EXPERIENTIA

Vol. XVI – Fasc. 11 Pag. 473–520 15. XI. 1960

Chemical Modifications of Virus infectivity: Reactions of Tobacco Mosaic Virus and its Nucleic Acid

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In many discoveries concerning viruses in general, the tobacco mosaic virus has been a forerunner. It was the first virus ever to be crystallized (STANLEY[2]), but later the crystallization of many other viruses, including animal viruses such as poliomyelitis and Coxsackie virus, have also been achieved (SCHAFFER and SCHWERDT[3], MATTERN[4]). The tobacco mosaic virus was furthermore the first virus whose composition of two components, namely its protein and its ribonucleic acid, has been demonstrated (BAWDEN and PIRIE[5], STANLEY[6]) while later it was recognized that many viruses are built of the same two constituents. And, furthermore, it was the nucleic acid of the tobacco mosaic virus which was first found to be infective. This discovery has to be attributed to GIERER and SCHRAMM[7], as well as to FRAENKEL-CONRAT[8], who were able to demonstrate that the tobacco mosaic virus can be split into its two components and that the isolated ribonucleic acid still carries the infectivity. Many other infective ribonucleic acids from a variety of plant and animal viruses have since been found (COLTER[9]) and it seems a fairly well established fact now that the ribonucleic acids represent the infective part of viruses in general.

The tobacco mosaic virus, therefore, seems to be representative of many viruses. Since it is the virus whose chemical structure is best known, and since it can be obtained in large quantities and is absolutely harmless for the experimentator, it might be justified to use it as a model in order to study some characteristic reactions of viruses in general. In the present study it was used to obtain information on the mechanism of some of the chemical processes which lead to an alteration of the biological properties of viruses.

Structure of the tobacco mosaic virus. The tobacco mosaic virus is a rod about 300 μm long and 15 μm wide (Fig. 1). It grows in such large quantities in infected tobacco plants that over 1 g of pure virus can be obtained from 1 l of sap from infected leaves. It can easily be purified by differential centrifugation from dilute salt solutions. It can also be crystallized by the addition of ammonium sulfate. It is very stable in solution and can be kept for years.

Chemically the tobacco mosaic virus (TMV) consists of about 95% protein and 5% ribonucleic acid. The protein part is made up of a large number of probably identical subunits with a molecular weight of about 18000. Since the TMV particle has a molecular weight of around 4×10^7, there are about 2000 protein subunits present in each virus particle. The nucleic acid molecule, by contrast, is much larger than the protein subunits. GIERER[10] and BOEDTKER[11] have presented physico-chemical and biological evidence indicating that the nucleic acid has a molecular weight of around 2×10^6. This would mean that the whole nucleic acid of a virus particle is contained in one single molecule which consists of about 6000 nucleotides.

The existence of a single polynucleotide chain of 6000 units per particle is not a particular feature of the tobacco mosaic virus but of a large number of viruses. FRISCH-NIGGEMEYER[12] has determined the absolute amount of ribonucleic acid in a variety of viruses of different size and shape and has found it to be a constant value of about 2×10^6, whether the whole virus has

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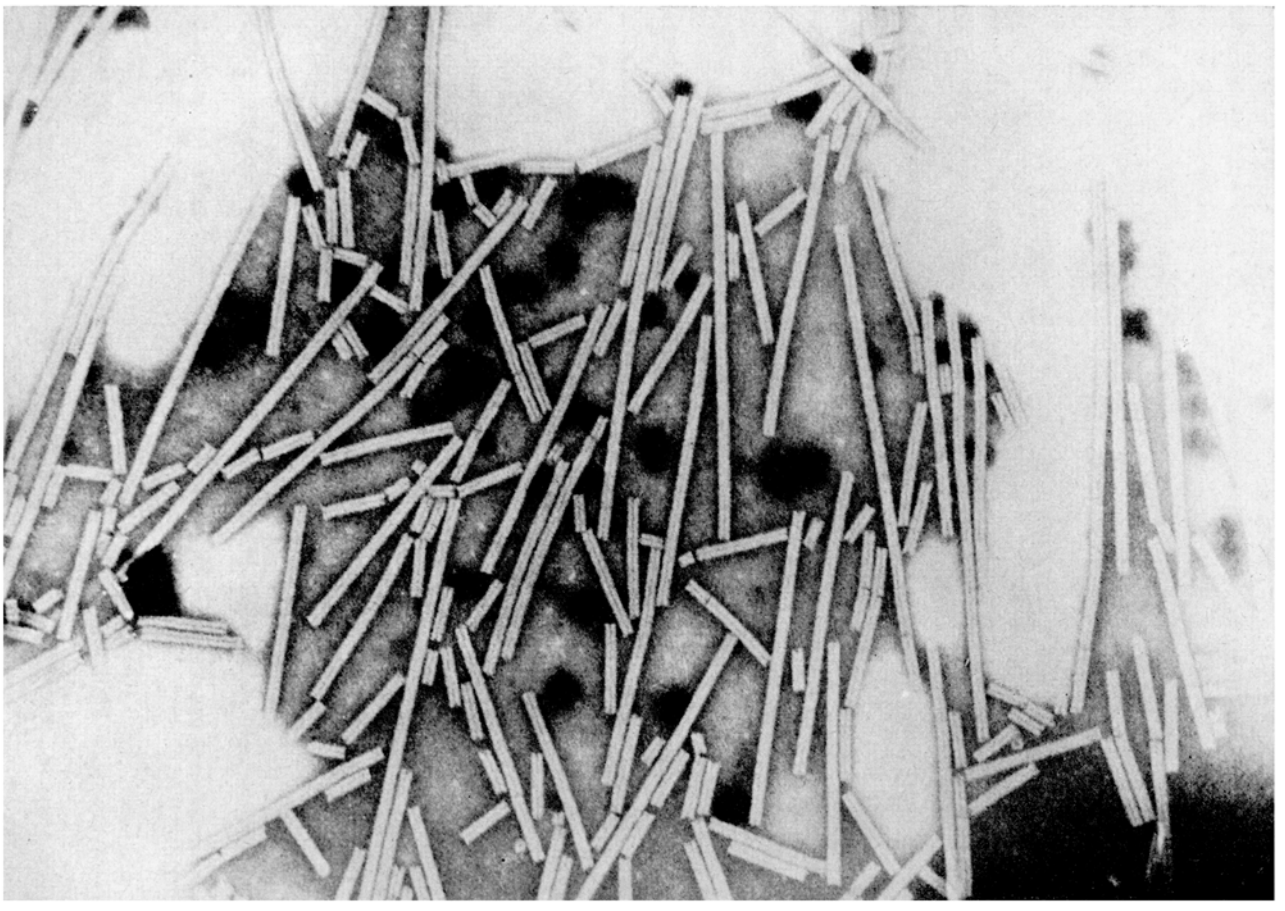

Fig. 1. Electron micrograph of tobacco mosaic virus (Electron micrograph by G. J. HILLS, courtesy of Dr. K. M. SMITH, Cambridge).

a total molecular weight of only 6 millions like the poliomyelitis virus or of 300 millions like the influenza virus. It should be noted, furthermore, that ribonucleoprotein particles with a similar amount of nucleic acid have also been found in the microsomes of uninfected cells (CHENG[13]). This nucleic acid content of 2×10^6, although present in many viruses, therefore does not seem to be a unique property of viruses.

