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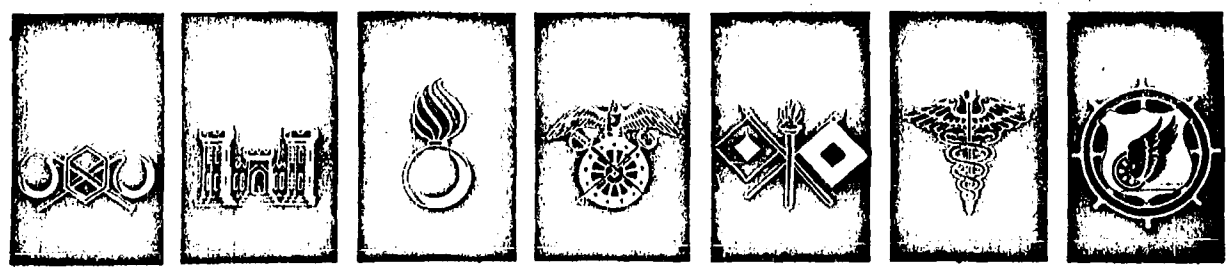


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GEN SESSION 3

TITLE: Effects of Mustard Gas (H) on T2 Bacteriophage and DNA Synthesis

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ABSTRACT: The reaction of only a few molecules of H with T2 bacteriophage both in vitro and in vivo causes the virus to rapidly lose its ability to reproduce and to initiate viral DNA synthesis. Most of the lethal reactions are localized in the genetic material (DNA). Individual genetic markers as well as the ability of the virus to initiate the biosynthesis of new enzymes are about 5-10% as sensitive to H as is the ability of the particle to reproduce. It is suggested that DNA replication and DNA function may have similar sensitivities to the agent.

The effect of H on the in vitro synthesis of DNA was also investigated. The only component of the reaction significantly affected by H is the DNA primer. Although DNA synthesis eventually recovered when H-treated primer and purified polymerase are employed, considerably less recovery took place with H-treated primer and impure preparations of polymerase. The later enzyme preparation was seriously contaminated with phosphodiesterase and other nucleases which degraded H-treated DNA primers prior to recovery. Secondary degradation of DNA was also observed with H-treated *E. coli*. It is suggested that the nuclease-mediated, secondary degradation of H-damaged DNA is responsible for a large segment of the H-toxicity to cells.

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EFFECTS OF
MUSTARD GAS* ON T2 BACTERIOPHAGE AND DNA* SYNTHESIS

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The investigation of mustard gas has covered more than half a century and has engaged the efforts of many investigators in many laboratories. Initially, interest was directed to the vesicant and protoplasmic poisoning actions of this compound. But in the past 15 years, the scope of its interest has been broadened considerably by the discovery that H and other alkylating agents are mutagens^{1,2} and anticarcinogens^{3,4,5} and have biological actions similar to those produced by ionizing radiations. In spite of repeated observation and extensive review^{5,6,7,8,9} of these effects, the mechanism of cellular toxicity of H is still unknown.

While the elucidation of the mechanism of action of H is an enormous task, it must precede the logical development of a therapy for the mustard burn and the reversing of systemic intoxication with the agent, problems of continuing concern to the Chemical Corps. Although there are certain gross similarities between the vesicant action of H on skin and the radiomimetic behavior of H on other cells and organisms, much more must be learned before a valid correlation between the two can be made. With the goal of augmenting the limited knowledge concerning the radiomimetic actions of H, the authors undertook the present investigation of the mechanism of inhibition of T2 bacteriophage by H.

* Mustard gas ($\text{Cl-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-Cl}$) is also known as sulfur mustard and yperite, and it will be denoted in the present report by the symbol H.

DNA--deoxyribonucleic acid.

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The present discussion is concerned mainly with matters of general importance for elucidating the mechanism of the inhibition of T2 bacteriophage by H and also **includes** some observations on the toxicity of the agent for DNA synthesis in vivo and in vitro.**

Summary of Effects of H on T2 Bacteriophage

The effects of H on some properties of the T2 bacteriophage system are graphically summarized in figure I. To provide a more meaningful basis for comparing the sensitivities of H of the various properties, the information in figure I is divided into two categories: phage particles that were H-treated before infection (dashed lines) and after infection (solid lines). This distinction is necessary because extracellular H-treatment produces secondary damages that prevent some of the particles from penetrating susceptible hosts and limits the progress of the intracellular events of the infective cycle one may wish to investigate. Although some of these complications are overcome when particles are exposed to H after adsorption (when many of the H-sensitive steps associated with penetration are already completed), the interpretation of results obtained with phage-bacterium complexes is more difficult because of the concomitant exposure of the host cell. Recognizing that exact quantitation is not possible in every instance, the authors feel that certain meaningful conclusions nevertheless can be drawn.

By examining the relative slopes of the inactivation curves in figure I, one will note the following features with respect to the action of H on the T2 bacteriophage system:

1. When singly infecting bacteria, the abilities of T2 bacteriophage to reproduce (curves 1 and 3) and to synthesize viral DNA (curve 2) are equally inhibited by H and are the most H-sensitive properties found.

