STUDIES ON BACTERIAL VIRUSES ACTIVE AGAINST *XANTHOMONAS PRUNI*

By

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TABLE OF CONTENTS

TABLE OF CHARTS AND FIGURES

I. INTRODUCTION

Viruses which attack bacteria have become convenient tools for the study of several biological phenomena. They have been valuable aids in investigations involving genetics, bio-chemical synthesis, virus-host relationships, and ecological factors in microbial populations. Because of the distinct specificity of these viruses, they have become useful in bacterial classification and identification, and may even someday prove their worth as therapeutic agents. Their particular value lies in the ease with which they lend themselves to experimentation. In contrast to the difficulties encountered with most other organisms, bacterial viruses, or bacteriophage, may be cultured conveniently in large numbers under known, measured conditions.

Quantitative phage research has dealt primarily with those viruses which attack Escherichia coli. However, the study of bacteriophage has broadened to the point where investigations of other phage-host systems might be profitable. This was the main reason that the bacteriophage of Xanthomonas pruni was chosen.

The X. pruni virus was first isolated by Dr. H. W. Anderson in 1928 and preliminary investigations of specificity, lysis, plaque formation, electron microscopy, effect of some chemical and physical agents on the stability of the virus and of its burst time have been carried out (Thornberry, et al, 1948, Thornberry, 1937, Eisenstark and Thornberry, 1950, and Crownover, 1950). It is the purpose of this paper to extend the investigations of these workers with particular intention of providing further material for the study of bacterial and perhaps viral genetics. The work is concerned with the isolation of different strains of bacterial viruses, all active against Xanthomonas pruni, and establishment of the individual identity of each virus by various means. These means include plaque morphology, indicator hosts, serology, burst time, and adsorption rate.

II. EXPERIMENTAL

A. Plaque Morphology

Eisenstark and Thornberry (1950) reported that differences observed in plaques of pruniphage on Xanthomonas pruni suggested the possibility of distinct genetic varieties such as those described for Escherichia coli phage (Demerec and Fano, 1945). They also mentioned that, except for an increase in the halo type of plaque when it was subcultured, attempts at isolating genetic varieties were unsuccessful. Since the initiation of the present investigation, additional work and altered techniques have yielded a number of plaque types which appear to breed true.

1. Materials and methods.

Standard plating technique. The procedure used for assay of phage by the plaque method was modified from that used by other workers (Thornberry, et al, 1948, Thornberry, 1937, Eisenstark and Thornberry, 1950 and Crownover, 1950). The bacterial host was an American Type Culture, No. 10016. The medium used for routine work and maintenance of stock cultures of the host organism was as follows:

Peptone	5 g.
Beef extract	3 g.
Glucose	5 g.
NaCl	5 g.
Agar	15-20 g.
Water	1 liter

The host organism was either grown for 36 hours on a slant and the growth emulsified in 100 ml. of sterile broth, or it was grown in 100 ml. of broth for 5 hours with agitation. However prepared, 4.5 ml. of host suspension was added to test tubes. These tubes served to dilute the phage stock in ten-fold dilutions. For plating, the host organism and diluted phage were rapidly mixed with 1.5 ml. of 2% agar which had been previously measured into tubes, melted and cooled to 50° C. The mixture was then poured over a sublayer of solid medium containing 1.5-2.0% agar. The plates were

incubated at 27° C. for 2-3 days.

Experiments were conducted in which this procedure was varied to determine the effect of age of culture, size of inoculum, agar concentration, salt concentration, and temperature on the plaques produced.

Effects of age of culture and size of inoculum. In the determination of the effects of age of culture and size of inoculum on plaque formation, a phage suspension with a titer of 5×10^7 was diluted serially. Samples of X. pruni cultures were taken at intervals of 2 hours from 0 to 22 hours and dilutions $1:10^6$, $1:10^7$, and $1:10^8$ of the phage were plated with each sample. After 3 days of incubation at 27° C., a calibrated ocular and stage micrometer were used to measure 100 plaques from each age culture, and the mean diameter was calculated in millimeters (see Chart 1).

The effect of size of inoculum was tested by adding 1.0, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 ml. of a turbid emulsion of the host organism to tubes containing 4.5 ml. of broth and then adding 100-200 phage particles in 0.5 ml. of phage suspension to each tube. These were plated in the routine manner and incubated at 27° C. for 2 days.

Effect of agar concentration. The procedure used to determine the effect of agar concentration on plaque formation was slightly different from that described for routine plaque counts. Flasks of nutrient broth containing various concentrations of agar were prepared by adding respectively 1.0, 1.5, 2.0, 3.0 and 4.0 g. of agar to six 100 ml. portions of broth in flasks. The phage was diluted to $1:10^9$ and 1 ml. of each of the last five dilutions was added to 4 ml. of a young cell suspension, 5 ml. of each concentration of agar was added and the mixture poured over a sublayered plate. The final concentrations of agar in the tubes were 0.50, 0.75, 1.00, 1.25, 1.50 and 2.00%.

Effect of salt concentration. To determine the effect of salt concentration on plaque formation, 0.0, 0.5, 1.0, 2.0 and 3.0% NaCl was incorpora-

ted into 5 sets of broth. A suspension of host cells was added to each of the five flasks. Ten tubes of 4.5 ml. each were prepared from each of the flasks. The tubes of broth were inoculated in duplicate with about 200 particles each of five strains of the phage. The plates were then poured in the routine manner over a sublayer of agar containing matched salt concentrations.

Effect of temperature. The effect of temperature was tested by incubating plates both at 30° C. and 25° C. for 2 days.

Isolation of plaque types. The method used in isolation of the different strains of pruniphage consisted of plating out the original stock phage to obtain plaques, placing differently appearing ones into broth, centrifuging the mixture and plating the centrifugate to obtain another set of plaques. This procedure was repeated numerous times to determine if the plaques picked retained the same plaque morphology on continued transfer.

2. Results

Effect of age of culture and size of inoculum. Chart 1 illustrates the effect of age of culture on the size of plaques produced. After an initial increase in the size of plaques, possibly during the lag phase of growth of the bacterial population, there occurs an almost linear decrease in the size of plaques produced on older cultures until only pin-point plaques are observed on a 22 hour old culture.

The effect of size of inoculum is illustrated in Figs. 22 and 23. The largest plaques were obtained with the smallest inoculum, and the plaques appeared smaller as the inoculum was increased, appearing as pin point plaques with the largest inoculum tested.

