ON THE INHIBITION OF PHAGE PRODUCTION BY 2-THIOURACIL AND 8-AZAGUANINE IN AN INDUCED LYSOGENIC *BACILLUS MEGATERIUM*

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SUMMARY

Thiouracil exerts an inhibitory influence on induced lysogenic *Bacterium megaterium* that is much more extensive on phage protein and phage DNA synthesis than on bacterial protein and DNA synthesis. The number of infectious phage particles produced decreases more than does the amount of phage protein. The action of thiouracil is reversible by uracil, but not by thymine nor thymidine.

Ultraviolet irradiation suppresses the inhibitory effect of thiouracil unless the cells have been in contact with the latter for 30–60 min preceding irradiation. Thiouracil incorporation into RNA is also suppressed by ultraviolet irradiation. Nevertheless, a considerable amount is incorporated if the thiouracil is added 30 min before irradiation.

The parallelism which seems to exist between incorporation of thiouracil into RNA and its inhibitory action on phage protein synthesis is consistent with the hypothesis that a particular fraction of RNA participates in the synthesis of phage constituents and that thiouracil influences phage synthesis by being incorporated into this fraction.

Though the phenomenon is not so readily observed, azaguanine also seems to exert a selective inhibitory action on the synthesis of phage protein and phage DNA in induced lysogenic *Bacterium megaterium*. This analogue is also incorporated into RNA and it is assumed that its mode of action is similar to that of thiouracil.

INTRODUCTION

Two separate facts support the view that ribonucleic acid (RNA) is involved in phage protein synthesis. In *Escherichia coli* there appears, immediately after phage infection, an RNA fraction of a particular constitution presenting a high metabolic activity (HERSLEY[1], VOLKIN AND ASTRACHAN[2]; KIHO AND WATANABE[3]). On the other hand, phage protein synthesis in an induced lysogenic *B. megaterium* is inhibited by ribonuclease (RNase) (JEENER[4,5]).

The role of this RNA presumably implicated in phage protein synthesis might be the transfer of activated amino acids onto the templates where proteins are assembled (HOAGLAND *et al.*[6]), or alternatively this RNA might constitute these

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templates themselves (JEENER[4,7], HERSHEY[8]). In the latter case, we should have to assume that the genetic information capable of monitoring phage protein synthesis is transmitted from desoxyribonucleic acid (DNA) to the proteins via a specific RNA intermediate.

These questions have already been discussed in a study of the inhibitory action of RNase on phage protein synthesis[5]. The present paper reports the study of an analogous inhibition of phage production in u.v.- or H_2O_2-induced lysogenic *B. megaterium* by thiouracil and azaguanine[4].

The essential facts that we wish to stress are: the selective character of thiouracil inhibition on the synthesis of phage constituents, the fact that this action is reversible only by uracil and not by thymine nor thymidine, and the existence of a correlation between the action of thiouracil and its incorporation into RNA.

A tentative explanation of these facts is proposed on the basis of the hypotheses (1) that thiouracil acts by modifying the structure of a specific RNA which is synthesized after induction, (2) that this RNA is necessary both for the transmission of genetic information from DNA to protein and also for the reduplication of the DNA molecule.

MATERIALS AND METHODS

The lysogenic strain of *B. megaterium* and culture methods are described elsewhere[5].

Induction is effected by irradiation of 2-mm layers of liquid cultures with the aid of a short-wave u.v. lamp (Mineralight Model S.L. 2537, output of $19 \cdot 10^2$ ergs/mm^2/min at a distance of 28 cm[16]).

The [^{35}S]thiouracil and the $^{32}PO_4$ were obtained from the Radiochemical Centre, Amersham.

The method of liberation of the cellular contents by lysozyme and the assay of phage specific proteins by means of antiphage sera have been described previously[5].

Assay of protein, RNA and DNA is effected as reported in the preceding paper[5].

Paper electrophoresis is carried out under the conditions described by MATTHEWS[10] in an apparatus similar to that employed by SMITH AND MARKHAM[9].

The DNA is isolated by the Schmidt-Thannauser procedure from the specific phage precipitate obtained with antiphage serum. It is then hydrolysed by *N* HCl and assayed by the Ceriotti method. The radioactivity of the incorporated ^{32}P is determined in a liquid counter.

RESULTS

(a) *Action of thiouracil on the synthesis of specific phage proteins*

Two facts that are already known must first be recalled.

In the lysogenic system studied, induction of phage synthesis by u.v. irradiation or peroxide treatment does not interrupt the synthesis of most of the normal bacterial constituents. Optical density of the cultures approximately doubles before lysis begins and phage is liberated.

It is also known that addition of thiouracil to a non-induced *B. megaterium* culture during exponential growth imposes a linear growth which is maintained for several hours. During the latter phase, normal ratios are maintained between RNA, DNA and protein (HAMERS[11]).

