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Title THE INHIBITORY EFFECT OF CYSTEINE ON STREPTOMYCES GIRSEUS PHAGE REPRODUCTION

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Cysteine inhibition of phage multiplication in Escherichia coli has been described by a number of workers. More recently cysteine has been found to lower the yield of Streptomyces griseus phage. The present study sought a more complete description of the "cysteine effect" as it has been noted in the S. griseus phage-host system.

The influence of cysteine was studied by measuring changes in the one-step growth curve of the system or in the relative phage yield. The influence of cysteine concentration, exposure time and site of action were examined.

A slight lengthening in the latent period of the S. griseus one-step growth curve and a 75 percent decrease in relative phage yield were observed when 50 μg/ml of cysteine was present throughout the entire growth period. A short exposure time of 20 minutes to 50 μg/ml cysteine during the early and middle portion of the latent

period also gave maximum inhibition of phage yield. Resuspension of infected cells in fresh medium did not remove the inhibitory effect. This indicated that the ionic balance of the system was not altered. Cysteine did not appear to influence the release of phage particles from the infected host, since the addition of this amino acid during the latter portion of the latent period did not result in a significant decrease in phage yield. In addition, no changes in phage yield were observed when cysteine was present during germination of the host spores or during the period of phage adsorption. A disturbance in the production of an essential amino acid was ruled out since cysteine did not compete with the production of homoserine, methionine, threonine or isoleucine. Other sulfhydryl compounds such as homocysteine, glutathione, and sodium thioglycollate were also found inhibitory to the phage-host system. These findings suggested that the cysteine inhibitory effect was centered at some site in the synthesis of phage protein.

THE INHIBITORY EFFECT OF CYSTEINE ON *STREPTOMYCES GRISEUS* PHAGE REPRODUCTION

by

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Typed by Susan Carroll

To Hiro --- my husband, colleague, and companion

To Bronwen --- our daughter

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For the years of patience and immeasurable assistance on the part of my husband --- thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE INHIBITORY EFFECT OF CYSTEINE ON *STREPTOMYCES GRISEUS* PHAGE REPRODUCTION

INTRODUCTION

The sequence of events which occur in a phage-infected cell has been established for coliphages (Stent, 1963). These events lead to the formation of new progeny phage. The presence of certain compounds in the growth medium may influence any step in the development of vegetative phages. These compounds may prevent the adsorption of the phage by the host, inhibit the penetration of the phage duplicating material (DNA), interfere with the syntheses of phage components or assembling of these components, or even block the release of mature phage. In many cases, the particular stage at which the interfering compound acts has been determined but the exact mechanism of the inhibitory action of the compound has not always been shown.

The cysteine inhibition of phage multiplication has been described by a number of workers. In 1951, Spizizin, Hampil, and Kenney reported the inhibition of phage T2r^{+} (*Escherichia coli*) growth by cysteine and the experimental evidence presented favored an interference mechanism in which cysteine was postulated to bind certain essential metal ions. T1 and T2 *E. coli* phage inhibition by cysteine, reported by Joklik (1952) also favored an interference

mechanism. Joklik's mechanism postulated the inhibition of the release of mature phage since plaque forming units were found to be present in both cysteine treated and non-treated cells. To account for the loss of infecting units when maturing lambda phage of E. coli K12 were exposed to cysteine, Gots and Hunt (1953) hypothesized that cysteine interfered with the synthesis of threonine.

In 1959, Gilmour, Noller, and Watkins reported that cysteine inhibited multiplication of the actinophage of Streptomyces griseus. It is the purpose of this study to provide a more complete description of the cysteine effect in the S. griseus 514-3 phage-host system.

HISTORICAL REVIEW

The Streptomyces griseus phage under study was originally isolated and investigated by Gilmour and Buthala (1950). The growth habits of this rather unique system have been examined. This system differs from the extensively investigated T-series phages of Escherichia coli, particularly because of the growth habit of S. griseus. The latter organism exhibits a filamentous, mold-like form of growth which makes difficult the accurate assessment of the multiplicity of infection. During germination, each spore may produce one, two or even more germ tubes, all of which are susceptible to infection but yet may form a single plaque when plated out. The occurrence of multiply infected host cell increases in such a system and often produces poor one-step growth curves. However, Gilmour, Noller, and Watkins (1959) have used newly germinated spores to simulate single cells and utilized the one-step growth procedure of Ellis and Delbruck (1939) from which quantitative determinations for the present studies have been made possible.

Electron microscopic examinations of this host-phage system have been undertaken primarily by Gilmour and Bradford (1965). The septated host is subject to infection by a particle whose hexagonal head measures about 100 mμ in diameter and whose tail is 360 mμ in length. The mode of attachment or infection has not yet

been established for this system; however, intracellular development of phage bodies have been observed in ultrathin sections of infected cells.

Processes involved in the synthesis of phage progeny have not been investigated for the actinophages. Since the phage pattern of S. griseus generally follows the one-step growth curve as established for the T-series of E. coli phages (Gilmour, Noller, and Watkins, 1959), a discussion of the metabolic events ascertained for the coliphages would be useful in the study of phage 514-3 and for centralizing the cysteine effect in this phage system.

It has been established for the coliphages that a sequential and orderly occurrence of events follow the exposure of susceptible host cells to infective phage particles. Almost immediately, phage particles adsorb by their tails to the cell surfaces of homologous bacteria. The kinetics of this adsorption process has been extensively studied by Schlesinger(1932). He was able to determine the fraction of input phage that remained unadsorbed after phage and bacteria were brought into contact and derived a first-order reaction for phage adsorption.

Following adsorption of the phage, the desoxyribonucleic acid (DNA) penetrates the host cell wall and causes a breakdown of bacterial DNA, the products of which enter a nucleotide pool. The classic work of Hershey and Chase (1952) has beautifully demonstrated

that only DNA is injected into the susceptible host leaving the phage protein at the cell surface. Later, however, a minor component of the infecting phage, the internal protein, has been found to enter the host (Hershey, 1957). This protein is not transferred to progeny viruses (Minagawa, 1961) but is synthesized de novo at a very early stage in the latent period (Murakami, Van Vunakis, and Levine, 1959).

