GIBBERELLIN METABOLISM IN HIGHER PLANT TISSUES

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ABSTRACT

GIBBERELLIN METABOLISM IN HIGHER PLANT TISSUES

By

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The objectives of the investigations were: (1) to determine whether gibberellic acid (GA_3) and gibberellin A_1 (GA_1) are converted to other gibberellins or metabolites in aleurone layers; (2) to study the properties and, if possible, to determine the structures of any resulting compounds; (3) to ascertain the metabolic pathway by which GA_3 is inactivated or conjugated in aleurone layers; and (4) to determine whether the metabolism of the phytohormones is related to their ability to evoke responses in the tissues.

GA_3 was rapidly inactivated by barley aleurone layers when it was applied and it was the only biologically active compound detected at any time (5 minutes to 24 hours after application) in the tissue. Bioassays of sections of chromatograms of ethanol extracts revealed one area, corresponding to GA_3, which was active in the barley endosperm assay.

In studies on GA_3 metabolism by barley (Hordeum vulgare cv. Betzes) and wheat (Triticum aestivum cv. Genesee) aleurone layers, tissues were incubated with radioactive phytohormone, removed from

the medium, thoroughly washed with water and subsequently boiled with methanol. The layers were ground with methanol, the extract was evaporated to dryness, and the residue dissolved in borax buffer, pH 8.4. An aqueous phase resulted after this solution was partitioned successively against ethyl acetate at pH 8.4 and 2.4. The incubation and wash solutions were combined and extracted similarly. The radioactivity present in the acidic ethyl acetate phases of GA_3 treated tissue as well as in the combined medium and wash solutions was primarily unmetabolized GA_3. Two polar metabolites that were produced within 15 minutes and accumulated over a 24 hour period were found in the aqueous phase of the layers.

The major highly polar metabolite (M_1) remained at the origin of paper chromatograms developed with isopropanol-water (4:1, v/v) whereas the minor metabolite (M_2) migrated behind the GA_3 area.

Acid treatment of the minor metabolite, M_2 of GA_3, and of GA_3 gave allogibberic and gibberic acids. M_2 gave a positive test for hexoses and for pentoses. ^{14}C-U-glucose was incorporated into the metabolite, ^{14}C-U-galactose was not. A diasaccharide resulting from the partial acid hydrolysis of M_2 migrated at the same rate as 3-O-β-D-glucopyranosyl-D-xylose. Complete acid hydrolysis of M_2 resulted in the production of glucose and xylose, apparently in equal amounts. Radioactive xylitol was detected on paper chromatograms after the sequential periodate oxidation, acid hydrolysis and reduction with $NaBT_4$ of M_2 of GA_3. The results, collectively, were indications that M_2 is very likely O-β-D-glucose-(1→3)-O-β-D-xylopyranosyl-(1→3)-O-GA_3.

Regarding the structure of M_1 of GA_3: the basic GA_3 structure appears to be modified in it since it does not give the same products as GA_3 on acid treatment. Alanine, glycine and serine were identified and their corresponding dinitrophenyl derivatives (DNP-alanine, DNP-glycine and DNP-serine) were prepared after hydrolysis of M_1 of GA_3. The N-terminal amino acids of M_1 of GA_3 are glycine and serine.

^{3}H-GA_1 was metabolized to GA_8 and to compounds tentatively identified as a GA_1-glycoside (M_2 of GA_1) and a GA_8-glycoside (M_3). These are thought to be congeners of M_2 of GA_3. Hence, they are suspected to be O-3-β-D-glucosyl-xylosyl-GA_1 and the corresponding glycoside of GA_8, respectively, the sugars being attached to C-3 of the ent-gibberellane ring.

Additionally, ^{3}H-GA_1 was converted to a compound having properties similar to the major highly polar metabolite, M_1 of GA_3. Evidence was also obtained that GA_8 is an intermediate in the formation of M_3 and M_1 of GA_1 since ^{3}H-GA_8 was metabolized by aleurone tissues to these compounds.

A cell-free enzyme system from Phaseolus vulgaris L. used to hydroxylate ^{3}H-GA_1 to ^{3}H-GA_8 also converted it to a less polar product. The enzyme system converted ^{14}C-GA_3 to two metabolites that are probably homologs of the GA_1 products. Additionally, a compound more polar than the two metabolites, that is very likely more highly hydroxylated, was formed from gibberellic acid.

In comparative studies on the conversion of gibberellins to polar metabolites by intact tissues from various plants, the

following results were obtained. Four-day-old seedlings of Pharbitis nil Choisy that were devoid of roots converted ^{14}C-GA_3 to two polar metabolites that are unlike those formed by aleurone layers.

^{3}H-GA_1 was metabolized by four-day-old germinating Zea mays to two unidentified polar products. This gibberellin was converted to two radioactive substances less polar than M_2 of GA_1 or M_3 by immature seeds of Scarlet Runner bean (Phaseolus coccineus L.). One of the metabolites appeared to be GA_8-glucoside. The polar metabolites formed by P. coccineus seeds had identical properties as compounds formed by germinating Phaseolus vulgaris seeds.

When mung beans (Phaseolus aureus) which were imbibing water were incubated with ^{3}H-GA_1, the hormone was converted to a major polar compound whose chromatographic and electrophoretic properties were similar to M_3 of GA_1. The highly polar metabolites, the M_1-like compounds, were only detected in aleurone layers.

These studies indicate that reactions for the formation of the conjugated forms of the gibberellins are probably mechanisms for regulating the endogenous level of the phytohormones inasmuch as the metabolites are inactive in bioassays. In aleurone layers, the gibberellins themselves rather than metabolites derived from them appear to be the biologically active hormonal species.

GIBBERELLIN METABOLISM IN HIGHER PLANT TISSUES

By

Bibhuti Narayan Singh

A DISSERTATION

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To my parents,

Raja Ram Singh

and

Shakuntala Devi Singh

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INTRODUCTION

The gibberellins have fascinated investigators for many years. Some have been concerned with the physiological effects of the gibberellins, others with the biochemical aspects of this group of hormones, and still others with the relationship of the structures of the various gibberellins with their function in the growth and the development of plants. Although the hormonal actions of gibberellic acid (GA_3) in barley aleurone tissues have been studied extensively, the question still remains, how do these phytohormones really act?

Studies on the metabolism of labeled gibberellins may provide significant information concerning their mode of action and the pathways of their degradation and inactivation. They may also explain the specificity of plant responses to different gibberellins. The metabolic fate of labeled gibberellins has been investigated in relation to germination and maturation of seeds (Barendse et al., 1968; Sembdner et al., 1968; Barendse and de Klerk, 1975), dwarfism (Kende, 1967; Davies and Rappaport, 1975a), and flowering (Railton and Wareing, 1973; Van Den Ende and Zeevaart, 1971; Durley et al., 1975). In general, plants convert biologically active gibberellins to less active polar metabolites. The disappearance of endogenous gibberellin activity during certain stages of plant development that has been observed in several instances may be due to the inactivation of the hormones themselves or to their conversion to bound forms.

The metabolic fate of gibberellic acid (GA_3) in aleurone layers, a tissue that has been used extensively to study its hormonal action, is unknown. The metabolism of ^{3}H-gibberellin A_1 (GA_1) the more readily available radioactive gibberellin, has been studied in several laboratories. The first report on gibberellin metabolism in cereal aleurone layers appeared from Kende's laboratory after he successfully prepared and purified tritium labeled GA_1 by catalytic reduction of GA_3 (Kende, 1967). Musgrave et al. (1972) treated barley aleurone layers with ^{3}H-GA_1, ^{3}H-GA_5 and the biologically inactive ^{3}H-GA_5 methyl-ester. A major highly polar metabolite which remained at the origin of chromatograms was formed from each of the labeled gibberellins. Aleurone layers accumulated only 4% of the ^{3}H-GA_1 counts originally added, whereas layers treated with ^{3}H-GA_5 and ^{3}H-GA_5-methyl-ester accumulated 18 and 22%, respectively. The aqueous phase of the layers contained most of the radioactivity which consisted of polar compounds. Unmetabolized GA_1 was present in the acidic ethyl acetate phase. The accumulation of radioactivity by the layers was inhibited in the cold as well as by dinitrophenol and sodium fluoride.

Nadeau et al. (1972) tentatively identified three polar metabolites resulting from the incubation of ^{3}H-GA_1 with barley aleurone layers as ^{3}H-GA_1-glucoside, ^{3}H-GA_8 and ^{3}H-GA_8-glucoside. A highly polar compound was produced in the greatest quantity. This compound was referred to as ^{3}H-GA-X. The tentative identification of the metabolites was based on comparing their properties and that of their methyl ester-trimethyl silyl ethers with authentic standards

on thin layers and in gas-liquid chromatographic systems, respectively. It is important to point out that in none of these studies was it shown that the sugar attached to the GAs is glucose.

Treatment of the major highly polar metabolite with 1 N H_2SO_4 or 1 N NaOH for 4 hours at 90°C did not liberate a gibberellin-like compound. This metabolite migrated faster than GA_1 or GA_8-glucoside, each of which contains only one free carboxyl group, when electrophoresed in a buffer containing pyridine, acetic acid, and water (10:1:90) at pH 6.0. It was suggested, therefore, that the compound, ^{3}H-GA-X, probably contains more than one carboxylic acid group. Another interesting point mentioned in the paper was that abscisic acid (ABA) enhanced the uptake of ^{3}H-GA_1 and increased the amount of labeled metabolites formed.

Later, Nadeau and Rappaport (1974) reported some of the properties of the major metabolite, ^{3}H-GA-X, and renamed it ^{3}H-ampho-GA_1. The formation of ampho-GA_1 in aleurone layers was proportional to the logarithm of the ABA concentration. There was a lag period of 2.5 hours in the formation of ampho-GA_1. They suggested that it may be necessary to induce an enzyme system responsible for the formation of the conjugate. Ampho-GA_1 was isoelectric at pH 2.2. It also formed a derivative with dinitrofluorobenzene. Ampho-^{3}H-GA_1 migrated faster than ^{3}H-GA_1 toward the anode upon electrophoresis at pH 5.9, indicating that it contained more carboxyl groups than GA_1. It was also said that the treatment of the major metabolite with 1 N HCl at 100°C for 12 hours resulted in the formation of compounds expected to be formed from the acid treatment of GA_1, indicating

that the conjugate was formed directly from GA_1. They suggested that the highly polar ^3H-GA_1-X formed from GA_1 is amphoteric and is probably conjugated with a peptide. They concluded that the hydroxyl group at carbon-3 of the GA ring is in a "free" form and is not the site of conjugation. The treatment of ampho-GA_1 with strong alkali did not produce an ethyl acetate-soluble radioactive substance, hence the possibility of having an amide or ester linkage was ruled out. Recently, Davies and Rappaport (1975a) have shown that a dwarf mutant of maize metabolized ^3H-GA_1 to ^3H-GA_8, ^3H-GA_8-glucoside and the unknown metabolite referred to as ^3H-GA_1-X. The ^3H-GA_1-X metabolite formed from the maize plant was similar to the ampho-^3H-GA_1 formed when aleurone layers were incubated with ^3H-GA_1.

The biologically inactive pseudo GA_1 (3α-OH-GA_1), which differs from GA_1 only in the orientation of the 3-OH group, was taken up by barley half seeds but was not metabolized (Stolp et al., 1973). Abscisic acid enhanced the accumulation of GA_1 and pseudo GA_1 when incubated with barley half seeds, but it only affected the metabolism of the biologically active GA_1. The important point mentioned in this paper was the significance of the steriochemistry of the 3-OH position in relation to the uptake and metabolism of ^3H-GA_1. It is possible that the enzymes which are responsible for the hydroxylation or glycosylation of GA_1 at the 3-OH position are very specific and are unable to react with pseudo GA_1.

Barendse and de Klerk (1975) studied the metabolism of ^{14}C-GA_3 in Pharbitis nil Choisy. A single metabolite, tentatively identified as GA_3-glucoside, was observed when ^{14}C-GA_3 was applied

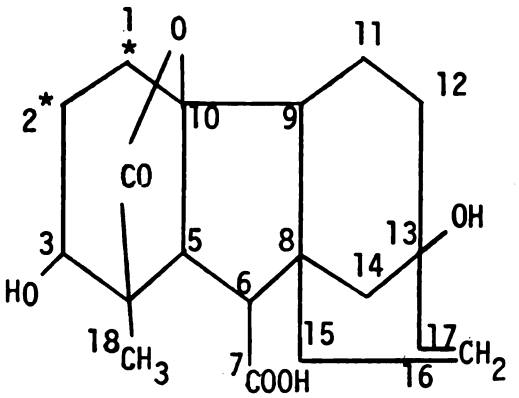
to seedlings of Pharbitis. The identification was made by cochromatographing the metabolite in different solvent systems with authentic 3-O-β-D-glucopyranosyl-GA_3. It gave GA_3 and gibberic acid on mild acid hydrolysis as determined by thin layer chromatography. The GA_3-glucoside was also hydrolyzed by enzyme preparations of cellulase and β-glucuronidase whereas β-glucosidase had no effect on it.

The formation of 2-O-β-glucosyl gibberellin A_3, 2-O-β-glucosyl-isogibberellin A_3, 2-O-β-glucosyl-gibberellinic acid and the β-glucoside of an unknown gibberellin-like substance has been reported by Asakava et al. (1974) in dwarf kidney bean plants treated with GA_3. Yamane et al. (1975) studied the pathways in which tritium labeled gibberellins (GA_1, GA_4, GA_5, GA_8 and GA_{20}) are metabolized in maturing and germinating bean seeds (Phaseolus vulgaris cv. Kentucky Wonder). It was found that each of the ^3H-GAs fed to the seeds was mainly converted to ^3H-GA_8-glucoside. GA_4 and GA_{20} were both converted to GA_1, whereas GA_1 and GA_5 were converted to GA_8. Furthermore, these gibberellins were converted to products tentatively identified as the corresponding glucosides and glucosyl-esters. The important point mentioned in this paper was that GA_4 and GA_{20} act as precursors of GA_8-glucoside, and the intermediate products were GA_1 and GA_8; whereas GA_5 was metabolized to GA_8-glucoside, which involved the formation of GA_8 as a free intermediate.

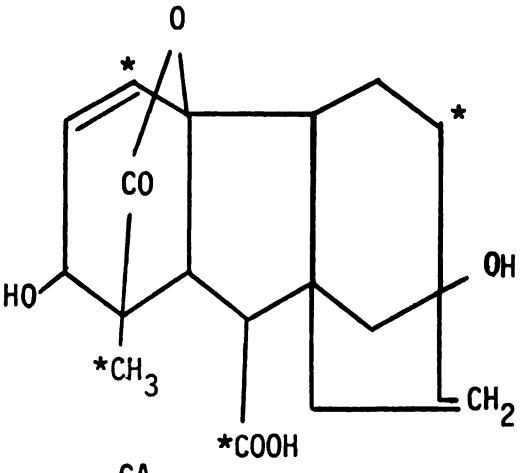
The interconversion of gibberellins has also been studied in other plants. Usually these transformations take place through hydroxylation reactions at carbon 2, 3, 12 or 13 of the gibberellane

ring. Thus, GA_5 was converted to GA_3 (hydroxylation on carbon 3) in seedlings of dwarf pea (Durley et al., 1973). GA_4 was converted to GA_1 (hydroxylation on carbon 13) and GA_{34} (hydroxylation on carbon 2) by dwarf rice (Durley and Pharis, 1973). GA_1 was converted to GA_8 (hydroxylation on carbon 2) by dwarf rice seedlings and seedlings of Scarlet Runner bean (Railton et al., 1973; Reeve et al., 1975). GA_{20} was converted to GA_{29} (hydroxylation on carbon 2) by etiolated shoots and germinating seeds of dwarf pea (Railton et al., 1974) and also by the plant Bryophyllum daigremontianum (Durley et al., 1975). GA_9 was converted to GA_{20} (hydroxylation on carbon 13), GA_{10} (hydroxylation on carbon 16), and 2, 3 dihydro GA_{31} (hydroxylation on carbon 12) by dwarf pea (Railton et al., 1974).

The objectives of the research reported here were: (a) to determine whether GA_3 and GA_1 are converted to other active gibberellins or metabolites in aleurone layers; (b) to study the properties and the structures of any resulting compounds; (c) to ascertain the metabolic pathway by which gibberellic acid is inactivated or conjugated in aleurone layers, and (d) to relate, if possible, the metabolism of the hormone with its ability to evoke responses in the tissue.



GA_1



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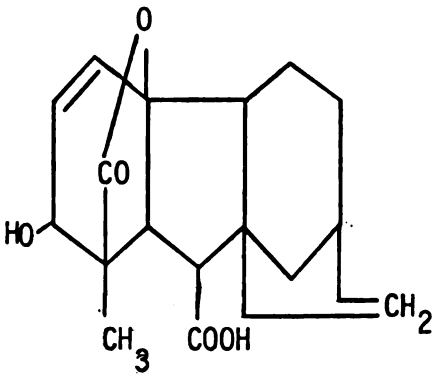

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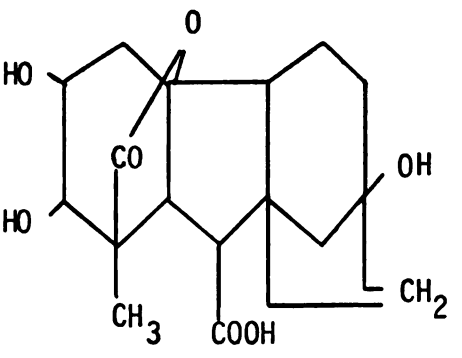


GA_8

Figure 1.--Structural formulas of some gibberellins discussed in the dissertation and the location of radioactivity (*) in the labeled compounds used.

MATERIALS AND METHODS

I. Preparation and Incubation of Tissues

Barley seeds (Hordeum vulgare cv. Betzes) were obtained from the Michigan Crop Improvement Association, East Lansing, Michigan. The seeds were treated with 50% H_2SO_4 for 25-30 minutes by shaking them at medium speed on a mechanical shaker in order to remove the husk. They were then washed extensively with distilled water, sterilized with 1% hypochlorite for 2-3 minutes, rewashed and allowed to imbibe water by shaking on a mechanical shaker at room temperature for 16-20 hours. The seeds were then placed at room temperature for an additional 16-20 hours before use.

The seeds were cut transversely with a razor blade to remove the anterior half containing the embryo. The extreme tip of the remaining posterior half was also cut off. The aleurone layers were made by making a superficial incision longitudinally passing through the median plane of the posterior halves of the seed in order to remove the starchy endosperm.

Aleurone layers were prepared from deembryonated seeds of wheat (Triticum aestivum cv. Genesee, obtained from Dr. Albert Ellingboe) that had imbibed water for 16 hours. All tissues were sterilized with hypochlorite immediately prior to use.

The aleurone layers were shaken with the hormone on a mechanical shaker at room temperature in incubation media containing

3.4×10^{-4} M streptomycin sulfate and 0.02 M calcium chloride. As a control in some experiments, layers were boiled for 2-3 minutes in water before incubation with the hormone, and were subjected to the extraction procedure given experimental tissues.

II. Bioassays

Several of the responses given by barley half seeds after application of gibberellic acid to them were the basis of bioassays in these studies, in addition to the commonly used amylase bioassay (Filner and Varner, 1967). Barley half seeds were prepared by merely removing the anterior embryo halves from imbibed seeds with a razor blade. To establish standard curves, 8 half seeds were shaken for 16-24 hours on a mechanical shaker in stoppered test tubes with 1 ml of logarithmic concentrations of gibberellic acid ranging from 1×10^{-3} to 10 μg per ml, each containing 250 μg streptomycin sulfate per ml and 0.02 M $CaCl_2$. At the end of the incubation, 5 ml of distilled water were added to the tubes, they were shaken thoroughly and aliquots assayed for phosphomonoesterase or phosphodiesterase activity. Typically, 0.5 ml of extracts from the half seeds, 0.5 ml of 0.05 M sodium acetate buffer, pH 4.8, and 0.1 ml of either p-nitrophenyl phosphate or bis-p-nitrophenyl phosphate (0.11 mg/ml) were incubated at 37°C for 5-10 minutes. After the addition of 1 ml of 10% NaOH, the p-nitrophenol produced was read at 405 nm in a spectrophotometer.

III. Chromatography and Paper Electrophoresis

Unless otherwise stated, paper chromatography was carried out on Whatman 3 MM paper by the descending method and thin layer chromatography on precoated silica gel G (E. Merck or Macherey-Nagel Co., Germany) of 0.25 mm thickness by the ascending technique. The paper chromatographic solvents (by volume) were: (A) isopropanol-water (4:1), (B) methanol-ethanol-n-butanol-water-acetic acid (4:3:1:1.5:0.5), (C) propanol-methanol-34% NH_4OH (6:4:3), (D) ethyl acetate-acetic acid-water (9:2:2), (E) nitromethane-acetic acid-ethanol-water saturated boric acid (8:1:1:1), (F) n-butanol-pyridine-water (6:4:3) and (G) propanol-ethyl acetate-water (7:1:2). The thin layer solvents (by volume) were: (H) benzene-n-butanol-acetic acid (70:25:5), (I) acetone-acetic acid (97:3), (J) chloroform-methanol-acetic acid-water (40:15:3:2), (K) isopropanol-6 N NH_4OH (5:1), (L) propanol-ethyl acetate-water (7:2:2), (M) propanol-ethyl acetate-water (7:2:1), (N) n-butanol-acetic acid-water (4:1:1) and (O) propanol-methanol-34% NH_4OH (4:3:3).

During the purification and identification of the metabolites and related compounds, the paper was initially washed with distilled water and dried, and thin layer plates were washed by developing them in a solvent system consisting of chloroform-methanol (1:1) before they were used for experimental purposes. All solvents used were redistilled.

Thin layer solvents used for the identification of amino acids were: (P) phenol-water (3:1), (Q) n-butanol-acetone-triethylamine-water (10:10:2:5) and (R) 2-butanol-methyl ethyl

ketone-dicyclohexylamine-water (10:10:2:5). The solvents used for the separation of DNP-amino acids were: (S) chloroform-tertiary amyl alcohol-acetic acid (70-30-3) and (T) toluene-2-chloro-ethanol-pyridine-25% NH_4OH (100:70:30:14). The composition of all solvent systems, by volume, will be given in parentheses hereafter in this dissertation.

The electrolytes used for paper electrophoresis were: (A) buffer composed of 0.01 M tetrasodium EDTA containing 0.34 ml pyridine per liter and adjusted to pH 3.5 with glacial acetic acid; (B) 0.05 M ammonium borate buffer, pH 9.3 (Coombe and Tate, 1970); (C) buffer containing 2.5% formic acid and 8% acetic acid, in water, pH 2.2; and (D) 0.1 M ammonium carbonate buffer, pH 8.9; (E) buffer containing 0.5 ml pyridine plus 2.5 ml acetic acid per liter of water and adjusted to pH 5.6. Electrophoresis (with a Durrum type unit and Whatman 3 MM washed strips) was of 8 hours duration when buffer A was used, 4 hours with buffer B and 6 hours with buffers (C), (D), and (E), all at 400 volts potential.

IV. Extraction of the Metabolites

At the end of the incubation period, the medium was decanted and the layers were washed on a mechanical shaker for 40 minutes, two changes of water being made. The wash solution was combined with the medium. The layers were boiled for 1-2 minutes in methanol and the tissues were subsequently ground with methanol successively in a mortar and pestle or with a Waring blendor. The methanol extracts were decanted and filtered under suction pressure after

each extraction. Although the methanol insoluble residue was routinely dried and assayed for radioactivity, very few counts were ever found. The methanol extract was evaporated to dryness under reduced pressure, the residue was taken up in borax buffer, pH 8.4, and the solution was extracted three times with ethyl acetate. The resulting top layer, the basic ethyl acetate phase, never contained significant amounts of radioactivity. The pH of the aqueous layer was adjusted to 2.4 with HCl and reextracted 4-5 times with ethyl acetate. The top layer was designated as the acidic ethyl acetate phase and the bottom layer as the aqueous phase. The aqueous phase was further extracted two times with petroleum ether to remove any remaining acidic, non-polar labeled compounds. The pH of the aqueous phase was adjusted to 7.0, evaporated to dryness and the residue taken up in a known volume of water. The incubation media and combined wash solutions from the layers were similarly adjusted to the pH values given above and were partitioned 4-5 times against ethyl acetate. The ethyl acetate phases were dried over anhydrous Na_2SO_4 before being evaporated to dryness and the residues were dissolved in a known volume of 80% ethanol and chromatographed on paper in system A. A small amount of sample was taken from each resulting phase (basic ethyl acetate, acidic ethyl acetate and aqueous phases of both layers and medium) to determine the amount of radioactivity in the total sample.

The aqueous phase of the layers was treated with charcoal. The adsorbent was washed 5-6 times with water by filtration with a Buchner funnel and the products were desorbed with a solution of

0.6% NH_4OH in 50% ethanol. The ethanolic solution was evaporated to dryness, the residue was taken up in a small portion of 50% ethanol and applied to paper for chromatography in solvent system A.

Unlabeled metabolites were processed similarly by incubating a large number of layers with unlabeled hormone. In the time course experiments, layers were taken out at different intervals, washed and extracted as described above.

V. Detection of Unlabeled Metabolites

For detecting M_2 metabolites and other gibberellins, the paper and thin layer chromatograms were sprayed with an ethanolic solution of H_2SO_4 (95 ml of ethanol containing 5 ml of conc. H_2SO_4) and the fluorescence developed by heating the chromatograms with a hot hair dryer was examined under a short wave length ultraviolet lamp. For detecting M_1 metabolites, chromatograms were sprayed with ninhydrin reagent (0.3 g ninhydrin was dissolved in 100 ml n-butanol and mixed with 3 ml glacial acetic acid) and the color was developed by heating the chromatograms (10-15 minutes for thin layer, 8-10 minutes for paper) in an oven at 105°C.

VI. Acid and Alkali Treatment of the Gibberellins and Metabolites

The experimental sample was treated with conc. HCl (or NH_4OH) either in a round bottom evaporating flask with a stopper or in a small Pyrex glass tube with a Teflon cap. The reaction mixture was heated in a boiling water bath or in an oven at 100°C for the desired time. The sample was allowed to cool to room temperature

and then evaporated to dryness. The evaporation process was repeated 3-5 times after adding more water. The resulting residue was dissolved in a known volume of 80% ethanol and applied to chromatograms.

VII. Enzyme Treatment of the Metabolites

A cellulase preparation from Aspergillus niger (Sigma), β-glucosidase (Sigma) from almonds, emulsin (E. Merck, Darmstadt) from almonds, and pectinase (Nutritional Biochemicals Corporation) were used in attempts to hydrolyze M_2 of both gibberellins and M_3 from GA_1.

The metabolites and 1-2 mg of the enzyme preparations in 0.4 ml of 0.05 M acetate buffer, pH 4.8, were incubated for 24 hours in a water bath at 37°C. The reaction was stopped by heating the sample in a boiling water bath for 6-10 minutes. The reaction mixture was centrifuged, the resulting supernatant solution was evaporated to dryness, and the residue was taken up in a known volume of water which was chromatographed on paper and thin layers along with standards.

VIII. Purification and Characterization of the M_2 Metabolite of GA_3

A. Purification

After M_2 was located on the paper chromatogram developed in solvent system A, it was eluted with water and subjected to adsorption on charcoal and desorption with NH_4OH in ethanol as described in section IV. The ethanol extract was evaporated to dryness, the

residue was dissolved in a known volume of 80% ethanol and was chromatographed further on thin layers in systems I, J, K and L; and on paper in system A.

B. Detection of Sugars in M_2 of GA_3

The diphenylamine-aniline-phosphoric acid reagent (Bailey and Bourne, 1960) and the ammoniacal silver nitrate reagent (Brown and Serrow, 1953) were used to locate all sugars on the paper and thin layer chromatograms. The diphenylamine reagent was made by dissolving 2 g diphenylamine, 2 ml aniline and 10 ml 80% ortho-phosphoric acid in 100 ml of acetone. The ammoniacal silver nitrate reagent was made as follows: 5 g silver nitrate was dissolved in 50 ml of water and conc. NH_4OH was then added until the precipitate just redissolved. The dried chromatograms were sprayed with the diphenylamine reagent and heated to develop colors at 100°C for 1-2 minutes (Whatman No. 4 paper), 2-3 minutes (3 MM paper) or 10-25 minutes (thin layers).

The silver nitrate dip technique (Robyt and French, 1963) was also used to detect sugar alcohols and sugars on paper chromatograms. The dried chromatogram was dipped in solvent I (1 ml of saturated silver nitrate solution in 200 ml redistilled acetone); air dried, then dipped in solvent II (1 ml of 40% NaOH in 100 ml methanol) to develop the color.

C. Chemical Tests for Pentose in M_2 of GA_3

The first test was performed as follows. To 0.5 ml of a solution containing metabolite of GA_3 in a test tube 0.5 ml of orcinol

reagent (made by adding 10 ml conc. HCl to 0.1 g orcinol and 0.05 g ferric chloride) was added. The tube was heated in a boiling water bath for 20 minutes to develop the color.

In the phloroglucinol test 0.2 ml of a solution containing the M_2 metabolite of GA_3 was added to 2.5 ml of phloroglucinol reagent (Ashwell, 1966) which was made in the following manner: 110 ml of glacial acetic acid, 2 ml of concentrated HCl and 1 ml of 8% glucose were mixed with 5 ml of 5% phloroglucinol in ethanol. The tube was heated for 15 minutes in a water bath to develop the color.

D. Identification of Sugars in M_2 of GA_3 by Thin Layer and Paper Chromatography

The purified M_2 metabolite of GA_3 from 4000 layers, isolated from experiments where 1000 layers/group were incubated in medium containing 500 µg GA_3/ml in a total volume of 30.0 ml, was hydrolyzed with 0.5 ml of 2.5 N HCl in a hot water bath at 95°C either for 40 minutes for partial hydrolysis or for 90 minutes for complete hydrolysis. The reaction was stopped by neutralizing the reaction mixture with potassium bicarbonate. The neutralized sample was treated first with Dowex 50W-X8 cation exchange resin (H^+ form), filtered and then treated with Dowex 1-X8 anion exchange resin (Cl^- form) to remove any positively and negatively charged materials. The resin was filtered, the filtrate evaporated to dryness and the residue dissolved in water.

A known volume of the above sample and a standard mixture of glucose, galactose, xylose, arabinose and ribose were spotted on

a thin layer of silica gel G (E. Merck) or on Whatman No. 4 paper. The thin layer chromatogram was developed 3-4 times in solvent system M and the paper was developed in solvent system D for 24 hours. The dried chromatograms were sprayed with ammoniacal silver nitrate reagent or diphenylamine reagent to develop colors, as described earlier.

E. Sequential Periodate Oxidation, Acid Hydrolysis and Borohydride Reduction of M_2 of GA_3

The metabolite M_2 of GA_3 from 1200 aleurone layers was treated with 2 mg of sodium metaperiodate in 0.2 ml water for 12 hours in the dark at room temperature. The reaction was stopped by adding a few drops of ethylene glycol and water was removed under reduced pressure. The mixture was hydrolyzed with 0.2 ml 2.5 N HCl for 40 minutes at 105°C. It was then cooled and HCl was removed by evaporating the sample to dryness. The residue was dissolved in 0.1 ml water, a few crystals (1-2 mg) of radioactive sodium borohydride ($NaBT_4$) were added, and the mixture incubated for 90 minutes at room temperature. The reaction was stopped by the addition of acetone and the mixture was evaporated to dryness under reduced pressure. This process was repeated 7-8 times by adding successive portions of acetone and methanol. The residue was finally extracted 3-4 times with benzene and diethyl ether, these extracts being discarded. The residue was dissolved in a known volume of water and applied to the paper and chromatographed in system E with authentic xylitol along with a standard mixture of glucose, galactose, glucitol,

xylitol and xylose. The radioactive area corresponding to xylitol, which was located on paper by the silver nitrate dip technique, was eluted with water and filtered. The filtrate was treated successively with cation exchange (H^+ form) and anion exchange (OH^- form) resins to remove any inorganic material, as described earlier (section VIII-D). The filtrate was evaporated to dryness, the residue was taken up in water and further chromatographed on paper in solvent systems F and G.

F. Incorporation of ^{14}C-U-Glucose into M_2 by GA_3 Treated Aleurone Layers

The aleurone layers (720) were incubated with 10 μc of glucose in the incubation medium containing GA_3 (500 μg/ml) for 24 hours at room temperature on a mechanical shaker. The washing and extraction of the layers were carried out as described earlier. The resulting aqueous phase of the layers was chromatographed on paper in solvent system A alongside of authentic M_2. The area corresponding to M_2 on the paper chromatogram was eluted with 80% ethanol, evaporated to dryness and chromatographed further two times on a thin layer plate with system L.