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(1) *Induction obtained by addition of H_2O_2.* The inhibitory action of thiouracil on phage protein synthesis in *B. megaterium* is most readily analysed after addition to the culture of the smallest amount of hydrogen peroxide that is sufficient to induce lysis in the vast majority of the cells.

When thiouracil (160–320 μg/ml) is added before induction, inhibition of lysis and of phage protein synthesis (measured as protein precipitated by antiphage serum) is complete.

Thiouracil added immediately after induction is just as active. If, however, addition of thiouracil is further postponed, its effect lessens, and, after 50 min, when a decrease in O.D. indicates that lysis is beginning, is no longer detected.

The action of thiouracil is thus similar to that of RNase (JEENER[5]): the sooner it is added, within a period extending from induction to the beginning of phage protein synthesis, the more extensive is its action, although partial inhibition still remains detectable if the thiouracil is added at the beginning of phage protein synthesis. The only difference, probably of no great importance, is that thiouracil inhibition is more decreased with delayed addition of the inhibitor than RNase inhibition.

But the most striking resemblance between the action of thiouracil and that of RNase is that the selective action of the inhibitor is equally marked in both cases.

For instance, in the absence of thiouracil, the O.D. of the induced control culture rises after induction from 0.215 to 0.395; then the culture lyses completely, liberating considerable amounts of phage protein. A similar culture treated with thiouracil (320 μg/ml) immediately after induction increases from O.D. 0.215 to O.D. 0.450. In this culture, no lysis or phage production is detectable.

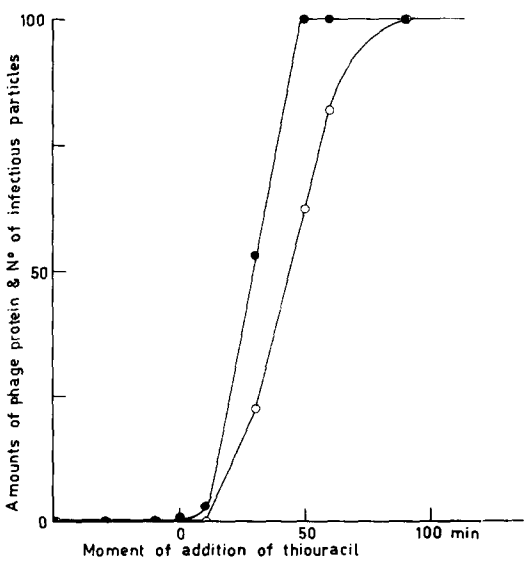

Fig. 1. Variation in the production of phage protein and of infectious particles in H_2O_2-induced *Bacillus megaterium* cultures as a function of the time of addition of thiouracil. The moment of addition of thiouracil in each culture is defined as the time elapsed between induction (0 min) and this addition. The amounts of phage protein and infectious particles produced are expressed as percentage of the amounts produced in control cultures in the absence of thiouracil. ●—● amount of protein precipitable by antiphage serum; ○--○ number of infectious particles.

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Control experiments show that the increase in O.D. corresponds to an increment in protein N precipitable by cold 10 % trichloroacetic acid.

In presence of thiouracil, the amounts of bacterial proteins synthesized are thus higher than in its absence, whereas phage protein synthesis is completely inhibited. If the addition of thiouracil is postponed, its inhibitory action is apparently less selective but nevertheless quite noticeable.

(2) *Induction obtained by ultraviolet irradiation.* Although thiouracil inhibits phage protein synthesis, whether induced by ultraviolet light or hydrogen peroxide, there are nevertheless striking differences in these two cases. This is shown in Fig. 1 (H_2O_2 induction) and Fig. 2 (ultraviolet induction), in which the amount of phage synthesized is plotted against the time of addition of thiouracil.

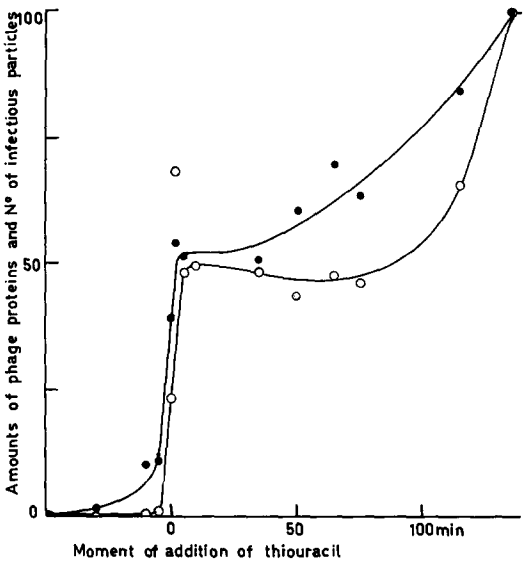

Fig. 2. Variation in the production of phage protein and of infectious particles in u.v.-induced *Bacillus megaterium* cultures as a function of the time of addition of thiouracil. Induction is performed at the time 0 min by u.v. irradiation of 50 sec. The moment of addition of thiouracil in each culture is defined as the time elapsed between induction (0 min) and this addition. The amounts of phage protein and infectious particles produced are expressed as percentage of the amounts produced in control cultures in absence of thiouracil. ●——● amount of protein precipitable by antiphage serum; ○——○ number of infectious particles.

