ISOLATION, PURIFICATION, AND CULTURE OF ANther CALLUS PROTOPLASTS FROM GOSSYPIUM HIRSUTUM

by

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A Thesis Submitted to the Faculty of the COMMITTEE ON GENETICS In Partial Fulfillment of the Requirements For the Degree of MASTER OF SCIENCE In the Graduate College UNIVERSITY OF ARIZONA

1980
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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

[Signature] 17 June 1980
F. R. H. Katterman
Date
Professor of Plant Sciences
ACKNOWLEDGMENTS

Affectionately to my family and friends, who helped and encouraged me in this process, my thanks. Particularly to my wife Lisa, whose support, patience, and love has allowed this fulfillment of a dream. I, as well, pledge my understanding, fortitude, and praise towards her adventure.
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ABSTRACT

With the capacity for selective haploid cell proliferation in *Gossypium hirsutum* anther callus culture (Barrow, Katterman, and Williams, 1978) and evidence for cytodifferentiation in cotton anther callus (Thomas, Katterman, and Williams, 1980), protoplast techniques have now been established for the efficient isolation and purification of large numbers of viable cotton anther callus protoplasts. Parameters of this study include: osmoticum, pecto-cellulase sources and concentrations, pH, callus growth conditions, and protoplast gradient separation.

Of five-day-old anther callus cultures subjected to digestion, 96% of all callus cells were converted to protoplasts using Miles TV Developmental Cellulase and Miles Experimental Pectinase. After gradient separation using 0.6M sucrose with 15mM arginine·HCl, 30% of these protoplasts were recovered. Further investigation into the culture of cotton protoplasts has led to a culture medium where cell wall deposition and cellular proliferation permit 56% of all observed cells to be viable after three weeks in culture.
INTRODUCTION

Agriculture has had a great influence on the economic and political development of the United States. Cotton, a major crop of the southern states and Arizona, maintains a rich tradition in this regard. Intimately linked in the past with opulence and as a major factor in the War Between the States, this crop currently plays an important role in the multi-billion dollar apparel industry.

Recently, the development of anti-bacterial cotton fiber has permitted the production of towels and sheets used in hospitals and other institutions to restrict infectious transmission (Smith, 1976). Other products, such as cotton-seed oil, are used in margarine and popcorn oil. With a high demand for energy, byproducts of cotton may, for example, be employed in cellulose conversion to alcohol. Consequently, the future contribution of cotton towards an improved quality of life will depend upon unique and continuing investigations into such areas.

The genomic constitution of the species of cotton that is grown to produce seed and fiber, (Gossypium hirsutum and G. barbadense) have been described as allo-tetraploids (Saunders, 1961). It is suggested by Hutchinson, Silow, and Stephans (1947) and Phillips (1963) that these species are a
result of a hybridization of the wild diploid cottons, *G. herbacium* and *G. raimondi*. Genetic drift and demographic separation then contributed to speciation.

Regardless of the evolutionary background, cultivated varieties of cotton continually meet with environmental and economic challenges. For example, chilling injury, the increasing cost of irrigation, pink bollworm, cotton rust, and other bacterial and fungal molestations plague cotton producers and result in restricted production. As traditional breeding techniques require time, and have been only partly successful in overcoming these problems, alternative procedures must be sought. The expansion of cultivated plant genetic pools using alternative breeding techniques has been suggested (Smith, 1971) followed by standard selection for adaptable plants.

To establish new variability within the genetic constraints of economically valuable cotton varieties one gene source would be from wild diploids. Unfortunately, Brown (1951) found high degrees of sexual incompatibility in crosses between many wild cotton species.

The utilization of tissue culture selection and protoplast fusion techniques, in support of conventional breeding methods, may provide solutions to the above problems. Tissue culture selection has provided insight into certain aspects of plant growth such as; disease resistance
(Helgeson, Haberlach, and Upper, 1976; Carlson, 1973), chilling effects (Breidenbach and Waring, 1977), salt tolerance (Dix and Street, 1975), and herbicide tolerance (Oswald, Smith, and Phillips, 1977). Current reports state that in vitro selection can increase the concentration of amino acids within certain plants. Such studies may have a significant bearing on the nutritive status of future human populations. Work in this area is presently being pioneered by Widholm (1976; 1977). Hence, selective techniques in tissue culture may allow vigor and resistance to become assimilated into the commercial cotton varieties.

Concurrent with selection techniques, methods in protoplast isolation, fusion, and culture are of particular interest in future breeding efforts. Inter- and intragenic somatic hybridization has been described (Kao et al., 1974; Potrykus, 1971). As demonstrated by Schieder (1978), sexual incompatibility normally encountered between species can be circumvented by somatic cell fusion. Gene transfer in vitro need not involve whole cell-cell fusion. Any subcellular or cellular component with intact genetic information may be introduced into plant protoplasts. Chloroplast transplantation (Bonnet and Banks, 1976) and bacterial gene uptake (Doy, Gressoff, and Rolfe, 1973) have demonstrated this possibility. Therefore, with additional genetic information, the arising cell will contain DNA both of the protoplast and the donor DNA.
The capacity of "totipotent" plant cells to give rise to whole plants in vitro represents the greatest challenge in experiments of this nature. Although callus growth conditions for a variety of Gossypium species have been described (Williams, 1978; Price, Smith, and Grumbles, 1977), plantlet regeneration from callus continues to be elusive.

Because of cotton's economic importance and the recent success of somatic embryogenesis in G. klotzschianum (Price and Smith, 1979), this work was undertaken to clarify and define previous methods for cotton protoplast isolation (Bhojwani, Power, and Cocking, 1977), to increase yield of viable protoplasts, and to devise a more effective purification technique. This information, combined with work now in progress to establish maximum culture conditions for protoplasts, will permit subsequent studies in somatic fusion, transformation, and regeneration of the Gossypium group.
LITERATURE REVIEW

Structure and Digestion of Cell Wall

Protoplastic space, in callus or plant, is normally accommodated within the complex matrix termed the "cell wall". This fiberous array of cellulose, hemicellulose, pectin, and lignin allows the plant cell the capacity to tolerate large osmotic changes while permitting molecular exchange and growth. Consequently, removal of the cell wall demands consideration of wall constructs and enzymes used to degrade them.

Information regarding the compositional arrangement of the cell wall is inconsistent. A model of wall structure based on the preponderance of certain oligopolymeric forms (as determined by selective enzyme digestion) has been presented by Albertscheim (1975). Multiple cellulose strands arranged in a staggered array all being parallel to one another were suggested. Upon these strands, colinear xyloglucan is situated. The xyloglucan is linked with vertically inclined arabinogalactan which is associated with rhamnogalacturonan at the opposite end. From the rhamnogalacturonan, (arranged parallel to the cellulose fibers), the complex is reversed. Another vertical arabinogalactan links the rhamnogalacturonan underneath a second cellulose fiber.
Although with merit, the above model does not comply with other information on the overall cell wall composition and may merely reflect a characteristic of the in vitro grown cells used to deduce the model. Monro, Penny, and Bailey (1976), after chemical fractionation of cell walls, suggest a non-covalent interaction of much of the pectin, hemicellulose, and glycoprotein residues. These results also imply a more intimate covalent interaction between glycoproteins and cellulose microfibers. In compromise, Lamport (1978) suggests a soluble glycoprotein of two types. One glycoprotein is thought to covalently link arabinogalactan while the other is passively associated with the polymer.

Information as to the cell wall of cotton is limited to fiber compositional analysis. Meinert and Delmer (1977) have examined \textit{G. hirsutum} L. fiber at various times during development. Fiber compositional change was observed with age. At ten days post-anthesis, cellulose, uronic acid, and protein made up 25\% of the cell wall. From days 5 to 18 post-anthesis, cellulose content increased 7 fold while uronic acid and protein content decreased 4 and 10 fold respectively. Recently, Huwyler, Franz, and Meir (1978) reported the presence of beta-1,4 and beta-1,3 linkages in fiber of \textit{G. arboreum}. These linkages are thought to be in close proximity to each other without ionic interaction. These findings suggest a complex fibular matrix of varied composition depending on the age of cotton cell wall.
Mahadevan and Tatum (1965) found the composition of cell wall polymer of mutant strains of *Neurospora* correlated with the type of culture spreading and other structural aspects of growth. Therefore, morphology of a growing walled cell may be a function of the stage of development and the cell wall composition. Callus cells appear different from their original plant cells too. However, while the overall cellular morphology of callus and whole plants is different, the substituent groups making up each cell wall may not be.

For digestion of the plant cell wall, enzymes secreted by some fungi are utilized. Of these, *Myrothecium verrucaria* cellulase was used in the first enzymatic mediated isolation of protoplasts (Cocking, 1960). At present cellulase of *Tricoderma verdi* is frequently used.

Jones (1976), in a review of fungal cellulases, suggests cellulase is a many component inducible enzyme making purification difficult. Cellulase of *T. verdi* demonstrates; cleavage of cellulose to linear anhydrous polymer (G1 activity), endoglucanase activity (CX), cellobiase, and other activities such as xylanase. Copper and manganese have been implicated as inhibitors of these activities while phosphate, magnesium, cobalt, and calcium each may stimulate activity. "Onozuka" cellulase can hydrolyze xylan, mannan, galactomannan, and polygalacturonic acid residues with a pH optima of 4-5 (Yakult Biochemical, 1978a). An associated
enzyme, "Macerozyme R-10" (a pectinase) demonstrates; poly-
galacturonic acid, xylan, galactan, and protein hydrolyzing
ability. These activities demonstrate a collective pH optima
of between 4.5 and 6.5 (Yakult Biochemical, 1978b).

In an earlier study of *M. verrucaria* cellulase
(Halliwell, 1961) a crude cellulase preparation demonstrated
a differential activity dependent upon the degree of pre-
swelling of substrate and substrate type. A pH optima of 3-6
and 20-54°C temperature optima were also found. With the
inclusion of cellobiose, a 10% inhibition of cellulase act-
vity was observed. Schilde-Rentschler (1977) found cell
wall deposition on regenerating tobacco protoplasts to be
dramatically reduced in the presence of 0.05M cellobiose.
This may suggest plant cellulase enzymes may be more sensi-
tive to cellobiose inhibition than cellulases of fungal
origin.

Other factors influencing digestion of plant cell
walls have been enumerated on by other workers. Richards
(1976) suggests that the configuration and packing of cell-
ulose beta-1,4 linkages may create a polymer where more than
half of the ordered regions are resistant to penetration and
attack by small molecules. While loosely packed pectin res-
adues may digest easily, cellulose degradation would likely
dictate total digestion kinetics. Denium (1976) demonstrated
digestion to be temperature and substrate age dependent. As
The temperature of plant growth was increased up to 25°C day and 20°C night, a decline in digestibility was observed. Plant growth temperatures above these values did not alter digestibility. Digestion was more complete in upper younger leaves than lower leaves. These effects were attributed to increased lignification with increased temperature and low carbohydrate concentration in the younger as compared to older leaves.

The Plasmalemma and Cell Fusion

Following protoplast isolation, the remaining cell membrane alone is responsible for the viability of the plant cell. Membrane structure is generally based upon the fluid mosaic model of Singer and Nicolson (1972). Briefly, this model suggests the membrane to be a lipid bilayer where individual lipid moieties are free to vibrate, rotate, and translocate. Imposed upon and within the membrane are proteins of varying polarity. The fluid nature of the membrane also allows the proteins free movement.

