EFFECTS OF HALOGENATED PYRIMIDINES ON THE GROWTH OF TOBACCO MOSAIC VIRUS

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SUMMARY

The inhibition of TMV formation and the incorporation of 5-fluorouracil into viral RNA occurs in *Phlox drummondi* as well as in the Turkish tobacco host. The inhibitory action of 5-fluorouracil on the formation of TMV in isolated leaf disks of Turkish tobacco plants is dependent on the time interval which has elapsed between infection and exposure to 5-fluorouracil. 5-fluoroorotic acid, 5-fluorouridine, and 2-thio-5-fluorouracil are also inhibitors of the formation of TMV, and the first two compounds also lead to the formation of viral nucleic acid which contains 5-fluorouracil. Uridine will reverse the action of 5-fluorouracil, while thymidine is without effect. Halogenated pyrimidines, which inhibit the formation of DNA in other systems, have little effect on the formation of TMV.

INTRODUCTION

The halogenated pyrimidine, 5-fluorouracil (FU), has been shown to be an inhibitor of growth in diverse biological systems[1] including the growth of phage T2 in *Escherichia coli*[2], and the formation of tobacco mosaic virus (TMV) in cultured Turkish tobacco leaves[3,4]. In a previous study[5] it was shown that the inhibition of the formation of TMV was accompanied by the replacement of up to 47 % of the uracil of the virus ribonucleic acid (RNA) by fluorouracil. The infectivity of the FU containing virus was identical to that of normal virus when assayed on several host plants in which tobacco mosaic virus gives rise to local lesions. In Turkish tobacco plants, where the virus gives a systemic infection, the rate of virus growth following infection by the FU containing virus was smaller than the corresponding rate following infection by the normal virus.

The mechanisms of the inhibitory action of FU and some of its derivatives have been studied in various bacterial and mammalian systems[6–9]. The present paper is concerned with attempts to elucidate the mechanism of the inhibition of TMV formation by FU. A correlation of the yield of virus with structural variations in the FU molecule gave some idea as to the sites of action of FU. A study of a few of the variables involved in the growth of TMV in the presence of fluorouracil has yielded

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information about the early stages of the biosynthesis of the virus. In addition the action of FU on the ribonucleic acid metabolism in uninfected leaves was studied*.

EXPERIMENTAL

Materials

The 5-fluoropyrimidine derivatives were a gift from Dr. R. DUSCHINSKY, Hoffmann-LaRoche, Inc., Nutley, New Jersey. The 5-bromouracil was a gift from the Sigma Chemical Company, St. Louis, Missouri. The 5-chlorouracil was prepared by Dr. G. RUSHIZKY of these laboratories, and the 5-iodouracil was obtained from Dr. G. HITCHINGS, Burroughs Wellcome, Inc., Tuckahoe, New York.

Assays and culture techniques

Except where otherwise noted, leaves of Turkish tobacco plants were infected with TMV, and after 18 h the leaves were removed from the plants. Disks were punched from the leaves and floated on aqueous solutions of the materials to be tested. After 7–8 days of incubation the disks were washed and frozen, and the virus was isolated. Details of these procedures are given in a report[5].

RESULTS

Effect of FU on the yield of TMV under various conditions

The effect of increasing concentrations of FU on the amount of TMV produced in excised disks of Turkish tobacco leaves has been described[5]. These results demonstrated that incubation of infected leaf disks on a 0.1 % solution of FU decreased the yield of virus by about 50 % (Table I) and, furthermore, some of the uracil of the

TABLE I

EFFECTS OF 5-FLUOROURACIL ON THE FORMATION OF TMV IN TWO DIFFERENT HOSTS*

*Host plant***	*Inhibition by fluorouracil Virus yield-percent of water control*	*Fluorouracil content of virus RNA Percent of normal uracil content*
Turkish tobacco	50 ± 10	28–47***
Phlox drummondi	57 ± 10	38