The first peculiarity observed in the case of ultraviolet induction is that an extensive inhibition of phage protein synthesis can be obtained only if the inhibitor is added before induction. The inhibition observed increases with the time that elapses between addition of thiouracil and induction and is completed only when this period reaches 30–60 min (Fig. 3). Inhibition of phage protein synthesis goes parallel with the inhibition of cellular lysis.

In the second place, if thiouracil is added at the time of irradiation or immediately afterwards, one notices that the curve indicating the amounts of phage produced rises very steeply, the inhibitory action of the thiouracil being decreased by 50 % after only a few minutes delay in adding the inhibitor.

If the addition of thiouracil is still further delayed, the amounts of phage proteins

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produced increase gradually and finally reach the level obtained in the controls in the absence of thiouracil.

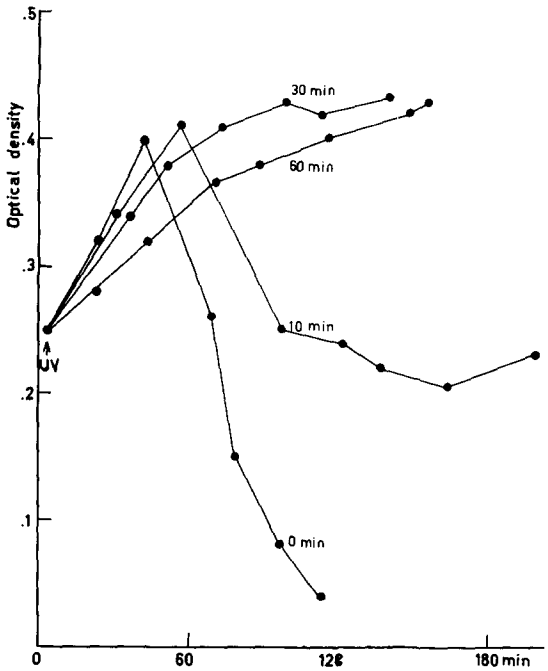

Fig. 3. Evolution in the optical density of u.v.-induced cultures. The cultures are induced at the moment indicated by an arrow. Thiouracil is added 60, 30, 10 and 0 min before induction.

The interpretation of these facts necessitates a short discussion.

The sudden inflexion of the curve in Fig. 2 when u.v. irradiation follows the addition of thiouracil might be due to a simple suppression of the induction of prophage into vegetative phage (for instance the thiouracil acting as a u.v.-absorbing screen) and not to an inhibition of phage synthesis in an induced system.

This hypothesis must be completely refuted. The addition of uracil to a culture irradiated by u.v. light in the presence of thiouracil restores an abundant phage production. Uracil being incapable of inducing lysogenic bacteria, it is therefore beyond doubt that induction takes place in presence of thiouracil under influence of u.v. irradiation, and that thiouracil prevents phage protein synthesis only in a system potentially still capable of it.

If such is the case, the observed facts can be interpreted by assuming (1) that the thiouracil, during the period of 30–60 min preceding u.v. induction, brings about a phenomenon which is a condition of the inhibitory action that it will exert after the induction on the synthesis of the phage constituents, (2) that this phenomenon is inhibited by u.v. irradiation.

In the case of H_2O_2 induction, preliminary contact of the cells with thiouracil is not required for its subsequent inhibitory action. We admit therefore that H_2O_2 exerts little or no inhibitory influence on the above phenomenon.

The nature of this phenomenon brought about by thiouracil will be discussed

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presently. For the moment, we merely note that in our experiments u.v. light exerts a very complex influence: it induces prophage, transforming it into vegetative phage, and it inhibits a preliminary reaction produced by thiouracil and conditioning its subsequent inhibitory action.

The gradual increase of phage proteins which appears when thiouracil is added between the 50th and the 130th minute following induction is readily accounted for by the amounts of phage proteins already present at that time.

A last but very important fact: the inhibitory effect of thiouracil, completely reversible by uracil in equimolar amounts, cannot be reversed either by an excess of 20 moles thymine, or by an excess of 2 moles thymidine.

