THE PRESERVATIVE EFFECT OF SOME REDUCING SYSTEMS ON THE VIRUS OF TOMATO SPOTTED WILT[1]

by

RUPERT J. BEST

(From the Waite Agricultural Research Institute, University of Adelaide, South Australia).

(Submitted for publication 5th November, 1938.)

INTRODUCTION.

Suspensions of the virus of tomato spotted wilt (T.S.W.) in buffer solutions at pH values favouring virus activity become non-infectious after a few hours' exposure to air at room temperatures. In a previous paper (Best and Samuel, 1936) the effects of a number of reducing agents in prolonging the period of activity of the inocula under controlled conditions at pH 7 were described. The preservative effect on the virus of some of the systems there described has since been studied over longer time periods, and some previously untried systems such as ascorbic acid, glutathione, thioglycollate and adrenaline have also been tested. The results of these tests are recorded in the present paper.

EXPERIMENTAL METHODS.

Methods of preparing and handling inocula were essentially the same as those described in previous papers of this series. A modified Holmes-Samuel primary lesion method, using *Nicotiana tabacum* (var. Blue Pryor) as test plant, was used to estimate relative concentrations of active virus (Best, 1937). The initial concentration of infective juice was chosen as 1 in 100 in order that any fall in lesion numbers could (within limits) be interpreted as a proportionate fall in active virus units.

All solutions were made up in a composite phthalate-phosphate-borate buffer (0·04 M) of pH 7. In cases where the test substance altered the pH value of the solution this was adjusted to pH 7 by the addition of NaOH or HCl before adding the virus.

To determine redox potentials, platinum gauze electrodes were used in an atmosphere of nitrogen free from oxygen. The nitrogen was purified as previously described (Best and Samuel, 1936).

Unless otherwise stated the test plants were transferred to the laboratory 24 hours before being inoculated, and the lesions were counted after the rate of their appearance had slowed down and before their spreading rendered counting difficult.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research.

EXPERIMENTAL RESULTS.

Platinised Platinum-Hydrogen System.

It had been found (Best and Samuel, 1936) that when infective juice was added to a buffer solution (pH 7) containing platinised platinum gauze and through which pure hydrogen was passed, the concentration of active virus remained unchanged over the four-hour period of the experiment, whereas the control suspension had become inactive in that time.

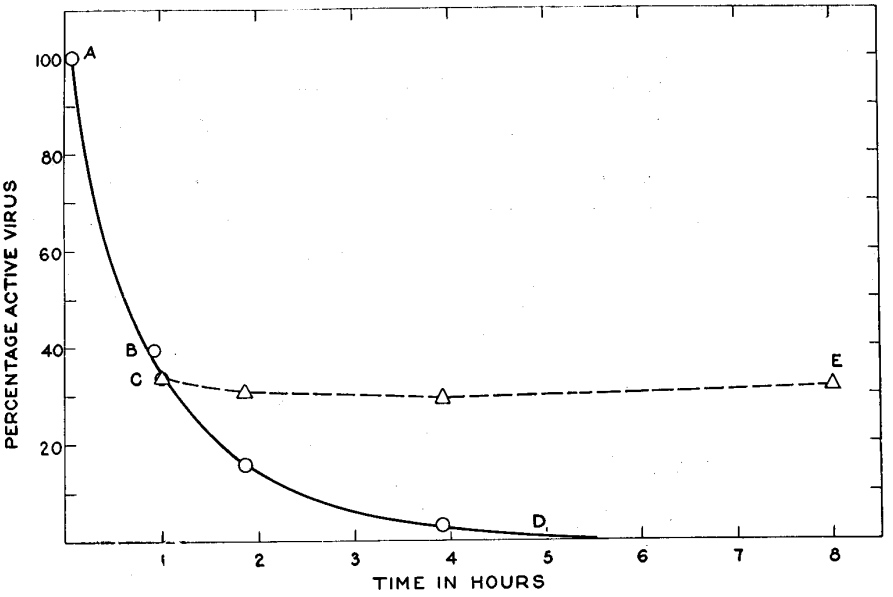

Figure 1. Illustrating (1) the rate of inactivation of a suspension of tomato spotted wilt virus (buffered at pH 7) when exposed to air (A, B, C, D) and (2) the arresting of the inactivation process when a portion was transferred to a container through which hydrogen was bubbling in the presence of platinised platinum gauze (C, E). [Juice] = 1/100. Temperature 21°C.

The experiment now to be described was designed to determine whether the normal oxidative inactivation of the virus could be arrested by the above treatment after it had begun, and to test the possibility of reactivating virus which had been inactivated during exposure to air.

