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FINAL REPORT

A Multidisciplinary Research Program Directed Toward Utilization of Solar Energy Through Bioconversion of Renewable Resources

MASTER

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DISCLAIMER

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Publications Issued During Total Term of Contract

Publications (Brown) 1976-1979)

1. Brown, C.L. 1976. Forests as energy sources in the year 2000: What man can imagine, man can do. Jour. For. 74:7-12.
2. Steinbeck, K. and C.L. Brown. 1976. Yields and utilization of hardwood fiber grown on short rotations. Appl. Polymer Sym. #28, 393-401. Removed
3. Blum, R.B. 1978. Carbohydrate levels in sycamore coppice roots over a seven month period. MS Thesis, University of Georgia, 68pp.
4. Brown, C.L., T.R. Clason, and J.L. Michael. 1976. Paraquat induced changes in the reserve carbohydrates, fatty acids, and oleoresin content of young slash pines, p. 8-19. In M.H. Esser (ed.), Proc. Lightwood Res. Coord. Coun. U.S.F.S. S.E. Forest Expt. Sta., Asheville, N.C. Removed
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10. Sommer, H.E. and C.L. Brown. 1979. Application of tissue culture to forest tree improvement. In: Eds. W.R. Sharp, P. Larsen, E. Paddock, and V. Raghavan. Plant Cell and Tissue Culture Principles and Application. Chapter 25: pp. 461-491. Ohio State University Press. Columbus. Removed

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2. Finnerty, W.R. 1976. Comparison of primary products with respect to energy conversion. Microbial Energy Conversion pp. 85-96. UNITAR.
3. Finnerty, W.R. and P. Kerr Falco. 1977. Seasonal variation in resin acids and other metabolites in Pinus elliottii. Lightwood Research Coordinating Council and U.S. Forest Service. pp 44-50.
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3. Legall, J., D. DerVartanian, H.D. Peck. 1979. Flavo proteins, Iron Proteins, and Hemoproteins as Electron-Transfer Components of the Sulfate Reducing Bacteria.

SUMMARY OF RESEARCH FOR

TOTAL CONTRACT PERIOD

Program A - Genetic selection, physiological basis of vigor, tissue culture.

Professional personnel

Claud L. Brown - no salary from grant, each worker 15% time.

*Klaus Steinbeck - *Steinbeck did not participate after 1977 because of his DOE grant.

**Harry Sommer - **Has conducted most of the tissue culture plantlet studies during 1977-1978-1979.

Technicians - Debbie Fortner, 50% Program A, 50% Program B.
Justin Coile, 100% Program A - 1976-77, 1977-78.

Graduate Students - Mark Blum, M.S. Degree. Part of 1976-77, 1977-78.

Major Accomplishments

1. Phenotypic selections have been made of exceptionally vigorous, fast growing sycamore trees in young plantations for cloning and outplanting on fairly uniform sites. Approximately 20 fast growing clones have been established for testing clonal responses at age 3 and 5 years and for use in tissue culture screening studies for possible assays indicating inherent vigor. In one such study conducted in vitro, strong positive correlations were obtained in the amount of callus produced by 3rd internode tissue segments at the end of 30 days between paired phenotypic selections (low vs. high vigor clones). Currently similar clones are being screened in vitro for response to varying levels of nitrogen nutrition, and at different temperature regimes (23°-30°-38°C).

2. Techniques were developed for mass Cloning American sycamore using small internodal stem segments from dormant and active twigs in mist beds and/or growth chambers. These same techniques are applicable to other woody dicots which root without difficulty, e.g., Populus deltoids, Acer rubrum, and Robinia pseudoacacia.

3. Seasonal carbohydrate content of sycamore rootstock was determined on field plots which at being coppiced (harvested followed by sprouting) on one or two-year rotations for the preceding eight years. Concentrations of starch and sugar varied significantly during the season, but non-significantly between treatments. Total root biomass and total reserve carbohydrate stored in root systems is probably more important in regrowth potential of sprouts than concentration per se.

Program A

Major Accomplishments (cont.)

4. Considerable success has been attained in studies of organogenesis leading to bud and root differentiation or ultimately plantlet formation in several woody species having potential use in short rotation biomass production. Starting with various types of explant material (shoot apices, leaf axils, hypocotyl and cotyledon sections of embryos or young plants) plantlets have been produced with fairly good success in sweetgum (Liquidambar styraciflua) and black locust (Robinia pseudoacacia), a legume, currently being used in mixed outplantings of sweetgum and sycamore in field trials in an effort to reduce the future use of nitrogenous fertilizers in short rotation biomass production. More recently somatic embryogenesis has been achieved in suspension cultures of sweetgum which is a major step toward developing a practical system of mass cloning this species and other woody dicots for commercial production of superior genotypes.

5. In numerous other studies, pollen cultures have been established from several trees, but no haploid plantlets have yet been produced.

Program B - Physiology of paraquat induced oleoresin biogenesis.

Professional personnel

Claud L. Brown - no salary, 12 month appointment. Time devoted to project: 15%.

Technicians - Debbie Fortner, full time, 50% Program A, 50% Program B.

Graduate Students - None

Major Accomplishments

1. Developed suspension culture system for batch cultures of clonal lines of slash (P. elliottii) and longleaf pines (P. palustris) on chemically defined media for use in physiological, biochemical, and ultrastructural studies in this program (B) and in Program C (Finnerty).

2. Determined physiological effects of sub-lethal paraquat levels (0.1-1.0 mg/l) in pine suspension cultures, e.g., inhibition of cell division, increased respiratory rates, decreases in soluble and total protein.

3. Cytological studies of paraquat treated cells show evidence of accelerated aging, loss of membrane integrity and death corresponding to levels of paraquat and length of time in culture (3 to 30 days). Destructive effects of paraquat on vacuolar and organelle membranes, as well as cytoplasm, are observable within one day in cells supplied with 10.0 mg/l; in 14 days when supplied at 1.0 mg/l.

4. In cytological and ultrastructural studies of pine cells in paraquat treated trees it was shown that all cells of the symplast, including those not directly associated with resin ducts begin to synthesize excessive oleoresin and secrete or lose it initially through pits into the lumen of contiguous tracheids, thus causing the phenomenon of "resin or pitch soaking".

5. In field studies using 25-year-old slash pine trees, judicious blocking of downward translocation of current photosynthate by imposing deep transverse saw-cuts at 1.0 and 0.25 meters immediately above the paraquat treated xylem faces did not prevent resin soaking of the xylem at and above the treated face. These studies and others indicate that a good portion of the carbon being utilized in the syntheses of oleoresin is being mobilized by paraquat affected cells from previously formed reserve foods (starch, sugars, possibly amino acids, or other sources) and not altogether from current photosynthate.

PROJECT SUMMARY - Finnerty

YEAR ONE

Seasonal fluctuation of soluble metabolite pools in Pinus elliottii indicate that carbon from carbohydrate reserves was mobilized successively into reducing sugars, acids and amino acids during winter and early spring. Peak production and accumulation of terpenoid compounds occurred after cessation of growth in early-mid summer. Mechanical wounding, cutting and/or treatment with the herbicide, paraquat, resulted in a comparable flow of carbon into the terpenes. No unique metabolic pathways were associated with paraquat induced terpene synthesis, only an acceleration of carbon flow into these secondary metabolites.

SECOND YEAR

Since data accumulated during year one indicated that tissue cultures of Pinus elliottii could not synthesize resin acid, laboratory models utilizing phloem and xylem tissue slices were developed to study the incorporation of radiolabeled substrates into the terpenes. The seasonal fluctuation in resin acid synthesis observed in the field studies was duplicated in the laboratory model. Monoterpenes appeared accounte for from 90-95% of the incorporated ^{14}C, irrespective of the substrate. Carbon from glucose and the amino acids arising from pyruvate contributed more ^{14}C to resin acids than any other substrate supplied, including melavonate. ^{14}C recovered from the purified methyl resin acid area of TLC plates never exceeded 5% of the supplied substrates and in >95% of the cases was <.01%.

THIRD YEAR

Emphasis was switched from terpene production in slash pine to identification of neutral, phospho- and glycolipids extracted from stem and tap root tissue of Platanus occidentalis.

The usual phospholipid spectrum was identified plus an as yet unidentified compound which in these trees is the predominate phospholipid. Mono- and digalacytosyl diglyceride were identified and several other glycolipids were visualized on TLC, but are as yet unidentified. The extracted neutral lipid fraction is very complex and has been characterized only to the classes of compounds present, i.e., fatty acid, mono-, di-, triglyceridies, etc., by TLC.

FOURTH YEAR

Seasonal fluctuation of lipids extracted from stem and root tissues of American sycamore indicate that while certain neutral, phospho-, and glycolipids do serve as storage forms over winter, lipid storage was not significant in these tissues. Preliminary data indicate that the predominate phospholipid also contained nitrogen. Since the concentration of this compound(s) was maximal during winter and decreased from spring through summer, it could function as storage form of C, N, and P.

The Microbiology and Physiology of Anaerobic Fermentations of Cellulose

Abstract

Cellulytic bacteria, cellobiose fermentors, sulfate-reducing bacteria and methanogenic bacteria have been isolated from established anaerobic mesophilic and thermophilic cellulose-methane fermentations and these isolates, plus known laboratory strains, have been employed to partially reconstitute highly active cellulose fermentations. These mixed cultures will be utilized as model systems to study the parameters required for the maximum production of CH_4, H_2 and chemical feedstocks such as acetate, ethanol, propionate, etc., from cellulose. Thus, the physiology of these reconstituted cultures will be investigated as regards cultural conditions, microbial types, inoculum size, interspecies H_2 transfer and specific regulatory phenomena, the accumulation of cellobiose and acetate. Other bacteria will be isolated as indicated by experimental results and a major effort will be made to isolate microorganisms from extreme environments in order to utilize growth conditions which will enhance the rate of formation, yield or recovery of products. When additional information is available regarding the microbial types and physiology of reconstituted cultures, it should be possible to "taylor-make" microbial populations with regard to substrate, conditions and desired products. With single culture the biochemistry physiology and bioenergetics of the various types of bacteria included in the fermentation will also be investigated. Emphasis will be placed on the mechanism and bioenergetics of acetate formation by species of Clostridium and Acetobacterium, the bioenergetics of sulfate-reduction and interspecies H_2 transfer by species of Desulfovibrio and Desulfotomaculum and electron transfer and proton translocation by the H_2-utilizing methanogenic bacteria.

Program A: Genetic selection of superior trees, physiological basis of vigor, tissue culture systems leading to cloning of diploid and haploid cell lines.

Investigators: Claud L. Brown and Klaus Steinbeck

Scope of investigations over 3 year period

The concept of managing higher plants for their fuel value as well as associated or derived by-products has been proposed by biologists and engineers for several years. Only recently, with the rapid escalation of petroleum prices brought about by increased shortages America has the feasibility of using renewable sources of biomass to lessen the use of petrochemicals been given serious consideration.

Over ten years ago, we recognized the pressures impinging on our natural resources as a result of a shrinking land base for forests and agriculture and the need for more intensive silvicultural practices for enhancing above ground yields of fiber with minimal inputs of energy. Through the efforts of several scientists in the School of Forest Resources at the University of Georgia this concern led to the concept of short-rotation forestry by which rapidly growing plantations of young hardwoods could be planted at row-crop spacings and harvested at frequent intervals. Upon harvest, a new stand is assured by sprouts from the severed root system of the original trees which may be repeatedly harvested at 3 to 6 year intervals. Periodic fertilization at the time of harvest is required to maintain levels of soil productivity, however, cultivation or replanting is not required because of rapid sprout growth. Such a system of producing renewable biomass, because of its low energy inputs, appears to be one of the feasible alternatives for reducing our almost complete dependence on petrochemicals for future sources of plastic feedstocks and fuels.

Research objectives under Program A (Brown and Steinbeck) in this multidisciplinary program were (1) the selection of exceptionally fast growing phenotypes from wild populations in previously established plantations, and cloning of these initial selections to provide future sources of material for assessing their genetic potential through field performance; (2) to initiate studies relating to the physiological basis of vigor in an attempt to screen for inherent vigor in woody plants at an early age; and (3) to study growth and differentiation of cells and tissues in vitro ultimately leading to practical systems of cloning either by (a) plantlet formation or (b) somatic embryogenesis in suspension cultures.

Significant Results - Program A

During the past 3 years considerable progress has been made in all of these areas which will ultimately lead to practical applications for enhancing biomass yields from woody plants. Major accomplishments are listed under each objective above.

Genetic Selection

1. Most of our phenotypic selections have been restricted to sycamore (Platanus occidentalis) because of its good yield performance on upland sites and its ease in propagation in establishing clonal lines for field testing. Approximately 20 fast growing clones have been established in field plots at the Whitehall Forest for assaying their performance in the field and to provide material for future studies. Twelve selected clones were multiplied by stem cuttings and outplanted at two widely different sites (one a dry upland, the other a moist, fertile bottomland) to obtain genotype x site interactions and the relative performance of the selected clones.

Dr. Klaus Steinbeck, one of the initial investigators on Project A during the first two years, was primarily responsible for the field aspects of selection, propagation, and testing of different clonal lines under field conditions. Because he was the recipient of another DOE contract in the Fuels from Biomass Systems Branch, Solar Energy Division during 1977 he withdrew from active participation in this program to extend the concept and to develop sizeable acreages of short-rotation hardwood plantations in the Piedmont and Coastal Plain Provinces in Georgia. The extension of this phase of field research by Dr. Steinbeck has in many ways supplemented this program because of continued close collaboration between his current program and our program during the past two years.

2. Techniques, were developed for rooting American sycamore using internodal stem segments from dormant and active twigs in mist beds or growth chambers. These same techniques can be used in cloning other species which root with considerable ease, e.g., Populus deltoides, Acer rubrum, Robinia pseudoacacia and Alnus glutinosa, the latter two being nitrogen fixers which could help circumvent the need for nitrogen fertilization in future short-rotation plantations by using species mixtures now being tested by Dr. Steinbeck in his ongoing field studies.

Physiological Basis of Vigor

Although the final length attained by internodes in an elongating stem is a function of cell length x cell number, previous stem growth analyses with sweetgum (Liquidambar styraciflua) and other hardwoods have shown that cell multiplication or final cell high number correlates exceptionally with final length of internodes, whereas, final cell length was not significantly different between the longest and shortest internodes of a given shoot. Because growth (fresh and dry weights) undifferentiated

callus cells and/or cells in liquid suspension culture are primarily functions of cell division rather than final cell size we attempted in several laboratory trials to screen putative high and low vigor phenotypic selections on the basis of their initial and sustained callus growth rates on chemically defined growth media. We realized of course, that to extrapolate from the frequency of cell division in a highly artificial *in vitro* system to the *in vivo* processes of cell multiplication and shoot growth under highly variable environmental conditions in the field involves high risk speculation, nevertheless several studies were performed to test these relationships resulting in the following observations.

1. In one of the initial studies strong positive correlations were obtained in the amount of callus produced *in vitro* (fresh and dry weights) with initial phenotypic selections of high and low vigor sycamore trees grown at 4 x 4 feet spacing on a relatively uniform bottomland site.

2. In another study, where paired phenotypic selections were made for high and low vigor clones in a plantation which had been repeatedly harvested at 2-3 year intervals to permit rapid sprout (coppice growth), there were very weak positive correlations or even negative correlations between callus formation in laboratory trials and observed field performance.

3. In a more definitive study 5 high vigor and 5 low vigor clones of sycamore were selected on the basis of their relative growth performance in field plots after 7 growing seasons and screened for relative growth response *in vitro*. From each clone 1.0 cm sterile stem explants were taken from the third internode of 25 actively elongating shoots, weighed aseptically, and explanted to a chemically defined agar medium specifically formulated for rapid callus growth of sycamore cells. At the end of 30 days explants were harvested to obtain fresh and dry weights of callus

produced in culture. The results of this carefully performed study were highly variable and non-significant with respect to percent increases in fresh and dry weights between the selected high and low vigor clones. In fact, only two of the five high vigor clones possessed greater increases in fresh and/or dry weights than the means of the low vigor clones, and conversely two of the five low vigor clones out performed the best of the high vigor clones.

4. Additional experiments were initiated using the same clones of known field performance. Stem explants were again taken from the young, immature distal third internode of elongating shoots and grown in liquid shake culture under varying experimental conditions. These were: (1) A standard defined basal medium with three levels of nitrogen nutrition (NH_4 and NH_2 sources) comprising low, medium, and high levels of total nitrogen (ca. 70, 200, and 600 mg/l^N respectively) and (2) the best basal medium used for the growth of sycamore callus with the stem explants grown under 3 rigidly controlled temperature regimes, namely 23, 30, and 38°C in plant growth chambers. The objectives of these studies were (1) to test the response of high and low vigor clones in vitro to optimum and stress levels of nitrogen, an environmental variable often limiting field performance; and (2) a similar attempt to screen for normal and supraoptimal temperature effects on overall metabolic processes affecting respiration and assimulation rates, hence net cell proliferation, callus formation and dry weight.

The overall results of these studies conformed to what one might expect, i.e., in all clones there was a favorable response to increased nitrogen nutrition, and an optimum growth response, at the lower temperature regime (continuous 28°C) callus growth was slightly less in cultures maintained

at 34°C and severely inhibited in all cultures at 38°C. There were no significant differences in clonal response due to extremely high within clone variation. The within clonal variation was greater in liquid suspension culture than in stem explants grown on nutrient agar which was totally unexpected because in the former all portions of the explants were continuously bathed in a thin well-aerated layer of liquid medium. In agar solidified cultures the size and extent of callus formation imposes transport gradients among cells far removed from contact with the nutrient agar so that callus growth is frequently assymetrical and highly variable. Hence, it is difficult to explain why, for example, 6 out of 10 original explants of the same clone may proliferate uniformly along their entire surface resulting in a rather uniform increase in callus growth and dry weight, whereas the remaining 4 might exhibit wide variation in their growth response in the same culture flask when all original explants were essentially uniform in size and age, viz., from the thrid immature internode of comparable one-year-old sprouts of the same root stock. Herein lies the difficulty of screening genotypic responses in vitro with known in vivo field performance which ultimately involves releasing the totipotency of individual cells from numerous spatial biochemical and biophysical restraints. Obviously, we have only touched upon one approach to the difficult problem of screening for physiological traits contributing to the genotypic expression of vigor in woody plants.

Tissue Culture

Organogenesis and Cloning by Plantlet Formation

For short rotation biomass production hardwoods are the major candidate because they exhibit rapid juvenile growth, typically allowing harvest at

age 3 to 5 years for wood chips which can be converted to fuel or used as chemical feed stock. The short rotation age allows greater stocking rates per acre, more vigorous growth, hence greater yields than conventional forestry. The next crop develops from stump sprouts, rather than replanting which results in reduced energy inputs and higher net energy outputs than most agronomic crops.

Numerous species of hardwoods have been suggested for use in short rotation biomass production. A general strategy for attempting to clone these species via tissue culture has been developed. First, sterile seedlings of the species are grown or juvenile material is collected from the field. In general there is a greater probability of obtaining the differentiation of shoots, roots or embryoids from juvenile material. Second, the explants to be placed in culture are chosen. These may be any living part of the plant, but particularly the shoot tips, axillary buds, shoot sections, hypocotyl sections, and root sections are most often used. Third, is the choice of tissue culture media or media system to be used. Many hundreds of media have been published, however based on the literature, 5 were chosen for the initial screening for differentiation of organs or embryoids in culture. These are Murashige's A medium and B medium, Anderson's rhododendron medium, Halperin & Wetherell's system, and Kolehbach's system. It should be noted that all these media contain Murashige and Skoog's major salts or modification thereof. In event these attempts proved unsatisfactory a second group of media or media systems was selected for further screening for the differentiation of organs or embryoids. The second battery of screening media included the substitution of Blaydes' improved salts, Risser and White's salts and modifications thereof, or Heller's salts for the Murashige and Skoog basal salts.

The other approach used in obtaining bud and/or root differentiation and sometimes used in combination with the change in nutrient salts was to vary the hormones, or their concentrations. For experiments on variations in auxin concentration NAA was commonly used and for cytokinin different concentrations of 6-benzyladenine (BA) was used. The concentrations of these growth factors varied from 0 to 32 mg/l .

Fourth, was the choice of environmental conditions. For most organogenic studies a temperature of $25^{\circ} \pm 2^{\circ}C$ with about 500 ft candles of light from flourescent tubes for 15 hours a day was used. The species we attempted to clone by tissue culture were black locust (Robinia pseudoacacia), honey locust (Gleditsia sp.), European black alder (Alnus glutinosa), and sweetgun (Liquidambar styraciflua). Sweetgum has performed remarkably well in short rotation yields of cellulose whereas black locust, honey locust, and black alder are being tested not only for biomass yields, but also for their ability to replace part of the fertilizer requirements by nitrogen fixation. To achieve maximum yields short rotation plantations are generally fertilized at each harvest, i.e., at 3 to 5 year intervals.

During the past year with considerable effort devoted to this segment of research program A, we have obtained highly significant results in cloning several forest species for the first time in vitro and considerable progress has been made toward developing a practical system for ultimate use in selecting and propagating superior genotypes for short rotation biomass production. Briefly these results are:

1. European black alder has rarely shown organogenesis. Shoot tips have regenerated on Murashige's A medium and Anderson's rhododendron medium. Plantlets were obtained from the shoots by rooting on modified Morel's medium with IBA. Only one European black alder plantlet has been successfully developed to the stage of field planting. Screening of this species

on the second set of media has not been completed so it is too early to conclude that it will not be amenable to propagation by tissue culture.

2. The results with honey locust have been more encouraging. Frequently multiple buds and shoots were produced from the apex, leaf axils, and cotyledon axils on Murashige's A and Anderson's rhododendron media. Polar structures with a root can be obtained in suspension culture. Further investigations are needed to determine the exact nature of these structures. Todate they have only produced additional roots.

3. Black locust (Robinia pseudoacacia) has been the most intensively investigated nitrogen fixer in our studies. On Murashige's A and Anderson's rhododendron media both shoot tips and leaf axils formed callus and regenerated shoots. These shoots readily rooted on media with modified Morel's media and IBA. The major problem encountered was that callus generally outgrew the shoots on Murashige's A and Anderson's rhododendron media. However, 25 plantlets obtained from these experiments are ready for planting this winter. In the second media screening for black locust both the mineral composition and hormones were varied. Four basic media were tried, each with varying hormone concentrations. Briefly, the four are similar to Murashige's A or B except for changes in salts and hormones, and currently we have been able to regenerate shoots in approximately 50% of the cultures explanted on several media. Organogenesis has not yet been obtained in cell suspension cultures of black locust in our first set of screening media.

4. Sweetgum is one of the most highly regarded species for short rotation forestry due to its rapid growth, high wood density and adaptability to a wide range of sites. Field tests with it show from 8-16 tons per acre yield at age 3 are possible, even without the advantage of genetic selection and cloning superior genotypes.

Our initial organogenic studies with sweetgum were most discouraging because all explants from sweetgum died on the initial screening media. In other studies (Brown, unpublished data) sweetgum did not produce callus from stem explants. However, in anther culture experiments sweetgum had produced abundant callus and a few roots on media using Blaydes' major salts. Apparently Murashige and Skoog's salts are lethal to sweetgum tissue at the concentration used. Thus the second part of the media screening was conducted using Blaydes' salts, and varying the concentration of NAA from 0.1 to 8.0 mg/l and BA from 0.1 to 8.0 ppm. At high auxin to cytokinin ratios roots differentiated from the callus with a frequency of up to 80%, while at low auxin to cytokinin ratios shoots differentiated from the callus with a frequency of up to 40%. During the first 2-3 months in culture calluses showing differentiation bore either shoots or roots but never both. Shoots obtained from the calluses have been rooted on a modified Morel's medium with IBA, but considerable more research will be required to develop a practical cloning system for sweetgum.

More recently sporadic somatic embryogenesis has been achieved by Dr. Harry Sommer in a few cell suspension cultures of sweetgum which may well be a major step toward developing a practical system of mass cloning this species as well as other difficult to propagate forest trees.

Publications (Research Program A - Brown and Steinbeck)

Brown, C. L. 1976. Forests as energy sources in the year 2000: What man can imagine, man can do. Jour. For. 74:7-12.

Steinbeck, K. and C. L. Brown. 1976. Yields and utilization of hardwood fiber grown on short rotations. Appl. Polymer Sym. #28,. 393-401.

Blum, R. B. 1978. Carbohydrate levels in sycamore coppice roots over a seven month period. MS Thesis, University of Georgia, 68pp.

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Sommer, H. E. and C. L. Brown. 1979. Application of tissue culture to forest tree improvement. In: Eds. W. R. Sharp, P. Larsen, E. Paddock, and V. Raghavan. Plant Cell and Tissue Culture Principles and Application. Chapter 25: pp. 461-491. Ohio State University Press. Columbus.

Research Program B: Physiology of paraquat induced oleoresin biogenesis.

Investigator: Claud L. Brown

Scope of investigation over 3 year period

Initially this program was conceived to study the physiological basis of enhanced oleoresin formation in southern pines when treated with sub-lethal concentrations of the herbicide paraquat (1,1' dimethyl-4,4' bipyridylium dichloride). The bipyridylium ion was shown to increase oleoresin and pitch soaking in boles of slash and longleaf pines by as much as 10-15 fold by USDA-USFS workers at the Olustee Naval Stores Laboratory, Olustee, Florida in 1972. This highly significant induction of oleoresin by controlled chemical application in pine forests of the Southeast could comprise a vast potential in our overall energy budget by (1) supplying increased renewable sources of organic substances for diversified uses in the resin and plastic industries thereby freeing up petroleum products for more critical demands and uses and (2) possible conversion of mono- and di-terpenes into direct sources of liquid fuels. Because of present economics in conversion to fuels the first option appears to hold more promise than the latter one.

The overall objectives of this program (Program B) were to (1) develop an *in vitro* culture system for studying certain physiological parameters of oleoresin formation under controlled laboratory conditions; (2) study the effects of paraquat on increased oleoresin biosynthesis in field-grown trees with emphasis on uptake, translocation,

mobilization, and possible sources of carbon utilized in the process of resin soaking; and (3) observe histological and cytological changes induced by the paraquat ion at the light microscope and ultrastructural levels. In addition, this program supplied necessary cell suspensions and other plant materials required for certain phases of research under Program C (The biochemical basis of paraquat induced oleoresin production in pines) conducted by Dr. W. R. Finnerty. Substantial progress and contributions were made to each of the categories enumerated above and in the best interest of the overall ERDA-DOE funded research at Georgia, Programs B and C (Brown and Finnerty) were combined during the second year with each investigator still carrying out this research in his own area of expertise.

Significant Results - Program B

1. A reliable and productive system was developed for batch culture of clonal lines of slash and longleaf pines in suspension culture on chemically defined media for use in physiological, biochemical and ultrastructure studies in both programs (B and C).
2. It was shown that paraquat at sub-lethal levels exerts a pronounced inhibition on cell division, increases respiratory rates, and causes a decrease in fatty acids, soluble and total protein is pine suspension cultures at concentrations of 0.1 - 1.0 mg/l.
3. Cytological observations also show evidence of accelerated aging, loss of membrane integrity and death corresponding to levels of paraquat used and length of time (3 to 30 days) in culture.
4. At the ultrastructure level destructive effects of paraquat on vacuolar and organelle membranes and cytoplasm are observable in one

day in cells supplied with 10.0 mg/l. At 1.0 mg/l paraquat the effects are not as drastic but the cultures continually decline undergoing senescence and death at 14 days.

5. Unfortunately, paraquat-treated pine cells in liquid culture, do not synthesize sufficient oleoresin to be quantified in batch cultures, probably because of its profound deleterious effect on membrane permeability and subsequent loss of precursors into cellular free space and into the liquid substrate bathing individual cells and/or clumps of cells. Because of this fortuitious result it was necessary to turn to the use of intact twigs or stems of young trees for other physiological studies.

6. Paraquat fed into terminal leaders of 5-year-old slash pine trees through lower severed lateral branches (10 ml of 200 mg/l) show weekly decreases in starch levels and a corresponding increase in content of oleoresin using standard histochemical techniques for light microscopy. Quantitative analyses of extractives from treated shoots indicate a decrease in starch and accompanying increases in fatty acids, monoterpenes, and resin acids.

7. In studies using 25-year-old slash pine trees, judicious blocking of downward translocation of current photosynthate by imposing deep transverse saw-cuts at 1.0 and 0.25 meters above paraquat treated xylem faces did not prevent resin soaking of the xylem at and above the treatment site indicating that at least a portion of the carbon being mobilized by paraquat affected cells comes from previously formed, reserve foods (starch, sugars, possibly amino and fatty acids, or other sources) and not altogether from current photosynthate.

8. The above hypothesis was strengthened by subsequent defoliation experiments of young paraquat treated and control (water treated) trees. Defoliation of 7-year-old trees followed by paraquat application to the bole significantly reduces the amount of oleoresin produced at and above the treatment site; however, the completely defoliated trees still produced over 50 per cent as much oleoresin as the treated foliated controls during the 5 month period following treatment.