The X-ray crystallographic studies of FRANKLIN, KLUG *et al.* (FRANKLIN[14], FRANKLIN and HOLMES[15], FRANKLIN and KLUG[16]) have made it possible to design a model showing the way in which the different constituents of the virus are linked together (Fig. 2). From this it appears that the nucleic acid lies like a helical coil inside the virus with an empty space in the center, whereas the protein subunits form a cover around the nucleic acid.

Electron micrographs of tobacco mosaic virus treated in several ways seem to confirm this structure. The existence of the hollow space inside has been proved by the fact that it can be filled with a contrast medium (HUXLEY[17]). Figure 1 shows a virus preparation treated this way where every virus particle shows a dark area in the center due to the presence of the contrast medium.

The protein can also be removed by various chemical treatments such as weak alkaline solution (SCHRAMM *et al.*[18]) or sodium dodecyl sulfate (HART[19]). If this treatment is carried out very briefly the protein is only partially removed and various intact parts of the virus appear to be linked only by a thin thread in the center which corresponds to the nucleic acid (Fig. 3). In the absence of the protein coat, the virus loses its rigidity and becomes flexible at the places where the protein has been removed (Fig. 4).

NIXON and WOODS[20], furthermore, were able to demonstrate by electron microscopy the helical structure of the protein subunits as portrayed by FRANKLIN *et al.* by their x-ray studies.

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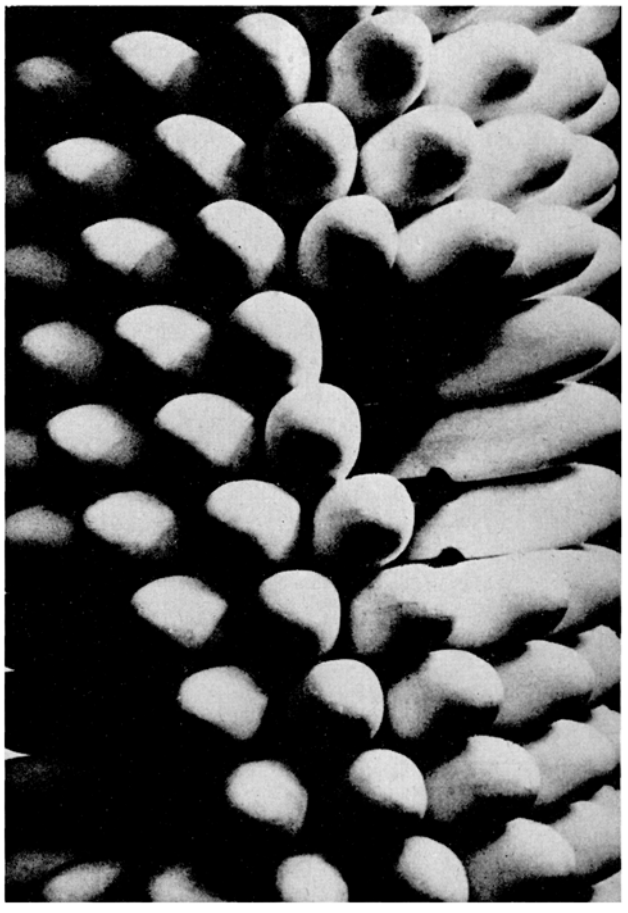
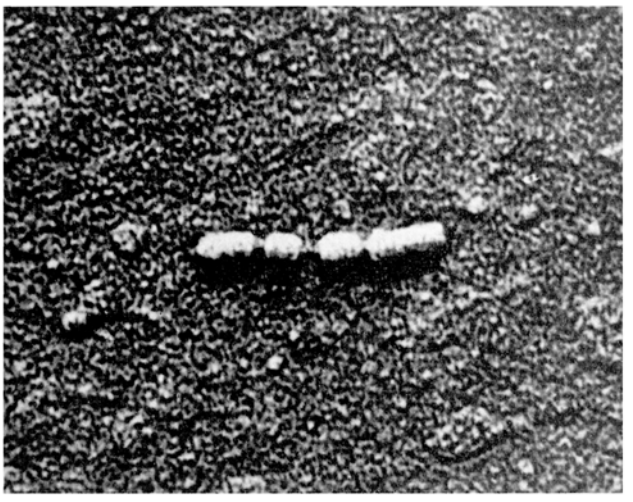


Fig. 2. Model of tobacco mosaic virus (FRANKLIN *et al.*[21] 1956).



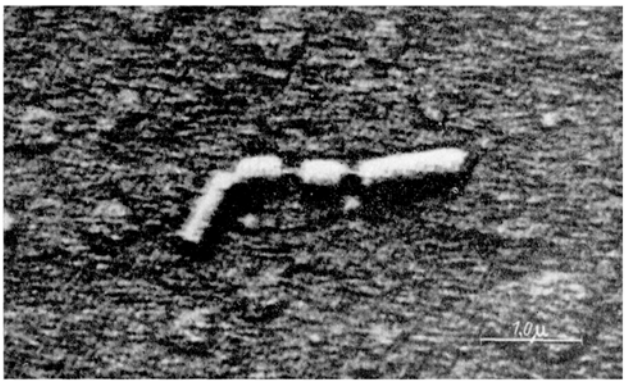


Fig. 3 and 4. Tobacco mosaic virus after partial removal of its protein at pH 10·3. (Courtesy of Prof. G. SCHRAMM, Tübingen.

Pathological changes caused by TMV. The tobacco mosaic virus causes two kinds of pathological changes according to the nature of the host. Some species of plants, such as *Nicotiana glutinosa*, react by the formation of a number of local lesions on each infected leaf. These local lesions appear after a few days and expand only very slowly (Fig. 5). The number of lesions is dependent on the concentration of the virus in the solution which has been rubbed on the leaf. The infectivity of a virus preparation or a virus nucleic acid can, therefore, be measured by the number of necroses which appear on a number of half leaves after infection as compared to the number of necroses caused by a control solution of known virus concentration.

In contrast, in some other varieties of tobacco plants, such as the 'Turkish tobacco', *Nicotiana tabacum*, the infection spreads rapidly over the whole plant causing a systemic infection. The leaves show diffuse discolorations rather than local lesions (Fig. 6). These so-called systemic hosts are preferentially used for the preparation of large quantities of virus. They can also be used to study the time-course of virus production by measuring the virus present in identical leaf disks at various time intervals.

Infectivity of tobacco mosaic virus nucleic acid. It was found by GIERER and SCHRAMM[7], as well as by FRAENKEL-CONRAT[8], that the application of the isolated ribonucleic acid on leaves of local lesion hosts does also result in the formation of local necroses and causes the formation of new virus. Not only the intact virus but also its isolated nucleic acid are therefore capable of initiating a viral infection.

Since then, the infectivity of many other virus nucleic acids has been described (COLTER[9]). Even though the preparations of many animal viruses were not quite pure, the residual activity after phenol extraction was sensitive to ribonuclease which indicated that it was due to the free virus nucleic acid. Infectious nucleic acid preparations have also been isolated from three other purified plant viruses (KAPER and STEERE[22], RUSHIZKY and KNIGHT[23]).

The infectivity of the isolated nucleic acid from tobacco mosaic virus is quite appreciably lower than the

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[22] J. M. KAPER and R. L. STEERE, Virology *7*, 127 (1958), *8*, 527 (1959).

