Effects of certain antiviral compounds on symptoms and infectivity of cowpea chlorotic mottle virus in cowpea and soybean plants.

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EFFECTS OF CERTAIN ANTIVIRAL COMPOUNDS ON SYMPTOMS AND INFECTIVITY OF COWPEA CHLOROTIC MOTTLE VIRUS IN COWPEA AND SOYBEAN PLANTS

A Thesis Presented
By
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A large number of antiviral compounds are currently being studied for possible use against viral infections of humans and animals. Very little is known about the effects of antiviral compounds on viral infections of plants.

Plant virus chemotherapeutants are needed as an alternative to the preventative means of plant virus control currently used to avoid crop losses. Ideally, a viral chemotherapeutant would selectively inhibit the viral infection process without having toxic effects on the host cell metabolism. The compound would also have to move systemically throughout the plant while retaining its activity for a reasonable period of time to be effective.

Antiviral compounds inhibit virus infectivity by affecting different stages of the viral infection process. Therefore, plants were treated with the different compounds at various times before, during, and after inoculation, so that their inhibitory effects would be more likely to be expressed.

To best observe the effects of each antiviral compound on systemic and hypersensitive virus infection, it was necessary to work with a virus-host system in which systemic symptom development and the hypersensitive response could be easily observed. Such a system is provided by the cowpea chlorotic mottle virus (CCMV) which causes systemic infection in cowpea plants, Vigna unguiculata, variety "California
Blackeye," and hypersensitive response on soybean plants, *Glycine max*,
variety "Harasoy." In addition, purified CCMV possesses good antigenic
properties thereby making it possible to determine the effects of cer¬
tain antiviral compounds on the presence of CCMV antigen by immunolog¬
ical techniques.

This study was undertaken to determine the effects of certain
antiviral compounds on the presence and infectivity of CCMV in hosts
known to exhibit systemic symptoms or the hypersensitive response, and
thus to gain information on the possibilities for use of such compounds
as a means of control against plant virus infections.
CHAPTER II
LITERATURE REVIEW

Crop losses due to plant virus diseases generally occur in three situations: 1) where perennial crops develop viral symptoms after much time and land have been invested; 2) where annual seed crops become infected due to cultural or environmental conditions which favor virus infection; and 3) where mild but widespread infections of vegetatively propagated plants occur leading to small losses every year (Matthews, 1970). Currently, only evasive control measures are available for the prevention of crop losses due to plant virus diseases. These measures include removal of virus-infected sources, control of vectors, use of virus-free seed and vegetative stocks, modifications of cultivation procedures, and ideally, use of resistant crop varieties (Agrios, 1978).

So far no antiviral compounds have been found which would directly protect or cure a crop from virus infection. However, certain antiviral compounds are known to inhibit virus infections in human, animal, and plant hosts (Maugh, 1976). Any one of these compounds may have potential for use in the prevention or cure of virus infections in plant crops.

2-Thiouracil is a pyrimidine nucleoside analog which has been shown to have several different effects on virus infectivity. According to Steele and Black (1967), 2-thiouracil inhibits the absorption of poliovirus onto rhesus kidney cells by inactivating functional capsid sulfhydryl groups. When 2-thiouracil ($10^{-3} M$) was added to poliovirus-
infected rhesus kidney monolayers, virus titre (PFU/ml) was less than 10% of the titre of the control cultures containing no 2-thiouracil.

Multiplication of tobacco mosaic virus (TMV), potato virus X (PVX), potato virus Y (PVY), and henbane mosaic virus was reduced in leaves of tobacco, *Nicotiana tabacum*, variety "White Burley," floated in 2-thiouracil (Bawden and Kassanis, 1954). $^{35}\text{S}$-labelled 2-thiouracil was incorporated into both host and viral RNA of TMV infected tobacco leaves. The $^{35}\text{S}$-labelled 2-thiouracil suppressed the incorporation of $^{32}\text{P}$ into viral RNA with little effect on $^{32}\text{P}$ incorporation into host RNA (Ralph, et al., 1965). Therefore, it was thought that 2-thiouracil may decrease TMV infectivity by incorporating itself into viral RNA, thereby interfering with virus replication (Kado and Agrawal, 1972).

Contrary to these findings, Dawson and Kuhn (1972) observed an enhancement of cowpea chlorotic mottle virus (CCMV) infectivity by 2-thiouracil. Sizes of local lesions on soybean plants, *Glycine max*, variety "Bragg," increased up to 13 times after primary leaves were inoculated and floated in a solution of 2-thiouracil (0.005 M). Sap infectivity and $^{32}\text{P}$ incorporation into viral RNA also increased when primary leaves of cowpea plants, *Vigna unguiculata*, variety "Early Ramshorn," were sprayed daily with 2-thiouracil.

Addition of uracil counteracts both the inhibitory effect of 2-thiouracil on TMV multiplication (Commoner and Mercer, 1951) and the enhancement effect of 2-thiouracil on CCMV multiplication (Kuhn, 1971). Plants sprayed with 2-thiouracil exhibit symptoms of toxicity such as leaf yellowing, curling, and growth inhibition (Matthews, 1970).
Addition of uracil had no effect upon alleviation of these toxic symptoms (Bawden and Kassanis, 1954). Due to the toxic nature of 2-thiouracil, its use in the control of virus diseases has been avoided.

Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a nucleoside analog active against a variety of RNA and DNA viruses (DeClercq and Luczak, 1975; Maugh, 1976). The multiplication of several plant viruses is also known to be inhibited by ribavirin. Lerch (1977) reported that the multiplication of PVX in 4-week-old tobacco plants, Nicotiana tabacum, variety "Xanthi NC," was inhibited when roots were placed in a nutrient solution containing ribavirin (5 ug/ml). Inhibition was measured by performing infectivity assays in which sap from PVX-infected tobacco leaves was inoculated onto detached leaves of Gomphrena globosa. Inhibition ranged from 23 to 78%, increasing in upper tobacco leaves as the distance from the lowest inoculated leaves increased. Inoculated tobacco plants treated with ribavirin remained symptomless and grew more vigorously than the control plants.

A 40% reduction in the development of systemic symptoms was reported when tomato plants, Lycopersicon esculentum, were sprayed with ribavirin (500 mg/l) immediately after inoculation with tomato white necrosis virus (deFazio, et al., 1978). Virus replication, as measured by infectivity assays on Chenopodium amaranticolor, was reduced by 89% in inoculated, ribavirin-treated plants.

Multiplication of apple chlorotic leafspot virus in Chenopodium quinoa was completely inhibited when ribavirin was applied between two days before and eight hours after inoculation (Hansen, 1979).
Infectivity was measured by inoculating sap from ribavirin-treated C. quinoa onto fresh C. quinoa. Applications of ribavirin (250 ppm) by leaf spray, soil drench, and brief sand culture were equally effective. Concentrations of ribavirin in excess of 500 ppm were phytotoxic, causing marginal waviness and twisting of young leaves.

Shepard (1977) found that when calli derived from PVX-infected protoplasts of tobacco, *Nicotiana tabacum* variety "Xanthi NC," were exposed to ribavirin (10 mg/l) in a medium that promoted the onset of shoot morphogenesis, the regenerated tobacco plants showed an almost complete absence of PVX infection. Exposure of calli to ribavirin concentrations of 100 mg/l resulted in morphological abnormalities including leaf narrowing and underdevelopment and a weakening of stem and horizontal growth.

The mechanisms of action of ribavirin was investigated by Streeter, et al. (1978). Ribavirin is converted to its 5'-phosphate derivative within the host cell. It then becomes a potent competitive inhibitor of inosinate dehydrogenase which is involved in the conversion of inosinate to xanthylate, the immediate precursor of guanylate and guanosine 5'-monophosphate. This inhibition directly interferes with the synthesis of viral RNA or DNA. The use of ribavirin as a chemotherapeutant has so far been limited due to its toxic effects which include teratogenicity when pregnant mammals are exposed to it (Oxford, 1979; Maugh, 1976).

Amantadine (1-adamantanamine hydrochloride), a tricyclic amine, is the only compound currently used in the prevention of viral infections
of the respiratory tract caused by influenza A virus (Whitley and Alford, 1978). Amantadine appears to interfere with a virus-cell membrane interaction such as penetration, uncoating, or release (Hermans, 1977; Maugh, 1979). Kato and Eggers (1969) observed an inhibition of the uncoating of fowl plaque virus in chick embryo cell cultures when amantadine (25 ug/ml) was present 1 hour before or at the time of inoculation. Inhibition was characterized by a delay in the hemagglutination of chicken red blood cells and appearance of infective virus (PFU/ml) and a decrease in virus yield by 80 to 90%.

Oxford (1979) observed that amantadine (50-100 ug/ml) had no effect on the absorption, penetration, uncoating, or RNA transcripase activity of the amantadine-sensitive influenza A virus. Instead, decreases in polypeptide synthesis were seen using polyacrylamide gel electrophoresis with $^{35}$S-methionine labelled polypeptides. Influenza A recombinants derived from resistant and susceptible parents showed that gene 7, which codes for the virion matrix protein, also carries the property of amantadine resistance. Matrix protein has been shown to affect late uncoating and the subsequent transcription of influenza A virus RNA necessary for cell infection. Therefore, amantadine may inhibit late uncoating (or some unknown event accompanying this) through its effects on matrix protein.

The molecular basis for the mechanism of action of amantadine is still unknown (Oxford, 1979; Prusoff and Ward, 1976). Amantadine has not yet been tested for its effects on plant viruses.

Methisazone (N-methylisatin-B-thiosemicarbazone) is a potent
inhibitor of the pox viruses (Maugh, 1976; Whitley and Alford, 1978; Hermans, 1977). However, the virtual eradication of smallpox has eliminated the need for methisazone. Methisazone is thought to inhibit an intracellular event necessary for virus replication (Prusoff and Ward, 1976). Woodson and Jolik (1965) observed a reduction in the yield (PFU/ml) of vaccinia virus grown on HeLa cell cultures in the presence of methisazone (3.3 ug/ml). Methisazone appears to reduce the amount of "late" viral messenger RNA (present from 3 hours after infection on) thereby resulting in a reduction of 80 to 90% in the synthesis of viral protein and mature viral progeny (Woodson and Jolik, 1965; Whitley and Alford, 1978). It has been speculated that methisazone may also inhibit viral replication by inhibiting the RNA-dependent-RNA polymerase necessary for the replication of RNA viruses (Oxford, 1979). The RNA-dependent-RNA polymerase of influenza virus may be a zinc-activated or zinc metallo-enzyme (Oxford and Perrin, 1974). Certain thiosemicarbazones are chelators of heavy metal ions and have been shown to inhibit RNA-dependent-RNA polymerases (Gingras, et al., 1962). The precise mechanism of action of methisazone is still unknown (Whitley and Alford, 1978) and it has yet to be tested for its effects on plant viruses.