A suspension of the virus was prepared by adding 1 ml. of freshly-expressed infective juice to 99 ml. of buffer solution (pH 7). The suspension was used as the control and was exposed to air. Five minutes after adding the juice a portion

was withdrawn and was inoculated on the appropriate leaves of two previously arranged 5 x 5 Latin squares. After 56 minutes' exposure to air another portion was withdrawn and was inoculated on one half of each of the appropriate leaves of the same squares (this inoculation took three minutes to perform).

Immediately after this inoculation (60 minutes from the beginning of the experiment) one-half of the remaining control suspension was transferred to a flask through which pure hydrogen was passing, and which contained platinum gauze which had been coated electrolytically with platinum black. This was now designated the "test" suspension and was inoculated on the opposite halves of the same leaves as the 56-minute control inoculum had been. The test inoculum (under hydrogen) and the control inoculum (exposed to air) were again inoculated against each other on opposite halves of the appropriate leaves after lapses of 52 minutes, 2 hours 55 minutes, and 7 hours. During the inoculation process the inocula were stored in dropping funnels under an atmosphere of hydrogen. Inoculum was dropped from the funnels on to the ground glass spatulas as required.

TABLE 1.

Showing the Protective Effect on a Suspension of the Virus of Tomato Spotted Wilt in Buffer Solution at pH 7, of Hydrogen in the Presence of Platinised Platinum.

[*Juice*] *of Inocula = 1/100. Temperature, 21° C.*

C = Control inoculum exposed to air.
T = Test inoculum protected by the H_2–Pt system.

Time.	5 minutes.		56 mins.	60 mins.	1 hr.	52 mins.	3 hrs.	55 mins.	8 hrs.	
Inoculum	C	C	C	T	C	T	C	T	C	T
No. of lesions on 10 half leaves	137	147	56	48	15	44	5	42	0	46
	n.s.		n.s.		**		**		**	
	**			n.s.		n.s.		n.s.		n.s.
Per cent of control (mean initial value 142)	100		39·5	33·8	10·6	31·0	3·5	29·6	0	32·4

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two 5 × 5 Latin squares on 25 tobacco plants trimmed to two leaves per plant.

The results of the subsequent lesion count are summarized in Table 1 and Fig. 1. From these it is clear that although rapid inactivation of the virus occurred in the suspension exposed to air no significant changes took place in the activity of the portion which was subjected to the reducing conditions of the hydrogen-platinum system.

Long Range Storage Trials with Cystein as Preservative (Oxygen Excluded).

That virus activity may be preserved *in vitro* for at least 48 hours by the presence of cystein in T.S.W. inocula, provided the pH value is also favourable, was shown in a previous paper (Best and Samuel, 1936). It was of some importance for its own sake and also from the point of view of keeping the virus active during attempts to purify it, to determine the length of time it was possible to maintain the virus in an active state.

Trial No. 1.

The freshly expressed juice of infected tomato plants was used to prepare two suspensions, one (the control) in 0·04 M composite buffer solution of pH 7·5 and another (the test) in a similar solution but 0·01 M in respect to cystein.[2] The concentration of infective juice was 1 in 100. Pure hydrogen was bubbled continuously through both suspensions. Purification of the hydrogen and precautions to exclude oxygen were as previously described (Best and Samuel, 1936). At suitable intervals over a period of six days, portions of the inocula were withdrawn (through a tap at the bottom of the specially designed vessels) and were inoculated against each other on opposite halves of 20 tobacco leaves. The temperature of the inocula was maintained at about 0°C. by a packing of crushed ice.[3]

The 80 test plants required for inoculation purposes were drawn from a single potting of 100 tobacco plants. These were divided into 8 groups of 10 plants per group. Actually the test plants which received the last inoculum were 6 days older than the first group were at the time of inoculation. To obtain some sort of check on a possible susceptibility gradient with age within this main batch, the last inoculum was also applied to a group of 10 plants (8B) from a batch which had been sown and potted a week later than the main batch. The plants of group 8B would, therefore, be more nearly comparable (in respect to their condition) with the plants which received the first inoculum than the plants of the last group (8A) of the main batch would be. Each group of plants was trimmed to two leaves per plant and was transferred to the laboratory 40 hours before the plants were inoculated. The main difficulty in carrying out tests of this nature over periods which extend beyond 2 or 3 days is the liability of the test plants to undergo changes in suceptibility. As shown previously (Samuel, Best and Bald, 1935) variability in the susceptibility of plants at the moment of inoculation is reduced by the above procedure. It has also been shown (Best, 1936) that the conditions of light and temperature to which test plants are exposed, after they have been inoculated with one and the same suspension of virus, affect the number of lesions which finally develop. Any possible differential effects due to these causes were reduced as far as possible by keeping the inoculated plants in the laboratory for 48 hours before transferring them back to the glasshouse. By this means the conditions in respect to temperature and light were made very uniform for all groups during the early stages of lesion formation. These favourable conditions were further enhanced by the chance that a period of cool, cloudy weather (autumn of 1935) set in at the beginning of the experiment and continued to the end, thus making conditions in the glasshouse also more uniform than usual.

