A STUDY OF THE GENETIC IMPROVEMENT OF QUAKING AND BIGTOOTHE ASPEN BY SELECTION, HYBRIDIZATION, AND THE EXPLOITATION OF POLYPLOIDY

BIOCHEMICAL CHARACTERIZATION OF ASPEN AND ASPEN HYBRIDS

Project 2412
Report Three
A Progress Report

LOUIS V. AND MAUD HILL FAMILY FOUNDATION

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SUMMARY

The present report covers a study of the antigens obtained from *Populus tremuloides*, triploid, leaves and *Populus canescens*, diploid, leaves, the preparation of which was described in Progress Report One, Fig. 11 and 14. The balance of the report covers the development of tissue culture techniques, the use of callus tissue as a source of antigens, the preparation of antisera, and the evaluation and modification of immunoelectrophoresis, immunodiffusion, disk* electrophoresis, and immunodisk* electrophoresis techniques.

*Spelling of the word "disk" throughout this report follows usage in Webster’s Third International Dictionary rather than the alternative spelling – "disc."*
INTRODUCTION

The objectives of the present work were discussed in detail in Progress Report One. The first objective, "to extend the range of genetic markers to the biochemical level," was defined broadly as a study of protein differences between trees. The second objective, "to develop rapid, efficient techniques for the detection of biochemical markers," was defined broadly as the design, development, and application of techniques capable of detecting genetically significant differences in the macromolecular components of aspen. The work is essentially a feasibility study to establish the major techniques and variables which must be used or taken into account if biochemical information is to be exploited within the framework of a tree improvement program.

LEAF ANTIGENS

ANTISERA PRODUCTION

Rabbits were injected in groups of two with the four antigen preparations described in Fig. 1 and 2, *P. tremuloides* antigens (TF and TS) 1 ml. per rabbit per injection, *P. canescens* antigens (CF and CS) 1.6 ml. per rabbit per injection. Two control rabbits were not injected. Abdominal subcutaneous injections were made on May 22, 25, 27, 29, June 1, 3, 4, and 5. On June 12 all rabbits were bled from the marginal ear vein by shaving the ear in the location of the vein, rubbing xylene on the skin, clamping the vein near the base of the ear, lancing the vein with a razor blade and allowing the blood to drip directly into sterile test tubes which were then stoppered and placed in an ice chest. Twenty-five to 75 ml. of blood were collected by this method from each rabbit. The blood was allowed to clot overnight, the clot removed and the serum centrifuged,
Lyophilized products (combined, 6 g. total)  
(See Fig. 11, Report One)  
\[ \text{26 g. aqueous solution} \]  
\[ \text{Diluted to 40 ml.} \]  
\[ \text{10 p.p.m. merthiolate added} \]  
\[ \text{(8 mg. protein/ml.)} \]  
\[ \text{25 ml. for rabbit immunization} \]  
\[ \text{15 ml. for double diffusion experiments (Fraction T-P)} \]

\[ \text{12.5 ml. plus 12.5 ml.} \]  
\[ \text{Freund's adjuvant (Fraction TF)} \]  
\[ \text{12.5 ml. plus 12.5 ml. physiological saline (Fraction TS)} \]

Figure 1. Preparation of \textit{P. tremuloides}, Triploid, Antigens for Rabbit Immunization

285 ml. final dialyzed volume  
(3.33\% dry weight, 0.6\% N)  
(See Fig. 14, Report One)  
\[ \text{10 p.p.m. merthiolate added} \]  
\[ \text{Concentrated to 70 ml. (approximately 350 mg. protein)} \]  
\[ \text{(dialysis sac in contact with dry Sephadex G-200)} \]  
\[ \text{50 ml. for rabbit immunization} \]  
\[ \text{20 ml. for double diffusion experiments} \]

\[ \text{25 ml. plus 25 ml.} \]  
\[ \text{Freund's adjuvant} \]  
\[ \text{(approximately 2.5 mg. protein/ml.) (Fraction CF)} \]  
\[ \text{25 ml. plus 25 ml. physiological saline (approximately 2.5 mg. protein/ml.) (Fraction CS)} \]

Figure 2. Preparation of \textit{P. canescens} Antigens for Rabbit Immunization
20,000 x g. for 10 minutes. Ten p.p.m. merthiolate was added to each antiserum and all stored at 2 to 4°C.

On July 23 the same rabbits received one additional injection and were bled again on August 6. The antisera were prepared and preserved as described above. On August 10 the antisera were lyophilized (Table I).

