ESCHERICHIA COLI CONTAINING UNNATURAL PYRIMIDINES IN ITS DEOXYRIBONUCLEIC ACID[1]

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Received for publication June 13, 1955

Following preliminary observations of Weygand *et al.* (1952) on 5-bromouracil, the incorporation of 5-bromouracil and 5-iodouracil into deoxyribonucleic acid (DNA) has been independently established by British and American investigators (Dunn and Smith, 1954; Zamenhof and Griboff, 1954*a*, 1954*b*; Zamenhof, 1954). These halogenated pyrimidines are structural analogues of thymine and are known to compete with thymine and to inhibit the growth of *Lactobacillus casei* (Hitchings *et al.*, 1945). When special strains of *Escherichia coli* were grown on 5-bromouracil, a considerable portion of thymine in the highly polymerized DNA of these cells was replaced by 5-bromouracil. Also, 5-iodouracil could be incorporated but to a lesser degree.

This study has recently been extended (Zamenhof *et al.*, 1956*a*) and 5-chlorouracil (but not 2-thiothymine or uracil) has been introduced into DNA; the extent of introduction in relation to the halogen substituent and strains used has been determined. Finally, the introduction of 5-bromouracil into DNA of non-dividing cells has been studied.

The present paper deals mainly with the following problems: (1) Can the DNA containing such halogenated pyrimidines still undergo reproduction? (2) What are the effects of such halogenated DNA components on the phenotype and the genotype of the cell?

MATERIALS AND METHODS

STRAINS. The strains used in this study were Strains I and II described previously (Zamenhof and Griboff, 1954*b*). Strain I was isolated from American Type Culture Collection No. 11117; it requires thymine for growth on a synthetic medium. Strain II is one of the "back-mutants" (of strain I) to complete thymine independence. Strain I/Br ("5-bromouracil resistant") was isolated from a papilla on a colony of Strain I grown on nutrient agar containing 100 μg 5-bromouracil per ml. Strain I/TT ("2-thiothymine resistant") was isolated from a papilla on a colony of Strain I grown on nutrient agar containing 200 μg 2-thiothymine per ml. Strain B (wild) was used as host in T-series phage experiments. Strain W (wild), was from Amer. Type Culture Collection No. 9637.

CULTURES. Strains I, II, B and W were maintained by daily transfer into nutrient broth (Difco). Strains I/Br and I/TT were maintained by daily transfer into "basal broth" (Roepke *et al.*, 1944) containing 50 μg 5-bromouracil per ml (Strain I/Br) or 800 μg 2-thiothymine per ml (Strain I/TT).

The study of growth promotion and growth inhibition was performed in basal broth.

The "enriched medium," referred to further on, was Difco nutrient broth enriched by addition of 2 per cent Proteose Peptone No. 3 (Difco) and 5 per cent (final) dextrose solution (added as $\frac{1}{10}$ vol. 50 per cent solution, autoclaved separately). 0.01 vol. of 0.2 per cent phenol red solution in 0.2 N NaOH was also added, to serve as a pH indicator. When this medium was used, the incubation was done under constant rotation (for aeration). After the turbidity became appreciable, the pH was maintained at 7.2 (indicator) by dropwise addition of NaOH solution for at least 10 hr.

When large amounts of cells were desired (Zamenhof *et al.*, 1956*a*), 1300 ml of the enriched medium were incubated as mentioned above. 10 ml of the 18-hr-old stock culture served as inoculum.

Viable cell count was performed on poured and on streaked nutrient agar plates.

[1] This investigation was supported by the research grant C-1760 from the National Institutes of Health, Public Health Service, the research grant G-8 from the American Cancer Society and an Institutional Grant from the American Cancer Society to Columbia University.

TABLE 1

Correlation between the growth characteristic and the introduction of 5-bromouracil into deoxyribonucleic acid (DNA) of Escherichia coli

	Strain			
	I	I/Br	II	B
Minimal amount of thymine to support growth (μg/ml)*	0.2	0.1	0	0
Minimal amount of 5-bromouracil to inhibit growth (μg/ml)†	5	100‡	2000	no inhibition by 2000
Thymine replaced by 5-bromouracil in DNA§ (molar per cent)	48	32	14	4.4

* In basal broth; 24 hr, 37 C.