Many benzimidazole compounds are known to inhibit virus multiplication (Matthews and Smith, 1955). Tamm and Eggers (1963) reported that 2-hydroxybenzyl-benzimidazole selectively inhibits the RNA polymerases of certain RNA animal viruses. Methyl benzimidazol-2yl-carbamate (MBC) is the fungitoxic principle of benomyl, a systemic
fungicide widely used in agriculture (Clemons and Sisler, 1969). MBC (2 grams per plant) was shown to suppress symptom expression of TMV in tobacco plants, *Nicotiana tabacum* variety "White Burley," and beet western yellows virus in lettuce, variety "Cobham Green" (Tomlinson, et al., 1976). MBC may affect the host metabolism by preventing the degeneration and loss of chlorophyll which is commonly caused by TMV infection (Waygood, 1965).

Fraser and Whenham (1978) observed that TMV multiplication in tobacco plants, *Nicotiana tabacum*, varieties "White Burley" and Samsun," was inhibited when MBC (0.5 grams per plant) was applied as a solution to the roots, MBC reduced the accumulation of TMV RNA (ug TMV RNA/gram fresh weight) in upper, systemically infected leaves by 98% of that of the control plants, as seen by absorbance of purified extracts at 265 nm. MBC was shown to decrease TMV infectivity and eliminate the inhibition of leaf growth resulting from TMV infection. The inhibition of TMV infectivity and stimulation of leaf growth caused by cytokinins (Fletcher, et al., 1968) suggests that MBC may indirectly suppress TMV RNA accumulation, infectivity, and symptom expression by its known cytokinin activity (Thomas, 1974). MBC is known to be systemically translocated throughout the plant and is stable after absorption at concentrations which are nontoxic thereby making it a potentially useful plant virus chemotherapeutant.

Huang, et al. (1966) reported that formycin, a base analog of adenosine, caused an inhibition of TMV multiplication and symptom development without toxicity to the host plants, *Nicotiana tabacum*
variety "Bright Yellow," and Phaseolus vulgaris variety "Pinto." Leaf disks of N. tabacum variety "Bright Yellow," inoculated with TMV and floated on a medium containing formycin (100 ppm), exhibited a complete absence of systemic symptoms and an 80% inhibition of TMV multiplication. Complete inhibition of local lesion formation was observed on P. vulgaris variety "Pinto," when formycin (100 ppm) was sprayed within 12 hours before and 12 hours after TMV inoculation.

Wawrosh and Sarkar (1974) observed an inhibition of TMV biosynthesis in tobacco plants, Nicotiana tabacum variety "Samsun," by formycin (50 ug/ml). Although, specific infectivity of TMV and TMV RNA were reduced in the presence of formycin, no incorporation of formycin into TMV or host RNA could be detected. The mechanism of action by which formycin inhibits TMV biosynthesis is still unknown.

Several methods are commonly used for the application of antiviral compounds onto plant tissue. Floating leaves or leaf disks on a solution containing the compound has the advantage of uniformly exposing the leaf tissue to a precise amount of the compound (Huang, et al., 1966; Franki, 1962; Kuhn, 1971). However, this method does not permit observations on the effects of a compound on plant growth, virus multiplication, and the development of systemic symptoms over a long period of time. In addition, leaves removed from a host plant exhibit metabolic changes which may influence virus multiplication and response to the antiviral compound.

Leaf sprays (Dawson and Kuhn, 1972; Bawden and Kassanis, 1954) and soil drench (Fraser and Whenham, 1978; Hansen, 1979) are two
methods of application which are useful for screening tests in addition to having potential for practical use. Both methods permit the assessment of the effects of a compound on plant growth, virus multiplication, and the development of systemic symptoms throughout the life of the plant. Leaf sprays have an additional advantage of requiring less compound than soil drench when small plants or small numbers of plants are used.

Cowpea chlorotic mottle virus (CCMV) was discovered by Kuhn in 1964. Since then, its structure and activity have been studied extensively. CCMV is an icosahedral RNA virus with a particle diameter of approximately 25 nm and a molecular weight of $4.6 \times 10^6$ daltons (Maramorosch, 1977). The virus primarily infects legumes and is transmitted by inoculation with infected sap onto healthy host plants. Symptoms include the development of a bright yellow mottle in systemically infected cowpea plants (Vigna sp.) approximately two weeks after inoculation and the formation of dark brown local lesions on the hypersensitive host soybean, Glycine max, from 24 to 48 hours after inoculation.

Purified CCMV nucleoprotein can be obtained in relatively high concentrations of approximately 0.4 mg of virus per gram of infected cowpea leaf tissue (Verduin, 1978). CCMV is most stable at a pH of 4.7. Therefore, CCMV purification is usually carried out in pH 4.7 acetate buffer by the use of chloroform-butanol extraction and differential ultracentrifugation (Bancroft, 1971).

The hypersensitive response of local lesion formation on soybean provides a useful system for assaying the viral infectivity of plant sap
from infected hosts. In addition, CCMV is a good antigen (Maramorosch, 1977), making it possible to use immunological assays for the detection of viral antigen. Immunofluorescence was used to detect the presence of CCMV in the cytoplasm of protoplasts isolated from tobacco plants, *Nicotiana tabacum* variety "White Burley" (Motoyoshi, et al., 1973).

The enzyme-linked immunosorbent assay (ELISA) has been recently used for the highly sensitive detection of plant viruses. The use of ELISA avoids many of the limitations previously imposed by the use of serological techniques and infectivity assays (Clark and Adams, 1977). ELISA enables results to be obtained quickly and quantitatively when a colorimeter is used for the spectrophotometric detection of hydrolyzed substrate. Low virus concentrations can be detected and virus inactivators commonly present in plant extracts do not affect the detection of viral antigen by ELISA. ELISA was found to be 100 times more sensitive than the infectivity assay for detecting maize dwarf mosaic virus in extracts of infected maize leaves (Sum, et al., 1979). ELISA was found to be the only reliable method for the year round detection of chrysanthemum B virus in *Chrysanthemum indicum*, when compared with other plant virus assays (Koenig, et al., 1979).

Since ELISA does not indicate the infectivity of the virus particles, it is necessary to use both the infectivity assay and ELISA to provide a system for determining infectivity and concentration of the virus. Both of these methods have been useful for studying the effects of antiviral compounds on CCMV.
CHAPTER III
MATERIALS AND METHODS

The Plants

Cowpea (*Vigna unguiculata*, variety "California Blackeye") and soybean (*Glycine max*, variety "Harasoy") plants were grown in the greenhouse in sterilized soil consisting of a 1:1:1 mixture of soil, sand and peat moss. A solution of soluble fertilizer (16-32-16) was applied to the soil once a week. Plants were watered daily by direct application of water to the soil.

When the primary leaves were fully expanded (9-11 days after planting), the plants were inoculated with the appropriate dilution of virus-infected cowpea sap. The plants and their response to various treatments were then observed and recorded.

In each experiment, one flat of plants was used for each treatment. Although 12 seeds per flat were planted, the 8 most uniform plants in each flat were selected for treatment. Four replications of each treatment were performed.

The Virus

Maintenance. Cowpea chlorotic mottle virus (CCMV) was maintained in cowpea plants for use in subsequent inoculations of cowpea and soybean plants. Plant sap inoculum was prepared by grinding with a mortar and pestle a 1:10 weight/volume ratio of systemically infected cowpea trifoliate leaves and cold 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M
sodium diethyldithiocarbamate (NaDIECA) and 0.01 M cysteine hydrochloride. The inoculum was strained through several layers of cheesecloth and then stored on ice at 0°C while inoculations were performed.

Inoculation was carried out by first dusting the primary leaves of plants with 600-mesh carborundum. The inoculum was applied by gently rubbing a bent glass rod dipped in inoculum once over the surface of each primary leaf. Inoculated leaves were then rinsed with tap water. Chlorotic mottling appeared on the trifoliate leaves of cowpea plants within 10-14 days after inoculation. Local lesions appeared on the primary leaves of soybean plants within 24-48 hours after inoculation and were counted on half leaves using a hand lense.

**Purification.** Purified CCMV was obtained by differential centifugation according to a modified procedure of Gay and Kuhn (1968). Approximately 200 grams of systemically infected trifoliate leaves were harvested from cowpea plants 10-14 days after the plants were inoculated. The plant tissue was homogenized for 3 minutes in a Waring blender with 2.0 M, pH 5.0 sodium acetate buffer (containing 0.01 M NaDIECA and 0.01 M cysteine hydrochloride), chloroform, and butanol (1.0 ml of each per gram of tissue). All solutions were chilled prior to purification and the preparation was kept on ice in order to maintain it between 0° and 4°C. After straining the homogenate through 8 layers of cheesecloth, it was centrifuged for 10 minutes at 1600 g in a Sorvall RC-2B refrigerated centrifuge. The supernatant was removed and centrifuged for 10 minutes at 25,000 g. The supernatant was once again removed and centrifuged for 1 hour at 98,000 g in a Beckman L2-65B ultracentrifuge.
Each pellet was suspended in 1 ml of cold 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M MgCl\textsubscript{2} (Dawson and Kuhn, 1972), using a glass homogenizer. The homogenate was then centrifuged for 10 minutes at 20,000 g. The supernatant was removed and centrifuged for 1 hour at 138,000 g. Each pellet was suspended in 0.1 M, pH 5.0, sodium acetate buffer, containing 0.01 M MgCl\textsubscript{2}. The homogenate was centrifuged at 20,000 g. The supernatant was removed and centrifuged for 1 hour at 138,000 g. Each pellet was again suspended in 0.01 M, pH 5.0, sodium acetate buffer, containing 0.01 M MgCl\textsubscript{2}. The homogenate was centrifuged once more at 20,000 g. The supernatant contained purified CCMV in buffer. The amount of virus nucleoprotein was estimated by measuring the optical density at 260 nm using a Beckman DB-G grating spectrophotometer. A virus nucleoprotein concentration of 1 mg/ml has an optical density of 6.0 (Dawson and Kuhn, 1972). Yields of purified CCMV ranged from 0.2 to 0.6 mg per gram infected plant tissue. The purified CCMV preparation was stored at 4°C until used.

