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PHENOTYPIC CHARACTER OF PHAGE PROTEIN ABNORMALITIES INDUCED BY BROMOURACIL

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Abstract—The incorporation of bromouracil, a thymine analogue, into the DNA of bacteriophage T_2 stimulates the synthesis of significant quantities of abnormal proteins. Their abnormal character is deduced from the study of certain properties of phages produced in the presence of bromouracil: stability of structure, adsorption on a sensitive bacterial strain and immunologic specificity.

The modifications studied are not transmitted to the progeny synthesized in the absence of the base analogue.

In the presence of bromouracil, the synthesis of phage DNA and protein is much less affected than the synthesis of virus particles. This difference is attributed to the fact that the abnormal proteins cannot be integrated into the phage particles.

INTRODUCTION

Incorporation of base analogues into nucleic acids, either in RNA (thiouracil[1, 2] or fluorouracil[3, 4]) or in DNA (uracils halogenated in position C_{-5}[5, 6]), was reported by many authors to stimulate the synthesis of abnormal proteins.

The proteins of several viruses synthesized in the presence of bromouracil, a thymine analogue, appear to be abnormal[7, 8]. In particular, bacteriophages T_2 produced in the presence of bromouracil are reported to show abnormal properties: a reduced "efficiency of plating",[9, 10] a partial inability to "kill" sensitive bacteria[10] and an increased sensitivity to any kind of radiations, X, u.v. and visible light.[10, 11]

Our aim, in this work, was to study, on the one hand, certain properties of T_2 phages produced in the presence of bromouracil and, on the other, the total proteins synthesized by *E. coli* T^-, a strain which is auxotrophic for thymine, after infection.

Our results suggest that the abnormal character of the phage proteins synthesized in the presence of bromouracil is essentially a phenotypic one and cannot be explained by a mutagenic effect caused by substitution of bromodeoxyuridylic acid for thymine in DNA.

MATERIAL AND METHODS

Bacterial strains *E. coli* T^- and *E. coli* B/r were used. The bacteriophage strain was $T_{2r}{}^+$.

The media used have been described by Dunn and Smith.[9]

Bacteria were grown at 37°, with constant agitation. Thymine was present in the cultures at a concentration of $4 \cdot 10^{-6}$ M. Bromouracil "Light" ($2 \cdot 10^{-3}$ M) was added

Abbreviations: For convenience, T designates the control phages while b represents the phages grown in the presence of bromouracil. PTA, phosphotungstic acid; HMC, hydroxymethylcytosine; RNAase, ribonuclease.

to the cultures when the bacterial concentration reached about $1{\cdot}5.10^8$ cells/ml. After 1·5 generation times, the bacteria were infected with phages, the multiplicity of infection being comprised between 1 and 5.

Purified phages were obtained by submitting completely lysed cultures to three successive cycles of centrifugation (30 min at 4,500 rev/min and 30 min at 20,000 rev/min).

Non-specific anti-bacterial antibodies present in the anti-T_2 rabbit antiserum used were eliminated by precipitation with an excess of bacterial proteins (obtained after disruption of a concentrated bacterial suspension in a sonic oscillator Raytheon, 250 watt, 10 kc, at maximum intensity during 3 min).

The structure of phages was observed with the Siemens Elmiskop I electron microscope. Three different methods have been used: the "PTA negative staining" technique,[12] the "agar-filtration" method[13] and, finally, the spreading of a drop of phage suspension in doubly distilled water followed by dehydration on the electron microscope grids. The latter two preparations were shadowed with an alloy, Au/Pd.

The infective phage yield was determined by plating on nutrient agar according to the method of Adams.[14]

Estimation of DNA, proteins and lysozyme were carried out by the methods of Ceriotti,[15] Folin[16] and Weidel and Katz[17] respectively.

Other experimental procedures are described in "RESULTS".