2. Most of the properties of the virus particle directly or indirectly attributable to functions of viral protein are inhibited by H to similar extents (curves 4, 5, 6, 7, and 8). An exception is the ability of the particle to adsorb on susceptible hosts (curve 15). However, these properties are much less sensitive than the ability of the virus to reproduce (curve 1).

** For experimental details the reader is referred to CRDL Special Publication 2-45, October 1961, "On the Mechanism of Inhibition of T2 Bacteriophage by Mustard Gas."¹⁰ Reprints are available from the author on request.

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3. The ability of singly infected cells to reproduce T2 phage is much less sensitive to H when the agent is administered 9 minutes after infection (curve 9) than when applied at an earlier stage (curve 3).

4. Limited mainly by the action of H on the cell's capacity to support phage development (curve 12), the abilities of multiply infected cells to reproduce T2 bacteriophage (curve 10) and to synthesize viral DNA (curve 11) are fairly resistant to H.

5. The sensitivity to H of a genetic marker, i.e., the ability of an H-inactivated parental particle to contribute the marker to progeny in a mixed infection with viable carrier phage, is only about 8% of the sensitivity of the particle to reproduce (compare curves 13 and 3).

6. The ability of an H-inactivated particle to initiate the biosynthesis of an enzyme characteristic of the infected cell is only about 5% as sensitive to H as is the ability of the particle to reproduce (compare curves 14 and 3).

The possible meaning of these and some other findings and their bearing on the mechanism of action of H will now be discussed.

The Site of Action of H in T2 Bacteriophage

The current findings support the idea that the most H-sensitive target in T2 bacteriophage is viral DNA and that protein functions are also affected. The evidence is the following:

1. Particles are rapidly inactivated by low doses of H either when free or when preadsorbed on host cells (figure I, curves 1 and 3), indicating a relatively small effect on protein functions. This conclusion was reinforced by determining that individual properties associated with the viral protein such as killing ability, injection of DNA, adsorption, etc., are much less sensitive to H than the ability of the virus to reproduce (figure I, curves 4, 5, 6, 7, 8, and 15).

2. At an H dose corresponding to 1 phage-lethal-hit, a minimum of 45 molecules of H react with viral DNA and approximately 30 molecules alkylate viral protein (table I).

3. H-treated DNA is unstabilized to heating at 74°C, a temperature that does not affect normal DNA.¹⁰

4. The capacity of the bacterium to support phage reproduction and to synthesize viral DNA is not especially sensitive to H (figure I, curve 12), showing that the enzymic machinery of the host is not seriously impaired by the agent.

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5. Vegetative phage becomes abruptly resistant to H after 6 to 9 minutes of metabolism (figure I, curve 9), resembling findings with other agents that are thought to act on viral DNA (ultraviolet or X irradiations, decay of incorporated P^{32} , etc.).

6. Phage particles that contain H-treated DNA but functional protein are noninfective. Such particles are in many ways similar to particles that have been exposed to H while intact.¹⁰

7. H produces damages that are localized in the genetic material of T2 and T4 bacteriophages. An individual marker is about 1/5 as sensitive as is the ability of the particle to reproduce (figure I, curve 13).

8. H-inactivated T2 particles can be made to multiplicity reactivate with high efficiency, showing that a genetic reconstitution process can be invoked to reconstitute a viable genome from undamaged portions surviving the H-treatment.¹⁰

9. H significantly modifies the priming activity of DNA in the in vitro DNA synthesizing reaction catalyzed by E. coli polymerase (table II). Since DNA synthesis is also inhibited by H in vivo (figure I, curve 2), the finding provides independent evidence that H acts on a "template" function of DNA.

The chemical reaction(s) of H with DNA that may actually be responsible for toxicity of the agent is(are) not clear at the present time. Although alkylations of guanine, adenine primary phosphate groups, etc., have been reported,^{11, 12, 13, 14, 15} Rutman, et al.,¹⁶ suggest that the extent of alkylation probably does not bear any quantitative relation to the biological effectiveness of mustards and that the type of reaction appears to be more important than the extent. This view is supported by the author's finding (table I) that, with an H dose sufficient to produce one phage-lethal-hit, there occur 40 or more combinations per phage equivalent of DNA. It is probable that only one of these combinations is responsible for toxicity and that, consequently, most of the other reactions are nonlethal, although some of them may "mutate" the DNA molecules.

Polyfunctional vs. Monofunctional Agents

Polyfunctional mustards of both the sulfur and nitrogen types are several orders of magnitude more toxic to T2 bacteriophage than the monofunctional mustards, supporting a cross-linking mechanism.¹⁰ Aside from considerations of dose, the two agent types appear to differ also in the mechanism by which they inhibit phage reproduction. Polyfunctional agents cause only very localized lesions in the phage genome and leave the greater portion of the genome intact, available for rescue by genetic recombination processes such as multiplicity and cross-reactivations.¹⁰ On the other hand, some

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single-armed agents inactivate phage particles largely by inhibiting protein function that are essential for infection. A single-armed mustard that acts in a different way is β -chloroethyldiethylamine (semi-HN). While still retaining its preferential action on viral DNA and in this respect resembling the action of a polyfunctional agent, it produces lethal damages to phages that are, however, not multiplicity reactivatable (unpublished observation). This finding suggests that the lethal damages produced by semi-HN, in contrast to those produced by the polyfunctional agents, may not be highly localized within the genetic structure and that viral DNA might be inactivated only after extensive reactions involving the greater portion of the genome.

