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Selective chemical antagonism of lactic streptococcus bacteriophage

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SELECTIVE CHEMICAL ANTAGONISM OF LACTIC STREPTOCOCCUS BACTERIOPHAGE

by

Dee McDonald Graham

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Dairy Bacteriology

Approved:

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Dean of Graduate College

Iowa State College
1954

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INTRODUCTION

Bacteriophage proliferation begins upon contact of a virus particle with a susceptible host cell. The initially reversible adsorption quickly changes, and the virus becomes firmly bound. Shedding its protein carrier at this point, virus nucleic acid enters the host cell. This step may involve an enzymatic activity of the virus on a component of the cell wall. Very quickly now the organization of the host cell is changed. The genetic structure of the virus appears to merge with mechanisms already existing in the cell. During an interval (latent period) characteristic of the infecting virus under the experimental conditions prevailing, the complex accomplishes the formation of new virus. Synthesis of virus protein begins immediately, and production of nucleic acid begins a little later. Complete infectious particles begin to appear in the cell after about one-half of the latent period has elapsed. Lysis of the host cell at the end of the latent period results in a sudden release of the newly-formed virus particles, thus completing one cycle of virus growth.

Conditions of nutritional deficiency which delay or prevent virus development usually affect the host in the

same way. Recently, some success has been found in the search for inhibitors capable of preventing virus replication without injuring the growth of the host organism. Present information indicates that this is the first method by which the processes leading to virus synthesis can be divorced from the normal host metabolism sufficiently to permit mechanistic studies of virus multiplication.

Considerable economic loss is incurred each year in the cheese and cultured milk industries as a result of bacteriophage action on lactic cultures. Discovery of an effective inhibitor would have a definite practical application. Such an inhibitor would have to be non-toxic to the host bacteria and to human beings, and effective in a complex natural medium such as milk.

The influence of various inhibitors on lactic streptococcus bacteriophage has been studied in this work. A condensed version of these results has been published previously (Graham and Nelson, 1953).

STATEMENT OF THE PROBLEM

The specific aims of this study were:

A. To screen a variety of compounds for possible selective inhibition of lactic streptococcus bacteriophage.

B. To analyze the action of any effective inhibitors found.

REVIEW OF LITERATURE

Consideration of the different phases of virus growth leads to the suggestion that inhibition of virus might be accomplished at any one of three stages: (1) during the extracellular stage, (2) during the process of invasion of the host cell and (3) during intracellular multiplication of the virus. Included in the following review will be pertinent works illustrating either inhibition or systems potentially susceptible to inhibition in each of these stages of virus growth.

Extracellular Virus

Theoretically it should be possible to destroy extracellular virus without gross destruction of host cells. However, knowledge of the differences between virus and cell structures has not yet permitted the development of any good, selective virucides. Various bactericides have been found to inactivate virus particles. Wells and Sherwood (1934) observed a relationship between the selective action of dyes on bacteria and their respective phages. In general, viruses attacking Gram negative bacteria were more resistant to deleterious agents than were viruses which attacked Gram positive bacteria.

Anderson (1943) found that penatin markedly reduced the concentration of plaque-forming particles of coliphage. The penatin-inactivated phage particles retained their original morphology as well as their ability to adsorb to and kill susceptible bacteria. An antibiotic isolated from a Streptomyces species inhibited a large number of phages (Asheshov et al., 1952). The agent, which was named phagolessin A58, inactivated unadsorbed particles but did not affect intracellular virus. Phage inactivated by A58 could not multiply but retained the ability to kill host cells. Depolymerized desoxyribose nucleic acid (DNA) completely protected phage from inactivation by A58, but inactivated phage was not reactivated by DNA. In general, bacteria were much less sensitive to A58 than were bacteriophages. A58 was very toxic to white mice.

Certainly the most selective and efficient method known for inactivation of virus particles is neutralization with the homologous antibody. Almost all viruses are good antigens (Burnet et al., 1937). Hershey et al. (1943) have studied in detail the antigenic properties of coliphage. In rabbits the phage induced formation of a specific antibody capable of precipitating the virus and neutralizing its infectivity. Each lytic unit could absorb several thousand molecules of antibody, although only two or three molecules appeared necessary for neutralization of infectivity. The rate of reaction of a single phage particle in

undiluted antiserum was considerably less than the rate of the pneumococcus-antipneumococcus reaction. Delbrück (1945a) reported that phage adsorbed to or multiplying on susceptible cells was not affected by the specific anti-phage serum. Wilkowske (1949) found that cross-neutralization tests with phage antisera were of value in differentiating strains of lactic streptococcus phage. An instance in which the destruction of extracellular virus with antiserum has become of clinical importance is that of the administration of human gamma globulin to inactivate measles virus (Lilly Research Laboratories, 1952).

Invasion of Host Cells

The concept of cell receptors involved in the early stages of virus infection was introduced by Hirst (1942). The combination of influenza virus with chicken erythrocytes appeared very analogous to the adsorption of virus by susceptible host cells. The virus, however, did not multiply on the red cells. Instead, the adsorbed virus gradually was eluted in a manner suggesting an enzymatic action of the virus on a component which bound the virus to the cell. Destruction of the erythrocyte receptor substance by periodate indicated that it was carbohydrate in nature (McCrea, 1948). Recognizing that in many cases of competition between structurally similar substances, inhibitory

analogs appeared to compete with their related metabolites for specific enzymes, Green and Wooley (1947) studied the ability of various carbohydrates to inhibit agglutination of chicken red blood cells by influenza A virus. Several polysaccharides were effective. Apple pectin, one of the most efficient inhibitors, also prevented multiplication of influenza virus in embryonated eggs.

The enzymatic nature of the reaction between erythrocytes and influenza virus was elucidated in a series of very excellent reports from Burnet's laboratory (Anderson, S.G., 1948; McCrea, 1948; Burnet, 1948a, b and c; Anderson et al., 1948). The red cell receptor groups and a similar substance (Francis inhibitor) present in normal serum were shown to be mucoprotein-polysaccharide molecules which were attacked by several viruses and by the receptor-destroying enzyme (RDE) isolated from Vibrio cholerae. In both instances the reaction was activated by calcium and optimum activity occurred at about pH 6.2. Presence of the receptor groups on the surface of cells was necessary for adsorption and invasion of influenza virus. To explain this enzymatic action of the virus, the authors hypothesized that a firm adsorption to the cell surface was needed to allow entry of the virus into the cytoplasm; subsequent to entry, the further processes leading to multiplication of the virus could not proceed until the virus had freed itself from the

adsorbed mucoid. From the standpoint of therapy, destruction of cell receptors by RDE or poisoning of the virus enzyme appeared of limited value due to difficulty of dispersion of such materials about the surface of susceptible tissue cells. Isolation from various glands and tissues of receptor-like mucoprotein suggested a role of this material in the natural defense mechanisms of the host. Eaton (1950) stated that intranasal application of RDE decreased the severity of influenza infections in mice.

The existence of more than one type of receptor group on the surface of a bacterial cell was suggested by the work of Miller and Goebel (1949). Based on differences in behavior toward Phase I and II Shigella sonnei, the authors concluded that coliphages T3, T4 and T7 combined with a type-specific lipo-carbohydrate complex on the surface of the bacterial cell; this same substance, however, did not serve a similar function for T2 and T6 viruses. The chemical nature of the receptor groups of the latter viruses was not apparent.

The presence of a receptor-destroying enzyme in bacteriophage has not been reported. Possible inferences of such an enzymatic function may be drawn from several studies. Hershey et al. (1944) observed an influence of univalent cations on infectivity of several phages attacking Escherichia, Salmonella, Eberthella and Shigella. About 90

per cent or more of the bacteria which adsorbed phage in the absence of electrolyte did not yield a plaque when plated on salt-free agar; adsorption in the presence of electrolyte resulted in plaque formation by about half of the bacteria which adsorbed phage. Adams (1949) observed that in the absence of calcium, coliphage T5 was adsorbed but did not multiply. Addition of the ion after adsorption resulted in a normal latent period before virus was produced, even when addition of the ion was delayed as much as 30 minutes after adsorption. Thus it appeared that calcium was necessary for a reaction occurring very early in the multiplication of this virus. The question, however, of whether the ion was needed throughout the latent period or only for a short interval during invasion was not investigated. Potter (1953) has shown that with lactic streptococcus bacteriophage, the need for calcium is manifested for only a few seconds following the initial contact between virus and host cell. Calcium was not required for adsorption or for virus multiplication subsequent to invasion of susceptible cells.

Intracellular Multiplication

Horsfal (1950) has pointed out that the key to chemotherapy of virus infections lies in the finding of

substances which are capable of inhibiting intracellular virus growth. Virus infection usually is not detected until a large proportion of the virus population is in an intracellular position. Thus, agents which affect free virus or the adsorption or invasive processes are of limited value for treatment of virus infections, although such agents might be of definite preventive value. In the following sections, conditions and other factors which appear to suppress intracellular virus growth will be discussed.

Physicochemical factors

Influenza A virus can be propagated successfully in chick embryos incubated at 37 C., but young chicks do not support growth of the virus. Enders and Pearson (1941) studied this resistance and found that the high body temperature of the young chicks (40 to 41 C.) prevented development of the virus. The possible presence of neutralizing sera was excluded. The virus grew normally in isolated chick tissue incubated at 37 C. Luria (1943) obtained growth of susceptible bacteria in the presence of coliphage at above-optimum temperatures. Between 15 C. and 40 C., growth of the virus and host bacteria was affected in a parallel manner. At 43 C. or 45 C. the bacteria grew at reduced rates. Cells infected with virus at 37 C., then transferred to

either 43 C. or 45 C. did not liberate virus. Cells returned to 37 C. produced a small amount of virus after a delay which increased with the time held at high temperature. Similar findings with lactic streptococcus phage were reported by Turner (1948) and Ford and Babel (1950). By selection of a critical temperature, bacterial growth was able to proceed, whereas virus increase was inhibited completely in some phage-host combinations.

Suppression of phage multiplication at levels of pH which permitted growth of host cells was observed by Overcast et al. (1951). Variations were observed in the effect of extreme pH levels on different strains of the virus. Optimum proliferation was at about pH 6.5; four of the five phages tested did not multiply below pH 5.0. Kerby et al. (1949) observed that at extremes of pH (below 5 or above 10) fragments appeared to be split off from T7 particles, as judged by two boundaries obtained in differential centrifugation studies. The pH range for infectivity of this virus lay in the narrow range of 6 to 8.

The precise effects on virus multiplication of such non-specific factors as extremes of temperature and pH are difficult to interpet. Such effects probably are best taken simply as indications of differences in the processes of virus multiplication and normal cell growth.

Physiology of host cells

One of the most generally observed characteristics of virus growth is that young, actively growing cells are necessary for optimum virus proliferation. Bacteria in their lag phase usually produce less virus than will organisms during their exponential phase of growth (Cohen, 1949). Much work has been done on the relationship between virus growth and the multiplication of host cells prior to infection.

Price (1947a) has reported the increase of phage in the presence of staphylococci prevented from multiplying by the presence of penicillin. Later results using the penicillin-treated system indicated a constant competition between the bacteria and phage for essential building elements (Price, 1947b). The author suggested that factors which upset the equilibrium would favor the increase of one component at the expense of the other. High concentrations of bacteria suspended in Locke's solution formed phage when penicillin was present; in the absence of penicillin no phage was formed. Addition of niacin resulted in phage increase without penicillin. These results were interpreted as indicating that niacin was essential for phage growth and that, in the presence of penicillin, utilization of niacin by the cells was prevented, thus making more niacin available for phage synthesis. Elford (1948) also studied the effect of penicillin on phage-infected staphylococci and confirmed the finding that

some virus was produced by cells unable to multiply. That the phage-producing ability of the cells possibly was injured by the antibiotic, however, was shown by the greatly reduced yield of virus from cells suitably treated with penicillin. Synthesis of metabolic intermediates essential for both phage and cell growth appeared to be interrupted. The combined action of penicillin and phage resulted in more rapid lysis of host cells than was accomplished by either agent alone.

Cohen and Anderson (1946a) observed that adsorption of coliphage T2 or T4 resulted in inability of the host to multiply. The rate of oxygen consumption and the respiratory quotient remained at the values observed just before infection. Adsorption of irradiated virus produced the same effects. The possibility of growing phage on host cells damaged just sufficiently to prevent their multiplication was investigated (Anderson, T.F., 1948). Ultraviolet irradiation of host cells appeared to result in damage limited to areas of molecular dimensions at or near the sites of absorption of individual quanta of radiant energy, thus leaving much of the cell mechanism intact. Multiplication of T2 virus on Escherichia coli killed in this manner was obtained. Anderson concluded that only a portion of the cellular mechanism of the host cell was required for multiplication of T2 phage.

From these reports it seems that production of phage is not dependent upon multiplication of the host cells. Young, physiologically active cells probably are needed to provide the proper enzymatic machinery for virus growth. The extreme importance of this factor suggests that the systems first affected by a decline in the physiological condition of host bacteria are intimately related to phage multiplication.

Interference

Prevention of the growth of a virus in an infected cell by introduction of a different virus is called mutual exclusion (Delbrück and Luria, 1942). Only one of the infecting virus strains multiplies, and the infecting particles of the excluded strain are not recovered upon lysis. This effect points out a method whereby the growth of virus in a susceptible cell can be influenced by the addition of a specific agent. When the chemistry of the virus particle and of virus-host interactions is better understood, synthetic replicas of the factor responsible for the interference effect may provide a method for controlling certain virus infections. A complete explanation of the mechanism involved, furthermore, may permit greater understanding of virus growth.

In studying a case of interference between two bacterial viruses, Luria and Delbrück (1942) were able to dissociate the

interfering from the reproducing capacity of the virus. Adsorption of a single particle of ultraviolet-irradiated coliphage gamma prevented multiplication of virus alpha on the same host cell. The irradiated virus was unable to reproduce itself but retained its ability to prevent multiplication of host bacteria. The authors hypothesized that interference between the two viruses resulted from competition for a "key-enzyme" present in limited amount in each bacterial cell. This enzyme appeared essential both for virus reproduction and for cell growth. Effects similar to the interference resulting from the use of irradiated virus were produced by 5-methyltryptophan (Cohen and Anderson, 1946b). The only difference was that the effects of irradiated virus were not reversible, whereas the effects of 5-methyltryptophan were readily reversed by removal of the tryptophan analog.

In later work, the "key-enzyme" hypothesis was discarded in favor of a new "penetration" hypothesis which seemed to account more adequately for the interference effects (Delbrück, 1945b). The "penetration" hypothesis assumed, among other things, that penetration of a virus into a cell makes that cell impermeable to any other virus, just as the fertilization of an egg by one spermatazoon excludes other spermatazoa. In the case of the viruses, however, the exluded virus exerted an influence revealed by a reduced yield of the successful

virus. This effect on yield was called the "depressor effect" and appeared to result from competition of the excluded and the successful virus for a common substrate. Diminution of the "depressor effect" by antivirus serum, which does not affect intracellular virus, indicated that the excluded virus was located at the surface of the infected cell.

Lysogenic bacteria can be made to produce a normal yield of phage by treatment with ultraviolet light (Lwoff et al., 1950). The presumably intracellular position of the pro-phage appeared to offer a means of testing the "penetration" hypothesis (Weigle and Delbrück, 1951). A lysogenic coliphage, on its way to maturity as a result of ultraviolet irradiation, was excluded by a different phage introduced at various stages during the maturation period. The authors concluded that mutual exclusion, at least in this case, did not result from a barrier to penetration but more likely from a block near the terminal stage in maturation of the phage particle.

A practical application of the interference phenomenon was reported by Green and Stulberg (1946). A modified ferret-passage strain of distemper virus appeared to block the development of a related virulent strain of the same virus in the fox. When inoculation was made intranasally, as occurs in natural infection, the modified strain interfered with the development of the virulent strain regardless of the order of

introduction of the two strains. The modified virus appeared to have a definite therapeutic effect during the incubation period and in the stage of early symptoms.

Natural cellular resistance

Strain specificity has been observed in all classes of viruses studied. Nelson et al. (1939) found that strains of lactic streptococci freshly isolated from natural sources were not sensitive to the available strains of phage. Enders (1948) concluded that native cellular resistance accounts for a major portion of the observed natural immunity to mammalian viruses. Tissue trophism, i.e., preference for certain specialized tissue, may indicate inability of certain tissues to support virus growth. Detailed study of the characteristics of naturally resistant cells and virus-resistant mutants may reveal what factors are responsible for the inability of viruses to attack these cells.

Benzer et al. (1950) reported that mutation of E. coli to complete resistance to the T phages was associated with failure of the resistant bacteria to adsorb the phage to which they became resistant. In one case, using an unusual phage variant obtained from mixed infection experiments, reproduction of T2 activity was demonstrated in cells which could not adsorb the normal T2 particles. In this case it appeared that the resistance mutation altered only the ability

of the cells to adsorb and permit invasion of the virus. The intracellular synthetic mechanism seemed to be unaffected.

Anderson (1946) studied the ability of virus-resistant mutants of E. coli to grow in a synthetic minimal medium. The 27 mutant strains obtained from the independent action of three phages on the same host represented four distinct resistance patterns. Many of the mutants were unable to develop in the minimal medium, although heavy growth of the parent strain was obtained. Resistance to different virus strains appeared to be related to a lack of specific synthetic abilities on the part of the host. All tryptophan-less mutants were resistant to T1 but sensitive to T5. Resistance to T7 was associated with loss of ability to synthesize proline. The amination capacity of some of the resistant strains appeared to be altered.

An additional link between the metabolic pattern of the host and its resistance to virus was established by the observations of Delbrück (1946). Using phages T4 and T6 which require tryptophan for their adsorption, indole was found to antagonize the activating effect of tryptophan. Since the bacteria used could produce indole from tryptophan, there existed in this system a mechanism whereby an activator of the virus was converted into an inhibitor by the host.

An opposite approach to the question of virus specificity was taken by Stanley and Lauffer (1948). Analysis of variant strains of tobacco mosaic virus revealed that mutation of the virus was accompanied by changes in the amino acid composition of the particles. Presence, absence or differences in amount of individual amino acids were accompanied by marked changes in virulence. Among eight strains of virus studied, the differences in composition involved 16 of 19 amino acids determined. Despite the marked differences in composition and certain properties of these virus strains, the particles all appeared to have the same size and shape. The highly purified preparations obtainable with these plant viruses permitted studies on the effect of altering specific chemical groupings of the virus particle. The results indicated that certain alterations modified the virulence of the derivative particles toward one host but not another.

Nutritional requirements for virus synthesis

Benzer et al. (1950) pointed out that oxidizable substrate in the medium containing infected cells is necessary for phage multiplication. Proliferation does not occur in cells suspended in buffer or if the respiration of the infected cells is blocked in any way.

Fowler and Cohen (1948) found that synthesis of virus depended on the presence of simple compounds in the medium. For multiplication, coliphage T2 required a source of C (lactate), N (NH_4) and P (PO_4). The yield of virus per infected cell was less in simple than in complex media, indicating a greater number of synthetic reactions necessary for virus formation in the simple medium. No single carbon- and nitrogen-containing compound was found which when added to the simple medium gave a yield of virus equal to that obtained in nutrient broth. This rate of synthesis of virus was approximated only with a complex mixture of L-amino acids and purine and pyrimidine bases.

In minimal media, Spizizen (1943a) observed differences in the rate of utilization of certain metabolites for coliphage synthesis. An especial preference for glycine, glycine anhydride and hippuric acid was found. These compounds supported limited phage multiplication in the absence of demonstrable cell multiplication. The effect was not produced by various dipeptides, other amino acids or degradation products of glycine. Phage production was inhibited by amino-methane sulfonic acid, an analog of glycine, in the presence of alpha-amino acids and closely related compounds. In the presence of xanthine, however, virus increase occurred. Only partial inhibition occurred in broth. The authors sug-

gested that other substances in addition to alpha-amino acids and closely related compounds contributed to phage multiplication.

