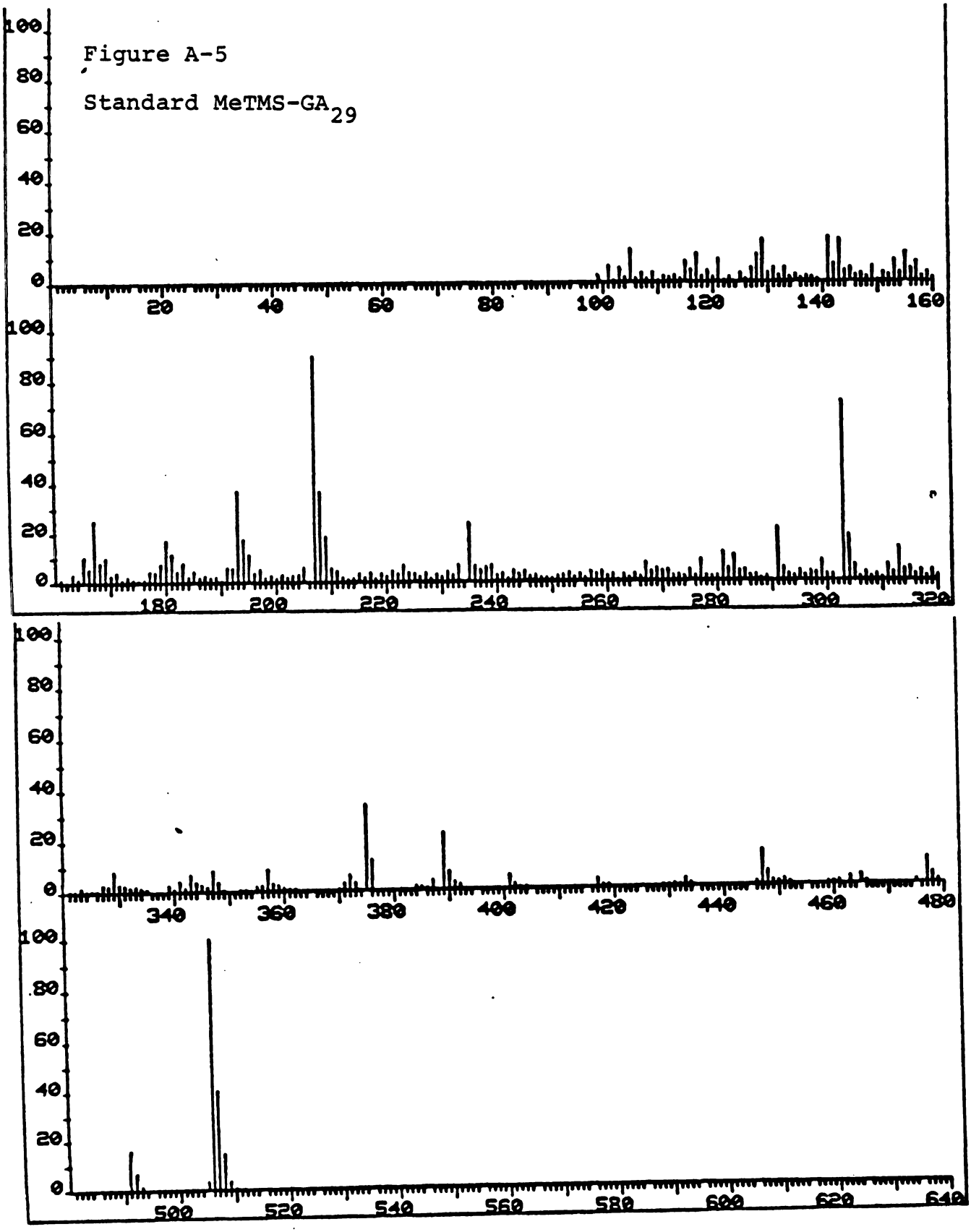


Figure A-6

Spinach MeTMS-GA_{19}