9. These observations along with light and electron microscope studies of paraquat treated trees indicate that reserve foods contribute a significant amount of carbon precursors utilized in paraquat induced oleoresin formation.

10. In additional cytological and ultrastructural studies of paraquat treated slash pine stems it was shown that all cells of the xylem symplast including those not directly associated with resin ducts begin to synthesize excessive oleoresin and secrete or lose it initially through pits into the lumen of contiguous tracheids.

11. At the ultrastructual level oleoresin is observed in plastids, mitochondrial envelopes, vacuoles, and ER cisternae in both control and treated trees. Cells near the site of paraquat treatment show extreme damage to membrane integrity, depletion of starch, and loss of cellular organization.

12. The transport of oleoresin across organelle and plasma membranes does not appear to be achieved by transporting vesicles or by a mechanism of membrane exocytosis. From these studies it appears that oleoresin, a lipophilic substance, could be synthesized at a particular site such as the ER cisternae and by random movement come in contact

with other organelles where it could move across membranes and appear compartmentalized. Although this is an alternative to the concept of synthesis by various organelles, this possibility has not been ascertained with certainty.

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Summary of Studies Related to Paraquat

Associated Resin Acid Synthesis in Pinus elliottii.

Data collected from both field and laboratory studies indicate that the soluble metabolites; carbohydrates, keto acids, amino acids, and peroxyacids are mobilized when tissues of P. elliottii are injured. The data indicate little qualitative or quantitative difference between control and paraquat-treated tissue mascerates for periods up to 48 hours. Data presented in the 1976 ERDA Progress Report indicated that cut controls and paraquat-treated intact field grown 5-year old trees responded essentially the same for periods up to 28 days. Non-cut controls were qualitatively and quantitatively different from the injured trees. Seasonal fluctuations observed in untreated 5-year old pine trees indicate that comparable mobilization of carbon accompanies resin acid synthesis under natural conditions. The most pronounced differences between cut and paraquat-treated tissues was the apparent inability of paraquat-treated material to regulate amino acid metabolism over the long term. In composite, these data would indicate a mobilization of starch with accompanying increased pool sizes of reducing sugars, keto acids, and amino acids which appear to be available for resin acid synthesis. Paraquat treatment did not alter the percent composition of individual resin acids. Thus, paraquat apparently effected the rate of resin acid synthesis, but did not appear to alter the normal biosynthetic pathway for resin acid synthesis.

Data reported by Wolter at the 1976 and 1977 Lightwood Research Council could explain the comparable initial response of cut and paraquat-treated tissues. Increased ethylene production accompanied injury. Both Wolter and Peters reported data indicating that ethylene treatment induced increased accumulation of resin acids in pines. The differences apparent in paraquat-treated materials at longer time periods could be a result of the herbicide action. A representative of the Imperial Chemical Company reported that increased resin acid synthesis was associated only with compounds exhibiting herbicidal activity.

Paraquat may effect both the rate and amount of monoterpenes produced by P. elliottii; Proceeding LRCC (1976,1977). Loss of large amounts of radioactivity by volatization following paraquat treatment could be indicative of loss of monoterpenes.

This loss of radioactivity and the recovery of isotope and terpenes in diethylether extracts of the incubation medium indicate the importance of isotope and mass balances to experiments of this type. In longer experiments the loss of this material would constitute the greater amounts of both volatile materials and resin acids.

SEASONAL VARIATION OF RESIN ACIDS AND OTHER METABOLITES IN PINUS ELLIOTTII

W. R. Finnerty and Pat C. Kerr[1]

ABSTRACT

Seasonal fluctuations in pool sizes of peroxyacids, reducing sugars, keto acids, amino acids, and total and individual resin acids were observed in Pinus elliottii (slash pine). Generally, pool size of the soluble metabolite are maximal in winter and spring prior to the initiation of rapid vegetative growth. Pools are reduced during the growth period, and synthesis and accumulation of total resin acid increased after cessation of rapid growth. Resin acid composition appeared to fluctuate and guttation is postulated as a possible mechanism to explain the variation in total resin acid concentration.

INTRODUCTION

Diel and/or seasonal fluctuations of metabolic pool sizes can be of upmost importance in interpretation of data from many physiological studies. Seasonal fluctuations of several classes of compounds; peroxyacids, reducing sugars, keto acids, amino acids, and resin acids in Pinus elliottii Engelm. var. elliottii (slash pine) were observed incidental to a study of the effect of paraquat (methyl viologen), on terpene synthesis. Such base line data appear critical for accurately assessing specific treatment regimes in content of optimizing product yield as well as affecting data interpretation as to whether a treatment (in this case, paraquat) is reproducible in magnitude as well as season.

METHODS

Bark, phloem, and xylem tissues from node and stem sections of terminal leaders of 5-year old slash pine (P. elliottii Engelm. var. elliottii) were analyzed monthly to ascertain seasonal variation in soluble metabolites and resin acids. The tissues were minced and extracted with boiling 70% ethanol and water or with chloroform:methanol (2:1). The ethanol-water extracts were assayed for peroxyacids as thiobarbituric acid-positive reacing material (Heath and Packer, 1968), amino acids as ninhydrin-positive reacting material (Rosen, 1957), carbohydrate as free reducing sugars by the anthrone procedure

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(Ashwell, 1957), and keto acids as the 2,4-dinitrophenylhydrozenes) (Friedemann, 1957). The chloroform:methanol extracts were methylated with "Methyl 8" (Pierce Chemical Company) and the methylated resin acids were determined by gas chromatography on a 6 ft x 1/8" glass column of 5% DEGS-PS at 200°C.

RESULTS

Seasonal fluctuations in slash pine were observed in a number of biochemical parameters as well as total and individual resin acids. Quantitative differences in total TBA-positive materials, carbohydrates, keto acids, amino acids, and total resin acids as well as individual resin acids were observed in needles, bark-phloem, and xylem tissues of node and stem sections at given sampling periods. To simplify the graph, data for needles are not presented in Figs. 1-5. Quantitatively, needles contained 2-3 times more soluble products than xylem tissue, but the seasonal fluctuation of materials in needles was similar to that observed in xylem.

Total TBA-positive materials (Fig. 1), carbohydrate (Fig. 2), keto acids (Fig. 3), and amino acids (Fig. 4), were generally higher per unit weight of tissue in winter and early spring. TBA-positive material (Fig. 1) was observed to increase in January and was maximal (15-16 units/gm wet wt) at the January sampling period. Values generally remained high (10-11 units/gm wet wt) through the April sampling period and decreased to 4-5 units/gm wet wt during May, June and July.

Free carbohydrates (Fig. 2) increased approximately 10-fold in bark-phloem and xylem tissues of both node and stem sections from the November to the January sampling periods (Fig. 2). Maximum values of 100-115 mg/gm wet wt observed during January and February decreased to approximately 45 mg/gm wet wt in bark-phloem tissue at the March sampling period. The downward trend continued through April to low values of 10-20 mg/gm observed from May through November. The bark-phloem contained approximately 10 times more carbohydrate than the xylem. Maximum values of 10 mg/gm observed from January through March decreased to 4 mg/gm during April. Values of 1-3 mg/gm were observed from May through November.

Keto acid (Fig. 3) content in the bark-phloem sample increased 3-fold from November to January-February. Maximum values of 20-24 units/gm wet wt observed during March decreased to 5 units/gm during April. Values varying between 1-5 units/gm were observed May through November. Xylem tissues contained 10-20 times less keto acid (0.1 to 1.0 units/gm wet wt) than bark-phloem. Maximum values of 1-1.5 units/gm were observed in March with a decrease to 0.2 units/gm in April.

Amino acid (Fig. 4) content of bark-phloem and xylem tissues appeared to be biphasic with increased concentrations observed from June through October and from January through March. The absolute maxima observed in the two tissues occurred at different times during the year; i.e., bark-phloem maximum occurred in winter-spring (February, March, April) while the maximum for xylem tissue occurred in summer-fall (June, July, September, October).

Although the maximum observed concentration of total amino acids (1100-1150 µg/gm was the same in bark-phloem tissues of node and stem sections, this

value was observed one month earlier in the stem section (February) and persisted through the March sampling period. Amino acid content decreased in both the node and stem sections from March to the June sampling period (node 1150 to 450 µg/gm; stem 1150 to 250 µg/gm). Increased values from 600 (node) to 800 (stem) µg/gm were observed from July to the October sampling period.

Amino acid concentration observed in xylem tissue from node sections was approximately 2.5 to 10-fold less than that observed in bark-phloem tissue. This same differential was apparent in xylem tissue obtained from stem sections with the exception of the June sampling period when 10-fold (2600 µg/gm) more amino acid content was observed in the xylem sample than in the bark-phloem sample.

The concentration of total resin acid (Figs. 5,6) exhibited biphasic maxima in January-February samples (1.5-2.5 mg/gm) and May-June samples (2.2-4.5 mg/gm) in both bark-phloem and xylem tissues of node and stem sections. Both the rate and magnitude of fluctuation in total resin acid concentration was greatest in tissues of the stem section. The seasonal fluctuation observed in xylem tissues generally are similar in node and stem sections (decreases from January through March-April flowed by increased through May-June), but the data indicate accumulation of resin acids in the node bark-phloem during summer-early fall concomitant with a decreasing concentration in the stem bark-phloem. Table 1 is a tabulation of resin acid fluctuation observed over the entire period of study.

The concentration of individual resin acids (Figs. 6,7) differed with the tissue at given sampling periods and within the same tissue at different sampling times. In general, pimaric (Fig. 7) and sandaracopimaric acids (Fig. 6) were most abundant during winter and spring in all of the tissues. Palustric acid (Fig. 6) was highest in the needles during summer, in the phloem in January and April, and in the xylem during the winter. Isopimaric acid (Fig. 6) concentration was highest in xylem and phloem during the spring-summer, but highest in the needles during winter-spring. Abietic acid (Fig. 7) concentrations exhibited less seasonal fluctuation than the other resin acids. Concentrations were most constant (17-30%) in the xylem with the maxima occurring in the needles during winter-spring and in the phloem during spring-summer. Dehydroabietic acid (Fig. 7) was highest in needles during the summer-fall season, in phloem during the winter season, and in the xylem during the summer season.

DISCUSSION

Data collected in this study indicate all measured parameters exhibit seasonal fluctuation. Maximum concentration of soluble metabolites was observed in winter and early spring (Figs. 1-4) before the onset of vegetative growth observed in late March and April. Rapid and extensive vegetative growth ceased in late June-early July. Resin acid accumulation generally was higher in late spring and summer (Figs. 5,6) coincident with and following the period of extensive vegetative growth. Accumulation of individual resin acids in different tissues did not follow the same seasonal pattern as total resin acid concentration (Figs. 6,7).

Some possible metabolic interaction may be indicated by these data. Concentration of soluble carbohydrate increased in January-February (Fig. 2) presumably due to starch degradation and increased photosynthesis. Soluble reducing sugars decreased rapidly and extensively from February to March (just prior to initiation of vegetative growth). Maximum concentration of keto acids was observed in March (Fig. 3); i.e., concomitant with the period of carbohydrate depletion. Soluble amino acids (Fig. 4) also exhibited a maximum concentration in March. This periodicity suggests a conversion of carbohydrate carbon through keto acids to amino acids.

In general, mass accumulation of amino acid carbon (Fig. 4) correlated with accumulation of total resin acid (Figs. 5,6). Total resin acid content also increased during late winter and spring just prior to rapid vegetative growth. TBA-positive material (Fig. 1) increased during January-April and decreased from April through July concomitant with the increased accumulation of total resin acids (Figs. 5,6). These same fluctuations in soluble metabolites were observed following paraquat treatment of slash pine (Finnerty et al., 1966) indicating that no new enzymatic pathways are induced following paraquat treatment.

Fluctuations of individual resin acids (Figs. 6,7) as well as the seasonal fluctuation of total resin acids observed in this study indicate that these compounds may not be static secondary metabolites. Total resin acid content also decreased during late winter and spring just prior to rapid vegetative growth. Metabolic studies performed with tissue slices in this laboratory (unpublished data) indicate that resin acid synthesis was minimal prior to and during the period of rapid vegetative growth. While mobilization and utilization of resin acid carbon in the grwoth process could explain the decreased pool size of resin acid, guttation of resin acid was very apparent during late winter and early spring. (This period coincides with the time that the Gum Naval Stores Industry collected sap.) comparable reductions in monoterpene accumulation and synthesis have been reported in mint by Loomis (1967). Globules of gummy material could be removed from needles and stems of 5-year old slash pine, and exudate could be collected easily from pores on the stems of lateral branches as well as boles. Chromatography of these materials in neutral lipid solvents on silica gel G plates indicated the presence of resin acids as well as many more neutral lipids (mono-, di-, triglycerides, free fatty acids, wax esters, etc.). Materials remaining at the origin after development were assumed to be polar lipids. Thus, pine guttation fluid may be similar to the latex of rubber plants which contain large amounts of terpenoid compounds.

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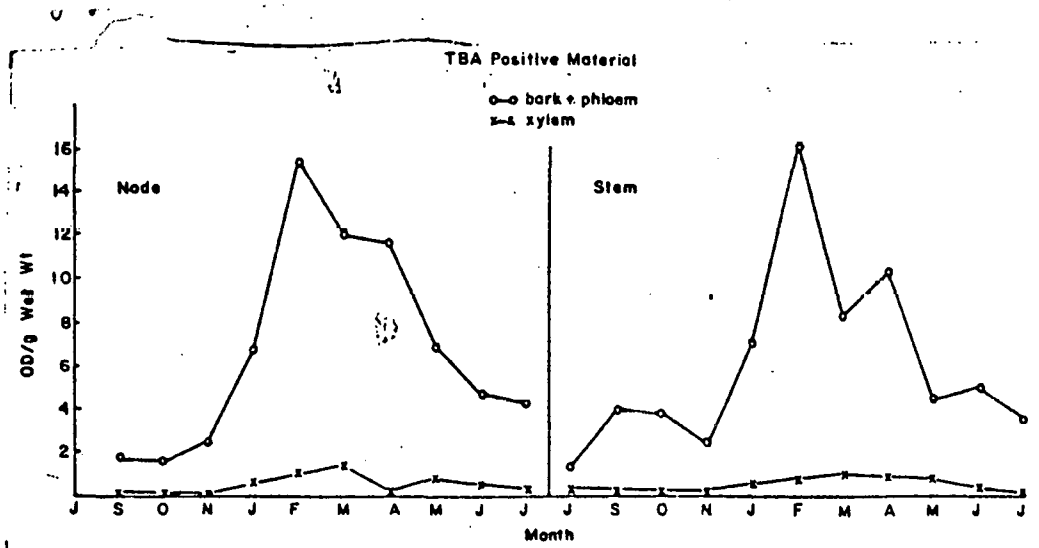

Figure 1.
Variation of thiobarbituric acid positive material in slash pine tissues.

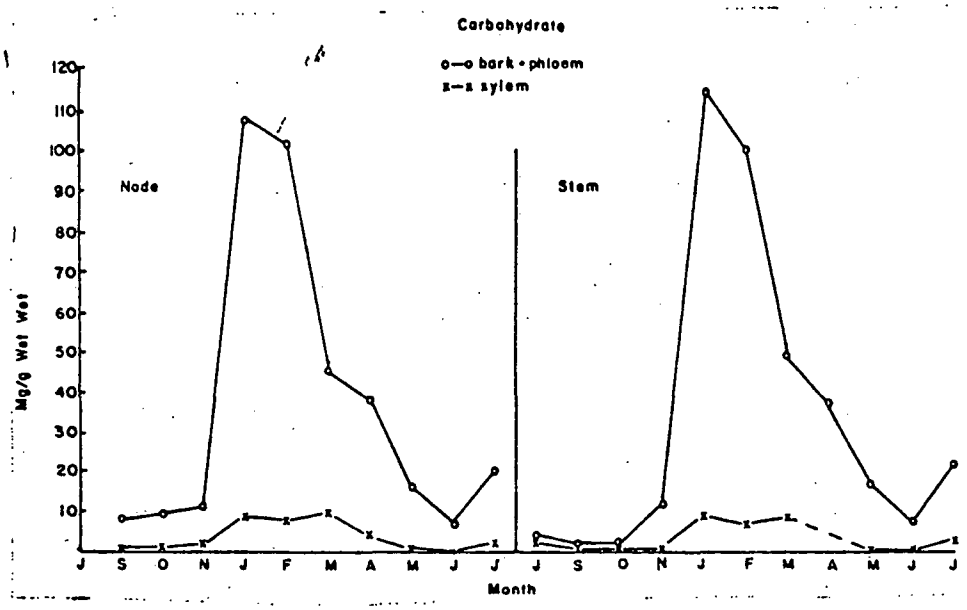

Figure 2.
Variation of free carbohydrate in slash pine tissues.

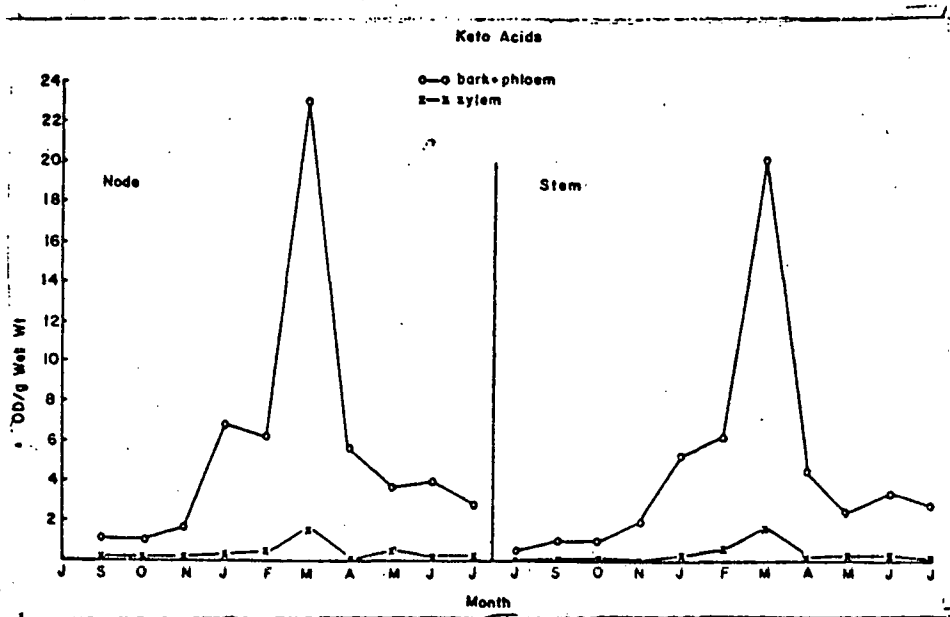

Figure 3.
Variation of keto-acids in slash pine tissues

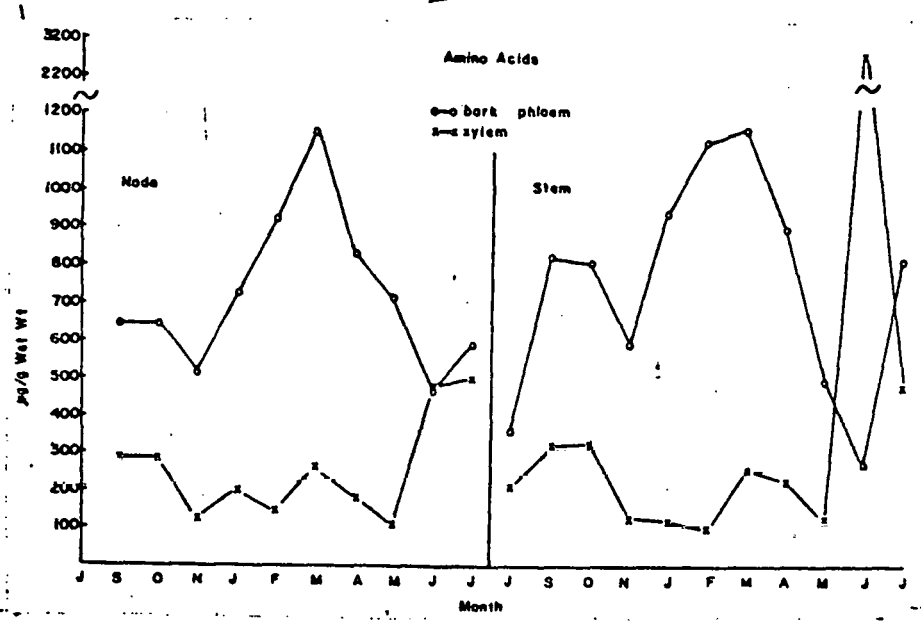

Figure 4.
Variation of amino acids in slash pine tissues.

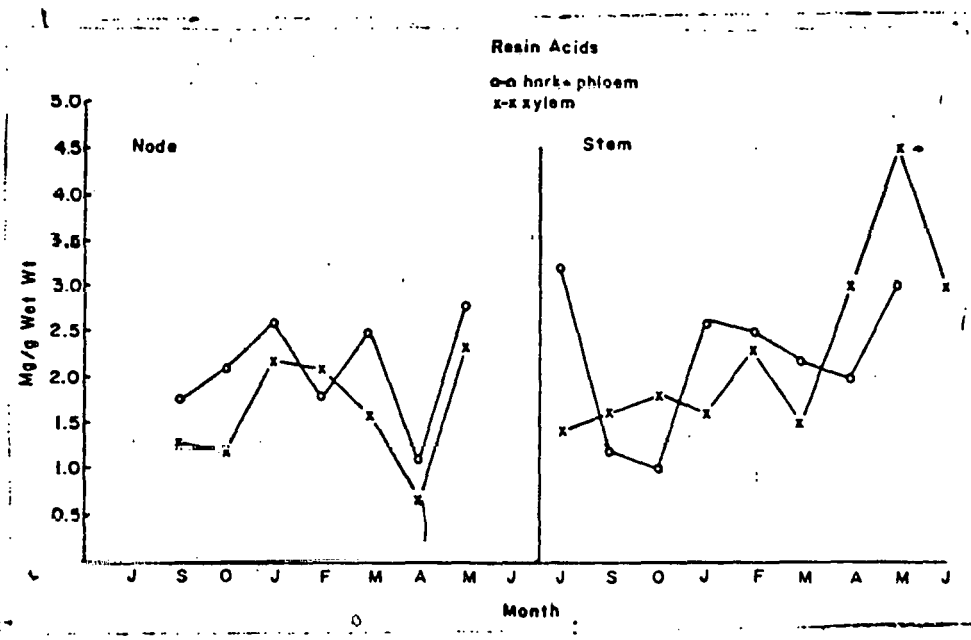

Figure 5.
Variation of resin acids in slash pine tissues.

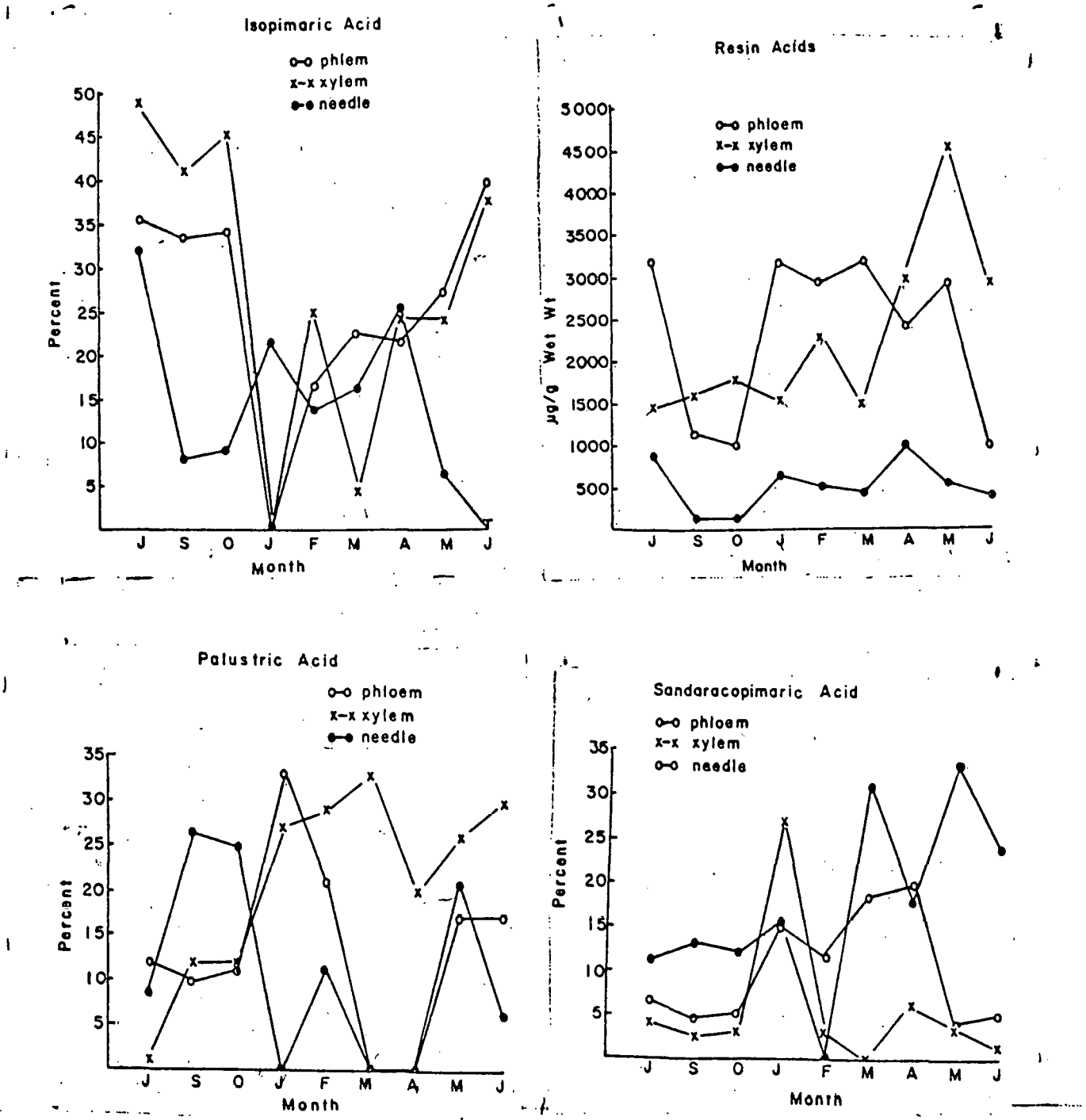

Figure 6.
Variation of isopimaric, sandaracopinaric, palustric, and total resin acids in stem tissue of slash pine.

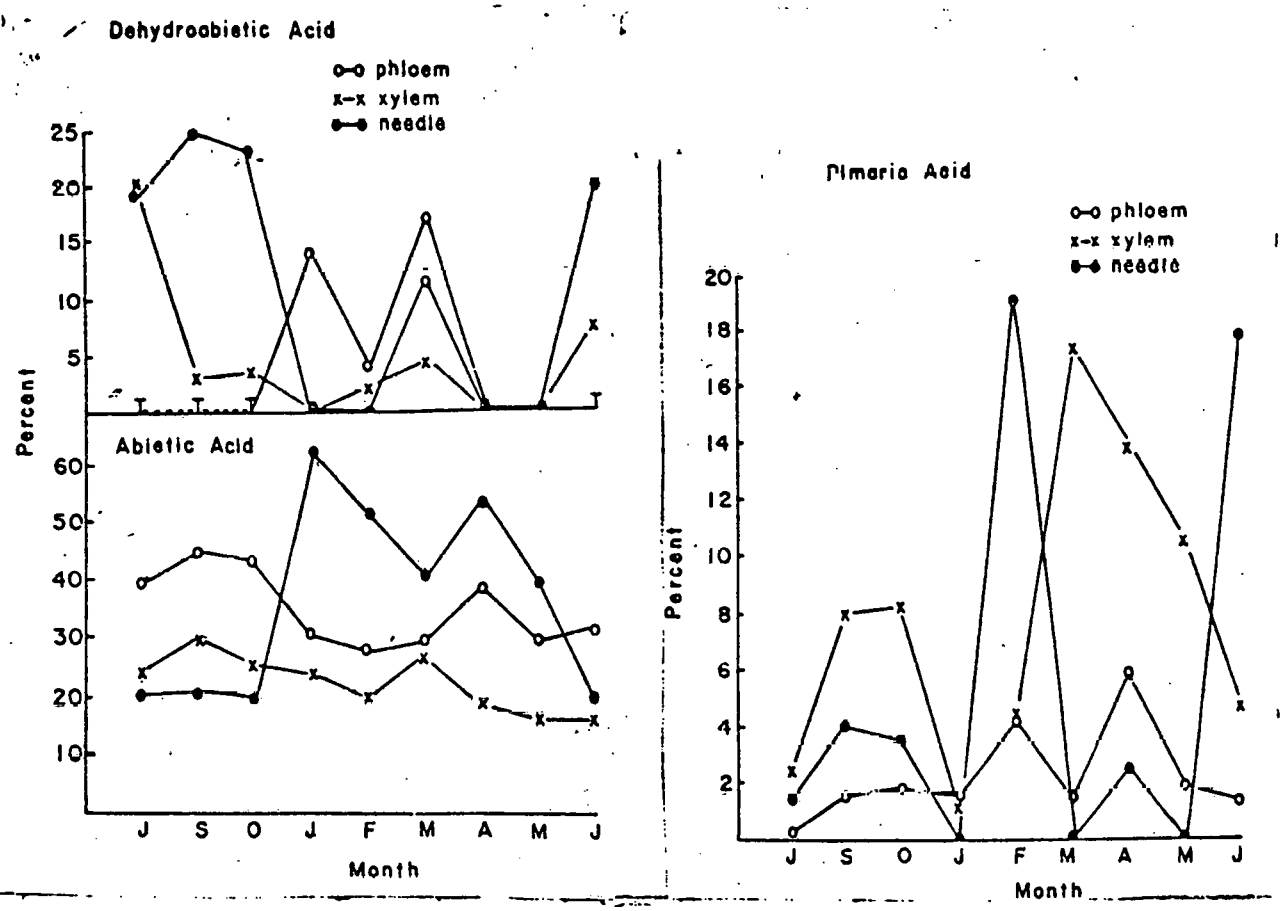

Figure 7.
Variation of abietic, dehydroabietic and pimaric acids in stem tissue of slash pine.

