THE BACTERIOPHAGE

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THE BACTERIOPHAGE

INTRODUCTION

D'Herelle (1926) applied the term bacteriophagy to the phenomenon (commonly called the Twort-D'Herelle phenomenon) which consists essentially in a "dissolution" of bacteria through the operation of a principle he named "bacteriophage". According to him transmissible lysis is the result of an acute infectious disease of bacteria in which the etiological factor is an "autonomous ultra-microscopical corpuscle--"Bacteriophagum intestinale". It is quite generally accepted, however, that the phenomenon was first observed and described by Twort in 1915, although some believe it was first observed by Hankin who, in the latter part of the last century, found that the water of the Jumna river, below the town of Agra, India, was bactericidal for various micro-organisms, and particularly the cholera vibrio. To the agent responsible for bacteriophagy D'Herelle gave the name "bacterio-phage" (to eat bacteria). Recently upon D'Herelle's request, because of a dispute of long standing between himself and Twort as to the relationship of the transmissible autolysis of Twort and the bacteriophagy, a committee was chosen to investigate the question and settle the dispute. Flu and Renaux (1932) were chosen to meet the qualifications demanded by D'Herelle and, after a thorough study had been made, it was decided that the phenomenon of transmissible autolysis of Twort and bacteriophagy

are identical phenomenon.

Twort (1915), while experimenting with calf vaccinia, conceived the idea that, since there are ultra-microscopical viruses that are pathogenic there must be some that are non-pathogenic; and he began searching materials from the soil, dung, grass, hay, and water from ponds for such a virus. Growth of the filter-passing viruses was not obtained on the artificial media nor by animal inoculations; but one day while he was working with glycerinated calf vaccina which had been inoculated on agar tubes, he observed micrococcic growths in which watery looking areas appeared. In some of the cultures of micrococci that grew, it was found that not all of the colonies could be sub-cultured, but if kept, they became "glassy" and transparent. On examination of these glassy areas nothing but minute granules could be seen. In a review of his previous study Twort (1930) stated that "under suitable conditions the 'glassy' or clear spots increased in size." This change in appearance, according to him, begins at the edge of the colony, and sometimes gradually spreads. It is evident from a review of the literature that these areas are now generally recognized as plaquès or places where lysis of the bacteria has occurred. Action seemed to be more rapid and complete with vigorously growing young cultures than with old ones and very little action could be observed on dead cultures even though young heat killed cultures were used. The

observed transparent material was passed through the finest porcelain filters and one drop of the filtrate, pipetted over an agar slant, was sufficient to make the media unsuitable for the growth of the micrococci. In some cases growth was stopped and the culture turned transparent directly. This condition or disease of the micrococci, when transmitted to pure cultures of the micrococcus, was conveyed to fresh cultures for an indefinite number of generations, but the transparent material did not grow by itself on any media tried. Heating the substance to 60°C. for one hour seemed to kill it. Some action could be noted on Staphylococcus aureus and albus isolated from boils of man but it had no action on the coli group, streptococci, tubercle bacilli or yeasts. When Twort plated out what he thought was a pure culture of the micrococcus it seemed free of the disease for months but eventually the transparent material would reappear and it could be obtained once again from subcultures. These results made it appear probable that the material was produced by the micrococci although it might have been due to contamination. Twort also obtained similar results from organisms of the intestinal tract. The following three explanations were given by him as to the nature of the transparent material (1) "It may be living bacteria that will grow only on living material; or it may be a tiny amoeba; (2) it may be living protoplasm that forms no definite individuals or an enzyme with power of growth; (3) if it is a part of the micrococcus it must be either a stage in its life history which will not grow on ordinary media

but stimulates fresh cultures of the micrococcus to pass into the same stage, or an enzyme secreted by the micrococcus which leads to its own destruction and the formation of more enzyme." He also suggests that it may be an acute infectious disease of the bacteria. It is rather interesting to note that each of Twort's explanations has some adherents among the present day workers. Prominent among these are: D'Herelle, who believes the lytic principle is a living parasite of bacteria. Hadley, who believes it is a stage in the life cycle of the organism, and Bordet and Bronfenbrenner who think it is a product of the bacterial cell.

It is interesting to note in the following main points of Twort's findings that they are, in general, believed to be true:

1. The transparent (lytic) material can be separated from the organism from which it is derived by filtration.

2. It can be transmitted indefinitely from one culture to another.

3. It is most active against young growing cultures.

4. There is no action on dead bacteria.

5. To be transmitted in series it must be in the presence of the living organisms.

6. It is active in dilutions of one to one million.

7. It resists heat to 60°C. for thirty minutes.

8. Action is not hastened by anaerobiosis.

9. It increases in quantity when acting on young growing

cultures of susceptible bacteria.

10. It is not very active on old cultures.

11. It acts to lesser degree on closely related organisms.

12. It does not act on unrelated organisms.

D'Herelle (1917) reopened experimentation on this subject when he isolated a b'phage active against the Shiga bacillus from a case of dysentery in an adult. Attempts to isolate the agent at various stages of the disease were not successful, except during convalescence. Sinse the phage seemed to appear only as the disease subsided, D'Herelle (1926) concluded that recovery somewhat depended upon the stage in the course of the disease and the amount of increase of the virulence of the active material.

EXISTENCE IN NATURE

It is the belief of many workers that bacteriophage may be encountered any where there are bacteria actively reproducing. Hadley (1927) states, "The phenomenon of transmissible bacterial autolysis is widespread in the bacterial world." It is probably found more often among the intestinal and pyogenic groups of organisms although it has been isolated from a wide variety of bacteria. Larkum (1929) in speaking of the value of bacteriophage vaccines says that in general no phage has been discovered for the infectious agents of diseases for which we have no prevention. This, he thinks, is unfortunate because of its possibilities for therapeutic purposes. Kuttner (1923) reports finding the lytic material in the normal tissues of the guinea pig and rabbit.

According to D'Herelle (1926) the normal habitat of the bacteriophage is the intestinal tract of animals and it may be found where contamination from the intestinal excreta occurs. It seems to be true that sewage and surface water are likely to contain some kind of a lytic substance for certain bacteria that are commonly found in such places. This may be due to the ability of the bacteria to reproduce in such materials. He also says that there is just one species of bacteriophage and that the different "races" or strains are different from one another because of adaptation, just as a "horse streptococcus" may become a "rabbit streptococcus", or a "guinea pig streptococcus" by passage through a series of animals. D'Herelle possibly was working with a polyvalent phage where the "adaptation" was a result of an increase of the bacteriophage, for the susceptible bacterium, caused by regeneration at the expense of the particular bacterium studied with subsequent loss of the phages that were active against other bacteria. There is also evidence to make one believe that an adaptation takes place, or possibly merely the regeneration of new material that is more potent. Schwartzman (1927) reports the development of a powerful bacteriophage against hemolytic streptococci of erysipelas origin by adaptation, using a B. Coli phage, a Staphylococcus phage, and a rabbit hemolytic streptococcus phage to begin with. Bronfenbrenner (1933) states that polyvalence is usually due to

the presence of several independent phages; but he cites evidence to show that pure single phages may exhibit activity against several more or less closely related species of bacteria. He gives no evidence to support the assumption that adaptation has taken place. Hitchner (1930) isolated a race of bacteriophage from the root nodules of red clover in which he found the agent to be specific for its own homologous strain of organisms, but no lysis was produced on a stock culture from the nodules of red clover, or on strains isolated from peas, beans, alfalfa and vetch. Hadley (1931) thinks he converted a B. paratyphosus A organism from the "S" to the "R" type and then caused the "R" type to develop the bacteriophage spontaneously. Stark and Stark (1932) found an anti-aerogenes phage on lettuce, carrots, corn silks and other plant tissues. The author isolated a bacteriophage for A. cloacae from river water, one for B. coli from the intestine of a cat, and one for an Achromobacter organism from sewage. It would seem superfluous to mention any more instances where the lytic principle has been found, for the literature contains numerous reports of this nature.

NATURE OF THE LYTIC AGENT

The nature of the bacteriophage as explained by the original discoverer was presented above. D'Herelle (1926) believes the

bacteriophage, from a physical point of view, to be a colloidal micella which is a living parasite of bacteria. It multiplies, he thinks, at the expense of the bacterium, heterologous with respect to itself, and transfers this heterologous substance into a homologous substance, a substance distinctly its own; therefore, it (phage) is endowed with the power of assimilation which he considers one of the most important criteria of life. He assumes the corpuscular nature of the active agent of transmissible autolysis on the basis that minute granules were observed by him in the filtrates of bacterial cultures, subjected to the action of phage, upon examination with an ultra-microscope. The identification of these particles with the active agent is supported by the observation that on long standing or by intensive centrifugation of the active filtrates, the particles tend to settle to the bottom of the container; and, at the same time, the active principle collects in the lower portion of the container. He found that serial dilution gives no gradual diminution of activity of phage, as exhibited by antiseptics; but the resulting dilution either completely lysed the bacteria inoculated into the tube or there was no lytic effect as dilution was carried beyond the point at which a certain amount (1×10^{-10} c.c.) of the agent is present. According to Topley and Wilson (1929) this latter view is now

widely accepted and the dilution method is also considered the best means of isolating pure races of bacteriophage. The author found that the limiting dilution depends upon the concentration and virulence of the material used. Bronfenbrenner (1929) raises the question as to the nature of the small amounts of the active agent. He believes it is possible that the lytic principle is present in the media in a state of true solution, or as a highly dispersed colloid, and that it is merely adsorbed on bacterial debris and other particles which determine its distribution in the medium. It is possible also, according to Bronfenbrenner, that the particles in the filtrate represent the corpuscle of autonomous organised parasites, and can be identified with the granules, observed by D'Herelle, within the infected bacteria under an ultra-microscope; but "Later investigations have indicated that these particles do not represent autonomous units of the active agent, but merely serve as a vehicle on which the active principle is adsorbed, and from which, under proper experimental conditions, it can be detached." To do this Bronfenbrenner deposited the particles carrying the phage on an ultra-filter through which they could not pass and washed them repeatedly with water and buffer solution but the particles did not give up the lytic substance. When these same particles however were washed with broth the phage reappeared in the filtrate. Hetler and Bronfenbrenner (1931) were

able to dislodge the bacteriophage from some of the larger particles and cause them to be redistributed to smaller particles. Diffusion cells made of thin porous powered glass discs fused together into glass vessels and standardized by Hydrochloric acid diffusion, were used in this work. They found that the particles on which the active agent is carried varied in size, ranging from .6 $\mu\mu$ to 11.4 in radius. Bronfenbrenner (1929) questioned the above theory and attempted to determine the living nature of the phage by experimentation on the carbon-dioxide production of the lytic principle. He used a micro-respirometer which would measure .005 c.c. of CO_2 but none of the gas could be detected in 10^{12} active units of the bacteriophage during a period of ninety-six hours. If the phage did respire, except in quantities too small to be detected, it was calculated that its rate of respiration would have to be at least ten thousand times slower than that of an equal number (10^{12}) of bacterial spores not to be detected in ninety-six hours. He further says it is generally observed that the smaller the living organism, and consequently the greater its surface per gram mass, the higher is its rate of respiration. Therefore, he concludes, from these experiments that the phage did not respire. Bronfenbrenner further states that when the "Bacteriophagum intestinale" is in the process of active "multiplication" as is assumed to be the case in the presence of susceptible bacteria, the rate of respiration of the whole culture

(when corrected for the higher rate of multiplication of bacteria in the presence of bacteriophage) was found to be the same as that of a control culture without the phage. He and others writers, in studying the independent metabolism of the bacteriophage, as as expressed in terms of its reducing power, found that the rate of reduction by the culture of susceptible bacteria, grown in the presence of phage, is entirely independent of the concentration of the active agent; but the rate rises and falls with the number of intact bacteria present during the various phases of the process. It will be seen later this author who has done so much work on the subject in this country believes the phenomenon to be an autolytic process in which the active substance is a product of bacterial metabolism.

Bordet and Ciuca explained bacteriophagy on an autolytic basis in 1920. Briefly Bordet's view is expressed in the following quotation: "Bacteriophagy represents the pathological exaggeration of a process which is physiological to bacteria, and is the result of a vitiation which has occurred in the accomplishment of a normal process". Lisbonne and Carrere in 1922 showed that different strains of B. Coli may bring about lysis of dysentery bacilli when cultivated with them in bouillon; moreover, the filtrate of such a culture caused lysis of dysentery bacilli. The process could be transmitted in series according to Bordet (1931) who claims to have accomplished similar results with an organism of

the same species. He found that in repeated isolations one does not succeed in depriving a lysogenic colon bacillus of its aptitude for starting lysis of the dysentery bacillus.

Hadley (1927) regards the bacteriophage merely as a stage in the life cycle of an organism undergoing dissociation in which he describes three types or characteristics of the organism in the process of change -- a "mutation-like phenomenon". The first of these types he calls the normal or S (smooth), the second the intermediate, O (transitional), and the third the abnormal, R (rough). Through the functioning of the O type which is a highly unstable and sensitive phase in the life of the culture, the R type is produced and frequently the transitional stage cannot be observed, according to him. The lytic areas, O colonies, then (representing the phage) are, in reality, "vanishing" secondary colonies arising and quickly disappearing in the mother culture, thereby becoming a filterable stage in the cyclogeny of the same bacterial species, or perhaps a closely related species. The filterable substance then acts as a stimulus, when added to new cultures, to the susceptible bacterial cells which produce more of the lytic agent.

Rivers (1929) suggested the possibility of the bacteriophage being a latent virus. Fowl tumors and carcinomas, he says, are transmissible in series. Schultz and others (1929) conclude, after having made a series of studies on the antigenic properties of other ultra-viruses, including herpes, vaccinia, variola,

rabies, and foot-and-mouth disease, that the bacteriophage possesses all the essential attributes of an ultra-virus.