[23] G. W. RUSHIZKY and C. A. KNIGHT, Virology *8*, 448 (1959).

infectivity of the intact tobacco mosaic virus. This appears especially if the comparison is not carried out on a weight basis, but if identical amounts of free nucleic acid and of nucleic acid in the intact virus are compared. The activity of free nucleic acid compared with that of an intact virus containing the same amount of ribonucleic acid ranges from 0·1–2%. This lower infectivity does not appear to represent a lower intrinsic capacity of the nucleic acid to cause an infection but is rather due to the fact that it is much more accessible to destruction by cellular enzymes than the nucleic acid inside the virus which is protected by its protein coat.

Highly purified infectious nucleic acid preparations have a protein content of less than 0·2% (RAMACHANDRAN and FRAENKEL-CONRAT[24]). The infectivity therefore seems to lie entirely in the nucleic acid. Whether there is a small peptide attached to the nucleic acid which might be of some importance, cannot, however, be excluded with certainty. An interesting biological difference in the infectivity of the free nucleic acid and the intact virus has been described by SCHRAMM and ENGLER[25] and by FRAENKEL-CONRAT *et al.*[26]. After infection with the free nucleic acid, there is a shorter latent period until new virus is produced and until the necroses appear. The free nucleic acid, therefore, seems to initiate an infection faster than the intact virus.

Splitting the virus. The protein and the nucleic acid of the virus can be separated in various ways:

a) Treatment with sodium dodecyl sulfate: By adding about 1% sodium dodecyl sulfate to a virus solution at a slightly alkaline or acid pH the two components separate. By adding ammonium sulfate to a concentration of 33% the protein is precipitated already at room temperature and can be removed by centrifugation, whereas the nucleic acid precipitates only later upon standing in the cold (FRAENKEL-CONRAT *et al.*[27]).

b) Phenol extraction: If phenol saturated with water is added to a virus solution with rapid stirring an emulsion is formed. Upon centrifugation the protein is dissolved in the phenol layer or settles as a layer between the two phases, whereas the nucleic acid remains in the water phase (SCHUSTER, SCHRAMM and ZILLIG[28]).

c) Upon short heating of a virus in a solution of salts, the protein denatures and can be removed by centrifugation, whereas the nucleic acid remains in solution (COHEN and STANLEY[29], KNIGHT[30]).

d) Acetic acid: If two volumes of glacial acetic acid are added to a virus solution, in contrast to the previous methods, the protein stays in solution and the nucleic acid is precipitated (FRAENKEL-CONRAT[31]).

Only the first two methods have provided infectious nucleic acids from tobacco mosaic virus. Heating has been successful in the preparation of an infectious ribonucleic acid from other plant viruses (KAPER and STEERE[22]). The last method does not give an infectious nucleic acid but the protein seems to be particularly well preserved and native.

Chemical modifications of the infectivity of tobacco mosaic virus nucleic acid. Chemical alterations in the virus nucleic acid can be induced in two ways, either by mo-

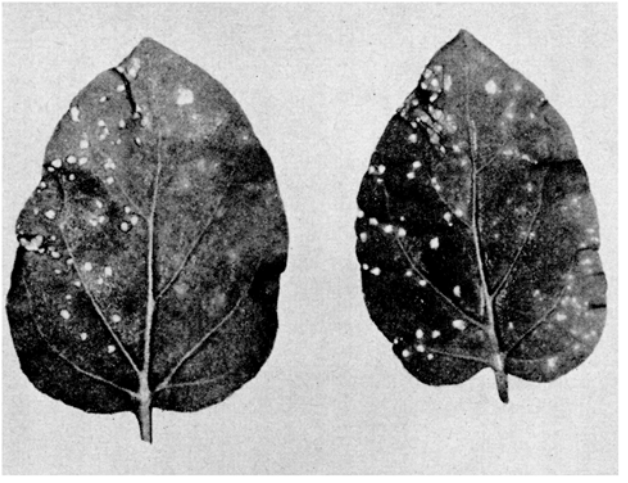

Fig. 5. Tobacco leaves infected with TMV, local lesion host (*Nicotiana glutinosa*). Courtesy of Dr. K. M. SMITH, Cambridge.

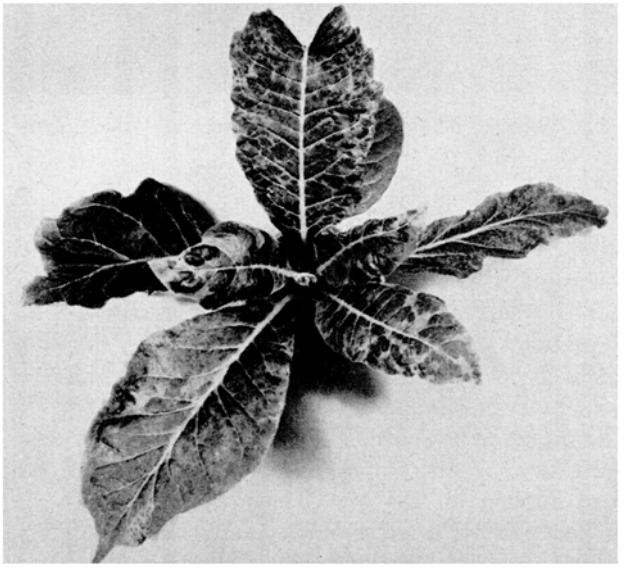

Fig. 6. Tobacco plant infected with TMV, systemic host (*Nicotiana tabacum*). Courtesy of Dr. K. M. SMITH, Cambridge.

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[31] H. FRAENKEL-CONRAT, Virology *4*, 1 (1959).

difying the nucleic acid acid after it has been formed or by incorporating an unnatural base into the nucleic acid while the virus is growing. The first way is the general mechanism for the inactivation of a virus, whereas the second is usually encountered upon the addition of structural analogs to infected plants. In the following text, examples will be presented for both these mechanisms of chemical modifications which change the infectivity of tobacco mosaic virus.

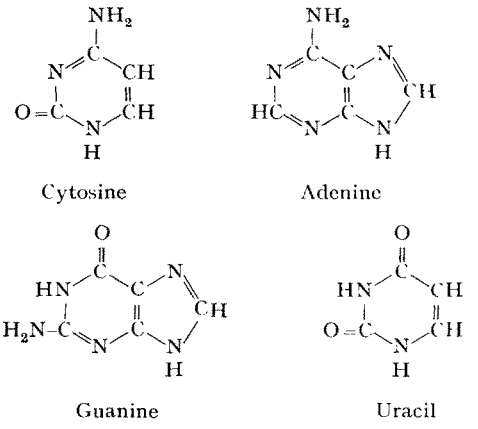
Reaction with formaldehyde. One of the chemicals used most widely for the inactivation of viruses is formaldehyde. It has found extensive application in the preparation of various vaccines, e.g. the Salk vaccine against poliomyelitis virus. Formaldehyde is a very reactive substance with a particular tendency towards polymerization. It has also been widely used in the tanning of skins, and the chemical reactions between formaldehyde and proteins have been extensively studied (FRENCH and EDSALL[32]). Especially the studies of FRAENKEL-CONRAT *et al.*[33]) have provided evidence for a two step reaction. In a first step formaldehyde reacts with an amino group in the protein molecule to form a methylol derivative (Reaction I). In a second step the hydroxyl group of this methylol derivative reacts with another amino group or any active hydrogen atom and thus forms a methylene bridge (Reaction II).

$$\text{I}\quad R\text{-}NH_2 + HCHO \rightleftharpoons R\text{-}NH\text{-}CH_2OH$$

$$\text{II}\quad R\text{-}NH\text{-}CH_2OH + H_2N\text{-}R \longrightarrow R\text{-}NH\text{-}CH_2\text{-}NH\text{-}R$$

Reactions of this kind are certainly encountered when formaldehyde is allowed to act upon a virus. It is doubtful, however, whether it is the reaction with the virus protein which leads to inactivation, since there are a number of very active protein reagents which, although they react with the virus, do not cause inactivation. It seems not unlikely, therefore, that the principal action of formaldehyde might be its chemical reactivity towards the nucleic acid rather than towards the protein.