Requirement of tryptophan for multiplication of coliphage T2 was shown by Cohen and Fowler (1947). The requirement for tryptophan was manifested throughout the latent period, although tryptophan was not required for the adsorption or liberation of T2. This requirement of tryptophan for multiplication of T2 is distinct from the adsorption cofactor function of tryptophan for T4 and T6. The tryptophan requirement of T2 normally is supplied by the synthetic mechanisms of the host.

Certain nutritional deficiencies alter the susceptibility of mice to virus infection. The effect of specific deficiency varies with different virus infections. Riboflavin deficiency increased the resistance of mice against the Lansing strain but not Theiler's poliomyelitis virus (Rasmussen et al., 1944), whereas with pantothenic acid deficiency the reverse was true (Lichstein et al., 1944). In one instance a multiple deficiency of phosphorus, calcium and vitamin D increased the susceptibility of mice to the Lansing virus (Foster et al., 1949). These results suggest differences in the metabolic pathways used for proliferation of these viruses.

Single deficiencies of nine essential amino acids resulted in prolongation of incubation periods and reduced incidence of paralysis in mice infected with Theiler's GD VII encephalomyelitis virus (Pond et al., 1952). Tryptophan, isoleucine, valine and methionine were most effective in altering the course of infection. Fatality of the disease was not reduced by any of the amino acid deficiencies. Similar effects of amino acid deficiencies on Lansing poliomyelitis virus in mice were found by Davies et al. (1952). Quantitative studies revealed slower and less extensive multiplication of virus in the nervous tissue of tryptophan-deficient than non-deficient mice.

Clark et al. (1949) have summarized similar results of several additional investigations, showing clearly that the typical clinical picture of many virus diseases can be altered markedly by a variety of dietary deficiencies. A rather specific nutritional environment appears necessary for a host to respond in the usual way to a virus infection.

Metabolic functions important for virus growth

Numerous investigators have searched among the metabolic products of molds, actinomycetes and bacteria to determine whether these organisms produce antiviral as well as antibacterial substances (Jones et al., 1945; Asheshov et al., 1949). Other workers have tested large numbers of different compounds for antiviral activity (Fitzgerald and Babbitt,

1946; Dickinson, 1948; Czekalowski and Dolby, 1949; Wooley et al., 1952). In many of these experiments, virus-infected chick embryos and bacterial virus systems have been used for preliminary screening. These simpler systems provide definite experimental advantages over the use of virus-infected animals. The similarities between the reproduction of phage and of mammalian viruses have led to the hope that some agents effective against phage may be useful against mammalian viruses as well. In certain instances, as with Streptomyces griseus phage which poses a threat to streptomycin production (Perlman et al., 1951), and lactic streptococcus phage which is a constant danger in the manufacture of cheese and cultured milk (Elliker, 1951), the importance of the microorganisms involved has stimulated direct efforts to control phage. In all of these investigations, most of the agents were not effective in preventing virus multiplication. In some instances, however, sufficient knowledge has been gained that reference can be made to certain phases of metabolism in connection with virus growth.

Carbohydrate metabolism. Inhibition of coliphage by the analog desoxypyridoxine was observed by Wooley and Murphy (1949). Growth of the host bacteria was unaffected by the virustatic concentration of desoxypyridoxine. The

antiviral effect was counteracted by pyridoxine, glucose-6-phosphate and several other intermediates of carbohydrate metabolism, acetic acid and several other short-chain fatty acids. The authors suggested that desoxypyridoxine possibly interfered, in some manner, with utilization of glucose.

Cohen and Roth (1953) have studied intermediate carbohydrate metabolism in E. coli in an attempt to understand the mechanism of inhibition of ribose nucleic acid (RNA) synthesis under conditions of viral infection with T-even phages. Normally, E. coli has been found to make extensive use of the oxidative (phosphogluconate) pathway in the metabolism of glucose. Since the ribose of RNA appears to be derived from the oxidative pathway, a possible inhibition of this route in the case of virus infection was suspected. However, virus was produced, though more slowly and in lesser yield, upon cells metabolizing typical intermediates of the oxidative pathway (gluconate, ribose, D-arabinose or nucleosides) as the sole source of carbon. Apparently no enzymatic step of the oxidative pathway is inhibited during the multiplication of virus. They concluded that inhibition of RNA synthesis by T-even phage infection is due to redirection of metabolism at steps not directly involving steps of carbohydrate metabolism.

Protein metabolism. Several reports have pointed out

the synthesis of a specialized virus protein by infected cells. Bacterial antisera do not cross-react with homologous phage particles (Benzer et al., 1950). Phage antisera previously exhausted of bacterial antibodies by exposure to host cells retain full activity toward the phage with which they were prepared. This indicates that the protein moiety of the phage particle is different from proteins normally existing in the host bacteria. Kozloff et al. (1951) presented data which suggested that synthesis of virus protein occurred after infection of the cells and that about 80 per cent of the nitrogenous constituents of the virus were assimilated from the medium after infection. A limited amount of the original bacterial N was used in synthesis of the protein of the first phage particle but apparently this source of N soon was exhausted.

Spizizen et al. (1951) found that salicylate and gentisate, antagonists of tryptophan and tyrosine, respectively, prevented growth of coliphage T2r^{+}. Six- to ten-times the virustatic concentration of salicylate and 100-times that of gentisate were required to delay growth of the host bacteria. Eaton et al. (1951) observed the inhibition of influenza and mumps viruses by basic amino acids. Although several amino acids reduced the rate of virus multiplication in tissue culture, those having two

amino groups were the most effective. Arginine appeared to reduce virus multiplication through an effect upon the virus-host complex. No effect upon the infective or hemagglutinating properties of free virus particles was observed. The virustasis was not due to toxicity of the amino acids to the host tissue. Rafelson et al. (1951) showed that the presence of Theiler's GD VII encephalitis virus in minced mouse brain tissue stimulated incorporation of radioactive carbon from glucose into most of the amino acids but inhibited the incorporation of glucose fragments into lysine and histidine. The amounts of these two amino acids were reduced in virus-infected tissue, whereas the amounts of other acids were unchanged. Lysine and histidine prevented virus growth and uptake of $P^{32}O_4$ in the same system.

The propagation of several viruses, therefore, appears to be related to the metabolism of certain amino acids. Although not definitely established in any of the cases cited, inhibition in these systems would appear to result from an interference with the synthesis of virus protein.

Nucleic acid metabolism. A relationship of the metabolism of nucleic acids and related compounds to virus multiplication has been established by numerous investigators. Cohen and Fowler (1947) found that 5-methyltryptophan completely blocked the stimulated synthesis of DNA occurring

during infection with T2 phage. The authors suggested that this effect possibly resulted from inhibition of synthesis of virus peptides essential for the reception and organization of virus nucleotides. Wooley et al. (1952) observed the inhibition of T2 by furacin. Synthesis of nucleoprotein possibly was blocked through interference of furacin with the xanthine-oxidase-hypoxanthine or a similar system.

The relatively simple and known composition of plant viruses affords the opportunity of correlating the structure of an inhibitor with that of virus constituents. Investigating the formation of tobacco-mosaic virus in isolated tobacco leaf tissue, Commoner and Mercer (1951) found that thiouracil was a potent inhibitor in extremely low concentrations. The inhibiting effect was reversed by uracil, which is one of the constituent pyrimidines of tobacco mosaic virus nucleic acid. Similarly, Moore and Friend (1951) reported that 2,6-diaminopurine inhibited Russian encephalitis virus in mice.

Inhibition of coliphage by levels of acridines which permitted normal development of host cells was reported by Fitzgerald and Lee (1946). The antiviral effect was overcome by RNA. The acridine compounds did not prevent infection of susceptible cells but appeared to prevent

intracellular growth of the virus. This observation was confirmed by the finding of Dickinson (1948) that an acridine compound, proflavine, reduced multiplication of a lysogenic and a normal strain of Pseudomonas phage. Additional studies on the effect of proflavine on coliphage were carried out by Foster (1948). Results of one-step and single infected cell experiments indicated that proflavine reduced the amount of virus produced per infected bacterium, apparently by blocking a late reaction necessary for the formation of active phage. Some of the earlier processes leading to phage production were completed in the presence of the drug. Lysis of infected cells was not prevented. DNA was more effective than RNA in reversing the inhibition of phage multiplication. The author suggested that the chemical step subject to proflavine inhibition might be related to the formation or utilization of DNA for phage synthesis. Green et al. (1946) found that a different acridine compound (nitroakridin) suppressed growth of influenza virus in embryonated eggs.

The nucleic acid fraction of the virus appears to be related to infectivity in the case of certain plant viruses. Bawden and Kleczkowski (1948) observed that ribonuclease combined readily with potato virus X and inhibited the infectivity of the virus; however, little or no destruction of the virus particles occurred. Incubation of the virus-

enzyme complex in borate buffer freed the virus and restored its infectivity. Markham et al. (1948) found that crystalline preparations of turnip yellow mosaic virus contained two components separable by ultracentrifugation. One component was nucleoprotein and was a typical, infective plant virus. The other compound was devoid of nucleic acid, non-infective and smaller by 50 per cent than the infective particle. Composition of the protein fraction, antigenic properties, crystalline form and electrophoretic mobility of the two particles were identical. The infective particle contained 28 per cent pentose nucleic acid, the non-infective particle, none. The authors concluded that combined nucleic acid was essential for the ability of the virus to reproduce itself in susceptible tissue.

Based on the results of numerous investigators, Evans (1952) concluded that in the synthesis of coliphages, a considerable portion of bacterial DNA is incorporated into the phage particle. The bacterial DNA is not transferred intact, however, but appears to be degraded to nucleotide or nucleoside fragments and, in some instances, supplemented by the inclusion of appreciable quantities of material synthesized from constituents present in the medium at the time of infection. The author further suggested that the disturbance of nucleic acid metabolism as a result of virus infection is manifested primarily in reactions concerned

with the organization of large nucleic acid fractions. Synthesis of the smaller sub-units (purines and pyrimidines) probably is accomplished by mechanisms normally present in the host.

Therefore, differentiation between metabolic reactions normally occurring in host cells and reactions involved primarily in virus synthesis probably begins in the organization of high molecular weight nucleic acid fractions. Inhibition of such reactions appears to be the most probable means of suppressing virus multiplication without interfering with normal growth of host cells.

METHODS

Preparation of Media

Enriched skim milk was prepared by supplementing grade-A commercially pasteurized skim milk with ten per cent filtered V-8 juice[1]; 150 ml. of the enriched milk were dispensed into 6-ounce screw-cap bottles. Litmus milk was prepared in exactly the same way except that litmus was added before dispensing 8 ml. into 12 by 120 millimeter test tubes. Both media were autoclaved at 15 lb. pressure for 15 minutes. These and all other media used were stored in the dark at 3 to 5 C.

Trypticase soy agar[2] (TSA) was prepared from the individual ingredients by dissolving 5 g. trypticase, 5 g. phytone, 4 g. sodium chloride, 1 g. sodium citrate, 0.2 g. L(-)cystine, 5 g. dextrose and 15 g. agar agar in 1000 ml. of distilled water. The cystine was dissolved in about 100 ml. of the water with the aid of approximately 1.5 ml. of 2.5 N NaOH. Solution of the other ingredients was accom-

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[1]A combination of eight vegetable juices prepared by the Campbell Soup Co., Camden, N. J.

[2]After a formula suggested by Baltimore Biological Laboratories, Inc., Baltimore, Md.

plished by heating with flowing steam. The medium was adjusted to pH 6.7, resulting in a pH after sterilization of approximately 6.6. The completed medium, dispensed in 150 ml. quantities into 6-ounce screw cap bottles, was autoclaved at 15 lb. pressure for 15 minutes.

Trypticase soy broth (TSB) was prepared exactly the same as TSA except for omission of agar agar, reduction of glucose from 5 to 2.5 g., addition of 52×10^{-4} M $CaCl_2.2H_2O$ and adjustment of the reaction to pH 7.0 before autoclaving. The calcium was autoclaved in the medium which, prior to sterilization, was dispensed into tubes receiving 4.5 ml. and bottles receiving 45 ml. Sterilization was accomplished by autoclaving at 15 lb. pressure for 15 minutes.

The simplified medium of Niven (1944) plus acetate and oleate (Collins and Nelson, 1950) and reticulogen[3] (SM) was prepared as directed by Niven, using the hydrolysed casein. A further simplified modification (FSM) was prepared exactly the same except for omission of the purine and pyrimidine bases, riboflavin, reticulogen, acetate and oleate. The K_2HPO_4 was reduced in both media from 40 to 5 mg. per 10 ml. of medium to avoid precipitation upon the addition of the $CaCl_2.2H_2O$ needed for phage proliferation (Potter and Nelson, 1952b). SM containing 30×10^{-4} M calcium

[3]See table 1, footnote*.

was used for experiments with combinations F6/H1-11 and F68/IP5. FSM without added calcium was used for combination F54/565. For combination F56/712, FSM containing 100 x 10^{-4} M calcium was used. Stock solutions of calcium, autoclaved separately, and reticulogen from the sterile commercial preparation were pipetted aseptically into the sterile media. Thiamine, glutamine and asparagine were sterilized by passing through a Selas #03 microporous porcelain filter, and were added after autoclaving the medium. The remainder of the ingredients were assembled from stock solutions; the mixture was adjusted to pH 7.0, dispensed in 6-ounce bottles in quantities to make 100 ml. of complete medium and autoclaved at 15 lb. for 11 minutes. After combining the sterile components, the media were stored in the dark at 3 to 5 C. for not more than 2 weeks. Immediately before using, 4.5 ml. of medium were dispensed with a pipette into test tubes. The compositions of the two media are listed in table 1.

Selection and Maintenance of Cultures

The cultures of phage and their host bacteria were selected from the Dairy Bacteriology Laboratory collection. Phage-host combinations F4/H1-11, PF11/122-1, F56/712 and F63/E8-1 were chosen on the basis of differences in the host range, heat resistance and antigenic character of the phage

Table 1

Composition of the highly purified basal media

	SM
	micrograms
Riboflavin	10
Calcium pantothenate	10
Nicotinic acid	10
Pyridoxine	10
Thiamine HCl	1
Biotin (crystalline free acid)	0.01
Reticulogen*	2 microliters
Xanthine	50
Adenine SO_4	50
Guanine HCl	50
Uracil	50
	milligrams
Vitamin free acid hydrolysed casein	5
Glutamine	1
Asparagine	1
Dextrose	50
Sodium thioglycolate	1
Sodium acetate	20
Sorbitan monooleate	20
Salts	**
Water to make	10 ml.

FSM
SM minus:
Riboflavin
Reticulogen*
Xanthine
Adenine SO_4
Guanine HCl
Uracil
Sodium acetate
Sorbitan monooleate

*A parenteral liver extract with vitamin B_{12}, obtained from Eli Lilly and Co., Indianapolis, Indiana.

**Salts consisted of 0.8 mg. $MgSO_4.7\ H_2O$, 40 micrograms $FeSO_4.7\ H_2O$, 12 micrograms $MgCl_2$, 5 mg. $K_2HPO_4.3\ H_2O$ per 10 ml. of medium, plus 0, 30 or 100 x 10^{-7} M $CaCl_2.\ 2\ H_2O$, depending on the phage-host combination used.

(Wilkowske, 1949). F54/565 was chosen because of the ability of phage F54 to develop in SM without added calcium, and F6/H1-11 was selected in the latter part of the work because of certain similarities to F56/712. Prior to use, purity of the cultures was insured by plating the bacteria and reisolating a single colony three times in succession; the phage then was plated using the purified host bacteria and three serial single-plaque isolations were made.

The bacteria were maintained in litmus milk. The cultures were incubated at 32 C. until coagulated, then stored at 3 to 5 C. between transfers. Cultures in regular use were transferred daily; otherwise, fresh propagations were made at weekly intervals.

Bacteriophage was propagated in enriched skim milk. The milk was inoculated with about 10^5 cells and 10^3 phage particles per ml. After incubation at 32 C. for 8 to 10 hours, the milk was coagulated with 5.5 ml. of sterile ten per cent lactic acid per 150 ml. of milk. The clear whey, obtained by filtering through sterile, coarse filter paper, was passed through a Selas #03 microporous porcelain filter to remove bacterial cells. The resultant whey filtrate was diluted one-to-ten into sterile skim milk, TSB or other medium in which the phage was to be used for experiments. The titer of such phage preparations usually was 10^7 or 10^8 per ml. and the keeping quality was very good when stored at

3 to 5 C. Fresh propagations of phage were made only after the titer had decreased about 100-fold as a result of storage for several months.

Experimental Procedures

Counts of bacteria and bacteriophage

Bacteria were counted by the agar plate method (American Public Health Association, 1948), except that TSA was used as the nutrient medium. Bacteriophages were enumerated with the two-layer plaque-plate method of Potter and Nelson (1952a) except that tomato juice agar was replaced by TSA and one per cent $CaCl_2.2H_2O$ was the cell diluent.

Screening and testing methods

Agar plate method for screening antibiotic-producing organisms. Spores were grown on slants of Carvajal's oat meal agar (65 g. rolled oats plus 1000 ml. H_2O, boil, filter through cheesecloth, restore volume to 1000 ml. with H_2O, add two per cent agar and sterilize at 15 lb. pressure for 20 minutes; Raper, 1952) or TSA. For testing, a basal layer of about 20 ml. of agar was poured into a petri dish, solidified, inverted and dried by incubating overnight at 32 C. A needle then was used to inoculate the center of the plate very lightly with spores. The plate was incubated for 4 to 6 days at 32 C. to allow for production of the antibiotic

and its diffusion into the basal layer.

The antibiotic then was tested on the same plate in the following manner. Overlay suspensions of bacteria and of phage plus bacteria were prepared as described subsequently for the paper disc plate method. About 1.5 ml. of the bacterial overlay was pipetted onto a small area near one edge of the basal layer. By carefully tilting and rotating the plate the overlay was flowed toward the center until the giant colony was partially surrounded but not quite touched by a semi-circle of overlay material. After allowing a few minutes for solidification of the bacterial overlay, the same procedure was repeated in the other half of the plate with the overlay of phage plus bacteria. Thus a single large colony of antibiotic-producing organism served to test for host toxicity and antiphage activity.

Emmerson's agar (Emmerson et al., 1946), Brewer's medium (Brewer, 1943) modified by substituting phytone for the soy bean meal and TSA were used for basal layers. TSA was used in the overlay in all cases.

Paper disc plate method. A two-layer plaque-plate was prepared by the regular procedure for enumerating phage except that an excess of phage was included in the overlay to produce complete clearing of the agar. A uniform quantity of test solution was taken up with a 0.01 ml. standard loop which then was touched to a sterile paper disc in a dry petri

dish. The disc, now wetted with the drop of test solution and adhering to the loop by surface tension, was deposited on the solidified overlay of the plaque plate. A control plate was prepared in a similar manner except that the overlay contained bacteria only, the phage dilution being replaced by sterile water. Three dilutions of each of two test solutions, or a total of six paper discs, were examined on each plate.

The plates were observed after incubation for 15 to 17 hours at 32 C. Inhibition of phage was indicated by a zone of bacterial growth around the disc on an otherwise completely lysed plate. Host toxicity was indicated by a clear zone around a disc on the control plate which otherwise was turbid from growth of the bacteria. On the phage plates, a double zone frequently was seen when the test solution inhibited phage but also was very toxic to the host bacteria. In these cases, the disc was surrounded first by a clear zone where the bacteria were inhibited by the high concentration of test solution. Further out, where the concentration of inhibitor was less, a concentric circle of cell growth was seen and still further out phage action again cleared the plate. For this reason, the width of the band of growth rather than the outside diameter of the zone was recorded in all cases. Since this complication did not exist on the bacterial plates, inhibition was recorded in terms of zone diameters. All

measurements were expressed in millimeters. No quantitative comparison of different inhibitors can be made due to unknown differences in the diffusion rates of different substances; however, a rough indication of the threshold values for phage and host inhibition of a given compound can be obtained with this method.

