THE INHIBITION AND MUTAGENESIS OF AN RNA BACTERIOPHAGE BY 5-FLUOROURACIL

By C. I. Davern*

[*Manuscript received February* 24, 1964]

Summary

The yield of the RNA bacteriophage $f_{Can\ 1}$ is strongly inhibited by 5-fluorouracil (FU). The production of non-infective phage particles in the presence of FU was demonstrated. The infective phage produced in the presence of FU have a relative plating efficiency lower than normal phage when assayed at supra-optimal temperatures, and FU was shown to be mutagenic for $f_{Can\ 1}$. Mutants sensitive to high temperature were isolated from infected cultures incubated in the presence of 2×10^{-5}M FU. Complementation among these mutants could not be investigated because of strong interference preventing mixed infection.

I. Introduction

The mutagenic activity of the halogenated uracils 5-chlorouracil, 5-bromouracil, and 5-iodouracil (Litman and Pardee 1956, 1960*a*, 1960*b*; Benzer and Freese 1958) for organisms containing DNA has been attributed to the electronegativity of the halogen substituent occasioning an increase in errors of incorporation and replication during DNA duplication (Freese 1959; Terzaghi, Streisinger, and Stahl 1962).

In support of this hypothesis, Lawley and Brookes (1962) observed that the pK_a for 5-bromodeoxyuridine is 8·1 as compared to a pK_a of 9·8 for thymidine. The substitution of hydrogen on C_5 by fluorine in uridine also lowers its pK_a from 9·3 to 7·6 (Kahan and Hurwitz 1962; Goldberg, Dahl, and Parks 1963).

Since 5-fluorouracil (FU) is readily incorporated into RNA in the place of uracil (Heidelberger *et al.* 1957; Gordon and Staehelin 1958; Horowitz and Chargaff 1959; Munyon and Salzman 1962), one would expect it to behave as a mutagen for organisms containing RNA, and also to cause phenotypic miscoding in organisms containing DNA when it is incorporated into messenger RNA. Such phenotypic effects of incorporation of FU into the messenger RNA of organisms containing DNA have been observed in the form of "phenotypic reversion" for some T4 phage mutants (Champe and Benzer 1962) and λ phage mutants (Campbell 1962). A similar interpretation has been put forward to explain the changes in amino acid composition of *Escherichia coli* protein when this organism is cultured in the presence of FU. At the functional level, FU-induced coding disturbances are indicated by the production of inactive β-galactosidase, and also a heat-labile alkaline phosphatase (Bussard *et al.* 1960; Naono and Gros 1960). The interference with messenger RNA coding integrity is also indicated by the inhibition of T2 bacteriophage yield by FU in the presence of thymidine (Goodman, Saukkonen, and Chargaff 1960; Saukkonen, Goodman, and Chargaff 1960; Shug, Mahler, and Fraser 1960; Aronson 1961*a*).

* Division of Plant Industry, CSIRO, Canberra; present address: Laboratory of Quantitative Biology, Cold Spring Harbour, Long Island, New York.

However, such FU effects are not consistently observed. For example, the amino acid composition of yeast protein is not affected by incubation of yeast in the presence of FU (Kempner and Miller 1962) and Aronson (1961*b*) did not observe any FU-induced changes in *E. coli*. More significantly, both tobacco mosaic virus (Gordon and Staehelin 1958) and polio virus (Munyon and Salzman 1962) can tolerate as much as 47% replacement of their uracil by FU without losing infectivity. Furthermore, detectable changes in the amino acid composition of their protein coats were not observed, and Holoubek (1963) concluded that FU incorporation into viral RNA does not lead to coding errors.

Nevertheless the yields of these viruses are reduced in the presence of FU. Polio virus cultured in the presence of 10^{-3}M FU yields 12–21% of the control (Munyon and Salzman 1962). This level of FU leads to 35% replacement of uracil by FU. Similar results for tobacco mosaic virus at the same level of substitution have been observed by Gordon and Staehelin (1958). The RNA phage f_2 (Loeb and Zinder 1961) seems to be even more sensitive to inhibition of yield by FU, in that culture of this phage in the presence of 4×10^{-4}M FU reduced the yield to 1–3% of the control (Cooper and Zinder 1962).

In the following paper, FU is shown to be a potent inhibitor of the RNA phage $f_{Can\,1}$ (Davern 1964) and, in contrast to the results obtained with tobacco mosaic virus and polio virus, $f_{Can\,1}$ cultured in the presence of FU is shown to be considerably reduced in its specific infectivity. The mutagenic activity of FU so indicated for this phage is further demonstrated by the induction of mutants which are sensitive to high temperatures.

II. Materials and Methods

(*a*) *Bacteriophage*

The RNA bacteriophage, $f_{Can\,1}$, isolated from Canberra sewage (Davern 1964) was used.