FRAENKEL-CONRAT[34] has demonstrated that formaldehyde causes very marked changes in the ultraviolet spectrum of ribonucleic acids. Using formaldehyde labelled with C^{14}, it has been possible to study quantitatively the reaction of formaldehyde with ribonucleic acid (STAEHELIN[35]). Figure 7 shows schematically the linking of a few nucleotides as it occurs in ribonucleic acids in which, however, many hundreds of nucleotides are linked in this manner. The data of GIERER[10] and BOEDTKER[11] actually suggest that the ribonucleic acid of tobacco mosaic virus has a molecular weight of about 2×10^6 and consists of about 6000 nucleotides linked together in one chain. The only groups capable of reacting with formaldehyde are the amino groups of the bases, i.e. of adenine, guanine and cytosine since uracil does not carry an amino group.



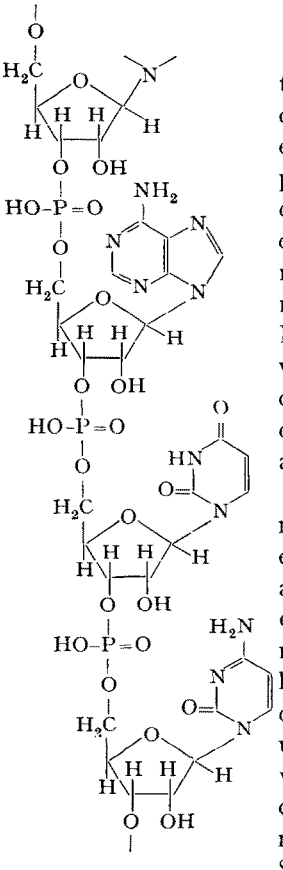


Fig. 7. Linking of nucleotides in ribonucleic acids.

This was demonstrated by the use of synthetic polynucleotides which are formed by the enzyme polynucleotide phosphorylase (GRUNBERG-MANAGO *et al.*[36]) and which contain only one base. Whereas polynucleotides containing adenine and cytosine (Poly A and Poly C) react very readily with formaldehyde, polynucleotides containing only uracil (Poly U) do not react at all (STAEHELIN[35]).

Intact ribonucleic acids also react accordingly. In order to express the extent of the reaction quantitatively, the binding of formaldehyde to the ribonucleic acid has been related to the number of nucleotides. It can be seen in Figure 8 that the reaction stops when about 5·5–6% formaldehyde have been bound to the nucleic acid on a weight basis. Since the molecular weight of formaldehyde is less than one tenth of that of an average nucleotide, it can be said on a molar basis that somewhere between 60 and 70 molecules of formaldehyde have reacted with about 100 nucleotides. According to the base composition of the tobacco mosaic virus nucleic acid, the bases which carry an amino group, i.e. adenine, guanine and cytosine, correspond

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[33] H. FRAENKEL-CONRAT and H. S. OLCOTT, J. Amer. chem. Soc. *70*, 2673 (1948).

[34] H. FRAENKEL-CONRAT, Biochim. biophys. Acta *15*, 307 (1954).

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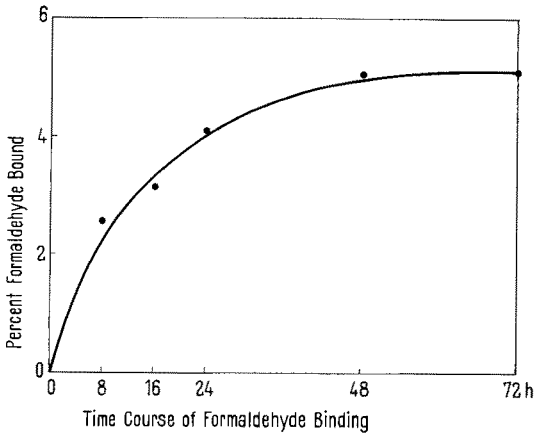


Fig. 8. Reaction of formaldehyde with nucleic acid from tobacco mosaic virus. The reaction was carried out according to STAEHELIN[35] (1958) with formaldehyde C^{14} and the amount of formaldehyde bound expressed in mg of formaldehyde/100 mg of nucleic acid.

Tab. I. Inactivation of Nucleic Acid[a] (STAEHELIN[41])

Inactivating agent	Concentration %	Activity %
Formaldehyde	0·1	0
	0·05	4
	0·025	34
Glyoxal	0·1	6
	0·05	23
	0·025	81
	0·0125	100
Kethoxal	0·02	1
	0·01	8
	0·005	32
	0·0025	73

[a] TMV-RNA (1 mg/ml) was incubated as in Table I for 30 min at 23°. After the reaction the nucleic acid was precipitated 6 times with alcohol to remove all free inhibitor and then assayed.

Tab. II. Inactivation of Intact TMV[a] (STAEHELIN[41])

Inactivating agent	Concentration %	Temperature	Time h	Activity % of original
Formaldehyde	1	23°	8	41
			16	33
			24	9
Glyoxal	2	23°	8	52
			16	19
			24	11
Kethoxal	2	23°	8	60
(β-ethoxy-α-keto-butyraldehyde)			16	63
			24	57
	4	23°	8	81
			16	53
			24	21
	4	37°	8	31
			16	29
			24	20

[a] TMV (1 mg/ml) was incubated at room temperature in 0·001 M phosphate buffer pH 6·8 with the concentrations of inhibitors and for the times indicated. After the reaction the solutions were dialyzed overnight against the same buffer in the cold and assayed.

to about 71% of the total bases. The binding of 60–70 molecules of formaldehyde per 100 nucleotides indicates that almost every amino group has reacted with one molecule of formaldehyde, probably in a manner very similar to the mechanism described for proteins (Reactions I and II).

In the presence of higher salt concentrations, or already of low concentrations of divalent kations, the reaction is less extensive because a great number of the amino groups have become unreactive, probably through the formation of hydrogen bonds between the bases (STAEHELIN[37]).

The reaction proceeds very slowly at low formaldehyde concentrations (Fig. 8). Inactivation of the nucleic acid, however, occurs very fast and the infectivity of the free nucleic acid is lost when only a few molecules of formaldehyde have reacted with the many thousand amino groups present in the nucleic acid (Table I). This great sensitivity of the isolated ribonucleic acid is in contrast to the relative resistance of the intact virus towards formaldehyde treatment. Tobacco mosaic virus is inactivated only at about 100 times greater concentrations of formaldehyde and after longer times of treatment (Table II). This seems rather surprising since the virus contains the nucleic acid as its active component. One has to assume, therefore, that the amino groups of the ribonucleic acid are not accessible in the intact virus. This inaccessibility could be brought about in two ways:

a) The amino groups could be involved in strong hydrogen bonding in the intact virus, as in deoxyribonucleic acid, which also does not react readily with formaldehyde, or

b) The presence of the protein coat surrounding the nucleic acid could prevent access of the formaldehyde to the nucleic acid.

