STUDIES IN PARTIALLY RESOLVED BACTERIOPHAGE-HOST SYSTEMS

VI. THE INVOLVEMENT OF RIBONUCLEIC ACID IN VARIOUS ASPECTS OF THE REPRODUCTION OF BACTERIOPHAGE T2

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SUMMARY

A variety of inhibitors has been shown to have pronounced effects on the replication of T2 bacteriophage in protoplasts of *E. coli* B. Ribonuclease applied in 5 or 10 min pulses exerts a maximum effect (40 % reduction of burst size) when applied between 5 and 10 min after infection. *p*-chloromercury benzoate shows a sharp maximum of effectiveness and inhibits the burst completely when added 15 to 20 min after infection. Of a variety of purine and pyrimidine antagonists, 5-fluorouridine and 2′-deoxy-5-fluorouridine showed the most striking effects. The latter compound appears to have a specific inhibitory action on DNA synthesis and the inhibition (some 98 %) is essentially completely reversed by thymidine. The action of 5-fluorouridine appears to be considerably more complex. In part its effect is also directly on DNA synthesis and is reversible by thymidine. During the first 10 min of infection, however, this inhibitor, especially when tested in the presence of thymidine, appears to exert its primary effect on RNA synthesis with resulting effects on the synthesis of "early protein" and hence DNA. These effects can be overcome by uridine. A combination of thymidine and uridine completely abolishes the inhibition by 5-fluorouridine even at quite high concentrations. These conclusions have been reached on the basis of measurements of phage yield and also of incorporation of $^{35}SO_4$ and $^{32}PO_4$ into the appropriate macromolecules.

INTRODUCTION

Current studies of the inter-relationships of genetically specific DNA, RNA and protein involve many different systems. The one which we are considering—T2 in-

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; π, the protoplast-infecting agent derived from T2 (see previous papers); RNase, ribonuclease; FUR, 5-fluorouridine; dFUR, 2′-deoxy-5-fluorouridine; UR, uridine; TR, thymidine; UMP, uridine-5′-phosphate; dUMP, deoxyuridine-5-phosphate; Tris, tris-(hydroxymethyl) aminomethane; PCA, perchloric acid; TCA, trichloroacetic acid; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; BSA, bovine serum albumin; PCMB, *p*-chloromercury benzoate; BBSA, nutrient broth containing 2 % BSA.

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fected *Escherichia coli*—offers many advantages. It is clear that phage DNA is the initiating carrier of specificity (see review by HERSHEY[1]) and that proteins and, presumably, RNA are involved specifically and obligatorily. The proteins now known to be required in the replication of T-even phages include the following: (a) The "early protein" apparently essential for DNA synthesis[2–5]. (b) Certain enzymes the synthesis of which is induced by phage infection[6–10], some or all of which may be identical with "early protein". (c) The various head, tail, and internal proteins of the phage particle[11,12]. The RNA of *E. coli* seems to be typical of RNA as involved in protein synthesis in a wide variety of biological systems in that it occurs in two forms, soluble and particulate, with distinct functions. The soluble RNA appears to serve as the carrier of individual amino acids during their initial activation[13,14]. The fixed RNA seems to be an integral part of the site of protein synthesis[15] and to be concerned with the control of the arrangements of individual units in polypeptide sequences. This last RNA, then, is presumably the bearer of the primary information during the process now called "patternization"[16–18]. There is as yet no unequivocal answer to the question of whether in the phage—*E. coli* system well-known to be novel in a number of particulars, there occurs direct transfer of information from phage DNA to new protein. The weight of evidence, however, now seems to favor RNA intermediacy. We have found a polyribonucleotide to be directly concerned in an apparent first step of the infection of protoplasts of *E. coli* by π[19]. A very early step, possibly succeeding this one, appears to involve activation of polynucleotide phosphorylase[20]. An RNA fraction which is capable of rapid incorporation and turnover of $^{32}PO_4$ occurs as a direct and immediate consequence of infection of *E. coli* by bacteriophage T2 as shown by VOLKIN AND ASTRACHAN[21,22].

The present report is concerned with attempts to establish additional relationships between the synthesis of RNA and protein at the various stages of phage reproduction. A variety of experimental approaches has been employed for this purpose. We have investigated (a) the effect on phage reproduction of PCMB and of RNase applied in short pulses; (b) the effect on phage reproduction of a variety of inhibitors of nucleic acid synthesis—notably purine, pyrimidine, and nucleoside analogues and antagonists, (c) the effect on the rates of synthesis of DNA, RNA, and protein of some of these same inhibitors applied during the first few minutes after infection.

METHODS AND MATERIALS

Except for the isotope experiments to be described below, the techniques used were either standard phage procedures[23], or, when involving π, our own methods which have already been described[24–26]. The particular details for each experiment are listed in the legend of the appropriate table or figure. The isotope experiments were performed essentially as described by HERSHEY AND MELECHEN[4] with the following modification: *E. coli* B was grown through 6 transfers in HERSHEY's low phosphate, low sulfate medium (herein abbreviated as H medium); the sixth growth flask was then used as a source of cells for all isotope experiments. Bacteria in 250 ml of medium were allowed to grow to a concentration of $2.0 \cdot 10^8$ cells/ml (actual Petroff-Hausser count), centrifuged and washed twice with cold 0.1 *M* Tris buffer pH 8.1. They were then suspended in 25 ml (concentration: $2.0 \cdot 10^9$ cells/ml) of modified H medium freed

of sulfate (for the ^{35}S experiments) or phosphate (for the ^{32}P experiments). 2.5 ml of each of these cell suspensions were mixed with an equal volume of a solution, consisting of the modified H medium plus the following (final concentration after mixing): in tube 1 – 300 μg FUR/ml; tube 2 – 300 μg FUR and 300 μg uridine/ml; tube 3 – 300 μg FUR and 300 μg thymidine/ml; tube 4 – 300 μg FUR, 300 μg uridine and 300 μg thymidine/ml; in tube 5 – 300 μg dFUR; in tube 6 – 300 μg dFUR and 300 μg thymidine; in tube 7 – 25 μg chloramphenicol/ml and in tube 8 – no inhibitor. After 5 min at 0° the reaction tubes were placed in a 37° bath and aeration was started. T2 bacteriophage was then added in a serial fashion to give an average multiplicity of 10 T2/cell. (Absorption was 99 % complete and over 90 % of all cells were shown to be infected in parallel experiments.) After precisely 1 min $Na_2{}^{35}SO_4$ was added to one set and $Na_2H^{32}PO_4$ to the other. The amount of phosphate added to each tube corresponded to 8 μg P and, in our counter, showed $3.55 \cdot 10^8$ counts/min on the day of the experiment. The amount of sulfate added was approx. 10 μg S and, on the day of the experiment, registered $1.07 \cdot 10^7$ counts/min. After addition of the radioisotopes phage growth at 37°, with constant aeration, was allowed to proceed for precisely 6.5 min. The various sets were then treated as follows:

