Archives of Virology 70, 215—223 (1981)

Archives of Virology



# The Purification and Nature of an Antiviral Protein from *Cuscuta reflexa* Plants

By

L. P. AWASTHI

Plant Virus Laboratory, Department of Botany, Lucknow University, Lucknow, India

With 3 Figures

Accepted August 18, 1981

## Summary

An antiviral substance, showing highly significant virus inhibiting property, has been isolated from the aqueous extract of *Cuscuta reflexa* plants. The biologically active virus inhibitor was purified by fractionation with organic solvents followed by precipitation with a saturated solution of ammonium sulphate, dialysis, ultracentrifugation and filtration through a Sephadex G-200 column. It shows characteristics of proteins with a molecular weight around 14,000—18,000 daltons. The antiviral activity of the purified material was increased several folds. It has prevented the infection of several unrelated isometric as well as anisometric viruses in their hypersensitive and systemic hosts. Systemic resistance induced in lower treated as well as upper nontreated leaves of host plants, whose lower leaves had been treated with inhibitor, was significantly reversed in presence of Actinomycin D.

## Introduction

In the present paper the isolation and characterization of the virus inhibitor from *Cuscuta reflexa* extract (3) has been discussed. It has been also studied whether the systemic resistance induced in host plants by purified *Cuscuta reflexa* inhibitor, like crude inhibitor (3), was sensitive to Actinomycin D. Actinomycin D inhibits DNA-dependent cellular RNA synthesis (16), but does not inhibit viral RNA multiplication (20). Reversal of systemic resistance in the presence of Actinomycin D therefore lends support to the hypothesis that some protective substance(s) is produced in plants (9, 6, 23, 24, 25).

## Materials and Methods

Raising of test hosts, maintenance of virus cultures, preparation of virus inoculum, and inoculation procedure were the same as described earlier (3).

### *Extraction of Virus Inhibitor*

Two hundred grams fresh filaments of the vigorously growing *Cuscuta reflexa* Roxb. plants (parasitizing on *Zizyphus jujuba*) were used as the source of inhibitor.

0304-8608/81/0070/0215/$ 01.80

The frozen filaments of *C. reflexa* were thawed, ground in a meat grinder with distilled water (1 ml/g) and pressed through cheese cloth. The expressed juice was centrifuged at $3000 \times g$ for 15 minutes. The supernatant fluid was centrifuged at $120{,}000 \times g$ for 1 hour. Then the more or less opalescent brown supernatant fluid was immediately filtered to remove some lipid material. The filtrate was dialysed against 100 times its volume of distilled water for 48 hours at 4° C. The non dialysable fraction (bag contents) was centrifuged at $3000 \times g$ for 15 minutes.

*Purification*

The supernatant thus obtained was mixed with petroleum ether in equal amounts. The mixture was shaken vigorously in a separatory funnel for 15 minutes and allowed to settle. The two layers (upper solvent and lower aqueous) were collected separately. Lower aqueous layer was taken in another separatory funnel and was mixed in a series with diethyl ether, chloroform and benzene. The final aqueous fraction was evaporated to dryness in a waterbath at 40—50° C. The residue obtained was suspended in 25 ml of distilled water and centrifuged at $3000 \times g$ for 15 minutes. An equal volume of a saturated solution of ammonium sulphate was added to this supernatant and after 12 hours the mixture was centrifuged at $3000 \times g$ for 15 minutes. The precipitate obtained was dissolved in 10 ml of distilled water. The solution was centrifuged at $3000 \times g$ for 15 minutes and the supernatant was dialysed at 4° C against distilled water by continuous stirring for 48 hours. The non-dialysable fraction (bag contents) was centrifuged at $3000 \times g$ for 15 minutes and the supernatant thus obtained was centrifuged at $120{,}000 \times g$ for 120 minutes. The supernatant, after testing its antiviral activity, was freeze dried and further purified by passing it through a Sephadex G-200 column.

