THE INDUCTION OF MUTANTS OF BACTERIOPHAGE T2 BY 5-BROMOURACIL

III. NUTRITIONAL AND STRUCTURAL EVIDENCE REGARDING MUTAGENIC ACTION*

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(Received October 16th, 1959)

SUMMARY

A study has been made of the induction of mutants of bacteriophage T2 by thymine analogues, in particular by 5-bromouracil (BrU) and 5-bromouracil deoxyriboside. BrU was mutagenic only when the host bacteria were subjected to thymine deficiency brought about in the present work by the addition of sulfanilamide. In media containing sulfanilamide and BrU, *E. coli* B exhibited an absolute requirement for uracil, which was eliminated upon phage infection. The induction of phage mutants by BrU was greatly influenced by the various nutrients provided in the medium. To obtain reproducible results, a defined medium was derived which contained salts, glucose, sulfanilamide, valine, methionine, xanthine, and uracil. In this medium three substances prevented BrU mutagenesis, adenine and serine partially and thymidine completely; these substances are involved in DNA synthesis.

BrU was incorporated into phage DNA in place of thymine in large amounts (nearly 100% in two experiments). This incorporation was always accompanied by the appearance of a high proportion of mutants and a yet higher proportion of non-infective phage, which were also unable to kill *E. coli*. The infective population of phages grown in sulfa and BrU were more sensitive to u.v. light than were normal phages. Of the many pyrimidine analogues tested, only chloro-, bromo-, and iodouracil (not fluorouracil) and especially the deoxyriboside of BrU, exhibited mutagenic activity, which was in all cases specifically prevented by thymidine. All four of these compounds can substitute for thymine in phage DNA. The hypothesis is put forward that BrU mutagenesis is mediated through its incorporation into DNA, but that the incorporation *per se* is insufficient to cause the mutagenesis.

INTRODUCTION

At the present time, there are many lines of evidence which link the chemistry of the material carrying genetic information to the chemistry of deoxyribonucleic acid

* Taken from a thesis by R.M.L. submitted to the Graduate Division of the University of California in 1957 in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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(DNA). To study the chemistry of mutations, therefore, use should be made of substances known to have a direct action on DNA. One class of such substances are the halouracil analogues of thymine, which have been shown to be incorporated into the DNA of several microorganisms[1-3]. We have observed that, when the coli-bacteriophage T2 was grown under conditions in which it could incorporate one of these analogues, 5-bromouracil (BrU) into its DNA, large numbers of mutants appeared among the progeny. The proof that these mutants arose as a result of mutagenic induction and not by selection of preexisting mutants and some preliminary experiments on BrU-induced mutagenesis have been reported in a previous publication[4]. It has furthermore been shown that protein synthesis is not essential for the initiation of the series of events leading to mutation by BrU[5,6].

Because of its direct connection with DNA, therefore, BrU would appear to be an ideal mutagenic agent to utilize for the study of the chemistry of genetic alterations. This paper is concerned with the demonstration of the direct connection between BrU-induced mutagenesis and thymine metabolism and with a description of the chemical and physiological properties of bacteriophages grown in the presence of BrU. The accompanying paper[7] presents the results of kinetic investigations aimed at the elucidation of the mechanism of the BrU-induced mutagenesis.

MATERIALS AND METHODS

Compounds

Amino acids: Two vitamin-free casein hydrolysates were employed in this study. The first, casein hydrolysate I, was prepared in this laboratory and contained principally aspartic acid, threonine, glycine, alanine, serine, glutamic acid, valine, leucine, tyrosine, and lysine. The second was purchased from Difco Laboratories and contained, in addition to the amino acids found in casein hydrolysate I, histidine and arginine. All of the individual L-amino acids were obtained commercially.

Purines: Adenine, guanine, xanthine, and hypoxanthine were all purchased from the Nutritional Biochemicals Corp. 6-methylaminopurine was kindly supplied by the Wellcome Research Laboratories, and all of the purine nucleosides by Dr. C. A. DEKKER.

Pyrimidines: Uracil, 5-aminouracil, and 5-bromouracil were purchased from the Nutritional Biochemicals Corp.; [2-^{14}C]uracil from the Research Specialities Co.; thymine and thiothymine from Dougherty Chemicals; 5-nitrouracil, 5-hydroxyuridine, 5-hydroxyuracil deoxyriboside, 5-bromouridine, 5-bromouracil deoxyriboside, and thymidine from the California Foundation for Biochemical Research. 5-diazouracil, 5-ethyluracil, 6-azauracil, and 6-azathymine were gifts from Dr. E. SASSENRATH; 5-iodouracil was a gift from Dr. P. NEWMARK and 5-fluorouracil from Hoffman-LaRoche Inc.