In the ^{35}S experiment the tubes were chilled, a sample of each was removed for radio-assay of total ^{35}S present (0.05 ml of a 1:3000 dilution per planchet), the remainder was centrifuged in the cold (Servall). The pellets were washed twice by re-suspension and re-centrifugation in 0.1 *M* Na_2SO_4 to dilute out any $^{35}SO_4^{=}$ not incorporated into the cellular material. Each final pellet was then taken up in 2.5 ml of cold protoplast medium (final concentration $2.0 \cdot 10^9$ cells/ml). A 1-ml aliquot of this suspension was removed, an equal volume of 1.0 *M* PCA was added, the mixture was allowed to stand in the cold for a period of 2 h and then centrifuged. The precipitates were resuspended in 1.0 ml of cold 0.5 *M* PCA and re-centrifuged. This process was repeated once more. After a dilution of 1:2000, 0.05-ml aliquots of each sample were spread on planchets and counted to obtain the total sulfur incorporated into cell proteins. The cells in a second 1-ml aliquot were osmotically shocked by conversion to protoplasts followed by a rapid dilution into 0.01 *M* NaCl with strong agitation (cell lysis > 99 % complete by direct microscopic count or disappearance of zero-time infective centers). The lysate was then centrifuged at 4500 × *g* for 10 min to obtain a low speed residue (P_1) and a supernatant. The latter was centrifuged at 15,000 × *g* for 20 min to yield a high-speed pellet (P_2) and the final supernatant (S). P_1 and P_2 were suspended in 1.0 ml of cold 0.5 *M* PCA and treated as described above for whole cells. The final dilutions for radio assay were 1:400 and 1:40 respectively. The S fraction was precipitated with an equal volume of 1.0 *M* PCA and then treated as above; final dilution for counting 1:400.

In the ^{32}P experiments the tubes were chilled, 0.5 ml of unlabeled 0.150 *M* KH_2PO_4 was added to each, an 0.1-ml aliquot was removed for radioassay of total ^{32}P (final plating – 0.05 ml of a $1:4 \cdot 10^5$ dilution) and the remainder was centrifuged. The pellets were washed twice with 0.1 *M* Tris containing 0.01 *M* KH_2PO_4 and taken up in 2.5 ml each of protoplast medium. Sample treatment, cell lysis, centrifugal fractionation and PCA precipitation were then performed as described above. The nucleic acids thus obtained were fractionated by a modified Schmidt-Thannhauser procedure as follows: the precipitates were dissolved in 2.0 ml of 1 *N* NaOH and incubated for 18 h at 36°. The solutions were then neutralized with 0.20 ml of 10 *M*

HCl and made 0.5 *M* in PCA by addition of 2.2 ml of *M* PCA. The residues were taken up in 4.4 ml each of PCA and plated; these constitute the "DNA" fractions. The supernatants, also in a volume of 4.4 ml, constitute the "RNA" fractions.

The bovine serum albumin (BSA) was Armour 30 % sterile solution. The RNase used was Worthington Laboratories twice recrystallized product, the PCMB was purchased from Sigma Chemical Company and the various purines and pyrimidines were obtained from Schwarz Laboratories or from the California Corporation for Biochemical Research and were chromatographically pure. 8-azaguanine, 2-thiouracil and 5-bromouracil all were products of the California Corporation for Biochemical Research. 5-hydroxyuridine was given to us by courtesy of Professor VISSER of the University of Southern California; generous supplies of FUR, dFUR, were provided through the courtesy of Drs. AESHLIMANN, RUBIN AND SCOTT of Hoffmann-La Roche, Inc., and of DRB through that of Dr. FOLKERS of Merck-Sharp and Dohme. Phosphate ^{32}P and sulfur ^{35}S were obtained from Oak Ridge National Laboratories.

RESULTS AND DISCUSSION

Phage replication in protoplasts

Since our original report on the reproduction of T2 in protoplasts infected with π (see ref. 25) our procedures have undergone several modifications. A typical growth curve of the virus under the exact conditions used in the present experiments is, therefore, reproduced in Fig. 1a. The data show not only the free phage released spontaneously but also the total obtained upon osmotic shock of the infected protoplasts. Several features appear to be of interest. (a) The infected protoplasts behave like infected cells to some extent in that phage appear first within the protoplasts, and are released only after some delay. At 25 min the first internal phage appear. The differential between internal and released phage is at maximum at about 60 min. (b) Meanwhile phage are being released at an exponential rate between 25 and 120 min. The mechanism for this release is unknown to us but we believe that it may be a spontaneous destruction of the somewhat unstable protoplasts. (c) After some 150 min almost all of the protoplasts seem to have lysed (direct microscopic observation) and, indeed, there is no increase in phage numbers upon osmotic shock of the system. (d) The continued production of phage after this time is not immediately explicable. We have reason to believe that it is possible that replication may occur within a more highly resolved particulate system no longer containing intact protoplasts. It is also possible, however, that the effect is only apparent and due to unavoidable observational artifacts of the rather delicate protoplast system.

Phage replication in protoplasts, as in cells, is dependent on the maintenance of an adequate supply of oxygen and is sensitive to the ordinary inhibitors of respiration and phosphorylation (Table I).

