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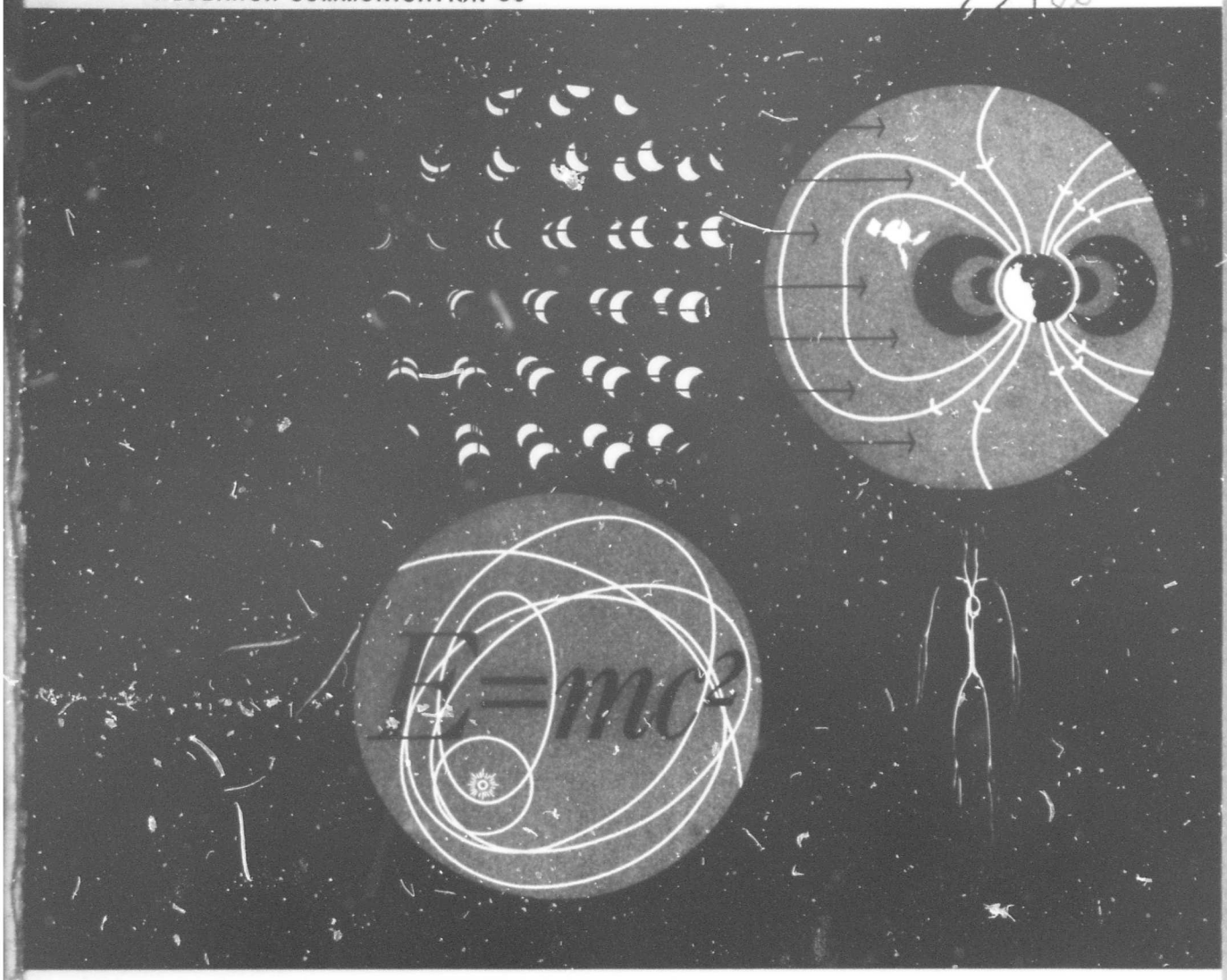


# MIBT AND BACTERIOPHAGE MULTIPLICATION

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## CREDIT

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## ABSTRACT

N-methyl isatin-β-thiosemicarbazone, a known inhibitor of pox-virus *in vitro* and *in vivo*, is without effect upon the virulent bacteriophage T4 , T4B , T4Bol , or the temperate phage λ grown in *E. coli*. At concentrations which show striking anti-viral activity against the pox-virus in tissue culture, this compound exhibits no activity in the processes of adsorption, penetration, or replication of the bacteriophage studied.