Although cross-alkylation of DNA appears to be an important first step in inhibiting the reproduction of T2 bacteriophage, a different mechanism is probably responsible for other biological actions of the mustards. Single-armed agents are very efficient as mutation producers,^{1, 2, 17} and also retain vesicant properties.¹⁸ There is limited evidence that some of these effects may also originate from an initial attack of the agent on DNA. Thus one could suppose that a single alkylating attack (by a single-armed agent or by a half-reacted double-armed agent) may modify a base such as guanine, altering its base-pairing characteristics, and in this way producing a mutation.^{15, 17} A single alkylation of a base in DNA probably does not affect the structural integrity of the macromolecule but merely modifies the genetic information, whereas a single cross-link would produce a profound change in DNA, putting the molecule out of action and finally killing the organism.¹¹

Unfortunately, little can be said at present regarding the underlying basis for the vesicant action of the mustards and, indeed, a great deal more information than is now available is required to relate a particular biological effect of H to a specific reaction of H with cellular components.

Interaction of H-treated DNA with Biologically Important Proteins

An indication that H affects the structural integrity of the DNA molecule is the observation that H-damaged DNA loses its ability to associate with various types of proteins. The evidence that protein attachment to DNA is impaired by H was derived from 3 different experiments, and a biologically important process was inhibited in every case. The results of these experiments were:

1. When viral protein synthesis was resumed following treatment with a moderate dose of H of phage precursor DNA (formed in the absence of protein synthesis), intact particles were not formed.¹⁰ In similar experiments employing ultraviolet irradiation instead of H, Tomizawa¹⁹ observed efficient production of finished phages, although most of them were noninfective. These results demonstrate that,

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although UV-irradiated DNA can, H-treated DNA cannot, interact productively with viral protein.

2. When singly infected cells are exposed to H 9 minutes after infection, the ability of the virus to reproduce is more resistant to H than when the agent is administered at an earlier time (compare figure I, curves 9 and 3). Uchida and Stent^{20,21} obtained a similar result with phages that were killed by decay of incorporated P^{32} , and they showed that the acquisition of resistance at the ninth minute might be due to the association with viral DNA of a newly synthesized protein. It is probable that a similar mechanism is responsible for protecting viral DNA from being lethally damaged by H when the agent is applied at the ninth minute. However, no protection is afforded at the earlier times when the protective protein has not yet been synthesized. The question of whether H-inactivated particles are capable of synthesizing the protective protein has not been answered, but it should be recalled that such particles do initiate the biosynthesis of several enzymes (figure I, curve 14). Even if the protective protein were similarly synthesized, it might fail to associate with H-damaged DNA and thus fail to protect.

3. The ability of DNA to prime the in vitro synthesis of DNA that is catalyzed by E. coli polymerase is significantly modified by H (table II, also see reference 10). When H-treated DNA was used as primer, DNA synthesis was inhibited for a time but was then renewed at a rate similar to that observed with untreated DNA. The findings suggest that H-treated DNA has a lower affinity for polymerase than does normal DNA. If it should develop that the association of DNA and polymerase are also impaired by H in vivo, the mechanism of the temporary inhibition of DNA synthesis would be explained. Although the DNA synthesis finally recovers, cells would be killed because of "unbalanced growth."²²

Some Genetic Consequences

A significant proportion of the H-induced lethal damages to T2 bacteriophage appear to be located in the genetic structure of the particle. When particles are exposed to the agent before adsorption, only a maximum of 30% of the lethal damages are sustained by viral protein, some of which may prevent the particle from penetrating the host bacterium (figure I, compare curves 1 and 8). When particles are treated after adsorption, only a very small fraction of the lethal damages result in inhibition of the cell's capacity to support phage development (figure I, compare curves 3 and 12). These results suggest that the majority of the lethal damages to T2 bacteriophage probably occur in the viral genome, and the author was able to confirm this conclusion by more direct evidence. Results from marker rescue experiments show that H inactivates individual genetic markers and that the sensitivity of a given marker is about 8% of the sensi-

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tivity of the whole genome (figure I, compare curves 3 and 13). Because several markers at different sites within the genome are about equally affected, H seems to produce genetic damages randomly along the chromosomal structure. The highly localized nature of the genetic lesions produced by H means that inactive particles contain considerable stretches of undamaged genetic material, a notion that is compatible with the observed high efficiencies of multiplicity and cross reactivations.¹⁰ It should be stressed that such reactivations probably are not due to chemical reversal of H damages but rather are due to genetic mechanisms that are capable of reconstituting a viable genome from undamaged genetic sections derived from several parents. A similar mechanism can also reactivate particles that have been killed by ultraviolet or X irradiations, agents that produce their lethal damage by different reactions.²³