Inhibition by RNase

In previous publications we have shown that protoplasts of *E. coli*, especially in the presence of BSA and in a nutrient medium, may be exposed to the action of RNase without any apparent loss in numbers of bodies or deleterious effects on their gross morphology[24]. These observations were confirmed in the present instance for periods up to 15 min, even at 37° and at RNase levels as high as 500 μg/ml with

10^9 protoplasts/ml. In the experiments shown in Fig. 1-B infected protoplasts at $1 \cdot 10^7$/ml were exposed to the enzyme at 50 μg/ml for pulses of only 5 min during different stages of phage replication. The enzyme was then rendered innocuous by a 500-fold dilution, phage reproduction was allowed to continue for a total of 120 min, and the extent of inhibition was determined. It will be seen that the period of

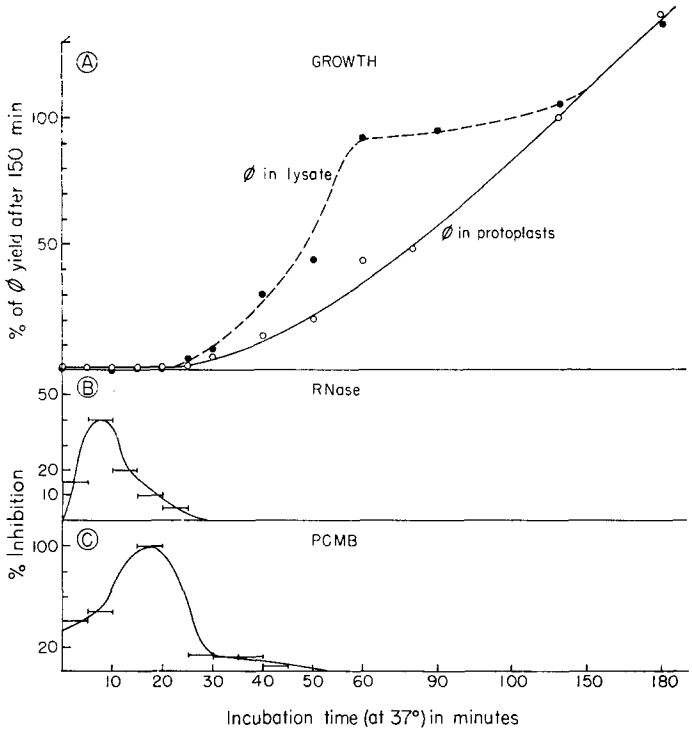

Fig. 1A. Growth of bacteriophage T2 in protoplasts of *E. coli* B, infected with π: Protoplasts at $2 \cdot 10^9$/ml were infected with an equal volume of a concentrated π preparation (nominal infective titer $2 \cdot 10^9$ infective units/ml). After 5 min at room temperature an aliquot of the mixture was diluted 1:1000 into a growth tube containing BBSA, held at 37°, and aerated vigorously. At the times indicated, samples were diluted 1:100 into BBSA ("phage in protoplasts") or distilled water ("phage in lysate") and plated. Actual plaque counts (calculated back to original infection tube, *i.e.* a dilution factor of $10 \cdot 10^2 \cdot 10^3$ for the zero time experiment) were: 0-time, $3.20 \cdot 10^8$/ml; 150 min (protoplasts) $3.40 \cdot 10^{10}$/ml; 150 min (lysate) $3.49 \cdot 10^{10}$/ml. Fig. 1B. RNase pulse experiment: Infected protoplasts (at 10^9/ml) were diluted 1:10 into BBSA and aerated vigorously at 37°. At the times indicated at the left hand margin of each set, samples from the concentrated growth tube were diluted 1:10 into BBSA containing 50 μg/ml of RNase. After 5 min exposure to the enzyme a dilution of 1:500 was made to stop enzyme action. (In separate experiments it was shown that RNase at this level (0.1 μg/ml) had no effect on overall phage production in protoplasts.) In this dilute growth tube, aeration was continued for a total of 90 min. An aliquot was then diluted and plated for phage. In a typical experiment, *e.g.* that represented by the third set, the protoplasts would have remained 10 min in the concentrated growth tube, 5 min (10-15 min from 0) in the RNase tube, and then an additional 75 min (15–90 min from 0) in the dilute growth tube. Data are expressed as percent inhibition of the phage yield in a control sample treated identically but devoid of RNase. Data for this control (calculated back to the original infected protoplasts) zero time infective centers—$3.00 \cdot 10^8$/ml; final phage yield—$2.40 \cdot 10^{10}$/ml; burst size, 80. Fig. 1C. PCMB pulse experiment: Similar to experiment 1B, except that the inhibitor used was PCMB at the level of $2 \cdot 10^{-4}$ *M*. The time of exposure was 5 min, as before; the dilution to stop inhibitor action was 1:1000; total incubation time —60 min. Data for the no-inhibitor control (calculated back as in Expt. 1B): zero time infective centers —$1.06 \cdot 10^8$/ml; final phage yield $2.30 \cdot 10^9$/ml; burst size, 22.

TABLE I

EFFECT OF METABOLIC INHIBITORS ON DEVELOPMENT OF T2 IN π-INFECTED PROTOPLASTS

Protoplasts were infected as described in the legend to Fig. 1. After 5 min an aliquot was diluted $1:2.5 \cdot 10^4$ into a growth tube containing the respective inhibitor at the concentration shown, and incubated at 37° for 2 h. At the end of this period an aliquot was suitably diluted, plated for phage, and the burst size compared to a control devoid of inhibitor. Controls for zero time infective centers in the presence of the various inhibitors were also plated—no inhibition was found. Data for control: zero time infective centers—$2.15 \cdot 10^8$/ml; final phage yield—$2.69 \cdot 10^{10}$/ml; burst size, 125.