The synthesis of phage components occurs after DNA penetrates the cell wall. The growth of intracellular phage particles takes place within the eclipse period, a time during which the infected cell contains no material capable of initiating a plaque (Luria, 1950). This interval occupies about the first half of the latent period (measured by one-step growth experiments) at which time there is no detectable net increase of phage titer. The development consists of a sequential order of events and the precise time schedule for the expression of the structural genes has been described as "clock" by Jacob and Wollman (1961). The synthesis of internal protein is detectable first in T2 infected E. coli at two to three minutes after infection (Murakami, Van Vunakis, and Levine, 1959) but these proteins are not involved in the growth process of vegetative phage. Another group of proteins that is synthesized early in the latent period is the requisite enzymes for the synthesis of vegetative phage particles. They have been referred to as "early-proteins"(Stent, 1963) and unlike the phage specific internal proteins, they do not appear in the

infective virus, but are essential to phage growth. These proteins are enzymes involved in reactions necessary for replication of viral DNA (Barner and Cohen, 1959; Flaks and Cohen, 1959; Keck, Mahler, and Fraser, 1960; Kornberg, 1960; Kornberg, et al. 1959). The syntheses of these enzymes proceed prior to the appearance of or in the absence of viral DNA synthesis.

The components for DNA synthesis are obtained from a pool of precurser DNA of the phage (Hershey, 1953). The phosphorus is derived from the breakdown products of host DNA and from the growth medium, and is built up in the bacterium within the first ten minutes of intracellular growth. This DNA is later withdrawn at random for incorporation into intact progeny virus.

The next series of events during the latent period involve the appearance of structural elements for progeny phage. Proteins of the head, core, sheath, tail fibers, and tail plates are synthesized and appear about eight minutes after infection with the T-even phages (Stent, 1963). The bulk of the material for protein synthesis is obtained from the external medium. Little, if any contribution is made by the host cell protein (Siddiqui, et al. 1952).

Well before the end of the eclipse period, phage maturation, the process involving the assembling of structural components, occurs. Kellenberger (1962) envisages this process as a series of events, the first of which involves the condensation of viral DNA

macromolecules from the precurser pool into polyhedral bodies. The second step is the growth of the protein head membrane around the DNA condensate. In the third step, the phage is endowed with a tail and completed with the addition of the base plate and tail fibers. The assembled phage particles are then ready to be released from the host.

It was found that half way through the latent period, a phage induced lysozyme makes its appearance (Stent, 1963). The phage lysozyme causes erosion of the cell wall and lysis of the infected cell thereby permitting the release of progeny viruses. As a consequence, a rise in titer, represented by the rising pattern of the one-step growth curve is observed.

Having reviewed the nature of phage growth as described for the coliphages, it may be seen that interference with any of the above functions may result in changes in the ultimate phage yield. One cannot, however, disregard the metabolic pattern of the host. Since the host provides the environment for phage synthesis, a nutritional environment detrimental to the host may very well interfere with normal phage infection and propagation.

Infection may be prevented by the interference of either phage adsorption or penetration in the presence of compounds which compete with necessary organic cofactors or which remove specific cation requirements. In a number of phage systems, organic or

inorganic compounds are necessary for phage adsorption. Tryptophan or its analogs have been found to be necessary for certain strains of T-even phages (Anderson, 1945). For the optimal adsorption of phage T1, a concentration of 0.001 M of divalent or 0.01 M monovalent cations are required and in T-even strains, adsorption takes place most rapidly in the presence of 0.1 M monovalent cations (Puck, Garen, and Cline, 1951). Delbruck's (1948) T4 mutant phage stock 12 requires both trytophan and calcium. However, with several of the mutant strains of the T4 coliphages examined, indole, which is produced from tryptophan by the host, interferes with the action of tryptophan. Brenner, et al. (1962) have been able to show that cofactor molecules prevent an interaction between the tail sheath and tail fibers rather than promote the reaction between the cell surface and tail fibers. Reaction of the tail fibers with tryptophan cofactor molecules frees the tail fibers which normally embrace the tail sheath and renders the phage adsorbable.

Another stage of virus invasion, established by Garen and Puck (1951) is an irreversible enzymatic transformation allowing the penetration of the infectious phage unit. In the presence of $CaCl_2$ or $MgCl_2$, T1 virus can attach to the host. However, the addition of zinc ions inhibits the penetration of the virus into E. coli B cells. The virus is still capable of attachment to the host but propagation of progeny does not occur. In this case zinc ions compete

with calcium or magnesium ions for sites on the surfaces of bacterial cells. Thus, the presence of zinc ions affects the cell and not the virus and thereby prevents the penetration of units necessary for phage duplication.

Hershey and Chase (1952) have also been able to prevent penetration of phage T2 of E. coli by the use of dilute formaldehyde. This time, the phage itself is inactivated by the compound. The capacity to adsorb is maintained, but the phage cannot inject its DNA into the cell.

Similar results have been obtained with polyvalent metal ions. Using 32-P labeled T2r virus, Kozloff and Henderson (1955) infected the susceptible host cells in nutrient broth and ethylenediaminetetraacetic acid (EDTA, a compound which binds all polyvalent cations). Phage particles are adsorbed, the host cells are killed, but progeny phage is not produced. Using a Waring blender, 90 percent of the viral 32-P (DNA) can be taken off the host cell. Polyvalent metal ions are complexed by EDTA and penetration of the viral DNA is inhibited.

Following infection with a virulent phage, interference with the synthesis of early enzymes or any phage specific compounds could result in decreasing the ultimate yield of progeny virus. The presence of purine or pyrimidine analogs in phage nucleotide pools influences the chemical composition of DNA synthesized. For example,

2-aminopurine and 5-fluorodeoxyuracil can substitute for adenine and thymine respectively thereby affecting the synthesis of normal DNA (Cohen, et al. 1958; Freese, 1959). Analogs of amino acids also interfere with normal phage growth. Studies conducted on the effect of the tryptophan analog, 5-methyltryptophan (5MT) on phage growth indicated that the analog is not simply being incorporated in the place of tryptophan. Since the inhibition of virus multiplication could be reversed by adding tryptophan, and since the latter compound produced no lag period in overcoming 5MT action, it was believed that 5MT interfered with specific stages of tryptophan metabolism or utilization (Cohen, 1947).