The 5-bromouracil (BrU) obtained commercially was found to contain a 5 % by weight impurity of uracil; pure BrU was synthesized by the method of WHEELER AND MERRIAM[8] from uracil and bromine. 5-chlorouracil was synthesized in this laboratory by the method of BARRETT, GOODMAN AND DITTMER[9].

Antibiotics: Sulfanilamide (sulfa) was purchased from Mallinckrodt Chemical Works.

Media

The basic synthetic medium used was the glucose-salts medium, M-9 (NH_4Cl, 1 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.2 g; and glucose, 4 g/l of of water[10]. The various additions to this medium will be mentioned in the text, but sulfa when employed was present to the extent of 1 mg/ml. The defined medium developed experimentally, VM medium, contains per liter of M-9: sulfa, 1 g; xanthine, 10 mg; uracil, 2.5 mg; valine, 12 mg; and methionine, 9 mg. The glycerol-casamino acids medium of FRASER AND JERREL[11] was employed for the growth of seed cultures and for bacteria used for phage assays. Adsorption medium consisted of the following per liter of distilled water: NaCl, 4 g; K_2SO_4, 5 g; KH_2PO_4, 1.5 g; Na_2HPO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 5.5 mg; and gelatin, 10 mg; it was used for making bacterial and phage dilutions.

Bacterial strains

Unless otherwise noted *E. coli* B will refer specifically to a substrain *E. coli* B (American), which was found to support a larger production of phage in sulfa containing media than any of the other *E. coli* B strains tested.

Bacteriophage strains

The T2r^+ and T2r_2 phage stocks were obtained from Dr. G. S. STENT. The T2r_2 stock employed throughout most of this work exhibited a 0.1 % background of plaque-type mutants. In general standard phage techniques as described by ADAMS[12] were employed. Phages were always plated on nutrient agar. When desired, high titer phage stocks were prepared according to the method of HERRIOTT AND BARLOW[10] and purified by differential centrifugation.

Experimental procedure for the growth of bacteria and bacteriophage

The incorporation of BrU into the DNA of bacteriophages occurs most efficiently when the bacteria to be infected have been subjected to thymine deficiency. Such conditions can be achieved either by the employment of thymine-requiring bacterial mutants[1,3] or by the addition of an inhibitor of thymine synthesis such as sulfa[2] to the growth medium of normally thymine sufficient organisms. Sulfa has been employed throughout this work, because it is applicable to various strains of *E. coli* and because it provides the opportunity for considerable variation in the experimental conditions. Sulfa reduced the infective phage yield about 10 times in VM medium.

In most experiments, an inoculum of *E. coli* B grown overnight in M-9 was added to the desired experimental medium (containing sulfa) to give a concentration of about $2 \cdot 10^7$ bacteria/ml. Growth with aeration at 37° for four generations was followed by infection with bacteriophage. This extent of growth was sufficient to establish a partial sulfa inhibition, but the resultant bacteria were completely viable and did not differ in appearance from those grown normally. Unless otherwise noted, the bacteria were always infected with a phage multiplicity of above one. Under these conditions lysis inhibition ensued, even with a T2r phage strain. The mature phages could be released by lysing the infected cells with chloroform.

The effects on phage will be reported in terms of infective phage yields and percentage of plaque-type mutants. (Only plaque-type mutants were scored in the present work.) The infective phage yield is the number of phage particles capable of

forming plaques produced per infected bacterium, and the percentage of plaque-type mutants represents the number of mutant plaques observed × 100 divided by the total number of plaques. For each determination of the percentage of plaque-type mutants, at least 1,000 plaques were counted.

Determination of DNA base composition

For the determination of BrU incorporation into the DNA of bacteriophage T2, phage lysates were prepared in large volumes of various media. After four generations of growth, the bacteria were infected with a low phage multiplicity, and the infection allowed to proceed overnight. The resultant phage particles were purified by differential centrifugation and then hydrolysed with 88 % formic acid in a nitrogen atmosphere[13]. The DNA bases were separated by two dimensional chromatography on Whatman No. 1 paper, with isopropanol-HCl[14] and n-butanol-NH_3[15] as the first and second solvents, respectively. Thymine and BrU had the same R_f in the first dimension, but they were separated by the second solvent, thymine having an R_f of 0.44 and BrU of 0.35. The bases were eluted from the paper with 0.1 *N* HCl and their concentration determined from optical density readings.

Technique for u.v. irradiation

The phages to be irradiated were suspended in adsorption medium, 3 ml placed on a 10-cm watch glass and irradiated at a distance of 82 cm with 350 ergs/sec/cm^2 of u.v. from 0.35 amp. Rad-i-air lamp, whose main output was at 2537 Å. After each consecutive 8-sec irradiation, aliquots were withdrawn for later dilution and plating for phage survivors. The platings were performed in semidarkness to prevent photoreactivation. In each case, the experimental samples which were progeny of T2r_2 were admixed with approximately equal numbers of a control phage stock of a different plaque-type, T2r^+. Due to the presence of T2r^+ in the same irradiation vessel, the resultant u.v. inactivation curves could be corrected for the protection against u.v. afforded by the presence in the medium of any nucleic acid derivative.