Inhibitor	*Concentration (M)*	*Per cent inhibition of burst size**
Cyanide	$5 \cdot 10^{-3}$	100
	$5 \cdot 10^{-4}$	100
Azide	$5 \cdot 10^{-3}$	78
	$5 \cdot 10^{-4}$	13
p-chloromercuribenzoate	$2 \cdot 10^{-3}$	100
	$2 \cdot 10^{-4}$	100
2,4-dinitrophenol	$5 \cdot 10^{-4}$	91

* Plaques after 2 h/plaques at zero time, under identical conditions; compared to the same ratio for an uninhibited control.

maximum sensitivity to RNase subsequent to infection[19] occurs during the first 10 min of phage development with a peak between 5 and 10 min. This is precisely the time of synthesis of the proteins mentioned as sets (a) and (b) in the introduction and of maximum turnover of RNA. By the use of 10-min pulses 80 % inhibition was observed when the system was exposed to RNase during the first 10 min, as compared to 20 % when exposure took place during the second 10-min period. The pattern may be shifted to longer times (as may phage synthesis in general) by the use of lower temperatures. At 22° the extent of inhibition after enzyme treatment for the first 10 min was only 5 %; this was increased to 39 % during the second 10 min. This selective action of RNase on some process specific for phage replication, and presumable related to the synthesis of some specific protein(s) confirms recent findings and hypotheses advanced by JEENER[27] who used an induced, lysogenic strain of *Bacillus megaterium*.

Inhibition by PCMB

A similar pulse experiment with PCMB, a known inhibitor of many respiratory enzymes as well as of infection by intact T2 (see ref. 19), is shown in Fig. 1C. Although its inhibitory action, at $2 \cdot 10^{-4}$ *M* concentration is exerted throughout the first 25 min or so of phage development, there is a sharp and pronounced maximum between 15 and 20 min after infection. Experiments with 10-min pulses of exposure to inhibitor covering the entire period of phage development have yielded qualitatively similar results with maximum inhibition between 10 and 20 min. The greatly decreased and ever decreasing inhibition in the subsequent period (when phage is assembled at a maximal rate) is especially surprising. The result suggests tentative exclusion of phage protein itself as a possible locus of action of the inhibitor. Excluded also are the

energy yielding enzyme sequences of the host, since they too would be expected to be taxed to their full capacity during the period of active phage synthesis and assembly commencing at 25 min. The action of the inhibitor may therefore be localized first of all on some protein or proteins concerned with the initial phases of phage replication, and, even more profoundly, on one (or more) which makes its appearance at about 15 min, just prior to the appearance of the first progeny phage at a time when the pool of phage DNA and proteins is known to be already fully formed. It would be tempting therefore to speculate that this protein might be concerned with the assembly of the various components of the bacteriophage into an intact virus.

Inhibition by purine, pyridimidine and nucleoside antagonists

When this study was initiated, relatively little had been published about the effects on phage replication of a variety of inhibitors of nucleic acid biosynthesis§.

TABLE II

EFFECT OF VARIOUS PURINE AND PYRIMIDINE ANALOGUES ON DEVELOPMENT OF T2 IN π-INFECTED PROTOPLASTS

Protoplasts were infected as described in the legend to Fig. 1. After 5 min an aliquot was diluted 1:500 into a growth tube containing the respective inhibitor at the concentration shown. After 30 min each sample was divided into three parts, one, labeled "30 min", was diluted 1:500 into BBSA; the second, labeled "60 min-diluted", was diluted 1:50 into BBSA; the third was retained in the original growth tube. Thus the first contained the inhibitors at 0.002 the original concentration, the second at 0.02 and the third at the original level. Incubation was then continued for an additional 30 min. Samples were then suitably diluted, plated for phage, and compared to controls treated similarly but devoid of inhibitor. Controls for effect of inhibitors on zero time infective centers were also plated—no significant effect was found. The table represents data collected in several different experiments. Plaque counts in a typical experiment for the controls: zero time infective centers $-2.88 \cdot 10^8$/ml; "30 min" $-2.10 \cdot 10^{10}$/ml; "60 min diluted" $-2.10 \cdot 10^{10}$/ml; "60 min undiluted" $-1.96 \cdot 10^{10}$/ml.

Inhibitor	*Concentration (mM)*	*% inhibition* after exposure for*		
		30 min	*60 min*	
			Diluted	*Undiluted*
8-azaguanine	1	0	43	53
	5	76	81	95
2-thiouracil	1	0	29	32
	5	72	75	90
5-OH uridine	1	0	13	32
	5	84	83	90
5-Br uracil	0.5	0	53	—
	1	90	99	99
dFUR	0.5	67	22	72
	1	—	—	98
FUR	0.5	76	56	90
	1	90	—	95
DRB	1	82	82	95

* Plaques at end of incubation period/plaques at zero time, in this particular experiment; compared to control treated identically but devoid of inhibitor.

§ These investigations were undertaken before the publication of the brilliant study of COHEN *et al.*[37] which localized the main bacteriocidal locus of 5-fluorouracil, FUR and dFUR at the level of the thymidylate synthetase, with dFUR-5'-phosphate as the actual inhibitory species.

We therefore decided to screen a variety of such agents using the π-protoplast system, which has the particular advantages of accessibility of the metabolic apparatus to charged molecules, *e.g.* ATP. The results of these experiments are summarized in Table II. Among the agents tested, the two fluorinated nucleosides, FUR and dFUR appeared to exert their action at the lowest concentration and were therefore selected for further study. It also seemed of interest that of all the inhibitors studied only the effects exerted by the fluoropyrimidine nucleosides could be reversed, at least in part, by further incubation in nutrient media. (Compare the per cent inhibition after "30 min" with that after "60 min diluted".) There was, therefore, the possibility that in these instances we were dealing with true inhibition of nucleic acid biosynthesis at the nucleoside or nucleotide levels, rather than with the formation of a "unnatural" nucleic acid which contained the analogue in its actual base structure. A survey of the various agents tested here with respect to their probable mechanism of action has recently been published by MATTHEWS[28]. Of these 8-azaguanine, 5-thiouracil, and 5-bromouracil all have indeed been shown to give rise to "unnatural" or modified nucleic acids, while 5-bromouracil, especially, has been reported to be incorporated into *E. coli*[29-31] and its bacteriophages[29] with powerful and specific mutagenic action[32,33]. The other compounds tested were 5-hydroxyuridine, reported by ROBERTS AND VISSER[34] and by SPIEGELMAN[35] to block RNA synthesis in competition with uridine. DRB, which has similar action as an antagonist, has been reported by TAMM to be a competitive inhibitor of the incorporation of adenosine into RNA[36].

