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DEPARTMENT OF ECOLOGY AND
BEHAVIORAL BIOLOGY

MASTER

UNIVERSITY OF MINNESOTA

MINNEAPOLIS, MINNESOTA 55455

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VERTEBRATE BEHAVIOR AND ECOLOGY

Progress Report

for Period July 1, 1978 - June 30, 1979

John R. Tester and Donald B. Siniff

University of Minnesota

Minneapolis, Minnesota 55455

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Institution and Address:

Department of Ecology and Behavioral Biology
University of Minnesota
Minneapolis, Minnesota 55455

Principal Investigators:

Dr. John R. Tester, Professor
Dr. Donald B. Siniff, Professor

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- (2) Application of Radiotelemetry to Selected Problems
in Vertebrate Censusing and Population Study

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SUMMARY

Our efforts in engineering design and development this year emphasized improvement in the quality and versatility of radio transmitters, use of microprocessors in an automated fish tracking system and construction of an automated system to monitor movements and activities of aquatic mammals in response to water temperature. Improved radio transmitters were used on a variety of species including sea otters, manatees, and wall-eyed pike. The fish tracking system, installed at Monticello, Minnesota, will next be modified for monitoring marine mammal movements. The temperature data logging system was tested on manatees in the St. John River near Blue Springs, Florida.

The long-term goal in the subproject on evaluation of census methods is to utilize the extensive experience, technology, and equipment developed over the years in radio telemetry to resolve basic problems in animal census and population study methods. More immediate goals are to use radio telemetry to develop satisfactory methods for censusing and measuring other important parameters of the sea otter populations in Alaska and California. During the past year, our efforts have resulted in information on activity patterns and the behavioral repertoire of sea otters and on the response of otters to contamination by Alaskan crude oil. Three preliminary manuscripts presented as part of this report describe these aspects in detail.

SUBPROJECT ONE

ENGINEERING DESIGN AND DEVELOPMENT

Engineering design and development during this year concentrated on improving the quality of instrumentation for use in biological studies. A high powered transmitter and a recording system to monitor water temperature in a manatee study were developed. We continued to develop aircraft tracking techniques and completed and installed a microprocessor-based system for monitoring the effects of power plant effluents on the location of fish in experimental channels. Two papers, "Use of a microprocessor to locate fish in experimental channels" and "Automatic radio telemetry recording of fish temperature and depth", are being published as part of the 2nd International Conference on Wildlife Biotelemetry. A third paper, "State of the art and needs of the earth platform for animal location systems," was given as an invited paper at the 13th Symposium on Remote Sensing of the Environment at Ann Arbor, Michigan in April 1979.

Transmitter Design

In most telemetry studies quality of data is limited by the signal to noise ratio of the received signal. Since receiver design has reached a point of theoretical limit, any further increase in signal to noise ratio must come from an increased transmitter power level. Consequently, design efforts by a number of groups have concentrated on attempting to increase the power output of the transmitter. The primary problem for all groups has been maintaining power output while being able to maintain the stability of the transmitter circuit. In most designs where sufficient power output was achieved; transmitter adjustment and component choice were critical resulting in a transmitter which was unstable. In particular, when an efficient design was achieved problems with parasitic oscillations became apparent. Additionally, since transmitters used in biological telemetry must be small and all are subject to a highly variable environment, packaging and size become important. These additional requirements influence stability of the circuit. It would also be highly desirable that the transmitter remain stable when it is potted. Incapsulation is the least costly and lowest weight method to ensure protection from environmental factors. The alternative is to use a heavy hermetic case.

Unfortunately very little published data are available on design of transmitters suitable to our application. Particularly unfortunate is the lack of device characterization data for the transistors used in most animal transmitters. Most transistors are characterized for use in either 12 volt or 28 volt systems; whereas, in biotelemetry 3 or 6 volt systems are generally used. Hence our first task was to develop techniques to measure the parameters of the transistors at the voltages we were going to use. In order to optimize power transfer, devices must be properly matched between stages. This requires knowledge of the device's input and output resistance and capacitance. These parameters

along with the device gain, must be evaluated for different supply voltages and input power levels. The measurement system is shown in block diagram form in Figure 1-1. The power gain of the circuit is maximized with the transistor in the circuit while the power reflection is minimized using the reflectometer bridge. The device was then removed from the circuit and the circuit parameters measured with the input terminated at 50 ohms. A Boonton 260A impedance bridge was used to measure the resistance and capacitance. A computer program was written to determine the value of the components for conjugate match. A summary of results for the 2N4427 and 2N4027 transistors is given in Figure 1-2. R_s , C_s and Gain were 22 ohms, 30 pt and 9.5 db for 2N4427 and 44 ohms, 21 pt and 8.0 db for 2N4072. It should be noted that the gain falls off rapidly for voltages less than 9 volts. As a consequence we find it impractical to use power supply voltages below 6 volts.

The test data collected were used to develop a high-powered, stable transmitter circuit. We found that it was impractical to develop a transmitter with power output of greater than 100 milliwatts unless we used a minimum of 3 stages. The first stage of the transmitter is a conventional oscillator circuit operating at 1/2 our output frequency (Figure 1-3). In this case our output frequency is 164 MHz with the oscillator operating at 82 MHz. The oscillator and the entire transmitter circuit are turned on and off via the Vcont line on the oscillator. Using this input control line only low power levels need to be controlled. No problems with self biased oscillation occurred. The second stage acts as a doubler and driver for the output stage. This stage uses emitter stabilization to reduce self-biased oscillation. The third stage is used as a power amplifier stage. Chokes and resistors are used in the base circuits to reduce parasitic oscillation in the biasing circuit, a problem in earlier designs. Values of L12 and L13 and C12 are chosen depending on the length of antenna used. In all cases the values of L12 and L13 are fixed once the antenna length is chosen. C12 is kept accesible for final tuning after the transmitter is potted and the antenna attached. The transmitter has been used on polar bear, sea turtles, and manatees with no apparent problems. All of these transmitters were operated at 6 volts using 2 lithium cells.

Microprocessor based fish tracking system

The system was designed to track fish as they swim freely in experimental channels (Fig. 1-4). The channels are constructed with alternating pool and riffle areas each 33 meters long, and allow manipulation of effluent characteristics near a power plant. Since the design requirement was to locate each fish in the nearest pool or riffle area every 15 minutes a scheme was chosen where the signal strengths from transmitters on the fish are measured at each pool and riffle area.

The measurement system consists of an antenna at each pool and riffle area, and a receiver which feeds a signal into an analog to digital (A/D) converter. The converter supplies a digital representation of the signal level or in the absence of a signal a representation of the noise level. The digital value for the signal at each antenna is stored in the

FIGURE 1-1. TRANSISTOR PARAMETER MEASUREMENT DIAGRAM

FIGURE 1-2. GAIN AND EFFICIENCY VERSUS SUPPLY VOLTAGE FOR

DATE	SYM	REVISION RECORD	H	DR.	CK.

FIGURE I-3. HIGH POWER TRANSMITTER.

TOLERANCES (EXCEPT AS NOTED)		CEDAR CREEK LAB - U of M	
DECIMAL		SCALE	DRAWN BY
FRACTIONAL			APPROVED BY
ANGULAR		TITLE 3 STAGE TRANSMITTER	
	DATE 7-3-79	DRAWING NUMBER

FIGURE I-4. EXPERIMENTAL CHANNEL SCHEMATIC

computer memory. At the end of each pass the antenna with the highest signal value is computed. Since signal strength falls off rapidly with distance, the antenna with the highest signal serves to locate the fish. The maximum signal level is also compared to the average signal level for all antennas to give an index of the signal to noise level, and thus gives an indication of the reliability of the data. Data are displayed using a thermal printer. The computer prints out the location of the antenna with the maximum signal along with the index of signal to noise ratio, and a receiver channel number which identifies the frequency of the radio tag on the fish. After all the receiver channels are scanned, the day number and time of day are printed out.

A typical data printout is shown in Figure 1-5. The entire system is under control of a RCA COSMOS 1802 Microprocessor. This microprocessor was chosen because its CMOS circuitry is compatible with circuitry in the Cedar Creek receiver and because it has inherent noise immunity. Its ready availability and its low power consumption also contributed to its selection. System control and programming are done via a calculator like microterminal notepad supplied by RCA for the 1802 system. The microprocessor provides all control functions for the system including keeping track of time of day. It also determines which antenna has the maximum signal and calculates the signal to noise ratio. Sampling times and choice of fish to be tracked can be chosen by the user in the field by inputting codes via the notepad. A complete description of the system is included with this report as a preprint. Copies of the hardware and software documents are available to other users. The system has been installed at the Monticello field site, and is meeting all the design goals.

Temperature data logging system

A system was developed to measure and record the temperature of the water as manatees swim up and down the St. John River near Blue Springs, Florida. The goal of the study was to determine the amount of time manatees spend in various water temperatures. The simplest way to do this was to attach a temperature measuring transmitter to the manatee and record the output. The range of the transmitters and the expected travel route of the manatees required placement of a number of portable receiving stations along the river route. Since no line power was available, battery power was required. Battery operation of the receiver and pulse decoder presented no problems; however, data recording without line power is more difficult. Rustrak recorders have been used in a number of battery powered recording applications, however, a separate recorder is needed for each animal tracked. A printer would be the ideal recording media in this situation; however, standby power requirements for printers are high, and would require the recharging of the battery supply on a daily basis. As an alternative, a system was designed to sample and hold the data in an intermediate storage device and print out the data in a batch mode when the intermediate storage was full.

FIGURE I-5. DATA OUTPUT

The system (Fig. 1-6) uses a standard memory receiver and pulse decoder. A clock was added to provide sampling control as well as time of day. After 255 data samples are received the recorder is turned on and the data are printed out. Thus the high power demand recorder is on only for a short period. With this scheme the battery lasts a week or longer depending on the recording rate, and standard line reel tape recorders can be used instead of higher cost, low voltage, special recorders. The data are stored in a CMOS CDP 1822 4 x 256 random access memory chip. One chip is used for each printer digit. Using this system power during the data store mode is approximately 100 milliamps. The system could be used to store and print out any type of digital data.

Transmitter standardization

We have continued engineering efforts in trying to develop standard methods to evaluate transmitters and to reliably predict the performance to be expected in the field. Our first goal is to develop a reliable measurement technique to determine the power output of the transmitter. This process is difficult with animal telemetry applications because the antenna is an integral part of the transmitter and is not easily specified. As a consequence direct power output measurements are meaningless. Additionally the animal's presence in the antenna field greatly affects the transmitter characteristics. We are trying to develop standard measuring techniques that will take all of these variables into consideration.

The method we have chosen is to use a standard range measurement technique. This consists of placing a transmitter at a known location and measuring the signal strength or field strength at a known distance from the transmitter. By knowing the range characteristics, the gain of the antennas, and the calibration of the measurement system, an absolute value for the equivalent power output can be obtained. In our system we use a high frequency spectrum analyzer as the power measuring device. This allows a use of a single gain standard over the entire frequency range. Although this technique is quite simple, slight variations anywhere in the system still cause a high degree of variability in the measurements.

In addition to the power output level of the transmitter, knowledge of signal attenuation versus distance is required for accurate range prediction. A number of theoretical calculations for attenuation versus distance are available. The first is the free space path loss calculation which assumes a 20db per decade signal attenuation. Unfortunately these calculations hold true only for free space conditions. These conditions are approximately met for air to air transmissions. However, air to air communications rarely occur in wildlife tracking situations. A second theoretical path loss calculation that is available is the one for ground to ground communications where both transmitter and receiver are on the ground. This assumes a path loss of approximately 40db per decade. Unfortunately these calculations do not make allowances for terrain factors or for vegetative losses. We

FIGURE 1-6. TEMPERATURE DATA LOGGING SYSTEM

are attempting to develop a bank of data that will allow us to predict ranges of path loss under various types of conditions. In developing this data bank we are making measurements under varying conditions, under varying terrain types and under varying vegetative conditions. We expect that as this bank of data is developed we will be able to predict typical reliable ranges and also to indicate what the worst case conditions may be. We expect to build up this data base over the next several years.

Turn on delay transmitter

In September 1978, turn on delay transmitters were constructed for use on sea otters in Prince William Sound, Alaska. Four delayed transmitters were attached to sea otters in October 1978. Two were to turn on after three months delay and two after six months delay.

The otters were not located after the appropriate time had elapsed. A number of factors may account for this problem. The otters may have moved out of the area searched, the transmitters could have fallen off or been physically damaged by the otter, the otter may have died or the system may have malfunctioned. System malfunction could have been caused by any of three factors: 1) battery failure, 2) CMOS timing circuits not operating reliably at the voltage level used or 3) high impedance leakage paths between circuit leads caused by contamination. Battery failure is a distinct possibility as we have had some leakage problems in the past with the type of battery used (Power Conversion Model 400-5).

The CMOS timing circuits are specified by the manufacturer to operate from 3 to 18 volts DC. The operating voltage of the lithium battery is 2.8 volts DC; thus it is operating at slightly below the specified voltage for the circuit. However, discussions with the manufacturer indicate that the circuit should operate reliably below 3 volts. Initial lab tests also indicated that the circuits would operate reliably at 2.8 volts, and our current tests also indicate the circuits should operate satisfactorially at 2.8 volts. A contamination problem was observed in the laboratory when excessive or misplaced amounts of adhesive were used to glue the CMOS flat packs together. Epoxy is now being used to glue the units. The location and amount of epoxy can be precisely controlled, and circuits thus assembled have operated satisfactorily. Additional field tests of the turn on delay transmitters are scheduled for August and September 1979 at Prince William Sound, Alaska.

SUBPROJECT TWO

APPLICATION OF RADIO TELEMETRY
TO SELECTED PROBLEMS
IN VERTEBRATE CENSUSING AND POPULATION STUDY

General objectives of this subproject are to take advantage of new techniques in radio telemetry to obtain information on census methods and population problems. The work is being done in close cooperation with research supported at DOE's Pacific Northwest Laboratory under the project titled "Analysis of Natural Systems", directed by Dr. L. L. Eberhardt. An important part of the PNL program has been devoted to developing the improved quantitative methods in ecology required for DOE's environmental missions. In the process of this long term effort, it has become abundantly evident that improvements in census methods are of key importance, and require ecological advances of the kind developed under the present research program.

Our long-term goal is to utilize the extensive experience, technology and equipment developed over the years in radio telemetry to resolve basic problems in animal census and population study methods, as required to support environmentally oriented DOE missions. Our more immediate goals are to use radio telemetry to develop satisfactory methods for censusing and measuring other important parameters of the sea otter and to apply these methods to otter populations in Alaska and along the California coast.

Sea otters are particularly sensitive to environmental disturbances because of their high metabolic rate and sensitivity to contaminations such as hydrocarbons. We are particularly concerned about oil contamination which is likely to occur as shipping increases from the terminus of the Alaska pipeline at Valdez, Prince William Sound, Alaska. We feel that it is important that a long term research program be initiated to: (1) develop population monitoring techniques and procedures and to shed light on population regulatory factors, (2) begin data collection to evaluate possible influences of environmental changes and disturbances and (3) to conduct experiments which will yield information relative to the influence of various contaminants on sea otter populations.

The above paragraphs are repeated from earlier progress reports and generally set the logic for our research efforts. The Progress Report this year is made up of three papers which are in manuscript form and ready to be reviewed and then submitted for publication. These papers are presented in their entirety. Since telemetry techniques were used in all papers there is some duplication of figures from one paper to the next. These three papers report on: (a) sea otter activity patterns; (b) behavioral repertoire, and (c) oiling experiments.

Activity patterns of male sea otters (*Enhydra lutris*)
in male rafting areas in Prince William Sound, Alaska.

Introduction

Sea otters are often described as being crepuscular in nature, feeding primarily in the early morning and late afternoon. This pattern of daily activity has been reported for otters inhabiting the coastal waters of California (Fisher 1939; Hall and Schaller 1964; Sandegren et al. 1973; Miller et al. 1954: 40; Shimek and Monk 1977) and Amchitka Island in the Aleutian Chain (Kenyon 1969: 59, Estes and Smith 1973: 14, Estes et al. 1978). The cycle is apparently not affected by changes in tide and changes in season (Hall and Schaller 1964; Kenyon 1969: 60; Estes and Smith 1973: 14; Estes et al. 1978), although it may be temporarily disrupted by bad weather (Kenyon 1969: 60; Miller et al. 1975: 40-42). Estes et al. (1978) postulated that the activity pattern of sea otters near Amchitka Island may have developed in response to the activity cycle of fish which constitute an important component of their diet. In contrast, otter populations off the coast of Oregon and near Attu Island in the Aleutians were less dependent on the temporal availability of a particular prey item and exhibited a pattern of foraging that was uniform throughout the day.

Sessile invertebrates are also the major food source for sea otters in California, and there is some contention as to the crepuscular nature of these animals. A study of sea otters in California using radio-telemetry to detect activity contradicted previous findings on the activity pattern of this population (Loughlin 1977: 21-28). No apparent rhythm was observed, and substantial variability was exhibited among individuals. In addition, activity at night was documented. Night activity, especially feeding, has been suggested by others (Barabash-Nikifirov et al. 1947: 86; Hall and Schaller 1964; Estes and Smith 1973: 14; Miller et al. 1975: 43, Shimek and Monk 1977) but these reports were based on limited observations or theoretical considerations of energy requirements and hence were somewhat speculative. For example, Sandegren et al. (1973) and Estes et al. (1978) found that females with young pups spent less time feeding during daylight hours than other otters, presumably to protect their young from predators. It may be inferred that these animals spent more time foraging nocturnally. Except for Loughlin (1977), the previous studies have relied on visual counts of otters in various activity categories such as feeding, resting, grooming, interacting, or locomoting to determine the activity pattern of the population.

This paper reports on recent studies using radio-telemetry which offers several advantages over visual monitoring. These are: (1) Activity can be recorded over a 24 hour period. This is needed in order to gain further insights into the nocturnal activities of this species; (2) Activity can also be recorded during adverse weather conditions. This is particularly important in areas where such conditions prevail for considerable periods of time (e.g., Aleutian Islands). (3) Otters can be

individually distinguished, so variability in activity among individuals (and for each individual) is readily determined. (4) Visual observations may be biased if the observer views an area used principally for one particular activity. Certain areas used primarily for foraging and other areas used chiefly for resting have been recognized in both California and Alaska (Estes and Smith 1973: 16; Miller et al. 1975: 40; Shimek and Monk 1977). Each of these may have a pattern of temporal use not representative of the activity pattern of the population as a whole. The ability to individually identify otters by radio-telemetry virtually eliminates this bias.

Methods

This study was carried out in Prince William Sound, Alaska during the summers of 1977 and 1978. From June to July 11, 1977, the work was concentrated in Sheep Bay and from July 12 to August 14, 1978, the work was in Simpson Bay (Fig. 1). Animals were captured using floating gill nets of 8 3/4" stretch mesh size, anchored at one end and without weights in the lead line. Captured animals were instrumented with small radio frequency transmitters (Fig. 2) and released at the point of capture. A remote automatic recording station was established on a prominent location overlooking the bay and the pattern of the radio signal was received at the station. A strip-chart recorder marked each time a pulse from a transmitter was received. The activity pattern which results is due to the r-f transmitters not being receivable when the antenna was below the water.

We attempted to find the "best" sampling rate or that rate which would give us the most realistic picture of the actual activity of the otter. During 1977 telemetry animals were scanned so that each animal was monitored for 10 seconds. We summarized the data into 10 minute periods and coded for activity based on the number of marks recorded. Eight marks per 10 minute interval were recorded for each animal for the number of channels scanned if the transmitter was always above the surface. Thus, we assigned 6, 7 or 8 marks to resting, 3, 4 or 5 marks to active, and 1 or 2 marks to feeding (Fig. 3). These data were put in a computer format and actiograms of the 24 hour activity pattern were constructed based on these determinations.

In 1978, we decided to record the signal from individual animals for longer periods so that we could measure dive times for feeding. Thus, a scan rate of four minutes per animal was generally chosen, and eight animals were monitored at any one time, giving thirty-two minutes between samples of individuals. In some instances one individual was tuned to two channels; thus, sampling on some individuals was more frequent. The speed of the chart on the Esterline Angus was such that each pulse from a transmitter made a unique line and the general picture which emerged was a thick line approximately 0.8 cm in length if every pulse was recorded during a four minute period (Fig. 3). When breaks occurred in this line, a dive had taken place and we measured the duration of the dive by measuring the distance of the blank (Fig. 3). For activity, these

Fig. 1. Map of study area showing locations of camps during the 1977 and 1978 field seasons.

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DIRECTOR'S REPORT

The Research Program

I

As explained in the Acting Director's Report in last year's "Plant Research", several changes were decided upon in the research program of the PRL. Two of the existing projects or "tasks" were to be expanded and two new ones to be initiated; two existing tasks were to be terminated. The immediate reason for these decisions was the transition of our funding from the U.S. Atomic Energy Commission (AEC) to the new U.S. Energy Research and Development Administration (ERDA). The PRL program should reflect the new, broader-based agency's expanded objectives and needs, it should include both research on plants as converters and as users of energy, and research on effects on plants--crop and other--of environmental stresses that may be caused by expanded energy production, whether deriving from growing reliance on new energy technologies, or the increased use of old ones, e.g. the growing consumption of coal which is projected well into the 21st century. In the period covered by the present report, those decisions have been implemented, and it is only appropriate to devote the Director's pages to the activities of the expanded and the newly initiated tasks. However, some of these activities did not get under way until late in the reporting period and are not yet reflected in the section on individual research reports. We will therefore to some extent anticipate next year's report, including certain results and developments which were not accomplished before 1976.

II

Our report may begin by stating, as is sometimes necessary, a triviality. Plants convert solar radiation into chemical energy; man uses this stored energy as food, fiber and fuel. The conversion of solar energy by plants involves the absorption and reduction of CO_2 . The inescapable consequence of the uptake of CO_2 by land plants is loss of water to the atmosphere. Plants moderate this water loss through the

action of the stomata--adjustable microscopic pores present on the surface of leaves and other, younger parts of the plant--but this action also reduces the supply of CO_2 . The stomatal apparatus is the plant's prime and specific site of control for the regulation of the exchange of water vapor and CO_2 between the plant and its aerial environment. Stomatal functioning affects the efficiency by which plants use sunlight, atmospheric CO_2 , water and, indirectly, nutrients found in the soil or supplied as fertilizer. Knowledge of stomatal functioning is necessary for an estimation of the minimal water requirement for plant production and for an assessment of the potential of plants as energy converters for man.

The question of how stomata regulate gas exchange is the subject of PRL Task #11, headed by K. Raschke. In the reporting period, three approaches to obtaining answers were made: (i) A theoretical framework was built (by G. D. Farquhar) in which the notion of environmental gain was applied to the treatment of stomatal feedback in the steady state as well as during transients. Conversion of these expressions to state space variable notation allows application of optimal control theory, including the inverse of optimal control, i.e. what index of performance is being minimized by stomatal action? (ii) The existing computer-controlled air-conditioning and gas analysis system was used to measure gains in the stomatal feedback loop involving CO_2 in the absence and presence of abscisic acid, the phytohormone which is formed in the leaves under water stress and which may cause stomatal closure. The equipment was modified and programs were written enabling us to perturb the leaf's environment in such a fashion that the effect of stomatal movement on one kind of gas exchange (e.g. that of CO_2) is continuously being compensated while the feedback loop involving the other gas exchange (e.g. that of H_2O) remains fully functional. (iii) The osmotic mechanism of the stomatal valve which controls these gas exchanges was further analyzed during Raschke's sabbatical leave in Munich, Germany, where he investigated, with P. Dittich of the University of Munich, the metabolism of malate and carbohydrates in leaf epidermes. We hope that the results of these studies will lead to elucidation of the mechanism by which guard cells respond to the feedback messenger

to the stomata, CO_2 , and perhaps also to the messenger of stress, abscisic acid; this in turn should help us to understand how plants regulate the gain of CO_2 and the loss of water. This knowledge may lead to modifications of existing cultivation procedures, directed at the saving of energy and water by crops, to new breeding criteria, and thus to an optimization of energy conversion by plants for the benefit of man.

III

While plants convert solar into chemical energy, the cultivation of plants requires an investment of additional energy to manufacture the implements of plant culture, to till the soil, to transport, store and process the produce. Crops require energy subsidies. A large portion of the energy which has thus to be invested goes into the production of fertilizers, with nitrogenous fertilizers accounting for the bulk of this energy subsidy. For instance, at least one third of the energy invested in a maize crop is made through the application of nitrogenous fertilizers; of the natural gas presently consumed in the U.S., 2 percent are used for the reduction of atmospheric nitrogen gas to ammonia for use as fertilizer. A side effect of the high use of fertilizers in current production practices for most crops is run-off which results in pollution problems (eutrophication of bodies of water). There are, however, plants, mainly the legumes which include some important crops, that are capable of assimilating nitrogen directly from the air. If other crops, particularly the cereals, could be made to do the same, the expenditure of energy for production of nitrogenous fertilizers could be greatly reduced, and so could the pollution caused by such fertilizers.

The enzyme, nitrogenase, responsible for biological nitrogen fixation, is oxygen-labile. The only organisms known to be capable of fixing nitrogen (N_2) at normal ambient levels (0.2 atm) of oxygen are blue-green algae and Azotobacter. Fixation by Azotobacter is dependent upon very high rates of respiration of carbohydrate supplied to them. Blue-green algae fix N_2 while producing O_2 by photosynthesis, and require only light as energy source.

These algae have three kinds of cells--vegetative cells, heterocysts, and spores--which differ morphologically, biochemically, and functionally. Nitrogen fixation is primarily localized in the heterocysts, but involves a cooperation between these and the vegetative cells. The latter supply the heterocysts with products of photosynthesis and receive, in return, products of N_2 fixation.

If--as we are attempting in PRL Task #09, headed by C. P. Wolk--we can identify the interactions between vegetative cells and heterocysts, we should achieve a much better understanding of how N_2 fixation might proceed in association with the photosynthetic tissue of a crop plant. Our experiments with ^{13}N -labeled nitrogen gas have shown unequivocally that the atmospheric nitrogen fixed by the blue-green alga, Anabaena cylindrica, is metabolized by the glutamine synthetase-glutamate synthase pathway. Suspensions of heterocysts from this alga couple fixation of nitrogen to uptake of exogenous glutamate, to form glutamine. Wolk and his co-workers are seeking to determine whether it is principally glutamine which moves out of heterocysts as a vehicle for net transfer of fixed nitrogen into vegetative cells.