Later investigations by other workers (Burton, 1955; Hershey and Melechen, 1957) have shown that the synthesis of phage-specific DNA is inhibited by the presence of an inhibitory amino acid analogue or the antibiotic chloramphenicol. However, if the inhibitors are added after the start of intracellular phage development, synthesis of phage DNA does proceed. Infective phage particles are produced only if the inhibitor is removed.

Pardee and Prestidge (1958) have obtained similar results with 7-azatryptophan (7AT), another tryptophan analog. The analog is incorporated into proteins and permits syntheses of proteins and nucleic acid but bacteriophage T1 and T2 do not appear in active form. When 7AT is added immediately following infection, the rate of phage

DNA synthesis is decreased. Addition of the analog after infection also inhibits phage development. Proteins are produced in the presence of 7AT but these do not exhibit the properties of normal phage proteins. These workers felt that the analog caused major disorganizations in the entire process of phage protein synthesis. They deduced that early proteins are necessary for phage DNA formation. By a similar line of reasoning, Ebisuzaki (1963) felt that early enzymes are also necessary for the formation of structural proteins. He examined the effect of 7AT on deoxycytidylic hydroxymethylase, one of the early proteins, and on the formation of serum blocking proteins and found that the synthesis of late proteins are prevented if the early proteins are defective (containing 7AT or 5MT).

Natural amino acids can also act as inhibitory antimetabolites of other amino acids. A repression in the synthesis of an essential amino acid may result from the presence of another amino acid. For example, serine and leucine can inhibit T2 multiplication and the inhibition by leucine may be reversed by isoleucine, valine, or norleucine (Fowler and Cohen, 1948). In studies conducted on the nutritional requirements for the development of lamdba phage in E. coli K12, Gots and Hunt (1953) noted that although valine is essential for optimal maturation of lambda phage its presence prevents maturation if isoleucine is not present in the medium. In the absence of isoleucine, valine represses isoleucine synthesis. In the same

system, abortive loss of infective units also arises if cysteine is present in the medium. In this case, cysteine interferes with the synthesis of another amino acid, threonine, which is essential for lambda phage development. An excess of threonine reverses the effect. Since it was found that the threonine requirement could be replaced by homoserine, a threonine and methionine precurser (Gots and Koh, 1950), it was postulated that cysteine preferentially reacts with homoserine to ultimately form methionine, thus retarding threonine biosynthesis.

Other than the involvement of cysteine in threonine biosynthesis, cysteine inhibition in other systems has been suggested to involve binding of metal ions (Spizizin, Hampil, and Kenney, 1951; and Joklik, 1952). Using 2 μg/ml of cysteine in the E. coli T2r+ system Spizizin postulated that cysteine binds metal ions necessary for the maintenance of the optimal ionic balance. This in turn inhibits either multiplication or release of phage progeny. By artificially rupturing infected cells prior to its natural time of burst, Joklik has shown that normal numbers of infective phage were present in E. coli in the presence or absence of cysteine. He therefore postulated that cysteine interfered with the release of phage by combining with some essential factor such as a metal ion.

Any interference with cellular energy metabolism influences the formation of new phage progeny (Price, 1947). For example,

iodoacetate, fluoride, and azide, known inhibitors of certain reactions in host carbohydrate dissimilation pathways, prevent phage multiplication in penicillin-treated Staphylococcus muscae. Since iodoacetate, fluoride and azide act at the triose phosphate hydrogenase, enolase, and cytochrome system sites respectively, ATP formation is presumably blocked. Gramicidin also inhibits phage multiplication by preventing the uptake of inorganic phosphate from the medium attesting to the fact that energy rich phosphate is necessary for phage synthesis.

Even if all the components constituting a phage particle were synthesized without interruption, infective particles would not appear if the assembling of the components, or the release of assembled particles were obstructed. Foster (1948) showed that if proflavine were present when phage T2 infects E. coli B, lysis occurs at the normal time but no infectious phage is released. Infective progeny phages are released if the dye is removed before the onset of lysis. In addition, all the known major constituents of the infective progeny are released upon lysis (DeMars, 1955). It appears from these studies that proflavine inhibits the maturation of vegetative phage.

Since phage lysozyme is involved in the lytic process (Streisinger, Mukai, and Miller, 1960), any interference with the enzyme or with cofactors involved in release of phage would result in loss of infective units. In this regard, cysteine inhibition of

phage release as postulated by Joklik (1952) has been discussed earlier.

We have thus seen that decreases in the number of progeny viruses may be due to a variety of effects of inhibitory compounds acting upon various stages of the developmental cycle of the bacteriophage. In the light of these possibilities, it is the aim of the present study to ascertain the nature of the cysteine inhibition of phage production in the S. griseus phage-host system.

MATERIALS AND METHODS

Culture and Phage Growth

Strain 3475 of Streptomyces griseus, the original Waksman streptomycin producer was used as the host for all experiments. Spores for inocula were harvested from a semi-synthetic medium. The glycerol-asparagine agar slants consisted of glycerol, 10 ml per 1000 ml of water; glucose, 0.1 percent; K_2HPO_4, 0.1 percent; asparagine, 0.1 percent. Spores were harvested and suspended in 0.25 percent peptone broth, filtered through 30 layers of cheese-cloth, and diluted to give a standard optical density. This value corresponded to approximately 3-5 x 10^8 spores/ml by plate count on glucose nutrient broth (glucose, 0.5 percent; peptone 0.5 percent; beef extract, 0.3 percent; yeast extract, 0.01 percent). Prior to each experiment, the spores were diluted with synthetic medium to 1 x 10^8/ml. The synthetic medium used was a slightly modified version of the medium developed by Folston (1956). This medium (designated CSM) consisted of the following compounds: $K_2HPO_4 \cdot 3H_2O$, 0.1 percent; $(NH_4)_2HPO_4$, 0.2 percent; $CaCl_2$, 0.001M; glucose, 0.5 percent; glutamic acid, alanine and aspartic acid - 250 µg/ml; arginine and lysine - 100 µg/ml; valine, isoleucine, leucine, and histidine, 50 µg/ml.

