Effect of 5-Fluorouracil on the Growth of Bacteriophage R17

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ABSTRACT

GRAHAM, A. F. (The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.), and CLARE KIRK. Effect of 5-fluorouracil on the growth of bacteriophage R17. J. Bacteriol. 90:928-935. 1965.—When added to Escherichia coli within 2 min after phage R17, 5-fluorouracil (FU), at a concentration of 10^{-5} M, completely inhibited the synthesis of infectious ribonucleic acid (RNA) and phage. If the addition of FU was made later than 5 min after infection, infectious RNA synthesis was blocked but infectious phage was still formed; the infectious RNA made before the addition of FU continued to be incorporated into mature phage. These properties of the inhibitor were used to determine the kinetics of phage RNA synthesis and the size of the phage precursor RNA pool. At a concentration of 2.2 \times 10^{-4} M FU, the yield of phage was reduced to 15\% of that in an uninhibited control, 28\% of the phage RNA uracil was replaced with FU, and the specific infectivity of the phage was unaltered.

The uracil analogue, 5-fluorouracil (FU), may be incorporated into ribonucleic acid (RNA) in a number of replicating systems where it replaces uracil (Horowitz and Chargaff, 1959; Gros and Nanno, 1961; Gordon and Staehelin, 1959; Monyon and Salzman, 1962; Holoubek, 1963). For RNA-containing viruses, up to 47\% of the uracil of tobacco mosaic virus (Gordon and Staehelin, 1959) and 36\% of the uracil of poliovirus (Monyon and Salzman, 1962) could be replaced by FU without change in the specific infectivities of the viruses. In this paper, we present some results on the incorporation of FU into phage R17. It is shown that substitution of 28\% of the uracil by FU leads to no change in the infectivity of the virus. It was found that the formation of infectious RNA was blocked by an extracellular concentration of 10^{-5} M FU. This inhibitory effect was utilized to determine the kinetics of synthesis of phage precursor RNA. Shimura and Nathans (1964) and Davern (1964) also investigated some of the inhibitory effects of FU on their RNA phage systems, and their results will be referred to later.

MATERIALS AND METHODS

The bacterial strain Escherichia coli K-12, Hfr, the phage R17, and the tria(hydroxymethyl)aminomethane-maleate medium (TMM) were previously described (Paranchych and Graham, 1962; Ellis and Paranchych, 1963). Prior to infection, bacteria were grown to a concentration of 3 \times 10^{8} per milliliter in TMM, centrifuged, and suspended in TMM at 10^{9} per milliliter at 37 C. Phage was added to the desired multiplicity (m). Adsorption was at least 80\% complete in 2 min. At 2 min after adding the phage, the culture was chilled and centrifuged, and the cells were suspended in TMM to a density of 3 \times 10^{8} per milliliter. On some occasions, the infected culture was merely diluted to 3 \times 10^{8} bacteria per ml with TMM instead of being centrifuged. Infected cultures were artificially lysed at will by shaking them with 10 \mu g/ml of lysozyme and one drop of chloroform for 15 min at 37 C (Cooper and Zinder, 1962).

Infectious RNA was extracted and assayed as described by Paranchych (1963). Phage antisem was kindly supplied by A. Hagopian. It was prepared by subcutaneous injection of purified phage and adjuvant into rabbits. A booster injection was given intravenously after 3 weeks, and the rabbit was bled after 1 month. Such antisem had k = 4,500 to 6,500 (Adams, 1959).

FU was obtained through the kindness of Hoffman-La Roche, Inc., Nutley, N.J. FU-C^{14} was supplied by Calbiochem at a specific activity of 20 mc/m mole. Before use, FU-C^{14} was tested for radioactive purity by one-dimensional paper chromatography in butanol - water - ammonia (Gordon and Staehelin, 1959).

Radioactive isotopes were assayed with a Tricarb liquid scintillation counter as previously described (Ellis and Paranchych, 1963).

Purification of phage. Infected cultures (20 to 100 ml) were lysed artificially 120 min after infection, and solid ammonium sulfate was added to a concentration of 2 M. After being left for 18 hr at 4 C, the mixture was centrifuged at 10,000 \times g.
for 20 min. The sediment was stirred for 10 min with 5 ml of TMM and centrifuged at 10,000 × g for 20 min to remove nonviral particulate material. An equal amount of nutrient broth and 0.1 ml of a 1% solution of gelatin were added to the supernatant solution which was then centrifuged at 100,000 × g for 3 hr. Broth (0.2 ml) containing 100 µg of crystalline trypsin was added to the pellet, and the mixture was left at 4°C for 18 hr. Crystalline ribonuclease (100 µg) was then added and, after 2 hr at 37°C, the mixture was layered over a 5 to 20% linear sucrose gradient. The gradient was centrifuged in the SW39 head of the Spinco model L centrifuge for 90 min at 35,000 rev/min, the tube was punctured, and two-drop samples were collected into 0.5 ml of broth. The three samples comprising the major part of the phage peak were combined and used for the necessary assays. In some instances, to be described later, the phage was further sedimented in a cesium chloride gradient of average density 1.43 for 24 hr at 35,000 rev/min.