*Filtration Through Sephadex Gel*

Sephadex G-200 (Pharmacia, Fine chemicals, Uppsala, Sweden), was prepared and packed upto 45 cm into a $2.8 \times 90$ cm column (1), which allowed a flow rate of 0.2 ml/min. The column was eluted with distilled water. All operations were performed at 4° C. The partially purified and freeze dried inhibitor (25 mg) was dissolved in 1 ml distilled water, applied to the top of the gel and 40 fractions, 5 ml each, were collected. The absorbance of each fraction was recorded at 280 nm. The elution volume (ve) of the fractions showing maximum inhibition was calculated and the ultra-violet light absorption spectrum was recorded at 220—340 nm. The antiviral activity of all the fractions was tested on *N. glutinosa* leaves against tobacco mosaic virus (TMV). The fractions were applied by forefinger dipped in the solution on to the upper surface of 2 lower leaves of test plants (2 upper leaves rubbed with distilled water), 24 hours before virus challenge. The virus was challenge inoculated in all the four leaves. By exactly the same procedure 2 lower leaves on control plants were rubbed with distilled water.

*Molecular Weight of the Virus Inhibitor*

The molecular weight of the virus inhibitor in the purified preparation of *C. reflexa* was determined by Sephadex gel filtration method (1, 21, 27, 29). The inhibitor solution (25 mg in 1 ml distilled water containing 2 mg of Blue Dextran 2000) was layered under the distilled water on the top of the column of Sephadex G-200 as described earlier and 40 fractions, of 5 ml each, were collected. Four proteins of known molecular weight (Cytochrome C., Vallabh Bhai Patel Chest Institue, New Delhi, Mol. wt. 12,500; Lysozyme, Serva Feinbiochemica, Germany, Mol. wt. 14,000; Chymotrypsin, BDH, India, Mol. wt. 24,500 and Bovine serum albumin, C.D.R.I., Lucknow, India, Mol. wt. 68,000) were also applied through the same column, and 40 fractions, of 5 ml each, for each reference protein, were also collected. The void volume (vo) of the column was determined by measuring the elution volume (ve) of the Blue dextran 2000 band front.

The elution volume and the absorbance at 280 nm of all the fractions of each reference protein and the virus inhibitor from *C. reflexa* was determined. A standard graph was prepared by the plots of Ve/Vo ratios of these proteins against their logarithmic molecular weight, and the molecular weight of the virus inhibitor was derived from it (21, 27, 29).

*Qualitative Tests for Proteins, Carbohydrates, Phosphorus and Nucleic Acid Sugars*

The presence of protein in the purified sample was confirmed with the biuret reaction, Millon's reaction and Xanthoproteic reaction; Carbohydrates by Molisch's test, Fehling's solution test, Bardfoed's reagent test and Feigl spot test; phosphorus by ammonium molybdate reagent; and nucleic acid sugars (RNA or DNA) by the orcinol reaction and diphenyl amine test (27).

The purified samples were hydrolysed with 6N HCl in a sealed vessel for 36 hours and then evaporated to dryness under reduced pressue to remove excess HCl. The residue obtained was dissolved in 2 ml distilled water and centrifuged at $3000 \times g$ for 15 minutes. The supernatant solution (20—40 µl) was spotted on glass plates coated with thin layers of silica gel and chromatographed. The chromatograms were developed in n-butanol: acetic acid: water (4:1:5), and amino acids were located with 0.2 per cent ninhydrin in acetone (15).

*Quantitative Analysis of Proteins and Nitrogen*

The proteins in the purified sample were estimated quantitatively by Folin phenol reagent (12), and nitrogen by Koch-McMekin Micro Kjeldahl method (7). The nitrogen content was determined by referring the optical density readings to a standard curve constructed previously with ammonium sulphate solution of known nitrogen content. The protein value was calculated by multiplying the nitrogen value by 6.25 (30).

*Effect of the Inhibitor on the Infection of Viruses in Their Hypersensitive and Systemic Hosts*

Earlier tests indicated that rubbing *C. reflexa* extract on lower leaves of test hosts invariably rendered upper leaves refractory to virus infection. Hence in all experiments reported here only the basal two leaves, out of four of a plant, were treated by rubbing their upper surface with a 20 ppm solution of *C. reflexa* inhibitor (CR inhibitor); the upper 2 untreated leaves were rubbed with distilled water. Twenty four hours later residual CR inhibitor was washed off the leaf surface with distilled water. The leaves were blotted dry and sprinkled with 600 mesh carborundum powder. Ten minutes later all leaves were inoculated by forefinger dipped in virus inoculum. With control plants lower 2 leaves were rubbed with distilled water instead of CR inhibitor (Table 1).