The protoplast membrane has been shown capable of pinocytotic uptake of latex spheres (Mayo and Cocking, 1969). They suggest the absorption of these uncharged, inert spheres is mediated by an attachment stage followed by uptake only in "active" regions of the plasmalemma. Membrane alterations due to herbicide application have also been observed in protoplasts (Boulware and Camper, 1972).
Agents which induce agglutination have been used to study protoplast membranes. Information of Glimelius, Wallin, and Eriksson (1974) and Chin and Scott (1974b) suggest concanavaline A, wheat germ agglutinin, and phytohemagglutin all aggregate protoplasts and initiate protein, RNA, and DNA synthesis in a manner akin to animal cells.

Agglutination has been demonstrated by using; antiserum, pH increase, polyethylene glycol 6000, and poly-L-lysine (Burgess and Fleming, 1974). Protoplast fusion was also noted in this work when polyethylene glycol (PEG) was used. Yamada et al. (1979) suggests fusion leads to a bilayer mixture and rearrangement of the membranes. Fluidity in this case seemed associated with the phospholipid entity of protoplast cell membranes. Where increased fusion was observed, a corresponding increase in fluidity and concommitant decrease in viscosity was noted. Higher temperatures demonstrated increased fluidity and also resulted in increased cell fusions.

Frye and Edidin (1970) suggested cell fusion was not related to metabolic turnover or cytoplasmic interaction. The phenomena appeared related to cell approximation to another cell followed by membrane mixing. Ahkong et al. (1975) suggested aggregation of membrane proteins (due to disorganization of the lipid fraction after treatment with lysolecithin or a treatment of elevated temperature) may
induce fusion. These authors suggest the fusion progression to begin with a perturbation of the bilayer which results in membrane fluidity and aggregates intermembrane proteins. This process of interaction involving intermixing of closely apposed membranes with local areas denuded of protein may result in cell fusion.

Charged particles such as proteins may indeed restrict protoplast fusion. Grout and Coutts (1974) subjected protoplasts to electrophoretic separation in the presence and absence of agglutinating agents such as; concanavalin A, poly-L-ornithine, and protamine sulfate. A neutralization of surface charges caused aggregation, yet membrane intermixing was not mentioned. Therefore, with the loss of membrane charge produced by proteins, aggregation is possible. Nevertheless, a further explanation for the mechanism of fusion is needed.

It is apparent from the above discussion that the protoplast cell membrane appears as a complex dispersion of lipid bilayer interspersed with protein. The protein elements of the membrane surface allow aggregation of membranes of different cells using specific and non-specific agglutinins. Aggregation of protein substituents of the plasmalemma may allow specific regions of the membrane to become juxtaposed and permit membrane mixing. Regardless of specificity, fluidity does stimulate fusion and is related to the lipid composition of the fused cells.
**Cell Wall Resynthesis**

After protoplast isolation, given suitable conditions, cell wall regeneration and division may occur. Resultant cells may divide forming cell masses leading to organogenesis. Most often, callus formation occurs.

Resynthesis of cell wall material upon protoplast membranes generally takes on an aberrant character. Horine and Ruesink (1972) demonstrated an electron dense and atypical amorphous concentration of cell wall surrounding protoplasts after three days in culture. This material was verified as cell wall by digestion with "cellulase" and "pectinase". Resynthesis of such a cell wall was not dependent upon protein or RNA synthesis as cyclohexamide, puromycin, and actinomycin D had no effect. Pojnar, Willison, and Cocking (1967) also found thickening of protoplast surfaces three or four days after isolation.

Electron microscopy has been applied to the study of protoplast cell wall formation. Grout (1975) found a 16 hour lag period prior to initial fiber deposition. This material was not associated with any structural particle or differentiated region of the membrane. Regenerated microfibers were observed to have tapering ends or were rising through the membrane surface with no short fragments present. Evidences collected in these experiments suggest that cell wall formation is similar to crystallization or precipitation of
cell wall precursors, these being promoted by ionic conditions of the membrane. Robenek and Peveling (1977), using membrane fracture techniques, have found what appear to be hexagonal arrays of particles arranged in two rows of fourteen on the plasmalemma. Their orientation on the cell surface suggests a specialized region used for outward passage of cellulose precursors. These arrays are envisioned as a collective enzyme complex for fiber biosynthesis and orientation. Concurrent with such development, mitochondrial and polysome numbers increased. Once the protoplast has formed a cell wall the sugar composition of the extra cellular wall material is similar to that of the original cell (Takeuchi and Komamine, 1978).

It is important to be cautious with regard to generalizations of cell wall and membrane conditions as different plants have individual requirements for protoplast isolation and culture. At best, the protoplast membrane can be considered the formost constraint in environmental separation. This degree of partitioning will be subject to a number of variables, each with their importance imparted by the type of plant.

**Protoplast Methodology**

Protoplast isolation and culture has been described for a number of plant groups. Table 1 provides a survey of some of the variables with respect to; cell origin, enzyme
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<th>Origin</th>
<th>Regeneration</th>
<th>Enzyme Mixture</th>
<th>Ref.#</th>
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| Atropa belladonna   | callus      | plants             | 0.5M mannitol
1.5% Cellulase R-10
0.75% Macerozyme R-10 | 12    |
| Asparagus officinalis| calode      | roots and shoots   | 0.9M mannitol
3% Onozuka Cellulase
0.75% Macerozyme | 2     |
| Avena sativa        | leaf        | cell clusters      | 0.7M mannitol or sorbitol
4% Meicelase
1% Macerozyme
1% KDS               | 10    |
| Brassica napus      | leaf        | plants             | 4.5% mannitol
4.5% sorbitol
0.5% Onozuka Cellulase
0.5% Rhozyme HP
0.5% Driselase
0.5% Hemicellulase   | 14    |
| Citrus sinesis      | callus      | embryos            | 0.14M sucrose
0.28M mannitol
0.28M sorbitol
1% Cellulase
1% Pectinase
0.3% KDS              | 26    |
| Cucumis sativas     | leaf        | cell clusters      | 11% mannitol
0.3% Cellulase
0.4% Pectinase
0.5% KDS               | 6     |
<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Regeneration</th>
<th>Enzyme Mixture</th>
<th>Ref. #</th>
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<tr>
<td>Dacus carota</td>
<td>callus</td>
<td>embryos</td>
<td>0.6M sorbitol, 1.5% Cellulase</td>
<td>11</td>
</tr>
<tr>
<td>Dacus carota</td>
<td>callus</td>
<td>callus</td>
<td>0.4M sorbitol, 5% Onozuka Cellulase, 1% Macerozyme, 0.05M citrate buffer</td>
<td>27</td>
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<tr>
<td>Datura innoxia</td>
<td>leaf</td>
<td>plants</td>
<td>0.5M mannitol, 3% Onozuka Cellulase, 1% Macerozyme</td>
<td>23</td>
</tr>
<tr>
<td>Glycine max</td>
<td>callus</td>
<td>cell clusters</td>
<td>0.55M sorbitol, 0.5% Driselase, 0.25% Pectinase</td>
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<tr>
<td>Gossypium hirsutum</td>
<td>callus</td>
<td>cell clusters</td>
<td>11% mannitol, 4% Meicelase, 0.25% Driselase, 0.4% Macerozyme</td>
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<td>Hordeum vulgare</td>
<td>leaf</td>
<td></td>
<td>0.6M sorbitol, 10mM CaCl₂, 2% Cellulysin, 0.5% Macerozyme, 1% Hemicellulase</td>
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<td>Hyoscyamus niger</td>
<td>leaf</td>
<td>callus</td>
<td>2.5% KCl and 1% MgSO₄, 2.5% Onozuka Cellulase, 0.02% Pectinol</td>
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<td>Species</td>
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<td>Regeneration</td>
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<td>Malus sp.</td>
<td>pericarp</td>
<td>single cells</td>
<td>0.6M sorbitol, 0.5% Cellulase, 0.5% Pectinase, 0.5% Rhozyme</td>
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<td>Medicago sativa</td>
<td>leaf</td>
<td>cell clusters</td>
<td>0.55M sorbitol, 0.5% Driselase, 0.25% Pectinase</td>
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**Symbols**

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<thead>
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<tr>
<td>KDS</td>
<td>potassium dextran sulfate</td>
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<tr>
<td>PVP</td>
<td>polyvinyl pyrrolidone</td>
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<tr>
<td>Ca-THO</td>
<td>calcium tetrahydrogen orthophosphate</td>
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mixture, and growth response. Rather than a description of each experiment, an outline of general procedures for protoplast usage will follow.

Generally plant material is grown aseptically or surface sterilized with household bleach. Antibiotics may be used in the isolation solutions where they may affect the cellular metabolism of the protoplasts. On occasion in vitro preculture of tissues may improve protoplast yield as well as test plant material for sterility (Donn, 1978). In vitro plasmolysis of tissue previous to protoplast isolation has been a common practice in the literature (Gosch and Reinert, 1978; Kanai and Edwards, 1973a; Shepard and Totten, 1977). Together, these procedures are instituted not only for sterilization purposes, but also to "prepare" the tissue for protoplast isolation and culture in a liquid media.

Uchimiya and Murashige (1974) outlined a sequence of experimental steps to determine the optimal conditions for protoplast isolation from tobacco callus. The variables examined were: osmotic concentration, osmotic form, cellulase and pectinase concentrations, time of digestion, growth phase of the callus, pH, agitation rate, and volume of enzyme to a given cell substrate unit.

In general, reports demonstrate the use of inert sugars such as mannitol and sorbitol stabilize the osmotic concentration. Mannitol has been found toxic to one species of tobacco (Nagy and Maliga, 1976) and may not be inert.
Temperature and activity of pecto-cellulase enzymes appear in agreement with information presented earlier. The pH optima of "Onozuka" cellulase seems coincident with the suggestion of separate cellulase iso-enzymes, each with a different pH optima. This point will be discussed later.

Age related cell wall compositional change, being mentioned before, may play a role in protoplast isolation. Using detached leaves of Avena sativa, Martin and Thimann (1972) demonstrated repressed proteolysis of tissues in the presence of "senescence retardants" whereas the addition of serine promoted proteolysis. Such lytic behavior has been shown inhibited by cyclohexamide, kinetin, dibasic amines, and basic amino acids in oat protoplast isolation (Kaur-Sawhney et al., 1976; 1977). At concentrations of 10-50mM, arginine·HCl and lysine promoted $^3$H-uridine incorporation into RNA and stimulated other metabolic processes.

Following isolation of protoplasts further purification must be performed in order to remove cell wall digesting enzymes, cell wall debris, and walled cells. Many workers have suggested the use of sucrose gradients for this purpose. In an elaborate display of sucrose gradient technology, Harms and Potrykus (1978) developed an iso-osmotic density gradient where protoplasts were dispersed within a discontinuous gradient. Following centrifugation, protoplast banding patterns were according to their buoyant densities.
regardless of application at the top or bottom of the gradient. Within this work, specific banding density as related to plant type, plant organ, and tissue is demonstrated. Positional rebanding within a second gradient resulted in a 86-98% recovery of protoplasts at the original density.

Other forms of gradients have been used with varying degrees of success. These gradient systems utilize; sucrose (Cocking et al., 1974), Lymphoprep\textsuperscript{R} (Larkin, 1976), and polyethylene glycol (PEG) with dextran (Kanai and Edwards, 1973b). Unfortunately Lymphoprep has been designed with the low osmotic requirements of mammalian cells in mind. PEG, on the other hand, promotes cell fusion. Therefore, sucrose remains the gradient material of choice.

