Characterization of intercellular wash fluid from tobacco mosaic virus infected pinto bean primary leaves

Susan M. Geske
The University of Montana

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Characterization of Intercellular Wash Fluid from Tobacco Mosaic Virus Infected Pinto Bean Primary Leaves

by

Susan M. Geske

B.S., B.A. University of Montana, 1984
Presented in partial fulfillment of the requirement for the degree of Masters of Arts
University of Montana 1987

Approved by:

M. Cheverin
Chairman, Board of Examiners

E. Murray
Dean, Graduate School

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Characterization of intercellular wash fluid from tobacco mosaic virus infected pinto bean primary leaves. (66 pp.).

Director: Dr. Meyer Chessin

Intercellular wash fluid (IWF) was obtained from tobacco mosaic virus (TMV) infected primary leaves of Phaseolus vulgaris var. Pinto, on selected days after inoculation, via in vacuo infiltration of phosphate buffer and centrifugation. Tests for the presence of the intracellular enzyme glucose-6-P-dehydrogenase in the IWF extracts showed that the extraction procedure did not cause major cell damage.

As an inhibitor of virus establishment IWF had limited effect when mixed with virus prior to inoculation. But when infiltrated 6-9 hrs. after TMV inoculation, IWF reduced lesion numbers by 60-69% and diminished the appearance of larger lesion size classes indicating it acts as an inhibitor of virus replication.

IWF proteins produced distinct peaks in concentration during a 10 day period after virus inoculation. Peaks occurred 12 hours, 4 days, 7 days, and 10 days after infection. There also appears to be a slight periodicity over time in the levels of intercellular proteins after wounding only one-half of a single primary leaf with buffer and Celite. IWF extractions taken 7 days after inoculation contained the greatest protein concentrations.

IWF samples were dialyzed, in washed tubing, for 24 hrs. before testing inhibitory activity against virus establishment or replication. Inhibition results confirm that a substance responsible for virus inhibition, greater than 10,000-14,000 d molecular weight, is present in IWF extracts.

Increased quantities of 8 IWF proteins were the direct result of virus infection. These protein bands stained darker than other proteins with Coomassie brilliant blue on SDS-PAGE gels. The apparent molecular weights of these proteins were: 36,500 d; 34,000 d; 33,000 d; 28,750 d; 26,500 d; 18,200 d; 16,800 d; and 15,200 d. SDS-PAGE and immuno-blot experiments indicate the three lower molecular weight enhanced proteins were produced de novo, but the others were not.

High performance liquid chromatography tests of Day 7 IWF samples shows the appearance of a new protein in the TMV infected half-leaf and, to a lesser extent, in the adjacent half. The protein peak is detectable slightly or not at all in the opposite primary leaf.

IWF can function in inhibiting virus replication, although a specific antiviral protein has not been isolated in this study. The presence of enhanced, specific proteins in IWF from infected tissue indicates that cells are selectively releasing or allowing proteins to enter the intercellular spaces after TMV infection. General cell leakiness after virus infection apparently did not seem to have occurred.
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INTRODUCTION

Viruses and plants have long been associated with one another. Plant viruses are known to infect nearly all species of higher plants, that have been studied to date, either naturally or artificially (Hammond 1982). Cucumber Mosaic Virus (CMV) can infect plants found in almost all Angiosperm families, and Alfalfa Mosaic Virus (AMV) can infect 393 dicot and 8 monocot species (Beczner and Schmelzer 1972). Because viruses do not have a free living phase in their life cycles, they must rely on living plants year around. In many cases crop destroying viruses can survive adverse conditions or overwinter in weed species without causing harm or noticeable symptoms; yet when introduced into a susceptible crop, they can cause extensive damage. Crop yields can be reduced dramatically; in the case of Potato Virus X, reduced potato yields will not be indicated by disease symptoms on the above ground plant parts. Since there is a great potential for virus induced damage to a variety of crops and a lack of chemical means to control such diseases, it is imperative to study how plants are capable of defending themselves naturally. Enhancing a plant's natural defense mechanisms may then be the effective way to control viral diseases.

Plant Responses to Virus

Plants respond to virus infection in a few distinct ways. Few infections result in total destruction of a plant, since this could lead to the disappearance of that particular virus by eliminating or reducing the spread or appearance of
the host plant species. More often subliminal, systemic, and localized infections can occur, to a less detrimental degree, depending on the specific host-virus combination.

According to Sulzinski and Zaitlin (1982), subliminal infections are restricted to only a few cells. It is believed that within a population of plant cells, most cells are capable of carrying out virus replication. Yet, in subliminal infections the virus is unable to move from the infection center. Although the cells that are infected contain virus which is replicating at a normal rate, the host appears to be asymptomatic. The plant, in a sense, is resistant. New evidence (Taliansky et al. 1982) support Sulzinski's and Zaitlin's hypothesis that the host plant has an ability to destroy a "translocation protein", coded by the virus, which allows cell to cell movement. An addition of a "helper" virus to a host plant can induce the systemic spread of a subliminal infection by another virus. The helper virus may suppress the host plant's natural defense.

Another reaction to virus infection is a systemic response. From the original site of infection, a virus multiplies and spreads throughout the entire plant. Mottling and mosaic symptoms are often the result of a systemic spread (Sela 1981 c). Green islands, which are areas of cytologically normal cells that contain low concentrations of virus, are surrounded by chlorotic areas containing high titers of virus. These islands are resistant to subsequent virus infection (Chessin 1983) and are thought to be unable
to support TMV replication (Atkinson and Matthews 1970). The host-virus combination will determine whether a systemic infection will lead to stunted growth, low yields or a predisposition to other diseases (Matthews 1981).

The other common response of plants to virus infection is the localization of virus particles by the plant. Often localization is accompanied by necrotization of leaf tissue (Sela 1981 c). This is referred to as a hypersensitive response. Resistance to virus spread in the genus Nicotiana is conferred by a single gene (Sela 1981 and Otsuki et al. 1972) called the N-gene. The hypersensitive response is best known in tobacco (Nicotiana spp.) with TMV, although it occurs in many plant species. Hypersensitivity is a general, non-specific response to disease agents, but the response does have a temperature sensitive step. Tobacco varieties known to carry the dominant allele for hypersensitivity do not develop necrotic lesions at 28 C (Otsuki et al. 1972). This suggests that the N-gene itself does not control a cell's inherent ability to contribute to TMV synthesis. It also does not appear to affect cell to cell virus movement by altering cell structural or physiological properties.

Various cellular changes do occur after the onset of viral infection in a hypersensitively responding plant. Generally, there are starch accumulations near infection sites (Helms and McIntyre 1962) and increases in mitochondrial numbers, mitochondrial partial volume, and in membrane amounts of rough endoplasmic reticulum (Reunov et al. 1986). Reunov also noticed that lytic processes were
stimulated along with intensified golgi apparatus activity and a decrease in cell wall synthesis. Cellular metabolism is heightened as a result of TMV infection, leading to the eventual destruction of virus particles by hydrolases within cells undergoing autolysis. There is still debate whether phenols and toxic quinones are responsible for necrotization or are just a by-product of the process (Sela 1981c). In any case, cellular collapse follows and produces necrotic lesions. There tends to be very little virus in lesion centers, but the edges contain high titers. In tissues encompassing the lesions, no virus is detected (Yarwood 1960).

Virus particles multiply and spread at similar rates during the initial phases of their life cycle whether the particles are in systemic or hypersensitive plants (Otsuki et al. 1972). Lesion size and shape, however, may be determined by physiological and morphological differences between the heterogenous cells found in a leaf (Helms and McIntyre 1962). Lesions reportedly stop enlarging a few days after infection signalling a decline in virus multiplication (Sela 1981c; Otsuki et al. 1972; and Yarwood 1960). It is at this point that virus spread in a hypersensitive plant differs from spread in a systemic host. Average lesions may involve only 15.5 bean epidermal cells and microlesions may involve about one-fifth that number (Helms and McIntyre 1962). Lesions in tobacco plants may be somewhat larger. Thus, virus spread is effectively halted since only a small portion of leaf cells
are involved. Sela (1981 c) claims that localization of virus is a means of limiting virus replication and that necrosis of leaf tissue is a result of localization and not the cause. One effect associated with local lesion formation is the phenomenon of local acquired resistance (LAR). Tissues immediately surrounding local lesions are resistant to subsequent virus infection to varying degrees (Loebenstein 1972).