One of the ways that have been proposed to transfer N_2 -fixing capability to non-leguminous crop plants would simultaneously render the nitrogenase immune to ambient O_2 . That way would be to establish a blue-green alga or an Azotobacter as an endosymbiont within the cells of the plant. The algae have the advantage that their nitrogenase activity could be powered by sunlight, rather than by using up carbohydrates which are synthesized by the host and which might otherwise be transferred as nutrient to the seeds or other storage organs. A necessary condition for stability of such a symbiosis would be, however, that the alga would have to be in some way dependent upon the host. The attempt is therefore being made to isolate auxotrophic mutants of the N_2 -fixing blue-green alga, Anabaena variabilis, i.e. mutants which are unable to produce some compound essential for their growth (e.g. an amino acid) and would therefore depend on the host for supply of this "factor". If such mutants can be isolated, we shall try to determine whether they can reproduce stably within plant cells, supplying the host cells with fixed nitrogen.

IV

While the activities of Tasks #09 and #11 have been expanded, they are "old" tasks that have been active for several years, producing many exciting results. Two other Tasks are new. One, Task #14, headed by A. D. Hanson, is investigating problems of plant responses to environmental stress. The importance of stress research derives from two considerations, one short-term and the other longer-term. Increases in energy production and the use of new technologies, or the increased use of old ones like the predicted increased use of coal of which we have spoken, will result in additional environmental stresses. The effects of these stresses on plants needs to be studied and, as far as possible, anticipated. But no less importantly, efforts at reducing the energy subsidies to crops, of which we have also spoken, and efforts at expanding crop production to new areas, mean that crops will have to perform in higher-stress environments.

Stress research is not among the most active areas of current plant research. Existing methods for identifying the capacities of plants to tolerate environmental stresses are mostly time-consuming and not very reliable (unlike, e.g., methods for measuring responses to nitrogenous fertilizers, or protein quality). We know little about those traits that endow a plant with tolerance to stress, and about their inheritance; the metabolic responses of higher plants to various environmental stresses are also poorly understood. The first objective of Task 014 was therefore the identification and delineation of meaningful research areas. A literature survey was undertaken during 1975 and 1976 (in consultation with agronomists, plant breeders and environmental scientists) to identify problems which increased energy costs and increased energy production may raise in relation to plants, especially crops. The essential parts of this survey have been summarized in a formal report, soon to be available. Research on the metabolism of plants subjected to water stress appears to have the greatest theoretical and practical value, for two reasons: first, because water stress (drought) is a major determinant of vegetation patterns and crop yields; and second, because water stress is intimately interwoven with several other important environmental stresses, including

stresses caused by salinity, ion toxicity, and freezing.

Experimental work (started in 1976) has centered on the accumulation of free proline in the leaves of drought-stressed cereals and range-grasses. These experiments were carried out in collaboration with the Wheat Breeding Program at MSU, because proline accumulation in cereal seedlings has been proposed as a biochemical selection test with a positive correlation to drought resistance in the field. Reevaluation of this claim, using one "drought-resistant" and one "drought-susceptible" barley cultivar, showed that the two cultivars did differ markedly in their rates of proline accumulation when under drought-stress. However, this difference could be fully explained by their relative rates of decline in water status: The more drought-resistant cultivar showed a slower decline in water status and accumulated proline at a lower rate than the less resistant one. In general, proline accumulation was found to occur in barley leaves and leaf zones as a symptom of moderate to severe water stress; the lower the water status, the higher the proline accumulation rate, regardless of cultivar. Within single leaves of moderately drought-stressed barley there were gradients both of water status and of proline accumulation rate, with the lowest water status and highest proline accumulation rate at the leaf tip. In leaf zones which had accumulated proline during severe water stress, and then died and became totally desiccated, the proline was retained in the dead leaf zones and was thus lost totally to the living parts of the plant. About 30% of the original soluble protein N may be converted to proline and walled off in barley leaves which die during severe water stress. The finding that the highest levels of proline are in dead or dying tissue casts considerable doubt on the proposed use of proline accumulation as a simple test showing positive correlation with drought resistance in cereals.

Preliminary work on the incorporation of ^{14}C from ^{14}C -formate into glycine betaine in barley leaves showed that this incorporation was increased ten-fold in moderately drought-stressed tissue. The increased rate of ^{14}C -formate incorporation was accompanied by a two- or three-fold increase in the total amount of betaine in the stressed leaves.

The second new PRL Task, #015, was initiated in direct response to ERDA's need for information on the impact on plants of expected increases in atmospheric sulfur dioxide (SO_2) pollution as a byproduct of the expected increases in burning of coal for energy. P. Filner and L. G. Wilson formed a team to study how SO_2 injures plants, and the mechanisms available in plants for resisting SO_2 injury. Filner had worked previously on regulation of sulfate assimilation, and Wilson on enzymatic mechanisms of sulfate reduction; they thus bring a great deal of potentially applicable knowledge to the project. The real backbone of the project, however, is a Research Associate, Ray Bressan, who was recruited specially for it. The first goal of the project was to find a plant species in which two cultivars, organs, or cell types differed in sensitivity to SO_2 . By comparing the physiological and biochemical properties of such paired biological material, it should be possible to discern factors responsible for the difference in SO_2 sensitivity. Cucurbit species were chosen for screening because of indications in surveys made 40 years ago that cucurbits exhibited wide variations in SO_2 sensitivity. An apparatus was designed and constructed to permit exposure of whole plants or plant parts to SO_2 at specified concentrations, with continuous monitoring of the amount of SO_2 absorbed by the plants. With this apparatus, which has been in operation virtually 7 days a week, 24 hours a day, differences in SO_2 sensitivity were found among cucurbit species (pumpkin being particularly resistant) and also among cultivars of cucumber. The differences in sensitivity between cucumber cultivars has been shown to be based on differences in rate of SO_2 absorption, in accord with an old and previously untested speculation as to why some species are more resistant than others. On the other hand, a marked increase in SO_2 sensitivity with leaf age was observed which cannot be accounted for by differences in SO_2 absorption, either per unit leaf area or per leaf cell. Thus there is now reason to believe for the first time that there is a form of SO_2 resistance which functions after SO_2 enters the plant, i.e. which has a biochemical basis, and which is under developmental control.

One quite unanticipated finding of the SO_2 project was that plants which are fed sulfate through slightly damaged roots while under high light intensities (a condition comparable to that in a cultivated field) emit hydrogen sulfide (H_2S) for several hours. In addition to

the question of function--does the plant do it for a reason?--this finding has raised the intriguing possibility that normal plants may under some circumstances be a significant source of atmospheric sulfur pollution! This discovery was possible only because of the availability of the extremely sensitive monitor of volatile sulfur, obtained for the SO_2 project. Comparable results are quite difficult to obtain by wet chemical methods because the low rate of H_2S emission by plants requires long trapping periods, while the trapped H_2S oxidizes fairly rapidly.

IV

The changes which have been made in the PRL's research program in 1974-75 are the most conspicuous ones to date but they are not without precedent. Several faculty positions have changed hands, resulting in entirely new tasks; reevaluation and modification of the entire program have been going on continuously, aided by internal reviews of the faculty and input from an external advisory committee, and reflecting the advances in our own work as well as developments made in other institutions. And this process of self-regulation will and should continue, as there will be other personnel changes, more new developments will have to be integrated into the program, and the needs of the supporting agency, ERDA, will undoubtedly change, too. At the same time, the continuity of the Laboratory's research program should not be jeopardized; additions and other changes should on the one hand be grounded in existing strengths, and on the other should feed back into these strengths. The changes which were planned in 1974-75 and implemented in 1975-76 have, we feel, struck such a balance. For example, the expanded work on nitrogen fixation in blue-green algae, apart from its intrinsic significance, has provided valuable leads to the understanding of the chemical interdependence of the different cell types of these organisms and has thus strengthened this older and previously predominant project of Task 09. Similarly, the work on SO_2 effects, in addition to providing some information about the sites of SO_2 resistance in plants--information of potential significance for identifying and perhaps alleviating SO_2 damage--has led to the realization of metabolic activities of plants (H_2S release) the existence of which had not been realized before. Thus, those changes in the research program of the PRL can serve as models or guides in further modifications within, and additions to, this program.

Teaching

The following faculty members participated on a regular basis in instruction (scheduled courses, excluding research):

Deborah Delmer

Biochemistry 200. Introduction of Biochemistry (5 credits)*

Biochemistry 855. Plant Biochemistry (3 credits)**

Philip Filner

Biochemistry 452. Biochemistry (4 credits)*

Alfred Haug

Biophysics 402. Introductory Biophysics (3 credits)***

Biophysics 890. Readings in Biophysics (3 credits; taught 2 terms)

Hans Kende and Jan Zeevaart

Botany 415. Plant Physiology: Growth and Development (5 credits)

Kenneth Poff

Microbiology 900. Topics in Microbiology (3 credits)*

Klaus Raschke

Botany 954. Advanced Plant Physiology IV (3 credits)***

Jan Zeevaart

Botany 953. Advanced Plant Physiology III (3 credits)***

* Jointly with another instructor

** Jointly with two other instructors

*** Jointly with several other instructors

Training

The following graduate students (graduate assistants) working in the Laboratory obtained their degrees:

<u>Name</u>	<u>Present location and position</u>
(in parentheses, thesis supervisor and MSU Department conferring degree)	
Liliana Cardemil, Ph.D. (C.P. Wolk; Botany & Plant Pathology)	Facultad de Ciencias, Universidad de Chile, Santiago, Professor
David T. H. Ho, Ph.D. (J.E. Varner; Biochemistry)	Department of Biology, Washington University, St. Louis, Missouri, Research Associate
Maureen Meinert, M.S. (D.P. Delmer; Biochemistry)	Department of Biochemistry, Michigan State University, East Lansing, Michigan, Specialist
Ziva Reuveny, Ph.D. (P. Filner; Biochemistry)	Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, Research Associate

The following research associates completed their periods of work and training in the Laboratory; * indicates an investigator who was on leave from and has returned to his home institution, and who had his personal support:

<u>Name</u>	<u>Present location and position</u>
(in parentheses, supervising faculty member)	
Robert Fisher (C.P. Wolk)	Department of Biology, Virginia Commonwealth University, Richmond, Assistant Professor
Ursula Heiniger (D.P. Delmer)	Institut de Physiologie végétale et de Phytochimie, Université de Fribourg, Switzerland, Research Biologist
Philip Nordin* (D. T. A. Lamport)	Department of Biochemistry, Kansas State University, Manhattan, Professor

Faculty activities, functions, honors, etc.

H. Kende became a member of the Advisory Panel for Developmental Biology, National Science Foundation. *A. Lang* was elected an Honorary Vice President of the XII International Botanical Congress, Leningrad, USSR, July 1975. He started a sabbatical leave at the K. A. Timiryazev Institute of Plant Physiology of the Academy of Sciences of USSR, Moscow, under the Scientific Exchange Program between the National Academy of Sciences and the Academy of Sciences of USSR (August 11, 1975).

K. Raschke returned from a sabbatical leave, spent at the Botanical Institute, Technical University, Munich, Germany with support from the John Simon Guggenheim Foundation (August 31, 1975).

PERSONNEL

Faculty, Including Joint Appointments

Deborah P. Delmer, Assistant Professor of Biochemistry
 Philip Filner, Professor of Biochemistry
 Alfred Haug, Professor of Biophysics
 Michael Jost, Associate Professor of Botany and Plant Pathology
 Hans J. Kende, Professor of Botany and Plant Pathology
 Derek T. A. Lamport, Professor (Research)
 Anton Lang¹, Director and Professor of Botany and Plant Pathology
 Kenneth L. Poff, Assistant Professor of Botany and Plant Pathology
 Klaus Raschke, Professor of Botany and Plant Pathology
 Lloyd G. Wilson, Associate Professor of Botany and Plant Pathology
 (Research)
 C. Peter Wolk, Professor of Botany and Plant Pathology
 Jan A. D. Zeevaart, Professor of Botany and Plant Pathology

Research Specialists (including supervising faculty member)

Graham Farquhar (K. Raschke) From July 1, 1975
 Andrew Hanson (A. Lang) From July 1, 1975

Research Associates (including supervising faculty member)

Peter Beutelmann² (H. Kende) From September 1, 1975
 Mary Ericson (D. Delmer)
 Graham Farquhar (K. Raschke) Until June 30, 1975
 Stuart Flashman (P. Filner) From November 1, 1975
 Robert Fisher (P. Wolk) Until August 31, 1975
 Gary Gardner³ (H. Kende)
 Andrew Hanson (H. Kende) Until June 30, 1975

¹On sabbatical leave from August 11, 1975

²Supported by Max Kade Foundation

³Partially supported by a grant from the National Science Foundation
 to H. Kende

Ursula Heiniger (D. Delmer) Until December 31, 1975
 Katsushi Manabe (K. Poff) From February 16, 1975
 John Meeks (P. Wolk) From August 16, 1975
 Harry Phillips (H. Kende) From September 16, 1975
 Hartmut Quader¹ (P. Filner) From October 1, 1975
 Robert Saftner (K. Raschke) From October 1, 1975
 Werner Schmidt¹ (K. Poff) From October 1, 1975
 Joseph Thomas² (P. Wolk)
 Ludo Van Rompuy (J. A. D. Zeevaart) From January 1, 1975

Graduate Assistants (including departmental affiliation and supervising faculty member)

Kent Bradford (Horticulture) - H. Kende and D. R. Dilley
 (Department of Horticulture)

*Charles Caldwell (Botany and Plant Pathology)

Liliana Cardemil de Balboa (Botany and Plant Pathology) - P. Wolk
 Until October 15, 1975

Judith Cherniack (Biochemistry) - P. Filner

Dean Dubbe (Botany and Plant Pathology) - K. Raschke

Takayasu Hirose (Botany and Plant Pathology) - P. Wolk

David Tuan-Hua Ho (Biochemistry) - J. E. Varner

Until October 15, 1975

Jean-Cheui Hsung (Biophysics) - A. Haug

*David Kavon (Botany and Plant Pathology)

*Cynthia Kennedy (Botany and Plant Pathology)

*Anita Klein (Biochemistry)

Kenneth Leonards (Botany and Plant Pathology) - A. Haug and

E. Cantino (Department of Botany and Plant Pathology)

*David Maltby³ (Biochemistry)

Maureen Meinert (Biochemistry) - D. Delmer, Until September 15, 1975

James Metzger (Botany and Plant Pathology) - J. A. D. Zeevaart

¹Supported by Deutsche Forschungsgemeinschaft (DFG)

²Partially supported by a grant from National Science Foundation to C. P. Wolk

³Supported by a training grant from the National Institutes of Health to
 the Department of Biochemistry

*Has not yet chosen a major advisor

Andrew Mort (Botany and Plant Pathology) - D. T. A. Lamport

Margaret Pierce (Botany and Plant Pathology) - K. Raschke

*Louis Ptak (Botany and Plant Pathology)

Ziva Reuveny (Biochemistry) - P. Filner, Until August 1, 1975

Shirley Rodaway (Botany and Plant Pathology) - H. Kende

Tom Skokut (Botany and Plant Pathology) - P. Filner, P. Wolk

Michael Sussman¹ (Botany and Plant Pathology) - H. Kende

Jeffrey Suttle (Botany and Plant Pathology) - H. Kende

Carol Van Kirk (Botany and Plant Pathology) - K. Raschke

Herman Weller² (Biophysics) - A. Haug

Bruce Whitaker (Botany and Plant Pathology) - K. Poff

Narendra Singh Yadav (Botany and Plant Pathology) - P. Filner, H. Kende

Li Lillian Yang (Biophysics) - A. Haug

Technical Personnel (including supervising faculty member; if none listed -- general service)

Susan Atkins, Senior Technician (P. Filner)

James Brede, Senior Technician (J. A. D. Zeevaart) Until January 9, 1975

Michael Caughey, Senior Technician (D. T. A. Lamport)

Charles Cetas, Senior Technician (J. A. D. Zeevaart)

Alma Clark, Senior Laboratory Assistant,

Renate de Zacks, Principal Technician (H. Kende)

Robert Geyer, Instrument Maker and Shop Supervisor

Alicia Green, Librarian, Until September 30, 1975

Marina Kao, Senior Technician (K. Raschke)

Robert Kever, Equipment Technician

Carl Kulow, Senior Technician (D. Delmer)

Elliot Light, Supervisor Research

Anna Owens, Senior Laboratory Assistant

Raymond Sculley, Principal Technician (M. Jost) Until August 1, 1975

¹Partially supported by a predoctoral fellowship from the National Science Foundation

²Supported by a training grant from the National Institutes of Health to the Department of Biophysics

*Has not yet chosen a major advisor

Paul Shaffer, Senior Technician (P. Wolk)

Thomas Sharkey, Senior Technician (K. Raschke)

Michiyo Skokut, Senior Technician (K. Poff)

Gary Smith, Senior Technician (A. Haug)

Henry Wieferich, Mechanical Technician

Elizabeth Wojciuch, Senior Technician, Until February 7, 1975

Administrative and Clerical Personnel

Karen Cline, Senior Departmental Secretary

Homer K. Curtis, Administrative Assistant

Kathryn Harmon, Principal Clerk

Tawn Jarvis, Senior Clerk Stenographer

PUBLICATIONS

- *1) Austin, S., A. Galonsky, J. Bortins and C. P. Wolk: A batch process for the production of ^{13}N -labeled nitrogen gas. Nuclear Inst. Meth. 126:373-379 (1975).
- 2) Baumgartner, B., H. Kende and P. Matile: Ribonuclease in senescing morning glory: purification and demonstration of *de novo* synthesis. Plant Physiol. 55:734-737 (1975).
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- 4) Durley, R. C., R. P. Pharis and J. A. D. Zeevaart: Metabolism of [^3H]-Gibberellin A₂₀ by plants of *Bryophyllum daigremontianum* under long- and short-day conditions. Planta (Berl.) 126:139-149 (1975).
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MEETINGS, SEMINARS, LECTURES

Annual Meeting of the American Society of Plant Physiologists, Corvallis, Oregon (August 1975): Papers were given by D. P. Delmer on "Utilization of UDP-glucose by detached cotton fibers" (co-authored with C. Kulow); U. Heiniger on "Utilization of UDP-glucose by a particulate fraction of cotton fibers" (co-authored with D. Delmer); P. Filner on "Apparent derepression of ATP sulfurylase in response to restriction of sulfur supply in cultured tobacco cells" (co-authored with Z. Reuveny); G. Farquhar on "Dynamic stomatal behavior"; M. Pierce on "Stomatal closure in response to xanthoxin and abscisic acid" (co-authored with R. D. Firn and K. Raschke); A. Mort on "Evidence for polysaccharide attachment to extensin in cell walls obtained from tomato cell suspension cultures" (with D. T. A. Lampert); G. Gardner on "Cytokinin binding to particulate fractions from moss protonemata" (co-authored with H. Kende and M. Sussman); A. D. Hanson on "The biosynthetic pathway of ethylene in excised flower tissue of *Ipomoea tricolor*" (co-authored with H. Kende); H. Kende on "The regulation of aging in excised flower tissue of *Ipomoea tricolor*" (co-authored with A. D. Hanson); M. Sussman on "Cytokinin binding to particulate fractions from tobacco callus" (co-authored with H. Kende); R. W. Fisher on "A substance stimulating sporulation of a blue-green alga" (co-authored with C. P. Wolk); K. Poff on "Thermotaxis by pseudoplasmodia of *Dictyostelium discoideum*"; J. A. D. Zeevaart on "Comparative studies on phloem exudation from induced and non-induced *Perilla* leaves."

Annual Meeting of the Midwest Section of the American Society of Plant Physiologists, Bradley University, Peoria, Illinois (June 1975): Papers were given by M. R. Sussman on "Synthesis of high specific activity benzyladenine-benzyl- p^3H " (co-authored with R. Firn and H. Kende); A. Hanson on "Ethylene production and aging in excised flower tissue of *Ipomoea tricolor*" (co-authored with H. Kende); S. Rodaway on "Effect of 5-fluorouracil on the thermolability of α -amylase from barley half-seeds" (co-authored with H. Kende); M. Ericson on "Lipid intermediates involved in the synthesis of a plant glycoprotein" (co-authored with D. Delmer); U. Heiniger on "UDP-glucose utilization in cotton fibers"; M. Meinert on "The primary cell wall of the cotton fiber" (co-authored with D. Delmer); M. Pierce on "Stomatal closure in response to xanthoxin and abscisic acid" (co-authored with K. Raschke and R. Firn); L. Wilson on "Enzymatic sulfate reduction in yeast"; A. Mort on "Evidence for the attachment of a polysaccharide including a polyrhamnogalacturonan to 'extensin', the hydroxyproline-rich cell wall protein"; L. Cardemil on "Structural analysis of the polysaccharides from spore and heterocyst envelopes of a blue-green alga" (co-authored with C. P. Wolk); G. Farquhar on "Resistance to evaporation of the mesophyll cell wall"; R. Fisher on "A substance stimulating sporulation of a blue-green alga" (co-authored with C. P. Wolk); K. Manabe on "Absorbance changes induced by light in corn coleoptiles" (co-authored with K. Poff).

XII International Botanical Congress, Leningrad, USSR (July 1975):

A. Lang chaired the symposium "The role of the USSR Academy of Sciences in the development of botany". Papers were presented by D. Delmer on "The possible role of lipid intermediates in glycoprotein synthesis in higher plants" (co-authored with M. Ericson); D. Delmer on "The utilization of UDP-glucose by developing cotton fibers" (co-authored with U. Heiniger); A. Haug on "Membrane structure of *Thermoplasma acidophila*" (co-authored with J. C. Hsung); A. Lang on "Environment and internal mechanisms of autogenesis regulation" (Symposium "General aspects of plant ontogenesis"); D. T. A. Lampion on "Evidence for galactosyl serine as a major site of polysaccharide attachment in extensin"; K. Raschke on "The stomatal feedback system responses to carbon dioxide and abscisic acid" (Symposium "The mechanism of stomatal movements").

L. Cardemil presented a paper on "Structural analysis of the backbones of the polysaccharides from spore and heterocyst envelopes of a blue-green alga" (co-authored with C. P. Wolk) at the Annual Meeting of the American Society for Microbiology, New York City, N.Y. (May 1975).

P. Filner gave seminars on "Regulation of two functionally convergent pathways in higher plants: nitrate and sulfate assimilation" and "Taking apart and putting together microtubules in vitro" at Washington State University, Genetics Program, Pullman (April 14, 15 1975) and on "Interactions of colchicine and microtubules" at the University of Washington, Department of Botany, Seattle (April 16, 1975).

G. Gardner gave seminars on "Molecular characteristics of phytochrome" at the University of Massachusetts, Department of Botany, Amherst (January 16, 1975); "Properties of phytochrome" at Arizona State University, Department of Botany, Tempe (February 19, 1975) and at Rutgers University, Department of Biochemistry and Microbiology, New Brunswick, New Jersey (May 8, 1975); "Hormone receptors in higher plants" and "Proteolysis of phytochrome" at Kansas State University, Division of Biology, Manhattan (May 19 and 20, 1975); "Cytokinin binding in a particulate fraction from moss extracts" at Princeton University, Department of Biology, Princeton, New Jersey (December 2, 1975) and at Smithsonian Institution, Radiation Biology Laboratory, Rockville, Maryland (December 5, 1975); "Cytokinin binding in moss extracts" at the University of North Carolina, Department of Botany, Chapel Hill (December 8, 1975).

A. Hanson gave a seminar on "Ethylene and senescence in morning glory flowers," at Monsanto Agricultural Division, St. Louis, Missouri (February 24, 1975).

A. Haug gave a seminar on "Regulation on membrane lipid fluidity in Mycoplasma," at the V. L. Komarov Botanical Institute, Myxomycetes Group, USSR Academy of Sciences, Leningrad, USSR (July 10, 1975).

H. Kende presented a paper at the Gordon Research Conference on Postharvest Physiology, Santa Barbara, California on "The effect of ethylene on compartmentation during senescence" (June 23-27, 1975); and gave seminars on "On the regulation of plant senescence by ethylene" at Harvard University, Biological Laboratories, Cambridge, Massachusetts (May 29, 1975); and "On the role of ethylene in the senescence of morning glory flowers", at the U.S. Department of Agriculture, Beltsville, Maryland (October 15, 1975).

A. Lang during a sabbatical leave in the USSR, gave seminars on "Plant hormones--advances and open questions" at the Institute of Plant Physiology and Biochemistry, Siberian Division, Academy of Sciences of USSR, Irkutsk (August 26, 1975); the Institute of Agrochemical Problems and Hydroponics and the Botanical Institute of the Academy of Sciences of the Armenian SSR, Erevan (September 11 and 12, respectively), the Botanical Institute, Academy of Sciences of the Ukrainian SSR, Kiev (September 23, 1975) and the K. A. Timiryazev Institute of Plant Physiology, Academy of Sciences of USSR, Moscow (November 19, 1975); talks on the organization and research program of PRL at the Nikita State Botanical Garden, Yalta, Crimea (September 17, 1975) and the K. A. Timiryazev Institute, Moscow (December 16, 1975); and a lecture on "Early stages in plant development" also at the latter institute (November 21, 1975).

D. T. A. Lamport gave seminars on "Extensin eliminated: new evidence for the role of a hydroxyproline-rich macromolecule in plant cell walls" at Kansas State University, Department of Biochemistry, Manhattan, (April 27, 1975); and "Do the hydroxyproline-rich glycoproteins of plant cell walls throw any light on the origin of multicellular eukaryotes?" at Iowa State University, Department of Botany and Plant Pathology, Ames (November 9, 1975).

K. Poff presented papers "Effects of light on *Dictyostelium discoideum*" at the Slime Mould Workshop, University of Essex, Colchester, England (April 1975); "Thermotaxis in *Dictyostelium*" and "The photoreceptor pigment for phototropism" at the Gordon Conference on Sensory Transduction in Microorganisms, Santa Barbara, California (December 1975); "Absorbance changes associated with the blue light photoreceptor pigment" (co-authored with W. Butler) at the Annual Meeting of the American Society of Photobiologists, Louisville, Kentucky (June 1975); and gave seminars on "Photoreceptors of *Dictyostelium discoideum*" at the Max-Planck Institut für Ernährungsphysiologie, Dortmund, Germany (April 1975); "The photoreceptor pigment associated with phototropism in *Phycomyces*", at the Max-Planck Institut für Molekulare Genetik, Berlin, Germany (April 1975); "Photoreceptors in *Dictyostelium*, *Neurospora* and corn" at the University of Freiburg, Institut für Biologie II, Freiburg, Germany (April 1975); "The photoreceptor pigment of phototaxis in the cellular slime mold, *Dictyostelium discoideum*" at the State University of Antwerp, Department of General Botany, Antwerp, Belgium (April 1975); "Thermosensory physiology" at the Carnegie Institute of Washington, Plant Biology Department, Stanford, California (August 1975).