The ability of H to produce mutations was not investigated in the present work. However, in a recent report Bautz and Freese¹⁷ indicated that the ethylation by alkylating agents (presumably of the 7-N position of guanine) of DNA from T4 bacteriophage increased the mutation rate. These investigators suggested that the eventual elimination from DNA of a base by secondary hydrolysis of the N-glycosidic bond might have been responsible for the mutation. They further hinted that reactions with primary phosphoryl groups of the DNA backbone probably do not have mutagenic consequences for they would fracture the molecule by breaking the sugar phosphate bond that links two adjacent nucleotides. Although such a mechanism might be involved when lethal DNA damages are produced by the decay of incorporated P³², it has not been demonstrated to be mutagenic. It is interesting that Bautz and Freese found single-armed alkylating agents to be quite potent as mutagens while the author showed that similar monofunctional mustards are not nearly as toxic to these same particles as polyfunctional compounds. It therefore appears that alkylating agents can modify the DNA molecule in at least two distinct ways: one type of reaction, e.g., a single alkylating attack on guanine, leads to a point mutation; and the other type of reaction, e.g., a crossalkylating attack by a polyfunctional mustard, culminates in a lethal event and entirely inactivates the affected gene.

Some Metabolic Consequences

1. The effect of H on the host cell and its capacity to support phage development.

The most H-sensitive properties of *E. coli* B bacteria are the abilities of cells to reproduce and to synthesize DNA. However, H-treated bacteria are still able to increase in size,²⁴ synthesize RNA and protein,²⁵ carry out respiratory metabolism, and produce adaptive enzymes.²⁶ Coupled with the observed inhibition of DNA synthesis (which may be only temporary²⁵), the otherwise normal metabolism apparently leads to an imbalance, which may ultimately

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kill the cell by "unbalanced growth."²² The death-by-unbalanced-growth theory is compatible with the author's recent finding (unpublished) that significantly larger bacterial survivals can be obtained by permitting H-treated E. coli B to metabolize at a slower rate. It is suggested that cells have a limited ability to repair or circumvent some (although not all) of the lethal H damages.

Although sterilized by H and unable to synthesize bacterial DNA, E. coli bacteria remain capable hosts for T2 bacteriophage, supporting viral reproduction and synthesizing almost normal amounts of viral DNA.¹⁰ The findings confirm similar observations by Herriott²⁴ and show that the enzymic machinery of the host cell is not damaged sufficiently by H to prevent it from making its necessary contributions to sustaining the viral infection. Exactly how T2 bacteriophage is able to overcome the block in the DNA synthetic mechanism is not known, but it should be recalled that the virus supplies a new set of functional genes, which establish new synthetic pathways especially geared to the synthesis of phage-specific DNA.²⁷

2. The inhibition by H of DNA synthesis in vivo and in vitro, and its bearing on the toxic mechanism.

The inhibition of DNA synthesis by H may be the metabolic block primarily responsible for the cytotoxicity of the agent. In bacterial cells, even a temporary interruption of the process could lead to the unbalanced growth situation, culminating in the eventual death of the organism.²² In the T2 bacteriophage system, the ability of the virus to reproduce itself when singly infecting a susceptible host and the capacity of the virus to initiate the synthesis of viral DNA are equally sensitive to H (figure I, compare curves 1 and 2), possibly indicating a causal relationship between these two properties. It is to be noted, however, that the infectivity of the particle and viral DNA synthesis become more resistant to H when the agent is administered at a later stage of infection (figure I, curve 9) or when cells are infected with more than one particle (figure I, curve 11). Although the mechanism presently is not understood, resistance is in some way associated with maintaining the integrity of the parental genome, which may become associated at about the ninth minute with a protective protein or which,^{20,21} once damaged by H, can be reconstituted by multiplicity reactivation. The in vivo evidence therefore leads to the conclusion that H inhibits DNA synthesis by damaging the "DNA template", and an intact template is need for DNA replication.

H also affects DNA synthesis in vitro, a reaction that is catalyzed by E. coli polymerase and which requires a DNA primer.¹⁰ The demonstration (table II) that the priming ability of DNA is the most H-sensitive component of the reaction confirms the notion that the agent interferes with a template function of DNA. The lag before the in vitro DNA synthesis finally renews is strikingly

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similar to that observed in the proposed reconstitution of an active genome by multiplicity reactivation. The results suggest that the effects of H on the in vitro synthesis of DNA are very similar to the effects observed in vivo and therefore reinforce the belief that a molecular basis for the cellular toxicity of H is the ability of the agent to damage DNA.

Another possibility for explaining the inhibition of DNA synthesis by H would be that the agent, by damaging gene function, prevents the formation of enzymes critical to DNA synthesis. (It should be recalled that the catalytic activities of such enzymes are not especially sensitive to H.¹⁰) The author demonstrated¹⁰, however, that the ability of T2 particles to initiate the biosynthesis of one such critical enzyme (phage-specific deoxyguanylate kinase) is only 5% as sensitive to H as is the infectivity (figure I, compare curves 3 and 14). This implies that most inactive particles probably are not prevented from carrying out the gene functions associated with enzyme formation. In spite of this discovery and even if H-inactivated particles were similarly capable of forming other enzymes, one could not rule out entirely the possibility that DNA synthesis might be inhibited by a damage to the enzyme-forming apparatus. Thus, one could suppose that inactive particles harbor damages at different genetic sites, remain capable of synthesizing most enzymes, but fail to produce the complete set of enzymes need for DNA synthesis. Such a mechanism might be invoked to explain the phenomenon of multiplicity reactivation, which restores DNA synthesis. In multiply infected cells, in contrast to singly infected cells, several inactive particles may pool their enzyme-forming potentials to establish a complete enzyme set, and the inhibited viral DNA synthesis recovers (figure I, curve 11).