The other effect, which may just be a variation of LAR is systemic acquired resistance (SAR) (Sela 1981 c). Systemic resistance is induced by virus infection in one part of the plant which causes another part to become resistant to subsequent virus infection. Even leaves at a considerable distance from the original infection site become resistant (Roberts 1984; Ross 1966; Bozarth and Ross 1964; and Ross 1961). Wounding treatments do not induce SAR. Ross suggested that one or more substances formed during local lesion production, moved systemically to uninfected areas and activated mechanisms that normally limited lesion size or localized virus infection. Further work done by Sela and Applebaum (1962) demonstrated that a substance inhibitory to virus infection was present in virus infected tissues. They called this substance an antiviral factor. Since that time, a number of virus inhibitors have been isolated and characterized from diverse plant species.

Antiviral Substances

During the last 25 years, many substances were extracted from virus infected and healthy plants that appeared to be
antiviral in nature. Various workers, using different plant host-species and diverse extraction procedures, were able to demonstrate that an antiviral substance(s) of some sort was present in both. Ragetli and Weintraub (1962) and Grasso (1978) found endogenous inhibitors in Dianthus caryophyllus and Phytolacca americana along with 13 other species tested. Even though endogenous inhibitors are effective antiviral agents, they tend not to be protective of the host species which produces them. The potential for a viricide based on endogenous inhibitors is slim because most endogenous inhibitors have not been adequately shown to be inhibitors of virus replication. Since systemic infections are the most common response of crop plants to virus infection, a viricide which inhibits mostly virus establishment would not give adequate protection to a susceptible plant unless 100% inhibition is attained. More important are the induced inhibitors of virus establishment or replication, for they are substances that naturally defend the host species in which they are found and from which possible viricides may eventually be made.

Induced antiviral substances have been found in Chenopodium amaranticolor (Faccioli and Capponi 1983), Datura stramonium (Loebenstein and Ross 1963), Phaseolus vulgaris var. Prince (Kimmins 1969), Nicotiana glutinosa, N. tabacum var. Samsun NN, and other hypersensitive Nicotiana spp. (Mitra 1985; Loebenstein and Gera 1981; and Sela 1981 b). Not entirely understood, these substances can inhibit virus
Perhaps the first antiviral substance to be studied was the one isolated by Sela and Applebaum (1962) from *N. glutinosa* and appropriately called an antiviral factor (AVF). Sela and his co-workers have since purified and characterized AVF (Mozes et al. 1978; Antignus et al. 1977; and Sela et al. 1966). AVF, extracted from homogenized TMV-infected tobacco, was found to be a phosphoglycoprotein with a molecular weight of 22,000 d. It was stable in SDS and at pH 2.5. AVF does not complex with TMV particles nor does it affect the uncoating of the virus itself. AVF does not cause local lesion formation, yet it certainly is present in those tobacco species which respond in a hypersensitive manner; i.e., containing the N-gene.

A mode of AVF action has been proposed by Gat-Edelbaum et al. (1983) and Sela (1981 b). Their working model places AVF in the role of a messenger of antiviral activity (or a messenger stimulator) which is passed on to uninfected cells. AVF may then trigger defense mechanisms, which in turn confer virus resistance upon the cells.

A series of steps have been outlined to show how AVF production is stimulated. First, because AVF is released so quickly after virus inoculation (Gat-Edelbaum et al. 1983) there is thought to be a pre-existing AVF precursor within the cell. The N-gene is responsible for coding for processing enzymes which convert pre-AVF into active AVF in a coupled reaction with enzyme phosphorylation of some unknown component. Phosphorylation can be triggered by TMV or other
viral infections, fungal infections, TMV coat protein, polyacrylic acid, ethylene, dsRNA, synthetic nucleotides such as poly I: poly C, and tRNA among others (Gat-Edelbaum et al. 1983, Sela 1981 b and Kassanis 1980).

There is evidence that the infecting virus actually turns on the plant's defense mechanisms. AVF activity can only be shown after 1 or 2 days after inoculation. Presumably this allows time to accumulate enough TMV-dsRNA to stimulate AVF production. Once in its active form, AVF is then transported to healthy cells. Sela has not yet elucidated how translocation takes place or exactly what happens once AVF is present in an uninfected cell.

A second antiviral substance, distinct from AVF, has been isolated from virus infected N. tabacum var. Samsun NN protoplast medium and has been given the name IVR (Inhibitor of Viral Replication) (Loebenstein and Gera 1981). Loebenstein (1985, unpublished data), Gera and Loebenstein (1983), and Gera et al. (1983) purified and characterized this substance and differences were noted between IVR and AVF. General IVR properties include 2 biologically active principles – one a 26,000 d and the other a 57,000 d substance. The 57,000 d substance may of course be a dimer of the smaller substance. IVR is thought to be a protein, but whether it is a phosphorylated glycoprotein has not been ascertained. It is stable to pH 2.5 and at 80 C. IVR has not been detected in intact plants, although it does inhibit virus replication within intact plants (Gera and Loebenstein
1983). IVR inhibitory activity is not restricted to the inducing virus as Cucumber Mosaic Virus and Potato Virus X were two other viruses that IVR inhibited in the protoplast studies.

IVR is thought to suppress virus replication via stimulation of virus localizing mechanisms. IVR does not alter TMV directly nor does it affect the plasmodesmata number, indicating that physically restricting virus within the cell is not the mode of action of IVR.

Recently, a new antiviral substance has been isolated from TMV infected *N. glutinosa* protoplast medium (Mitra 1985). Two substances, designated AVS-30 and AVS-65, have antiviral properties. AVS-30 was determined to be endogenous; whereas, AVS-65 was an induced substance which showed extreme potency against viral RNA-directed protein synthesis. AVS-65 is similar to AVF in that it is a phosphorylated glycoprotein with a molecular weight of 24,000 d. It is also heat stable to 64 C. Mitra's extensive study demonstrated that AVS-65 is more inhibitory than AVS-30 and that AVS-65 can induce further production of AVS-65 in other cells. Thus, resistance can be transferred from cell to cell. AVS-65 is associated with dramatic decreases in TMV coat protein synthesis in infected protoplasts, but still has not been determined to be the immediate cause of that decrease.

AVS-65 is an end-product of a specific methodology and it is uncertain if the substances extracted from three hypersensitive varieties of tobacco are indeed the same.
However tests have been made using AVS-65 obtained from different plant species. AVS-65 isolated from *N. glutinosa* is more effective at inhibiting virus replication in protoplasts than in whole plants, possibly due to the presence of cell walls in intact plants which may limit AVS-65 access. It is also better at inhibiting virus replication in protoplasts when compared to the AVS-65s obtained from *N. Nicotiana* vars. Samsun NN and Xanthi nc. An AVS-65 from tobacco cultivar Samsun NN, however, works better at inhibiting virus establishment compared to the other two.

All three antiviral substances (AVF, IVR, and AVS-65) have been compared amongst themselves as well as with mammalian interferon. IVR and AVF are serologically weakly cross reactive but IVR and interferon are not reactive at all (Loebenstein 1985, unpublished data). Similarities include temperature and pH stability, and precipitation with zinc acetate. In addition, AVF and AVS-65 do not complex directly with virus particles. These molecules are active inhibitors at extremely low concentrations. AVS-65 has not been detected by common protein assays, although antibody production to AVS-65 has not been undertaken. Yet, it is highly potent (Mitra 1985). AVF may be active at only a few molecules per cell (Sela 1981 a) as compared to IVR and interferon which may also work at such relatively low concentrations (Gera and Loebenstein 1983 and Orchansky et al. 1982).

Pierpoint (1983), Chessin (1983), Reichman et al. (1983), and Rosenberg et al. (1985) describe current
similarities and differences between induced antiviral substances found in plants and interferon. Similarities include:

1. proteinaceous nature
2. similar molecular weights
3. antiviral activity
4. released by virus infected tissues
5. dependence on host transcription and translation for activity
6. dsRNA presence is mandatory for activity

An interesting report by Rosenberg et al. (1985) states that purified human leukocyte interferon inhibits TMV replication in tobacco protoplasts and that tobacco was 10-1000 times more responsive to interferon than the animal cell systems tested. However, this is only one isolated incidence, and plant antiviral substances have not been conclusively proven to inhibit animal viruses in animal cell systems (Sela 1981 a).