Separation on 8um filters has also been described (Howland, Hart, and Yette, 1975).

For the determination of protoplast viability the ideal technique would allow this distinction while cells remain undisturbed. As their divisions are not synchronous and difficult to quantitate, most methods rely on differential staining techniques. Larkin (1976) found that the stain fluorescein diacetate will be absorbed by cells without a cell wall. Kanai and Edwards (1973b) have used Evan's Blue exclusion dye to test for membrane integrity. Ferrari, Palmer, and Widholm (1975) described the use of a protein
stain (phenolsafranine) in conjunction with sodium dodecyl-sulfate (SDS) to test for protoplast viability. As SDS solublized the cell membrane, the stain reacted with the protein contained within the protoplast. If cell walls were present, the stained protein remained within them whereas without cell wall, protein was stained and distributed in the surrounding media. Use of phenylsafranine to determine non-viable cells can be accomplished in the absence of SDS as membranes of such cells would (perhaps) be broken. Other methods of viability determination may use different dyes or oxygen uptake (Taiz and Jones, 1971).

Precursor uptake and incorporation has been thought of as a valid criteria for protoplast viability. Due to the artificial nature of protoplast culture and inherent problems in the use of $^3$H-thymidine incorporation, results of such incorporation into DNA of protoplasts has been suspect. Howland and Yette (1975) included 5-fluorodeoxyuridine during $^3$H-thymidine exposure. This technique allowed for a linear incorporation of $^3$H-thymidine into protoplast DNA but also represents an altered metabolic process. Using cells pre-labeled in callus culture with $^3$H-thymidine, Radojevic and Kovoor (1978) used $^{32}$P-orthophosphate to follow DNA synthesis in protoplasts. The dual labeling technique allows a sensitive quantitation of pre-existing DNA as compared to newly synthesized DNA without cytotoxic effects.
In long term experiments, culture media of approximate composition is essential for the maintenance and growth of protoplasts. Using standard techniques, Uchimiya and Murashige (1976) have evaluated parameters such as carbon source and concentration, osmotic concentration, and phytohormone concentration in the culture of tobacco callus protoplasts. Use of 0.6mg/l naphthaleneacetic acid and 0-0.1 mg/l 6-benzyladenine in the nutrient salts of Murashige and Skoog, found in Gamborg et al. (1976), was optimal for tobacco protoplast growth. Potrykus, Harms, and Lorz (1979) describe a multiple-droplet-array technique for the large scale evaluation of media parameters. This technique is rapid and allows accurate determinations of optimal media.

Unlike cells of animal and yeast origin, plant cells require a rather high plating density of between 1 X 10^4 to 1 X 10^5 cells/ml unless various additives or conditions can be manipulated to lower this value. An example of such manipulation can be found in lowered osmotic conditions (Shepard and Totten, 1975). Raveh and Galun (1975) have irradiated protoplasts as "feeder" cells which permit a plating density of 1 X 10^2 cells/6cm petri dish of non-irradiated protoplasts. This technique relies upon metabolic processes of DNA replication impaired protoplasts to buffer and introduce unknown growth factors into the culture medium, thus allowing for a lowered plating density.
Therefore, protoplast isolation, purification, and culture parameters are as varied as the plant sources used. For the successful utilization of protoplasts these techniques must be maximized in order to provide an accurate dimension to a particular investigation.

**Application of Protoplasts**

The study of numerous physiological and biochemical processes have been facilitated using protoplasts. Kanai and Edwards (1973a) have presented a technique for the separation of bundle sheath cells from mesophyll protoplasts. After tissue digestion, an effective separation of these cell types is achieved by collecting bundle sheath cells upon an 80um filter which permits the mesophyll protoplasts to pass through. Following assays of enzymes usually associated with photosynthesis, the authors were able to assign specific enzymes to either or both cell fractions. The technique presented here was shown applicable to the study of other C₄ plants. Edwards et al. (1978) have demonstrated various photosynthetic processes with intact chloroplasts of *Triticum aestivum* using isolated protoplasts. This particular technique circumvents problems of photosynthetic assessment following traditional mechanical disruption for chloroplast isolation.
Utilization of protoplasts in virus studies has allowed a more critical observation of the resultant aberrations following infection. Examples of such studies are tobacco protoplasts infected with; cow-pea chlorotic mottle virus (Motoyoshi et al., 1973), cucumber mosaic virus (Otsuki and Takebe, 1973), and tobacco mosaic virus (Takebe and Otsuki, 1969). Tobacco mosaic virus has also been studied in tomato protoplasts (Cocking and Pojnar, 1969). Demonstration of viral RNA mediated infection has been documented using a protoplast system (Aoki and Takebe, 1969). Using a protoplast infection assay, actual burst sizes can also be measured (Takebe and Otsuki, 1969).

While the process of DNA repair in eucaryotic cells remains an ongoing topic of investigation in animal cells (Yoshida, Ungers, and Rosenberg, 1977; Castellot et al., 1979), investigation into DNA repair in plant cells has been delayed due to the cell wall. Studies in dark repair (Howland, 1975), X-ray effects (Galun and Raveh, 1975), and UV induced damage (Ohyama, Pelcher, and Gamborg, 1974), have recently used protoplasts. It has been shown that at least 50% of the DNA in carrot protoplasts was repaired 5 minutes after 20 krads of gamma irradiation treatment.
Transgenesis, or the transfer of vitality using chloroplasts fused with plant protoplasts has been suggested (Bonnet and Banks, 1976). A green algae, Codium fragile, chloroplast preparation was incorporated into carrot protoplasts with the aid of PEG. Intracellular localization of these chloroplasts was confirmed with the electron microscope. If cytoplasmic associated male sterility pertains to the chloroplast genome, as the above authors suggest, then chloroplast transplantation could be applied to the construction of new male sterile lines in any plant material.

Enucleation and nuclear transplantation into protoplasts also offers a potential for genetic modification in higher plants (Wallin, Glimelius, and Eriksson, 1978; Potrykus and Hoffmann, 1973). Such procedures may offer alternative methods for DNA extraction as well.

Somatic hybridization is a current technique using fused cells of different origins for the study of biochemical and genetic interactions. The importance of the technique in mammalian genetics is unsurpassed (Marx, 1973), but has not achieved similar importance in plant studies as yet.

The earliest reports of protoplast fusion found the event may be spontaneous (Cocking, 1972), or induced with; NaNO₃ (Power, Cummins, and Cocking, 1970), dextran sulfate (Kameya, 1975), high pH and high calcium (Binding, 1974), and tapping (Ito and Maeda, 1973). Presently, PEG has been routinely used for protoplast fusion.
The mechanism of action of PEG induced fusion, as presented by Kao and Wetter (1976), may be supported by the previous discussion on fusion events. These workers suggest PEG \((\text{HOCH}_2\text{-O-CH}_2)_n\text{-CH}_2\text{OH}\) has a slight negative polarity allowing hydrogen bonding to water, proteins, etc. Through the attraction of PEG to the membrane proteins a molecular bridge may form between two adjacent protoplasts. Washing with calcium ions allows these ions to bind PEG quickly and the resultant complex then elutes away from the cells. With the loss of PEG, membrane proteins are apposed to one another creating an electronic distribution on the protoplast cell membrane. This charge is the direct result of the loss of PEG and such perturbation has been suggested earlier to induce fusion. The cell membranes now mix and result in a heterokaryocyte (or somatic hybrid).

A procedure often cited as a method of fusion was presented by Kao et al. (1974). These results indicate that in soybean X soybean fusions, efficiency ranged from 22-53% fusion events. Factors such as PEG concentration, length of "cellulase" treatment, and commercial source of "cellulases" used in protoplast isolation affected the fusion frequency. These authors suggest the presence of a protease in the "cellulase" preparations may assist fusion by the removal of excess charged proteins within the plasmalemma.
Large scale identification of fusion products is often difficult and represents one explanation as to the problems of agricultural promotion using somatic fusion. Potrykus (1971) demonstrated intra- and interspecific fusion of protoplasts isolated from *Torenia bailloni* and *T. fournier*. Since this experiment involved petal derived protoplasts, petal color could be used to quantitate fusion events. Recently, Gosch and Reinert (1978) used red pigment of callus protoplasts of *Dacus carrota*, green mesophyll cells from *Petunia hybrida*, and the dense cytoplasm of *Atropa belladonna* as indices of fusion. Using this system, these authors achieved 33 shoot cultures from the *D. carrota X P. hybrida* fusions which did not appear similar to either parent.

Successful fusion selection on the basis of rapid hybrid growth has been suggested (Melchers, 1976). Selection of hybrids using this criteria has been applied to the study of sexual incompatibility where rapid growth selected fusion products in the genus *Datura* resulted in hybrid plantlets (Schieder, 1978). Carlson, Smith, and Dearing (1972) used accelerated growth of hybrids in the selection of fusion products of *Nicotiana glauca X N. langsdorffii*. Resultant peroxidases, flowers, and fertile seed appeared identical to the naturally obtained hybrid. Kung et al. (1975) has demonstrated Fraction I protein may also be used as a
criteria for selection of fusion products. In the *N. glauca* X *N. langsdorffii* cross described above, the small polypeptide fractions of nuclear origin were of both parental types. However, the large polypeptide subunits of the chloroplast were of *N. glauca* only. With absorption of *N. tabacum* chloroplast preparations into *N. suaveolens* protoplasts, both nuclear and chloroplast coded peptides of each parent were revealed. This final result was suggested as a result of "contamination" in the chloroplast preparation.

Complementation selection may be an effective criterion for fusion product selection and proliferation. Giles (1974) used actinomycin D resistance as a criteria for somatic hybridization. Glimelius, Wallin, and Eriksson (1978) demonstrated the promotion of PEG mediated fusion with concanavalin A using auxotrophic *N. tabacum* protoplasts. In an elegant display of hybridization and selection of hybrids employing a combined visual and kanamycin resistant line, Maliga et al. (1977) hybridized *N. sylvestris* with *N. knightiana*. Each parental cell type had lost regeneration ability, yet hybrid plants were obtained.
MATERIALS AND METHODS

Cellulase Enzyme Assay

Sample preparation. The procedure of Worthington Biochemicals (1978) with modification was performed with enzyme activity being expressed as glucose equivalent reducing sugars (Caraway, 1970). Enzymes assayed were; Cellulysin (Cal Biochemical Lot# 400297), TV Developmental Cellulase (Miles Biochemicals Lot# TSL76.353), and Cellulase Concentrate (Miles Biochemicals Lot# 48119). Sigma Cell Type 100 powder (Sigma Biochemical Co.) was used as the cellulose substrate.

Prior to assay, powder was equilibrated in 0.1M acetic acid-acetate buffer at various pH values in a stock solution of 5% w/v concentration. After 16 hours incubation at 4°C the substrate was agitated, 4ml withdrawn, and placed in a 50ml flask. To this, 1ml of cellulase enzyme prepared in buffer as 1mg/ml was added to the flask. The reaction was allowed to proceed for 2 hours at 29°C rotating on an orbital shaker at 100/rpm. Samples were then decanted and centrifuged at 530xg for 10 minutes. Supernatant was withdrawn, mixed with an equal volume of 0.3N Ba(OH)₂·8H₂O and allowed to stand on ice for 10 minutes.
After precipitation the tube was mixed and an equal volume of 5% w/v ZnSO₄·7H₂O was added. Following 10 minutes on ice the tube was centrifuged and triplicate aliquots of supernatant were tested for reducing sugars by the Smogyi-Nelson technique (Caraway, 1970).