IX. Purification and Characterization of the M_1 Metabolite of GA_3

A. Purification

The major metabolite (M_1) that remained at the origin of the paper chromatogram in solvent system A was eluted with water and the solution filtered. The filtrate was subjected to adsorption onto

charcoal and desorption with NH_4OH in ethanol. The ethanolic extract was evaporated to dryness and the residue dissolved in 50% ethanol. This was applied to paper and successively chromatographed on paper in systems B and C, and on thin layer in systems N and O. It was then electrophoresed successively in buffers C and D. The purity of the unlabeled compound was assessed by the presence of a single ninhydrin positive spot on the chromatogram which migrated along with radioactive M_1.

B. Hydrolysis

The major metabolite, M_1 of GA_3, was transferred to a thick Pyrex glass tube designed for evacuation and sealing. The sample was evaporated to dryness and 200 µl of 6 N HCl were added to it. The liquid was then frozen by immersing the tube in a dry ice-ethanol bath. After replacing the air with N_2, the tube was evacuated to approximately 1 mm of mercury and sealed subsequently. Hydrolysis was carried out for 16-18 hours in a temperature regulated heated block (Lab Line Instrument, Melrose Park, Ill.) at 110°C. HCl was removed after hydrolysis by slow evaporation in a vacuum desiccator containing solid NaOH.

The residue was dissolved in water and a portion of the hydrolyzed sample was chromatographed on thin layers of silica gel G (Macherey-Nagel) and cellulose plates (Brinkmann) of 250 µ thickness in several solvent systems (N, P, Q, and R) along with amino acid standards. The dried chromatograms were sprayed with the ninhydrin reagent and heated to develop the color. Chromatograms developed in

systems Q and R were dipped in petroleum ether for 20-30 seconds to remove excess amines, dried and then sprayed with the ninhydrin reagent. The purified M_1 metabolite of GA_3 from 4000 layers, isolated from experiments where 1000 layers/group were incubated in medium containing 500 μg GA_3/ml in a total volume of 30 ml, was used in each of the following experiments on M_1 identification.

C. Identification of Amino Acids by Dinitrophenylation

The dinitrophenylation technique was carried out in the following manner. The reaction was performed in a small glass stoppered test tube containing 0.2 ml sample (a known concentration of amino acids or acid hydrolyzed M_1), 0.5 ml buffer (8.4 g $NaHCO_3$ and 2.5 ml of 1 N NaOH made up to 100 ml with distilled water, pH 8.8), 0.02 ml of 10% 1-fluoro-2-4 dinitrobenzene (FDNB) solution in absolute ethanol and 0.4 ml absolute ethanol. This was mixed thoroughly and the reaction mixture was incubated in an oven in the dark for 1 hour at 65°C. After the reaction mixture was taken out of the oven and allowed to cool at room temperature, the pH was adjusted to approximately 12.0 with NaOH solution. Water (1 ml) was added to the mixture and it was extracted with 2 ml portions of diethyl ether four times, the ether phase being discarded. The pH of the aqueous phase was adjusted to around 1.0 with dilute HCl and it was reextracted 7-8 times with diethyl ether. The aqueous phase was discarded. The resulting ether extracts were combined and evaporated to dryness and the residue was taken up in a known volume of acetone.

These are the standard steps in the procedure for dinitrophenylation although they have been slightly modified (Pataki et al., 1967).

The DNP-derivatives of hydrolyzed products of M_1 were spotted on thin layer silica gel G (Macherey-Nagel) and a standard DNP-amino acid mixture of alanine, serine, and glycine was spotted alongside them on each chromatogram. The chromatograms were then developed. Solvent system S was used for the development of the chromatogram in one dimension. For the two dimensional chromatography the thin layer plate was developed first in solvent system T, the chromatogram was dried with a hair dryer for 15-30 minutes and rechromatographed in the second dimension in solvent system S. All these operations (preparation and chromatography) were carried out under diffuse light conditions.

D. Identification of the Terminal Amino Acids by Dansylation

1. Preparation of dansyl (DNS) derivatives.--The technique for dansylation was based on the method of Parke (1975) and Weiner et al. (1972), with some modifications.

To a glass stoppered test tube 0.1 ml of a solution containing amino acids or M_1 metabolite was added and the solution was subsequently evaporated to dryness. Then, 20 µl of 0.2 M sodium carbonate buffer pH 9.8 was added to the sample and it was evaporated to dryness again. The residue was dissolved in 15 µl of water. To the reaction mixture, 30 µl of dansyl chloride stock solution was added and the test tube was sealed with Parafilm. Dansyl chloride was prepared by dissolving it in acetone (15 mg/ml) by

vortexing; the solution was centrifuged and an equal volume of water added to the supernatant solution. The reaction mixture was incubated at 37°C for an hour and then evaporated to dryness.

After the completion of the dansylation of M_1, the product was transferred to a thick Pyrex glass tube designed for evacuation and sealing. The reaction mixture was evaporated to dryness and it was hydrolyzed with 0.2 ml of 6 N HCl at 110°C for 8 hours using a similar procedure as that described earlier (section IX-B) for the identification of amino acids in M_1. Finally, the residue was dissolved in a known volume of 50% aqueous pyridine (by volume) and stored in the freezer until it was ready to be used. A similar procedure was utilized for the dansylation of the M_1 metabolite of GA_3.

2. Chromatographic separation of DNS-derivatives.--The double-layer polyamide plate (Cheng-Chin Trading Co., Ltd.; distributed by Gallard-Schlesinger) was cut with scissors to 5 cm x 5 cm and 5 cm x 10 cm sizes from the full 15 cm square plate. The standard dansyl amino acids (DNS-alanine, DNS-serine and DNS-glycine; 1 µl) were spotted onto one side of the plate by touching the tip of a Hamilton microsyringe containing them to the polyamide sheet. The plate was dried with a hair dryer between applications. The experimental sample (3 µl of hydrolyzed DNS-M_1) was spotted on the back of the plate, opposite the standards. The plate was placed vertically in a small beaker and dried with a medium hot air stream from a hair dryer for a few minutes. Then the polyamide plate was chromatographed at room temperature in three successive solvents in

an ascending manner according to the method given by Weiner et al. (1972) and Parke (1975). The chromatographic solvents were: (I) 1.5% formic acid in water; (II) acetic acid-benzene (1:9), and (III) acetic acid-methanol-ethyl acetate (1:1:20). The plate was first developed in solvent I for the first dimension and then successively in the other two solvents (II and III) in the second dimension. The chromatogram was dried after it was developed in each solvent under a stream of air from a hair dryer. The dried chromatogram was examined under a short wave length ultraviolet lamp and the results interpreted as described by Weiner et al. (1972) and Parke (1975). The solvents were freshly prepared each time 2-3 hours prior to chromatography. The chromatogram developing jars from Eastman Kodak were used for chromatography. All of the operations, including preparation and chromatography of DNS derivatives, were carried out under dim light conditions.

X. Hydroxylation of ^{14}C-GA_3 and ^{3}H-GA_1 by a Cell Free System

A cell free system was prepared by a method described by Patterson et al. (1975), with a few modifications. The seeds used in this experiment were runner beans, Kentucky Wonder pole variety, purchased from a local store. Bean seeds (5 g) were scratched, surface sterilized and thoroughly washed with water before being allowed to imbibe water for 20 hours at room temperature. After imbibition the seeds were decoated, sterilized, washed and ground in a mortar with a pestle for 5 minutes in the cold (3-4°C) with sand (200-400 mg), polyvinylpyrrolidone (1.25 g) and 40 ml of a solution

of 0.05 M Tris-maleate buffer, pH 6.5, containing 0.2 M sucrose. The slurry was filtered through cheesecloth and the filtrate was centrifuged at 12,000 x g for 15 minutes. The supernatant fraction was decanted and recentrifuged at 95,000 x g in a Beckman model L ultracentrifuge (SW40 rotor) for 2 hours at 0°C. The resulting supernatant fraction was used. The unused portion of the fraction was stored in 10% glycerol in a freezer at -15°C for later use.

The radioactive hormones, ^{14}C-GA_3 (8.2 µg in 0.3 ml water) and ^{3}H-GA_1 (0.044 µg in 0.8 ml water), were incubated separately in stoppered test tubes with 2 ml enzyme, 1.5×10^{-3} g ferric chloride, 8×10^{-4} g ascorbic acid, 1×10^{-3} g NADPH, 1×10^{-3} g streptomycin sulfate and enough 0.05 M Tris-maleate buffer, pH 6.5, to make a final volume of 3 ml. The reaction mixtures were incubated on a mechanical shaker for 16-18 hours at room temperature. The reaction was stopped by heating the tubes in a boiling water bath for 7-10 minutes, cooled, and centrifuged at 1,000 x g for 5 minutes. The residue was discarded, the pH of the supernatant solution was adjusted to 2.5 with dilute HCl and then extracted 4-5 times with ethyl acetate. The resulting ethyl acetate and the remaining aqueous phase (whose pH was adjusted to 7.0) were evaporated to dryness. The residue was dissolved in 80% ethanol and electrophoresed in the borate buffer system.

XI. Chemicals

Gibberellic acid (8-methylene-^{14}C-GA_3), which was synthesized by the method of Hanson and Hawker (1973), was obtained from

Amersham/Searle Corporation, Arlington Heights, Illinois. It had a specific activity of 4.45 μCi/mg. Gibberellic acid (1, 7, 12, 18-^{14}C-GA_3), also obtained from Amersham/Searle, was used in some experiments and it had a specific activity of 4.9 μCi/mg. The radioactive gibberellin A_1 [1,2-^{3}H(N)] with a specific activity of 31 Ci/m mole (5.2×10^3 cpm/2.8×10^{-2} μg) was purchased from New England Nuclear Corporation, Boston, Massachusetts.

The unlabeled gibberellic acid (GA_3) was obtained from Eastman Kodak Co., Rochester, N.Y.; gibberellin A_7 (GA_7) from ICN Pharmaceuticals, Inc., Cleveland, Ohio; gibberellin A_1 (GA_1) was a gift from Professor Hans Kende and abscisic acid was kindly donated by Professor A. De Hertogh.

The radioactive GAs were as active, biologically, as the unlabeled hormones in barley half seed bioassays. The purity of the compounds was assessed by thin layer and paper chromatography. The single peaks, located on chromatograms by counting the area or located with a short wave length ultraviolet lamp after spraying with ethanolic sulfuric acid and heating, corresponded to areas that exhibited biological activity. Hence, they were very pure.

The radioactive gibberellin A_8 (^{3}H-GA_8) was prepared from ^{3}H-GA_1 by utilizing the cell free system reported by Patterson et al. (1975).

D-glucose-U-^{14}C was obtained from International Chemical and Nuclear Corporation, Irvine, California, and sodium borohydride [^{3}H] ($NaBH_4$) from New England Nuclear; whereas 2,4-dinitrofluorobenzene (DNFB) and dansyl chloride (5-dimethylamino-napthalene-1-sulfonyl

chloride) were kindly donated by Professor Derek Lamport. All other chemicals were purchased commercially.

XII. Determination of Radioactivity

Segments of paper chromatograms and electrophoretograms were cut off, and zones from thin layer chromatograms were scraped off and counted directly in scintillation counting fluid which was made by dissolving 5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in a liter of toluene. Portions of liquid samples were counted directly in Bray's (1960) scintillation fluid. Radioactivity was determined with a Packard Liquid Scintillation Spectrometer (Model 3003).

RESULTS

I. Attempts to Find Biologically Active Metabolites of GA_3

The only biologically active form of the hormone present in ethanolic extracts at any time (5 minutes to 24 hours) after incubation of gibberellic acid with barley aleurone layers was the compound itself. Thus, bioassays of sections of paper and thin layer chromatograms of extracts in systems A and H revealed only one area, corresponding to gibberellic acid, which was active in the barley endosperm assay at R_F 0.51 after paper chromatography in system A and R_F 0.54 after thin layer chromatography in system H (Figure 2). Radioactivity was also located there when ^{14}C-GA_3 was utilized. Additionally, the material was shown to be gibberellic acid by thin layer chromatography in systems K (R_F 0.41) and I (R_F 0.81). Hence there was no evidence that gibberellic acid was converted to any other biologically active form of the hormone.

II. The Inactivation of Gibberellic Acid by Aleurone Layers

The inactivation of the hormone by barley aleurone layers progresses with time although inspection of the data in experiment 1 of Table 1 revealed an apparent lag in the destruction during the first 30 minutes of incubation. The rate of destruction approached linearity thereafter. A plot of the rate of loss of the hormonal activity between hours 14 and 22 of experiment 2 also gave a straight

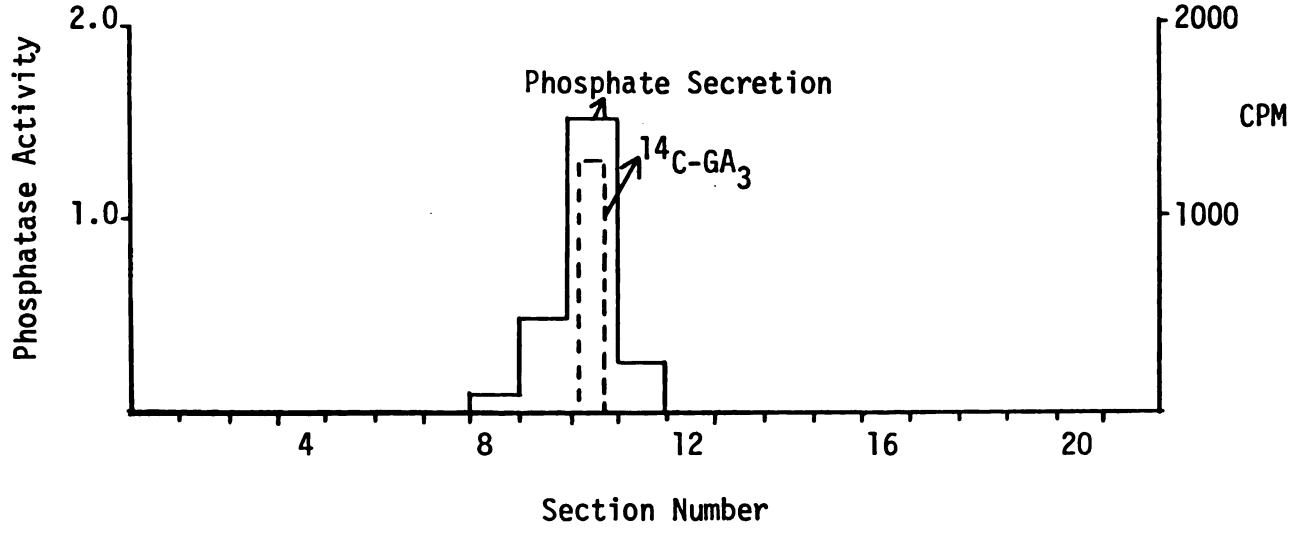


Figure 2.--Correspondence of the migration of ^{14}C-GA$_3$ with the biologically active area of extracts from barley aleurone layers treated with GA$_3$. The layers (60/group) were incubated in medium containing 8.5 µg GA$_3$/ml in a total volume of 5.0 ml for 5 minutes at room temperature on a mechanical shaker and then extracted with hot ethanol. After the extract was applied, the thin layer chromatogram was developed in the benzene-n-butanol-acetic acid (70:25:5) system. Sections of 8 mm were eluted with 1 ml of water and the extracts incubated with barley half seeds (8/group) to bioassay for GA$_3$. Phosphatase secretion was measured.

TABLE 1.--Inactivation of gibberellic acid by barley aleurone layers. Four groups of aleurone layers (50/group in experiment 1 and 15/group in experiment 2) were exposed to solutions containing 5 µg/ml and 10 µg/ml GA_3 for 15 and 30 minutes, respectively. The layers were then washed with distilled water at 0°C for 3 hours. One group was removed and extracted with boiling ethanol, others were allowed to incubate in flasks without solution for the times given, and subsequently extracted. The ethanol was removed, the residue dissolved in streptomycin sulfate-$CaCl_2$ solution and the solution bioassayed for GA_3.

	Time of Incubation (hours)	µg GA_3 Present
Experiment 1	0	5.00
	0.5	5.00
	2.0	3.00
	4.5	1.00
Experiment 2	0	5.25
	14.0	3.75
	16.0	3.00
	22.0	1.20

line. Anaerobiosis did not affect the rate in which hormonal activity was lost. It is unlikely that it was bound to polymers or converted to inextractable forms since it can be shown that virtually all of the radioactivity is extractable from the tissue when labeled GA_3 is used (see section IV in Materials and Methods).

III. The Metabolism of Gibberellic Acid

A. Distribution of Radioactivity in Extracts of ^{14}C-GA_3 Treated Layers

Table 2 summarizes the distribution of radioactivity in extracts of aleurone layers treated with ^{14}C-GA_3. Experiments 1 and 2 in Table 2 are comparable. Experiment 2 differed from experiment 1 in that three times the number of layers were used and there was

TABLE 2.--Distribution of radioactivity in various extracts of ^{14}C-GA_3 treated aleurone layers.

	Experiment			
	Control	1	2	3
Number of layers	53	53	180	420
Incubation time (hours)	24	24	26	24
Total volume (ml)	4	4	12	13
Total cpm ^{14}C-GA_3 initially added	60,000	60,000	1.8×10^5	5.8×10^5
GA_3 concentration, µg/ml	2.75	2.75	2.75	6.15
Cpm found in medium plus wash solution	52,160	49,000	140,000	4.8×10^5
Cpm found in methanolic extracts	7,240	5,150	12,600	88,900
Cpm found in acidic ethyl acetate phase of layers	6,840	3,700	8,070	55,000
Cpm found in basic ethyl acetate phase of layers	200	150	540	1,400
Cpm in aqueous phase (polar metabolites)	None	1,140	3,570	28,000
Percentage of GA_3 converted to polar metabolites	None	1.90	1.98	4.82

a corresponding increase in volume. Hence, the hormonal concentration was the same. Notably, in both experiments almost 2% of the applied hormone was metabolized. However, in experiment 3, in which more layers were present and the hormonal concentration was higher than in the previous experiments, 4.82% of the applied hormone was metabolized.

Extracts from boiled aleurone tissues (control experiment) incubated with the labeled hormone contained only gibberellic acid and there was no evidence of decomposition or degradation as a consequence of the experimental procedure. Most of the radioactivity was found in the acidic ethyl acetate phase, both from the medium and from extracts of layers.

B. Chromatography of Extracts

Paper chromatography (system A) and thin layer chromatography (system H) of the acidic and basic ethyl acetate phase of both layers and medium showed that all of the radioactive counts were localized in the GA_3 area. The aqueous phase of the combined medium and wash solutions contained virtually no radioactivity. About 70-85% of the radioactivity of the medium and the wash solution was unmetabolized GA_3.

There were two significant radioactive zones of polar metabolites present on paper chromatograms developed in system A (Figure 3A) of the resulting aqueous phase of aleurone layers. The major component, which contained 75-85% of the radioactivity of the metabolites, appeared at the origin of the paper chromatogram

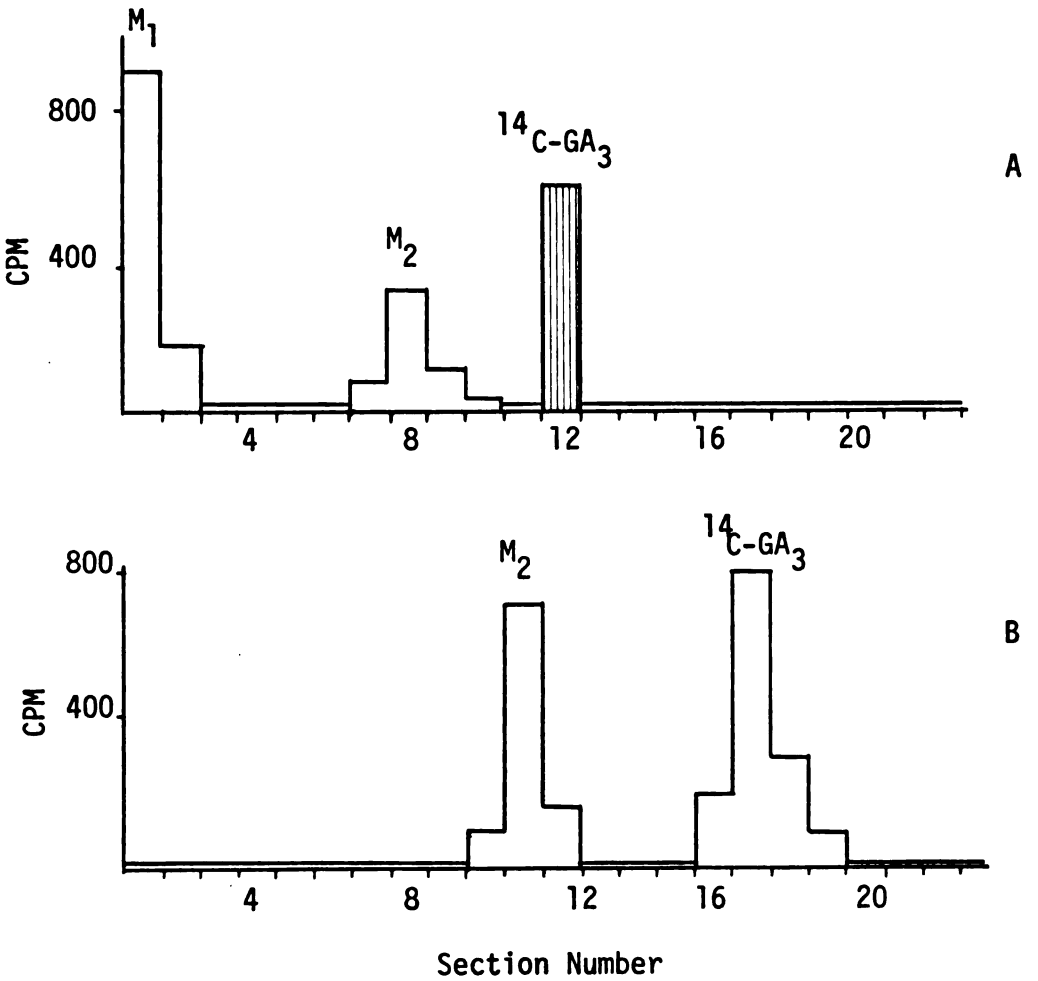


Figure 3.--Pattern of distribution of radioactivity on paper chromatograms of the polar metabolites present in the aqueous phase of GA_3-treated barley aleurone layers. The aqueous phase had been partitioned against ethyl acetate both at pH 8.4 and 2.4. The radioactive metabolites were adsorbed onto charcoal, desorbed with ammoniacal ethanol, the solutions concentrated, placed on paper and chromatographed.

A. Distribution of radioactivity in the metabolites and in GA_3 after chromatography for 18 hours.

B. Distribution of radioactivity of the minor metabolite, M_2 of GA_3, when it was cochromatographed with ^{14}C-GA_3 for 43 hours. Chromatograms were developed in the isopropanol-water (4:1) system.

(system A) whereas the other component which contained 15-25% of the total radioactivity migrated slower than GA_3 upon chromatography for 18-24 hours (Figure 3A).

The major metabolite that remained at the origin of the paper chromatogram will be referred to as M_1 and the minor metabolite that migrated behind the GA_3 area, as M_2.

Significant separation of the minor metabolite from GA_3 was obtained when they were cochromatographed in system A for 43 hours (Figure 3B). This metabolite can also be easily separated from GA_3 on thin layer plates of silica gel G in solvent systems H, I, J, K and L (Figure 4). Values for the R_F of M_2 from GA_3 and GA_3 in these systems are given in Table 3.

The major highly polar metabolite that remained at the origin of the paper chromatogram (Figure 3A) can also be separated from GA_3 and other metabolites by electrophoresis in buffers B and C (Figure 15), by paper chromatography in systems B and C (Figures 5 and 6) and by thin layer chromatography in system N (Figure 6).

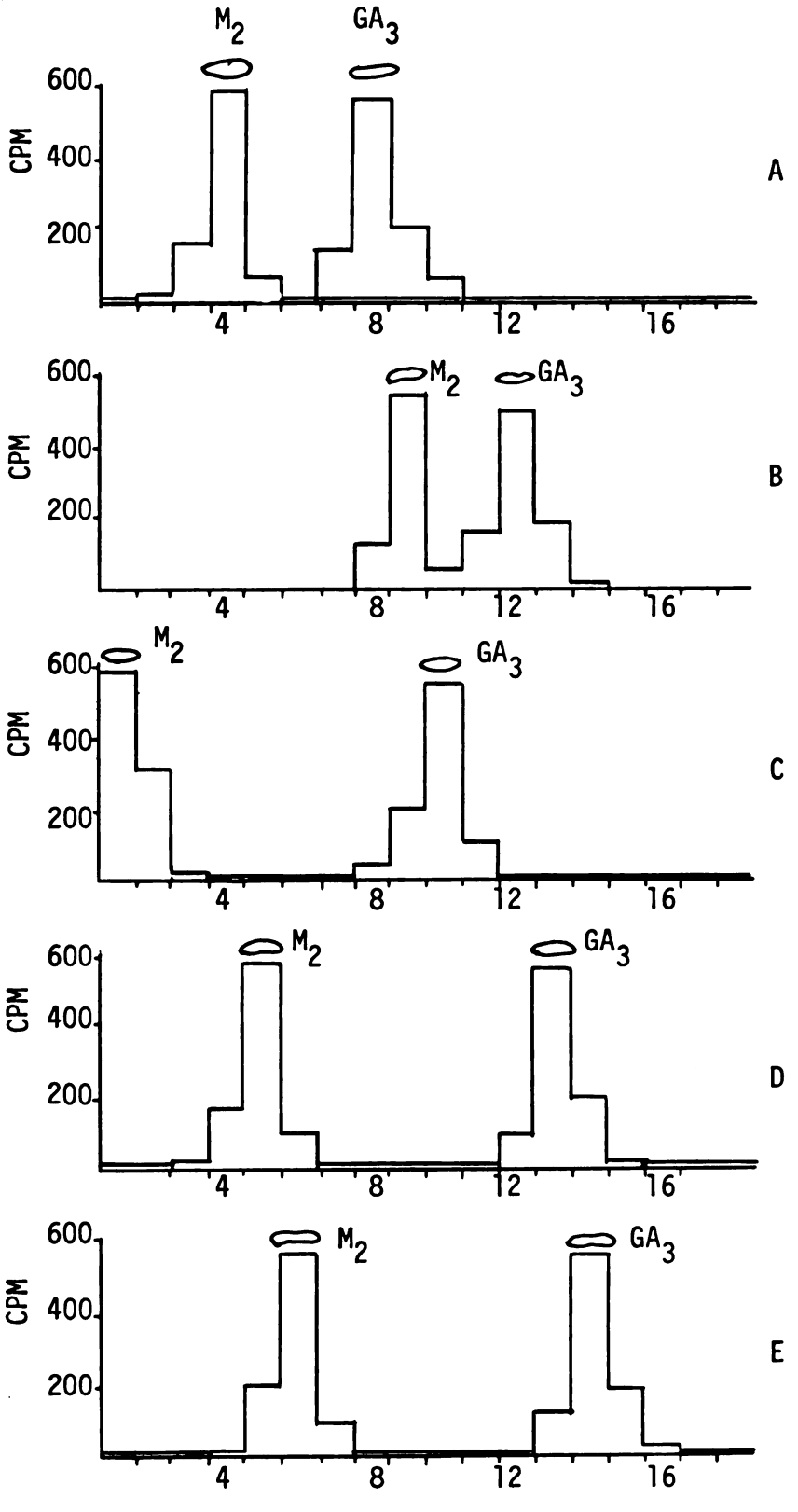
TABLE 3.--R_F values of GA_3 and M_2 of GA_3 in various thin layer chromatographic systems.

Solvent System	R_F Values	
	GA_3	M_2
Isopropanol-6 N NH_4OH (5:1)	0.41	0.20
Propanol-ethyl acetate-acetic acid (7:2:2)	0.67	0.51
Benzene-n-butanol-acetic acid (70:25:5)	0.53	0.08
Acetone-acetic acid (97:3)	0.73	0.24
Chloroform-methanol-acetic acid-water (40:15:3:2)	0.77	0.31

Figure 4.--Distribution of radioactivity of M_2 of GA_3 on thin layer chromatograms after development in various solvent systems.

A. Isopropanol-6 N NH_4OH (5:1).
B. Propanol-ethyl acetate-acetic acid (7:2:2).
C. Benzene-butanol-acetic acid (70:25:5).
D. Acetone-acetic acid (97:3).
E. Chloroform-methanol-acetic acid-water (40:15:3:2).

The oval drawings at the top represent the yellowish-green fluorescent spots of unlabeled M_2 and GA_3 in ultraviolet light after the plates were sprayed with 5% H_2SO_4 in ethanol and heated.


M2
GA3
600
400
200
CPM
A
B
C
D
E
4
8
12
16
Section Number

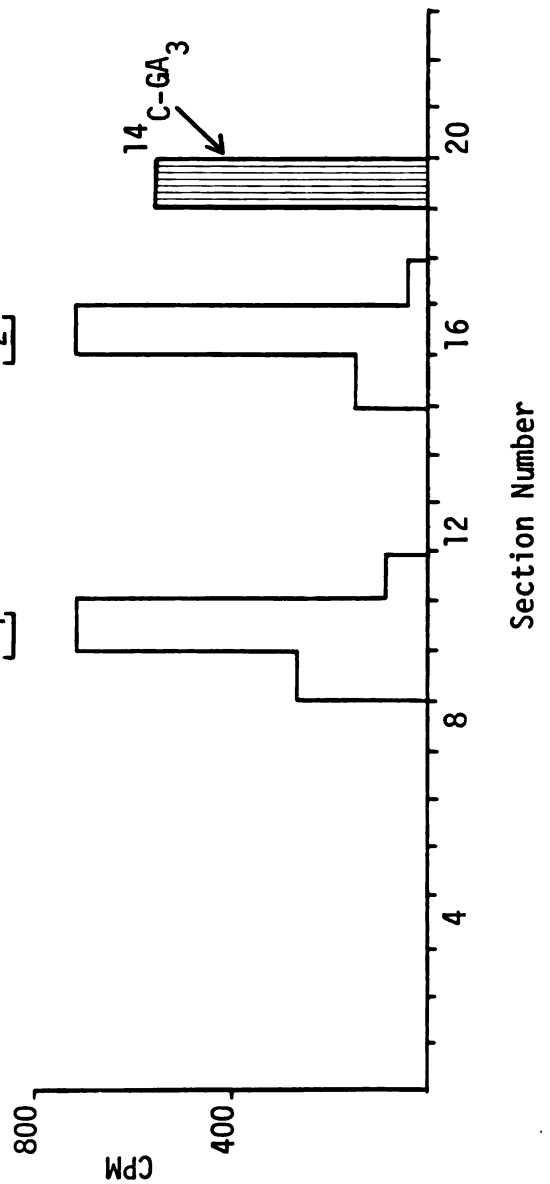


Figure 5.--Migration patterns of radioactivity on a paper chromatogram of M_1 and M_2 of GA_3 in relation to GA_3. The chromatogram was developed in the methanol-ethanol-n-butanol-water-acetic acid (4:3:1:1.5:0.5) solvent system.