There is good evidence in favour of the second mechanism, and, especially by comparing the effect of various inactivating chemicals with similar properties but of different size, it was possible to show the importance of the problem of penetration into the virus. This will be shown in the following experiments.

Reaction with glyoxal derivatives. Glyoxal derivatives have been shown by various investigators to inactivate viruses *in vitro* (DE BOCK *et al.*[38], MCLIMANS *et al.*[39]). A few have also had a chemoprophylactic effect on virus infections in eggs. Lately some glyoxal derivatives have been reported to have chemoprophylactic activity in animals (CAVALLINI *et al.*[40]).

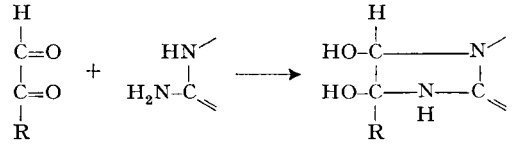
[37] M. STAEHELIN, Exper. *15*, 413 (1959 a).

[38] C. A. DE BOCK, J. BRUG, and J. N. WALOP, Nature, Lond. *179*, 706 (1957).

[39] W. F. MCLIMANS, G. E. UNDERWOOD, E. A. SLATER, E. V. DAVIS, and R. A. SIEMS, J. Immunol. *78*, 104 (1957).

[40] G. CAVALLINI and E. MASSARANI, J. med. pharm. Chem. *1*, 365 (1959). – G. CAVALLINI, E. MASSARANI, D. NARDI, F. MAGRASSI, P. ALTUCCI, G. LORENZUTTI, and U. SAPIO, J. med. pharm. Chem. *1*, 601 (1959).

Glyoxal derivatives react with nucleic acids in a way quite different from formaldehyde. In contrast to the marked shift of the maximum to higher wave lenghts and the general increase in the height of the absorption maximum caused by formaldehyde, there is only a very slight shift of the maximum in the opposite direction, i. e. towards shorter wave lengths, and no increase in the absorption upon treatment with glyoxal derivatives (STAEHELIN[41]). Formaldehyde causes changes in the ultraviolet spectrum similar to those with nucleic acids also with free nucleotides, i.e. adenylic, guanylic, and cytidylic acids, but glyoxal derivatives only change the spectrum of one nucleotide, namely guanylic acid. By using a radioactive glyoxal derivative, it can also be shown that only guanylic acid out of all four nucleotides reacts. Figure 9 shows the electrophoretic separation of a mixture of four nucleotides after it has reacted with a radiaoctive glyoxal derivative (Kethoxal). From the radio-autogram to the left, it can be seen that only guanylic acid has become labelled but that the reaction with this nucleotide has been complete, since after the reaction the entire guanylic acid band, as seen in the ultraviolet light, migrates with a velocity corresponding to the reaction product with the glyoxal derivative rather than to free guanylic acid. Since glyoxal derivatives react with all compounds containing the diamine structure shown below, it appears most likely that they react with guanine in the following manner under the formation of a stable 5-membered ring (Reaction III).



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This reaction takes place much more readily and much faster than the formaldehyde reaction. Accordingly, the inactivation of the isolated ribonucleic acid from tobacco mosaic virus occurs much faster, especially by stable glyoxal derivatives such as Kethoxal (β-ethoxy-α-ketobutyraldehyde), than by formaldehyde. Glyoxal itself is less active because of its marked instability (Tab. II).

Interestingly, towards the intact virus Kethoxal is much less active than glyoxal, whereas formaldehyde is the most active compound of all (Table I). The order of activity towards the intact virus, therefore, follows the size of the compounds, the activity being greater the smaller the compound. This is probably most noteworthy in the case of glyoxal and Kethoxal, which have the same mechanism of action but whose order of activity is reversed according to whether they are assayed on the free nucleic acid or on the intact virus.

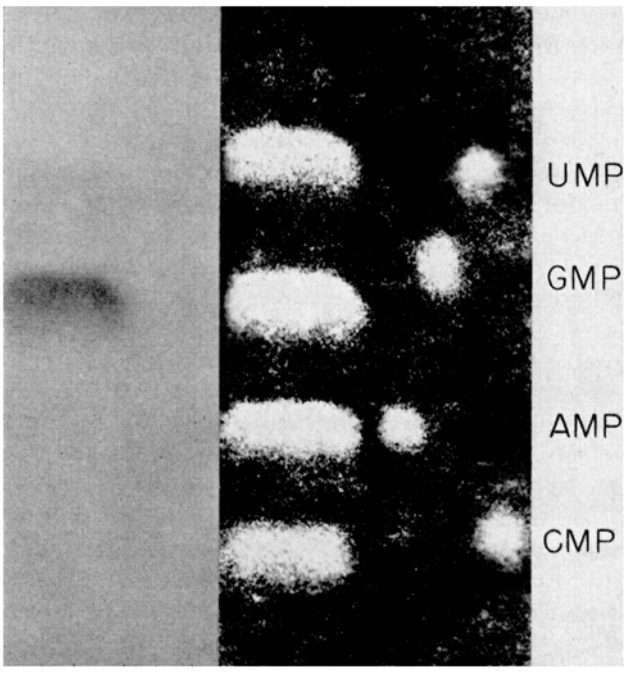


Fig. 9. Reaction of nucleotides with Kethoxal-C^{14}. Adenylic, guanylic, cytidylic and uridylic acids (all 0·005 M) were incubated in 0·01 M phosphate buffer pH 7 with 1% Kethoxal-C^{14} for 16 h at room temperature. The mixture was then separated by paper electrophoresis at pH 3·5 in 0·05 M ammonium formate. The ultraviolet absorption of the four separated bands is shown on the right hand side together with the four markers. The radioautogram of the same experiment is shown on the left hand side.

It appears that towards the free nucleic acid Kethoxal is more active than glyoxal because of its greater stability, but that its larger size might render it less active towards the intact virus because it prevents it from penetrating easily towards the nucleic acid in the center of the virus. The finding that the very small molecule formaldehyde, although being less active towards the free nucleic acid, is the most active compound towards the intact virus, also favours the concept that the ability of a compound to penetrate to the nucleic acid through the protein coat determines to a great extent its power to inactivate a virus. It might be noted that the much higher concentrations of inactivating compounds needed to inactivate the intact virus also indicates that the nucleic acid is highly protected in the intact virus but that these very high concentrations might ultimately result in its chemical inactivation.

Reconstitution of an inactive virus. The inactivating agents mentioned so far react with proteins as well as with nucleic acids. It is difficult, therefore, to show the effect on the nucleic acid in the intact virus separately. But it has been possible to prepare a virus in which only the nucleic acid moiety has been modified chemically.

[41] M. STAEHELIN, Biochim. biophys. Acta *31*, 448 (1959 b).