(*b*) *Host Bacterium*

Hfr_1, a methionine-requiring strain of *E. coli* K-12, originally isolated by Dr. A. Garen and supplied by Dr. A. F. Graham, was used routinely for phage assay, and for the investigation of the effect of FU on phage yield, infectivity, and mutagenic activity.

(*c*) *Media*

For the study of the effects of FU, a glucose–mineral salts medium supplemented with 10 μg/ml of methionine was used. This medium consists of the following ingredients per litre: Na_2HPO_4 6 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 0·5 g, glucose 4 g, 0·01M $CaCl_2$ 10 ml, 0·1M $MgSO_4$ 10 ml, 0·01M $FeCl_3$ 0·1 ml, and 1% gelatin 1 ml.

Nutrient broth (containing Difco bacto-nutrient broth 8 g, NaCl 5 g, and $CaCl_2$ 0·44 g per litre) was used for culturing host bacteria for phage assay, and for routine culturing of phage stocks.

(*d*) *Phage Assay*

Phage titres were determined by plaque formation; the agar layer method of Adams (1959) was used. The compositions of the top and base agar layer media have been described (Davern 1964).

(*e*) *Phage Antiserum*

Phage $f_{Can\,1}$ antiserum was taken from rabbits inoculated with a course of intravenous injections of a highly purified phage preparation. The K value (Adams 1959) of this phage antiserum was 4000.

(*f*) *Immunodiffusion Analysis*

Ouchterlony's agar gel immunodiffusion technique was used for the estimation of the quantity of phage-specific antigen synthesized in FU-treated and control cultures. The agar gel contained 0·1M NaCl, 0·08M Tris, pH 7, 0·75% agar, and 0·025% NaN_3. The centre well contained undiluted phage antiserum, while the peripheral wells contained dilutions of ultrasonically disrupted bacterial cells, derived from both infected and uninfected cultures. The plates were allowed to develop for 2–3 days at 4°C before being photographed.

(*g*) *Characterization of Temperature-sensitive Mutants*

Single plaque clones were characterized by plating them at both 37 and 44°C, either as spot tests on an agar plate pre-seeded with host bacteria, or by the standard agar layer method used in routine phage assays. In the spot test, the phage titre of the clone tested must be less than 10^7 phage/ml, otherwise a temperature-sensitive mutant will give a plaque at 44°C since, in the time the agar plate takes to reach the restrictive temperature in the incubator, the cells initially infected are able to complete one infection cycle and lyse. Thus, if the phage titre is too high, the first cycle of phage growth will lyse most or all of the host bacteria, giving a clear spot on the lawn of indicator bacteria.

(*h*) *Complementation Tests*

Two drops (about 0·1 ml) of Hfr_1 cells in the log phase of growth in broth at 10^8/ml were added to tubes containing 0·1 ml phage (titre: 10^{10}/ml). The phage inoculum was either a 1 : 1 mixture of two temperature-sensitive mutants, or a pure suspension of one temperature-sensitive mutant. The input multiplicity of 50 for each phage should ensure mixed infection for practically all the cells. A control study of input multiplicity versus proportion of cells infected, under the conditions used for the mixed infection experiments, was made. It showed that the input multiplicity was essentially the multiplicity of infection, at least in respect to the Poisson expectation for the proportion of cells escaping infection for any given input multiplicity.

(*i*) *Ultrasonic Disruption of Cells*

Bacterial suspensions (5 ml) were frozen rapidly in a dry ice–methanol freezing mixture, then thawed and disintegrated by ultrasonic vibration with a Mullard sonic

drill for a succession of three 60-sec periods, interspersed with cooling to 0°C. Aliquots of the disintegrated cell suspensions were assayed for infective phage, and analysed by immunodiffusion for the presence of phage-specific antigen.

III. Results

(a) *Inhibition of Phage Yield by FU*

Hfr_1 cells in the log phase of growth in glucose–mineral salts medium at 10^8/ml were infected at an input multiplicity of 10 (i.e. equivalent input phage titre $= 2\times10^7$/ml) with $f_{Can\,1}$ phage for 5 min at 37°C and then diluted 50-fold into glucose–mineral salts media containing various concentrations of FU. The phage yields after incubation for 60 min with aeration at 37°C are given in the following tabulation:

FU concentration (M):	Nil(control)	10^{-3}	2×10^{-4}	10^{-4}	4×10^{-5}
Phage yield (infective phage/ml):	$1\cdot4\times10^9$	$5\cdot8\times10^6$	$9\cdot3\times10^6$	$1\cdot1\times10^7$	$1\cdot4\times10^7$

The phage yields obtained at the highest FU concentration are probably residual, non-adsorbed input phage (Hfr_1 cells grown in glucose–mineral salts medium absorb phage poorly—for example, cells at 10^8/ml infected at an input multiplicity of 2 for 5 min at 37°C and then diluted into phage antiserum are only infected to the extent of 1 cell in 50).