RESULTS

Nutritional requirements for the sulfa and bromouracil system

The growth of *E. coli* in the presence of sulfa requires the addition of certain amino acids, a purine, and a pyrimidine[16]. In our[4] early studies of BrU-induced mutagenesis, the medium of DUNN AND SMITH[7] which contains a vitamin-free casein hydrolysate, xanthine, and thymine, respectively was employed. The results obtained with one preparation of casein hydrolysate differed from those obtained with another, and the substitution of xanthine and thymine by other naturally occurring bases also caused variations in the number of phage and phage mutants produced. A series of investigations was therefore undertaken to determine which of the amino acids, purines and pyrimidines gave the greatest yield of mutants, to develop a simple defined medium that gave reproducible results, and to obtain information on the mechanism of mutagenesis.

Effects of various amino acids on mutagenesis: The amino acid requirements for mutant production and phage development were investigated by two methods: supplementation of a synthetic medium by individual amino acids, and amino acid

elimination from a complete medium. In all of these experiments, the purine requirement was fulfilled by xanthine and the pyrimidine requirement by uracil.

In the tests of the effects of addition of individual amino acids, valine, methionine, and serine were first chosen for study because these three had been found by WINKLER AND DE HAAN[16] to support the growth of *E. coli* in a sulfa containing medium. It can be seen from Table I that T2 grew and was caused to mutate in the absence of added

TABLE I

EFFECTS OF THE ADDITION OF VALINE, METHIONINE, AND SERINE ON PHAGE AND PHAGE MUTANT PRODUCTION

E. coli B was inoculated into M-9 medium supplemented with 1 mg/ml sulfa, 25 μg/ml xanthine, 50 μg/ml BrU, 2.5 μg/ml uracil, and the desired amino acid mixture. After four generations of growth, the bacteria were infected with a T2r_2 multiplicity of two, and 2–3 h later the infected cells were caused to lyse by the addition of chloroform. The progeny phages were then diluted and plated on *E. coli* B, and the results registered in terms of infective phage yield per bacterium and the percentage of plaque-type mutants.

Amino acid			*Infective phage yield*	*% Plaque-type mutants*
Serine (25 μg/ml)	*Valine (40 μg/ml)*	*Methionine (30 μg/ml)*		
—	—	—	50	2.5
+	—	—	87	1.1
+	+	—	130	1.2
+	—	+	87	1.0
+	+	+	150	1.1
—	+	—	45	3.8
—	—	+	30	1.9
—	+	+	100	3.9

amino acids. Serine alone or in combination with the other amino acids stimulated T2 production, but greatly reduced the fraction of mutants. The highest yields of mutants and total phage were obtained in the presence of valine plus methionine. Addition of a few other amino acids, notably leucine, did support increases in infective phage production, but none significantly increased the mutagenic power of BrU. The above observations led to the synthetic medium (VM-medium, described under METHODS) which was used in most of the experiments to be reported. A number of variations of this medium were studied, but in general they showed little effect or were not beneficial to mutant production.

Very similar results were obtained when, in VM medium, BrU was added at the time of phage infection instead of at the time of bacterial inoculation. With varying BrU concentrations the percentage of mutants rose to a maximum at 50 μg/ml BrU and then decreased at higher concentrations of BrU (Fig. 1).

At a multiplicity of 2 T2 per bacterium, BrU inhibited phage production by about 60% (Fig. 1); but at a multiplicity of 6 T2, the BrU was not inhibitory (see Table IV).

In all of these experiments, the phage yields were higher and the percentage of mutants somewhat lower than at the same BrU concentration in media rich in amino acids. This discrepancy was investigated by elimination of groups of amino acids from a complex medium. Results obtained with media containing Difco casein hydrolysate were not changed greatly when a mixture of 18 amino acids[17] was

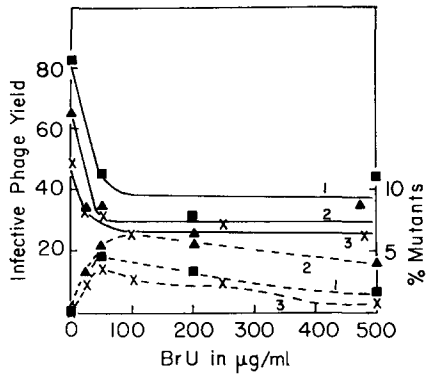

Fig. 1. Pattern of action of varying concentrations of BrU on phage and phage mutant production in valine and methionine media. Expt. 1: Valine 40 μg/ml and methionine 30 μg/ml. BrU added at time of bacterial inoculation. Expt. 2: Valine 12 μg/ml and methionine 9 μg/ml. BrU added at time of bacterial inoculation. Expt. 3: Valine 12 μg/ml and methionine 9 μg/ml. BrU added at time of phage infection. In all three experiments, the bacteria were infected with 2 phages per bacterium and lysed with chloroform 2 h later. ——, infective phage yield; – – –, percentage of plaque-type mutants.