Reagents for the Smogyi-Nelson Test. The copper sulfate solution was prepared by dissolving 29g NaHPO₄ and 40g KNaC₄H₄O₆·4H₂O in 700ml of water. Following the addition of 100ml 1N NaOH, 80ml 10% CuSO₄·5H₂O are slowly added with stirring. Once dissolved, 180g NaSO₄ are added and the solution diluted to one liter. After standing for 2 days at room temperature the solution was filtered through glass wool and stored at room temperature.

A solution, hence known as arsenomolybdate solution, was prepared by dissolving 42ml of H₂SO₄ and 50g of (NH₄)₆Mo₇O₂₄·4H₂O in 900ml of water. In a separate container 6g of Na₂HASO₄·7H₂O are dissolved in 50ml of water and added to the first solution. After 48 hours of incubation at 37°C the solution was stored in a brown bottle at room temperature.

Glucose standards were originally prepared as one gram glucose and 0.2g of benzoic acid dissolved in 50ml of water. This solution was then further diluted to provide standards between 1000-10μg/ml. Standards were stored at 0-5°C.
**Procedure of Reducing Sugars Test.** From standards, supernatant, and water blanks 1ml was withdrawn in triplicate and placed in 150 X 25mm tubes. Following the addition of 1ml of copper sulfate solution all tubes were covered with foil and placed in a boiling waterbath for 10 minutes. After incubation, tubes were removed and cooled in a low temperature water bath. Once cooled, 1ml of arsenomolybdate solution was added, the tubes mixed, and allowed to stand for at least 2 minutes. Water was then added to each tube (usually 1ml each) and the relative absorbances recorded at 490nm in a "Spectronic 20" (Bausch and Lomb). Final glucose equivalents were calculated as:

\[
\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{standard}}} \times 4(\text{dilution}) = \text{ug/ml glucose reducing equivalent units}
\]

**Osmotic Determination**

According to the method of Ross (1974), a salt and icewater mixture was prepared. Samples were applied in 3ml aliquots in 150 X 25mm tubes. A thermometer was introduced into the sample and the tube placed in the ice mixture. At intervals of 10 seconds temperature was recorded. Osmolality was then calculated on the basis of sample freezing point depression as compared to water using the calculation stated by the above author.

\[
\frac{\text{Freeze Pt. sample} - \text{Freeze Pt. water}}{1.84} = \text{Osmolality in Cs/kg water}
\]
Callus Production and Maintenance

Gossypium hirsutum var. Stoneville 2-B anther callus was initiated two years ago on Linsmaier and Skoog media with 5mg/l napthalene acetic acid (NAA) and 1mg/l 6-benzyladenine (6BA). After 3-4 weeks callus was transferred and subsequently maintained for 1½ years on Murashige and Skoog salts with 1.0g/l inositol, 1mg/l thiamine, 3% glucose, 1% agar, 2mg/l NAA, and 0.1mg/l 6BA (maintenance medium).

With friable and dark growing callus in mind, 2mm of anther callus was subcultured and placed in the dark at 20°C. Following four monthly transfers, growth was observed. After an additional transfer and growth in the dark at 20°C, the resultant callus (white and dry) had grown about three times the original inoculum. A 2mm portion of this tissue was then transferred and grown at 29°C with 18 hours light at 3-6uEm^-2sec^-2. Tissue proliferated rapidly and the resultant callus was maintained on maintenance medium as before.

Callus Growth and Differentiation

Original anther callus (SV) has been shown capable of cytodifferentiation when cultured for 45 days on similar media to that of maintenance medium, with the deletion of phytohormones as stated and the use of 5mg/l 6-(4-hydroxy-3-methyltrans-2-butenylamino)purine (zeatin). After incubation on the zeatin rich medium (preinduction medium), 9% of the
cultures placed on maintenance media with 1.5mg/l NAA and 0.1mg/l 6BA developed roots after 45 additional days in culture. Likewise, cotton shoot tips and stem explants have been shown capable of plantlet development on similar media (Thomas, Katterman, and Williams, 1980). In order to compare the original callus (SV) with that acquired after dark and temperature selection (SV-10), the above experiments were repeated.

**Protoplast Isolation**

Generally 2% w/v Miles TV Cellulase and 0.5% w/v Miles Experimental Pectinase were dissolved in freshly prepared 0.71M mannitol containing 15mM arginine•HCl and a pH adjustment to 5.6 with either 1N HCl or KOH. Following centrifugation at 200xg for 10 minutes, 2.5ml of supernatant were added to 0.5g fresh weight of peripheral callus tissue (10 days old), in a Falcon No. 1006 tight lid petri dish. Digestion was performed in the dark at 29°C for 2 hours. In the case of protoplasts isolated for culture, the enzyme solution was centrifuged and filter sterilized by passage through a Millex™ 0.45um filter (Milipore Corp.).

The mixture was then retrieved after 2 hours of static incubation and the mixture expressed with a pipette. After expressing the solution into a 12ml conical centrifuge tube, the dish was washed with 0.71M mannitol containing 15mM arginine•HCl of pH 8.0.
After centrifugation at 100xg for 10 minutes the supernatant was removed below the meniscus. Protoplasts were resuspended in the previous wash solution with an appropriate amount of Evan's Blue stain to result in a final dye concentration of 1.25%.

Protoplasts were counted in a Hauser "Hi Lite" hemocytometer at 100X magnification with dye excluding cells counted as viable (Kanai and Edwards, 1973b). Total cells were estimated by incubating 0.5g fresh weight of callus tissue in 5ml of 5% w/v chromium trioxide for 3 hours on a gyrotyor shaker at 100/rpm at 20°C. Resultant cells were counted with a hemocytometer (Uchimiya and Murashige, 1974).

**Protoplast Purification**

An iso-osmotic density gradient was used to determine a purification scheme (Harms and Potrykus, 1978). Protoplasts were isolated as described except callus cells were harvested at day 5 after transfer. After incubation in enzymes, protoplasts were washed with 10ml of KCM-1 buffer (0.35M KCl, 0.245M CaCl$_2$·2H$_2$O, and 0.254M MgCl$_2$·6H$_2$O) prepared by mixing each solution as 1:1:1 with the pH adjusted to 6.0 with 1N HCl or KOH. Protoplasts were centrifuged at 100xg for 5 minutes, the pellet washed with 5ml of buffer, and the protoplast pellet adjusted with buffer to a final concentration of 1 X 10$^6$ protoplasts/ml.
Gradients were prepared at room temperature by mixing 0.56M sucrose to KCM-1 buffer in the proportions: 0:7, 1:6, 2:5, 3:4, 4:3, 5:2, 6:1, and 7:0. The discontinuous gradient was constructed from top to bottom by passing 1ml of the light fraction (containing protoplasts) through a heat extended Pasteur pipette into the bottom of a 100 X 16mm tube. Successive heavier fractions were then added in 1ml amounts, the pipette gently removed, and gradients centrifuged at 100xg for 10-15 minutes. The resulting bands were pipetted off and counted. A modified buffer, KCM-2 (0.409M KCl, 0.28M MgCl$_2$·6H$_2$O, and 0.277M CaCl$_2$·2H$_2$O), was mixed 1:1:1 and evaluated also.

Additional gradients examined were; Lymphoprep$^R$ (Nyegaard A/S, Norway), 0.56M sucrose with 0.14M mannitol, 0.6M sucrose, and a 5:1 mixture of 0.7M MgSO$_4$·7H$_2$O to KCM-2 buffer.

**Culture of Protoplasts**

For culturing experiments, callus digested protoplasts were recovered from the dark and mixed with 2 volumes of 0.6M sucrose containing 15mM arginine·HCl at pH 5.6. Two volumes of this solution were layered beneath one volume of KCM-2 buffer and centrifuged at 200xg for 2-5 minutes. After centrifugation the protoplasts banded at the gradient inter-phase. These were removed and washed with 9 volumes of KCM-2 buffer and centrifuged at 100xg for 1-2 minutes. The pellet
was resuspended in a mixture of KCM-2 buffer protoplast culture medium (3:1), and centrifuged at 100xg for 2-5 minutes. The resultant pellet was diluted with protoplast culture media, Table 2.

After protoplast density was determined, protoplast medium was added to yield a final density of 4 x 10^5 protoplasts/ml. From this solution, 1ml was withdrawn and placed in a Falcon No. 1006 tight lid petri dish and 3ml of medium added. Dishes were sealed with "Parafilm" (American Can Co.) and incubated at 29°C in the dark. Observation was performed weekly.

Micro-droplet culture as described by Potrykus, Harms, and Lorz (1979) was also evaluated in order to determine the relative effectiveness of variations on the protoplast growth. Usually protoplasts were purified and suspended in protoplast medium without additives at 2 x 10^5 protoplasts/ml. A series of 5x5 and 6x6 10ul droplets were arranged on the underside of a Falcon No. 1029 petri dish lid. Various media additives had previously been prepared in protoplast media at 2 times the desired final concentration. (These samples had been placed in tubes and autoclaved earlier). For each protoplast containing droplet on the underside of the lid, a media additive dilution was
Table 2. Optimum Nutrient Medium for Culture of Cotton Anther Callus Protoplasts

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/l</th>
<th>moles/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN03</td>
<td>2500.0</td>
<td>25.0mM</td>
</tr>
<tr>
<td>CaCl2·2H2O</td>
<td>750.0</td>
<td>5.1mM</td>
</tr>
<tr>
<td>MgSO4·7H2O</td>
<td>250.0</td>
<td>1.0mM</td>
</tr>
<tr>
<td>NH4NO3</td>
<td>250.0</td>
<td>3.1mM</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>134.0</td>
<td>1.0mM</td>
</tr>
<tr>
<td>NaH2PO4·H2O</td>
<td>150.0</td>
<td>1.1mM</td>
</tr>
<tr>
<td>Na2EDTA</td>
<td>18.7</td>
<td>50uM</td>
</tr>
<tr>
<td>FeSO4·7H2O</td>
<td>13.9</td>
<td>50uM</td>
</tr>
<tr>
<td>Minor Salts of Murashige and Skoog (in Gamborg et al., 1976)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myo-inositol</td>
<td>100.0</td>
<td>555.0uM</td>
</tr>
<tr>
<td>Thiamine·HCl</td>
<td>1.0</td>
<td>2.9uM</td>
</tr>
<tr>
<td>Pyridoxine·HCl</td>
<td>0.1</td>
<td>0.4uM</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.1</td>
<td>0.8uM</td>
</tr>
<tr>
<td>Glucose</td>
<td>130,000.0</td>
<td>710.0mM</td>
</tr>
<tr>
<td>NAA</td>
<td>0.5</td>
<td>2.7uM</td>
</tr>
<tr>
<td>2iP</td>
<td>0.5</td>
<td>2.4uM</td>
</tr>
</tbody>
</table>

The pH is adjusted to 5.6 with 1N KOH or HCl
added resulting in a final volume per droplet of 20µl. When droplets were completed, 20ml of KCM-2 buffer was poured into the petri dish bottom, the lid inverted, placed upon the petri dish bottom, and sealed with Parafilm. All culture dishes were grown as described before.