Effect of agar concentration. Increased agar concentration in the layer containing the growing bacteria and virus decreased the size and distinctness of the plaques. The effect was not quite as pronounced as that of age of culture. The bacterial film did not contain as luxuriant growth on the higher

concentrations of agar and the plaques were indistinct as a result of this decreased growth.

Effect of salt concentration. Varying the salt concentration in the growth medium appeared to affect all the five phages similarly. On 0% salt, the plaques were slightly smaller than on 0.5%. On increasing concentrations, the plaques were enormously larger and were largest at the upper limit of salt concentration in which the host organism would grow. The morphology of the plaques was also affected. The halos and central lytic areas on 3% salt were enlarged all out of proportion to that observed on 0.5% salt, making it impossible to distinguish the five types by their plaque morphology as they appear in the lower concentration. Fig. 20 and 21 illustrate the remarkable difference in size and morphology of plaques produced on 0.5% and 3.0% salt, respectively.

Effect of temperature. The effect of incubation temperature on plaque size is illustrated in Figs. 24 and 25. An increase in temperature from 25° C. resulted in decreased plaque size. At 30° plaques were tiny, disfigured, and indistinct. Above 33° plaque formation was not observed, even though the host organism grew well at this temperature.

Isolation of plaque types. Five different plaque types produced on 0.5% agar and 0.5% salt concentration were chosen from plates containing the original phage suspension obtained from Dr. Thornberry. Figs. 1-15 illustrate the morphology of the five types. XP1, the "XP" standing for Xanthomonas pruniphage, forms a large, somewhat irregular plaque with an indistinct central lytic area and often has a concentrically ringed appearance. The central lytic area is faint and sometimes difficult to discern. This plaque type characteristically varies in size from minute to large, all the sizes, however, having the same detailed appearance. Regardless of what size plaque of XP1 type is selected for subculturing, a plate exactly like this one as to range

of sizes is obtained. XP2 forms a medium-sized plaque with a large, well defined central lytic area and a rather narrow zone of secondary lysis. XP3 forms a medium-sized plaque with a small but distinct central lytic area and a wide, characteristically light-appearing halo of secondary lysis. XP4 forms a small plaque with an ill-defined margin that appears fuzzy when closely examined macroscopically. The last of the series, XP5 also forms a small sized plaque which is similar to XP2, except that the zone of secondary lysis is wider in proportion to the central clear zone.

3. Discussion

Introduction. In order to make use of plaque morphology, i.e., relative size, shape, definition of margin, and width of halo of the plaques, as a criterion for differentiation of phage types, it was desirable to know the effect of various factors influencing the characteristics of the plaques. This was necessary in order to obtain reproducible and comparable results with the experiments. For this reason, a cursory study of the factors influencing plaque morphology was made solely to determine optimum, standard conditions for best showing plaque differences. There was no attempt made to study these factors exhaustively.

Effect of age of culture and size of inoculum. As reported in the results of the effect of age of culture on plaque size, the older the culture, the smaller the plaques. This is in accord with the findings of Kleczkowska (1945) working with Rhizobium bacteriophage. She reported that plaques 1.8 mm. in diameter are produced when a one-day old culture was used, while the plaques were only 0.8 mm. in diameter with a culture 7 days old. Her explanation for this involved the physiological aging of the bacterial clones forming the background of the plaques, and this idea was supported with evidence that clones 19 hours old were scarcely susceptible to plaque formation. Furthermore, once plaques were formed, they had never been seen to increase in size. While

plaques of pruniphage have also been observed to decrease in size with increased age of culture, the situation here does not appear to be the same, for plaques have been observed to increase in size. Plaques of a phage active against mycobacteria also have been reported to increase in size, and as much as 6-7 fold over a period of a few days (Zampiere, 1950). Another possible explanation may be found, then, on consideration of the number and activity of cells present when the plaques are being formed. It was observed with the pruniphages that an increased inoculum would cause a decrease in the size of the plaques. This is in agreement with Cherry and Watson (1949), working with the Streptococcus lactis host-virus system, who also reported a decrease in plaque size with increased size of inoculum. Bronfenbrenner and Korb (1925) observed similarly that when the number of susceptible bacteria was increased, the size of the plaques produced decreased. Furthermore, when using mixtures of young and old cells, the size of plaques decreased as the proportion of old cells was increased. This may help to explain the decreased plaque size with increased age of culture in the case of the pruniphages. The diminishing of the plaques with increased age of culture may be attributable to two factors: (1) the increased size of the inoculum and (2) the increased proportion of old and dead cells which would adsorb phage without allowing its multiplication, thus decreasing the effective titer. This is certainly in accord with Anderson's (1948) statement that one of the factors influencing the formation of plaques is the ratio of the time of lysis of infected cells to the time of division of uninfected cells.

Effect of agar concentration. One of the first reports of the effect of agar concentration on plaque size was by Bronfenbrenner and Korb (1925). It was observed that the average size of plaques increased as the concentration of agar decreased. This observation was also made with the pruniphages.

The decrease in size of plaque was thought to be caused by adsorption of the phage on the agar. In a later publication Bronfenbrenner and Hetler (1928) using spot inoculations on different concentrations of agar and gelatin, attributed the inhibition of lysis with 4% agar or 50% gelatin to the prevention of water from entering and disrupting the bacterial cells. Kleczkowska (1945) also reported that increased agar concentration decreased the size of plaques, and in addition, has claimed on the basis of experimental evidence that unattached phage can diffuse through agar and produce plaques, making it unnecessary for the phage to be attached to the bacteria before pouring the plates. It may be surmised, then, that increased agar concentration effects a decreased plaque size by slowing the rate of virus diffusion--an important factor in plaque formation (Anderson, 1948)--and possibly by adsorbing some of the phage, although this seems unlikely in view of the above mentioned evidence of Kleczkowska (1945). Increased agar concentration has been reported not only to affect plaque size, but also the formation of a halo (Hershey and Bronfenbrenner, 1945). Coliphage PC which produced halo plaques on 0.6% agar, produced plaques without halos on 1% agar. Increased agar concentration appeared only to decrease the width of the halos in the case of the pruniphages.