In referring to the lytic agent as a product of bacterial metabolism, Bronfenbrenner suggests the fact, that accumulation of the active agent in the medium is independent of lysis, but is intimately connected with the active growth of the bacteria, strengthens the view expressed earlier that the phage may be some product of bacterial metabolism. He also says "although facts concerning the phenomenon of bacteriophagy leave an impression that the active agent of transmissible lysis is a bacterial product, the reports of successful production of phage from bacteria directly without the intermediary of any biological material have thus far not been convincing." Koser (1927) concludes after experimenting with a bacteriophage active against a thermophilic organism at 57° to 58°C. that the lytic principle is not a distinct species of living organisms; but that a close relation between the principle and organism itself is suggested. Many other workers too numerous to mention in a paper of this kind have come to conclusions similar to the ones just mentioned relative to the possibility of the bacteriophage being a product of bacterial metabolism.

Some workers suggest the possibility that the active substance in transmissible lysis may be an enzyme or a toxin. Kuttner (1923) gives the following reasons for believing phage to be an enzyme; (1) Extremely small amounts will dissolve

a large number of bacteria. (2) The lytic principle was still active after remaining in a sealed tube for four years. (3) It resists heating to 70°C. for one hour. She concludes, however, that it is not an enzyme because it acts on living rather than dead cells; and that the phage represents a secretion of bacteria of the nature of an autolysin which acts as a catalyst on the actively growing bacteria, destroying the equilibrium between constructive forces and destructive forces in favor of the latter. Potency in itself means little because, as Rivers (1929) says, very small amounts of filterable viruses are sometimes sufficient to kill a rabbit in every case. The same is true of rabies -- it is always fatal if infection occurs. Downs (1931), working in this laboratory with B. tularense found that one one-millionth of a c.c. of blood from an infected guinea pig is sufficient to kill a normal pig. Resistance to heat or viability after standing long periods of time can not be taken as criteria because the bacteriophage compares favorably with some of the ultra-viruses and some bacteria in these respects. Weiss and Arnold (1924) who have done considerable work on the antigenic properties of the bacteriophage found that phage reacts antigenically like a ferment. Muckenfuss (1928) and Schultz (1929) found that the phage antiserum neutralisation of phage is closely analogous to that of the neutralization of a toxin by an antitoxin. They do not conclude, however, that the bacteriophage must necessarily be a toxin or ferment. Northrop and Krueger (1930) in a report

on the kinetics of the bacterium-bacteriophage reaction remark that the lytic agent does not react like a living organism.

HOW TO OBTAIN THE BACTERIOPHAGE AND SOME OF ITS PROPERTIES

A very simple method of obtaining the lytic agent is given by Arnold (1925). Briefly, it consists of filtering the water, sewage, or other material in which he expected to find the phage and, if weak in lytic activity, an equal amount of the filtrate was added to double strength broth and the mixture was subsequently inoculated with the bacteria. After twenty-four hours incubation at 37°C. the culture was filtered and the process repeated. If there are very small amounts of the phage present, or if it is of low virulence, it may be necessary to repeat the procedure several times before any lytic action can be observed. The author used similar methods in isolating races of the lytic principle mentioned previously, except that sub-cultures from the mixtures were made, every three or four hours, into plain broth tubes and onto 1% plain agar plates. This was done so that a small amount of lysis might be detected in case there might not be enough material present to lyse the whole culture, as is often the case.

As a general rule, bacteriophagy will take place to the greatest extent in media and under conditions which provide optimum growing conditions for the bacteria that are to undergo dissolution. It is generally conceded that young actively reproducing bacteria that are susceptible to the particular

phage in question are a requisite for lysis to occur; but Northrop and Krueger (1930) found that lysis without growth will take place with young susceptible bacteria when the ratio of phage to bacteria (P/B) equals 125 or more (or when log P/B = 2.1 or more). Findings in this investigation indicate that media with a reaction of pH 7.6 to 7.8 gave the best results with the phages, except a diphtheria race of phage which seemed to be more active at about pH 7.2--7.4. However, lysis was obtained over a fairly wide range of hydrogen ion concentration. Sugars or substances which may permit a lowering of the pH to any great extent should be avoided, if possible, remembering of course, the above statement on the matter. A sugar medium could not be used at the beginning of this study, but, after the virulence of the phages had increased, a small amount of sugar was used apparently with better results.

The lytic effect can be noted on solid media, providing the percentage of agar used is not too high. Bronfenbrenner has demonstrated that lysis will not occur on anything above 4% agar. The amount of available water seems to play a great part in bacteriophagy. D'Herelle (1926) suggests .8 to 1% agar as being optimum. The activity of phage on plain agar is manifested by the formation of small, clear, transparent areas from .5 m.m. to 8 m.m. in size, if very high dilutions are used. Otherwise, it may appear as clear irregularly shaped areas. The single circular areas have been given the name, "plaque" by D'Herelle

who thinks they represent colonies of bacteriophage corpuscles, derived from individual corpuscles. Partial anaerobiosis does not (Schwartsman 1925) favor regeneration of the phage by modifying the rate of bacterial growth, but it has some direct relation to the regeneration of the principle. In this sense it enhances the phage, provided the restriction be produced within the first three hours of growth of the bacteria.

The temperature range at which the agent will regenerate, as reported by various workers, is 8°C. to 76°C., depending upon the race and virulence of the bacteriophage, also the kind of media used. It is evident that a thermophilic organism would be involved if phagic action occurs at 76°C. A temperature of 70°C. for one hour usually destroys the phages of the ordinary (mesophilic) bacteria. Rivers (1927) studying the effect of repeated freezing (-185°C.) and thawing on colon bacilli, virus III, vaccinia virus, herpes virus, bacteriophage, complement, and trypsin found no great difference in the resistance of the various substances. Nanavutty (1930) found heat susceptibility of the bacteriophage to be dependent in large measure on the substrate in which it is suspended, perhaps owing to an alteration in its physical state, and also upon the dilution of the material when heated. For example, B. Coli phage diluted 1-10,000 and heated to 65°C. became inactive in 3 minutes. Undiluted phage (otherwise same conditions as diluted) withstood 65°C. for 30 minutes without any reduction in activity. The lytic activity

was destroyed quicker in K_2HPO_4 buffer solution than in bouillon. The author (1930) found an anticoli phage was destroyed at 65°C. in 30 minutes when heated in a protein-free, K_2HPO_4 buffer medium.

Resistance of the bacteriophage to chemicals is intermediate between the vegetative and spore forms of Bacillus subtilis according to D'Herelle (1925); and the young corpuscles are more resistant than the old ones. A detailed discussion on this subject will be given in part II.

As regards the therapeutic properties of the bacteriophage, Bronfenbrenner (1929) states that a review of the clinical reports undoubtedly supports D'Herelle's conclusion that in dysentery and Staphylococcus infections the results seem very encouraging. Larkum (1929) believes there is as much justification for the use of phage for therapeutic purposes as ever existed for the use of scarlet fever toxin or antitoxin. He has been using it to some extent for vaccination purposes as well as for direct application in Staphylococcus infections. In one case where Larkum (1929) used a typhoid bacteriophage as a substitute for typhoid vaccine, he found the bactericidal power of the blood and the opsonic index of those inoculated with phage were higher than those where the ordinary bacterin was used. Six months later the antibody titer, produced by one inoculation of the bacteriophage was higher than where three vaccine inoculations had been made. Reactions subsequent to injection of phage were not nearly so marked as when bacterins were administered. Walker (1930)

compared the toxicity and germicidal properties of phage with mercuric chloride, phenol, formalin, iodine and chloramine. The results of this comparison show that Staphylococcus bacteriophage had a much wider range of dilution (full strength to 1-512) over which the resulting lesions were partially supressed without necrosis of the tissues. Patterson and Albee (1930) got gratifying results through the introduction of an antistaph phage into the wound of an osteomyelitis case in which staphylococcus aureus, along with B. Coli, pyocyaneous, and diphtheroid organisms were found. British workers (1931) expressed the belief that when three types or races of anticholera phage are mixed together they will cure any case of cholera that receives early treatment. One phage developed at the expense of six strains of dysentery organisms and another developed at the expense of five strains of cholera organisms were mixed and used on both cholera and dysentery cases with good results. They found the mixture kept better than if the dysentery phage were kept separate from the cholera. In the oral treatment of fifty-nine cases of cholera, seven deaths resulted, while twelve out of thirteen cases died where no phage was used. Applebaum and Mac Neal (1932) found that purulent exudate, when diluted exerts an interfering influence on the lytic action of phage. Blood and Serum were also found to exert an inhibiting influence upon the action of the phage. Similar results were found to be true for both coli and staphylococcus bacteriophages.

These experiments indicate that the therapeutic value of the lytic material would be diminished in pyogenic infections. Cowles (1931) obtained no protective action of b'phage in cases of experimental anthrax when the organisms and lytic agent were injected simultaneously into white mice. Moreover he did not demonstrate any immunising value for phage when it was used as an antigen for anthrax vaccinations in mice, guinea pigs or rabbits.

The antigenic properties of the bacteriophage were first detected by Bordet in 1921 (quoting from Weiss 1927). His findings have since been confirmed by many workers. Much work has been done upon the antigenic properties of phage, and also upon the resulting antiserum properties, not only for the interest which the field affords but also because of the fact that it seems to be a good method of approach in trying to determine the nature of the bacteriophage. Some of the most delicately known protein tests, complement-fixation for example, can be utilized in a study of this kind. Schultz (1929) reports that D'Herelle and Eliava in seeking to demonstrate the unicity of phage, tested an antiserum for a Shiga phage against filtrates containing lytic agents for the organism of plague and barbone; and cross-reactions were obtained. These investigators concluded that the bacteriophage was a common antigen responsible for their nonspecific reactions.

Gratia and Jaumain (Schultz 1929) were first to question that "common antigen" actually represented the phage. They noted that, where as in the neutralization tests the antiphagic sera behaved in a highly specific manner in inactivating different phages, in the complement-fixation tests, the antiphagic sera,

when tested repeatedly against the same bacteriophage suspensions, but freshly prepared for each test, some times yielded results which appeared specific; but at other times were clearly non-specific. Those observations led them to the conclusion that the cross-fixation must be due to the presence of some substance in the broth either formed during the process of bacterial metabolism or set free during the lysis of the organisms -- an antigenic substance common to all bacteria.

Arnold and Weiss (1925) were the first to successfully eliminate the non-specific reactions which served to more or less obscure the apparently unique antigenic behavior of the phage. This they apparently did by trypsinization of the phage suspension. Schultz (1929) states, "That phages, with rare exceptions, withstand the action of trypsin indefinitely is well known." Animals injected with these trypsinized bacteriophage suspensions yielded antiphagic sera of marked activity, but these sera produced no agglutinins, precipitins or complement-fixing antibodies against the bacteria or bacterial proteins of the homologous strains nor for the bacteriophage itself. On the basis of these observations they came to the conclusion that only neutralizing antibodies were produced against the phage. Schultz and his co-workers (1928) could not demonstrate any specific complement-fixing antibodies when immune sera, produced in rabbits with pure vaccinia brain virus, were tested against antigens prepared from lesions of cutaneous vaccinia or against brain virus antigens. Immune sera also failed to present evidence of specific precipitating anti-

bodies, but virucidal antibodies were found in all the sera tested. They also found (1928) the same facts were true of herpes virus and later (1929) report that no complement-fixing antibodies, precipitins or agglutinins were produced in their experiments upon rabbits in which Staphylococcus phage was used as the antigen. They did demonstrate specific neutralizing antibodies for the phage -- hence their conclusion that bacteriophage possesses all the essential characteristics of an ultra-virus.

In a study of the antilytic antibody Schultz (1929) quoting from Bail and others, states that the neutralizing antibody exhibits a high degree of specificity but he believes that exact limits of this specificity still remain to be worked out. Some investigators suggest that the specificity is such that an antiserum prepared against a given phage will neutralize only the corresponding phage, and this in the case of a polyvalent phage quite independently of the bacterial substrate at the expense of which the particular polyvalent bacteriophage is developed. Observations by other workers seem to indicate that the specificity may be even more restricted, being, in the case of polyvalent phages, often influenced by the bacterial species at the expense of which the phage has been regenerated (Weiss and Arnold 1924); and Muckenfuss (1928) thinks the antibody acts on the lytic agent by inactivation of individual units -- a reduction in the number of plaques rather than their size as reported by some. The author (1930) confirmed Muckenfuss' findings. Most investigators agree

that the speed of reaction in the neutralisation of phage by its specific antiserum is slow. Schultz (1928), Muckenfuss (1928) and others claim that the neutralisation of the bacteriophage by the antiphagic serum is closely analogous to that of neutralisation of toxin by antitoxin. According to Weiss and Arnold the reaction is influenced by the same factors of time, temperature and Berkfeld filtration, using mixtures of toxin-antitoxin of similar concentrations.

For purposes of purifying the bacteriophage before using it as an antigen Weiss and Arnold (1926) resorted to tryptic digestion of the lysate to remove the antigenic bacterial proteins in order to produce only neutralising antibodies for the phage after injecting trypsinised material into rabbits. Their results show that only antilytic antibodies, with as high a titer as the untreated produced, were obtained.

Schultz (1929) working with a staphylococcus bacteriophage was unable to demonstrate an antilytic antibody after tryptic digestion of the lysate. He observed in previous work that some strains of Staphylococcus b'phage were not very resistant to the action of trypsin while the bacteriophages for the colon-typhoid group of organisms were quite resistant. The author (1930) was able to demonstrate only an antilytic antibody for a B. coli b'phage after the antibacterial antibodies had been absorbed from the b'phage immune serum. There was no chance for the introduction of protein into these tests, other than from the bacteria them-

selves, because protein-free synthetic media were used. Kligler and Olitsky (1932) using protein-free suspensions of a coli bacteriophage, prepared by the adsorption-elution technic, to immunise rabbits were able to demonstrate that the pure bacteriophage is a definite antigenic entity. They also found that the union of the agent and its specific antibody takes place very slowly; and that the addition of complement accelerated the reaction. This latter result agrees with the finding reported by the author in 1930.