The inhibition by 10^{-3}M FU is completely suppressed by the simultaneous addition of 10^{-4}M uridine, whereas the addition of 5×10^{-5}M thymidine is without effect. Since 5-fluorodeoxyuridine in the presence of uridine does not inhibit this RNA phage (Davern 1964), it may be concluded that FU is interfering with some aspect of uridine metabolism or function.

(b) *Kinetics of FU Inhibition*

Aliquots of an infected culture were transferred to media containing 2×10^{-4}M FU at various times after infection and the growth curves of the phage were followed in the presence of FU. These growth curves are plotted in Figure 1. The phage yield continued to rise with time throughout the experiment for the control culture, whereas upon transfer to FU medium, the phage yield rose for no more than 45 min.

A closer study of the effect of transferring infected culture aliquots to FU media at various times after infection is shown in Figure 2. The yield of phage was estimated, after lysis, both at the time of transfer and after incubation for at least 65 min in the presence of FU for each of a succession of samples taken from the infected culture. Here, where the infected cells were washed almost free of unadsorbed phage, it can be seen that the yield of phage is lower after incubation in FU for the earliest transfer time, and higher for later transfer times than the yield obtained at the time of transfer. These results indicate that the FU blocks or interferes with an early event in the infection cycle.

At the earliest transfer time, a significant proportion of the titratable phage probably represents a residuum of recoverable absorbed phage, which on further incubation in the presence of FU proceeds with the infection process and thus becomes lost to further assay.

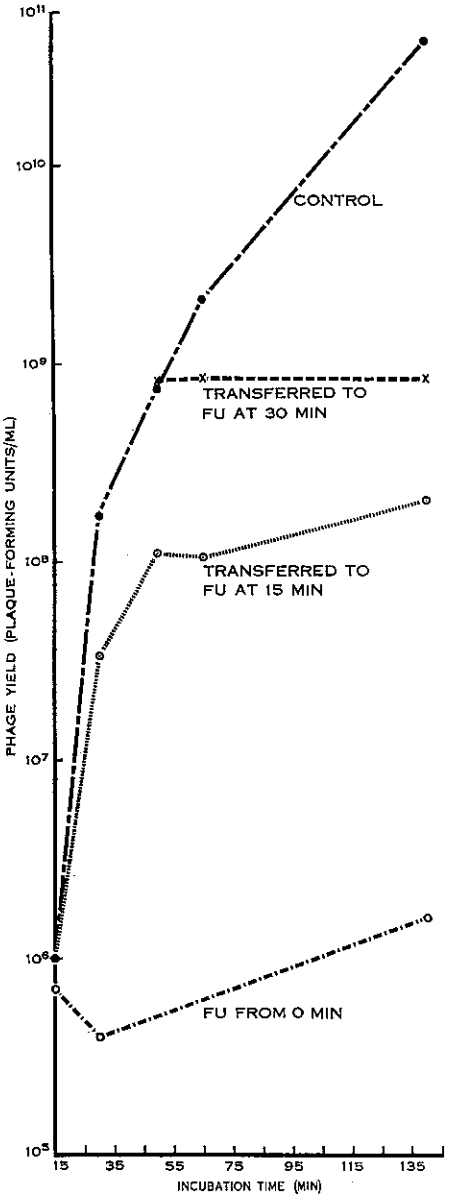

Fig. 1

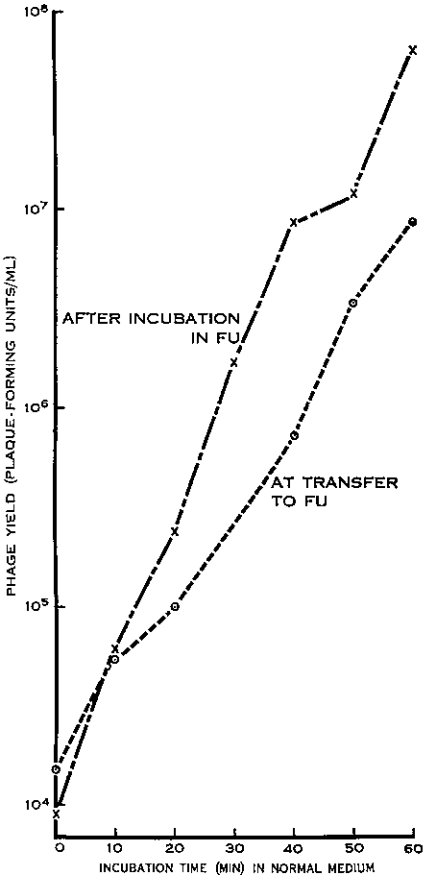

Fig. 2

Fig. 1.—Effect on phage yield of transfer of phage-infected cells to FU-containing medium at various times during the phage growth cycle.