*Sensitivity of Systemic Resistance Induced by* C. reflexa *Inhibitor to Actinomycin D*

To determine whether Actinomycin D would reverse the resistance induced in upper non treated leaves of test hosts by CR inhibitor, a 20 µg/ml solution of Actinomycin D (Merck sharp and Dohm, U.S.A.) was applied (on to the same 2 lower leaves of *N. glutinosa* which had been treated earlier with inhibitor) at 0, 6, 12, 18 and 24 hours following inhibitor treatment. An equal number of identical lower two leaves, on each plant, in control sets were treated with AD alone, inhibitor alone or distilled water. Two upper leaves, in each case, were rubbed with distilled water. The virus (TMV) was challenge inoculated in all the four leaves, 24 hours after inhibitor treatment (Table 2).

*Physical Properties of the Inhibitor in* C. reflexa

The physical properties of the purified virus inhibitor like thermal inactivation point or longevity *in vitro* were studied in the same way as described for crude inhibitor (3).

## Results

The elution profile of the inhibitor from the Sephadex G-200 column shows that fractions 25—33 were most inhibitory, in which protein concentration was also high (Fig. 1). Fraction 28 which was most inhibitory, showed the maximum and minimum absorption of ultra-violet light at 280 nm and 245 nm respectively (Fig. 2).

Chemical tests revealed that inhibitor contained only proteins and no carbohydrates, phosphorus and nucleic acids. Thin layer chromatography of the hydrolysed sample also confirmed presence of animo acids. Upon spraying with ninhydrin reagent 16 spots developed on TLC plate, these matched with those produced by alanine, arginine, aspartic acid, cystein, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. Upon spraying with aniline phthalate no spots for carbohydrates were formed.

In the purified preparation of the virus inhibitor, protein percentage was 92—96. The nitrogen value which was 15—16 per cent when multiplied by 6.25 also yielded the same amount of protein.

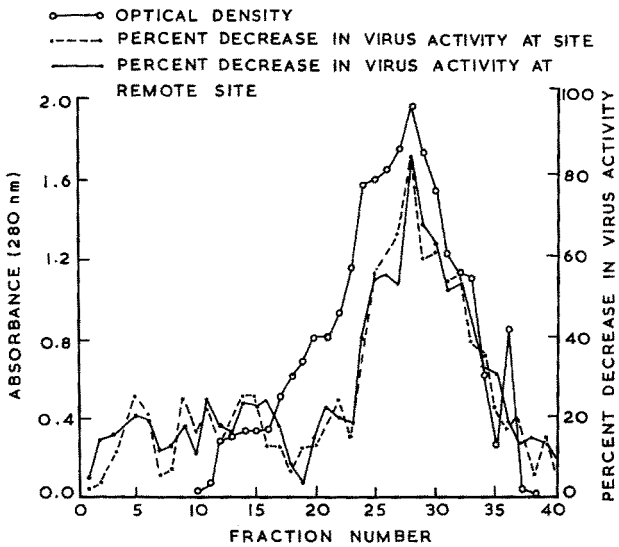


Fig. 1. Sephadex gel filtration of virus inhibitor in *C. reflexa* and percent decrease in virus infectivity at site and remote site by effluents in *N. glutinosa* in relation to optical densities of the fractions

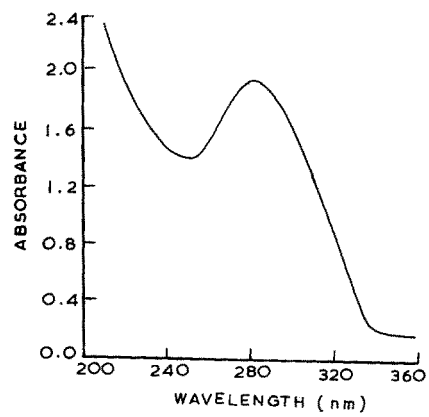


Fig. 2. Ultraviolet light absorption spectrum of the purified virus inhibitor (Fraction 28)

*Molecular Weight of the Virus Inhibitor in* C. reflexa

The void volume (Vo) of the column was 40 ml (determined by Blue Dextran 2000) and the elution volumes (Ve) of cytochrome C, Lysozyme, Chymotrypsin, Bovine serum albumin and virus inhibitor in *C. reflexa* were 150 ml, 145 ml, 125 ml, 85 ml and 140 ml respectively. A plot of Ve/Vo ratios of standard proteins (of known molecular weight) against their logarithmic molecular weight suggest the molecular weight for the virus inhibitor in *C. reflexa* is between 14,000—18,000 daltons (Fig. 3).