Lesion counts were made daily after a reasonable number had appeared. Each group was counted on at least four successive days. In all cases the count recorded for the seventh day after

2 The cystein used was a particularly pure sample of the hydrochloride prepared from Australian merino wool by Mr. H. R. Marston, of the C.S.I.R.'s Division of Animal Nutrition, to whom my thanks are due.

3 This required the attendance of some one during the whole of the six day period to renew the ice packing every few hours. My thanks are due to Mr. V. A. Stephen for taking his share of these long vigils.

inoculation was taken as the basis of comparison as at this stage the rate of increase had slowed down considerably and was optimal for the purpose in hand. The results are summarized in Table 2.

TABLE 2.

Showing the Activity of Suspensions of the Virus of Tomato Spotted Wilt over a Period of Six Days. [*Juice*] = *1/100. Temperature of Inocula* = 0° *C.*

Control suspension in buffer solution.
Test suspension in buffer solution 0·01 M. in respect to cystein.
Both suspensions had an initial pH value of 7·5 which changed very little during the course of the experiment.
Pure hydrogen was bubbled continuously through both suspensions.

Time stored (in hours)	1	12	24	48	72	96	120	144	
Plant Group No.	1	2	3	4	5	6	7	8A	8B
Tests for significance between controls			n.s.	n.s.	**	**	**	**	**
Lesions on 20 half-leaves: Control	401	512	321	369	159	20	3	0	0
	n.s.	n.s.	n.s.	*	*	*	*	*	*
Test	407	654	404	516	449	264	274	315	512
	**	**						**	
Tests for significance between tests			n.s.	n.s.	n.s.	**	**	n.s.	n.s.

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

In addition to the two suspensions described in the foregoing, two other suspensions were prepared at the same time from the same stock of infective juice. One of these (No. 3) was a duplicate of the cystein test suspension but it was kept in total darkness and was left undisturbed for 6 days. The other (No. 4) was a suspension in 0·01 M solution of sodium sulphite buffered at pH 7·5. These suspensions were also kept at 0°C. and in an atmosphere of hydrogen. After 144 hours' storage each of these suspensions was inoculated on five two-leaved tobacco plants drawn from the same batch as group 8B. The former suspension had produced (seven days after inoculation) 447 lesions and the latter 90 lesions on twenty half-leaf replicates.

Reference to Table 2 reveals that after 48 hours the virus in the control suspension was not significantly different from its original value, but thereafter a significant fall in active virus concentration took place. After five days' storage the proportion of active virus had been reduced to less than 1 p.c. of its original value, and by the sixth day the suspension was non-infective. Under the circumstances, the number of lesions produced by the test suspension remained remarkably constant over the whole of the six day period. There was no significant difference between the lesion numbers of the first and final inoculations. The number of lesions from the fourth and fifth day inoculations was in each case significantly lower than that from the first inoculation, but since there was no significant difference between the numbers for the first and last inoculations the above differences

appear to be due to a lower susceptibility of the test plants. This deduction is strengthened by the fact that the number of lesions on group 8B was significantly higher than that on group 8A, although all plants in these groups were treated with the same inoculum (all inocula were protected against inactivation during inoculation and the plants of these two groups were alternated during the inoculation procedure). It is clear that the plants of group 8A had a significantly lower susceptibility than the plants of group 8B. The number of lesions on group 8B was actually higher than that on the group which had received the first inoculum six days previously, and, although this difference is shown as not significant in Table 2, it was nearly significant. In view of these considerations *we may conclude that the concentration of active virus was as high after six days' storage as it was at the beginning of the experiment.*

An interesting point brought out by the table is that the number of lesions at the 12-hour inoculation was significantly higher than those at the 1-hour and 24-hour inoculations. This applies to both test and control suspensions, although the increase in the number over that of the first inoculation is proportionately higher for the test inoculum. The inoculation after 12 hours' storage was performed at night (9.40 p.m., mean inoculation time). All other inoculations were performed during the morning (9.40 a.m., mean inoculation time). On a number of other occasions when a series of inoculations of a protected suspension of the virus has included an inoculation performed at night the same phenomenon of a rise in lesion numbers has been observed. A parallel case is provided by another experiment recorded in this paper (Table 7, page 11) where the final inoculation was performed at night. Increases which are probably due to changes in the degree of dispersion of the virus do sometimes take place during the early stages of an experiment, but these have reached an end point in less than one hour, and it was to eliminate this effect that the first inoculation was performed one hour after adding the juice to the media. In other experiments not reported here the lesions number has remained constant for several inoculations performed at intervals during the day, and has then increased suddenly for the night inoculation. We may therefore rule out any possible dispersion effect and ascribe the increase to a more favourable environment for virus multiplication at night. It would be difficult to determine how much of this would be due to changes in the internal and how much to changes in the external environment.