The Antiserum Production. Purified CCMV was suspended in 0.01 M, pH 7.0, phosphate buffer, containing 0.85% NaCl, (PBS) for injection into rabbits. A preparation of purified CCMV nucleoprotein, suspended in acetate buffer, was centrifuged for 1 hour at 138,000 g. The pellets were suspended and homogenized in PBS. The homogenate was centrifuged at 20,000 g. The supernatant contained purified CCMV nucleoprotein suitable for use as antigen in the production of CCMV antisera. Two rabbits were immunized
and bled according to the procedure of Otsuki and Takebe (1969). Each rabbit was injected intravenously with 10 mg of purified CCMV antigen in 1 ml PBS. Four days later, the rabbits received intramuscular injections of 50 mg CCMV in 1.2 ml PBS emulsified with an equal volume of Freund's complete adjuvant. After 1 month a final intravenous injection of 10 mg CCMV in 0.8 ml PBS was injected into each rabbit. The rabbits were bled by cardiac puncture using a 50 cc sterile plastic syringe 10 days after the last immunization. Food was withheld from the rabbits for 16 hours before bleeding to reduce serum lipids.

The antiserum was prepared according to the procedure of Williams and Chase (1967). The blood was allowed to clot for 1 hour at 37°C in sterile 50 ml plastic centrifuge tubes. The tubes were rimmed with a wooden applicator stick and refrigerated overnight at 4°C for clot contraction. The clot was removed and the serum was centrifuged for 20 minutes at 300 g in a Sorvall RC-2B refrigerated centrifuge to remove blood cells. Complement was inactivated by incubating the serum at 56°C for 20 minutes. The serum was stored in 3 ml serum vials and frozen until used.

**Titer and specificity.** The antiserum from each rabbit was titered by using the microprecipitin test according to a modified procedure of Ball (1974). Grid patterns were drawn on microprecipitin plates so that serial dilutions of CCMV-antiserum (from each rabbit) or normal rabbit serum and antigen (purified CCMV) could be placed in each square. Normal rabbit serum was included to be certain that a nonspecific precipitin reaction was not occurring between CCMV antigen and rabbit
serum proteins. Each serum was serially diluted in 0.01 M, pH 5.0, sodium acetate buffer. One ul drop of each serial dilution of antiserum and CCMV antigen was placed in each square on a microprecipitin plate. The drops of antiserum and antigen in each square were gently mixed with wooden applicator sticks. The same procedure was carried out with normal rabbit serum and CCMV antigen. The plates were incubated overnight at 4°C in a moisture chamber. They were observed the following day under a light microscope for the presence of a cloudy, white precipitate. The greatest antiserum dilution at which a precipitate was seen represented the titer of the antiserum. The antiserum titers for both rabbits were between 1/800 and 1/1600.

Specificity of the antisera for CCMV antigen, as opposed to normal plant tissue antigens, was demonstrated by the Ouchterlony gel double diffusion test and the viral neutralization test.

Ouchterlony plates were prepared by dissolving 0.9 g Agarose agar in 100 ml 0.01 M, pH 7.0, PBS. The agar was autoclaved for 18 minutes and preserved by the addition of 0.025% sodium azide. The agar was poured into sterile disposable plastic petri plates. After the agar had solidified, 7 wells were cut using a size 5 cork borer. The center well contained CCMV antiserum diluted 1:10 in PBS. The six surrounding outer wells contained either purified CCMV antigen (100 ug/ml in sodium acetate buffer), CCMV-infected plant sap, or healthy plant sap (both diluted 1:10 in sodium acetate buffer). The plates were incubated in a moisture chamber for one week at 4°C. Precipitin bands formed only between purified CCMV antigen and CCMV antiserum and
between CCMV-infected plant sap and CCMV antiserum but never between healthy plant sap and CCMV antiserum.

For virus neutralization, CCMV antiserum was serially diluted with PBS. One ml of each serial dilution was incubated with 1 ml purified CCMV antigen (100 ug/ml) for 30 minutes. Each mixture was then inoculated onto the primary leaves of soybean plants (6 leaves/treatment). After 48 hours, the presence of antiserum (diluted up to 1:1600) in the inoculum was seen to reduce local lesion numbers. Neutralization of virus infectivity suggested that the antiserum contained antibodies specific for CCMV.

The Antiviral Compounds

Preparation of antiviral compounds. Each compound was applied onto cowpea and soybean plants at the highest concentration determined to be nontoxic. 2-Thiouracil (supplied by Sigma Chemical Co., St. Louis, Missouri) and ribavirin, trademark named Virazole, (supplied by ICN Pharmaceuticals, Irvine, California) were suspended at a concentration of 500 mg per liter distilled, deionized water. Amantadine (supplied by Pfaltz and Bauer, Inc., Stamford, Connecticut) was suspended at a concentration of 250 mg per liter distilled, deionized water. Methisazone, trademark named Marboran, (supplied by Burroughs Wellcome Co., Research Triangle Park, North Carolina) was suspended in 1 N NaOH which was further diluted 1:10 with distilled, deionized water to give a final methisazone concentration of 250 mg per liter. Formycin, trademark named Formycin B, (supplied by Sigma Chemical Co., St. Louis,
Missouri) was suspended at a concentration of 25 mg per liter distilled, deionized water. Bavistin (supplied by B.A.S.F., Aktiengesellschaft, West Germany), a commercial preparation containing 50% (w/w) methyl benzimidazol-2-yl-carbamate in non-active filler, was suspended at a concentration of 1,000 mg per liter distilled, deionized water.

Method of application. Both cowpea and soybean plants were sprayed with the antiviral compounds using DeVilbiss Non-Corrosive 251 Atomizers. The plants were sprayed with a fine mist until droplets began to coalesce to assure even coverage of the leaves.

Timing of compound application. Six spray regimes (Treatments A-F) were followed for the application of the antiviral compounds. The compounds were sprayed as follows:

- Treatment A—2 days and 1 day before inoculation and at the time of inoculation.
- Treatment B—2 days and 1 day before inoculation, at the time of inoculation, and 1 day after inoculation.
- Treatment C—At the time of inoculation.
- Treatment D—At the time of inoculation and 1 day after inoculation.
- Treatment E—At the time of inoculation and 1 day, 2 days, 3 days, and 4 days after inoculation.
- Treatment F—1 day, 3 days, 6 days, and 8 days after inoculation.

Plants were always inoculated in the morning. When plants were sprayed on the day of inoculation, spraying took place immediately after inoculation.
Experimental Design

Direct in vitro effects of antiviral compounds on CCMV infectivity. The direct or in vitro effects of the antiviral compounds were tested to determine whether any of the compounds directly influenced the infectivity of the CCMV inoculum or the infection process. Systemically infected tissue from cowpea plants was homogenized in 0.1 M, pH 5.0, sodium acetate buffer containing 0.01 M NaDIECA and 0.01 M cysteine hydrochloride to give a 1:5 plant sap dilution. Double strength spray solutions were made for each of the six compounds. Three ml of plant sap were mixed with 3 ml of each spray solution. The mixtures were allowed to incubate for 1 hour at 4°C. The pH of each mixture was determined using a pH meter. Each mixture was inoculated onto 8 soybean primary leaves. Control plants were inoculated with a mixture containing 3 ml of plant sap and 3 ml of distilled, deionized water. Local lesions were counted after 48 hours.

Effects of antiviral compounds on local lesion formation on soybean plants. Preliminary experiments were done to examine the effects of 2-thiouracil, ribavirin, amantadine, methyl benzimidazol-2yl-carbamate, methisazone, and formycin on local lesion formation on soybean plants. The six compounds were applied using spray treatments A, B, C, and D. The control consisted of plants which were inoculated without being sprayed. Eight soybean primary leaves were inoculated and local lesions were counted after 48 hours.

Further testing of ribavirin, amantadine, formycin, and methisazone
was accomplished by spraying the soybean plants three times on the day of inoculation. Plants were sprayed at 8:00 A.M., 12:00 A.M., and 5:00 P.M. while inoculation was carried out at approximately 11:00 A.M. A spreader-sticker was added to each spray solution at a concentration of 50 ul in 30 ml of solution. Controls consisted of inoculated soybean plants which were unsprayed, sprayed with distilled water, or sprayed with distilled water containing spreader-sticker. Inoculation of counting of local lesions were done according to the procedures previously described.

Effects of antiviral compounds on systemic CCMV infection of cowpea plants. Preliminary experiments were done to determine what effects, if any, the six antiviral compounds exhibited on systemic infection of cowpea plants. The six compounds were applied using spray treatments, A, B, C, D, E, and F. The positive control consisted of plants which were inoculated with CCMV without being sprayed. The negative control consisted of plants which were sprayed without being inoculated. To determine the effects of the antiviral compounds on the infectivity of CCMV within the systemically infected plants, infectivity assays were performed on soybean plants using cowpea tissue from each treatment as an inoculum source. To prepare the inoculum, six primary leaves were randomly selected from cowpea plants receiving a given treatment. The six leaves were shredded into small 3 mm pieces with a razor blade. Using a mortar and pestle, 0.5 g of shredded leaf tissue was homogenized with 4.5 ml of pH 5.0, 0.1 M., sodium acetate buffer containing 0.01 M NaDIECA and 0.01 M cysteine hydrochloride. Eight soybean primary leaves
were inoculated and local lesions were counted according to the procedures previously described. These assays were performed 10 days after the cowpea plants were inoculated. Visual observations were recorded 10 days and 42 days after inoculation to determine the effects of each compound on symptom development and plant growth.

A second set of experiments were done to further test four of the antiviral compounds which appeared to have an effect on systemic CCMV infection of cowpea plants in the preliminary experiments. 2-Thiouracil, ribavirin, amantadine, and methisazone were applied using spray treatments A, C, and E. The control plants were inoculated without being sprayed. Infectivity assays were performed according to the procedure described in the preliminary experiment. Two infectivity assays were performed 10 days after inoculation using either the primary leaves or the trifoliate leaves as an inoculum source. Two additional infectivity assays were performed 16 and 22 days after inoculation using trifoliate leaves as an inoculum source.

