MASTER

SRO-0888-2

A MULTIDISCIPLINARY RESEARCH PROGRAM
DIRECTED TOWARD UTILIZATION OF
SOLAR ENERGY THROUGH BIOCONVERSION
OF RENEWABLE RESOURCES

Progress Report

William R. Finnerty, Project Officer
Office of Energy Research
Biological Sciences, Room 831
University of Georgia
Athens, Georgia 30602

November, 1977

PREPARED FOR THE
DEPARTMENT OF ENERGY
UNDER CONTRACT EY-76-S-09-0888

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

PROGRESS REPORT CONTENTS
(EY-76-S-09-0888)

PAGE

1. Disposition Document

2. Progress Report (SRO-0888-1)

a) Disclaimer Statement-----	1
b) Abstract to Program A - Brown & Steinbeck-----	2
c) Program A Progress Report - Brown & Steinbeck-----	4
d) Program B Abstract - Finnerty-----	12
e) Program B Progress Report - Finnerty-----	13
f) Program C Abstract - Peck and Ljungdahl-----	61
g) Program C Progress Report - Peck and Ljungdahl-----	62
h) Reprints	
1) SRO-0888-2	
2) SRO-0888-3	
3) SRO-0888-4	
4) SRO-0888-5	

DISCLAIMER STATEMENT

"This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately-owned rights."

Abstract of Progress Report

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

Investigators - Brown and Steinbeck

1. Techniques were developed for mass cloning American sycamore (Platanus occidentalis) using single buds from dormant and active twigs of one- and two-year-old sprouts and young trees for use in physiological studies and for multiplying selected genotypes in clone banks.
2. Strong positive correlations were obtained between callus formation and growth of cells (fresh and dry weights) on a defined basal medium with the growth performance of 5-year-old trees in the field previously selected on the basis of phenotypic expression of vigor (slow and fast growing clones).
3. Weak positive correlations were obtained between callus growth and vigor of repeatedly coppiced clones from putative fast and slow growing field selections (paired observations from the same field plots).
Tissue culture explants were all taken at the distal, third internode from one-year-old sprouts growing on 10-year-old root stocks. The trees had been harvested three previous times at 3-year-intervals.
4. A mixed plantation of Alnus glutinosa and sycamore was established at a 4 x 8 foot spacing to study the future effects of the nitrogen fixing European black alder on total biomass production.
5. Seasonal levels of reserve foods (sugars and starch) were followed in root stocks of American sycamore to learn more about the pattern of carbohydrate recycling in repeated harvested (coppiced) young plantations.

6. Greenhouse studies of interactions between a slow and fast growing clone of sycamore with photoperiod (long and short night periods) had to be abandoned during the summer months because of failure in control of daily temperatures. The study is being re-established.
7. Sporadic root differentiation was obtained on sterile x-sectional stem discs of sycamore in vitro after testing 25 various combinations of IAA and cytokinin. No intact plantlets have been formed todate, but studies are still in progress.
8. Anther cultures of sycamore and cottonwood were attempted on Gresshoff and Doy's and Nitsch's haploid media. Some callus was observed in various cultures, but no haploid plantlets were differentiated in these early attempts.

PROGRESS REPORT

Research Program A: Genetic selection, physiological basis of vigor, tissue culture, and haploid cell lines.

Date: December 31, 1977

Investigators: Brown and Steinbeck

1. Work relative to "genetic selection" and other field studies.

Sixteen additional clones of American sycamore were selected for phenotypic expression of poor or high vigor for use in tissue culture screening tests described below under section 2b.

Although sycamore stem cuttings from one- and two-year-old coppice (root sprouts) can be rooted with a high degree of success during the dormant season from most selected clones, it becomes important to develop cloning techniques for rapidly multiplying selected genotypes in clonal banks during various seasons of the year. Likewise, while tissue culture techniques are being developed for producing plantlets from callus cultures, or embryoids and plantlets from suspension cell cultures, we sought and developed a technique for multiplying selected clones by using single axillary buds attached to a small 5-10 cm internode segment. Buds were collected at 3 month intervals during the season and placed in small fiber containers (tubes) about 25 x 150 mm in length) in growth chambers under long day (18 hrs) with intermittent mist and supplied with complete inorganic nutrients weekly. Dormant buds taken just before spring growth gave 90-100% rooting success and could be transplanted to the greenhouse or lath-house for hardening off before field planting. Axillary buds formed in early spring could likewise be propagated in this manner, but later in the season sycamore buds failed to extend without 30 or more days

of 5°C cold prior to placing in the growth chamber. Interestingly enough, this cold requirement does not exist for several other hardwoods under test, namely, Liriodendron (tulip poplar), cottonwood (Populus deltoides), red maple (Acer rubrum), and others. Information of this sort and techniques for rapid, successful propagation are essential to both genetics research and future commercial production of high vigor clones.

In order to determine the effects of the nitrogen fixing European black alder (Alnus glutinosa) on the biomass production of American sycamore (Plantanus occidentalis), a plantation was established at the Whitehall Forest on an upland site in February 1977. Each species is grown in control plots by itself and two mixing strategies, alternate rows of each species and alternate trees within rows, are being tried. Four replications of these four species possibilities were established at a constant 4 x 8 foot spacing. Plot size is 64 x 44 feet. The summer drought caused high mortality in the plantation, especially among the alder. During the winter of 1977/78 the dead trees will be replaced. This plantation is too young to provide data.

2. Work relative to "physiological basis of vigor."

(a) Seasonal Levels of Food Reserves

A graduate student, Mr. Mark Blum, has spent essentially full-time research this year on seasonal aspects of carbohydrate levels in coppiced root stocks of sycamore. Quantitative measurements of soluble sugars and starch content are being completed and this work will constitute the basis of a masters thesis and publishable results within the next few months.

(b) Attempts to Screen for Inherent Vigor Using Tissue Culture
Techniques

As a follow-up on last year's observations of the proliferative ability (initial callus formation) of phenotypically selected putative fast and slow growing clones of sycamore on chemically defined nutrient agar, additional experiments were conducted this year on two types of field selections, namely (1) trees previously selected and propagated as either high or low vigor clones and grown for 3 to 5 years in the field without coppicing (no cutting), and (2) 2-3 year old sprouts from ten-year-old stumps repeatedly coppiced at 3 year intervals, where putative high and low vigor clones were selected in pairs from the same field plots.

Twenty-four replicates, using 1.0 cm stem segments from the third internode of currently elongating shoots, were explanted from each of the above selected clones (total 16 clones). The sterile explants were each weighed aseptically and placed on a chemically defined medium previously adopted for rapid callus production of sycamore. These were maintained under relatively uniform cultural conditions (e.g., $25^{\circ} \pm 3C$, ca. 500 f.c. cool-white fluorescent light, 16-hr photoperiod) for 30 days to allow for adequate callus formation, then sacrificed to obtain fresh and dry weights.

Briefly, the increase in fresh and dry weights of the explants from the first group of previously selected and tested clones correlated exceptionally well with field performance, i.e., the clonal response in the laboratory conformed to the observed

vigor rating in field trials in 5 out of 6 clones. The aberrant clone had to be discarded because of error in recording initial fresh weights. In fact, the dry weights of this clone fell well within the expected values, suggesting that the vigor of all 6 clones could be quantitatively screened by increase in cell number (callus formation). Increase in dry weight also appears to be a more reliable index than either increase in fresh weight or increase in volume because of inherent clone differences in cell size on the same medium.

In the second group of coppiced trees selected on the basis of putative high and low vigor clones growing side by side in the same field plots, the increase in fresh and dry weights of each cultured clone (callus) corresponded to the vigor of the original selected clone in only 6 out of 10 clones, viz., slightly better than a 50/50 random chance prediction. In fact, the mean weight of two slow growing clones exceeded the mean weight of all 5 high vigorous clones; whereas, the means of 2 fast growing clones were less than the mean value of all 5 slow growing clones. These observations substantiate the long recognized complexities of identifying genotypic-environmental interactions over time involving such parameters as shoot and root competition between clones, number of sprouts per clone, effects of repeated coppicing, changes in leaf area indices, nutrient and water availability, etc.

The good correlations obtained between previously field tested clones and subsequent growth of these clones in vitro

appears to hold promise in identifying the most promising genotypes at an early age in making phenotypic selections of young trees. However, one has to recognize the limitations of such an artificial system and supplement it by subsequent, well-replicated, field trials.

