PATTERNS OF CELLULAR CONTROLS OPERATING IN BACTERIOPHAGE REPRODUCTION

II. EFFECT OF 5-FLUOROURACIL ON METABOLIC EVENTS IN BACTERIA INFECTED WITH COLIPHAGE T2r+

JUSSI J. SAUKKONEN*, FRED GOODMAN AND ERWIN CHARGAFF

Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

The formation of protein and deoxyribonucleic acid in *E. coli* cells infected with T2r+ phage in the presence of 5-fluorouracil without or with the addition of pyrimidine supplements (uracil, thymidine) was studied. The fluoro compound completely inhibited the synthesis of deoxyribonucleic acid in infected cells; protein still was produced, though at a diminished rate. In the presence of this inhibitor, uracil stimulated only the synthesis of protein, thymidine also that of deoxyribonucleic acid; with both supplements figures surpassing those of control preparations were recorded.

The purine and pyrimidine composition of the deoxyribonucleic acid present in the various systems was determined and the fate of the nucleic acid of the infecting phage was followed through the use of ^{14}C-labeled phage and the estimation of total and specific radioactivity of the nucleic acid components.

INTRODUCTION

The effects of 5-fluorouracil on the multiplication of even- and odd-numbered T phages were studied in the preceding paper[1]. In the present communication an attempt is made to gain some insight into the biochemical phenomena underlying the inhibition caused by the fluoro pyrimidine. The formation of protein and nucleic acids in *E. coli* cells infected with phage T2r+ was followed under conditions simulating those observed in the preceding study and the composition of the deoxyribonucleic acid remaining or synthesized in these systems was determined. In some of the experiments ^{14}C-labeled phage was employed.

EXPERIMENTAL

Materials

The pyrimidines employed have been mentioned before[1]. The purity of all preparations was checked spectrophotometrically and by means of paperchromato-

* Present address: State Serum Institute, Helsinki, Finland.

graphy in several solvent systems. In the case of 5-fluorouracil use was also made of chromatography on an anion exchange column (Dowex-2, formate; gradient elution with 0.2 *M* ammonia–0.2 *M* ammonium formate) which permits a good separation of fluorouracil and uracil.

Strains and media

The bacterial strain used was *E. coli*, strain B. In all experiments phage T2r$^+$ was employed which was obtained through the courtesy of Prof. F. J. RYAN. The synthetic medium and the general methodology were mentioned in the preceding paper[1].

Radioactive phage was prepared by growing the bacteria in the presence of [^{14}C]-glucose (255,000 counts/min/μmole; 6.5 μmoles/ml of culture) followed by infection with a low multiplicity of phage. Marked lysis occurred within 4 to 5 h, when the phage preparation was concentrated and purified by several cycles of alternating centrifugations at low and high speeds[2]. The purified phage had a titer of $8.4 \cdot 10^{11}$/ml and gave 35,600 counts/min/ml.

Experimental arrangements

The bacterial cells were grown in the synthetic medium with aeration at 37°. When a density of about $2 \cdot 10^8$ cells/ml was reached, the culture was divided into portions to which the various supplements, as specified later, were added and the incubation at 37° was continued for 30 min. Infection was then carried out with T2r$^+$ at a ratio of 10 to 15 phage per bacterium. The samples removed at intervals for various determinations were chilled, brought to a 5 % concentration with respect to trichloroacetic acid by the addition of a 50 % solution, and kept at 4° for 1 h.

For the estimation of total protein and nucleic acids the precipitates were collected by centrifugation and washed once with cold 5 % trichloroacetic acid, twice with ethanol–ether (1:3, v/v) and once with ether[3]. The residues were dissolved in 0.2 *M* sodium hydroxide and kept at 30° for 18 h. Samples of the centrifuged solutions served for the estimation of the nucleic acids and of protein.

For the study of the composition of the deoxyribonucleic acid encountered under various conditions the arrangement of HERSHEY *et al.*[4] was followed. Bovine serum albumin was added to the infected cultures to a final concentration of 0.3 % before precipitation with cold trichloroacetic acid.