Phage strain 514-3 isolated by Gilmour and Buthala (1950),

specific for S. griseus 3475 was maintained in 0.25 percent peptone broth. A high titered phage stock was obtained by the soft-agar layer method as described by Adams (1959). One tenth of a milliliter of phage stock containing about 1×10^6 phage/ml was inoculated into three ml of semi-solid agar (glucose nutrient broth plus 0.7 percent agar) containing 0.2 ml of S. griseus spores from a stock of 5×10^8 spores/ml titer and poured directly over a plate of glucose nutrient agar. Ten plates so inoculated were allowed to incubate for 15 hours after which five ml of peptone broth were added to each plate. The confluently lysed cells on the agar were collected and centrifuged to remove cellular debris and agar. The supernatant was filtered through a Seitz filter to obtain a sterile preparation of phage. The titers of the cultures prepared by this method averaged between 1×10^{10} to 3×10^{10} plaque forming units. For experimental purposes, stocks were diluted with synthetic medium to a titer of 1×10^7 plaque forming units/ml.

One-step Growth Studies

The cysteine effect was studied by observing changes in the one-step growth curve as described by Gilmour, Noller, and Watkins (1950). The general procedure was as follows: one ml containing 1×10^8 spores was germinated in eight ml of synthetic medium for four hours after which 1×10^7 phage particles contained in one ml

were added. Adsorption was allowed to proceed for ten minutes and unadsorbed phage was removed by centrifuging the mixture at low speed to sediment the bacteria, leaving the free phage in the supernatant fluid. The supernatant was assayed by the plaque count method to determine the number of unadsorbed phage particles. The difference between input phage and phage count of the supernatant represented the number of adsorbed phages. The sedimented cells were washed with phosphate buffer (0.625 M, pH 6.86), and diluted with CSM so as to obtain low plate counts during the latent period. By doing so, readsorption to uninfected cells or cellular parts was kept at a minimum. Incubation was allowed to proceed for varying lengths of time up to about 200 minutes at 31 C. One-tenth milliliter aliquots were removed at appropriate times and assayed for phage by the soft-agar layer technique. Cysteine was added in various concentrations at selected time intervals in the growth cycle and the resulting relative phage counts determined. The term "relative increase" designates the ratio of the average count at burst to the average count obtained during the latent period. In all experiments, the synthetic medium, CSM was employed and a phage-spore input ratio of 0.1 used. With this ratio, approximately 20-25 percent adsorption was obtained.

Inhibition Studies

As indicated in the introduction and historical survey, several events in the cycle of phage growth may be influenced by inhibitory compounds such as cysteine. Thus, experiments were designed to measure the effect of the agent at a specific stage in the S. griseus phage infection cycle.

First a quantitative analysis of the cysteine effect was undertaken to determine the least concentration of cysteine capable of producing the maximum effect (minimum relative increase of phage yield). The following range in cysteine levels was used: 5, 25, 50, 100, 250 and 500 µg/ml. The cells were exposed to phage in the CSM medium containing the appropriate concentration of the inhibitory agent. After removal of the free phage, the infected cells were resuspended in fresh CSM containing the same cysteine concentration. The compound was allowed to remain during the entire growth cycle. Aliquots of 0.1 ml were removed at different time intervals for phage assay.

S. griseus spores may incorporate cysteine along with other essential amino acids during the germination period. Thus, there exists the possibility that incorporated cysteine may exhibit an intracellular inhibitory effect. To test this idea, spores were allowed to germinate in the presence of cysteine for 10, 30, 60, and 210

minutes. Prior to the adsorption by infective phage, the cells were washed with 0. 625 M phosphate buffer at pH 6. 86 and resuspended in 9. 0 ml of CSM. One ml of stock phage was added and growth proceeded as described for an infected system.

An examination of possible cysteine interference with the normal adsorption process was pursued by exposing infective phage particles to cysteine throughout the adsorption period. After germination of spores in a cysteine-less medium, phage particles were added immediately after the addition of 500 μg cysteine and allowed to remain in contact for 10 and 20 minutes. The cells were then centrifuged and washed free of unadsorbed phage. After resuspension in fresh CSM the one-step growth experiment was allowed to proceed as usual.

A sulfhydryl grouping is present in the molecule of cysteine and can function in the reduction of certain compounds. To ascertain whether the observed inhibition was due to the sulfhydryl radical, a number of compounds which also contained sulfhydryl groups were tested. Among the compounds investigated were homocysteine, sodium thioglycollate, and glutathione. All of these compounds were added at the same molar concentrations so that they could be compared on an equal basis. The compounds remained in the medium throughout the experiment and phage was assayed in the usual manner.

Vegetative phage particles are synthesized during the latent period. Two experiments were performed to determine the specific time interval within the latent period of 110-130 minutes that is most affected by cysteine.

First, the exposure period to cysteine necessary to produce the maximum effect was determined. Cells were infected, washed free of unadsorbed phage, then exposed to cysteine for 10, 15, 20, and 25 minutes. The washing and resuspension of infected cells consumed 30 minutes between the end of infection and the onset of exposure to cysteine. After the cysteine exposure for the time intervals indicated above, the cells were washed free of cysteine and resuspended in fresh CSM. Then aliquots were removed at regular intervals for phage assay.

Next, the time interval within the latent period wherein exposure to cysteine resulted in the greatest inhibition of phage development was investigated. Fifty μg/ml cysteine was added at 30, 50, 70, and 90 minutes after adsorption and allowed to remain with infected cells for 20 minutes. Cysteine was removed by centrifuging the cells and resuspending the pellet in fresh cysteine-less medium. Then growth of the cells was allowed to continue and samples removed for phage assay up to the 200 minute period.

In order to examine the effect of cysteine as a competitor in respect to the production of another amino acid, cysteine was added

to the CSM to which an excess of either homoserine, methionine, threonine, or isoleucine was added. The above amino acids were added 30 minutes after the phage adsorption period and were allowed to remain in the system for 20 minutes. At the fiftieth minute, the infected cells were removed from the amino acid medium. Samples for phage assays were collected between the 70 to the 200 minute time period. Changes in the relative phage counts were determined.