Effect of salt concentration. There have been numerous references in the literature to the effect of sodium chloride on bacteriophagy. Adams (1949) reported that the T-series of coliphages are more rapidly inactivated in 0.1 N. sodium salts than in nutrient broth and that this effect is decreased by 10^{-3} M. divalent cations. This aspect of the sodium chloride effect was further discussed by Gratia (1940, 1940a) and Sertic (1936, 1936a). Fong and Krueger (1949) and Fong (1948) reported a protective action of sodium chloride and increased activity at high temperatures in its presence with Staphylococcus bacteriophage. Schribner and Krueger (1937) stated that the presence of 0.25 M. sodium chloride with Staphylococcus bacteriophage resulted in 5-10 fold

increase in phage production. On the other hand, Ohashi (1938) reported that inorganic salts decreased phage activity. The effect of sodium chloride on phage activity appears to be one concerned with adsorption and surface phenomena associated with adsorption. Cherry and Watson (1949) studied the effect of electrolytes on virus adsorption and concluded that they were most stimulating for both cellular lysis and virus adsorption at concentrations ranging from 0.005 M. to 0.05 M.. Krueger and Strietman (1938), Scribner and Krueger (1937) and Gest (1943) also studied the effect of salts on the bacterium-virus reaction. Hershey, Kalmanson and Bronfenbrenner (1944) not only found that NaCl increased adsorption of the virus, but also stated that when infectivity was low and small plaques were produced, a very small increase in the salt concentration increased the size of the plaques. As shown in the experimental results of the present investigation, increased salt concentration has been observed also to increase the plaque size of the pruniphages. Here, however, the plaques increased tremendously over a range up to a limit of about 3% salt. It is suspected that the action of salt in this case is not greatly influencing the adsorption of the phage, but is merely slowing down the rate of growth of the host cells in the increased salt concentrations. This idea is supported by the fact that Xanthomonas pruni respires at a slower rate in 3% salt than in 0.5% salt in the medium and will produce more cells in the lower concentration of salt than in the higher in a given length of time (Goucher and Mandell, 1951).

Effect of temperature. The effect of temperature of incubation on the plaques produced by pruniphages was to decrease the size. This is in agreement with the work of Anderson (1948) and Kleczkowska (1945). Kleczkowska (1945) did not observe the formation of plaques with Rhizobium bacteriophage at temperatures above 30° C. This conforms very closely with the present observations on pruniphage. The reason for the diminished size of plaques

with increased temperature and lack of plaque formation above 30-33° C. is obscure since it is well below the inactivation temperature of, at least, the pruniphage (Crowmover, 1950), and the host organism grew well at this temperature. This phenomenon may be explained in part by the increased rate of growth of the host organism at the higher temperature, since plaque formation has been reported to be a function of the time of division of the uninfected cells (Anderson, 1948).

Plaque types. The separation of distinct strains of bacteriophage on the basis of plaque characteristics has been used by a number of workers. Levine (1938) reported the separation of two Shigella phages by plaque formation and specific adsorption. Kleczowska (1946) reported the separation of a large and a small plaque-producing strain of Rhizobium phage. Hershey (1945), working with coliphage PC, noticed that 1% of the halo plaques produced on 0.6% agar had primary lytic spots in the halo. From these spots he obtained a different phage which showed interference phenomena between adjacent plaques of the two types which took the form of suppression of lysis in the area receiving phage of both types.

The outstanding example of the separation of strains of bacteriophages is the T-series of coliphages. These phages were separated by their reaction on indicator hosts and by the appearance of the plaques (Demerec and Fano, 1945). This series consists of seven distinct phages which have been studied in great detail by numerous workers.

With the pruniphages, five different plaque types were chosen as described in a previous section of this report. Investigations have been limited to these five types; however, it should be noted that a second type has been observed in the XP5 plaque suspensions. It has been isolated and will be called XP6. It is not known whether XP6 is a contaminant or arose from XP5 by mutation, but it appears to be different from the other five phages and will be

studied further. Recently, another phage active against Xanthomonas pruni was isolated from a rabbit fecal sample and when additional data are obtained for it, it may become XP7.

It should be mentioned that an interesting sidelight during the process of selection of the different plaques of pruniphage was the observation that the lytic areas of plaques, stained directly with methylene blue, appeared reddish and the background of growth, a bluish green. This phenomenon was also reported by Hadley (1924) who attempts to explain the difference in color to increased alkalinity at the lytic sites from the alkaline debries from lysed bacterial cells. This does not quite seem reasonable, yet no other reference to this phenomenon has been found in the literature and no explanation is offered.

Standard plating technique. In the course of experiments, certain difficulties were encountered which required modification of standard procedures. One of these concerned the fact that Xanthomonas pruni produced a gummy material and tended to grow in a ropy manner (Eisenstark and Thornberry, 1950), thus making test-tube cultures a difficult procedure. The technique described above eliminated this difficulty and afforded several advantages. It assured a uniform suspension in all the tubes. The volume in the tubes was accurate for dilution since it was not subject to autoclaving and storage losses before use. Finally, chance of contamination ruining the plates was diminished because the culture was dispensed after growth had taken place. The purpose of the 1.5 ml. of 2% agar being added to 4.5 ml. of broth was to obtain the smallest workable volume of culture medium in which the growth took place--a volume which would distribute itself evenly in a thin film over the sublayer and which contains only 0.5% agar final concentration. This concentration was chosen from the results of the experiment on the effect of agar concentration. The standard medium contained 0.5% salt, since this concentration was the one

which best showed up the differences in plaque morphology between the five phage types in the experiment on the effect of salt concentration. The age and size of inoculum were chosen from the results of the experiments on the effect of these two factors, and finally, the plates were incubated at 27° C. for best plaque differentiation.

4. Conclusions

1. Plaques of Xanthomonas pruniphage are best obtained with detailed plaque morphology by the use of a modified plating technique which includes the use of a small inoculum of young cells, a thin sublayered film of 0.5% agar concentration for growth in a medium containing 0.5% salt in plates incubated at 27° C.

2. Five strains of X. pruni bacteriophage have been established on the basis of distinct differences in their plaque morphology.

B. Indicator Hosts

Differentiation of phage types may be accomplished in several ways. The use of plaque morphology for this purpose has just been discussed. Another means of differentiation is the isolation of variants of the original host organism which are susceptible to the lytic action of some of the bacteriophage variants, but resistant to others. The search for and establishment of such variant strains of Xanthomonas pruni are described in this section.