The paper discs were cut from Blue Bird blotter paper[4], with a 6 millimeter office punch. About 250 discs were placed in a small screw cap bottle and autoclaved at 15 lb. pressure for 30 minutes. The standard loop was cleaned thoroughly with distilled water and sterilized by flaming.

Tube method. To one of two tubes each containing 4.5 ml. of broth, 0.5 ml. of inhibitor solution was added. The other tube received 0.5 ml. of sterile water and served as a control. Both tubes were inoculated with about 10^5 cells and 10^3 phage particles per ml. After incubation, the tubes were observed visually. The control tube was cleared as a result of phage action; turbidity in the other tube indicated that the inhibitor prevented mass lysis. A second pair of tubes was inoculated with cells only. Turbidity in the control of this pair indicated normal growth. Less or no turbidity in the other tube indicated toxicity of the inhibitor to the bacteria.

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[4]Made by the Wrenn Paper Co., Middletown, Ohio.

With slight modification, this procedure also was used for testing the ability of metabolites to counteract the effect of inhibitors. For these tests, an inhibitor solution 100-fold more concentrated than necessary to prevent mass lysis was diluted one to 100 into TSB. Four and one-half ml. of the resultant inhibitory broth were dispensed into each of two test tubes. To one tube was added 0.5 ml. of the metabolite solution. The other tube received 0.5 ml. of sterile water and served as a control. Both tubes were inoculated with phage and cells. After incubation, the control tube was turbid because mass lysis was prevented by the inhibitor. Clearing of the tube containing added metabolite indicated that the antiviral inhibitor was counteracted by the metabolite. Additional controls in TSB containing no inhibitor insured normal activity of the phage and lack of toxicity from the added metabolite.

Cultures of bacteria were grown in the test medium for at least three transfers prior to use. The phage was suspended in the same medium as described under selection and maintenance of cultures. Both the cells and phage were diluted in sterile medium so that an inoculum of one drop of each gave the desired initial population. Incubation was at 32 C. for 10 hours in the case of TSB and 18 to 24 hours for SM and FSM.

Long-time growth experiments. Generally the procedure was to add 5 ml. of inhibitor solution to 45 ml. of medium. The 50 ml. of medium then were inoculated with cells and phage. Plate counts of each were made periodically for periods up to 10 to 24 hours. Mass lysis, phage increase, cell multiplication and virucidal effects were studied with this method. Mass lysis in these experiments was considered to be initiated at the time when the bacterial counts began to decrease sharply; the magnitude and rate of decrease in count are a measure of the extent of mass lysis. The minimum concentrations of inhibitor which significantly reduced growth of bacteria(MBIC) and phage (MPIC) were determined by the following criteria. The MBIC was the concentration of inhibitor which lowered by 50 per cent the count of host bacteria after incubation for 10 hours in the absence of phage. The concentration which reduced proliferation of phage 100-fold after similar incubation in the presence of host cells was the MPIC. The reduction of count in each case was determined by comparison with a control incubated without inhibitor. The inhibition ratio (IR) was equal to the ratio MBIC/MPIC. As suggested by Spizizen et al. (1951), inhibitors having a ratio greater than one are considered more active against the phage system than against bacterial growth. Conversely, inhibitors with a ratio of one or less probably reduce phage growth primarily by limiting the amount of cellular substrate produced.

One-step growth experiments. The one-step growth method of Delbrück and Luria (1942) was applied in the following way. Host bacteria were grown at 32 C. for 4 to 5 hours, depending on the experiment. The physiological state and size of inoculum were controlled to reproduce closely the cell counts for a given period of incubation; these ranged from about 10^7 to 10^8 per ml. Five ml. of a culture then were mixed with 5 ml. of phage diluted in similar medium at 32 C. to give a ratio of phage to cells between one to 50 and one to 500. In all cases, the adsorption time was 10 minutes. The multiplicity of infection, which is the number of phage particles per bacterium, was approximately equal to the above ratio as more than 90 per cent adsorption occurred in all cases. Under these conditions practically no bacterium is infected by more than one phage particle. After adsorption, the mixture was diluted 10^4-fold in the particular medium to prevent further adsorption and reduce the phage concentration to a countable number (about 20 to 200 plaques per plate). In certain experiments, only a 10^3-fold dilution was made immediately after adsorption in order to permit further dilution necessary for introduction or removal of inhibitor during the latent period. After incubation for 20 minutes, a 10-fold dilution in addition to the 10^4-fold dilution was made to reduce readsorption following the burst

and to retain a countable number of particles. The total time of incubation following adsorption was 60 to 120 minutes, depending on the experiment. Aliquots were removed from the mixture following the adsorption period (zero time) and at 5 or 10 minute intervals throughout the critical portion of the curves and titered by the regular plaque method. Cell counts were made at the beginning and near the end of the experiment.

Single infected cell method. Adsorption was accomplished exactly as in the one-step growth method, then the mixture was diluted to a point at which less than one infected bacterium was present in 3 ml. Three ml. samples of this mixture were dispensed into each of a series of test tubes, incubated at 32 C. for 55 minutes and placed in an ice bath to keep further changes at a minimum during the time required to titer the tubes for phage by the regular plaque count method. In the plating procedure, the 3 ml. of growth medium replaced the phage dilution normally used. Three ml. of the usual cell suspension in the calcium diluent and 3 ml. of agar were added, then 6 ml. of the resultant 9 ml. mixture were divided between two plates. The burst size was calculated by multiplying the total plaque count of the two plates by 1.5.

Preparation of inhibitor solutions. The general practice was to prepar 1 M or less concentrated aqueous

solutions, depending on the solubility of the compound. In some instances neutral solvents or alkali were required to help dissolve the material. Sterile water was used for dissolving the compounds and in making dilutions. The solutions were not sterilized, with the exception of the antimetabolites and a few non-toxic substances, all of which were sterilized by passage through a Selas #03 microporous porcelain filter. Controls for sterility of the solutions were included in all experiments. For tests in liquid media, the solutions were prepared ten- or 100-fold more concentrated than desired in the medium, then added in the proportion necessary to give the desired concentration in the growth medium.

Spectrometric determinations. Spectrometric determinations were made with a Coleman model no. 11, 8-volt spectrophotometer equipped with a Coleman universal filter PC4. Maximum absorption occurred with crystal violet at 562.5 millimicrons, and all measurements of concentration were made at this wave length. Maximum absorption with DNA occurred at 350 millimicrons, in the range 300 to 800 millimicrons. Dilute solutions of DNA (0.4 g. per 100 ml.) gave greater than 90 per cent transmittance at 562.5 millimicrons.

RESULTS

This investigation developed in two phases. First, a diverse group of agents was screened for antiphage activity. Crystal violet was found to selectively inhibit two strains of phage. An analysis of the mechanism of inhibition by crystal violet comprises the second part of this report.

Screening

The 110 agents tested are listed in table 2. Included are eleven antibiotic-producing organisms. Good antibiotic production was obtained with all of the five bacilli and six actinomycetes, as indicated by inhibition of lactic streptococci; none of these antibiotics prevented mass lysis of the host bacteria by phage on agar plates. Of the 99 other agents tested, ten reduced the extent of mass lysis with one or more of four phage-host combinations used in the paper-disc, agar-plate test. The effect of three dilutions of each of these inhibitors on mass lysis (a) and the host bacteria (b) is shown in table 3. Numerous other agents were toxic to the host bacteria but did not prevent mass lysis in any concentration employed.

Table 2

Agents tested for virustatic activity

Number	Name	Group
		Alkaloids
1.	Caffeine	
2.	Nicotine	
3.	Quinine	
4.	Strychnine	
5.	Tannic acid	
		Antibiotics
6.	Aureomycin	
7.	Chloromycetin	
8.	Penicillin G	
9.	Streptomycin	
10.	Terramycin	
		Organisms producing antibiotics
	Organism	Antibiotic produced commercially
11.	*Bacillus brevis*	Tyrothrycin
12.	*Bacillus licheniformis*	Bacitracin
13.	*Bacillus polymyxa*	Polymyxin
14.	*Bacillus subtilus*	Bacillin
15.	*Bacillus subtilus*	Subtilin
16.	*Streptomyces aureofaciens*	Aureomycin
17.	*Streptomyces fradiae*	Neomycin
18.	*Streptomyces griseocarnus*	Hydroxystreptomycin
19.	*Streptomyces griseus*	Streptomycin
20.	*Streptomyces rimosus*	Terramycin
21.	*Streptomyces* species	Actinomycin
		Antimetabolites
	Analog	Metabolite antagonized
22.	3-Acetylpyridine	Nicotinic acid
23.	Adenosine	Cytidine
24.	4-Amino-N-methylfolic acid	Folic acid
25.	Barbituric acid	Uracil
26.	γ-Benzenehexachloride	Inositol
27.	Benzimidazole	Adenine, guanine and uracil

(Continued)

Table 2

(Continued)

Number	Name	Group
		Antimetabolites (ctd.)
	Analog	Metabolite antagonized
28.	2-Chloroaminobenzene	PABA
29.	8-Chloroxanthine	Purines and nucleic acid
30.	Desoxypyridoxine hydrochloride	Pyridoxine
31.	dl-Desthiobiotin	Biotin
32.	Dicumarol	Menadione
33.	Ethionine	Methionine
34.	N^{10}-Methylpteroylglutamic acid	Folic acid
35.	Pantoyltaurine	Folic acid
36.	Pyridine-3-sulfonic acid	Nicotinic acid
37.	Quercetin	Rutin
38.	β-2-Thienylalanine	Phenylalanine
39.	Thiouracil	Uracil
	Dyes, dye intermediates and other organic materials	
40.	Acetylsalicylic acid	
41.	Auramine (base)	
42.	Brilliant green	
43.	Bromcresol green	
44.	α-Bromobutyric acid	
45.	α-Bromopropionic acid	
46.	α-Bromovaleric acid	
47.	Bromthymol blue	
48.	Butter yellow (dimethylaminoazobenzene)	
49.	Chloroacetic acid	
50.	Citrus pectin	
51.	Crystal violet	
52.	3,6-Diaminoacridine sulfate	
53.	2,4-Dinitrophenol	
54.	Eosine	
55.	Ethyl carbamate (urethan)	
56.	Fuchsin (basic)	
57.	Hexamethylenetetramine	
58.	Hydroquinone	
59.	8-Hydroxyquinoline sulfate	
60.	Indigo	
61.	Janus green	

(Continued)

Table 2

(Continued)

Number	Name	Group
		Dyes, dye intermediates and other organic materials (ctd.)
62.	Meldola blue	
63.	Methylene blue thiocyanate	
64.	Methyl orange	
65.	Methyl red	
66.	Neutral red	
67.	Nile blue A	
68.	ρ -Nitrobenzoic acid	
69.	Phenosafranine	
70.	ρ -Phenylenediamine	
71.	Resazurin	
72.	Rhodamine B	
73.	Roccal (alkyldimethylbenzylammonium chlorides)	
74.	Safranine O	
75.	Salicylic acid	
76.	Salicylaldehyde	
77.	Sodium alizarin sulfonate	
78.	Streptokinase-streptodornase	
79.	Sulfanilic acid	
80.	Sulfosalicylic acid	
81.	Tartrazine	
82.	4,4'-Tetramethyldiaminotriphenylmethane (leuco malachite green)	
83.	Tetramethyl-ρ -phenylenediamine hydrochloride	
84.	Toluidine blue O	
85.	Trypan blue	
		Enzyme poisons
86.	Iodoacetic acid	
87.	Malonic acid	
88.	Sodium azide	
89.	Sodium cyanide	
90.	Sodium fluoride	
		Metallic salts
91.	$Al_2Cl_6 \cdot 12H_2O$	
92.	$CoCl_2 \cdot 6H_2O$	
93.	$CuCl_2 \cdot 2H_2O$	

(Continued)

Table 2

(Continued)

Number	Name	Group
		Metallic salts (ctd.)
94.	$Fe_2(SO_4)_3$	
95.	$FeSO_4$	
96.	$HgCl_2$	
97.	$La(NO_3)_3 .6H_2O$	
98.	$LiCl_2 . H_2O$	
99.	$NiCl_2 . 6H_2O$	
100.	$PbOH(C_2H_3O_2)_3$	
101.	$SbCl_3$	
102.	$SnCl_2$	
103.	$ZnSO_4$	
		Sulfa drugs
104.	Sulfadiazine	
105.	Sulfasuxidine	
106.	Sulfamerazine	
107.	Sulfanilamide	
108.	Sulfaguanidine	
109.	Sulfathalidine	
110.	Sulfathiazole	

Table 3

Results with agents which prevented mass lysis of one or more phage-host combinations in the paper-disc, agar-plate test

Concentration added to the paper disc	Width of zone of inhibition (mm.) F4/H1-1 (a)*	(b)	PF11/122-1 (a)	(b)	F56/712 (a)	(b)	F63/E8-1 (a)	(b)
Aureomycin (no. 6)								
1000 mg %	-	27	-	30	-	28	-	20
100 "	-	22	-	25	-	26	9	20
10 "	-	16	-	20	-	19	5	12
Benzimidazole (no. 27)								
Saturated	3	-**	-	10	2	-	2	-
0.1 "	-	-	-	-	-	-	-	-
0.01 "	-	-	-	-	-	-	-	-
Chloromycetin (no. 7)								
100 mg %	-	20	-	13	9	23	-	-
10 "	3	15	-	25	2	14	-	14
1 "	7	-	-	-	-	-	-	-
Crystal violet (no. 51)								
1000 mg %	-	-	-	-	3	20	-	-
100 "	-	-	-	-	2	18	-	-
10 "	-	-	-	-	-	-	-	-
3,6-Diaminoacridine sulfate (no. 52)								
0.01 molar	3	27	4	30	6	18	5	25
0.001 "	-	22	-	23	-	15	-	20
0.0001 "	-	16	-	18	-	-	-	15
8-Hydroxyquinoline sulfate (no. 59)								
0.1 molar	18	45	-	40	3	40	-	35
0.01 "	13	25	3	20	3	20	5	20
0.001 "	5	12	4	10	4	13	3	10
Iodoacetic acid (no. 86)								
0.1 molar	-	34	-	-	6	17	-	35
0.01 "	-	21	-	-	-	-	-	20
0.001 "	-	-	-	-	-	-	-	-

(Continued)

*a=effect on mass lysis; b=effect on host bacteria.
**Negative sign indicates no inhibition.

Table 3

(Continued)

Concentration added to the paper disc	Width of zone of inhibition (mm.) F4/H1-1 (a)	(b)	PF11/122-1 (a)	(b)	F56/712 (a)	(b)	F63/E8-1 (a)	(b)
Salicylaldehyde (no. 75)								
1000 mg %	12	20	-	-	8	-	-	-
100 "	5	-	-	-	-	-	-	-
10 "	-	-	-	-	-	-	-	-
Terramycin (no. 10)								
100 mg %	5	22	-	28	8	26	-	23
10 "	-	17	-	23	6	20	-	18
1 "	-	12	-	12	2	13	-	12
Tetramethyl-ρ-phenylenediamine·HCl (no. 83)								
0.1 molar	7	-	7	-	7	-	-	-
0.01 "	-	-	3	-	-	-	-	-
0.001 "	-	-	-	-	-	-	-	-

The agents which prevented mass lysis on plates were tested further with the tube method, regardless of the degree of toxicity indicated on the control plates. In addition, several completely ineffective agents of limited solubility were retested at higher levels by dissolving the inhibitor in the broth medium. This avoided the dilution resulting from adding an aqueous solution of inhibitor to the medium, as was necessary in the plate method. All of the antimetabolites and metallic salts were tested by the tube method in both TSB and SM media.

In the first series of tube tests, decimal dilutions of inhibitor were used to establish the approximate concentration which prevented growth of the host bacteria. Then a 100-fold range immediately below this level was tested for toxicity to the host bacteria and prevention of mass lysis, using two-fold increments of inhibitor throughout the range. In this way an indication of the selectivity of the inhibitor was obtained. Inhibition ratios estimated from these tests generally were greater than those subsequently determined by plate counts.

All of the ten agents which were effective on agar plates also prevented mass lysis when tested by the tube method. However, most were extremely toxic to the host bacteria. The effect of these and other compounds was very similar in TSB and SM media, although the selectivity tended

to be slightly greater in TSB. A lower level of inhibitor was required in the more highly purified SM medium. The inhibition ratio (IR) and minimum bacterial inhibitory concentration (MBIC) determined in TSB for these ten agents are given in table 4. For nine of these compounds, the IR ranged from less than one to two with the most sensitive phage, indicating considerable toxicity to the host bacteria. However, crystal violet had an IR of three to four with F56/712. With other combinations, the IR of crystal violet ranged from one to three. At least with F56/712, crystal violet appeared to selectively inhibit multiplication of the phage.

The representative data presented in tables 5 through 10 illustrate the type of experiment used for determination of inhibition ratios and show in detail the effect of the most selective agents. The counts of free phage particles incubated without and with inhibitor in the absence of host cells show virucidal effects. Counts of bacteria incubated in the absence of phage indicate the degree of toxicity to the host cells. Phage and bacteria counts of mixtures containing cells and phage reveal the effects on mass lysis and phage proliferation.

Table 5 shows the influence of salicylaldehyde on F56/712. A slight inactivation of phage particles resulted from incubation in the presence of this compound. The MBIC

Table 4

Inhibition ratio (IR) and minimum bacterial inhibitory concentration (MBIC) of the ten agents which prevented mass lysis on agar plates

Name of compound	IR in TSB**		MBIC*** in: TSB	SM	Concentration (units)
Aureomycin*	1	(F56)	0.01		mg. %
Benzimidazole	1-2	(F56)	100	50	x 10^{-4} M
α-Bromopropionic acid*	1	(F56)	64	10	x 10^{-4} M
Crystal violet	3-4	(F56)	16	10	x 10^{-7} M
3,6-Diaminoacridine-SO_4*	1	(PF11)	10	5	x 10^{-6} M
8-Hydroxyquinoline-SO_4*	1	(F68)	500	10	x 10^{-6} M
Iodoacetic acid	1-2	(F56)	240	5	x 10^{-6} M
Salicylaldehyde	0.7	(F56)	14		mg. %
Tetramethyl-ρ-phenylene-diamine.2 HCl*	1	(PF11)	50		x 10^{-6} M
Terramycin	1-2	(F4)	40	10	mg. %

*The IR and MBIC for these five compounds were estimated from results of tube tests.
**Results with the most sensitive phages which are indicated in parentheses.
***Average value for four strains of host bacteria.

Table 5

Effect of salicylaldehyde on phage-host combination F56/712 in TSB

System tested	Count of control at 0 time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor: 0	6.0	12	16	24	32	48
F56 (no cells)	3×10^3	49×10^1		3×10^2		5×10^2		31×10^1
712 (no phage)	55×10^5	97×10^7	116×10^7	102×10^7	56×10^7	31×10^7	165×10^6	49×10^6
F56/712 (cell count)	57×10^5	197×10^3	92×10^4	35×10^6	271×10^6	330×10^6	203×10^6	60×10^6
F56/712 (phage count)	3×10^3	11×10^7	10×10^7	15×10^7	80×10^5	31×10^5	31×10^3	3×10^1

*Mg. per 100 ml. of broth.

was between 16 and 24 mg. per cent salicylaldehyde, whereas the MPIC was between 24 and 32 mg. per cent. This gives for salicylaldehyde an IR slightly less than one. Some proliferation of phage occurred in the presence of toxic levels of this compound. However, an inhibitor concentration of 24 mg. per cent permitted the development of a normal cell count in the presence of phage. When incubated in the presence of host cells, the phage was more susceptible to inactivation by salicylaldehyde than when the phage was incubated without cells present. A similar effect was seen in several instances with other agents. After phage is adsorbed to host cells, the virus-host complex represents phage in a rapidly developing state, as contrasted to the dormant nature of free phage particles in the absence of host cells. Actively growing bacteria are more susceptible than are physiologically inactive bacteria to a variety of deleterious conditions. These data suggest that this may be equally true in the case of viruses.

