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INVESTIGATIONS INTO THE CHEMOTHERAPEUTIC ACTIVITY OF NITROSO HYDROXYARYL COMPOUNDS TOWARDS SOME VIRUS DISEASES

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I. INTRODUCTION

The economic importance of virus diseases in plants is often very considerable on account of the serious losses these diseases may cause. This refers to plants which are perennial or are propagated vegetatively, but also to annual and biennial crops raised from seed when they are infected in an early stage. Viruses disturb the normal metabolic processes in the whole plant and once they have penetrated they persist indefinitely in its vegetative parts, as there is no recovery from most virus diseases. Moreover, the losses thus incurred are increased by the viruses spreading very rapidly through insect vectors.

A most effective way of avoiding virus diseases would be growing virus-resistant varieties, but only a few cases of real immunity are known. Another possibility is growing varieties with a high degree of virus tolerance, so that the yield of the crop is not much affected by the infection. The results obtained with this method have been more encouraging. The use of virus-free plant material and variation of normal cultural practice can give better results, but only if sufficient precautions are taken to isolate the plants from the sources of infection. By far the most effective way is to destroy the virus-diseased plants and to kill the insect vector with insecticides. Much work has been done in this field with good results.

Nevertheless, none of these methods has led to complete elimination of the virus diseases. Attempts have therefore been made to destroy the virus in the cells of the host plant without impairing the viability of these cells. In this respect various physical treatments, e.g. ultra-violet, infra-red or X-ray radiation, or heat treatment might offer possibilities. Heat therapy against viruses with a low thermal inactivation point has in some cases been successful, although this method is unlikely to be generally applicable. A curing effect of the radiation of infected plants has not been reported.

Such methods, however, involve the risk of experimental error. Under the influence of the treatment symptoms may be masked, when the virus remaining unaffected. Thus, THUNG (1949) found that, tobacco mosaic virus was inoculated, at certain elevated temperatures the symptoms were completely masked, but the virus remained active and the symptoms appeared as soon as the plants were kept at lower temperatures.

STODDARD (1942) was one of the first to point to the possibility of chemotherapeutic treatment of plants. He had found that bud wood from peach trees infected with X disease can be cured by soaking it in solutions of quinhydrone, urea or sodium thiosulphate, but this experiment still needs confirmation. Although the chance of finding substances that are indeed chemotherapeutically active may be considered rather small, it seemed worth while to investigate various compounds in this respect.

Chemicals can have a preventive or a curative effect. A compound is preventive when it prevents the virus from penetrating into the plant or from spreading if it does penetrate. Only when a compound

attacks the virus itself and inhibits its synthesis, which results in the plant being cured, it has a curative effect. A disadvantage of curative chemotherapeutics is that the disturbing influence of the virus may remain visible at the end of the treatment, particularly in the full-grown parts of the plants, since symptoms as necrosis, leaf roll or mosaic patterns cannot be expected to disappear altogether.

Compounds with preventive activity are the most attractive, as the plant remains healthy. From the results obtained by other investigators and of personal experience it does not seem likely that such compounds have already been found; mostly, the virus multiplication is retarded, which makes the consequences of an infection less serious.

When a chemical proves to have chemotherapeutic activity, it is often difficult to demonstrate which of the two effects plays a role.

BAWDEN (1954) distinguishes between inhibitors of infection and inhibitors of virus increase. The former prevent infection when inoculated into leaves simultaneously with virus; the latter retard the multiplication rate of the virus in the plant when applied to leaves already infected.

This distinction is similar to that between substances with a preventive and with a curative effect. Table I lists a number of compounds, natural or synthetic, that are inhibitors of infection.

Some, if not all, of these compounds may have inactivated the virus in vitro already, before it reaches the plant, as a result of interaction between virus and chemical. They may also act at the time

TABLE I

Some inhibitors of infection mentioned in the literature

Compounds	Virus	Host plant	Authors
A. *Natural compounds*			
trichotecine roseum	TMV	tobacco French bean	BAWDEN and FREEMAN (1952) BAWDEN (1954)
leaf extracts containing tannin, tannic acid, geraniatannic acid, extracted from strawberry, raspberry and other leaves	TMV tobacco necrosis virus rattle virus	tobacco	HIRTH (1953) TRESH (1956) THUNG and VAN DER WANT (1951) PFANKUCH (1943)
enzymes, sera, extracts from bacteria fungi higher plants insects (65 substances mentioned)	TMV	tobacco	BARTELS (1955 *a*, *b*, 1956)
extracts from carnations	TMV	tobacco	NOORDAM, THUNG and VAN DER WANT (1951) VAN DER WANT (1951) RAGETLI (1957)

TABLE I (continued)

Compounds	Virus	Host plant	Authors
B. *Synthetic compounds*			
thiouracil	TMV	tobacco	BAWDEN (1954)
trisodium phosphate	potato virus X TMV	potato tobacco	BROCK (1952)
cyanide hydroxylamine iodoacetate formaldehyde	TMV	tobacco	CHIBA and Coworkers (1954) CARTWRIGHT and LAUFFER (1954) HIRAYAMA (1954) KNIGHT (1951)
sodium chloride potassium chloride	potato virus X	potato	KANNGIESSER and DEUBNER (1954)
iodine phenyl isocyanate carbobenzoxy chloride (benzochloro formate) p-chlorobenzoyl chloride benzenesulphonyl chloride	TMV	tobacco	KNIGHT (1951)
victoria blue Browning No. 47 and other dyes tannins serum globuline octyl-X decyl-X dodecyl-X, etc. X = dimethylbenzyl ammonium chloride	TMV	tobacco	PFANKUCH (1943)
lysine polypeptides	TMV	tobacco	BURGER and STAHMANN (1951)
several chemicals, including: dyes proteins and protein derivatives amino acids nucleic acid derivatives plant growth substances polypeptides enzymes	TMV	tobacco	DALE and THORNBERRY (1955)

the virus just comes into contact with the plant, by taking the receptor place and thus preventing infection (THUNG 1952, RAGETLI 1957).

The investigations described in this publication were set up with the aim of finding compounds having an "in vitro" antivirus activity, i.e. in the plant. Greenhouse experiments with young plants were done to establish what compounds displayed chemotherapeutic

activity without being phytotoxic. The best of these were further tested in the field, where the plants could be examined till their mature stage. The results of both the greenhouse and the field tests are discussed in the last chapter in the light of additional data on "in vitro" effect, serology and chemical properties.

As the virus synthesis (which has not been investigated) is of a different pattern in various plants, it was desirable to include in this investigation as many different viruses and plants as possible in order to obtain the greatest possible validity of results. For practical reasons, however, this number had to be reduced considerably. Still, to make the spectrum as wide as possible, four different types of virus, viz. tobacco mosaic virus, yellow virus and two bean viruses, and seven types of plants belonging to different families of the Dicotyledones were used. With this selection diseases having their strongest influence on either leaf-, root- or seed production were generated.

The choice of these viruses and plants was determined by their economic importance in Western Europe.

The losses caused by tobacco mosaic virus (TMV) are very considerable. For tobacco BAWDEN (1950) estimates them at as much as 4.10^6 pounds per year in the USA alone; in England, the yield of greenhouse tomatoes is reduced by at least 10 % every year. The total loss caused by TMV cannot be given because this virus is spread throughout the world.

Virus yellows in sugar beet is spread over the whole Western and Southern part of Europe and is even reported to occur in Turkey, Syria and Persia (SCHLÖSSER, 1952); it has also been found in the USA (COONS, 1952) and recently in the USSR (GORYUSKIN and KOKHAN, 1958). The damage caused by this disease varies every year. In the Netherlands and Belgium the losses vary from 10–40 % of the total sugar production; in one area the damage is often much more serious than in another. According to HULL (1953) diseased plants produce weekly 4.5 % less sugar than healthy ones. In years of heavy attack the weight of the root crop drops considerably and losses of 40–60 % may be found; it is remarkable that mostly the sugar content does not decrease by more than 1 %.

The bean viruses cause losses every year. For the Netherlands exact data are not available; in the USA the annual losses due to bean mosaic diseases are estimated at several million dollars (ZAUMEYER and REX THOMAS, 1953).

II. MATERIAL AND METHODS

1. PLANT MATERIAL

For the investigation the following plants were selected:

a. Tobacco

1. *Nicotiana tabacum L.* var. White Burley or var. no. 11
2. *Nicotiana rustica L.*
3. *Nicotiana glutinosa L.*

The seeds had been obtained from our own plants and showed great uniformity in germination.

b. Sugar beet, *Beta vulgaris L.* var. Kuhn P.[1])
This variety has been grown from crossings of different families in order to adapt it to geographical and climatological circumstances, so that the seedlings always grow under optimum conditions.

c. Beans
1. French bean, *Phaseolus vulgaris L.* var. Beka [2])
2. Broad bean, *Vicia faba L.* var. Driemaal wit [3])

d. Tomato, *Solanum lycopersicum L.* var. Ailsy Craig [3])

2. Cultivation of plants

The plants were cultivated in the greenhouse in a soil mixture containing leaf mould, farmyard manure, black soil and coarse sand (2:2:2:1). This mixture was left for periods varying from about 3 months to one year, after which it was again thoroughly mixed and left undisturbed for another 3 months; it was then ready for use.

The day was from sunrise till sunset during summer time; from September till the end of April the day was extended till midnight by means of light supplied by two rows of Philips TL tubes (fluorescent lamps) of 65 Watts each, placed parallel at about 1 metre above a table carrying plants, table width about 1 metre. If in winter daylight was insufficient these TL tubes were lighted. Sometimes mercury vapour lamps were used (Philips HO 450 Watts): one per 4 square metres, placed 1–1.5 metres above the table.

The temperature was kept at 20–22° C, except during the night, when it was lowered to 16–18° C. The relative humidity was about 70 %.

The tobacco, tomato and sugar beet seedlings were grown in seed pans and transplanted in boxes accomodating 100 of them. Here they remained till they were 10 centimetres high, when they were singled out into pots. A few days later the plants were used for tests. Care was taken that all plants included in a test were similar.

With beans the procedure was somewhat different, five seeds being placed in a pot, so that no transplanting was necessary.

As it is known that virus multiplication depends on the age of the host plant, a few details must be added as to the stage of development reached by the test plants before being infected. In all seasons the plants were used for the experiments when the fourth leaf was about 3 cm (4–leaf stage); they were then about 10 centimetres high. In early summer (May–June) sugar beet reaches this stage in about 3 weeks, Rustica tobacco in 4 and White Burley in nearly 5 weeks, bean plants in 2–3 weeks and tomato plants in about 4 weeks. In winter it took them 2–3 months to reach this stage of development.

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[1]) Received from the "Koninklijke Beetwortelzaad-Cultuur Kuhn and Co.", Naarden.

[2]) Purchased from the "Coöperatieve Aan- en Verkoopvereniging Centraal Bureau Rotterdam".

[3]) Purchased from "Turkenburg's Zaadhandel", Bodegraven.

In summer the first visible symptoms of virus yellows in sugar beet appear after about 3 weeks; TMV virus produces symptoms in a little more than one week, the bean viruses and TMV in tomato plants in 1–2 weeks. In winter these periods are 2–3 times longer; with yellow virus in sugar beet only mild symptoms appear, if any.

Generally the winter season is not ideal for these experiments, as certainly the light intensity is a limiting factor and probably also the temperature. To our amazement, for tobacco plants September is too unfavourable for symptoms to be developed.

3. Viruses

As explained in Chapter I the viruses and plants were so chosen as to produce diseases having their strongest influence on the production of either leaves, roots or seeds.

The major part of the work was done in the greenhouse with:

a. Tobacco mosaic virus (Nicotiana virus 1) in tobacco; leaf damage.
b. Yellow virus (Beta virus 4) in sugar beet; damage especially to the roots.
c. Common and yellow bean mosaic virus (Phaseolus viruses 1 and 2) in French bean and broad bean; reduction of seed production.

Tobacco mosaic virus (TMV) is very stable. A mixture of a few strains obtained from the Institute for Phytopathological Research (I.P.O.) at Wageningen was investigated. In some cases it was necessary to purify the virus (especially for the methods described under 4), which was done according to the directions of Bawden (1950), K. M. Smith (1951) and Thung (1949).

Sugar beet yellow virus is rather unstable; it cannot be stored outside the plant, therefore it was not purified. For this investigation a mixture of strains was used, as it seemed desirable to test the antivirus activity of the compounds against several strains available in nature.

The bean viruses isolated lose infectivity within two days (Beemster and Van der Want, 1951; Van der Want, 1954), so that purification is impossible. A mixture of a few strains received from I.P.O. was used.

4. Methods of infection

The plants were inoculated with TMV and the bean viruses in the usual way by rubbing the leaves with carborundum powder and injecting them with diluted cell sap. Only one half of the leaf thus treated was inoculated. If the whole leaf should be treated, it might be killed by the inoculum before the virus had become systemic in the plant. The crude TMV sap can be diluted considerably with water without losing its infectivity. Generally, dilutions of 1:1000 and even higher still cause the plants to become diseased, however, only dilutions of about 1:10 were used.

Sugar beet yellow virus cannot be inoculated with this technique. Here the insect vector, *Myzus persicae* (*Sulz.*), had to be used. This aphid was bred on diseased plants throughout the year. To be sure of infection on every plant 10 of such aphids were placed (mostly

6 will be sufficient). Two days later they were killed with an insecticide that had no residual effect.

5. Determination of degree of virus infection in plants

As a chemotherapeutic compound cannot be expected to suppress virus multiplication altogether in all cases and considering that the virus, once it has become capable of overcoming the chemotherapeutic action, will continue to multiply, the differences between the treated and untreated plants will be greatest in the early phases of the disease. Therefore it was important to determine the degree of virus infection as soon as possible after the anti-virus compound had been applied and infection started.