† In basal broth containing 1 μg thymine per ml; 24 hr, 37 C.

‡ Mutations to a lower degree of resistance also occur.

§ In enriched medium containing 100 μg 5-bromouracil per ml; 24 hr, 37 C.

Total cell count was performed by means of a Petroff-Hausser bacteria counting chamber, using 1 vol. of 1:10 dilution (in water) of a saturated ethanol solution of crystal violet per 1 vol. of bacterial culture. Two counts were made: (1) counting double (non-separated) cells as one; (2) counting them as two cells.

Viability was estimated as ratio of the viable cell count to total cell count, counting non-separated cells as one.

DNA content per cell was estimated by determining total DNA in washed cells from a known volume of culture, by a modification of the Schneider procedure (Schneider, 1945; Zamenhof *et al.*, 1953); the value so obtained was then divided by the total number of cells in this volume (counting double non-separated cells as two).

Other (special) methods are described under appropriate headings.

THE REPRODUCTION OF DNA CONTAINING UNNATURAL PYRIMIDINES. *The inhibition and the introduction of 5-bromouracil.* The correlation between the growth inhibition by 5-bromouracil (in the presence of 1μg thymine per ml), and the amount of introduction of 5-bromouracil into DNA (Zamenhof *et al.*, 1956*a*) is represented in table 1. It will be seen that the strain which is more inhibited by 5-bromouracil will also introduce more of this compound (i.e., replace more thymine by 5-bromouracil in the DNA). However, this correlation does not always follow for a strain grown in different conditions. When the Strain I was grown on non-enriched medium (considerable inhibition by 100 μg 5-bromouracil per ml) the amount of introduction was only 18 to 28 per cent (Zamenhof and Griboff, 1954*a*); when it was grown on enriched medium with aeration (practically no inhibition[2] by 100 μg 5-bromouracil per ml) the amount of introduction was 48 per cent (Zamenhof *et al.*, 1956*a*); when the amount of 5-bromouracil in the enriched medium was increased to 1 mg per ml, 99 per cent cells died in 7 hr, yet no 5-bromouracil was found in the DNA.

In general, it should be concluded that the growth inhibition by an unnatural base does not imply introduction into DNA, and *vice versa*. Even wild Strain B, which is not inhibited at all, shows some incorporation of 5-bromouracil; on the other hand, when cells were grown on enriched medium containing 500 μg 2-thiothymine (per ml), which is in this concentration almost as inhibitory as 5-bromouracil (Zamenhof and Griboff, 1954*b*), no 2-thiothymine could be detected in the DNA (Zamenhof *et al.*, 1956*a*; compare also Dunn and Smith, 1955).

A certain correlation between the amount of introduction and thymine requirement of the strain has been established. Strain I requires for growth at least 0.2 μg thymine per ml, Strain I/Br requires at least 0.1 μg thymine per ml, whereas Strains II and B require none (table 1); correspondingly, the replacement of

[2] This lack of inhibition and a good introduction of 5-bromouracil may be due to larger amounts of thymidine in the enriched medium; in the presence of this nucleoside, the 5-bromouracil does not inhibit growth (Zamenhof and Griboff, 1954*b*).

TABLE 2

Comparison of deoxyribonucleic acid (DNA) content and viability of cells grown with and without 5-bromouracil. 37 C; enriched medium

Incubation	Strain							
	I			I/Br			II	
	R_{DNA}	R_V	Repl.	R_{DNA}	R_V	Repl.	R_V	Repl.
hr								
7	1.05	0.95	7					
24	1.1	0.7*	48	1.05	0.8	32	0.72	14

R_{DNA} = ratio of DNA per cell (based on total count) in cells grown with (100 μg per ml) and without 5-bromouracil.

R_V = ratio of viability of cells grown with (100 μg per ml) and without 5-bromouracil.

Repl. = molar per cent thymine replaced by 5-bromouracil.