In table 6 the effects of iodoacetic acid on F56/712 are presented. No destruction of phage occurred in the absence of host cells with levels of iodoacetic acid up to 100×10^{-7} M, which was eight times the MBIC. The MPIC was between 6 and 12×10^{-7} M, giving an IR of one to two. Mass lysis was prevented by 12×10^{-7} M iodoacetic acid. A possible

Table 6

Effect of iodoacetic acid on phage-host combination F56/712 in TSB

System tested	Count of control at 0 time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor: 0	2	6	12	18	24	36	100
F56 (no cells)	43×10^2	31×10^2			36×10^2		43×10^2		43×10^2
712 (no phage)	91×10^5	103×10^7	102×10^7	100×10^7	45×10^7	73×10^7	41×10^7	65×10^6	41×10^5
F56/712 (cell count)	75×10^5	1000×10^2	520×10^2	34×10^4	43×10^7	64×10^7	35×10^7	89×10^6	39×10^5
F56/712 (phage count)	53×10^2	13×10^7	25×10^7	23×10^7	69×10^3	288×10^3	45×10^3	428×10^1	52×10^1

*$\times 10^{-7}$ M.

slight stimulation of both cell and phage counts occurred with an inhibitor concentration of 18 x 10^{-7} M. This finding was not repeated, however, and may not be valid. As the counts indicated only slight toxicity with 24 x 10^{-7} M iodoacetic acid, further experiments were carried out using the more sensitive one-step growth method. The data plotted in figure 1 indicate that iodoacetic acid did not selectively inhibit F56. In fact, an appreciable increase of phage occurred in spite of the extremely toxic or possible slight bactericidal effect of the inhibitor on the host cells. Inactivation of some infected cells apparently is shown by the decreasing phage counts during the latent period.

In table 7 is shown the effect of benzimidazole on F56/712. A slight virucidal effect which increased with the inhibitor concentration is seen from the counts of phage incubated without cells. Toxicity to the host bacteria increased similarly, the MBIC being between 4 and 8 x 10^{-3} M benzimidazole. In contrast to salicylaldehyde, which even at very toxic levels permitted an appreciable increase of phage, benzimidazole in relatively less toxic concentrations markedly reduced proliferation of the phage. The MPIC for benzimidazole was between 4 and 8 x 10^{-3} M, giving an IR of about one. Definite inactivation of virus in the presence of host cells occurred with 10 x 10^{-3} M benzimidazole. Mass lysis was prevented by 8 x 10^{-3} M. These results were con-

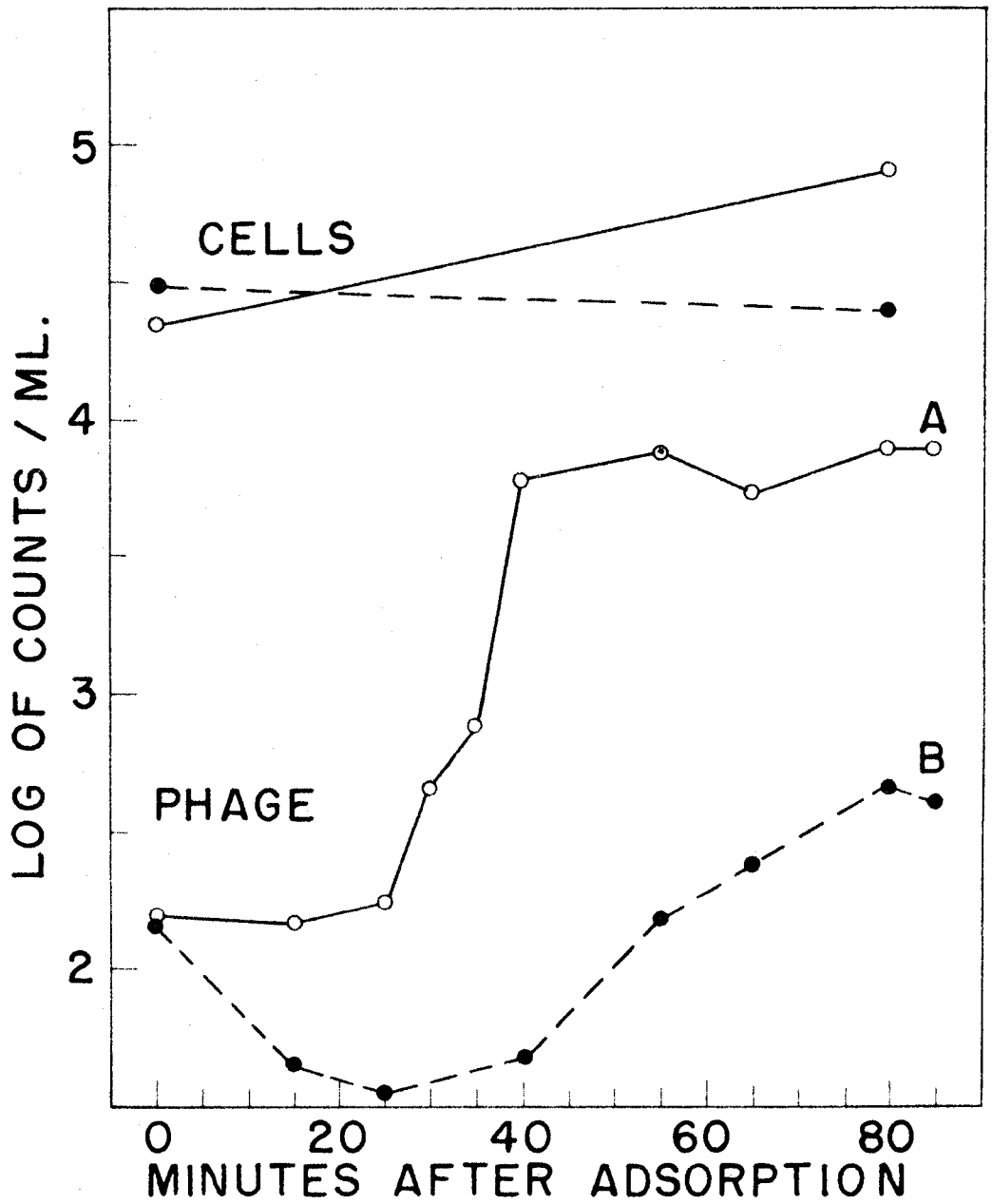

Figure 1. Effect of iodoacetic acid on the one-step growth curve of F56/712.

A=0, B=1.2 x 10^{-7} M iodoacetic acid.
Cells: open circles = 0, closed circles = 1.2 x 10^{-7} M iodoacetic acid.

Table 7

Effect of benzimidazole on phage-host combination F56/712 in TSB

System tested	Count of control at o time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor: 0	2	4	8	10
F56 (no cells)	102×10^2	135×10^2	96×10^2	88×10^2	81×10^2	74×10^2
712 (no phage)	107×10^5	82×10^7	84×10^7	57×10^7	29×10^7	202×10^6
F56/712 (cell count)	102×10^5	52×10^4	51×10^5	49×10^6	270×10^6	236×10^6
F56/712 (phage count)	73×10^2	9×10^7	316×10^5	333×10^5	147×10^1	3×10^1

*$\times 10^{-3}$ M.

firmed upon repeated trials.

Tables 8 and 9 show the effect of crystal violet on F56/712 and F68/IP5, respectively. Both F56 and F68 incubated in the absence of host cells were not inactivated by the levels of dye used. Normal multiplication of both host cultures occurred in levels of crystal violet which hindered proliferation of the homologous phage. The IR for F56/712 was about three and for F68/IP5 slightly greater than one. These experiments were repeated several times and the values presented in table 4 are representative findings for the various runs. Furthermore, the effect upon F56/712 was confirmed with the one-step growth method as shown in figure 2. Crystal violet very effectively prevented increase of the phage, whereas the rate of multiplication of the host cells was not altered. No inactivation of adsorbed phage occurred in this experiment.

Crystal violet apparently was the only one of the entire group of agents tested which selectively inhibited phage. Screening tests with 41 other phage-host combinations revealed that most of the additional strains of virus were less susceptible to crystal violet than were F56 and F68. Mass lysis of F6/H1-11 and F72/R1 was prevented on agar plates; however, further tests showed that the IR was one or less for these combinations. The data presented in table 10 show that crystal violet definitely was not a selective

Table 8

Effect of crystal violet on phage-host combination F56/712 in TSB

System tested	Count of control at 0 time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor: 0	0.26	1.0	2.1	3.1	4.1	6.2	16.0
F56 (no cells)	28 x 10^2	34 x 10^2	33 x 10^2		27 x 10^2		38 x 10^2		44 x 10^2
712 (no phage)	79 x 10^5	76 x 10^7	74 x 10^7	65 x 10^7	87 x 10^7	57 x 10^7	44 x 10^7	35 x 10^6	232 x 10^4
F56/712 (cell count)	76 x 10^5	186 x 10^3	75 x 10^4	46 x 10^7	53 x 10^7	55 x 10^7	43 x 10^7	58 x 10^6	181 x 10^4
F56/712 (phage count)	30 x 10^2	8 x 10^7	10 x 10^7	47 x 10^5	190 x 10^1	115 x 10^1	357 x 10^1	204 x 10^1	37 x 10^0

*x 10^{-7} M.

Table 9

Effect of crystal violet on phage-host combination F68/IP5 in TSB

System tested	Count of control at 0 time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor:					
		0	1.0	2.1	3.1	4.1	6.2
F68 (no cells)	59×10^{0}	63×10^{0}	53×10^{0}	61×10^{0}	62×10^{0}	63×10^{0}	60×10^{0}
IP5 (no phage)	47×10^{5}	53×10^{7}	48×10^{7}	38×10^{7}	185×10^{6}	40×10^{6}	
F68/IP5 (cell count)	47×10^{5}	109×10^{4}	187×10^{6}	34×10^{7}	267×10^{6}	44×10^{6}	
F68/IP5 (phage count)	63×10^{0}	890×10^{7}	25×10^{7}	62×10^{5}	7×10^{3}	1×10^{2}	

*$\times 10^{-7}$ M.

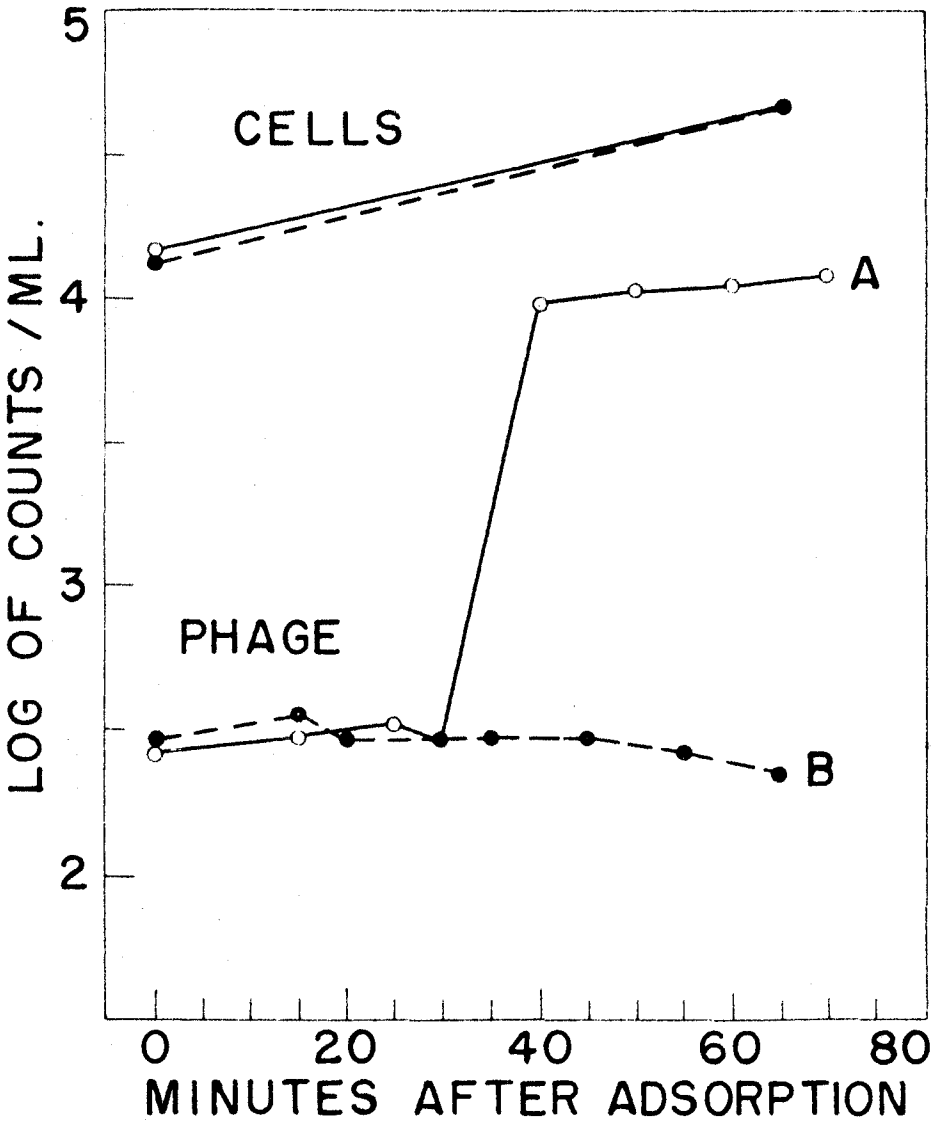

Figure 2. Effect of crystal violet on the one-step growth curve of F56/712.

A=0, B=1.0 x 10^{-7} M crystal violet.
Cells: open circles = 0, closed circles = 1.0 x 10^{-7} M crystal violet.

Table 10

Effect of crystal violet on phage-host combination F72/R1 in TSB

System tested	Count of control at 0 time	Count after incubation for 10 hours in TSB containing the following concentrations* of inhibitor: 0	1.0	2.1	4.2
F72 (no cells)	51×10^2	62×10^2			55×10^2
R1 (no phage)	23×10^4	22×10^7	14×10^7	130×10^6	14×10^5
F72/R1 (cell count)	24×10^4	3×10^1	1×10^3	5×10^3	8×10^3
F72/R1 (phage count)	42×10^2	52×10^7	317×10^7	395×10^7	27×10^3

*$\times 10^{-7}$ M.

inhibitor for F72. With many other combinations, of which F55/573 was typical, on agar plates mass lysis occurred in levels of crystal violet markedly toxic to the host bacteria. Thus F68, F6 and F72 represented types intermediate between F56 and F55 in susceptibility to inhibition by crystal violet.

In addition to the tests in TSB and SM, the effect of crystal violet in milk also was determined. The IR was almost identical in all three media. However, approximately 1000-fold more dye was required in milk than in TSB. The MBIC in milk was about 1.0×10^{-4} M, in TSB 16×10^{-7} M and in SM about 10×10^{-7} M.

Analysis of the Action of Crystal Violet

Having established that crystal violet selectively reduced the increase of F68 and particularly F56 on their respective hosts IP5 and 712, an investigation of the inhibition mechanism was initiated. The previous finding that crystal violet did not inactivate unadsorbed phage particles suggested a possible effect upon the process of virus multiplication.

Effects on growth and mass lysis

The minimum bacterial inhibitory concentrations (MBIC) and inhibition ratios (IR) reported in table 4 of the preceding section were based on counts made after incubation for 10 hours at 32 C. In the more detailed experiments described here, periodic counts were made throughout the incubation period.

Figure 3 shows the effect of crystal violet on multiplication of S. lactis 712 in TSB. No toxicity is seen in the case of 1.0 and 2.1 x 10^{-7} M crystal violet, whereas 3.1 x 10^{-7} M definitely was toxic to the bacteria. The toxicity is manifested by extension of the lag phase and a reduced rate of increase during the logarithmic phase. The bacterial counts at 10 hours are about equal in all cases. These findings were confirmed in numerous other experiments.

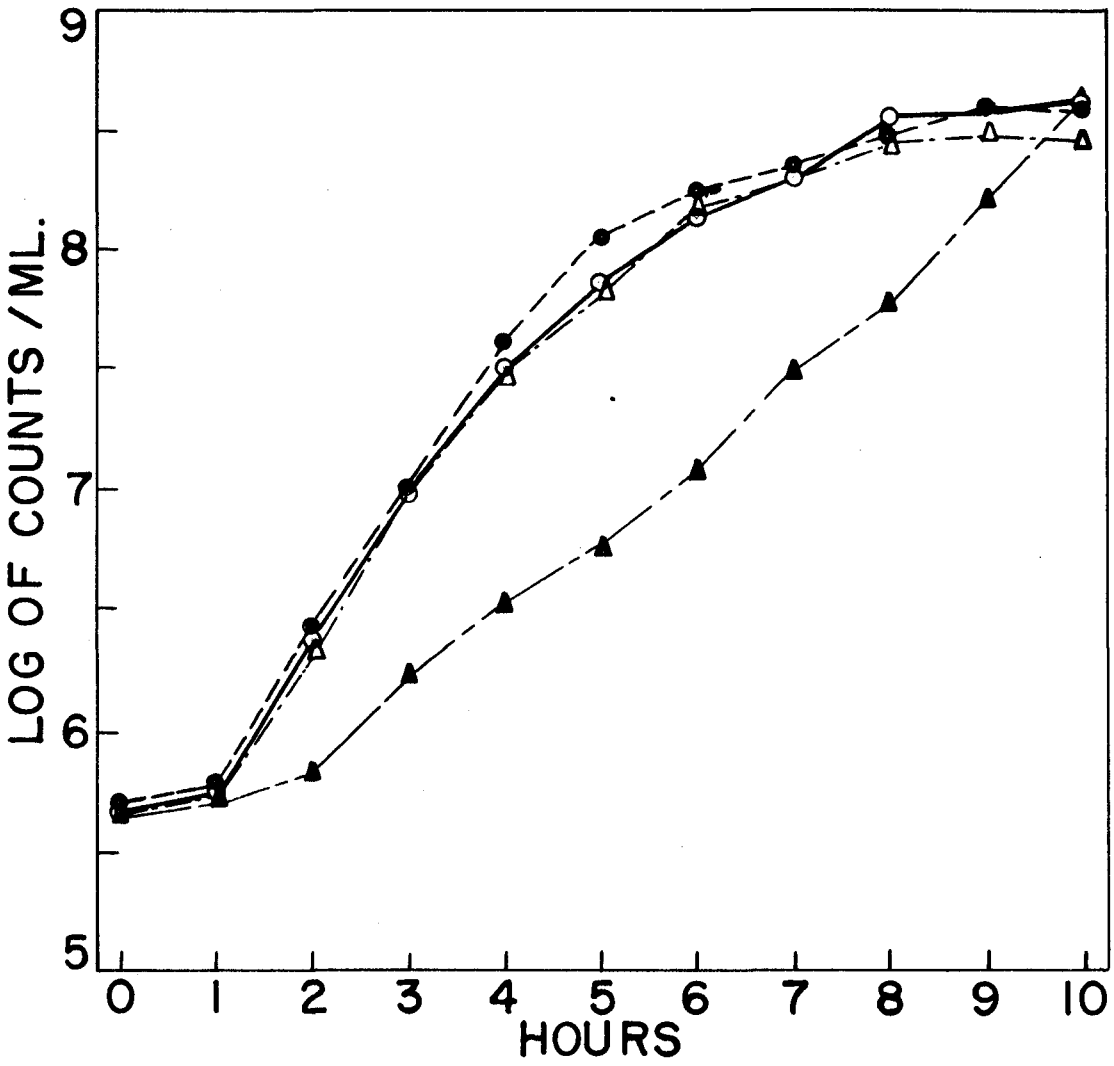

Figure 3. Counts of host bacteria (712) in TSB containing crystal violet.

Open circles = 0, closed circles = 1.0, open triangles = 2.1, closed triangles = 3.1 x 10-7 M crystal violet.