A final set of experiments was done to determine the effects of two of the antiviral compounds on the presence of CCMV antigen within systemically infected cowpea plants. Ribavirin and amantadine were applied using spray treatment E. The controls consisted of healthy plants which were not inoculated or sprayed and inoculated plants which were not inoculated or sprayed and inoculated plants which were not sprayed. The enzyme-linked immunosorbent assay (ELISA) was performed 2, 4, 8, and 15 days after inoculation. Randomly selected primary leaves (at 2, 4, and 8 days after inoculation) and trifoliate leaves (at
15 days after inoculation) from each treatment were homogenized in the ELISA virus (conjugate) buffer (see below) using a mortar and pestle to give 1:10 (w/v) dilutions of plant sap in which the presence of viral antigen could be detected. Separate ELISA tests were performed on each of the 4 replications of each antiviral compound spray treatment.

**The Enzyme-Linked Immunosorbent Assay**

The enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the procedures of Clark and Adams (1977).

**Preparation of buffers and glassware.** Several buffers and solutions were prepared for ELISA. Coating buffer was prepared by adjusting the pH of 0.05 M sodium carbonate buffer to 9.6 with HCl. PBS-Tween washing solution was prepared by adding 0.15 M NaCl and 0.05% (v/v) Tween 20 to 0.02 M, pH 7.4, phosphate buffer. Conjugate (virus) buffer was prepared by adding 2.0% (w/v) polyvinylpyrrolidone (MW 10,000) and 0.2% (w/v) ovalbumin to PBS-Tween. Substrate buffer was prepared by adjusting the pH of 10% (v/v) diethanolamine to pH 9.8 with HCl. A solution of 3 M NaOH was prepared to stop enzyme activity before the ELISA plates were read. Sodium azide (0.02% w/v) was added to all buffers to prevent contamination.

Any glassware which contained antibodies was siliconized with Sigmacote to prevent loss of protein by absorption.

**Immunoglobulin G purification.** One ml samples of CCMV antiserum were diluted with 9 ml distilled, deionized water. Ten ml of saturated
ammonium sulphate solution were added slowly while stirring to 10 ml of diluted antiserum. After the mixture was allowed to stand for 1 hour at room temperature, it was centrifuged for 5 minutes at 4,000 g to collect the precipitate. The precipitate was dissolved into 2 ml of half-strength PBS. The sample was then dialyzed overnight at 4°C against 500 ml of half-strength PBS with 2 changes of the buffer during the dialysis.

Further purification of IgG was achieved by passing the sample through a short chromatographic column which was packed to a height of 5 cm with DE 22 cellulose (Whatman, Inc.). DE 22 cellulose was pre-equilibrated in half-strength PBS and prepared for use according to the manufacturer's instructions. Malachite green dye was passed through the packed column to make certain the column was properly packed before the IgG sample was loaded onto the column. Once a sample was loaded onto the column, the column was washed with half-strength PBS until purified IgG protein was eluted. Two ml fractions were collected and monitored spectrophotometrically at 280 nm. The fraction containing purified IgG (unabsorbed protein) was eluted soon after the void volume. The peak fraction had an optical density of 2.84 at 280 nm. The presence of purified IgG was confirmed by a positive precipitin reaction with purified CCMV antigen. The purified IgG was diluted in half-strength PBS until an optical density of 1.4 was obtained to give a concentration of approximately 1 mg/ml. It was then stored in siliconized glass tubes at 4°C.

Conjugation of enzyme with IgG. A liquid preparation containing 2 mg of
Sigma No. P-4502 Type VII alkaline phosphatase in 0.001 M MgCl₂ and 0.0001 M ZnCl₂ was centrifuged at 4,000 g for 5 minutes. The supernatant was discarded and the precipitate was dissolved by direct addition of 1 ml (=1 mg) of purified IgG to the centrifuge tube. The preparation was dialyzed against 500 ml PBS overnight at 4°C with 2 changes of the buffer during dialysis. After dialysis, 25 % glutaraldehyde solution was mixed with the IgG preparation to give a final glutaraldehyde concentration of 0.05% (v/v). The mixture was incubated for 4 hours at room temperature after which time a very faint sandy brown color developed. The conjugate preparation was dialyzed overnight at 4°C against 500 ml PBS with 0.01% sodium azide with 2 buffer changes during the dialysis. After the addition of 5 mg/ml bovine serum albumin, the conjugate was stored at 4°C in a siliconized glass test tube.

ELISA procedure. Purified IgG was diluted 1:10,000 in coating buffer to give an optimum concentration of 0.1 µg/ml coating IgG. The wells of the ELISA plates (Gilford Instruments, Inc.) were each coated with 200 ul of coating IgG. Outer wells which were not used were filled with distilled water throughout this procedure. The plate was covered with Saran Wrap and incubated in a moisture chamber for 4 hours at 37°C or overnight at 4°C. The wells were washed by flooding them with PBS-Tween and allowing them to sit for 3 minutes before emptying the plate. After the wells were washed 3 times, test samples diluted in virus (conjugate) buffer and filtered through 2 layers of Miracloth were added in 200 ul aliquots to the coated wells. The plate was incubated and washed as described above. Alkaline phosphatase conjugated IgG was
diluted 1:1600 in conjugate buffer to give an optimum concentration of conjugated IgG. The conjugate was added in 200 ul aliquots to each well. The plate was incubated and washed as described above. P-nitrophenyl phosphate tablets were dissolved into substrate buffer to give a final substrate concentration of 1 mg/ml. Substrate was added in 300 ul aliquots to each well. After a 1-hour incubation at room temperature, the reaction was stopped by the addition of 50 ul 3 M NaOH to each well. The results were determined within a half hour by quantitating the absorbance of the hydrolyzed substrate at 405 nm using a Gilford EIA processor/reader.

Determination of optimum coating IgG and enzyme-conjugated IgG concentrations. The ELISA procedures described above were used to determine optimum concentrations of coating and enzyme-conjugated IgG. The wells of an ELISA plate were coated with serial dilutions of coating IgG (10, 1.0, and 0.1 ug/ml) in coating buffer. Three replicates of each coating IgG concentration were used. Each of the three replicates received either a 1:200, a 1:800, or a 1:3200 dilution of enzyme conjugated IgG. The following test samples were used: 1:10, 1:100, 1:1,000 (w/v) dilutions of virus-infected tissue in virus (conjugate) buffer: 1:10 and 1:100 (w/v) dilutions of healthy tissue in virus (conjugate) buffer; and virus conjugate buffer. Optimum concentrations were determined spectrophotometrically as those concentrations of coating and enzyme-conjugated IgG which produced $A_{405}$ readings between 0 and 2.0.
Statistical Analysis

Statistical analysis of local lesion numbers and ELISA A_{405} readings was done by a one-way analysis of variance for the variable experimental treatment (Zar, 1974). For analysis of local lesion numbers, the mean values of 8 local lesion numbers (from 8 primary half leaves of soybean plants) were used to represent each experimental treatment. Since 4 replications of each experimental treatment were performed in all experiments, the group mean of the 4 replications for each experimental treatment were determined. Analysis of ELISA A_{405} readings was accomplished by using the group mean values from 4 replications of each experimental treatment. The Duncan new multiple range test was used for a comparison of the group means to determine which experimental treatments produced significantly different effects on CCMV infectivity at the 0.05 level of significance (Zar, 1974).

Since local lesion numbers deviate greatly from a normal frequency distribution, analysis of variance by the Duncan new multiple range test was also performed on local lesion numbers which were transformed according to the procedure of Kleczkowski (1955). The transformation \( z = \log_{10}(x+c) \) was used when local lesion numbers were greater than 10, where \( x \) = number of lesions and \( c = 5 \). Numbers less than 10 were transformed according to the formula \( z = \log_{10} \left( \frac{1}{2} (x+c+\sqrt{x^2+2cx}) \right) \). However, transformed local lesion numbers are only presented when they exhibit differences among experimental treatments which were not evident from the analysis of untransformed local lesion numbers.
Effects of Antiviral Compounds on the Development of Viral Symptoms and Plant Growth

Six antiviral compounds namely 2-thiouracil, ribavirin, amantadine, formycin, methisazine, and methyl benzimidazol-2yl-carbamate, were applied on cowpea leaves according to spray treatment regimes A, B, C, D, E, or F (described in Materials and Methods). Their effects on CCMV symptom expression and their toxicity to plant growth were determined by visual observations recorded 10 and 42 days after the inoculation of cowpea plants. Symptom development and plant growth were compared to that of unsprayed, inoculated and sprayed, uninoculated control plants.

Cowpea plants inoculated with CCMV and sprayed with 2-thiouracil, treatments A, E, and F, exhibited severe CCMV symptoms consisting of chlorotic mottling 10 days after inoculation, while plants sprayed with 2-thiouracil, treatments B, C, and D, exhibited slight chlorotic mottling when compared with the unsprayed, inoculated control plants (Fig. 1 and Fig. 2). Inoculated and uninoculated plants sprayed according to any of the treatment regimes (A through F) exhibited symptoms of toxicity which included stunting of plant growth, bleaching of primary and trifoliate leaves, and puckering of trifoliate leaf margins (Fig. 2). Forty-two days after inoculation, sprayed cowpea plants still exhibited puckering of young, newly expanded, trifoliate leaf margins. Symptoms
Fig. 1. Chlorotic mottling on trifoliate leaves of cowpea plants systemically infected with cowpea chlorotic mottle virus observed ten days after inoculation.

Fig. 2. Reduction (left) and increase (right) in symptom development of cowpea chlorotic mottle virus-infected cowpea plants caused by different 2-thiouracil treatments within ten days after inoculation. Stunting, bleaching, and puckering of trifoliate leaves were toxic effects caused by 2-thiouracil application.
of CCMV continued to remain severe with treatments A, E, and F while with treatments B and D symptoms became severe on young trifoliate leaves of plants observed 42 days after inoculation. Symptoms developed only slightly on plants sprayed according to treatment C and observed 42 days after inoculation.