(c) Photoperiodic Screening for Vigor

Earlier proposed research relating to the use of photoperiod as a tool in screening for inherent vigor (genotypic response) in woody plants has twice fallen to fortuitious technical difficulties. In the first instance inadequate numbers of individual remets of a single "slow" and "fast" growing clone were propagated for meaningful analysis; during the past growing season when the problem of clonal propagation was solved, an intensive period of summer heat in our "wet-pad" cooled greenhouses wiped out any valid comparison of growth responses between potted seedlings of "high" and "low" vigor clones under long- and short-day treatments. The concept is still viable, and new propagules of selected clones will still be tested under hopefully corrected, semi-controlled greenhouse studies. The basic concept proposed is that an enhanced 2-3 fold increase in height growth of seedlings receiving a low light intensity interruption of the dark period vs. normal day length conditions is far above the normal photosynthetic fixation of CO_2 and hence specific genotypes may be identified for their ability or capacity to mobilize and redistribute current photosynthate in a more "efficient" manner than other seedlings fixing comparable amounts of CO_2 under similar light intensities. If the more

"vigorous clones" respond to increased dry matter production under both short and long-day conditions it may be that specific genotypes could be recognized at an early age. Certainly this response needs more investigation, and new clones are being established for this purpose.

In Vitro Studies of Plantlet Formation in Sycamore

Young, primary tissues of the uppermost elongating internodes of sycamore from 3 selected clones were explanted to 25 different combinations of bud and/or root differentiation media using a modified Murashige and Skoogs basal medium. The primary variables in factorial design were: IAA (0 to 10 mg/l) and BAP (benzyl aminopurine) at (0 to 10 mg/l). Varying degrees of organogenesis have been observed in various cultures (mainly root initiation) but no embryoids or plantlets have been obtained thus far. The problem in working out techniques for mass propagation or cloning of sycamore and other short-rotation hardwoods lies in formulating the proper medium and system for each species. Experimental approaches are still largely empirical and final systems will likely require environmental manipulation of light and temperature along with hormonal responses to ellicit mass in vitro cloning responses. These procedures are in progress.

Haploid Cell Lines

Only two attempts were made this past Spring (1977) to induce haploid plantlet formation in two species; namely cottonwood (Populus deltoides) and sycamore (Platanus occidentalis) on two media (Gresshoff and Doy's and Nitsch's) both previously used for the production of haploids in

tomato and tobacco respectively. Both failed. Anthers were explanted prior to meiosis, at the tetrad stage, and at later stages of microspore maturation (mature pollen). Callus was observed in some cultures, but bud or root differentiation failed to occur. The genetic potentialities for use of haploid plantlets and possible development of homozygous lines in forest trees is obvious, hence we plan to intensify and extend this effort to several other woody species which hold promise in biomass production.

Publications and other Activities

Steinbeck, K., and C. L. Brown. 1976. Yield and utilization of hardwood fiber grown on short rotations. In: Applied Polymer Symposium No. 28, 393-401. John Wiley and Sons, Inc.

Brown, C. L. 1977. Potentialities of biomass as sources of energy. In: *The Energy Crisis: overview and analysis. Colloquium on Energy, the Georgia Institute of Technology and the University of Georgia.*

Sommer, H., and C. L. Brown. 1977. Application of tissue culture to forest tree improvement. Paper presented at IV Biological Sciences Colloquium on Plant Cell and Tissue Culture, Principles and Application. Ohio State University.

Birchem, R., and C. L. Brown. 1977. Identification of oleoresin in epoxy-embedded slash pine tissue. Submitted to *Stain Technology*.

Brown was invited to present seminar and participate in discussion of short-rotation hardwoods as sources of biomass and fuelwood for developing countries with scientists from A.I.D., State Department and NAS, Washington, D. C. October 14, 1977.

Brown was invited participant and discussant on Wood Food and Energy Symposium at Ohio State University, Sept. 7, 1977, held at IV Annual Biological Sciences Colloquium.

PROGRAM B - FINNERTY

The research program for the past 15 months consisted of the following personnel:

W. R. Finnerty, principal investigator

Mrs. P. Kerr Falco, research associate, 100%

Mrs. Maria Stanton (Jan. 1977-Aug. 1977), 100%

Mrs. Mary Singer (Oct. 1, 1977 to present), 100%

Publications

1. Seasonal Variation of Resin Acids and other Metabolites in Pinus elliotii. W. R. Finnerty and P. Kerr Falco. 1977. Proceedings of the Annual Lightwood Research Council. in press.

Meeting Presentations

1. Seasonal Variation of Resin Acids. W. R. Finnerty and P. Kerr Falco. 4th Annual Lightwood Research Council Meeting, January, 1977, Jacksonville, Florida.

Project B. The Biochemical Basis of Paraquat Induced Oleoresin Production
in Pines. Principal Investigator, W. R. Finnerty.

ABSTRACT

Data collected from both field and laboratory studies during 1977 confirm and extend those observations and conclusions drawn from the data reported in 1976. Soluble metabolites; carbohydrates, keto acids, amino acids, and peroxyacids are mobilized when tissues of P. elliotii are injured. Little qualitative or quantitative difference between control and paraquat-treated tissue mascerates or cut controls and paraquat-treated intact field grown 5-year old trees were observed during these studies. 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 appeared to be available for resin acid synthesis. Paraquat treatment did not alter the percent composition of individual resin acids. Thus, paraquat apparently affected the rate of resin acid synthesis, but did not appear to alter the normal biosynthetic pathway for resin acid synthesis.

Paraquat may effect both the rate and amount of monoterpenes produced by P. elliotii. Loss of up to 90% of the ^{14}C fixed by volatilization following paraquat treatment could be indicative of loss of monoterpenes.

METHODS

Methods used are as described in the 1976 ERDA Progress Report and/or the preprint on metabolite seasonal variation included with this progress report.

Thin layer chromatography, silicic acid column chromatography (Malula et al., 1975) and high pressure liquid chromatography were used to separate and concentrate resin acids. The latter separation was performed by Micromeritics Corporation of Norcross, Georgia, utilizing a silver loaded Partisil SCX column, hexane/THF/MeCN (80/20/0.02) as solvent (flow rate 1 ml/min) at 400 psi pressure, and ambient temperature. After initial separation utilizing one or more of the above procedures, samples were methylated, developed by TLC, and the methyl resin acid area eluted, counted, and placed on GC to determine the qualitative and quantitative composition of the sample.

Methodology has received increased time and attention this year because of the appearance of problems with recovering radioactivity by liquid scintillation spectroscopy in plant extracts after development with neutral lipid solvents on TLC silica gel G plates. Tables 1, 2, and 3 contain data indicating recovery efficiency for ^{14}C from the same extract after development on silica gel G-TLC plates and silicic acid column chromatography. Nine to 15 fold more activity was recovered from the plates than was placed on the original origin. The count obtained from the crude extract was reproducible, and 11,000,000 of the original 15,000,000 CPM placed on a silicic acid column were recovered (Table 2). When these fractions were placed on the origin of silica gel G plates, recoveries varied from 50% to 28X more than was placed on the original origin (Table 3). Of these seven samples, 4 to 28 fold more activity was recovered from 5 of them than had been placed on the

origin. I would estimate that this ratio of 5:7 would hold for all the samples analyzed since spring, 1977. Data included in Table 4 indicate recovery of phospho- and glycolipids placed on a silicic acid column (2,485,000 CPM recovered from the original 3,000,000).

Data in Table 5 indicates another problem. The resin acid area was eluted from a neutral lipid plate (increase in activity on this plate was observed) and contained an apparent 5000 CPM. After elution, 43,882 apparent CPM were obtained from the column. Activity moving to the resin acid area was present in all column fractions (Table 5). Although the data is not shown, this was true in all extracts tested.

Since the counts present in the original samples are reproducible, and 75-80% of the activity could be eluted from silicic acid columns there appears to be some interaction between the extract, the silica gel G, and the solvents resulting in the formation of fluorescent material(s). When crude plate extracts, fractions from silicic acid columns, and samples eluted from silica gel G-TLC plates were developed on a liquid chromatograph, negative absorption peaks (indicative of fluorescent materials) were apparent only in those samples that had been developed with neutral lipid solvents on silica gel G plates (LC scans - Appendix this report).