Results on proliferation of F56 on its host 712 in TSB containing crystal violet are seen in figure 4. The lag phase was 1, 2, 3 and 6 hours in the presence of 0, 1.0, 2.1 and 3.1 x 10^{-7} M crystal violet, respectively. After the lag phase, the rates of phage increase were very similar. The final yield of virus was normal with 1.0 x 10^{-7} M crystal violet, but was considerably less when higher levels of dye were employed. Considerable reduction of the initial phage titer occurred during the lag period in the presence of 3.1 x 10^{-7} M crystal violet. This inactivating effect on adsorbed phage was observed in several other instances, although a five-fold greater concentration of crystal violet did not reduce the count of free virus particles, even after incubation in the presence of the dye for 10 hours at 32 C.

In figure 5 are representative data showing the effect of crystal violet on population changes of the host in combination F56/712. Mass lysis was delayed and partially prevented by 1.0 x 10^{-7} M crystal violet. Additional counts not plotted were made at 13 hours and showed that mass lysis had not occurred by that time in the presence of 2.1 and 3.1 x 10^{-7} M dye. Again toxicity to the host bacteria is indicated by a slower rate of increase in the presence of 3.1 x 10^{-7} M crystal violet, although the total number of cells present after 10 hours was not reduced.

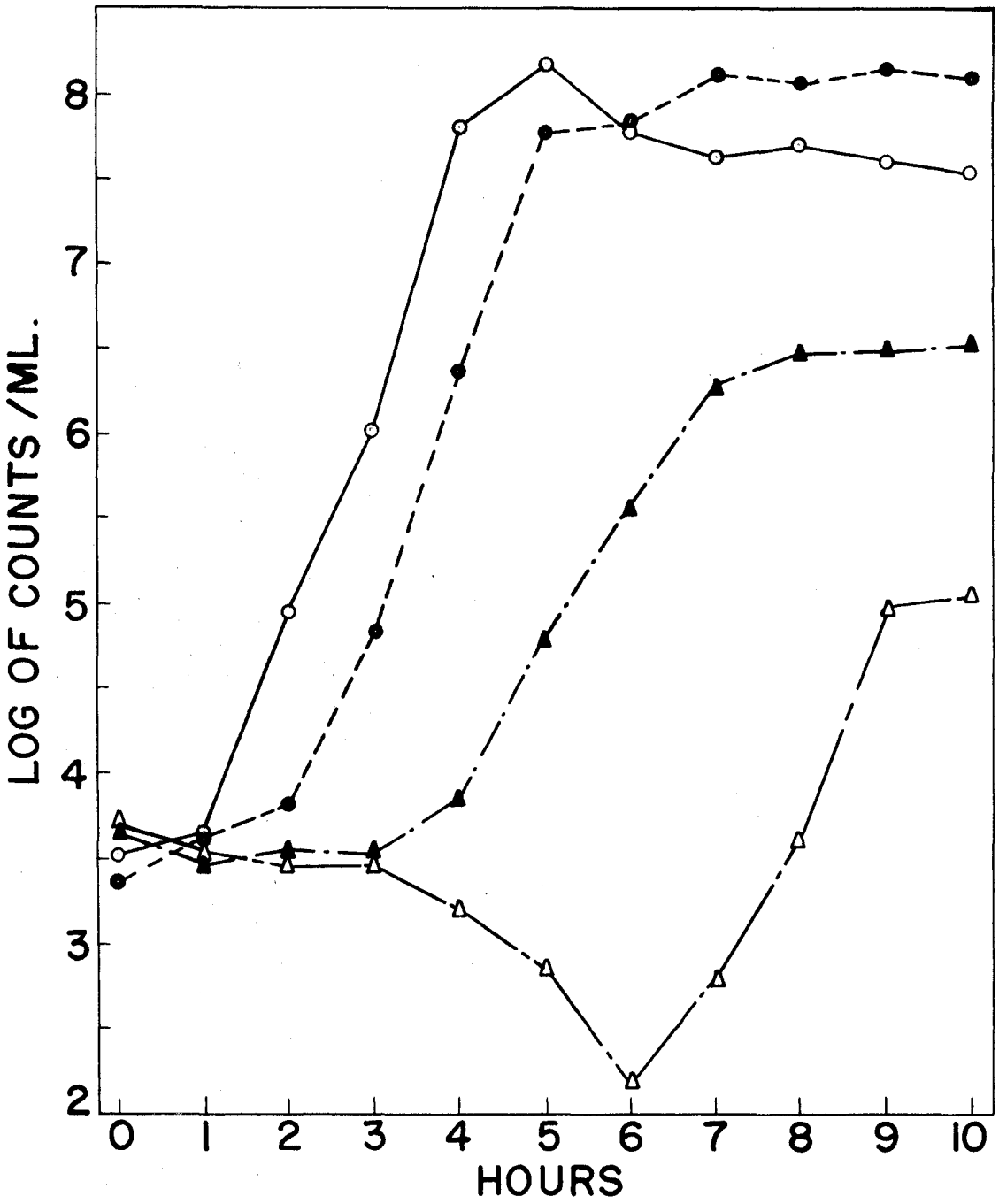

Figure 4. Counts of phage F56 increasing on organism 712 in TSB containing crystal violet.

Open circles = 0, closed circles = 1.0, closed triangles = 2.1, open triangles = 3.1×10^{-7} M crystal violet.

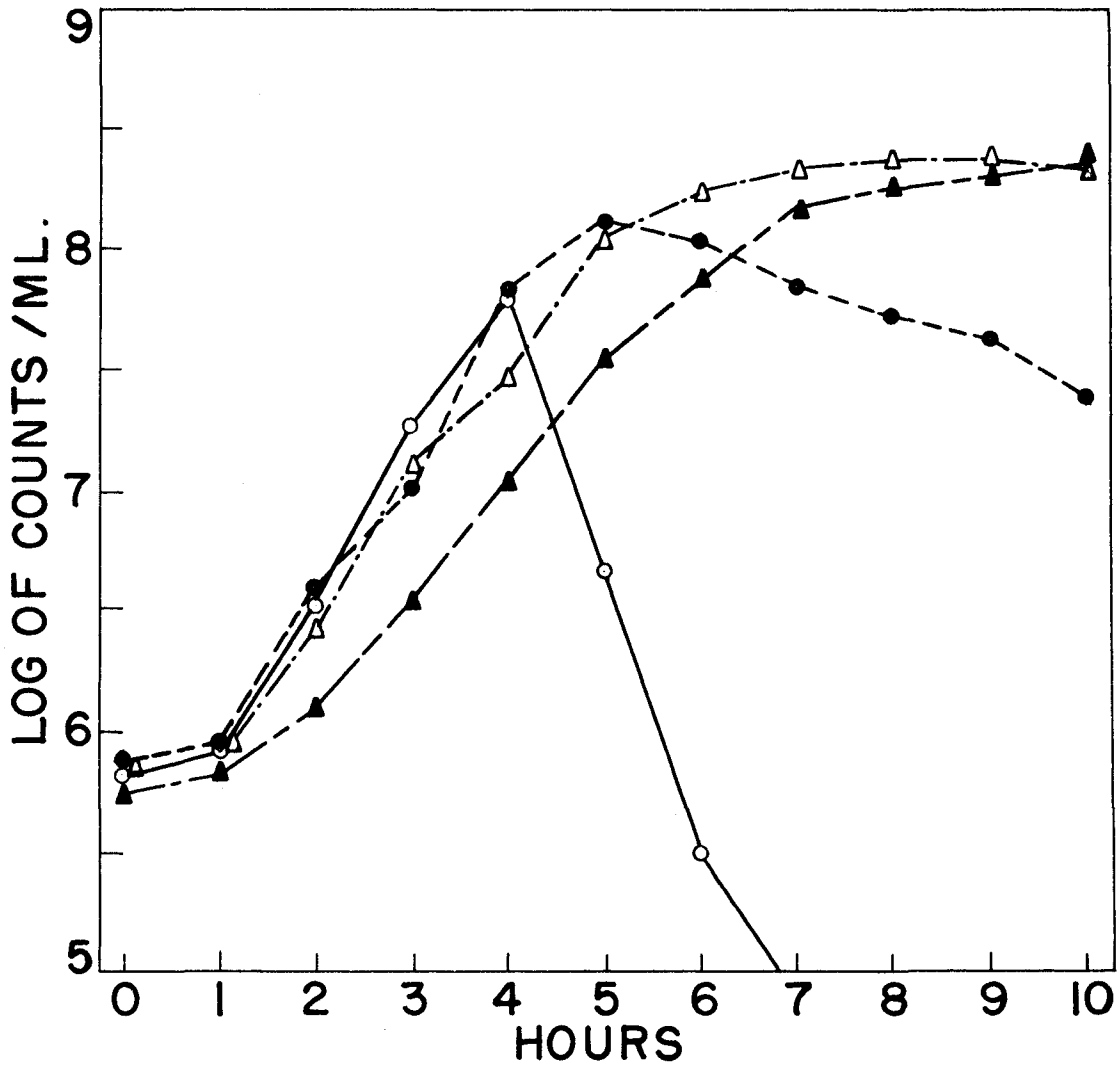

Figure 5. Counts of organism 712 in combination with F56 in TSB containing crystal violet.

Open circles = 0, closed circles = 1.0, open triangles = 2.0, closed triangles = 3.1×10^{-7} M crystal violet.

Similar effects of crystal violet on counts of S. lactis IP5 and the homologous phage F68 in the presence of IP5 are seen in tables 11 and 12. These data indicate very little toxicity to the host bacteria and an extension of the lag phase of virus increase similar to that seen in the case of F56. However, in more extended trials such as the one shown in table 13, mass lysis was delayed but not prevented by 1.0 x 10^{-7} M crystal violet. Moreover, appreciable toxicity to the host bacteria was revealed, thus preventing the use of higher levels of dye. In all instances, the rate of phage increase was normal after the lag period. Because of the very narrow inhibition ratio (IR) for F68 and even lower values for some other combinations (tables 9 and 10), only F56 was employed in most of the following experiments.

Factors influencing the lag effect of crystal violet

The inhibitory effect of crystal violet appeared to result primarily from an extension of the lag phase of virus growth. The possible influence of several factors on this lag effect was investigated.

Hoffman and Rahn (1944) described a bacteriostatic effect of higher levels of crystal violet on S. lactis; this action was believed to result from an unfavorable poising of the oxidation-reduction potential, which was reduced to normal by cellular activity during the lag phase. To determine if the suppression of phage growth by crystal violet

Table 11

Effect of crystal violet (CV) on counts of *S. lactis* IP5 in TSB

Concn. of CV ($\times 10^{-7}$ M)	Counts per ml. at various intervals			
	0*	1.5	3.0	7.0
0	510×10^3	65×10^4	47×10^5	275×10^6
0.5	465×10^3	62×10^4	32×10^5	280×10^6
0.75	462×10^3	63×10^4	44×10^5	267×10^6
1.0	511×10^3	65×10^4	49×10^5	238×10^6

*Incubation time in hours.

Table 12

Effect of crystal violet (CV) on counts of phage F68 in the presence of IP5 in TSB

Concn. of CV ($\times 10^{-7}$ M)	Counts* per ml. at various intervals			
	0**	1.5	3.0	7.0
0	38 $\times 10^2$	182 $\times 10^2$	82 $\times 10^4$	139 $\times 10^7$
0.5	26 $\times 10^2$	44 $\times 10^2$	65 $\times 10^2$	40 $\times 10^7$
0.75	32 $\times 10^2$	46 $\times 10^2$	86 $\times 10^2$	317 $\times 10^7$
1.0	29 $\times 10^2$	34 $\times 10^2$	40 $\times 10^2$	256 $\times 10^5$

*The bacterial counts at 0 hours for 0, 0.5, 0.75 and 1.0 $\times 10^{-7}$ M concn. of CV were 44, 26, 31 and 32 $\times 10^4$ per ml., respectively.

**Incubation time in hours.

Table 13

Effect of crystal violet (CV) on counts of S. lactis IP5 and of phage F68 increasing on IP5 in TSB

Concn. of CV (x 10^{-7} M)	Counts per ml. at various intervals								
	0*	1	2	4	6	8	10	16	26
IP5**									
0	32 x 10^4	39 x 10^4	53 x 10^4	48 x 10^5	195 x 10^5	80 x 10^6	197 x 10^6	175 x 10^6	145 x 10^6
1.0	37 x 10^4	34 x 10^4	51 x 10^4	128 x 10^4	72 x 10^5	205 x 10^5	67 x 10^6	129 x 10^6	57 x 10^6
F68/IP5***									
0	8 x 10^3	-	242 x 10^2	536 x 10^4	12 x 10^8	9 x 10^8	7 x 10^8	11 x 10^8	8 x 10^8
1.0	5 x 10^3	-	60 x 10^2	68 x 10^2	168 x 10^2	20 x 10^6	50 x 10^8	48 x 10^8	43 x 10^8

*Incubation time in hours.
**Bacterial counts.
***Phage counts.

resulted from a poising effect, 0.5 per cent sodium thioglycolate was added to the medium to insure strongly reducing conditions. Toxicity of crystal violet to S. lactis IP5 was lessened slightly but the yield of F56 and F68 in one-step growth experiments was not increased by thioglycolate. This indicates that the virustasis probably was not due to an effect of crystal violet on the oxidation-reduction potential of the medium.

Stearn and Stearn (1926) reported that increased pH augmented bacteriostasis by crystal violet. Hoffman and Rahn (1944) observed the same effect; a 2.5-fold greater concentration of dye was required to inhibit S. lactis at pH 5.1 than at pH 8.1. Acid production by the host bacteria conceivably might reduce the virustatic ability of crystal violet and account for the increase of phage at a normal rate after the lag period. To test this possibility, phage was proliferated in the presence and absence of crystal violet in TSB adjusted initially to several pH levels between 5.5 and 7.5. No effect other than the usual influence of pH on phage growth could be established in repeated trials.

Selection or adaptation of host bacteria toward a greater tolerance for crystal violet also was investigated. Plates inoculated with about 10^6 bacteria were poured with agar containing up to 5.1×10^{-6} M crystal violet. Serial isolations of surviving colonies from plates prepared in this

way failed to produce strains of host bacteria capable of proliferating phage in greater-than-normal concentrations of crystal violet. Resistance of the bacteria to high levels of dye was increased slightly.

A portion of the crystal violet used would be expected to be bound by cellular constituents. Normal growth of host bacteria during the phage lag period might result in a cell mass sufficient to "dilute" and thus reduce to a non-inhibitory concentration the very small amount of dye employed in these experiments. F56 was proliferated in the presence and absence of crystal violet, using different inoculum levels of phage and bacteria. The results of a representative experiment are in figure 6.

Periodic counts of the host bacteria indicated identical rates of increase in the presence and absence of crystal violet (cf. figure 3, 1.0×10^{-7} M crystal violet). However, the virus lag period in every case is greater in the presence than in the absence of crystal violet. Virus increase did not occur in the presence of crystal violet until the cell population reached about 2 to 4 $\times 10^6$ per ml., regardless of the inoculum level used. Thus with a high initial cell population (A), virus increase began after about 1 hour, whereas low initial cell populations (C and D) resulted in a delay of 3 to 6 hours before virus increase occurred in the presence of crystal violet. In each case the virus lag period

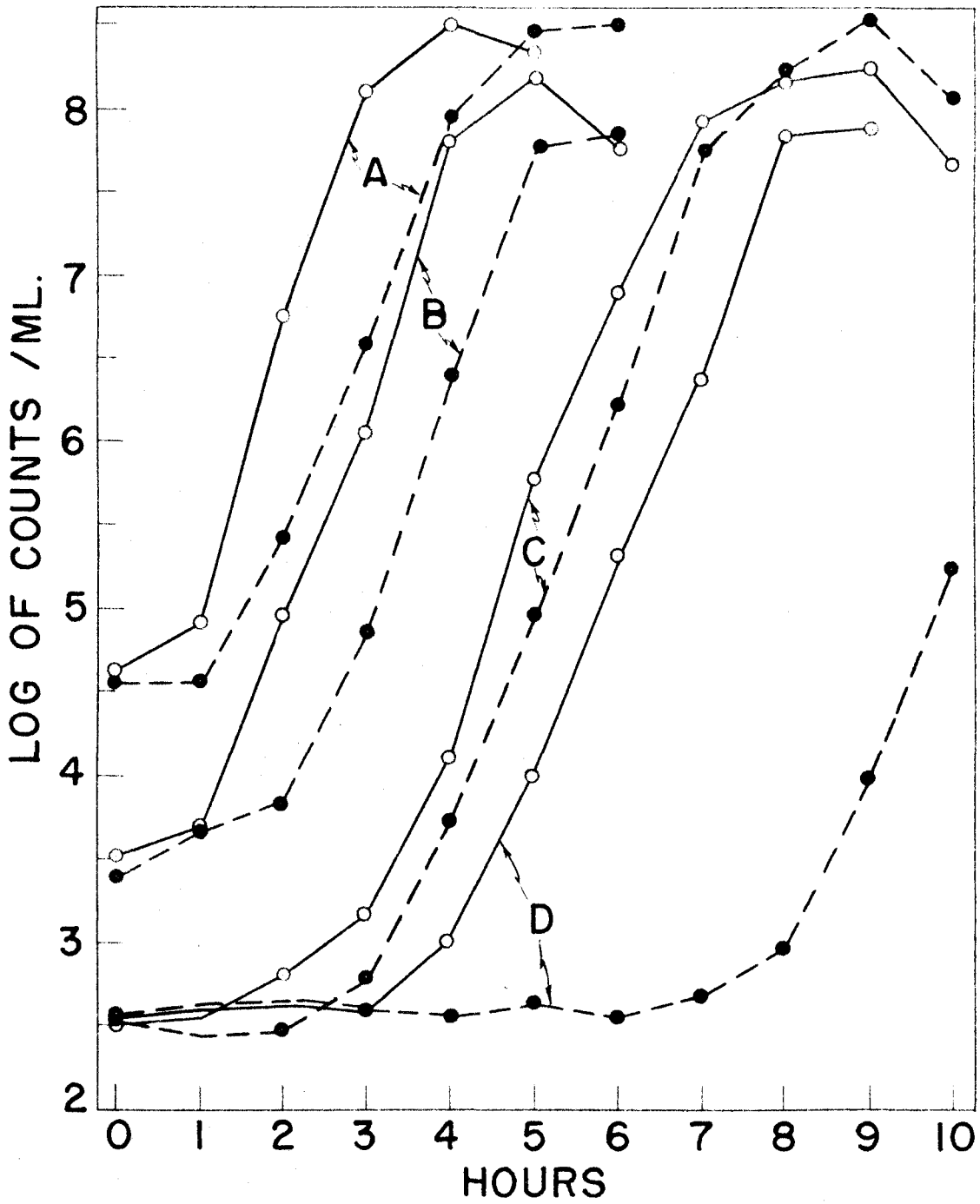

Figure 6. Effect of the initial concentration of host bacteria (712) on proliferation of F56 in the presence and absence of crystal violet (CV).

Open circles = 0, closed circles = 1.0×10^{-7} M CV. Bacterial counts at 0 hours were 10^6, 10^5, 10^4, and 10^3 per ml. for paired curves A, B, C and D, respectively.

with crystal violet present corresponds closely to the time required for the cell population to reach about 2 to 4 x 10^6 per ml. The extension of the lag period in the absence of crystal violet when low inocula are used results from a slower rate of adsorption of the virus in the dilute systems.

In table 14 are data calculated from figure 6, showing the proportion of phage to bacteria initially and at the end of the virus lag period. When phage increase began, the bacteria had increased to a substantially greater proportion in the presence than in the absence of crystal violet. These trials were repeated with essentially the same results, and control curves of other experiments always showed the same trend. Therefore, it appears that the quantity of cell mass present in the medium exerts a direct influence on the virus lag period, possibly by reducing the effective dye concentration to a non-inhibitory level.

Effects on growth characteristics of virus

The effect of crystal violet on adsorption of phage to host bacteria is seen in table 15. When the dye was associated with either the phage or the cells for 15 minutes before and then with the mixture throughout the 10-minute adsorption period, there was no difference in the per cent of virus adsorbed in the presence of 0, 1.0 and 2.1 x 10^{-7} M crystal violet.