RESULTS

1. *Properties of bacteriophages grown in the presence of bromouracil*

Efficiency of plating. The efficiency of plating, a measure of the infectious character of the particles, was determined by a comparison between the number of viable phages, measured by plating on nutrient agar with sensitive bacteria, and an estimate of total phages obtained by measuring the DNA content either in specific serum precipitates or in the pellets obtained after centrifugation of purified phages in such conditions that sedimentation of particles is total (30 min at 20,000 rev/min). Both methods of assaying the total number of phage particles were comparable, the immunological precipitation of intact particles being always complete for b as well as for T.

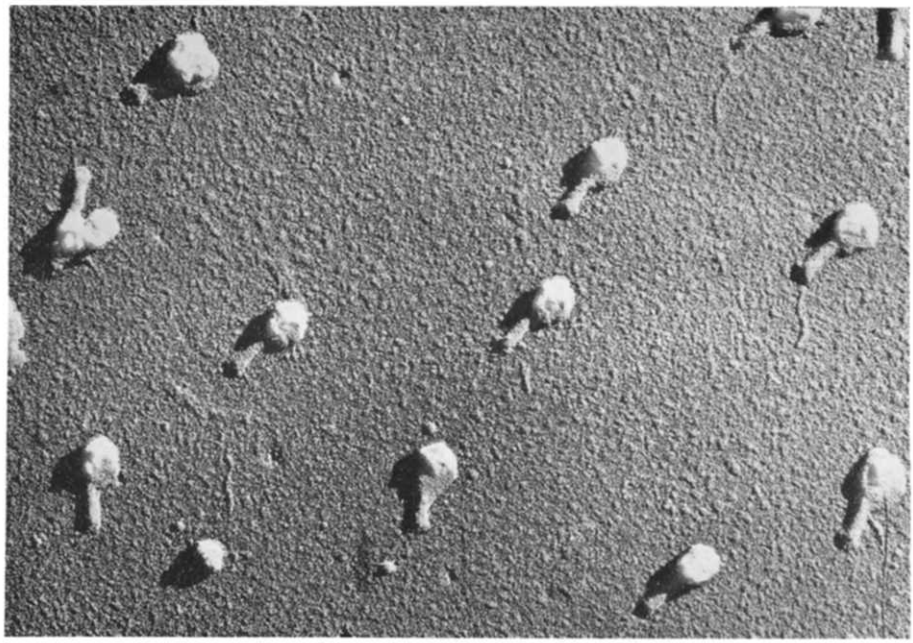
We have observed, in agreement with a number of authors,[9, 10] that the efficiency of plating of phages grown in the presence of bromouracil is generally modified, but in a variable manner from one experiment to another. The fact that this phenomenon is irregular and difficult to interpret led us to a more extensive investigation.

Electron microscopy

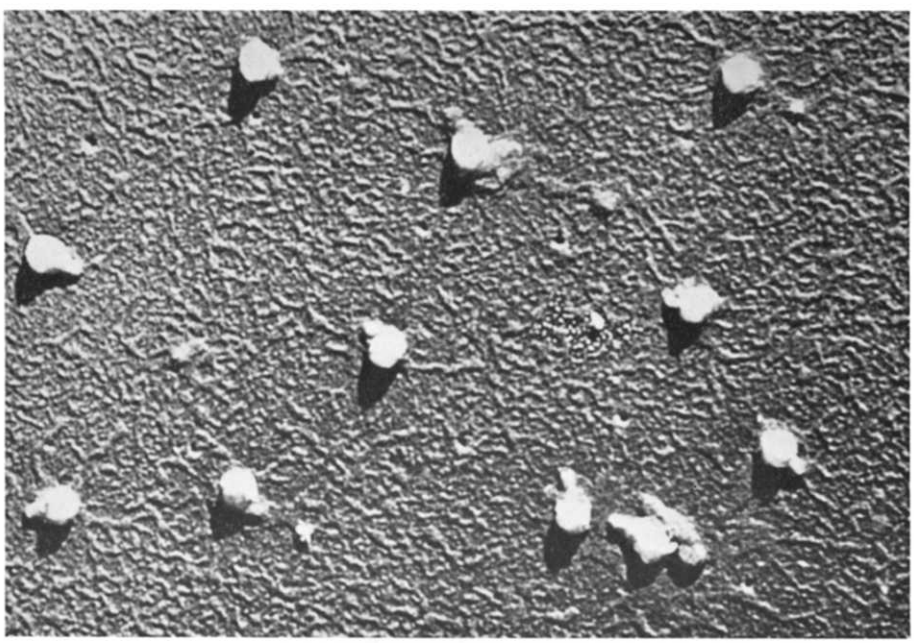
The first result suggesting the existence of abnormal proteins is the instability of the bacteriophage structure as observed by electron microscopy. We have compared structure of phages T and b; Fig. 1 shows that the structure of phages b is altered.