RESULTS

Effect of different concentrations of FU on formation of phage. Figure 1 shows the kinetics of phage multiplication in bacteria in which FU was added shortly after infection. Bacteria were infected at m = 10, and 2 min later the culture was chilled and centrifuged, and the bacteria were suspended in fresh medium and divided into three portions. One portion was left as a control, and FU was added to the other two portions to concentrations of 1.5 × 10⁻⁵ and 10⁻⁴ M. The three cultures were placed at 37°C, and samples were taken at intervals, artificially lysed, and assayed for phage.

There was no production of phage at 10⁻⁴ M FU. Similar results were obtained by Cooper and Zinder (1962), Shimura and Nathans (1964), and Davern (1964) at somewhat higher concentrations of FU. With a concentration of 1.5 × 10⁻⁵ M FU, phage production lagged considerably behind that of the control culture. In the following sections, some properties of the system at each of these FU concentrations will be considered.

Reversal of FU inhibition by uracil. Inhibition of phage synthesis by FU can be reversed by uracil (Cooper and Zinder, 1962; Davern, 1964). The following experiment shows that the extent of reversal depends on the time of addition of uracil. FU was added to a bacterial culture to 10⁻⁴ M, and immediately afterwards phage was added to m = 10. At intervals, a portion of this culture was withdrawn and made 10⁻³ M with uracil. All cultures were kept at 37°C until 100 min after infection, lysed, and assayed for phage. It is clear from the results in Fig. 2 that inhibition by FU was completely reversible only until approximately 20 min after infection. This result would be explicable if it had taken 20 min to saturate the bacterial pools with FU. The following experiment shows that this was not the case. FU (10⁻⁴ M) was added to several bacterial cultures at different times before infection. Phage was then added at time-zero. After 15 min, uracil was added to each culture to 10⁻³ M, and after a further 120 min the cultures were lysed. The
lysate titer of each culture was similar to that of a control without FU. Thus, the inhibition in cultures pretreated with FU for as long as 30 min before infection was still fully reversible by uracil added 15 min after infection. The following experiment shows that inability to reverse this inhibition completely 20 to 30 min after infection is undoubtedly related to a decrease in infectious centers caused by FU.

Change in infectious centers in the presence of FU. Two cultures were infected at \( m = 10 \). FU (\( 10^{-4} \) m) was added to one culture at the time of infection. The same concentration of FU was added to the other culture 13 min after infection. At intervals, infectious centers were determined in each culture by titration through antiserum that contained \( 10^{-4} \) m uracil. The results (Fig. 3) show that when FU was added 13 min after infection there was little or no decrease in infectious centers until 40 min. This later decrease in infectious centers resulted from lysis of bacteria by phage that had matured in the presence of FU, as shown later. However, when the FU was added at the time of infection, there was a rapid decline in infectious centers beginning at 15 to 20 min. Davern (1964) also observed a decrease in infectious centers in the presence of FU. Other experiments showed that even when added 2 min after infection FU did not cause as rapid a disappearance of infected cells as when added at time-zero. The rate of loss of infectious centers thus depended on how late after infection the FU was added. Some early reaction occurs in infected cells that prevents their loss as infectious centers when FU is added later.

It was also found, in other experiments, that when FU (\( 10^{-4} \) m) was added at the time of infection there was a rapid decrease in the amount of parental infectious RNA that could be extracted from the cells. In some cases, this loss amounted to over 95% during the first 20 min after infection. The possible relationship between the results presented in this section will be discussed later.