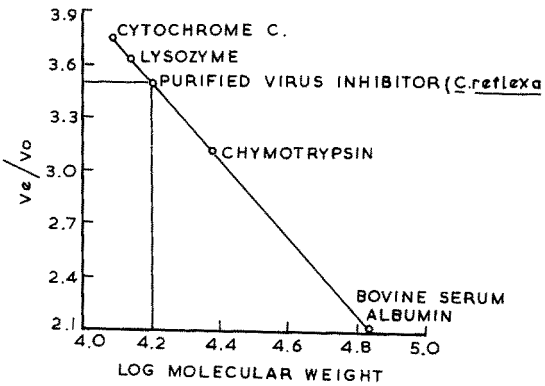


Fig. 3. Estimation of mol. wt. of the inhibitor, plot of log molecular weight against Ve/Vo ratios of proteins (of known molecular weight) in Sephadex G-200 column

The results clearly indicate that CR inhibitor prevented infection of all four viruses in both hypersensitive as well as in systemic hosts. The inhibition of isometric (GMV and TRSV) and anisometric (TMV and SRV) viruses was highly significant when inhibitor was applied on to the two basal leaves of test hosts, 24 hours prior to virus challenge. Application of the inhibitor induced resistance in non-treated regions, showing that the protection was systemic (Table 1). The systemic resistance induced by CR inhibitor in *N. glutinosa* plants was completely reversed by simultaneous application of Actinomycin D, 20 μg/ml. If Actinomycin D was applied upto 12 hours after inhibitor treatment, the reversal was partial and after 18 hours Actinomycin had no effect (Table 2).

*Physical Properties of the Purified Virus Inhibitor in C. reflexa*

The inhibitor in *C. reflexa* was inactivated by heating at 70° C for 15 minutes and by storage for 6 months at 4—10° C.

## Discussion

Inhibitors of virus infection and multiplication isolated from plant extracts have different physico-chemical characteristics. Majority of them were proteinaceous, glycoproteinaceous, or polysaccharides while, a few others were phenolics or alkaloides (26).

Table 1. *Prevention of virus infection and multiplication in their hypersensitive or systemic hosts by* C. reflexa *inhibitor*

| Virus | Host | % reduction of virus infection | |
|---|---|---|---|
| | | 24 h before virus challenge | 24 h after virus challenge |
| TMV | *C. amaranticolor* | 92.7[a] | 9.2 |
| (Anisometric) | *D. metel* | 87.1[a] | 2.8 |
| | *N. glutinosa* | 90.8[a] | 8.0 |
| | *N. tabacum* var. KY 58 | 97.2[a] | 16.5 |
| | *N. tabacum* var. NP 31[a] | 76.9[a] | 21.4 |
| SRV | *C. amaranticolor* | 94.7[a] | 32.5 |
| (Anisometric) | *C. tetragonoloba* | 88.9[a] | 11.7 |
| | *V. sinensis* | 96.5[a] | 15.4 |
| | *C. juncea*[a] | 72.6[a] | 8.6 |
| GMV | *C. amaranticolor* | 78.2[a] | 2.7 |
| (Isometric) | *S. oleracea* | 75.2[a] | 11.6 |
| | *V. sinensis* | 69.5[a] | 17.3 |
| | *G. globosa*[a] | 46.3[b] | 14.1 |
| TRSV | *C. amaranticolor* | 72.6[a] | 22.9 |
| (Isometric) | *N. glutinosa*[a] | 41.5[b] | 14.8 |

Differences due to treatment with inhibitor are significant, a = at 1 per cent level, b = at 5 per cent level

[a] Active virus from systemic host was assayed, 15 days after inoculation, on *C. amaranticolor* leaves

Table 2. *Sensitivity of systemic induced resistance in* N. glutinosa *by* C. reflexa *inhibitor to Actinomycin D (AD) 20* μg/*ml*

| Two upper/lower leaves treated with | Average number of local lesions from 10 leaves ± SEM |
|---|---|
| Distilled water | 173.5±8.25 |
| Distilled water | 182.0±9.24 |
| Distilled water | 161.2±2.12 |
| Actinomycin D (AD) | 168.7±3.58 |
| Distilled water | 4.6±1.16 |
| CR Inhibitor | 3.0±1.00 |
| Distilled water | 174.9±6.25 |
| CR inhibitor; then AD soon thereafter | 186.4±9.26 |
| Distilled water | 153.2±7.46 |
| CR inhibitor; AD after 6 hours | 147.8±4.82 |
| Distilled water | 94.2±5.26 |
| CR inhibitor; AD after 12 hours | 89.8±6.15 |
| Distilled water | 24.6±4.16 |
| CR inhibitor; AD after 18 hours | 19.7±1.12 |
| Distilled water | 6.1±1.12 |
| CR inhibitor; AD after 24 hours | 2.3±1.14 |