Inhibition of phage synthesis by FUR and dFUR

In order to define the locus or loci of action of the fluorinated pyrimidine nucleosides we have examined their effect on the reproduction of T2 in cells and protoplasts and in various media. These include a nutrient broth—BSA medium (BBSA) standard for our experiment with protoplasts, a synthetic medium (D) and a minimal synthetic medium (H), developed by HERSHEY specifically for incorporation studies with inorganic phosphate and sulfate. We have also attempted to localize their mode of action by means of a study of the reversibility of the inhibition by a variety of different compounds such as nucleosides, etc. Some typical experiments are summarized in Table III. Several different conclusions have emerged from these studies: (a) With the inhibitor added in millimolar concentration, inhibition of phage synthesis can be observed in all media and in both cells and protoplasts. (b) As might be expected the inhibition is more pronounced in a synthetic than in a broth medium; experiments with the minimal synthetic medium show the most dramatic effects. (c) At equal concentration, the riboside and deoxyriboside give rise to approximately equal inhibition, but the pattern for the reversal of this inhibition is grossly different for the two agents. (d) Among the compounds tested, only pyrimidine nucleosides appear to be capable of overcoming the inhibition exerted by the fluorinated derivatives. (e) Inhibition by FU is overcome in an apparently competitive manner by uridine and in a non-competitive manner by thymidine; addition of both gives a synergistic effect. (f) Inhibition by dFU is reversed completely by the addition of thymidine; uridine has little or no effect if added either alone or in conjunction with thymidine. Since thymidine reverses FUR inhibition only partially but dFUR inhibition completely, it would seem that FUR affects two different sites and dFUR only one of these. This latter site is probably concerned with DNA synthesis.

TABLE III

EFFECTS OF FUR AND dFUR ON DEVELOPMENT OF T2 IN CELLS INFECTED WITH T2 AND ON PROTOPLASTS INFECTED WITH *n*

The experiments with protoplasts were performed in a manner similar to that described in the legend to Table II (but with the inhibitor present throughout the growth period). Incubation in the presence of the inhibitor plus reversing agent shown was for a total of 90 min; there were no significant effects of the addition of these agents on the burst size of the control, average ~ 60, determined separately for each experiment. In the case of cells, growth was in the medium indicated to a concentration of $2.0 \cdot 10^8$ bacteria/ml. After washing with 0.1 *M* Tris, the cells were resuspended in the growth medium to $1.0 \cdot 10^9$ cells/ml and infected with T2 at an average multiplicity of 8 phages/bacteria. Adsorption was measured in each experiment by plating the supernatants after centrifuging and shown to be 99% complete. Infection was allowed to proceed for 5 min. The cells were then chilled, centrifuged, washed twice with 0.1 *M* Tris and resuspended in 25 ml of cold medium. Plating for infective centers indicated that all the cells (10^9/ml) had become infected. Aliquots were then diluted 1:200 into growth tubes containing the respective inhibitors plus reversing agents. Incubation was for 90 min at 37° and the values shown are the percentage of burst size when compared to controls devoid of inhibitor. Burst size of control in D, 130; in H, 75. In the experiment in H medium which constituted the phage growth control for the isotope experiments, the following procedure was followed: after centrifuging and washing the cells were resuspended in cold H medium to a concentration of $2 \cdot 10^9$ cells/ml. 1.0-ml samples were then mixed with equal volumes of the various agents to give the final concentrations shown. Aliquots were plated for zero time infective centers, and the tubes placed in a 37° bath with constant aeration. After 90 min samples were removed, diluted $1:10^6$ and $1:10^7$ and plated for phage. Calculation of % inhibition was done as before on the basis of burst size: burst size of control, 50.

System				*Cells*		*Protoplasts*
Medium				*D*	*H*	*B-BSA*
Inhibitor		*Reversing agent*				
Compound	*Concentration (M)*	*Compound*	*Concentration (M)*	*% of untreated control**		
FUR	$1.0 \cdot 10^{-3}$	None		10		
		Uridine (UR)	$1.0 \cdot 10^{-3}$	24		
		Thymidine (TR)	$1.0 \cdot 10^{-3}$	57		
		TR + UR	$1.0 \cdot 10^{-3}$	100		
	$1.14 \cdot 10^{-3}$	None			0.27	
		UR	$1.23 \cdot 10^{-3}$		1.2	
		TR	$1.24 \cdot 10^{-3}$		0.30	
		TR + UR	$1.23 \cdot 10^{-3}$ ea.		95	
	$7.68 \cdot 10^{-4}$	None				4.7
		UR	$1.0 \cdot 10^{-3}$			11
			$3.0 \cdot 10^{-3}$			15
			$5.0 \cdot 10^{-3}$			24
			$1.0 \cdot 10^{-2}$			36
		TR	$1.0 \cdot 10^{-3}$			15
			$3.0 \cdot 10^{-3}$			17
			$5.0 \cdot 10^{-3}$			20
			$1.0 \cdot 10^{-2}$			15
		Adenosine	$3.0 \cdot 10^{-3}$			6.7
		Guanosine	$3.0 \cdot 10^{-3}$			5.8
		Cytidine	$3.0 \cdot 10^{-3}$			5.2
	$1.0 \cdot 10^{-3}$	None				10
		UR	$5.0 \cdot 10^{-3}$			45
		TR	$5.0 \cdot 10^{-3}$			55
		Uracil	$5.0 \cdot 10^{-3}$			20
		Thymine	$5.0 \cdot 10^{-3}$			10

TABLE III (continued)

System				Cells		Protoplasts
Medium				D	H	B-BSA
Inhibitor		Reversing agent		% of untreated control*		
Compound	Concentration (M)	Compound	Concentration (M)			
dFUR	$1.0 \cdot 10^{-3}$	None		2.1		
		UR	$1.0 \cdot 10^{-3}$	3.3		
		TR	$1.0 \cdot 10^{-3}$	93		
		TR + UR	$1.0 \cdot 10^{-3}$	98		
	$1.14 \cdot 10^{-3}$	None			0.22	
		TR			59	
	$1.0 \cdot 10^{-3}$	None				10
		UR	$5.0 \cdot 10^{-3}$			9.6
		TR	$5.0 \cdot 10^{-3}$			75
		Uracil	$5.0 \cdot 10^{-3}$			11.2
		Thymine	$5.0 \cdot 10^{-3}$			22

* Plaques at end of incubation/plaques at zero time, under identical conditions; compared to the same ratio for a control devoid of inhibitor.