1. Materials and methods.

Obtaining possible host variants. Four different procedures were used in attempts to establish host variants. First, single plaques of the phages were beaten in nutrient broth and incubated until overgrowth occurred. Usually, a day or two was required. Samples of this overgrowth were then streaked out to obtain colonies. In the second procedure, a suspension of the host organism was plated out with 0.5 ml. of high titer phage according to the plating procedure described under "Plaque Morphology". Third, a mixed sus-

pension of phage and host was smeared over the surface of nutrient agar plates. The fourth procedure involved streaking of the overgrowth from a lysed culture to obtain colonies.

Colonies from all four procedures were then subcultured on slants.

Testing of possible host variants. Once cultures of possible host variants were obtained, they were tested by the spot inoculation technique. This technique involved pouring layered plates without any virus according to the routine procedure previously described, except that in this case, the 4.5 ml. of host suspension was prepared by emulsifying some growth from a slant of the host to be tested in 4.5 ml. of sterile broth. Small drops of each of the five phage suspensions were then placed on the semisolid surface. Growth or lack of growth on the spots were interpreted as resistance or susceptibility to the corresponding phage. (See Figs. 16, 17, 18, and 19)

2. Results

The overgrowth from suspended plaques yielded only strains of the host organism resistant to all five phages, while growth from a mixed suspension of host and phage smeared on the plate proved to contain only susceptible hosts. Cultures obtained from overgrowth of poured plates and lysed cultures, however, showed a remarkable range of quantitative differences in resistance to the five phages. Examples of two such cultures showing partial overgrowth of some of the spots are shown in Figs. 18 and 19. From a total of 220 cultures tested, there was one obtained which was susceptible to XP5 and resistant to the other four phages. This variant is illustrated in Fig. 17, as compared to a completely susceptible host strain in Fig. 16.

3. Discussion

Use of indicator hosts. A set of indicator host strains serves as an excellent tool to the geneticist for studies of mutation in both bacteria and viruses, and to the virologist for studies of virus-host interaction. Such

a set of indicator hosts has been established by Demerec and Fano (1945) for Escherichia coli, and has been used for the study of mutation of the bacterial host. A review of the particular advantages of this experimental tool is also to be found in their paper. Delbruck (1946) has described the usefulness of the indicator host-virus system for studying the host-virus relationship.

Obtaining host variants. When a culture of a susceptible bacterium is attacked by the homologous active phage, the culture clears as lysis of the cells occurs, and then after a time the culture becomes turbid again with growth of resistant organisms (Luria and Delbruck, 1943). It was first believed that the growth of the resistant bacteria in a lysed culture was induced by direction action of the phage (d'Herelle, 1926). Since then, the idea has been proposed that bacterial variants occurred in the culture by spontaneous mutation before the virus was added and the virus merely served to eliminate the sensitive organisms (Gratia, 1921 and Burnet, 1929). This hypothesis has been supported by more recent experimental data (Luria and Delbruck, 1943). A detailed analysis of the possibilities of how resistant variants may have arisen is present in the same paper. This lysis-resistant overgrowth sequence seems to be well established in the case of coliphages. Kleczcowska (1945) working with Rhizobium bacteriophage, however, reported two kinds of overgrowth after lysis of a culture. One type of overgrowth described was especially interesting, since it consisted of clones of sensitive host cells growing in the presence of excess active phage. This same phenomenon has been observed with the pruniphages, both on an agar surface and in liquid culture, which may account for the paucity of indicator hosts obtained. Kleczkowska (1945) explained this growth of susceptible bacteria in an excess of phage by demonstrating that a 14 day lysed culture contained a substance which prevented phage interaction with the bacteria. There was no such sub-

stance found in a 14 day culture to which no virus had been added. It is possible that a similar situation exists with the pruniphages. Extracts of susceptible cultures of staphylococci which would inhibit bacteriophage has also been reported by Rakieten, Rakieten and Doff (1936).

Bronfenbrenner and Korb (1925) reported that the presence of resistant bacteria merely clouded plaques with overgrowth and did not affect the size or count. If this fact may be applied to the clearing of spot inoculations as well as plaques, then the quantitative differences of the strains of X. pruni reported above may be explained by the presence of varying proportions of resistant bacteria in the host populations. This in turn may be attributed to a high mutation rate of the susceptible host culture which would cause a different proportion of the population to be resistant.

4. Conclusion.

One indicator host was obtained which was susceptible to XP5 and resistant to the other four phages. This host served to show that XP5, at least, was different from the other four phages. Also, hosts showing a range of quantitative differences in resistance to the five phages were obtained.

C. Serology

1. Materials and methods.

Preparation of antisera. Five rabbits were injected with centrifuged plaque suspensions of individual plaques intravenously. The injections were given at intervals of 2 and 3 days over a period of 5 weeks with increasing doses of virus centrifugate ranging from 0.5 ml. to 3.5 ml. A week after the final injection the rabbits were bled, the serum separated, sterilized by filtration and stored in the refrigerator.

Determination of serum constants. The protocol used in determining the inactivation constants of the antisera was essentially that employed by Adams (1950), the only difference being in the plating technique employed. The

stock virus suspensions were diluted to yield 1000 plaques when 0.5 ml. was plated according to the method already described for plating of the pruniphages.

2. Results.

The experimental data of serum constants of phage inactivation are presented in Chart 2. This chart, while incomplete, is evidence implying serological differences among the five phages.

3. Discussion

Originally, five antisera were prepared, one against each of the five phages, XP1-XP5. As mentioned in a previous section of this report, XP5 was observed to contain another plaque-type variant now isolated and called XP6. Unfortunately, it is suspected that the rabbit supposedly immunized against XP5 was actually injected with a mixture of XP5 and XP6, since the injections were completed before XP5 and XP6 were separated. Therefore, the data concerning antiserum against XP5 and/or XP6 may not be significant.

Bacterial viruses, like most plant and animal viruses, will effect the production of antibodies in the blood of experimental animals when injected over a period of time (Adams, 1950, and Wahl and Lewi, 1939). The antiserum from different bacteriophages has been studied by many investigators and has been used to advantage in several ways. Immune serum can be used to eliminate free phage in studies of virus-host interaction, since adsorbed and intracellular virus is unsusceptible to antiserum action (Delbruck, 1945). It has been used to group and separate various bacteriophages (Rountree, 1949, and Sertic and Boulgakov, 1935). Clifton, Mueller and Rogers (1935) have studied the effect of antiserum on lysis. The theory of the action of antiserum on phage, the factors influencing the phage-antiphage reaction, and the reactivation of neutralized phage by enzyme action have been discussed by Hershey (1941), Kalmanson, et al. (1942), and Kalmanson and Bronfenbrenner (1942). Andrewes and Elford (1933) have reported that a given strength of antiserum will neutralize

over a wide range only a definite percentage of phage, irrespective of the quantity of phage present. This phenomenon they have termed the "percentage law." Additional information about this law is to be found in a second paper by Andrewes and Elford (1933a) and one by Hershey, et al. (1943).