For all methods described below test and control plants had to be of the same age and stage of development. Often a few leaves were sufficient for a test; these leaves had to be taken from similar parts of the plants included in that test. In those experiments where leaf discs could be used they were neither cut from the small top leaves nor from the oldest bottom leaves; nor were large veins included. This technique was preferred as the plants themselves remained intact and could be used for a further determination.

The following methods were applied:

a. *Serology*

An anti-serum was obtained from rabbits injected intravenously with a purified virus solution. This anti-serum was used to establish, by means of the precipitation reaction, the presence of virus in the purified sap extracted from the plants under test. Equivalent amounts of virus and anti-serum have to be used, as an excess of either of them may inhibit precipitation (prozone effect). For the precipitation reaction a rather impure sap could also be used; the sap extracted from the plants by crushing leaves in a mortar was heated at 60° C for about 10 minutes, after which the bulky green precipitate of plant material was removed by centrifuging.

The agglutination reaction was also performed with crude plant sap. Any virus present in the sap produces agglutination of the anti-serum and the chloroplasts.

Routine serological tests were done to investigate tobacco mosaic virus and yellow virus in sugar beet.

With TMV the following procedure was used:

Anti-serum was obtained by injecting rabbits intravenously with 3–5 ml of a purified virus solution. After 4–8 injections the titer of the anti-serum was usually sufficiently high. Rabbit's blood was then collected and left for some hours to clot. The anti-serum was poured off and centrifuged to remove any blood cells left. It is not sterile and has to be stored frozen.

Infected plant material was crushed and 1 ml of the sap thus obtained was heated for about 10 minutes at 60° C and centrifuged

at low speed, i.e. 3000 r.p.m.[1]) The supernatant liquid was stored, if necessary at 0° C. Before use it was diluted with 0.85 % NaCl solutions in ratios of 1:1, 1:4, 1:8, etc., as was also done with the anti-serum.

The bottom of a Petri dish was coated with a hydrophobic film of "Vinylite" (a mixture of polyvinyl acetate and polyvinyl chloride, applied as a 1 % solution in chloroform). With a drawnout pipette a drop of the virus extract was placed on the bottom of the Petri dish, and a drop of anti-serum on the same spot. This was repeated with the dilute virus and the anti-serum dilutions. Besides, a complete set of controls was run with healthy plant sap and normal rabbit serum.

When all drops were in the desired position a paraffinic oil ("Shell Ondina" oil 29 or Oleum paraffinicum) was carefully poured into the dish, until they were just covered, without disturbing them. The dishes were incubated at 37° C for 15 minutes and were then examined for flocculae under the microscope (method described by VAN SLOGTEREN, 1955).

Table II gives an example of a serological determination of the concentration of a virus solution. For various concentrations of the anti-serum the maximum dilutions of TMV in the solution to be tested which still gave a normal serological reaction were determined. This was repeated with a standard virus solution. The ratios of the two dilutions were determined and the mean values calculated (for the relative anti-serum concentrations of 1, $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$ the ratio was 2, 2.5, 1, 1, and 2 for the + signs in the table and 2, 4, 4, 1, and 1.3 for the $\pm$ signs).

The concentration of the unknown TMV solution was thus found to be 0.25 %.

The incubation period, i.e. the time interval (in days) between the moment of infection and the appearance of the first symptoms, was at least 16 days for White Burley tobacco in experiments performed in January and February 1954. Serologically, however, it could be demonstrated that the virus was present in the top leaves as soon as 12 days after inoculation. Somewhat later in the season, in March, when the rate of growth increases, the first symptoms of the disease were noticeable after an incubation period of about 10 days and serologically the virus was shown to be present 7 days after inoculation. It may be expected that later in the season this incubation period will be shorter still.

The concentration of TMV in *Nicotiana rustica* is low, the highest found being 0.006 % .The reason for this is that the disease causes a top necrosis resulting in the growing point dying off on account of which the virus multiplication decreases rapidly. Owing to this low concentration it was impossible to obtain exact data on the virus propagation during the incubation time. Often a few days before the end of the incubation time a slight serological reactivity was found.

[1]) Number times gravity: 1000–1500.

TABLE II

Example of a serological determination of the titer of a TMV solution as compared with a standard TMV solution of 0.1%

The unknown virus solution was isolated from White Burley tobacco plants, purified according to K. M. SMITH (1951), BAWDEN (1950) and THUNG (1949). Both the virus extract and the anti-serum (prepared from rabbit's blood) were diluted with 0.85 % NaCl solution. Incubation for precipitation reaction: 15 min. at 37° C. Duplicate determinations were performed as microtests in Petri dishes; for every reaction one drop of anti-serum and one drop of virus solution were mixed. Controls with the physiological salt solution (0.85 % NaCl) did not give precipitations. ++, + = positive serological reaction; ± = just perceptible reaction; 0 = no reaction.

Relative concentr. of anti-serum	Virus solution to be tested														Standard virus solution of 0.1 %											
	Dilutions														Dilutions											
	1 ×		2 ×		4 ×		8 ×		16 ×		32 ×		64 ×		1 ×		2 ×		4 ×		8 ×		16 ×		32 ×		64 ×	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1/1	++	++	++	++	++	++	+	+	++	+	±	±	0	0	++	++	+	++	++	++	+	+	±	±	0	0	0	0
1/2	++	++	++	++	++	++	++	++	+	+	±	±	0	0	++	++	++	++	++	++	±	+	0	0	0	0	0	0
1/4	++	++	++	++	+	+	±	+	±	+	±	±	0	0	++	++	++	++	++	++	±	+	0	0	0	0	0	0
1/8	++	++	++	++	+	+	±	±	0	0	0	0	0	0	+	+	+	+	+	+	±	±	0	0	0	0	0	0
1/16	++	++	++	++	+	+	±	0	0	±	0	0	0	0	±	±	+	+	±	±	±	±	0	0	0	0	0	0
1/32	±	±	±	±	0	0	0	0	0	0	0	0	0	0	±	±	0	±	0	0	0	0	0	0	0	0	0	0
1/64	0	0	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/128	0	0	±	±	0	0	0	0	0	0	0	0	0	0														
1/256	±	0	±	0	0	0	0	0	0	0	0	0	0	0														
1/512	0	0	0	0	0	0	0	0	0	0	0	0	0	0														

The routine serological test to investigate sugar beet yellow virus was somewhat different. Plant sap from diseased leaves was diluted with its own volume of 0.85 % NaCl solution and centrifuged for 15 minutes at low speed (3000 r.p.m.). The supernatant liquid was stored at 0° C. Before use (usually the next morning) it was once more centrifuged at the same low speed for 15 minutes and diluted further if required.

The anti-serum was obtained from the Laboratory of Bulb Research at Lisse [1]). It had been prepared from the blood of rabbits injected with crude sap. The anti-serum was received frozen-dried in small ampullas. Upon being dissolved in 1 ml 0.85 % NaCl solution it was ready for use.

The experimental procedure was similar to that with TMV, the incubation period of the Petri dishes now being 2–4 hours at 37° C before they could be examined for flocculae under the microscope. Owing to the instability of the sugar beet yellow virus it is not possible to prepare a standardized virus solution. Consequently, the absolute amount of virus present in the host plants treated with a chemotherapeutic compound and in the untreated control plants could not be established and the investigations had to be restricted to determining the ratio of the two values at the end of the experiment.

Beet serology is rather difficult and is more time-consuming than tobacco serology. Although several tests were done it was impossible to demonstrate the presence of the virus earlier than 1–2 days before the end of the incubation period, when the leaves were already somewhat crisp. The distribution of the virus over a plant is rather uneven, as already found by Booij and Coworkers (1944). There are even indications that the distribution of the virus over one leaf is variable.

An objection to these methods of virus determination may be that virus particles may lose their activity, but not their reactivity. It seems fairly certain that the infectivity is localized in the ribonucleic acid part of the particle and not in its protein part, the latter being responsible for the serological reaction (Fraenkel Conrad (1955), Matthews and Smith (1955), Markham (1953), Gierer and Schramm (1956 a and b)).

b. *Reducing sugar test according to* Van Duuren (1955)

This is a rough test for the presence of yellow virus in sugar beet. When the plants are infected the transport of metabolites to the roots is disturbed, which leads to a surplus of reducing sugars in the leaves. This could be determined by punching, preferably in the morning, a disc of 6 millimetres diameter from the central interveinal tissue of four outer leaves of a plant. These four discs were placed in a tube containing 4.5 ml water and 0.5 ml of a 0.5 % solution of 3.5-dinitrosalicylic acid in 1 N sodium hydroxide. Several of such samples were kept at 100° C in a water bath for 30 minutes. Immediate-

[1]) The author wishes to express his thanks to Professor Van Slogteren and his collaborators for supplying this anti-serum.

ly after cooling optical density readings were taken at 500 mμ with a spectrophotometer, the transmission of the control tube without plant material being adjusted at 0. The colour was not stable and had to be measured within 2 hours.

The optical density values with young healthy leaves was nearly zero: with older uninfected leaves from greenhouse plants they were somewhat higher, but always less than 0.13. With mature leaves from field-grown sugar beet plants even higher values may be found. Leaves with symptoms of infection gave optical densities higher than 0.23. Values between 0.13 and 0.23 were difficult to interpret; they probably refer to infected leaves in the incubation period.

In Table III an example of this test is given.

TABLE III

Van Duuren test on sugar beet yellow virus

Every sample consists of 4 discs (dia. 6 mm) punched from outer leaves. It is placed in a tube containing 4.5 ml water and 0.5 ml of a 0.5% solution of 3.5-dinitrosalicylic acid in 1 N sodium hydroxide; the tube is kept in a water bath of 100° C for 30 minutes.

Plant material	Days after infection	Incubation time, days	Optical density at 500 mμ
Normal healthy plants	0	0	0.08; 0.10; 0.11; 0.11
Infected plants			
a. during incubation period	18	24, 25	0.09; 0.12; 0.18; 0.32
b. after incubation period	22, 44	21, 21	0.35; 0.41; 0.46; 0.96

c. *Reso blue test*

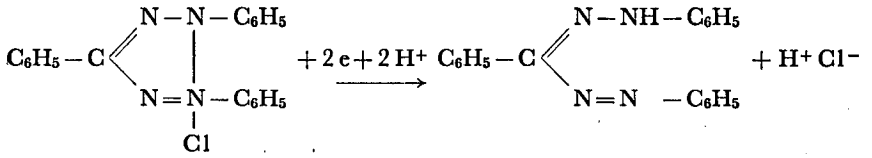
This test, introduced by Igel and Lange to determine leaf roll virus in potatoes, is described by MÜNSTER (1955) and SCHUSTER (1956). It demonstrates callus formation by a blue colouration in the phloem. Diseased plants form more callus than healthy ones. The same holds for sugar beet plants affected by virus yellows. Sections (1–2 millimetres thick) from the hypocotyl of a sugar beet plant were dipped in a reso blue [1]) solution (100 mg/l) for 3 minutes, after which they were immersed in a 1 % $CaCl_2$ solution to fix the dye. The microscope revealed blue callus stains 10–80 μ long and about 15 μ broad in the phloem. It was simple enough to distinguish severely diseased from healthy material, judging by the amount of callus formed. It proved to be difficult, however, to decide on the presence of virus during the incubation period. The results obtained in that period were often unreliable. The uneven distribution of the callus in the sections provided another difficulty. Therefore the selectivity of this test was not high. BROADBENT and HEATHCOTE (1957) came to the same conclusion when they used this method to investigate leaf roll virus in potatoes.

d. 2, 3, 5-*triphenyl tetrazolium chloride* (*TTC*) *test*

According to F. E. SMITH (1951) the insoluble red triphenyl

[1]) Supplied by E. Gurr, Germany.

formazone is formed in the colourless solution of TTC in the presence of tissue by the following reaction:



The TTC acting as an electron acceptor for many pyridine nucleotide dehydrogenases. In 1955 BEAL and Coworkers used TTC to detect the presence of viruses in plants. In virus-infected tissue (roots, stems, petioles or leaves) a relatively rapid change in colour from colourless via green, rust and amber to red occurs, whereas in virus-free tissue a similar but much slower change can be observed.

The maximum differences were usually observed at 35° C within 15–30 minutes after the sections had been immersed in $1\frac{1}{2}$–2 ml of a 0.1% aqueous solution. In the experiments described below, however, the reaction was performed at low pressure (9 centimetres Hg) in order to evacuate the air from the plant tissue, owing to which it sinks. As tissue five leaf discs of 6 millimetres diameter were used. Contrary to the common view it is of great advantage to note, not the colour changes of the sunken tissue itself, but those of the solution, as in the medium the reaction proceeds at a much lower rate. The discs need about 20–30 hours to sink. This is done in the dark; afterwards they are placed in daylight and the vacuum is removed. The colour change can be measured spectrophotometrically at 550 mμ within 10 hours.

Leaf discs from healthy plants impart a red colour to the solution, while discs from diseased plants which have had symptoms for some time give a pink colour. Leaf discs examined during incubation

TABLE IV

TTC (2, 3, 5-triphenyltetrazolium chloride) test on yellow virus in sugar beet and on TMV in tobacco (White Burley)

For each test 5 leaf discs of 6 millimetres diameter were used. They were placed in a 0.1 % solution of TTC at low pressure (9 cm Hg). About 48 hours later the change in colour was measured.

