Technical Report

PhosphorImager and PD Densitometer Imaging System Network
DOE-#9202-1281. Status and Use of the Equipment

The equipment is in good condition. Several software problems were encountered, but were corrected by the manufacturer at no cost to us. The system is used on a regular basis (see specific descriptions below).

2. New Research Projects Undertaken as a Result of Equipment Availability

Rob Alba, Graduate Student, Department of Botany and Complex Carbohydrate Research Center.

This equipment has significantly increased the rate at which I can address my questions; specifically, does the HBG or 13mer stimulate *in vitro* membrane protein phosphorylation (or dephosphorylation) events. Typically, these experiments take two to seven days, most of which was accounted for by autoradiogram exposure time. With the PhosphorImager, I am able to run an entire experiment in one day. I did approximately 50 experiments in a four to five month period of time; thus, the PhosphorImager saved me six to eight months. In essence, the PhosphorImager has allowed me to rapidly and thoroughly investigate one possible signal transduction mechanisms of the HBG→phytoalexin defense response.

Phillip Buckhaults, Graduate Student, Department of Biochemistry.

Regulation of expression of a very low abundance mRNA (GlcNAc transferase V). This glycosyl transferase is regulated by oncogenes which influence the steady state mRNA levels. It is a very low abundance transcript, and the study of its regulation would be very difficult without the extreme sensitivity of the PhosphorImager.

Wenwen Chien, Graduate Student, Department of Biochemistry.

The cell surface oligosaccharide structure is regulated by the level of the expression of glycosyltransferases. A change in the oligosaccharide structure may contribute to a different metastatic potential and altered adhesive properties of various B16 mouse melanoma cell lines. The project is to study the relationship among factors: glycosyltransferase enzyme activity, cell surface sugars, and metastatic potentials.

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François Côté, Postdoctoral Research Associate, Complex Carbohydrate Research Center and Department of Botany.

Our laboratory's research interests are to study the perception and the transduction of the signals leading to the induction of defense responses in plants. The biological model system being used is the elicitation of phytoalexin accumulation in soybean by oligoglucosides released from cell walls of the phytopathogenic fungus, *Phytophthora megasperma* f.sp. *glycinea*. Phytoalexins are substances known to inhibit pathogen growth. We have data supporting the presence of a binding site for the fungal elicitor in soybean (Cheong and Hahn, 1991, *The Plant Cell*, 3: 137-147). We have already begun the purification of elicitor binding protein(s) (EBPs) from soybean root membrane extracts. In order to demonstrate the physiological function of the EBPs and their role in signal transduction, the corresponding genes have to be isolated. I have established a strategy for the functional screening of a cDNA expression library, utilizing the radiolabeled hepta- β -glucoside as the screening ligand. The system that I am using is based on the expression of foreign genes by transfection of mammalian cells. The detection of individual cells expressing positive cDNAs was done on special glass slides that were ultimately coated with an photographic emulsion. The PhosphorImager being available has allowed the very important step of pre-screening these glass slides for the presence of a positive cluster of cells. The identification and the characterization of a plant receptor recognizing an oligosaccharin (oligosaccharide with a biological activity) will be a major advance in the study of the biological activities regulated by other members of this new class of plant signal molecules. Our limited knowledge of the structure and function of plant binding proteins or receptors will also benefit from this project.

Jamie DeStefano, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

We have been using the densitometer to scan our SDS-PAGE gels of *Pneumocystis carinii* cell wall glycoprotein fractions obtained from preparative electrophoresis. This was an ongoing project when the densitometer was first made available. Before the densitometer, we had to manually calculate the molecular weights of these cell wall glycoproteins, but with the arrival of the densitometer, the molecular weight of each band was accurately obtained in a fraction of the time. We were also able to compare many gels from various preparative electrophoresis runs and determine which fractions contained identical glycoproteins. The equipment has helped us to easily obtain molecular weights for ten *P. carinii* cell wall glycoproteins, of which only one has been well characterized.

Marly Eidsness, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Identification of putative polygalacturonase genes in *Rhizobium meliloti* genomic DNA.

Edward Korzus, Graduate Student, Department of Biochemistry.