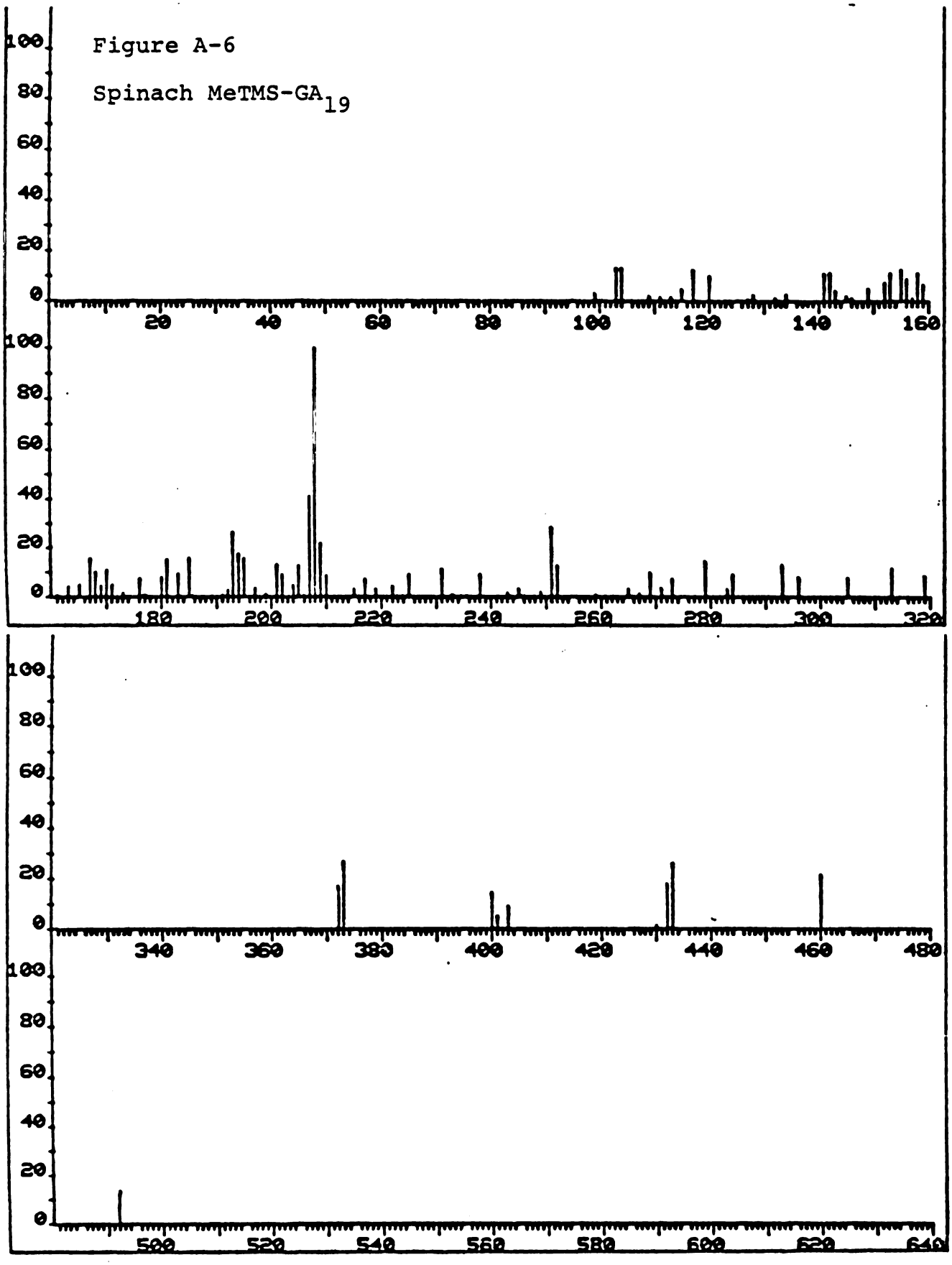


Figure A-7

Standard MeTMS-GA_{17}