MECHANISM INVOLVED IN THE PROCESS OF LYSING BACTERIA

"The fact that the regeneration of the active agent depends wholly on the presence of living, susceptible bacteria suggests that it may be itself a product of some phase of bacterial activity. At all events, it is clear that when traces of active agents are introduced into a growing culture of susceptible bacteria, the regeneration of the agent takes place during the phase of active growth, and precedes the lysis of the bacteria (when the latter takes place at all). Thus the possibility that the active agent is liberated as a result of lysis of bacteria seems to be excluded. -- As to the nature of the active substance and the mechanism of its production by bacteria, there exists great divergence of opinion. As to the nature of the effect of this hypothetical substance on normal bacteria, resulting in the one hand in its own regeneration and on the other in the disappearance of visible bacterial growth there exists two views. The majority of workers

think this effect consists in the vitiation of bacterial metabolism accompanied by the accumulation of products which under normal conditions are not formed at all, or appear only temporarily as intermediary links in the chain of metabolic reactions. The accumulation of these products in turn causes disfunction of new generations of bacteria, and thus perpetuates the disease leading finally to autolysis of the bacteria." -- Bronfenbrenner 1929. According to this worker and others there seems to be an accelerated multiplication of the bacteria when in the presence of phage followed by a swelling of the affected cells and finally a rupture of the cell wall and immediate liberation of the contents of the cell into the solution. If this were a process of digestion, chemical examination of the solution of lysed material should show the presence of products of digestion; but so far, the literature reveals no findings where this has been the case, except that given by Bronfenbrenner in which he reports lysis of bacteria in a protein-free medium which he says "gave unmistakable evidence of hydrolysis of bacterial protein." He thinks the reason others have not been able to show hydrolytic action is probably due to the fact that the material subjected to analysis consisted of ordinary media too rich in various products of hydrolysis of protein to permit the detection of possibly a small increase due to hydrolysis of bacteria. He does not believe that the active agent plays any part in the actual dissolution, but that it is a result of intracellular digestion brought about by normal endoferments. He therefore concludes, "The phenomenon of trans-

missable lysis can thus be divided into two stages. The early stage depends on the presence of the phage which stimulates bacteria. As a result, the activity of intracellular ferments is increased and the rate of multiplication is exaggerated. Some of the products escape from the cells into the medium, and in turn produce a stimulating effect on newly formed cells. These products represent the active agent (bacteriophage)." How these endoferments function to bring about bursting of the bacterial cells is explained on a physical basis in which the materials within the cells are broken up into numerous smaller particles; thereby greatly increasing the total number of particles within the cell membrane. Then due to the larger number of particles the phenomenon of osmosis begins to exert its effects, resulting in an imbibition of H_2O by the bacteria to the point of rupture and a consequent clearing of the medium in which the bacteria are growing.

This is somewhat similar to Bordet's explanation of the phenomenon; namely, that bacteriophagy represents the pathological exaggeration of a process which is physiological to the bacteria and which is the result of a vitiation that has occurred in the accomplishment of a normal process.

Northrop and Krueger (1930) however, believe that there is a normal growth curve of the bacteria up to a certain point at which lysis begins explosively. They also think that lysis will occur with growth of bacteria when the bacteriophage and bacteria are in a ratio of P/B = 125 where "P" = b'phage and "B" = bacteria. They further state, that probably the internal

phage is responsible for the lysis. At any rate, it was found (Northrop and Krueger 1932) that there must be a concentration of 110 units of phage within the cell or of 12×10^8 units in each ml. of surrounding solution in order for lysis to occur.

D'Herelle (1926) believes that bacteriophagy takes place by the phage corpuscle first becoming fixed to the bacterium to exercise its action, after which the corpuscles enter the bacterial cells where they multiply and are always liberated with the rupture of the cell. He claimed to have observed the process of multiplication within the cell with the aid of an ultra-microscope, the particles being visible as small granules within the bacteria about to be lysed.

Bayne-Jones and Sandholzer (1933) made motion photomicrographic analyses of the mechanism of lysis. Their measurements of the increase in size of the bacteria show an increase in the size of B. coli, under the influence of phage, of two to eight times the original volume; while normal bacteria increased not more than four times the original size. Not all the coli cells were seen to enlarge and none of the B. megatherium increased in size before lysis. For B. megatherium it seemed to be a slow disintegrating process, extending over a period of two to ten minutes, but for B. coli rupture required only about one-half second. It did not appear to be a bursting of the cells due to osmotic pressure. Based on measurements of cellular changes and pictures of cellular debris, the following conclusion was reached:

"Our observations suggest to us that reduction of surface tension at the cell medium interface and at the interfaces of the particles of constituents within the cells may be an important factor in the mechanism of lysis." It would seem to the author that possibly a combination of the latter theory and of Bronfenbrenner's would give a more logical explanation of the phenomenon than anything offered up to the present time.

A STUDY OF THE SELECTIVE ACTION OF DYES, OF PHENOL, AND OF SODIUM HYDROXIDE ON BACTERIOPHAGES

Koch in 1881 (Smith 1922) was the first to investigate a systematic study of the effect of disinfectants upon bacteria. However, the first observation of the bactericidal properties of dyes is usually accredited to Rozsahegyi (1887) who called attention to the specificity or selective action of certain dyes in inhibiting different bacteria. Penzoldt (1890) again called attention to the fact that some dyes, especially methyl violet, are very destructive to bacteria, and he suggests their use in the treatment of wounds. Drigalski and Conradi (1902) were apparently first to suggest the practical utilization of the selective action of dyes in isolating pure cultures of bacteria. They used crystal violet to inhibit the cocci for the isolation of B. typhosus from feces. They also noted that B. coli was more readily inhibited by malachite green and brilliant green than was B. typhosus. Loeffler (1906) used malachite green for the same purpose, but Conradi (1908) showed that brilliant green was better. The

bactericidal action of methyl-violet was again mentioned by Dreyer, Kriegler and Walker (1911) who demonstrated that Staph aureus was more susceptible to the effects of the dye than B. typhosus.

The first systematic investigation of the selective action of dyes was made by Churchman (1912). While making an experimental study of bacteriuria from a specimen of urine thought to contain prodigiosus organisms which had been injected into the circulation, he got a contamination with B. subtilis. In an effort to rid the material of this contamination, he studied the effect of a number of dyes on these organisms and a striking selective action of gentian violet was observed. The dye (5 drops of saturated aqueous solution) was added to a 48 hours old broth culture, mixed and allowed to stand for one hour, after which agar streaks were made from each broth culture. After 24 hours incubation at 37°C. it was found that prodigiosus was unaffected while the stained subtilis failed to grow. On further study it was found that bacteria could be divided into two groups according to their behavior toward gentian violet. The same sort of selective action was noted when the gentian violet was put into agar medium on which the organisms were grown; but more constant results were obtained. Churchman studied 130 species and 318 strains of organisms and found that about 90% of the Gram-positive organisms were also gentian violet positive (inhibited) while about 10% of the Gram-positive organisms were gentian violet negative (no inhibition). However work up to the present time indicates that Churchman's figures are too low for the number of Gram-positive bacteria that are inhibited. As regards precision and constancy there can be

no comparison whatever, according to Churchman, between this reaction and the Gram stain. The dye reaction is further distinguished from the Gram stain by the fact that an organism reacts in the same way toward the dye no matter what its age. It is believed that the bacteriostatic action of gentian violet is like that of other bactericides -- a quantitative one. In sufficient strength it will kill all bacteria. The interval, however, between the dilution necessary to completely prevent growth of the Gram-positive bacteria and that required to have an appreciable effect upon the Gram-negative ones is usually so wide that the selective action may almost be thought of as independent of any quantitative element. The Gram-positive bacteria never grow in a dilution of 1 - 100,000, while the Gram-negative organisms were uninfluenced by that dilution. The Gram-negative organisms grow regularly in a 1 - 10,000 dilution, irregularly in 1 - 5000, and often in 1 - 1000 dilution of gentian violet. Incidentally, the author noted among the Gram-negative bacteria a considerable variation in resistance to the action of gentian violet -- one B. coli, for instance, would not grow in a 1 - 300,000 dilution of the dye. A selective action similar to that of gentian violet is exhibited to a greater or lesser degree by other basic dyes of the triphenylmethane group.

Simon and Wood (1914) and Krumwiede and Pratt (1914) were the next investigators to do any extensive work in this field. Simon and Wood showed that the "inhibitory action upon the growth of certain bacteria which has been shown to be common to all triamine-triphenyl methanes is not an exclusive property of

this group of aniline dyes, but manifested also to a greater or lesser extent by other strongly basic dyes, and is dependent upon the presence of basic auxochromic groups and the absence of corresponding acid groups (ie., a preponderance of the former over the latter) in association with the existence of certain chromophoric radicles." It would seem superfluous to cite any more literature on this particular field for the work, in general, has been well established. The specific field that we are interested in at present is to determine the correlation between the action of dyes upon the bacteria and their respective phages.

Little work has been done to show the effects of dyes on the b'phages, and the experiments that have been performed are of a very general nature. D'Herelle (1926) speaks of the action of various dyes, including gentian violet, as being similar to other disinfectants. He found lysis to take place anywhere bacteria would grow. Reference has been made to the effect that the resistance of the lytic principle to disinfectants is intermediary between the vegetative and spore stages of B. subtilis. Schultz and Kreuger (1928) found that .002% (1 - 50,000 dilution) of methylene blue inactivated a Staphylococcus phage. They used two Staph b'phages that were susceptible to trypsin and tested them with carmine, congo red, methyl red, neutral red, methyl green, brilliant green, brilliant cresyl blue, trypan blue, basic fuchsin, crystal violet, gentian violet, orange G, eosin B, bismark brown and aniline violet, in addition to methylene blue. High concentrations

(they do not say what a high concentration is) were incubated with the phages for 24 hours. The temperature of incubation was very indefinite -- sometimes in the incubator and sometimes at room temperature. No appreciable influence was exerted on any of the bacteriophages tested with the exception of the affect produced by methylene blue on the Staph. phages. These particular phages were completely inactivated within 6 to 12 hours by concentrations of the dye as low as .002%. Eight other races of phages, including anti-coli, anti-dysentery, anti-typhoid, and anti-proteus did not appear to be influenced in the least by the dyes used. Clifton and Lawler (1930) tested the effects of toluidine blue, methylene violet, methylene green, methylene azur, thionin, eosin B and phenol red upon a Staphylococcus phage. The dyes were added in relatively high concentrations (not given definitely) to the phagic filtrates which were then incubated at 37°C. for 24 hours. Only toluidine blue inactivated the lytic agent. Inactivation was not obtained with either methylene blue or toluidine blue when added to a coli phage. This, they conclude, indicates that the inactivation is a specific phenomenon, affecting particularly the Staph. phage. Clifton (1931) also reported the inactivation of a Staphylococcus bacteriophage with methylene blue when the dye-phage mixture was first exposed to the sunlight for a short time before incubation. Inactivation did not take place if the dye and phage were mixed, incubated, and tested in the dark. It is significant for the work that is to follow to note that inactivation was only obtained with phages that lyse Gram-positive

bacteria.

In view of these negative results with some of the stronger dyes, it occurred to the author that the use of dyes in concentrations far in excess of that required for the inhibition of bacteria, correlated with variable periods of incubation and variable temperatures, might produce results that otherwise could not be obtained; it further seemed advisable to test certain phages, including those active against Gram-positive bacteria, which have not previously been studied. Moreover, there appeared to be a need for testing several brands of dyes and other disinfectants (e.g. sodium hydroxide and phenol) in order to determine if the bacteriophages that lyse Gram-positive bacteria are not more susceptible to deleterious substances in general than are those that lyse Gram-negative organisms.

The assumption has been made that the bacteriophage represents a stage in the life cycle of bacteria or is perhaps a metabolic product. Should this assumption be true a somewhat similar action of the dyes upon the bacteria and their respective lysates might be found. Furthermore, if this is found to be true in general, it would suggest a close relationship between the two; and hence, some evidence of the bacterial origin of the hypothetical substance may be obtained. It seems that a study of the effect of dyes upon bacterial enzymes would give some valuable information along these lines in a way of comparison; and such a study has been made. A detailed report of such an investigation will be given in another part of this paper.

EXPERIMENTS

Twelve races of lytic principles, five that are lytic for Gram-positive bacteria and seven that lyse Gram-negative organisms were employed in this work. They include phages of Staphylococcus, Aerobacter cloacae, Bacillus megatherium, B. coli, Achromobacter, C. diphtheria, B. typhosus, and B. shiga. At least two phages that lyse Gram-positive bacteria and two that lyse Gram-negative bacteria were tested simultaneously in order to have comparable conditions. Freshly lysed bacterial cultures yielding phage of high potency were prepared for each experiment. Since many of the phages acted upon related species of organisms, they were taken through a series of cultures of the particular organism in question before being used for the experimental work; and subsequently the active principle carried the name of the organism at the expense of which it was regenerated. The megatherium phage 1 was obtained from Cowles of Yale, the coli 2 phage from D'Herelle's stock, the Staph phage 1 from Lilly & Co., and the phages of Staphylococcus 2, B. coli 3, B. typhosus, B. shiga, Bacillus megatherium 2, and C. diphtheria were obtained through the courtesy of J. Bronfenbrenner. The other three, coli 1, cloacae, and Achromobacter, were isolated by the author as given above. B. coli phage 1 and coli 2 phage were developed at the expense of individual strains of bacteria. When starting each series of experiments fresh isolations of the phages were made by the single plaque method and the bacteria were streaked and single colonies picked.

For media, plain beef broth, adjusted to pH 7.6 and one per cent plain agar were employed. Colorimetric methods, using phenol red, were used in most of the work to determine the pH. The potentiometer was used to standardize the color standards and to check the accuracy of the color determinations of pH. Not more than .2 pH variations was detected at any time.

The dyes, crystal violet and brilliant green (National Aniline Co.), and two brands of gentian violet (Coleman and Bell, and Grubler's) were employed. A stock solution was made by dissolving 2.5 gms. of the dye in 95% alcohol and making up to a dilution of 1-40 by addition of physiological saline. This original solution was kept to make all subsequent dilutions for work with the phage and enzyme experiments. The latter dilutions were prepared with the plain broth as the diluent. Crystaline (C.P.) phenol and stick (C.P.) sodium hydroxide were purchased to prepare those respective solutions. The preliminary work with the disinfectants was done with varying dilutions before the optimum strengths were found. The dye concentrations that were found satisfactory for the phage work were 1-2500, 1-5000, and 1-10,000 for gentian violet or crystal violet; and 1-1200, 1-2500, and 1-5000 for brilliant green.