We may cite two reasons for suspecting a lack of stability rather than an abnormal structure of phages b.

First, when mild methods of spreading phages on the electron microscope grids are used, either the "agar-filtration" or the "PTA-embedding" methods, the structure of phages b remains identical to that of the control. Secondly, when the preparations are obtained by simple dehydration on the grids, fragments resulting presumably from a



(A)



(B)

FIG. 1. Preparation by simple dehydration of phages purified by 3 cycles of centrifugation, resuspended in H_2O, fixed by formol vapors and shadowed by Au/Pd. A, control phages (T); B, phages grown in the presence of bromouracil (b).

facing page 216 B.P.

partial destruction of the phage tails cover the support film and bacteriophages b show only incomplete tails.

Adsorption onto sensitive bacteria

The adsorption experiments were performed in the following way. Phages T and b, in which DNA was uniformly labelled with ^{32}P, were added to a growing bacterial culture of *E. coli* B/r in the proportion of 1 phage to 1 bacterium. At intervals between 0 and 6 min, samples of the cultures were removed, chilled and diluted to stop the adsorption reaction. The bacteria were centrifuged immediately at 4,500 rev/min for 5 min. The total quantity of non-adsorbed phages was determined by the serum-precipitable radioactivity of the supernatant, and the amount of non-fixed infective phages was measured by plating on nutrient agar in the presence of sensitive bacteria.

It is clearly shown in Fig. 2 that a great proportion of phages b detected by the ^{32}P incorporated into DNA, is not adsorbed onto sensitive bacteria.

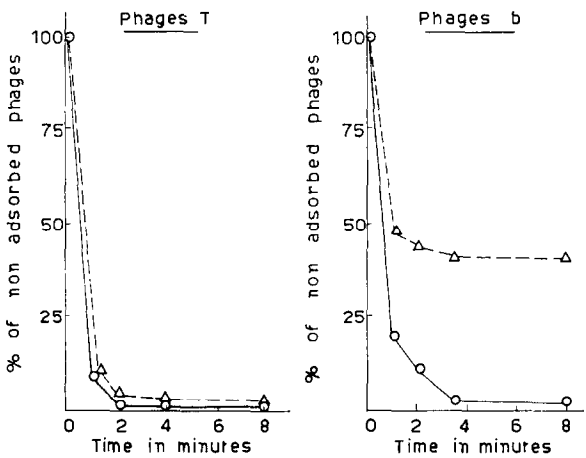


FIG. 2. Adsorption on sensitive bacteria of normal phages (T) and of phages grown in the presence of bromouracil (b). Phages T and b, uniformly labelled with ^{32}P, are added to an exponentially growing culture of *E. coli* B/r at 37°, in the proportion of 1 phage/bacterium. At intervals between 0 and 6 min, the adsorption reaction is stopped by chilling and dilution and the bacteria are centrifuged. The total amount of non-adsorbed phages (– – △ – – △ – –) is measured by the serum precipitable radioactivity of the supernatant. The infective phages not fixed (—○——○—) are determined by plating on nutrient agar.