Plant material	Days after infection	Incubation time, days	Optical density at 550 mμ
Normal healthy sugar beet plants	0	0	0.36; 0.29; 0.25
Infected sugar beet plants			
a. during incubation period	8, 16, 18	20, 21, 20	0.26; 0.60; 0.51
b. after incubation period	19, 23, 19	18, 20, 19	0.23; 0.24; 0.24
Normal healthy tobacco plants	0	0	0.36; 0.35; 0.41
Infected tobacco plants			
a. during incubation period	2, 4, 4	7, 7, 6	0.40; 0.48; 0.59
b. after incubation period	14, 12, 10	7, 8, 7	0.44; 0.59; 0.54

produce a darker red colour of the solution than healthy ones. Table IV gives the corresponding optical density values.[1])

e. *Determination of plant (tissue) respiration according to* BLOM *and* EDELHAUSEN (1955)

With the aid of an improved spectroscopic method OWEN (1955a, b, 1956, 1957a, b) determined the respiration rate of tobacco plants infected with TMV, especially during the incubation period. In leaves the respiration rate increased within an hour after inoculation, being considerably higher than in the uninfected control plants. When the plants showed visible symptoms and the leaves contained the maximum amount of virus the respiration rate fell below that of the controls. As the increase in respiration rate started shortly after the leaves had been inoculated, long before virus multiplication was detectable by serology or by the glutinosa test, it was investigated whether this rate could be determined according to the method of direct CO_2 titration as described by BLOM and EDELHAUSEN (1955). This method is based on the relatively great solubility of CO_2 in acetone. The CO_2 production of plants was determined in the following way.

Through a closed container, in which plant material was kept in the dark, CO_2 free air was passed for a certain period of time and, together with the CO_2 produced by the plant, was led into acetone. The volume of air thus passed through was measured with a flow meter. The amount of CO_2 entrained was determined by titrating the CO_2 absorbed by the acetone with a 0.01 or 0.1 N solution of sodium methanolate (CH_3ONa) in methanol, thymol blue being used as the indicator.

Although this method was found to be not very reliable, indications were obtained that during the incubation time White Burley plants have a higher respiration rate than uninoculated plants. When after the incubation time the diseased plants have distinct symptoms, their respiration rate is probably less than that of healthy plants. The same seems to be true for sugar beet plants, although not to such a pronounced degree.

f. *Nicotiana glutinosa test*

To determine the amount of TMV some infected material was passed through a mincer and the sap used for inoculating one half of leaves of healthy *Nicotiana glutinosa* plants, the other halves being inoculated with a standard TMV solution of known virus content. The number of local lesions formed after a few days was counted. The content of tobacco mosaic virus can be determined by comparing the number of local lesions in the test half of the leaf with that of the control half.

[1]) As it is difficult to prepare uniform samples from discs an improvement was made later on by using plant sap after centrifuging at 7000 r.p.m. during 25 minutes. Also it is better to keep the reaction continuously in the dark as a TTC solution is light-sensitive.

However, the results are influenced by the age of the *Nicotiana glutinosa* leaves and, moreover, by the fact that these leaves are not uniform in shape and size; besides, it is impossible to calculate exactly the virus content in the plant if only a small part of this plant is used as test material.

Testing chemicals for their chemotherapeutic activity on *Nicotiana glutinosa* has the drawback that this compound has to act merely on the spot where virus penetrates into the plant. Consequently, its action on a virus causing local lesions must be more vigorous than on a systemic virus.

It must be pointed out that none of these methods is entirely trustworthy. Therefore, in investigating the chemotherapeutic effect of various compounds all the methods mentioned above were applied. As it proved to be too time-consuming to examine the whole range of compounds in this way, only those were tested further which influenced the virus multiplication at least in such a way that the visible symptoms became less distinct. It must be remarked, however, that the seriousness of the symptoms does not always correlate with the amount of virus present, as COONS (1954) described for virus yellows in sugar beet.

A chemotherapeutic effect was considered positive only when the results of all the tests mentioned were in agreement.

6. APPLICATION OF COMPOUNDS EXPECTED TO HAVE CHEMOTHERAPEUTIC ACTIVITY

The chemicals to be tested were always applied before infection took place, either by spraying the leaves or through absorption of a solution via the roots.

a. *Spraying*

Fifty mg of the chemical to be tested was dissolved in 50 ml water, or, in several instances, in 2 ml acetone containing 0.1 % Triton X 155[1]) as wetting agent and poured into 48 ml water. The plants, placed in an area of 0.5 square metre, were evenly sprayed with this solution at 3 atmospheres in high volume (50 ml/0.5 square metre is 1000 litres/hectare). The same number of control plants were sprayed with water containing acetone and wetting agent.

Two days after spraying the plants were inoculated with the exception of a number of them, which was the same in the two sets of plants. Thereupon, if the results were promising the plants were examined by the methods just described, either once more during the incubation time or at the end of this period. Compounds which showed no chemotherapeutic activity in a 0.1 % w solution were considered to have no anti-virus properties. If a 0.1 % w solution appeared to have a phytotoxic effect on the uninfected control plants lower concentrations were tested.

[1]) Rohm and Haas, USA.

b. *Absorption via the roots*

The compound enters the plant through the roots and is transported to the leaves and the upper growing point.

Healthy plants were lifted from their pots. Their roots with adhering soil were kept in an 0.1 % w aqueous solution (mostly without acetone and wetting agent) for 24 hours. Subsequently they were potted up again and 24 hours later were inoculated with the virus. This is called the dipping method. As to the controls the same precautions were taken as with the spraying technique.

In modern practice nursery plants are often transplanted with so-called "soil blocks"; in this case the dipping method may be of more practical value than the spraying treatment.

A drawback of the dipping method is that an unknown, maybe sometimes large, quantity of the substance is absorbed by the adhering soil, which may delay the absorption by the plant to a point of time too late to prevent virus multiplication.

To prevent this disturbing effect the plants, after having germinated, were transplanted in 1½–2 kilogrammes of sterilized sand in red plastic "Vinidur" boxes (20 × 20 × 10 centimetres; the red colour was chosen because it contrasts well with the plant material). The roots of the test plants were rinsed with tap water; 8 plants were planted in each box. Then the chemical under test was added, in a concentration of 0.001–0.01 % w, to 150–200 ml of Knopp's solution and with this mixture the sand was wetted evenly (nutrient technique). Usually two days later six plants were inoculated with the virus in the common way, the other two remaining uninfected. The controls were the same as with the spraying technique.

III. EXAMINATION OF ANTI-VIRUS SUBSTANCES IN THE GREENHOUSE

1. Literature

A compound will be regarded as an antivirus factor when, upon penetrating into the plant tissue, it can inactivate the virus in vivo or inhibit the virus multiplication; besides, it must not produce a phytotoxic effect in the concentration used.

In Table V a survey is given of the various antivirus compounds (column 1) mentioned in the literature (column 5).

The concentrations used are not recorded; they were always low, varying from 0.001 %–1 %. In columns 2 and 3 the plants and viruses are recorded on which the influence of the chemotherapeutic was investigated; it can also be found when the local lesion method was used to determine the degree of activity (TMV with as host plant *Nicotiana glutinosa* or bean, Southern bean mosaic virus and its host Pinto bean). As pointed out on page 377 this method has serious drawbacks as an in vivo test.

Sometimes it is not clear whether an inhibitor activity is described or whether the compound displays real, i.e. in vivo antivirus activity. Therefore in column 4 of the table the methods used to apply the compounds are indicated.

TABLE V

Antivirus compounds mentioned in the literature

Method: 1. absorption of the chemotherapeutic substances through the root system; — 2. vacuum infiltration, mostly of the leaves; — 3. spraying; — 4. floating the detached leaves or leaf discs on the solution of the chemical under test, whether or not solidified with agar; — 5. wrapping a cotton pad drenched with the compound round the shoots; applying the chemical at a certain spot of the plant in a lanoline paste, or through a woollen thread passed into the stem; dipping an intact bud or leaf in a solution of the compound for a certain time; injecting the plants with the chemical, etc.; — 6. treatment of the seeds, seed potatoes or beet stecklings with a solution of the compound; — 7. tissue cultures, the compound added to the tissue nutrient solution.

Compound	Virus	Host plant	Method	Author(s)
Thiouracil	TMV	tobacco	4	COMMONER, MERCER (1951)
			3	MERCER, LINDHORST, COMMONER (1953)
			1, 4	NICHOLS (1953; 1954)
			4	JEENER, ROSSEELS (1953)
			4	SCHLEGEL, RAWLINS (1953; 1954*b*)
			1	HOLMES (1954; 1955)
	lucerne mosaic virus	tobacco	1, 3	MATTHEWS (1953)
		beans		
	TMV	tobacco spp.	3, 4	BAWDEN, KASSANIS (1954)
	several other viruses	several other plants		BAWDEN (1954)
	stone fruit virus	cucumber	2	KIRKPATRICK, LINDNER (1954)
	TMV	tomato		
	ring spot virus	plum tree	5	MILIKAN, GUENGERICH (1957)
	cucumber mosaic virus	tobacco	1	PORTER, WEINSTEIN (1957)
guanazolo (8-azaguanine)	lucerne mosaic virus	clover	1, 3	MATTHEWS (1951; 1952; 1953*a*, *b*; 1954)
	TMV	tobacco (also Nic. glutinosa)		
		tomato		
	stone fruit virus	cucumber	2	KIRKPATRICK, LINDNER (1954)
	TMV	tomato		
	virus yellows	sugar beet	1, 3	RUSSEL, TRIM (1957)
	ring spot virus	plum tree	5	MILIKAN, GUENGERICH (1957)

TABLE V (continued)

Compound	Virus	Host plant	Method	Author(s)
guanazolo (8-azaguanine) 2-thiocytosine 2-thiothymine 2.6-diaminopurine 6-methylpurine 6-chloropurine	TMV	tobacco	3	MERCER, LINDHORST, COMMONER (1953)
	TMV	tobacco	7	KURTZMAN and Coworkers (1957*a*, *b*)
pyrimidines thiazole malonic acid nicotinic acid	TMV	tobacco	4	RYZHKOV, MARCHENKO (1954, 1955)
diazouracil dl- and l-isoleucine several other chemicals	TMV	tobacco	4	SCHLEGEL, RAWLINS (1953; 1954*b*)
malachite green	TMV	tobacco	4	TAKAHASHI (1948; 1957)
	TMV potato virus X	tomato	3	WILLIAMS and Coworkers (1952)
	potato virus X	potato var. Green Mountain (Early Carmen)	7	NORRIS (1954, 1955*a*, *b*)
malachite green other triphenylmethane dyes, as: victoria blue, night blue	TMV	tobacco	4?	KAUSCHE, HAHN, SCHLEITH (1950)
methylene blue crystal violet malachite green neutral red	tissue tumour virus [1])	Rumex acetosa	7	NICKELL (1951)
chloramphenicol	stone fruit virus TMV	cucumber tomato	2	KIRKPATRICK, LINDNER (1954)
naphthalene acetic acid	tissue tumour virus [1])	Rumex acetosa	7	NICKELL (1950)
	TMV	tobacco	7 3	HILDEBRANDT, RIKER, WATERTOR (1952) NICHOLS (1952*a*, *b*)
	lettuce big vein virus	luttuce seedlings	1	RICH (1954)
indolyl acetic acid	tissue tumour virus [1])	Rumex acetosa	7	NICKELL (1951)
colchicine				

coconut milk naphthalene acetic acid	TMV	tobacco	7	SEGRETAIN (1952)
coconut *milk* aspartic acid glutamic acid	TMV	tobacco	7	SEGRETAIN, HIRTH (1956)
2,4-dichlorophenoxy acetic acid methoxine naphthalene acetic acid	TMV	tobacco	7	VAN OVERBEEK (1952)
2,4-dichlorophenoxy acetic acid	chrysanthemum mosaic virus	chrysanthemum	1	HOWLES (1953; 1955)
2,4,6-trichlorophenoxy acetic acid indole acetic acid	lettuce big vein virus	lettuce seedlings	1	RICH (1954)
ammonium salt of 2,4-dichloro-phenoxy acetic acid 3-chlorophenoxy acetic acid	TMV tomato aspermy virus	tobacco chrysanthemum	1, 3, 6	HOWLES (1957)
cortisone	TMV	tobacco	4	HIRTH, STOLKOWSKI (1957)
calcium chloride	carnation mosaic virus	carnation	5	THOMAS, BAKER (1950)
			1, 3	RUMLEY, THOMAS (1951)
	lettuce big vein virus	lettuce seedlings	1	RICH (1954)
potassium bromide	TMV	tobacco	1	SARDINA (1951, 1953)
sodium bromide ammonium bromide			1	CORDON (1953)
sodium chloride potassium chloride	potato virus X	seed potatoes	6	KANNGIESSER, DEUBNER (1954)
trisodium phosphate	TMV	tomato seed	6	JOHN, SOVA (1956)
sodium hydroxide	TMV cucumber mosaic virus	tomato seed	6	MILINKO (1956)
mineral salts	TMV	Pinto bean	1, 6	PANZER (1957)
zinc	TMV	tobacco	1, 4	HELMS, POUND (1955)
zinc sulphate	peach X virus	peach tree	5	STODDARD (1944)
	carnation mosaic virus	carnation	1, 3	RUMLEY, THOMAS (1951)
	lettuce big vein virus	lettuce seedlings	1	RICH (1954)
zinc chloride	TMV	tobacco	4	SCHLEGEL, RAWLINS (1953; 1954*b*)
zinc chloride	TMV	tobacco	4	WEINTRAUB, GILPATRICK, WILLISON (1952)
zinc sulphate	carnation mosaic virus	Dianthus barbatus		
	strawberry virus 2	strawberry	1	FULTON (1954)
nitrous acid (HNO_2)	TMV	tobacco	5	KASAHARA, KAKUMIZU (1953)
milk	TMV	tomato	3	Anonymus (BAWDEN, 1954*a*, *b*)

[1]) tissue tumour virus Aureogenus magnivena Black.