Reversal of FU inhibition: kinetics of phage and infectious RNA formation. The following experiment was done to determine whether the synthesis of phage components commenced immediately on reversal of FU inhibition. At 2 min after infection of a culture at \( m = 10 \), FU was added to 10\(^{-4} \) m. At 20 min after infection, uracil was added to 10\(^{-3} \) m. Samples were removed at intervals for assay of infectious RNA and total phage. Infectious RNA began to form 4 to 5 min after reversal, and mature phage appeared after an
additional 10 min. Both continued to form at very close to the normal rate (Paranchych, 1963). The presence of FU thus shortened the total eclipse period to 6 to 7 min for infectious RNA and to 16 to 17 min for phage. These estimates for the eclipse period are about 3 min less than usually found in uninhibited cultures but are perhaps not significantly less. This is additional evidence that the step inhibited by FU is an early one. Since an RNA synthetase is formed in infected cells in the presence of FU (Lodish, Cooper, and Zinder, 1964), FU may block one of the preliminary stages in replication of phage RNA.

Kinetics of infectious RNA and phage synthesis after late addition of FU. FU (10^-4 M) was added to one-half of an infected culture 17 min after addition of phage at m = 10. Samples were removed from both portions at intervals and assayed for infectious RNA. The results (Fig. 4) show that there was an immediate and complete inhibition of infectious RNA synthesis. In another experiment, a culture was infected at m = 10 and divided in two parts after 20 min. FU was added to one portion at 10^-4 M, and samples were taken at intervals from both portions for assay of total phage (Fig. 5). After addition of FU, mature phage continued to form for a further 15 min at almost the same rate as in the uninhibited control, as Davern (1964) also found. Apparently, after the late addition of FU at 20 min the already-formed infectious RNA continued to be incorporated into mature phage.

Use of FU inhibition to determine the kinetics of formation of phage RNA. The results obtained with FU inhibition suggested a method of estimating the kinetics of phage RNA synthesis. Thus, if 10^-4 M FU is added at any time to an infected culture and phage formation is then allowed to go to completion, the final amount of phage formed should indicate the total amount of phage RNA present in the cell at the time FU was added. A similar technique with other inhibitors was employed by Flanagan and Ginsberg (1962) to determine the kinetics of viral DNA and protein synthesis during infection of KB cells with adenovirus.

The kinetics of phage RNA synthesis determined by the FU method were first compared with those for the formation of infectious RNA. A culture of bacteria was infected at m = 10. At intervals, FU was added to small portions of this culture to 10^-4 M, and at the same times, samples were withdrawn from the main culture for extraction and assay of infectious RNA. Those small portions containing FU were incubated until 90 min, when phage production was practically complete, lysed, and assayed for phage. It was observed that phage RNA determined by the FU technique increased in parallel with infectious RNA. Essentially, the two methods measure the same quantity. Sanders (1960) found a similar situation with the encephalomyocarditis virus-Krebs 2 cell system.

In further experiments, phage was added to bacteria; after 2 min the cultures were chilled and centrifuged, and the bacteria were suspended in fresh medium and placed at 37 C. At intervals, samples were taken for assay of total phage. At similar times, portions of the cultures were made.

FIG. 5. Kinetics of phage formation when FU was added 20 min after infection to a concentration of 10^-4 M. Control, no FU, ○; after addition of FU, ●.

FIG. 6. Kinetics of formation of phage RNA determined by the FU inhibition technique, and of mature phage, under conditions of multiple infection (m = 10). RNA, ●; phage, ○.
10^{-4} \text{m} \text{ in FU, incubated until 120 min, lysed, and assayed for phage. Figures 6 and 7 show the results of experiments carried out with multiplicities of 10 and 0.1, respectively.}

The early course of RNA synthesis is clearer in Fig. 7 because of the low background of unadsorbed phage in the singly infected culture. With multiply infected cultures, there is a significant elution of adsorbed virus at early times after infection that raises this background level (Paranchych and Graham, 1962). Nevertheless, in both cultures phage maturation commenced at 20 min and phage RNA started to form at approximately 5 to 7 min after infection. The time at which phage RNA began to form is somewhat earlier than was generally found by assay of infectious RNA, probably because the FU technique is more sensitive at earlier periods. It is clear from these data, as it was from infectious RNA measurements (Paranchych, 1963; Davis, Pfeifer, and Sinsheimer, 1964) and the kinetics of P32 incorporation (Cooper and Zinder, 1962), that a considerable pool of phage precursor RNA accumulates prior to phage maturation. To determine the size of this pool in phage-equivalent units, it was necessary to know the ratio of non-infectious to infectious particles for R17 phage.

Table 1. Ratio of phage particles to infectious particles at different times after infection

<table>
<thead>
<tr>
<th>Time after infection (min)</th>
<th>Lysate titer (phage/ml)</th>
<th>Phage particles per infectious particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>7.4 \times 10^{11}</td>
<td>7.3</td>
</tr>
<tr>
<td>120</td>
<td>1.7 \times 10^{12}</td>
<td>8.7</td>
</tr>
<tr>
<td>180</td>
<td>1.5 \times 10^{11}</td>
<td>6.4</td>
</tr>
</tbody>
</table>

The following experiment yielded this information.