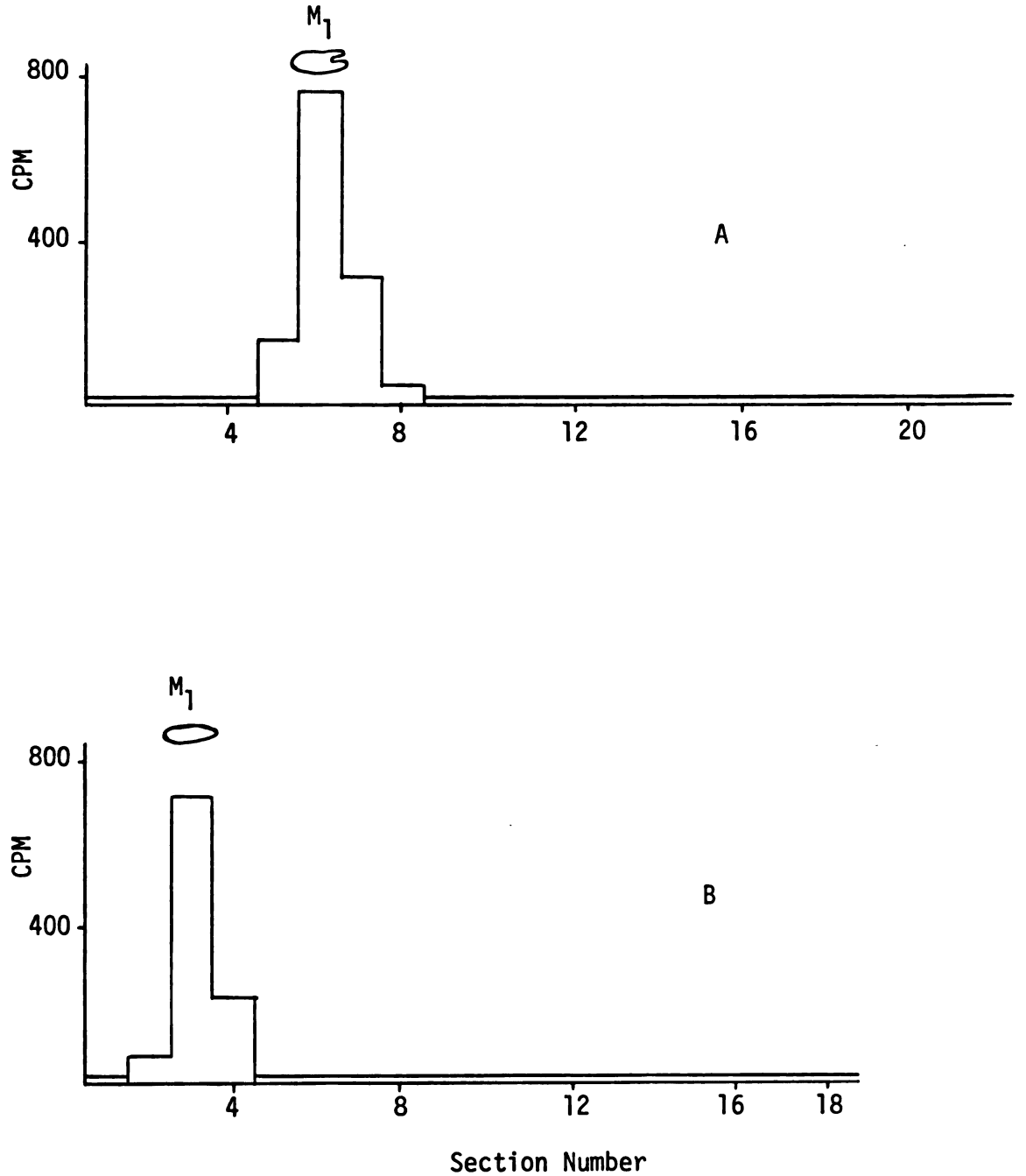


Figure 6.--Migration pattern of radioactivity on chromatograms of the major metabolite, M_1 of GA_3.

A. Paper chromatogram developed in n-propanol-methanol-35% NH_4OH (4:3:3).

B. Thin layer chromatogram developed in n-butanol-acetic acid water (8:2:2).

The drawings at the top represent the pinkish purple spots of M_1 of GA_3 after the chromatograms were sprayed with ninhydrin reagent and heated.

Perfecting chromatographic systems in which the highly polar metabolite would migrate away from the origin was of some significance in these studies since they afforded procedures for purifying it.

The incubation of wheat aleurone layers with ^{14}C-GA_3 also resulted in the formation of similar M_1 and M_2 metabolites (Figure 40B).

IV. The Metabolism of Gibberellin A_1

A. Distribution of Radioactivity in Extracts of ^{3}H-GA_1 Treated Layers

1. In the aqueous phase.--The aqueous phase of ^{3}H-GA_1 treated layers which had been partitioned against ethyl acetate at pH 8.4 and 2.4 contained materials that appeared as three distinct radioactive peaks on paper chromatograms developed in system A (Figure 7). One of these remained at the origin of the paper chromatogram and it had properties similar to the major highly polar metabolite M_1 from GA_3. It is referred to as M_1 of GA_1. The second metabolite, referred to as M_2 of GA_1, migrated slower than GA_1. The third metabolite, referred to as M_3 of GA_1, migrated slower than M_2 of GA_1 on the paper chromatogram in system A. M_1 and M_2 of GA_1 were formed almost in equal amounts, whereas the amount of M_3 differed from experiment to experiment (Figure 7).

2. In the acidic ethyl acetate phase.--The acidic ethyl acetate phase of the aleurone layers contained material that appeared as two significant peaks of radioactivity on paper chromatograms in system A (Figure 8A). One of them was unmetabolized

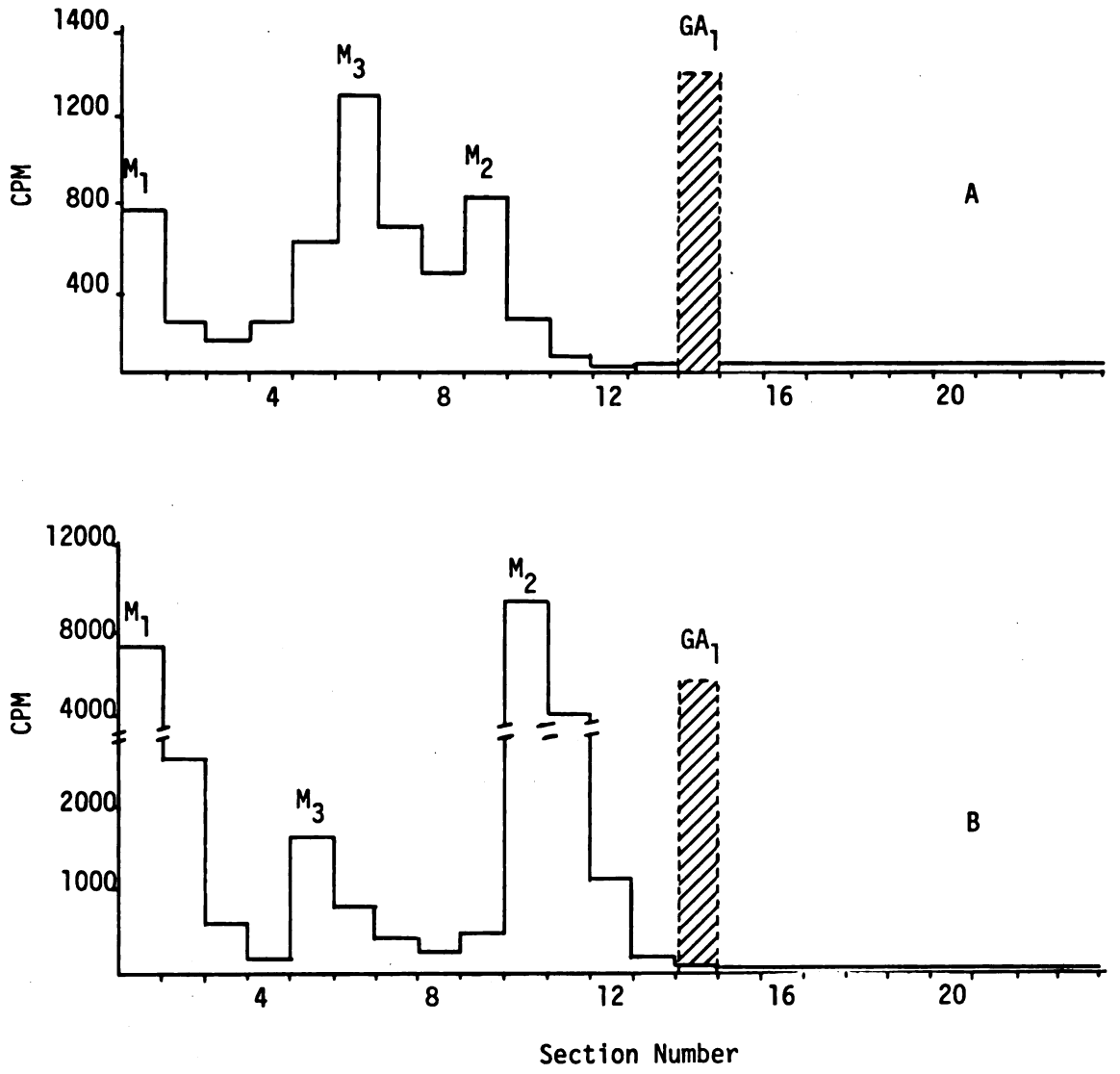


Figure 7.--Distribution of radioactivity on paper chromatograms of the polar metabolites present in the aqueous phase of ^{3}H-GA_1 treated barley aleurone layers. The aqueous phase had been partitioned against ethyl acetate at alkaline and acidic pH values. Chromatograms were developed in isopropanol-water (4:1).

A. Aleurone layers (250) were incubated in medium containing 2.9×10^{-3} µg ^{3}H-GA_1/ml in a total volume of 9.5 ml.

B. Aleurone layers (220) were incubated in medium containing 7×10^{-1} µg ^{3}H-GA_1/ml in a total volume of 6.75 ml.

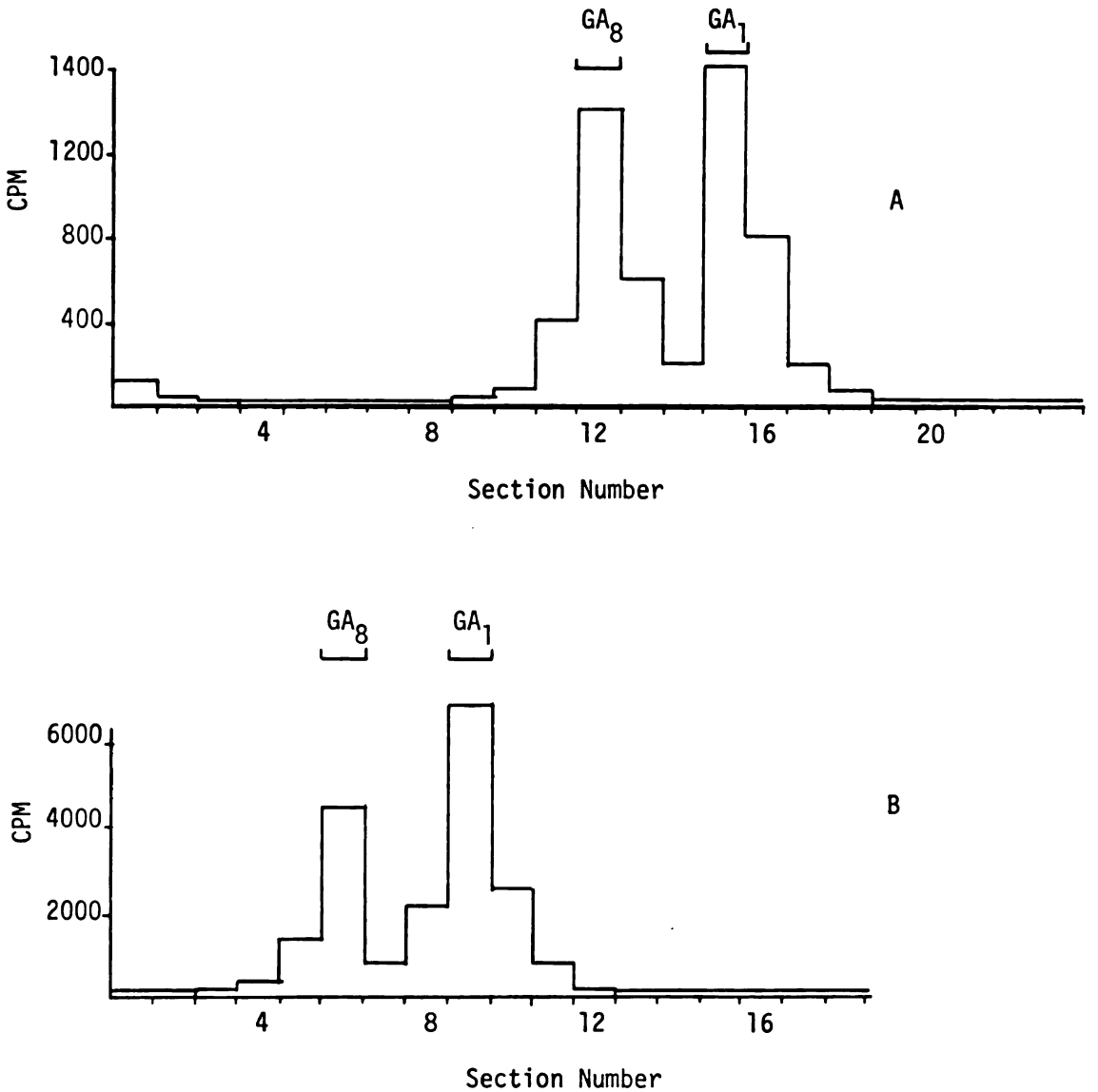


Figure 8.--Distribution of radioactivity on chromatograms of the acid ethyl acetate phase of ^{3}H-GA_1 treated aleurone layers.

A. Paper chromatogram developed in the isopropanol-water (4:1) solvent system.

B. Thin layer chromatogram developed in the isopropanol-6N NH_4OH (5:1) solvent system.

GA_1. The other radioactive component migrated just behind GA_1 and in the GA_8 area on paper chromatograms. Upon electrophoresis in borate buffer the more polar product migrated in front of GA_1 and with GA_8 (Figure 8B). It was further separated from other metabolites but migrated with GA_8 on thin layers in systems H, J and K. It was concluded that the acidic metabolic product was GA_8 (Figure 9). The R_F values of GA_1, GA_8, the acidic metabolite and, additionally, M_3 in several solvent systems are given in Table 4.

Most of the radioactivity from the medium and wash solutions was located in the GA_1 area and consisted of the unmetabolized hormone.

V. Incubation of GA_1 and GA_3 with a Hydroxylating Enzyme System from Phaseolus vulgaris

A. Incubation of ^3H-GA_1 with the Enzyme

The conversion of GA_1 to GA_8 (hydroxylation on C-2 of the GA ring) by an enzyme from Phaseolus vulgaris has been reported by Patterson and Rappaport (1974) and Patterson et al. (1975).

TABLE 4.--R_F values for GA_1, M_3 of GA_1, the metabolite from the acidic phase of the layers and authentic GA_8.

Solvent System	R_F Values			
	GA_1	GA_8	Acidic Metabolite	M_3
Benzene-n-butanol-acetic acid (70:25:5, by volume)	0.50	0.36	0.36	0.05
Isopropanol-6 N NH_4OH (5:1, by volume)	0.46	0.30	0.30	0.10
Chloroform-methanol-acetic acid-water (40:15:3:2, by volume)	0.71	0.52	0.52	0.15

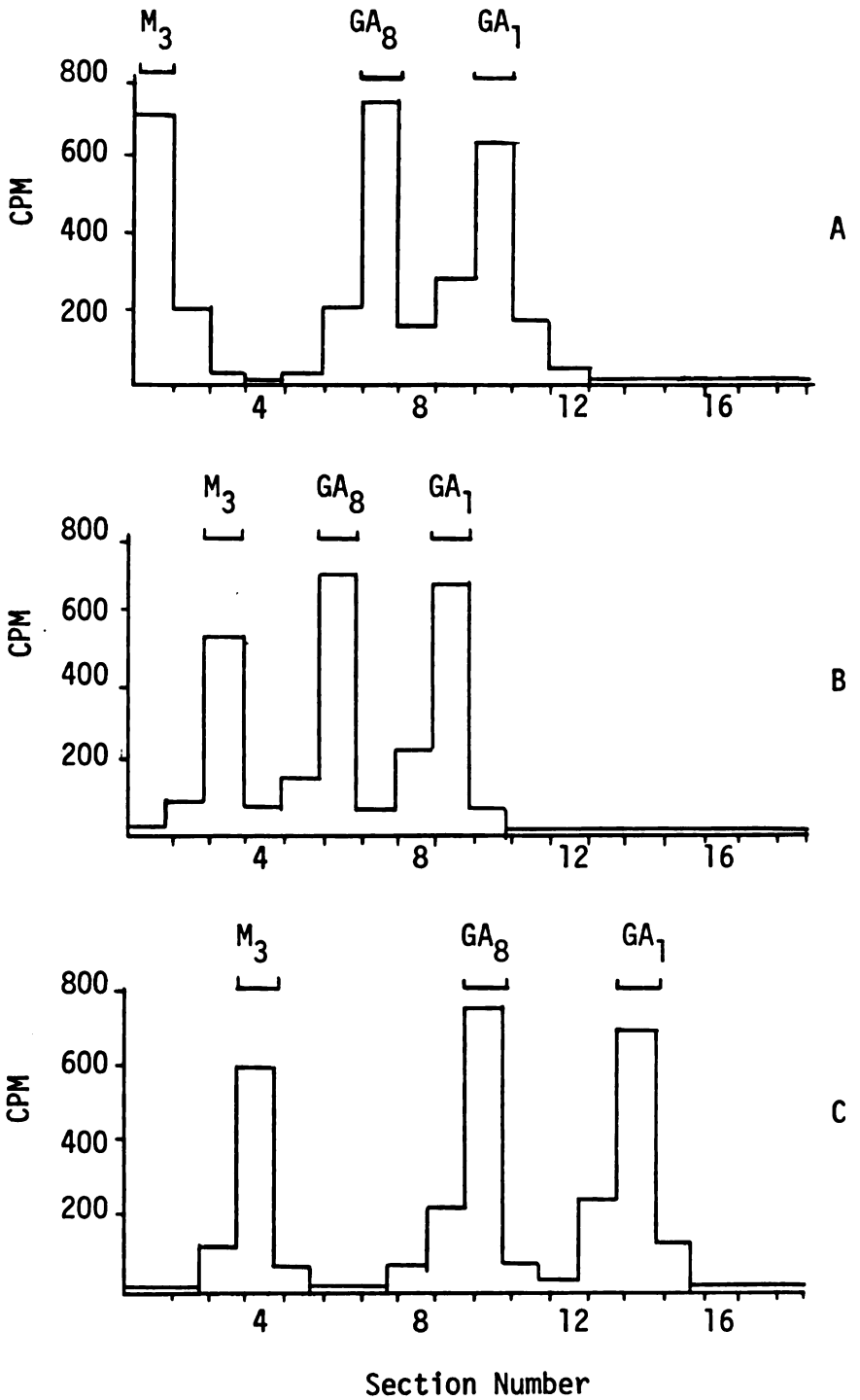


Figure 9.--Migration pattern of radioactivity on thin layer chromatograms of the M_3 metabolite of GA_1, in relation to the products present in the acidic ethyl acetate phase of ^3H-GA_1 treated layers. The plates were developed in the following systems:

A. Benzene-n-butanol-acetic acid (70:25:5);
B. Isopropanol-6 N NH_4OH (5:1);
C. Chloroform-methanol-acetic acid-water (40:15:3:2).

In the present studies two radioactive products were located on paper electrophoretograms (Figure 10A) of the acidic ethyl acetate phase when ^{3}H-GA_1 was incubated with the enzyme system prepared from Phaseolus vulgaris seeds. One of the products migrated behind GA_1 and the other major product migrated in front of GA_1 on the electrophoretograms (Figure 10A). The slower moving product was further chromatographed on paper in system A where it migrated faster than GA_1. Attempts were not made to characterize it further. The major product, GA_8, was also chromatographed on paper in system A and on thin layer in systems H and K where it migrated slower than GA_1 (Figure 9) but as expected of GA_8 in all systems tested.

B. Incubation of ^{14}C-GA_3 with the Enzyme

Incubation of ^{14}C-GA_3 with the enzyme from Phaseolus vulgaris resulted in the formation of three products which were separated on paper electrophoretograms (Figure 10B). One of the products was located behind GA_3 (and is probably similar to the product from GA_1) and the other two migrated in front of GA_3. One of the latter migrated in a manner very similar to GA_8. This compound probably possesses adjacent hydroxyl groups which are capable of forming a borate complex, as is true of GA_8. To the best of my knowledge, hydroxylation of GA_3 has never been reported although this seems possible. The other compound migrated faster than the GA_8-like product on an electrophoretogram and probably possesses more than two adjacent hydroxyl groups on the gibberellane ring.

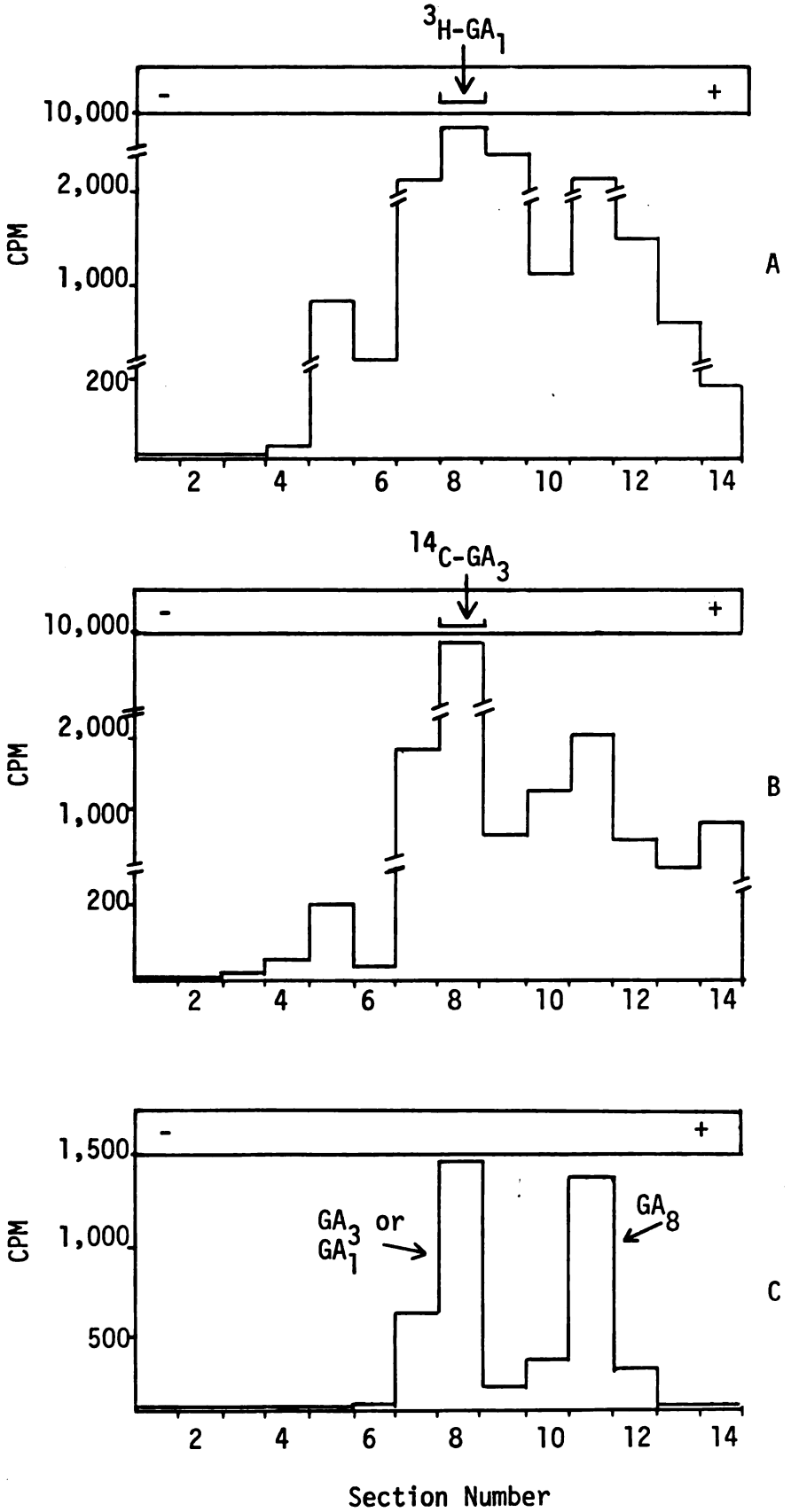
Figure 10.--Pattern of distribution of radioactivity on paper electrophoretograms of the acidic ethyl acetate phase resulting from the incubation of GA_1 and GA_3 with a hydroxylating enzyme system from Phaseolus vulgaris.

A. ^{3}H-GA_1 was incubated with the enzyme for 17 hours.

B. ^{14}C-GA_3 was incubated with the enzyme for 17 hours.

C. The rates of migration of GA_1, GA_3 and GA_8 are shown.

Electrophoresis was carried out in ammonium borate buffer, pH 9.3 for 4 hours.


^{3}H-GA$_1$
-
+
10,000
2,000
1,000
200
CPM
A
2
4
6
8
10
12
14
^{14}C-GA$_3$
-
+
10,000
2,000
1,000
200
CPM
B
2
4
6
8
10
12
14
-
+
1,500
1,000
500
CPM
GA$_3$ or GA$_1$
GA$_8$
C
2
4
6
8
10
12
14
Section Number

VI. Metabolism of Radioactive GA_8 by Aleurone Layers

Two metabolites were located on paper chromatograms developed in system A after ^3H-GA_8 was incubated with aleurone layers (Figure 11). A highly polar metabolite very similar to M_1 from GA_3 remained at the origin of the paper chromatogram and the other metabolite migrated at the same rate as M_3 from GA_1. Like M_3 from GA_1 and the M_2 metabolites, but unlike the simple gibberellin glucosides, it remained at the origin when subjected to thin layer chromatography in the benzene-n-butanol-acetic acid (70:25:5) system. It appears from these results that the M_3 metabolite is derived from GA_8. It is likely that GA_8 is an intermediate in the formation of M_1 of GA_1 since it was metabolized to a very highly polar product similar to M_1 of GA_1.

VII. Time Course of Formation of the Metabolites

In experiments with ^3H-GA_1, all of the metabolites (M_1, M_3 and M_2) could be detected within 15 minutes. Their formation was linear with time and there was no lag period in their production (Figure 12). A plot of the time course of the formation of the polar metabolites from ^{14}C-GA_3 (the radioactive counts appearing in the aqueous phase of the aleurone layers) was also about linear with time for 24-32 hours (Figure 13).

Incubation of aleurone tissues with ^{14}C-GA_3 for a long period of time (48-72 hours) resulted in the disappearance of the polar metabolites (M_1 and M_2) from the aqueous phase of the layers (Table 5). Chromatography of the aqueous phase of the medium and

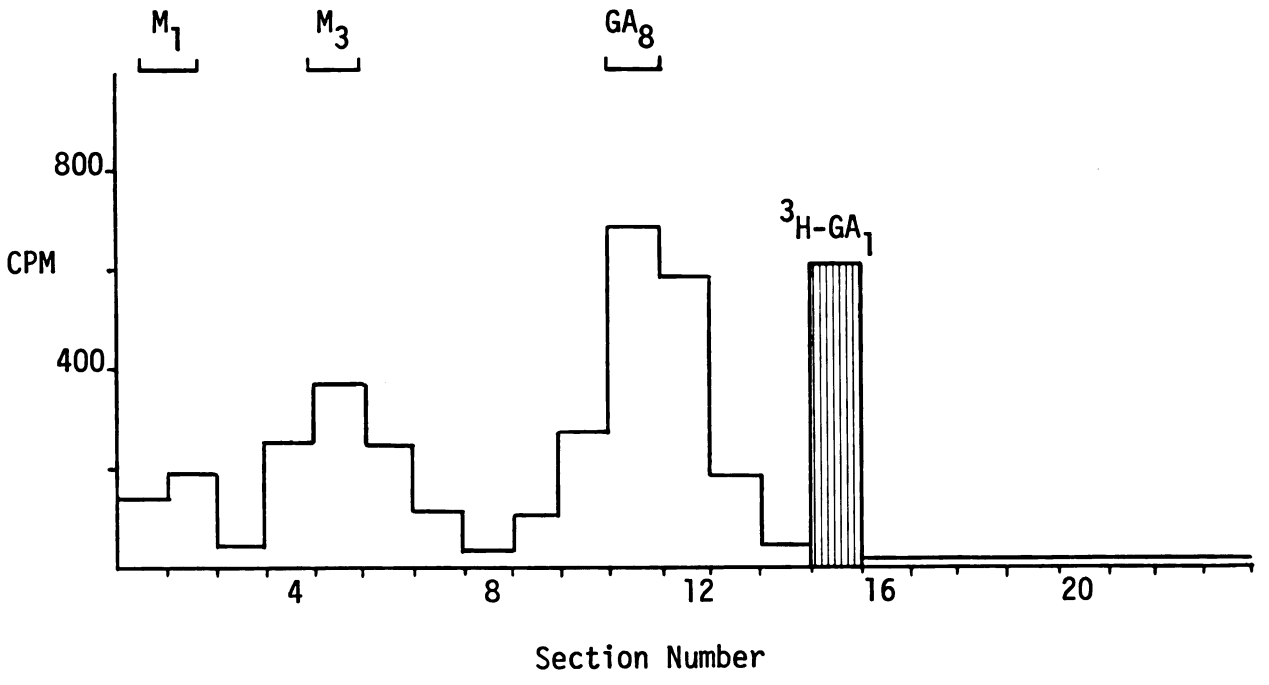


Figure 11.--Distribution of radioactivity from the aqueous phase of ^{3}H-GA_8 treated aleurone layers on a paper chromatogram. Aleurone layers (175) were incubated in medium containing ^{3}H-GA_8 (approximately 33,000 CPM) in total volume of 0.6 ml at room temperature on a mechanical shaker for 18 hours. The aqueous phase had been partitioned against ethyl acetate at pH 8.4 and 2.4. The chromatogram was developed in the isopropanol-water (4:1) system.

Figure 12.--Time course of the formation of polar metabolites (M_1, M_2 and M_3) from ^3H-GA_1 by aleurone layers. Aleurone layers (62/group) were incubated in media containing 6.3×10^{-3} µg ^3H-GA_1/ml in a total volume of 3.95 ml, on a mechanical shaker at room temperature. Some layers were removed at different intervals. They were washed, extracted with methanol and the extract evaporated to dryness. The residue was dissolved in a known volume of borax buffer, pH 8.4, which was partitioned against ethyl acetate at pH 8.4 and 2.4. The aqueous phase was treated with charcoal which was subsequently eluted with ammoniacal ethanol and the extract chromatographed on paper in the isopropanol-water (4:1) system.

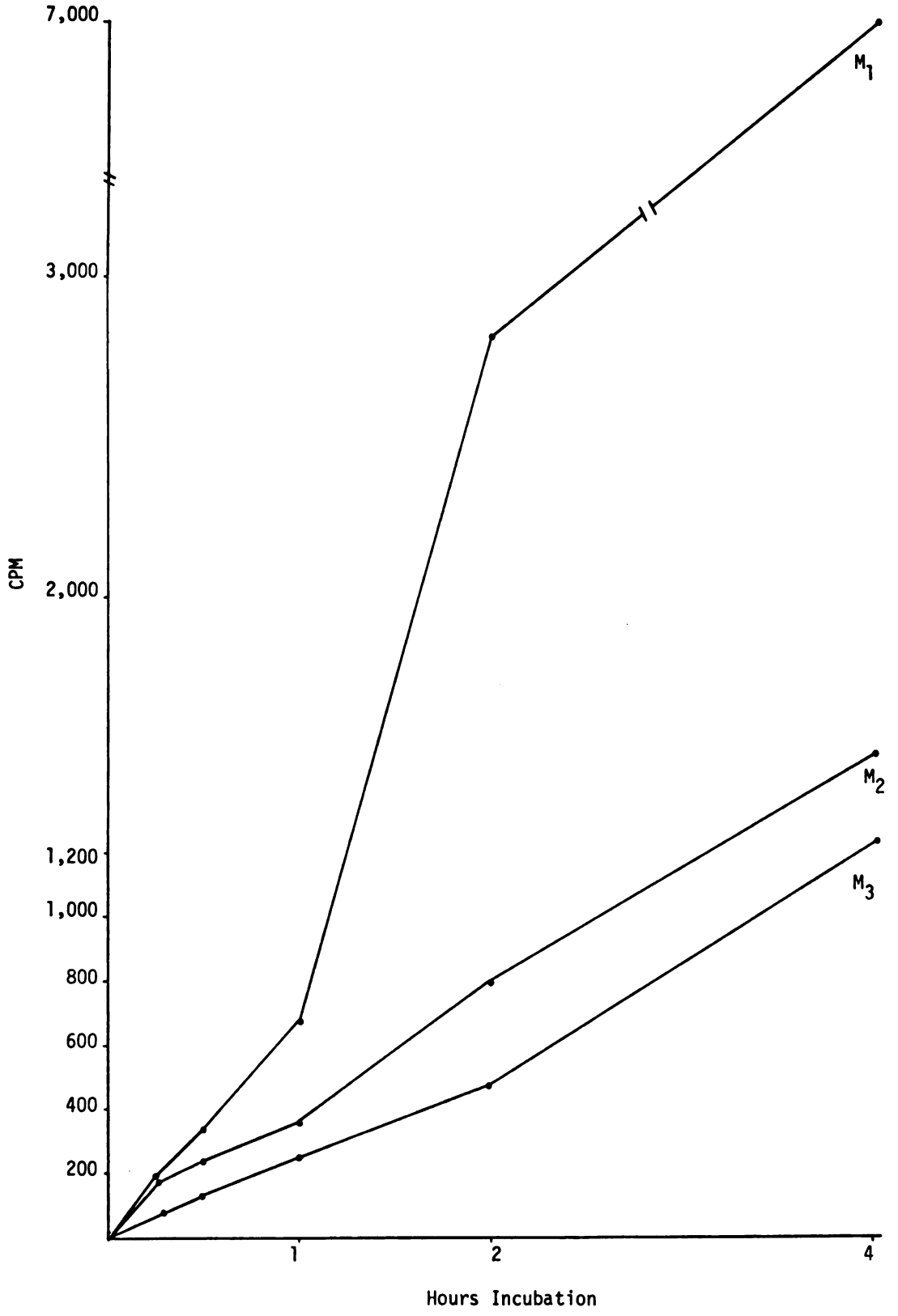

7,000
3,000
2,000
1,200
1,000
800
600
400
200
CPM
M1
M2
M3
1
2
4
Hours Incubation

Figure 13.--Time course of the formation of polar metabolites of ^{14}C-GA_3. Aleurone layers (50/group) were incubated in media containing 2.71 µg ^{14}C-GA_3/ml in a total volume of 4.05 ml on a mechanical shaker at room temperature. Some layers were removed at different intervals, washed with distilled water and extracted with methanol. The extracts were evaporated to dryness, the residue dissolved in a known volume of borax buffer, pH 8.4, and the solution partitioned against ethyl acetate at pH 8.4 and 2.4. A known volume of the resulting aqueous phase was counted in Bray's solution.