TABLE 1

Seasonality of Resin Acid Content

	RESIN ACIDS (mg/g fresh tissue weight)																			
	1975			1976							1977*									
TISSUE	7	9	10	1	2	3	4	5	6	7	2/7	2/10	2/21	2/28	4/1	4/12	4/28	5/9	5/17	6/15	8/29
phloem	3.2	1.2	1.0	3.2	3.0	3.2	2.4	3.0	11.0	31.7	8.9	16.9	2.5	12.6	1.0	0.94	0.98	0.90	3.0		
																				100.6+	16.8
xylem	1.4	1.6	1.8	1.6	2.3	1.5	3.0	4.5	31.0	65.4	13.3	15.6	16.6	12.5	9.5	5.7	1.8	1.2	7.5		
	4.6	2.8	2.8	4.8	5.3	4.7	5.4	7.5	42.0	97.1	22.2	32.5	19.1	25.1	10.1	6.6	2.7	2.1	10.5	100.6+	16.8

BIOCHEMICAL CHANGES IN PARAQUAT-TREATED STEMS OF SLASH PINES

W. R. Finnerty, P. C. Kerr, R. A. Makula,
Claud Brown and Terry Clausen

ABSTRACT

Slash pine was examined in context of the specific metabolic changes occurring following the in vivo application of paraquat. Increased amounts of resin acids, monoterpenes, amino acids, keto acids, and thiobarbituric acid-positive substances were determined with concomitant decreases in starch and soluble carbohydrate. A slight increase in total phospholipid content as well as a transient increase in total fatty acid occurred following paraquat treatment.

INTRODUCTION

The application of paraquat to pines was observed to induce significant increases in the amount of oleoresin produced during a specified growing season (Roberts, 1973). The biochemical basis for this increased oleoresin biogenesis in pines has yet to be determined. A variety of physiological and biochemical effects have been described following the treatment of various tissue with paraquat. Harris and Dodge (1972) have described the generalized disorganization of membrane integrity and loss of cellular lipid in flax cotylelon leaves treated with paraquat. Baker and Wilson (1966) demonstrated the degradation of long-chain polyunsaturated fatty acid hydroperoxides to malondialdehyde. This report describes biochemical changes that occur in slash pine stems following paraquat treatment.

MATERIALS AND METHODS

Twenty-four, five-year-old slash pine trees growing in a plantation at 8'x10' spacings were selected for treatment during late July. Trees of comparable size and vigor, possessing heatlhy terminal leaders approximately 24 inches in length subtended by several strong lateral branches were randomly assigned to paraquat and control treatments.

Treated trees were fed 10 ml of 0.02 percent (2 mg) paraquat solution through a severed lateral branch at the base of the terminal leader through latex tubing fitted to a graduated 10 ml pipette. The pipette was attached temporarily to the terminal leader vertically above the severed branch to permit gravity flow of solution for uptake into the transpiration stream. The lateral branches were severed early in the morning to allow exudation of

oleoresin, and recut at the time of fitting the latex tubes to prevent pluging of the cut xylem surface with exuded resin. After all trees were prepared, the pipettes attached to 12 trees were filled with 10 mls of paraquat. Normally all of the solution would be taken up into the transpiration stream of the leader within 6-8 hours. Non-cut and cut (fed water only) trees were used as controls in this study. Following treatment, the terminal leaders of 3 treated and control trees were harvested at the specified times.

In early September, the same technique was used to treat trees with 20 mg of paraquat. Stems from both studies were excised, minced and extracted with boiling 70% ethanol or with chloroform:methanol (2:1, v/v). The ethanol extracts were assayed for perotyacids as thiobarbituric acid-positive reacting material (Heath and Packer, 1968), amino acids as ninhydrin-positive material (Rosen, 1957) reducing sugars as anthrone-positive materials (Ashwell, 1950), and keto-acids as 2-4-dinitrophenylhydrazine-positive materials (Friedemann, 1957). The chloroform-soluble lipid was analyzed by silica gel G thin-layer chromatography in a solvent system consisting of petroleum ehter:diethylether:glacial acetic acid (80:20:1, v/v/v). The resin acids and free fatty acids were removed from the silica gel, methylated with "Methyl 8" and analyzed by gas chromatography. Resin acids were analyzed by ASTM method D3008-72 using methyl benhemic acid as the internal standard. Fatty acids were analyzed using a SP-222-PS column (Supelco, Inc.) with methyl heptadecanoic as the internal standard. Monoterpenes were extracted in closed flasks 4 times with 30 ml of anhydrous diethyl ether with vigorous shaking. Monoterpenes were separated on a Carbowax 20M column; quantification was by external standard for each monoterpene. The total chloroform-soluble lipid was saponified by refluxing in 10% methanolic-KOH, methylated, and analyzed by gas chromatography. Phospholipids were quantitated by the method of Bartlett as quoted by Dittmer and Wells (1969).

Values as reported represent averages of at least 3 trees per time point. While tree-to-tree variation of all measured parameters was observed, overlapping standard deviations were observed only at day 7 in monoterpenes and resin acids and at those times that the plotted data would indicate no differences between control and treated trees.

RESULTS

Basic physiological changes resulting from the application of paraquat to slash pine were examined in context of the quantitative changes occurring in starch, total soluble carbohydrate, amino acids, keto acids, fatty acid peroxidation, phospholipid, fatty acid, resin acid, and monoterpenes, respectively.

Differences in both magnitude and rate of disappearance of soluble carbohydrate were observed following the application of both 2 and 20 mg of paraquat (Fig. 1B,2B). Within 24 hours anthrone positive material had increased approximately 1.5 times in paraquat-treated tissue and then decreased 5-7 fold by the 48-hour sampling period. Carbohydrate levels in control plants decreased over a 96-hour period following paraquat application. Carbohydrate levels in treated and control plants were essentially identical from 4-28 days.

Changes in keto acid content were observed in tissues treated with 2 and 20 mg paraquat, but the rates of change were different (Fig. 1C,2C). Hydrazone-positive material was double that of the zero time control (1.7 to 3.8 units/gm wet wgt) in tissue treated with 20 mg of paraquat at the end of 24 hours (Fig. 2C). This value was maintained to 48 hours, and had declined by 96 hours to 1.8 units. This value remained constant from 4-14 days. Fluctuations in the controls varied from 1.7 to 3.1 units/gm during the 14-day study. In the 2 mg paraquat study, no difference between control and treated tissues were observed until 7 days after treatment (Fig. 1C). Five times more keto acids (1 unit/gm vs 0.2 unit/gm) were observed in treated tissues at seven days and continued to increase linearly throughout the 28-day experiment with a maximum of 8.1 units/gm (control value 1.5 units/gm). On day 28 control values fluctuated from 0.2 to 1.5 units/gm during the 28-day experiment.

Rates of change of ninhydrin-positive materials were different in tissues treated with 2 and 20 mg paraquat. After treatment with 20 mg paraquat, amino acid content increased 4-fold from the zero time control (0.5 to 2.3 mg/gm wet wgt) at the 24-hour sampling period. Maximal amino acid content (4.1 mg/gm) was observed at 48 hours and remained essentially constant through day 7. Amino acid content of control and treated tissues were the same (2 and 2.4 mg/gm) at day 14. Control values fluctuated from 0.5 to 2.4 mg/gm during the 14 day experiment. In the 2 mg paraquat study, no difference was observed until 4 days after treatment (Fig. 1D). Amino acid content doubled (3.25 vs 1.5 mg/gm) on day 4 and remained constant to day 7. Concentrations of 1.5 to 2.0 mg/gm were observed in treated tissue from day 14 to day 28. Control values were constant (1-1.8 mg amino acids/gm) throughout the 28 day study.

Rates of change of 2-thiobarbituric acid-positive material were different in tissues treated with 2 and 20 mg of paraquat (Fig. 1A,2A). Peroxyacid content increased 3 times over the zero time control (2.5 vs 8.5 units/gm) 24 hours after treatment with 20 mg paraquat (Fig. 2A). Concentrations of peroxyacids declined steadily from 4.6 units/gm on day 2 to 2.5 units/gm on day 14. Control values fluctuated from 2.5 units/gm at zero time to 4.2 units/gm on day 7 and 14. Three-fold increase in peroxyacid content (1.3 vs 3.5 units/gm) was observed 7 days after treatment with 2 mg paraquat (Fig. 1A). Peroxyacid content continued to increase to a maximum of 7.5 units/gm on day 28. Control values fluctuated from 0.7 units to 3 units/gm during the 28 day experiment.

Starch content was estimated only in the 2-mg paraquat study (Fig. 1E). Starch content in treated tissues was 1/2 that of control tissues on day 7 (1.55 vs 3.55 mg/gm wet wgt). Concentrations were similar in both tissues on day 14 (2.8 and 3.3 mg/gm in control and paraquat treated, respectively). Starch content was approximately 60% that of control tissues (2.5 vs 4.2 and 2.4 vs 3.5 mg/gm) on days 21 and 28.

Increased resin acids concentration was observed in tissues treated with 2 and 20 mg paraquat (Fig. 1F,2E). Fluctuations in resin acid concentration varied from 2 to 16 mg/gm tissue in the controls of the 2 mg paraquat experiment. Resin acid content of paraquat treated tissue was 6, 8, 3, and 9 times greater than that of the control on days 7, 14, 21 and 28, respectively (Fig. 1F). No linearity was observed in either the control or treated data.

Control values in the 20 mg paraquat experiment (Fig. 2E) varied from 1.5 mg/gm at zero time to 4.1 mg/gm on days 7 and 14. Resin acid concentration in paraquat-treated tissues was 10 and 20 mg/gm on days 7 and 14, respectively. Paraquat treatment did not alter the composition of the total resin acid pool, i.e., the percent distribution of individual resin acids was the same in treated and untreated trees (Table 1).

Monoterpene concentrations was estimated only in the 2 mg paraquat study (Fig. 1G). Control values varied from 3 to 8 mg/gm from day 7 to day 28. Monoterpene concentrations in paraquat-treated tissues was double that of the control on days 7, 14 and 21. Maximal concentrations of 72 mg monoterpene/gm was observed in treated material on day 28. This value was 9 times that of the control.

Thin-layer chromatography of the non-polar lipid fraction of slash pine revealed a complex lipid composition. The solvent system employed allowed for the co-migration of free resin acid and free fatty acid (R_f = 0.35). The quantitation of the polar lipid fraction obtained from paraquat-treated stems are compared to control stems (Table 2). The amount of polar lipid appeared to be generally increased in paraquat-treated stems after 4 days.

Total fatty acids were obtained by saponification of the crude lipid extract, methylated and analyzed by gas chromatography. The control samples contained low amounts of fatty acids (Table 3). An increase in total fatty acid was observed in 7 day paraquat-treated stems. At the 14-day sampling total fatty acid decreased. The free fatty acid was separated from the total extractable lipid by thin-layer chromatography. The amount of free fatty acid comprised less than 0.05% of the total fatty acid in paraquat-treated stems.

DISCUSSION

When compared to uncut control trees, the metabolic patterns observed in these studies were similar at both treatment levels, i.e., transitatory increase of soluble carbohydrate, followed by depletion of this pool associated with increased amounts of peroxy-, keto- and amino acids. The rate of change of the soluble carbohydrate pool appeared the same at both treatment levels (Fig. 1B,2B), while the rate of increase of peroxy- (Fig. 1A,2A), keto- (Fig. 1C,2C) and amino acids (Fig. 1D,2D) was faster with the 20 mg paraquat treatment. All 3 parameters had increased within 24 hours of treatment with 20 mg paraquat, but increased peroxy-, keto- and amino acids were observed 96 and 168 hours, respectively, after treatments with 2 mg paraquat.

Starch depletion following paraquat treatment has been documented chemically (Fig. 1E) and cytologically (Brown *et al.*, 1976), and could account for the increased soluble carbohydrate following paraquat treatment.

The source of the increased amino acid pools is unknown at this time. Decreased protein synthesis, increased protein hydrolysis, and/or conversion of carbohydrate or other soluble components into amino acids are possible pathways.

Protein synthesis appears to be inhibited when plant tissues undergo dessication (Dhindsa and Bewley, 1975). In general, paraquat treatment of *P. elliottii* tended to reduce the water content of affected areas; and could

thereby inhibit protein synthesis. Label from glycine-2-^{14}C and uniformly ^{14}C-labeled algal protein hydrolyzate was recovered in acid hydrolyzates of ethanol and water-insoluble plant residues. Radioactivity was recovered at the R_f of several common amino acids after development in butanol-acetic acid-water on Whatman #1 filter paper. These data could indicate that some protein synthesis was occurring in paraquat-treated tissue, but there was no attempt to estimate exchange.

Attempts to estimate proteolysis have been unsuccessful. Materials that interfere with the Lowry protein determination were extracted from the tissues with both 70% ethanol and water. Azoalbumin and several other artificial substrates cannot be used because colored compounds absorbing at the same wavelength as that of the artificial substrates were extracted from pine tissues by the buffers, salts, and solvents used for protein extraction.

Hydrolysis of starch to glucose and its conversions into amino acid carbon via keto acids is a possible pathway.

Keto acids (2,4-dinitrophenylhydrazine reactive materials) increased following paraquat-treatment *in vivo* (Fig. 1C,2C). Extracts from *in vitro* experiments were analyzed on a Beckman Amino Acid Analyzer (unpublished data). The qualitative and quantitative changes in amino acid composition and concentration following paraquat treatment are associated with those amino acids which can derive carbon from pyruvic acid.

TBA reactive materials are assumed to be related to the peroxidation of fatty acids (Baumgartner *et al.*, 1975; Baker and Wilson, 1966). TBA-reactive materials increased *in vivo* following paraquat-treatment (Fig. 1A,2A). Autooxidation of fatty acids and polyunsaturated esters produced compounds that induced both reversible and non-reversible biochemical and morphological damage (Baker and Wilson, 1966; Schausenstein, 1967). Inhibition of two SH-enzymes, glyceraldehydephosphate dehydrogenase and lactate dehydrogenase, by these peroxidation products were reported by Schausenstein (1967). Neither fructosediphosphate aldolase, hexokinase, or glucose-6-phosphate dehydrogenase were inhibited by the peroxidiation products. These data could indicate that specific blocks in carbohydrate metabolism could result from paraquat-treatment if peroxidation of acids occurred. Morphological changes were observed by Schausenstein (1967) as a result of peroxidationproducts, and Dodge and Harris (1972) reported extensive structural damage in flax cotyledons following paraquat treatment.

Brown *et al.* (1976) observed structural damage in paraquat-treated *P. elliottii*. Phospholipid analysis of control and paraquat-treated trees *in vivo* did not support the concept of extensive structural damage in these trees (Table 1). If extensive membrane destruction occurred, the phospholipid content would probably be much lower than that of the control.

Pool sizes of all measured parameters were generally lower in September than in July, reflecting the seasonal variation of metabolites observed in these studies. Even though the absolute amount of resin acids were 8 times lower in September, paraquat treatment (2 and 20 mg) was followed by a 12-fold increase in maximum measured resin acids (Fig. 1F,2E).

In summary, *in vivo* paraquat treatment of 5-year old pine trees resulted in apparent decreased water and starch content of affected tissues with increased amounts of amino acids, keto acids, and TBA-reactive materials in association

with increased oleoresin synthesis. The transitory increase in free reducing sugars, and maintenance of low pool size could indicate rapid turnover of soluble carbohydrate. Paraquat treatment of P. elliottii was associated with mobilization of storage compounds and apparently increased carbon flow into secondary metabolites. Carbon flow appears to be through soluble carbohydrates, keto acids, and amino acids into the structurally more complex terpenes.

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TABLE 2

Percent Distribution of each Resin Acid of Total Resin Acids Extracted from Paraquat-Treated and Control Tissue of Pinus elliottii.

	CONTROL		PARAQUAT	
RESIN ACID	mg	% of Total	mg	% of Total
Palustric	4.10	26.1	2.84	25.8
Isopi aric	5.24	33.3	3.69	33.5
Abietic	2.76	17.6	1.99	18.1
Dehydroabietic	1.22	7.8	0.82	7.4
Neoabietic	2.40	15.2	1.70	15.4
Total	15.72	100.1	11.04	100.2

Table 2.--Total phospholipid following paraquat treatment

Days	Control	Paraquat-treated
	µmoles Lipid phosphorus/gm (dry wt)	
1	1.1	1.0
2	0.9	0.9
4	2.0	3.3
7	2.0	2.4
14	1.7	2.0
21	1.5	2.8

Table 3.--Fatty acid and resin acid composition of slash pine

Sample	Total		Free	
	Fatty Acid	Resin Acid	Fatty Acid	Resin Acid
	mg/gm dry weight		µg/gm dry weight	
Control	6.48	4.16	5.63	161.00
July-Aug				
7 days	54.70	10.00	5.63	1214.31
14 days	18.64	20.34	2.00	3453.79
September				
7 days	44.11	3.36	43.14	454.00
14 days	23.00	95,00	30.00	4726.50

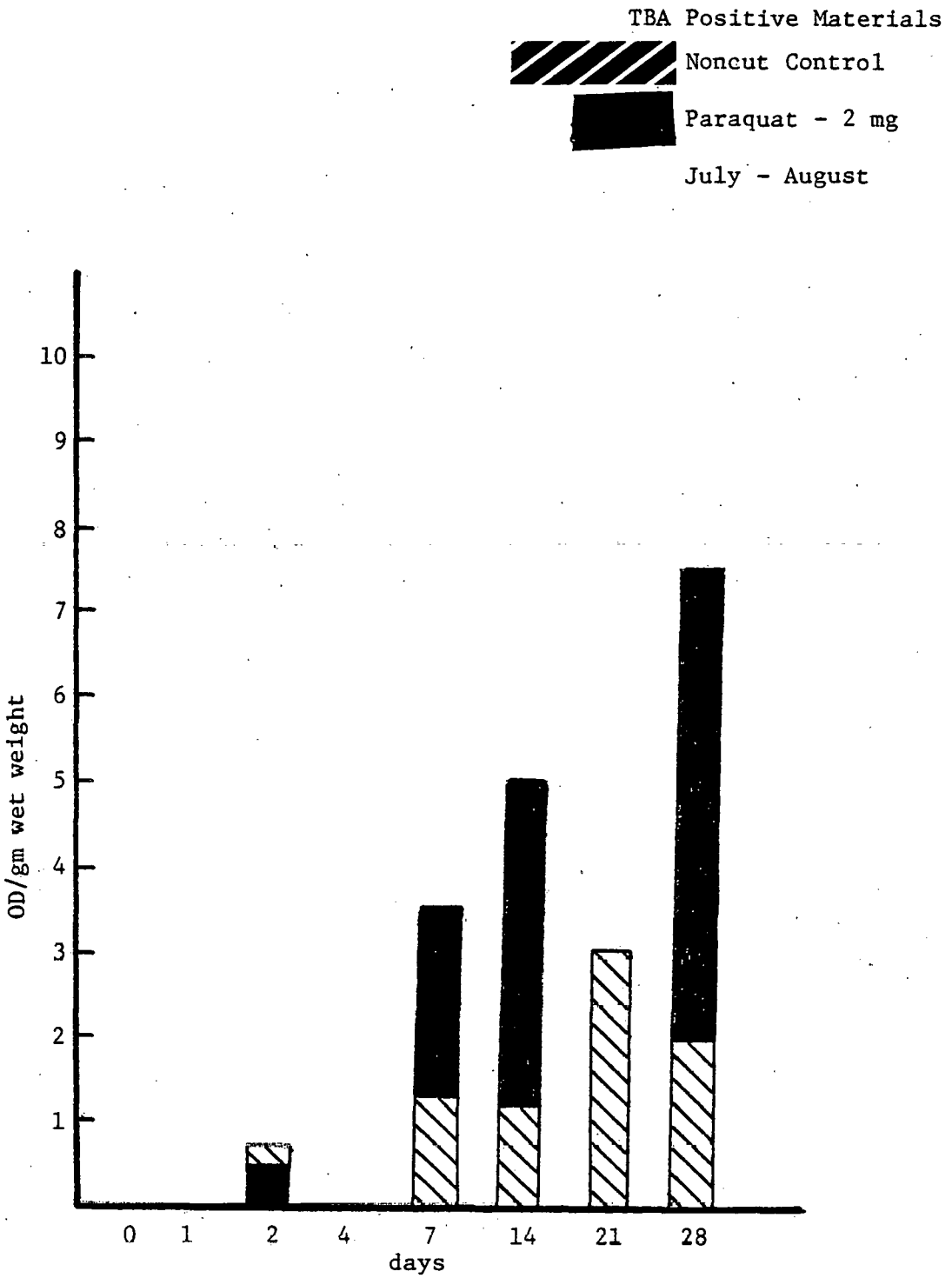
TBA Positive Materials
Noncut Control
Paraquat - 2 mg
July - August
OD/gm wet weight
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
21
28
days

Fig. 1A

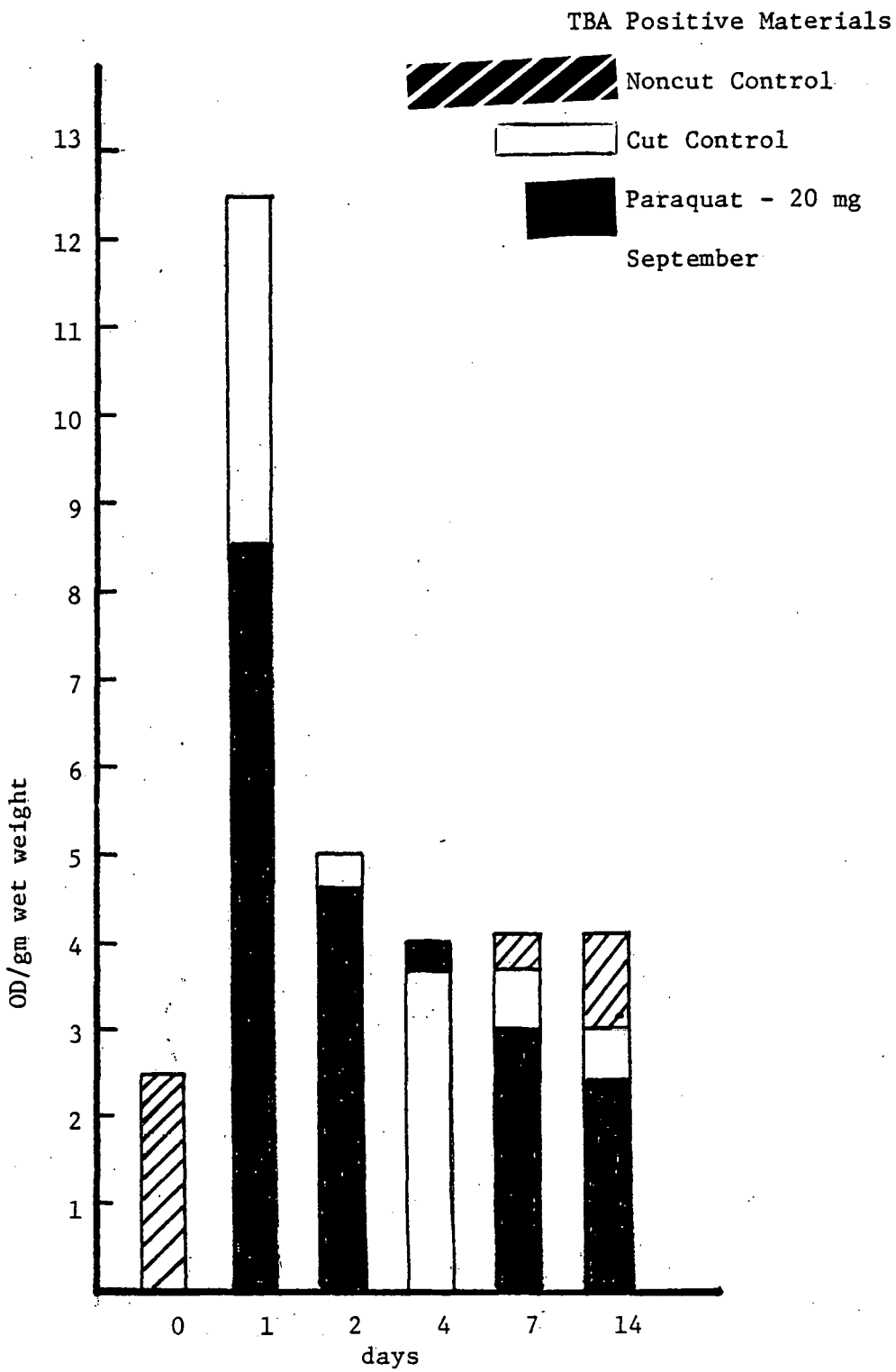
TBA Positive Materials
Noncut Control
Cut Control
Paraquat - 20 mg
September
OD/gm wet weight
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
days

Fig. 1B

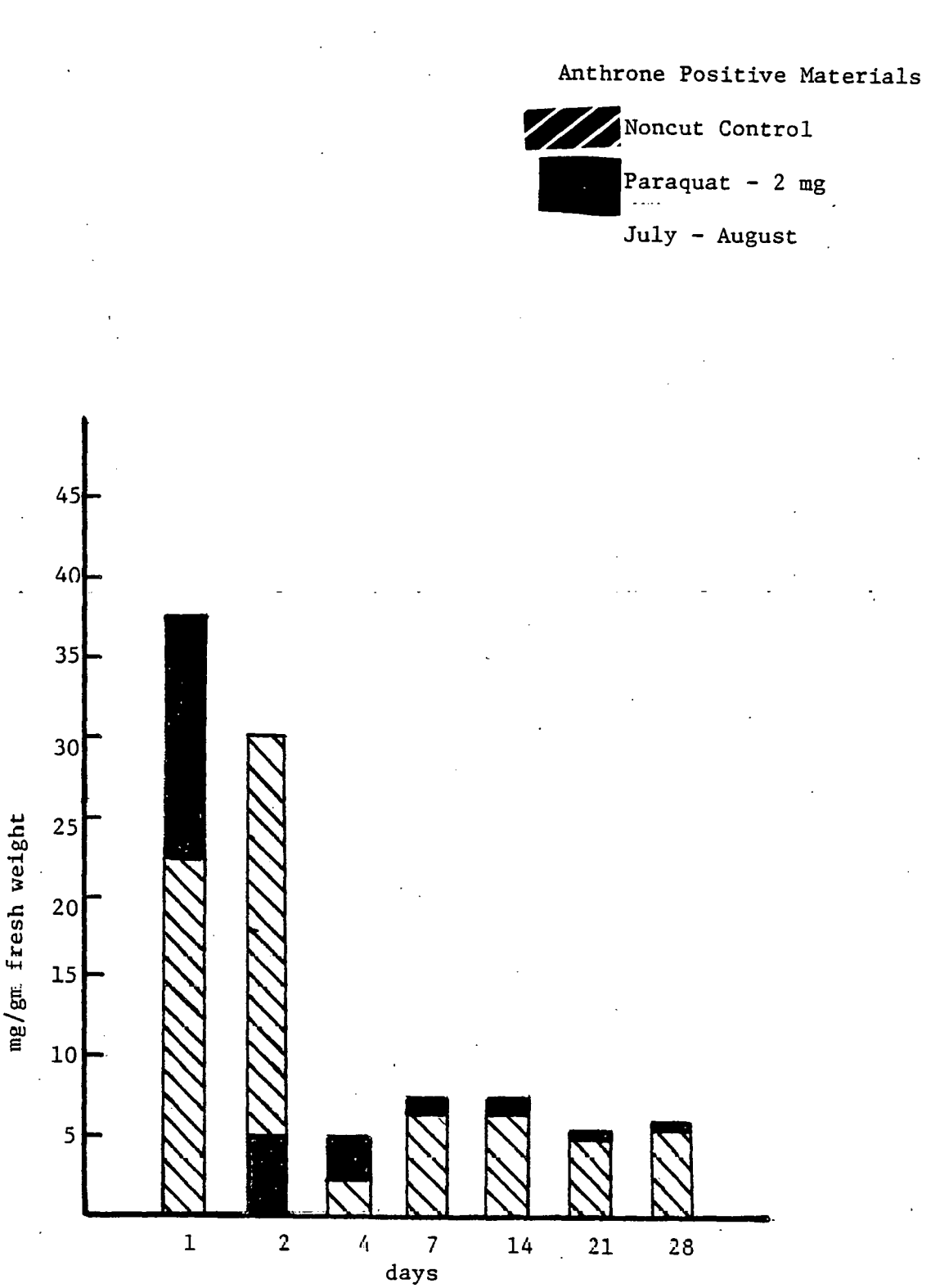
Anthrone Positive Materials
Noncut Control
Paraquat - 2 mg
July - August
mg/gm. fresh weight
45
40
35
30
25
20
15
10
5
1
2
4
7
14
21
28
days