**Protease Assay**

Assay conditions were 1mg/ml of bovine serum albumin (BSA) with 0.1mg/ml of cellulase enzyme in 0.05M phosphate buffer of pH 6.0. The reaction time was one hour at 30°C. Following termination of the reaction with 5% trichloroacetic acid (final) the remaining protein content was determined with the Biuret method of Chaykin (1966).
RESULTS

Cellulase Enzyme Assay. At the onset of this study various cellulase enzymes were available which had been stored at 5-10°C for over a year. The cellulase assay previously described, demonstrated a linear relationship of optical density to glucose concentration (Figure 1). This assay was then judged as acceptable for cellulase activity determination. The results of enzyme assays indicates the most effective enzyme for cellulose powder digestion was Miles Cellulase Concentrate (Table 3). This enzyme was most effective within the physiological pH values of 5.0 to 5.6. The effect of a salt mixture on the enzyme mediated digestion of cellulose powder was examined. A solution of 2.0mM CaCl₂·2H₂O, 2.0mM KNO₃, and 0.4mM KH₂PO₄ was selected as the salt mixture to test. These concentrations are not unlike those used by Coutts and Wood (1977) for protoplast isolation. Results of salt effects on cellulase activity are presented in Table 4. A general stimulation of activity was observed at lower substrate concentrations.
Figure 1. Proportional Relationship in Reducing Sugar Analysis of Glucose Concentration to Optical Density at 490nm. Each point represents the mean of 3 separate standard curves prepared in triplicate.
Table 3. Cellulose Powder Digestion by Cellulase Enzymes at Varied pH. Data expressed as ug/ml of glucose reducing sugars ± standard deviation.

<table>
<thead>
<tr>
<th>pH</th>
<th>Cellulyisin</th>
<th>TV Cellulase</th>
<th>Cellulase Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>149.86 ± 7.23</td>
<td>156.03 ± 8.10</td>
<td>150.11 ± 7.95</td>
</tr>
<tr>
<td>4.2</td>
<td>169.19 ± 13.81</td>
<td>200.86 ± 1.92</td>
<td>174.49 ± 3.94</td>
</tr>
<tr>
<td>4.8</td>
<td>184.85 ± 14.25</td>
<td>185.62 ± 6.15</td>
<td>234.68 ± 11.90</td>
</tr>
<tr>
<td>5.2</td>
<td>208.99 ± 5.89</td>
<td>212.83 ± 5.75</td>
<td>278.96 ± 14.97</td>
</tr>
<tr>
<td>5.6</td>
<td>200.65 ± 13.05</td>
<td>214.75 ± 4.78</td>
<td>273.13 ± 8.74</td>
</tr>
</tbody>
</table>

Table 4. Varied Substrate Concentration and the Effects of a Salt Mixture on Cellulase Concentrate Digestion of Cellulose Powder. All data represents the mean of duplicate experiments of three replications each. Numerical values are as ug/ml of glucose reducing sugars.

<table>
<thead>
<tr>
<th>Substrate Concentration</th>
<th>+salt</th>
<th>-salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% (w/v)</td>
<td>88.50</td>
<td>45.66</td>
</tr>
<tr>
<td>1.0%</td>
<td>111.63</td>
<td>67.39</td>
</tr>
<tr>
<td>1.5%</td>
<td>146.51</td>
<td>115.10</td>
</tr>
<tr>
<td>2.0%</td>
<td>159.43</td>
<td>122.51</td>
</tr>
<tr>
<td>2.5%</td>
<td>201.16</td>
<td>159.52</td>
</tr>
<tr>
<td>3.0%</td>
<td>239.02</td>
<td>180.87</td>
</tr>
<tr>
<td>4.0%</td>
<td>281.65</td>
<td>275.29</td>
</tr>
</tbody>
</table>
Osmotic Requirement. As isolated protoplasts are by definition without a cell wall and thus exposed to rapid exchange with their environment, a proper osmoticum is imperative in protoplast viability. Results represented in Figure 2 demonstrate that maximum protoplast yield occurred when mannitol concentrations were between 0.65M and 0.87M, regardless of the enzyme regiment used to isolate them.

These results were derived from a non-centrifuged preparation of protoplasts because centrifugation in 0.71M mannitol with 15mM arginine·HCl partitions protoplasts into floating and pelleting types. All other experiments were based on protoplasts pelleting after a 100xg centrifugation for 5 minutes where supernatant was withdrawn below the meniscus in order to preserve the floating protoplasts. Protoplasts found floating in 0.71M mannitol were often observed as nonviable as judged by Evan's Blue exclusion stain. Hence, these protoplasts are counted in the total yield but not the counting of viable protoplasts.

Activities of Cellulases. Determinations of cellulose powder digestion had indicated Miles Cellulase Concentrate to be the most effective cellulase in this reaction, Table 2. To examine the correlation between cellulase activity and protoplast yield, different brands of cellulase were allowed to digest callus to protoplasts. All enzymes tested were of a 2% w/v concentration.
Figure 2. Effects of Osmotic Concentration on Protoplast Yield Using Two Different Enzyme Combinations. Miles Cellulase Concentrate (2% w/v) and Macerozyme R-10 (0.5% w/v) - - - Miles TV Developmental Cellulase (2% w/v) and Miles Experimental Pectinase (0.5% w/v) - - -
The often used "Onozuka" or Cellulysin cellulase (Cal Biochem.) showed good yield capacity, yet Miles TV Cellulase demonstrated superior yield and viability of protoplasts, Figure 3. Miles Cellulase Concentrate, Driselase (Kyowa Hakko Co. Ltd., Japan), and any combination of cellulases all proved less effective in total yield and viability. Hence, Miles TV Cellulase was used for further experiments.

Sources of Pectinase. The purpose of a pectinase in protoplast isolation is generally considered to digest polygalacturonic acid residues which become impacted between adjacent cell walls. By separating cells with a pectinase, the action of a cellulase can be more evenly distributed and thereby result in uniform protoplast production from experiment to experiment. Macerozyme R-10 had been used up to this point in the study, irrespective of the presence of "protease" within this enzyme preparation (Yakult Biochem. Co. Ltd., Japan, 1978b).

Miles TV Cellulase as 2% w/v with 0.71M mannitol and 15mM arginine·HCl at pH 5.6 was then supplemented with 0.5% w/v of various pectinases. Evaluation of total and viable protoplast yields with respect to a given pectinase was then preformed.
Figure 3. Cellulase Sources and Protoplast Yield.
Cellulysin; CEL, TV Developmental Cellulase; TV, Cellulase Concentrate; CC, Driselase; DR. All enzymes were tested at a final concentration of 2%w/v. Total cells; T, and viable cells; V. All groups contained 0.5%w/v R-10 Pectinase.
Figure 4. Effects of Pectinases and BSA on Protoplast Yield. Macerases: MA, Miles's Experimental Pectinase; EP, Macerozyme R-10; R-10, Pectinol; PE, bovine Serum Albumin; BSA. Total cells: T, and viable cells: V. Enzymes were tested at a final concentration of 0.5%w/v with 2%w/v TV Cellulase.
Results described in Figure 4 indicate that the Macerozyme R-10 was less effective in total production with a greater percentage of protoplasts remaining viable as compared with Miles Experimental Pectinase. However, the latter pectinase showed greater net yield of viable protoplasts and was used in further experiments. Macerase (Cal Biochem,) and Pectinol (Rhom Hass) reflected poor yields of protoplasts. Neither pectinase could be recommended for cotton protoplast isolation.

Macerozyme R-10 has already been associated with "protease" activity. Further results (in Figure 4) demonstrate that mixtures of BSA in addition to 15mM arginine\*HCl in the enzyme solution did not significantly improve protoplast yield or viability.

**Enzyme Concentration.** The pecto-cellulase mediated cell wall hydrolysis does require a balance of enzyme to substrate in a given environment. Enzyme concentrations were then varied to derive their maximum concentration for optimal protoplast yield and viability. Maximum conditions corresponded to 2% w/v TV Cellulase and 0.5% w/v Experimental Pectinase (Figure 5). This data also suggests that protoplasts will not be isolated from cotton anther callus with negligible cellulase and/or pectinase.
Figure 5. Protoplast Yield Affected by Cellulase and Pectinase Concentrations. Varied Experimental Pectinase with 2% w/v TV Cellulase; ● --- ●, Varied TV Cellulase with 0.5% w/v Experimental Pectinase; ○ --- ○.
Substrate Concentration. To further establish the optimum protoplast isolation conditions, varied substrate (callus) fresh weight was digested with constant enzyme concentration and volume. The resultant peak of isolation efficiency between 0.6g and 0.8g fresh weight was observed (Figure 6). In preliminary experiments with callus fresh weight, that in excess of 0.8g resulted in irregular yields. These experiments were conducted as those before except the mannitol concentration was lowered to 0.55M.

"Protease" Activity of Cellulase. Of the cellulase and pectinase enzymes assayed for protease activity, the pecto-cellulase enzyme mixture of choice for protoplast isolation (determined earlier as TV Cellulase and Experimental Pectinase) represents the lowest combined protease activity, Table 5. All enzymes tested were lowed in protease activity than Pronase B (Cal Biochem.).

Additions to the Pecto-cellulase Mixture. Preliminary experiments had shown that when mannitol was the only non-protein constituent of the pecto-cellulase mixture, yield of protoplasts was low. Therefore, various additives were included in the enzyme mixture and their effects recorded. The additives tested made up three categories: amino acids and polyamines, proteins, and inorganic salts.
Figure 6. Callus Fresh Weight and the Effects on Protoplast Yield. All samples contained constant enzyme concentration and volume.
Table 5. Preliminary Evidence for the Presence of Protease Activity within Some Cellulase and Pectinase Enzymes. Data represents the mean of triplicate samples.

<table>
<thead>
<tr>
<th>Enzyme Tested</th>
<th>mg BSA Digested hr$^{-2}$mg$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV Cellulase</td>
<td>0.28</td>
</tr>
<tr>
<td>Cellulysin</td>
<td>0.68</td>
</tr>
<tr>
<td>Cellulase Concentrate</td>
<td>0.93</td>
</tr>
<tr>
<td>Macerozyme R-10</td>
<td>0.48</td>
</tr>
<tr>
<td>Experimental Pectinase</td>
<td>0.00</td>
</tr>
<tr>
<td>Pronase B (Cal Biochem.)</td>
<td>18.00</td>
</tr>
</tbody>
</table>
Results described in Figure 7 present the tested concentrations of the additives as compared to the 15mM arginine·HCl control. Concentrations of all additives were not unlike those of similar compounds tested in other protoplast systems (Kaur-Sawhney et al., 1976; 1977; Galston, Altman, and Kaur-Sawhney, 1978).

Generally amino acids and polyamines were beneficial towards protoplast yield production. Unlike data presented earlier where BSA mixed with arginine·HCl did not improve protoplast yield (Figure 4), BSA in the absence of the amino acid showed amenable effects on protoplast yield. Some other proteins promoted protoplast yield as well (histone II and trypsin inhibitor). Alternatively, crude yeast extract was not found beneficial and proved the poorest of all additives tested.

Magnesium sulfate at 10mM aided in protoplast isolation as much as arginine·HCl, whereas most other salt and salt mixtures did not appreciably promote protoplast yield. Additionally, the presence of light also proved detrimental towards protoplast yield. Therefore, 15mM arginine·HCl was used in further experiments.

**pH Considerations.** Successful protoplast isolation demands a combination of enzymatic and physiological integrity within similar pH ranges. Likewise the effects of the culture media used to grow the donor callus may affect the relative isolation capacity.
Figure 7. Chemical Additives and Their Effect on Protoplast Yield as Compared to 15mM Arginine·HCL Control. All groups tested were digested with 2% w/v TV Cellulase and 0.5% w/v Experimental Pectinase in 0.71M mannitol at pH 5.6 in the dark except where noted.