Divided plates in which the dye was in one-half of the plate and plain agar on the other half, were used in the preliminary work along with tubes of the broth containing the desired dilutions of dye. Plain broth controls were also made. One drop of the suspension (phage plus bacteria) was added to the various tubes and mixed

and then one drop of this mixture was streaked on the divided plates. Dilutions of dye ranging from 1-2000 to 1-300,000 were tried. After the proper incubation periods, it was found that lytic action took place in every instance where the bacteria grew.

It was evident from such results that it would be necessary to employ much higher concentrations of dye and incubate the phage in the dye mixtures much longer, if inhibitory action were to be manifested. This procedure necessitated making a very great dilution of the dye-phage mixtures before bacterial growth could take place in testing for the presence of any remaining active agent. Consequently high concentrations of the dyes were mixed with equal amounts of the phage suspensions to give the desired inhibitive dilutions (1-2500, 1-5000, and 1-10,000 of violet dyes. 1-1200, 1-2500, and 1-5000 of brilliant green) which were then incubated at 37°C. for varying periods of time indicated below. Dye-broth and phage controls were also included. At the end of each period of incubation one drop of the mixture in each case was diluted in plain broth to make a dilution of 1-25,000,000 or higher of the gentian violet. Since the dye dilution was mixed with an equal amount of the phage suspension, the procedure gave final dilutions of about 1-40,000 of the lytic agent. Such final dilutions of dye did not have any inhibitory action on the bacteria. In the controls where no dye was present, it was found that this amount of dilution (1-40,000) permitted great phagic action. One drop of the organism suspension made from an agar slant to which 1 c.c. of broth had been added, was now added

to each of the final dilution tubes. Then one drop of this latter mixture was added to one side of an agar plate and spread with a sterile bent glass rod over one-half of the plate. The other half of the plate served as a control for the organism with no lytic principle present. Both the final dilution tubes and plates were incubated at 37°C. and readings of the broth tubes taken at three and six hour, and over-night periods. Plates were read after over-night incubation only. The reason for reading results in the broth cultures as indicated is that one may be able to observe lysis in three to six hours that would not show the following day because of secondary growths of bacteria. Since plates give a somewhat permanent record of what has occurred, they were made the criterion as to whether lysis had taken place, although the tubes and plates checked with surprising constancy. As is well known, a plate may not show any signs of lytic activity while the tube from which it was made may appear to be lysed. Occasionally just the reverse has been found to be true. The controls include a dye-broth control made by using the highest concentration of dye in order to be sure the absence of bacterial growth is not due to inhibitive action of dye rather than bacteriophagic action. A phage control is needed as a standard for comparison as well as to check the lytic activity in each instance, while the organism control is needed mainly for comparison but also to show that the medium is desirable for the particular organism used. That means that the dye-broth and organism controls should show normal bacterial growth (cloudy) and the phage

control should exhibit a clear broth tube or much lytic activity on the plates (clear areas). Unless all these checks were definitely discernable no record was made of any of the results.

CRYSTAL VIOLET

Results obtained by following the procedure just described are reported below. It may be seen from Table I that the megatherium and Staphylococcus phages were made completely inactive by dilutions of the dye which did not affect the Achromobacter, cloacae and two coli phages. A summary of the reactions is given in the following table.

TABLE I.

Results of tests (in broth tubes and on agar plates) for presence of active phage after being acted upon by crystal violet (N.A.C.) for periods of time shown. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of dye action	Inhibiting dilutions of crystal violet 1-2500	1-5000	1-10,000	Controls dye-broth	plain broth	Phage
Megath-	1.Broth	24 hrs.	cloudy	cloudy	cloudy	cloudy	cloudy	clear
erium-1	tubes	48 hrs.	"	"	"	"	"	"
	2.Agar	24 "	N.growth	N.growth	N.growth	N.growth	N.gr.	Lysed
	plates	48 "	"	"	"	"	"	"
Achromo	1.Broth	24 "	clear	clear	clear	cloudy	cloudy	clear
bacter	tubes	48 "	S.cloudy	"	"	"	"	"
	2.Agar plates	24 "	Lysed	Lysed	Lysed	N.Growth	N.gr.	Lysed
		48 "	N.Growth	"	"	"	"	"
Cloa-	1.Broth tubes	24 "	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
cae		48 "	"	"	"	"	"	"
	2.Agar plates	24 "	N.Growth	Lysed	Lysed	N.Growth	N.gr.	Lysed
		48 "	"	"	"	"	"	"
Coli-2	1.Broth tubes	24 "	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	S.Cloudy	"	"	"	"	"
	2.Agar plates	24 "	N.Growth	Lysed	Lysed	N.Growth	N.gr.	Lysed
		48 "	"	"	"	"	"	"
Staphy-lococcus-1	1.Broth tubes	24 "	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
	2.Agar plates	24 "	N.Growth	N.Growth	N.Growth	N.Growth	N.Gr.	Lysed
		48 "	"	"	"	"	"	"

			1-2500	1-5000	1-10,000	d.broth	broth	Phage
Coli-1	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Lysed
		48 "	"	"	"	"	"	"
	2.Agar plates	24 "	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"

S = slight, N.Growth = normal bacterial growth.

By referring to the above table, one can see in addition to the selective action mentioned above, that inhibition is more pronounced after 48 hours than at the end of the 24 hour period. Also some of the phages that lyse Gram-negative bacteria were inhibited in the higher concentrations while the phages of megatherium and Staphylococcus were completely inhibited in all dilutions of the dye in 48 hours time. The Achromobacter and coli 2 phages were made inactive in the highest concentration (1-2500) but the coli-1 phage did not seem to be affected. Data gathered from the above work suggested that a longer incubation period might be advisable; and, therefore, the suggestion was carried out in the experiments that follow.

Table 1-b gives the remainder of the group of b'phages tested as above except the inhibition time was extended to one week.

TABLE I-b

Results with phages where crystal violet was used but incubation for inhibition was extended to one week. Other conditions same as Table I.

PHAGES TESTED	Test	Time of dye action	Inhibiting dilutions of crystal violet 1-2500	1-5000	1-10,000	Controls Dye-broth	Plain broth	Phage
Coli-3	1. Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	Cloudy	"	"	"	"
	2. Agar plates	24 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Gr.	Lysed
		48 "	"	S. "	"	"	"	"
		1 wk.	"	N.Growth	"	"	"	"
Shiga	1. Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 hrs.	"	Cloudy	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2. Agar plates	24 hrs.	N.Growth	S.Lysis	Lysed	N.Growth	N.Gr.	Clear
		48 "	"	N.Growth	"	"	"	"
		1 wk.	"	"	"	"	"	"
Megatherium-2	1. Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	Cloudy	"	"	"
	2. Agar plates	24 hrs.	N.Growth	N.Growth	N.Growth	N.Gr.	N.Gr.	Lysed
		48 hrs.	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"

			1-2500	1-5000	1-10,000	d.broth	broth	phage
Staphylococcus-2	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	N.Growth	N.Gr.	N.Gr.	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Typhosus	1.Broth tubes	24 hrs.	S.Cloudy	S.Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"

S = slight, N.Growth = normal bacterial growth

The data in Table I-b show a similar division of reactions of the dye upon phages of Gram-positive and Gram-negative organisms, as was shown for the first group. (Table I) The Gram-positive, megatherium-2 was made completely inactive in all dilutions of gentian violet in one week's time although it took one week's incubation to inactivate it in the 1-10,000 dilution. Staphylococcus-2 was inactivated in all dilutions in 24 hours. Shiga phage became inactive in the two highest concentrations of dye in 48 hours, but remained active in the 1-10,000 dilution. Coli-3 phage was inactivated in 1-2500 and 1-5000 dilutions within one week but the 1-10,000 dilution remained active. The phage of B. typhosus was inhibited completely

in 48 hours in the first two dilutions while the highest dilution (1-10,000) was unaffected within one week. It may be noticed that if inhibition were accomplished, it took place within 48 hours, with the exception of the megatherium phage.

GENTIAN VIOLET (C.B.)

The experiment shown in Table II was conducted in the same manner as nearly as possible, as the one just given except gentian violet from Coleman and Bell Co. was the dye employed. May I repeat here that the dye-phage mixtures were made, using dye dilutions prepared from the original stock solution by employing the plain broth as diluent, and incubating for 24 hours, 48 hours, and 1 week respectively before taking a test to see if inactivation were accomplished. At the same time a plain broth-dye control (1-2500 dilution of dye) and phage control were made and carried along with the dye-phage mixtures in test tubes. The phage control was prepared by mixing an equal amount of plain broth and the desired phage filtrate. This means that the phage was in a 1-2 dilution in all tubes that contained it. In order to test for the presence of active phage at times stated, one drop of each mixture was diluted approximately 1 in 20,000 giving final dilutions of dye of 1-25,000,000 or higher and final dilutions of bacteriophage of 1-40,000. One drop of bacterial suspension of the desired organism was then added to each of the latter dilution tubes and to one plain broth tube which served as a bacterial control. Now one drop from each tube containing the bacterial suspension was added to a plain agar plate

and spread with a sterile, bent glass rod after which both tubes and plates were incubated over-night and results recorded as found in the tables. Sometimes, as mentioned before, results were recorded at three and six hour periods. In case a tube cleared before the next day, the record stood because secondary growths may occur and mask the actual occurences in the broth tubes. Of course this does not hold for the plates.

TABLE II

Results of tests for presence of active phage after being acted upon by gentian violet (G.B.) for periods of time shown. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of dye action	Inhibiting dilutions of gentian violet 1-2500	1-5000	1-10,000	Controls dye-broth	plain broth	phage
Megatherium-1	1. Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	Cloudy	"	"	"
	2. Agar plates	24 hrs.	N.Growth	N.Gr.	S.Lysis	N.Growth	N.Gr.	Lysed
		48 "	"	"	N.Growth	"	"	"
		1 wk.	"	"	"	"	"	"
Achromobacter	1. Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	– "
		1 wk.	"	Cloudy?	"	"	"	"
	2. Agar plates	24 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Gr.	Lysed
		48 "	"	N.Growth	"	"	"	"
		1 wk.	"	S.Lysis	"	"	"	"
Cleaceae	1. Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	S. Cloudy	"	"	"	"
		1 wk.	"	Clear	"	"	"	"
	2. Agar plates	24 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"

TABLE II Con't.

			1-2500	1-5000	1-10,000	d.broth	broth	phage
Coli-2	1.Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 hrs.	"	Cloudy	"	"	"	"
		1 wk.	"	S. "	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	S.Lysis	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	N.Growth	S.Lysis	"	"	"
		1 wk.	"	"	"	"	"	"
Staphylococcus-1	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	N.Gr.	N.Gr.	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Coli-1	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		48 hrs.	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Coli-3	1.Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	Cloudy	"	"	"	"
	2.Agar	24 hrs.	N.Growth	S.Lysis	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	N.Growth	"	"	"	"

TABLE II Con't.

			1-2500	1-5000	1-10,000	d.broth	broth	phage
Shiga	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Megath erium-2	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	S.Cloudy	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Staphy-lococcus-2	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
		48 hrs.	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	N.Growth	N.Gr.	N.Gr.	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Typh-osus	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar	24 hrs.	N.Growth	Lysis	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	N.Growth	"	"	"	"
		1 wk.	"	"	"	"	"	"

TABLE II Con't.

			1-2500	d. broth	broth	phage
Diphtheria	1. Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Clear
		48 hrs.	"			
		1 wk.	"			
	2. Agar plates	24 hrs.				
		48 hrs.				
		1 wk.				

Legend: Same as Table I.

An inspection of Table II reveals the same sort of selective action as was exhibited by the crystal violet and the reactions in each instance are very similar, if not identical. There appears to be an irregularity in the 1-5,000 dilution tube of the cloacae phage at the 48 hour period. This may be explained, as has been mentioned before, on the basis that the tube had cleared and secondary growth developed some before the results were recorded. Occasionally a broth tube containing active phage does not entirely clear. No doubt the reason for this phenomenon can be more fully explained by additional experimentation. Frequently results were recorded similar to these when there was a positive evidence of lysis but not so much as was evident in the lytic control. None of the phages that lyse Gram-negative organisms were inhibited by the highest dilution (1-10,000) of gentian violet while all those that are lytic for Gram-positive bacteria were inactivated by that dilution in one

week's time or less. The phages of Bacillus megatherium seem to be more resistant than are the Staphylococcus and diphtheria agents.

The data in Table III show no results for the diphtheria phage after a period of 24 hours. There is no conclusive evidence to show that nothing will happen after this period, if the experimental factors are made to include all possible variation. However, the conclusions here are drawn from the results of the investigations as reported.

GENTIAN VIOLET (Grubler's)

Table III shows the same type of experiments, using the same lytic agents as before, but Grubler's gentian violet is the inhibiting agent. Other conditions were duplicated in so far as possible.

TABLE III.

Results of tests for presence of active phage after being acted upon by Grubler's gentian violet for period of time shown. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of dye action	Inhibiting dilutions G. gentian violet 1-2500	1-5,000	1-10,000	Controls dye-broth	plain broth	phage
Megatherium-1	1. Broth tubes	24 hrs.	Cloudy	Cloudy	S.Cloudy	Cloudy	Cloudy	Clear
		48 "	"	"	Cloudy	"	"	"
		1 wk.	"	"	"	"	"	"
	2. Agar plates	24 hrs.	N.Growth	N.Growth	Lysis	N.Growth	N.Growth	Lysed
		48 "	"	"	N.Growth	"	"	"
		1 wk.	"	"	"	"	"	"
Achromobacter	1. Broth tubes	24 hrs.	Clear	S.Cloudy	Clear	Cloudy	Cloudy	Clear
		48 hrs.	"	Clear	"	"	"	"
		1 wk.	Cloudy	"	"	"	"	"
	2. Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	Lysis	"	"	"	"	"
Cloacae	1. Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2. Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"

TABLE III Con't.