The inhibitory effect of daylight on the development of T.S.W. lesions (Best, 1936) is a factor which must be taken into account. Although this is primarily connected with the external environment its ultimate effects are concerned with the internal environment. This effect has been discussed in the paper referred to (p. 236–237) and similar explanations may be applied to the present case. There are also possibilities of differences in the pH value as well as oxygen tension of plant cells (or their contents) during the dark as opposed to the light period.

The number of lesions produced by suspension No. 3 from which light was

excluded was not significantly different, after six days' storage, from the corresponding suspension exposed to light. There was therefore no inactivation of virus *in vitro* by daylight of laboratory intensity.

Although the virus in suspension No. 4 which contained sodium sulphite as a preservative was still active after six days, its concentration had been reduced to about 20 p.c. of its original value.

Trial No. 2.

Having maintained the virus in an active state for six days an experiment was designed to determine how long the virus would remain active in sealed tubes. A suspension of infective juice was made up in a phthalate-phosphate-borate buffer solution of pH 7·5 containing cystein (0·01 M) exactly as described for the previous experiment. Pure hydrogen was bubbled through the suspension for four hours. Ten-millilitre aliquots were then transferred to specially constructed Pyrex tubes through which pure hydrogen was passing. The tubes were sealed off and stored in a refrigerator whose temperature varied little from 1°C. Three hours from the beginning of the experiment the first tube was opened, the suspension was transferred to a dropping funnel through which hydrogen was passing and was then inoculated on the leaves of ten tobacco plants which had been trimmed to two leaves per plant. These plants had been transferred to the laboratory thirty hours before being inoculated and were taken back to the glasshouse forty-two hours after being inoculated. They were stored in a compartment which had been fitted with calico shades to keep off direct sunlight. Other tubes were opened at suitable intervals over a period of forty-two days and the contents inoculated on batches of ten tobacco plants treated in the same way as the first batch. The lesions produced were counted on three successive days (for each batch) and the counts recorded in Table 3 are, in all cases, those made six days after inoculation. The twenty-eight day and thirty-five day inoculations were made on two batches of plants differing in age by one week and for the thirty-five day inoculation the contents of two tubes were bulked before inoculation.

TABLE 3.

Longevity of the Virus of Tomato Spotted Wilt in Sealed Tubes in a Suspension Buffered at pH 7·5 and 0·01 M in respect to Cystein. The space above the Suspension was taken up by Pure Hydrogen. The Temperature of Storage was about 1° C. [Juice] = 1/100.

Time stored.	3 hours.	7 days.	15 days.	21 days.	28 days.		35 days.		42 days.
Age of test plants in weeks	6	5	6	5	5	6	5	6	5
No. of lesions on 40 half-leaves	1,393	1,144	665	154	8	91	1,025	690	0
	*	n.s.	**	**	**		**		

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

The virus was still quite active after thirty-five days' storage, but was inactive after the forty-second day. It is difficult to form a reliable estimate of the quantitative relationships of the various inocula in respect to active virus content, because of the variation from week to week of the susceptibility of the host plants

and the differences in temperature and light intensity prevailing at the time of lesion development.

Even where these latter conditions were the same, as for the plants at the 28-day and at the 35-day inoculations, groups of plants differing in age by only one week reacted very differently in respect to the number of lesions produced by the same inoculum. Of the two groups inoculated on the twenty-eighth day, the six-weeks-old plants developed more than eleven times as many lesions as the five-weeks-old plants, although both groups had received the same inoculum. For the thirty-five-day inoculation the five-weeks-old plants developed nearly twice as many lesions as the six-weeks-old plants. These differences which were clearly due to differences in susceptibility of the test plants, were significant in both cases.

The number of lesions produced by the first inoculation was significantly higher than that produced by any of the subsequent inoculations. In view of the regular falling-off in lesion numbers during the first twenty-eight days, and the fact that after forty-two days the suspension was non-infective, we may conclude that there was a falling-off in the concentration of active virus. It is interesting to note, however, that the number of lesions produced by the thirty-five-day inoculation was not significantly different from that produced by the seven-day inoculation, was significantly lower than that produced by the first inoculation, and was significantly higher than those produced by the fifteen-day, twenty-one-day and twenty-eight-day inoculations. Either the falling-off in virus activity was greater in some tubes than in others or the test plants varied greatly in susceptibility. The former is very unlikely, and we may conclude that after thirty-five days' storage the concentration of active virus has fallen significantly but not very greatly, but that after forty-two days' storage the inoculum was no longer infective. The maintaining of virus in an active state for thirty-five days represents a great advance when it is remembered that the virus in unprotected suspensions in buffer solution alone becomes non-infective in less than twelve hours.