15mM arginine·HCL; AR, 15mM serine; SE, 15mM lysine; LY, 15mM glycine; GY, 15mM spermidine; Sp, 15mM putrascene; Pu, 0.5% BSA; BS, 0.5% trypsin inhibitor; TI, 0.5% histone fraction II; H2, 0.5% yeast extract; YE, no additive; W, Wild Carrot salts; WC*, Murashige and Skoog salts; MS, 10mM CaCl2·2H2O; Ca, 10mM MgSO4·7H2O; Mg, 15mM arginine·HCL with digestion in light; AL.

Reports of kinetin mediated protoplast preservation during isolation (Kaur-Sawhney et al., 1976) suggested the following experiments. Anther callus was transferred to maintenance medium as described with either 0.1mg/l or 0.01mg/l 6BA. Cultures were grown for five transfers (two weeks per transfer of incubation) on a given cytokinin concentration. After appropriate incubation, protoplasts were then isolated under different pH conditions.

Figure 8 indicates no significant deviation in protoplast yield until pH 6.0. A pH maxima for the cotton protoplast system can be represented as a range between 5.6 and 6.0 with specific pH optima relating to the cytokinin concentration of the callus medium. All further experiments were conducted at pH 5.6 because adequate protoplast yields were obtained from callus grown on either cytokinin amount.

Other Considerations. Temperature can affect enzymatic reactions of many types, yet with regard to cellulase other workers (Halliwell, 1961; Uchimiya and Murashige, 1974) suggest that cellulase activity is rather indiscriminate within physiological confines. Therefore only 29°C was used and held constant for all experiments.

Clearly, the volume of enzyme solution to the substrate concentration within a given container must have an effect on protoplast yield. Preliminary tests using a Falcon No. 1005 petri dish with 2.5ml of enzyme solution
Figure 8. Effects of pH and Varied Cytokinin Concentration in Callus Growth Media to Protoplast Yield. Callus medium with 0.1mg/l 6BA; O--O, with 0.01mg/l 6BA; -.-. pH of enzyme solution adjusted with 1N HCl or 1N KOH.
to 0.5g fresh weight of callus showed negligible protoplast yield. With the addition of 5ml of enzyme solution the usual yields of protoplasts were obtained (the volume covers the bottom of the dish).

With respect to time of incubation, conditions as stated earlier (2 hours of digestion time) provided maximum yields with incubation for 2 additional hours having no effect on protoplast yield. By reducing the cellulase and pectinase concentrations to 0.2% and 0.05% respectively, usual yields were obtained after 16-20 hours of incubation.

Callus Growth and Protoplast Yield. The amount of isolated protoplasts was dependent on the age of the callus culture, Figure 9. Previous experiments were performed on 10 day old callus which may explain the poor yields obtained. Maximum protoplast yield was achieved when callus was digested by pecto-cellulase enzymes after 5 days in culture.

At the peripheral regions of callus expansion (the origin of cells for protoplast conversion), an overall increase in cell number was not significantly higher at any point on the growth curve. Although fresh weight increased with time in culture, cells/g fresh weight ranged from a minimum of $3.8 \times 10^6$ cells/g at day 14 to $4.0 \times 10^6$ cells/g at day 7. This may suggest a rather low level of active dividing cells in this agar grown callus.
Figure 9. Effects of Culture Age on Protoplast Yield. Callus Growth: •—•, Protoplasts Obtained; O---O.
Purification of Protoplasts. The necessity of removing the pecto-cellulase enzymes from isolated protoplasts is apparent. One means of purification would allow a constant osmotic pressure with variable densities within a discontinuous gradient. This gradient would allow cotton protoplasts to be separated from the enzyme mixture on the basis of buoyant density exclusively.

Figure 10 illustrates the banding pattern of 5 day old anther callus protoplasts. Although Harms and Potrykus (1978) found no more than 4 bands in any protoplast preparation during the original description of the technique, up to six bands were observed for cotton anther callus protoplast preparations. This may be due to a slightly larger tube for fractionation in this case, thus allowing larger amounts of protoplasts to be applied to the gradient and thereby produce a more vivid banding pattern.

For the routine culture of cotton protoplasts, simpler systems are desirable. Experiments with Lymphoprep\textsuperscript{R} showed superior yield, 72% as compared to the iso-osmotic gradient with 53% recovery, yet neither preparation was free of broken protoplasts and debris. Further gradient application did not improve protoplast recovery amounts.

Because of data presented in Figure 2 suggesting cotton protoplasts capable of withstanding high osmotic concentrations of mannitol, KGW-2 buffer was compared to
Figure 10. Iso-Osmotic Density Gradient Separation of 5 Day Old Anther Callus Protoplasts.

<table>
<thead>
<tr>
<th>Calculated Density (g/cm³)</th>
<th>0.56M Sucrose: KCM-1</th>
<th>% Protoplasts Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.017</td>
<td>0.7</td>
<td>none</td>
</tr>
<tr>
<td>1.025</td>
<td>1.6</td>
<td>trace</td>
</tr>
<tr>
<td>1.032</td>
<td>2.5</td>
<td>9.85 ± 1.06</td>
</tr>
<tr>
<td>1.039</td>
<td>3.4</td>
<td>20.93 ± 1.73</td>
</tr>
<tr>
<td>1.047</td>
<td>4.3</td>
<td>25.31 ± 2.31</td>
</tr>
<tr>
<td>1.054</td>
<td>5.2</td>
<td>15.88 ± 1.35</td>
</tr>
<tr>
<td>1.061</td>
<td>6.1</td>
<td>11.18 ± 2.60</td>
</tr>
<tr>
<td>1.069</td>
<td>7.0</td>
<td>16.65 ± 1.69</td>
</tr>
</tbody>
</table>
KCM-1 buffer in terms of protoplast recovery (or yield) after centrifugation. The osmolal concentration of KCM-1 and KCM-2 were 0.66 Os/kg water and 0.81 Os/kg water respectively as determined by freeze point depression.

Figure 11 demonstrates the effectiveness of the modified buffer (KCM-2) on protoplasts sedimented after digestion by pecto-cellulase enzymes of callus tissue. With centrifugation time tripled, KCM-2 was clearly more effective than KCM-1 in protoplast recovery.

Higher osmolal concentrations of gradients (as compared to 0.56M sucrose) were also tested for protoplast recovery. Gradients of 0.6M sucrose and 0.56M sucrose with 0.14M mannitol showed approximately 50% recovery of protoplasts applied to the gradient (Figure 12) with a final wash in KCM-2 resulting in an average recovery of 32%.

The adopted procedure for sterile isolation and purification of cotton protoplasts will follow. Between 0.5g and 0.8g fresh weight of callus is digested in 0.71M mannitol with 15mM arginine·HCl, 2% TV Cellulase, and 0.5% Experimental Pectinase at pH 5.6. After 2 hours of dark digestion, the solution is mixed with 2 volumes of 0.6M sucrose with 15mM arginine·HCl. Of this solution, 2 volumes are layered beneath 1 volume of KCM-2 and centrifuged for 5-10 minutes at 200xg. Protoplasts are then removed from the
Figure 11. Comparison of KCM-1 and KCM-2 Buffers upon Protoplast Recovery Following Centrifugation at Different Times.

Time of centrifugation (100xg) in minutes.
Figure 12. Higher Osmotic Concentrations of Gradients and Protoplast Recovery.

0.7M MgSO\textsubscript{4}·7H\textsubscript{2}O and KCM-2 mixed 5:2 and centrifuged at 100xg; Mg, 0.6M sucrose centrifuged at 100xg; S1, 0.6M sucrose; S2 and 0.56M sucrose with 0.14M mannitol; SM both centrifuged at 200xg.
interphase and washed with 9 volumes of KCM-2. After samples are sedimented at 100xg for 1-2 minutes, supernatant is removed and the protoplast pellet resuspended in culture media. Yields have approximated 30% of the total protoplasts recovered with this method.

A condensation of the above technique has been applied to large scale isolation of protoplasts. Upon introduction of enzymes and protoplasts to 2 volumes of 0.6M sucrose with 15 mM arginine-HCl, the solution is gently mixed and allowed to stand for one hour. Separation of protoplasts was evident by the banding at the meniscus of the mixture. Protoplasts (now concentrated) were pipetted off, washed, and cultured without a gradient centrifugation step. Yields have approximated the former method, however the reduced manipulation and reduced number of sterile vessels required for purification is advantageous.

Protoplast Culture. Preliminary results of protoplasts cultured in tight lid petri dishes using Murashige and Skoog salts (as presented by Gamborg et al., 1976), KCM-2 buffer with added KNO₃, and modified B-5 salts (Bhojwani, Power, and Cocking, 1977) all with 1 mg/l NAA, 0.1 mg/l 6BA, and 10% w/v mannitol, indicated that only the B-5 modified medium was capable of supporting growth. Protoplasts cultured in Murashige and Skoog salts turned brown while those cultured in KCM-2 with KNO₃ lysed prior to 3
weeks in culture. Attempts using agar in protoplast culture did not support growth in identical media. Also, cultures grown in the dark and shaken at 100/rpm lysed after an hour.

In order to increase the number of testable media variations for protoplast culture, the hanging droplet technique of Potrykus, Harms, and Lorz (1979) was applied. Using protoplast medium of Table 2 with substitutions of 1mg/l NAA and 0.1mg/l 6BA for the phytohormones stated, amino acid effects on protoplast growth were tested. Concentrations of 5mM and 10mM; arginine·HCl, lysine·HCl, glycine, glutamine, asparagine, and cysteine·HCl as well as mixtures of these amino acids supported less growth of protoplasts than media without them.

Cytokinins and auxins were then evaluated in protoplast growth promotion. Kinetin and 6BA at; 2, 1, 0.5, 0.25, 0.1, and 0mg/l were examined, each with varied NAA concentrations of; 5, 2, 1, 0.5, 0.1, or 0mg/l. Only cultures of 1 and 0.5mg/l NAA with either 0.25 or 0.1mg/l 6BA demonstrated limited cell proliferation. Cell wall formation was not restricted to a given phytohormone level as judged by ellipsoid cell shapes with peripheral wall thickening.
Identical concentrations of NAA (as before) when tested against 10, 5, 2, 1, 0.5, 0.1, and 0mg/l of 6-(8-8-dimethylallylamino)-purine (2iP) demonstrated more encouraging results. Table 6 indicates the relative effectiveness of cellular proliferation with respect to phytohormone concentrations after 3 weeks in culture. Based on the above information, 0.5mg/l NAA and 2iP were chosen for further experiments.

Protoplast density had an effect on the ability of cell division in the above culture medium in Falcon No. 1006 tight lid petri dishes. In duplicate experiments with 10 cultures per cell density, final densities of $1 \times 10^4$ protoplasts/ml did not sustain growth. By increasing the cell density to $5 \times 10^4$ protoplasts/ml between 5-20 microcalli colonies were evident after 3 weeks in culture. When final densities corresponded to $1-2 \times 10^5$ protoplasts/ml, greater than 50 microcalli colonies were observed after 1 week in culture. These colonies were comprised of as many as 35 cells/colony and were usually floating in the culture medium with a trailing portion anchored to the bottom of the dish. After a week in culture many ellipsoid shaped cells were present and swelling of overall cell size was observed. Such aggregates were observable without magnification.
Table 6. Hanging Drop Array in Testing Optimal Auxin and Cytokinin Concentrations

<table>
<thead>
<tr>
<th>NAA mg/l</th>
<th>5</th>
<th>2</th>
<th>1</th>
<th>0.5</th>
<th>0.1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>0.5</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>0.1</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

This data represents the tested variables possible on one petri dish. Results are representative of the concentrations tested where + signifies growth and − little or no growth.
In order to procure viable callus cultures derived from protoplast culture, additional culture feeding is needed. Cultures examined thus far are able to continue growth when the medium of Table 2 with 10% glucose is added (1ml to 3ml of original media) after 3-4 weeks after initial culture.