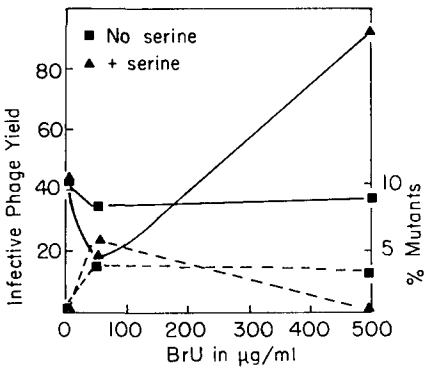

Fig. 2. Pattern of action of varying concentrations of BrU on phage and phage mutant production in 13 amino acid medium or in the same medium without serine. BrU added at time of bacterial inoculation. The bacteria were infected with 2 phages per bacterium and lysed with chloroform 2 h later. ——, infective phage yield; – – –, percentage of plaque-type mutants.

employed (or even with 7 amino acids including serine, although in this case only 3 % mutants appeared). Data obtained in a medium containing 13 amino acids, including serine, found by FOWLER AND COHEN[18] to support the growth the growth of T2 as satisfactorily as more complex media, gave results like those obtained with casein hydrolysate. Upon removal of serine the data took on a very different appearance (Fig. 2); and the results were now similar to those obtained with VM medium (Fig. 1). In the presence of serine a marked dependence of the percentage of mutants and phage yield on BrU concentration was noted, whereas in the absence of serine the results were relatively independent of BrU concentration.

An explanation of the partial prevention of the mutagenic action of BrU by serine in the simple VM medium (Table I), is that the β-carbon of serine is normally utilized for the synthesis of the 5-methyl group of thymine and the 5-hydroxymethyl group of 5-hydroxymethylcytosine of phage DNA[19] therefore, serine was undoubtedly utilized as a precursor for the synthesis of the natural phage pyrimidines, since the block of one carbon transfer reactions by sulfa was not complete. Thus the effect of the one amino acid which partially prevented the action of BrU can be related to the synthesis of thymine.

Purine supplementation and mutagenesis: In the experiments reported so far, the purine, xanthine, had always been added to the media; but the addition of other naturally occurring purines greatly affected the action of sulfa and BrU (Table II). The best combination of high phage yields and high percentages of mutants were obtained when xanthine was provided. All of the adenine compounds inhibited the production of phage and phage mutants and also bacterial growth when added singly or in combination with other purines. In the presence of purine nucleosides, the phage yields were always slightly lower with the ribose compounds than with the corresponding deoxyribonucleosides. 6-methylaminopurine, which increases in concen-

tration under conditions of thymine deficiency[20], did not reverse bromodeoxyuridine action and probably did not function as a methyl donor, nor did it behave like an adenine derivative.

On the basis of the results of Table II, it would appear that the most desirable of the purines for the expression of mutagenesis by BrU is xanthine, and that adenine compounds counteract BrU action.

TABLE II

EFFECTS OF THE ADDITION OF PURINE COMPOUNDS ON THE PRODUCTION OF PHAGE AND PHAGE MUTANTS

E. coli B was inoculated into M-9 medium supplemented with the following per ml: 1 mg sulfa, 12 μg valine, 9 μg methionine, 2.5 μg uracil, and 0.65 μmoles of the desired purine. After four generations of growth, the bacteria were infected with a high multiplicity of T2r_2, and the infected cells caused to lyse by the addition of chloroform 2 and 3 h later for Expts. A and B, respectively. In Expt. A, 50 μg/ml BrU was added at the time of bacterial inoculation, and in Expt. B, 50 μg/ml bromodeoxyuridine at the time of phage infection.