EXPERIMENTAL RESULTS

Effect of Cysteine on Phage Growth Cycle

A typical representation of the cysteine inhibitory effect is illustrated in Figure 1. In this case, 50 μg/ml of cysteine was added 30 minutes after adsorption and allowed to act for 20 minutes. Earlier experiments in which cysteine was present throughout the experiment gave similar results. The effect is characterized by a slight lengthening of the latent period from an average of 110-130 minutes to 130-150 minutes--an increase of about 20 minutes. There is also approximately 75 percent decrease in the relative phage yield. In the absence of cysteine there is about a twenty-fold increase in the plaque count. Whereas, when infection proceeds in a medium containing cysteine, there is only a five-fold increase. In terms of burst size, the system infected in the absence of cysteine gave a burst size of about 100 phage particles per spore while the same system grown in the presence of this amino acid produced a burst size of about 25. Results of the following experiments have been expressed in tables in terms of relative phage increases, with the maximum increase of infected cells in the cysteine-less medium set at 20-fold.

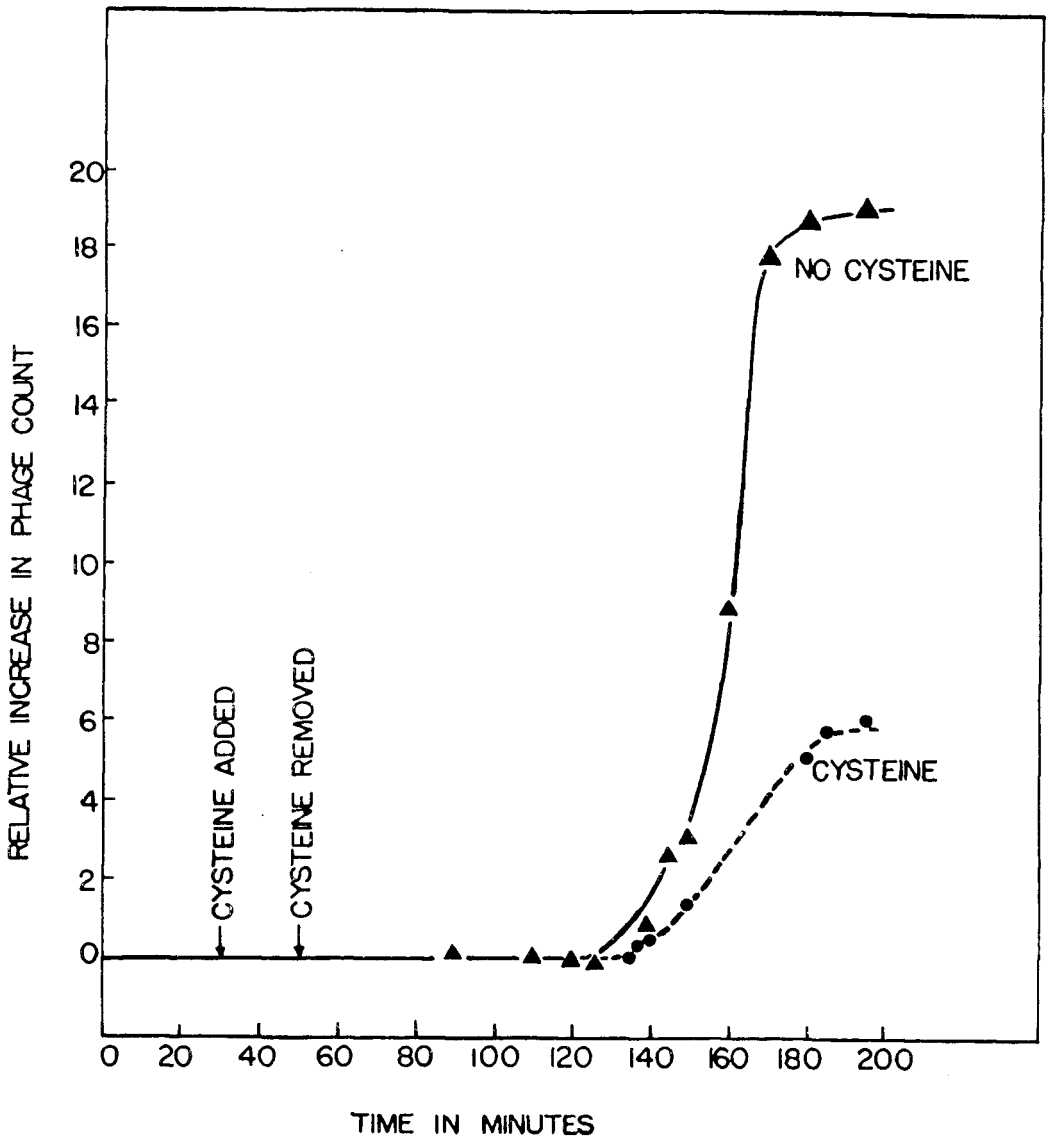

Figure 1. Observed cysteine inhibition of S. griseus phage-514-3.

Cysteine Concentration

The data obtained for the effect of 5, 25, 50, 100, 250, and 500 μg/ml cysteine on the relative phage increases are shown on Table 1. In the range of 5-50 μg/ml the amount of inhibition is directly proportional to cysteine concentration used in the medium. From 50 μg/ml to 250 μg/ml, no significant increases in phage inhibition are observed. In all previous experiments, the presence of cysteine has never completely prevented the production of phage progeny. As shown in Table 1, a very high concentration of 500 μg/ml is necessary before complete suppression is attained. Thus, 50 μg/ml appears to be the minimum concentration necessary to produce the maximum effect without resulting in complete inactivation which probably would result from toxification of the entire system.

Table 1. Effect of cysteine concentration on phage multiplication.

Concentration of Cysteine in μg/ml	Relative Increase in 180 minutes	Percent inhibition
0	20.0	0
5.0	9.6	52
25.0	6.6	67
50.0	4.0	80
100.0	3.2	84
250.0	4.0	80
500.0	0	100

Spore Germination

It is possible for a very small percentage of host protein to be utilized for the synthesis of phage progeny (Siddiqui, et al. 1952). During spore germination, it is also possible that cysteine along with other amino acids may become a part of the host amino acid pool. Therefore the possibility that the cysteine present in the host pool might exert an inhibitory effect on the subsequent production of phage progeny was examined. Using the minimum concentration which caused the maximum inhibition of phage synthesis (50 μg/ml), S. griseus spores were allowed to germinate in the presence of cysteine for various time intervals prior to adsorption with phage. Comparisons of the data from spores exposed to cysteine during germination and samples germinated in the absence of cysteine show no difference in phage production (Table 2). It is evident that regardless of the length of spore exposure to the amino acid, no inhibition is produced.

