Effects of 5-Halogenated Uracils on the Growth of *Escherichia coli* and their Incorporation into Deoxyribonucleic Acids

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A variety of structural analogues of purines and pyrimidines are known to affect the growth of viruses, bacteria and other micro-organisms, plants and animals. Some of these may exert their action through being incorporated into nucleic acids in place of the natural purine or pyrimidine. Such examples are: 8-azaguanine, a structural analogue of guanine, which has been shown to replace guanine in the ribonucleic acid (RNA) of tobacco-mosaic virus (Matthews, 1953), and is incorporated into the RNA of turnip-yellow-mosaic virus (Matthews, 1955), of several micro-organisms (Henrich, Dewey, Parks & Kidder, 1952; Lasnitzki, Matthews & Smith, 1954; Matthews & Smith, 1956), of both mouse tumour and normal tissues (Mitchell, Skipper & Bennett, 1950; Mandel, Carlo & Smith, 1954; Lasnitzki *et al.* 1954), and 2-thiouracil which is incorporated into the nucleic acid of tobacco-mosaic virus (Jeener & Rosseels, 1953; Matthews, 1956).

Thymine

5-Bromouracil

5-Bromouracil (whose structure is shown above), 5-iodouracil and 5-chlorouracil may be considered as structural analogues of thymine where the methyl group has been replaced by a halogen atom. 5-Bromouracil inhibits the growth of *Lactobacillus casei* (Hitchings, Falco & Sherwood, 1945; Hitchings, Elion & Falco, 1950), *Streptococcus faecalis* R (Weygand & Wacker, 1952; Weygand, Wacker & Dellweg, 1952; Prusoff, 1954), *Lactobacillus leichmannii* and *Lactobacillus arabinosus* (Bardos, Levin, Herr & Gordon, 1955) and vaccinia virus (Thompson *et al.* 1949), and in most instances the inhibition is reversed by thymine. Weygand and his co-workers (1952) showed that, after the growth of *Strep. faecalis* R in a medium containing

5-bromouracil labelled with the radioactive isotope ^{82}Br , a radioactive substance with some of the properties of 5-bromouracil could be isolated from the nucleic acids after hydrolysis. They concluded that 5-bromouracil was incorporated into the bacterial deoxyribonucleic acid (DNA), although they did not actually demonstrate which nucleic acid contained the analogue.

We have studied the effects of the 5-halogenated uracils on the growth of two strains of *Escherichia coli* and their bacteriophages and have shown that they are incorporated into the DNA of both the bacteria and viruses, quantitatively replacing thymine residues (Dunn & Smith, 1954). At the same time Zamenhof & Griboff (1954) were independently studying the effect of 5-bromouracil on several strains of *E. coli* and also showed that this analogue was incorporated into the bacterial deoxyribonucleic acid.

In this communication we shall describe in detail the effects of the halogenated uracils on the growth of bacteria and bacteriophage and the isolation of the nucleotides and nucleosides of these substances from deoxyribonucleic acids.

As Weygand & Wacker (1952) found that a small proportion of 5-bromouracil was incorporated into *E. coli* under normal conditions, we examined the effect of the analogues in conditions where the bacteria were dependent on a supply of thymine for their growth. For this we used either *E. coli* strain B/r, which normally does not utilize thymine but which may be made thymine-dependent for its growth by cultivation in a sulphanilamide-containing medium (Winkler & de Haan, 1948), or a thymine-requiring strain of *E. coli*, 15T⁻, which has been isolated and studied by Barner & Cohen (1954); Cohen & Barner (1954, 1955).

EXPERIMENTAL

Growth of bacteria and bacteriophages

Basal medium. *Escherichia coli* strain B/r and strain B were grown in the following medium (A): KH_2PO_4 , 13.6 g.; $(\text{NH}_4)_2\text{SO}_4$, 3.3 g.; NaOH, 2.4 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g.; CaCl_2 , 0.01 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005 g., made up to 1 l. with water. Sterile 40% (w/v) glucose was added after autoclaving to give a final concentration of 1%. *E. coli* 15T⁻

was grown on the same medium supplemented with various amounts of thymine, 2.5 mg./l. being the minimum requirement of this organism under our conditions.

Bacteria were grown at 37°, either in 5 l. bottles with vigorous aeration from an air compressor, or in flasks on a rotary shaker (175 rev./min. with a 2 in. diam. movement). On harvesting, the culture was chilled to 4° and the bacteria were sedimented by centrifuging and washed either in 0.14M-NaCl or in 0.1M-sodium citrate, pH 7.3.

Bacteria inhibited by sulphanilamide. Whereas the inhibition of *E. coli* B/r by 5-bromouracil was observed in a synthetic medium similar to that described by Rutten, Winkler & de Haan (1950) (Table 1), it was found preferable to replace the amino acids by a vitamin-free casein hydrolysate as this modification considerably increased the growth rate of the bacteria (Table 1). *E. coli* B/r was grown therefore in medium S, which consisted of medium A supplemented as follows: sulphanilamide, 1 mg./ml.; vitamin-free casein hydrolysate (Difco, or prepared as described by Barton-Wright, 1952), 1 mg./ml.; xanthine, 25 mg./l.; and varying amounts of thymine. For some of the bacteriophage experiments the xanthine in medium S was replaced by 25 mg. of adenine/l. Under these conditions adenine is slightly inhibitory to the growth of the bacteria (Shive & Roberts, 1946; Winkler & de Haan, 1948), but it was found that the growth rate of the bacteria was similar to that in the xanthine medium if 5 mg. of DL-tryptophan/l. was also added to the medium. This apparent antagonism between adenine and tryptophan does not appear to have been observed previously.

As it was found that *E. coli* B/r inhibited by 5-bromouracil in medium S continued to grow to their normal maximum density, even when the inhibitor was added at about 10⁸ cells/ml., it was possible to grow cultures of the organism in solid medium in Roux flasks, the analogue being incorporated into the agar medium before autoclaving. This method did not give higher yields per volume of medium than the liquid culture (approx. 3 mg./ml.) and required 72 hr. incubation to give maximum yields, so it was used only for one preparation.