CONTENTS

## Section 1

## INTRODUCTION

Living cells have control mechanisms to ensure that proteins are synthesized in the required amounts. The rate of synthesis of a protein is partly under specific internal genetic control and partly under the general control of the external chemical environment.

From both the structural and functional standpoint, proteins are a most important chemical constituent of cells. An understanding of regulatory mechanisms controlling the synthesis of specific proteins is fundamental to an understanding of growth control in the living cell.

In a previous paper (Ref 7), we proposed that the Ehrlich ascites carcinoma cell may harbor a latent virus which allows uncontrolled synthesis of a growth factor, resulting in the neoplastic nature of the cell. Experimentally, N-methyl isatin-β-thiosemicarbazone (MIBT) killed the Ehrlich cell in vitro. It was suggested that MIBT, an analog of isatin (a plant growth regulator), killed the Ehrlich cell by acting as a corepressor with a viral repressor molecule of the virus and/or host cell to halt synthesis of a vital growth factor. Because the antiviral activity of isatin-β-thiosemicarbazone and its derivatives, such as MIBT, appears to be limited to the deoxyribonucleic acid (DNA) viruses of the pox group (Ref 16), it was also suggested that the latent virus in the Ehrlich cell may be a DNA virus. To prove the existence of a latent DNA virus in the Ehrlich cell and to locate the site of action of MIBT is a monumental

task because of the complexity of the animal cell -- physiologically and genetically. An attempt was therefore made to learn if the MIBT action can be expressed through a gene regulatory system. The system chosen for such inquiry was the bacterial cell Escherichia coli (E. coli).

E. coli has a number of DNA viruses which attack it. These viruses are called bacteriophage, or phage. The infectious particle consists of DNA, which contains all the information necessary for synthesis of a new phage, and a rigid protein coat. The protein coat is organized into a polyhedral head, which contains the DNA, and a tail. The tail has the major role in the process of infection. The phage adsorbs to the bacterium by its tail. The DNA is then injected into the bacterium, where it directs the synthesis of all phage components. This process culminates in the formation of infectious particles which are released by the lysis of the host. If MIBT has any effect on the DNA viruses of E. coli, it could be exhibited in one of several ways: (1) the phage may not be able to adsorb to the bacterium; (2) the phage may not be able to penetrate the bacterium; (3) the number of new phage released from a bacterium may be lower than the average untreated bacterium; (4) there may be no new phage released, or; (5) the new phage released may not be capable of reproducing because of an inability to adsorb, penetrate, or initiate synthesis of new progeny.

Section 2

# MATERIALS AND METHODS

## ORGANISMS

*E. coli* B, *E. coli* K 12(λ), and *E. coli* C600G were given to us by Drs. J. Holland and D. L. Wulff, University of California, Irvine. Bacteriophage T4Bo1 was sent to us by Dr. L. M. Kozloff, University of Colorado. Bacteriophage T4 was obtained from the American Type Culture Collection, Rockville, Maryland.

The following media were used in these experiments:

| | | |
|---|---|---|
| Nutrient broth: | Difco nutrient broth | 8 gm/l |
| | NaCl | 5 gm/l |
| Medium M-9: | $NH_4Cl$ | 1 gm/l |
| | $MgSO_4 . 7H_2O$ | 0.27 gm/l |
| | $KH_2PO_4$ | 3 gm/l |
| | $Na_2HPO_4 . 7H_2O$ | 11 gm/l |
| | Glucose | 2 gm/l |
| Tryptone broth: | Tryptone | 10 gm/l |
| | NaCl | 5 gm/l |
| Lambda diluent: | Potassium phosphate buffer, pH 7.0 | 0.01 Molar |
| | $MgSO_4$ | 0.01 Molar |

When solid media were used, the concentrations of agar were 15 gm/l and 7 gm/l.