* 0.94 when 2-thiothymine (500 μg per ml) was used instead of 5-bromouracil.

thymine by its analogue (5-bromouracil) is highest in Strain I, somewhat lower in Strain I/Br, and much lower in Strains II and B.

It has been shown recently that in special conditions 5-bromouracil appears to be introducible into DNA of non-dividing cells (Zamenhof *et al.*, 1956*a*; compare also Weygand *et al.*, 1952).

The unchanged DNA content. Although in Strain I almost half of the thymine can be replaced by 5-bromouracil (table 1), objections could still be raised against the concept that such unnatural (i. e., 5-bromouracil-containing) DNA can be reproduced. For instance, it could be argued that the cell now manufactures more DNA molecules, of which those which contain 5-bromouracil are inactive and unable to reproduce. If this were true, then to have an average of approximately half of the thymine replaced by 5-bromouracil the cell should now manufacture at least two times as much DNA, of which one half has *all* its thymine replaced by 5-bromouracil, and the other half none. This situation is highly improbable; still, to exclude such possibility, the DNA content per cell containing 5-bromouracil and per control cell was determined. The results are represented in table 2. It will be seen that at 7 hr as well as at 24 hr the cells having 5-bromouracil in their DNA practically had the same DNA content as control cells; this indicates that, if the cells containing 5-bromouracil can still reproduce, they utilize for reproduction the 5-bromouracil-containing DNA.[3]

The viability.[4] It could also be argued that the cells containing 5-bromouracil in their DNA cannot reproduce; for instance, the culture could contain two kinds of cells: cells which have some thymine replaced by 5-bromouracil and are "dead" (cannot reproduce), and the cells which have no 5-bromouracil in DNA at all and therefore are viable. If this were true for Strain I (in which practically half of the thymine is replaced by 5-bromouracil), then all the following conditions would have to be fulfilled: (1) viability of such a culture should be not more than 50 per cent of the control culture (without 5-bromouracil); as can be seen from table 2, this does not happen, since the viability is not much lower than in the control. Similar reasoning can be suggested for Strain I/Br, for which the decrease of viability is very insignificant indeed. (2) 5-bromouracil would have to be distributed unevenly so that one half of the cells contains *all* of it, and the other half none; such condition is highly improbable. (3) In such cells containing 5-bromouracil in the DNA, *all* the thymine in *all* the DNA molecules would have to be replaced by 5-bromouracil. Only then the average replacement as high as one-half of the total could be accounted for; such condition is again highly improbable. (4) If the cell with 5-bromouracil in its DNA cannot divide any more, then this 100 per cent replacement of thymine would have to be accomplished in a single division—which is again a rather improbable situation.

Thus it appears that the introduction of 5-bromouracil cannot be satisfactorily correlated with the "death" of the cells. The following

[3] It could be argued that a part of the DNA molecules even in a normal bacterial cell is genetically inert and that only those molecules introduce 5-bromouracil. Against such a hypothesis speaks the fact that in some bacterial cells there are less than 200 molecules of DNA (Zamenhof *et al.*, 1953) so that *one* DNA molecule may have to carry *several* hereditary functions (for a recent discussion, see Zamenhof, 1956); however, it is to be stated that at present no rigid proof can be offered that every DNA molecule is an active heredity determinant or that only DNA molecules are heredity determinants.

[4] The discussion refers to the haploid stage through which these cells are believed to pass, like any normal cell of *E. coli*.

alternative hypothesis is in agreement with the experimental data: 5-bromouracil is gradually introduced into the DNA of the majority of cells or of all cells, so that the average amount of introduction in the DNA of the entire population is representative of the actual amount of introduction in the DNA of each cell. Upon reaching a certain amount of introduction, characteristic of the strain used and enhanced by good growing conditions (as mentioned above in the discussion of the inhibition), the cell division on 5-bromouracil medium cannot proceed any longer; this situation may occur partly because the DNA molecules are now sufficiently different from the normal ones. Further incubation of essentially non-dividing cells in enriched medium containing 5-bromouracil results in further introduction of 5-bromouracil (Zamenhof *et al.*, 1956*a*), but also in a slight loss of viability, as compared with the culture in which 5-bromouracil is absent or replaced by 2-thiothymine (table 2); however, the great majority of the cells resume divisions when placed on a 5-bromouracil-free medium (viability test); in these conditions, 5-bromouracil is again replaced by thymine (as discussed further on).