Application of ribavirin, treatments A through F, caused cowpea plants to remain almost completely free of CCMV symptoms during the 10 days after inoculation. No toxic effects were exhibited on the plants when sprayed according to any of the six treatment regimes (Fig. 3). When the cowpea plants were observed 42 days after inoculation, they still did not exhibit any symptoms of toxicity. In addition, young trifoliate leaves of plants sprayed with treatments E and F were completely free of CCMV symptoms 42 days after inoculation while those sprayed with treatment A exhibited only very faint symptoms. Young trifoliates of plants sprayed according to treatments B, C, and D exhibited more severe symptoms at 42 days after inoculation than did trifoliates at 10 days after inoculation but the symptoms were milder than those exhibited 42 days after inoculation by unsprayed, control plants.

Amantadine, applied according to treatments A through F, reduced the chlorotic mottling symptoms of CCMV-infected cowpea plants observed 10 days after inoculation. Treatments A, B, C, and D resulted in almost complete suppression of CCMV symptoms, while treatments E and F allowed mild symptom development during the first 10 days after inoculation. Trifoliate leaves showed minimal margin necrosis when amantadine was applied according to treatments E and F on both sprayed inoculated and
Fig. 3. Inhibition of cowpea chlorotic mottle virus symptom development and absence of toxic effects observed ten days after inoculation when cowpea plants were sprayed with ribavirin.
sprayed, uninoculated control plants (Fig. 4) when observed 10 days after inoculation. Forty-two days after inoculation, some leaf margin necrosis was still observed on trifoliate leaves of cowpea plants sprayed according to treatments E and F. Young trifoliate leaves on plants sprayed according to treatments A, B, C, and D were almost completely free of CCMV symptoms when observed 42 days after inoculation. Treatments E and F allowed the development of some symptoms on young trifoliate leaves 42 days after inoculation but these were much less severe than the symptoms of unsprayed, inoculated control plants.

Formycin, although nontoxic to the directly sprayed primary leaves of cowpea plants, was highly toxic to young, newly expanding, trifoliate leaves of sprayed, inoculated plants and sprayed, uninoculated control plants. The toxic effects included leaf margin necrosis, leaf puckering and curling, and the formation of necrotic 'shot-hole' lesions on trifoliate leaves. The development of CCMV symptoms was inhibited by formycin treatments A, B, C, D, and E when evaluated 10 days after inoculation (Fig. 5). At 42 days after inoculation, the toxic effects caused by formycin were less severe than at 10 days after inoculation. CCMV symptoms in young trifoliate leaves observed 42 days after inoculation were reduced by treatments A, B, C, and D just as they were 10 days after inoculation; however, treatment E was no longer as effective in reducing symptom development. Young trifoliates of plants sprayed according to treatments E and F exhibited CCMV symptoms as severe as the symptoms on unsprayed, inoculated control plants 42 days after inoculation.
Fig. 4. Amantadine induced reduction in cowpea chlorotic mottle virus symptom development of cowpea plants within 10 days after inoculation.

Fig. 5. Severe phytotoxicity but reduction in cowpea chlorotic mottle virus symptom development observed on cowpea plants ten days after inoculation as a result of formycin application.
Application of methisazone treatments A through F inhibited CCMV symptom development of cowpea plants observed 10 days after inoculation. All methisazone treatments induced severe toxic effects consisting of striation and narrowing of trifoliate leaves in both sprayed, inoculated and sprayed, uninoculated control plants observed 10 days after inoculation (Fig. 6). Forty-two days after inoculation, these toxic effects were no longer observed on trifoliate leaves. CCMV symptoms were absent in young trifoliates of plants sprayed with treatments A through F and observed 42 days after inoculation.

Cowpea plants sprayed with methyl benzimidazol-2yl-carbamate according to treatments A through F, exhibited CCMV symptoms that were comparable to the symptoms on unsprayed, inoculated control plants with no evidence of any toxic effects throughout the 42 days of observation (Fig. 7).

**Direct In Vitro Effects of Antiviral Compounds on CCMV Infectivity**

Mixtures of each antiviral compound and plant sap from CCMV-infected cowpea leaves were incubated for 1 hour at 4°C to determine the direct effects, if any, of each compound on the infectivity of CCMV. The in vitro effects were determined by performing infectivity assays using the antiviral compound-CCMV infected sap mixtures as the inoculum. When local lesion numbers were compared only methisazone directly reduced CCMV infectivity significantly. This was apparently due to the elevation of the pH of the inoculum when mixed with methisazone. When transformed local lesion numbers were compared, treatment of CCMV in sap
Fig. 6. Formation of narrow, striated trifoliate leaves and reduced cowpea chlorotic mottle virus symptom development observed on methisazone-treated cowpea plants observed ten days after inoculation.

Fig. 7. Absence of toxic effects and presence of normal cowpea chlorotic mottle virus symptoms on trifoliate leaves of cowpea plants sprayed with methyl benzimidazol-2yl-carbamate and observed ten days after inoculation.
with either methisazone or ribavirin reduced CCMV infectivity significantly (Table 1).

### Effects of Antiviral Compound Sprays on Formation of Local Lesions on Soybean Plants

The six antiviral compounds namely 2-thiouracil, ribavirin, amantadine, formycin, methisazone, and methyl benzimidazol-2yl-carbamate were sprayed on soybean plants according to treatment regimes A through D and the soybean primary leaves were inoculated with CCMV-containing sap from systemically infected cowpea leaves. The number of local lesions produced on the soybean primary leaves 48 hours after inoculation was taken as a measure of the effect of each antiviral compound on local lesion formation of CCMV. Although each of the compounds, with all treatment regimes, reduced the number of local lesions formed, the lesion numbers were not significantly lower than those on unsprayed, inoculated control soybean plants (Table 2). Transformed local lesion numbers yielded no further statistically significant differences. Local lesion size remained the same in the control plants and in plants receiving the various treatments.

### Effects of Antiviral Compounds on Formation of Local Lesions on Soybean Plants Receiving Three Spray Applications on Day of Inoculation

Ribavirin, amantadine, formycin, and methisazone were applied on soybean plants three times (at 8:00 A.M., 12:00 A.M., and 5:00 P.M.) on the day of their inoculation with CCMV in order to test further the effect of these compounds on local lesion formation. Each compound was
### TABLE 1

Effect of Antiviral Compounds on pH and Infectivity of Cowpea Chlorotic Mottle Virus Inoculum

<table>
<thead>
<tr>
<th>Antiviral Compound</th>
<th>Inoculum pH</th>
<th>Inoculum Infectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Local Lesion Transformation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Thiouracil</td>
<td>5.7</td>
<td>73.00 ± 66.03</td>
<td>1.73</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>5.7</td>
<td>48.97 ± 46.96</td>
<td>1.58*</td>
</tr>
<tr>
<td>Amantadine</td>
<td>5.2</td>
<td>65.38 ± 44.60</td>
<td>1.77</td>
</tr>
<tr>
<td>Formycin</td>
<td>5.3</td>
<td>104.94 ± 17.09</td>
<td>2.04</td>
</tr>
<tr>
<td>Methisazone</td>
<td>11.0</td>
<td>0.03*+ 0.07</td>
<td>0.41*</td>
</tr>
<tr>
<td>Methyl benzimidazol-2yl-carbamate</td>
<td>6.3</td>
<td>85.47 ± 28.57</td>
<td>1.94</td>
</tr>
<tr>
<td>Control--No Compound</td>
<td>5.5</td>
<td>85.72 ± 29.50</td>
<td>1.94</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean leaves.

<sup>b</sup>Each number represents a logarithmic transformation of the mean number of local lesions (Kleczkowski, 1955).

*Statistically significant difference in infectivity between antiviral compound and control when analyzed by the Duncan new multiple range test (*P*=0.05).
TABLE 2

Effect of Antiviral Compound Sprays on the Number of Cowpea Chlorotic Mottle Virus Local Lesions on Soybean Plants

<table>
<thead>
<tr>
<th>Antiviral Compound</th>
<th>Spray Treatment Regime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>102.19 ± 86.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>71.63 ± 35.47</td>
</tr>
<tr>
<td>Amantadine</td>
<td>86.22 ± 66.12</td>
</tr>
<tr>
<td>Formycin</td>
<td>111.61 ± 39.15</td>
</tr>
<tr>
<td>Methyl benzimidazol-2yl-carbamate</td>
<td>66.69 ± 11.13</td>
</tr>
<tr>
<td>Methisazole</td>
<td>73.13 ± 20.64</td>
</tr>
<tr>
<td>Control--No Compound</td>
<td>117.41 ± 49.30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean half leaves. The local lesions numbers were statistically analyzed by the Duncan new multiple range test (P=0.05).
mixed with a spreader-sticker before it was sprayed on the plants. The numbers of local lesions produced on plants sprayed with the four antiviral compounds were significantly lower than those produced on inoculated control plants that were left unsprayed, sprayed with distilled water, or sprayed with distilled water containing spreader-sticker. Furthermore, the number of local lesions produced on methisazone-treated plants was significantly lower than the number of local lesions produced on plants treated with any of the other antiviral compounds. Transformed local lesion numbers yielded no further significant differences. Local lesion numbers were also lower on inoculated control plants sprayed with a mixture of distilled water and spreader-sticker, but these numbers were not significantly lower than the number of local lesions produced on unsprayed plants (Table 3). None of the antiviral treatments affected the size of individual local lesions.

**Effects of Antiviral Compound Sprays on Infectivity of CCMV in Cowpea Plants**

The antiviral compounds 2-thiouracil, ribavirin, amantadine, formycin, methisazone, and methyl benzimidazol-2yl-carbamate were sprayed on CCMV-infected cowpea plants according to spray treatment regimes A, B, C, D, E, and F. The treated plants were assayed for virus infectivity, as determined by the number of local lesions produced on soybean plants (Fig. 8), by harvesting the primary leaves of the treated cowpea plants 10 days after inoculation and using them as a source of inoculum for the inoculation of soybean plants.