* Half leaves were cultured either on 0.1% FU solution or water for 7 days.
** In the case of both species about 50% of the FU was absorbed by the leaf segments from the culture medium.
*** Extreme range of values observed in several experiments.

virus was replaced by FU. The following experiment was carried out to ascertain whether this inhibition and concomitant incorporation of FU took place in a different, unrelated host for TMV production. For this purpose leaves of plants of *Phlox drummondi* (Burpee's Giant Tetra Red, W. A. Burpee Company, Riverside, California) two months old were infected with a 100 μg/ml solution of TMV in 0.1 *M* sodium phosphate buffer, pH 7.0. After 18 h equal weights of infected leaf segments were floated either

* The following abbreviations are used in this manuscript: 5-fluorouracil, FU; tobacco mosaic virus, TMV; ribonucleic acid, RNA; deoxyribonucleic acid, DNA.

on water or on a 0.1 % solution of FU. The leaves were incubated, and the virus was isolated as previously described for tobacco leaves[5]. It was found that FU causes similar reductions in the yield of virus in leaves of *Phlox drummondi* and Turkish tobacco. Moreover, FU was incorporated into the viral RNA in both of these unrelated plant hosts (Table I).

In order to ascertain the effect of exposure to FU at various time intervals after infection, disks were punched from Turkish leaves and floated on water 6 h after infection of the leaves. At the various times indicated in Fig. 1, the water in three dishes was replaced by a 0.1% solution of FU. All of the disks exposed to the FU

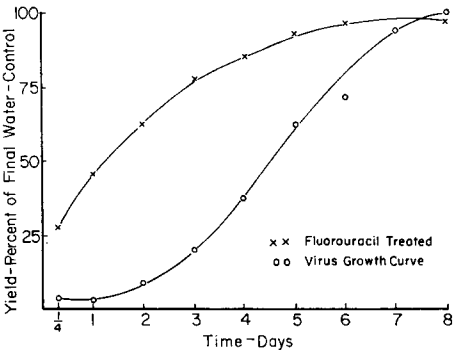


Fig. 1. Yield of TMV as a function of the time of addition of FU in days. Turkish tobacco leaves were infected at o time. At 6-h disks were punched from the leaves and floated in water. At the times indicated by the X designation, 3 sets of leaf disks were placed in a 0.1% solution of FU. The disks exposed to the FU solution were all harvested after 8 days. At the same time a growth curve was determined by harvesting infected disks that had been incubated on water for periods of time indicated by the o designation.

solution were incubated for a total of eight days after infection. The disks were then harvested, and the amount of virus produced was determined. The average amount of virus produced in each set of three dishes is expressed as the percent of the virus yield in a water control that was incubated for eight days. The results are plotted in Fig. 1 as the curve labeled "Fluorouracil Treated". These results show that when the disks are exposed to a 0.1% solution of FU at times immediately after infection (as the 1/4 and 1 day points in Fig. 1) and then incubated in the presence of FU for a total period of eight days subsequent to infection, the yield of virus is markedly reduced. On the other hand, if the disks are incubated on water for several days and then exposed to the FU solution for the remainder of the eight day incubation period (for example, the 5 and 6 day points in Fig. 1), the yield of virus as compared to that of the water control is not substantially reduced. In this experiment it was also important to determine how much virus was present in the leaf disks at the time of exposure to the FU solution. For this purpose three dishes of leaf disks, that had been incubated on water, were harvested each time another set of disks was exposed to the FU solution. The average amount of virus present in each set of dishes that had been incubated on water was expressed as the percent of the amount of virus present in the water control that had been incubated for eight days. These results are plotted in Fig. 1 as the curve labeled "Growth Curve". It is important to note that the points plotted as the "Growth Curve" refer to disks which had been incubated only on water, and the total time of incubation of these disks after infection varied

from 1/4 to 8 days. The points plotted as the "Fluorouracil Treated" curve refer to disks which were exposed to both water and FU solution for varying periods of time, but the total time of incubation after infection in this case was eight days. The results of this experiment, as shown in Fig. 1, indicate that exposure of infected disks to a 0.1 % solution of FU at times shortly after infection results in a definite decrease in the final yield of virus. The effect of exposure to FU is greatest at the times when the virus content of the disks is small.