Fig. 2A

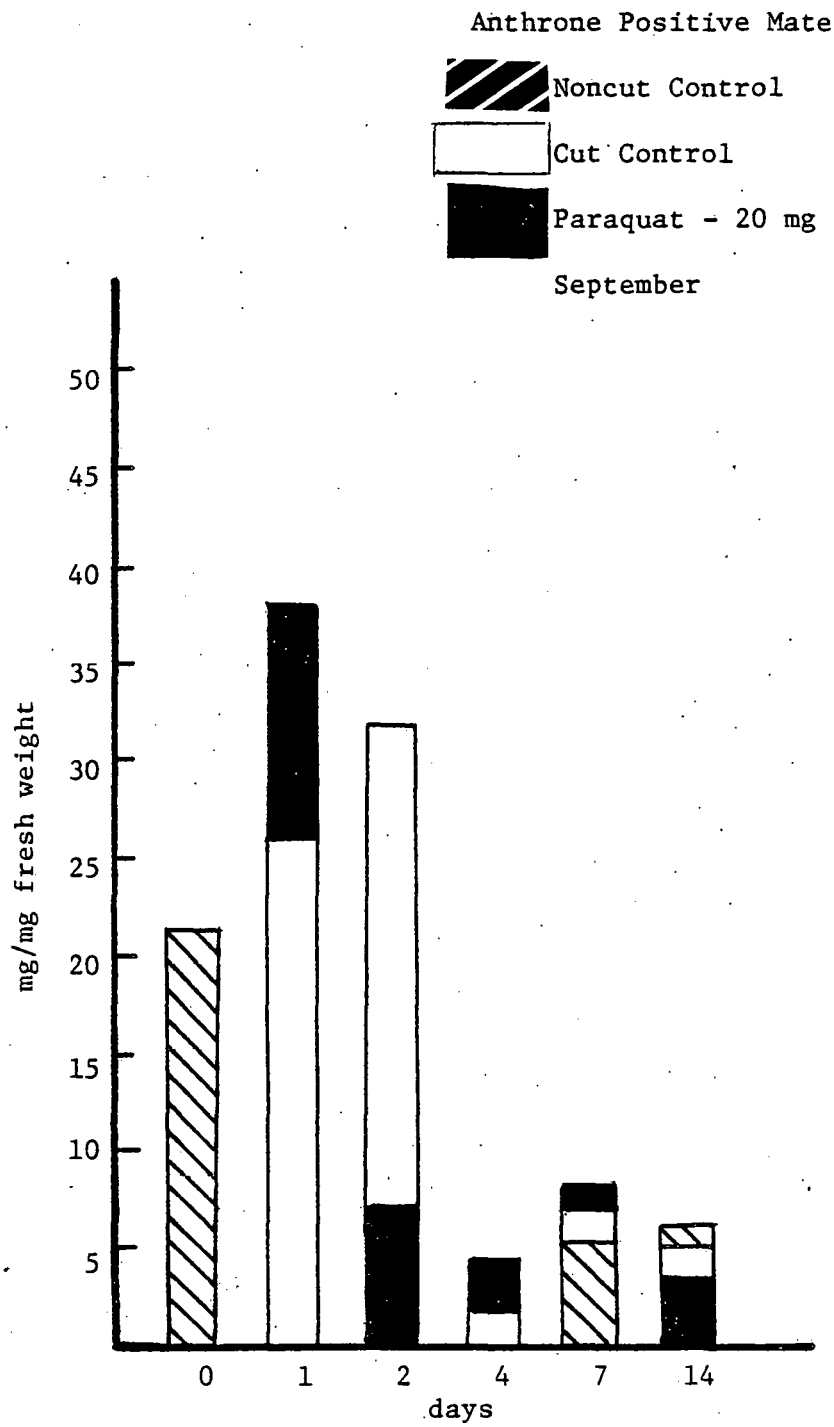
Anthrone Positive Materials
Noncut Control
Cut Control
Paraquat - 20 mg
September
mg/mg fresh weight
50
45
40
35
30
25
20
15
10
5
0
1
2
4
7
14
days

Fig. 2B

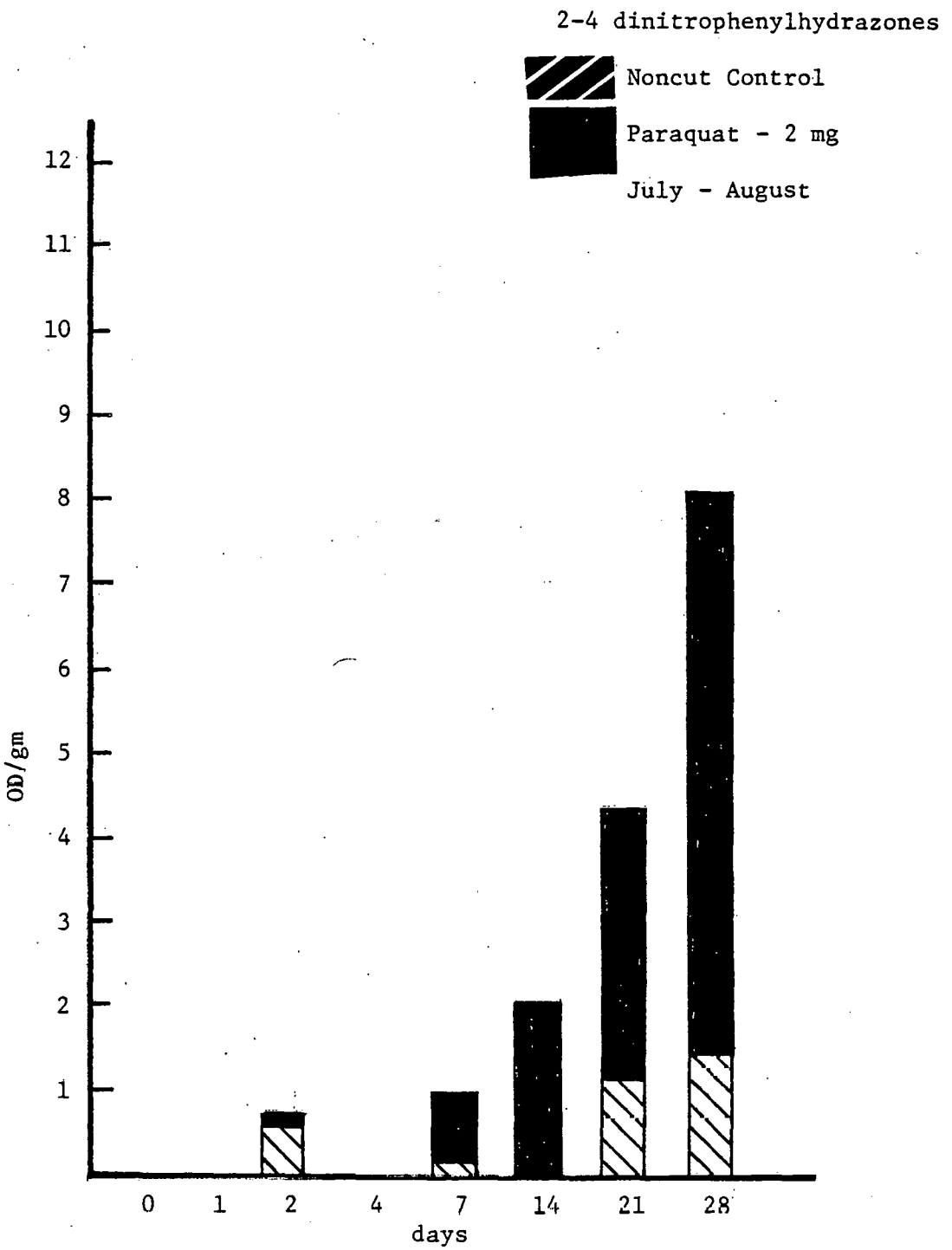
2-4 dinitrophenylhydrazones
Noncut Control
Paraquat - 2 mg
July - August
OD/gm
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
21
28
days

Fig. 3A

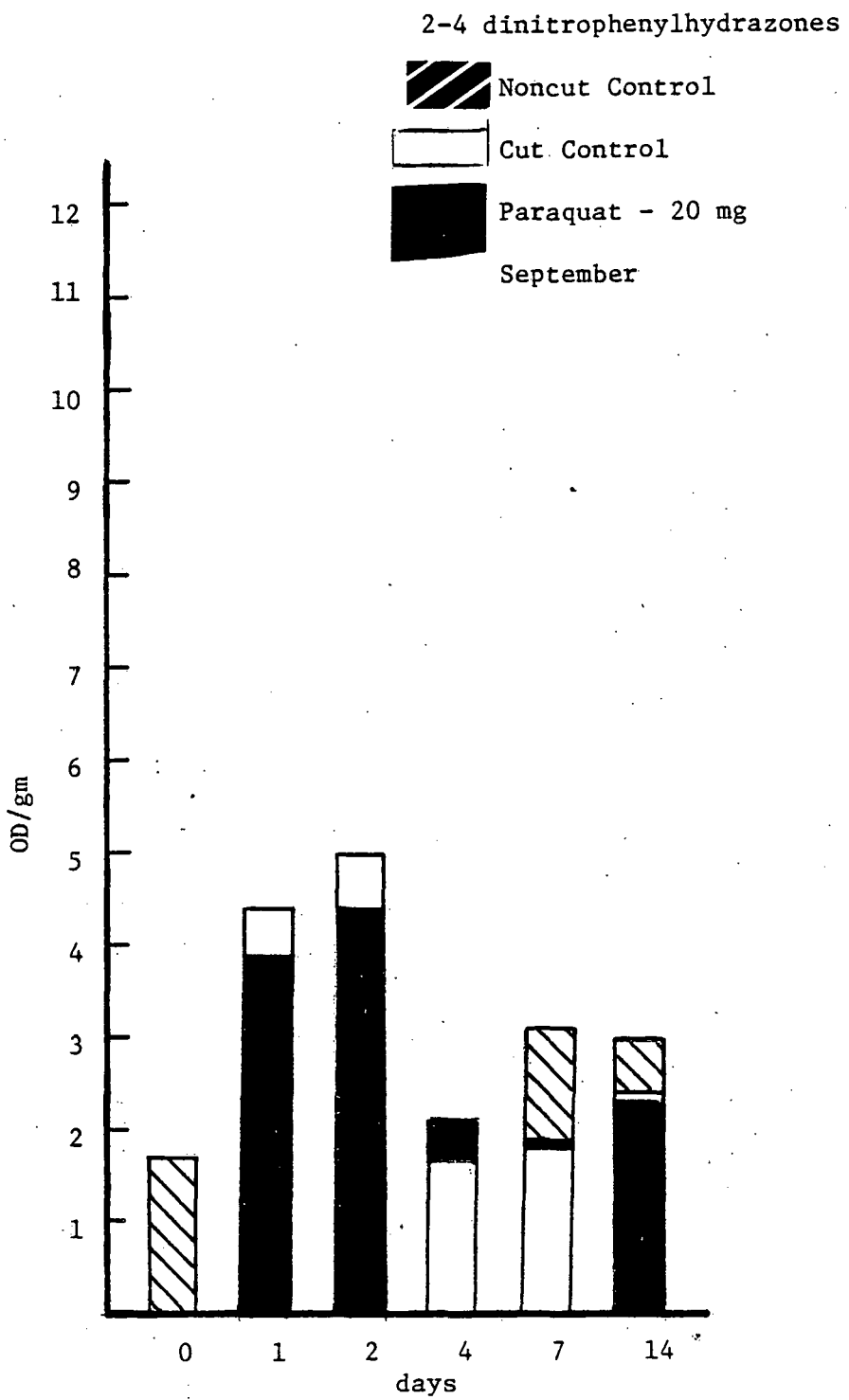
2-4 dinitrophenylhydrazones
Noncut Control
Cut Control
Paraquat - 20 mg
September
OD/gm
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
days

Fig. 3B

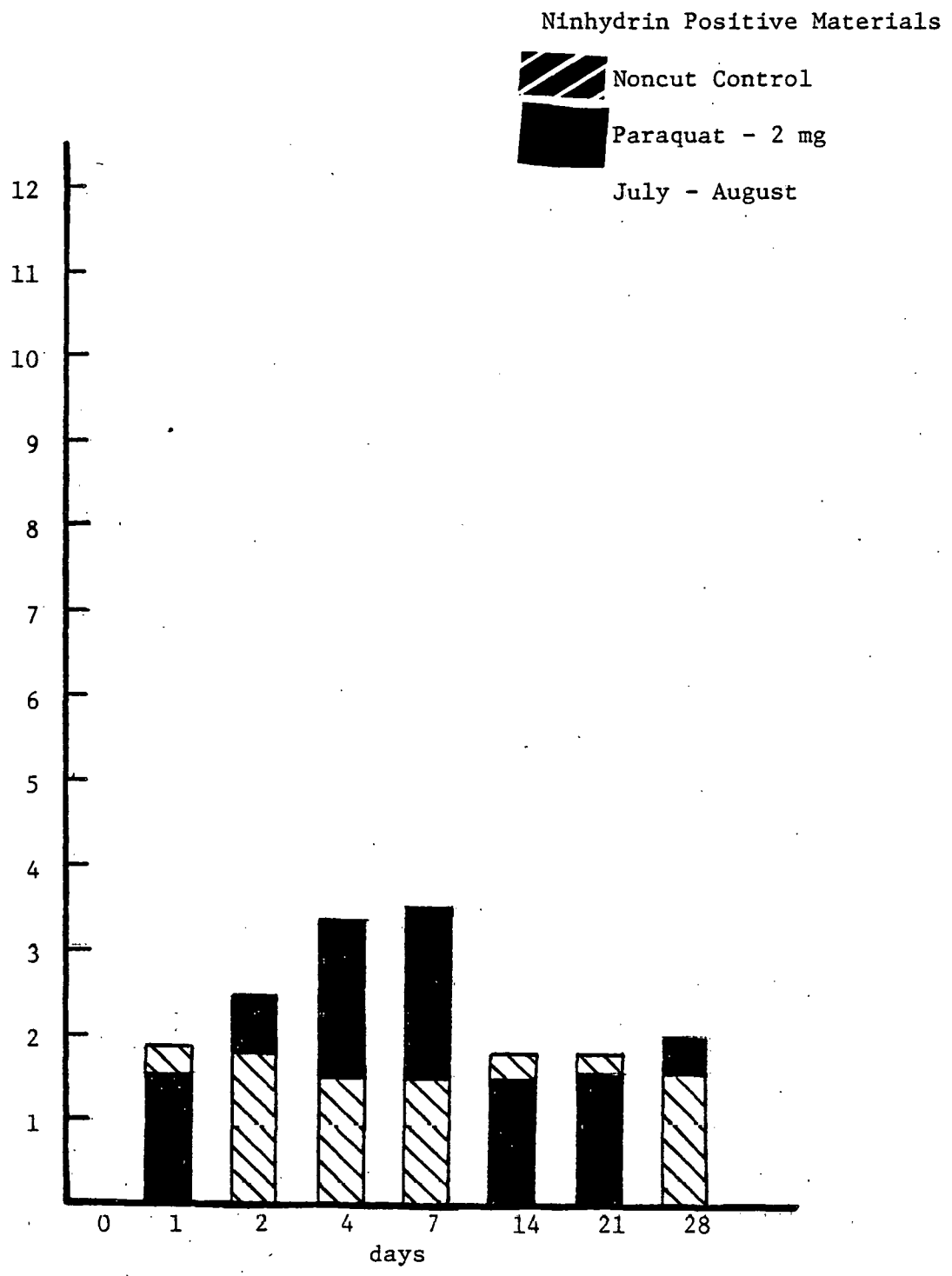
Ninhydrin Positive Materials
Noncut Control
Paraquat - 2 mg
July - August
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
21
28
days

Fig. 4A

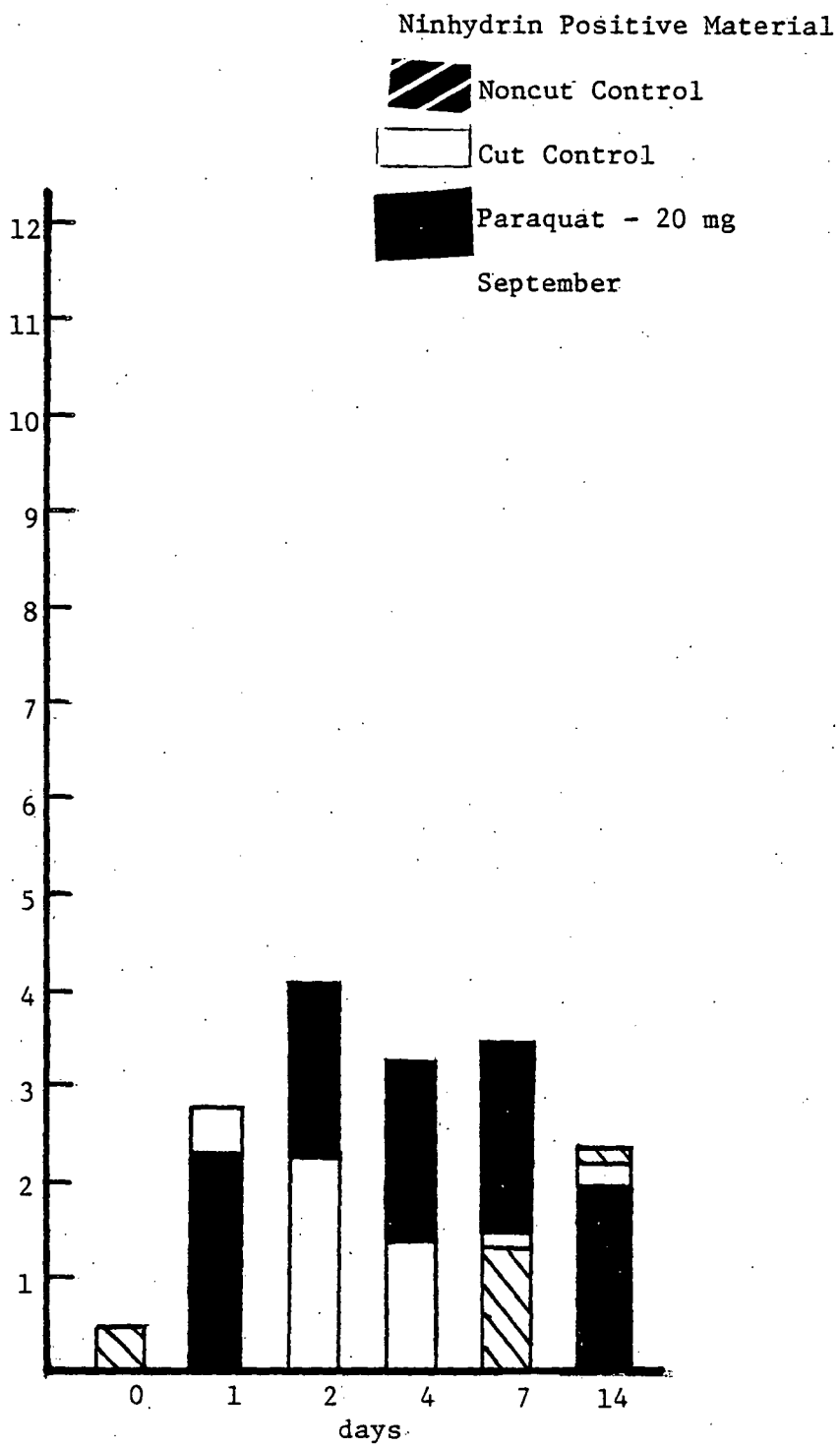
Ninhydrin Positive Material
Noncut Control
Cut Control
Paraquat - 20 mg
September
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
days

Fig. 4B

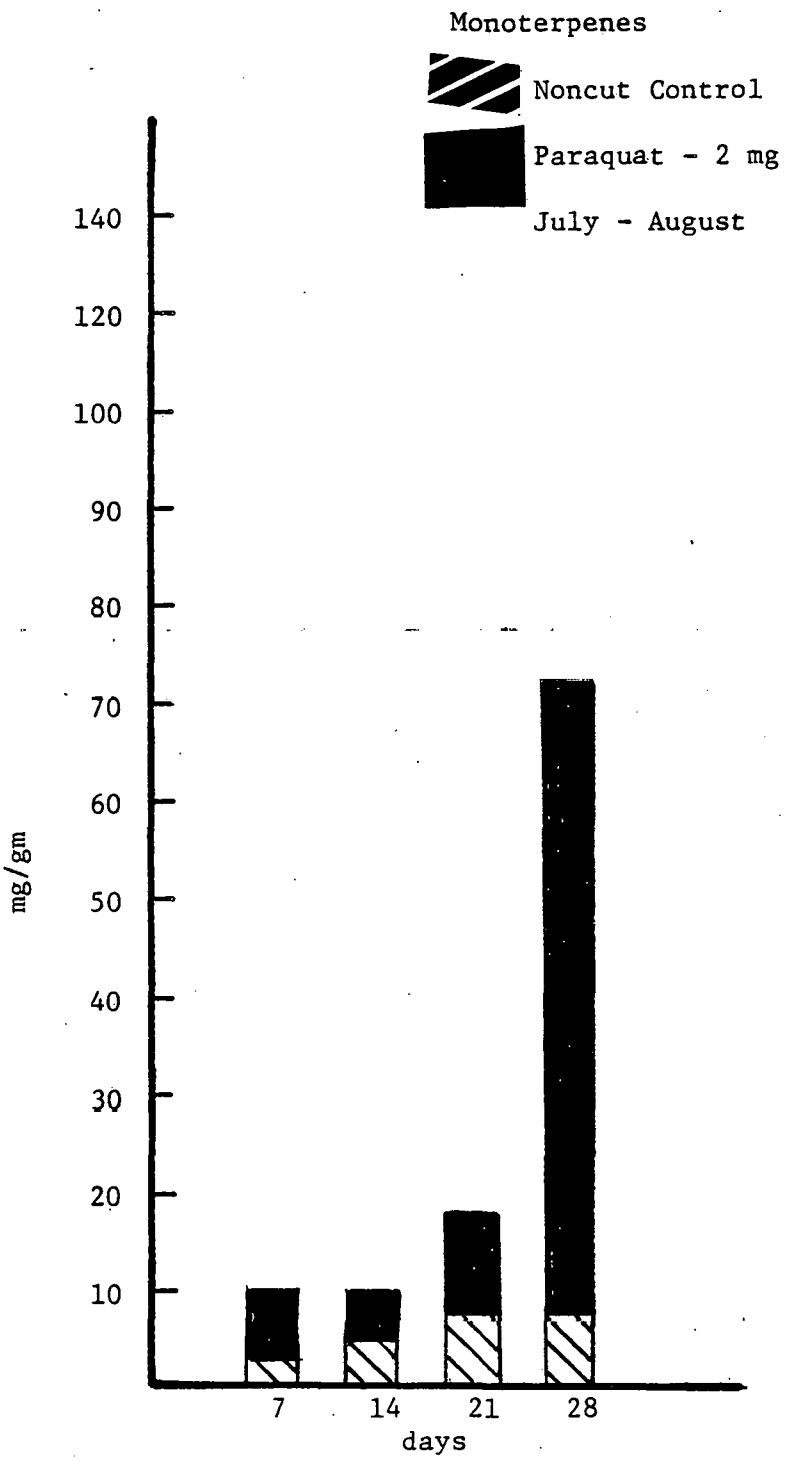
Monoterpenes
Noncut Control
Paraquat - 2 mg
July - August
140
120
100
90
80
70
60
50
40
30
20
10
mg/gm
7
14
21
28
days

Fig. 6A

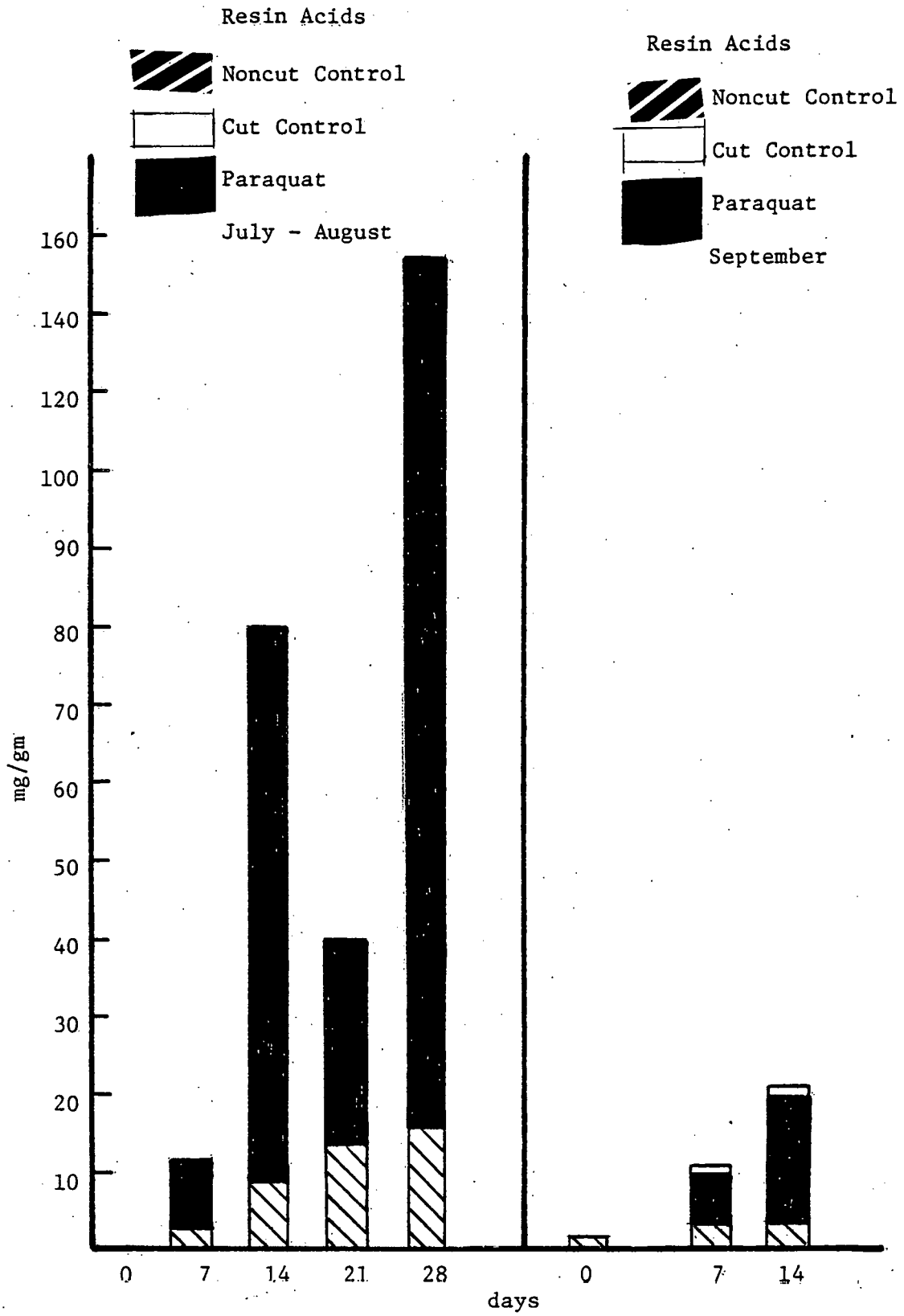
Resin Acids
Noncut Control
Cut Control
Paraquat
July - August
Resin Acids
Noncut Control
Cut Control
Paraquat
September
160
140
120
100
90
80
70
60
50
40
30
20
10
mg/gm
0
7
14
21
28
0
7
14
days