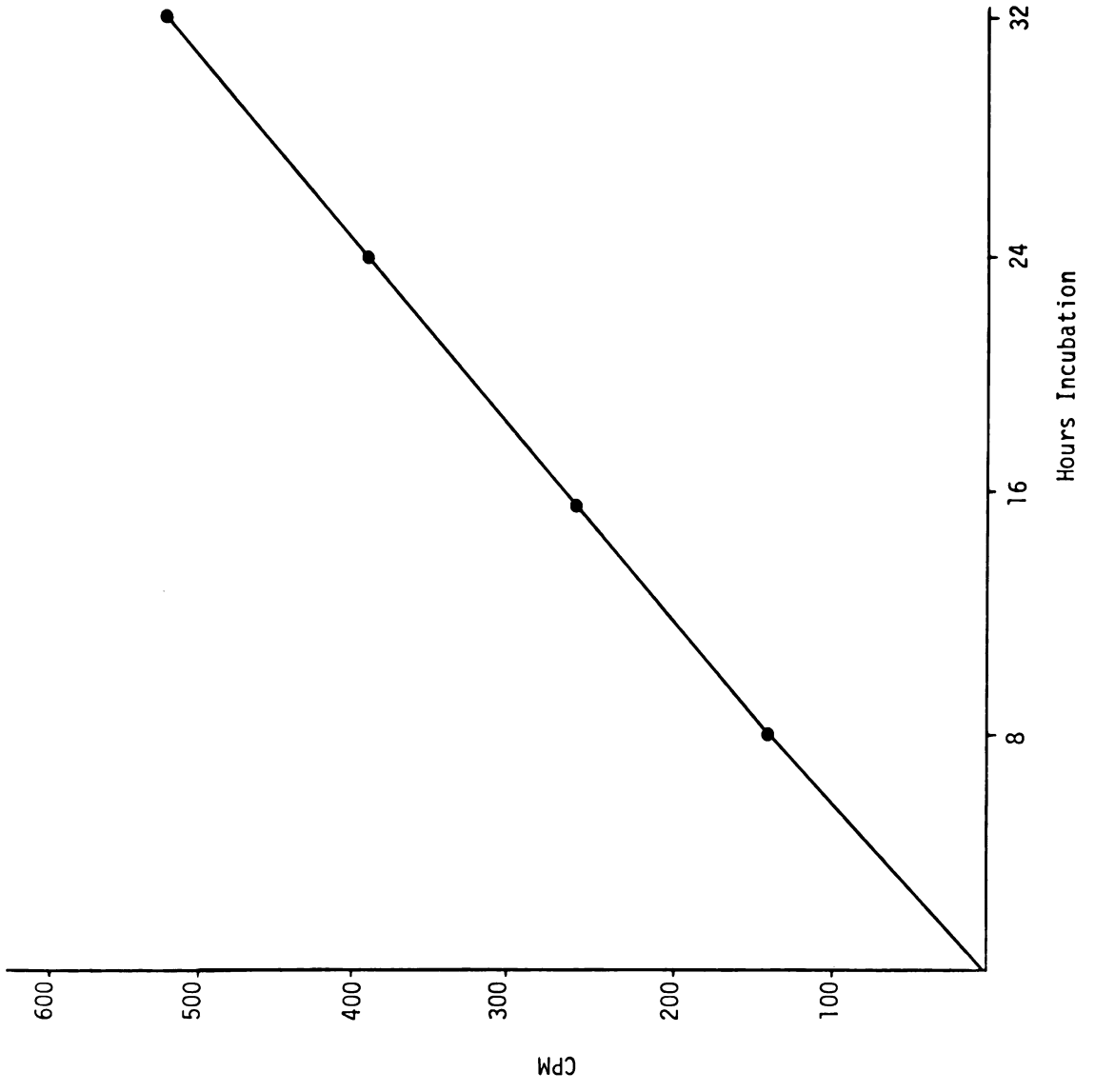

600
500
400
300
200
100
CPM
8
16
24
32
Hours Incubation

TABLE 5.--Effect of prolonged incubation on the amount of radioactivity present in polar metabolites (M_1 and M_2) in the aqueous phase of ^{14}C-GA_3 treated aleurone layers. The tissues (154 layers/group) were incubated in media containing 2.75 µg ^{14}C-GA_3/ml in a total volume of 5 ml at room temperature on a mechanical shaker. At the end of the incubation periods, the layers were washed and extracted with methanol. The aqueous phase of the layers (which had been partitioned against ethyl acetate at pH 8.4 and 2.4 and treated with charcoal) was chromatographed on paper in isopropanol-water (4:1).

Incubation Period (hours)	Radioactivity (cpm) in Polar Metabolites on paper chromatograms	
	M_1	M_2
24	2497	451
48	2162	511
72	375	26

wash solution, which had been partitioned against ethyl acetate at pH 8.4 and 2.4, resulted in the appearance of three significant peaks of radioactivity on paper chromatograms in system A (Figure 14). One radioactive peak was present at the origin of the chromatogram, presumably M_1; the second was located in the M_2 area, so is thought to be that compound; and the third migrated in front of the radioactive zone at the origin. It did not appear to be any of the metabolites observed earlier.

The acidic ethyl acetate phase of the layers contained very little radioactivity. The acidic and basic ethyl acetate phase of the medium contained most of the radioactive counts. Upon paper chromatography of both of these phases in system A, all the counts were located in the GA_3 area.

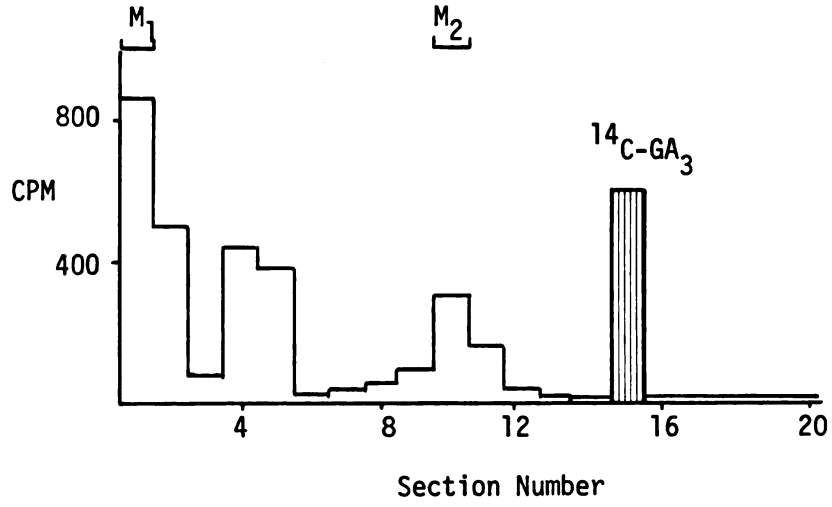


Figure 14.--Distribution of radioactivity on a paper chromatogram of the aqueous phase of the combined medium and wash solution from aleurone layers incubated with ^{14}C-GA_3 for 72 hours. The paper chromatogram was developed in the isopropanol-water (4:1) solvent system.

Thus, it is evident from the results that the formation of these polar metabolites from gibberellins by the aleurone tissues is linear with time and it appears that after prolonged incubation the layers lose their ability to retain, and possibly to form, the polar metabolites.

VIII. Electrophoretic Properties of the Metabolites

The rates of migration of all the metabolites from GA_1 and GA_3 toward the anodes were about the same as GA_1/GA_3 in buffer A, pH 3.5, and D, pH 8.9 (data not presented). M_2 from both GAs and M_3 from GA_1 migrated at about the same rate as GA_1 or GA_3 in ammonium

borate buffer at pH 9.3 (data not presented). The similarity in mobility of these metabolites of GA_3/GA_1 in these buffers suggests that they are charged to about the same extent as the GAs. They probably contain only one free carboxyl group.

The major highly polar metabolites (M_1) from GA_3 and GA_1 migrated faster than the phytohormones upon electrophoresis in ammonium borate buffer, pH 9.3 (Figure 15A). This strongly suggests that the M_1 metabolites possess adjacent hydroxyl groups which are capable of forming borate complexes, as is true of GA_8 and GA_{32} (Coombe and Tate, 1970). M_1 from GA_1 migrated a little faster than GA_8 upon electrophoresis in borate buffer for a long period of time (5 hours); this might indicate that M_1 possesses an additional hydroxyl group (Figure 16).

GA_3 and GA_1 migrated toward the anode and M_1 toward the cathode upon electrophoresis in buffer C at pH 2.2 (Figure 15B). This suggested that M_1 contains highly charged positive group or groups (assumed to be amino groups since M_1 gave a positive test with the ninhydrin reagent). Although M_1 migrated similarly to the GAs in buffers A and D, unexpectedly, it migrated faster than GA_3 when it was electrophoresed in pyridine-acetic acid buffer, pH 5.6. Possibly this is due to the presence of extra hydroxyl or carboxyl groups.

Gibberellinic acid was prepared by a method described by Grove and Mulholland (1960) by heating a mixture of ^{14}C-GA_3 and hydrazine sulfate plus NaOH (1:1) in a boiling water bath for 40 minutes. This solution was acidified and extracted with ethyl

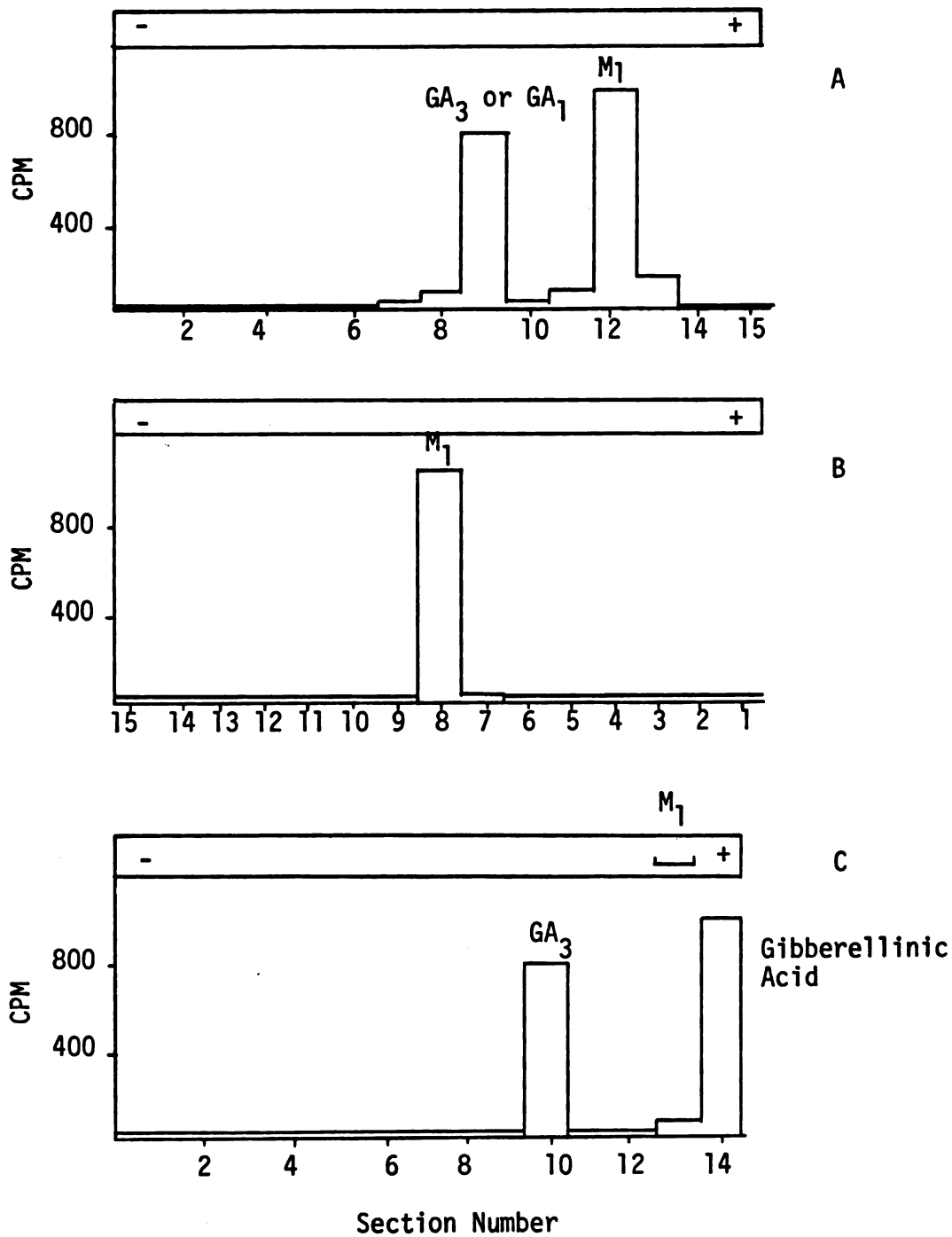


Figure 15.--Distribution of radioactive M_1 metabolites on paper electrophoretograms.

A. M_1 of both GA_1 and GA_3 electrophoresed for 4 hours with 0.05 M ammonium borate buffer, pH 9.3.

B. M_1 of GA_1 electrophoresed for 6 hours with formic-acetic acid buffer, pH 2.2.

C. M_1 of GA_3 and gibberellinic acid electrophoresed for 6 hours with the pyridine-acetic acid buffer, pH 5.6.

A Durrum type unit and Whatman 3 MM strips were used at 400 volts potential.

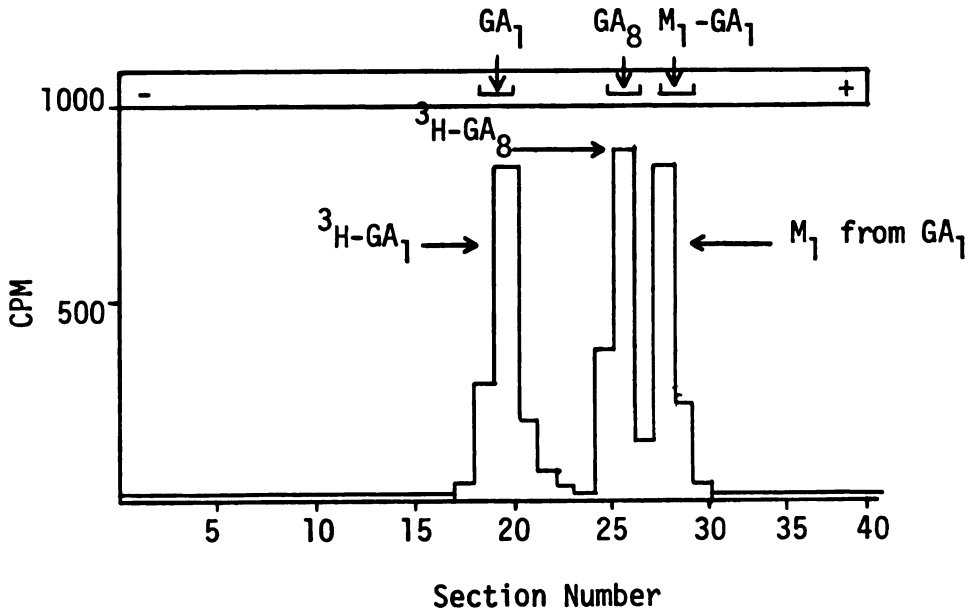


Figure 16.--Distribution of radioactivity in ^{3}H-GA_1, ^{3}H-GA_8 and M_1 of GA_1 on an electrophoretogram. Electrophoresis was carried out on 40 cm strips of 3 MM paper for 5 hours in ammonium borate buffer, pH 9.3.

acetate and the extract chromatographed on paper in system A. Gibberellinic acid migrated behind GA_3 on this chromatogram. This compound, which contains an extra carboxyl group, migrated much faster than M_1 of GA_3 in the pyridine-acetate buffer, pH 5.6 (Figure 15C). Thus, it seems unlikely that M_1 contains extra carboxyl groups.

IX. Effect of Anaerobiosis, N_2 and KCN on the Formation of Polar Metabolites from GA1

Upon incubation of aleurone layers with the phytohormone ^{3}H-GA_1 under anaerobic condition, the formation of M_1 and M_3 metabolites was inhibited, however, M_2 production was not affected.

Incubation under an atmosphere of N_2 or with KCN inhibited the formation of all the polar metabolites (Table 6). The formation of M_1 was inhibited more significantly than was the other metabolites. The inhibition of M_3 formation under anaerobic conditions probably means that the conversion of GA_1 to GA_8 was prevented. Hence, GA_8 was not available for the formation of the GA_8-glycoside (M_3).

X. Abscisic Acid (ABA) and the Formation of Gibberellin Metabolites

It has been reported (Nadeau et al., 1972) that the inclusion of ABA in the medium increases the production of polar metabolites from GA_1-treated aleurone layers. In the present experiments, abscisic acid enhanced the formation of polar metabolites from both GA_1 and GA_3 by the layers.

A. Influence on GA_3 Metabolism

The formation of M_1 and M_2 from GA_3 was 23% and 18.6% higher in the treated tissues than in the controls, respectively (Table 7). The formation of more polar metabolites from GA_3 in ABA treated layers may account for the presence of less radioactivity in the combined medium and wash solutions of the experimental sample than was present in the control (Table 7).

B. Influence on GA_1 Metabolism

In experiments with GA_1, the presence of more radioactive counts in the aqueous phase of ABA treated layers than in controls was also noted (Table 8). This was due to an increase in the

TABLE 6.--Effects of anaerobiosis, KCN, and incubation in an atmosphere of nitrogen on the formation of the polar metabolites of ^{3}H-GA_1 by layers.

Expt. No.	Content of the Flask		Incubation Time (Hours)	Condition	Radioactivity (cpm) Present in Polar Metabolites on Paper Chromatograms Developed in System A		
					M_1	M_3	M_2
I	Number of aleurone layers	192	18	Control	792	841	1,709
	^{3}H-GA_1 conc. (µg/ml)	2.1×10^{-3}		Anaerobic	419	677	2,475
	Total volume (ml)	10.5					
II	Number of aleurone layers	75	5	Control	1,407	568	861
	^{3}H-GA_1 conc. (µg/ml)	9.3×10^{-3}		Anaerobic	870	535	1,206
	Total volume (ml)	2.4					
III	Number of aleurone layers	103	5	Control	997	501	944
	^{3}H-GA_1 conc. (µg/ml)	3.7×10^{-3}		Anaerobic	596	461	1,007
	Total volume (ml)	6		KCN (1×10^{-3}M)	376	305	460
IV	Number of aleurone layers	104	5	Control	1,619	1,126	1,137
	^{3}H-GA_1 conc. (µg/ml)	1.12×10^{-2}		Anaerobic	1,110	799	1,434
	Total volume (ml)	3		Nitrogen	250	497	867

TABLE 7.--Effect of abscisic acid on ^{14}C-GA_3 metabolism in aleurone layers. Aleurone layers (300/group) were incubated in media containing 5.21 μg ^{14}C-GA_3/ml in a total volume of 11.5 ml. When used, the ABA concentration was 1.9 x 10^{-5}M. Incubation was done at room temperature on a mechanical shaker for 24 hours. The amount of radioactivity on paper chromatogram (developed in System A), in M_1 and M_2 metabolites was determined by counting the areas of the chromatograms.

	^{14}C-GA_3 (Control)	^{14}C-GA_3 + Abscisic Acid
Cpm GA_3 initially added	430,000	430,000
Cpm in aqueous phase of layers	19,530	31,210
GA_3 metabolized on the basis of counts present in the aqueous phase, %	4.54	7.26
Cpm in aqueous phase after charcoal treatment	16,000	21,000
Total counts (cpm) in M_1 on chromatogram	8,400	10,370
Total counts (cpm) in M_2 on chromatogram	2,360	2,800
GA_3 metabolized to M_1, %	1.95	2.41
GA_3 metabolized to M_2, %	0.55	0.65
GA_3 metabolized--Total M_1 + M_2, %	2.50	3.06
Cpm in basic ethyl acetate phase of layers	1,500	300
Cpm in acidic ethyl acetate phase of layers	50,400	52,800
Cpm in medium and wash solutions	303,120	262,500

TABLE 8.--Effect of abscisic acid on the amount of radioactivity from ^3H-GA_1 found in the aqueous and acidic phases of aleurone layers. Aleurone layers (250/group in experiment I and 50/group in experiment II) were incubated in media containing 2.9×10^{-3} µg ^3H-GA_1/ml (for expt. I) or 2.6×10^{-3} µg ^3H-GA_1/ml (for expt. II) in total volumes of 9.5 ml and 2.1 ml, respectively. When used, the ABA concentration was 1.9×10^{-5} M. Incubations were carried out at room temperature for 24 hours on a mechanical shaker.

Expt.	Content of the Incubation Medium	Radioactivity (cpm)	
		Aqueous Phase	Acidic Phase
I	^3H-GA_1 (control)	110,960	154,980
	^3H-GA_1 + abscisic acid	152,000	132,514
II	^3H-GA_1 (control)	19,100	32,448
	^3H-GA_1 + abscisic acid	25,000	21,764

TABLE 9.--Effect of abscisic acid on the formation of the polar metabolites (M_1, M_3 and M_2) from ^3H-GA_1 in the experiments given in Table 8. This table gives the amount of radioactivity (cpm) present in each of the metabolites on paper chromatograms in the isopropanol-water (4:1) system.

Expt.	Content of the Incubation Media	Radioactive Counts (cpm) on Paper Chromatograms		
		M_1	M_3	M_2
I	^3H-GA_1 (control)	1,042	2,735	1,410
	^3H-GA_1 + abscisic acid	3,730	2,852	2,618
II	^3H-GA_1 (control)	670	498	370
	^3H-GA_1 + abscisic acid	1,550	509	456

production of the polar metabolites (Table 9, page 60). This may account for the presence of less radioactivity in the acidic ethyl acetate phase in abscisic acid treated layers than was present in the control extract (Table 8). The ABA effect was characterized by a significant increase in the formation of M_1 and M_2 from GA_1, however, M_3 was not affected (Table 9). In one experiment the amount of M_1 formed was 258% higher than the control and the amount of M_2 formed was 86.5% higher than control. In the second experiment, which contained fewer layers, M_1 and M_2 were formed in amounts that were 131% and 23% higher than the control, respectively.

XI. The Effect of GA_1 and GA_7 on the Formation of Metabolites from ^{14}C-GA_3 and of GA_3 on the Formation of Metabolites from ^{3}H-GA_1

There was a decrease in the amount of radioactivity found in the polar metabolites when aleurone tissues were incubated with ^{14}C-GA_3 plus excess unlabeled GA_1 or GA_7 (Table 10). Their formation was reduced 88% by GA_7, whereas 71% reduction resulted by the inclusion of GA_1. The formation of polar metabolites from ^{3}H-GA_1 treated layers was also reduced by the inclusion of GA_3 (Table 11). The reduction was 12% for M_1, 78% for M_2 and 77% for M_3.

The chemical structures of all these three GAs are very similar (Figure 1). GA_7 differs from GA_3 in not having a hydroxyl group at C-13 of the GA ring. GA_1 differs from GA_3 in that it contains an unsaturated bond between carbons 1 and 2. The inclusion of GA_7 or GA_1 in the medium probably competitively inhibits the formation of metabolites from GA_3.

TABLE 10.--Effect of GA_1 and GA_7 on the amount of radioactivity from ^{14}C-GA_3 found in the methanol extract and polar metabolites of aleurone layers. Aleurone layers (55/group) were incubated in media containing 2.75 µg GA_3/ml in a total volume of 4.05 ml. When they were used, GA_1 and GA_7 were added to final concentrations of 30.86 and 24.69 µg/ml, respectively. Incubations were for 24 hours on a mechanical shaker at room temperature.

Content of the Flask	Radioactivity (cpm)	
	Methanol Extract	Metabolites
^{14}C-Gibberellic acid	5,150	1,150
^{14}C-Gibberellic acid + GA_1	4,500	330
^{14}C-Gibberellic acid + GA_7	2,750	130

TABLE 11.--Effect of GA_3 on the formation of the polar metabolites (M_1, M_3 and M_2) from ^{3}H-GA_1, by aleurone layers. Aleurone layers (220/group) were incubated in media containing 7×10^{-3} µg GA_1/ml in a total volume of 6 ml. When it was used, GA_3 was added to a final concentration of 5 µg/ml. Incubations were for 24 hours on a mechanical shaker at room temperature.

Content of the Flask	Radioactivity (cpm)		
	M_1	M_3	M_2
^{3}H-Gibberellin A_1	7,184	1,431	9,363
^{3}H-Gibberellin A_1 + GA_3	6,294	328	2,047

XII. Studies on the Identification of the M_2 Metabolites

A. Effect of Enzymes on the M_2 Metabolites from Both GA_3 and GA_1 and on M_3 of GA_1

The M_2 metabolites from both GA_1 and GA_3 and M_3 from GA_1 were not hydrolyzed by β-glucosidases from a variety of sources. After treatment with pectinase, whose active component appears to be β-xylosidase, M_2 from both GAs was hydrolyzed to some extent. The products migrated to the area of GA_3/GA_1 on the chromatograms (Figures 17 and 19). M_3 from GA_1 was also hydrolyzed by pectinase to a product which migrated in the area of GA_8 on thin layer and paper chromatograms (Figure 18A). The product obtained after enzymatic hydrolysis of M_3 on the chromatograms was further electrophoresed in borate buffer, pH 9.3, where it migrated in front of GA_1 and with GA_8 (Figure 18B). M_3 from GA_1 was hydrolyzed to a greater extent with pectinase than either M_2 of GA_1 or M_2 of GA_3.

The enzyme β-xylosidase from the crude pectinase preparation was partially purified on a Sephadex G-200 column. Incubation of it with M_2 from GA_1 resulted in the formation of GA_1 (Figure 19).

In order to ascertain that the simple glucosides such as GA_3-, GA_1- or GA_8-glucoside could in fact be hydrolyzed with β-glucosidases it was necessary to obtain some of these and to test the effect of the enzyme on them. GA_3-glucoside was prepared by Koenigs-Knorr glucosidation of the GA_3 via tetra-o-acetyl-glucopyranosyl bromide and subsequent deacetylation with sodium methoxide in methanol (Bárczai-Martos and Körösy, 1950; Murray, 1971). The product

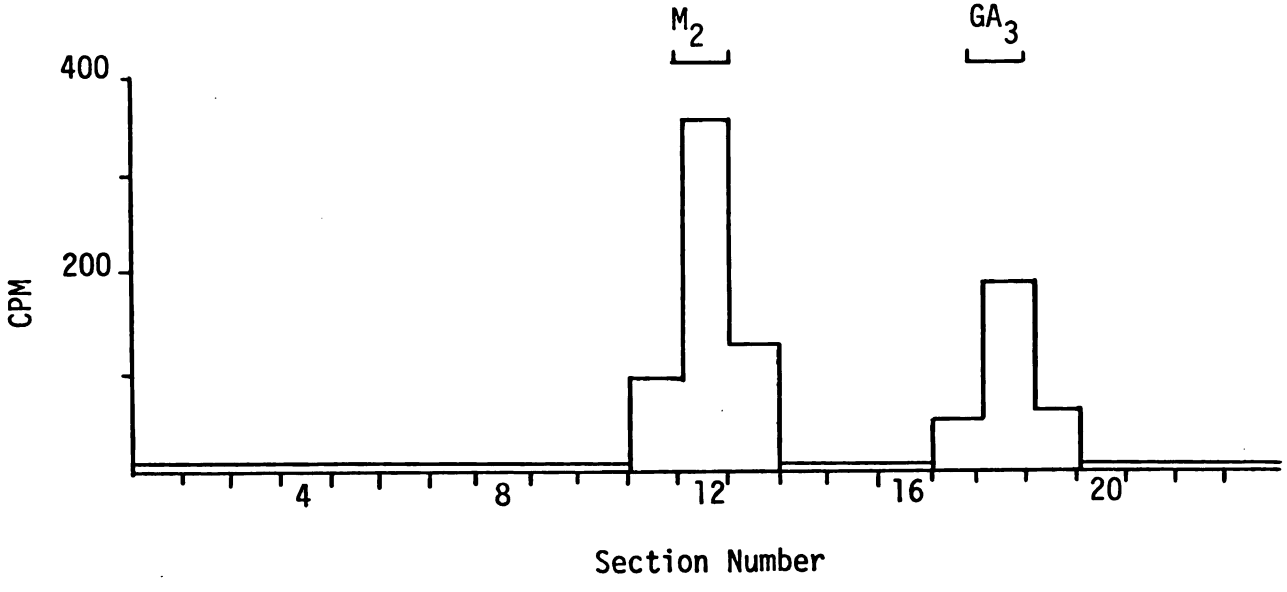


Figure 17.--Distribution of radioactivity on a paper chromatogram of the M_2 metabolite treated with pectinase. M_2 was incubated at 37°C for 22 hours with pectinase (1-2 mg) in 1.5 ml of acetate buffer, pH 5.4. The reaction was stopped by heating the mixture in a boiling water bath for 5 minutes, the mixture was concentrated and chromatographed in isopropanol-water (4:1).

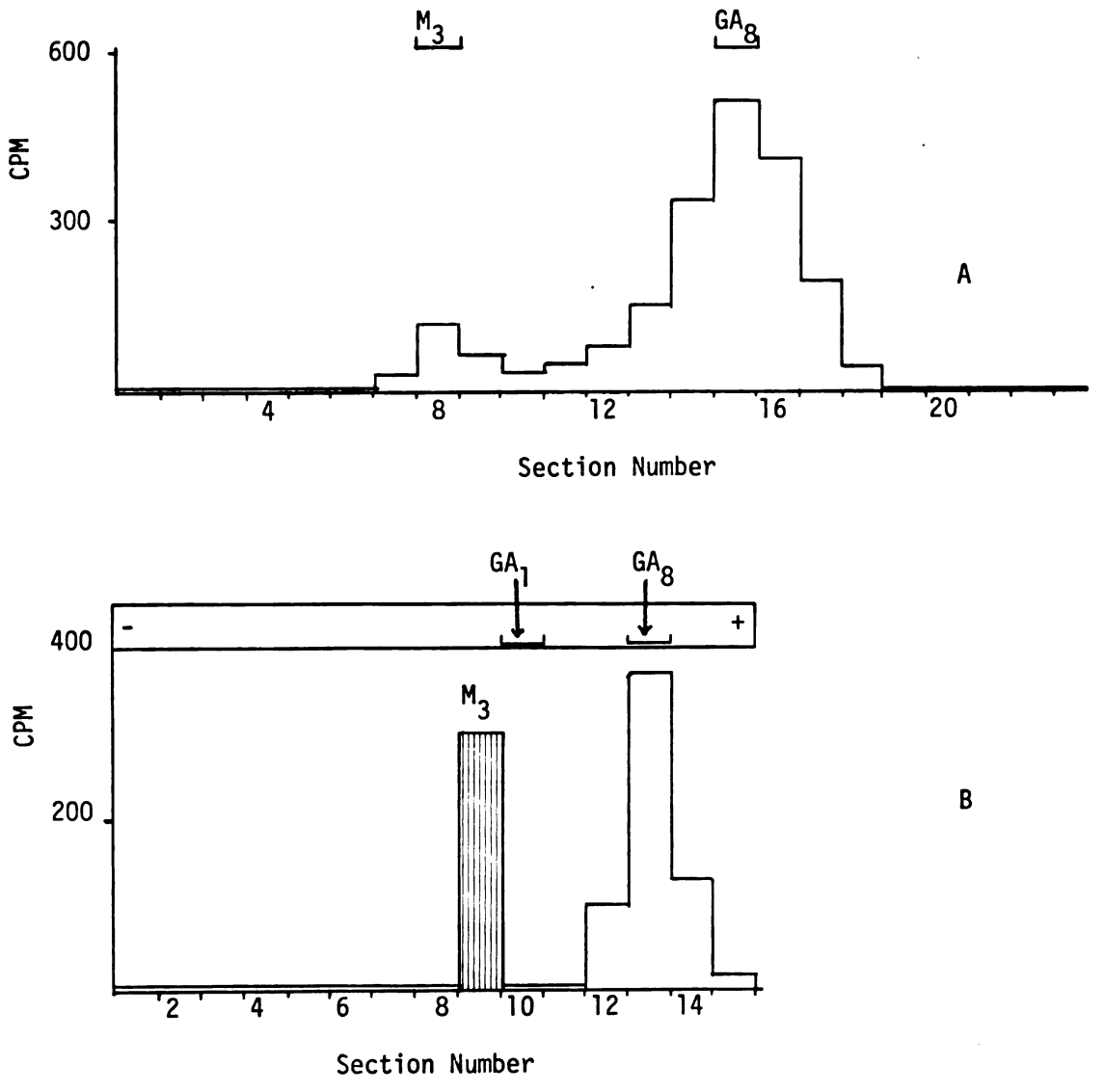


Figure 18A.--Distribution of radioactivity on a paper chromatogram of the M_3 metabolite treated with pectinase. M_3 was incubated at 37°C for 36 hours with pectinase (1-2 mg) in 1.7 ml of 0.1 M acetate buffer, pH 5.4. The reaction was stopped by heating the mixture in a boiling water bath for 5-7 minutes. It was centrifuged, the supernatant solution concentrated, spotted on paper, and chromatographed in the isopropanol-water (4:1) system.