Since *E. coli* grown on nutrient agar or nutrient broth was

expected to contain considerable quantities of *p*-amino-benzoic or folic acids, which can reverse the sulphanilamide inhibition (Winkler & de Haan, 1948), the bacteria were cultured on medium A and then on medium S before being used for the experiments. This method resulted in bacteria dependent on thymine for their growth, but by using only one cycle of growth in medium S, we avoided the selection of mutants resistant to sulphanilamide. The following procedure was that adopted for the preparation of inocula. Two drops from an 18 hr. culture of *E. coli* B/r in medium A were added to 4.5 ml. of one-quarter strength Ringer solution (Locke), resulting in a dilution of approx. 1/100. Ten drops of this dilution (containing about 2 × 10⁸ bacteria) were added to 100 ml. of medium S containing 10 mg. of thymine/l. The bacteria were grown aerobically at 37° for 24 hr., in which time the density reached approx. 10⁹ bacteria/ml. This culture was used as the inoculum for growth and incorporation experiments, being added to give about 10⁸ cells/ml. (The growth rate of the bacteria in medium S was greatly increased for 0.5–1.5 hr. after inoculation. The reason for this increase in growth rate has not been determined but the increase was found to be more pronounced when the inoculum had been grown under partial anaerobic conditions in non-shaken flasks.)

Bacteriophages T2r and T5. These were prepared by lysis of *E. coli* B/r in medium S. The proportions of thymine and 5-halogenated pyrimidine present during phage and bacterial growth will be described in the next section. Bacteria were infected with an average of two bacteriophages/cell at a bacterial density of 1.5 × 10⁸ cells/ml.

Bacteriophage T2r⁺. Bacteriophage T2r⁺ produced only partial lysis of *E. coli* B/r in medium S, but complete lysis was obtained in a similar medium containing only 0.25 mg. of sulphanilamide/ml. and 5 mg. of thymine/ml. (medium S1). Similarly Rutten *et al.* (1950) noted that the bacteriophages T2, T4 and T6 would not multiply on *E. coli* B in the presence of more than 0.25 mg. of sulphanilamide/ml. They do not indicate whether they used the r or r⁺ strain. These observations are true only of bacteria grown in medium S for a sufficient length of time to become thymine-dependent.

Table 1. *Growth of E. coli B/r in the presence of variable amounts of thymine and 5-bromouracil*

Tubes containing 10 ml. quantities of medium were inoculated with one drop each of a dilution of *E. coli* B/r grown in medium A (about 2 × 10⁸ bacteria/tube were added) and incubated at 37°. The amount of growth, determined from the optical density, is expressed as the equivalent bacterial density (cells/ml. × 10⁻⁸). Inhibited cultures contained 10⁻⁸M-5-bromouracil.

		Control cultures			Cultures with 5-bromouracil		
Thymine concn.		Incubation time (hr.)					
Medium*	(mm)	24	48	72	24	48	72
A	0	15.9	23.5	—	—	—	—
SS	0	0.0	0.0	0.0	—	—	—
SS	0.08	—	2.6	14.7	—	—	0.8
S	0	0.0	2.0	15.9	—	—	—
S	0.04	6.1	22.0	—	—	7.4	13.6
S	0.08	7.9	18.3	—	1.1	13.6	—
S	0.24	13.1	21.2	—	3.0	14.2	—
S	0.40	13.6	21.2	—	4.3	15.3	—
S	0.79	13.1	17.7	—	7.0	15.9	—

* Medium SS = medium A supplemented with (per l.): sulphanilamide (1 g.), xanthine (25 mg.), DL-methionine (35 mg.), DL-serine (15 mg.), DL-valine (20 mg.).

For media A and S see above.

T2 was purified by the method of Herriott & Barlow (1952). T5 was isolated by alternate cycles of low-speed centrifuging (15 min. at 3300 rev./min.) to sediment bacterial debris, and high-speed centrifuging (45 min. at 12 000 rev./min. in the Sorvall SS1 centrifuge) to sediment the virus. After sedimentation, the virus was resuspended in one-quarter strength Ringer's solution. Both preparations were incubated with ribonuclease and deoxyribonuclease to remove non-viral nucleic acids before the final cycle of centrifuging.

Addition of analogues

The 5-halogenated uracils were dispensed as 0.2M-solutions in 0.4N-KOH and added to the media at bacterial densities from $0.5-5.0 \times 10^8$ bacteria/ml. to give the required concentrations. As the media contained 0.1M-phosphate the small quantities of alkali added made a negligible change in the pH.

Bacterial growth. This was measured by determining the optical density of cell suspensions with a Gallenkamp Direct-Reading photoelectric colorimeter (no. 3615) and an Ilford violet-light filter (no. 621). These density measurements were converted into equivalent bacterial counts by means of a calibration curve constructed from values obtained with dilutions of a culture of *E. coli* B/r grown on medium A, and microscopic counts on the same culture. A similar calibration curve was constructed to convert the density values into bacterial dry weights.

Colony counts. Colonies were counted by the spreading technique on nutrient-agar plates and bacteriophage plaques by the double-layer technique of Gratia (1936). Viable-cell counts were also made by the urea-agar method of Valentine & Bradfield (1954).

Isolation of deoxyribonucleic acids

E. coli strains. Some preparations were isolated by a modification of a method described by Gandelman, Zamenhof & Chargaff (1952). The cells were washed three times in 0.1M-sodium citrate buffer, pH 7.3, and frozen. The thawed cells were ground with glass (Pyrex) powder at 4° in the citrate buffer. The extract was centrifuged for 30 min. at 12 000 rev./min. in the Sorvall SS1 and the pellet extracted in 2M-NaCl at 4° for 18 hr. After centrifuging, a second extraction with 2M-NaCl sufficed to remove most of the DNA from the cell debris. The combined extracts were precipitated with 2 vol. of neutral ethanol, the fibrous precipitate was dissolved in 2M-NaCl and protein removed by chloroform-gel formation (Sevag, Lackman & Smolens, 1938). Two volumes of ethanol were added to the deproteinized solution and the precipitated nucleic acids dissolved in 0.14M-NaCl and incubated with ribonuclease at 37° for 1 hr. The solution was dialysed against M-NaCl to remove the products of ribonuclease digestion. The DNA within the cellophane bag was then precipitated with 2 vol. of neutral ethanol and the fibres were washed in 75% (v/v) ethanol-water.

Preparations of DNA were also made by the method of Smith & Wyatt (1951). As the removal of protein by chloroform-gel formation has been found to produce some fractionation of DNA from *E. coli* 15T⁻ (Dunn, unpublished work), in some of the preparations of DNA from this organism protein was removed by purified trypsin. Commercial trypsin (Hopkin and Williams Ltd.) was dissolved in 0.25N-HCl and then fractionally precipitated twice with

(NH₄)₂SO₄, the fraction precipitated by 70% saturation being retained but not that precipitated by 40% saturation (Kunitz & Northrop, 1936). The resulting material was free from significant amounts of ultraviolet-absorbing material and from deoxyribonuclease. The enzyme was dissolved in water (50 mg./ml.) and stored at -30°. The DNA extracts were incubated for 18 hr. at 30° with the purified trypsin in 0.1M-sodium citrate buffer, pH 7.5, chloroform being added to prevent bacterial contamination. After freeing the citrate solution of insoluble material by centrifuging, the DNA was precipitated by adding an equal vol. of 0.1M-MgSO₄, sufficient acetic acid to bring the mixture to below pH 6.0 and then twice the resulting volume of ethanol. It was washed twice with 60% (v/v) ethanol-water, dehydrated in absolute ethanol and acetone, and dried in air.

