EFFECT OF ENZYMES ON INFECTION OF BEAN LEAVES BY TOBACCO MOSAIC VIRUS, *MARMOR TABACI*, H.

H. H. THORNBERRY, J. L. DALE AND Y. L. NENE
University of Illinois, Urbana

This paper is a continuation of a study on the effect of some compounds and biological products upon infection by tobacco mosaic virus. The previous study by Dale and Thornberry (1955) reported the findings from investigations on eight groups of substances and biological products (dyes, amino acids, polypeptides, proteins and protein derivatives, nucleic acid derivatives, plant growth substances, miscellaneous substances, and enzymes.) Since 13 of the 16 tested enzymes inhibited or reduced the amount of infection at some pH, it seemed worthwhile to ascertain the effect of many other enzymes on infection. The results of such an investigation are reported and discussed.

Review of Literature

Literature in addition to the previous paper by Dale and Thornberry (1955) has been reviewed by Bawden (1954).

Materials and Methods

The enzymes used were commercial preparations purchased from the Nutritional Biochemicals Corporation. They were tested at 0.1% concentration in the inoculum unless otherwise stated. Snake venom from the western rattle snake, *Crotalus atrox,* and obtained from the Ross Allen Company, was tested at concentrations indicated in tables.

The buffer system was 0.1 M sodium phosphate; NaH_2PO_4 for pH 4.5, 1:9 mixtures of 0.1 M Na_2HPO_4 and NaH_2PO_4 for pH 6.0, and Na_2HPO_4 for pH 8.5. The solutions were adjusted to the desired pH with 1.0 N HCl or NaOH.

The virus was a subculture of tobacco mosaic virus, *Marmor tabaci* var. *vulgare* Holmes, Johnson's strain No. 1, secured from W. C. Price in 1938 and maintained in living plants of Turkish (Samsun) tobacco, *Nicotiana tabacum* L. Partially purified preparations of the virus were used at a concentration to provide about 50 lesions per leaf from inoculations with nontreated virus.

Assays for most of the tests were on primary leaves of 12- to 14-day-old Scotia beans, *Phaseolus vulgaris,* L. However, Pinto beans were substituted while we were restocking with seed of a satisfactory strain of Scotia bean. Opposite leaves were inoculated with treated and nontreated virus and washed with tap water soon after the application of virus by stroking the upper surface of the leaves with a cheesecloth pad moistened with the inoculum. Ten half leaves were inoculated with each inoculum.

Experiments and Results

The influence of some additional enzymes and similar substances on infection was ascertained at three

TABLE 1.—Effect of Enzymes and Related Substances on Infection of Primary Leaves of Bean by Tobacco Mosaic Virus in 0.1 M Sodium Phosphate Buffer. (Concentration at 0.1% except where noted.)