Callus Selection and Differentiation. As haploid cell production is the objective of anther callus culture, attempts at any selective pressure may provide a preponderance of such cell types within a callus culture. The SV anther callus does not grow well in the dark and not at room temperature (25°C) whereas SV-10 was isolated under these conditions.

In comparison, both calli were tested for growth in suspension culture. SV-10 was far superior to SV callus in culture initiation and growth rate. The latter callus grew in a manner similar to haploid callus growth (anther callus of cotton) described by Barrow, Hatterman, and Williams (1978).

Growth characteristics in agar culture of the calli were also different. In maintenance media (as described earlier) SV callus becomes hard, compact, and very green. Conversely, SV-10 grows to a friable, wet, and faint green color. Growth indexes on such media were $5.42 \pm 0.70$ and $3.01 \pm 0.37$ respectively after 14 days in culture.
Using the root preinduction media of Thomas, Katterman, and Williams (1980) growth of SV callus was 33.39 ± 6.82 whereas that of SV-10 was 19.88 ± 2.42 after 45 days in culture. Appearance of SV-10 was of numerous meristematic nodules (100/culture) with a hard and green constitution. Agar in this media seemed partially hydrolyzed with a final consistency similar to 0.6% agar. Conversely, SV callus had formed some nodules (5-10/culture), was very green on the apex of the callus while white on the underside. The agar was also rather concentrated after the 45 day incubation.

Both calli were then transferred to root induction medium described in the above reference. Of the SV-10 cultures, nearly all formed rootlike structures using the phytohormones described with B-5 salts (Gamborg et al., 1976). None of the SV cultures produced such structures.
DISCUSSION

The primary objective at first focused on factors contributing to protoplast yield. Determination of the rate limiting aspects of cotton anther callus digestion to pro-oplasts were thoroughly investigated in terms of cell wall digestion and the maintenance of protoplast integrity during this process. The effects of callus growth conditions were also incorporated into this portion of the study with the overall objective of high rates of cell conversion to pro-oplasts.

The digestion of purified cellulose, a major component of plant cell walls, was initially performed to identify the most active cellulase enzyme at hand. Once determined to some degree, can cellulose powder digestion be equated to the ability of a cellulase enzyme to yield cotton anther callus protoplasts?

Holm (1978) suggests the cell wall of plant material may contain fractions of chemically modified cellulose. These modifications may produce an inaccurate estimate of cellulase activity when comparing cell wall digestion to cellulose powder digestion. Reese, Siu, and Levinson (1950)
had earlier stated that two separate enzymes were interacting in the digestion of cellulose; a C1 activity and a CX activity. In a review, Jones (1976) states the C1 component measured by cellulose powder digestion may be inactive without the presence of a CX element (usually measured as the digestion of carboxymethyl cellulose).

Therefore, a simple estimate of cellulase enzyme activity using a cellulose powder substrate may not be an accurate measurement of the C1 function, but can be considered indicative of cellulose specific hydrolysis. Findings represented in Table 3 demonstrate that Cellulase Concentrate, when compared to TV Cellulase and Cellulysin, contains the greatest activity in cellulose powder digestion between pH 4.8 and 5.6.

Jones (1976) has suggested that cellulose digestion by cellulase enzymes may be stimulated by the addition of magnesium, cobalt, or phosphate. Halliwell and Griffin (1973) have shown that copper, zinc, or iron may inhibit cellulase activity. Therefore, Cellulase Concentrate was tested for stimulation of cellulose powder digestion in a mixture of CaCl2, KNO3, and KH2PO4 (Table 4). From these results, it is unclear as to the effect of the salt mixture in this reaction. However, a slight stimulation may be present at low substrate concentration. The stimulation of cellulose digestion was not of apparent importance in the isolation of protoplasts and was not pursued further.
Once cellulose powder digestion had been determined with the cellulase enzymes at hand, a comparison of protoplast yielding ability for each was performed. These experiments were also conducted to establish whether or not cellulose digestion was the rate limiting step in protoplast isolation. Data presented in Table 3 and Figure 3 indicates that there is no clear relationship between cellulose powder digestion and the amount of protoplasts isolated from callus tissue.

Cells used for protoplast isolation were selected from callus 10 days after subculture (Callus was transferred to fresh media every 14 days). It can be argued that cellulose synthesis prior to day 10 is not of sufficient quantity to represent a rate limiting step in cell wall digestion during protoplast isolation. However, later experiments, Figure 9, demonstrate that 10-12 day old callus resulted in less protoplasts obtained after digestion in comparison to all other ages tested. Evidences of cellulose content of plant calli increasing with time after subculture (Uchimiya and Murashige, 1974) and in the developmental progression of cotton fibers (Meinert and Delmer, 1977) have been recorded. Cellulase Concentrate was the most effective enzyme tested in cellulose powder digestion. In aged plant callus cells (with presumably substantial cellulose within the cell wall) why is this enzyme least effective in protoplast isolation?
In mixtures of Cellulase Concentrate with TV Cellulase the viability of cotton anther callus protoplasts decreases dramatically. A significant decrease in total and total viable protoplasts obtained can also be observed when Cellulysin is mixed with Cellulase Concentrate (Figure 3). These findings suggest a component of the latter two enzymes allows the protoplast to retain a spherical shape, yet does damage to the cell membrane permitting entry of Evan's Blue dye. The resultant blue protoplast would then be counted as inviable. Alternatively, in mixtures of TV Cellulase with Cellulysin only a slight depression of protoplast viability was observed as compared to the Cellulysin alone treatment. Therefore, the loss of protoplast viability is not simply a result of mixing two different cellulase enzymes.

Albertscheim (1975) has indicated that the plant cell wall may be comprised of interlocked polymeric units intimately positioned near the plasma membrane. Protein and glycoprotein projections, as discussed by Singer and Nicholson (1972), may protrude from the membrane into the cell wall creating further passive interactions in the proper osmoticum.

If membrane pressure against the cell wall is high, any removal of the cell wall material may induce membrane infusion into the now exposed opening in the wall. It can be conceived that a cellulase enzyme with a random pattern
of monomeric digestion will create small breaks in the cell wall with equally small amounts of membrane infiltration. Likewise, a cellulase with the capacity for linear digestion of monomers will result in large gaps along the cell wall. With membrane pressing through these gaps, the potential for membrane impalement upon the remaining insoluble cell wall polymer increases. Repressed viability as measured by a membrane exclusion dye as well as a loss of total protoplasts obtained after digestion may be the result of such a puncture.

The effect of osmoticum is pertinent to the above discussion. As osmotic concentration decreases the protoplasmic space will increase. Thus, by lowering the osmotic concentration, increased water uptake will result in added pressure on the cell wall. Any repression of protoplast viability and/or yield due to the above mentioned process may be detected. As demonstrated in Figure 2, Cellulase Concentrate may induce lysis in this manner when callus digestion is performed in 0.55M mannitol. The yield of protoplasts using Cellulase Concentrate as compared to TV Cellulase increases rapidly between 0.55M and 0.65M mannitol. However, the explanation as to why Cellulase Concentrate can be effective in cellulose powder digestion, yet unable to yield large numbers of protoplasts regardless of osmotic concentration requires further consideration.
One possibility for the lack of correlation of cellulose digestion and protoplast yield among the enzymes tested may be the presence of antagonistic contaminants present in the cellulase preparations. Kao et al. (1974) has shown Driselase to be the cellulase enzyme of choice for the preparation of protoplasts for fusion studies. He attributes this ability to the presence of protease enzymes within the Driselase preparation. Protease presence may then explain why Driselase produces few viable anther callus protoplasts (Figure 3).

Although formal analysis of protease-like activity will not be included in this thesis, preliminary data suggests protease presence in all but one of the enzymes tested in this experiment (Table 5). The optimum cellulase for protoplast isolation, TV Cellulase, demonstrates only 0.28 mg of protein digested per mg hour. Cellulase Concentrate contains protease activity nearly four times higher than TV Cellulase. These concentrations of cellulase enzyme to BSA substrate might not relate directly to the process of protoplast isolation due to kinetic considerations. However, this evidence does suggest that while Cellulase Concentrate may digest large amounts of cellulose powder and callus cell wall, the contaminant protease may destroy the resultant protoplast. Therefore, total yield and protoplast viability may relate to the amount of protease within commercial cellulase preparations.
Like cellulase enzymes, pectinases may have various enzymatic activities associated with them. In order to examine a range of pectinases, each pectinase was mixed as 0.5\% w/v with 2\% w/v TV Cellulase, 0.71M mannitol, and 15mM arginine·HCl and allowed to digest callus to protoplasts. Of the four pectinases tested, only Macerozyme R-10 and Experimental Pectinase improved protoplast yield (Figure 4).

With the previous suggestion that protein degradation on the membrane surface affects protoplast yield, BSA was added to the digestion mixture. No improvement of protoplast yield was observed when Experimental Pectinase was used, with perhaps an increase in protoplast yield in the Macerozyme R-10 tests. These results as well as information on the protease content of the enzyme preparations (Table 5) suggest protease activity may in part account for the differences in yield between the two effective pectinases. Macerase and Pectinol did not demonstrate the ability to promote protoplast isolation and were investigated no further.

The kinetic aspects of protoplast digestion were performed using TV Cellulase and Experimental Pectinase. Concentrations of cellulase, pectinase, and callus when varied demonstrated optimal conditions for protoplast isolation to be 2\% TV Cellulase, 0.5\% Experimental Pectinase, and 0.6g-0.8g of callus tissue (Figures 5 and 6).
It may be pointed out that in trial experiments the size of the reaction vessel dictated protoplast yield regardless of other considerations. In future studies with other cotton species, protoplast production may relate to the data in these experiments only when the volume of displacement of the enzyme solution, (or dish size), can be equated.

Protoplast maintenance during isolation and the effects of callus growth conditions play a role in the net protoplast yield. While results presented in Table 3 demonstrate cellulose powder hydrolysis to have a pH maxima of 5.2 to 5.6, digestion of callus to protoplasts appears more complex with regard to pH maxima.

As demonstrated in Figure 8, anther callus when grown on maintenance media with a low cytokinin concentration (0.01mg/L 6BA) has a pH optima of 5.0 and 5.6 with respect to protoplast yield. Conversely, by increasing the 6BA concentration to 0.1mg/L the pH optima becomes 6.0. The net yield of protoplasts isolated under varied pH conditions then may somehow relate to the effects of cytokinins in the callus growth medium as well as any pH requirements imposed by the pecto-cellulase enzyme combination used.
Evidence provided by Halmer and Thorpe (1976) may provide insight into cytokinin mediated changes in the cell wall. These authors found cytokinin induced changes in the callus texture and in composition of the hemicellulose and hydroxyproline-containing-protein fractions of the cell wall. Cellulases and pectinases are comprised of a variety of enzymatic activities, some with differing pH optima (Yakult Biochemicals, 1978a; 1978b). The alteration in pH optima for the digestion of callus to protoplast may then reflect a distinctly different cell wall structure in the two cytokinin concentrations tested. Thus, the digestion of callus in the two cases may require different enzymatic activities with different pH optimums.