**TABLE I**

<table>
<thead>
<tr>
<th>Antiserum Identification</th>
<th>Antiserum Weight, g.</th>
<th>Lyophilized Antiserum Weight, g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-1</td>
<td>12.09</td>
<td>0.87</td>
</tr>
<tr>
<td>TS-2</td>
<td>Lost: rabbit died during period of injection</td>
<td></td>
</tr>
<tr>
<td>TF-1</td>
<td>30.69</td>
<td>2.33</td>
</tr>
<tr>
<td>TF-2</td>
<td>17.91</td>
<td>1.41</td>
</tr>
<tr>
<td>CS-1</td>
<td>20.28</td>
<td>1.37</td>
</tr>
<tr>
<td>CS-2</td>
<td>25.49</td>
<td>1.67</td>
</tr>
<tr>
<td>CF-1</td>
<td>25.52</td>
<td>1.97</td>
</tr>
<tr>
<td>CF-2</td>
<td>20.41</td>
<td>1.50</td>
</tr>
<tr>
<td>Control</td>
<td>20.26</td>
<td>1.40</td>
</tr>
</tbody>
</table>

*Antiserum identified by antigen preparation used in its production, see Fig. 1 and 2. Numbers identify individual rabbits from which antiserum was obtained. Aliquots of the sera were not lyophilized.*

**EXAMINATION OF ANTIGEN PREPARATIONS AND ANTISERA BY DOUBLE DIFFUSION**

Double diffusion experiments were carried out in 100-mm. Petri dishes as described in Progress Report One, except that Oxoid Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) was used and that the results were recorded photographically without washing the agar gel. A low angle illumination
unit was constructed using a 12-inch circular fluorescent light source mounted in a black box in such a manner that it was vertically adjustable. The Petri dish to be photographed was floated on water in a shallow Plexiglas dish the bottom of which extended into the box approximately one-quarter inch centered with respect to the fluorescent light. The sides of the Plexiglass dish were blackened in order to reduce undesirable reflections. With this apparatus the precipitin bands were clearly visible. The illumination apparatus was mounted under a photographic enlarger equipped with a shutter and a 4 by 5-inch plate holder. The results were recorded photographically as follows: (1) with Polaroid Type 52 Pola Pan 200, (2) by reproduction of the Polaroid positive by making a copying negative on Gravure copy film and contact printing on Polycontrast paper, (3) and with Contrast Process Panchromatic Film contact printed on Polycontrast paper. Two copies of the present report contain Polaroid positives and reproductions from Polaroid positives as indicated in the figures. (One such copy marked "primary copy" was retained by the Institute and one forwarded to the co-operator.) All other copies of the report are fabricated using prints from the Contrast Process Panchromatic negatives except for Fig. 3, A and B, which in all copies of the report are reproductions from Polaroid positive prints. The Polaroid film gave superior definition of the precipitin bands and closely approximated the visual appearance of the plates in all major characteristics. Some loss of detail was apparent.

Double diffusion experiments were carried out using the *P. tremuloides*, triploid, antigen preparation, Fig. 1, and *P. canescens* antigen preparation, Fig. 2. The organization of the experiments and results are shown in Fig. 3 and 4. The results with the *P. canescens* antigens which had been prepared by alcohol precipitation were essentially negative because of the poor solubility of the antigen preparation. This was believed to be the result of the alcohol
precipitation technique and not an inherent difference in the *P. canescens* protein.

No photographic record was made of the *P. canescens* experiments. It was apparent, however, that the preparation was antigenic as indicated by the precipitin reactions with the *P. tremuloides* antigen preparation. The experiments were carried out with the antisera described in Table I.

From the results, Fig. 3, it was apparent that the use of adjuvant during immunization resulted in antisera which were superior qualitatively and quantitatively (with respect to numbers and clarity of precipitin bands) to those induced with antigen preparations administered in saline without adjuvant. Figure 3, C and E, indicated clearly the adjuvant effect. An examination of the precipitin bands also revealed differences in the antisera from different rabbits immunized with the same antigen preparation. Figure 3, G and H, indicated that lyophilized antisera produced a larger amount of nonspecific precipitation but was clearly superior with respect to freedom from contamination. The white zones spreading outward from the antisera cups, Fig. 3, H, are colonies of micro-organisms which were present in the sera. The essential features of the patterns obtained with lyophilized and original liquid sera are the same. Some bands are more distinct with the lyophilized sera.

Differences and similarities between *P. tremuloides* and *P. canescens* antigen preparations can be judged by comparing the patterns obtained with the two respective antisera when diffused against *P. tremuloides* antigen preparation. Figure 3, A, B, C, and D, shows common bands (as evidenced by the formation of continuous, interconnecting, qualitatively similar patterns) and a number of different bands (as evidenced by discontinuous, noninterconnecting, or crossing, qualitatively different patterns) between the cups containing "tremuloides" and "canescens" antisera. Because of the insolubility of the "canescens" antigen preparation the alternative comparison was not successful. It was noted that at least one
Figure 3. Double Diffusion. *Populus tremuloides*, Triploid, Antigen in Center Wells. Outer Wells Contain Antisera as Indicated (See Table I)
Figure 3 Continued. Double Diffusion. *Populus tremuloides*, Triploid, Antigen in Center Wells. Outer Wells Contain Antisera as Indicated (See Table I)
band was formed with the control serum but a comparable band did not appear with all antisera. Figure 4 shows the effect of dilution of the antigen preparation on the character of the precipitin bands. It is probable that some of the apparent differences in Fig. 3 can be attributed to concentration effects. The results also indicate that protein concentration is a major variable in double diffusion experiments.