Table 2. Effect of cysteine on spore germination

Cysteine exposure time (min)	Relative Increase	Percent inhibition
210	20.5	0
60	21.0	0
30	20.5	0
10	22.0	0
0	20.0	0

Phage Adsorption

Since cysteine had no apparent effect on spore germination and subsequent phage production, the adsorption process of the phage growth cycle was examined as a possible site of cysteine inhibition. When cysteine is present during the ten-minute adsorption period, there is no observable inhibitory effect (Table 3). Also, exposure for an extended 20 minutes does not produce any changes in phage yields. Thus, it appears that cysteine neither exerts its effect during spore germination nor during the period of phage adsorption.

Table 3. Effect of cysteine on phage adsorption

Adsorption time (min)	Cysteine	Relative increase	Percent inhibition
10	present	20.8	0
10	absent	20.0	0
20	present	19.5	2.5
20	absent	20.0	0

Latent Period

Since the above experiments indicated that cysteine did not affect germination or adsorption, it was thought that some stage in the latent period may be inhibited by this amino acid. It was first necessary to find the minimum exposure time required to give a maximum

inhibition using 50 μg/ml cysteine. Figure 2 illustrates that when phage infected cells are exposed to cysteine for 20 minutes at an early stage in the latent period, a high level of inhibition is obtained. A 10 to 15 minute exposure time, conducted over essentially the same portion of the latent period does not effectively inhibit phage synthesis. A 20 minute exposure time with 50 μg/ml of cysteine produces the same effect as when cysteine remains throughout the experiment.

After establishing the minimum exposure time of 20 minutes, the interval in the latent period wherein the exposure to cysteine was most effective was determined. Cysteine was added at 30, 50, 70, and 90 minutes after the ten minute adsorption period and the infected cells were maintained in the cysteine for the already described 20 minute exposure period. The long latent period, characteristic of the S. griseus phage system lent itself well to this type of experiment. Ample time was available for sampling prior to phage release without having to resort to chilling the cells to slow down the release of progeny phage.

The data in Table 4 show that between the 30 and 50 minute span of the latent period, a high level of inhibition is obtained. Exposure of infected cells to cysteine between the 70 to 90 minute period results in a decreased inhibitory effect. In general, no inhibition is observed when cysteine is added during the final minutes of the latent period.

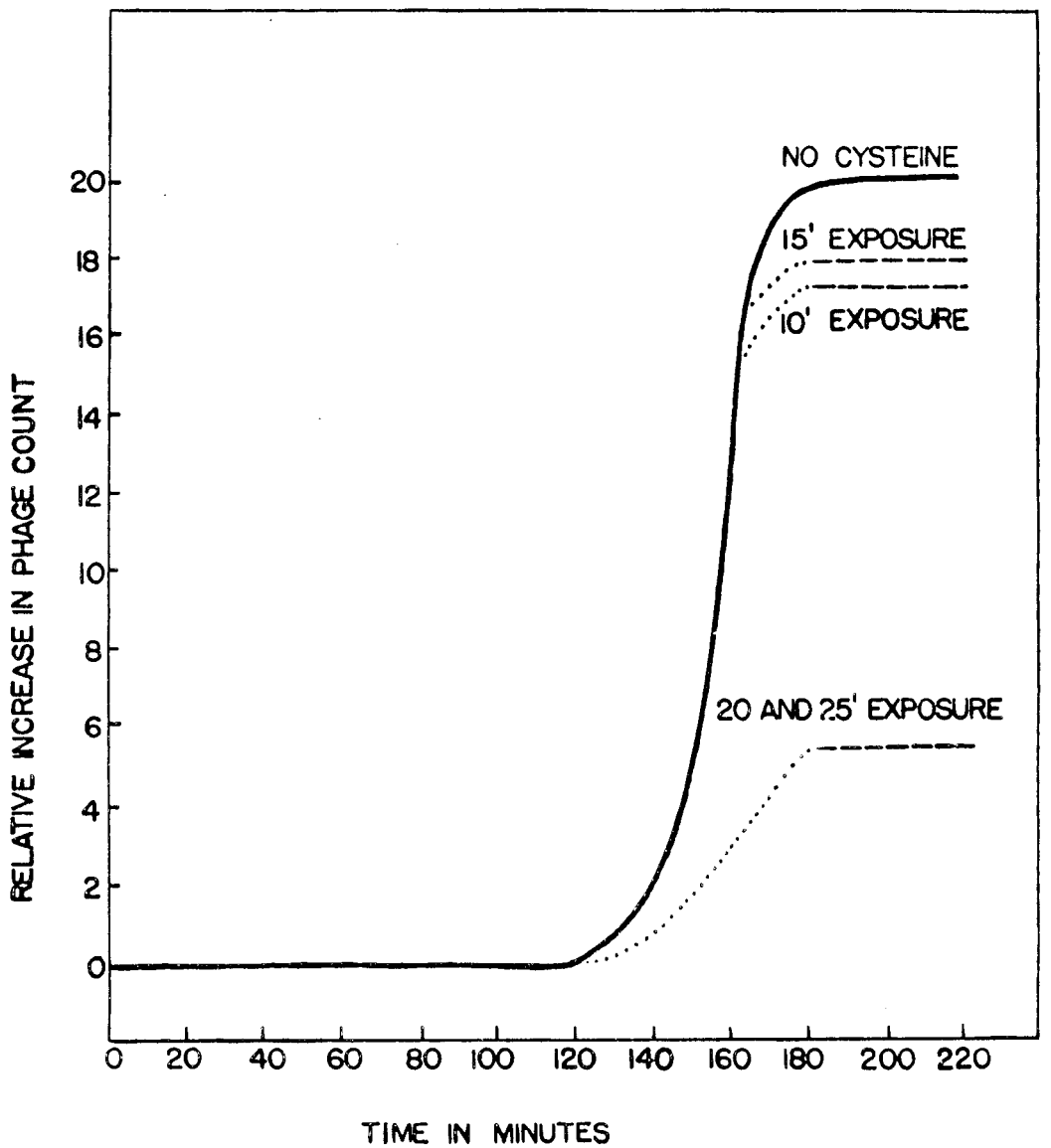

Figure 2. Effect of cysteine exposure time on S. griseus phage system.