Fig. 2.—Phage titres at various times of transfer to FU-containing medium, and after incubation for at least 60 min in the presence of FU, for samples taken at various times after infection.

(c) Loss of Ability of Infected Cells to Yield Progeny with Time of Incubation in FU

The effect of incubation of infected cells in the presence of FU on their subsequent ability to produce infectious progeny was investigated by following the change in the ability of infected cells to register as infective centres in the plaque assay. A 5-ml cell culture, in glucose–mineral salts medium grown to $2{\cdot}4\times10^8$ cells/ml, was infected at an input multiplicity of 1. After phage adsorption for 10 min at 37°C, the culture was chilled, and the cells centrifuged and resuspended in 1 ml cold fresh medium. An aliquot of this infected cell suspension (0·5 ml) was then dispensed into 4·5 ml medium containing 10^{-3}M FU and a 5×10^{-2}-fold dilution of phage antiserum to inactivate unadsorbed phage. Then a 0·1-ml aliquot of this suspension was diluted 50-fold into each of two tubes of medium containing 10^{-3}M FU.

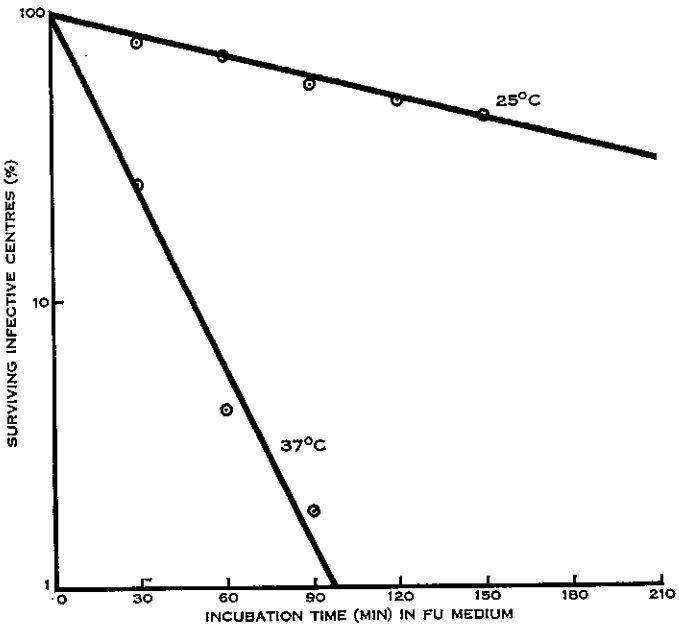

Fig. 3.—Decline in the ability of phage-infected cells to yield infective progeny phage with time of incubation in the presence of 10^{-3}M FU. The decline is recorded for two incubation temperatures.

These dilute cell suspensions were then incubated at 37 and 25°C respectively. Samples were taken throughout the incubation period for the assay of cells as infective centres. The 2000-fold dilution of the sample for the assay reduced the concentration of phage antiserum to an ineffective level and the dilution, coupled with the plating of the cells on the broth agar of the assay plates, served to reverse the FU inhibition. The decline in the ability of the infected cells to act as infective centres with time in the presence of FU is logarithmic with a half-time of 15 min at 37°C and 118 min at 25°C (Fig. 3).

Since FU treatment has been reported to induce cell-wall degeneration with consequent cell lysis in a strain of *E. coli* K-12 (Tomasz and Borek 1959, 1960), the

possibility that lysis may have been responsible for the infective centre decline observed in the present work was checked by following the viable count of uninfected Hfr_1 cells cultured in the presence of 10^{-3}M FU. No change in viable cell count was observed over an incubation period of 2 hr in the FU medium.

If, as seems likely, the initiation of phage replication in a population of infected cells is asynchronous (Davern 1964) and the probability of initiation is constant for some time after infection, then just such a logarithmic decline in infective centre titre would be expected if cells that initiated phage multiplication in the presence of FU were consequently to lose their ability to produce infective phage. Technical limitations preclude an investigation of the nature of the decline in infective centres in the first 15 min. Thus the maximum time that an initiated cell can spend in the presence of FU before it loses its ability to produce phage on reversal can only be estimated as something less than 15 min.

(*d*) *Immunodiffusion Analysis for Phage Antigen from FU-inhibited Cultures*

The possibility that non-infectious phage particles were formed in the presence of FU was tested by assaying for the formation of phage antigen by the agar gel immunodiffusion test.