Figure 4. Double Diffusion. Triploid Antigen Dilution Series in Outer Wells. TF-2 (Table I) Antiserum in Center Well

TISSUE CULTURE

OBJECTIVES

One of the major problems of selecting genetic markers, especially at the biochemical level, is the identification of markers which appear consistently under the conditions of the experiment; that is, markers the presence or absence of which clearly relate to genetic changes rather than environmental changes. The basic mechanisms for macromolecular synthesis in the cell are
understood, at least in broad outline. Both the qualitative and quantitative aspects of macromolecular synthesis are strongly affected by environment. Changes in both the macroenvironment and the microenvironment (which changes as cell populations differentiate) are important. The sampling of natural populations of cells in higher plants (e.g., the leaf tissues used in the work discussed above) can be expected to show variation of macromolecular content from young to old leaves from site to site, and from season to season. A comprehensive statistical study would be required to distinguish environmental from genetic variation.

Steward, Lyndon, and Barber (3) have demonstrated clearly changes in macromolecular (protein) content with respect to age of the pea root. The alternative is to control the environment so that differences expressed are necessarily based on genetic differences. This is not easily achieved with higher plant cells because they tend to grow slowly and to produce clumps of cells possessing a strong tendency to differentiate. One of the basic objectives is to establish conditions of growth where each cell will be equally affected by the environment. Ideally, this would require completely dispersed cell growth; that is, conditions where every cell would differentiate identically. This state is most closely approached with micro-organisms, and becomes more difficult to achieve as the complexity of the organism increases. The natural disposition of plant cells to aggregate makes serious the problem of standardizing growth in tissue culture. Callus tissue showing a nonpolarized or generalized differentiation may prove to be sufficiently reproducible to be usable for comparative purposes. Ultimately, it may be possible to induce truly dispersed single cell growth and achieve the stability of growth response which is now possible with certain tissue cultures of higher animal origin.
PROCEDURE

P. tremuloides, triploid, callus tissue (referred to as triploid callus tissue) stock culture was maintained on coconut milk Medium No. 23, on a rotary shaker as described in Progress Report One. A large reciprocating shaker capable of carrying 36 three-liter Erlenmeyer flasks was constructed. The rate of oscillation was continuously variable and the length of the stroke variable in 1-cm. increments from 1 to 6 cm. The stroke and rate of shaking were adjusted to give a nearly splashing agitation of the liquid medium to insure good aeration. Five-hundred milliliters of medium per flask was found to give a satisfactory yield per flask.

Tissue clumps (irregular, approximately 3/8 inch in diameter) were transferred aseptically to sterile Petri dishes and chopped into small pieces (approximately 1/16 inch in diameter) with sterile razor blades. The chopped tissue was then used as inoculum. Table II gives the results of a preliminary experiment on the reciprocal shaker using 1000 ml. of coconut milk Medium No. 23 per three-liter flask at 29 to 30°C. for 24 days.

It was desirable in the present investigation to be able to examine the protein (antigenic) components of the growth medium which derived from the growing triploid callus tissue. For this reason, in large-scale callus production only the dialyzable fraction of coconut milk was used in the preparation of the growth medium. A preliminary experiment demonstrating the suitability of the method is given in Table III. The loss of activity was significant but the yield was adequate and the physical texture of the tissue satisfactory for use of the medium in large-scale production. The procedure used for sustained culture of triploid callus tissues is given in Fig. 5.
### TABLE II
YIELD OF TRIPLOID CALLUS TISSUE IN LIQUID CULTURE

<table>
<thead>
<tr>
<th>Flask</th>
<th>Inoculum, g.</th>
<th>Final Weight, g.</th>
<th>Net, g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.98</td>
<td>contaminated</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.20</td>
<td>59.1</td>
<td>56.9</td>
</tr>
<tr>
<td>3</td>
<td>2.32</td>
<td>29.9</td>
<td>27.6</td>
</tr>
<tr>
<td>4</td>
<td>2.41</td>
<td>37.7</td>
<td>35.3</td>
</tr>
<tr>
<td>5</td>
<td>1.37</td>
<td>38.6</td>
<td>37.2</td>
</tr>
<tr>
<td>6</td>
<td>1.25</td>
<td>contaminated</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.84</td>
<td>55.9</td>
<td>54.1</td>
</tr>
<tr>
<td>8</td>
<td>1.75</td>
<td>59.9</td>
<td>58.1</td>
</tr>
<tr>
<td>Total</td>
<td>15.12</td>
<td>Yield = 18.6 fold weight increase</td>
<td>281.1</td>
</tr>
</tbody>
</table>