K. Raschke presented papers on "How stomata resolve the dilemma of opposing priorities" at the Meeting for Discussion on Water Relations of Plants, The Royal Society, London (January 1975); and on "Transfer of ions and products of photosynthesis to guard cells" at the U.S.-Australia Science Agreement Conference on Transport and Transfer Processes in Plants, Canberra, Australia (December 1975). He also gave seminars on "Stomatal regulation of water loss: hydraulics and harmonics" at the University of Lancaster, Department of Biological Sciences, Lancaster, England (February 1975); "Die Regelung des Wasserhaushaltes in der Pflanze" at Ciba-Geigy Ltd., Basel, Switzerland (March 1975); "Stomata im Dilemma zwischen Durst und Hunger" at Kleinwanzlebener Saatzucht A.G., Einbeck, Germany (April 1975); "Spaltöffnungsbewegung: Alkali, Apfelsäure, Abszissinsäure" at University of Göttingen, Botanische Anstalten, Göttingen, Germany (April 1975); "Stomabewegung: Mechanik und Metabolik", Ruhr-Universität Bochum, Germany (June 1975); "Zum Mechanismus der stomatären Regelung von Transpiration und Photosynthese", University of Tübingen, Institut für Biologie, Germany (June 1975); "Neue Untersuchungen über den Mechanismus der Stomatabewegungen", Colloquium of the Botanical Institutes of the University and the Technical University, Munich, Germany (June 1975); "Ionentransport und Säurestoffwechsel als Ursachen der Stomabewegung", Kernforschungsanlage Jülich, Institut für physikalische Chemie, Germany (December 1975); "Stomatal movement: mechanics and metabolics" at Volcani Center, Bet Dagan, Israel (December 1975); "Stomatal regulation of photosynthesis and transpiration" at the India Meteorological Department, Agricultural Meteorology Division, Poona, India (December 1975).

C. P. Wolk gave seminars on "Differentiation and pattern formation in filamentous blue-green algae" at Kettering Research Laboratory, Yellow Springs, Ohio (March 12, 1975); and "Developmental biology of the blue-green algae" at the University of Minnesota, School of Medicine, Department of Microbiology, Minneapolis (November 12, 1975).

J. A. D. Zeevaart gave a seminar on "Hormonal control of flower formation", Washington University, Department of Biology, St. Louis, Missouri (April 21, 1975).

PRL SEMINAR SERIES

<u>Date</u>	<u>Speaker and Institution</u>	<u>Subject</u>
January 8, 1975	Dr. R. J. Huskey ¹ University of Virginia Charlottesville, Virginia	Genetic control of development in <i>Volvox</i>
January 15, 1975	Dr. J. A. D. Zeevaart MSU/ERDA Plant Research Lab	Metabolism of abscisic acid in bean seedlings
January 22, 1975	Dr. R. Hertel University of Freiburg Freiburg, Germany	<i>In vitro</i> auxin binding
January 29, 1975	Dr. L. Bogorad Harvard University Cambridge, Massachusetts	Complementary chromatic adaptation -- a problem in developmental biology
February 5, 1975	Dr. M. Zaitlin ² Cornell University Ithaca, New York	Replication of tobacco mosaic virus
February 11, 1975	Dr. R. Lozier ³ University of California San Diego, California	Light energy transduction by the purple membrane of <i>Halobacterium halobium</i>
February 12, 1975	Dr. D.P.S. Verma McGill University Montreal, Canada	The regulation of leghemoglobin gene expression during symbiotic nitrogen fixation
February 19, 1975	Dr. R. Hertel University of Freiburg Freiburg, Germany	The nature of the blue-light receptor pigment in plants
February 26, 1975	Dr. J. A. Bassham ⁴ Lawrence Berkeley Laboratory University of California Berkeley, California	Metabolic regulation of photosynthetic carbon metabolism
March 5, 1975	Dr. J. E. Varner Washington University St. Louis, Missouri	Control of synthesis and secretion of the yeast glycoprotein enzymes
March 20, 1975	Dr. S. Roseman ⁵ The Johns Hopkins University Baltimore, Maryland	Studies on a bacterial phosphotransferase and its role in sugar transport

<u>Date</u>	<u>Speaker and Institution</u>	<u>Subject</u>
March 26, 1975	Dr. A. A. Lev ⁶ Institute of Cytology Academy of Sciences of USSR Leningrad, USSR	Possible influence of ionic composition on the structure of bilayer lipid membranes
April 2, 1975	Dr. W. R. Cummins University of Toronto Toronto, Ontario, Canada	The role of turgor pressure in growth and growth storage
April 10, 1975	Dr. C. F. Fox University of California Los Angeles, California	Biological effect of membrane lipid physical state in cultured mammalian fibroblasts
April 16, 1975	Dr. B. A. Palevitz State University of New York Stony Brook, New York	Microtubules and microfilaments: their role in motility and morphogenesis of plant cells
April 17, 1975	Dr. F. Gaertner Biology Division Oak Ridge National Laboratory Oak Ridge, Tennessee	Why is a multienzyme complex?
April 23, 1975	Dr. D. S. Fensom ⁷ Mount Allison University Sackville, New Brunswick, Canada	Can you take the pulse of turnips? (A contractile model of phloem transport)
April 30, 1975	Dr. D. D. Harpstead Department of Crop and Soil Sciences Michigan State University	Genetic engineering for nutritional quality in food crops
May 5, 1975	Dr. A. A. Nichiporovich ⁸ K. A. Timiryazev Institute of Plant Physiology Academy of Sciences of USSR Moscow, USSR	The properties of photosynthesis affecting crop productivity
May 7, 1975	Dr. J. DeMoss University of Texas Houston, Texas	Assembly and maturation of the nitrate reductase in <i>E. coli</i>
May 14, 1975	Dr. J. Dainty University of Toronto Toronto, Ontario, Canada	Osmotic and turgor regulation in plant cells
May 21, 1975	Dr. G. G. Laties ¹⁰ University of California Los Angeles, California	Possible role of ethylene in cyanide-resistant respiration of plant mitochondria

<u>Date</u>	<u>Speaker and Institution</u>	<u>Subject</u>
May 28, 1975	Dr. L. Margulis ² Boston University Boston, Massachusetts	Evolutionary consequences of symbiosis and the origin of cells
May 29, 1975	Dr. C. E. Ballou ⁹ University of California Berkeley, California	Recent developments on the structure and biosynthesis of yeast cell wall mannan
June 16, 1975	Dr. J. DeGreef State University of Antwerp Antwerp, Belgium	Rapid physiological changes controlled by phytochrome
July 3, 1975	Dr. U. P. Hansen University of Kiel Kiel, Germany	Time constants in the effect of light on membrane potential of <i>Nitella</i>
August 8, 1975	Dr. K. Mothes Institut für Biochemie der Pflanze Halle, German Democratic Republic	Secondary metabolism in plants
September 9, 1975	Dr. J. Eber University of Colorado Boulder, Colorado	Enzymes of phenylpropanoid metabolism in cell suspension cultures of plants
September 26, 1975	Dr. W. W. Schwabe ¹⁰ Wye College University of London Kent, England	Apical growth and leaf positioning in higher plants
October 1, 1975	Dr. J. Boyer University of Illinois Urbana, Illinois	Nitrate reduction in plants having low water potentials
October 8, 1975	Dr. M. H. Goldsmith Yale University New Haven, Connecticut	Does a pH gradient drive polar auxin transport?
October 17, 1975	Dr. R. C. Valentine Plant Growth Laboratory University of California Davis, California	Molecular biology of nitrogen fixation
October 22, 1975	Dr. W. R. Briggs Carnegie Institution of Washington Stanford, California	Investigations on the blue light photoreceptor

<u>Date</u>	<u>Speaker and Institution</u>	<u>Subject</u>
November 5, 1975	Dr. T. Hodges Purdue University West Lafayette, Indiana	Selectivity of ion transport in roots
November 12, 1975	Dr. L. Mortenson Purdue University West Lafayette, Indiana	Studies on the role of molybdenum in dinitrogen fixation
November 14, 1975	Dr. O. Björkman ⁵ Carnegie Institution of Washington Stanford, California	Photosynthetic response and adaptation to extreme environments
November 19, 1975	Dr. D. Weeks Institute for Cancer Research Philadelphia, Pennsylvania	Regulation of microtubulin synthesis in <i>Chlamydomonas reinhardtii</i>
December 3, 1975	Dr. R. Keates McMaster University Hamilton, Ontario, Canada	The accessory protein of microtubules
December 10, 1975	Dr. J. S. Lovett Purdue University West Lafayette, Indiana	Regulation of RNA and protein synthesis in the zoospores and during zoospore germination in <i>Blastocladiella</i>

¹Joint seminar with the Genetics Group

²Joint seminar with the Department of Crop and Soil Sciences

³Joint seminar with the Department of Microbiology and Public Health

⁴Arranged in cooperation with the Department of Botany, University of Michigan, Ann Arbor

⁵Sponsored by the Plant Research Laboratory Graduate Assistants

⁶Joint seminar with the Departments of Biophysics and of Chemistry

⁷Joint seminar with the Department of Botany and Plant Pathology

⁸Joint seminar with the Departments of Crop and Soil Sciences and of Horticulture

⁹Joint seminar with the Department of Biochemistry

¹⁰Joint seminar with the Department of Horticulture

RESEARCH REPORTS

The following reports, dealing with work much of which is still in progress, are not intended as publications, and should not be cited without specific permission by the responsible investigator.

STOMATAL REGULATION OF THE GAS EXCHANGE BETWEEN PLANT AND ENVIRONMENT

This project deals with the regulation of CO_2 uptake and water-vapor loss of plants by the stomata. Virtually all the carbon dioxide taken up by plants during the conversion of solar energy into chemical energy passes through the stomata; almost three-quarters of the water precipitated on the terrestrial U. S. returns to the atmosphere through stomata. The stomatal control system responds, through the action of several feedback loops, to environmental factors (CO_2 , humidity, light, temperature, pollutants) and to physiological factors within the plant (the intercellular CO_2 concentration, water potential, concentration of abscisic acid, a plant hormone formed rapidly under stress). We are attempting to determine functions which describe the responses of the components of the feedback system to the above inputs, and by applying physical and biochemical principles and methods we hope to gain an understanding of the underlying physiological mechanisms of stomatal regulation.

Stomata are rarely, if ever, in a steady state in the field. The stomatal control mechanisms are not homeostatic; the opposing priorities of increased assimilation and decreased transpiration see to that. We have begun to develop tools to study stomatal function while stomata move, and to measure stomatal performance. The first three of the following contributions report on such tools and their use, while the last three report on groundwork necessary to identify the biochemical transducer and effector mechanisms in guard cells.

1. Computer Control of Air-Conditioning and Gas Analysis System

G. D. Farquhar, D. Phillips, T. D. Sharkey, K. Raschke

Measurement of plant environment and performance requires facilities for accurate measurement, multiple, fast computations and display of data. We require the results of these computations even as the plant is responding, and an on-line minicomputer is the simplest means of achieving this. Last year we reported plans for interfacing a minicomputer to our gas analysis system (Plant Research 74, p. 34). We have now written the necessary "drivers",

programs necessary for interfacing the computer to its peripherals, and the programs required for calibration of equipment and immediate display of data. Computer control of the air-conditioning and gas analysis system is now used routinely.

We have also modified equipment and written programs that allow us to perturb the leaf environment in predetermined fashions. The humidity of the air can be varied by varying the temperature of water flowing through the jacket of a condenser. The computer determines the mixing ratio of hot and cold water in this flow. This has enabled us, as planned, to break the feedback loop involving transpiration, by compensating changes due to stomatal aperture with changes in ambient humidity. Maintenance of constant transpiration contributes to a reduction in the ambiguity associated with experiments on stomata. We have also written a program that produces sinusoidal variations in the humidity of the air passing over a leaf. A modification of this program changes humidity in a manner that evaporation now follows a sinusoidal trace.

2. Quantitative Framework for the Study of the Effects of Stress on Feedback Loops Involving Stomata

G. D. Farquhar

The degree to which stomata affect the fluxes of CO_2 to the leaf and water and sensible heat from the leaf depends on the environment imposed on those stomata. For example, in an atmosphere with a high ambient concentration of CO_2 , a small change in stomatal conductance gives rise to a larger change in the influx of CO_2 than in an environment in which the $[\text{CO}_2]$ is low. Extending the notion of stomatal environment to include the photosynthetic tissue, we also know that in an environment in which efficient CO_2 fixation is occurring (e.g. plants with the C_4 pathway) a small change in stomatal conductance gives rise to a bigger change in the influx of CO_2 than does an 'inefficient' environment. The sensitivity of the flux of water vapor to changes in stomatal conductance, $\frac{dE}{dg}$, has been termed "environmental gain". An analogous concept exists for the flux of CO_2 but one can choose to divide the CO_2 feedback loop slightly differently and to call the sensitivity, $\frac{dC_i}{dg}$, of the concentration of CO_2 inside the leaf, C_i , to changes in stomatal conductance the environmental gain. The remaining halves of the CO_2 feedback loops involving water and CO_2 are the sensitivities of stomatal conductance to small changes in evaporation rate and internal $[\text{CO}_2]$, respectively.

The sensitivities, $\frac{dg}{dE} C_i$ and $\frac{dg}{dC_i} E$ depend on the physiology of the stomata and are called physiological gains.

The dimensionless product of an environmental and a physiological gain is called a loop gain, G . Its magnitude is a measure of the importance of that loop and is a measure of what happens to a disturbance travelling around the loop. A positive loop gain means that positive feedback is occurring and a negative loop gain means that negative feedback is occurring. Consider a disturbance which would cause a change, n , in stomatal conductance if acting alone, i.e., with the feedback loop inactivated. It may be shown simply that the change in conductance that occurs in the presence of a feedback loop, with a loop gain G , is $\frac{n}{1-G}$. Thus with a loop gain of -1 (negative feedback) the effect of an external disturbance is halved. The loop gain should also affect the speed of stomatal responses and the stability of the system.

The notion of physiological gain may be extended to include the time dependence of transient physiological processes. The physiological transfer function is the ratio of the Laplace transformations of the stomatal response to the small perturbation of flux (or C_i in the case of CO_2). Knowing the physiological transfer functions, the stomatal responses to arbitrary (small) environmental perturbations can be predicted.

There is evidence that the hydraulic physiological transfer function is of third order,

$$\text{i.e. } \bar{g}/\bar{E} = H_0 \frac{(1 - s\tau_1)(1 + s\tau_1)}{(1 - s\tau_3)(1 - s\tau_4)(1 - s\tau_5)} \quad (1)$$

where $\bar{g} = \int_0^\infty e^{-st} (g - g_0) dt$ and g is stomatal conductance

$\bar{E} = \int_0^\infty e^{-st} (E - E(g_0)) dt$ and E is evaporation rate

s is the Laplace operator, and the τ_i are time constants. To convert this to state space variable notation

$$\dot{x} = Ax + Bu \quad (2)$$

where x is the state of the system, u is the control vector, A is the system matrix and B is the associated matrix of the input vector; we note that if h is the effective boundary layer conductance and p is the evaporation potential

$$E = \frac{ghp}{g + h}$$

$$\bar{E} = \frac{\partial E}{\partial p} \bar{p} + \frac{\partial E}{\partial g} \bar{g} \quad (3)$$

The form (2) is achieved by taking the inverse transforms of Eqs. (1) and (3), combining them and putting

$$x = (g \dot{g} \ddot{g})^T$$

$$u = (p \dot{p} \ddot{p})^T$$

The responses of stomata to perturbations of the CO_2 feedback loop can be described in a similar manner, with the control vector depending on changes of light intensity or of concentrations of CO_2 in the ambient air.

These two loops may also be combined using state and control vectors of greater dimension.

Using state space variables notation single, a n^{th} order system is then reduced to n first order equations, which expedites analysis using digital computers. The main advantage, however, is that it allows the solution of certain problems of optimal control. For example, one could ask, in the language of optimal control: "Knowing the system and associated matrices, A and B , and choosing as an index of performance, J , the time taken to move from one state (conductance) to another, what controls (humidity and $[CO_2]$) should be implemented to minimize J ?"

The more fundamental problem is the inverse problem (Kalman, 1964). Knowing the control laws, what index of performance is being minimized? The challenge is to choose a state vector, depending on assimilation and transpiration and perhaps conductance itself, use control laws related to the transfer functions and determine what aspects of this summary of dynamic plant behaviour are being optimized. The work is continuing in order to solve this problem.

Reference

Kalman, R. E., Trans. ASME, J. Basic Eng., 86:51 (1964)

3. Measurement of CO₂ Loop Gains and the Effects of Abscissic Acid on these Gains

D. Dubbe, G. D. Farquhar, K. Raschke

A key process in the response of plants to many different kinds of stress is the production of abscissic acid. It was reported earlier (Plant Research '73, p. 25) that increased levels of ABA increase the sensitivity of stomata of *Xanthium strumarium* to [CO₂] (i.e. the magnitude of the physiological gain is increased). We have commenced studies using the previously outlined approach and measured the environmental, physiological and loop gains of the CO₂ feedback loops in three species, *Gossypium hirsutum* (cotton), *Xanthium strumarium* (cocklebur), and *Zea mays* (corn), with and without the addition of (±)-ABA (akin to stressed and non-stressed plants respectively).

In each case the leaf temperature was held at about 23 C while the dewpoint of the air was 18 C, yielding a small environmental gain of the feedback loop associated with water. Together with the use of detached leaves this minimized the hydraulic feedback. The quantum flux density was 130 nE cm⁻² s⁻¹.

Some data for *X. strumarium* are shown in Fig. 1. The conductance to diffusion of vapor is that of the leaf in series with its boundary layer. In graphs A and B the physiological and environmental gains are given by the slopes of the curves. The appropriate operating points are the intersections of these curves, as shown in graphs C and D, where the 'environmental response' has been replotted as independent vs. dependent variable. The loop gain is then the product of the slope of the physiological response curve and the inverse slope of the replotted environmental response. Values for these various gains are given in Table 1.

The addition of (±)-ABA to the irrigation water resulted in an increase in the magnitudes of all three gains for *Xanthium strumarium* and *Gossypium hirsutum*, the loop gains changing from zero to -0.5 and -1.0, respectively, with normal ambient CO₂ concentrations. *Zea mays* has a high loop gain of -1.0 in the absence of (±)-ABA. The increases in the environmental gains resulted from stomatal closure and not from direct effects on photosynthesis. We conclude that the CO₂ control loop is unimportant in unstressed *Xanthium* and *Gossypium*, but important when these species are

Fig. 1. A indicates the physiological response of the stomata of *Xanthium strumarium* in the absence (solid line) and presence (dashed line) of 10^{-5} M ($\pm$)-ABA in the irrigation water of leaves with trimmed lamina. B indicates the dependence of intercellular [CO₂] on conductance at various ambient CO₂ concentrations, calculated from the diffusion equation and the photosynthetic response curves. In C and D the physiological and "environmental" curves are superimposed to find appropriate operating points.

Table 1. Values for the physiological, environmental and total loop gains in *Xanthium strumarium*, *Gossypium hirsutum*, and *Zea mays* at an ambient $[CO_2]$ of $300 \mu\ell \ell^{-1}$.

	ABA added ($\checkmark$)	Physiological gain $cm \ s^{-1} (\mu\ell \ell^{-1})^{-1}$	Environmental gain $s \ cm^{-1} (\mu\ell \ell^{-1})$	Loop gain
<i>Xanthium</i>	-	0	16.7	0
	$\checkmark$	-0.005	100	-0.50
<i>Gossypium</i>	-	-0.0001	100	-0.01
	$\checkmark$	-0.002	500	-1.00
<i>Zea</i>	-	-0.0025	417	-1.04

stressed and important in *Zea* all the time. We conclude further that in *Zea* and in *Gossypium* leaves supplied with 10^{-5} M ($\pm$)-ABA, the intrinsic responses to external perturbations appear halved by the action of the CO_2 feedback loop.

4. Control of Stomatal Aperture in Leaves by Abscissic Acid (ABA): Dependence on Transpiration Rate, Presentation Time, and Temperature

T. D. Sharkey, K. Raschke

This was in continuation of a study in the time lags of stomatal responses to ($\pm$)-ABA and in the quantities of this messenger substance needed to elicit a stomatal response (Plant Research '74, p. 25-28). As before, the delay between the addition of ABA to the irrigation water of detached leaves and a 5% reduction in stomatal conductance was measured and the amount of ($\pm$)-ABA was determined which during this time had entered the leaf tissue. This amount was called the 5% dose. In all three species tested (*Xanthium strumarium*, *Gossypium hirsutum*, *Zea mays*), the 5% dose was found to have been independent of the rate of solution uptake (= transpiration rate), although the latter was varied up to threefold. There were, however, differences in the 5% doses between species and between leaves of different ages. After application of 10^{-5} M ($\pm$)-ABA the 5% doses were (in pmole ($\pm$)-ABA per cm^2 leaf lamina): *X. strumarium*, 98; *G. hirsutum*, 43; *Z. mays*, 17; young leaves of *G. hirsutum*, 30. We do not yet know, whether these differences in the 5% doses were caused by differences in leaf anatomy or had another cause.

If ($\pm$)-ABA was presented for limited times only, smaller doses than the 5% doses were sufficient to produce (transient) stomatal closing movements. Presentation of 10^{-5} M ($\pm$)-ABA for 5, 10, and 15 min to leaves of *X. strumarium* reduced the triggering doses to 22, 45, and 72 pmoles cm^{-2} , respectively. In theory, pulses of ABA sent out by the mesophyll would, therefore, suffice to signal water stress to the guard cells; continuous supply of ABA would not be required for the stomatal moderation of water loss.

The temperature dependence, if any, of stomatal responses to ABA was thought to indicate whether the magnitude of these responses was determined predominantly by physical processes (like adsorption) or metabolic ones. Five-percent doses were determined for all three species at 15, 20, and 25 C leaf temperature. Transpiration rates were kept equal at all temperature levels by adjusting the moisture content of the air. In CO_2 containing air ($300 \mu\text{l l}^{-1}$) the 5% doses increased slightly with temperature in *X. strumarium*, and decreased slightly in *G. hirsutum*. In CO_2 -free air, the 5% doses were larger in both species and they declined with temperature in either. We ascribe this enhancement by temperature of the effect of ($\pm$)-ABA to the increasing evolution of CO_2 with increasing temperature. Between 15 and 25 C, respiration of leaves of *X. strumarium* increased 20-fold, that of *G. hirsutum* 3-fold. We know from earlier experiments (Plant Research '73, p. 25-28) that CO_2 enhances the effect of ABA on guard cells. There was hardly any influence of temperature on the response of maize stomata to ABA. At all temperatures between 15 and 25 C the 5% doses were smaller in the presence of $300 \mu\text{l CO}_2 \text{ l}^{-1}$ than in CO_2 -free air. At 15 C, the dose was 1/2 of that in CO_2 -free air; at 25 C, it was <1/3. We concluded (a) that the temperature dependence of stomatal responses to ($\pm$)-ABA was small in the three species tested, (b) that the 5% dose declined with the increase in the CO_2 content of the air (confirming and extending earlier results), and (c) that CO_2 evolved by the leaf tissue during respiration enhanced the action of ($\pm$)-ABA on guard cells.

5. Synthesis and Metabolism of Malate in Epidermal Tissue^{*}

(a) *Assimilation of CO₂ by Epidermes*

K. Raschke; P. Dittrich (University of Munich, Germany)

This investigation was conducted to answer two questions: (i) Is [carbon-4] of malate in guard cells derived from CO₂? (ii) Are guard cells able to fix CO₂ at all times, or only during stomatal opening? If CO₂ is fixed independently from the state of stomatal aperture, then the rate of malate formation could be used by the guard cells to sense the CO₂ concentration in the air (Raschke, 1975).

Epidermes were pulled off leaves of *Commelina communis* and *Tulipa gesneriana* and cleaned from debris of the mesophyll (using a microscope for inspection). Then the epidermal samples were exposed to ¹⁴CO₂ in the gas phase. After various periods of exposure the epidermal samples were killed and extracted, and the residue was hydrolyzed. Extracts and hydrolysates were chromatographed after Bassham and Calvin. Autoradiograms of the chromatograms were evaluated, following conventional procedures.

Isolated epidermes of *Commelina communis* and *Tulipa gesneriana* assimilated ¹⁴CO₂ in the dark as well as in the light; whether stomata were open or closed (Fig. 2). In all cases, the main assimilation products were malic and aspartic acids. Both acids together accounted for more than 90% of the assimilated radioactive carbon. Phosphoglyceric acid appeared in the light only in tulip epidermis, but contained even in this case less than 2% of the label. The same was true for sugar phosphates; no sugar phosphates occurred in epidermal tissue of *Commelina* (Table 1). Guard cells of *C. communis* and *T. gesneriana* therefore do not

^{*} This work was performed during K.R.'s sabbatical leave at the Botanisches Institut der Universität München, Germany.

Fig. 2. Products of CO_2 fixation by epidermal tissue.

Table 1. Assimilation of CO_2 by isolated epidermis of *Commelina communis*.
Stomata open.

Time of exposure (min)	15		90	
Illumination	light	dark	light	dark
Radioactivity (cpm cm ⁻²)				
Total	760	960	4,440	2,530
Hydrolyzable residue ¹	0	0	0	50
Radioactivity (% of soluble fraction)				
Malate	70	58	49	62
Aspartate	24	23	22	18
Citrate	6	6	5	6
Fumarate	0	0	1	1
Ser, gly	0	0	20	10
Glutamate	0	13	2	2
Sugar phosphates	0	0	0	0
Glucose	0	0	0	0
Sucrose	0	0	0	0

¹ yielded only glucose

possess the reductive phosphate pathway; if it exists, it is only rudimentary. (The small amounts of phosphoglyceric acid occurring in tulip epidermis may have been because of contamination with a few mesophyll chloroplasts). Epidermes of both species were however in any physiological state able to incorporate $^{14}\text{CO}_2$ into malate. During the past 20 years it has been demonstrated repeatedly by autoradiography that the ability of the epidermis to assimilate CO_2 resides in the guard cells.

Reference

Raschke, K., Planta 125, 243 (1975)

(b) *Metabolism of Malate- $\text{U-}^{14}\text{C}$ and Malate-4- ^{14}C by Epidermal Tissue*

K. Raschke; P. Dittrich (University of Munich, Germany)

After stomatal closure, usually starch reappears in guard cells. It is likely that gluconeogenesis occurs in guard cells. This possibility was tested by feeding labelled malate to epidermal strips and following the appearance of radioactivity in other acids and carbohydrates.