The effect of incubation of leaves with FU prior to infection with TMV was ascertained by culturing leaves of Turkish tobacco plants on water or a 0.1 % solution of FU for 7 days. At the end of this period the leaves cultured on FU were not quite as green as the water control, but both sets of leaves were still turgid. The leaves were then infected with TMV, and 7 mm disks were excised. The disks from the FU grown leaves were divided into two groups. One group was again cultivated on a 0.1 % solution of FU and the other group on water. The disks from the leaves cultured on water were similarly treated. Thus, four sets of infected disks were cultured; *i.e.*, (a) disks incubated before and after infection on water; (b) disks incubated on water before, and on FU, after infection; (c) disks incubated on FU before, and on water after, infection; and (d) disks incubated on FU before and after infection. After five more days of cultivation, the disks were harvested, and the yields of TMV were determined. The results (Fig. 2) show that pre-incubation of leaves with FU has a pronounced effect on the final yield of virus. It is also to be noted that the effects of pre-incubation on FU are not reversed by placing the infected plant tissues on water.

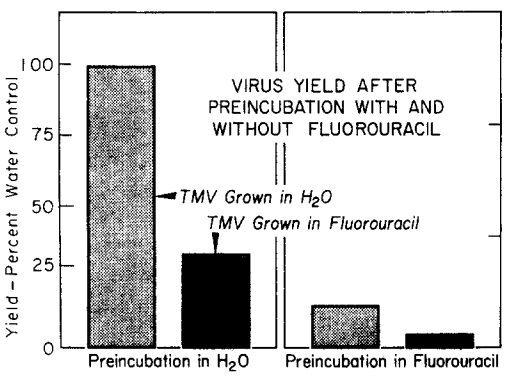


Fig. 2. Inhibition of virus formation by FU under various conditions. Half leaves of Turkish tobacco were cultured on water or FU for one week, infected, and 7-mm disks were excised. An equal number of disks from each set of leaves was then floated on water or 0.1 % FU and cultured for 5 days. The amount of virus formed after 5 days was determined.

Effect of FU on the ribonucleic acid metabolism of normal leaves

The effect of FU on the metabolism of the various leaf ribonucleic acid fractions was investigated in the following experiment. Corresponding quarter leaves were incubated either in water or in a 0.1 % solution of FU for 7 days. Then *ca.* 36 μC of ^{32}P (sodium phosphate, pH 7) was added to each set, and incubation was continued for an additional 24 h. The leaves were then thoroughly washed with water and ground with sand in the presence of an equal wt. of sucrose-phosphate buffer (0.25 *M* sucrose–0.02 *M* phosphate buffer pH 6.8). The different cell fractions were separated by differ-

ential centrifugation according to the procedure of ZAMECNIK[10]. Each fraction was purified by one additional cycle of differential centrifugation, but some contamination of the mitochondrial and microsomal fractions by chloroplast fragments was unavoidable in view of the fragility of tobacco leaf chloroplasts. The RNA was isolated by the hot salt extraction procedure of DAVIDSON AND SMELLIE[11]. The radioactivity of known amounts of RNA was determined, and it was found that incubation in a 0.1 % solution of FU resulted in a 3–5 fold decrease in the incorporation of inorganic phosphate into the RNA of various subcellular fractions (Table II).