### TABLE III
THE EFFECT OF DIALYSIS ON THE ADEQUACY OF COCONUT MILK FOR ASPEN TISSUE CULTURE

<table>
<thead>
<tr>
<th>Medium</th>
<th>Inoculum, g.</th>
<th>Final Tissue, g.</th>
<th>Net, g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 23</td>
<td>0.097</td>
<td>4.69</td>
<td>4.59</td>
</tr>
<tr>
<td>No. 23 without coconut milk</td>
<td>0.097</td>
<td>0.92</td>
<td>0.00</td>
</tr>
<tr>
<td>No. 23 with dialyzable fraction of coconut milk</td>
<td>0.098</td>
<td>3.74</td>
<td>3.64</td>
</tr>
<tr>
<td>No. 23 with nondialyzable fraction of coconut milk</td>
<td>0.103</td>
<td>1.28</td>
<td>1.18</td>
</tr>
</tbody>
</table>
Triploid callus tissue grown in coconut milk medium* at 29°C. for 28 days in the dark with continuous reciprocal shaking.

\[ \text{Callus tissue separated from growth medium on a Buchner funnel without filter paper} \]

\[ \text{Callus tissue washed with distilled water (see Fig. 8)} \]

\[ \text{Growth medium (see Fig. 6)} \]

**Figure 5. Production of P. tremuloides, Triploid, Callus Tissue in Coconut Milk Liquid Medium**

**PREPARATION OF ANTIGENS**

**GROWTH MEDIUM ANTIGENS**

Figure 6 details the procedure employed in the isolation of the high molecular weight, water-soluble components of the growth medium. In the utilization of coconut milk growth medium as a source of antigenic material, the question arose as to whether any of the antigens (GM-P) found in the growth medium may have been components originally present in coconut milk. An experiment as outlined in Fig. 7 was carried out to test this possibility.

A micro-immunodiffusion test was run on Samples 1, 2, and 3 according to procedures described under micro-immunodiffusion. The five antisera listed.

*Medium, (coconut milk, Medium No. 23, Progress Report One) was used except that no agar was included and the coconut milk was treated differently. Fresh coconut milk was heated to 60°C., filtered and dialyzed in cellulose casing against cold distilled water (1 volume of coconut milk diluted to 10 volumes after dialysis). This dialyzate was incorporated in the medium and the fraction remaining in the dialysis casing discarded. This procedure prevented the carry-over of high molecular components into the medium which was dispensed into 3000-ml. Erlenmeyer flasks, 500 ml. per flask, and autoclaved. Each flask was inoculated with several 28-day callus tissues cut aseptically into small pieces.*
Growth medium (see Fig. 5)  
↓  
Filtered Whatman No. 1  
↓  
Dialyzed in cellulose casing against distilled water  
↓  
Concentrated at room temp. by pervaporation to about 1/10 original volume  
↓  
Lyophilized  
↓  
Dissolved in 45 ml. distilled water  
↓  
Centrifuge, 20,000 X G, 10 min.  
↓  
Sephadex G-100 (Fig. 28) GM  
↓  
Fractions combined  
↓  
Sephadex G-25 (Fig. 26) GM  
↓  
Excluded fraction, 800 ml.  
↓  
Lyophilized, 626 mg. GM-P  
↓  
Salt fraction, 200 ml.  
↓  
Lyophilized, 2.67 g.  
↓  
Trailing salt-free fraction, 1000 ml.  
↓  
Concentrate by pervaporation  
↓  
Lyophilized, 33 mg.  
↓  
Balance:  
250 mg. +12.5 ml. saline  
+12.5 ml. Freund's adjuvant  
Immunoelectrophoresis  
Disk electrophoresis  
Sephadex G-100 (Fig. 27)  

Figure 6. Preparation of Antigens From Growth Medium Used in Production of *P. tremuloides* Callus Tissue
Fresh coconut milk, 166 ml.

- 50 ml. lyophilized 4.109 g., (Sample 1)
- Immunodiffusion

116 ml. heated to 60°C.

- Cooled and filtered on Whatman No. 1 paper

- 50 ml. dialyzed in cellulose casing against 300 ml. distilled water

50 ml. lyophilized 4.088 g. (Sample 2)

- Immunodiffusion

Portion inside dialysis sac, discarded

Dialyzate diluted to 500 ml.