How might other additives affect protoplast isolation? In the case of leaf detachment, protease and nuclease enzymes have been induced from within the excised tissue (Wyen et al., 1972). With the release of protease the potential for membrane deformation and protoplast injury similar to that observed by Weiss (1966) is increased. Udvardy, Farkas, and Marre (1969) found cytokinins were able to repress protoplast lysis after excision. Even in the case of oat protoplast stabilization the inclusion of cytokinins, polyamines, and amino acids have been shown to inhibit protease and nuclease induction (Kaur-Sawhney et al., 1976; 1977).
Data presented in Figure 7 supports the contention that amino acids and polyamines benefit protoplast yield when included in the isolation mixture. Serine, sometimes considered a senescence inducing factor (Martin and Thimann, 1972), was beneficial in protoplast isolation.

When protein solutions are present in the isolation mixture a promotion of protoplast yield is evident when compared to the control (no additive). However, protoplast yields were not as great as those obtained in the presence of amino acids and polyamines. Earlier, (Figure 4) it was demonstrated that BSA in the presence of 15mM arginine·HCL was minimally effective in protoplast yield preservation. When BSA is tested without arginine in the isolation mixture, protoplast preservation greater than the control results (Figure 7). This is true for trypsin inhibitor and histone II as well. Alternatively, crude yeast extract proves poorest of all additives tested with regard to protoplast yield. Likewise, with the exception of MgSO₄, salt mixtures did not improve protoplast yield when compared to the control (no additive).

The common feature of some additives found beneficial towards protoplast preservation is the potential for additive to membrane binding. Lehninger (1975) has discussed the presence of amino acid receptors, a Mg dependent ATPase, and other protein components located within the plasmalemma.
If (upon cell wall release) the naked cell membrane becomes exposed to the altered environment, then normal membrane perturbation may become exaggerated and lead to membrane destabilization. Internal membranes, such as those surrounding the lysosomes, conceivably may also be affected. As lysosome structures become ruptured, proteases and nucleases are released and destroy the cell. Amino acids, polyamines, and Mg, by binding to the cell and/or internal membranes may induce membrane stabilization in a manner akin to that suggested by Grout and Coutts (1974). These authors found that with the addition of poly-L-ornithine, neutralization of the cell membrane charge and protoplast stability results. Therefore, by binding to the membrane, the beneficial additives neutralize the normal membrane charges. This allows a slowing of the membrane charge dispersion and transport to a level tolerated by the protoplast.

In the presence of excess protein, protoplast preservation may be related to the aforementioned process. When protease degraded protoplasts (in the absence of amino acids and polyamines) release their cellular contents into the surrounding medium, excess protein may engage the degradative enzymes and protect intact protoplasts. Support for this hypothesis is based on the observation that all protein types tested were less effective than amino acids and usually more beneficial than salts and the control tests.
In addition, Figure 7 indicates the presence of light (3-5 uEm\(^{-2}\)sec\(^{-2}\)) decreases protoplast yield to half that obtained in the dark digestion. Whether this effect is due to free radical generation or other unknown factors leading to membrane instability is unclear. However, these results point out the importance of low light conditions for protoplast isolation and any further manipulation.

The effects of callus culture age on protoplast yield is significant and may represent the most important aspect of yield with respect to protoplast isolation. Uchimiya and Murashige (1974) attribute the repression in yield of protoplasts isolated from older suspension grown tobacco callus to the accumulation of secondary cell wall with culture age. Mazliak and Kader (1978) have also pointed out that with increased age, the exchange properties of cell membranes becomes altered. Maximum yield of cotton anther callus protoplasts at day 5 (Figure 9) may indicate either the lack of secondary cell wall and/or maximum cytoplasmic density as related to membrane structure in the absence of substantial starch deposits.

Therefore, the digestion and preservation of protoplasts is dependent on their cellular makeup and the enzymes used to isolate them. Cellulose powder digestion cannot be equated to protoplast isolation efficiency due to presumed differential substrate specificity within the particular
pecto-cellulase mixture used, including any protease contamination. Normal kinetic aspects of enzyme activity with regard to enzyme and substrate concentration will reflect protoplast isolation efficiency to a degree. Integrated within the isolation process, variables including culture age, cytokinin concentration of the donor callus, pH, and requirements pertaining to membrane stabilization via inhibition of endogenous protease activity all play a significant role in the final net yield of viable protoplasts. These considerations have permitted the conversion of nearly all cotton anther callus cells to viable protoplasts on the fifth day after transfer.

Protoplast purification was based upon the density of the cell types in concert with osmotic requirements. In preliminary experiments with various forms of osmotica, 0.71M glucose or mannitol was acceptable for protoplast isolation whereas 0.71M sucrose gave irregular yields of protoplasts. This observation may be related to the densities of these sugars at 0.71M concentration. According to Weast (1975) the densities of mannitol and sucrose at the above concentration are 1.043g/cm³ and 1.090g/cm³ respectively. As demonstrated in Figure 10, when 0.56M sucrose is mixed in various proportions with KCM-1 buffer, an array of protoplast bands occur after centrifugation. The average density of cotton anther callus protoplasts is about
1.043-1.050g/cm³. Therefore, in the sucrose solution of 0.71M, the protoplasts will float making uniform dispersion for cell counting impossible. A mannitol solution of 0.71M allows this dispersion and was used for estimates of protoplast numbers.

Uchimiya and Murashige (1974) found protoplasts of tobacco suspension callus capable of a range of osmotic tolerance between 0.4M and 0.85M mannitol. As described in Figure 2, cotton anther callus protoplasts prefer a range between 0.67M and 0.85M mannitol. These considerations are important when a purification procedure is to be constructed.

It is of note that the osmolal concentration of the pecto-cellulase enzyme mixture is not exclusively a function of the sugar used. The enzymes and digested cell wall do contribute to the osmolal concentration. After protoplast separation, freeze point depression determination of this solution indicated an osmolal concentration of 1.20 Os/kg water.

Attempted protoplast purification using 0.56M sucrose incorporated into the iso-osmotic gradient and the commercially available Lymphoprep® resulted in low yields of purified protoplasts. One explanation may be the osmolal concentration of each gradient (0.66 and 0.40 Os/kg water) may be too low for cotton protoplasts to tolerate.
For the above reasons, KCM-2 buffer was developed. Although with a higher osmolal concentration than KCM-1, the density remained low. In a comparison of the two buffers in protoplast dilution and washing, KCM-2 was superior in terms of protoplasts obtained (Figure 11).

Higher osmolal concentrations of gradients (0.60M sucrose or 0.56M sucrose with 0.14M mannitol) also proved effective in the purification of cotton anther callus protoplasts in large numbers. Centrifugal speeds of 100xg and 200xg were found acceptable for protoplast purification. A gradient of MgSO₄ was attempted, but protoplast yields were not substantial (Figure 12).

The relationship of osmolal concentration and the correspondent densities of gradients and wash solutions play an important part in the purification of protoplasts. Most plant protoplasts are able to pellet during centrifugation within the pecto-cellulase enzyme mixture. However, cotton anther callus protoplasts float in this solution. A simple purification procedure for cotton anther callus protoplasts can be to mix 0.60M sucrose with the pecto-cellulase enzyme solution after 2 hours of callus digestion. After allowing equilibrium to be reached (one hour) in this solution, protoplasts will float to the top of the mixture and thereby decrease the amount of manipulation needed.
While great efforts have been made towards the regeneration of macroscopic calli and/or plantlets from protoplast culture, progress has been slow. In particular, variations of media are necessary as callus and protoplast growth requirements differ. Uchimiya and Murashige (1976) have examined a number of media variations and have optimized the auxin, cytokinin, and carbohydrate concentrations for tobacco callus protoplasts. Donn (1978) has used amino acids in addition to salts, carbohydrates, and phytohormones to regenerate protoplasts of *Vicia narbonensis*. Bhojwani, Power, and Cocking (1977) have suggested 250mg/l NH₄NO₃ with 750mg/l CaCl₂·2H₂O together with the salts of B-5 to promote cotton hypocotyl callus protoplast growth. The medium of Table 2 has demonstrated 56% of all cells observed after 3 weeks in culture to be viable. In comparison, identical media altering only 0.71M glucose to 0.08M glucose with 0.63M mannitol resulted in 34% of the cells remaining viable after 3 weeks in culture. The medium suggested by Bhojwani, Power, and Cocking (1977) only contained 22% viable cells after the same culture duration.
CONCLUSION

This study was performed to devise a method for the maximum yield, purification, and culture of cotton anther callus derived protoplasts. Parameters of; pecto-cellulase enzyme source and concentration, osmoticum, pH, organic and inorganic digestion additives, culture age, gradient separation, and constituents of culture media were examined.

Optimal conditions for cotton protoplast isolation corresponded to 0.6g-0.8g of 5 day old callus digested with; 2% w/v TV Developmental Cellulase and 0.5% w/v Experimental Pectinase in 0.71M mannitol with 15mM arginine·HCl at pH 5.6 for 2 hours in the dark at 30°C. Afterwards, the solution is mixed with 2 volumes of 0.6M sucrose with 15mM arginine·HCl and allowed to stand for 1 hour. Protoplasts are then removed from the meniscus and washed in 9 volumes of KCM-2 buffer. After a 1-2 minute centrifugation at 100xg, the protoplast pellet is resuspended in culture medium of Table 2 and cultured at 1-2 X 10^5 protoplasts/ml in 3ml of either a Falcon No. 1006 tight lid petri dish or a 50ml screwcap flask in the dark at 30°C. After 3-4 weeks, macroscopic calli will be observed.
Protoplast technology has been a salient feature of recent advances in photosynthesis (Kanai and Edwards, 1973a; 1973b), plastid transfer (Bonnet and Banks, 1976), sexual incompatibility (Power et al., 1974; Schieder, 1978), and somatic hybridization (Kao et al., 1974). Anther culture coupled with protoplast techniques and plant regeneration have permitted the rapid production of homozygous plant lines with concomitant selection against sub-lethal genes in Solanum tuberosum (Wenzel et al., 1979). These authors found in microspore derived plants that the full genetic power present in the heterozygote was expressed. However, in protoplast derived plants from mesophyll callus slight alterations in the geno- and phenotype were observed. Such changes in the genetic expression of protoplast derived plants seemed related to the age of the mesophyll callus.

The importance of plant production by protoplast hybridization in agricultural development has been elaborated on by Nickell and Torrey (1969) and Carlson and Polacco (1975). Using normal plant tissues (leaf) such hybridizations are possible (as has been mentioned above). With future developments into genetic stability and expression in callus cultures, protoplast technology may further benefit the agricultural process.
Future protoplast mediated biochemical and breeding application in plants may be surpassed in importance by direct application to the human condition. Protoplast fusion with cytoplasmic mixing and cell wall deposition in human and other animal cells has been demonstrated (Dudits et al., 1976; Jones et al., 1975; Lima-De-Faria, Eriksson, and Kjellen, 1977; Willis, Hartman, and Lamater, 1977). As mammalian cells are sensitive to temperature in culture (Nelson et al., 1971) with temperature affecting in vitro aging (Thompson and Holliday, 1973) plant calli are not affected. A combined plant-animal hybrid then offers a unique eucaryotic system for such investigation. It is hoped that this methodology of cotton protoplasts will contribute to these and other future scientific inquiries.


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