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Effects of Oxalate on the Infectivity of Tobacco Mosaic Virus and its Ribonucleic Acid

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由崎俊道：タバコ・モザイク・ウイルスおよびその核酸の感染性に及ぼす蓚酸塩の影響
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Abstract

In *Nicotiana glutinosa* L. and French beans, a substance dialyzed from Chenopod plant extracts was found to cause an increase in the number of local lesions produced by TMV or TMV-RNA when simultaneously inoculated. The increase-causing substance proved to be an oxalate. A maximum increase of TMV infection was obtained at a concentration of 0.02 M sodium oxalate in a 0.1 M phosphate buffer at pH 7.0. By contrast, the oxalate did not increase the infectivity of TMV in *Datura stramonium* L. and *Chenopodium album* L. The increasing action of the mixture of TMV and the oxalate decreased with dilution. A pretreatment of the leaves of *N. glutinosa* with the oxalate solution increased the infectivity of TMV and TMV-RNA when the viruses were inoculated immediately after the treatment. However, other treatments of the leaves with the oxalate after inoculation did not increase the infectivity of TMV. The oxalate had no counteractive effects on the activity of a pancreatic ribonuclease and inhibitors of TMV and TMV-RNA. On the basis of these results it was assumed that the oxalate affected the susceptibility of the host rather than the viruses. In conclusion, the use of a phosphate buffer mixed with 0.02 M oxalate was more effective on assay of TMV and TMV-RNA infection in both *N. glutinosa* and French bean than that of a phosphate buffer used singly, regardless of the application of bentonite and Carborundum.

Introduction

Juices extracted from various plants have been reported to inhibit tobacco mosaic virus (TMV) infection. Many of the inhibitors in the plant juices have a proteinaceous nature in that they are destroyed by heat treatment and can not be dialyzed through a cellophane membrane. The inhibitor isolated from *Chenopodium album* L. has been identified as a glycoprotein (Sako and Hidaka, 1967). On the other hand, Kuntz and Walker (1947) reported that spinach juice contains two kinds of inhibitors. One is an inhibitor of TMV infection and has the nature of a protein. The other is a small particle that withstands boiling and dialyzes through a cellopane

membrane, and is the inhibitor of the cabbage black-ringspot virus.

This paper reports a study on the increase of TMV and its ribonucleic acid (TMV-RNA) infection by the oxalate from juices of plants which can be dialyzed through a cellophane membrane, especially the juices from *C. album*.

Materials and Methods

Leaves of *C. album* were collected from the field, and the other plants used in this experiment were grown in pots and cultivated in a greenhouse. The extracted juices were obtained from leaves macerated with an amount of distilled water equal to the weight of the leaves. TMV was purified by differential centrifugation (Steere, 1959) from the juice of Samsun tobacco plants infected with TMV. TMV-RNA was prepared from the purified TMV by the phenol method (Gierer and Schramm, 1956). Concentrations of TMV and TMV-RNA were estimated from optical densities (Takahashi, 1951). Absorbance values of 2.7 and 24.0 at 260 nm were taken as equivalent to 1 mg TMV and 1 mg TMV–RNA/ml, respectively. The infectivity of TMV and TMV–RNA was measured by the number of local lesions produced on the leaves of *Nicotiana glutinosa* L. or the other test plants. The leaves were generally dusted with 600-mesh Carborundum and inoculated by rubbing with a moistened gauze pad. Immediately after rubbing, each bunch of leaves was washed gently with tap water. Each inoculum was finally adjusted at pH 7.0 and 0.1 M of phosphate by using a 0.2 M phosphate buffer, unless otherwwise stated. Bentonite was prepared from commercial bentonite according to the clarification procedure reported by Fraenkel-Conrat *et al* (1961).

Results

Extracted juices were obtained from the leaves of several kinds of plants. Each juice sample was placed in a cellophane tube, and immersed in distilled water for two days. The dialyzable

Table 1. Effect of dialyzable and non-dialyzable fraction obtained from plant extracts on the infectivity of TMV