(b) *Action of thiouracil on phage DNA synthesis*

The DNA of this *B. megaterium* phage contains no specific constituent comparable to the 5-hydroxymethylcytosine of the T-even coli phages. It is therefore impossible, by current methods, to assay phage DNA in the presence of host DNA. Nevertheless, isotopic methods do permit an analysis of the kinetics of phage DNA synthesis, as is shown by the following example.

Lysogenic *B. megaterium* cells are uniformly labelled by growth on ^{32}P-containing medium. The specific radioactivity of the ^{32}P-containing constituents is then equal to that of the inorganic PO_4.

The cells are rapidly centrifuged and resuspended in culture medium containing unlabelled phosphate. This treatment decreases the specific activity of the inorganic phosphate 1280 times.

After a short lapse of time not exceeding 15 sec the cells are induced to lyse by u.v. light. When lysis is complete, the phages are precipitated by the specific antiserum. The precipitate is washed. The DNA is separated by the Schmidt-Thannauser procedure and the specific activity of its P is determined. The P of the phage DNA will be labelled only in as far as it derives from bacterial ^{32}P.

In such an experiment the specific activity of the phage DNA P was 17,391 dis/min/mg. This amounts to 2 % of the specific activity of the total P of the bacterial constituents before induction (907,907 dis/min/mg). This implies that 2 %, at the utmost, of the phage DNA P derives from bacterial constituents present before induction and that the remaining 98 % derives from the phosphate in the culture medium.

In contrast, if labelled P is added only immediately after induction, the specific radioactivity of the phage DNA P will be practically identical to that of the inorganic phosphate of the culture medium (JEENER[12]).

Thus if one adds the radioactive P to a culture during phage DNA synthesis, the phage DNA synthesized after addition of the isotope will have the specific activity of the culture medium phosphate, whereas the phage DNA synthesized before will be unlabelled. The ratio of the specific activities of the phage DNA P to the culture medium inorganic P will give an estimate of the amount of phage DNA synthesized after addition of the ^{32}P*.

* The action of ultraviolet light is not accompanied by the appearance of a pool of DNA precursors large enough to render this estimate incorrect, at least not in the case where DNA synthesis is inhibited (JEENER[12]). As a matter of principle, the objection might be made that such a pool might nevertheless appear when DNA synthesis is not inhibited. There is no evidence to support this.

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This method, which has been used to establish the kinetics of phage DNA synthesis under normal conditions (JEENER[12]), is equally useful for determining whether or not DNA is synthesized in the presence of all inhibitors of phage multiplication, provided that their effects on phage particle production are reversible, and that the DNA produced after reversion is included in particles precipitable with the antiphage serum.

The following results are obtained in the presence of thiouracil. Cultures treated with thiouracil 78 min before u.v. induction do not lyse and do not produce phage particles, not even several hours after irradiation. Similar cultures to which equimolar amounts of uracil are added at different times (0–265 min) after induction, undergo a partial lysis accompanied by phage production. If ^{32}P is added at the same time as the uracil, the specific activity of phage DNA P equals that of the inorganic P (Table I). From what has been said before, we conclude that all phage DNA is synthesized after addition of the ^{32}P and the uracil; or in other words, that phage DNA is not synthesized in the presence of thiouracil if uracil is not added.

TABLE I

SPECIFIC RADIOACTIVITY OF DNA OF PHAGES PRODUCED AFTER ADDITION OF URACIL IN U.V.-INDUCED CULTURES OF *B. megaterium*

Thiouracil is added to all the cultures 60 min before induction at a concentration inhibiting all phage production. Labelled phosphate is added at the same time as the uracil. The specific radioactivity of the inorganic phosphate of the culture medium is 26,343 dis/min/mg.

Time between induction and addition of uracil and ^{32}P (min)	*DNA precipitated by antiphage serum (μg)*	*Specific activity of phage DNA P (dis/min/mg)*
0	336	24,370
70	288	27,560
100	288	24,120
190	137	28,910
265	84	22,570

Nevertheless, during the 265 min which elapse between induction and the final addition of uracil, bacterial growth does continue and the O.D. of the culture doubles. Although growth slows down in presence of thiouracil, the ratio bacterial DNA/bacterial proteins remains normal and a considerable amount of bacterial DNA is synthesized.

Thiouracil thus appears here as a very selective inhibitor, blocking phage DNA synthesis completely.

It can be seen that thiouracil dissociates the process by which the prophage becomes a potentially active vegetative phage following u.v. irradiation from the subsequent process by which phage DNA molecules are built. But, the latter of these two processes is inhibited, as is phage protein synthesis (see above).

One reservation to this interpretation remains to be made: if phage DNA synthesized in presence of thiouracil were not integrated into the particles precipitated by the specific antiphage serum, it would have been completely overlooked by the methods used. A hypothesis of this type is, however, entirely gratuitous.