Table 4. Phage inhibition as a function of time of cysteine addition during the latent period.

Time of Cysteine Addition (min) (after 10 min. adsorption)*	Relative Increases	Percent Inhibition
no cysteine added	20	0
30	4.6 - 5.1	76
50	4.8 - 5.0	75
70	8.0 - 14.0	44
90	20 - 26	0

*In each case, the phage-host system was exposed to 50 μg/ml of cysteine for 20 minutes.

Mechanism of Cysteine Inhibition

Sulfhydryl Effect

To ascertain whether the observed cysteine inhibition was due to the sulfhydryl component, a number of sulfhydryl containing compounds such as homocysteine, sodium thioglycollate, and glutathione were tested. It is evident from the data in Table 5 that each compound exerts a significant reduction in phage reproduction. The inclusion of homocysteine, thioglycollate, and glutathione produces between 50-60 percent inhibition. It appears however, that cysteine is about 50 percent more effective as an inhibitor than the other

Table 5. Observed effect of sulfhydryl compounds on phage yield.

Sulfhydryl compound	Concentration in ug/ml	Concentration in umoles/ml	Relative Increase (in 180 min.)	Percent inhibition
Cysteine	50.0	0.285	6.8	76
Homocysteine	38.5	0.285	10.2	49
Sodium thioglycollate	32.8	0.285	8.2	59
Glutathione	87.0	0.285	9.5	53
CSM (control)	----	-----	20.0	0

tested compounds. Thus it may be concluded that the observed interference of phage production by cysteine primarily involves the sulfhydryl group and is not due to cysteine per se. This conclusion is also supported by observations made in earlier experiments in which the use of old solutions of cysteine failed to produce a maximum inhibitory effect. Since in solutions, cysteine readily oxidizes to cystine, solutions had to be prepared fresh prior to use for the experiments.

Involvement in Amino Acid Synthesis

Homoserine is a common precurser in the biosynthesis of methionine and threonine in E. coli and other organisms (Gots and Hunt, 1953), and isoleucine may be formed from threonine (Greenberg, 1960). In the methionine pathway, homoserine functions as a precurser via its reaction with cysteine. Homoserine may also be phosphorylated to form phosphohomoserine and be subsequently converted to threonine. Isoleucine may be formed from threonine by the formation of alpha-ketobutyrate and the condensation of the latter with acetaldehyde. If the presence of cysteine had shunted the biosynthetic pathway in favor of methionine formation, hence decreasing the synthesis of isoleucine and threonine, then the addition of isoleucine and threonine should reverse the effect of cysteine. In Table 6, it may be noted that despite the addition of homoserine, threonine, methionine and isoleucine, cysteine readily maintains its inhibitory

effect. The isoleucine concentration of 0. 31 μM designated in Table 6 is in addition to the amount normally present in the synthetic medium. This addition causes only a slight decrease in the inhibitory effect. The addition of homoserine and threonine slightly enhances the inhibition by about 20 percent. This may suggest that added methionine relieves cysteine from functioning as a methionine precurser and increases the availability of cysteine for its inhibitory activity. This possibility is illustrated in Figure 3. The inhibitory activity may involve the sulfhydryl components in the syntheses of phage proteins and will be discussed later.

Table 6. Inhibitory effect of cysteine in the presence of homoserine, threonine, isoleucine, or methionine.

Amino acid(s)	Concentration of added amino acid	Relative increase	Percent inhibition
No addition		20. 0	0
Cysteine	0. 28 μM	5. 2	74
Cysteine + homoserine	0. 42 μM	4. 8	76
Cysteine + threonine	0. 42 μM	3. 8	81
Cysteine + methionine	0. 27 μM	2. 4	88
Cysteine + isoleucine	0. 31 μM	6. 7	67