Substance	Infection index at		
	pH 4.5	pH 6.0	pH 8.5
Enzymes:			
Acetyl cholinesterase	0.88	1.13	0.015
Acid phosphatase	0.02	0.00	0.00
Alcohol dehydrogenase	0.76	0.76	0.66
Aldolase	0.40	0.64	0.74
Alkaline phosphatase	0.00	0.00	0.00
D-amino acid oxidase	0.00	0.00	0.00
Amylopsin	0.07	0.01	0.01
Arginase	0.29	0.53	0.03
Carbonic anhydrase	0.85	0.74	0.043
Carboxylase	0.45	0.33	0.32
Carboxypeptidase	0.34	1.17	0.81
Carnosinase	1.08	0.77	0.11
Chymotrypsinogen	0.00	0.00	0.00
Desoxyribonuclease	0.59	0.63	0.31
Emulsin	0.01	0.02	0.04
Erepsin	0.02	0.00	0.001
Ficin	0.02	0.005	0.008
Fumarase	0.77	1.40	3.8
B-glucosidase	0.66	0.67	0.105
Glyceraldehyde phosphate dehydrogenase	0.65	0.72	1.22
Hexokinase	0.17	0.03	0.009
Invertase (mellibiase free)	0.01	0.02	0.02
Lactic acid dehydrogenase	1.00	0.95	0.27
Lipoxidase	0.44	0.44	0.57
Malic acid dehydrogenase	0.76	0.56	1.41
Mylase P	0.02	0.04	0.004
Pancreatin	0.07	0.06	0.01
Papain crystalline 2x	0.48	0.056	0.004
Pectin esterase	0.28	0.10	0.14
Penicillinase	0.71	0.86	0.78
Pepsin	0.67	0.18	0.32
Pepsinogen	0.04	0.02	0.005
Peroxidase (horseradish)	0.04	0.03	0.01
Protease	0.00	0.00	0.30
Proteinase	0.09	0.08	0.09
Rhodanase	0.00	0.02	0.02
Ribonuclease	0.00	0.00	0.00
Trypsinogen	0.008	0.0008	0.00
Tyrosinase	2.15	1.14	0.10
Uricase	0.05	0.03	0.02
Xanthine oxidase	1.82	0.57	0.22
Cytochrome C	0.03	0.08	0.15
Di-(p-chlorophenyl)-phosphoryl chymotrypsin	0.00	0.00	0.00
Properdin, 10 units per ml	[a]	...	0.00
Snake venom, 0.01%	...	...	0.00
Snake venom, 0.01% boiled for 10 minutes	...	...	0.00
Triphosphopyridine nucleotide	0.59	0.80	1.28
Trypsin inhibitor, Lima bean	0.30	0.37	0.29

[a] No t st.

TABLE 2.—Effect of Concentration of Venom from *Crotalus atrox*, on Infection of Primary Leaves of Bean by Tobacco Mosaic Virus and on the Virus at 22°C. and pH 7.0 for 60 Minutes. (Assay of virus in 0.1 M sodium phosphate buffer at pH 8.5.)

Venom concentration, %	No. lesions per leaf	
	0 minutes	60 minutes
0.5	0.0	0.0
0.1	0.0	0.0
0.01	0.0	0.0
0.001	6.8	1.0
0.0001	15.5	22.6
None	42.6	60.5

H-ion concentrations. The assay plants were inoculated within 30 minutes from the time of mixing the viral suspension and the solution of enzyme or substance. In Table 1, the infection indices, each a ratio of the number of lesions from the treated virus to that from the nontreated virus, show that 21 of the 41 enzymes strongly inhibited infection at all three H-ion concentrations. Properdin and boiled and nonboiled snake venom were tested only at pH 8.5; they inhibited infection. Five enzymes inhibited or reduced the amount of infection at one or two H-ion concentrations. Trypsin inhibitor, triphosphorpyridine nucleotide, and 13 enzymes had little or no effect on the amount of infection. Tyrosinase at pH 4.5 and fumarase at pH 8.5 increased the amount of infection. Infection indices between 0.5 and 1.5 are probably within the experimental error since the results are from one test.

The influence of the concentration of snake venom in the inoculum on infection was determined by mixing the venom and virus just prior to inoculation. To detect any action of enzymes in the venom on the infectivity of the virus, samples of the virus-venom mixture were adjusted to pH 7.0 and incubated at 22°C. for 60 minutes, then adjusted to pH 8.5 for inoculation. The results given in column 2 of Table 2 show that the venom is a strong inhibitor of infection. Even as little as 0.0001% inhibited infection about 40%; 0.001% about 83%. The results listed in column 3 of Table 2 indicate no action of the enzymes in the venom on the infectivity of the virus, since the amount of inhibition was about that from the venom without incubation.