Epidermes of *Commelina communis* were floated on solutions of labeled malate. The largest portion of malate taken up by epidermes was found to remain malate (60...85%). A smaller portion appeared in the citric acid cycle and was partially transaminated. When uniformly malate was offered, radioactivity was found in carbohydrates (glucose, fructose, sucrose). This was not observed after feeding malate the 4-C of which was the only labeled one (Tables 1,2). Only traces of radioactivity were found in sugar. Epidermal cells, and in particular guard cells, are therefore able to perform gluconeogenesis. The slight radioactivity of the sugars observed after the application of 4-C-labeled malate can be explained as resulting from an equilibration between malate and fumarate during which some label moved from position 4 to position 1. Label in position 1 was, of course, not lost during decarboxylation of malate, and carried into the carbohydrates.

The appearance of radioactivity in sugars as well as in citrate indicates that during stomatal closure part of the malate is converted into sugar, another part is burnt up.

Table 1. Metabolism of malate in epidermis of *Commelina communis* in darkness. (Percent of total activity).

	Malate-U- ¹⁴ C			Malate-4- ¹⁴ C		
	10	30	120	10	30	120
Exposure (min)						
Radioactivity (cpm cm ⁻²)	1,240	1,610	3,470	25,100	13,500	17,420
Malate	91	84	61	96	95	86
Aspartate	2	2	4	0.3	0.5	0.9
Citrate	7	6	4	2	3	6
Glucose	0	0	6	0	0	0
Fructose	0	0	5	0	0	0
Sucrose	0	0	14	0	0	0
Hydrolyzable residue	1	<1	2	0	0	0.6

Table 2. Metabolism of malate in epidermis of *Commelina communis* in the light. (Percent of total activity).

	Malate-U- ¹⁴ C			Malate-4- ¹⁴ C		
	10	30	120	10	30	120
Exposure (min)						
Radioactivity (cpm cm ⁻²)	1,130	1,170	3,150	17,400	17,500	29,400
Malate	87	92	78	96	86	84
Aspartate	0	1	2	0.3	0.5	1.1
Citrate	7	4	4	2	8	7
Glucose	0	0	10	0	0	0.3
Fructose	0	0	0	0	0	0
Sucrose	0	0	0	0	0	0.4
Hydrolyzable residue	0	4	3	0	0.7	2

6. Uptake and Metabolism by Epidermal Tissue of Radioactively Labeled Carbohydrates*

P. Dittrich (University of Munich, Germany); K. Raschke

It is well-known that, in general, starch disappears from the chloroplasts of guard cells during stomatal opening. Most probably, starch and sugar supply carbon-1 through -3 of the malic acid which is required to take up K^+ and balance the positive charges of K^+ . This work was conducted to see whether the radioactivity of labeled carbohydrates offered to guard cells would appear in malate. At the same time, the experiments would provide a test whether guard cells are able to use assimilates of the mesophyll to replenish their carbon (and possibly energy) resources. We showed in a preceding report (p. 36) that guard cells appear to be unable to fix CO_2 photosynthetically. ^{14}C -labeled glucose, glucose-1-phosphate (G-1-P), sucrose and maltose were offered to epidermal strips of *Commelina communis* and *Tulipa gesneriana* in concentrations between 40 μM and 10 mM. The uptake rates varied between 4×10^{-11} mole cm^{-2} h^{-1} (from a 40 μM solution of sucrose) and 4×10^{-8} mole cm^{-2} h^{-1} (from a 40 μM solution of sucrose) and 4×10^{-8} mole cm^{-2} h^{-1} (from a 10 mM solution of G-1-P). These rates were equivalent to 10^{-14} to 10^{-11} mole stoma $^{-1}$ h^{-1} , if all label taken up was accumulated in the guard cells. The carbohydrates taken up were metabolized rapidly in the light as well as in darkness; the resulting labeling pattern was very similar to that obtained by green leaf tissue after a short period of photosynthesis: label appeared in sucrose; glucose, fructose, maltose and in sugar phosphates (an example is given in Table 1). Two hours after the exposure to the labeled carbohydrates the relative intensity of labeling of malate began to increase. A pulse of 1 hr duration of ^{14}C -G-1-P chased by 1 hour ^{12}C -G-1-P forced up to 42% of the initially absorbed label into malate. Overnight, up to 82% of the label was directed into the insoluble fraction. Hydrolysis resulted in glucose only, indicating formation of starch (which could also be shown histochemically). Strong formation of starch was observed also after feeding glucose, sucrose, and maltose.

* This work was performed during K.R.'s sabbatical leave at the Botanisches Institut der Universität München, Germany.

Table 1. Metabolism of ^{14}C -labeled sugars by epidermal tissues after 4 hours of incubation in darkness, stomata originally open.
(Percent of radioactivity in epidermal sample).

Metabolite	Glucose		Sucrose	Maltose
	<i>C. communis</i>	<i>T. gesneriana</i>	<i>C. communis</i>	<i>C. communis</i>
Starch	81	47	62	34
Glucose	6	2	10	2
Sucrose	2	8	14	2
Maltose	0	0	1	40
Mal, asp	2	31	2	6
Citric cycle acids	1	1	1	4
Sugar phos- phates	1	1	3	2

It was thought possible that not only the guard cells but all epidermal cells make starch from carbohydrates of their phosphates. In order to test this possibility ^{14}C -G-1-P was fed to epidermal samples. These samples were embedded in glycol methacrylate (after washing) and sectioned. Autoradiograms of the sections were found blackened over the guard cells only; starch formation was therefore thought to have been concentrated in the guard cells.

The epidermis of leaves is thus able to absorb assimilation products from the mesophyll; most probably, guard cells, jointly with the ordinary epidermal cells, "parasitize" on the mesophyll. Carbohydrates are being used by the guard cells to make starch and malate simultaneously. ^{14}C also enters the citric-acid cycle and appears in the corresponding transamination products.

Epidermes of *C. communis* and *T. gesneriana* appear not to be able to perform the reductive pentose phosphate cycle, in spite of the presence of chloroplasts in the guard cells. Guard cells seem to have to depend on an import of carbohydrates from the mesophyll. This suspicion was supported by the result of the following experiment.

Epidermal samples of *C. communis* were exposed to $^{14}\text{CO}_2$ for 10 min, along with whole leaves of this species. The ^{14}C contents of the epidermes on the leaves were then compared with those of the isolated epidermes. Epidermes which had remained in contact with the mesophyll contained 17 times more ^{14}C than the isolated epidermes. In a following experiment, epidermal samples were put on mesophyll which had been assimilating $^{14}\text{CO}_2$ for 10 min before it was brought into contact with the isolated epidermis. Three hours later, mesophyll and the epidermal sample were extracted and chromatographed. The labeling pattern of the epidermis corresponded with that of the mesophyll (sucrose, glucose, fructose, malate, glutamate) with the exception of the share of malate; epidermal tissue contained, in relative terms, about twice as much malate as the mesophyll (22% of the total label versus 10%) (Table 2).

Table 2. Labeled compounds found 3 hours after a 10 min exposure of leaves of *Commelina communis* to $^{14}\text{CO}_2$. (% of radioactivity in ethanol extract).

Compound	Mesophyll	Epidermis	(Isolated epidermis after 90 min exposure to $^{14}\text{CO}_2$)
Sucrose	60	40	0
Glucose	6	12	0
Fructose	7	4	0
PGA and sugar phosphates	1	4	0
Malate and aspartate	11	30	72
Citric cycle acids	8	5	7
Serine and glycine	0	0	20

INHIBITORY EFFECTS IN FLOWER FORMATION

Anton Lang*

As summarized, e.g., in Lang (1965), there is extensive physiological evidence supporting the idea that flower formation is regulated by a hormonal messenger (the "floral stimulus" or "florigen") which is produced mainly in the leaves, and the production of which may in turn be regulated by certain environmental conditions, namely, low temperature (vernalization) or length of day (photoperiodism). However, quite extensive attempts at extracting and identifying the flower hormone have so far largely failed. On the other hand, there is some evidence for the presence of flower inhibitory effects in plants or in individual leaves maintained under non-flowering conditions. Many of these effects can be explained on the basis that the transport of the flower hormone from leaf to bud is interfered with. But in some cases this explanation is not particularly convincing. Thus, Lang and Melchers (1943) showed over 30 years ago that if the leaves of the long-day plant henbane (*Hyoscyamus niger*) are removed the plant forms flowers regardless of daylength: on long days, on short days, and even in continuous darkness. On the other hand, presence even of a single leaf speeds up flower formation, as compared to a defoliated plant, but only if the leaf is exposed to long days, whereas presence of a leaf on short days prevents flower formation as long as the leaf stays viable (unpublished observations). This indicates that flowering may be regulated not only by flower-promoting substances, but rather by an interplay of promotive and inhibitory materials, and this possibility was thought to deserve further exploration, since if confirmed it would add a new aspect to regulation of flowering.

* Conducted during a sabbatical leave at the K. A. Timiryazev Institute of Plant Physiology of the Academy of Sciences of the USSR, under the scientific exchange program between the National Academy of Sciences and the Academy of Sciences of USSR, in cooperation with Professor M. Kh. Chailaklyan, Head, Dr. I. A. Frolova, Jr. Scientist, and other coworkers of the Institute's Laboratory of Growth and Development.

To determine the possibility of the existence of flower-inhibiting substances which can be translocated within the plant and between graft partners, grafts were made opposite to the type of grafts which provided one of the principal supports for the existence of "florigen", i.e. the flower promoting substance(s). In the latter grafts, a plant capable of flowering is grafted together with a plant that is maintained under non-flowering environmental conditions, and it is observed whether the latter graft partner forms flowers. We grafted a non-flowering partner onto one that was capable of flowering by itself, and observed whether the flowering response of the latter was reduced. As the partner capable of flowering we used a dayneutral tobacco variety, "Trapezond" (T), that is, a plant in which flowering is largely environment-independent and occurs spontaneously once the plant reaches a certain age. As non-flowering partners, a short-day tobacco variety, "Maryland Mammoth" (MM) and a long-day tobacco species, *Nicotiana sylvestris* (NS), were used. MM flowers on short days and does not on long days, NS behaves in the opposite manner. The grafts with NS failed to give results, because of unfavorable conditions. The results of the grafts between T and MM are summarized in Table 1. The T partners flowered in all cases. However, when the grafts were maintained on long days, the flowering of T was delayed as compared with control grafts (T on T). In contrast, when the grafts were kept on short days, that is, MM was induced to flower formation, T on which MM had been grafted flowered sooner than T-on-T controls. Thus, the results indicate that while a flowering graft partner may hasten flowering in a dayneutral partner, supporting the existence of flower-promoting substances in plants maintained under environmental conditions favorable for flowering, a non-flowering partner of the same species and variety may delay flowering, supporting the idea of transmissible flower-inhibiting substances in plants maintained under environmental conditions unfavorable for flowering. It must be pointed out that the differences were of marginal statistical significance, and the experiments will have to be repeated and expanded, including analogous grafts between long-day and dayneutral plants.

Table 1. Flowering response of Trapezond tobacco (T; dayneutral) in grafts with Maryland Mammoth (MM; short-day plant).

Photoperiod	Graft combination	No. of successful grafts	Flowering response of Trapezond		
			No. flowering	Days to visible buds	No. leaves prior to flowers
Short day	T on T	10	10	86	29
	MM on T	4	4	67	24
Long day	T on T	10	10	62	24
	MM on T	7	7	73	33

Prior to grafting, MM and T were maintained on long days. Scions (MM on T) had ca. 5 leaves and were disbudded; stocks (T) were defoliated and disbudded except for one axillary bud, and the flowering response was "read" on the side shoot developing from this bud.

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ENVIRONMENTAL CONTROL OF PLANT GROWTH AND DEVELOPMENT THROUGH HORMONES

We are interested in control of plant growth and development by environmental factors such as photoperiod and temperature. Hormones often function as intermediaries between perception of an environmental factor and the eventual response (flower formation, stem growth etc.). Thus a complete understanding of environmental effects on plants must include knowledge of the nature of the hormones involved, and how their rates of biosynthesis and metabolism are controlled by the environment. We have established in earlier work that the hormone content of whole plants is usually not correlated with the physiological response under study. We are attempting therefore to refine hormone analysis by determining the hormonal distribution of gibberellins as well as of abscisic acid (ABA) and its metabolites among the various organs and cell fractions to see if these hormones are compartmentalized.

Since recent reports suggest that "estrogen-like" substances play a role in flower formation, we have analyzed the estrogen fraction of several plants in the vegetative and flowering stage to verify this claim. Several other sources, such as apple and date seeds, which reportedly contain estrone, have also been analyzed. The nature of long-day inhibition of flowering in *Xanthium* leaves has been studied further by correlating assimilate transport with the flowering response.

Previous work on the role of gibberellins in stem growth and flower formation of the photoperiodically sensitive plants *Bryophyllum* and *Silene* has been continued to determine which gibberellins are physiologically active in these plants.

In most plants the young leaves and shoot tips have a much higher level of ABA, or of its metabolites, than do the mature leaves. ABA could either be synthesized in situ, or exported with the assimilates from the mature leaves. This question has been investigated in *Ricinus* since ABA transport in this plant can be readily studied by analysis of phloem exudate.

1. Analysis of the Estrogen-like Fraction in Higher Plants

L. L. L. Van Rompuy, J. A. D. Zeevaart

The general occurrence of steroids in plants has led to the suggestion that these compounds play a hormonal role in reproduction just as they do in animals (Heftmann, 1975). Cholesterol, the precursor of steroid hormones, has been found in a wide variety of plants. Furthermore, estrogens have been reported to be present in several plant sources (Dean et al., 1971), and the appearance of estrogen-like compounds has been associated with the formation of flower buds (Kopcewicz 1971, 1972). However, estrogens or estrogen-like compounds were never identified by unambiguous criteria in any of these reports. We have therefore analyzed the fraction that would contain estrogens from several plant sources by gas chromatography-mass spectrometry in order to identify the compounds reportedly involved in the flowering process.

Plant material was extracted with toluene and methanol, and the extract hydrolyzed with hydrochloric acid. The acidic fraction was isolated by partitioning in sodium hydroxide, and further purified and analyzed by column-, thin-layer-, and gas chromatography. Extracts from the following sources were analyzed by mass fragmentography: shoots of *Phaseolus*, *Perilla* and *Chenopodium*, and seeds of apples (*Malus*) and dates (*Phoenix*).

The selected fragments were characteristic for steroids containing a phenolic A ring and saturated B, C and D rings. Estrone, estradiol, estriol, or closely related metabolites were not found in any of the plant sources examined. The detection limit of the mass fragmentographic search was determined by adding known amounts of estrogens to apple seed extracts prior to analysis. A quantity of 50 ng of an estrogen could be detected, corresponding to a level of 1.3 µg estrone/250 g apple seeds. The reported level of 32 µg/250 g seeds (Gawienowski and Gibbs, 1969) could not be confirmed.

Following silylation the acidic fraction was analyzed by gas chromatography. The major constituent showed a retention time slightly longer than that of estradiol (Fig. 1). The structure of this substance was determined by mass spectrometry. Comparison of the silylated and the deuteriosilylated spectra indicated the presence of two derivatized hydroxyl functions. The shift of m/e 147 to m/e 162 in the deuteriosilylated spectrum could only be explained by the presence of fragment $(CH_3)_3Si-O^+=Si(CH_3)_2$, characteristic for vicinal hydroxyl groups. The ethylene glycol structure was confirmed by

Fig. 1. Gas chromatogram of the estrogen-like fraction after silylation from *Phaseolus*, analyzed on a 3% SP-2100 column at 225°C, isothermal (—). Superimposed is the chromatogram of a mixture of estrone, estradiol and estriol, analyzed under the same conditions (---).

the formation of an acetone, in turn characterized by a prominent $m/e = 101$ in its mass spectrum. The molecular formula of the underivatized substance was determined by accurate mass measurement of the molecular ion ($M^+ = 352$) as $C_{21}H_{36}O_4$, corresponding to the monoglyceride of linoleic acid.

In order to prove the identity of the unknown substance with the monoglyceride, the latter was synthesized from glycerol and linoleic acid. 1-Monolinolenin was separated from the reaction mixture and yielded a mass spectrum identical with that of the unknown substance, both in the free form and as the silylether (Fig. 2). The monoglyceride of linolenic acid was found in *Phaseolus*, *Perilla* and *Chenopodium* shoots, while the monoglycerides of linoleic acid and of oleic acid were found in apple seeds and date seeds, respectively. Monostearate was found in date seeds.

Fig. 2. Mass spectra of the synthetic and of the natural monoglyceride of linoleic acid, isolated from *Phaseolus*. The spectra were obtained by combined gas chromatography-mass spectrometry after silylation.

In conclusion, the occurrence of estrogens in plants remains to be demonstrated. Caution should be exerted to distinguish estrogens from compounds such as monoglycerides that have similar chromatographic properties.

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2. Floral Induction and Translocation of Labeled Assimilates in Partially Induced *Xanthium* Leaves

C. Cetas, J. A. D. Zeevaart

When the tip half of a *Xanthium* leaf is induced, flowering will not occur unless the basal half under LD conditions has been removed (Plant Research '74, p. 45). We have continued our studies of the nature of this LD inhibition by correlating the flowering response induced by the apical leaf half with translocation of ^{14}C -assimilates from this induced part.

Removal of the basal (non-induced) leaf half, as well as merely severing the basal part along the midrib, resulted in normal flower formation, and also significantly increased the amount of radioactivity that accumulated in the shoot tip (Table 1). On the other hand when the induced and non-induced leaf halves were separated by cutting the mesophyll perpendicular to the midrib, no flowering resulted. In this case the labeled assimilates moved out of the apical half via the midrib as shown by autoradiography. It is unlikely therefore that the LD inhibition is due to inactivation of the floral stimulus moving through non-induced tissue. A more important factor appears to be export of assimilates from the basal (non-induced) leaf half: shading this part after induction of the tip had taken place, did result in flower formation.

Table 1. Translocation of ^{14}C -assimilates from apical halves of *Xanthium* leaves to the shoot tips as affected by treatment of the non-labeled basal half.

Treatment	Radioactivity per shoot tip,
	dmp x 10^{-3}
Leaf blade intact	41
Basal half removed	216
Leaf blade intact	43
Basal half cut parallel to midrib	196

3. Flower Formation and Stem Growth in Two Strains of *Silene armeria*

J. Suttle, J. A. D. Zeevaart

Silene armeria is a long-day plant which grows as a rosette under short-day (SD) conditions. Experiments were conducted with two strains obtained from Professor S. J. Wellensiek, Agricultural University, Wageningen, The Netherlands: a dwarf strain (Wellensiek, 1972), and the 1.2 (=late) strain (Wellensiek 1973). Under our experimental conditions (8 hr light of high intensity from fluorescent lamps supplemented for the remaining 16 hr with light from incandescent lamps) the 1.2 strain required at least 6 LD for a 100% flowering response. Additional LD treatment further increased stem elongation to a final height of approximately 40 cm. The dwarf strain flowered in response to 4 LD with a compact inflorescence but the plants showed hardly any stem growth.

Neither strain responded with flower formation to applied GAs under non-inductive conditions, but differences were observed in the stem growth pattern. Application of 100 μ g GA to the shoot tips of 1.2 plants resulted in considerable stem elongation, GA₃ being more effective than GA₇. The dwarf strain responded only slightly to GA application. Following equal GA₃ treatments the final plant heights of control and treated plants were 5 and 10 cm in the dwarf, and 6.5 and 43 cm in the 1.2 strain, respectively.

Because of the unresponsiveness of plants of the dwarf strain to applied GA, it became of interest to see if this strain is capable of producing GAs. To examine this, plants of both strains were exposed to 0, 1, 6, or 12 LD, and the GA content of each group was determined by extraction. As measured in the *d*-5 corn bioassay both strains had the same GA content. It appears therefore that dwarfism in *Silene* is not caused by absence of GAs, but to the inability of this genotype to utilize GA.

The relationship between stem growth and flower formation was further studied in strain 1.2 by treatment with the growth retardant AMO-1618. Plants were treated for 0, 2, or 4 weeks prior to exposure to inductive cycles. The plants treated for 4 weeks remained initially in the rosette stage in LD while the control group began to elongate. Eventually the treated plants showed some stem growth and all groups

flowered profusely. Thus, although AMO-1618 retarded stem growth, it had no effect on floral induction as was established previously with a different *Silene* strain (Cleland and Zeevaart, 1970).

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4. The Role of Gibberellin A₂₀ and its Metabolites in Stem Growth and Flower Formation in *Bryophyllum daigremontianum*

C. Cetas, J. A. D. Zeevaart

Induction of flower formation in the long-short-day plant *Bryophyllum daigremontianum* by the transfer from long days (LD) to short days (SD) is accompanied by an increase in stem elongation as compared to plants under SD, or in plants grown continuously under LD conditions. Differences in growth rate are correlated with the levels of extractable GA₂₀ (Zeevaart, 1973). However, while 100 µg of exogenously applied GA₂₀ were required to cause flower formation and stem growth under SD, only 5 µg of GA₃ were needed to induce a comparable response (Gaskin et al., 1973). The possibility was therefore considered that GA₂₀ itself has little or no biological activity in *Bryophyllum*, but must be converted to a more active GA. This was investigated by application of 2,3-[³H]-GA₂₀ and studying its metabolism. In order of decreasing yields GA₂₉, 3-*epi*-GA₁, and C/D-ring-rearranged GA₂₀ were identified as the metabolites (Durley et al., 1975). All three compounds have now been tested by injection into the leaves of *Bryophyllum* plants grown under SD. As shown in Table 1, none of the compounds had any effect on stem growth or flower formation at the levels tested. It is concluded therefore that GA₂₀ per se, and not its metabolites, is responsible for stem growth and flower formation in *Bryophyllum*.

Table 1. The effect of various dosages of four different gibberellins on stem growth and flower formation when injected into mature leaves of *Bryophyllum daigremontianum* plants grown under short-day conditions. Five plants per treatment.

GA applied per plant (μ g)		Stem growth after 84 days (cm)	Flowering response ^a
Control		14.6	V
GA ₃	5	56.4	F
	20	73.2	F
GA ₂₉	10	15.0	V
	30	14.9	V
	100	15.4	V
3- <i>epi</i> -GA ₁	10	13.1	V
	30	12.9	V
	100	13.0	V
C/D-RR GA ₂₀ ^b			
	10	13.0	V
	70	12.6	V

^aV = vegetative; F = flower buds

^bC/D-RR GA₂₀ = C/D-ring-rearranged GA₂₀

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5. Sites of Absciscic Acid Synthesis and Metabolism in *Ricinus communis*

C. Cetas, J. A. D. Zeevaart

The content of absciscic acid (ABA) of young leaves and buds is usually quite high as compared to that of mature leaves (Plant Research '74, p. 53). It is not clear, however, whether this is due to a high rate of ABA synthesis in situ, or to import from older leaves. This question has been investigated in *Ricinus communis*, because phloem exudate can be easily collected in this plant (Hoad, 1973), thus enabling

one to determine transport in the phloem of ABA and its metabolites, phaseic acid (PA) and dihydrophaseic acid (DPA).

The acidic fraction prepared from phloem exudate of *Ricinus* was purified by thin-layer chromatography, and ABA, PA, and DPA were measured quantitatively by gas chromatography with electron capture detector (Zeevaart and Milborrow, 1976). Following water stress the concentration of all three compounds in the phloem increased over that in turgid control plants (Table 1). The largest increase in the mature leaves was in the ABA content, with smaller rises in the levels of PA and DPA. Most striking was the very high DPA content of the shoot tips, even when the plants were never under water stress.

Table 1. The effect of water stress on the contents of abscisic, phaseic, and dihydrophaseic acids in phloem exudate, shoot tips, and mature leaves of *Ricinus communis*. Material harvested 24 hr after end of stress period.

Compound	Phloem exudate		Leaves		Shoot tips	
	Turgid	Stressed	Turgid	Stressed	Turgid	Stressed
	$\mu\text{g/ml}$		$\mu\text{g/g fresh wt}$			
ABA	0.2	2.3	0.3	2.7	0.1	0.4
PA	0.3	0.8	0.2	0.7	0.5	0.8
DPA	0.4	3.6	1.2	2.8	18.7	34.8

When mature or young leaves were detached from the plants and wilted, the level of ABA increased in leaves of both ages, thus demonstrating that young leaves are capable of producing ABA.

It appears from these results that ABA is predominantly produced in the mature leaves, and that ABA itself as well as its metabolites are translocated in the phloem to the shoot tips (sinks) where DPA accumulates as the end product of the metabolic pathway. Thus, ABA metabolism can take place both in young and mature leaves, while synthesis would take place

mainly in mature leaves since these leaves show symptoms of water stress first. To what extent ABA synthesis also occurs in young leaves in situ remains to be determined.

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STUDIES ON HORMONE ACTION

The action of plant hormones has been studied further by investigating the interaction of cytokinins with cellular components and by characterizing biochemical responses to hormones. Studies on the site of action of cytokinins were conducted with tobacco cells cultured in liquid medium. These cells are absolutely dependent on added cytokinin for continued proliferation. Experiments on cytokinin binding were also performed with moss protonemata which respond to added cytokinin by initiation of gametophyte development. It is hoped that such experiments will eventually lead to the elucidation of the site of action of plant hormones.

Work on the action of ethylene in senescing flower tissue was also pursued further. The ultimate aim of these experiments is to find out how ethylene induces its own synthesis during the aging process. According to our working hypothesis, ethylene causes a loss of cellular compartmentation which leads to ethylene generation through intermixing of components of the ethylene generating system and eventually to cell death. In accordance with this hypothesis, we have further investigated the pathway of ethylene biogenesis and have investigated the effect of ethylene on membrane composition. We hope to identify the process(es) affected by ethylene that lead to membrane aging and we hope to be able to localize and characterize the components of the ethylene generating system. The action of gibberellic acid on hydrolase synthesis in barley aleurone layers was also studied within the program of hormone-mediated biochemical responses.

1. Regulation of Senescence by Ethylene in Flower Tissue

(a) *Relationship between Ethylene Evolution and Aging in Morning Glory Flower Tissue*

H. Kende, A. D. Hanson

Rib segments excised from the corolla of morning glory (*Ipomoea tricolor* Cav.) flowers were used to study the relationship between senescence and ethylene production. Such segments, isolated one or two days (day -1 or day -2) before flower opening (day 0) passed through the same developmental

phases as did the corresponding tissues of the intact organ. When excised on day -1 and incubated overnight, the rib segments turned from purple to blue and changed from a slightly curled to a flat configuration. On day 0, these segments rolled up during the afternoon and turned purple again; the rolling up coincided with an increased rate of ethylene production. Premature rolling up and associated ethylene evolution were induced by ethylene or propylene treatment. When segments were excised on day -2 and incubated overnight, there were no changes in color or shape; during day -1, no spontaneous rolling up and little ethylene evolution occurred. Application of ethylene or propylene to these immature segments elicited rolling up but did not stimulate endogenous ethylene production.