Mass recovery of resin acids from silica gel G-TLC plates appears to be more dependable than the count recovery. Ninety to 95% of the total resin acids placed on the origin of a TLC plate could be recovered from the resin acid area, but differences in individual resin acids were observed (palustric acid was converted to isopimaric acid and abitic acid to dehydroabitic acid). Mass recovery from the original extracts was reproducible for periods of several weeks and there was negligible degradation of individual resin acids. Mass recovery of methyl resin acids averaged 41% (range 33.3 to 50%) of the

resin acids in the original extract. No residual resin acids could be detected by gas chromatography in the resin acid area of the plates from which the methyl resin acids were eluted. Apparent radioactivity was present in both the resin acid and the methyl resin acid areas..

While gas chromatography (single column) was used as a qualitative and quantitative technique in these studies, difficulties associated with this technique have been discussed by Zinkle (1977). Differential methylation of individual resin acids intramolecular transformation on various columns, and differential sensitivity and recovery of individual resin acids on different columns make quantitative recovery of all of the resin acid difficult and would entail the use of multiple columns.

Liquid chromatography appears to be the method of choice. Micromeritics Corporation of Norcross, Georgia, has separated resin acid standards (none of which are pure) and some of our experimental samples at ambient temperature with no prior derivatization of the samples. Scans of separation from crude extracts of slash pine and tissue culture cells, of fractions eluted from silicic acid columns, and of materials eluted from silica gel G-TLC plates included in an appendix to this report, indicate that liquid chromatography could be used as both the preparative and analytical procedure for resin acid separation and purification. Implementation of the proposed ^{13}C -NMR work is dependent upon the successful separation and purification of the resin acids.

RESULTS

Data presented in Fig. 1-8 and Tables 6-7 show incorporation of ^{14}C -labeled precursors into various metabolites, and fluctuation in pool sizes following treatment of phloem and xylem tissues of Pinus elliotii with paraquat from 0 to 48 hours. Phloem and xylem (5 g wet weight each) were incubated in 50 ml of 10^{-3} M phosphate buffer, pH 6.8, containing $10\text{ }\mu\text{C}$ glucose- $\text{U-}^{14}\text{C}$ in the presence and absence of 1 mg paraquat.

Data presented in Fig. 1 show that the amount of ^{14}C -incorporated was the same in both control and treated tissue (ca 2000×10^3 CPM/gm phloem and 800×10^3 CPM/gm xylem). The rate of incorporation approached linearity for the first twenty-four hours in both phloem samples and in control xylem. Conversely, linear incorporation of label into paraquat treat xylem samples was observed from 12 to 48 hours. Total incorporation was higher in phloem than in xylem (maximum of 2000 vs 900 CPM/gm wet wgt).

Loss of ^{14}C during evaporation in vacuo was observed in all control and treated samples (Fig. 2). Loss of ^{14}C was highest in paraquat-treated tissues (65% in phloem, 30% in xylem), but the time course was different in phloem and xylem. At six and 12 hours, 65% of the label was lost from paraquat-treated phloem, while only 25% was lost from the 24 and 48 hour samples. Loss from control phloem tissue varied from 20-30% at all sampling periods. Loss from paraquat-treated xylem was highest at 24 and 48 hours (ca 30% vs 15-20%).

Data presented in Fig. 3 show an increase from 6-48 hours in ether-soluble ^{14}C which could be extracted from the acidified incubation mixture after the tissue had been removed. Fig. 4 contains data showing the ^{14}C incorporated into resin acids/gm wet tissue weight in the ethanol extracts

of the tissues and in the diethyl ether-soluble material from the incubation mixture. Maximum incorporation of ^{14}C was observed at 24 hours in phloem (126×10^3 CPM in paraquat-treated tissue, 60×10^3 CPM in control tissue) and at 12 hours in xylem (10×10^3 CPM in paraquat-treated tissue, 25×10^3 CPM in control tissue).

Fluctuation in mass of resin acids/gm wet wgt (Fig. 5) does not correlate to the incorporation of ^{14}C into resin acids (Fig. 4). Specific activity data (Fig. 5) indicate that when the mass of resin acids was high, the specific activity was low and vice versa.

Free fatty acids were essentially identical in control and paraquat-treated xylem during the entire study (Fig. 6), but decreased by a factor of 3 between 24 and 48 hrs. Larger fluctuation in free fatty acid between control and treated tissue and from time to time was apparent in phloem tissue. In control tissue, variation between 34 and 43 units were observed from 6-24 hours. During the same period, fatty acids increased from 6 to 36 units/gm in paraquat-treated phloem. Fatty acid concentration decreased to 4 and 14 units/gm in control and paraquat-treated tissues, respectively, from 24 to 48 hours.

Fig. 7 and 8 contain data showing the fluctuation in soluble ninhydrin; 2,4-dinitrophenylhydrazine; anthrone; and 2-thiobarbituric acid reactive materials in ethanol extracts of slash pine with and without paraquat treatment. All soluble components, except the ninhydrin positive material, decreased from time 0 in both treated and control tissue. Amino acids increased in control and treated tissue to the same maximal value (30-35 mg in phloem, 15 to 17 mg in xylem) but reached this maximal value at different times (Fig. 7). Paraquat-treated phloem reached its maximal concentration at 6 hours, the control at 12 hours. Amino acid concentration in both

treated and control xylem samples was maximal at 12 and 24 hours. At 48 hours, ninhydrin positive material had increased from 14 to 48 mg in the control phloem, while the concentration in paraquat-treated material remained constant at 12 mg.

Tables 6 and 7 contain data on the concentration of individual amino acids in these samples. In general, the changes observed were associated with those amino acids which can derive carbon from pyruvic acid.

Table 8 contains data from an 11 day tissue incubation with and without paraquat that showed a decrease in mass of individual and total resin acids in paraquat-treated tissue. While the mass changed, the percent distribution of each resin acid of the total was the same in control and treated tissues.

Tables 9-11 contain ^{14}C and mass data obtained from 24 hour incubation experiments with various substrates in the presence and absence of 1 mg paraquat. Table 9 contains data from several glucose experiments performed from February to June of 1977. From February to May 17, ca 1% of the total incorporated label was recovered from the methyl resin acid area of a neutral lipid TLC plate. On May 17, 5% and 2% of the total activity was recovered in the methyl resin acid area of the phloem and xylem samples, respectively. On June 15, 21% of the total activity was recovered in the methyl resin acid area of a combined phloem-xylem sample. Mass recovery of methyl resin acids averaged 41% (range 33.3 to 50%) of the resin acids in the original extract. Recovery of radioactivity in methylated resin acids averaged 22% (range 9 to 33%) of the activity recovered from the resin acid area of a neutral lipid plate. To restate, while 33 to 50% of the mass of resin acid was recovered from the methylated resin acid area, only 9 to 33% of the ^{14}C -activity in the resin acid area was recovered in the methyl resin acid area.

Several amino acids including serine (Table 10), glycine, leucine, glutamic, and an amino acid hydrolyzate (Table 11) as well as acetate and palmitate (Table 11) could contribute label to materials with the chromatographic mobility of resin acid and/or methyl resin acid. Since free and methyl fatty acids have essentially the same chromatographic mobility as resin acid and methyl resin acid, respectively, the radioactivity in these samples could be from unconverted substrate. Phenol, DOPA and hydroxymethylglutaryl-CoA (Table 11) were also incubated with pine tissues. ^3H - from DOPA was recovered in fairly large amounts (32.7% phloem and 7.0% xylem), but no data are available for the methyl resin acids from these preparations.

Suspension tissue cultures of P. elliotii obtained from Claud Brown were incubated with various radioactive substrates (Tables 12-13). 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 in 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 doubled 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. 9). 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 wgt). 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.

Seasonal variation of soluble metabolic pools have been observed in field grown untreated P. elliotii. Data for ninhydrin; anthrone; 2,4-dinitrophenylhydrazine; and 2-thiobarbituric acid (TBA) reactive materials; total resin acid and certain individual resin acids obtained over a 9-12 month period are presented in the preprint included with this progress report. Table 14 contains a comparison of total resin acids isolated from phloem and xylem of stem sections for certain months of 1975, 1976, and 1977. Resin acids were maximal during summer months (June and July), declined during the fall months and appeared minimal during the winter months. Depending upon weather conditions, accumulation began in spring and reached a maximum again in summer (after the cessation of new vegetative growth).