In experiments designed to detect the synthesis of small amounts of phage precursor deoxyribonucleic acid, the cells were grown to a density of 3 to $4 \cdot 10^8$/ml and the culture was divided into portions which, after receiving the various pyrimidine supplements, were kept at 37° for 30 min. The samples then were centrifuged and the sediments suspended in the adsorption medium[2] (one-quarter of the original volume) containing the same concentrations of pyrimidine supplements. After the addition of ^{14}C-labeled phage in a ratio of 15 phage per bacterium, the suspensions were kept for 10 min at 37° and the cells were collected by centrifugation and suspended in the growth medium (about three-quarters of the original volume) again containing the pyrimidines employed in the particular experiment. Samples removed at the beginning of the experiment and 3 h later served for the study of deoxyribonucleic acid composition. The individual purines and pyrimidines, separated by paper chromatography, were eluted with 0.01 *N* HCl; the extracts were, after spectrophoto-

metry, neutralized with NaOH, transferred quantitatively to stainless steel planchets, evaporated to dryness, and their radioactivity was determined.

Analytical procedures

The bacterial and phage assays were mentioned in the preceding paper[1]. Deoxyribonucleic acid was estimated by the DISCHE procedure[5] with a preparation from calf thymus as the reference standard, ribonucleic acid by means of the orcinol reaction[6], an ox liver preparation serving as the standard. The modification of the biuret reaction for the estimation of protein[7] employed bovine serum albumin as the standard. Phosphorus was determined colorimetrically[8]. The radioactive specimens were counted at infinite thinness in a Nuclear-Chicago D47 gas flow counter equipped with a Micromil end window.

RESULTS AND DISCUSSION

Changes in nucleic acid and protein contents

The global effects of the presence of 5-fluorouracil in the growth medium on the synthesis of deoxyribonucleic acid and protein are shown in Fig. 1. The fluoro compound completely inhibits the formation of deoxyribonucleic acid by cells infected with T2r$^+$ phage during the observation period of 4 h. This block is not abolished by supplementation with uracil; but thymidine permits a four-fold increase in this nucleic acid: an increase which is, however, significantly lower than in the untreated culture. The addition of both uracil and thymidine to the system containing fluorouracil results in the restoration of the capacity to make deoxyribonucleic acid in amounts that, in fact, often surpass those formed under comparable conditions in untreated phage-bearing cultures. Whether the slight initial rise observed even under conditions of inhibition (Sections II and III of Fig. 1) is meaningful cannot be said.

Protein is accumulated in the cells treated with 5-fluorouracil at a much slower rate than in the absence of the inhibitor, the total amounting to only about one-half of that formed by the untreated cells. The presence of thymidine improves the yield slightly, that of uracil appears to increase both rate of synthesis and total amount considerably. When both supplements are added, a complete reversal of the inhibition, or even an increase over the control cultures, is again recorded.

The observation that thymidine as the only supplement of fluorouracil is able to stimulate the synthesis of considerable amounts of phage deoxyribonucleic acid (Section IV of Fig. 1 and Table I to be discussed below) and even of phage particles[1] could lead to the surmise that the administration of thymidine made some uracil available to the cellular system. We are, however, not aware of any evidence of such a demethylation reaction, except for a recent, equally indirect, suggestion with respect to *Bacillus cereus*[9]. That such a conversion, if it occur indeed, cannot be massive is demonstrated by the inability of thymidine to replace uracil effectively in the uracil-deficient *E. coli* mutant 63–86 (see ref. 10, 11). (Unpublished observations by Dr. J. HOROWITZ.) It should also be remembered that, as we have shown in the preceding paper[1], thymidine is unable to replace uracil as a requisite for the multiplication of T3 phage in cells treated with fluorouracil.

We have omitted, from Fig. 1, observations on changes in ribonucleic acid

content under the conditions of this experiment. It is generally stated that the synthesis of this component ceases immediately upon infection of the bacterium by phage[12], although a metabolically active species of ribonucleic acid[13], differing in composition from the ribonucleic acid of the host cell[14], has been observed in infected cells. (Compare the interesting discussion of this problem on p. 56 of ref. 15.) We noticed occasionally slight increases in ribonucleic acid in the initial stages of infection (within 60 min), especially in the systems containing, in addition to fluorouracil, either uracil alone or uracil and thymidine.