SEM = standard error mean

The biophysical and chemical properties of the virus inhibitor in *C. reflexa* revealed that it is proteinaceous in nature. The protein nature is also confirmed by the ultraviolet light absorption spectrum (maximum absorption at 280 nm) and no characteristic maximum at 260—265 nm, which suggests the absence of nucleic acids or purines and pyrimidines. The tests for nucleic acid sugars were also negative.

As indicated by high speed centrifugation and Sephadex gel filtration, the molecular weight of the inhibitor appears to be low (14,000—18,000 daltons). Several proteinaceous inhibitors reported earlier have slightly different molecular weight. Bovine pancreatic ribonuclease has a mol. wt. 13,700 (11), *Dianthus Caryophyllus* inhibitor has mol. wt. about 10,000 (14), *Phytolacca americana*, inhibitor has a mol. wt. 13,000 (32), *Chenopodium album*, *C. amaranticolor*, *Atriplex nitens* and *Amaranthus caudatus* inhibitors have mol. wt. 25,000—38,000 (21), and *C. amaranticolor* inhibitor has a mol. wt. of 15,000 (22).

Like the virus inhibitors from pepper (13, 2), brinjal (28) and *Boerhaavia diffusa* (24, 27), inhibitor in *C. reflexa* also did not pass through cellophane membrane, showed protein nature and induced systemic resistance (resistance at non-treated sites) in host plants. There is also a systemic effect expressed in the phenomenon that the inhibitor applied to the lower leaves (two basal leaves) reduces virus infection in upper non-treated leaves, too. This type of systemic response (systemic acquired resistance) induced in host plants by local lesion producing viruses (17, 18, 8, 4, 19) Polyacrylic acid (5, 6), acetyl salicylic acid (31) and a number of viral and non-viral agents (10) has been observed earlier. The induction of resistance can be reversed by the application of Actinomycin D, up to 6 hours after inhibitor treatment. These results suggest that development of systemic induced resistance depends on the transcription mechanism of the cell from DNA to RNA, thereby producing a resistance-inducing substance. If Actinomycin D is applied close in time to the inhibitor treatment, when the hypothetical resistance inducing agent is beginning to be produced interference is completely prevented. However, if AD is applied at a later stage when such a substance has already been produced, interference is not affected (9, 6, 24).

## Acknowledgements

I would like to express my sincere gratitude to Dr. H. N. Verma for his inspiring interest and expert guidance. I am grateful to Dr. K. C. Saxena, Assistant Director, Central Drug Research Institute, Lucknow, for the help in biochemical analysis. Thanks are due to the Council of Scientific and Industrial Research, New Delhi for awarding me a Research Associateship.

## References

1. Andrews, P.: Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem. J. **91**, 222—223 (1964).
2. Apablaza, G. E., Bernier, C. C.: Inhibition of tobacco mosaic virus infection by plant extracts. Canad. J. Bot. **50**, 1473—1478 (1972).
3. Awasthi, L. P.: Characteristics and mode of action of a virus inhibitor from *Cuscuta reflexa* plants. Arch. Virol. (communicated) (1981).