Bacteriophage T5. DNA was liberated from the virus in the following way: 3 ml. of a suspension of T5 in 0.14M-NaCl (containing approx. 10^{13} phage particles/ml.) was mixed with 1 ml. of 0.5M-sodium citrate, pH 6, and incubated at 50° for 40 min. The removal of bivalent ions, in particular Ca²⁺ by complex formation with the citrate, liberates the DNA (Lark & Adams, 1953). The DNA was precipitated by 2 vol. of neutral ethanol and washed in 50% (v/v) ethanol-water. No attempt was made to remove the virus protein.

Hydrolysis of nucleic acids

Hydrolysis to purines and pyrimidines. DNA was hydrolysed for 1 hr. in 72% (w/w) HClO₄ at 100° (Marshak & Vogel, 1950).

Hydrolysis to 5'-nucleotides. Deoxyribonuclease (10 µg./ml.) was added to an aqueous solution of DNA (20 mg./ml.) in 0.002M-MgSO₄ and adjusted to pH 7.6. The mixture was incubated at 37° for 6 hr., the pH being maintained constant by periodic additions of aq. NH₃ soln. Glycine buffer, pH 9, was then added to the deoxyribonuclease digest to give 0.02M-glycine. Snake-venom phosphodiesterase (4 mg. of enzyme preparation free from 5'-nucleotidase activity/ml.) was added and incubation continued for 5 hr. During this time the mixture was maintained at pH 9 by occasional additions of aq. NH₃ soln.

Hydrolysis to nucleosides. The procedure was as above, except that crude Russell-viper venom, containing 5'-nucleotidase, was used instead of the purified phosphodiesterase.

Separation of nucleic acid components

The halogenated uracils were separated from the natural purines and pyrimidines in nucleic acid hydrolysates by paper chromatography. Table 2 lists the *R_F* values of these substances in the following solvent systems:

- (1) Propan-2-ol (680 ml.), 11.6N-HCl (176 ml.), water to 1 l. (Wyatt, 1951).
- (2) Propan-2-ol (700 ml.), water (300 ml.) with NH₃ in the vapour phase (Markham & Smith, 1952).
- (3) Butan-1-ol (770 ml.), water (130 ml.), 98% formic acid (100 ml.) (Markham & Smith, 1949).
- (4) Butan-1-ol (860 ml.), water (140 ml.) with NH₃ in the vapour phase (Markham & Smith, 1949).

For the routine analysis of DNA containing these substances we chose a two-dimensional chromatographic method. A perchloric acid hydrolysate of the DNA was placed about 8 cm. from the edge of a sheet of Whatman

Table 2. R_f values of thymine, uracil, 5-halogenated uracils and their deoxyribosides in four solvent systems

Compound	R_f value in			
	Solvent 1	Solvent 2	Solvent 3	Solvent 4
Uracil	0.68	0.62	0.28	0.20
Thymine	0.78	0.70	0.42	0.42
5-Chlorouracil	0.71	0.63	0.43	0.18
5-Bromouracil	0.75	0.65	0.44	0.21
5-Iodouracil	0.76	0.66	0.47	0.26
5-Chlorouracil deoxyriboside	—	—	0.42	0.10
5-Bromouracil deoxyriboside	—	—	0.40	0.15
Thymidine	—	—	0.38	0.38

Table 3. Maxima and minima in the absorption spectra of the 5-halogenated uracils, their nucleosides and nucleotides

Values are given in $m\mu$.

Compound	pH 1		pH 13	
	Max.	Min.	Max.	Min.
5-Chlorouracil	273	237	289	252
5-Chlorouracil deoxyriboside	275	240	—	—
5-Chlorouracil deoxyribonucleotide	276	238	274	247
5-Bromouracil	276	240	290	255
5-Bromouracil deoxyriboside	278	244	—	—
5-Bromouracil deoxyribonucleotide	279	244	—	—
5-Iodouracil	281	246	—	—
5-Iodouracil deoxyribonucleotide	285	246	—	—

no. 1 filter paper approx. 30 cm. $\times$ 48 cm. This was then run in solvent 1, which separates adenine, guanine, cytosine (uracil if present) and thymine. The 5-halogenated uracils run just behind thymine (Table 2). A strip was cut across the paper to include only the spots of thymine and halogenated pyrimidines. This was run in the solvent 4, which separates thymine and the halogenated uracils. The compounds were located by the method of Markham & Smith (1949), eluted in 0.1 N-HCl and their ultraviolet absorption spots measured against appropriate blanks in a Beckman Model DU or a Unicam Model SP. 500 spectrophotometer. Table 3 gives the spectral maxima and minima of the 5-halogenated uracils. For estimation the following molar extinction coefficients were used: 5-bromouracil, 7.01×10^3 at 276 $m\mu$; 5-chlorouracil, 8.08×10^3 at 273 $m\mu$; 6-methylaminopurine, 15.1×10^3 at 267 $m\mu$; all in 0.1 N-HCl. Other extinction coefficients used were those of Wyatt (1951).

The discovery that the DNA from *E. coli* contains the additional base 6-methylaminopurine (Dunn & Smith, 1955) led us to estimate this base also in some of the samples of DNA from *E. coli* 15T⁻. This base was estimated together with cytosine in spots separated by solvent 1, but the ratio of the two bases was determined by eluting them and rechromatographing in solvent 2. The amount of 5-halogenated uracil present was also determined by this method, the analogue and thymine being estimated jointly after separation of the hydrolysate with solvent 1. In this instance solvent 4 was used for the second chromatogram.

One property of the 5-halogenated uracils proved singularly useful in separating these substances and their derivatives. At pH 9 halogenated uracils and their derivatives bear a negative charge, probably due to the ionization of the 6-hydroxyl group on the pyrimidine. The

Table 4. Electrophoretic mobilities of the 5-halogenated uracils, their nucleosides and nucleotides

Mobilities, expressed as cm./hr. at 20 v/cm., were determined in 0.05 M-borate, pH 9. Mobilities of corresponding components of normal nucleic acid are included for comparison.

	Mobilities (cm./hr. at 20 v/cm.)		
	Free base	Deoxy-riboside	Deoxyribo-nucleotide
5-Chlorouracil	8.7	—	11.0
5-Bromouracil	8.0	5.3	10.4
5-Iodouracil	6.8	—	9.9
Normal DNA bases	0.2-0	0.2-0	6.8-9.4

acidic groups of guanine, adenine, uracil and thymine are only partially ionized at pH 9, whereas cytosine has no charge. Consequently on paper electrophoresis in 0.05 M-borate buffer, pH 9 (Markham & Smith, 1952), the 5-halogenated uracils migrate rapidly towards the anode, whereas the movement of the natural purines and pyrimidines is slight (Table 4).

