THE DIFFERENTIATION OF BOVINE STAPHYLOCOCCI BY BACTERIOPHAGE

by

PING HANG FUNG, B.Sc.

THE UNIVERSITY OF MANITOBA

A Thesis

submitted to

The Faculty of Graduate Studies and Research

The University of Manitoba

In partial fulfilment

of the requirements for the degree

of

Master of Science

1960

ACKNOWLEDGMENTS

The author is pleased to express his thanks to the late Dr. T.M.B. Payne, Professor and Chairman of the Department of Microbiology, under whose direction this study was carried out.

Grateful acknowledgment is likewise expressed to the Provincial Laboratory of Animal Pathology under the direction of Dr. J. Isa, for sympathetic encouragement and advice.

Finally sincere appreciation is extended to Dr. Norman James, Professor Emeritus of Microbiology, for his advice and assistance in the preparation of the manuscript.

THE DIFFERENTIATION OF BOVINE STAPHYLOCOCCI BY BACTERIOPHAGE

By Ping Hang Fung

ABSTRACT

A preliminary study was carried out on the optimum time for incubating phages produced by staphylococci. This was done by comparing the growth of each of several strains of Staphylococcus with the growth of the same strain plus its homologous phage in trypticase soy broth at 37°C at 15-minute intervals for nine hours, with an additional final colorimetric reading at 22 hours. A titer of the concentration of phage particles in the lysed culture was made at one hour and thereafter at half-hour intervals until the reading on the colorimeter remained stationary for one hour. The optimum time proved to be different for different groups of phages. In one group the titer remained at a high level overnight; in a second the titer was lowered appreciably; and in a third group the titer was practically lost. On the basis of this finding the time of incubation in the studies reported hereafter was varied from 2 to 6 hours, the time in each case depending on the reaction with the individual phage.

Three hundred and twenty-five isolates were obtained from udders of cows in nine areas adjacent to Winnipeg. Of these 166 were typable by using the 21 phages in the basic set together with six others. Most of the typable isolates were susceptible to phage 81. The majority of these isolates were susceptible also to one or more of four other phages--42B, 47B, 80 and 82. One phage-type was predominant in each area, but different predominant phage-types occurred in different areas. The majority of the isolates could not be grouped on the basis of the basic set alone.

Seven of 45 isolates obtained from pooled (cans) milk in one of these areas were typable. Two of the types from the pooled milk were the same as two of the 18 types obtained from individual cows in that area. Two other types were different from the types isolated from the milk of individual cows. In a second area investigated none of the isolates from the pooled milk was typable.

One of the seven isolates from bulk (tank) milk obtained from the latter two areas was typable. This type was the same as the predominant type isolated from herds in the first of the two areas.

In trials with 30 bottles of milk staphylococci were not found.

TABLE OF CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

INTRODUCTION

INTRODUCTION

Staphylococci are widespread. Their presence in air and in the immediate environment of man is common. Under normal conditions they may be present in the nose, the throat or on the skin of the human and certain domestic animals. Due to their high adaptability staphylococcal infections have become of increasing concern, particularly during the past decade. They can adjust themselves to new environments such as human tissue bathed in antimicrobials, and most pathogenic strains presently predominant are antibiotic-resistant (16).

Of the acute infections in man staphylococcal infections present the most urgent need for basic treatment, especially following operations (2). This need results from the fact that penicillin-resistant strains have steadily risen from 50 to 80% of the strains currently carried by selected groups of individuals within hospitals, and to a lesser extent in the population at large (16). Staphylococcal

infections are endemic in the general population, since outbreaks have been reported repeatedly (2,19).

Immunization against staphylococcal diseases has not proven promising. Further, the development of strains resistant to antibiotics and drugs, currently accepted as effective, limits optimism that a permanent effective antibiotic or synthetic drug will appear in the immediate future. Therefore, control remains of primary importance. A prime requisite in this control is the ability to differentiate pathogenic from non-pathogenic strains.

The heterogeneity of this group of micro-organisms confounds both the medical man and the biologist. In the medical field, a narrow view widely held is based on the dogma of the fixity of bacterial species, allowing plasticity within the species as regards virulence but failing to recognize variation due to genetic instability. The biologist, caring little about disease, aims to fit a species into a phylogenetical scheme and is bedevilled by the overlapping of characters such as coagulase reaction, toxin production,

pigment formation and tolerance to high salt concentration. However, methods of differentiating strains on the basis of one or more of these characters have not proven completely satisfactory (22). Bacteriophage typing appears to be stable and consistent. It provides an arbitrary method for the segregation of strains. The staphylococci that cause an epidemic in one area always fall into one phage pattern (17,19).