Four 200 ml cultures of Hfr_1 in the log phase of growth were grown to 2×10^8 cells/ml in glucose–mineral salts medium. Three of the cultures were infected with the RNA phage at input multiplicity of 10, and 10^{-3}M FU added to one of these infected cultures. The uninfected culture and one of the infected cultures were immediately centrifuged and the cell pellets resuspended in 5 ml of neutral saline and frozen in dry ice–acetone. The infected culture containing the FU was incubated for 4 hr, while the control infected culture was incubated for 2 hr. The cultures were then centrifuged and processed in the same way as the first two cultures. The four frozen 5-ml cell suspensions were then disrupted by ultrasonic vibration. An aliquot from each infected cell suspension was taken for phage assay, and another aliquot was taken from each of the four suspensions for immunodiffusion analysis. The results of the immunodiffusion analysis are shown in Figure 4. Only the infected control which had been incubated for 2 hr and the FU-treated infected cell preparations which had been incubated for 4 hr contained phage-specific antigen.

To obtain an estimate of the amount of phage antigen produced in the presence of FU relative to the amount produced in the absence of FU, a second gel diffusion plate was set up containing an undiluted sample of the FU preparation and a series of twofold dilutions of the control infected preparation. From Figure 5 it can be seen that the infected culture exposed to FU produced about one-eight of the phage-specific antigen found in the control culture. Since the ratio of infectious phage yield from the FU culture to the control culture is 1/152, the FU culture contained 19 times more phage-specific antigen than would be expected from its infectious phage titre. Furthermore, the phage-specific antigen could be completely sedimented from both control and FU culture preparations by centrifugation at 50,000 r.p.m. for 90 min in the Spinco model L ultracentrifuge (No. 50 head). Thus the phage-specific antigen produced in the presence of FU is particulate, and hence is probably non-infectious phage.

(e) *Induction of Mutants Sensitive to High Temperatures by FU*

The production of non-infective phage particles in the presence of FU suggests that FU acts as a mutagen. This possibility was tested in the following experiments.

The induction of a special class of mutant, the "conditional" lethal, was chosen as the criterion of FU mutagenic activity. These mutants are phenotypically normal in one environment and lethal in another. While they are not as simply detected as straight morphological mutants, they have the advantage that any gene coding for an essential protein, be it enzymic, structural, or regulatory in function, should be susceptible to mutational modification to a conditional lethal state. In the present work the phenotypic character assayed for was the malfunction of the mutant protein under high temperature conditions.

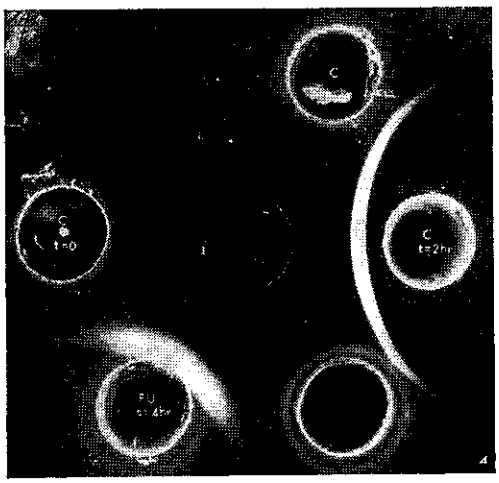

Fig. 4

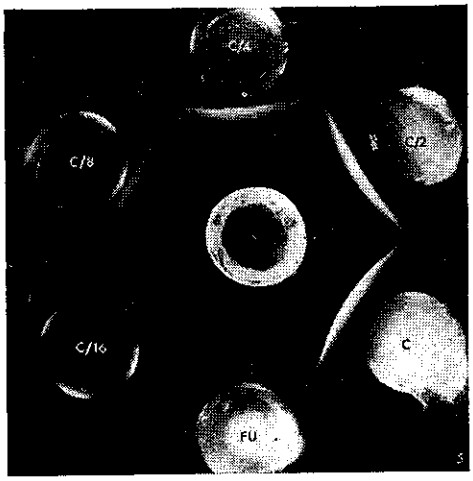

Fig. 5

Fig. 4.—Immunodiffusion analysis for phage-specific antigen production in the presence of FU. The sample wells contained cell suspensions (cells disintegrated by ultrasonic vibration) of uninfected cells (C), infected cells disrupted immediately after infection (C, $t = 0$), infected cells disrupted 2 hr after infection (C, $t = 2$ hr), and infected cells incubated in the presence of 10^{-3}M FU for 4 hr (FU, $t = 4$ hr).

Fig. 5.—Estimation of the relative amounts of phage-specific antigen production in the presence and absence of FU by matching precipitin band intensities of the culture containing FU and a twofold dilution series of the control infected culture.