Source plant of extracts	Number of local lesions*					
	Mixture of non-dialyzable fraction(M)**	Control(C)	M/C×100	Mixture of dialyzable fraction(M)**	Control(C)	M/C×100
Chenopodium album L.	3	1139	0.3	3979	977	407.3
Beta vulgaris L.	0	148	0 0	435	150	291.3
Spinacia oleracea L.	0	66	0.0	2947	1213	243.0
Brassica oleracea L. var. *capitata* L.	7	98	7.1	227	140	162.1
Cucumis sativus L.	5	100	5.0	164	286	57.3
Zea mays L.	60	375	16.0	218	203	107.4
Phytolacca americana L.	1	278	0.4	2185	774	282.3
Nicotiana glutinosa L.	106	247	42.9	2910	1501	194.0
N. tabacum L. var. White Burley	56	493	11 4	387	150	258 0
Datura stramonium L.	345	457	75.5	252	150	168.0
Solanum melongena L. var. *esculentum* Nees.	42	109	38.5	306	443	69.1
Lycopersicon esculentum Mill.	38	300	12 7	122	156	78.2

*The total number of local l lesions produced on 16–24 half-leaves of *N. glutinosa* is shown.

**The mixture of TMV and franction was used.

fraction and the non-dialyzable fraction were examined in order to learn the effects on the formation of local lesions in *N. glutinosa* by TMV infection. To each fraction of juices was added the purified TMV suspension in the presence of a 0.1 M phosphate buffer at pH 7.0. As a control, the TMV was likewise added to the phosphate buffer. Both preparations were inoculated to the leaves of *N. glutinosa* by the half-leaf method (Holmes, 1929). The total number of local lesions produced on half leaves were compared. As shown in Table 1, the non-dialyzable fraction showed an inhibitory effect in many kinds of juices, but some of the dialyzable fractions stimulated the TMV infection. Notably, the dialyzable fraction, obtained from plants belonging to Chenopodiaceae and *Phytolacca americana* L. which contain a strong inhibitor(s) of the virus, produced an apparent increase of virus infection. The following experiments were carried out to study the nature of the increase-causing substance obtained from extracts of *C. album*.

1) Effects of the oxalate isolated from the dialyzable fraction of *Chenopodium album* L.

Samples of the dialyzable fraction of *C. album* extracts were heated at either 100 °C or 180 °C for 24 hours in order to determine the stability of the increase-causing agent in TMV infection. Each of the treated samples was examined for the effects of TMV infection by the same method as mentioned above. The increase-causing agent was not affected by heating at 100 °C or 180 °C, although the agent was destroyed by heating at 500 °C for 30 min. in an electric oven. Further experiments were made to determine the absorption of the increase-causing agent to an activated carbon. One gram of the activated carbon was mixed with 10 ml of the dialyzable fraction and, after stirring, the activated carbon was removed by filtration. The treated sample showed the same stimulatory effect as the untreated sample. Since the agent was regarded as stable in respect to the heat and activated carbon treatments, as shown in Table 2 , the dialyzable fraction was concentrated by means of evaporation by exposure to infra-red light and clarified by using activated carbon. Finally, the clarified fraction was dried in an oven at 100 °C. The solution of the dried materials showed an increasing activity comparable to the original fraction. The dried material was analysed to determine its chemical components. As a result, large amounts of oxalic acid or oxalates were found in the material. Therefore, sodium oxalate of different concentrations

Table 2. Effect of heat treatment and activated carbon on the stimulatory agent in dialyzable fraction

Treatment	Number of local lesions*		
	Mixture (M) **	Control(C)	M/C×100
Heated			
at 100℃ for 24 h.	736	193	381.3
at 180℃ for 24 h	1335	358	370.1
at 500℃ for 30 min.	101	162	62.3
Activated carbon	1360	375	362.7

*The total number of local lesions produced on 24–32 half-leaves of *N. glutinosa* is shown.
**The mixture of TMV and the treated fraction was used.

Table 3. Effect of oxalate on the infectivity of TMV

Source of oxalic acid	Concentration of oxalic acid (mol.)	Number of local lesions*		
		Mixture (M)**	Control(C)	M/C×100
Precipitate obtained from dialyzable fraction	0.002	442	244	181.1
	0.004	720	324	221.9
	0.006	1694	803	211.0
	0.008	1401	782	239.0
	0.01	2790	1220	249.9
	0.02	1167	267	394.3
	0.05	1783	779	228.9
Sodium oxalate	0.002	760	463	164.1
	0.004	1282	568	225.7
	0.006	1494	666	224.3
	0.008	1219	510	239.0
	0.01	941	322	292.2
	0.02	681	211	324.2
	0.05	453	224	202.2

*The total number of local lesions produced on 24 half-leaves of *N. glutinosa* is shown.
**The mixture of TMV and the dialyzable fraction or sodium oxalate was used.