The infectivity of bacteriophage particles containing 5-bromouracil or 5-iodouracil has been studied by Dunn and Smith (1954). According to these authors it seems likely that particles containing these halogenated pyrimidines are infective but that the probability of successful infection by a virus particle decreases with increasing incorporation of the analogue.

The above explanation is offered merely as a working hypothesis; more direct proof that a single dividing cell may contain 5-bromouracil in its DNA cannot be offered at present.

CHANGES CAUSED BY INTRODUCTION OF 5-BROMOURACIL INTO DNA. If one accepts the premise that the cells containing 5-bromouracil in their DNA can still divide, it is of interest to study the effects of such a drastic change in the DNA on the phenotype and genotype of the cell. It may be recalled that changes (deamination or depurination) affecting less than 0.2 per cent of the amino groups in DNA having transforming activity result in complete inactivation (Zamenhof *et al.*, 1953).

Phenotype changes. On nutrient agar containing 100 μg 5-bromouracil per ml, Strain I forms very flat faint colonies, while the cells introduce 5-bromouracil into DNA. However, it will form such colonies even when grown in the presence of substance which is not introduced into DNA, such as 2-thiothymine; the changed appearance of colonies must therefore be considered to be the result of inhibition rather than of introduction of 5-bromouracil into DNA. The change is not permanent: upon transfer to normal nutrient agar, normal colonies appear.

On basal agar containing 25 μg 5-bromouracil per ml, Strain I/Br forms mucoid colonies; the individual cells can be shown to have large capsules (india ink "negative staining"). The colonies on nutrient agar are not mucoid; however, they are mucoid on MacConkey agar free of 5-bromouracil, which again indicates that the change (mucoid growth) is not an effect of introduction of 5-bromouracil into DNA.

On nutrient agar containing 100 μg 5-bromouracil per ml, Strain II forms *normal* colonies while the cells introduce 5-bromouracil into DNA.

In summary, then, there is no evidence of any phenotypical changes caused by the phenomenon of introduction of 5-bromouracil into DNA (if one does not count this introduction itself as a phenotypical change).

It must be pointed out that when a small inoculum of cells containing 5-bromouracil in DNA is put on a medium containing no 5-bromouracil, the thymine in the medium again replaces 5-bromouracil when the cells reproduce and the supply of 5-bromouracil in the cells has been exhausted. This is obvious for the synthetic medium which does not contain bromine; but even if the nutrient medium should contain traces of bromine, the cell is unlikely to carry an enzyme to brominate uracil in the 5 position.

Genotype changes. If the DNA is a heredity determinant (for a recent discussion, see Zamenhof, 1956) then the changes in its structure, if permanent and reproducible, may be called a mutation. As shown above, the introduction of 5-bromouracil is not a mutation because it is not permanent: the structural change disappears upon removal of the environmental cause of the change (5-bromouracil).[5]

[5] It could, however, be argued that, irrespective of nomenclature, the facts indicate that the structural changes in this heredity determinant (appearance or disappearance of 5-bromouracil in DNA) are merely a function of the environment.

However, as mentioned above, a typical stable[6] mutant Strain I/Br (5-bromouracil "resistant") was isolated in the conditions in which in Strain I 5-bromouracil was being introduced into DNA. This mutant has now been investigated to establish whether its appearance is caused by such introduction of 5-bromouracil into DNA.

It will first be seen (table 1) that the introduction of 5-bromouracil into DNA of this strain is not higher but lower than in the case with non-resistant Strain I. Thus, higher resistance to 5-bromouracil of Strain I/Br does not seem to be correlated with the fact that this compound now forms part of the DNA molecule and therefore is less "unnatural."