Inorganic phosphate. This was estimated by the method of Allen (1940).

Materials

5-Bromouracil. This was prepared by the method of Wheeler & Johnson (1907).

5-Iodouracil. This was prepared according to Johnson & Johns (1906). Neither product contained any uracil and each gave a single ultraviolet-absorbing spot in solvents 1, 2 and 3.

5-Chlorouracil. This and a further sample of 5-bromouracil were gifts from Imperial Chemical Industries Ltd.

Snake venom phosphodiesterase. This was a gift from Dr L. Astrachan and had been prepared from *Crotalus adamanteus* venom by the method of Butler (1955).

RESULTS

Effect of halogenated uracils on bacterial growth

Escherichia coli B/r in medium A. A concentration of 0.01M-5-bromouracil was found to be inhibitory to the growth of *E. coli* B/r in medium A, but this inhibition was not annulled by the presence of 0.01M-thymine or a similar concentration of uracil in the medium. 5-Bromouracil (0.001M) produced no detectable inhibition of *E. coli* B/r in this medium. 5-Chlorouracil, however, does inhibit the growth of *E. coli* B/r at a concentration of 0.001M, the inhibition commencing immediately on the addition of the inhibitor. This inhibition was completely annulled by adding uracil (9×10^{-4} M) before the addition of the analogue. A similar concentration of thymine did not affect this inhibition.

Escherichia coli B/r in medium S. In the sulphanimide medium (S) containing 10–100 mg. of thymine/l. the mean generation time of *E. coli* B/r is about 2 hr. as compared with 50 min. in medium A. In medium S without thymine the mean generation time of the bacteria was never less than 4 hr.,

indicating that in this medium the cells were partly dependent on the external supply of thymine for growth. Fig. 1 shows the effects of 10^{-3} M-5-bromouracil on the growth of bacteria in medium S at two levels of thymine concentration. With 8×10^{-5} M thymine the bacterial growth rate [the slope of the curve relating log (bacterial density) with time] begins to decrease about 3 hr. after the addition of the 5-bromouracil, and thereafter remains approximately constant. Increasing the thymine concentration to 8×10^{-4} M partially reverses the growth inhibition. Uracil (8×10^{-4} M) did not affect the growth inhibition by 5-bromouracil under these conditions.

Growth inhibition by 5-iodouracil was essentially similar, although at equivalent concentration 5-bromouracil was the more effective in inhibiting growth.

A proportion of the bacteria (5% at 4 hr. and 15% at 12 hr. after the addition of the inhibitor) grown in the presence of 10^{-3} M-5-bromouracil and 8×10^{-5} M-thymine were distinctly longer (three to eight times) than those grown in the absence of 5-bromouracil (Fig. 2). Colony counts made 6–7 hr. after the addition of the inhibitor were considerably lower (about 25%) than the total counts

Fig. 1. Inhibition of growth of *E. coli* B/r by 5-bromouracil. Bacteria were grown in medium S containing: 8×10^{-5} M-thymine (A and C); 8×10^{-4} M-thymine (B). At the time indicated by the arrow, 5-bromouracil was added to cultures B and C to give final concentrations of 10^{-3} M. Bacterial dry weights were obtained from optical-density measurements as described in the text.

Fig. 2. Normal and elongated cells in a culture of *E. coli* B/r inhibited by 5-bromouracil in medium S. Electron micrograph, $\times 1800$.

calculated from the measurements of optical density. This does not necessarily imply that 75 % of the cells were non-viable, as no allowance has been made in the optical-density measurements for the elongated cells mentioned above. By the urea method approx. 50 % of the cells were found to be viable. (The elongated cells already present are included as viable cells with this method.)

Escherichia coli 15T⁻. The inhibiting effect of 5-bromouracil on the growth of the thymine-requiring strain 15T⁻ has already been reported by Cohen & Barner (1954, 1956). Fig. 3 shows the relation between turbidity and time of *E. coli* 15T⁻ growing in medium A supplemented with 10^{-3} M concentrations of the 5-halogenated uracils and 8×10^{-5} M-thymine. Approx. 1-2 hr. after the addition of the inhibitor the bacteria are about twice the length of normal cells and continue to increase in size, forming long filaments (Fig. 4). The later points on the turbidity curves obtained under these conditions are thus proportional, not to bacterial numbers, but rather to the weight of organisms per unit volume. As with *E. coli* B/r in S medium, 5-bromouracil and 5-iodouracil do not appear to inhibit the growth until about 1.5 mean generation times after their addition. 5-Chlorouracil, however, inhibited the growth immediately upon its addition but did result in a further marked decrease in the growth rate corresponding with the onset of inhibition in the other two cultures.

The inhibitions due to 5-bromouracil and 5-iodouracil were largely annulled when the thymine concentration was increased to 8×10^{-4} M before they were added. This concentration of thymine had no observable effect on the growth inhibition produced by 10^{-3} M-5-chlorouracil.

As shown in Fig. 5, the addition of 9×10^{-4} M-uracil before the addition of 5-chlorouracil annulled the immediate inhibition, but did not prevent the growth rate rapidly decreasing after about 1 hr. In other experiments 2×10^{-4} M-uracil was sufficient to produce this effect. If 8×10^{-4} M-thymine was present in addition to the uracil, then all phases of the inhibition due to 5-chlorouracil were largely annulled.

In the presence of 4×10^{-5} M-thymine, 2×10^{-4} M-uracil added before the addition of 10^{-3} M-5-bromouracil or 10^{-3} M-5-iodouracil had no observable effect on the inhibition produced.

Incorporation of 5-halogenated uracils into deoxyribonucleic acid

The various conditions used for the growth of *E. coli* B/r and *E. coli* 15T⁻ in the presence of the analogues are given in Table 5.

Some of the conditions used in the preparation of bacteriophages T2r, T2r⁺ and T5 containing the halogenated uracils are given in Table 6. The phages

Fig. 3. Inhibition of growth of *E. coli* 15T⁻ by 5-halogenated uracils. Bacteria were grown in medium A containing 8×10^{-5} M-thymine. At the time indicated by the arrow 5-halogenated uracils were added to give final concentrations of 10^{-3} M. Bacterial dry weights were obtained from optical-density measurements.