Table 4. Effect of oxalic acid and oxalate on the infectivityof TMV

Oxalic acid and oxalate	Number of local lesions*		
	Mixture (M)**	Control(C)	M/C×100
Oxalic acid	1771	576	307.5
Ammonium oxalate	858	268	320.1
Potassium oxalate	1226	573	214.0
Sodium oxalate	684	211	324.2

*The total number of local lesions produced on 24–32 half-leaves of *N. glutinosa* is shown.
**The mixture of TMV and 0.02 M oxalic acid or oxalate was used.

from 0.002 M to 0.05 M was tentatively tested for the effectiveness in the increase of TMV infection. Each of the oxalate solutions was mixed with TMV inoculum in the presence of a 0.1 M phosphate buffer, and the infectivity was compared with the control by means of the half-leaf method on *N. glutinosa*. The same test was carried out by using solutions of the dried material after adjustment to a given concentration of oxalic acid. As shown in Table 3, the greatest increase of TMV infection was shown at a concentration of 0.02 M oxalic acid in both preparations of commercial sodium oxalate and the dried material obtained from the dialyzable fraction. Several kinds of oxalates and oxalic acid were similarly tested for the increasing effect on TMV infection, as shown in Table 4. Each of the oxalates and the oxalic acid of 0.02 M increased TMV infection. From these results, the increase of TMV infection was considered to be due to the presence of the oxalate in the inoculum. To confirm this, a solution of calcium chloride was added to the inoculum containing oxalic acid or sodium oxalate in order to remove it. It was clearly shown that no increase of TMV infection was produced by the inoculum from which the oxalic

acid had been removed.

2) Effects of the oxalate on the infectivity of TMV-RNA

It had been reported that the infectivity of TMV-RNA was remarkably increased with the addition of bentonite to the inoculum (Singer and Fraenkel-Conrat, 1961). Sarkar (1963) demonstrated that the infectivity of TMV-RNA was promoted to 5 percent of TMV by an application of bentonite, Tris phosphate-HCl buffer, and *N. tabacum* L. var. Xanthi nc. The effect of the oxalate on TMV-RNA infection of *N. glutinosa* was examined regarding two buffers namely, (a), 0.1 M phosphate buffer at pH 7.0 and (b), 0.05 M Tris-phosphate-HCl buffer at pH 8.7. TMV-RNA of 10 μg/mg was prepared in each buffer containing sodium oxalate of 0.02 M or the dried materal obtaind from extracts of *C. album* equivalent to 0.02 M oxalic acid. As a control, TMV-RNA was respectively suspended in two buffers without oxalate. To all preparations was added the suspension of bentonite at a concentration of 1 mg/ml. The results are shown in Table 5. The infectivity of TMV-RNA was increased by the addition of oxalate to both buffers. The

Table 5. Effect of oxalate on the infectivity of TMV-RNA suspended in Tris-phosphate-HCl and phosphate buffer

Diluent solution of TMV-RNA		Number of local lesions*		
(A)	(B)	(A)	(B)	A/B×100
P-B with sodium oxalate	P-B**	2615	768	340.5
P-B with dialyzable fraction	P-B	1942	639	303.9
P-B with dialyzable fraction	P-B with sodium oxalate	899	914	98.4
Tris-B***	P-B	382	235	162.6
Tris-B with sodium oxalate	Tris-B	591	278	212.6
Tris-B with dialyzable fraction	Tris-B	996	539	184.8
Tris-B with dialyzable fraction	Tris-B with sodium oxalate	930	760	122.4

*The total number of local lesions produced on 16–24 half-leaves of *N. glutinosa* is shown.
**P-B; 0.1 M phosphate buffer.
***Tris-B; 0.05 M Tris-phosphate-HCl buffer.
The dialyzable fraction and sodium oxalate were mixed with the inoculum as epuivalent to 0.02 M oxalic acid.

increases were similarly produced in the case of the dried material obtained from extracts of *C. album*. Compared with the control the increase obtained with the phosphate buffer (3.0–3.4 fold) was higher than that of the Tris-phosphate-HCl buffer (1.8–2.1 fold), although the TMV-RNA infection in Tris-phosphate-HCl buffer without oxalate was about 1.6 or 1.7 fold of that of the control phosphate buffer. The results suggest that the oxalate can increase the infectivity of TMV-RNA to a given degree in both buffers.