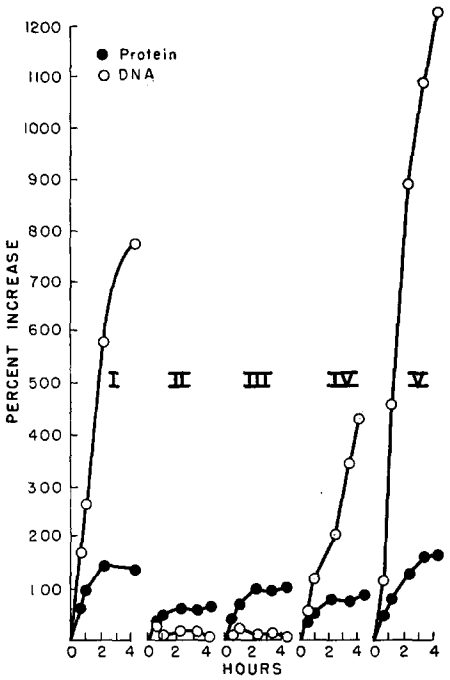

Fig. 1. Formation of protein and deoxyribonucleic acid in *E. coli*, strain B, infected with T2r+ phage in the presence of various pyrimidines (5-fluorouracil, 50 μg/ml; uracil, 20 μg/ml; thymidine, 50 μg/ml). See text for experimental conditions. The addition of phage occurred at zero time. The systems represented are: I, no additions; II, fluorouracil; III, fluorouracil and uracil; IV, fluorouracil and thymidine; V, fluorouracil, uracil and thymidine.

It should be mentioned that, under the experimental conditions followed here, approx. 99 % of the cells were infected within 10 min after phage addition.

The findings on T2r+ described here may be correlated with the results on the production of T2r phage particles summarized in Table III of the preceding communication[1]. In that table it was shown that under conditions of high phage input only about 0.5 % of the normal phage yield was obtained in the presence of 5-fluorouracil; that this figure did not change materially when uracil was added; but that the supplementation of the fluoro compound with thymidine raised the yield to about one-fifth of the normal. These observations are in good agreement with the trend in the formation of deoxyribonucleic acid under the various conditions studied in the present paper. A rise in protein, on the other hand (compare Section III of Fig. 1), is not in itself indicative of the ability to produce phage particles.

TABLE I

COMPOSITION OF DEOXYRIBONUCLEIC ACID IN *E. coli* CELLS INFECTED WITH T2r+ IN PRESENCE OF 5-FLUOROURACIL AND PYRIMIDINE SUPPLEMENTS

The values for composition are reported as μmoles/100 ml culture, with the exception of the parenthetical figures which indicate the particular constituent in mole % of total bases recovered. Abbreviations: Ø, phage T2r+; FU, 5-fluorouracil (50 μg/ml); U, uracil (20 μg/ml); dTR, thymidine (50 μg/ml). The times indicate hours after infection.

Experiment No.	*I*	*II*	*III*	*IV*	*V*	*VI*
Conditions of experiments	*At start, no additions*	*Ø; 3 h*	*Ø, FU; 3 h*	*Ø, FU, U; 3 h*	*Ø, FU, dTR; 3 h*	*Ø, FU, U, dTR; 3 h*
"DNA-P" in total preparation*	0.94	16.32	0.91	0.98	4.64	13.73
DNA-P in extract*	0.73	10.80	0.51	0.56	3.36	10.54
Sum of recovered purines and pyrimidines	0.73	9.22	0.56	0.60	3.47	9.07
			Composition of DNA			
Adenine	0.18 (25)	2.80 (30)	0.16 (29)	0.17 (28)	1.04 (30)	2.81 (31)
Guanine	0.18 (25)	1.83 (20)	0.10 (18)	0.11 (18)	(0.60 17)	1.50 (17)
Cytosine	0.17 (23)	0.03	0.06 (11)	0.05 (8)	0.10 (3)	0.08 (1)
5-Hydroxymethylcytosine		1.62 (18)	0.06 (11)	0.07 (12)	0.51 (15)	1.49 (16)
Thymine	0.20 (27)	2.94 (32)	0.18 (32)	0.20 (33)	1.22 (35)	3.19 (35)
			Molar proportions			
Purines to pyrimidines	1.0	1.0	0.9	0.9	0.9	0.9
Adenine to thymine	0.9	1.0	0.9	0.9	0.9	0.9
Guanine to cytosine + hydroxymethylcytosine	1.1	1.1	0.8	0.9	1.0	1.0