TABLE V (continued)

Compound	Virus	Host plant	Method	Author(s)
trichothecin	TMV	tobacco	3, 4	BAWDEN (1954)
ribonuclease	TMV	tobacco	2	CASTERMAN, JEENER (1955, 1956)
4-chloro-3,5-dimethylphenoxy-ethanol	TMV	tobacco	1	DAVIS (1952)
tannic acid	TMV	tomato	3	WILLIAMS and Coworkers (1951)
streptomycine	TMV	tobacco	4	LEBEN, FULTON (1952; 1953)
	necrosis virus	cow pea		
	ring spot virus	cow pea		
vitamin B 1	TMV	tobacco	4	WEINTRAUB, GILPATRICK, WILLISON (1952)
		Nic. glutinosa		
MK 61 (antibiotic from Nocordia species)	TMV	tobacco	4	SCHLEGEL, RAWLINS (1954*a*)
noformicin (antibiotic)	TMV	tobacco	1, 3	GRAY (1955)
	Southern bean mosaic virus	Pinto bean		
cytovirin (antibiotic)	TMV	tobacco	1, 3	GRAY (1957)
	Southern bean mosaic virus	Pinto bean		
quinhydrones 8-hydroxyquinoline calcium hydroxyquinoline magnesium hydroxyquinoline o-nitrophenol urea sodium thiosulphate	peach X virus	peach tree	5	STODDARD (1942)
p-aminobenzene-sulphanylamide do. mixed with maltose or dextrose p-toluenesulphanylamide hydroquinone maltose dextrose	peach X virus	peach tree	5	STODDARD (1944)

sulphaguanidine *sulphasuxidine* *sulphathalidine* *sulphamerizine* p-aminobenzenesulphoxylamide sodium diethyldithiocarbamate	carnation mosiac virus	carnation	5	THOMAS, BAKER *(1950)*
hydroxyquinoline sulphate sulphathiazole sulphaguanidine sulphasuxidine sulphathalicidine sulphamerizine	carnation mosaic virus	carnation	1, 3	RUMLEY, THOMAS (1951)
ethanol containing croton-aldehyde and possibly small quantities of other aldehydes, higher alcohols and/or methanol and/or pyridine bases	several viruses	several plants	1, 3	NYMAN (1952)
sodium salt of 4-sulphonamide-phenyl azo-7-acetylamino-1-oxynaph-thaline 3,6-disulphonic acid (= prontosil)	TMV	tobacco	1	SARDINA (1951; 1953)
radiophosphorus P_{32}	TMV	tobacco	4	SCHLEGEL, GOLD, RAWLINS (1953)
furfuryl-5-chloro-2-methyl carbanilate furfuryl carbanilate	Southern bean mosaic virus	Pinto bean	5	PORTER and Coworkers (1957)
nemagon carbon bisulphide methyl bromide DD mixture Vapam	peach yellow bud mosaic virus	peach tree	1	WAGON, TRAYLOR (1957)

MATTHEWS and SMITH (1955) and ROLAND (1955) have given reviewing articles on this subject; WEINTRAUB and KEMP (1955), who investigated Nicotiana glutinosa, and SCHLEGEL and RAWLINS (1953; 1954 a, b) who applied the leaf disc technique, have given extensive lists of all chemicals screened by them.

It appears that most investigations have been done with 2-thiouracil, guanazolo, the triphenylmethane dyes (malachite green), growth substances, and inorganic nickel, zinc and calcium salts. The other compounds mentioned have scarcely been examined and it seems desirable to verify their activity by more research.

2–Thiouracil, a representative of the pyrimidines, is first described as an antivirus factor by COMMONER and MERCER (1951); they used TMV in tobacco with the leaf disc method. Antivirus activity was found at concentrations of down to $4.3.10^{-5}$ mols per litre. According to these authors thiouracil is incorporated in the virus particle where it replaces uracil and inhibits virus propagation. The same view is held by JEENER and ROSSEELS (1953), who worked with ^{32}S labelled thiouracil, by MATTHEWS (1953 a, b; 1954), and by MATTHEWS and SMITH (1955). The first-mentioned authors were able to trace the radio-activity in the virus particle. NICHOLS (1953) also accepts this view, but he points out that this substance is phytotoxic, as it causes chlorosis of the plant and thus inhibits virus multiplication.

PORTER and WEINSTEIN (1957) also stress the harmful effect (interveinal chlorosis) of thiouracil on the plants and point out the antimetabolic relationship between thiouracil and uracil.

The antivirus activity of guanazolo (8-azaguanine or 5-amino-7-hydroxy-1-V-triazolo(d)-pyrimidine) and related compounds mainly towards lucerne mosaic virus and TMV, obtained by spraying white clover or tobacco plants, or drenching the soil in which these plants grow, with concentrations up to 1 % is mainly described by MATTHEWS (1951, 1953 b, 1954) and by MATTHEWS and SMITH (1955). MATTHEWS states that guanazolo had already been described as a strong inhibitor of the growth of microorganisms (see also CHANTRENNE and DEVREUX (1958)), fungi and algae, and of virus tumours (NICKELL and Coworkers (1950), CHANTRENNE and DEVREUX (1958)), even of malignant cells in mice. It was suggested that the growth inhibiting effect should be attributed to the interference of guanazolo with the incorporation of guanine in the nucleic acids. A similar conclusion was drawn from the experiments on the effect of guanazolo on the reproduction of lucerne mosaic virus; 3–4 % of the guanine in the virus nucleic acid was replaced by 8-azaguanine. This incorporation reduced the infectivity of the virus.

Other investigators (BAWDEN (1954); RUSSEL and TRIM (1957)) do not sustain this theory, as both thiouracil and 8-azaguanine have a marked phytocidal effect. As these compounds affect the metabolism of plants enough to change their appearance there is, according to BAWDEN (1954), obvious reason to suppose that they inhibit virus multiplication by affecting some host-cell mechanism rather than by acting directly on the virus particle.

The activity of the triphenylmethane dye malachite green was first mentioned by TAKAHASHI (1948). He floated tobacco leaves inoculated with TMV on malachite green solutions and observed that in concentrations as low as 2 ppm the virus synthesis was inhibited. He suggested that the compound acts as a block in enzyme reactions, leading to virus formation, but it was already known that malachite green inhibits the activity of a dehydrogenase in *Escherichia coli* and also affects the respiration of tobacco stem tissue. Its technical use is hampered by its very poor penetration ability. Malachite green also greatly reduces intercellular growth of influenza virus and it is suggested that this effect may be due to interference with ribonucleic acid metabolism (HOYLE, 1951).

Besides, it is known from the work of BRAUN (1951), RYZHKOV and Coworker (1950) and KAUSCHE and Coworkers (1950) that triphenylmethane dyes react with proteins, particularly with the nucleoproteins of TMV in vitro. In particular, an inactivating effect was found for alkaline triphenylmethane dyes.

The chemotherapeutic activity of the growth substances is difficult to interpret, because HARTMAN and PRICE (1950) even found that under certain circumstances growth regulators of the 3-naphthoxy acetic acid group increased Southern bean mosaic virus synthesis in Pinto bean.

The antivirus activity of nickel, zinc and calcium salts has been investigated by several research workers, but has not been explained satisfactorily either.

2. COMPOUNDS TESTED FOR CHEMOTHERAPEUTIC ACTIVITY

Several chemicals were examined in order to determine their antivirus activity. A choice was made from the most important compounds mentioned in Table V and related substances; in addition, benzimidazoles, thiourea derivatives, sulphanilamides and nitroso compounds were included in the investigation. In the group of the nitroso compounds antivirus activity could fairly soon be demonstrated with p-nitroso-dimethyl-aniline on all plants used. This was an incentive to examine a series of this type of compounds. A survey of all the compounds tested for their chemotherapeutic activity is given in Table VI.

In order to obtain a rough measure of the chemotherapeutic effect the incubation periods of the virus-infected plants, both with and without application of the compound under test, were determined. From these two values the percentage change of the incubation period brought about by the compound was calculated. This was done for all the virus-host plant relations tested. Each experiment was repeated at least 3 times in the summer season, care being taken that the various virus-host plant relations participated in the calculations of the average in the same ratio. Thus, this procedure provided a sound basis for estimating chemotherapeutic potency. It is true that this average value does not make the best possible use of the information available, as it ignores the specific differences in effect of a compound

TABLE VI

Compounds tested for their chemotherapeutic activity

Virus-host relations investigated: sugar beet yellow virus—sugar beet; TMV—tobacco (White Burley, no. 11, *Nicotiana glutinosa*); TMV—tomato; Phaseolus virus 1—French bean; Phaseolus virus 2—broad bean.

The compounds were applied according to the methods described on pages 377 and 378 in a 0.1 % solution for spraying and dipping and in a 0.001 % to 0.01 % solution for the nutrient technique (with and without 0.04–0.4 % acetone and wetting agent). The plants were infected with the virus 2–3 days after they had been treated with the compound.

The incubation periods of the virus-infected plants, both with and without application of the compound tested, were determined. From these two values the percentage change of the incubation period brought about by this compound was calculated. This was done for all the virus-host plant relations tested; each experiment was repeated at least three times in the course of 1–3 years. The average values of these percentage changes are given in column 3.

The phytotoxicity of the compounds was roughly reduced from the observed injurious effects and the retarded growth of the treated, non-infected plants as compared with the untreated controls.

++: marked, +: mild, and —: no phytotoxicity. (see column 4)

A. COMPOUNDS WITH CHEMOTHERAPEUTIC ACTIVITY

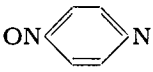
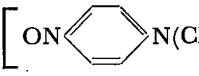
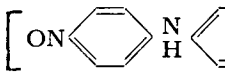
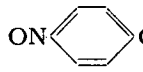
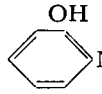
Compound	Chemical structure	Chemo-therapeutic activity	Phyto-toxicity
a. *Nitroso compounds*			
1. *Nitrosoaminoaryl compounds*			
p-nitrosodimethylaniline		10	++
— .HCl salt		10	+
— $.H_3PO_4$ salt		10	—
— $.ZnCl_2$ complex		8	—
— $.CoCl_2$ complex		7	—
— $.H_4Fe(CN)_6$ complex		4	+
— $.Ni(ClO_4)_2$ complex		3	—
p-nitrosodiphenylamine $.CuCl_2$ complex		8	—
2. *Nitrosohydroxyaryl compounds*			
p-nitrosophenol		13	—
— $.NiCl_2$ complex		7	—
o-nitrosophenol $.NiCl_2$ complex		15	—
— $.CuSO_4$ complex		8	+
— $.ZnSO_4$ complex		7	—

TABLE VI (continued)

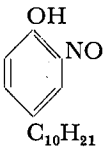
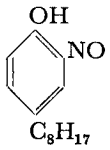
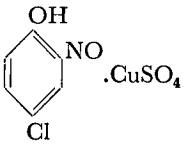
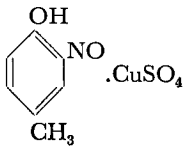
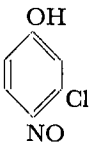
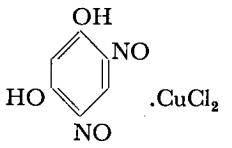
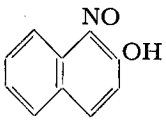
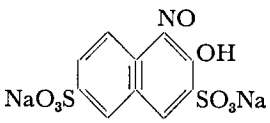
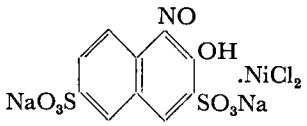
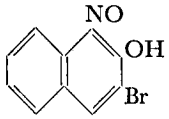
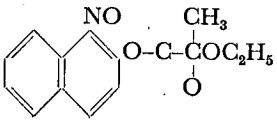
Compound	Chemical structure	Chemo-therapeutic activity	Phyto-toxicity
o-nitroso-p-sec-cetylphenol		4	—
o-nitroso-p-sec-octylphenol		2	—
o-nitroso-p-chlorophenol .$CuSO_4$ complex		12	—
o-nitroso-p-cresol .$CuSO_4$ complex		4	—
m-chloro-p-nitrosophenol		4	+
dinitrosoresorcinol .$CuCl_2$ complex		3	—
1-nitroso-2-naphthol		13	—
— .$NiCl_2$ complex	— .$NiCl_2$	7	—
1-nitroso-2-naphthol-3,6-disulphonic acid (Na salt)		13	+
1-nitroso-2-naphthol-3,6-disulphonic acid .$NiCl_2$ complex		6	—
1-nitroso-3-bromo-2-naphthol		3	+
1-nitroso-2-(1-carboxy-ethyl)ethoxynaphthalene		5	—

TABLE VI (continued)

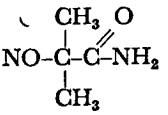
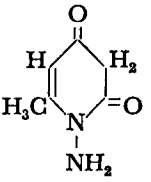
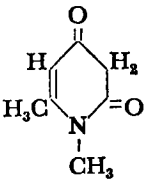
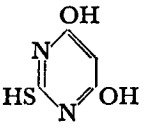
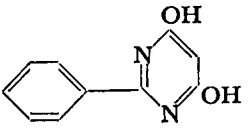
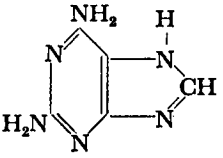
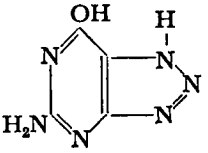
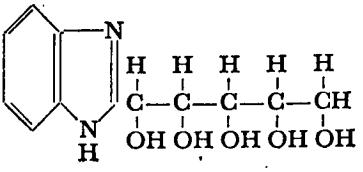
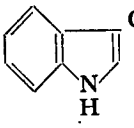
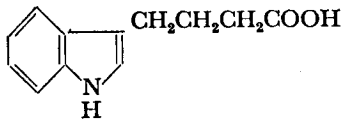
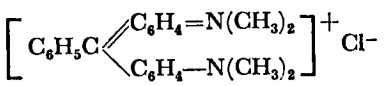
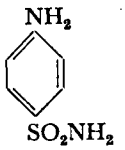
Compound	Chemical structure	Chemo-therapeutic activity	Phyto-toxicity
3. *Other types of nitroso compounds*			
α-nitrosoisobutyric amide		6	+
b. *Pyridones*			
1-amino-4-oxy-6-methylpyridone		4	—
1,6-dimethyl-4-oxypyridone		4	—
c. *Pyrimidines*			
2-thiouracil		>10	++
2-phenyl-4,6-dihydroxy-pyrimidine		4	+
2,4-diaminopurine		3	+
8-azaguanine (guanozolo)		4	+
d. *Benzimidazoles*			
1-(benzimidazole-2)-1,2,3,4,5-pentahydroxypentane		3	—