Because plant and animal systems are not routinely thought of as comparable, the differences between interferon and plant antiviral substances could be extremely important in delineating the universality of antiviral materials. As stated before, AVS-65 can induce more AVS-65 but interferon can not induce more interferon in other cells. Interferon is species specific in that it acts against all viruses to which a particular species is susceptible. Interferon is active only in its source species. AVF and IVR are not species specific.

Overall, there appears to be more than one class of antiviral substances that are induced in hypersensitive plants exposed to virus, much like the multiple classes of
antiviral substances found in mammals - the interferons. Certainly the plant species used and the type of extraction procedure followed plays a role in the actual antiviral substance found. A plant probably has many defense mechanisms to help it localize virus infections.

Pathogenesis Related (b) Proteins

Pathogenesis related (PR) proteins are a relatively new discovery. These proteins were originally thought to be strictly associated with viral or fungal pathogen induced diseases in tobacco (Antoniw et al. 1978; Gianinazzi et al. 1977; and Van Loon 1976). Since these new proteins are readily produced in virus infected, local lesion forming tobacco cultivars, it was believed that they were responsible for or at least associated with localizing mechanisms, systemic acquired resistance or inhibition of virus multiplication. Further work done throughout Europe and in Canada revealed some of the chemical and physical make-up of these proteins extracted from homogenized tissues, intercellular fluids, and protoplasts.

PR, PS, p or b proteins, as they are variously called, are low molecular weight substances, generally not exceeding 18,000 d (Redolfi 1983). They are soluble under acid conditions (pH 2.5 - 3.0), resistant to a range of proteases, and mobile in PAGE gels under non-denaturing conditions (Antoniw and White 1983). They appear at the onset of local lesion formation caused by virus or viroid infection (Fortin et al. 1985 and Abu-Jawdah 1983, 1982); however, they also
are induced by a wide diversity of chemicals or by natural ageing stresses such as flowering or leaf senescence (Van Loon 1983 and Fraser and Clay 1983). They are reported to be produced only in green tissues, not roots or inner stems (Asselin et al. 1985). There is still inconclusive evidence for de novo production of all b proteins (DeTapia 1986; Conejero et al. 1983; and Van Loon 1983), but it is agreed that these proteins are coded for by the host plant.

b - Proteins have not been proven to have any antiviral activities, but the tobacco species in which they are found also carry the N - gene; i.e. respond in a hypersensitive manner to TMV infection (Asselin et al. 1985 and Ahl et al. 1985, 1982). Their highest concentration can be found in a ring near lesion margins. A hypersensitive response is temperature sensitive. Above 30 C, necrotic lesions are not formed and neither are b-proteins (Van Loon 1985).

Ahl and her co-workers (1985) have extensively studied the presence of b - proteins in tobacco to devise a phylogeny for the genus Nicotiana. The phylogeny produced is remarkably similar to other phylogenies based on chromosomal and cytological studies. Her results indicate that protein bl is sexually transmitted via a monogenic inheritance. The ability to synthesize b - proteins in healthy tobacco hybrids is associated with high resistance to TMV and Tobacco Necrosis Virus infection (Ahl et al. 1983) and can be passed on to offspring.

Induced, b - protein like substances have been demonstrated in a variety of plant species. Because of the
complexity of extracting, analyzing, and comparing these induced proteins, each set of researchers has given distinct names to the proteins they have found. Redolfi et al. (1983) outlines 17 plant species and their unique induced proteins. Van Loon et al. (1983) did compare a few of these proteins with antisera to tobacco cultivar Xanthi nc and Samsun NN b - proteins. It was found that the b - proteins from Nicotiana species are serologically related, and perhaps identical, but b - proteins from all other plant species tested were not serologically reactive. They concluded that b - proteins from different genera are sufficiently different to be considered genus specific.

Conceivably, PR or b proteins are necessary in plant defense mechanisms. They can be found in intercellular fluids, where, it is hypothesized, they initiate enzymatic changes in the chemical composition or environment of the cell walls (Parent et al. 1984). Others believe they are affiliated with the recovery phenomenon of diseased plants (Nuecken and Nienhaus 1983), acquired resistance phenomena (Antoniw et al. 1980 and Coutts 1978) or are just a general phytopathological response of the plant to infection and symptom development (Comacho-Henriquez et al. 1983).

Kassanis (1983), nevertheless, questions the importance of in-depth studies of PR (b) proteins as a way to solve the question of how natural resistance in plants is attained. In many cases PR (b) proteins can be induced by non-pathogenic means and resistance to disease is not normally produced.
after the proteins are present. But it is important to realize that because b proteins are found in all N - gene carrying plants and in the intercellular spaces of virus infected plants, they may indeed be part of a defense mechanism pathway, even if they are not antiviral themselves.
Research Goals

Research for this work was designed in such a way to show whether or not an antiviral substance could possibly travel in the intercellular spaces of tobacco mosaic virus infected primary leaves of pinto beans. This idea has been addressed only recently by Wierenga-Brants (1983) and Mitra (1985, unpublished data) with tobacco cultivars. Past research into antiviral substances has centered on using plant homogenates or protoplasts to extract inhibitors. Even though it has been proposed that AVF may travel to neighboring cells, little work has been done with intercellular fluids and the possibility that antiviral substances may be present there. It has been suggested that a chemical signal is responsible for inducing systemic acquired resistance (Chessin, personal communication; Ross 1961). If so, it is probable that such a signal may travel through the intercellular spaces to reach uninfected plant tissues. It is also of interest to determine if b - proteins can be found in pinto bean intercellular fluids. The presence of b - proteins in intercellular fluids may substantiate their possible role in plant defense mechanisms.

The aim was not to isolate a specific substance, but rather, to characterize the entire intercellular wash fluid as to its biological properties and its protein profile. The proteins in the intercellular wash fluid were to be compared to the pathogenesis - related (b) proteins.

Pinto beans were chosen because of the convenience of their fast growth rate and because little work has been done
with this species as a source of IWF.
MATERIALS AND METHODS

I. Chemicals

All chemicals for IWF extraction, electroblotting and SDS-PAGE, with the exception of SDS, were obtained from Sigma Chemical Co. (St. Louis, MO). SDS was obtained from Gallard - Schlesinger Chem. Mfg. Corp. (Carle Place, NY). Molecular weight markers and the kit for protein analysis were obtained from Bio - Rad Laboratories (Richmond, CA).

II. Plants

Phaseolus vulgaris var. Pinto was used in all bioassay experiments as well as being the source plant for IWF extracts. Seeds were sown in Jiffy mix, three per 4" pot, with a single application of a general plant fertilizer (RA.PID.GRO. Corp., Dansville, NY 14437). Plants were kept in either a controlled growth room or environmental chamber or in the greenhouse. Temperature remained at 20 - 23 C, and the photoperiod was 16 hours per day. Standard greenhouse conditions were maintained, although temperature ranges fluctuated more during some summer days.

Primary leaves of comparable sized eleven to twelve day pinto bean plants were used for virus assay or IWF extraction. Meristematic tissue and the trifoliate leaf bud of each plant was pinched off prior to treatment.

III. Virus

Tobacco mosaic virus was isolated according to the method of Gooding and Hebert (1967) from systemically infected Nicotiana tabacum var. Samsun leaves and from two
infected, hypersensitive tobacco species -- *N. tabacum* var. Xanthi - nc and *N. tabacum* var. Samsun NN. Virus inoculum was diluted with buffer (see below) so that an average of 100 - 200 lesions per Pinto bean half-leaf were produced.

IV. **Inoculation and Extraction of IWF**

The two primary leaves of a typical bean plant was divided into four halves and inoculated in the following manner (see Fig. 1).