Table 14

Proportion of phage to cells at the beginning of the rise* in the presence and absence of crystal violet (CV) using different inoculum levels of F56/712

Trial no.	Cells per ml. at 0 time	Concn. of CV ($\times 10^{-7}$ M)	Phage lag period (hours)	Phage/cell proportion 0 time	Phage/cell proportion rise time
A**	4.4×10^6	0	0-1	0.011	0.013
		1.0	1-2	0.007	0.007
B	7.0×10^5	0	0-1	0.008	0.011
		1.0	2-3	0.008	0.007
C	3.4×10^4	0	1-2	0.010	0.011
		1.0	2-3	0.009	0.002
D	7.5×10^3	0	3-4	0.60	0.0025
		1.0	7-8	0.80	0.0005

*Determined from the data of figure 6 plus hourly counts of the bacteria.
**The letters refer to similarly lettered pairs of curves in figure 6, showing the phage increase throughout the experiment.

Table 15

Adsorption (ads.) of phage to host cells in the presence* and absence of crystal violet (CV)

Concn. of CV (x 10^{-7} M)	Per cent adsorption in 10 minutes		
	F56/712	F68/IP5	
	Phage in presence of CV before ads.	Phage in presence of CV before ads.	Cells in presence of CV before ads.
0	93, 98**	91, 98	92, 98
1.0	98	93, 97	92, 96
2.1	98	89, 96	91, 96

*Phage or cells, as indicated, were mixed with CV 15 minutes before preparing the adsorption mixtures. The level of CV was maintained during the adsorption period in each case.

**Duplicate values represent separate determinations.

Figure 7 shows the effect of 1.0×10^{-7} M crystal violet on several phases of a single growth cycle of F56. Three important points are revealed in this experiment. (1) The identical latent period with no crystal violet (curve A) and with crystal violet present only during the 10-minute adsorption period (curve B) indicates that the dye did not block invasion. (2) The removal of crystal violet by dilution immediately after adsorption (curve B) resulted in a virus yield identical to that obtained in the control (curve A). This again indicates that invasion was not prevented and, furthermore, that any dye possibly irreversibly bound to the virus-host complex was not inhibitory. (3) Curves C and D show the effect of crystal violet present during the latent period. Again the latent period was identical to that of the control, but the yield of phage was reduced considerably. Thus the presence of crystal violet in the medium during the latent period apparently decreased intracellular multiplication of the phage. A similar effect on multiplication of F68 is seen in figure 8, where increasing concentrations of crystal violet resulted in progressively lower yields of phage. The findings of both experiments were confirmed upon repeated trials.

Average burst size as determined by the one-step method represents an arithmetic average derived from the collective yield from about 20 to 200 infected cells, as employed in

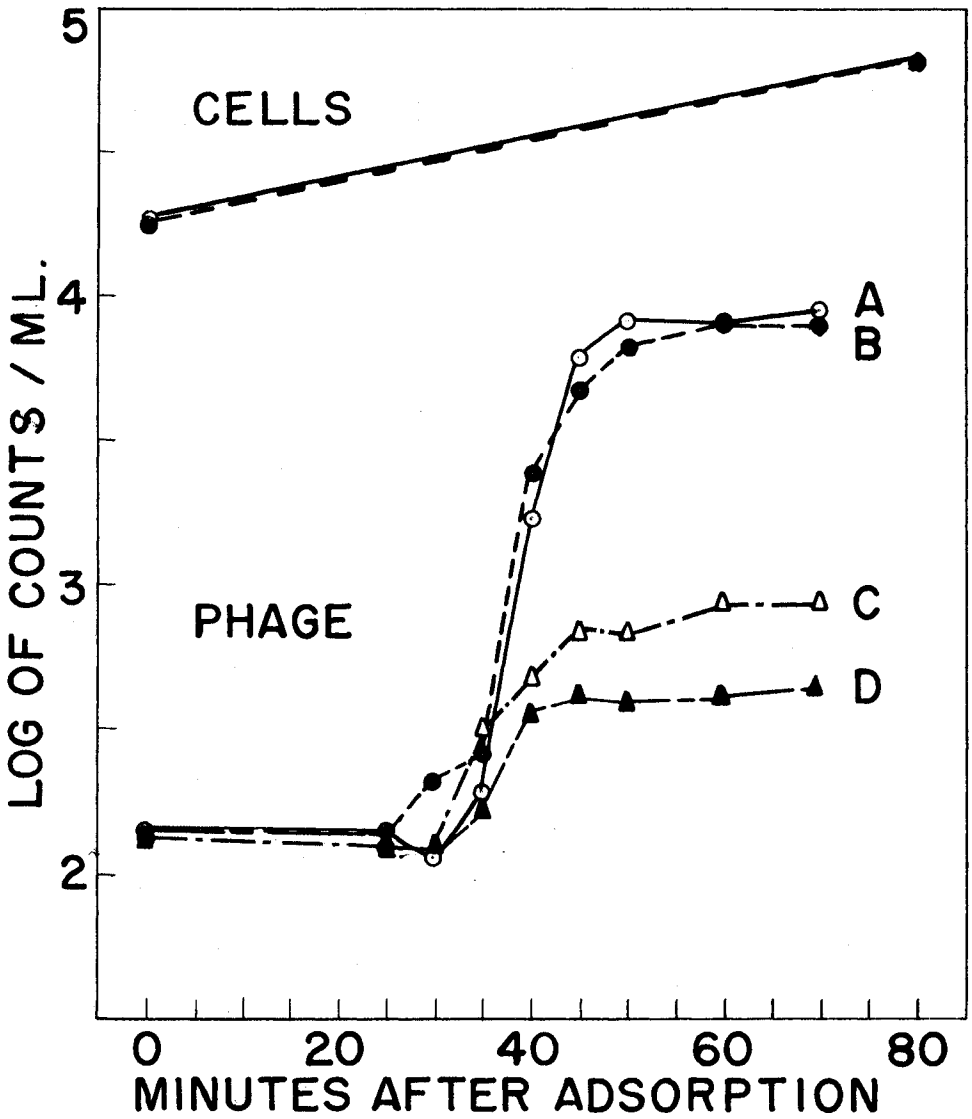

Figure 7. Effect of crystal violet (CV) on invasion and multiplication of F56/712.

A= no CV; B= CV present during adsorption (removed by dilution); C= CV present after adsorption; D= CV present throughout. Cells: open circles = 0, closed circles = 1.0×10^{-7} M CV.

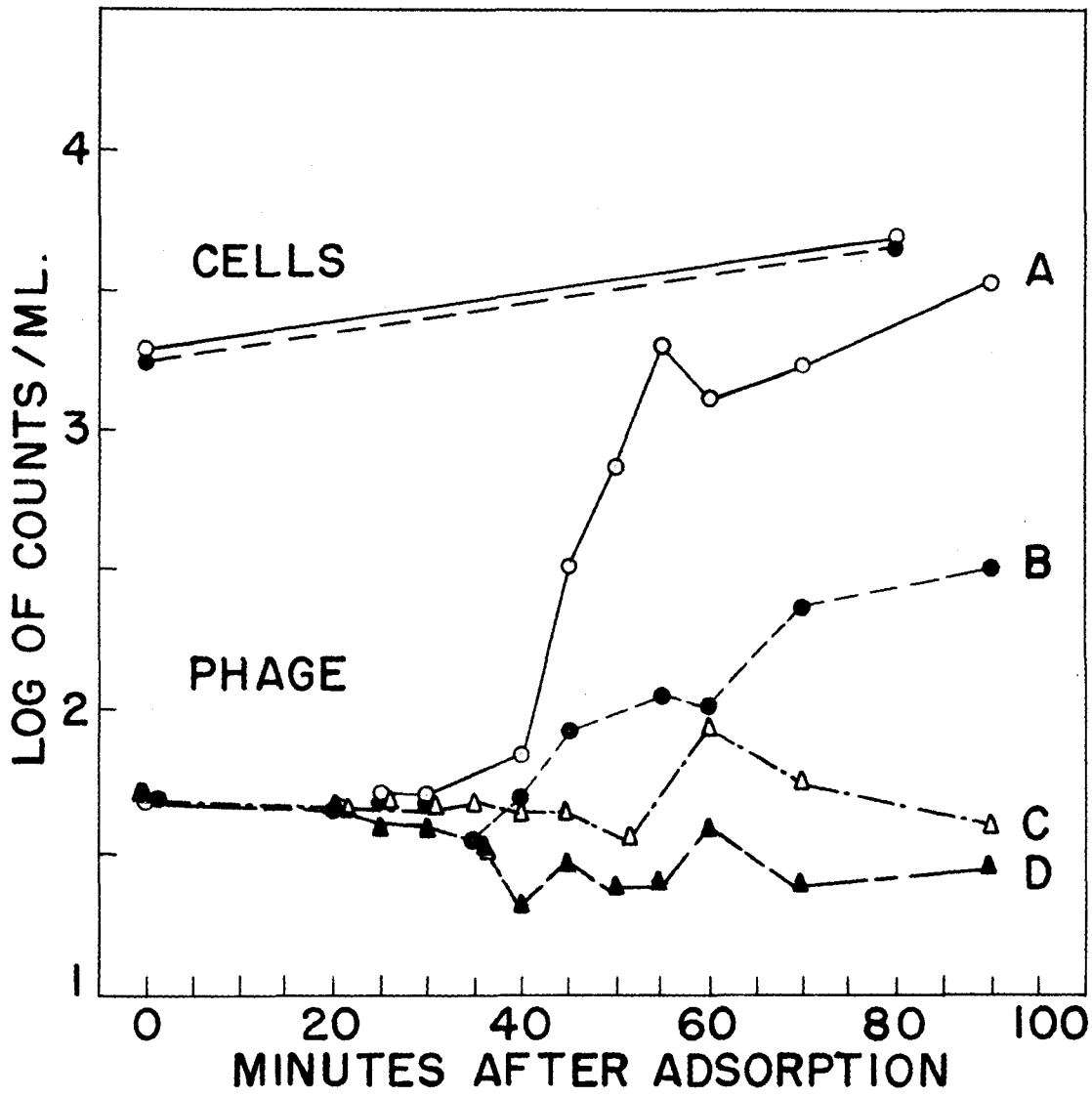

Figure 8. Effect of crystal violet (CV) on the one-step growth curve of F68/IP5.

A= 0, B= 0.5, C= 1.0, D= 1.5 x 10^{-7} M CV added after adsorption.
Cells: open circles = 0, closed circles = 1.5 x 10^{-7} M CV.

this work. Reduction of the average can result either from a smaller yield of phage from each of the infected cells, or from a portion of the infected cells not bursting and the remainder giving normal size bursts. Determination of the burst size of individual infected cells (table 16) revealed that crystal violet added 5 minutes after adsorption reduced the yield of phage per infected bacterium. Trial I also indicates a reduction in the number of bursts, although only the yield per burst is reduced in trial II. These data appear to support the earlier indications that crystal violet interferes principally with intracellular multiplication, rather than the invasive process.

Kinetic studies were undertaken to establish the stage at which crystal violet blocked the multiplication process. Figure 9 shows the effect of adding 1.0×10^{-7} M crystal violet during the latent period of F56. Identical latent periods resulted when the dye was added at any time up to 20 minutes after adsorption; however, the yield of phage increased as the interval between adsorption and the addition of crystal violet was increased. A similar experiment in which crystal violet was added immediately after adsorption, then removed by dilution with fresh broth at various times during the latent period is seen in figure 10. Again the latent period was unchanged and the yield of phage increased as the time of exposure to crystal violet decreased. Both of

Table 16

Burst size of individual infected cells in the presence and absence of crystal violet (CV)

Trial no.	Concn. of CV* ($\times 10^{-7}$ M)	Burst size of individual infected cells of combination F56/712	No. of bursts observed
I	0	66, 66, 95, 101, 143, 153, 256, 267	8
	1.0	6, 9, 23, 39, 53	5
II	0	50, 60, 62, 81, 128	5
	1.0	11, 17, 20, 24, 30	5

*Added 5 minutes after adsorption (cf. curve C, figure 9).

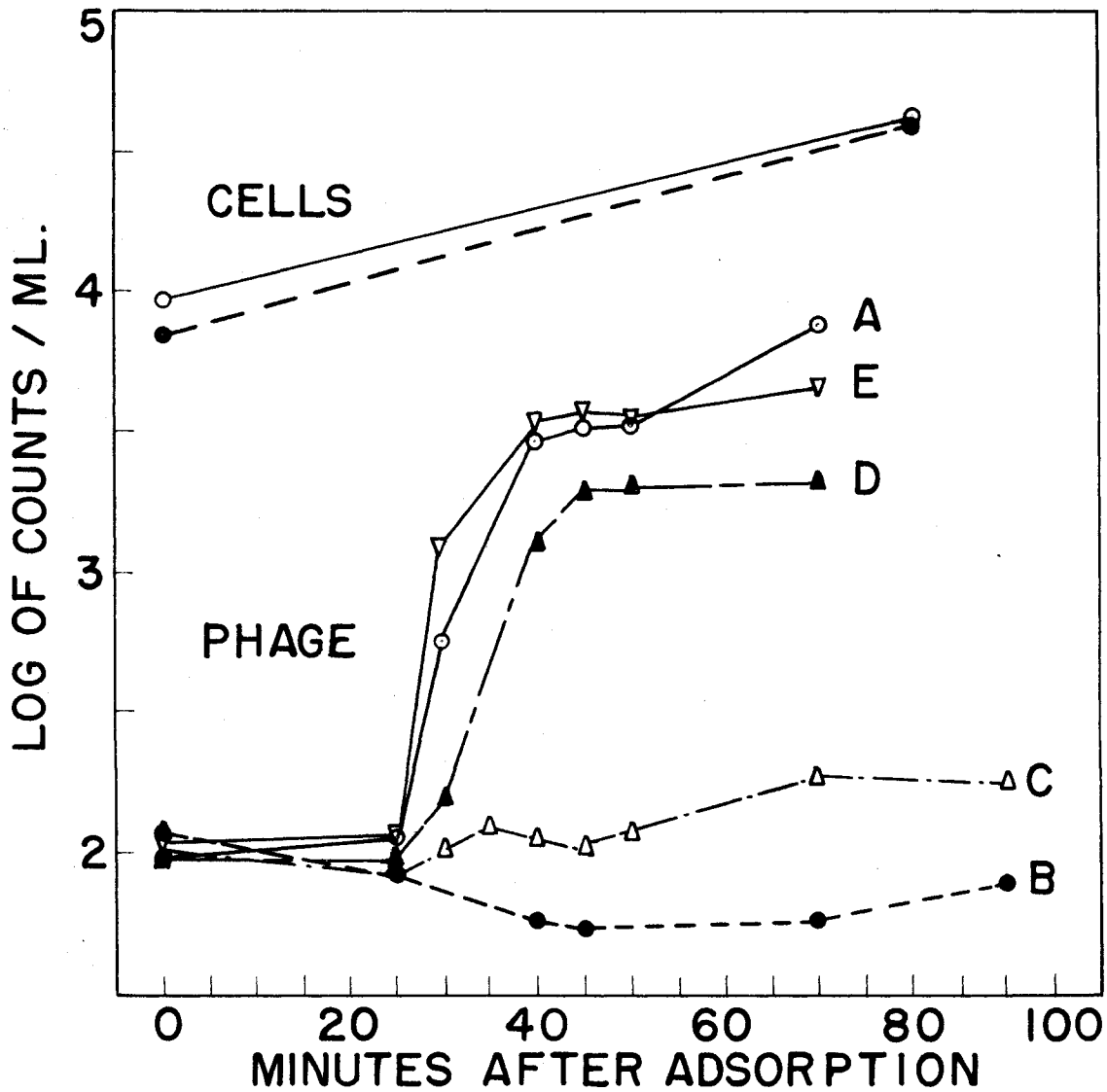

Figure 9. Effect of adding crystal violet (CV) during the latent period of F56/712.

A= no CV, B= CV added at 0 minutes (min.), C= CV added at 5 min., D= CV added at 10 min., E= CV added at 20 min.
Cells: open circles = 0, closed circles = 1.0×10^{-7} M CV.

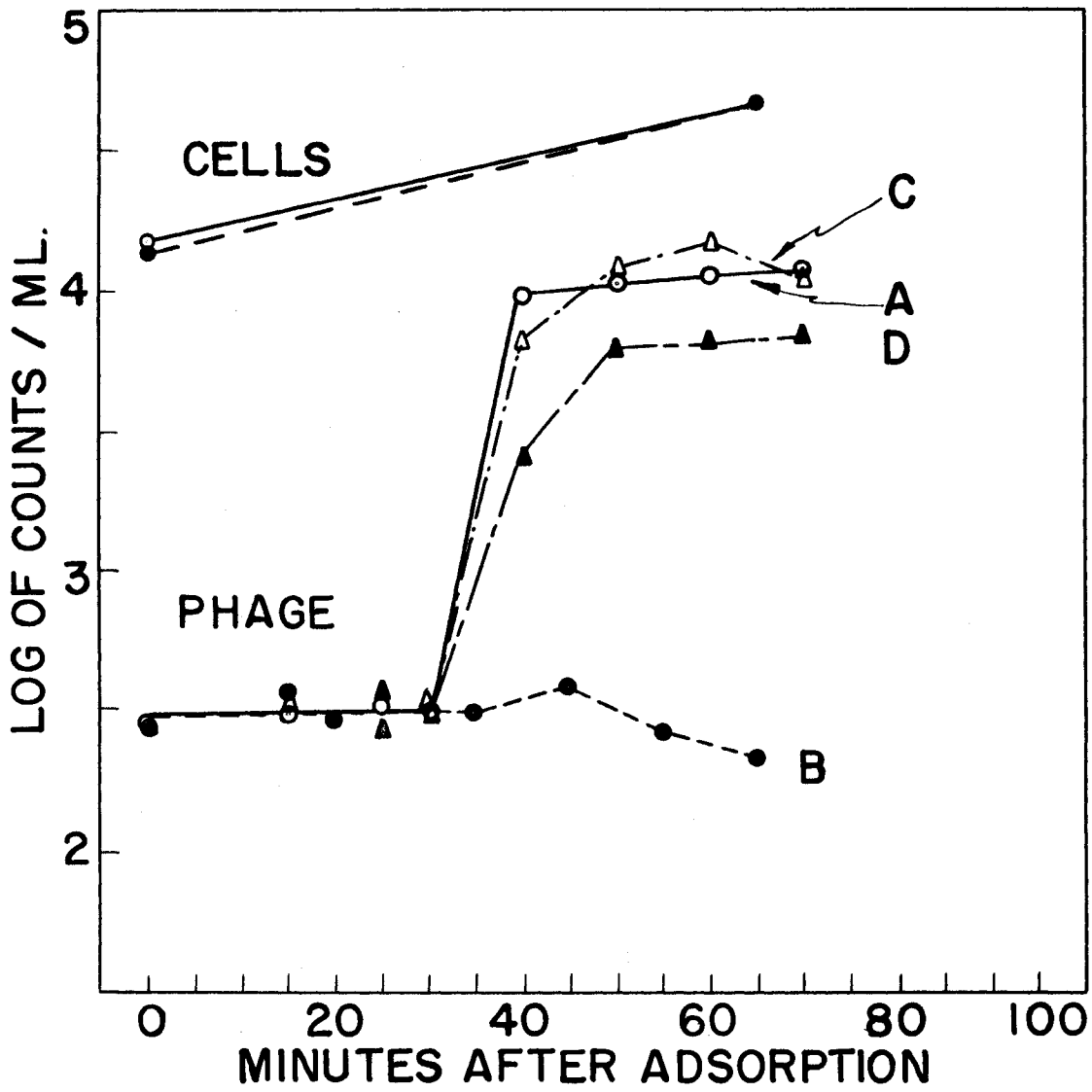

Figure 10. Effect of adding at 0 minutes (min.) and subsequently removing crystal violet (CV) during the latent period of F56/712.