It was of interest to see whether resistance to halogenated pyrimidines can be demonstrated also in cases where the pyrimidine is not introduced into DNA. 2-Thiothymine seems to be non-introducible into DNA of *E. coli* (Dunn and Smith, 1955; Zamenhof *et al.*, 1956*a*). Yet, as mentioned above (description of strains), Strain I/TT resistant to 2-thiothymine has been isolated. Strain I/TT is resistant also to 5-bromouracil (25 to 50 μg per ml); Strain I/Br is resistant also to 2-thiothymine (200 μg per ml) (however, the statement that the two strains are identical cannot be made at the present moment).

Thus it appears that the resistance to halogenated pyrimidine is not correlated with its introduction into DNA. The nature of resistance is not well understood: it may have relation to the production of large capsule (mucoid colonies) by these strains. It must, however, be pointed out that the permeability (for thymine) does not seem to be reduced, since Strain I/Br can grow on even less thymine than Strain I (table 1).

It seemed of interest to study whether the mutation to 5-bromouracil resistance is a spontaneous one, what the mutation rate is, and whether it can be increased by the presence of 5-bromouracil. These studies were facilitated by the fact that, when Strains I and I/Br are grown together, the proportion of I/Br cells neither decreases very considerably in the absence of 5-bromouracil nor does it increase in the presence of 100 μg 5-bromouracil per ml (table 3).

[6] "Back-mutants" to 5-bromouracil-sensitivity can be isolated from Strain I/Br grown on enriched medium without 5-bromouracil. They can form normal size or small colonies, smooth or rough.

A fluctuation test (Luria and Delbrück, 1943) was made on a series of 9 individual cultures (1 ml each, approx. 2.7×10^9 cells) and a series of 9 samples from one culture (10 ml). Each culture (enriched medium, aeration by shaking) was centrifuged, washed, resuspended in 0.1 ml saline and streaked on basal agar containing 25 μg 5-bromouracil per ml. A detailed study similar to that reported by Ryan (1952) has shown that the colonies appearing within 24 hr represent mutants which were present in original cultures; any mutants resulting from the background (faint) growth on the agar itself appeared only after 48 hr and were disregarded.

The results are represented in table 4. High χ-square and low P for samples from independent cultures (as compared with samples from single culture) clearly indicate that the mutation to resistance to 5-bromouracil is a spontaneous one.

The spontaneous mutation rate (table 4) was calculated according to Luria and Delbrück's formula (Luria and Delbrück, 1943; New-

TABLE 3

Growth of mixed populations of I and I/Br cells. 37 C; enriched medium with and without 5-bromouracil

Incubation	Per Cent I/Br Cells	
	No 5-bromouracil	100 μg 5-bromouracil per ml
hr		
0	48	48
7	31	31
24	6	25

TABLE 4

Fluctuation test and mutation rate I → I/Br. 37 C, 24 hr, enriched medium without 5-bromouracil*

	Samples from Independent Cultures	Samples from Single Culture
Total cells per sample	2.73×10^9	2.76×10^9
Average resistant cells per sample	3.0	3.37
χ-square	37.96	8.26
P	0.00001	0.3
Mutation rate per viable cell	3×10^{-10}	

* See text for details and references.

combe, 1948): The mutation rate (3×10^{-10} per viable cell) is of the same order of magnitude as are the reported rates of mutation to resistance to antibiotics (for a recent review, see Braun, 1953). It is realized that all these estimates may be too low (Kraft and Braun, 1955).

The mutation rate in the presence of 100 μg 5-bromouracil per ml was similarly determined; it was found to be 4×10^{-10} per viable cell; thus 5-bromouracil, in conditions in which it is introduced into DNA, is neither a mutagen nor an antimutagen for mutation I → I/Br. On the other hand, this mutation rate can be easily increased by known mutagens (U.V. 3×10^4 ergs per mm², survival 2×10^{-3} per cent, mutation rate 0.5 to 3×10^{-4} per viable cell; Fe^{++} 0.4 M, 2 hr at 37 C, survival 3.5 per cent, mutation rate 3×10^{-6} per viable cell).

In summary, then, the mutation I → I/Br is not correlated with the introduction of 5-bromouracil into DNA. No other typical mutations during or after this introduction have been observed.