Purine added	*Expt. A*		*Expt. B*	
	Infective phage yield	*% Mutants*	*Infective phage yield*	*% Mutants*
None	50	2.0	66	3.0
Xanthine	50	4.0	44	6.0
Hypoxanthine	25	3.0	25	6.5
Xanthine + hypoxanthine	23	1.5	—	—
Guanine	50	3.5	58	4.5
Guanine + xanthine	55	2.5	—	—
Guanine riboside	—	—	36	5.0
Guanine deoxyriboside	—	—	62	3.0
Adenine	30	2.0	28	1.5
Adenine + xanthine	10	1.0	—	—
Adenine + guanine	3	0	31	3.0
Adenine riboside	—	—	28	1.0
Adenine deoxyriboside	—	—	78	1.0

TABLE III

EFFECTS OF THE ADDITION OF PYRIMIDINES ON THE PRODUCTION OF PHAGE AND PHAGE MUTANTS

E. coli B was grown in VM medium for four generations and then infected with a high multiplicity of T2r_2. The pyrimidine compounds were all added at the time of phage infection, and the infected bacteria were all lysed by the addition of chloroform 2 h after infection.

Expt.	*Pyrimidine added*	*Pyrimidine concentration in μg/ml*	*Infective phage yield*	*% Plaque-type mutants*
A	None	—	57	0.1
	Thymine	50	73	0.1
	Thymidine	25	80	0.1
	BrU	50	57	1.9
	BrU + thymine	50 + 50	67	2.3
	BrU + thymidine	50 + 25	51	0.1
B	None	—	106	0.1
	BrUDR	50	39	4.5
	BrUDR + thymidine	50 + 25	91	0.1

Pyrimidine supplementation and mutagenesis: The *E. coli* B strain used in these experiments grew in VM medium in the absence of added thymidine or other pyrimidines, but upon the addition of BrU it exhibited an absolute requirement for uracil. The added uracil was shown by tracer techniques to be utilized for the synthesis of all of the pyrimidines of the RNA and DNA of the bacteria. This uracil requirement, however, disappeared upon phage infection. As uracil proved not to be inhibitory to phage development, it was left in the medium during the infectious process and was shown (using [2-^{14}C]uracil) to be utilized for the synthesis of the phage DNA pyrimidines.

Of the naturally-occurring pyrimidine compounds, only thymidine was able to prevent the mutagenic action of BrU (Table III). The specific reversal of the action of the BrU mutagenesis by thymidine, a compound not known to be utilized for anything other than DNA synthesis, provides strong evidence in support of a direct connection between BrU-induced mutagenesis and DNA metabolism.

Survey of pyrimidines for mutagenic action: A number of pyrimidine derivatives were tested for their ability to induce the appearance of plaque-type mutants of bacteriophage T2. The compounds exhibiting mutagenic action in VM medium were the 5-halouracil derivatives: 5-chlorouracil, 5-bromouracil, 5-iodouracil, and especially 5-bromouracil deoxyriboside (BrUDR) (Table IV) (see also[21]). BrUDR was both the

TABLE IV

COMPARISON OF THE EFFECTS OF 5-HALOURACIL DERIVATIVES ON PHAGE AND PHAGE MUTANT PRODUCTION

E. coli B was grown in VM medium for four generations and then infected with 6 T2r_2 per bacterium. All of the 5-halouracil derivatives were added at the time of phage infection and at a concentration of 0.26 μmoles/ml. The infected bacteria were caused to lyse 3 h after infection by the addition of chloroform.

Halouracil	*Infective phage yield*	*% Plaque-type mutants*
None	115	0.1
5-chlorouracil	192	1.9
5-bromouracil	106	2.5
5-iodouracil	159	0.5
5-bromodeoxyuridine	33	7.7
5-bromouridine	73	0.4
5-fluorouracil	6	0.1

most powerful mutagen and the most inhibitory to the production of infective phage. Even at a concentration as low as 0.02 μmoles/ml, BrUDR could induce the appearance of at least 1.5 % mutants. Of the free halouracil bases, BrU induced the highest proportion of mutants, but a larger total number of mutants was obtained with chlorouracil. Iodouracil was the least effective mutagen of the three. None of the bases were inhibitory to phage growth and all are known to be incorporated into phage DNA in place of thymine[22]. Finally, the mutagenic action of all four compounds was prevented specifically by the simultaneous addition of thymidine.

Bromouridine had a slight mutagenic effect (Table IV), but most likely it was converted by the infected bacteria into BrUDR, and the 0.4 % mutants obtained was an expression of the extent of this conversion.

The halopyrimidine 5-F-uracil was not at all mutagenic and was very inhibitory to phage growth (see Table IV). Of the other compounds tested in VM medium the following had no demonstrable mutagenic action: uracil, 5-aminouracil, 5-diazouracil, 5-ethyluracil, 5-nitrouracil, 6-azauracil, 6-azathymine, 2-thiothymine, 5-hydroxyuridine, and 5-hydroxyuracil deoxyriboside. Most of these compounds did reduce phage yields.