A= no CV, B= CV present throughout.
Time CV removed (by dilution): C= 10 min., D= 20 min.
Cells: open circles = 0, closed circles = 1.0×10^{-7} M CV.

these experiments were repeated several times with very similar results, indicating that the virus yield was related directly to portion of the latent period which elapsed with no inhibitory level of crystal violet in the medium.

The effect of removing crystal violet by dilution with fresh medium at various intervals after the normal latent period is seen in figure 11. Prolonged exposure of the virus-host complex to crystal violet resulted in decreased yields of phage upon subsequent removal of the dye. The rise began about 5 minutes after removal of crystal violet at 30 and 40 minutes. This temporary delay in the release of phage probably represents the time required for completion of the steps previously blocked by crystal violet. A delay approximately equal to the latent period occurred after removal at 50 and 60 minutes. A small rise between 50 and 60 minutes apparently took place in the presence of crystal violet (curve B), possibly as a result of the cells reaching a population sufficient to lower the concentration of dye. The increased amount of virus present at the time of removal at 60 minutes may account for the apparently inverted positions of curves F and G. Some secondary adsorption of phage probably occurred during this extended experiment, resulting in a tendency for the curves to drift upward following the rise. These findings were confirmed

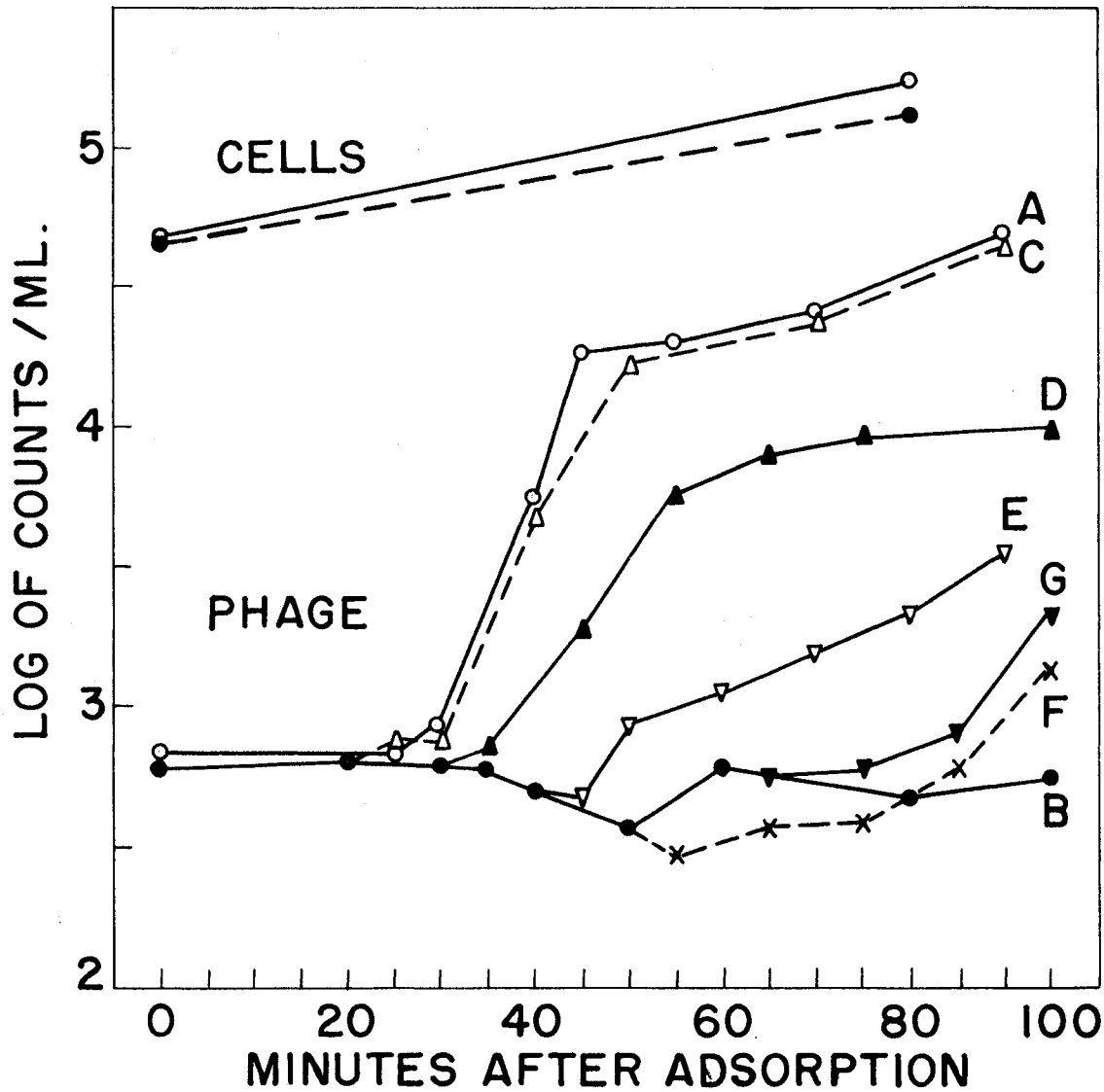

Figure 11. Effect of adding at 0 minutes (min.) and subsequently removing crystal violet (CV) after the normal latent period of F56/712.

A= no CV, B= CV present throughout.
Time CV removed (by dilution): C= 20 min., D= 30 min., E= 40 min., F= 50 min., G= 60 min.
Cells: open circles = 0, closed circles = 1.0×10^{-7} M CV.

upon repeating the experiment.

These data indicate that in many instances the steps blocked by crystal violet could be completed quickly upon removal of the dye up to 10 minutes beyond the normal latent period; removal at later times appeared to result in a second cycle of virus growth, possibly in newly infected cells. Completion of the crystal violet-inhibited steps at this late time may indicate that the dye interferes with a late reaction in the synthesis of phage particles.

Competitive antagonism of crystal violet and desoxyribose nucleic acid

The following materials were tested for ability to permit mass lysis in the presence of 2.1×10^{-7} M crystal violet: calcium chloride, choline chloride, sodium thioglycolate, hydrolysed casein, tryptophan, reticulogen, yeast extract, ribose nucleic acid (RNA) and desoxyribose nucleic acid (DNA). Decimal dilutions embracing a 1000-fold range of each nutrilite, all in considerable excess of crystal violet, were tested. Growth of phage and bacteria was tested in controls containing the nutrilite without crystal violet. Only DNA permitted mass lysis of combination F56/712 in the presence of the dye. The plaque counts of F56 after growth for 10 hours in TSB containing 2.1×10^{-7} M crystal violet, no crystal violet and 2.1×10^{-7} M crystal violet plus 0.16 per cent DNA were 1.3×10^{6}, 1.9×10^{8} and 1.1×10^{8} per ml.,

respectively. The constituent purine and pyrimidine bases of DNA (adenine, guanine, cytosine and thymine) as well as D-ribose did not counteract the inhibitory effect of crystal violet.

Table 17 gives the relationship between different levels of crystal violet and DNA in TSB. Prevention of mass lysis by 1.0, 2.1 and 3.1 x 10^{-7} M crystal violet was overcome by 0.04, 0.16 and 0.32 per cent DNA, respectively. This indicates competition between the two compounds throughout the range of concentrations tested.

Apparent competition between an inhibitor and a metabolite can result from competition for specific enzyme systems as in typical antimetabolite functions. However, direct reaction between an inhibitor and a metabolite may result in inactivation of the compound present in a lower equivalent concentration, giving a similar competitive effect. The possibility of direct combination between crystal violet and DNA *in vitro* was investigated.

An electrometric titration was not feasible, due to the small differences in pH of crystal violet and DNA solutions. A precipitate, which became visible only after several hours, was obtained with the dye-protein titration procedure of Chapman *et al.* (1927). The lowest dye concentration which caused a precipitate to form in the presence of 0.8 g. DNA per 100 ml. was 8.0 x 10^{-4} M crystal violet. An excess of

Table 17

Competitive antagonism of crystal violet (CV) and desoxyribose nucleic acid (DNA) with combination F56/712

Concn. of CV ($\times 10^{-7}$ M)	Concn. of DNA (g. per 100 ml. TSB)							
	0	0.01	0.02	0.04	0.08	0.16	0.32	0.64
0	+*	+	+	+	+	+	+	+
1.0	-	-	±	+	+	+	+	+
2.1	-	-	-	-	-	+	+	+
3.1	-	-	-	-	-	-	+	+

*+= mass lysis, ±= partial lysis, - = no mass lysis. All tubes recorded were inoculated with virus and cells; control tubes inoculated with cells only contained growth in all cases.

dye was present in the proportion of 0.8 g. DNA per 100 ml. and 65 x 10^{-4} M crystal violet. Assuming a molecular weight of 100,000 for DNA, which probably is a minimum value, the molar concentration of 0.8 g. DNA per 100 ml. is 8.0 x 10^{-5}. Therefore, the concentration of crystal violet equivalent to 8.0 x 10^{-5} M DNA appears to be between 8.0 and 65 x 10^{-4} M. These data suggest that each molecule of DNA may be capable of binding several molecules of crystal violet.

Spectrometric measurements at 562.5 millimicrons of mixtures of 2.0 x 10^{-6} M crystal violet and various proportions of DNA revealed increasing transmittance as the relative concentration of DNA was increased in proportions which antagonized crystal violet in the biological system (table 18). Extraction with tetrachloroethane of the dye from similar crystal violet-DNA mixtures resulted in recovery of less crystal violet as the proportion of DNA was increased (table 19).

Therefore, the data strongly suggest that crystal violet and DNA do combine *in vitro*. This result is in agreement with the findings of Stearn (1930), who reported a covalent linkage between crystal violet and nucleic acid, and Mirsky and Ris (1951) who have described a stoichiometric reaction between crystal violet and the phosphoric acid groups of DNA.

Table 18

Per cent transmittance (T) at 562.5 millimicrons of crystal violet (CV) and various proportions of desoxyribose nucleic acid (DNA)

Concn. of DNA (g./100 ml.)	Per cent T of: DNA	Per cent T of: DNA plus CV*	Corrected per cent T** for DNA plus CV
0	100.0	89.9	89.9
0.1	96.9	78.8	81.9
0.2	94.9	67.6	72.7
0.4	89.8	64.1	74.3
0.6	88.2	62.2	74.0
0.8	84.0	60.9	76.9
1.2	77.4	55.1	77.7
1.6	70.6	50.6	80.0
2.4	61.2	43.5	82.3
3.2	53.3	38.3	85.0

*2.0×10^{-6} M CV.

**Corrected for DNA absorption as follows: (% T of DNA + CV) + (100 - % T of DNA).

Table 19

Extraction of crystal violet (CV) from desoxyribose nucleic acid (DNA) mixtures*, using $C_2H_2Cl_4$

Contents of mixture CV**	DNA***	Per cent transmittance of the extract, determined at 562.5 millimicrons
2.1	0	89.1 (89.9 before extraction)
0	3.2	100.0
0	0.4	100.0
2.1	3.2	67.9
2.1	0.4	65.2

*Aqueous mixtures containing the indicated concentrations of solute were prepared by combining stock solutions in a 100 ml. volumetric flask. To a 50 ml. separatory funnel, 10 ml. of the mixture and 10 ml. of $C_2H_2Cl_4$ were added. After shaking 50 times, about 3 minutes were allowed for the bubbles to rise, then approximately 9.5 ml. of the lower layer were withdrawn. Moisture was removed with 0.5 g. anhydrous Na_2SO_4.

**x 10^{-6} M.

***g. per 100 ml.

DISCUSSION

Screening

Of the entire group of agents tested, only crystal violet selectively inhibited phage multiplication. This finding was disappointing but is similar to the results obtained in comparable studies by other investigators using different virus-host systems (Fitzgerald and Babbitt, 1946; Dickinson, 1948). The screening system used appears adequate for the detection of any reasonably selective inhibitors of phage. The four phage-host combinations were selected for diversity so as to represent the varied types of lactic streptococcus phage. Several additional combinations employed in many of the early tests gave essentially the same results as the four principal test phages. The concentration gradient resulting from diffusion of test solutions into the agar in the paper disc method constitutes a very sensitive test for effective levels of inhibitor. Opportunity for inhibition during all of the three phases of virus growth (adsorption, invasion and multiplication) was present in the paper disc and tube tests.

Prevention of mass lysis was the criterion of phage inhibition in all of the screening procedures. Therefore, agents which only slightly reduced the rate of virus increase

were not detected. In addition, proliferation of virus on cells unable to multiply would not be revealed. Although such agents would not be suitable for practical application, their use in mechanistic studies might be of some value. By proliferating phage on host bacteria unable to multiply because of nutritional deficiency, Spizizen (1943b) developed the concept that virus multiplication is associated with certain specific cellular reactions. Both stimulation and inhibition of phage multiplication was obtained through the use of various compounds involved in cellular metabolism. Similarly, Price (1947b) found that niacin was required for growth of phage in staphylococci inhibited by penicillin.

Appreciable phage increase occurred in the presence of toxic levels of salicylaldehyde and iodoacetic acid. In contrast to these were agents such as benzimidazole, which in relatively less toxic levels completely prevented phage proliferation. These findings indicate that certain portions of the metabolic activity of the host cells differ in their importance for phage growth. However, the relationship between phage formation and normal metabolism of the host is so close that none of these agents was sufficiently selective to inhibit only the phage system.

Many of the agents tested have been reported effective inhibitors of one or more different virus systems. Failure of these compounds to prevent development of lactic

streptococcus bacteriophage possibly reflects fundamental variations in the multiplication mechanisms of different viruses. Smith (1949) found marked differences when the same inhibitor was applied to coli, dysentery and staphylococcus phages. The data emphasized the specificity of different phage strains toward inhibitors. Varied reactions were obtained with the phages used in the present study. In tests of all inhibitors, F56 usually was the most sensitive and F68 was intermediate among several virus strains. The greater sensitivity to crystal violet of strains F56 and F68 than any of 41 other phage-host combinations tested suggests at least quantitative differences in the multiplication mechanisms of the different strains. Based on dissimilar resistance to ultraviolet light of phages T2r and T7, Benzer (1952) has suggested that the modes of reproduction of different viruses on the same host may vary greatly.

Elion and Hitchings (1951) reported that several pteridine compounds, inhibitory to Lactobacillus casei in a minimal medium, were ineffective regardless of concentration when the level of adenine sulfate in the medium exceeded 10 micrograms per ml. Their effectiveness also was decreased when optimum amounts of folic acid were present. In view of these findings, the complex trypticase soy medium used might be expected to obscure the antiphage effect of certain com-

pounds. However, tests in SM and the minimal medium for F56 (FSM) gave essentially the same results as were obtained in TSB. In fact, there was some tendency for greater selectivity of the inhibitors in the richer medium. For crystal violet, the selectivity was greater in milk than in TSB, which in turn gave slightly better results than FSM. Fitzgerald and Lee (1946) suggested that selective inhibition of virus may result from greater ability of the cell mechanism to resist inhibitors through the use of alternate metabolic pathways not available to the virus. Possibly in the case of crystal violet, such alternate routes were utilized more readily in the richer medium.

All of the compounds which prevented mass lysis also were toxic to the host cells, thus emphasizing the close relationship between the mechanism of virus growth and that of normal cell multiplication. The hope of finding selective inhibitors of virus depends upon the existence of differences in these mechanisms. Prior to the discovery of the sulfonamides and antibiotics, very few selective inhibitors of animal parasites had been found and more did not appear to be obtainable. The metabolism of the parasites and their hosts was considered too closely related to permit selective inhibition. Now we know that this is not true. Possibly similar findings eventually will be obtained with the viruses.

Analysis of the Action of Crystal Violet

Inhibition of phage increase by levels of crystal violet not demonstrably toxic to the host bacteria indicates interference with mechanisms more important for virus multiplication than for normal cell growth. Non-specific effects resulting in subtle changes in the normal metabolism of the host not revealed by differences in rate of growth of the bacteria cannot be excluded but do not appear likely under the conditions of these experiments. With numerous other inhibitors tested, mass lysis occurred in the presence of levels definitely toxic to the host bacteria. In the case of iodoacetic acid, appreciable phage increase was recorded in the presence of bactericidal levels of inhibitor. Strains of host bacteria selected from large populations by means of resistance to relatively high levels of crystal violet did not differ from the original type in the level of dye required to inhibit phage increase on the different strains. However, the tolerance of the bacteria for high levels of crystal violet was increased slightly. This suggests that the characteristics involved in inhibition of the host may differ from those responsible for inhibition of the virus.

Failure of virustatic levels of crystal violet to destroy unadsorbed phage particles, reduce adsorption or prevent invasion strongly suggests that the effect is due almost entirely to inhibition of either intracellular multi-

plication or release of phage particles. Reduction of the yield from individual infected cells supports this observation. The relationship of virus yield to the portion of the latent period elapsed in the absence of crystal violet indicates that the phase of synthesis interrupted by the dye normally is operative during a considerable portion of the latent period; however, in about one-half of the particles formed, this phase usually is completed within the first 10 minutes. Removal of crystal violet after its presence throughout the normal latent period results in release of phage about 5 minutes later. This indicates that the step blocked by crystal violet can be delayed until late in the synthetic process and then completed very quickly in the absence of the dye. In this respect the crystal violet effect resembles the finding of Foster (1948) that a late reaction in the formation of coliphage is blocked by proflavine. However, proflavine was not inhibitory to lactic streptococcus phage. Prolonged exposure of the phage-host complex to crystal violet resulted in a second latent period before phage was produced following removal of the dye. Whether the phage produced under these conditions came from the original phage-host complex or from newly infected cells is not apparent.

A portion of the crystal violet used would be expected to be bound by cellular constituents. Evidence in favor of

this is seen in the inverse relationship between the lag period of phage growth and the number of cells present in the medium plus crystal violet (figure 6 and table 14). The bound crystal violet either is not inhibitory to phage or is very loosely bound, as indicated by removal of the inhibitory effect upon dilution of the inhibited phage-host complex into fresh broth. Possibly, free dye must be present in order to combine with certain phage components as they are synthesized by the phage-host complex.

The antagonism of crystal violet by DNA but not by RNA or any of the other nutrilites tested suggests that inhibition of the phage results from interference with some phase of DNA metabolism. In the partially chemically defined minimal medium containing no purines, pyrimidines or riboflavin, F56 was not inhibited by the purine and pyrimidine analogs benzimidazole, barbituric acid and thiouracil. Analogs of PABA and folic acid, which are believed to be involved in the synthesis of purine and pyrimidine bases (Gots and Chu, 1952; Snell, 1951), also did not prevent phage multiplication, indicating that these early stages of nucleic acid metabolism are equally important for normal cell growth and phage multiplication. Differentiation between the two functions appears to involve reactions of more highly organized structures. 8-Chloroxanthine, analog of purines and nucleic acid, and adenosine, the only available

nucleoside analog, did not prevent phage multiplication; other nucleoside or nucleotide analogs might reveal inhibition in these areas.

Combination of crystal violet with DNA *in vitro* indicates that the inhibition may result from direct combination of the dye with phage DNA or perhaps fractions thereof at a stage in the synthetic process prior to final incorporation of nucleic acid into the structure of the phage particle. Chemical analysis of coliphage particles has revealed a high proportion of DNA. Cohen (1948) and Cohen and Arbogast (1950b) have shown that DNA is synthesized much more rapidly by the phage-host complex than by uninfected host cells. Through premature lysis of infected cells, Doermann (1951) has observed the appearance of virus at a rate very similar to the rate of synthesis of nucleic acid. Almost all of the nucleic acid formed by the phage-host complex can be accounted for in the virus progeny (Cohen and Arbogast, 1950a). Coliphage T2 appears to consist of a nucleic acid functional unit attached to a sulfur-containing protein carrier (Hershey and Chase, 1952). If these findings with coliphage are applicable to the lactic streptococcus phages, then crystal violet conceivably might combine with the nucleic acid fraction and prevent its attachment to the protein carrier.

SUMMARY AND CONCLUSIONS

1. Most of the 110 agents tested were toxic to the host bacteria but did not retard phage multiplication sufficiently to prevent mass lysis. Several compounds which prevented mass lysis were extremely toxic to the host bacteria.