This presentation deals with an attempt to establish types of staphylococci by the bacteriophage method. It involves the flora in milk aseptically drawn from the cow and in milk at various stages of handling. The finding at a stage of handling of a type not present in the aseptically drawn sample could provide a basis for measuring sanitation in the handling of milk, since the new type could represent contamination. Conceivably, as an alternate probability, the new type could represent a genetical variant.

HISTORICAL

HISTORICAL

The first investigation involving results that seemed to suggest that strains of staphylococci could be divided into groups according to their susceptibility to a series of bacteriophages was reported by Epstein and Fejgin (6). These investigators noted that staphylococci from lesions and from other sources did not respond in the same way to the phages studied. Burnet and Lush (3) divided different staphylococcal phages into different groups according to lytic action on different staphylococci. Williams and Timmins (24) attempted to use this phenomenon to differentiate the strains of staphylococci causing acute osteomyelitis.

However, it was Fisk (7,8) who initiated the present practice of phage typing of staphylococci. This investigator demonstrated that lysogenic staphylococci are widespread. He used the cross culture method to obtain different phages from the lysogenic strains of staphylococci and found that the reaction of these phages on different strains of staphylococci is selective. This reaction of a

staphylococcal strain to a given phage is not readily altered by changes in environment. Soon this method of typing proved of value in two small epidemiological studies (9,11).

Except for the addition of new phages and certain modifications in technique, the current methods used in phage typing of staphylococci are based on those used by Wilson and Atkinson (25). These workers demonstrated that phages could be separated from cocci by filtration. The earlier method depended upon inhibition of the cocci by the quaternary ammonium disinfectant, zephiran (7). They isolated 18 phages and devised a method for routine testing of different types of staphylococci on the basis of phage pattern. They divided their typable staphylococci into 21 types.

A comprehensive review of the methods currently in use has been reported by Williams and Rippon (23).

The main use of phages from human staphylococci has been in tracing the sources of epidemics of infections in man, but their use has been extended to the typing of staphylococci of animal origin.

MacDonald (12) was the first to report the

typing of staphylococci of bovine origin by phages. This investigator, working with phages obtained from Wilson and Atkinson, reported that the majority of the coagulase positive strains isolated from 280 samples of accredited milk, and all the 34 strains from bovine mastitis, were fully susceptible to phage 42D.

Smith (20), noting the value of the phage-typing in the investigation of outbreaks of staphylococcal infections in human subjects, attempted to ascertain whether the method of Wilson and Atkinson (25) might be of value in the epidemiological study of staphylococcal infections in animals. This investigator obtained 21 phages from G. S. Wilson, and isolated 11 additional phages. He reported that 25 of these could be grouped, as follows:

3A Group: Phages 3A, 3B, 3C and 51.
6 Group: Phages 6, 7, 42B, 47 and 47C.
29 Group: Phages 29, 29A, 31, 42C, 44, 52 and 52A.
42D Group: Phages 42D, E174/16, 14/94, 1363/14, 129/16, 30/16 and P42D/E193.
88 Group: Phages 88 and 88A.

He showed that 93.3% of 1016 isolates obtained from

milk were typable by this grouping, whereas the remaining 6.7% proved to be susceptible to the 32 test phages. Over 83% were susceptible to one or more of the 42D group, and 9.5% to one or more of the 29 group. He divided the typable staphylococci from bovinemilk into 25 types. Strains isolated from the milk of cows suffering from acute or chronic mastitis did not differ in phage type from the strains isolated from the milk of cows with apparently normal udders. One phage type was predominant in the strains of staphylococci examined from one herd but in other herds the predominant types were different. Different phage types in different quarters of the same cow were not unusual but strains isolated from one quarter were generally of the same type, with few exceptions.

With a larger number of phages, Smith (20) typed a greater percentage of strains of staphylococci than did MacDonald (12) who used only the phages obtained from Wilson and Atkinson. Further, the percentage of strains fully susceptible to 42D alone (now allocated to Group IV) was not as large as that reported by MacDonald (12).