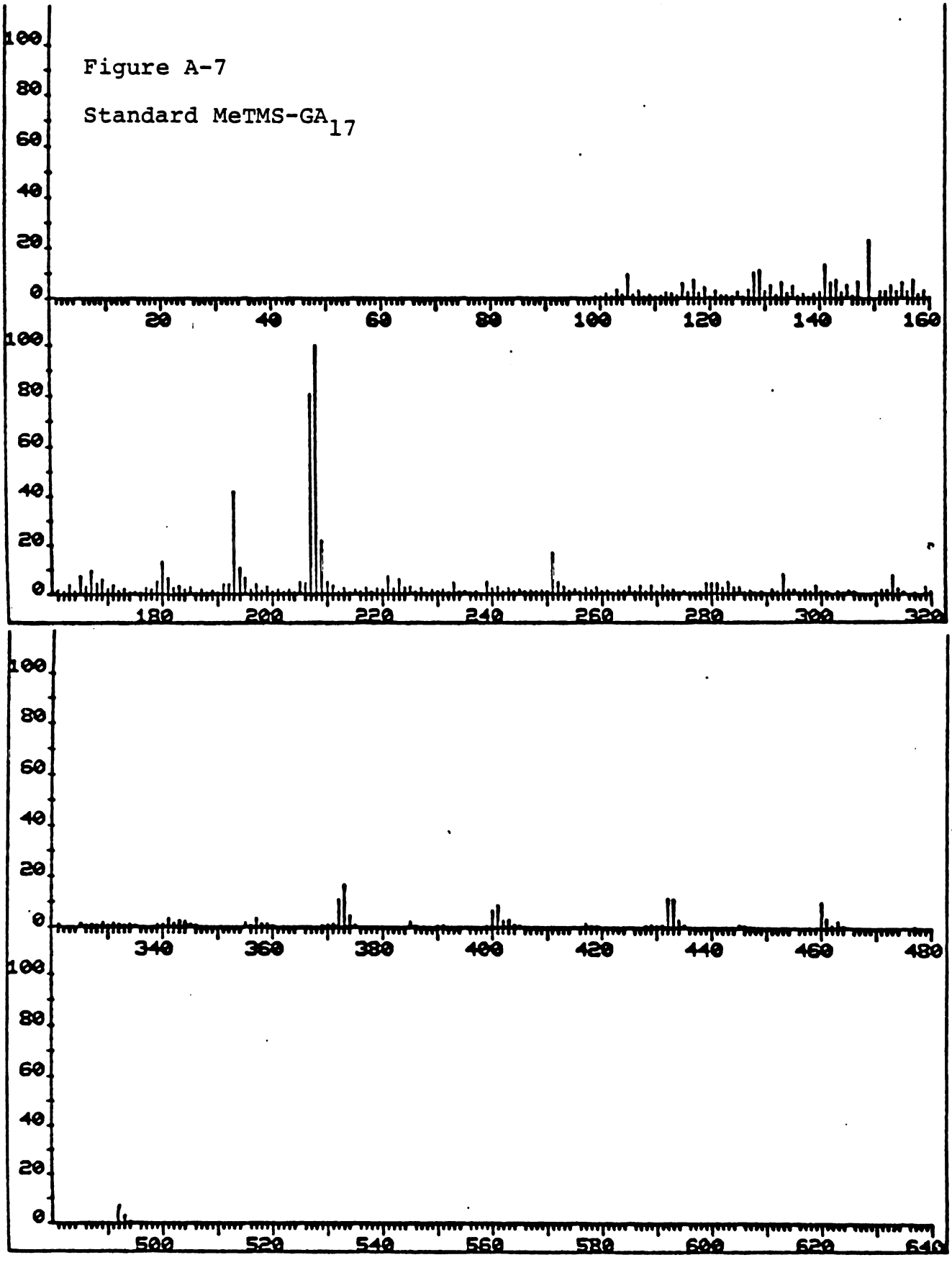


Figure A-8

Spinach MeTMS-GA_{44}