Properties of bacteriophage grown in the presence of bromouracil

Incorporation of BrU into the DNA of T2: Base ratios: In agreement with DUNN AND SMITH[2], it was found that BrU affects the chemical composition of phage DNA only by replacing some of the thymine. Several examples of the molar base ratios of the DNA of T2r_2 grown in sulfa and BrU are presented in Table V and compared to

TABLE V

MOLAR BASE RATIOS OF T2r_2 PHAGES GROWN IN SULFA AND BROMOURACIL
Lysate numbers refer to preparations mentioned in Table VI.

Base	*Molar base ratios*			
	Lysate 6	*Lysate 7*	*Lysate 8*	*Normal T2r*[13]
Guanine	1.06	1.15	1.05	1.09
HMC*	0.78	0.85	0.87	1.03
Adenine**	2.00	2.00	2.00	2.00
Thymine	1.03 } 2.06	0.85 } 2.10	0.87 } 2.19	2.07 } 2.07
BrU	1.03 }	1.25 }	1.32 }	0.00 }

* HMC = 5-hydroxymethylcytosine.
** Value assigned arbitrarily.

the values obtained by WYATT AND COHEN[13] for normal T2*r* phages. Within experimental error, the ratios of adenine to guanine were normal, and the ratios of adenine to thymine + BrU were equivalent to the normal adenine to thymine ratio. The hydroxymethylcytosine values were always low, but as it is difficult to recover 100% of this base, these low values probably were due to experimental losses and not to an effect of BrU. In the subsequent discussion, therefore, the per cent BrU incoporated will refer to BrU/(Thymine + BrU) × 100.

Extent of BrU incorporation and mutagenic action: A number of experiments designed to measure the amount of BrU incorporation into the DNA of T2, the per cent plaque-type mutants, and per cent infectivity of these phages are presented in Table VI. The growth media employed all had in common the presence of sulfa, xanthine, and uracil, but differed in the source of amino acids listed in the second column and in the BrU concentration listed in the third column. It can be seen that the actual amount of BrU incorporated was influenced by both the amino acid content and the BrU concentration in the medium. Sufficient data are not available to explain these differences.

Of the biological properties of the phages which had incorporated BrU, the most striking was that they all exhibited a high proportion of plaque-type mutants (listed in the fifth column of Table VI). There was no obvious relationship between the amount of thymine replaced by BrU and the percentage of plaque-type mutants produced. Even when almost all of the thymine was replaced by BrU, most of the

TABLE VI

INCORPORATION OF BrU INTO THE DNA OF BACTERIOPHAGE T2

Concentrations of amino acids: both casein hydrolysates, 1 mg/ml; methionine 30 μg/ml and valine 40 μg/ml.

Lysate No.	*Amino acid source*	*BrU in μg/ml*	*% BrU incorporated*	*% Plaque-type mutants*	*% infectivity*
1	Casein Hydrolysate I	50	100	11.5	9
2	Casein hydrolysate I	500	100	13	2
3*	Casein hydrolysate I	2000	67	11.5	24
4	Difco casein hydrolysate	50	54	11	6
5	Difco casein hydrolysate	50	55	8	6
6	Methionine + valine	25	50	4	53
7	Methionine + valine	50	60	7	54
8	Methionine + valine	50	60	6	39
9	Methionine + valine	200	78	7	30

* $T2r^+$; all other lysates started with $T2r_2$.

viable phages were not plaque-type mutants. This result would indicate that the incorporation of BrU alone does not explain the mutagenic property of this agent.

BrU incorporation and production of inactive phage: Another biological property affected by growth in the presence of BrU was the ability of the phage particles to form plaques. The infectivity of the purified lysates (listed in the last column of Table VI) was calculated from a comparison of the plaque titer and the total number of phages, as determined by the O.D. at 260 mμ. The phages grown with casein hydrolysate I (lysates 1 to 3) and with valine and methionine (lysates 6 to 9) exhibited an approximate reciprocity between BrU incorporation and phage infectivity (in agreement with the results of DUNN AND SMITH[2]). However, this relationship did not hold for the phage lysates grown with the Difco casein hydrolysate (lysates 4 and 5), in which the percentage of the phage population remaining infective was always smaller than the percentage of thymine in the phage. Therefore, phage lysates were partially inactivated by the incorporation of BrU into their DNA, but the extent of this inactivation was not always proportional to their content of BrU.

In the two preparations listed as having 100 % BrU incorporation and 9 and 2 % infectivity (lysates 1 and 2, respectively of Table VI) no thymine had been observed on the chromatograms of the DNA hydrolysates, but the ratios of adenine to BrU were 0.93 and 0.95, respectively. Normally, there is less adenine than thymine + thymine analogue (see Table V). It is quite possible, therefore, that a small quantity of thymine may have been overlooked. But even if the 100 % values were in error by 5 or 10 %, it would seem extremely unlikely that 90 to 95 % of the phage populations had incorporated all of the BrU while the remainder had none. Thus, although the results of lysates 1 and 2 are not absolutely conclusive, they do provide evidence that phages can be infective while containing BrU in their DNA.