Price et al (14) studied the phage types of

staphylococci isolated from cows in England. These investigators divided most of the 896 isolates from 276 quarters of 15 herds into 12 fairly characteristic patterns when phages at routine test dilutions were used. These were designated by the letters A to M, as follows;

Code Letter	Phage susceptibility pattern determined with phages at routine test dilution
A	42D, 7/42D/44/44A, and other combinations of these four phages
B	Numerous phages of groups 1 and 111
C	Lysed by one or more phages of group 1 and also by 42B
D	7 or 7/52A +
E	3C/7/29/42B/52/52A/54 (and 7/52/54+)
F	7/42B/54 +

Code Letter	Phage susceptibility pattern determined with phages at routine test dilution
H	47A
I	42C/42D/44/44A/54+
J	54+
K	6/47/53/54+
L	6/7/47/53/54+
M	53/54

Three hundred and thirty nine colonies fell into pattern C; 183 into A; and 181 into L. In heavily infected herds infections were due to more than one phage type. Two phage types obtained from one quarter were uncommon, though different types from different quarters of the same cow were encountered quite frequently. This finding was in agreement with that obtained by Smith (20).

Barnum and Fuller (1) used the bacteriophage typing of staphylococci initiated by Smith (20) to

differentiate pathogenic from non-pathogenic strains in milk, along with other characteristics of the strains, in studies on the epidemiology and the efficacy of treatment and control measures.

These investigators isolated phages from 42 lysogenic cultures of staphylococci. Using these phages, they tested 648 isolates of staphylococci from milk, and found that most of the isolates belonged to one of five groups. One hundred and ninety five belonged to Group A; 179 to Group A_1; 76 to Group B; 45 to Group B_1; 17 to Group B_2; 123 were not susceptible to any of the 42 phages; and 13 were unclassifiable by these phages. Further, this method of phage typing did not distinguish between strains isolated from healthy and diseased udders. This result confirmed the finding of Smith (20). However, it did afford definite evidence that cows served as carriers of strains that could cause infection in the mammary gland. One phage group of staphylococci was predominant in one herd and other groups in different herds. This is in agreement with the results obtained by other workers (14).

Edwards (4) found that most strains from 35 cows of one herd were lysed by phage 42D at routine test dilution, but also were lysed by stronger concentrations

of phages 54, 77 and 79. Later this investigator working with Rippon (5) reported similar findings, in this case stronger concentrations of a larger number of phages proving to be lytic. They divided the pathogenic staphylococci isolated from these herds into five types, as follows:

Type	Diluted Phage	Phage Pattern undiluted
I	42D	29/52/79/42D/54/77
Ia	42D	42D/6/7/42E/47
2	—	3A/3B/3C/71
3	52B	7/75/77/52B
4	52A	52/52A
5	78	54/76/77/78

All the above types were found in cases of clinical mastitis in cows. This finding confirmed the results of Price et al (14) mentioned earlier. These results likewise confirmed those of Smith (20) and Price et al (14) that one type was predominant in each herd but the predominant type in one herd differed from that in

another.

In a study of 57 isolates of staphylococci from milk of a mastitis-affected herd, Rountree et al (17) found that 33 were lysed by phage 42D or by other phages not attacking human strains. Twenty two isolates belonged to phage pattern 29/42D/52.

Thatcher and Simon (21) reported that the predominant type of staphylococci isolated from dairy products in Canada was 42D.

In Wisconsin, Seto and Wilson (18) isolated six staphylococcal phages designated as S1, S2, S3, S4, S5, and S6. The phages were propagated on micrococci of bovine origin. These investigators found that of 379 coagulase positive staphylococcal cultures tested 93% were sensitive to one or more of the above six phages and to phage 42D. All cultures of phage type S2 were sensitive to phage 44A-- this type was reported by McLean (13) to be the predominant type in staphylococci isolated from milk in Australia-- and 80% of the 44A phage type cultures were sensitive to phage S2. Thus, the predominant type of staphylococci of bovine origin isolated in Wisconsin was the same as that in Australia but differed from the predominant type of bovine origin isolated in England (20) and in Canada (21). In the

latter two countries the predominant type was 42D.