Overnight treatment of segments cut on day -1 with benzyladenine (10^{-6} M) markedly retarded spontaneous rolling up and ethylene evolution, although the response to applied ethylene was only slightly slowed. Overnight treatment of segments cut on day -1 with the ethoxy analog of rhizobitoxine (10^{-5} to 10^{-4} M) resulted in almost complete (>99%) inhibition of both spontaneous and propylene-induced ethylene evolution. Although spontaneous rolling up was delayed, it was not abolished, and ethylene-induced rolling up was almost unaffected.

These data indicate that an ethylene-generating system develops as an integral part of the aging process in flower tissue. Ethylene hastens aging of the flower, but may not play an obligatory role in flower senescence.

(b) *Ethylene Biosynthesis and Methionine Metabolism in Senescent Flower Tissue of Morning Glory*

A. D. Hanson, H. Kende

As in a number of ripening fruits, ethylene produced by senescent morning glory flower tissue has been shown to be derived principally from carbon atoms 3 and 4 of methionine (Plant Research '74, p. 59). The metabolism of tracer quantities of ^{14}C -, ^3H - and ^{35}S -methionine was investigated at various stages in the development of the flower, in order to correlate rolling up, increase in membrane permeability and the onset of ethylene evolution with changes in methionine metabolism (Hanson and Kende, 1976).

In immature flower rib segments, cut from buds on day -2 and incubated overnight on tracer ^{14}C -methionine, the major ethanol-soluble ^{14}C -metabolite present throughout day -1 was a strongly cationic product identified as S-methylmethionine (SMM). In segments prepared on day -1 and incubated overnight on ^{14}C -methionine, SMM was the major soluble metabolite present in the morning of day 0 before rolling up and ethylene production had begun; as these segments senesced during day 0, ^{14}C was progressively lost from SMM and appeared in free methionine. Immature segments contained about 4 nmoles of free methionine and about 16 nmoles of SMM per 30 segments. As the segments senesced the methionine content increased about ten-fold while the SMM content remained unchanged. During this time about 0.8 nmole of ethylene was released per 30 segments.

The paradox of a decline in ^{14}C -label in SMM with concomitant production of ^{14}C -methionine, at a time when the chemical concentration of SMM was constant, was resolved with tracer experiments using L-methionine- $\text{U-}^{14}\text{C}$, L-methionine-methyl- ^3H and L-homocysteine thio-lactone- ^{35}S . In both senescent and immature tissue, SMM was capable of acting as a methyl donor; only as senescence began, however, was the methyl group transferred to homocysteine, to give two molecules of methionine. Of the two product methionine molecules, one was re-methylated to SMM, the other contributed to the rise in free methionine level. These reactions are summarized in Fig. 1.

Because SMM was a prominent metabolite of methionine in both immature and senescing rib segments, a possible role for SMM as a closer precursor of ethylene than methionine was investigated. Following overnight incubation of segments on $10\text{ }\mu\text{M}$ L-methionine- $\text{U-}^{14}\text{C}$, the specific activity of the ^{14}C -ethylene evolved was compared with that of C3 + C4 of free methionine and SMM. The specific activity of C3 + C4 of methionine and SMM extracted from the tissue was determined using a microscale adaption of the FMN-mediated photochemical degradation of methionine described by Yang *et al.* (1967). As SMM is not an active substrate for this reaction it was first converted to methionine by conc. HCP treatment (Lavine *et al.*, 1954). Figure 2 shows that the specific activity of the ethylene evolved during senescence closely paralleled the specific activity of C3 + C4 of free methionine, confirming that free methionine is the main precursor of ethylene in this tissue. Because the specific activity of SMM was about

Fig. 1. A scheme for methionine metabolism in relation to ethylene biosynthesis in immature and senescent flower tissue of morning glory.

Fig. 2. Specific activities of $^{14}\text{C}_2\text{H}_4$ and C3 + C4 of methionine and SMM during senescence. Ethylene was collected during 3 consecutive periods; and at the end of each period a sample of segments was extracted. Δ --- Δ : time course of ethylene production; the 3 collection periods are indicated with vertical broken lines and the specific activities of ethylene collected in each period are shown as bars; o-o: specific activity of C3 + C4 of methionine; ●-●: specific activity of C3 + C4 of SMM.

twice that of methionine and ethylene, it is very improbable that SMM is a closer precursor of ethylene than methionine itself. That the specific activity of C3 + C4 of SMM is double that of methionine is consistent with the operation of the pathways in Fig. 1.

In summary, the SMM concentration in juvenile morning glory flower tissue is several times greater than that of free methionine. As senescence begins, SMM transfers methyl groups to homocysteine giving rise to a ten-fold increase in the free methionine level; some fraction of this free methionine is then available for conversion to ethylene. Two lines of evidence suggest that the synthesis of methionine via this route may limit the rate of ethylene production. First, although exogenous methionine was unable to influence ethylene production and senescence, homocysteine thiolactone (supplied overnight at 3×10^{-4} M) consistently provoked premature rolling up and ethylene evolution. Tracer experiments demonstrated that supply of homocysteine thiolactone stimulated methionine synthesis from SMM in both senescent and immature tissue. Second, ethylene applications which advance the onset of rolling up and breakdown in cellular compartmentation (Hanson and Kende, 1975) also stimulate ethylene production and the synthesis of methionine from SMM. It is tempting to speculate that ethylene biosynthesis during aging is regulated by the availability of homocysteine to a compartment in which methionine is first produced from SMM and then is consumed in ethylene production.

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(c) Biosynthesis of Stress-Induced Ethylene in Immature Morning Glory Flower Tissue

A. D. Hanson, H. Kende

Although carbon atoms 3 and 4 of methionine are known to give rise to the ethylene evolved during the senescence of several fruits (Yang, 1974), the involvement of the methionine pathway in the synthesis of stress-induced

ethylene is less well documented, and available data suggest that another pathway or pathways could be involved. Abeles and Abeles (1972) were able to account for about one-half of the ^{14}C -ethylene produced by chemically stressed leaves in terms of a methionine pathway. Fruits of the non-ripening rin tomato mutant, however, produce almost no ethylene spontaneously but evolve the same amount of ethylene in response to cutting injury as do normal fruits (Herner and Sink, 1973). Because the ethylene produced by senescing morning glory flower tissue is derived from methionine, the same tissue was used prior to senescence to assess the contribution of the methionine pathway to stress-induced ethylene synthesis (Hanson and Kende, 1976).

Rib segments cut from buds on day -2 evolved a small quantity of ethylene on day -1, principally during the morning. When such segments were stressed mechanically (by cutting into small pieces, by crushing or by multiple perforations) early in the afternoon, ethylene production increased more than ten-fold within 1 hr and subsided to a low rate after about 3 hr (Fig. 3).

Fig. 3. Effect of wounding on the specific activities of ethylene and C3 + C4 of methionine and SMM. Ethylene was collected during two periods from untreated segments (A) and from segments wounded (arrow) by multiple perforations (B). The specific activities of the ethylene evolved during the two collection periods are shown as bars. Δ ----- Δ : time course of ethylene evolution; o—o: specific activity of C3 + C4 of methionine; ●—●: specific activity of C3 + C4 of SMM.

Evolution of ethylene by both untreated and stressed segments was inhibited more than 95% by overnight pretreatment with the ethoxy analog of rhizobitoxine (3×10^{-5} M); rhizobitoxine and its analogs are regarded as specific inhibitors of the utilization of methionine in the pathway of ethylene synthesis.

After overnight exposure of segments to tracer (9 μ M) L-methionine- $U-^{14}C$, the specific activity of the ethylene evolved by untreated and stressed segments was determined and compared with the specific activities of C3 + C4 of methionine and SMM extracted from the tissues (Fig. 1). The specific activity of the ethylene produced by untreated segments was close to that of SMM, while in stressed tissue the specific activity of the ethylene evolved was somewhat lower, although still above that of methionine.

The rhizobitoxine analog and ^{14}C data are consistent with the synthesis of the bulk of the stress-induced ethylene from methionine, possible from a small pool of relatively high specific radioactivity which is not in rapid equilibrium with other pools of free methionine in the tissue.

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(d) Senescence and Phospholipid Content of Membranes of Morning Glory Flower Tissue

P. Beutelmann, H. Kende

Earlier results on aging in morning glory flower tissue indicate that the senescence process will proceed even if over 99% of the ethylene evolution has been inhibited. From such data, we concluded that ethylene production may be a fairly late event in aging, and that prior biochemical processes must proceed it. Since available evidence supports the hypothesis that ethylene synthesis is a consequence of loss of cellular compartmentation, we decided to investigate membrane composition of morning glory flower tissue prior to and during senescence. Initially, the level of phospholipid in total membrane preparation has been investigated, but an extension of these experiments to other lipid components and to specific membrane fractions is planned.

Before the onset of senescence, there is a continuous, slow decline in the content of phospholipid. At the time when rib segments roll up and when they start to produce ethylene, the rate of phospholipid loss is accelerated by about 10-fold, resulting in a 50% loss within 8 hr (Fig. 4).

Fig. 4. Loss of phospholipids, ethylene production and rolling up of rib segments of morning glory flower tissue.

Exposure of mature tissue to ethylene on the day of flower opening accelerates rolling up, ethylene production and phospholipid degradation. In juvenile tissue, ethylene treatment causes rolling up and enhancement of phospholipid loss. The ratio of the major phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) and the associated fatty acids remains constant during the period of degradation.

These results are consistent with the hypothesis that ethylene production is preceded by membrane changes which are accelerated once ethylene synthesis commences.

(e) Senescence of Flower Tissue of *Tradescantia hirsuticaulis*

J. Suttle, H. Kende

Flowers of *Tradescantia* are ephemorous; they open early in the morning and fade on the same day. In pursuing our hypothesis that aging and ethylene production are due to loss of cellular compartmentation, we have studied senescence of *Tradescantia* flower petals, as this can be monitored by following the loss of anthocyanin from the cell. Since this pigment is originally localized in the vacuole, its efflux from the cell signifies a gross loss of cellular integrity. Flower petals were excised and floated on water in a closed vessel stoppered with a serum vial cap and equipped with a side arm. Anthocyanin loss in the bathing medium was monitored spectrophotometrically by transferring the bathing medium into the side arm which could be inserted into a colorimeter. Simultaneously, ethylene generation was monitored by withdrawing gas samples from each flask. When flower petals were treated with ethylene, premature senescence and anthocyanin efflux were observed (Fig. 5).

Fig. 5. Effect of a pretreatment with 10 ppm ethylene for 1 hr on ethylene evolution and anthocyanin efflux in isolated *Tradescantia* petals. Seventeen petals per 5 ml 5 mM KCl.

In naturally aging petals, release of anthocyanin was accompanied by rising ethylene production. This was taken as further evidence that ethylene synthesis is intimately connected to loss of cellular compartmentation. Carbon dioxide, an antagonist of ethylene action in many systems, inhibited aging of the flower tissue. Similarly, an analogue of rhizobitoxine almost completely inhibited fading of the petals and ethylene evolution. The latter result points to the methionine pathway as the origin of ethylene. This was further verified by showing that the specific radioactivity of ethylene very closely matched the specific radioactivity of the carbon atoms 3 and 4 of methionine extracted from the tissue. Like in *Ipomoea*, applied ^{14}C -methionine was converted to S-methylmethionine which was reconverted to methionine during aging. This is further confirmation of the fact that the S-methylmethionine $\rightarrow$ methionine pathway must be operating when ethylene is generated.

2. Hormone Binding in Plants

(a) *Cytokinin Binding to a Membranous Fraction from Cultured Tobacco Cells*

M. R. Sussman, R. deZacks, H. Kende

Using a liquid suspension culture of cytokinin-dependent tobacco cells, we have continued our investigations of an *in vitro*, particulate, 'high'-affinity ^3H -benzyladenine (^3H -BA) binding site (see Plant Research '74, p. 60). In order to more rigorously determine whether binding activity correlates with biological activity, we have synthesized and tested a series of benzyladenine analogues differing by a single halogen substitution in the benzene ring. Ideally, the best such analogues for this study would be those where the most subtle chemical change would produce the most dramatic change in biological activity. It was originally hoped that *trans*- and *cis*-zeatin would be suitable for this purpose but, as shown in Figure 1, *trans*-zeatin is significantly less active than BA, and the two stereoisomers are only marginally different in their biological activity. The *ortho*-, *meta*-, and *para*- derivatives of fluoro-, chloro-, or bromo-benzyladenine analogues do, however, show gross differences in biological activity (Fig. 1). It is evident that (i) loss of activity correlates with increasing bulkiness of the halogen rather than its electronegativity (e.g., compare BA, *p*Cl-BA and *p*F-BA) and (ii) substitution in the *ortho*-position least affects biological activity.

Fig. 6. Tobacco callus bioassay of *ortho*-, *meta*- and *para*- fluoro-, chloro-, and bromo-benzyladenine derivatives and other known cytokinins. Fresh weight was measured after 2 weeks growth in liquid culture. Depicted is the average with standard error of the results of at least 5 experiments.

These data are in agreement with results of Okumura *et al.* (1959) using the chloro-derivatives of BA in the radish leaf assay. Most important, however, the differences in biological activity correlate well with the ability of the analogues to compete with ^3H -BA in the binding assay (Table 1). This is as expected if the observed binding were physiologically significant.

Table 1. Binding assay (at 0-4°C) of cytokinin analogues using an 80,000 x g (30 minutes) particulate membrane fraction of the liquid tobacco suspension cultures. The results of a single experiment are shown as the average with standard error of triplicate samples. Absolute values vary between experiments, but the general relative order of activity does not.

<u>Compound Tested*</u>	<u>Competible Bound CPM per Pellet ± S.E.</u>
Benzyladenine (BA)	318 ± 27
<i>o</i> -Fluoro-BA	279 ± 20
<i>m</i> -Fluoro-BA	249 ± 26
<i>p</i> -Fluoro-BA	271 ± 27
<i>o</i> -Chloro-BA	227 ± 25
<i>m</i> -Chloro-BA	118 ± 34
<i>p</i> -Chloro-BA	72 ± 28
<i>o</i> -Bromo-BA	200 ± 32
<i>p</i> -Bromo-BA	34 ± 24
Kinetin	284 ± 27
<i>cis</i> -Zeatin	134 ± 16
<i>trans</i> -Zeatin	166 ± 16

* All tubes contain 5×10^{-9} M ^3H -BA. The non-radioactive compounds tested above were added at 5×10^{-7} M.

When ^3H -BA binding assays are done on the various membrane fractions separated by equilibrium sucrose density gradient centrifugation (15-45% w/w sucrose), there is considerable high-affinity saturable binding in all fractions. However, of crucial importance is our finding that heat-labile high-affinity saturable binding is exclusively limited to a fraction equilibrating at 30-40% (w/w) sucrose. Unfortunately, this region of the gradient contains many of the major membrane and organelle fractions, and it has not yet been possible to determine specifically which membrane fraction is involved in cytokinin binding. The heat lability of cytokinin binding activity of this membrane fraction is in direct contrast with the

low-affinity-saturable and non-saturable binding which actually increases as much as three-fold after a heat pre-treatment. This was the main reason for concluding that the ^{14}C -BA binding reported earlier (Plant Research '72, p. 67) was physiologically probably not significant.

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(b) *Binding of Cytokinins to Particulate Fractions from Moss Protonemata*

G. Gardner, M. R. Sussman, H. Kende

We reported in Plant Research '74 (p. 61) preliminary experiments on the binding of ^3H -benzyladenine (BA) to a 13,000 - 80,000 x g particulate fraction of moss extracts. During 1975 our major effort was directed toward characterization and purification of the active material with the hope that we might be able to identify the sub-cellular fraction(s) containing binding activity. As we reported, active cytokinins competed for the binding of ^3H -BA, whereas the ribosides of the free bases, which are inactive in causing bud formation in moss, competed poorly. These studies with cytokinin analogues were expanded, and additional attempts were made to optimize binding. In addition, it was found that ^3H -zeatin would bind to these fractions, although the extent of binding was not as great as with ^3H -BA.

Two approaches were taken to isolate the active fractions. The first utilized sucrose equilibrium density gradients. Initial experiments indicated that the binding activity might be concentrated in certain gradient fractions; however, later experiments yielded different gradient profiles. It was felt that the variability observed was inherent in the procedures necessary for cell breakage and extraction of the tough-walled moss protonemata, and no valid conclusions about the localization of the binding activity could be drawn. The second approach was to solubilize the binding activity with the detergent Triton X-100 and attempt to detect competition in the Triton-soluble supernatant using gel filtration techniques. When Triton-solubilized material was mixed with ^3H -BA and chromatographed on a Bio-Gel P-6 column, radioactivity was found in the exclusion volume of the column. The amount of radioactivity in this high molecular weight

fraction was reduced if the column was eluted in the presence of non-radioactive BA. While it thus appears as if we had shown specific BA binding in the detergent solubilized material, the possibility exists that the hormone was interacting with the detergent (see below). Until the question of non-specific detergent interactions is resolved, we will not be able to determine whether we have successfully solubilized specific hormone binding activity.

A report of cytokinin binding to ribosomes (Fox and Erion, 1975) made it imperative to determine whether we were observing the same phenomenon. The 80,000 x *g* supernatant was centrifuged for 2 hr at 95,000 x *g*, and the resultant pellet was used for the binding assay. It was found that the binding activity was much greater in the 13,000-80,000 x *g* pellet than in the higher speed pellet which putatively contained ribosomes. These two pellets were analyzed for RNA, and it was clear that the binding was not correlated with the RNA content of the fraction. Thus, the binding activity which we have observed is probably different than the binding of cytokinins to ribosomes reported by others.

It had been pointed out by Cuatrecasas and Hollenberg (1975) that the animal hormones insulin and adrenalin can bind to nonspecific adsorptive materials and that this binding can exhibit saturability and even stereospecificity. We have found this to be a serious problem with cytokinins. We had observed that BA binds to glass, and this effect could be quantified with talc powder. We found that ^3H -BA bound to a 1 mg/ml suspension of talc, and the bound counts were displaced competitively by non-radioactive BA, zeatin, or isopentenyladenine. Zeatin-riboside and isopentenyladenine-riboside were less effective competitors. ^3H -zeatin also bound to talc in the same manner. The detailed properties of the binding of BA to talc were found to be different than those to the biological fractions, e.g., the binding to moss fractions was much more susceptible to heat treatment than binding to talc. However, our major concern, which applies as well to all other studies in the area of cytokinin binding, is to distinguish between binding that is biologically meaningful, and hopefully reflects receptor interactions, and binding which is simply non-meaningful physical adsorption.

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(c) In Vivo Binding of ^3H -Benzyladenine in Moss Protonemata

G. Gardner, H. Kende, R. deZacks

Moss protonemata were incubated for 24 hr on a medium containing 10^{-8}M ^3H -benzyladenine (BA) plus varying concentrations of non-radioactive BA. The plants were then weighed, and total radioactivity in the tissue was determined. The data, expressed as radioactivity per fresh weight, indicated that over half of the ^3H -BA uptake can be competed by non-radioactive BA. Competition could be observed after as little as 4.5 hr; one hour was insufficient. Thin-layer chromatographic analyses indicated that the radioactivity was still associated with BA.

Adenine, which does not cause bud formation, did not compete for the uptake. BA-riboside, which also does not cause bud formation, successfully competed with ^3H -BA. The fact that BA-riboside competed in the in vivo experiments but not in the in vitro binding assay suggests that these are two separate phenomena and that the in vivo experiments may represent a cytokinin uptake system. If so, we reasoned that we might be able to separate these systems by comparing the effect of BA-riboside on uptake at room temperature and at 4° . At the lower temperature, one might expect the uptake system, if active, to be less important and the (putative) receptor interaction to be more obvious. The experimental results were consistent with this prediction. BA competed well at either 19° or 4° , whereas BA-riboside only competed at 19° .

3. Effects of 5-Fluorouracil on RNA and α -Amylase Synthesis in Barley Aleurone

S. Rodaway

Gibberellic acid induces the de novo synthesis of α -amylase and some other hydrolytic enzymes by the barley aleurone layer. We attempted to determine whether the mRNA coding for α -amylase was also synthesized de novo in response to GAs or whether it existed in some form where it was unavailable

for translation prior to addition of the hormone. It seemed that the method of choice for determining the time of the onset of the α -amylase mRNA synthesis would be that employed earlier by Carlson (1972) which used the base analogue 5-fluorouracil (FU) to produce "faulty" mRNA which, in turn, caused differences in thermal stability of the enzyme synthesized after various pre-incubations with the drug.

Last year we reported (see Plant Research, '74, pp. 66-7) that we had been unable to find α -amylase populations with different thermal stabilities after lengthy incubation with FU and we have now extended those observations. α -Amylase was purified from barley half-seeds (cv. Betzes) to the stage where one molecular weight component ($MW\ 41,400 \pm 1,000$) was visible after electrophoresis on polyacrylamide gels containing 1% SDS. The enzyme, which represented 3% of the original half-seed protein had two peaks of activity in a preparative isoelectric focusing system (about pH 5.0 and pH 6.1), two peaks of activity following chromatography on DEAE-cellulose and six electrophoretic isozymes distinguished by activity under non-denaturing conditions. The isozymes were present in roughly the same ratios before and after purification, and the purified enzyme was essentially free of carbohydrate and protease. Lengthy FU treatment of the half-seeds had little or no effect on the net amount of α -amylase synthesized, the recoverability of enzyme at any stage of the purification, the appearance of isozyme patterns, or the final specific activity of the enzyme. The thermal denaturation process itself was found to be independent of enzyme concentration at least up to 440 $\mu\text{g/ml}$ and not to occur in the presence of high levels of calcium. The denaturation kinetics were the same for α -amylase from half-seeds incubated in 10^{-3}M FU for 3.5 days out of a total imbibition time of 4.5 days with GA_3 added for the last 36 hr. Other experiments including FU for the entire 4.5 days of treatment also produced α -amylases with the same stabilities.

10^{-2}M FU was found to inhibit at least 90% of rRNA and 4-5 S RNA synthesis in aleurone layers while only 10% of the amylase activity was affected. 10^{-4}M FU had a lesser effect (about 30% inhibition). Thus FU was able to enter the aleurone tissue of the half-seeds and to exert its effect, i.e. the inhibition of rRNA synthesis. These results imply either (i) that FU did not sufficiently alter mRNA nucleotide sequences to cause changes in the α -amylase primary structure which would be evidenced by changes in the enzyme thermal stability or (ii) that the α -amylase mRNA pre-existed in

the dry half-seed or was synthesized during the time it took to build up the FU-ribosyl triphosphate pool, the precursor for incorporation into RNA. New rRNA or 4-5 S RNA was not required for α -amylase synthesis.

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DEVELOPMENT AND NITROGEN FIXATION IN FILAMENTOUS BLUE-GREEN ALGAE

Filamentous blue-green algae (cyanobacteria) form multicellular patterns by mechanisms termed "fields of inhibition" and "inductions" in developmental biology. Thus, differentiated cells called heterocysts inhibit nearby vegetative cells from becoming heterocysts (Wolk, 1967) and, in *Anabaena cylindrica*, induce adjacent vegetative cells to sporulate (Wolk, 1966). Because these algae have three types of cells (vegetative cells, heterocysts, and spores or akinetes), they permit comparison of alternative differentiation processes within an organism which has a simple structure. Studies of pattern formation in these morphologically simple organisms may serve as models for investigations of pattern formation in higher organisms, both plants and animals.

Aerobic fixation of dinitrogen (N_2) is known to take place to a substantial extent, and perhaps only, in the heterocysts, and is dependent on a cooperation between these cells and the vegetative. The latter supply heterocysts with products of photosynthesis and receive, in return, products of N_2 fixation.

The goals of this research project are (i) to elucidate the biochemical bases of pattern formation, and (ii) to analyze the mechanisms underlying photosynthetic nitrogen fixation by these algae.

In order to elucidate the intercellular interactions which control the patterns, we are presently trying to identify algal products which can control the differentiation of spores (1, below), and we are seeking by use of ^{13}N to identify substances which move from cell to cell (2, below). The latter project is also a means to identify certain of the intercellular interactions underlying photosynthetic nitrogen fixation by heterocysts. Our efforts to determine the principal biochemical differences which distinguish the three cell types continue to concentrate on the envelopes of heterocysts and spores (3, below), because these envelopes are early, major products of differentiation. Detailed study of the intracellular control of differentiation must await detailed knowledge of the interactions, and of the biochemical differences they control.

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1. A Substance Stimulating the Differentiation of Spores in the Blue-Green Alga *Cylindrospermum licheniforme*

R. W. Fisher, T. Hirose, P. W. Shaffer, C. P. Wolk

Filaments of *Cylindrospermum licheniforme* cultured in phosphate-free medium (sporulation medium) sporulate adjacent to heterocysts. Centrifugal supernatant fluids from sporulated cultures stimulate sporulation of freshly inoculated algae. Thus, filaments grown in sporulation medium show no acceleration of sporulation until the fifth day of culture, whereas the acceleration of sporulation of filaments grown in conditioned sporulation medium starts essentially immediately (Fig. 1).

Fig. 1. Time course of sporulation of *C. licheniforme* in sporulation medium (o) and in sporulation medium conditioned by prior growth and sporulation of the alga (o).

The final percent sporulation is approximately the same in the two cultures. Growth and sporulation of *C. licheniforme* in sporulation medium therefore modifies that medium in a way that makes it stimulatory to spore formation upon reinoculation. Conditioning has no significant effect upon growth, or upon the pH of the medium.

Supplementation of conditioned sporulation medium with all of the constituents of sporulation medium does not affect the sporulation-stimulation which results from conditioning (Table 1).

Table 1. The effect of supplementing conditioned medium with components of fresh sporulation medium.

Medium	% Heterocysts with spores	
	Expts. A ^a	Expts. B ^a
Sporulation medium	6.4 $\pm$ 2.7 ^b	5.8 $\pm$ 2.6 ^b
Conditioned sporulation medium	66.0 $\pm$ 6.1	64.8 $\pm$ 6.6
Conditioned, supplemented sporulation medium	68.0 $\pm$ 13.8	67.2 $\pm$ 12.8

^aIn experiments A, constituents of sporulation medium were added to liquid, conditioned sporulation medium. In experiments B, liquid sporulation medium was added to lyophilized, conditioned sporulation medium.