			1-2500	1-5000	1-10,000	d.broth	broth	phage
Coli-2	1.Broth tubes	24 hrs.	S.Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	Cloudy	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	Lysis	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	Lysis?	"	"	"	"
		1 wk.	"	"	"	"	"	"
Staphy-lococcus-1	1.Broth tubes	24 hrs.	Cloudy	S.Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	Cloudy	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	S.Lysis	N.Growth	N.Grweth	Lysed
		48 "	"	"	" ?	"	"	"
		1 wk.	"	"	"	"	"	"
Coli-1	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Coli-3	1.Broth tubes	24 hrs.	S.Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	Cloudy	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysis	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 hrs.	"	"	"	"	"	"
		1 wk.	N.Growth	"	"	"	"	"

TABLE III Cont.

Organism	Medium	Time	1-2500	1-5000	1-10,000	d.broth	broth	phage
Shiga	1.Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		48 "	"	Cloudy	"	"	"	"
		1 wk.	"	S.Cloudy	"	"	"	"
	2.Agar plates	24 hrs.	Lysis	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	"	Lysis	"	"	"	"
		1 wk.	N.Growth	"	"	"	"	"
Megatherium-2	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	Lysis	N.Growth	N.Growth	Lysed
		48 "	"	"	Lysed	"	"	"
		1 wk.	"	"	N.Growth	"	"	"
Staphylococcus-2	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth	N.Growth	Lysis	N.Growth	N.Growth	Lysed
		48 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Typhosus	1.Broth tubes	24 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		48 "	"	Clear	"	"	"	"
		1 wk.	"	Cloudy	S.Cloudy	"	"	"
	2.Agar plates	24 hrs.	Lysis	Lysed	Lysed	N.Growth	N.Growth	Lysed
		48 "	N.Growth	"	"	"	"	"
		1 wk.	"	Lysis	"	"	"	"

TABLE III cons.

		1-2500	1-5000	1-10,000	d.broth	broth	phage
Diphth-1. Broth tubes	24 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
eria	48 "	"	"	"	"	"	"
	1 wk.	"	"	"	"	"	"

Legend: same as Table I.

Data in Table III indicate a division between the phages that lyse Gram-positive bacteria and those that are lytic for Gram-negative organisms, the former being inhibited by concentrations of dye that do not seem to influence the activity of the latter. However there is a very noticeable difference in the reactions obtained with Grubler's gentian violet and the two former dyes used in that Grubler's dye appears to be a much weaker disinfectant. The phages of cloacae and coli-1 were not inhibited by any of the concentrations of dye. Coli-1 stands in a class by itself since it apparently has not been inhibited by any of the violet dyes. Other unusual properties of coli-1 phage are its ability to constantly form very large plaques ([illegible] mm. dia.) and bring about disassociation of the organism. One of the Staphylococcus phages was not completely inactivated in the highest dilution in this experiment while it always has been before. Megatherium-1 phage was inhibited to a greater extent than before. Frequently there appears to be a limiting dilution beyond which no inhibiting action takes place as is shown in the highest dilution (1-10,000) of crystal violet which caused no diminished activity of the phage of B. cloacae within one week.

A convenient reference to the essential findings with the three violet dyes is found in Table IV, which summarizes the data.

TABLE IV

Results obtained with crystal violet and two brands of gentian violet as phage-inhibiting agents. Incubation temperature 37°C.

Phages Tested	Dye	Inhibiting dilutions of dye 1-2500	1-5000	1-10,000
Staphylococcus-1	Crystal violet	+ in 24 hr.	+ in 24 hr.	+ in 24 hr.
Megatherium-1	"	+ in 24 hr.	+ in 24 hr.	+ in 24-48 hr.
Cloacae	"	+ in 24 hr.	- in 24-48 hr	- in 1 wk.
Coli-1	"	- at 1 wk.	- at 1 wk.	- at 1 wk.
Coli-2	"	+ in 24 hr.	-,+ in 48 hr.	- in 48 hr.
Achromobacter	"	+ in 24-48hr.	- in 48 hr.	- in 48 hr.
Coli-3	"	+ in 24 hr.	- at 1 wk.	- at 1 wk.
Shiga	"	+ in 24 hr.	+ in 48 hr.	- at 1 wk.
Megatherium-2	"	+ in 24 hr.	+ in 24 hr.	+ in 48 hr.
Staphylococcus-2	"	+ in 24 hr.	+ in 24 hr.	+ in 24 hr.
Typhosus	"	+ in 24 hr.	+ in 48 hr.	- at 1 wk.
Staphylococcus-1	Coleman and Bell Gentian Violet	+ in 24 hr.	+ in 24 hr	+ in 24 hr.
Megatherium-1	"	+ in 24 hr.	+ in 24 hr.	+ in 48 hr.
Cloacae	"	+ in 24 hr.	- at 1 wk.	- at 1 wk.
Coli-1	"	+ at 1 wk.	- at 1 wk.	- at 1 wk.
Coli-2	"	+ in 24 hr.	+ in 48 hr.	- at 1 wk.
Achromobacter	"	+ in 24 hr.	+ in 48 hr.	- at 1 wk.
Coli-3	"	+ in 24 hr.	+ in 1 wk.	- at 1 wk.
Shiga	"	+ in 24 hr.	+ in 1 wk.	- at 1 wk.

TABLE IV Conc.

		1-2500	1-5000	1-10,000
Megatherium-2	Coleman and Bell	+ in 24 hr.	+ in 24 hr.	- at 1 wk.
Staphylococcus-2	Gentian Violet	+ in 24 hr.	+ in 24 hr.	+ in 24 hr.
Typhosus	"	+ in 24 hr.	+ in 48 hr.	+ at 1 wk.
Diphtheria	"	+ in 24 hr.	+ in 24 hr.	+ in 24 hr.
Staphylococcus-1	Grubler's gentian violet	+ in 24 hr.	+ in 24 hr.	- in 1 wk.
Megatherium-1		+ in 24 hr.	+ in 24 hr.	+ in 1 wk.
Cloacae	"	- at 1 wk.	- at 1 wk.	- at 1 wk.
Coli-1	"	- at 1 wk.	+ at 1 wk.	- at 1 wk.
Coli-2	"	+ in 24 hr.	- at 1 wk.	- at 1 wk.
Achromobacter	"	- at 1 wk.	- at 1 wk.	- at 1 wk.
Coli-3	"	+? in 1 wk.	- at 1 wk.	- at 1 wk.
Shiga	"	+ in 1 wk.	- at 1 wk.	- at 1 wk.
Megatherium-2	"	+ in 24 hr.	+ in 24 hr.	+ in 1 wk.
Staphylococcus-2	"	+ in 24 hr.	+ in 24 hr.	- at 1 wk.
Typhosus	"	+ in 48 hr.	- at 1 wk.	- at 1 wk.
Diphtheria	"	+ in 24 hr.	+ in 24 hr.	+ in 24 hr.

+ complete inhibition
- no or incomplete inhibition
-,+ sometimes negative and sometimes positive in time given

BRILLIANT GREEN

It was found in the preliminary work that brilliant green had to be used in more concentrated solutions than the other dyes. Therefore the concentrations were doubled; but only two of the phages were inhibited with the strong solutions (1-1200, 1-2500, and 1-5000) used. However, stronger solutions could not be used conveniently for with a dilution of 1-40,000 the brilliant green seemed to slightly inhibit the cloacae organisms as may be seen in controls of Table V. Nevertheless the effect was not enough to invalidate the results in the least for only the broth tubes were so affected. The increased surface area of the agar plate was the main factor in overcoming this hindrance because in reality a greater dilution is made by spreading the suspension over the agar.

Table V summarizes the data from experiments performed by the same methods and under identical conditions, as nearly as possible, except brilliant green was substituted for the violet dyes.

TABLE V

Results of tests for presence of active phage after being acted upon by brilliant green for periods of time shown. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of dye action	Inhibitive dilutions of brilliant green 1-1200	1-2500	1-5000	Controls dye-broth	plain broth	phage
Megath	1.Broth tubes	24 hrs.	Clear?	Clear	Clear	Cloudy	Cloudy	Clear
erium-1		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Achromo	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
bacter		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Cloa-	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy?	Cloudy?	Clear
cae		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"

TABLE V con't.

			1-1200	1-2500	1-5000	d.broth	p.broth	phage
Coli-2	1.Broth tubes	24 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed	Lysed	N.Growth	N.Growth	Lysed
		96 "	"	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Staphy-lococcus-1	1.Broth tubes	24 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		96 "	Clear	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
	2.Agar plates	24 hrs.	N.Growth?	Lysed	Lysed	N.Growth	N.Growth	Lysed
		96 "	Lysed	"	"	"	"	"
		1 wk.	"	"	"	"	"	"
Coli-1	1.Broth	24 hrs.	Clear	Clear	Clear	S.Cloudy	Cloudy	Clear
		96 "	"	"	"	"	"	"
		1 wk.	"	"	Clear	"	"	"
	2.Agar plates	24 hrs.	Lysed	S.Lysis	S.Lysis	N.Growth	N.Growth	Lysed
		96 "	"	Lysed	"	"	"	"
		1 wk.	"	"	Lysed	"	"	"
Coli-3	1.Broth tubes	24 hrs.	Clear	Clear		Cloudy	Cloudy	Clear
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"

TABLE V Con't.

			1-1200	1-2500	1-5000	d.broth	broth	phage
Shiga	1.Broth tubes	24 hrs.	Clear	Clear		Cloudy	Cloudy	Clear
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
Megath-erium	1.Broth tubes	24 hrs.	Clear	Clear		Cloudy	Cloudy	Clear
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
Staphy-lococcus-2	1.Broth tubes	24 hrs.	S.Cloudy	Clear		Cloudy	Cloudy	Clear
		96 "	Clear	"		"	"	"
		1 wk.	"	"		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
Typh-osus	1.Broth tubes	24 hrs.	Clear	Clear		Cloudy	Cloudy	Clear
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
		1 wk.	"	"		"	"	"

TABLE V Conc.

			1-1200	1-2500	1-5000	d.broth	broth	phage
Diph-	1.Broth tubes	24 hrs.	Clear	Clear		Cloudy	Cloudy	Clear
theria		96 "	"	"		"	"	"
		1 wk.	Cloudy	Cloudy		"	"	"
	2.Agar plates	24 hrs.	Lysed	Lysed		N.Growth	N.Growth	Lysed
		96 "	"	"		"	"	"
		1 wk.						

Legend: same as Table I.

Brilliant green under conditions of the experiments did not inhibit but two of the b'phages, Staphylococcus and coli-1, within one week and the former very irregularly, Coli-1 though, seemed to be affected each time it was checked but the inhibition was not complete. Since brilliant green gave mostly negative results in the experiments just cited, it was decided to try the effect of a water bath temperature of 50°C. for one hour immediately following the check made after one week's incubation at 37°C. The point of thermal inactivation of bacteriophage, as determined by us and as observed by many other workers, is much above 50°C. When this procedure was carried out, one of the phages of B coli (1) became completely inactive in the two higher concentrations, while the condition of the other phages was apparently unchanged. This data is not shown in table form.

It is interesting to note that the two phages of B. coli developed at the expense of the same organism reacted in opposite manner to the two dyes. Whereas crystal violet gave negative results with B. coli phage 1 and brilliant green produced definite inhibition

crystal violet inhibited B. coli phage-2 and brilliant green gave negative results. It might be further stated that the latter lytic principle is capable of lysing a strain of the typhoid bacillus while the former is not. It was stated above that coli-1 phage produced much larger plaques than any of the other phages of B. coli; and it also constantly brought about disassociation of B. coli while the others did not. In fact is was very seldom that anything showed on the plates after lysis had occurred, except a few isolated rough colonies of B. coli, which, from macroscopic examination, appeared to be contaminants.

DISCUSSION

The nature of the bacteriophage and the mechanism involved in the process of lysis of bacteria by the hypothetical substance has been discussed in detail above. Therefore it is sufficient at this time to mention only facts which are pertinent to problems at hand. The author has suggested a combination of the Bronfenbrenner (1929) theory of the bacteriophage and the mechanism involved and that of Bayne-Jones, both of whom believe the lytic agent to be a product of bacterial metabolism. Bronfenbrenner explains the phenomenon as being initiated by the agent catalysing the endo-ferments of susceptible bacteria thereby bringing about a change in osmotic forces upon the cell which in turn causes imbibition of water and subsequent rupture of the cell, liberating more of the stimulating substance, bacteriophage. However, it has been

shown that much of the active agent is liberated before rupture of the cell. Bayne-Jones does not believe the bursting of the cell is entirely due to osmotic pressure but that a reduction in the surface tension at the cell medium interface and at the interface of particles of constituents within the cell may be an important factor in the mechanism of lysis.

If the bacteriophage is a bacterial product; it is possible that some light might be shed upon the question by making a study of the effects of dyes upon the bacteria and their respective phages. It would at least suggest a chemical relationship between the bacteria and their respective lytic agents if some correlation of the effects of the dyes upon them could be shown.

With the above ideas in mind twelve lytic principles, six that lyse Gram-positive organisms and six that lyse Gram-negative bacteria were obtained for the investigation.

Preliminary work indicated that the bacteriophage was much more resistant to deleterious substances than the vegetative cells of the bacteria; so it was decided to mix high concentrations of the dye with the lysates and incubate for variable periods of time at various temperatures before adding the bacteria for the detection of active bacteriophage. In order to make the latter test it was necessary to highly dilute the dye-phage mixtures in order to permit uninhibited bacterial growth. Final dilutions of the violet dyes of one to twenty-five million or higher seemed to work perfectly and still the dilution (1-40,000) of phage which was effected by the procedure was not sufficient to prevent active lysis in the

control.