In a subsequent study in Wisconsin, Reid and Wilson (15) found the same predominant phage type of staphylococci in cows with acute mastitis, with chronic mastitis and with clinically normal udders as that reported by Seto and Wilson (18). Forty-four per cent of the typable isolates belonged to phage type 44A, and 30% belonged to type 42D. Further, type 44A staphylococci occurred more frequently in udders of acute and chronic mastitis-affected cows than did type 42D. However, the 42D type occurred slightly more frequently in normal udders than did the 44A type. A greater number of strains was phage typable from cows with acute mastitis than from those with chronic mastitis or from those with normal udders.

MATERIAL AND METHODS

MATERIAL AND METHODS

Milk Samples

The milk samples used in this study were collected under the supervision and with the cooperation of Dr. Norman E. Stanger, Assistant Pathologist of the Animal Pathology Laboratory located on the University campus. The first set of samples was obtained aseptically from each of the four quarters of each cow. Before sampling, the udders of the cow were washed with a warm chlorine compound followed by 70% ethanol. The second set was obtained from cans of pooled milk from each herd; the third set from bulk tank which contained the milk from a number of herds; and the fourth from the bottled milk after pasteurization.

These cows were from herds in the vicinity of Winnipeg, namely: St. Vital, Tuxedo, the University of Manitoba, Haywood, Grunthal, Gunton, Kenton, Stonewall, and West Kildonan. Can samples, tank

samples and bottled milk samples were obtained from herds in the first two areas.

Isolation of Staphylococci

Each sample was streaked onto a plate containing bovine blood in tryptose agar base and incubated at 37°C overnight. Typical staphylococcal colonies were isolated on nutrient agar slopes and incubated at 37°C overnight. The cultures from these slopes were then sub-cultured in trypticase soy broth at 37°C for 4-6 hours, at which time they were typed.

Sources of Bacteriophages

The phages used comprised the basic set of 21 recommended by the International Committee on Phage Typing of Staphylococci (10), as follows:

Gr. I. 29, 52, 52A, 79, 80.
Gr. II. 3A, 3B, 3C, 55, 71.
Gr. III. 6, 7, 42E, 47, 53,54, 73, 75, 77.
Gr. IV. 42D.
Misc. 187.

and the following five phages obtained from and by the courtesy of Dr. E. T. Bynoe, Laboratory of Hygiene, Ottawa, Ontario:

42B, 47B, 81, 82, 971.

One additional phage designated phage W was obtained from the Manitoba Provincial Bacteriological Laboratories, Department of Public Health and Welfare, Winnipeg by courtesy of Dr. L. P. Landsdown.

Culturing the Propagating Strains of Staphylococci

Each strain of Staphylococcus from the freeze-dried vial was suspended in 0.5 ml nutrient broth, and sub-cultured on a blood-agar plate and in a tube of broth, and incubated at 37°C overnight. If growth was obtained on the blood-agar plate, a typical staphylococcal colony was picked and streaked on a nutrient agar slope in a screw-capped tube, and stored at 4°C. However, if staphylococci failed to grow on the blood-agar plate, a transfer from the broth culture was streaked on a second blood-agar plate and after incubation at 37°C a discrete colony was cultured on an agar slope. This was incubated at 37°C overnight and stored at 4°C.

The cultures thus obtained were replated on blood-agar plates at three-week intervals and the new isolates cultured at 37°C overnight on nutrient agar slopes and stored at 4°C. Each propagating strain of Staphylococcus was designated by placing PS before the number of the homologous phage. Thus, the propagating strain for phage 3A was labelled PS3A.

For use in propagating a phage, a propagating strain was sub-cultured from the agar slope in trypticase soy broth. This broth culture was discarded after two weeks, at which time a fresh culture in broth was prepared from the agar slope.

Broth Method of Propagating Bacteriophages

A propagating strain of Staphylococcus was sub-cultured in 8 ml trypticase soy broth at 37°C for two hours. One milliliter of the homologous phage was then added and incubation at 30°C continued for 2-6 hours, depending on the phage. Generally a maximum titer was obtained when the broth started to clear up. The lysed culture was centrifuged at 4,000rpm., and the supernatant titered. The method of titering will be given later.

The supernatant was stored at 4°C.

If the supernatant had a titer less than 1/1,000, it was used as phage for repeating the process described immediately above until the supernatant gave a titer of 1/1,000 or more. The supernatant with the required titer was passed through a sintered glass filter (Hysil, grade 5), and the filtrate titered and checked for lytic activity on the other propagating strains. The lytic spectrum will be referred to later.