* Determined by means of the diphenylamine reaction.

TABLE II

COMPOSITION OF DEOXYRIBONUCLEIC ACID AND DISTRIBUTION OF RADIOACTIVITY IN *E. coli* CELLS INFECTED WITH ^{14}C-LABELED T2r$^+$ IN PRESENCE OF 5-FLUOROURACIL AND URACIL

The values describing the distribution of individual purines and pyrimidines are reported in μmoles/100 ml culture (with the particular constituent as mole % of total bases recovered in parentheses). The total radioactivity refers to counts/min/100 ml culture (with the specific radioactivities given in brackets as counts/min/μmole). For conditions of experiments see Table I.

Experiment No. / *Conditions of experiments*	*VII* Ø, *FU*, o *h*		*VIII* Ø, *FU*, 3 *h*		*IX* Ø, *FU*, *F*, o *h*		*X* Ø, *FU*, *U*, 3 *h*	
	Composition	*Total radioactivity*	*Composition*	*Total radioactivity*	*Composition*	*Total radioactivity*	*Composition*	*Total radioactivity*
Sum of recovered purines and pyrimidines	1.40	568 [410]	0.68	380 [560]	1.53	606 [400]	0.96	500 [520]
Adenine	0.39 (28)	177 [450]	0.19 (28)	95 [500]	0.42 (27)	192 [460]	0.26 (27)	135 [520]
Guanine	0.30 (21)	94 [310]	0.14 (21)	78 [560]	0.33 (22)	91 [280]	0.19 (20)	69 [360]
Cytosine	0.28 (20)	8	0.09 (13)	6	0.31 (20)	10	0.16 (17)	12
5-Hydroxymethylcytosine	0.04 (3)	89 [2230]	0.06 (9)	56 [930]	0.05 (3)	94 [1880]	0.06 (6)	91 [1520]
Thymine	0.39 (28)	200 [510]	0.20 (29)	145 [730]	0.42 (27)	219 [520]	0.29 (30)	193 [670]
	Molar proportions							
Purines to pyrimidines	1.0		0.9		1.0		0.9	
Adenine to thymine	1.0		1.0		1.0		0.9	
Guanine to cytosine + hydroxymethylcytosine	0.9		0.9		0.9		0.9	
Adenine + thymine to guanine + cytosine + hydroxymethylcytosine	1.3		1.3		1.2		1.3	
Proportion of phage DNA in total DNA, %*	16		50		16		34	

* The figures for hydroxymethylcytosine served for the calculation of the percentage of phage DNA, with the assumption that the latter contained 32 mole % each of adenine and thymine and 18 mole % each of guanine and hydroxymethylcytosine.

Composition of deoxyribonucleic acid

The nature of the deoxyribonucleic acid found in the bacterial cells under various conditions was studied by analyzing the base composition of the preparations; the results are summarized in Table I. The findings on the nucleic acid composition in untreated bacteria (Expt. I) are in good agreement with previous work in which the same, relatively crude analytical method was used[4], as well as with observations on the composition of the intact deoxyribonucleic acid of different *E. coli* strains[16,17]. The recovery of purines and pyrimidines was very satisfactory when compared with the figures for the nucleic acid contents of the extracts (based on the determination of deoxy sugar). Much less satisfactory was the agreement between the deoxy sugar values of the total preparations and the extracts, particularly in the experiments (Nos. III and IV) employing fluorouracil alone or together with uracil.