The treatments were evaluated for their effects on the infectivity of
TABLE 3

Effect of Antiviral Compound Sprays, Applied 3 Times on the Day of Inoculation, on the Number of Cowpea Chlorotic Mottle Virus Local Lesions on Soybean Plants

<table>
<thead>
<tr>
<th>Spray Treatment</th>
<th>Number of Local Lesions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methisazone</td>
<td>$3.07^a \pm 1.42$</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>$6.57^b \pm 2.61$</td>
</tr>
<tr>
<td>Amantadine</td>
<td>$8.38^b \pm 1.81$</td>
</tr>
<tr>
<td>Formycin</td>
<td>$9.03^b \pm 4.43$</td>
</tr>
<tr>
<td>Control—Distilled H$_2$O and Spreader-Sticker</td>
<td>$16.72^c \pm 2.54$</td>
</tr>
<tr>
<td>Control—Distilled H$_2$O</td>
<td>$27.51^c \pm 2.26$</td>
</tr>
<tr>
<td>Control—No Spray</td>
<td>$29.38^c \pm 2.86$</td>
</tr>
</tbody>
</table>

*Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean half leaves. Means followed by a common letter are not significantly different. The local lesion numbers were statistically analyzed by the Duncan new multiple range test ($P=0.05$).
Fig. 8. Local lesions on primary leaves of soybean plants observed 24 to 48 hours after inoculation.
CCMV in cowpea plants by comparing the infectivity of CCMV in sap from sprayed plants to that of unsprayed, inoculated control plants (Table 4). Although many of the treatments caused some decrease in CCMV infectivity, none of these were significantly lower than the infectivity of sap from unsprayed, inoculated control plants. The six compounds reduced CCMV infectivity in cowpea plants when applied according to the following spray treatment regimes: 2-thiouracil, A, B, C, and D; ribavirin, A, B, C, D, E, and F; amantadine, A, B, C, D, and E; formycin, A, B, and C; methisazone, A, B, C, D, and E; methyl benzimidazol-2yl-carbamate, A, B, C, E, and F. Eight of the treatments caused some increase in CCMV infectivity. These were: E and F for 2-thiouracil; F for amantadine; D, E, and F for formycin; F for methisazone; and D for methyl benzimidazol-2yl-carbamate. 2-Thiouracil treatments E and F increased infectivity significantly (Table 4). Transformed local lesion numbers yielded no further significant differences.

Effects of Antiviral Compounds on Infectivity of CCMV in Cowpea Plants at 10, 16, and 22 Days After Inoculation

Four of the antiviral compounds namely 2-thiouracil, ribavirin, amantadine, and methisazone which appeared to have the greatest effects on CCMV infectivity and on symptom development 10 days after inoculation, were further tested for their effects on CCMV infectivity in cowpea plants. Infectivity assays were performed 10 days after inoculation using as inoculum either the inoculated primary leaves or the first trifoliate leaves. The purpose of this experiment was to determine whether CCMV infectivity in the younger trifoliates was more or less sensitive
Effect of Antiviral Compound Sprays on Infectivity of Cowpea Chlorotic Mottle Virus in Cowpea Plants, Assayed 10 Days after Inoculation

<table>
<thead>
<tr>
<th>Antiviral Compound</th>
<th>Spray Treatment Regime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>73.16\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>±14.14</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>62.72</td>
</tr>
<tr>
<td></td>
<td>±37.14</td>
</tr>
<tr>
<td>Amantadine</td>
<td>60.03</td>
</tr>
<tr>
<td></td>
<td>±20.08</td>
</tr>
<tr>
<td>Formycin</td>
<td>52.56</td>
</tr>
<tr>
<td></td>
<td>±32.41</td>
</tr>
<tr>
<td>Methisazone</td>
<td>56.72</td>
</tr>
<tr>
<td></td>
<td>±40.55</td>
</tr>
<tr>
<td>Methyl benzimidazol-2yl-carbamate</td>
<td>78.75</td>
</tr>
<tr>
<td></td>
<td>±13.64</td>
</tr>
<tr>
<td>Control—No Compound</td>
<td>79.66</td>
</tr>
<tr>
<td></td>
<td>±12.72</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean half leaves. The local lesion numbers were analyzed by the Duncan new multiple range test (P=0.05).

*Statistically significant difference between antiviral compound and control.
to the effects of the antiviral compounds than CCMV infectivity in the inoculated primary leaves. Trifoliate leaves were also used as inoculum when infectivity assays were performed at 16 and 22 days after inoculation to determine whether the antiviral compounds become more or less effective against the virus at later stages of infection. The compounds were applied according to spray treatment regimes A, C, and E. The treated cowpea plants were assayed for virus infectivity (as determined by the number of local lesions produced on soybean plants) by using tissue from treated plants as inoculum for inoculation of soybean plants. Local lesion numbers produced by sap from sprayed plants were compared to those produced by sap from unsprayed, inoculated control plants (Table 5). Transformation of local lesion numbers yielded no additional significant differences.

When the primary leaves of treated cowpea plants were assayed, ribavirin, amantadine, and methisazone reduced virus infectivity but these reductions were not statistically significant (Table 5). 2-Thiouracil decreased virus infectivity when applied according to treatment A, but when it was applied according to treatments C and E, 2-thiouracil increased virus infectivity. 2-Thiouracil treatment E increased virus infectivity considerably over the virus infectivity in unsprayed control plants (Table 5).

When infectivity of virus in trifoliate leaves was assayed 10 days after primary leaf inoculation, all of the antiviral compound treatments reduced CCMV infectivity in the sprayed cowpea plants. However, only plants sprayed with ribavirin treatment E had significantly lower virus
TABLE 5

Effect of Antiviral Compounds on Infectivity of Cowpea Chlorotic Mottle Virus in Cowpea Plants at 10, 16, and 22 Days After Inoculation

<table>
<thead>
<tr>
<th>Antiviral Compound</th>
<th>Treatment</th>
<th>Days After Inoculation--Tissue Assayed&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 - Pri.</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>A</td>
<td>43.13 ± 16.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>63.66 ± 21.69</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>71.53 ± 23.04</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>A</td>
<td>59.22 ± 17.74</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>59.19 ± 23.06</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>37.69 ± 7.73</td>
</tr>
<tr>
<td>Amantadine</td>
<td>A</td>
<td>50.03 ± 9.03</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>54.78 ± 17.15</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>45.72 ± 17.47</td>
</tr>
<tr>
<td>Methisazole</td>
<td>A</td>
<td>48.35 ± 18.58</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>45.28 ± 21.77</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>44.35 ± 18.34</td>
</tr>
<tr>
<td>Control</td>
<td>No Spray</td>
<td>62.39 ± 20.54</td>
</tr>
</tbody>
</table>

<sup>a</sup>Primary leaf tissue (Pri.) and trifoliate leaf tissue (Tri.) were assayed.

<sup>b</sup>Each number represents the mean number of local lesions of 4 replications ± the standard deviation. Each replication consists of 8 soybean half leaves. The local lesion numbers were analyzed by the Duncan new multiple range test (P=0.05).

<sup>*</sup>Statistically significant difference between antiviral compound and control.
infectivity than unsprayed, inoculated control plants (Table 5).

When infectivity of virus in trifoliate leaves was assayed 10 days after primary leaf inoculation, all of the antiviral compound treatments continued to reduce CCMV infectivity in the sprayed cowpea plants. However, none of these reductions were statistically significant when compared to the infectivity of inoculated control plants (Table 5).

Infectivity assays performed 22 days after inoculation showed that CCMV infectivity was reduced in unsprayed, inoculated control plants as well as in plants sprayed with each of the antiviral compound treatments. Although CCMV-infected plants sprayed with 2-thiouracil, treatments A, C, and E; ribavirin, treatments A, and E; and methisazone, treatment E, had less infectivity than unsprayed, inoculated control plants, plants sprayed with ribavirin, treatment C; amantadine, treatments A, C, and E; and methisazone, treatments A and C had greater infectivity than inoculated, unsprayed control plants. The infectivity in the plants sprayed with these treatments, however, was not significantly different from that of inoculated, unsprayed control plants (Table 5).

Effects of Ribavirin and Amantadine on the Presence of Viral Antigen in CCMV-Infected Cowpea Plants

The presence of viral antigen was detected by the enzyme-linked immunosorbent assay (ELISA) in CCMV-infected cowpea plants sprayed with ribavirin or amantadine according to spray treatment E. The ELISA tests were carried out at 2, 4, 8, and 15 days after inoculation. The absorbance of the ELISA reaction at 405 nm indicated the relative amount
of viral antigen present. In all ELISA tests, sap from healthy control plants gave significantly lower or zero absorbance readings due to background absorbance as a result of nonspecific reactions (Table 6).

At 2 days after inoculation, sap from inoculated cowpea plants sprayed with ribavirin contained slightly less viral antigen than unsprayed, inoculated control plant sap while sap from plants sprayed with amantadine contained slightly more viral antigen than unsprayed, inoculated control plant sap. However, neither one of the viral antigen concentrations were significantly different from the viral antigen concentration in unsprayed, inoculated control plants (Table 6).

At 4 days after inoculation, sap from inoculated cowpea plants sprayed with ribavirin contained slightly less viral antigen than sap from unsprayed, inoculated control plants while sap from plants sprayed with amantadine contained slightly more viral antigen than sap from unsprayed, inoculated control plants. However, these differences in viral antigen concentration were also not statistically significant (Table 6).

Sap from inoculated cowpea plants sprayed with ribavirin and amantadine and assayed by ELISA 8 days after inoculation, contained less but not significantly lower viral antigen than inoculated control plants.

When cowpea sap was assayed 15 days after inoculation, sap from plants treated with ribavirin and amantadine contained more viral antigen than inoculated control plants, but these differences were not statistically significant either (Table 6).
TABLE 6

Effect of Ribavirin and Amantadine on the Presence of Viral Antigen in Cowpea Chlorotic Mottle Virus-Infected Cowpea Plants detected by the Enzyme-Linked Immunosorbent Assay (ELISA) at 2, 4, 8, and 15 Days After Inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days After Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Healthy Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>0.319 ± 0.055</td>
</tr>
<tr>
<td>Amantadine</td>
<td>0.388 ± 0.175</td>
</tr>
<tr>
<td>Inoculated Control</td>
<td>0.361 ± 0.116</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each number represents the mean light absorption reading at 405 nm (A<sub>405</sub>) of 4 ELISA wells ± the standard deviation. The A<sub>405</sub> readings were analyzed by the Duncan new multiple range test (P=0.05).

<sup>b</sup>Statistically significant differences were detected only between healthy control and other treatments at each time of ELISA.
CHAPTER V

DISCUSSION

Many of the antiviral compounds were very effective in suppressing the development of CCMV symptoms on inoculated cowpea plants. However, statistical analysis of the infectivities of CCMV in plants receiving the various antiviral compound treatments and in inoculated control plants seldom revealed statistically significant differences between the effects of the treatments and the untreated inoculated controls. This was probably due to the fact that the local lesion numbers obtained for each experimental treatment exhibited great variability which resulted in large standard deviations among the treatment means. Transformation of local lesion numbers according to Kleczkowski (1955) before statistical analysis also resulted in large standard deviations. In spite of the lack of statistically significant differences, certain consistent trends were observed in the effects of the antiviral compounds on CCMV infectivity.