Human α -1-proteinase inhibitor (α -1-PI) and α -1-antichymotrypsin (α -1-Achy) are plasma proteins belonging to a superfamily of proteins referred to as Serpins (SERine Proteinase InhibitorS). Their complexes with HLE and Cat G, respectively, are rapidly removed from the circulation by binding to specific receptors located on the surfaces of a variety of cell types; including macrophages, monocytes, and fibroblasts. In a previous study, it was found that these complexes could stimulate the production of IL-6 and, indirectly, acute phase protein synthesis. Studies on both the structure and function of the serpin:enzyme complex receptor were expanded in order to better understand the primary events involved in the maintenance of the proteinase inhibitor potential in interstitial fluids, as well as the involvement of these complexes in the cell signalling network.

Lee Pratt, Research Professor, and Bernard Hauser, Graduate Student, Department of Botany.

We are using the phosphorimager to quantitate expression of individual members of the phytochrome gene family in tomato, as a function of seedling/plant age and of distribution of each member throughout a plant. ^{32}P -labelled riboprobes specific to each phytochrome mRNA are being used to probe Northern blots.

Neil Price, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Biosynthesis of lipid A (endotoxin) in *Rhizobium leguminosarum*

Biosynthesis of nodulation factors in *Bradyrhizobium japonicum*

The equipment has been invaluable for monitoring radioenzyme assays of steps in biosynthetic pathways.

Myron Williams, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Cloning of antibody genes. Screening of putative λ phage antibody expression library for binding of radiolabelled antigen (^{125}I).

Sheng-Cheng Wu, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Induction of defense-related genes of rice by *M. grisea* and its secreted cell wall-degrading enzymes. Because of a large number of quantitative Northern hybridization experiments, this project would not be possible without taking the advantage of the PhosphorImager (e.g. high sensitivity and quantitativity).

3. Financial Data on Operation and Maintenance Expenses Provided by the Institution

No operation and maintenance expenses have been necessary. Several software problems were corrected by the manufacturer at no cost to the institution.

4. Number of Faculty, Postdoctoral Research Associates, and Graduate Students who Utilize Equipment

Faculty: 2
Postdoctoral Research Associates: 7
Graduate Students: 6
Technicians: 1

5. Results of Experiments and Important Breakthroughs in Using the Equipment

Rob Alba, Graduate Student, Department of Botany and Complex Carbohydrate Research Center.

I have been investigating ABG-(+ 13mer) specific, plasma membrane protein phosphorylation (or dephosphorylation) events. I feel confident that I have thoroughly investigated this hypothesis *in vitro* and have obtained no evidence to date in support of the hypothesis. I am not sure if this is because such a signal transduction mechanism does not exist in this system, or because it is not detectable *in vitro*. Because I feel that I had the assays working well and others have been able to detect similar such phosphorylation events, it is my current belief that the initial events, following ligand-receptor binding, do not involve protein kinases.

All my experiments were done using 1-D gels. It will be necessary to repeat similar experiments using 2-D gel electrophoresis. These experiments will be done in the next two months and a final conclusion on this hypothesis will have to wait until then.

As these are negative results *in vitro*, I would by no means call them an "important breakthrough." Negative results in biochemistry, particularly *in vitro*, simply do not hold that much water.

Kevin Brady, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

The equipment has been used to rapidly analyze radiolabelled hybridization experiments. The speed of a "quick and dirty" analysis this equipment provides is very helpful, but the quality of the output is not anywhere near "publication quality".

Phillip Buckhaults, Graduate Student, Department of Biochemistry.

Two papers:

A cDNA clone for the complete cloning sequence for α -1,3(6)-mannosylglycoprotein β -1,6-*N*-acetylglucosaminyltransferase V (GlcNAc-T V, EC 2.4.1.155) was isolated and expressed in COS-7 cells. Degenerate oligonucleotide primers for polymerase chain reaction were synthesized based on the amino acid sequence of three tryptic peptides isolated from affinity-purified GlcNAc-T V. Polymerase chain reaction amplimers were isolated from rat and mouse mRNA. A cDNA-encoding full-length enzyme was isolated from a rat 1 cell (EJ-*ras*-transformed) library and sequenced. Transient expression of this clone in COS-7 cells, followed by enzymatic activity assays, demonstrated that this cDNA sequence encodes GlcNAc-T V. Northern analysis of rat kidney mRNA revealed a single band corresponding to a length of about 7 kilobases. Sequence analysis of the cDNA clone demonstrated an open reading frame that encoded a type II membrane protein of 740 amino acids. (*J. Biol. Chem.* (1993) 268: 15381-15385)

Manuscript in preparation.