- Autoclaved

- Cooled and filtered on Whatman No. 1 (Sample 3)

- Immunodiffusion

Figure 7. Preparation of Coconut Milk for Micro-Immunodiffusion Control Experiment
Fresh callus tissue (see Fig. 5) frozen in buffer
(0.2M K$_2$HPO$_4$, 0.05M NaCl, pH 7.7)

\[ \downarrow \]

Slotted tissue press
(see Fig. 9, 10, 11)

\[ \downarrow \]

Centrifuge 10,000 X G, 20 min.

\[ \begin{align*}
\text{Sediment} \\
\text{Water added to 800 ml.,} \\
\text{extracted in cold for 24 hr.} \\
\text{Centrifuge 10,000 X G, 20 min.} \\
\text{Sediment} \\
\end{align*} \]

\[ \begin{align*}
\text{Supernant} \\
\text{Add buffer to 900 ml.,} \\
\text{extracted 72 hr.} \\
\text{Centrifuge 10,000 X G, 20 min.} \\
\text{Sediment} \\
\end{align*} \]

\[ \begin{align*}
\text{Supernant} \\
\text{Concentrated to 150 ml.} \\
\text{by pervaporation} \\
\text{Centrifuge 20,000 X G, 10 min.} \\
\text{Sediment} \\
\end{align*} \]

\[ \begin{align*}
\text{Dialyze in cellulose} \\
\text{casing against saline} \\
\text{Sephadex G-25 (Fig. 26)} \\
\text{SED-P} \\
\end{align*} \]

\[ \begin{align*}
15 \text{ ml. + 15} \\
\text{ml. Freund's adjuvant} \\
\text{Rabbit immunization} \\
\text{(see Table V)} \\
\end{align*} \]
Figure 9. Large-Capacity Stainless Steel Tissue Press With Air-Actuated Hydraulic Pressure Regulator. Nitrogen Tank at Left of Press is Used to Purge Collecting Train of Air (See Fig. 10)
Figure 10. Large-Capacity Tissue Press Showing Collection Train in Deep Freeze. Note Frozen Mass of Tissue Removed From Cylinder
a permanent plain face. The slot width employed in the present work and found to be highly satisfactory was 0.0126 inch. The procedure for tissue disruption was as follows: The bottom of the cylinder was plugged with a liquid-tight nylon plug which extended into the cylinder to the same depth as the slotted base when the cylinder is mounted in the press. Dry ice was packed around the cylinder and a slurry of buffer and callus tissue poured into the cylinder to within approximately 1-1/4 inches from the top to allow room for the introduction of the piston. After freezing, the nylon plug was removed and the cylinder mounted on the base in the press as shown in Fig. 9. A pressure of approximately 3000 pounds per square inch was maintained on the press by means of a manually operated hydraulic pump, later replaced by an air-actuated hydraulic pressure regulator shown in Fig. 9 and 10. In approximately 15 minutes, sufficient heat was transferred to the frozen cylinder of tissue to start melting at surfaces. As the buffer melted the tissue was crushed between the ice and the walls of the press and subjected to a shear stress as the liquid was forced out the slot in the base. As the liquid emerged from the press, the release of pressure caused the melting point to return to normal (above the temperature of the emerging cell paste). The cell paste immediately refroze resulting in the extrusion of a frozen ribbon of cell paste which was collected in the cold to prevent melting and to preserve it until the next step, centrifugation. Figure 10 shows a collection train (developed since the completion of the present work) which allows direct collection of sequential samples in the deep freeze under a stream of nitrogen gas to retard oxidative reactions in the tissue preparation. Microscopic examination of the cell paste revealed satisfactory cell breakage.
Figure 12. Utilization of T-10-P Antigens Prepared From P. tremuloides, Triploid, Callus Tissue
T-14-P
(Excluded fraction, Sephadex G-25, 305 ml.)

\[ \downarrow \]

Lyophilized, 176 mg.

91 mg.

\[ \downarrow \]

61 mg.

\[ \downarrow \]

Sephadex G-100 (Fig. 27)

Fractions combined

Concentrated by pervaporation

Lyophilized, 350 mg.

42 mg. Disk electrophoresis

308 mg. + 7.5 ml. saline

Immunodiffusion Immunelectrophoresis Disk electrophoresis

\[ \downarrow \]

Figure 13. Utilization of T-14-P Antigens Prepared From P. tremuloides, Triploid, Callus Tissue
T-15-P
(Excluded fraction, Sephadex G-25, 275 ml.)

Lyophilized, 51 mg.

↓

35 mg.
+ 3.5 ml. saline
+ 3.5 ml. Freund's adjuvant

Rabbit immunization
(see Table V)

↓

16 mg.

Sephadex G-100 (Fig. 27)

Effluent lyophilized,
16 mg.