## PREPARATION OF MIBT

The initial concentration of MIBT was dissolved in hot 1N-NaOH. The solution was diluted tenfold in distilled water and was neutralized 40% (v/v) by the addition of 0.1N-HCl. A total of 1.4 ml was added to 48.6 ml of Medium M-9 to give the desired final concentration. The final pH of the medium was 7.2.

## PREPARATION OF L-TRYPTOPHAN

L-Tryptophan was dissolved in hot distilled water to a final concentration of 0.2 mg/ml. The solution was cooled and brought up to the proper volume. One ml was added to 49 ml of medium to give a final concentration of 4 μg/ml.

## METHOD OF INOCULATION

To assure a constant inoculum, one ml of a stationary phase culture (Ref 12), diluted tenfold, was added to 49 ml of medium, giving a final concentration of approximately $2 \times 10^7$ cells/ml. The flask was incubated at 37°C with shaking for 18 hours. This culture was kept in the refrigerator and was used to inoculate new cultures for the week of experiments.

## PREPARATION OF PHAGE STOCK

One-tenth ml of phage stock was added to a faintly turbid nutrient broth culture of E. coli B. The broth was incubated overnight at 37°C

with shaking. The culture was centrifuged at 1470g for ten minutes to allow the cells to settle. The supernatant containing the free phage was used as a stock culture. The number of phage was determined by the procedure described below.

## ASSAY OF PHAGE

The host bacteria and virus particles were mixed in three ml of warm (46°C) 0.7% agar. The mixture was poured over the surface of a 1.5% agar plate (15 ml/plate) and allowed to harden, forming a thin layer. The bacteria grow as tiny subsurface colonies in this layer and are nourished by the deep 1.5% agar layer. Plaques, caused by lysis of bacteria, appear as clear holes in the opaque layer of bacterial growth. Each plaque arises from the infection of a bacterium by a phage, thus providing a way for enumerating the number of infectious particles present.

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## Section 3

## EXPERIMENTAL RESULTS AND DISCUSSION

In testing the possible effects of MIBT on the DNA phage of E. coli, we first examined the direct effect of MIBT on the growth of the bacteria. Medium M-9 containing MIBT at 10 μg/ml, 1 μg/ml, and 0.1 μg/ml, was inoculated with E. coli B. After 18 hours' incubation, no MIBT effect was observed. All the cultures had reached the stationary phase of growth. In addition, kinetic studies were performed. The cells were incubated at 37°C with shaking. At desired time intervals, aliquots were removed from the flasks, diluted, and plated on nutrient agar to determine the number of viable cells. An initial depression in the growth of the cells in the presence of MIBT was noted, but this was lost after two hours. This depression corresponded to a lag in the growth of the untreated cells, although the MIBT effect was slightly greater (Figure 1). It was proposed that this depression was due to inoculation of the flasks with a culture which had been refrigerated. Since cold depresses cell activity, the cells needed time to adjust to the temperature change from 4°C to 37°C and to activate metabolic systems required for growth. In this case, MIBT inhibited the activation process. This hypothesis was confirmed by allowing the cells to incubate one hour at 37°C before MIBT was added. No depression of growth was observed (Figure 2).

The effect of MIBT on the coliphage T4 was then tested. An aliquot of the stock culture was diluted in Medium M-9 containing 1 μg/ml MIBT to give $2.4 \times 10^7$ phage/ml. After fifteen minutes,