Tracings of elution patterns of standard resin acids (none of which are pure) and experimental samples of slash pine tissue from a liquid chromatograph, wet chemical data comparing pool sizes of various metabolites in pine and sycamore, and qualitative distribution of lipids in pine and sycamore after development of samples with neutral and phospholipid solvents on TLC plates are included in an appendix to this report.

DISCUSSION

Data collected from both field and laboratory studies during 1977 confirm and extend those observations and conclusions drawn from the data reported in 1976. Soluble metabolites, carbohydrates, keto acids, amino acids, and peroxyacids are mobilized when tissues of P. elliotii are injured. Data presented in Fig. 1-8 and Tables 6-7 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 (Finnerty et al.) 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 (Finnerty et al., ERDA '76; Seasonal Variation Preprint included with this progress report). 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 (Finnerty et al., ERDA, 1976; Fig. 7). In composite, these data would indicate a mobilization of starch (Brown et al., ERDA, 1976), with accompanying increased pool sizes of reducing sugars, keto acids, and amino acids which appeared to be available for resin acid synthesis. Paraquat treatment did not alter the percent composition of individual resin acids (Finnerty et al., ERDA, 1976; Table 8). 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 (1976, 1977) could explain the comparable initial response of cut and paraquat-treated tissues. Increased ethylene production accompanied injury. Both Wolter (1977) and Peters (1977) 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. As reported last year (Finnerty et al., ERDA, 1976) a representative of the Imperial Chemical Company reported that increased resin acid synthesis was associated only with compounds exhibiting herbicide activity.

Paraquat may effect both the rate and amount of monoterpenes produced by P. elliotii (Finnerty et al., ERDA, 1976; Fig. 2; Proceedings Lightwood Research Coordinating Council, 1976; 1977). Loss of large amounts of radioactivity (Finnerty et al., ERDA, 1976; Fig. 2) by volatilization following paraquat treatment could be indicative of loss of monoterpenes.

This loss of activity and the recovery of isotope and terpenes in diethylether extracts of the incubation medium (Fig. 3) indicate the importance of isotope and mass balances to experiments of this type. In longer experiments (data not included) the loss of this material would constitute the greater amounts of both volatile materials and resin acids.

The seasonal variation of glucose-U-¹⁴C incorporated into resin acid reported in 1976 was also observed in 1977 (Table 9). Comparison of data in Table 9 with that in Table 14 indicate low recovery of ¹⁴C in resin acid (0.06 to 1.4%) from February until May 17 when 5% and 2% of the total ¹⁴C was recovered from the methyl resin acid area from phloem and xylem, respectively. On June 15, 21% of the total ¹⁴C was recovered from the methyl resin acid area of a combined phloem-xylem macerate. These May-June

dates correspond to times of increased accumulation of resin acids in field grown untreated 6-year old pine trees. As reported last year, increased resin acid accumulation and increased incorporation of ^{14}C into resin acid occurred after flowering and completion of rapid vegetative growth.

Accumulation of mass of resin acid and ^{14}C from several substrates did not occur simultaneously (Fig. 5, data for glucose only). Generally, the mass continued to increase while the total ^{14}C -count remained constant so that the specific activity decreased. Whether this decrease is due to a dilution of ^{14}C by the increased pool size of glucose as the starch is hydrolyzed or to a mixed synthetic pathway of resin acids is not known.

Carbon from amino acids, fatty acids, phenol, DOPA, and hydroxymethyl-glutaryl-CoA (Table 9, 10, 11) can be isolated from the resin acid and/or methyl resin acid areas of TLC plates. The percentage incorporated into methyl resin acid from these substrates also varied with time. While the percent ^{14}C incorporation was fairly low in this report, data on terpene synthesis reported in the literature indicate as low or lower values.

In general, amino acid and glucose carbon appeared to be most effective in labeling resin acid. One set of data obtained from serine- ^{14}C , glycine- ^{14}C , and glucose- ^{14}C incubations on June 15 (Tables 9, 10, 11) may or may not have any real significance. The phloem-xylem macerate used in these incubations was taken from a single source (zero time resin acid content of 75 mg/gm fresh wgt). After 24 hours incubation, the 3 different treatments apparently resulted in 3 different resin acid masses; glucose 78 mg/gm, glycine 98 mg/gm, and serine 126 mg/gm. Technique difficulty prevented estimation of ^{14}C recovered in the methyl resin acid area from the serine and glycine incubations, but 21% of the ^{14}C from the glucose was recovered in the methyl resin acid area. Since no mass of any substrate

was added with the ^{14}C , the results are somewhat puzzling, but perhaps merit further consideration.

Incubation of suspension tissue cultures of P. *elliottii* with radioactive substrates resulted in ^{14}C label from glucose, acetate, palmitate and serine being recovered from the resin acid area of TLC plates. No resin acids were detected in any of these samples by gas chromatography. The data indicated an increase incorporation of ^{14}C into this area from glucose and palmitate in the presence of paraquat. Total uptake of ^{14}C from glucose (556,339 to 103,129) and serine (1,610,665 to 136,459) was inhibited by the paraquat treatment. ^{14}C -label recovered from the resin acid area of TLC plates following serine incubation was reduced from 1.9 to 0.5%. Total uptake of acetate (304,036 to 786,310) and palmitate (45,947 to 837,024) was increased in the presence of paraquat. Data in Fig. 9 indicated an increased uptake of acetate-2- ^{14}C in the presence of 1 and 2 mg paraquat/50 ml incubation medium. Response following treatment with 4 and 8 mg paraquat/50 ml was comparable to the untreated controls. No attempt has been made to characterize the compounds moving into this area of TLC plates from tissue culture extracts at the present time.

TABLE 1

Recovery of ^{14}C in Chloroform Extracts of *P. elliottii* Incubated with Acetate-2- ^{14}C in the Presence and Absence of Paraquat after Development in Neutral Lipid Solvent on Silica Gel G TLC Plates.

SAMPLE	CPM PLACED ON ORIGIN	CPM RECOVERED FROM PLATE	EFFICIENCY OF RECOVERY
Control-1	57,100	617,200	+ 10.8
Control-2	86,160	737,700	+ 8.6
0.5 mg	23,142	272,320	+ 11.8
1.0 mg	152,000	2,191,780	+ 14.4
2.0 mg	324,375	4,879,020	+ 15.0
4.0 mg	83,080	1,152,180	+ 13.9
8.0 mg	40,000	523,860	+ 13.1

TABLE 2

Recovery of ^{14}C in Combined Chloroform Extracts of P. elliottii Incubated with Acetate-2- ^{14}C in the Presence and Absence of Paraquat After Elution from a Silicic Acid Column (15,000,000 CPM Placed Column).

FRACTION	CPM	FRACTION	CPM
100% PE	103,040	90:10	59,177
99% PET	13,220	100% Et ₂ O	612,940
1% Et ₂ O			75.9% Recovery
98:2	97,776	CBM 1:1	9,068,000
97:3	473,776	MeOH	<u>462,960</u>
96:4	267,904		
95:5	220,800	TOTAL CPM RECOVERED	11,380,000

TABLE 3

Recovery of ^{14}C in Fraction Eluted from a Silicic Acid Column after Development in Neutral Lipid Solvent on Silica Gel G TLC Plates.

FRACTION	CPM PLACED ON ORIGIN	CPM RECOVERED FROM PLATE	EFFICIENCY OF RECOVERY
100% PE	25,760	12,788	49.6%
99% PE+1% Et ₂ O	3,305	91,548	+27.7
98:2	19,555	105,720	+ 5.4
97:3	118,444	466,082	+ 3.9
96:4	66,976	61,852	92.3%
95:5	55,200	218,116	+ 4.0
90:10	14,794	54,272	+ 3.7

TABLE 4

¹⁴C Recovered in Phospholipid and Glycolipid Fractions from Combined Phloem, Xylem, and Needle Sample after Elution from Silicic Acid Column (Total CPM Placed on Column 3,000,000).