^bMean value, plus or minus standard deviation, from five experiments, each of which was assayed after three days of culture.

Supplemented conditioned sporulation medium contains all of the components of sporulation medium in concentrations equal to or greater than their concentration in sporulation medium. The stimulation of sporulation which is effected by conditioning of sporulation medium is not, therefore, the result of a depletion of the medium. Rather, the filaments produce a substance which is released into the medium and which stimulates the differentiation of spores in newly inoculated filaments. Sporulation of the original inoculum may result from an increasing concentration of this same substance.

Conditioned sporulation medium is diluted, 1:19, with fresh sporulation medium sporulation is still accelerated 50% as much as by undiluted conditioned medium.

Our current efforts are directed toward purification and identification of the sporulation-stimulatory substance(s) released into the medium by sporulating cultures and possibly involved, within filaments, in

an induction, by heterocysts, of the sporulation of adjacent vegetative cells.

2. Products of the Fixation of Nitrogen Gas by Filaments and Heterocysts*

C. P. Wolk, J. Thomas, J. Meeks; S. Austin, A. Galonsky (MSU Cyclotron Laboratory)

In order to determine the products of fixation of nitrogen gas by heterocysts, we have with intact filaments developed techniques for identifying products of nitrogen fixation, using ^{13}N -labeled nitrogen gas [$^{13}\text{N-N}_2$]. The experiments represent a means of elucidating certain of the interactions whereby heterocysts and vegetative cells cooperate in the fixation of atmospheric nitrogen, and which may possibly be involved also in the intercellular control of pattern formation.

Suspensions of algal filaments are exposed to $^{13}\text{N-N}_2$ for varying periods of time, then combined with four volumes of methanol, and the filaments sedimented by centrifugation. The 80% methanolic supernatant extract is concentrated under vacuum, spotted on a cellulosic thin layer plate, and subjected to electrophoresis at different pH's, and sometimes also to chromatography (Thomas *et al.*, 1975).

As illustrated in Fig. 2, substances coelectrophoresing in borate buffer, pH 9.2, with (and which have by means of additional experiments been identified as) glutamine and glutamate are the principal initial organic products of fixation of $^{13}\text{N-N}_2$, with glutamine labeled much more rapidly than glutamate. Distillation experiments have shown that even after 1 min of fixation, the amide nitrogen of glutamine is much more extensively labeled than is its α -amino nitrogen (ratio ca. 6:1). Pulse-chase experiments showed clearly that radioactivity from glutamine is transferred to an acceptor molecule to form ^{13}N -labeled glutamate. Consistent with this observation, azaserine (an inhibitor of glutamine amide transferases) permits the synthesis of glutamine, but totally blocks the formation of glutamate. That (as expected) ammonia serves as the precursor of glutamine amide nitrogen was shown by the kinetics of labeling of these two products during short periods of labeling (Fig. 3), and by the observation that methionine sulfoximine permitted continued formation of $^{13}\text{NH}_3$ while preventing ^{13}N -labeling of glutamine and glutamate.

* Supported in part by National Science Foundation.

Fig. 2. Scan of radioactivity from ^{13}N in thin layer electrophoretograms of organic compounds extracted from *Anabaena cylindrica* with 80% methanol after (A) 120 s, (B) 60 s, and (C) 20 s of fixation of ^{13}N -labeled nitrogen gas. Unlabeled amino acids, coelectrophoresed as markers, were localized with ninhydrin. 0 = origin; L = lipids, displaced from the origin by chromatography. The ^{13}N -containing substances in (B) and (C) co-electrophoresed with glutamine and glutamate, as did the two major ^{13}N -containing substances in (A).

Fig. 3. Time course of incorporation of ^{13}N into pools of NH_3 and amide nitrogen, after fixation of $^{13}\text{N}-\text{N}_2$ for 15, 5 and (approximately) 1.25 s. Eighty percent methanolic extracts were subjected to vacuum distillation at pH 10, to determine $^{13}\text{NH}_3$ (—o—), and then to steam distillation in the presence of 40% NaOH, to determine amide- ^{13}N (—•—). The ordinate represents cpm $^{13}\text{NH}_4^+$ or cpm amide- ^{13}N in the fixation vial, corrected to the time of the start of fixation, and normalized to equal μCi of ^{13}N in the vial.

Our observations demonstrate unequivocally that N_2 -derived nitrogen fixed by this organism is metabolized by the glutamine synthetase-glutamate synthase pathway.

In the presence of ATP, dithionite (Stewart *et al.*, 1969) and light (Wolk and Wojciuch, 1971), suspensions of heterocysts couple fixation of nitrogen to uptake of exogenous glutamate, to form glutamine. We shall seek to determine whether glutamine and/or glutamate move out of heterocysts as vehicles for net transfer of fixed nitrogen into vegetative cells.

References

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3. Envelope Polysaccharides of Heterocysts and Spores

L. Cardemil de Balboa, C. P. Wolk

The only known biosynthetic process associated with sporulation which appears to differ qualitatively from processes of growth of vegetative cells is deposition of the spore envelope. The envelope, after removal of lipid, is 68% carbohydrate by weight in *A. cylindrica*. In the same alga, the envelope produced when a vegetative cell differentiates into a heterocyst is approximately equal in mass to the vegetative cell, and after extraction of lipid is 92% carbohydrate by weight.

Smith degradation (periodate oxidation followed by reduction with sodium borohydride and mild acid hydrolysis) cleaved off all branches from the envelope polysaccharides, but left the backbones intact. The backbones consist of 1,3-linked glucose (Glc) and mannose (Man), in a ratio of 3:1. Disaccharides, trisaccharides and tetrasaccharides obtained from the backbone polysaccharides by partial acid hydrolysis were fractionated by column chromatography and separated by high-voltage paper electrophoresis.

The presence of both Glc and Man in oligosaccharides derived from partial acid hydrolysis implies that the backbones do not consist of separate glucan and mannan chains. The fact that no di-, tri-, or tetrasaccharide contains two mannosyl residues implies that the backbones do not consist of a random distribution of glucosyl and mannosyl moieties. The presence of a single mannosyl residue within each of the four tetrasaccharides implies that the backbones consist of a repeating unit Glc→Glc→Glc→Man, an interpretation which is consistent with the structures and relative frequencies of di-, tri- and tetrasaccharides which were observed.

The disaccharides were identified as Glc→Man, Man→Glc, and Glc→Glc, ratio 1.0:1.1:1.6, and the trisaccharides as Glc→Glc→Man, Glc→Man→Glc, Man→Glc→Glc and Glc→Glc→Glc, ratio 1.0:1.0:1.0:1.0. Because Glc→Man, Glc→Glc, and Glc→Glc→Glc were completely hydrolyzed by β -glucosidase but not measurably by α -glucosidase, and because Man→Glc was hydrolyzed by β -mannosidase but not by α -mannosidase, we conclude that all of the linkages in the backbones are in the β -configuration.

We have found no differences between the envelope polysaccharides from heterocysts and spores. As discussed above, the backbone of both consists of $\beta(1\rightarrow3)$ -linked glucose and mannose with a repeating sequence of Glc→Glc→Glc→Man.

Moreover, all of their other glycosidic linkages are present in the same frequency, within experimental error. Although it remains to be determined whether or not the branches are linked with the same anomeric configurations to the same sugar residues in the backbones of the two polysaccharides, the evidence available to date seems sufficient to suggest, as a working hypothesis, that the envelope polysaccharides from the two types of differentiated cells are essentially identical. We propose that, if the polysaccharides from the two sources are essentially identical, their structures resemble, to a first approximation, the structure presented in Fig. 4.

Fig. 4. Possible structure of the polysaccharides from the envelopes of heterocysts and spores of *Anabaena cylindrica*. Linkages to terminal sugars (T-S) indicated by dashed arrows can be present in only certain of the "basic" units illustrated. On the basis of approximate stoichiometry, it may be suggested that the 2,3,4-Glc in the polysaccharide has, as regular substituents, T-Glc and (linked to the C-2 or C-4) Glc-(1 $\rightarrow$ 4)-Glc. Because the sequence of substitutions of backbone glucosyl residues is unknown, an arbitrarily chosen sequence is illustrated. Unlinked carbons-6 are omitted from the drawing for the sake of simplification.

Only a portion of the mannosyl residues in the backbone is substituted, and certain of the terminal sugars are present in amounts not stoichiometric with the backbone sugars. Therefore, even if the sequence of substitutions of glucosyl residues in the backbone were correct, the "basic" structural unit illustrated would not be, in the strict sense, a "repeating" subunit. It is also possible that the envelopes contain a mixture of slightly differing polysaccharides, each of which has a strictly repeating subunit.

THE PRIMARY CELL WALL GLYCOPROTEIN EXTENSIN AND RELATED MATTERS

We are interested in the structure, function and metabolism of extensin, the hydroxyproline-rich protein present in the primary cell walls of most green plants. The importance of this material arises especially from the demonstration that it is a glycoprotein in which the hydroxyproline hydroxyl group is glycosidically linked to short arabinose oligosaccharides, while the serine hydroxyl group is glycosidically attached to galactose. Both of these glycopeptides linkages could function as cross links between wall polysaccharides. Thus we have postulated that the primary cell wall can be looked at as a protein-glycan network (analogous to the peptidoglycan of bacterial cell walls) in which the extensin polypeptide plays an integral structural role and is therefore also involved, directly or indirectly, in cell wall plasticity and cell growth. Most of the current work is devoted to the determination of the structure of extensin (including polysaccharide attachment) from cell walls of higher plants, using in vitro cell cultures. Ultimately we hope to describe how the extensin polypeptide backbone relates to the rest of the plant cell wall structure. For this reason we are also attempting to study the structure of soluble extensin precursors (and other soluble hydroxyproline-rich glycoproteins), so that we can determine which polysaccharides (if any) are attached to extensin before insertion into the wall structure.

1. Extensin Structure

(a) *Solubilization of Extensin via β -Elimination Under Semi-Aqueous Conditions*

D.T.A. Lamport, M. Caughey

We continue to study the effect of semi-aqueous alkaline conditions on the β -elimination of serine and release of hydroxyproline-rich material from isolated tomato primary cell walls. Although this method always solubilizes >60% of the hydroxyproline, the chromatographic profiles were irreproducible, and turned out to be very dependent on the reaction temperature. Thus, running the reaction of 45°C, solubilized hydroxyproline-

rich macromolecules most of which were well retarded on Ultrogel AcA54, and fractionated further on Sephadex g-50 and by iso-electric focusing (pH 7-10). The amino acid analyses of these fractions are interesting because of their distinct resemblance, when written as an empirical formula, to the sum of the compositions of the tryptic peptides previously isolated from tomato cell walls:

	HYP	SER	LYS	VAL	TYR	THR	TYR DERIV.	ASP	GLU	ILU	LEU
Sum of tryptic peptides	27	8	6	2	2	1	2	=	-	-	-
G-50 Fraction I	27	6.4*	5.8	2.6	2.7	0.8	1.9	1.3	0.8	0.4	0.6
G-50 Fraction II	27	5.8*	4.6	2.3	1.1	1.0	0.3	1.5	1.2	0.4	0.7

* This figure is the sum of Ser + Gly + Ala because Gly and Ala are derived from serine during the elimination reaction.

It is therefore reasonable to consider the possibility that the hydroxyproline-rich material released by β -elimination may be intact or near-intact extensin polypeptide subunits.

(b) *Solubilization of Extensin via Sodium Chlorite Oxidation*

A. Mort, D.T.A. Lamport

Boiling the tomato walls for 2 hours in a 5% ammonium oxalate solution (pH 6.5) containing a little sodium dithionite to prevent oxidation, solubilizes about 40% of the weight of the wall, although virtually all of the hydroxyproline remains with the insoluble wall residue. Sodium chlorite oxidation (75° for 30 minutes in 1% NaClO₂ w/v in 1% acetic acid) of these extracted walls then solubilizes most of the macromolecular hydroxyproline. The material solubilized accounts for ca. 10% of the wall dry weight and consists almost exclusively of glycopeptide fragments. These glycopeptides and peptides have a very broad size range, but most of the hydroxyproline-rich glycopeptides void a Sephadex G-50 column. The G-50 void contains over 50 mole percent hydroxyproline (with respect to amino acids). The peptides retarded on G-50 are much less rich in hydroxyproline and have an amino acid composition more like general cytoplasmic proteins. Fragments of non-hydroxyproline-containing proteins

would not be expected to be heavily glycosylated and therefore, would probably not be large and so are retained on G-50.

The mechanism by which NaClO_2 releases protein from cell walls is still not clear, just as the way in which cell wall protein is retained in the wall is unclear. We do know that NaClO_2 cleaves some peptide bonds, although which ones remains to be determined. Sodium chlorite oxidation does not affect polysaccharides.

(c) *Fractionation of Sodium Chlorite Solubilized Glycopeptides*

A. Mort, D.T.A. Lamport

The glycopeptides split into two fractions when chromatographed on a Bio-Gel A.5 M or A.115 M column, a void and a retarded peak. These two fractions were further purified by DEAE cellulose chromatography.

The large molecular weight fraction (A.5 M void) gives a single relatively sharp peak on DEAE cellulose. (An additional uronic acid peak may also elute somewhat later if the ammonium oxalate treatment is not followed by thorough washing.) The uronic acids, neutral sugars and hydroxyproline co-chromatograph in this single retarded peak on DEAE cellulose. If this material is then chromatographed on a CL Sepharose 6B column there appear to be two fractions only partially resolved. The more retarded of the two has the composition:

HYP₃₀ ARA_{1.3} SER_{7.1} VAL_{1.0} TYR_{0.5} LYS_{2.3} α -aminoadipic acid_{1.1},
 trace amounts of other amino acids, arabinose₁₅₇ galactose₂₀
 galacturonic acid₃₂ glucose₄.

It is possible that the larger of the two fractions is a slightly less degraded fragment which contains an additional region somewhat poorer in hydroxyproline.

On an Aca22 column (fractionation range 60,000 to 1 million daltons) the DEAE retarded peak is anomalously retarded, eluting almost with the salt. This was interpreted as a strong interaction between the column material and the glycopeptides. Nevertheless, there is a very good correspondence in the elution of the hydroxyproline and the uronic acids.

After iso-electric focusing of the DEAE retarded peak the hydroxyproline and uronic acids again show coincidence. From all these attempts to purify the uronic acids from the protein we conclude that the two are probably

covalently attached, although conclusive evidence has not yet been obtained. For example attempts to retain the protein at low pH on cation exchange columns (CM cellulose and phospho cellulose) were unsuccessful. However so far only degradative procedures have succeeded in separating the uronic acid from the protein. Attempts to fractionate on the basis of density in a caesium chloride gradient are planned.

The low molecular weight material released by chlorite oxidation (and retarded on an A.5 M column) can also be chromatographed on DEAE cellulose giving the elution profile in Fig. 1. Amino acid analyses

Fig. 1. G-50 void+ on DEAE cellulose pH 5.2 50 mM Na Acetate. Linear salt gradient 0-0.5M.

show that these smaller fragments differ from the larger fragments. They have less serine but much more lysine and valine, while sugar analyses show that they contain arabinose, galactose and glucose, but no galacturonic acid. It is quite possible therefore that there are at least two regions in the wall protein, one rich in serine but relatively poor in lysine, while the other is richer in lysine and valine.

(d) *β -Elimination of Selected Fractions Obtained by Sodium Chlorite Oxidation of Tomato Cell Walls*

A. Mort, D.T.A. Lamport

In a further attempt to show covalent linkage between the uronic acid-containing polysaccharide and the protein, we subjected the high molecular weight DEAE material to a β -elimination reaction in 50% DMSO, 10% EtOH, 40% H₂O, 0.2N NaOH, 1 M NaBH₄ at 37°C stirred continuously for 6 hours, followed by neutralization with acetic acid. After this reaction the uronic acids still appeared in the void volume of an A.5 M column but the hydroxyproline was retarded by a G-50 column (ca. 2 x void). Little information is available on the effect of base or protein under these conditions and so it is difficult to interpret these results. It appears that some bonds must have been broken by the alkali as the same fraction totally deglycosylated by liquid HF voids a G-50 column. However this experiment does show that conditions which cleave a polysaccharide from serine residues in the protein do allow separation of the uronic acids from the protein.

2. Soluble Hydroxyproline-Rich Glycoproteins from Higher Plants

D.T.A. Lamport, M. Caughey

Relatively small amounts of hydroxyproline-rich glycoproteins occur soluble in the cytoplasm as well as in the growth medium of suspension cultures of higher plants, and also as we recently discovered, in the xylem sap of the sugar maple (*Acer saccharum*). We have been able to purify the peptide component of one of these glycoproteins utilizing our HF-deglycosylation method (see below). Thus the hydroxyproline-rich glycoprotein secreted into the medium by sycamore-maple (*Acer pseudoplatanus*) cells normally voids a Sephadex G-100 column. After HF-deglycosylation the deglycosylated protein has an elution volume of about 2x void and the amino

acid composition shows a dramatic enrichment in hydroxyproline. An even more interesting feature now appears if one compares the amino acid composition of this protein from the growth medium with the composition of a glycoprotein obtained simply by evaporation and dialysis of sugar maple sap. Both glycoproteins have as their most abundant amino acid residues, hydroxyproline, serine, and alanine:

	MAPLE SAP POLYSACCHARIDE	DEGLYCOSYLATED MEDIUM GLYCOPROTEIN
HYP	30	30
ASP	23.1	20.5
THR	14.7	18.6
SER	27.0	25.8
GLU	18.7	13.5
GLY	19.8	13.3
ALA	27.8	24.1
VAL	8.6	11.4
MET	1.1	-
ILU	7.0	6.1
LEU	18.2	11.0
TYR	1.6	0.6
PHE	5.9	5.0
LYS	11.2	8.5
HIS	3.1	3.2
ARG	4.8	3.0

These data lead to two important conclusions: First, the two glycoproteins, one secreted by cells in culture, the other present in xylem sap, may well be identical. Second, these glycoproteins are extensin-like in their high proportion of residues existing as hydroxyproline and serine, but distinctly unlike extensin in having so much alanine. This leads to the interesting possibility that these glycoproteins are amphipathic - i.e. have two distinct regions, the hydrophilic one of which could for example be extensin per se, while the hydrophobic region would contain the bulk of the alanine. Such an arrangement of hydrophilic-hydrophobic domains typifies some membrane proteins.

3. Hydroxyproline-Rich Glycoproteins from *Volvox*

D.T.A. Lamport, M. Caughey

We have previously reported that the extracellular matrix of *Volvox carteri* is extremely rich in hydroxyproline and glycine. This indicates the possibility of a single protein with a composition intermediate between that of extensin and collagen with the rather interesting possibility that

they are homologous proteins. Recently Kochert (1974) has commented on these data suggesting the possibility that the glycine component might be an artifact arising from the glycyglycine present in the growth medium. We therefore re-examined some of our original material (which had been obtained by 70% ethanol precipitation of the growth medium) after passage through Sephadex G-25 to remove any small molecules possibly occluded or adsorbed during the ethanol precipitation step. After acid hydrolysis of the Sephadex void fraction we reconfirmed the high glycine content, by high pressure liquid chromatography on Chromobeads C2 cation exchange columns, and by gas chromatography of the amino acid isobutyl esters as their N-heptafluorobutyrate. Whether or not this glycine is an integral component or just a macromolecular contaminant of the *Volvox carteri* matrix is an important question for the future.

Reference

Kochert, G., PNAS 71:1211 (1974)

4. Characterization of Possible Extensin Precursors from Sycamore-Maple Cells

P. Nordin, D.T.A. Lamport

We considered the possibility that polygalacturonides are attached to extensin during its biosynthesis. Therefore we incubated log phase cells of sycamore-maple with ^3H -methyl-labelled methionine and ^{14}C -proline, and then fractionated the organelle matrix proteins by iso-electric focusing. Although some fractions were ^3H -labelled, the ^3H -label was not saponifiable. This could mean that pectin is not attached to extensin during its biosynthesis. Alternatively it might indicate that attached pectin was methylated only very late in biosynthesis, or that there is extensive demethylesterification (e.g. via pectin methyl esterase) during our extraction procedures.

5. The Use of Hydrogen Fluoride for Deglycosylation of Glycoproteins and for Sugar Analysis

(a) *Glycoprotein Deglycosylation*

A. Mort, D.T.A. Lamport

Cell wall glycopeptides containing galactose, arabinose and galacturonic acid are completely deglycosylated after 1 hour in liquid

hydrogen fluoride at 0°C (Plant Research '74, p. 102). However this treatment does not completely deglycosylate all glycoproteins so far examined:

(i) *Fetuin*. According to Spiro and Bhoyroo (1974) native fetuin contains three asparaginyl-linked oligosaccharides; each contains 3 residues of sialic acid, 3 galactose, 5 N-acetyl glucosamine and 3 mannose. In addition there are 3 serine- or threonine-linked oligosaccharides each containing 1-2 residues of sialic acid, 1 galactose and 1 N-acetyl galactosamine. We treated fetuin with HF for 1 hour and then gel filtered it on a P100 column. After this treatment the fetuin contained approximately 3 N-acetyl glucosamine residues, 3 N-acetyl galactosamine, 1.5 galactose, 1 NANA, and no mannose. Although neutral sugars were reduced drastically the amino sugars directly linked to the protein (Asn-GlcNAc and Ser- or Thr-GalNAc) were not removed. This is especially clear for galactosamine which is present only as a direct link to protein (Ser- or Thr-GalNAc) and is entirely unaffected by HF at 0°. Thus treatment of fetuin with HF at 0° for 1 hour (in the absence of the scavenger anisole) released virtually no free galactosamine.

(ii) *Pig submaxillary Mucin (PSM)*. This glycoprotein according to Carlson (1968) contains many short oligosaccharides linked to threonine and serine residues, e.g.

Thr(or Ser)-O-N-acetylgalactosamine-galactose-N-acetylgalactosamine.

Treatment of PSM with liquid HF at 0°C for 1 hour removes at least 95% of the galactose, glucose and N-acetyl neuraminic acid, although 70% of the N-acetyl galactosamine remains with the protein. This again indicates that HF at 0° does not cleave Ser or Thr Gal. NAc.

(iii) *Crab Shells*. Crab shells contain a mixture of chitin (poly 1-4 N acetyl-glucosamine), pigments, protein, and calcium carbonate. HF at 0°C for 1 hour partially solubilizes the shell but the soluble N-acetyl-glucosamine remains polymeric. After 3 hours in HF at room temperature the N-acetyl-glucosamine becomes monomeric and so, with no additional hydrolysis, can be subjected to gas chromatography as the TMS derivative. As such it chromatographs with N-acetyl glucosamine, not with glucosamine. Thus HF does not remove the N-acetyl group.

From these experiments we conclude that HF does not appreciably cleave glycosidic linkages of amino sugars at 0°C, yet does at room temperature and therefore could be very useful in elucidating the sequence and linkages of amino sugars in glycoproteins. For example it should be possible to obtain disaccharides in good yield with the amino sugars at their non-reducing end. It should also be possible to simplify the elucidation of glycopeptide linkages as all the neutral sugars can be cleaved off, leaving only the one or two amino sugars attached to the peptide.

References

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(b) Total Sugar Analysis

A. Mort, D.T.A. Lamport

Some polysaccharides such as cellulose and polygalacturonides are exceptionally resistant to acid hydrolysis, while the methods generally in use lead to losses because of incomplete hydrolysis and partial degradation of the hydrolysis products. We therefore considered the use of HF (in the absence of scavengers* to cleave glycosidic linkages. We compared the yield of monomers after treatment of Larch arabinogalactan** (using mannitol as inert internal standard) with anhydrous HF (0° 1 hr.) and 2N trifluoroacetic acid (121° 1 hr.) respectively, followed by gas chromatography of the alditol acetates or trimethylsilyl methyl glycosides (Table 1). From experiments in progress where HF completely solubilizes all the cell wall sugars we conclude that the HF technique is highly satisfactory especially for analysis of polysaccharides resistant to the usual acid hydrolytic methods.

* Scavengers, such as anisole, are normally added to the HF reaction mixture to prevent alkylation of tyrosine and other reactive amino acids by the fluorinated compounds produced during HF addition across the bonds which are cleaved.

Linn (1975) has patented a method of generating alkylated aromatic compounds by reacting them in high concentrations of cellulose in HF.

** A polysaccharide obtained by extracting larch sawdust with water.

Table 1. Sugar yields from arabinogalactan as a percentage of total weight

<u>Derivatives prepared:</u>	<u>Alditol acetates*</u>	<u>TMS methyl glycosides</u>
<u>Treatments</u>		
HF	100	96
2N TFA (121° 2 hr.)	90	-
1.5 M HCl-MeOH (80° 8 hr.)	-	70

* Based on a secondary standard

Reference

C. B. Linn, U.S. Patent 2,798,100 (1957)

ENZYMIC MECHANISMS AND CONTROL OF POLYSACCHARIDE AND GLYCOPROTEIN
SYNTHESIS IN HIGHER PLANTS; CELL WALL STRUCTURE

This project continues to place major emphasis on a study of the enzymic mechanisms and control of polysaccharide synthesis in higher plants. At present, this work centers on the biosynthesis, composition, and structure of cell wall polysaccharides, with special emphasis on cellulose biosynthesis. Work has also been initiated on a second, related problem, glycoprotein synthesis in developing seeds of the legumes.

Cellulose is the world's most abundant bio-organic compound, but its biosynthesis is still one of the major unresolved problems in plant biochemistry. There are numerous claims of in vitro synthesis of β -(1 $\rightarrow$ 4)-glucan by enzyme systems in plants but in many cases, either the physiological significance of the reaction or the identity of the product can be questioned. We have chosen as our experimental material the developing cotton (*Gossypium hirsutum*) fiber since it offers a number of experimental and practical advantages (see Plant Research '74, p. 103). This year's work in this area included (i) a determination of the in vivo rate of cellulose deposition during fiber development, (ii) in vivo studies using coumarin, a specific inhibitor of cellulose synthesis, (iii) a detailed characterization of a highly active UDP-glucose: glucan synthetase in cotton fibers, and (iv) a preliminary characterization of non-cellulosic glucan in cell wall fractions isolated from cotton fibers.

We have also continued studies on the composition and structure of the primary cell wall of the developing cotton fiber. This work is being pursued for two reasons: (i) to complement our enzymatic studies on polysaccharide synthesis in cotton fibers, and (ii) with the hope that the developing cotton fiber can eventually be exploited as a model system for studying cell elongation in plants. No complete understanding of how a plant cell elongates in response to hormones can be obtained without knowledge of the structure of the cell wall and of how that structure is altered during wall extension. The cotton fiber, obtainable in sufficient quantity for biochemical analyses, is a single cell which elongates synchronously over an extended period of time.