TABLE II

EFFECT OF 5-FLUOROURACIL ON PHOSPHATE METABOLISM OF SUBCELLULAR FRACTIONS

Cellular fraction	*Radioactivity of RNA (counts/min/mg)*	
	Control	*5-Fluorouracil grown*
Chloroplasts	1400	280
Mitochondria	240	92
Microsomes	24	8.8

Possible incorporation of FU into chloroplast RNA

Since a large amount of moderately radioactive nucleic acid was obtained from the above chloroplast fraction, the chloroplast nucleic acid was degraded with alkali, and the resulting nucleotides were submitted to electrophoresis at pH 9.2. Radioautography revealed the presence of a band of radioactive material which migrated with the velocity of a fluorouridylic acid. The amount of this rapidly moving material was too small to be observed under u.v. light, and, consequently, no definite identification was possible by means of its u.v. absorption spectrum. Attempts to demonstrate the incorporation of FU into the RNA of separated tips of Turkish tobacco plants by culture in the presence of FU and ^{32}P labeled inorganic phosphate were inconclusive. These results indicate that the magnitude of any possible FU incorporation into any RNA fraction examined was less than about 4 % of the uracil present.

TABLE III

EFFECT OF 5-HALOGENOPYRIMIDINES ON THE FORMATION OF TMV IN EXCISED LEAF DISKS

Compound	*Concentration %*	*Virus yield-percent of control*
H_2O	—	100
5-Fluorouracil	0.1	50 ± 5
5-Fluorouridine	0.01	50 ± 5
5-Fluoro-2-thiouracil	0.1	52 ± 10
5-Fluoroorotic acid	0.05	41 ± 5
5-Fluoroorotic acid	0.025	52 ± 5
5-Fluoroorotic acid	0.01	58 ± 5
5-Fluoroorotic acid	0.001	88 ± 5
5-Chlorouracil	0.1	100 ± 5
5-Chlorouracil	0.2	100 ± 5
5-Bromouracil	0.1	93 ± 5
5-Iodouracil	0.1	90 ± 5

Inhibitory effects of other halogenated pyrimidine derivatives

The ribonucleoside of FU, 5-fluoro-1-β-D-ribofuranosyluracyl (fluorouridine), was found to be an inhibitor of the formation of TMV. The molar concentration of the nucleoside required for 50 % inhibition was about one-tenth of the equivalent concentration of the aglycone (Table III and Fig. 3). Fluorodeoxyuridine (5-fluoro-2'-

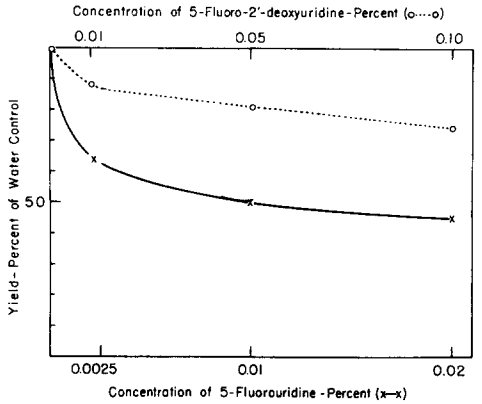


Fig. 3. Effects of 5-fluorouridine and 5-fluorodeoxyuridine on the formation of TMV in isolated disks of Turkish tobacco leaves.

deoxyuridine) was, comparatively, an ineffective inhibitor. These results are shown in Fig. 3. 5-fluoroorotic acid was also found to be an inhibitor of the formation of TMV under the conditions of the present assay (Table III).

The replacement of the oxygen in the 2-position of FU by sulfur did not decrease the inhibitory activity of the molecule. Incubation of leaves on a 0.1 % solution of 2-thio-5-fluorouracil also caused about a 50 % reduction in the yield of TMV (Table III).

The introduction of halogens other than fluorine into the 5-position of the uracil molecule resulted in virtually complete disappearance of inhibitory properties (Table III).