	CPM	% OF TOTAL	% RECOVERY FROM COLUMN
glycolipid (Acetone)	1,085,260	36.2	
Phospholipid C:M (1:1)	1,400,000	46.7	82.8
	<hr/> 2,485,260		

TABLE 5

Silicic Acid Chromatography of Resin Acid Area Eluted from Silica Gel G Plate (Apparent 5000 CPM Placed on Column)

FRACTION	CPM	% OF ACTIVITY RECOVERED FROM RA AREA OF 80:20:1 PLATE
100% PE	100	1.0
99% PE, 1% Et ₂ O	30,000	7.0
98:2	12,000	1.2
97:3	200	26.0
96:4	185	82.2
95:5	719	1.0
90:10	478	24.3
100% Et ₂ O	200	51.0
TOTAL	43,882	

TABLE 6

Amino Acid Analysis (Percent Distribution) from Phloem Tissue of Pinus elliotii in Presence and Absence of 1 mg Paraquat

AMINO ACID	HOURS				
	0	6	12	24	48
tryptophan-control	0	0	0.69	NS	NS
paraquat	0	0	2.2	0	0.5
lysine-control	T	T	1.5	NS	NS
paraquat	T	20.5	0.9	T	4.1
histidine-control	0	0	0.11	NS	NS
paraquat	0	0	0	T	0.4
arginine-control	0	0	2.2	NS	NS
paraquat	0	2.4	7.9	T	1.0
aspartic-control	8.2	3.5	2.6	10.7	1.4
paraquat	8.2	1.2	3.9	2.0	2.3
threonine-control	3.0	3.7	1.8	2.3	2.5
paraquat	3.0	1.6	1.7	1.9	2.3
serine-control	1.5	6.7	10.3	7.5	2.3
paraquat	1.5	3.2	10.0	10.7	10.9
glutamic-control	19.7	19.9	50.4	33.4	11.2
paraquat	19.7	10.5	37.2	39.1	32.4
glycine-control	7.3	2.4	1.1	2.1	1.3
paraquat	7.3	0.7	0.6	2.3	3.4
alanine-control	10.8	10.0	19.3	30.4	6.7
paraquat	10.8	7.4	26.2	26.4	22.8
valine-control	36.8	7.6	1.1	1.6	8.1
paraquat	36.8	6.1	2.3	5.8	8.2
methionine-control	T	8.6	2.9	2.1	4.9
paraquat	T	4.8	2.8	4.5	2.9
isoleucine-control	12.7	6.0	0.7	1.1	7.4
paraquat	12.7	6.9	0.6	1.5	1.8
leucine-control	0	27.9	1.8	1.5	47.2
paraquat	0	31.7	0.8	3.5	4.1
tyrosine-control	0	0	1.0	2.6	1.5
paraquat	0	0.5	0.8	0.7	1.0
alanine-control	0	3.8	2.5	4.7	5.5
paraquat	0	2.4	2.1	1.9	1.9
Total MG Amino Acids Recovered/Sample:					
CONTROL	3.292	13.89	34.997	14.058	47.704
PARAQUAT	3.292	29.747	18.991	10.548	14.184

TABLE 7

Amino Acid Analysis (Percent Distribution) from Xylem Tissue of Pinus elliotii in Presence and Absence of 1 mg Paraquat.

AMINO ACID	HOURS				
	0	6	12	24	48
tryptophan-control	NS	0	0	1.0	0
paraquat	NS	0	0	4.7	1.4
lysine-control	NS	T	2.9	1.7	0.8
paraquat	NS	T	1.8	0.5	2.8
histidine-control	NS	0	0	0.6	0.3
paraquat	NS	0	0.4	0	0.7
arginine-control	NS	0	36.2	22.3	2.2
paraquat	NS	0	36.7	0	29.1
aspartic-control	NS	2.2	3.6	3.4	2.2
paraquat	NS	4.2	2.6	1.7	2.2
threonine-control	NS	1.9	1.2	1.1	1.3
paraquat	NS	T	0.7	1.0	0.8
serine-control	NS	5.2	5.5	10.7	10.6
paraquat	NS	10.5	3.4	7.1	8.5
glutamic-control	NS	16.9	13.2	37.6	41.0
paraquat	NS	17.2	29.1	47.6	19.9
glycine-control	NS	2.1	0.7	0.6	2.1
paraquat	NS	T	0.2	0.8	1.2
alanine-control	NS	7.8	17.5	19.3	32.4
paraquat	NS	4.4	17.6	31.6	24.9
valine-control	NS	6.8	1.9	1.8	6.2
paraquat	NS	0	0.8	2.2	1.9
methionine-control	NS	16.3	2.2	0	0.8
paraquat	NS	57.2	2.7	0.7	2.4
isoleucine-control	NS	5.0	2.1	0	0
paraquat	NS	6.6	0.6	0.4	0.6
leucine-control	NS	32.3	11.6	0	T
paraquat	NS	0	2.0	0.6	2.4
tyrosine-control	NS	T	0.4	0	T
paraquat	NS	0	0.3	0.3	0.3
Ø alanine-control	NS	3.6	1.0	0	T
paraquat	NS	0	1.2	0.8	0.8

Total MG amino acids
recovered/sample

CONTROL	NS	3.655	16.303	15.686	3.581
PARAQUAT	NS	1.2	14.174	12.407	8.599

TABLE 8

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

RESIN ACID	CONTROL		PARAQUAT	
	mg	% of Total	mg	% of Total
Palustric	4.10	26.1	2.84	25.8
Isopinaric	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	<u>2.40</u>	<u>15.2</u>	<u>1.70</u>	<u>15.4</u>
Total	15.72	100.1	11.04	100.2

TABLE 9

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

SAMPLE	% RA AREA OF TOTAL	% METHYL RESIN AREA OF RA. TOTAL	% METHYL RESIN AREA OF TOTAL
2/28			
phloem	1.0	NS	NS
xylem	7.9	NS	NS
3/9			
xylem radioactivity	13.6	100%*	--
mass mg/gm wet wgt	NS	NS	NS
3/28			
phloem	6.2	22.0	1.4
	NS	NS	NS
xylem	1.6	100%	--
	NS	NS	NS
4/12			
phloem	10.0	14.0	1.4
	0.003 mg	0.001 mg	33.3%
4/28			
phloem	5.0	100%	--
	NS	NS	NS
xylem	5.0	28.0	1.4
	17.85 mg	8.0	44.8%
5/9			
phloem	1.5	38.0	0.06
	0.9 mg	0.3	33.3%
xylem	5.0	17.5	0.9
	1.2 mg	0.6	50.0%
5/17			
phloem	34.0	15.0	5.1
	0.033	0.012	36.4
xylem	22.0	9.0	2.0
	0.346 mg	0.16	46.2
6/15			
phloem	64.0	33.0	21.1
+ xylem	78 mg	34 mg	43.6%

TABLE 10

Percent Recovery of ^{14}C and Mass of Resin Acids and Methyl Resin Acids
Following 24-Hour Incubation of Tissues with Serine- $\text{U-}^{14}\text{C}$ in the Presence
and Absence of 1 mg Paraquat

SAMPLE	% RA OF TOTAL	% METHYL RESIN ACID	
		OF RA	OF TOTAL
2/29			
phloem	0.3	NS	NS
xylem	0	NS	NS
3/9			
phloem radioactivity	1.4	100%	--
mass - mg/gm	NS	NS	
5/17			
phloem	16.0	11.0	1.8
	0.033 mg	0.014 mg	42.4%
xylem	17.0	7.0	1.2
	0.346 mg	0.14 mg	40.5%
6/15			
phloem	12.5	100%	-
+ xylem	125 mg	50 mg	39.7%

TABLE 11

Percent Recovery of ^{14}C and Mass or Resin Acids and Methyl Resin Acids
Following 24-Hour Incubation of Tissues with Miscellaneous Substrates
in the Presence and Absence of 1 mg Paraquat

SAMPLE	SUBSTRATE	% RA OF TOTAL	% METHYL RESIN ACID OF RA	OF TOTAL
3/9	Acetate			
xylem radioactivity		2.2	100	-
Mass mg/gm		NS	NS	NS
4/12	Acetate			
phloem		10.0	60.0	6.0
		0.003 mg	0.001 mg	33.3%
xylem		5.0	48.0	2.4
		0.59 mg	0.24 mg	48.0%
5/12	Acetate			
phloem		9.0	3.0	0.30
+				
xylem		0.208 mg	0.10 mg	48.1%
	Palmitate			
phloem		10.0	26.0	2.6
xylem		5.0	100%	-
		0.33 mg	0.16 mg	48.5%
6/15	Glycine			
phloem		32.6	100%	-
+				
xylem		98 mg	45 mg	45.9%
	Phenol- ^{14}C			
phloem		7.9 (5/17)	0.5 (2/18)	NS
xylem		4.2 (5/17)	3.3 (2/18)	NS
	DOPA ^3H			
phloem		NS (5/17)	32.7 (2/18)	NS
xylem		7.0 (5/17)	0.0 (2/17)	NS
	Amino Acid Hydrolyzate			
phloem		0.2	NS	NS
xylem		0.7	NS	NS
	Leucine			
phloem		0.5	NS	NS
xylem		0.0	NS	NS
	Glutamic			
phloem		0.04	NS	NS
xylem		0.0	NS	NS
	Hydroxymethyl-glutaryl-CoA			
phloem		0.03	NS	NS
xylem		0.0	NS	NS

TABLE 12

Tissue Culture - Uptake of Radioactive Substrate - Glucose-U-¹⁴C
(CPM/gm air dry wgt.)