- **Side #1** - TMV + Celite
- **Side #2** - No rubbing
- **Side #3** - Buffer + Celite
- **Side #4** - No rubbing

The buffer was a 0.067M, pH 7.0 Na-K phosphate solution which was kept at 4°C. Celite was added to both buffer and TMV solutions at 10 mg/ml total volume as an abrasive. Leaves were rinsed in lukewarm tap water within 5 minutes after inoculation and then placed in the greenhouse, controlled growth room or environmental chamber until extractions were made.

Intercellular wash fluid was extracted at selected intervals after inoculation from at least 18 - 24 plants per extraction day using a modified procedure compiled from various sources (Parent and Asselin 1984, de Wit and Spikeman 1982, and Klement 1965). Leaves were rinsed, just prior to extraction, in room temperature distilled water and blotted dry. The mid-veins were removed and the half-leaves then placed in cold buffer with 0.1% (v/v) 2-mercaptoethanol. Glass and rubber weights prevented floatation of the leaves during evacuation. Vacuum infiltration of buffer was
Fig. 1. Representation of pinto bean primary leaves showing treatment divisions.
achieved using a Nevaco, model 911 vacuum pump (Nelson Vacuum Pump Co., Berkeley, CA) connected to a glass dessicator. Leaves were under vacuum for 2.5 minutes, after which the vacuum was released, over a 25-30 second period, allowing the buffer to infiltrate the plant intercellular spaces. Leaves were quickly blotted dry, rolled and placed into polycarbonate centrifuge tubes (Nalge Co., Rochester, NY). The tubes were spun at 2800 - 2900 rpm (1000 - 1150 g) for 8 to 10 minutes in a Servall Angle Table-top centrifuge (Ivan Sorvall, Inc., Norwalk, CN). The fluid was visually checked for clarity and either used immediately or stored in glass, 10 x 75mm disposable culture tubes at -17 C for future use.

V. IWF Tests

A. Presence of Glucose 6-P Dehydrogenase

To determine whether cellular damage occurred due to the extraction procedure itself, glucose 6-P dehydrogenase was measured in fresh IWF samples and crushed, healthy leaves. 400 ul IWF or crushed leaf samples were assayed for enzyme activity using a procedure modified from Sigma Diagnostics Kit No. 345-UV. Absorbance readings were taken at one minute intervals for five minutes immediately after the addition of G-6-P dehydrogenase substrate solution. A Bausch and Lomb Spectronic 1001 split beam spectrophotometer (Rochester, NY) was used.

Leaf homogenate was prepared mixing 2.38 grams of tissue and 20 ml of buffer solution with a mortar and pestle. The mixture was spun at 1150 g for 20 minutes and the supernatant was assayed.
B. **IWF as Inhibitor of Virus Establishment**

Intercellular wash fluid taken at selected times from 0 to 10 days after inoculation was mixed in a 1:1 or 1:0.5 ratio with TMV inoculum, with the addition of 10 mg/ml Celite. Mixtures stood for 5 minutes before inoculation using the primary leaf divisions stated above. Inoculated leaves were rinsed in tap water after 24 hours. After 5 to 7 days, lesions were counted.

C. **IWF as Inhibitor of Virus Replication**

Whole pinto bean primary leaves were completely inoculated with TMV 6 to 9 hours prior to treatment. Leaves were prepared as for IWF extraction and randomly placed in beakers containing Day 7 IWF extracts and buffer in a 1:4 ratio. Control leaves were placed in buffer only. Infiltration of the mixtures was accomplished as previously stated. Infiltrated leaves were put on paper towelling, covered, and placed in a controlled growth chamber for 5 days. Lesion numbers were then counted and diameters measured.

D. **IWF Dialyzibility**

Day 7 extracts were dialyzed against distilled water for 24 hours at 4°C using Spectropor 4 (Spectrum Medical Industries, Inc., L.A., CA) dialysis tubing with a molecular weight cut-off point of 10 - 14 kd. The tubing was prepared in a boiling water bath using sodium bicarbonate followed by washing in distilled water. Biological activity was checked by testing the extracts as inhibitors of viral...
establishment. Day 10 extracts were tested for activity against virus replication.

E. Determination of Protein Contents

Protein contents of IWF extracts were routinely determined using the protein assay kit supplied from the Bio-Rad Labs. that employs the Bradford method (1976). The standard assay procedure was followed with bovine serum albumin used to determine the standard curve. IWF extracts taken from plants in which only one-half of a single primary leaf had been rubbed with buffer and Celite were also assayed for protein contents. In this case, IWF was extracted daily from 3 to 7 days after inoculation.

F. SDS - PAGE Gels

Extracts were dialyzed, in prepared dialysis tubing, against distilled water overnight, after which they were concentrated in a Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY) at 45 C heat until dry. Added to each pellet was 50 ul of solubilizing solution containing 10% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.1M Tris (pH 6.8), and 8% 2-mercaptoethanol. Samples were vortexed and heated in 100 C water for 5 minutes.

SDS-PAGE was carried out according to the procedure outlined by Laemmli (1970) and Judd (1982). Separating gels were generally 15% acrylamide (acrylamide/N,N' -methylenebisacrylamide ratio, 30:0.8), although 10%, 12.5%, and 17.5% gels were also used. Stacking gels were 3% acrylamide. Table 1 lists the chemical constituents of a 15% gel.
One hundred to 150 ug of IWF sample protein were added to each lane. Included in each gel were the low molecular weight markers from Bio - Rad Labs. These markers were Phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.3K). Electrophoresis was done at 10 watts with constant power for 2 to 3 hours. Gels were fixed in 40% methanol, 7% acetic acid overnight, stained with 0.25% Coomassie brilliant blue in fixing solution for 1 hour, followed by destaining in fixing solution. Apparent molecular weights of protein bands were ascertained by band location in relation to low molecular weight markers.
Table 1. Chemical Constituents of a 15% SDS-PAGE Gel.

<table>
<thead>
<tr>
<th></th>
<th>Stacking Gel</th>
<th>Separating Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide:BIS</td>
<td>2.5 ml</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>1M Tris (pH 6.8)</td>
<td>1.88 ml</td>
<td>6.0 ml</td>
</tr>
<tr>
<td>0.2M EDTA</td>
<td>0.15 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Water</td>
<td>10.3 ml</td>
<td>8.4 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.0075 ml</td>
<td>0.015 ml</td>
</tr>
<tr>
<td>Ammonium persulfate (10% wt/vol)</td>
<td>0.15 ml</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>
G. Immuno-Blot Analysis

1. Preparation of Antiserum

A New Zealand rabbit was bled prior to 4 subcutaneous injections of a dialyzed and concentrated Day 7, side #1 IWF extraction in Complete Freund's adjuvant. Intramuscular and intravenous injections were then made for two months with Day 7, side #1 IWF extractions resuspended in PBS (0.01M phosphate buffer, pH 7.4, with 0.85% NaCl). Antiserum was collected 4 times.

2. Electroblotting

Intercellular wash fluid samples were run on a 15% SDS-PAGE gel and electroblotted onto nitrocellulose paper (Millipore; Millipore Corp., South San Francisco, CA) at 25 volts and 1 amp. for 1.5 hours using a method modified from Judd (1986). The nitrocellulose paper blots were blocked for 1 hour in Dulbecco's modified PBS (dPBS) - Tween, 100mM PBS, 0.5% Tween 20, pH 7.4, and then exposed to IWF antibodies for 1 hour. Two washes in dPBS - Tween for 10 minutes each followed antibody exposure. Blots were then incubated with 10ul protein A - horseradish peroxidase in 50ml dPBS - Tween for 1 hour to reveal antibody binding. A 20 minute wash in TBS (20mM Tris, 500mM NaCl, peroxidase buffer, pH 7.2) preceded protein band development. Developing solution was 60mg 4-chloro-1-naphthol in 20ml methanol added to 100 ml TBS with 100 ul of 30% hydrogen peroxide. Distilled water stopped development and increased contrast.