Ability of non-infective phage to kill bacteria: A few studies were made to determine whether the non-infective phages could retain their ability to kill *E. coli* B. Varying numbers of phage were adsorbed to bacteria and the cultures were plated to determine bacterial colonies. From the fraction of surviving bacteria at any phage concentration, equal to e^{-M} according to the Poisson distribution formula, the multiplicity M of the killing particles can be calculated. The ratio of M to the known multiplicity of

TABLE VII

BACTERIAL KILLING ABILITY OF T2 GROWN IN SULFA AND BrU

E. coli B was grown to about 10^8 cells per ml in M-9 medium, then centrifuged and resuspended in the same volume of adsorption medium. This suspension was distributed into individual tubes, and kept on ice. Then serial dilutions of the phage lysates to be tested were added to the separate tubes after prewarming to 37°, and adsorption allowed to proceed for 10 min before dilution and plating for numbers of surviving bacteria. A was a purified lysate grown in BrU and shown to contain 98 % non-infective phage particles; B was a crude lysate.

Lysate	*Fraction of bacterial survivors* e^{-M}	*Multiplicity of killing particles* M	*Multiplicity of infective phage added* N	*Ratio of killing to infective particles* M/N
A	0.12	2.1	1.9	1.1
	0.225	1.5	1.1	1.35
	0.385	0.95	0.8	1.2
	0.61	0.45	0.4	1.1
	0.86	0.15	0.16	0.9
	0.96	0.05	0.08	0.6
B	0.00	—	3.0	—
	0.16	1.8	0.6	3.0
	0.76	0.27	0.13	2.1
	0.905	0.1	0.03	3.3
	0.95	0.05	0.013	3.9

infective, plaque-forming phages added, N, gives the number of killing particles present per infectious particle.

Two different results were obtained (Table VII) with these phage preparations, depending on their purity. In lysate A, a purified preparation of phages, the bacterial killing ability could be entirely accounted for by the infective phage population. This preparation was known to contain 98 % non-infective phages. Therefore, the phage particles rendered incapable of forming plaques had also lost the ability to kill bacteria. In lysate B there were about three times as many killing particles as infective phages. The phages were still suspended in their original growth media, and the excess in bacterial killing power must have been due to the presence of particles of non-viral or incomplete viral nature[23].

Sensitivity of phages to inactivation by u.v. light: In order to determine whether the infective phages resulting from growth in sulfa, or in sulfa and BrU, could be distinguished from infective phages grown in the absence of these compounds, their sensitivities to inactivation by u.v. light were measured. In each case, the experimental samples ($T2r_2$) to be irradiated were admixed with an approximately equal number of normal $T2r^+$ (see METHODS). With phages grown in sulfa alone (Fig. 3A), the initial shoulder always observed with T2 was greatly reduced, and, although the exponential part of the inactivation curve exhibited the same slope as the control, it extrapolated to only 1.4 times the initial titer rather than the normal 2 times. Thus sulfa grown phages were inactivated by u.v. light at the same rate as the control phages, but were more sensitive in terms of absolute survival at any one dose.

In Fig. 3B, the u.v. sensitivity of $T2r_2$ phages grown in the presence of sulfa and BrUDR is compared to that of normal $T2r^+$. The shape of the inactivation curve of the former is quite different from that of the control. The shoulder was diminished in sharpness as for the sulfa grown phages, but the straight part of the curve could be

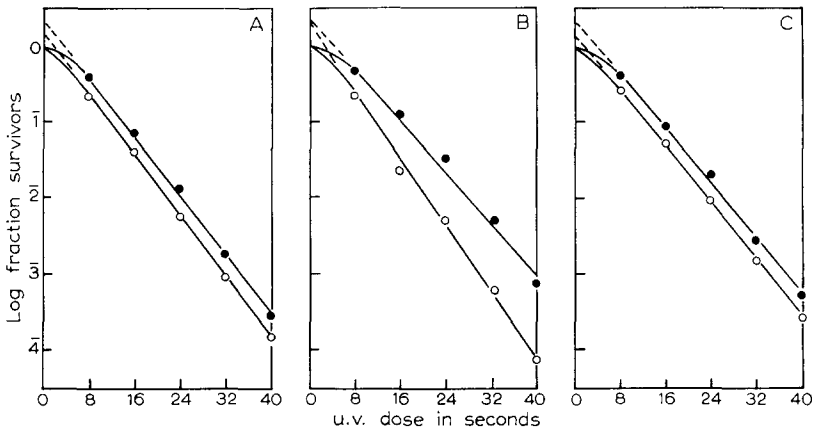

Fig. 3. u.v. inactivation curves of T2r_2 grown under various experimental conditions. A. T2r grown in sulfa alone. B. T2r_2 grown in sulfa and BrUDR. C. T2r_2 grown in sulfa, BrUDR and thymidine. O = experimental T2r_2; ● = normal T2r^+. Ordinate: log of the fraction of infective phage survivors.

extrapolated back to about twice the initial titer. The slope of this line, however, differed from that of T2r^+ by a factor of 1.27, indicating that the infective progeny of T2r_2 grown in sulfa and BrUDR are inactivated by u.v. light about 25 % more rapidly than are normal phage.