SAMPLE				% CPM recovered in RA area on 80:20:1 TLC plate		
	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 13

Tissue culture incubated 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
Control acetate	304,036	4.9
Paraquat acetate	786,310	1.7
Control glucose	445,339	1.6
Paraquat glucose	103,129	3.4
Control palmitate	45,947	0.6
Paraquat palmitate	837,024	4.2
Control serine	1,610,665	1.9
Paraquat serine	136,459	0.5

TABLE 14

Resin Acids - mg/gm Fresh Wgt.

TISSUE	1975			1976							1977*										
	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	2.3	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

* Extraction procedure changed from $\text{CHCl}_3:\text{CH}_3\text{OH}(2:1)$ to boiling 70% $\text{C}_2\text{H}_5\text{OH}$.

[†] phloem and xylem combined.

CPM glucose - d - C 1 gm act ugt

Fig 1

phloem

Xylem

Control

pano gnt

Percent ^{14}C - of total Ethanol extract loss under vacuum

3/8/77

phloem

xylem

Fig 2

□ control
■ paraquat

100

90

80

70

60

50

40

30

20

10

NS

0

6

12

24

36

48

NS

0

6

12

24

36

48

40

Time (hours)

20 Squares to the Inch

50

phloem

xylem

Resin Acids -
phloem

CPM/gm wet wt
xylem

Fig. 1

Phloem

Xylem

see surface -

10/gm wet wt.

Fig 6

5/18/76

□ control
■ paraquat

phloem

xylem

3/18/77

Fig 7

phloem
 □ control
 ■ paraquat

Xylem Fig 8

3/18

Anthrone Reactive material

2-2 thiobarbituric acid Reactive material

Fig. 9

LITERATURE CITED

1. Brown, C. 1976. Progress Report ERDA.
2. Finnerty, W. R. 1976. Progress Report ERDA.
3. Makula, R. A., P. J. Lockwood, and W. R. Finnerty. 1975. Comparative analysis of the lipids of Acinetobacter species grown on hexadecane. J. Bacteriol. 250-258.
4. Peters, W. 1977. Ethylene-diquat synergism. Proceedings Lightwood Research Council. in press.
5. Wolter, K. E. and D. F. Zinkel. 1976. Metabolic, hormonal, photosynthetic, and ligatering response of red pine treated with paraquat. Proceedings Lightwood Research Council 31-40. 1976.
6. Wolter, K. E. 1977. Potential alternative to bipyridilium herbicides for inducing lightwood. Proceedings Lightwood Research Council. in press.
7. Zinkel, D. F. 1977. Anal. Biochem. 37.

APPENDIX

SYCAMOREPINE

Ninhydrin g/gm Fresh Wgt

1,500
4/29/771,100
6/2/771,500
5/761,500
5/4/77

Root	812	264	NS	NS
Stem	339	220	614	330
Leaf	1,203	124	150	679

Hydrazone - OD/gm Wet Wgt

Root	1.6	1.2	NS	NS
Stem	0.95	3.4	2.7	1.8
Leaf	12.8	12.0	0.76	4.8

Anthrone - g/gm Fresh Wgt

Root	6,211	7,100	NS	NS
Stem	5,905	8,800	17,244	4,760
Leaf	23,564	11,750	1,641	5,674

Phospholipids - m Lipid P/gm Wet Wgt

Root	NS	2.16 (50%)*	NS	NS
Stem	NS	1.80 (69%)	NS	NS
Leaf	NS	16.0 (58%)	NS	NS

*recovery of lipid phosphorus development CHCl_3 :meoh () TLC.

Qualitative Distribution of Lipids in Sycamore and Pine - TLC

Neutral Lipids

LIPID	Sycamore	Pine (Xylem)
monoglycerides	✓	-
diglycerides	✓	✓
triglyceride	✓	✓
free fatty acids	✓	✓
resin acids	-	✓
methyl esters	✓	✓
wax esters	✓	✓

Phospholipids

PC	✓	
PG	✓	ERDA progress report - 1976
Cardiolipid	?

Perine

^{14}C glucose

$X = 202, 100 \text{ CPM}$
 $1 = 236 \text{ CPM}$
 $2 = 180 \text{ CPM}$
 $3 = 323 \text{ CPM}$
 $\text{Rest} = 78 \text{ CPM}$

24/11

27

glucose

abietic?

25 1/2 cm

	(1 min count)	5 min
1 =	0 CPM	0
A =	21 CPM	<u>44</u>
2 =	0 CPM	<u>8</u>
3 =	7 CPM	<u>12</u>
4 =	0 CPM	<u>14</u>
Rest =	60 CPM	

Originally Isopimaric
and abietic acid present

X on GC no dehydro-
abietic seen

dehydro?

④

A

①

②

③

abietic?

24 cm

not
on GC
culture

C^{14} glucose

Sample ②
from HPLC-Like

1 = 20 CPM
2+3 = 7 CPM
3 = 27 CPM
4 = 24 CPM
5 = 9 CPM
6 = 7 CPM
7 = 15 CPM
Rest = 20 CPM

② abietic 2

on TLC (80:20:1)
area eluted
2 Etac and
on HPLC ↓

1	-	11 CPM	
2	-	0 CPM	$\left(\begin{array}{l} 0 \text{ CPM} \\ 6 \text{ CPM } 2^{\text{nd}} \text{ time} \\ 8 \text{ CPM} \end{array} \right)$
3	-	6 CPM	
4	-	0 CPM	
Rest	-	28 CPM	

④

dehydro 2

dehydro 2

①

abietic 2

241 nm

TC
C¹⁴ acetate

originally
no R.A. ^{seen} on GC.

$$1+2 = 15 \text{ CPM}$$

$$3 = 24 \text{ CPM}$$

$$3+4 = 334 \text{ CPM ?}$$

$$4 = 13 \text{ CPM}$$

$$\text{Rest} = 304 \text{ CPM}$$

sample
diluted
analysis

C^{14} acetate

① ② ③

Originally
no R.A. seen
on GC.

257 nm

1 - 10 CPM

2 - 12 CPM

2+3 - 14 CPM

5 - 32 CPM

Rest - 2,330 CPM

⑤ abietic 2

④

241 nm

C.

PROPOSAL: BIOCHEMISTRY OF METHANOGENESIS

Principal Investigators: Drs. Peck and Ljungdahl

ABSTRACT

Cellulytic bacteria, cellobiose fermentors and methanogenic bacteria have been isolated from established anaerobic cellulose-methane fermentations and employed to partially reconstitute highly active cellulose methane fermentations. These mixed cultures will be utilized as model systems to study the parameters required for maximum production of CH_4 , H_2 , and chemical feedstocks such as acetate, formate, ethanol, propionate, etc. The physiology of these reconstituted cultures will also be investigated as regards interspecies H_2 transfer, cellobiose accumulation, inoculum size, microbial types and cultural conditions. Other bacteria will be isolated as indicated by experimental results and a major effort will be made to isolate and characterize a bacterium converting acetate to methane. Finally, it should be possible to completely reconstruct this important food chain from pure cultures. The biochemistry of enzymes involved in one and two carbon metabolism will continue to be investigated with emphasis being placed on the role of selenium and tungsten in the active site of formate dehydrogenase and the possible role of formate and formate dehydrogenase in methane formation. The bioenergetics of methane formation will continue to be investigated utilizing the hydrogenase/fumarate reductase couple for the detection of cell-free electron transfer coupled phosphorylation and the isolation of low molecular weight electron transfer proteins. The goal of this aspect of the research is to obtain an understanding of the physiology and pathway of methane formation in methanogenic and related anaerobic bacteria.

C.