Considerable correlation has been noted between serological groupings and groupings based upon other properties, such as particle size and plaque morphology. However, in some cases, phages which were separable by other means were antigenically indistinguishable (Hershey, 1943, and Luria, 1945). Phages may be considered distinctly different, then, if they are serologically different, but no conclusion can be drawn if they are serologically identical. The relatedness or unrelatedness of phages can be deduced by a consideration of their rates of inactivation with an antiserum prepared against one of them as measured by their serum constant (Adams, 1950 and Hershey, et al., 1943). The more closely two viruses are related serologically the closer will their serum constants agree (Adams, 1950).

Unfortunately, the experimental data obtained were not adequate to establish identity of each phage type. However, the data indicate that perhaps an additional determination might reveal significant results. The reaction constants obtained, however, seem to show that there definitely were differences in the rate of inactivation of the five pruniphages with each antiserum. Anti XP1 serum had the highest serum constant with XP1, which was to be expected. XP4 and XP5 were inactivated at the same rate by anti XP5 and the value of XP2 with anti XP2 serum was much lower than would be expected. These determinations, although far from conclusive, suggest that a repeated determination might reveal significant results.

4. Conclusions.

No definite statements can be made from the data available, but the preliminary serum constant values obtained show differences in the rate of inactivation of the five pruniphages by an antiserum prepared against one of

them. These differences in rate of inactivation may indicate serological differences among the five phages. The phages, however, appear to represent a closely related group serologically.

D. Burst Time

1. Materials and methods.

The procedure used in determination of the burst times of the five pruniphages was adapted from that described by Crownover (1950). The virus-host mixture was assayed at 30 minute intervals over a period of 3 hours. At each time interval, assay was made of the total phage, i.e., adsorbed phage plus free phage, and also of the free phage alone by centrifuging down the host cells. The plates were poured according to the routine plating procedure already described.

2. Results

The burst time curves of the five pruniphages studied are shown in Chart 3. It may be seen in this chart that, while there are definite rises in all the curves between one and two hours, no clear-cut jumps indicating initial bursts are discernible.

3. Discussion

The process of the multiplication of bacterial viruses may be considered as a cycle of stepwise events. There are three main steps recognized in this cycle as follows: (1) adsorption, the attack of the virus on the susceptible bacterial host, probably involving chance collisions to bring together the phage and bacterial cell, (2) the latent state in which the virus is replicated inside the cell. This phase ends with burst of the bacterial cell and liberation of newly formed phage particles, (3) the dormant free form of the virus persisting between contacts with the host cells (Anderson, 1946, Horsfall, 1949 and Price, 1948).

The time required for adsorption plus the time for virus replication

inside the cell is known as the burst time. Every virus strain lyses the cell at its own characteristic time. This time, at which the virus is liberated, is called the minimum latent period (Price, 1948) and is independent of the multiplicity of infection (Delbruck, 1946). Each member of the T-series of coliphages, in addition to producing different types of plaques and exhibiting differences in infectivity of indicator hosts, has a distinct burst time which is different from the others (Ellis and Delbruck, 1939, Delbruck, 1940, 1946). It was expected that the pruniphages might also be separated by differences in their burst times. The knowledge of the approximate burst time of the phages is also of considerable value in experiments involving quantitative assay of the phage present in a sample at a given time. The assay procedure must obviously not take longer to perform than the burst time of the virus if a significant determination is to be made.

The curves in Chart 3 show definite rises in number of phage present for all five phages between one and two hours. This is in agreement with Crowner (1950) who reported a burst time between 1.5 and 2 hours. Chapman et al. (1951), working with the bacteriophage of Erwinia carotovora, an organism related to Xanthomonas pruni, also reports a burst time of about 1.5 hours. In the case of pruniphage, it is suspected that assay of the host-virus mixture at shorter time intervals would reveal more precise burst times. It may be observed from the chart that the curves for XP1, XP4 and XP5 first start to rise sharply after one hour while the curves for XP2 and XP3 do not start to rise sharply until after two hours. This is an indication that the five phages might be separated into at least two groups.

4. Conclusion

The burst time data for the pruniphages indicate that bursts occur for all of them somewhere between one and two hours. While differentiation of the phage types by burst time is suspected from the information available, no definite separation can be made without additional experimental data.

III. SUMMARY

Bacterial viruses have become convenient tools for the study of several biological phenomena. The study of bacteriophage has broadened to the point where investigations of phage-host systems other than Escherichia coli might be profitable; therefore, this investigation was undertaken. This report is concerned with the isolation of different strains of bacterial viruses, all active against Xanthomonas pruni, and establishment of the identity of each virus by means of plaque morphology, indicator hosts, serology and burst times.

A standard plating technique for the pruniphages has been worked out and described. The effects of age of culture and size of inoculum, agar concentration, salt concentration, and temperature were determined. It was concluded that plaques of Xanthomonas pruniphage are best obtained with detailed plaque morphology by the use of a modified plating technique which included the use of a small inoculum of young cells, a thin sublayered film of 0.5% agar concentration, 0.5% NaCl in the medium, and incubation at 27° C. Five strains of X. pruni bacteriophage were established on the basis of distinct differences in their plaque morphology.

The isolation of host variants susceptible to action of some bacteriophage types, but resistant to others, is another means of differentiation of phage variants. The procedure for obtaining and testing of possible host variants is described. An indicator host susceptible to XP5 and resistant to the other four phages was obtained. In addition, hosts showing a range of quantitative differences in resistance to the five phages were obtained.

Antisera were prepared against each of the five phages and were used to test for serological relationships between them. Preliminary serum constant values obtained show differences in the rate of inactivation by an antiserum prepared against one of them, but they appear to represent a closely related serological group.

Since every virus strain lyses the host cell at its own characteristic time, it was expected that the pruniphages could be differentiated by this means. Burst time data obtained indicate that all five pruniphages have a burst time between one and two hours. Differences in burst time are suspected from the data, but no definite separation can be made without additional experimentation.