Current studies are designed to obtain more information on the effect of H on enzyme biosynthesis. Of special concern is the ability of H-inactivated T2 particles to initiate the synthesis of phage-specific polymerase,²⁷ an enzyme peculiar to the infected cell and primarily responsible for catalyzing the viral DNA synthesis.

3. The sensitivity to H of phage-bacterium complexes at different stages of the latent period.

Four major hypotheses (which are not mutually exclusive) may be offered to explain the development of resistance to H at the ninth minute after single infection: (1) the parental DNA, in matings with progeny DNA, may reconstitute a viable genome by multiplicity reactivation; (2) the parental DNA may associate with a newly synthesized protein, which might protect the DNA from being lethally damaged by an otherwise lethal agent; (3) the genetic information originally carried by the sensitive parental DNA may be transferred to nonsensitive structures, thus making the parental DNA dispensable for sustaining the infection; and (4) all H-sensitive

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functions required for DNA replication may be completed by the ninth minute, so the agent is no longer lethal when administered at this time, i.e., all enzymes necessary for viral DNA synthesis are already present at this stage of the latent period.

Although there is some experimental evidence to support each of the 4 proposals, one cannot presently attribute the 9-minute resistance to any one, or any combination, of them.

Multiplicity reactivation is an efficient process when multiply infected bacteria are exposed to H at a very early stage of development, but has not yet been shown to occur when the agent is administered at the ninth minute. Also, whereas a normal multiplicity reactivation process involves matings between several parental particles, in the present instance matings would have to be between a parental and one or more progeny genomes or between several of the progeny. It should be stressed that these are unsolved problems and do not eliminate multiplicity reactivation as a possible agency responsible for making monocplexes resistant to H at the ninth minute.

The formation by infected cells of a protein capable of protecting parental T2 particles from being killed by the decay of incorporated P^{32} has been inferred from studies by Uchida and Stent.^{20,21} These investigators also demonstrated that this protein protects specifically only those parental particles responsible for its formation. It does not protect secondarily infecting particles they would have to synthesize their own protein to become resistant to P^{32} decay. These results may mean that such protein becomes physically associated with the DNA of the parent particle. It may exert its protective action by bracing the backbone of parental DNA and thus preventing a complete scission of the macromolecule by P^{32} decay or, possibly, by other agents. Another possibility is that the protein protects DNA by separating the complementary strands (preparatory to DNA synthesis, which begins at approximately this time), thus preventing simultaneous damages from occurring at complementary sites of the duplex (i.e., a damage to only one of the two complementary sites may not be lethal since the genetic information is still available on one strand). To test some of these notions, current investigations are under way in this laboratory to determine if polymerase can protect the ability of DNA to prime the in vitro synthesis of DNA from being damaged by H.

Little can be said regarding the proposed transfer of genetic information from the H-sensitive parental DNA to H-resistant molecules. The most logical candidate substance would be RNA which, because of its structural differences from DNA, is more resistant to H.²⁸ Although the synthesis of "phage-specific RNA," which mimics the base composition of parental DNA, has been reported²⁹ and is apparently required for phage reproduction, such RNA has not been

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shown to be a template for the synthesis of progeny DNA. Recent in vitro findings also point to a definite role of DNA in the synthesis of RNA; but, again, a requirement for an RNA primer for DNA synthesis has not been found. These results suggest that phage-specific RNA represents an aspect of DNA function rather than DNA replication, and it may be an important intermediate in the translation of genetic information from parental DNA into the amino acid sequence of a protein or enzyme. Proteins that are generally more resistant to H and similar agents than are the nucleic acids are not well suited for carrying the genetic code and will not be considered here as an intermediate template in the synthesis of progeny DNA. However, the denial of a genetic role for proteins should in no way minimize the importance of these molecules as catalysts for DNA or, perhaps, even as having a function in the determination of the size of the DNA polymer.

An intriguing possibility is that the resistance at the ninth minute may be due to the fact that all the enzymes necessary for viral DNA synthesis are present by this time. When further protein synthesis is inhibited by chloramphenicol added at the ninth minute, subsequent DNA synthesis is not appreciably affected although the antibiotic does prevent the initiation of DNA synthesis when given at an earlier time.³⁰ The similarities in the time spectrum relative to the action of chloramphenicol (an agent that inhibits protein synthesis) and of the radiations or H (agents that affect DNA directly) lead one to suspect that the resistance of monocplexes to H is also related, in an as yet obscure manner, to an inhibition of the biosynthesis or function of one or more of the important proteins or enzymes required for viral DNA synthesis. In this connection it was found, however, that most H-inactivated T2 particles retain their ability to initiate the biosynthesis of several of such enzymes.¹⁰ Current studies are designed to investigate further the biosynthesis of proteins and their importance for the development of the H-resistance at the ninth minute.