PROPOSAL: BIOCHEMISTRY OF METHANOGENESIS

Principal Investigators: Peck and Ljungdahl

Introduction

The anaerobic fermentation of cellulose is ecologically and economically the most important microbial transformation occurring in nature (1). The fermentation is responsible for the decomposition of plant materials in marine environments, fresh water sediments and swamps, submerged soils, sewage digestors and the rumen. The fermentation also has great potential as a commercial source of methane and has already been utilized on a small scale to provide energy under limited conditions such as in sewage treatment plants and in remote communities. The fermentation itself is complex in that it requires two or more microorganisms and has been most extensively studied in ruminants (2). Although the microbial flora of the rumen is highly variable, it is possible to isolate pure cultures of methanogenic bacteria and cellulose fermenting bacteria but there does not appear to be large numbers of specific microbes always associated with these activities (3). Nevertheless, this experience with the microbiology of the rumen clearly indicates that the microorganisms responsible for the methanogenic fermentation of cellulose are not restricted to a symbiotic relationship with each other and that the fermentation can be analyzed by conventional anaerobic bacteriological techniques.

More recently, new insights into the relationships among microorganisms involved in this and other fermentations have been gained from the concept of interspecies hydrogen transfer (4). In this physiological process, fermentation patterns in mixed cultures are dramatically altered by the transfer of molecular hydrogen between different physiological types of bacteria and a methanogenic bacterium (5). Alternatively, methane formation can be utilized as an electron "sink" in lieu of the normal electron acceptor as with the sulfate reducing bacteria (6). Superficially, the formation of methane appears to be energetically wasteful but it is believed to thermodynamically "pull" reactions which are energetically un-

favorable. The methanogenic bacteria are in extremely diverse groups of bacteria of two physiological types: those capable of forming methane from H_2 and CO_2 (and in some instances formate or methanol) and those capable of forming methane from acetate. This latter group has not as yet been isolated in pure culture but they appear to be extremely important for methane production from sewage (7) and fresh water sediments (8). Generally 70% of the methane is derived from acetate and 30% from CO_2 and CO_2 is produced in excess of CH_4 rather than in the equimolar amounts theoretically expected. These observations emphasize the importance of acetate formation and utilization in anaerobic environments.

In natural environments, cellulose can be decomposed to CO_2 , methane and cell material. Under anaerobic conditions the fermentation can be utilized for the production of commercial amounts of methane. In addition, the fermentation can be modified to produce chemical feedstocks such as acetate, ethanol, butyrate, etc. 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.

Activities and Accomplishments

Stable mesophilic and thermophilic methane-cellulose fermentations have been established and are maintained in our laboratory. Microbiological analysis of the mesophilic fermentations has been most successful but growth of these isolates is extremely slow and the establishment of cultural purity very tedious. The following physiological types of bacteria were isolated on selective media: cellulose decomposers, cellobiose fermentors, glucose fermentors, sulfate reducing bacteria and methanogenic bacteria (H_2 - CO_2). Three cultures of cellulose decomposers have been isolated and, although all are gram negative curved rods, they can be differentiated on their abilities to produce molecular hydrogen. One produced no H_2 , another, traces of H_2 and the third, rather large amounts of H_2 . Sixteen cultures were isolated which had the ability to degrade cellobiose and these proved to be

a more diverse group of organisms but could be divided into two physiological groups on the basis of their ability to produce H_2 or not in the fermentation of cellobiose. The glucose fermentors were simple to isolate and have not been studied as yet. Two cultures of sulfate reducing bacteria were isolated as well as two cultures of methanogenic bacteria.

Biochemical techniques have now been established in our laboratories to analysis most putative products of these fermentations; gases, H_2 , CO_2 , CH_4 ; alcohols, methanol thru pentanol; volatile fatty acids, formic acetic thru valeric; and non-volatile acids. Two cultures of the cellulose decomposers have been analyzed, (1a) which forms molecular hydrogen during the fermentation of cellulose and (1b) which does not produce molecular H_2 during the break down of cellulose. Strain (1a) degrades cellulose (indicated as glucose) by the following equation:

Strain 1b grows more slowly on cellulose and it has not yet been possible to obtain a good analysis of fermentation products.

With pure isolates and the techniques for analyzing fermentation products available, we have attempted to reconstitute the methane-cellulose fermentation with some success as shown in Table 1. All cultures were isolated in our laboratory from a cellulose enrichment culture and it should be noted that the cellulytic bacterium had the ability to form significant amounts of H_2 while the cellobiose fermentor did not produce significant amounts of H_2 . The addition of a methanogenic bacterium or a cellobiose fermenting bacterium to the cellulytic bacterium had little effect on the total formation of products; however, combination of the three physiological types of bacteria resulted in large increases in the formation of CH_4 , acetate and CO_2 . The methane formed in the three membered culture is equivalent to 64 μ mole of H_2 and on this basis, represents a 20 fold stimulation of H_2 formation compared to the cellulytic bacterium alone. Although it has been demonstrated that the presence of a methanogenic bacterium can modify the

products formed by a cellulytic bacterium (9). This is the first demonstration of the stimulation of a cellulose decomposition by the presence of other bacteria. The role of the cellobiose fermentor is probably to remove cellobiose (and possibly glucose) which are inhibitors of cellulose formation and the methanogenic bacteria to "pull" the reaction toward completion by the formation of methane. It should be noted that all the H_2 was converted to CH_4 . Purely nutritional relationships seem unlikely as we have not been able to increase the growth of the cellulytic bacteria by various supplements to the media. Changes in the composition of products are evident and it is clear that the cellobiose fermenting bacterium is able to utilize H_2 for the formation of reduced products, although not forming H_2 during the fermentation of cellobiose. The data indicate that we have partially reconstituted a cellulose-methane food chain and can analyze the various biochemical and microbiological parameters required for the optimal formation of products such as methane, ethanol, acetate, etc.

In carrying out these experiments, we now analyze duplicate cultures at 12 and 25 days for a total of four complete fermentation balances per bacterium or mixture of bacteria. A major unresolved problem involves adjusting inocula and media so as to support reasonable growth of the mixtures of bacteria. An example of this problem is shown in Table 2. This is a similar type of experiment to that shown in Table 1; however, the cellulytic bacterium does not form H_2 but the cellobiose fermentor does form H_2 - just the opposite of the physiological types in Table 1. The methanogenic bacterium is Methanobacterium, strain MOH, which is a well characterized laboratory strain of the methane bacteria. The cellulytic bacterium grew rather poorly even in the presence of the methanobacterium; however, in the presence of the cellobiose fermentor there was a good stimulation of growth as evidenced by cellulose utilization and the production of hydrogen, acetate and CO_2 . This differs from the previous result and may be due to the different physiological type of microorganisms employed in the experiment.

Table I

Microbial Requirements for Optimum Gas Formation from Cellulose (cellobiose fermenting bacterium)

Microbial Type	Products (μ moles/ml)				Other Products
	<u>H₂</u>	<u>CH₄</u>	<u>CH₃COOH</u>	<u>CO₂</u>	
Cellulytic bacterium (1a)	2.69	0	3.1	4.33	formate, propionate (0.9), ethanol (2.0), isovaleric, succinate, oxalacetate
(1a) plus Methanogenic bacterium (6)	0	1.75	3.53	11.2	formate (.2), ethanol (0.3), propionate (.05), succinate (0.7)
(1a) plus Cellobiose	0	0	1.24	10.15	formate (.2), ethanol (1.0), propionate (.5), isobutyric (.3), butyric (.3), isovaleric (0.5), pyruvate (0.9), lactate (0.5) fumarate
(1a) plus (3) plus (6)	0	16.1	16	18.0	formate, ethanol (3.0), propionate (0.8), isobutyric, butyric, isovaleric

Table 2

Microbial Requirements for Optimum Gas Formation from Cellulose (25 days) - Methanobacterium, strain MOH

Microbial Type	Products (μ moles/ml)				Cellulose Utilized (μ moles glucose/ml)	Other Products
	H_2	CH_4	CH_3COOH	CO_2		
Cellulytic bacterium (1b)	0	0	0.8	3.0	3.2	--
(1b) plus Cellobiose fermentor	51	0	13	29	18.2	Ethanol, 4; propionate, 4.0; butyric 4.0.
(1b) plus methanogenic bacterium (MOH)	0	0.7	2.35	0	4.1	--
Cellobiose fermentor plus MOH	0	0.5	1.5	5.6	2.0	--
(1b) plus (MOH) plus Cellobiose fermentor	2.7	9.8	23.0	29.0	25.9	Ethanol, 5; propionate, 1.5; butyric 5.8.