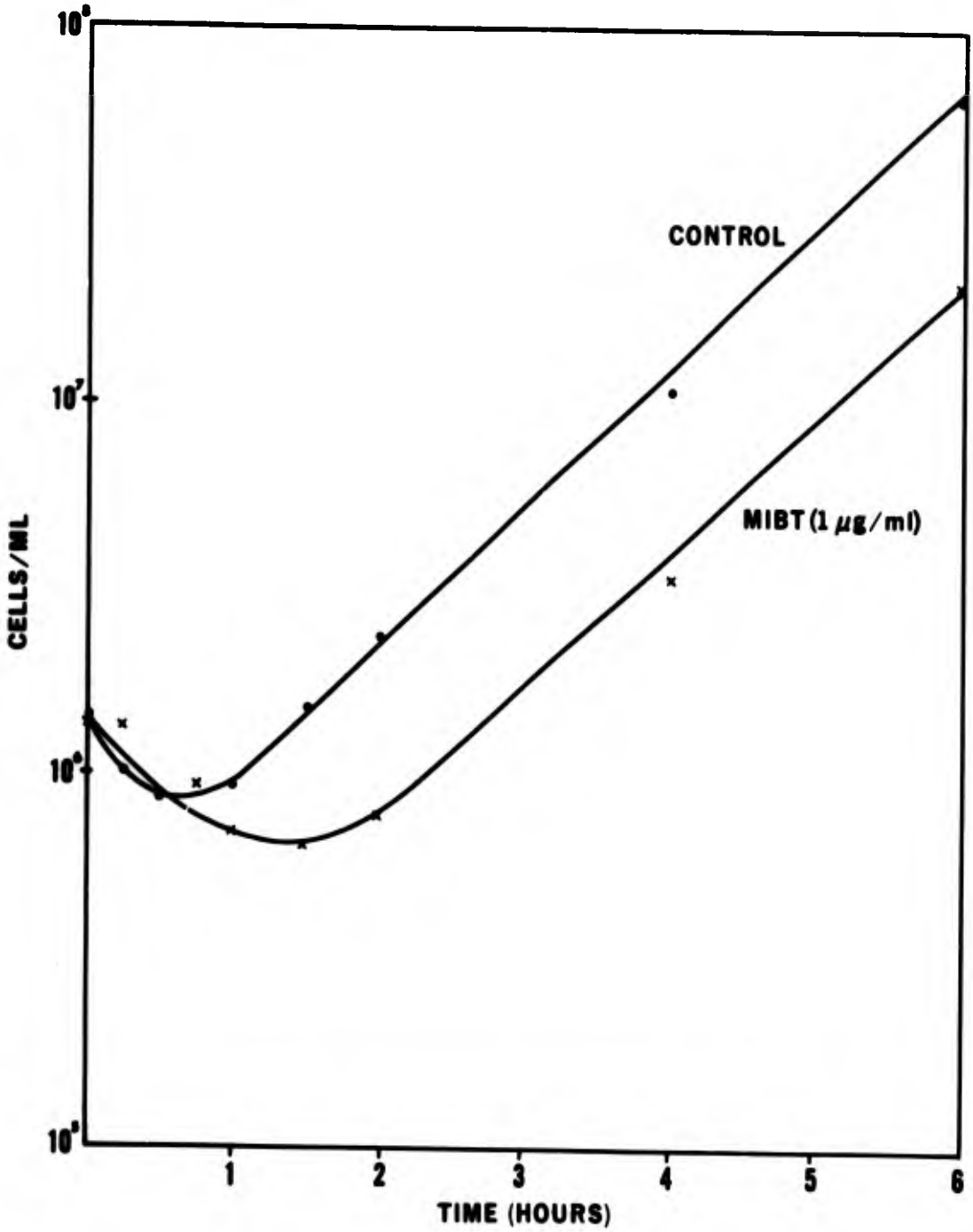


Figure 1. The effect of MIBT on the growth of E. coli B at 37°C. Inoculum was taken from a stationary phase culture stored at 4°C. The points represent the mean values of at least two experiments with each condition carried out in duplicate.