Unclassified changes. I. Change in cell morphology. When Strain I was grown for 4 to 7 hr on enriched medium containing 100 μg 5-bromouracil or 5-chlorouracil[7] per ml, up to 98 per cent of the cells became up to 30 times longer and up to 1½ times larger in diameter than the normal cells of *E. coli* (figure 1). These abnormally large cells are not related to filaments or "snakes" (rough colonies) often observed when *E. coli* is grown under adverse conditions: such filaments are simply strings of cells which did not separate after division (for a review see Hinshelwood, 1946) and whose DNA content is several times the normal. On the other hand, the elongated forms encountered in this study are *single* cells, as evidenced by a practically normal DNA content (tables 2 and 5).

The elongation might be the result of competition with thymine rather than of introduction of 5-bromouracil itself into DNA. Indeed, similarly elongated cells can be observed when Strain I is grown in basal medium containing a limited amount of thymine (0.2 μg per ml).[8] Such cells

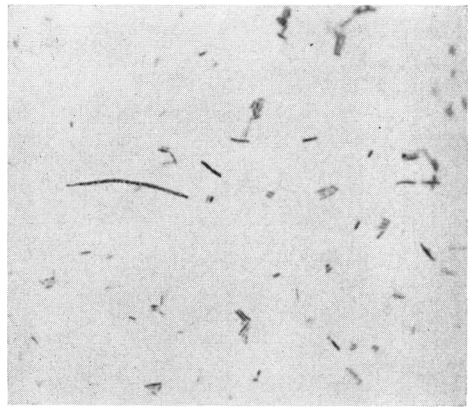

Figure 1. Elongated cells and normal cells (short dividing rods) of *Escherichia coli* Strain I, cultured for 7 hr in enriched medium containing 100 μg of 5-bromouracil per ml Methylene blue; × 400.

were first described by Roepke and Mercer (1947) in their thymine-deficient strain from which Strain I was isolated (Zamenhof and Griboff, 1954*b*). Roepke and Mercer noticed that such elongation does not occur for strains deficient in other nitrogenous bases. This phenomenon of elongation was also studied by Jeener and Jeener (1952) in *Thermobacterium acidophilus* R 26, which requires both thymidine and uracil for growth. These authors have shown that, while the omission of uracil merely inhibits growth without changing cell morphology, the omission of thymidine results in elongated cells in which the ratio of cytoplasm to "nucleoids" is 35 to 40 times greater than that in normal cells. The addition of a thymidine source (DNA) is followed by a rapid increase in the number of "nucleoids" and by cell division. The authors conclude that in such elongated cells only the DNA synthesis is impaired, whereas the RNA and protein synthesis still goes on.

Similar conclusions have been reached by Cohen and Barner (1954, 1955) in their thorough

[7] Similar cells can also be observed when growing Strain I in enriched medium containing 100 μg uracil riboside per ml; this nucleoside strongly inhibits growth, but neither uracil nor nucleoside is incorporated into DNA (Zamenhof *et al.*, 1956*a*).

[8] Even in these conditions, the DNA structure might have undergone a change: it has recently been reported that in the DNA of these cells the 6-methylaminopurine partially replaces thymine (Dunn and Smith, 1955). This purine is reported to be present in a small amount also in DNA of cells grown on normal medium and therefore it might be more persistent in DNA than 5-bromouracil when the cells are transferred from adverse to a normal medium.

TABLE 5

Elongated cells of Escherichia coli. Robinow (Giemsa-HCl) stain

	Strain			
	I		I/Br	
	Medium			
	A†	B	A	C
Cell length (μ)				
Average*	3.8	8.5	2.7	59
Max.	7	16	4	225
Cell diameter				
Average*	1.0	1.2	1.0	1.0
Cell volume (μ^3)				
Average*	3	10	2.1	46.2
Max.	5.5	18	3.1	177
Number of "nuclear bodies"				
Average*	2.1	3.3	2	17
Max.	4	9	2	61
Length of cytoplasm devoid of "nuclear bodies" (μ)				
Average*	0.9	1.9	0.99	2.4
Max.	2	5	2.5	12
Deoxyribonucleic acid (DNA) per cell (μg $\times 10^8$)				
Average	0.9	0.8	0.9	1.1
DNA per unit cell volume (μg $\times 10^8$/μ^3)				
Average*	0.3	0.14	0.43	0.024

* Average of 6 determinations.