For this purpose, use was made of the ability of the two constituents of TMV. i.e. the protein and the nucleic acid, to reconstitute again and form an intact virus particle. FRAENKEL-CONRAT and WILLIAMS[42] have first made the extremely interesting observation that upon combining a solution of pure nucleic acid with a solution of pure protein, under suitable conditions, intact virus particles are formed again from the two constituents. This reconstitution can proceed to the extent of 30–80% of the material present (FRAENKEL-CONRAT *et al.*[43]). The reconstituted virus is fully infective and in the electron microscope appears to have the same structure as the original tobacco mosaic virus. The splitting and the reconstitution of the virus are shown schematically in Figure 10. The same drawing also shows the processes involved in the production of reconstituted virus from inactivated nucleic acid. In this experiment the virus is first split and the nucleic acid isolated. This nucleic acid is then inactivated by some chemical treatment and recombined with untreated protein to form intact virus again.

Table III shows the results of such an experiment using formaldehyde as an inactivating agent of the nucleic acid. It can be seen that the reconstitution proceeds to a similar extent with the inactivated nucleic acid as with untreated nucleic acid, and that the infectivity of the reconstituted virus closely parallels the infectivity of the nucleic acid used for reconstitution. In addition to formaldehyde, a number of different inactivating agents can be used (FRAENKEL-CONRAT, STAEHELIN and CRAWFORD[44]), e.g. epoxydes, β-propiolactone, HNO_2, ultraviolet light, etc. After treatment with all these agents, the nucleic acid has still the ability to reconstitute intact but inactive virus. The only requirement appears to be the intactness of the entire chain length of the nucleic acid strand. If the nucleic acid is inactivated by ribonuclease, for instance, the ability to reconstitute rapidly diminishes.

The characteristics of this inactive reconstituted virus are shown in Figure 11. Its special advantage over the formaldehyde inactivated virus is its unaltered antigenic property. Whereas in the intact virus the protein also reacts extensively with formaldehyde which alters its antigenic structure, in the reconstituted virus the inactivated nucleic acid is surrounded by a native protein. This makes it an antigen which resembles the untreated virus much more closely than does the formaldehyde treated virus. This seems to be confirmed by its serological reactions. Cross reactions of sera obtained by injecting rabbits with native TMV and with inactive reconstituted TMV have indicated that the two viruses behave as identical antigens. Formaldehyde inactivated TMV, by contrast, was precipitated only at a somewhat higher titer by normal ANTI-TMV serum than intact TMV and inactive reconstituted TMV.

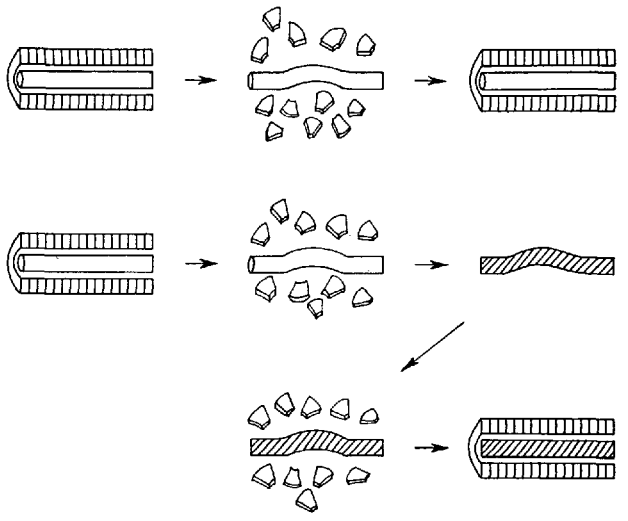


Fig. 10. Splitting of tobacco mosaic virus and reconstitution of active (top) and inactive virus (bottom).

Tab. III. Reconstitution of formaldehyde inactivated nucleic acid with native protein

Time of inactivation	Activity of nucleic acid % of controls	Activity of reconstituted virus % of controls	Yield of reconstitution % of theory
0	100	100	33
1	81	85	27
3	6	7	30
6	2	3	28

(from STAEHELIN[45])

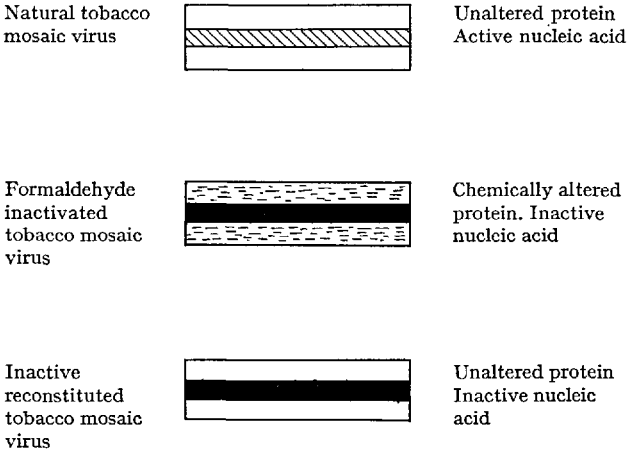


Fig. 11. Characteristics of natural TMV, formaldehyde inactivated TMV and inactive reconstituted TMV.

[42] H. FRAENKEL-CONRAT and R. C. WILLIAMS, Proc. nat. Acad. Sci. Wash. *41*, 690 (1955).

[43] H. FRAENKEL-CONRAT and B. SINGER, Biochim. biophys. Acta *33*, 359 (1959).

[44] H. FRAENKEL-CONRAT, M. STAEHELIN, and L. CRAWFORD, Proc. Soc. exp. Biol. *102*, 118 (1959).

[45] M. STAEHELIN, Helv. physiol. Acta *17*, [C] 40 (1959 c).

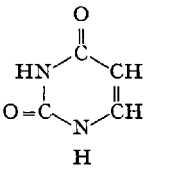
Reaction with nitrous acid. SCHUSTER and SCHRAMM[46] have studied the effect of nitrous acid on the biological properties of tobacco mosaic virus nucleic acid. Nitrous acid causes a deamination of the purine and pyrimidine bases to their respective hydroxyl derivatives. Thus, cytosine is converted into uracil, guanine into xanthine and adenine into hypoxanthine. From the kinetics of the reaction, they concluded that the deamination of one of 3300 nucleotides of the nucleic acid results in an inactivation of the molecule.

GIERER and MUNDRY[47] made the extremely interesting observation that, upon progressing inactivation, the relative proportion of mutants steadily increased. After an inactivation of over 95% of the molecules, more than 50% of the survivors turned out to be mutants. This finding is unique for the inactivation with nitrous acid and can possibly be correlated with the fact that one of the three reactions taking place, i.e. the conversion of cytosine to uracil does not result in the formation of an unnatural derivative but in a transformation of one natural base into another. The kinetics of the reaction indicate that one single transformation is sufficient to cause a mutation. It would appear, therefore, that the mechanism of mutation consists in the alteration of only one of the 6000 nucleotides into another natural nucleotide.

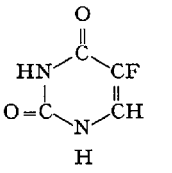
Incorporation of purine and pyrimidine analogs. A chemical modification of the virus infectivity can also be obtained by incorporating structural analogs into the nucleic acid of viruses. In this case the virus is not altered after it is formed but is already produced in the cell as an unnatural modification. The incorporation of structural analogs has been studied quite extensively in tobacco mosaic virus. At least three unnatural bases, i.e. 8-azaguanine (MATTHEWS and SMITH[48]), 2-thiouracil (JEENER[49]) and 5-fluorouracil (GORDON and STAEHELIN[50]), have been shown to be incorporated into tobacco mosaic virus. In all three instances the virus containing the analog differed in its biological characteristics from normal TMV.