Glutathione and Thioglycollic Acid as Protective Agents.

In the experiments just described in which cystein was used as a preservative, oxygen was excluded. However, cystein will protect the virus against inactivation even when suspensions are exposed to air (Best and Samuel, 1936), although in this case the protective action does not last so long. It was of some interest to test the effect of other substances whose reducing properties were centred in sulphydryl groups. Glutathione and thioglycollic acid were two such substances which were available. The results of tests carried out with these two substances are summarized in Tables 4 and 5.

In both cases the test substance was dissolved in 0·04 M buffer solution of pH 7 to give a 0·01 M solution. Sufficient NaOH solution was then added to neutralize the acidity of the substances and bring the pH value back to 7 before adding the infective juice. In accordance with the usual practice, test and control suspensions were in both cases divided into as many aliquots as there were to be time-inoculations. All suspensions were exposed to air throughout.

TABLE 4.

Showing the Preservative Effect on the Virus of Tomato Spotted Wilt of Reduced Glutathione (0·01 M) in Buffer Solution of pH 7.

Control = suspension in buffer solution (0·04 M) of pH 7.

Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to reduced glutathione.

All suspensions were exposed to air.

Temperature 18°C. [Juice] = 1/100.

Test and control suspensions were inoculated on opposite halves of the same leaves.

Time exposed to air.	5 mins.	1·5 hours.	3 hours.	6·3 hours.	12 hours.
No. of lesions on 8 half-leaves:					
Control	458	172	50	20	0
	n.s.	*	*	*	*
Test	403	435	355	311	223
	V	n.s.	n.s.	n.s.	*

n.s. = no significant difference.

* = difference is significant at the 0·02 probability level.

** = difference is significant at the 0·01 probability level.

TABLE 5.

Showing the Preservative Effect on the Virus of Tomato Spotted Wilt of Sodium Thioglycollate (0·01 M) in Buffer Solution of pH 7.

Control = suspension in buffer solution (0·04 M) of pH 7.

Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to neutralized (to pH 7) thioglycollic acid.

All suspensions were exposed to air.

Temperature 17°C. [Juice] = 1/100.

Time exposed to air	10 mins.	1·6 hrs.	3·1 hrs.	6·2 hrs.	12·1 hrs.	24 hrs.
No. of lesions on 12 half-leaves:						
Control	179	54	22	10	0	0
	n.s.	*	*	*		
Test	240	227	191	153	18	0
	V	n.s.	n.s.	n.s.	**	**

n.s. = no significant difference.

* = difference is significant at the 0·02 level of probability.

** = difference is significant at the 0·01 level of probability.

This experiment was performed with two 6 × 6 Latin squares on 36 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

Both reduced glutathione and thioglycollate at pH 7 protect the virus against inactivation on exposure to air, but glutathione is a more efficient preservative than thioglycollate.

Ascorbic Acid (Reduced Form).

Ascorbic acid was chosen as a test substance for several reasons. It contains no sulphur, the redox potential of its solutions falls within the range where a protective action on the virus is to be expected if this action is simply connected with the reduction potential, and the tomato plant is particularly rich in the vitamin.

A preliminary test was performed in May, 1934, with a small quantity of the crystalline vitamin prepared from natural sources (B.D.H. product). After five minutes' exposure to air the concentration of active virus in the test suspension (0·01 M in respect to ascorbic acid and buffered at pH 7) was significantly higher than in the corresponding control. The numbers of lesions produced on eight half-leaves were 279 and 135 respectively, However, at the second inoculation performed after a further four hours' exposure to air, neither suspension was infective.

The lower price of the synthetic vitamin made it possible to perform an experiment covering a longer time period, and during June of 1935 two further experiments were carried out—one with ascorbic acid alone and the other with ascorbic acid in the presence of potassium cyanide. The purpose of the cyanide was to inhibit the catalytic oxidation of ascorbic acid by copper and ferric compounds. That such an oxidation of ascorbic acid is catalysed by copper compounds has been shown by Robertson (1934) who also found that it could be arrested by KCN. The results of these experiments are summarized in Tables 6 and 7.

TABLE 6.

Showing the Protective Effect of Ascorbic Acid on the Virus of Tomato Spotted Wilt.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 molar in respect to ascorbic acid (neutralized to pH 7).
All suspensions were exposed to air.
Temperature 16°C. [Juice] = 1/100.

Time exposed to air.	5 mins.	1·5 hours.	3 hours.	6 hours.
No. of lesions, 8 half-leaves:				
Control	—257	— 81	— 19	— 4
	n.s.	*	*	*
Test	—331	—223	—122	—76

(** spans 5 mins. and 1·5 hours.)

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

TABLE 7.

A Comparison of the Protective Effects on the Virus of Tomato Spotted Wilt of a Mixture of Ascorbic Acid and KCN, and KCN alone.