Figure 18B.--Distribution of radioactivity on a paper electrophoretogram of the products obtained after pectinase hydrolysis of M_3. The product from the paper chromatogram (given above) was eluted with 80% ethanol, and electrophoresed for 4 hours in ammonium borate buffer, pH 9.3.

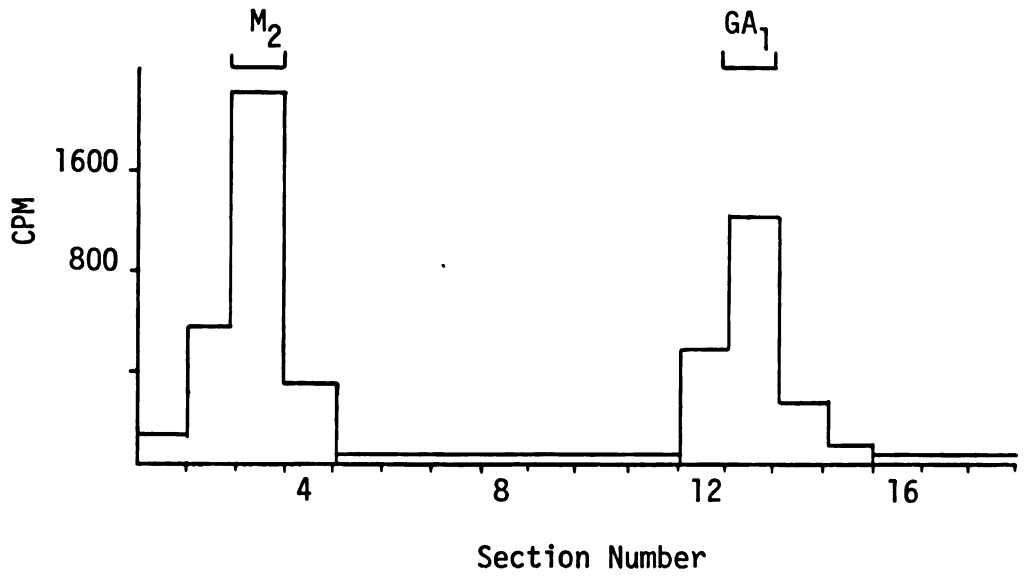


Figure 19.--Distribution of radioactivity on a thin layer chromatogram of products from the treatment of M_2 from GA_1 with β-xylosidase. Pectinase was a source of β-xylosidase which was separated from the β-glucosidase and β-galactosidase present by chromatography on a Sephadex G-200 column. The enzyme was incubated with M_2 from GA_1 for 24 hours at 37°C. The reaction was stopped by heating in a boiling water bath for 5 minutes; the mixture was then concentrated and chromatographed in the acetone-acetic acid (97:3) solvent system.

obtained was tentatively identified as a GA_3-glucoside. Upon incubation with β-glucosidase in 0.05 M acetate buffer, pH 4.8, for 15-20 hours at 37°C it was converted to GA_3 (as identified on a thin layer chromatogram in system H). It had an R_F value of 0.28 on a thin layer chromatogram in system H and upon enzymatic hydrolysis the product migrated to the GA_3 area (R_F value of 0.52).

To form GA_8-O(2)-β-D-glucopyranoside from ^{3}H-GA_1 (Sembdner, 1968, 1970), immature seeds (7/group) of P. coccineus were incubated with ^{3}H-GA_1 for 20 hours. The seeds were extracted with methanol as described earlier for the layers. The methanol extract was

evaporated to dryness, the residue dissolved in a known volume of borax buffer, pH 8.4, and extracted with ethyl acetate at pH 8.4 and 2.4. The resulting aqueous phase was chromatographed on paper in system A (Figure 20A). The product migrated in front of M_3 of GA_1 on paper in system A and on thin layer chromatography in system J. Upon treatment with β-glucosidase (Sigma) it gave GA_8. The resulting GA_8 was also identified by its behavior during TLC in system J as well as on a paper electrophoretogram in ammonium borate buffer, pH 9.3, where it migrated faster than GA_1. Thus the enzyme hydrolyzed the simple gibberellin glucosides.

It should be noted, however, that this enzyme (almond β-glucosidase-Sigma) had no effect on M_3 or M_2 of GA_1 under the same conditions. This indicates that M_2 and M_3 from GA_1 and M_2 from GA_3 are probably not simple glucosides.

B. The Effect of Acid Treatment on GA_3 and M_2 of GA_3

Two spots were located on paper and thin layer chromatograms with a short wave length ultraviolet lamp when non-radioactive GA_3 was treated with acid. One had a bright greenish fluorescence and a R_F value of 0.61 on paper (system A) and 0.64 on thin layers (system H). The other had a bright blue fluorescence and a R_F value of 0.90 on paper (system A) and 0.80 on thin layers (system H). Radioactivity was found solely in these products when ^{14}C-GA_3 was employed. The treatment of M_2 of GA_3 with strong acid gave three products. One was located in the GA_3 area and the other two in the

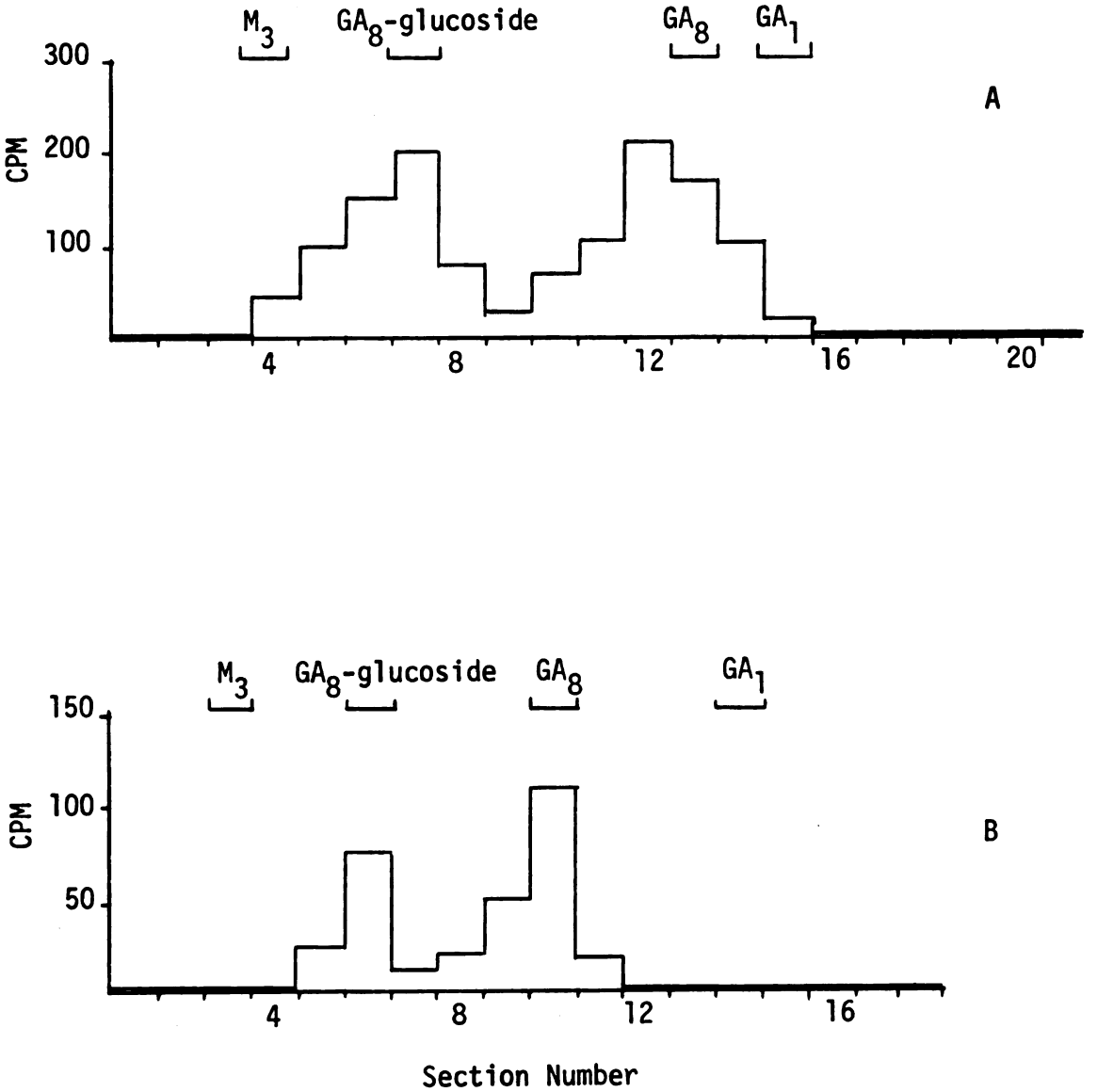


Figure 20A.--Distribution of radioactivity on a paper chromatogram of the aqueous phase of extracts from ^{3}H-GA_1 treated Phaseolus coccineus seeds. Immature seeds (7/group) were incubated with 7.8×10^{-3} µg ^{3}H-GA_1/ml in 2.6 ml of incubating medium on a mechanical shaker at room temperature for 20 hours. The material was chromatographed in the isopropanol-water (4:1 system).

Figure 20B.--Distribution of radioactivity on a thin layer chromatogram after treatment of the putative GA8-glucoside with β-glucosidase. The product was eluted from paper with 80% ethanol, the solution evaporated to dryness, the residue taken up in a known volume of acetate buffer, pH 5.4, containing β-glucosidase and incubated for 20 hours at 37°C. The mixture was concentrated after stopping the reaction, and spotted on TLC. The chromatogram was developed in chloroform-methanol-acetic acid-water (40:15:3:2).

same areas as the products resulting from acid treatment of GA_3 (Figure 21). Thus, it is evident that M_2 is a GA_3 derivative.

C. Detection of Sugars in M_2 of GA_3

The metabolite M_2 gave a bluish grey color that is characteristic of hexoses on chromatograms that were sprayed with diphenylamine. A positive test for pentose with orcinol reagent (greenish color) as well as with phloroglucinol (yellowish red color) was also given by the M_2 metabolite.

D. Incorporation of ^{14}C-U-Glucose into the Minor Metabolite, M_2 of GA_3

^{14}C-glucose was incorporated into M_2 of GA_3. Although radioactivity was widely distributed on the paper chromatogram after the first chromatographic separation, there was a large amount concentrated in the M_2 area. The material residing in the M_2 area on the paper chromatogram was further chromatographed on thin layer in system L where only one peak was observed, it being in the M_2 area (Figure 22).

Radioactivity from ^{14}C-U-galactose was not incorporated into M_2 of GA_3.

E. Identification of the Sugars in M_2 of GA_3

1. Partial hydrolysis of M_2.--M_2 was treated with 1 N HCl for 2 hours at 90°C in a water bath and the reaction mixture was subsequently taken to dryness. The evaporation process was repeated 3-4 times successively by adding water. The residue was dissolved

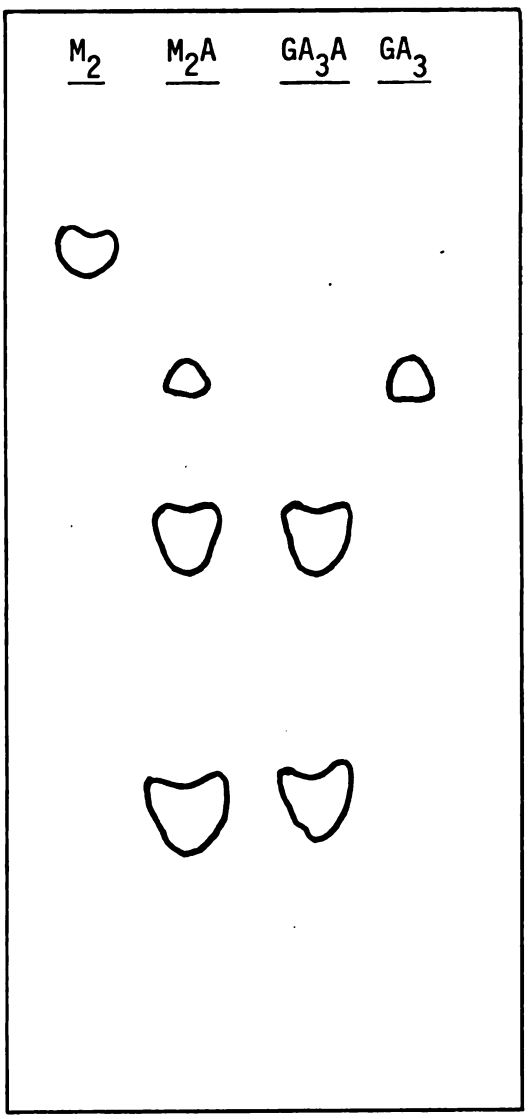
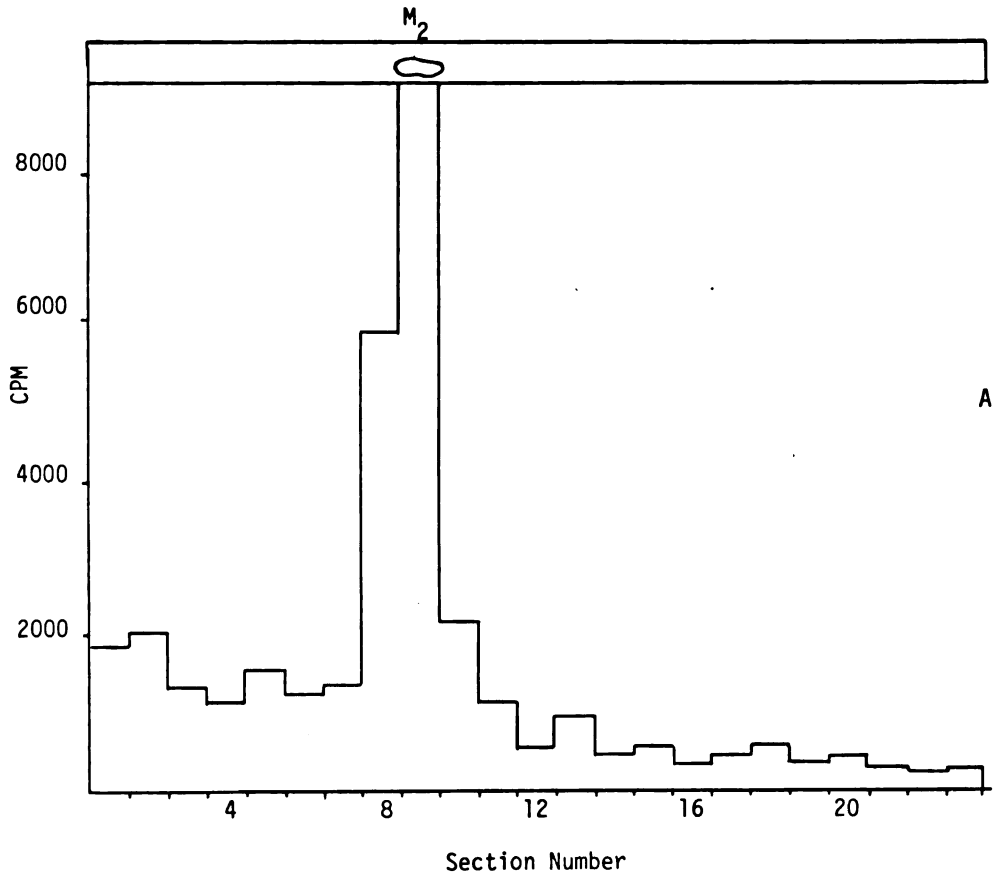


Figure 21.--Diagrammatic representation of the paper chromatographic separation of the products formed after acid treatment of M_2 of GA_3, and GA_3. The gibberellin and the metabolite were treated with strong HCl for 30-40 minutes in a boiling water bath. The reaction mixtures were evaporated to dryness under reduced pressure and this process was repeated 3-4 times by successively adding small amounts of water. The residue was taken up in a known volume of 80% ethanol and spotted on chromatographic paper which was developed in the solvent system consisting of isopropanol-water (4:1). The dried chromatogram was sprayed with ethanolic sulfuric acid and heated to locate the compounds under UV light.

M_2A--acid treatment of M_2 of GA_3 and GA_3A--GA_3 treated with acid.

Figure 22A. Distribution of radioactivity on a paper chromatogram of the aqueous phase of aleurone layers which were incubated with GA_3 and ^{14}C-U-glucose. The chromatogram was developed in the isopropanol-water (4:1) system.

Figure 22B.--Pattern of distribution of radioactivity on a thin layer chromatogram of purified M_2 of GA_3 after incorporating ^{14}C-U-glucose into it. The radioactive area corresponding to M_2 on the paper chromatogram in Figure 22A was eluted with water and then treated with charcoal. The M_2 product was desorbed with ethanolic NH_4OH, the solution evaporated to dryness and the residue taken up in a known volume of 80% ethanol for chromatography. The chromatogram was developed in the propanol-ethyl acetate-water (7:2:2) system. The drawings at the top of the figure represent standard M_2 of GA_3.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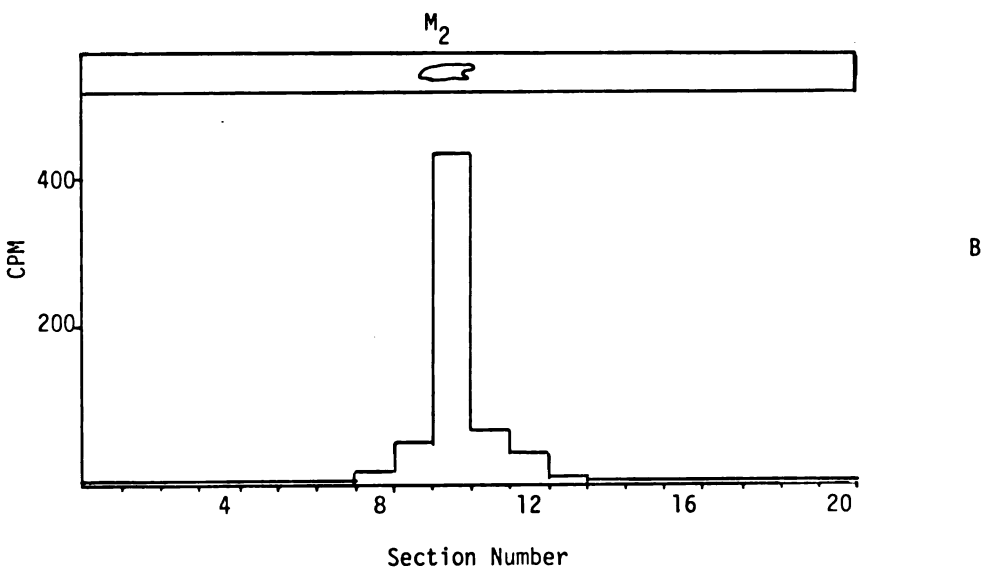
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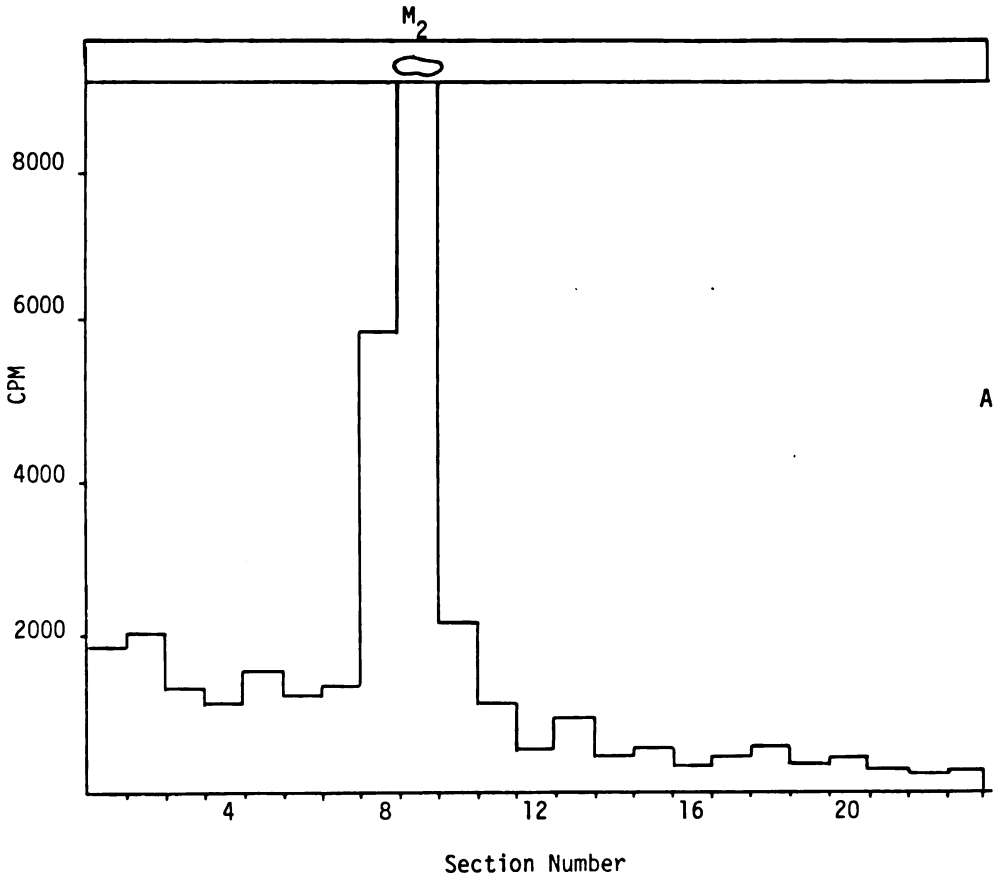
Figure 22A. Distribution of radioactivity on a paper chromatogram of the aqueous phase of aleurone layers which were incubated with GA_3 and ^{14}C-U-glucose. The chromatogram was developed in the isopropanol-water (4:1) system.

Figure 22B.--Pattern of distribution of radioactivity on a thin layer chromatogram of purified M_2 of GA_3 after incorporating ^{14}C-U-glucose into it. The radioactive area corresponding to M_2 on the paper chromatogram in Figure 22A was eluted with water and then treated with charcoal. The M_2 product was desorbed with ethanolic NH_4OH, the solution evaporated to dryness and the residue taken up in a known volume of 80% ethanol for chromatography. The chromatogram was developed in the propanol-ethyl acetate-water (7:2:2) system. The drawings at the top of the figure represent standard M_2 of GA_3.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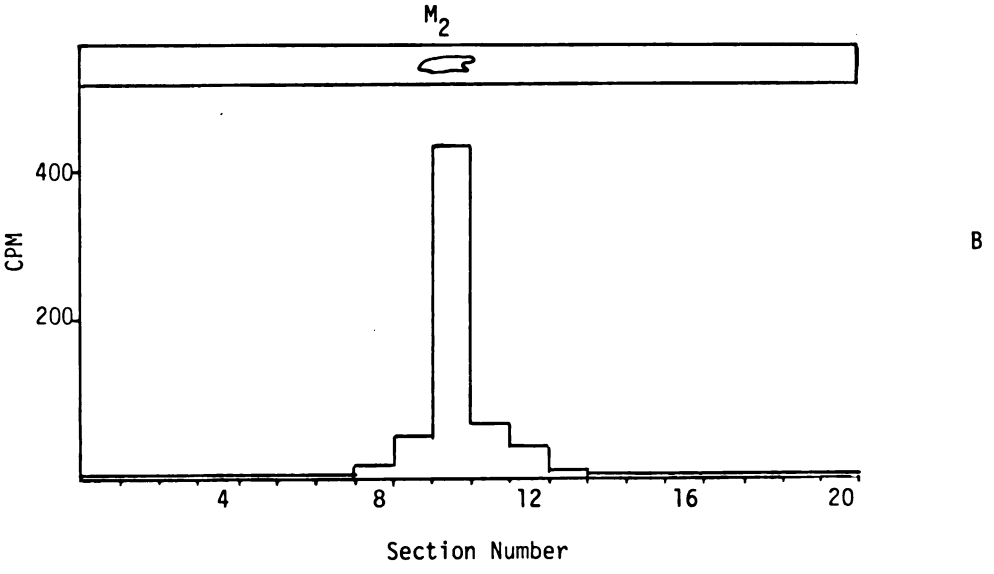
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in a known volume of water and chromatographed on Whatman No. 4 paper in a system containing propanol-ethyl acetate-water (4:5:1) for 30 hours. Three spots were located on this chromatogram after it was sprayed with the diphenylamine reagent. One spot was present just behind the galactose area, probably representing a disaccharide, a second spot was located in the glucose area and the third spot migrated in front of glucose in the xylose area (Figure 23).

The resulting disaccharide migrated faster than cellobiose, laminaribose, gentiobiose, maltose and sucrose but slower than hexoses on paper chromatograms. The migration pattern of this disaccharide was compared with that of 3-O-β-D-glucopyranosyl-D-xylose. They migrated at the same rate during paper and thin layer chromatography. The glucopyranosyl-xylose was prepared by incubating a mixture of cellobiose and xylose with cellulase from Aspergillus niger in acetate buffer pH 5.4 for 20-30 hours at 37°C (Barker et al., 1957). It migrated just behind galactose on paper chromatograms in the propanol-ethyl acetate-water (6:1:3) system after developing the paper for 26 hours.

After M_2 of GA_3 was partially hydrolyzed with 2.5 N HCl for 40 minutes, two monosaccharides were present on thin layer chromatograms, one in the xylose area which gave a bluish green color with diphenylamine and the other in the glucose area which gave a bluish grey color with diphenylamine (Figure 24). These characteristic colors were produced by xylose and glucose standards. There was another spot just behind glucose but in front of galactose, in the area of glucosyl-xylose. It gave a blue green color tinged with

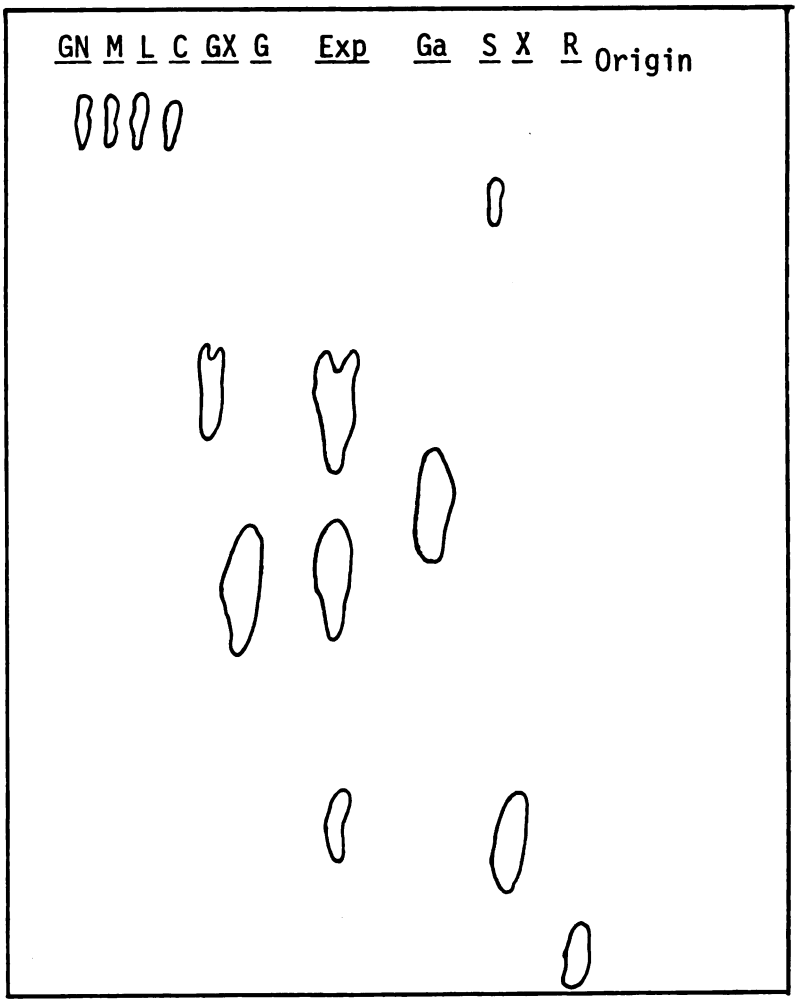


Figure 23.--Diagrammatic representation of the paper chromatographic separation of the sugars obtained after the minor metabolite, M_2 of GA_3, was partially hydrolyzed with dilute HCl. M_2 was treated with 1 N HCl for 2 hours at 90°C in a water bath. Chromatography was performed on Whatman No. 4 paper for 30 hours in the propanol-ethyl acetate-water (4:5:1) system. The dried chromatogram was sprayed with the diphenylamine reagent to locate the sugars. GN--gentiobiose, C--cellobiose, L--laminaribose, M--maltose, S--sucrose, GX--glucosyl-xylose, Ga--galactose, G--glucose, Exp--M_2 hydrolysis products, X--xylose, R--ribose.

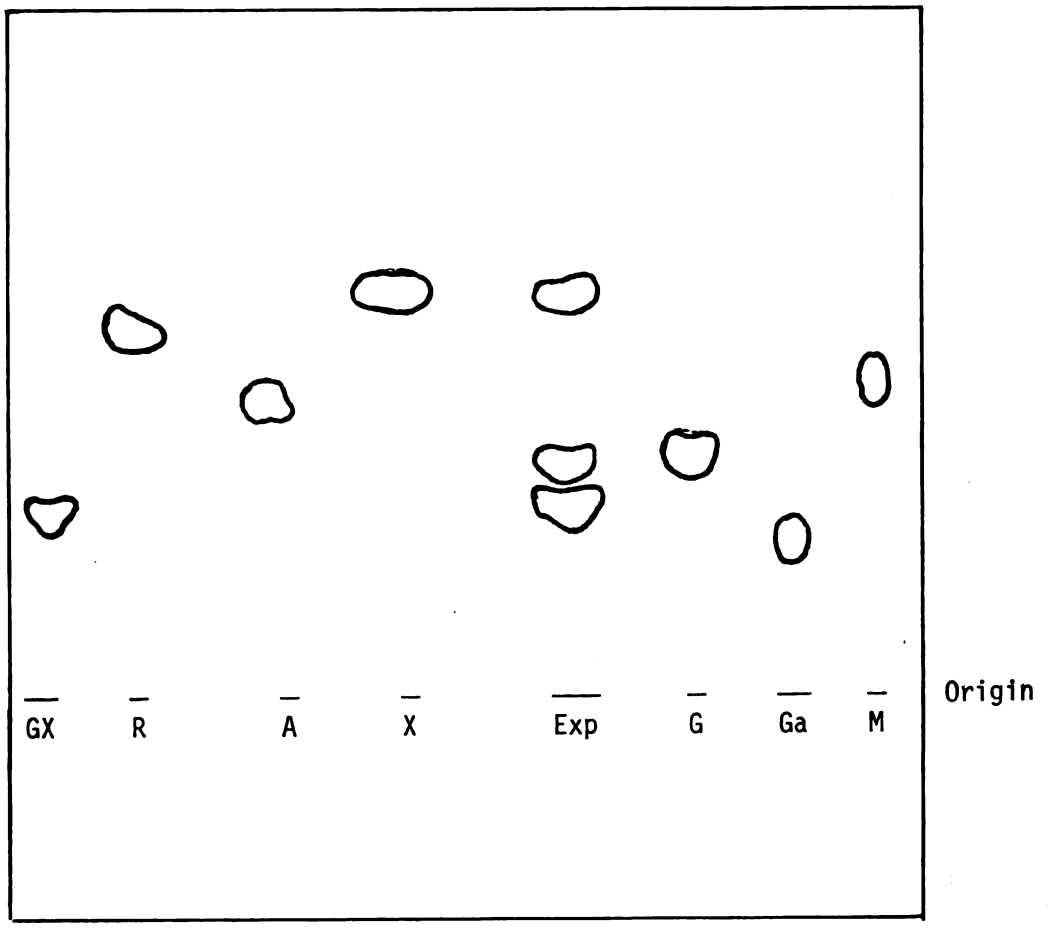


Figure 24.--Thin layer chromatographic separation of the sugars obtained after the minor metabolite, M_2 from GA_3, was partially hydrolyzed with dilute HCl. The metabolite was treated with 2.5 N HCl for 40 minutes at 95°C in a water bath. The chromatogram was developed 3 times in propanol-ethyl acetate-water (7:2:1), dried and sprayed with either the diphenylamine reagent or ammoniacal silver nitrate reagent to locate the sugars. M--mannose, Ga--galactose, G--glucose, Exp--M_2 hydrolysis products, X--xylose, A--arabinose, R--ribose, GX--glucosyl-xylose disaccharide.

grey when treated with diphenylamine, similar to the color given by the cellulase product (Figure 24).