† A, nutrient or synthetic broth; B, synthetic broth with 0.2 μg of thymine per ml; C, synthetic broth with 1 μg of thymine and 50 μg of 5-bromouracil per ml.

and elegant study of another thymine-requiring mutant of *E. coli*. These authors also studied the elongation of cells in the complete absence of thymine, and concluded that the resulting unbalanced growth of the cytoplasm leads to the irreversible loss of power to multiply, i.e., that this organism "adapts to die."

In the present study the cells did not lose their power to multiply. A 7-hr culture containing 98 per cent of greatly elongated cells (either because of limited amount of thymine or because of the presence of 5-bromouracil) had over 90 per cent viability. When such greatly elongated cells were deposited on normal nutrient agar and observed through a microscope, they were found to continue to elongate and eventually to divide in the center or nearer the ends to give rise to two or three greatly elongated cells. After normal incubation they gave rise to normal-sized colonies still containing 10 per cent of elongated cells. After one more transfer on normal nutrient agar 2 per cent of elongated cells could still be found. The unchanged proportion of greatly elongated cells can be maintained indefinitely by transfers into a synthetic medium containing 0.2 μg of thymine per ml every 7 hr. However, when left for further incubation in this medium or in the enriched medium containing 100 μg of 5-bromouracil per ml, the proportion of elongated cells at 24 hr becomes less than one tenth of the proportion at 7 hr; this clearly indicates that the change from elongated back to normal cells is not a typical rare mutation, because no selection of a hypothetical "back mutant" could have taken place: in the enriched medium containing 100 μg of 5-bromouracil per ml the number of cells at 7 hr (2.57×10^9 per ml) was practically the same as at 24 hr (2.52×10^9 per ml). The change (breaking into normal cells) could perhaps be called "de-adaptation" or rather "adaptation to normal conditions." However, as mentioned above, some cells remain elongated even after two transfers on normal nutrient agar (approximately 60 generations); it might be, therefore, that the "persistent modification" into elongated cells is a different phenomenon (of a cytoplasmic nature?) (compare Spray and Lodge, 1943).

When elongated cells obtained by growing strain I for 7 hr in basal broth containing 0.2 μg of thymine per ml were stained by the Robinow method (Giemsa-HCl) (Robinow, 1944), they were found to contain fewer "nuclear bodies" per unit length than the normal cells. The average and the maximal dimensions and numbers of nuclei are represented in table 5 (medium B). It can be seen that although the average volume of the cell is 3 times higher than that of the average "normal" cell of this age (medium A), the average number of nuclei is only 1.5 times higher. The DNA content per one elongated cell (table 5) in a culture containing 98 per cent of such cells was even less than normal. Thus, the DNA:cytoplasm ratio in such cells is on the average four times lower than in normal cells of this age.

Still longer cells can be obtained by growing Strain I/Br for 7 hr in basal broth containing 1 μg

of thymine and 50 μg of 5-bromouracil per ml (table 5, medium C). It can be seen that in this case the average number of discrete "nuclear bodies" is more than 8-fold the normal, yet the amount of DNA per cell is practically the same as in the normal cell, so that the DNA per unit volume is now only about 1/20 of normal. This speaks against the possibility that such cells have a higher ploidy than the normal cells. The cause of the increased number of discrete "nuclear bodies" is not clear: each must contain on the average seven times less DNA. In some cases these nuclear bodies were indeed smaller, weaker stained or divided into smaller Giemsa-stainable fragments, scattered along the cell.

In extreme cases the cells were 0.22 mm long, i. e., over 80 times longer than the normal cell.