DISCUSSION AND CONCLUSION

Strong inhibition by many of the enzymes tested confirms the earlier report by Dale and Thornberry (1955) that enzymes of different specificities inhibit infection. Under these conditions it seems unlikely, therefore, that specific and generally recognized enzymatic reactions of a chemical nature are involved in the mechanism of inhibition. Nonspecific physical alteration of the surface of virus or the injured cell seems likely. The inhibition is surely physical in nature, since enzymatic chemical reactions are excluded. Since all enzymes tested do not inhibit infection and since the proteins in casein, skimmed milk, and beef blood serum do inhibit infection (Dale and Thornberry, 1955), the inhibiting proteins and enzymes probably have some particular physical property which accounts for the inhibiting

action. Proteins are surface active and adsorb to sites of surfaces with appropriate electrical charge; therefore, the mechanism may be one involving surface interactions between the inhibiting protein and the virus, the injured cell, or both, although the data do not indicate which component might be involved. Thus, inhibition of infection by enzymes may be due to a competitive mechanism in which the inhibiting protein competes with the viral nucleoprotein for the surface of the injured cell or with the substances on the surface of the injured cell for the viral nucleoprotein. If nucleic acid is released from the viral protein for infection, the inhibiting protein might protect the viral particle and thus prevent the separation of nucleic acid for subsequent infection.

It is of interest that properdin, a protein of blood serum, inhibits infection since it protects animals against viral infections (Pillemer *et al.*, 1954) and interacts with polysaccharides on the surface of cells (Pillemer *et al.*, 1955). Likewise it is of interest that a specific protein on the surface of cells of one mating strain of a heterothallic yeast interacts with a complementary polysaccharide on the surface of cells of the opposite mating strain in the primary union of the two cells (Brock, 1959).

Snake venom, which contains several enzymes, strongly inhibited infection. Since boiling an aqueous solution of the venom for 10 minutes did not destroy the inhibitive property of the venom, either the enzymes are resistant to the treatment or stable substances other than enzymes in the product inhibited infection. However, such enzymes as chymotrypsin may be reactivated upon cooling after being boiled (Northrup, 1939). Substituted chymotrypsin, di-(p-chlorophenyl)-phosphoryl chymotrypsin, also inhibited infection strongly.

Increase in the amount of infection from two enzymes, fumarase at pH 8.5 and tyrosinase at pH 4.5, but inhibition at pH 8.5, also appears to be associated with properties other than enzymatic activity, since the favorable pH for activity of these enzymes, fumarase pH 6.6 (Spector, 1956) and tyrosinase pH 5.5-7.0 (Spector, 1956), is not the pH at which increased infection occurred.

Summary

The data support the conclusion that enzymes inhibit infection by a mechanism other than their catalytic chemical action on specific substrates. It is conjectured that a particular physical mechanism, probably surface interactions, is involved.

Acknowledgments

1. These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Illinois, NR 131-193.
2. The studies, in part, were conducted by the junior authors in connection with a graduate research course in plant pathology.
3. Properdin was supplied by the late Louis Pillemer (private commun.) November 16, 1955.
4. Substituted chymotrypsin, di-(p-chlorophenyl)-phosphoryl chymotrypsin was provided by A. K.

Balls (private commun.) January 16,1957.

Literature Cited

BAWDEN, F. C. 1954. Inhibitors and plant viruses. *In* Advances in virus research, Ed. by K. M. Smith and M. S. Lauffer, Vol. 2:31-57.

BROCK, T. D. 1959. Biochemical basis of mating in yeast. Science, 129:960-961.

DALE, J. L., and H. H. THORNBERRY. 1955. Effect of some compounds and biological products upon infection by tobacco mosaic virus. Trans. Ill. St. Acad. Sci., 47:65-71.

NORTHRUP, J. H. 1939. Crystalline enzymes, the chemistry of pepsin, trypsin, and bacteriophage. Columbia Univ. Press, New York, 176 pp.

PILLEMER, LOUIS, LIVIA BLUM, I. H. LEPOW, O. A. ROSS, E. W. TODD, and A. C. WARDLOW. 1954. The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immunity. Science, 120:279-285.

PILLEMER, LOUIS, M. D. SCHOENBERG, LIVIA BLUM, and LEONA WURZ. 1955. Properdin system and immunity. II. Interaction of the properdin system with polysaccharides. Science, 122: 545-549.

SPECTOR, W. S. 1956. Handbook of biological data. W. B. Saunders Co., Phila., 584 pp.

Manuscript received April 30, 1959.