Fig. 4. Elongated cells in a culture of *E. coli* 15T⁻ grown in medium A in the presence of 5-bromouracil for 6 hr. Photograph of stained preparation in light microscope, $\times 780$.

were added at a bacterial concentration of 1.5×10^9 cells/ml., as this gave maximum phage yields in medium S under our conditions. 5-Bromouracil or 5-iodouracil ($0.002M$) added 0–9 hr. before infecting each of the bacteria with an average of 1.5–3 phage particles did not prevent the lysis of *E. coli* B/r in this medium. The lysis normally occurred in about 90 min. in medium S and incubation was continued for a further 6 hr. to ensure that lysis was complete. The lysates were stored for 18 hr. at 5° before the phages were purified.

Isolation of halogenated uracils from deoxyribonucleic acid

Stability of 5-halogenated uracils to hydrolysis by perchloric acid. 5-Chlorouracil and 5-bromouracil were each found to be unchanged after heating with $HClO_4$ for 1 hr. at 100° , both in the absence and in the presence of DNA. 5-Iodouracil was unchanged by heating with 72% (w/w) $HClO_4$ alone. However, in the presence of DNA it was quantitatively converted into a compound identified chromatographically and spectroscopically as uracil. Another method used to hydrolyse DNA to the bases [90% formic acid at 175° for 0.5 hr. (Wyatt, 1951)] also converted 5-iodouracil into uracil even in the

absence of DNA. When estimating the amounts of 5-iodouracil in various preparations of DNA we used the amount of uracil detected in the $HClO_4$ hydrolysate, as the conversion of 5-iodouracil into uracil under these conditions appeared to be quantitative.

By two-dimensional chromatography, as described above, ultraviolet-absorbing material with the chromatographic behaviour of 5-bromouracil was isolated from hydrolysates of DNA prepared from *E. coli* B/r, *E. coli* 15T⁻ and bacteriophages T 2r, T 2r⁺ and T 5, all of which had been grown in the presence of the inhibitor. The ultraviolet-absorption spectrum of the additional base (Fig. 6) and its electrophoretic mobility at pH 9 were used to identify it as 5-bromouracil. In the same way 5-chlorouracil was shown to be incorporated into the DNA of *E. coli* 15T⁻.

Perchloric acid hydrolysates of DNA from *E. coli* B/r and *E. coli* 15T⁻ which had been grown in the presence of 5-iodouracil were found to contain a compound identified as uracil. This uracil was taken as an indication of incorporation of 5-iodouracil, as RNA had been removed from these bacteria by treatment with alkali and hydrolysates of DNA isolated from control bacteria in the same manner showed no detectable amount of uracil. Uracil was also detected in perchloric acid hydrolysates of

Fig. 5. Effect of thymine and uracil on the inhibition of growth of *E. coli* 15T⁻ by 5-chlorouracil. Bacteria were grown in medium A containing: $4 \times 10^{-5}M$ -thymine (A and E); $8 \times 10^{-4}M$ -thymine (B); $4 \times 10^{-5}M$ -thymine, $9 \times 10^{-4}M$ -uracil (C); $8 \times 10^{-4}M$ -thymine, $9 \times 10^{-4}M$ -uracil (D). At the time indicated by the arrow, 5-chlorouracil was added to cultures A–D to give final concentrations of $10^{-3}M$. Bacterial dry weights were determined from optical-density measurements.

Fig. 6. Ultraviolet-absorption spectra of 5-bromouracil, its nucleoside and nucleotide. All spectra are for compounds in $0.1N-HCl$. —, Synthetic 5-bromouracil, (λ_{max} . 276 $m\mu$, λ_{min} . 240 $m\mu$); ●, 5-bromouracil isolated from an $HClO_4$ hydrolysate of DNA from *E. coli* B/r; ---, deoxyriboside of 5-bromouracil isolated from the DNA of *E. coli* B/r (λ_{max} . 278 $m\mu$, λ_{min} . 244 $m\mu$); ····, deoxyribonucleotide of 5-bromouracil isolated from the DNA of *E. coli* B/r (λ_{max} . 279 $m\mu$, λ_{min} . 244 $m\mu$).

Table 5. Proportions of 5-halogenated uracils incorporated into the deoxyribonucleic acids of *E. coli* B/r and *E. coli* 15T⁻ under various conditions

Strain of <i>E. coli</i>	Medium	10 ⁻⁶ M-Concn. in medium of			Bacterial density (mg./ml.)		Length of incubation with halogenated uracil (hr.)	Proportion of halogenated uracil in DNA. Moles/100 moles (thymine + halogenated uracil)	Reference no. of DNA preparation†
		Halogenated uracil	Thymine	Uracil	On adding halogenated uracil	On harvesting			
B/r	A	100	0	0	—	—	—	<2	1
B/r	S	5-Bromouracil	0	0	0.17	1.7	21	N.E.†	2
B/r	S*	5-Bromouracil	1.6	0	0.02	3.1	72	35	3
B/r	S	5-Bromouracil	0.8	0	0.21	1.0	8.7	N.E.	4
B/r	S	5-Iodouracil	0.8	0	0.22	1.2	8.7	17	5
15T ⁻	A	5-Bromouracil	0.4	0	0.54	2.4	—	41.5	6
15T ⁻	A	5-Bromouracil	0.2	0	0.50	2.1	4.0	31	7
15T ⁻	A	5-Iodouracil	0.2	0	0.27	—	—	57	8
15T ⁻	A	5-Iodouracil	0.2	0	0.46	1.8	4.0	29	9
15T ⁻	A	5-Chlorouracil	0.2	0	0.27	—	—	70	10
15T ⁻	A	5-Chlorouracil	0.2	0	0.50	1.7	4.0	64	11
15T ⁻	A	5-Chlorouracil	0.2	9.0	0.47	2.0	4.0	40	12
15T ⁻	A	5-Chlorouracil	8.0	0	0.58	2.2	4.0	30	13

* Grown on Roux flasks (Bacterial concentration is per ml. of solid medium).

† N.E. The analogue was detected in the DNA but its proportion was not estimated.

‡ Reference nos. here and in Table 6 related these preparations to their complete analyses in Table 7.

Table 6. Conditions used in the preparation of bacteriophage containing various amounts of incorporated 5-halogenated uracils and the proportions of infective virus particles present

The infectivity of the preparations is based on comparison of the ratio plaque titre:optical density at 260 m μ with that of control virus. As this ratio for control T 2 was often considerably below the values obtained by Herriott & Barlow (1952), the proportion of viable particles are also expressed relative to their value. The thymine concentrations in media S and S1 were 8×10^{-6} M and 4×10^{-6} M respectively.