Our opinion of the function of the remaining site of FU inhibition is indicated by the following discussion based on the reactions of Fig. 2 which represents the currently accepted pathways of incorporation of uridine into RNA and DNA: FUR, or its nucleotide (FUR-5'-P) cannot cause inhibition solely at step (e) since in this case thymidine should reverse the action completely and competitively. Also there would then be no difference in action between FUR and dFUR. Conversely if action were exclusively at step (d), the action of thymidine would then be to reverse the

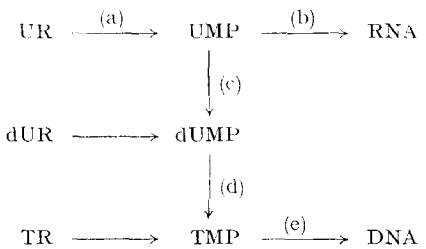

Fig. 2

inhibition non-competitively and completely. This indeed was the effect observed with dFUR. This then pinpoints the site of action of dFUR and is consistent with the hypothesis of COHEN[27] for the mode of action of fluorinated pyrimidines. Furthermore uridine should reverse inhibition of step (d) by FUR completely and competitively which is observed, and this reversal should be strictly additive to that exhibited by thymidine, which is not. Step (d) is the one postulated above, however, as being affected by both FUR and dFUR. If the second site for FUR action were steps (a) (as suggested by SKOLD[38]) and/or (c), thymidine should again reverse the inhibition non-competitively, and an additive effect of thymidine and uridine would be expected.

This hypothesis also is not capable therefore of explaining the powerful synergistic effect observed in H medium. This last effect implicates some reaction between UMP and RNA (*i.e.* sequence (b)) as the additional locus of FUR inhibition[38,39], and of necessity suggests that the product (RNA) is essential for some step in phage replication. In order to establish this phenomenon with more precision, the following two sets of experiments have been performed.

Effects of delayed addition of dFUR and FUR

As can be seen from Table IV, if the addition of the fluoropyrimidine nucleosides was delayed for 5–10 min several interesting effects were observed: the extent of inhibition of phage replication was the same, but the reversal of FUR inhibition by thymidine was enhanced and the effect now appeared quantitatively similar to that obtained with dFUR. Thus after the first few minutes, inhibition of phage replication by FUR becomes more similar to that by dFUR and appears to be due largely to interference with DNA synthesis. As a corollary the main effect of FUR on RNA-metabolism, then, must occur during the period of synthesis of the "early protein".

TABLE IV

EFFECT OF DELAYED ADDITION OF FUR AND dFUR

Experiment similar to that described in Table II (except for the dilutions after 30 min) for the samples marked "0"; in another set of samples those marked "+10" or "+5", the infected protoplasts were diluted 1:50 into BBSA and were incubated for 10 or 5 min at 37°. At the end of this period they were then diluted 1:10 into BBSA containing the agents shown and incubation was continued for 90 min. In Expt. I, the burst size was 50; in Expt. II, 65; in Expt. III, 62.

Expt.	*Inhibitor*	*Time added (min)*	*% Inhibition* Nucleosides added (at $5.0 \cdot 10^{-3}$ M)*		
			None	*Uridine*	*Thymidine*
I	FU	0	95	75	72
		+10	95	—	51
	dFU	0	95	93	52
		+10	95	—	53
II	FU	0	94	—	66
		+ 5	90	—	31
III	FU	0	96	—	74
		+ 5	93	—	66
	dFU	0	98	—	56
		+ 5	90	—	56

* Plaques at end of incubation/plaques at zero time under identical conditions; compared to the same ratio for a control devoid of inhibitor.

Incorporation experiments

The above hypothesis predicts a strong effect of FUR on incorporation into infected cells, during the first few minutes, of [^{35}S]sulfate into protein and [^{32}P]phosphate into nucleic acids. Experiments to test the hypothesis are summarized in Table V. The experimental procedure used was very similar to that pioneered by

TABLE V

INCORPORATION OF $^{35}SO_4$ AND $H^{32}PO_4$ INTO CELLULAR CONSTITUENTS IN THE PRESENCE OF FUR AND dFUR

Experiment described in the text. Amount of label fixed was calculated on a basis of 10^9 cells/ml.

Label added		*[^{35}S]sulfate*		*[^{32}P]phosphate*			
Inhibitor	*Competitor*	*Fraction of label rendered PCA insoluble* ($\times 10^{-3}$)	*% of uninhibited control*	*Fraction of label fixed in*		*% of control*	
				DNA ($\times 10^5$)	*RNA* ($\times 10^4$)	*DNA*	*RNA*
None	None	190	(100)	159	151	(100)	(100)
FUR	None	123	65	46	63	29	40
FUR	UR	183	96	57	109	36	72
FUR	TR	126	66	70	76	44	50
FUR	UR + TR	187	99	93	112	58	74
dFUR	None	142	75	62	110	39	73
dFUR	TR	127	66	79	79	50	53
Chloramphenicol	None	56	29	92	171	58	113

HERSHEY *et al.* Cells were multiply infected by incubation with 10 T2/cell for 1 min at 37° in a medium containing both the inhibitor (plus ordinary pyrimidine nucleoside where indicated) and the labeled substrate. Phage development was allowed to take place for an additional 6.5 min, at this temperature. Each sample was then chilled and analyzed. The effect of chloramphenicol at a level of 25 μg/ml was also studied in order to have available a comparison with an inhibitor of reasonably certain mode of action. It will be seen that FUR interferes, albeit to a varying extent, with all three of the incorporation proceses under investigation. Incorporation here is presumably to be equated with net synthesis of the respective components. The inhibition of RNA synthesis by FUR is, as demanded by the hypothesis advanced earlier, more effectively reversed by uridine than by thymidine. A similar inhibition and reversal pattern is also observed in the case of protein synthesis, presumably as a consequence of primary action on RNA synthesis. In agreement with the results of KIHO AND WATANABE[40,41] and those of ASTRACHAN AND VOLKIN[22], published while this manuscript was in preparation, there is no inhibition, but rather a slight stimulation of the entry of phosphate into RNA in the presence of chloramphenicol. The small amount of DNA synthesis occurring during these early stages of virus replication (amounting to approximately only 0.1 the net synthesis of RNA) shows quite a different pattern. This pattern is, however, qualitatively in agreement with the results observed in the above experiments in which phage assay was used as a measure of inhibition. The effects include: partial reversal by thymidine of both dFUR and FUR inhibition; partial reversal by uridine of FUR inhibition; more complete and synergistic reversal by addition of both thymidine and uridine.