Plate Method of Propagating Bacteriophages

Freshly prepared trypticase soy agar was poured in 15 cm Petri dishes to a depth of 5 mm. The plates with the lids slightly opened were then dried at 37°C for one hour and with the lids on for 10 hours. Each plate was flooded with one of the overnight trypticase soy broth cultures and allowed to stand at 20°C for five minutes. Excess broth was drawn off with a sterile Pasteur pipette, and the agar surface dried for one half hour with the lid slightly opened. The homologous phage was spread over all but a small segment of

the agar surface. This segment served as a control on the growth of the bacteria. Incubation was either at 37°C for 3-6 hours or at 30°C for 10-15 hours.

Incubation at 30°C generally gave higher titers. However, at either temperature close observation should be carried out at three hours and after to detect overgrowth of lytic areas by secondary colonies. The control segment was then removed with a sterile knife, and the preparation held at -20°C overnight. This was followed by leaving the plate slightly tilted at 20°C to thaw. The fluid expressed on thawing was drawn off with a Pasteur pipette and centrifuged at 4,000 rpm. A portion of the supernatant was titered and the remaining portion stored at 4°C. If a satisfactory titer was obtained (1/1,000 or higher) the supernatant was passed through a sintered glass filter. The filtrate was titered, and checked for lytic activity on other propagating strains. If the supernatant failed to give a satisfactory titer, the process was repeated using the supernatant as phage.

The plate method was found to be essential

for the propagation of phages 29, 52, 52A and 79 and to be superior for phage 187. However, it was much more convenient in terms of labour and equipment to propagate the others by the broth method, which method was used for the propagation of the larger group.

Optimum Incubation Time for Phage Production

During the propagation of phages, it was observed that time of incubation was important in obtaining high titer. Over incubation very often gave an unsatisfactory titer (less than 1/1,000). The following experiment was set up in an attempt to find the optimum time of incubation.

Two test tubes of trypticase soy broth were sub-cultured with a propagating strain of Staphylococcus in the same way as in the propagation of phage by the broth method. To one tube 0.5ml of homologous phage suspension having a titer of 10 was added. The other tube served as a control on the growth of the propagating strain. These cultures were incubated in a water bath at 37°C. To test for growth and lysis, readings at 15-minute intervals on the percent light transmittance

with a Beckman colorimeter (Model C) were taken. One hour after the phage had been added, an aliquot was taken from the tube containing phage and the aliquot centrifuged at 4,000 rpm and titered. Thereafter, aliquots were taken every half hour, and the aliquots centrifuged and titered until the percent transmittance remained stationary or decreased steadily for one hour. The remaining portion of the culture containing the phage was then incubated at 37°C overnight. After incubation, it was centrifuged and the supernatant titered.

Titration of Phage-Containing Broth

Serial ten-fold dilutions of the filtrate were prepared. Freshly prepared trypticase soy agar was poured in Petri dishes and dried at 37°C overnight. Each plate was then flooded with a six-hour culture of the homologous propagating strain, and the excess liquid drawn off. The surface of the medium was permitted to dry, the lid being slightly opened. When dry, the surface of the medium was spotted with one drop of each dilution on different areas of the plate. Incubation was at 30°C overnight. The routine test dilution (R. T. D.) was the highest dilution at which the

phage produced confluent lysis on the propagating strain. A titer of 1/10 indicated 10 times as strong as the routine test dilution.

Testing of Bacteriophages

At the outset it was essential to test each phage for contamination or slight variations otherwise undetected. This was carried out by comparing its lytic activity with the standard lytic spectrum issued by the Staphylococcal Reference Laboratory, Colindale, England, and the Laboratory of Hygiene, Ottawa, Canada.

Each new phage was tested on the following propagation strains for lytic activity:

PS29, PS29A, PS42B/47C, PS42C, PS42E,
PS44A, PS47, PS51, PS52, PS53, PS54,
PS80, PS2009.

The undiluted phage suspension was spotted on each propagating strain, and the phage was titered on those strains which were lysed. The highest dilution of the phage suspension that gave 50 or more plaques on the homologous propagating strain

was given the arbitrary number of 5. The lytic activity of this phage on the other propagating strains listed was graded relative to this reaction, as follows:

5 = maximum titer.
4 = 10^1 - 10^2 of maximum titer.
3 = 10^3 - 10^4 of maximum titer.
2 = 10^5 - 10^6 of maximum titer.
1 = Occasional or very weak lysis.
0 = Inhibition.