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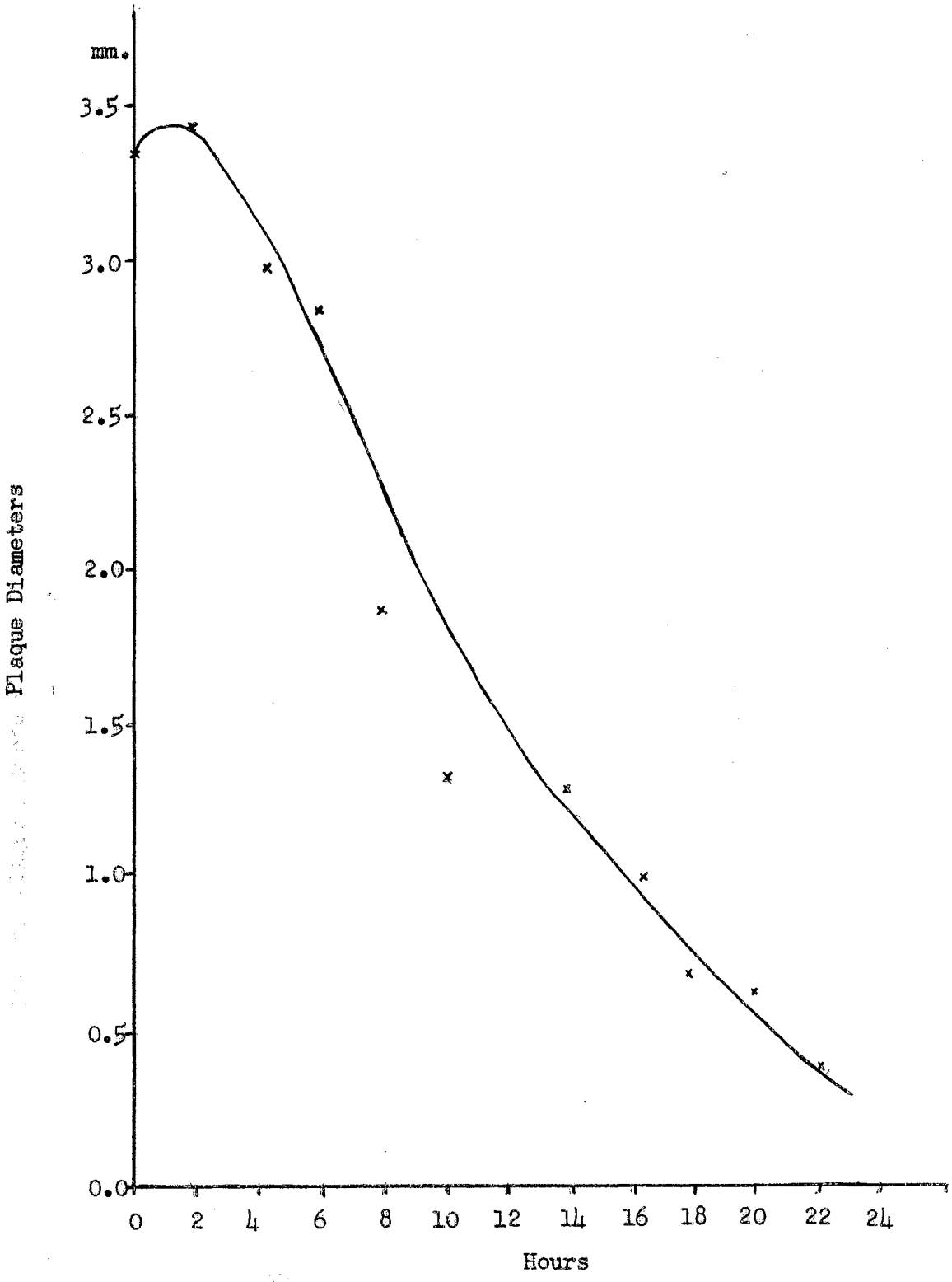


Chart 1 -- Effect of Age of Culture on Plaque Size

Chart 2. Serum Constants of Phage Inactivation

	XP1	XP2	XP3	XP4	XP5
Anti XP1	25.4	17.9	18.1	11.3	- -
Anti XP2	19.1	12.8	18.7	12.7	- -
Anti XP3	- -	- -	16.1	16.6	- -
Anti XP4	- -	- -	- -	16.0	- -
Anti XP5	30.8	24.0	50.6	59.7	59.7