Control = suspension in buffer solution (0·01 M) which was also 0·01 M in respect to KCN.
Test suspension in buffer and KCN as for the control but 0·01 M in respect to ascorbic acid as well. Both test and control suspensions were adjusted to pH 7.
All suspensions were exposed to air.
Temperature = 16°C. [Juice] = 1/100.

Time exposed to air.	5 mins.	3 hours.	6·1 hours.	12·5 hours.
No. of lesions, 8 half-leaves:				
Control	285	245	287	430 *
Test	190 n.s.	304 n.s.	288 n.s.	341 n.s.
		**		*

n.s. = no significant difference.
* = difference is significant at the 0·02 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants from the same potting as those used for the experiment summarized in Table 6, and trimmed to 2 leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

From Table 6 it is evident that ascorbic acid protected the virus against inactivation on exposure to air. However, the protective effect is not nearly as marked as in the case of cystein or even glutathione, and is more nearly akin to that of thioglycollate. Notwithstanding the protective effect of ascorbic acid, the virus in the test suspension had undergone a progressive inactivation, and at first sight it seemed possible that the protective effect may have been due to an initial reduction of inactivating material present in the juice. However, an examination of the data shows that the inactivation of test and control suspensions proceeded at very different rates. The velocity constants[4] were 0·014 for the control and 0·0048 for the test. The "half life" periods were 50 and 144 minutes respectively. Virus in the control was therefore being inactivated nearly three times as fast as virus in the presence of ascorbic acid. From Table 7 it is evident that when KCN was present as well as ascorbic acid no inactivation at all took place over a period of twelve hours (the duration of the experiment).

In the experiment summarized in Table 7 which covered a period of twelve and a half hours, no virus was inactivated either in the control suspension (KCN) or in the test suspension (KCN + ascorbic acid). In this instance the protective action of KCN alone was more prolonged than in previously reported experiments (Best and Samuel, 1936). There was no significant difference at any inoculation between the test and control inocula.

[4] A study of the reaction velocity of the inactivation processes under various conditions will form the subject matter of a later paper.

Adrenaline.

It was of some interest to determine the effect on the virus of this derivative of catechol. Catechol brings about a practically instantaneous inactivation of the virus in suspensions exposed to air. Adrenaline operates at a more negative redox potential, and as shown by Ball and Clark (1931) its reversibly oxidized form is unstable and has only a transient existence in solution. It seemed likely therefore, that adrenaline would react differently from catechol and that it might even have some protective effect. One experiment was carried out with inocula exposed to air and another with air excluded. The results are summarized in Tables 8 and 9.

TABLE 8.

Showing the Effect of Adrenaline on the Activity of the Virus of Tomato Spotted Wilt.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 M in respect to adrenaline.
All suspensions were exposed to air.
Temperature 16°C. [Juice] = 1/100.

Time exposed to air.	6 mins.	1·5 hours.	3 hours.	6 hours.
No. of lesions on 8 half-leaves:				
Control	201	209	123	33
	n.s.	n.s.	n.s.	n.s.
Test	163	196	116	19

n.s. = no significant difference.

This experiment was performed with two 4 × 4 Latin squares on 16 tobacco plants trimmed to two leaves per plant. Test and control inocula were applied on opposite halves of the same leaves.

TABLE 9.

Effect of Adrenaline on the Virus of Tomato Spotted Wilt when Air was Excluded.

Control = suspension in buffer solution (0·04 M) of pH 7.
Test = suspension in buffer solution (0·04 M) of pH 7 and 0·01 in respect to adrenaline.
Both suspensions were maintained in an atmosphere of pure hydrogen.
Temperature 16°C. [Juice] = 1/100.

Time:		12 minutes.	5 hours.
		**	
No. of lesions on 12 half-leaves:	Control	205	107
		n.s.	†
	Test	220	178
		n.s.	

n.s. = no significant difference.
† = difference is significant at the 0·05 probability level.
** = difference is significant at the 0·01 probability level.

This experiment was performed with two groups of six tobacco plants taken at random from a uniform batch of twelve plants. Test and control inocula were applied on opposite halves of the same leaves.

From Table 8 it is seen that there was no significant difference between test and control suspensions at any inoculation. The test suspension was becoming inactive at the same rate as the control.

In the absence of oxygen, however, there was no significant change in the concentration of active virus units after five hours' storage in the presence of adrenaline, whereas the concentration in the control suspension had fallen significantly in this time. This action is similar to that observed with some other reducing agents, and may be attributed to the reduction by adrenaline of the small quantity of oxidant in the juice.

After a few minutes' exposure to air the test suspensions containing adrenaline became brown whereas the test suspension protected against air did not. The reason why the adrenaline did not protect the virus against aerobic inactivation is therefore probably due to an opposing action of the oxidation products of adrenaline (cf. pyrogallate).

Redox Potentials.