↓

Add 2 ml. saline

Immunodiffusion
Immunoelectrophoresis
Disk electrophoresis

Figure 14. Utilization of T-15-P Antigens Prepared From P. tremuloides, Triploid, Callus Tissue

DISK ELECTROPHORESIS

The techniques and apparatus used were essentially those described by Davis (1) with minor modifications.

APPARATUS

The basic apparatus, Fig. 15, followed the design of Davis except that the buffer vessels were constructed of 3/8-inch Plexiglas, the electrodes were high purity carbon arc electrodes which were discarded when etched or dirty, and the adjustable support for the upper buffer vessel was designed to allow the
rotation of the upper reservoir and to conserve bench space. The lower electrode extended through the bottom of the lower reservoir to allow connection with the positive lead of the power supply underneath the reservoir. This permitted rotation of the upper unit for observational and photographic purposes without interference with the lower lead. The carbon electrode of the upper reservoir was mounted in a vertically drilled center hole in the bottom of the reservoir to allow the upper electrode to remain stationary during rotation of the upper reservoir. A RECO Model E 800-2 power supply (Reco Instrument Corporation, Oakland, California) with a 750-volt, 200 ma., maximum output was used in the disk electrophoresis experiments cited in this report. Preliminary experiments were carried out using the power supply of a Spinco, Model H, Electrophoresis-Diffusion apparatus (Beckman Instruments Incorporated, Palo Alto, California).

PROCEDURE

The procedure employed was identical to that of Davis (1) except for the order and sequence of the steps in formation of the acrylamide gel columns. Glass tubes 5 mm. I.D. x 100 mm. long were mounted in B-D Vacutainer caps. Small pore gel, 1.35 ml., was added to the tube and overlaid with water using a micrometer pipet to prevent surging and mixing at the monomer-water interface. After polymerization of the small pore gel, the water was removed, the surface of the gel washed twice with large pore gel and 0.1 ml. of large pore spacer gel photopolymerized after overlayering with water. The water was again removed and the surface of the spacer gel washed twice with large pore gel. Sample gel, 0.1 ml., was added and photopolymerized without adding a water layer. This technique requires less time than the Davis technique between the preparation of the sample gel and electrophoresis. It also eliminates the necessity of a two-step formation of the spacer gel. After the gel column was removed from the B-D Vacutainer cap
and mounted in the grommets of the upper reservoir, if the sample gel polymerization was inhibited, the sample was overlayered (micropipet) with the buffer-bromphenol-blue solution to be used in the upper reservoir. Buffer-bromphenol-blue solution was added to the upper reservoir and buffer without bromphenol blue to the bottom reservoir which was filled very close to the top. The gel columns were mounted so that their ends extended approximately one-quarter inch below the surface of the buffer in the lower reservoir and were bubble free. With this arrangement, the entire gel column, except for the lower one-quarter inch, was visible and the tubes could be rotated to the front for inspection. (Caution: The power should be turned off before touching the apparatus.)

The staining and destaining procedure followed that of Davis (1) except that in the destaining procedure a crystallizing dish was substituted for the upper reservoir and U-shaped 8-mm. tubing was used to form the buffer bridge between the destaining tube and the upper reservoir, Fig. 16. This arrangement allowed the addition or removal of gels at any time.

MICRO-IMMUNODIFFUSION

PROCEDURE

Immunodiffusion experiments were carried out using a Standard Immunodiffusion Set LKB* 6800A-7. This equipment was designed for micro-immunodiffusion tests in a 1-mm. thick agar gel layer on microscope slides. Microscope slides were held in three plastic frames (six slides per frame) mounted on a leveling board during formation of the agar layer. A hot (85 to 90°C.) agar solution (1% Oxoid Ionagar No. 2, 1% sodium chloride, 1% sodium

*LKB Produkter AB, Stockholm, Sweden.
azide) was layered on the slides and, after 15 minutes, when the agar solution had gelled, the frames were transferred to a holder which was placed in a humid chamber for at least 30 minutes.

A modification of the LKB diagonal immunodiffusion pattern was used for this work. A trough 2-mm. wide was punched in the center of each microscope slide using a die designed for immunoelctrophoresis. The inner well cutters of the diagonal die were removed and the outer well cutters used to punch five wells, outside diameter 3 mm., on each side of each trough. The gel was removed from the wells with a suction needle attached to a filter flask, and removed from the troughs with a gel knife. Antisera were placed in the troughs and antigens in the wells.