One of the simplest ways of incorporating structural analogs into TMV is to infect leaves of a systemic host plant and to let them float on a solution of the structural analog. Virus growth proceeds readily also in detached leaves, especially under strong illumination. This method can also be used quantitatively to determine the effect of a structural analog on the growth of virus by excising identical disks from a large number of leaves (e.g. with a cork borer). The amount of virus produced in a certain number of leaf disks in the presence of various concentrations of the analog can then be determined spectrophotometrically after some preliminary steps of purification. The effect of the analog is expressed by comparing the virus yield in the leaf disks grown in a solution of the analog with those grown in water.

5-Fluorouracil. 5-Fluorouracil being a close analog of uracil has been examined in this manner. It was found to inhibit the growth of TMV in leaf disks, although rather high concentrations of the analog were required (GORDON and STAEHELIN[50]).



Uracil



5-Fluorouracil

The riboside, 5-fluorouridine, was somewhat more active than the free base. It was found, furthermore, that the extent to which the virus growth was inhibited was not only a function of the nature and the concentration of the analog but also of the time at which the analog was added. The inhibition was more pronounced the sooner after the infection the analog was added (Fig. 12), although there was only very little virus produced during the first few days.

The inhibition, even at very high concentrations of 5-fluorouracil, was never complete and it was of special interest to study the virus grown in the presence of the analog. This virus was found to contain 5-fluorouracil to a rather high extent (GORDON and STAEHELIN[50]). The analog replaced the normal base uracil since the sum of 5-fluorouracil and uracil always corresponded to the uracil content of normal TMV. The presence of 5-fluorouracil during the growth of the virus, however, resulted in a replacement of 30–50% of the uracil by the the structural analog. The nucleic acid of this virus,

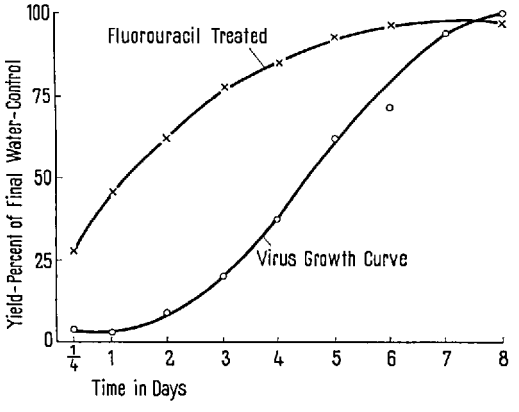


Fig. 12. Inhibition of the growth of TMV by the addition of 5-fluorouracil at various times (from GORDON and STAEHELIN[50]). O virus growth curve. × virus yield on the eighth day after addition of 5-fluorouracil at various times in percent of the virus yield in the absence of 5-fluorouracil.

[46] H. SCHUSTER and G. SCHRAMM, Z. Naturf. *13b*, 697 (1958).
[47] A. GIERER and K. W. MUNDRY, Nature, Lond. *182*, 1457 (1958).
[48] R. F. C. MATTHEWS and J. D. SMITH, Adv. Virus Res. *3*, 49 (1955).
[49] R. JEENER, Biochim. biophys. Acta *23*, 351 (1957).
[50] M. P. GORDON and M, STAEHELIN, Biochim. biophys. Acta *36*, 351 (1959).

therefore, contains in its nucleic acid about 500–800 molecules of 5-fluorouracil. It would be expected that this high extent of replacement of a natural base by an unnatural analog would result in some profound changes of its biological properties.

Infectivity of TMV containing 5-fluorouracil. The infectivity of a tobacco mosaic virus can be tested in either of two ways. When applied to leaves of a local lesion host (Fig. 5), the number of local necroses is proportional to the number of infective particles present in the solution. On local lesion hosts, the infectivity of the virus containing 5-fluorouracil appeared to be identical with that of normal TMV, i.e. solutions of both viruses in equal concentrations caused the same number of local lesions on the leaves. This test, however, does not give any information about the rate at which the virus is formed unless the time of the appearance of the lesions is very accurately measured. In a more accurate way, the rate of virus growth can be followed by infecting leaves of a systemic host (Fig. 6). Opposite half leaves were infected with normal and with 5-fluorouracil containing virus. Disks of identical size were then prepared and floated on water, and the amount of virus produced in a certain number of disks was determined at different times. When assayed this way on a systemic host, the virus containing 5-fluorouracil was definitely less infective than ordinary TMV (Fig. 13). These apparently conflicting results can be summarized as follows:

a) The virus containing the analog has the same number of infective virus particles as the natural virus since it causes the same number of necroses on local lesion hosts.

b) After infection with the unnatural virus, growth of new virus proceeds less rapidly since the virus yield at given times after the infection in systemic hosts is smaller than after infection with normal TMV.

These findings can be explained if we depict the processes which lead to virus infection in the following manner (Fig. 14). From analogy with phages, we assume that at one point on the surface or inside the cell nucleic acid is set free from the virus. This nucleic acid then transfers its information to the cell and provokes a series of highly specific processes in the cell, about which very little is known at present but which finally lead to the production of new virus. It would be expected that the presence of an unnatural base in the nucleic acid would exert its effect at the time when the nucleic acid reacts first with the cell. If the unnatural base very closely resembles the natural base, it can well be imagined that the nucleic acid is not inactive but the time required to give the information to the cell is prolonged. This would cause a prolongation of the latent period, i.e. the time before the onset of production of new virus (Fig.14). This assumption is supported by the finding that the incorporation of 5-fluorouracil causes a quantitative but never a qualitative change in the virus infection. In spite of a very thorough study, we were never able to detect the formation of mutants after infection with unnatural virus, although GIERER and MUNDRY[47] have shown that the alteration of one single base into another natural base might result in the production of a new mutant. This would indicate that the cell finally recognizes the 5-fluorouracil molecule as uracil.

This retardation of the onset of a new infection caused by the incorporation of 5-fluorouracil into the virus might also explain the strong time dependence of the inhibiting effect of 5-fluorouracil on the growth of TMV. This time dependence is most marked at the very early stages after infection, i.e. in the latent period

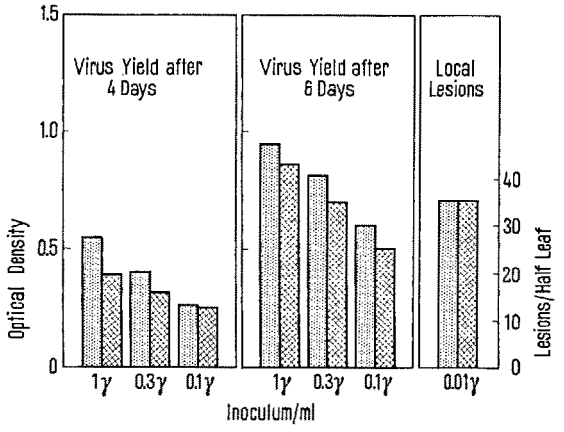


Fig. 13. Infectivity of TMV containing 5-fluorouracil. Left columns: normal TMV. Right columns: 5-Fluorouracil containing TMV.