The value of the redox potential of a suspension of infective juice in 0·01 M cystein has been reported previously (Best and Samuel, 1936). The values for suspensions of infective juice in 0·01 M solutions of the sodium salts of glutathione, thioglycollic acid and ascorbic acid, and of adrenaline are set out in Table 10. From the literature on the subject it is evident that difficulties have been experienced in obtaining steady potentials with pure solutions of these four substances. In conformity with these experiences the systems containing glutathione and adrenaline gave potentials which became more negative with time, but after five hours what may be regarded as "equilibrium" values were set up.

TABLE 10.

Redox Potentials of Suspensions of the Juice of Tomato Plants Infected with the Virus of Tomato Spotted Wilt, in Solutions of Various Reducing Substances. All Suspensions were Buffered at pH 7 and were 0·01 M in Respect to the Test Substance. For Glutathione the Concentration of Infective Juice was 1 in 50, and for the Rest it was 1 in 100.

A. Oxygen excluded throughout.			B.* After exposure to air.		
Test substance	E_h (volts) at 18°C.		Time exposed	E_h (volts) at 18°C.	
	at 4′.	at equilibrium.	(in hrs.).	at 4′.	at equilibrium.
Glutathione	+ 0·029	— 0·113	12	+ 0·147	+ 0·006
Na thioglycollate	— 0·096	— 0·102	12	+ 0·028	— 0·055
Ascorbic acid (Na salt)	+ 0·062	+ 0·062	6	+ 0·165	+ 0·096
Adrenaline	+ 0·209	+ 0·107	5	+ 0·268	+ 0·123†

* The suspensions were exposed to air for the times specified. Pure nitrogen was then bubbled through the suspensions and the potentials were measured at intervals.

† The exposed adrenaline system was originally a deep red brown but after passing nitrogen for a few hours this colour vanished and the suspension became light green, as when first prepared. All other systems remained bright green even after long exposure to air and still retained their power to reduce phenol indo 2–6 di bromo phenol.

The ascorbic acid system did not vary more than one millivolt over a six-hour period, and the 22-hour (final) reading of the thioglycollate system was only six millivolts more negative than the initial, four-minute, reading. When potentials take several hours to arrive at an equilibrium value it is not always easy to decide whether the drift is due to a slow approach to a true equilibrium in a stable system or to changes in the redox potential of the system. As far as the glutathione and cystein are concerned the work of Dixon and Quastel (1923) and Michaelis (1930) and others has established the fact that a platinum electrode takes some hours to come to equilibrium.

The initial redox potential is in all cases except that of adrenaline, more negative than the critical value for the virus. Even after exposure to air the potentials of the first three on the list are still below the critical value although ascorbic acid is not far below. As expected the potentials are more positive after exposure to air than before. The behaviour of glutathione, thioglycollate and ascorbic acid in respect to the virus fits in quite well with what we would expect from a knowledge of their redox potentials.

The values obtained for adrenaline are difficult to interpret in view of the fact that the potential begins at a value above the critical for the virus but falls fairly rapidly to a value below the critical value. The oxidation products are known to be unstable. Also the suspensions reduced phenol indo 2–6 di bromo phenol, even after exposure for some hours, showing that they still retain the capacity to reduce suitable substrates.

It must be remembered that the redox potential alone does not define the status of a system (in regard to oxidation or reduction). The value of the potential will determine whether a certain reaction will go and in what direction. It is a measure of the intensity factor. However, given a favourable potential, the capacity of a system to oxidize (or reduce) other systems will depend on the amount of active oxidant (or reductant) present.

The rate at which the reaction takes place is another important factor, although this is to some extent connected with the intensity factor.

If we keep these points in mind the behaviour of adrenaline fits in quite well with the known facts.

DISCUSSION.

Before the whole of the differences in lesion numbers recorded in previous sections is ascribed to a preservative effect of the added substance it is necessary to consider other possibilities such as (for example) possible effects of the test substances on the reaction of the host plants.

At inoculation the virus is presumably held to the cell protoplasm by an adsorption process, but it is very probable that some of the test substances enters along with the virus. In the case of a reducing substance this may well provide an environment which is more favourable than usual for multiplication of the virus.

Such action (and the reverse of it in the case of inactivants) must be small, and may in any case be considered ultimately as an action on the virus in the sense that the action is qualitatively similar to the *in vitro* action.