TABLE VI (continued)

Compound	Chemical structure	Chemo-therapeutic activity	Phyto-toxicity
e. *Indole derivatives*			
β-(3-indolyl)propionic acid		3	—
γ-(3-indolyl)butyric acid		4	—
f. *Dyes*			
malachite green		8	+
g. *Sulphanilamides*			
Sulphanilamide		7	+
h. *Inorganic compounds*			
zinc chloride	$ZnCl_2$	7	—
zinc sulphate	$ZnSO_4$	7	—
nickel chloride	$NiCl_2$	8	—

B. COMPOUNDS LACKING CHEMOTHERAPEUTIC ACTIVITY

Nitroso compounds

1. *Nitrosoaminoaryl compounds*
p-nitrosodimethylaniline.$CdCl_2$, $MgCl_2$, $FeCl_2$, $CuCl_2$ or urea complexes; p-nitrosomonomethylaniline, p-nitrosodiphenylamine, p-nitroso-N-cyclohexyl-N-ethyl-aniline, p-nitrosotriphenylamine, N-nitroso-N-methylaniline, potassium salt of phenylnitrosoamine, 4-nitroso-1-naphthylamine sulphate, 1-nitroso-2-naphthylamine, p-nitrosodimethylamine, p-chloronitrosobenzene, methylphenyl-N-nitrosoamine.
2. *Nitrosohydroxyaryl compounds*
1 hydroxy-2-methyl-4-nitrosobenzene, 1-hydroxy-3-methyl-4-nitrosobenzene, 2,5-dinitrosoresorcinol, 5-nitroso-2-oxydiphenyl, 1-nitroso-4-naphthol, 2-nitroso-1-naphthol, 1-nitroso-6-methylnaphthol-2, 5-nitroso-8-hydroxyquinoline, 2-nitrosophenol, 5-nitroso-1-naphthol, 2-nitroso-5-chlorophenol and its copper chloride complex, 2,4-dichloro-6-nitrosophenol, 1-nitroso-3-chloro-2-naphthol, 8-nitroso-1-naphthol, 8-nitroso-2-naphthol, 5-nitroso-2-naphthol, 2-bromo-4-nitrosophenol, 2-chloro-4-nitrosophenol, acetylated 1-nitroso-2-naphthol, acetylated 1-nitroso-4-naphthol, 1-nitroso-2-naphthol and its copper chloride complex, nitrosoorthocresol, nitrosometacresol.
3. *Heterocyclic N-nitroso compounds*
N-nitrosopiperidine, N-nitrosomorpholine.
4. *Other types of nitroso compounds*
Potassium salt of nitrosoformamidoxine, α-nitrosoisobutyroamidine hydrochloride, N-nitrosomethylurethane, m-nitrosotoluol, nitrosation product of diphenylether, nitroso-p-ethoxyacetophenone, nitrososalicylic aldehyde and its copper chloride complex, 4-nitrososalicylic acid and its copper chloride complex.

on the various virus-host plant systems. However, it is of practical use, the more so as a large number of virus-host plant relations are concerned.

Compounds with a marked chemotherapeutic activity (arranged in order of decreasing activity) are:
o-nitrosophenol.$NiCl_2$ complex, 1-nitroso-2-naphthol, p-nitroso-phenol, 1-nitroso-2-naphthol-3,6-disulphonic acid (sodium salt), o-nitroso-p-chlorophenol.$CuSO_4$ complex, 2-thiouracil, p-nitroso-dimethylaniline, p-nitrosodimethylaniline.HCl salt, p-nitroso-dimethylaniline.H_3PO_4 salt, p-nitrosodimethylaniline.$ZnCl_2$ complex, p-nitrosodiphenylamine. $CuCl_2$ complex, o-nitrosophenol.$CuSO_4$ complex, malachite green, nickel chloride, p-nitrosodimethylaniline.$COCl_2$ complex, p-nitroso-phenol.$NiCl_2$ complex, o-nitrosophenol.$ZnSO_4$ complex, 1-nitroso-2-naphthol. $NiCl_2$ complex, sulphanilamide, zinc chloride, zinc sulphate, 1-nitroso-2-naphthol-3,6-disulphonic acid (sodium salt). $NiCl_2$ complex, and α-nitrosoisobutyric amide.

One of the most active antivirus compounds so far investigated is p-nitrosophenol. m-Nitrosophenol, on the other hand, had no activity at all. As o-nitrosophenol could not be used owing to its instability its metal salts were examined. It could be demonstrated that especially its nickel salt had an attractive chemotherapeutic activity. *Nicotiana rustica* plants treated with this salt and, two days later, inoculated with TMV did not develop any symptoms of the disease. These plants did not contain the virus in an amount that could be detected serologically. Unfortunately, in further experiments it appeared that these metal salts of o-nitrosophenol are still too unstable to expect that they will be of any use in practice. Therefore, their activity was not further investigated.

The active nitroso compounds are poorly soluble in water; it was investigated whether this could be improved by solving 50 milligrams of the chemical in 2 millilitres of acetone mixed with 0.01 % Triton X 155, as already described in II 6a. This procedure gave a better result. Alcohol could not be used as a solvent on account of its harmful effect. 1-Nitroso-2-naphthol proved to be better soluble in the form of its sodium or potassium salt. without losing its anti-virus activity.

It was tried to improve the formulation by adding: ethyl acetate, dioctyl phosphate, trichloropropane, some paraffinic oils, orthoboric acid, glucose, potassium or sodium hydrocarbonate or sodium phthalate, alkaline sulphite, T-pol or Triton X 155, but of these only Triton X 155 had a favourable effect. This detergent improves spreading and wetting of the plant without being phytotoxic.

The compounds tested differ widely in phytotoxicity. Those having marked phytotoxic properties, such as thiouracil, had to be excluded from further investigations, as they would be of no importance for practical use.

The most active chemotherapeutic compounds which are not phytotoxic are:
o-nitrosophenol.$NiCl_2$ complex, 1-nitroso-2-naphthol, p-nitrosophenol, o-nitroso-p-chlorophenol.$CuSO_4$ complex, p-nitrosodimethylaniline. H_3PO_4 salt, p-nitrosodimethylaniline.$ZnCl_2$ complex, p-nitrosodi-phenylamine.$CuCl_2$ complex, nickel chloride, p-nitrosodimethyl-

aniline.$CoCl_2$ complex, p-nitrosophenol.$NiCl_2$ complex, o-nitrosophenol.$ZnSO_4$ complex, 1-nitroso-2-naphthol-3,6-disulphonic acid (sodium salt).$NiCl_2$ complex.

It was a disappointment that p-nitrosodimethylaniline proved to be too phytotoxic; even with a solution of this compound that just showed a slight chemotherapeutic activity the symptoms of its toxic effect appeared. Therefore it was investigated whether complexes formed by p-nitrosodimethylaniline and metal salts, such as $CoCl_2$, $Ni(ClO_4)_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$ and $CuCl_2$, would give better results. The experiments showed that the $CoCl_2$ and $Ni(ClO_4)_2$ complexes had a positive chemotherapeutic activity against TMV in *Nicotiana tabacum* (var. no. 11), while the $ZnCl_2$ complex suppressed TMV in *Nicotiana rustica*. No activity was observed with these compounds in a 0.1 % solution. However, the chemotherapeutic activity of these salts generally remained so far behind that of other nitroso compounds that these salts had to be excluded from further research.

It seemed worth while to determine whether these compounds were not toxic for rabbits either. It was demonstrated that comparatively large amounts (1–3 grams) of these compounds could daily be mixed with the food for 6 weeks without having any harmful effect on the animals. Examination of their organs (liver, spleen, kidney, etc.) did not reveal visible aberrations. These observations justified a more extensive investigation into the effect of these compounds.

3. Influence of thiouracil and the non-toxic chemotherapeutic compounds

First of all the influence of the non-toxic chemotherapeutic nitroso compounds on the incubation period of several viruses was determined (Table VII); this influence is expressed as the average of the percentage increase in the incubation period.

The normal incubation time strongly depends on the environmental and developmental conditions of the host plants. Moreover, the increase in incubation period due to treatment with a chemotherapeutic compound showed an approximately proportional variation rather than independence of the length of this period. As generally the results vary considerably from one occasion to another it was necessary to take the following precautions.

All the experiments had to be performed during the period from April to the end of August with young plants, in the four-leaf stage of development which had been grown under favourable conditions in a greenhouse and whose incubation time remained within the following lower and upper limits: 18 and 22 days for sugar beet, 8 and 10 days for tobacco and 10 and 12 days for beans and tomato, respectively.

In addition, the experiments with the most active compounds were repeated many times in the course of a period of 2 to 3 years. The calculation of the percentage increase of the incubation period approximately eliminates the small differences remaining between the incubation periods and renders the results better comparable.

TABLE VII

Influence of some non-phytotoxic nitroso compounds with chemotherapeutic activity

The compounds were applied according to the methods described in II. 6. The plants were infected with the virus 2 days after application of the chemical. S: spraying of a 0.1 % solution of the compound on the leaves; D: dipping of the roots in a 0.1 % solution of the compound for 24 hours; N: adding of 15 mg of the compound to 150 ml of Knopp's nutrient solution. I = 2-nitrosoisobutyric amide; II = o-nitroso-p-chlorophenol.$CuSO_4$ complex; III = p-nitrosophenol; IV = o-nitrosophenol.$NiCl_2$ complex; V = 1-nitroso-2-naphthol.

Virus	Host plant	Method of application	Average percentage increase in incubation period after adding				
			I	II	III	IV	V
Yellow virus	Sugar beet	S	0	10	4	6	8 ± 1.3
		D or N	0	8	10	12	10 ± 1.9
TMV	Nicotiana rustica	S	10	30	14	26	21 ± 4.4
		D or N	10	30	16	18	14 ± 4.1
	Nicotiana tabacum	S	16	6	10	40	8 ± 2.4
		D or N	14	14	22	38	19 ± 4.1
	Nicotiana glutinosa	S	0	10	4	6	(25 repli-
		D or N	0	0	6	6	cates)
Phaseolus virus 1	French bean	S	10	10	26	4	
		D or N	12	6	20	10	
Phaseolus virus 2	Broad bean	S	0	4	14	10	
		D or N	0	10	16	4	

Table VII confirms that α-nitrosoisobutyric amide has a moderate chemotherapeutic activity, whereas 1-nitroso-2-naphthol, o-nitroso-p-chlorophenol. $CuSO_4$ complex, p-nitrosophenol and o-nitrosophenol. $NiCl_2$ complex belong to the most active compounds tested.

The question may arise whether the effect of a chemotherapeutic compound depends on the type of virus, the host plant and the way this compound entered the plant. Now the individual observations show considerable variation and this prevents making a general inference. Table VII, however, enables the conclusion to be drawn that the activity of a chemotherapeutic substance may depend on the factors mentioned. It appeared that 2-nitrosoisobutyric amide in 0.1 % solution does not interfere with the development of yellow virus in sugar beet, of TMV in *Nicotiana glutinosa* or of Phaseolus virus 2 in broad bean; p-nitrosophenol strongly inhibits the development of the last-mentioned virus, whereas it hardly interferes with the propagation of TMV in *Nicotiana glutinosa*. In some cases the way in which the chemotherapeutic substance enters the plant is of great importance to its activity. However, it must be admitted that it is not easy to draw conclusions from our experiments, as the quantity of active compound penetrating into the cells is not known. Therefore, the differences in activity may be attributed to differences in permeability as well as to specific reactions of the viruses and host plants.

TABLE VIII *Relation between the chemotherapeutic activity and toxicity of some of the most active compounds*

A = minimum concentration with chemotherapeutic activity; B = maximum concentration not toxic in the host plant. D = dipping method; S = spraying of the leaves. < = concentration just phytotoxic.

Virus	Host plant	p-Nitrosophenol				1-Nitroso-2-naphthol (Na or K salt)				o-Nitroso-p-chlorophenol (copper complex)				Thiouracil				Mixture of thiouracil and p-nitroso-phenol or 1-nitroso-2-naphthol			
		A		B		A		B		A		B		A		B		A		B	
		D	S	D	S	D	S	D	S	D	S	D	S	D	S	D		D	S	D	S
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%				
TMV	Nicotiana rustica or Nicotiana tabacum	0.1	0.1	0.20	0.25	0.1	0.1	0.15	0.25	0.1	0.1	0.20	0.25	0.05 [1]	0.1 [1]	0.01 [1]	0.05	0.05/0.05 %	0.05/0.05 %	< 0.05/0.05 %	0.05/0.05 %
	Nicotiana glutinosa	0.1	0.1	0.20	0.25	0.1	0.1	0.15	0.2	0.1	0.1	0.20	0.25	0.05 [1]	0.1 [1]	0.01	0.05	0.05/0.05 %	0.05/0.05 %	< 0.05/0.05 %	0.05/0.05 %
	tomato	0.1	0.1	0.20	0.25	0.1	0.1	0.15	0·	0.1	0.1	0.20	2. 25	0.05 [1]	0.1 [1]	0.01	0.05	0.05/0.05 %	0.05/0.05 %	< 0.05/0.05 %	0.05/0.05 %
Phaseolus virus 1 or 2	French bean or broad bean	0.05	0.1	0.20	0.25	0.05	0.1	0.1	0.25					0.05 [1]	0.1 [1]	0.005	<0.1	0.05/0.05 %	0.05/0.05 %	< 0.05/0.05 %	< 0.05/0.05 %
yellow virus	sugar beet	0.1	0.1	0.20	0.25	0.1	0.1	0.25	0.25	0.1	0.1	0.20	0.25	0.05 [1]	0.1 [1]	0.01	0.05				

[1]) In experiments with toxic compounds it is often difficult to note the minimum concentrations with a chemotherapeutic effect.