Fig. 5A

Fig. 5B

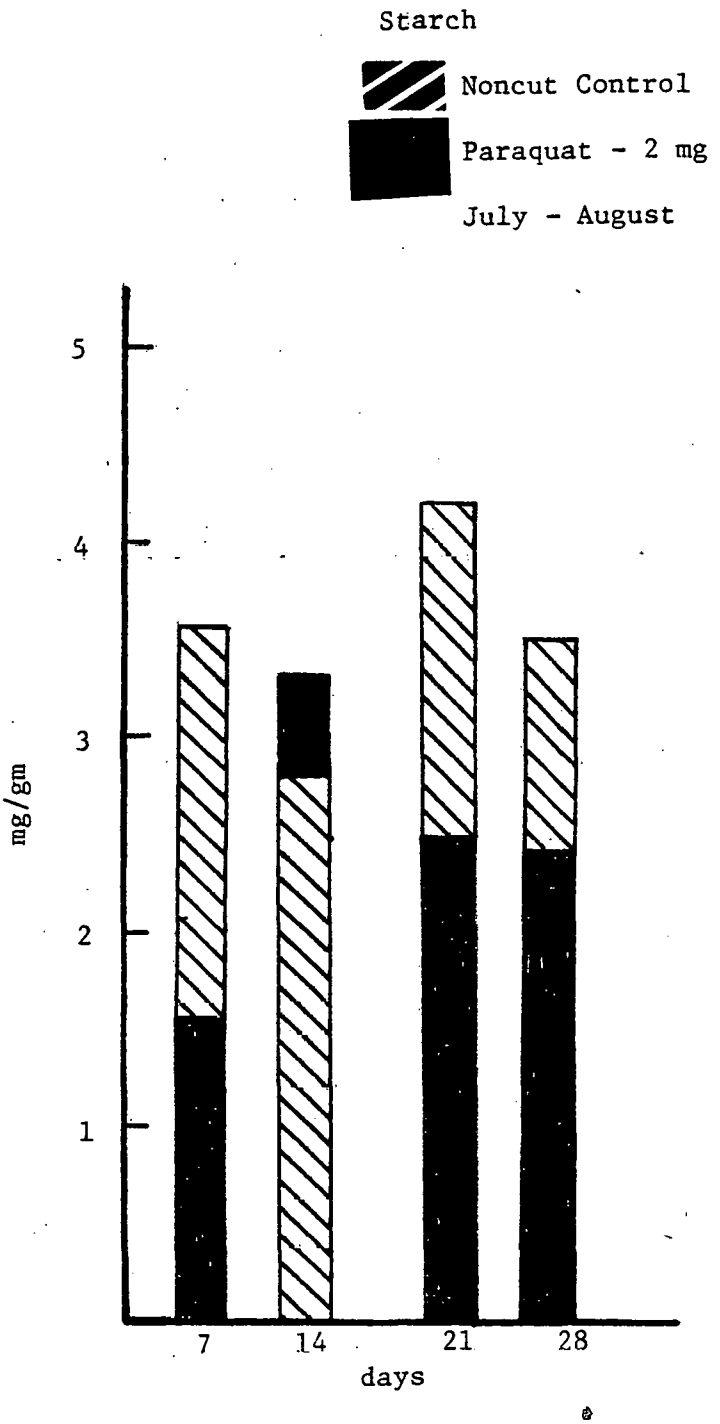
Starch
Noncut Control
Paraquat - 2 mg
July - August
5
4
3
2
1
mg/gm
7
14
21
28
days

Fig. 7A

USE OF PHLOEM AND XYLEM TISSUE SLICES AS A LABORATORY MODEL FOR STUDYING PARAQUAT ASSOCIATED- AND NORMAL-TERPENE SYNTHESIS IN PINUS ELLIOTTII

W. R. Finnerty and P. C. Kerr

ABSTRACT

Data collected in these studies indicate that phloem and xylem tissue slices can be used to study terpene synthesis in slash pine. Neither mevalonate-4,5^{3}H nor acetate-2-^{14}C contributed as much label to materials with the chromatographic mobilities of resin acids and/or methyl resin acids as glucose, glycine, serine, and several other compounds of diverse chemical structure. Incorporation of label from these substrates and mass of total resin acids extracted from untreated trees varied seasonally with minima during the growth season (spring) and maxima during summer-early fall. Depending upon substrate and season, from 0 to 90% of the ^{14}C extracted by boiling 70% ethanol and water was lost under vacuo indicating that the volatile monoterpenes could be very important in the carbon flow of this species. While these data are not definitive, they are in agreement with those reported on monoterpene synthesis in Minta by Loomis. To date, the efficient functioning of the mevalonate pathway for terpene synthesis in pines does not appear to have been demonstrated either in vivo or in vitro.

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridylium salt) application increased oleoresin production in many species of the genus Pinus, in vivo (Peters and Roberts, 1976; Rowe et al., 1976). Conversely, no appreciable resin soaking (lightering) was observed after treating eastern hemlock, balsam fir, tamarack, Douglas-fir, or Norway spruce with the same amounts of paraquat (Rowe et al., 1976).

Morphological, anatomical (Rowe et al., 1976; Brown et al., 1976) and biochemical (Finnerty et al., 1976) changes were observed following in vivo application of paraquat to coniferous species. Paraquat application killed the cambium behind the treatment area extending from slightly below the wound to a height above the wound that was concentration dependent. Treatment resulted in a wedge of dead dry wood and bark that followed the spiral grain upward in all trees. Traumatic resin ducts were observed commonly at the edges of the wound in those species that did not readily lighter.

Accumulation of resin in treated tissues was observed visually at both the micro- and macroscopic level and chemically, but there appeared to be no change in resin acid composition. Monoterpene accumulation increased after in vivo paraquat treatment (Brown et al., 1976) and certain data indicate that the monoterpine fraction was stimulated more than the resin acid fraction (LRCC Proceedings, 1977). Starch hydrolysis (Brown et al., 1976) followed in vivo paraquat treatment with associated increased pool sizes of

2-thiobarbituric acid, anthrone, 2,4-dinitrophenylhydrazine, and ninhydrin reactive materials (Finnerty et al., 1976).

Since the weather can not be controlled and use of radioisotopes is difficult under field conditions, tissue slices of phloem and xylem from Pinus elliottii (slash pine) were incubated with various labeled substrates under laboratory conditions to ascertain the usefulness of this method as a model system to study biosynthesis of terpenes. Data collected during these experiments indicate the utility of the method and are reported in this paper.

MATERIALS AND METHODS

Terminal leaders from 5- (1976) and 6- (1977) year old plantation-grown slash pine were cut 2-3 hours after solar noon and brought into the laboratory. Needles and bark were removed, the underlying phloem and xylem were separated and cut into slices approximately 2x5x5 mm. Five grams each of phloem and xylem were incubated in 50 ml of sterile 10^{-3} M phosphate buffer, pH 6.5, in 250 ml flasks. At zero time, the radioactive substrate and 1 mg paraquat in 1 ml sterile water or 1 ml sterile water, respectively, were added to the tissues and shaken at 200 rpm on a Gyro-shaker under normal laboratory lighting at room temperature (20-22°C).

After the specified time periods, the tissues were removed and extracted 2 times in boiling 70% ethanol (EtoH) followed by a hot water extraction. In certain instances tissues were extracted in chloroform:methanol (2:1) prior to the EtoH extraction. Resin acids were estimated from aliquots of the ethanol extract. Other soluble components were estimated from aliquots of the combined ethanol and water extracts.

The tissue free incubation media (supernatant) was acidified to pH 4 with HCl and extracted 3 times with diethyl ether (Et_2O). The Et_2O extracts were combined and aliquots were used to determine radioactivity, volatile components and total lipid.

Peroxy acids were estimated as 2-thiobarbituric acid (TBA) reactive material (Heath and Packer, 1967); amino acids as ninhydrin reactive material (Rosen, 1957; free carbohydrate as anthrone reducing materials (Ashwell, 1957); and keto acids as 2,4-dinitrophenylhydrazine reactive materials (Friedemann, 1957). Resin acids (RA) were methylated with "Methyl 8" (Pierce Chemical Co.) and estimated by gas chromatography (GC) on a 6'x1/8" glass column of 5% DEGS-PS at 200°C. Radioactivity determinations were done on a Searle Delta 300 liquid scintillation counter. Data are corrected for quench (when it exceeds 10%) by the external standard procedure. Radioactivity in RA was estimated in eluted samples after development on silica gel G thin-layer chromatography plates (TLC) with petroleum ether (PE):Et_2O:glacial acetic acid (80:20:1, v/v/v). During 1977, aliquots eluted from the RA-area were methylated and developed by TLC as before. The methyl resin acids (CH_3-RA) were eluted and counted as before. Mass determinations on aliquots of eluted RA and CH_3-RA were done by GC. Mass recovery of RA after development on TLC was always from 95-98% of that in the original extract, though degradation of palustric to isopimaric and abietic to dehydroabietic acids occurred. Mass recovery of CH_3-RA averaged 41% (range 33.3 to 50%) of that in the original extract. Recovery of radioactivity in the CH_3RA area averaged 22% (range 9 to 33%) of the activity

recovered from the RA-area of a neutral lipid TLC plate. Free fatty acids and methyl fatty acids co-chromatograph with RA and CH_3-RA, respectively. The loss of RA mass during the second development on TLC as CH_3-RA does not appear to be due to incomplete methylation alone since no RA could be detected by GC analysis from the eluted RA area of the plate. Radioactivity could be detected in this eluted RA area.

RESULTS

Data included in Tables 1 and 2 indicate that label from diverse classes of compounds; sugars, amino acids, fatty acids, and phenols; can be recovered from the RA and/or CH_3-RA areas of TLC plates after development in neutral lipid solvents. Some of these same substrates (Tables 3,4) can contribute radioactivity to volatile components (presumably monoterpenes). Comparison of the data in Tables 1 and 2 with those in Tables 3, 4 and 5 indicate that generally more label from all substrates was incorporated into the volatile fraction. The loss of ^{14}C from paraquat-treated material was always equal to, and in most cases, exceeded that loss from control tissues.

Neither mevalonate-4,5-^{3}H (0-3%) nor acetate-2-^{14}C (0-10%) (Table 2) contributed large amounts of radioactivity to the RA area. Even less activity was recovered from the CH_3-RA area (0.3 to 6%) of these same samples.

Carbon from glucose (Table 1) and amino acids (Table 2) appeared to be most efficient in labeling both RA and the volatile compounds (Tables 3,4) Seasonal fluctuation in label incorporated into RA (table 1) and volatile compounds (Table 3) was documented with glucose-U-^{14}C. Decreasing radioactivity was isolated from the RA- and CH_3-RA areas of TLC plates from early winter to spring. Minimal label was recovered in both 1976 and 1977 during the period of rapid vegetative growth (March-May). Maximal incorporation of radioactivity from exogenous substrates, and accumulation of mass of resin acid in field-grown slash pines (Finnerty and Kerr, 1977) occurred during the three months (June-Aug.) following cessation of rapid vegetative growth.

Data shown in Fig. 1A-1N are from two incubation experiments with exogenous glucose-U-^{14}C. One experiment was performed on Feb. 17, 1976, and label was recovered in resin acids. The second experiment was performed on Apr. 5, 1976, and no label was recovered in resin acids. Experiments performed between these two dates indicate that there was a continium between the two experiments. Although not shown, comparable experiments performed during 1977 indicate that the seasonal trend was reproducible, but slightly displaced in time depending probably upon weather conditions (winter temperatures in Athens, GA during 1977 were the 100-year low).

Certain differences and similarities are apparent in the data with the exception of resin acids which were identical within experimental error. Zero time pool sizes of all measured parameters were 2 to 6 times lower in April than in February. Anthrone (6 times lower) and ninhydrin (5 times lower) reactive materials (Fig. 1C,D,G,H) exhibited the greatest differences between the two dates. Keto-acids were 4 times lower in April (Fig. 1E,F) while TBA-positive materail (Fig. 1A,B) was only 2 times lower. With the exception of ninhydrin-positive material in paraquat treated tissues in February (Fig. 1G) no differences were observed in pool size of control and treated tissues. Amino

acid content of paraquat-treated tissue was approximately 2 times that of control tissue from 17 to 48 hours after treatment. In February, concentration of sugars, peroxy-, keto- and amino-acids of the paraquat-treated tissues was approximately 1/2 that of the zero time value 17 hours after paraquat treatment. Amino acid concentration of control tissue was 1/4 that of the zero time control after 17 hours.

In contrast to the February data, peroxy-, keto-, and amino-acid concentration increased approximately 2-fold within 24 hours over that of the zero time control. Concentration of anthrone positive material was approximately 1/2 that of the zero time control at 24 hours. After 48 hours, soluble carbohydrate was 1/3 the concentration of the zero time control, while the keto- and amino-acid concentrations were approximately 2 times greater than those at zero time.

During the February study, resin acid concentration decreased by a factor of approximately 10 in treated and control phloem tissue, but only by a factor of 3 in control and treated xylem tissue (Fig. 1L). Resin acids and volatile compounds were recovered from Et_2O extracts of the acidified supernatant (Fig. 1N,0). Paraquat treatment did not appear to stimulate resin acid accumulation in either phloem or xylem in these studies (Fig. 1L). Increased (190 to 520 µg/gm) resin acid accumulation was observed in the control phloem samples from 17 to 72 hours (Fig. 1L). The percent of ^{14}C recovered from the RA area of TLC plates was not different between treated and control tissues at 17 and 72 hours (Fig. 1I). The percent distribution of ^{14}C in control and treated tissue did vary with time and with each other from 17 to 48 hours. In paraquat treated tissues, the percent of ^{14}C decreased from 17% at 17 hours, to 14% at 24 hours to 9% at 48 hours, while in control tissue it increased from 20% at 17 hours to 40% at 24 hours and decreased to 17% at 48 hours (Fig. 1I). Mass and specific activity data are not available for the 24 and 48 hour sample, but data for 17 and 72 hours (Fig. 1K) are representative of the trend in specific activity seen in all of the experiments performed with all the substrates tested to date; i.e., increases in mass of resin acid were associated with decreases in specific activity and vice versa.

During the February incubation, ^{14}C from glucose was recovered in volatile compounds (Fig. 1M). Label was recovered in the volatile fraction in only the 48-hour incubation during the April experiment (data not shown). No differences between control and treated tissues were apparent in the volatile fraction (Fig. 1M). Extensive loss of ^{14}C (50-80%) by evaporation was observed during the first 24-hours, while losses varied from 20 to 40% from 24 through 72 hours.

Increased amounts of Et_2O soluble ^{14}C was extracted from the acidified supernatant in both the February and April experiments with increasing incubation time (Fig. 1-N,0). Larger amounts of Et_2O soluble material (low of 1% at 17 hours to a high of 7% at 72 hours) were recovered in February than in April (low of 0.03% at 6 hours to a high of 4.7% at 48 hours). During February, paraquat treatment appeared to increase the rate of release of Et_2O soluble material from phloem (control 1%; paraquat, 4.4% at 17 hours) but not the amount since 3.8% of the ^{14}C in the supernatant from control phloem was Et_2O soluble at the 24-hour period. No differences between control and treated phloem was detected at 48- and 72-hours, though the total Et_2O soluble material continued to increase to approximately 5% in paraquat and 6.5% in control tissue. The amount of Et_2O soluble ^{14}C increased with time of

incubation in both control and treated xylem tissue from 1.5% at 17 hours to 4.5% at 72 hours.

During April (Fig. 1N,O) Et_2O soluble ^{14}C increased with time of incubation from 6 to 48 hours. Control and treated phloem and xylem samples were identical through 24 hours. Between 24- and 48-hours, Et_2O soluble ^{14}C increased at approximately the same rate and to the same extent in control and treated phloem, but both the rate and amount of ^{14}C recovered from the xylem samples increased. Control xylem increased from 0.5% at 24 hours to 2.7% at 48 hours; paraquat xylem increased from 0.3% at 24 hours to 4.7% at 48 hours (Fig. 1O). Up to 95% of this Et_2O soluble 14C was lost under vacuo.

DISCUSSION

Carbon-14 from diverse classes of compounds; i.e., sugars, fatty acids, amino acids, phenols, etc.; can be recovered from the RA and CH_3-RA areas of TLC plates. Neither mevalonate-4,5-^3H nor acetate-2-^{14}C contributed appreciable label to components with the chromatographic mobilities of the RA or their derivatives. In general, glucose, glycine and serine appeared to be the most efficient carbon donors to the terpenes of P. elliottii. Similar results were reported and discussed by Loomis (1976) from studies of monoterpene synthesis in Minta. Limited uptake of exogenous mevalonate is certainly possible, but limited uptake of acetate does not seem too likely since label was recovered from protein hydrolyzates and all soluble components. Perhaps the 0.01 to 1% of the total label incorporated from acetate that could be recovered from the RA-area represents de novo synthesis, while the higher percentage from other substrates was not only in terpenoid components. It is noteworthy though, that in these studies with pine and Loomis' studies with Minta, glucose, glycine and serine appeared to contribute more carbon to terpenes than either mevalonate or acetate.

In vitro enzyme assays of mevalonic acid kinase from Pinus radiata (Beytia et al., 1969; Nascimento et al., 1969) and from Agave americana (Garcia-Peregrin et al., 1972; Suarez and Garcia-Peregrin, 1977) indicate low recovery (0.05-0.1%) of activity incorporated from mevalonate into hydrocarbons. These extracts contained very active unspecific phosphatases (activity exceeded that of mevalonic kinase 3-10 times under experimental conditions to minimize phosphatase activity) which could account for the low recovery in the terpenes.

The amount of label recovered from the RA-area of TLC plates fluctuated seasonally; i.e., decreasing from fall to spring with minimal incorporation of label corresponding with minimal mass of RA in untreated trees during the time of rapid tree growth (March-May). Increasing amounts of label and increasing mass of resin acids were observed during the summer (June-Sept.). Loomis (1967) also reported cessation of monoterpene synthesis in Minta during the time of flowering and rapid growth. The volatile fraction (presumably monoterpenes) from slash pine also appeared to follow the same seasonal pattern as indicated by decreasing label in this volatile fraction and inability to detect the typical aroma of these compounds in the field during this time. Presumably, carbon is being cycled into new cellular material at the expense of secondary metabolites such as the terpenes at this time. After growth was completed, synthesis of the secondary metabolites was maximal.

The lack of recovery of ^{14}C- from the RA-area during the April ^{14}C-glucose study may be related to the decreased pool size of carbohydrate which could effect the rate and extent of RA-synthesis. While the percent of Et_2O soluble material recovered from the supernatant was much lower during the April experiment, it increased during the 48 hours of incubation from approximately 0.05% to 4.7% of the total counts remaining in the supernatant. Essentially all of the ^{14}C at 48 hours was loss under vacuo indicating that monoterpene synthesis could be occurring. Perhaps ^{14}C could have been isolated from the RA-area if the experiment had been of longer duration, since data (both control and paraquat treated) from field and laboratory studies indicate a flow of carbon from carbohydrate to terpenes.

Perhaps the most surprising observation during these studies is the amount of ^{14}C recovered in volatile compounds (Tables 3,4,5) which could be monoterpenes. Depending upon the time of year and substrate from 0 to 90% of the total radioactivity recovered in hot 70% EtoH and H_2O extracts of the tissues and Et_2O soluble materials extracted from the supernatant was lost when the samples were reduced under vacuo. In 50% of the experiments, >50% of the ^{14}C was lost by evaporation. Since amino acids, carbohydrates, keto acids, etc., would be extracted into the EtoH and water, these data indicate that these volatile components are very important in the carbon economy of slash pine. The metabolic fate of these compounds; i.e., whether they serve as precursors for more complex terpenes; whether their biosynthetic pathways are reversible and the carbon can re-enter the flow of carbon to soluble and/or structural components, or whether all or some carbon is lost from the trees by volatilization; is unknown at this time. Certainly the latter pathway does play some role as is evident by the "aroma" of pine forests.

Paraquat, in general, tended to increase the amount of volatile ^{14}C, especially in xylem. Data collected by several paper companies and the U.S. Forest Service from extensive field tests (LRCC 1967,1977) indicate that paraquat treatment stimulated production of the terpene fraction (monoterpenes) more than the RA fraction. These laboratory studies also indicate a comparable trend. To date, no analysis has been performed on these volatile components.

Data collected in these studies indicate that isolated tissue systems can be used to study terpene synthesis in slash pine. Neither mevalonate-4,5^3-H nor acetate-2^{14}C contributed as much label to materials with the Rf of RA and/or CH_3-RA as glucose, glycine, serine and several other compounds of diverse chemical structure. Since these areas on TLC plates after development in neutral lipid solvents do contain at least free and methyl fatty acid, as well as RA and CH_3-RA, respectively, just how much of the total radioactivity was in resin acids is unknown. The specific activity data are confusing in that it increased when the mass of RA is decreasing and vice versa. In fact, the count remained stable after 24-36 hours, but the same continued to fluctuate. This decrease can be partly explained by dilution of the radioactivity as starch was hydrolyzed (Brown et al., 1976; Finnerty et al., in preparation) and the soluble carbohydrate was mobilized to all soluble pools.

While these data are not definitive, they are in agreement with those reported on monoterpene synthesis in Minta by Loomis. The same questions on the mevalonate pathway that were raised (and discussed) by him are applicable to this study. To date, the efficient functioning of the mevalonate pathway for terpene synthesis in plants does not appear to have been demonstrated either in vivo or in vitro.

TABLE 1

Percent recovery of ^{14}C and mass of resin acids and methyl resin acids following 24-hour incubation of tissues with glucose-U-^{14}C in the presence and absence of 1 mg paraquat - data collected during winter-summer, 1976-1977.

SAMPLE	% RA Area of Total Fixed		% CH_3-RA Area of RA Area		% CH_3-RA Area of Total Fixed	
	phloem	xylem	phloem	xylem	phloem	xylem
1976						
Feb. 19						
Control	35	19	NS	NS	NS	NS
Paraquat	4	11	NS	NS	NS	NS
Mar. 18						
Control	6	9	NS	NS	NS	NS
Paraquat	10	5	NS	NS	NS	NS
May 5						
Control	0	0	0	0	0	0
Paraquat	0	0	0	0	0	0
May 10						
Control	0	0	0	0	0	0
Paraquat	0	0	0	0	0	0
June 1						
Control	10	3	NS	NS	NS	NS
1977						
Feb. 28						
Control	1	8	NS	NS	NS	NS
	12.6*	12.5*	NS	NS	NS	NS
Mar. 9						
	NS	13.6	NS	NS	NS	NS
Mar. 28						
	6	2	22	NS	1.4	NS
Apr. 12						
	10	NS	14	NS	1.4	NS
	0.003*	NS	0.001*	NS	33.3	NS
Apr. 28						
	5	5	NS	28	NS	1.4
	0.9*	17.85*	0.3*	8*	33	45
May 9						
	1.5	5	38	17.5	0.06	0.9
	0.9*	1.2*	0.3*	0.6*	33.3	50
May 17						
	34	22	15	9	5	2
	0.033*	0.346*	0.012*	0.16*	36.4	46.2
June 15						
	64		33		21	
	78*		34*		44	

*Mass of RA and CH_3-RA mg/gm fresh wgt.
NS = no sample.

TABLE 2

Percent Recovery of ^{14}C and Mass of Resin Acids and Methyl Resin Acids Following 24-hour Incubation of Tissues With Miscellaneous Substrates in the Presence and Absence of 1 mg Paraquat; Data Collected During Winter-Summer, 1976-77.

	% RA AREA OF TOTAL		% CH_3-RA AREA OF RA AREA		% CH_3-RA AREA OF TOTAL FIXED		
SAMPLE	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	SUBSTRATE
1976							
Jan. 10	0		0		0		Mevalonate 4,5-^3H
Jan. 20	0		0		0		Mevalonate 4,5-^3H
Feb. 24							
Control	3	3	NS	NS	NS	NS	Mevalonate 4,5-^3H
Paraquat	3	3	NS	NS	NS	NS	
Apr. 20							
Control	0.01	0.1	NS	NS	NS	NS	Mevalonate 4,5-^3H
Paraquat	0.015	0.2	NS	NS	NS	NS	
1977							
Feb. 28	0.1	0.15	NS	NS	NS	NS	Mevalonate 4,5-^3H
Apr. 12	0.05	0.1	NS	NS	NS	NS	Mevalonate 4,5-^3H
1976							
Jan. 20	0		0		0		Acetate 2-^{14}C
Feb. 24							
Control	1	2.2	NS	NS	NS	NS	Acetate 2-^{14}C
Paraquat	1.5		NS	NS	NS	NS	
1977							
Mar. 9	NS	2	NS	NS	NS	NS	Acetate 2-^{14}C
Apr. 12	10	5	60	48	6	2.4	Acetate 2-^{14}C
	0.003*	0.59*	0.001*	0.24*	33.3	48	
May 12	9		3		0.3		Acetate 2-^{14}C
	0.208*		0.10*		48		
Apr. 12	10	5	26	NS	2.6	NS	Palmitate ^{14}C
	NS	0.33*	NS	0.16*	NS	48.5	
1976							
Jan. 20	2	9.5	NS	NS	NS	NS	Cinnamic acid ^{14}C
Jan. 20	3	21	NS	NS	NS	NS	Phenol U-^{14}C
1977							
Feb. 18	0.5	3.3	NS	NS	NS	NS	Phenol U-^{14}C
May 17	7.9	4.2	NS	NS	NS	NS	Phenol U-^{14}C
1976							
Jan. 20	1	1	NS	NS	NS	NS	DOPA-^3H
1977							
Feb. 18	32.7	0	NS	NS	NS	NS	DOPA-^3H
May 17	NS	7.0	NS	NS	NS	NS	DOPA-^3H
1977							
June 15	0.03	0	NS	NS	NS	NS	Hydroxymethylglutyrl COA-^4C
1976							
Jan. 10	4.0		NS		NS		Amino acid hydrolyzate-^{14}C
1977							
June 15	0.2	0.7	NS	NS	NS	NS	Amino acid hydrolyzate-^{14}C
1976							
Jan. 10	0.5		NS		NS		Leucine-4,5-^3H
1977							
June 15	0.5		NS		NS		Leucine-4,5-^3H
1976							
Jan. 10							
Control	3	3	NS	NS	NS	NS	Glycine-2-^{14}C
Paraquat	8	6	NS	NS	NS	NS	
1977							
June 15	32.6		NS		0		Glycine-2-^{14}C
	98*		45*		46		
1977							
Feb. 28	0.3	0	NS	NS	NS	NS	Serine-U-^{14}C
Mar. 9	1.4	NS	NS	NS	NS	NS	Serine-U-^{14}C
May 17	16	17	11	7	1.8	1.2	Serine-U-^{14}C
	0.033*	0.346*	0.014*	0.14*	42.4	40.5	
June 15	12.5		NS		NS		Serine-U-^{14}C
	125		50		39.7		
May 17	0.04	0	NS	NS	NS	NS	Glutamic-U-^{14}C

NS = no sample.

*Mass resin acid and methylated resin acid mg/gm fresh weight.

Table 3

Percent of total radioactivity lost *under vacuo* from extracts of tissues incubated 24 hours with glucose-U-^{14}C in the presence and absence of 1 mg paraquat

Sample	2/17/76	3/18/76	4/5/76	5/2/76	6/1/76	6/27/77
Phloem						
Control	57	25	0	0	95	61
Paraquat	56	28	0	0	NS	
Xylem						
Control	59	27	0	0	83	85
Paraquat	78	32	0	0	NS	

TABLE 4

Percent of total radioactivity lost under vacuuo from extracts of tissues incubated 24 hours with miscellaneous substrates in the presence and absence of 1 mg paraquat

SAMPLE	SUBSTRATE	% LOST
2/24/76	Glycine-U-^{14}C	
phloem		
Control		71
Paraquat		86
xylem		
Control		56
Paraquat		86
2/24/76	Acetate-2-^{14}C	
phloem		
Control		27
Paraquat		30
xylem		
Control		32
Paraquat		36
2/24/76	Mevalonate-4,5-^{3}H	
phloem		
Control		46
Paraquat		49
xylem		
Control		55
Paraquat		22
2/24/76	Leucine,4,5-^{3}H	
phloem		
Control		52
Paraquat		67
xylem		
Control		65
Paraquat		70
6/1/76	phenol-^{14}C	
phloem		84
xylem		94
6/1/76	cinnamic acid*	
phloem		78
xylem		52
6/1/76	DOPA*	
phloem		95
xylem		93

TABLE 5

Total radioactivity (CPM) and percentage activity of each extract loss to evaporation. Experiment of 6/2////

SAMPLE	$CHCl_3$:CH_3OH (2:1)		Boiling 70% ETOH		Ether Extract of Medium		TOTAL	
	CPM LOST	% LOST	CPM LOST	% LOST	CPM LOST	% LOST	CPM LOST	% LOST
Control	16,080	11.1	196,000	40.1	763,330	93.3	975,410	67.2
Paraquat	28,300	40.8	414,400	74.5	1,430,175	98.9	1,872,875	90.4

TABLE 6

Tissue slices incubated for specified times with 10 µc glucose-U-^{14}C in presence and absence of 1 mg paraquat. Percentage counts per minute from resin acid area of the plates of total c/m recovered.