Incorporation of derivatives of FU into TMV-RNA

It was of interest to ascertain whether the inhibition of TMV growth by 5-fluoroorotic acid and 5-fluorouridine was accompanied by the incorporation of FU into the RNA of the virus. Accordingly, 114-mg and 89-mg lots of TMV were prepared from infected Turkish leaf disks incubated in the presence of 0.05 % 5-fluoroorotic

TABLE IV

INCORPORATION OF DERIVATIVES OF 5-FLOROURACIL INTO TMV–RNA

*Compound**	*Concn. in incubation medium*	*Fluorouracil content of virus RNA***
5-Fluoroorotic acid	0.05%	43%
5-Fluorouridine	0.01%	19%

* The concentrations of compounds used were found to result in an inhibition of about 50 % in the yield of virus.

** Expressed as percent of normal uracil content.

acid and 0.01 % fluorouridine solutions, respectively. Samples of RNA prepared from these lots of virus were hydrolyzed with alkali and examined for the presence of the 2′ and 3′ phosphate of 5-fluorouridine as previously described[5]. The results, as given in Table IV, indicate that each of these inhibitory compounds leads to the formation of TMV-RNA which contains 5-fluorouracil.

There was some indication of the formation of an abnormal TMV-RNA when infected leaves were incubated on a solution of 2-thio-5-fluorouracil; however, the results were complicated by the instability of the 2-thiosubstituent. No incorporation (less than 4 % of the uracil) was observed in the case of 5-chlorouracil[12].

Reversal of FU inhibition

Attempts were made to reverse the action of a 0.1 % solution of FU by the simultaneous addition of various compounds. It was found that uracil and thymidine at molar concentrations up to 10 times that of the FU had little or no effect on the formation of TMV. On the other hand, uridine had a definite effect on the production of virus, and at high concentrations of uridine the effect of FU was completely reversed as shown in Table V.

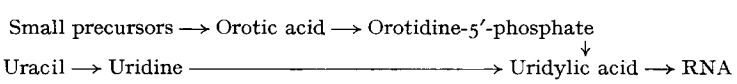
TABLE V

EFFECT OF URIDINE ON THE INHIBITION OF TMV FORMATION BY 5-FLUOROURACIL

Sample No.	*5-Fluorouracil concn. μ moles/ml*	*Uridine concn. μ moles/ml*	*Virus yield percent of control*	
			Expt. 1	*Expt. 2*
1	0	0	100	100
2	7.7	0	60 ± 5	53 ± 5
3	0	7.7	100 ± 5	101 ± 5
4	7.7	7.7	73 ± 5	74 ± 5
5	0	77	100 ± 5	103 ± 5
6	7.7	77	100 ± 5	101 ± 5
7	0	154	—	105 ± 5
8	7.7	154	—	101 ± 5

DISCUSSION

The synthesis of the uridylic acid moiety of tobacco leaf RNA and TMV-RNA is probably similar to the overall pathway which has been demonstrated in mammals and bacteria[13]. This scheme may be presented as follows:



The levels of phosphorylation at which some of these reactions take place is unknown. The pathway from uracil is probably of minor importance except in the presence of exogenous uracil or uracil analogues. The 5-fluoro-analogues of orotic acid and uridine are closely related to the intermediates of the *de novo* synthesis of uridylic acid and should be readily converted into nucleotides of fluorouridine. It has been found that FU, 5-fluoroorotic acid, and 5-fluorouridine are inhibitors of TMV formation (Tables I and III) and lead to the formation of TMV-RNA containing FU (Tables I and IV). According to the above scheme it may be postulated that these compounds are metabolized to a common pool of fluorouridine nucleotides which are

responsible for the similar behavior of these three compounds in the present system. It should be noted that synthetic nucleotides of FU have been prepared[14], so that it should be possible directly to observe their effects in cell free systems. The close relation of FU to the above scheme of *de novo* biosynthesis of uracil is also shown by the reversal of FU inhibition of TMV production by high concentrations of uridine (Table V). At the time these experiments were in progress the fluoro compounds labeled with radioactive isotopes were not available, so that the question of the relative permeability of the plant cells to these compounds could not be readily ascertained.