H. High Performance Liquid Chromatography
High performance liquid chromatography was performed using a Perkin – Elmer (Norwalk, CT) Series 4 solvent delivery system. An HPLC gradient profile, as described by Judd (1987), was employed to separate the proteins in an Alltech reverse-phase C18 column (Alltech Associates, Deerfield, IL). Elution times were recorded on a Perkin – Elmer LCI-100 computing integrator. Fifty microliter samples of fresh Day 7 IWF extracts from all 4 treatment sides were examined. The resulting protein fractions were not isolated because of time constraints.
RESULTS AND DISCUSSION

A. Glucose-6-Phosphate Dehydrogenase

Results from testing for glucose-6-phosphate dehydrogenase activity show that only a small amount of the enzyme is present in intercellular fluids as compared to the amount within leaf cells (Table 2). Although these results are similar to those found by Rathmell and Sequeira (1974), they reported a difference in specific activity (mU/mg protein) of enzyme between homogenized leaves and intercellular fluids, indicating two isozymes of G-6-P-Dehydrogenase were present. My results, based on similar enzyme specific activities, indicate that the same G-6-P-Dehydrogenase is present both within and outside of the cell.

Many differences in testing systems could account for the differences in enzyme activities reported. Rathmell and Sequeira (1974) tested tobacco cultivar Bottom Special and in this study Pinto bean was tested. These results still, however, indicate that the extraction procedure does not cause major cell breakdown. What trace amount of cell leakage that may occur due to cut leaves, would not account for the large differences in biological activity, protein concentrations, and protein profiles found in IWF from the four inoculation treatments.

B. IWF as Inhibitor of Establishment

IWF, when mixed with TMV and inoculated onto 11 - 12 day old Pinto beans, produced variable results in inhibiting virus establishment (Table 3). At most, a 50% reduction in
Table 2. Presence of Glucose-6-Phosphate-Dehydrogenase in Leaf Homogenates and Intercellular Fluid.

<table>
<thead>
<tr>
<th>Sample</th>
<th>G-6-P-DH (mU)</th>
<th>Protein (mg/ml)</th>
<th>Specific Activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IWF- Buffer/celite rubbed</td>
<td>1.09 mU</td>
<td>2.14</td>
<td>0.51</td>
</tr>
<tr>
<td>IWF- No rubbing</td>
<td>0.93 mU</td>
<td>2.07</td>
<td>0.45</td>
</tr>
<tr>
<td>Healthy Leaf Homogenate</td>
<td>30.60 mU</td>
<td>46.70</td>
<td>0.65</td>
</tr>
</tbody>
</table>

\( \text{a} \) - mU=milliunits, One International Unit (U) = the amount of enzyme activity that will convert 1 micromole of substrate per minute at 23 C.
Table 3. Percent Inhibition of Virus Establishment by IWF Extracts Taken at Selected Intervals After Inoculation.

<table>
<thead>
<tr>
<th>Extraction Day</th>
<th>Percent Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>+20 (n=3)</td>
</tr>
<tr>
<td>Day 2</td>
<td>-14 (n=2)</td>
</tr>
<tr>
<td>Day 3</td>
<td>-29 (n=3)</td>
</tr>
<tr>
<td>Day 4</td>
<td>-9 (n=6)</td>
</tr>
<tr>
<td>Day 5</td>
<td>+20 (n=4)</td>
</tr>
<tr>
<td>Day 7</td>
<td>-5 (n=9)</td>
</tr>
<tr>
<td>Day 8</td>
<td>+8 (n=2)</td>
</tr>
<tr>
<td>Day 9</td>
<td>-44 (n=3)</td>
</tr>
<tr>
<td>Day 10</td>
<td>-3 (n=5)</td>
</tr>
</tbody>
</table>

a - Based on the lesion number ratio 1:2/3:4. A positive number indicates a stimulation of lesion production. A negative number indicates percent inhibition.

b - n = Number of experiments performed per extraction day.
lesion numbers was observed, with IWF extracts taken three and nine days after inoculation consistently producing at least 30% virus inhibition. Often lesion numbers were increased. Using Day 7 IWF extracts, four of nine experiments produced an average inhibition of 26% whereas, five of the nine experiments produced an average increase of 13%. The low percentage of inhibition varied widely depending on which day the IWF was extracted, but there was generally no trend. These particular results infer that Pinto bean IWF has little, if no, ability to inhibit virus establishment. Contrasting these results, are the results obtained by Wieringa-Brants (1983), Mitra (1985, unpublished data), and Spiegel (1985, unpublished data). Even though their methods for testing inhibitory activity against virus establishment and replication were different from my methods, and they used only IWF from tobacco cultivars Samsun NN and Xanthi nc, all were able to produce a range of 30% - 100% reduction in lesion numbers.

A lag time may be necessary for an inhibitor in IWF to stimulate cell defense mechanisms in Pinto bean. Thus, when IWF is introduced at the same time with TMV inoculum, it may not act quickly enough or at a rate faster than TMV replication. This may explain the varied inhibition results. Therefore, Pinto bean IWF is not an inhibitor of virus establishment.

C. IWF as Inhibitor of Replication

Overall, lesion numbers were decreased 60 - 69% by the
four treatment extracts compared to the control (Table 4). Average lesion diameters changed little as compared to the control, although the appearance of the larger lesion size classes was diminished in the treatments using side #1 and side #2 IWF extracts (Table 5). This strongly implies that IWF extracts from virus infected Pinto bean leaves is an inhibitor of virus replication.

Interestingly, all four IWF extractions showed some biological activity in inhibiting lesion numbers when IWF is infiltrated 6-9 hours after TMV inoculation. This is evidence that by at least 7 days after virus inoculation, an antiviral substance is located within the intercellular spaces of non-infected plant tissues. It is yet unknown whether an antiviral substance has actually moved from the infected sites to the uninfected areas or whether a chemical signal has moved to the uninfected areas and stimulated the production of an antiviral substance.

It is possible that IWF may inhibit virus replication even though average lesion diameters changed only slightly in this experiment. Infiltrating IWF into leaves exposes many plant cells to an antiviral inhibitor at the same time. An inhibitor of replication could still effect lesion numbers if it was introduced at a crucial point in the virus multiplication cycle, which may then result in the production of non-visible microlesions. Perhaps inhibiting the development of necrotic lesions by an inhibitor in the intercellular wash fluid would account for the drastic decrease in lesion numbers.
Table 4. Comparison of Average Lesion Number and Diameters Using Day 7 IWF Extracts Applied 6-9 Hours After Inoculation with TMV.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Lesion Number (a)</td>
<td>60.0</td>
<td>22.1</td>
<td>23.9</td>
<td>18.4</td>
<td>18.3</td>
</tr>
<tr>
<td>Average Lesion Diameter</td>
<td>3.3</td>
<td>3.2</td>
<td>2.9</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>(x 10 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) - See Table 5 for experimental sample sizes.
Table 5. Lesion Size-Class Frequency Percentages Using Day 7 IWF Extracts Infiltrated in Pinto Bean Leaves 6-9 Hours After TMV Inoculations.

<table>
<thead>
<tr>
<th>Size Class (x 10 mm)</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.9</td>
<td>16.6</td>
<td>10.9</td>
<td>11.6</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>26.7</td>
<td>22.1</td>
<td>32.7</td>
<td>22.3</td>
<td>17.3</td>
</tr>
<tr>
<td>3</td>
<td>20.0</td>
<td>23.5</td>
<td>32.3</td>
<td>26.5</td>
<td>26.9</td>
</tr>
<tr>
<td>4</td>
<td>11.5</td>
<td>14.7</td>
<td>11.7</td>
<td>12.1</td>
<td>16.5</td>
</tr>
<tr>
<td>5</td>
<td>10.7</td>
<td>13.4</td>
<td>8.2</td>
<td>13.5</td>
<td>19.2</td>
</tr>
<tr>
<td>6</td>
<td>5.2</td>
<td>5.1</td>
<td>2.7</td>
<td>7.4</td>
<td>5.4</td>
</tr>
<tr>
<td>7</td>
<td>6.3</td>
<td>3.2</td>
<td>0.8</td>
<td>2.3</td>
<td>5.0</td>
</tr>
<tr>
<td>8</td>
<td>3.3</td>
<td>1.4</td>
<td>0.4</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>9+</td>
<td>4.4</td>
<td>0.0</td>
<td>0.4</td>
<td>1.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Average of 6 trials.
Total leaves used in all 6 trials: Control (N=36), Treatments (N=47-48)
Total lesion diameters measured in all 6 trials: Control (N=270) Treatments (N=215-260).
D. Dialyzibility

Biological activity was retained by IWF solutions after 24 hours dialysis. This experiment did not directly compare dialyzed IWF extracts with non-dialyzed IWF extracts, although the results for both are similar. Virus establishment was reduced 29% with a Day 7, side #1 IWF extract compared to a side #3 extract. Testing for an effect on viral replication with a Day 10 side #1 extract, lesion numbers were decreased by 73% but diameters were decreased only by 15%. These results indicate that an inhibitor of greater than 10 - 14 kd molecular weight is present in intercellular wash fluids.