Typing of Isolates

Routine test dilutions (R. T. D.) in trypticase soy broth were prepared for each of the 27 phages used. Each R.T.D. was checked twice weekly by spotting it on its homologous propagating strain. If the spot showed less than 50 plaques, this R.T.D. was discarded and new R.T.D. was prepared from stock phage suspension.

Isolates growing in the trypticase soy broth mentioned earlier were checked for purity and morphology by Gram staining. Only those cultures showing cocci, less than one micron and arranged in grape-like clumps were typed.

A freshly prepared and dried trypticase soy agar plate was flooded with one of the six-hour broth cultures referred to earlier. Excess liquid was drawn off with a sterile Pasteur pipette, and the medium surface permitted to dry with the lid slightly opened. The plate was placed on a labelled spinned wheel as shown in Fig. 1, and each of the 27 phages was spotted with a loop on the proper area. One loopful of phage suspension contained approximately 0.005 ml. After the spotted areas dried, the plate was incubated at 30°C overnight with the plate inverted. The lytic activity of the different phages on the isolate were recorded, as follows:

++ = confluent lysis, or 50 or more plaques.

\+ = 20 - 50 plaques.

± = weak lysis or less than 20 plaques.

If confluent lysis occurred with 3A, 3B and 3C only the result was recorded as 3A/3B/3C. If on the contrary in addition weak lysis occurred with some of the other phages the result was recorded as 3A/3B/3C+.

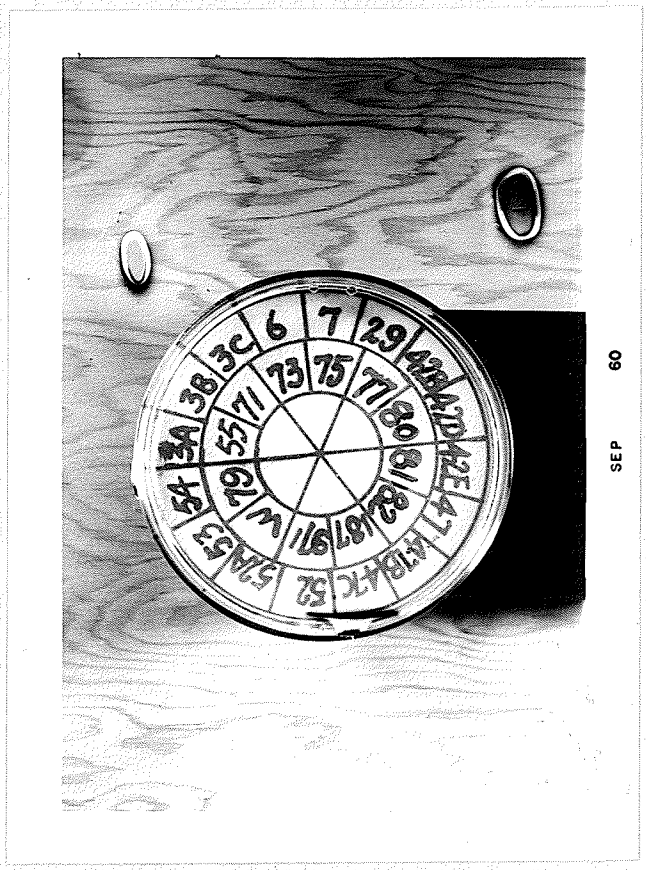

Fig. 1.

Spotting of isolates with phages. Trypticase soy agar plate, flooded with broth culture and dried, was placed on the spinning wheel. Each phage was spotted on the proper area.

EXPERIMENTAL

EXPERIMENTAL

Optimum Time of Incubation for Phage Production

The phages studied (3A, 3B, 3C, 6, 7, 42, 47, 51 ,53, 54, 55, 71, 73, 75, 77, 80, 81 and 82), could be divided into three groups according to the relationship between time of incubation and concentration of phage particles produced. The first group included phages 3A, 3B, 7, 42E, 47, 71, 73, 80, 81 and 82, and is represented in Fig. 2 by phage 3A. It may be observed that the staphylococci increased in number for about two hours after which time lysis has a greater effect than increase in number of cells. The titers of the aliquots showed that at one hour the phage particles had increased in number, and the number of phage particles kept on increasing for three to five hours, when a slight drop in titer occurred. This titer remained approximately the same thereafter, even after incubation overnight. The detailed data concerning colorimetric readings and titers of the aliquots on the individual phages may be found in appendix A.