Two cultures of Hfr_1 in the log phase of growth and at 4×10^8 cells/ml were infected with $f_{Can\,1}$ at an input multiplicity of about 1, and 10^{-4}M FU was added to one of the cultures. After incubation for $4\frac{1}{2}$ hr at 37°C the cultures were lysed and assayed for infective phage at 37°C. The control culture yielded $1{\cdot}6 \times 10^{12}$ infective phage/ml, while the FU culture yielded $1{\cdot}8 \times 10^9$ infective phage/ml.

The effect of assay temperature on relative plating efficiency for phage from the control and FU cultures was examined by plating equal volumes of a suitable dilution of each phage preparation for assay at 31, 37, and 43·5°C. Plaque yield for phage from control cultures was, respectively, 694, 609, and 820 for these temperatures,

whilst the corresponding yields for the FU cultures were 217, 405, and 93. These results indicate that while the relative plating efficiency of the phage from control cultures is, if anything, best at 43·5°C, it is worst at this temperature for phage from FU-cultures, where a fourfold drop in plating efficiency was observed relative to the 37°C assay. This latter phage also seems to plate relatively poorly at 31°C.

Forty plaques were picked at random from three plates which had been inoculated with dilute aliquots of the phage from FU cultures and incubated at 37°C. Each plaque was picked into 10 ml of neutral saline containing chloroform to kill any bacteria carried over. On plaque assaying suitably dilute aliquots of these phage clones at 37 and 43·5°C, 10 of them failed to give plaques at the higher temperature. By comparison none of 104 plaques tested from a control phage stock failed to give plaques at the higher temperature. The temperature-sensitive clones were recultured and retested. All of them proved to be temperature-sensitive mutants. Thus the proportion of temperature-sensitive mutants which arose in the presence of 10^{-4}M FU was 25%. The technique of screening used here would overlook delayed mutants which may arise on the assay plates. This possibility, coupled with carryover of unadsorbed normal input phage, would tend to lead to an underestimation of the mutagenic activity of FU in this system.

In another experiment where FU was used at a concentration of 2×10^{-5}M, and 146 single plaque clones were tested, the proportion of temperature-sensitive mutants was 9%.

(*f*) *Stability of FU-induced Temperature-sensitive Mutants*

Stock cultures of some of the temperature-sensitive mutants were assayed at 37 and 44°C to detect the proportion of temperature-insensitive revertants. This proportion only provides an approximate estimate of reversion rate, since it is a function of both the true reversion rate and the selective advantage that the revertants may have had during the preparation of the phage stocks. Eight of the mutant stocks tested contained from 5 to 30 revertants per 10^4 phage.

One of the mutant stocks (td 95) contained only 1 in 10^6 revertants. In the absence of genetic analysis, it cannot be decided whether the latter mutant is more stable than the others, or is a double mutant.

(*g*) *Complementation Tests*

With such a small organism as this RNA phage, it may be a feasible proposition to count the number of protein-coding cistrons it contains by means of complementation tests among independently derived, FU-induced, single-site, temperature-sensitive mutants, provided recombination is rare enough not to obscure the complementation assay. In complementation tests involving seven and six mutants respectively, no evidence of complementation was observed. Either all these mutants involved the same gene or they involved genes among which complementation is not possible. Alternatively some kind of interference may have been preventing mixed infection. The latter possibility was tested by determining the character of the progeny phages derived from a 1 : 1 mixture of cells infected with wild-type

phage and the relatively stable temperature-sensitive mutant (td 95). Tubes of mixed and pure phage suspensions were adsorbed to broth-cultured cells in the log phase of growth, at an input multiplicity of 100, for 5 min at 37°C. Then $\frac{1}{3}$ vol. of 1 in 100 phage antiserum was added to each tube. After incubation for 10 min in the antiserum, the infected cell suspensions were diluted and plated at 37 and 44°C. The infective centre counts at each temperature for each kind of phage infection are given in the following tabulation:

	Plaque Count	
	37°C	44°C
Cells infected with:		
Wild-type phage only	360	308
td 95 phage only	431	0
1 : 1 mixture	341	181

Nineteen single-plaque progenies were picked from the 37°C assay plate of the mixed infection and spotted onto two sets of agar plates seeded with indicator bacteria. One set of these plates was then incubated at 37°C and the other set incubated at 44°C. Six of the 19 plaques proved to contain only temperature-sensitive phage. The data from the tabulation above, together with the result of this progeny characterization, indicate the occurrence of interference.

Further studies on this interference phenomenon, where the effect of sequential mixed infection on the character of isolated plaque progenies was examined, confirmed the extent of this interference. For example, in an experiment where the second phage was added 5 min after the first phage, then, when td 95 is first, 15 out of the 20 plaque progenies tested were temperature-sensitive, whereas when td 95 was the second phage, only 1 out of 20 plaque progenies tested was temperature-sensitive.