It seemed desirable to investigate for the most active chemotherapeutic compounds whether there is a large difference between the minimum concentration bringing about an anti-virus effect and the maximum concentration that does not cause any symptoms of damage to the host plants. Table VIII gives the results of one of these experiments; thiouracil was included as a phytotoxic substance. The lowest concentration of this compound that still counteracted the virus always had a marked injurious effect on the host plants, whereas with p-nitrosophenol and the copper complex of o-nitroso-p-chlorophenol there was a definite difference between the critical concentrations: the toxic effect appeared at a concentration 2–2.5 times as high as the one leading to a chemotherapeutic effect.

Solutions containing both thiouracil and p-nitrosophenol or thiouracil and 1-nitroso-2-naphthol were also tested. It might be expected that with these solutions, considering the much lower concentration of thiouracil, the chemotherapeutic effect of this compound would be relatively more pronounced than its injurious effect. This was definitely the case, but the effect was insufficient, the mixtures still being too toxic, particularly for French and broad beans.

IV. FIELD EXPERIMENTS

1. Introduction

The experiments described in the preceding chapter were carried out with seedlings a few weeks old. It may be expected, however, that the response of such young plants, grown in a greenhouse and artificially infected with virus, to a treatment with chemotherapeutic compounds will differ considerably from that of older or full-grown plants in the field which have been infected with virus under natural conditions. The compounds may have a stronger effect on seedlings than on full-grown plants; moreover, the consequences of a virus infection may be more harmful to young plants, when they are insufficiently protected by the chemotherapeutic agents. On the other hand, in field experiments these compounds have to remain active for some considerable time, as infection may occur on several occasions; this prolonged activity can be obtained by more frequent application of the chemical. For these reasons it seemed appropriate to perform several series of field experiments in the course of five years.

The response to chemotherapeutic treatment was determined by comparing the yields of virus-infected plants in treated and in untreated field plots. It is obvious that only those compounds were chosen which in the experiments described in Chapter III had produced the best results.

In 1953, the first year of our field experiments, 0.1 % solutions of p-nitroso-phenol, the potassium salt of 1-nitroso-2-naphthol and of thiouracil, as well as 50/50 mixtures of thiouracil and one of the nitroso solutions mentioned were sprayed in high volume (1000 litres/hectare), once or twice before and once; twice or three times

after inoculation at intervals of about two weeks. The chemotherapeutic activity was tested on Phaseolus virus 2 in broad beans, Phaseolus virus 1 in French beans, and on TMV in *Nicotiana tabacum* (var. no 11 and White Burley) and *Nicotiana rustica*.

The first results obtained were so promising that in the following years the investigation was continued with more extensive field experiments. Thiouracil and its mixtures were not further examined on account of their phytotoxicity to the bean plants, which resulted in a considerable decrease in seed production. Experiments on the effect of 1-nitroso-2-naphthol-3,6-disulphonic acid (Na salt), o-nitrosophenol.$NiCl_2$ complex and o-nitroso-p-chlorophenol.$CuSO_4$ complex were now included.

The effect of all the compounds sprayed on virus development was investigated by varying the concentration range from 0.05 % to 2.0 %, by repeating the spraying 3–8 times or by varying the spraying period (before, at the time of or after natural infection by aphids).

The investigations were extended to comprise yellow virus and sugar beet; in fact, with these plants by far the most trials were performed.

A few experiments were done in which the above-mentioned compounds were used as seed dressings. The experimental conditions remained otherwise unchanged.

The field plots were situated in different regions of the Netherlands: in the experimental garden of the Koninklijke/Shell Laboratory at Amsterdam; in the Eemnesserpolder[1]); at Renkum; at Well, Bergen and Siebengewald in the Northern part of Limburg—one of the areas where virus yellows causes most serious damage to the sugar beet

TABLE IX

Climatological conditions during the years of the field experiments

Deviations from the average temperature (° C) and average rainfall (in millimetres) in the Netherlands (de Bilt) from 1953–1958. The standard values used are those calculated for 1955 over the period 1921–1950.

Season	Average temp., ° C	Deviation (° C) from average in				
		1953	1954	1955	1957	1958
March–May	8.6	+ 0.3	— 0.1	— 2.0	+ 0.5	— 1.6
June–August	16.4	+ 0.1	— 1.4	+ 0.3	+ 0.4	— 0.2
September–November	9.8	+ 1.3	+ 0.9	+ 0.3	— 0.2	+ 0.9

Season	Average rainfall, mm	Deviation (%) from average in				
		1953	1954	1955	1957	1958
March–May	150	— 34	— 14	0	— 10	— 6
June–August	214	+ 38	+ 52	— 35	+ 35	+ 7
September–November	218	— 69	+ 20	— 5	+ 25	— 16

[1]) The author is very much endebted to the management of the "Koninklijke Beetwortelzaad Cultuur Kuhn and Co", Naarden for the use of a part of their experimental field, and for the harvest analysis, free of charge, performed by them in 1953.

crop—and in the experimental garden of I.P.O. at Wageningen.

In the following sections the experiments carried out to limit the effect of the virus infections mentioned and their results are described. A survey of the climatological conditions during the years these experiments were done is given in Table IX.

2. Bean viruses in broad bean and French bean

In 1953 and 1954 the effect of p-nitrosophenol, of 1-nitroso-2-naphthol (K-salt) and of o-nitroso-phenol ($NiCl_2$ complex) was determined, in small experiments; the 1954 results on broad bean are recorded in Table X.

TABLE X

Preliminary experiments on the effect of chemotherapeutic compounds on the yield of broad bean plants inoculated with Phaseolus virus 2

Two experiments, 20 plants per plot of 2½ square metres. Exp. A: Seedlings planted on 12-4-1954; Sprayed on 14-5, 19-5, 3-6 and 17-6-1954; Inoculated by hand on 21-5-1954. Exp. B: Seeds sown on 12-4-1954. Sprayed on 3-3, 14-6, 17-6 and 24-6-1954; Inoculated by hand on 21-6-1954. Natural infection by aphids also occurred; insects killed with a parathion spray (0.05 %) on 11-6-1954. Harvest on 22-7-1954. Yield on infected untreated plants: Exp. A: 100 seed pods, weight 810 g; Exp. B: 235 seed pods, weight 2540 g.

Spraying compound	Experiment	Concentration of spraying solution			
		0.1 %		0.2 %	
		% increase of yield as compared with infected untreated plants			
		nr. of pods	weight	nr. of pods	weight
p-nitrosophenol	A	+ 130	+ 160	+ 70	+ 140
	B	+ 20	+ 40	— 30	— 20
1-nitroso-2-naphthol (K salt)	A	— 10	+ 20	+ 80	+ 100
	B	0	— 10	+ 20	+ 20
1-nitroso-2-naphthol-3,6-disulphonic acid (Na salt)	A	+ 30	+ 50	+ 80	+ 110
	B	0	0	+ 20	+ 30
o-nitrosophenol ($NiCl_2$ complex)	A	+ 10	+ 90		
	B	0	+ 10		

These experiments were only of a tentative nature, the number of replicates was too small. The same applies to the experiments in which the effect of these chemicals on French bean was determined.

All these results go to show that p-nitrosophenol may have chemotherapeutic activity in comparatively low concentrations (0.1 %). The nitrosonaphthols had a favourable effect rather at higher concentrations (0.2 %), although when applied on French bean these compounds were also active at 0.1 %. It will be seen that the figures of Table X show a considerable variation. This is also due to the fact that a small favourable effect of spraying will result in a relatively high percentage increase when the plants are heavily attacked by the virus, whereas this value is relatively low when the virus disease does not seriously affect the plants.

3. YELLOW VIRUS IN SUGAR BEET PLANTS

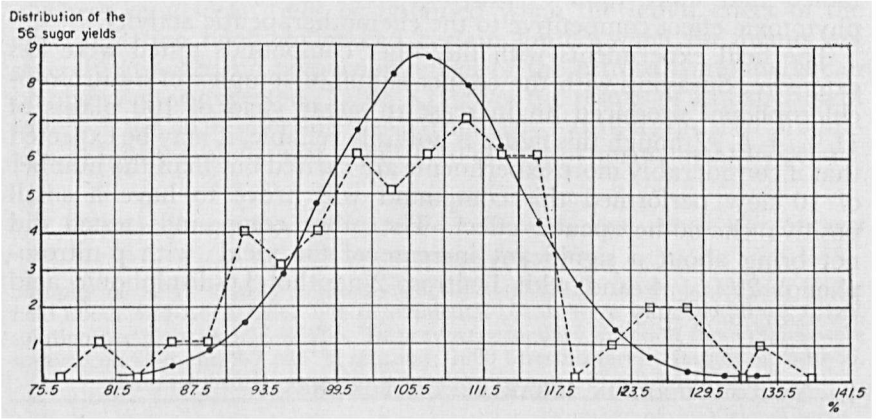
In the period of 1954–1958 experiments were undertaken in order to establish the influence of spraying sugar beet infected with yellow virus with nitroso compounds. This was done by determining the sugar yield of sprayed and unsprayed plots of sugar beet.

The seeds were drilled in rows of 35 or 40 centimetres and singled out at distances of 20–32 centimetres to obtain the same density as in a field with 70,000 plants/hectare.

It has often been suggested (LÜDECKE 1945, SCHOENE 1954, SIMON 1954) to grow sugar beet crops at a higher density, even up to 100.000 plants/hectare. The area is sooner covered by the crop and this is attractive. For the aphids prefer a crop with open spots, which often occur in fields containing 50–60.000 beet plants/hectare. At this density, however, the root crop develops better.

It is worth mentioning that the trial plots in the northern part of Limburg were situated in a field of a few hectares on which sugar beets were grown. The plants were infected with yellow virus in the natural way by aphids. In 1954 the aphids appeared rather late due to the cold weather. Hence the first symptoms of the disease did not appear until 10th July, about a fortnight later than in normal years.

In the other experiments, viz. those at Amsterdam, in the Eemnesserpolder, at Renkum and in the I.P.O. garden in Wageningen, the plants were cultivated in experimental fields. They were infected by placing 6–10 aphids on every plant, as it was not quite certain that the natural infection would be sufficient. A few days later the aphids were killed by a Systox spray in order to prevent the aphids from migrating to other crops in the neighbourhood. The symptoms of the disease became visible at the expected time, about three weeks after the date of infection.



Graph. 1 Frequency distribution of yield of sugar beet plants infected with sugar beet yellow virus and sprayed with 0.1% solution of 1-nitroso-2-naphtol (potassium salt)

Yield of sprayed plants expressed in % of yield of untreated control plants.
•——• normal distribution curve
□-----□ experimental distribution curve

All experiments were performed in blocks with plots of 14 or 28 square metres (100–200 beet plants). These plots were randomized to enable a variance analysis to be made at harvest time according to the FISCHER (1951)—KUIPER (1952) methods. Invariably 3–5 replicates were investigated at the same place and time and the average value of the analysis was regarded as its results.

In all, 56 field experiments in which sugar beet plants were sprayed with 1-nitroso-2-naphthol in 0.1 % solution were carried out in 5 years. The sugar yield of 100 virus-infected plants treated with this compound was 107 % ± 1.5 of the yield of the infected but untreated control plants. The increase, though small, was significant. Graph 1 shows the frequency distribution curve of the sugar yield together with the normal theoretical frequency distribution curve best fitting it. The two curves show a fair agreement.

Treating the sugar beet plants with more dilute solutions gave less favourable results: spraying with 0.05 % solutions also increased the sugar yield, but this effect was less marked than that obtained with 0.1 % solutions. Treating the plants with solutions of 0.2 % or higher gave decidedly poor results; phytotoxic phenomena were observed, which often neutralized the expected chemotherapeutic effect. That is the reason why, considering all the field experiments (68 in all) in which 1-nitroso-2-naphthol was sprayed in a concentration range of 0.05–0.2 %, the average increase in sugar yield of 100 plants was only 5 % ± 1.5. From these experiments it can be concluded that the minimum concentration limits having a positive chemotherapeutic and an injurious effect do not differ much. It is not altogether precluded that the two phenomena spring from a common source, the more so because strong indications were obtained that more than 3 sprayings with 0.1 % also resulted in a phytotoxic effect competitive to the chemotherapeutic activity.

The field experiments with the other compounds tested were less extensive. Spraying with the copper sulphate complex of o-nitroso-p-chlorophenol produced an increase in sugar yield of 100 plants of 11 % ± 4. Although this figure is not fully reliable, it may be expected that if considerably more experiments are carried out than the number of 10 now performed this compound will prove to have a small positive chemotherapeutic effect. The other compounds tested did not bring about a significant increase of the yield: with p-nitrosophenol 2 % ± 4 and with 1-nitroso-2-napthol-3,6-disulphonic acid (Na salt) 6 % ± 5.

4. YELLOW VIRUS IN SUGAR BEET STECKLINGS

A few small experiments were performed to investigate whether the seed production of diseased stecklings can be increased by dipping them for 18½ hours in a 0.05 % solution of nitroso compounds before they are planted out in spring. Nine stecklings, to be used as controls, were dipped in water for 18½ hours.

By this procedure the solution penetrates 1–2 centimetres into the

root tissues, the root showing the brownish-yellow colour of the nitroso compounds over this distance. During the growing period of the stecklings they are sprayed 3 times with a 0.1 % solution of the compound tested in order to reduce the effect of secondary infections.