The incorporation of radiophosphorus into the subcellular components was also inhibited by FU (Table II). This finding indicates that the rates of turnover and synthesis of RNA in isolated leaf segments are decreased by the presence of a pool of fluorouracil nucleotides. In the case of chloroplast RNA some indication of the incorporation of a small amount of FU was obtained. It is not known whether this questionable and small incorporation of FU may be ascribed to a slower rate of synthesis and/or turnover of plant RNA as compared to viral RNA, or to the operation of more selective mechanisms.

Some insight into the mode of virus production was obtained with the use of various halogenated uracil analogues. Compounds such as 5-chloro, 5-bromo, and 5-iodouracil[15,16], which are known to produce abnormal DNA molecules in *E. coli*, were virtually without effect on the formation of TMV in isolated disks of Turkish tobacco. The deoxyribonucleoside, 5-fluoro-2′-deoxyuridine, had only a slight inhibitory action under the conditions of the present assay in contrast to the striking activity of the corresponding ribonucleoside, 5-fluorouridine (Fig. 3). In mammalian[6] and bacterial[8] systems it has been found that 5-fluoro-2′-deoxyuridine-5′-phosphate is an extremely potent inhibitor of the thymidylate synthetase system. The action of FU or its derivatives in some of the latter systems may be partially[8], or completely[7], reversed by thymine or thymidine. In tobacco leaves infected with TMV, thymidine at 10 times the molar concentration of 0.1% FU has no effect on the inhibitory action of the uracil analogue. Uridine, on the other hand, at high concentrations produces a complete reversal of the inhibition by FU (Table V). Although there is need for more direct evidence, the results obtained with the above series of compounds suggest that the formation of new molecules of deoxyribonucleic acid does not play an important role once the TMV infection is initiated in a tobacco leaf.

The results obtained with the 5-fluoropyrimidines in the present studies in some respects are similar to those obtained by HEIDELBERGER and colleagues[6] in an extensive series of investigations aimed at elucidating the mechanism of the inhibition of the growth of some neoplastic tissues by these compounds. These workers found that FU, 5-fluoroorotic acid, and 5-fluorouridine were converted into a pool of 5-fluorouridine nucleotides and, moreover, some conversion to 5-fluoro-2′-deoxyuridine monophosphate was observed. In a variety of tissues FU, 5-fluoroorotic acid, and 5-fluorouridine were incorporated into RNA (but not the DNA) as FU in amounts which approached a maximum of 6% of the uracil content. Under certain conditions FU inhibited the incorporation of [^{32}P]phosphate into a variety of tissues *in vivo*. The results of the studies by the Wisconsin workers indicate that the mechanism of action of the 5-fluoropyrimidines in mammalian cells is complex and possibly involves the formation of acid soluble nucleotides, interference with the synthesis and function

of RNA, and inhibition of DNA synthesis by blockage of the methylation of thymidine precursors.

The production of TMV in the isolated leaf system used in the present experiments was very sensitive to the time interval between infection and exposure to FU (Figs. 1 and 2), and the effect of FU decreases before the bulk of virus is synthesized. Since the yield of TMV in normal as well as in FU cultivated leaf disks reaches a maximum after 6–7 days, the inhibition of virus formation by FU seems to be absolute and not merely the reflection of a decrease in the rate of virus production. The experiments of SIEGEL AND WILDMAN[17] indicate that on leaves of *Nicotiana glutinosa* a period of 5 h is required before a decrease of the u.v. sensitivity of the system indicates that the initial infecting TMV particle has given rise to a multiple number of infectious centers. In the case of systemic infection in Turkish tobacco, u.v. sensitive centers will probably be associated with the emergence of new loci of TMV formation during the spread of the infection. The presence of fluorouracil nucleotides during these crucial early stages would be expected to inhibit or completely abolish the formation of sites for virus synthesis. Once the mechanism for TMV formation is established in a site, newly introduced fluorouracil ribonucleotides would merely be polymerized into molecules of TMV-RNA.

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