Immunological properties

The precipitation curves were obtained in the following way. Purified phages, uniformly labelled with ^{35}S and sonicated (12 min), were added in increasing amounts to a constant quantity of anti-T_2 purified antiserum. When reaction was complete (2 hr at 37° followed by 48 hr at 5°), the precipitates were centrifuged, the supernatants were precipitated by trichloracetic acid (10%) in the presence of a carrier and the radioactivity of these precipitates was determined.

Results of this type of experiment are shown in Fig. 3.

These experiments have clearly shown that, after sonication, a great proportion (up to 50 per cent) of phage b proteins is unable to react with specific antibodies.

Phenotypic character of the phage protein modifications

The immunological properties of phages T and b (first passage) and of the descendants produced in the absence of the base analogue (second passage) have been studied simultaneously by the method described above.

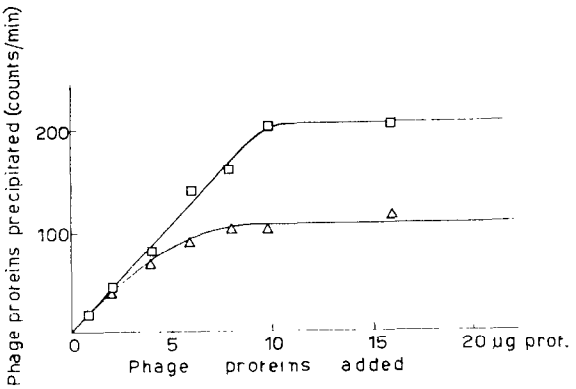


FIG. 3. Antiphage serum precipitation curves of labelled proteins of control phages (T)—□——□— and of phages grown in the presence of bromouracil (b)—△——△—. The protein preparations have the same specific radioactivity (^{35}S). These labelled proteins are added in increasing amounts to a constant quantity of anti-T_2 antiserum. The radioactivity of precipitates is determined after complete reaction (2 hr at 37° followed by 48 hr at 5°).

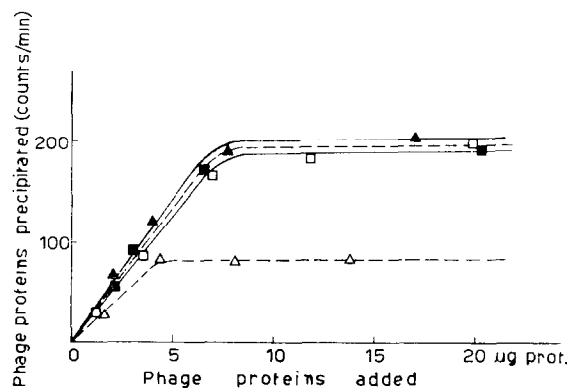


FIG. 4. Phenotypic character of the immunologic modifications undergone by phage proteins synthesized in the presence of bromouracil. Antiphage serum precipitation curves for phages T and b (first passage) and for their progeny synthesized in the absence of the base analogue (second passage). (See legend of Fig. 3 for the technique used.) Dotted lines represent the precipitation curves of the first passage phages. (T) – – □ – – – □ – –; ((b) – – △ – – – △ – –. Continuous lines represent the precipitation curves of the second passage phages. (T)—■——■—; (b)—▲——▲—.

As pointed out previously, the efficiency of plating of phages grown in the presence of bromouracil varies in an irregular manner. The first passage phage b stock used in this experiment appears to contain many abnormal phages, since only 50% of the isolated phage protein was specifically precipitable by anti-T_2 antiserum, but the

efficiency of plating was identical to that of the control stock. Each particle, whether it contains bromouracil-substituted DNA or normal DNA, is thus able to give rise to a descendance.

The results shown in Fig. 4 suggest that the abnormal character of phage proteins is not transmitted to the progeny and thus cannot be attributed to mutations.

2. *Total synthesis of viral components integrated or not in mature phage particles*

We have studied the synthesis of a specific protein coded by the phage genome (lysozyme) and of the total protein produced after infection.

The experimental principle is the following.