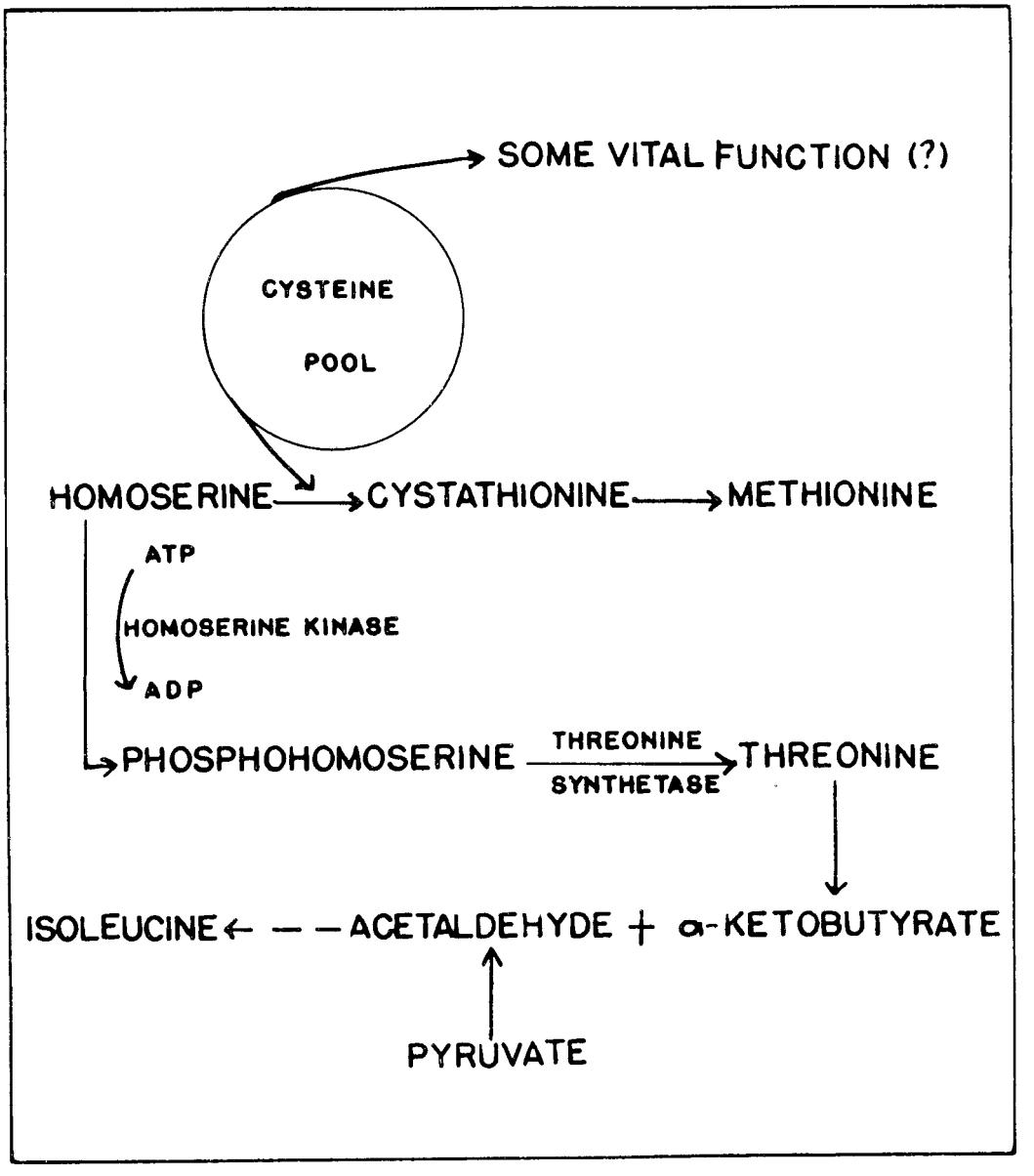

Figure 3. Possible interrelationship of cysteine inhibition of phage growth and biosyntheses of several amino acids.

DISCUSSION

Examination of the cysteine effect on the different stages of the phage growth cycle indicates that this amino acid interferes with the biosynthesis of phage components. The presence of cysteine during spore germination and phage adsorption has no effect on phage yields. The maximum inhibition occurs during the first half of the latent period with little or no effect observed toward the end of the latent period. Assuming that the general events occurring in the latent period for coliphages also holds true for actinophages, it would appear that cysteine neither affects maturation nor release of progeny phage since these events take place after the midway point of the latent period. Unfortunately all attempts at disrupting infected cells with lysozyme or chloroform to release mature phage, if present, were not successful. If release of mature phage is truly affected, one would expect to find the normal amount of phage particles upon artificial lysis of the infected cells.

The first half of the latent period is involved with the biosynthesis of phage components. However, whether the production of early proteins are affected could not be determined since experiments in which cysteine was present at the onset of the latent period could not be undertaken using methods available for the present study.

An exceedingly high cysteine concentration of 500 µg/ml is required before complete cessation of phage synthesis occurs whereas lower concentrations allow a degree of phage synthesis. Whether the observed concentration effect occurs because of binding of essential cations or instead represents an effect centered at some point or points in the growth cycle remains open to conjecture. The comparison of cysteine with other compounds containing sulfhydryl radicals (Table 5) indicates that the inhibition is a sulfhydryl effect. In view of this, Spizizin, in whose system the sulfhydryl effect has been also noted, postulates that the possible mechanism of inhibition involves the binding of metal ions which would disrupt the optimal ionic balance and in turn influences either multiplication or release of progeny phage. Joklik (1952) has also offered a similar mode of inhibition by cysteine. The data obtained in the present study do not readily support this mechanism. Inhibition is obtained at an early stage in the latent period when infected cells are exposed for 20 minutes, after which cysteine is removed. If the effect centers on a specific interference in ionic balance of the external medium, then we might have expected little or no inhibition upon the early removal of cysteine from the medium. It has been observed that the ten and 15 minute exposure time experiments conducted over essentially the same span of the latent period does not produce the pronounced effect exhibited by a 20

minute exposure time. One would expect a ten or 15 minute exposure time to be sufficient to immobilize essential cations.

In consideration of the data obtained, it is suggested that the overall biosynthesis or biosynthetic rate of phage reproduction is affected by the presence of cysteine. One can only speculate as to the mode of this biosynthetic inhibition. Considering that the inhibition is a sulfhydryl effect and possibly involves the synthesis of structural phage entities, one may examine the problem from the view of protein configuration. Besides peptide bonds, disulfide bonds are involved in the primary structure of a protein molecule. An excess of sulfhydryl groups may cause the reduction of disulfide bonds or may prevent the formation of these bonds and stereoconfiguration of a protein molecule might be altered. Once the disulfide bonds are reduced at a critical period, mere removal of free cysteine in the medium should not alleviate the effect. In these studies, inhibition occurred at times when phage proteins were probably being synthesized. The fact that we are involved with a sulfhydryl effect and that the effect is not reversible when cysteine is removed lends some support to the above hypothesis.

SUMMARY

The inhibitory effect of cysteine in the Streptomyces griseus 514-3 phage-host system has been described and studied. The data derived from this study may be summarized as follows:

1. We are concerned here with a sulfhydryl effect which at a concentration of 50 μg/ml causes a 75 percent decrease in the relative phage yield.

2. The presence of cysteine during germination of the host cells and during adsorption of the host by the phage does not affect multiplication.

3. A relatively short exposure time of 20 minutes with 50 μg/ml cysteine during the early and middle portion of the latent period decreases the phage yield. Resuspension into fresh medium does not remove the effect; an indication that we are not concerned with an alteration of ionic balance.

4. A slight inhibition, if any, occurs if the system is exposed to cysteine toward the latter part of the latent period, thus favoring an interference mechanism with the biosynthesis of phage rather than release of mature phage particles.

5. Presence of additional concentrations of homoserine, methionine, threonine, or isoleucine does not alleviate the cysteine inhibitory effect indicating that cysteine is not affecting protein synthesis by competing for the synthesis of another essential amino acid.

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