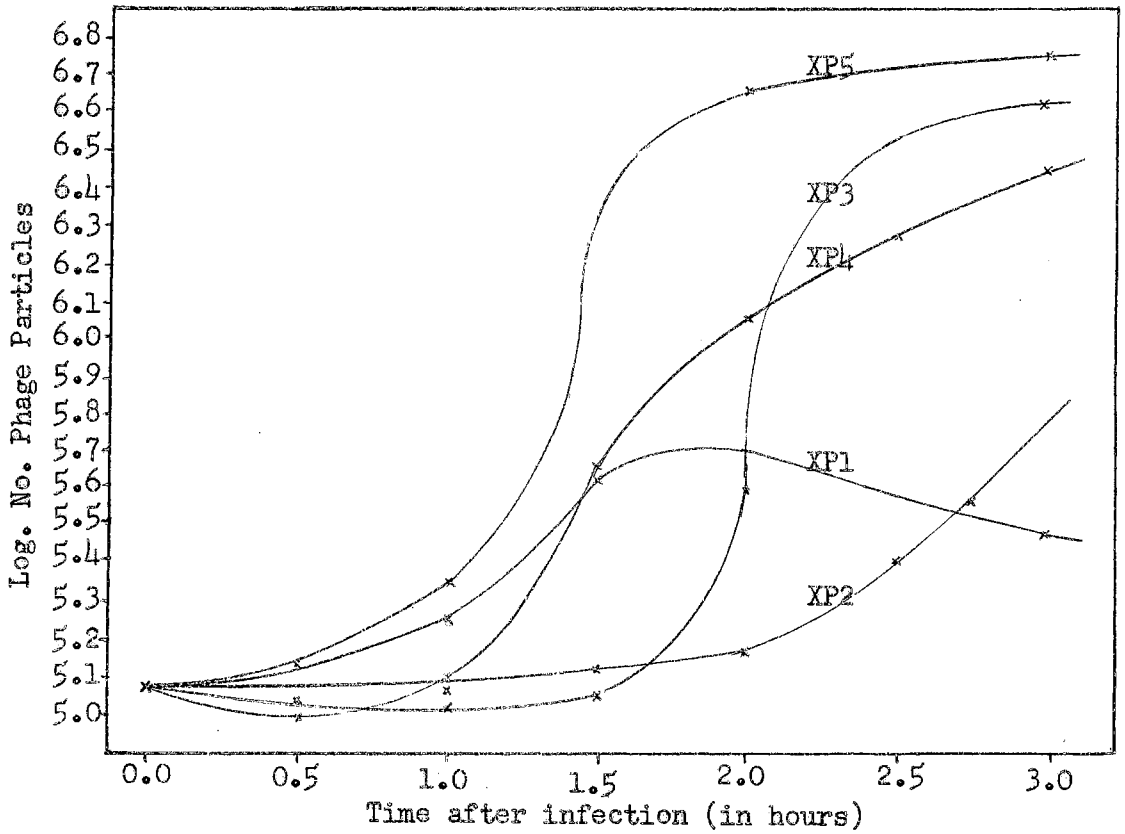


Chart 3. Pruniphage Burst Time Curves

Figs. 1,2,3 Plaque morphology of XP1.

Figs. 4,5,6 Plaque morphology of XP2.

Figs. 7,8,9 Plaque morphology of XP3.

Figs. 10,11,12 Plaque morphology of XP4.

Figs. 13,14,15 Plaque morphology of XP5.

Figs. 1,4,7,10,13 are diagrammatic representations of plaque morphology as described in the text.

Figs. 2,5,8,11,14 are single, enlarged plaques.

Figs. 3,6,9,12,15 are sections of petri dishes which show the plaques.

All photographs were made as positive prints by placing the petri plates themselves in the enlarger.

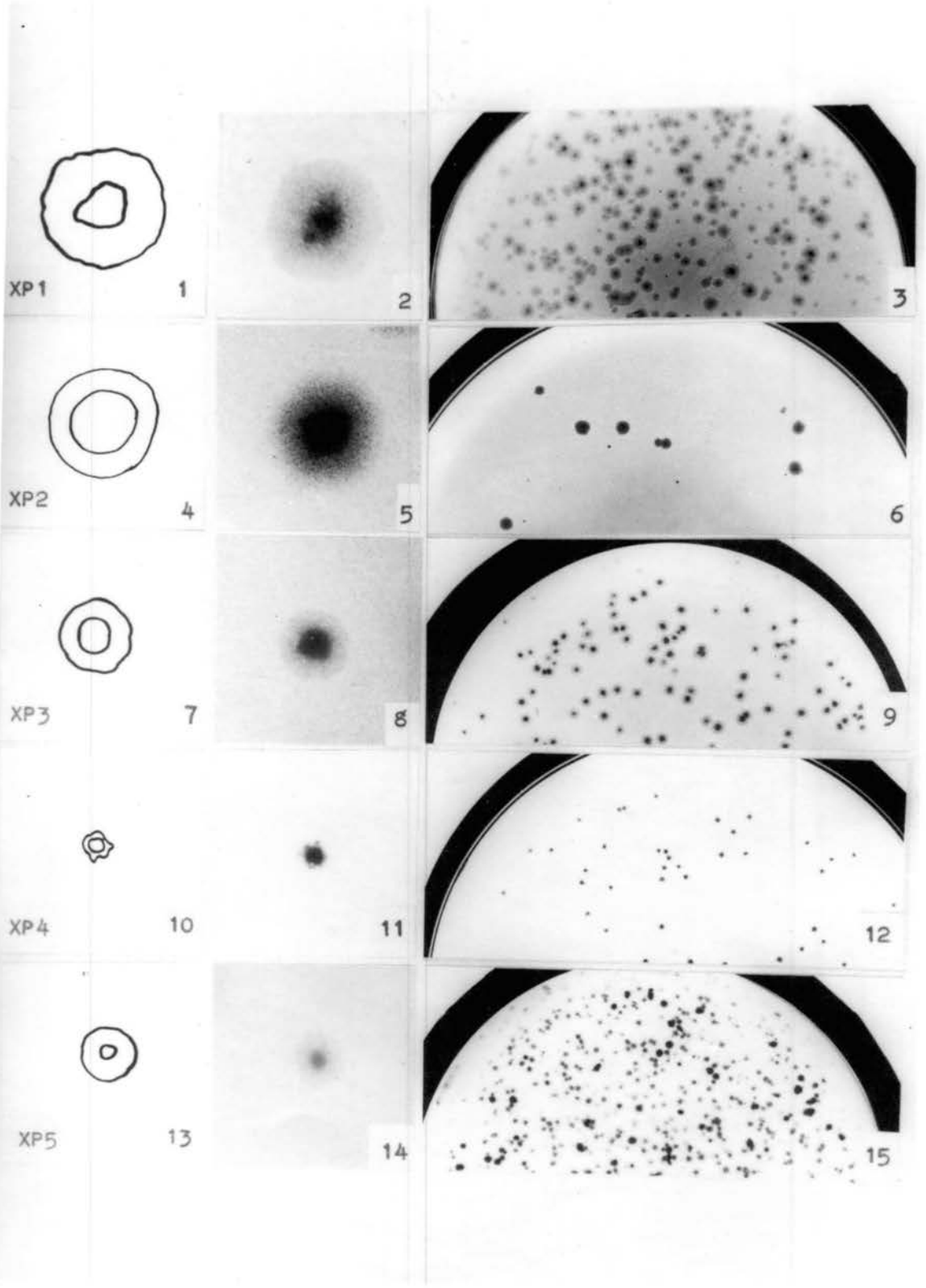
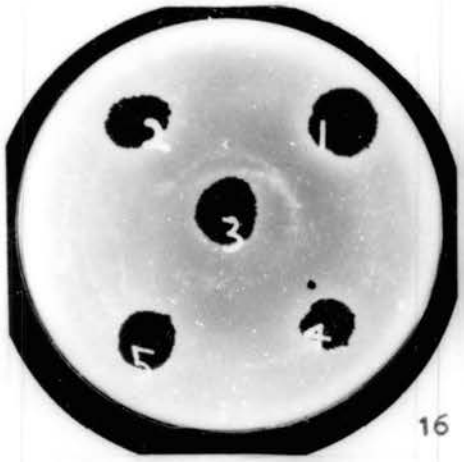

XP1
1
2
3
XP2
4
5
6
XP3
7
8
9
XP4
10
11
12
XP5
13
14
15

Fig. 16 Spot inoculation of XP1-XP5 on a completely susceptible host.