When a radioactive protein precursor and T_2 bacteriophages are added simultaneously to a bacterial culture, only the viral proteins and the proteins necessary to bacteriophage growth are labelled. Indeed the fact that, immediately after infection, the synthesis of bacterial protein is stopped while phage protein are produced is generally accepted.[18-20]

The infection procedure being such that all bacteria are infected together, the radioactivity of a precursor incorporated in the proteins of total lysates is a measure, at least comparative, of the phage protein synthesis if the free precursor concentration existing in the cells at infection time is constant or may be neglected.

[^{35}S]-sulfate (carrier free) and T_2 phages (multiplicity of 5) were added simultaneously to a bacterial culture of *E. coli* T^-. The fact that 99% of the bacteria are killed 5 min after infection was verified. After one night at 37°, the cells were spontaneously lysed and the following characteristics were determined:

infective phage yield,

amount of bacteriophages measured by estimation of DNA in immunological precipitates,

total protein synthesis determined by the incorporation of [^{35}S]-sulfate,

synthesis of a specific protein, lysozyme.

Results of this type of experiment are summarized in Table 1.

It is shown in this table that the total protein synthesis and that of the specific protein (lysozyme) are reduced in the same proportions, namely 4·5 times; the total

TABLE 1. COMPARISON BETWEEN THE AMOUNTS OF TOTAL PHAGE PROTEINS, OF A SPECIFIC PHAGE PROTEIN (LYSOZYME), OF MATURE AND INFECTIVE PHAGES PRODUCED BY *E. coli* T^- AFTER INFECTION IN THE PRESENCE OR IN THE ABSENCE OF BROMOURACIL

	Control phages (T)	Phages grown with bromouracil (b)	Ratio T/b
Infective phages/ml lysate	$5{\cdot}4.10^{11}$	$1{\cdot}7.10^{10}$	1/32
Total amount of phages (μg DNA of immunologic ppt./ml)	104	8·5	1/12
Total protein synthesis (counts/min/ml lysate)	5,282	1,175	1/4·5
Synthesis of lysozyme (E/ml)	4·2	0·86	1/4·8

(^{35}S) sulfate and T_2 phages with a multiplicity of 5 are added simultaneously to exponentially growing *E. coli* T^- at 37° and with constant agitation. The following characteristics (see text) have been determined after spontaneous lysis of the cells (one night at 37°).

amount of phage particles is reduced 12 times. In phages T_2, the amount of DNA and proteins are nearly the same. It can thus be concluded that only one third of the proteins synthesized in the presence of bromouracil are integrated into phage particles.

Experiments performed after inducing lysis by addition of chloroform to the bacterial culture 20 min after infection have given identical results.

On the other hand, the total amount of bacteriophage particles (DNA of immunological precipitates), of protein (radioactivity of the labelled precursor incorporated) and of DNA synthesized in the presence of broumouracil have been determined simultaneously. This last experiment is easily performed since T_2 and bacterial DNA differ by the presence in the former of a particular base replacing cytosine, namely 5 HMC. Base composition of non-infected bacterial DNA and of total (bacterial + viral) DNA synthesized in T and b lysates was determined by two dimensional paper chromatography[21] and the total amount of phage DNA produced was evaluated.

Results of this type of experiment are presented in Table 2.

TABLE 2. COMPARISON BETWEEN THE TOTAL AMOUNTS OF PHAGE PARTICLES, PROTEINS AND DNA SYNTHESIZED AFTER INFECTION IN THE PRESENCE OR IN THE ABSENCE OF BROMOURACIL

	Control phages (T)	Phages grown with bromouracil (b)	Ratio T/b
Total bacteriophages (μg DNA of immunologic ppt./ml)	14·7	4·3	1/3·4
Total phage protein synthesis (counts/min/ml lysate)	3,735	3,756	1/1·1
Total phage DNA synthesis (μmole phage DNA/0·1 μmole bacterial DNA)	0·830	0·550	1/1·5

These characteristics have been determined after artificial cell lysis induced by the addition of chloroform 25 min after infection (see text for other details).