Crystal violet suppressed growth of two phage strains at very low levels (1.0×10^{-7} M) which permitted normal growth of the host cells. Failure of crystal violet to prevent multiplication of many other phage strains suggests possible variations in the multiplication mechanisms of different strains of virus.

2. Virustatic levels of crystal violet did not destroy unadsorbed virus, reduce adsorption or prevent invasion. Increase of phage was reduced in one-step growth experiments, and mass lysis was prevented or delayed in long-time experiments. Phage yield was related directly to the portion of the latent period during which no crystal violet was present. Removal of crystal violet following its presence during most of the latent period resulted in a slight delay in the release of virus. Crystal violet reduced the yield of plaque-forming particles per infected bacterium, whereas the proportion of infected bacteria giving rise to active

progeny did not appear to be influenced to a significant degree. The evidence indicates that crystal violet interferes with a late phase of intracellular multiplication of the phage.

3. Inhibition of phage by crystal violet was counteracted by DNA but not by RNA or other reagents tested. Precipitation of DNA by crystal violet was demonstrated *in vitro*. The data indicate that inhibition of phage by crystal violet may result from combination of the dye with DNA at some critical stage in the incorporation of DNA into the phage particle.

4. The limited number of phage strains selectively inhibited and the rather narrow inhibition ratios in these cases make crystal violet unsuitable as a phage inhibitor in commercial dairy fermentations.

BIBLIOGRAPHY

Adams, M. H. 1949 The calcium requirements of coliphage T5. J. Immunol., 62:505-516.

American Public Health Association. 1948 Standard methods for the examination of dairy products, 9th edition. New York.

Anderson, E. H. 1946 Growth requirements of virus-resistant mutants of Escherichia coli strain "B". Proc. Nat. Acad. Sci. U. S., 32:120-128.

Anderson, S. G. 1948 Mucins and mucoids in relation to influenza virus action. I. Inactivation by RDE and by viruses of the influenza group, of the serum inhibitor of haemagglutination. Australian J. Exp. Biol. Med. Sci., 26:347-354.

Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F. and Stone, J. D. 1948 Mucins and mucoids in relation to influenza virus action. VI. General discussion. Australian J. Exp. Biol. Med. Sci., 26:403-411.

Anderson, T. F. 1943 The action of an antibiotic substance (penatin) on bacteriophage. J. Bacteriol., 46:110.

Anderson, T. F. 1948 The growth of T2 virus on ultraviolet-killed host cells. J. Bacteriol., 56:403-410.

Asheshov, I. N., Strelitz, F. and Hall, E. A. 1949 Antibiotics active against bacterial viruses. Brit. J. Exp. Pathol., 30:175-185.

Asheshov, I. N., Strelitz, F. and Hall, E. A. 1952 Phagolessin A58: a new antibiotic active against bacterial viruses. Antibiotics and Chemotherapy, 2:366-374.

Bawden, F. C. and Kleczkowski, A. 1948 Variations in the properties of potato virus X and their effects on its interactions with ribonuclease and proteolytic enzymes. J. Gen. Microbiol., 2: 173-185.

Benzer, S. 1952 Resistance to ultraviolet light as an index to the reproduction of bacteriophage. J. Bacteriol., 63:59-72.

Benzer, S., Delbrück, M., Dulbecco, R., Hudson, W., Stent, G. S., Watson, J. D., Weidel, W., Weigle, J. J. and Wollman, E. L. 1950 A syllabus on procedures, facts and interpretations in phage. In Delbrück, M. Viruses 1950, pp. 100-147. Pasadena, Calif., Calif. Inst. Technol.

Brewer, J. H. 1943 Vegetable bacteriological media as substituted for meat infusion media. J. Bacteriol., 46:395-396.

Burnet, F. M. 1948a Mucins and mucoids in relation to influenza virus action. III. Inhibition of virus heamagglutination by glandular fluids. Australian J. Exp. Biol. Med. Sci., 26:371-379.

Burnet, F. M. 1948b Mucins and mucoids in relation to influenza virus action. IV. Inhibition by purified mucoid of infection and haemagglutination with the virus strain WSE. Australian J. Exp. Biol. Med. Sci., 26:381-387.

Burnet, F. M. 1948c Mucins and mucoids in relation to influenza virus action. V. The destruction of "Francis Inhibitor" activity in a purified mucoid by virus action. Australian J. Exp. Biol. Med. Sci., 26:389-402.

Burnet, F. M., Keogh, E. V. and Lush, D. 1937 The immunological reactions of the filtrable viruses. Australian J. Exp. Biol. Med. Sci., 15:226-368.

Chapman, L. M., Greenberg, D. M. and Schmidt, C. L. A. 1927 Studies on the nature of the combination between certain acid dyes and proteins. J. Biol. Chem., 72:707-729.

Clark, P. F., McClung, L. S., Pinkerton, H., Price, W. H., Schneider, H. A. and Trager, W. 1949 Influence of nutrition in experimental infection. Bacteriol. Rev., 13:99-134.

Cohen, S. S. 1948 The synthesis of bacterial viruses. I. The synthesis of nucleic acid and protein in Escherichia coli B infected with T2r+ bacteriophage. J. Biol. Chem., 174:281-293.

Cohen, S. S. 1949 Growth requirements of bacterial viruses. Bacteriol. Rev., 13:1-24.

Cohen, S. S. and Anderson, T. F. 1946a Chemical studies on host-virus interactions. I. The effect of bacteriophage adsorption on the multiplication of its host, Escherichia coli B. J. Exp. Med., 84:511-523.

Cohen, S. S. and Anderson, T. F. 1946b Chemical studies on host-virus interactions. II. The chemical simulation of the interference phenomenon by 5-methyl tryptophane. J. Exp. Med., 84:525-533.

Cohen, S. S. and Arbogast, R. 1950a Chemical studies in host-virus interactions. VII. A comparison of some properties of three mutant pairs of bacterial viruses, T2r^{+} and T2r, T4r^{+} and T4r, T6r^{+} and T6r. J. Exp. Med., 91:619-636.

Cohen, S. S. and Arbogast, R. 1950b Chemical studies in host-virus interactions. VIII. The mutual reactivation of T2r^{+} virus inactivated by ultraviolet light and the synthesis of desoxyribose nucleic acid. J. Exp. Med., 91:637-650.

Cohen, S. S. and Fowler, C. B. 1947 Chemical studies on host-virus interactions. III. Tryptophane requirements in the stages of virus multiplication in the Escherichia coli-T2 bacteriophage system. J. Exp. Med., 85:771-784.

Cohen, S. S. and Roth, L. 1953 The phosphogluconate pathway of carbohydrate metabolism in the multiplication of bacterial viruses. J. Bacteriol., 65:490-495.

Collins, E. B. and Nelson, F. E. 1950 Acetate and oleate requirements of the lactic group of streptococci. J. Bacteriol., 59:69-74.

Commoner, B. and Mercer, F. 1951 Inhibition of the biosynthesis of tobacco mosaic virus by thiouracil. Nature, 168:113-114.

Czekalowski, J. W. and Dolby, D. E. 1949 Effect of enzyme inhibitors on the genesis of phage. Nature, 163:719-720.

Davies, W. L., Pond, W. L., Smith, S. C., Rasmussen, A. F., Jr., Elvehjem, C. A. and Clark, P. F. 1952 The effect of certain amino acid deficiencies on Lansing poliomyelitis in mice. J. Bacteriol., 64:571-582.

Delbrück, M. 1945a Effects of specific antisera on the growth of bacterial viruses (bacteriophages). J. Bacteriol., 50:137-150.

Delbrück, M. 1945b Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect. J. Bacteriol., 50:151-170.

Delbrück, M. 1948 Biochemical mutants of bacterial viruses. J. Bacteriol., 56:1-16.

Delbrück, M. and Luria, S. E. 1942 Interference between bacterial viruses. I. Interference between two bacterial viruses acting on the same host, and the mechanism of virus growth. Arch. Biochem., 1:111-114.

Dickinson, L. 1948 The bacteriophage of Pseudomonas pyocyanea. I. The effect of various substances upon their development. J. Gen. Microbiol., 2:154-161.

Doermann, A. H. 1951 Intracellular phage growth as studied by premature lysis. Federation Proc., 10:591-594.

Eaton, M. D. 1950. Chemotherapy of virus and rickettsial infections. Ann. Rev. Microbiol., 4:223-246.

Eaton, M. D., Magasanik, B., Perry, M. E. and Karibian, D. 1951 Inhibition of influenza and mumps viruses in tissue culture by basic amino acids. Proc. Soc. Exp. Biol. Med., 77:505-508.

Elford, W. J. 1948 The influence of antibacterial substances on the interaction of bacteria and bacteriophages. I. The influence of penicillin. J. Gen. Microbiol.,2:205-219.

Elion, G. B. and Hitchings, G. H. 1951 Antagonists of nucleic acid derivatives. V. Pteridines. J. Biol. Chem., 188:611-621.

Elliker, P. R. 1951 The problem of bacteriophage in the dairy industry. J. Milk and Food Technol., 14:13-16.

Emmerson, R. L., Whiffen, A. J., Bohonos, N. and DeBoer, C. 1946 Studies on the production of antibiotics by actinomycetes and molds. J. Bacteriol., 52: 357-366.

Enders, J. F. 1948 Remarks on cellular resistance to mammalian viruses. Federation Proc., 8:625-630.

Enders, J. F. and Pearson, H. E. 1941 Resistance of chicks to infection with influenza A virus. Proc. Soc. Exp. Biol. Med., 48:143-146.

Evans, E. A. 1952 Biochemical studies of bacterial viruses, Chapter II. Chicago, The University of Chicago Press.

Fitzgerald, R. J. and Babbitt, D. 1946 Studies on bacterial viruses. The effect of certain compounds on the lysis of Escherichia coli by bacteriophage. J. Immunol., 52:121-126.

Fitzgerald, R. J. and Lee, M. E. 1946 Studies on bacterial viruses. Observations on the mode of action of acridines in inhibiting lysis of virus-infected bacteria. J. Immunol., 52:127-135.

Ford, H. F. and Babel, F. J. 1950 Effect of incubation temperatures on the retention of bacteriophage by a culture of Streptococcus lactis. J. Dairy Sci., 33:466-472.

Foster, C., Jones, J. H. Henle, W. and Brenner, S. A. 1949 Nutrition and poliomyelitis. The effects of deficiencies of phosphorus, calcium, and vitamin D on the response of mice to the Lansing strain of poliomyelitis virus. J. Infectious Diseases, 85:173-179.

Foster, R. A. C. 1948 An analysis of the effect of proflavine on bacteriophage growth. J. Bacteriol., 56: 795-809.

Fowler, C. B. and Cohen, S. S. Chemical studies in host-virus interactions. IV. A method of determining nutritional requirements for bacterial virus multiplication. J. Exp. Med., 87:259-274.
1948

Gots, J. S. and Chu, E. C. Studies on purine metabolism in bacteria. I. The role of p-amino benzoic acid. J. Bacteriol., 64:537-546.
1952

Graham, D. M. and Nelson, F. E. Inhibition of lactic streptococcus bacteriophage by crystal violet and other agents. J. Gen. Physiol., 37:121-138.
1953

Green, R. G. and Stulberg, C. S. Cell-blockade in canine distemper. Proc. Soc. Exp. Biol. Med., 61: 117-121.
1946

Green, R. H., Rasmussen, A. F., Jr. and Smadel, J. E. Chemoprophylaxis of experimental influenza infections in eggs. Public Health Repts. (U. S.), 61:1401-1403.
1946

Green, R. H. and Wooley, D. W. Inhibition of certain polysaccharides of hemagglutination and of multiplication of influenza virus. J. Exp. Med., 86:55-64.
1947

Hershey, A. D. and Chase, M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol., 36:39-56.
1952

Hershey, A. D., Kalmanson, G. and Bronfenbrenner, J. Quantitative relationships in the phage-antiphage reaction: unity and homogeneity of the reactants. J. Immunol., 46:281-299.
1943

Hershey, A. D., Kalmanson, G. M. and Bronfenbrenner, J. Coordinate effects of electrolyte and antibody on the infectivity of bacteriophage. J. Immunol., 48:221-239.
1944

Hirst, G. K. Adsorption of influenza hemagglutinins and virus by red blood cells. J. Exp. Med., 76:195-209.
1942

Hoffman, C. E. and Rahn, O. The bactericidal and bacteriostatic action of crystal violet. J. Bacteriol., 47:177-186. 1944

Horsfal, F. L., Jr. Chemotherapy in viral infections. Am. J. Med. Sci., 220:91-102. 1950

Jones, D., Beaudette, F. R., Geiger, W. B. and Waksman, S. A. A search for virus-inactivating substances among microorganisms. Science, 101:665-668. 1945

Kerby, G. P., Gowdy, R. A., Dillon, E. S., Dillon, M. L., Csaky, T. Z. and Beard, J. W. Purification, pH stability and sedimentation properties of T7 bacteriophage of Escherichia coli. J. Immunol., 63:93-107. 1949

Kozloff, L. M., Knowlton, K., Putnam, F. W. and Evans, E. A., Jr. Biochemical studies of virus reproduction. V. The origin of bacteriophage nitrogen. J. Biol. Chem., 188:101-116. 1951

Lichstein, H. C., Waisman, H. A., Elvehjem, C. A. and Clark, P. F. Influence of pantothenic acid deficiency on resistance of mice to experimental poliomyelitis. Proc. Soc. Exp. Biol. Med., 56:3-5. 1944

Lilly Research Laboratories. The multiplication of viruses. Research Today, 8:26-50. 1952

Luria, S. E. Dissociation of the growth of bacterial viruses and of their host by means of temperatures above optimum. Proc. Indiana Acad. Sci., 53: 28-29. 1943

Luria, S. E. and Delbrück, M. Interference between inactivated bacterial virus and active virus of the same strain and of a different strain. Arch. Biochem., 1:207-218. 1942

Lwoff, A., Siminovitch, L. and Kjelgaard, N. Induction de production de phage dans une bacterie lysogene. Ann. inst. Pasteur, 79:815-858. 1950

Markham, R., Matthews, R. E. F. and Smith, K. M. Specific crystalline protein and nucleoprotein from a plant virus having insect vectors. Nature, 162:88-90. 1948

McCrea, J. F. 1948 Mucins and mucoids in relation to influenza virus action. II. Isolation and characterization of the serum mucoid inhibitor of heated influenza virus. Australian J. Exp. Biol. Med. Sci., 26:355-370.

Miller, E. M. and Goebel, W. F. 1949 Studies on bacteriophage. I. The relationship between the somatic antigens of Shigella sonnei and their susceptibility to bacterial viruses. J. Exp. Med., 90:255-265.

Mirsky, A. E. and Ris, H. 1951 The composition and structure of isolated chromosomes. J. Gen. Physiol., 54: 475-492.

Moore, A. E. and Friend, C. 1951 Effect of 2,6-diaminopurine on infection of Russian encephalitis in the mouse. Federation Proc., 10:365-366.

Nelson, F. E., Harriman, L. A. and Hammer, B. W. 1939 Slow acid production by butter cultures. Iowa Agr. Exp. Sta. Research Bull. No. 256.

Niven, C. F., Jr. 1944 Nutrition of Streptococcus lactis. J. Bacteriol., 47:343-350.

Overcast, W. W., Nelson, F. E. and Parmelee, C. E. 1951 Influence of pH on proliferation of lactic streptococcus bacteriophage. J. Bacteriol., 61:87-95.

Perlman, D., Langlykke, A. F. and Rothberg, H. D., Jr. 1951 Observations on the chemical inhibition of Streptomyces griseus bacteriophage multiplication. J. Bacteriol., 61:135-143.

Pond, W. L., Davies, W. L., Smith, S. C., Elvehjem, C. A., Rasmussen, A. F., Jr. and Clark, P. F. 1952 The influence of amino acid deficiencies on Theiler's GD VII encephalomyelitis of mice. J. Bacteriol., 64:583-592.

Potter, N. N. and Nelson, F. E. 1952a Effects of calcium on proliferation of lactic streptococcus bacteriophage. I. Studies on plaque formation with a modified plating technique. J. Bacteriol., 64:105-111.

Potter, N. N. and Nelson, F. E. Effects of calcium on
1952b proliferation of lactic streptococcus bacteriophage. II. Studies of optimum concentrations in a partially defined medium. J. Bacteriol., 64:113-119.

Potter, N. N. and Nelson, F. E. Role of calcium and
1953 related ions in proliferation of lactic streptococcus bacteriophage. J. Bacteriol., 66:508-516.

Price, W. H. Bacteriophage formation without bacterial
1947a growth. I. Formation of Staphylococcus phage in the presence of bacteria inhibited by penicillin. J. Gen. Physiol., 31:119-126.

Price, W. H. Bacteriophage formation without bacterial
1947b growth. II. The effect of niacin and yeast extract on phage formation and bacterial growth in the presence of penicillin. J. Gen. Physiol., 31:127-133.

Rafelson, M. E., Jr., Winzler, R. J. and Pearson, H. E.
1951 A virus effect on the uptake of C^{14} from glucose *in vitro* by amino acids in the mouse brain. J. Biol. Chem., 193:205-217.

Raper, K. B. (Personal Communication.) Northern Regional
1952 Research Laboratory, Peoria, Illinois.

Rasmussen, A. F., Waisman, H. A. and Lichstein, H. C.
1944 Influence of riboflavin on susceptibility of mice to experimental poliomyelitis. Proc. Soc. Exp. Biol. Med., 57:92-95.

Smith, A. G. The effect of acriflavine and phosphine GRN
1949 on lysis of bacteria by bacteriophage. J. Immunol., 61:57-63.

Snell, E. E. Bacterial nutrition-chemical factors. In
1951 Werkman, C. H. and Wilson, P. W. Bacterial Physiology, p. 230. New York, Academic Press, Inc.

Spizizen, J. Biochemical studies on the phenomenon of
1943a virus reproduction. I. Amino acids and the multiplication of bacteriophage. J. Infectious Diseases, 73:212-221.

Spizizen, J. 1943b Biochemical studies on the phenomenon of virus reproduction. II. Studies on the influence of compounds of metabolic significance on the multiplication of bacteriophage. J. Infectious Diseases, 73:222-228.

Spizizen, J., Kenney, J. C. and Hampil, B. 1951 Biochemical studies on the phenomenon of virus reproduction. III. The inhibition of coliphage $T2r^{+}$ multiplication by sodium salicylate and sodium gentisate. J. Bacteriol., 62:323-329.

Stanley, W. M. and Lauffer, M. A. 1948 Chemical and physical procedures. In Rivers, T. M. Viral and rickettsial infections of man, pp. 18-66. Philadelphia, J. B. Lippincott Company.

Stearn, A. E. 1930 Compound formation of crystal violet with nucleic acid and gelatin and its significance in dye bacteriostasis. J. Bacteriol., 19: 133-143.

Stearn, E. W. and Stearn, A. E. 1926 Conditions and reactions defining dye bacteriostasis. J. Bacteriol., 11:345-357.

Turner, G. E. 1948 Dynamics of the Streptococcus lactis-bacteriophage relationship. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library.

Weigle, J. J. and Delbrück, M. 1951 Mutual exclusion between an infecting phage and a carried phage. J. Bacteriol., 62:301-318.

Wells, A. Y. and Sherwood, N. P. 1934 Selective action of dyes and of other disinfectants on bacteriophages. J. Infectious Diseases, 55:195-198.

Wilkowske, H. H. 1949 Characterization of bacteriophage active against lactic streptococci. Unpublished Ph. D. Thesis. Ames, Iowa, Iowa State College Library.

Wooley, J. G. and Murphy, M. K. 1949 Metabolic studies on T2 Escherichia coli bacteriophage. I. A study of desoxypyridoxine inhibition and its reversal. J. Biol. Chem., 178:869-875.

Wooley, J. G., Murphy, M. K., Bond, H. W. and Perrine, T. D.
1952 The effect of certain chemical compounds on the multiplication of T2 bacteriophage. J. Immunol., 68:523-530.

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