(c) *Effects of thiouracil on the number of infectious phage particles produced*

In the preceding experiments, the number of infectious particles produced was

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in many cases determined at the same time as the quantity of phage antigen (see for example Figs. 1 and 2).

These determinations confirm by a more classical method that the action of thiouracil on phage production results in a selective inhibition of the latter.

This inhibition is complete if thiouracil is added sufficiently soon after induction of lysis, or incomplete if addition of thiouracil is delayed (Figs. 1 and 2). Reversibility of the inhibitory effects of thiouracil by uracil, and not by thymine nor thymidine, is also confirmed by infectious particle counts.

Figs. 1 and 2 show that the effect of thiouracil on infectious particle counts is throughout more important than its effect on the amount of phage protein produced.

This is substantiated by the data listed in Table II obtained after partial inhibition by thiouracil. Phage protein is decreased by a factor of 2, phage DNA by a factor of 6.7 and infectious particles by a factor of 77.

TABLE II

CHARACTERISTICS OF PHAGE MATERIAL PRODUCED BY THIOURACIL-TREATED CULTURES OF *B. megaterium* UNDER CONDITIONS IN WHICH INHIBITION IS NOT COMPLETE

Time of addition of thiouracil	*Protein N precipitated by antiphage serum (μg)*	*DNA precipitated by antiphage serum (μg)*	*Number of infectious particles per 0.1 ml culture*
5 min before u.v. irradiation	41.8	24	$8 \cdot 10^5$
5 sec after u.v. irradiation	44.8	50	$140 \cdot 10^5$
30 min after u.v. irradiation	62.7	120	$266 \cdot 10^5$
Control (no thiouracil)	82.7	160	$620 \cdot 10^5$

Thiouracil, added to a lysate containing phage particles produced under normal conditions does not cause any decrease in the number of infectious centers assayed by plating. Thiouracil thus can have no direct influence on the complete phage particles.

It is therefore obvious that thiouracil exerts a two-fold action; firstly it inhibits the synthesis of phage proteins and phage nucleic acid, and secondly it prevents these materials from acquiring a normal infectious character.

A hypothesis that should be considered is that the particles synthesized in presence of thiouracil are built up of protein of abnormal structure and that, consequently, these phage particles having abnormal tail proteins are incapable of absorption onto sensitive bacteria. We have indeed shown that this is probably the cause of the reduced infectivity of phage particles synthesized in presence of RNase.

This hypothesis can be eliminated by the following experiment (Table III). Normal phage and phage synthesized in presence of thiouracil (under conditions where inhibition is not complete) are labelled with ^{32}P. These phages are added to a suspension of sensitive *B. megaterium*. After 30 min the suspension is centrifuged at low speed. The amount of ^{32}P centrifuged along with the bacteria is always proportional to the amount of ^{32}P-labelled phage added. Thiouracil therefore does not interfere with the capacity of the DNA-containing phage particles grown in its presence to be absorbed onto sensitive cells.

We still have to consider whether the low infectivity of these particles might not be due to their DNA being present in abnormal amounts, or having an abnormal structure, or perhaps not even being injected into the host cell. No experiments have been made as yet to discriminate between these possibilities.

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TABLE III

CAPACITY OF ADSORPTION OF LABELLED PHAGE PARTICLES GROWN IN THE PRESENCE OF THIOURACIL

	Protein N precipitated by antiphage serum (µg/10 ml lysate)	*DNA precipitated by antiphage serum (µg/10 ml lysate)*	*Number of infectious particles per 10 ml lysate*	*Radioactivity of DNA fixed onto sensitive cells (dis/min)*
Control culture	27.8	56	$3942 \cdot 10^8$	593
Thiouracil-grown culture	23.5	18	$588 \cdot 10^8$	186

Phage was purified by several centrifugation cycles and treated with DNase and RNase.

The phages cultured in the presence of thiouracil and the phages produced by the control culture contained DNA of the same specific radioactivity.

Equal volumes of each of the two phage solutions are added to equal volumes of the same culture of sensitive *B. megaterium*. The adsorption process is allowed to proceed for 30 min. Separation of adsorbed from free phage is effected by centrifugation.