Structural localization of effects

In a previous paper of this series we have advanced a hypothesis of bacteriophage synthesis which assigns a paramount role to certain structural and functional elements of the bacterial cell, most notably the cell (or protoplast) membrane and the RNA

associated therewith[19]. This hypothesis has since received added support by the findings of NISMAN[42] that the protoplast membrane of *E. coli*, as of other organisms, or possibly a class of ribosomes attached thereto[15] is implicated as the site of active protein synthesis. It therefore appeared to be of interest to see whether there were any readily discernible differences in the extent of isotope incorporation, subsequent to phage infection, into various fractions of a lysate (prepared as in our earlier papers by conversion of the cells to protoplasts by means of lysozyme–Versene and osmotic shock of the protoplasts*). Three fractions were examined: (a) P_1, a low speed residue, obtained after centrifuging for 10 min at 4500 $\times$ *g*. This fraction presumably contains any particulate cell-wall materials remaining, any intact cells and protoplasts, and intact protoplast ghosts and membranes. (b) P_2, a high speed residue obtained after centrifuging the remaining supernatant at 15,000 $\times$ *g* for 20 min. This fraction contains fragments of protoplast membranes and of wall material. (c) S_2, the supernatant remaining after (b). This contains all small particulate material and the soluble portion of the cytoplasm, including components falling into these classes liberated from the cells by action of lysozyme plus Versene.

In discussing the results obtained, which are summarized in Table VI, we shall deal separately with those bearing on protein and those bearing on nucleic acid synthesis. The more extensive data on protein synthesis (^{35}S incorporation) may be

TABLE VI

DISTRIBUTION OF ^{35}S AND ^{32}P IN VARIOUS FRACTIONS OF A PROTOPLAST LYSATE

Experiment described in the text. Amount of label fixed was calculated on a basis of 10^9 cells/ml.

Label added	*Incorporation into*	*Inhibitor*	*Competitor*	*Fraction of label fixed*			*% of uninhibited control*		
				P_1 ($\times 10^3$)	P_2 ($\times 10^4$)	S_2 ($\times 10^3$)	P_1	P_2	S_2
[^{35}S]sulfate	Protein	None	None	19.0	16.0	34.0	(100)	(100)	(100)
		FUR	None	12.3	4.67	29.6	65	29.4	87.0
		FUR	UR	23.0	18.1	41.8	120	111	123
		FUR	TR	11.5	5.20	31.6	60	32.5	93.0
		FUR	UR + TR	15.8	14.7	34.0	83	93	100
		dFUR	None	14.5	6.72	36.0	76	42.0	106
		dFUR	TR	10.5	8.27	22.0	55	52.0	64.7
		Chloramphenicol	None	10.0	0.02	26.6	53	0.12	78.2
[^{32}P]phosphate	RNA	None	None	4.30	3.87	2.43	(100)	(100)	(100)
		FUR	None	2.24	1.90	1.80	52	49.0	74.0
		dFUR	None	2.20	1.80	1.80	51	46.3	74.0
		Chloramphenicol	None	6.25	2.15	16.5	145	55.7	677
	DNA	None	None	0.25	0.29	0.11	(100)	(100)	(100)
		FUR	None	0.27	0.24	0.040	108	83	36
		dFUR	None	0.29	0.23	0.066	116	80	60
		Chloramphenicol	None	0.32	0.17	0.077	128	58	70

* Under the conditions of the experiment, conversion to protoplasts could be shown to be 99.9% complete, while shocking into 0.01 *M* NaCl led to the disruption of $>99\%$ of the protoplasts, as indicated by direct microscopic count, disappearance of zero-time infective centers, and liberation of material absorbing ultraviolet light at 260 mμ.

summarized as follows: Qualitatively the picture is similar to that obtained for gross incorporation into phage-infected cells—both FUR and dFUR inhibit, but the inhibition by the former agent is more complete and may be reversed by uridine but not by thymidine. Reversal by uridine alone, however, is now more effective than that exerted by a combination of this agent with thymidine. Quantitatively there is a striking gradation of all of these effects in the order $P_2 > P_1 \gg S_2$. Although no precise determinations of protein or RNA content have been made in these particular experiments, our experience has shown that the P_2 fraction never contains more than 10% of the total protein or RNA of the cells. Thus the incorporation, inhibition, and reversal picture with respect to total incorporation is a reflection of a similar, but possibly even more pronounced pattern of specific activities. It is also of interest that the experiment with added chloramphenicol shows a similar distribution; again the effect is more pronounced in the particulate fraction—here the incorporation into the P_2 protein appears by far the most sensitive. These observations are of interest in at least two contexts: (a) The differential sensitivity to chloramphenicol at 25 μg/ml appears to give strong support to the contention of ARONSON AND SPIEGELMAN[43] that this level of the antibiotic is insufficient to eliminate all protein synthesis. (b) The differential inhibition pattern appears to confirm the hypotheses advanced among others by BUTLER *et al.*[44], by BROWN AND BROWN[45], and by NISMAN[42] that relatively large particulate elements, probably identical with the protoplast membrane system, are the site of active protein synthesis in bacteria.