Another probability is that the reducing substance may increase the degree of dispersion of the virus in the suspension. This may result in an increase or a decrease in the number of resultant lesions according to the size of the critical infective unit. The presence of reducing agents does, in fact, result in a greater dispersion of the juice as a whole, but the evidence is against the virus component of the juice being affected in this way. If the above factors and others such as changes in surface tension were operative, we would expect that lesion numbers for the test suspension would be higher than those for the controls by the same proportion in any series of inoculations. However, the ratio $\frac{\text{lesions from test}}{\text{lesions from control}}$ increases progressively (and significantly) with time. Also in a large majority of experiments where the control inoculum is undergoing slow inactivation there is no significant difference between the lesion numbers produced by the test and control inocula at the first inoculation. Further, in those cases where the difference between test and control inocula at the first inoculation is significant, extrapolation of the numbers for the control to zero time shows that the whole of the difference may be accounted for by the greater rate of inactivation of the control suspension.

In those experiments carried out with reducing substances in the absence of air there can be no doubt that these substances are responsible for the preservation, and we may also draw the same conclusion concerning their action in the presence of air.

In a previous paper (Best and Samuel, 1936) the conclusion was drawn that the preservative effect was due to the reducing action as such, provided the redox potential was below $+ 0{\cdot}1$ volt. It was also pointed out that "it so happens that most of the easily accessible reducing agents operating over this potential range which are at the same time not open to objection on account of some other possible action, are sulphur compounds, but since the pyrogallate and hydrogen-platinum systems also protect the virus . . . . the effect may be ascribed to their common action as reducing agents". The sulphur compounds in question were cystein, sodium sulphite, sodium hydrosulphite and sodium sulphide. To these must now be added reduced glutathione and thioglycollate. Pyrogallate, ascorbic acid, H_2 — Pt and KCN are the non-sulphur-containing systems which have a definite protective action. Adrenaline gives only partial protection. The action of KCN may be partly through its action on disulphides to produce sulphydryl compounds, and some evidence for this view was presented in the paper referred to above. However, the instantaneous action of the platinum-hydrogen system and the action of the other non-sulphur-containing systems together with the demonstration of a critical redox potential for inactivation by oxidants makes it evident that the main

effect of the reducing substances is due to their power of acting as reducing agents at potentials more negative than the critical potential for the virus.

However, there are marked differences in the relative efficiencies of the various substances as protective agents in the presence of air. The possibility of some of these differences being due to specific effects cannot be overlooked. Sulphydryl compounds in general and cystein in particular are the most efficient preservatives. Mow much of this is due to the –SH group and how much to other properties remains to be determined. However, it was pointed out previously (Best and Samuel, 1936) that some of these differences may be ascribed to the fact that the oxidation products of the sulphur-containing-compounds are not active oxidants whereas those of pyrogallate are. In the case of ascorbic acid we have another substance which undergoes fairly rapid oxidation in air in solutions at pH 7, under the influence of catalysts. The removal of the protective agent through aerobic oxidation could therefore account for the lower efficiency of ascorbic acid, although the evidence of Table 10 is against this. There is also the possibility that a secondary reaction, in which the oxidised form of ascorbic acid may be involved, may result in virus becoming inactivated, and so opposing reactions would be set up. That such a reaction may inactivate tobacco mosaic virus has been shown by Lojkin (1937).

Finally, there is the possibility of inactivating effects being due to decomposition products. On several occasions hydrogen sulphide has been detected (by odour and the lead acetate test) in samples stored in the presence of cystein.

SUMMARY.

The effect of various reducing systems on the activity of suspensions of the virus of tomato spotted wilt has been tested. Hydrogen in the presence of platinised platinum arrested the normal aerobic inactivation of the virus, and thereafter maintained the activity at a constant level for the duration of the experiment (eight hours).

Suspensions of the virus in the presence of cystein and absence of oxygen have been kept in an active state for 35 days as compared with the normal *in vitro* life of a few hours.

The sodium salts of glutathione, thioglycollic acid and ascorbic acid (all buffered at pH 7) preserved the virus against rapid aerobic inactivation.

Adrenaline had no significant effect on the activity of suspensions exposed to air, but protected the virus against the slow inactivation which takes place in the absence of oxygen.

Redox potentials of the test systems are recorded and discussed in relation to the relative efficiencies of the protective agents.

REFERENCES.

Ball, E. G. and Clark, W. M. (1931) : Proc. Nat. Acad. Sci., 17, p. 347.
Best, Rupert J. and Samuel, Geoffrey (1936) : Ann. appl. Biol., 23, p. 759.
Best, Rupert J. (1936) : Austral. J. exp. Biol., 14, p. 223.
Best, Rupert J. (1937) : Austral. J. exp. Biol., 15, p. 65.
Dixon, M. and Quastel, J. H. (1923) : J. Chem. Soc., 123, p. 2943.
Lojkin, Mary (1937) : Contr. Boyce Thompson Inst., 8, p. 445.
Michaelis, L. (1930) : "Oxidation-Reduction Potentials". J. B. Lippincott Co.
Robertson, E. B. (1934) : J. Soc. chem. Ind., 53, p. 277.
Samuel, G., Best, R. J. and Bald, J. G. (1935) : Ann. appl. Biol., 22, p. 508.