Until some way is devised to overcome this interference no further progress can be made in the genetic analysis of this phage.

IV. Discussion

The inhibition of $f_{Can\,1}$ phage by FU, unlike that of polio and tobacco mosaic viruses, can be explained in terms of FU-induced lethal mutations as well as by possible secondary effects on the bacteria in its capacity as a host.

There are a number of possible reasons why organisms vary in their response to the incorporation of FU into their RNA. The probability of tautomeric shift or ionization of FU could vary from organism to organism because of pH variation. In this connection, Goldberg, Dahl, and Parks (1963) found a pH-dependence for the reaction of FU as a substrate with uridine diphosphate glucose dehydrogenase, which could be related to the ratio of the un-ionized form to the ionized form of FU. Freese (1963) emphasizes the possible influence of adjacent bases on the probability that FU in a particular location in an RNA molecule will act as a mutagen. With the considerable species variation in RNA base composition, this "nearest neighbour" effect could be an important source of variation in FU mutagenic activity.

In addition, the phenotypic consequences of FU-induced mutations are expected to vary with the nature and the position of the change in any particular gene, or gene message. The effect of FU on polio virus is significant in this con-

nection, for, while the plating efficiency of polio virus culture in the presence of FU is normal at normal assay temperatures, Cooper (1964) found that its plating efficiency is reduced by a factor of 3–10 relative to that of normal virus when assayed at high temperatures. The FU-free progeny of the FU-substituted polio virus DNA retained this high-temperature sensitivity, and at least 10% of the clones isolated from this progeny proved to be mutants sensitive to high temperatures. Thus FU, while showing high mutagenic activity for at least part of the polio genome, induces mutations which are of trivial phenotypic consequence under normal assay conditions.

Stock preparations of most of the FU-induced temperature-sensitive mutants contain about 10^{-3} revertants. Since the preparation of these stocks involves an increase in phage titre of about 10^4, the reversion frequency per replication event is at least an order of magnitude less, allowing that the revertants may have a selective advantage over the mutants.

The attempts to make a genetic analysis of the phage $f_{Can\,1}$ with these temperature-sensitive mutants have, so far, been frustrated by interference.

V. Acknowledgments

The skilled technical assistance of Mrs. H. Brzostowska is gratefully acknowledged. The author also wishes to thank A. F. Hoffman-La Roche and Co., Switzerland, for a gift of 5-fluorouracil.

VI. References

Adams, M. H. (1959).—"Bacteriophages." (Interscience Publishers, Inc.: New York.)

Aronson, A. I. (1961*a*).—The inhibition of bacteriophage protein synthesis by 5-fluorouracil. *Biochim. Biophys. Acta* **49**: 89–97.

Aronson, A. I. (1961*b*).—The effect of 5-fluorouracil on bacterial protein and ribonucleic acid synthesis. *Biochim. Biophys. Acta* **49**: 98–107.

Benzer, S., and Freese, E. (1958).—Induction of specific mutations with 5-bromouracil. *Proc. Nat. Acad. Sci., Wash.* **44**: 112–19.

Bussard, A., Naono, S., Gros, F., and Monod, J. (1960).—Effects d'un analogue de l'uracil sur les proprietés d'une protéine enzymatique synthetisée en sa précence. *C. R. Acad. Sci., Paris* **250**: 4049–51.

Campbell, A. (1962).—Effect of 5-fluorouracil on suppressor sensitive mutants of bacteriophage lambda. *Bact. Proc.* **1962**: 145.

Champe, S. P., and Benzer, S. (1962).—Reversal of mutant phenotypes by 5-fluorouracil: an approach to nucleotide sequences in messenger-RNA. *Proc. Nat. Acad. Sci., Wash.* **48**: 532–46.

Cooper, P. D. (1964).—The mutation of poliovirus by 5-fluorouracil. *Virology* **22**: 186–92

Cooper, S., and Zinder, N. D. (1962).—The growth of an RNA bacteriophage. The role of DNA synthesis. *Virology* **18**: 405–11.

Davern, C. I. (1964).—The isolation and characterization of an RNA bacteriophage. *Aust. J. Biol. Sci.* **17**: 719–25.

Freese, E. (1959).—The specific mutagenis effect of base analogues on phage T4. *J. Mol. Biol.* **1**: 87–105.

Freese, E. (1963).—The molecular mechanisms of mutations. In "Biological Structure and Function at the Molecular Level". (Symp. Fifth Int. Congr. Biochem., Moscow, 1961.) (Ed. V. A. Engelhardt.) I.U.B. Symp. Series Vol. 21. pp. 204–26.