Bacteriophage	Strain of <i>E. coli</i> used as host	Medium	Analogue	Concn. in medium of analogue (mm)	Proportion of		Percentage of viable		Reference no. of DNA preparation
					Time interval between adding inhibitor and adding bacteriophage (hr.)	halogenated uracil in DNA. Moles/100 moles (thymine + halogenated uracil)	Control (present work)	Results of Herriott & Barlow (1952)	
T2r	B/r	S	5-Iodouracil	2	8.5	48	<2	—	14
T2r	B/r	S	5-Bromouracil	2	6.5	79	33	6.7	15
T2r	B/r	S	5-Bromouracil	2	8.5	60	22	4.7	16
T2r+	B/r	S1	5-Bromouracil	2	1.0	43	—	—	17
T2r+	B/r	S1	5-Bromouracil	2	0.0	50	8	3.4	18
T2r+	B/r	S1	5-Bromouracil	2	3.3	63	—	—	19
T2r+	B/r	S1	5-Bromouracil	2	5.3	39	12	4.1	20
T2r+	B/r	S1	5-Bromouracil	6	10.5	62	13	4.5	21
T2r+	B/r	A	5-Bromouracil	10	1.25	34.9	60	55.5	22
T2r+	B	A	5-Bromouracil	10	1.25	31.7	60	55.5	23
T2r+	B	A	5-Bromouracil	10	1.25	14.5	99	90	24
T5	B/r	S	5-Bromouracil	2	6.0	Approx. 20	<2	—	—

bacteriophage T2r grown on bacteria inhibited by 5-iodouracil.

As an 0.01 M-concentration of 5-bromouracil had been found to be inhibitory to *E. coli* B/r in medium A, bacteria were grown under these conditions and the DNA was examined for the presence of incorporated 5-bromouracil. None was detected, although the amount of DNA used was sufficient for us to detect the analogue if it had represented 2% of the thymine [cf. Zamenhof, Reiner, de Giovanni & Rich (1956b), who find that in similar conditions 5-bromouracil replaces 4.4% of the thymine residues in the DNA of *E. coli* B].

Replacement of thymine in the nucleic acid. The quantitative analysis of the purines and pyrimidines showed that when 5-bromouracil, 5-chlorouracil or 5-iodouracil is incorporated into DNA the proportion of thymine in the nucleic acid is decreased such that the molar proportion (halogenated pyrimidine + thymine)/(total bases) equals the molar proportion (thymine)/(total bases) in the corre-

sponding normal DNA (Table 7). Some deviations from this rule were observed with DNA from *E. coli* 15T⁻, but in this instance it has since been found that the proportion of 6-methylaminopurine also increased (Table 7) and this was estimated as cytosine in the earlier analyses.

The 5-halogenated uracils are thus utilized by the bacteria in the place of thymine and quantitatively replace thymine residues in the DNA. It is thus convenient to express the amount of incorporation as moles of thymine replaced by the analogues. The amounts of the analogue incorporated under various conditions, expressed in this way, are given in Tables 5 and 6.

Isolation of the deoxyribonucleotides

The isolation of purine or pyrimidine analogues from DNA as the free base is by no means a good proof of their incorporation, as the analogue may not have been completely removed during the preparation of the nucleic acid (Lasnitzki *et al.* 1954).

Table 7. *Molar proportions of bases in some deoxyribonucleic acid preparations containing 5-halogenated uracils*

All preparations were hydrolysed in HClO₄. 5-Hydroxymethylcytosine was not estimated in the T2 bacteriophages, but the proportion of this base present was calculated from the adenine:5-hydroxymethylcytosine ratio given by Wyatt & Cohen (1953) (these values are given in black). Except where 6-methylaminopurine has been separately determined, the values for cytosine include 6-methylaminopurine estimated as cytosine. 5-Iodouracil was converted into uracil and estimated as this (see text).

Deoxyribonucleic acid	Moles/100 moles of total bases							Reference no. of DNA preparation†
	Adenine	Guanine	Cytosine	Thymine	5-Halogenated uracil	Thymine + analogue	6-Methylamino-purine	
<i>E. coli</i> B/r								
Normal	23.1	26.4	25.5	25.0	0	25.0	—	—
'Bromouracil'	22.7	25.7	26.7	15.9	8.8	24.7	—	3
'Iodouracil'	24.0	26.4	25.8	19.6	4.1	23.7	—	5
<i>E. coli</i> 15T ⁻								
Normal	23.6	27.5	26.6	22.4	0	22.4	—	—
'Bromouracil'	23.4	27.5	27.3	12.7	9.1	21.8	—	6
'Chlorouracil'	21.9	30.2	28.6	5.8	12.1	17.9	—	10
'Iodouracil'	23.6	30.2	30.9	6.6	8.7	15.3	—	8
Normal	23.6	27.5	26.1	22.4	0	22.4	0.42	—
'Bromouracil'	23.6	25.8	25.5	16.7	7.4	24.1	0.7	7
'Iodouracil'	22.8	26.6	25.2	18.0	6.5	24.5	0.7	9
'Chlorouracil'	23.4	27.3	26.0	7.9	14.2	22.1	1.4	11
'Chlorouracil'	21.8	26.5	26.8	14.0	9.4	23.4	1.5	12
'Chlorouracil'	24.1	26.5	27.3	14.7	6.5	21.2	0.6	13
Bacteriophage T2r								
Normal	32.6	19.4	17.3	30.7	0	30.7	—	—
'Bromouracil'	32.4	19.6	17.1	6.5	24.4	30.9	—	15
'Iodouracil'	30.9	22.9	15.9	15.6	14.7	30.3	—	14
Bacteriophage T2r†								
Normal*	32.5	18.2	16.7	32.6	0	32.6	—	—
'Bromouracil'	31.4	19.3	16.4	21.6	11.5	33.1	—	22
'Bromouracil'	31.8	18.6	16.3	22.7	10.5	33.2	—	23
'Bromouracil'	32.5	18.8	16.7	15.9	16.1	32.0	—	18
'Bromouracil'	32.0	19.8	16.4	12.2	19.6	31.8	—	21

* T2r†. Normal DNA values are those of Wyatt & Cohen (1953).

† Reference nos. of these preparations are given in Tables 5 and 6.

By isolating compounds corresponding to the deoxyribosides and deoxyribonucleotides of the 5-halogenated uracils, we showed that they probably formed an integral part of the DNA structure.

DNA from *E. coli* B/r containing 5-bromouracil was hydrolysed successively with deoxyribonuclease and snake-venom phosphodiesterase to yield the nucleoside 5'-phosphates. These were run on paper electrophoresis in 0.05M-borate buffer, pH 9. In addition to the single band containing deoxyguanylic, deoxyadenylic, deoxycytidylic and thymidylic acids, the hydrolysate contained a faster-migrating component having an ultraviolet-absorption spectrum in 0.1N-HCl with a maximum at 279 m μ and a minimum at 244 m μ (Fig. 6). The maximum is shifted 3 m μ to the longer wavelength compared with that of 5-bromouracil, and corresponds to the difference in spectrum between thymine and thymidylic acid. When hydrolysed with HClO₄, this substance gave 5-bromouracil (identified by its *R_F* values on paper chromatography in solvents 1 and 4, and spectroscopically) and inorganic phosphate in the molar ratio 0.98:1. The deoxyribonucleotide of 5-bromouracil was also isolated from the DNA of T5 phage.