The procedures used in the present study seem to have a tendency to yield too high values for thymine, but even so the characteristic, nearly equimolar base distribution in the deoxyribonucleic acid of *E. coli* is apparent in the results of Expt. I. After 3 h, in the untreated infected cells (Expt. II) about 17 times as much nucleic acid is found as in noninfected bacteria at the start, but this nucleic acid now has nearly the composition of that of phage[18], and the almost complete disappearance of polymer-bound cytosine demonstrates the destruction of the bacterial nucleic acid during this period. In the systems infected with phage in the presence of 5-fluorouracil and supplemented with both uracil and thymidine or only with the latter (Expts. V and VI) 85 to 90 % of the deoxyribonucleic acid shows the characteristics of phage nucleic acid although, as expected, the absolute amount of nucleic acid formed is much greater in the first instance. The decrease in the cytosine content of these preparations was somewhat smaller in Expt. II; and this may mean that the breakdown of host nucleic acid did not quite go so far in the inhibited systems.

Under conditions of infection in the presence of fluorouracil alone or together with uracil (Expts. III and IV) no increase in deoxyribonucleic acid beyond the control value is recorded; and in these systems both cytosine and hydroxymethylcytosine, in almost equal quantities, are found*. From the figure for the latter pyrimidine it can be calculated that more than one-half of the total deoxyribonucleic acid consisted of phage nucleic acid. There must, consequently, have occurred considerable degradation of bacterial nucleic acid under these circumstances.

It appeared of some interest to reinvestigate the stages in which no net synthesis of deoxyribonucleic acid was apparent, *i.e.* in cells infected with phage in the presence of fluorouracil alone or together with uracil, by a different technique permitting a more direct decision about the fate of the components of the infecting phage. In this series of studies ^{14}C-labeled phage was employed and special care was taken to remove excess phage, as described above. The results are shown in Table II. We shall first consider the findings on the composition of the total nucleic acid of the preparations and then those relating to the fate of the infecting phage nucleic acid. Expts. VII and IX describe the state of the internal deoxyribonucleic acid immediately after the uptake of phage in the presence of fluorouracil either alone or together with uracil. On the basis of the hydroxymethylcytosine content, about one-sixth of the

* A minor component, probably identical with the one mentioned by HERSHEY *et al.*[4], was frequently seen on the chromatograms of the hydrolysates of these preparations. It moved in propanol–HCl together with adenine, in propanol–NH_3 with cytosine.

total nucleic acid is attributable to the phage. This proportion increases considerably after 3 h (Expts. VIII and X) which could, superficially, be taken to mean that more bacterial than phage nucleic acid is destroyed during this period; it could, however, be simply due to a more complete extraction of the latter in these preparations. But the content in deoxyribonucleic acid unquestionably is much lower after 3 h. The figures for hydroxymethylcytosine can also be used to compute the composition of that portion of the total nucleic acid that is attributable to the host; and when this is done, proportions are found that are not too far from those expected for *E. coli* nucleic acid. We have also included in this table some of the characteristic molar proportions of the nucleic acid which, considering the crudeness of the methods, are not unsatisfactory.

Turning now to the findings on radioactivity we notice first in Table II that some of the total radioactivity is lost in the course of 3 h: about 33 % when going from Expt. VII to VIII; 17 % when Expts. IX and X are compared. This drop is, however, inferior to that shown by the balances of total recovered purines and pyrimidines which amounts to 51 and 37 %, respectively, in the two sets of experiments. This may again be taken to signify that relatively more bacterial than phage nucleic acid has disappeared during the experimental period. With one exception, the specific activities of the individual components rose during the interval of 3 h. Only the specific activity of hydroxymethylcytosine decreased; and this, could, perhaps, indicate that some synthesis of this pyrimidine took place even in the presence of the inhibitor: a conclusion to be treated with caution in view of the low concentration of this component. Some differences between cells treated with fluorouracil alone and those supplemented also with uracil are noticeable, but need not be discussed in detail.