The antiviral compounds appeared capable of altering CCMV infectivity depending on the times at which compounds were applied and at which virus assays were performed in relation to the time of plant inoculation. Changes in CCMV infectivity in soybean plants sprayed with treatment regimes A, B, C, and D, prior to or immediately after inoculation, may indicate a compound effect on an early stage of the viral infection process (absorption, penetration, uncoating, or de novo RNA synthesis) since these effects were apparent within 48 hours after
inoculation (at about the time local lesions appeared) (Table 2). Similar conclusions may perhaps be drawn from experiments in which CCMV-inoculated soybean plants were sprayed with the antiviral compounds 3 times on the day of inoculation (Table 3). Changes in CCMV infectivity that become evident in virus assays (Tables 4 and 5) or from observations of virus symptom development within 10 days after inoculation (when peak CCMV infectivity is normally reached) probably indicate that the antiviral compound affects a later stage of the viral infection process (replication, maturation, or long distance movement). Changes in CCMV infectivity that become evident after 15 or more days from inoculation of sprayed plants probably indicate that virus breakdown or stability was affected by the antiviral compound. Such changes were monitored by infectivity assays performed at 16 and 22 days after inoculation (Table 5) and observation of symptoms at 42 days after inoculation. In addition, since ribavirin and amantadine were most successful at suppressing virus symptom development without causing phytotoxicity, their effects on viral antigen concentration in sprayed cowpea plants were quantitatively determined by ELISA performed at 2, 4, 8 and 15 days after inoculation to ascertain whether the compounds may have affected early or late stages of the viral infection process or virus breakdown.

Of the six antiviral compounds tested for their effects on CCMV infectivity, only methisazone and ribavirin may have directly affected the infectivity of CCMV in the inoculum (Table 1). Methisazone appears to have directly inactivated CCMV by elevating the pH of the inoculum
(Table 1) since the CCMV virion becomes unstable and noninfectious when exposed to a pH above 7.0 (Verduin, 1978). Although the pH of the inoculum containing methyl benzimidazol-2-yl-carbamate was somewhat elevated at 6.3 (Table 1), there was no inhibitory effect on the infectivity of CCMV in the inoculum. The direct effect of ribavirin on the infectivity of CCMV could not be a pH effect since the addition of ribavirin did not change the pH of the inoculum to one in which CCMV would be unstable. It is possible that it is not the CCMV in the inoculum that is directly affected by ribavirin but rather the infectivity of CCMV in the plant possibly due to the entrance of ribavirin into the soybean plant during inoculation and subsequent interference of ribavirin with the CCMV infection process. Of the six antiviral compounds tested for their direct effects on CCMV infectivity, 2-thiouracil, ribavirin, and amantadine were previously determined by others to have no direct effects on the infectivity of certain viruses (Dawson and Kuhn, 1972; Lerch, 1977; Kato and Eggers, 1969), while no such information was available for formycin, methisazone, and methyl benzimidazol-2-yl-carbamate.

The early infection processes of CCMV in soybean plants may have been affected by all six antiviral compounds when applied prior to or immediately after inoculation according to spray treatment regimes A, B, C, and D (Table 2), and by methisazone, ribavirin, amantadine, and formycin when applied 3 times on the day of inoculation (Table 3). Although local lesion numbers on soybean plants were reduced by application of antiviral compounds in both experiments (Tables 2 and 3),
statistically significant reductions in local lesion numbers were only observed when soybean plants were sprayed 3 times on the day of inoculation with antiviral compounds (Table 3). The significant reductions in local lesion numbers shown in Table 3 are probably the result of increased uptake of the antiviral compounds due to greater amounts of compound on the leaf surface from 3 applications on the day of inoculation, abrasion of the leaf surface during inoculation, and the presence of a spreader-sticker. Although a spreader-sticker may possess detergent activity causing solubilization of viral protein (Matthews, 1970), significant reductions in local lesion numbers due to the presence of spreader-sticker were not observed. Spreader-sticker was not used for the application of antiviral compounds onto cowpea plants because it caused primary leaves sprayed with many of the compounds to exhibit toxic effects possibly resulting from increased compound absorption. Besides, the absence of leaf hairs on cowpea plants assured better spray coverage by the antiviral compounds than was possible on the primary leaves of soybean plants which are covered with leaf hairs.

Although the differences in local lesion numbers were not statistically significant, local lesion numbers on inoculated soybean plants were consistently greater on plants sprayed with 2-thiouracil (all treatment regimes) than with any of the other antiviral compounds and similar to the lesion numbers of control plants (Table 2). Local lesions on some of the 2-thiouracil-treated primary leaves were so numerous that they coalesced and gave the appearance of large necrotic areas, thereby making it difficult to count individual local lesions. Numbers
of individual local lesions on 2-thiouracil-treated plants may therefore have been even greater than what was actually counted. On the other hand, Kuhn (1971) observed significant increases in local lesion size when CCMV-inoculated soybean plants were sprayed with 2-thiouracil within 24 hours after inoculation. It is possible that some of the increases in local lesion size observed by Kuhn (1971) may have been due to local lesions becoming so numerous that they could no longer be distinguished from each other and were observed as single large local lesions. Francki (1962) and Kuhn (1971) observed that 2-thiouracil affects virus synthesis and not the host response to infection. Therefore, the results presented here along with the results of others suggest that 2-thiouracil may affect an early stage of the virus infection process.

The early effects of ribavirin on the CCMV infection process may be attributed to the inhibition of general RNA synthesis by ribavirin through its ability to competitively inhibit an enzyme necessary for the synthesis of guanine ribonucleotides in virus-infected cells (Browne, 1979; Byhan, et al., 1978; Goswami, et al., 1979). A slight but non-significant decrease in viral antigen concentration in the primary leaves of cowpea plants sprayed with ribavirin according to treatment regime E was detected 2 days after inoculation by ELISA (Table 6); this again suggests that ribavirin may inhibit an early stage of the viral infection process or possibly early viral protein synthesis. The inhibitory effects of ribavirin are thought to occur during the early synthesis of viral nucleic acid (DeClercq and Luczak, 1975; Simon, et al.,
1973) regardless of whether ribavirin is applied before or immediately after virus inoculation.

The early effects of amantadine on the CCMV infection process may be attributed to the inhibition by amantadine of viral uncoating (Kato and Eggers, 1969) or penetration into the host cell (Grunert, et al., 1965). The slight increase in viral antigen concentration in the primary leaves of cowpea plants sprayed with amantadine according to the treatment regime E and detected by ELISA at 2 days after inoculation (Table 6), if significant, may represent an increase in viral coat protein rather than complete virions. However, amantadine has also been reported to reduce the synthesis of viral polypeptides (Oxford, 1979).

Formycin may inhibit the virus infection process by exhibiting an early effect on viral RNA synthesis when applied immediately after inoculation (Wawrosch and Sarkar, 1975; Huang, et al., 1966). White and Dawson (1979) found that cordycepin, which is an adenosine derivative like formycin, inhibited in vitro formation of CCMV-RNA by CCMV replicases.

Methisazone was reported to be an effective inhibitor of RNA viruses (Woodson and Jolik, 1965; Pearson and Zimmerman, 1969) possibly through its effect on the RNA-dependent RNA polymerase responsible for transcription of the viral genome early in the infection process (Tonew, et al., 1974). The significant reduction of local lesion numbers on soybean plants sprayed with methisazone 3 times on the day of inoculation, when compared to the local lesion numbers on control plants as well as
those on plants receiving the other antiviral treatments, may be partially due to an elevation of the pH at the leaf surface from repeated methisazone application (Table 3).

Although the reductions in local lesion numbers on plants sprayed with methyl benzimidazol-2yl-carbamate prior to or immediately after inoculation were not significant (Table 2), the results were in accordance with those of Fraser and Whenham (1978) who found that early application of methyl benzimidazol-2yl-carbamate onto tobacco plants (up to 30 days before inoculation) was most effective for inhibiting TMV infectivity. It is uncertain whether methyl benzimidazol-2yl-carbamate affects early viral RNA synthesis (Fraser and Whenham, 1978) or indirectly affects virus synthesis through an early effect on host RNA synthesis (Tamm and Eggers, 1963).

With the exceptions of 2-thiouracil (Tables 4 and 5) and ribavirin (Table 5), application of the antiviral compounds onto inoculated cowpea plants failed to significantly affect the infectivity of CCMV within 10 days after inoculation. This suggests that later stages of the viral infection process occurring within 10 days after inoculation are not directly affected by these chemicals. The systemic infection in CCMV-inoculated cowpea plants may have been less sensitive to the effects of the antiviral compounds than the hypersensitive response of local lesion formation on CCMV-inoculated soybean plants. Since the systemic infection process in virus-infected plants occurs over a much longer period of time than local lesion formation, differences in the infectivity of CCMV in systemically infected cowpea plants may be more subtle than the
differences in local lesion numbers produced on soybean plants. This is because virus replication in cowpea plants may have a chance to recover from the effects of the antiviral compounds within the 10 days after inoculation and then proceed at an almost normal rate. Therefore, the same effects that were induced by the antiviral compounds early in the infection process of soybean plants may also have occurred in inoculated cowpea plants treated with the antiviral compounds. However, the effects on systemically infected cowpea plants were not as evident either because of the lesser susceptibility of the systemic viral infection process to the effects of the antiviral compounds or because of recovery of viral replication within 10 days after inoculation. Similarly, expression of systemic symptoms in CCMV-inoculated cowpea plants appeared to be more sensitive to the effects of the antiviral compounds than was CCMV infectivity in inoculated cowpea plants since many of the antiviral compounds suppressed viral symptoms in inoculated cowpea plants while infectious virus was still present.