A substance having the expected properties of 5-chlorodeoxyuridylic acid was isolated in a similar way from the DNA of *E. coli* 15T⁻ grown in the presence of 5-chlorouracil. The ultraviolet-absorption spectrum of the deoxyribonucleotide and that of the free base are shown in Figs. 7 and 8, and its electrophoretic mobility is given in Table 4. On hydrolysis with HClO₄ it yielded 5-chlorouracil.

Enzymic hydrolysates of DNA from *E. coli* B/r grown in the presence of 5-iodouracil also gave a rapidly migrating component (Table 4) on electrophoresis at pH 9. This had the expected ultraviolet-absorption spectrum of 5-iododeoxyuridylic acid (Table 3). On hydrolysis in HClO₄ it yielded uracil. This is consistent with the breakdown of 5-iodouracil when heated with HClO₄ in the presence of DNA.

Isolation of deoxyribosides

Treatment of 5-bromouracil-containing DNA with deoxyribonuclease and crude snake venom and separation of the products by two-dimensional chromatography with solvents 3 and 4 showed the presence, in addition to the four normal nucleosides, of a substance with the expected *R_F* values (Table 2) and ultraviolet-absorption spectrum (Fig. 6) of 5-bromouracil deoxyriboside. On hydrolysis with HClO₄ this was converted into 5-bromouracil.

Similarly, the deoxyriboside of 5-chlorouracil was isolated from the DNA of *E. coli* 15T⁻. Its *R_F* values and spectral characteristics are given respectively in Tables 2 and 3.

Fig. 7. Ultraviolet-absorption spectra of 5-chlorouracil deoxyribonucleotide isolated from the DNA of *E. coli* 15T⁻; —, in 0.1N-HCl (λ_{max} 276 m μ , λ_{min} 238 m μ); ---, in 0.1N-KOH (λ_{max} 274 m μ , λ_{min} 247 m μ).

Fig. 8. Comparison of spectrum of synthetic 5-chlorouracil with that of the compound isolated from the DNA of *E. coli* 15T⁻. —, Synthetic 5-chlorouracil; ●, material isolated from the deoxyribonucleotide after HClO₄ hydrolysis; both spectra for the compounds in 0.1N-HCl, (λ_{max} 273 m μ , λ_{min} 237 m μ); ---, synthetic 5-chlorouracil in 0.1N-KOH, (λ_{max} 289 m μ , λ_{min} 252 m μ).

*Properties of bacteriophage containing
5-bromouracil and 5-iodouracil*

Preparations of bacteriophage containing 5-bromouracil or 5-iodouracil had physical properties similar to those of normal preparations. In its purification T2 containing either 5-bromouracil or 5-iodouracil precipitated at pH 3.9, and it was isolated by the centrifuging procedure used in the preparation of normal T2. Under the electron microscope the particles in preparations of T2 containing 5-bromouracil could not be distinguished from those in control preparations. The ultraviolet-absorption spectra of normal and bromouracil-containing T2 suspensions are almost identical, showing that they contained similar proportions of nucleic acid (Fig. 9).

This observation justified the use of the ratio plaque counts:optical density at 260 $m\mu$ to give the relative proportions of infective particles in control preparations and those containing halogenated uracils. For our control preparations of T2 grown on medium S this ratio was lower than the average value of 1.21×10^{11} reported by Herriott & Barlow (1952). T2 grown in media A, S and S1 (Table 6) gave values of 1.1, 0.21 and 0.34×10^{11} respectively. Compared with control preparations the 'bromouracil' and 'iodouracil' bacteriophage preparations contained a high proportion of particles (in some more than 98 %) which would not give plaques when assayed on *E. coli* B/r (Table 6). The preparations listed in Table 6 were not grown under comparable

conditions and so are not suitable for determining the relation between the bromouracil content and the proportion of infective particles.

DISCUSSION

The results presented extend our original observations on the incorporation of 5-bromouracil and 5-iodouracil into the DNA of *E. coli* B and its bacteriophages, and show that all three 5-halogenated uracils are readily incorporated into the DNA of *E. coli* 15T⁻. Zamenhof *et al.* (1956*b*) have described the incorporation of all three halogenated uracils into the DNA of a similar thymine-requiring strain of *E. coli*, and Wacker, Trebst, Jacherts & Weygand (1954) have shown that 5-bromouracil can be incorporated into the DNA of several other species of bacteria.

For significant incorporation of the 5-halogenated uracils into the DNA of *E. coli* it appears necessary for the cells to be dependent on an external supply of thymine for their growth. The sulphanilamide medium allowed a slow growth of *E. coli* B/r in the absence of thymine, whereas *E. coli* 15T⁻ is dependent on externally supplied thymine both for its growth and survival in medium A (Cohen & Barner, 1954). These two systems differed in the type of inhibition by the 5-halogenated uracils. Growth inhibition (as measured by the optical density of suspensions) of *E. coli* B/r and *E. coli* 15T⁻ did not commence until about 1.5 generation times after the addition of 5-bromouracil or 5-iodouracil. The growth rate of *E. coli* B/r then decreased and remained approximately constant for 10 hr. or more, whereas that of *E. coli* 15T⁻ decreased progressively and almost ceased 4–5 hr. after addition of the analogue. 5-Chlorouracil differed from the other two halogenated uracils in causing a decrease in growth rate immediately upon its addition to *E. coli* 15T⁻, but after 1.5 hr. this was followed by further progressive decrease in growth rate. The time interval before the onset of this progressive inhibition with *E. coli* 15T⁻ was the same for all three analogues and appeared to be independent of the extent of inhibition. Cohen & Barner (1956) have reported a similar type of inhibition of *E. coli* 15T⁻ by 5-bromouracil. The morphological changes in *E. coli* 15T⁻ accompanying the inhibition are also found with cells grown in a thymine-deficient medium (Cohen & Barner, 1954) or in the presence of other thymine analogues (Dunn & Smith, 1955; Zamenhof, de Giovanni & Rich, 1956*a*). Complete thymine starvation causes the rapid death of *E. coli* 15T⁻ (Barner & Cohen, 1954). Cohen & Barner (1956) have shown that addition of 5-bromouracil to *E. coli* 15T⁻ under these conditions allows a doubling of the number of viable cells, and delays but does not prevent their subsequent death.