Unclassified changes. II. Change in colony size. When Strain I is grown in basal broth containing a limited amount of thymine (0.2 μg per ml) (one or several transfers) and then streaked on nutrient agar, one observes the appearance of colonies of all sizes, ranging from normal down to pinpoint. The phenomenon does not seem to be related to the appearance of elongated cells (as described above) since the small colonies contain, if anything, less of these cells than the large colonies. The decrease in colony size seems to be here the result of a non-permanent injury to the cell grown in adverse conditions (limited DNA synthesis): indeed, upon transfer of the cells from a pinpoint colony onto fresh nutrient agar, only normal size colonies appear (adaptation to normal conditions?). Similar results were obtained when the adverse conditions were created by growing strain I/Br in basal broth containing 50 μg of 5-bromouracil per ml, or by growing Strain I for 7 hr in enriched broth containing 100 μg of 5-bromouracil per ml. However, in certain conditions the change to pinpoint colonies appears to be more permanent. When Strain I/Br, grown as above, was passed through enriched medium (24 hr, 37 C) prior to streaking on nutrient agar, the resulting colonies remain pinpoint even after the total of 4 transfers on normal nutrient agar and 2 in normal broth (approximate total of 140 generations). The pinpoint colonies contain cells that give rise to not only pinpoint but also normal size colonies; the proportion of cells forming normal colonies increases on passage through nutrient broth because of the constant change to these normal cells and because of their selective advantage (faster growth) over cells forming pinpoint colonies. Also, as expected, some such normal size colonies often contain cells that give rise to both normal and pinpoint size colonies. The cells of both normal and pinpoint colonies were either 5-bromouracil-resistant (like Strain I/Br) or 5-bromouracil-sensitive; they were either smooth or rough; this indicates that all these changes are independent.

The pinpoint colonies as described above are probably not related to small colonies known to arise as a result of a "suppressor" mutation in *E. coli* (Demerec *et al.*, 1954). However, mutants similar to the latter were also encountered: they can be isolated from papillae on faint colonies of Strain I grown on normal agar containing 100 μg of 5-bromouracil per ml (Zamenhof and Griboff, 1954*b*). Such cells were 5-bromouracil-resistant but mutated back to 5-bromouracil-sensitivity;[6] however, the change from small back to normal size has not been observed.

At present it cannot be said whether the pinpoint colonies described in this work are related to the cytoplasmic change described in yeast by Ephrussi and Hottinguer (1951), or to the change described in *E. coli* by Weed and Longfellow (1954).

DISCUSSION

In summarizing all the above findings one can say that cells containing 5-bromouracil in their DNA still seem capable of dividing and that thus far this drastic change in heredity determinants does not result in any detectable *typical* mutation. It may be recalled that the substitution of hydrogen by deuterium in Drosophila genes also did not result in an increased mutation rate (Zamenhof and Demerec, 1943); the substitution of active hydrogen atoms by deuterium in DNA having transforming activity also did not result in any inactivation of the transforming principle (Zamenhof and Leidy, *unpublished experiments*). It may be that certain seemingly drastic changes in the molecule of the heredity determinant do not affect its function. On the other hand, the changes affecting less than 0.2 per cent of amino groups in the DNA molecule result in total inactivation (Zamenhof *et al.*, 1953).

It must be pointed out that, despite unchanged function, the DNA molecules on reproduction must not produce true duplicates of themselves since they may replace their thymine by 5-bro-

mouracil and *vice versa*. The evidence of changes in DNA molecules on reproduction has also been obtained in the study of transforming principle (Zamenhof *et al.*, 1956*b*).

The adverse conditions (limited DNA synthesis) and/or replacement of thymine by an unusual base, and *vice versa*, give rise to certain modifications (elongated cells, pinpoint colonies) which thus far cannot be called typical mutations, yet appear too persistent to be classified as adaptations or deadaptations. It is still conceivable that this persistence is related to the persistence of an unusual base in DNA[8] thus representing a new (genetical?) phenomenon. The study of these changes is being continued.

SUMMARY

Study of *Escherichia coli* containing 5-bromouracil in its deoxyribonucleic acid (DNA) suggests that the organism is capable of reproduction and contains an unchanged amount of DNA. The replacement of thymine by 5-bromouracil and *vice versa* need not be accompanied by changes in phenotype or by typical mutations. However, these and other adverse conditions may produce modifications (greatly elongated cells poor in DNA, pinpoint colonies) which persist on normal media and as yet defy classification.

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