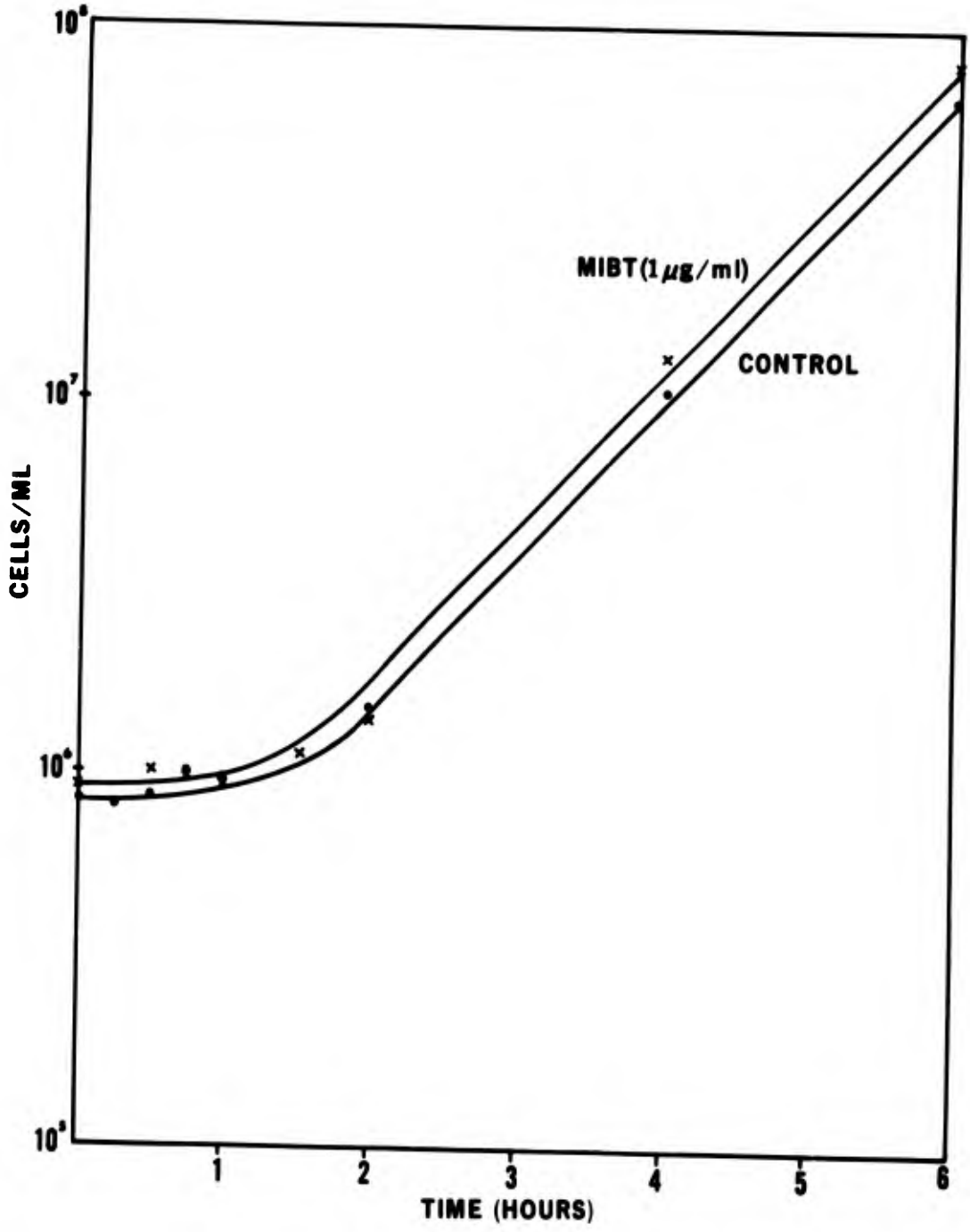


Figure 2. The effect of MIBT on the growth of E. coli B at 37°C. Inoculum was taken from a stationary phase culture at 37°C. The points represent the mean values of at least two experiments with each condition carried out in duplicate.

the phage were assayed as outlined in Section 2. No difference was observed between the number of plaques formed by the MIBT-treated phage and the number formed by the untreated controls.

Bacteriophage T4Bol , a mutant of phage T4 which requires tryptophan for attachment to the bacterium (Ref 1), was the next phage to be tested. Removal of L-tryptophan from the medium deactivates the phage and results in an inability to attach to the bacterium (Refs 10, 11, 14, and 15). This phage was selected because of a previous observation in which L-tryptophan was found significantly to inhibit the cytocidal action of MIBT on the Ehrlich ascites cells (Ref 7).