There was only questionable stimulation of the cellulose fermentation in the presence of Methanobacterium; however, as H_2 was detected in the gas phase, it appears that strain MOH did not grow in sufficient numbers to act as a hydrogen sink to "pull" the fermentation. These data illustrate some of the problems involved in these types of experiments and emphasize the importance of analysis of fermentation products for the interpretation of the overall results.

Although the reconstituted methane-cellulose fermentation grow at a rate comparable to natural mesophilic enrichments, other products accumulate such as ethanol, propionate, butyrate lactate, etc., which in natural enrichments are converted to methane. Other microorganisms such as the sulfate reducing bacteria, the "acetate" methanogenic bacteria, and an unknown group which convert propionate and butyrate to methane are probably required for these conversions.

An attempt was made to increase the yield of methane by utilizing Methanosarcina barkeri as the methanogenic bacterium. This organism is the only characterized laboratory culture which forms methane from both acetate and H_2 plus CO_2 at appreciable rate and the results are shown in Table 3. The yield of methane was approximately doubled and only low amounts of acetate accumulated in the media. The accumulation of H_2 suggests that M. barkeri, as with strain MOH did not pull the fermentation. It is curious that other products of the fermentation were not significantly altered and suggests that in these partially reconstituted culture, interspecies H_2 transfer may not play a significant role.

In summary, cellulose and cellobiose fermentors and methanogenic bacteria have been isolated from natural mesophilic cellulose enrichments and utilized to partially reconstitute methane-cellulose fermentations. Both cellobiose fermentors and methanogenic bacteria can be required for the rapid fermentation of cellulose and it is proposed that the cellobiose fermentors remove cellobiose (and probably glucose) which are inhibitors of cellulose formation and that methane formation "pulls" the fermentation of cellulose. By selecting various physiological types

Table 3

Comparison of the Fermentation Products formed (25 days) by Reconstituted mixed Cultures containing either Methanobacterium strain MOH or Methanosarcina barkeri.

Mixed Culture	H ₂	CH ₄	CO ₂	<u>Products (μmole/ml)</u>					Cellulose Utilized (μmole glucose/ml)
				<u>Acetate</u>	<u>Ethanol</u>	<u>Butanol</u>	<u>Propionate</u>	<u>Butyrate</u>	
Cellulytic plus Cellobiose fermentor plus strain MOH	2.7	9.85	29	23.07	4.9	0.4	1.56	5.84	25.93
Cellulytic plus Cellobiose fermentor plus <u>M. barkeri</u>	5.7	17.2	44.6	0.8	4.0	1.1	3.2	6.3	20.0

of anaerobic bacteria for use in these mixed cultures, it should be possible to adjust the fermentation of cellulose for a number of desired products such as methane, hydrogen, ethanol, acetate, etc.

Formate dehydrogenase has been found to be widely distributed in methanogenic bacteria and is of high interest because of the possible role of this enzyme in methane formation. Tungsten has been found to be an essential component of the formate dehydrogenase of C. thermoaceticum and this is the first time that an enzymatic role for this metal has been demonstrated (10). The requirement of selenium and tungsten for the growth of Methanococcus vanniellii suggests that formate dehydrogenase may be involved in methane formation (11). Extracts of C. thermoaceticum contain three components which are labeled by radioactive tungsten. One of these is the formate dehydrogenase and the others appear to be a protein of about 60,000 molecular weight and a low molecular weight peptide. The second protein now appears to be a storage-protein for tungsten and the low molecular weight peptide is probably a cofactor form of tungsten analogous to that found in Mo-requiring enzymes such as nitrate reductase (12) and nitrogenase (13).

The pathway of acetate formation by the "Balch" isolate, Acetobacterium woodii (14), has continued to be investigated. This organism is widespread in nature and can obtain its energy and cell carbon anaerobically either by oxidizing H_2 and reducing CO_2 to acetate or by fermenting fructose to three acetate. All of the folate enzymes and formate dehydrogenase involved in the net synthesis of acetate have been demonstrated in both hydrogen and fructose grown cells and the presence of B_{12} proteins and several low molecular weight electron carriers has been demonstrated but not yet identified. In addition to this organism being a new physiological type of anaerobe which must obtain its energy for growth from electron-transfer phosphorylation coupled to the oxidation of H_2 and reduction of CO_2 to acetate, it is the first instance where the pathway of net acetate is

clearly involved in autotrophic carbon dioxide formation. We are particularly interested in this microorganism because it may be useful in enhancing the yield of acetate in the fermentation of cellulose.

We have continued our attempts to isolate low molecular weight electron transfer components from extracts of the methane bacteria, mainly Methanobacterium thermoautrophicum, as an approach to the bioenergetics of these unique anaerobes. These studies have been limited by the lack of sufficient amounts of bacteria but over the past year we have modified one of our 400 liter fermentors so that it can now safely be used for the mass culture of the methane bacteria. Working with modest amounts of cells, (50-100 gm wet weight) we have seen nothing that resembles flavodoxin, rubredoxin, ferredoxin or cytochromes except for a small amount of membrane-bound cytochrome b when the bacteria are grown with hemin. A "ferredoxin-like" component has been observed that appears to be a small polypeptide capable of complexing iron, and is being characterized. The soluble fraction contains large amounts of two compounds which have spectra similar to NADH and NADPH but do not appear to be chemically identical to NADH and NADPH. These are also being identified. The soluble protein fraction contains a number of high molecular weight non-heme iron proteins which exhibit 4Fe-4S EPR signals but these have not been further characterized. These observations suggest that the methane bacteria do not have a conventional electron transfer chain and may form ATP only from protons generated on the outside of the cell during the initial step in the oxidation of H_2 by hydrogenase.

Progress has continued on the electron transfer chain involved in the net formation of acetate by C. thermoaceticum. In addition to a menaquinone and a cytochrome b (15), this Clostridium has been shown to contain a four-iron, four-sulfide ferredoxin rather than the eight-iron, eight-sulfide ferredoxin usually found in Clostridia (16). Two types of rubredoxin have also been isolated and characterized as individual species on the basis of their amino acid composition

and EPR signals. The physiological role of the rubredoxins remains unknown; however, a NADH(NADPH) rubredoxin reductase has been purified which reduces both species of rubredoxin. Work is also continuing on the mechanisms of the folate enzymes involved in the net synthesis of acetate and their possible relationship to the mechanism of methane formation.

Publications

- Ferry, J. G., D. W. Sherod, H. D. Peck, Jr. and L. G. Ljungdahl. Levels of Formyl Tetrahydrofolate synthetase and methylene tetrahydrofolate Dehydrogenase in Methanogenic Bacteria in "Microbial Production and Utilization of Gases", p. 151-155. Akademie der Wissenschaften zu Gottingen, West Germany, 1976.
- Yang, S-S., L. G. Ljungdahl and J. LeGall. A four-iron, four sulfide ferredoxin with high thermostability from C. thermoaceticum. J. Bacteriol. 130:1084-1090 (1977).
- Ferry, J. G. and H. D. Peck, Jr. Relationship Between Sulfate Reduction and Methane Production in Salt Marsh Sediments. Ann. Meeting Am. Soc. Microbiol., 1977.
- Dilworth, G. and H. D. Peck, Jr. Studies on the Reduction of Nitrate by a Sulfate Reducing Bacterium. Ann. Meeting Am. Soc. Microbiol., Abstract, 1977.
- Ljungdahl, L. G. and Sherod, D. Proteins from Thermophilic Microorganisms. In "Extreme Environments Mechanisms of Microbial Adaption" (M. R. Heinrich, ed.), pp. 147-187. Academic Press, N.Y. (1976).
- Ljungdahl, L. G. and Andreesen, J. R. Reduction of CO₂ to Acetate in homoacetate fermenting Clostridia and the involvement of tungsten in formate dehydrogenase. In "Microbial Production and Utilization of Gases" (H. G. Schlegel, G. Gottschalk, and N. Pfennig, eds.), pp. 163-172. Akademie der Wissenschaften zu Gottingen. E. Goltze K. G. Gottingen (1976).

Yang, S.-S and Ljungdahl, L. G. Properties of two rubredoxins and a NAD(P)H-rubredoxin reductase isolated from Clostridium thermoaceticum. Ann. Meeting Am. Soc. Microbiol., Abstract, 1977.