The frames were then placed in a humid incubation chamber at room temperature (+ 2°C.) for 20 to 30 hours. During this time, counterdiffusion of antigens and antibodies produced visible arcs of precipitation where homologous antigens and antibodies met. Unprecipitated protein was removed by washing in a 1% NaCl bath for 6 hours followed by 16 hours in a fresh 1% NaCl solution. The arcs of precipitation were best detected by drying, staining and destaining; the staining solution was: 6% Buffalo black in methyl alcohol, acetic acid and water in the proportions 45:10:45. The staining time was 5 minutes followed by 10 minutes in each of four rinsing baths (methyl alcohol-acetic acid-water 45:10:45). The slides were removed from the plastic frames, allowed to air dry and labeled.
IMMUNOELECTROPHORESIS

PROCEDURE

An Immunophor Standard Conversion Set LKB 6800A-2 was used to obtain the micro-immunoelectrophoresis results presented in this report. Immunoelectrophoresis differs from immunodiffusion in that an electrophoretic separation of antigens precedes diffusion against specific antisera.

The preparation of agar films on microscope slides was the same as for immunodiffusion; the agar solution consisted of Ionagar, buffer solution and distilled water 1:25:75 (g.:ml.:ml.). The buffer solution was sodium diethylbarbiturate 29.34 g., sodium acetate·3H2O 19.42 g., 0.1N HCl 180 ml. and water to 3000 ml. (ionic strength 0.1, pH 8.6).

A 2-mm. wide trough was cut in the center of each slide and a small well, outside diameter 1.5 mm., for antigens on each side and near the middle of each trough. The diffusion distance, or distance between the wells and the trough, was 1.6 mm. After removal of gel, 1 to 2 µl. antigen were placed in each well. The frames were then placed in an electrophoresis chamber similar to LKB model 3276 BN. Electrical contact between the gel layer and the buffer solution in the electrode vessels was achieved with rayon wicks. A current of 50 ma. (Reco, Model 800-2 power supply) was applied and electrophoretic separation was allowed to proceed for about 1-1/2 hours. The gel was then removed from the troughs, antiserum was added and diffusion allowed to proceed at room temperature for about 24 hours. Washing, drying, staining and destaining of the gel was carried out as described for immunodiffusion.
IMMUNODISK ELECTROPHORESIS

APPARATUS

Disk electrophoresis was carried out in the manner described above. An apparatus was designed to mold an agar gel layer to receive the extruded cylindrical acrylamide gels and to receive antisera in troughs for immunodiffusion experiments, Fig. 17 and 18. It consisted of a Plexiglas base on which 3-1/4 by 4-inch lantern slides were mounted side by side against a straight edge (1/8-inch Plexiglas strip), Fig. 17. The mold, shown resting on its side in Fig. 17, was made up of four 5 mm.-glass rods which ran the full length of the mold and four rows of 1.5 by 65 mm. Plexiglas trough molds, best seen in Fig. 18, located on center between the glass rods and centered over the lantern slides.

PROCEDURE

With the lantern slides mounted as shown in Fig. 17, the outer edges of the slides were lightly coated with petroleum jelly to prevent the flow of agar over the edge when forming the film. The base was then placed on a leveling board and sufficient agar (1.2% Ionagar No. 2, 1% NaCl) poured over the lantern slides to form a film 1 mm. deep. While the agar was still in the liquid state the mold was placed on the lantern slides, the agar allowed to solidify, and the entire assembly placed in the cold room (2 to 4°C.) for about an hour after which the mold was removed by carefully rotating the glass rods to loosen them and lifting one end to affect its release. At this point the extruded, cylindrical acrylamide gels were placed in the round bottom troughs formed by the glass rods, the desired antiserum placed in the adjacent rectangular troughs, Fig. 19, the assembly incubated at room temperature in a humid chamber for 24 hours prior to washing, drying,
staining, and destaining in the manner described for immunoelectrophoresis on microscope slides.

RESULTS OF IMMUNODIFFUSION, IMMUNOELECTROPHORESIS, AND IMMUNODISK ELECTROPHORESIS

The organization and results of immunodiffusion experiments carried out on microscope slides with the above-described antigen and antisera preparations are summarized in Fig. 20. All possible pairs of antigens and antisera were directly compared. Where triploid leaf antigens (T) were diffused against triploid serum, several bands developed; the observed number is a minimum estimate of the number of antigen-antibody systems since some may interfere or superimpose. Precipitation patterns should be examined at various antigen concentrations if comparisons are to be made.