Regardless of mechanism, the discovery that not all stages of phage development are equally sensitive to H emphasizes the importance of studying the action of a nucleotoxic substance in a synchronized system. Perhaps some of the conflicting results in the literature relative to the effects of H in other systems are due to a failure to work with synchronized cell populations.

4. The ability of H-inactivated T2 particles to initiate the biosynthesis of enzymes and the importance of this phenomenon for relating gene function to gene replication.

Following infection of E. coli B with T2 bacteriophage, several new enzymes appear that are not present in uninfected cells.²⁶ It appears likely that these enzymes represent products of the

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phenotypic function of viral DNA. Even in single infection (i.e., in the absence of multiplicity reactivation) H-inactivated phage are capable of initiating the biosynthesis of several of these enzymes. For example, when enzyme activity was assayed 15 minutes following infection, the ability of the particle to synthesize phage-specific deoxyguanylate kinase³¹ was never found to be greater than 5% as sensitive to H as were the abilities to reproduce and to synthesize DNA (figure I, compare curves 2, 3, and 14). Whether or not these enzyme assays quantitatively reflect the biosynthetic potentials of H-inactivated virus, it is apparent (assuming that these enzymes are products of gene function) that gene function precedes gene replication and may even be manifested in its absence.

The separation of the 2 most important attributes of genetic material, gene function and gene replication, may explain the nucleotoxicity of H. To wit, most H-treated cells, although sterile, continue to grow in size, synthesize protein and RNA, and carry out almost normal respiratory activities. At least some of these metabolic processes are directly or indirectly controlled by the genetic material. While a single H damage to DNA is sufficient to inhibit the reproduction of all the genetic material, thus preventing cell multiplication, the remaining undamaged portions of the genome continue to carry out their phenotypic functions, providing the cell with almost all the factors needed for growth.

The finding that gene function precedes gene replication (all enzymes needed for viral DNA synthesis are present by the ninth minute) may mean that double-stranded DNA is responsible for phenotypic action. Contrariwise, soon after parental DNA becomes single-stranded (at about the ninth minute), DNA synthesis begins and enzyme biosynthesis ceases. The possibility that enzyme biosynthesis is therefore controlled by the state of the parental DNA is supported by the discovery that particles killed by UV irradiation cannot synthesize DNA but continue to produce enzymes for much longer periods and in much larger amounts than do viable phages.³² The author is presently studying these phenomena using H-treated particles.

It is of interest that the ability of a single genetic marker to reproduce itself in matings with viable carrier phage (marker rescue experiments) was only slightly more sensitive to H than was the ability of the particle to initiate the synthesis of an enzyme (figure I, compare curves 13 and 14). This finding suggests that a single lethal event may be responsible for inhibiting gene replication and gene function, and that a particle harboring such a lethal damage is rendered sterile. However, the ability of the particle to reproduce itself can be restored when damaged genetic sections are replaced by functional components through matings among several particles (multiplicity or cross-reactivations). Thus, for phage viability, one requires a complete set of gene functions (enzymes, phage proteins, etc.) as well as at least one intact genome to serve as a primer for its own duplication.

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Reversal of the H-Damages

There are a number of indications that E. coli B bacteria, T2 bacteriophage, and DNA have a limited capacity for repairing or circumventing H-induced damages to the replicating mechanisms. The survival of H-treated E. coli B partially depends on the medium on which organisms grow following exposure. Unpublished results demonstrate that highest bacterial survivals are obtained on a purely synthetic medium (inorganic salts and glucose) and lowest survivals on a nutritionally rich medium (yeast extract). In addition, chloramphenicol added to either medium has a small beneficial effect on survival, but a mixture of ribonucleotides (although not of deoxyribonucleotides) added to the synthetic medium lowered the survival of H-treated cells. Generally speaking, the findings suggest that when the growth rate of H-treated bacteria is slowed, survival is favored by delaying the onset of "unbalanced growth"²² while permitting some repair of the H-damages to proceed.

T2 bacteriophages can efficiently repair the lethal H-damages by multiplicity reactivation when several inactive particles pool their undamaged genetic segments to reconstitute a new viable genome. Furthermore, T2 bacteriophage (even in single infection) becomes significantly more resistant to H after some 6-9 minutes of intracellular development, indicating that particles have passed some H-sensitive stage or are now capable of repairing or circumventing the H-damages. All chemotherapeutic attempts on H-inactivated T2 particles were unsuccessful, although some of the compounds used (notably aminoethylthiouronium bromide [AET] and mercaptoethyl amine [MEA]) had a prophylactic, dose reducing action when administered prior to or simultaneously with, H.