E. Determination of Protein Contents

Protein contents of IWF extracts were measured at different intervals after virus inoculation. Peak increases in protein content occurred in the 12 hour, Day 4, Day 7, and Day 10 extracts. Treatment #1 of Day 7 IWF extracts usually contained the greatest amount of total protein (Table 6). Total protein markedly decreased from Day 0 to 12 hours after inoculation.

Preliminary protein content studies of IWF extracts taken three to seven days after inoculation treatments from pinto bean plants that were only rubbed with buffer and Celite gave interesting results. A slight periodicity over time still appears in the levels of intercellular proteins after wounding just half of a single primary leaf on a plant. The results represent just one trial using 12 to 15 plants
Table 6. Comparison of IWF Protein Contents (ug x10 /ml) Taken at Selected Times After TMV Inoculation.

<table>
<thead>
<tr>
<th>Extraction Time</th>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>1:3</th>
<th>1:2</th>
<th>2:4</th>
<th>3:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs (5)</td>
<td></td>
<td>38.6</td>
<td>32.6</td>
<td>37.7</td>
<td>50.9</td>
<td>+ 2</td>
<td>+18</td>
<td>-36</td>
<td>-25</td>
</tr>
<tr>
<td>12 hrs (2)</td>
<td></td>
<td>14.4</td>
<td>15.2</td>
<td>9.2</td>
<td>9.5</td>
<td>+57</td>
<td>-5</td>
<td>+60</td>
<td>-3</td>
</tr>
<tr>
<td>24 hrs (2)</td>
<td></td>
<td>12.3</td>
<td>---</td>
<td>12.0</td>
<td>10.2</td>
<td>+3</td>
<td>---</td>
<td>---</td>
<td>-13</td>
</tr>
<tr>
<td>36 hrs (2)</td>
<td></td>
<td>16.3</td>
<td>13.5</td>
<td>20.8</td>
<td>15.1</td>
<td>-22</td>
<td>+21</td>
<td>-11</td>
<td>+38</td>
</tr>
<tr>
<td>48 hrs (2)</td>
<td></td>
<td>15.2</td>
<td>17.9</td>
<td>23.0</td>
<td>13.6</td>
<td>-34</td>
<td>-15</td>
<td>+32</td>
<td>+69</td>
</tr>
<tr>
<td>4 days (5)</td>
<td></td>
<td>57.0</td>
<td>36.7</td>
<td>15.7</td>
<td>10.5</td>
<td>+263</td>
<td>+55</td>
<td>+249</td>
<td>+49</td>
</tr>
<tr>
<td>5 days (1)</td>
<td></td>
<td>26.9</td>
<td>28.1</td>
<td>28.6</td>
<td>17.5</td>
<td>-6</td>
<td>-4</td>
<td>+61</td>
<td>+63</td>
</tr>
<tr>
<td>7 days (11)</td>
<td></td>
<td>63.0</td>
<td>34.7</td>
<td>30.0</td>
<td>19.5</td>
<td>+110</td>
<td>+82</td>
<td>+78</td>
<td>+54</td>
</tr>
<tr>
<td>8 days (1)</td>
<td></td>
<td>35.2</td>
<td>23.5</td>
<td>21.4</td>
<td>22.5</td>
<td>+64</td>
<td>+50</td>
<td>+4</td>
<td>-5</td>
</tr>
<tr>
<td>9 days (1)</td>
<td></td>
<td>35.8</td>
<td>18.5</td>
<td>17.3</td>
<td>16.1</td>
<td>+107</td>
<td>+94</td>
<td>+15</td>
<td>+7</td>
</tr>
<tr>
<td>10 days (6)</td>
<td></td>
<td>55.1</td>
<td>19.7</td>
<td>18.0</td>
<td>14.3</td>
<td>+206</td>
<td>+180</td>
<td>+38</td>
<td>+26</td>
</tr>
</tbody>
</table>

(x) = Number of trials. IWF extracted from 18-24 plants per extraction day.
per extraction day. Treatment letters, i.e., side a, b, c, and d correspond to the same sides of pinto bean primary leaves as in the other experiments using sides 1, 2, 3, and 4 respectively. Side c was the only side rubbed (Table 7).

Both experimental results support the hypothesis that proteins are being made by the host plant after a stress—be it viral or wounding. These findings are apparently novel since it has not been previously reported that plants have a three day cyclicity in protein levels. It is not known why plants would produce bursts of proteins and release them into the intercellular spaces. Perhaps it can be related to the replication cycle of the virus or the development of lesions. But that would not explain the protein cycle shown in non-infected plants. Rosenberg et al. (1985), Reichman et al. (1983), and Orchanisky et al. (1982) have noticed that AVF can maintain cells in a state of resistance longer if given in supplemental doses after virus infection. TMV infected Pinto beans may also be producing an antiviral substance, as well as other defense proteins, and releasing them into the intercellular spaces throughout the course of infection to remain in a state of higher resistance.

**SDS-PAGE Protein Profiles**

IWF protein profiles were elucidated by running samples through an SDS-PAGE gel. Eight bands showed increased protein levels in lane 1, containing side #1 IWF, compared to all the other lanes (Fig. 2). The apparent molecular weights of the eight bands were: 36,500 d; 34,000 d; 33,000 d;
<table>
<thead>
<tr>
<th>Extraction Time</th>
<th>Treatment a</th>
<th>Treatment b</th>
<th>Treatment c</th>
<th>Treatment d</th>
<th>Percent Difference a:c</th>
<th>Percent Difference a:d</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>312</td>
<td>239</td>
<td>314</td>
<td>277</td>
<td>-1</td>
<td>+13</td>
</tr>
<tr>
<td>4 days</td>
<td>379</td>
<td>408</td>
<td>342</td>
<td>249</td>
<td>+11</td>
<td>+52</td>
</tr>
<tr>
<td>5 days</td>
<td>363</td>
<td>309</td>
<td>405</td>
<td>366</td>
<td>-10</td>
<td>-1</td>
</tr>
<tr>
<td>6 days</td>
<td>376</td>
<td>401</td>
<td>206</td>
<td>159</td>
<td>+83</td>
<td>+136</td>
</tr>
<tr>
<td>7 days</td>
<td>198</td>
<td>289</td>
<td>154</td>
<td>273</td>
<td>+29</td>
<td>-27</td>
</tr>
</tbody>
</table>

Side "c" was rubbed only.
IWF extracted from 12-15 plants per extraction day.
28,750 d; 26,500 d; 18,200 d; 16,800; and 15,200 d. Occasionally, a band near 10,000 d molecular weight would show up in the gels in lane 1, however, there were no comparable bands in the other lanes. The 36,500 d band was composed of two protein bands, both of which increased in staining. The single band representing the 33,000 d protein was composed of three protein bands. This information was attained by underloading SDS-PAGE gel lanes with the IWF samples. These bands migrate similarly in an SDS-PAGE gel indicating that their molecular weights are alike, but no conclusions can be drawn about whether they are polypeptide subunits of the same protein. All enhanced bands, produced with IWF extracts taken three or more days after inoculation, had corresponding bands appear in the three other lanes. However, those were either stained more lightly or were barely visible. SDS-PAGE gels run with Day 0 or 12 hour extracts revealed that the five larger molecular weight proteins were not produced de novo after virus infection, but the three smaller molecular weight proteins may have been. The evidence was not conclusive. Bands tended to increase or decrease in staining intensity depending on the day of extraction (Fig. 3). In addition, a 20,500 d protein band was apparent in only lane #1 in Day 0 and 12 hour extracts but its appearance diminishes by Day 4.