The problems of identification, discrimination, and quantification in gel immunodiffusion are discussed by Feinberg (2). Comparisons of precipitin bands are frequently described in terms of identity, nonidentity, or partial identity. If antigens are identical, their precipitin bands are formed in the same relative position and join laterally forming continuous symmetrical arches. If antigens are the same but differ in concentration, the arch will be distorted toward the well containing the weaker antigen. Nonidentical antigens produce precipitin bands which cross without interference, i.e., they do not join laterally to form continuous arches. A reaction of partial identity produces a spur where the arches join laterally. In Fig. 20, examples of partial identity can be seen, i.e., where T-10 and T-15 antigens are paired and diffused against triploid (T) leaf antiserum. The numbers of apparent antigens is greatest for the triploid leaf preparation.
Figure 18. Immunodisk Electrophoresis (Ager Gel Mold: Shown in Position on Lantern Slides but Without Ager Film)
Figure 19. Immunodisk Electrophoresis (Immunodiffusion Step, Run 3). As Viewed, Electrophoresis was From Left (-) to Right (+) in the Acrylamide Gels Except for the Lower Right Gel Which was Oppositely Oriented When Placed on the Molded Agar Film
Figure 20. Micro-Immunodiffusion. (Antigens Were Placed in Wells and Antisera in Troughs)
It is clear, however, that the resolution achieved by double diffusion is inferior to immunoelectrophoretic resolution, although in the present work, electrophoresis in agar gels on microscope slides was only slightly better than double diffusion; Fig. 21. Distinctly superior resolution was achieved with immunodisk electrophoresis, Fig. 22, 23, and 24. The superiority of the immunodisk electrophoresis is believed to be the result of distinctly superior electrophoretic separation, the use of higher absolute amounts of antigen, and the selective concentration of antigens during electrophoresis. The disk electrophoretic resolution can be seen in Fig. 25; the gels shown are replicates of the gels used in the experiment shown in Fig. 19 and 24.

The gels (shown in Fig. 25, in the electrophoretic destaining tubes) were stained and destained as described above. In the first tube (triploid), a remnant of the large pore spacer gel can be seen between the small pore gel (which occupies approximately the lower three-quarters of the tube) and the darkly stained cotton plug which was inserted into the top of the destaining tube to retard convection during destaining. The sample and spacer gels were usually lost during extrusion because of their soft texture and incomplete fusion with the small pore gel. Figure 19 is a photograph taken a short time after the extruded disk electrophoresis gels were placed on the agar for immunodiffusion. On the left end of some of the acrylamide gels can be seen the remnants of sample and spacer gels. Precipitin bands which appear in the vicinity of the spacer or sample gels are difficult to interpret because lack of mobility can be the result of nonspecific factors such as solubility or denaturation during photopolymerization of the sample gel. Taking into account the differences in photographic magnification in Fig. 25, 24, and 19 (roughly 1:1) the identification of some of the precipitin bands, Fig. 24, with specific disks (protein fractions) in Fig. 25, is possible. It is clear that even the improved electrophoretic separation
achieved with the disk technique falls far short of direct electrophoretic resolution of the immunologically identifiable components.

The lack of deeply staining protein disks in the first (triploid) gel, Fig. 25, can be accounted for in terms of the poorer resolution of the components as indicated by the long, shallow precipitin bands obtained with T-P, Fig. 24. Tailing in the small pore gel and poor steady state stacking in the initial separation in the large pore gel are the most likely causes of such bands. Generally, the width of the disk in the acrylamide gel is proportional to the concentration of the component(s) in the disk. The width of the disk is in turn related to the width (length of arc) of the precipitin band observed and the distance of the precipitin band from the acrylamide gel; the wider the disk the darker the band (concentration effect), and the greater the distance of the band from the acrylamide gel the wider and shallower the band (dilution effect). Where there are very light, long, shallow bands, the proteins were probably denatured (as evidenced by inhomogeneity of physical properties) during their preparation or electrophoresis. The relatively crude method of cell breakage (Waring Blendor) employed in the preparation of the triploid leaf protein is consistent with this possibility. Experiments described in Fig. 22 and 23, employed the same variables as the experiment described in Fig. 24, except that the times of electrophoresis and diffusion against antisera were slightly different (Table VI) and SED-P was omitted from subsequent experiments when it was found that the preparation contained insufficient soluble antigenic material to produce visible precipitin bands under the conditions of the experiment. It was noted, however, that SED-S (the antiserum obtained with SED-P) produced a number of precipitin bands with the other antigenic preparations. The dark zones around the serum troughs resulted from incomplete removal of "unreacted" antiserum. The first experiment, Fig. 22, and the second experiment, Fig. 23, were washed for 24 hours (one change of wash
Figure 24. Immunodisk Electrophoresis (Run 3). Lower Right Gel Reversed Relative to all Other Gels When Placed on Molded Agar Film
solution after 6 hours) prior to drying and staining. The cause of the poor
results in the second experiment is now known. In the third experiment, the agar
gel was washed for 72 hours (wash solution changed after 6 and 48 hours) which
gave satisfactory clearing and over-all superior resolution compared to the first
two runs.

Figure 25. Disk Electrophoresis
Figure 26. Elution of Fractions T-10, T-14, T-15 and GM From Sephadex G-25 Column (LKB Uvicord).

(See Fig. 6 and 8)
Figure 28. Elution of Fraction OM from Sephadex G-100 Column (Ultraviolet Absorption relative)
LITERATURE CITED