H-treated DNA spontaneously reacquires the ability to prime the in vitro synthesis of DNA which is catalyzed by E. coli B polymerase.¹⁰ A similar lag before DNA synthesis renews spontaneously has also been reported in vivo with H-treated E. coli.²⁵ It is proposed that, with the aid of polymerase, new and functional primers are reconstituted from the polydeoxynucleotide sections that were produced by the action of H on DNA. The finding that repair of the H-induced inhibition of the ability to synthesize DNA occurs spontaneously is encouraging from a therapeutic standpoint, although it must be remembered that such newly synthesized DNA has not been analyzed for genetic competence.

Probably the potentially most fruitful approach to eventual therapy of H-induced lesions to cells and viruses is the elucidation of the biochemical mechanisms responsible for the H-resistance of certain mutant strains of E. coli B. Since mutants such as E. coli B/r are similarly resistant to all agents that are presumed to exert their toxic effects through an action on DNA, the molecular basis for resistance is presently quite obscure. On the other hand, retaining

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the basic assumption that DNA is the primary target, it is feasible that damaged DNA might be secondarily degraded by nucleases, which are present in sensitive, but may be absent in resistant strains, thus permitting repair to take place in the resistant organisms. A similar mechanism was invoked to explain the author's discovery that the priming ability of heated H-treated DNA does not recover when impure *E. coli* polymerase is used to catalyze the *in vitro* synthesis of DNA.¹⁰ Presumably, the contaminating phosphodiesterase is able to degrade H-damaged DNA. However, when the contaminating phosphodiesterase activity is reduced by further purification of polymerase, the priming ability of H-treated DNA recovers after a lag. Recent findings indicate that secondary breakdown of DNA also occurs with H-treated *E. coli* B and *E. coli* 15T⁻A⁻U⁻ bacteria (sensitive strains) but is somewhat less with H-treated *E. coli* B/r organism (resistant strains). With the hope of clarifying the biochemical basis for resistance by some mutants of *E. coli*, the author is presently investigating nuclease levels in these organisms.

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LEGEND FOR FIGURE I

- 1 - Reproduction of T2 following single infection.
- 2 - Viral DNA synthesis by singly infected cells.
- 3 - Reproduction of T2 by singly infected cells.
- 4 - Lysis of bacterial hosts by phage ghosts.
- 5 - Ability of phage to kill host cells.
- 6 - Ability of phage to exclude other phages.
- 7 - Ability of phage to inhibit bacterial RNA synthesis.
- 8 - Ability of phage to inject its DNA.
- 9 - Sensitivity of singly infected cells 9 minutes after infection.
- 10 - Reproduction of T2 by multiply infected cells.
- 11 - Viral DNA synthesis by multiply infected cells.
- 12 - Capacity of bacteria to support phage reproduction.
- 13 - Sensitivity of a host-range genetic marker.*
- 14 - Ability to initiate the biosynthesis of an enzyme peculiar to the infected cell.*
- 15 - Adsorption on host bacteria.

* These data are corrected for loss of bacterial capacity. The multiplicity of infection was 0.1 T2 particle per cell.