(d) *Relation between the inhibitory effects of thiouracil and its incorporation into ribonucleic acid*

As HAMERS[11] has shown, *B. megaterium* cells cultured in the presence of [^{35}S]-thiouracil incorporate appreciable amounts of it into their RNA. Chromatographic tests used by HAMERS led him to believe that thiouracil was incorporated as thiouridylic acid. Paper electrophoresis (pH 3.5) of the nucleotides of RNA after a preliminary purification as barium salts (THOMAS AND SHERRATT[13]) shows that all ^{35}S activity travels within the uridylic acid band*. This band is eluted and then submitted to electrophoresis at pH 9 when the ^{35}S component separates from the uridylic acid. Contrary to our expectations, this substance, separated at pH 9, presents a mobility at this pH of only 12 % in excess of that of uridylic acid. If it were thiouridylic acid, its mobility ought to have been far greater (MATTHEWS[10]). The radioactive region of the paper is eluted. The eluate presents an ultraviolet absorption spectrum different from what we might expect for thiouridylic acid, knowing that of thiouridine obtained by STROMINGER AND FRIEDKIN[14]. It remains none the less possible that the eluted spot contains other constituents than that bearing the ^{35}S label.

One possible explanation for the reduced electrophoretic motility at pH 9 of the ^{35}S-labelled component is that the thiouracil is not incorporated as such into the RNA. This possibility must be considered, in view of the work of SARCIONE AND SOKAL[15] in which they reported methylation of thiouracil into 2-methylthiouracil in the rat. The corresponding nucleotide would have a very different electrophoretic mobility.

As it is, the ^{35}S is incorporated into RNA, is precipitated together with the nucleotides, and presents an electrophoretic motility at pH 3.5 corresponding to uridylic acid. If we admit that we are dealing with a nucleotide containing one atom of ^{35}S per molecule then the radioactivity incorporated in three hours by a culture of *B. megaterium* corresponds to an amount of abnormal nucleotide that can be as high as 20 % of the uridylic acid.

* Electrophoresis at pH 3.5 shows that thiouracil does not move, whereas SO_4 migrates much faster than the components studied. These two possible contaminants are therefore eliminated in the first step of the analysis.

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For the sake of convenience, the ^{35}S activity incorporated in the RNA will be expressed in percentage of uracil presumably replaced by thiouracil.

In Table IV are listed the results of experiments described above in which incorporation of [^{35}S]2-thiouracil has been studied along with its inhibitory effect on phage protein and phage DNA synthesis.

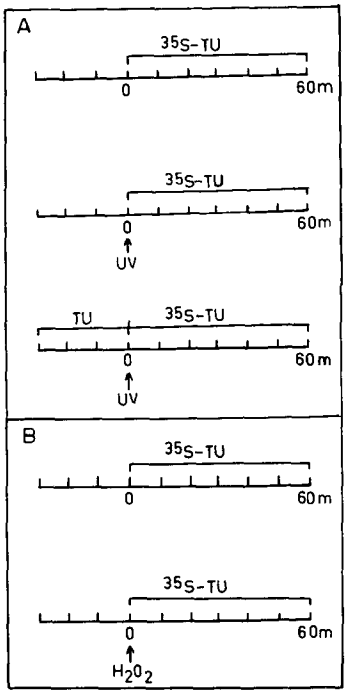
These results, which were quite unexpected, lead us to the following conclusions.

1. Ultraviolet irradiation applied in small doses to obtain induction suppresses thiouracil incorporation if the latter is added after irradiation.

2. If, on the other hand, the cells have been in contact for 30 min with unlabelled thiouracil before irradiation, then this irradiation is without effect on subsequent incorporation.

TABLE IV

INCORPORATION OF [^{35}S]THIOURACIL IN *B. megaterium* RNA

Conditions	*Incorporation into total RNA*	*Incorporation into newly synthesized RNA (calculated)*
A 	1.1 %	2.1 %
	0.02 %	0.03 %
	0.24 %	1.04 %
B	3.9 %	8.26 %
	1.13 %	4.77 %

Induction by u.v. light or H_2O_2 is always performed at time zero. [^{35}S]thiouracil (^{35}S-TU) is added immediately after induction. Incorporation is studied after 60 min. In one case, the cells were in contact with unlabelled thiouracil (TU) for 30 min preceding induction.

The amounts of thiouracil incorporated are expressed as percentage of the amounts of uracil found (first column); or as percentage of the amounts of uracil present in the RNA synthesized between time zero and time 60 min (second column).

Since it is likely that thiouracil incorporation occurs mainly or exclusively in the new RNA synthesized in its presence, the comparison between the different results is probably the most significant when the amounts of thiouracil incorporated are related to the amounts of RNA synthesized in its presence (2nd column). The rate of incorporation of thiouracil is very different from experiment A to experiment B. The data obtained after a short time are comparable only if they are taken from the same series of experiments (same strain, same conditions).

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3. H_2O_2 in amounts sufficient to induce lysis in the bacteria has only a slight effect on thiouracil incorporation.

It appears as if the ability of the cells to incorporate thiouracil during phage synthesis and the inhibitory action of the latter on this synthesis, are correlated. Indeed, thiouracil exerts an extensive effect only if added 30 min before u.v. induction, in which case incorporation takes place; it has hardly any effect if added immediately after u.v. induction, in which case incorporation into RNA is impaired. In the case of H_2O_2 induction, which is without effect on thiouracil incorporation, the effect of the latter is as extensive immediately after induction as before.