	Fig. 1-I 2/17/76					Fig. 1-J 4/5/76				
Control	NS	20.0	40.0	12.0	9.0	NS	0	0	0	0
Paraquat	NS	16.0	9.0	9.0	8.0	NS	0	0	0	0

TABLE 7

Fig. 1-K. Specific activity of resin acids extracted from tissue slices after incubation for specified times with 10 µc glucose-U-^{14}C in presence and absence of 1 mg paraquat. Experiment performed 2/17/76.

	17 hrs		72 hrs	
	Phloem	Xylem	Phloem	Xylem
Control	0.19	0.09	0.13	0.37
Paraquat	0.10	0.05	0.30	0.18

TABLE 8

Mass of resin acid per unit wet weight of tissue (µg RA/mg wet wgt) recovered from tissues slices after incubation for specified times with 10 µc glucose-U-^{14}C in presence or absence of 1 mg paraquat. Experiment performed 2/17/76.

	0 hr		17 hr		72 hr	
	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem
Control	3000	2300	190	1000	520	990
Paraquat	-	-	300	970	230	860

TABLE 9

Percentage of total radioactivity in volatile compounds loss under vacuuo. Experiment performed 2/17/76.

	17 hrs		24 hrs		48 hrs		72 hrs	
	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem	Phloem	Xylem
Control	47	57	57	59	34	39	21	23
Paraquat	50	55	56	79	30	38	12	17

TABLE 10

Fig. 1-N. Percentage radioactivity in diethylether soluble material of total radioactivity in incubation medium after incubation with 10 μc glucose-U-^{14}C for specified times in presence and absence of 1 mg paraquat.

	2/17/76				4/5/76			
	0	17 hr	24 hr	48 hr	72 hr	0	6 hr	12 hr	24 hr	48 hr
Control										
Phloem		1.0	3.8	5.8	7.0		0.04	0.06	0.11	0.04
Xylem		1.6	3.0	4.6	4.8		0.07	0.07	0.15	2.7
Paraquat										
Phloem		4.4	2.0	5.0	6.6		0.06	0.05	0.12	0.9
Xylem		1.4	2.6	1.6	3.6		0.05	0.06	0.13	4.7

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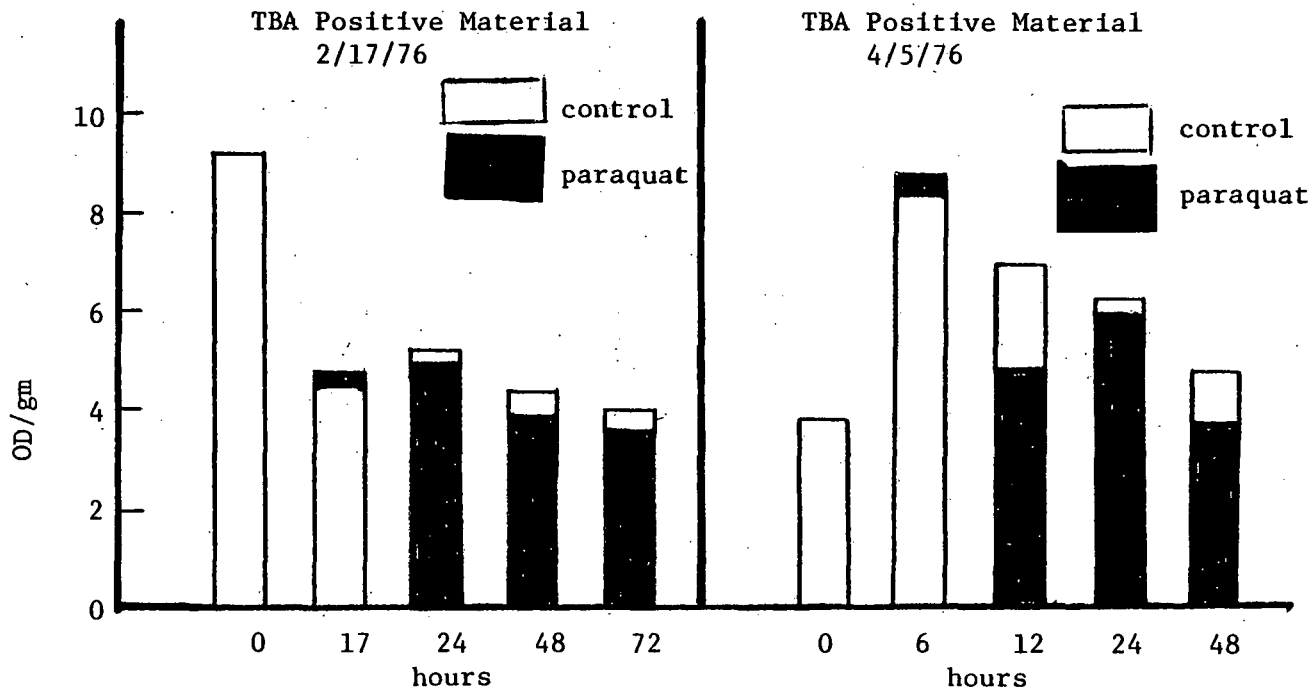
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paraquat
OD/gm
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8
6
4
2
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17
24
48
72
hours
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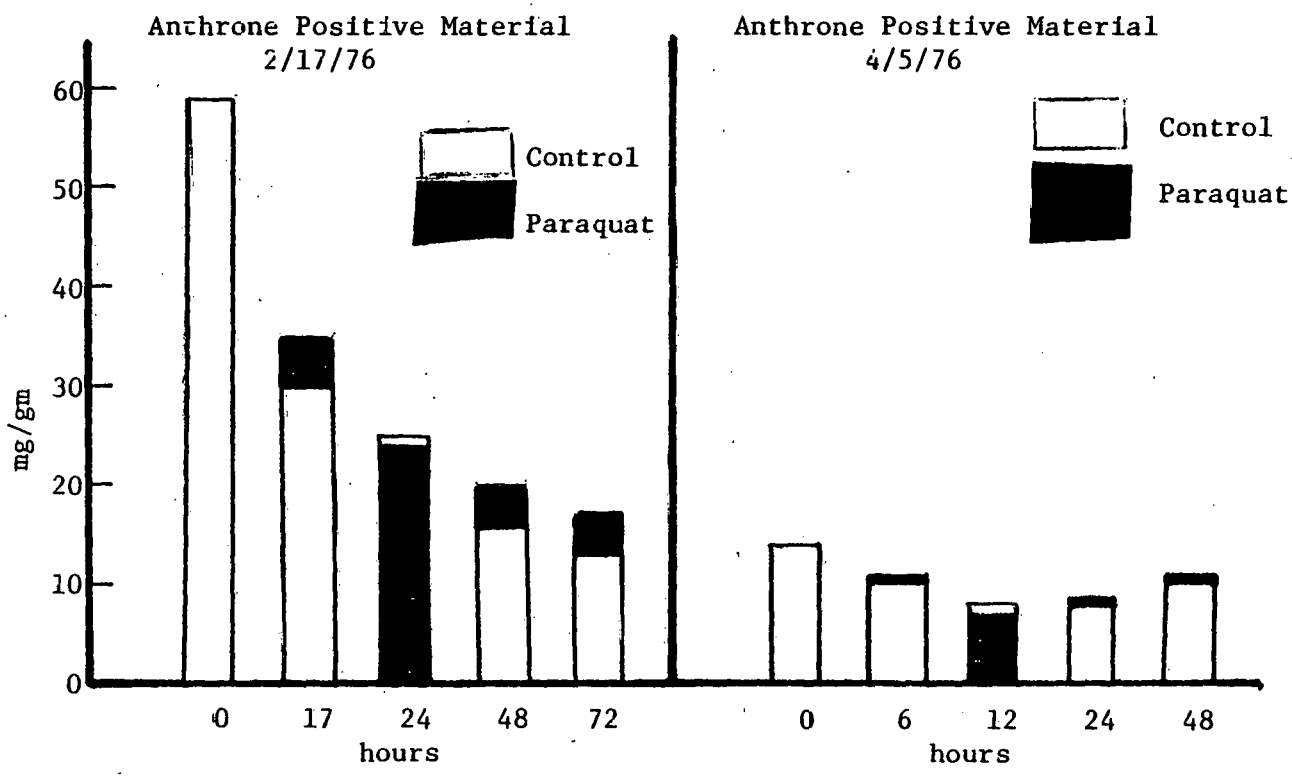
4/5/76
control
paraquat
0
6
12
24
48
hours

Fig. 1A

Fig. 1B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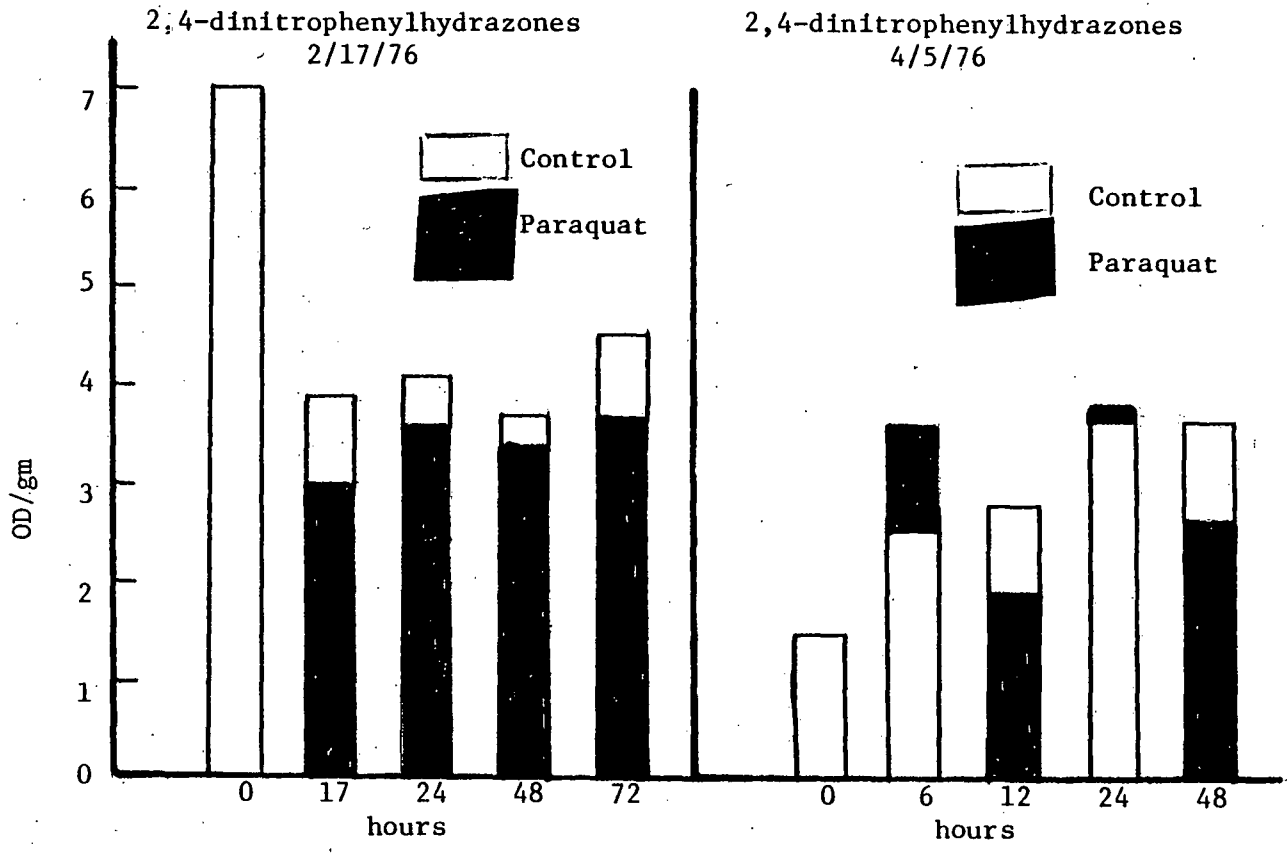
hours

Fig. 1C

Fig. 1D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4/5/76
Control
Paraquat
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Fig. 1E

Fig. 1F

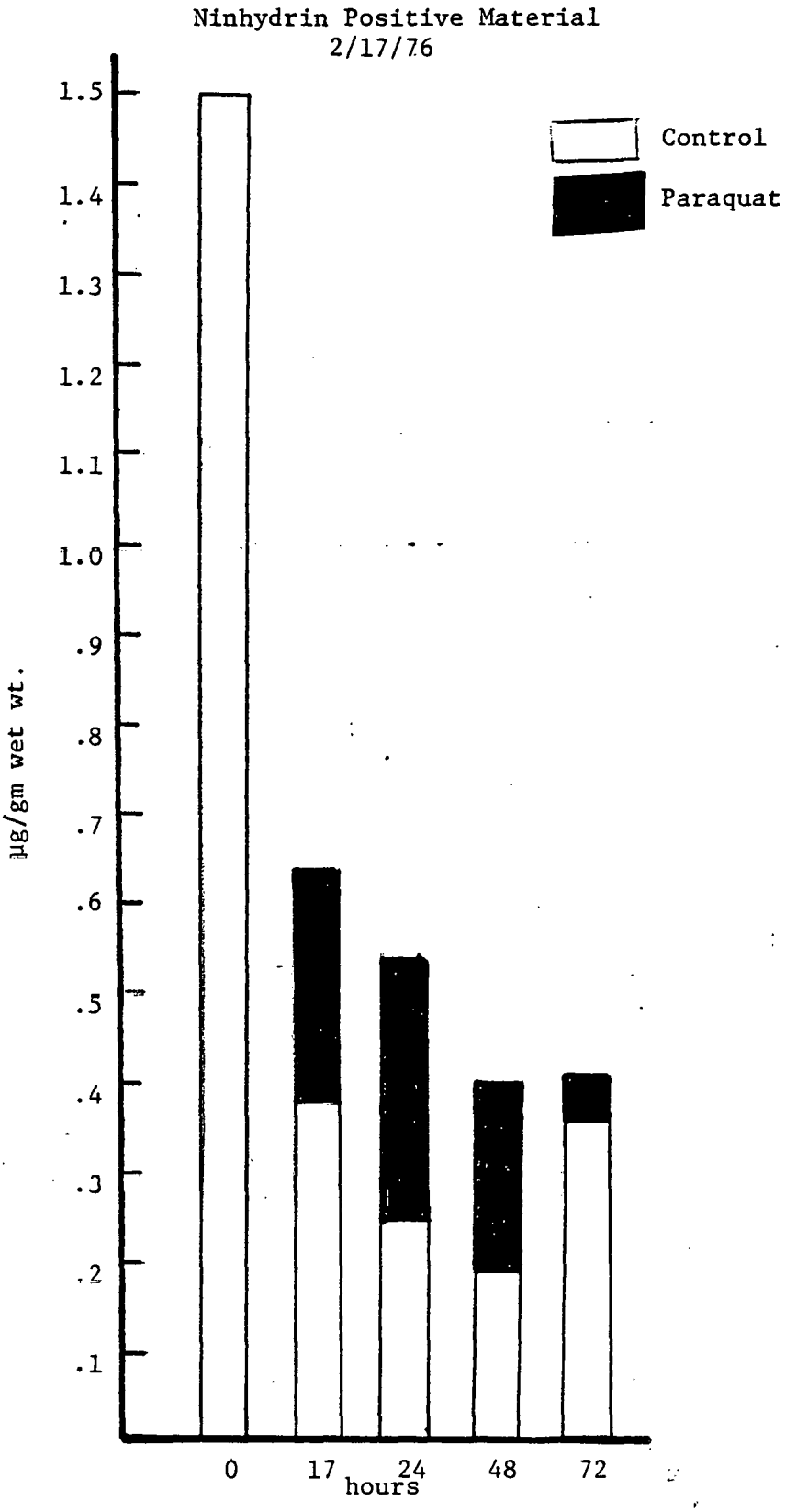
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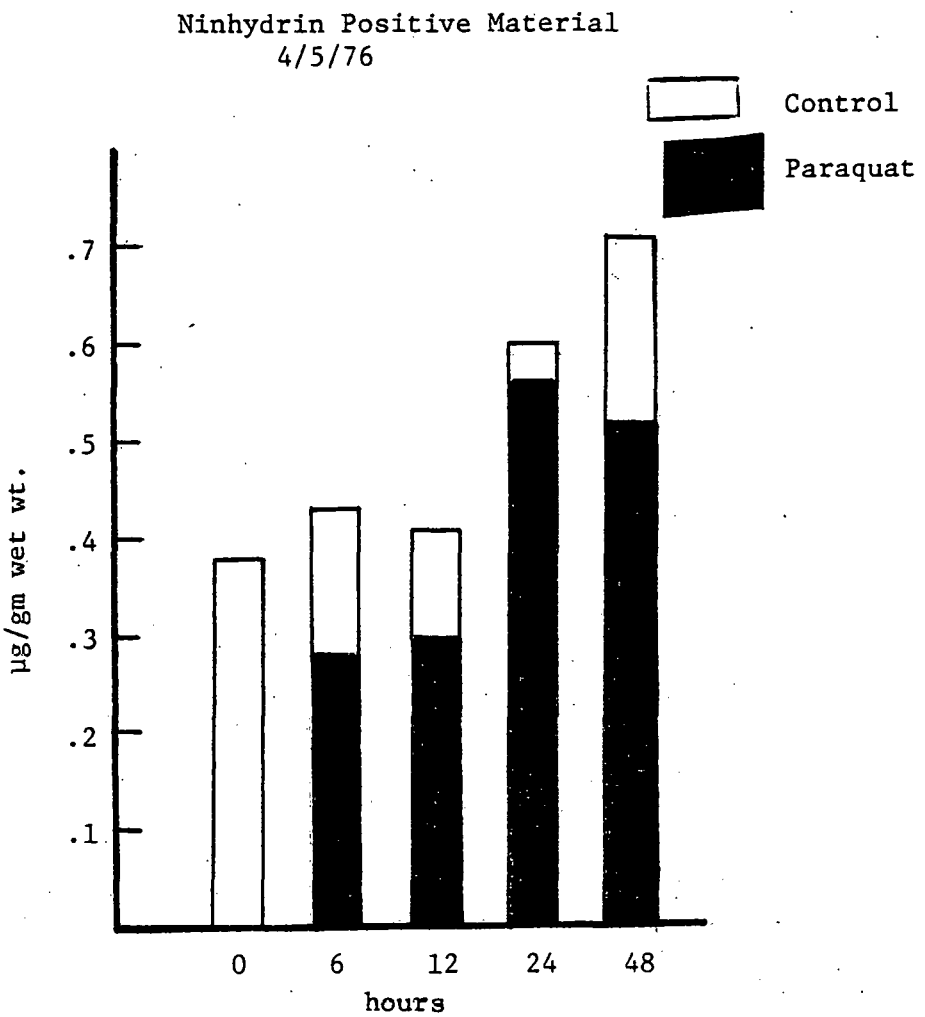
Fig. 1G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12
24
48
hours

Fig. 1H

SUMMARY

Characterization of Lipid Fractions of *Platanus accidentalis*, *Pinus elliottii* and *Pinus palustris*

Characterization and estimation of total neutral lipids, glycolipids and phospholipids from stem and tap root tissues of *Platanus occidentalis* (American sycamore) indicated that lipids were not predominant or major storage compounds in this tree.

Characterization of the phospholipid fraction showed the presence of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidic acid (PA), plus two unknown compounds, PL-A and PL-B. PL-A constituted approximately 50% of the phospholipid in certain tissues at certain times of the year. The fluctuation in the concentration of this lipid could explain the summer minima of total phospholipid observed in stem tissue.

Characterization of the glycolipid fraction showed the presence of mono- and digalactosyldiglycerides plus several other presently unidentified compounds. Two of these unknown compounds (Rfs of 0.50 and 0.55) fluctuated seasonally in root tissue. This fluctuation could explain the apparent seasonal variance of total glycolipids in this tissue.

The neutral lipids were compositionally very complex. This complexity, and the relative insensitivity of the quantitative method used to estimate the separated components, resulted in only the qualitative description of these lipids.

Characterization of the phospholipid and glycolipids obtained from stem tissue, whole cells and isolated colorless plastids and mitochondria from tissue cultures of *Pinus elliottii* and from whole cells and isolated green plastids and mitochondria from tissue cultures of *Pinus palustris* showed similar compounds to those observed in sycamore. PC and PE comprised approximately 75% of the total phospholipid fraction of tissue culture cells. The unknown, PL-A, comprised roughly 17% of the phospholipid fraction. Mono- and digalactosyldiglyceride were present in the glycolipid fraction.

Scatter in the data between the estimations of individual glycolipids and neutral lipids from whole cells versus isolated plastids and mitochondria can be explained by the inherent error associated with char density measurements.

MATERIALS AND METHODS

Plant Material

Plant materials used in this study were: 1) tissue cultures of Pinus palustris (longleaf pine) and Pinus elliottii (slash pine), 2) stem tissue of Pinus elliottii, and 3) stem and root tissues of Platanus occidentalis (American sycamore).

Cell Cultures

Liquid suspension cultures of P. elliottii and P. palustris derived from the cambium of slash and longleaf pine trees were grown and maintained as described by Birchem et al. (1978) with the following exceptions: (i) the basal medium was modified by the addition of 100 mg/l glutamine; (ii) cells were subcultured routinely every 14 days instead of 28 days. Fourteen-day cells were considered to be in the exponential phase of growth (Birchem et al., 1978).

Isolation of Plastids

Pine tissue culture cells were harvested by filtration through Whatman #1 filter paper. Cold grinding buffer A (4-6 C, 400 ml) which contained 10 mmol Tris, 10 mmol $MgCl_2$, 0.25 M sucrose, 0.5% PVP-K-30, and 0.1 ml/l β-mercaptoethanol, pH 7.0, was added to 120 g of fresh cell weight. Cells were broken at room temperature with two 5 sec. bursts in a Waring blender. The suspension was filtered through several layers of cheesecloth and the pH of the filtrate was adjusted to pH 7.0 with 1 N NaOH. The filtrate was centrifuged at 500 rpm for 15 min in an IEC International centrifuge to remove whole cells and cell debris. The supernatant fluid was centrifuged at 1,000 x g for 15 min in a Sorvall RC2-B centrifuge to sediment the plasmid fraction. Plastids were suspended in Buffer B which contained 10 mmol Tris, 10 mmol $MgCl_2$, 0.25 M sucrose, pH 7.0, at 4 C, and were centrifuged at 1,000 x g for 15 min. The final pellet was suspended in Buffer B for extraction of lipids in 300 ml of chloroform:methanol (1:2, v/v) and backwashed with 0.9% NaCl.

Isolation of Mitochondria

Pine tissue culture cells were harvested by filtration through Whatman #1 filter paper and 120 g of fresh cell weight were suspended in 400 ml of cold (4-6 C) grinding Buffer C which contained 10 mmol Tris, 10 mmol Na_2EDTA, 0.3 M sucrose, 0.1% bovine serum albumin (BSA), 0.5% PVK-K-30, 0.1 ml/l of β-mercaptoethanol, pH 7.2. Cells were broken by two 5 sec. bursts in a Waring blender and were filtered through cheesecloth. The filtrate was adjusted to pH 7.2 with 1 N NaOH and was centrifuged 15 min at 1,000 x g in a Sorvall RC2-B centrifuge to remove whole cells, cell debris, and plastids. The supernatant fluid was centrifuged 15 min at 10,000 x g in a Sorvall RC2-B centrifuge to sediment the mitochondria. The mitochondria pellet was suspended in Buffer D which contained 10 mmol Tris, 10 mmol Na_2EDTA, 0.3 M sucrose and 0.1% BSA, pH 7.2, at 4 C and was centrifuged at 250 x g for 10 min. The supernatant fluid was centrifuged at 6,000 x g to sediment the mitochondria. The pellet was suspended in a small volume of Buffer D for extraction with 3000 ml of chloroform:methanol (1:2, v/v) at 6 C as described for the plastid fraction.

Plastid and mitochondrial preparations were judged to be relatively free of whole cell and particulate contamination by phase contrast microscopy and by sucrose density gradient centrifugation.

Slash Pine Stem Sampling

A lateral branch was removed from between the 1st and 2nd node of an 8-year slash pine tree, divided into 2x2 cm pieces and extracted for total lipids.

Platanus occidentalis

Stem and tap root tissues were harvested from field growth American sycamore coppice for the seasonal distribution study. Trees were grown at 1'x4' spacing and were fertilized in March 1978. Shoots were 1-2 years old and roots were 10 years old when harvested from outer rows of the plantings. Whole trees (inclusive of the apical 23 cm of tap root) were destructively sampled (dug up) in mid-afternoon (approximately 1430-1500) and returned to the laboratory for processing.

In March 1979, an experiment was begun in collaboration with K. Steinbeck, School of Forest Resources, University of Georgia, to ascertain pool size(s) and the fluctuation in pool sizes of carbohydrates (Steinbeck), nitrogenous (Steinbeck) and lipoidal (Finnerty) storage compounds in tap roots of American sycamore in uncut (control) and cut (above-ground biomass harvested) coppice stands. North- and south-facing plants grown in a single experimental field plot, i.e., same spacing, fertilization, etc., are being used in the experiment. Root tissues were removed each month from each root with a wood corer. Coppice was harvested each month, and root cores were removed before coppicing and at monthly intervals thereafter. Data was collected relative to the monthly fluctuation of pool sizes of the storage compounds in control (uncut) roots and any variation in these pool sizes associated with removal and/or regrowth of above-ground biomass.

Ten plants were sampled in March, but because of difficulty in sampling, and the ultimate number of samples to be processed, only 4 plants (2 north-facing and 2 south-facing) were coppiced in April, May and June, resulting in a total of 24 samples in June, the last month included in this report. Data will be reported as the average and range of the samples as indicated.

Lipid Extraction

Pooled stem and pooled root tissues (200-300 g/each) were cut into 2x2 cm pieces and extracted 4 times with chloroform:methanol (1:2, v/v). The tissues were extracted again with fresh chloroform:methanol (2:1, v/v). Lipid extracts were filtered through Whatman #1 filter paper, pooled and back-washed with 0.9% NaCl. Tissues from root cores (1 gm dry weight) were extracted in the same manner.

Lipid Fractionation

Neutral lipid, glycolipid and phospholipid were separated by silicic acid column chromatography (20x2 cm). Neutral lipids were eluted with 10 column volumes of $CHCl_3$. Glycolipids were eluted with 10 column volumes of acetone and phospholipids with 10 column volumes of chloroform:methanol (2:1, v/v)

followed by absolute methanol. Neutral lipids were further fractionated on a silicic acid column (20x2 cm) employing discontinuous gradients of petroleum ether containing increasing amounts of diethyl ether. Phospholipids and glycolipids were fractionated on a 2.5x18 cm column of Whatman DE-23 DEAE-cellulose according to the method of Rouser et al. (1969).

TLC

TLC plates, 0.4 mm thick, prepared with either silica gel G or with silica gel H impregnated with 0.1% sodium tetraborate were activated at 110 C and used within 30 min. Development of TLC plates was in the following solvent systems unless otherwise specified: (i) solvent A, chloroform:methanol: 5 N NH_4OH (65:30:5, v/v/v); (ii) solvent B, chloroform:methanol:H_2O (95:35:5, v/v/v); (iii) solvent C, petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v).

Lipids were detected with iodine vapors or by spraying with 0.005% Rhodamine 6G or 50% H_2SO_4 followed by charring at 130 C. Phospholipids were detected by the phosphate spray reagent of Dittmer and Lester (1964). Glycolipids were detected by spraying with 0.2% orcinol in 75% H_2SO_4 followed by heating at 110 C for 15 min. Sterols were detected by spraying with 50% H_2SO_4 and heating at 110 C for 5 min; or by the Liebermann-Burchard reagent (4 ml acetic anhydride and 1 ml H_2SO_4 and heating at 110 C for 5 min).

After detection of iodine vapors, phospholipid and glycolipid were eluted from the silica gel with chloroform:methanol:H_2O (0.45:0.45:0.1, v/v/v). After detection with iodine vapors or 0.005% Rhodamine 6G, neutral lipids were eluted from silica gel with diethyl ether.

Analytical Methods

Lipid-phosphorus was determined by the method of Barlett (as quoted in Dittmer and Wells, 1969). Acyl ester was determined by the method of Snyder and Stephens (1959). Total glycolipid content, based on total lipid sugar, was determined by the anthrone method of Ashwell (1957) using glucose as a standard. Sugars were acetylated after acid hydrolysis of the purified lipid by the method of Albersheim et al. (1969). The acetylated sugars were analyzed on a 1/8" x 10' stainless steel column containing 3% ECNSS-M on 80/100 gas Chrom Q at 200 C in a Varian 1200 gas chromatography.