When the experiments were so conducted, using three brands of dyes namely, one crystal violet and two makes of gential violet, a selective phagiostatic action of the dyes on the bacteriophages was demonstrated, and the results suggest that there is a correlation between the selective action of the dyes on bacteria and their respective phages. By using proper concentrations of the dyes the phages that lysed Gram-positive bacteria were completely inhibited while those that lysed Gram-negative bacteria were apparently not diminished in their activity. Only one of the lytic agents, coli-1 was resistant to the action of the strongest solution (1-2500) of the violet dyes. This agent was much different in several respects from the other phages of B. coli. It produced much larger plaques (7 mm.) and constantly brought about disassociation of the bacteria when in their presense. The results in all experiments tended to show complete lysis or none at all, although that was not always the case. One would expect such results in the broth tubes because one uninactivated corpuscle of phage should be sufficient to bring about complete lysis of the bacteria. The dilution method of isolation depends upon this principle. That fact, it would seem, should not apply to the plates; but it is surprising how well the tubes and plates checked. Rather disconnected results were obtained with the diphtheria phage because it was difficult to keep it going in the media used. A few tests were made with it in media of pH 7.2 and apparently more successfully.

Almost identical results were gotten with crystal violet and Coleman and Bell's gentian violet, but Grubler's gentian violet did not exhibit as much inhibitive action although the same sort of selective action was manifested. The only explanation that can be offered for the difference is that perhaps a larger percentage of methyl violet is found in Grubler's dye.

Brilliant green was prepared in twice the concentrations of that in the violet dye experiments and then only one Staphylococcus phage and coli-1 phage were influenced in the highest concentration (1-1200) used. The Staphylococcus phage was very irregularly affected while the coli agent was constantly inhibited to some extent, but not completely. In view of the negative results with this dye, it was decided to try the effect of a water bath temperature of 50°C. for one hour immediately following the check made after one week's incubation at 37°C. The point of thermal inactivation of the bacteriophage, as determined by us and as observed by many other workers, is much above 50°C. When this procedure was carried out, the coli-1 phage became completely inactive in the two higher concentrations, while the condition of the other phages was apparently unchanged. It may be noted that, in addition to differences mentioned above, the two phages of B. coli developed at the expense of the same organism reacted in opposite manner to the two dyes. Whereas crystal violet gave negative results with B. coli phage-1 and brilliant green produced definite inhibition, crystal violet inhibited B. coli phage-2 and brilliant green gave negative results.

CONCLUSIONS

Evidence has been cited which suggests a chemical relationship exists between bacteria and their respective phages.

A selective phagiostatic action of crystal violet, gentian violet, and brilliant green on the twelve bacteriophages tested has been demonstrated, and the results suggest that there is a correlation between the selective action of the violet dyes on bacteria and their respective phages.

High concentrations of dyes were required to effect inhibition of the phages.

A high dilution of the primary dye solutions (inhibiting dilutions) to the point where there would be no inhibition of bacterial growth was a requisite in demonstrating the activity or inactivity of the lytic principles after each period of incubation.

Under the conditions of the experiment, the phages that lysed Gram-positive bacteria were completely inhibited by concentrations of the violet dyes which did not appear to diminish the activity of the phages that lysed Gram-negative bacteria.

The reactions of crystal violet and Coleman and Bell's gentian violet gave almost identical results; but Grubler's gentian violet, while showing the same general selective action did not give the pronounced inhibition that the other two dyes exhibited.

The inhibitive property of brilliant green was extremely variable when compared with that of crystal violet, but nevertheless

distinct with one phage of the colon bacillus in particular. The latter phage, coli-1, was the only one of the twelve which was not influenced by the violet dyes.

Twelve bacteriophages, six that lysed Gram-positive bacteria and six that lysed Gram-negative organisms, were included in this investigation.

PHENOL AND SODIUM HYDROXIDE

Another series of experiments was performed with phenol and sodium hydroxide as phagiostatic agents in order to discover whether or not certain phages,are, in general, more susceptible to deleterious substances than are others. It was found that the phenol and sodium hydroxide must be used in much higher concentrations than the violet dyes to produce inhibitive effects. Another difference noted was that less dilution of the inhibiting agent, permitted uninhibited bacterial growth in comparison to that used with the dyes. For inhibitive action phenol dilutions of 1-20,1-80, and 1-160, and sodium hydroxide dilutions of 1-500, 1-1000, and 1-1500 were employed. After the disinfectant-phage mixtures had been made and subjected to the proper conditions of incubation,they were diluted so as to give dilutions of phenol of 1-2800 and dilution of sodium hydroxide of 1-70,000 or higher. Otherwise these experiments were conducted as with the dyes.

The results given in Tables V and VI also indicate that in general, the b'phages may be put into two groups. Although

this was especially true of the action of phenol, several variations were observed with sodium hydroxide. In both instances more uniform results followed. However, there was not so much of a margin for the dividing line between the two groups as when the dyes were used.

PHENOL

A summary of the results obtained with phenol as inactivating agent is found in the following table.

TABLE VI

Results of tests for presence of lytic material after being acted upon by phenol for time given. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of phenol action	Inactivating dilutions of phenol 1-20	1-80	1-160	Controls phenol broth	plain broth	phage
Clea-	1.Broth tubes	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
cae		1 wk.	"	"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	"	"	"	"	"	"
Staphy-	1.Broth tubes	48 hrs.	Cloudy	Clear		Cloudy	Cloudy	Clear
lococcus-1		1 wk.	"	Cloudy	Clear	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	"	N.Growth	"	"	"	"
Coli-2	1.Broth tubes	48 hrs.	Cloudy	Clear		Cloudy	Cloudy	Clear
		1 wk.	"	Cloudy	Clear	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed		N.Growth	N.Growth	Lysed
		1 wk.	"	Lysis	Lysed	"	"	"
Coli-1	1.Broth tubes	48 hrs.	Cloudy	Clear		Cloudy	Cloudy	Clear
		1 wk.	"	"	Clear	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed		N.Growth	N.Growth	Lysed
		1 wk.	"	"	Lysed	"	"	"
Achromo	1.Broth tubes	48 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
bacter		1 wk.		"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth	N.Growth	Lysed	N.Growth	N.Gr.	Lysed
		1 wk.		"	"	"	"	"

TABLE VI cont..

			1-20	1-80	1-160	phenol broth	broth	phage
Staphylococcus-2	1. Broth tubes	48 hrs.	Cloudy	S. Cloudy	S. Cloudy	Cloudy	Cloudy	Clear
		1 wk.	"	"	Cloudy	"	"	"
	2. Agar plates	48 hrs.	N. Growth	N. Growth	Lysed	N. Growth	N. Gr.	Lysed
		1 wk.	"	"	N. Growth	"	"	"
Shiga	1. Broth tubes	48 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		1 wk.		"	"	"	"	"
	2. Agar plates	48 hrs.	N. Growth	N. Growth	Lysed	N. Growth	N. Growth	Lysed
		1 wk.		"	"	"	"	"
Megatherium-1	1. Broth tubes	48 hrs.	Cloudy	Cloudy	Clear	Cloudy	Cloudy	Clear
		1 wk.				"	"	"
	2. Agar plates	48 hrs.	N. Growth	N. Growth	Lysed	N. Growth	N. Growth	Lysed
		1 wk.				"	"	"

Legend: same as Table I.

Table VI shows that all the phages lysing Gram-positive bacteria were completely inhibited by phenol in a dilution of one to eighty within one week's incubation, while those lysing Gram-negative bacteria were found to be active under the same conditions. A one to twenty dilution of phenol produced complete inhibition in all the lytic agents tried within twenty-four hours, while only the Staphylococcus-2 phage was made inactive by the 1-160 dilution of the disinfectant. It is believed that, since coli-1 phage had been more resistant to deleterious substances than any of the lytic agents, a dilution of phenol could be found that would permit lysis by this agent when under the same conditions all the others would be inactive.

SODIUM HYDROXIDE

Results with sodium hydroxide as the phagiostatic agent are shown in Table VII.

TABLE VII

Results of tests for presence of lytic material after it was acted upon by sodium hydroxide for periods of time given. Incubation temperature 37°C.

PHAGES TESTED	Test	Time of action NaOH	Inactivating dilutions NaOH 1-500	1-1000	1-1500	NaOH broth	Controls Plain broth	Phage
Cloa-cae	1.Broth tubes	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		1 wk.	S.Cloudy	"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	"	"	"	"	"	"
Staphy-lococcus-1	1.Broth tubes	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		1 wk.	"	"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	"	"	"	"	"	"
Coli-2	1.Broth tubes	48 hrs.	Cloudy	Clear		Cloudy	Cloudy	Clear
		1 wk.	"	"		"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed		N.Growth	N.Gr.	Lysed
		1 wk.	"	"		"	"	"
Coli-1	1.Broth tubes	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		1 wk.	" ?	"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth	Lysed	Lysed	N.Growth	N.Gr.	Lysed
		1 wk.	"	"	"	"	"	"
Achromo-bacter	1.Broth tubes	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
		1 wk.	"	"	"	"	"	"
	2.Agar plates	48 hrs.	N.Growth?	Lysed	Lysed	N.Growth	N.Gr.	Lysed
		1 wk.	"	"	"	"	"	"

TABLE VII Cont.

Organism	Medium	Time	1-500	1-1000	1-1500	NaOH broth	plain broth	phage
Staphy-	1.Broth tubes	48 hrs.	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Clear
lococcus		1 wk.	"	"	"	"	"	"
	2.Agar plates	48 hrs.	S.Lysis	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	N.Growth	"		"	"	"
Shiga	1.Broth tubes	48 hrs.	Clear	Clear	Clear	Cloudy	Cloudy	Clear
		1 wk.	S.Cloudy	"		"	"	"
	2.Agar plates	48 hrs.	S.Lysis	Lysed	Lysed	N.Growth	N.Growth	Lysed
		1 wk.	"	"		"	"	"
Megath	1.Broth plates	48 hrs.	Cloudy	Clear	Clear	Cloudy	Cloudy	Clear
orium-2		1 wk.						
	2.Agar	48 hrs.	N.Growth	N.Growth	Lysis	N.Growth	N.Gr.	Lysed
		1 wk.						

Legend: same as Table I.

Only two of the phages (megatherium-2 and one race of the Staphylococcus) that lyse Gram-positive bacteria were found to be more susceptible to the action of sodium hydroxide than are some of the agents lytic for Gram-negative organisms. Coli 1 and shiga phages were, however, more resistant to the effects of sodium hydroxide than any of the other tested. Sometimes they appeared to be inhibited within a week while at other times no inhibition of lysis could be detected. With the two exceptions, all the phages were inhibited by sodium hydroxide in a dilution of 1-500 within

one week. Four of the lysates, cloacae, coli-2, megatherium-2, and Staphylococcus-1 appeared to be completely inactive within twenty-four hour's time when incubated with sodium hydroxide in a dilution of 1-500.

Table VIII gives the results for both phenol and sodium hydroxide.

TABLE VIII

A summary of the results with phenol and sodium hydroxide as the phage-inhibiting agents. Incubation temperature 37°C.

PHAGES TESTED	Disinfectant	Dilution of inhibiting substance 1-20	1-80	1-160
Megatherium-1	Phenol	+ in 24 hrs.	+ in 24 hrs.	- in 24 hrs.
Staphylococcus-1	"	+ in 24 hrs.	+ in 1 wk.	- in 1 wk.
Staphylococcus-2	"	+ in 24 hrs.	+ in 1 wk.	- in 1 wk.
Cloacae	"	+ in 24 hrs.	- at 1 wk.	- at 1 wk.
Coli-1	"	+ in 24 hrs.	- at 1 wk.	- at 1 wk.
Coli-2	"	+ in 24 hrs.	- at 1 wk.	- at 1 wk.
Achromobacter	"	+ in 24 hrs.	- at 1 wk.	- at 1 wk.
Shiga	"	+ in 24 hrs.	- at 1 wk.	- at 1 wk.
		1-500	1-1000	1-1500
Megatherium-2	NaOH	+ in 24 hrs.	+ in 24 hrs.	- in 24 hrs.
Staphylococcus-1	"	+ in 24 hrs.	- at 1 wk.	
Staphylococcus-2	"	+ in 1 wk.	- at 1 wk.	
Cloacae	"	+ in 24 hrs	- at 1 wk.	
Coli-1	"	-,+ at 1 wk.	- at 1 wk.	
Coli-2	"	+ in 24 hrs.	- at 1 wk.	
Achromobacter	"	+ in 1 wk.	- at 1 wk.	
Shiga	"	+ at 1 wk.	- at 1 wk.	

\+ complete inhibition
\- no or incomplete inhibition
-,+ sometimes negative and sometimes positive

DISCUSSION

After demonstrating a selective action of dyes upon the bacteriophage it was decided to include phenol and sodium hydroxide in the investigation to discover whether or not certain phages are, in general, more susceptible to deleterious substances than others. It was found that phenol and sodium hydroxide had to be used in much higher concentrations than the violet dyes if inhibitive effects were to be produced. A lesser dilution of inhibiting solutions, permitted uninhibited bacterial growth, when compared to the dyes. Otherwise the conditions were kept the same as those employed in the dye experiments.

Under these experimental conditions it was demonstrated that the bacteriophages again fell into two groups on a similar basis to that given above. This was more especially true of phenol than of sodium hydroxide. The results obtained with phenol were very uniform but those secured with sodium hydroxide were very irregular. It has been known for some time that Gram-negative organisms are more easily hydrolised by sodium hydroxide than are the Gram-positive organisms. If that fact is taken into account, it will in part explain the reactions of sodium hydroxide on the bacteriophages though directly opposite results would be expected. However, in the various experiments worked, phages that lysed Gram-positive bacteria did show as much, if not more, resistance to the action of sodium hydroxide than any other lytic principles, except coli-1 phage and the Shiga agent. Very likely dilutions of phenol could be found which would show more variation

in resistanse of the various lysated but the range in dilution would be very small when compared to that of the dye.

CONCLUSIONS

The selective action of phenol and sodium hydroxide on bacteriophages is exhibited in the above experiments.

While the action of phenol upon the bacteriophages seems to divide the lytic agents into two groups as with the dyes, the effects of the disinfectant appears to be more uniform, the margin for the dividing line between the two is smaller.

Sodium hydroxide gave much more irregular results than any other of the inhibiting substances used, although two of the lytic agents that lysed Gram-negative bacteria were more resistant to its action than any of the others.

With the two exceptions mentioned the phages that lysed Gram-positive bacteria were as resistant to the effects of sodium hydroxide as any others tested.

Much greater concentrations of phenol and sodium hydroxide were required to bring about inhibition of the phages than was necessary for the dyes. It was also necessary to have a higher concentration of phenol than sodium hydroxide.