The second group included phage 77, 75 and 51 and is represented in Fig. 3 by phage 77. In this group,

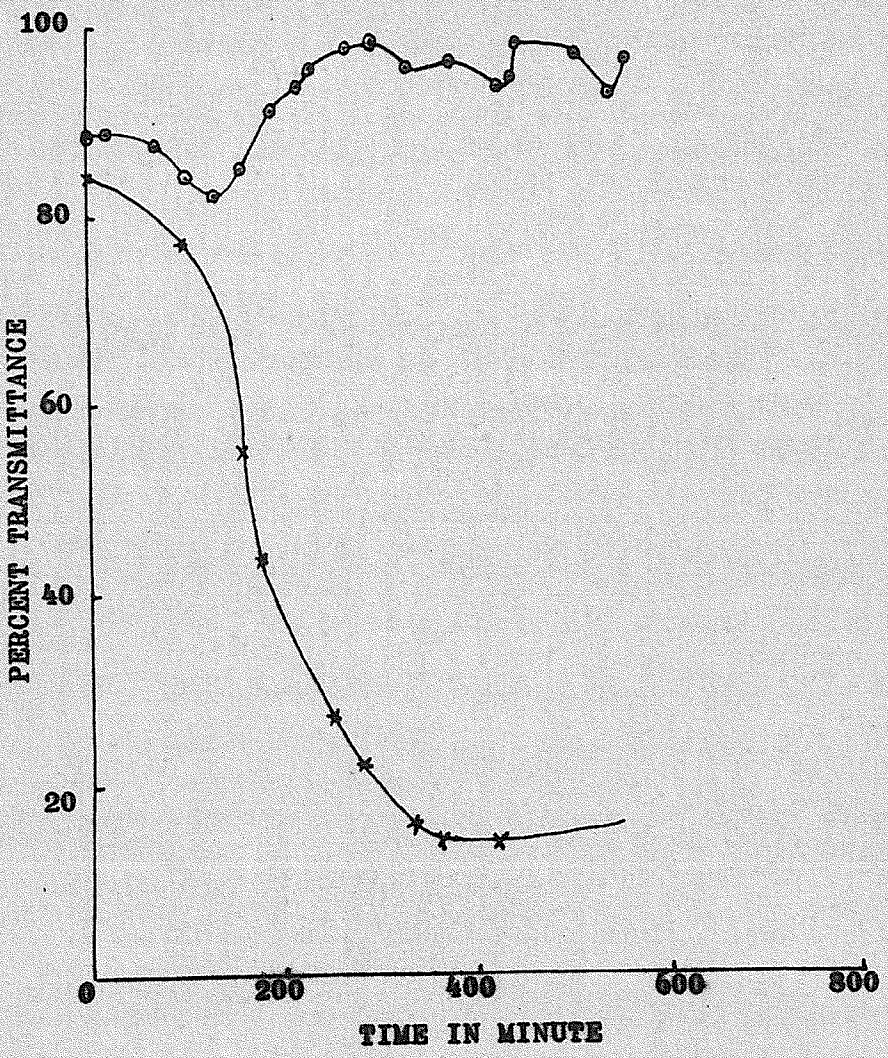

Fig. 2. Lysis of a strain (3A) of Staphylococcus areus by its homologous phage in trypticase soy broth at 37°C, measured with a Beckman Colorimeter.

x——x Growth of Staphylococcus areus.

o——o Lysis of Staphylococcus areus by its homologous phage.