Fig. 9. Comparison of ultraviolet-absorption spectrum of 5-bromouracil containing bacteriophage T2 with that of normal bacteriophage. Smooth curve is for normal T2 and the filled-in points for the 'bromouracil' preparation no. 15, in which the 5-bromouracil had replaced 79% of the thymine. The two preparations had the same optical density at 260 $m\mu$.

During this period the DNA content of the culture increases, presumably accompanied by incorporation of 5-bromouracil. Evidently 5-bromouracil can only temporarily delay the biological effects of thymine starvation.

More detailed examination of the composition of the abnormal bacterial and bacteriophage DNA has confirmed that, in almost all cases, the halogenated uracils quantitatively replace part of the thymine normally present. However, in *E. coli* 15T⁻ the introduction of 5-bromo-, 5-chloro- or 5-iodo-uracil into the DNA is accompanied by an increase in the proportion of 6-methylaminopurine. Thus whereas the halogenated uracils may replace thymine in the DNA, they do not prevent the increase in the proportion of 6-methylaminopurine in the DNA characteristic of thymine deficiency (Dunn & Smith, 1955). In some of the preparations of *E. coli* 15T⁻ DNA, the proportion of halogenated uracil plus thymine is significantly less than the normal proportion of thymine. Further examination will be necessary before it can be concluded that an incomplete replacement of thymine residues may occur.

5-Chlorouracil differs from the other halogenated uracils in that both thymine and uracil are necessary to annul its growth inhibition of *E. coli* 15T⁻. It is therefore necessary to consider that the bacteria utilize the compound as an analogue of uracil as well as of thymine, and Dunn (1957) has shown that 5-chlorouracil is incorporated to a small extent into the RNA of *E. coli* 15T⁻. However, the requirement of uracil to annul the growth inhibition due to this analogue cannot be entirely attributed to the effect of the natural pyrimidine in preventing the incorporation of the analogue into the RNA, because uracil as well as thymine markedly reduces the proportion of 5-chlorouracil incorporated into the DNA (Table 5, preparations 11-13).

The halogenated uracils provide a series with respect to the size of the 5-substituent atom and other properties. It is of interest to compare their ability to replace thymine in DNA. Zamenhof *et al.* (1956*b*) found, after addition of equivalent (w/v) concentrations of the halogenated uracils to *E. coli* (strain I), that the extent of thymine replacement increased in the order iodouracil, chlorouracil, bromouracil, which was also the order of increasing growth inhibition. With *E. coli* 15T⁻ in medium A, supplied with 8×10^{-5} M-thymine and equivalent concentrations (10^{-3} M) of the halogenated uracils, we found the order of increasing inhibition to be bromouracil, iodouracil, chlorouracil (Fig. 3). Chlorouracil was also incorporated into the DNA of the bacteria to a greater extent than the other two analogues (Table 5, preparations 7, 9, 11). Even in the presence of excess of uracil, which it was hoped would prevent the analogue being utilized by the uracil-sensitive pathway, 5-chlorouracil was found

to be the most inhibitory of the three analogues and was still incorporated to the greatest extent (Table 5, preparation 12). Studying the growth inhibition of *Lactobacillus casei* by the 5-halogenated uracils, Hitchings *et al.* (1950) found that whereas 5-chlorouracil was the most inhibitory when the bacteria were supplemented with thymine, 5-iodouracil was the most inhibitory in the presence of folic acid. The relative effects of the three 5-halogenated uracils on the growth of bacteria and the relative extent of their incorporation into DNA thus appears to depend on the growth conditions or bacterial strain and cannot be correlated with properties, such as the size of the 5-substituent atom.

Under comparable conditions in medium S considerably more 5-bromo- and 5-iodo-uracil was incorporated into DNA of bacteriophage than into DNA of uninfected bacteria. 5-Bromouracil supplied at high concentrations was also incorporated into T2 grown on *E. coli* B/r in the basal medium A. Under these conditions no incorporation of 5-bromouracil could be detected in DNA of uninfected *E. coli* B/r. The rate of DNA synthesis during bacteriophage development is about four times that in uninfected bacteria. Possibly the rate of thymine synthesis under these conditions becomes a limiting factor in DNA synthesis, thus favouring the incorporation of externally supplied 5-bromouracil.

The preparations of bacteriophage containing 5-bromo- or 5-iodo-uracil contained a high proportion of non-infective particles with normal physical properties. The incorporation of 8-azaguanine into the RNA of tobacco-mosaic virus and turnip-yellow-mosaic virus also results in virus with decreased infectivity (Matthews, 1954, 1955). It seems probable from the results presented in Table 6 that some of the T2 particles containing 5-bromouracil are able to produce plaques, although other experiments are necessary to establish this. 5-Bromouracil had been added to the bacteria grown in medium S before infection and had presumably been incorporated into the host DNA. It is possible that the infective particles in the phage progeny contain no 5-bromouracil but this explanation seems inadequate. Litman & Pardee (1956) have described a preparation of T2 in which all the thymine had been replaced by 5-bromouracil and which yet contained 9% of infective particles. They have also made the very interesting observation that T2 stocks, grown in the presence of 5-bromouracil under conditions where the analogue is incorporated into the DNA, may contain a high proportion of mutant types.

SUMMARY

1. 5-Bromouracil, 5-iodouracil and 5-chlorouracil were each found to inhibit the growth of *Escherichia coli* 15T⁻, and the first two compounds were shown

to inhibit *E. coli* B/r rendered thymine requiring by growth in the presence of sulphanilamide. With 5-bromouracil and 5-iodouracil there was a lag of 1.5 mean generation times between the addition of the inhibitor and the onset of the growth inhibition, and this lag was also noted with 5-chlorouracil in the presence of uracil.

2. Increased concentrations of thymine were found to annul the inhibitions due to 5-bromouracil and 5-iodouracil, but both thymine and uracil were required to annul the inhibition of *E. coli* 15T⁻ by 5-chlorouracil.

3. All three analogues were shown to be incorporated into the deoxyribonucleic acid (DNA) of the inhibited bacteria. 5-Bromouracil and 5-iodouracil were also shown to be incorporated into the DNA of bacteriophages T2 and T5 grown on *E. coli* B/r in the presence of the analogues. Under comparable conditions a higher proportion of the analogues was incorporated into the bacteriophage than into the bacteria. In almost every instance the analogues appeared to be quantitatively replacing thymine residues.

4. Compounds with the expected properties of the deoxyribonucleotides and deoxyribosides of the 5-halogenated uracils were isolated from the DNA. This strongly suggests that the unusual pyrimidines did form an integral part of the DNA structure.

5. In the DNA from *E. coli* 15T⁻ an increase in the proportion of 6-methylaminopurine was found to accompany the incorporation of the analogues.

6. Whereas the physical properties and appearance of bacteriophage particles containing incorporated 5-bromouracil appeared normal a proportion was found to be non-viable.

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