The T4Bol stock culture, prepared as previously described, was diluted in M-9 to a concentration of approximately $1 \times 10^3$ phage/ml . The phage were allowed to deactivate for ten minutes. A 0.2 ml aliquot was then added to log phase bacterial cells in Medium M-9 containing MIBT at 4 μg/ml . Five-tenths ml was assayed for plaque-forming ability. MIBT appears to have no direct effect on the phage, since the number of plaques on the control and experimental plates were similar.

In an attempt to determine whether MIBT influences attachment and/or penetration due to a change in the host's cell wall, E. coli B was grown to stationary phase in Medium M-9 containing three concentrations of MIBT (40 μg/ml, 4 μg/ml, and 0.4 μg/ml). These cultures were used to inoculate new flasks with their respective MIBT concentrations. The new cultures were grown at 37°C with shaking to approximately $1 \times 10^8$ cells/cc. At this point, 0.01M KCN

was added to stop the cell processes (Ref 2). L-tryptophan (4 μg/ml) and T4Bo1 ($1 \times 10^7$ phage/ml) were also added to the cells. The mixture was incubated at room temperature, and 4.5 ml aliquots were removed at 10 minutes, 20 minutes, and 30 minutes. Chloroform at 0.5 ml was added to lyse the cells and halt any further attachment (Refs 3 and 8). The lysate was then assayed for free phage. If MIBT interfered with adsorption, the plaque count would be greater in the MIBT-treated flasks than in the untreated control. No difference was observed.

The possibility of MIBT affecting the growth of phage inside the bacterium was investigated by growing E. coli B cells to approximately $1 \times 10^8$ cells/ml in Medium M-9 containing L-tryptophan (4 μg/ml) and MIBT (40 μg/ml and 4 μg/ml). One set of the MIBT flasks was inoculated with cells which had been grown in MIBT for one week prior to the experiment, thus allowing the cells to complete any adaptation necessary before adding phage. T4Bo1 was added to a final concentration of $1 \times 10^7$ phage/ml. The cultures were grown overnight at 37°C with shaking. Aliquots were removed from each flask, and chloroform was added (20% v/v) to lyse the cells. The lysate was assayed. No difference was observed between the control and experimental flasks.

The next experiment was performed to determine whether a saturation of the system with MIBT would produce any kind of effect. The cells and phage used in this experiment had previously been grown in the presence of MIBT. Nutrient broth was inoculated with E. coli B and grown to approximately $1 \times 10^7$ cells/ml. T4Bo1 was added to a final concentration of $1 \times 10^4$ phage/ml. The flasks were incubated

at 37°C with shaking. Aliquots were removed at zero time, 1 hour, 2 hours, 3 hours, and 24 hours. Chloroform was added, as before, to lyse the cells; and the lysate was assayed. No effect was observed

MIBT appears to have no effect on the virulent DNA bacteriophage of E. coli. It was felt that perhaps this was due to the fact that the phage used have no intimate relationship with the host cell, but merely use the host as a supplier of the necessary building blocks for new phage synthesis and as a protection from any detrimental external factors during this period of synthesis. This may not be the case with the proposed latent virus in the Ehrlich ascites cell. In fact, it is more likely that the latent virus has a very intimate connection with the Ehrlich cell and its life cycle. This type of "symbiotic" relationship, termed lysogeny, also exists between bacteria and their temperate phage (Ref 9).