These results are similar in some respects to the results obtained by Abu-Jawdah (1983, 1982). He found that the virus induced protein p1 had a molecular weight of about 18,500 daltons under denaturing conditions. The 18,200
Figure 2. 15% SDS-PAGE gel showing eight enhanced proteins from a Day 7 IWF extraction.
Figure 3. Comparison of proteins from IWf extractions taken at selected days after TMV infection.
dalton protein in this study may correspond to Abu-Jawdah's pl protein. Bean cultivars used in his study were Brittle Wax and Immuna and he used an acid extraction from leaf homogenates to obtain only four induced proteins from them. Redolfi and Cantisani (1984) also found only four PS proteins, all of which had a molecular weight of 13,900 +/- 600 daltons from acid extracts of homogenized leaves of *Phaseolus vulgaris* var. Saxa.

Very little research has been done with bean intercellular fluid and its protein contents. None of the proteins in this study could be automatically identified as PS proteins because extractions were not made under acid conditions, but at pH 7.0. This does not preclude the possibility, though, that these proteins may indeed be PS proteins. These proteins were produced in a plant exhibiting the hypersensitive response to virus infection and were also seen, albeit in low concentrations, in leaves which showed systemic acquired resistance.

Infection with TMV may be causing the plant to increase the manufacture of proteins, for defense, or selectively release cellular proteins into the intercellular spaces. The gels show that protein staining in lane 1 is increased selectively for specific proteins compared to the staining in the other three lanes; suggesting that general cell leakiness is not occurring after TMV infection, contrary to some reports (Matthews 1981).

**Immuno-Blot Analysis**
Immuno-blots were produced from Day 0 and Day 10 IWF extracts. Protein bands appeared on the nitrocellulose paper at the locations where the five, larger molecular weight enhanced proteins appeared on SDS-PAGE gels in the Day 0 IWF extracts. All four lanes, representing the four different inoculation treatments, showed protein bands on the nitrocellulose paper. The 20,500 d protein also appeared in all lanes of the Day 0 immuno-blot, although the bands were very light in samples from treatments #2, #3, and #4. Only the three smaller molecular weight proteins did not appear in the Day 0 immuno-blots indicating that those particular proteins were not present in healthy leaf tissue. It appears, then, that the 18,200 d, 16,800 d, and the 15,200 d proteins were produced de novo after infection with tobacco mosaic virus.

Ten days after virus infection, all of the enhanced proteins are present throughout the Pinto bean primary leaves. Immuno-blots taken of Day 10 IWF extracts showed protein bands, representing all of the enhanced proteins, in each sample. The presence of all protein bands in the Day 10 immuno-blot would seem to indicate that the rabbit was capable of producing antibodies to IWF proteins and that the lack of antibodies was not the reason for the lack of protein band development of the three proteins in the Day 0 IWF extracts.

High Performance Liquid Chromatography

High performance liquid chromatography of IWF samples produced unexpected results. Fewer total protein peaks show
up in this experiment as opposed to the total number of protein bands found in SDS-PAGE gels, perhaps indicating that some of the stained protein bands in SDS-PAGE gels represent polypeptide subunits.

Comparing side #1 to side #3 there appears to be only an increase in one protein in side #1, but a diminishing or complete loss of two to four other proteins (Fig. 4). When comparing Fig. 4 to Fig. 5, there is a noticeable trend in the protein peak which has a retention time of 4.228 minutes in the side #1 IWF extract. It is at its highest in side #1 and gradually decreases to where it is almost undetectable in the side #4 IWF extract. This may indicate that the protein is increased by TMV infection within a leaf and may be a part of the defense mechanism of the plant. Just the opposite appears to happen with the protein peaks with a retention time of 3.684 minutes and 6.756 minutes in side #3. These peaks are undetectable in side #1. It is unknown why this is. However, these results do support the other experimental evidence presented that show there are protein changes in the intercellular spaces in plants infected with TMV.
Figure 4. Comparison of HPLC samples from side #1 and side #3 Day 7 IWF extracts.
Figure 5. Comparison of HPLC samples from side #2 and side #4 Day 7 IWF extracts.
SUMMARY AND CONCLUSIONS

Extracting intercellular proteins, via in vacuo infiltration and centrifugation, has many advantages. It is a simple procedure and avoids some of the disadvantages of leaf homogenization. The technique is gentle to the plant cells and does not cause major cell damage. It is necessary, though, to routinely test the general technique to assure no major cell damage is occurring, especially if leaves are undergoing multiple infiltrations prior to centrifugation as other workers do. Many researchers depend on visual inspection of their extraction samples to determine cellular damage (Parent and Asselin 1984; deWit and Spikeman 1982). Even low concentrations of chlorophyll, indicative of cell damage, may be masked by tannins or other substances present in the sample. Based on the difference between glucose-6-P-dehydrogenase found in the intercellular wash fluids and leaf homogenates, the single infiltration technique used in this study did not cause detectable damage.

A virus inhibitor is apparently present in the intercellular spaces of TMV infected Pinto bean leaves. Although inhibition of virus establishment was highly variable when IWF was mixed with TMV inoculum prior to inoculating, IWF did significantly reduce lesion numbers when infiltrated 6-9 hours after TMV infection. This suggests that IWF contains an inhibitor of virus replication. According to Loebenstein and Gera (1981), an inhibitor of virus replication is active when added 5-12 hours post virus
inoculation, which ensures that virus establishment has taken place. Additional evidence for an effect on replication was provided by IWF which produced a slight increase in the frequency of small lesion size classes and a diminished appearance of the larger lesion size classes. IWF extracts from TMV infected Pinto bean half-leaves produced the greatest effects, but extracts from the three other inoculation treatments also showed antiviral activity. Generally an antiviral substance which acts against virus multiplication will have an effect on lesion diameters, but effects on lesion numbers are much more variable. In my experiments, lesion numbers were altered the most.

It appears that the inhibitor in my extracts is larger than 10,000-14,000 d molecular weight based upon the results obtained from dialysis experiments. It has not been determined whether any of the induced proteins shown in SDS-PAGE gels is the inhibitor, though.

TMV infected Pinto beans showed a strange three day cyclicity in the levels of their intercellular proteins. The largest difference in protein contents between TMV rubbed leaves and buffer rubbed leaves occurred in Day 4 IWF extracts, which showed an increase of 263% total protein content. Day 7 IWF extracts from TMV infected leaves contained the greatest amounts of protein per milliliter of IWF extract. Wounding also tended to induce a cyclical response in protein levels, though the periodicity was shorter—generally only two days, and the percent difference
between extracts from virus and buffer rubbed leaves was much smaller.

Eight enhanced proteins were detected in the intercellular spaces of TMV infected Pinto bean leaves. Of these, the five largest molecular weight proteins were not produced de novo; but according to the immuno-blot experiments, it appears that the three smaller proteins were. A 20,500 d protein was present in SDS-PAGE gels using Day 0 and 12 hour extracts, but only from TMV infected plant tissues (side #1). By three days after inoculation, staining of this band was reduced although it was still slightly visible. From Day 3 to Day 10 the 20,500 d protein was occasionally found in IWF extracts from buffer rubbed leaves (side #3), but again, the staining intensity of this band was very light. Some of the enhanced protein bands increased in staining intensity over time, peaking at Day 4 or Day 7 depending on the protein.

High performance liquid chromatography experiments reinforced the previous evidence which showed qualitative and quantitative protein changes in Pinto bean leaves after inoculation with virus.

It is well known that plants undergo many changes in cellular protein levels when diseased (Van Loon 1982) and it is generally assumed that many of these substances can travel from cell to cell via the plasmodesmata since this would be more energy efficient (Matthews 1981). Recent evidence (Fortin and Asselin 1985) suggests that certain proteins are increased in tobacco intercellular spaces after
exposure to virus or other stress producing agents. In addition to the above study, my study with Pinto beans has shown that protein profiles change in the intercellular spaces over time after virus infection. Travel through the intercellular spaces by direct viral inhibitors or other proteins, which may be involved in such defense mechanisms as wound responses, stimulation of systemic acquired resistance or induction of other toxic substances, may be just another mechanism by which the plant can effectively battle virus infections.