Ratio of noninfectious to infectious particles in mature phage. Bacteria were infected at m = 10, washed, and transferred into medium containing P32 of known specific activity. After 60, 120, and 180 min at 37 \text{C}, 20-ml samples were removed and lysed, and the phage was purified. Each purified preparation was assayed for infectivity and P32. Phage phosphorus is obtained entirely from the exogenous supply (Ellis and Paranchych, 1963). Thus, the number of phage particles in each preparation could be calculated from the P32 content, appropriate phage constants (Enger et al., 1963; Mitra, Enger, and Kaesberg, 1963), and the specific activity of the medium, and then referred to the infectivity titer. The average number of particles per infectious unit from this experiment (Table 1), and two others shown later (Table 2), was seven. In addition, Table 1 shows that phage produced after 60 min, amounting to 60 to 70\% of the total phage yield, had a constant specific infectivity.

Size of the phage RNA pool. The best estimates for the early size of the phage RNA pool are made from the data of Fig. 7. The total amount of phage RNA and the amount of RNA contained in mature phage were plotted in Fig. 8a in terms of phage-equivalent units per infected cell. One unit of RNA, whether precursor RNA or mature phage RNA, is that amount contained in one phage particle, or 1.96 \times 10^{-13} \mu g (Mitra et al., 1963). At 5 min after infection, there were about 15 units of phage RNA per cell, and at 20 min, just at the time phage maturation commenced, there were 1,500 units. This latter estimate is in agreement with the result of Davis et al. (1964), if it is assumed that their MS2 phage contained seven particles per infectious unit as with R17. Since lysis and reinfection of cells after 40 min or so confuse the later picture in a single infection experiment, data from the multiple infection experiment are shown in Fig. 8b. There is some uncertainty in these latter calculations, since the number of infected cells in a multiply infected culture changes with time (Paranchych and Graham, 1962). This is clearly shown in Fig. 3,
where there is almost a twofold increase in infectious centers between 5 and 15 min. In a number of such experiments, about 70% of the bacteria finally became infected by 20 min, and there was then little further increase. In plotting the lower curves of Fig. 8, it was assumed that the culture contained $2 \times 10^6$ infected cells throughout the course of infection. At 20 min after infection, the precursor pool of phage RNA was about 2,000 units ahead of mature phage. The infected cell produced about 40,000 units of phage RNA altogether, and this production was almost complete in 60 min. At this time, the pool was about 15,000 units ahead of mature phage, and then started to decrease as phage maturation continued.

Incorporation of FU into phage. As shown in Fig. 1, phage multiplication took place in the presence of $1.5 \times 10^{-4}$ M FU, although at a much decreased rate from the uninhibited control. This inhibition could have been explained if incorporation of FU into phage RNA had decreased the specific infectivity of the phage. To answer this question, experiments were carried out as follows. Phage was added to bacteria at $m = 10$. After 2 min for adsorption, the infected cells were centrifuged, washed, suspended, and distributed into several flasks. Each flask contained the same amount of P32 and different amounts of FU-C14, both of known specific activity. After being shaken at 37°C for 150 min, the cultures were lysed artificially and assayed. Phage from each culture was then purified and assayed for P32, C14, and infectivity. The number of particles per infectious phage was calculated, as described in the preceding section, and from the known specific activity of FU-C14 the amount of FU in each preparation could be determined. Results from two such experiments are shown in Table 2. It should be noted that these results were obtained after the sucrose gradient sedimentation step of the purification; they were unchanged after a further banding of the phage preparations in cesium chloride gradients as described in Materials and Methods. Clearly, 28% of the uracil of phage RNA can be replaced by FU without decreasing the specific infectivity of the phage.

DISCUSSION

When added to infected cells at a concentration of $10^{-4}$ M or greater, FU inhibits completely the further formation of infectious RNA. The time of addition of the inhibitor is important. If added within 2 min of phage, there is no synthesis of infectious RNA or phage. If added later, after 5 min, mature phage continues to form.