4. Bozarth, R. F., Ross, A. F.: Systemic resistance induced by localized virus infections: extent of changes in uninfected plant parts. Virology **24**, 446—455 (1964).
5. Gianinazzi, S., Kassanis, B.: Virus resistance induced in plants by polyacrylic acid. J. gen. Virol. **23**, 1—9 (1974).
6. Kassanis, B., White, R. F.: Inhibition of acquired resistance to tobacco mosaic virus by Actinomycin D. J. gen. Virol. **25**, 323—324 (1974).
7. Koch, F. C., Hankee, M. E.: Practical methods in biochemistry, 5th ed. Baltimore: The Williams and Wilkins Company 1948.
8. Loebenstein, G.: Further evidence on systemic resistance induced by localized necrotic virus infections in plants. Phytopathology **53**, 306—308 (1963).
9. Loebenstein, G., Rabina, S., Van Praagh, T.: Sensitivity of induced localized acquired resistance to actinomycin D. Virology **34**, 264—268 (1968).
10. Loebenstein, G.: Localization and induced resistance in virus infected plants. Ann. Rev. Phytopathol. **10**, 177—206 (1972).
11. Loring, H. S.: The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease. J. gen. Physiol. **25**, 497—505 (1942).
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurements with the Folin phenol reagent. J. biol. Chem. **193**, 265—275 (1951).
13. McKeen, C. D.: The inhibitory activity of extract of *Capsicum frutescens* on plant virus infections. Canad. J. Bot. **34**, 891—903 (1956).
14. Ragetli, H. W. J.: Behaviour and nature of virus inhibitor occurring in *Dianthus caryophyllus* L. Tijdschr. Plantenziekten **61**, 245—344 (1957).
15. Randerath, K.: Thin layer chromatography. New York: Academic Press 1963.
16. Reich, E., Goldberg, I. H.: Actinomycin and nucleic acid function. Progr. Nucleic Acid Res. **3**, 183—234 (1964).
17. Ross, A. F.: Localized acquired resistance to plant virus infection in hypersensitive hosts. Virology **14**, 329—339 (1961).
18. Ross, A. F.: Systemic acquired resistance induced by localized virus infections in plants. Virology **14**, 340—358 (1961).
19. Ross, A. F.: Systemic effects of local lesion formation. In: Beemster, A. B. R., Dijkstra, J. (eds.), Viruses of plants, 127—150. Amsterdam: North-Holland Publishing Company 1966.
20. Sänger, H. L., Knight, C. A.: Action of actinomycin D on RNA synthesis in healthy and virus-infected tobacco leaves. Biochem. Biophys. Res. Commun. **13**, 455—461 (1963).
21. Smookler, M. M.: Properties of inhibitors of plant virus infection occurring in the leaves of species in the chenopodiales. Ann. Appl. Bio. **69**, 157—168 (1971).
22. Taniguchi, T., Goto, T.: Purification of an inhibitor of plant virus infection occurring in leaves of *Chenopodium amaranticolor*. Ann. Phytopathol. Soc. Japan **42**, 42—45 (1976).
23. Verma, H. N., Awasthi, L. P.: Prevention of virus infection and multiplication by leaf extract of *Euphorbia hirta* and the properties of the virus inhibitor. New Bot. **6**, 49—59 (1979).
24. Verma, H. N., Awasthi, L. P.: Antiviral activity of *Boerhaavia diffusa* root extract and the physical Properties of the virus Inhibitor. Canad. J. Bot. **57**, 926 to 932 (1979).
25. Verma, H. N., Awasthi, L. P.: Occurrence of a highly antiviral agent in plants treated with *Boerhaavia diffusa* inhibitor. Canad. J. Bot. **58**, 2141—2144 (1980).
26. Verma, H. N., Awasthi, L. P., Mukerjee, K.: Characteristics and mode of action of natural inhibitors of virus infection. In: Misra, P. C. (ed.), Vistas in mycology and plant pathology, published by Prof. K. S. Bhargava's 60th Birth Anniversary Felicitation Committee, Gorakhpur University, India (1979).
27. Verma, H. N., Awasthi, L. P., Saxena, K. C.: Isolation of the virus inhibitor from the root extract of *Boerhaavia diffusa* inducing systemic resistance in plants. Canad. J. Bot. **57**, 1214—1217 (1979).
28. Verma, H. N., Mukerjee, K.: Brinjal leaf extract induced resistance to virus infection in plants. Ind. J. expt. Biol. **13**, 416—417 (1975).

29. VERMA, H. N., MUKERJEE, K., AWASTHI, L. P.: Determination of molecular weight of a polypeptide inducing resistance against viruses. Naturwissenschaften **67**, 364 (1980).
30. WEST, E. S., TODD, W. R., MASON, H. S., VAN BRUGGEN, J. T.: Text Book of biochemistry, 4th ed. New York: Macmillan 1966.
31. WHITE, R. F.: Acetylsalicylic acid (asprin) induces resistance to tobacco mosaic virus in tobacco. Virology **99**, 410—412 (1979).
32. WYATT, S. D., SHEPHERD, R. J.: Isolation and characterisation of a virus inhibitor from *Phytolacca americana*. Phytopathology **59**, 1787—1794 (1969).

Authors' address: Dr. L. P. AWASTHI, Plant Virus Laboratory, Department of Botany, Lucknow University, Lucknow-226007, India.

Received August 11, 1981