The observation that synthesis of some 5-hydroxymethylcytosine may occur even in the presence of 5-fluorouracil (Table II) and the effect of thymidine in permitting a limited resumption of the synthesis of phage precursors and of phage could, perhaps, be reconciled by the assumption that fluorouracil did not completely block the formation of "deoxycytidylate hydroxymethylase" and "thymidylate synthetase"[19,20], but only the action of the latter enzyme.

It may be of some interest to tabulate the percentage contribution of the individual nucleic acid constituents to the total radioactivity observed in the various

TABLE III

DISTRIBUTION OF TOTAL RADIOACTIVITY AMONG DEOXYRIBONUCLEIC ACID CONSTITUENTS IN *E. coli* CELLS INFECTED WITH ^{14}C-LABELED T2r$^+$ IN PRESENCE OF 5-FLUOROURACIL AND URACIL

Compare Table II for details. For the computation, the counts of cytosine were deducted from the total radioactivities listed in Table II.

DNA constituent	*% of total radioactivity*			
	Experiment No.			
	VII	*VIII*	*IX*	*X*
Adenine	32	25	32	28
Guanine	17	21	15	14
5-Hydroxymethylcytosine	16	15	16	19
Thymine	36	39	37	40

experiments; and such a comparison is provided in Table III. In the samples taken immediately after infection, *i.e.*, in Expts. VII and IX, essentially the proportions characteristic for T2r$^+$ phage nucleic acid[18] are observed, though here again (compare the discussion of Table I) the contribution of thymine is somewhat high. After 3 h (Expts. VIII and X) the radioactivity patterns become severely distorted; but it cannot be decided whether the apparently greater conservation of pyrimidine nucleotides is meaningful. In any event, it may be conjectured that the phage nucleic acid is not preserved intact during this period.

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REFERENCES

1 F. GOODMAN, J. J. SAUKKONEN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 44 (1960) 458.
2 A. D. HERSHEY AND M. CHASE, *J. Gen. Physiol.*, 36 (1952) 39.
3 D. ELSON, T. GUSTAFSON AND E. CHARGAFF, *J. Biol. Chem.*, 209 (1954) 285.
4 A. D. HERSHEY, J. DIXON AND M. CHASE, *J. Gen. Physiol.*, 36 (1953) 777.
5 K. BURTON, *Biochem. J.*, 62 (1956) 315.
6 W. MEJBAUM, *Z. physiol. Chem., Hoppe-Seyler's*, 258 (1939) 117.
7 C. F. CRAMPTON, R. LIPSHITZ AND E. CHARGAFF, *J. Biol. Chem.*, 206 (1954) 499.
8 E. J. KING, *Biochem. J.*, 26 (1932) 292.
9 B. H. SELLS, *Biochem. Pharmacol.*, 2 (1959) 255.
10 J. HOROWITZ, J. J. SAUKKONEN AND E. CHARGAFF, *Biochim. Biophys. Acta*, 29 (1958) 222.
11 J. HOROWITZ AND E. CHARGAFF, *Nature*, 184 (1959) 1213.
12 S. S. COHEN, *Advances in Virus Research*, 3 (1955) 1.
13 A. D. HERSHEY, *J. Gen. Physiol.*, 37 (1953) 1.
14 E. VOLKIN AND L. ASTRACHAN, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Johns Hopkins Press, Baltimore, 1957, p. 686.
15 A. D. HERSHEY, *Advances in Virus Research*, 4 (1957) 25.
16 E. CHARGAFF, H. M. SCHULMAN AND H. S. SHAPIRO, *Nature*, 180 (1957) 851.
17 A. N. BELOZERSKY AND A. S. SPIRIN, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. III, Academic Press Inc., New York, in the press.
18 G. R. WYATT AND S. S. COHEN, *Biochem. J.*, 55 (1953) 774.
19 S. S. COHEN, J. G. FLAKS, H. D. BARNER, M. R. LOEB AND J. LICHTENSTEIN, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1004.
20 J. G. FLAKS AND S. S. COHEN, *J. Biol. Chem.*, 234 (1959) 2981.