The identity of xylose was further confirmed by chromatographing the partially hydrolyzed product on Whatman No. 4 paper in system D for 23 hours. This system distinctly separates xylose from ribose (Bourne et al., 1963). Three sugar areas were detected on the paper chromatogram; one was in the disaccharide area behind galactose, a second migrated with glucose and the third migrated with xylose (Figure 25).

2. Complete hydrolysis of M_2.--The product was treated with 2.5 N HCl for 80-90 minutes in order to get complete hydrolysis. Upon chromatography on thin layer in system M only two sugars, one in the xylose area and the other in the glucose area, were located with the silver nitrate spray (Figure 26). Thus, M_2 metabolite from GA_3 contains xylose and glucose. These sugars appeared to be present in equal amounts.

F. Determination of the Linkages Involved in the Sugars of the M_2 Metabolite of GA_3

The paper chromatographic separation of the products obtained after the sequential periodic acid oxidation, acid hydrolysis and reduction with $NaBT_4$ of the minor metabolite, M_2 of GA_3, resulted in radioactivity being distributed all over the chromatogram (Figure 28A).

Further chromatography of the radioactive zone corresponding to the xylitol area (in system F and G) revealed the presence of

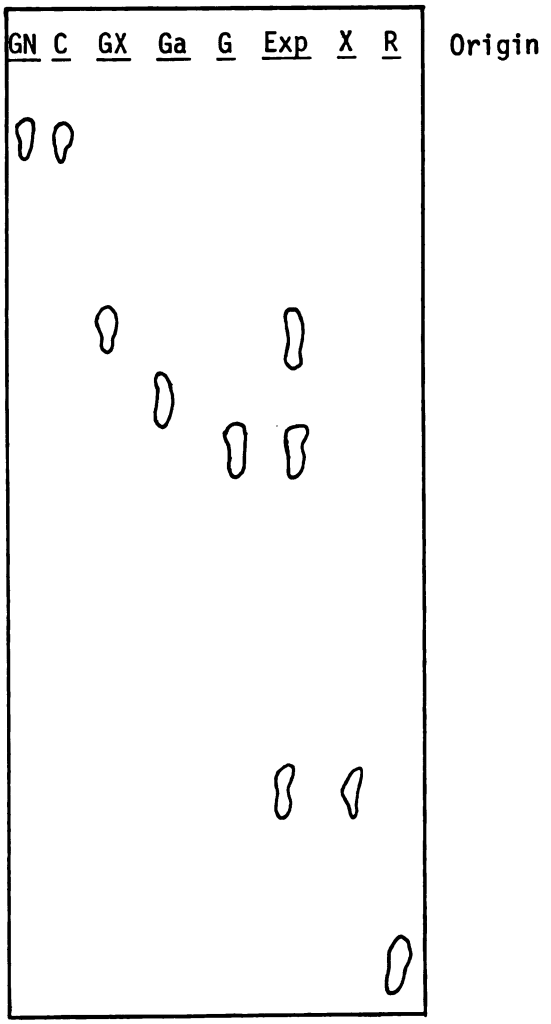


Figure 25.--Paper chromatographic separation of the sugars obtained after M_2 from GA_3 was partially hydrolyzed with dilute HCl. The M_2 metabolite of GA_3 was treated with 2.5 N HCl for 45 minutes at 95°C in a water bath. Whatman No. 4 chromatographic paper was used and it was developed for 24 hours in ethyl acetate-acetic acid-water (9:2:2). The dried chromatogram was sprayed with the diphenylamine reagent to locate all the sugars. GN--gentiobiose, C--cellobiose, GX--glucosyl-xylose, Ga--galactose, G--glucose, Exp--M_2 hydrolyzed products, X--xylose, R--ribose.

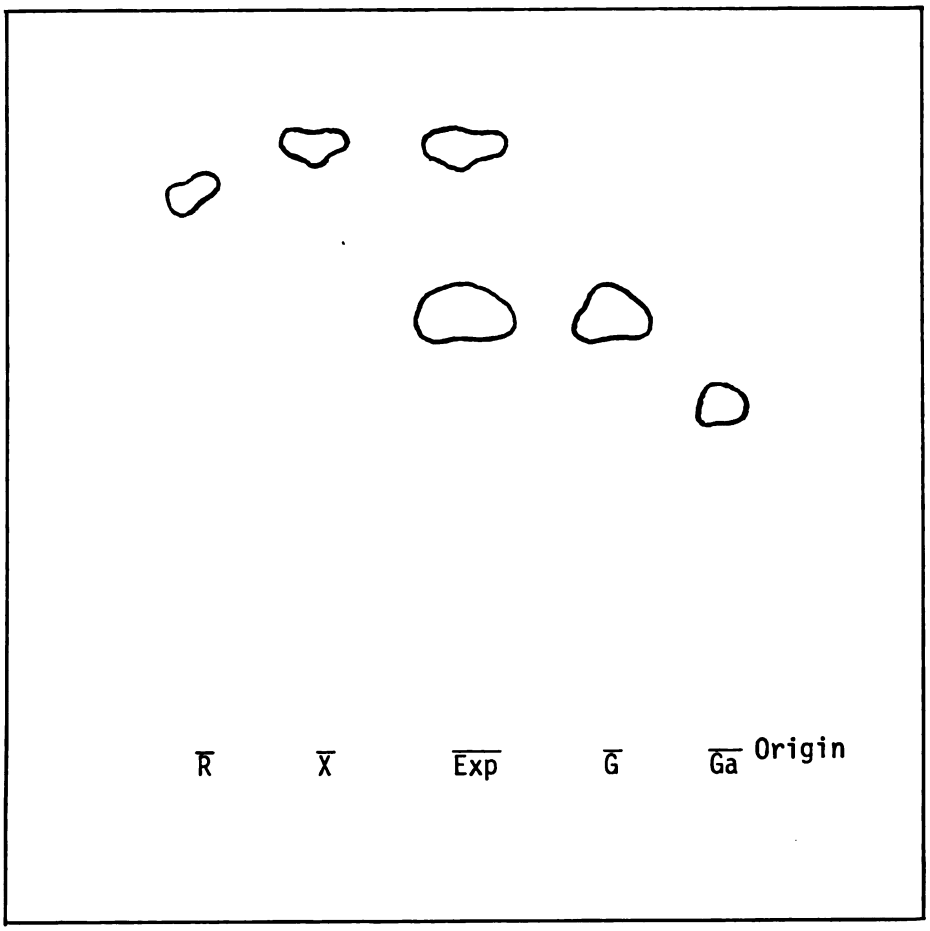


Figure 26.--Diagrammatic representation of the thin layer chromatographic separation of the sugars obtained after the minor metabolite, M_2 of GA_3, was completely hydrolyzed with dilute HCl. The metabolite was treated with 2.5 N HCl for 80-90 minutes at 95°C in a water bath. The chromatogram was developed four times in the propanol-ethyl acetate-water (7:2:1) solvent system. Sugars were located with either the diphenylamine reagent or the ammoniacal silver nitrate reagent. R--ribose, X--xylose, Exp--M_2 hydrolysis products, G--glucose, Ga--galactose.

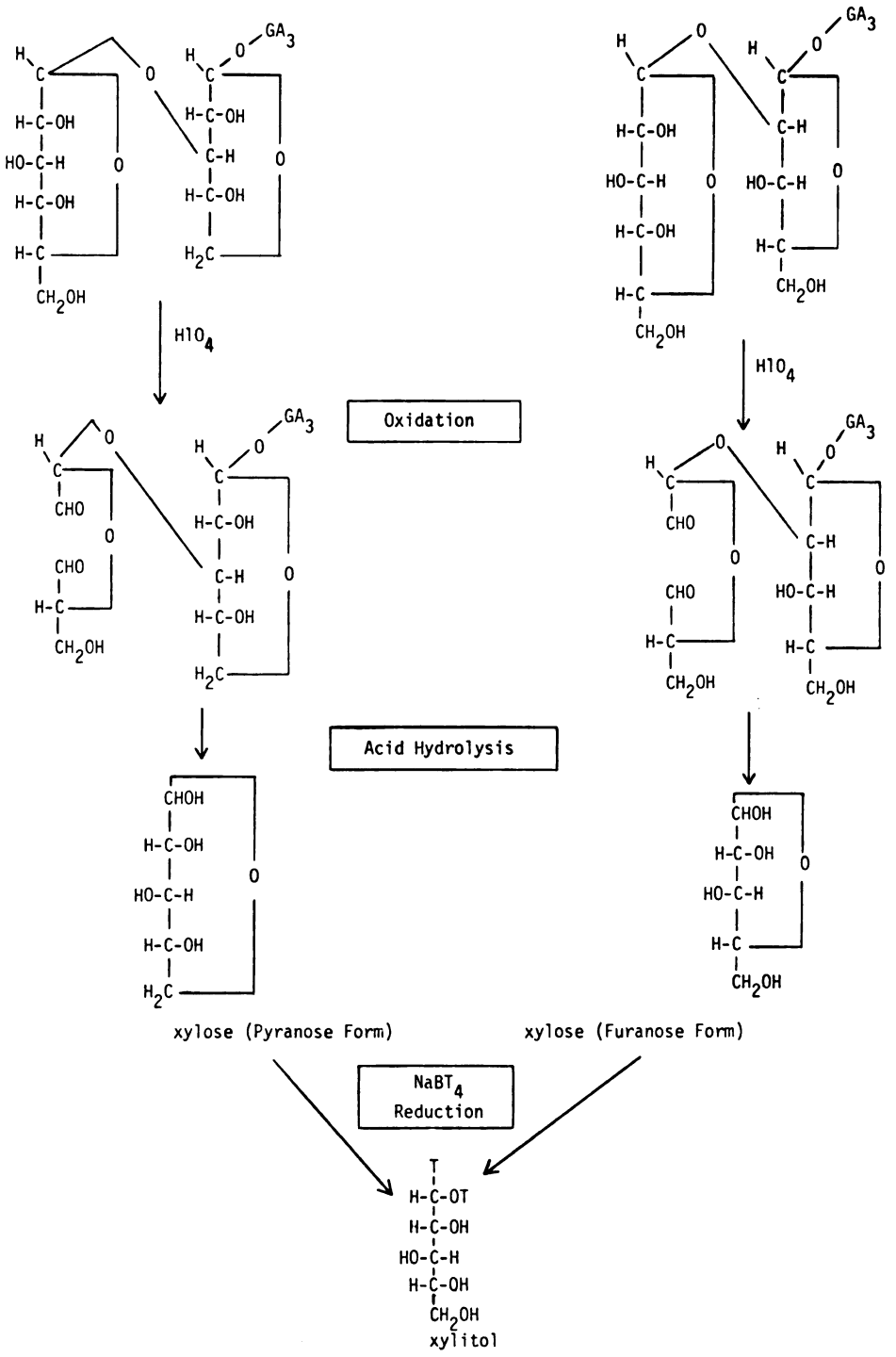
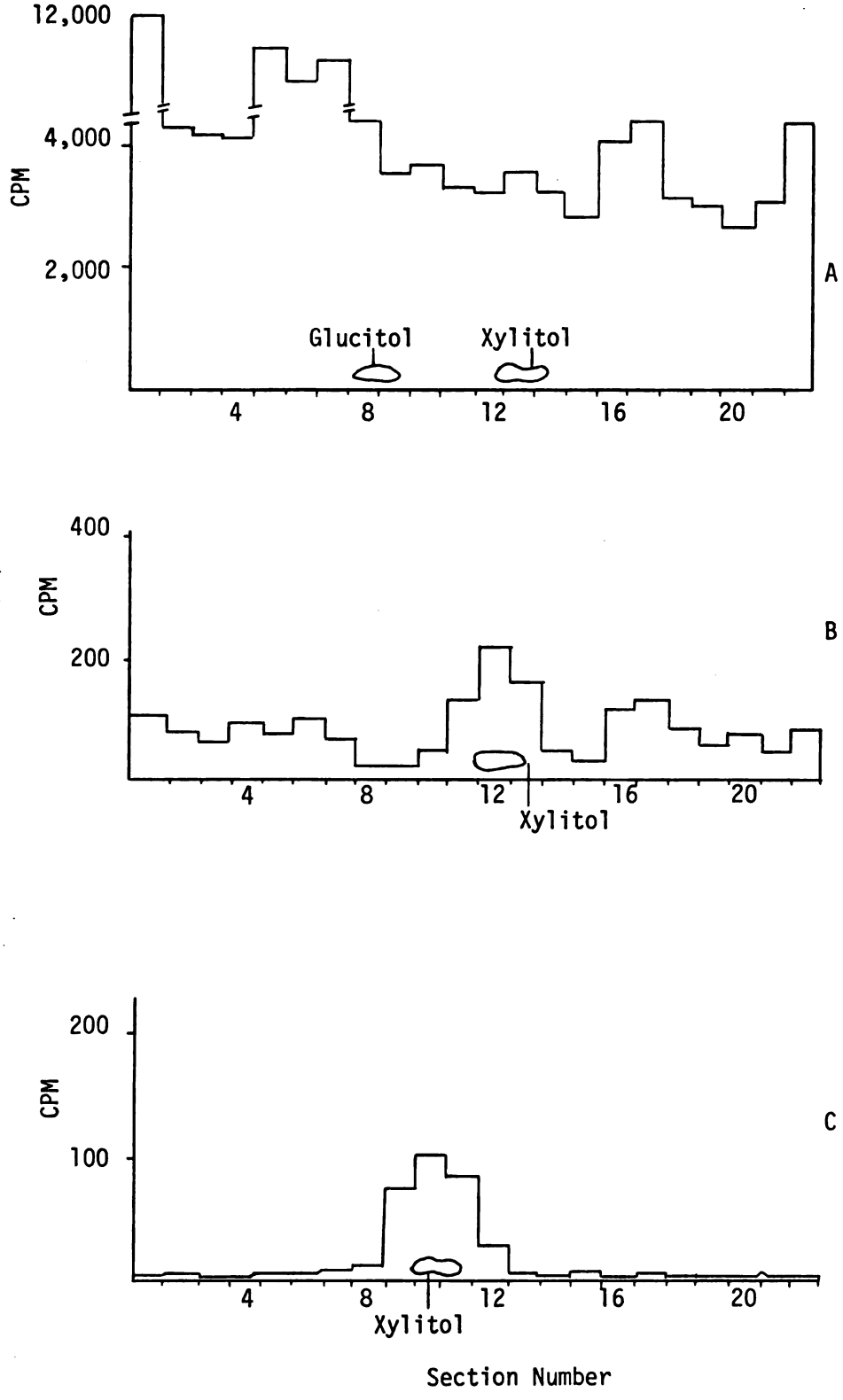


Figure 27.--Linkages of glucosyl-xylose-GA_3 that can give xylitol after sequential periodate oxidation, acid hydrolysis and reduction with $NaBT_4$ of M_2 of GA_3.

Figure 28A.--Distribution of radioactivity on a paper chromatogram of the products resulting from sequential periodate oxidation, acid hydrolysis and reduction with radioactive $NaBH_4$ of M_2 of GA_3. The chromatogram was developed in nitromethane-acetic acid-ethanol-water saturated boric acid (8:1:1:1) for 15 hours. The drawing at the bottom represents areas of the standards located by the silver nitrate dip technique.

Figure 28B.--Distribution of radioactivity on a paper chromatogram of the product obtained in the xylitol area on the paper chromatogram of system E and rechromatographed in system F. The radioactive zone corresponding to xylitol was eluted with water and treated successively with Dowex cation (H^+ form) and anion exchange resins (OH^-form) to remove any inorganic components. The filtrate was concentrated and placed on chromatograms that were developed in n-butanol-pyridine-water (6:4:3) system for 20 hours. The drawing at the bottom represents the area of unlabeled xylitol located by the silver nitrate dip technique.

Figure 28C.--Distribution of radioactivity on a paper chromatogram of the product obtained in the xylitol area on the paper chromatogram of system F and rechromatographed in system G. The radioactive area corresponding to xylitol was eluted with water, concentrated and placed on the chromatogram. The chromatogram was developed for 20 hours in propanol-ethyl acetate-water (7:1:2).


12,000
4,000
2,000
CPM
A
Glucitol
Xylitol
4
8
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16
20
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CPM
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20
Xylitol
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100
CPM
C
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16
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Xylitol
Section Number

only one major radioactive peak, it being located in the xylitol area (Figure 28B and 28C). Therefore, the final product obtained after oxidation, hydrolysis and reduction of M_2 of GA_3 was xylitol.

Xylitol can be formed only if M_2 is either (a) 2-O-β-D-glucose-β-D-xylofuranosyl-GA_3, where C-1 of glucose is linked to xylose at C-2 and C-1 of xylose to GA_3 (probably at C-3 of GA_3); or (b) a 3-O-β-D-glucose-β-D-xylopyranosyl-GA_3, where C-1 of glucose is linked to xylose at C-3, and C-1 of xylose to GA_3 (probably at C-3 of GA_3).

It seems likely that the M_2 metabolite of GA_3 is O-β-D-glucose-(1→3)-O-β-D-xylopyranosyl-(1→3)-O-GA_3 since a disaccharide (3-O-β-D-glucose-O-β-D-xylopyranosyl) resulted from the partial acid hydrolysis of the GA_3-glycoside which migrated identically with 3-O-β-D-glucopyranosyl-D-xylose on paper and thin layer chromatograms.

XIII. Studies on the Identification of the M_1 Metabolites

A. Effect of Enzymes on M_1 Metabolites

The major very highly polar metabolites, M_1 from GA_1 and GA_3, were not affected by treatment with either emulsin, bromelin, papain, trypsin or lipase.

B. Effect of Acid Treatment on M_1 of GA_3

A single broad radioactive peak was observed after M_1 from GA_3 was treated with 11.7 N HCl. It had a R_F value of 0.76 on paper chromatograms developed in system A (Figure 29).

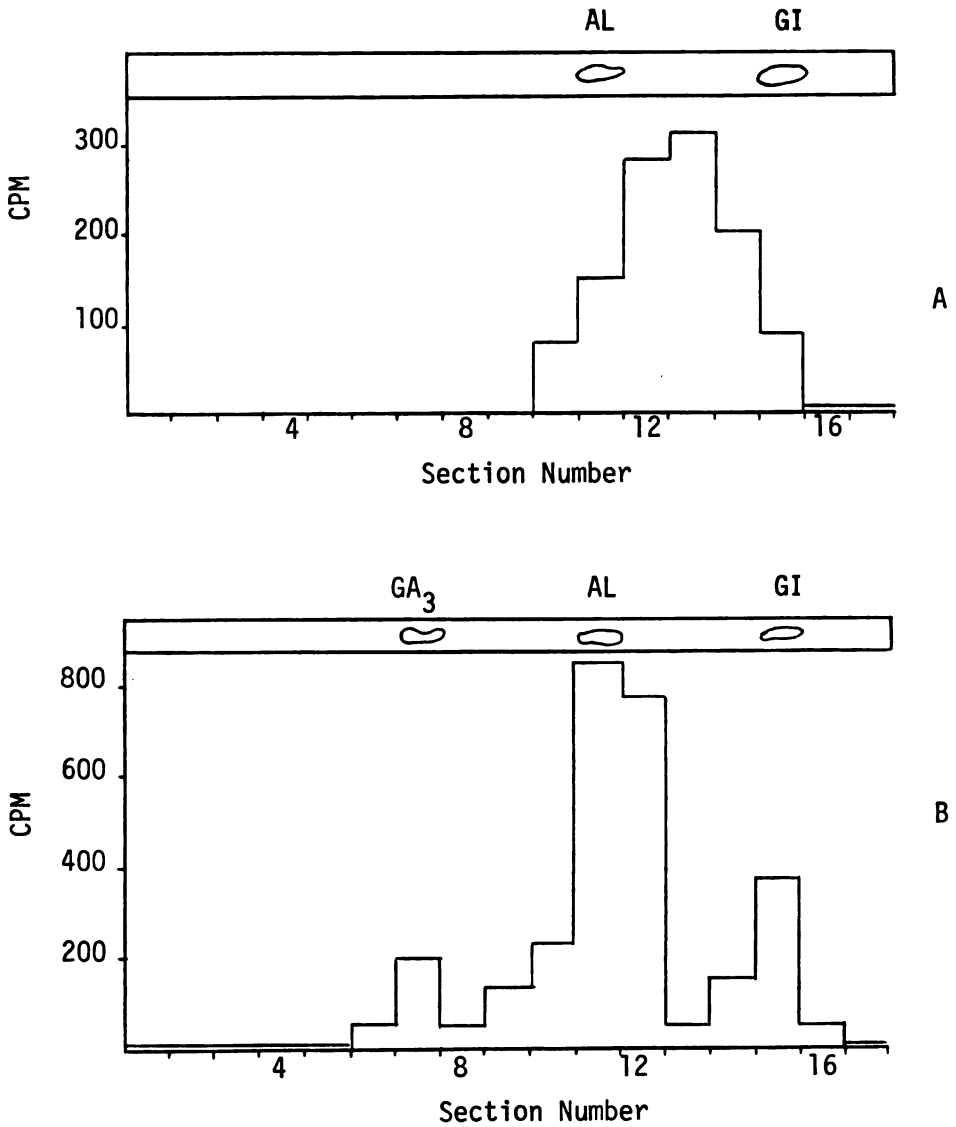


Figure 29.--Distribution of radioactivity on paper chromatograms of the products formed after acid treatment of ^{14}C-GA_3 and M_1 from ^{14}C-GA_3. The labeled M_1 (A) and GA_3 (B) were treated with strong HCl for 30-40 minutes in a boiling water bath. The chromatogram was developed in the isopropanol-water (4:1) solvent system. The drawings at the top represent the fluorescent spots of unlabeled GA_3 and its acid treatment products, allogibberic (AL) and gibberic (GI) acids located under ultraviolet light after the papers were sprayed with 5% H_2SO_4 in ethanol and heated.

C. Effect of NH_4OH Treatment on the Metabolites of GA_3 and GA_1

The hydrolysis of M_1 with strong NH_4OH resulted in the formation of products that were less polar than M_1 and which migrated in front of M_1 and behind the GA_3 area on the paper chromatogram in solvent system B (Figure 30A). One of the compounds may represent the partially hydrolyzed product. The products of NH_4OH treatment of M_1 of GA_3 migrated in front of GA_3 (Figure 30C). Treatment of M_1 from GA_1 with NH_4OH resulted in the formation of a similar less polar product which migrated in front of M_1 and behind GA_1 (Figure 30B).

D. Effect of Sodium Metaperiodate Treatment on M_1 of GA_1

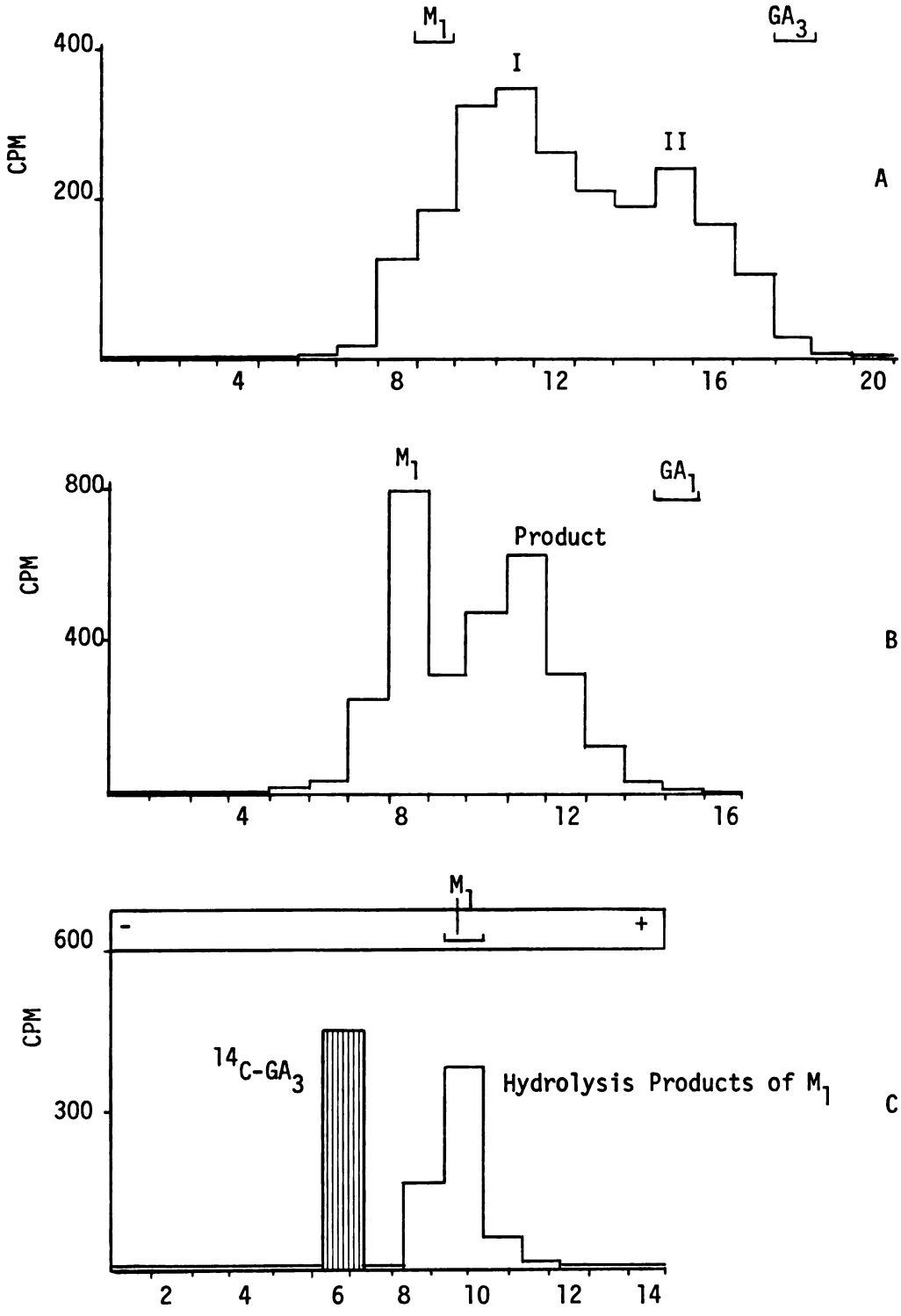
The product obtained after periodate treatment of M_1 migrated in front of M_1 on thin layer chromatograms in methanol-ethanol-water-acetic acid (3:3:3.5:0.5) and when electrophoresed in borate buffer, the rate of its migration was less than M_1; it migrated with GA_1 (Figure 31). This suggested that periodate treatment oxidized vicinal hydroxyl groups present in M_1.

E. Identification of the Amino Acids Present in the Major Metabolite, M_1 of GA_3

Three distinctive areas that gave a pinkish purple color with ninhydrin spray resulted after thin layer chromatography of the acid hydrolyzed products of M_1 in system N. These spots were located in the areas corresponding to glycine, serine and alanine/threonine (Figure 32). Thin layer chromatography in system P gave a better

Figures 30A and 30B.--Distribution of radioactivity on paper chromatograms of products resulting from the treatment of M_1 of GA_3 and M_1 of GA_1 with NH_4OH for 30 minutes at 100°C. Reaction mixtures were evaporated to dryness and the residues were taken up in a known volume of 80% ethanol. Chromatograms were developed for either 6 hours in the case of M_1 of GA_3 (A) or 4.5 hours in the case of M_1 of GA_1 (B) in methanol-ethanol-n-butanol-water-acetic acid (4:3:1:1.5:0.5).

Figure 30C.--Distribution of radioactivity on a paper electrophoretogram of the product obtained after NH_4OH hydrolysis of M_1 of GA_3. The products, I and II from the paper chromatogram of Figure 30A, were eluted with 80% ethanol and electrophoresed in ammonium borate buffer, pH 9.3, for 4 hours.


M1
GA3
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I
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M1
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8
12
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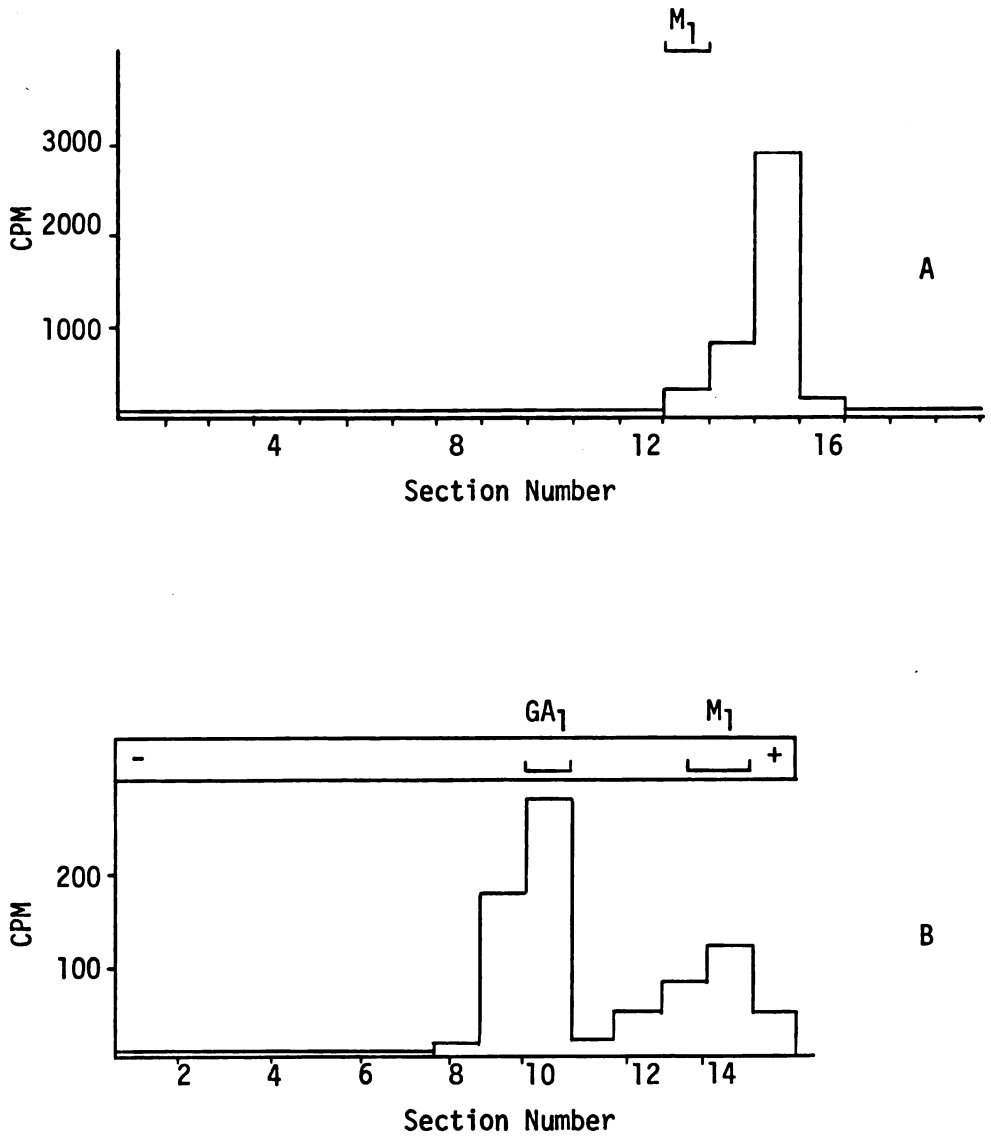


Figure 31.--Distribution of radioactivity on a thin layer chromatogram and a paper electrophoretogram of the product formed from M_1 of GA_1 after periodate treatment. The thin layer chromatogram (A) was developed in methanol-ethanol-water-acetic acid (3:3:3.5:0.5). The resulting product from TLC was eluted with 80% ethanol, evaporated to dryness, the residue was dissolved in 80% ethanol and electrophoresed in ammonium borate buffer, pH 9.3, for 4 hours (B).