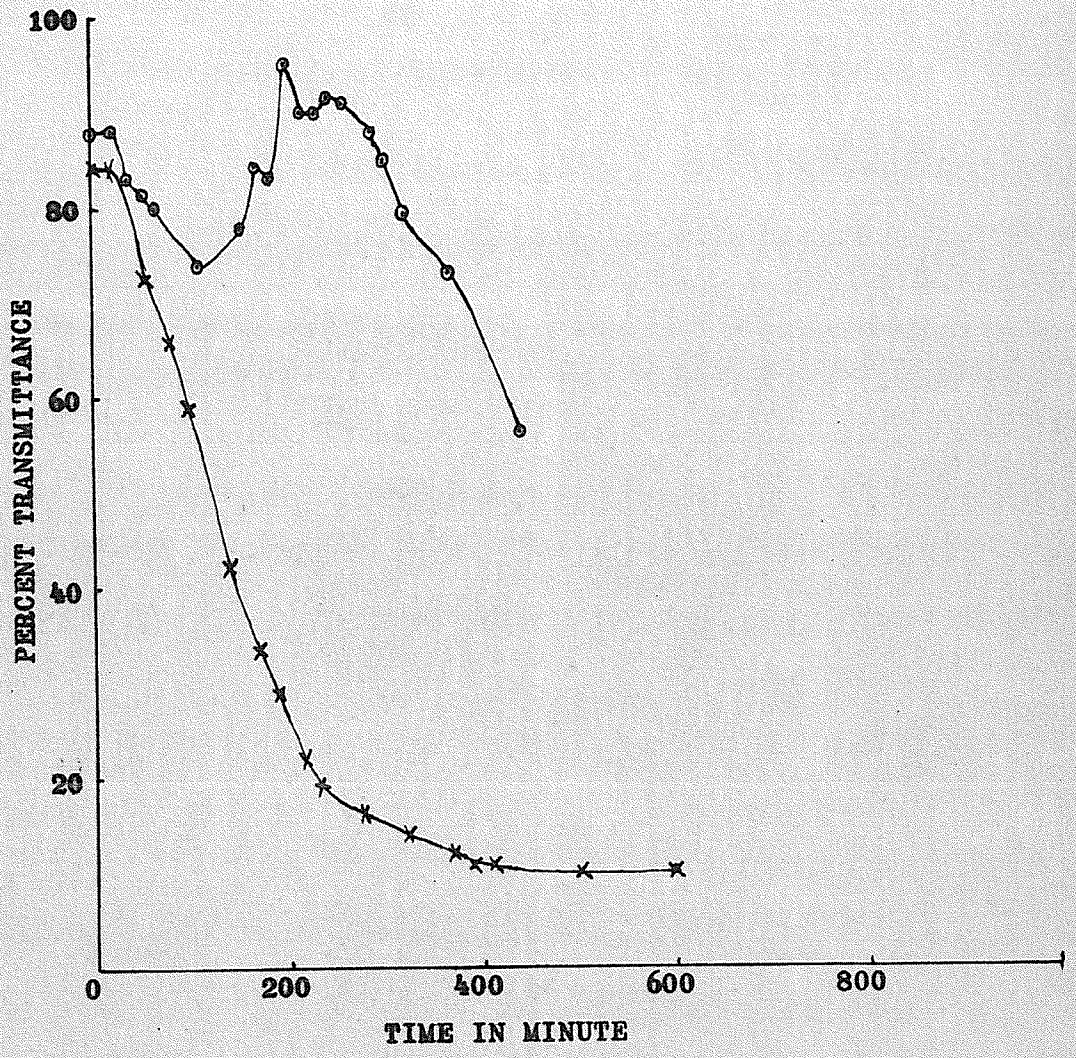

Fig. 3. Lysis of a strain (77) of Staphylococcus areus by its homologous phage in trypticase soy broth at 37°C, measured with a Beckman Colorimeter.

×——× Growth of Staphylococcus areus.

⊙——⊙ Lysis of Staphylococcus areus by its homologous phage.

the first two to three hours of incubation gave approximately the same result as did the first group. However, with each phage of this group soon after the maximum titer had been reached, the staphylococci increased and the phage particles decreased rapidly as shown by the titers of the aliquots. After incubation overnight no reaction or only very weak lysis occurred when the undiluted supernatant was spotted on the homologous strain.

The third group included phages 3C, 6, 42D, 53, 54 and 55 and is represented in Fig. 4 by phage 3C. In this group the result during the first two to three hours of incubation was much the same as in the previous two groups. However, when the maximum titer had been reached, it remained stationary for two to four hours. Then, the titer decreased slowly, and if incubation was continued overnight, a satisfactory titer (10^3 or more) was not obtained.

The data on these three groups indicated that the optimum time of incubation for the production of these phages was from two to six hours.