The stecklings were never cured by dipping, but already during growth it appeared that the untreated control plants were more severely attacked than the treated ones. These differences became very pronounced at harvest time: the treated plants produced more seeds (Table XI). The potassium salt of 1-nitroso-2-naphthol had stimulated the seed production of the infected seedlings considerably, although it did not exceed 45 % of that of normal uninfected plants.

TABLE XI

Seed production of infected sugar beet stecklings after treatment with various nitroso compounds

Stecklings dipped in 0.05 % solutions of nitroso compounds for 18½ hours. Control stecklings dipped in water. Stecklings planted after one day (3-5-1955). Spraying on 9-6, 1-7 and 15-7-1955 with 0.1 % solution (1000 litres/hectare). Harvest: 22-9-1955. Seed production of uninfected plants: about 240 g/plant.

	Number of stecklings	Yield of seeds, g/plant
Untreated stecklings	9	50
Stecklings treated with:		
p-nitrosophenol	9	67
1-nitroso-2-naphthol (K salt)	8	108
o-nitroso-p-chlorophenol ($CuSO_4$ complex)	9	57

Probably the increase in yield after treatment with p-nitrosophenol, although less marked, is also significant.

These results can only be obtained when the lethal effect of the virus infection does not exceed certain limits; when the plants are severely attacked no favourable effect of the nitroso compounds can be observed or expected. Table XII gives the results of an experiment in which the virus infection was particularly harmful; the seed production of infected plants was about 1/10 of that of normal healthy plants.

TABLE XII

Seed production of sugar beet stecklings severely attacked by virus yellows after treatment with various nitroso compounds

Samples of 54–79 heavily infected sugar beet stecklings were planted out in the field about mid-April 1956. The experimental plants were watered (50 ml, 0.1 % solution per plant) on 27-4-1956. To prevent secondary infection these plants were sprayed on 22-6 and 9-7 (0.1 % solution; 1000 litres/hectare). Harvest on 9-10-56. Normal healthy stecklings produce about 240 g seeds/plant.

	Number of stecklings	Yield of seeds, g/plant
Untreated stecklings	79	25
Stecklings treated with:		
p-nitrosophenol	54	26
1-nitroso-2-naphthol (K salt)	55	26

5. TMV IN TOBACCO

In several series of experiments TMV-infected tobacco plants (*Nicotiana rustica*, *Nicotiana tabacum* var. no. 11; *Nicotiana tabacum* var. White Burley) were sprayed in 0.1 or 0.2 % concentrations with solutions of p-nitrosophenol or the potassium salt of 1-nitroso-2-naphthol.

Slight indications of a chemotherapeutic effect of both compounds were obtained; especially the results with the potassium salt of 1-nitroso-2-naphthol in 0.2 % solution against TMV in *Nicotiana rustica* were rather convincing.

The experiments with *Nicotiana tabacum* var. White Burley were not so successful, as the plants grew slowly and consequently the propagation of the virus was not very fast.

Experiments with thiouracil alone were not carried out on account of the phytotoxicity it had shown in greenhouse experiments. Instead, thiouracil mixed with a nitroso compound was used.

Positive results were obtained with TMV-infected plants of *Nicotiana tabacum* var. no. 11 sprayed with solutions containing thiouracil (0.05 %) and p-nitrosophenol (0.05 %) or 1-nitroso-2-naphthol (0.05 %). Table XIII lists the results of an experiment in which the influence of spraying on the incubation time was determined in relation to the growth season.

TABLE XIII

Increase of the incubation time by spraying Nicotiana tabacum var. no. 11 with solutions containing thiouracil and a nitroso compound

Each exp. comprised 12 plants of Nicotiana tabacum var. no. 11. The plants were sown in various months and had a length of about 30 cm at the beginning of the exp. In the first and second exp. the plants were sprayed 6 times, with intervals of 3 days. Inoculation with TMV after the third spraying. In the third, fourth and fifth exp. the plants were sprayed 2–3 times, with intervals of 3–7 days. Inoculation with TMV after the first spraying. Spraying solution: thiouracil (0.05 %) and a nitroso compound (0.05 %).

Period during which the experiment was carried out	Incubation time of unsprayed plants, days	Incubation time (days) of plants sprayed with	
		p-nitrosophenol + thiouracil	1-nitroso-2-naphthol + thiouracil
April–May	25 ± 0.5	—	30 ± 1.1
May–June	14 ± 0.5	34 ± 1.5	—
July–August	11 ± 0.5	25 ± 1.–	25 ± 1.–
August–September	22 ± 1.–	31 ± 2.–	31 ± 1.5
September–October	28 ± 1.5	37 ± 0.8	37 ± 0.8

The plants grew very well when sown early (spring, early summer), but the growth rate decreased considerably when sown later (mid-summer, autumn). The experiment started when the plants had a height of about 30 cm. The incubation time of TMV-infected plants decreased for those sown during the period of April to July, but increased considerably in the case of those sown later on; without

doubt this was due to environmental conditions, particularly as regards light and temperature. Spraying of the plants with the solutions already mentioned resulted in a marked increase in incubation time; the seasonal decrease of the incubation time displayed by the unsprayed plants disappeared almost entirely. Therefore, spraying was most effective in the period of May till August. As a consequence of the inhibitory effect of these compounds on the development of TMV, the visible symptoms were less pronounced in all the experiments.

The influence of spraying with these solutions was also investigated on *Nicotiana rustica* (Table XIV).

TABLE XIV

Influence of spraying with solutions containing thiouracil and a nitroso compound on the development of TMV-infected Nicotiana rustica plants

Each exp. comprised 12 plants of Nicotiana rustica. The plants had a length of about 30 cm at the beginning of the exp. Spraying 3 times (9, 21 and 24-7-1953) with a spraying solution containing thiouracil (0.05 %) and a nitroso compound (0.05 %). Control plants sprayed with water. Plants inoculated with TMV on 16-7-1953.

Sprayed with	Percentage of diseased plants				Length of plants on 12-8-1953, cm	Number of leaves larger than 20 by 25 cm on 1-9-1953	
	31-7	3-8	10-8	17-8		no visible TMV symptoms	with TMV symptoms
A. *Non-infected plants*							
water	0	0	0	0	85 ± 2.6 (flowers)	11 ± 0.2	
p-nitrosophenol + thiouracil	0	0	0	0	83 ± 3.– (a few fl.)	11 ± 0.3	
1-nitroso-2-naphthol + thiouracil	0	0	0	0	79 ± 1.8 (no flowers)	11 ± 0.6	
B. *TMV-infected plants*							
water	100	100	100	100	57 ± 2.6	4 ± 0.4	4 ± 0.3
p-nitrosophenol + thiouracil	0	25	83	92	68 ± 2.1	7 ± 0.3	2 ± 0.4
1-nitroso-2-naphthol + thiouracil	0	33	75	92	68 ± 3.3	8 ± 0.5	2 ± 0.6

Here, too, spraying did not prevent the outbreak of the disease, but it reduced its rate of development considerably. Consequently, the plants grew better than the control ones. There was a marked increase in the number of full-grown leaves, which seem to be of some inportance with regard to the industrial use of tobacco leaves. This can easily be explained. TMV causes top necrosis and dying off of the growing point. As, according to the preceding experiment, spraying delays the outbreak of the disease the plant reaches a later stage of development in the meantime. The total number of leaves increases somewhat and the number of leaves with visible symptoms decreases. Therefore, the consequences of the infection are less serious.

Surprisingly enough, these solutions, although they contained

thiouracil (if only 0.05 %), had no appreciable injurious effect on the tobacco varieties tested. It should be borne in mind, however, that in these experiments it is the production of ful-grown leaves that counts, whereas in the bean experiments the seed production was determined.

V. GENERAL DISCUSSION

1. Introduction

In the experiments described in Chapters III and IV the performance of several nitroso compounds and of thiouracil in reducing the harmful and growth-inhibiting effect of several virus infections was investigated. It appeared that 1-nitroso-2-naphthol had a positive, though not very considerable chemotherapeutic effect on the plants tested: sugar beet infected with yellow virus, bean species with the bean viruses. Other nitroso compounds (p-nitrosophenol, 1-nitroso-2-naphthol-3,6-disulphonic acid (Na salt) and the copper sulphate complex of o-nitroso-p-chlorophenol) also proved to have a favourable effect. Thiouracil was too toxic to be of any value. It could be demonstrated, however, that solutions containing thiouracil and p-nitrosophenol, in which the concentration of each of these substances was so low (0.05 %) as to have only a slight influence on the development of the virus, brought about a considerable increase in incubation period and growth of *Nicotiana rustica* and *Nicotiana tabacum* var. no. 11 plants infected with TMV. In addition, these solutions were not noticeably harmful to these plants.

Unfortunately, field experiments on a scale large enough to warrant significant results have only been carried out with sugar beet plants infected with yellow virus and sprayed with 1-nitroso-2-naphthol (K salt). These experiments showed a small, but positive chemotherapeutic effect of the compound. Small-scale field experiments on TMV-infected tobacco species with solutions of thiouracil and a nitroso compound also resulted in a significant positive effect.

It could be demonstrated that 1-nitroso-2-naphthol and p-nitrosophenol have no perceptible influence on the virus in vitro. Equal volumes of 0.2 % TMV and of 0.2 % of one of the nitroso compounds investigated were mixed and kept at room temperature (18° C) for different times, varying from 2–24 hours. At the end of these periods five tobacco plants were inoculated with the mixture in the ordinary way. Control plants were treated in the same way with a TMV solution of the same concentration, without a nitroso compound being added. No differences in incubation time and severity of attack by the disease were found between the plants inoculated with these mixtures and the control plants. It would have been better if the tests had been done with a series of dilutions of the mixtures, because only with the smallest amount of virus capable of producing infection one can really prove that the nitroso compounds have no influence on the virus in vitro. Nevertheless these experiments gave strong indications that the virus is not inactivated, the more so as it must be

expected that the concentration of these nitroso compounds in the plant cells is much less than in the solutions tested. Moreover, electron microscopic examination of the virus particles with the aid of the shadow casting technique with Pd did not substantiate the assumption of a reaction in vitro: virus particles remaining in the nitroso solutions did not differ in any respect from those of the untreated virus, a particular point being that the virus particles were not disintegrated.

2. Effect of nitroso compounds on the virus alone

The experiments described in Section V.1 have shown that 1-nitroso-2-naphthol and p-nitrosophenol have no perceptible influence on the virus in vitro. It must be assumed, therefore, that the compounds tested act inside the plant cells. Here they can either inhibit the virus production or stimulate the plant to destroy the virus particles by producing "antigenic substances". The latter hypothesis was not investigated as it would have required extracting these antigenic substances and testing them for antivirus activity.

The virus propagation is different according as the nitroso compounds are applied to the plant before, simultaneously with or after the virus infection. Application of the chemotherapeutic compounds several days before the incubation date has no effect; the compound has already been inactivated. Therefore the application of the chemotherapeutic compound was standardized: in all the experiments the plants were sprayed 2 days before infection. During this time the chemical has ample opportunity to penetrate into the plant.

Spraying three or more days after infection with p-nitrosophenol or with 1-nitroso-2-naphthol was, in most cases, ineffective. With *Nicotiana rustica* and often with White Burley, spraying one or two days after inoculation still produced a chemotherapeutic effect. These results suggest that the incubation time of TMV in *Nicotiana rustica* only increases under the influence of the antivirus factor, when the virus propagation has not yet reached its optimum rate and the translocation of the virus from the inoculated leaf towards the top has only just started.

No anti-virus effect can be expected when the concentration of the spraying solution is too low. On sugar beet plants 0.1 % solutions of p-nitrosophenol or 1-nitroso-2-naphthol had a marked inhibiting effect on the virus multiplication; plants sprayed with 0.05 % solutions gave a lower yield than those treated with 0.1 % solutions. These results could be confirmed serologically with 1-nitroso-2-naphthol treated plants. Therefore, as a rule 0.1 % was used, the more so as concentrations exceeding 0.1 % often led to phytotoxicity in field experiments. From the experiments described on page 394 and recorded in Table VIII it can be derived that for these compounds the highest permissible concentration at which no phytotoxicity occurs is nearly 0.2–0.25 %. Here a difference in behaviour between plants grown in the greenhouse and in the field manifests itself. Serological tests demonstrated a reduction in virus content of greenhouse and field

plants treated with 0.15 and 0.2 % solutions. This reduction proved to be at least similar to that obtained with a 0.1 % solution.

Application of too much of the chemical by spraying too frequently (6–8 times) is often harmful; it was not found to decrease the virus content perceptibly.

During the incubation time the rate of virus production in White Burley tobacco plants treated with 1-nitroso-2-naphthol drops considerably. It was determined serologically that this compound may reduce the virus content to $^1/_2$–$^1/_{64}$ of the amount in treated control plants. Complete destruction of the virus hardly ever occurred, so that the visible symptoms of the disease still appeared, though the incubation time had increased, sometimes by as much as 10 days. But even when these symptoms are perceptible the virus content is still lower than in the untreated plants. A similar effect can be expected on *Nicotiana rustica* and sugar beet.

It is a well-known fact that few virus particles suffice to infect a plant systemically. However, infecting with a small number of virus particles cannot be done, because only few virus particles of an inoculum "strike", as is known from the experiments with the local lesion method on *Nicotiana glutinosa*. Therefore, in the greenhouse experiments and in the field experiments with artificial infection the quantities of infecting material used were on the safe side to warrant infection (see II.4), thus always a hundred to a thousand times larger than the required minimum. The practice to use the appearance of the first visible symptoms as a yardstick for anti-virus activity is, therefore, not quite reliable. It may be expected that often many virus particles are destroyed by a chemical, but the few that remain will cause the plant to become diseased all the same.