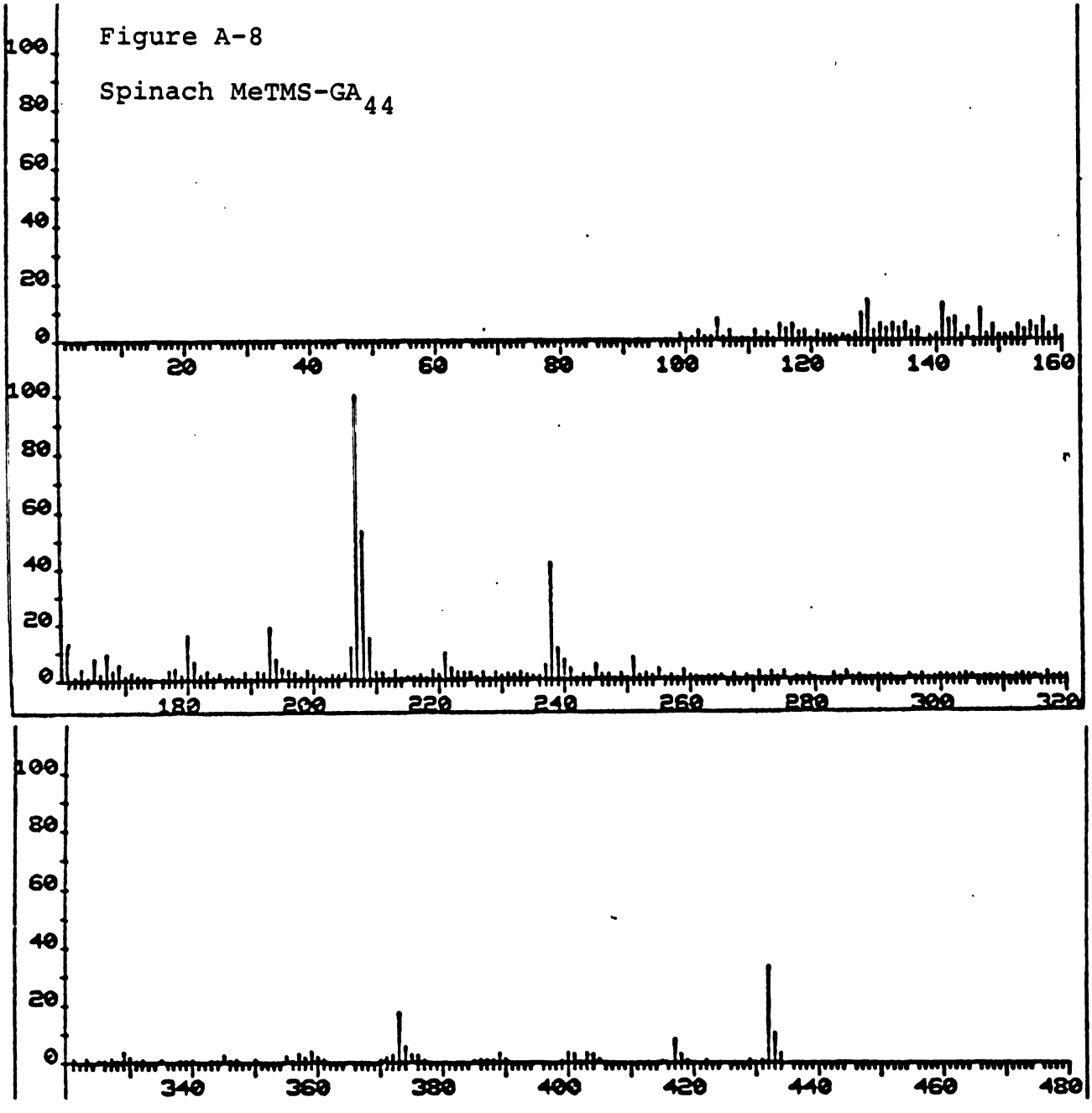
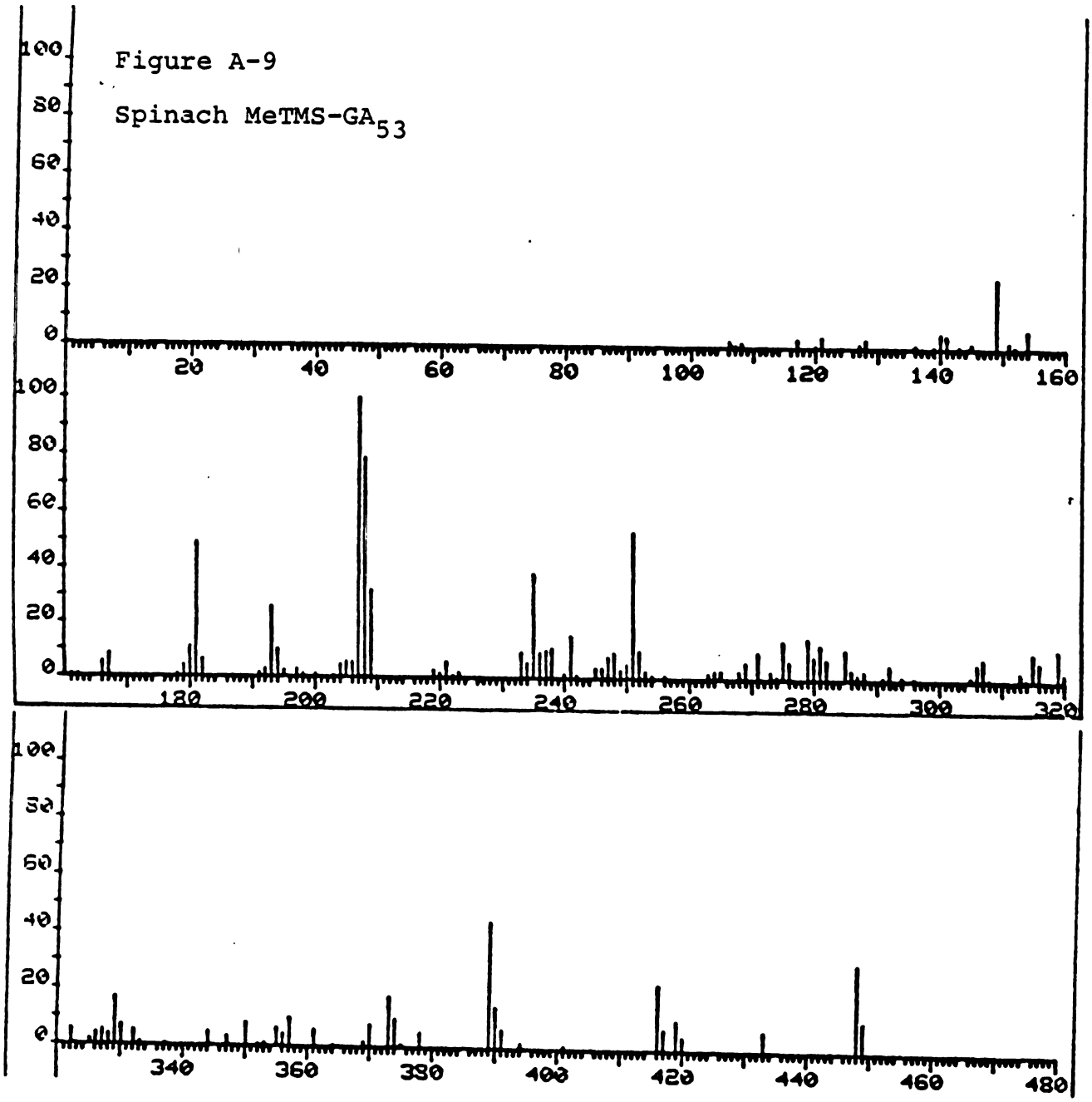


Figure A-9

Spinach MeTMS-GA_{53}



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