3) The mechanism of the increase of TMV and TMV-RNA infections by oxalate

a) The effect of oxalate on the infectivity of TMV in several host plants

The increase of TMV infection by oxalate was tested in several host plants; including *N. glutinosa* L., French bean, *C. album* L.. and *Datura stramonium L.*. The oxalate effect was found in *N. glutinosa* and French bean, but not in *D. stramonium*, as shown in Table 6. The result suggests that the effect of oxalate is primarily in increasing the susceptibility of the leaves, rather than in increasing the infectivity of TMV in the inoculum.

Table 6. Effect of sodium oxalate on the infectivity of TMV in different host plants

Host plant	Number of lesions*		
	Mixture(M)**	Control(C)	M/C×100
Nicotiana glutinosa L.	684	211	324.2
Phaseolus vulgaris L. (Ōtebo)	1253	455	275.4
Datura stramonium L.	201	875	23.0
	325***	318***	102.2
Chenopodium album L.	1111	1201	108.1

*The total number of local lesions produed on 24–32 half-leaves of *N. glutinosa* is shown.
**The mixture of TMV and 0.02 M sodium oxalate was used.
***This case was used 0.002 M sodium oxalate, others were used 0.02 M sodium oxalate.

b) Dilution of the mixture of TMV and oxalate

The mixture of TMV and sodium oxalate was diluted with a phosphate buffer at a given concentration. As a control, the TMV suspension was equally diluted with the phosphate buffer. The infectivity of the diluted mixture was compared with that of the control by the use of inculation to *N. glutinosa*. The results are shown in Table 7. The increasing action was reduced with the increase in dilution. The result indicates that the oxalate affects the host plant, rather than the virus infectivity directly.

Table 7. Dilution of the mixture of TMV and sodium oxalate

Dilution	Number of local lesions*		
	Mixture(M)**	Control(C)	M/C×100
1	1417	423	335.0
10^{-1}	839	451	186.0
10^{-2}	199	134	148.5
10^{-3}	20	19	105.3

*The total number of local lesions produced on 22–24 half-leaves of *N. glutinosa* is shown.
**The mixture of TMV and 0.02 M sodium oxalate was used.

c) Comparison of the effect of oxalate with that of bentonite on the infectivity of TMV and TMV-RNA

It has been known that clarified bentonite prevents the action of ribonuclease (RNase) and resultantly increases the infectivity of TMV-RNA (Singer and Fraenkel-Conrat, 1961). Further, it

has been reported that the bentonite inhibits the action of inhibitors of TMV and TMV-RNA (Yoshizaki and Murayama, 1968). The experiments were designed in order to determine whether the oxalate would act in a similarly fashion to bentonite in preventing the action of RNase and other inhibitors. The TMV-RNA preparations of 10 μg/ml were incubated in a phosphate buffer containing 0.02 M sodium oxalate, and bentonite of 1 mg/ml for either 6 or 24 hours. Each preparation was inoculated to the leaves of *N. glutinosa* after bentonite was added to each of the preparations, in order to prevent the inhibition by RNase. The results are shown in Table 8. The oxalate did not affect the action of RNase. Similar results were obtained from the experiments by

Table 8. Effect of oxalate and bentonite on the inactivating action of RNase

Suspension of the mixture of TMV-RNA and RNase	Time of incubation (hour)	Number of local lesions*
Phosphate beffer	6	0
	24	0
Phosphate buffer containing 0.02 M sodium oxalate	6	0
	24	0
Bentonite	6	2884
	24	907

*The total number of local lesions produced on 8 leaves of *N. glutinosa* is shown.

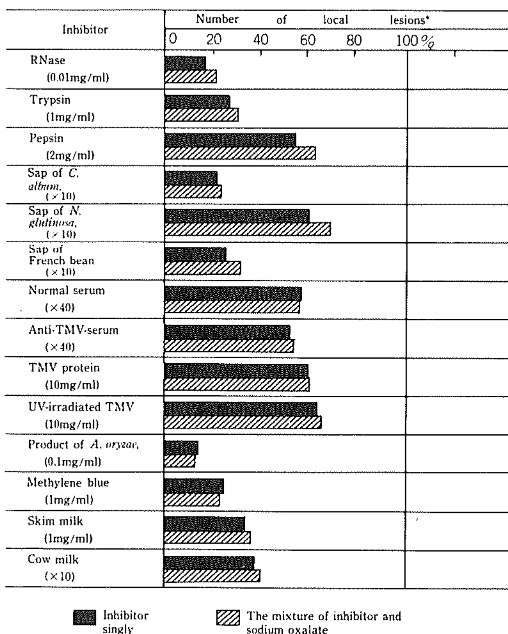

Fig. 1. Effect of oxalate on the inhibitory substances of TMV
*The total number of local lesions produced on *N. glutinosa* by TMV, compared with that of control
The concentrations of inhibitors are shown in ( )