How the composition and structure of the fiber cell wall changes during the elongation process has been investigated. Since cultured fibers respond to hormones (auxin and gibberellin) during growth, and may potentially be used for studies relating biochemical changes in the wall to the process of hormone-regulated growth, we have also compared the wall composition of cultured fibers (i.e. fibers growing on excised ovules cultured *in vitro*) to that of plant-grown fibers to see whether the culture system sufficiently mimics the normal developmental process to be useful as an experimental system.

The second area of investigation, the mechanism and control of glycoprotein synthesis in higher plants, serves as a complement to the studies of cell wall polysaccharide synthesis since many of the questions we can ask with this system are similar to those for cell wall synthesis: What nucleotide sugars are the substrates?, Are lipid intermediates involved?, Where are the sites of synthesis?, What are the controls? Glycoprotein synthesis has been intensively investigated in animal systems, and recent evidence has shown that the carbohydrate moieties are often transferred to the protein via lipid intermediates. Glycoproteins also exist in plants, but except for one recent paper by Forsee and Elbein (1975), very little is known of their mode of biosynthesis. Vicilin, one of the major storage proteins in the seeds of legumes, has recently been characterized (Ericson and Chrispeels, 1976) and shown to be a glycoprotein with sugar composition (N-acetylglucosamine and mannose) analogous to that of many animal glycoproteins. For this reason, we are currently studying the process of glycoprotein synthesis in developing legume seeds.

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1. Studies on Cellulose Synthesis

(a) *The in Vivo Rate of Cellulose Deposition During Fiber Development*

M. Meinert, D. P. Delmer

Last year we reported values for the cellulose content of the cotton fiber wall expressed as percent of the weight of the cell wall during the course of fiber development (Plant Research '74, p. 105). These data showed that the cellulose content of the wall, on this basis, undergoes an abrupt increase between 16 and 18 days post-anthesis. During this time, which represents the onset of secondary wall synthesis, the percent by weight of cellulose in the wall increases from 25% to 75%. Recently we have adopted a new means of expressing changes in cell wall composition during fiber development. By collecting data on fiber length, fiber yields, cell wall yields, and yields of individual wall components (in this case, cellulose), we are now able to calculate the content of cellulose (or other components; see report 2, below) per unit length of fiber wall. Such a calculation is much more representative of absolute changes in wall composition than are values based on weight percent, since weight percent values are dependent upon the relative changes in other fractions of the wall as well. Fig. 1 shows how the content of cellulose per mm of fiber length varies during fiber development. By taking the first derivative of this plot (with corrections for increase in fiber length), we have also calculated the apparent in vivo rate of cellulose deposition as a function of fiber age. Surprisingly, the data show that there are two peaks in the rate of cellulose deposition--one which occurs at the onset of secondary wall deposition, and a second one which occurs much later, at 27 days post-anthesis. Therefore, the concept of secondary wall cellulose synthesis as a continuous event merits further examination.

Fig. 1. Changes in cell wall cellulose content per mm fiber length during fiber development. The solid line represents the grams of cellulose per mm fiber length and was calculated as follows: gm cellulose per mm fiber length = % cellulose x gm cell wall per mm fiber length where gm cell wall per mm fiber length =

$$\frac{\text{gm cell wall}}{\text{gm fibers}} \times \frac{\text{gm fibers}}{\text{boll}} \times \frac{\text{boll}}{\# \text{ fibers}} \times \frac{1}{\text{fiber length (mm)}}$$

using the assumption that the number of fibers per boll is constant throughout development and equals 384,000 (C.A. Beasley, personal communication).

The calculation for the apparent "rate" of cellulose deposition (dashed line) is given below and involves a correction for changes in fiber length during elongation:

$$\text{"rate"} = \frac{(G_n \times L_n) - (G_{n-1} \times L_{n-1})}{L_n - 0.5}$$

where G_n = grams cellulose per mm fiber length at day n

and L_n = mm fiber length at day n

(b) *The Effect of Coumarin on the Synthesis of Primary and Secondary Wall Cellulose in Developing Cotton Fibers*

T. Skokut, D. P. Delmer

Last year we reported that coumarin, in short term in vivo labeling experiments with cultured fibers, appears to be a specific inhibitor of cellulose synthesis (Plant Research '74, p. 111). We have now used coumarin to investigate the question of whether primary and secondary wall cellulose are synthesized by the same or different enzyme systems. Cultured fibers of various ages and in varying stages of primary or secondary wall synthesis were pulsed with ^{14}C -glucose $\pm$ coumarin and incorporation into the cellulosic fraction was measured. Using 1.0 mM coumarin, specific inhibition of cellulose synthesis ranged from 62 to 85% of control values, independent of fiber age. Thus, coumarin was equally effective in inhibiting primary and secondary wall cellulose deposition, indicating that the coumarin-sensitive step in the process is the same for both primary and secondary wall cellulose synthesis.

(c) *Properties of a UDP-glucose: Glucan Synthetase in Cotton Fibers*

U. Heiniger, C. Kulow, D. P. Delmer

We reported, in Plant Research '74 (pp. 107-110), the presence of an active glucan synthetase in cotton fibers which uses UDP-glucose as substrate and which has the unusual property of requiring both cellobiose and UTP for activity at low substrate concentrations, while requiring only cellobiose for maximal activity at high substrate concentrations. The activity could be measured either using a detached fiber assay (see Plant Research '74, p. 107 for description) or in isolated membrane preparations. Those results were preliminary, and at that time we had only limited information about the kinetic properties of the enzyme and did not yet know the linkages of the glucose residues in the glucan, although it resembled cellulose in its solubility properties. This year we have devoted a major effort to a detailed characterization of this enzyme.

The kinetics of the reaction, as assayed in detached fibers, have been studied in detail, including experiments designed to measure optimal cation requirements (Mg^{++} at 5 mM gives optimal activity), optimal cellobiose concentrations (>5 mM), optimal UTP concentrations

(0.6 mM), and optimal pH (broad optimum from pH 6.5 to 8.4). Other β -linked glucosides (laminaribiose or β -methyl-glucoside) can effectively substitute for cellobiose as activators, whereas α -linked glucosides are relatively ineffective. The kinetics with respect to UDP-glucose concentration are complex at lower substrate concentration, indicating activation by the substrate; the range in which this activation occurs can be shifted to lower substrate concentrations by the presence of cellobiose. At higher concentrations of UDP-glucose (>0.1 mM), the kinetics exhibit a more standard Michaelis-Menton form, allowing one to calculate an apparent K_m for UDP-glucose of 5.0 mM without cellobiose present and 1.7 mM in the presence of cellobiose.

The unusual requirement for UTP at substrate concentrations below 50 mM has been investigated further, and we have now come up with a plausible explanation of this phenomenon: The glucan synthetase, which is stimulated by cellobiose and having a high K_m for UDP-glucose, requires high substrate concentrations for significant activity. At low UDP-glucose concentrations, the UTP serves as a substrate for another enzyme, UDP-glucose pyrophosphorylase, which converts UTP plus endogenous glucose-1-P to pyrophosphate and UDP-glucose, thus raising the substrate concentration to activating levels. A variety of experiments, including the demonstration that ^{14}C -glucose-1-P (+UTP) can be substituted for UDP- ^{14}C -glucose as substrate, fully support this conclusion.

The glucan synthetase, when assayed in detached fibers, is highly active throughout fiber development (Fig. 2), having activities in the range of our calculated rates of in vivo cellulose deposition, and in several respects has properties expected of a true cellulose synthetase. However, detailed studies on the linkage of the product produced by this enzyme have shown that the product clearly is not cellulose, but rather a non-cellulosic β -linked glucan. Results of partial acid hydrolyses, periodate oxidation, specific enzyme digestion, and also of methylation analyses show that the product is predominantly a β -(1 $\rightarrow$ 3)-glucan. The product appears identical regardless of whether it is produced under high or low UDP-glucose concentrations, either with the detached fiber assay or by isolated membrane preparations. Because the enzyme in so many respects resembles a cellulose synthetase, but clearly is not, we feel such results should serve as a caveat emptor to other workers in this field.

Fig. 2. Activity of UDP-glucose: glucan synthetase as a function of fiber age. Initial rates of incorporation were assayed by incubation for 10 min of 50 mg fresh weight of buffer-washed fibers in 250 μ l of 0.05 M TES buffer pH 7.5 containing 5 mM $MgCl_2$, 10 mM cellobiose, and 1.0 mM UDP- ^{14}C -glucose (148,000 dpm). Following incubation, the fibers were extracted with chloroform/methanol, followed by successive washes in water. Radioactivity remaining in fibers was designated as glucan product. These initial rates were then converted to ng glucose equivalents incorporated per mm fiber length per day by the following formula:

$$(6 \text{ I.R.}) \times \frac{24 \text{ hrs}}{\text{day}} \times \frac{1}{\text{D.W.}} \times \frac{\text{total D.W.}}{\text{boll}} \times \frac{\# \text{ fibers}}{\text{boll}} \times \frac{1}{L}$$

where I.R. = initial rate

D.W. = dry weight fibers corresponding to 50 mg fresh weight fibers at age assayed

L = mm fiber length at age assayed

The method for calculation of in vivo rate of cellulose deposition is given in the legend of Figure 1.

(d) *Cellulose Synthesis in Pea Stem Sections*

D. P. Delmer, C. Kulow

The similarity of the glucan synthetase described above in cotton fibers to a recently reported "cell surface cellulose synthetase" in pea stem sections (Shore and MacLachlan, 1975; Shore et al., 1975) prompted us to investigate in more detail than that published, the linkages of the product formed by this pea stem enzyme. Using techniques similar to those described in the previous section, we find that in our hands, the product of the reported pea stem "cellulose synthetase" when produced under conditions as close as possible to those described by Shore and coauthors, is not cellulose, but rather a predominantly β -(1 $\rightarrow$ 3)-linked glucan, with properties indistinguishable by our techniques from the product of the cotton fiber glucan synthetase.

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(e) *The Non-Cellulosic Glucan of Fiber Cell Walls*

M. Meinert, D. P. Delmer

Last year (Plant Research '74, p. 105) we reported the presence of a non-cellulosic glucan component of the cell wall which increased in content coincident with the earliest stages of secondary wall cellulose deposition in cotton fibers. In view of our discovery of a non-cellulosic glucan synthetase in these fibers, we have begun to focus attention on this natural glucan in the wall as it could represent the product synthesized in vivo by this glucan synthetase. Preliminary methylation analyses of unfractionated walls indicate that there is a rise in 3-linked glucose in the wall which roughly parallels the increase in non-cellulosic glucan in the wall. Current studies are directed at determining whether this glucan exhibits turnover in the wall, and, if so, whether it might serve as a precursor to cellulose.

2. Biochemical Characterization of the Cotton Fiber Cell Wall During Development: The Elongating Fiber Cell Wall is a Dynamic Structure

M. Meinert, D. P. Delmer

The elongating cotton fiber is a material in which it is possible to examine the biochemical changes in cell wall composition associated with the development of one single higher plant cell throughout the entire period of elongation and secondary wall formation. Such compositional analyses show that the elongating cotton fiber cell wall is a dynamic structure. Table 1 presents a summary of the results of our compositional analyses of cell walls of plant grown fibers harvested at various stages of development.

Table 1. Summary of cotton fiber cell wall composition during development.

The first line of figures represents the percent by weight of that component; the second line of figures represents the $\text{g} \times 10^{-10}$ of component per mm fiber length. ND = not determined. Numbers in parentheses refer to the totaled percent of analyzed components under conditions where not all components were determined.

Component	Fiber Age (Days Post-Anthesis)										
	5	8	10	12	14	16	18	20	22	25	29
Cellulose	9 1.9	10 4.2	23 10.8	24 14.4	25 23.0	31 49.9	74 170	82 245	87 362	90 594	94 1363
Neutral sugars	33 6.9	25 10.5	25 1.3	20 12.0	17 15.6	14 22.5	12 27.5	10 29.9	9 37.4	7 46.2	4 58.0
Uronic acids	10 2.1	16 6.7	22 10.2	21 12.6	16 14.7	9 14.4	3 6.9	2 6.0	2 8.3	ND	ND
Protein	40 8.4	24 10.1	22 10.4	22 13.2	25 23.0	21 33.6	3 16.1	4 12.0	ND	ND	ND
Ash	ND	3 1.3	3 1.4	3 1.2	11 10.1	11 17.6	4 9.2	2 6.0	ND	ND	ND
Total Cell Wall	(91) 21	78 42	95 47	89 60	94 92	86 160	100 230	100 299	(98) 416	(97) 660	(98) 1450

The data are expressed both as a percent by weight of the wall (upper figures) or as grams of component per mm fiber length (figures in italics), since while relative changes in cell wall composition (%) are of interest, a more useful way of expressing the data is in absolute terms of grams per unit length of cell wall (see above, report 1a).

The relative proportions of cellulose, neutral sugars, uronic acids, and protein in the cotton fiber cell wall continually change throughout the elongation process. When expressed as grams component per mm fiber length, the protein and uronic acid content of the cell wall rise until just prior to the time of maximal rate of cell elongation (16 to 19 days post-anthesis; elongation ceases between 22 and 24 days post anthesis), and then sharply decline at a rate consistent with actual removal from the wall. The total neutral sugar fraction increases continually throughout elongation; the relative ratios of the individual sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, and non-cellulosic glucose) continually change, with arabinose and non-cellulosic glucose undergoing the greatest change in content (data not shown). The initial rise in total neutral sugars can be primarily attributed to a rise in arabinose content. In later stages of elongation, the arabinose content per unit length of fiber declines; at this time (approximately coincident with the earliest stages of secondary wall cellulose synthesis), a significant increase in the level of non-cellulosic glucose occurs in the wall (see also report 1e, above).

Results obtained for compositional analyses of cell walls derived from culture-grown fibers indicate that these walls are quite similar to those derived from fibers grown on the plant, both in terms of composition and in terms of relative changes in composition during development, indicating that the culture system should be quite useful for further studies on the mechanism of cell elongation and maturation, and on turnover of wall components. However, one major difference was observed: the sequence of developmental changes which occur in the cell wall of culture-grown fibers are compressed in time relative to plant-grown fibers.

A comparison of data available in the literature on cell wall compositions with our data for the cotton fiber cell wall (Table 2), indicates that the primary cell wall of the fibers of cotton, a dicotyledonous angiosperm, is similar to that of other reported dicot primary walls (and even more closely

resembles the composition of a cultured gymnosperm, Douglas fir). This conclusion is further supported by our linkage analyses of the carbohydrates of the fiber cell walls as determined by preliminary methylation analyses of unfractionated cell walls. To cite one example, we find that fiber cell walls contains significant amounts of 4-linked glucose, 4,6-linked glucose and terminal xylose residues indicative of a xyloglucan structure characteristic of dicots, and lack branched xylosyl residues indicative of the monocot arabinosylan (Burke et al, 1974).

Table 2. Composition by weight percent of cell walls from various plant sources.

	<u>Wheat</u> ¹	<u>Rye Grass</u> ¹ <u>Endosperm</u>	<u>Sycamore</u> ¹ <u>maple</u>	<u>Douglas</u> <u>fir</u> ¹	<u>Cotton</u> <u>fiber</u> ²
Total neutral sugars	63.1	73.7	49.8	37.2	25.3
Rhamnose	0.6	0.6	3.1	2.5	1.9
Fucose	0.1	0	1.3	1.0	0.3
Arabinose	20.7	20.3	21.0	12.7	8.2
Xylose	24.8	19.8	7.6	5.0	2.6
Mannose	1.0	0.1	0.3	0.5	1.0
Galactose	12.0	5.9	12.8	7.6	4.3
Glucose	3.9	27.0	3.7	7.9	7.0
(non-cellulosic)					
Cellulose	14.0	13	23	22	23
Uronic acids	12	7	13	18	22
Protein	11	7	10	18	22
Hydroxyproline	0.14	<.05	2.0	0.4	0.3

¹ Taken from the data of Burke et al. (1974) on cell walls from cultured cells of these plants.

² From plant grown fibers harvested 10 days post-anthesis (mid-phase of elongation).

Reference

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3. Glycoprotein Synthesis in Developing Cotyledons of *Phaseolus vulgaris*

M. C. Ericson, D. P. Delmer

The enzymatic processes involved in glycoprotein synthesis have been studied using extracts obtained from developing cotyledons of *Phaseolus vulgaris* harvested at the time of active deposition of vicilin, the only major storage protein in this species. Using crude extracts, radioactivity from GDP- ^{14}C -mannose can be incorporated into a single chloroform:methanol-soluble product as well as into insoluble product(s) (Fig. 3). The kinetics of these reactions, as determined by pulse and pulse-chase experiments, are consistent with the radioactive glycolipid serving as an intermediate in the synthesis of the insoluble product(s).

Fig. 3. Kinetics of Pulse and Pulse Chase Experiments. Reaction mixtures contained (in a final volume of 0.55 ml): 0.5 ml crude cotyledon extract; GDP-D- ^{14}C -mannose, 3.00×10^{-4} μmol (35,000 cpm); and MnCl_2 , 5.0 μmol . Incubations were allowed to continue for various lengths of time at 15°C. After 10 min, 0.1 μmol unlabeled GDP-D-mannose was added to "chased" samples. Reactions were terminated by the addition of 1.5 ml of $\text{CHCl}_3:\text{CH}_3\text{OH}$ (1:2). Samples were then incubated at 37°C for 20 min. After incubation, 0.5 ml CHCl_3 and 0.5 ml 0.9% NaCl in 0.01 M HCl was added to each tube. Samples were separated by centrifugation into three phases: an aqueous phase which contained unused substrate and was discarded; an interface layer designated as insoluble product; and an organic phase designated as CM-soluble product. Solid curves = pulsed products, broken curves = chased products.

The characteristics of the glycolipid, as determined by its mobility in three different thin-layer chromatographic systems, its resistance to alkaline saponification, and its extreme acid lability, are consistent with it being a mannosyl-phosphoryl-polyprenol. Treatment of the radioactive glycolipid with mild acid (0.01 N HCl, 100°, 10 min) results in the release of 91% of the radioactivity which is exclusively recovered as ^{14}C -mannose. The incorporation of mannose from GDP- ^{14}C -mannose into mannosylphosphorylpolyprenol is reversible by the addition of excess GDP, indicating that the mannose is attached to the lipid via a monophosphate linkage.

Of the radioactivity in the insoluble product(s), about 20% is pronase-digestible during a "pulse experiment", and about 40% is pronase-digestible after a chase with unlabeled GDP-mannose (Table 3).

Table 3. Solubilization of the radioactive interface product by pronase digestion.

Pulsed interface product was obtained from samples incubated for 20 minutes with GDP- ^{14}C -mannose. Chased interface product was obtained from samples incubated for 5 minutes with GDP- ^{14}C -mannose and for an additional 15 minutes with unlabeled GDP-mannose.

Sample	Radioactivity (dpm)	% Solubilized
Pulsed interface product (before proteolytic digestion)	11,380	-
Pulsed interface product (after proteolytic digestion)	9,550	-
Counts solubilized by proteolytic digestion	1,020	19.3
Chased interface product (before proteolytic digestion)	4,700	-
Chased interface product (after proteolytic digestion)	2,720	-
Counts solubilized by proteolytic digestion	1,980	42.0

The other 60% is as yet uncharacterized but could be a mannan. The difference in susceptibility to pronase digestion in product obtained from either a pulse or a chase is best explained by the presence of a radioactive lipid-oligosaccharide present in the insoluble product fraction during the pulse; this radioactivity is then transferred to protein during a chase, thus increasing the percent of insoluble product that is glycoprotein after a chase. This conclusion is supported by the fact that a radioactive product soluble only in a mixture of chloroform:methanol:H₂O (1:1:0.3) may be extracted from the insoluble residue obtained during a pulse, but this radioactivity decreases during a chase. This product stays at the origin during TLC in lipid solvents. Thus we suggest that this product may be a lipid oligosaccharide, the final intermediate in glycoprotein synthesis.

Preliminary data indicate that radioactivity from UDP-N-acetyl-[¹⁴C]-glucosamine can be incorporated into both chloroform:methanol-soluble and insoluble products. Results with both GDP-¹⁴C-mannose and UDP-N-acetyl-¹⁴C-glucosamine clearly support a role for lipid intermediates in the glycosylation of protein in this higher plant system and indicate that the mechanisms of glycoprotein synthesis in higher plants are quite similar to those reported for mammalian systems.

MOLECULAR STUDIES OF MEMBRANE SYSTEMS

We investigate physico-biochemical properties of differentiating and non-differentiating biological membranes and how they respond to environmental stress. Since we have found that the membrane lipid fluidity of Mycoplasmas is regulated and kept virtually constant despite changes of growth temperature and food supply, we henceforth have to ask the question whether the membrane lipid fluidity is regulated during morphogenetic modifications of the membrane, or during adaptive changes of the cell exposed to environmental stress. Therefore we are continuing to study the simple membrane from Mycoplasmas which can serve as a valuable model system to understand mechanistically how membranes of eucaryotic cells function. Morphogenetic alterations of the plasma membrane are investigated in the aquatic fungus *Blastocladiella emersonii*.

As in previous years we continue to study fast photoprocesses in the photosynthetic light transducer. To investigate such photoprocesses, the emission of delayed light is recorded after turning off the exciting light. The delayed light intensity at a particular point in time seems to depend on an energized state of the chloroplast membranes.

1. Membrane Potential of *Thermoplasma acidophila*

J.-C. Hsung, A. Haug

Thermoplasma acidophila is a mycoplasma-like organism which grows optimally at 59 C and pH 2. We demonstrated previously that the intracellular pH of this organism is close to neutral as measured by the distribution of a weak organic acid, 5,5-dimethyl-2,4-oxazolidinedione, across the plasma membrane. We had also demonstrated that the pH gradient is maintained passively by a Donnan equilibrium. Now we have demonstrated that part of the pH gradient is maintained by a membrane potential. The potential was measured by the distribution of radioactive KS^{14}CN , which almost completely dissociates at $\text{pH} \geq 2$. The measured membrane potential lies between 105 and 118 mV, positive inside the cell. Increasing the external KSCN concentration to 0.01 N with cold KSCN, the potential is lowered to 55 mV. When the external pH is changed to six, the potential is practically diminished to values smaller than 15 mV. These results show that at least part of the pH gradient across the cell membrane is maintained by a non-metabolic membrane potential.

2. Thermotropic Mesomorphism and Membrane Lipids in the Membrane of *Thermoplasma acidophila*

L. L. Yang, H. Weller, A. Haug

As part of its adaptation to growth at different temperatures, *Thermoplasma acidophila* apparently adjusts certain physico-biochemical parameters of its cell membrane. Cells were adapted from growth at 59 C to growth at 37 C, and the temperature dependence of membrane lipid fluidity of isolated cell membranes and membranes of intact cells from adapted and non-adapted cells was studied by paramagnetic spin probes. Regardless of growth temperature, two membrane lipid phase transitions can be observed, one below 25 C, and one at about 45 C. The membrane lipid fluidity throughout this temperature range depends, however, on the growth temperature. The low temperature transition (Fig. 1) increases with increasing Mg^{2+} or Ca^{2+} concentration of the cell

Fig. 1. ESR-determined microfluidities for isolated *T. acidophila* cell membranes suspended in various concentrations of Mg^{2+} . Cells grown at 56 C. 5-Nitroxyl-stearate spin label.

suspension. In the presence of bivalent ions the membrane becomes more rigid, the permeability of the cell membrane is probably impaired, and the activity of membrane-bound enzymes may possibly be modified.

There are less lipoproteins and glycolipids in membranes isolated from cells grown at 37 C than in membranes from cells grown at the higher temperature. Cells grown at these two temperatures differ with respect to two major and several minor protein bands as determined by gel electrophoresis. Both types of cells contain a membrane-bound ATPase which has a pH optimum in the neutral region. This enzyme may therefore face the cytoplasm. All phospholipids are choline positive as determined by thin-layer chromatography.

3. Physico-Biochemical Properties of Developing Zoospores from *Blastocladiella emersonii*

K. Leonards, A. Haug

Membranes from the uniflagellate zoospore of the aquatic fungus *Blastocladiella emersonii* are at a higher level of organization than those from Mycoplasmas. These zoospores allow a study of alterations in membrane lipid fluidity during cellular development. It is already known that ultrastructural and physiological changes occur during the development of this cell, and also that these changes can be appreciably affected by environmental factors. In the past year we refined the technique for growing cells of *Blastocladiella* in large, synchronous batch cultures. Selective isolation and spin labeling of zoospore plasma membranes is being attempted in order to study biochemical properties of membrane components, physico-chemical parameters of the membrane, and possible control mechanisms of membrane lipid fluidity during morphogenesis.

4. Microsecond Delayed Light Emission and Ion Accumulation in Spinach Chloroplasts

A. Haug; D. Bell, N. E. Good (MSU Department of Botany and Plant Pathology)

Delayed light emission is believed to originate from a back reaction of photochemical species of photosystem II. After turning off the exciting light, nanosecond and microsecond delayed light originates in photosystem II, similar to slower components of delayed light. We had demonstrated previously that delayed light is not a fast component of prompt fluorescence. The

persistence of the photochemical back reaction for millisecond delayed light, as detected by delayed light as a probe, seems to be dependent on an energized state of the chloroplast membranes, which in turn is closely related to their ability to make ATP. Previous experiments had shown that ammonia concentrations which uncouple phosphorylation from electron transport increase slightly the emission of delayed light in the millisecond region. Other uncouplers nearly abolish millisecond delayed light emission. It appears that an increased membrane potential is involved in the enhancement of delayed light emission in the presence of ammonia.

In order to obtain further insight into the relationships between delayed light emission and membrane potential, we studied possible correlations in the microsecond region of delayed light emission. We found that virtually all uncouplers enhance the intensity of microsecond delayed light. A possible explanation lies in the availability of back-reacting species in the presence of the uncouplers tested. Additional studies are required to elucidate the mechanism(s) involved in the enhancement of microsecond delayed light emission.

SENSORY TRANSDUCTION IN PLANTS

This project is directed toward an understanding of the mechanisms whereby information concerning the state of the environment is received by an organism. Toward this goal we are studying the sequence of events in information acquisition through non-visual light reception or the measurement of temperature.