Conversely, it was not necessary to make the final dilutions of the two latter substances so high as it was for the dyes in order to permit uninhibited bacterial growth.

One might conclude from the results obtained that, in general, the phages which lysed Gram-negative bacteria are more resistant to deleterious substances than are those which lyse Gram-positive bacteria.

SELECTIVE ACTION OF GENTIAN VIOLET ON ENZYMES

Since several investigators have suggested the enzyme-like nature of the bacteriophage, and because of the many similar properties between the two biological substances; a study of the effect of dyes on enzymes seemed to be a logical investigation to follow the above work, of dye-action on the b'phage.

Properties in which enzymes and b'phages are similar may be summarized as follows: (1) both are sensitive to 70°C. for any length of time; (2) there is an optimum temperature for the most activity; (3) they are antigenic; (4) sunlight and ultra-violet rays are said to injure both; (5) an optimum pH exists; (6) they are specific in reactions; (7) small amounts ofeither substance will affect large amounts of substrate; (8) both are considered catalysts; (9) they gradually lose strength on standing; (10) they are difficult to isolate in pure state; (11) either will withstand freezing; (12) there is optimum concentration for best reaction; and (13) both substances apparently are adsorbed onto their substrate. On the other hand, they differ in that (1) the bacteriophage acts best on living cells; (2) it increases in amount as reaction progresses whileenzymes do not; and (3) enzymes are rapidly destroyed when in solution especially at temperatures of 50°C. to 80°C. (Waksman 1926); but the bacteriophage is not. Although no special study has been made, it has

been observed that some of the bacterial enzymes, especially those from Gram-positive bacteria, lose their potency in a few days at room temperature when in an alkaline median, while the bacteriophage remains potent until the solution evaporates to dryness; and then, if redisolved, will exhibit active lysis. Some of the bacterial enzymes, perhaps would exhibit similar resistanse to evaporation and drying if tested.

The chief objective in this experimental work was to see if there might not be a similar relationship existing between the action of dye upon bacterial enzymes as was found with the bacteriophages. Literature dealing directly upon this question has not been found. The following observations were also noted:: the effect of variousreactions of the media on enzyme activity, the effect of room and ice box temperatures relative to rapidity of deterioration, and the quantity and speed of liquefaction of gelatin.

EXPERIMENTS

Bacteria that are rapid gelatin liquefiers were secured for this work by two methods; (1) by exposing gelatin plates in a dusty room for a short time and incubating; and (2) by spreading heavily polluted river water onto gelatin; and, after incubation at 20°C. for 24 hours, the colonies which showed the most liquefaction were chosen from both sources. Then two stock cultures, prodigiosus and Achromobacter organisms that were known to be good liquefiers, were used. No attempt was made to identify all the organisms, the purpose being primarily to get an equal number of Gram-positive and Gram-negative bacteria. Twelve organisms, six Gram-negative and six Gram-positive, were thus collected. Although there may have been duplicate species, all were different strains. However judging from general characteristics, not more than two of the strains appeared to belong to any one species and there was not more than two instances where this occurred.

Ordinary twelve per cent gelatin and plain beef broth were the mediums employed. One series of tests was run with media adjusted to a pH of 7.6 to 7.8 and the other was about pH 6.0. Coleman and Bell's gentian violet was the inhibiting agent in these experiments.

Enzymes were prepared by spreading a suspension of each organism over the surface of a gelatin plate and incubating at 20°C. for 3 to 4 days after which the liquefied gelatin was

passed through Berkfeld filters; these filtrates constituted the enzyme preparations, or perhaps we might say suspension (stock solutions). Broth culture filtrates were experimented with but were found far inferior, relative to enzyme potency, to the gelatin culture filtrates. Even tho the organisms were inoculated on gelatin of a pH 6.0, the resulting enzyme filtrate was found to be alkaline in reaction due to the hydrolysis of protein material and the consequent liberation of ammonia. On account of the change in pH of the media, no attempt was made to keep the material very definitely at a certain pH. This explains why it is said to be acid medium or alkaline medium in the tables. Sufficient acid broth was used to make dilutions of filtrates from acid gelatin to insure an acid reaction for the test. Alkaline (about pH 7.8) was used to make the dilutions for the alkaline series of filtrates. Occassionally the filtrates were not sterilized by passing them thru Berkfeld V and N filters. It seemed to occur more often when filtering cultures three or more days old; but if the filtrates were again filtered immediately after bacterial growth appeared the second filtrate was almost invariably sterile. It is the belief of the writer, as many others (Seastone and Lawrence, 1933, Wyckoff, 1933) that filterable forms develop in old cultures and it is these forms thatoccasionally pass thru the filters. Trouble of this nature was not experienced when working with bacteria under the influence of bacteriophage although many authors think

that it does cause the production of filterable forms of bacteria. The enzyme, trypsin (Digestive Ferments Co., Liquid) was tested along with the bacterial enzymes as a means of comparison. Pepsin was also tried but it was found that neither the Gram-positive organisms nor their enzymes would work in a pH of 5.0 or below and pepsin would not act much above that. Such result suggest thatthe two groups of organisms could be separated relative to the hydrogen ion concentration.

Divided plates were first tried but results were too variable and all the enzymes were able to partically liquify the gelatin in the highest concentrations of the dyes it was possible to make. Hydrolytic reactions were probably catalysed before the dye had time to inactivate the enzyme. In one instance when adding the Achromobacter suspension to a gelatin plate to spread for preparation of enzyme filtrate, it was noticed that a small depression had been "eaten" out in the medium during the time that elapsed between the adding of a drop of the bacterial suspension and the spreading of the drop with a sterile glass rod -- not more than thirty seconds.

Table 9 gives a summary of results taken from experiments where the enzyme filtrates were transferred directly to divided gelatin plates. Plates were prepared by pouring one side of a slanting plate and solidifying in the ice box before pouring the other side. Symbols have been used to indicate the bacteria from

which the enzymes were derived and the Gram reaction of each organism is given in table 9.

These organisms having an "A" as part of the symbol are from the air isolations whilethose having an "R" are from the river. "Meg" is megatherium, "38" is Achromobacter, "88" is vulgaris and "prod" is prodigosus.

TABLE IX

Results obtained from divided gelatin plates. Incubation temperature 20°C.

Enzymes From Bacterium	Gram	Divided Plates Concentrations of Dye 1-400	1-1000	1-2000	Control
Predig.	-	0	0	0	Liq.
A 2	+	75	50	25	"
A 4	+	50	50	0	"
A 5	+	50	0	50	"
A 6	+	90	25	25	"
R 1	-	25	0	0	"
R 2	-	25	0	0	"
R 4	+	25	25	0	"
R 6	-	0	0	0	"
Vulg.	-	75	50	25	"
3 B	-	0	0	0	"

Liq. = definite liquefaction of gelatin
Numerals indicate per cent inhibition of enzyme action when compared to control where no dye is present.

Results on the divided plate show a division of the enzymes of Gram-negative and Gram-positive organisms into two groups; those enzymes of the former appear to be much more resistant than the others. On the other hand the results appear quite variable and one gelatinase from a Gram-negative organism (vulg) was inhibited as much or more than any of the enzymes. It is possible, not only in this experiment but also in those which follow, that the recorded numeral represents as much as twenty five per cent error because of the inability to transfer equal portions of the various solutions to the gelatin with the loop, even tho the same loop of uniform size was used. If complete inactivation could be obtained this source of error might be in a large measure eliminated. Therefore the plate method was soon dropped and the following method similar to that used with the bacteriophage employed.

Methods of preparing the inactivating dye-enzyme solutions similar to that of the bacteriophages were then followed by making the proper dilutions of dye in plain broth. It is known that plain broth cuts down the germicidal action of the dyes more than water would but one can control the pH of the system better by so doing. Results will also be comparable with the bacteriophage results. Equal quantities of dye solution and enzyme filtrates were mixed to produce the desired inactivating solutions and a control was prepared by adding an equal amount of plain broth to the enzyme filtrate. These solutions were

incubated at 20°C. and tests made at various times (usually 24 hours; 48 hours; and 72 hours) to see if inactivation had occurred. Twenty degree centigrade incubation was used because of such rapid deterioration of some of the enzymes at higher temperatures and of course the gelatin had to be incubated at a low temperature because of liquefaction due to temperature alone. Because of the different incubation temperature, results entirely comparable to the phage experiments were not obtained.

Tests to determine the degree of inactivation of the enzymes were performed by transferring one loopful of each mixture to a gelatin plate and incubating these for three to six days. Four day periods of incubation were made, before taking the final reading of results, where possible. The latter period was found to be necessary to insure plenty of time for enzyme action in all instances. Results were also more reliable and easier to read than when resorting to shorter incubation periods. A ten-penny nail was provided to prepare the loop on the inoculating needle for making all transfers of material. Provision was thereby made for uniformity in the work. Means of providing entire uniformity in the matter of transferring equal quantities of enzyme mixtures was very difficult. Data were not recorded unless the enzyme control had produced a definite concave area in the gelatin. A single loopful of dye alone and one of plain broth were added to each plate as controls for purposes of comparison in reading results.

Results of experiments in which a combination of the divided plate and dye-filtrate inhibiting solutions were employed are shown in Table X. The proper dilutions of dye for the solutions had not yet been found. It may be noted that higher concentrations of dye were used than that used with the bacteriophages. The difference in temperature of incubation should not be disregarded here but will be taken up elsewhere in the discussion.

TABLE X.

Results obtained with a combination of divided plate and test-tube methods. Incubation temperature 20°C.

Organism	Gram	Tubes after 42 hours inhibitive action			Divided plates		
		Concen. of dye		Control	Concen. of dye		Control
		1-800	1-8000		1-200	1-400	
R 2	-	0	0	Liq.	50	25	Liq.
3 S	-	90	50	"	25	25	"
A 4	+	100	90	"	75	25	"
R 1	-				50	50	"
A 2	+	100	90	"	75	25	"
S 8	-	75	50	"	95	90	"
A 6	+	100	90	"	50	25	"
Meg	+	90	25	"	25	0	"
R.4	+	25	0	"	0	0	"
A 5	+	100	75	"	25	25	"
Trypsin		25	0	"	25	25	"

Legend: same as for Table IX

Divided plates in the above table reversed the selective action for the two groups of enzymes, confirming the previous findings, that much variation in results was obtained by employing such a method of procedure. Many trial experiments were run before finally reaching the above conclusion. Directly opposite results were obtained when the dye was mixed with the filtrates and incubated for 42 hours before testing for active enzyme. Without exception the enzymes from Gram-positive bacteria were inactivated by dilutions of gentian violet that did not inactivate the enzymes from Gram-negative organisms when using the latter type of test. The last statement was made on a basis of group reactions for there are some individual exceptions which will be discussed below.

Acid and alkali media were both employed in the next series of work, the results of which are given in Table XI. Divided plates were omitted.

TABLE XI

Results from experiments after inhibitive action of gentian violet in acid and alkali liquid media for periods of time shown. Incubation temperature 20°C.

Enzyme from	Gram	Acid Medium, Inhibiting time shown, 24 hrs., Con. of dye 1-400	1-800	Control	72 hrs., Con. of dye 1-400	1-800	Control	Alkali Medium, Inhibiting time shown, 24 hrs., Con. of dye 1-400	1-800	Control	72 hrs., Con. of dye 1-400	1-800	Control
A 5	+	100	95	Liq.	100	50	Liq.	100	100	Liq.	100	100	Liq.
R 2	-	0	0	"	25	0	"	0	0	"	25	0	"
3 S	-	90	75	"	90	50	"	75	75	"	75	75	"
A 4	+	90	75	"	100	100	"	75	50	"	25	50	"
R 1	-	50	75	"	0	0	"	0	0	"	25	0	"
A 2	+	75	75	"	100	75	"	100	100	"	100	100	"
S 8	-	100	100	"	75	50	"	100	90	"	100	100	"
A 6	+	100	50	"	100	75	"	50	50	"			
Meg	+	75	50	"				100	0	"	100	75	"
R 4	+	0	0	"	0	0	"	0	0	"	0	25	"
R 6	-	100	75	"				25	0	"	0	0	"
Prod.	-				100	50	"	100	75	"	100	75	"
Trypsin		75	50	"	0	0	"	0	0	"	50	0	"

Liq. = definite liquefaction of gelatin.
Numerals indicate per cent inhibition of enzyme action when compared to control where no dye is present.

Table XI shows a very striking selective action of gentian violet upon the enzymes. In general the enzymes that were produced by Gram-positive bacteria were inactivated by the dilutions of dye used (1-400 and 1-800) while those produced by Gram-negative organisms were not. However, the division into two groups on the basis of the Gram reaction is not nearly so marked as it was with the bacteriophages. Three definite exceptions (S 8, Prod., and R 4) and one partial exception (3 B) occurred. Enzymes of the Gram-negative bacteria, S 8 and Prod. were completely inactivated by the 1-400 dilution of gentian violet and enzyme from 3 B was almost inactivated. The enzyme from the Gram-positive bacterium, R 4 was not inhibited in the least in the highest concentration of dye. All the enzymes produced by Gram-positive bacteria, except the R 4 mentioned, were inactivated in the 1-400 dilution. There appears to be an inconsistency in the acid medium for trypsin. Trypsin appeared to be as resistant to the action of the dye in most experiments as any of the bacterial enzymes. By calculating the total per cent inhibition as shown in the table, slightly more inhibition is recorded for the alkaline medium; but if the ones omitted from the data were taken into account, the acid side would probably show a little higher figure. The difference is so slight that it is not safe to say which reaction is more favorable for inactivation of the enzymes. A very decided difference was noted in the amount of liquefaction that was produced by the various enzymes upon the

two kinds (acid, alkali) of media, the alkaline medium being the more favorable. Especially did those produced by Gram-negative bacteria exhibit a much greater liquefaction on the alkaline side of neutrality. The pH range at which the latter enzymes would work was also larger. Enzymes from the Gram-positive bacteria failed to function in a medium as high in acid content as the enzymes from the Gram-negative organisms.