The hypothesis that the inhibitory action of thiouracil on phage synthesis is related to its incorporation into RNA deserves further notice and will be discussed later.

A better knowledge of the mode of action of u.v. light on thiouracil incorporation is not essential for the present work. We note here the different hypotheses that can be put forward. This will avoid having to recall them in the final discussion.

One hypothesis is that u.v. light determines the accumulation within the cell of a substance capable of competing with thiouracil for one or other step in the metabolic pathway leading to its incorporation. Indeed, KANAZIR AND ERRERA[16] have shown that u.v. irradiation on *E. coli* provokes an accumulation of nucleic acid precursors, including uridine diphosphate. Nevertheless, this hypothesis does not account for the importance of the pretreatment of the cells with thiouracil before ultraviolet irradiation which is necessary for post-irradiation incorporation and inhibition of phage synthesis.

Another hypothesis is that u.v. light inhibits the formation of an inducible enzyme system necessary for the incorporation of thiouracil into RNA. An inhibitory effect of u.v. light on adaptive enzyme formation has indeed been reported several times[17,18]. This hypothesis has the advantage of accounting for all the known facts and more especially for the importance of the pretreatment with thiouracil before u.v. induction.

(e) *Comparison between the effects of thiouracil and those of azaguanine on the synthesis of phage constituents in* B. megaterium

Just like thiouracil, azaguanine appears to be a selective inhibitor of the synthesis of phage constituents whether induction is brought about by u.v. light or by H_2O_2.

Nevertheless, this selective character is not so easily shown in the case of azaguanine.

Indeed, the limits of concentrations between which the selective character is apparent are far narrower. Several concentrations of azaguanine must be tried in each series of experiments and the most favorable concentrations are then chosen. In the experiments reported here these vary between 10 and 25 μg/ml.

On the other hand, the moment of addition of azaguanine is very important and it has always been necessary to add this inhibitor to the culture sufficiently early to allow a 2–3-fold increase in the O.D. in its presence before inducing the cells.

In the most successful experiments, it is possible to obtain, after induction, an increase in the O.D. of the azaguanine-treated cultures that is larger than in the absence of azaguanine but is not followed by lysis or phage production.

As a rule, the selective character of azaguanine action on phage protein synthesis

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is revealed by a decrease in phage protein synthesized four to five times greater than the decrease in bacterial protein synthesis.

Although azaguanine does not exert a direct effect on the infectious properties of completed phage particles, nevertheless the phage material synthesized in its presence is 4–12 times less infectious per weight unit than in the untreated controls. Thus azaguanine, like thiouracil, exerts its influence simultaneously on the amount of phage protein synthesized and on the structure of the infectious particles.

Equimolar amounts of guanine are capable of completely suppressing the azaguanine inhibition. As with thiouracil, reversion and phage protein production are still possible three hours after induction, that is to say at a time at which in control cultures all phage syntheses are completely finished. Thus azaguanine, like thiouracil, permits a dissociation between induction (being the modification of prophage into potential vegetative phage) and production of phage proteins.

When labelled phosphate is added together with guanine, the P of the phage DNA always has the specific activity of the inorganic phosphate. It is thereby apparent that this DNA is synthesized after addition of guanine up to which moment DNA synthesis had been inhibited by azaguanine. This inhibition is very likely to be selective since the O.D. of the culture rose from 0.110 to 0.250 in the interval between induction and addition of guanine. An increase of O.D. of this magnitude is always accompanied by a very noticeable synthesis of DNA.

Azaguanine and thiouracil exert therefore a selective inhibitory influence on phage protein synthesis and on phage DNA synthesis.

The inhibitory action of azaguanine on synthesis by uninfected bacteria has already been studied by MATTHEWS[19]. CHANTRENNE AND DEVREUX[20,21] have recently insisted on the complex effects of azaguanine in *B. cereus* in which it produces a remarkable dissociation between protein synthesis, which is inhibited, and RNA synthesis, which is enhanced.

Under the conditions reported here, azaguanine provokes a decrease in the growth rate of induced lysogenic *B. megaterium*. It affects protein synthesis (precipitable by cold 10% trichloroacetic acid) more than DNA synthesis and DNA synthesis more than RNA synthesis. These differences are slight and do not involve a profound alteration in the proportion of cellular constituents (for an overall increase of 700% in protein N in the presence of azaguanine, the ratio RNA/protein N is increased by only 20%).