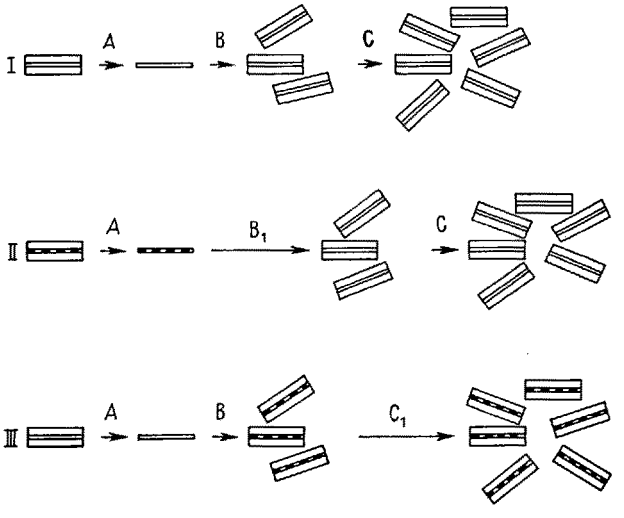


Fig. 14. Effect of 5-fluorouracil incorporation on the processes leading to virus production.
A) Liberation of nucleic acid. B) Transfer of information from nucleic acid to infected cell and formation of first new virus particles. C) Spreading of virus infection by first newly formed virus particles. I. Normal virus infection. II. Infection with virus containing 5-fluorouracil. Steb B delayed. III. Infection in the presence of 5-fluorouracil (incorporation of 5-fluorouracil into the first newly formed virus particles resulting in a delayed further spreading of the infection). Step C delayed.

long before the bulk of virus is formed. If 5-fluorouracil is so rapidly incorporated into TMV, it would be expected that upon very early addition of 5-fluorouracil already the earliest new forms of virus contain the analog. But it is these very first new viruses, possibly even nucleic acids only, which are responsible for the spreading of the infection from cell to cell. Since the the incorporation of 5-fluorouracil results in a slower rate of virus growth, the spreading of the infection by these early stages would be inhibited. This would result in a reduction of the virus yield which is in accord with the experimental findings (Fig. 14),

This delayed spreading of the virus infection does not explain the inhibition of the virus growth by 5-fluorouracil entirely, however, since 5-fluorouracil in addition causes an inhibition of the synthesis of ribonucleic acid in general (STAEHELIN and GORDON[51]). But it might be responsible for the very marked time dependence of the addition of 5-fluorouracil very soon after the infection.

Zusammenfassung

Die biologischen Eigenschaften des Tabakmosaikvirus können durch chemische Umsetzungen seiner Nukleinsäure in verschiedener Weise beeinflusst werden:

1. Formaldehyd reagiert mit den Aminogruppen der Basen, Glyoxal und seine Derivate speziell mit der Diaminstruktur des Guanins. Beide Reaktionen bewirken eine Inaktivierung des Virus.

2. Durch Behandlung der isolierten Nukleinsäure und nachherige Rekonstitution mit nativem Eiweiss konnte ein Virus gewonnen werden, dessen antigene Eigenschaften denjenigen des unbehandelten Virus entsprechen, das jedoch inaktiv ist.

3. Reaktion mit salpetriger Säure, bei der unter anderem Cytosin in Uracil übergeführt wird, führt ausser zu Inaktivierung auch zu einer grossen Anzahl von Mutanten.

4. Der Einbau strukturanaloger Basen, wie z.B. des 5-Fluoruracils, führt zu einem Virus, das zwar eine Infektion hervorrufen kann, bei der jedoch die Wachstumsgeschwindigkeit des neuen Virus verzögert ist.

[51] M. STAEHELIN and M. P. GORDON, Biochim. biophys. Acta *38*, 307 (1960).

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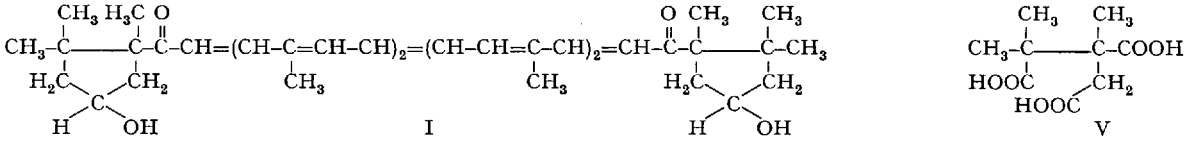
Bemerkungen zur Struktur des Capsorubins

Es wurde bereits berichtet, dass die richtige Bruttoformel des Capsanthins $C_{40}H_{56}O_3$ und die des Capsorubins $C_{40}H_{56}O_4$ beträgt[1]. Vor kurzem haben ENTSCHEL und KARRER[2] sowie BARBER, JACKMAN, WARREN und WEEDON[3] die Struktur des Capsanthins und des Capsorubins (I) festgestellt, doch konnte die Lage der Substituenten am Cyclopentanring (3 CH_3- und 1 OH-Gruppe) nicht als vollkommen gesichert angesehen werden. Auch ENTSCHEL und KARRER zogen die Möglichkeit in Betracht, dass der Cyclopentanring in beiden Farbstoffen nicht am C_3, sondern an C_4 OH-Gruppen tragen könnte. Nunmehr gelang es uns, die Lage der OH- und CH_3-Gruppen der Cyclopentanringe eindeutig festzulegen. Capsorubinacetat



I

wurde ozonisiert, das Ozonid mit H_2O_2 behandelt und zuletzt nach Entfernung der Acetylgruppen mit Chromsäure oxydiert. Aus dem Säuregemisch wurden nach präparativer Papierchromatographie folgende Säuren kristallin dargestellt und identifiziert: 1,1-Dimethylbernsteinsäure (II), Smp. 140°C, $R_f = 0{,}37$; Trimethylbernsteinsäure (III), Smp. 149°C, $R_f = 0{,}67$; 1,1,2-Trimethylglutarsäure (IV), Smp. 110°C, $R_f = 0{,}94$; Camphoronsäure (V), Smp. 165°C, $R_f = 0{,}06$. Ausserdem wurde noch Dimethylmalonsäure (VI) nachgewiesen, jedoch reichte die Menge nicht zur Kristallisation. III, IV und V legen die Methylgruppen eindeutig fest; IV und V liefern ausserdem den Beweis, dass sich die OH-Gruppen nicht in Position 4 befinden können, und II schliesst das Vorhandensein einer OH-Gruppe an C_2 aus. Es kann so als bewiesen betrachtet werden, dass sich die OH-Gruppen in Lage C_3 und C_3' befinden.

Es erscheint wahrscheinlich, dass die OH-Gruppe des Cyclopentans auch im Capsanthin und Kryptocapsin an C_3 steht. Diesbezügliche experimentelle Befunde werden demnächst mitgeteilt.

V

[1] L. CHOLNOKY, D. SZABÓ und J. SZABOLCS, Liebigs Ann. *606*, 194 (1957).

[2] R. ENTSCHEL und P. KARRER, Helv. chim. Acta *43*, 89 (1960).

[3] M. S. BARBER, L. M. JACKMAN, C. K. WARREN und B. C. L. WEEDON, Proc. chem. Soc., Lond. *1960*, 19.

[4] L. CHOLNOKY, K. GYÖRGYFY, E. NAGY und M. PÁNCZÉL, Acta chim. Acad. Sci. hung. *6*, 143 (1955); vgl. Nature *178*, 410 (1956).