In the case of RNA synthesis we find an analogous pattern. The inhibitory action by the fluorinated pyrimidine nucleosides is exerted primarily on the RNA of the particulate fractions. This is in agreement with some preliminary data cited by VOLKIN AND ASTRACHAN[46] which indicated that such a fraction was the site of the RNA turnover during the early phases of phage synthesis. The present experiments also confirm their more recent finding that there is no inhibition but a stimulation of net incorporation of phosphate into RNA under the influence of chloramphenicol[47] (see also ref. 40, 41). Our data, however, suggest a definite functional heterogeneity of the various RNA fractions studied: The presence of the antibiotic led to a depression of RNA synthesis and/or turnover in the P_2 fraction. This effect is parallel to the complete inhibition of protein synthesis, shown above. Since it appears to be fairly well established that chloramphenicol does not interfere with RNA metabolism *per se*[48–51], this observation must then imply that the synthesis of some of the polyribonucleotide appearing during the period immediately after phage infection is dependent on protein synthesis in an obligatory manner. It is of interest that an amino acid dependent ATP–^{14}C incorporation induced by phage DNA has recently been discovered by G. BROWN*. The very pronounced stimulation of phosphate incorporation into the P_2 and especially the S_2 fraction is of interest in view of the hypothesis advanced by a number of workers[48–51] that the primary function of chloramphenicol is the "uncoupling" of protein and RNA synthesis with a block exerted in such a fashion as to lead to the accumulation of an unstable nucleic acid rather than a stable ribonucleoprotein. This unstable nucleic acid may then well be dis-

* Private communication: *cf.* also G. BROWN in *Structure and Function of Genetic Elements*, Vol. 12. Brookhaven National Lab. Symposia in Biology, 12, 1959, p. 47.

charged into and accumulate in the soluble portion of the cytoplasm*. In agreement also with our own data on gross incorporation into the RNA of cells there is a pronounced stimulation of net incorporation under the influence of chloramphenicol.

Relatively little can be said about the distribution data for DNA in view of the low total levels of activity observed. Yet the occurrence of a dFUR and FUR-insensitive DNA synthesis in some of the fractions and their differential sensitivity to these agents may be of more than passing interest.

CONCLUDING REMARKS

The present observations permit one to make some comments concerning two points: the possible mode of inhibition of T2 reproduction in *E. coli* by fluorinated pyrimidines, and the possible role of RNA and protein synthesis in phage replication. In agreement with the hypothesis of COHEN *et al.*[37] dFUR probably blocks DNA synthesis selectively and hence prevents phage reproduction. FUR, however, appears to produce at least two distinct effects[39]. One is analagous to the effect of dFUR and blocks phage reproduction by interference with DNA synthesis. The other is concerned with RNA metabolism and is competitively overcome by uridine.

There would seem to be two obvious ways in which this last effect could occur; faulty, non-functional RNA may be synthesized with fluorouracil in place of uracil or else the entry of UMP, and hence the synthesis of RNA itself, may be blocked. Incorporation of fluorouracil into RNA has been reported to occur in certain tumors[39] and tobacco mosaic virus (TMV)[52]. But in the latter case even substitution of 32 % of the uracil did not lead to impairment of the specific infectivity although the yield of virus was lowered. Thus the synthesis of RNA and/or protein was partially blocked, but the RNA produced was fully functional. The alternative explanation postulates a block of RNA synthesis by interference with the utilization of uridine with a concomitant or resultant decrease in protein synthesis. This interpretation is rendered more likely, not only by the results with TMV, and our own data, but also by observations on two additional systems. HOROWITZ *et al.*[53], working with a uracil-requiring mutant of *E. coli*, found that fluorouracil severely depressed the net synthesis of RNA and the incorporation of [^{14}C]adenine. Although some protein synthesis appeared to occur, this protein was probably non-functional as indicated by a complete absence of formation of inducible β-galactosidase. Fluorouracil also caused a block of RNA synthesis in a hepatoma[54]. In our system the FUR block on RNA synthesis probably manifests itself during two separate and distinct periods of phage reproduction: during the period immediately after infection, *i.e.* the time when the early proteins are synthesized, and addition of the agent leads to eventual interference with DNA synthesis as well, and at a period subsequent thereto when the phage proteins proper are laid down[1], and the agent presumably no longer affects the synthesis of DNA**. Thus FUR mimics the action of chloramphenicol and of certain amino acid analogues.

* A very similar phenomenon has also been observed in our laboratories by Dr. A. HUNT. *Pseudomonas fluorescens* KB_1, while adapting to nicotinate also accumulates RNA in the S_2 fraction in the presence of chloramphenicol—in this system it could be shown that initial synthesis occurred in the protoplast membrane, followed by liberation of the freshly synthesized RNA into the S fraction.

** To eliminate all effects of FUR on DNA synthesis it will be advisable to carry out all future experiments in the presence of saturating levels of thymidine.

*** See note added in proof.

Unlike these agents, however, its primary action is presumably on RNA synthesis or on a joint precursor of protein and RNA.

The present report provides some additional evidence for some of the postulates advanced in a previous paper of this series[19] and the scheme presented there. Various fractions isolated from infected lysates show markedly different rates of incorporation of ^{35}S and of ^{32}P. It appears even more likely now that the protoplast membrane system, which may include associated particulate elements, possesses complete functional autonomy for the synthesis of the proteins and nucleic acids required for phage reproduction. Our data here also indicate that the ribonucleoside polyphosphate pool postulated must be in very rapid metabolic equilibrium with some of its component entities, since free pyrimidine nucleosides and inorganic phosphate appear to be capable of entering the polyribonucleotide required for the synthesis of "early protein". Resort to the principle of Occam's razor suggests identity of this polyribonucleotide to the "RNA" of our scheme.

NOTE ADDED IN PROOF

More recent reports by Horonitz et al indicate, however, that the first alternative, *i.e.* incorporation, may be responsible for the observed effects. A very considerable portion of the total RNA-uracil (97 %) of *E. coli* B was replaceable by FU when the cells were grown for 21 h in the presence of the inhibitor. Concomitantly with this synthesis of RNA there was formation of protein; DNA synthesis was inhibited completely. As with the uracil-auxotroph the ability of lactose to induce β-D-galactosidase was almost entirely blocked in the presence of FU as was any further rise in the enzyme in cells which had been pre-induced; cells of a constitutive strain were also unable to form additional enzyme. Some of these effects were reversible by uracil[55,56]. Whether or not incorporation of the analogue into an "early RNA" is sufficiently rapid must, however, still remain an open question. In the case of aza-uracil, another pyrimidine analogue recently shown to interfere with phage replication at an early stage[57] the block is definitely believed to occur *prior* to the formation of RNA.

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