Each bacterium of a lysogenic strain harbors and maintains a non-infective structure called a prophage, which endows the cell with the ability to give rise to infective phage without intervention of exogenous phage particles. The prophage is perpetuated as part of the bacterial hereditary apparatus. In a small fraction of a population of growing lysogenic bacteria, the prophage becomes "induced" to produce a crop of infective phage (Refs 5, 6, and 13). Prophage induction leads to death and ultimate lysis of the cell. Induction can occur spontaneously or can be produced through use of physical and chemical treatments such as ultraviolet light, x-rays, hydrogen peroxide, or nitrogen mustard (Ref 9). The events that ensue in the lysogenic cell after induction of its prophage are entirely analogous to those of the vegetative phase of phage development following

infection of a sensitive bacterium with a free phage particle. There first occurs an eclipse during which no infective phage can be detected in the cell but during which the DNA and protein of the progeny are synthesized. Later, about halfway through the latent period, these various phage precursors begin to combine to constitute mature, infective progeny viruses. These progeny appear in the culture medium after spontaneous lysis of the bacterial cell at the end of a latent period.

Experiments were performed to determine whether MIBT affects a typical temperate phage of E. coli. MIBT may act in several ways: (1) it may induce the prophage, (2) it may prevent induction, (3) it may alter the number of phage progeny produced by induction, or (4) it may produce altered phage progeny which are not capable of further reproduction. The first possibility would result in an increase in the number of plaques formed by the MIBT-treated cells. The last three possibilities would result in a lower plaque count than that of the nontreated, control lysogens.

The lysogen E. coli K-12 ($\lambda$), which carries the lambda prophage, was grown overnight at 37°C with shaking in tryptone broth, with and without 4 $\mu$g/ml MIBT. Aliquots of each of these stocks were diluted and inoculated into fresh tryptone broth with and without 4 $\mu$g/ml MIBT to give a final concentration of $1 \times 10^7$ cells/ml. These cultures were grown up to $1 \times 10^8$ cells/ml. Aliquots were spun down at 8700g for ten minutes and were resuspended in an equal volume of lambda diluent. One ml of each culture was added to separate petri dishes (60 × 15 mm). The cells were irradiated with a total dose of $2.4 \times 10^4$ ergs/cm$^2$ of ultraviolet light. The

irradiation and subsequent manipulations were carried out under yellow light to prevent photoreversals (Ref 4). After irradiation, the cells were put into 4 ml tryptone broth with, and without, 4 μg/ml MIBT and were incubated in a 37°C water bath for 60 and 90 minutes to allow development of the prophage into infective virus particles. At the designated times, chloroform was added to the broth to lyse the cells and release any intracellular phage particles. The lysate was diluted and assayed with an indicator strain (E. coli C600G) which was sensitive to the lambda phage and nonlysogenic. No difference was observed in the number of plaques formed by the MIBT-treated and untreated cultures. MIBT apparently has no effect on the lysogenic DNA phage of E. coli.

It is obvious from these experiments with the virulent bacteriophage T4Bol and the temperate λ phage that the anti-viral agent and plant-growth regulating analog MIBT has no discernible effect on these viruses or their relationship to the host E. coli cell. Although compounds exhibiting activity against animal viruses are not necessarily capable of activity against a bacterial virus, the likelihood of such activity from MIBT was interesting enough to investigate. The possibility does remain that other bacteriophage not examined here might be regulated in some manner by the presence of this plant-growth regulating analog. There is the argument, however, that the mechanisms for plant and animal growth control are fundamentally different from those obtaining in the bacterial cell or the bacterial virus. These differences, which relate to the stability and heterogeneity of mRNA, as well as to the regional separation of transcription and translation, suggest that regulation of enzyme synthesis in animal and plant cells may operate at levels of translation as well as transcription of mRNA.

If this is the case with the neoplastic Ehrlich cell, a genetic repressor at the site of protein translation, namely the ribosome, would be devoid of activity at a DNA site of transcription, and hence no regulatory activity against a virulent or temperate bacteriophage would be expected.

## Section 4

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13. ABSTRACT

N-methyl isatin-β-thiosemicarbazone, a known inhibitor of pox-virus in vitro and in vivo, is without effect upon the virulent bacteriophage T4, T4B, T4Bol, or the temperate phage λ grown in E. coli. At concentrations which show striking anti-viral activity against the pox-virus in tissue culture, this compound exhibits no activity in the processes of adsorption, penetration, or replication of the bacteriophage studied.

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