My results indicate that the intercellular wash fluid from virus infected bean leaves has an antiviral nature. It is inhibitory toward virus replication. The more studied antiviral substances, AVF, IVR, and AVS-65, also show this antiviral property although AVF is usually associated with inhibition of virus establishment rather than viral replication. Because of the experimental differences in extraction procedures used in my study and those used by Sela (1981 c), Gera and Loebenstein (1983), and Mitra (1985), the inhibitory activity present in IWF extracts can not be directly related to the other antiviral substances. IWF extracts were neither concentrated nor purified prior to experiments testing inhibition of virus multiplication or establishment as the other workers have done with their respective antiviral substances. This may explain why the inhibitor in IWF did not produce inhibition exceeding 75% in any of the experiments as have the other known antiviral
substances. Although Wieringa-Brants' (1983) and Mitra's (1985, unpublished data) work with tobacco intercellular fluids from virus infected leaves was not based on using purified IWF, they were able to achieve up to 100% inhibition of virus establishment and multiplication. Perhaps the larger protein amounts present in tobacco intercellular fluids (Parent and Asselin 1984) may account for the difference between our studies. The difference may also stem from the possibility that Pinto bean plants are inherently different in their defense mechanisms than tobacco plants. Necrotic lesions expand continuously, albeit slowly, in hypersensitive bean plants, whereas they appear to stop expanding after 3-4 days in hypersensitive tobacco plants. Concentrations of inhibitory substances found naturally within intercellular spaces may be much lower than those found within cells, thus making travel through intercellular space of secondary importance.

The three day cyclicity of protein levels produced in TMV infected bean leaf intercellular spaces appear to be unique. No other research, that I am aware of, has reported such findings and it is not known why this would be advantageous for a virus infected or stressed plant to produce proteins in bursts.

Of these eight enhanced proteins, at least one is comparable to a known PS protein. The pathogenesis related (b) proteins are not serologically related to the induced PS proteins found in bean plants (Van Loon et al. 1983), but they may all be representatives of a larger class of defense
proteins with each genus producing a unique set.

It is thought that a chemical signal, possibly proteinaceous in nature, may play a role in the acquired resistance phenomenon of virus infected plants (Chessin, personal communication; Ryan 1984). At first, the 20,500 d protein present in Day 0 and 12 hour extracts may appear to be such a signal. However, the SDS-PAGE band representing this protein diminishes greatly over time. Systemic acquired resistance can be demonstrated in non-infected leaves 10 days after inoculation of other leaves on the plant (Ross 1961). If the 20,500 d protein is a signal protein which induces systemic resistance, it would be expected to be readily apparent in all Day 10 IWF extracts. It was not.

Matthews (1981) has reported that virus particles and even some virus coat protein may still be left on the leaf surface after washing with water. TMV coat protein is composed of 17,500 d molecular weight sub-units. It is possible that the 20,500 d and the 17,500 d proteins are the same but that would not explain the presence of the 20,500 d protein in leaves that were not infected.

Ryan (1984) also reports that wound signals responsible for inducing proteinase inhibitors have molecular weights of 5,000 - 10,000 d, but these would not be apparent in my SDS-PAGE gels since all samples were dialyzed before being electrophoresed. Ryan's wound signals were smaller than the molecular weight cut-off point of the dialysis tubing. He likens the proteinase inhibitor proteins to the induced
tobacco PR (b) proteins on the basis of molecular weights and stability under acid or proteolytic conditions. Proteinase inhibitors have sub-unit molecular weights of 8,000-12,000 d. Bean IWF protein profiles rarely showed any induced proteins in the 10,000 -14,000 d range.

Another interesting result from SDS-PAGE gels indicates that general cell leakage is not occurring after plant cells are inoculated with virus. The presence of enhanced, specific proteins in IWF from infected tissue indicates that cells are selectively releasing or allowing proteins into the intercellular spaces after TMV infection. But it is unknown whether these proteins are directly involved in defense mechanisms or are just the result of physiological changes the plant is undergoing. Past studies (Matthews 1981) report increased cell membrane leakiness after virus infection. Non-specific leakage of proteins from cells may be detrimental to the plant not only because they contain many hydrolytic enzymes but because a general exodus of proteins may effect cellular metabolism.

In conclusion, a virus inhibitor of multiplication is present in the intercellular spaces of TMV infected Pinto bean leaves. Based on virus replication studies it is also present, to a lesser extent, in leaves which have acquired systemic resistance inferring that an antiviral substance can travel through the intercellular spaces of plant leaves. Virus infection is responsible for the qualitative and quantitative protein changes found in the intercellular spaces. Although these proteins have not been directly shown
to be antiviral in themselves, they may function in overall plant defense mechanisms such as systemic acquired resistance. Intercellular spaces may simply serve as an alternate route for antiviral substances or associated proteins to travel. This study has elucidated some of the similarities, differences, and properties between proteins and fluids obtained from various virus infected plant species, but more research needs to be done to expand upon the results obtained thus far.

bFuture Studies

To further understand the inter-relationships between virus infection, induced inhibitors, acquired resistance, and protein production by Pinto bean host plants, more experimental research is needed.

Experiments which test membrane integrity during IWF extraction procedures are necessary to determine if this technique would be truly helpful in answering the question of whether intercellular spaces are involved in antiviral defense mechanisms. This could be accomplished by testing for glucose-6-P-dehydrogenase in IWF samples taken from leaves which have been treated with heat or chemicals, such as acetone or other organic solvent, that are known to increase cell membrane permeability but which are much less drastic in their effects than homogenization. By testing IWF extracts from variously treated Pinto bean leaves, a more accurate conclusion could be drawn about relative membrane damage incurred by the extraction procedure presented in this
Further testing of IWF samples as an inhibitor of virus replication should include testing them in systemically responding host plants, followed by an assay of virus titer either by ELISA methods or a bioassay on hypersensitively responding plants. This "double assay" gives a better indication of inhibitory activity than testing IWF extracts directly in a hypersensitive plant and measuring lesion number and size. In addition, experiments to test the physical properties of IWF in respect to its activity as a virus replication inhibitor would allow comparisons to be made between IWF and the three other characterized antiviral substances - AVF, IVR, and AVS-65.

An acid extraction of Pinto bean intercellular fluids may demonstrate the presence of PS proteins in a bean cultivar that, as yet, has been untested. This would determine whether PS proteins are strictly cellular proteins or if they may travel in the intercellular spaces. The presence or absence of PS proteins in IWF extracts may have implications for the role of PS proteins in acquired resistance, since PS proteins have only been detected in leaf homogenates so far.

The question of why Pinto bean plants undergo a periodicity of protein levels after virus infection is of physiological interest. It would be necessary to assay protein contents of IWF samples, on a daily basis, from completely healthy, unrubbed bean plants to determine if periodicity is inherent. Complementary experiments would
include assaying protein levels of the homogenized leaves from which IWF extracts had already been taken to show protein periodicity within the cells and assaying TMV levels of infected leaves over time.

Many possibilities exist for study of the proteins found in the intercellular spaces of virus infected Pinto bean plants. Radioactive tracers could be employed to determine de novo production of proteins. Extensive immuno-blot experiments may also produce more conclusive evidence for de novo production of proteins. Isolation of the 20,500 d protein band which appears in SDS-PAGE gels and testing its activity as an inducer of virus resistance may yield promising results in answering the question of whether a signal may be involved in systemic acquired resistance. Basic research could also be accomplished with the proteins found in intercellular wash fluids and elucidated with HPLC experiments. Each protein peak fraction could be extracted and run on an SDS-PAGE gel to not only demonstrate if the protein is composed of polypeptide subunits, but also to compare the bands produced with the SDS-PAGE gel protein bands presented in this study. Additionally, each protein peak shown in HPLC experiments could be tested for its antiviral activity against virus replication and for its ability to induce resistance.

High performance liquid chromatography experiments could also be done with fresh IWF extracts taken on a daily basis after TMV infection to show trends in concentration levels of
distinct proteins in order to give a better understanding of how proteins are changing in response to virus infection.

Much knowledge has already been gained in the last 20 years about acquired resistance in plants after infection with disease agents. However, more studies need to be undertaken in order to produce or lead to the production of a marketable viricide or inducer of resistance which would enhance a plant's natural defense against virus infection.
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