Some field experiments were performed with diseased sugar beet stecklings. Satisfactory results were obtained with 1-nitroso-2-naphthol (K salt) and with p-nitrosophenol when the plants were only mildly infected. Severely attacked stecklings, however, did not respond to treatment with these compounds (see Tables XI and XII).

Another condition that must be fulfilled for a positive effect to be obtained is that the plants are young and growing at optimum rate. In spring the increase in incubation time due to treatment with nitroso derivatives is greater than in late summer and in autumn under otherwise similar conditions (see Table XIII). This must be attributed to the plants growing under sub-optimum conditions in the latter case. A similar effect is observed with sugar beet and the two bean species.

The climatological conditions also have some, though usually only little, influence; the differences observed are to be regarded as secondary consequences of the changing growth rates of the plants.

The results of spraying with chemotherapeutic compounds were derived from yield analyses, from observations on the moment of appearance and the development of the visible symptoms of the disease, and from serological determinations of the virus content of the plants. The last two methods are direct ones. In addition, the

influence of the compounds on virus development was also traced with the aid of the following indirect methods for determining the virus activity: the reducing sugar test, the reso blue test, the 2, 3, 5-triphenyl tetrazolium chloride (TTC) test and the respiration test. The variability of all these tests, however, proved to be so great that no reliable results could be obtained. These tests, particularly the reducing sugar test, did demonstrate the presence of virus in the infected plants, often during the incubation period already, but on account of the large spread in results the improvement caused by treatment with 1-nitroso-2-naphthol could not be proved significantly. The TTC, the reso blue and the respiration test were so insensitive that during the incubation time not even differences between healthy and infected plants could be demonstrated significantly. Under these circumstances it was of no avail to use these methods to investigate the effect of the nitroso compounds on the test plants.

3. Stability of the chemical in the plant

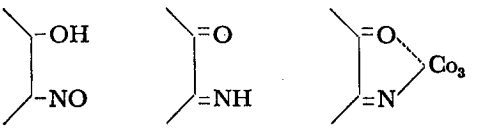
It is important to investigate whether the nitroso compounds remain unchanged in the plant tissues or whether they are decomposed. Therefore microchemical tests were performed with treated tobacco, sugar beet and even tulip plants. They have been borrowed from Feigl (1956); four of them proved to give reliable results.

Briefly, the procedure of these tests, performed with chemical but without plants, is as follows:

(a) A few milligrams of the powdered 1-nitroso-2-naphthol is melted in a microcrucible along with some milligrams of phenol. After cooling, a drop of concentrated sulphuric acid is added. The liquid turns dark chery-red. After diluting with water, the solution is made alkaline with a few drops of 4 N sodium hydroxide. It then turns deep blue (Liebermann test). By means of this test 0.4 μ grams of p-nitrosophenol or 0.5 μ grams of 1-nitroso-2-naphthol can be identified.

Generally aliphatic nitroso and iso-nitroso compounds and aromatic nitroso compounds give this reaction. The sulphuric acid probably saponifies the nitroso compounds; the nitrous acid produced nitrosates the phenol in the unoccupied p-position.

(b) A drop of 0.1 % cobalt nitrate solution is added to about 0.1–1.0 ml of 0.1 % 1-nitroso-2-naphthol. A positive response is signalled by a brown-red colour, which is resistant to 2 N sulphuric acid. In this reaction the o-nitrosophenols (I) are in equilibrium with their tautomeric o-quinone oxime forms (II), which combine with trivalent cobalt ions containing this metal in the chelate ring (III):

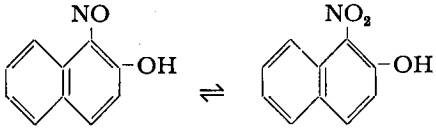


I II III

The test reveals 1 μ gram of 1-nitroso-2-naphthol and 5 μ grams of 1-nitroso-2-naphthol-3,6-disulphonic acid (Na salt).

(c) A quantity of 0.1–1.0 ml of 0.1 % 1-nitroso-2-naphthol is mixed in a test tube with a drop of an aqueous tyrosine solution of about 100 ppm and heated.

A drop of concentrated nitric acid is added to the hot solution. A bright purple colour appears which gradually fades. The chemistry of this reaction is unknown. An essential step seems to be that the nitric acid apparently oxidizes the 1-nitroso-2-naphthol (I) to 1-nitro-2-naphthol (II), and this reaction product reacts with tyrosine.



I II

Limit of identification: 0.5–1.0 μ grams of 1-nitroso-2-naphthol. This test was described by FEIGL as a tyrosine test.

(d) A drop of 1-nitroso-2-naphthol (0.1 %) or a few milligrams of this chemical is mixed with 1 or 2 millilitres of a freshly prepared 1 % solution of sodium pentacyanoammine ferroate (prussic salt). After about 5 minutes an intense green colour is formed. With sodium pentacyanoaquo ferroate the same reaction takes place. This colour reaction depends on the exchange of the water or ammonia molecule of the prussic salt for a molecule of the nitroso compound:

$$Na_3[Fe(Cn)_5NH_3] + RNO \rightarrow NH_3 + Na_3[Fe(CN)_5RNO]$$
$$Na_3[Fe(CN)_5H_2O] + RNO \rightarrow H_2O + Na_3[Fe(CN)_5RNO]$$

It is noteworthy that the entrance of the RNO molecule into the inner sphere of the prussic salt, with expulsion of the NH_3 or H_2O molecule, occurs only in the light; it is a photoreaction. Limits of identification: 1 μ gram for 1-nitroso-2-naphthol and 0.15 μ grams for p-nitrosophenol.

These four tests, especially reactions (b) and (c) gave in vitro, good response. They could equally well be applied when the compounds were added to ground plant material immediately before the determination. Ground plant material in 1-nitroso-2-naphthol gave a weaker reaction than the corresponding control solution. Any substances interfering with the determinations were practically absent in the plant.

In plants sprayed with a 0.1 % solution of 1-nitroso-2-naphthol in the usual way (50 mg/$\frac{1}{2}$ sq. metre) a long time or just before they were examined by one of these tests, not a trace of this chemical could be detected; thus, less than 1μ gram was available in the solution of the tested plant material. As $\frac{1}{2}$ square metre is just covered by 18 plants, which should receive all 50 mg of the compound dissolved in 50 ml water containing acetone and wetting agent, one plant will have absorbed 3 mg = 3000 μ grams. It can hardly be imagined that 3000 times the amount of 1 μ gram is lost because it did not reach the plants or did not penetrate into them.

Since it is difficult to determine how much of the spraying substance is received by a plant, the dipping method was used in the further experiments. The volume of solution taken up by the plants is easier to determine. The plants to be tested were dipped with their roots or stems in a solution of 1-nitroso-2-naphthol (0.1–0.2 % w) for varying times (1-5 days). During this period relatively large amounts of the compound were absorbed: in 5 days about 5 ml or 5000 μ grams of

its solution per plant. The stem, also the large veins and even the smaller ones in the leaves were coloured dark brown. At the end of the experiment always one plant was rinsed with water and ground, the pulp being extracted with water or acetone. The total volume of the extracts was about 50 ml, which was tested. For each determination according to one of the four microchemical reactions 2–10 ml of the extracts were used. The reaction was invariably negative, like the reaction on the pulp residue. Thus, no nitroso compound could be detected. This proves that less than 1/5000 of the amount of chemical that has been absorbed remains as such in the plant.

Negative results were also obtained when after dipping the plant material was dried first or deep-frozen at the end of the experiment, or when the water or acetone extract was evaporated, preferably in vacuo, to a very small volume. These results can only be explained by assuming a rapid decomposition of the substance in the plant tissue.

The distribution of the brown colouring substance found in the stems and veins of the leaves was investigated microscopically. This substance could be followed over a great distance in the smaller veins. In the vessels the brown colour was darker than that of the nitroso compounds used. This substance was found exclusively in the xylem, expecially in the ring- and net vessels. It could not be established whether the phloem parts of the stele also contained this substance; the perecambium and the parenchym cells of the roots were free from it. It was important to determine whether the chemical reaches the cells of the inoculated leaves. The presence of the nitroso substance or its decomposition products in the leaf cells could not be demonstrated, either microscopically or microchemically.

It must thus be assumed that 1-nitroso-2-naphthol or its decomposition products exert an extremely strong inhibiting influence on the virus production, as the amount in the leaf cells must be very small. The second possible assumption, viz. that these compounds block the transport of the virus through the phloem, seems highly improbable, as in this tissue none of these substances could be detected.

4. Concluding remarks

These nitroso compounds, especially 1-nitroso-2-naphthol, greatly reduce the harmful effect of the virus infection.

Yet it is not fully established that they are antivirus factors, as it could not be demonstrated that they inactivate the virus either by chemical reaction or by interacting with the plant cell system producing the virus particle. As to thiouracil, it is often assumed that this compound interferes with the virus production through substituting the uracil. The effect of the nitroso-thiouracil mixtures as described on pp. 400 and 401 (Tables XIII and XIV) can be explained in the same way.

The activity of the nitroso compounds, however, is no less specific than that of thiouracil. An attractive supposition is that they act in a similar fashion and to speculate that a nitroso compound could react

with uracil, or any other indispensable virus component available in the plant cells, forming e.g. a nitroso-uracil compound. In this way either inactive virus particles containing these nitroso compounds instead of the indispensable components might be formed or the virus synthesis would be inhibited. As soon as the nitroso compound is entirely decomposed the uracil (or the other indispensable virus component) formed in the plant again becomes available for virus synthesis and the plant is attacked by the disease after all; only the incubation time has increased considerably. If this hypothesis should be true the nitroso compound would act as an antivirus factor. On the other hand, such a hypothesis would also explain the fact that the phytotoxic and antivirus activities of these compounds are almost the same, as the plant itself needs uracil as well.

Further investigations into the problem of antivirus factors would be desirable. To be of practical use the antivirus activity of such substances should be higher and they would have to be more specific than the nitroso compounds tested in this investigation.

SUMMARY

(1) The object of the search described in this publication was to find "inhibitors of virus increase", not "inhibitors of infection".

(2) The investigations were performed with the following plants and viruses: tobacco species with tobacco mosaic virus (TMV); the disease causes leaf damage; sugar beet with virus yellows; the disease decreases root production; French bean and broad bean with Phaseolus viruses 1 and 2; the disease reduces seed production.

The cultivation method, the age of the plants tested, the virus, its purification and properties, and the techniques applied to infect the plants are briefly described.

The virus influence was investigated by means of direct methods, such as serology, and indirect ones, such as a reducing sugar test according to VAN DUUREN (1955), a reso blue test, (MÜNSTER (1955), SCHUSTER (1956)), to determine the callus production in the phloem (these two for sugar beet only), and a test with 2,3,5-triphenyl tetrazolium chloride (TTC) (see F. E. SMITH, 1951, and BEAL *et al.*, 1955). All the indirect tests, as well as the various tests with Nicotiana glutinosa, proved to be of little value (Tables II, III and IV).

(3) The chemicals were applied via the leaves (spraying, preferably as a 0.1 % solution, at a rate of 100 ml per square metre) or via the roots (dipping in a 0.1 % solution, or nutrient technique, 0.001–0.01 %w added to 150–200 ml Knopp's solution).

Table V lists the chemicals with antivirus properties described in the literature. Some of the most important are discussed separately (thiouracil, 8-azaguanine, malachite green, growth regulators and inorganic salts). On the basis of these data several types of chemicals were investigated. The nitroso hydroxyaryl compounds displayed such an activity that closer examination was justified.

The antivirus activity of a chemical was expressed as the percentage increase in incubation time; for this purpose the exact moment at which the first visible symptoms of the disease appear must be determined.

p-Nitrosophenol, 1-nitroso-2-naphthol, and the copper sulphate complex of o-nitroso-p-chlorophenol proved very attractive, being not too phytotoxic (Tables VI, VII and VIII); the potassium salt of 1-nitroso-2-naphthol-3,6-disulphonic acid showed good activity, but also some phytotoxicity. Thiouracil and its mixtures with p-nitrosophenol and 1-nitroso-2-naphthol displayed a particularly good activity on tobacco without being phytotoxic in the concentrations used (0.05 % + 0.05 %; Table VIII).

(4) The four nitroso compounds mentioned were used in field experiments.

In some tentative trials on French bean and broad bean the antivirus properties of p-nitrosophenol and 1-nitroso-2-naphthol were again observed (Table x). Most experiments, however, were performed on sugar beet. Indications of antivirus activity were obtained in all cases. Taking all the results obtained in 4 years (56 experiments) together, an average improvement of 7 % ± 1½ as compared with untreated plants was obtained for 1-nitroso-2-naphthol, mostly applied as its potassium salt (Graph 1). The other nitroso derivatives mentioned did not show any significant antivirus activity. The nitroso-thiouracil mixtures had a very reliable activity on tobacco, but on other plants their phytotoxicity proved to be too high (Tables XIII and XIV).

(5) It was demonstrated that these nitroso compounds only have a good effect when they are applied in a 0.1 % concentration. Two or at most three sprayings are permissible. A higher concentration or more frequent spraying produces phytotoxic effects, often to such an extent that the resulting loss exceeds the improvement brought about by the antivirus compound.

The influence of the chemical could be demonstrated with serological tests, but not with the other tests mentioned under (2). The chemical itself is decomposed in the plant. It may be that it reacts with uracil, just as thiouracil does, or with any other indispensable virus component available in the plant cells. It can thus be expected that there is not enough uracil left for the virus synthesis in the cells. As soon as the nitroso compound is entirely decomposed the uracil formed in the plant again becomes available for virus synthesis and the plant is attacked by the disease after all; only the incubation time has increased considerably. If this hypothesis should be true the nitroso compound would act as an antivirus factor. On the other hand, such a hypothesis would also explain the fact that the phytotoxic and antivirus activities of these compounds are almost the same, as the plant itself needs uracil as well.

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