Glycerol was quantitated after mild alkaline methanolysis by the following method: 10-100 nmol of the glycerol sample were evaporated to dryness and 200 µl of H_2O, 50 µl of 2.5 N H_2SO_4 and 100 µl of 0.1 M sodium periodate were added. This reaction mixture was incubated for 5 min at room temperature followed by the addition of 100 µl of 1 M sodium arsenite. The liberated formaldehyde was determined by adding 5 ml of the chromatropic acid reagent (200 mg 4,5-dihydroxy-2,7-naphthalene-disulfonic acid was dissolved in 20 ml H_2O, filtered and diluted in 100 ml with 12.5 M H_2SO_4) and heating in a boiling water bath 30 min. Sample absorbance was determined at 570 nm.

Phospholipid Quantitation

Approximately 250-350 nmol lipid-phosphorus were applied in triplicate to a silica gel G plate and developed in solvent A. One lane was sprayed with the phosphate spray reagent. After location of phosphate positive spots, the remainder of the plate was treated with iodine vapor and the phospholipids were transferred to glass tubes for phosphorus determination. Known amounts of each phospholipid were developed and analyzed concurrently with the tree samples. Blank areas of the TLC plates were used as blanks. Recovery of lipid phosphorus applied to the plate ranged from 93 to 98%.

Mild Alkaline Methanolysis

Phospholipids were deacylated by mild alkaline methanolysis at 4 C as described previously (Makula and Finnerty, 1974). The water-soluble glyceryl-phosphoryl esters were separated on pre-coated cellulose TLC plates (Eastman 13255, Eastman Kodak Co., Rochester, NY) in the solvent system: ethyl alcohol: 0.5 M ammonium acetate, pH 7.5 (7:3, v/v). Glycerylphosphoryl esters were visualized with the phosphate spray of Hanes and Isherwood (1949).

Calculation of Distribution Profiles of Individual Glycolipids, Phospholipids and Neutral Lipid Components

Glycolipid. Total carbohydrate content of the glycolipids following silicic acid chromatography was determined by the anthrone method. The percentage distribution of each glycolipid was estimated by densitometry of the separated glycolipids after development on silica gel TLC.

Phospholipid. Total lipid phosphorus was determined on the phospholipids following silicic acid chromatography. The percentage distribution of each phospholipid was estimated by determining the phosphorus content of the separated phospholipids after development on silica gel TLC. Individual phospholipid concentrations were calculated using the total lipid phosphorus and relative percentage distribution data.

Neutral Lipid. Total neutral lipid was determined by weighing the residue resulting from the neutral lipid fraction following silicic acid chromatography. The percentage distribution of each neutral lipid was estimated by densitometry after separation and visualization by charring of the separated neutral lipids after development on silica gel TLC.

RESULTS AND DISCUSSION

American Sycamore Total Lipids

Table 1 is a tabulation of the data for total neutral lipid, glycolipid and phospholipid content obtained from stems and roots of American sycamore from December 1977 through May 1979. All lipid classes are expressed on a weight basis (mg lipid/g fresh weight tissue). Glycolipid and phospholipid are expressed also in μmol glucose equivalents and μmol lipid P/g fresh weight, respectively.

Total lipid content of stem tissue averaged 7.1 mg/g (range 5.2-9.1 mg/g) during the nine sampling periods and did not appear to fluctuate seasonally. Conversely, total lipid content of root tissue appeared to fluctuate around

the yearly average of 5.3 mg/g with a minimum content of 3 mg/g during May and July and a maximum content of 9.7 mg/g during December 1978. Total lipid of stem and root tissues were similar on a weight basis except during May, July and September, 1978, and March and May, 1979, when the concentration of total lipid was 1.5-2.7 times higher in stem tissue.

Neutral lipid content of stem tissue averaged 3.8 mg/g (range 2.4-5.4 mg/g) and did not appear to fluctuate seasonally. Neutral lipid content of root tissue averaged 2.2 mg/g (range 1.4-3.6 mg/g), but the concentration of neutral lipid appeared to double when comparing the summer-fall concentration of 1.4-1.9 mg/g with that measured during December and March (2.5-3.6 mg/g). Neutral lipid content of stem and root tissue were similar except during September through November, 1978, and March-May, 1979, when stem tissue contained 2 times more neutral lipid.

Glycolipids

The average glycolipid content of stem tissue was the same based on both weight (2.1 mg/g) and glucose molar equivalents (2.1 μmol glucose equivalents/g). The range of the weight determinations were 1.5-2.8 mg/g, that of the carbohydrate determinations (1.3-3.1 μmol/g). The average glycolipid content of the root was 2.1 mg/g based on weight and 2.4 mol glucose equivalent/g based on carbohydrate determinations.

While the fluctuation around the average values in terms of μmol of glucose equivalents/gm for both stem and root tissues might indicate a spring-summer minima of glycolipid, this observation could also be explained by the higher absolute values observed in December 1978 and March and Ma7 1979 over comparable values obtained in 1977 and 1978. The estimated concentrations of glycolipid was approximately 2 times larger during the later sampling period, and more samples during the summer of 1979 would be needed to ascertain if a minima would again be apparent during the summer months. Concentrations of glycolipid in stem and root tissues did not appear to differ during the sampling period.

Estimation of glycolipid content on a weight basis indicated more pronounced fluctuation and differences in concentration between stem and root tissues than the carbohydrate determinations. Concentrations in roots during May and July, 1978, were 1/2 of that observed in March 1978 (0.7 mg/g vs 1.8 mg/g) and 3-5 times higher during September-December 1978, than in July. Concentration of glycolipid in stem and root tissues were similar by carbohydrate determination, but on a weight basis, stem tissue contained 3-4 times more glycolipid in May and July than root tissue. In December, the concentration in stem tissue remained constant and was 1.5 times lower than the root concentration which increased 1.5 times when compared with the values observed during July through November.

Data expressed in Table 2, relative to the distribution of individual glycolipids, indicate that much of the fluctuation in total glycolipid of both stem and root was associated with fluctuations in compounds with R_f values of 0.5 and 0.55. These two glycolipids and the one with an R_f of 0.89 all contain glucose. Digalactosyldiglyceride (R_f, 0.39) constituted one of the major glycolipids in both stem and root and was present in equal amounts in both tissues with the exception of December 1977. Monogalactosyldiglyceride constituted, on the average, about 1/2 the concentration of glycolipid as the digalactosyldiglyceride.

Sycamore stem and root contained 5 major glycolipids, and several minor glycolipids which have not been characterized. Glycolipids were purified by chromatography on DEAE-cellulose columns. Chloroform:methanol (98:2, v/v) eluted glycolipids I (R_f, 0.89) and II (R_f, 0.68). Glycolipid (II) co-chromatographed on silica gel in solvent A and B with spinach leaf monogalactosyldiglyceride. Glycolipid (I) contained glucose and glycolipid (II) contained galactose. Chloroform:methanol (90:10, v/v) eluted 3 glycolipids from DEAE-cellulose-glycolipid (III) (R_f, 0.55), (IV) (R_f, 0.50), and (V) (R_f, 0.39). Glycolipid (V) co-chromatographed on silica gel in solvents A and B with spinach leaf digalactosyldiglyceride. Glycolipid (III) and (IV) contained glucose and glycolipid (V) contained galactose. Glycolipid (III) may contain a sterol moiety as determined by spraying with several steroid sprays.

Phospholipids

The average phospholipid content of stem tissue was 1.2 mg/g based on weight and 1.0 μmol lipid P/g based on lipid phosphorus determination. Little, if any, fluctuation was apparent on a weight basis, but, concentrations of lipid P during May through September was approximately 50% the concentrations observed during Spring and Winter. The average phospholipid content of root tissue was similar (0.9 mg/g and 0.8 μmol lipid P/g) to the concentration observed in the stems. However, the phosphorus determinations in root tissue indicated an essentially flat distribution profile, while the weight data indicated fluctuation with lows during March through July and a high in December 5 times that determined in Spring. Concentrations of phospholipids were similar in both stem and root tissues with the exception of May and July when stem concentrations were 2-3 times higher on a weight basis and in December and March when observed stem concentration exceed root concentration 2-fold.

Data tabulated in Table 3 on the distribution of individual phospholipids indicate that most of the seasonal fluctuation in total phospholipid was due to one compound, the unknown PL-A. Little, if any, fluctuation in the concentration of any other individual phospholipids were observed.

Sycamore stem and root tissue was characterized by 6 major phospholipids and several trace phospholipids.

The major phospholipid in both sycamore root and stem tissues, PL-A, has not been identified. PL-A was purified on a DEAE-cellulose column by elution with 0.01 M ammonium acetate in methanol, followed by TLC on silica gel G in solvent A. Products obtained after acid hydrolysis (2 N HCl, 2 hours, 110 C), mild alkaline methanolysis (4 C), and attempts to determine the lipid P-glycerol-ester molar ratio have proven to be inconclusive in structure proofs.

PE and PC were purified on a DEAE-cellulose column by elution with chloroform/concentrated acetic acid (3:1, v/v), followed by TLC on borate-impregnated silica gel H in solvent B. PE and PC co-chromatographed with authentic PE and PC on silica gel G in solvent A and borate-impregnated silica gel H in solvent B.

PE was ninhydrin-positive and yielded a water-soluble backbone identified as glycerylphosphorylethanolamine following mild alkaline methanolysis determined by TLC on cellulose in solvent system D.

PC yielded a positive reaction for choline with the Dragendorff reagent as described by Kates (1972). Mild alkaline methanolysis yielded a water-soluble glycerylphosphoryl ester which was identified as glycerylphosphoryl-choline by cellulose TLC in solvent system D.

PG was purified from a DEAE-column by elution with 0.01 M NH_4 acetate in methanol followed by TLC on silica gel G in solvent A. PG co-chromatographed with authentic PG on silica gel G in solvent A and on borate-impregnated silica gel H in solvent B. PG gave s positive staining reaction for vicinal hydroxyl groups by the periodate-Schiff reagent as described by Kates (1972). The water-soluble backbone derived from mild alkaline methanolysis of PG was identified as glycerylphosphorylglycerol by cellulose TLC in solvent D.

PL-B was purified on a DEAE-column by elution with .01 M NH_4 acetate and .06 M NH_4OH in $CHCl_3$/MeOH (4:1, v/v). PL-B has been tentatively identified as a phosphoglycolipid containing phosphate, glucose, galactose and inositol. Sugar moieties were identified by gas chromatography. The glycerylphosphoryl ester obtained upon mild alkaline methanolysis has not been identified.

PA was purified with DEAE-column chromatography by elution with 0.05 M NH_4 acetate, 0.3 M NH_4OH in $CHCl_3$/MeOH (4:1, v/v). PA co-chromatographed with authentic PA on silica gel G and borate-impregnated with silica gel H in solvents A and B, respectively. Mild alkaline methanolysis at 0 C yielded a glycerylphosphoryl ester which co-chromatographed with α-glycerophosphate on cellulose TLC in solvent D.

Neutral Lipids

The neutral lipid fraction of root and stem tissues of American sycamore was very complex. Data presented in Table 4 indicates the elution characteristic of the neutral lipid fraction from silicic acid columns; the R_f, co-chromatography, and the chemical reactivity of the separated neutral lipids after development by TLC.

Relative percentage distribution of the neutral lipids after development by TLC is given in Table 5. Because of the complexity of this fraction, and the error inherent in densitometry, these data are qualitative only.

Data presented in Tables 6, 7, 8 and 9 indicate that, on the average, removal of above ground biomass was not associated with changes in total lipid and/or phospholipid pool sizes when compared with uncut tap root tissue. On a weight basis, the average total lipid content of uncut trees was 5.1 mg/g and that of cut trees was 5.2 mg/gm (Table 8). North- and south-facing uncut trees contained 5.1 and 5.5 mg/gm, respectively; north- and south-facing cut trees contained 5.0 and 5.4 mg/gm, respectively (Table 8).

The lipid-P values indicate that cutting was not associated with changing pool sizes (uncut trees 3.5 μmol lipid P/gm; cut trees 3.5 μmol lipid P/g (Table 8). These data do indicate a possible difference in phospholipid content of north- and south-facing uncut trees (uncut - 2.7 μmol lipid P/g; uncuts - 4.0 μmol lipid P/g (Table 8)) and in the monthly response to cutting (Table 7). North-facing trees harvested in March appear to contain a higher concentration of lipid P one month after harvesting (3.2 μmol/g) than uncut controls (1.7 μmol/g). Also, these trees appear to exhibit some seasonal fluctuation in lipid P from 2.2 μmol/g in March to a high of 4.4 μmol/g in May. Conversely, south-facing trees did not exhibit this apparent fluctuation in lipid-P since the data ranged from 3.7 to 4.2 μmol/g during the

4-month sampling period. Variation among and between trees at each sampling period was high and could easily explain this apparent difference between north- and south-facing trees.

Taken collectively, the data presented in Tables 1 through 9 indicates that lipids do not play a prominant role as storage compounds in American sycamore stem and root tissues. Isolation and identification of the individual phospholipids and glycolipids indicate that the common phospholipids, PG, PE, EC and PA, are present in the tissue. The predominant phospholipid, PL-A, is unknown to date, and fluctuation in the pool size of this lipid in the stem largely accounts for the seasonal Summer low observed in total phospholipid. PL-B also is unknown, but appears to be a phosphoglycolipid.

Mono- and digalactosyldiglyceride comprise a large percentage of the total glycolipid content of sycamore tissue. Compounds with R_f of 0.50 and 0.55 largely accounts for the seasonal fluctuation observed in total glycolipid.

Characterization and Estimation of Lipid Components Extracted From Tissues of Pinus elliottii and Pinus palustris

Data presented in Tables 10 through 15 indicate that the glycolipids and phospholipids extracted from pine tissues are similar to those observed in sycamore.

Total glycolipid (Table 10) was higher in the green tissue culture cells of Pinus palustris (1.35 µmol/g) than in the colorless cells of Pinus elliottii (0.75 µmol/g). Mono- and digalactosyldiglyceride constituted 50% or more of the total glycolipid.

Conversely, the phospholipid (Table 11) content of the green and colorless tissue culture cells was the same, 2.7 and 2.3 µmol/gm, respectively. PC (50%) and PE (25%) accounted for the majority of the total phospholipid pool. The unknowns, PL-A and PL-B, accounted for approximately 17% of the total phospholipid.

Table 12 contains data relative to the distribution of glycolipids in isolated plastids and mitochondria from tissue cultures of slash and longleaf pine. In slash pine, the individual glycolipids with R_f values of 0.39, 0.50, and 0.55, appear to be concentrated in the plastids, while compounds with R_f values of 0.68 and 0.89 were concentrated in the mitochondria. In longleaf pine the compound with an R_f value of 0.89 was concentrated in the mitochondria, while the remaining compounds were evenly distributed in both subcellular organelles.

Table 13 contains comparable data on the distribution of phospholipids. In slash pine, PL-B, PE, and PL-A were concentrated in mitochondria; PA, PC, and PG were evenly distributed in plastids and mitochondria. In longleaf pine, PA was concentrated in the plastids; PL-B, PC, PE and PG were evenly distributed in plastids and mitochondria. PL-A was isolated from tissue culture whole cells, but was not detected in the extracts of the isolated particulates.

Silicic acid column elution characteristics and TLC characteristics of the neutral lipid fraction from tissue culture whole cells and isolated subcellular organelles are shown in Tables 14 and 15. All of these data are qualitative as was the case with these compounds from sycamore. In general, the neutral lipid fraction from the tissue cultures (Table 15) was not as complex as that extracted from sycamore (Table 5).

Table 1. Total lipid, neutral-, glyco-, and phospholipid content of stem and root tissues of American sycamore expressed on weight (mg lipid/gm fresh weight), on lipid P (μmol lipid P/gm fresh weight) and on glucose equivalents (μmol glucose equivalents/gm fresh weight).

	TOTAL LIPID	NEUTRAL LIPID	GLYCOLIPID		PHOSPHOLIPID	
DATE	mg/gm	mg/gm	mg/gm	μm glucose/gm	mg/gm	μm/gm
Dec. 77						
stem	-	-	-	-	-	1.3
root	-	-	-	1.0	-	0.5
Mar. 78						
stem	6.5	4.2	2.0	1.3	0.34	1.1
root	5.3	3.0	1.8	1.4	0.5	0.7
May 78						
stem	7.1	2.9	2.8	1.6	1.4	0.6
root	2.9	1.9	0.7	1.5	0.4	0.7
July 78						
stem	5.2	2.4	2.0	2.5	0.8	0.7
root	3.1	1.9	0.8	2.5	0.4	0.7
Sept. 78						
stem	7.2	4.2	1.8	1.9	1.2	0.6
root	4.7	1.8	2.1	2.2	0.8	0.5
Oct. 78						
stem	5.5	2.8	1.5	1.7	1.2	0.9
root	4.4	1.4	2.2	1.9	0.9	1.0
Nov. 78						
stem	6.8	3.7	2.1	2.3	1.0	1.0
root	5.4	1.7	2.8	2.6	0.8	0.8
Dec. 78						
stem	9.1	4.5	2.7	3.1	1.9	1.4
root	9.7	3.6	4.1	2.7	2.0	0.8
Mar. 79						
stem	8.9	5.4	1.9	2.6	1.5	1.3
root	6.2	2.5	2.5	3.7	1.1	1.0
May 79						
stem	8.0	4.5	2.4	2.5	1.5	0.7
root	5.6	2.6	2.2	2.4	0.8	0.8
AVERAGE						
stem	7.7	3.8	2.1	2.1	1.2	1.0
root	5.3	2.2	2.1	2.4	0.9	0.8

Table 2. Glycolipid content of stem and root tissues of American sycamore. Total glycolipid content expressed as μmol glucose equivalents/gm fresh weight. Each individual glycolipid expressed as percentage of total glycolipid fraction eluted from silicic acid columns.

	μm Glucose Equivalents/gm Fresh Weight									
	Dec. 77	Mar. 78	May 78	July 78	Sept. 78	Oct. 78	Nov. 78	Dec. 78	Mar. 79	May 79
R_f	Stem 2.9	Stem 1.3	Stem 1.6	Stem 2.5	Stem 1.9	Stem 1.7	Stem 2.3	Stem 3.1	Stem 2.7	Stem 2.5
Compound	Root 1.0	Root 1.4	Root 1.5	Root 2.5	Root 2.2	Root 1.9	Root 2.6	Root 2.7	Root 3.6	Root 2.4
0.39(V)[b]										
stem	34.4	28.5	24.8	31.1	28.8	28.3	30.7	18.5	23.8	23.5
root	18.8	39.6	31.7	31.0	30.1	18.1	18.2	21.9	23.8	23.3
0.50(IV)[b]										
stem	12.5	11.3	9.9	7.2	4.7	17.4	-	-	4.3	tr
root	6.8	24.8	9.7	8.8	6.7	-	a	a	11.7	tr
0.55(III)[b]										
stem	21.3	13.4	20.2	22.2	12.1	14.1	16.7	17.6	12.6	17.4
root	8.7	35.6	21.0	23.9	9.6	21.6	27.3[a]	22.0[a]	14.3	17.6
0.68(II)[b]										
stem	12.5	19.7	21.9	25.6	12.1	13.3	16.4	14.5	11.2	16.5
root	17.9	tr	19.7	21.6	17.3	16.1	12.9	13.1	11.6	16.5
0.75(GL-A)[b]										
stem	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND
root	34.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucose[b] 0.89(I)										
stem	19.4	27.2	23.0	13.9	18.0	18.4	12.6	15.1	9.2	10.8
root	13.3	tr	17.8	14.6	20.2	25.0	15.4	18.0	7.7	11.2
Rest[b]										
stem					24.0	18.0	24.0	23.0	39.0	31.0
root	0.3				16.0	19.0	25.0	25.0	31.9	31.4

[a]Combination of compounds with R_fs of 0.50 and 0.55.

[b]Data expressed as percentage distribution of each individual glycolipid of total glycolipid fraction eluted from silicic acid columns.

[c]Not detected.

Table 3. Phospholipid content of stem and root tissues of American sycamore. Total phospholipid and each individual phospholipid expressed as μmol lipid P/gm fresh weight.

	Dec. 77	Mar. 78	May 78	July 78	Sept. 78	Oct. 78	Nov. 78	Dec. 78	Mar. 79	May 79
Total PL										
stem	1.3	1.1	0.6	0.7	0.6	0.9	1.1	1.4	1.3	0.7
root	0.5	0.7	0.7	0.7	0.5	1.0	0.8	0.8	1.0	0.8
PL-A										
stem	0.8	0.7	0.3	0.4	0.4	0.5	0.7	0.9	0.8	0.4
root	0.2	0.3	0.3	0.3	0.3	0.4	0.3	0.4	0.4	0.3
PG										
stem	0.1	0.09	0.06	0.05	0.04	0.05	0.09	0.1	0.03	0.04
root	0.04	0.05	0.04	0.06	0.1	0.04	0.04	0.03	0.03	0.04
PE										
stem	0.1	0.2	0.08	0.09	0.03	0.07	0.1	0.09	0.1	0.05
root	0.06	0.1	0.08	0.06	0.04	0.1	0.1	0.1	0.1	0.08
PC										
stem	0.1	0.1	0.04	0.06	0.03	0.1	0.09	0.1	0.2	0.08
root	0.07	0.1	0.1	0.1	0.09	0.2	0.2	0.1	0.2	0.1
PL-B										
stem	0.07	0.04	0.02	0.04	0.03	0.05	0.05	0.06	0.07	0.05
root	0.03	0.06	0.06	0.07	0.03	0.07	0.05	0.05	0.1	0.1
PA										
stem	0.09	0.05	0.06	0.07	0.07	0.08	0.06	0.07	0.09	0.1
root	0.06	0.04	0.09	0.1	0.06	0.1	0.09	0.08	0.1	0.1

Table 4. Elution characteristics of neutral lipid fractions of American sycamore root and stem tissues from silicic acid columns and the R_f reactivity, and co-chromatography of separated components after development on silica gel TLC plates.

NL #	% Ether in Pet Ether	R_f	Co-Chromatographed with Following Standards After TLC	Other Chemical Reactivity Observed After TLC
1	2%	0.91	wax and sterol ester	sterol positive
1a	2%	0.84	-	-
2	2%	0.82	TG	-
3	4%	0.78	-	sterol positive
4	4%	0.67	-	sterol positive
5	4%	0.56	ubiquinone	-
5a	6%		free fatty acid	-
6	6%	0.44	-	sterol positive
7	6%	0.36	alcohol	-
8	25% & 100%	0.28	DG	-
8a	25% & 100%	0.27	cholesterol	sterol positive
9	25%	0.23	-	-
10	50%	0.21	-	-
11	50%	0.16	-	-
12	50%	0.08	-	-
13	50%	0.04	MG	-
14	100%	0.00	-	-

Table 5. Neutral lipid content of stem and root tissues of American sycamore. Total neutral lipid expressed as mg lipid/gm fresh weight. Each individual neutral lipid expressed as percentage of total neutral lipid fraction eluted from silicic acid column.

	mg/gm Fresh Weight									
	Dec. 77	Mar. 78	May 78	July 78	Sept. 78	Oct. 78	Nov. 78	Dec. 78	Mar. 79	May 79
TOTAL NL:										
stem		4.2	2.9	2.4	4.2	2.8	3.7	4.5	5.4	4.5
root		3.0	1.9	1.9	1.8	1.4	1.7	3.6	2.5	2.6
NL# & R_f:										
1 - 0.91[a]										
stem	2.0	12.8	10.7	17.9	15.0	11.5	10.9	22.3	16.9	6.1
root	11.7	13.5	15.5	13.7	19.4	20.3	25.3	28.0	17.1	22.1
1a- 0.84[a]										
stem	3.9	-	-	-	-	-	-	4.1	-	-
root	-	-	-	-	-	-	-	-	-	-
2 - 0.82[a]										
stem	14.8	17.3	10.4	12.4	9.2	8.8	9.1	5.6*	9.9	9.2
root	7.7	15.7	10.9	13.7	11.8	15.1	15.8	10.8	14.1	14.3
3 - 0.78[a]										
stem	4.3	2.3	-	-	-	-	-	*	4.6	-
root	-	2.2	1.6	1.9	-	-	-	3.1	5.6	-
4 - 0.67[a]										
stem	4.6	tr	4.1	4.0	3.8	3.1	4.5	2.8	5.2	5.0
root	2.3	1.9	1.5	1.9	3.9	tr	-	-	4.7	5.0
5 - 0.56[a]										
stem	4.2	3.2	-	tr	2.1	3.3	1.3	-	tr	-
root	1.6	-	tr	tr	tr	tr	-	-	2.1	-
5a- [a]										
stem	-	-	2.4	-	-	-	-	-	-	-
root	tr	-	-	-	-	-	-	-	-	-
6 - 0.44[a]										
stem	2.1	1.5	2.4	7.7	5.6	6.6	4.8	6.1	7.6	3.6
root	2.8	3.1	3.4	2.7	3.3	2.5	-	5.0	4.0	4.5
7 - 0.36[a]										
stem	3.7	6.0	6.3	6.9	tr	-	3.0	3.0	6.4	6.3
root	3.0	0.9	4.6	2.2	3.1	4.8	-	5.9	5.6	5.9
8 - 0.28[a]										
stem	4.7	4.4	9.1	9.9	-	-	-	2.0	3.4	7.5
root	10.7	4.0	3.6	8.4	6.1	4.8	-	1.7	4.0	4.3
8a- 0.27[a]										
stem	tr	tr	-	-	4.4	3.7	47.5*	-	-	-
root	-	-	4.6	-	-	-	-	-	-	-
9 - 0.23[a]										
stem	13.4	26.3	38.3	34.7	36.2*	40.8*	-	50.1*	28.2*	35.3*
root	50.5	28.7	45.7	37.1	37.7*	47.1*	58.9*	43.4*	38.5*	30.0*
10- 0.21[a]										
stem	4.6	3.2	-	-	-	-	-	-	-	-
root	tr	6.7	3.1	-	-	-	-	-	-	-
11- 0.16[a]										
stem	6.6	9.3	-	-	2.9	10.6	10.6*	3.9	6.2	7.8
root	tr	6.3	-	11.0	3.8	3.5	-	1.9	2.3	4.9
12- 0.08[a]										
stem	2.5	3.5	-	-	7.0	2.4	-	-	1.6	-
root	tr	8.9	-	-	4.1	1.4	-	-	-	2.8
13- 0.04[a]										
stem	7.0	5.8	3.5	3.5	3.6	2.8	5.8	-	2.4	16.1
root	4.3	5.2	3.7	4.2	5.7	tr	-	-	-	0.9
14- 0.00[a]										
stem	3.6	4.4	6.3	3.1	10.3	6.3	2.5	-	7.5	3.1
root	5.6	2.7	1.6	3.2	1.3	tr	-	-	2.1	5.5

[a] Percentage distribution of each neutral lipid total eluted from silicic acid column neutral lipid fraction.

Table 6. Total lipids extracted from root borings of American sycamore expressed as mg lipid/gm dry weight.

	Zero Time	1st Month After Cutting		2nd Month After Cutting		3rd Month After Cutting	
		Uncut		Uncut		Uncut	
	March	Control	Cut	Control	Cut	Control	Cut
All trees	5.2	4.0	4.0	6.8	6.3	5.7	5.2
N	5.5	3.2	3.2	6.8	6.6	4.5	5.1
S	4.4	4.8	4.6	6.8	5.8	7.0	5.4
	April						
All trees	4.0	6.8	5.4	5.7	5.4		
N	3.2	6.8	5.5	4.5	4.3		
S	4.8	6.8	5.3	7.0	6.4		
	May						
All trees	6.8	5.7	4.4				
N	6.8	4.5	3.7				
S	6.8	7.0	5.9				

Table 7. Total phospholipid extracted from root borings of American sycamore expressed as µmol lipid P/gm dry weight.

Sample	Zero Time March	1st Month After Cutting		2nd Month After Cutting		3rd Month After Cutting	
		Uncut Control	Cut	Uncut Control	Cut	Uncut Control	Cut
All trees	3.4	2.9	3.4	4.0	3.9	3.6	3.6
N	2.2	1.7	3.2	4.4	3.9	2.9	3.6
S	4.2	4.1	3.6	3.7	3.9	4.2	3.5
	April						
All trees	2.9	4.0	4.2	3.6	3.0		
N	1.7	4.4	4.3	2.9	2.5		
S	4.1	3.7	4.2	4.2	3.4		
	May						
All trees	4.0	3.6	3.0				
N	4.4	2.9	2.6				
S	3.7	4.2	4.0				

Table 8. Average lipid extracted from root tissue of American sycamore from March through June, 1979.

Sample	Uncut Trees		Cut Trees	
	mg lipid/gm dry wgt	µmol lipid P/gm dry wgt	mg lipid/gm dry wgt	µmol lipid P/gm dry wgt
All trees	5.1	3.5	5.2	3.6
N	5.1	2.7	5.0	3.5
S	5.5	4.0	5.4	3.7

Table 9. Monthly average lipid extracted from root tissue of cut and uncut trees from March through June, 1979.