### Table 2. Effect of substitution of FU for uracil on the specific infectivity of R17 phage

<table>
<thead>
<tr>
<th>Expt</th>
<th>Conc of FU (M)</th>
<th>Lysate titer (phage/ml)</th>
<th>Phage particles per infectious particle</th>
<th>Uracil substituted by FU %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>$10^{13}$</td>
<td>6.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$4.3 \times 10^{-7}$</td>
<td>$10^{13}$</td>
<td>7.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>$8.5 \times 10^{-7}$</td>
<td>$9.2 \times 10^{11}$</td>
<td>7.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>$1.7 \times 10^{-6}$</td>
<td>$7.5 \times 10^{11}$</td>
<td>7.5</td>
<td>1.9</td>
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<tr>
<td></td>
<td>$4.3 \times 10^{-6}$</td>
<td>$4.7 \times 10^{11}$</td>
<td>7.9</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>$8.5 \times 10^{-6}$</td>
<td>$3.8 \times 10^{11}$</td>
<td>12.8</td>
<td>12.5</td>
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<tr>
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</tr>
<tr>
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<td>$2.2 \times 10^{-6}$</td>
<td>$1.8 \times 10^{11}$</td>
<td>7.7</td>
<td>28</td>
</tr>
</tbody>
</table>
added goes on to be incorporated into infectious phage. These properties of FU were used to determine the kinetics of the phage precursor RNA pool, but, for the method to be legitimate, there must be evidence that sufficient phage protein was synthesized to encapsulate all the precursor RNA present at the time the inhibitor was added. That this was the case is shown by the observation that the formation of phage RNA measured by the inhibition technique, paralleled the production of infectious RNA. If all the precursor RNA were not incorporated into infectious phage, its synthesis would have appeared to lag behind that of infectious RNA, particularly at the early times. Most of the required protein is probably synthesized after addition of FU, since other experiments have shown (Hagopian, unpublished data) that phage antigenic protein is formed no earlier than phage RNA, and that there is only a very small pool of phage precursor protein. Furthermore, Davern (1964) found that phage antigenic protein can form, and be incorporated into noninfectious particles, even in the presence of $10^{-3} \, \text{M} \, \text{FU}$.

The amount of FU incorporated into R17 phage increased with the extracellular concentration of FU, as might be expected from the observation that phage RNA is formed entirely de novo (Ellis and Paranchych, 1963). Increasing concentrations of FU slowed the rate of phage formation (Fig. 1) and decreased the final yield (Table 2), as Davern (1964) and others have found. With a concentration of $2.2 \times 10^{-5} \, \text{M} \, \text{FU}$, the yield of phage was about 15% of that in the control. Phage produced under these conditions had incorporated FU to the extent of 28% of the uracil normally present, and its specific infectivity was unaltered by the substitution. Under these conditions, FU would appear to have exerted an inhibitory effect by inhibiting some reaction involved in the synthesis of phage RNA rather than by inducing lethal mutations as suggested by Davern (1964). At higher concentrations of the analogue, sufficient FU is incorporated into phage RNA to produce mature, noninfectious particles (Davern, 1964; Shimura and Nathans, 1964), although such particles retain their antigenicity (Davern, 1964) and the yield is low. As yet it is unknown whether these heavily substituted phages can still adsorb to the host bacteria.

The decrease in infectious centers some 20 min after infection in the presence of $10^{-4} \, \text{M} \, \text{FU}$, the inability to reverse completely this inhibition after 15 to 20 min by the addition of uracil, and the decrease in amount of parental infectious RNA that could be extracted from infected bacteria during the period of inhibition are probably related aspects of the same phenomenon and could be explained as follows. It is known that parental RNA enters into a double-stranded complex in the infected cells with newly synthesized minus-strands of RNA and that this duplex begins to form not later than 5 min after infection (Kelly and Sinshheimer, 1964; Erikson, Fenwick, and Franklin, 1964; Weissmann et al., 1964). In the presence of FU added at time-zero, all the newly synthesized minus strands would incorporate FU. There is evidence that the parental strand is normally displaced from the double-stranded form during the replicative cycle (Weissmann et al., 1964). If it is supposed that the FU-containing double-strand is an uncommonly stable one, the normal cycling of parental RNA would be slowed down to an extent depending on the content of FU. The slower rate of formation and the decreased yield of phage in the presence of $1.5 \times 10^{-3} \, \text{M} \, \text{FU}$ may be thus explained. At higher concentrations of FU ($10^{-3} \, \text{M}$), the double-stranded form may be so heavily substituted and stable that the already-combined parental RNA strands cannot be displaced from the duplex. Accordingly, when all parental RNA has combined with FU-containing minus strands and can no longer be extracted from the cells as an infectious entity, the inhibition cannot be reversed with uracil, and the cell is lost as an infectious center.

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**Literature Cited**


5-FLUOROURACIL AND GROWTH OF PHAGE R17