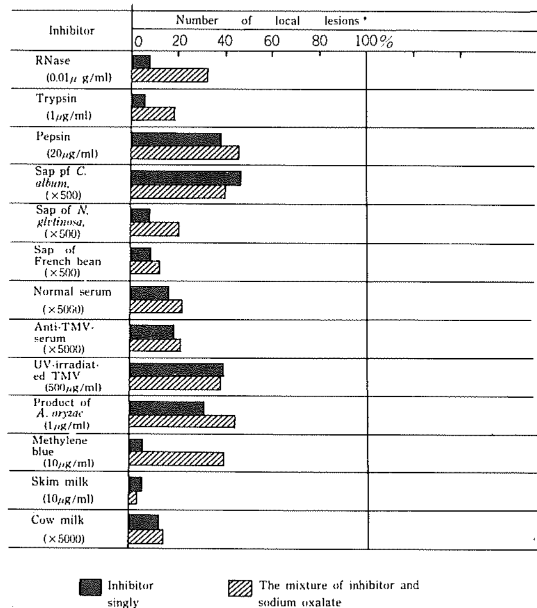

Fig. 2. Effect of oxalate on the inhibitory sustances of TMV-RNA
*The total number of local lesions produced on *N. glutinosa* by TMV-RNA, compared with that of control.
The concentrations of inhibitors are shown in ( ).

the use of inhibitors of TMV and TMV-RNA; including trypsin, pepsin, the sap of *C. album*, of French beans, and of *N. glutinosa,* normal or anti-TMV serum obtained from rabbits, TMV inactivated by exposure to ultraviolet light, a growth product of *Aspergillus oryzae*, methylene blue, skim milk, and cow milk. To the mixture of TMV or TMV-RNA and each of the inhibitors was added a sodium oxalate of 0.02 M. As a control, the phosphate buffer was likewise added to the mixture. The infectivity of both preparations was compared to each other on *N. glutinosa*. The results are summarized in Figures 1 and 2 . The effect of oxalate on inhibitors was not shown in every case of this experiment. It is obvious that the mechanism of the increase of the infectivity of viruses by oxalate is different from that of bentonite.

d) The susceptibility of leaves of *N. glutinosa* treated with sodium oxalate

Experiments were made to determine whether the increase of the virus infections could be

Table 9. Increasing effect of leaves of *N. glutinosa* treated previously with sodium oxalate on the infectivity of TMV-RNA

Virus	Days after treatment	Number of local lesions*		
		Treated leaves(T)**	Control leaves(C)	T/C×100
TMV	0	718	333	215.6
	1	2369	2043	116.0
	2	2273	1975	115.1
	3	2133	2188	97.5
	5	2881	2471	116.6
TMV-RNA	0	348	171	203.5
	1	269	228	118.0
	2	219	201	109.0
	3	184	190	96.8
	5	234	226	103.5

*The total number of local lesions produced on 24–32 half-leaves of *N. glutinosa* is shown.

**The leaves treated with 0.02 M sodium oxalate was used.

Table 10. Increasing effect of leaves of *N. glutinosa* treated after inoculation with sodium oxalate on the infectivity of TMV and TMV-RNA

Virus	Time after inoculation (hour)	Number of local lesions*		
		Treated leaves (T)**	Control leaves(C)	T/C×100
TMV	0	539	481	112.1
	6	563	549	102.6
	24	549	504	108.9
TMV-RNA	0	128	107	119.6
	6	136	140	97.1
	24	123	120	102.5

*The total number of local lesions produced on 24 half-leaves of *N. glutinosa* is shown.

** The leaves treated with 0.02 M sodium oxalate was used.

induced in the leaves of *N. glutinosa* by the treatment before or after inoculation with a solution of 0.02 M sodium oxalate by means of the same procedure as used in inoculation. The other half-leaves were similarly treated with the phosphate buffer solution as the control leaves. Then, TMV or TMV-RNA were inoculated to all surfaces at given intervals. As shown in Table 9, it was found that the increasing effect was only induced in the leaves inoculated immediately after treatment. Further, no effect was induced in the leaves treated with oxalate after inoculation, as shown in Table 10. No effects of oxalate were induced when the mixture of TMV or TMV-RNA and each of the inhibitors described above was inoculated to the leaves of *N. glutinosa* treated with a sodium oxalate of 0.02 M before or after inoculation.