Month	mg lipid/gm dry wgt	µmol lipid P/gm dry wgt
March	5.2	3.4
April	4.0	3.3
May	6.2	4.0
June	5.2	3.4

Table 10. Glycolipid content from field grown stem tissues of slash pine and from tissue culture cells of slash and long leaf pines. Data expressed as μmol glucose equivalents/gm fresh weight.

	Slash Pine		Long Leaf Pine
	Lateral stem tissue 8 yr old tree	Whole cell tissue culture	Whole cell tissue culture
Total Glycolipid	0.11	0.75	1.35
0.39(V)	0.03	0.2	0.5
0.50(IV)	0.01	0.1	0.3
0.55(III)	0.02	0.2	0.3
0.68(II)	0.03	0.1	0.3
0.89(I)	0.01	0.08	0.05

Table 11. Phospholipid content from field grown stem tissues of slash pine and from tissue culture cells of slash and long leaf pine. Data expressed as µg lipid P/gm fresh weight.

	Slash Pine		Long Leaf Pine
	Lateral stem tissue 8 yr old tree	Whole cell tissue culture	Whole cell tissue culture
Total Phospholipid	0.84	2.3	2.7
PA	0.08	0.1	0.3
PL-B	0.07	0.2	0.2
PC	0.3	1.0	1.1
PE	0.1	0.6	0.7
PG	0.07	0.2	0.1
PL-A	0.2	0.2	0.2

Table 12. Glycolipid content of whole cells and isolated particulates from tissue cultures of slash and long leaf pines. Data expressed as µg/mg lipid.

	SLASH PINE			LONG LEAF PINE		
	Whole Cells	Plastids	Mitochondria	Whole Cells	Plastids	Mitochondria
Total Glycolipid	232	325	187	208	230	250
0.39(V)	74	115	46	72	58	75
0.50(IV)	39	76	46	42	44	30
0.55(III)	53	93	36	40	58	70
0.68(II)	42	33	51	48	55	48
0.89(I)	23	7	13	8	16	28

Table 13. Phospholipid content of whole cells and isolated particulates from tissue cultures of slash and long leaf pines. Data expressed as µg/mg lipid.

	SLASH PINE			LONG LEAF PINE		
	Whole Cells	Plastids	Mitochondria	Whole Cells	Plastids	Mitochondria
Total Phospholipid	564	360	585	480	477	406
PA	44	53	43	58	147	25
PL-B	49	23	43	42	36	37
PC	244	168	255	194	158	193
PE	143	67	148	126	120	127
PG	49	43	50	20	19	29
PL-A	37	5	45	31	ND	D

Table 14. Elution characteristics of neutral lipid fraction of tissue culture cells and isolated particulates of slash and long leaf pines from silicic acid column and the R_f reactivity and co-chromatography of separated components after development on silicic gel TLC plates.

NL #	T Ether in Pet Ether	R_f	Staining Characteristics	Matching Standard*
1	2%	0.91	-	wax & sterol ester
2	2%	0.82	-	triglyeeride
3	4%	0.79	steroid	-
4	4%	0.67	-	
5b	4%		steroid	-
5c	4%		-	resin acid
6	6%	0.44	steroid	-
7	6%	0.36	-	alcohol
7a	6%		-	-
8	25%	0.28	-	diglyceride
8a	25% & 100%	0.27	steroid	cholesterol
9	25%	0.23	-	-
10	50%	0.21	-	-
11	50%	0.16	-	-
12	50%	0.08	-	-
13	50%	0.04	-	monoglyceride
14	100%	0.00	-	-

*Co-chromatographed in solvent C on silica gel G.

Table 15. Relative percentage of individual neutral lipids of total neutral lipid fraction of tissue culture cells and isolated particulates from slash and long leaf pines. Individual lipids were separated on silica gel TLC plates after elution of neutral lipid fraction from silicic acid columns.

NL #	R_f	Slash T.C. Whole Cell	Slash T.C. Plastids	Slash T.C. Mitochondria	Longleaf T.C. Whole Cell	Longleaf T.C. Plastids	Longleaf T.C. Mitochondria
1	0.91	24.9	26.9	39.4	29.3	33.2	16.8
2	0.82	21.9	13.5	10.7	30.3	16.9	20.9
3	0.78	8.7	8.8	5.4	14.1	13.2	22.5
4	0.67	5.7	ND	ND	ND	ND	ND
5b		ND	ND	ND	ND	ND	ND
5c		ND	ND	ND	ND	ND	ND
6	0.44	8.8	8.8	8.5	ND	9.4	5.2
7	0.36	2.9	12.2	6.9	ND	6.9	5.3
7a		ND	ND	ND	ND	ND	ND
8	0.28	2.4	ND	ND	ND	ND	ND
8a	0.27	31.5	13.1	24.6	26.3	13.2	20.2
9	0.23	ND	2.1	1.0	ND	1.3	ND
10	0.21	ND	ND	ND	ND	ND	ND
11	0.16	ND	5.5	1.5	ND	2.8	3.0
12	0.08	ND	4.0	ND	ND	ND	3.5
13	0.04	ND	1.9	ND	ND	ND	ND
14	0.00	ND	3.1	1.9	ND	3.3	2.6

ND = not detected

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CARBON FIXATION BY STEM TISSUE OF PLATANUS OCCIDENTALIS AND STEM AND TISSUE CULTURE CELLS OF PINUS ELLIOTTII

W. R. Finnerty and P. Kerr Falco

American sycamore has a very rapid growth rate. It is not unusual for sprouts to grow 10-20 feet during the first growing season. The main and lateral stems of this tree are green and significant amounts of chlorophyll can be extracted from 4-5 year-old stems. The question(s) arose if this chlorophyll was photosynthetically active, and if so, if stem photosynthesis could partially explain the rapid growth rate.

Stems were removed from lateral and main stems of field-grown stands of American sycamore at monthly intervals. Leaves were removed when present. Approximately 1 gm fresh weight of stem tissue was placed in 0.2 ml 10^{-3}M phosphate buffer, pH 6.8, in a 50 ml glass photosynthesis chamber. $^{14}CO_2$ (50-100 μc) was released in the gaseous form in the closed chamber by injecting acid into a solution of Na $H^{14}CO_3$. Dark controls were treated in the same manner. At the end of the incubation period (1-4 hours), the tissues were extracted in boiling 70% ethanol followed by extraction in boiling water. The alcohol and water extracts were combined and total radioactivity was estimated using a Searle scintillation spectrometer.

Stems, needles and stems, whole cells and isolated colorless plastids from tissue cultures of slash pine were also used in similar studies.

Light intensities used during the fixation studies are relative to one another in terms of wattage.

Tables 1 through 3 show the tabulated ^{14}C-fixation data collected during these studies. The average values reported are obtained by averaging at least 5 samples except where indicated (Table 1). The average rate of $^{14}CO_2$ fixation in the light by stems of American sycamore was 1.7 μg carbon fixed/g/hr (range 0.7-2.7). The average rate of $^{14}CO_2$ fixation in the dark under comparable conditions was 0.5 μg carbon fixed/g/hr (range 0.3-0.7). Increasing the light intensity 5-fold at the same concentration of CO_2 (1.5-1.8 μg CO_2/ml) increased the fixation rate from 0.4 to 1.5 μg carbon/g/hr. Increasing the CO_2 concentration from 1.8 to 3.0 μg CO_2/ml at the higher light intensity did not change the rate of light fixation (1.5 and 1.1 μg carbon/g/hr), but doubled the rate of dark fixation from 0.4 to 0.7 μg/carbon/g/hr. These data indicate that concentrations of CO_2 in air (0.6 μg CO_2/ml) would be rate-limiting to CO_2 fixation by stems of American sycamore. These data also indicate that the highest intensity light source available to us was rate-limiting at the lowest concentration of CO_2 used. Thus, we used 1.8 μg CO_2/ml and the higher light intensity source for the studies to ascertain the yearly average rate. This rate was limited by light so the fact that the monthly observed rate was constant throughout the year may not be realistic under conditions where photosynthetic energy generation would be maximal.

The average CO_2 fixation rates for the pine tissues are shown in Table 2. Stems of 3-month old seedlings fix carbon at least 15 times faster (40.0 vs 2.7 μg carbon/g/hr) than stems from 7-year old trees under the same laboratory

conditions. A five-fold increase in light intensity was associated with an apparent 8-fold increase in the rate of carbon fixation (0.35 to 2.7 μg carbon/g/hr) at the same concentration of CO_2 (1.9 μg CO_2/ml). Although the CO_2 concentration was not varied in the pine stem studies, the fact that stem slices exhibited a 6-fold higher rate of fixation than intact stems at the same light intensity and CO_2 concentration was indicative of a diffusional limitation on the rate of CO_2 fixation.

CO_2 concentration was varied in the tissue culture studies. The rate of CO_2 fixation by whole cells at 3.5 μg CO_2/ml (0.35 μg carbon/g/hr) increased approximately 40-fold (16.0 μg carbon/g/hr) at 86.5 μg CO_2/ml. Carbon fixation by isolated colorless plastids from the tissue culture cells was also increased by increasing amounts of CO_2. Aliquots from the same plastid preparation fixed carbon at the rate of 0.06 μg carbon/hr at 3.5 μg CO_2/ml and 0.82 μg carbon/hr at 86.5 μg CO_2/ml.

Calculated hourly rates of carbon fixation by stem tissue of field-grown slash pine after the indicated times of exposure to $^{14}CO_2$ are shown in Table 3. The time of harvesting the tissue varied from immediately before to 1.5-2 hours after sunrise. The calculated average rate of light fixation irrespective of the length of incubation was 2.7 μg carbon/g/hr (range 1.2-3.3 μg carbon/g/hr). The calculated average rate of dark fixation irrespective of the length of incubation was 1.1 μg carbon/g/hr. The time of harvest apparently did not effect the average rate of light fixation (2.0 vs 2.8 μg carbon/g/hr) but did effect the average rate of dark fiaation (1.7 vs 0.5 μg carbon/g/hr) as would be expected. Higher rate of dark fixation would be expected in tissues previously exposed to light because of increased amounts of TPNH, etc. This same response was apparent in the pine seedlings data reported in Table 2. The rate of dark fixation by the seedlings which were grown under continuous light was from 2 to 18 times faster than those observed from field grown trees harvested 1.5-2 hours after sunrise.

These experiments demonstrate only that stems of sycamore and pine can fix $^{14}CO_2$ and that the rate of fixation in the light exceeded the rate observed in the dark. Due to experimental limitations in light sources, it was impossible to obtain definitive data relative to a possible seasonal variation in the biological potential of sycamore stems to fix CO_2 and the maximal rate of fixation of CO_2 by sycamore stems at any time of the year.

Wiebe (1975) reported light enhanced CO_2 uptake in wood of first and fourth year twigs of Betula pendula Roth., Populus tremuloides Michx, Syringa vulgaris L., and Tilia americana L., but not in Gleditschia triacanthos. In Betula and Syringa, photosynthesis occurred in both primary and secondary xylem. Pearson and Lawrence (1958) had earlier demonstrated photosynthesis in Populus tremuloides during the winter months in Minnesota. Kriedemann and Buttrose (1971) demonstrated photosynthesis by woody shoots of Vitis vinifera L., and the role of woody shoots in the refixation of respiratory CO_2.

Primary fixation of atmospheric CO_2 during periods when the stems are bare and refixation of respiratory CO_2 at all times (but again especially during winter) could be of prime importance to tree maintenance if not the actual growth process.

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Table 1. CO_2 fixation by tissues of Platanus occidentalis

Tissue	µg CO_2 ml	Relative light intensity	µg carbon fixed/gm/hr light	µg carbon fixed/gm/hr dark	Previous light history
Stem	1.5	1	avg.0.4 range: 0.3-0.6	avg.0.06 range: 0.02-0.09	All samples harvested from field grown trees 2-3 hours after sunrise
Stem	1.8	5	avg.1.5 range: 0.8-2.7	avg.0.4 range: 0.3-0.5	
	3.0	5	avg.1.1* range: 1.1-1.3 yearly	avg.0.7* range: 0.7-0.8 yearly	
	1.8	5	avg.1.7 range: 0.7-2.7	avg.0.5 range: 0.3-0.7	

*Two samples only.

Table 2. CO_2 fixation by tissues of Pinus elliottii

Tissue	µg CO_2/ml	Relative light intensity	µg carbon fixed/gm/hr		Previous light history
			light	dark	
Needle & stem seedling	1.9	5	97.6[a]	1.4	laboratory grown under continuous light at intensity 5
Stem seedling	1.9	5	40.0[a]	11.0	
Stem - 7 year old trees	1.9	1	0.35	0.02	Field grown harvested
	1.9	5	2.7 -avg. range: 1.2-3.3	0.60-avg range: 0.5-0.8	2 hours after sunrise[b]
Stem slices - 7 year old tree	1.6	1	2.5	0.63	
Tissue culture whole cells	3.5	5	0.35	NS	Continuous light
	86.5	5	16.0	NS	
Tissue culture Isolated Colorless Plastids	3.5	5	0.06[b]	NS	Continuous light
	86.5	5	0.82[b]	NS	

[a]Value may be underestimated since >50% of total carbon was recovered in ethanol plus water extracts of tissue.

[b]Sample weight unknown. Each sample consisted of 1 ml aliquots from the same plastid preparation.

NS = no sample.

Table 3. Calculated hourly rate of carbon fixation by stem tissues of field grown Pinus elliottii after the indicated times of $^{14}CO_2$ fixation.

Time	Light	Dark
1 hour[a]	2.7	2.5
3 hours[a]	1.2	0.8
4.5 hours[b]	3.3	0.63
7 hours[b]	2.2	0.46

[a]Harvested 1.5-2.0 hours after sunrise.

[b]Harvested before sunrise.

INCORPORATION OF ^{14}C BY TISSUE CULTURES OF PINUS ELLIOTTII IN PRESENCE AND ABSENCE OF PARAQUAT

Suspension tissue cultures of P. elliottii obtained from Claud Brown were incubated with various radioactive substrates (Tables 1-2). The cells do not appear to produce resin acid under the present culture conditions, but in the presence of paraquat, increased ^{14}C was isolated and the resin acid area on neutral lipid TLC plates when glucose and palmitate were incubated with the cells. Total incorporation of radioactivity by the tissues was inhibited from glucose (5 to 10-fold inhibition), while palmitate uptake appeared to be stimulated by a factor of approximately 20. Total uptake of ^{14}C from acetate doulbed in the presence of paraquat, but the percentage of activity in the resin acid area was 3-fold lower than the control. Both total uptake and the percentage of the activity in the resin acid area decreased when serine-^{14}C was incubated with tissue cultures in the presence of paraquat.

Since acetate and palmitate uptake appeared to be stimulated by paraquat, experiments were performed with paraquat concentration ranging from 0 to 8 mg paraquat/50 ml incubation medium (Fig. 1). Significant differences were observed between 2 control flasks during each experiment (e.g., control 1 - 2,958,549, control 2 - 5,512,476 CPM/gm dry wt). It appears that additions of 1 and 2 mg paraquat did stimulate total incorporation (9,200,000 and 17,900,000 CPM, respectively) of acetate carbon into pine cell suspension culture cells, while total incorporation with 4 and 8 mg paraquat were equivalent to the controls.

TABLE 1

Tissue Culture - Uptake of Radioactive Substrate - Glucose-U-^{14}C
(CPM/gm air dry wt)

	Total CPM extracted by C:M (2:1 v:v)			% CPM recovered in RA area on 80:20:1 TLC plate		
SAMPLE	3 hrs	7 hrs	14 hrs	3 hrs	7 hrs	14 hrs
Control	52,615	98,000	116,500	2.3	1.5	3.6
Paraquat	178,666	44,739	17,000	3.6	2.4	7.3

TABLE 2

Tissue culture indubated for 6 hrs with glucose-U-^{14}C, serine-U-^{14}C, palmitate-^{14}C, and acetate-2-^{14}C.

SAMPLE	CPM/gm air dry wgt	% CPM recovered in RA area on 80:20:1 TLC plate
Acetate		
Control	304,036	4.9
Paraquat	786,310	1.7
Glucose		
Control	445,339	1.6
Paraquat	103,129	3.4
Palmitate		
Control	45,947	0.6
Paraquat	837,024	4.2
Serine		
Control	1,610,665	1.9
Paraquat	136,459	0.5

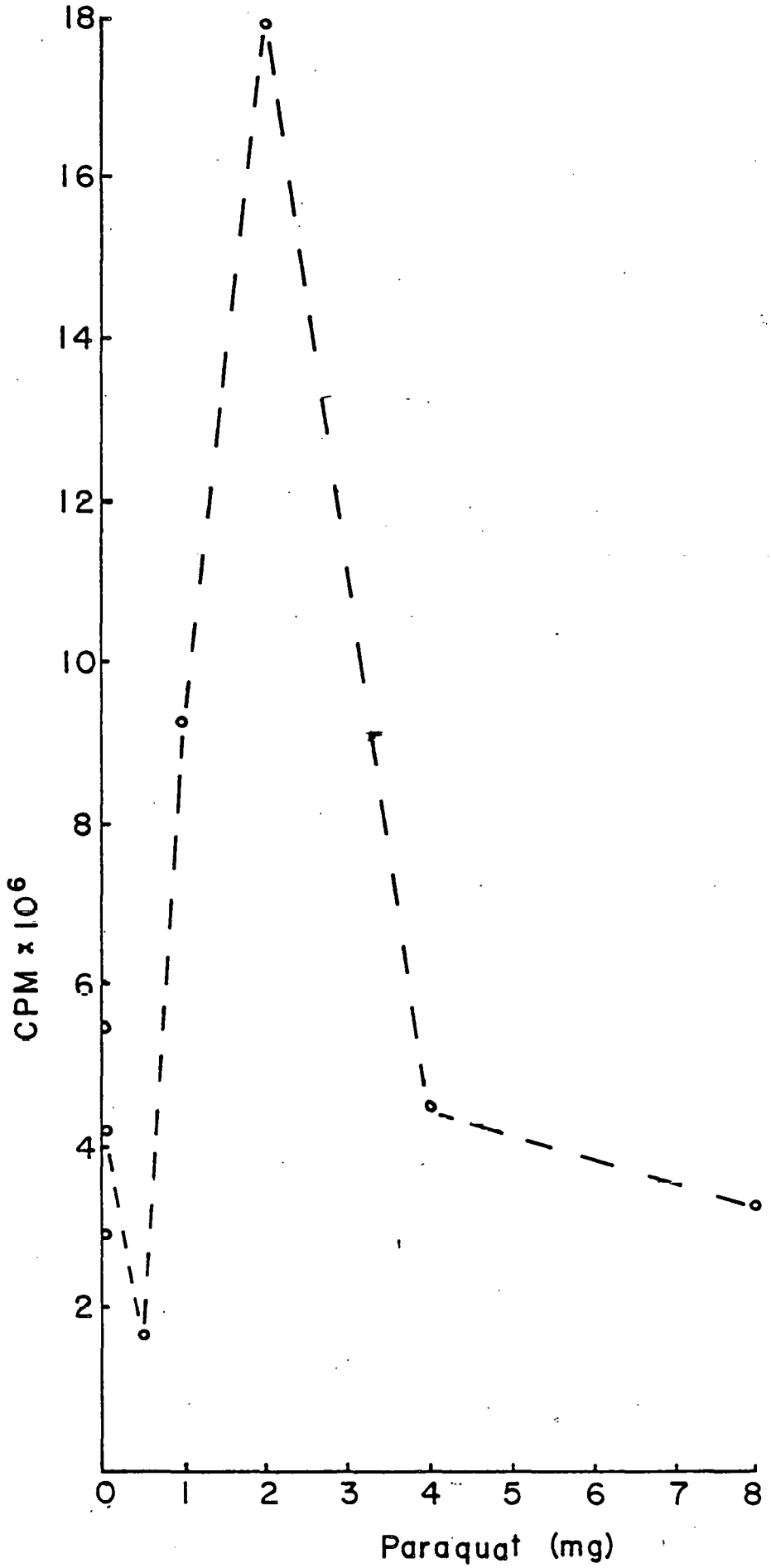

Fig. 1

FINAL REPORT

BIOCHEMISTRY AND PHYSIOLOGY OF METHANE FORMATION FROM CELLULOSE

(Drs. Ljungdahl and Peck)

The anaerobic fermentation of cellulose to methane and carbon dioxide is viewed as a complex fermentation involving at least four physiological types of microorganisms. The first stage is the conversion of cellulose to products, i.e., CO_2, H_2, acetate, ethanol, lactate, etc., and the second stage, the conversion of the fermentation products to methane and carbon dioxide. The first stage requires the presence of a primary bacterium, a cellulase-producer, plus a secondary bacterium, usually a cellobiose fermentor, to remove reducing sugars which appear to be inhibitory to cellulase activity and biosynthesis. The second stage also requires two basic groups of microorganisms; ancillary bacteria responsible for the oxidation of fermentation products to substrates for methanogenesis, i.e., acetate, CO_2, H_2, and finally the formation of methane by the H_2-utilizing and acetate-utilizing methane bacteria. Taxonomically, the microbiological flora involved in these fermentations can be quite variable depending upon the temperature, cellulose source, fermentation patterns in the first stage and pH, but their function and physiology in the process is expected to be quite comparable in different fermentations.

Cellulytic bacteria, cellobiose fermentors, sulfate reducing bacteria and H_2-utilizing methanogenic bacteria have been isolated from established mesophilic and thermophilic cellulose-methane fermentations and these isolates, plus known laboratory strains, have been utilized to partially reconstitute highly active cellulose fermentation to products such as ethanol, acetate, butyrate and methane. The critical observation appears to be the requirement that the secondary bacterium have the capability of fermenting (removing) cellobiose which is both a product and inhibitor of cellulase activity. A second important

observation involves the products formed by the reconstituted cellulose fermentations. In all cultures studied, the products are those of the secondary bacterium (a cellobiose fermentor) rather than the cellulytic organism. Thus, when a single strain of a primary organism (cellulase producer) which forms a mixture of fermentation products is grown with a secondary bacterium which forms only acetate, one observes the conversion of cellulose to acetate. Similarly, a mixed culture of the primary organism with a secondary bacterium which form mainly ethanol catalyzes the conversion of cellulose to ethanol. From these general considerations, one can conclude that this anaerobic fermentation has high potential for the conversion of cellulosic materials to chemical feedstocks, i.e., acetate, ethanol, propionate, butyrate, etc., in the first stage and methane in the second stage. The variability and diversity of the microorganisms involved in the fermentation should make it possible to arrange or manipulate the fermentations to produce a single product such as ethanol under desirable conditions such as high temperature. However, before the fermentation can be utilized extensively in a practical sense, the microorganisms involved, their physiology and biochemistry must be understood in order that the fermentation can be controlled and maximum yields of products may be obtained under optimal conditions for product recovery.

Anaerobic thermophiles have not been extensively studied and little information is available concerning their temperature range, taxonomy or physiology. As the thermophilic fermentation of cellulose exhibits properties which may make it economically the process of choice, we have been investigating organisms which produce high proportions of ethanol and acetate from cellobiose and glucose for use in reconstituted fermentations. A thermophilic ethanol-forming anaerobe was recently isolated in our laboratory from mud samples taken from hot springs in the Yellowstone National Park. Its morphology and fermentation pattern

differ from all known extreme thermophilic bacteria, and we are quite convinced that this is a new type of bacterium, which has not been described earlier. The name Thermoanaerobium ethanolicum, which we have suggested, describes its properties. "Thermo" indicates that it is a thermophile. The temperature characteristics are $T_{min} = 37^{\circ}$, $T_{opt} = 65^{\circ}C$, and $T_{max} = 78^{\circ}C$. "Anaerobium" indicates that it grows and multiplies only under anaerobic conditions, and "ethanolicum" indicates that ethanol is the main product. When the pH is controlled, the yield of ethanol is as high as 1.8 moles per mole of glucose. The fermentation may then be summarized as follows:

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$$

This stoichiometry resembles the fermentation of glucose to ethanol by yeast, and as it seems to have high potential for the commercial production of ethanol, we have filed for a patent.

T. ethanolicum ferments cellobiose as well as a wide range of hexoses and pentoses and we have been able to couple this new bacterium with a thermophilic cellulose-fermenting clostridia with enhanced yields of ethanol.

Employing mixtures of first and second stage organisms we have been able to obtain conversion of most but not all of the fermentation products to methane. The microbiology of the second stage, i.e., the conversion of fermentation products to methane and CO_2 has been a very active area of research both in the U.S. and abroad and we have been studying the individual physiologies of these organisms in an effort to better understand their interactions in mixed cultures. Emphasis has been placed on the mechanism and bioenergetics of acetate formation by species of Clostridium and Acetobacterium, the bioenergetics of sulfate-reduction and interspecies H_2 transfer by species of Desulfovibrio and Desulfotomaculum and electron transfer and proton translocation by the H_2-utilizing methanogenic bacteria. The major results are summarized in the following

paragraphs.

Microorganisms that carry out fermentations and form acetate as essentially the only product are called "homoacetate fermenting" and the best biochemically characterized homoacetate fermenting organism is C. thermoaceticum.

A new type of acetate-forming microorganism Acetobacter woodii has been isolated by others which derives the energy required for growth from the oxidation of H_2 coupled to the reduction of CO_2 to acetate. It is of interest because it could be extensively involved in interspecies hydrogen transfer in a manner analogous to the methane bacteria and could amplify the formation of acetate in the anaerobic cellulose fermentations. In addition, it is possible to conceive of a fermentation in which all the methane was produced from acetate using this new physiological type. We have demonstrated that the pathway of acetate formation with minor exceptions is identical to that occurring in C. Thermoaceticum involving B_{12} and folate enzymes.

In an attempt to resolve the problem of the pathway of respiratory sulfite reduction in the sulfate reducing bacteria, we have purified the three bisulfite reductases from these bacteria and characterized them with regard to siroheme, siroporphyrin, non-heme iron and other properties. Their similarities are inescapable. Utilizing EPR and optical spectroscopy, the reduction of the heme component with methyl viologen reduced by hydrogenase and the reduction of hon-heme iron by a mixture of dithionite and methyl viologen has been observed with all three enzymes. Apparent non-reactivity and variability toward dithionite is postulated to be due to the formation of sulfite in dithionite solutions and different reactivities of the chromophores. We have concluded that methyl viologen functions by donating electrons primarily to the siroheme and in the presence of excess sulfite, imposes a two and/or four electron reduction on the enzyme resulting in the formation of trithionate and

thiosulfate. The physiological pathway presumably involves the six electron reduction of sulfite to sulfide and considerably simplifies our ideas concerning electron transfer in these organisms.

An alternative approach to the study of the bioenergetics of respiratory sulfate reduction has been the investigation of a strain of D. vulgaris which is capable of respiratory reduction of nitrate to ammonia.

As both the reduction of sulfite and nitrite are six electron reductions, we anticipated that the nitrite reductase might produce some insights into the bioenergetics of sulfite reduction as it appeared to be a more simple system and possibly contain a siroheme as prothetic group. The enzyme has now been purified and has a molecular weight of 66,000. In contrast to the bisulfite reductases, this reductase exhibits a typical cytochrome c spectrum, is membrane-bound and is unique in containing six hemes per mole. From its heme content it appears to be a new-type of nitrite reductase particularly suited for the six electron reduction. It differs from other characterized nitrite reductases in lacking cytochrome d, absence of siroheme and the number of hemes.

Our laboratory has been highly successful in isolating low molecular weight electron transfer proteins and respiratory enzymes from the sulfate reducing bacteria and characterization of these proteins has produced new insights regarding the physiology, phylogeny and ecology of these bacteria. This approach has been employed with the thermophilic H_2-utilizing methanogenic bacterium M. thermoautotrophicum and the preliminary results can be summarized as follows:

1. A simple technique was devised for isolating the low molecular weight cofactors, F_{420}, F_{430}, F_{340}, without destroying the proteins of the cells by boiling.

2. F_{430} was purified and found to undergo a redox cycle.

3. At least one new cofactor appears to be present in the low molecular weight fraction.

4. Catalase is absent but extracts contain a single superoxide dismutase. The absence of catalase is interesting and supports the idea that methane bacteria can not synthesize heme.

5. High levels of a soluble hydrogenase stable to oxygen were found and the enzyme purified. It appears to be a non-heme iron protein similar to that found in C. pasteurianum in physical properties and is being further characterized.

6. A low molecular weight protein was isolated which appears to be a rubredoxin from its spectral properties and the fact that it is reduced by sodium ascorbate.

7. A low molecular weight non-heme iron protein was also isolated which may be ferredoxin as it is active in the phosphoroclastic reaction; however, further characterization is required before it is conclusively identified as a ferredoxin.

8. A new low molecular weight protein with an absorption maximum at 430 nm was also isolated but we were unable to detect a redox cycle.

These observations are extremely exciting from the point of view of the bioenergetics, mechanism of CO_2 reduction to methane and the phylogeny of the methane bacteria and this aspect should be actively pursued over the next few years.

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