WenWen Chien, Graduate Student, Department of Biochemistry.

Products of enzyme activities were analyzed by thin layer chromatography. The plate was then scanned by Bioscan which gave only signals along one direction. Ambiguous results were obtained. By using the PhosphorImager, the whole picture of the plate can be shown. This really clarifies the results. One major breakthrough for me!

François Côté, Postdoctoral Research Associate, Complex Carbohydrate Research Center and Department of Botany.

With glass slides supporting cells expressing control plasmids, positive cells organized in clusters were detected as dark spots on a relatively clear background. The slides were then coated with a photographic emulsion and exposed for a period of time compatible with the intensity of the signal obtained with the PhosphorImager. After development and observation under the microscope, the clusters of cells previously identified were confirmed to contain between two and six cells of an average diameter of 50 microns. Unfortunately, when these experiments were repeated with the cDNA library, no positive clones were identified.

In addition to these experiments, the PhosphorImager/Densitometer combination has allowed significant improvements in two other areas of my research:

Polyacrylamide Gel Electrophoresis (PAGE): The new technology produces high quality hard copy of gels, reducing the costly need for photographs. It allows semiquantitative detection of single band, and very accurate determination of the molecular weight of unknown protein species.

Radiolabeled Hepta- β -glucoside Binding Assays: The phosphorImager is a very valuable alternative to the use of a gamma counter for the detection of bound radiolabeled (125 I) molecules. It is as quantitative, and allows solid phase assay and tissue localization in addition to the liquid phase binding assay.

Marly Eidsness, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Rhizobium meliloti Southern blot shows two hybridization bands (in all restriction enzyme digests) when probed with *Agrobacterium tumefaciens* biovar 3 polygalacturonase gene fragments.

The PhosphorImager reduced the time required to observe hybridization bands on Southern blot from 24 hours (for conventional X-ray film exposure) to 3 hours (PhosphorImager screen).

Edward Korzus, Graduate Student, Department of Biochemistry.

In the experiment, measurement of IL-6 increased levels of the mRNA in stimulated fibroblasts and U937 using Northern hybridization analysis. Here the cells were incubated with a variety of stimulants, including native and proteolytically fragmented inhibitors, complexes, and peptide fragments representing sequences within the reactive site loop of the inhibitor. The data indicate that the carboxy-terminal portion of human α -1-P1 carries at least part of the binding for inhibitor:enzyme complexes to the receptor on fibroblasts. Native inhibitors presumably have part of this moiety buried and it becomes better exposed after either complex formation or proteolytic inactivation. The data given clearly confirms that certain sequences on the Serpin are intimately involved in cytokine stimulation in fibroblasts. These data are consistent with our approaches to isolate and characterize the receptor for Serpin:enzyme complexes, as well as to determine its biological significance in different cells.

Karen Liljebjelke, Technician, and Debra Mohnen, Assistant Professor, Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center.

The phosphorimager has been instrumental in our studies aimed at identifying, characterizing, and purifying polygalacturonate 4- α -galacturonosyltransferase, the enzyme responsible for synthesizing the polygalacturonan portion of the plant cell wall polysaccharide pectin. The ability of the phosphorimager to detect and quantitate very low levels of radioactivity has allowed us to characterize pectin biosynthesis products synthesized *in vitro* using total membranes from suspension cultured plant cells. Specifically, the size and nature of the synthesized product(s) was analyzed by TLC separation of *in vitro* synthesized ^{14}C -labeled product and autoradiography using a Molecular Dynamics PhosphorImager. The migration of radiolabeled total product, and product that was chemically (base) or enzymatically (EPGase) treated, was compared to the migration of authentic oligogalacturonides of various sizes. The results showed that 34-50% of the product synthesized in a 30 min reaction was digested into monopentagalacturonide when base and EPGase treated, suggesting that at least 34-50% of the product is homogalacturonan and that part of the homogalacturonan is methylesterified. These results confirm that the synthesized product consists largely of α -1,4-linked galacturonides.

Lee Pratt, Research Professor, and Bernard Hauser, Graduate Student, Department of Botany.