Discussion

Various reports regarding the increase of the infectivity of plant viruses by chemicals in inoculum are available, for examplê, as reviewed by Yarwood (1957). Phosphate is generally effective in increasing the infectivity of viruses or their nucleic acids. Both Thornberry (1935) and Yarwood (1952) found that phosphate caused the greatest in the infectivity of TMV in beans when the pH of the inoculum was 8.5 and the concentration of 0.0068 M to 0.1 M K_2HPO_4. However, Takahashi (1956) reported that such a high pH causes an appreciable inactivation of the virus within a short time. The highest pH to which the phosphate effect safely could be measured was pH 7.0. The most important improvement for increasing the number of local lesions by virus infection assay are the use of Carborundum and suspension of the inoculum in a phosphate buffer. The present experiment demonstrates the increasing action of dialyzable fraction prepared from several plant extracts upon the infectivity of TMV suspended in a 0.1 M phosphate buffer at pH 7.0, notwithstanding the fact that non-dialyzable fractions contain an inhibitor(s) of the virus. The increasing agent in the fraction of *C. album* extracts was confirmed to be the presence of the oxalate. It was obvious that the oxalate caused an increase of TMV and TMV-RNA infections over those of the control which were suspended in a 0.1 M phosphate buffer at pH 7.0. Several workers (Stanley, 1935; Ross, 1953; Paul, 1954; Yarwood, 1952) suggested that the effect of phosphate is primarily in increasing the susceptibility of the leaves, rather than increasing the infectivity of the virus, because the phosphate increases infection more in beans than in other hosts. The oxalate in the inoculum increased the infectivity of TMV on *N. glutinosa* and French beans,but did not increase the virus infections of other hosts. This result and other results, namely (a) dilution of the mixture of TMV and oxalate and (b) treatment with oxalate before and after inoculation, indicate that the oxalate affects the host rather than the viruses. The low infectivity of purified TMV-RNA has been generally attributed to the action of plant RNases (Bawden and Pirie, 1959; Fraenkel-Conrat and Singer, 1959; Singer and Fraenkel-Conrat, 1961). It has been shown that bentonite binds and inhibits the action of RNase (Brownhill *et al*, 1959; Singer and Fraenkel-Conrat, 1961) The treatment of TMV-RNA with bentonite was found to protect, stabilize, and enhance the infectivity of TMV-RNA (Singer and Fraenkel-Conrat, 1961). Similarly, bentonite in a sufficient concentration in the inoculum almost completely inhibits the action of proteinaceous inhibitors and rusults in a remarkable increase in the infectivity of TMV and TMV-RNA mixed with inhibitors. (Yoshizaki and Murayama, 1968). Oxalate was proved not to

have. the stabilizing effect of bentonite on the infectivity of TMV and TMV-RNA. It is apparent that the mechanism of increasing infection by oxalate is different from that of bentonite. Other methods to increase TMV-RNA have been reported; TMV-RNA inoculum with an alkaline buffer (Lippincott, 1961; Sarkar, 1963), high salt concentration (Singer and Fraenkel-Conrat, 1961), certain metal ions (Singer and Fraenkel Conrat, 1962), and sugar (Kongsvik and Santilli, 1970). The effects of these methods were different upon the hosts used for assay. Some of these methods were not effective in the increase of TMV infection. Others have found that the Pinto bean was more sensitive to TMV-RNA than *N. glutinosa*. or *N. tabacum*. var. Xanthi nc (Commoner, 1957,1959, Singer and Fraenkel-Conrat, 1961), and Kado (1964) reported that the *Chenopodium amaranticolor* Coste and Reyn. was more sensitive to TMV-RNA when phosphate was not used. Although the details of the mechanism of the oxalate effect on virus infection is unknown, the oxalate effect seems to be independent of these factors.

More detailed information on this aspect is to be considered in a future report. In conclusion, it is clear that the use of a phosphate buffer and oxalate as a virus diluent are more effective in the assay of the infectivity of TMV and TMV-RNA than that of the phosphate buffer alone in both *N. glutinosa* and French beans, regardless of the application of bentonite and Carborundum in inoculation.

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