The incorporation of azaguanine as azaguanylic acid into RNA of several bacteria has been reported. This is also the case with *B. megaterium*. The uninduced cells are cultured in presence of azaguanine (200 μg/ml). RNA is extracted and hydrolysed, and the azaguanylic acid isolated by paper chromatography and electrophoresis at pH 9 (SMITH AND MATTEWS[22]). The spectrum of the compound isolated was that of azaguanylic acid. It amounted to 18% of the guanylic + azaguanylic acids. From this point of view azaguanine is comparable to thiouracil.

DISCUSSION

The theory put forward in a preceding paper[5] to account for the inhibitory action of RNase on phage protein synthesis could be summarized as follows.

When RNase enters into a bacterial cell, it inhibits protein synthesis by altering the structure of RNA template molecules upon which these proteins are assembled.

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In the case of a phage-infected bacterium or an induced lysogenic one, the genetic information, initially carried by phage DNA, is transferred to newly synthesized RNA molecules. These new molecules exert the same action on phage protein synthesis that the preexisting RNA molecules exert on bacterial protein synthesis. If RNase is added to a phage–bacterium system immediately before the transfer of genetic information from phage DNA to phage RNA, the action of the enzyme will be more extensive on phage RNA being synthesized than on the bacterial RNA already present and protected within cellular structures. Under these conditions, the inhibitory action of RNase will be selectively exerted on phage protein synthesis, which is blocked, whereas bacterial protein synthesis is only slowed down.

We shall not recall here the arguments supporting this theory. We shall only examine the extent to which a similar hypothesis may be applied to the inhibitory action of thiouracil and azaguanine.

Since both thiouracil and azaguanine are incorporated in large amounts in the RNA in the present system it is very tempting to assume that the inhibition of phage protein synthesis is due to the resulting structural modification of the RNA. This idea is corroborated by the parallelism that seems to exist between the incorporation of thiouracil into RNA and its inhibitory action on phage protein synthesis, this being especially demonstrated by the fact that both are simultaneously suppressed by u.v. irradiation.

The particular fraction of RNA which we presume appears after induction and plays a role in phage protein synthesis would be entirely synthesized after addition of thiouracil or azaguanine in the above experiments. By contrast, most of the bacterial RNA is formed before the addition of the analogues.

Most or perhaps all incorporation of these analogues occurs during synthesis of RNA, which is itself practically not renewed. Owing to this fact, the fraction of RNA determining phage-protein specificity would undergo structural modification on a much larger scale than the RNA determining the specificity of bacterial proteins. The selective action of thiouracil and azaguanine on phage protein synthesis would thus be easily explained.

This interpretation has moreover the advantage of accounting for the action of the three agents, RNase, thiouracil and azaguanine in terms of the one property they have in common, their effect on RNA.

A number of objections must be considered. The first is that thiouracil and azaguanine might inhibit phage protein synthesis by being incorporated into low-molecular-weight RNA, which apparently transfers the activated amino acids onto the templates on which they are assembled into proteins (HOAGLAND *et al.*[6]).

Such a hypothesis might account for a general action of these two inhibitors on all protein synthesis but is inadequate to explain their selective action on phage protein synthesis.

The same answer can be given to another objection: thiouracil or azaguanine might interfere with the synthesis of coenzymes directly or indirectly linked with protein synthesis. Moreover, in this case, we would even have to assume that thiouracil, azaguanine and RNase provoke very similar effects although acting on different mechanisms.

A more serious difficulty results from the fact that both thiouracil and azaguanine selectively block phage DNA synthesis. Two problems present themselves.

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The first is whether this action is direct or indirect. The only direct mechanism one can think of is that thiouracil prevents uracil utilization in the synthesis of thymidylic acid[24,25].

This would seem to be ruled out by the fact that neither thymine nor thymidine suppress the thiouracil inhibition of phage production. Nor does such a suggestion account for the selective action of thiouracil on phage DNA synthesis.

However, it is possible that the action of thiouracil on DNA synthesis is indirect and exerted on a particular fraction of RNA intervening in this synthesis. The participation of RNA in phage DNA synthesis has already been postulated to account for different experimental results (STENT[25], VOLKIN AND ASTRACHAN[26]). On the other hand, proteins are also involved in phage DNA synthesis[25-28] and RNA might be required for their synthesis. This last hypothesis would explain the selective character of the inhibitory action exerted by thiouracil on phage DNA synthesis.

Finally, whatever the mode of action of thiouracil on phage DNA synthesis may be, it appears possible at first sight that this inhibitory action on DNA might in itself explain the simultaneous inhibition of phage protein synthesis. Nevertheless, phage protein synthesis can proceed either when DNA synthesis is blocked by u.v. light (WATANABE[29]), or when DNA synthesis is absent as a result of a genetic modification of the prophage (JACOB, FUERST AND WOLLMAN[30]). It seems therefore that the action of thiouracil must be independently exerted on phage DNA synthesis and on phage protein synthesis.

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