Figure I

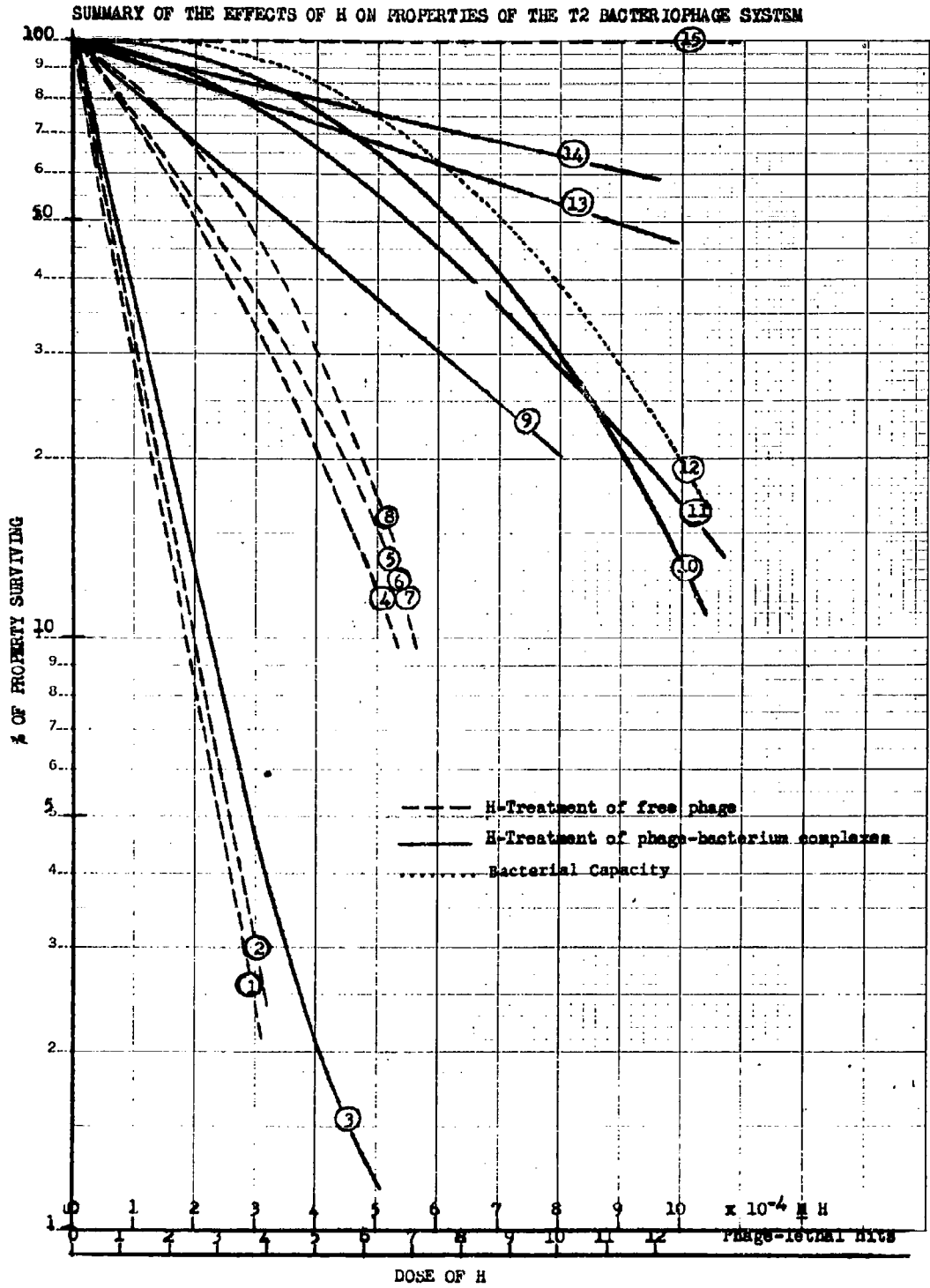


TABLE I
DISTRIBUTION OF H^{35} ON PROTEIN AND DNA OF T2 BACTERIOPHAGE

SAMPLE	INITIAL $[H^{35}]$	PREHYDRO- LYZED $[H^{35}]$	P/Po	NUMBER OF PHAGE- LETHAL HITS (EXTRACELLULAR)	C.P.M. PER PHAGE	MOLECULES* OF H^{35} BOUND PER PHAGE	MOLECULES OF H^{35} BOUND PER PHAGE PER PHAGE-LETHAL-HIT
10 ¹² T2	0	5×10^{-4}	1.0	0	3.6×10^{-11}	3.4	
10 ¹² T2	2×10^{-4}	3×10^{-4}	0.018	4.3	3.2×10^{-9}	306	71
10 ¹² T2	5×10^{-4}	0	7.9×10^{-5}	10.4	6.6×10^{-9}	630	61

C.P.M. PER PHAGE AFTER SHOCKING AND DNAASE (TCA- INSOLUBLE)	C.P.M. PER PHAGE AFTER HEATING TCA MIXTURE 95°C (TCA-INSOLUBLE)	% OF C.P.M. ON PHAGE PROTEIN	MOLECULES H^{35} BOUND TO PROTEIN PER PHAGE PER PHAGE-LETHAL HIT	H^{35} BOUND TO PHAGE DNA	
				C.P.M. PER PHAGE AFTER SHOCKING AND DNAASE (TCA- SOLUBLE)	C.P.M. PER PHAGE AFTER HEATING TCA MIXTURE AT 95°C (TCA- SOLUBLE)
3.3×10^{-11}	3.8×10^{-11}			5.1×10^{-12}	2.0×10^{-12}
1.3×10^{-9}	1.4×10^{-9}	41-44	29-31	2.0×10^{-9}	2.1×10^{-9}
2.7×10^{-9}	2.8×10^{-9}	43-45	26-28	3.8×10^{-9}	4.2×10^{-9}

* 1.06×10^{-11} C.P.M. = 1 MOLECULE H^{35} .

TABLE I
EFFECT OF H ON THE DNA SYNTHESIS CATALYZED BY *E. COLI* POLYMERASE

Experiment	DNA Synthesized in 30 min (% of control)
1. Complete system (control)	100.0
2. Complete system less dTTP	5.9
3. Complete system, H-treated dATP	81.2 ^{2/}
4. Complete system, H-treated dGTP	21.7 ^{2/}
5. Complete system, H-treated dTTP	112.0 ^{2/}
6. Complete system, H-treated dTTP	121.0 ^{2/}
7. Complete system, H-treated polymerase	95.3, ^{2/} 91.0, ^{4/} 82 ^{2/}
8. Complete system, H-treated primer ^{3/}	16.8 ^{2/}

^{1/} Complete system (0.3 ml final volume) contained 20 μ moles glycine buffer, pH 9.2; 2 μ moles MgCl₂; 0.3 μ moles 2-mercaptoethanol; 10 μ g calf thymus DNA (highly polymerized, obtained through the courtesy of Dr. R. M. Haytlett); 5 μ moles dATP; 5 μ moles dTTP; 10 μ moles of dGTP; 5 μ moles of dCP-3TP (2 x 10⁶ d.p.m. per μ mole); 0.06 units partially purified *E. coli* polymerase.

^{2/} Initial H

^{2/} 1 x 10⁻³ M

^{2/} 2 x 10⁻⁴ M

^{4/} 5 x 10⁻⁴ M

^{5/} Highly polymerized calf thymus DNA

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