It is shown there that, in the presence of bromouracil, the phage DNA synthesis is reduced 1·5 times, approximately in the same proportion as the protein synthesis (1·1 times), whereas the reduction of bacteriophage yield is 3·4 times. Thus, the non-integration of phage proteins into viral particles cannot be attributed to a deficient DNA synthesis but probably to an abnormal structure of the elementary protein units.

DISCUSSION

Our observations on some properties of phages b and on the synthesis of proteins in the presence of bromouracil can be explained by an abnormal structure of these proteins.

Indeed, the partial inability of phages b to adsorb onto sensitive bacteria could be attributed to the existence of tail proteins sufficiently abnormal to prevent attachment of the phages to the extremely specific bacterial receptors.

In the same way, the reduced immunological precipitation observed for phages b may be attributed to the presence of modified antigenic sites unable to react with the complementary active sites of antibody molecules.

We have also shown that an appreciable amount of protein synthesized in the presence of the base analogue were not integrated into mature phages, probably because the elementary protein units were so abnormal that they could not be associated.

The observations on some properties of phages synthesized in the presence of bromouracil, described in the first part of this work, are in agreement with those of Dunn and Smith[9] and of Litman and Pardee[10] on T_2 phages and those of Easterbrook and Davern[7] on vaccinia virus.

There is also close parallelism between the effect of bromouracil on phages T_2 and that of RNAase on lysogenic phages of *B. megaterium* synthesized after UV induction.[22, 23] Both phage preparations show abnormal properties (instability of structure, reduced adsorption on sensitive bacteria and altered immunologic specificity) which may be attributed to the presence of abnormal proteins. This abnormal character has been demonstrated for the proteins of lysogenic phages of *B. megaterium* by fingerprints of tryptic peptides.[23] Moreover, Dupont–Mairesse[22] has observed an accumulation, in the presence of RNAase, of significant amounts of proteins incapable of being integrated into virus particles but possessing their immunologic specificity.

The existence of phenotypic modifications produced in the proteins of phages T_2 grown in the presence of bromouracil was suggested by Litman and Pardee[10] in 1960. They observed that the increased sensitivity to inactivation by UV light of phages b was not transmitted to the progeny. However, this interpretation must be considered with much caution. Indeed, it has been found since that the mode of action of UV light is different for DNA molecules containing bromodeoxyuridylic[25] rather than thymidylic[24] nucleotides.

Our immunological experiments have shown that bromouracil induces phenotypic modifications of the protein structure and thus presumably affects the RNA fractions, either transfer or messenger, involved in protein synthesis.

Two types of bromouracil action on these RNAs may be visualized. First, the fact that many errors in protein synthesis may be induced by the eventual incorporation of bromouracil in the RNAs should not be neglected; indeed the existence of a thymine ribonucleotide has recently been demonstrated.[26] However, Fleisner and Borek[27] have suggested that the "rare bases" of soluble RNA, including thymine, are enzymatically methylated after their incorporation into the nucleotide chains. This objection is not sufficient to discard definitively an eventual incorporation of bromouracil in RNA. Indeed, a bacterial strain, thymine-deficient and able to transform external thymine into ribosylthymine[28] was used. Furthermore, incorporation of some methylated bases into transfer RNA has been reported.[29] If, on the other hand, it were demonstrated that bromouracil cannot be incorporated either in messenger or in transfer RNA, then, to explain the fact that many reading errors of the genetic code (involving phenotypic modifications in proteins) could occur without errors in DNA duplication (the origin of mutations), we would have to postulate that the mechanism of messenger RNA synthesis is different of that of DNA duplication.

If this last interpretation were true, new arguments would be brought in favour of the idea that mechanisms more complex than simple pairing by hydrogen bonds between the complementary bases of the Watson and Crick[30] model are involved in the synthesis of specific polynucleotides (Loftfield[31]).

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