2-Thiouracil may inhibit or enhance the CCMV infection process depending on the time of its application. CCMV infectivity in inoculated cowpea plants sprayed prior to or immediately after inoculation (treatment regimes B, C, and D) (Table 4) was slightly but nonsignificantly reduced. The reduction in infectivity of CCMV in inoculated cowpea plants sprayed with 2-thiouracil prior to or immediately after inoculation coincides with a decrease in the development of systemic symptoms on plants sprayed with treatment regimes B, C, and D (Fig. 2); this again suggests that 2-thiouracil may inhibit an early stage of the virus
infection process. In contrast, the infectivity of CCMV was significantly increased when inoculated cowpea plants were sprayed with 2-thiouracil after inoculation (treatment regimes E and F) (Tables 4 and 5) and the increase in CCMV infectivity paralleled an increase in the development of systemic symptoms (Fig. 2). Holmes (1955) and Francki (1962) observed that 2-thiouracil exhibited a preventative effect against TMV multiplication in tobacco when applied before or immediately after inoculation. Steele and Black (1967) observed that poliovirus was inhibited by 2-thiouracil early in the infection process due to an inhibition of viral absorption. In contrast, Dawson and Kuhn (1972) observed that when 2-thiouracil was applied daily from inoculation for up to 40 days after inoculation infectivity of CCMV from cowpea plants increased beginning 4 to 5 days after inoculation. Therefore, 2-thiouracil may have two different sites of action: one responsible for virus inhibition early in the infection process and another responsible for virus enhancement which occurs later in the infection process. Both the inhibitory and enhancing effects of 2-thiouracil on virus infectivity can be counteracted by uracil (Dawson and Kuhn, 1972; Bawden and Kassanis, 1954) which suggests that 2-thiouracil probably affects virus replication by interfering with the incorporation of uracil into viral RNA.

The slight reductions in the infectivity of CCMV in inoculated cowpea plants sprayed with all ribavirin treatment regimes evident within 10 days after inoculation (Tables 4 and 5) and the reduction of viral antigen from 4 to 8 days after inoculation (Table 6) coincide
with an almost complete absence of viral symptoms on ribavirin-treated cowpea plants (Fig. 3) and the early inhibitory effects on local lesion formation on inoculated soybean plants (Tables 2 and 3). In addition to the early inhibitory effects of ribavirin on viral RNA synthesis ribavirin may also indirectly affect virus synthesis through possible effects on the resistance of the host (deFazio, et al., 1980). Ribavirin may induce increased tolerance or host resistance of cowpea plants to CCMV infection since ribavirin-treated plants are almost free of viral symptoms although they still contain infectious CCMV after 10 days from inoculation (Tables 4, 5, and 6). Since a significant reduction in the infectivity of CCMV from trifoliate leaves of cowpea plants sprayed with treatment regime E was observed within 10 days after inoculation (Table 5), CCMV in younger trifoliate leaves may have been more sensitive to the inhibitory effects of ribavirin regardless of whether ribavirin affected early viral RNA synthesis or host resistance to viral infection.

Application of amantadine also did not significantly reduce the infectivity of CCMV in inoculated cowpea plants within 10 days after inoculation. However, the slight reductions in the infectivity of CCMV in plants sprayed with treatment regimes A through E (Tables 4 and 5) and the slight reduction of viral antigen at 8 days after inoculation (Table 6) coincide with an almost complete absence of viral symptoms in cowpea plants sprayed with treatment regimes A through E (Fig. 4). Treatment regime F which did not begin until 1 day after inoculation was not effective in reducing CCMV infectivity (Table 4) which again
suggests, along with the early inhibitory effects on local lesion formation on soybean plants (Tables 2 and 3), that amantadine inhibits virus infectivity early in the infection process (absorption or uncoating) when applied prior to or immediately after inoculation.

Although formycin, methisazone, and methyl benzimidazol-2yl-carbamate failed to significantly reduce the infectivity of CCMV in inoculated cowpea plants within 10 days after inoculation (Tables 4 and 5) formycin prevented the development of viral symptoms when applied according to treatment regimes A through E (Fig. 5) and methisazone prevented the development of viral symptoms when applied according to treatment regimes A through F (Fig. 6). These reductions in symptom development coincide with the reductions in local lesion numbers on inoculated soybean plants (Tables 2 and 3) and may be due to possible early effects of formycin and methisazone on viral RNA synthesis. This again suggests that symptom development of inoculated cowpea plants may be more sensitive to the effects of the antiviral compounds than the infectivity of CCMV in the inoculated cowpea plants or that symptom development could be affected if the antiviral compound increases host resistance to virus infection rather than exhibiting a direct effect on the virus. Methyl benzimidazol-2yl-carbamate, in addition to having little effect on CCMV infectivity in primary leaves of cowpea plants (Table 4), did not affect symptom development on CCMV-inoculated cowpea plants (Fig. 7). Since virus in the younger, upper leaves of the plant may be more sensitive to the effects of methyl benzimidazol-2yl-carbamate (Fraser and Whenham, 1978), it may be necessary to assay the
younger trifoliate leaves of inoculated cowpea plants to detect possible effects of methyl benzimidazol-2-yl-carbamate on CCMV infectivity.

None of the antiviral compounds significantly reduced CCMV infectivity when compared to the infectivity of CCMV in inoculated control plants after 15 or more days from inoculation of sprayed cowpea plants suggesting that virus breakdown or stability was not affected by any of the antiviral compounds (Tables 5 and 6). The reduced infectivity of CCMV in all inoculated cowpea plants at 22 days after inoculation when compared to the infectivity of CCMV in inoculated cowpea plants at 10 and 16 days after inoculation and the extreme variability in infectivity of CCMV 22 days after inoculation in all inoculated cowpea plants (Table 5) may be attributed to the fall in virus concentration frequently observed with increasing age of the infected leaves (Matthews, 1970). Therefore, further comparisons of the effects of the antiviral compounds on the infectivity of CCMV at 22 days after inoculation may be meaningless. However, the reduced infectivity of CCMV observed 22 days after inoculation in cowpea plants sprayed with 2-thiouracil treatment regime E when compared to the infectivity of CCMV in control plants and plants treated with all other antiviral treatments (Table 5) may be due to increased breakdown of CCMV since Dawson and Kuhn (1972) observed an increased breakdown of CCMV in 2-thiouracil-treated cowpea plants beginning 14 days after inoculation. The infectivity of CCMV remained considerably greater in amantadine and some methisazone-treated cowpea plants assayed 22 days after inoculation (Table 5) than in control plants or in plants treated with the other antiviral compounds. This suggests
that amantadine and perhaps methisazone may help to prevent virus breakdown and to prolong its infectivity.

No further reductions in the development of symptoms on CCMV-inoculated cowpea plants treated with any of the antiviral compounds occurred after the 10th and up to the 42nd day after inoculation. This indicates that virus stability was not affected by the antiviral compounds after relatively long periods of time from inoculation. Moreover, the young trifoliate leaves of inoculated cowpea plants sprayed with 2-thiouracil treatment regimes B and D; ribavirin treatment regimes A, B, C, and D; amantadine treatment regimes E and F; and formycin treatment regime E, which exhibited suppression of symptoms within 10 days after inoculation, showed increased symptom development 42 days after inoculation indicating that the antiviral effects are not always permanent.

The toxicity of 2-thiouracil, formycin, and methisazone to the virus-infected host plant (Fig. 2, 5, and 6) may render them useless as plant virus chemotherapeutants. It is not likely that methyl benzimidazol-2yl-carbamate would be useful as a plant virus chemotherapeutic due to its inability to suppress symptom development on the virus-infected host plant (Fig. 7). The lack of toxicity of ribavirin and the presence of only mild toxicity of amantadine to the virus-infected host plants along with the ability of both antiviral compounds to suppress viral symptoms (Fig. 3 and 4) suggest that these two chemicals may have potential for use as plant virus chemotherapeutants.

Although the local lesion assay generally results in considerable
variability and consistently yields a large standard deviation when local lesion numbers are statistically analyzed, it is so far the most accurate, if not the only, assay for detecting the infectivity of plant viruses. The eight replications per treatment used here is the normal number of replications employed in experiments of this type. However, due to the extreme variability in the system perhaps a considerably greater number of treatment replications would have resulted in a more normal distribution of the local lesion numbers and would have lowered the standard deviations among treatment means so that significant differences would become apparent (Zar, 1974). For these reasons, only general trends in the effects of these antiviral compounds on the infectivity of CCMV can be reported. These trends in infectivity are also generally supported by visual observations of the reduction in symptoms caused by the antiviral compounds. Alternative methods of compound application or more frequent or prolonged applications of these compounds on the plant might also have made the effects of these antiviral compounds on CCMV infectivity more evident. Finally, although the virus-host system of CCMV in cowpea and soybean plants provided a rapid means for screening the antiviral compounds, it may not have been the most suitable system for studying the effectiveness of these chemicals as antiviral agents. It is possible that other viruses with different nucleoprotein composition and replication may be more sensitive to these compounds than the isometric, single-stranded RNA virion of CCMV.
Six compounds all previously shown to inhibit certain animal or other plant viruses, were sprayed on the primary leaves of cowpea and soybean plants to determine their effects on symptom development on the virus-infected plant and on the infectivity of the virus, cowpea chlorotic mottle virus (CCMV). The compounds sprayed were as follows: 2-thiouracil, ribavirin, amantadine, formycin, methisazone, and methyl benzimidazol-2yl-carbamate.

Inoculated cowpea plants sprayed with ribavirin and amantadine exhibited almost complete suppression of symptom development, but only slight decreases in CCMV infectivity, and little or no toxic effects. Inoculated cowpea plants sprayed with 2-thiouracil exhibited somewhat increased symptom development and increased CCMV infectivity, along with severe phytotoxicity. Inoculated cowpea plants sprayed with formycin exhibited a slight reduction in the development of viral symptoms, little or no decreases in CCMV infectivity, and severe phytotoxicity. Methisazone suppressed symptom development on inoculated cowpea plants and caused slight reductions in CCMV infectivity, along with a hormonal-like phytotoxic effect on the plants. Methyl benzimidazol-2yl-carbamate had no toxic effects on inoculated cowpea plants but symptom expression and CCMV infectivity remained comparable to that of the inoculated control plants.

All six antiviral compounds caused some but statistically non-
significant reductions in local lesion formation on CCMV-inoculated soybean plants. However, when ribavirin, amantadine, formycin, and methisazone were sprayed on soybean plants three times on the day of inoculation, they all reduced local lesion numbers significantly.

Quantitative determination of viral antigen by ELISA showed that ribavirin and amantadine caused slight, but statistically nonsignificant reductions in viral antigen in sap from infected cowpea plants compared to the inoculated control plants.
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