Goldberg, N. D., Dahl, J. L., and Parks, R. E. Jr. (1963).—Uridine diphosphate glucose dehydrogenase pH dependence of the reactions with 5-fluorouracil and 6-azauracil analogues of uridine diphosphate glucose. *J. Biol. Chem.* **238**: 3109–14.

GOODMAN, F., SAUKKONEN, J. J., and CHARGAFF, E. (1960).—Patterns of cellular controls operating in bacteriophage reproduction. 1. Effect of 5-fluorouracil on the multiplication of several coliphages. *Biochim. Biophys. Acta* **44**: 458–68.

GORDON, M. P., and STAEHELIN, M. (1958).—The incorporation of 5-fluorouracil into the nucleic acid of tobacco mosaic virus. *J. Amer. Chem. Soc.* **80**: 2340–1.

HEIDELBERGER, C., ET AL. (1957).—Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. *Nature* **179**: 663–6.

HOLOUBEK, V. (1963).—The composition of tobacco mosaic virus protein after the incorporation of 5-fluorouracil into the virus. *J. Mol. Biol.* **6**: 164–6.

HOROWITZ, J., and CHARGAFF, E. (1959).—Massive incorporation of 5-fluorouracil into a bacterial ribonucleic acid. *Nature* **184**: 1213–15.

KAHAN, F. M., and HURWITZ, J. (1962).—The role of deoxyribonucleic acid synthesis. IV. The incorporation of pyrimidine and purine analogues into ribonucleic acid. *J. Biol. Chem.* **237**: 3778–85.

KEMPNER, E. S., and MILLER, J. H. (1962).—Alteration of carbon metabolism by a base analog. *Biophys. J.* **2**: 327–37.

LAWLEY, P. D., and BROOKES, P. (1962).—Ionization of DNA base analogues as a possible explanation of mutagenesis, with special reference to 5-bromodeoxyuridine. *J. Mol. Biol.* **4**: 216–19.

LITMAN, R. M., and PARDEE, A. B. (1956).—Production of bacteriophage mutants by a disturbance of deoxyribonucleic acid metabolism. *Nature* **178**: 529–31.

LITMAN, R. M., and PARDEE, A. B. (1960*a*).—The induction of mutants of bacteriophage T2 by 5-bromouracil. III. Nutritional and structural evidence regarding mutagenic action. *Biochim. Biophys. Acta* **42**: 117–30.

LITMAN, R. M., and PARDEE, A. B. (1960*b*).—The induction of mutants of bacteriophage T2 by 5-bromouracil. IV. Kinetics of bromouracil-induced mutagenesis. *Biochim. Biophys. Acta* **42**: 131–40.

LOEB, T., and ZINDER, N. D. (1961).—A bacteriophage containing RNA. *Proc. Nat. Acad. Sci., Wash.* **47**: 282–9.

MUNYON, W., and SALZMAN, N. P. (1962).—The incorporation of 5-fluorouracil into poliovirus. *Virology* **18**: 95–101.

NAONO, S., and GROSS, F. (1960).—Effects d'un analogue de base nucléique sur la biosynthèse de protéines bactériennes. Changements de la composition global des protéines. *C.R. Acad. Sci., Paris* **250**: 3527–9.

SAUKKONEN, J. J., GOODMAN, F., and CHARGAFF, E. (1960).—Patterns of cellular controls operating in bacteriophage reproduction. II. Effect of 5-fluorouracil on metabolic events in bacteria infected with coliphage T2r. *Biochim. Biophys. Acta* **44**: 469–77.

SHUG, A., MAHLER, U. R., and FRASER, D. (1960).—Studies in partially resolved bacteriophage-host systems. VI. The involvement of RNA in various aspects of the reproduction of phage T2. *Biochim. Biophys. Acta* **42**: 255–71.

TERZAGHI, B. E., STREISINGER, G., and STAHL, F. W. (1962).—The mechanism of 5-bromouracil mutagenesis in the bacteriophage T4. *Proc. Nat. Acad. Sci., Wash.* **48**: 1519–24.

TOMASZ, A., and BOREK, E. (1959).—An early phase in the bacterial action of 5-fluorouracil on *E. coli* K12: osmotic imbalance. *Proc. Nat. Acad. Sci., Wash.* **45**: 929–32.

TOMASZ, A., and BOREK, E. (1960).—The mechanism of bacterial fragility produced by 5-fluorouracil: The accumulation of cell wall precursors. *Proc. Nat. Acad. Sci., Wash.* **46**: 324–7.

WATSON, J. D., and CRICK, F. H. C. (1953).—The structure of DNA. *Cold Spring Harb. Symp. Quant. Biol.* **18**: 123–31.