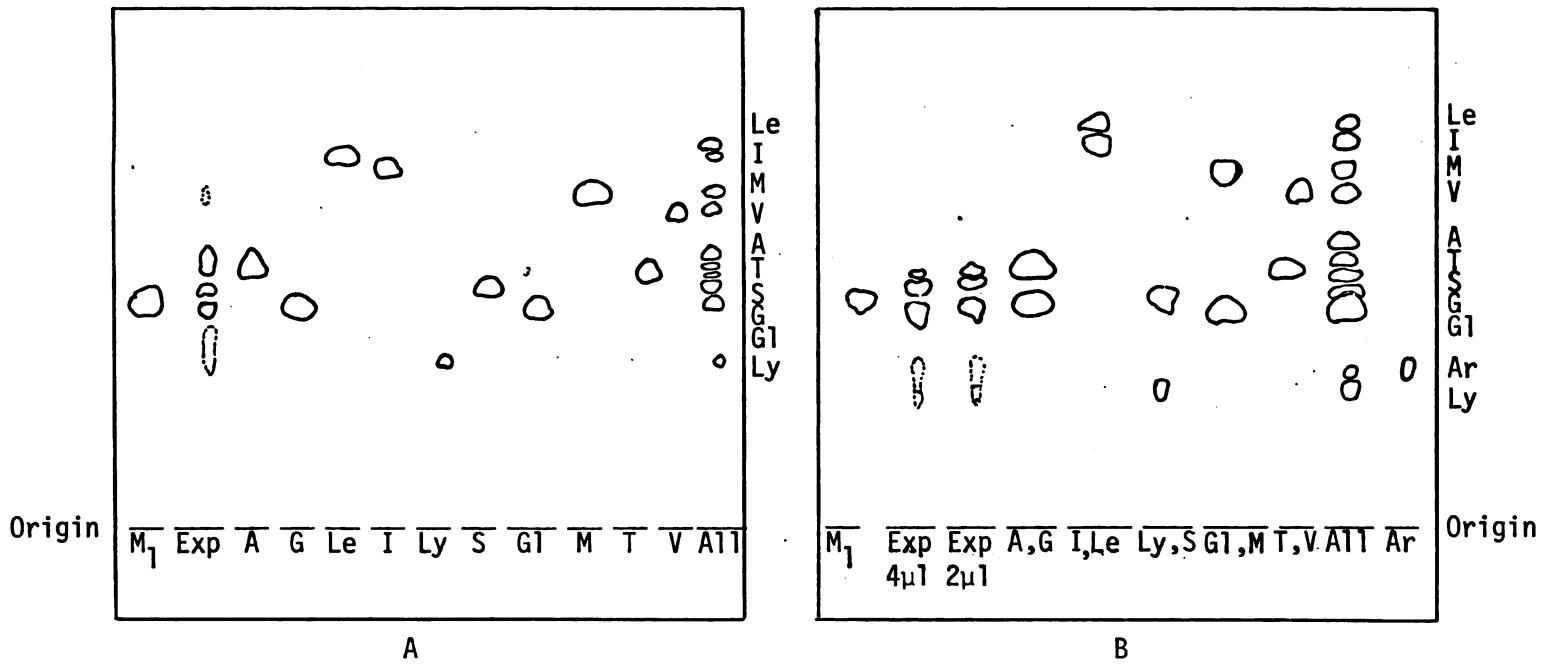


Figure 32.--Diagrammatic representation of the thin layer chromatographic separation of standards and the amino acids obtained after M_1 of GA_3 was hydrolyzed with HCl. Hydrolysis was effected with 6 N HCl in a temperature regulated heating block for 18 hours at 110°C. The chromatograms were developed either two times (Figure A) or three times (Figure B) in n-butanol-acetic acid-water (4:1:1). The dried chromatograms were sprayed with the ninhydrin reagent to locate all the amino acids. M_1--major metabolite of GA_3, Exp--acid hydrolyzed M_1, A--alanine, G--glycine, Le--leucine, I--isoleucine, Ly--lysine, S--serine, Gl--glutamine, M--methionine, T--threonine, V--valine, All--all the standards and Ar--Arginine.

separation of these compounds, revealing the presence of alanine, glycine and serine (Figure 33). The best separations of these amino acids were obtained when the hydrolyzed material from M_1 was chromatographed on thin layers of silica gel G and cellulose in systems R and Q (Figures 34 and 35). The amino acids from M_1 were identified in these systems solely as alanine, glycine and serine.

A tentative identification of serine resulting from the hydrolysis of M_1 was also made by using periodate/Nessler's reagent (Brenner et al., 1965). It gave a positive yellowish orange color with the reagent. The same color was obtained with M_1.

The presence of these three amino acids in M_1 was further confirmed by preparing their corresponding DNP-derivatives from the products of hydrolysis of M_1 and separating them by chromatography. DNP-alanine, DNP-glycine and DNP-serine were observed on thin layer chromatograms (Figures 36 and 37).

F. Terminal Amino Acids Present in M_1 of GA_3

Two yellowish green fluorescent spots were seen under ultraviolet light on polyamide plates when M_1 was dansylated, subsequently hydrolyzed and chromatographed. One corresponded to DNS-serine and the other to DNS-glycine (Figure 38). On 5 cm x 5 cm polyamide plates, a yellowish blue fluorescent spot was present in front of standard DNS-alanine. This was identified as DNS-amine. A distinct separation was obtained when a 5 cm x 10 cm polyamide plate was used (Figure 38B). It was concluded that M_1 has two N-terminal amino acids: serine and glycine. Thin layer chromatography on silica gel G

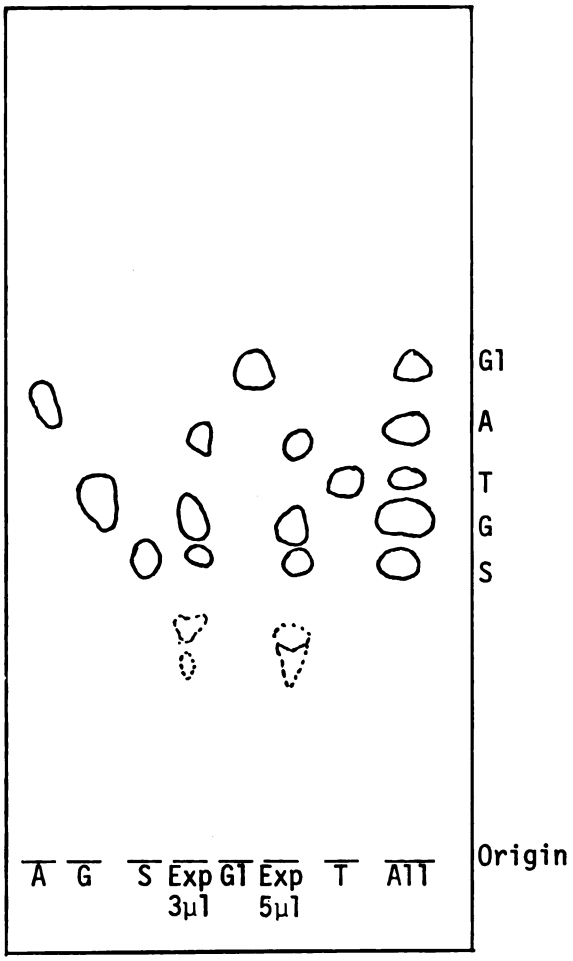


Figure 33.--Chromatographic separation of the amino acids obtained after M_1 of GA_3 was hydrolyzed with 6 N HCl for 18 hours at 110°C. The chromatogram was developed two times in the phenol-water (75:25) system, dried, and sprayed with ninhydrin reagent to locate the amino acids. Gl--glutamine, A--alanine, T--threonine, G--glycine, S--serine.

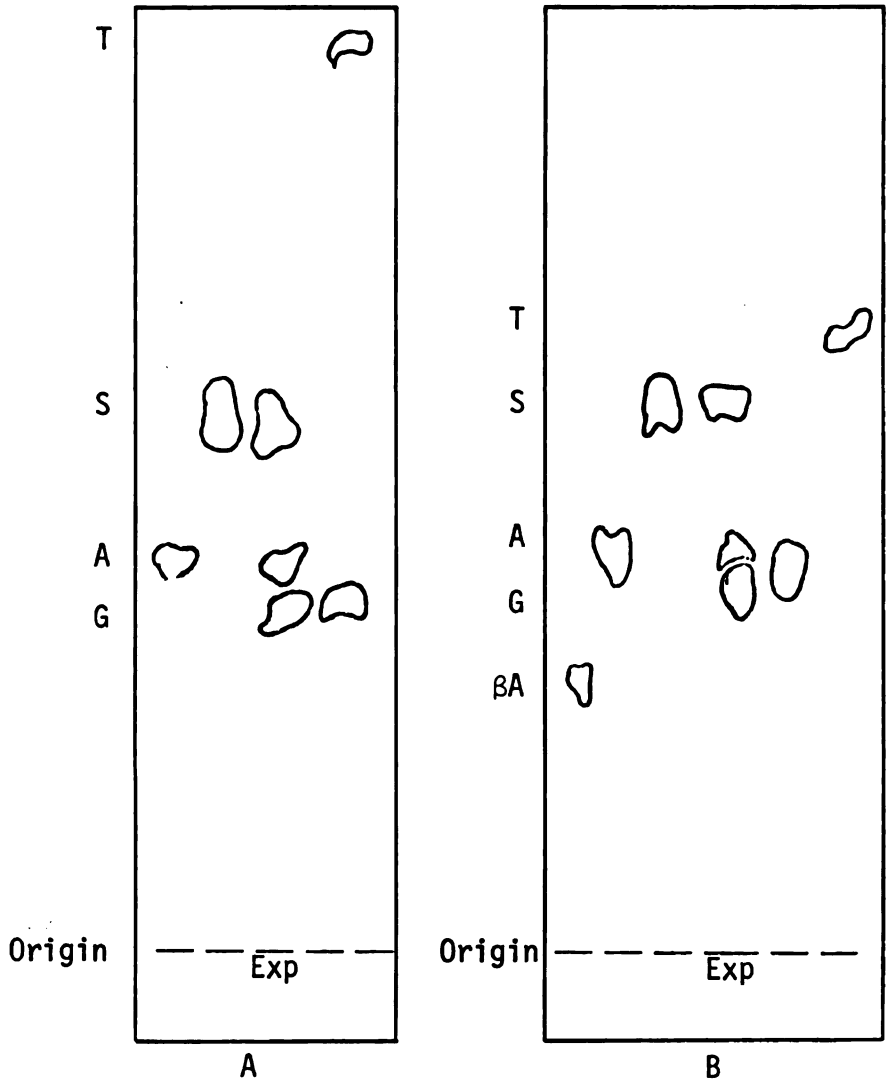


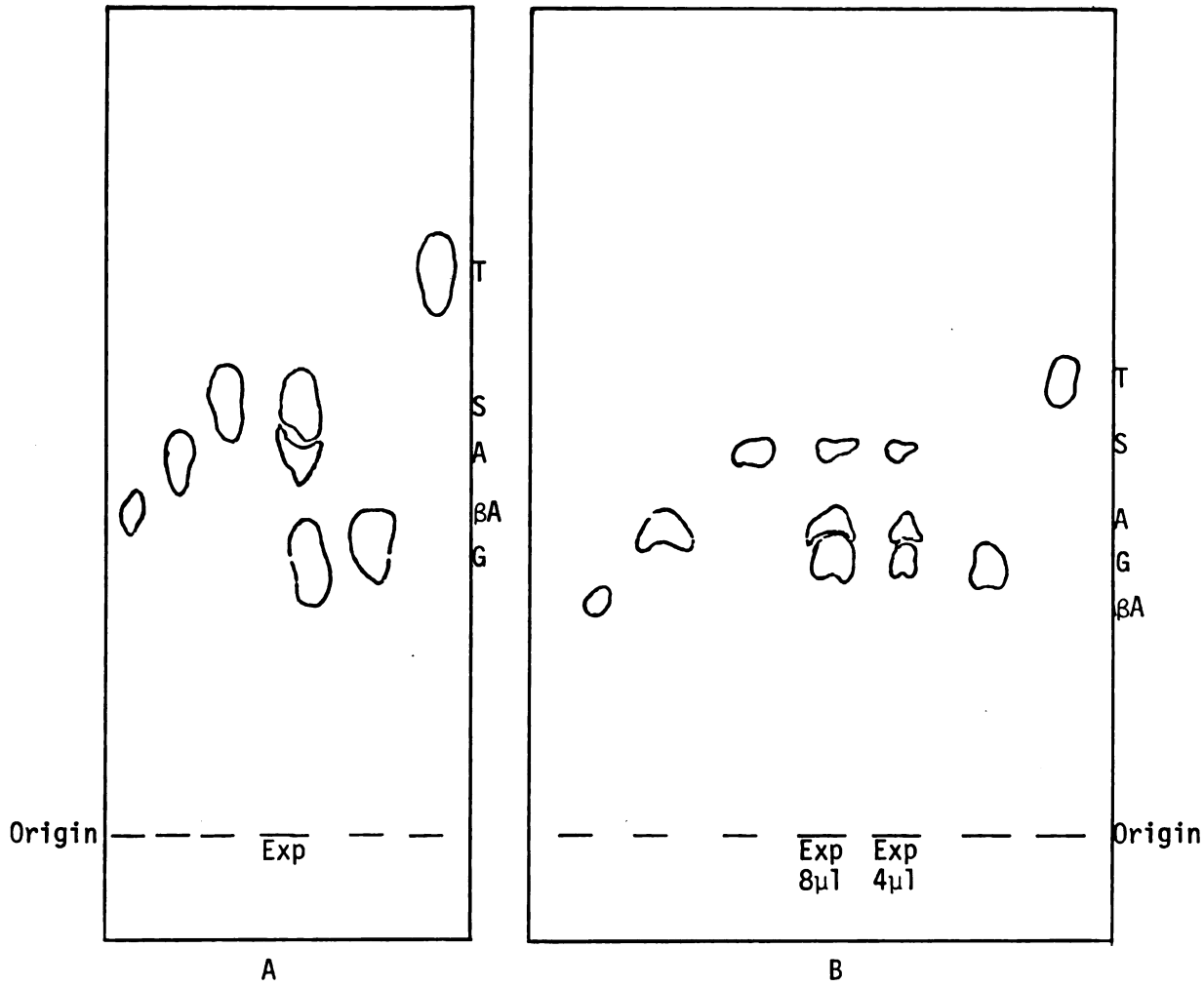
Figure 34.--Diagrammatic representation of the thin layer chromatographic separation of the amino acids obtained after the major metabolite, M_1 of GA_3, was hydrolyzed with 6 N HCl for 18 hours at 110°C.

(A) A cellulose (Brinkmann) plate developed two times in the 2-butanol-methyl ethyl ketone-dicyclohexyl amine-water (10:10:2:5) solvent system.

(B) A silica gel G plate developed in the 2-butanol-methyl ethyl ketone-dicyclohexyl amine-water (10:10:2:5) system. Amino acids were located with the ninhydrin reagent spray.

A--alanine, βA--β-alanine, G--glycine, S--serine, T--threonine, Exp--acid hydrolyzed M_1.

Figure 35.--Diagrammatic representation of the thin layer chromatographic separation of the amino acids obtained after the major metabolite, M_1 of GA_3, was hydrolyzed with 6 N HCl for 18 hours at 110°C. Thin layer plates of celluose (Brinkmann, Figure A) and silica gel G (Figure B) were developed in the solvent system n-butanol-acetone-triethylamine-water (10:10:2:5). The dried chromatograms were sprayed with the ninhydrin reagent to locate the amino acids. A--alanine, βA--β-alanine, G--glycine, S--serine, T--threonine, Exp--acid hydrolyzed M_1.


T
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A
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T
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βA
Origin
Exp 8µl
Exp 4µl
B

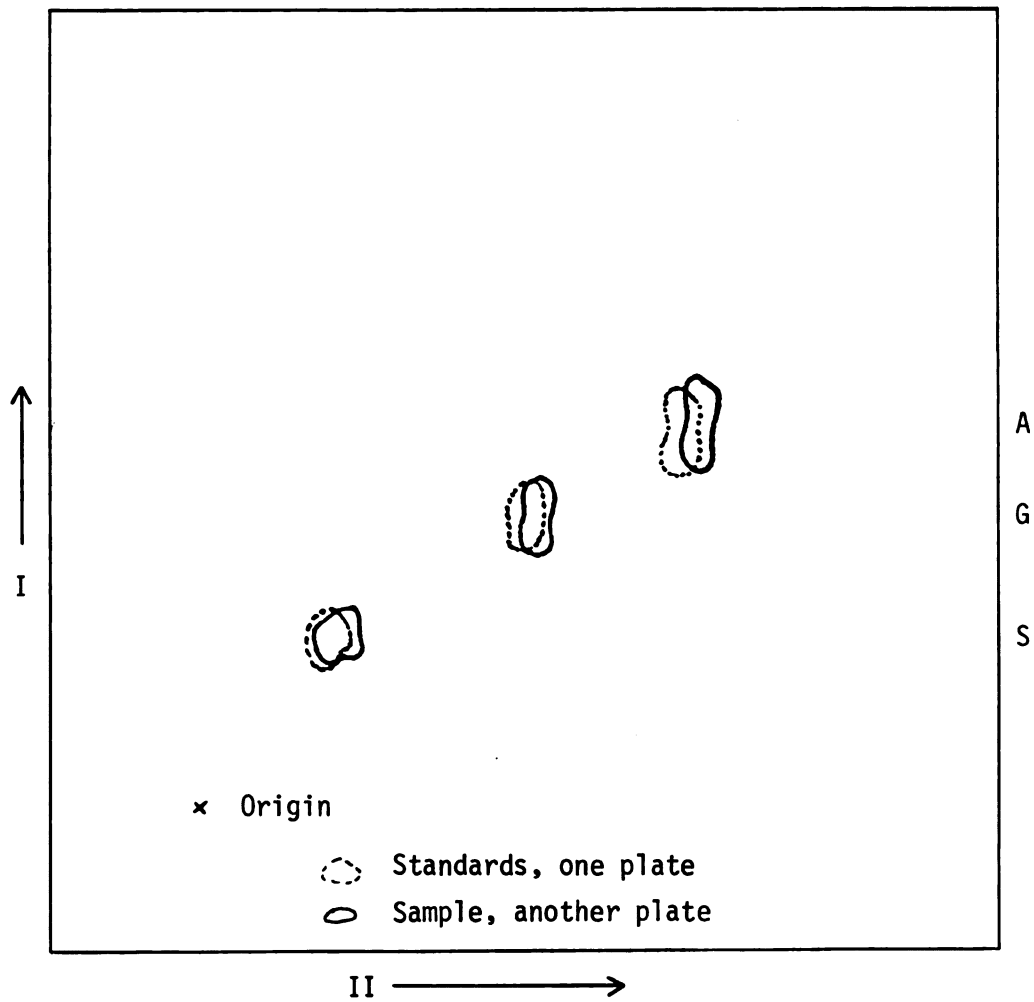


Figure 36.--Two-dimensional thin layer chromatographic separation of the DNP-amino acids obtained after the dinitrophenylation of the products of acid hydrolysis of M_1 of GA_3. The chromatogram was developed by the ascending technique in direction I with the toluene-2-chloroethanol-pyridine-25% NH_4OH (100:70:30:14) solvent system; the dried chromatogram was developed in direction II with the solvent system containing chloroform-tert.-amyl alcohol-acetic acid (70:30:3). A--DNP-alanine, G--DNP-glycine, S--DNP-serine.

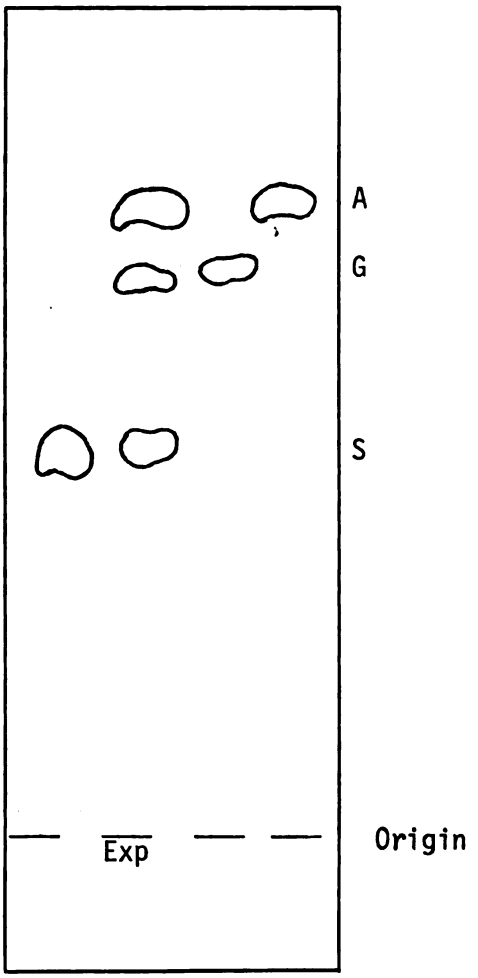


Figure 37.--Diagrammatic representation of a thin layer chromatographic separation of the DNP-amino acids obtained after the dinitrophenylation of the products of hydrolysis of M_1 of GA_3. The chromatogram was developed in the chloroform-tert.-amyl alcohol-acetic acid (70:30:3) system, under diffused light condition. A--DNP-alanine, G--DNP-glycine, S--DNP-serine, Exp--DNP derivatives of acid hydrolyzed M_1.

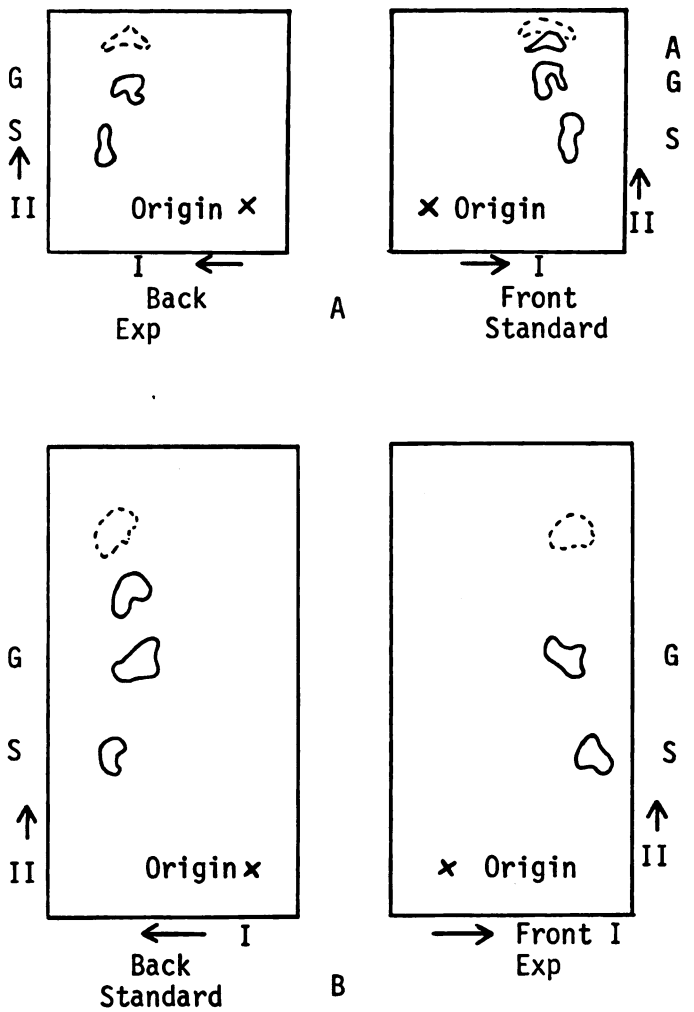


Figure 38.--Polyamide thin layer chromatographic separation of the DNS-amino acids obtained after the dansylation and subsequent acid hydrolysis of M_1 of GA_3. The experimental sample was applied on the back of the plate, opposite the standards. For one experiment a 5 cm x 5 cm size polyamide plate (Figure A) was employed and for another experiment a 5 cm x 10 cm size plate (Figure B) was used. The polyamide plate was developed by the ascending technique in dimension I with the solvent consisting of 1.5% formic acid in water (w/v). The dried chromatogram was developed in dimension II with acetic acid-benzene (1:9) first. It was dried, then developed with acetic acid-methanol-ethyl acetate (1:1:20). The chromatogram was examined under a short wave length ultraviolet lamp in order to locate the dansylated amino acids. Exp--acid hydrolyzed dansylated M_1, A--DNS-alanine, G--DNS-glycine, S--DNS-serine.

of the DNS-M_1 hydrolyzed products, also showed only DNS-serine and DNS-glycine under ultraviolet light (Figure 39). Only a very small quantity (nanomoles) of the material was required for detection on polyamide plate, but ten times this amount was required on thin layers. The polyamide plates were very sensitive to overloading of the material, smearing being produced.

XIV. Comparative Study on Gibberellin Metabolism

A preliminary study of the nature of the polar products resulting from the metabolism of radioactive gibberellins by various plants (as assessed by the properties of labeled material from the aqueous phase on chromatograms) was made. Four-day-old morning glory seedlings, devoid of roots, converted ^{14}C-GA_3 to two compounds that are unlike those formed by barley and wheat aleurone layers (Figure 40C). The product which migrated immediately behind GA_3 on paper in system A was further chromatographed on a thin layer in system H where it migrated like GA_3-glucoside (R_F 0.23). The isolation of a single polar metabolite (tentatively identified as GA_3-glucoside) from ^{14}C-GA_3 treated seedlings of Pharbitis nil Choisy, has been reported by Barendse and de Klerk (1975).

^{3}H-GA_1 was metabolized by intact four-day-old germinating corn seeds to metabolites having the properties of GA_8 and M_3 formed by barley and wheat. But, unlike what occurs in the other grasses, M_1 of GA_1 was not formed (Figure 41B).

^{3}H-GA_1 was converted to two compounds, both less polar than M_2 and M_3 of GA_1, by immature seeds of Scarlet runner bean (Phaseolus

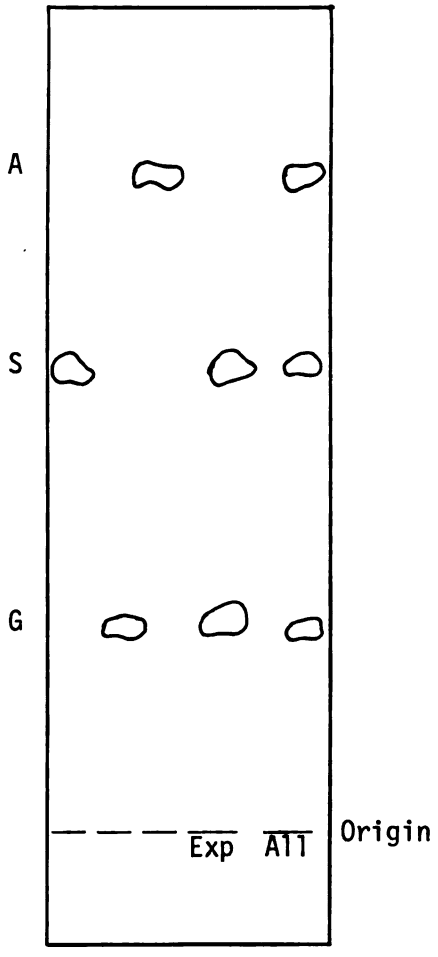
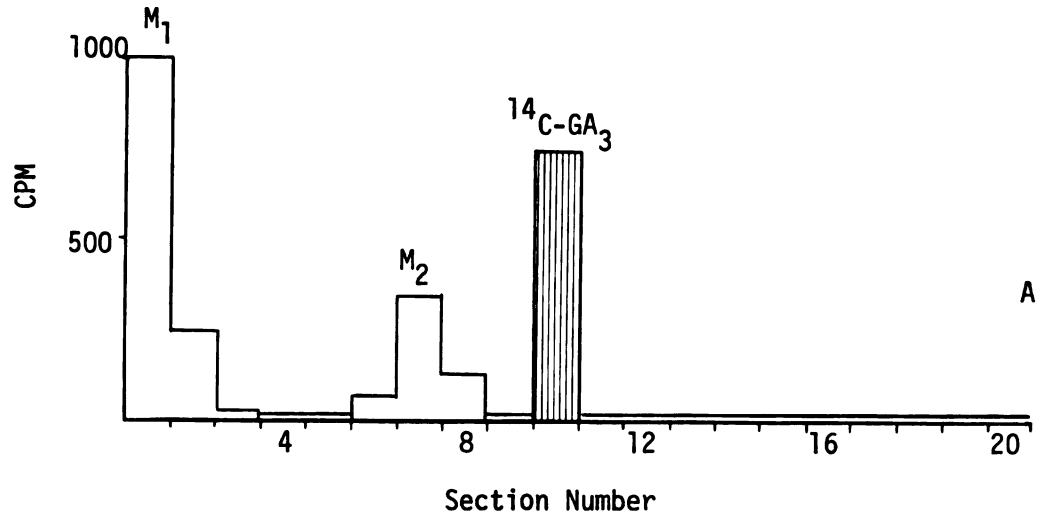


Figure 39.--Diagrammatic representation of a thin layer chromatographic separation of the DNS-amino acids obtained after the dansylation and subsequent acid hydrolysis of M_1 of GA_3. The chromatogram was developed in the benzene-pyridine-acetic acid (16:4:1) system. The dried chromatogram was examined under a short wave length ultraviolet lamp in order to locate the dansylated amino acids. A--DNS-alanine, G--DNS-glycine, S--DNS-serine, Exp--acid hydrolyzed dansylated M_1.

Figure 40.--Distribution of radioactivity on paper chromatograms of the polar metabolites present in the aqueous phase of GA_3 treated tissues. The aqueous phase had been partitioned against ethyl acetate at pH 8.4 and 2.4. The radioactive metabolites were adsorbed onto charcoal, desorbed with ammoniacal ethanol, the solutions concentrated, placed on paper and chromatographed. Chromatograms were developed in isopropanol-water (4:1).

A. Barley aleurone layers.
B. Wheat aleurone layers.
C. Morning glory, 4-day-old seedlings devoid of roots.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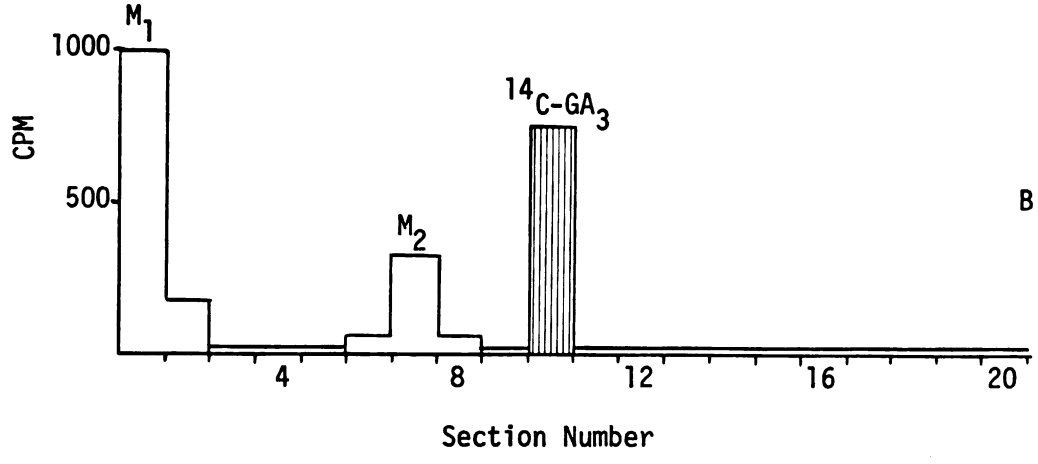
1000
14C-GA3
CPM
500
M2
A
4
8
12
16
20
Section Number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1000
14C-GA3
CPM
500
M2
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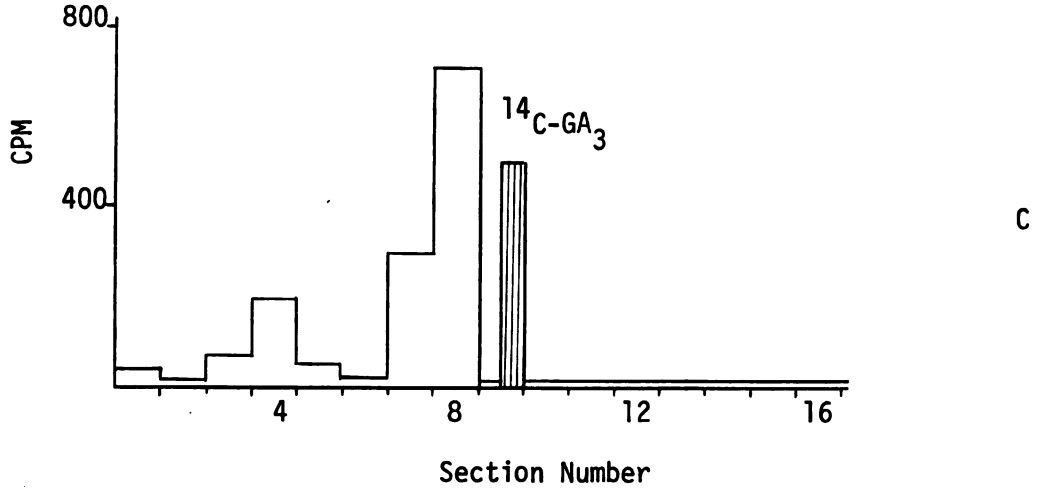
800
14C-GA3
CPM
400
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Section Number

coccineus) (Figure 41E). One compound was hydrolyzed by β-glucosidase to GA_8 and appears to be a GA_8-glucoside. The other product migrated just behind GA_1 on the paper chromatogram and was not identified. Kentucky Wonder beans which were imbibing water converted ^3H-GA_1 to metabolites whose properties were identical to those of the compounds formed by the Scarlet runner beans (Figure 41D).