Thymidine was able to abolish the increased u.v. sensitivity brought about by growth in BrUDR. Fig. 3C presents the u.v. inactivation curve of phages grown in the presence of sulfa and both BrUDR and thymidine, and shows that thymidine prevented any change brought on by the mutagen. The shape of this curve was identical to that of sulfa grown phages (Fig. 3A), indicating once more that thymidine specifically reversed only the action of BrUDR and not the effect of sulfa (see Table III).

To determine whether BrU induced a heritable increase in u.v. sensitivity, an aliquot of phages grown in the presence of BrU was allowed to infect *E. coli* B in a normal synthetic medium, and the resultant progeny were tested for their reaction to u.v. These progeny no longer exhibited the u.v. sensitivity characteristics of their parents and their u.v. inactivation curve was identical in form to that of T2r^+.

On the basis of these observations one can state that the *viable* phages grown in sulfa and BrUDR (or BrU) are modified phenotypically in their structure. But it is not possible to decide whether the increased u.v. sensitivity of these phages is due to the presence of BrU in their DNA or to an alteration in the structure of other parts of the phage.

DISCUSSION

These studies have three objectives. The first is to provide a simple system for the study of BrU mutagenesis, in which one can reproducibly obtain an easily countable number of mutants. The second is to study the properties of phages containing BrU; and the third is to gather evidence from nutritional and analytical data regarding the manner in which BrU causes mutation. The first objective has been accomplished: a simple synthetic medium has been devised in which, under well-specified conditions,

5 to 10 % plaque-type mutants can be routinely obtained in a single cycle of reproduction. It should be stressed that BrUDR, a mutagen superior to BrU, is needed to obtain this percentage of mutants. FREESE[21] has independently found BrUDR to be an excellent mutagen even in the absence of sulfa.

The principal conclusion regarding the nature of the mutagenic action is that reactions of thymidine are involved. The evidence for this view is several-fold: thymidine reverses the mutagenic action of BrU compounds completely; BrUDR, an anologue of thymidine, is the most effective mutagen; sulfa, which inhibits the synthesis of thymidine is required for the mutagenic action of BrU; serine which stimulates the synthesis of thymine decreases the mutagenic action of BrU.

The incorporation of BrU in place of thymine in DNA is most probably involved in the mutagenic action. With incorporation near 100 % in some cases, it is likely that viable phages contain BrU—a suggestion supported by the finding that viable phages grown in the presence of BrU are more sensitive to u.v. light and to x-rays[24] than are normal phages. Perhaps the strongest piece of evidence in favor of this hypothesis is that only those pyrimidines that are incorporated in place of thymine (*e.g.*, the Cl-, Br-, and I-uracils) are mutagenic, although other pyrimidines have equally strong inhibitory effects on phage production.

The data presented here do not rule out an alternative hypothesis that a limited supply of thymine (caused by the combined action of sulfa and BrU) creates mutagenic alterations in the phage DNA; for example, substitutions or deletions could be brought about at sites normally occupied by thymine. Indeed, thymine deficiency has been shown to produce mutations in *E. coli*[25]. In the present experiments the lack of proportionality between the amount of BrU incorporated and the per cent mutants suggests that mutagenesis is not simply due to incorporation. Also, the nutritional complexities observed (effects of amino acids and adenine compounds and the *decrease* in the per cent mutants when high concentrations of BrU are added), and also the production of numerous inactive particles and lethal subunits in the presence of BrU suggest that the action of BrU is not restricted to its simple substitution into DNA in place of thymine; but whether these metabolic alterations have anything to do with the primary steps of mutagenesis is not clear.

Even though incorporation of BrU may be an essential step in the mutagenic process, it is not at all certain that this is the step at which the mutation is completed; *i.e.*, BrU in DNA might act in every way like thymine, and only when it is replaced by another base, perhaps HMC, would a hereditary change be established. Further investigations of these matters will be presented in the following paper[7].

ACKNOWLEDGEMENTS

We wish to thank Dr. G. S. STENT for valuable discussions and suggestions and for space in his laboratory during part of the execution of this work. This work was supported (in part) by cancer research funds of the University of California and USPHS Training Grant.

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