As we have been using the phosphorimager for only a few months, our work is far from complete. In fact, it is only beginning. Nonetheless, we have established that the probes we are using are highly specific for the corresponding mRNA, and we can obtain precise, quantitative data over more than four orders of magnitude from a single imaging of a Northern blot. We can also obtain absolute mRNA levels by reference to standard curves (placed on each blot) that include known, absolute amounts of mRNA for each phytochrome and for actin as an internal standard. It is evident that without the phosphorimager, it would be impractical, if not impossible, to obtain the same data with the same degree of precision. The phosphorimager will be used rather intensively over the next few months in order to complete an initial description of phytochrome mRNA distributions in tomato seedlings and mature plants.

Neil P.J. Price, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

Biosynthesis of lipid A precursors in *Rhizobium leguminosarum*. Comparison with glucosamine metabolism in *Escherichia coli*. Bacterial lipopolysaccharide (LPS) is the major antigen of the outer membrane of Gram negative bacteria and, in *Rhizobium*, it is a determinant of the nitrogen-fixing symbiosis with legumes. LPS is anchored through attachment to lipid A, the principal lipid component of the outer membrane. Several important structural differences exist between the lipid A from *E. coli* and that from *Rhizobium leguminosarum*, suggesting that diverse biosynthetic pathways must exist. In *Rhizobium*, UDP-*N*-acetylglucosamine is a key intermediate in the biosynthesis of lipid A, peptidoglycan, and, after flavanoid induction, Nod factors. We have isolated and utilized [³²P]-labeled intermediates from the *E. coli* lipid A biosynthetic pathway as substrates for cell-free preparations of *Rhizobium leguminosarum* bv. *phaseoli* and *viceae*. Enzyme activities were monitored and compared to cell-free preparations of *E. coli*. The early pathway is conserved in both *R. leguminosarum* biovars and *E. coli*, and the rhizobia are capable of supporting all six enzymatic steps necessary to synthesize 6'-(Kdo)₂-tetraacyldisaccharide-1,4'-bis-phosphate [(Kdo)₂IVa] from GlcNAc-UDP. These steps include (i) UDP-GlcNAc 3-*O*-acyltransferase; (ii) UDP-3-*O*-(R-3-hydroxymyristoyl)-GlcNAc de-*N*-acetylase and (iii) *N*-acyltransferase; (iv) lipid A disaccharide synthase; (v) tetraacyldisaccharide-1-phosphate ATP-dependent 4'-kinase; and (vi) 6'-Kdo transferase. Our data suggests that divergence in lipid A biosynthesis leading to rhizobial lipid A must occur at a later stage in the pathway, and presumably not until after attachment of the Kdo residues.

A novel phosphatase from *Rhizobium leguminosarum* involved in the biosynthesis of bacterial endotoxin with reduced toxicity.

Bradley Reuhs, Graduate Student, Department of Botany.

The cell-associated and extracellular polysaccharides of rhizobia are known to be important in the symbiotic infection of the leguminous host plants by the nitrogen-fixing bacteria. We have been studying a novel capsular polysaccharide (CPS) that is

essential for the successful infection of alfalfa by *Rhizobium meliloti* AK631. Although the CPS is produced by infective as well as non-infective strains, only those strains that carry a specific gene (*sxbX*), which is responsible for the modification of the polysaccharide, are able to infect the host plant. We have recently found that the activity of the *sxbX* gene is regulated, in a host-specific manner, by the addition of alfalfa root extract to the growth media of *R. meliloti* AK631. The modified form of acidic polysaccharide, which is important for infection, is readily visualized on PAGE gels by staining with a cationic dye (alcian blue). By the careful addition of various amounts of the alfalfa root extract to the culture medium of *R. meliloti* AK631, followed by cell disruption, PAGE analysis, alcian blue staining of the gels, and quantification of the degree of staining by analysis of the gels on the Molecular Dynamics Densitometer, we were able to establish the induction kinetics associated with the *sxbX* gene. The validity of these studies has since been verified by chemical means; however, the PAGE/densitometry protocol remains the most important tool available for the further analysis of the *sxbX* gene activity.

Myron Williams, Postdoctoral Research Associate, Complex Carbohydrate Research Center.

About 10^4 λ phage plaques were screened by probing with ^{125}I -labelled endopolygalacturonase proteins. Not positive plaques were found.