It should be remembered that the numerals in the tables represent the degree of inhibition when compared to the control of the same enzyme and not to any of the others. For example fifty per cent inhibition in each of two enzymes does not mean that corresponding tests of the two show the same amount of liquefaction; but, on the contrary, one may show twice as much liquefaction as the other.

Table XII shows the results for the same type of test as the previous one, except the concentrations of gentian violet were doubled. One can see at a glance that the results are similar to those shown before but inhibitive action was a little more pronounced.

TABLE XII

Results from experiments after inhibitive action of gentian violet in acid and alkali media for periods of time shown. Incubation temperature 20°C.

Enzyme from	Gram	Acid Medium, Inhibiting time shown: 24 hrs. Con. of dye 1-200	24 hrs. Con. of dye 1-400	24 hrs. Control	72 hrs. Con. of dye 1-200	72 hrs. Con. of dye 1-400	72 hrs. Control	Alkali Medium, Inhibiting time shown: 24 hrs. Con. of dye 1-200	24 hrs. Con. of dye 1-400	24 hrs. Control	72 hrs. Con. of dye 1-200	72 hrs. Con. of dye 1-400	72 hrs. Control
A 5	+	100	100	Liq.	100	100	Liq.	100	100	Liq.	100	100	Liq.
R 2	-	25	25	"	50	25	"	0	0	"	25	0	"
3 S	-	50	25	"	75	50	"	100	50	"	100	100	"
A 4	+	100	100	"	100	100	"	100	100	"	100	100	"
R 1	-	0	0	"	25	0	"	25	0	"	25	0	"
A 2	+	100	100	"	100	100	"	100	100	"	100	100	"
S 8	-	100	75	"	100	100	"	90	75	"	100	100	"
A 6	+	90	75	"	100	100	"	100	75	"	100	100	"
Meg.	+	100	100	"	100	100	"	90	75	"	100	100	"
Z 4	+	25	25	"	25	0	"	0	0	"	0	0	"
R 6	-	75	50	"	50	50	"	25	0	"	50	25	"
Pred.	-	100	100	"	100	100	"	75	25	"	100	100	"
Trypsin		0	0	"	25	25	"	0	0	"	25	0	"

Liq. = definite liquefaction of gelatin.
Numerals indicate per cent inhibition of enzyme action when compared to control where no dye is present.

These data indicate a division of the enzymes as in the other experiments but the higher concentrations of gentian violet increased the degree of inhibition of liquefaction. Three enzymes, A 4, A 2, and A 5, secreted by Gram-positive bacteria were inactivated in all the tests and the others in this group materially reduced in activity except the R 4 which again was only slightly affected. Gelatinases from the Gram-negative bacteria, 3 S, S 8, and Prod. were inactivated in the 1-200 dilution of dye after the incubation period had been extended to 72 hours in the alkaline medium. Taken as a whole there was a little more inhibition produced in the acid medium. Trypsin was little affected.

DISCUSSION

In general the bacterial enzymes fell into two groups when considered on the same basis as the bacteriophages. The enzymes secreted by Gram-positive bacteria were inactivated by concentrations of gentian violet that did not affect to the same degree the enzymes from Gram-negative bacteria. Four exceptions to the rule were found, three on the Gram-negative side and one on the Gram-positive in which the opposite reaction took place. No such exceptions were found with the bacteriophages. The results suggest a close relationship between the bacteria and their respective (products?) enzymes or bacteriophages. Higher concentrations of dye were required to inactivate the enzymes; but perhaps the difference in incubation temperature would account

for the difference, in part at least. This is a point that should be substantiated or disproved by further experimentation. While no special study was made, it was observed that the bacteria and their enzymes could be similarly grouped with reference to their reactions to hydrogen ion concentrations, the Gram-positives being more readily inhibited by the higher concentration of hydrogen ions.

Divided plate methods were found unsatisfactory for the enzyme work as was true with the phage experiments. Similar methods of procedure were employed in each instance. The agent to be inactivated was incubated in high concentrations of dye in plain broth for varying lengths of time before actual tests were made to determine the extent of inhibition obtained. It is probable that the enzymes catalysed hydrolytic processes on the dye plates before inactivation could be accomplished and this may account for the unsatisfactory results on the plates. This was not the case with the phage work for here the bacteria were inhibited in growth before the lytic agents were.

Evidence has been given to show that filterable forms of the bacteria may be produced in cultures three or four days old. If the same bacterial cultures were filtered while young, no "contaminations" occurred. The bacteriophage experiments gave little, if any, evidence of filterable forms; almost invariably the filtrates remained sterile for periods of time up to three years. However, the potency of the enzymes was rapidly lost in

alkaline media while that of the phages was not, even though its virulence does gradually decrease. One race of coli-phage that has been kept in the ice-box in a liquid synthetic medium was found active after about two and one-half years. Some of the bacterial enzymes lost their ability to liquefy gelatin after remaining in plain broth (pH 7.6) in the ice-box for a few weeks.

No attempt was made to keep a definite pH for the enzymes on the acid side because of reversion to the alkaline reaction due to the hydrolysis. The filtrate was made acid (phenol red and litmus used) before mixing with the dye for inactivation. Organisms were grown on the alkaline (pH 7.8) media and no adjustment made for these filtrates. They no doubt remained decidedly alkaline in reaction. In fact trial tests were made to confirm this hypothesis. The pH range at which the enzymes worked was rather wide although the alkaline media was more favorable for their action. Enzymes secreted by Gram-negative bacteria worked over a wider range of hydrogen ion concentration than did those produced by Gram-positive bacteria. The trypsin-like nature of the bacterial enzymes is suggested by the fact that they actively liquefy gelatin on either acid or alkaline medium as the trypsin did. Trypsin compared very favorably with the enzymes from the Gram-negative organisms in its reactions to the gentian violet. Pepsin could not be used because it was inactive in the pH range required by the other enzymes.

CONCLUSIONS

A selective action of gentian violet on enzymes similar to that found for bacteriophages has been demonstrated.

Higher concentrations of dye were employed to effect inactivation of the enzymes than with the phages.

Under the conditions of the experiment, the enzymes (in general) that were secreted by Gram-positive bacteria were inactivated by dilutions of dye that only partially inhibited those produced by Gram-negative bacteria.

Four exceptions to the above rule were observed, three on the Gram-negative and one on the Gram-positive side in which the opposite reaction occurred.

An incubation temperature of 20°C was found satisfactory for the work with enzymes.

The evidence indicates that filterable forms of bacteria were encountered.

The pH range is rather wide for the bacterial enzymes, including both acid and alkaline reactions.

Evidence of the trypsin-like nature of the bacterial enzymes has been given.

Trypsin exhibited properties, with reference to dye, similar to the bacterial gelatinases from Gram-negative bacteria.

The divided-plate method of study was found unsatisfactory.

Pepsin could not be employed in this investigation because

of the high pH used.

Gram-positive bacteria and their enzymes were inhibited to a greater degree in a low pH than the Gram-negative.

A larger quantity of material was liquefied in alkaline medium by the enzymes.

GENERAL DISCUSSION

The data show that the bacteriophages fall into two groups, when classified by their reactions to certain chemical substances and environmental conditions. The division on the one hand includes all those lytic agents which are developed at the expense of Gram-negative bacteria while on the other hand we find those principles which are developed at the expense of Gram-positive organisms. Crystal violet, gentian violet, phenol, and sodium hydroxide when used in the proper concentrations and under the proper experimental conditions will bring about the inhibition of the phages which lyse Gram-positive bacteria while those that are lytic for Gram-negative organisms are not made inactive.

Similarly bacterial enzymes have fallen into two groups with reference to the Gram-reaction of the respective bacteria. However, gentian violet was the only substance tested against the enzymes. It was observed that the enzymes can be similarly grouped with respect to their reactions toward hydrogen ion concentrations, the enzymes that were produced by Gram-positive bacteria being more unfavorably influenced by the lower pH values. Apparently greater quantities of gelatin were hydrolysed by the enzymes from Gram-negative bacteria in either acid or alkaline medium. In the two experiments run, the enzymes produced by Gram-positive bacteria seemed to lose their potency more rapidly at room temperature (in alkaline medium) than did the enzymes

of the other group. This may be an apparent rather than a real loss because, according to Waksman and Davis, the greater the maximal enzyme content of a culture the longer will the enzyme remain active. It, also, is possible that this may explain the greater hydrolytic properties of the Gram-negative group. Granting that the latter is true, there would still be a difference in the two groups in that greater quantities of enzymes were more easily obtained from Gram-negative bacteria. The quantity of enzymes present may be a factor in the results with gentian violet, since all the enzymes seemed to be inhibited to some extent. Certainly this does not account for all the selective action observed. The Achromobacter enzyme was the most active of any and it liquefied greater quantities of gelatin; yet it was inhibited much more than several of the others in the experiments performed.

That the bacteria themselves can be grouped into groups according to their staining reactions is well known. Not only can the bacteria be grouped into one of two groups by the Gram stain but also by their reactions to various other substances. For example, Churchman (1928) lists twenty-two different ways in which the Gram-negative bacteria react differently from the Gram-positive. The rule can not be applied in a hard and fast manner for exceptions on either side are found. However, the same thing was found to be true of the enzymes.

Since the bacteria and their respective ensymes and bacteriophages can be similarly grouped relative to their reactions to various reagents, it would not seem entirely out of place to assume that there may be some chemical relationship between the bacteria and their respective phages. There is no question about there being a definite relationship existing between the bacteria and the ensymes. Churchman (1928) states, in speaking of the Gram reaction, that on the whole, the tendency is to emphasise the chemical rather than the physical factors of this reaction. Possibly this lends some support to the assumption just made, providing the bacteriophage is a metabolic product of the bacteria. The experimental evidence in this work and that of many others supports this view.

The b'phage may be an ensyme or an ensyme-like substance as many workers (Davison 1922, Kutluer 1925, Weiss and Arnold 1924) have suggested. Perhaps it is a catalase or catalase-like substance. The phage has many properties in common with ensymes, and a few in particular with the ensyme, catalase. Properties of special importance are that both catalyse reactions in one direction (mainly so, at least) and in both cases definite chemical substances are formed. Specificity might be mentioned as another important property of the two -- they act on material of a definite chemical nature and cause the production of definite chemical substances. It is true that the b'phage acts upon a

more complex substance but so do enzymes in general. While catalase reacts with H_2O_2 to form H_2O and O_2, it is not unreasonable to assume that the phage reacts with the bacterial cell and causes a specific chemical change in which a substance identical to the catalyser is produced. We know that enzymes in general bring about (or at least catalyse) specific changes in the substrate. Different enzymes acting on the same substrate will cause the production of different split products, e.g. zymase splits glucose to form two molecules each of alcohol and carbon dioxide, but lactacidase splits glucose to form two molecules of lactic acid (Buchanan 1915). Even though these may not illustrate all the steps involved, the above named substances are nevertheless always formed. The observation that each of two races of b'phage acting on the same strain of bacteria and causing the production of more of the lytic agent is identical in every respect to the one used, but different from the other in all respects in which the two differed to begin with, could be explained on the above basis. The main difference in each case being that a substance identical to the catalyser was being formed in bacteriophagy, but with the enzymes new substances were produced. Thus an explanation of the fact that coli-1 phage and coli-2 phage, both of which were developed at the expense of the same organism, exhibited several distinctly different properties might be given. The few differences that might be mentioned between the enzymes and the b'phage need not invalidate such conclusions because enzymes themselves differ in many

respects. Waksman states that proteases of plants are distinctly different in their action from the proteases of micro-organisms and it has been found that the proteases of the various species of bacteria may differ markedly from one another in their properties.

The author has attempted throughout this investigation to find a dividing line between phages and enzymes of Gram-negative bacteria and those of Gram-positive organisms, which in general has been done.

Stating an hypothesis on the mechanism of inhibition of the various substances is unwarranted by the data of this experiment for the following reasons: first, the limited knowledge of the chemical reactions of dye; and second, the chemical composition of the materials used in this investigation is unknown.

GENERAL CONCLUSIONS

A selective phagiostatic action of crystal violet, gentian violet, brilliant green, phenol, and sodium hydroxide on bacteriophages tested has been demonstrated; and the results suggest that there is a correlation between the selective action of crystal violet, gentian violet, and phenol on bacteria and their respective phages.

A similar selective action was found to obtain for bacterial enzymes when gentian violet was employed as the inhibiting agent.

Evidence has been cited which suggests a close relationship between the bacteria and their respective bacteriophages.

High concentrations of the disinfectants were required to effect inhibition of the phages and enzymes -- higher for the enzymes than for the phages under the experimental conditions. Higher concentrations of phenol and sodium hydroxide were required than for the violet dyes in the phage experiments.

A high dilution of the primary disinfectant solutions (inhibiting dilutions) to the point where there would be no inhibition of bacterial growth was a requisite in demonstrating the activity or inactivity of the lytic principles after each period of incubation. However, a higher dilution of the dyes was necessary for this purpose than when phenol and sodium hydroxide were used.

Under the conditions of the experiment, the phages that lysed Gram-positive bacteria were completely inhibited by

concentrations of crystal violet, gentian violet, phenol, and sodium hydroxide which did not appear to diminish the activity of the phages that lysed Gram-negative bacteria.

Similarly, enzymes from Gram-positive bacteria were in general inactivated by dilutions of gentian violet that did not prevent hydrolysis of gelatin by enzymes from the Gram-negative group of bacteria.

The group of enzymes from Gram-positive bacteria do not function so well in media with a low pH as do those from Gram-negative bacteria. The former also appeared to be more unfavorably influenced at room temperature than the latter.

The enzymes from Gram-negative bacteria exhibited greater hydrolytic properties.

The inhibitive property of brilliant green was extremely variable when compared with that of the other disinfectants, but nevertheless distinct with one phage of the colon bacillus in particular.

Twelve bacterial enzymes, six from Gram-negative bacteria and six from Gram-positive organisms, were employed in this work.

Trypsin of animal origin was also included in the enzyme experiments. It compared favorably with the more resistant group (Gram-negative) of enzymes in its reactions toward gentian violet.

Twelve bacteriophages, five that lysed Gram-positive bacteria and seven that lysed Gram-negative bacteria, were tested in this investigation.

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