When mung beans were incubated with ^3H-GA_1, the phytohormone was converted to one major polar product (Figure 41C), which migrated in a manner similar to M_3 of GA_1 on paper in system A as well as on thin layer chromatograms in system J (R_F 0.15). Upon electrophoresis in ammonium borate buffer, pH 9.3, it migrated in a manner similar to M_2 and M_3 of GA_1. Like M_3 of GA_1 it was not hydrolyzed by β-glucosidase. All of these properties indicate that it may not be a simple glucoside. Thus, it appears that the mung beans probably have a mechanism for metabolizing GA_1 that differs from the other two beans studied. The highly polar metabolites (M_1-like compounds) were not detected in any other plant tissues studied here; only in aleurone layers.

DISCUSSION

I. The Metabolism of the Gibberellins and Hormonal Action

The results indicate that GA_3 was not converted to any other form that is more active than the applied gibberellin itself. The hormone was inactivated by aleurone layers and its destruction was almost linear with time. Extrapolation of the results of the time course (Figure 12) of the formation of metabolites demonstrated that the hormone was metabolized and inactivated during early stages of incubation. In preliminary results (data not presented) none of the gibberellin metabolites detected in aleurone layers (except GA_8) was biologically active in the barley half seed bioassay. It appears that when the metabolites are formed hormonal activity is lost.

II. On the Pathways of Gibberellin Metabolism

The reduction by GA_1 and GA_7 of the accumulation of radioactivity in barley aleurone layers incubated with ^{14}C-GA_3 and the reduction by GA_3 of the formation of polar metabolites in layers incubated with ^{3}H-GA_1 indicate that the pathways for the metabolism of these gibberellins are similar (Tables 10 and 11). GA_7 contains only one hydroxyl group, at C-3. If the conjugation site in M_2 is C-3 of the GA ring, GA_7 will compete with the small amount of labeled giberellin. Therefore, the inclusion of it in the medium

should inhibit the formation of metabolites from GA_3. The possibility exists, however, that GA_7 might be converted to some unknown compound which in turn inhibits the formation of these metabolites.

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The effect of ABA on the production of the polar metabolites appeared to be due to increased uptake of the hormone by aleurone tissues rather than an effect on the formation of a specific metabolite (Tables 7, 8 and 9). Abscisic acid might affect the permeability of the aleurone layers to the hormone. It has been shown that it increases the permeability of carrot root cells and stem discs of Pelargonium to water (Glinka and Reinhold, 1971, 1972).

IV. The M2 Metabolites

A. M_2 of GA_3

The metabolite M_2 of GA_3 migrated very close to GA_3 upon electrophoresis, an indication that it contained one free carboxyl group. It was not hydrolyzed by glucosidases from a variety of sources and its rate of migration in chromatographic systems was less than an authentic GA_3-glucoside. M_2 of GA_3 was hydrolyzed by a preparation of pectinase (whose active component appeared to be β-xylosidase) to give gibberellic acid (Figure 17). Treatment of GA_3 and M_2 (from GA_3) with hot mineral acid gave allogibberic and gibberic acid (Figure 21). Therefore, M_2 is a GA_3 derivative. This metabolite gave a positive test for hexoses, and a positive test for pentoses with orcinol and phloroglucinol reagents. Radioactive glucose was incorporated into M_2 of GA_3 (Figure 22);

radioactive galactose was not. Partial acid hydrolysis of M_2 resulted in the production of a product that migrated slower than hexoses and faster than hexose disaccharides on paper chromatograms; a characteristic feature expected of a disaccharide consisting of a hexose and a pentose. This product of partial acid hydrolysis of M_2 migrated in the same manner as the disaccharide, 3-O-β-glucopyranosyl-D-glucose. Complete acid (2.5 N HCl) hydrolysis and subsequent chromatography of the products on paper as well as on thin layers showed that the minor metabolite contained glucose and xylose in apparently equal amounts (Figures 23-26).

Xylitol was a major radioactive product obtained after the sequential reaction of M_2 of GA_3 with periodate, acid hydrolysis and $NaBT_4$ reduction (Figure 28). It appears that glucose is the terminal sugar in M_2 and xylose is in the pyranose form where C-1 of glucose is linked to xylose at C-3 and C-1 of xylose to GA_3 (probably at C-3 of the ent-gibberellane ring). Thus, it seems likely that the minor metabolite, M_2 of GA_3, is O-β-D-glucose-(1→3)-O-β-D-xylopyranosyl-(1→3)-O-GA_3.

To the best of my knowledge, this is the first report that GA_3 is conjugated with xylose and glucose. The simple glucosides of gibberellins, such as 3-O-β-glucosyl-gibberellin A_3 (Tamura et al., 1968), 2-O-β-glucosyl-gibberellin A_8 (Schreiber et al., 1967, 1970), 11-O-β-glucosyl-gibberellin $_{35}$ (Yamane et al., 1971) etc. and a partially characterized GA_1-β-D-glucoside (Hemphill et al., 1973) have been reported as metabolic products of the corresponding gibberellins in many dicotyledonous plants.

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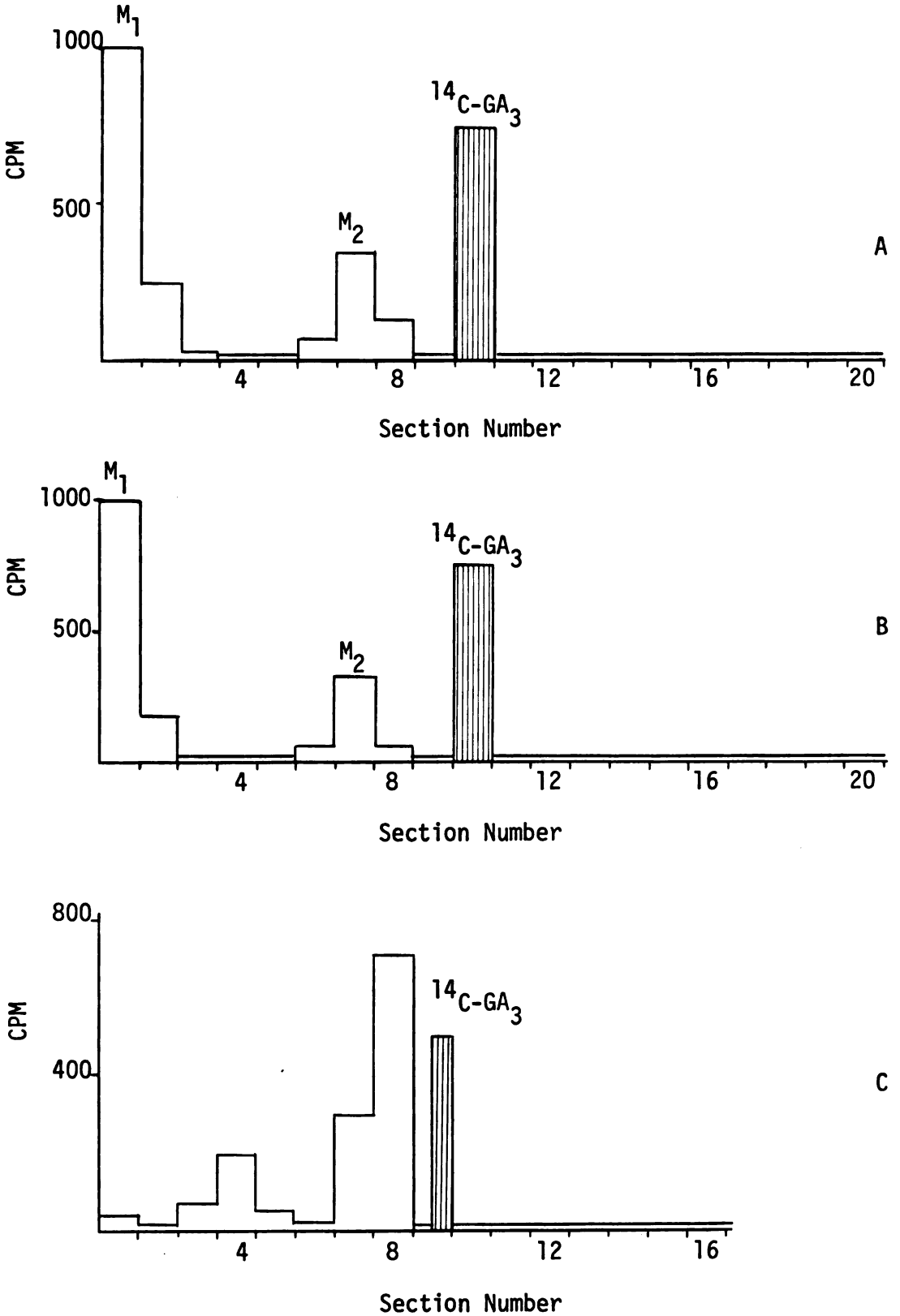
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M2
B
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Section Number
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14C-GA3
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Section Number

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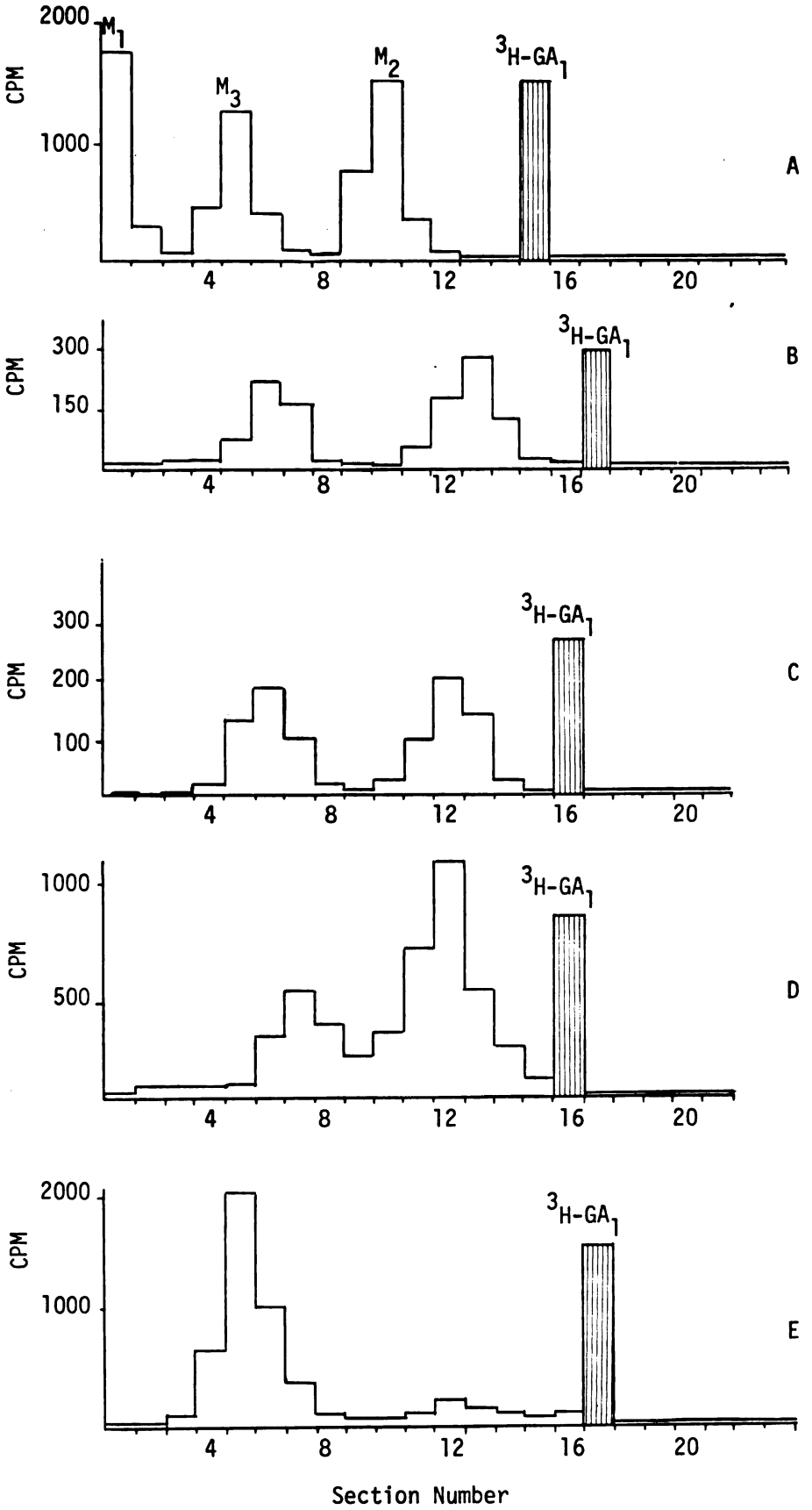
A. Barley aleurone layers.
B. Four-day-old germinated corn.
C. One-day-old germinated mung bean.
D. One-day-old germinated Kentucky Wonder bean.
E. Immature seeds of Phaseolus coccineus.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D
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8
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16
20
2000
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CPM
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Section Number

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B. M_2 of GA_1 and M_3

The metabolites M_2 and M_3 of GA_1, like M_2 of GA_3, were not affected by treatment with β-glucosidases although they were hydrolyzed by a preparation of β-xylosidase to GA_1 and GA_8, respectively. Incubation of ^3H-GA_8 with aleurone layers gave M_3, indicating that the M_3 of GA_1 is a GA_8 derivative (Figure 11).

M_2 from GA_1, and M_3 (derived from GA_1) are probably congeners, along with M_2 of GA_3. Since none of these metabolites was hydrolyzed by β-glucosidases from various sources and since acid hydrolysis of M_2 of GA_3 gave xylose and glucose, it appears that these are not simple glucosides. Very likely all of them are glycosides consisting of O-3-β-glucosyl-xylopyranose attached to C-3 of ent-gibberellane ring. The aglycone moieties are gibberellins GA_1, GA_8 and GA_3, respectively.

Nadeau et al. (1972) have reported that the material referred to here as M_3 (from GA_1) is a GA_8-glucoside. No evidence was provided by these workers that the products studied by them are simple glucosides nor was the identity of the sugar conjugated with the gibberellins determined.

The variations in the activity of β-glucosidases from a variety of sources against gibberellin glucosides have been discussed by Sembdner et al. (1973). The enzyme, β-glucosidase or emulsin isolated from almond and obtained from Darmstadt, Germany, was highly active in hydrolyzing GA_1 and GA_8 glucosides; however, β-glucosidase obtained from L. Light & Co., Ltd., England, and cellulase from Aspergillus niger (obtained from Heidelberg) were less active. None

of these enzymes gave significant hydrolysis of GA_3 glucoside. GA_1 and GA_8 glucosides were hydrolyzed completely and GA_3-glucoside was hydrolyzed partly by the enzyme cellulase, a commercial product from Aspergillus niger. Whether any stereo-chemical differences that may exist between the glucopyranosides of GA_1 and GA_3 might be responsible for the different results obtained in enzymatic hydrolysis could not be determined.

V. Electrophoretic Properties of the M_1 Metabolites

The results obtained here indicate that the major highly polar metabolite (M_1) is probably charged to the same extent as GA_3, i.e., there is probably one free carboxyl group present. Since M_1 migrates faster than GA_3 on electrophoretic paper in ammonium borate buffer (pH 9.3), the presence of vicinal hydroxyl groups which are capable of forming a borate complex is suggested (Figure 15A). The formation of a borate complex has been reported (Coombe and Tate, 1970) by GA_8 and the water-soluble apricot gibberellin A_{32}, both of which contain hydroxyl groups adjacent to each other.

The fact that M_1 migrates faster than GA_3 in electrophoretic buffer E, pH 5.6, towards the anode may be due to the presence of extra hydroxyl or carboxyl groups. It has been observed (Pridham, 1959) that phenolic hydroxyl groups are highly dissociated at higher pH values and have a profound effect on mobilities during electrophoresis. These compounds, in most instances, migrated faster in alkaline than in acidic buffers because of the increased charge due to hydroxyl dissociation. Gibberellenic acid, which contains two

carboxyl groups migrated even faster than M_1 in pyridine-acetic acid buffer, pH 5.6 (Figure 15C).

M_1 from GA_1 migrates slightly faster than GA_8 when electrophoresed in borate buffer, indicating that it is slightly more negatively charged (Figure 16).

The migration of M_1 towards the cathode at pH 2.2 is probably attributable to the presence of the amino acids (Figure 15B). A similar migration pattern for ^3H-GA_1-X was observed earlier (Nadeau and Rappaport, 1975). Since M_1 contains three amino acids, it was assumed that it would migrate faster than serine, glycine and alanine upon electrophoresis in the low pH (2.2) buffer. The only simple explanation for its failure to do so is that the slow mobility of M_1 may be due to the presence of many hydroxyl groups. Hydroxy lysine migrated slower than lysine. The mobility of some amino acids in this buffer (< = slower than) can be represented in the following manner: dopa < hydroxy proline < phenyl alanine < proline < serine < alanine < glycine < hydroxy lysine < lysine. Thus, the amino acids containing a hydroxyl group migrated slower than those that possess no hydroxyl group upon electrophoresis in buffer C.

VI. Effect of Acid, NH_4OH and Periodate Treatment on the M_1 Metabolites

Acid treatment of the major metabolite, M_1, resulted in the formation of a new product which was unlike the two degradation products (allogibberic and gibberic acid) obtained after GA_3 was treated with acid (Figure 29). It appears that the basic structure

of GA_3 has been modified in the major metabolite since it does not give the same products as GA_3 on acid treatment.

The M_1 metabolite of GA_3/GA_1 was hydrolyzed by NH_4OH to a product that was more polar than the parent gibberellins. This hydrolyzed product migrated faster than the GAs in ammonium borate buffer upon electrophoresis indicating the presence of adjacent hydroxyl groups which probably are not involved in the conjugation site of the M_1 metabolite (Figure 30).

The oxidation of M_1 of GA_1 with sodium metaperiodate also indicated that this metabolite contained hydroxyl groups adjacent to each other.

VII. Possible Structure of M_1 of GA_3

Acid hydrolysis of the major metabolite, M_1 of GA_3, gave rise to three amino acids: alanine, glycine and serine (Figures 32-35). The DNP derivatives of alanine, glycine and serine were also formed upon dinitrophenylation of M_1 acid hydrolysis products (Figures 36 and 37). The ratio of these amino acids was not determined. Dansylation of the terminal amino acid of this metabolite resulted in the production of two DNS-derivatives, DNS-glycine and DNS-serine, which indicated the presence of glycine and serine as terminal amino acids (Figures 38 and 39).

The fact that M_1 is oxidized by sodium metaperiodate and its subsequent behavior when electrophoresed in ammonium borate buffer, pH 9.3, suggest that it might be a highly oxidized compound.

The formation of the major metabolite M_1 probably involves hydroxylation. Since hydroxylation reactions involve the incorporation of an atom of O_2 from atmospheric O_2 into the product, this may explain why M_1 production was inhibited under anaerobic conditions.

From the above findings it seems probable that the formation of M_1 involves hydroxylation first; the resulting products rapidly become conjugated with amino acids. M_1 from GA_1 and M_1 from GA_3 are probably congeners. They probably are compounds which contain two or more hydroxyl groups adjacent to each other on the gibbane ring.

GA_8 is formed from GA_1 by hydroxylation at C-2 of the gibbane ring. Incubation of GA_8 with aleurone tissues resulted in the formation of a compound similar to the M_1 metabolites of GA_1/GA_3 (Figure 11), which suggests that GA_8 might be a precursor of the highly polar metabolite from GA_1.

The conversion of GA_1 to GA_8 through hydroxylation at C-2 has been well documented in higher plants (Railton et al., 1973; Reeve et al., 1975; Yamane et al., 1975). In aluerone layers GA_1 was metabolized to GA_8 and it was further metabolized to a product similar to the M_1 compounds as well as to a glycoside (M_3).

Inhibition of the formation of M_3 under anaerobic condition indicates that its formation depends on the availability of GA_8 for glycosidation (Table 6); hydroxylation reactions are inhibited under anaerobic conditions.

VIII. Hydroxylation of GA_3

Incubation of ^{14}C-GA_3 with the cell free preparation from beans resulted in the formation of three products. One migrated slower than GA_3 upon electrophoresis in buffer B but was not characterized further. The other two were probably formed through a hydroxylation reaction since they migrated faster than the parent GA_3 in borate buffer upon electrophoresis. They probably contain adjacent hydroxyl groups, which are capable of forming a borate complex. One of the hydroxylation products migrated in a manner similar to GA_8 on electrophoretograms and paper chromatograms. The second hydroxylation product migrated slightly faster, indicating that it is more negatively charged than the first (Figure 10).

IX. Study of the Highly Polar Metabolite of GA_1 from Rappaport's Laboratory in Relation to the Findings Reported Here

There was no lag period in the formation of the metabolites in barley aleurone layers from ^{3}H-GA_1 or ^{14}C-GA_3. The polar metabolites (M_1, M_3 and M_2) of GA_1 were detected within 15 minutes (Figure 12). The reports (Nadeau and Rappaport, 1974) from Rappaport's laboratory indicated that they were not able to detect ^{3}H-ampho-GA_1 (M_1) during the first hour of incubation in leaf sections of maize (Davies and Rappaport, 1975b) and only after 2.5 hours in barley aleurone tissues. The difference in the rate of production of these metabolites in barley aleurone tissue is probably due to the use of different varieties of barley seeds.

Some of the evidence provided by these workers on characterizing the amphoteric conjugate, M_1 of ^{3}H-GA_1, from barley aleurone layers is confusing and misleading. First of all, there was only one indication of the use of chromatographic systems where ^{3}H-ampho-GA_1 (M_1 of GA_1) migrated away from the origin: the organic phase of butanol-acetic acid-water (4:1:4). In the present studies M_1, the highly polar metabolite from GA_3 or GA_1, remained very close to the origin of thin layer (silica gel G) chromatograms (R_F ^{3}H-GA_1 0.72 and R_F M_1 0.09) when this system was used, although Nadeau and Rappaport (1974) observed the migration of ^{3}H-ampho-GA_1 away from the origin (R_F 0.53) in the same system. The R_F value for GA_1 was not given. A simple question can be raised: If ^{3}H-ampho-GA_1 (similar to M_1 metabolite) is a highly polar compound, how can it move in this solvent system which is not very polar?

It was suggested (Nadeau and Rappaport, 1974) that ampho-GA_1 is a GA_1 derivative, the nitrogen containing groups being conjugated with GA_1 through an ether linkage. It was said further that the C-3 hydroxyl group is not the site of conjugation. These findings were based on the following reasonings. The mixture containing ^{3}H-ampho-GA_1 plus unlabeled gibberellin A_1 upon treatment with 1 N HCl at 100°C for 12 hours gave four similar products: two radioactive products IIb and IIIb that came from ^{3}H-ampho-GA_1, and two similar non-radioactive products IIa and IIIa. The product IIIa,b which appeared to be more polar than GA_1 and IIa,b migrates faster

than GA_1 in a non-polar solvent system consisting of ether-benzene-acetic acid (135:65:10). The R_F values were: GA_1, 0.29; IIa,b, 0.59; IIIa,b, 0.73, when they are chromatographed on TLC. Logically, GA_1 which is more lipoidal than IIIa,b, should have migrated faster than the IIIa,b product in this solvent on TLC.

Amide and ester linkages were ruled out since strong alkali treatment of a mixture of ^3H-ampho-GA_1 and unlabeled GA_1 did not

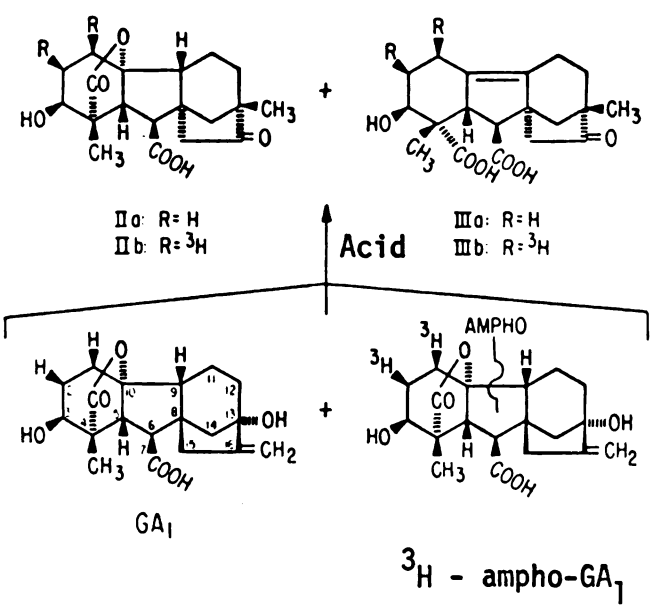

Figure 42.--Acid hydrolysis of GA_1/^3H-ampho-GA_1 mixture. From Plant Physiol. 54:811 (Nadeau and Rappaport, 1974).

form a radioactive compound, however, non-radioactive 3αOH-GA_1 (pseudo GA_1) was observed, as expected from GA_1 (the C-3 hydroxyl group in GA_1 undergoes epimerization in presence of mild alkali). The authors mentioned that this could happen (epimerization) only when the C-3 hydroxyl group is not conjugated. The question can be raised again, If no radioactive component was present in the fraction after the strong alkaline treatment of the mixture, how can they be sure that the C-3 hydroxyl was not the site of conjugation in ^{3}H-ampho-GA_1? Further statements were made by these workers: "Treatment of a mixture of I (ampho-GA_1) and GA_1 with 0.1 N NaOH did not cleave the conjugate . . . . However, the mild alkali treatment did cause a change in I (^{3}H-ampho-GA_1) as did subsequent acid hydrolysis . . . ." Once it was claimed that alkali treatment had no effect on ^{3}H-ampho-GA_1 but it was stated later that mild alkali did cause a change in ^{3}H-ampho-GA_1. Thus, the validity of the interpretation of the results published in this paper seems to be questionable.

Sembdner et al. (1973) have indicated that they have prepared conjugates of gibberellins not only with glucose and other carbohydrates, but also with amino acids or peptides; however, they have not provided any evidence for the existence of naturally occurring gibberellin metabolites conjugated with amino acids or peptide, or with any carbohydrates other than glucose in plants. In this paper they mentioned that gibberellin-amino acid conjugates have been synthesized in which the C-7 carboxyl group of GA_3 is amide linked with the amino group of either glycine, L-leucine,

L-serine, L-phenylalanine or L-proline. The GA_3 amino acid conjugates have been shown to be biologically inactive in the dwarf pea, dwarf maize and the α-amylase bioassays.

X. Gibberellin Metabolism in Tissues of Other Plants

GA_3 was metabolized by morning glory to two polar compounds which were unlike the products obtained from aleurone layers. Incubation of ^3H-GA_1 with four-day-old germinated corn gave two unidentified polar substances. It has been reported that ^3H-GA_1 was metabolized to a highly polar compound like the M_1 metabolites, to GA_8 and to GA_1 and GA_8 glucosides when d-5 dwarf maize seedlings were treated with GA_1 (Davies and Rappaport, 1975a). ^3H-GA_1 was metabolized to one polar product in mung bean. Its chromatographic and electrophoretic properties were similar to M_3 of GA_1. Two polar metabolites were detected in ^3H-GA_1 treated immature seeds of Phaseolus coccineus and Kentucky Wonder runner bean. They appeared to be less polar than M_2 or M_3 of GA_1 and the metabolite formed from mung beans.

These studies appear to underscore the variations in the manner in which gibberellins are metabolized among different plants; differences may also exist in the same plant at various physiological age and state of maturity (Figure 41).

XI. Significance of the Research

Figure 43 outlines probable pathways by which GA_1 and GA_3 are inactivated in aleurone layers, based on observations made in these studies.

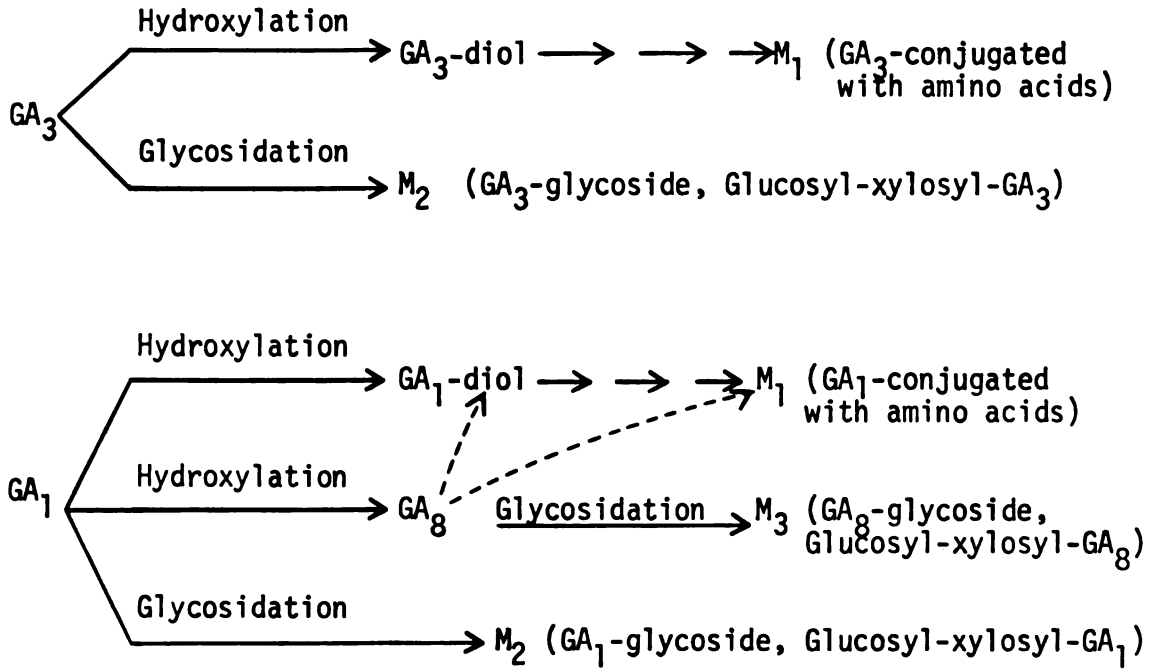

Figure 43.--Postulated pathways of gibberellin metabolism in aleurone layers.

The significant aspects of this research are probably the following:

1. The revelation that the inactivation of the hormonal activity of the gibberellins in aleurone layers is probably effected by a series of reactions involving glycosidation or hydroxylation/oxidation, with subsequent glycosidation or conjugation.

2. The finding that there is no apparent relationship between the metabolism of the hormones and their biological effects in layers other than the fact that the gibberellins are inactivated when they are metabolized by the tissue.

3. The revelation that, at least in the case of GA_3, the gibberellins are glycosidated with glycosyl xylose and that glycine, alanine and serine are attached to a metabolite. This basic information should be invaluable in efforts to establish, unequivocally, the structure of the metabolites.

4. The finding of the uncharacterized products observed in the reaction mixtures after the hydroxylating enzyme preparation reacted on GA_1 and on GA_3 (which to my knowledge has not been studied before) probably opens up new avenues of research on the metabolism of the gibberellins. Studies on the biochemical mechanism in which the gibberellins are glycosidated and conjugated would also seem to be fruitful areas in which to direct future research.

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