Our work on light reception is directed at the identification and isolation of the photoreceptor pigment controlling the numerous physiological responses collectively known as the "blue-light" responses because the action spectra for these responses show a maximum in the blue region of the spectrum, from 450 to 480 nm. This problem is being approached in two major ways: (i) We are proceeding with the isolation and purification of the components of the "blue-light" photoreceptor pigment system from the cellular slime mold, *Dictyostelium discoideum*. This pigment system exhibits a light-induced absorbance change, the action spectrum for which is similar to the "blue-light" action spectra. (ii) Much of the recent data concerning the "blue-light" photoreceptor pigment are most easily interpreted based on the hypothesis that the pigment is localized (at least in its active form) on the plasma membrane. We are therefore working to isolate and spectrally characterize purified plasma membranes from "blue-light" responding organisms.

For our work on thermosensory transduction, we are also using *Dictyostelium* the pseudoplasmodia of which are positively thermotactic. Our work includes: (i) the development of the conceptual basis for the study of the sensory transduction of a parameter (heat) which is inherently non-specifically absorbed; and (ii) the measurement of characteristics of the thermotransducer mechanism to permit an identification of the thermotransducer.

1. Photosensory Transduction

(a) *Photoreducible Cytochrome b of Dictyostelium*

K. Manabe, K. Poff

Poff and Butler (1974, 1975) and Munoz and Butler (1975), have described the photoreduction of a specific *b*-type cytochrome for which the action spectra are similar to action spectra for the physiological blue light responses. This similarity of action spectra is taken as evidence that the photoreceptor pigment for the reduction of the cytochrome is a flavin. The present work was undertaken to purify the *b*-cytochrome in preparation for adding flavin to the *b*-cytochrome to reconstitute the postulated system in vitro.

The procedure used for purification of the *b*-cytochrome is given in Table 1. Using ammonium sulfate precipitation, gel filtration by Sephadex G-100, DEAE chromatography, a second gel filtration by Sephadex G-100 and finally chromatography over brushite, the cytochrome was purified about 1,900 fold. The ratio of absorbance at 280 nm to absorbance of the Soret peak of the oxidized form of the final preparation was 0.3. A room temperature absorption spectrum of the cytochrome shows peaks in the reduced form at 556-560 nm, 528 m, and 424 nm, and in the oxidized form at 413 nm. Spectral analysis of the pyridine haemochrome formed from the final preparation indicates that the cytochrome has a non-covalently bound heme. The molecular weight of the cytochrome is 14-17 K by gel filtration and SDS gel electrophoresis.

Preliminary data indicate that the purified *b*-cytochrome is reducible by light in the presence of added FMN or added crude flavoprotein from *Dictyostelium* in the presence of an electron donor such as EDTA or NADH.

Table 1. Purification of the photoreducible *b*-type cytochrome of *Dictyostelium discoideum*

Purification step	Total protein* (mg)	Total cyt <i>b</i> ** (μmoles)	Specific activity (10 ³ x μmoles cyt <i>b</i> /mg protein)	Purification (fold)	Yield (percent)
Crude homogenate	5576	0.139	0.025	1	100
Ammonium sulfate ppt. (0.4 - 0.85 satd) Sephadex G-100	426.5	0.106	0.248	9.9	76
DEAE Ultrafiltration	18.2	0.044	2.42	96.8	32
Sephadex G-100 Brushite chromatography	0.5	0.024	48	1920	17

* Protein was assayed by Lowry method.

** Extinction coefficient of α peak of the reduced form was presumed to be 25.6/mM/cm.

References

- Munoz, V. and W. L. Butler, Plant Physiol. 55:421 (1975)
 Poff, K. L. and W. L. Butler, Nature 248:799 (1974); Plant Physiol. 55:427 (1975)

(b) Light-induced Absorbance Changes in Corn Coleoptiles

K. Manabe, K. Poff

We have sought blue light-induced absorbance changes in coleoptiles of corn, selecting this material because corn has frequently been used in phototropism experiments and there is a large amount of information on the isolation of sub-cellular organelles from corn coleoptiles, and because blue light-induced absorbance changes have not previously been described in material from flowering plants. Coleoptiles grown in darkness show blue light-induced absorbance changes identifiable as phytochrome changes. These may be decreased by an incubation under red light, or circumvented by transforming the phytochrome with red light prior to irradiation with blue light.

Coleoptiles incubated under red light show absorbance changes identifiable as the protochlorophyll-to-chlorophyll phototransformation. This can be circumvented by transforming all of the protochlorophyll with red light prior to irradiation with blue light.

Coleoptiles treated in such a manner that neither the phytochrome phototransformation nor the protochlorophyll-to-chlorophyll phototransformation can be induced by blue light (prior exposure to red light) show a blue light-induced absorbance change. This light-induced increase in absorbance at 420 nm does not correspond to the blue light-induced absorbance change thought to be correlated with the "blue-light" photo-receptor pigment in *Phycomyces* (Poff and Butler, 1974), and *Neurospora* (Munoz and Butler, 1975).

References

- Munoz, V., and W. L. Butler, Plant Physiol. 55:421 (1975)
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(c) *Inhibition of Phototropic Responses of Corn Coleoptiles*

W. Schmidt, P. Filner, K. Poff

The question of the identity of the photoreceptor (flavin versus carotenoid) for the physiological blue light responses such as phototropism has not yet been answered satisfactorily. In essence this results from the fact that no single avenue of investigation can provide a definitive answer to such a question; the answer will be derived from the results of diverse experiments. We have extended the work by Hart and Filner (Plant Research '67, p. 28-30) who used inhibitors specific for phototropism in corn seedlings to obtain further information on the identity of the photoreceptor pigment of phototropism. Potential inhibitors were applied in aqueous solution to the roots of 144 hr dark grown corn seedlings for one hour before application of the test stimulus (light, gravity). The phototropic stimulus consisted of a weak ($\sim 3 \mu\text{W cm}^{-2}$) blue (440 nm defined by interference filter with a 10 nm half-band) horizontal light beam for 6 hr applied to the vertical coleoptile. In the case of dual beam irradiations, a second monochromatic light beam ($\sim 200 \mu\text{W cm}^{-2}$) was applied orthogonal to the blue light. The geotropic stimulus was presented by laying the coleoptile horizontal for one hr, and the response was then permitted to develop in darkness for 5 hr with the coleoptile in the vertical position.

We assume that the paths in geotropism and phototropism in the same organism (corn seedling) differ mainly in that section of the path close to the input receptor. Thus, geotropism is used as a control for the specificity of any potential inhibitor of the photoreceptor pigment of phototropism.

Potassium iodide, phenylacetic acid and sodium azide inhibit phototropism more than geotropism (Fig. 1a, b, c). The potential osmotic influence of the inhibitors can be dismissed; potassium chloride had no effect on either geotropism or phototropism at concentration as high as 10^{-1}M (Fig. 1d). Based upon the known interaction of phenylacetic

Fig. 1. Phototropism and geotropism in the presence of potential inhibitors. Each point represents the mean of measurements on twenty coleoptiles with the error bars representing the standard error of the measurement. Response expresses coleoptile bending as a percentage of that in the control.

- Phenylacetic acid added in aqueous solution to the roots of the seedling.
- Potassium chloride added in aqueous solution to the roots of the seedling.
- Sodium azide added in aqueous solution to the roots of the seedling.
- Potassium iodide added in aqueous solution to the roots of the seedling.

acid and the iodide ion with flavins the results are interpreted as support for the hypothesis that a flavin is the photoreceptor pigment for phototropism. Similarly, azide has been reported to inhibit in vitro electron transport to flavin (Schmidt and Butler, 1976), in addition to its inhibitory effects on mitochondrial electron transport by cytochrome a_3 to oxygen. The specificity of inhibition of phototropism by these inhibitors may result from localization of the photoreceptor pigment

on the plasma membrane giving easier access to inhibitors by the photoreceptor pigment than by intracellular flavins (e.g. mitochondrial flavins).

Red light does not induce phototropism but inhibits the phototropic response to blue light. This inhibitory effect has a broad action maximum (from at least 650 to 750 nm) which may be evidence that neither phytochrome in either form nor protochlorophyll is the photoreceptor pigment for the inhibition. Another potential candidate for this photoreceptor pigment is the triplet state of the postulated flavin photoreceptor for phototropism. According to this hypothesis, the triplet state of the flavin would be induced by blue light and would be physiologically active in the phototropic response. Red light could serve to inhibit phototropism by exciting and thus de-populating, this triplet state.

(d) *Plasma Membrane-bound Cytochrome b and Flavin in Phycomyces*^{*}

W. Schmidt

The photoreceptor pigment for phototropism in *Phycomyces* is probably bound to the plasma membrane and the polarity of this environment may well affect the absorption spectrum of the photoreceptor pigment and thereby affect the action spectrum for the physiological response. Thus, spectral identification of the photoreceptor pigment should be accompanied by subcellular localization of the pigment. The goal of this work was to isolate reasonably pure plasma membranes from *Phycomyces* using the technique devised by W. Schmidt, K. Thomson, W. L. Butler and M. Delbrück (unpublished) and to characterize these membranes through electron microscopy and visible spectroscopy.

* This work was initiated by W. S. in the laboratory of W. L. Butler at the University of California, San Diego in cooperation with K. Thomson in the laboratory of M. Delbrück at the California Institute of Technology.

The isolation technique is based on gentle breakage of the mycelium through shaking on a rotary shaker with large (5 mm diameter) glass beads, minimizing breakage of the internal organelles. Breakage of 15 hr spore germlings proceeds exponentially with more than 95% of the mycelia broken within 60 min. Most of the organelles other than wall fragments with attached plasma membrane are removed by washing. Electron micrographs of such washed wall fragments clearly show some plasma membrane still attached to the wall.

Ultrasonic disruption of the wall-membrane preparation permits removal of more than 90% of the plasma membranes, as indicated by comparative spectral analysis of the wall-membrane preparation, and the resultant wall preparation and membrane preparation (Fig. 1).

Fig. 1. Spectral characteristics of *Phycomyces* cell wall, wall with membrane attached, and purified membrane. Each spectrum is a dithionite-reduced-minus oxidized difference spectrum at 22°C.

Following disruption, the 1 Kg supernatant contains mainly plasma membrane vesicles which are pelleted between 8 Kg and 40 Kg. These membranes band at $\rho = 1.2$ to 1.3 g/cm^3 on a CsCl equilibrium gradient. Electron micrographs show the membrane-like structure of the vesicles from the 37 Kg pellet (Fig. 2) and the presence of few membranes left attached to the walls from the 1K pellet.

Fig. 2. Electron photomicrograph of purified plasma membrane vesicles from *Phycomyces* spore germlings. Preparation procedure given in Fig. 1. Fixation and staining with osmium. Final magnification x 55,000.

A dithionite reduced-minus-oxidized difference spectrum of the crude plasma membrane preparation clearly shows at least one *b*-type cytochrome based on the Soret band at 430 nm and the α band at 560 nm. A similar spectrum at -196°C of the purified plasma membrane preparation (Fig. 3) shows that the α -band is split into at least 3 bands, probably representing 2 or more cytochromes. In addition to the *b*-type cytochrome(s) there is a small but significant amount of flavin based on fluorescence emission spectra of a lumiflavin derivative of the purified membrane fraction.

Fig. 3. Dithionite-minus reduced difference spectrum of purified *Phycomyces* membrane (center). A 4th derivative of the spectrum is given below ($dx = 2$ nm), and wavelength calibration lines from a mercury lamp above.

(e) *Light-induced Changes in Adenylate Levels in Neurospora crassa and Dictyostelium discoideum*

B. Whitaker, K. Leonards, D. Delmer, K. Poff

Delmer and Brody (1975) have reported that light induces changes in adenosine monophosphate (AMP) levels in *Neurospora*. This fungus responds to blue light by a phase shift in its circadian banding rhythm. If it could be demonstrated that such changes in AMP levels were under control of the "blue-light" photoreceptor pigment, it would provide an entry into the early physiology of the "blue light" physiological responses. We sought to repeat these observations, to measure an action spectrum for any blue light effect on AMP levels, and to see whether such light-induced changes in adenylate levels can also be found in *Dictyostelium*.

Adenylates were converted to ATP and measured using the firefly luciferin-luciferase reaction. In *Neurospora*, both the circadian rhythm in AMP levels and the light-induced changes in AMP levels described by Delmer and Brody (1975) could be observed but the magnitude of these

light-induced changes was not sufficiently large to permit the determination of an action spectrum. No consistent light-induced adenylate level changes were found in *Dictyostelium*. Any assay for adenylates in *Dictyostelium* is complicated by the rapid turnover and interconversion of ATP, ADP, AMP, cyclic AMP and inorganic phosphate. We have concluded that quantitative measurements of light effects on adenylate levels in *Neurospora* and in *Dictyostelium* using the luciferin-luciferase assay are presently impractical.

Reference

Delmer, D. and S. Brody, J. Bact. 121:548 (1975)

2. Thermosensory Transduction: Thermotaxis by Pseudoplasmodia of *Dictyostelium discoideum*

K. Poff, M. Skokut

We have continued our work on thermotaxis in *Dictyostelium* with the goal of developing a research approach which will permit the identification of the thermoreceptor controlling thermotaxis. Temperatures and/or temperature gradients may be controlled across a petri dish by setting that dish on an aluminum block through each side of which is directed a flow of water from a constant temperature water bath. With the temperature in the center of a petri dish kept constant at 24 C, the gradient across the dish was varied up to 10 C/9 cm. Thermotactic orientation increases rapidly with an increasing thermal gradient, saturating at about 4 C/9 cm. Pseudoplasmodia will respond easily to a gradient of 0.5 C/9 cm which corresponds to a thermal gradient across a pseudoplasmodium of 0.00056 C.

The temperature response curve for the migration rate was measured (Fig. 1) to see if a temperature dependent change of migration rate is sufficient to explain thermotaxis; i.e., does migration rate decrease with temperatures over the range of thermotaxis? Migration rate indeed decreases with increasing temperature, but only from 27.5 C to 29 C. Over the temperature range for thermotaxis (22 C to 28 C from Fig. 2) there is no decrease in migration rate with increasing temperature.

Fig. 1. Relative slug migration rate (% of a control at 23.5 C) as a function of temperature.

Fig. 2. Relative ability of pseudoplasmodia to respond to a 0.1 C/cm thermal gradient as a function of the midpoint temperature of that gradient.

Thermotactic orientation was also measured with a constant thermal gradient (0.11 C/cm) as a function of the midpoint temperature of the gradient. The results (Fig. 2) show that pseudoplasmodia are thermotactically sensitive over the temperature range from 22 to 28 C but not above or below the range.

The total range for thermotaxis is thus less than 6 C. The low thermotactic sensitivity over the temperature range from 19.5 to 21.5 C (Fig. 2) where migration rate is relatively unimpaired (Fig. 1) is evidence that thermotaxis does not operate via a general thermal effect on the metabolism involved in migration. The narrow response range for thermotaxis is indicative of a single specific transition being used as the thermosensory transducer.

One possible transition which could be used as a thermosensory transducer is a change in fluidity of a lipid component of a cellular membrane. Such a transition might be expected to occur at a different temperature if the cells were incubated at that different temperature long enough for the membrane lipids to be modified by the cells. The cells used to form the pseudoplasmodia, the thermotactic sensitivity curve of which is plotted in Fig. 2 were raised at 23.5 C, and thermotactic sensitivity was maximal at 23.5 C. We measured such a curve for cells grown at 20 C and found the curve to be shifted to lower temperatures by 1 C. The cells grown at 20 C were developed for 16 hr at 23.5 C before plating to determine thermotactic sensitivity, and the 16 hr incubation at 23.5 C may account for the 1 C curve shift instead of the anticipated 3.5 C shift.

These data are consistent with the hypothesis that the thermotransducer is a specific substance or structure such as a lipid in a membrane matrix, and that this substance (lipid) undergoes a phase transition over a relatively narrow temperature range.

REGULATION OF FORMATION OF PROTEIN COMPLEXES AND ENZYMES IN
HIGHER PLANT CELLS

Two key kinds of processes which occur during development of eucaryotes such as plants are (i) coherent, orderly changes in spatial relations, and (ii) coherent, orderly changes in the spectrum of metabolic activities. Microtubules have been implicated in a wide range of developmental and physiological events where changes in spatial relations occur. Consequently, we are seeking clues to the mechanisms which govern the formation and disassembly of microtubules. The nitrogen and sulfur assimilation pathways of plants converge at protein synthesis, quantitatively the major fate of both nitrogen and sulfur. We therefore are studying regulation of the enzymes by which nitrate, ammonia and urea are assimilated, as well as the enzymes by which sulfate and organic sulfur compounds are assimilated, with the emphasis on characterization of interconnections in the regulation of nitrogen and sulfur assimilation.

1. Microtubules and Tubulin

(a) *Interactions of Tobacco Cell Components with Polymerizing Brain Tubulin*

P. Filner, S. Hooper

Although colchicine binding protein can be extracted from cultured tobacco cells and was shown to resemble animal tubulin in almost all respects, attempts to isolate microtubules from tobacco or other plant sources by methods which work with animal cells have failed. Also, attempts to cause tobacco tubulin to polymerize into microtubules in crude extracts have failed. Two possible sources of difficulty have been explored: (i) an inhibitor of polymerization or activator of depolymerization could exist in tobacco extracts, (ii) the tubulin in tobacco extracts could be too dilute to polymerize (a critical concentration has not been reached). We have obtained evidence for the existence of an inhibitor of polymerization of brain tubulin in tobacco extracts. The inhibitor appears to be titratable with brain tubulin. Addition of brain tubulin results in polymerization proportional to protein concentration after a certain threshold is exceeded. By prelabeling the tobacco proteins with a radioactive amino acid, it is possible to ask if any tobacco proteins are selectively associated with the polymerizing brain tubulin.

We have obtained indications of association of tobacco protein with the polymerizing brain tubulin, but it remains to be determined whether or not these tobacco proteins are tubulins.

(b) *Characterization of the Reaction between Tubulin-Colchicine Complex and Microtubules*

P. Filner, S. Hooper

The binding reaction between microtubules and tubulin-colchicine complex, which we discovered last year, has been found to resemble the polymerization reaction of tubulin quite closely. The major difference is that there are a limited number of binding sites for tubulin-colchicine complex in a microtubule preparation. Concentrations of complex sufficient to approach saturation of the binding sites do not prevent tubulin from polymerizing, nor do they cause massive depolymerization of microtubules, at least in the presence of glycerol, which stabilizes microtubules. We have proposed that the interaction of colchicine with microtubules proceeds in two steps: first, a binding of colchicine by free tubulin; second, a binding of tubulin-colchicine complex by microtubules, the latter by a reaction which is an analog of tubulin polymerization. It remains to be determined whether the binding of complex to microtubules has a quantitative influence on microtubule depolymerization or tubulin polymerization. The binding of radioactive complex to brain microtubules has proven to be a very convenient means of assaying "polymerization" and effects of factors on "polymerization". This assay is the first one of which we are aware that has the potential for detecting microtubules and tubulin polymerization in crude extracts in which the microtubules are too dilute substantially to affect either viscosity or light scattering, the two assays most commonly used for polymerization of relatively pure brain tubulin.

(c) *Mechanism of Resistance of Colchicum to Colchicine, an Alkaloid which It Synthesizes*

N. Yadav, P. Filner

Colchicum autumnale, the autumn crocus, tolerates very high concentrations of exogenously applied colchicine, as well as the high concentration of endogenously synthesized colchicine. The mechanism of resistance to this alkaloid is of some theoretical interest since colchicine interacts with a specific binding site of tubulins that has been highly

conserved during evolution and is therefore thought to be an essential part of tubulin. Does *Colchicum* have tubulin with a colchicine binding site or not? If it lacks the binding site, then the site is not essential for the function of tubulin. If it possesses the binding site, how is the colchicine kept from the tubulin?

A protein fraction found in very high concentrations in leaves of *Colchicum autumnale* has associated with it a chromophore that it tightly bound and closely resembles colchicine in its spectral characteristics. The protein is composed of subunits of molecular weight close to 55,000, as is tubulin from other sources. Current work is designed to determine if this is a tubulin-like protein, or if it is RU5P carboxylase, the most abundant protein in leaves, which also has a subunit of the observed size.

A major difficulty in working with *Colchicum autumnale* is the very brief natural period of vegetative growth, about six weeks. In order to have plant material all year long, we are attempting to accelerate and retard the onset of vegetative growth by varying the length of the cold treatment.

(d) Preparation of ^{125}I Labeled Tubulin

H. Quader, P. Filner

There is a need for radioactive tubulin of high specific activity so that the kinetics of tubulin polymerization and other tubulin interactions can be readily quantitated in the presence of other materials. The only readily available source of polymerizable tubulin is mammalian or avian brain, but it is exceedingly difficult to obtain highly radioactive tubulin from brain via biological labeling, i.e. feeding of radioactive precursors. Therefore, we have been exploring chemical labeling. Direct iodination with ^{125}I , using ^{125}I -KI and an oxidizing agent, is unsatisfactory because the oxidizers and the iodine denature tubulin. Bolton and Hunter (1973) devised an indirect iodination procedure, using ^{125}I -iodophenylpropionyl-N-succinimide, which acetylates epsilon amino groups of lysine at neutral pH in aqueous solutions. With this reagent, it was possible to prepare tubulin with one ^{125}I per tubulin molecule. This tubulin would not polymerize. However, when only one ^{125}I was bound per ca. 10 tubulins, the radioactive tubulin would polymerize into microtubules. This finding indicates that the constraints on tubulin structure

imposed by the interactions which occur in microtubules are so great that the polymerizing property of the protein is greatly modified by acetylation of a single amino group, and the microtubule can tolerate only about 1 modified tubulin for every 10 in the microtubule.

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Bolton, A. E. and W. M. Hunter, Biochem. J. 133:529 (1973)

2. Regulation of Nitrogen and Sulfur Assimilation in Plants

(a) *Urea and Ammonia Assimilation in Tobacco Cells*

T. Skokut, P. Filner, C. P. Wolk

Ammonia and urea are the major nitrogenous fertilizers currently used throughout the world. Synthesis of these compounds is a major fraction of the energy investment in Agriculture. It is usually taken for granted that these compounds are converted to nitrate by soil bacteria before they are assimilated by plants. However, plants can assimilate these compounds directly. Very little is known about the direct assimilation of urea and ammonia by plants, especially about how plants regulate these processes.

We have initiated a study of the paths of ammonia and urea assimilation and their regulation in cultured tobacco cells. The major findings to date are: (i) extracts of tobacco cells lack the enzymes of the urea→allophanate→ammonia pathway, which is important in some algae and fungi; tobacco cell extracts possess the enzyme urease, which is sufficient for the urea + H₂O→ammonia pathway. Using ¹³N-NH₄⁺, it has been found that ammonia is incorporated into glutamine by intact tobacco cells much more rapidly than into glutamate. This finding is consistent with recent findings of others that point to glutamine rather than glutamate as the first product of ammonia assimilation in plants.

Growth of tobacco cells on ammonia or urea is inhibited by methylamine to a greater extent than growth on nitrate. However, methylamine, which is considered an analog of ammonia, does not strongly inhibit ammonia uptake, urease activity or urease development in the tobacco cells. Since the pathways of assimilation of nitrate, ammonia and urea are considered to be identical after the formation of ammonia, the methylamine effect is paradoxical.

We believe that the methylamine effect merits closer examination, since it may be a manifestation of different pathways or intracellular localizations of ammonia assimilation, depending on the nitrogen source.

(b) *Selection of Mutants of the Sulfur and Nitrogen Pathways in the Plant Kingdom*

S. Flashman, P. Filner

Results obtained earlier in this laboratory by Reuveny (1975) suggested that it might be possible to use selenate on plants in a manner analogous to the way in which penicillin is used on bacteria by geneticists: to kill growing cells, while allowing non-growing mutant cells to survive. This seemed plausible because: (i) sulfur starvation of cultured tobacco cells derepresses ATP sulfurylase, and presumably the rest of the sulfate pathway; (ii) selenate, an analog of sulfate, is very toxic to tobacco cells in which ATP sulfurylase is derepressed, but not to cells in which the enzyme is repressed; (iii) the enzyme cannot be derepressed in cells lacking an exogenous assimilatable nitrogen source. These observations led to the prediction that cells deprived of a nitrogen source would be protected against selenate toxicity. This prediction has been confirmed experimentally. It seems likely, therefore, that if the cells are "deprived" of nitrogen as a result of a genetic lesion rather than a deficiency of the environment, such mutants might also be protected against selenate toxicity.

The minimal concentration of selenate and minimal exposure time necessary for killing close to 100% of tobacco cells with a nitrogen supply but which permit survival in the absence of nitrogen, have been determined. Following the killing treatment, an antidote containing a variety of nitrogenous compounds is administered, in order for the hypothetical mutant survivors to grow.

The same selection scheme is being tested on *Chlamydomonas reinhardtii* and yeast. At the time of this writing, we do not yet know if the selection works.

Reference

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MECHANISM OF SULFUR DIOXIDE TOXICITY IN PLANTS

R. Bressan, L. Wilson, P. Filner

As the world's supplies of oil and natural gas rapidly diminish and become depleted during the next three decades, it is expected that coal will become the major energy source for the U.S. Much of this coal has a high sulfur content, and so a substantial increase in atmospheric SO_2 pollution is anticipated. Although the concentrations of SO_2 at which plants suffer damage are known, neither the mechanism of toxicity, nor of resistance is understood. In response to ERDA's need for information on the effects of SO_2 , we recently initiated a study of these mechanisms.

Wide variation in sensitivity to SO_2 was evident among the cucurbits, in a survey done many years ago (Thomas et al., 1950). Consequently, we are concentrating on the cucurbits. We have found that the classic symptoms of SO_2 toxicity can be elicited in detached leaves by exposing them to bisulfite salt solutions. The leaves respond when floated on, vacuum infiltrated with, or fed through the cut petiole with, bisulfite solutions. Using these techniques, we have confirmed the occurrence of a wide range of sensitivity in the cucurbits, and have found the range of sensitivity among cultivars of *Cucurbita pepo* (squash) to be particularly striking.

Quantitation of SO_2 injury has traditionally been done by making subjective judgements of the appearance of damaged leaves. We are seeking an objective quantitative method. The decrease in chlorophyll content was found to be a poor indicator of leaf injury, because the magnitude of the change was small. Ethylene production increases greatly in slightly injured leaves, but declines in severely injured leaves. The ethylene production response correlates with sensitivity to SO_2 among cucurbits. Therefore, ethylene production may be a useful indicator of injury by SO_2 .

An apparatus for exposing plants to atmospheres of defined SO_2 concentration, for measuring SO_2 absorption by the plants, and for measuring emission of gaseous sulfur compounds by plants, is under construction. A system for analysis of ^{35}S -labeled sulfur compounds formed from ^{35}S - SO_2 is

being developed. Comparisons of the metabolic fates of SO_2 in resistant and sensitive plants are planned.

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