Fig. 17 Spot inoculation of XP1-XP5 on an indicator host, as described in the text.

Figs. 18,19 Hosts showing quantitative differences in resistance to the five pruniphages.



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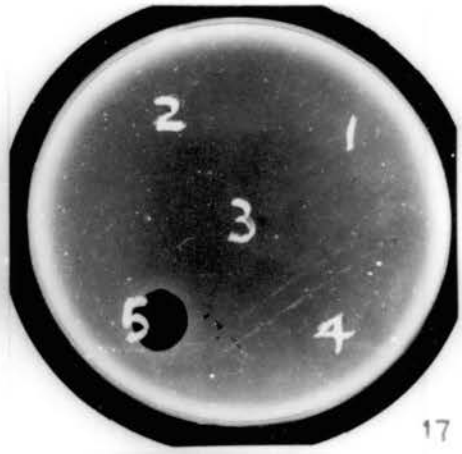

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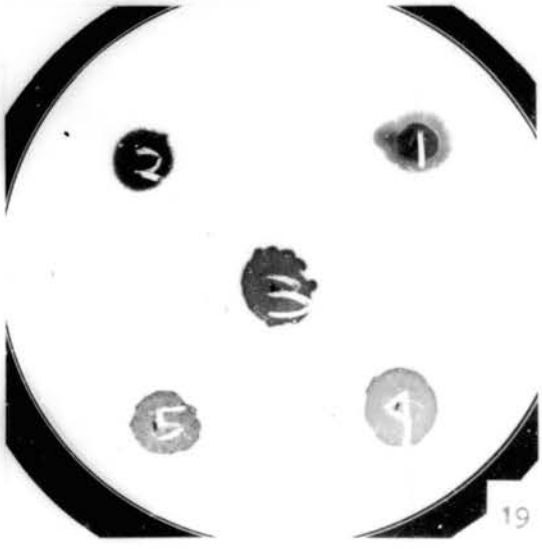

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Fig. 20 XP4 plaques produced in presence of 0.5% NaCl.

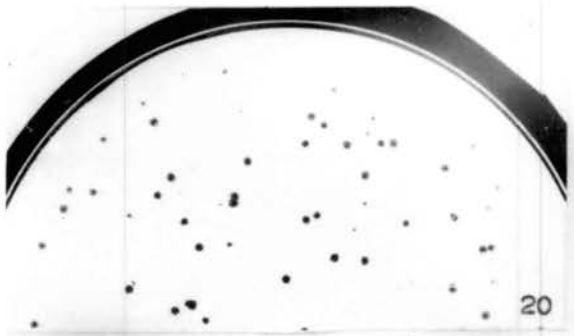
Fig. 21 XP4 plaques produced in presence of 3.0% NaCl.

Fig. 22 XP3 plaques produced with 0.5 ml. turbid host suspension.

Fig. 23 XP3 plaques produced with 0.05 ml. turbid host suspension.

Fig. 24 XP4 plaques incubated at 30 - 33° C.

Fig. 25 XP4 plaques incubated at 25° C.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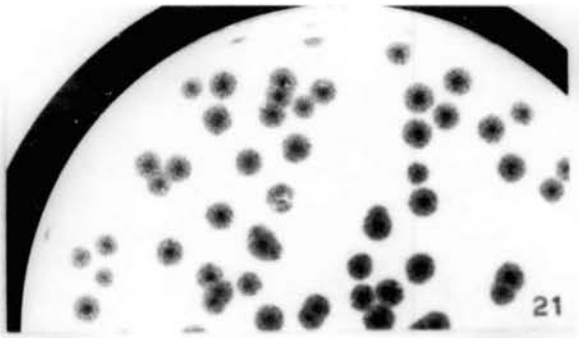
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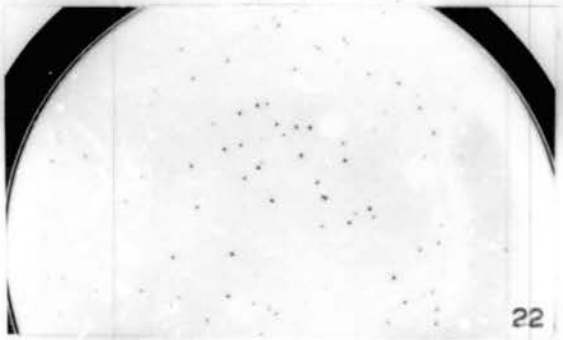
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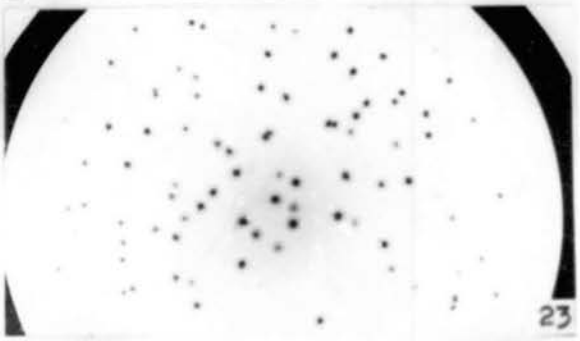
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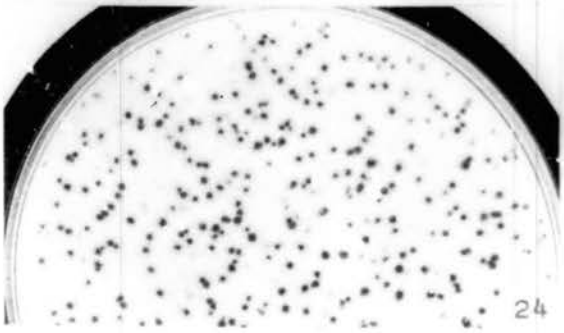
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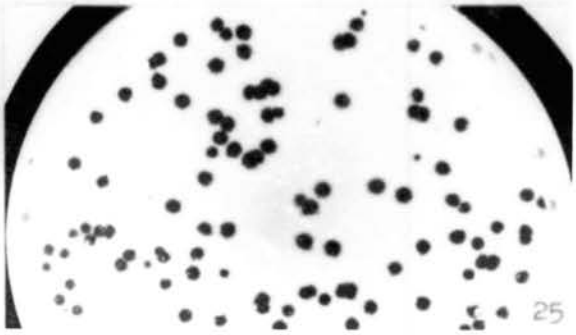
25

TYPIST PAGE

THESIS TITLE: Studies on Bacterial Viruses Active Against *Xanthomonas pruni*

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